

# The multiple antibacterial activities of the bladder epithelium

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**Abstract:** The urinary tract is subject to frequent challenges from the gut microflora. Indeed, up to 40% of women will experience at least one urinary tract infection (UTI) during their lifetime. Uropathogenic *Escherichia coli* (UPEC) contribute to an overwhelming majority of these cases and they typically initiate UTIs by invading the superficial epithelium that lines the bladder lumen. In addition to serving as an effective barrier to noxious agents found in urine, bladder epithelial cells (BECs) play a key physiological role in regulating bladder volume to accommodate urine flow. UPEC appear to coopt this latter property to circumvent this normally impregnable epithelial barrier. However, in spite of this shortcoming, recent studies suggest that BECs possess several immune mechanisms to combat bacterial invasion including expulsion of invading bacteria back into the bladder lumen following infection. These antibacterial activities of BECs are triggered and coordinated by sensory molecules located on the epithelial cell membrane and within the cells. Although, they are the primary targets of microbial attack, BECs appear to be equipped with a diverse repertoire of defense schemes to fend off many of these microbial challenges.

**Keywords:** Urinary tract infections (UTIs); bladder epithelial cells (BECs); antibacterial activities

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## Introduction

With the aging of the population, urinary tract infections (UTIs), which primarily afflict females, is increasingly becoming a clinical challenge. In the United States, the cost of UTI treatment is in excess of \$2 billion annually (1,2). Uropathogenic *Escherichia coli* (UPEC) contribute to 70–90% UTIs in non-immunocompromised individuals, while other pathogens such as *Pseudomonas aeruginosa*, *Kebsiella pneumoniae* and *Proteus mirabilis* account for most of the remaining (1-4). Although the urinary tract comprises of the urethra, bladder, ureters and kidneys, the most commonly targeted site is the bladder (1,2). Here we describe how UPEC circumvent the powerful

barrier functions of the bladder epithelium as well as the many antibacterial activities of the BECs before and after infection has been initiated.

## Bacteria invasion

Following contamination of the urethra by bacteria usually originating from the gut, the prospective pathogens reach the bladder by progressive ascending colonization (5). Since the bladder is routinely occupied by urine, a rich bacterial growth medium, these bacteria can reach exceedingly high numbers within a relatively short period of time in this organ. Although most of these bacteria are promptly eliminated when the urine is voided, bacteria

that are capable of binding tightly to epithelial cells lining the bladder will be able to resist this flushing action of urine and persist (3,6-9). Thus, adhesive bacteria will have a selective advantage in colonizing the bladder. Indeed, most uropathogens are richly endowed with fimbrial organelles such as type I fimbriae that specifically promote avid bacterial attachment to the bladder epithelium (7-10). The multilayered bladder epithelium comprises of basal, intermediate, and superficial epithelial cells. The superficial epithelial layer is composed of large octagonal shaped cells which are held together by tight junctions and are covered with an array of scallop-shaped plaques (composed of uroplakin Ia, uroplakin Ib, uroplakin II and uroplakin III) on the apical surface of these cell (11). These superficial epithelial cells present a highly impervious barrier to the toxic agents in urine and to any prospective pathogens.

While attachment to the bladder walls helps bacteria to transiently escape elimination with urine during voiding, there is a necessity to find a protected niche for proliferation and colonization. A potential niche for this activity is intracellular sites within the superficial epithelial cells lining the bladder. Since most UPEC isolates do not have specialized organelles or mechanisms (e.g., the type III secretion system) to gain entry into these host cells, how these bacteria achieve this feat of penetrating the highly impervious superficial bladder epithelial cells (BECs) is of interest. Studies by Bishop *et al.* revealed that UPEC gain entry into superficial BECs by coopting their unique physiologic activity of regulating bladder volume (12). Each of the superficial epithelial cells lining the bladder contain numerous intracellular vesicles called fusiform vesicles which are linked to Rab27b, a small GTPase regulating intracellular vesicle movement. These Rab27b<sup>+</sup> fusiform vesicles serve to store the extra membrane necessary for bladder expansion when urine accumulates. As urine distends the bladder, the resulting stress force imposed on the apical surface of these cells triggers a spike of intracellular cAMP which in turn induces exocytosis of these Rab27b<sup>+</sup> vesicles resulting in their collapse into the apical cell surface, allowing bladder expansion. When urine is voided and the bladder contracts, these collapsed membranes are once again internalized as intracellular vesicles in superficial epithelial cells (13). Apparently, UPEC coopt this bladder volume-regulating property of superficial epithelial cells by triggering localized exocytosis of fusiform vesicles at the site of bacterial attachment, and when these membranes are subsequently retracted into cells, the adherent bacteria are internalized along with them. These

internalized bacteria become encased in Rab27b<sup>+</sup> fusiform vesicles within the cytosol of the superficial epithelium (12). By gaining entry into BECs, uropathogens are able to conveniently escape the inhospitable environment of the bladder lumen and possibly any immune cells in the vicinity.

### Extracellular immune responses

Seemingly in recognition of UPEC's ability to coopt some of its normal cellular activities to gain entry, superficial BECs have evolved a variety of extracellular and intracellular antimicrobial activities to resist or minimize this threat. First of all, the cells are amply endowed with receptors such as toll-like receptor (TLR) 4 that are able to promptly recognize intruding bacteria. TLR4 is highly expressed on the apical surface of superficial epithelial cells as well as in Rab27b<sup>+</sup> membranes encasing intracellular bacteria (14,15). These receptors are wired to distinct signaling circuits which activate multiple antimicrobial activities within and outside these superficial epithelial cells. Although the antimicrobial peptides  $\beta$ -defensin 1 and cathelicidin are constitutively secreted by BECs, their level of expression is greatly enhanced upon activation of TLR4 and other receptors (16-19). These bactericidal peptides work by intercalating into and disrupting bacterial membranes (18), which is especially effective in limiting the survival of prospective pathogens in the early phase of infection and they represent one of the earliest extracellular antimicrobial responses mediated by epithelial cells (17).

Concurrent with the enhanced secretion of antimicrobial peptides by superficial BECs is secretion of multiple chemokines and pro-inflammatory cytokines. The chemokines include CXCL1 (20) and IL-8 (21), and the early pro-inflammatory cytokines include IL-1 $\beta$ , IL-6 and TNF (14,19). Many of the cytokines are released via a canonical NF- $\kappa$ B pathway mediated by the adaptor MyD88 (14), while a rapid IL-6 response (22) appears to be mediated by a non-canonical cAMP pathway involving Ca<sup>2+</sup> flux and the cAMP response element-binding (CREB) protein. Cumulatively, these mediators promote the early recruitment and activation of immune cells such as neutrophils, macrophages, mast cells and lymphocytes into the bladder (23-25). Neutrophils are one of the first immune cells recruited into the bladder, they typically reach the infected sites within 2 hours and are largely responsible for much of the bacterial clearance during the acute phase of infection (23-25). This neutrophil response, which is typically very vigorous, involves extravasation of large bolus

of cells from blood vessels in the lamina propria, followed by penetration of the basement membrane. Thereafter, these cells cross multiple layers of intermediate epithelial cells before reaching the superficial epithelium where most of infecting bacteria are found. In many cases, neutrophils also enter the bladder lumen to engage extracellular bacteria, although it is unclear how effective these cells are in the presence of urine (25). Bacterial clearance by neutrophils is achieved by direct phagocytosis of bacteria (26) as well as through extracellular burst of oxygen reactive species which are highly toxic to bacteria (27). Since these released oxygen radicals are also highly toxic to host tissue, neutrophils are also largely responsible for the pathology associated with bladder infections. The contributions of other recruited immune cells such as macrophages and mast cells to bacterial clearance have been well-documented in previous reviews (25,28).

### Intracellular immune responses

As indicated above, in order to avoid the inhospitable extracellular environment of the bladder, UPEC have evolved mechanisms to invade BECs. Typically, when bacteria gain entry into host cells, they are encased in endocytic vesicles comprising of membranes derived from the cell surface. These bacteria-encasing vesicles are subsequently shuttled into lysosomes where they are killed and degraded (29,30). Recent studies indicate that very few UPEC immediately suffer this fate upon entry into superficial epithelial cells because these bacteria are mostly encased in Rab27b<sup>+</sup> compartments that fail to fuse with lysosomes (31), and even if these bacteria eventually reach the lysosome, they avoid being killed because of their innate capacity to neutralize the lysosomes (12,32). Nevertheless, relatively few bacteria are able to persist in BECs following invasion. This is because of the powerful capacity of these BECs to detect intracellular bacteria and initiate their prompt expulsion (15,31,32). The fusiform vesicles that encase internalized UPEC are highly enriched in TLR4 molecules, which promptly detect intracellular UPEC and initiate expulsion of these bacteria back into the extracellular medium without any loss in cellular viability (15,31). This capacity of BECs to rapidly expel invading bacteria is a highly effective mechanism to control intracellular bacterial number. Indeed, within 24 h of invasion, more than 90% of the intracellular bacteria are expelled (12).

At least two apparently distinct TLR4-dependent mechanisms of expulsion of bacteria housed in Rab27b<sup>+</sup>

vesicles appear to be present in BECs. The first appears to be highly sensitive to the intracellular level of cAMP (12,31). When this level is increased by adenylyl cyclase 3, intracellular bacteria are spontaneously expelled via a pathway involving an adaptor protein MyRIP (31). The second pathway involves TRAM and TRIF, two classical adaptors in the intracellular TLR4 signaling pathway, which upon ligation to TLR4 on bacteria containing vesicles (BCVs) recruit another adaptor protein, TRAF3 (15). Previous studies of TLR4 signaling pathway have revealed that this molecule is readily ubiquitinated and the nature of ubiquitination on TRAF3 dictates what the cellular response is to a particular TLR4 signal (33). Bacterial expulsion is triggered because signals from TLR4 on BCVs induce K33-ubiquitination of recruited TRAF3, which promotes the binding of TRAF3 to a guanine nucleotide exchange factor RalGDS. The K33-ubiquitination on TRAF3 was found to occur on lysine 168 of the molecule and mutating this specific site abrogated bacterial exocytosis (15). Recruited RalGDS was found to mobilize a cellular transport system, typically employed for the export of hormones and various secretory proteins housed in secretory vesicles, called the exocyst complex. This aggregate of proteins forms an octameric protein complex that is implicated in the traffic of secretory vesicles and in tethering of these vesicles to the plasma membrane prior to SNARE-mediated fusion (34). Although exocyst complex appears to be involved in the export of BCVs, exactly how this octameric protein complex is marshalled to transport cytosolic BCVs to plasma membrane remains to be determined. Since this exocyst complex mediated bacterial expulsion is observed as early as 1 hour following bacterial entry (15), this appears to be a pivotal mechanism to prevent UPEC escaping into the cytosol.

Remarkably, intracellular UPEC that are not promptly expelled appear to be capable of escaping from Rab27b<sup>+</sup> BCVs into the cytosol of the BEC. Indeed, about two hours after infection, appreciable amounts of UPEC are present in cytosolic space of BECs (32). Interestingly, these cytosolic bacteria are promptly detected by components of the autophagy signaling pathway, which initiates the capture and processing of these cytosolic bacteria within autophagosomal compartments. Typical autophagy signaling proteins such as LC3 and ATG5 (35) were found to associate with bacteria containing autophagosomes (32). These bacteria containing autophagosomes were subsequently transported to multivesicular bodies in the cell before they eventually fused with lysosomes. Thus,

bacteria-containing lysosomal membranes were enriched in markers of both autophagosomes and multivesicular bodies such as LC3 and CD63, respectively (32). Remarkably, upon reaching the lysosomes, these UPEC were not degraded, but instead, were expelled in a viable state into the extracellular medium while still encased within lysosomal membranes (32). Thus, bacteria, which appeared to escape the initial Rab27b mediated expulsion and were subsequently entrapped by the autophagy pathway, were also expelled from infected BECs.

Why are UPEC that are delivered into the lysosomes not destroyed, as this compartment contains a variety of hydrolases such as cathepsins which readily degrade proteins under the acidified condition of lysosomes (29)? It is now known that upon the entry of UPEC into the lysosome, the bacteria actively neutralize the pH of this compartment so that it is no longer acidic and degradative (12,32). Apparently, in sensing dysfunction and its inability to destroy intracellular UPEC, the spontaneously expelled lysosomes release its contents including bacteria into the extracellular medium. This extraordinary action is mediated by a  $\text{Ca}^{2+}$  channel TRPML3 localized on the membrane of lysosome (32). This channel is activated only when the pH of the compartment is altered from its normal acidic condition to pH 7.4. Upon activation of this channel,  $\text{Ca}^{2+}$  stored in the lysosome rapidly flux out of this compartment into the cytosol, which then triggers the spontaneous expulsion of the defective lysosomes and its contents out into the extracellular space. This lysosome-mediated bacterial expulsion appeared to be especially pronounced between 4–6 hours post infection, after which persistent but less pronounced expulsion was observed for at least 24 hours (12,32).

### The last resort

If all of these intracellular export mechanisms fail to completely clear all of the bacteria and intracellular bacteria persist, a last resort activity is activated by BECs. A common observation associated with acute infections of the bladder is the shedding of large numbers of superficial epithelial cells and their removal during voiding of urine (3,25). Many of these exfoliated BECs appear to be covered with adherent bacteria. This capacity of various epithelial cells to deliberately exfoliate has been found to be a powerful host defense mechanism to rapidly reduce bacterial load on many mucosal sites (3,36). Since shedding of the superficial epithelium of the bladder exposes the underlying tissues

to the toxic contents of urine, exfoliation is accompanied by rapid renewal of superficial BECs through active proliferation of basal progenitor cells, which is regulated by bone morphogenetic protein 4 pathway (37) and Hedgehog/Wnt pathway (38). The newly formed superficial epithelium restores the barrier function of the bladder, protecting this organ not only from the toxic contents of urine but also harmful bacteria. While underlying mechanisms leading to the cell death and subsequent exfoliation remain to be identified, a number of bacterial factors produced by UPEC have been implicated in promoting exfoliation of BECs. One such factor is  $\alpha$ -hemolysin, which induces caspase 1/caspase 4 dependent inflammatory signaling pathways resulting in cell death (39). Conceivably, exfoliation of BECs is a double edged sword. Albeit widely regarded as a host defense activity, it may offer some opportunities to promote dissemination of bacteria into deeper tissue. Indeed, certain virulent UPEC actively cause death of the superficial epithelium so that they can reach intermediate BECs in deep tissue where they form quiescent intracellular reservoirs (QIRs) and persistent for extended periods of time (36,40). Actually, one of the major reasons for the high rate recurrence of infections (>30%) in the bladder (1,41) is associated with the presence of QIRs within subepithelium (42).

### Conclusions

The BECs have a powerful capacity to recognize infecting bacteria and to actively initiate intracellular and extracellular actions to reduce bacterial load in the bladder. The extracellular activities include direct secretion of antibacterial peptides, and the secretion of chemokines and cytokines that mediate the early recruitment of immune cells capable of clearing bacteria. The intracellular activities are primarily directed at expelling bacteria encased in Rab27b<sup>+</sup> vesicles. And for those bacteria have escaped into the cytosol, they are quickly snared by the autophagy pathway and taken to the lysosomes for degradation. However, because UPEC have the intrinsic capacity to neutralize the pH of lysosome, bacterial degradation fails to occur. Consequently, the lysosomes spontaneously exocytose and discharge their cargo of bacteria into the bladder lumen. Together, these extracellular and intracellular actions are activated by TLR4 and other pattern recognition receptors (PRRs) in BECs and they seem to act concurrently.

Since UTIs have a high frequency of recurrence, these antibacterial activities of BECs are not always successful in eliminating bacterial bladder infections. There is

now growing recognition that UPEC have evolved specific mechanisms to avoid or overcome many of these antibacterial responses of BECs and other immune cells in the bladder. For example, it is known that UPEC isolates specifically express an  $\alpha$ -hemolysin to decrease cytokine production by BECs (43). Thus, studies targeted at elucidating the mechanisms of bacterial virulence activities developed to inhibit BECs antibacterial responses might reveal novel strategies to counter these bacterial virulent actions during UTIs.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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