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Abbreviations: Atg16l1−/− 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 infection

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.1,2 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).3-7 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 (Arg16l1−/−; Arg16l1 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...
mucosal lining results in formation of intracellular bacterial communities (IBC s) 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 humans) 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 ATG16L1T300A 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.24 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 (uroplakin 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
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<sup>−/−</sup>* and *Atg16l1<sup>−<sub>1H1M</sub>−/−</sup>*
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<sup>−/−</sup> mice. n = 20 for control; n = 14 for nod2<sup>−/−</sup>; **P < 0.01. (C and D) H&E staining of kidneys from control (C) and nod2<sup>−/−</sup> (D) mice. Arrow indicates a region of neutrophil influx. Scale bar: 63 m. (E–H) Immunofluorescence staining of UPEC (green), E-cadherin (cyan), and nuclei (blue) in kidneys of control (E and G) and nod2<sup>−/−</sup> (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 ± SEM of the Log10 value at 1 at 14 dpi, n = 4 to 9 mice/group, n = 3 experiments.

We observed that the double-mutant mice were protected to the same extent as the Atg16l1<sup>hm</sup> mice and that loss of Nod2 did not abrogate the ATG16L1-deficiency-induced protection (Fig. 3).<sup>14</sup> NOD2 polymorphisms are not associated with the incidence of UTI. It has been shown that NOD2 polymorphisms are associated with susceptibility to CD.<sup>5,6,29</sup> 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).
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 \textit{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., which shows that \textit{nod2}\textsuperscript{−/−} mice are more vulnerable than wild-type mice to oral infection with \textit{Citrobacter rodentium} because of impaired recruitment of monocytes to the intestine. Kobayashi et al.\textsuperscript{31} also show that \textit{nod2}\textsuperscript{−/−} mice challenged orally with \textit{Listeria monocytogenes} are susceptible to infection; however, no significant difference in survival between \textit{nod2}\textsuperscript{−/−} and wild-type mice was noted upon systemic challenge, suggesting a tissue-specific effect. Similarly, Geddes et al.\textsuperscript{32} demonstrate that NOD2 can modulate inflammation and mediate efficient clearance of bacteria from mucosal tissue during \textit{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.\textsuperscript{33} show that NOD2 is not critical for initiating the innate immune response to \textit{Yersinia enterocolitica} infection as bacterial clearance and serum cytokine production levels upon systemic infection are indistinguishable between \textit{nod2}\textsuperscript{−/−} and control mice. However, Meinzer et al. show that NOD2 contributes to the susceptibility to \textit{Y. pseudotuberculosis} in mice\textsuperscript{34} and that \textit{Y. pseudotuberculosis} subverts NOD2 signaling to promote dissemination.\textsuperscript{35} NOD2 is also dispensable for the control of \textit{Brucella abortus} during systemic in vivo infection.\textsuperscript{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

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

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

Data provided for males and females separately. All \( p \) for Pearson chi-square or Fisher exact test > 0.05.
ATG16L1-deficient mice clear UPEC faster than wild-type mice, nod2−/− mice were not protected from UTI. Thus, in doubly mutant Atg16l1HM 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. Atg16l1HM mice were generated as described previously.40 Atg16l1HM nod2−/− mice were generated by crossing nod2−/− mice12 to Atg16l1HM 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

Histological 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 the 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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