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Caihong Wang

Washington University School of Medicine in St. Louis

Xuejun Yuan

Washington University School of Medicine in St. Louis

Emily Ma

Washington University School of Medicine in St. Louis

Graziella R. Mendonsa

Washington University School of Medicine in St. Louis

Theo R. Plantinga

Radboud University

See next page for additional authors

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Authors

Caihong Wang, Xuejun Yuan, Emily Ma, Graziella R. Mendonsa, Theo R. Plantinga, Lambertus A. Kiemeney, Sita H. Vermeulen, and Indira U. Mysorekar

NOD2 is dispensable for ATG16L1 deficiency-mediated resistance to urinary tract infection

Caihong Wang,¹ Xuejun Yuan,¹ Emily Ma,¹ Graziella R Mendonsa,¹ Theo S Plantinga,² Lambertus A Kiemeny,³ Sita H Vermeulen,⁴ and Indira U Mysorekar^{1,5,*}

¹Department of Obstetrics and Gynecology; Washington University School of Medicine; St Louis, MO USA; ²Department of Medicine; Radboud University Nijmegen Medical Centre; Nijmegen, Netherlands; ³Department for Health Evidence/Department of Urology; Radboud University Nijmegen Medical Centre; Nijmegen, Netherlands; ⁴Department for Health Evidence/Department of Human Genetics; Radboud University Nijmegen Medical Centre; Nijmegen, Netherlands; ⁵Department of Pathology and Immunology; Washington University School of Medicine; St Louis, MO USA

Keywords: uropathogenic *E. coli*, NLR, bladder, kidney, pyelonephritis, Crohn disease, L1007finsC, R702W, G908R

Abbreviations: *Atg16l1*^{HM} mice, *Atg16l1* hypomorphic mice; CD, Crohn disease; CFU, colony-forming units; H&E, hematoxylin and eosin; hpi, hours post inoculation; dpi, days post inoculation; IBC, intracellular bacterial community; LAMP1, lysosomal-associated membrane protein 1; MVB, multivesicular bodies; NOD2, nucleotide-binding oligomerization domain containing 2; QIRs, quiescent intracellular reservoirs; TEM, transmission electron microscopy; UPK3, uroplakin 3; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infections

NOD2 (nucleotide-binding oligomerization domain containing 2) functions as a pathogen sensor and is involved in development of Crohn disease, a form of inflammatory bowel disease. NOD2 functions in concert with the autophagy protein ATG16L1, which is also implicated in Crohn disease. Recently, we identified a novel protective role of ATG16L1 deficiency in uropathogenic *Escherichia coli*-induced urinary tract infections (UTIs), which are common infectious diseases in humans. Given the known roles of NOD2 in recruiting ATG16L1 to the bacterial entry site, autophagy induction, and Crohn disease, we hypothesized that NOD2 may also play an important role in UTI pathogenesis. Instead, we found evidence that NOD2 is dispensable in the pathogenesis of UTIs in mice and humans. First, loss of *Nod2* did not affect the clearance of bacteriuria and the recruitment of innate immune cells to the bladder. Second, we showed that, although *nod2*^{-/-} mice display increased kidney abscesses in the upper urinary tract, there were no increased bacterial loads or persistence in this niche. Third, although a previous study indicates that loss of *Nod2* reverses the protection from intestinal infection afforded by loss of ATG16L1 in mice, we found NOD2 deficiency did not reverse the ATG16L1-deficiency-induced protection from UTI. Finally, a population-based study of a cohort of 1819 patients did not reveal any association of *NOD2* polymorphisms with UTI incidence. Together, our data indicated that NOD2 is dispensable for UTI pathogenesis in both mice and humans and does not contribute to ATG16L1-deficiency-induced resistance to UTI in mice.

Introduction

NOD2, a member of the Nod-like receptor family of leucine-rich repeat proteins, is expressed in epithelial and immune cells, where it functions as an innate pathogen sensor with specificity for bacterial muramyl dipeptide.^{1–3} The fact that NOD2 plays an important role in host inflammatory immune responses was highlighted by the finding that several *NOD2* polymorphisms are associated with Crohn disease (CD),^{4–7} a type of inflammatory bowel disease. Three polymorphisms (L1007finsC, R702W, and G908R) in *NOD2* that are associated with CD have been shown to impair cellular responses to bacterial peptidoglycans.^{5,6,8} NOD2 interacts with and recruits the autophagy protein ATG16L1/autophagy-related 16-like 1 to sites of bacterial entry at the plasma membrane in cultured human epithelial cells.⁹ Notably, the most common *NOD2* polymorphism

associated with CD results in impaired recruitment of ATG16L1 to the bacterial entry site and much less bacterial autophagy,^{9,10} suggesting an epistatic relationship between NOD2 and the autophagy pathway.^{11,12} Other evidence for a connection between NOD2 and ATG16L1 comes from recent data demonstrating that whereas mice deficient for ATG16L1 (*Atg16l1*^{HM}; *Atg16l1* hypomorphic mice) are protected from infection with a common intestinal pathogen, *Citrobacter rodentium*, this protection is reversed in mice that also lack *Nod2*.¹³

This unexpected role for ATG16L1 in infection—loss of the gene-conferring protection rather than increased susceptibility—was first reported by our group in the case of urinary tract infections (UTIs),¹⁴ one of the most common infectious diseases in humans. UTIs are primarily caused by uropathogenic *Escherichia coli* (UPEC), and their progression has been well described in a murine model.¹⁵ UPEC invasion into the bladder

*Correspondence to: Indira U Mysorekar; Email: indira@wustl.edu
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mucosal lining results in formation of intracellular bacterial communities (IBCs) within the epithelial cells. The host defense response includes exfoliation of these cells into the bladder lumen (where they are noted in the urine of infected mice and humans¹⁶) to reduce bacterial load, an increase in expression of proinflammatory cytokines, and influx of innate immune cells. We observed that mice deficient for ATG16L1 cleared their bacterial load more rapidly than their wild-type counterparts and displayed faster epithelial regeneration and an enhanced innate immune response.

As with *NOD2*, T300A polymorphism in *ATG16L1* is linked to CD.¹⁷⁻²⁰ Studies in cultured human epithelial cells suggest that the *ATG16L1*^{T300A} allelic variant leads to a defect in xenophagy, or antibacterial defense.²¹⁻²⁴ Given the interplay of *NOD2* and *ATG16L1* in linking bacterial sensing and induction of autophagy and their significant roles in both CD pathogenesis and the response to an intestinal pathogen, we hypothesized that *NOD2* functions in regulating the UPEC pathogenic cycle in UTIs. Instead, we show here that *NOD2* is dispensable for the murine host response to UTI in the bladder. Furthermore, loss of *NOD2* does not reverse the *ATG16L1*-deficiency-induced protection from UTI. Finally, we observed no association between *NOD2* polymorphisms and UTI incidence in humans. Our data indicated that, contrary to the situation in the intestine, *NOD2* and *ATG16L1* play distinct roles in disease development in the urinary tract mucosa.

Results

***NOD2* is dispensable for the clearance of bacteriuria in the bladder mucosa**

To determine whether the absence of *Nod2* affects the UPEC pathogenic cycle in a similar manner as *ATG16L1* deficiency, we induced UTI in adult female *nod2*^{-/-} and wild-type mice by transurethrally inoculating them with 10⁷ colony-forming units (CFU) of a clinical cystitis UPEC isolate, UTI89.^{25,26} The progress of UPEC-induced UTI was monitored by measuring shedding of bacteria into the urine (i.e., bacteriuria). We found that *nod2*^{-/-} mice exhibited a similar urine CFU profile to control mice during the whole time course (Fig. 1A).

We previously showed that *ATG16L1*-deficiency-induced fast clearance of UTI was associated with increased recruitment of monocytes to infected bladders and a potent proinflammatory response.¹⁴ To examine whether *Nod2* deficiency alters the recruitment of innate immune populations upon infection, we examined urine of *nod2*^{-/-} mice before and 6 h post inoculation (hpi) and found that urine from infected *nod2*^{-/-} mice contained similar numbers of neutrophils (Fig. 1B) and monocytes (Fig. 1C) as infected control mice, though we noted a trend toward reduced influx.

Upon infection and during the acute stage of infection (0 to 72 h), UPEC invade the superficial uroepithelial cells where they replicate rapidly and establish IBCs.²⁷ A subset of UPEC survives and establishes long-term reservoirs within the urothelium. These quiescent intracellular reservoirs (QIRs) are enclosed within vesicular compartments decorated with late endosomal/

lysosomal markers such as LAMP1 (lysosomal-associated membrane protein 1); UPEC within QIRs can re-emerge to seed recurrent UTIs.²⁵ To examine whether *Nod2* deficiency affects colonization of the bladder and the establishment of IBCs and QIRs, we quantified the number of IBCs in the bladder at 6 hpi. We observed that bladders from *nod2*^{-/-} mice had similar numbers of IBCs as those from wild-type mice (Fig. 1D). We next examined control and *nod2*^{-/-} bladders at 14 d post inoculation (dpi) to determine whether absence of *Nod2* affects the establishment of QIRs. We found that bladders of *nod2*^{-/-} mice harbored slightly fewer LAMP1-positive QIRs than bladders of control mice, but the difference was not statistically significant (Fig. 1E).

Previous work has shown that bacterial QIRs occupy autophagosomal compartments²⁸ and that *ATG16L1* deficiency results in reduced QIR formation and significant alterations in the urothelial cell architecture.¹⁴ In contrast, transmission electron microscopy (TEM) analysis revealed only subtle differences in ultrastructural architecture between bladders of wild-type and *nod2*^{-/-} mice (Fig. 1F–H).

Urothelial tissue regeneration is an important step in the resolution of UTIs.²⁶ To determine whether or not *NOD2* plays a role in this process, we analyzed bladder tissue sections from control and *nod2*^{-/-} mice at 14 dpi. Hematoxylin and eosin (H&E) staining revealed that the bladders of both control and *nod2*^{-/-} mice displayed newly regenerated, terminally differentiated, nonproliferating superficial cells and resolution of inflammation (Fig. 1I). Immunolocalization of the luminal surface protein UPK3 (urolipin 3) demonstrated that these bladders also displayed regenerated superficial cells (Fig. 1J). Together, our data show that *NOD2* is dispensable for the clearance of bacteriuria and does not affect UPEC's ability to establish an acute infection or persist in the bladder.

Kidneys of UPEC-infected *Nod2*-deficient mice have high inflammatory scores and increased incidence of abscesses

After bacterial colonization of the bladder, UTIs can at times ascend to the kidneys, although in C57BL/6 mice this is relatively uncommon. We found that a large percentage of kidneys from *nod2*^{-/-} mice than from wild-type mice had abscesses at 14 dpi (Fig. 2A), a time when the bladders have cleared infection. Furthermore, the inflammation scores were significantly higher in kidneys of *nod2*^{-/-} mice than in those from controls (Fig. 2B). H&E staining of kidneys from *nod2*^{-/-} mice revealed abscessed areas with neutrophil influx (Fig. 2C and D). Immunofluorescence analysis revealed collections of bacteria tightly packed between tubular epithelial cells of the kidney (Fig. 2E and F) in multiple kidneys. Large extracellular bacterial biofilm-like communities were observed filling renal tubules in *nod2*^{-/-} but not in control mice (Fig. 2G and H). Given these data, we next determined whether *Nod2* deficiency was associated with increased bacterial titers and bacterial persistence in the kidneys. However, we found no significant difference between control and *nod2*^{-/-} mice in bacterial loads in the kidney tissue at 14 dpi (Fig. 2I).

***Nod2* deficiency does not reverse the *ATG16L1*-deficiency-induced protection from UTI**

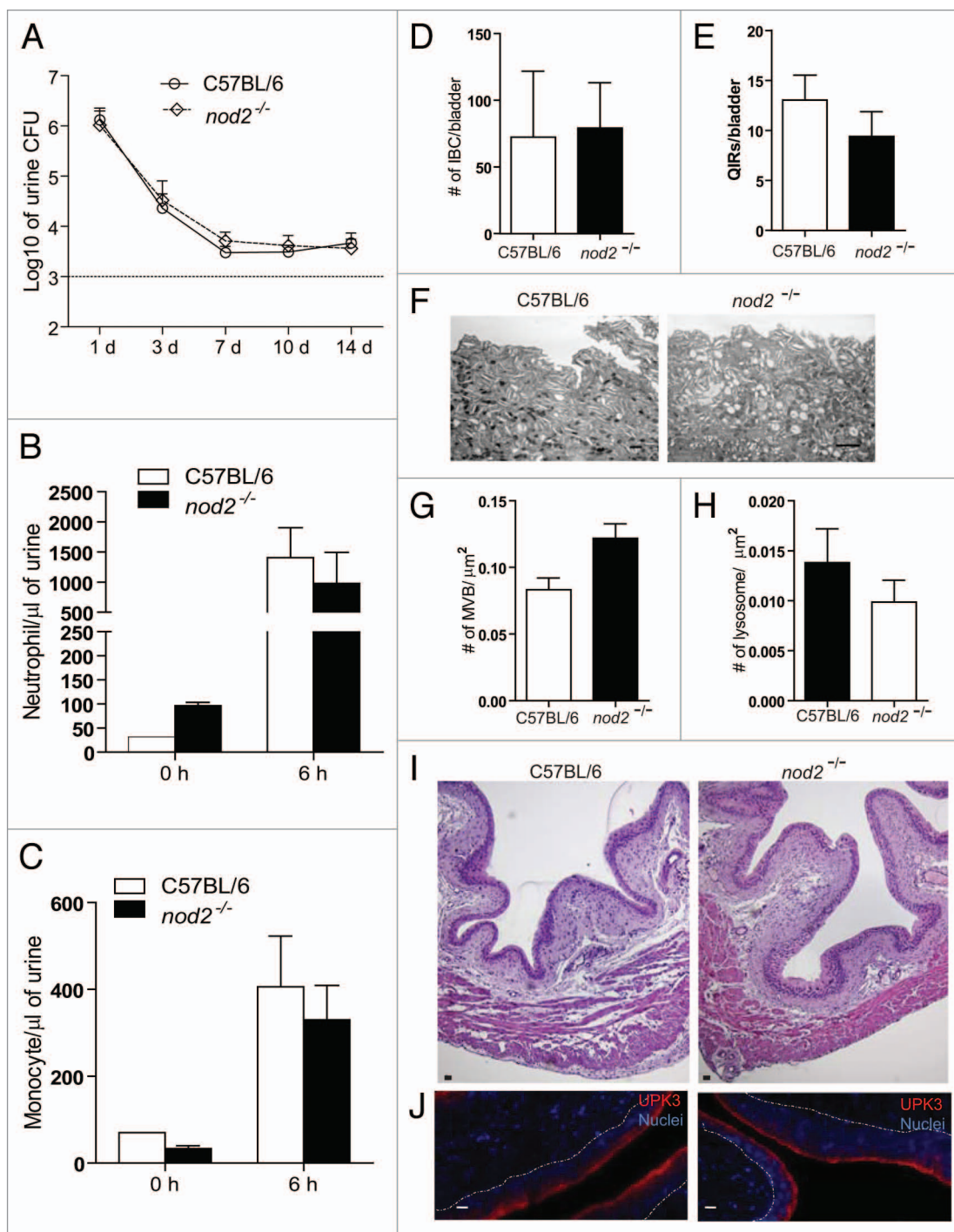


Figure 1. Loss of *Nod2* does not affect the course of UTI. (A) CFU counts of bacteriuria plotted as mean \pm SEM of the Log10 value at 1–14 dpi with UT189. * P < 0.05 by 2-way ANOVA with Bonferroni post-test. n = 5 to 15 mice/timepoint/genotype; n = 5 experiments. (B) Neutrophil counts in the urine. Bars represent mean \pm SEM; n = 4 or 5 mice/time point; n = 2 experiments. (C) Monocyte counts in the urine. Bars represent mean \pm SEM; n = 4 to 5 mice/time point; n = 2 experiments. (D) Quantification of IBCs in bladders from control vs. *nod2*^{-/-} mice at 6 hpi. (E) Quantification of QIRs in bladders from control vs. *nod2*^{-/-} mice at 14 dpi. n = 8 sections/bladder; n = 32 and 20 bladders from C57BL/6 and *nod2*^{-/-} mice, respectively. (F) TEM of control and *nod2*^{-/-} superficial cell ultrastructure. Panels representative of 10- to 15-sq μm regions were examined in n = 3 mice. Scale bar: 1 μm. (G and H) Quantification of MVBs (G) and lysosomes (H) in TEM images. Bars represent mean \pm SEM (I) H&E stained bladders from control and *nod2*^{-/-} mice at 14 dpi. Scale bar: 10 μm. (J) IF imaging analysis of control and *nod2*^{-/-} urothelium stained with an antibody to UPK3 (red), and biz-benzimide to highlight nuclei (blue). Scale bar: 10 μm.

A recent study demonstrates that whereas mice deficient for ATG16L1 are protected from infection with a common intestinal pathogen, *Citrobacter rodentium*, this protection is reversed in

mice that also lack *Nod2*.¹³ To explore the possibility that loss of *Nod2* might reverse the ATG16L1-deficiency-induced protection from UTIs, we compared bacteriuria in *Atg16l1*^{HM} and *Atg16l1*^{HM}

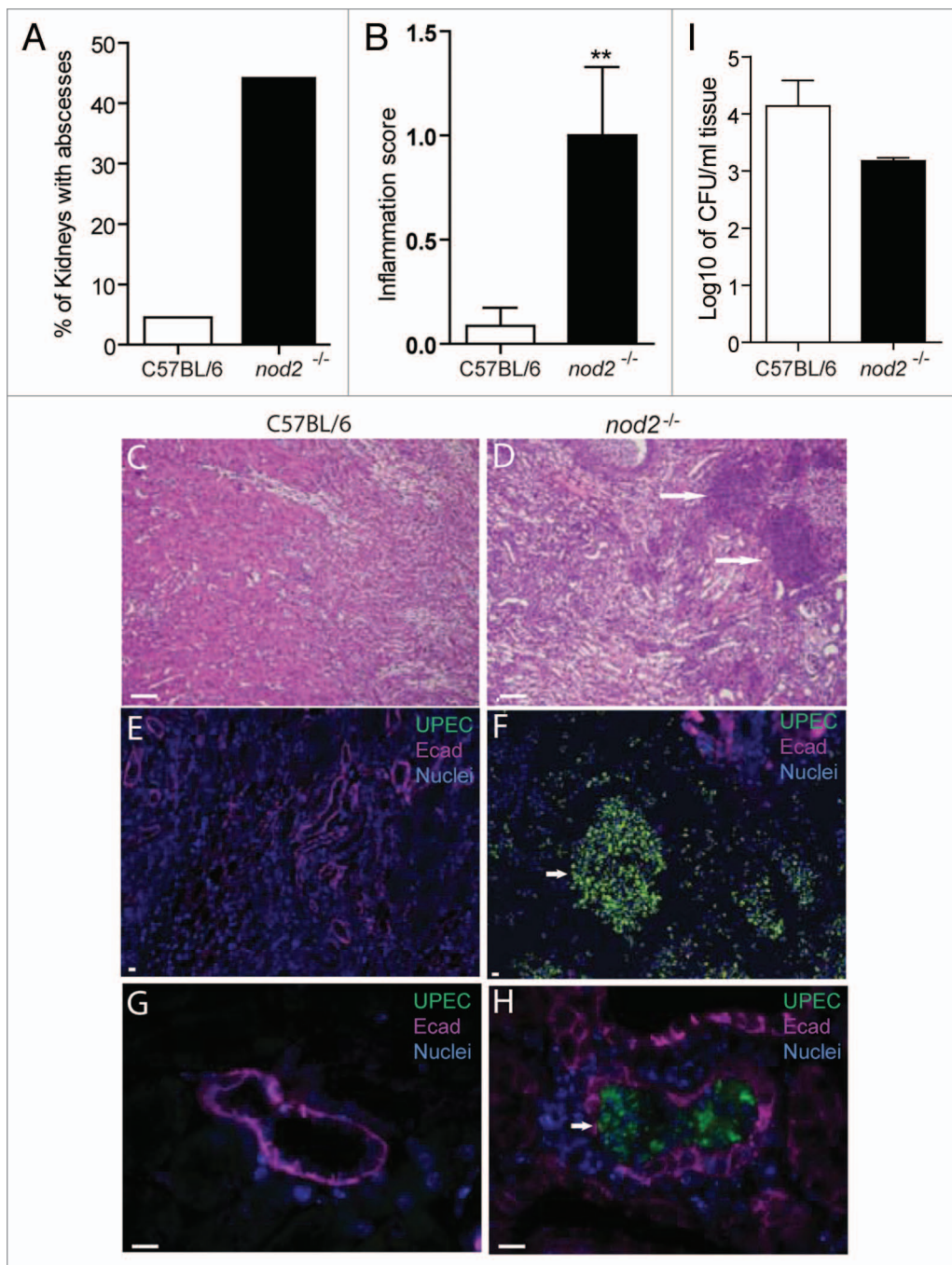


Figure 2. *Nod2* deficiency is associated with kidney abscess formation during UTI. (A) Quantification of the percentage of mice with kidney abscesses at 14 dpi. (B) Inflammation scores of kidneys from control and *nod2*^{-/-} mice. *n* = 20 for control; *n* = 14 for *nod2*^{-/-}; ***P* < 0.01. (C and D) H&E staining of kidneys from control (C) and *nod2*^{-/-} (D) mice. Arrow indicates a region of neutrophil influx. Scale bar: 63 μ m. (E–H) Immunofluorescence staining of UPEC (green), E-cadherin (cyan), and nuclei (blue) in kidneys of control (E and G) and *nod2*^{-/-} (F and H) mice. Arrow indicates clumps of bacteria and neutrophils inside renal tubules. (I) CFU counts of bacterial load in the kidney plotted as mean \pm SEM of the Log10 value at 14 dpi, *n* = 4 to 9 mice/group, *n* = 3 experiments.

nod2^{-/-} double-mutant mice. We observed that the double-mutant mice were protected to the same extent as the *Atg16l1*^{HM} mice and that loss of *Nod2* did not abrogate the ATG16L1-deficiency-induced protection (Fig. 3).¹⁴

***NOD2* polymorphisms are not associated with the incidence of UTI**

It has been shown that *NOD2* polymorphisms are associated with susceptibility to CD.^{5,6,29} To examine whether there is an association between *NOD2* polymorphisms and the incidence of UTI, we performed an association study of *NOD2* polymorphisms in a large

Dutch population cohort. Data on 3 *NOD2* polymorphisms, rs2066844 (R702W), rs2066845 (G908R), and rs2076756, and questionnaire data on UTIs, including those treated with antibiotics, were extracted for 1,819 human subjects. We found that these *NOD2* variants show no association with UTI incidence (Fig. 4; Table 1).

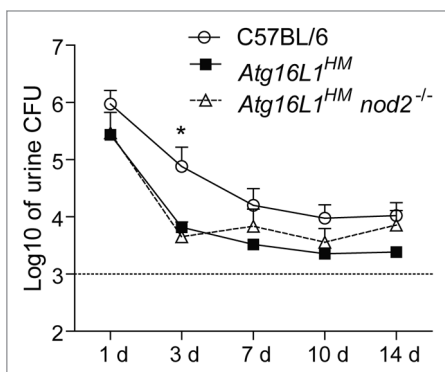


Figure 3. *Nod2* deficiency does not reverse the ATG16L1-deficiency-induced protection from UTI. CFU counts of bacteriuria plotted as mean \pm SEM of the Log10 value at 1–14 dpi with UTI89. **P* < 0.05 by 2-way ANOVA with Bonferroni post-test. *n* = 5 to 15 mice/timepoint/genotype; *n* = 5 experiments.

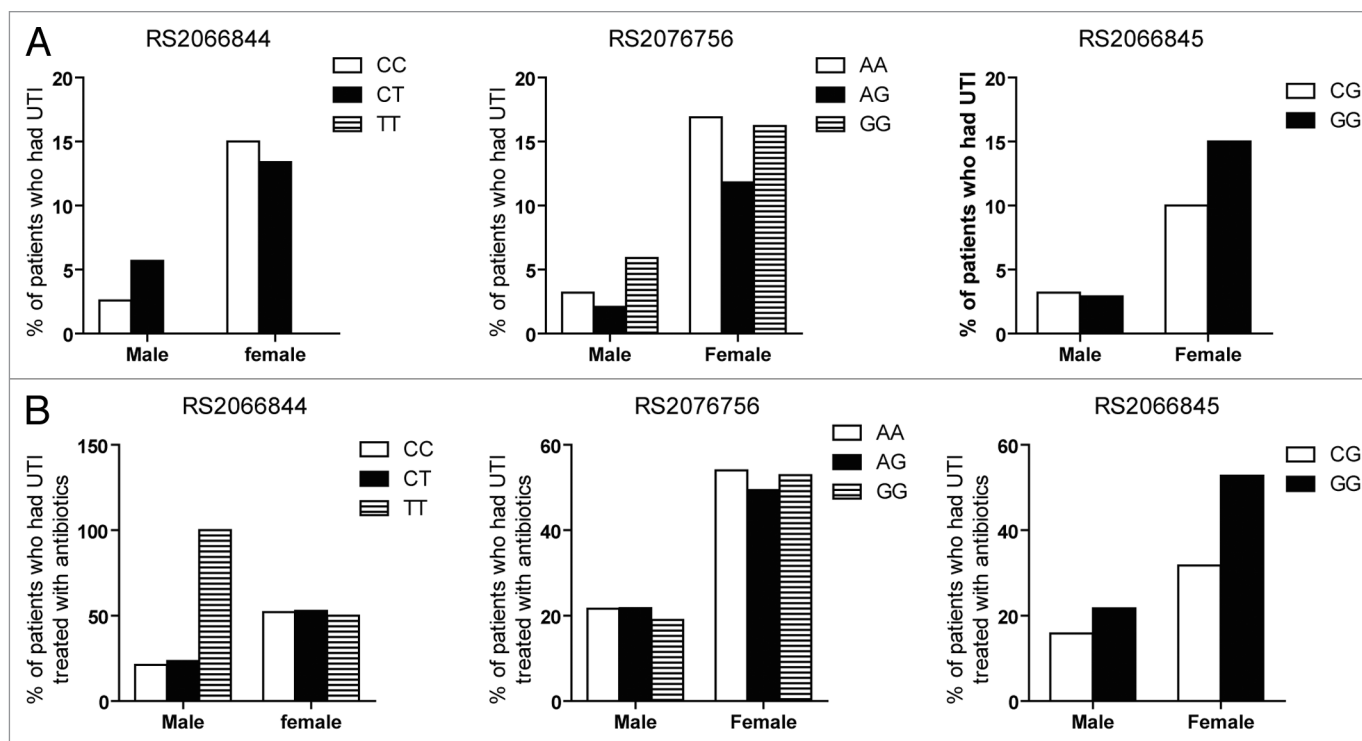


Figure 4. *NOD2* polymorphisms are not associated with the incidence of UTI. (A) Percentage of patients with regular bladder infections among *NOD2* SNP variant groups. (B) Percentage of patients with UTIs treated with antibiotics among *NOD2* SNP variant groups. Person Chi-Square test and Fisher Exact Test indicated that none of the differences were statistically significant.

Discussion

Here, we used a murine model to demonstrate that *NOD2* is dispensable for the host response to UPEC-induced infections in the bladder and does not affect recruitment of myeloid cells to combat infection. Furthermore, although our data suggest that *NOD2* may be required for prevention of ascending infection into the kidney, overall, loss of *Nod2* did not affect bacterial load and persistence in the kidneys. Thus, *NOD2* is dispensable for both lower and upper urinary tract infection. This is in contrast to the study by Kim et al.,³⁰ which shows that *nod2*^{-/-} mice are more vulnerable than wild-type mice to oral infection with *Citrobacter rodentium* because of impaired recruitment of monocytes to the intestine. Kobayashi et al.³¹ also show that *nod2*^{-/-} mice challenged orally with *Listeria monocytogenes* are susceptible to infection; however, no significant difference in survival between *nod2*^{-/-} and wild-type mice was noted upon systemic challenge, suggesting a tissue-specific effect. Similarly, Geddes et al.³² demonstrate that *NOD2* can modulate inflammation and mediate efficient clearance of bacteria from mucosal tissue during *Salmonella typhimurium*-induced colitis.

Other studies suggest that *NOD2* is not essential for host defense to pathogens in general. For example, studies from Jeong et al.³³ show that *NOD2* is not critical for initiating the innate immune response to *Yersinia enterocolitica* infection as bacterial clearance and serum cytokine production levels upon systemic infection are indistinguishable between *nod2*^{-/-} and control mice. However, Meinzer et al. show that *NOD2* contributes to

Table 1. Absolute number (% within genotype group) of subjects with regular bladder infections/UTI treated with antibiotics among *NOD2* variant groups.

Variant genotype	Regular bladder infection		UTI treated with antibiotics	
	Male	Female	Male	Female
rs2066844 CC	20 (2.6)	124 (15.0)	115 (21.2)	304 (52.0)
rs2066844 CT	6 (5.7)	11 (13.4)	15 (23.4)	28 (52.8)
rs2066844 TT	0 (0)	0 (0)	1 (100.0)	1 (50.0)
rs2076756 AA	14 (3.2)	82 (16.9)	66 (21.6)	183 (54.0)
rs2076756 AG	8 (2.1)	42 (11.8)	56 (21.8)	123 (49.4)
rs2076756 GG	4 (5.9)	11 (16.2)	8 (19.0)	27 (52.9)
rs2066845 CC	-	-	-	-
rs2066845 CG	1 (3.2)	3 (10.0)	3 (15.8)	7 (31.8)
rs2066845 GG	25 (2.9)	132 (15.0)	128 (21.7)	326 (52.8)

Data provided for males and females separately. All p for Pearson chi-square or Fishers exact test > 0.05.

the susceptibility to *Y. pseudotuberculosis* in mice³⁴ and that *Y. pseudotuberculosis* subverts *NOD2* signaling to promote dissemination.³⁵ *NOD2* is also dispensable for the control of *Brucella abortus* during systemic in vivo infection.³⁶ Thus, *NOD2* mainly plays key roles in intestinal pathogenesis, and this regulation may be pathogen-specific as well as tissue-specific.

Our findings revealed that *NOD2* and *ATG16L1* play different roles in the UPEC pathogenic cycle; whereas

ATG16L1-deficient mice clear UPEC faster than wild-type mice, *nod2*^{-/-} mice were not protected from UTI. Thus, in doubly mutant *Atg16l1*^{HM} *nod2*^{-/-} mice, the fast clearance of bacteriuria is mediated by ATG16L1 deficiency but not the lack of *Nod2*. Our findings suggest that *Nod2* deficiency does not affect invasion and colonization of the bladders, and the absence of *Nod2* does not affect the formation of protected niches containing UPEC during the latent stage of infection. This again is in direct contrast to the striking phenotypes elicited with ATG16L1 deficiency, suggesting that NOD2 and ATG16L1 play divergent roles in the UPEC pathogenic cycle.

Cadwell and colleagues have recently shown that ATG16L1-deficient mice are resistant to *Citrobacter rodentium*-induced infection,¹³ consistent with our observation that ATG16L1 deficiency confers protection from UTI.¹⁴ However, unlike our observation that NOD2 is dispensable for ATG16L1-deficiency-mediated protection from UTI, loss of *Nod2* abrogates the protective effect of ATG16L1 deficiency in *Citrobacter rodentium* infection.¹³ Thus, the outcome of the interaction between NOD2 and ATG16L1 may also be tissue- or pathogen- specific. A recent study using an influenza virus infection model suggests that NOD2-RIPK2-signaling limits immunopathology through autophagy-mediated removal of damaged mitochondria. This study indicates that the intersection between these pathways can be immuno-suppressive rather than directly antibacterial.³⁷ NOD2 signaling is involved in other important modulatory functions such as secreting antimicrobial peptides and activating adaptive immunity.^{31,38,39}

There is a large body of evidence suggesting interactions between NOD2 and ATG16L1 in host defense against intestinal infection in cultured cells, mouse models, and human association studies. Plantinga et al. show that the *ATG16L1* polymorphism modulates proinflammatory cytokine response selectively upon activation of NOD2.¹¹ However, our study highlights that this is not a universal paradigm as *NOD2* polymorphisms were not associated with a common infection of the urinary tract mucosa. Thus, mucosa-specific studies of *NOD2* and *ATG16L1* polymorphisms associated with inflammatory disease are warranted to elucidate the complex factors, including genetic differences, which are required in different disease models and in different mucosal niches.

Materials and Methods

Mice

All animal protocols were approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance, A-3381-01). Mice were maintained under specified pathogen-free conditions in a barrier facility and under a strict 12 h light/dark cycle. *Atg16l1*^{HM} mice were generated as described previously.⁴⁰ *Atg16l1*^{HM} *nod2*^{-/-} mice were generated by crossing *nod2*^{-/-} mice³¹ to *Atg16l1*^{HM} mice. C57BL/6 mice were used as the wild-type strain.

Bacterial strains, mouse inoculations, and urinalysis

Adult female mice (8 to 10 wk old) were anesthetized and inoculated via transurethral catheterization with 10⁷ CFU of

UTI89 in phosphate-buffered saline. Urinalysis and bacterial titering of urine and tissues were performed as described previously.¹⁵

Histochemical and immunofluorescence analyses

Bladders were processed as described previously.²⁶ The following primary antibodies were used: 1) rabbit polyclonal antibody to *E. coli* (United States Biological, E3500-20), 2) mouse monoclonal antibody to CDH1 [cadherin 1, type 1, E-cadherin (epithelial)] (BD Bioscience, 610182), 3) rat monoclonal antibody to LAMP1 (clone ID4B; Developmental Studies Hybridoma Bank), and 4) antibody to UPK3 (Fitzgerald, 10R-U103a). Antigen-antibody complexes were detected with Alexa Fluor 488, 594, and 647-conjugated secondary antibodies (Invitrogen, A21206, A-11007, A20990). Images were obtained with a Zeiss Apotome microscope (Zeiss) at 10 to 20× and 40 to 63× magnifications.

QIR quantification

Eight separate 5-μm serial sections over a thickness of 300 μm were immunostained with antibodies against *E. coli*, LAMP1, and E-cadherin and imaged at 63× on a Zeiss Apotome microscope (Zeiss). The total number of LAMP1-positive UPEC reservoirs per bladder was counted.

Transmission electron microscopy

The whole bladder was processed as described previously.¹⁴ The numbers of lysosomes and multivesicular bodies (MVBs) were determined and normalized to total surface area of the region examined by using JEOL 1200 EX II transmission electron microscope (JEOL USA, Inc) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques).

Neutrophil and monocyte counts in urine

Urine was collected before and after infection. The diagnostic lab in the Department of Comparative Medicine, WUSM used a HEMAVET® 950, Veterinary 5 part WBC Hematology System (Americas Drew Scientific Inc) to determine neutrophil and monocyte counts.

NOD2 polymorphism analysis

Nijmegen Biomedical Study (NBS) (<http://www.nijmegen-biomedischestudie.nl>) is a population-based survey conducted by the Department for Health Evidence and the Department of Clinical Chemistry of the Radboud University Nijmegen Medical Centre, The Netherlands. 21,756 age- and sex-stratified randomly selected inhabitants of the municipality of Nijmegen, the Netherlands, received an invitation to fill out a postal questionnaire on lifestyle and medical history, and to donate a blood sample. For this study, we used the subset of 1,980 participants from the NBS that was selected to serve as controls in genome-wide association studies and were genotyped by using the Illumina HumanHap370CNV-Duo BeadChip. Genotype data were imputed (IMPUTE software version 0.5) by using the CEU HapMap Phase II data as reference.⁴¹ Imputed genotype probabilities for those 1,819 participants that passed quality control were extracted for *NOD2* variants rs2066844 (R702W) and rs2066845 (G908R) and transformed into hard calls using a genotype probability threshold of ≥ 0.9; measured genotype data was extracted for rs2076756. All *NOD2* variants followed Hardy-Weinberg equilibrium. QN data on i) recurrent UTIs

diagnosed by a physician (available for n = 1790) and ii) UTIs treated with antibiotics (available for n = 1248), were extracted for this study.

Statistical analyses

To assess the significance of a difference between groups, Graph Prism software was used to perform 2-sample, unpaired *t* tests. For time-course studies, the standard error (SE) used in the *t* test was estimated by ANOVA, and 2-sample tests were performed at individual time points. To control for false positives, Bonferroni adjusted *P* values at individual time points are reported. A *P* value of less than 0.05 was considered to be significant. To evaluate the association between overall UTIs or UTIs treated with antibiotics and the *NOD2* variants in the NBS, cross-tabs were generated and Pearson chi-square or Fisher exact tests performed, for men and women separately [IBM SPSS Statistics for Windows 20 (IBM Corp.)].

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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