NLRP3 promotes diabetic bladder dysfunction and changes in symptom-specific bladder innervation

by

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Abstract

The NLRP3 inflammasome senses diabetic metabolites and initiates inflammation implicated in diabetic complications and neurodegeneration. No studies have investigated NLRP3 in diabetic bladder dysfunction (DBD), despite a high clinical prevalence. *In vitro*, we found that numerous diabetic metabolites activate NLRP3 in primary urothelial cells. *In vivo* we demonstrate NLRP3 is activated in urothelia from a genetic Type 1 diabetic mouse (Akita) by week 15. We then bred a NLRP3^−/− genotype into these mice and found this blocked bladder inflammation and cystometric markers of DBD. Analysis of bladder innervation established an NLRP3-dependent decrease in overall nerve density and Aδ-fibers in the bladder wall along with an increase in C-fiber populations in the urothelia, which potentially explains the decreased sense of bladder fullness reported by patients and overactivity detected early in DBD. Together, the results demonstrate the role of NLRP3 in the genesis of DBD and suggest specific NLRP3-mediated neuronal changes can produce specific DBD symptoms.
**Introduction**

Diabetic bladder dysfunction (DBD) affects a large proportion of diabetic patients and there is currently no specific therapy to treat it (1,2). With approximately 422 million diabetics worldwide in 2014 (3), and projections that as many as 1 in 3 people will be affected in the United States by 2050 (4), this represents a highly prevalent pathological condition without a targeted therapy. Patients with DBD may present with urinary frequency and urgency (i.e. overactive bladder or OAB) or detrusor underactivity and bladder decompensation (5) or some combination thereof. While definitive longitudinal studies in humans are lacking, several experimental animal models suggest progression from OAB to decompensation (6-8). Regardless, until now the mechanism by which the metabolic dysregulation translates into physiological dysfunction has been unclear.

It is now appreciated that diabetes is not just a disease of high blood sugar but also a disease of deranged metabolism resulting in hyperglycemia and the production of numerous metabolites such as uric acid and free fatty acids (9). These metabolites trigger inflammation that damages susceptible tissues with a resulting loss of function (10). Recent breakthroughs in other diabetic complications (nephropathy, retinopathy, cardiomyopathy) have demonstrated this inflammation results from activation of the Nod-Like receptor NLRP3, which forms a supramolecular complex known as an inflammasome (11).

NLRP3 is the best studied of the NOD-like receptor (NLR) family of pattern receptors. In general, pattern receptors recognize molecules released from damaged or dying cells (or those with deranged metabolism), known as damage (or danger) associated molecular patterns (DAMPS) or components of pathogens known as pathogen associated molecular patterns
(PAMPs). NLRP3 is by far the best understood NLR to recognize DAMPS and has been implicated in many diseases with a sterile inflammatory component, including diabetic complications. Upon recognition of DAMPS, via a poorly understood mechanism, NLRP3 oligomerizes and triggers enucleation of an adaptor protein known as Apoptosis-Associated Speck-like Protein C (ASC). ASC in turn interacts with procaspase-1 which is cleaved and activated through an auto-proteolytic process. Caspase-1, in turn, catalyzes the enzymatic maturation IL-1β, IL-18 and gasdermin D. Gasdermin D forms a pore in the plasma membrane, triggering a programmed necrosis called pyroptosis which releases IL-1β and IL-18 that act as proinflammatory cytokines to initiate the inflammatory response. Recently we have shown that NLRP3 plays an important role in the urinary tract of the rodent (12,13). In the rat bladder, NLRP3 is localized to the urothelium (14,15) where it mediates sterile inflammation in several important bladder pathologies including bladder outlet obstruction and cyclophosphamide-induced hemorrhagic cystitis (15,16). Experimental models have also implicated NLRP3 in the response to urinary tract infections (17,18). Based on the central role for NLRP3 in other diabetic complications, coupled with its importance in sterile cystitis, we hypothesized that NLRP3-mediated inflammation is a crucial element in the development of DBD.

To address this hypothesis we bred a genetic mouse model of Type 1 diabetes (the Akita mouse) (19) with a non-diabetic NLRP3$^{-/-}$ strain to create a novel strain of diabetic mice lacking NLRP3. These mice were then assessed for DBD. Mechanistically, DBD results, in part, from peripheral neuropathy (20,21). Therefore, we also evaluated a role for NLRP3 in the variation of nerve cell populations that could explain functional deficits in diabetic patients, specifically diminished sensation (Aδ-fibers) and bladder overactivity (C-fibers).
Research Design and Methods

Experimental Approach

Our approach in this study is three-pronged: 1) \textit{in vitro} analysis of the activation of the inflammasome in normal mouse urothelia by diabetic-associated DAMPS; 2) \textit{in vivo} urinary function (cystometry) in diabetic mice with a genetic deletion of NLRP3 and 3) quantitation of nerve densities in the bladders of these mice to assess potential changes in specific nerves thought to mediate specific DBD symptoms.

Animals

All protocols adhere to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center. Founder mice from The Jackson Laboratory (Bar Harbor, MA) consisted of Akita (C57BL/6J-Ins2Akita/J mice; stock number: 003548) (22) and NLRP3\textsuperscript{-/-} (B6.129S6-Nlrp3\textsuperscript{tm1Bhk}/J (stock number: 021302) mice (23). While the strain of origin for the NLRP3\textsuperscript{-/-} mice (129S6/SvEvTac) is different from the Akita background (C57BL/6J) these mice have been backcrossed to C57BL/6J for >11 generations (https://www.jax.org/). Mice were bred by the Breeding Core Facility at Duke University through an independently approved protocol and only female mice were used. All animals were genotyped by Transnetyx, Inc. (Cordova, TN) and provided to the laboratory around 4 weeks of age.

The results of genotyping were used to assign them to one of the 4 experimental groups. Nondiabetics are “nondiab” and diabetics are “diab”. The groups are
1. NLRP3\(^{+/+}\), nondiab,– homozygote wt NLRP3 genes, homozygote wt Ins2 genes - i.e. control mice

2. NLRP3\(^{+/+}\), diab – homozygote wt NLRP3 genes; heterozygote for Akita mutation at the Ins2 gene – i.e. Akita diabetic control.

3. NLRP3\(^{-/-}\), nondiab – both NLRP3 genes knocked out, homozygote wt Ins2 genes – i.e. NLRP3 knockout control.

4. NLRP3\(^{-/-}\), diab,– both NLRP3 genes knocked out, heterozygote for Akita mutation at the Ins2 gene. This is the experimental mouse generated for this study.

Animals were received at 5 weeks of age. Blood glucose becomes high in Akita mice (200-300 mg/dL) around 4 weeks of age and remains high thereafter (19,24). Mice were grown to 15 weeks when DBD becomes apparent (25). No changes in urinary dysfunction were found at previous time points (25).

**In vitro experiments**

NLRP3\(^{+/+}\), nondiab mice (i.e. control littermates) were used at 7-8 weeks of age. Urothelial cells were isolated (26) and plated (black-walled 96 well plates) at 50,000 cells/well in 90 µl complete media [F-12K media, 10% low-endotoxin dialyzed fetal bovine serum, 10 µM non-essential amino acids (all HyClone Laboratories, Logan, UT), 1.0 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 10 µg/ml insulin, 5 µg/ml transferrin and 6.7 ng/ml selenium (ITS, Gibco, Gaithersburg, Maryland). Following a 24 hr incubation (37\(^{\circ}\)C, 95% air/5% CO\(_2\)), DAMPs (10 µl) were added and incubated as indicated. 1 h prior to harvest 1.25 mM ATP was added to untreated wells. In studies that examined ATP doses, cells were plated for 24 h, then
treated with 1 µg/ml LPS (E Coli 055:B5; Sigma) in PBS or PBS alone for 24 h before treatment with the indicated doses of ATP for 1 h. Caspase-1 activity was then measured as previously described (14). Control fluorescence (0 mM DAMP) was subtracted from all wells and results normalized to the ATP response (except for the ATP dose response studies).

**Histological preparation**

Bladders were formalin-fixed and paraffin-embedded in a transverse orientation. Sections (5 µm) from the lower third of the bladder were stained with anti-NLRP3 (1:100; cat# LS-C334192; Life Span BioSciences, Inc., Seattle, WA), anti-PGP9.5 (1:200; cat# 381000; ThermoFisher, Waltham, MA), anti-Neurofilament 200 (NF-200; Aδ-fibers; 1:200, cat# N4142, Sigma-Aldrich, St. Louis, MO) or anti-Calcitonin Gene Related Peptide (CGRP; C-fibers; 1:80, cat# PC205L, Calbiochem, Burlington, MA) antibodies using standard methods and citrate antigen retrieval. Staining was visualized with secondary antibodies conjugated to either Alexa Fluor 488 (NLRP3, NF-200 and CGRP) or HRP (PGP9.5; developed with Vectastain ABC Staining Kit; Vector Laboratories, Burlingame, CA). All sections were imaged on a Zeiss Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) running Zen software (Zeiss). Tiling micrographs encompassing the entire cross section were captured by the software and stitched into a continuous image. Calibration bars were inserted and images exported as TIFF files.

**FAM-FLICA caspase-1 assay**

Caspase-1 activity was assessed using the FAM-FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN, USA) and the manufacturer’s
recommended protocol. Cells were analyzed on a FACSCalibur flow cytometer (BD-Bioscience; San Jose, CA) (excitation 488 nm, emission 533 nm) and dot plots of forward versus side scatter were used to gate on single cells. Histograms were created and gates were drawn to allow quantitation of the mean florescent intensity (MFI). The geometric mean of the MFI (the Geo Mean) of each sample was used for comparisons.

**Blood glucose**

Blood from the submandibular vein was assessed with the AimStrip Plus blood glucose testing system (Germaine Laboratories, San Antonio, TX).

**Evans Blue dye extravasation**

Extravasation of Evans blue dye is a direct measurement of vascular permeability which is increased during inflammation. Thus, movement of this dye into a tissue is used as an indirect measurement in inflammation (15-17,25). In this study mice were injected (i.v.) with 10 mg/kg dye in saline and 1 h later sacrificed. Bladders were weighed and incubated overnight (56°C) in 1 ml formamide and the absorbance (620 nM) of the formamide measured. Dye amounts were calculated from a standard curve and normalized to bladder weight.

**Cystometry**

Awake restrained cystometry was performed (15-17,25). One week prior, suprapubic tubes (PE-10 tubing with a flared end) were implanted in the bladder and secured with a purse string suture (6-0 silk). The tube was externalized at the back of the neck. One week later
animals were placed in a Ballman-type restrainer (Natsume Seisakusho Co., Tokyo, Japan) inside of a Small Animal Cystometry Lab Station (Med Associates, St. Albans, VT) and positioned above an analytical balance to measure voided volume. The catheter was connected to a syringe pump via an in-line pressure transducer and sterile saline infused at 15 µl/min for 60 - 120 min. Scale and pressure readings were continuously recorded with Med-CMG software (Med Associates, St. Albans, VT). After voiding cycles stabilized (typically 3-4 cycles) an additional 3-8 cycles were recorded for quantitation. Immediately after the last void, infusion was stopped, the catheter attached to a 3 ml syringe and the plunger was withdrawn for 10-15 sec to recover any PVR. CMG Analysis software (version 1.06; Med Associates, St. Albans, VT) was used to analyze voiding cycles, defined as the time intravesicular pressure returned to baseline after a previous void until it returned to baseline following the next void. Voiding pressure is defined as the peak intravesical pressure, void volume as the amount of change on the scale and frequency as the number of voids per hour. Voiding efficiency was calculated as 100 x the voiding volume divided by the bladder capacity (void volume + PVR).

Analysis of nerve densities

Quantitation of PGP9.5 and Aδ-nerve density in the bladder wall was carried out exactly as previously described (27) while quantitation of C-fibers in the urothelium required only minor modifications. Briefly, TIFF files were imported into NIS-Elements software (Nikon Co., Tokyo, Japan), calibrated and the bladder wall or urothelia layer demarcated (the ROI) and area calculated. PGP9.5+ neurons were defined as black/brown spots >50 um². Aδ-fibers were defined as fluorescent areas >50 um² that stained positive with a nuclear co-stain (DAPI). C-
fibers were defined as continuous fluorescent fibers >1 µm. Neuronal density in a given section was calculated by dividing the number of nerves by the µm² of the ROI.

Statistical analysis

All parameters were assessed by either a two-tailed Students T-test or a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc analysis. Both analyses used GraphPad InStat software (La Jolla, CA) and statistical significance was defined as p<0.05.

Results

Diabetic DAMPS activate the inflammasome in vitro

To assess the ability of diabetic DAMPS to trigger inflammasome activation, urothelial cells were treated in vitro and caspase-1 activity measured (14). ATP, the quintessential NLRP3-activating DAMP, elicited a classic dose response (Figure 1A) and is subsequently used to compare other DAMPs. In most cells, NLRP3 activation requires priming with an agent such as LPS (28-34). However, LPS priming had no effect on these cells (Figure 1B). Streptozotocin poisoning of beta cells is a widely used to create a type 1 model of diabetes. However, we found in Figure 1C that streptozotocin directly activates the inflammasome in urothelial cells, clearly contraindicating that model for these DBD studies. Finally, Figures 1D-G demonstrates activation of caspase-1 by four separate diabetic DAMPS (9); monosodium urate (MSU), high mobility group box 1 protein (HMGB-1), C6-ceramide and advanced glycation end products (AGEs).
*NLRP3 is activated during diabetes*

To explore a role for the inflammasome in DBD it is essential to demonstrate that it is activated in the bladder by diabetes. Figure 2 demonstrates a significant increase in active caspase-1, the enzymatic readout for active inflammasomes, in urothelia from the 15 week diabetic animals compared to nondiabetic controls.

*NLRP3 is expressed in the mouse urothelia and its distribution does not change with diabetes.*

Although documented in rat (14,15), NLRP3 has never been examined in the mouse bladder. As shown in Figure 3 (top left), expression of NLRP3 in the nondiabetic bladder was localized to the urothelial layer, identical to the rat. An indistinguishable distribution was noted in the diabetic strain (Figure 3, top right). Isotype controls showed little background staining.

*The NLRP3+/− genotype does not affect blood glucose in the diabetic*

To assess a role for NLRP3 in DBD we have explored numerous endpoints both nondiabetic and diabetic animals with intact NLRP3 (NLRP3+/+) and nondiabetic and diabetic mice with NLRP3 genetically deleted (NLRP3−/−). Blood glucose levels in these groups is shown in Figure 4. As expected, blood glucose levels were considerably greater in the diabetic compared to the nondiabetic mouse with NLRP3 present (NLRP3+/+) (Figure 4A). A similar increase with diabetes is seen with the NLRP3+/− strains (Figure 4B). No significant differences were detected between the nondiabetic or diabetic based on NLRP3 expression (i.e. comparing the NLRP3+/−, nondiabetic to the NLRP3−/−,
nondiabetic, and likewise with the diabetics). Thus, deletion of NLRP3 has no effect on blood glucose levels in either the nondiabetics or the diabetics.

**Inflammation is present in the diabetic bladder and is mediated through NLRP3**

While there is general evidence that inflammation is present in many tissues during diabetes, there is little or no evidence in the bladder. Therefore, we have used the Evans blue dye extravasation assay (15-17), a direct measure of vascular permeability and an indirect measure of inflammation, to gain insight into inflammation in the diabetic bladder. As shown in Figure 5A, there was a significant increase of dye extravasation in the 15-week diabetic mouse compared to the nondiabetic (both NRP3+/+), indicating substantial inflammation at this time point. This increase in extravasation associated with diabetes was completely blocked in the NLRP3−/− mouse (Figure 5B).

**NLRP3 is responsible for bladder dysfunction associated with DBD**

Previously we demonstrated that the diabetic Akita mice develop DBD by 15 weeks of age (25). To investigate the effects of NLRP3 on bladder dysfunction we performed cystometry at this time point on the four experimental groups (35). Figure 6 shows representative tracings, for each of the four groups, of the changes in pressure (cm H2O) in the bladder lumen during several micturition cycles. Peaks in pressure correspond with voids and are marked with an asterisk (*). Tracing align the first micturition to illustrate differences in the time between voids (the intercontraction interval) which is used to calculate voiding frequency. Not shown are tracings of the scale aligned under the rat that measure voided volume. Quantitative summaries
are shown in Figure 7. Figure 7A demonstrates a decrease in void volume in the diabetic mice compared to the nondiabetic when NLRP3 is present (NLRP3^{+/+}). Figure 7B shows an increase in voiding frequency in these same mice. In animal models, decreased void volume coupled with increased frequency are often considered synonymous with OAB, which in humans requires subjective measures (such as urgency) that cannot be measured in animals. These results are also consistent with our previous findings (25). Importantly, neither of these diabetic changes were apparent in the absence of NLRP3 (NLRP3^{-/-}) (Figure 7B and D).

The development of DBD, while complex, is thought to progress from an early, OAB phenotype to a later stage underactive bladder (UAB) characterized by increased post-void residual (PVR) volumes and decreased voiding efficiency (5). This UAB phenotype is indicative of a decompensated bladder. In the diabetic mouse at 15 weeks (Figures 7E and G) we detected a significant increase in PVR and decrease in voiding efficiency, indicating the transition to UAB and decompensation has begun (Figure 7E and G and (25)). However, these alterations are reduced in the NLRP3^{-/-} animals (Figure 7F and H).

_NLRP3 controls changes in the densities of nerves related to specific DBD symptoms_

DBD is associated with peripheral neuropathy and one gauge of neuropathy in a tissue is the alteration in nerve number and/or density which would be expected to decrease in diabetes and may be dependent on the NLRP3 inflammasome. To examine neuropathy in the bladder we have quantitated total nerves using PGP9.5 as a pan neuronal marker in the bladder wall (36). Representative staining is shown in Figure 8A. Arrows indicate positive staining while the block arrow indicates nonspecific, or at least non-neuronal staining of the urothelia. While the
significance of the urothelial staining is unknown, it has been previously reported (27,37). Quantitatively, the total number of nerves in the bladder wall was decreased in the diabetic mouse (Figure 8B) in the presence of NLRP3, but this effect was not significant in the NLRP3−/− strain (Figure 8C). There was no change of bladder wall size in any group (Figure 8D and E) so changes in nerve density (Figure 8F and G) directly reflect the changes in nerve number.

Next we assessed specific nerve types thought to underlie individual bladder symptoms in diabetics. First, bladder fullness is relayed to the CNS via Aδ-fibers and patients often report a reduced sensation of bladder fullness. Thus one may postulate there may be a decrease in the number and/or density of these fibers in our diabetic mice which may be driven by the NLRP3 inflammasome. Because Aδ-fibers are predominantly in the bladder wall we quantitated them in this compartment. Representative staining is shown in Figure 8H. As shown in Figure 8I, there was a significant decrease in the number of Aδ-fibers (NF-200+ cells) in the bladder wall of the diabetic mouse when NLRP3 was present. This decrease was not detected in the NLRP3−/− diabetics (Figure 8J). Bladder wall size (Figure 8K and L) remained constant, so changes in Aδ-fiber densities (Figure 8M and N) reflect changes in fiber number.

C-fibers are associated with an OAB phenotype (38) which is common in early stage diabetic patients and also apparent in our mice at 15 weeks of age (Figure 7). Thus one may postulate there may be an increase in the number and/or density of these fibers with diabetes and this change may be driven by NLRP3. C-fibers are predominately in the urothelia and lamina propria and so we have focused on this tissue layer. Representative staining is shown in Figure 8O. As shown in Figure 8P, the number of C-fibers (CGRP+) in the urothelium was significantly increased in the diabetic bladders when NLRP3 was intact. This increase did not occur in the
diabetic NLRP3−/− mice (Figure 8Q). Urothelium did not change size (Figure 8R and S) so density results (Figure 8T and U) again reflected changes in cell numbers.

Discussion

The diabetic bladder is unique in that tissue damage can be caused by two independent mechanisms; 1) polyuria and 2) hyperglycemia. Previous studies (39) concluded that polyuria produced muscle hypertrophy while the hyperglycemia caused tissue damage from oxidative stress. Oxidative stress often triggers inflammation and we have recently shown diabetic bladder inflammation is due to hyperglycemia, not polyuria (25). Here, we show that it is the NLRP3 inflammasome, located within the urothelium, senses and responds to metabolic dysregulation by initiating an inflammatory response. Most importantly, diabetic mice lacking the NLRP3 gene do not develop diabetic bladder dysfunction.

Numerous diabetic DAMPS activated the NLRP3 inflammasome in vitro, demonstrating their pro-inflammatory potential. Interestingly, activation of NLRP3 did not require priming as in most cell types (15). While atypical, this has been reported (40) and suggests urothelia either do not require priming or are already primed when isolated, possibly through exposure to the commensal microbiome (40). We also found streptozotocin to be an activator of NLRP3. Streptozotocin is not a diabetic metabolite but rather a pancreatic toxin commonly used to induce diabetes in experimental models. Our results discouraged the use of that model in DBD studies.

We have recently shown that bladder inflammation and DBD develop in the Akita diabetic mouse by 15 weeks (25). In that study (and this), extravasation of Evans blue was pronounced. This study also demonstrated a concurrently activation of the inflammasome. To
investigate the role of NLRP3, we crossed the Akita mouse with an NLRP3\textsuperscript{−/−} strain to create a unique substrain of diabetic mice lacking this inflammasome. Deletion of NLRP3 did not affect serum glucose levels but it did abolish the inflammatory response in the diabetic. Therefore, it appears urothelial NLRP3 is indeed capable of sensing the metabolic dysregulation of diabetes and promoting an inflammatory response.

Cystometrically, our diabetic animals demonstrate clear signs of early DBD at 15 weeks with decreased voiding volume, increased frequency and increased PVR, consistent with several prior studies (5,41). In the absence of NLRP3, diabetes did not change the frequency or void volume, unequivocally demonstrating that NLRP3 is responsible for the urinary changes of DBD. Interestingly, the diabetic bladder retained a significant PVR typically associated with later stage DBD and underactive bladder, thus suggesting that the transition towards a decompensated state has begun. Importantly, in the absence of NLRP3, the bladder maintained normal voiding volumes and efficient emptying, showing the importance of NLRP3 in the transition to decompensation where there is a much greater risk of complications such as infection and stone formation.

While traditional concepts of DBD postulated that the sole pathological cause was autonomic neuropathy (42), more conventional views recognize multifactorial disturbances (43). Considering the well-known association between peripheral neuropathy and the development of DBD (44), we hypothesized that various DBD-related symptoms result from deleterious effects on the nerves within the bladder and we found decreased nerve density in the bladder wall in the diabetic mice. Furthermore, the effects on different types of nerves vary. The $\alpha$-fibers, which sense fullness in the bladder, were decreased in the bladder wall and this may explain why a diminished sense of fullness is often reported with diabetic patients. On the other hand, the C-
fiber population in the urothelium increased in the diabetic bladders. C-fibers normally sense pain but they are also associated with the emergence of an overactive bladder phenotype (38) which is common in early stage diabetic patients. Thus, the differential effects of inflammation on these two types of nerves provide a possible explanation for the specific symptoms associated with DBD.

The current study provides a convincing mechanism whereby a plethora of diabetic insults converge on NLRP3 in the urothelia and translate into inflammation and damage to the bladder. These insults include ATP and numerous metabolites but also likely include additional insults such as reactive oxygen species, created from excessive oxidative phosphorylation, and ischemia which is a well-known activator of NLRP3 that recent studies suggest play a role in DBD (45). The signal emanating from the urothelia to trigger effects in the other bladder tissues has unidentified but likely attributable to the major products of the inflammasome, IL-1β and IL-18, acting in a paracrine fashion. Indeed we have shown that IL-1β is responsible for the decrease in PGP9.5+ nerves in the bladder wall in a rat model of bladder outlet obstruction (27) while others have implicated it in bladder smooth muscle hypertrophy (46).

The central role of NLRP3 in development of DBD suggests a possible strategy for the prevention and management of this diabetic complication. According to the DCCT trial, only 58% of patients are able to maintain the strict glycemic control favored by the American Diabetic Association (47). While strict regulation does prevent retinopathy, nephropathy and other diabetic complications, bladder dysfunction still remains a problem for these patients (48-50). The present study suggests that NLRP3 inhibitors could serve as a simple means to prevent or treat DBD and possibly other diabetic complications where this pathway plays a central role.
The results clearly show that activation of the NLRP3 inflammasome, possibly by diabetic metabolites, underlies bladder dysfunction and denervation during DBD in mice and therefore may serve as a critical pharmacological target for combating this complication in humans.

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Figure legends

**Figure 1. Analysis of various DAMPS on inflammasome activation in urothelial cells in vitro.** Cells were isolated, plated and treated with the doses of the various compounds indicated. Following incubation periods described below, caspase-1 was measured. A. ATP dose-response. ATP was prepared as a 25 mM stock in complete media (pH adjusted with 0.5 N NaOH) and dilutions made with complete media. The indicated final doses of ATP (in 10 µl) were then added to cells for 1 h prior to caspase-1 analysis. Each point represents the mean ± SEM. n = 5 for each dose. Asterisks indicate significant differences from O mM control. ***p<0.001 by ANOVA followed by Tukey’s post-hoc test. B. Effects of LPS on the ATP dose-response. Urothelial cells were plated for 24 h, then LPS (1µg/ml) in 10 µl PBS (or PBS alone) added for an additional 24 h. Then the indicated doses of ATP were added for 1 h prior to caspase-1 analysis. It should be noted that the –LPS samples (closed triangles) are the exact same samples shown in A, but are included in B for ease of comparison. Each point represents the mean ± SEM. n = 5 for each dose. Student’s two-tailed t-test was used to compare the –LPS and the + LPS sample at each dose of ATP. C. Streptozotocin dose response. Streptozotocin was prepared as a 200 mM stock in 0.1 M citrate buffer (pH 4.4) and dilutions made in media before being
added (10 µl) to the wells at the indicated final concentrations. Cells were incubated 24 h before the addition of 1.25 mM ATP for 1 h and subsequent analysis. Each point represents the mean ± SEM. n = 5 for each dose. *p<0.05 by ANOVA followed by Tukey’s post-hoc test. D. Monosodium urate crystals (InvivoGen, San Diego, CA) were received at 5 mg/ml and dilutions prepared in complete media just prior to addition to the well (in 10 µl). Cells were incubated 24 h before the addition of 1.25 mM ATP for 1 h and analysis. Each point represents the mean ± SEM. N = 6 for each dose. *p<0.05 **p<0.01 by ANOVA followed by Tukey’s post-hoc test. E. High mobility group box 1 protein (HMGB-1; ProSci, Poway, CA) was resuspended and diluted in complete media just prior to addition (10 µl) to the wells at the indicated final concentrations. Cells were incubated 24 h before the addition of 1.25 mM ATP for 1 h and subsequent analysis. Each point represents the mean ± SEM. n = 6 for each dose. **p<0.01 by ANOVA followed by Tukey’s post-hoc test. F. N-hexanoyl-D-erythro-sphingosine (C6-Ceramide; Alfa Aesar, Haverhill, MA) was dissolved in DMSO and diluted in complete media prior to addition to the wells (10 µl). Cells were incubated 24 h before the addition of 1.25 mM ATP for 1 h and analysis. Each point represents the mean ± SEM. n = 6 for each dose. *p<0.05 **p<0.01 by ANOVA followed by Tukey’s post-hoc test. G. Advanced Glycation Endproduct-BSA (AGE; Calbiochem, Millipore Sigma, Burlington, MA) was prepared and diluted in complete media prior to addition to the wells. Cells were incubated 24 hour before the addition of 1.25 mM ATP for 1 h and analysis. Each point represents the mean ± SEM. n = 6 for each dose. **p<0.01 ***p<0.001 by ANOVA followed by Tukey’s post-hoc test.

Figure 2. NLRP3 is activated in the urothelium during diabetes. Inflammasome activity (caspase-1) is increased in urothelia from diabetic mice. Urothelia were isolated and stained with
a FAM-FLICA Caspase-1 Assay Kit (Immunochemistry Tech., Bloomington, MN) as described. All mice were examined at 15 weeks of age. Bars are mean ± SEM. n=18 (nondiabetic), 17 (diabetic). *p<0.05 by Student’s two tailed t-test.

**Figure 3. NLRP3 is present in mouse urothelia and its distribution is not effected by diabetes.** All mice were examined at 15 weeks of age and all scale bars = 50 µm. Sections of bladder (5 µm) from the indicated mice were stained for NLRP3 using standard immunocytochemistry and antigen retrieval protocols along with an Alexa Flour 488 conjugated secondary antibody. Isotype controls used normal rabbit serum instead of primary antibodies. n = 3 (nondiabetic), 4 (diabetic).

**Figure 4. Blood glucose is not effected by knocking out NLRP3.** Blood glucose levels were assessed at week 15 using the AimStrip Plus blood glucose testing system. A. Blood glucose is very significantly increased in the diabetic mouse with a NLRP3+/+ genotype. Bars are mean ± SEM. n=27 (nondiabetic), 12 (diabetic). ***p<0.0001 by paired Student’s t-test. B. Blood glucose is significantly elevated in the diabetic mouse with a NLRP3-/- genotype. Bars are mean ± SEM. n=18 (nondiabetic), 21 (diabetic). ***p<0.0001 by Student’s two tailed t-test. ANOVA followed by Tukey’s post-hoc test was also used to compare all groups. No additional significant differences were found.

**Figure 5. Inflammation is present in the diabetic bladder and is mediated through NLRP3.** The effect of diabetes on the induction of inflammation in the bladder was assessed in the presence and absence of NLRP3 using the Evans Blue dye extravasation assay described in the
Methods section. A. The amount of Evans Blue dye in the bladder was increased in diabetic mice compared to nondiabetic (both NLRP3+/+). Bars are mean ± SEM. n=5 (nondiabetic), 12 (diabetic). ***p<0.0001 by Student’s two tailed t-test. B. Diabetes did not affect the movement of Evans Blue into the bladder in the absence of NLRP3. Bars are mean ± SEM. n=6 (nondiabetic), 16 (diabetic). ANOVA followed by Tukey’s post-hoc test was also used to compare all groups. No additional significant differences were found.

Figure 6. Representative tracings of changes in intravesicular pressures over time from the cystometry study used to demonstrate that NLRP3 is responsible for bladder dysfunction associated with DBD. Shown is a single representative tracing of the changes in luminal pressure (cm H₂O) in the bladder over several micturition cycles, obtained during cystometry, for each of the 4 experimental groups used in this study. These tracings were recorded using an inline pressure transducer that made measurements every 0.25 s during the course of the experiment. The tracings were chosen to represent only several micturition cycles and do not display the entirety of the recording which was typically much longer. Voidings correspond with the large peaks in pressure and are indicated with asterisks (*). The tracings from the 4 groups were arranged vertically to align the first voiding volume of each while continuing the recording for the same length of time. This was to allow easy comparison and judgement of voiding frequency (number of voids, indicated by peaks in pressure, over time). Typically 3-8 micturition cycles were quantitated per animal. A. representative tracing from the NLRP3+/+ strains. B. representative tracings from the NLRP3-/- strains.
**Figure 7. NLRP3 is responsible for bladder dysfunction associated with DBD.** The results of various parameters measured through cystometry are shown for nondiabetic and diabetic mice that either express NLRP3 (NLRP3+/+) or have that gene deleted (NLRP3-/-). All studies were performed at 15 weeks of age and animals were implanted with a suprapubic catheter one week prior to analysis. A. voiding volume in nondiabetic and diabetic mice (both NLRP3+/+), B. voiding volume in nondiabetic and diabetic mice with NLRP3 deleted (NLRP3-/-). C and D. Frequency of voiding in the indicated animals. E and F. The post-void residual (PVR) volume, or volume of urine remaining in the bladder immediately after the last void, in the indicated animals. No PVR was ever detected in any of the nine Nondiabetic/NLRP3+/+ mice examined. G and H. The voiding efficiency of the indicated animals calculated as 100(voided volume)/(voided volume + PVR). For all graphs, bars are mean ± SEM. n=9 and 7 for nondiabetic and diabetic mice, respectively, that are NLRP3+/+. N = 10 and 9 for nondiabetic and diabetic mice, respectively, that are NLRP3-/. **p<0.01, ***p<0.001 by a Student’s two-tailed t-test. ANOVA followed by Tukey’s post-hoc test was also used to compare all groups for each endpoint. The only additional significant differences found were in void volume comparing NLRP3+/+ diabetic to NLRP3-/- diabetic (p<0.05) and voiding efficiency comparing NLRP3+/+ diabetic to NLRP3-/- nondiabetic.

**Figure 8. NLRP3 controls changes in the densities of nerves related to specific DBD symptoms.** A. Representative micrographs of PGP9.5 staining (i.e. all neurons) in the bladder used to quantify nerves. The left micrograph depicts the entire transverse cross section stained and scanned into a TIFF file used for quantitation, as described in the methods section. The yellow box indicates the area zoomed in on the right micrograph to allow better visualization.
Block arrow points at urothelia that stain non-specifically for PGP9.5, or at least are of non-neuronal origin. Arrows indicate the brown staining in the bladder wall considered to stain positive for this antigen. B. The number of PGP9.5⁺ nerves in bladder wall of 15 week mice from nondiabetic (non diab) and diabetic (diab) mice that express NLRP3 (NLRP3⁺/⁺). C. Same analysis as B in mice that have the NLRP3 gene deleted (NLRP3⁻/⁻). D and E. The size of the bladder wall in the same sections and groups quantitated in B and C, respectively. F and G. Density of PGP9.5⁺ neurons in the same sections and groups quantitated in B and C, respectively. H. Representative micrographs of NF-200 staining (Aδ-fibers) in the bladder used to quantify nerves. The left micrograph depicts the entire transverse cross section while the yellow box indicates the area zoomed in on the right and arrows point at staining in the bladder wall considered to be positive for this antigen. I. The number of Aδ-fibers in bladder wall of 15 week mice from nondiabetic (non diab) and diabetic (diab) mice that express NLRP3 (NLRP3⁺/⁺). J. Same analysis as I in mice that have the NLRP3 gene deleted (NLRP3⁻/⁻). K and L. The size of the bladder wall in the same sections and groups quantitated in I and J, respectively. M and N. Density of PGP9.5⁺ neurons in the same sections and groups quantitated in I and J, respectively. O. Representative micrographs of CGRP staining (C-fibers) in the bladder used to quantify nerves. The left micrograph depicts the entire transverse cross section while the yellow box indicates the area zoomed in on the right and arrows point at staining in the bladder wall considered to be positive for this antigen. This section is also stained with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) in the coverslipping material to allow easier visualization. P. The number of C-fibers in bladder wall of 15 week mice from nondiabetic (non diab) and diabetic (diab) mice that express NLRP3 (NLRP3⁺/⁺). Q. Same analysis as P in mice that have the NLRP3 gene deleted (NLRP3⁻/⁻). R and S. The size of the bladder wall in the same
sections and groups quantitated in P and Q, respectively. T and U. Density of PGP9.5+ neurons in the same sections and groups quantitated in P and Q, respectively. For all graphs bars represent mean ± SEM. For B, D and F, n = 11. For C, E and G, n = 10. For I, K and M, n = 6. For J, L and N, n = 7. For P, R and T, n = 4 (non diab) and 6 (diab). For Q, S and U, n = 3 (non diab) and 4 (diab). *P<0.05, **p<0.01, ***p<0.001 by a Student’s two-tailed t-test. ANOVA followed by Tukey’s post-hoc test was also used to compare all groups for each endpoint. The only additional significant differences found were in Aδ-fiber nerve number comparing NLRP3+/+ diabetic to NLRP3−/− diabetic (p<0.05), bladder wall size in the Aδ-fiber study comparing NLRP3+/+ nondiabetic to NLRP3−/− nondiabetic (p<0.05), Aδ-fiber nerve number comparing NLRP3+/+ diabetic to NLRP3−/− diabetic (p<0.05). In the C-fiber studies the NLRP3+/+ diabetic was significantly different from both NLRP3−/− strains in the nerve number and density graphs (p<0.05)
Active Caspase-1 (Geo Mean)

- non diab
- diab

*
Serum Glucose

A. NLRP3^{+/+}

B. NLRP3^{-/-}
Evans blue extravasation

A. NLRP3+/+

B. NLRP3−/−
NLRP3

**Nerve number in bladder wall**

B. NLRP3+/–

C. NLRP3–/–

Bladder wall size

D. NLRP3+/–

E. NLRP3–/–

**Aδ-fiber number in bladder wall**

I. NLRP3+/–

J. NLRP3–/–

C-fiber number in urothelium

P. NLRP3+/–

Q. NLRP3–/–

**Urothelium size**

R. NLRP3+/–

S. NLRP3–/–

Nerve density

F. NLRP3+/–

G. NLRP3–/–

**Aδ-fiber density**

M. NLRP3+/–

N. NLRP3–/–

C-fiber density

T. NLRP3+/–

U. NLRP3–/–