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A Tissue-specific Role for Nlrp3 in Tubular Epithelial Repair following Renal Ischemia Reperfusion

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Abstract

Ischemia/reperfusion injury is a major cause of acute kidney injury. Improving renal repair would represent a therapeutic strategy to prevent renal dysfunction. The innate immune receptor Nlrp3 is involved in tissue injury, inflammation and fibrosis however its role in repair following ischemia/reperfusion is unknown. The current study addresses the role of Nlrp3 in the repair phase of renal ischemia/reperfusion and investigates the relative contribution of leukocyte- versus renal-associated Nlrp3 by studying bone marrow chimeric mice. We found that Nlrp3 expression was most profound during the repair phase. Although Nlrp3 expression was primarily expressed by leukocytes, both leukocyte- and renal-associated Nlrp3 was detrimental to renal function following ischemia/reperfusion. The Nlrp3-dependent cytokine IL-1β remained unchanged in kidneys of all mice. Leukocyte-associated Nlrp3 negatively affected tubular apoptosis in mice which lacked Nlrp3 expression on leukocytes which correlated with reduced macrophage influx. Nlrp3 deficient mice (Nlrp3KO) mice with wild-type bone marrow showed an improved repair response as seen by a profound increase in proliferating tubular epithelium which coincided with increased hepatocyte growth factor expression. In addition, Nlrp3KO tubular epithelial cells had an increased repair response in vitro as seen by an increased ability of an epithelial monolayer to restore its structural integrity. In conclusion, Nlrp3 shows a tissue-specific role where leukocyte-associated Nlrp3 is associated with tubular apoptosis whereas renal-associated Nlrp3 impaired wound healing.
Introduction

Ischemia reperfusion (IR) injury is a major cause of acute kidney injury (1) and increases the risk to develop chronic kidney disease (CKD) (2). Following injury, wounded tissue organizes an efficient response that aims to combat infections, clear cell debris, re-establish cell number and reorganize tissue architecture. First, necrotic tissue releases danger-associated molecular patterns (DAMP) such as high mobility group box-1 (3) or mitochondrial DNA (4) which leads to chemokine secretion (5) and subsequent influx of leukocytes. Neutrophils and macrophages clear cellular debris but also increase renal damage since depletion of neutrophils (6) or macrophages within 48 hours of IR will reduce renal damage (7). Around 72 hours of reperfusion, the inflammatory phase transforms into the repair phase and is characterized by surviving tubular epithelial cells (TEC) that dedifferentiate, migrate and proliferate to restore renal function (8).

Previously, we have shown that TLR2 and TLR4 play a detrimental role following acute renal IR injury (9–11). In addition, TLR2 appeared also pivotal in mediating tubular repair in vitro following cisplatin-induced injury (12) indicating a dual role for TLR2. The cytosolic innate immune receptor Nlrp3 is able to sense cellular damage (13) and mediates renal inflammation and pathology following IR (14–16) or nephrocalcinosis (17). Next to the detrimental role of Nlrp3 in different renal diseases models and in line with the dual role of TLR2, Nlrp3 was shown to protect against loss of colonic epithelial integrity (18). We therefore speculate that Nlrp3 which contributes to sterile renal inflammation during acute renal IR injury might also drive subsequent tubular repair.

To test this hypothesis, we investigated the role of leukocyte- versus renal-associated Nlrp3 with respect to tissue repair following renal IR. We observed that both renal- and leukocyte-associated Nlrp3 is detrimental to renal function following renal IR injury however through different mechanisms. Leukocyte-associated Nlrp3 is related to increased tubular epithelial apoptosis whereas renal-associated Nlrp3 impairs the tubular epithelial repair response. Our data suggest Nlrp3 as a negative regulator of resident tubular cell proliferation in addition to its detrimental role in renal fibrosis and inflammation (14,19).
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Material & Methods

Mice
Pathogen-free 8–12 week-old male wild type C57BL/6 mice were purchased from Charles River Laboratories. The generation of Nlrp3-deficient mice was described previously (20). Nlrp3-deficient mice were backcrossed onto a C57BL/6 genetic background for nine generations and bred in the animal facility of the Academic Medical Center in Amsterdam, The Netherlands. The CD45.1 positive allotype 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. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Bone marrow transplantation
Transplantation was carried out as described previously (10). Briefly, tibia, femur and spleen were harvested of wild-type CD45.1 or Nlrp3KO 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 Nlrp3KO 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 five days 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.

Renal ischemia reperfusion
Renal IR injury was induced as described previously (9). Briefly, both renal pedicles were clamped for 35 minutes using microaneurysm clamps through a midline abdominal incision under general anesthesia (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 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.

Biochemical analysis
Plasma urea and creatinine were measured using standardized clinical diagnostic protocols of the Academical Medical Center Amsterdam.

Quantitative 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 quantified in real-time on a Roche LightCycler 480 (Roche Diagnostics) using LightCycler 480 DNA SYBR Green (Roche Diagnostics). To quantify gene expression, cyclophilin G was used as an endogenous reference. The following primer sequences were used: Nlrp3 forward -ccacagtgaaccttgacagaagc-, reverse -ggtgtgtgaagttctggtg-; KIM-1 forward -ttggtgcctccgctctctct-, reverse -tcagctgggaatgcacaa-; NGAL forward -gcctcaaggacgacaacatc-, reverse -ctgaaccattgggtctctgc-; HGF forward -ccaagttgacgttgatcaagtg-; reverse -tggtccagacatagtcggtg-.

Cytokine expression
Snap-frozen kidneys were lysed using Greenberg Lysis Buffer (75 mM NaCl, 7.5 mM Tris, 0.5 mM MgCl₂.H₂O, 0.5 mM CaCl₂, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Sigma Aldrich) according to weight of the kidney and subsequently, homogenates were used to determine cytokine levels for IL-1β, IL-6, KC and MCP-1 according to manufacturers’ instructions (R&D Systems).

Immunohistochemistry
For PAS-D, paraffin-embedded tissue sections were deparaffinized and incubated with 0.25% amylase solution (Sigma Aldrich). Subsequently, slides were incubated with 1% periodic acid (Merck) followed by Schiff reagents (Merck). Counterstaining was carried out using haematoxyline (Sigma Aldrich). For cleaved caspase 3, Ki67 and F4/80, sections were boiled in 0.01M pH 6.0 citrate
buffer and subsequently exposed to a rabbit anti-mouse cleaved caspase 3 (Asp175) (Cell Signalling), rabbit anti-mouse Ki67 (Neomarkers) or rat IgG2b anti-mouse F4/80 (Serotec) respectively. Staining was visualized using a power anti-rabbit poly HRP (Dako) and additionally a rabbit anti-rat antibody (Dako) in case of F4/80.

**Quantification of immunohistochemistry**

Tubular necrosis was assessed on PAS-D sections by a renal pathologist using a semi-quantitative score as follows: 0: 0%, 1: 1-10%, 2: 10-25%, 3: 25-50%, 4: 50-75% and 5: 75-100% per high power field (400x magnification) (HPF). Cleaved caspase-3 and Ki67 positive tubular epithelial cells were counted in 10 randomly selected high power fields (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.

**Immunoprecipitation of IL-1β**

To detect IL-1β, we immuno-precipitated 1 mg of protein derived from snap-frozen kidneys by lysis of renal tissue with isolation buffer (20 mM Tris-Cl pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 10% Glycerol, 1% Triton X100). Subsequently, all IL-1β was isolated using an anti-IL-1β rat IgG1 monoclonal antibody (MAB401, R&D Systems) followed by precipitation using Protein G-Sepharose beads (Biovision). Subsequently, all precipitated IL-1β was visualized with Western blot using an anti-IL-1β polyclonal goat IgG (BAF401, R&D Systems) and streptavidin-poly HRP (Sanquin). As a positive control for immuno-precipitation or Western blot, we added 100 pg of recombinant IL-1β (R&D Systems) to 1 mg of protein or 50 pg of recombinant IL-1β (R&D Systems) respectively. A band at 18 kDa was interpreted as active IL-1β.

**In vitro hypoxia assay**

Proximal tubular epithelial cells were isolated from kidneys as described previously 9 and cultured using HK2 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen) until cells became 70-80% confluent. Hypoxia was induced following a protocol described previously (21). Ringer Ischemia solution (NaHCO₃ 4.5 mM, Na₂HPO₄ 0.8 mM, NaH₂PO₄ 0.2 mM, NaCl 106.0 mM, KCl 5.4 mM, CaCl₂ 1.2 mM, MgCl₂ 0.8 mM, morpholinoethanesulfonic acid 20 mM; pH 6.6) was made
anoxic by mixing 100% N2 gas with Ringer Ischemia solution for 5 minutes. Subsequently, HK2 medium was replaced by Ringer Ischemia solution and cells were transferred to a hypoxic chamber which was flushed for 1 minute with 100% N2 gas. Subsequently, cells were incubated for 4 hours at 37°C.

**In vitro wound healing assay**
Proximal tubular epithelial cells were cultured using HK2 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen) for 8 days. Subsequently, cells were harvested and seeded at 2*10⁵ cells/ml and cultured for an additional 2 days to obtain a confluent epithelial monolayer. Finally, a scratch was made using a p1000 pipette tip and cells were monitored for the following 72 hours using in-house designed software and a phase-contrast microscope. Recovery was assessed by measuring non-recovered wound area at marked points where the scratched area at t=0 was set at 100%.

**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 or the parametric one-way ANOVA with Bonferroni’s multiple comparison test in case of equal variances to test for multiple group comparison. The non-parametric Mann Whitney test was used for two group comparisons. For all analyses, p < 0.05 was considered significant.

**Results**
**Nlrp3 gene expression associates with renal repair phase**
We first investigated Nlrp3 gene expression in ischemic kidneys after one, five and ten days of reperfusion. Nlrp3 gene expression peaked after five days reperfusion with a five-fold increase compared to sham levels (Fig 1A). Similar to Nlrp3 expression in macrophages upon LPS stimulation (20), TECs upregulated Nlrp3 gene expression following hypoxia or upon LPS stimulation (Fig 1B). In order to determine the role of renal- versus leukocyte-associated Nlrp3 during the repair phase, i.e. after five days of reperfusion, we generated bone marrow chimeric mice made by reconstituting sub-lethally irradiated wild-type or Nlrp3KO mice with bone marrow taken from either wild-type or Nlrp3KO mice. After five days of reperfusion, we found that Nlrp3 is primarily expressed by infiltrating...
leukocytes as seen by almost similar Nlrp3 gene expression in Nlrp3KO mice transplanted with wild-type bone marrow (Wt->KO) compared to wild-type mice transplanted with wild-type bone marrow (Wt->Wt) (Fig 1C). Next, we found renal parenchymal Nlrp3 expression as seen by an increased expression in wild-type mice transplanted with Nlrp3KO bone marrow (KO->Wt) compared to Nlrp3KO mice transplanted with Nlrp3KO bone marrow (KO->KO) (Fig 1C). In conclusion, Nlrp3 is expressed both by leukocytes and renal parenchymal cells after five days of reperfusion although leukocyte-associated Nlrp3 gene expression is dominant.

Both renal- and leukocyte-associated Nlrp3 mediates renal dysfunction

Since Nlrp3 is expressed in both compartments, we investigated the role of leukocyte- versus renal-associated Nlrp3 expression on renal function following IR. Both renal-associated Nlrp3 as leukocyte-associated Nlrp3 mediated loss of renal function as seen by decreased levels of plasma urea (Fig 2A) or plasma creatinin (Fig 2B) in Wt->KO, KO->Wt and KO->KO mice compared to Wt->Wt mice after five days of reperfusion. Bone marrow chimeras were also subjected to ischemia followed by one day of reperfusion to investigate the early contribution of renal- versus leukocyte Nlrp3 on renal function. Here, we observed that only renal Nlrp3 mediates renal dysfunction based on plasma creatinine levels (Sup Fig 1A,B). We next investigated the renal damage markers KIM-1 (22) and NGAL (23) to investigate proximal versus distal damage. Both
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chimeras showed an expression pattern similar to plasma urea and creatinin with respect to KIM-1 (Fig 2C) and NGAL (Fig 2D) confirming that both leukocyte- and renal-associated Nlrp3 is detrimental following renal IR injury and indicating that distinct Nlrp3 expression has no differential effect on proximal versus distal tubular damage.

**Nlrp3 does not lead to a differential pro-inflammatory cytokine production**

To determine if the beneficial effect of Nlrp3 deficiency following renal IR injury was due to inhibition of cytokines and chemokines, we additionally investigated renal levels of KC, MCP-1, IL-1β and IL-6. The renal chemokines KC and MCP-1 remained unchanged in Wt->KO, KO->KO mice but were decreased in KO->Wt mice compared to Wt->Wt animals (Fig 3A,B). We observed that the pro-inflammatory cytokines IL-1β and IL-6 were not differentially expressed in kidneys of chimeras after five days of reperfusion (Fig 3C,D). To differentiate

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**Figure 2.** Both renal- and leukocyte-associated Nlrp3 deficiency protects against renal dysfunction. Renal function was assessed after five days of reperfusion using the markers plasma urea (A) and creatinin (B). Proximal and distal tubular damage was investigated using the proximal marker KIM-1 (C) and distal marker NGAL (D). Results are expressed as mean with SEM (n = 7-11 animals/group). Statistics were performed using Kruskal Wallis with Dunn’s multiple comparison test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus Wt->Wt mice).
pro- versus active IL-1β, we performed immunoprecipitation followed by blotting of kidney homogenates for active IL-1β. Although levels are overall low, we observed that Wt->KO mice had an increase in active IL-1β versus Wt->Wt mice whereas KO->KO and KO->Wt show a heterogenous amount of active IL-1β in homogenates (Fig 3E).

**Leukocyte-associated Nlrp3 mediates tubular apoptosis**

As the Nlrp3 inflammasome is also implicated in the induction of cell death (15,24), we next investigated tubular necrosis and apoptosis as an underlying cause for renal dysfunction using PAS-D and cleaved caspase-3 stained sections (Fig 4A,C). KO->Wt mice showed a strong reduction in the amount of tubular
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necrosis (Fig 4B) which was in line with improved renal function. Unexpectedly, we observed a similar degree of tubular necrosis in Wt->KO mice as compared to Wt->Wt mice which did not correlate with improved renal function suggesting a different underlying cause (Fig 4B). Cleaved caspase-3 staining of renal sections (Fig 4C) showed a pattern similar to necrosis however here, all mice which lacked Nlrp3 expression on leukocytes showed a reduction in apoptotic TECs (Fig 4D).

Macrophage influx correlates with tubular cell death

Depletion of macrophages following IR reduces renal damage (25). In this light, we examined the presence of macrophages using the macrophage marker F4/80 (Fig 5A). Macrophage numbers were decreased in KO->KO and KO->Wt groups compared to Wt->Wt mice (Fig 5B). Wt->KO mice had similar numbers of macrophages as Wt->Wt mice. Finally, we correlated macrophage influx to tubular necrosis (Fig 5C) and apoptosis (Fig 5D) and observed that macrophage influx was highly significantly related to tubular cell death.

Figure 4. Leukocyte-associated Nlrp3 regulates tubular apoptosis. PAS-D stained renal sections (A) were semi-quantitatively scored for tubular necrosis (B). Renal sections were stained for the apoptosis marker cleaved caspase-3 (C) and positive tubular cells were quantified (D). Results are expressed as mean with SEM (n = 7-11 animals/group). Statistics were performed using Kruskal Wallis with Dunn’s correction (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus Wt->Wt mice).
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Increased tubular epithelial proliferation in Nlrp3KO mice transplanted with wild-type bone marrow

As the amount of tubular cell death and renal inflammation in Wt->KO mice did not correlate with improved renal function, we next investigated the tubular repair response using the proliferation marker Ki67 (Fig 6A). Remarkably, we observed a 2-fold increase in proliferating TECs in Wt->KO mice suggesting an improved repair response. In line with tubular necrosis, we observed a decrease in KO->Wt mice and a trend in KO->KO mice compared to Wt->Wt (Fig 6B). Although growth factors contribute to renal repair (26), we did not observe an increase in gene expression of IGF-1, FGF-1 or FGF-7 in Wt->KO mice compared to Wt->Wt mice (data not shown). In contrast, renal hepatocyte growth factor (HGF) gene expression was profoundly upregulated in Wt->KO mice (Fig 6C).

Figure 5. Macrophage accumulation correlates with tubular cell death. Immunohistochemistry of the murine macrophage marker F4/80 (A) and quantification of renal sections (B). Correlation of F4/80+ area with semi-quantitatively scored necrosis (C) or amount of cleaved caspase-3+ tubular epithelial cells (D). Results are expressed as mean with SEM (n = 7-11 animals/group). Statistics were performed using an one-way ANOVA with Dunnett’s multiple comparison test (B) or Spearman’s test for correlation (C,D) (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus Wt->Wt mice).
In addition to extrinsic factors such as HGF, we also investigated whether intrinsic differences in repair exist between wild-type versus Nlrp3KO tubular epithelium. Using an in vitro wound healing assay, we observed that Nlrp3KO tubular epithelium had an increased capacity to restore integrity of an epithelial monolayer after an insult (Fig 7A). Time to initiate a repair response was decreased in Nlrp3KO tubular epithelium (Fig 7B). During repair, few cells lost cell-cell contact and migrated as single cells into the damaged area (Supplemental Movie 1, 2). We quantified migrating single cells and observed increased numbers in wild-type tubular epithelium after 20 hours compared to Nlrp3KO tubular epithelium (Fig 7C). In conclusion, Nlrp3KO tubular epithelium showed a better repair response following an insult which correlates with the increased proliferation of TECs we found in Wt->KO mice.
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Discussion

Improving repair following renal IR would represent a therapeutic strategy to reduce renal dysfunction. The innate immune receptor Nlrp3 is involved in the induction of renal damage, inflammation (14) and fibrosis (19) however its role on repair following IR is unknown. Here, we show that both renal- and leukocyte-associated Nlrp3 mediates loss of renal function although through different mechanisms. Leukocyte-associated Nlrp3 mediates tubular apoptosis whereas renal-associated Nlrp3 diminishes the repair response as seen by increased numbers of proliferating TECs in vivo and the ability to restore the integrity of an epithelial monolayer in vitro.

Nlrp3 has been shown to be detrimental to renal function after one day of reperfusion (14,15). However a role for Nlrp3 at the repair phase of renal IR injury was not investigated. We found that Nlrp3 gene expression (this study) and tubular proliferation (10) was highest after five days of reperfusion suggesting an important role for Nlrp3 during the repair phase. We therefore investigated the role of Nlrp3 on renal repair. We show that TECs express Nlrp3 (this study) as macrophages do (20). Current research shows a pro-fibrotic role for Nlrp3 in renal epithelium (27) and a pro-inflammatory role in macrophages (20), indicating cell type-specific effects of Nlrp3 on cellular function. To differentiate leukocyte-from renal-associated Nlrp3, we generated Nlrp3KO chimeras. Leukocyte-associated Nlrp3 gene expression is dominant over renal-associated Nlrp3 after five days of reperfusion which is in line with the observation that renal function is least impaired in wild-type mice which lack Nlrp3 expression on leukocytes. This protective effect seems dependent on the stage of disease as Shigeoka et al. and we found that leukocyte-associated Nlrp3 expression does not mediate renal dysfunction after one day of renal IR (15) (this study). To extent the study of Shigeoka et al., we created bone marrow chimeras to investigate the early contribution of renal-associated Nlrp3 to renal dysfunction after one day of ischemia-reperfusion. Here, renal-associated Nlrp3 mediated renal dysfunction. The observed protection became more clear in the repair phase (day 5) despite low renal-associated Nlrp3 gene expression. Our data indicates that the role of Nlrp3 is dependent on the stage of disease and Nlrp3 has a tissue-specific role.

To understand why Nlrp3 deficiency of leukocytes and renal parenchymal cells results in less impaired renal function in the late phase of renal IR injury we next analysed inflammation and tubular cell death. The first consequence of renal IR is tubular cell death leading to renal dysfunction and cytokine release (28). Indeed,
we observed that KC and MCP-1 were reduced in wild-type mice transplanted with Nlrp3KO bone marrow which also experienced low levels of necrosis. Since Nlrp3 is able to form the Nlrp3 inflammasome and subsequently activate the pro-inflammatory cytokine IL-1β (canonical pathway), we speculated that a reduction in a pro-inflammatory response might underlie improved renal function in chimeric mice. However, we did not find differential expression of the pro-inflammatory cytokines IL-1β or IL-6, nor decreased levels of active IL-1β in Nlrp3 KO mice transplanted with wild-type or Nlrp3 KO bone marrow. This suggests that alternative mechanisms might be involved in IL-1β maturation upon renal ischemia reperfusion injury. These results are consistent with other reports showing non-canonical effects of Nlrp3 in the kidney (15,16,27,29).

Next to cytokines, renal function is influenced by tubular damage and repair. Interestingly, we observed that renal apoptosis was reduced in mice lacking Nlrp3 expression in leukocytes suggesting that leukocyte-associated Nlrp3 influences tubular epithelial apoptosis and thereby renal dysfunction. One day after reperfusion, the time point at which neutrophil influx peaks (5), chimeric mice which leukocytes are Nlrp3 deficient did not show reduced renal damage (15) and function (this study). Therefore, we speculated that Nlrp3 in macrophages, of which influx peaks at day 5 (10), rather than neutrophils is important for differences in renal damage in the late phase of renal IR injury. Indeed, macrophages can induce tubular cell apoptosis in models of tubulo-interstitial inflammation. Here, polarization status of the macrophage and the production of pro-inflammatory cytokines and cytotoxic products is linked to tubular injury. In line, we observed that the level of renal macrophage influx and the amount of apoptotic TECs were both reduced in mice without Nlrp3 on their leukocytes. Additional analysis showed that macrophage influx indeed correlated with the amount of tubular epithelial apoptosis.

Conversely, deficiency of Nlrp3 on renal parenchymal cells did not lead to differences in tubular epithelial apoptosis or necrosis five days after renal IR. It was therefore of particular interest that despite similar levels of necrosis and apoptosis, mice deficient for renal parenchymal Nlrp3 with wild-type bone marrow showed an increased repair response as seen by a two-fold increase in proliferating TECs compared to control Wt->Wt mice. This suggests that renal-associated Nlrp3 is detrimental to renal repair, an observation that was supported by our in vitro wound healing assay. Apparently, Nlrp3 does not drive tubular repair after an insult as TLR2 does in vitro following cisplatin-induced injury (12).
We speculate that KO->KO mice did not show increased numbers of proliferating TECs due to reduced tubular damage needs to be repaired. We additionally observed reduced levels of proliferating TECs in wild-type mice which lacked Nlrp3 expression on leukocytes which probably reflects the severely reduced level of tubular necrosis that was found in these mice.

Several factors might explain the increased tubular epithelial proliferation in Wt->KO mice. The observed increased renal HGF expression could account for increased tubular epithelial proliferation. Indeed, in a model of HgCl2-induced renal injury, infused HGF increased tubular epithelial proliferation (30). The HGF gene is regulated by IL-6 (31). However, IL-6 levels remained unchanged. Nlrp3 regulates IL-1β (32) and it was shown that addition of IL-1β enhances HGF secretion of epithelial cells (33). Given that active IL-1β was elevated in kidneys of Wt->KO mice that show increased proliferation might suggest a role for IL-1β in renal HGF expression. In addition, epithelial Nlrp3 can have intracellular effects on proliferation. Indeed, wild-type tubular epithelium has a decreased capacity to restore integrity of a monolayer after an insult. Repair in both wild-type and Nlrp3KO epithelium initiated in a similar fashion however Nlrp3KO epithelium showed faster closure of the scratched area. The pattern of migration can be used to distinguish fibroblast-like cells as loosely connected migrating cells from epithelial cells which migrate as a sheet (34). We observed two patterns of migration where wild-type epithelium showed increased numbers of fibroblast-like cells after 20 hours whereas Nlrp3KO epithelium maintained epithelial-like behaviour. This observation is in line with papers demonstrating that Nlrp3 enhances renal epithelial-mesenchymal transition (EMT) (27) and fibrosis in a model of unilateral ureteral obstruction (19). In addition, mice that lack Nlrp3 in renal cells show increased levels of KC and MCP-1 suggesting a prolonged inflammatory phase which could be introduced by a reduced responsiveness to TGF-β (27). Based on these findings and the results of our study, we speculate that epithelial Nlrp3 is an important regulator of proliferation versus fibrosis during repair. Whether enhanced epithelial proliferation diminishes fibrosis following long-term IR remains to be investigated. However, a recent report showed that Nlrp3 deficiency does not regulate fibrosis following UUO (35). More research is warranted to investigate the role of Nlrp3 on ischemia-induced fibrosis.

In conclusion, our research extends the role of Nlrp3 in non-canonical pathways in the diseased kidney. We show here using Nlrp3KO chimeras that leukocyte-associated Nlrp3 negatively affects tubular epithelial apoptosis during the
repair phase of renal IR injury. Renal-associated Nlrc3 is detrimental to the repair response as reflected by enhanced HGF levels, an increased amount of proliferating TECs and an enhanced ability of epithelium to restore structural integrity in the absence of Nlrp3. Since acute kidney injury is associated with increased CKD incidence (36–39), we speculate that increasing renal repair following IR through modulation of renal Nlrp3 might offer therapeutic potential in reducing IR-induced CKD.

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Supplemental figures
Supplemental figures and movies can be found online.
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