Inflammasomes are important mediators of cyclophosphamide-induced bladder inflammation

Francis M. Hughes, Jr.,1 Nivardo P. Vivar,1 James G. Kennis,1 Jeffery D. Pratt-Thomas,1 Danielle W. Lowe,2 Brooke E. Shaner,3 Paul J. Nietert,4 Laura S. Spruill,5 and J. Todd Purves1,2,6

1Department of Urology, Medical University of South Carolina, Charleston, South Carolina; 2Department of Pediatrics, Medical University of South Carolina, Charleston, South Carolina; 3Department of Pharmaceutical and Biomedical Sciences, Medical University of South Carolina, Charleston, South Carolina; 4Department of Public Health Sciences, Medical University of South Carolina, Charleston, South Carolina; 5Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina; and 6Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, South Carolina

Submitted 22 May 2013; accepted in final form 22 November 2013

Inflammation underlies numerous bladder pathologies and is elicited by a plethora of agents such as urinary tract infections, bladder outlet obstruction, chemotherapies, and catheters. Pattern recognition receptors [Toll-like receptors (TLRs) and Nod-like receptors (NLRs)] that recognize pathogen- and/or damage-associated molecular patterns (PAMPs and/or DAMPs, respectively) are key components of the innate immune system that coordinates the production (TLRs) and maturation (NLRs) of proinflammatory IL-1β. Despite multiple studies of TLRs in the bladder, none have investigated NLRs beyond one small survey. We now demonstrate that NLRP3 and NLRC4, and their binding partners apoptosis-associated speck-like protein containing a COOH-terminal caspase recruitment domain (ASC) and NLR family apoptosis inhibitory protein (NAIP), are expressed in the bladder and localized predominantly to the urothelium. Activated NLRs form inflammasomes that activate caspase-1. Placement of a NLRP3- or NLRC4-activating PAMP or NLRP3-activating DAMPs into the lumen of the bladder stimulated caspase-1 activity. To investigate inflammasomes in vivo, we induced cystitis with cyclophosphamide (CP, 150 mg/kg ip) in the presence or absence of the inflammasome inhibitor glyburide. Glyburide completely blocked CP-induced activation of caspase-1 and the production of IL-1β at 4 h. At 24 h, glyburide reduced two markers of inflammation by 30–50% and reversed much of the inflammatory morphology. Furthermore, glyburide reversed changes in bladder physiology (cystometry) induced by CP. In conclusion, NLRs/inflammasomes are present in the bladder urothelium and respond to DAMPs and PAMPs, whereas NLRP3 inhibition blocks bladder dysfunction in the CP model. The coordinated response of NLRs and TLRs in the urothelium represents a first-line innate defense that may provide an important target for pharmacological intervention.

IN THE BLADDER, inflammation underlies, or is a contributing factor in, the majority of bladder pathologies, including voiding dysfunction, pelvic pain syndromes, and cancer. By far the most common bladder pathology is urinary tract infection, which is the second most common type of infection, behind only respiratory infections (i.e., the common cold). Urinary tract infections affect at least 60% of all women in their lifetime and account for between 8 and 10 million visits to outpatient clinics (and $3.5 billion) annually in the United States alone (21, 24). Copious studies have delineated the molecular interactions between the host and various pathogens that elicit this malady (for reviews, see Refs. 27, 38, and 57), but the bladder is also susceptible to sterile inflammation induced by numerous noninfectious agents such as bladder outlet obstruction, bladder stones, stents, and chemotherapies [with the best studied being cyclophosphamide (CP)]. Given the prevalence and healthcare costs of sterile and nonsterile bladder inflammation, there is considerable interest in understanding the common underlying mechanism. Work in other tissues has begun to cast light on the mechanisms of this innate response. Central to this work has been an understanding of pattern recognition receptors, specifically, Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (for reviews, see Refs. 2, 12, 19, 56, and 62).

Cell membrane-bound TLRs and cytoplasmic NLRs coordinate to initiate an inflammatory response through the recognition of patterns found on pathogens [pathogen-associated molecular patterns (PAMPs)] or various molecules that represent damage to a given tissue [damage-associated molecular patterns (DAMPs)] (45, 72). PAMPs represent well-known immunogenic components of bacteria, such as lipopolysaccharide (LPS) and flagellin (45), whereas DAMPs such as ATP are often released from necrotic cells. Each TLR recognizes their specific ligand. For example, TLR4 recognizes LPS and flagellin (45), whereas TLR5 binds to flagella. Binding of the ligand activates a constellation of effector pathways including NF-κB, which stimulates the transcription of pro-IL-1β (58).

Numerous NLRs are known or have been predicted, although few have been extensively studied (12, 43, 76). The best known is NLRP3 (NALP3), which responds to DAMPs such as ATP as well as the PAMP LPS. Activation of NLRP3 stimulates the binding of apoptosis-associated speck-like protein containing a COOH-terminal caspase recruitment domain (ASC) through homotypic binding of pyrin domains. ASC has a caspase recruitment domain and serves as an adaptor that binds NLRP3 to procaspase-1 to form a structure known as an inflammasome (43, 64), which has been referred to as the “central processing unit” of the inflammatory response (14). Formation of the inflammasome cleaves procaspase-1 to caspase-1, and the mature caspase-1 then processes pro-IL-1β,
leading to the release of mature IL-1β by pyroptosis. Inflammamasomes are also responsible for the cleavage and release of IL-18. IL-1β and IL-18 then act as proinflammatory cytokines, triggering the inflammatory response. Another well-known NLR, NLRC4, functions in an identical manner except that it contains a caspase recruitment domain and thus does not need an adaptor. However, its ligand specificity is determined by another protein, one of the NLR family apoptosis inhibitory proteins (NAIPs) (23, 54). For example, NAIP5 bridges flagellin binding to NLRC4, whereas NAIP2 triggers activation by proteins that compose the bacterial type 3 secretion systems. The net result, release of mature IL-1β and IL-18, is the same.

TLRs have been extensively studied in the bladder (57, 68, 69), where they are thought to play important roles in sterile and infectious inflammation. Given the interdependency of TLR and NLR systems in other tissues, it is somewhat surprising that very little has been published regarding NLRS in the bladder. The only direct evidence that these receptors exist in the bladder is from a tissue survey by Kummer et al. in 2007 (42). Indirect evidence has been put forth by Martins et al. (50), who demonstrated that the well-known cystitis-inducing agent CP activated P2X7 channels, which are required for the activation of NLRP3 by DAMPs (16). In this report, we begin a more systematic exploration of the presence, activation, and importance of NLRS/inflammamasomes in the bladder. We explored the presence and localization of NLRP3 and NLRC4, and their respective binding partners ASC and NAIP, using immunohistochemistry while examining their activation with relevant PAMPs and DAMPs. One well-established rodent cystitis model with relevant clinical implications is induced by CP.

CP is a nitrogen mustard alkylating agent that has been widely used for >50 yr as a chemotherapeutic agent to combat various neoplasms (25, 73). While highly effective, patients undergoing treatment with CP frequently experienced acute hemorrhagic cystitis (HC), a diffuse inflammation of the bladder leading to dysuria, hematuria, and hemorrhage (for reviews of HC, see Refs. 13 and 31). Treatment with this drug also led to physiological changes reminiscent of overactive bladder, which resulted in its adaptation as an animal model of this disorder (6, 22, 61). A metabolite of CP, acrolein, was found to trigger the inflammatory response. Another well-known protein, one of the NLR family apoptosis inhibitory proteins (NAIPs) (23, 54). For example, NAIP5 bridges flagellin binding to NLRC4, whereas NAIP2 triggers activation by proteins that compose the bacterial type 3 secretion systems. The net result, release of mature IL-1β and IL-18, is the same.

TLRs have been extensively studied in the bladder (57, 68, 69), where they are thought to play important roles in sterile and infectious inflammation. Given the interdependency of TLR and NLR systems in other tissues, it is somewhat surprising that very little has been published regarding NLRS in the bladder. The only direct evidence that these receptors exist in the bladder is from a tissue survey by Kummer et al. in 2007 (42). Indirect evidence has been put forth by Martins et al. (50), who demonstrated that the well-known cystitis-inducing agent CP activated P2X7 channels, which are required for the activation of NLRP3 by DAMPs (16). In this report, we begin a more systematic exploration of the presence, activation, and importance of NLRS/inflammamasomes in the bladder. We explored the presence and localization of NLRP3 and NLRC4, and their respective binding partners ASC and NAIP, using immunohistochemistry while examining their activation with relevant PAMPs and DAMPs. One well-established rodent cystitis model with relevant clinical implications is induced by CP.

CP is a nitrogen mustard alkylating agent that has been widely used for >50 yr as a chemotherapeutic agent to combat various neoplasms (25, 73). While highly effective, patients undergoing treatment with CP frequently experienced acute hemorrhagic cystitis (HC), a diffuse inflammation of the bladder leading to dysuria, hematuria, and hemorrhage (for reviews of HC, see Refs. 13 and 31). Treatment with this drug also led to physiological changes reminiscent of overactive bladder, which resulted in its adaptation as an animal model of this disorder (6, 22, 61). A metabolite of CP, acrolein, was found to trigger the inflammatory response. Another well-known protein, one of the NLR family apoptosis inhibitory proteins (NAIPs) (23, 54). For example, NAIP5 bridges flagellin binding to NLRC4, whereas NAIP2 triggers activation by proteins that compose the bacterial type 3 secretion systems. The net result, release of mature IL-1β and IL-18, is the same.

TLRs have been extensively studied in the bladder (57, 68, 69), where they are thought to play important roles in sterile and infectious inflammation. Given the interdependency of TLR and NLR systems in other tissues, it is somewhat surprising that very little has been published regarding NLRS in the bladder. The only direct evidence that these receptors exist in the bladder is from a tissue survey by Kummer et al. in 2007 (42). Indirect evidence has been put forth by Martins et al. (50), who demonstrated that the well-known cystitis-inducing agent CP activated P2X7 channels, which are required for the activation of NLRP3 by DAMPs (16). In this report, we begin a more systematic exploration of the presence, activation, and importance of NLRS/inflammamasomes in the bladder. We explored the presence and localization of NLRP3 and NLRC4, and their respective binding partners ASC and NAIP, using immunohistochemistry while examining their activation with relevant PAMPs and DAMPs. One well-established rodent cystitis model with relevant clinical implications is induced by CP.

CP is a nitrogen mustard alkylating agent that has been widely used for >50 yr as a chemotherapeutic agent to combat various neoplasms (25, 73). While highly effective, patients undergoing treatment with CP frequently experienced acute hemorrhagic cystitis (HC), a diffuse inflammation of the bladder leading to dysuria, hematuria, and hemorrhage (for reviews of HC, see Refs. 13 and 31). Treatment with this drug also led to physiological changes reminiscent of overactive bladder, which resulted in its adaptation as an animal model of this disorder (6, 22, 61). A metabolite of CP, acrolein, was found to trigger the inflammatory response. Another well-known protein, one of the NLR family apoptosis inhibitory proteins (NAIPs) (23, 54). For example, NAIP5 bridges flagellin binding to NLRC4, whereas NAIP2 triggers activation by proteins that compose the bacterial type 3 secretion systems. The net result, release of mature IL-1β and IL-18, is the same.

TLRs have been extensively studied in the bladder (57, 68, 69), where they are thought to play important roles in sterile and infectious inflammation. Given the interdependency of TLR and NLR systems in other tissues, it is somewhat surprising that very little has been published regarding NLRS in the bladder. The only direct evidence that these receptors exist in the bladder is from a tissue survey by Kummer et al. in 2007 (42). Indirect evidence has been put forth by Martins et al. (50), who demonstrated that the well-known cystitis-inducing agent CP activated P2X7 channels, which are required for the activation of NLRP3 by DAMPs (16). In this report, we begin a more systematic exploration of the presence, activation, and importance of NLRS/inflammamasomes in the bladder. We explored the presence and localization of NLRP3 and NLRC4, and their respective binding partners ASC and NAIP, using immunohistochemistry while examining their activation with relevant PAMPs and DAMPs. One well-established rodent cystitis model with relevant clinical implications is induced by CP.

CP is a nitrogen mustard alkylating agent that has been widely used for >50 yr as a chemotherapeutic agent to combat various neoplasms (25, 73). While highly effective, patients undergoing treatment with CP frequently experienced acute hemorrhagic cystitis (HC), a diffuse inflammation of the bladder leading to dysuria, hematuria, and hemorrhage (for reviews of HC, see Refs. 13 and 31). Treatment with this drug also led to physiological changes reminiscent of overactive bladder, which resulted in its adaptation as an animal model of this disorder (6, 22, 61). A metabolite of CP, acrolein, was found to be responsible for a large portion of the detrimental effects of this drug (8, 11). Consequently, oncologists now concomitantly administer 2-mercaptoethane sulfonate (Mesna), an orally available drug that accumulates in urine and acts as an acrolein chelator. This practice has reduced the incidence of acute HC arising from chemotherapy but not eliminated it (30).

Indeed, in current practice, HC remains a clinically relevant, potentially devastating, and challenging entity for physicians to treat. It is particularly prevalent in leukemia and lymphoma patients undergoing CP conditioning in preparation for stem cell transplant (17, 28, 31, 47, 66). In this study, we used the CP-induced model of cystitis and the inflammmasome inhibitor glyburide (Gly) (46) to assess the contribution of NLRP3 to bladder inflammation and the in vivo physiological bladder dysfunction caused by this drug. Excitingly, Gly is a well-tolerated Federal Drug Administration (FDA)-approved drug used in the treatment of diabetes. Its efficacy in combating CP-induced HC in rodent models suggests immediate translational relevance for treating a disorder in need of effective management.
washed, counterstained briefly with hematoxylin, coverslipped, and analyzed by light microscopy.

Caspase-1 Assay
At the indicated times, animals were given an intraperitoneal injection of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg). The abdominal cavity was then opened, and the bladder was exposed. Urine was removed by needle puncture. Bladders were then removed, weighed, and placed in ice-cold PBS. Animals were euthanized by thoracotomy. Using techniques similar to what have been reported elsewhere by us and others (26, 33, 51), urothelia were obtained by scraping. Briefly, the bladder was opened from the sphinctor to the dome, pinned down, and gently scraped with a size 11 scalpel blade, with the blade twisted to lift cells. Cells were placed in ice-cold PBS, pelleted (800 g, 10 min), resuspended with an equal volume of 40 mM HEPES (pH 7.4), 20 mM NaCl, 2 mM EDTA, and 20% glycerol, and stored at −20°C until analyzed.

Caspase-1 activity was measured using a fluorometric assay modified from previous studies (20, 29, 34). Briefly, cytoplasmic extracts were thawed, and protein concentrations were assessed by Bradford assay (7). Extract (20 μl) was then combined with 50 μl assay buffer [25 mm HEPES, 5% sucrose, 0.05% CHAPS (pH 7.5), 10 μl of 100 mM DTT, and 20 μl of 1 mM substrate N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVDF-AFC) in blacked-walled 96-well plates. Plates were then loaded onto a plate reader, fluorescence (excitation: 400 nm and emission: 505 nm) was measured every 30 s for 15 min, and the slope was determined. A standard curve of fluorescence versus free AFC (free AFC was obtained from Sigma) was then used to calculate the specific activity of caspase-1.

Urine Chemokine Analysis
To obtain urine following the indicated treatments, rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg), the bladder was externalized, and urine was recovered by needle puncture. Urine was stored at −70°C until analyzed by multiplex assays for levels of IL-1β and IL-18. Briefly, urine was diluted (typically 1:2) on a 96-well filter plate and analyzed in duplicate on the BioPlex platform (BioRad, Luminex Technology, Hercules, CA) as previously described (36, 37). Concentrations (in pg/ml) were then extrapolated from a seven-point standard curve for each cytokine using a five-parameter logistic fit using Bioplex software. Creatinine levels were assayed (typically with a 1:5 dilution) using a urinary creatinine kit obtained from Cayman Chemical (Ann Arbor, MI) and the manufacturer’s recommended procedures. Concentrations were extrapolated from an eight-point standard curve, and cytokine levels were then adjusted for creatinine levels and reported as picograms of cytokine per milligram of creatinine.

Evans Blue Extravasation
Evans blue extravasation was measured as previously described (35). Briefly, rats were injected with 25 mg/kg Evans blue dye (intravenously in 200 μl). One hour later, bladders were dissected, weighed, placed in formamide (1 ml), and incubated in the dark at 56°C overnight. The absorbance of the formamide was measured (620 nm) and compared with a standard curve. Results were calculated as nanograms of Evans blue per milligram of tissue (49, 63).

Urodynamics
Catheter implantation. Suprapubic catheters were implanted into rats as previously described (35). Briefly, animals were injected intraperitoneally with ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) and subsequently given carprofen (2.5 mg/kg sc) after induction. The bladder was then exposed, and a polyethylene (PE-50) catheter with a flared end inserted into a hole created in the dome of the bladder by a scalpel. The catheter was then secured in place with a purse string suture, and the other end of the catheter was externalized at the back of the neck. The rat was fitted with a SAI Quick Connect Harness from Strategic Applications (PHM-119-1, SAI Infusion Technologies, Lake Villa, IL), and the catheter was plugged with a urethral lock connector and stopper. The rat was given >72 h to recover.

Cystometry. After the indicated treatments, the SAI Quick Connect harness was removed, and each rat was placed into a restrainer (modified from item HLD-RM, Kent Scientific, Torrington, CT) with a small hole near the rear, which was fitted with a funnel. The urethra was positioned above the funnel, and the entire restrainer placed inside a Small Animal Cystometry Lab Station (Catamount Research and Development, St. Albans, VT). The catheter was attached to an infusion pump and in-line pressure transducer, and sterile isotonic saline (0.9% NaCl) was continuously infused into the bladder at a rate of 80 μl/min. An analytic balance beneath the wire-bottom animal cage measured the amount of urine voided during cystometry. Bladder pressure and infused and voided bladder volumes were continuously measured using MED-CMG software provided by Catamount. A single cystometrogram (CMG) is defined as the simultaneous recording of these three parameters during a single filling-voiding cycle. The infusion was continued for 60–120 min until at least three CMGs were measured. Data were analyzed using CMG Analysis software (version 1.06, Catamount).

For every CMG, voiding pressure is measured as the peak intravesical pressure during voiding, threshold pressure is the intravesical pressure just before the initiation of micturition, void volume is the amount of urine voided, the intercontraction interval (ICI) is the time between successive micturitions, nonvoiding contractions (NVCs) are defined as rises in intravesical pressure that exceed 7 mmHg but are not associated with voiding of urine, and NVC amplitude is the peak pressure associated with a given NVC.

Statistical Analysis
Comparisons between experimental groups in caspase-1, IL-1β, IL-18, bladder weight, and Evans blue dye extravasations experiments were performed using ANOVA. Since comparisons in urodynamic parameters (i.e., voiding pressure, threshold pressure, void volume, ICI, and NVCs) involved repeated (correlated) measurements within rats over time, general linear mixed models were used. General linear mixed models are ideal for clustered data analysis, as they account for between- and within-subject variation (52). All statistical analyses were conducted using SAS (version 9.3, Cary, NC).

RESULTS

NLRP3 and NLRC4 Inflammasomes Are Present in the Bladder Epithelium
The presence of NLRP3 and NLRC4 inflammasomes in the bladder was assessed by immunocytochemistry. As shown in Fig. 1, top (×20 digital micrographs scaled to fit the figure frame; 9.3% of the original size), NLRP3 and its adaptor protein ASC were present in the bladder and localized predominately to urothelia (arrows). This was also seen with NLRC4 and its binding protein NAIP. Virtually no expression of NLRP3 or ASC was found in the smooth muscle (detrusor) layer, although some staining was apparent with NLRC4 and NAIP (arrowheads), particularly in vascular elements (oval regions). The areas indicated by rectangles were cropped from the original micrographs and are presented in full scale in Fig. 1, middle. These images suggest that staining for these molecules is primarily cytoplasmic. No staining was apparent with

AJP-Renal Physiol doi:10.1152/ajprenal.00297.2013 • www.ajprenal.org
isotype antibody controls (presented as scaled micrographs; Fig. 1, bottom).

**NLRP3 and NLRC4 Are Activated by PAMPs and DAMPs**

To determine if NLRP3 and NLRC4 inflammasomes could be activated by relevant stimuli, a variety of DAMPs and PAMPs were placed in the lumen of the bladder and allowed to remain indwelling for 2 h. Afterward, caspase-1 activity in urothelia was assessed as described in MATERIALS AND METHODS. As shown in Fig. 2, LPS (a NLRP3-activating PAMP), ATP and MSU (NLRP3-activating DAMPs), and flagellin (a NLRC4-activating PAMP) all stimulated caspase-1-specific activity to a statistically significant degree compared with control ($P < 0.05$ for all).

**Gly Blocks CP Induction of Caspase-1 Activity and Release of IL-1β and IL-18**

CP treatment is widely used to induce cystitis in rodent models, where a single intraperitoneal injection can induce profound symptoms within 24 h (1, 40). The main activating agent has been shown to be the metabolite acrolein, which induces necrosis and apoptosis in urothelia (39, 41, 60, 65), resulting in sterile inflammation. Because necrosis releases DAMPs, CP would be predicted to activate the NLRP3 inflammasome. To assess this possibility, rats were treated with the NLRP3 inhibitor Gly (or vehicle) and CP (or saline) according to the dosing regimen shown in Fig. 3, and a variety of endpoints were measured. Briefly, rats were given Gly (2.5 mg/kg po) or vehicle (1 ml of 10% ethanol in PBS) 20 h and again 4
Fig. 3. Dosing regimen used to study the effects of glyburide (Gly) on various parameters in the cyclophosphamide (CP)-induced model of cystitis. This regimen was used for the results shown in Figs. 4–8 and Table 1. Vehicle, Gly, and CP were administered at the indicated doses, and animals were harvested for various analyses where indicated.

h before the intraperitoneal injection of CP (150 mg/kg) or saline (3.75 ml/kg). Four hours later, end points related to inflammasome activation (caspase-1 and urinary IL-1β and IL-18 levels) were measured. This time point was chosen based on the study by Smaldone et al. (67), which indicated that IL-1β and IL-18 levels were maximal at this time after CP injection and decreased thereafter. This transient rise is consistent with these cytokines functioning as proinflammatory cytokines and working to initiate the inflammatory reaction. As shown in Fig. 4A, CP treatment stimulated more than a twofold increase (P < 0.001) in caspase-1 activity after 4 h. Pretreatment with Gly alone had no effect on basal levels but completely blocked the activation of caspase-1 by CP (P < 0.001). A similar response was seen with urinary IL-1β, in which CP stimulated an increase (P < 0.02), and this was blocked by Gly treatment (P < 0.02). Interestingly, Gly alone reduced basal IL-18 levels, but the amount was not significant (P < 0.078). IL-18 showed a high constitutive production that was not significantly enhanced by CP (P < 0.87). While Gly did reduce IL-18 production in both saline- and CP-treated groups, the variability prevented these changes from reaching significance (P < 0.11 and P < 0.17 vs. vehicle controls and P < 0.06 and P < 0.10 vs. CP treatment, respectively).

Gly Reduces Bladder Inflammation

To assess the effects of Gly on the resulting inflammation and bladder function, a second set of animals was given additional Gly 4 and 16 h after CP, as shown in Fig. 3. Twenty-four hours after CP, animals were assessed for bladder weight, Evan’s blue extravasation, histological changes, and urodynamics. Twenty-four hours is a common time point used in studies of CP-induced inflammation with a single bolus injection. Moreover, the longer time period is necessary for the accumulating inflammation to exert demonstrable effects on bladder physiology (i.e., urodynamics). Figure 5 shows the resulting bladder weights, a common marker of bladder inflammation and a marker often used in the CP model (1, 9, 55). As shown in Fig. 4, CP treatment induced a greater than twofold increase (P < 0.0001) in bladder weight, whereas Gly had no effect (P = 0.90). However, Gly reduced the gain in wet weight stimulated by CP by 29% (P = 0.01).

To confirm this result using a more direct measurement of inflammation, we examined the extravasation of Evan’s blue dye into bladder tissue in the experimental groups shown in Fig. 3. As shown morphologically in Fig. 6A and quantitatively...
in Fig. 6B, very little Evans blue dye moved into bladder tissue in vehicle- and Gly only-treated samples. In contrast, CP induced a nearly 100-fold influx of dye ($P < 0.001$), with 50% of this influx being blocked by Gly treatment ($P = 0.02$).

Finally, we directly examined inflammation via histology with hematoxylin and eosin-stained sections. As shown in Fig. 7, histology of the bladder mucosa is normal after vehicle treatment and becomes markedly edematous with urothelial mucosal ulceration (large arrow) and a brisk neutrophilic inflammatory cell infiltrate (small arrows) after CP treatment. Changes were most severe in the dome (data not shown). Bladders of rats treated with Gly were histologically identical to those of vehicle-treated rats. Histological sections from rats treated with both CP and Gly demonstrated an improvement in the overall bladder architecture, displaying variable and focal improvement with a subjective decrease in edematous changes and areas of decreased neutrophilic infiltrate.

Gly Blocks the Negative In Vivo Effects of CP in the Bladder

To assess the contribution of inflammasomes to the in vivo bladder dysfunction associated with CP-induced cystitis, we performed cystometry on 24 h-treated animals from each of the four experimental groups shown in Fig. 3. Figure 8 shows representative micturition cycles from each of these groups, and Table I shows all relevant urodynamic parameters for the four groups and the between-group comparisons as assessed using general linear mixed models. As shown in Table I, CP treatment significantly increased the voiding pressure and significantly reduced the void volume and ICI of these animals. Gly alone significantly increased the mean void volume and ICI. Gly completely prevented the decreases induced by CP. In
INFLAMMASOMES IN THE BLADDER

DISCUSSION

Cystitis is a critical problem in urology, but understanding its underlying mechanisms is hampered by the heterogeneity of agents that induce the same physiological response patterns. Such agents necessarily engage a myriad of initiating pathways, all of which provide interesting and useful targets for pharmaceutical intervention in individual maladies. The identification of pathways common to all inflammatory stimuli would allow an interventional strategy that does not require the identification and understanding of each potential causative agent. As discussed above, the innate immune system is uniquely geared to respond to numerous insults and infectious agents by engaging a common pattern of pattern recognition receptors that recognize a multitude of DAMPs and PAMPs to initiate an inflammatory response. Central to the innate immune system is the production of proinflammatory cytokines such as IL-1β and IL-18. Studies have now clearly delineated the involvement of TLRs in the bladder as a significant number of bladder stones are generated by bladder distension. Together, these results demonstrate inflammasome activation upstream of NLRP3 and downstream of the P2X7 ion channel (46).

Table 1. Effect of glyburide on CP-induced urodynamic parameters

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Voiding Pressure, cmH2O</th>
<th>Threshold Pressure, cmH2O</th>
<th>Void Volume, ml</th>
<th>Intercontraction Interval, s</th>
<th>Number of Nonvoiding Contractions per Cystometrogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>59.7 ± 3.5</td>
<td>18.5 ± 2.2</td>
<td>0.90 ± 0.08</td>
<td>679 ± 50</td>
<td>1.61 ± 0.41</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>68.7 ± 3.9*</td>
<td>19.8 ± 2.4</td>
<td>0.68 ± 0.09*</td>
<td>506 ± 56*</td>
<td>1.46 ± 0.64</td>
</tr>
<tr>
<td>Glyburide</td>
<td>64.3 ± 4.5</td>
<td>21.8 ± 2.7</td>
<td>1.28 ± 0.10*</td>
<td>862 ± 66*</td>
<td>2.33 ± 0.88</td>
</tr>
<tr>
<td>Glyburide + cyclophosphamide</td>
<td>74.7 ± 4.1*</td>
<td>21.0 ± 2.5</td>
<td>1.21 ± 0.09*</td>
<td>897 ± 60*</td>
<td>2.28 ± 0.68</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 18 for vehicle, 7 for cyclophosphamide, 4 for glyburide, and 7 for glyburide + cyclophosphamide treatment. Rats with suprapubic catheters implanted were subjected to the treatment regimen shown in Fig. 3. At the end of the experiment, urodynamics were performed as described in MATERIALS AND METHODS. For each rat in each group, three to eight filling-voiding cycles were analyzed. Each parameter was quantitated for each cycle, and the mean was determined. The mean of each parameter from each rat was averaged with the means from the other rats in the group, and this (the mean of the means) is shown here. *Significant differences from control as assessed with a general linear mixed model (P < 0.05).

Inflammasome activation in various tissues often arises from resident or invading immune cells, although these structures have been found in epithelial cells such as the lung (75). Since the bladder epithelium represents a direct anatomic barrier to bacteria and irritative chemicals collected for excretion, it would seem likely that NLRs are present in this layer. Indeed, immunocytochemistry clearly demonstrated the presence and near exclusive localization of these structures to the urothelium. The presence of NLRP3 in this tissue is critical, for it is the only NLR thus far shown to respond to DAMPs. Consequently, it is likely to mediate sterile inflammation in the bladder and the effects of many nonbacterial cystitis-inducing agents including CP, as used in this study. The presence of NLRC4 is also significant as one of the ligands for this NLR is flagellin (23, 54), a well-known virulence factor found in the flagella of common uropathogens (74). We also showed the presence of ASC, which serves as an adaptor between NLRP3 (through its PYD domain) and caspase-1 (through its caspase recruitment domain). In contrast, NLRC4 has an endogenous caspase recruitment domain. In this NLR, NAIP proteins are thought to impart ligand specificity to NLRC4 activation with NAIP5 mediating the interaction with flagella (23) and NAIP2 facilitating the interaction with the type 3 secretion system present on many pathogenic bacteria (10, 15, 32, 54). Unfortunately, antibodies to individual rodent NAIPs are not available, although the presence of NAIP5 can be inferred by the response of urothelia to flagellin in this investigation. The presence of NAIP2 can also be predicted by studies implicating type 3 secretion systems in the virulence of uropathogenic bacteria (15), although additional studies will be necessary to demonstrate this.

The presence of inflammasome components in the urothelium suggest that they serve in a defensive capacity against attack from pathogens within the lumen of the bladder. Activation of inflammasomes was, indeed, demonstrated by the instillation of DAMPs and PAMPs well known to activate inflammasomes in other tissues. For example, inflammasomes were activated by intravesical instillation of LPS, a well-known contributor to uropathogenic virulence (18, 57, 71) and an activator of the NLRP3 inflammasome (12, 43, 44, 64). We also stimulated caspase-1 activity by two NLRP3-activating DAMPs, ATP and MSU. While both ATP and MSU crystals are prototypical NLRP3 DAMPs, MSU is particularly relevant in the bladder as a significant number of bladder stones are composed of this material (53). In addition, flagellin, an NLRP4 ligand (23, 54) and uropathogenic virulence factor (74), also activated caspase-1 when placed in the lumen. Together, these results demonstrate inflammasome activation by their cognate PAMPs and DAMPs that are known to be present in the bladder lumen in pathological conditions. To demonstrate the importance of these structures to bladder inflammation, it was critical to block them in vivo in a common animal model of cystitis. Gly is a FDA-approved drug used in the treatment of type II diabetes. An inhibitor of the ATP-sensitive K+ channel, Gly has been shown to block inflammasome activation upstream of NLRP3 and downstream of the P2X7 ion channel (46).
negative effects of CP treatment in the bladder have been attributed to the metabolite acrolein. The inclusion of Mesna in the treatment regime to bind acrolein in the urine has drastically reduced the appearance of HC but not prevented it completely, particularly in leukemia and lymphoma patients undergoing CP conditioning before stem cell transplant. In the present study, we administered Gly at doses similar to a previously published study (59) twice before CP administration and examined inflammasome activity 4 h after CP, a time period previously shown to coincide with the maximal production of IL-1β and IL-18 (67). Analysis of caspase-1, IL-1β, and IL-18 levels confirmed complete inactivation of the inflammasome in this model.

The success of this drug in blocking inflammasome activation allowed us to subsequently assess the contribution of the inflammasome/IL-1β/IL-18 pathway to inflammation and physiological dysfunction in the bladder and to dissect its influence from other systems implicated in this model (such as TNF-α and IL-6 (48, 67)). Thus, after blockade of the inflammasome pathway, the remaining inflammation and bladder dysfunction in a given model must be attributable to additional pathways. To do this, animals were provided additional Gly after CP (to ensure continued inactivation) and assessed 24 h later, a time period often used in studies of CP-induced inflammation. The extended period is also necessary for the accumulating inflammation to exert demonstrable effects on bladder physiology (i.e., urodynamics). Analysis of bladder weight and Evans blue extravasation showed that inflammasomes are responsible for approximately half of the changes due to CP-induced inflammation in this tissue. Consistent with that, histological examination demonstrated an improvement in the overall bladder architecture with Gly treatment compared with CP only-treated samples, although the improvement was not complete. The lack of a complete block to inflammation is not surprising given that other studies have implicated additional proinflammatory cytokines in the initiation of cystitis, especially TNF-α and IL-6 (48, 67). Thus, the results indicate that inflammasomes play a critical, but not exclusive, role in inducing inflammation in the bladder in response to CP.

Humans suffering from cystitis complain not only of pain but also from urinary urgency, increased frequency, and urinary incontinence. We, therefore, sought to explore the contribution of inflammasomes and the IL-1β pathway to the in vivo bladder dysfunction associated with cystitis. Urodynamics revealed that Gly completely blocked the reduction in void volume and ICI stimulated by CP, although it did not affect the increase in voiding pressure. The totality of the void volume/ICI response was somewhat surprising given that significant inflammation (bladder weight, Evans blue extravasation, and histological changes) remained after our dosing regimen. The results suggest that inflammasomes and/or the IL-1β pathway are central to bladder filling dysregulation (or the sensing of fullness) caused by CP. Interestingly, Gly alone increased void volume and ICI, which suggests that basal inflammasome/IL-1β levels in the bladder contribute to normal bladder function. While the mechanism underlying this remains to be explored, IL-1β has been shown to directly bind and stimulate the bladder detrusor muscle (3–5), clearly indicating cross-talk between these two tissues that could conceivably regulate volume sensing. Moreover, synergistic interaction of IL-1β with other cytokines directly on the detrusor has been proposed to be involved in orchestrating the local inflammatory response in interstitial cystitis (4). Conversely, IL-1β could be acting directly on sensory nerves (70).

Beyond Kummer et al.’s original detection of NLRP3 in the bladder (42), this study is the first to identify two of the best-studied inflammasomes, NLRP3 and NLRC4, in the bladder. We localized them primarily to the bladder epithelium and demonstrated that they can be activated in vivo by relevant PAMPs and DAMPs placed in the lumen of the bladder. Importantly, in vivo bladder inflammation in the CP-induced model of HC was reduced, whereas physiological dysfunction was ameliorated, by the inflammasome inhibitor Gly, suggesting that inflammasomes are important mediators of bladder inflammation and represent prospective targets for pharmacological intervention. Moreover, the efficacy of Gly in this model shows the immediate translation potential of this well-tolerated FDA-approved drug in treating what is often a challenging disorder for physicians. Its use in other models/incidences of cystitis remains to be explored.

GRANTS

This work was supported by National Center for Research Resources Grants UL1-RR-029882 and UL1-TR-000062.

DISCLAIMER

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.M.H. and J.T.P. conception and design of research; F.M.H., P.J.N., and J.T.P. manuscript; F.M.H., P.J.N., and J.T.P. edited and revised manuscript; F.M.H., D.W.L., P.J.N., and J.T.P. analyzed data; F.M.H. and P.J.N. interpreted results of experiments; F.M.H. prepared figures; F.M.H., D.W.L., P.J.N., and J.T.P. approved final version of manuscript.

REFERENCES


56. Moghimpour Bijani F, Vallejo JG, Rezaei N. Toll-like receptor signal-
    ing pathways in cardiovascular diseases: challenges and opportunities. *Int
57. Nielubowicz GR, Mobley HL. Host-pathogen interactions in urinary tract
58. O'Neill LA. Signal transduction pathways activated by the IL-1 receptor/
    2002.
59. Ohmasa F, Saito M, Oiwa H, Tsounapi P, Shomori K, Kitatani K,
    Dimitriadis F, Kinoshita Y, Satoh K. Pharmacological preconditioning
    of ATP-sensitive potassium channel openers on acute urinary retention-
60. Ohtani T, Nakamura T, Toda K, Furukawa F. Cyclophosphamide
    enhances TNF-α-induced apoptotic cell death in murine vascular endo-
61. Parsons BA, Drake MJ. Animal models in overactive bladder research.
63. Saitoh C, Yokoyama H, Chancellor MB, de Groat WC, Yoshimura N.
    Comparison of voiding function and nociceptive behavior in two rat
    models of cystitis induced by cyclophosphamide or acetone. *Neurourol
64. Schwartz PS, Waxman DJ. Cyclophosphamide induces caspase 9-de-
    pendent apoptosis in 9L tumor cells. *Mol Pharmacol* 60: 1268–1279,
65. Silva Lde P, Patah PA, Saliba RM, Szewczyk NA, Gilman L, Neum-
    mann J, Han XY, Tarrand J, Ribeiro R, Gulbis A, Shpall EJ, Jones R,
    Popat U, Walker JA, Petropoulos D, Chiattonne A, Steward J, El-
    Zimaity M, Anderlini P, Giralt S, Champlin RE, de Lima M. Hemor-
    rhagic cystitis after allogeneic hematopoietic stem cell transplants is the
    complex result of BK virus infection, preparative regimen intensity and
66. Song J, Abraham SN. Innate and adaptive immune responses in the
67. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs:
signal 0s that spur autophagy and immunity. *Immunol Rev* 249: 158–175,
    2012.
68. Wright KJ, Seed PC, Hultgren SJ. Uropathogenic Escherichia coli
    flagella aid in efficient urinary tract colonization. *Infect Immun* 73:
69. Yeretssian G. Effector functions of NLRs in the intestine: innate sensing,
70. Zambetti LP, Laudisi F, Licandro G, Ricciardi-Castagnoli P, Mortel-
    laro A. The rhapsody of NLRPs: master players of inflammation...and a lot