From infection to immunotherapy: host immune responses to bacteria at the bladder mucosa

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The pathogenesis of urinary tract infection and mechanisms of the protective effect of *Bacillus Calmette–Guerin* (BCG) therapy for bladder cancer highlight the importance of studying the bladder as a unique mucosal surface. Innate responses to bacteria are reviewed, and although our collective knowledge remains incomplete, we discuss how adaptive immunity may be generated following bacterial challenge in the bladder microenvironment. Interestingly, the widely held belief that the bladder is sterile has been challenged recently, indicating the need for further study of the impact of commensal microorganisms on the immune response to uropathogen infection or intentional instillation of BCG. This review addresses the aspects of bladder biology that have been well explored and defines what still must be discovered about the immunobiology of this understudied organ.

INTRODUCING AN OFTEN-OVERLOOKED MUCOSAL BARRIER TISSUE

Despite recent interest in immune responses at mucosal surfaces, the bladder is a frequently overlooked tissue. The luminal surface of the mammalian bladder is lined by a urothelium composed of three to six cell layers that is organized as follows: a basal layer that juxtaposes the lamina propria, one or more intermediate layers, and a superficial layer composed of unique, typically binucleated cells referred to as facet or umbrella cells¹ (Figure 1). Urothelial stem cells can be found in the basal layer where they have the capacity to differentiate into the other urothelial layers of the bladder.^{2,3} Underlying the urothelium is the submucosa, containing the blood and lymphatic vasculature. The function of the bladder is the containment and storage of urine until voiding. The bladder mucosa is noteworthy in that it is the most impenetrable barrier in the body, protecting tissues from toxins accumulated in the urine.⁴ This barrier is maintained by the tight junctions and unique apical membranes of the superficial umbrella cells, which are covered by plaques formed by four integral membrane proteins called uropodins (UP1a, UP1b, UPII, and UPIIIa).^{5–7}

Historically, the bladder has been considered to lack colonizing microflora,⁸ but more recent evidence suggests that similar to the gut or skin, commensal bacteria populate the

bladder mucosal surface.^{9–12} From the perspective of pathology, colonization by uropathogenic *Escherichia coli* (UPEC) or other uropathogens can result in bladder (i.e., cystitis) or kidney (i.e., pyelonephritis) infection. In the context of therapy for nonmuscle invasive bladder cancer, the tuberculosis vaccine strain, *Bacillus Calmette–Guerin* (BCG), is iatrogenically instilled into the bladder. Herein, we review the defense mechanisms employed by the bladder; we explore natural, pathologic, and intentional colonization of the bladder and the resulting immune response; and we highlight strategies to exploit bladder immunity for improved disease management.

INNATE DEFENSES OF THE BLADDER

The bladder mucosa is contiguous with the external environment and as such is routinely exposed to microorganisms. The proximity of the urethral opening to that of the gastrointestinal tract, with the latter being colonized with $>10^{14}$ microbes, poses a great risk. In addition, in women, the urethral opening is close to the vaginal mucosa, which hosts its own microbiota.^{13–15} Despite this, the bladder normally remains uninfected, due, in part to nonspecific defense strategies. Micturition is a passive defense mechanism; however, certain bacteria have exploited the sheer forces of urination to strengthen their attachment to the bladder surface.^{16–18} For example, single-molecule atomic force microscopy has revealed that type 1 pili, used by UPEC to

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Figure 1 Proposed schematic representation of the resident immune cells of the bladder urothelium. The bladder urothelium is composed of 3–6 urothelial cell layers. Stem cells, in dark pink, are found in the basal layer, juxtaposed to the lamina propria (delineated by the blue line). The bladder contains resident $\gamma\delta$ T cells (blue) and phagocytes such as macrophages (yellow) and dendritic cells (blue-green). Figures were generated with images from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (<http://creativecommons.org/licenses/by/3.0/>).

bind to the urothelial cell surface, can unravel from their typically helical structure.¹⁷ The authors suggest that pilus extension is necessary to counteract the shear forces encountered during urine expulsion and possibly extend the duration of bacterial attachment.¹⁷ More recently, modeling of urine shear flow has demonstrated that extension of the pilus optimizes the force on the adhesin protein to best trigger the catch bond employed by the adhesin to adhere to its receptor.¹⁸ As a second defense strategy, similar to the gut and other mucosal surfaces, a mucin layer composed of glycosaminoglycans impedes bacterial access to the urothelial cell surface.¹⁹ Removal of the mucin layer by chemical means, such as protamine sulfate, ammonium chloride, or hydrochloric acid, increases bacterial adhesion to the bladder surface in a number of model species, including rabbits, rats, and mice.^{20–24} In the bladder, however, the mucin layer is comparatively thin, suggesting that a thick mucus layer is not necessary for protection when the tissue is not exposed to high concentrations of commensal microbiota, such as in the gut.²⁵

In addition to micturition and a mucin barrier, the bladder is protected by constitutive and induced expression of secretory immunoglobulin A and cationic antimicrobial peptides, such as β -defensins and cathelicidin (also known as LL-37 or CAMP in humans and CRAMP in mice).^{26–34} Specific defenses against uropathogens include Tamm–Horsfall protein (also known as uromodulin) and iron sequestering lactoferrin and lipocalin.^{8,35–37} Secretory immunoglobulin A is produced locally in mucosal tissues and neutralizes pathogens and toxins while regulating signals induced by commensal microorganisms.³⁸ Secretory immunoglobulin A from the urine of infected patients can inhibit bacterial binding to urothelial cells and patients with an acute urinary tract infection (UTI) or those with a history of UTI have statistically significantly lower levels of urine secretory immunoglobulin A as compared with healthy, naive individuals.^{26,27} Mouse and human β -defensins have variable antimicrobial activity against Gram-negative and

Gram-positive organisms, but appear to protect the bladder against colonization by uropathogens.^{28–31,34} Indeed, uninfected mice deficient for β -defensin-1 were reported to have significantly more Gram-positive bacteria present in their urine as compared with wild-type mice.²⁸ Defensins are produced in the kidneys and female genital tract, and may be constitutively expressed (e.g., human β -defensin-1, human β -defensin-5) or upregulated upon infection (e.g., human β -defensin-2).^{29–31,34} Similar to the defensins, cathelicidin expression is upregulated in renal urothelial cells of humans and mice and exhibits antimicrobial activity toward Gram-negative bacterial species.³² Notably, CRAMP-deficient mice are more susceptible to uropathogenic bacterial colonization.³² Interestingly, recent work has demonstrated that vitamin D supplementation in postmenopausal women may decrease the risk of UTI secondary to the upregulation of cathelicidin expression upon infection.³³ In contrast to antimicrobial peptides, Tamm–Horsfall protein protects the bladder by physically blocking bacterial binding to the urothelium.^{36,37} “TammMice,” deficient for Tamm–Horsfall, have significantly higher levels of bacteria in their urine and bladders after infection and, in some instances, infection was shown to result in death, an unusual outcome in mouse UTI models.³⁵

As in most tissues, the bladder contains resident immune cells poised for encounter with invading microorganisms. In the early 1980s, major histocompatibility complex class II⁺ antigen-presenting cells, referred to as Steinman’s cells, were reported to reside in the bladders of mice, pigs, and humans.^{39–41} More recently, CD11c⁺ and F480⁺ cells have also been observed in the steady state in mouse bladders.^{42,43} Given the significant overlap of surface marker proteins, it is difficult to definitively assign these cells to a specific lineage, but data suggest that both macrophages and dendritic cells reside in the naive bladder.^{44,45} Notably, little is known about the frequency, phenotype, or role of resident antigen-presenting cells in the bladder mucosa. In addition to antigen-presenting cells, the

bladder contains resident $\alpha\beta$ and $\gamma\delta$ T cells.⁴⁶ In response to UTI, interleukin-17 (IL-17) is expressed by $\gamma\delta$ T cells that contribute to the upregulation of additional proinflammatory cytokines.^{47,48} Interestingly, although T cell receptor δ -chain knockout mice are more susceptible to UTI, IL-17 does not appear to play a role in the development of adaptive immune responses to the bacteria, suggesting that $\gamma\delta$ T cells contribute to innate defenses against infection in the bladder.^{48,49} Additional innate lymphocytes, such as innate lymphoid cells or mucosal-associated invariant T cells, reside in other mucosal tissues and play an important role in the maintenance of colonizing microbiota and the defense against infection;^{50–53} thus, it is reasonable to hypothesize that they play a critical role in the bladder, although this possibility has not been explored. Currently, predictions regarding steady-state immune cell populations in the bladder can only be extrapolated from more detailed descriptions of resident cells of other mucosal surfaces such as the gut or lung. Ultimately, to define the immune cell populations of the steady-state bladder, an extensive and detailed study must be undertaken.

URINARY TRACT INFECTION AND THE HOST RESPONSE

UPEC is the causative agent in ~85% of uncomplicated UTIs,^{54–56} followed in frequency by other Gram-negative and Gram-positive uropathogens, such as *Klebsiella pneumoniae* and *Staphylococcus saprophyticus*, respectively.⁵⁷ As part of their virulence repertoire, UPEC and *Klebsiella* express hair-like appendages called type 1 pili or fimbriae capped by the adhesin FimH.⁵⁸ Biochemical and crystallography studies have demonstrated that FimH specifically binds the uroplakin protein UP1a expressed on umbrella cells.^{59–61} FimH deficiency detrimentally affects bacterial colonization of the urinary tract, as the bacteria can no longer bind to the surface of the bladder, rendering them susceptible to expulsion by urination.^{62–66} FimH also plays a role in invasion of the urothelium. Martinez *et al.*⁶⁴ described a FimH-dependent zipper mechanism employed by the bacteria to invade cultured urothelial cells, which is dependent upon FAK (focal adhesion kinase) phosphorylation and actin rearrangement. This pathway was further elaborated to show that invasion requires Rac and Cdc42 activity and microtubules at the site of lipid rafts.^{67–69} Invasion has also been shown to occur via clatherin-coated cup pathways involving endothelial nitric oxide synthase (eNOS) and dynamin.^{70,71} Whereas UPEC invasion mechanisms have been elucidated primarily in cultured urothelial cell models, the role of eNOS was demonstrated using an *ex vivo* bladder urothelium invasion assay from eNOS-deficient mice, which demonstrated that in the absence of eNOS, bladder tissue was protected from bacterial invasion.⁷¹

After UPEC invades the bladder urothelium, it initiates a pathogenic cycle of intracellular growth and biofilm formation, and subsequent reservoir formation in mice.^{65,72–74} This pathogenic mechanism, termed the intracellular bacterial community (IBC) pathway, is not unique to UPEC. *Klebsiella pneumoniae* also employ this mechanism during UTI.⁷⁵

Reservoir formation has been observed in humans and is therefore thought to be an important contributor to recurrent infection.^{72,76–78} However, more recent data suggest that UPEC are adapted to inhabit both the gut and the urinary tract equally well and isolates may, in fact, move between these two locations.⁷⁹ It is interesting to note that C57Bl/6 mice harboring bacterial reservoirs do not exhibit bacteriuria, yet they maintain a burden of $\sim 10^3$ colony-forming units for months.⁸⁰ These bacteria are protected from conventional antibiotic treatments, and periodically emerge to cause fulminate infection, although the mechanism behind the emergence is unknown.⁸¹ Thus, following acute infection, the bladder can tolerate colonization by a pathogen for months without an apparent immune response; however, it remains to be determined if the bacteria are hidden from detection, or if there is a lack of surveillance mechanisms in the bladder mucosa.

Although UPEC cause the majority of community-acquired UTIs, several other uropathogens contribute to community and nosocomial infections. Gram-positive species such as *S. saprophyticus* and *Enterococcus faecalis* exhibit a tropism for the kidney.^{82,83} In the case of *S. saprophyticus*, persistence is mediated by the virulence factors Ssp, a lipase, and SdrI, an adhesion.⁸³ Gram-negative species such as *Pseudomonas aeruginosa* and *Proteus mirabilis* are associated with nosocomial infections, frequently because of colonization of indwelling catheters.^{84,85} Importantly, *Proteus* infections can result in the development of urinary stones (e.g., struvite and apatite crystals), as the bacteria convert urea to ammonia as a means of raising urinary pH levels.⁸⁶ Gram-positive group B *Streptococcus* are infrequently associated with UTI and normally colonize the female genital tract.⁸⁷ However, during UPEC-mediated UTI, group B *Streptococcus* can suppress the neutrophil oxidative burst and may render individuals more susceptible to recurrent UTI.^{88,89} In addition to bacteria, other pathogens can cause UTI. Primarily associated with indwelling catheters, *Candida* species can colonize both the upper and lower urinary tracts via antegrade or retrograde pathways.⁹⁰ Little is known regarding the bladder mucosal response to *Candida* infections, with the exception of a single study demonstrating that pretreatment of *Candida albicans* with the antimicrobial peptide LL-37 diminished its ability to achieve bladder colonization.⁹¹ Given the infrequency of *Candida* infections in the urinary bladder and the paucity of published studies, the relevance of this finding is unclear.

The host response to UPEC infection is the best characterized, and thus will be the primary focus here. Innate sensing of infection is a critical step in the immune response to infection, and deficiencies in the expression of Toll-like receptors (TLRs) 4, 5, and 11 have been described to be detrimental to the host response in experimental bladder infection.^{43,92–101} Furthermore, children asymptotically colonized with UPEC have lower TLR4 expression levels than children with no history of UTI, and adults carrying associated TLR4 polymorphisms have fewer neutrophils and decreased cytokine expression.^{102–104} However, the authors of these studies hypothesize lower expression levels may protect against more severe forms of

disease.^{102–104} Emphasizing its central role in pathogenesis, FimH binding and entry trigger a complex pathway leading to host immune activation. Electron microscopy studies from the Kong lab have demonstrated that FimH binding induces major structural changes in the extracellular domains, which are subsequently translated to the transmembrane domains of uroplakin proteins, likely inducing intracellular signaling pathways.¹⁰⁵ FimH-mediated entry induces apoptosis in superficial facet cells shortly after infection, resulting in the sloughing of this cell layer.⁶⁵ Apoptosis requires caspase-3 activation and mitochondrial membrane depolarization, both of which occur in a matter of hours.¹⁰⁶ Little is known regarding uropathogen stimulation of additional pathogen receptors (e.g., nod-like or C-type lectin receptors).

Early studies identified IL-6 and IL-8 in humans or macrophage inflammatory protein 2 (MIP-2) in mice in urine from infected bladders, which mediate neutrophil recruitment to the site of infection.^{107–110} More recently, using luminex-based assays to analyze multiple analytes simultaneously, two studies demonstrated that Gram-negative UPEC infection is highly inflammatory, inducing cytokines such as tumor necrosis factor- α , IL-6, IL-17a, and granulocyte colony-stimulating factor (G-CSF), whereas the response to the Gram-positive organism *S. saprophyticus* is comparatively silent in the bladder.^{47,83} The reasons for this are unclear, but likely bacterial colonization is required to trigger an inflammatory response in the bladder. For example, *S. saprophyticus* infection predominantly occurs in the kidneys, with infection in the bladder being a transient event.⁸³ As the cellular source of the inflammatory cytokines observed during infection and the stimuli that induce their secretion remain to be defined, it is difficult to speculate the reasons behind the differential cytokine expression profiles. Adding to the complexity of the innate immune response, variation in cytokine expression appears to occur with different experimental UPEC strains. One study has reported that mast cell-derived IL-10 induces an “immunosuppressed” environment in the bladder during infection with the J96 pyelonephritis isolate,¹¹¹ and another reported IL-10 upregulation after infection with CFT073, also derived from a pyelonephritis infection.¹¹² In contrast, IL-10 was not detected during a 2-week time course of infection with the cystitis isolate UTI89.⁴⁷ Similar to the *Staphylococcus* example cited above, tissue tropism and virulence factors may explain the differences in cytokine expression observed among these studies as UPEC isolates from pyelonephritis express a different set of virulence genes as compared with cystitis strains.¹¹³

Robust neutrophil infiltration is a hallmark of infection, and pyuria (i.e., pus present in the urine) is used as a clinical diagnostic of UTI.¹¹⁴ The importance of neutrophil infiltration was initially demonstrated by depletion experiments, where treatment with a Gr1-depleting antibody achieved using clone RB6-8C5 resulted in increased bacterial burden.⁹⁴ Complicating the interpretation of these findings, however, is that the RB6-8C5 clone also eliminates the Gr1⁺ monocyte subset (~50% of all circulating monocytes).¹¹⁵ As monocytes infiltrate the bladder in response to infection, they too must

be considered as the source of chemokine expression and contributors to bacterial clearance.^{47,116} A second study, in which neutrophil circulation was reduced by treating animals with anti-G-CSF antibody, found the bacterial burden in the bladder to be decreased after infection.⁴⁷ Based on these findings, the authors suggested that neutrophils may initially exacerbate infection by disturbing cell–cell junctions in a manner similar to that observed in the gut during *Shigella* infection.¹¹⁷ Indeed, intensity of the innate response to UTI can determine the severity of disease and the propensity for recurrence.¹¹⁸ Although these studies begin to dissect the role of inflammatory mediators and infiltrating cells, additional studies that take advantage of improved genetic models and specific agents are needed.

Although scant, there is some evidence that cellular immunity develops during UTI. Infection of SCID (severe combined immunodeficient), athymic, and T cell receptor- δ -deficient mice suggests that $\gamma\delta$ T cells play an early protective role, but these studies only assessed bladder colonization 7–14 days after bacterial instillation and did not include a challenge model.^{49,119} It has been shown that IL-10 and IL-4 deficiencies have no impact on colonization or bacterial clearance, providing indirect evidence that a T helper type 2 (Th2) host response is not a major determinant of the immune response.⁴⁹ Several studies suggest that there is a humoral response generated in response to bladder infection.^{120–127} For example, experimental vaccination with bacterial iron acquisition proteins, the type 1 fimbrial protein FimH, or attenuated strains confers modest antibody-mediated protection against UPEC infection in both humans and mice.^{120–126} Potential cellular responses to UTI have only been addressed in one study, which used UPEC engineered to express ovalbumin. Infection induced detectable antibody against ovalbumin in the serum and proliferation of transgenic CD4⁺ and CD8⁺ T cells in infected mouse spleens.¹²⁸ The study falls short of demonstrating that the primed T cells are specific for UPEC as only ovalbumin-specific responses were measured; however, challenge infection resulted in a lower bacterial burden.¹²⁸ This study is intriguing, as it provides evidence that cellular immune responses may be generated by intravesical bacterial infection. Many important questions about the generation of these responses remain unanswered: How do antigen-presenting cells present pathogen antigens? Are UPEC-specific T cells activated? What kind of immunological memory is generated? Can memory T cells be generated to achieve sterilizing immunity? Answers to these questions will require new infection models, the definition of immunodominant UPEC antigens, and the establishment of new immunologic tools for the study of UTI.

COMMENSAL COLONIZATION OF THE BLADDER

Although the bladder had been considered a sterile mucosa, application of sequencing technologies has revealed there to be commensal microbial communities.^{9–12} These studies challenge the notion that the bladder is sterile, a supposition that relied upon urine culture-based assays. It is well known

that the gut contains numerous genera of bacteria that resist typical culture applications. Therefore, it is reasonable that the bladder also contains noncultivable microorganisms. Indeed, viable but nonculturable bacteria can be detected in women with or without a history of UTI.⁹ Clean catch urine samples from healthy volunteers confirmed the presence of bacteria from >45 genera, across 11 different phyla.¹² Diversity was found among donors, suggesting that bladder commensal populations are polymicrobial and variable.¹² It remains unknown how stable the populations are in single individuals and if the commensal populations drift over time. In a small study encompassing healthy donors and spinal cord injury patients asymptomatic for UTI, 16S RNA sequencing analysis identified distinctions in genera between healthy individuals and patients.¹⁰ Catheter type and duration affected the microbial makeup of the urine, with a significant shift toward organisms known to be uropathogenic and away from “protective” commensals.¹⁰ That spinal cord injury patients showed a different colonizing population is not surprising, as these patients frequently require catheterization to void urine, a known risk factor for UTI.¹²⁹ In addition, divergence was also noted between men and women.¹⁰ Women have a much higher susceptibility to UTI than men, and although many explanations have been put forth to explain this bias,¹¹³ differing resident microflora between the sexes may contribute to the susceptibility to pathogenic strains.

Caution should be exercised in the interpretation of these pioneering studies, as samples contaminated by microbes

colonizing surrounding tissues may bias results. Urine was primarily collected by clean catch and may have contained bacteria from the proximal and distal urethra, as well as the bladder.^{10,12} The distal urethra can be colonized with Gram-positive bacterial species, such as staphylococci and lactobacilli, strains commonly found associated with vaginal mucosa.^{13–15} Moreover, it is possible to detect nonculturable bacteria from urine collected by transurethral and/or suprapubic aspiration, supporting the existence of a bladder microbiota.¹⁰ Integrating this new information into concepts of bladder immunobiology and disease pathogenesis remains an exciting challenge.

INTENTIONAL COLONIZATION AS A MEANS TO MODULATE IMMUNITY

Although it may seem counterintuitive, intentional instillation of bacteria into the bladder of patients with bladder disease can modulate the immune response of the host in beneficial ways. Colonization of individuals with subvirulent uropathogens may protect against recurrent UTI; and instillation of the vaccine strain for tuberculosis, BCG, has been the standard treatment for nonmuscle invasive bladder cancer for nearly four decades (Figure 2).

Asymptomatic bacteriuria strains as a treatment for recurrent UTI

UTIs annually affect more than 130 million people worldwide,^{54,55} with health costs of over \$3.5 billion in the United States and likely similar expenditures in Europe.^{113,127} The

Figure 2 Bacterial colonization as a therapeutic approach. (a, left side of bladder) Intentional colonization of the bladder with an avirulent strain of bacteria (blue) may induce an asymptomatic bacteriuria state that inhibits the ability of virulent strains (black) to infect the bladder. This strategy may effectively prevent further urinary tract infections. (b, right side of bladder) Although the mechanisms of Bacillus Calmette–Guerin (BCG)-mediated tumor immunity are not well understood, BCG-specific CD8⁺ T cells play a critical role in the response to therapy. BCG (light blue) is instilled after tumors are resected (red is healing urothelium). Recently, we demonstrated that the presence of BCG in regional lymph nodes correlated with a robust BCG-specific CD8⁺ T-cell response. Whether this represents live BCG that have disseminated, or whether the BCG have been transported inside of a phagocytic cell remains to be determined. Dotted lines depict the hypothesis that BCG are carried to regional lymph nodes and presented to CD8⁺ T cells. Primed T cells then migrate to the bladder to participate in tumor immunity. ABU, asymptomatic bacteriuria; UPEC, uropathogenic *Escherichia coli*. Figures were generated with images from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (<http://creativecommons.org/licenses/by/3.0/>).

increasing prevalence of antibiotic resistance in clinical uropathogen isolates^{113,127} complicates treatment and escalates costs.^{92,107} The Urologic Diseases Statistics published by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) indicates that UTI was the primary diagnosis in >8 million physician visits, of which 80% were women.^{113,130} Approximately one in two women will have at least one UTI in their lifetime,^{127,131} with the highest risk being in 16–35-year olds, a period during which women are 35 times more likely than men to become infected.^{113,132} In addition, 25 to 44% of all infected women will experience recurrent UTI (rUTI) within 6 months of the index infection.^{54,113,131} Thus, there is a pressing need for improved therapeutics to reduce recurrent infection and reduce healthcare-related costs.

Intentional colonization of the bladder of rUTI patients—bacterial interference—is one means of preventing recurrent infection.¹³³ Bacterial interference refers to a situation where a particular strain can prevent colonization or inhibit growth of another strain or species.¹³⁴ Support for this strategy is based on studies of patients with asymptomatic bacteriuria (ABU). For clarification, ABU reflects colonization by cultivatable bacteria and is distinct from the recent data on the bladder microbiota described above. Reported ABU rates for women are in the range of 2–10%.^{135,136} It is noteworthy that ABU strains do not completely lack virulence genes, but do commonly encode mutated or truncated virulence genes typically associated with UPEC.^{137–140} ABU status was thought to precede infection.¹⁴¹ However, more recent work demonstrates this status is not predictive of the likelihood of an impending UTI,¹⁴² refuting the idea that ABU status defines a population of patients more at risk for UTI.¹⁴³ Furthermore, antibiotic treatment of women with asymptomatic carrier status results in increased incidence of recurrent acute infection, suggesting that bladder colonization by commensal-like strains are protective against re-infection.¹⁴⁴

Several studies have explored the viability of intentional colonization with ABU strains for patients who suffer from frequent rUTI. Early studies have established that colonization with ABU strains is safe with few side effects.¹³³ Additional clinical trials have assessed the impact of colonization on the frequency of recurrent infection after colonization with ABU *E. coli* strains, such as 83972.^{145,146} In a patient population with incomplete bladder emptying—a risk factor for UTI—ABU colonization significantly extended the time to next rUTI.¹⁴⁵ In a slightly different strategy, patients were deliberately colonized using catheters coated with ABU *E. coli* 83971 and, here too, colonization appeared to decrease the frequency of UTI.¹⁴⁶

One complication of these studies is that colonization can be variable in patients, and in some situations multiple instillations were required to achieve a colonized state.¹⁴⁵ One reason for this may be genetic variability of the host, which in turn affects resistance to colonizing bacteria.¹⁴⁷ Consistent with this interpretation, susceptibilities to UTI and ABU carrier status have been correlated with polymorphisms in *TLR4*, *TLR2*, *CXCR1*, and *IRF3*.^{102,103,148–150} ABU frequently goes undetected because patients do not exhibit overt symptoms of

infection, but there is little information about the role of the immune response in shaping colonization.^{151,152} A recent analysis of the immune response to individuals intentionally instilled with ABU *E. coli* 83971 revealed that although the neutrophil chemokines IL-8 and growth-related oncogene- α are significantly induced as compared with levels found in the urine from noncolonized patients, other inflammatory cytokines are not induced, suggesting that ABU strains induce a modest inflammatory response.¹⁵² In addition, although extensive studies have investigated the localization of pathogenic bacteria within the bladder, demonstrating, for instance, that UPEC invades the urothelial cell layer and quiescent bacteria reside in vacuoles,^{65,76,80} little data exist regarding the localization of ABU strains. Still to be examined is whether ABU strains also invade the urothelium; or whether they reside within the lumen of the bladder in a manner that protects them from expulsion by micturition. Further human trials will be necessary to refine the patient population that benefits most from intentional ABU colonization.

BCG immunotherapy for bladder cancer

A second major disease of the bladder is urothelial carcinoma. Bladder cancer is one of the most prevalent cancers, with reported rates of >72,500 people per year in the United States and >100,000 individuals in Western Europe.^{153,154} It is noteworthy that, and in contrast to the prevalence of UTI,^{113,132} bladder cancer is much more common in men.¹⁵⁴ The most recent statistics in the United States report that 75% of new bladder cancer patients are male and that bladder cancer is the fourth most common cancer among men, as opposed to eleventh among women.¹⁵⁴ Interestingly, bladder cancer is treated by intentionally instilling bacteria into the lumen of the bladder to induce inflammation in the bladder mucosa. In 1976, Morales *et al.*¹⁵⁵ described an immunotherapeutic protocol of six weekly intravesical instillations of BCG (the vaccine strain against tuberculosis) shortly after tumor resection for the treatment of bladder cancer. This protocol remains the standard of care for high-risk nonmuscle invasive urothelial carcinoma, and succeeds in >50% of patients to reduce recurrence and diminish the risk of disease progression.^{156,157} In contrast, tumors that have infiltrated the connective tissue or muscle wall do not respond to BCG therapy. Despite the success of BCG immunotherapy, much remains to be discovered regarding its mechanisms of inducing tumor immunity in the bladder.

From the perspective of bladder biology, BCG immunotherapy can be viewed as an intentional UTI.¹⁵⁵ Direct and repeated instillation of live bacteria is required for successful tumor immunity;¹⁵⁸ resulting in a small but significant risk of systemic infection (referred to as BCGosis).^{87,158} Upon instillation, BCG is thought to bind the bladder surface via fibronectin, based on the ability to block adhesion with antifibronectin antibodies.¹⁵⁹ *In vivo* studies using rodent models of BCG instillation suggest that binding may only occur in areas of tissue damage,^{159–161} possibly exposed in the healing bladder after tumor resection. *In vitro*, BCG can invade tumor-derived urothelial cells, but

how well this reflects the *in vivo* situation is unclear.¹⁶² A recent survey of multiple urothelial cancer cell lines has revealed that only a subset of these lines can internalize BCG, via Rac-1 and Cdc42-mediated macropinocytosis.¹⁶³ Mutations in the PTEN/PI3K (phosphatase and tensin homolog/phosphatidylinositol 3-kinase) pathway confer increased levels of macropinocytosis in these cells.¹⁶³ Although this study reports *in vitro* data, it is intriguing, as a majority of bladder cancers harbor mutations in PTEN and Ras,^{164,165} and one may speculate that residual tumor cells, to their own detriment, selectively take up BCG during immunotherapy. Whether BCG invades healthy or tumor urothelial cells *in vivo* is under debate. In one study, microscopic examination of bladder washings after BCG therapy in humans revealed that BCG was found either alone or inside neutrophils, but not found in association with urothelial cells.¹⁶¹ In contrast, a separate study has reported BCG inside urothelial cells from patient bladder washings and in experimentally instilled mouse bladder urothelium.¹⁶⁶ Thus, it is possible that BCG is taken up into the bladder urothelium, but the consequences of uptake by normal urothelium vs. cancer cells requires further study.

BCG instillation into the bladder induces similar, but delayed, host responses as compared with UPEC-mediated UTI. Instillation induces an inflammatory infiltration, including neutrophils and monocytes, followed by the influx of Th1-biased T lymphocytes.^{167,168} Strikingly, multiple intravesical BCG instillations are required in order to observe neutrophils and monocytes robustly migrating to the bladder in humans and mice.^{169–172} Indeed, in an observational clinical trial conducted by our laboratory, we observed a “prime/boost” response pattern during repeated rounds of intravesical BCG instillation,¹⁷⁰ similar to that observed in earlier trials.^{169,173} Urine samples from week 3 BCG-treated patients contained >100-fold increase in monocytes and a 200-fold increase in neutrophils as compared with cellular infiltration at week 1 after BCG instillation.^{169,170} This was accompanied by increasing concentrations of proinflammatory cytokines and other innate mediators.¹⁷⁰ It is noteworthy that our study not only confirmed earlier observations of increasing cytokine expression over the course of therapy,^{169,173} but also greatly expanded the list of cytokines found in urine after BCG therapy.¹⁷⁰ Neutrophils are proposed to directly kill tumor cells because of their nonspecific release of granule proteins and molecules such as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), and play a role in recruiting monocytes and lymphocytes to the bladder mucosa.¹⁷⁴ Only a few studies have addressed the impact of tumor-associated macrophages on prognosis in bladder cancer, but they have all drawn the conclusion that increased macrophage number in tumors, before BCG therapy, correlates with an increased risk of recurrence.^{175–177} Complicating the interpretation of these data, the “increased risk” cutoff value for macrophage infiltration is different for each study. Although these studies suggest that macrophages may contribute to disease pathogenesis and treatment outcome, further studies are warranted to define the mechanisms by which macrophages

influence tumor growth and/or the response to intravesical BCG therapy.

Correlates between the magnitude of T-cell infiltration and clinical response have been reported in patient populations.¹⁵⁷ Our own group has explored the kinetic of immune cell infiltration in relation to BCG persistence and T-cell priming, recently demonstrating that subcutaneous immunization with BCG, before intravesical instillation, induced a boosted immune cell infiltration in the bladder after only one instillation as compared with nonimmunized mice.¹⁷¹ Given these findings, further studies into the mechanisms of T-cell priming during bladder cancer immunotherapy are warranted.

Using an orthotopic tumor mouse model, several groups have reported that BCG-mediated antitumor activity requires a functional immune system, including, but not limited to, CD4⁺ and CD8⁺ T lymphocytes.^{178–181} Both Th1- and Th2-associated cytokines have been detected in the urine of patients undergoing BCG therapy; however, it is generally thought that a Th1 T-cell response is required for productive antitumor immunity and the bias of the cytokine response may be predictive of response to therapy.^{170,182,183} IL-4 mRNA is reportedly decreased in tumor-bearing mice upon treatment with BCG, whereas tumor necrosis factor- α and interferon- γ (IFN γ) mRNAs remain present in tissues, suggesting that BCG therapy preferentially induces Th1 cytokines.¹⁸⁴ Use of mice deficient for key host response factors (e.g., IFN γ , IL-12, and IL-10) have indicated the importance of a Th1 immune response during BCG therapy.^{183,185} Specifically, IFN γ ^{-/-} and IL-12^{-/-} mice succumbed more rapidly to tumor challenge, whereas IL-10^{-/-} mice exhibited an enhanced response to BCG therapy.¹⁸⁵ Thus, strategies aimed at augmenting the IFN γ /IL-12 axis, or diminishing the role of IL-10 signaling, would likely increase Th1 differentiation and BCG treatment response. Accordingly, mice instilled with BCG and treated with neutralizing IL-10 receptor antibodies showed an enhanced expression of Th1 cytokines as compared with control IgG-treated mice.¹⁸⁶ Interestingly, these results were observed in both tumor-bearing and nontumor-bearing mice.¹⁸⁶ In addition, in mice treated with BCG and IL-10R neutralizing antibodies, more mice were tumor free at the end of the experiment.¹⁸⁶

Induction of Th1 cytokine expression, and in particular IL-2, has also been correlated with positive patient prognosis.^{187–191} Low IL-2 levels at the end of BCG induction cycles correlated with a greater likelihood of tumor recurrence,¹⁸⁹ whereas high levels of IL-2 may be indicative of a positive response to therapy.¹⁹⁰ Indeed, a recent literature review indicates that to date, IL-2 is the best prognostic marker available to predict patient response and recurrence after BCG therapy.¹⁸⁸ Additional cytokine or immune mediators have been investigated as treatment companion and/or surrogate biomarkers,^{192–194} but have yet to be validated in large clinical studies. Identification of one or more immune modulators and/or indicators of patient response would improve overall patient follow-up care.

Consistent with experimental results, genetic polymorphisms in cytokine and receptor genes confer an increased risk of

bladder cancer.^{194–196} Analysis of 60 patients revealed that specific polymorphisms in transforming growth factor- β , IL-4, and IL-10 were found to be significantly overrepresented in BCG nonresponder patients as compared with patients who responded to intravesical therapy,¹⁹⁶ whereas a separate study identified chemokine receptor polymorphisms, such as those in CCR2 and CCR5, that correlated with increased risk for development and progression of bladder cancer.¹⁹⁴ Larger validation cohorts are needed to replicate data for associating genetic polymorphisms and bladder cancer recurrence, progression, or response to therapy.

IMPROVING UPON THE HOST RESPONSE IN THE BLADDER DURING THERAPY

Current approaches to treating UTI and bladder cancer can be improved. Finding curative therapeutic options that circumvent the need for antibiotics while boosting the host response to UTI is desirable to prevent increased antibiotic resistance and to provide long-lasting immunity for recurrent UTI patients. Despite its success, 30–50% of patients treated with BCG experience tumor recurrence.^{168,197} Moreover, immunotherapy does not work in patients with muscle invasive disease. Thus, developing approaches that enhance the immune response may provide improved therapeutic options.

UTI therapeutics

Various therapeutic options have been proposed in recent years for the treatment and prevention of UTI. One option, which takes advantage of the host immune response, demonstrated the benefit of gene therapy to induce β -defensin-2 expression by the urothelium in an experimental rat model.¹⁹⁸ This strategy resulted in a statistically significant decrease in bladder-associated colony-forming units and inflammatory scores during the first 24 and 48 h of infection, suggesting that induction of antimicrobial molecules may be a relevant strategy to reduce the severity of recurrent infection.¹⁹⁸ Chemical treatments of the bladder have been proposed as a means to eradicate reservoirs protected from the immune response or limit infection. Small-molecular-weight mannosides, which specifically inhibit FimH-mediated bacterial binding to the surface of the bladder, show great promise for treatment and prevention of rUTI but their impact on host adaptive immunity remains unknown.¹⁹⁹ A single dose of intravesically instilled protamine sulfate has been shown to induce urothelial cell shedding and elimination of established bacterial reservoirs.⁸⁰ As a word of caution, several studies have demonstrated that protamine sulfate treatment increases bacterial adhesion to the bladder surface and enhances infection when administered before inoculation, suggesting that this approach would necessitate careful observation and proper consideration regarding the timing of treatment.^{22,23} Forskolin treatment, given at the onset of infection, raises cyclic adenosine monophosphate levels and induces the exocytosis of urothelial fusiform vesicles.²⁰⁰ Mice treated with forskolin at 6, 24, and 48 h after primary infection had lower bacterial burdens compared with untreated mice and less IL-6 in their urine.

Although it has not been experimentally demonstrated, the success of these chemical approaches may be a result of bacteria being forced out of their protective intracellular niche, and thus exposed to the host immune system. Further testing of these approaches, as well as development of additional immune-modulating therapies, are needed to find alternatives to antibiotic treatment.

Optimizing BCG therapy

Although BCG immunotherapy is one of the most successful cancer therapeutic approaches used today, a significant percentage of patients do show recurrence. Thus, much work has focused on strategies to improve the efficacy of BCG treatment of urothelial carcinoma. One common approach is the use of immune stimuli or cytokines, alone or coadministered with BCG, to induce a Th1 T-helper cell bias, as this is generally held to be necessary for successful therapy. Immunostimulatory approaches include the use of CpG oligodeoxynucleotides to increase cytokine expression and BAMLET (bovine α -lactalbumin made lethal to tumor cells) to induce tumor cell apoptosis in rat orthotopic bladder cancer models.^{201,202} Interestingly, BAMLET treatment exhibited a level of protection comparable to that of BCG treatment.²⁰² *In vitro*, IL-12 and IFN α_{2b} have been shown to induce splenocytes or peripheral blood mononuclear cells, respectively, to express Th1-associated cytokines such as IFN γ ,^{203,204} whereas IFN α_{2b} efficiently reduced the incidence of tumors in mice treated with combination therapy as compared with BCG alone.²⁰⁵ Thus, clinical trials aimed at testing improved therapeutic efficacy have investigated the benefit of coadministration of IFN α_{2b} with BCG.^{204,206} Despite reports that coadministration of IFN α_{2b} with BCG is well tolerated²⁰⁶ and induces earlier expression of IFN γ in patient urine,²⁰⁴ a larger clinical trial did not find added efficacy with concomitant IFN α_{2b} therapy; rather, the treatment increased side effects as compared with BCG alone.²⁰⁷

As an alternative approach, genetically modified BCG have been developed as a means of improving treatment effectiveness.²⁰⁸ For example, recombinant BCG expressing IFN α_{2b} induces more IFN γ , and IFN γ -induced protein-10 as well as greater cytotoxic activity in peripheral blood mononuclear cells against tumor cell lines as compared with stimulation with the parental BCG strain.^{209,210} Whether use of this recombinant strain in humans will be more effective than coadministration of type I IFN remains to be determined. BCG engineered to express the listeriolysin O protein confers superior protection against *Mycobacterium tuberculosis* challenge in mice.²¹¹ Listeriolysin O, from *Listeria monocytogenes*, allows BCG to escape from the phagosome and the authors speculate that once the bacteria gain access to the cytosol of the cell, they are more likely to be cross-presented via class I major histocompatibility complex molecules and thereby induce greater CD8⁺ T-cell priming.²¹¹ Although this hypothesis remains to be tested in the bladder, increased CD8⁺ T-cell priming would likely lead to improved response to therapy.¹⁷¹

Finally, our group has shown that vaccination with BCG before initiation of an orthotopic tumor challenge improved the

response to intravesical BCG therapy, with 100% of animals surviving tumor challenge, as compared with > 60% lethality in unvaccinated mice.¹⁷¹ Importantly, analysis of clinical data revealed that, similarly, patients with pre-existing immunity to BCG, as measured by purified protein derivative positivity, had a greater likelihood of achieving recurrence-free survival after BCG immunotherapy.¹⁷¹ This work is in accordance with prior findings that patients who seroconvert (i.e., become purified protein derivative positive) during therapy have a better response to therapy.^{158,212} Thus, boosting the immune response may provide real benefit to patients with bladder cancer, perhaps by limiting treatment failure or extending the time to recurrence.

CONCLUDING REMARKS

The bladder, long regarded as just a vessel to hold urine until expulsion, is now taking its rightful place as an important mucosal surface. As has been observed for other mucosae, the immune response is expected to be complex, and future work will help to contextualize it with respect to the pathogenesis of uropathogen infection and the success of BCG immunotherapy. Much focus has been on the necessity and nature of T-cell responses during BCG, but little is known for UPEC infection. The innate response to UPEC and BCG are better characterized and appear to proceed with very different kinetics. Defining the mechanisms of host response in the case of UTI will provide valuable insight into those of BCG and vice versa. In sum, a better understanding of the naive bladder and how immunity is initiated in different instances from this tissue will support disease management and treatment innovations for UTI and bladder cancer.

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DISCLOSURE

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