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PROPHYLACTIC nCMT-3 ATTENUATES SEPSIS-INDUCED ACUTE KIDNEY INJURY IN ASSOCIATION WITH NLRP3 INFLAMMASOME ACTIVATION AND APOPTOSIS

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Abstract

Background: The kidney is the most common extrapulmonary organ injured in sepsis. The current study examines the ability of aerosolized nano-chemically modified tetracycline-3 (nCMT-3), a pleiotropic anti-inflammatory agent, to attenuate acute kidney injury (AKI) caused by intratracheal lipopolysaccharide (LPS).

Methods: C57BL/6 mice received aerosolized intratracheal (IT) nCMT-3 (1 mg/kg) or saline, followed by IT LPS (2.5 mg/kg) to induce ALI-induced AKI. Tissues were harvested at 24 h. The effects of nCMT-3 and LPS on AKI were assessed by plasma/tissue levels of BUN, creatinine, NGAL, KIM-1 and renal histology. Renal MMP level/activity, cytochrome C, Bax, Bcl-2, caspase-3, p38 MAPK activation, NLRP3 and caspase-1 were also measured. Apoptotic cells in kidney were determined by TUNEL assay. Renal levels of IL-1β and IL-6 were measured to assess inflammation.

Results: ALI-induced AKI was characterized by increased plasma BUN, creatinine, injury biomarkers (NGAL, KIM-1) and histologic evidence of renal injury. LPS-treated mice demonstrated renal injury with increased levels of inflammatory cytokines (IL-1β, IL-6), active

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Competing interests

MKS has received an educational research grant from Dräger Medical Systems, Inc. MKS has lectured for Intensive Care On-line Network, Inc. (ICON) and Dräger Medical, Inc. JL is the founder of Tantargo Therapeutics LLC, which has no conflict of interest with this study.

MMP-2 and MMP-9, pro-apoptotic proteins (cytochrome C, Bax/Bcl-2 ratio, cleaved caspase-3), apoptotic cells, inflammasome activation (NLRP3, caspase-1) and p38 signaling. Intratracheal nCMT-3 significantly attenuated all the measured markers of renal injury, inflammation, and apoptosis.

Conclusions: Pre-treatment with aerosolized nCMT3 attenuates LPS-induced AKI by inhibiting renal NLRP3 inflammasome activation, renal inflammation, and apoptosis.

Keywords

Acute kidney injury (AKI); Acute respiratory distress syndrome (ARDS); Acute lung injury; MMPs; Apoptosis; Inflammation; Inflammasome; nano-chemically modified tetracycline-3 (nCMT-3)

Introduction

Development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are frequently associated with the subsequent damage and dysfunction of multiple organs (1). The phenomenon of multiple organ dysfunction (MODS) is common in sepsis and associated with increased mortality in intensive care unit (ICU) patients (1, 2). The kidney is particularly susceptible to injury with acute kidney injury (AKI) occurring in 45% of ICU patients admitted with ARDS. Furthermore, AKI in patients with ARDS carries a mortality rate of 42% compared with 20% for those with ARDS alone (1). There is crosstalk between the lung and kidney such that ALI can lead to renal injury through a variety of mechanisms including ventilator-induced lung injury resulting in systemic inflammation, capillary permeability changes, and neurohormonal dysregulation resulting in hypoxemia, hypoperfusion and renal injury (3). ALI has been shown to increase circulating levels of inflammatory cytokines including: IL-1β, TNF-α, plasminogen activator inhibitor-1 (PAI-1), IL-6 and IL-8 is associated with higher rates of AKI (1). The etiology of ALI-induced AKI is multifactorial and includes a combination of inflammation, dysregulated renal microcirculation, altered endothelial/epithelial integrity, mitochondrial injury, renal cell apoptosis and NLRP3 inflammasome activation (3–5).

Chemically-modified tetracyclines (CMT) are a drug class that was modified to eliminate the antimicrobial activity associated with tetracyclines while retaining their anti-inflammatory properties. CMT-3 (6-demythyl-6-deoxy-4dedimentylamino-tetracycline) has demonstrated a significant reduction in ARDS incidence and mortality in both a rat cecal ligation and puncture model (6, 7) and porcine gut perfusion/reperfusion injury model (8, 9). Although CMT-3 has demonstrated efficacy in halting the progression of lung injury, its effect on pulmonary and subsequent renal injury has not been as well-studied. The beneficial anti-inflammatory effects of CMT-3 have, however, been well-elucidated. CMT-3 is recognized as a potent inhibitor of matrix metalloproteinases (MMPs) which are activated by inflammatory mediators during organ damage, including lung and kidney injury. MMP-9 has been implicated in the activation and migration of neutrophils, with elevated levels associated with ALI in bronchoalveolar lavage fluid (BALF) (10) whereas MMP-2 has been associated with AKI which is attenuated by treatment with minocycline (11). CMT-3 has also been shown to attenuate elastase and gelatinase proteases as well as multiple

inflammatory mediators including: soluble triggering receptor expressed on myelocytes-1 (sTREM-1), TNF-α, IL-1, IL-6, IL-18, and the NLRP3 inflammasome in experimental sepsis-induced ARDS. (12).

Despite its efficacy as a pleiotropic inflammation modulator with downstream organ protection (6–9, 12, 13), CMT-3 has not been popularized for clinical use. This is in part because it has only been available as an oral formulation due to high hydrophobicity and poor solubility, limiting administration ease and systemic absorption in critically ill patients with poor gut function. We recently developed a nanoformulation of CMT-3 in a 20–30 nm telodendrimer nanoparticle (nCMT-3), with reduced cytotoxicity for intratracheal (IT) administration (12). In our previous work, nCMT-3 was shown to be effective in attenuating LPS-induced ALI (12). Our previous study investigates the effect of IT administration on lung injury and explores the mechanisms by which nCMT-3 attenuates lung injury. The current study examines the ability of prophylactic IT nCMT-3 to attenuate AKI in the same murine model of LPS-induced ALI. This study was thus designed to interrogate the pulmonary-renal interaction, and to further elucidate the redundant inflammatory pathways that are shared by both the lung and kidney, offering a pathophysiologic mechanism and a therapeutic target for patients with sepsis, inflammation, lung injury and ALI-induced AKI.

Materials and Methods

nCMT-3 preparation, pharmacokinetics and tissue biodistribution

nCMT-3 synthesis and nanoformulation have been previously described and included pharmacokinetic and tissue biodistribution studies in C57BL/6J mice after intravenous or IT administration (12). IT dosing resulted in peak nCMT-3 plasma levels 30 min after administration as well as higher and more sustained pulmonary and renal levels of nCMT-3 compared with intravenous administration (12).

Animals and animal care

8-week old C57BL/6 mice were acquired from Jackson Laboratories (Bar Harbor, ME) and housed under controlled conditions (temperature 22°C, photoperiod 12-h light and 12-h dark cycle) with unlimited access to food and water. Animal studies were approved by SUNY Upstate Medical University's Institutional Animal and Use Committee (IACUC #344). Experiments were performed in concordance with the National Institute of Health and ARRIVE guidelines concerning proper use of laboratory animals.

Acute kidney injury induction by ALI, nCMT-3 treatment and tissue harvest

Non-invasive tracheal installation by aerosolization with either nCMT-3 (1 mg/kg) or a vehicle was completed 2 h prior to induction of lung injury with LPS (2.5 mg/kg) or saline, as previously described (12). Mice in both LPS and control groups were anesthetized with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail via intraperitoneal injection. Following induction of anesthesia, mice were placed in a supine position on the intubation platform. A fiber-optic illuminator was placed over the trachea and the tongue was carefully retracted in an upward and leftward position for visualization of the larynx.

Instillation of LPS, nCMT-3, and saline solution (with volume not exceeding 70 μl per mouse) was completed with MicroSprayer Aerosolizer (Cat. #: YAN30012, Shanghai Yuyan Instruments Co., Ltd) insertion into the tracheal lumen. 24 h post LPS instillation, surviving mice were sacrificed under anesthesia. Blood (EDTA as anticoagulant) and kidney tissue (fixed with 10% formalin for histology or frozen for protein analysis) were collected for further analysis.

Western blot and ELISA

Frozen kidney samples were homogenized in RIPA buffer and extracted protein was used for Western blot analysis. Total protein concentrations obtained from kidney samples were determined by the BCA micro assay kit (Cat. #: 23235, Thermo Scientific, Rockford, IL). 20 μg of protein were separated by SDS-PAGE gel and transferred to PVDF membranes (Cat. #: IPVH00010, Millipore Co., Ltd.). Membranes were incubated with 5% non-fat milk (Cat. #: 1706404, Bio-Rad Laboratories) in Tris-buffered saline with 0.5 % Tween-20 (TBS-T) at room temperature for 1 h, and then overnight at 4°C with primary antibodies purchased from Santa Cruz Biotechnology, including MMP-2 (Cat. #: sc-13594, 1:500 dilution), Bcl-2 (Cat. #: sc-7382, 1:500 dilution), Bax (Cat. #: sc-7480, 1:500 dilution), and β-actin (Cat. #: sc-47778, 1:500 dilution), Cell Signaling, including Phospho-p38 MAPK (14: 9211S, 1:1000 dilution), NLRP3 (14: 15101, 1:1000 dilution), Caspase-1 (14: 24232, 1:1000 dilution), Cleaved caspase-1 (14: 89332, 1:1000 dilution), and Abcam, including cleaved caspase-3 (Cat. #: ab49822, 1:1000 dilution). Secondary antibodies linked to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Cat. #: sc-516102, 1:2000 dilution, Cat. #: sc-2357, 1:2000 dilution) was applied for 1 h at room temperature. Antibody-antigen complexes were visualized using ECL (Cat. #: 34580, Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. All images were analyzed by densitometry with Image J software; relative density of immunoreactive bands was normalized to the density of the corresponding β-actin bands.

Homogenized kidney samples were also used for IL-1β (Cat. #: 88–7013-88, Invitrogen), IL-6 (Cat. #: 88–7064-88, Invitrogen), NGAL (14: MLCN20, R&D Systems) and KIM-1 (14: MKM100, R&D Systems). All cytokines and the markers of renal injury were measured using commercial ELISA kits according to the manufacturer's instructions.

Histological assessment of kidney injury

Kidney samples were fixed with 0.5 ml of 10 % neutral formalin via tracheal instillation for histology analysis and subsequently embedded in paraffin. 0.3 μm kidney tissue sections were obtained and stained with hematoxylin and eosin (H&E). Histopathology evaluation of acute kidney injury was completed in a blinded fashion by two independent pathologists using a modified 0–5 scoring system as previously described (15, 16). Briefly, histological changes caused by acute tubular necrosis (ATN) were assessed in H&E-stained tissue and quantified by counting the percentage of tubules showing cellular necrosis, brush border loss, 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 5 fields (200×) were reviewed per slide.

TUNEL assay for apoptotic cells

For detecting renal apoptotic cells, deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) kit (Cat. #: ab206386, Abcam) was used according to the manufacturer's instructions. Briefly, the sections were deparaffinized with xylene, dehydrated through a graded alcohol series (90%, 80% and 70%), and treated with permeabilization solution (proteinase K). The labeling reaction was performed using a solution containing terminal deoxynucleotidyl transferase (tdt). The DAB solution was used to detect apoptotic cells. After staining, the sections were mounted by mounting medium. Apoptotic cells were quantified by counting TUNEL-positive cells from four randomly selected consecutive fields at 400X magnification in a blinded manner by two experienced researchers. The apoptosis index is expressed as the percentage of the number of TUNEL positive cells in the total cells.

Matrix metallopeptidase activity by Gelatin zymography

MMP-9 activity in kidney tissue was quantified with gelatin zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing 0.1% gelatin were used for effective MMP digestion using 10 μg of total protein. Gels were then incubated for 24 h and stained with Coomassie Blue. Activity was determined through quantification of cleared substrate lysis bands and MMPs were isolated through analysis of molecular weight and degree of inhibition by ethylenediaminetetraacetic acid or phenanthroline. Quantification achieved through scanning densitometric analysis with NIH Image J software.

Statistical analysis

Analyses of the data was generated using GraphPad Prism software (version 5.0), with data expressed as mean ± SEM. Sample size of each experimental group (n= 3–7) is presented on the figure legend. Group differences were determined using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test. Significance was determined as $P<0.05$ and data was obtained from at least three independent experiments.

Results

Effects of nCMT-3 on renal function, injury and inflammation in LPS-induced ALI

Serum blood urea nitrogen (BUN) and creatinine are commonly used in the clinical setting to assess the presence and severity of renal dysfunction. Plasma levels of both BUN and creatinine (Fig. 1A & B) were increased in the LPS-treated mice ($P < 0.05$ vs. Vehicle), a finding consistent with ALI-induced renal dysfunction. nCMT-3 treatment had no effect on baseline plasma BUN and creatinine levels. However, pretreatment with nCMT-3 significantly attenuated the increase in BUN and creatinine observed in the LPS treated mice ($P < 0.05$ vs. LPS).

Renal injury was assessed by measuring plasma and renal levels of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). NGAL is a lipocalin expressed in renal tubular cells which serves as a sensitive biomarker for the development of early AKI (17). KIM-1 is another commonly used biomarker of AKI which is more

specific to acute nephrotoxic injury (17). The LPS-treated mice demonstrate increased plasma and renal levels of both NGAL and KIM-1 (Fig. 1 C, D, E & F, $P < 0.05$ vs. Vehicle). nCMT-3 had no effect on plasma or renal levels of NGAL or KIM-1 in healthy mice. In contrast, pretreatment with nCMT-3 ameliorated the LPS-induced increase in plasma and renal NGAL and KIM-1 (Fig. 1 C, D, E & F, LPS vs. LPS/nCMT-3, $P<0.05$). These results provide evidence that prophylactic administration of IT nCMT-3 significantly attenuates kidney injury in the ALI-induced kidney injury model.

Histologic assessment of acute tubular necrosis

Sepsis-induced AKI is characterized by histologic injury termed acute tubular necrosis (ATN). The histologic findings of ATN (tubular epithelial attenuation, loss of brush borders, detachment of tubular epithelial cells and necrotic debris) were assessed and quantified in a blinded fashion using a modified 0–5 scoring system as described in Methods. LPS-treated mice demonstrated a significant increase in renal injury index (Fig 2 A & B, $P<0.05$ vs. Vehicle) compared to controls. nCMT-3 alone had no effect on renal injury index, however nCMT-3 significantly attenuated the LPS-induced increase in renal injury index (Fig 2 A & B, LPS vs. LPS/nCMT-3, $P<0.05$).

Effect of nCMT-3 on matrix metallopeptidase levels and activity

MMPs (especially MMP-2 and MMP-9) are endopeptidases that are upregulated in the setting of AKI and modulate renal microvascular permeability (11). Furthermore, the expression of MMP-9 is positively associated with the levels of neutrophil gelatinase-associated lipocalin (NGAL) (18). Our previous study has shown that nCMT-3 reduces LPS-induced MMP-2 and MMP-9 in the lung, and the levels of neutrophil elastase (NE) in plasma and bronchoalveolar fluid (BALF) (12). In this study, we measured the levels of MMP-2 and MMP-9 in the kidney and the results show the LPS-induced increase in renal MMP-2 and MMP-9 levels was significantly reduced following treatment with nCMT-3 (Fig. 2A & B, LPS vs. LPS/nCMT-3, $P<0.05$).

nCMT-3 attenuates mitochondrial dysfunction and renal apoptosis

Mitochondria, which regulate energy metabolism are increasingly recognized in the pathogenesis of AKI. Mitochondrial damage resulting in the release of cytochrome C and p38 mitogen-activated protein kinase (MAPK) activation are pro-apoptotic signals in AKI. To assess the pathogenesis of LPS-induced AKI, renal cytochrome C levels, p38 MAPK phosphorylation, pro-apoptotic proteins (cleaved caspase-3, Bax/Bcl-2 ratio), and apoptotic cells were measured. Renal levels of cytochrome C and p38 phosphorylation (Fig. 4A & B) were increased in LPS-treated mice ($P<0.05$ vs. Vehicle). Consistent with these findings, the Bax/Bcl-2 ration, cleaved caspase-3 (Fig. 4 C & D) and apoptotic cells (Fig. 4E) were all increased in LPS-treated mice ($P<0.05$ vs. Vehicle). All of these parameters (cytochrome C, p38 MAPK phosphorylation, Bax/Bcl-2 ratio, cleaved caspase-3, and renal apoptosis) were attenuated in the LPS/nCMT-3 treated mice (Fig. 4, $P<0.05$ vs. LPS).

Effects of nCMT-3 on renal NLRP3 inflammasome/caspase-1 and inflammatory cytokines

A critical aspect of AKI involves activation of the NLRP3 inflammasome by pathogen- and damage-associated molecular pattern molecules (e.g. LPS). The resulting cleavage of caspase-1 activates the secretion of pro-inflammatory cytokines IL-1β and IL-18, which further regulate the assembly of downstream proinflammatory cytokines such as IL-6. Renal levels of the NLRP3 inflammasome and cleaved caspase-1 were significantly increased in LPS-treated mice (Fig 5. A & B, $P < 0.05$ vs. Vehicle). Consistent with this observation, renal levels of IL-1β and IL-6 were also increased in the LPS-treated mice (Fig. 5 C & D, $P < 0.01$ vs. Vehicle). All of these parameters (NLRP-3, Caspase-1, IL-1β and IL-6) were attenuated in the LPS/nCMT-3 group (Fig. 5, $P < 0.05$ vs. LPS).

Discussion

The COVID-19 pandemic has drawn attention to the limited treatment options available for ARDS as well as the significant morbidity and mortality associated with this disease. Current management strategies for ARDS include treatment of the underlying cause (sepsis, gastric aspiration, inhalation injury, etc.), avoiding ventilator-induced lung injury with protective mechanical ventilation, prone positioning, steroids, and neuromuscular blockade. Despite these supportive therapies, the mortality associated with ARDS has remained unchanged (19). Furthermore, ARDS may propagate the development of extrapulmonary organ injury leading to significantly worse clinical outcomes. The presence of either renal or pulmonary injury significantly increases sepsis-associated mortality (1, 20). Importantly, ARDS independent of sepsis can propagate renal injury by influencing renal circulation, vasomotor tone, and inflammation (1, 3, 21, 22). The pathogenesis of ARDS includes a combination of increased capillary permeability, alveolar flooding, pulmonary surfactant deactivation, and mechanical injury (14). The reasons for associated extrapulmonary organ injury are multifactorial. The evolution of AKI is particularly complex and involves injury-mediated signaling between the lung and kidney, highlighting the importance of studying the lung-kidney axis as a system rather than as uncoupled organs in order to understand the pathophysiology of injury and develop targeted therapeutic strategies.

The current study has several important findings that provide insight into how nCMT-3 attenuates AKI in mice with LPS-induced ALI. First, prophylactic nCMT-3 attenuates ATN and LPS-induced increases in plasma creatinine and BUN, as well as biomarkers of renal injury, NGAL and KIM-1, in the plasma and kidney. Second, nCMT-3 ameliorates LPS-induced p38 MAPK activation, mitochondrial dysfunction and apoptosis in renal tissue. Finally, prophylactic nCMT-3 attenuates renal MMP (MMP-2 and MMP-9) expression, NLRP3 inflammasome activation, and inflammation in LPS-induced AKI. Collectively, these findings support the pleiotropic anti-inflammatory mechanisms of nCMT-3 in attenuating AKI in this model.

Tetracyclines are MMP modulators that inhibit pathologically excessive collagenolysis, allowing for broad use as a prevention and treatment strategy in diseases that are mediated by upregulated MMP activation. CMT-3 has gained widespread use in treating a breadth of diseases with the particular advantage of chemical modification to eliminate the antibiotic properties. CMT-3 has further been used in experimental models of ARDS by mitigating

acute lung injury and subsequent mortality (6, 9). The most recent modification to CMT-3 involves a novel nanoformulation that allows it to be administered as an aerosol. IT administration of nCMT-3 results in enhanced delivery to both the lung and kidney tissues (vs. IV administration) (12). In addition to protecting the lung from LPS-induced injury, nCMT-3 is gradually released from the lung providing sustained circulating levels of nCMT-3 which appear to effectively protect downstream organs (like the kidney) from inflammation and subsequent organ injury.

Through measurement of commonly used histological evaluation and biomarkers such as creatinine and BUN, in addition to AKI-specific biomarkers such as NGAL and KIM-1, we established a therapeutic value for prophylactic nCMT-3 treatment. NGAL is a component of the innate immune system, acting as an early marker for nephrotoxic and ischemic acute kidney injury (23). Furthermore, there is a well-established relationship between MMP and NGAL whereby NGAL can bind to MMP-9 to form a complex and potentiate MMP activity by preventing degradation (18). KIM-1 is another biomarker specific for ischemic and nephrotoxic- AKI, with higher levels indicating worse clinical outcomes (24–26). Both biomarkers were included in the analysis of nCMT-3 efficacy pertaining to kidney injury, establishing that pretreatment with our novel nCMT-3 formulation attenuates ALI-induced AKI.

The key to preserving renal integrity in patients with lung injury, inflammation, and sepsis is to maintain renal cell survival. Understanding the mechanisms that promote tubular cell apoptosis is therefore critical to developing preventive and treatment strategies (27). Cytokine upregulation, MMP activation, and p38 MAPK and NLRP3 inflammasome activation have all been implicated in AKI pathogenesis. MMPs have received particular attention because of their ability to degrade matrix components involved in cell survival and their expression from renal collecting ducts (28), and proximal tubules (18). In a mouse model of AKI induced by ischemia-reperfusion, MMP-2 and MMP-9 levels increased following injury and correlate with tubular apoptosis and necrosis, where treatment with MMP inhibitors reduced tubular injury and improved renal function (11). Proximal tubular MMP-2 has been shown to be sufficient to induce epithelial-mesenchymal transition by disruption of basement membranes leading to spontaneous tubular atrophy even in the absence of superimposed injury (29). By corollary, MMP-2 gene deletion has been shown to protect against tubular injury (11). Similarly, MMP-9 gene deletion was found to prevent microvascular density loss in a model of ischemic kidney injury (30). These findings have been supported by other studies demonstrating MMP inhibition decreases histologic damage (31), apoptosis, cytokine release, and renal injury in experimental models of AKI (32, 33).

We demonstrated that prophylactic nCMT-3 not only significantly decreased the expression of active MMP-2 and MMP-9, which are consistent with our previous findings in the lung (12), but also decreased expression of inflammatory and pro-apoptotic signaling in the kidney following LPS-mediated lung injury, particularly activation of the p38 MAPK and NLRP3 inflammasome pathways. MAPK signaling has been implicated in the development of ALI and AKI, as the suppression of this signaling through blockage of p38 phosphorylation reduces progression of lung and kidney injury due to sepsis (34, 35). Apoptosis and NLRP3 inflammasome activation have been associated with the pathogenesis

of AKI (36, 37). where inhibition of the NLRP3 inflammasome pathway can alleviate AKI (38). nCMT-3 was found to attenuate increased expression of NLRP3, caspase-1, and IL-1β, which are all upregulated following activation of the NLRP3 inflammasome (12). The protective effect of nCMT-3 on ALI-induced AKI was further supported by a reduction in apoptotic proteins (caspase-3, Bax/Bcl-2 ratio, and cytochrome C) and apoptotic cells. Thus, nCMT-3 was found to inhibit the renal NLRP3 inflammasome pathway and downstream apoptosis, consistent with our previous findings in the lung (12). The NLRP3 inflammasome pathway has been shown by others to be involved in regulated AKI in the cecal-ligation and puncture (CLP) model of sepsis (39). In this study, genetic deletion of NLRP3 and caspase-1 inhibition with Z-YVAD-FMK attenuated CLP-induced AKI and apoptosis (39). The finding that genetic deletion of NLRP3 or its inhibition attenuates sepsis-induced, contrast induced, ischemic and cisplatin-induced AKI are consistent with our results and confirms an important role for the NLRP3 inflammasome in multiple forms of AKI (40).

Our results provide evidence that prophylactic treatment with aerosolized nCMT-3 not only ameliorates the development of lung injury but also kidney injury. Based on the connection of lung and kidney in pathology and physiology, in this study, we used ALI-induced AKI to investigate the effect of nCMT-3 on renal injury. Our previous study showed that the concentration of CMT-3 in the kidney of IT-treated mice reached its peak (>20% ID/g) after 9 hours, which may be due to the fact that the lung acts as a drug reservoir and provides continuous drug release, which can effectively protect these remote organs such as the kidney in sepsis. Therefore, the improvement of kidney injury in this study is likely due to the ability of nCMT-3 to reduce ALI as well as the systemic/renal effects of nCMT-3 on renal injury/inflammation. Furthermore, the inflammatory pathways propagating renal injury were found to parallel those in lung injury, including MMP activation, cytokine elevation, and activation of the p38 and NLRP3 inflammasome, all of which were mitigated by nCMT-3 administration.

Despite our encouraging results, there are several limitations to our study. While LPS-induced ALI is a commonly used experimental model, the complexity of human ALI cannot be reproduced in a murine model, specifically the role of ventilator-induced lung injury. Although IT administration of aerosolized nCMT-3 proved efficacious in mice, this drug delivery method has not yet been established in more clinically relevant large animal or human models. Additionally, our study was based on previous work examining the effects of IT nCMT-3 on LPS-induced ALI at 24 hours (12). Therefore, in this study we only looked at potential mediators and pathways 24 hours after LPS administration and are unable to provide information on changes in these mediators over time. Finally, this study relied on a prophylactic dosing model to determine the efficacy of nCMT-3 treatment in ALI and subsequent AKI but did not establish the efficacy of post-injury nCMT-3 administration. However, experimental studies have shown that nCMT-3 treatment post injury is effective in preventing lung injury in a murine cecal ligation and puncture model (6) and in a porcine sepsis plus ischemia-reperfusion injury model (9). In summary, prophylactic IT administration of nCMT-3 is associated with improvements in experimental ALI-induced AKI, presumably via multiple mechanisms as described above.

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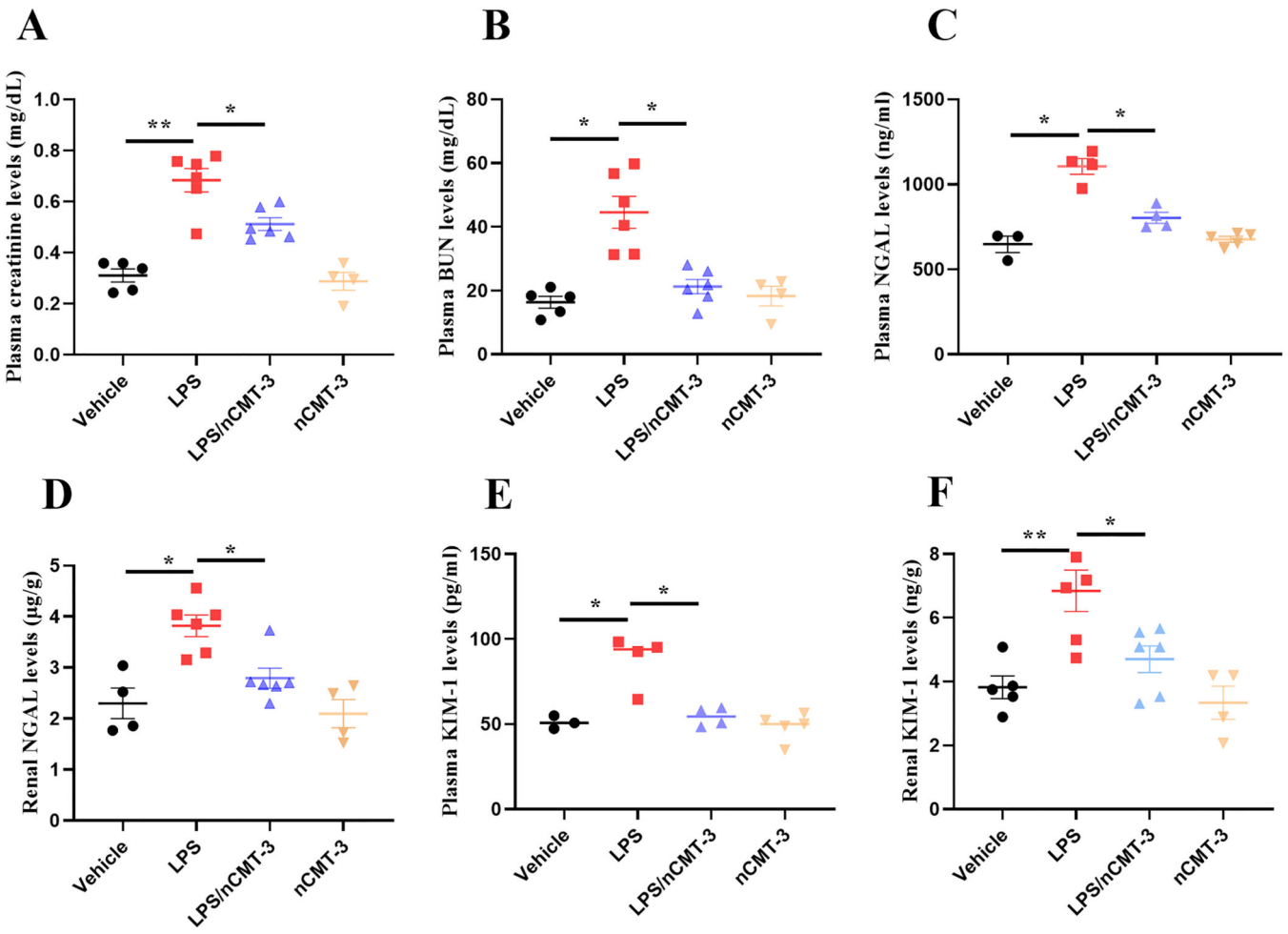

Fig. 1. Effects of nCMT-3 on renal function, injury and inflammation in LPS-induced ALI.

The mice were treated with nCMT-3 2 h prior to LPS-induced lung injury or PBS control. Plasma and kidney samples were harvested for protein analysis by ELISA. Plasma concentrations of creatinine and BUN (A & B), as well as the concentrations of neutrophil gelatinase-associated lipocalin (NGAL) (C & D) and kidney injury molecule-1 (KIM-1) (E & F) in plasma and kidney were examined. Data are presented as mean ± SE (n=3–6 per group). *: P<0.05, **: P<0.01.

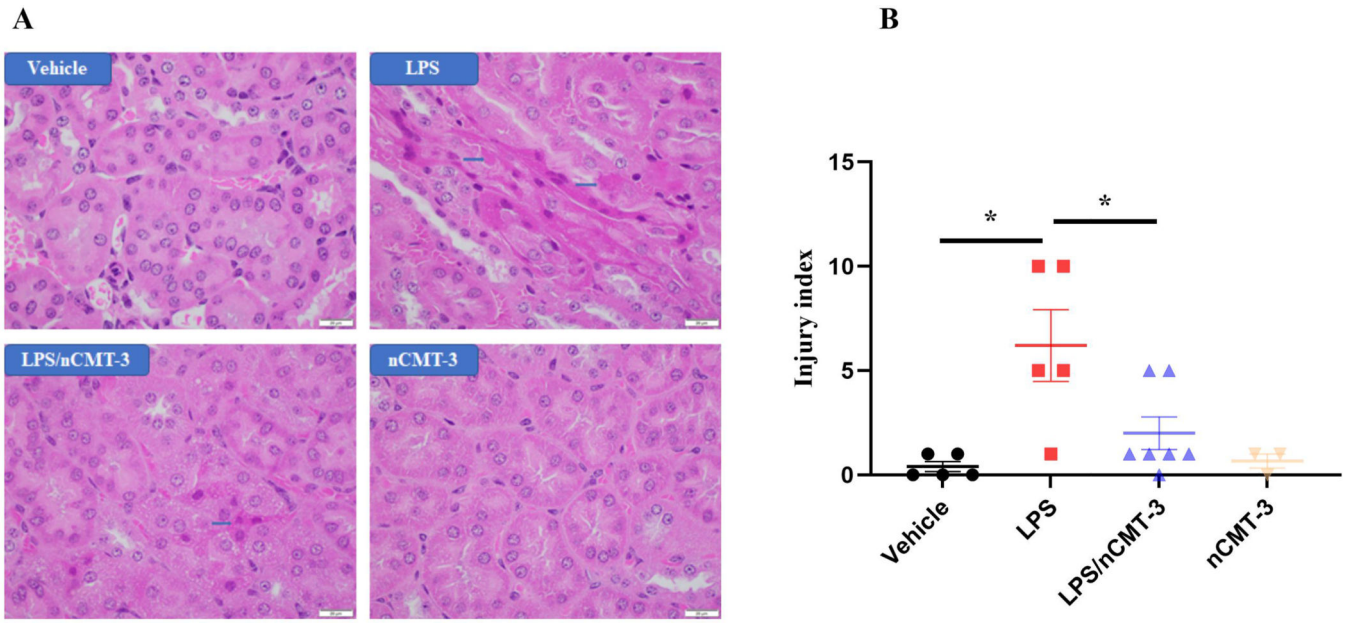

Fig. 2. Histological assessment acute tubular necrosis.

Mice were treated with nCMT-3 (1 mg/kg) or vehicle 2 h before induction of lung injury by LPS (2.5 mg/kg) or sham lung injury (by saline) using non-invasive tracheal installation by aerosolizer. Mice were sacrificed 24 h after LPS or saline administration, then kidney tissue was collected for H&E staining to evaluate histologic injury from each group (A). Kidney injury was characterized by acute tubular necrosis (ATN) (blue arrows). Semi-quantitative histological kidney injury score was assessed (B). Scatter dot plot represents mean values and standard error of mean (SE) (n=5–7 / group). *: P<0.05.

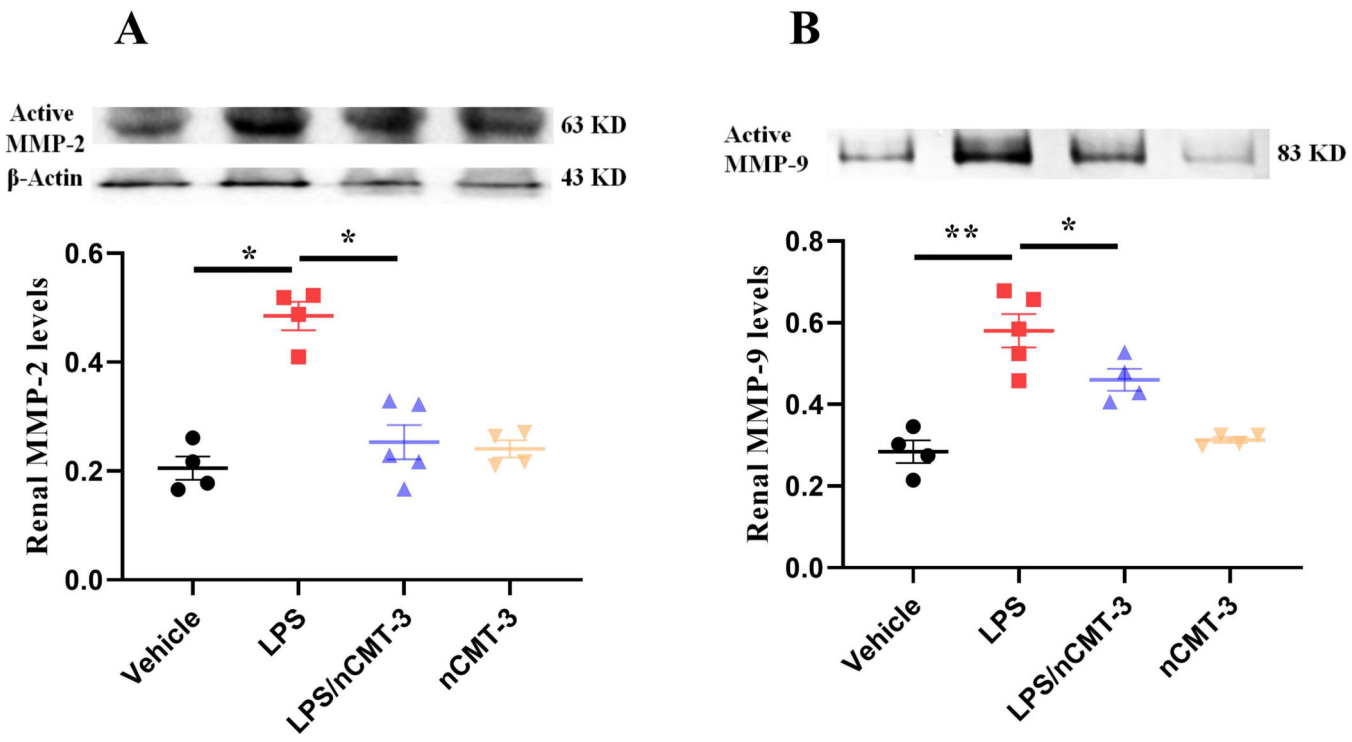

Fig. 3. Effect of nCMT-3 on matrix metallopeptidase levels and activity.

The mice were treated with nCMT-3 2 h prior to LPS-induced lung injury or saline control. Kidney samples were harvested for protein analysis by Western blot and gelatin zymography. MMP-2 (A) was examined by Western blot and MMP-9 (B) was examined by gelatin zymography. Scatter dot plot present mean values and standard error of mean (SE) (n = 4–5 / group). *: P<0.05, **: P<0.01.

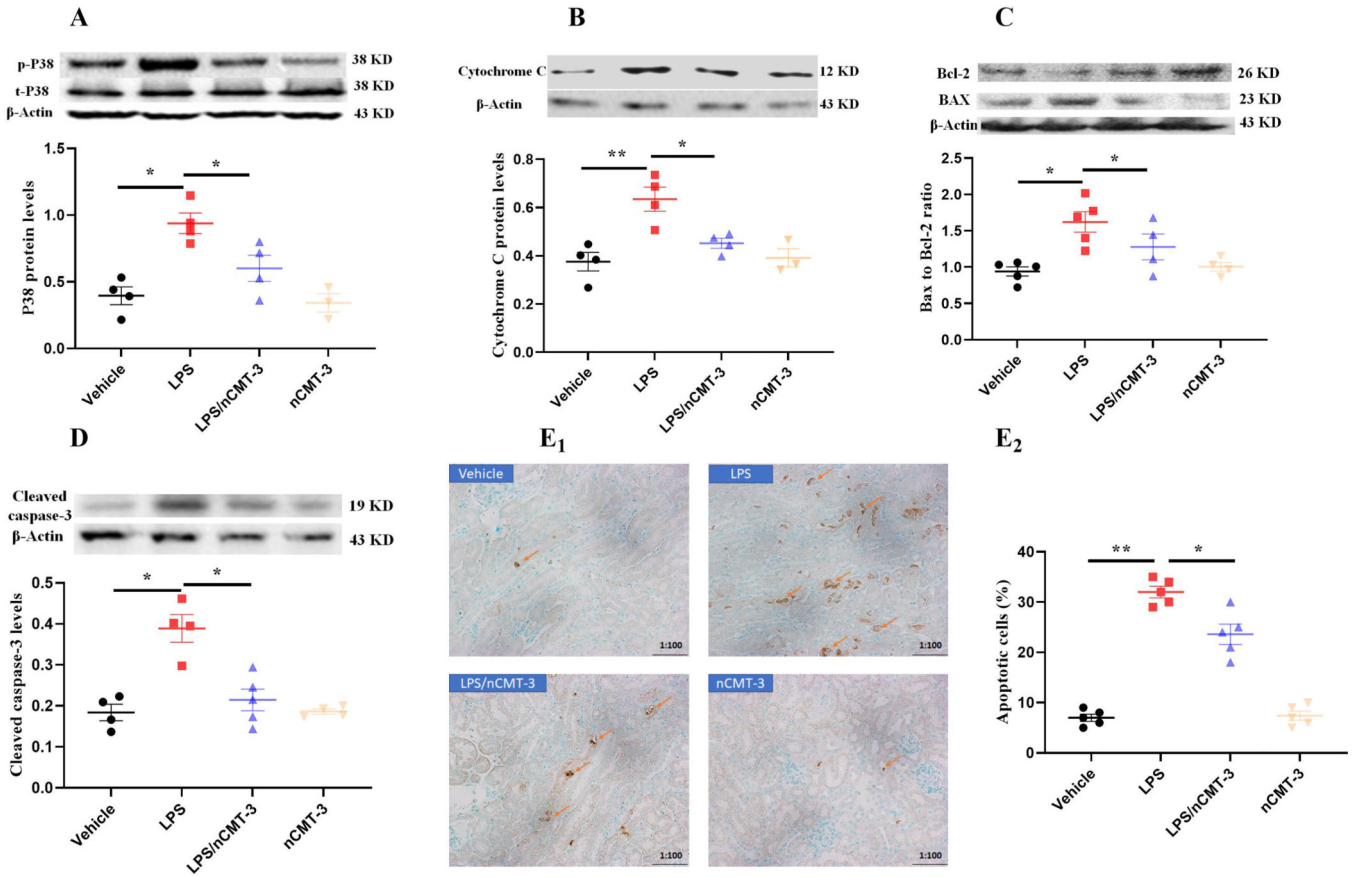

Fig. 4. nCMT-3 attenuates mitochondrial dysfunction and renal apoptosis.

Mice were treated with nCMT-3 (1 mg/kg) or vehicle 2 h before induction of lung injury with aerosolized non-invasive tracheal installation by LPS (2.5 mg/kg) or saline (sham). Mice were sacrificed 24 h after treatment. Kidney samples were harvested for protein analysis and TUNEL assay. p38 (A: phosphorylated p38/p-P38 and total p38/t-p38), cytochrome C (B), Bax/Bcl-2 (C), cleaved caspase-3 (D) were determined by Western blot. Apoptotic cells were determined by TUNEL assay (E_1 & E_2). Scatter dot plot present mean values and standard error of mean (SE) (n = 4–5 / group). *: P<0.05, **: P<0.01.

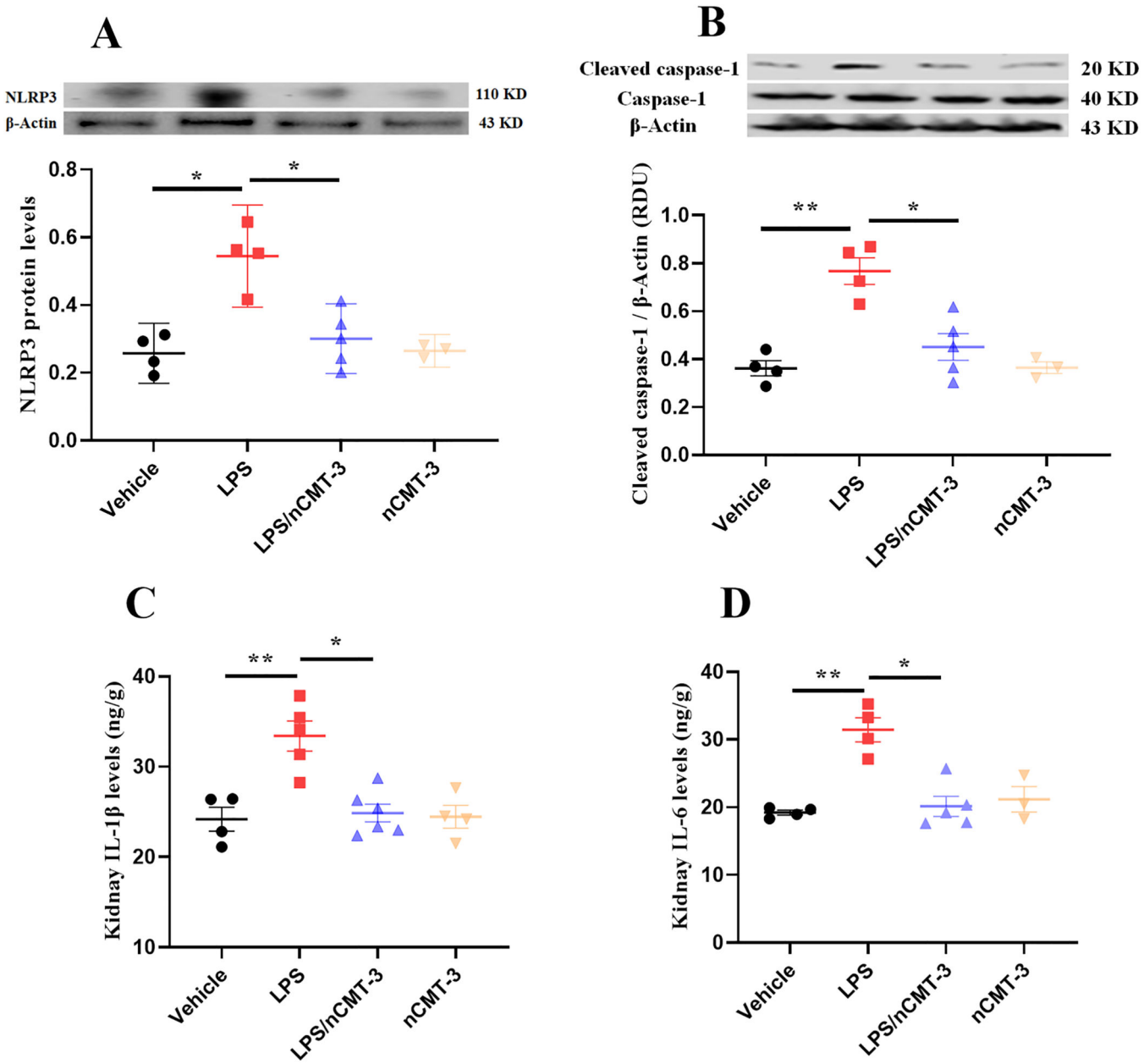

Fig. 5. Effects of nCMT-3 on renal NLRP3 inflammasome/caspase-1 and inflammatory cytokines.

Mice were treated with nCMT-3 (1 mg/kg) or vehicle 2 h before induction of lung injury with aerosolized non-invasive tracheal installation by LPS (2.5 mg/kg) or saline (sham). Mice were sacrificed 24 h after treatment. Kidney samples were harvested for protein analysis. NLRP3 (A) and caspase-1 (B) were determined by Western blot. IL-1β (C) and IL-6 (D) were assayed with ELISA. Scatter dot plot present mean values and standard error of mean (SE) (n = 4–5/ group). *: P<0.05, **: P<0.01.