

Toll Like Receptor 4-Mediated Immune Responses  
in the Bladder Epithelium

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
Molecular Genetics and Microbiology in the Graduate School  
of Duke University

2008

ABSTRACT

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## Abstract

The urinary tract is one of the most intractable mucosal surfaces for pathogens to colonize. In addition to the natural barriers at this site, potential pathogens have to contend with the vigorous local innate immune response that is initiated by engagement of surveillance molecule TLRs. TLR4 appears to be not only exclusively expressed on superficial BECs but also critical to triggering robust local innate immune responses. TLR4 recognizes Gram-negative bacterial component LPS and initiates a series of intracellular NF- $\kappa$ B associated signaling events resulting in a cytokine response. We examined intracellular signaling events in human BECs leading to the production of IL-6, a major urinary cytokine, following activation by *E. coli* and isolated LPS, and observed that, in addition to the classical NF- $\kappa$ B associated pathway, BEC TLR4 triggers a distinct and more rapid signaling response involving, sequentially, Ca<sup>2+</sup>, AC3 generated cAMP, and the transcriptional factor CREB. This capacity of BECs to mobilize secondary messengers and evoke a more rapid IL-6 response might be critical in their role as first responders to microbial challenge in the urinary tract.

Here, we also report two additional distinct TLR4-mediated defense mechanisms in BECs. First, BEC TLR4 inhibits bacterial invasion, a necessary step for successful infection. TLR4-mediated suppression of bacterial invasion was linked to increased intracellular cAMP levels which negatively impacted Rac-1 mediated mobilization of the

cytoskeleton. Additionally, we found that BECs continue to fight UPEC even after bacterial invasion by triggering bacterial exocytosis through a distinct TLR4-mediated mechanism following activation by LPS. In addition, we reveal that Caveolin-1, Rab27b, PKA, and MyRIP are components of the exocytic compartment and that they form a complex involved in the exocytosis of bacteria. The ability of TLR4 to mediate the rapid cytokine response, the inhibition of bacterial invasion, and the expulsion of intracellular bacteria from infected cells represents three previously unrecognized functions for this innate immune receptor.

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## List of Abbreviations

AC, adenylyl cyclase

BECs, Bladder epithelial cells

BHI, Brain Heart Infusion

6-Bnz-cAMP, N6- Benzoyladenosine- 3', 5'- cyclic monophosphate

8-CPT-2-cAMP, 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic  
monophosphate

CREB, cAMP response element-binding protein

dbcAMP, dibutyryl cyclic AMP

DMSO, dimethyl sulfoxide

Epac, Exchange protein activated by cAMP

Fsk, forskolin

Fura-2/AM, Fura-2 acetylmethyl ester

GI tract, gastrointestinal tract

16-HBE Human airway epithelial cells

HEK cells, human embryonic kidney (HEK) cells

Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP, horseradish peroxidase

IBC, Intracellular bacterial community

IFN, interferon

IRAK, interleukin-1 receptor-associated kinase

KD, knockdown

LPS lipopolysaccharides

LB, Luria-Bertani

MyD88, myeloid differentiation primary response protein 88

NF- $\kappa$ B, nuclear factor  $\kappa$ B

PAMPs pathogen associated molecular patterns

PDTC, pyrrolidine dithiocarbamate

PIPC, polyinosine-polycytidylic acid

PKA, protein kinase A

PKI, protein kinase A inhibitor

PMB, polymyxin B

PRRs pattern recognition receptors

RT, Room temperature

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TIR, Toll/Interleukin-1 Receptor

TLR, Toll like receptor

TNF, tumor necrosis factor

TRAF6, TNF receptor-associated factor 6

TRAM, TRIF-related adaptor molecule

TRIF, toll-interleukin receptor adaptor protein inducing interferon  $\beta$

UPEC, Uropathogenic Escherichia coli

UPIa, Uroplakin Ia

UTI, Urinary tract infection

WGA, wheat germ agglutinin

WT, wild-type

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I dedicate this dissertation to

My Mother and Father,

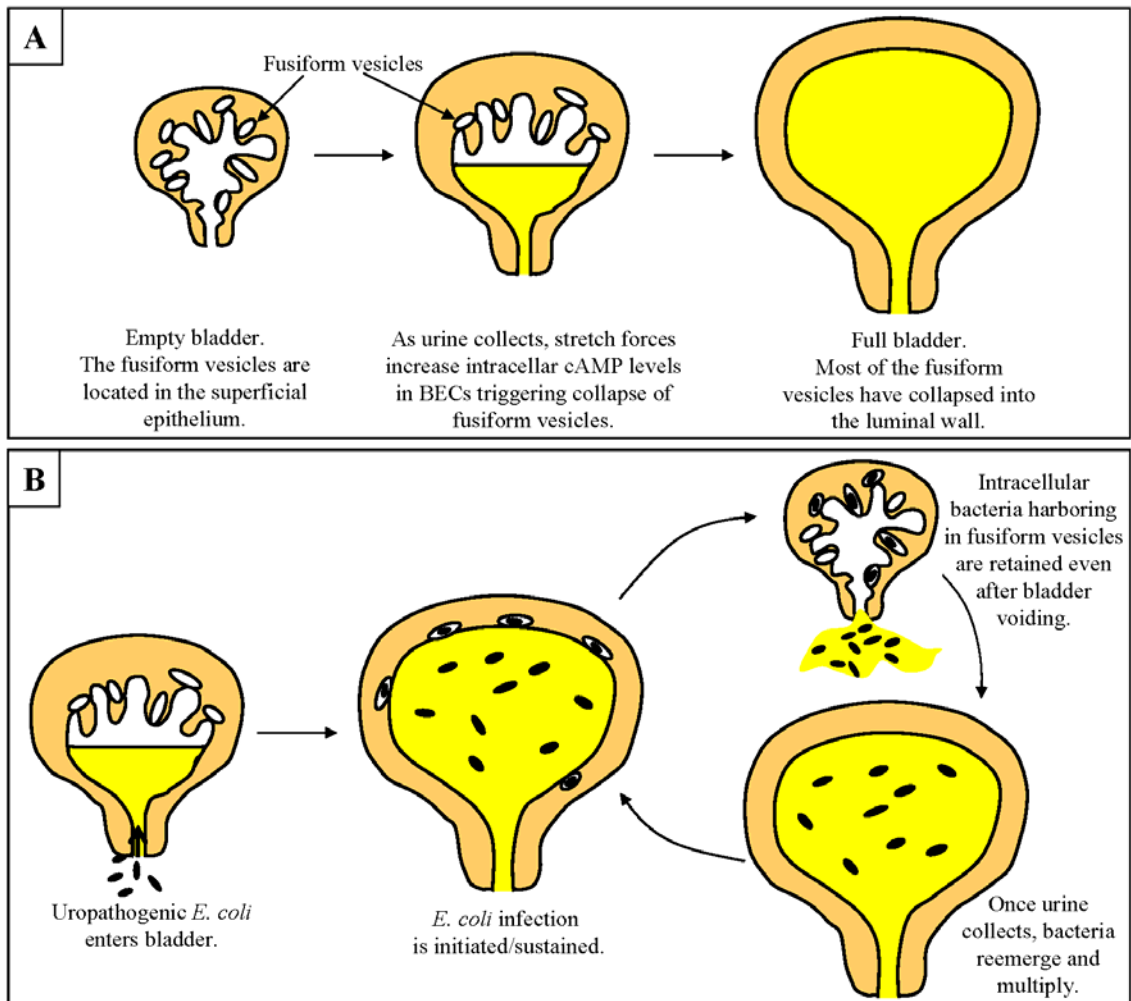
Husband, Jongmyeong,

and Son, Alexander Hyunsoo.

# Chapter 1: Introduction

## **1.1 Urinary tract infections (UTIs)**

During their lifetimes, 10-20% of females will receive medical attention for UTIs and nearly 3% will experience more than one infection per year (Andriole and Patterson, 1991; Patton et al., 1991). UTIs constitute the second leading cause of physician visits in the US, and the annual cost of treatment and management of this disease exceeds \$2 billion (Andriole and Patterson, 1991; Stamm, 1991). In addition, approximately a quarter of the yearly \$4 billion dollar cost attributed to nosocomial infections is a consequence of UTIs. These infections are a major complication among pregnant and elderly women with some estimates of the rate of bacteriuria among pregnant women as high as 25% (Andriole and Patterson, 1991). Once a female has had a UTI, there is a 1 in 4 chance of a recurrence and women with a history of recurring UTIs are at greater risk for developing pyelonephritis (Andriole and Patterson, 1991; Stamm, 1991). Intracellular bacteria in uroepithelial cells are believed to be a source for recurrent infection because most of the extracellular bacteria will be washed away when urine is voided. Once fresh urine collects in the bladder, these intracellular bacteria reemerge and rapidly multiply to initiate another round of infection (Martinez et al., 2000; Mulvey et al., 2001; Schilling and Hultgren, 2002) (Fig. 1.1).



**Figure 1.1: Cooption of fusiform vesicles of superficial BECs by UPEC.** **A**, Fusiform vesicles are intracellular vesicles that regulate bladder volume. When bladder volume increases as urine collects, fusiform vesicles collapse into luminal surface membrane providing the necessary membrane. **B**, A proposed model of how harboring of UPEC within fusiform vesicles sustains bladder infections. Once UPEC enter the lumen of bladder, they rapidly multiply in the urine. A fraction of these bacteria gain access into fusiform vesicles. When urine is voided all bacteria are eliminated except those within fusiform vesicles. Once urine collects in the bladder, bacteria reemerge from collapsing fusiform vesicles and rapidly multiply to previous levels.

### **1.1.1 Uropathogenic *E. coli* (UPEC) invasion**

Over 85% of the outpatient, community acquired UTIs are caused by *E. coli* (Johnson, 1991). Among several bacterial virulence factors that have been linked to UPEC, the single most common determinant of virulence is adhesion (Johnson, 1991; Mulvey et al., 2000). The capacity to bind avidly to the uroepithelial cells or to the mucus coat on these cells appears to be pivotal in determining whether or not bacteria are able to infect the urinary tract. UPEC are capable of expressing multiple cell surface organelles of adhesion including fimbriae. The most commonly expressed fimbria is the mannose binding type 1 fimbriae. Over 80% of all UPEC express type 1 fimbriae (Hagberg et al., 1981). This is not surprising in light of the fact that the colonizing bacteria must resist powerful innate immune responses in the urinary tract. In their intracellular location, these pathogens avoid the clearing actions of both urine flow and recruited phagocytes. Binding of FimH, a mannose binding lectin at the distal tip of the fimbria, to uroplakin 1a on the luminal surface of BECs triggers a distinct series of signaling reactions that culminates in the entry of the bacteria into the dynamic subapical pool of discoid vesicles called fusiform vesicles (Bishop et al., 2007). These fusiform vesicles are membrane-rich nondegradative compartments that serve to increase bladder volume by fusing with luminal plasma membrane of BECs (Truschel et al., 2002). Since the plasma membrane of BECs as well as fusiform vesicles are highly enriched in cholesterol, sphingolipids, and glycolipids (Apodaca, 2004), there is a

growing realization that UPEC invasion of BECs is lipid raft dependent. This notion has been supported by the finding that specific disruptors of cellular lipid raft structure inhibit UPEC invasion, and key cellular components implicated in bacterial invasion of BECs are typically localized with lipid raft structures (Duncan et al., 2004). One such lipid raft mediator of bacterial invasion is the Rho GTPase member, Rac-1, whose activation is critical because it enhances the accumulation of actin filaments at sites of bacterial entry (Duncan et al., 2004; Martinez and Hultgren, 2002). Several studies from the Hultgren laboratory have demonstrated that certain intracellular UPEC can multiply within their intracellular compartment to form “intracellular bacterial communities” (IBCs), some of which can then switch into a quiescent phase that persists intracellularly for indefinite periods of time (Anderson et al., 2003; Garofalo et al., 2007; Justice et al., 2004; Justice et al., 2006; Wright et al., 2007). It has been suggested that the recurrence of UTIs or the development of chronic UTIs can, at least in part, be attributed to these bacterial forms (Mysorekar and Hultgren, 2006).

## ***1.2 Innate Immune responses in the urinary tract***

The urinary tract including the bladder is typically sterile and most UTIs are thought to be initiated when uropathogens located in the GI tract, which is widely considered as a reservoir, are introduced into the urethra (Hooton, 2001). Because of the extreme impermeability of the bladder epithelium and the forceful nature of urine flow,

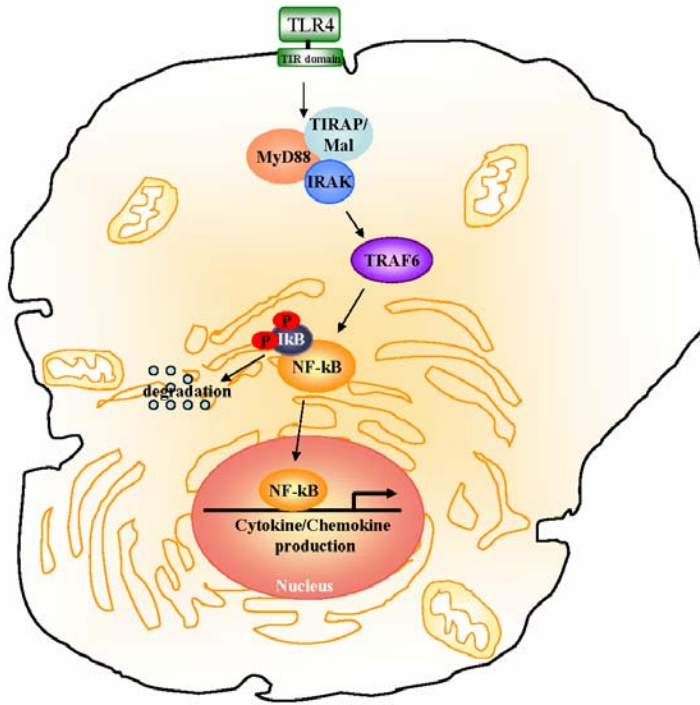
most prospective pathogens find it exceedingly difficult to establish a foothold in this organ (Cattell et al., 1970; Mackintosh et al., 1975). In addition to the natural defense actions, there exists a highly organized and effective innate and adaptive immune response that can counter and overcome bacterial attack.

### **1.2.1 TLRs and immunosurveillance**

Critical to any immune response mounted by the urinary tract is the presence of immune surveillance molecules. Like other mucosal surfaces, the lining of the urinary tract contains receptors capable of recognizing intruding pathogens by their invariant pathogen associated molecular patterns (PAMPs). Of the various immune surveillance molecules the Toll-like receptor (TLR) family is the best characterized (Andersen-Nissen et al., 2007; Samuelsson et al., 2004; Schilling et al., 2001; Zhang et al., 2004). Unlike the receptor for the FimH adhesin of UPEC, which promotes bacterial invasion and subsequent invasion of BECs (Martinez et al., 2000), the TLRs function by detecting different PAMPs and then mobilizing appropriate immune defenses. The common TLRs encountered in the urinary tract include TLR2 (recognizes bacterial lipoteichoic acid or lipoprotein), TLR3 (recognizes double stranded RNA), TLR4 (recognizes lipopolysaccharides (LPS)), TLR5 (recognizes flagellin), TLR9 (recognizes unmethylated CpG DNA of bacteria and viruses), and TLR11 (recognizes profiling of parasites). Perhaps, the best studied of these TLRs is TLR4 which is well expressed on epithelial

cells of the kidney and bladder (Samuelsson et al., 2004). TLR4 promotes the vigorous cytokine and chemokine responses to Gram negative bacteria in the urinary tract (Hagberg et al., 1984; Schilling et al., 2001). TLR5 and TLR11 are other TLRs that have been shown in *in vivo* studies to contribute to immune defense in the urinary tract (Andersen-Nissen et al., 2007; Zhang et al., 2004). TLR5 is predominantly expressed on bladder cells, whereas TLR11 is primarily found on kidney cells (Andersen-Nissen et al., 2007; Zhang et al., 2004).

TLR4 as well as its co-receptor, CD14, are amply expressed in the bladder of mice and humans, where it appears to be exclusively found on the superficial BECs lining the lumen (Miyazaki et al., 2006; Schilling et al., 2003; Song et al., 2007a). Upon recognition of bacterial LPS, TLR4/CD14 complexes on BECs are believed to follow the classical signaling pathway (Akira et al., 2006). In the classical pathway, TLR4 initiates a signaling cascade involving TIR adaptor molecules, TIRAM/Mal and MyD88, and substrates, IRAK and TRAF6, resulting in the activation of a transcriptional factor NF- $\kappa$ B (Akira et al., 2006). NF- $\kappa$ B first traffics to the nucleus and then stimulates transcription of various cytokines including IL-6 and IL-8, two of the major cytokines observed in the urinary tract following bacterial infection (Agace et al., 1993; Hang et al., 1998; Schilling et al., 2003) (Fig. 1.2).



**Figure 1.2: The canonical TLR4-mediated signaling.** TLR4 initiates a signaling cascade involving TIR adaptor molecules, TIRAP/Mal and MyD88, and substrates, IRAK and TRAF6, resulting in the activation of a transcriptional factor NF- $\kappa$ B. NF- $\kappa$ B first traffics to the nucleus and then stimulates transcription of various cytokines including IL-6 and IL-8, two of the major cytokines observed in the urinary tract following bacterial infection.

### **1.2.2 TLR4 and resistance to UTIs**

Over the past two decades, several independent observations in mice and humans have revealed a link between TLR4 and innate resistance to UTIs. It has been known for many years that the TLR4 mutant C3H/HeJ mice fail to clear UTIs once infected (Hagberg et al., 1984; Shahin et al., 1987), due to an inability to mount an adequate mediator response to the pathogen. The defect was subsequently linked to a mutation in the TLR4 gene (Hagberg et al., 1984; Shahin et al., 1987). In humans, patients with persistent asymptomatic bacteriuria have been found to have low levels of TLR4 expression relative to healthy age matched controls (Ragnarsdottir et al., 2007). A more direct link between a mutation in TLR4 and a predisposition to recurrent UTIs has recently been reported in children. Examination of polymorphisms in the TLR4 gene has revealed a remarkable relationship between the carrier status of the TLR4 A(896)G allele and the development of recurrent UTIs in childhood, which is independent of other renal abnormalities (Karoly et al., 2007). Cumulatively, these observations link defects in TLR4 or reduced expression of TLR4 to predisposition to recurrent UTIs with reduced symptoms.

### **1.3 Research objectives**

UTIs constitute the second most common type of infectious disease in humans, following respiratory tract infections. The majority of UTI patients are females ranging

in age from early teens to the elderly. A significant proportion (up to 25%) of these patients will subsequently be afflicted by recurring or persistent infection. The inability to eliminate UTIs in this segment of the population is attributable to many factors. In some cases, the patients have inherent anatomical abnormalities or immune deficiencies (e.g. defects in TLR4) that predispose them to re-infection. Whereas in others, antimicrobial therapy is ineffective because the bacteria either have developed resistance or have discovered an intracellular niche where they are impregnable to the antimicrobials. In all of these cases, conventional antibiotic therapy alone is unlikely to be sufficient for successful management of these infections. A possible emerging strategy for the management of intractable UTIs is to combine antimicrobial therapy with host immune system modulators. Since almost all UTIs are caused by Gram-negative bacteria that are recognized by an immunosurveillance molecule TLR4, employing TLR4 ligands or small molecules boosting TLR4-mediated pathway to bolster immunity in patients is of great interest. This strategy is likely functional, since reduced TLR4 expression and mutation are linked to patient susceptibility to UTIs. For these proposed emerging strategies to be effective, it is necessary to comprehensively characterize TLR4-mediated immune responses. Thus, my research focuses on elucidating the role of the immune system, especially TLR4-mediated responses, in the urinary tract.

In this dissertation, I will describe my research in three separate studies. Chapter 2 examines a novel TLR4-mediated intracellular signaling event in human BECs leading to the rapid production of IL-6 and IL-8, two major urinary cytokines. Chapter 3 details the novel TLR4-mediated mechanism to inhibit bacterial invasion, while Chapter 4 describes TLR4-mediated expulsion of bacteria from infected BECs. In the last Chapter, I will discuss the significance of the findings, especially the potential strategies to boost the host innate immune system.

## **Chapter 2: A Novel TLR4-Mediated Signaling Pathway Leading to IL-6 Responses in human BECs**

### ***2.1 Introduction***

The innate immune system is the first line of defense against infection and is thought to be primarily mediated by phagocytic immune cells such as macrophages and dendritic cells. These cells recognize microorganisms via a limited number of germline-encoded pattern recognition receptors (PRRs) that recognize microbial components known as PAMPs, which are essential for the survival of the microorganism and, therefore, difficult for the microorganism to alter (Akira et al., 2006). Several classes of PRRs, including TLRs and cytoplasmic receptors, recognize distinct microbial components and directly activate immune cells triggering intracellular signaling cascades that rapidly induce the expression of a variety of inflammatory cytokines that initiate a variety of overlapping immune responses. One of the best known PRRs is TLR4, which recognizes the major Gram negative bacterial surface component LPS (Akira et al., 2006). Studies of TLR4 signaling in monocytes, macrophages and dendritic cells have revealed that engagement of TLR4 by LPS triggers a signaling cascade involving several intracytoplasmic and nuclear transcriptional factors. TLR4 activation first engages a set of adaptor family members which link TLR4 to the serine/threonine kinases. These kinases mediate phosphorylation and ubiquitination of various substrates

eventually resulting in the activation of the transcriptional factor NF- $\kappa$ B, which regulates the expression of several immunomodulatory cytokines (Kawai and Akira, 2006).

The urinary tract is extremely intractable to infection by most pathogens. This is attributable to a large extent on the multifaceted innate immune defenses of the bladder and, in particular, BECs. These cells selectively exfoliate upon bacterial colonization and undergo re-epithelialization as a mechanism to reduce bacterial load in the bladder. They are also a major source of proinflammatory cytokines and chemokines in the urinary tract following bacterial infection (Agace et al., 1993; Schilling et al., 2003). These BECs derived mediators are responsible for the vigorous neutrophil response which is responsible for early clearance of infecting bacteria (Haraoka et al., 1999). A prominent mediator released by BECs is IL-6 and it is, by far, the single most prominent cytokine detected in the urine of infected patients (Otto et al., 1999). IL-6 is known to mobilize and amplify both local as well as systemic innate immune defenses against infection (Gabay, 2006). The production of some of the earliest indicators of inflammation in the body such as the acute phase proteins has been directly related to production of this cytokine (Gabay, 2006).

Considering the large number of pathogens capable of infecting the urinary tract, it is remarkable that UPEC account for over 85% of UTIs in patients without underlying

predisposing factors (Svanborg and Godaly, 1997). The singular success of UPEC in the urinary tract has been attributed to bacterial surface expression of filamentous fimbrial appendages, called type 1 fimbriae (Martinez et al., 2000). These structures promote avid bacterial binding to uroplakin 1a molecules on the surface of BECs triggering bacterial invasion of these cells (Duncan et al., 2004; Mulvey et al., 1998). In their intracellular location, UPEC avoid elimination by the flushing actions of urine (Roos et al., 2006). Recent studies have suggested additional traits on UPEC that account for their success as uropathogens. These include their capacity to block apoptosis and exfoliation of infected BECs (Klumpp et al., 2001) as well as inhibit the ability of BECs to mount a cytokine response (Hunstad et al., 2005). Although several genes on UPEC have been implicated in inhibiting cytokine production the underlying mechanism remains elusive, a problem exacerbated, at least partly, by the fact that most of our current understanding of TLR4 signaling is based almost exclusively on cells of hematopoietic origin (Akira et al., 2006). Here we sought to better define LPS/TLR4 signaling pathway in BECs. We were especially interested in defining the role, if any, of two second messengers,  $Ca^{2+}$  and cAMP, since these low-molecular weight diffusible molecules have been globally implicated in cellular signaling events including cytokine responses. We investigated the IL-6 responses of human BECs to *E. coli* and to isolated LPS. In this chapter, our studies demonstrated that the IL-6 response triggered by TLR4 in BECs involves not only the classical NF- $\kappa$ B associated pathway but also a distinct pathway involving  $Ca^{2+}$ , cAMP,

and CREB. Interestingly, the latter pathway resulted in a rapid IL-6 response which is evident at least 3 hrs before the NF- $\kappa$ B associated pathway.

## **2.2 Experimental procedures**

### **2.2.1 Bacteria and Cell lines**

A K-12 laboratory *E. coli* strain ORN103(pSH2) (80  $\mu$ g/ml Chloramphenicol) and a UPEC type 1 fimbriated and non-hemolytic strain CI5 were utilized in this study (Abraham et al., 1985; Orndorff and Falkow, 1984; Thankavel et al., 1997). Bacteria were grown statically in 10 ml of Luria-Bertani (LB) broth for 24 hours in the absence or, if indicated, presense of antibiotics. The human BEC line 5637 (ATCC HTB-9) was grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (HyClone) and incubated at 37° C with 5% CO<sub>2</sub>. Cells were seeded onto either a 24-well pate at a density of 1x10<sup>5</sup> cells per well or 6-well plate at a density of 1x10<sup>6</sup> cells per well and incubated overnight, if not otherwise specified. Human primary BECs were maintained in keratinocyte-SFM, containing recombinant epidermal growth factor and bovine pituitary extract (Invitrogen). For the experiments performed in this study, once the primary cells were confluent, cells were switched to keratinocyte medium containing 5% FBS and 2 mM CaCl<sub>2</sub>, and further incubated for additional three days (Cross et al., 2005). All experiments were performed using 5-7 passaged primary human BECs. Human airway epithelial cells (16-HBE) were cultured in DMEM plus 4 mM glutamine and 10% FBS,

and the human monocytic cell line (Mono Mac 6) was cultured as described previously (Wright et al., 1996).

### **2.2.2 IL-6 measurements**

BECs were seeded onto 24-well plates and grown overnight. The cells were infected with *E. coli* at a MOI of 100, or purified *E. coli* LPS was added to the cells at a concentration of 100 µg/ml, and incubated at 37° C for 6 h. Where indicated, *E. coli* or purified *E. coli* LPS were treated with 1 µg/ml polymyxin B for 30 min prior to use (Hedlund et al., 1999; Schilling et al., 2001). If specified, 2 mM NiCl<sub>2</sub> (Sigma), 5 µM BAPTA-AM (Sigma) or 0.4 mM MDL-12,330A (Calbiochem) was added to the cells for 30 min prior to infection. For cytokine induction mediated by cAMP analogs, 1 mM dibutyryl cAMP (Sigma) or 1 mM 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-3'-5'-cyclic monophosphate (Sigma) was added to the cells and incubated at 37° C for 6 h. IL-6 secretion was also measured after treating the cells for 6 h with an Adenylyl cyclase activator forskolin (50 µM; Sigma) in the presence or absence of NiCl<sub>2</sub> (2 mM) or polymyxin B (1 µg/ml), or with NiCl<sub>2</sub> (2 mM) or polymyxin B (1 µg/ml) in the absence of forskolin. Cell supernatants were collected and centrifuged to remove bacterial or cellular debris, and tested for IL-6 using the human IL-6 ELISA kit (R&D Systems) according to the manufacturer's protocol. Cell viability was not affected by the pharmacological agents employed, as assessed by trypan blue exclusion.

### 2.2.3. Ratiometric imaging

BECs were seeded onto a 22-mm square glass coverslip placed into a 6-well plate at a density of  $7.5 \times 10^5$  cells per well and grown overnight. Cells were incubated with 5  $\mu$ M Fura-2 acetylmethyl ester (Fura-2/AM) (Molecular Probes) in Hanks' Balanced Salt Solution (Invitrogen) for 30 min before the  $[Ca^{2+}]_i$  measurements. Ratiometric  $Ca^{2+}$  imaging was performed using a Nikon fluorescence microscope fitted with a 40x objective and digital CCD camera. Dual images (340- and 380-nm excitation, 510-nm emission) were collected, and pseudocolor ratiometric images were monitored during the experiments by using RatioTool software (Inovision Corporation, Raleigh, NC). In all samples, data were collected from regions of interest consisting of approximately 25 to 30 cells and acquired as a running ratio average. When cells were infected with *E. coli*, an MOI of 500 was used. To determine the role of bacteria-associated LPS, *E. coli* was treated with 1  $\mu$ g/ml of the antibiotic polymyxin B for 30 min prior to infection (Hedlund et al., 1999; Schilling et al., 2001). When cells were treated with purified *E. coli* LPS, 100  $\mu$ g/ml was used (Sigma). If specified, the cells were pretreated with 2 mM  $NiCl_2$  for 30 min. The Fura-2 calcium imaging calibration kit (Molecular Probes) was used to calibrate fluorimetric analyses to quantify intracellular calcium concentration.

## 2.2.4 Measurement of intracellular cAMP levels

BECs were seeded onto 6-well plates and grown overnight. Cells were left uninfected or infected with 100 MOI of *E. coli* for 1 h. To examine the role of soluble LPS, cells were treated with 100 µg/ml *E. coli* LPS for 6 h. After treatment, cells were washed four times with PBS to remove culture media and lysed in 250 µl of 0.1 M HCl. Cell lysate was used directly in a cAMP assay. Where indicated, cells were preincubated for 30 min with 2 mM NiCl<sub>2</sub>, 400 µM MDL-12,330A, or the bacteria were first incubated with 1 µg/ml polymyxin B for 30 min prior to use (Hedlund et al., 1999; Schilling et al., 2001). Intracellular concentrations of cAMP were determined using a cAMP enzyme immunoassay kit (Sigma) according to the manufacturer's instructions.

## 2.2.5 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using RNeasy purification system (Qiagen) according to the manufacturer's protocol. Two µg of total RNA was reverse transcribed and amplified with gene-specific primers using the RT-PCR System kit (Bio-Rad). The primer sequences for the genes and expected product sizes were as follows: 5'-CTGAGAGGATTTCCACAAG-3' (sense) and 5'-TTTGTGATATGAACCTTCCC-3' (antisense) for AC-1 (466 bp), 5'-CTTCCTCTTCATCATCTTCG-3' (sense) and 5'-TTCATTCTCCTTTGCAATTT-3' (antisense) for AC-2 (574 bp), 5'-TACTCGGTGGAGAAGGAGAAGCAG-3' (sense) and 5'-

CGAAAACGCTTGTGGTCGTATTC-3' (antisense) for AC-3 (554 bp), 5'-  
GACTTCAACCCACTGACACT-3' (sense) and 5'-AGTCTGATGATGTTGGGAAG-3'  
(antisense) for AC-4 (397 bp), 5'-GTACTCCAAGC AGGTAGACG-3' (sense) and 5'-  
GCTGTAGGTGAAGTACTCGG-3' (antisense) for AC-5 (484 bp), 5'-  
CATCTGCACACACTATCCAG-3' (sense) and 5'-ATGGCCTCAATCATGTCTAC-3'  
(antisense) for AC-6 (465 bp), 5'-CTCTCTGTGCTGATGTACGTCGAG-3' (sense) and 5'-  
TCTTGACGTAGAGGCTGTGGAAGT-3' (antisense) for AC-7 (587 bp), 5'-  
TTAGGAACCCCTCCTCCG-3' (sense) and 5'- TGCTAGGGGCACAGTCAAG-3'  
(antisense) for AC-8 (476 bp), 5'-GAAAACGGACGCCCACTTTG-3' (sense) and 5'-TCC  
TCCAGGAAGAACACCATCC-3' (antisense) for AC-9 (375 bp), 5'-  
AAAACCTGTCACCTCC AACG-3' (sense) and 5'-TCTAAAGCGTTGAG CCGAAT-3'  
(antisense) for soluble AC (556 bp), 5'-ATCCCATCACCATCTTCCAG-3' (sense) and 5'-  
CCTGCTTCACCACCTTCTTG-3' (antisense) for GAPDH (579 bp). The primers were  
synthesized by Integrated DNA Technologies, Inc. We confirmed that the other AC  
isotype-specific primers were functional by undertaking RT-PCR on total RNA from  
human embryonic kidney (HEK) cells (a positive control cell, where all ACs except AC4  
and 8 were expressed) (Ludwig and Seuwen, 2002). For PCR amplification of  
interleukins, 5' - ATGAACTCCTTCTCCACAAGCGC - 3' (sense) and 5' -  
GAAGAGCCCTCAGGCTGGACTG - 3' (antisense) for IL-6 (628 bp), 5'-  
GGACAAGAGCCAGGAAGAAACCACC-3' (sense) and 5'-

GCAACCCTACAACAGACCCACAC-3' (antisense) for IL-8 (460 bp), 5'-  
GTAAGCTATGGCCCACTCCA-3' (sense) and 5'-AGCAGCCGTGAGGTACTGAT-3'  
(antisense) for IL-1a (354 bp), and 5'-GGACAAGCTGAGGAAGATGC-3' (sense) and 5'-  
TCTTTCAACACGCAGGACAG-3' (antisense) for IL-1b (360 bp) were used.

## 2.2.6 Creation of Knockdowns Using RNA Interference

RNA interference vectors were generated using pQCXIN retroviral vector (BD Biosciences). Briefly, pQCXIN was digested by *Bam*HI and *Eco*RI and then was religated to generate pQCXIN1. Human U6 small nuclear RNA promoter was PCR-amplified from pTZ U6+ 1 (gift from John Rossi, Beckman Research Institute of the City of Hope, Duarte, CA) with added *Bgl*II site (5' ends), *Bam*HI, and *Xba*I sites (3' ends). The PCR product was cloned to the *Bgl*II and *Xba*I sites of pQCXIN1 to generate pQCXIN-U6. The following oligonucleotides were ordered from Integrated DNA Technologies, Inc.: AC3a, 5'-

GATCCGCTGTCTCCAGTACTACTTCAAGAGAGTGTAGTACTGGAGACAGCTT  
TTTTT-3', and AC3b, 5'-

CTAGAAAAAAGCTGTCTCCAGTACTACTCTCTTGAAGTGTAGTACTGGAG  
ACAGCG-3'; AC4a, 5'-

GATCCGTGTGTCCTCCATGATTTCTTCAAGAGAGGAAATCATGGAGGACACA  
CTTTTTTT-3', and AC4b, 5'-

CTAGAAAAAAGTGTGTCCTCCATGATTTCTCTCTTGAAGGAAATCATGGAG  
GACACACG-3'; AC6a, 5'-  
GATCCGTGACAGGTGTGAATGTGAATTCAAGAGATTCACATTCACACCTGTCA  
CTTTTTTT-3', and AC6b, 5'-  
CTAGAAAAAAGTGACAGGTGTGAATGTGAATCTCTTGAATTCACATTC  
CACCTGTCACG-3'; AC7a, 5'-GATCCGCTGGTGCTCGGTTCT TTGATTCAA  
GAGATCAAAGAACCGAGCACCAGCTTTTTTT-3', and AC7b, 5'-CTAGAAAAA  
AGCTGGTGCTCGGTTCTTTGATCTCTTGAATCAAAGAACCGAGCACCAGCG-3'.

The boldface and underlined sequences are forward and reverse sequences, respectively, which correspond to nucleotides 2324–2342 of the human AC-3 gene (AC3a and AC3b, GenBank™ accession number AF033861), nucleotides 2096-2114 of the human AC-4 gene (AC4a and AC4b, GenBank™ accession number AF497516), nucleotides 2085-2103 of the human AC-6 gene (AC6a and AC6b, GenBank™ accession number AF250226), and nucleotides 747-765 of the human AC-7 gene (AC7a and AC7b, GenBank™ accession number NM\_001114). The oligos were annealed to form double-stranded DNA and cloned into the *Bam*HI and *Xba*I sites of pQCXIN-U6 to generate pSi-AC3, pSi-AC4, pSi-AC6, and pSi-AC7. The Amphopack-293 Cell Line (BD Biosciences) was used to produce the viral particles. Production of viral particles, infection of target cell line (5637), and selection of viral infected cells were performed as recommended by the vendor of the pQCXIN vector (BD Biosciences). The geneticin-resistant stable-transfected cell lines

were named AC-3 KD, AC-4 KD, AC-6 KD, and AC-7 KD. Knockdowns were verified by RT-PCR using the AC isoform-specific primers listed above.

In order to create TLR4 knockdown, the following oligonucleotides were synthesized: TLR4a, 5'-  
GATCCGTTCCGATTAGCATACTTAGTTCAAGAGACTAAGTATGCTAATCGGAAC  
TTTTTTT-3', and TLR4b, 5'-  
CTAGAAAAAAGTTCCGATTAGCATACTTAGTCTCTTGAACTAAGTATGCTAAT  
CGGAACG-3'. The boldface and underlined sequences are forward and reverse  
sequences, respectively, which correspond to nucleotides 1026-1044 of the human TLR4  
gene (GenBank™ accession number U88880). Knockdowns were verified by RT-PCR  
using the gene-specific primers. The primer sequences for the gene were as follows: 5'-  
CGATTCCATTGCTTCTTG-3' (sense) and 5'-GCTCAGGTCCAGGTTCTT-3' (antisense)  
for TLR4.

### **2.2.7 Western blot analysis**

BECs were seeded onto 60-mm culture dishes and grown overnight. The cells were uninfected or infected with *E. coli* (MOI=100) for 1 h, or treated with 100 µg/ml LPS for 6 h. Cells were lysed in a RIPA buffer (Upstate) containing 1 mM PMSF and a 1:100 dilution of mammalian protease inhibitor cocktail (Sigma). The cell suspension was

passed 20 times through a 21-gauge needle and then sonicated briefly. The cell lysate was centrifuged at 10,000 rpm for 10 min with the precipitates then being discarded. Protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as the standard. Cellular proteins (100 µg per lane) were electrophoresed in 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane, which was blocked with 5% non-fat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1h, and then incubated overnight with 1:250-1:500 diluted anti-AC3 antibody (FabGennix, Inc.) in blocking solution at 4°C. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody for 1h at room temperature. The blots were then immunodetected with the enhanced chemiluminescence detection system (Pierce). As a loading control, 20 µg cellular proteins were loaded in 4-15% SDS-PAGE and blotted with anti-β-actin antibody (1:5000 dilution; Sigma). Image J software (National Institutes of Health) was used for densitometry to quantify protein expression for statistical analysis.

Anti-CREB and anti-phospho-CREB antibody were purchased from Cell Signaling technology and CREB western blotting was performed according to the manufacturer's instruction (Cell signaling technology). BECs were cultured onto 6-well plates overnight and left uninfected or infected with 100 MOI *E. coli* for 1 hr, or treated

with 50  $\mu$ M forskolin or 10  $\mu$ M calcium ionophore A23187 for 1 hr. When specified, BECs were treated for 6 hrs with either 2  $\mu$ g/ml of Lipoteichoic acid (TLR2 ligand) or 25  $\mu$ g/ml of polyinosine-polycytidylic acid (TLR3 ligand).

### **2.2.8 Detection of NF- $\kappa$ B nuclear translocation**

BECs were cultured onto 10-cm dish overnight and left uninfected or infected with 100 MOI *E. coli* for 1 or 2 hr. Nuclear extraction kit (Chemicon) were used for performing a nuclear extraction and the active form of NF- $\kappa$ B contained in the nuclear extract was detected using NF- $\kappa$ B p65 Transcription Factor assay system (Chemicon) according to the manufacturer's recommendation.

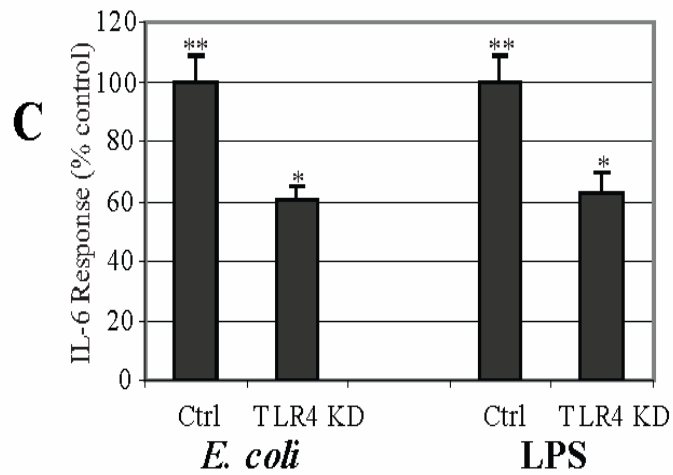
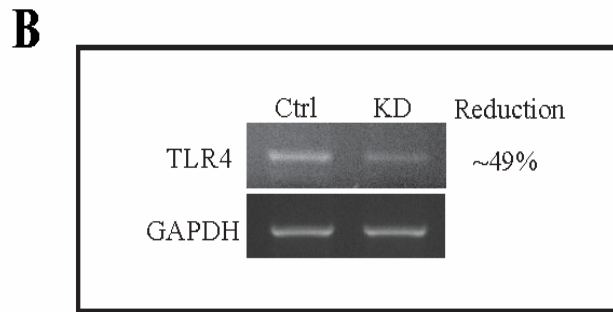
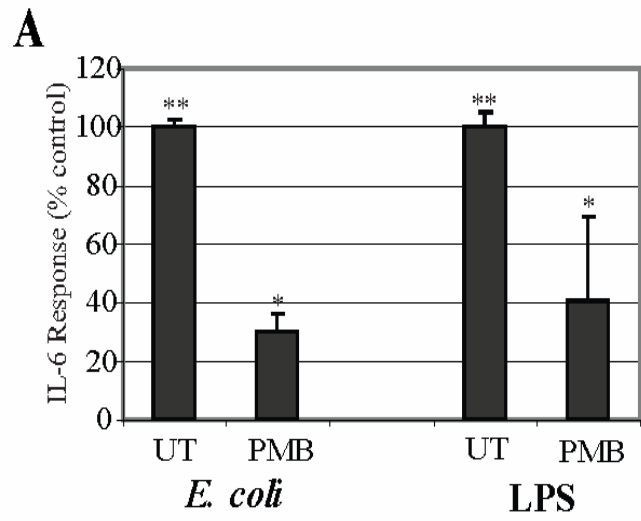
### **2.2.9 CREB Binding Assay**

We employed the Noshift transcription factor assay system (Novagen) to assay binding of CREB to CRE oligonucleotides. BECs were cultured and exposed for 1 h to *E. coli* ORN103(pSH2), and nuclear extracts were collected following the vendor's recommendation (Novagen). To detect the binding of CREB to the CRE site of the IL-6 promoter, CRE oligonucleotides from IL-6 promoter region were synthesized and end-labeled by biotinylation. The CRE oligonucleotide sequences utilized were the same as those used previously (Cao et al., 2006).

## **2.3 Results**

### **2.3.1. IL-6 response of BECs to type 1 fimbriated *E. coli* is largely elicited by LPS and involves TLR4**

Although there are several data implicating type 1 fimbriae and its adhesive subunit, FimH, as the determinant largely responsible on UPEC for triggering endocytic responses from BECs (Duncan et al., 2004; Mulvey et al., 1998) a recent study has reported LPS as the primary determinant on UPEC responsible for evoking the cytokine response from BECs (Schilling et al., 2001). We initiated our studies by examining the role of LPS in mediating the IL-6 response of BECs following exposure to *E. coli*. This was undertaken by comparing the IL-6 response of the human BEC line 5637 to *E. coli* in the presence and absence of polymyxin B (PMB), which binds to the lipid A portion of LPS and blocks its recognition by host cells (Schilling et al., 2001). The *E. coli* strain we selected for our studies was a well characterized laboratory strain of *E. coli*, ORN103(pSH2), expressing recombinant type 1 fimbriae, including the adhesive subunit, FimH. We employed this laboratory strain rather than a UPEC strain because UPEC strains express multiple genes capable of suppressing cytokine responses in BECs (Hunstad et al., 2005). We observed a strong IL-6 response from BECs following exposure to the laboratory *E. coli* that was significantly reduced following pretreatment of the bacteria with PMB (Fig. 2.1A). For comparative purposes, shown in Fig. 2.1A is the PMB mediated inhibition of the IL-6 responses of BECs to soluble *E. coli* LPS. Due to the



**Figure 2.1**

**Figure 2.1: IL-6 response of BECs to type 1 fimbriated *E. coli* is largely elicited by LPS and involves TLR4.** **A**, IL-6 secretion by BECs in response to *E. coli* (100 MOI) or purified LPS (100 µg/ml). When specified, *E. coli* and purified LPS were pretreated with 1 µg/ml polymyxin B (PMB) for 30 min. \*\*  $P < 0.001$  relative to values of untreated (UT) BECs; \* $P < 0.03$  relative to *E. coli* (EC) or LPS treated BECs. **B**, RT-PCR of control-transfected BECs (Ctrl) and TLR4 knockdown BECs (KD). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a loading control. **C**, IL-6 secretion of control-transfected BECs (Ctrl) and TLR4 KD BECs after *E. coli* and LPS stimulation. \*\* $P < 0.05$  relative to UT control; \* $P < 0.05$  relative to *E. coli* (EC) treated control or LPS control.

possibility of lipoprotein contamination of LPS prepared by TCA or PCP extraction, LPS ultra purified by ion-exchange chromatography and verified to contain <1% protein was used in this study (Sigma; *E. coli* 055:B5 LPS). To confirm that the LPS on *E. coli* was the primary determinant responsible for activating BECs, we sought to show that the activation of BECs involved TLR4, the signaling receptor for LPS. Using RNA interference techniques we generated BECs where expression of TLR4 was appreciably knocked down. Densitometric quantification of message levels in the knockdown (KD) BECs revealed that the expression of TLR4 was reduced by 49% (Fig. 2.1B). Shown in Fig. 1C is the IL-6 response of control (transfected with control vector) BECs and of the KD BECs to *E. coli* and LPS. Compared to control BECs, significant reduction in the IL-6 response to both *E. coli* and LPS was observed with the knock down cells (Fig. 2.1C). For the most part, the reduction in the IL-6 response paralleled the degree of knock down of TLR4 in the BECs (Fig. 2.1B-C). Taken together, these data confirm that LPS is the primary determinant on *E. coli* responsible for triggering the IL-6 response and the intracellular signaling triggered by LPS involves TLR4 on BECs.

### **2.3.2 IL-6 response of BECs to *E. coli* is preceded by an increase in intracellular Ca<sup>2+</sup>**

Since intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) has been implicated in important cellular processes including IL-6 secretion (Song et al., 2001; Uhlen et al., 2000), we examined the involvement, if any, of this second messenger in the IL-6 response of BECs to *E. coli*. We

investigated if exposure of BECs to *E. coli* induced an increase in  $[Ca^{2+}]_i$  by performing ratiometric imaging on Fura-2/AM-loaded 5637 BECs. A unique pattern of  $Ca^{2+}$  influx into exposed BECs to *E. coli* was observed (Fig. 2.2A). BEC  $[Ca^{2+}]_i$  was constant before bacterial exposure and increased rapidly, within 1 minute, after *E. coli* exposure returning to baseline levels within 5 min (Fig. 2.2A). To determine whether the *E. coli* induced increase of  $[Ca^{2+}]_i$  was essential for the BEC IL-6 response to bacterial exposure, we examined IL-6 secretion by BECs following bacterial exposure with or without pretreatment with  $NiCl_2$ , a general  $Ca^{2+}$  channel inhibitor (Belmeguenai et al., 2003), or BAPTA-AM, an intracellular  $Ca^{2+}$  chelator (Bissonnette et al., 1994) (Fig. 2.2B). Whereas BEC IL-6 secretion was readily induced after exposure to *E. coli*, pretreatment of BECs with  $NiCl_2$  or BAPTA-AM before bacterial exposure completely abolished IL-6 secretion by exposed BECs to *E. coli*. In addition, a general inducer of calcium influx, ionophore A23187, was able to directly induce BEC IL-6 production in the absence of *E. coli*, demonstrating the importance of  $[Ca^{2+}]_i$  increases in initiating this response.

We also investigated whether purified *E. coli* LPS was capable of inducing an increase in  $[Ca^{2+}]_i$  in BECs. LPS was seen to induce a similar, but delayed, increase in  $[Ca^{2+}]_i$  compared to that caused by *E. coli* (Fig. 2.2C and 2.2A). The LPS-induced  $[Ca^{2+}]_i$  peak occurred ~5 min after the addition of 100  $\mu$ g/ml LPS. Disrupting the LPS-induced  $[Ca^{2+}]_i$  increase with  $NiCl_2$  or BAPTA-AM pretreatment before LPS exposure greatly

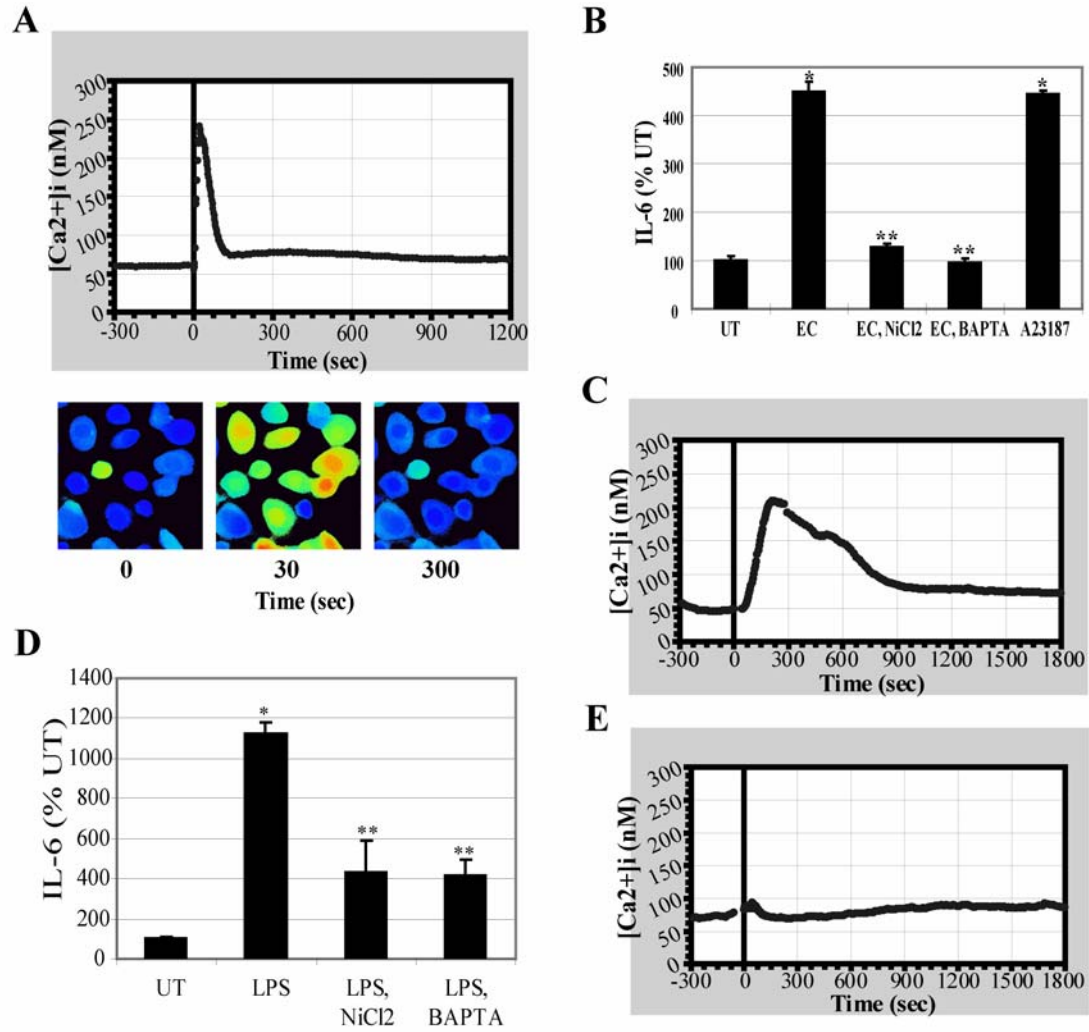


Figure 2.2

**Figure 2.2: IL-6 response of BECs to *E. coli* is preceded by an increase in  $[Ca^{2+}]_i$ .** **A**,  $[Ca^{2+}]_i$  tracing of BECs before and after *E. coli* exposure. *E. coli* was added at time 0. **B**, BEC IL-6 responses after *E. coli* exposure in the absence or presence of  $NiCl_2$  (2mM) or BAPTA-AM (5  $\mu$ M), or after calcium ionophore A23187 (1  $\mu$ M) treatment without bacterial exposure. \*  $P < 0.001$  relative to untreated (UT) BECs; \*\*  $P < 0.001$  relative to *E. coli* (EC) treated BECs. **C** and **E**, BEC  $[Ca^{2+}]_i$  tracing before and after purified LPS treatment (C) or polymyxin B pretreated LPS treatment (E). **D**, BEC IL-6 responses following exposure to LPS in the absence or presence of  $NiCl_2$  or BAPTA-AM. \*  $P < 0.001$  relative to UT BECs; \*\*  $P < 0.01$  relative to LPS treated BECs.

reduced IL-6 production by BECs (Fig. 2.2D). Pretreatment of LPS with PMB almost completely abrogated the  $[Ca^{2+}]_i$  response of BEC (Fig. 2.2E). Taken together, these observations provide strong evidence indicating that the IL-6 response of BECs to *E. coli* involves a sharp increase in  $[Ca^{2+}]_i$  levels. Although LPS appears to be the primary bacterial component responsible for elevation of  $[Ca^{2+}]_i$ , bacteria associated LPS evoked a faster  $[Ca^{2+}]_i$  response in BECs compared to soluble LPS.

### **2.3.3 IL-6 response of BECs to *E. coli* is associated with a significant increase in intracellular cAMP levels**

Intracellular cAMP is an important second messenger in several signaling pathways, including IL-6 response (Chio et al., 2004; Yadav et al., 2004; Zhang et al., 2002). Exposing BECs to *E. coli* for 1 hr demonstrated a 2.7-fold increase in intracellular cAMP, which was blocked by inhibiting adenylyl cyclase (AC) activity with the compound MDL-12,330A (MDL) (Fig. 2.3A). The increase in intracellular cAMP following bacterial exposure was dependent on both bacteria-associated LPS and an increase in  $[Ca^{2+}]_i$  as shown, respectively, by pretreating the bacteria with PMB or pretreating the BECs with  $NiCl_2$  (Fig. 2.3B). This *E. coli* induced  $[Ca^{2+}]_i$ -dependent cAMP production was found to be an important step in the cytokine response of BECs to bacterial exposure, since inhibition of ACs with MDL reduced BEC IL-6 expression by ~75% (Fig. 2.3C). In addition, a membrane-permeable cAMP analog, dibutyryl cAMP (dbcAMP), induced a greater than 3-fold increase in BEC IL-6 production in the absence

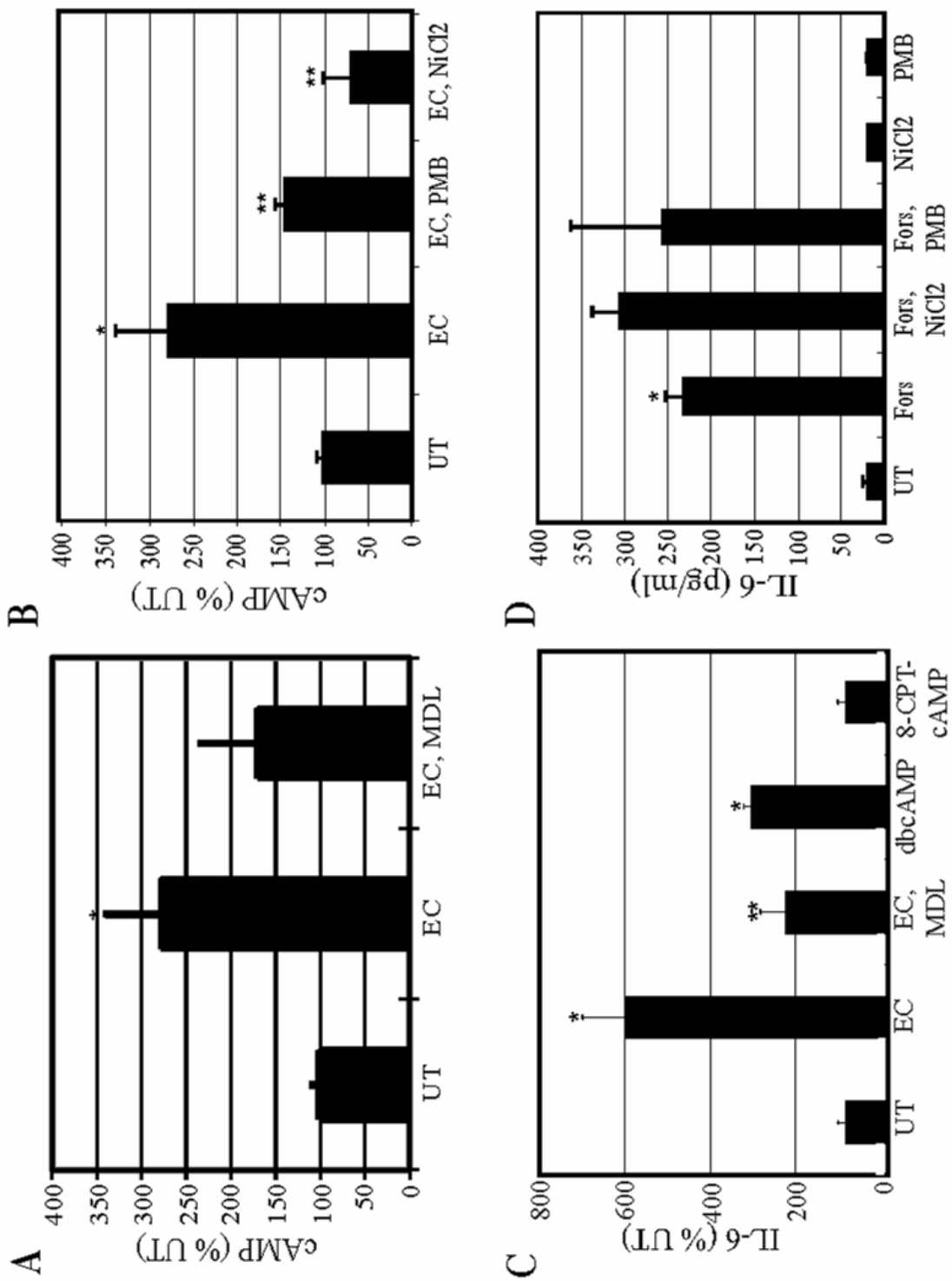


Figure 2.3

**Figure 2.3: IL-6 response of BECs to *E. coli* is associated with a significant increase in intracellular cAMP levels.** **A and B**, BEC cAMP production before and after *E. coli* exposure. When specified, BECs were pretreated with MDL-12,330A (0.4 mM) or NiCl<sub>2</sub> for 30 min, or *E. coli* was pretreated with polymyxin B (PMB) for 30 min. \**P*<0.03 relative to untreated (UT) BECs; \*\**P*<0.03 relative to *E. coli* (EC) treated BECs. **C**, IL-6 secretion by BECs was measured 6 hr after exposure to *E. coli*, in the absence (EC) or presence of MDL-12,330A (EC, MDL), or after 6 hr of treatment with 1 mM dibutyryl cAMP (dbcAMP), or 1 mM (8-(4-chloro-phenylthio)-2'-O-methyladenosine-3'-5'-cyclic monophosphate (8-CPT-cAMP) without bacterial exposure. \**P*<0.01 relative to UI; \*\**P*<0.02 relative to EC. **D**, IL-6 secretion by BECs incubated for 6 hr in the absence (UT) or presence of the AC-activator forskolin (50 μM) with or without NiCl<sub>2</sub> or PMB, or incubated with NiCl<sub>2</sub> or PMB in the absence of forskolin. \**P*<0.01 relative to untreated (UT) BECs.

of bacterial exposure, demonstrating the importance of intracellular cAMP in inducing BEC IL-6 production. However, a membrane-permeable cAMP analog (8-CPT-cAMP) that does not activate the classical cAMP-target protein, protein kinase A (PKA), but only activates the recently discovered cAMP-target protein Epac (Exchange protein activated by cAMP) (Enserink et al., 2002), did not induce the production of BEC IL-6, indicating that PKA is involved in the downstream induction of IL-6 production by exposed BECs to *E. coli* (Fig. 2.3C). Forskolin activates ACs, the enzymes that produce intracellular cAMP, by a direct mechanism (Insel and Ostrom, 2003), which should bypass the need for an increase in  $[Ca^{2+}]_i$  that is observed with *E. coli* induced intracellular cAMP production. As shown in Fig. 2.3D, direct activation of AC by forskolin led to a dramatic production of IL-6 that was not inhibited by  $NiCl_2$ , indicating that the increase in  $[Ca^{2+}]_i$  that occurred after *E. coli* exposure preceded the production of intracellular cAMP. Neither  $NiCl_2$  nor PMB treatment affected forskolin induced IL-6 production, demonstrating that these agents did not have a detrimental effect on protein synthesis in general (Fig. 2.3D). Thus, the IL-6 response to *E. coli* evoked by BECs involves another secondary messenger, cAMP, which acts downstream of the  $Ca^{2+}$  response.

### **2.3.4 AC-3 is responsible for mediating *E. coli* induced cAMP in BECs**

Because there are currently 10 known isoforms of mammalian ACs (Sunahara and Taussig, 2002), it was of interest to determine which AC was responsible for the *E. coli*-induced increase of intracellular cAMP in BECs. First, we determined which AC isoforms were actually expressed in BECs. RT-PCR was performed on total cellular RNA, using primers specific for each known AC isoform and only mRNA for AC isoforms 3, 4, 6, and 7 was detectable in BECs (Fig. 2.4A). We confirmed that the other AC isotype-specific primers used were functional by undertaking RT-PCR on total RNA from human embryonic kidney (HEK) cells, positive control cells, where all ACs except AC4 and 8 were expressed (Ludwig and Seuwen, 2002) (data not shown). RNAi was utilized to minimize the expression of each AC, which was verified by AC isotype-specific RT-PCR (Fig. 2.4B). Following *E. coli* exposure, intracellular cAMP, as well as IL-6 secretion, rose significantly in all of the KDs, except for the KD of AC-3, indicating that AC-3 is the BEC AC isoform linked to the IL-6 response following *E. coli* exposure (Fig. 2.4C and D). It is noteworthy that of the four AC isoforms expressed by BECs, only AC-3 is known to be activated by increases in  $[Ca^{2+}]_i$  (Choi et al., 1992; Cooper et al., 1994). The knockdown of AC-3 also abrogated the production of intracellular cAMP (Fig. 2.4E) and expression of IL-6 (Fig. 2.4F) following exposure of BECs to purified LPS. Interestingly, the AC-3 KD BECs, forskolin-induced IL-6 expression was largely unaffected (Fig. 2.4F). The appreciable IL-6 response to forskolin suggested that a general increase in

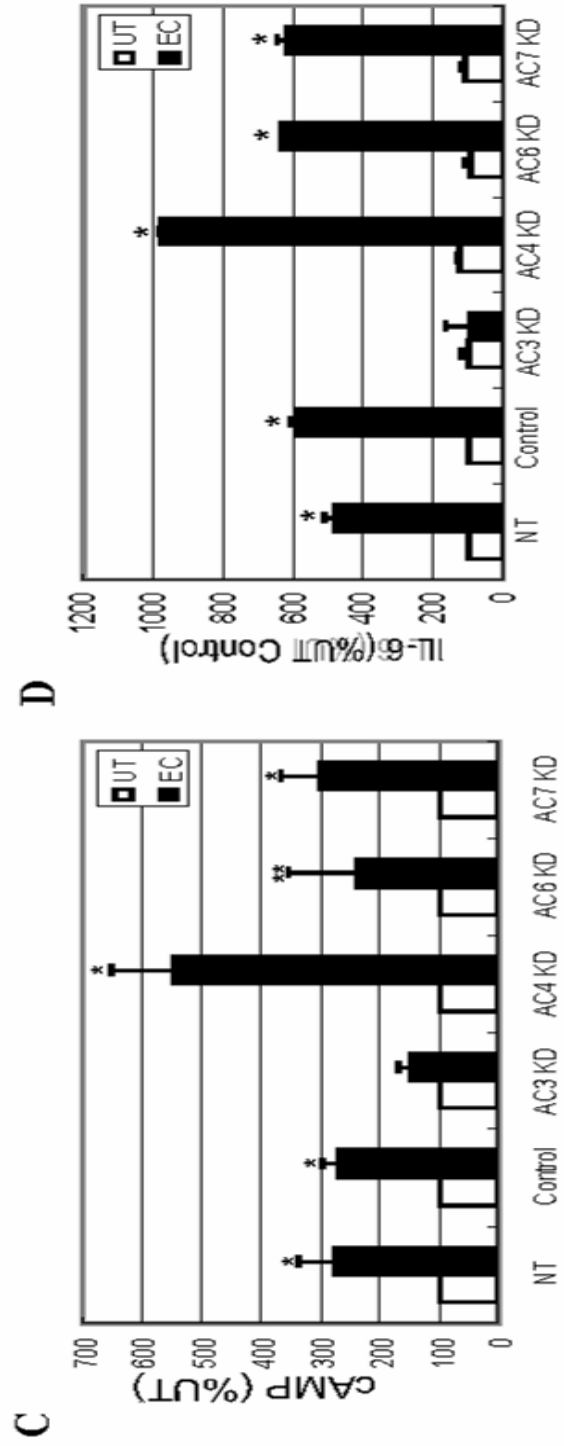
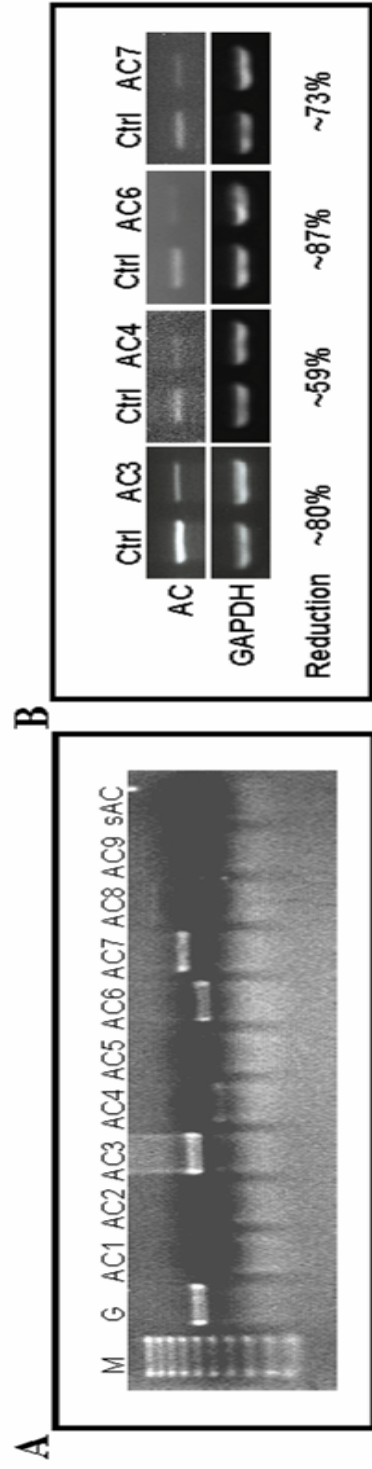


Figure 2.4

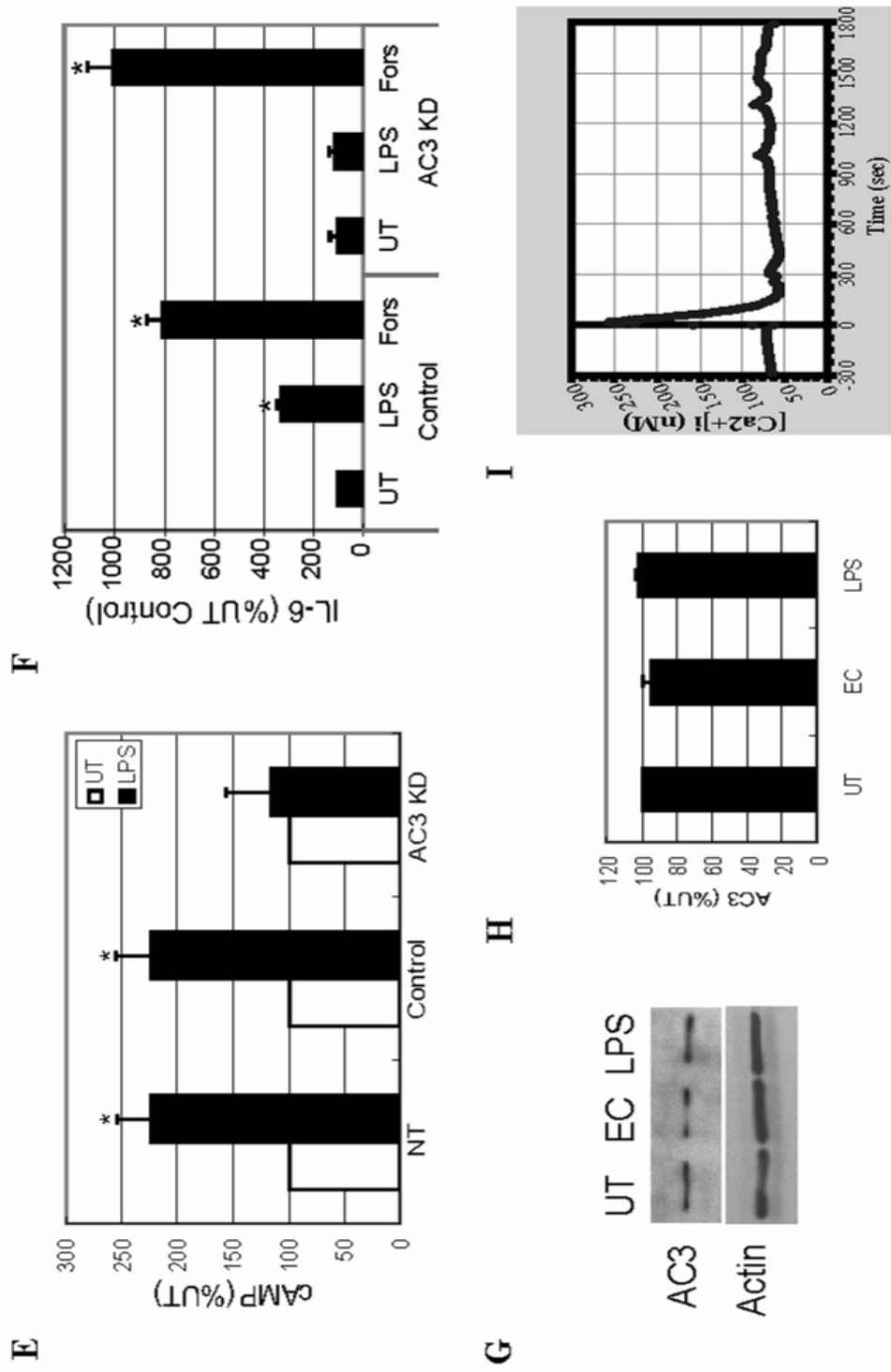


Figure 2.4 continued

**Figure 2.4: AC-3 is responsible for mediating *E. coli* induced cAMP production in BECs.** **A**, RT-PCR of BECs using primers specific for the 10 known mammalian AC isoforms. Only AC-3, AC-4, AC-6, and AC-7 mRNA was expressed. GAPDH-specific RT-PCR (Lane G) was used as a loading control. **B**, RT-PCR of control-transfected BECs (Ctrl) and AC-3, -4, -6, or -7 KD BECs. GAPDH-specific RT-PCR was used as a loading control. **C** and **D**, Intracellular cAMP production (**C**) and IL-6 secretion (**D**) by non-transfected BECs (NT), control-transfected BECs (Control), or AC-3, -4, -6 or -7 KD BECs left untreated (UT) or treated with *E. coli* (EC). \* $P < 0.005$  and \*\* $P < 0.02$  relative to respective UT values. **E** and **F**, Intracellular cAMP production (**E**) and IL-6 secretion (**F**) by non-transfected BECs (NT), control-transfected BECs (Control), or AC-3 KD BECs were measured in the absence (UT) or presence of *E. coli* LPS (LPS), or presence of forskolin (Fors) without LPS. \* $P < 0.003$  relative to respective UT values. **G**, BEC AC-3-specific Western blot before (UT) and after (EC) *E. coli* exposure for 1 hr, or exposure to *E. coli* LPS for 6 hr (LPS). An actin-specific Western blot was used as a loading control. **H**, Densitometric analysis of AC-3-specific Western blots, using ImageJ software. **I**,  $[Ca^{2+}]_i$  tracing in AC-3 KD BECs before and after *E. coli* exposure. *E. coli* was added at time 0.

intracellular cAMP was sufficient to signal IL-6 secretion in BECs. The absence of any reduction in the IL-6 response to forskolin in AC-3 KD BECs is attributable to the presence of other isoforms of ACs in these cells, which were directly activated by forskolin. Remarkably, when AC-3-specific Western blotting was performed on BECs before and after bacterial exposure or exposure to purified LPS, no discernible increase in the expression of AC-3 protein was observed, indicating that an increase in activity, rather than expression, of AC-3 occurred following infection (Fig. 2.4G and H). Finally, when we examined for increase in  $[Ca^{2+}]_i$  in the AC-3 KD BECs following exposure to *E. coli*, we found that it was comparable to that seen in wild-type (WT) BECs (Fig. 2.4I), which is consistent with the idea that the rise in  $[Ca^{2+}]_i$  preceded any rise in intracellular cAMP.

### **2.3.5 cAMP mediated phosphorylation of the CREB**

Next, we sought to connect the  $Ca^{2+}$  and cAMP dependent signaling events described in this study to the classical NF- $\kappa$ B associated signaling pathway mediated by TLR4. To link cAMP to the classical pathway we examined its effects on the translocation of the transcriptional factor, NF- $\kappa$ B, from the cytoplasm to the nucleus following bacterial exposure. Remarkably, when we examined control-transfected BECs and AC-3 KD BECs 1 hr following exposure to *E. coli* (which corresponds to the time we observed significant secondary messenger responses) for nuclear translocation, we

found little or no translocation of NF- $\kappa$ B in either cell type (Fig. 2.5A). However, when we increased the incubation time to 2 hrs following exposure to *E. coli* we detected a marked increase in translocation of NF- $\kappa$ B in control transfected BECs but an identical increase was also seen in AC-3 KD BECs (Fig. 2.5B). This finding revealed that (i) the cAMP responses in BECs preceded the nuclear translocation of NF- $\kappa$ B by significant amounts of time, and (ii) these cAMP responses did not appear to impact the NF- $\kappa$ B associated signaling pathway. These observations raised the intriguing possibility that the secondary messengers such as cAMP may be acting via an independent pathway. To see whether, the regulatory effect of cAMP on the IL-6 response was at the transcriptional level, we compared IL-6 mRNA levels in WT BECs, control-transfected BECs, and AC-3 KD BECs before and 1 hr after exposure to *E. coli*. We observed marked increase in IL-6 mRNA in WT BECs and control-transfected BECs but not in AC-3 KD BECs (Fig. 2.5C) indicating that the AC-3 mediated elevation in intracellular cAMP was regulating the IL-6 response at the transcriptional level. It is pertinent to also note the time frame of when these assays were undertaken. Here, mRNA for IL-6 was detected in control transfected BECs as early as 1 hr after exposure to *E. coli* (Fig. 2.5C).

Considering that nuclear translocation of NF- $\kappa$ B was detectable only after 2hr (Fig. 2.5B), this cAMP regulated pathway appears to be activated sooner than the classical pathway. Interestingly, when we examined AC-3 KD BECs for IL-6 mRNA 6hrs after exposure to *E.coli*, we detected similar amounts of message in AC3 KD BECs and control transfected

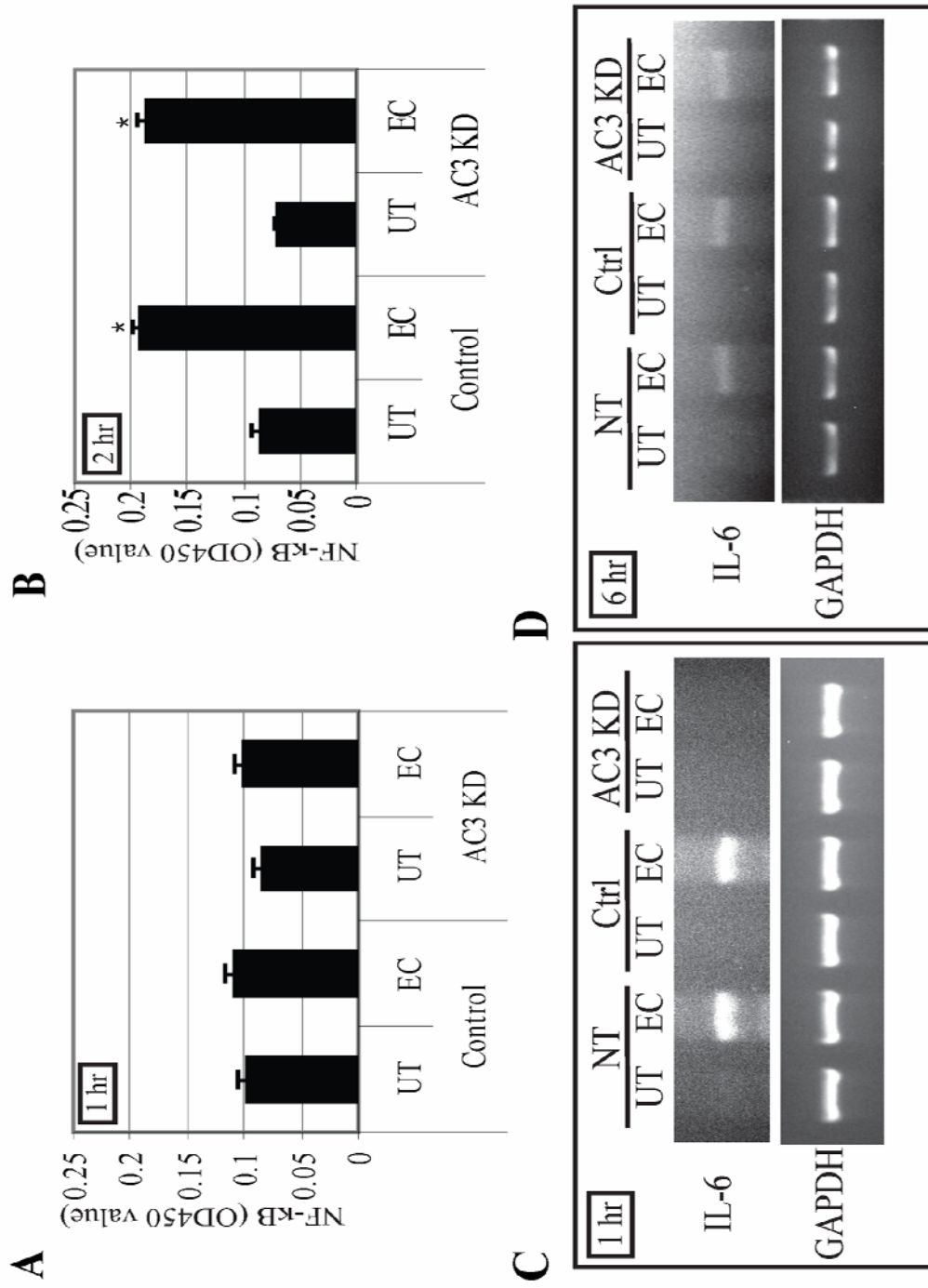


Figure 2.5

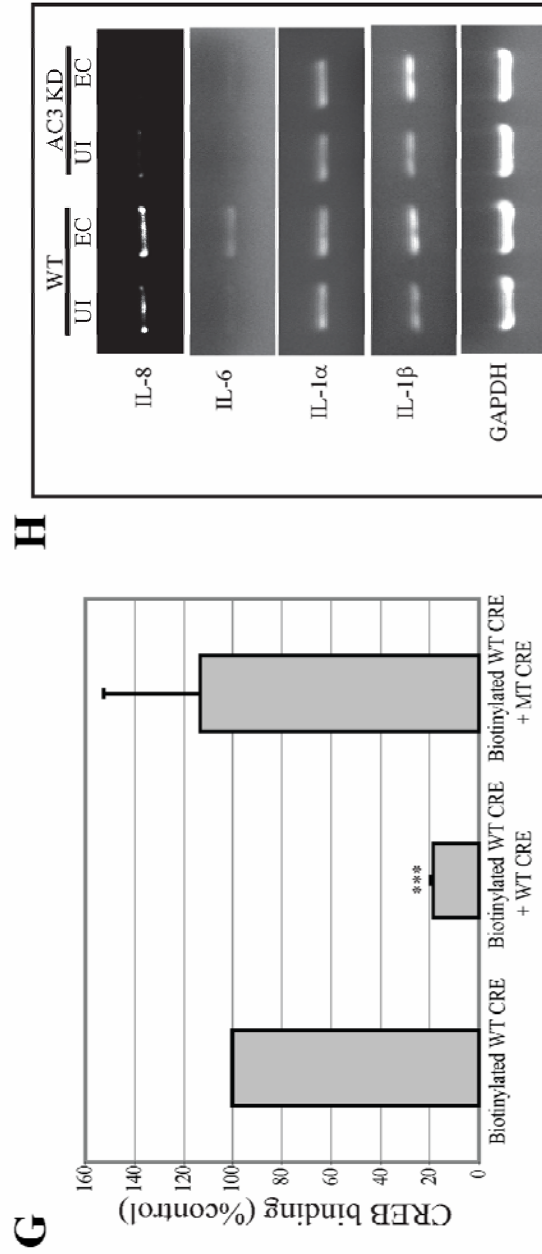
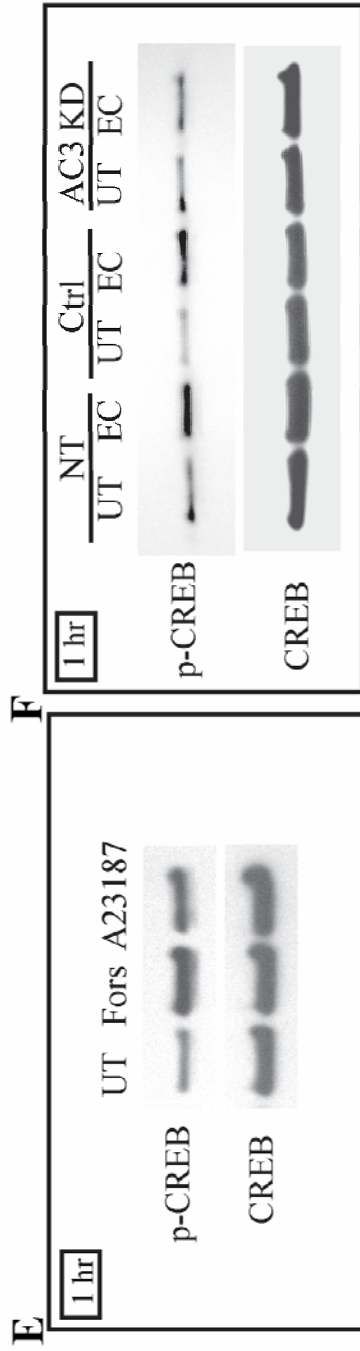


Figure 2.5 continued

**Figure 2.5: cAMP mediated phosphorylation of CREB and binding of CREB to CRE oligonucleotides.** **A and B**, NF- $\kappa$ B nuclear translocation in control-transfected BECs and AC-3 KD BECs before and after *E. coli* exposure for 1 hr (A) or 2 hr (B). **C and D**, IL-6 message levels in non-transfected (NT), control-transfected (Ctrl), and AC-3 KD BECs before and after *E. coli* exposure for 1 hr (C) or 6 hr (D) as measured by RT-PCR. **E**, Western blot of CREB phosphorylation levels in untreated (UT), and forskolin (Fors) or calcium ionophore A23187 (A23187) treated BECs. The treatment was for 1 hr. **F**, Western blot showing CREB phosphorylation of NT, Ctrl, and AC3 KD BECs before and after 1hr *E. coli* exposure. **G**, CREB binding to CRE site of the IL-6 promoter. Nuclear extracts of BECs exposed for 1 hr to *E. coli* ORN103(pSH2) were incubated with biotinylated wild-type CRE oligonucleotides in the absence (Biotinylated WT CRE) or presence of specific (Biotinylated WT CRE + WT CRE) or non-specific (Biotinylated WT CRE + MT CRE) oligonucleotide competitors. \*\*\* $P < 0.0001$ . **H**, Expression analysis of mRNA levels of various genes with CRE sites in their promoter. WT and AC3 KD BECs were incubated for 1 hr with *E. coli* ORN103(pSH2) and then total RNA was collected from untreated (UI) and bacteria treated BECs (EC) and subjected to RT-PCR. GAPDH was used as a loading control.

BECs (Fig. 2.5D) indicating that the classical NF- $\kappa$ B mediated pathway was still functional in AC3 KD BECs. Thus, the IL-6 response in BECs appears to originate from two distinct pathways: the NF- $\kappa$ B associated pathway and a separate but speedier pathway involving Ca<sup>2+</sup> and cAMP. One mechanism through which cAMP may directly affect transcription of IL-6 is by promoting phosphorylation of CREB which binds to CRE in IL-6 promoter region (Dendorfer, 1996). An increase in intracellular cAMP levels activates PKA whose catalytic subunits enter the nucleus and phosphorylates CREB (Shaywitz and Greenberg, 1999). Upon phosphorylation, CREB promotes the recruitment of various transcriptional co-activators which promote transcription of target genes with consensus sites for CREB, such as IL-6 (Andrisani, 1999; Mori et al., 1994). When we examined for CREB phosphorylation in BECs exposed to forskolin and calcium ionophore, A23187, two potent elevators of intracellular cAMP, we observed a marked increase in CREB phosphorylation on the western blots (Fig. 2.5E) consistent with the idea that CREB phosphorylation occurred following elevation of intracellular cAMP levels. To see if bacterial exposure also triggered phosphorylation of CREB, CREB protein from extracts of BECs before and after exposure to *E. coli* was probed for phosphorylation. Following *E. coli* exposure, we found an appreciable increase in phosphorylation of CREB in non-transfected BECs, and control transfected BECs, but not in AC-3 KD BECs (Fig. 2.5F). To extend these findings, we investigated the binding of CREB to the CRE sites on the IL-6 promoter region. Nuclear extracts of BECs were

obtained after 1hr incubation with *E. coli* ORN103(pSH2) and then incubated with the biotinylated oligonucleotides corresponding to CRE on the IL-6 promoter. Binding of CREB to CRE was assessed by a colorimetric assay. We found that CREB bound to CRE oligonucleotides but not to a scrambled oligonucleotide sequence of identical length (Fig. 2.5G). Thus, cAMP appears to be modulating IL-6 responses through the binding of the phosphorylated transcriptional factor, CREB to the CRE site on the IL-6 promoter region.

Since there are other inflammatory mediators such as IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 with consensus CRE sites in their promoter region that have known to be activated during UTI (Franz and Horl, 1999; Hunstad et al., 2005; Samuelsson et al., 2004), we examined if production of any of these mediators was modulated by the cAMP/CREB pathway following exposure to *E. coli*. We compared message levels for IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 in WT and AC3 KD BECs after 1 hr exposure to *E. coli* ORN103(pSH2). We found that mRNA levels for IL-8 but not IL-1 $\alpha$  or IL-1 $\beta$  appeared to be regulated by TLR4/cAMP/CREB pathway. Thus in addition to IL-6, production of IL-8 by BECs appears to be under the regulation of the novel signaling pathway.

### **2.3.6 The IL-6 response to *E. coli* of BECs is the product of two separate signaling pathways**

Based on the evaluation of transcriptional messages, the IL-6 response of BECs is mediated by two separate signaling pathways with different expression kinetics. To verify this observation, we compared the kinetics of IL-6 secretion in WT and AC3 KD BECs following exposure to *E. coli*. We found that whereas appreciable IL-6 secretion (arbitrarily defined as 4-fold over unstimulated controls) was observed as early as 6 hours in WT BECs, a comparable amount of IL-6 was only produced in AC3 KD BECs after about 9 hr (Fig. 2.6A). By 12 hrs, however, the amounts of IL-6 secretion were not significantly different between both cell types suggesting that the IL-6 responses of AC3 KD BECs eventually caught up to that of the WT BECs (Fig. 2.6A). A similar profile was obtained when we substituted the laboratory *E. coli* strain with a UPEC strain CI5 (Fig. 2.6B). To assess the relative contribution of the cAMP mediated pathway to the IL-6 response of BECs, we examined *E. coli* elicited IL-6 responses of WT BECs after selective inhibition of the NF- $\kappa$ B pathway with pyrrolidine dithiocarbamate (PDTC) (Liu et al., 1999b). This agent has been reported not to inhibit CREB activity (Liu et al., 1999a). We found that although the early kinetics of the IL-6 responses were not significantly different from untreated WT BECs, the amounts of IL-6 secreted, especially by the 12 hr period of incubation, was significantly reduced. Thus, while the cAMP/CREB mediated

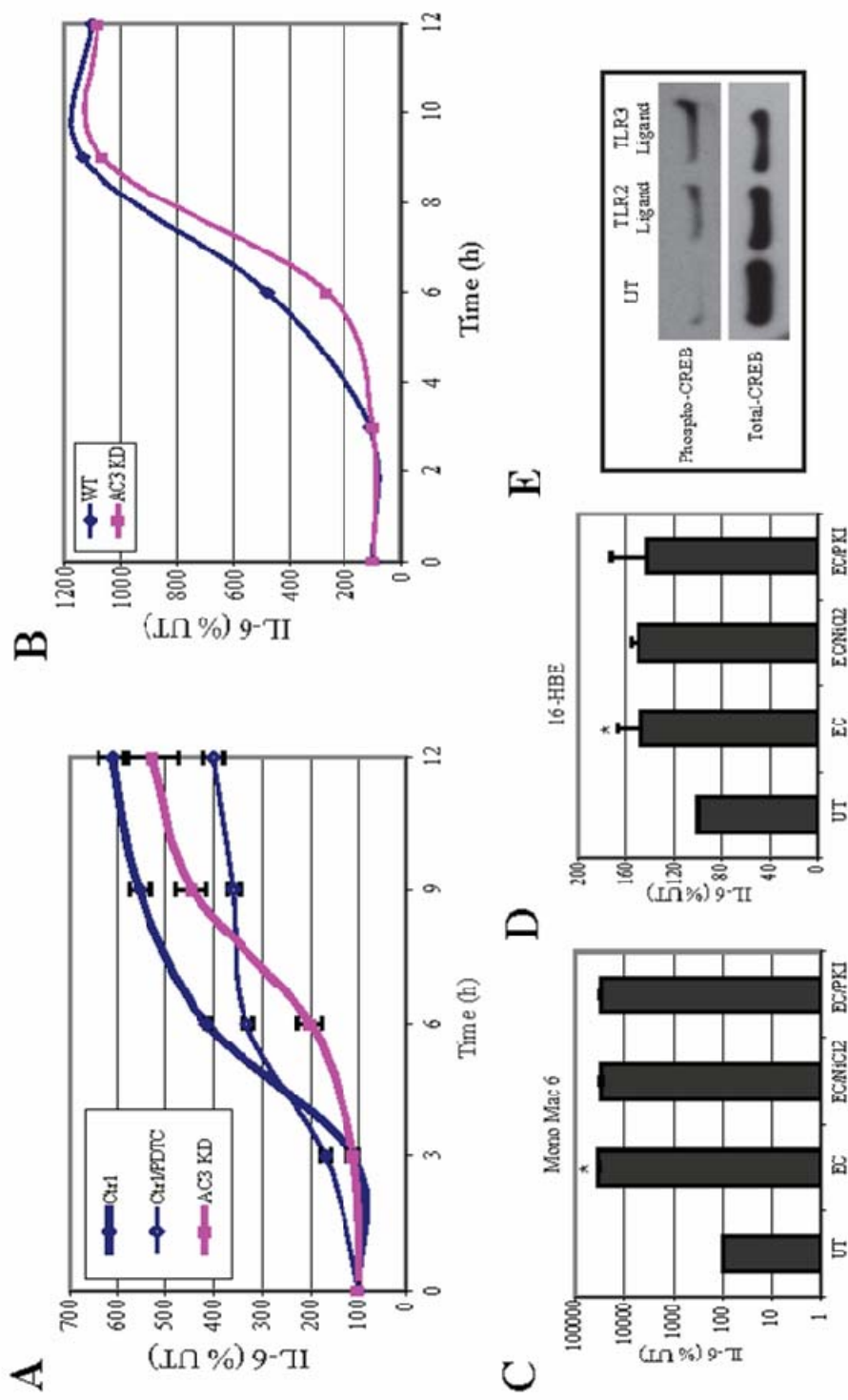


Figure 2.6

**Figure 2.6: The IL-6 response to *E. coli* of BECs is the product of two separate signaling pathways.** **A**, IL-6 secretion response to *E. coli* ORN103(pSH2) by WT and AC-3 KD BECs in the absence or presence of pyrrolidine dithiocarbamate (PDTC). **B**, IL-6 secretion response to UPEC CI5 by WT and AC-3 KD BECs. **C and D**, IL-6 secretion responses by Mono Mac 6 cell line (C) and 16-HBE cell line (D). Cells were treated for 12 hrs with *E. coli* ORN103(pSH2) in the absence (EC) or presence of NiCl<sub>2</sub> or PKI. \**P*<0.03 relative to untreated (UT) value. Identical results were obtained after 6 hrs of treatment with *E. coli* ORN103(pSH2). **E**, Increased CREB phosphorylation in BECs in response to TLR2 and TLR3 ligands. Western blotting for phospho-CREB and total-CREB in untreated BECs (UT) or BECs treated with either a TLR2 ligand (lipoteichoic acid) or a TLR3 ligand (polyinosine-polycytidylic acid).

IL-6 response was an early one, the amounts of IL-6 generated by this pathway were significantly less than that produced by the classical NF- $\kappa$ B associated pathway.

Additionally, we examined whether the IL-6 responses to *E. coli* mediated by other human cells involved the two second messengers, Ca<sup>2+</sup> and cAMP. Monolayers of the human monocytic cell line, Mono Mac 6, and the human bronchial epithelial cell line, 16-HBE, were exposed to *E. coli* ORN103(pSH2) as before, in presence of inhibitors of either Ca<sup>2+</sup> response or cAMP response and IL-6 secretion was measured. We found that whereas both cell lines evoked appreciable IL-6 responses to *E. coli* neither one of these responses were reduced by inhibitors of calcium (NiCl<sub>2</sub>) or cAMP (protein kinase A inhibitor PKI) signaling (Fig. 2.6C and D). Thus, the two secondary messengers, Ca<sup>2+</sup> and cAMP, appear to be important mediators of the IL-6 responses in BECs but not in other cell types.

Since BECs express other TLRs such as TLR2 and TLR3 (Backhed et al., 2001; Schilling et al., 2001), it was of interest to investigate whether known ligands for TLR2 and TLR3 also triggered the cAMP/CREB pathway. We assessed the phosphorylation levels of CREB before and 6hr after exposure to lipoteichoic acid (TLR2 ligand) or polyinosine-polycytidylic acid (TLR3 ligand) and found that both TLR ligands induced

significant phosphorylation of CREB (Fig. 2.6E). Thus, the CREB pathway appears to be activated by TLRs other than TLR4.

### **2.3.7 Involvement of intracellular Ca<sup>2+</sup> and cAMP in the IL-6 response of primary human BECs to UPEC**

Since the secondary messenger/CREB pathway was detectable only in immortalized human BECs it was important to validate our observation of the existence of a cAMP/CREB pathway in primary human BECs. Therefore, we investigated if freshly isolated and cultured human BECs would secrete IL-6 through a Ca<sup>2+</sup> and cAMP dependent mechanism, when exposed to UPEC strain CI5 (Abraham et al., 1985). We cultured primary bladder cells obtained from fresh bladder biopsies as described previously (Cilento et al., 1994). These cells exhibited characteristics of primary BECs including expression of uroplakin 1a, a marker of the asymmetrical unit membrane, the junctional complex protein ZO1, as well as cytokeratin, all of which are hallmarks of terminal differentiation in bladder umbrella cells (data not shown). We observed that, after the stimulation with UPEC strain CI5, primary BECs exhibited elevation in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2.7A). The intensity of the response was lower than that observed with the laboratory strain of *E. coli* (data not shown). This is consistent with the fact that UPEC express multiple virulence factors, some of which exhibit disparate effects on [Ca<sup>2+</sup>]<sub>i</sub> levels (Uhlen et al., 2000). As demonstrated previously with 5637 BECs, this response was significantly abrogated following pretreatment with the general Ca<sup>2+</sup> channel

blocker, NiCl<sub>2</sub> (Fig. 2.7B). Significant elevation in intracellular cAMP levels and IL-6 secretion was observed in primary BECs following exposure to UPEC (Fig. 2.7C and D). Both the increase in intracellular cAMP levels and IL-6 secretion were inhibitable by NiCl<sub>2</sub>, once again confirming the importance of [Ca<sup>2+</sup>]<sub>i</sub> to the BEC IL-6 response (Figs. 2.7C and D). Also shown in Fig. 2.7D is the IL-6 response of these primary cells to the laboratory *E. coli* strain ORN103. Notice that it is markedly higher than the response to the UPEC strain. Taken together, these findings support the notion that although the IL-6 response of primary BECs to UPEC is dampened, it involves a Ca<sup>2+</sup> and cAMP-dependent mechanism.

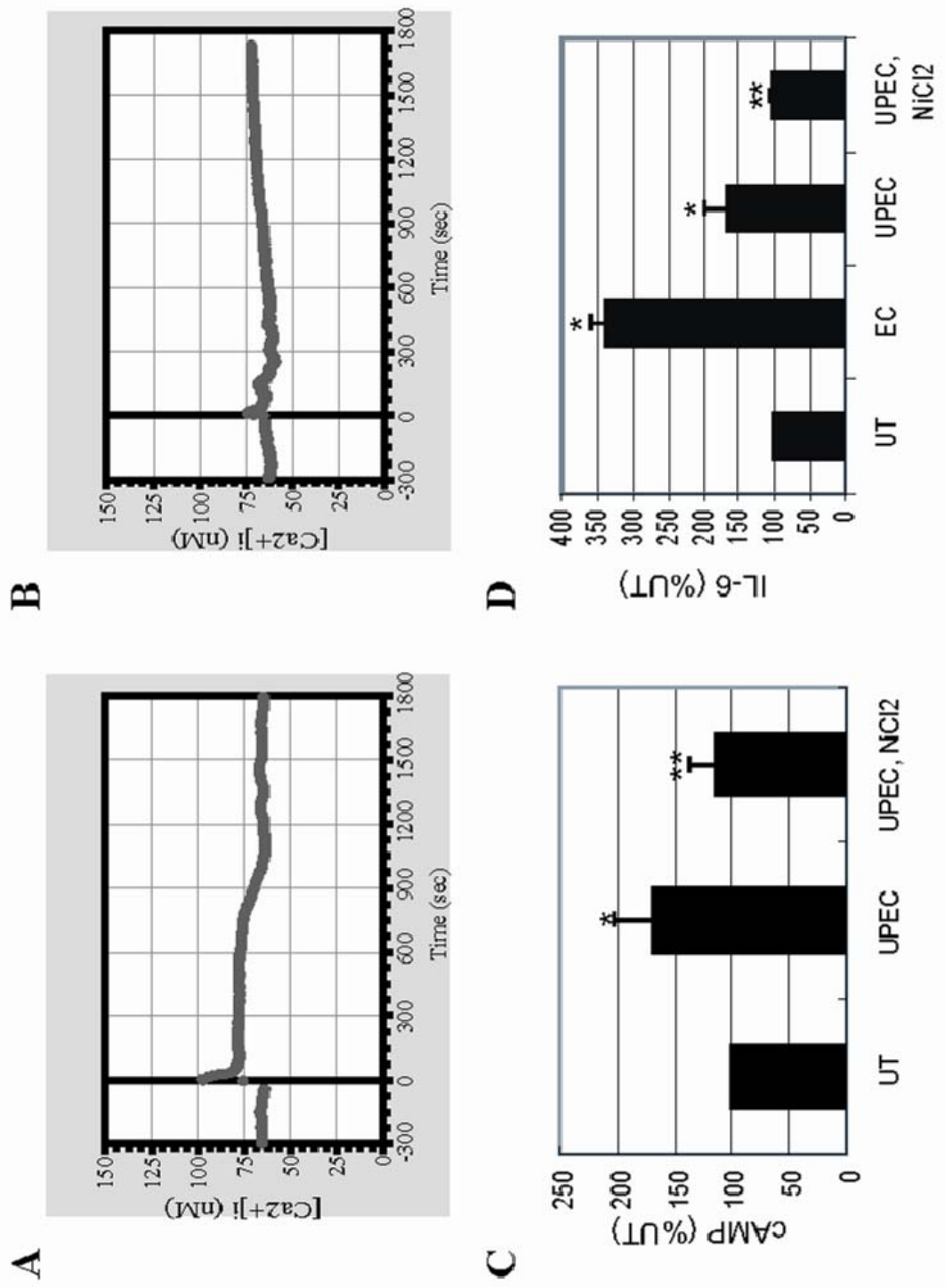


Figure 2.7

**Figure 2.7: The IL-6 response of primary human BECs to UPEC is linked to intracellular Ca<sup>2+</sup> and cAMP increase.** **A**, [Ca<sup>2+</sup>]<sub>i</sub> tracing before and after exposure of primary human BECs to UPEC strain CI5. **B**, [Ca<sup>2+</sup>]<sub>i</sub> tracing of the primary human BECs pretreated with NiCl<sub>2</sub> for 30 min. UPEC was added at time 0. **C** and **D**, Intracellular cAMP production (C), or IL-6 secretion (D) by the primary human BECs left untreated (UT) or treated with *E. coli* CI5 (UPEC) in the absence or presence of NiCl<sub>2</sub> (UPEC, NiCl<sub>2</sub>), or treated with *E. coli* ORN103 (EC). \**P*<0.03 relative to UT value. \*\**P*<0.03 relative to UPEC (CI5) treated value.

## **2.4 Discussion**

The bladder and the upper urinary tract are typically sterile which is attributable, at least in part, to the highly efficient immune system monitoring these sites. One of the principal effectors of immune-surveillance is the epithelial cell lining the urinary tract (Hedlund et al., 2001; Schilling et al., 2003). In addition to serving as a barrier against urine, BECs function as first responders, mobilizing multiple innate immune responses against microorganisms. Mediating microbial recognition on the surfaces of epithelial cells are PRRs which recognize specific microbial products and activate intracellular signaling events leading to secretion of various inflammatory and immunoregulatory cytokines (Backhed et al., 2001). That epithelial cells possess PRRs such as TLR4 and contribute to immune surveillance has only recently been recognized (Schilling et al., 2001; Svanborg et al., 2006). For a long time it was assumed that PRRs were exclusively found on immune cells of hematopoietic origin and, therefore, most of our current information regarding PRR mediated signal transduction is largely based on these cells (Akira et al., 2006; Pulendran et al., 2001). Although there is no conclusive data suggesting cell specific TLR4 signaling, there have been suggestions that LPS evoke intracellular signaling reactions in Kupffer cells (Seabra et al., 1998) and tracheal epithelial cells (Oshiro et al., 2004) that are absent in polymorphonuclear leukocytes (Rodeberg and Babcock, 1996). Here we report the existence of a distinct TLR4 mediated

signaling pathway leading to IL-6 secretion that is present in BECs but absent in other human cell types.

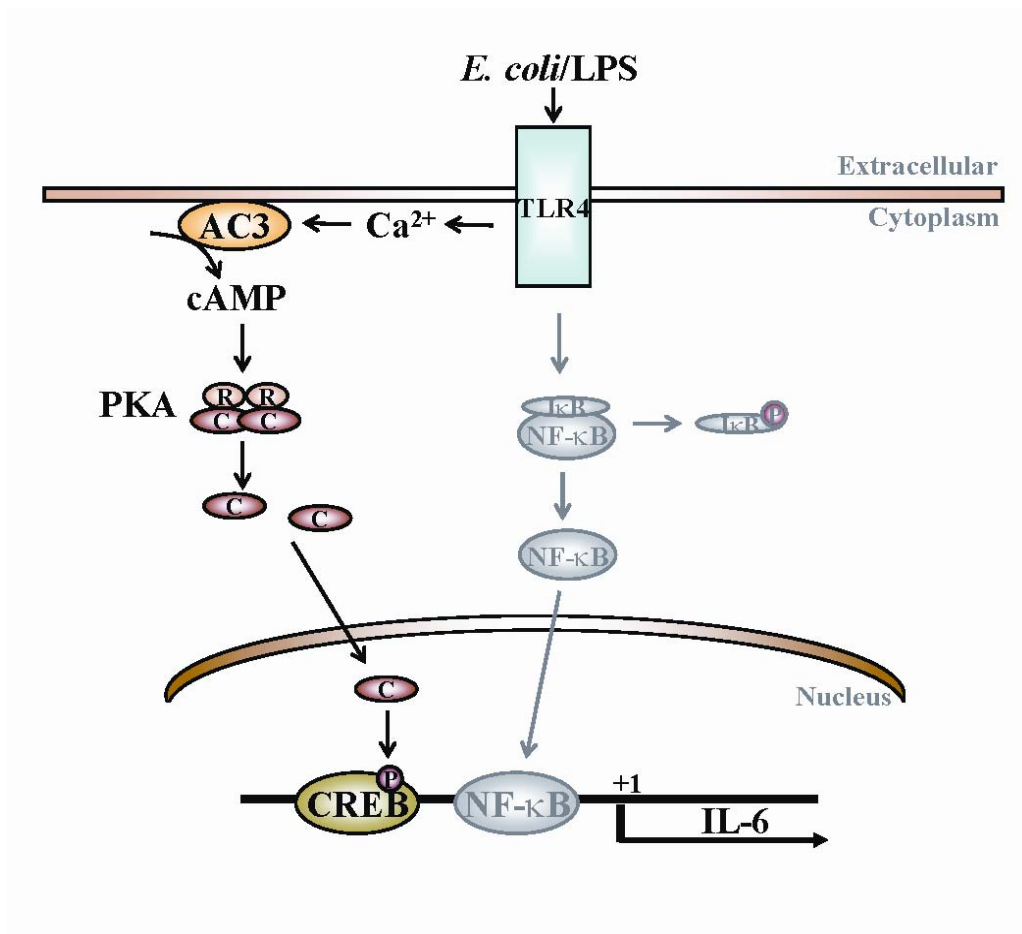
This novel signaling pathway detected in BECs is independent of the classical pathway involving the transcriptional element NF- $\kappa$ B and contains two well known secondary messengers,  $\text{Ca}^{2+}$  and cAMP, which mobilize a different transcriptional element, CREB. The existence of this pathway only became evident to us because of our focus on second messengers in TLR signaling and because our assays for IL-6 production were undertaken at earlier incubation periods than the more traditional 24-48hr incubation time points (Gabay, 2006). At the later incubation periods the contribution of this novel pathway to the IL-6 response is superceded by the traditional NF- $\kappa$ B mediated pathway. Evidence for the involvement of  $[\text{Ca}^{2+}]_i$  in the early BEC IL-6 response comes from the finding that a flux in  $[\text{Ca}^{2+}]_i$  was observed within a minute of exposure to *E. coli* and inhibiting this flux with  $\text{Ca}^{2+}$  channel inhibitors or intracellular  $\text{Ca}^{2+}$  chelators, inhibited the IL-6 response (Fig. 2.2B). Since a general inducer of calcium influx such as ionophore A23187 was able to induce IL-6 production from BECs even in the absence of *E. coli*, increase in  $[\text{Ca}^{2+}]_i$  appears sufficient to trigger the IL-6 response from BECs (Fig. 2.2B). Evidence for the role of cAMP in the IL-6 response comes from the finding that the IL-6 response to *E. coli* was closely associated with a 3-fold increase in intracellular levels of cAMP (Fig. 2.3A). In addition, inhibition of cAMP generating

ACs significantly reduced the IL-6 response of BECs to *E. coli* (Fig. 2.3C). As with the  $[Ca^{2+}]_i$  flux, merely enhancing intracellular levels of cAMP with a membrane-permeable cAMP analog, induced significant IL-6 release from BECs even in the absence of *E. coli* (Fig. 2.3C). Thus, the two secondary messengers are sufficient, as well as necessary, for the early IL-6 secretion in BECs.

That  $Ca^{2+}$  response preceded the cAMP production following bacterial stimulation was deduced from the findings that (i), the earliest detectable increase in intracellular cAMP levels was observed 15 mins following exposure to *E. coli* (data not shown), whereas  $Ca^{2+}$  responses could be seen within a minute of bacterial exposure (Fig. 2.2A) and (ii), the  $Ca^{2+}$  flux following exposure to *E. coli* remained largely unaffected in AC-3 KD BECs, while the cAMP response was abrogated (Fig. 2.4I). Since enhancement of intracellular cAMP specifically required an increase in  $[Ca^{2+}]_i$ , we suspected that a  $Ca^{2+}$  inducible form of AC was responsible. BECs were found to express mRNA for AC-3, 4, 6, and 7, but only RNAi knockdown of AC-3, a  $Ca^{2+}$ -inducible AC isoform, inhibited *E. coli*-induced intracellular cAMP production and subsequent IL-6 expression.

To identify where in the signaling cascade cAMP was exerting its effects, we compared IL-6 message levels in control and AC-3 KD BECs. The absence of IL-6 message in AC-3 KD BECs after 1 hr of exposure to bacteria suggested that cAMP was

regulating IL-6 production at the transcriptional level rather than at the levels of translation or cytokine secretion. Since translocation of NF- $\kappa$ B into the nucleus of BECs following exposure to *E. coli* was largely unaffected in the AC-3 KD BECs (Fig. 2.5B), cAMP may not be exerting its effect through altering NF- $\kappa$ B. Interestingly, one way that cAMP can directly promote expression of certain genes is to activate PKA, which translocates to the nucleus where it phosphorylates the transcriptional factor CREB (Shaywitz and Greenberg, 1999). Upon phosphorylation, CREB is believed to promote transcription of a number of genes, including IL-6, IL-8, IL-1 $\alpha$  and IL-1 $\beta$ , which possess consensus CRE sites on their promoter region (Andrisani, 1999; Gray et al., 1993; Iourgenko et al., 2003; Isshiki et al., 1990; Shaywitz and Greenberg, 1999). Interestingly, in BECs only IL-6 and IL-8 appear to be regulated by the cAMP/CREB pathway (Fig. 2.5). That cAMP was modulating phosphorylation of CREB was evident from CREB phosphorylation in control BECs following exposure to *E. coli* but not in AC-3 KD BECs. Thus, taken together, our cumulative data reveal the existence of a distinct TLR4 activated signaling pathway in BECs involving Ca<sup>2+</sup>, cAMP and phosphorylated CREB. A diagrammatic representation of the proposed TLR4 initiated Ca<sup>2+</sup>, cAMP and CREB dependent pathway as well as the NF- $\kappa$ B pathway in BECs is shown in Fig. 2.8. Although in the figure, we have indicated that the cAMP/CREB pathway in BECs is activated by TLR4, our data also suggest that TLR2 and TLR3 activation may also trigger this pathway (Fig. 2.6E).



**Figure 2.8: Proposed model for TLR4 signaling in BECs.** The proposed rapidly induced second messenger and CREB mediated pathway (dark line) as well as the classical NF-κB (grey-line) are shown. Both pathways are triggered by TLR4. R and C, regulatory and catalytic subunits of PKA.

Our analysis of the kinetics of IL-6 secretion in WT and AC3 KD BECs has revealed that this novel second messenger-CREB mediated pathway is mediating a faster IL-6 response than the classical NF- $\kappa$ B mediated pathway. Following exposure of BECs to *E. coli* ORN103(pSH2), marked phosphorylation of CREB was observed at least 1 hr before nuclear translocation of NF- $\kappa$ B was evident. Indeed, the earliest evidence of nuclear translocation of NF- $\kappa$ B in BECs following exposure to *E. coli* was at 2 hrs (Fig. 2.5B). Another piece of evidence implicating the second messenger-CREB pathway in a rapid and distinct IL-6 response was the observation that message for IL-6 was detectable in control BECs 1 hr following exposure to *E. coli* whereas no message for IL-6 was seen in AC3 KD BECs. However, by 6 hrs after the classical NF- $\kappa$ B signaling pathways had been activated there was very little difference in the amounts of mRNA in both cell types (Fig. 2.5D). Consistent with this finding, the kinetics of IL-6 secretion by WT BECs and AC3 KD BECs following exposure to *E. coli* ORN103(pSH2), revealed a 3hr hour lag in the latter's response but by 12 hrs the amounts of IL-6 secreted were comparable (Fig. 2.6A). Thus, the rapid and vigorous inflammatory responses to infection typically observed in the urinary tract may be attributable, at least in part, to this distinct cAMP dependent signaling pathway. The relevance of the early IL-6 response by BECs may be linked to their role as first responders. One of the primary cell types in the urinary tract reacting to BEC-generated IL-6 are also BECs. These cells possess IL-6 receptors (Meyers et al., 1991) and this cytokine, acting in autocrine fashion, may trigger various antimicrobial

responses such as production of antimicrobial peptides (Nemeth et al., 2004) and mucins (Li et al., 2003) as well as promoting exfoliation of infected BECs.

The existence of multiple pathways in BECs for triggering IL-6 responses could be an adaptation to avoid inactivation by UPEC. Several recent studies have suggested that host adapted pathogens possess the intrinsic capacity to block NF- $\kappa$ B activation in macrophages and cultured human epithelial cells through release of toxins or proteases (Collier-Hyams et al., 2002; Klumpp et al., 2001; Orth et al., 2000; Ruckdeschel et al., 2001; Tato and Hunter, 2002). Hultgren et al., have recently identified several genes: *rfa*, *rfb* and *surA*, in UPEC that contribute to suppressing the cytokine responses of BECs (Hunstad et al., 2005). Our observations that primary BECs (Fig. 2.7) evoked a more modest IL-6 response to clinical UPEC CI5 compared to the laboratory *E. coli* strain and that the cAMP/CREB mediated IL-6 response to UPEC CI5 in BEC lines was not striking compared to *E. coli* ORN103(pSH2) (Fig. 2.6A and B) could be manifestations of this phenomenon. Conceivably, depending on the nature of the BECs, the UPEC CI5 strain is able to partially diminish one or both of the two TLR4 mediated signaling pathways leading to IL-6 secretion.

Finally, because of the rapid emergence of multi-resistance among UPEC isolates, there is mounting interest in the development of alternate antimicrobial strategies. One

approach is to bolster innate immune defenses in the urinary tract either before or during infection. In this regard, our finding that small molecule enhancers of intracellular levels of  $\text{Ca}^{2+}$  and cAMP are sufficient to trigger early and vigorous cytokine responses from BECs is of interest. There are available many compounds capable of modulating the intracellular levels of both  $\text{Ca}^{2+}$  and cAMP (Elmslie, 2004; Insel and Ostrom, 2003; Pressman, 1976; Shafer et al., 1998). Judicious application of some of these agents for the treatment and prevention of UTIs is a possibility that will require further examination.

## **Chapter 3: TLR4-mediated inhibition of bacterial invasion**

### **3.1 Introduction**

In spite of its close proximity to the gastrointestinal tract which harbors a large reservoir of, primarily, Gram negative bacteria with the potential to cause infections, the urinary tract remains refractile to infection. Much of this resistance is attributable to the mechanical forces existing in the urinary tract and to the ability of the epithelial cells lining the tract to recognize incoming bacteria and to mount a rapid and effective immune response (Mulvey et al., 2000; Reid and Sobel, 1987).

The bladder, which comprises a major portion of the lower urinary tract, is a specialized structure employed for the storage of urine. Because of this role, the superficial BECs intrinsically are highly impermeable to pathogens (Truschel et al., 2002). On the apical surfaces of BECs are distinct proteins called uroplakins which along with a collection of lipids, cholesterol and sphingolipids, which constitute a distinct cellular entity called lipid rafts, provides a highly ordered lipid structure with very low membrane fluidity and permeability (Apodaca, 2004; Truschel et al., 2002). Another major impediment to bacterial colonization is the powerful flushing actions of urine which eliminates all bacteria that are not intimately associated with BECs (Reid and Sobel, 1987). In addition to these physical barriers, prospective pathogens have to

survive various powerful antimicrobial actions initiated by BECs and other mucosal cells (Mulvey et al., 2000; Samuelsson et al., 2004). Present in the plasma membrane of the epithelial cells are PRRs such as TLR4 that recognize LPS on Gram negative bacteria and activate a sequence of intracellular signals resulting in activation of the transcriptional factor, NF- $\kappa$ B, and the production of several NF- $\kappa$ B dependent cytokines including chemoattractants that recruit phagocytic cells to clear the infection (Schilling et al., 2003; Svanborg et al., 2006). The importance of TLR4 in the innate immune response is evident from the findings that TLR4 mutant mice mount a poor cytokine and neutrophil response to UTIs. Consequently, in contrast to isogenic control mice, these mice fail to resolve their infections (Hagberg et al., 1984; Schilling et al., 2001; Svanborg et al., 2006).

The TLR4 signaling mechanisms in BECs leading to cytokine and neutrophil responses during UTIs now appear to be more complex and distinct from that seen in other cell types. Initial studies revealed a signaling cascade consistent to that described in other cells namely, engagement of TLR4 by LPS triggers a signaling pathway involving several intracytoplasmic and nuclear transcriptional factors (Fischer et al., 2006; Schilling et al., 2001). TLR4 activation first engages a set of adaptor family members, which initiates a sequence of signaling events, resulting eventually in the activation of the transcriptional factor NF- $\kappa$ B, and the expression of several

immunomodulatory cytokines such as IL-6 and IL-8 (Kawai and Akira, 2006; Schilling et al., 2001). More recent studies now reveal that the vigorous and rapid TLR4 mediated IL-6 and IL-8 responses of BECs to UPEC is only partially attributable to the classical NF- $\kappa$ B pathway and that a second and more rapid signaling pathway is involved. This pathway possesses a number of prominent cellular secondary messengers, Ca<sup>2+</sup> and cAMP, as well as a transcriptional factor, cAMP response element-binding protein (CREB) (Song et al., 2007b). Although this distinct TLR4 mediated pathway appears to be present only in BECs (Song et al., 2007b), its specific contribution to bladder defense is unknown.

Uropathogens that overcome the defenses of the urinary tract typically do so by seeking refuge within the bladder epithelium. In their intracellular location, these pathogens avoid the clearing actions of both urine flow and recruited phagocytes. The most common mode of bacterial invasion of BECs involves type 1 fimbriae which are filamentous appendages expressed by *E. coli*, *K. pneumoniae* and various other uropathogenic enterobacteria (Abraham et al., 1988; Hagberg et al., 1983; Hagberg et al., 1981). Binding of FimH, a mannose binding lectin at the distal tips of the fimbriae to uroplakin 1a on the luminal surface of BECs, triggers a distinct series of signaling reactions that culminates in the entry of the bacteria into the dynamic subapical pool of discoid vesicles called fusiform vesicles found in BECs (Bishop et al., 2007). These fusiform vesicles are membrane-rich nondegradative compartments that serve to

increase bladder volume by fusing with luminal plasma membrane of BECs (Truschel et al., 2002). Since the plasma membrane of BECs as well as fusiform vesicles are highly enriched in cholesterol, sphingolipids, and glycolipids (Apodaca, 2004), there is growing realization that UPEC invasion of BECs is lipid raft dependent. This notion has been supported by the finding that specific disruptors of cellular lipid raft structure inhibits UPEC invasion and key cellular components so far implicated in bacterial invasion of BECs are typically localized with lipid raft structure (Duncan et al., 2004). One such lipid raft mediator of bacterial invasion is the Rho GTPase member, Rac-1, whose activation is critical because it enhances accumulation of actin filaments at sites of bacterial entry (Duncan et al., 2004; Martinez and Hultgren, 2002).

During infection of the urinary tract, both TLR4 mediated signaling and lipid raft mediated bacterial invasion of BECs are believed to occur. Here, we reveal the existence of crosstalk between the two pathways, where TLR4 signaling through increased production of the secondary messenger, cAMP, negatively regulates lipid raft mediated bacterial invasion.

## **3.2 Experimental Procedures**

### **3.2.1 *In vivo* invasion assay**

C3H/HeN and C3H/HeJ female mice were obtained from NCI and The Jackson Laboratory. 8-10-week-old female mice were anesthetized with sodium pentobarbital and inoculated transurethrally with  $1 \times 10^8$  of uropathogenic *E. coli* CI5 by urethral catheterization. After 1 hr, 0.1 mg of gentamicin was applied for 30 min to kill all extracellular bacteria, and bladders were washed briefly using 100  $\mu$ l of PBS. Bladders were aseptically removed and homogenized in 0.1% Triton X-100 in PBS. Homogenate dilutions were plated on LB plates and incubated overnight at 37°C for colony counts. n=10-20 per each experimental group. When indicated, a group of mice were pretreated intraperitoneally with 10 mg/kg forskolin and transurethrally with 100  $\mu$ M forskolin for 1 hr. All experiments were done according to protocols approved by the Duke Division of Laboratory Animal Resources and the Duke University Institutional Animal Care and Use Committee.

### **3.2.2 Immunofluorescence of bladder sections**

8-10-week-old female mice were anesthetized and inoculated transurethrally with  $1 \times 10^8$  of *E. coli* CI5. After 1 h, bladders were aseptically removed, embedded with OCT solution, and kept at -80 °C until use. Frozen sections of uninfected and infected

bladders were cut using standard methods. Each section was fixed in 4% paraformaldehyde for 10 min at RT and in precooled ethanol:acetic acid (2:1) for an additional 5 min at – 20 °C, blocked in PBS with 3% BSA for 30 min at RT, and incubated with primary antibodies diluted in PBS with 3% BSA for 30 min at RT. Sections were incubated for 30 min at RT with Alexa 488 conjugated secondary antibodies diluted in PBS with 1% BSA. To visualize epithelial cells, sections were incubated with wheat germ agglutinin (WGA) for 30 min at RT when the secondary antibodies were applied. Coverslips were mounted using Prolong Gold anti-fade reagent (Molecular Probes) and viewed. For TLR4 staining, sections were fixed as before, blocked in PBS containing 1% goat serum, and incubated with 1:100 diluted rat anti-mouse TLR4 antibody (R&D Systems) in blocking solution overnight at 4 °C. Same amounts of Rat IgG were used for the control staining. Sections were incubated with FITC-conjugated secondary antibody for 30 min at RT, mounted, and viewed.

### **3.2.3 Bacteria and BECs**

*E. coli* ORN103(pSH2) (Orndorff and Falkow, 1984), *E. coli* ORN103(pUT2002) (Minion et al., 1986), uropathogenic *E. coli* strain CI5 (Abraham et al., 1985; Orndorff and Falkow, 1984; Thankavel et al., 1997), *E. coli* MLK1067 (an insertional inactivation mutant of *msbB1*) (Clementz et al., 1997), *E. coli* W3110 (corresponding wild-type strain for MLK1067) (Clementz et al., 1997), uropathogenic *Klebsiella pneumoniae* strain 1236 (a

clinical isolate from Duke University Medical Center), and a Gram positive clinical isolate *Staphylococcus aureus* strain 54 (a clinical isolate from DUMC) were utilized in this study. Bacteria were grown overnight in LB broth or in Brain Heart Infusion (BHI) broth prior to use. 80 µg/ml of chloramphenicol was added for *E. coli* ORN103(pSH2) and *E. coli* MLK1067. The human BEC line 5637 (ATCC HTB-9) was grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (HyClone) and incubated at 37° C with 5% CO<sub>2</sub>.

### **3.2.4 *In vitro* bacterial invasion and adherence assays**

5637 human BECs were seeded onto 96-well plates at a density of 4x10<sup>4</sup> cells/well and incubated overnight. The cells were infected with 100 MOI bacteria for 1 h. The medium was replaced with fresh culture medium containing 100 µg/ml of the membrane-impermeable antibiotic gentamicin (Invitrogen) to kill extracellular bacteria and incubated at 37 °C for additional 1 hour. Each well was washed three times with PBS. In order to lyse the cells, 100 µl of 0.1% Triton X-100 in PBS was added to each well and incubated for 15 min. Cells were scraped, diluted, and plated onto LB agar plates. Colonies were counted to quantify the number of invading bacteria. To test the effect of various drugs on bacterial invasion, 50 µM forskolin (Sigma), 1 mM dibutyryl cAMP (Sigma), 1 mM N<sup>6</sup>- Benzoyladenosine- 3', 5'- cyclic monophosphate (6-Bnz-cAMP, Sigma), 1 mM 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate

(8-CPT-2-cAMP, Sigma), or 0.2  $\mu$ M Protein Kinase A inhibitor fragment (PKI, Sigma) in serum-free medium was added to the cells for 30 min prior to infection. The viability of the cells was not affected by any of the treatments used as determined by trypan blue exclusion. For the MTT adherence assay, cells were plated 96-well plates, incubated overnight, and fixed for 15 min with 3% paraformaldehyde. The monolayers were washed three times with sterile PBS and pretreated for 1 h at RT with blocking buffer (3% bovine serum albumin in PBS). 100  $\mu$ l of *E. coli* strain ORN103(pSH2) ( $A_{600} \sim 1.0$ ) in PBS, was incubated with cells for 1 h at 37 °C. Nonadherent bacteria were removed by washing the cell monolayers three times with PBS. Fifty microliters of LB was applied to each monolayer and incubated for 15 min at 37 °C. Fifty microliters of 2 mg/ml MTT (Sigma) in PBS was added, and the plates were incubated for 15 min at 37 °C to allow reduction of MTT to formazan by live bacteria. Next, 150  $\mu$ l of mixture of isopropyl alcohol and hydrochloric acid (24:1) was added to solubilize the formazan, and the absorbance was measured at 450 nm using a Tecan Sunrise remote microplate reader.

### **3.2.5 Creation of TLR4 knockdowns using RNA Interference**

TLR4 knockdown and control BECs were generated as described in Chapter 2.

### **3.2.6 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

Total cellular RNA was isolated using RNeasy purification system (Qiagen). Two  $\mu\text{g}$  of total RNA was reverse transcribed and amplified with gene-specific primers using the RT-PCR System kit (Bio-rad). The primer sequences for the genes were as follows: 5'-CGATTCCATTGCTTCTTG-3' (sense) and 5'-GCTCAGGTCCAGGTTCTT-3' (antisense) for TLR4 and 5'-ATCCCATCACCATCTTCCAG-3' (sense) and 5'-CCTGCTTCACCACCTTCTTG-3' (antisense) for GAPDH. The primer sequences for the AC genes and expected product sizes were described in Chapter 2. We confirmed that the AC isotype-specific primers were functional by undertaking RT-PCR on total RNA from HEK cells (a positive control cell, where all ACs except AC4 and 8 were expressed) (Ludwig and Seuwen, 2002).

### **3.2.7 Creation of BECs overexpressing constitutively activated, or dominant-negative Rac1**

Rac1 was PCR amplified and inserted into the pLEGFP-C1 (Clontech) to produce GFP-Rac1. The GFP-Rac1 construct was then used to generate the dominant-active GFP-Rac1 (Q61L), or the dominant-negative GFP-Rac1 (T17N) by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the vendor's instruction.

### **3.2.8 Rac1 activity assay**

For Rac1 activity assay, we used a glutathione-S-transferase (GST)-PAK-CD (p21-activated kinase (PAK) CRIB domain; which interacts with activated form of Rac1) fusion protein to selectively isolate activated form of Rac1 from human BECs. Multiple steps were involved in this approach; cloning and production of GST-PAK-CD fusion protein followed by pull down of activated form of Rac1 from BEC extracts employing the GST-PAK fusion protein.

#### **3.2.8.1 Cloning and Production of GST-PAK-CD fusion protein**

We first generated a plasmid containing GST-PAK-CD as described previously (Sander et al., 1998). Briefly, a DNA fragment encoding amino acids 56-141 from human PAK1B (GenBank accession number AF071884) was amplified by standard PCR and inserted into pGEX-4T-1 (Amersham Pharmacia Biotech) to produce pGST-PAK-CD. This plasmid was transformed into *E. coli* BL21. To purify GST-PAK-CD protein, *E. coli* harboring pGST-PAK-CD were grown overnight in LB broth with 100 µg/ml of ampicillin. On the following day, 1 ml of grown bacteria was added into 20 ml LB broth with ampicillin, and incubated for additional ~2 hours ( $A_{600}=0.6-0.8$ ). Expression of GST-PAK-CD protein was induced by addition of 0.1 mM IPTG for another 4 hours. Cells were harvested, resuspended in lysis buffer (0.1 M NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0, 100 µg/ml lysozyme, 5 mM DTT, protease inhibitor cocktail, 1.5%

Sarkosyl), and then passed in 27-G needle 10 times. The supernatant was taken and incubated with 50% Glutathione sepharose beads slurry (Amersham Pharmacia Biotech) for 30 min at 4°C. Fusion protein bound the beads washed and directly used for a pull-down assay described as follows.

### **3.2.8.2 Pull down and quantitation of activated Rac1**

5637 human BECs were seeded onto 10-cm culture dish at a density of  $4.2 \times 10^6$  cells/plate (~60% confluency) and incubated overnight. The medium was replaced with fresh culture medium with 0.2% FBS and incubated at 37 °C for additional 5 hrs. The cells were inoculated with *E. coli* ORN103(pSH2) (100 MOI), and incubated at 37 °C for experimental time frames. The cells were lysed in a RIPA buffer (Upstate Biotechnology) containing 1 mM PMSF and a 1:100 dilution of mammalian protease inhibitor cocktail (Sigma). The cell suspension was passed 20 times through a 27-gauge needle, and centrifuged for 5 min with the precipitates then being discarded. Protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. To pull down activated form of Rac1 from BEC extracts, 200 µg BEC extracts were mixed with purified GST-PAK-CD protein bound the beads. The mixture was incubated for 1 hr at 4°C with shaking, and pellets were collected by centrifugation and washed four times with 1 ml of lysis buffer. After final wash, 50 µl of 2 x Sample buffer

(Bio-Rad) was added and the samples were boiled for 5 min. Western blotting for Rac1 (BD Biosciences) was performed using 10  $\mu$ l of the samples.

### **3.2.9 Measurement of intracellular cAMP levels**

5637 human BECs were seeded onto 6-well plates and grown overnight. The cells were uninfected or infected with 100 MOI *E. coli* ORN103(pSH2), or treated with 100  $\mu$ g/ml *E. coli* LPS (Sigma) for indicated time points. The cells were washed four times with PBS to remove culture media, and lysed in 250  $\mu$ l of 0.1 M HCl for 10 min. After centrifugation, the supernatant was directly used for the cAMP assay. Intracellular concentrations of cAMP were determined using a cAMP enzyme immunoassay kit (Sigma) according to the manufacturer's instructions. In order to measure intracellular cAMP levels of BECs obtained from control HeN and TLR4 mutant HeJ mice, 8-10 week old female mice were anesthetized and sacrificed, and bladders were aseptically removed, bisected, turned epithelium-side out, lysed in 300  $\mu$ l of 0.1 M HCl for 20 min. Supernatant from centrifugation was directly used for the assay as before.

### **3.2.10 Statistics**

Two tailed Student's T-tests were performed in order to determine the statistical significance of experimental changes from control values.

### **3.3 Results**

#### **3.3.1 Increased invasion of mouse bladders by Gram negative bacteria when TLR4 signaling is abrogated**

One of the earliest *in vivo* examples revealing the critical role of PRRs in host defense was the observation made over 20 years ago that TLR4 mutant C3H/HeJ mice, in contrast to isogenic control C3H/HeN mice, were unable to clear experimental UTIs (Hagberg et al., 1984; Shahin et al., 1987). This observation was ascribed to the inability of TLR4 mutant mice to mount a local cytokine and neutrophil response (Hagberg et al., 1984; Shahin et al., 1987). Here, we examined if in addition to their inability to mount an adequate inflammatory response these TLR4 mutant mice exhibited increased susceptibility to bacterial invasion. A small volume (50  $\mu$ l) of saline containing  $1 \times 10^8$  of a type 1 fimbriated uropathogenic *E. coli* strain CI5 was introduced via catheter into the bladders of a group of anesthetized TLR4 mutant and isogenic control mice. One hour later, the bladders were emptied and instilled with gentamicin for 30 min to kill all extracellular bacteria. Thereafter, the mice were sacrificed and the bladders were removed, rinsed and the intracellular numbers of bacteria were determined by standard colony counts of bladder homogenates (Hagberg et al., 1984). The values obtained from each bladder homogenate represent the number of intracellular bacteria. Since the incubation time was limited to only 1 hr, the contribution of recruited immune cells to bacterial clearance was minimal. We found that the number of intracellular *E. coli* in the bladders of TLR4 mutant mice were at least 12 fold higher than the controls (Fig. 3.1A).

Immunomicroscopy of bladder cross sections of both groups of mice confirmed bacterial association with the superficial epithelium of the bladders of TLR4 mutant but not in control mice (Fig. 3.1B and 3.1C). Employing immunoprobes for TLR4, we sought to confirm earlier claims of the presence of TLR4 in bladder cells (Backhed et al., 2001; Samuelsson et al., 2004). Whereas no discernable staining was observed when an isotype antibody was employed (Fig. 3.1D), the relatively large basal level of TLR4 expression on superficial BECs in control mice was striking (Fig. 3.1E). Interestingly, neither the distribution nor expression levels of TLR4 appeared to change following bacterial infection (Fig. 3.1F). To demonstrate the specificity of this TLR4 mediated response for LPS, we examined the invasive capacity of another LPS producing bacteria, a type 1 fimbriated uropathogenic *Klebsiella pneumoniae* strain 1236, as well as a non LPS producer, a clinical isolate *Staphylococcus aureus* strain 54. Whereas no significant difference between the TLR4 mutant and control mice in staphylococcal invasion was detected, an 11-fold difference in invasion was observed with *K. pneumoniae* (Fig. 3.1G and 3.1H). These observations reveal that compared to controls, TLR4 mutant mice are highly susceptible to invasion by type 1 fimbriated Gram negative enteric bacteria. Thus, TLR4 markedly suppresses the invasion of BECs by type 1 fimbriated enterobacteria.

To further support this conclusion, we compared the ability of a LPS modified type 1 fimbriated K-12 *E. coli* strain MLK1067 (*msbB* mutant) and its parent strain W3110

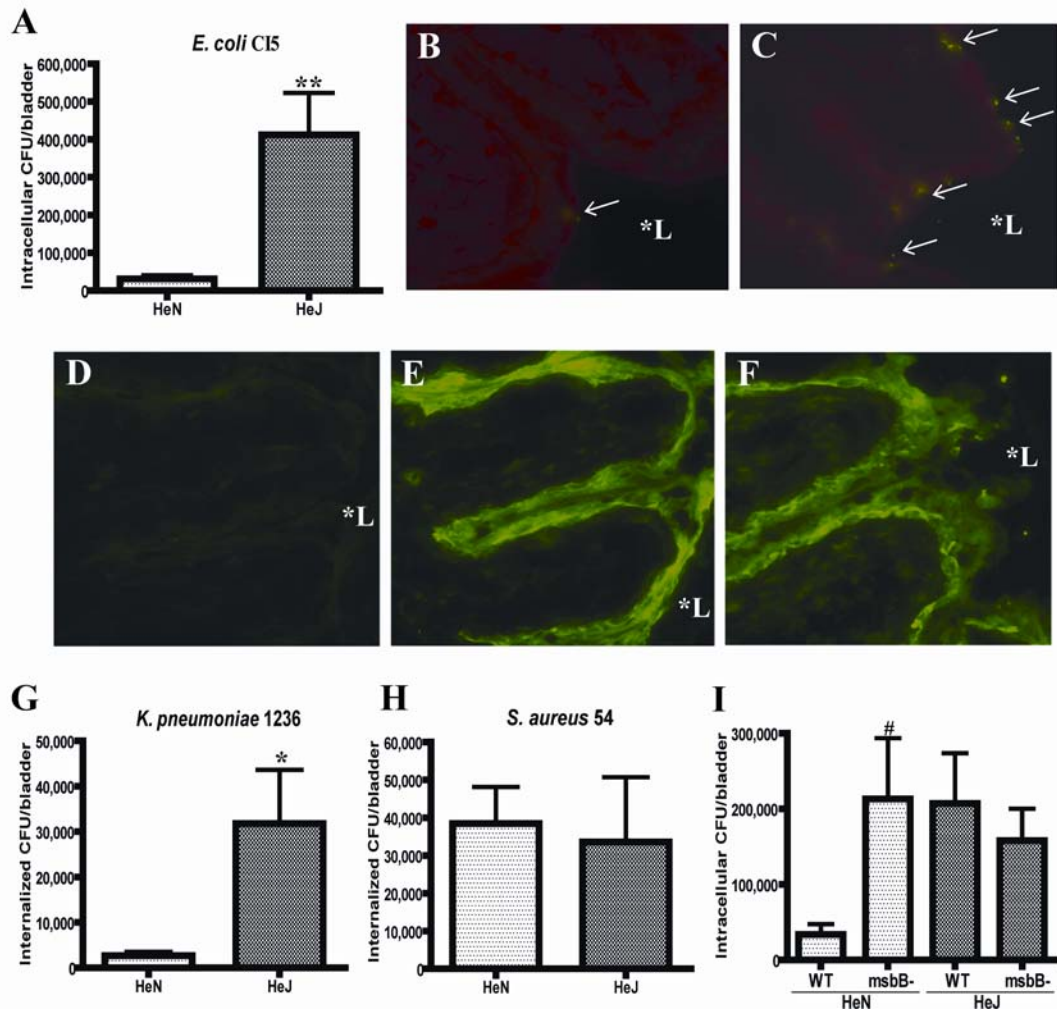


Figure 3.1

**Figure 3.1: Increased invasion of mouse bladders by Gram negative bacteria when TLR4 signaling is abrogated.** **A**, TLR4 mutant C3H/HeJ (HeJ) and isogenic control C3H/HeN (HeN) mice were inoculated transurethrally with  $1 \times 10^8$  of *E. coli* CI5 (A). Bacterial invasion after 1 hr was assessed by gentamicin protection assays followed by colony counts of bladder homogenates. **B-C**, Fluorescent images of bladder sections of HeN (B) and HeJ (C) mice 1 hr following instillation of  $1 \times 10^8$  *E. coli* CI5. Green, *E. coli*; Red, Wheat Germ Agglutinin (WGA). **D-F**, TLR4 immunostaining images of bladder sections of HeN mice before (D and E) and 1 hr after (F) instillation of  $1 \times 10^8$  *E. coli* CI5. E and F were stained with TLR4-specific antibody whereas D was stained with isotype control antibody. **G-H**, TLR4 mutant HeJ and control HeN mice were inoculated transurethrally with  $1 \times 10^8$  of type 1 fimbriated *K. pneumoniae* 1236 (G), or *S. aureus* 54 (H). Bacterial invasion after 1 hr was assessed as before. **I**, TLR4 mutant HeJ and control HeN mice were inoculated transurethrally with  $1 \times 10^8$  of type 1 fimbriated *msbB* mutant MLK1067 (*msbB*-) or corresponding wild-type (WT) *E. coli* W3110. Bacterial invasion after 1 hr was assessed as before. In A, G, H, and I, \*\*  $P < 0.0001$ ; \*  $P < 0.01$ , relative to values of HeN; #  $P < 0.05$ , relative to values of wild-type *E. coli* W3110 infected HeN. Bars represent the mean + S. D.. In B-F, magnification was 200X. \*L stands for lumen.

(Clementz et al., 1997) to invade bladders of wild type mice. The *msbB* mutant *E. coli* MLK1067 produces a penta-acylated lipid A that is poorly recognized by the TLR4 signaling complex and as a consequence this strain fails to trigger TLR4-LPS signaling in host cells (Coats et al., 2005). Predictably, unlike the parent strain, the *msbB* mutant will not activate TLR4 and therefore a significantly greater invasion of mouse bladders should result. Indeed, we found a 6.3-fold greater invasion of mouse bladders by the mutant MLK1067 strain compared to the parent strain (Fig. 3.1I). Consistent with this idea, when we compared bladder invasion by wild-type and *msbB* mutant *E. coli* in the TLR4 mutant mice, no significant difference in invasion between the two strains was seen (Fig. 3.1I). Cumulatively, these observations provide definitive evidence that TLR4-LPS signaling plays a key role in reducing bacterial invasion of bladder cells.

### **3.3.2 Recapitulation of *in vivo* observations using a human BEC line**

To elucidate the molecular basis for TLR4 mediated abrogation of bacterial invasion, we initiated studies employing a well established *in vitro* model, the 5637 human BEC line. It has previously been demonstrated that interactions of this cell line with Gram negative bacteria closely mimics *in vivo* behavior of BECs (Duncan et al., 2004; Schilling et al., 2001). We sought to recapitulate our *in vivo* observations of the modulatory role of TLR4 on *E. coli* invasion. First, we compared invasion of BECs by wild type *E. coli* W3110 and its *msbB* mutant derivative, and observed a 9-fold greater

invasion by the mutant *E. coli* compared to the parent *E. coli* strain (Fig. 3.2A) which is consistent with the *in vivo* data. Next, using RNA interference techniques, we generated BECs where expression of TLR4 was knocked down (KD). Densitometric quantitation of message levels in the TLR4 KD BECs, revealed that the expression of TLR4 was reduced by 49% (Fig. 3.2B). We sought to compare invasion of control (transfected with control vector) BECs and TLR4 KD BECs by the type 1 fimbriae expressing *E. coli* ORN103(pSH2). This K-12 laboratory *E. coli* strain expressing recombinant type 1 fimbriae rather than a UPEC strain was employed for most of our subsequent studies (Orndorff and Falkow, 1984). Typically, in addition to type 1 fimbriae, UPEC express several other virulent factors which could potentially confound our studies. As expected, we found that *E. coli* ORN103(pSH2) invaded TLR4 KD BECs in significantly higher numbers than control transfected or non-transfected BECs (Fig. 2C). It is noteworthy that the adherence of *E. coli* to both control BECs and TLR4 KD BECs were identical (Fig. 3.2D). It is also noteworthy that invasion of TLR4 KD BECs by *E. coli* ORN103(pSH2) was also FimH dependent because limited invasion of TLR4 KD BECs was observed when the isogenic FimH mutant *E. coli* ORN103(pUT2002) (Minion et al., 1986) was tested (data not shown). Thus, our *in vitro* data recapitulate our *in vivo* observations implicating TLR4 as a negative modulator of *E. coli* invasion into BECs.

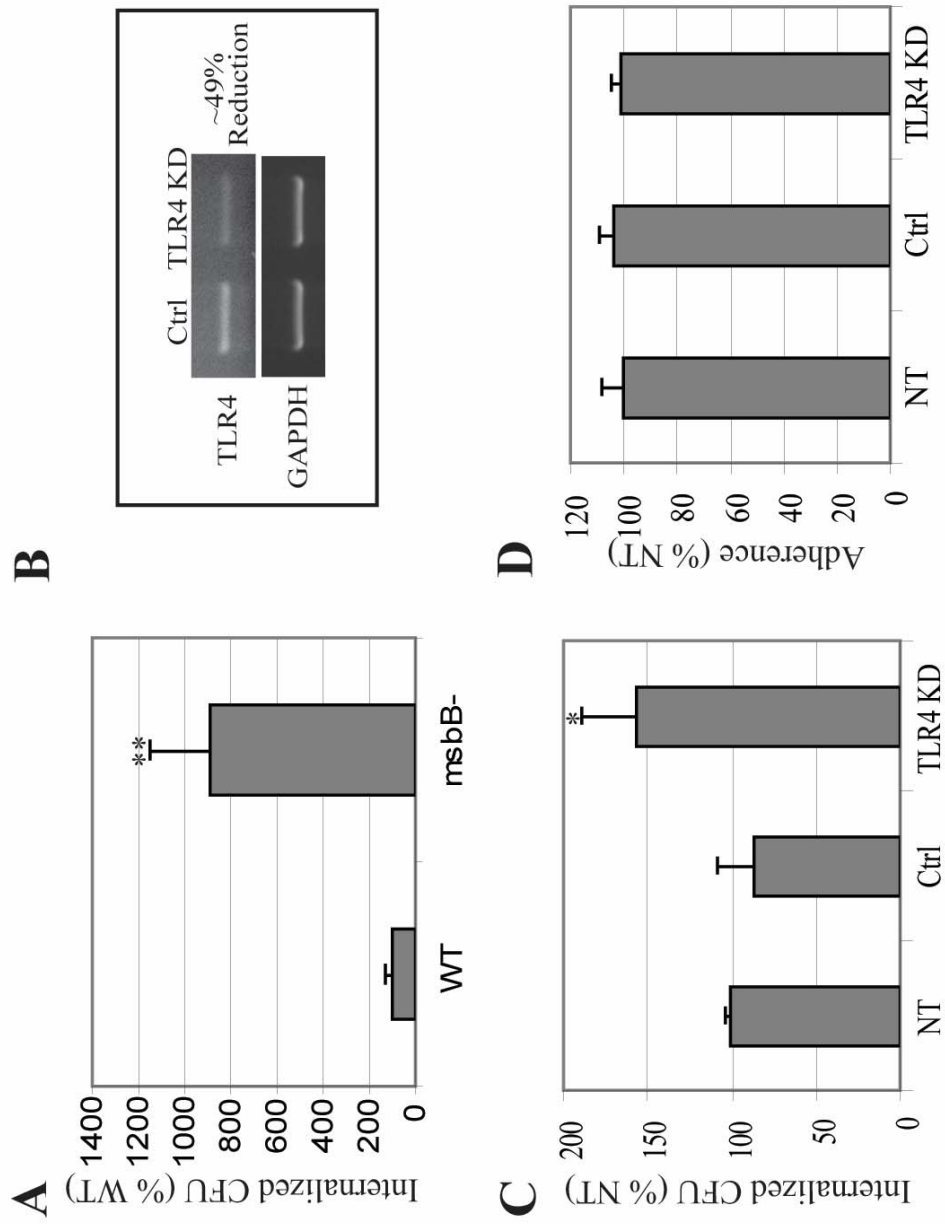
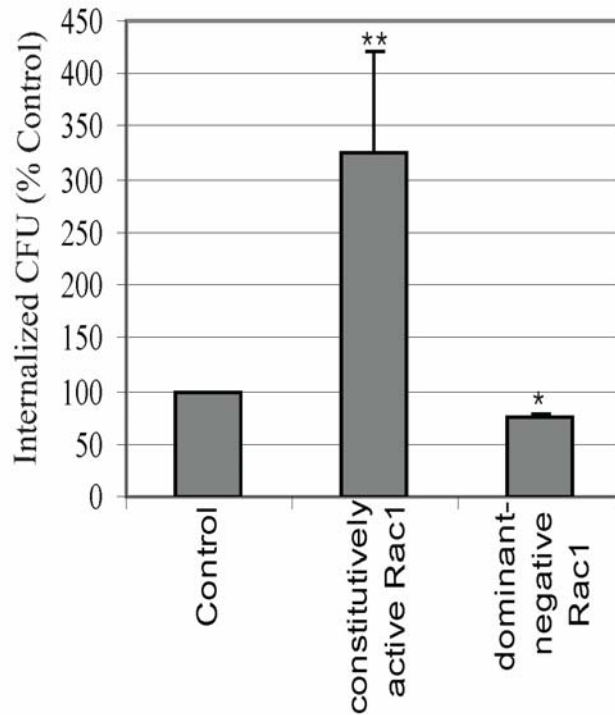
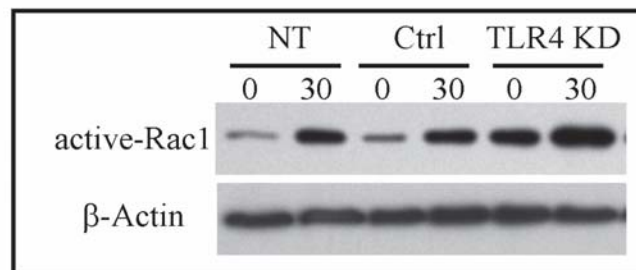


Figure 3.2

**Figure 3.2: TLR4-LPS signaling abrogates *in vitro* invasion of BECs by type 1 fimbriated Enterobacteria.** **A**, Bacterial invasion following exposure of BECs to wild-type *E. coli* W3110 (WT) or *msbB* mutant *E. coli* MLK1067 (*msbB*-). \*\* $P < 0.001$ , relative to values of wild-type *E. coli* infected BECs. **B**, RT-PCR of control-transfected BECs (Ctrl) and TLR4 knockdown BECs (TLR4 KD). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a loading control. **C-D**, Bacterial invasion (C) or bacterial adherence (D) following exposure of non-transfected BECs (NT), control-transfected BECs (Ctrl), and TLR4 KD BECs to type 1 fimbriated *E. coli* ORN103(pSH2). \* $P < 0.03$ , relative to control values. Bars represent the mean + S. D. in A, C and D.

### 3.3.3 TLR4 KD BECs exhibit an enhanced Rac-1 response to *E. coli*

To gain an understanding of how TLR4 signaling was negatively impacting bacterial invasion in BECs, we sought to compare the level of Rac-1 activation in TLR4 KD BECs and control BECs following exposure to type 1 fimbriated *E. coli* ORN103(pSH2). We and others have previously implicated Rac-1, a critical mediator of actin cytoskeletal dynamics and a component of lipid rafts, in the invasion of BECs by *E. coli* (Duncan et al., 2004; Martinez and Hultgren, 2002). Shown in Fig. 3.3A is a confirmatory experiment where invasion by type 1 fimbriated *E. coli* ORN103(pSH2) of control BECs, BECs overexpressing constitutively activated Rac1 and BECs overexpressing a dominant negative form of Rac-1 was examined. Compared to control BECs, bacterial invasion of BECs where Rac1 was constitutively activated was markedly higher. Conversely, bacterial invasion of BECs with the dominant negative form of Rac-1 was significantly lower than the controls (Fig. 3.3A). Both of these observations reiterate the idea that Rac-1 activation is essential to the invasion of BECs by type 1 fimbriated *E. coli*. To investigate the activation states of Rac-1 in TLR4 KD BECs, non-transfected BECs and control transfected BECs, we isolated activated Rac-1 from each of the BEC lysates before and 30 min following exposure to *E. coli* ORN103(pSH2). The assay we employed to detect the activated form of Rac-1 was a pull down assay employing a fusion protein comprising of GST-fused to PAK which specifically binds active GTP bound Rac-1 (Manser et al., 1994). An appreciable increase in activated Rac-1 was detected in each of

**A****B**

**Figure 3.3: TLR4 KD BECs exhibit an enhanced Rac-1 response to *E. coli* which correlates with enhanced bacterial invasion.** **A**, Invasion of control-transfected BECs, BECs overexpressing constitutively active Rac1, or BECs with dominant-negative Rac1 by type 1 fimbriated *E. coli* ORN103(pSH2). \*\* $P < 0.01$ ; \* $P < 0.05$ , relative to control values. Bars represent the mean + S. D.. **B**, GTP-bound Rac1 levels of non-transfected (NT), control-transfected (Ctrl), and TLR4 KD BECs before (0) and 30 min after (30) exposure to *E. coli*. An actin-specific Western blot was used as a loading control.

the cell types 30 min following exposure to bacteria, although the amounts of activated Rac-1 BECs in TLR4 KD BECs was markedly higher than in any of the control BECs (Fig. 3.3B). Indeed, the level of activated Rac-1 in each cell type (Fig. 3.3B) correlated closely with the extent of bacterial invasion seen (Fig. 3.2). These observations suggest that TLR4 mediated suppression of bacterial invasion is through inhibition of Rac-1 activation. It is noteworthy that even before exposure to bacteria, the levels of activated Rac-1 in TLR4 KD BECs was relatively high (Fig. 3.3B) suggesting that TLR4 may be constitutively suppressing Rac-1 in quiescent cells.

### **3.3.4 TLR4 KD BECs evoke a reduced cAMP response to *E. coli***

Since TLR4 has not previously been reported to suppress activation of Rac-1, we reasoned that the mechanism would involve one or more inhibitory intermediaries. Because secondary messengers can have profound and sometimes inhibitory effects on multiple cellular functions, we examined the possibility of cAMP as the candidate inhibitory substrate. cAMP has previously been shown to negatively regulating Rac-1 activity and subsequent Rac-1 mediated reorganization of actin cytoskeleton (Nagasawa et al., 2005). Rac-1 inactivation by cAMP has been shown to specifically involve PKA, whose catalytic domains are activated by cAMP binding (Howe, 2004; O'Connor and Mercurio, 2001; Waschke et al., 2004). Before determining whether cAMP was involved

in suppressing Rac-1 activation it was important to demonstrate cAMP production by BECs following exposure to type 1 fimbriated *E. coli* ORN103(pSH2). We examined if this was the case and if so, whether TLR4 KD BECs evoked a diminished cAMP response. We compared the levels of intracellular cAMP in control and TLR4 KD BECs before and 1 h after exposure to *E. coli* ORN103(pSH2). We observed a marked increase in intracellular cAMP in BECs following exposure to bacteria (Fig. 3.4A). This bacteria-induced increase in intracellular cAMP was significantly reduced in TLR4 KD BECs (Fig. 3.4A) indicating that TLR4 was mediating this cAMP response. A similar observation was made if the *E. coli* was replaced by purified LPS (Fig. 3.4A) which is consistent with the notion that the bacterial component responsible for activating TLR4 was indeed LPS. It should be noticed that bacteria induced increase in intracellular cAMP was seen as early as 15 min after exposure, but the 1 h time point was selected so as to maximize difference in cAMP levels under various conditions. To further investigate the relationship between intracellular cAMP and *E. coli* invasion of BECs, we compared bacterial entry into non-transfected, control-transfected and TLR4 KD BECs following treatment with 50  $\mu$ M forskolin (Fsk), a broad spectrum activator of adenylyl cyclases, or 1 mM dibutyryl cAMP (dbcAMP), a membrane permeable cAMP analog. In all cases, Fsk and dbcAMP treatments markedly reduced bacterial uptake indicating that increasing intracellular cAMP levels had a powerful effect in blocking *E. coli* invasion (Fig. 3.4B). We next investigated if the Fsk mediated increase in intracellular cAMP

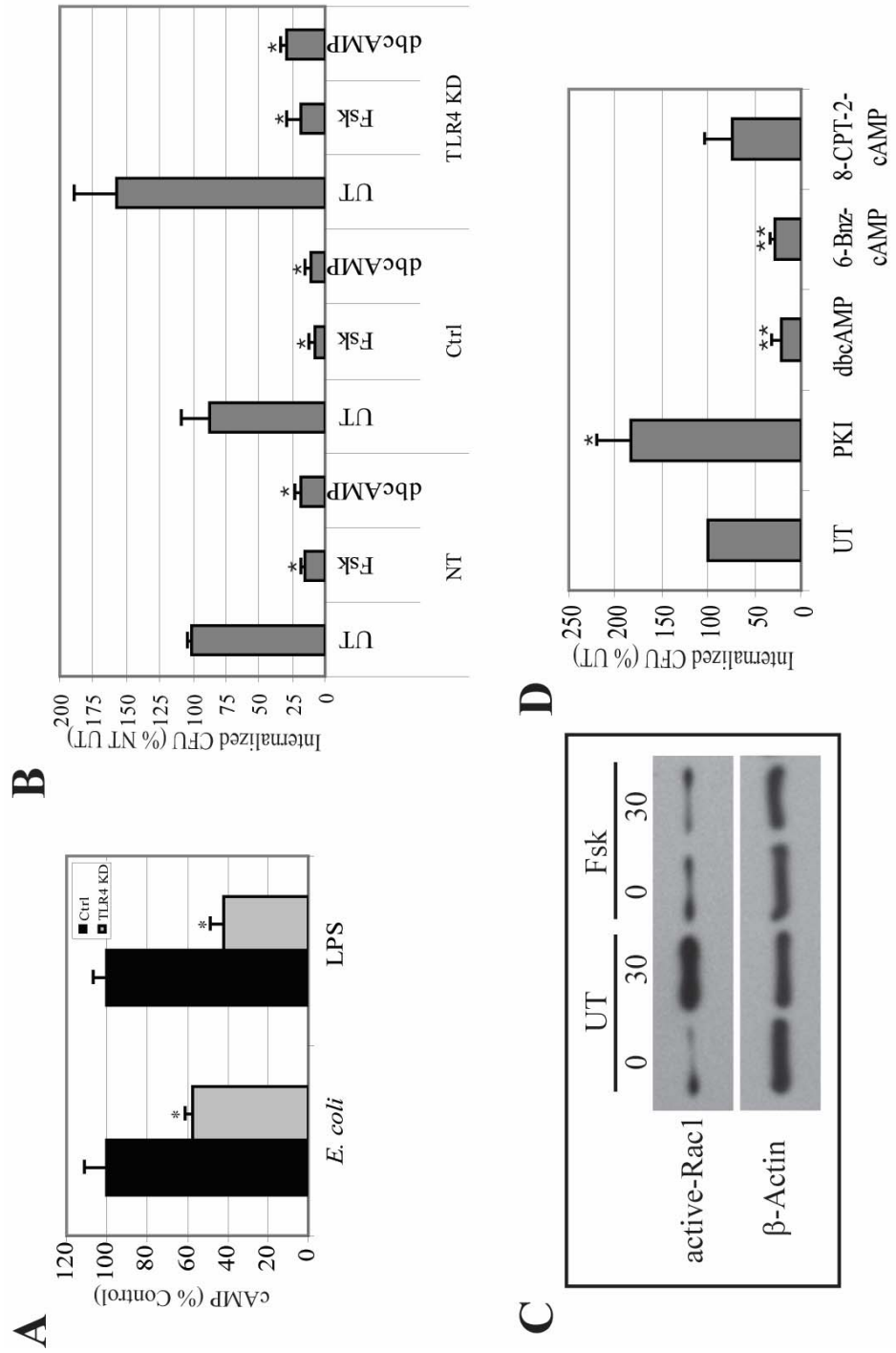


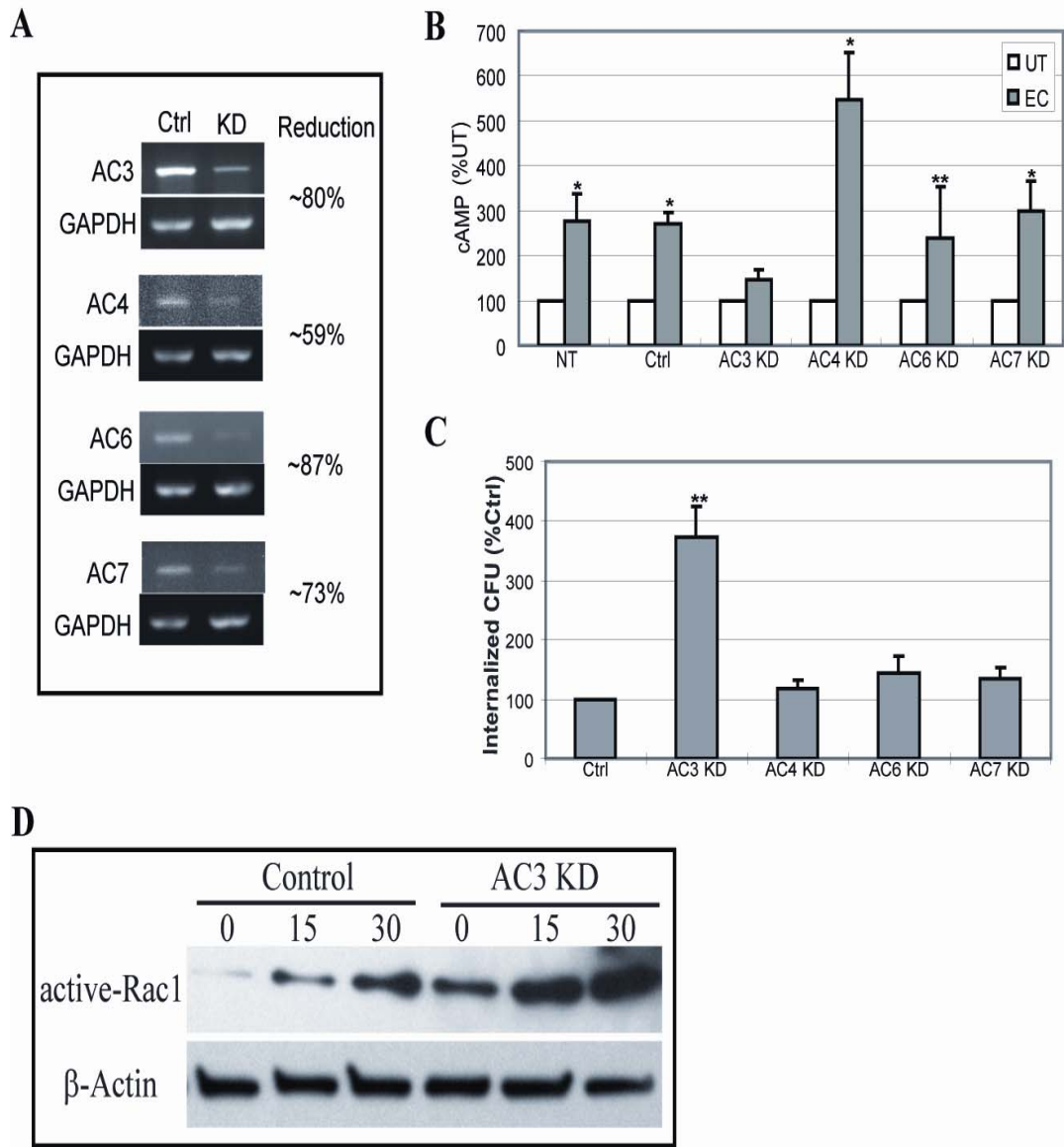
Figure 3.4

**Figure 3.4: TLR4 KD BECs evoke a reduced cAMP response to *E. coli* which correlates with increased bacterial invasion.** **A**, Intracellular cAMP levels of control-transfected BECs (Ctrl) and TLR4 KD BECs after exposure to type 1 fimbriated *E. coli* ORN103(pSH2) or *E. coli* LPS. \* $P < 0.05$  relative to *E. coli* (EC) or LPS treated control. **B**, *E. coli* invasion of non-transfected (NT), control-transfected (Ctrl), and TLR4 KD BECs left untreated (UT) or following treatment with 50  $\mu\text{M}$  forskolin (Fsk) or 1 mM dibutyl cAMP (dbcAMP). \* $P < 0.001$  relative to values of respective UT BECs. **C**, GTP-bound Rac1 levels in untreated (UT) and forskolin-treated (Fsk) BECs before (0) or after 30 min (30) exposure to *E. coli*. An actin-specific Western blot was used as a loading control. **D**, *E. coli* invasion of untreated (UT) BECs or BECs following 30 min treatment with 0.2  $\mu\text{M}$  PKA inhibitor peptide (PKI), 1 mM dibutyl cAMP (dbcAMP), 1 mM 6-Bnz-cAMP, or 1 mM 8-CPT-2-cAMP. \*\* $P < 0.001$ ; \* $P < 0.01$ , relative to UT values. Bars represent the mean + S. D. in A, B, and D.

would also negatively impact Rac-1 activation. We examined for bacterial induced Rac-1 activation in untreated and Fsk treated BECs. As shown in Fig. 3.4C, Fsk treated BECs failed to evoke any Rac-1 activation in response to bacteria which is consistent with the finding that Fsk significantly reduces bacterial invasion in BECs (Fig. 3.4B). In additional experiments, we compared the effects of two membrane-permeable cAMP analogs, dbcAMP and 8-CPT-cAMP on bacterial invasion. Whereas the former analog activates two downstream effectors PKA and the exchange protein directly activated by cAMP (Epac), the latter activates only Epac. We found that dbcAMP but not 8-CPT-cAMP significantly inhibited bacterial invasion (Fig. 3.4D) indicating that PKA but not Epac was involved in the uptake of bacteria. Consistent with this notion, treatment of BECs with a PKA specific inhibitor peptide (PKI) or another cell permeable cAMP analog, 6-Bnz-cAMP which specifically activates PKA revealed negative impacts of cAMP-PKA on *E. coli* invasion (Fig. 3.4D). Thus, the enhanced levels of bacterial invasion seen in TLR4 KD BECs compared to control BECs are directly attributable to their limited cAMP response to bacteria. Our observations cumulatively support the notion that TLR4 mediated increase in intracellular cAMP and its downstream effector, PKA, are largely responsible for suppressing Rac-1 mediated bacterial invasion.

### **3.3.5 The BEC cAMP response to *E. coli* is mediated by Adenylyl cyclase 3**

Next, we sought to determine how TLR4 was increasing intracellular cAMP levels in BECs. Because there are currently 10 known isoforms of mammalian adenylyl cyclases (ACs) (Sunahara and Taussig, 2002) it was of interest to identify the specific AC in BECs responsible for the TLR4 mediated cAMP response. First, we sought to determine which AC isoforms were actually expressed in BECs. RT-PCR was performed on total cellular RNA, using primers specific for each known AC isoform and only mRNA for AC isoforms 3, 4, 6, and 7 was detectable in BECs (Song et al., 2007b) (data not shown). We confirmed that the other AC isotype-specific primers used were functional by undertaking RT-PCR on total RNA from human embryonic kidney (HEK) cells, positive control cells, where all ACs except AC4 and 8 were expressed (Ludwig and Seuwen, 2002) (data not shown). RNAi was utilized to minimize the expression of each AC, which was verified by AC isotype-specific RT-PCR (Fig. 3.5A). To see which of the 4 ACs were involved in suppressing *E. coli* invasion, we exposed the respective KD BECs to *E. coli* ORN103(pSH2) and examined for cAMP production and bacterial invasion. We observed an increase in intracellular cAMP in all the KD cells except AC3 KD BECs (Fig. 3.5B). Similarly, the level of invasion into the various BECs was comparable with that of the control except AC3 KD BECs following *E. coli* exposure (Fig.



**Figure 3.5: The BEC cAMP response to *E. coli* and its subsequent effect on bacterial invasion is mediated by Adenylyl cyclase 3.** **A**, RT-PCR of control-transfected BECs (Ctrl) and AC-3, -4, -6, or -7 KD BECs. GAPDH-specific RT-PCR was used as a loading control. **B**, Intracellular cAMP levels in non-transfected BECs (NT), control-transfected BECs (Ctrl), or AC-3, -4, -6 or -7 KD BECs before (UT) or after exposure to *E. coli* ORN103(pSH2) (EC). \* $P < 0.005$  and \*\* $P < 0.02$  relative to respective UT values. **C**, *E. coli* invasion of control-transfected BECs (Ctrl), or AC-3, -4, -6, or -7 KD BECs. \*\* $P < 0.0001$  relative to control values. Bars represent the mean + S. D. in B and C. **D**, Active-Rac1 levels of control and AC3 KD BECs before and at 15 or 30 min after exposure to *E. coli*. An actin-specific Western blot was used as a loading control.

3.5C). Indeed, the level of bacterial invasion in AC3 KD BECs was over 3 fold higher than in controls. Consistent with these findings, there was a high level of Rac-1 activation in AC-3 KD BECs following exposure to *E. coli* ORN103(pSH2) relative to controls (Fig. 3.5D). Thus, AC3 is the BEC AC isoform linked to the intracellular cAMP response following *E. coli* exposure. This means that AC3 is also the isoform which is responsible for suppressing invasion of human BECs by *E. coli*. The high level of Rac-1 activation seen in quiescent AC-3 KD BECs is reminiscent of the situation in TLR4 KD BECs and suggests that the suppressive effects of TLR4 on Rac-1 activation in quiescent BECs is through AC-3 derived cAMP.

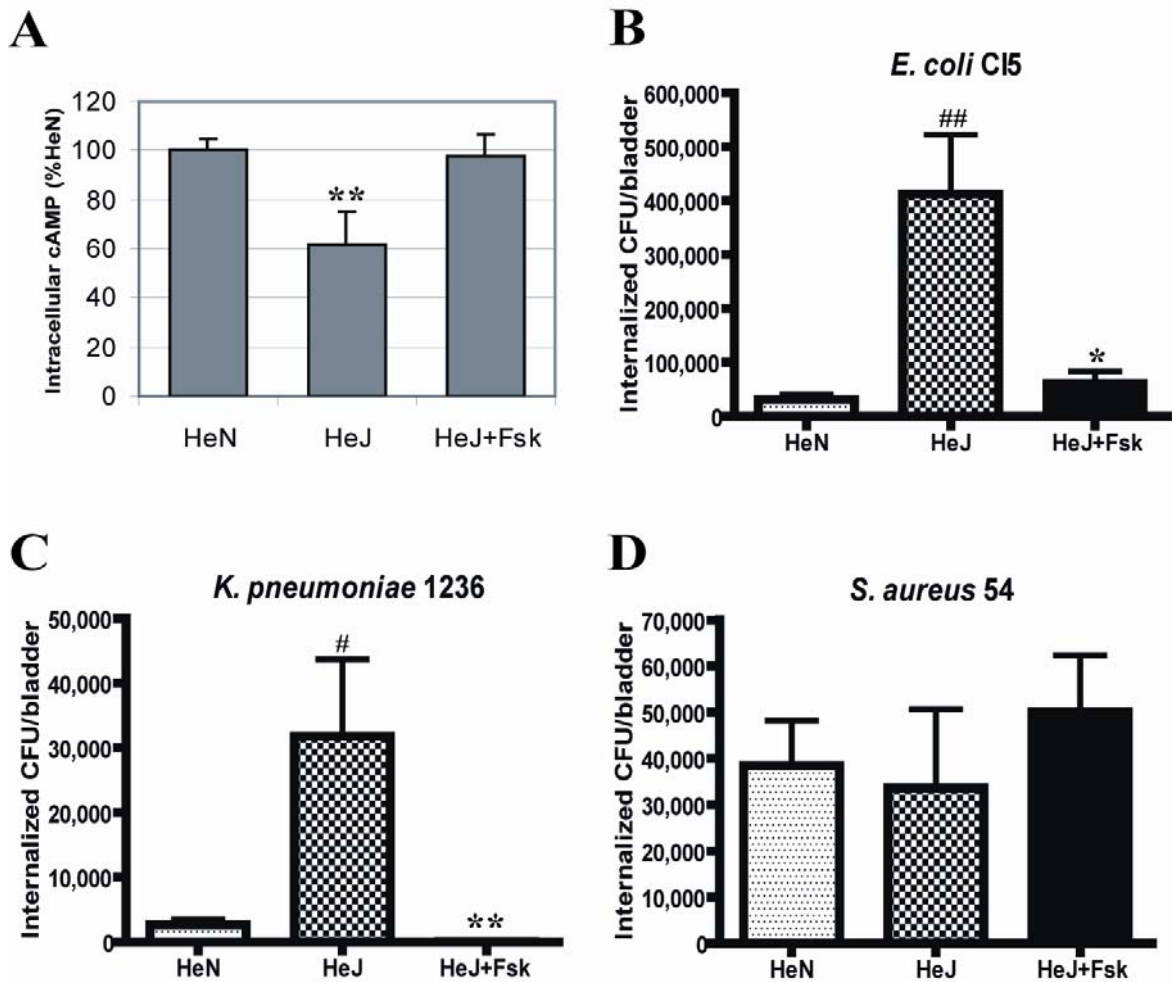
### **3.3.6 Application of a booster of intracellular cAMP in the urinary tracts of TLR4 mutant mice reduces bacterial invasion**

In our *in vivo* studies, we had observed that TLR4 mutant mice were highly susceptible to invasion by uropathogenic *E. coli* and *K. pneumoniae* (Fig. 3.1A and 3.1B). Our *in vitro* studies suggest the existence of a cAMP dependent innate mechanism in BECs for countering bacterial invasion. We sought to establish a link between intracellular cAMP in BECs and susceptibility to bacterial invasion *in vivo*. Our *in vitro* studies have suggested that TLR4 constitutively regulates cAMP levels in BEC in a positive fashion, if so, intracellular levels of cAMP in TLR4 mice would predictably be lower than that seen in control mice. We compared the basal cAMP levels in superficial BECs of bladders in TLR4 mutant and control mice and as predicted, intracellular cAMP

levels in TLR4 mutant mice were found to be significantly lower than in control BECs (Fig. 3.6A). Thus, the increased susceptibility of TLR4 mutant mice to enterobacterial invasion could be attributable to low levels of intracellular cAMP in BECs. If the increased susceptibility of TLR4 mutant mice to bacterial invasion is attributable to their inability mount an appropriate cAMP response, then application of the cAMP booster, Fsk, should compensate for the cAMP deficiency and reduce susceptibility to bacterial invasion. Indeed, when bacterial invasion and intracellular cAMP levels in BECs of TLR4 mutant mice were examined following combined intraperitoneal/intravesicular Fsk treatment, the levels were comparable to that seen in the wild type mice (Fig. 3.6A and 3.6B), indicating that the increased susceptibility could be reversed merely by treating the urinary tract with a booster of intracellular cAMP levels. Application of Fsk to TLR4 mutant mice also reduced invasion by type 1 fimbriated *K. pneumoniae* 1236 but not invasion by *S. aureus* 54 (Fig. 3.6C and 3.6D).

### **3.4 Discussion**

Invasion of host cells is the single most important mechanism that pathogens employ to avoid rapid clearance by the host's immune system. Here, we report a novel host cell adaptation for resisting this powerful microbial trait. The mechanism in BECs for resisting invasion by type 1 fimbriated *E. coli* involves TLR4, an immune surveillance molecule, which is well known for mobilizing a wide range of innate immune responses



**Figure 3.6: Reduced intracellular levels of cAMP in BECs of TLR4 mutant mice and use of Fsk to enhance resistance of BECs to invasion by type 1 fimbriated Enterobacteria.** **A**, Intracellular cAMP in superficial BECs obtained from control (HeN), TLR4 mutant (HeJ) mice, and TLR4 mutant mice treated with Fsk (HeJ+Fsk) (n=3-5). \*\*P<0.01, relative to HeN values as well as relative to HeJ+Fsk values. **B-D**, Invasion of bladders of control (HeN) mice, TLR4 mutant (HeJ) mice and TLR4 mutant (HeJ) mice pretreated with Fsk by  $1 \times 10^8$  of *E. coli* CI5 (B), *K. pneumoniae* 1236 (C), or *S. aureus* 54 (D). When indicated, the mice were pretreated for 1 h with forskolin via intravesicular catheter instillation and intraperitoneal (IP) injection. Bars represent the mean + S. D. in A-D. ##P<0.001; #P<0.01, relative to HeN values. \*\* P<0.01; \* P<0.03, relative to HeJ values.

against Gram negative bacteria including secretion of critical proinflammatory mediators and antimicrobial peptides (Saemann et al., 2005; Samuelsson et al., 2004; Schilling et al., 2003; Shahin et al., 1987). Blocking invasion of type 1 fimbriated enterobacteria represents a novel albeit surprising role for TLR4, considering that TLRs in macrophages have been implicated in promoting bacterial uptake and subsequent phagosome maturation (Blander and Medzhitov, 2004, 2006; Ozinsky et al., 2000; Underhill and Gantner, 2004).

Invasion of BECs by type 1 fimbriated UPEC involves a distinct but poorly defined endocytic pathway. What is currently known regarding UPEC invasion of BECs is that cellular lipid raft components are involved and that the internalized bacteria are retained within compartments resembling fusiform vesicles of BECs (Bishop et al., 2007; Duncan et al., 2004). A number of lipid raft components have been shown to be necessary for UPEC invasion of BECs including Uroplakin 1a, the FimH receptor, caveolin-1, a scaffolding protein, and Rac-1, an inducer of cytoskeletal remodeling (Duncan et al., 2004; Martinez and Hultgren, 2002). Interestingly, our studies reveal that Rac-1 is also a molecular target of TLR4 signaling and that Rac-1 represents the site where TLR4 signaling and lipid raft mediated phagocytosis intersect. Shown in Fig. 3.7 is a diagrammatic depiction of how TLR4 signaling converges with the bacterial invasion pathway.

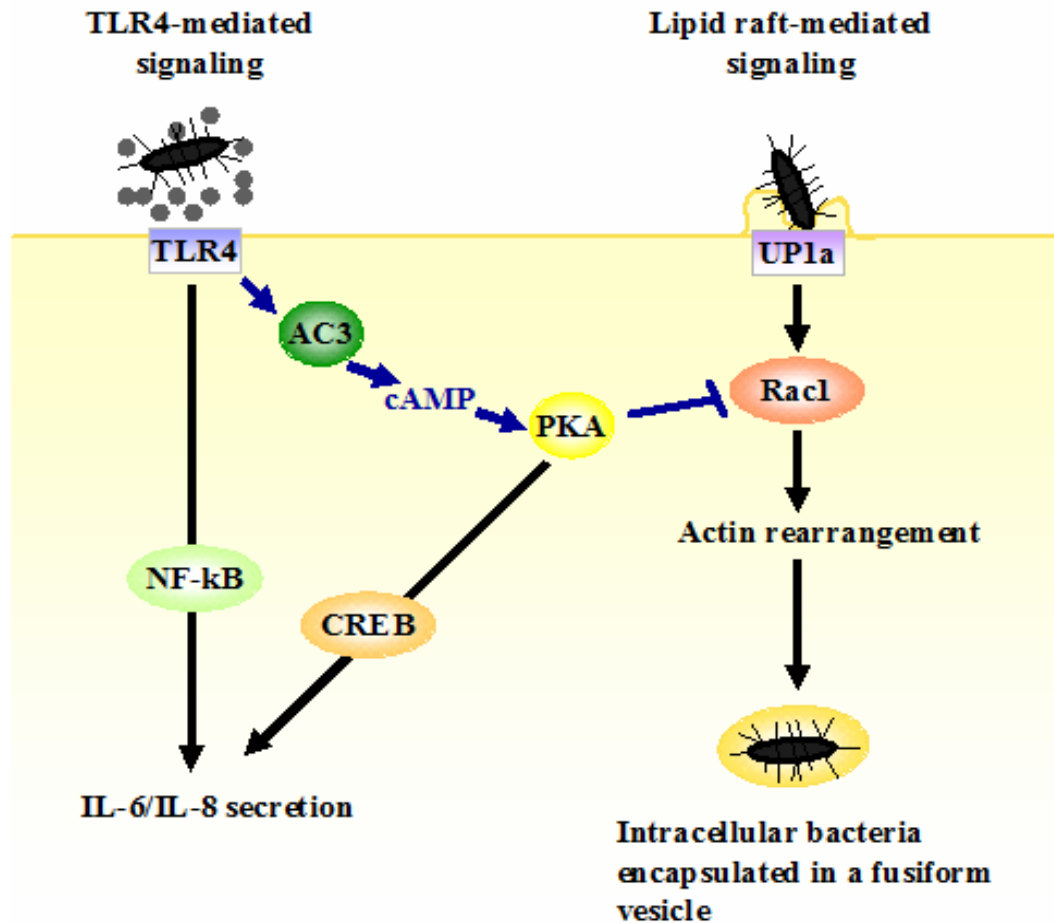


Figure 3.7: Diagrammatic depiction of the parallel signaling reactions occurring in BECs during infection by type 1 fimbriated Enterobacteria. Highlighted in the figure is the TLR4 initiated and AC-3, cAMP, and PKA dependent signaling pathway that dissects with and abrogates the lipid raft mediated endocytic pathway.

The connection between TLR4 and Rac-1 was revealed from the findings that the level of Rac-1 activation in TLR4 KD BECs was markedly higher relative to control BECs even in the steady state (Fig. 3.3B). Although the levels of activated Rac-1 correspondingly increased in each cell type following exposure to type 1 fimbriated *E. coli*, the amounts in TLR4 KD BECs was strikingly higher compared to that in control BECs (Fig. 3.3B). Thus, TLR4 negatively impacts Rac-1 activation, a necessary step for actin remodeling and bacterial invasion (Duncan et al., 2004; Martinez and Hultgren, 2002). TLR4 mediated suppression of Rac-1 appears to be the underlying basis for why bacterial invasion in TLR4 KD BECs is several fold higher than in control BECs (Fig. 3.2 and 3.3). Since previous reports in other cell types have shown that TLR4 regulation of Rac-1 activity is of a positive nature (Schmeck et al., 2006; Wissel et al., 2005; Zhang et al., 2005), our finding is consistent with the notion that TLR4 signaling circuitry in BECs is more complex than in other cell types.

Since TLR4 has not previously been reported to negatively impact activation of Rac-1, TLR4 mediated suppression of Rac-1 activity presumably involves a distinct set of ancillary molecules. Our studies point to a second messenger, cAMP and its downstream element, PKA, as critical intermediaries in the TLR4 circuitry responsible for suppressing Rac-1 activation and subsequent bacterial invasion. We showed that following ligation of TLR4, cAMP, derived primarily from AC-3, played a critical role in

impeding bacterial invasion. That AC-3 generated cAMP was critical for abrogating bacterial invasion was evident from the finding that both Rac-1 activation and bacterial invasion was markedly enhanced in the AC-3 KD BECs compared to control BECs. Indeed, the levels of Rac-1 activation and bacterial invasion in AC-3 KD BECs were comparable to the strikingly high levels observed previously in TLR4 KD BECs. Interestingly, in addition to regulating cAMP and Rac-1 activity during Gram negative infections, TLR4 appears to play a constitutive role in regulating these molecules even in quiescent BECs.

Since inhibitors of the transcriptional factor NF- $\kappa$ B such as MG-132 had limited effect on either intracellular production of cAMP or bacterial invasion (data not shown), these events appeared independent of the traditional TLR4 signaling pathway. Recently, it was reported that TLR4 activation in BECs resulted in signaling via the traditional NF- $\kappa$ B pathway as well as via a second and a “more rapid” pathway involving AC-3 derived cAMP and the transcriptional factor, CREB (Song et al., 2007b) (Fig. 3.7). That this TLR4 initiated and cAMP dependent pathway blocks bacterial invasion reveals an important and previously unsuspected physiological function for this pathway.

Presumably, the invasion-abrogating defenses in BECs lining the lumen are triggered when TLR4 molecules make contact with LPS shed from bacteria, an event

likely to be initiated as soon as bacteria enter the bladder. If the contaminating bacteria are not able to overcome cellular actions that impede penetration, they will be rapidly eliminated by the flushing forces of urine. That BECs are invaded by type 1 fimbriated *E. coli* in spite of their TLR4 mediated resistance argues that expression of type 1 fimbriae could be a trait specifically evolved by UPEC to invade BECs. Indeed, a survey of UPEC isolates has revealed that over 80% of UPEC express type 1 fimbriae (Hagberg et al., 1981; Langermann et al., 1997). The discovery of the central role of cAMP in modulating cellular defenses to bacterial invasion is also of particular interest because intracellular cAMP levels are readily regulatable *in vivo* with a variety of small molecule modulators (Elmslie, 2004; Insel and Ostrom, 2003; Pressman, 1976; Shafer et al., 1998), raising the possibility of boosting bladder defenses for therapeutic purposes. We have demonstrated that administering TLR4 mutant mice with the cAMP inducer, forskolin, can markedly boost both intracellular cAMP levels and resistance of BECs to bacterial invasion to levels typically found in wild type cells. Recently, it was reported that administering forskolin in the urinary tracts of UPEC-infected mice reduced bladder infections by triggering the exocytosis of UPEC from fusiform vesicles of infected BECs (Bishop et al., 2007). Our findings suggest that the protective properties ascribed to forskolin in the urinary tract could also be attributable to reducing bacterial invasion of BECs.

With the growing realization that most pathogens seek refuge in host cells at some stage of the infectious process, much of the research has focused on elucidating the various strategies employed by pathogens to penetrate and subsequently survive within cells. The intrinsic capacity of certain host cells to completely or partially abrogate microbial entry has gone largely unrecognized. Our studies demonstrating the intrinsic properties of BECs to counter bacterial invasion could partly explain the remarkable intractability of the urinary tract to infection in spite of frequent contamination by gut flora.

## **Chapter 4: TLR4-mediated expulsion of bacteria from infected BECs**

### **4.1 Introduction**

During their lifetimes, 10-20% of American females will receive medical attention for a UTI and nearly 3% will experience more than one infection per year (Andriole and Patterson, 1991; Patton et al., 1991). UTIs represent the second leading cause of physician visits in the US, costing the health care system over \$2 billion per year (Andriole and Patterson, 1991; Patton et al., 1991). Interestingly, when compared to other mucosal surfaces, the urinary tract is difficult to colonize. Much of the resistance of the urinary tract is attributable to the flushing actions of urine and to the impermeability of the epithelial lining. Because of their specialized role in storing urine, the apical surface of superficial BECs is lined by scalloped-shaped plaques comprising a tightly interlaced latticework of proteins called uroplakins (Apodaca, 2004). These proteins are closely associated with a collection of lipids, sphingolipids and cholesterol that cumulatively constitute a surface that is highly impregnable to urine, solutes and potential pathogens (Apodaca, 2004).

UPEC are uniquely successful in overcoming the bladder defenses, accounting for over 85% of all bladder infections. The singular success of UPEC in the urinary tract is ascribed primarily to its capacity to penetrate and harbor within the superficial BECs

(Duncan et al., 2004; Mulvey et al., 1998). *E. coli* invasion of BECs is achieved by employing a novel mechanism whereby bacteria directly enter specialized exocytic vesicles (Bishop et al., 2007). These compartments (or fusiform vesicles) in BECs perform an important physiologic function by providing the necessary membranes required for bladder expansion. As urine volume increases, these vesicles fuse with the apical bladder surface in a cAMP dependent manner allowing bladder expansion. That *E. coli* are indeed harbored in fusiform compartments in infected BECs was confirmed by immune microscopy which revealed intracellular bacteria encased in membranes closely associated with Rab27b, a marker of fusiform vesicles.

Surprisingly, a significant number of intracellular *E. coli*, harbored within exocytic compartments of BECs, were subsequently returned into the extracellular medium in piecemeal fashion (Bishop et al., 2007). Seemingly, BECs have the innate capacity to sense and expel infecting bacteria. This represents a novel cellular defense mechanism against intracellular bacteria. We sought to elucidate the underlying mechanism for this intriguing host cell response. We show that expulsion of UPEC from infected BECs is mediated by the immune surveillance molecule TLR4 and is triggered by bacterial LPS. We show that the expulsion of bacteria also requires the contribution of cAMP, protein kinase A (a product of cAMP signaling), MyRIP (a PKA anchoring protein), Rab27b (a GTPase associated with fusiform vesicles), and caveolin-1 (a

scaffolding protein also associated with the compartment harboring intracellular *E. coli*). The list of signaling substrates suggest that TLR4 employs a distinct signaling and exocytic mechanism for expelling bacteria from infected BECs.

## **4.2 Experimental Procedures**

### **4.2.1 Bacterial strains and cell lines**

*E. coli* ORN103(pSH2) (chloramphenicol; 100 µg/ml) (Orndorff and Falkow, 1984), *E. coli* CI5 (Abraham et al., 1985; Orndorff and Falkow, 1984; Thankavel et al., 1997), *E. coli* CI5 expressing GFP (ampicillin; 100 µg/ml) (in this study), *E. coli* W3110 (Clementz et al., 1997) , and *E. coli* MLK1067 (chloramphenicol; 100 µg/ml) (Clementz et al., 1997) were grown statically for 24 hours in LB broth with appropriate antibiotics prior to use.

The human BEC line 5637 (ATCC HTB-9), 5637 AC3 knock-down cells (Song et al., 2007b), and 5637 TLR4 knock-down cells (Song et al., 2007a) were grown in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (HyClone), 2.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate. 5637 cells were then incubated in RPMI 1640 (Sigma) supplemented with 2.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 1% BSA (Sigma) during the assays described here. Human primary BECs were maintained in keratinocyte-SFM, containing recombinant epidermal growth factor and

bovine pituitary extract (Invitrogen). All experiments were performed using 5-7 passaged human primary BECs. All cells were cultured at 37 °C with 5% CO<sub>2</sub>.

#### **4.2.2 Bacterial invasion assay**

5637 human BECs were seeded onto 96-well plates at a density of  $4 \times 10^4$  cells/well and incubated overnight. The cells were infected with 100 MOI bacteria for 1 h. The medium was replaced with fresh culture medium containing 100 µg/ml of the membrane-impermeable antibiotic gentamicin (Invitrogen) to kill extracellular bacteria and incubated at 37 °C for additional 30 min. Each well was washed three times with PBS. In order to lyse the cells, 100 µl of 0.1% Triton X-100 in PBS was added to each well and incubated for 15 min. Cells were scraped, diluted, and plated onto LB agar plates containing 80 µg/ml of chloramphenicol. Colonies were counted to quantify the number of invading bacteria.

#### **4.2.3 Bacterial exocytosis assay**

5637 human BECs were seeded onto 96-well plates at a density of  $4 \times 10^4$  cells/well and incubated overnight. Bacteria were diluted in serum free culture media and were added to the monolayer at a MOI 100:1. After 1 h, the cells were washed and treated with 100 µg/ml gentamicin and 100 mM methyl  $\alpha$ -D-mannopyranoside (Sigma) for an additional 30 min. The monolayer was then washed with fresh culture media containing

100 mM methyl  $\alpha$ -D-mannopyranoside. Additional culture media (100  $\mu$ l) plus methyl  $\alpha$ -D-mannopyranoside and bacteriostatic antibiotics 25  $\mu$ g/ml TMP and 125  $\mu$ g/ml SMZ to prevent bacterial growth were then added to each well and incubated with the monolayer for 4 hours. The culture medium was collected and the cell monolayer was washed with an additional 100  $\mu$ l of culture media with methyl  $\alpha$ -D-mannopyranoside, which was also collected and pooled. Twenty-five microliters of the pooled culture media was plated on LB agar plates. To make initial numbers of intracellular bacteria in siRNA BECs similar to the numbers in control BECs, 200 MOI bacteria for Rab27b siRNA BECs and 400 MOI bacteria for Cav1 siRNA BECs were used. For human primary BECs, 1000 MOI of bacteria were added to the wells. Similar initial intracellular numbers were verified by performing a bacterial invasion assay.

#### **4.2.4 Lactate dehydrogenase (LDH) and *E. coli* survival assays**

LDH release assays were performed to examine bladder cell membrane integrity, according to the manufacturer's instructions (Sigma Tox7). BECs were uninfected or infected with 100 MOI *E. coli* and cell viability was measured 4 hrs after infection. Additionally, it was examined that the exocytic compartment encasing *E. coli* did not possess bactericidal activity. Inhibitors of lysosome acidification including  $\text{NH}_4\text{Cl}$  (10 mM) and Bafilomycin (1  $\mu$ M), which neutralize bactericidal activity within lysosomes, caused no improvement in *E. coli* ORN103(pSH2) persistence within 5637 BECs.

#### **4.2.5 Time lapse microscopy**

In order to visualize bacteria, a plasmid expressing GFP (pSMC2) (Bloemberg et al., 1997) was transformed into *E. coli* CI5. Human primary BECs were cultured on a coverslip in 12-well plate and infected with 500 MOI of *E. coli* CI5 expressing GFP for 1 hour. In 1 hour, cells were washed with media containing 100 mM methyl  $\alpha$ -D-mannopyranoside (Sigma) and treated with 100  $\mu$ g/ml gentamicin for 30 min to kill extracellular bacteria. Cells were washed and incubated in freshly prepared media containing methyl  $\alpha$ -D-mannopyranoside to prevent re-entry of bacteria for 3 hours. Then, cells were set on a microscope to take a series of images. To distinguish extracellular bacteria from intracellular bacteria, infected cells were immuno-stained with an Alexa546-labeled *E. coli* antibody prior to time lapse microscopy. An *E. coli* antibody was prelabelled with Alexa546 dye using an antibody labeling kit (Molecular Probes).

#### **4.2.6 Measurement of intracellular cAMP levels**

Detailed experimental procedures were described in Chapter 2 and 3.

## **4.2.7 RNA Interference**

### **4.2.7.1 Creation of TLR4 and AC3 knockdowns**

Detailed information to create TLR4 and AC3 knockdown BECs was described in Chapter 2.

### **4.2.7.2 Knockdowns of Rab27b, Caveolin1, and MyRIP**

Negative control siRNA (siRNA ID # 120374), Rab27b siRNA (siRNA ID # 120374), Caveolin1 siRNA (siRNA ID # 10479), and MyRIP siRNA (siRNA ID # S24759) were purchased from Ambion and used for knocking down specific genes in BECs according to the vendor's recommendation (Ambion). Briefly BECs were seeded and incubated overnight. On the following day, BECs were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) reagents as recommended by the vendors (Invitrogen and Ambion). Cells were washed 6 hours after transfection and incubated in freshly culture media for additional 48 hours. For assays, cells were trypsinized and transferred to appropriate culture dishes one day before experiments performed.

### **4.2.8 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

Total cellular RNA was isolated using RNeasy purification system (Qiagen). Two  $\mu\text{g}$  of total RNA was reverse transcribed and amplified with gene-specific primers using

the RT-PCR System kit (Bio-rad). The primer sequences for the genes were as follows: 5'-CGATTCCATTGCTTCTTG-3' (sense) and 5'-GCTCAGGTCCAGGTTCTT-3' (antisense) for TLR4 and 5'-ATCCCATCACCATCTTCCAG-3' (sense) and 5'-CCTGCTTCACCACCTTCTTG-3' (antisense) for GAPDH, 5'-GTGGTTTCTTTGAACCCATA-3' (sense) and 5'-GAGTTAGGCTTTTGTGATGC-3' (antisense) for MyRIP, and 5'-TACTCGGTGGAGAAGGAGAAGCAG-3' (sense) and 5'-CGAAAACGCTTGTGGTCGTATTC-3' (antisense) for AC-3 (554 bp).

#### **4.2.9 Generation of BECs overexpressing GFP-Rab27b**

To generate the GFP-Rab27b, the forward primer 5'-GATCTCGAGCTATGACCGATGGAGACTATGAT-3' and reverse primer 5'-GGTGGATCCCTAGCAGATACATTTCTTCTCTG-3' (Integrated DNA Technologies) were used to amplify Rab27b from 5637 BECs by RT-PCR. The RT-PCR products were digested with XhoI and BamHI, and then ligated to XhoI/BamHI-digested pLEGFP-C1 (Clontech) to generate pGFP-Rab27B. The Amphopack-293 Cell Line (BD Biosciences) was used to produce the viral particles. Production of viral particles, infection of target cell line (5637), and selection of viral infected cells were performed as recommended by the vendor (BD Biosciences). The geneticin-resistant stable-transfected cell lines were generated and verified under a fluorescence microscope.

#### **4.2.10 Sucrose Density Fractionation of BECs**

BECs expressing GFP-fused Rab27b were grown to ~80% confluence on 15 cm cell culture dishes. Two plates were infected with 100 MOI *E. coli* ORN103(pSH2) in 25 ml of serum-free medium for 2 h. Infected plates, along with uninfected plates, were washed five times with ice-cold PBS and scraped off the plates using a rubber policeman in 2 ml of homogenization buffer (10 mM Tris (pH 7.2), 2 mM EDTA, 1 mM PMSF, and a 1:100 dilution of mammalian protease inhibitor mixture (Sigma)). The cell suspension was then passed 20 times through a 21-gauge needle, brought to 45% sucrose by the addition of an equal volume of 90% sucrose, and then overlaid with 4 ml each of 35, and 5% sucrose. The gradients were centrifuged for 18 h at 39,000 rpm in a SW41Ti rotor (Beckman Instruments, Palo Alto, CA), and 12 equal fractions were collected from the top of each gradient and assayed for Caveolin1 and Rab27b by Western blotting using polyclonal anti-caveolin-1 antibody (BD Transduction Laboratories) and monoclonal anti-GFP-antibody (Santa Cruz Biotechnology).

#### **4.2.11 Immunoprecipitation**

GFP or GFP-fused Rab27b were immunoprecipitated from 5637 BECs overexpressing GFP or GFP-Rab27b. Briefly, 5637 BECs were uninfected or infected with 500 MOI *E. coli* ORN103(pSH2). In 2 hrs, cells were washed and harvested in 0.5 ml of 1X RIPA buffer (Millipore) plus protease inhibitor cocktails (Sigma) and 1 mM PMSF. The

harvested cells were then passed 20 times through a 21-gauge needle and microcentrifuged for 10 min at 4 °C. The cell lysates were immediately used for immunoprecipitation assays. For the assay, 200 µl cell lysates were mixed with anti-GFP antibody and incubated with gentle rocking overnight at 4 °C. 50 µl of a 50% suspension of protein A-Sepharose (Upstate) was added, and the sample was further incubated for 2 h at 4 °C. The immunoprecipitates were washed five times with 1 ml of 1 x RIPA buffer (Millipore), resuspended in 60 µl of 2x Laemmli sample buffer (Bio-Rad) and boiled for 5 min. Immunoprecipitates were assayed for Caveolin-1 using polyclonal anti-Caveolin 1 antibody (BD Transduction laboratories).

In order to examine that Rab27b, MyRIP, PKA, and Cav1 are in the same binding complex in BECs, Rab27b, MyRIP, and PKA were immunoprecipitated from either uninfected or infected 5637 BECs. Bladder cell lysates were prepared as described above and immediately used for an immunoprecipitation assay. Primary antibodies used in this assay were a rabbit anti-Rab27b IgG (IBL), a goat anti-MyRIP IgG (Abcam), and a mouse anti-PKA RIIa IgG (BD Transduction laboratories). Immunoprecipitates were prepared as described above and assayed for Caveolin-1. Each sample was boiled for 5 min and run on a 4-20% Tris-glycine SDS-PAGE gel (Bio-Rad). Interactions among the proteins were revealed with a primary rabbit anti-Cav1 antibody (BD Transduction

laboratories) and either a secondary HRP-conjugated Clean-bot IP detection reagent (Thermo scientific) or a secondary HRP-conjugated mouse IgG (Bio-Rad).

#### **4.2.12 Immunofluorescence microscopy**

5637 BECs overexpressing GFP-Rab27b were seeded onto 12-mm diameter glass coverslips and grown overnight. The cells were fixed overnight in 1% paraformaldehyde in PBS. After removing the fixative, the cells were permeabilized and blocked with saponin buffer (0.05% saponin, 10 mM HEPES, 10 mM glycine, 10% goat serum). The cells incubated with primary antibodies diluted in saponin buffer for 30 min at RT, washed three times with saponin buffer, and incubated with secondary antibodies in saponin buffer for 30 min at RT. A primary polyclonal antibody against Caveolin (BD Transduction Laboratories) was revealed with donkey anti-rabbit IgG Cy5 (Molecular Probes). Coverslips were mounted with Prolong Gold antifade reagent (Molecular Probes) and examined using a Nikon confocal laser scanning instrument with appropriate filter sets.

#### **4.2.13 Statistical analysis**

Unpaired T-tests were performed on all data sets in order to determine the statistical significance of experimental changes from control values. A Fisher test was

performed on the 24 hr infection experiments to determine the statistical significance of the drop in infection rate percentage. The  $\alpha$ -level for significance was 0.05.

## **4.3 Results**

### **4.3.1 Expulsion of *E. coli* from infected human primary BECs**

In recent *in vivo* and *in vitro* studies, we reported that type 1 fimbriated *E. coli* invade BECs by usurping distinct cAMP regulated exocytic compartments named fusiform vesicles (Bishop et al., 2007). Surprisingly, a significant number of these bacteria were subsequently released into the extracellular medium with limited loss of host cell viability. When we assessed intracellular bacterial numbers at different time points after initial infection and following gentamicin treatment to kill extracellular bacteria, an appreciable (~20%) drop in intracellular bacterial numbers was observed in infected BECs by 4 hrs, and by 24 hrs this drop was over 75% of original intracellular numbers (Fig. 4.1A). It appeared that the loss of intracellular bacteria was not due to either death of bladder cells (Fig. 4.1C) or death of infected bacteria (Fig. 4.1D), but rather due to bacterial exocytosis from infected BECs. Intracellular bacterial numbers at 0 hr after the gentamicin treatment were similar to the sum of bacterial numbers of intracellular and extracellular location at 4 hrs after the treatment (Fig. 4.1B). In order to exclude complications due to extracellular bacterial growth, we incorporated bacteriostatic antibiotics TMP and SMZ in the culture media. We also examined for

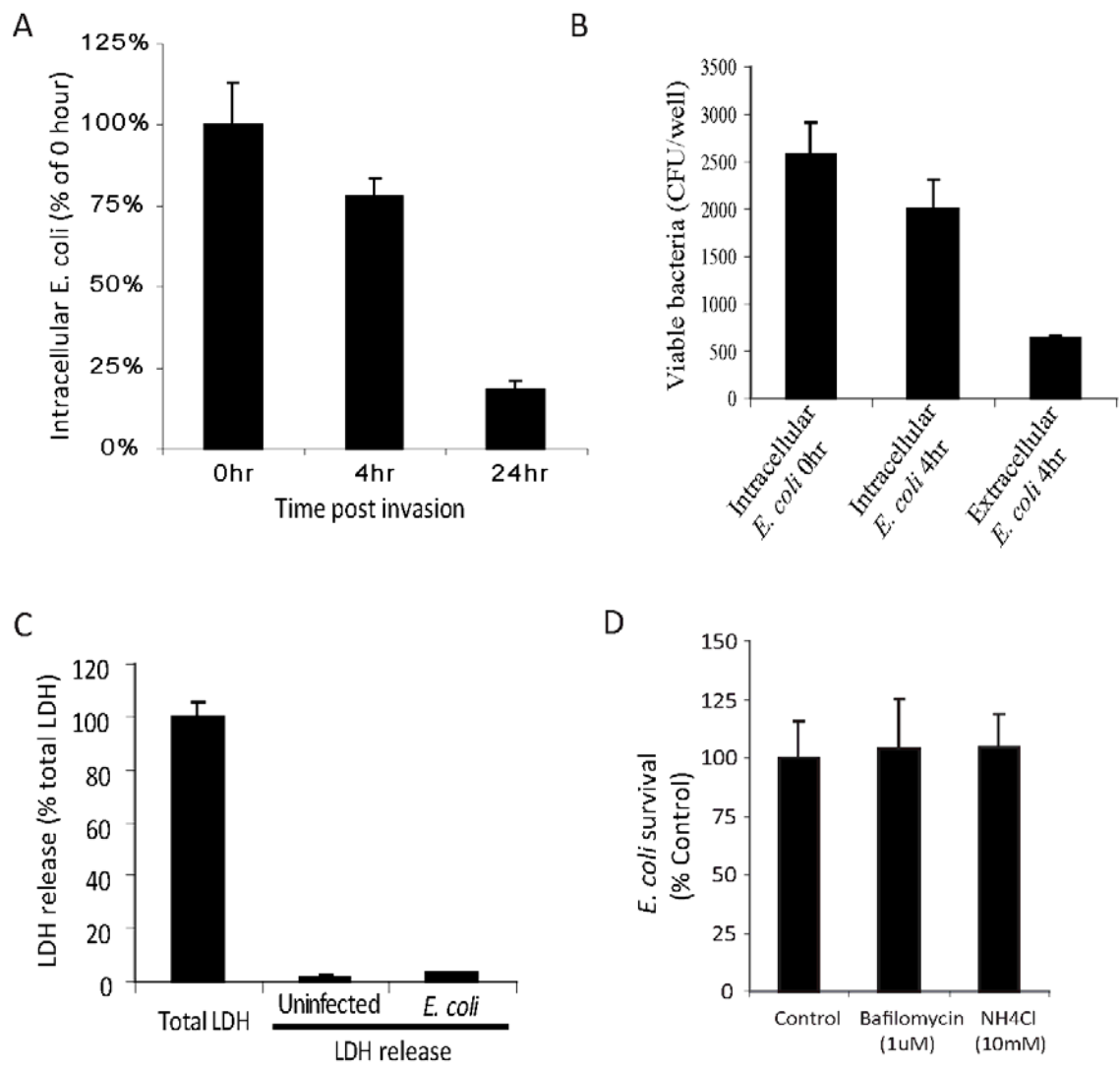
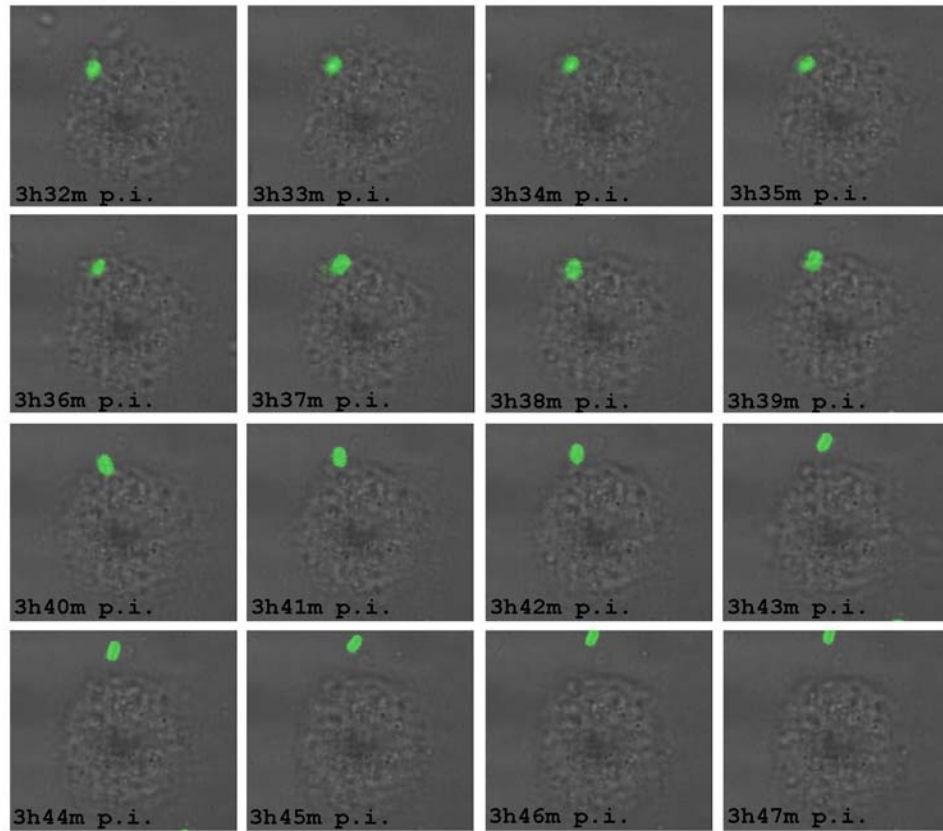


Figure 4.1

**Figure 4.1: *E. coli* exocytosis from infected BECs.** **A**, 5637 BECs were infected with *E. coli* ORN103(pSH2) for 1 h after which a standard gentamicin protection assay was performed to quantify the number of intracellular *E. coli* (0 hr). A significant decrease in intracellular *E. coli* was seen as early as 4 h after gentamicin treatment (4 hr) and continued until 24 h after the addition of gentamicin (24 hr). **B**, Numbers of intracellular and extracellular bacteria in BECs at 0 hr and 4 hr after gentamicin treatment. The sum of numbers of intracellular and extracellular bacteria at 4 hr after gentamicin treatment was similar to the numbers of intracellular bacteria at 0 hr, suggesting bacteria exocytosis from infected BECs. **C**, BEC membrane integrity was assessed by a lactose dehydrogenase (LDH) release assay. *E. coli* infection to BECs for 4 hrs caused no difference in LDH releases from uninfected BECs. **D**, Treatment of infected BECs with NH<sub>4</sub>Cl and Bafilomycin which neutralize bactericidal activity within lysosomes caused no change of numbers of intracellular bacteria, indicating the compartment harboring *E. coli* did not possess bactericidal activity.

possible bacterial growth within BECs and found no appreciable increase of bacteria numbers between 1 and 4 hrs post infection (data not shown). Cumulatively, *E. coli* are exocytosed from infected BECs. Since we used immortalized human BECs, it was important to validate our observation of bacterial exocytosis in primary human BECs. Therefore, we investigated if freshly isolated and cultured human BECs would be able to expel intracellular bacteria. We cultured primary bladder cells obtained from fresh bladder biopsies as described previously (Cilento et al., 1994). These cells exhibited characteristics of primary BECs including expression of uroplakin 1a, a marker of the asymmetrical unit membrane, the junctional complex protein ZO1, as well as cytokeratin, all of which are hallmarks of terminal differentiation in bladder umbrella cells (data not shown). We infected human primary BECs with UPEC strain CI5 expressing GFP, after which we killed all extracellular *E. coli* through gentamicin treatment for 30 min. The cells were then vigorously rinsed and fresh culture media including bacteriostatic antibiotics and D-mannose were added. In order to distinguish extracellular bacteria from intracellular bacteria, we added an Alexa546-conjugated *E. coli* antibody to immunostain extracellular *E. coli*. Videomicroscopy was employed to visualize exocytosis of bacteria from infected BECs. Shown in Fig. 4.2A are time-frames from 3 hr 32 min post infection depicting the exocytosis of intracellular *E. coli* from an infected BEC. We noticed the shedding of bacterial membranes, in the form of discrete vesicles, as the intracellular bacteria emerge into the extracellular environment.

A



B

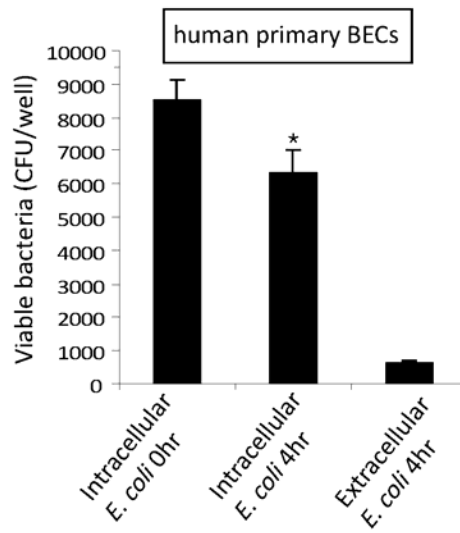


Figure 4.2

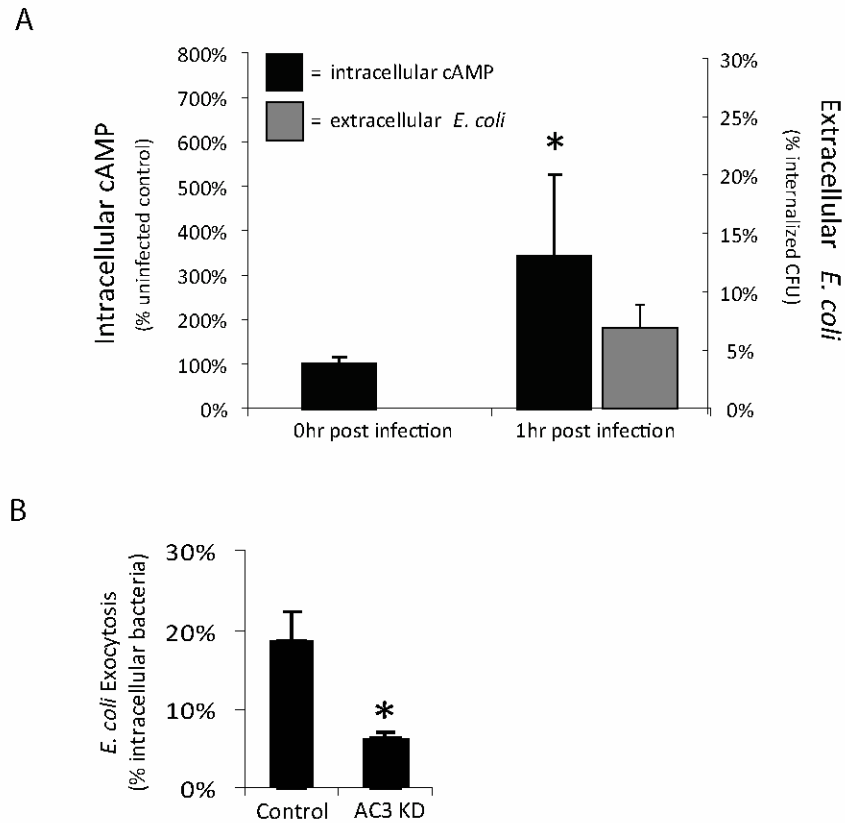
**Figure 4.2: *E. coli* exocytosis from human primary BECs.** **A**, Time lapse microscopy was performed to examine *E. coli* exocytosis from human primary BECs. Primary BECs were infected with *E. coli* CI5 expressing GFP for 1 hr after which gentamicin was added to the culture media to kill extracellular bacteria. Then, infected BECs were incubated in a fresh medium and used for time lapse microscopy. Videomicroscopy was employed to visualize exocytosis of bacteria from infected. Shown here is a movie depicting the exocytosis of intracellular *E.coli* within one minute time frame from an infected BEC. In this case, *E. coli* began to exocytose from infected primary BECs ~3.5 hrs after the gentamicin treatment. **B**, Quantitative bacterial exocytosis assays were performed using human primary BECs. Significant numbers of intracellular bacteria were expelled from infected primary BECs at 4 hrs after the gentamicin treatment. \*  $P < 0.05$  by unpaired T-test when compared to numbers of intracellular *E. coli* at 0 hr; Error bars represent S.D..

Quantitative analysis of these events revealed that ~11% of intracellular *E. coli* was expelled from infected human primary BECs during the first 4 hrs of incubation (Fig. 4.2B). Taken together, these data reveal that *E. coli* exocytosis by infected BEC is a dynamic and relatively rapid event.

### **4.3.2 Expulsion of *E. coli* from infected BECs is cAMP dependent**

Since regular exocytosis of fusiform vesicles is dependent on cAMP, we hypothesized that bacterial expulsion from infected BECs would also be dependent on intracellular levels of cAMP. To determine if there was a relationship between intracellular cAMP levels and bacterial expulsion, we assayed intracellular cAMP levels in *E. coli* infected BECs at 0, 1 and 4 hrs post antibiotic treatment (Fig. 4.3A, black bars). We found that cAMP levels in the infected BECs had significantly increased over baseline levels by 1 hr and were markedly higher levels (600% of baseline levels) by 4 hrs (Fig. 4.3A, black bars). We also assayed bacteria expulsion at the same time points (Fig. 4.3A, grey bars). There was a good correlation between intracellular cAMP levels in BECs and expulsion of *E. coli* (Fig. 4.3A, black and grey bars).

To further demonstrate the requirement of cAMP for bacterial expulsion, we used RNA interference techniques to knock down the enzyme responsible for cAMP



**Figure 4.3: Expulsion of *E. coli* from infected BECs is cAMP dependent. A,** Intracellular cAMP levels were determined by a cAMP enzyme immunoassay either before, or after 1 h of *E. coli* ORN103(pSH2) infection of 5637 BECs. At the same time, the number of exocytosed *E. coli* was determined by sampling the extracellular media 1 h after gentamicin treatment. The increase in extracellular *E. coli* correlated to the increase in intracellular cAMP. **B,** Control BECs and AC3 knockdown (kd) BECs were infected for 1h with *E. coli* ORN103(pSH2). Following a 30 min gentamicin treatment, the *E. coli* infected BECs were incubated with a fresh medium containing bacteriostatic antibiotics and D-mannose. After 4 h, the medium was cultured for extracellular *E. coli* and revealed that AC3 kd BECs were significantly less effective in expelling intracellular *E. coli* than the control BECs. \*  $P < 0.05$  by unpaired T-test when compared to uninfected controls; Error bars represent S.D. in A and S.E.M. in B.

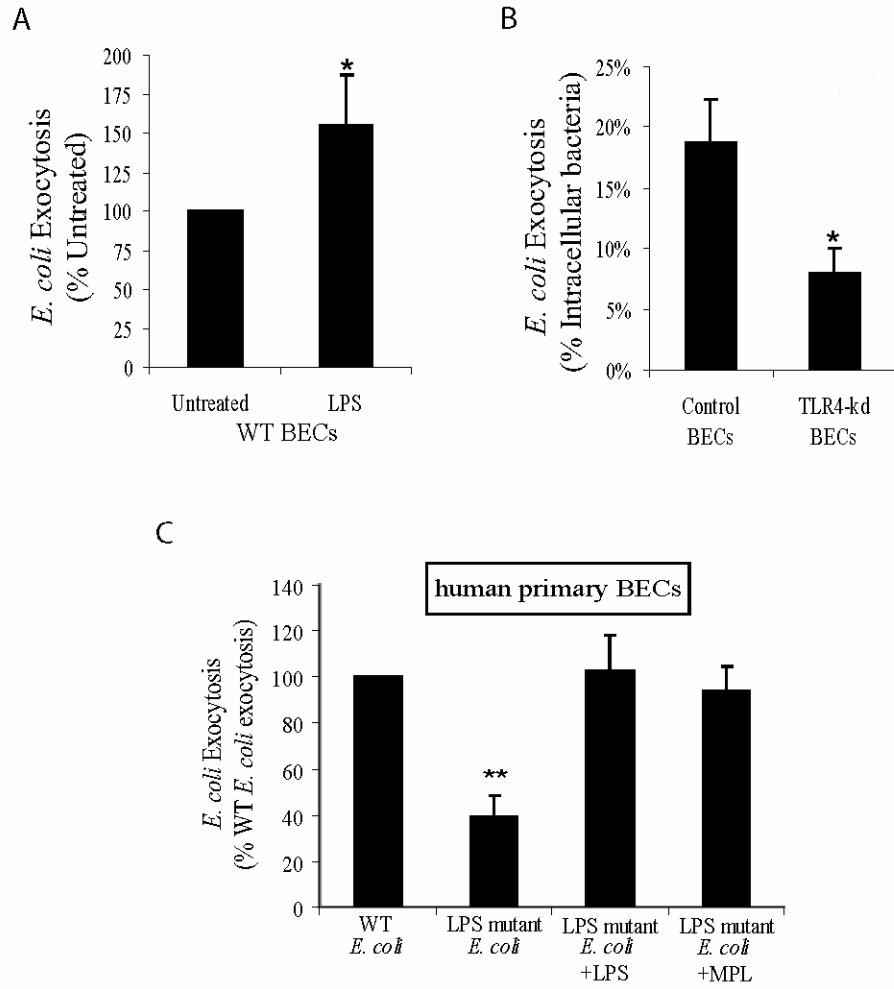
production during *E. coli* infection of BECs, adenylyl cyclase 3 (AC3) ((Song et al., 2007b)). The production of AC3 mRNA was decreased in AC3 knockdown (kd) BECs by approximately 80% (Fig. 2.4B). We compared the ability of both sham transfected BECs and AC3 kd BECs to expel *E. coli* 4 hrs following infection, which was the time of maximal cAMP production and bacterial expulsion as seen in Fig. 4.3A. AC3 kd BECs were significantly less effective in expelling intracellular *E. coli* than control BECs (Fig. 4.3B). Taken together, these observations suggest that expulsion of intracellular *E. coli* by infected BECs is dependent on intracellular cAMP levels.

#### **4.3.3 *E. coli* expulsion from infected BECs is initiated by TLR4**

In order to identify the cellular component initiating bacterial expulsion, we sought to investigate what factors were responsible for elevating intracellular cAMP levels. We have previously reported that following exposure to *E. coli* or LPS, TLR4 on BECs activates AC3 resulting in a powerful cAMP response (Song et al., 2007b). Conceivably, TLR4 could be the determinant on infected BECs responsible for cAMP mediated *E. coli* expulsion. To examine this possibility, we added ultra-purified soluble *E. coli* LPS to BECs already infected with *E. coli* to see if this agent would accelerate bacterial expulsion. Due to the possibility of lipoprotein contamination of LPS prepared by TCA or PCP extraction, LPS ultra purified by ion-exchange chromatography and verified to contain <1% protein was used in this study (Sigma; *E. coli* 055:B5 LPS).

Treatment of BECs with LPS resulted in a significant increase in *E. coli* expulsion (Fig. 4.4A). To confirm that the LPS on *E. coli* was the primary determinant responsible for activating BECs, we sought to show that the activation of BECs involved TLR4, the cognate receptor for LPS, we, again, employed RNA interference to generate BEC transfectants whose TLR4 mRNA levels were reduced by ~50% when compared to sham transfected BECs (Fig. 2.1B). As predicted, when compared to control BECs, bacterial expulsion from TLR4 kd BECs was markedly decreased by ~50%, which is consistent with the decrease in TLR4 mRNA levels (Fig. 4.4B).

To further implicate TLR4 in the expulsion of *E. coli* from infected human primary BECs, we compared the exocytosis of wild type *E. coli* W3110 and its LPS modified derivative *E. coli* MLK1067. *E. coli* MLK1067 contains a mutation in the *msbB* gene such that its LPS has limited ability to activate TLR4 (Clementz et al., 1997). We found that the level of expulsion of the *msbB* mutant by infected human primary BECs was markedly lower than the level observed with the wild type *E. coli* W3110, which was reversed by pretreatment of human primary BECs with soluble LPS or LPS analog MPL (Fig. 4.4C). Taken together, TLR4 modulates bacterial exocytosis from infected BECs and is likely mediated by the same LPS-initiated signaling mechanisms that have been reported to increase intracellular cAMP within BECs.



**Figure 4.4**

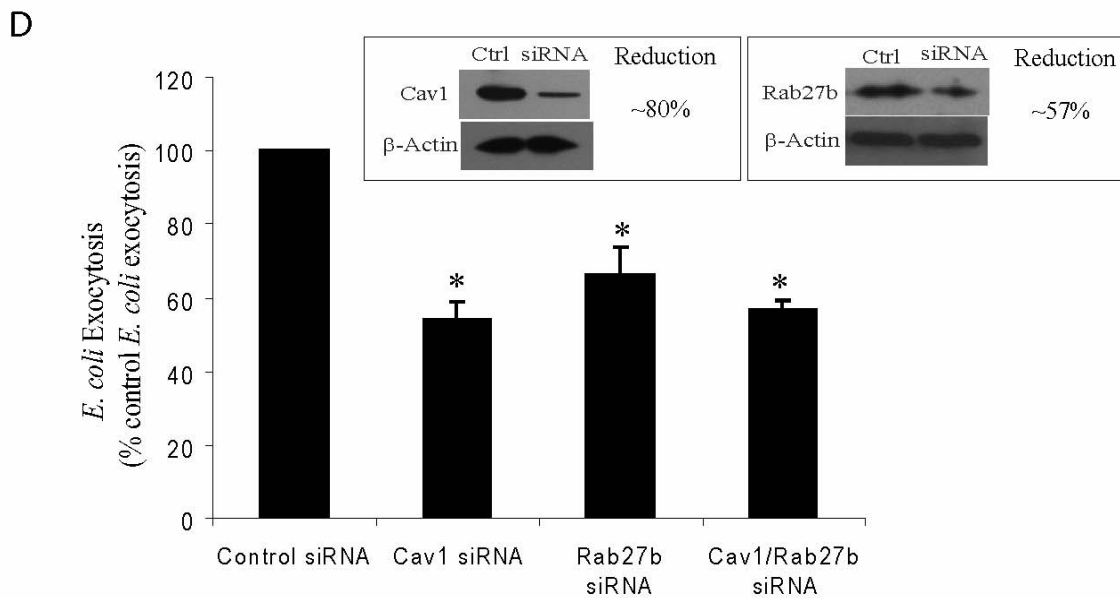
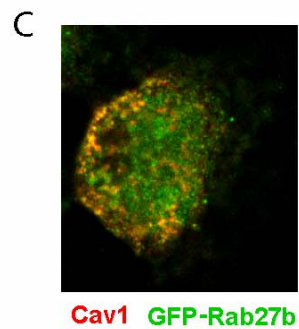
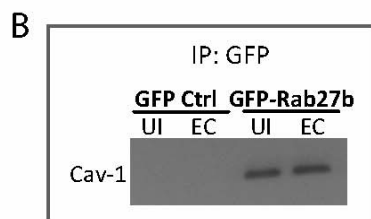
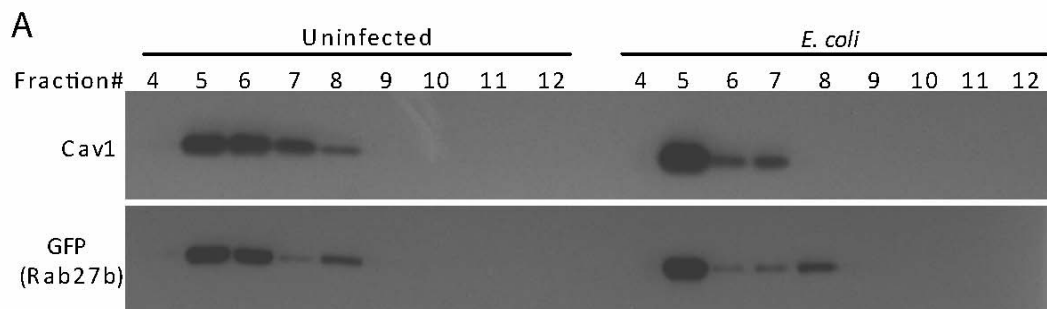
**Figure 4.4: *E. coli* expulsion from infected BECs is initiated by TLR4.** **A**, Wild-type BECs were infected for 1h with *E. coli* ORN103(pSH2). When cells were treated with gentamicin, 100 µg/ml LPS was added to the culture medium to examine the effect on bacterial exocytosis, when indicated. Following a 30 min gentamicin and LPS treatment, the infected BECs were incubated with fresh media containing bacteriostatic antibiotics and D-mannose. After 4 h, the medium was cultured for extracellular *E. coli*. Treatment of BECs with LPS (LPS) resulted in a significant increase in *E. coli* exocytosis compared to untreated BECs (Untreated). **B**, Exocytosis assays were performed using control and TLR4 kd BECs as described above except LPS treatment. TLR4 kd BECs were significantly less effective in expelling intracellular *E. coli* than the control BECs. \* P < 0.05 by unpaired T-test when compared to controls; Error bars represent S.E.M.. **C**, Human primary BECs were utilized for exocytosis assays. Primary BECs were infected with either WT *E. coli* (*E. coli* W3110) or LPS mutant *E. coli* (a msbB mutant *E. coli* MLK1067). When indicated, LPS or MPL were added to the media as described before. \*\* P < 0.0005 by unpaired T-test when compared to WT *E. coli* infected BECs. Error bars represent S.D..

#### **4.3.4 *E. coli* exocytosis by BECs is dependent on Rab27b and Caveolin-1**

In order to gain a better understanding of how TLR4 and cAMP were modulating bacterial exocytosis from infected BECs, we sought to identify the role, if any, of signaling substrates previously implicated in the entry of *E. coli* into BECs. Here, we focused on two of these substrates, Rab27b and Caveolin-1. It was previously shown that, following entry into BECs, *E. coli* became encased in fusiform vesicles, highly enriched in Rab27b (Bishop et al., 2007). Rab27 has previously been implicated in the regulated delivery and/or targeting of fusiform vesicles to the apical plasma membrane of BECs (Chen et al., 2003). Of the two Rab27 isoforms identified, human BECs express only the b isoform (data not shown) (Chen et al., 2003). It was hypothesized that fusion of Rab27b enriched fusiform vesicles into the plasma membrane was a preceding event in the entry of *E. coli* into BECs (Bishop et al., 2007). Indeed, knockdowns of Rab27b expression in BECs significantly reduced entry of *E. coli* into BECs (Bishop et al., 2007). Interestingly, Duncan et al. had previously shown that entry of *E. coli* into BECs was critically dependent on the scaffolding and signaling molecule Caveolin-1. Knockdowns of Caveolin-1 in BECs were found to markedly reduce entry of *E. coli* (Duncan et al., 2004).

First, we sought to examine if Rab27b and Caveolin-1 were in any way coassociated. In order to facilitate detection of Rab27b in BECs, we transfected these cells

with GFP-fused Rab27b. Since Caveolin-1 is typically found in cellular microdomains called lipid rafts, we homogenized uninfected and *E. coli* infected BECs, and fractionated cell extracts on a sucrose gradient. We investigated if Rab27b was detectable in the lipid raft microdomains where most of the Caveolin-1 is typically found. Shown in Fig. 4.5A is a Western blot of the various cellular fractions probed with a Caveolin-1-specific antibody. Caveolin-1 was distributed in fractions 5 to 8 before infection and interestingly, after infection the majority of this protein had shifted into fraction 5 (Fig. 4.5A). Remarkably, a similar distribution pattern was observed when the fractions were probed for Rab27b, employing a GFP-specific antibody, suggesting the possible colocalization of Caveolin1 and Rab27b both before and after infection (Fig. 4.5A). However, it is noteworthy that the colocalization between the two molecules seemed to increase following bacterial infection (Fig. 4.5A). To investigate if the two molecules were actually coassociated with each other, we performed a pull down assay using a GFP-specific antibody and observed specific coassociation between Rab27b and Caveolin1 since control cells expressing only GFP did not pull down Caveolin1 as a binding partner (Fig. 4.5B). Consistent with the Western blots of the cell fractions, coassociation of Rab27b with Caveolin1 was slightly increased after infection (Fig. 4.5B).



**Figure 4.5**

**Figure 4.5: *E. coli* exocytosis by BECs is dependent on Rab27b and Caveolin-1.** **A**, Cell extracts from uninfected and infected (*E. coli*) BECs were fractionated on a sucrose gradient. Rab27b and Caveolin-1 were distributed in fractions 5 to 8 in uninfected BECs and after infection the majority of these proteins had shifted into fraction 5. **B**, Coassociation between Rab27b and Caveolin-1 was investigated using a pull down assay. Specific coassociation between Rab27b and Caveolin1 was observed since control cells expressing only GFP (GFP Ctrl) did not pull down Caveolin1 as a binding partner. UI, uninfected; EC, *E. coli* infected. **C**, Indirect immunofluorescence showed colocalization between Caveolin-1 (Red) and Rab27b (Green). **D**, Rab27b and/or Caveolin-1 expression levels in regular BECs were reduced by using gene specific siRNAs. Compared to levels in sham siRNA transfected cells (Ctrl), protein expression levels of Rab27b and Caveolin-1 were reduced by ~57% and by ~80% in kd cells (siRNA), respectively (inset). We observed a significant decrease in bacterial exocytosis in Caveolin-1 kd BECs (Cav1 siRNA), in Rab27b kd BECs (Rab27b siRNA), and in double kd BECs (Cav1/Rab27b siRNA) compared to controls. \*  $P < 0.03$  by unpaired T-test when compared to control siRNA transfected BECs. Error bars represent SEM.

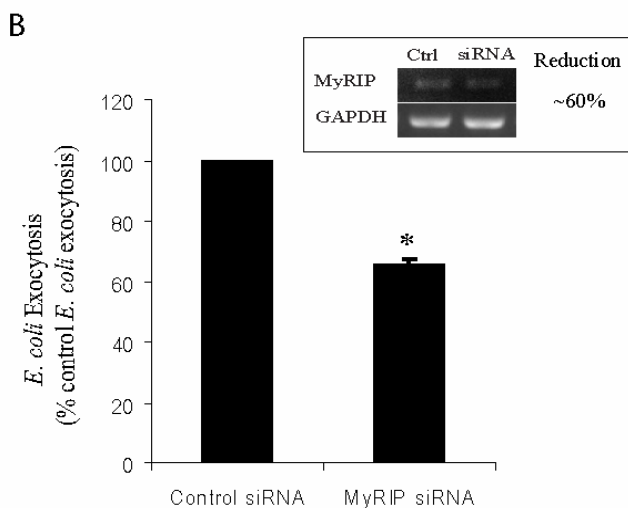
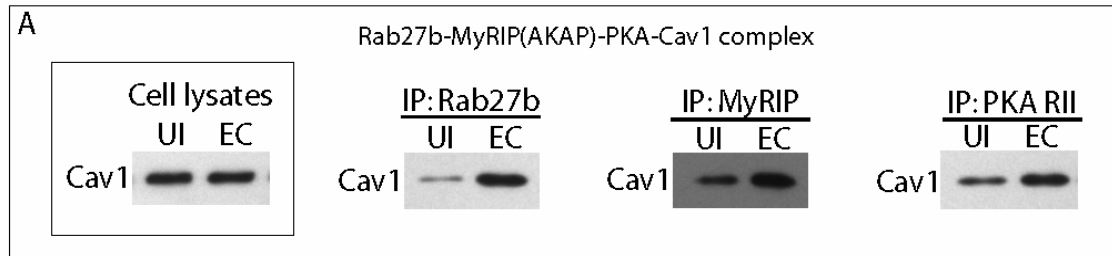
Using an indirect immunofluorescence assay, uninfected BECs expressing GFP-fused Rab27b were immunostained with a Caveolin1 specific antibody and found that a good proportion of Rab27b positive vesicles was enriched in Caveolin-1 (Fig. 4.5C).

In view of the coassociation of Rab27b and Caveolin-1, we sought to examine their involvement in bacterial exocytosis from infected BECs. We reduced Rab27b and/or Caveolin-1 expression levels in regular BECs by using gene specific siRNAs. Compared to levels in sham siRNA transfected cells, protein expression levels of Rab27b and Caveolin-1 were reduced by ~57% and by ~80% in kd cells, respectively (Fig. 4.5D, inset). We observed a significant decrease in bacterial exocytosis in Caveolin-1 kd BECs and in Rab27b kd BECs compared to controls (Fig. 4.5D). Consistent with this finding, when we knocked down both Rab27b and Caveolin1 we also found a decrease in *E. coli* expulsion (Fig. 4.5D). Taken together our observations indicate that the two molecules previously implicated in the entry of *E. coli* into BECs, Rab27b and Caveolin-1, not only contribute to bacterial exocytosis but also appear to do so through interactions with each other.

#### **4.3.5 *E. coli* expulsion by BECs requires MyRIP activity**

To connect TLR4-initiated and cAMP-mediated signaling with bacterial expulsion that was mediated by Rab27b-Caveolin1 complexes, we searched for known intermediates of TLR4/cAMP signaling that could potentially interact with either Rab27b

or Caveolin1. MyRIP/Slac2c is a candidate of interest, since it is a scaffolding protein that has been previously shown to tether protein kinase A (PKA), a downstream effector of the TLR4/cAMP pathway, to exocytic compartments by binding with Rab27b (Goehring et al., 2007). Interestingly, MyRIP/Slac2c has also been shown to mediate exocytic activities such as melanosome and insulin exocytosis and through its interaction with Rab27b (Fukuda and Kuroda, 2002; Waselle et al., 2003). Conceivably, MyRIP serves as the link between the TLR4/cAMP signaling and the Rab27b/Caveolin-1 complexes found localized on the the fusiform vesicle membranes encapsulating bacteria. To see if MyRIP associates with the Rab27b/Caveolin-1 complexes in BECs, we performed a pulldown assay using Rab27-, MyRIP-, and PKA RII-specific antibodies. Control immunoblots demonstrated that equal levels of Caveolin-1 protein were expressed in the cell extracts before and after infection (Fig. 4.6A inset). Interestingly, all these signaling molecules were coassociated with Caveolin-1 in BECs even before infection and after infection, the coassociation became markedly more increased (Fig. 4.6A), consistent with the remarkable shifts of Caveolin-1 and Rab27b to fraction 5 after *E. coli* infection (Fig. 4.5A). These coassociations were specific since when a pulldown assay was performed with a lysosomal marker Lamp1-specific antibody, no band was detected when the samples were probed for Caveolin-1 on a Western blot (data not shown). To further demonstrate the functional contribution of MyRIP in *E. coli* expulsion by BECs, we examined the effects of knocking down MyRIP expression



**Figure 4.6: *E. coli* expulsion by BECs requires MyRIP activity.** **A**, A pulldown assay was performed using Rab27-, MyRIP-, and PKA RII-specific antibodies. A control immunoblot revealed that equal levels of Caveolin-1 protein were expressed in the cell extracts before and after infection (inset). All these signaling molecules were coassociated with Caveolin-1 in BECs even before infection (UI) and after infection (EC), the coassociation became markedly more increased. **B**, An *E. coli* exocytosis assay was performed to examine effects of MyRIP on bacteria expulsion. MyRIP mRNA levels were reduced by ~60% in kd BECs (inset) and levels of *E. coli* exocytosis were significantly dropped by 40%. \*  $P < 0.002$  by unpaired T-test when compared to control siRNA transfected BECs. Error bars represent SEM.

employing a gene specific siRNA on *E. coli* expulsion. MyRIP mRNA levels were reduced by ~60% in MyRIP kd BECs (Fig. 4.6B inset) and levels of *E. coli* exocytosis were significantly dropped by 40% (Fig. 4.6B). Taken together, MyRIP plays a key role in modulating *E. coli* exocytosis by serving as a critical conduit for signaling between TLR4 and the exocytic compartment housing intracellular bacteria.

#### **4.4 Discussion**

With the growing realization that pathogens seek refuge in host cells at one or more stages in the infectious process, most research has focused on elucidating the various strategies employed by pathogens to enter and avoid microbicidal activity of infected host cells. Hence, any intrinsic capacity of host cells to sense and expel infecting pathogen has gone largely unnoticed. Here we report that BECs have the capacity to expel intracellular bacteria and that the critical sensory molecule responsible for bacterial expulsion is TLR4. Evidence implicating TLR4 comes from the observations that treatment of BECs with soluble LPS shows higher levels of *E. coli* expulsion compared to untreated BECs and that knocking down TLR4 expression in BECs markedly reduces *E. coli* expulsion compared to controls. In addition, the level of exocytosis of wild type *E. coli* is markedly higher than an LPS mutant that fails to activate TLR4 and when its failure is rescued by LPS or MPL, the levels of exocytosis of

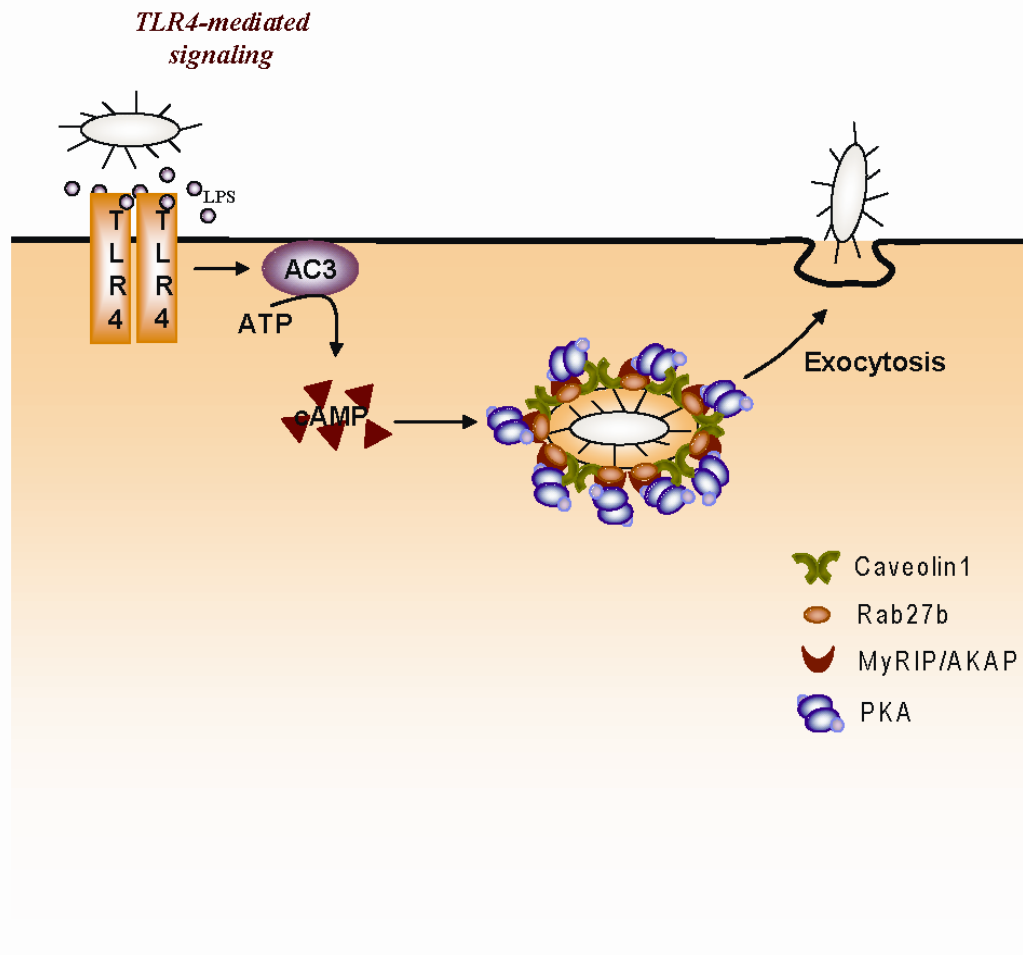
LPS mutant *E. coli* are returned to the levels seen in exocytosis of wild type *E. coli* (Fig. 4.4). TLR4 mediated expulsion of *E. coli* by infected BECs is possible because cAMP is a major byproduct of TLR4 signaling (Song et al., 2007b) and because the intracellular bacteria are harbored within specialized exocytic compartments that are primed to exocytose when intracellular cAMP levels become elevated (Bishop et al., 2007).

Expulsion of intracellular *E. coli* by infected BECs represents a novel function for TLR4. In the urinary tract, TLR4 is largely known for initiating the rapid and vigorous neutrophil responses to *E. coli* and other gram negative bacterial infections (Freundus et al., 2001; Svanborg et al., 2001; Svanborg et al., 2006). Upon sensing LPS on infecting bacteria, TLR4 molecules on BECs and other uroepithelial cells trigger the release of inflammatory cytokines including neutrophil chemoattractants (Freundus et al., 2001; Schilling et al., 2003; Schilling et al., 2001; Song et al., 2007b; Svanborg et al., 2001; Svanborg et al., 2006). While these recruited phagocytes are effective in clearing extracellular pathogens, they are largely ineffective in eliminating bacteria hidden within superficial BECs. Thus, TLR4 mediated expulsion of intracellular bacteria could be viewed as a mechanism to improve efficiency of neutrophil mediated bacterial clearance. Our findings may also help explain why TLR4 mutant mice are notoriously unable to clear *E. coli* bladder infections. This limitation was previously ascribed to the inability of these mice to mount a vigorous cytokine and neutrophil response following

bladder infection (de Man et al., 1989; Haraoka et al., 1999; Schilling et al., 2001).

However, in view of the role of TLR4 in expelling intracellular *E. coli* and the recently reported role of TLR4 in inhibiting invasion of BECs by *E. coli* (Song et al., 2007a), the inability of TLR4 mutant mice to clear *E. coli* infections may be attributable, at least in part, to increased numbers of bacteria harbored within BECs.

Although cAMP-dependent exocytosis of fusiform vesicles into the apical plasma membrane of superficial BECs is a normal physiological event, relatively little is known regarding the molecular machinery involved. We have demonstrated the critical role played by Rab27b and Caveolin-1, both of which have previously been shown to be constituents of the vesicle encasing intracellular *E. coli* and also shown to be critical for bacterial entry. How TLR4 on BECs regulates bacterial exocytosis begins to understand. Our studies reveal that MyRIP/Slac2c plays a critical role in linking TLR4 signaling to the secretion mechanism of fusiform vesicle encasing intracellular *E. coli* in BECs. MyRIP acts as a scaffolding protein, which binds to both PKA (a downstream effector of TLR4 signaling) and Rab27b on the bacteria encasing compartments to confine PKA activity to the secretion of the vesicles (Fig. 4.7). Understanding the mechanism how PKA coordinates a process of fusiform vesicle exocytosis is of great interest. Protein phosphorylation emerges as an important regulatory mechanism that controls the secretory pathway in many cell types including non-neuronal cells. Many studies show



**Figure 4.7: Diagrammatic depiction of the TLR4-mediated expulsion of bacteria from infected BECs.** The TLR4 initiated and AC-3, cAMP, and PKA dependent signaling pathway regulates bacteria exocytosis from infected BECs. Caveolin-1, Rab27b, MyRIP, and PKA are found in the same binding complexes.

that proteins implicated in membrane fusion such as SNAREs and SNARE regulators (e.g. Rab proteins and Sec1/Munc18) undergo posttranslational modification by phosphorylation to control membrane fusion (Snyder et al., 2006). Recent *in vivo* and *in vitro* studies showed that PKA phosphorylates t-SNAREs including Syntaxin 4 and SNAP-25 and other regulators in the secretory pathway (Foster et al., 1998; Risinger and Bennett, 1999). Conceivably, other signals mediate priming of fusiform vesicles to the plasma membrane and once the vesicle docking occurs, PKA regulates steps of membrane-membrane fusion by phosphorylation of t-SNAREs. One possible scenario is involvement of Caveolin 1 on the vesicle membrane in the priming step since it has been shown that phosphorylated Caveolin 1 induces flattening and fusion of caveolae-derived vesicles (e.g. fusiform vesicles) by unknown mechanisms (Nomura and Fujimoto, 1999). Thus far, one phosphorylation site of Caveolin 1 is identified at tyrosine 14 that is a substrate of Src tyrosine kinase. Interestingly we found that Src tyrosine kinase is also a component of the exocytic compartments since a pull-down assay using a Src-specific antibody identifies Caveolin 1 as a binding partner (unpublished data). In addition, Src tyrosine kinase activation has been identified as an intermediate of TLR4 signaling in human lung endothelial cells (Gong et al., 2008). Thus, phosphorylation is likely to occur on many proteins of the exocytic compartments simultaneously to promote secretion of the vesicles and combinatorial effects of those signalings with

temporal and spatial constraints seem to be important to regulate secretion of this distinct compartment.

Finally, our findings point to a novel strategy for the treatment of urinary tract infection, especially in cases that are refractile to antibiotic treatment. Conceivably, UTIs may be reduced by employing compounds that promote exocytosis of intracellular bacteria harboring in BECs. Indeed, we have demonstrated recently that treating infected mice with forskolin, a potent elevator of intracellular cAMP, can significantly reduce UTIs in mice (Bishop et al., 2007). The possibility of expelling intracellular bacteria as a form of treatment may have broad therapeutic implications as most pathogens tend to seek intracellular refuge in at least one stage of the infectious process.

## Chapter 5: Conclusions

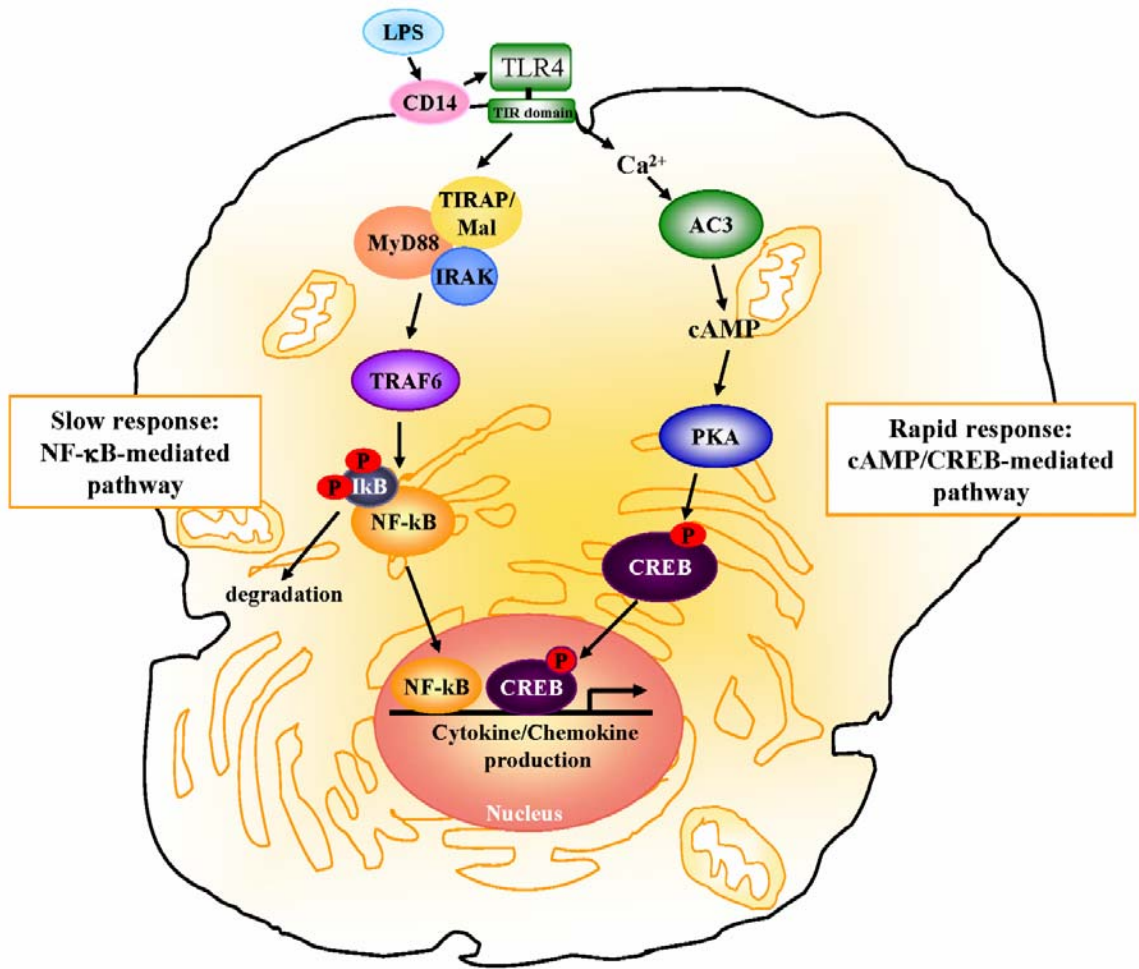
The urinary tract is one of the most intractable mucosal sites for bacteria to colonize. Considering its close proximity to the GI tract and the possibility of cross contamination by gut flora, together with the fact that urine provides a rich medium for bacterial growth, it is remarkable that UTIs are not more frequent. The primary factor for this remarkable refractivity to pathogens goes to impermeability of the bladder epithelium and its powerful flushing action of urine, which leads to elimination of unattached and loosely attached bacteria from the tract.

In addition, BECs are able to sense potential pathogens and evoke a powerful multifaceted immune responses aimed at the early elimination of the pathogen. Among the immune surveillance molecules on BECs, TLR4 is exclusively expressed and lined on superficial BECs. Consistent with high expression levels on superficial BECs, engagements of TLR4 by bacteria and their products appear to evoke multifaceted immune responses, the best known of which is cytokine secretion, which is important for the recruitment of neutrophils to clear bacteria. Upon recognition of Gram-negative bacterial LPS, TLR4/CD14 complexes on cells initiate a series of signaling events triggered by the association of its intracellular binding domain the so-called Toll/Interleukin-1 Receptor (TIR) domain within TLR4 with TIR containing domains on

adaptor molecules. The cytoplasmic adaptor proteins recruited to the TLR4 complex include TIRAP/Mal and MyD88. A serine/threonine kinase IRAK is subsequently recruited to the complex and phosphorylated, which results in dissociation of the signaling molecules from the receptor. This event is crucial for binding of the TLR complex to the key downstream signaling molecule TRAF6, which leads to the activation of a transcriptional factor NF- $\kappa$ B (Akira et al., 2006). NF- $\kappa$ B first traffics to the nucleus and then stimulates transcription of various cytokines and chemokines. This pathway has been implicated in the IL-6 and IL-8 cytokine response of BECs following stimulation by LPS (Agace et al., 1993; Hang et al., 1998; Schilling et al., 2003). However, our studies have revealed a second and parallel TLR4 activated signaling pathway that also contributes to the IL-6 and IL-8 response. In this second pathway, cAMP, and its associated transcriptional factor CREB, are important components. The sequence of TLR4 initiated reactions in this second pathway involves an influx of intracellular  $\text{Ca}^{2+}$ , triggering a rapid increase in intracellular cAMP levels by the action of AC3. The increase in intracellular cAMP causes the phosphorylation of CREB, which in turn, activates the expression of IL-6 and IL-8 (Song et al., 2007b). Although ligation of TLR4 on BECs by bacterial LPS activates both pathways, the kinetics of IL-6 secretion via the cAMP pathway is at least 2 hrs faster than the traditional pathway. The requirement of a second and more rapid cytokine response could be relevant in the urinary tract in view of the constant barrage of gut contaminants which are the source of most uropathogens.

A diagrammatic illustration of the two signaling pathways in BECs is depicted in Fig. 5.1.

Remarkably, we found that individual BECs are intrinsically able to actively resist bacterial invasion and that these resistance mechanisms involve TLR4. The role of TLR4 in inhibiting bacterial invasion of BECs is indicated from the finding that invasion of the bladders of TLR4 mutant (C3H/HeJ) mice by type 1 fimbriated *E. coli* or *Klebsiella pneumoniae* was 10-12 fold greater than that seen in wild type mice (C3H/HeN). As stated previously, when FimH protein on type 1 fimbriated UPEC couples with Uroplakin 1a, a major component of BEC plaques, this typically initiates the invasion of BECs. Uroplakin 1a is found within cellular entities called lipid raft microdomains which are dynamic assemblies of proteins and lipids that float freely within the liquid-disordered bilayer of cellular membranes (Duncan et al., 2004; Duncan et al., 2002; van der Goot and Harder, 2001). Upon ligation, these lipid raft microdomains cluster to form larger highly ordered platforms exhibiting endocytic ability (Duncan et al., 2004; Duncan et al., 2002; van der Goot and Harder, 2001). Bacterial FimH-mediated aggregation of Uroplakin 1a found within lipid raft domains of the apical surface of BECs is presumably the trigger for bacterial invasion. Several other components of lipid rafts on BECs such as Caveolin1 (a principal structural component of lipid rafts) and Rac1 have also been implicated in *E. coli* entry (Duncan et al., 2004). Not only are both lipid raft



**Figure 5.1: TLR4-initiated signaling pathways in BECs.** Upon ligation by LPS, TLR4 initiates the classical signaling pathway involving NF-κB as well as a second chain of reactions where cAMP is a major substrate. The latter pathway results in a markedly faster cytokine response than the former pathway. TLR (Toll-like receptor), BECs (Bladder epithelial cells), LPS (Lipopolysaccharide), NF-κB (Nuclear factor-κB), cAMP (cyclic AMP), AC (Adenylyl cyclase), PKA (Protein kinase A), CREB (cAMP response element-binding protein).

components essential for bacterial entry into BECs specific binding between Caveolin1 and Rac1 is a required step for bacterial uptake by BECs (Duncan et al., 2004).

Remarkably, the TLR4 initiated mechanism for resisting bacterial invasion through this pathway appears to involve cAMP dependent signaling. For instance, it was found that bacterial invasion was blocked when intracellular cAMP, through a downstream effector PKA, inhibited the activation of Rac1 in the lipid raft domains and a necessary element for actin remodeling (Fig. 3.7). Presumably, the invasion-abrogating defenses in BECs lining the lumen are triggered when TLR4 molecules make contact with bacterial LPS, an event likely to be initiated as soon as infecting bacteria reach the bladder. The contaminating bacteria are rapidly eliminated if they are not able to overcome the cellular actions that impede BEC penetration. An indication of the magnitude of this TLR4 mediated response can be obtained from the finding that there were 10-12 fold greater bladder invasion by bacteria in TLR4 mutant mice compared to controls (Song et al., 2007a). A number of recent reports now suggest that this property to block bacterial invasion may be a common property of other TLRs and cell types (Gonen et al., 2007; Tenor and Aballay, 2008).

This study has also revealed that TLR4 is still actively involved in combating invading bacteria, even after a successful bacterial invasion event. We have noticed that

a large portion of intracellular UPEC is forcibly expelled by BECs via a TLR4 mediated activity. If infected BECs were allowed to incubate over a period of 18 hrs, the majority of intracellular UPEC are eventually expelled in a piece-meal fashion without any appreciable loss of host cell or bacterial viability (Bishop et al., 2007). This activity was markedly reduced in infected BECs whose TLR4 expression was silenced by RNA interference. This novel TLR4 mediated activity is directly linked to the observation that UPEC are housed within distinct compartments of BECs (Bishop et al., 2007) that are positive for both endocytic marker Caveolin-1 and exocytic marker Rab27b. Previously, it had been reported that following lipid raft mediated uptake of UPEC, these bacteria were housed within nondegradative compartments of unknown composition (Bishop et al., 2007). Recently, *in vitro* and *in vivo* studies have revealed that these compartments share properties of fusiform vesicles, which are distinct cAMP regulated exocytic vesicles present in superficial epithelial cells of the bladder to regulate bladder volume (Apodaca, 2001; Bishop et al., 2007). When the bladder has to expand to accommodate urine, fusiform vesicles migrate to the apical surface of the cells and fuse into the apical plasma membrane providing the extra membrane required for bladder expansion. After urine has been voided from the bladder, the fusiform vesicles are reclaimed by the intracellular compartment (Fig. 1.1). A critical determinant of the fusion of fusiform vesicles with the apical surface of BECs is the increase in intracellular cAMP levels (Apodaca, 2001) which explain how TLR4 is able to mediate expulsion of intracellular

UPEC harbored within fusiform vesicles of BECs. Support for the notion that bacterial exocytosis is induced by increasing intracellular cAMP has been provided by studies demonstrating that knockdown of AC3 showed a reduced *E. coli* exocytosis from infected BECs. This notion is also supported by studies where the bacterial load in infected murine bladders could be significantly reduced by treatment with small molecule inducers of intracellular cAMP (Bishop et al., 2007). This finding also indicates that some of the TLR4 mediated cellular responses can be mimicked by modulating substrates in the signaling circuitry. The mechanism how TLR4 signaling modulates bacterial exocytosis from infected BECs begins to understand. Modulating secretion of the bacteria-encasing vesicles was mediated by activation of PKA, a downstream effector in the TLR4/cAMP pathway. PKA anchoring protein MyRIP anchors PKA to the exocytic compartment encasing infecting bacteria by its binding to Rab27b, a component of the secretory vesicles.

In summary, we reveal three previously unrecognized TLR4-mediated immune responses in BECs to UPEC. Although recruitment of neutrophils and other immune cells to the infection site appears to be the primary function of TLR4 in the tract, TLR4 on BECs appears to actively inhibit bacterial invasion and expel UPEC from infected BECs. These novel TLR4 initiated activities are mediated by a unique signaling pathway where the well known secondary messenger, cAMP, is a major substrate (Fig. 5.2 depicts

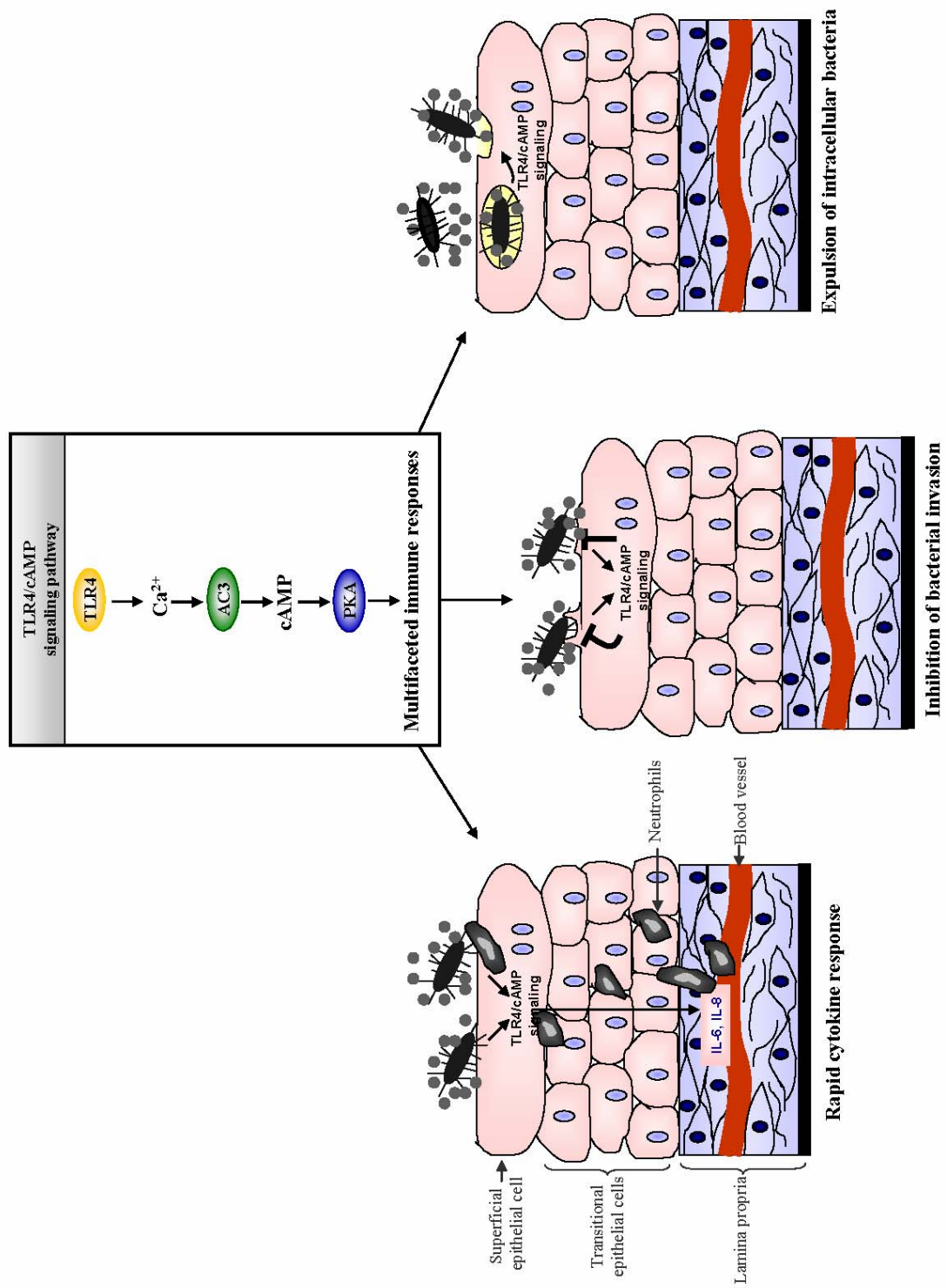


Figure 5.2

**Figure 5.2: A model depicting multiple immune outcomes of TLR4/cAMP signaling pathway in BECs.** TLR4/cAMP signaling triggers rapid cytokine/chemokine secretion, inhibition of UPEC invasion, and UPEC expulsion from infected BECs.

multiple TLR4/cAMP-mediated immune functions). Taken together, these activities could begin to explain the remarkable refractivity of the urinary tract to infection.

There is a growing need for the development of novel strategies for the treatment of UTIs. Antibiotics are becoming increasingly ineffective against uropathogens because of multiple drug resistance and their intrinsic ability to harbor within BECs as quiescent forms. A major focus of future study could be the development of small molecule compounds to boost local TLR initiated innate immune responses during infection. These could include TLR ligands or agents such as forskolin that boost intracellular levels of cAMP, a substrate of the TLR4 signaling pathway. In the future, strategies to boost the innate immune system of the urinary tract could complement or even replace antibiotic therapy. Unlike antibiotic treatment, however, this mode of therapy will not be broadly applicable. Instead, it will have to be tailored to each patient and must take into consideration, among other factors, the virulence and antibiotic resistance profile of the infecting bacteria as well as the age, immune competence and genetic make-up of the patient. For example, employing TLR4 ligands to boost immunity in patients with defective TLR4 genes will not be productive but the use of activators of downstream components of the pathway could be useful. Thus, for these proposed emerging strategies to be completely effective, comprehensive information regarding relevant traits of the pathogen and the host will become necessary.

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## Biography

Jeongmin Song, raised at a small town in South Korea, graduated in 1998 Magna Cum Laude from Hannam University with a Bachelor of Science degree in Microbiology. She immediately joined the Master's program in the Department of Biology at Yonsei University. Her thesis research was supervised by Dr. Eungbin Kim in the Department of Biology and her thesis title is "Biochemical and molecular analysis of regulatory mechanisms on aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1 (2000)". Before joining the Ph. D. program in the Department of Molecular Genetics and Microbiology at Duke University, she worked in the Department of Ophthalmology at Yonsei University Medical Center as a research assistant under supervision of Dr. Joon H. Lee. After learning researches on bacterial side as well as on mammalian cell side, in 2003 she joined the Ph. D. program in the Microbial Pathogenesis Section and began her dissertation research under supervision of Dr. Soman N. Abraham in the Departments of Pathology, Molecular Genetics and Microbiology, and Immunology. Her publications are listed below.

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**Song J.** (2000) Biochemical and molecular analysis of regulatory mechanisms on aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. *Master's thesis*.

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**Song J, Abraham SN.** Innate and Adaptive Immune Responses in the Urinary Tract. (2008) *European Journal of Clinical Investigation. Suppl2:21-8.*

**Song J, Bishop BL, Li G, Grady R, Stapleton A, Abraham SN.** TLR4-Mediated Expulsion of *E. coli* from Infected Bladder Epithelial Cells. (*manuscript in preparation*)