Mechanisms of renal injury and repair: role of stem cells, chemokines and the nodosome
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NOD1 and NOD2 are not involved in the development of renal injury and fibrosis during obstructive nephropathy

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Submitted
Abstract

The role of NOD1 and NOD2 in renal fibrosis was determined using a model for progressive renal disease in wildtype and NOD1/NOD2 double deficient (NOD1/2 DKO) mice. Minor changes in inflammatory response were observed in NOD1/2 DKO mice, while no effect was seen on renal injury or development of fibrosis.
Introduction

NOD1 and NOD2 are members of the intracellular pattern recognition receptor (PRR) family NLR (Nucleotide-binding domain and Leucine-rich repeat containing Receptor). PRRs are important in mediating a rapid response to pathogens via recognition of several highly conserved pathogen associated molecular patterns (PAMP). In addition to PAMPs various (endogenous) danger associated molecular patterns (DAMP) or stress signals have been identified that can initiate sterile inflammation. Upon renal injury DAMPs are released such as biglycan, high-mobility group box 1 (HMGB1), and hyaluronic acid that can signal via Toll-like receptors (TLRs) and NLRs1-4.

NOD1 and NOD2 detect specific substructures from bacterial peptidoglycan (PGN). NOD1 senses Gram⁻ derived PGN containing meso-DAP5;6, while NOD2 senses Gram⁻ and Gram⁺ derived PGN containing MDP7;8. In addition to bacterial structures, Sabbah et al. reported the activation of NOD2 by single-stranded RNA viruses9. Recently, activation of NOD1 and NOD2 by the non-pathogenic derived cell permeable small molecule DMXAA was reported10. As far as we know, no endogenous DAMPs for NOD1 and NOD2 are described. However, based on their structural and functional similarities with other NLR family members and TLRs it could be speculated that NOD1 and NOD2 are also activated by currently unknown endogenous ligands.

NOD1 is widely expressed in many cell types and organs including the tubular epithelial cells (TEC) in the kidney11-14. Although NOD2 expression is believed to be more restricted, it has been described in leukocytes and various epithelial cells including TEC13-17. The expression of NOD1 and NOD2 in the kidney and more specific in TEC, that actively participate in the immune response following kidney injury, suggests that these PRRs may participate in the inflammatory response and pathogenesis during renal damage. Several other PRRs like NLRP3, TLR2, and TLR4 have already been shown to play a role in obstructive nephropathy2;18;19. However, nothing is known about the role of NOD1 and NOD2 in inflammation and fibrosis during obstructive nephropathy. In the present study we therefore investigated the role of NOD1 and NOD2 in a model of permanent unilateral ureter obstruction.

Methods

Mice
Pathogen-free 8- to 12-week old C57Bl/6 (WT) mice were purchased from Janvier (Le Genest, France). NOD1/NOD2 double knockout (NOD1/2 DKO) mice were generated from NOD1 and NOD2 knockout mice and backcrossed to C57Bl/6 background at least 10 generations as described before20. NOD1/2 DKO mice were bred in the animal facility of the Academic Medical Center in Amsterdam, The Netherlands. Age- and sex-matched mice were used in all experiments. The animal and Use Committee of the University of Amsterdam approved all experiments.
Unilateral ureter obstruction
Female mice received a pre-operative dose of analgesia (0.15 mg/kg buprenorfine, subcutaneously) and were anesthetized by inhalation of 3% isoflurane, 0.2% N₂O and 2% O₂ during the whole surgical procedure. The right ureter was permanently ligated using 6-0 silk (Tyco, Gosport, UK). Mice were sacrificed 3, 7 and 14 days after surgery. Kidneys were snap frozen in liquid nitrogen and stored at -80°C or fixed in 10% formaline o/n prior to further processing. Contralateral non-obstructed kidneys served as control.

Quantitative real-time RT-PCR
Total RNA was extracted from kidney using the TRIzol® reagent (Invitrogen, Breda, The Netherlands) and converted to cDNA. Quantitative real-time RT-PCR was performed on a LightCycler® 480 System (Roche, Mijdrecht, The Netherlands) using LightCycler® 480 SYBR Green I Master mix (Roche). Specific gene expression was normalized towards the reference gene TATA box binding protein (TBP). Primer sequences are as follows: NOD1 forward 5’-tcagactcagcgtcaaccag-3’ and reverse 5’-taaacccaggaacgtcacga-3’, NOD2 forward 5’-gggagatgttgagtggaac-3’ and reverse 5’-agcgaagagcacactcaacc-3’, and TBP forward 5’-ggagaatcatggaccagaaca-3’ and reverse 5’-gatgggaattccaggagtca-3’.

Histology and immunohistochemistry
Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-µm thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid-Schiff reagents after diastase digestion (PasD). Injury to tubules was assessed by determining the percentage of affected tubules per 10 fields (magnification x400) semi-quantitatively on a scale from 0 to 4 (0 = 0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, and 4 = >75%) according to the following criteria: tubular dilatation, epithelial simplification, and interstitial expansion in the cortex.

For immunohistochemistry, sections were stained with FITC-labelled anti-mouse Ly-6G (Pharmingen, BD Biosciences, Alphen a/d Rijn, The Netherlands), rat anti-mouse F4/80 (Serotec, Oxford, UK), rabbit anti-mouse active caspase-3 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-human Ki67 (Neomarkers, Fremont, CA, USA), rabbit polyclonal to collagen type I (GeneTex, Irvine, CA, USA), or mouse anti-human αSMA (DAKO, Heverlee, Belgium) to detect granulocytes, macrophages, apoptosis, proliferation, collagen type I, and myofibroblasts respectively. The number of Ly6 and F4/80 positive cells and the number of caspase-3 and Ki67 positive TEC was counted in 10 non-overlapping fields (magnification x400). The percentage of positive staining for F4/80, collagen type I, and αSMA in obstructed kidneys was analyzed using a computer-assisted digital analysis program (Image Pro-plus®, Media Cybernetics). At least 15 visual fields were sampled from the cortex of each kidney (magnification x20).
Statistical analyses

All statistical analyses were performed using GraphPad Prism 5 software (San Diego, CA, USA). Data were analysed using the non-parametric Mann-Whitney U-test. Results are expressed as mean ± standard error of the mean (SEM). \( P<0.05 \) was considered statistically significant.

Results

The role of NOD1 and NOD2 in chronic renal disease was investigated using the mouse model unilateral ureter obstruction (UUO). First we analyzed the expression of NOD1 and NOD2 mRNA in WT kidney at several time points after UUO. We found a 10-fold higher expression of NOD1 compared with NOD2 in the kidney (figure 1).

![Figure 1](image)

**Figure 1.** Expression of NOD1 (a) and NOD2 (b) in the contralateral (ctr) kidney and in kidneys 3, 7, and 14 days following obstruction. Gene expression is normalized towards the reference gene TBP. Data are expressed as mean ± sem.

Both genes were not altered during the development of obstructive nephropathy. Tubular injury, as assessed by scoring PasD-stained kidney sections, increased markedly after UUO with a similar degree of damage in WT and NOD1/2 DKO at all time points examined (figure 2a). Tubulointerstitial injury in obstructed kidneys can result in an imbalance between TEC apoptosis and proliferation. Apoptosis and proliferation of TEC was increased at all investigated time points after obstruction (figure 2b-c). However, no difference between WT and NOD1/2 DKO mice was observed. Fibrosis was determined by collagen type I deposition. In both WT and NOD1/2 DKO obstructed kidneys fibrosis increased progressively, however no difference between the mouse strains was observed (figure 2d). Next we analyzed the amount of myofibroblast by \( \alpha \)SMA immunohistochemistry. In line with tubular injury and fibrosis, the amount of myofibroblasts increased after UUO. Myofibroblast accumulation was delayed in NOD1/2 DKO mice as less \( \alpha \)SMA positivity was found 3 days following obstruction compared to WT mice, while at 7 and 14 days \( \alpha \)SMA staining was comparable between both mouse strains (figure 2e).
Figure 2. (a) Tubular injury in WT (white bars) and NOD1/2 DKO (black bars) mice 3, 7, and 14 days after obstruction. Both mouse strains had similar levels of injury at all examined time points. There were also no differences observed in amount of apoptotic (b) and proliferating (c) TEC following obstruction between both mouse strains. Due to severe tubular atrophy it is impossible to identify TEC after 14 days, therefore at this time point the total amount of apoptotic or proliferating cells (both interstitial and tubular cells) is analyzed. (d) Collagen type I deposition in kidneys increased similar in WT (white bars) and NOD1/2 DKO (black bars) mice following obstruction. (e) Myofibroblast accumulation in kidneys, assessed by αSMA immunostaining, increased following obstruction. Three days following obstruction NOD1/2 DKO (black bars) mice had significant less renal myofibroblasts compared to WT (white bars) mice. Data are expressed as mean ± sem. *P<0.05 compared with WT.
One of the early events in progressive renal injury is the induction of chemokines and the subsequent recruitment of inflammatory cells. The granulocyte chemoattractant KC (figure 3a) and the monocyte chemoattractant MCP-1 (figure 3b) increased significantly following obstruction in both WT and NOD1/2 DKO kidneys. Except for a slight but significant higher MCP-1 level in kidneys from NOD1/2 DKO mice compared with WT 7 days following obstruction, no difference in KC and MCP-1 levels were observed between both mouse strains. The influx of granulocytes (figure 3c) and accumulation of macrophages (figure 3d) increased in the obstructed WT and NOD1/2 DKO kidneys, yet there were no differences in these parameters between both mouse strains 3 and 14 days post UUO. Seven days following obstruction there was a slight but significant decrease in granulocyte influx and a significant increase in macrophage accumulation in NOD1/2 DKO kidneys compared with WT kidneys. Together these results reveal that there are no or minor changes in the inflammatory response following renal obstruction in NOD1/2 DKO mice compared with WT mice.

Figure 3. Renal KC (a) and MCP-1 (b) levels were similar between WT (white bars) and NOD1/2 DKO (black bars) mice except for a slight increased renal MCP-1 level 7 days following obstruction in NOD1/2 DKO compared to WT mice. Granulocyte (c) and macrophage (d) influx increased following obstruction in both mouse strains. At day 7 significant less granulocytes and more macrophages were present in NOD1/2 DKO (black bars) obstructed kidneys compared to WT (white bars), while there were no differences at the other examined time points. Data are expressed as mean ± sem. *P<0.05 compared with WT.
Discussion

To investigate the role of NOD1 and NOD2 in chronic renal sterile inflammation and fibrosis we used UUO as a model for progressive renal injury. UUO initiates a sequence of events in the obstructed kidney, including interstitial inflammatory cell accumulation and TEC death, ultimately leading to renal fibrosis which is the final common pathway for numerous forms of progressive renal disease. Recently, the role of the PRRs TLR2, TLR4, and NLRP3 in progressive renal injury was investigated. Although TLR2 initiates the inflammatory response during obstructive nephropathy, it does not play a significant role in the development of renal progressive injury and fibrosis. On the other hand, TLR4 attenuates tubular damage and does contribute to renal fibrosis during obstructive nephropathy as demonstrated by decreased collagen deposition in TLR4 deficient mice. Other work implied a central role for NLRP3 in renal inflammation, fibrosis and tubular damage (Pulskens et al. submitted) at different phases of UUO. From our study we can conclude that NOD1 and NOD2 do not play a significant role in the development of fibrosis or the progression of renal disease after UUO-induced injury. No differences were observed between WT and NOD1/2 DKO obstructed kidneys regarding tubular injury, apoptosis, proliferation, and fibrosis. A marginal effect of NOD1/2 deficiency could be detected in the inflammatory response during obstructive nephropathy. Slightly more MCP-1 and concomitant increased macrophage accumulation was observed in the NOD1/2 DKO kidney 7 days following obstruction, while granulocyte influx was lower at this time point. The majority of infiltrating leukocytes into the UUO-damaged kidney are macrophages, which produce cytokines responsible for tubular apoptosis and fibroblast proliferation and activation. However, enhanced macrophage accumulation did not affect the progression of renal fibrosis in NOD1/2 DKO mice. Apparently, different PRR members have unique responses during obstructive nephropathy that lead to a profoundly different outcome of local injury, inflammation and fibrosis. It is additionally conceivable that NOD1 and NOD2 are not or marginally activated by endogenous ligands present in the kidney during obstructive nephropathy and therefore do not display an overt phenotype as compared to WT mice.

Overall, NOD1 and NOD2 do not make a significant contribution to the development of progressive renal injury, and fibrosis during obstructive nephropathy.

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