Tissue-specific roles of the pattern recognition receptors NLRP3, NLRX1 and TLR9 in sterile inflammatory kidney disease
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Renal-associated NLrX1 Suppresses Ischemia Reperfusion-Induced Tubular Cell Death

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Abstract

Ischemia reperfusion injury is an important contributor to acute graft failure after kidney transplantation and causes inflammation via toll-like receptor-dependent pathways. The nucleotide-binding leucine-rich repeat (NLR) family member Nlrx1 is involved in the negative regulation of toll-like receptor-mediated NF-κB activation however its role during ischemia reperfusion injury has not yet been investigated. Here, we studied the effect of Nlrx1 deficiency on renal function and pathology after one and five days of reperfusion. In addition, we assessed the contribution of Nlrx1 on renal- versus bone marrow-derived cells in the pathogenesis of renal ischemia reperfusion injury by creating bone marrow chimeras. We observed that Nlrx1 is expressed by bone marrow-derived cells and renal epithelial cells and is down regulated in the kidney after ischemia reperfusion injury. Nlrx1 deficiency is detrimental in ischemic acute kidney injury and led to increased mortality, tubular necrosis, apoptosis and renal KC, MCP-1 and TNF-α levels. Differences in tubular cell death between mice with or without Nlrx1 could not be explained by differences in leukocyte accumulation. Bone marrow chimeric mice revealed that renal-associated Nlrx1, rather than myeloid Nlrx1, suppressed tubular epithelial necrosis and apoptosis and consequently plasma LDH levels. These data show that, in contrast to other TLR and NLR family members, renal-associated Nlrx1 is crucial in conferring renal protection after ischemia reperfusion injury primarily through its anti-apoptotic and anti-necrotic effects in tubular epithelial cells.
Introduction

Ischemia reperfusion injury (IRI) is an important contributor to acute graft failure after kidney transplantation (1). Following ischemia reperfusion of the kidney intracellular reactive oxygen species (ROS) are generated which can damage renal epithelium (2). Subsequently, necrotic cells release intracellular components among which damage-associated molecular patterns (DAMP) (3). These DAMPs can induce local inflammation and damage during renal IRI through signalling via pattern recognition receptors (PRRs) such as TLR2 (4) and TLR4 (5,6) which results in the release of cytokines and chemokines through NF-κB (7). Released chemokines facilitate neutrophil and macrophage influx which can enhance tubular damage (8,9). In line with TLR2 and TLR4, Nlrp3, a member of the NLR family of PRRs, plays a detrimental role following renal IRI as well (10).

Nlrx1 is a mitochondrial NLR family member whose function remains elusive. It is involved in the negative regulation of cytokine expression after viral infection and LPS challenge (11–15), and controls apoptotic cell death sensitivity in transformed fibroblast cells (14). Upon LPS stimulation, Nlrx1 dissociates from TRAF6 and binds to IKK complex thereby preventing IKKα/IKKβ phosphorylation and subsequent downstream NF-κB activation (13). Contrary to previous findings, it was also shown that Nlrx1 does not inhibit (15) but enhances NF-κB activity through the induction of reactive oxygen species (ROS) (16). Both mechanisms play a role following renal IRI and seem contradictory at first glance but can be unified into one mechanistic overview where first, Nlrx1 is suppressing cytokine signalling to prevent hyper-inflammation when only few pathogens or damaged cells are present. Second, in case of a more severe infection or injurious insult Nlrx1 becomes an inducer of ROS which kills pathogens (17) or induces cell death in damaged cells.

Given the importance of ROS and inflammatory mediators at different phases of renal ischemia and the role of Nlrx1 in these processes, we investigated the effect of Nlrx1 deficiency on renal function and pathology both after one and five days of reperfusion. In addition, we created bone marrow chimeras to differentiate the role of renal- versus bone marrow-associated Nlrx1 on renal pathology following IR.

In this study, we found that Nlrx1 is down regulated in the kidney following renal IR. Nlrx1 deficiency has a detrimental impact on renal IRI as it leads to increased mortality, cytokine expression, plasma LDH levels and tubular necrosis.
and apoptosis. Using bone marrow chimeras, we established that Nlrx1 of renal parenchymal cells, rather than of bone marrow-derived infiltrating immune cells, is responsible for protection against ischemia reperfusion-induced cell death.

**Material & Methods**

**Mice**
The generation of Nlrx1 deficient mice was described previously (15). Male Nlrx1KO and wild-type C57BL/6 (Charles River), age 8-12 weeks, were used to apply bilateral and unilateral ischemia-reperfusion. CD45.1 positive allotype male wild type mice used to create chimeric mice were purchased from Charles River Laboratories (B6.SJL-PtprcaPepcb/BoyCrl strain). Only age- and sex-matched mice were used in all experiments. All procedures were approved by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam.

**Bone marrow transplantation**
Transplantation was carried out as described previously (4). Briefly, tibia, femur and spleen were harvested of wild-type CD45.1 or Nlrx1KO CD45.2 donor mice. Bone marrow was isolated from tibia and femur. Subsequently, a mixture of bone marrow and splenocytes was intravenously injected into lethally irradiated (2 pulses of 4.5 Gy) wild-type CD45.1 or Nlrx1KO CD45.2 recipient mice. Recipient mice received neomycin-containing water 1 week prior to transplantation up to 4 weeks after transplantation. Six weeks after transplantation renal ischemia was applied followed by one day of reperfusion. Blood collected via heart puncture prior to centrifugation was used to assess bone marrow transplantation efficiency. Erythrocytes were lysed and cells were stained using anti-CD45.1 PE (BD Pharmingen) and anti-CD45.2 FITC (BD Pharmingen). Expression was assessed using flow cytometry. Cut-off for a successful transplantation was set at >75% positivity.

**Ischemia Reperfusion**
Renal IR injury was induced as described previously (6). Briefly, both renal pedicles were clamped for 20 minutes or 25 minutes in case of bone marrow-transplanted mice using microaneurysm clamps through a midline abdominal incision under general anaesthesia (1.25 mg/ml midazolam (Actavis), 0.08 mg/
ml fentanyl citrate and 2.5 mg/ml fluanisone (VetaPharma Limited)). After clamp removal, kidneys were inspected for restoration of blood flow. The abdomen was closed in 2 layers and all mice received a subcutaneous injection of 0.1 mg/kg buprenorphin (Temgesic, Schering-Plough). To maintain fluid balance, mice were supplemented with a few drops of sterile 0.9% NaCl intraperitoneal before closing the abdomen. Mice were sacrificed after one or five days after surgery. Mice were sacrificed and blood was collected by heart puncture and plasma was collected by centrifugation. Kidneys were snap-frozen in liquid nitrogen or formalin-fixed followed by paraffin embedding.

**Cytokine Expression**

Snap-frozen kidneys were lysed using Greenberg Lysis Buffer (75 mM NaCl, 7.5 mM Tris, 0.5 mM MgCl$_2$·H$_2$O, 0.5 mM CaCl$_2$, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Sigma Aldrich) and subsequently, homogenates were used to determine cytokine levels for KC, MCP-1, TNF-α and IL-6 according to manufacturers’ instructions (R&D Systems).

**Immunohistochemistry**

To detect granulocytes, formalin-fixed renal sections were digested with 0.25% pepsin in 0.1 M HCl (Sigma Aldrich) followed by incubation with FITC labelled anti-mouse Ly6G (BD Biosciences), rabbit anti-FITC (Dako) and finally horse radish peroxidase-conjugated goat anti-rabbit IgG. To detect macrophages, formalin-fixed renal sections were boiled in 0.01M pH 6.0 citrate buffer subsequently exposed to a rat IgG2b anti-mouse F4/80 (Serotec). Staining was visualized using a power anti-rabbit poly horse radish peroxidase (Dako) and additionally a rabbit anti-rat antibody (Dako). Apoptotic tubuli were visualised through boiling formalin-fixed renal sections in 0.01M pH 6.0 citrate buffer and subsequently exposed to a rabbit anti-mouse cleaved caspase 3 (Asp175) (Cell Signalling). Staining was visualized using a power anti-rabbit poly HRP (Dako). Ly6G-positive cells and caspase-3-positive tubuli were counted in 10 randomly selected non-overlapping high power fields with a 400x magnification. F4/80 staining was quantified using a Dotpro Slidescanner to obtain digital images followed by ImageJ software to quantify the amount of positive pixels.
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Biochemical analysis
Plasma urea, creatinine and lactate dehydrogenase (LDH) were measured using standardized clinical diagnostic protocols of the Academic Medical Centre Amsterdam.

Real time and RT-PCR
Total RNA was isolated from frozen sections using Trizol (Life Technologies) following the recommended manufacturers’ protocol. Complementary DNA was made by ligation of oligo-dT primers and subsequent polymerization using Taq DNA polymerase (Invitrogen). Finally, cDNA was investigated with electrophoresis and an agarose gel or quantified in real-time on a Roche LightCycler 480 (Roche Diagnostics) using LightCycler 480 DNA SYBR Green (Roche Diagnostics). To quantify gene expression, PPIA was used as an endogenous reference.

Histochemistry
Periodic acid Schiff-diastase (PAS-D) stain was performed as follows. Paraffin-embedded sections were deparaffinized and incubated with 0.25% amylase solution (Sigma Aldrich). Subsequently, slides were incubated with 1% periodic acid (Merck) followed by Schiff reagens (Merck). Counterstaining was carried out using hematoxyline (Sigma Aldrich). Necrosis was assessed on PAS-D sections by a qualified pathologist using a semi-quantitative score where 0: 0%, 1: 1-10%, 2: 11-25%, 3: 26-50%, 4: 51-75% and 5: 76-100% of tubules are necrotic.

Statistics
All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the non-parametric Kruskal-Wallis test with Dunn’s correction to test for multiple group comparison. The non-parametric Mann Whitney test was used for two group comparisons. Correlation between variables was determined using Spearman. For all analyses, p<0.05 was considered significant.
**Results**

**Nlrx1 is down regulated following ischemia reperfusion**

Following IRI, we analysed mRNA expression of Nlrx1, the adaptor protein mitochondrial antiviral signalling (MAVS) and two genes of which transcription is regulated by Nlrx1: the matrix-facing protein of the respiratory chain complex III, UQCRC2\(^{15,18}\) and the pro-inflammatory cytokine IL-6\(^{11,13}\). Nlrx1 is down regulated after one and five days of reperfusion (Fig 1A). The mitochondrial antiviral signalling (MAVS) mRNA transcript is downregulated after five days of reperfusion (Fig 1B). Expression of the downstream effector cytokine IL-6 was increased after one and five days of reperfusion (Fig 1C) whereas UQCRC2 expression was decreased one day after reperfusion (Fig 1D).

![Figure 1](image-url)
**Nlrx1 deficiency is detrimental following renal ischemia reperfusion after five days**

Plasma urea and creatinine levels did not significantly differ between wild-type and Nlrx1KO mice following bilateral ischemia and one day of reperfusion (Fig 2A,B). KIM-1 expression was also not different (Fig 2C). Following bilateral ischemia, Nlrx1 deficiency reduced survival at five days of reperfusion since 5 out of 8 Nlrx1KO mice died while all 8 wild-type mice survived (data not shown).

To ensure survival at five days post-IR, we performed unilateral ischemia. We observed no difference in plasma urea levels after five days of reperfusion between wild-type and Nlrx1KO mice (Fig 2E). Plasma creatinine levels showed a significant increase after five days of reperfusion in Nlrx1KO mice versus wild-type mice (Fig 2E). In concordance with plasma creatinine, we observed a significant increase in KIM-1 expression in Nlrx1KO versus wild-type kidneys subjected to IR after five days (Fig 2F). We conclude that Nlrx1 deficiency has a detrimental impact on renal IRI after five days.

**Figure 2.** Nlrx1 deficiency is detrimental following five days of renal IR. Renal function as seen by plasma urea (A), creatinine (B) or KIM-1 expression (C) in case of sham-operated mice (n=4) or bilateral ischemia followed by one day of reperfusion (IR) (n=6-8). Renal function as seen by plasma urea (D), creatinine (E) or KIM-1 expression (F) of mice subjected to unilateral ischemia following five days of reperfusion (n=8). Statistics were performed using Mann-Whitney where * = p < 0.05
Nlrx1 deficiency reduces granulocyte numbers after five days

As granulocytes are able to cause renal injury following IRI (19), we next analysed the amount of granulocytes that were present in the kidney and observed no difference after one day of reperfusion (n = 7) (A,C) or unilateral ischemia followed by five days of reperfusion (n = 8) (B,D) and subsequently quantified in 10 randomly selected high power fields (HPF) (400x) (C,D). Statistics were performed using Mann Whitney where ** = p < 0.01

Nlrx1 deficiency induces a mild increase in macrophage accumulation after five days

Macrophages are important mediators of injury during the inflammatory phase but also contribute to cellular repair during the regenerative phase (9). Macrophage
accumulation was similar in both mouse strains during the inflammatory phase, i.e. after one day of reperfusion (Fig 4 A,C) whereas during the repair phase, Nlrx1KO mice showed an increased macrophage accumulation after five days of reperfusion (Fig 4B,D).

**Nlrx1 deficiency increases cytokine levels**
Since Nlrx1 regulates NF-κB activation following RIG-I or TLR4 stimulation (11,13) and NF-κB regulates an inflammatory signalling pathway, we investigated renal cytokine levels downstream of NF-κB. KC expression was enhanced in Nlrx1KO versus wild-type mice after one day of reperfusion. KC expression was also increased in contralateral kidneys of Nlrx1KO mice and not in the ischemic kidney after five days (Fig 5A). MCP-1 is enhanced in ischemic kidneys of Nlrx1KO mice versus wild-type mice after five days of reperfusion (Fig 5B).

**Figure 4.** Nlrx1 deficiency induces a mild increase in macrophage accumulation. Renal sections were stained for the macrophage marker F4-80 (A,B) of mice subjected to sham surgery (n=2) or bilateral ischemia followed by one day of reperfusion (n=7) (A,C) or unilateral ischemia followed by five days of reperfusion (n=8) (B,D) and subsequently quantified in 10 randomly selected high power fields (HPF) (400x) (C,D). Statistics were performed using Mann Whitney where ** = p < 0.01
TNF-α strongly increased in Nlrx1KO mice after one day of reperfusion whereas no differences were observed after five days of reperfusion (Fig 5C). Although IL-6 protein expression showed a small increase in kidneys of Nlrx1KO mice after one day of reperfusion, levels did not exceed those of sham mice which is, for unknown reason, in discrepancy with the difference in IL-6 transcripts (Fig. 1C). Remarkably was the increase in the control kidneys of Nlrx1KO mice after five days of reperfusion, of which levels in both strains were higher than in the ischemic kidneys (Fig 5D).
**Nlrx1 deficiency causes increased tubular apoptosis and necrosis**

Quantitative scoring of tubular apoptosis showed that Nlrx1KO mice have more caspase-3+ tubuli following 1 day of bilateral IRI (Fig 6A,C) and also after five days of IRI (Fig 6B,D). In addition, we observed using semi-quantitative scoring of PAS-D stained sections, an increase in tubular necrosis after both one and five days of reperfusion in Nlrx1KO mice as compared to wild-type mice following IRI (Fig 6E-H). Increases in tubular necrosis related to an increase in plasma LDH levels, a marker for cellular damage. Nlrx1KO mice showed a trend towards higher LDH levels after one day and a significant increase after five days of reperfusion in Nlrx1KO mice versus wild-type mice (Fig 6I).

**Figure 6.** Nlrx1 deficiency causes increased tubular apoptosis and necrosis. Caspase-3 (A-D)- and PAS-D (E-H) stained sections of mice subjected to bilateral ischemia followed by one day of reperfusion (A,E) or unilateral ischemia followed by five days of reperfusion (B,F). Tubular apoptosis was scored quantitatively in caspase-3+ tubuli per high-power field (400x magnification) (C,D). Tubular necrosis was scored in a semi-quantitative manner (G,H). To assess overall cellular damage, plasma LDH levels were obtained (I). AU = arbitrary units. Statistics were performed using Mann Whitney where * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.
Renal-associated Nlrx1 deficiency increases cellular damage

Wild-type bone marrow or bone marrow-derived cells such as macrophages and neutrophils show Nlrx1 gene expression. Interestingly, proximal tubular epithelial cells, which are most susceptible to ischemic injury, also express Nlrx1 (Fig 7A). To determine the role of renal- versus bone marrow-associated Nlrx1, we created bone marrow chimeras and applied bilateral ischemia followed by one day of reperfusion. Plasma urea and creatinine levels did not differ (Fig 7B,C). Cellular damage was investigated using plasma LDH levels. Here, Wt->KO but not KO->Wt levels were increased versus Wt->Wt mice (Fig 7D).

Figure 7. Renal-associated Nlrx1 deficiency increases cellular damage. Nlrx1 and TBP mRNA expression in several hematopoietic cells, bone marrow and renal primary tubular epithelial cells (A). Renal function of bone marrow-transplanted mice (n=7/group) as seen by plasma urea (B) and creatinine (C). Plasma LDH levels were measured as an indication of cellular damage (D). Statistics were performed using Kruskal Wallis with Dunn’s multiple comparison test where * = p < 0.05
Renal-associated Nlrx1 deficiency increases tubular necrosis and apoptosis

Both wild-type and Nlrx1KO chimeric mice showed extensive tubular damage in the cortico-medullary region which extended into the cortex. As tubular necrosis in the cortico-medullary region reached maximal injury values, we investigated cortical tubular necrosis and observed that Wt->KO mice showed significantly increased levels of tubular necrosis whereas necrosis in KO->Wt, although also higher, did not differ significantly from Wt->Wt mice (Fig 8A,B). We next investigated apoptosis to explore the effect of Nlrx1 on another type of cell death by staining for cleaved caspase 3. Similar to tubular necrosis we observed a sharp increase in Wt->KO mice whereas KO->Wt did not differ significantly from Wt->Wt mice (Fig 8C,D). TNF-α, KC and MCP-1 levels did not differ between groups (data not shown). Overall, we conclude that Nlrx1 of renal parenchymal cells, rather than of bone marrow-derived infiltrating immune cells, is responsible for protection against ischemia reperfusion-induced tubular cell death.

Figure 8. Renal-associated Nlrx1 deficiency increases tubular necrosis and apoptosis. Using PAS-D stained renal sections (A), cortical tubular necrosis was assessed in a semi-quantitative manner (B). In addition, tubular apoptosis was investigated using an antibody directed against cleaved Caspase 3 (C) and apoptotic tubuli were counted in the cortex (D). AU = arbitrary units, n=7/group. Statistics were performed using Kruskal Wallis with Dunn’s multiple comparison test where * = p < 0.05 and ** = p < 0.01.
Discussion

Preventing IR-induced renal injury and related pathology is an effective way to reduce graft loss following kidney transplantation. Nlrx1 regulates TLR-induced NF-κB signalling and the production of ROS (11,13,16), two important processes following IR, suggesting a potential role for Nlrx1. We showed here that Nlrx1 expression is down regulated in the kidney following IR. Nlrx1 deficiency has detrimental effects on renal IRI which associates with increased cytokine levels, macrophage accumulation, plasma LDH levels and tubular necrosis and apoptosis. Nlrx1 deficiency reduced granulocyte numbers at the late phase. Using bone marrow chimeras, we showed that Nlrx1 deficiency in renal parenchymal cells leads to increased tubular necrosis, apoptosis and plasma LDH levels. In contrast to other TLR and NLR family members (20), we now show that Nlrx1 plays a protective role in experimental renal ischemia reperfusion injury.

We observed that Nlrx1 mRNA is expressed by both hematopoietic cells and tubular epithelial cells and is down regulated in the kidney after IR suggesting that Nlrx1 plays a role following IR. IL-6 is a key protein regulated by Nlrx1 as shown by Allen et al. (11) and Xia et al. (13). In line we found an inverse correlation between Nlrx1 and IL-6 mRNA during renal IR. However, for unknown reasons IL-6 protein seems only marginally influenced by Nlrx1 deficiency at the time points we studied. IR furthermore led to a down regulation of the Nlrx1-associated molecules MAVS and UQCRC2.

Nlrx1KO mice showed a survival disadvantage when subjected to bilateral IR. This finding is in concordance with an increase in tubular cell death and plasma LDH levels, but not with a rise in urea and creatinine levels. Apparently urea and creatinine values are not necessarily specific for detecting tubular cell death. The most striking finding was that tubular necrosis and apoptosis was greatly enhanced in Nlrx1KO mice following either one or five days of reperfusion. Plasma LDH levels correlated with the observed increase in tubular necrosis suggesting that plasma LDH is derived from the kidney. In line with our data, a recent paper was published that showed convincingly that Nlrx1 protects SV40-transformed murine embryonic fibroblast cells from extrinsic apoptosis (14).

An increase in necrosis is accompanied by increased levels of DAMPs, such as HMGB1 (21), mitochondrial DNA (22) or ATP (10) that are able to activate pattern recognition receptors and induce cytokine expression. In line with this, we observed increased cytokine levels for KC, MCP-1 and TNF-α. In addition, Nlrx1KO mice showed higher IL-6 levels in kidneys of IR-operated mice after
one day of reperfusion and in contralateral kidneys after five days of reperfusion. IL-6 levels were also greatly enhanced in Nlrx1KO mice in a model of septic shock (11) or influenza virus infection (13).

Leukocyte influx is mainly determined by the expression of adhesion molecules and chemokines. Macrophage accumulation was greatly higher after five days of reperfusion compared to control kidneys which is consistent with the kinetics of macrophage influx following ischemia/reperfusion (7,9). Increased macrophage accumulation in Nlrx1KO mice after five days of reperfusion matches the increased expression of MCP-1 but is not in line with observed tubular damage. This suggests that macrophages are not critical for Nlrx1-associated tubular damage. Granulocyte numbers do not associate with KC expression nor with tubular cell death at day one. More research is warranted to investigate the role of Nlrx1 on granulocyte function and factors facilitating granulocyte influx. Possibly, the low amount of granulocyte influx in kidneys from Nlrx1KO mice after five days contributes to tubular damage through a reduced capacity to clear cellular debris (23). In summary, the differences in tubular cell death we found between mice with or without Nlrx1 at both time points can probably not be explained by differences in leukocyte numbers.

To further investigate the role of Nlrx1 in renal IRI, we created bone marrow chimeras to discriminate between the effect of renal- versus bone marrow-associated Nlrx1. Renal function parameters, i.e. plasma urea and creatinine, were again not significantly different at day one. However, mice that lack Nlrx1 on renal parenchymal cells show increased tubular necrosis, increased plasma LDH and in addition increased levels of cleaved caspase 3-positive tubular epithelial cells, a marker for apoptosis. These data suggest that Nlrx1 in renal parenchymal cells is responsible for suppressing cell death.

Nlrx1 is able to regulate cell death through intrinsic or extrinsic processes (14). Given that Nlrx1 deficient mice had enhanced TNF-α expression (this study) and TNF-α mediates renal injury following IR (19), we speculate that increased tubular cell death in Nlrx1KO mice could also be due to enhanced expression of TNF-α. We are currently investigating ROS in our model but based on previous observations which show that Nlrx1 could amplify ROS (16), one could expect that Nlrx1KO would have less ROS and as a consequence reduced IR-related pathology. We however observed increased IR-related pathology in Nlrx1KO mice suggesting that this Nlrx1-ROS linkage is not decisive for the outcome of renal IRI. Further research is needed to clarify the mechanisms behind Nlrx1-associated tubular cell death.
Collectively, we show that renal-associated Nlrx1 is important in conferring renal protection after ischemia reperfusion injury, primarily by its anti-apoptotic and anti-necrotic effects in tubular epithelial cells. Nlrx1 may therefore be an interesting therapeutic target in diseases where cell death contributes to pathology (24).
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References


