NLRP3 Inflammasome Knockout Mice are Protected Against Ischemic but not Cisplatin-Induced Acute Kidney Injury.

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Abstract

We have demonstrated that caspase-1 is a mediator of both cisplatin-induced AKI and ischemic AKI. As caspase-1 is activated in the inflammasome, the aim of the study was to further investigate the inflammasome in cisplatin-induced and ischemic AKI. Mice were injected with cisplatin or subjected to bilateral renal pedicle clamping. Immunoblot of whole kidney following cisplatin-induced AKI revealed: (1) an increase in ASC, the major protein that complex with NLRP1 or 3 to form the inflammasome, (2) an increase in caspase-1 activity, caspase-5 and NLRP1, components of the NLRP1 inflammasome, (3) a trend towards increased NLRP3. To determine whether the NLRP3 inflammasome plays an injurious role in cisplatin-induced AKI, NLRP3 -/- mice were studied. In cisplatin-induced AKI, BUN, serum creatinine, ATN score and tubular apoptosis score were not significantly decreased in NALP3 -/- mice compared to wild-type mice. We have previously demonstrated the injurious role of caspase-1 in ischemic AKI. NLRP3, but not ASC or NLRP1, is increased in ischemic AKI. NLRP3 -/- mice with ischemic AKI had significantly lower BUN, serum creatinine, ATN and apoptosis scores than wild type controls. The difference in protection against cisplatin-induced AKI compared to ischemic AKI in NLRP3 -/- mice was not explained by differences in proinflammatory cytokines IL-1β, IL-6, CXCL1 or TNF-α. In conclusion, the NLRP3 inflammasome is a mediator of ischemic AKI but not cisplatin-induced AKI. Further investigation of the NLRP1 inflammasome in cisplatin-induced AKI will be interesting.
Introduction

Cisplatin is one of the most widely used and most potent chemotherapeutic agents. The exposure of tubular cells to cisplatin activates complex signaling pathways that lead to tubular cell injury and death (Schrier, 2002) (Safirstein, 2007) and a robust inflammatory response further exacerbating renal tissue damage. The injurious role of inflammation in AKI is increasingly appreciated with the involvement of leukocytes, adhesion molecules, and cytokines (Bonventre and Zuk, 2004; Friedwald and Rabb H, 2004) (Devarajan, 2006) (Lee et al., 2011). The cellular inflammatory response in AKI is also evidenced by increased CD4 T cells (Akcay et al., 2011) (Faubel et al., 2005), macrophages (Lu et al., 2007) (He et al., 2009), and neutrophils (Faubel et al., 2007) (Melnikov et al., 2002) in the kidney. The inflammasome is a protein scaffold or molecular complex which contains NLRP (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing) proteins and an adaptor protein called ASC (Apoptosis-associated Speck-like protein containing a Caspase recruitment domain) (Franchi et al., 2009) (Schroder et al., 2010) (Schroder and Tschopp, 2010). The most fully characterized inflammasome is the NLRP3 inflammasome which contains the NLRP3 protein and the ASC protein (Schroder, Zhou, and Tschopp, 2010). Another less well-characterized inflammasome, the NLRP1 inflammasome, contains NLRP1 protein, ASC protein and caspase-5 (Martinon et al., 2009). As inflammation plays a role in cisplatin-induced AKI and the inflammasome plays a crucial role in inflammation, we developed the hypothesis that the inflammasome is activated in AKI.

Our published data demonstrate that caspase-1 is a mediator of cisplatin-induced AKI (Faubel et al., 2004) and ischemic AKI (Melnikov et al., 2001). Caspase-1 is activated in the inflammasome. It is known that NLRPs recruit the adaptor ASC that recruits caspase-1 resulting
in secretion of “leaderless” proteins via direct or indirect physical interaction (Keller et al., 2008). Active caspase-1 in the inflammasome activates IL-1β, IL-18 and is a regulator of the “unconventional” protein secretion of “leaderless” proteins like IL-1α (Keller et al., 2008) (Guegan et al., 2003). IL-1α is a proinflammatory cytokine that is markedly increased in the kidney after cisplatin injection and is notably reduced in the caspase-1 -/- mice (Faubel et al., 2007). As IL-1β, IL-18 and caspase-1 are increased in cisplatin-induced AKI (Faubel et al., 2007) and ischemic AKI (Melnikov et al, 2001 and 2002) and IL-1β and IL-18 are activated by caspase-1 in the inflammasome, we developed the hypothesis that crucial inflammasome proteins like NLRPs and ASC would be increased in AKI and that NLRP3 knockout would protect against cisplatin-induced and ischemic AKI.

**Materials and Methods**

**Cisplatin-induced AKI**

For all the mouse studies, 8-10 week-old male C57BL/6 mice weighing 20-25 grams were used. All experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado Denver. Mice were maintained on a standard diet and water was freely available. Mice were housed 5 per cage under a 12 hour light and dark schedule for at least one week prior to cisplatin administration. Six hours before cisplatin administration, food and water were withheld. Cisplatin [cis-Diamminedichloro-platinum (II)] (Aldrich, Milwaukee, WI, USA) was freshly prepared the day of administration in sterile normal saline at a concentration of 1 mg/mL. Mice were given 25 mg/kg body weight of cisplatin or vehicle (saline) intraperitoneally (IP), after which the mice again had free access to food and water. We have
described this model of cisplatin-induced AKI in detail elsewhere (Tao et al., 2005) (Faubel et al., 2007) (Lu et al., 2007). Briefly, after 25 mg/kg cisplatin injection, BUN and serum creatinine are normal on day 1 and slightly increased on day 2. On day 3 after cisplatin injection, renal dysfunction, renal neutrophil infiltration, renal tubular cell apoptosis and acute tubular necrosis scores are severe.

Ischemia Protocol

The operator was blinded to treatment groups. Mice were anesthetized with IP Avertin (2,2,2-tribromoethanol: Aldrich, Milwaukee, WI). A midline incision was made and the renal pedicles were bilaterally clamped for 24 min with microaneurysm clamps. The time of ischemia was chosen to obtain a reversible model of ischemic AKI and avoid animal mortality. Serum creatinine reaches a peak at 24–48 hr of reperfusion and then gradually returns to normal within 3-7 days. This model is well established in our laboratory (He et al., 2008). After clamp removal, kidneys were observed for restoration of blood flow by the return to their original color. The abdomen is closed in two layers. Sham surgery consisted of the same surgical procedure except that clamps were not applied. During the first 24 hr of the reperfusion period, the animals were kept in an incubator at 29°C. Animals were sacrificed at 24 hrs after ischemia for all the measurements performed in the study. Blood samples were obtained at sacrifice via cardiac puncture.

NLRP3 -/- mice
NLRP3 -/- mice in the C57BL/6 background were generated by Millenium pharmaceuticals Inc (Cambridge, MA) and obtained from Dr Fayyaz Sutterwala, (Univ. Iowa Carver College of Medicine). The mice were genotyped as previously described (Sutterwala et al., 2006). Age, sex and weight matched C57BL/6 mice obtained from Jackson labs (Bar Harbor, Maine) were used as controls.

**Histological examination**

Paraformaldehyde (4%)-fixed and paraffin-embedded kidneys were sectioned at 4 μm and stained with periodic acid-Schiff (PAS) by standard methods. All histological examinations were performed by the renal pathologist in a blinded fashion. Histological changes due to acute tubular necrosis (ATN score) were evaluated in the outer stripe of the outer medulla on PAS-stained tissue and were quantified by counting the percent of tubules that displayed cell necrosis, loss of brush border, cast formation and tubule dilatation as follows: 0 = none, 1 = <10%, 2 = 10-25%, 3 = 26-45%, 4 = 46-75% and 5 = >75%. At least 10 fields (x250) were reviewed for each slide.

Morphologic criteria were used to count apoptotic cells on PAS-stained tissue by the pathologist experienced in the evaluation of renal apoptosis. Morphologic characteristics included cellular rounding and shrinkage, nuclear chromatin compaction and formation of apoptotic bodies (Gobe et al., 2000). Apoptotic tubular cells were quantitatively assessed per ten HPF (x400) in the outer stripe of the outer medulla by the renal pathologist in a blinded fashion.
Neutrophil infiltration was quantitatively assessed on PAS-stained tissue by the renal pathologist by counting the number of neutrophils per ten high power fields (X400) as we have previously described (Faubel et al, 2004). At least 10 fields were counted in the outer stripe of the outer medulla for each slide.

**RAW 264.7 macrophages**

Mouse RAW 264.7 cells are a mouse monocyte/macrophage cell line from ascites fluid in a male mouse in which a tumor was induced by injection of Abelson leukemia Virus (A-MuLV) (American Type Culture Collection). Cells are grown in DMEM high glucose medium supplemented with Na₂HCO₃ (0.075%), L-glutamine, PCN/strep (1%), 0.5 mg/ml insulin and 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37°C. Cells are treated with cisplatin 10 or 50 μM for 6 or 12 hrs.

**Freshly isolated mouse renal proximal tubules**

Proximal tubules were isolated from kidney cortex of male C57BL/6 mice using collagenase digestion and Percoll centrifugation as we have described in detail (Edelstein, 2000). Six-milliliter aliquots of tubule suspension (approximately 1 to 2 mg/mL) were placed in siliconized 25-mL Erlenmeyer flasks for a “recovery” period, which included gassing with 95% O₂/5% CO₂ for 5 min on ice. Flasks were then capped with rubber stoppers and kept at room temperature for 5 min, then placed in a shaking water bath at 37°C for 10 min. After “recovery” period, PT cells were incubated with vehicle, 10 or 50 μM cisplatin. Cisplatin was freshly prepared and dissolved in DMSO (final concentration 0.1%) at the time of the experiment. Control PT cells were treated with the vehicle (0.1% DMSO). After adding the reagents, the flasks were re-gassed with 95% O₂/5% CO₂ for 5 min and then closed and kept in the shaking water bath for
25 min. At the end of the “preincubation and experiment” period, 1 mL of tubule suspension was sampled for measurement of lactate dehydrogenase (LDH), 5 mL for caspase activity, ELISA, or immunoblots.

Caspase Assay

The activity of caspases was determined on cytosolic extracts of whole kidneys or proximal tubules by the use of fluorescent substrates as we have described in detail (Faubel et al., 2004) (Dursun et al., 2006). Ac-Typ-Glu-His-Asp-AMC (Ac-WEHD-AMC) as a susceptible substrate for caspase-5 and Ac-Tyr-Val-Ala-Asp-AMC (Ac-YVAD-AMC) as a susceptible substrate for caspase-1. Peptide cleavage was measured over 1 hour at 30°C using a Cytofluor 4000 series fluorescent plate reader (Perseptive Biosystems, Framingham, MA, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Caspase activity was expressed in nmol AMC released per minute of incubation time per milligram of lysate protein.

ELISA

Mouse IL-1α, IL-1β, CXCL1 (also known as IL-8 or KC) and TNF-α immunoassay kits were obtained from R&D Systems (Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions.

Immunoblotting
Cytosolic extracts of whole kidney or proximal tubular cells were immunoblotted as previously described in detail (Dursun et al., 2006). Immunoblot analyses were performed with the following antibodies: 1) a purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of active caspase-1 p10 of mouse origin (1:200) (catalogue number sc-514) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), 2) a rabbit polyclonal antibody to caspase-5 (1:500) (Code No JM-3029-100, MBL, Woburn, MA) that detects pro-caspase-5 (47 kDa) and cleaved caspase-5 (38 kDa), 3) a purified rabbit polyclonal antibody to ASC (1:200) (catalogue number sc-22514) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), 4) a polyclonal antibody to NLRP1 (1:200) (catalogue number #4990, Cell Signaling Technology, Danvers, MA), and 5) a polyclonal antibody to NLRP3 (1:1000) (catalogue number ab91525, Abcam, Cambridge, MA).

Statistical analysis

Non-normally distributed data was analyzed by the nonparametric unpaired Mann Whitney test. Multiple group comparisons are performed using analysis of variance (ANOVA) with posttest according to Newman-Keuls. A $P$ value of $<0.05$ was considered statistically significant. Values are expressed as means $\pm$ SE.

Results

Increase in ASC in cisplatin-induced AKI

Immunoblot of whole kidney showed increases in ASC (Figure 1A) on day 2 of cisplatin-induced AKI. NLRP3 protein was increased on day 2 of cisplatin-induced AKI, but the increase was not
statistically significant (Figure 1B). In the cisplatin-induced AKI model, we have previously demonstrated that there is increased caspase-1 activity and tubular apoptosis on day 2 and increased ATN, BUN and creatinine on day 3 after cisplatin-administration (Faubel et al., 2004). Thus, the increase in ASC and caspase-1 on day 2 preceded the development of AKI on day 3.

**Caspase-1, IL-1α and IL-1β are increased in cisplatin-treated proximal tubules**

To determine the localization of NLRP3 and the effect of cisplatin-treatment on NLRP3 in macrophages and proximal tubules, macrophages in culture and freshly isolated proximal tubules were studied. On immunoblot, NLRP3 (118 kDa) was present in the isolated macrophages, but was not increased with 10 or 50 µM cisplatin treatment for 6 or 12 hours (Figure 2A). We further investigated the inflammasome in a model of freshly isolated proximal tubules treated with 10 or 50 µM cisplatin that has been shown to induce necrosis and apoptosis (Dursun et al., 2006). On immunoblot, NLRP3 (118 kDa) was present in the freshly isolated proximal tubules but not increased by cisplatin treatment (Figure 2B). A dose-dependent increase in active caspase-1 protein (10 kDa) (Figure 2C) and an increase in caspase-1 activity (Figure 2D) was seen in proximal tubules treated with cisplatin. Caspase-1 activates IL-1β and IL-1α in the inflammasome (Martinon et al., 2009) (Keller et al., 2008). IL-1α levels were significantly increased in 10 µM and 50 µM cisplatin-treated groups compared to vehicle-treated (Figure 2E). IL-1β levels were increased in the 50 µM cisplatin-treated group compared to cisplatin 10 µM and vehicle-treated groups (Figure 2F).

**NLRP3 -/- mice are not protected against cisplatin-induced AKI**

The decrease in serum creatinine in NLRP3 -/- mice compared to wild type mice with cisplatin-induced AKI did not reach statistical significance (Figure 3). BUN, ATN score and apoptosis
scores were not statistically different between wild type mice and NLRP3 -/- mice with cisplatin-induced AKI (Figure 3).

**Increase in NLRP1 and caspase-5 in cisplatin-induced AKI**

On immunoblot, there was a large increase in NLRP1 on day 3 of cisplatin-induced AKI (Figure 4A). NLRP1 was decreased in NLRP3 -/- mice on day 3 of cisplatin-induced AKI compared to wild type mice (Figure 4B). There was an increase in caspase-5 protein on days 2 and 3 of cisplatin-induced AKI (Figure 4C). Caspase-5 activity was significantly increased on day 3 of cisplatin-induced AKI (Figure 4D). Caspase-1 activity was increased in cisplatin-induced AKI in both wild type and NLRP3 -/- mice compared to vehicle treated mice (Figure 4E).

**NLRP3 -/- mice are protected against ischemic AKI**

We have previously demonstrated the injurious role of caspase-1-mediated production of IL-18 in ischemic AKI (Melnikov et al., 2001) (Melnikov at al., 2002). As caspase-1 and IL-18 are activated in the NLRP3 inflammasome, we determined whether NLRP3 -/- mice are protected against ischemic AKI. BUN, serum creatinine, ATN score and apoptosis scores were significantly decreased in NLRP3 -/- mice compared to wild type mice with ischemic AKI (Figure 5). Representative pictures of kidney histology are shown in Figure 6A, B and C.

Caspase-1, neutrophil infiltration, ASC, NLRP1 and NLRP3 in ischemic AKI

Caspase-1 activity was increased in ischemic AKI in both wild type and NLRP3 -/- mice compared to sham-operated treated mice (Figure 7A). Neutrophil infiltration in the kidney was increased in ischemic AKI in both wild type and NLRP3 -/- mice compared to sham-operated...
treated mice (Figure 7B). NLRP3, but not ASC or NLRP1, is increased in ischemic AKI (Figure 7C).

Cytokines in cisplatin-induced versus ischemic AKI (Table 1)
To determine the possible reason for the differences in protection against cisplatin-induced AKI versus ischemic AKI in NLRP3 -/- mice, the pro-inflammatory cytokines IL-1β, IL-6, CXCL1 and TNF-α were studied. IL-1β was increased in ischemic AKI but not cisplatin-induced AKI. IL-6 and CXCL1 were increased in both cisplatin-induced and ischemic AKI. TNF-α was increased in cisplatin-induced AKI but not ischemic AKI. None of the cytokines were significantly decreased in NLRP3 -/- mice with cisplatin-induced or ischemic AKI.

Discussion
Diseases associated with increased inflammasome activity include familial Mediterranean fever (FMF), adult onset Still’s disease, Behcet’s disease, vitiligo (mutation in NLRP1), gout and pseudogout (Martinon at al., 2009) (Lamkanfi and Dixit, 2012) (Strowig et al., 2012). An interesting report that cisplatin treatment triggers FMF attacks (Toubi et al., 2003) stimulated our interest to study the role of the inflammasome in cisplatin-induced AKI. This link between cisplatin treatment and FMF relapse lies in increased production of IL-6, IL-1, IL-8 and TNF-α that is common to both FMF and AKI. This is important clinically as cisplatin may aggravate the inflammatory response in patients with diseases characterized by NLRP3 mutations or NLRP3 activation.
The role of inflammation in AKI, especially in ischemic AKI, has been described previously with involvement of leukocytes, adhesion molecules, and endothelial injury (Bonventre and Zuk, 2004) (Friedwald and Rabb H, 2004) (Devarajan, 2006) (Ramesh and Reeves, 2004). There is also increasing evidence that cisplatin-induced AKI is an inflammatory process (Faubel at al., 2007) (Faubel et al. 2004) (Lu et al., 2007). Early inflammation is largely mediated by the innate immune system, which provides rapid non-adaptive responses against infections and injuries.

The innate immune system uses an array of germline encoded pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), to detect pathogen-associated molecular patterns (PAMPs). Damaged cells e.g. from trauma or ischemia release endogenous danger signals or danger-associated molecular patterns (DAMPs) that alert the innate immune system to imminent tissue damage (Iyer et al., 2009). NLRP1 and NLRP3 proteins act as PRRs to detect PAMPs and DAMPs (Schroder et al., 2010) (Schroder and Tschopp, 2010). Within the cytoplasm, NLRP proteins form a complex, the inflammasome, with ASC and inactive caspase-1. NLRP inflammasomes and caspase-1 are essential components of the inflammatory response. In the present study, there was an increase in crucial inflammasome proteins ASC and NLRP1 in the kidney and an increase in caspase-1 activity in the kidney in cisplatin-induced AKI. The increase in caspase-1 activity in cisplatin-induced AKI occurred in both wild type and NLRP3 -/- mice. The lack of a decrease in caspase-1 activity in NLRP3 -/- mice was associated with the lack of protection against cisplatin-induced AKI.

To determine the localization of inflammasome activation by cisplatin, we examined macrophages, the major source of NLRP3 (Anders and Muruve, 2011). NLRP3 was present in macrophages in culture, but was not increased by low or high dose cisplatin. In this regard, we have demonstrated that while macrophages increase on day 2 in cisplatin-induced AKI that macrophage depletion using liposomal encapsulated clodronate is not protective against
cisplatin-induced AKI (Lu et al., 2007). The lack of protection by macrophage depletion argues against the macrophage as a source of injurious the NLRP3 inflammasome in cisplatin-induced AKI. NLRP proteins are expressed in CD4 T cells (Wilmanski et al., 2008). We and others have demonstrated that CD4 T cells increase early in cisplatin-induced AKI and that CD4 T cell depletion markedly protects against cisplatin-induced AKI (Akcay et al., 2011). Thus future investigation of CD4 T cells as a source of the NLRP1 inflammasome in cisplatin-induced AKI would be interesting.

Next, to further determine the localization of inflammasomes in kidney, we examined proximal tubules, the major site of injury in cisplatin and ischemic AKI. There was an increase in caspase-1, IL-1α and IL-1β in cisplatin-treated proximal tubules that was not associated with an increase in NLRP3 suggesting that activation of caspase-1, IL-1α and IL-1β may be independent of the NLRP3 inflammasome in cisplatin-treated proximal tubules.

The role of the inflammasome is starting to emerge in kidney diseases (Anders and Muruve, 2011). A role of the NLRP3 inflammasome was demonstrated in a unilateral ureteric obstruction model of CKD (Vilaysane et al., 2010). NLRP3-deficiency protects mice against mortality, renal dysfunction, and neutrophil influx in a model of ischemic AKI (Iyer et al., 2009). In another study in ischemic AKI in mice, it was concluded that NLRP3 causes AKI by a direct effect on renal tubular epithelium and that the AKI was independent of inflammasome-induced pro-inflammatory cytokine production (Shigeoka et al., 2010). The present study demonstrates for the first time an increase in ASC, the major component of the NLRP3 and NLRP1 inflammasome, and NLRP1 protein in the kidney in cisplatin-induced AKI. However, the lack of a statistically significant increase in NLRP3 in the kidney in cisplatin-induced AKI and in proximal tubules treated with cisplatin and the lack of protection against cisplatin-induced AKI in NLRP3 -/- mice suggest that the NLRP3 inflammasome is not involved in cisplatin-induced AKI.
The NLRP1 inflammasome consists of NLRP1 protein, ASC protein and caspase-1 (Martinon et al., 2009). Both caspase-5 protein and activity and NLRP1-protein were increased in cisplatin-induced AKI suggesting a role for the NLRP1 inflammasome in cisplatin-induced AKI. In future studies, when NLRP1 -/- mice become available, study of the role of the NLRP1 inflammasome in cisplatin-induced AKI will be interesting.

Two previous studies have reported protection against ischemic AKI in NLRP3 -/- mice (Iyer et al., 2009) (Shigeoka et al., 2010). There are important similarities and differences between our study and these previously reported studies. The present study confirms the impressive decrease in serum creatinine and ATN scores in ischemic AKI reported in the previous studies. In addition, we describe an impressive decrease in BUN, another marker of kidney function. Our model of ischemia differed from the two previous studies in that we used bilateral renal pedicle (artery and vein) clamping whereas the previous studies used a model of clamping of the renal artery only. In the previous study, there was a decrease in IL-1β in the kidney in NLRP3 -/- mice. In our study, there was not a decrease in IL-1β or other cytokines, IL-6, CXCL1 or TNF-α in NLRP3 -/- mice. In one of the previous studies, there was a decrease in caspase-1 in the kidney in NLRP3 -/- mice (Shigeoka et al., 2010). In the present study, caspase-1 activity was increased in ischemic AKI in both wild type and NLRP3 -/- mice. These results indicate that the protection against ischemic AKI in NLRP3 -/- mice in the present study occurs despite the increase in caspase-1 activity. The previous study demonstrated a large decrease in TUNEL-positive tubular cells in NLRP3 -/- mice. Morphology is the gold standard for detection of apoptosis and TUNEL staining fails to discriminate between PT apoptosis and necrosis especially in vivo in the kidney and TUNEL staining grossly overestimates PT apoptosis in the kidney (Andrade L et al., 2000) (Grasl-Kraupp et al., 1995) (Gobe et al. 2000). In the present study, morphologic criteria e.g. cellular rounding and shrinkage, nuclear chromatin compaction
and formation of apoptotic bodies were used to detect apoptosis and demonstrated a less impressive decrease in apoptotic tubular cells than previously reported using TUNEL-staining in NLRP3 -/- mice.

In summary, there is an increase in the inflammasome components ASC and caspase-1 in the kidney in mice with cisplatin-induced AKI and caspase-1, IL-1β and IL-1α in proximal tubules treated with cisplatin. The increase in caspase-1 in kidney and proximal tubules was not associated with a statistically significant increase in NLRP3 protein and NLRP3 -/- mice were not protected against cisplatin-induced AKI suggesting that the NLRP3 inflammasome is not a mediator of cisplatin-induced AKI. In contrast NLRP3 -/- mice are protected against ischemic AKI confirming the role of the NLRP3 inflammasome in ischemic AKI. The difference in protection against cisplatin-induced AKI vs. ischemic AKI in NLRP3 -/- mice was not explained by differences in proinflammatory cytokines IL-1β, IL-6, CXCL1 or TNF-α. NLRP1 and caspase-5, components of the NLRP1 inflammasome were increased in cisplatin-induced AKI. Thus, the role of other inflammasomes merits future study in cisplatin-induced AKI.

Authorship Contributions

Participated in research design: Edelstein, Jani, Keys

Conducted experiments: Edelstein, Kim, Lee, Akcay, Nguyen, He, Ravichandran

Performed data analysis: Edelstein, Ljubanovic

Wrote or contributed to writing of manuscript: Edelstein, Kim, Lee, Keys, He, Ravichandran, Jani
References


Footnotes

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Legends for Figures

Fig. 1. Renal ASC and NLRP3 in cisplatin-induced AKI.

On immunoblot of whole kidney extracts, there was an increase in ASC (24 kDa) (A) in cisplatin-induced AKI. There was an increase in NLRP3 (118 kDa) (B) in cisplatin-induced AKI that was not statistically significant. In densitometric analysis of immunoblots, data are presented as protein/actin ratios plotted on the y-axis. β-actin used as a loading control was not different between the groups. Representative immunoblots of at least 3 separate experiments. *P<0.05 vs. Veh (Vehicle), **P=0.1 vs. Veh (Vehicle).

Fig. 2. NLRP3 in macrophages and NLRP3, caspase-1, IL1β and IL-1α in freshly isolated proximal tubules treated with cisplatin

NLRP3 was not increased in macrophages treated with cisplatin 10 or 50 μM for 6 or 12 hours (A) or proximal tubules (B) treated with cisplatin 10 or 50 μM for 25 minutes. Caspase-1 protein (C), caspase-1 activity (D), IL-1β (E) and IL-1α (F) were increased in proximal tubules treated with cisplatin. β-actin used as a loading control was not different between the groups. Representative immunoblots of at least 3 separate experiments.
Fig. 3. Serum creatinine, BUN, ATN score and apoptosis in NLRP3 -/- mice in cisplatin-induced AKI

Creatinine and BUN were increased in cisplatin-induced AKI (Cis) compared to vehicle treated (Veh) mice. Creatinine, BUN, ATN score and the number of apoptotic tubular cells were not significantly decreased in NLRP3 -/- mice with AKI compared to wild type (WT) mice with AKI. *P<0.01 vs. WT Veh, ‡ P<0.01 vs. WT Veh, NS vs. WT Cis. The number of animals per group is indicated on the figure. NS = not significant

Fig. 4. NLRP1 caspase-5 and caspase-1 activity in cisplatin-induced AKI

NLRP1 protein (A) was increased in kidney on day 3 of cisplatin-induced AKI. NLRP1 protein was decreased in NLRP3 -/- mice on day 3 of cisplatin-induced AKI compared to wild type mice (B). Caspase-5 protein (C) and caspase-5 activity (D) were increased in cisplatin-induced AKI. In densitometric analysis of immunoblots, data are presented as protein/actin ratios plotted on the y-axis. β-actin used as a loading control was not different between the groups. Representative immunoblots of at least 3 separate experiments. *P<0.05 vs. Veh (Vehicle). Caspase-1 activity was increased in cisplatin-induced AKI in both wild type and NLRP3 -/- mice compared to vehicle treated mice (E). n=4 per group. *p<0.01 vs. Veh.

Fig. 5. Serum creatinine, BUN, ATN score and apoptosis in NLRP3 -/- mice in ischemic AKI

Creatinine, BUN, ATN score and the number of apoptotic tubular cells were increased in ischemic AKI (AKI) compared to sham-operated (sham) mice. Creatinine, BUN, ATN score and
the number of apoptotic tubular cells were significantly decreased in NLRP3 -/- (-/-) compared to wild type (WT) mice with ischemic AKI. *P<0.01 vs. WT Sham or -/- sham mice. **P<0.001 vs. WT AKI. ***P<0.01 vs. WT AKI. ****P<0.05 vs. WT AKI. The number of animals per group is indicated on the figure.

Fig. 6. Representative pictures of kidney histology

Periodic acid-Schiff (PAS) -stained section of wild type sham-operated (A), wild type ischemic AKI (B) and NLRP3 -/- mouse ischemic AKI (C) are shown. In wild type sham-operated (A) kidneys the tubules have well-defined brush borders without necrosis or apoptosis. In wild type ischemic AKI (B) there is acute tubular necrosis (large arrows) and tubular cell apoptosis (small arrows). In NLRP3 -/- mouse ischemic AKI (C) acute tubular necrosis and tubular cell apoptosis is significantly reduced.

Fig 7. Caspase-1, neutrophil infiltration, ASC, NLRP1 and NLRP3 in ischemic AKI

Caspase-1 activity was increased in ischemic AKI in both wild type (WT) and NLRP3 -/- (-/-) mice compared to sham-operated treated mice (A). n=4 per group. *P<0.05 vs. sham. Neutrophil infiltration in the kidney was increased in ischemic AKI in both wild type (WT) and NLRP3 -/- (-/-) mice compared to sham-operated treated mice. n=8 per group. *P<0.05 vs. sham. NLRP3, but not ASC or NLRP1, is increased in ischemic AKI (Figure 7C). n=4-6 per group. *P<0.05 vs. sham. In densitometric analysis of immunoblots, data are presented as protein/β-actin ratios plotted on the y-axis.
Table 1: Cytokines in cisplatin-induced and ischemic AKI.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle WT</th>
<th>Cisplatin WT</th>
<th>Cisplatin NLRP3 −/−</th>
<th>Sham WT</th>
<th>Ischemia WT</th>
<th>Ischemia NLRP3 −/−</th>
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<tr>
<td>IL-1β (pg/mg) N=3-6</td>
<td>4.2 ± 1.1</td>
<td>6.2 ± 0.9</td>
<td>9.6 ± 1.3</td>
<td>4.2 ± 0.7</td>
<td>5.9 ± 0.3</td>
<td>11.4 ± 5.1</td>
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<tr>
<td>IL-6 (pg/mg) N=3-6</td>
<td>1.4 ± 0.4</td>
<td>4.0 ± 0.8*</td>
<td>2.0 ± 0.4†</td>
<td>1.9 ± 0.2</td>
<td>9.1 ± 0.2**</td>
<td>22.4 ± 13**</td>
</tr>
<tr>
<td>CXCL1 (pg/mg) N=3-6</td>
<td>2.4 ± 0.7</td>
<td>112.5 ± 7.2**</td>
<td>98.0 ± 24.5**</td>
<td>7.6 ± 0.8</td>
<td>116 ± 4.7**</td>
<td>107.6 ± 25**</td>
</tr>
<tr>
<td>TNF-α (pg/mg) N=3-6</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.1**</td>
<td>1.2 ± 0.1***</td>
<td>0.8 ± 0.2</td>
<td>0.2 ± 0.02</td>
<td>0.5 ± 0.05</td>
</tr>
</tbody>
</table>

WT= wild type. *p<0.05 vs. Vehicle/Sham WT, **P<0.01 vs. Vehicle/Sham WT, †P<0.05 vs. Cisplatin WT.
Figure 1

A

ASC

β-actin

24 kDa

43 kDa

Veh Day 1 Day 2 Day 3

ASC-CARD/actin

Veh Day 1 Day 2 Day 3

* *

B

NLRP3

β-actin

118 kDa

43 kDa

Veh Day 1 Day 2 Day 3

NLRP3/actin

Veh Day 1 Day 2 Day 3

**
Figure 3

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Figure 7

A. Caspase-1 activity (nmol/min/mg)

B. Neutrophils (per 10 HPF)

C. ASC, NLRP3, NLRP1, B-actin protein levels

Sham: WT, AKI: (−/−)