RESEARCH ARTICLE

NLRX1 is not involved in the host defense against *Escherichia coli* induced pyelonephritis [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (*E. coli*) are one of the most prominent infections that have serious impact on kidney functioning and the development of chronic kidney disease. NOD-like receptor (NLR)X1 is an innate immune receptor that is important for immune metabolism and regulation, with as yet an unknown role in UTI and the pathophysiology of pyelonephritis.

Methods: Wild-type (WT) and NLRX1 Knock-out (KO) female mice were subjected to UTI by intravesically inoculation of uropathogenic *E. coli* and sacrificed at 24h and 48h after infection after which bacterial burden and the inflammatory response in the bladder and kidney were studied. *Ex vivo* we studied the role of NLRX1 during the LPS induced pro-inflammatory cytokine response and phagocytosis of *E. coli* by granulocytes and monocytes.

Results: Here, we report that during early experimental UTI NLRX1 absence reduces bacterial clearance in the bladder and dampens the inflammatory cytokine response, whereas in the kidney NLRX1 does not affect bacterial burden or cytokine response. In addition, we found that NLRX1 is not essential for the pro-inflammatory cytokine secretion by granulocytes and monocytes in response to LPS nor for bacterial phagocytosis.

Conclusion: Together, we report that NLRX1 is important in enhancing the early host defense against uropathogenic *E. coli* in the bladder but does not affect the development of pyelonephritis.

Keywords

Innate Immune Receptor NLRX1, Lower and Upper UTI, Pyelonephritis, Animal model, *Escherichia coli*

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Introduction

Toll like receptors (TLRs) and NOD-like receptors (NLRs) are members of a large family of extracellular and intracellular pattern recognition receptors (PRRs) that trigger immune responses to prevent pathogen invasion and growth^{1,2}. Urinary tract infections (UTIs) are common bacterial infections in humans, that occur most commonly in women and children³. UTIs are caused by the presence of uropathogenic bacteria, usually *Escherichia coli* (*E. coli*), in the lower urinary tract (bladder) that overcome the host innate immune defense. When the infection ascends from the bladder via the ureters to the upper renal pelvis, lower UTI can lead to acute pyelonephritis. If untreated pyelonephritis can have serious implications for renal functioning and the development of damage and scarring^{4,5}. Antimicrobial resistance among UTIs are increasing^{6,7}, therefore new insights in host defense mechanisms are required to obtain new targets for therapy.

TLRs are known to play an important role in the host response to UTIs⁸, whereas the role of NLRs herein is unclear. NOD-like receptor X1 (NLRX1) is an ubiquitously expressed PRR in mitochondria that controls mitochondrial activity in tubular epithelial cells and hepatocytes, and in this way effects respectively ischemic acute kidney disease and liver steatosis^{9,10}. Other functions for NLRX1 include negative regulation of antiviral immunity¹¹, and inhibition of NF- κ B signaling by disrupting interaction of TRAF6 and IKK^{12,13}. Given these studies, NLRX1 could play a potential role during the pathophysiology of acute bacterial infections such as pyelonephritis.

To get more insight in NLRX1 functioning during bacterial infection we investigated in the present study the role of NLRX1 during uropathogenic *E. coli*-induced lower and upper UTI in mice. We found that although NLRX1 absence enhances bacterial burden in the bladder during the early phase of infection, NLRX1 is not involved in the host defense against pyelonephritis.

Methods

Animals

NLRX1 KO mice with a C57BL6/J background were generated as described previously¹⁴ and bred at the animal facility of the Academic Medical Center (AMC) in Amsterdam, The Netherlands. Age- and gender-matched C57BL6/J WT mice were obtained from Charles River (Maastricht, The Netherlands). Animals were housed in individual ventilated cages (IVCs) with bedding and cage enrichment that were kept under standard environmental conditions (temperature, humidity, ventilation, light/dark cycle) and under specific pathogen-free conditions (SPF) with *ad libitum* access to water and food. The mice were allowed to acclimatize for a week before starting the experimental procedures.

Animal experimental procedures

The *in vivo* study was performed with 2 experimental groups: 1) WT (n=8) and 2) NLRX1-KO (n=8) and 2 sham/control groups: 3) WT (n=4) 4) NLRX1-KO (n=4). Each experimental group was subjected for two time points (24h and 48h) to UTI as described previously¹⁵ and briefly explained later. The total

number of mice per *in vivo* experimental group was 16 and the total number per sham/control group was 4. To reach a statistical significant effect of NLRX1 deficiency the number of 8 mice per experimental group was based on a variation coefficient of 15%, a minimal relative effect of 30%, a P value of 5% and a power of 80%, that were based on previous studies done in our group¹⁵⁻¹⁷. For both experimental and sham/control groups 11–12 week old female mice (median weights: WT; 19,6 and NLRX1-KO; 21,3 grams) were used. Each experimental group was divided in 2 cages of 3 and 5 animals and in the sham/control group 4 animals per cage were kept. For the experimental group uropathogenic *E. coli* 1677, isolated from an uroseptic patient, was cultured in the laboratory in sterile Tryptic Soy Broth (TSB) overnight at 37°C, 5% CO₂. The next day, in the morning this suspension was diluted 1:100 in fresh TSB and in 2–3h cultured to optical density OD_{620nm} = 1 was reached (measured with a spectrophotometer (DU640, Beckman, USA)). Subsequently bacteria were spun down for 14 min at 4000 rpm at 4°C, washed three times and resuspended in 10 mL sterile PBS. The same day, in the animal facility, under general anaesthesia (10 μ l/1 g body weight of FFM mixture, containing 1.25 mg/ml midazolam (Dormicum®, Roche, Woerden, The Netherlands), 0.08 mg/ml fentanyl citrate/2.5 mg/ml fluanisone (Hypnorm, Veta Pharma Ltd., Leeds, UK)) that was given intraperitoneal, mice were via the urethra intravesically inoculated with 8*10⁸ CFU in a 100 μ l volume. Mice in the sham/control group underwent the same procedure with administration of 100 μ l sterile PBS. CFU concentrations in the inoculum were determined by plating 10-fold serial dilutions on blood-agar plates at 37°C, 5% CO₂ overnight. Mice were sacrificed 24 and 48 hour post-inoculation by cardiac puncture under 4% isoflurane/O₂ followed by cervical dislocation. Blood was collected in lithium-heparin containing tubes and kidneys and bladders were collected for further analysis. In the WT 24h group one animal reached, because of signs of severe sepsis, the human end point and was excluded from further data analysis. The animals used to study leukocyte composition and *ex vivo* granulocyte and monocyte functioning contained 2 experimental groups: 1) WT (n=6) and 2) NLRX1-KO (n=6) and were sacrificed as described earlier for the *in vivo* experiments. Mice used for the *ex vivo* experimental groups were 13–14 week old female mice (median weights: WT;26.5 and NLRX1-KO;26.6 grams). The total number of mice per *ex vivo* experimental group was 6. In the WT group one sample was excluded from further data analysis due to low blood gain.

Ethics statement

All animal procedures were ethically approved under DPA 25 AB-1 by the Animal Care and Use Committee of the Academic Medical Center Amsterdam and were conducted in compliance with the ARRIVE guidelines (NC3Rs).

Bacterial outgrowth determination

Bladder 20% (w/v) and left kidney 10% (w/v) tissues were homogenized in sterile PBS with a tissue homogenizer (Polytron PT1300D homogenizer, Kinematica AG). To determine bacterial loads 10-fold serial dilutions of bladder- and kidney-homogenates were plated onto blood agar plates. Colonies were counted 16h after incubation at 37°C.

Leukocyte composition and count

Absolute leukocyte number in blood was measured with a Coulter Counter (Beckmann Coulter Inc., Fullerton, CA). To assess relative leukocyte composition, 100 μ l whole blood erythrocytes were lysed by adding 2 ml lysis buffer (8.3g/L NH_4Cl , 1.0g/L KHCO_3 , 0.1 mM EDTA, pH 7.4) for 15 min at RT. The remaining cells, leukocytes, were washed once, centrifuged and resuspended in FACS buffer (0.5% BSA, 0.01% NaN_3 , 0.35mM EDTA in PBS) and were measured by flow cytometry on a FACS Canto (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo version 10 software. Living cells, lymphocyte, granulocyte and monocyte populations were gated based on forward-scattered light (FSC)/side-scattered light (SCC).

Whole blood stimulation

Whole blood of WT and NLRX1 KO mice was incubated at 37°C, 5% CO_2 in 10ng/mL LPS (cat no. 14391, Sigma-Aldrich, Zwijndrecht, The Netherlands) conditioned RPMI medium (Thermo fisher Scientific, Waltham, MA, USA) containing 10% FCS (Invitrogen, Carlsbad, CA, USA) with 2 mM L-glutamine and 100 U/mL penicillin/streptomycin (all from Thermo fisher Scientific, Waltham, MA, USA). After 14h, cells were spun down (5 min, 4000 rpm) and supernatants were collected and stored at -20°C prior to use for cytokine measurements.

Phagocytosis assay

Phagocytosis by granulocytes and monocytes was measured with the PHAGOTEST (cat no. 10–0100, Glycotope Biotechnology, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 100 μ l of heparinized whole blood was incubated with opsonized FITC- labeled *E. coli* for 10 minutes in a 37°C water bath, whereas the negative controls remained on ice. After phagocytosis was stopped the surface signal was quenched. After lysing the erythrocytes and fixation of the leukocytes, phagocytic capacity was measured by flow cytometry on a FACS Canto (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo version 10 software.

Enzyme-linked immunoabsorbance assay (ELISA)

Bladder 5% (w/v) and kidney 10% (w/v) tissues from 24h and 48h UTI subjected WT and NLRX1 KO mice were homogenized with a tissue homogenizer (Polytron PT1300D homogenizer, Kinematica AG) in Greenberger lysis buffer (GLB) (300mM NaCl, 30mM Tris, 2mM MgCl_2 , 2mM CaCl_2 , 1% (v/v) Triton X-100, pH set at 7.4, supplemented with Protease Inhibi-

tor Cocktail II (cat no. p8340, Sigma-Aldrich, Sigma-Aldrich, Zwijndrecht, The Netherlands). Levels of keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein 1 (MCP-1), interleukin 1 β (IL1 β), interleukin 6 (IL6), tumor necrosis factor alpha (TNF α) and mouse myeloid peroxidase (MPO) were determined in bladder and kidney homogenates and whole blood plasma (KC, TNF α and IL6 only) by duo set ELISAs (cat no. MKC00B, MM200, MJE00, MLB00C, M6000B, MTA00B, DY3667, R&D Systems, Abingdon, UK), performed according to the supplied protocols. ELISA data measured in bladder and kidney homogenates was adjusted for total protein concentration as determined by BCA protein assay (cat no. B9643, Sigma-Aldrich, Sigma-Aldrich, Zwijndrecht, The Netherlands) developed with 4% CuSO_4 .

Plasma biochemical analysis

From hepanarized blood, plasma was obtained from the upper phase after spinning the tube for 10 minutes at 2000 *rpm*. Urea and creatinine levels in plasma were determined at room temperature by colorimetric enzyme reactions involving creatinase and urease and analyzed on the Cobas c702 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN, USA) according to standard diagnostic procedures performed by the department of Clinical Chemistry of the Academic Medical Center Amsterdam.

RNA isolation and real time quantitative PCR (RT-qPCR)

Total RNA was extracted from snap-frozen -80°C stored bladder and kidney using TriReagent (cat no. T9424, Sigma-Aldrich, Zwijndrecht, The Netherlands) followed by chloroform phase separation to obtain the aqueous RNA containing upper phase and isopropanol precipitation according to the manufacturer protocol procedure description and converted to cDNA. cDNA was synthesized using M-MLV reverse transcriptase according to the procedure described in the manufacturer protocol (cat no. 28025, Thermo Scientific). Transcription was analyzed by RT-qPCR on a Roche LightCycler 480 using 2.5 μ l sensiFAST SYBR master mix (cat no. bio-98020, Bioline reagents, London, UK), 0.20 μ l forward primer, 0.20 μ l reverse primer (Table 1), 2.10 μ l distilled H_2O and 1 μ l cDNA per reaction. qPCR primers were synthesized by Eurogentec (Maastricht, The Netherlands) and described in the list below. qPCR data was analyzed based on linear regression using the LinRegPCR program, that is freely available^{18,19}. Briefly, the LinRegPCR program imports non-baseline corrected qPCR data, performs a baseline correc-

Table 1. qPCR Primers.

Gene	Full name	GenBank accession number	Forward primer	Reverse primer
<i>Nlx1</i>	NOD-like receptor X1	NM_001163742 NM_001163743 NM_178420	TTGCCATTGCCCAGGACCTCTT	GGATCAAGAAGGAGATATGCTCATCTGGTAG
<i>Tbp</i>	TATA box binding protein	NM_013684	GGAGAATCATGGACCAGAACA	GATGGGAATTCCAGGAGTCA

tion on each sample separately, determines a window-of-linearity and then uses linear regression analysis to determine the PCR efficiency per sample from the slope of the regression line. The mean PCR efficiency per amplicon and the C_q value per sample are used to calculate a starting concentration per sample, expressed in arbitrary fluorescence units (au)¹⁹.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM), bacterial outgrowth data are expressed on a logarithmic scale as median scatterplot. The non-parametric Mann Whitney U test was performed for two group comparison. For all analyses, values of $P \leq 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

Local NLRX1 expression in the bladder and kidney during experimental UTI

To determine whether NLRX1 expression is modulated in the murine bladder and kidney during urinary tract infection (UTI), wild-type (WT) mice were intravesically inoculated with uropathogenic *E. coli* and sacrificed at 24h and 48h after infection. Non-infected sham mice were used as controls. Real-time quantitative PCR revealed that *Nlr1* transcript levels were constitutively present in the bladder and kidney (Figure 1A and B). *Nlr1* transcript levels show a non-significant trend towards increased levels in the bladder after 24h, while levels are returned towards baseline sham levels at 48h (Figure 1A). In the kidney *Nlr1* transcript levels remained at baseline level 24h after infection while after 48h levels were significantly increased (Figure 1B). Together, these data show that in response to UTI, local *Nlr1* expression is increased upon *E. coli* infection.

NLRX1 deficiency enhances early bacterial burden from the bladder while it does not influence bacterial burden in the kidney during experimental UTI

To investigate whether NLRX1 plays a role in bladder and kidney during lower and upper UTI, we examined bacterial loads in these organs from WT and NLRX1 knock-out (KO) mice 24h and 48 h after inoculation with uropathogenic *E. coli*. This revealed that the bacterial outgrowth, as measured by colony forming units (CFU), in bladder tissue from NLRX1 KO mice was significantly higher at 24h after infection compared to WT while at 48h no differences in bacterial burden were found (Figure 2A). NLRX1 deficient mice had more improved bacterial clearance from the bladder at 48h as compared to 24h (Figure 2A). No differences in the amount of CFU were found between kidneys from WT and NLRX1 KO mice at both time points (Figure 2B). To monitor the local inflammatory response during infection, we next measured the levels of KC, MIP-2, MCP-1, IL-1 β , IL-6 and TNF α in kidney and bladder homogenates (Figure 2C and D). The production of MIP-2 in the bladder was in NLRX1 KO mice reduced at both 24h and 48h after infection compared to WT animals (Figure 2C). In addition, NLRX1 KO bladders show reduced levels of MCP-1 and TNF α compared to WT at 48h, while no differences were found in KC, IL-1 β and IL6 levels (Figure 2C). We identified an increase in renal KC levels at 24h in NLRX1 KO mice compared WT mice, whereas at 48h KC levels were similar between both groups (Figure 2D). At both 24h and 48h no differences were found in renal MIP-2, MCP-1, IL-1 β , IL-6 and TNF α levels between WT and NLRX1 KO mice (Figure 2D). In addition, upper UTI and NLRX1 have no significant influence on renal function as reflected by similar plasma levels of urea and creatinine between all mice (Supplementary Figure 1 A and B). Together, this indicates that the lack of NLRX1 is associated with an early

Figure 1. NLRX1 expression in bladder and kidney during experimental urinary tract infection (UTI). *Nlr1* mRNA transcript levels in wild-type (WT) (A) bladder and (B) kidney from sham, and uropathogenic *E. coli*-inoculated mice. All data are expressed as mean $\pm$ SEM, $n=4$ (sham) and $n=7-8$ (24h and 48h) animals per group. Statistical significance was determined by non-parametric Mann Whitney U test, * $P<0.05$.

Figure 2. Bacterial outgrowth and inflammatory response in WT and NLRX1 KO bladder and kidney during experimental urinary tract infection (UTI). Outgrowth of uropathogenic *E. coli* expressed in colony forming units (CFU) in (A) bladder and (B) kidney homogenates from wild-type (WT) and NLRX1 knock-out (KO) mice 24h and 48h after inoculation. Levels of KC, MIP-2, MCP-1, IL-1 β , IL6 and TNF α in (C) bladder and (D) kidney homogenates from the *E. coli* inoculated WT (white bars) and NLRX1 KO (black bars) mice. Data at A and B are expressed on a logarithmic scale as median scatterplot. Data at C and D are expressed as mean $\pm$ SEM. For all data $n=7-8$ animals per group and statistical significance between WT and NLRX1 KO was determined by non-parametric Mann Whitney *U* test, * $P<0.05$ and ** $P<0.01$.

impaired ability to clear uropathogenic *E. coli* from the bladder, probably due to an impaired pro-inflammatory cytokine response, while NLRX1 deficient mice had an improved bacterial clearance from 24h to 48h in the bladder as compared to WT. No differences were found in bacterial burden and cytokine response in the kidney when mice were deficient for NLRX1.

NLRX1 does not affect circulating leukocyte number and local presence of activated neutrophils during experimental UTI

By analyzing inflammatory cells in the circulation we observed that the numbers of granulocytes and monocytes were equal between uninfected WT and NLRX1 KO mice (Figure 3A). A non-significant trend towards an increased presence of lymphocytes in NLRX1 KO compared to WT is shown (Figure 3A). We observed that WT and NLRX1 KO mice 24h and 48h after inoculation, have similar numbers of circulating leukocytes (Figure 3B). Recruitment of neutrophils in the kidney and bladder are essential for the host defense against uropathogenic *E. coli*²⁰. Therefore, we determined active neutrophil presence by measuring kidney and bladder myeloperoxidase (MPO) concentrations. No differences in MPO levels were found in

bladder and kidney between WT and NLRX1 KO mice at 24h and 48h (Figure 3C and D), indicating a similar number of activated neutrophils.

NLRX1 is not essential for pro-inflammatory cytokine secretion in response to LPS and phagocytosis of *E. coli* by granulocytes and monocytes

Since we observed differences in bacterial outgrowth in the bladder while the number of local neutrophils after infection is equal between WT and NLRX1 KO, we investigated if NLRX1 absence causes functional changes to granulocytes and monocytes. This revealed that NLRX1 is not critical for the secretion of the pro-inflammatory cytokines KC, TNF α and IL6 after *ex vivo* whole blood LPS stimulation (Figure 4A, B and C). To investigate if NLRX1 is important for the phagocytic activity of granulocytes and monocytes, leukocytes from WT and NLRX1 KO mice were *ex vivo* incubated with fluorescein labelled opsonized *E. coli*, and phagocytosis was analyzed using flow cytometry. Granulocytes and monocytes from both WT and NLRX1 KO mice show increased phagocytic activity responses when challenged with *E. coli* at 37°C compared to 0°C (Figure 4D and E). However, no differences were observed in

Figure 3. Circulating and local leukocyte dynamics in naïve WT and NLRX1 KO mice and upon experimental urinary tract infection (UTI). (A) Total circulating leukocyte, lymphocyte, granulocyte and monocyte cell counts in blood from uninfected wild-type (WT) (white bars) and NLRX1 knock-out (KO) (black bars) mice $n=5-6$ animals per group. (B) Circulating leukocytes in blood from WT (white bars) and NLRX1 KO (black bars) mice 24h and 48h after uropathogenic *E. coli* inoculation. MPO levels in (C) bladder and (D) kidney homogenates from 24h and 48h inoculated WT (white bars) and NLRX1 KO mice (black bars). All data are expressed as mean $\pm$ SEM, $n=7-8$ (B–D) animals per group. Statistical significance between WT and NLRX1 KO was determined by non-parametric Mann Whitney *U* test.

Figure 4. Cytokine secretion in response to LPS and *E. coli* phagocytosis by WT and NLRX1 KO granulocytes and monocytes. Levels of (A) KC (B) TNFα and (C) IL6 after 14h *ex vivo* LPS whole blood stimulation from wild-type (WT) (white bars) and NLRX1 knock-out (KO) (black bars). ND = not detectable. Phagocytic activity responses of (D) granulocytes and (E) monocytes from WT (white bars) and NLRX1 KO (black bars) mice *ex vivo* challenged with *E. coli* at 0°C and 37°C. All data are expressed as mean ± SEM, *n*=5–6 animals per group. Statistical significance between all columns was determined by non-parametric Mann Whitney *U* test, **P*<0.05 and ***P*<0.01.

the percentage of granulocytes and monocytes that undergo phagocytosis between WT and NLRX1 KO (Figure 4E and F). Together, these results suggest that the early decreased bacterial clearance in the bladders from NLRX1 KO mice cannot be explained by an impaired granulocyte or monocyte response.

Dataset 1. NLRX1 expression data – This file contains the data underlying the analysis of the NLRX1 expression in bladder and kidney as shown in Figure 1

<http://dx.doi.org/10.5256/f1000research.15361.d212937>

Dataset 2. *In vivo* mice bladder and kidney colony forming units (CFU)-, cytokine-, general marker-data and leukocyte counts in sham and after 24h and 48h of infection – This file contains the data underlying the analysis of the data used in Figure 2, Figure 3 and Supplementary Figure 1

<http://dx.doi.org/10.5256/f1000research.15361.d212938>

Dataset 3. *Ex vivo* FACS output data - This file contains the FACS data underlying the leukocyte composition analysis as shown in Figure 3

<http://dx.doi.org/10.5256/f1000research.15361.d212939>

Dataset 4. *Ex vivo* cytokine data – This file contains the data underlying the cytokine determination in LPS stimulated whole blood as shown in Figure 4

<http://dx.doi.org/10.5256/f1000research.15361.d212940>

Dataset 5. *Ex vivo* FACS output data on granulocyte and monocytes phagocytosis - This file contains the FACS data underlying the granulocytes and monocytes phagocytosis as shown in Figure 4

<http://dx.doi.org/10.5256/f1000research.15361.d212941>

Discussion

Innate immune receptors like TLRs and NLRs are known to play pivotal roles in the first line of host defense against invading pathogens. NLRX1 is an innate immune receptor that can modulate inflammatory responses²¹ and cell metabolism^{9,10}. As such NLRX1 could play a potential role during the pathophysiology of UTI. To study this we investigated the role of NLRX1 during uropathogenic *E. coli*-induced lower and upper UTI in mice. Although NLRX1 enhances the inflammatory cytokine response and the bacterial clearance in the bladder during early experimental UTI, we found that this receptor does

not affect overall renal bacterial loads and inflammation during pyelonephritis. In addition, we observed that NLRX1 is not essential for pro-inflammatory cytokine secretion by granulocytes and monocytes in response to LPS nor for phagocytosis of *E. coli*.

In this study we investigated the role of NLRX1 in influencing bacterial burden and inflammation in bladder and kidney during experimental UTI. We found that the lack of NLRX1 is associated with an impaired ability to clear uropathogenic *E. coli* from the bladder at 24h only, probably due to an impaired pro-inflammatory MIP-2 cytokine response which is usually needed for the recruitment of granulocytes to the site of infection^{20,22} and the initiation of host defense during UTI²³. Surprisingly however, the levels of bladder MPO as an indicator of neutrophil influx and the *ex vivo* granulocyte phagocytic capacity to ingest *E. coli* are not affected by NLRX1 while local *Nlr1* expression in the bladder tended to be increased at 24h. Possibly, granulocyte influx is altered by NLRX1 at an earlier time point than 24h. Despite the impaired MIP-2, MCP-1 and TNF α cytokine response, bacterial burden in the bladder at 48h, is not different in NLRX1 deficient mice compared to WT. In fact, NLRX1 KO are able to clear *E. coli* bacteria faster since the CFU reduction from 24h to 48h is significantly while bacterial levels in bladder remained the same in WT. Whereas in the kidney, the outgrowth from 24h to 48h in WT and NLRX1 KO remains unchanged, indicating that despite the local *Nlr1* expression increase at 48h NLRX1 this does not affect bacterial outgrowth in the kidney. Our study demonstrates that due to NLRX1 absence, the MIP-2 cytokine release to recruit neutrophils is less pronounced and hence possibly attenuates the early phase of the host defense against *E. coli* in the bladder without affecting later bacterial bladder burden, innate myeloid cell phagocytosis and the promotion of pyelonephritis.

NLRX1 is on the one hand described to negatively regulate NF- κ B signaling^{12,13} and on the other to indirectly amplify the NF- κ B pathway²⁴. During *E. coli*-induced UTI infections the NF- κ B signaling pathway is via TLR4 activation one of the most important pathways for the pro-inflammatory cytokine release and the clearance of *E. coli* from the urinary tract²⁵. We observed in NLRX1 KO bladders that despite the increased bacterial burden 24h post infection, besides reduced MIP-2, the levels of pro-inflammatory cytokines TNF α , IL6 and IL1 β were equal in both mouse strains. Whereas at the later 48h time point in the bladder and at 24h and 48h in the kidney, NLRX1 has no effect on pro-inflammatory cytokine response and bacterial burden. This indicates that upon early UTI, NLRX1 absence leads to a suppressed pro-inflammatory cytokine response in the bladder. Whether this is caused by an altered neutrophil influx in the early onset of infection or the ability of NLRX1 to influence NF- κ B signaling warrants further study. From our study it is however clear that NLRX1 is neither essential for whole blood pro-inflammatory KC, TNF α and IL6 cytokine responses to LPS, nor for bacterial phagocytosis by granulocytes and monocytes. Similar observations were done in bone marrow-derived macrophages where TNF α and IL6 cytokine expression remained

similar in WT and NLRX1 deficient cells after a *Helicobacter pylori* (LPS positive) infection²⁶. In contrast, TNF α and IL6 levels were increased upon NLRX1 knockdown in LPS-stimulated peritoneal macrophages¹² and IL6 levels in LPS-stimulated bone marrow-derived macrophages¹³ indicating that NLRX1 attenuates NF- κ B signaling. In contrast, increased NF- κ B signaling upon LPS positive *Shigella flexneri* infection was observed in NLRX1 overexpressing epithelial cells²⁴. Possibly, the role of NLRX1 varies between cell types involved in host defense, such as myeloid cells and parenchymal cells, during different time points of infection and different ligands. Together, we observed that NLRX1 does not affect the pro-inflammatory cytokine response after LPS challenge in granulocytes and monocytes, whereas previous studies show that in macrophages and epithelial cells NLRX1 can behave differently^{12,13,24}. Indeed, during UTI epithelial cells are important in activating inflammation via various signaling pathways²⁵. Based on our results that granulocyte and macrophage functioning are not affected by NLRX1, we assume that during UTI NLRX1 plays a role in the early bacterial burden in the bladder by activating the pro-inflammatory cytokine response in parenchymal cells via NF- κ B.

Besides its role in immune regulation, we previously observed that NLRX1 functioning extends to the control of mitochondrial activity, oxidative stress and cellular metabolism in parenchymal cells of the kidney and liver^{9,10}. Macrophages and in particular neutrophils contribute during infections to the host defense via oxidative burst^{27,28}. From our data it is not clear if NLRX1 plays a role in the oxidative burst in myeloid cells during UTI. However, a previous study showed that NLRX1 plays no significant role in ROS production of LPS activated neutrophils and macrophages¹³. Together, our data indicates that there is a role for NLRX1 during UTI in the bladder by activating the pro-inflammatory cytokine response, while no direct role for NLRX1 is observed in myeloid cells.

Conclusion

We report that NLRX1 is important in attenuating the early bacterial burden in the bladder by enhancing the local pro-inflammatory cytokine response but has no effect on the development of pyelonephritis.

Data availability

Dataset 1: *NLRX1* expression data – This file contains the data underlying the analysis of the *NLRX1* expression in bladder and kidney as shown in Figure 1. [10.5256/f1000research.15361.d212937](https://doi.org/10.5256/f1000research.15361.d212937)²⁹

Dataset 2: *In vivo* mice bladder and kidney colony forming units (CFU)-, cytokine-, general marker-data and leukocyte counts in sham and after 24h and 48h of infection – This file contains the data underlying the analysis of the data used in Figure 2, Figure 3 and Supplementary Figure 1. [10.5256/f1000research.15361.d212938](https://doi.org/10.5256/f1000research.15361.d212938)³⁰

Dataset 3: *Ex vivo* FACS output data - This file contains the FACS data underlying the leukocyte composition analysis as shown in Figure 3. [10.5256/f1000research.15361.d212939](https://doi.org/10.5256/f1000research.15361.d212939)³¹

Dataset 4: *Ex vivo* cytokine data – This file contains the data underlying the cytokine determination in LPS stimulated whole blood as shown in [Figure 4](#). [10.5256/f1000research.15361.d212940](#)³²

Dataset 5: *Ex vivo* FACS output data on granulocyte and monocytes phagocytosis - This file contains the FACS data underlying the granulocytes and monocytes phagocytosis as shown in [Figure 4](#). [10.5256/f1000research.15361.d212941](#)³³

Competing interests

No competing interests were disclosed.

Grant information

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Supplementary material

Supplementary Figure 1. Renal function of wild-type (WT) and *NLRX1* knock-out (KO) mice during experimental urinary tract infections (UTI)

Plasma levels of renal function markers (A) urea and (B) creatinine from sham, 24h and 48h uropathogenic *Escherichia coli* inoculated WT (white bars) and *NLRX1* KO (black bars) mice. All data are expressed as mean $\pm$ SEM, $n=3-4$ (sham) and $n=7-8$ (24h and 48h) animals per group. Statistical significance was determined by non-parametric Mann Whitney *U* test.

[Click here to access the data.](#)

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Sheryl Justice

The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA

The manuscript by Kors *et al.* explores the role of the nod-like receptor X1 (NLRX1) during urinary tract infection (UTI). Given the intracellular populations during UTI, the potential role of intracellular immune regulators could be important and the investigations are understudied, making the work significant. Better understanding of the immune modulators could lead to new approaches to treat UTI. The investigators use a knock out mouse to evaluate the bacterial burden in the urinary tissues as well as the immune responses. In addition, the investigators evaluate the efficacy of phagocytosis of *E. coli* by granulocytes and macrophages. There are alternative conclusions that are more consistent with the published literature and better explain the data than the those provided by the investigators. The analysis of the circulating immune cells was important to exclude potential confounding differences in overall immune capacity.

Major concerns:

1. The use of 10^8 as the inoculum is known to induce early exfoliation of the superficial bladder epithelial cells in C57Bl/6 mice (at about 16 hours), and most groups use a lower inoculum to evaluate the acute phase of UTI. The bacterial burden observed in the wild type at 24 hours is consistent with prior studies that the early exfoliation leads to establishment of a quiescent intracellular reservoir between 10^3 and 10^4 . The similarity of this burden at 48 hours further supports this conclusion. In this scenario, the conclusion for the NLRX1 studies would be that the exfoliation is delayed. This would be a very interesting observation given that our understanding of the host responses that modulate exfoliation in response to infection is incomplete. Microscopic studies need to be performed to evaluate the exfoliation status to draw conclusions. Please provide the limit of detection on the graphs for the bacterial burden.
2. The immune response is known to induce morphological changes in UPEC that will reduce the CFU recovered. The loss of NLDX1 could alter these changes and an apparent increase in CFU could be observed, but the bacterial biomass could be similar. Given the bacterial burden of the parent, the conclusion proposed in point 1 is favored. Microscopic analysis would distinguish between these conclusions.

3. In the discussion, the statement that begins “In fact, NLRX1 KO mice are able to clear E. coli faster....” Is not consistent with what is known about the infection. Earlier time points (6 or 16 hours) would certainly reveal that there is a higher burden of UPEC in the WT and as such, the opposite conclusion would be made. It is highly unlikely that the burden in the WT mice would start at 10 e3 and remain there throughout the course of infection. Even if this were the case, the above conclusion would still not be supported since the burden is lower in WT mice at 24 hours. Inclusion of an earlier time point is needed.
4. Although statistically significant, IL-6 changes are modest and are observed at the later stages of the acute infection. There are multiple studies that have quantified IL-6 and correlated the effect of changes in IL-6 on the infection outcome. The results should be presented in context with the published literature.
5. Investigation for the potential role in myeloid cell function is intriguing. However, as the investigators indicated, there are potentially other functions that may be affected. The ability of the phagocytes to kill UPEC would be an interesting test and would provide mechanistic insight into the role of NLDX1 in this system.
6. Please provide power calculations for the immune studies, why were fewer mice included in this portion of the study? Was the burden determined for these mice?
7. The dataset for the FACs analyses were not included (the cytokine data was uploaded twice). Please provide an example of the gating strategy

Minor concerns:

1. In the methods for the animal experiments “human” should be “humane”.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: urinary tract infection, Escherichia coli, bacterial pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Apr 2019

Lotte Kors, UMC Amsterdam Location AMC, Amsterdam, The Netherlands

Dear Dr. Justice,

Thank you for the thoughtful comments. We have addressed the comments and questions raised and have detailed our response below.

1. The use of 10^8 as the inoculum is known to induce early exfoliation of the superficial bladder epithelial cells in C57Bl/6 mice (at about 16 hours), and most groups use a lower inoculum to evaluate the acute phase of UTI. The bacterial burden observed in the wild type at 24 hours is consistent with prior studies that the early exfoliation leads to establishment of a quiescent intracellular reservoir between 10^3 and 10^4 . The similarity of this burden at 48 hours further supports this conclusion. In this scenario, the conclusion for the NLRX1 studies would be that the exfoliation is delayed. This would be a very interesting observation given that our understanding of the host responses that modulate exfoliation in response to infection is incomplete. Microscopic studies need to be performed to evaluate the exfoliation status to draw conclusions. Please provide the limit of detection on the graphs for the bacterial burden.

Reply:

We appreciate the reviewer's alternative interpretation of the data and payed attention to this possibility in the second paragraph of the discussion as follows: "It is in addition possible that processes like delayed *E. coli*-attachment, invasion and modulated exfoliation or factor secretion of superficial bladder cells contribute to the increased presence of *E. coli* bacteria in NLRX1 KO bladders at 24h. Whether or not direct or indirect NLRX1-mediated modulation of bladder cells contribute to the bacterial burden has not been proven yet and warrants further study.". We agree that microscopic studies of the superficial bladder epithelial cells to evaluate exfoliation would be interesting to add however unfortunately we used all bladder material for the study of CFU counts. Instead we added this possibility as stated above. We finally would like to stress that 24h after exfoliation effective regeneration is induced making it probably difficult to convincingly show exfoliation at 48h (1). We provided the detection limit to the figure legends.

1. Niall F. Davis and Hugh D. Flood. The Pathogenesis of Urinary Tract Infections. In: *Clinical Management of Complicated Urinary Tract Infection*. 2011

2. The immune response is known to induce morphological changes in UPEC that will reduce the CFU recovered. The loss of NLDX1 could alter these changes and an apparent increase in CFU could be observed, but the bacterial biomass could be similar. Given the bacterial burden of the parent, the conclusion proposed in point 1

is favored. Microscopic analysis would distinguish between these conclusions.

Reply:

The assumption that the changes by NLRX1 loss will change the immune response and as such the UPEC is able to indirectly cause an increase in CFU without affecting bacterial biomass is interesting. We however showed in our paper by the *ex vivo* experiments in granulocytes and monocytes and by the various cytokine and chemokine levels in the bladder that when the bacterial outgrowth is increased, the immune response is not changed in absence of NLRX1. In addition, CFU counts and not bacterial biomass is as far we know the standard to measure bacterial burden in the used UTI model. This together with our main conclusion that NLRX1 is not involved in the host defense against *E. coli*-induced pyelonephritis we believe that further research on morphological changes to the UPEC is beyond the scope of the article.

3. In the discussion, the statement that begins "In fact, NLRX1 KO mice are able to clear *E. coli* faster...." Is not consistent with what is known about the infection. Earlier time points (6 or 16 hours) would certainly reveal that there is a higher burden of UPEC in the WT and as such, the opposite conclusion would be made. It is highly unlikely that the burden in the WT mice would start at 10^3 and remain there throughout the course of infection. Even if this were the case, the above conclusion would still not be supported since the burden is lower in WT mice at 24 hours. Inclusion of an earlier time point is needed.

Reply:

This is an excellent point raised by the reviewer. We agree with the reviewer that based on other studies the bladder bacterial burden in the WT is likely increased at an earlier time point than 24h and as such the results will be interpreted differently than described in the original discussion. Therefore we revised this part of the discussion as follows: "We found that the lack of NLRX1 is associated with an increased bacterial bladder burden at 24h. Previous studies by us (unpublished data) and others (2) show that the peak in uropathogenic *E. coli* outgrowth from C57BL6 mice bladders is observed before 24h. This, together with our observations, indicates that the bacterial clearance in the NLRX1 deficient bladder is delayed compared to WT". Inclusion of earlier time points will give insights in the kinetics of UPEC burden in the WT bladder, which has as indicated already been done previously. However, adding an earlier time point retrospectively in the current study to analyze CFU kinetics is not valid since there are always intra-experimental variations in the alive inoculum size that can only be verified in retrospect (a day after the inoculum is given to the animals). This would imply that we have to repeat all the *in vivo* experiments which is for ethical reasons not acceptable by our institute.

2. Mulvey MA, Schilling JD, Hultgren SJ. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. *Infect. Immun.* 2001;69(7):4572–4579.

4. Although statistically significant, IL-6 changes are modest and are observed at the

later stages of the acute infection. There are multiple studies that have quantified IL-6 and correlated the effect of changes in IL-6 on the infection outcome. The results should be presented in context with the published literature.

Reply:

As can be seen from figure 2C and D and figure 4C there is no statistical significant difference in IL-6 between WT and NLRX1 deficient kidney, bladder and whole blood. We however agree that putting our results in the context with the published literature will benefit the discussion and did this in the third discussion paragraph.

5. Investigation for the potential role in myeloid cell function is intriguing. However, as the investigators indicated, there are potentially other functions that may be affected. The ability of the phagocytes to kill UPEC would be an interesting test and would provide mechanistic insight into the role of NLRX1 in this system.

Reply:

We agree with the reviewer that investigating the oxidative burst of myeloid cells in response to UPEC would be interesting. However as our main message is that NLRX1 is not involved in the host defense against E. coli induced pyelonephritis we believe that adding such an experiment is beyond the scope of this paper. As an alternative we mention in the discussion that from our data it is not clear if NLRX1 plays a role in the oxidative burst in myeloid cells during UTI.

6. Please provide power calculations for the immune studies, why were fewer mice included in this portion of the study? Was the burden determined for these mice?

Reply:

Based on the protocol optimized in our institute we observed that *ex vivo* experiments have a lower variation-coefficient than *in vivo* experiments and for ethical reasons we therefore included less mice. In order to increase clarity on this point we now describe the calculation in the material and method section: "To be able to reach a statistical significant effect of NLRX1 deficiency the number of 6 mice per experimental group was assessed with an unpaired t-test based on a variation coefficient of 10%, a minimal relative effect of 30%, a significance of 5% and a power of 80%."

7. The dataset for the FACS analyses were not included (the cytokine data was uploaded twice). Please provide an example of the gating strategy

Reply:

Unfortunately the FACS and Cytokine data were mixed up and we changed this. We provided the FACS gating strategy in Supplemental figure 2.

Minor concerns:

In the methods for the animal experiments "human" should be "humane"

Reply:

We changed this.

Competing Interests: No competing interests were disclosed.

Reviewer Report 30 August 2018

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Sylvia Knapp

Department of Medicine I, Medical University of Vienna, Vienna, Austria

This report documents predominantly negative data by showing that NLRX1 does not seem to substantially alter the innate immune response during murine urinary tract infection. NLRX1 is a member of the Nod-like receptor family, which plays a role in mitochondrial activity and has mainly been implicated in negative regulation of anti-viral and TLR-triggered inflammation.

The report is well written, all data are explained in sufficient detail and presented well. By comparing wild type and *Nlrp1*-deficient mice that were infected with an uropathogenic strain of *E.coli*, the authors detected transient differences in the inflammatory response (chemokine) and bacterial clearance (bladder, early timepoint), without any consequence on later bacterial elimination (i.e. 48h) and progression towards pyelonephritis. Likewise, using whole blood assays (cytokine secretion and phagocytosis), NLRX1 did not confer any alterations in the inflammatory or phagocytic response to LPS or whole bacteria.

The "conclusion sentence" of the article sounds somewhat stronger than the conclusions drawn and discussed in the entire manuscript (including the title): stating that "NLRX1 is important in attenuating the early bacterial burden in the bladder by enhancing the local pro-inflammatory cytokine response" exaggerates the fact that only MIP2 levels were reduced in *Nlrp1* deficient bladders (without any consequence on MPO levels). I suggest rewording this sentence.

Comment to source data: Dataset 3 and 4 are mixed up (therefore I checked "partly").

It seems that these experiments were only performed once, i.e. no replicate experiments. I suggest stating this in the methods, possibly also providing an explanation for this.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Apr 2019

Lotte Kors, Amsterdam UMC location AMC, Amsterdam, The Netherlands

Dear Dr. Knapp,

Thank you for the thoughtful comments. We have addressed the comments and questions raised and have detailed our response below.

The "conclusion sentence" of the article sounds somewhat stronger than the conclusions drawn and discussed in the entire manuscript (including the title): stating that "NLRX1 is important in attenuating the early bacterial burden in the bladder by enhancing the local pro-inflammatory cytokine response" exaggerates the fact that only MIP2 levels were reduced in *Nlrp1* deficient bladders (without any consequence on MPO levels). I suggest rewording this sentence.

Reply:

We agree with the reviewer that the conclusion sentence is somewhat strong compared to the overall message of the article and we therefore reworded this sentence. The conclusion in the first version was based in addition to MIP2 levels on the observation (as discussed in the third paragraph of the discussion) that at 24h the bacterial burden is increased in the NLRX1 KO bladders while the pro-inflammatory cytokine levels remain at WT level.

Comment to source data: Dataset 3 and 4 are mixed up (therefore I checked "partly").

Reply:

We changed the titles of the datasets.

It seems that these experiments were only performed once, i.e. no replicate experiments. I suggest stating this in the methods, possibly also providing an explanation for this.

Reply:

The experiments were performed *in vivo* and *ex vivo*, the data shown is based on biological replicates and one animal is indicated in the figures by *n*. For ethical reasons these experiments were performed once.

Competing Interests: No competing interests were disclosed.

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