LL-37 induced cystitis and the receptor for advanced glycation end-products (RAGE) pathway

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ABSTRACT

To elucidate pathways in bladder inflammation, we employed our physiologically relevant LL-37 induced cystitis model. Based on inflammatory studies involving other organ systems implicating the receptor for advanced glycation end-products (RAGE), we first hypothesized that RAGE is critically involved in LL-37 induced cystitis. We further hypothesized that a common RAGE ligand high mobility group box 1 (HMGB1) is up-regulated in bladders challenged with LL-37. Finally, we hypothesized that NF-κB dependent inflammatory genes are activated in LL-37 induced cystitis. Testing our first hypothesis, C57Bl/6 mice were challenged with either saline (control) or 320 μM of LL-37 intravesically for 1 hr. After 12 or 24 hours, tissues were examined with immunohisto-chemistry (IHC) for RAGE, and both mRNA and protein isolation for respective qRT-PCR and Western Blot analysis. Our second hypothesis was tested by employing HMGB1 IHC. Testing our final hypothesis, qRT-PCR was performed investigating five genes: TNFα, IL-6, IL-1β, GM-CSF, COX-2. In control and LL-37 challenged tissues, IHC for RAGE revealed similar qualitative expression. Evaluation with qRT-PCR and Western Blot for RAGE revealed diminished expression at the mRNA and protein level within LL-37 challenged bladders. IHC for HMGB1 revealed a moderate qualitative increase within LL-37 challenged tissues. Finally, with the exception of TNFα, all NF-κB dependent inflammatory genes yielded substantial up-regulation. We have employed our LL-37 induced cystitis model to gain insight towards a possible mechanistic pathway involved in bladder inflammation. This work provides data for future studies involving the inflammatory ligand HMGB1, RAGE, and receptor pathways that activate NF-κB.

Keywords: LL-37; Cathelicidin; Bladder Inflammation; Bladder Fibrosis; Spina Bifida; Myelomeningocele; Interstitial Cystitis; RAGE; HMGB1; NF-Kappa B

1. INTRODUCTION

In order for the bladder to store urine it must be compliant (pliable). It is imperative that it can hold urine at low pressures. Failure of this results in elevated bladder pressure, transmitting urine to the kidney resulting in glomerular injury, renal parenchymal fibrosis and failure. Deposition of extracellular matrix (ECM) within the bladder wall is the main reason for loss of bladder wall pliability. What leads to excess ECM deposition and resultant fibrosis remains unclear, but accumulated evidence suggests that inflammatory cascades play a significant role. In response to inflammatory insult, bladder fibrosis occurs as part of a wound healing process and the accumulation of ECM proteins (collagen types I and III) [1]. Also, during chronic tissue damage fibroblasts undergo a process of activation and conversion to myofibroblasts that proliferate and produce ECM [2,3]. The end result is a non-compliant fibrotic bladder. Myelomeningocele (MMC)/spina bifida (SB) and interstitial cystitis (IC)/painful bladder syndrome (PBS) are both disease states that affect the urinary bladder. In both, chronic inflammation can lead to fibrosis and bladder non-compliance, further perpetuating the disease state. The underlying physiology involves inflammation, yet the exact mechanism is elusive; as a result, current treatment options are suboptimal [4-7].

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We have previously published a novel murine model of inflammatory bladder disease using the human cathelicidin LL-37 peptide to induce physiologic bladder inflammation [8]. The exact inflammatory mechanism of action for LL-37 is not completely understood and responses are elicited through the activation of a number of cell-surface receptors and signaling pathways, depending on the cell type being studied and the responses being assessed. In addition to the important antimicrobial role of the cathelicidin LL-37, it also demonstrates immuno-modulatory properties and triggers inflammation via the promotion of leukocyte chemotaxis, stimulation of mast cell degranulation, enhanced neutrophil function, induction of chemokines including IL-8, regulation of inflammatory responses (in part via NF-κB signaling), angiogenesis, and the increased expression of extracellular matrix components [9,10]. Relatively low concentrations of LL-37 (13 - 25 μM) can be cytotoxic against several eukaryotic cells, and pose a significant risk to human cells upon exposure [11]. We hypothesized that LL-37 induced bladder inflammation involves the receptor for advanced glycation end-products (RAGE) and a common RAGE ligand high mobility group box 1 (HMGB1), resulting in the activation of NF-κB dependent inflammatory genes.

To our knowledge, the RAGE pathway has not been described or implicated in inflammatory diseases of the bladder. RAGE is primarily involved in homeostasis and inflammation and is one of the primary receptors for high mobility group box 1 (HMGB1) [12]. RAGE, a multiligand receptor of the immunoglobulin (Ig) superfamily [13], is expressed on multiple cell types including polymorphonuclear leukocytes (PMNs), monocytes/macrophages, T and B lymphocytes, DC, EC, and mucosal epithelial cells [14]. There have been studies that implicate RAGE as a protective pathway in processes involving pulmonary fibrosis, specifically loss of RAGE signaling was found to yield more prominent fibrotic tissue responses [15-17]. Although most studies suggest that ligand activation of RAGE transmits cell surface signals to various intracellular pathways including NF-κB, a heterodimeric protein complex is responsible for a multitude of transcriptional programs that produce proinflammatory cytokines and enzymes such as TNFα, IL-1β, IL-6, and COX-2 [18-24]. The expression of RAGE also is controlled by the transcription factor activity of NF-κB [21] and activation results in increased cell surface expression of RAGE. This leads to amplification of the original signal, further exacerbating inflammation [25,26]. Despite studies identifying the role of NF-κB in the RAGE signaling pathway, the cellular effectors and molecular mechanisms of such activation remain unknown.

In the lower urinary tract, HMGB1 is ubiquitously expressed in urothelial cells and extracellularly liberated into urine [27]. During episodes of bladder tissue insult, high urinary levels of HMGB1 have been identified [27] signifying necrotic tissue injury. HMGB1 was first identified as a non-histone chromosomal protein involved in DNA binding [28] and later recognized as a pro-inflammatory cytokine mediating endotoxin lethality in mice [29]. It is predominantly a nuclear protein present in most eukaryotic cells where it stabilizes nucleosome formation and facilitates transcription [30-32]. However, others have reported that HMGB1 also functions extracellularly [29,33-36], although it lacks a classical secretion signal. HMGB1 can be liberated via two routes, either from necrotic cell death with passive leakage from the cell [37] and/or active secretion from activated monocytes, macrophages [29,38], mature dendritic cells [31], NK cells [39], and endothelial cells [34]. RAGE was first identified as the receptor for HMGB1. Thus, HMGB1 activation could both initiate and sustain a proinflammatory phenotype.

In this study we propose that LL-37 induced bladder inflammation involves RAGE, a common RAGE ligand HMGB1, and ultimately converges on NF-κB signaling. We investigated whether activation of these pathways in the bladder could be responsible for the sustained, proinflammatory phenotype.

2. MATERIALS AND METHODS

2.1. Bladder Inflammation and Tissue Collection

Experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. LL-37 induced bladder inflammation was performed as previously described [8,40]. Briefly, 8 to 12-week old female C57BL/6 mice were catheterized, bladders emptied, then washed with 150 μL of 0.9% sodium chloride and emptied. After washing, 320 μM of LL-37 or saline (controls) were infused slowly and left indwelling for 1 hour. Tissues were harvested at either 12 or 24 hours and the bladders were split longitudinally. One section was fixed in 4% paraformaldehyde for histology and the other was either snap frozen in liquid nitrogen then stored at −80˚C for protein extraction, or placed in RNAlater (QIAGEN, Germantown, MD, USA) overnight at 4˚C then proceeded to RNA isolation or stored at −80˚C for future use.

2.2. Immunohistochemistry (IHC) for RAGE and HMGB1

5 μm sections were deparaffinized then rehydrated through xylene and graded alcohols. Endogenous peroxidase activity was blocked with 1% Hydrogen peroxide in TBST for 20' and washed 3X in TBST for 3'. Antigen retrieval was performed (Vector laboratories, Burlingame, CA., Lot# V0421). To minimize non-specific anti-

body binding, sections were incubated for 60' in 5% FBS in TBS with 0.3% Triton X-100. Sections were incubated overnight at 4˚C with primary antibody (Goat-Anti Mouse RAGE 1:200 in blocking solution, R&D, Minneapolis, MN, Lot# AF1179; or Rabbit-Anti Mouse HMGB1, 1:1000 in blocking solution, Abcam, Cambridge, MA, Lot# ab18256). Following O/N incubation, slides were washed 3X in TBST for 3'. Sections were then incubated for 60' with biotinylated secondary antibody 1:2000, followed by Vectastatin Elite ABC Reagent (Vector Laboratories, Lot# PK-6100) diluted in TBST for 30'. Between incubations, sections were washed 3X for 3' in TBST. For visualization of immunoreactivity sections were incubated in DAB peroxidase substrate for 20 - 40 sec. Sections were washed in ddH$_2$O, counterstained, and dehydrated. Negative controls included incubation with TBST in place of the primary antibody and no immuno-reactivity was observed.

2.3. Protein Isolation and Western Blot

Bladder tissues stored at −80˚C were homogenized in ice-cold RIPA lysis buffer (Thermo Scientific, Rockford, IL) with the addition of protease inhibitor (Sigma Aldrich) and phosphatase inhibitor (Thermo Scientific, Rockford, IL) using a Mini-Bead Beater homogenizer (BioSpec Products, Inc. Bartlesville, OK, USA). Isolated total protein was quantified using the BCA method (PIERCE, Rockford, IL). Equal amounts of protein were electrophoresed on a 4% - 12% SDS-polyacrylamide gel (InVitrogen) and subsequently transferred to a nitrocellulose membrane (0.45 m, InVitrogen). The membrane was blocked in washing solution (0.1% Tween 20 in PBS) containing 5% nonfat dry milk. The same primary antibody utilized for RAGE IHC was utilized for the Western Blot experiments. Secondary antibodies were incubated at room temperature for 1 hour in blocking solution. Proteins were visualized via ECLplus chemiluminescence reagents (GE healthcare life sciences, Pittsburgh, PA).

2.4. RNA Preparation and Real-Time qRT-PCR

Tissues were homogenized on ice with a hand homogenizor, then total RNA was purified according to manufacturer's instructions (Qiagen RNeasy fibrous mini kit, Germantown, MD, USA). cDNA synthesis was performed following manufacturer instructions (ABI, Foster City, CA). Primer's for all genes of interest (RAGE, TNFα, IL-6, IL-1β, GM-CSF, COX-2) were synthesized and purchased from Applied Biosystems (Foster City, CA). Gene expression was quantified using the Taqman Gene Expression Assay (ABI, Foster City, CA) on an Applied Biosystems 7900 HT instrument. The data sets were analyzed with normalization, variance stabilization,

and log2 transformation. The $2^{-\Delta\Delta Ct}$ method was utilized in the analysis for differences in relative gene expression [41].

3. RESULTS

3.1. RAGE Immunohistochemistry Comparing Saline Control versus LL-37 Challenged Bladders

To establish if baseline RAGE expression was present in normal bladders and to test for potential changes in RAGE expression after LL-37 challenge, both saline control and those exposed to LL-37 were subjected to RAGE IHC. Saline instilled control bladders harvested after 12 and 24 hours are demonstrated in **Figures 1A** and **B** (U—urothelium, SBM—submucosa, LP—lamina propria, SMC—smooth muscle cell layer).

LL-37 instilled bladders harvested after 12 and 24 hours are demonstrated in **Figures 1C** and **D**. In saline controls, strong RAGE immunoreactivity (brown stain)
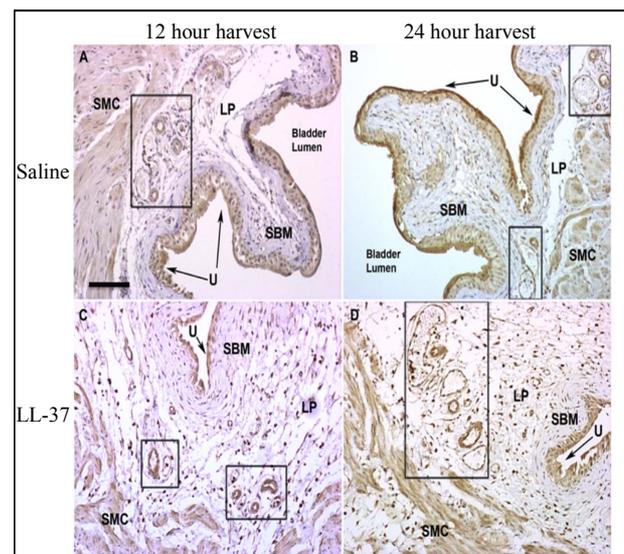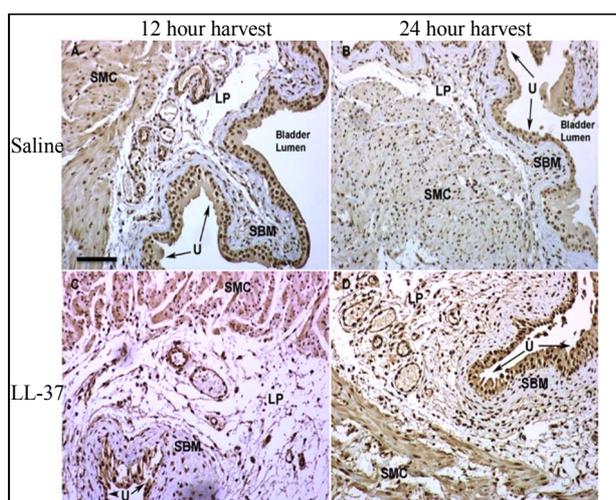

Figure 1. RAGE IHC comparing saline control vs. LL-37 challenged bladders. Saline controls after 12 h (A) and 24 h (B). LL-37 challenged bladders harvested after 12 h (C) and 24 h (D). U—urothelium, SBM—submucosa, LP—lamina propria, SMC—smooth muscle cell layer. In saline control bladders (A) & (B), strong RAGE immunoreactivity (brown stain) was observed along the umbrella cells, with moderate intensity within the cytoplasmic regions in the urothelial (U) layer. No RAGE signal was observed in the SBM layer and LP, except for the arteriolar vessels had a moderate signal (rectangle). RAGE was detectable in the SMC layer. In LL-37 instilled bladders (C) and (D), similar RAGE expression patterns were observed compared to saline control tissues except for a qualitative loss of RAGE signal along superficial umbrella cells, but increased RAGE detection in the endothelium lining venules (D) (rectangle). In addition, numerous acute inflammatory cells were RAGE positive in both (C) and (D). Images reduced from × 10, scale bar panel A represents 75 μM.

was observed along the umbrella cells, with moderate intensity within the cytoplasmic regions in the urothelial (U) layer. No RAGE signal was observed in the SBM layer and LP, except for the arteriolar vessels had a moderate signal (rectangle). RAGE was detectable in the SMC layer. In LL-37 instilled bladders, similar RAGE expression patterns were observed compared to saline control tissues except for a qualitative loss of RAGE signal along superficial umbrella cells, but increased RAGE detection in the endothelium lining venules (**Figure 1D**—rectangle). In addition, numerous acute inflammatory cells were RAGE positive.

3.2. HMGB1 Immunohistochemistry Comparing Saline Control versus LL-37 Challenged Bladders

To help understand the impact of HMGB1 on LL-37 induced bladder inflammation, IHC was employed to determine HMGB1 expression patterns in both saline control versus LL-37 challenged bladders (**Figure 2**). In saline controls, HMGB1 immunoreactivity was apparent (brown stain) in all nuclei of the urothelium, myofibroblasts in the submucosa, endothelial cells of arterioles and venules, fibroblasts in the lamina propria, and


Figure 2. HMGB1 IHC comparing saline control versus LL-37 instilled bladders. Saline instilled control bladders harvested after 12 h (A) and 24 h (B). LL-37 instilled bladders harvested after 12 h (C) and 24 h (D). U—urothelium, SBM—submucosa, LP—lamina propria, SMC—smooth muscle cell layer. In saline control bladders (A) & (B), HMGB1 immunoreactivity was apparent (brown stain) in all nuclei of the urothelium (U), myofibroblasts in the SBM, endothelial cells of both arterioles and venules, fibroblasts in the LP, and smooth muscle cells. In LL-37 instilled bladders (C) and (D), similar ubiquitous HMGB1 immunoreactivity was observed compared to saline control tissues, except for positive detection in all acute inflammatory cells (primarily PMNs) within the inflamed tissues. Images reduced from × 10, scale bar panel A represents 75 μM.

smooth muscle (**Figures 2A** and **B**). Analysis of the LL-37 instilled bladders showed similar ubiquitous HMGB1 immunoreactivity to saline control tissues, except for positive detection in all acute inflammatory cells (primarily PMNs) within the inflamed tissues, along with a qualitative increased staining pattern in the smooth muscle cell layer (**Figures 2C** and **D**).

3.3. RAGE qRT-PCR and Western Blot

In order to quantitate RAGE expression, both qRT-PCR and Western Blot methods were employed. In the qRT-PCR experiments, total RNA was isolated from both saline control and LL-37 inflamed bladders. Tissue and RNA isolation was performed 24 hours after either saline or LL-37 exposure. LL-37 inflamed tissues demonstrated a near 10-fold reduction in RAGE RNA levels (**Figure 3**, red bar) when compared to saline controls (**Figure 3**, blue bar).

To quantitate the amount of RAGE protein in LL-37 challenged bladders, Western Blot analysis was performed (**Figure 4**). Lung tissue served as our positive control (**Figure 4**, lane A) (based on known robust expression levels of RAGE) and we identified diminished RAGE protein levels in our LL-37 challenged samples (**Figure 4**, lane B) versus saline instilled controls (**Figure 4**, lane C). Two bands were observed based on our antibody profile that detected both membrane-bound RAGE (55 kDa) and soluble RAGE (48 kDa). Normalization was carried out via baseline actin content. The diminished RAGE RNA expression patterns observed from the qRT-PCR results were further validated with decreased RAGE protein expression from our Western Blot experiments. Expression patterns observed from the
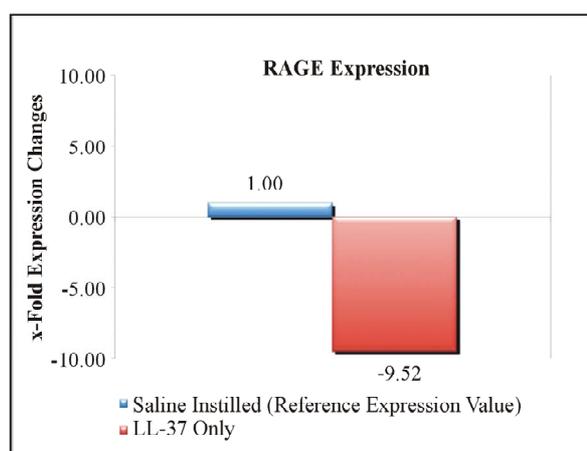
Figure 3. Quantitative RT-PCR of RAGE. Total RNA was isolated from both control and LL-37 inflamed bladders. Tissue and RNA isolation was performed 24 hrs after either saline or LL-37 exposure. LL-37 inflamed tissues demonstrated a near 10-fold reduction in RAGE mRNA levels (red bar) when compared to saline controls.
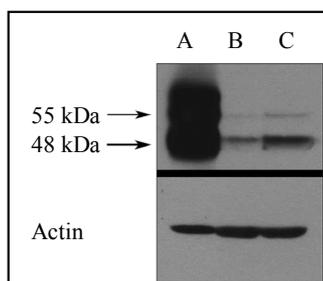
Figure 4. RAGE Western Blot. (A)—lung protein isolate (positive control), (B)—LL-37 instilled tissue harvested after 24 hours, (C)—saline instilled control tissue harvested after 24 hours. Two common RAGE bands were detected at 48 kDa and 55 kDa. A moderate reduction in RAGE protein levels were observed within the inflamed LL-37 instilled bladders (B). Normalization carried out via baseline actin content.

qRT-PCR results were further validated with decreased RAGE protein expression from our Western Blot experiments.

3.4. Downstream NF-κB Dependent Gene Expression

HMGB1 signaling via RAGE can lead to the activation of NF-κB. Therefore, we wanted to investigate five common downstream genes activated by NF-κB [18-24]. We hypothesized that LL-37 challenged tissues would yield up-regulation of all five genes (TNFα, IL-6, IL-1β, GM-CSF, COX-2) when compared to saline controls. To test this hypothesis, we utilized qRT-PCR from mRNA isolated in 12 & 24 h tissues, for both LL-37 challenged bladders and saline controls. **Figure 5** summarizes our

results. With the exception of TNFα, all genes at both time points yielded substantial up-regulation when compared to controls.

4. DISCUSSION

Using our previously established model, we created bladder inflammation using the cathelicidin LL-37 [8,40], which has been found to be elevated in urine samples obtained from pediatric patients with either cystitis or pyelonephritis [42]. Profound bladder inflammation was observed after LL-37 exposure yielding global erythema, edema, hemorrhage, and hypervascularity. More severe tissue inflammation occurred in 24-hour versus 12-hour tissues [8]. IHC for RAGE identified expression along cytosolic and non-nuclear cellular membrane portions of the urothelium and smooth muscle, with strong RAGE expression in the acute inflammatory cells within LL-37 inflamed tissues. Overall RAGE expression, however, was down-regulated in LL-37 inflamed tissues. Western Blot analysis confirmed basal expression levels for RAGE in control tissues, with a moderate RAGE reduction in inflamed bladders. As stated earlier, some lung studies have shown that a decrease in RAGE expression leads to fibrosis [15-17] and this appears to be true in the bladder between LL-37 and NF-κB, understanding only that LL-37 up-regulates inflammation through pathways that converge on NF-κB. We attempted to show that LL-37 induced bladder inflammation was connected to the signaling through RAGE and its various ligands.

Unexpectedly, a reduction in RAGE expression was noted in inflamed tissues compared to controls. Although, IHC for the RAGE ligand HMGB1 demonstrated moderate differences between control and LL-37 inflamed tissues. We mainly observed increased expression patterns in the acute inflammatory cells and smooth muscle cells within the inflamed samples. Despite diminished
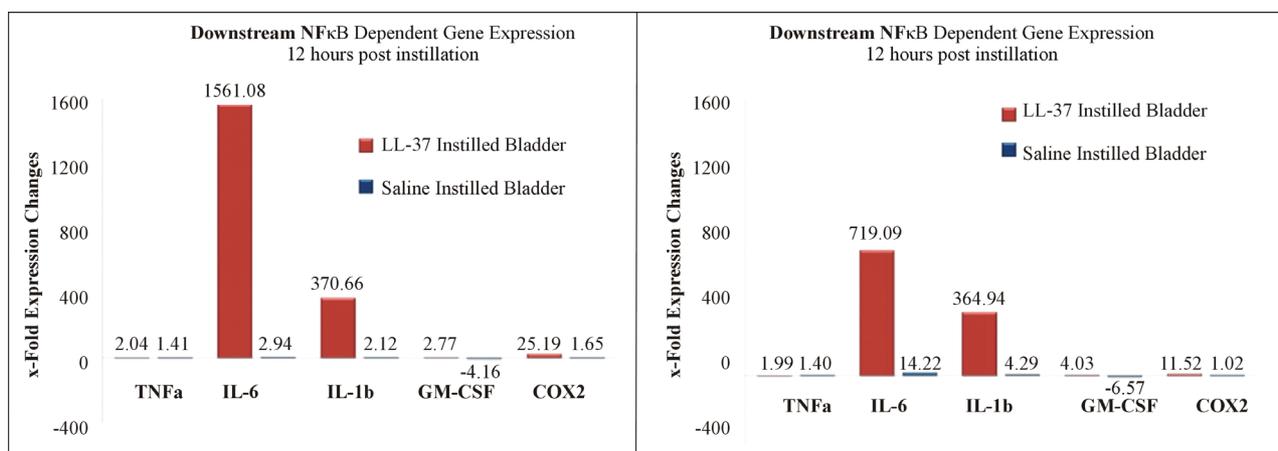
Figure 5. Quantitative RT-PCR for TNFα, IL-6, IL-1β, GM-CSF, & COX-2. With the exception of TNFα, all genes at both 12 h and 24 h time points yielded substantial up-regulation (red bars) when compared to controls (blue bars).

RAGE expression, the moderate qualitative increase in HMGB1 expression seen in the inflamed tissues would suggest alternative receptor pathways are involved in the profound activation of NF-κB dependent genes. Again, four of five common downstream genes activated by NF-κB were substantially up-regulated within our LL-37 challenged tissues.

It has been well described that Toll-like receptors (TLRs), specifically TLR2 and TLR4, are involved in HMGB1 signaling [43-45]. HMGB1 can act as a ligand and bind not only the RAGE receptor [46], but TLR2 and TLR4. TLRs are a family of conserved proteins that enable cells of the immune system to respond to microbial structures. TLR2 has been shown to respond to gram-positive components and fungi and TLR4 is the main receptor for endotoxin. Both TLR2 and TLR4 have been shown to be present in the lower urinary tract and bladder urothelium [27] and play a vital role in molecular defense mechanisms during urinary tract infection [47-49]. TLR2 also has been implicated in inflammatory responses in the urinary system and may play a role in progressive tissue fibrotic changes [50]. The signaling cascades initiated by TLR2 and TLR4 are complex. Similar to RAGE activation, the most prominent effect of TLR activation is the induction of NF-κB dependent gene expression. Overall, HMGB1 activation of TLR2 and/or TLR4 could both initiate and sustain a pro-inflammatory phenotype, ultimately explaining the increased expression of NF-κB dependent genes. With this knowledge, along with our data demonstrating such profound up-regulation of NF-κB dependent genes, further studies are needed to elucidate the role of TLRs.

5. CONCLUSION

We have innovatively employed our reproducible LL-37 induced cystitis model to gain further insight towards a possible mechanistic pathway involved in bladder inflammation. This work opens the door for future studies involving the inflammatory ligand HMGB1, RAGE, and the corresponding receptor signaling pathways that implicate NF-κB.

6. ACKNOWLEDGEMENTS

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