KSN 2020
FULLY VIRTUAL MEETING

Abstract Submission No. : IL-9030

Mechanisms of tissue inflammation and injury in the kidney

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Acute kidney injury (AKI) is associated with short-term and long-term complications. Sepsis is the most common cause of AKI in critically ill patients that is associated with high rates of morbidity and mortality. An episode of AKI may be linked to later development of chronic kidney disease (CKD). At present, there are no effective pharmacologic therapies for the treatment of AKI. Thus, understanding the mechanism of the pathogenesis of kidney injury is critical in developing therapies. We have uncovered a novel role of the necroptosis pathway as a key mediator of sepsis-induced AKI, that involves mitochondrial dysfunction, and the development of kidney fibrosis, which is a hallmark of progressive CKD.

Key mechanisms that underlie sepsis-induced organ injury are mediated via mitochondrial dysfunction. The NLRP3 inflammasome pathway leading to the activation of pro-inflammatory interleukins is well known. We have recently demonstrated the role of the necroptosis pathway, a genetically regulated form of necrotic cell death, that involves activation of Receptor Interacting Protein Kinases (RIPK1 and RIPK3), which in turn activate the mixed lineage kinase domain like (MLKL) pseudokinase. Apoptotic cell death is characterized by DNA fragmentation and cell shrinkage, whereas in necroptotic cell death, we see cell swelling and ultimately cell membrane rupture.

In our previous studies, we reported that the necroptosis mediator RIPK3 promotes kidney fibrosis, and this occurs independently of the classical MLKL-mediated necroptosis, but rather via an AKT-dependent fatty acid synthesis pathway to induce kidney fibrosis. To investigate sepsis-induced AKI, we employed the cecal ligation and puncture (CLP) model, which is a model of polymicrobial sepsis. Using the CLP-induced sepsis model, we examined the functional role of RIPK3 in acute kidney injury and the involvement of mitochondrial dysfunction.

Sepsis induces RIPK3 in kidney tubular epithelial cells, as demonstrated by stimulation with lipopolysaccharide (LPS) in cultured proximal tubular epithelial cells, and in the kidney after CLP. In the knockout mice, deficient in RIPK3, we observe attenuation of kidney tubular injury after CLP, as demonstrated by lower kidney injury score compared to the wildtype mice, and lower levels of urine lipocalin-2 and beta-2 microglobulin, as well as decreased KIM-1 (kidney injury molecule-1) expression compared to the wildtype mice. Therefore, Ripk3 deficiency protects against kidney tubular injury in experimental sepsis. On the other hand, MLKL deficiency fails to protect against kidney tubular injury.

We further show that RIPK3 regulates NADPH oxidase-4 (NOX4) during sepsis-induced kidney injury. NOX4 is a critical regulator of macrophage inflammatory responses and is localized primarily to the mitochondria of macrophages. Using human proximal tubular epithelial cells in which RIPK3 is knocked down by siRNA or by CRISPR/Cas9 genome editing, and using primary tubular epithelial cells isolated from *Ripk3* knockout mice show that NOX4 induction by LPS or bacterial DNA is attenuated upon RIPK3 knockdown. Furthermore, this RIPK3 regulation of NOX4 occurs via post-transcriptional mechanism, and the co-immunoprecipitation studies suggest that this involves intermolecular interactions between RIPK3 and NOX4. Similar findings of RIPK3-dependent NOX4 regulation were seen in vivo as shown in the *Ripk3* knockout mice subjected to CLP, compared to the wildtype mice. Accordingly, when we examined *Nox4* knockout mice, we found that Nox4 deficiency reduced kidney tubular injury in CLP-induced sepsis. RIPK3 deficiency also reduced ROS levels in the kidney during sepsis, as well as in human proximal tubular epithelial cells after LPS challenge. These results indicate that RIPK3 promotes oxidative stress.

We next examined the effects on mitochondrial function first in cell culture studies using human proximal tubular epithelial cells after LPS challenge and show that RIPK3 deficiency reduced mitochondrial dysfunction. Ultrastructural analysis of mitochondrial morphology by electron microscopy (EM) demonstrated evidence of mitochondrial damage in the wildtype mice after CLP (widening of mitochondrial cristae and swelling of mitochondria) which were absent in the *Ripk3* knockout mice after CLP. RIPK3 also regulates mitochondrial complex protein expression following CLP, particularly mitochondrial complex I and –III in sepsis-induced kidney injury.

Additionally, elevated mitochondrial DNA (mtDNA) levels, a marker of mitochondrial injury, were observed in the plasma of the wildtype mice 6 hours after CLP, relative to sham control This was not observed in *Ripk3* knockout mice. Thus, sepsis induces RIPK3, which in turn through NOX4, resulting in mitochondrial dysfunction and necroptosis in the proximal tubules of the kidney.

Unlike apoptotic cell death, necroptosis is characterized by cell swelling and plasma membrane rupture. Thus, mtDNA released from damaged mitochondria can be released extracellularly. These extracellular mtDNA can serve as DAMPS (damage-associated molecular patterns) to activate innate and adaptive immune responses to recruit immune cells, and the inflammatory response further exaggerates kidney injury.

In human samples obtained from patients admitted to our ICU with sepsis, we measured RIPK3 mRNA expression in the urinary cells, and RIPK3 protein expression in the urine and plasma, and show higher RIPK3 levels in patients with sepsis-associated AKI. Moreover, both urinary and plasma mtDNA levels were higher in patients with sepsis-associated AKI. Together, these results indicate that urine and plasma RIPK3 protein levels, as well as mtDNA copy number, are significantly associated with sepsis-induced AKI, and could serve as potential biomarker.

Maintaining healthy mitochondria is critical for normal kidney function. The removal of dysfunctional mitochondria by a conserved process known as mitophagy is crucial for mitochondrial quality control. Mitophagy is a form of selective autophagy that targets damaged mitochondria for degradation, and is controlled by several proteins including PINK1 and Parkin. PINK1 facilitates the mitochondrial recruitment of Parkin, which catalyzes the poly-ubitquitination of damaged mitochondria. Mitophagy together with mitochondrial fusion and fission are tightly interconnected processes, and the balance between these processes is essential in maintaining a healthy network of mitochondria. Alterations in mitochondrial dynamics are implicated in kidney injury and the progression of kidney diseases.

We noted that mitophagy is downregulated in CKD. Reduced expression of mitophagy regulators PINK1, mitochondrial fusion protein mitofusin 2 (MFN2), and Parkin is observed in human CKD kidneys. Similarly, expression of MFN2, Parkin, and LC3 II was downregulated in experimental model of kidney fibrosis induced by unilateral ureteral obstruction (UUO). Treatment with TGF-β1 downregulated these mitophagy-related proteins MFN2 and Parkin in mitochondrial fractions isolated from macrophages. PINK1-mediated mitophagy in macrophages is also suppressed in a model of chronic kidney injury induced by adenine diet (AD), and by TGF-β1 treatment.

We confirmed that deficiency of either of the mitophagy mediators PINK1 or Parkin promoted kidney fibrosis, as demonstrated by increased trichrome blue staining of the UUO kidneys in *Pink1* or *Prkn* knockout mice. Furthermore, myeloid-specific knockout of *Mfn2* aggravated kidney fibrosis

To summarize our findings, our proposed model for the mechanism of AKI and CKD and role of necroptosis pathway and mitochondrial dysfunction is as follows:
- In AKI, RIPK3-mediated necroptosis pathway induces NOX4 and mitochondrial dysfunction, resulting increased oxidative stress and reduced ATP production. Damaged mitochondria release mtDNA, and extracellular mtDNA may serve as a biomarker of kidney injury.
- With persistent kidney injury, and in progression of CKD, RIPK3-mediated necroptosis pathway via

the AKT and FA synthesis pathway induces kidney fibrosis, which is a hallmark of CKD.
- In CKD, there is inhibition of mitophagy with decreased levels of PINK1, MFN2, and Parkin to induce mitochondrial dysfunction, increased oxidative stress, and reduced energy production, which stimulates profibrotic macrophages to also induce kidney fibrosis.
-RIPK3 represents a promising potential new therapeutic target in AKI and CKD.