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Toll-Like Receptor 4 Activation Contributes to Diabetic Bladder Dysfunction in a Murine Model of Type 1 Diabetes

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COMPLICATIONS

Diabetic bladder dysfunction (DBD) is a common urological complication of diabetes. Innate immune system activation via Toll-like receptor 4 (TLR4) leads to inflammation and oxidative stress and was implicated in diabetes pathophysiology. We hypothesized that bladder hypertrophy and hypercontractility in DBD is mediated by TLR4 activation. Wild-type (WT) and TLR4 knockout (TLR4KO) mice were made diabetic by streptozotocin (STZ) treatment, and bladder contractile function and TLR4 pathway expression were evaluated. Immunohistochemistry confirmed the expression of TLR4 in human and mouse bladder. Recombinant high-mobility group box protein 1 (HMGB1) increased bladder TLR4 and MyD88 expression and enhanced contractile response to electrical field stimulation. Bladder expression of TLR4 and MyD88 and serum expression of HMGB1 were increased in STZ compared with control mice. Carbachol (CCh)-mediated contraction was increased in bladders from STZ mice, and TLR4 inhibitor CLI-095 attenuated this increase. Induction of diabetes by STZ in WT mice increased bladder weight and contractile responses to CCh and to electrical field stimulation. TLR4KO mice were not protected from STZ-induced diabetes; however, despite levels of hyperglycemia similar to those of WT STZ mice, TLR4KO STZ mice were protected from diabetes-induced bladder hypertrophy and hypercontractility. These data suggest that TLR4 activation during diabetes mediates DBD-associated bladder hypertrophy and hypercontractility.

Among the lower urinary tract complications of diabetes, diabetic bladder dysfunction (DBD) is one of the most common, affecting ~50% of patients with diabetes (1,2). DBD is characterized by a wide range of urinary symptoms (frequency, urgency, nocturia), which progress from initial overactive bladder to later urinary incontinence (1–5). Alterations of smooth muscle, urothelial, and neural functions underlie bladder dysfunction manifestations. Bladder hypertrophy is widely documented in DBD patients and in animal models of diabetes, including streptozotocin (STZ)-induced diabetes and *db/db* models (3–6). Although cystometry findings in patients with diabetes are more variable, depending on the duration of diabetes, hypercontractility of the bladder during the overactive phase of DBD was widely documented in animal models of both type 1 and type 2 diabetes (1,3–5,7). Increased reactive oxygen species (ROS) production due to chronic hyperglycemia, and Rho kinase and K channel alterations have been implicated in DBD (1,7,8); however, the definitive mechanisms mediating bladder hypertrophy and bladder smooth muscle hypercontractility are still unknown.

Activation of the innate immune system has recently been recognized as a mechanism mediating the pathogenesis of diabetes and other chronic sterile conditions characterized by inflammation. Toll-like receptor (TLR) 4 is an innate immune receptor classically activated by bacterial lipopolysaccharide (LPS) on immune cells (9). TLR4 typically signals through its downstream partner MyD88 to activate the nuclear factor-κβ (NF-κβ) pathway leading to ROS and cytokine production. However, TLR4 is also widely expressed on nonimmune cells and can be similarly activated by endogenous, noninfectious molecules, also termed damage-associated molecular patterns. Studies in patients with diabetes have shown

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increased TLR4 expression and signaling in various cell types and organs (10–12). One of the endogenous TLR4 ligands that may mediate TLR4 activation in diabetes is high-mobility group box protein 1 (HMGB1), a highly conserved nonhistone nuclear protein that is passively released from necrotic cells or secreted from immune cells (13). Strong evidence supports the role of HMGB1 in TLR4 activation during diabetes (10–12). Increased circulating levels of specific endogenous ligands, including HMGB1 have been observed in diabetes (10,11,14). Additionally, high-glucose conditions in vitro also induce TLR4 expression and activation of downstream inflammatory pathways (15). Importantly, the deletion of TLR4 leads to protection from some diabetic complications in animal models, despite similar levels of glycemia (16–18).

The findings highlighted above support the notion that TLR4 activation during diabetes, sustained either by chronic hyperglycemia or by continuous stimulation via the increased levels of its ligands, leads to increased production of ROS and cytokines, which in turn stimulate the proliferation of bladder smooth muscle cells and alter their contractile responses. Thus, in this study, we hypothesized that TLR4 activation mediates DBD. To test our hypothesis, we used a mouse diabetes model that clearly exhibits bladder hypertrophy and hypercontractility as well as pharmacological antagonism or genetic deletion of TLR4.

RESEARCH DESIGN AND METHODS

Animals

Age-matched male wild-type (WT) C57BL/6 and TLR4 knockout (TLR4KO) (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ) mice (The Jackson Laboratory) were used at 8–10 weeks. All mice were maintained on a 12-h light/dark cycle with ad libitum access to standard chow and water. Animals were euthanized by exsanguination under isoflurane anesthesia. All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health and were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University.

STZ-Induced Diabetes

Some WT C57BL/6 and TLR4KO mice were made diabetic by STZ administration following the American Diabetes Association protocol (STZ 50 mg/kg i.p. daily after 4 h fasting for 5 consecutive days). Control mice were infused with equal volumes of vehicle (citrate buffer). Diabetes was confirmed by fasted glycemia measurements.

Sample Collection

Whole blood was collected by cardiac puncture under isoflurane anesthesia. After 30 min at room temperature, clotted blood was spun (2,500*g*, for 10 min, twice) and supernatant (serum) was collected. Bladders were removed, sectioned into two longitudinal strips, and denuded of urothelium.

Isometric Contraction Measurements

Bladder strips were mounted on a muscle strip myograph (Danish Myo Technology) in warm and oxygenated physiological salt solution containing the following (in mmol/L): 130 NaCl, 4.7 KCl, 14.9 $NaHCO_3$, 5.5 dextrose, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 1.6 $CaCl_2$, and 0.026 EDTA. Tissues were subjected to 3 mN passive tension (predetermined in pilot experiments as optimal for mouse bladder contraction) and equilibrated for 1 h in physiological salt solution at 37°C with frequent buffer changes. Tissue integrity was verified by contraction to a 120 mmol/L KCl solution. Electrical field stimulation (EFS)-induced contractile responses were elicited to increasing current frequencies (1–32 Hz, 10 s each) Concentration-response curves to carbachol (CCh; 10^{-9} to 10^{-5} mol/L) were constructed. Maximal contractile responses to CCh in the presence of TLR4 antagonist CLI-095 (10 μmol/L) were normalized to KCl-induced contraction.

Ex Vivo HMGB1 Incubation

Mouse bladder strips denuded of urothelium were incubated for 6 h at 37°C in clear DMEM containing recombinant HMGB1 (100 ng/mL) or vehicle (PBS). Tissues were then rinsed and either used in myograph experiments or frozen for protein extraction.

Western Blotting

Protein expression was measured by standard Western blotting technique. Briefly, urothelium-denuded tissue strips were frozen in liquid nitrogen and homogenized by mortar and pestle pulverization followed by solubilization in T-PER lysis buffer containing the following protease inhibitors: 100 mmol/L sodium orthovanadate, 100 mmol/L phenylmethylsulfonylfluoride, and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After sonication and centrifugation (11,000*g* at 4°C for 15 min), supernatants were collected and protein concentration was measured using a bicinchoninic acid protein assay. Samples containing equal amounts of protein (50 μg for the bladder) or equal dilution factors (1:10 dilution for the serum) were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After the blocking of nonspecific binding in 5% milk and overnight incubation with primary antibodies at 4°C, membranes were exposed to horseradish peroxidase–conjugated secondary antibody, and bands were visualized using an enhanced chemiluminescence system. Band densitometry was quantified using ImageJ. The following antibodies were used: TLR4 (Abcam), MyD88 (Cell Signaling Technology), HMGB1 (Abcam), TRIF (Toll-like receptor adaptor molecule 1) (Abcam), p65 (Cell Signaling Technology), IRAK (Abcam), and β-actin (Cell Signaling Technology). Band density was normalized to β-actin band density, with the exception of quantification of HMGB1 in serum.

Human and Mouse Bladder TLR4 Immunohistochemistry

Commercially available paraformaldehyde-fixed paraffin-embedded normal human bladder cross sections (5 μm) were purchased (Abcam). Paraformaldehyde-fixed WT and TLR4KO mouse bladders were embedded in paraffin and cross-sectioned (5 μm) at the Augusta University Histology Core. After dewaxing using Histochoice Clearing Agent (Vector Laboratories) and repeated washes in isopropanol, epitope unmasking was performed by incubation in Deco Antigen Retrieval Solution (5 min, 95°C). Endogenous peroxidase activity was blocked (0.3% H_2O_2 in PBS) for 30 min. Sections were blocked for nonspecific binding by incubating for 30 min with competing serum (1.5% in PBS). In a humidified chamber, sections were incubated overnight at 4°C with competing serum (negative control) or with anti-TLR4 antibody (5 μg/mL; human bladder, Novus Biologicals; mouse bladder, Abcam). The remaining steps were performed according to the manufacturer protocol (Vector Laboratories). Sections were washed three times in PBS and incubated for 30 min with biotinylated secondary antibody. After washing three times in PBS and incubation for 30 min with Vectastain ABC Elite Reagent, sections were exposed to diaminobenzidine/H_2O_2, and staining was monitored and timed. Reactions were stopped by PBS. Sections were then counterstained with hematoxylin, dried, and mounted. Photographs were taken using a Zeiss Axioplan2 microscope.

Mouse Bladder Histological Analysis

Paraformaldehyde-fixed mouse bladder segments were embedded in paraffin, cross-sectioned, and stained with hematoxylin-eosin at the Augusta University Histology Core. Photographs were taken using a Zeiss Axioplan2 microscope, and bladder wall thickness and smooth muscle thickness were measured using ImageJ.

Data Analysis

Values are presented as the mean ± SEM, and n represents the number of animals used in each experiment. Statistical differences were calculated by Student t test (protein expression after HMGB1 incubation), one-way ANOVA followed by Bonferroni post hoc test (body weight, glycemia, bladder weight, maximal contractile response to CCh, bladder wall thickness, protein expression), and two-way ANOVA with repeated measures (EFS-induced contractile responses and concentration-response curves to CCh). All statistical tests were performed with GraphPad Prism (version 5.0; GraphPad Software). The significance level of all tests was set at $\alpha = 0.05$.

Reagents and Chemicals

STZ and CCh were obtained from Sigma-Aldrich. DMSO was obtained from Thermo Fisher Scientific (Waltham, MA). Recombinant human HMGB1 was purchased from R&D Systems (Minneapolis, MN). CLI-095 was purchased from InvivoGen (San Diego, CA).

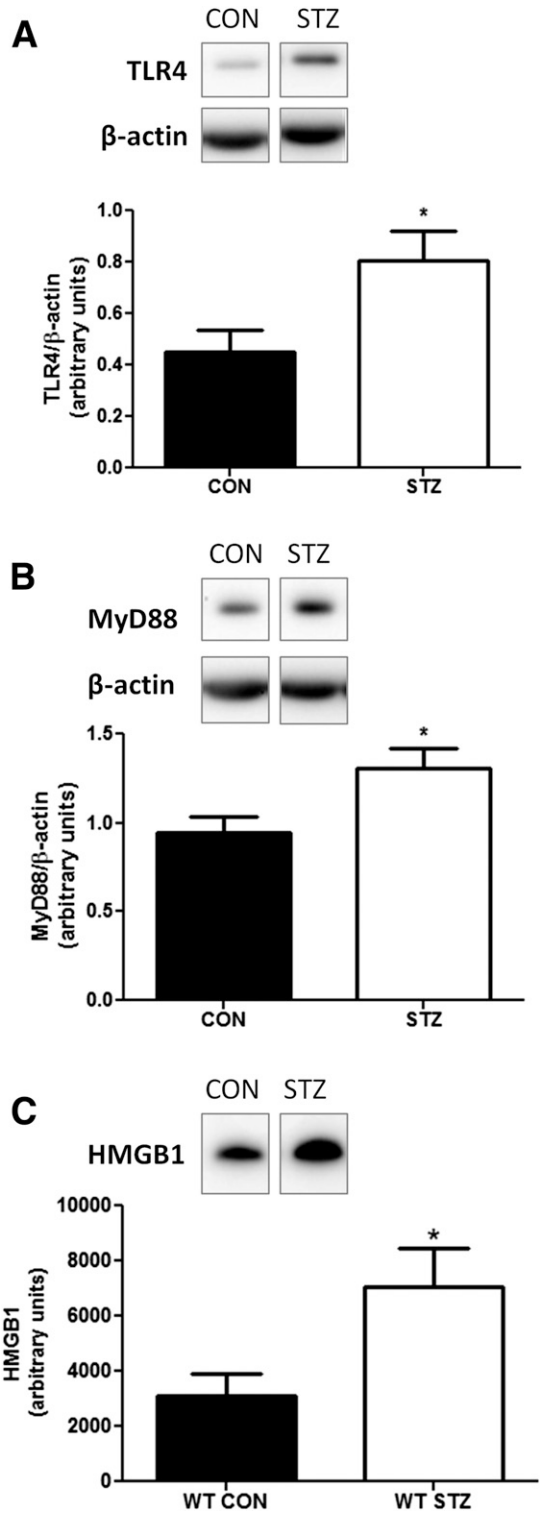

Figure 1—*A*: Protein expression of TLR4 in bladder tissue from control (CON) vehicle-treated mice and STZ-treated diabetic mice (top), quantified as TLR4 band density relative to β-actin (bottom). $n = 4$. *Statistically significant ($P < 0.05$) change. *B*: Protein expression of MyD88 in bladder tissue from control vehicle-treated mice and STZ-treated diabetic mice (top), quantified as MyD88 band density relative to β-actin (bottom). $n = 5$. *Statistically significant ($P < 0.05$) change. *C*: Protein expression of HMGB1 in serum from control vehicle-treated mice and STZ-treated mice (top), quantified as arbitrary units (bottom). $n = 5$. *Statistically significant ($P < 0.05$) change.

RESULTS

TLR4 Pathway Is Activated in the Mouse Bladder in STZ-Induced Diabetes

In the case of TLRs, it is generally accepted that their protein expression is an indication of their activation status. For instance, the stimulation of TLR4 in immune cells by specific ligands induces an increase in the expression of TLR4 via a positive-feedback loop. Thus, to investigate whether STZ-induced diabetes correlates with activation of the TLR4 pathway in bladder tissue, we measured the protein expression of TLR4 and its downstream signaling partner MyD88 (Fig. 1*A* and *B*). We observed that TLR4 and MyD88 protein expression was increased in homogenates of bladder tissue from mice after 4 weeks of STZ-induced diabetes.

Circulating Levels of HMGB1 Increase in STZ-Induced Diabetes

In addition to bacterial products, TLR4 is also activated by endogenous mediators, some of which may be released in higher amounts during states of chronic inflammation, such as diabetes. HMGB1 is a TLR4 ligand the levels of which are significantly elevated in the circulation of patients with both type 1 and type 2 diabetes. In order to test whether HMGB1 is also elevated in STZ-induced diabetic mice, we measured the protein expression of HMGB1 in serum from mice after 4 weeks of STZ-induced diabetes (Fig. 1*C*). We observed that serum protein expression of HMGB1 is significantly higher in STZ-treated mice compared with vehicle-treated mice.

Ex Vivo Incubation With HMGB1 Activates the TLR4 Pathway in Mouse Bladder Tissue

Although we observed increased circulating levels of HMGB1 and activation of TLR4 in the bladder, other TLR4 ligands that are increased in diabetes could also have mediated this effect. Thus, we tested the effects of ex vivo incubation of bladder tissue with HMGB1 (100 ng/mL, 6 h) (Fig. 2*A* and *B*). We observed that expression of TLR4 and MyD88 was significantly increased in tissue homogenates from HMGB1, compared with vehicle-treated bladder segments.

Ex Vivo Incubation With HMGB1 Increases EFS–Induced Contractile Response of Bladder Tissue

In order to evaluate whether the increase in TLR4 pathway activation in the presence of HMGB1 results in an increase in contractile responses of the bladder, we

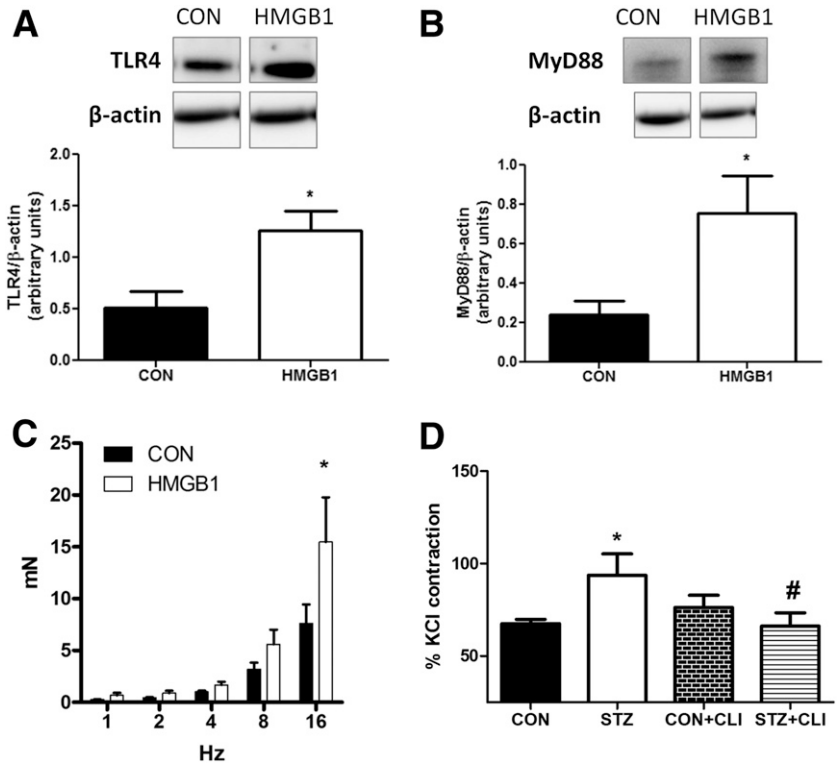

Figure 2—*A*: Protein expression of TLR4 in bladder tissue from control (CON) mice after 6 h of incubation with vehicle or 100 ng/mL recombinant HMGB1 (top), quantified as TLR4 band density relative to β-actin (bottom). $n = 4$. *Statistically significant ($P < 0.05$) change. *B*: Protein expression of MyD88 in bladder tissue from control mice after 6 h incubation with vehicle or 100 ng/mL recombinant HMGB1 (top), quantified as TLR4 band density relative to β-actin (bottom). $n = 4$. *Statistically significant ($P < 0.05$) change. *C*: EFS-induced contractile responses in bladder strips after incubation with HMGB1 (100 ng/mL, 6 h, $n = 6$) or vehicle (CON; $n = 4$). *Statistically significant ($P < 0.05$) change. *D*: Maximal CCh-induced contractions as a percentage of KCl-induced contraction in mouse bladder from control vehicle-treated mice and STZ-treated mice after 30 min of incubation with vehicle or TLR4 antagonist CLI-095 (CLI) (10 μmol/L). $n = 5$. *Statistically significant ($P < 0.05$) compared with control; #statistically significant ($P < 0.05$) compared with STZ.

performed EFS-induced contractile responses in bladder strips after incubation with HMGB1 (100 ng/mL, 6 h) or vehicle (Fig. 2*C*). We observed that the maximal response elicited by 16 Hz EFS is significantly increased in tissues after incubation with HMGB1.

TLR4 Inhibition Normalizes Bladder Hypercontractility in STZ-Induced Diabetic Mice

Contractile responses of the bladder are typically increased in patients with diabetes with overactive bladder as well as in STZ-induced diabetic mice. To test whether the activation of TLR4 plays a functional role in bladder contraction in diabetes, we used a pharmacological inhibitor of TLR4 (CLI-095) and measured the contractile responses of the bladder to CCh. We chose CCh because it is a cholinergic agonist that mimics the actions of acetylcholine, the physiological contractile agonist of the bladder, while being more resistant to cholinesterase than acetylcholine. As expected, CCh-induced contraction in bladder segments is significantly increased in STZ-induced diabetic mice compared with vehicle-treated control mice (Fig. 2*D*). In the presence of TLR4 antagonist CLI-095 (3 μmol/L, 30 min), the maximal CCh-induced contraction was unchanged in the control bladder, while it was significantly decreased in bladder segments from STZ mice.

Deletion of TLR4 Does Not Protect Mice From Diabetes Development

As previously mentioned, ligands of TLR4, such as HMGB1 may also induce effects via other TLRs. To determine clearly the role of TLR4 in the development of DBD, we used a genetically deficient mouse model and tested the effects of diabetes induction with STZ. Male WT and TLR4KO age-matched mice were treated with STZ or vehicle at 8–10 weeks of age. Glycemia and body weight 4 weeks after diabetes induction were not statistically different between the two mouse strains, suggesting that genetic deletion of TLR4 does not protect mice from STZ diabetes induction itself (Fig. 3*A* and *B*). Given the known correlation between hyperglycemia and bladder dysfunction, whether via TLR4 or other mechanisms, it is important that glycemia itself is not a variable in our model.

Diabetes-Induced Bladder Hypertrophy Is Attenuated in TLR4KO Mice

DBD is associated with bladder hypertrophy in both human patients with diabetes and in animal models of diabetes, with both smooth muscle and urothelium size increases independently contributing to the increase in whole-bladder tissue mass. Four weeks after diabetes induction with STZ, WT STZ mice exhibited the expected increase in relative bladder weight compared with vehicle-treated mice. However, despite levels of hyperglycemia similar to those in WT mice, TLR4KO STZ mice are at least partially protected from bladder hypertrophy, as evidenced by their relative bladder weight, which was no longer statistically different from that of TLR4KO vehicle-treated mice (Fig. 3*C*). Similarly, bladder wall and smooth muscle layer thickness were both significantly increased in STZ mice compared with vehicle-treated WT mice; however, no change was observed between TLR4KO vehicle-treated and STZ-treated mice (Fig. 4).

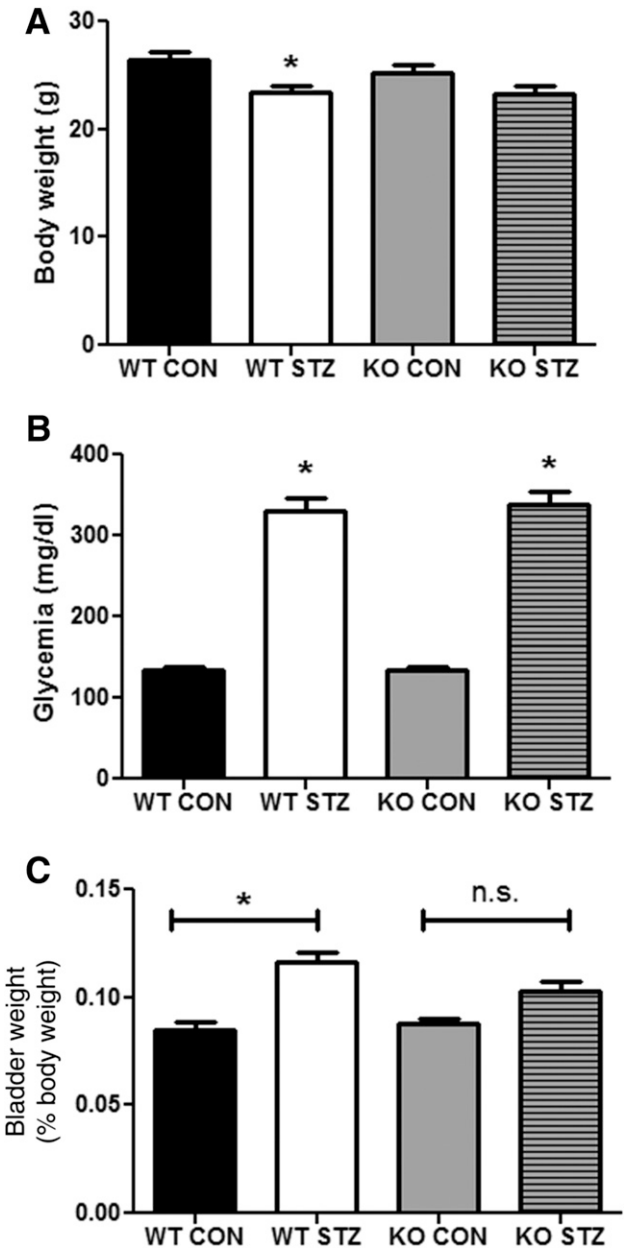

Figure 3—Body weight (*A*), glycemia (*B*), and relative bladder weight (*C*) of control vehicle-treated (CON) or diabetic STZ-treated (STZ) WT and TLR4KO (KO) mice at 4 weeks after diabetes induction. n = 15 WT CON; n = 14 WT STZ; n = 16 KO CON; n = 5 KO STZ. *Statistically significant (P < 0.05) compared with control. n.s., not significant.

Diabetes-Induced Bladder Hypercontractility Is Attenuated in TLR4 Knockout Mice

As stated above, DBD in patients with overactive bladder and early STZ-induced diabetic mice is manifested by increased contractile responses compared with nondiabetic

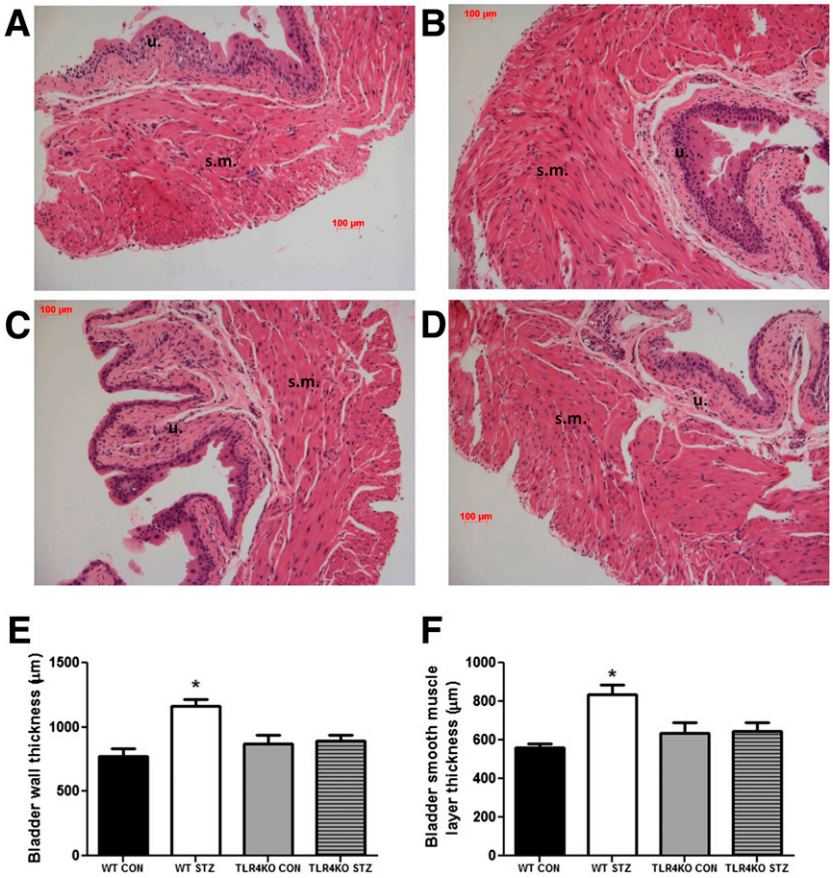

Figure 4—*A–D*: Representative images of hematoxylin-eosin–stained bladder cross sections from vehicle-treated (CON) or diabetic STZ-treated (STZ) WT and TLR4KO mice at 4 weeks after diabetes induction. WT CON (*A*), WT STZ (*B*), TLR4KO CON (*C*), TLR4KO STZ (*D*). Urothelium is indicated by "u.," and smooth muscle by "s.m." Total bladder wall thickness (*E*) and smooth muscle layer thickness (*F*) measurements of hematoxylin-eosin–stained bladder cross sections. $n = 5$. *Statistically significant ($P < 0.05$) change from WT CON.

controls. To determine whether bladder TLR4 is necessary and sufficient for diabetes-induced hypercontractitliy, we tested the contractile responses of bladder segments from WT and TLR4KO mice after 4 weeks of diabetes induction with STZ. Both the CCh and EFS of bladder segments resulted in augmented contractile responses in WT STZ mice compared with WT vehicle-treated mice (Fig. 5*A* and *B*). However, there was no statistical difference in contractile responses of bladder segments between TLR4KO STZ-treated and TLR4KO vehicle-treated groups (Fig. 5*C* and *D*).

Augmented Bladder TLR4 Signaling Is Attenuated in TLR4 Knockout Mice

To determine whether mediators downstream of TLR4 associate with the observed structural and functional alterations in mouse DBD, we measured protein expression of TRIF, IRAK, and the p65 subunit of NF-κB. STZ-induced diabetes resulted in increased expression of these mediators in the WT mouse, once again suggesting the potential contribution of innate immune-mediated inflammation in DBD. However, there was no difference in the bladder protein expression of these mediators between the TLR4KO vehicle-treated and STZ-treated groups (Fig. 6).

Human and Mouse Bladder Tissue Express TLR4

Although TLR expression is presumed to be ubiquitous in immune cells, nonimmune cell expression is not necessarily as clear. We measured TLR4 expression in mouse bladder tissue. However, because there are few reports on the expression of TLR4 or lack thereof in the human urogenital tract, we tested whether TLR4 is present in human bladder tissue by immunohistochemistry. Cross sections of normal human bladder were stained with a specific anti-TLR4 antibody. The presence of TLR4-positive staining was observed in both the urothelial and the smooth muscle layers of the human bladder (Fig. 7). Additionally, WT mouse bladder TLR4 expression was confirmed with immunohistochemistry staining of cross sections with a specific anti-TLR4 antibody. No TLR4 staining was observed in the TLR4KO mouse bladder (Fig. 8).

DISCUSSION

DBD is a common urological complication of diabetes with poorly understood pathogenic mechanisms and virtually lacking in effective treatments. In our study, we demonstrated for the first time the important role of the innate

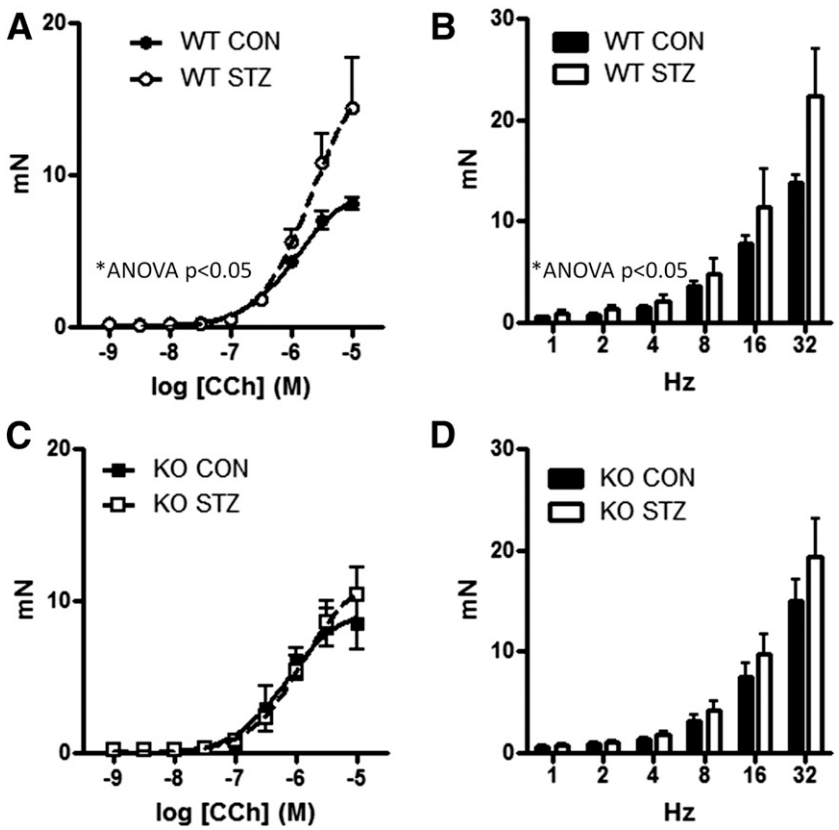

Figure 5—Contractile responses to CCh (*A*, *C*) and EFS (*B*, *D*) observed in bladder segments from control vehicle-treated (CON) or diabetic STZ-treated (STZ) WT and TLR4KO (KO) mice at 4 weeks after diabetes induction. n = 10 WT CON, WT STZ; n = 9 KO CON; n = 8 KO STZ. *Statistically significant (P < 0.05) compared with control.

immune receptor TLR4 in mediating bladder dysfunction in a STZ-induced mouse model of diabetes.

The goal of this study was to understand the role of TLR4 signaling in the development of DBD, specifically its initial overactive phase. Chronic low-grade inflammation mediated by cell injury, endogenous TLR4 ligands release, and sustained innate immune system activation are now believed to be involved in determining organ damage in various systemic diseases, including diabetes (14,19).

TLRs, including TLR4, are pattern recognition receptors involved in innate immune system activation (9). Although it was originally believed that the innate immune system is activated only in response to pathogens like bacteria and viruses, it is now widely accepted that endogenous molecules generated during sterile injury have equal capacities in activating immunity, which actually evolved to react to danger and not just to non–self-threats (9,20,21). Thus, activation of TLRs is a process that has now been documented as initiating or perpetuating the chronic low-level systemic inflammation that characterizes chronic sterile conditions, such as diabetes (9,14,19,22–24).

Activation of TLR4 by numerous endogenous molecules has been demonstrated. Our results in the STZ-induced diabetic mouse confirmed previous findings of higher circulating levels of HMGB1 in diabetes. Some of the other endogenous TLR4 ligands also have increased levels in diabetes, including HSP60 and HSP70, S100 proteins, oxidized LDL, hyaluronan, advanced glycation end products, and fibrinogen (10,11,14). Additionally, the increased permeability of the digestive tract to microbial components in diabetes mediates an increase in circulating LPS levels, which has been widely documented in patients with diabetes. In our study, we focused on HMGB1; however, it is not excluded that other endogenous mediators or LPS may induce additional stimulation of the TLR4 pathway. Several studies (14,22–26) show that TLR4 activation by HMGB1 administration in vivo caused or worsened organ damage and that HMGB1 inhibition ameliorated survival or disease phenotype in animal models of stroke, ischemia-reperfusion, sepsis, and shock. Our results suggest that HMGB1 is capable of inducing the expression of TLR4 pathway proteins in the mouse bladder. In a future study, we plan to address the specificity of HMGB1 activation of TLR4 by studying the effects of HMGB1 inhibition on DBD.

Corroborating our results, the increased expression and activation of TLR4 has also been demonstrated in patients with diabetes (10–12,14). TLR4 gene polymorphisms have also been associated with type 2 diabetes

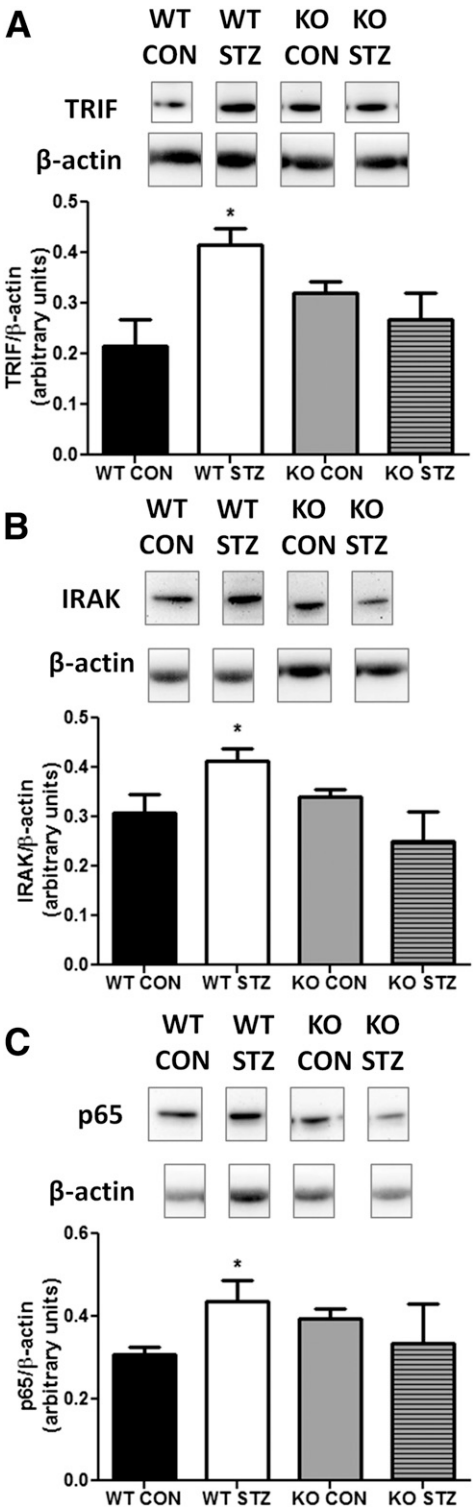

Figure 6—Protein expression of TRIF (*A*), IRAK (*B*), and p65 (*C*) in bladder tissue from vehicle-treated (CON) or diabetic STZ-treated (STZ) WT, and TLR4KO (KO) mice at 4 weeks after diabetes induction, quantified as band density relative to β-actin. $n = 5$. *Statistically significant ($P < 0.05$) change from WT CON.

in a human population (27). Importantly, several studies demonstrated that high-glucose conditions in vitro induce the increased expression of TLR4 and TLR4-mediated ROS production in immune and nonimmune cells; however, the mechanism for glucose-mediated TLR4 activation is as yet unknown. Interestingly, high glucose was shown to induce HMGB1-mediated activation of TLR4 in vascular smooth muscle cells (28). Furthermore, the implication of the TLR4 pathway in mediating some diabetic complications and organ damage has been established with rodent genetic models (16–18). Similar to our findings, these studies demonstrated that although hyperglycemia levels are not decreased by the lack of expression of TLR4, there is a significant improvement in cytokine levels and amelioration of the diabetic phenotype. Although several of TLR4 ligands, including HMGB1, may also activate other innate immune receptors, including TLR2 and RAGE (receptor for advanced glycation end products), our data in TLR4KO mice suggest that TLR4 deletion is sufficient for restoring bladder function and ameliorating hypertrophy in STZ-induced diabetes.

We have observed activation of the TLR4 pathway in the diabetic bladder and the upregulation of its signaling mediators TRIF and IRAK and of the p65 subunit of NF-κB. TLR4 signaling commonly leads to NF-κB pathway activation, production of proinflammatory cytokines, and increases in ROS production that are likely mediated by NADPH oxidase, all of which are known modulators of smooth muscle contraction (29). Our group has demonstrated that TLR4 is involved in vascular smooth muscle function during hypertension and that in vivo inhibition of this pathway attenuated the vascular contractile response to norepinephrine and decreased blood pressure in a genetic model of hypertension (30,31). There is currently no other study investigating the involvement of this important innate immunity modulator in bladder smooth muscle function, although the expression of TLR4 in human urothelium has been documented (32,33). Interestingly, a recent report identified LRRC33 as a negative physiological regulator of TLRs, and the striking phenotypical result of genetic deletion of LRRC33 is bladder hypertrophy (34,35). On the basis of the current knowledge and the data in this study, we believe that TLR4 activation during diabetes, sustained by continuous increased levels of its ligands, leads to increased production of ROS and cytokines, which stimulate the proliferation of bladder smooth muscle cells and alter their contractile responses.

DBD is characterized objectively by bladder hypertrophy and alterations in bladder contractility. Hypertrophy of the whole bladder and of individual layers has been consistently documented in humans and in animal models of diabetes (1–5). The wide range of clinical cystometric findings in patients with diabetes and the apparent discrepant results of contractile function measurements in rodent models of diabetes are best explained by the DBD temporal theory (1), which couples the early storage dysfunction with hypercontractility and overactive bladder, and the late voiding dysfunction, manifested as incontinence, with bladder atonia. Although increased diuresis and hyperglycemia-induced oxidative stress are widely

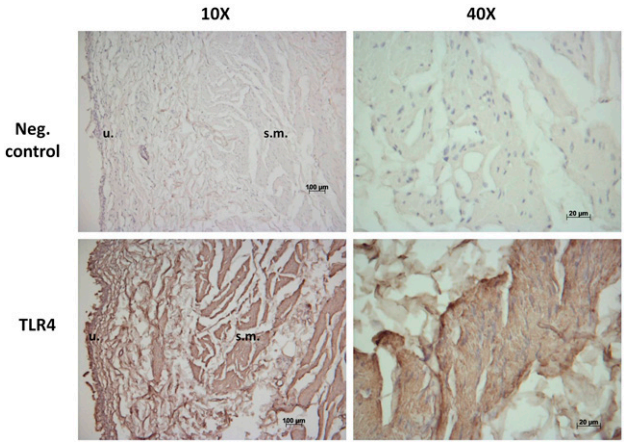

Figure 7—Representative image of immunohistochemical staining for TLR4 in human bladder cross sections (bottom). Negative (Neg.) control staining was obtained with secondary antibody alone (top). TLR4-positive staining is seen as brown, and hematoxylin nuclear counterstain is seen as blue. Urothelium is indicated by "u.," and smooth muscle by "s.m." on lower magnification images (left). Higher magnification highlights smooth muscle staining (right).

believed to cause bladder hypertrophy and contractile alterations, neither of these two factors can experimentally fully account for DBD, nor do they explain its clinical progression (1,36,37). An interesting report evaluated the separate roles of polyuria and hyperglycemia in DBD by performing urinary diversion in STZ animals to remove polyuria, but not hyperglycemia, and sucrose-induced diuresis in the absence of hyperglycemia (1,36,37). These authors concluded that, although polyuria is sufficient in inducing hypertrophy, hyperglycemia-induced oxidative stress is the main driver of contractile alterations. Knowing that hyperglycemia may induce both TLR4 activation and HMGB1 release, TLR4 activation may be the missing factor in DBD pathogenesis; and continuous HMGB1 release may drive chronic inflammation, detrusor remodeling and contractile dysfunction. Our results demonstrate that, despite unchanged hyperglycemia, genetic lack of TLR4 ameliorates bladder hypertrophy and prevents the increased bladder contraction observed in early STZ-induced diabetes. Moreover, acute pharmacological TLR4 inhibition also reduced contraction in bladder segments from STZ-induced diabetic mice. These data suggest that the potential mechanism for chronic hyperglycemia-induced ROS generation in DBD is actually the activation of TLR4.

A limitation of our study is the fact that since we used urothelium-denuded bladder strips, we did not account for the potential effects of TLR4 expression in the urothelium. The urothelium, via secretion of prostaglandins and other active mediators, is involved in the contractile function of the bladder (1,8,38), specifically exerting an inhibitory effect on contraction, much like the endothelium of blood vessels. We observed TLR4 expression in the urothelium, confirming results that urothelial TLR4 is expressed and functional (32,33,39). Activation of urothelial TLR4 by bacterial LPS during urinary tract infections, which are more frequent in patients with diabetes, may be a factor confounding manifestations of DBD. Although they are a potential aggravating factor, bacterial infections could not explain DBD in its entirety (40), since antibiotic treatment does not resolve sterile DBD, which is nonetheless present in a vast majority of patients and in diabetic animals. Although we focused on smooth muscle TLR4 in our study, the influence of urothelial TLR4 would have been similarly removed in the global TLR4KO model we used.

Another limitation of our study is the fact that we did not account for sex-specific effects. Although poorly explored in the DBD patient population (41), sex could play an important role in DBD, especially considering the known sex differences in innate immune activation. Studies on the sex specificity of TLR4 involvement in DBD will be the subject of a future endeavor.

To conclude, we have demonstrated that the activation of TLR4 is necessary and sufficient to mediate bladder

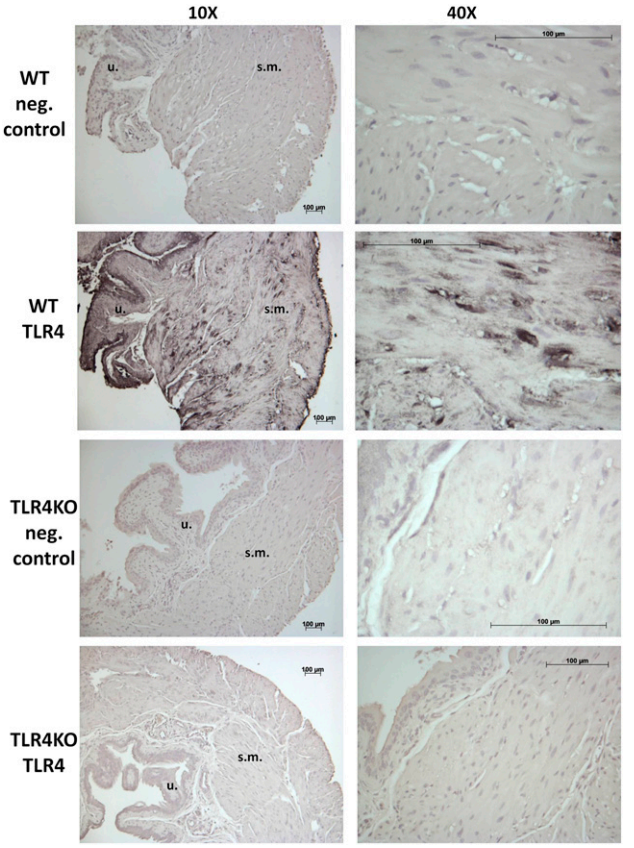

Figure 8—Representative image of immunohistochemical staining for TLR4 in WT (top) and TLR4KO (bottom) mouse bladder cross sections. Negative (neg.) control staining was obtained with secondary antibody alone. TLR4-positive staining is seen as brown, and hematoxylin nuclear counterstain is seen as blue. Urothelium is indicated by "u.," and smooth muscle by "s.m." on lower-magnification images (left). Higher-magnification images (right) highlight smooth muscle staining.

hypertrophy and hypercontractility in STZ-induced diabetic mice. Despite significant recent advances in understanding DBD, the underlying molecular pathways that initiate this dysfunction are poorly understood, hindering our ability to effectively treat it. We know that hyperglycemia-induced oxidative stress plays a pivotal role in the development of DBD. However, it is often difficult to achieve complete glycemic control with oral antihyperglycemic or insulin treatments, and slowing disease progression using other therapies, such as antioxidants, has also failed. Also important is the fact that patients with diabetes have a higher rate of bladder infections, and it may be that activation of the innate immune response by bacterial components plays a unique role in the infected diabetic bladder. Our study provides new molecular mechanisms associated with the development of DBD, as well as a basic research platform for the development of future therapies for DBD by manipulating molecules (i.e., the TLR4 pathway) that are upstream and temporally precede the increase in ROS or cytokine production and the following bladder hypertrophy and hypercontractility.

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