Long non-coding RNA Neat1 promotes acute kidney disease via activation of NLRP3 inflammasome

Sydney Tang (scwtang@hku.hk)
The University of Hong Kong, Queen Mary Hospital https://orcid.org/0000-0002-6862-1941
Rui Xue
The University of Hong Kong, Queen Mary Hospital
Wai Han Yiu
The University of Hong Kong, Queen Mary Hospital
Kam Wa Chan
The University of Hong Kong, Queen Mary Hospital https://orcid.org/0000-0002-3175-1574
Sarah Lok
The University of Hong Kong, Queen Mary Hospital
Yixin Zou
The University of Hong Kong, Queen Mary Hospital
Jingyuan Ma
The University of Hong Kong, Queen Mary Hospital
Hongyu Li
The University of Hong Kong, Queen Mary Hospital
Loretta YY Chan
The University of Hong Kong, Queen Mary Hospital
Kar Neng Lai
The University of Hong Kong, Queen Mary Hospital
Hui Yao Lan
Chinese University of Hong Kong https://orcid.org/0000-0003-4283-9755

Article

Keywords: LncRNA Neat1, inflammation, NLRP3 inflammasome, protein binding partner, Rack1

Posted Date: April 8th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1518831/v1

Abstract

Acute kidney injury (AKI) is common in hospitalized patients and is associated with high mortality. Inflammation plays a key role in the pathophysiology of AKI. Long non-coding RNAs (lncRNAs) are being increasingly recognized as regulators of the inflammatory and immune response. In this study, we first observed significantly increased urinary and circulating lncRNA Neat1 among 66 hospitalized patients with AKI versus control subjects from a primary care clinic; and among kidney transplant recipients, Neat1 levels were highest after surgery followed by a prompt decline to normal levels in parallel with recovery of kidney function. Next, in a mouse model of lipopolysaccharide (LPS)-induced septic AKI, in which kidney tubular Neat1 was increased, short hairpin RNA-mediated knockdown of Neat1 in the kidney preserved kidney function by lowering BUN and serum creatinine/cystatin C, protected against tubular apoptosis and suppressed overexpression of AKI biomarkers KIM-1 and NGAL, leukocyte infiltration and both intrarenal and systemic inflammatory cytokines IL-6, TNF-α and IL-1β. Finally, in LPS-treated C1.1 mouse kidney tubular cells, Neat1 was induced via the TLR4/NF-κB signaling pathway which promoted subsequent activation of NLRP3 inflammasomes via binding with another scaffold protein, Rack1. Silencing of Neat1 in C1.1 cells ameliorated LPS-induced upregulation of IL-6, whereas overexpressing Neat1 alone without LPS stimulation was sufficient to drive C1.1 cells into a proinflammatory phenotype. In conclusion, Neat1 exerts a prominent role in AKI by promoting NLRP3 inflammasome activation and is a potential novel biomarker and therapeutic target for AKI.

Main

Acute kidney injury (AKI) is commonly observed in hospitalized patients and is a potentially fatal complication of critical illness, including Covid-19.[1,2] Among hospitalized patients in the critical care setting, over 50% of AKI cases are sepsis-related with a high mortality.[3] AKI can cause permanent kidney damage with a high risk of subsequent chronic kidney disease (CKD),[4,5] imposing a major burden on healthcare resources.[5] Early detection and treatment improves the outcome of AKI and is the current focus of management.[5] Inflammation plays a pivotal role in the multi-factorial pathogenesis of AKI,[6] and delineating its underlying mechanisms will identify novel biomarkers and therapeutic targets to enhance clinical management.

Long non-coding RNAs (lncRNAs) are more than 200 nucleotides in length[7] and have emerged as important regulators of gene expression during disease development and progression.[8] LncRNA nuclear-enriched abundant transcript 1 (Neat1), one of the most extensively studied lncRNAs, maintains the structural integrity of the paraspeckles, a specific type of nuclear body in the interchromatin space whose function remains poorly understood.[9,10] There is accumulating evidence that Neat1 exerts a critical role in the progression of several diseases including those of the kidney.[9,11,12] Viral infection and other intracellular damage can increase the expression of Neat1 to activate inflammasomes.[13] Nonetheless the function of Neat1 in the regulation of inflammasome activation during AKI remains largely unknown.

The NOD-like receptor family protein 3 (NLRP3) inflammasome is a critical component of innate immunity[14] that comprises a sensor molecule NLRP3, the adaptor apoptosisassociated specklike protein containing a CARD (ASC), and the effector protease caspase-1.[15] It is capable of sensing cellular stress due to a wide variety of stimuli, including invading pathogens, toxins, endogenous danger signals and metabolic dysfunction.[16] Dysregulation of NLRP3 inflammasome activation is associated with many inflammatory diseases.[17] Two sequential steps are required for activation of NLRP3 inflammasome: priming and activation. The key priming event is the TLR4/NL-κB signaling mediated transcription of NLRP3, IL-1β and IL-18 and the activation step is characterized by recruitment of ASCs to assemble the NLRP3 inflammasome complex[18] with consequent cleavage of pro-caspase-1.[17] Cleaved caspase-1 induces maturation and secretion of proinflammatory cytokines and apoptosis.[19]

In this study, we first made a cross-sectional comparison of circulating and urinary Neat1 expression between patients with and without AKI, and followed its longitudinal changes over time in patients who were recovering from AKI after a planned ischemia-reperfusion injury, i.e. kidney transplantation surgery in the real world. We then used an experimental mouse model of lipopolysaccharide (LPS)-induced septic AKI and LPS-treated C1.1 mouse kidney tubular cells to confirm and dissect the mechanistic role of Neat1 in mediating inflammatory responses via TLR4/NF-κB signaling during AKI. Finally, we identified nuclear Rack1 as an interacting protein binding partner of Neat1 that provides a scaffold for NLRP3 inflammasome assembly and activation.

Results

Neat1 expression is increased in patients with AKI

In the cross-sectional study, urine and circulating Neat1 levels were significantly higher (Fig. 1A) among 66 hospitalized patients with AKI versus 152 control subjects without AKI recruited from a primary care clinic (Table 1). After adjusting for baseline estimated glomerular filtration rate (eGFR), smoking history and other comorbidities, AKI was associated with a 15.9- ($p = 0.006$) and 3.2- ($p < 0.001$) fold higher expression of Neat1 in the urine sediment and buffy coat, respectively (Supplementary Table 1). The association remained robust in sensitivity analyses.

In the prospective longitudinal study of six kidney failure patients (Table 2) who underwent kidney transplantation (and therefore deemed to have a scheduled ischemia-reperfusion AKI insult to the donor kidney), urinary and circulating Neat1 levels peaked immediately after transplant surgery and progressively declined to normal levels comparable to those observed among the non-AKI control subjects stated above. The decline of Neat1 paralleled recovery of kidney function as reflected by decreasing serum creatinine and rising eGFR (Fig. 1B). Data for individual patients are shown in Supplementary Figure S1.

Neat1 knockdown in the kidney ameliorates tubular injury and kidney dysfunction in a mouse model of LPS-induced AKI

In light of Neat1 induction in patients with AKI and reduction during AKI recovery, we investigated the kidney-specific role of Neat1 in a mouse model of LPS-induced septic AKI using small hairpin RNA-mediated knockdown of Neat1 (shRNA Neat1, Fig. 2A). Neat1 gene expression was significantly increased in the kidney 12 h and 24 h after LPS injection, which was significantly attenuated after treatment with shRNA Neat1 (Fig. 2B). These findings were confirmed by RNA *in situ* hybridization that also demonstrated Neat1's localization in tubular cell nuclei (Fig. 2C). Morphologically, LPS induced tubule dilatation and brush border loss which was ameliorated by shRNA Neat1. LPS also upregulated expression of the AKI biomarkers kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) which reduced by Neat1 knockdown (Fig. 3A and B). Biochemically, Neat1 knockdown improved kidney function by reducing increases in blood urea nitrogen (BUN), serum creatinine (SCR) and cystatin C levels by LPS treatment (Fig. 3C, D and E).

Neat1 induction is associated with kidney inflammation in LPS-induced AKI mice

LPS-injected kidneys showed increased kidney interstitial expression of F4/80 and Ly-6B.2, whereas shRNA Neat1-treated mouse kidneys had attenuated macrophage and neutrophil infiltration (Fig. 4A and B). Neat1 knockdown also significantly reduced LPS-induced circulating and kidney tissue pro-inflammatory cytokine gene expression including that of IL-6, TNF-α, NLRP3 and IL-1β in kidney tissue (Fig. 4C and D).

Neat1 knockdown attenuates apoptosis by suppressing NLRP3 inflammasome activation

LPS treatment activated intrarenal NLRP3 inflammasome and apoptosis as shown by superinduction of cleaved caspase-1 at 12 h, and phosphorylated poly-ADP ribose polymerase (p-PARP), Bcl-2-associated X protein (Bax) (and a corresponding reduction of Bcl-2), and the number of TUNEL-positive cells at 24 h. These changes were all reversed by knockdown of Neat1 (Fig. 5).

Neat1 regulates TLR4/NF-κB-mediated inflammatory responses in LPS-stimulated tubular epithelial cells

In cultured C1.1 kidney tubular cells, LPS upregulated Neat1 expression maximally at 60 min, which preceded that of pro-inflammatory cytokines CCL-2 and IL-6 at 1.5 and 2h, respectively (Fig. 6A). Transfection with antisense LNA GapmeRs in C1.1 cells knocked down Neat1 by 47% (Fig. 6B), which significantly suppressed LPS-induced IL-6 overexpression at both gene and protein levels (Fig. 6C, D and E). Conversely, cells overexpressing Neat1 after transfection with a pcDNA3.1-Neat1 plasmid (Fig. 6F) exaggerated expression of IL-6 and CCL-2 (Fig. 6G, H and I) versus control.

To unravel the mechanisms of Neat1 expression, we used Integrative Genomics Viewer (IGV) software and the online database AnimalTFDB 3.0 (http://bioinfo.life.hust.edu.cn/AnimalTFDB/#!/), and predicted four putative NF-κB binding sites on the promoter region of Neat1 (Fig. 7A). ChIP-qPCR demonstrated enrichment of p65 to the Neat1 promoter (Fig. 7B), suggesting that LPS-induced Neat1 transcription may be regulated by TLR4/NF-κB signaling. This was confirmed by prevention of LPS-induced Neat1 overexpression in C1.1 cells that were pretreated with the TLR4 inhibitor CIL-095 or NF-κB inhibitors (BAY11-7085, JSH-23) (Fig. 7C).

Silencing of Neat1 blocks NLRP3 inflammasome activation in LPS-stimulated tubular epithelial cells

To further investigate whether Neat1 could promote NLRP3 inflammasome activation, C1.1 cells were transfected with different plasmids and stimulated with the following treatments: 1) GapmeRs control, 2) GapmeRs control with LPS, 3) GapmeRs control with LPS and Nigericin, 4) GapmeRs Neat1, 5) GapmeRs Neat1 with LPS, 6) GapmeRs Neat1 with LPS and Nigericin. Silencing of Neat1 in LPS-primed C1.1 cells prevented nigericin, an activator NLRP3 inflammasome, from resulting in pro-caspase-1 cleavage (Fig. 8A). In addition, it also prevented the oligomerization of ASC despite concomitant stimulation with LPS and nigericin (Fig. 8B). Similarly, ASC speck formation was also significantly suppressed under the same inflammasome stimulatory conditions (Fig. 8C).

Neat1 interacts with Rack1 and regulates NLRP3 inflammasome

There is growing evidence that lncRNAs regulate biological and pathological processes through direct interaction with proteins. To identify potential binding partners for Neat1, we performed a biotinylated Neat 1 RNA pull-down assay followed by mass spectrometry in cell lysates from unstimulated and LPS-stimulated C1.1 cells. The protein-RNA complexes enriched by Neat1 pull-down were silver-stained on SDS-PAGE (Fig. 9A) and subjected to mass spectrometry. Potential Neat1 binding proteins were identified in LPS-stimulated cells compared with unstimulated control (Fig. 9B). Among them, Rack1 was selected for further validation of its functional role in NLRP3 inflammasome activation. Western blot analysis confirmed that Rack1 interacted with the biotinylated Neat1 probe in the LPS-stimulated pull-down sample, but not with the non-specific LacZ probe (Fig. 9C). The specific interaction between Neat1 and Rack1 was further validated by RNA immunoprecipitation (RIP) assay (Fig. 9D).

Rack1 serves as a scaffold protein and facilitates many biological processes, however, whether it interacts with Neat1 during the activation of NLRP3 inflammasome remains unknown. In human embryonic kidney 293T cells co-transfected with pcDNA3-Flag-NLRP3 and pcDNA3-Flag-ASC plasmids, co-immunoprecipitation assay revealed a NLRP3-ASC-Rack1 interactions in LPS-primed, nigericin-treated cells. Gene silencing of Neat1 disrupted these protein-protein interactions of the inflammasome complex (Fig. 10A). The role of Neat1 in Rack1-mediated NLRP3 inflammasome activation was further

investigated by knocking down Neat1 in C1.1 cells overexpressing Rack1. Under LPS/nigericin stimulation, both NLRP3 and cleaved caspase-1 expression were induced but significantly decreased after Neat1 knockdown. Overexpression of Rack1 could not accentuate NLRP3 inflammasome activation or rescue the inactivation of NLRP3 inflammasome consequent to Neat1 deficiency (Fig. 10B). On the contrary, cells with Neat1 overexpression further enhanced LPS/nigericin-induced NLRP3 expression and cleavage of caspase-1. The inflammasome activation was blunted by Rack1 knockdown although NLRP3 levels remained unchanged (Fig. 10C). Expression of ASC remained unchanged (Fig. 10B and C) during these transfection experiments regardless of the transfection strategy or efficiency (Fig. 10D and E). Altogether, our results suggest that Neat1 promoted the expression of NLRP3 inflammasome and its assembly, leading to cleavage of pro-caspase-1.

Discussion

Since its discovery, Neat1 has been widely investigated in various human malignancies including lung, colorectal and prostate cancer for its regulatory role in growth, proliferation, and apoptosis.[20–22] Until recently, Neat1 was recognized as an important immunomodulator in other diseases such as liver disease,[23] rheumatoid arthritis[24] and heart disease,[25] but its role in kidney disease has remained incompletely defined. Here, we provide 3 lines of solid evidence that highlights an indisputable role of Neat1 in AKI. First, our human studies from a cross-sectional cohort indicated that Neat1 was overexpressed during AKI which led to a > 15-fold and > 3-fold increase in its urinary and circulating levels, respectively, after adjusting for baseline eGFR and comorbidities. We then made use of the fact that kidney transplantation is one form of “planned” AKI from surgical ischemia-reperfusion injury and demonstrated a temporal relation between decline of heightened postoperative Neat1 in urine and blood to non-AKI levels and recovery of allograft kidney function following surgery.

Second, we adopted a mouse septic AKI model not only because LPS induction is a well-accepted AKI model but also because sepsis is among the commonest causes of AKI in human.[3,5] Indeed, AKI is recognized as a systemic disease that not only leads to kidney lesions, but also affects the immune system.[26] LPS administration to mice induces systemic inflammatory responses that mimic the clinical features of septic shock including increased leukocyte infiltration, tubular injury and kidney failure. The critical role of Neat1 in AKI was confirmed from 2 observations in our mouse model: 1) there was overexpression of Neat1 in the kidney of animals that developed LPS-induced AKI; and 2) knockdown of Neat1 specifically in the kidney, by ultrasound microbubble-mediated shRNA, ameliorated intrarenal inflammation, tubular cell injury and apoptosis, as well as improved kidney function versus control animals with intact Neat1.

Third, LPS stimulated overexpression of Neat1 in cultured mouse kidney tubular epithelial cells. This is a particularly important observation as AKI is usually caused by acute tubular necrosis leading to a rapid decline in kidney function.[5,27] Hence, our *in vitro* studies focused on this resident cell type which we also employed to further dissect the underlying cellular mechanisms for Neat1 to mediate the inflammatory

responses in the development of AKI by silencing or overexpressing it. We also showed for the first time that Neat1 expression is regulated by TLR4/NF-κB signaling and that it interacted with the protein Rack1 to activate NLRP3 inflammasome.

As key regulators of immune responses, lncRNAs are involved in the production of inflammatory mediators, cell differentiation and migration. Neat1 is a highly and ubiquitously expressed lncRNA that is enriched in the nucleus for paraspeckle formation. Here, we identified Neat1 as a downstream target of TLR4/NF-κB signaling, a major receptor-mediated pathway evoked by LPS.[28] TLR4/NF-κB signaling regulates various processes of the immune response via transcription of inflammatory genes.[29] Using loss- and gain-of-function approaches, we testified Neat1's proinflammatory role as its deficiency prevented LPS-induced overexpression of IL-6/CCL2, whereas its augmentation increased these inflammatory cytokines even without requiring LPS. Such finding corroborated our data in animals in which Neat1 mediated IL-6 expression in mice with LPS-induced AKI, and this is also in agreement with our previous observation that NF-κB mediates TLR4-induced IL-6 expression in tubular epithelial cells.[30] The involvement of Neat1 in TLR4/NF-κB signaling has been reported in pyroptosis, apoptosis and tumorigenesis,[31–33] but whether NF-κB activation is involved in Neat1-regulated inflammatory responses remains unknown. Here, we observed that the p65 subunit of NF-κB directly bound to the promoter region of Neat1 and that LPS-induced Neat1 expression was suppressed by blocking TLR4/NF-κB signaling. Collectively, these phenomena indicate that activation of TLR4/NF-κB signaling promotes the expression of Neat1 in tubular epithelial cells in LPS-induced sepsis while Neat1 modulates the downstream expression of inflammatory events.

lncRNAs regulate gene expression at the levels of chromatin remodeling, transcription and post-transcriptional processing.[34] Neat1 can act as miRNA sponges[35] or transcription regulators via protein binding partners[36,37] to activate or repress gene expression.[38] It remains unclear how Neat1 regulates inflammation during AKI. Inflammasomes are critical multiprotein complexes of the innate immune system that regulate caspase-dependent inflammation, cell death and other cellular pathways.[39] Activation of NLRP3 inflammasome triggers downstream inflammatory cytokines, with consequent cell apoptosis and ultimately AKI.[40] Our mechanistic studies indicated that Neat1 promoted the assembly of NLRP3 inflammasome in part through interaction with Rack1 in LPS-stimulated tubular epithelial cells. Knockdown of Neat1 reduced NLRP3 expression, caspase-1 activation and apoptosis, resulting in preserved kidney function in LPS-induced AKI mice. Furthermore, the systemic levels of TNF-α, IL-1β and IL-6 were suppressed in Neat1-deficient mice. *In vitro*, knockdown of Neat1 disrupted ASC oligomerization and protein-protein interaction between NLRP3 and ASC, suggesting a critical role of Neat1 in regulating NLRP3 inflammasome assembly.

lncRNAs regulate biological functions by binding to DNA, RNAs (mRNAs and microRNAs) or proteins.[34,41] Neat1 acts as a scaffold by interacting with RNA binding proteins (RBPs) to enhance miRNA processing.[42] On the other hand, Neat1 binds to an epigenetic inhibitory factor of EZH2 and guides it to the promoter of target genes.[36,43] Here, we identified Rack1 as a protein binding partner of Neat1. Their

interaction might facilitate NLRP3 inflammasome activation in LPS-stimulated tubular epithelial cells. Rack1, a multifunctional scaffold protein for kinases and receptors, mediates NLRP3 inflammasome activation in macrophages through direct binding to NLPR3 and NEK7.[44] Our results showed that Rack1 was co-immunoprecipitated with NLRP3 in cells stimulated with LPS plus nigericin, but not in those with Neat1 knockdown, suggesting that Neat1 is required for Rack1-mediated NLRP3 inflammasome activation. Absence of either Neat1 or Rack1 completely abrogated NLRP3 inflammasome activation in LPS-stimulated tubular epithelial cells, indicating the essential role of Neat1 and Rack1 for NLRP3 inflammasome activation. Interestingly, overexpression of Neat1, but not Rack1 alone, activated NLPR3 inflammasome, suggesting an overarching role of Neat1 in inflammasome complex formation. Given that Neat1 is an architectural lncRNA that acts as a scaffold for paraspeckle assembly, it might work together with Rack1 to assist various inflammasome components including NLRP3, ASC, and caspase-1 to form a compact structure. We also demonstrated that Neat1 regulated NLRP3 inflammasome activation at the nuclear level. The induced expression of NLRP3 and IL-1β transcripts was inhibited in LPS-treated Neat1-deficient mice. Neat1 sequesters the protein splicing factor proline/glutamine-rich (SFPQ) in paraspeckles, thereby enhancing the transcription of IL-8 from its promoter.[45] Future studies are warranted to elucidate the mechanism through which Neat1 regulates the transcription of NLRP3 and IL-1β.

In conclusion, we have presented a spectrum of human, animal and *in vitro* evidence for a novel role of lncRNA Neat1 in AKI and unraveled its regulatory mechanisms toward NLRP3 inflammasome activation. In response to the septic stimulus from LPS, Neat1 was upregulated in the kidney tubular compartment via the classical TLR4/NFκB signaling pathway and interacted with Rack1 to construct a scaffold for NLRP3 inflammasome assembly to trigger inflammatory responses that orchestrate the subsequent phenotype characterized by tubular injury, apoptosis and loss of kidney function (Fig. 11). Neat1 is a prospective novel biomarker and therapeutic target for AKI.

Methods

Clinical specimens

The use of human specimens for this study was approved by the Research Ethics Committee/ Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster. All patients and control subjects gave informed consent prior to collection of urine and blood samples. Serum/plasma were separated from blood cells by centrifugation at 3000 rpm for 10 min and stored at -80°C for further analysis. Leukocytes from blood samples were prepared by removal of red blood cells from the buffy coat fraction using ACK lysing buffer (Gibco, Grand Island, NY). Urine specimens were centrifuged at 3000 rpm for 10 min and urinary sediments obtained after discarding the supernatant. Relative Neat1 expression was measured by qRT-PCR in urine sediment and buffy coat of non-AKI and AKI subjects.

Cell Culture and stimulation

Mouse kidney tubular epithelial cells (C1.1) were cultured in Dulbecco's modified Eagle medium/nutrient mixture F12 (Gibco), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in 5% carbon dioxide atmosphere. Human embryonic kidney 293 cells (293T) (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium with high glucose and GlutaMAX (Gibco), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FBS at 37°C in 5% carbon dioxide atmosphere. For Lipopolysaccharide (LPS) priming, C1.1 cells were serum-starved for 24 h and stimulated with 200 ng/mL LPS (Sigma-Aldrich, St Louis, MO). To inhibit TLR4 and NF-κB signaling, cells were pre-treated with 5 µM CLI-095 (InvivoGen, San Diego, CA), 1 µM Bay11-7085 or 2.5 µM JSH-23 (Selleck Chemicals, Houston, TX) for 1 h prior to LPS stimulation. For inflammasome activation, cells were primed with 1000 ng/mL LPS for 3 h and stimulated with 5 µM nigericin (InvivoGen) for 1 h.

Transfection

To knock down Neat1, C1.1 cells were transfected with antisense LNA GapmeRs Neat1 or negative control (QIAGEN, Germantown, MD) using Lipofectamine® 2000 reagent (Invitrogen). For double transfection using X-tremeGENE™ siRNA Transfection Reagent (Roche, Basel, Switzerland), cells were co-transfected with pCMV-Rack1 or pCMV empty plasmids (OriGene, Rockville, MD) together with corresponding GapmerR, or co-transfected with pcDNA 3.1-Neat1 or empty plasmid (Invitrogen) and siRNA-Rack1 or scrambled siRNA (Invitrogen).

In co-immunoprecipitation experiment, 293T cells were transfected with pcDNA3-Flag-NLRP3 (Addgene#75127), pcDNA3-Flag-ASC plasmid (Addgene#75134) (a gift from Bruce Beutler) and siRNA Neat1 or negative control (Invitrogen) using Lipofectamine® 2000 reagent.

Mouse model of LPS-induced AKI

All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (HKU) and adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice weighing 20 to 25 g at 8 weeks of age were supplied by the Centre for Comparative Medicine Research, HKU. To specifically knock down Neat1 in mouse kidney, shRNA-pSuper.puro vector targeting Neat1 (200 µg/mouse) or pSuper.puro empty vector (OligoEngine, Seattle, WA) was mixed with Sonovue microbubbles (Bracco, Milan, Italy) at a ratio of 1:1 (vol:vol) and injected into mice via the tail vein. An ultrasound transducer (Therasonic, Electro Medical Supplies, Wantage, UK) was immediately applied on both kidneys with a pulse-wave output of 1 MHz at 2 W/cm^2 for 5 min on each side. Three days after ultrasound-mediated gene transfer, LPS (20 mg/kg),

dissolved in 0.9% saline, was injected into mice via intraperitoneal injection. Saline was given to control animals. All animals were sacrificed at 12 h and 24 h after LPS injection and kidneys harvested. For each kidney, half was snap-frozen in liquid nitrogen and stored at -80°C. The other half was fixed with 10% formalin and embedded in paraffin.

RNA extraction and Real-Time quantitative PCR

Total RNAs from urinary sediments, leukocytes and cultured cells were extracted by TRIZOL™ LS and TRIZOL™ reagent (Invitrogen), and that from kidney cortical tissues by NucleoSpin RNA/Protein kit (Macherey-Nagel, Duren, Germany). RNAs were then reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time qPCR was performed using SYBR™ Green reagent and specific primers on the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative gene expression was obtained after normalization with GAPDH, followed by comparison with respective control group using StepOne™ software v2.3 (Applied Biosystems). The primer sequence of target genes is listed in Supplementary Table 3.

Western blot analysis

Total proteins were prepared from cultured cells and kidney cortical tissue using RIPA lysis buffer (Millipore, Bedford, MA) with 10% Protease Inhibitor Cocktail (Sigma-Aldrich) and NucleoSpin RNA/Protein kit, respectively. Cytoplasmic and nuclear proteins were separated using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Waltham, MA). Protein lysates were resolved by SDS-PAGE in Bolt™ 4-12% gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were blotted with 5% non-fat milk and incubated with primary antibodies overnight at 4°C, followed by peroxidase-conjugated secondary antibodies (Dako, Carpinteria, CA) for 1 h. Proteins were visualized with Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA) using ChemiDoc XRS+ system (Bio-Rad). Relative expression of target protein was normalized with GAPDH expression using Image Lab 3.0 (Bio-Rad). Primary antibodies against p-PARP, p-p65, and p65 (Cell Signaling Technology, Beverly, MA); GAPDH (Sigma-Aldrich); Rack1 (Abcam, Cambridge, UK); Bax, Bcl-2, CCL-2, and IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA); ASC, pro-caspase-1, and cleaved caspase-1 (AdipoGene Life Sciences, San Diego, CA) and NLRP3 (Bioss Antibodies, Woburn, MA) were used.

ELISA

Human serum level of IL-6 and IL-8 was measured using a human ELISA kit (R&D Systems, Minneapolis, MN). The level of IL-6 and CCL-2 in supernatant from cultured cells and level of IL-6, IL-8, TNF-α, and IL-1β

from mouse serum was detected using a mouse ELISA kit (R&D Systems) according to the manufacturer's instructions.

Kidney function assessment

In human, serum creatinine and estimated GFR in AKI and control subjects, and kidney transplant recipients from day 1 to day 7 and day 14 were reported by service clinical biochemistry laboratories. In animals, serum creatinine (SCR), blood urea nitrogen (BUN), and Cystatin C levels were determined by Creatinine LiquiColor® Test (Stanbio Laboratory, Boerne, TX), QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA) and human Cystatin C ELISA kit (R&D Systems) respectively, according to the manufacturer's instructions.

Histological staining

Paraffin-embedded kidney tissue was sectioned (4 μm thickness), deparaffinized, rehydrated and followed by periodic acid-Schiff (Polysciences Inc., Warrington, PA) staining protocol. Tubular injury of the sections was scored according to the percentage of damaged renal tubules from 0 to 5: 0, normal; 1, tubular lesion <10%; 2, 10-20% lesion; 3, 20-30% lesion; 4, 30-40% lesion; 5, >40% lesion.

Immunohistochemical staining

Following deparaffinization and rehydration, paraffin-embedded kidney sections were heated in citrate buffer (10 mM, pH 6.0) or protease K solution (10 μg/mL, pH 8.0) for antigen retrieval. Sections were quenched by 3% hydrogen peroxide and blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich). Primary antibodies against NGAL (Abcam), F4/80 and Ly6B.2 (Bio-Rad) were applied on the sections for overnight incubation at 4℃, followed by peroxidase-conjugated second antibodies (Abcam). Sections were developed using DAB substrate (Dako) and counterstained with hematoxylin (Dako) before mounting. Staining was quantified using Image J analysis software (NIH, Bethesda, MD).

TUNEL assay

An ApopTag® Peroxidase *in situ* apoptosis detection kit (Millipore) was used according to the manufacturer's instructions. In brief, paraffin-embedded kidney sections were deparaffinized, rehydrated and subjected to Proteinase K treatment. Sections were labeled with digoxigenin (DIG)-conjugated nucleotide by terminal deoxynucleotidyl transferase at 37°C. Positive signals were developed by anti-DIG peroxidase, counterstained with hematoxylin and quantified using Image J analysis software (NIH).

RNA *in situ* hybridization

To determine the expression and localization of Neat1 in kidney tissue sections, RNA *in situ* hybridization was performed using RNAscope 2.5 HD assay (Advanced Cell Diagnostics, Hayward, CA) following the manufacturer's protocol. Briefly, deparaffinized sections were treated with protease K and hybridized with a horseradish peroxidase labelled Neat1 probe. The signal was developed by DAB substrate and nuclei were counterstained by hematoxylin. Quantification of positive staining was performed using Image J analysis software (NIH).

ASC oligomerization

After LPS priming and nigericin treatment, cells were washed with cold PBS and lysed with 0.5% Triton X-100 solution with protease inhibitor cocktail. Cell lysates were centrifuged and separated into supernatant (Triton-soluble) and pellet (Triton-insoluble). Triton-soluble fraction was used as input. The Triton-insoluble fraction was cross-linked with 2 mM disuccinimidyl suberate (Thermo Scientific) and boiled with SDS loading buffer (Bio-Rad) for Western blot analysis.

Formation of ASC speck-like aggregates

Transfected cells seeded on the Nunc® Lab-Tek II Chamber Slide (Thermo Scientific) were stimulated with LPS and nigericin. After that, cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked with 5% BSA. Cells were incubated with anti-ASC antibody (Novus Biologicals, Littleton, CO) overnight at 4°C, followed by Alexa Fluor 594-conjugated secondary antibody (Invitrogen) for 1 h. Nuclei were contained with DAPI (Thermo Scientific). Fluorescence images of ASC aggregates were visualized under a fluorescence microscope.

Chromatin immunoprecipitation (ChIP)

A SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology) was used to perform ChIP. In brief, LPS-stimulated cells were cross-linked with 37% formaldehyde (Sigma-Aldrich), followed by nuclease digestion and sonication. The protein-bound chromatin fragments were immunoprecipitated using antibodies against p65, Histone H3 (positive control) or normal IgG (negative control) (Cell Signaling Technology). The eluted DNA was purified and detected by qRT-PCR analysis using specific primers listed in Supplementary Table 3.

RNA pull-down and mass spectrometry

An RNA pull-down Kit (BersinBio, Guangzhou, China) was used according to the manufacturer's instructions. LPS-primed cells were lysed by RIPA lysis buffer with 10% Protease Inhibitor Cocktail. Magnetic bead complex with biotinylated Neat1 probe or negative control LacZ probe was added to protein lysates for incubation. The RNA-protein-bead complexes were washed, and proteins eluted in protein elution buffer and separated by SDS-PAGE, followed by Western blot analysis or subjected to mass spectrometry (Centre for PanorOmic Sciences, HKU).

RNA immunoprecipitation (RIP)

RIP assay was performed using an EZ-Magna RIP® (Cross-Linked) RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Briefly, cell lysate was prepared from LPS-primed cells and the protein-RNA complexes immunoprecipitated by incubation with Protein A/G magnetic beads (Santa Cruz Biotechnology) and anti-Rack1 antibody (Abcam). Normal IgG was used as negative control (Millipore). The immunoprecipitated RNAs were eluted and detected by RT-qPCR analysis. The results are presented as % Input Recovered. The primers used for detecting Neat1 or U1 snRNA are listed in Supplementary Table 3.

Co-immunoprecipitation (Co-IP)

293T cells transfected with pcDNA3-Flag-NLRP3, pcDNA3-Flag-ASC plasmids, siRNA-Neat1 and corresponding controls were stimulated with LPS/nigericin. The cells were lysed in RIPA lysis buffer with 10% Protease Inhibitor Cocktail and pre-cleared with normal IgG (AdipoGene) agarose beads. After centrifugation, the supernatants were incubated with anti-NLRP3 antibody or normal IgG (AdipoGene) overnight at 4°C, followed by incubation with protein A/G agarose beads. Beads were washed with lysis buffer and boiled in SDS loading buffer with β-mercaptoethanol for Western blotting analysis.

Statistical analysis

The association between acute kidney injury and urinary Neat1 expression was assessed by univariable and multivariable regression. Neat1 expression was log-transformed for statistical analysis. A sensitivity analysis was performed by re-categorizing the comorbidities into chronic kidney disease, metabolic-related disorders, cardiovascular disease, and neoplasms to account for collinearity of comorbidities. All inter-group comparisons are expressed as mean ± SEM. Differences between experimental groups were evaluated by one-way ANOVA or t-test using GraphPad Prism v.7 (GraphPad Software). $P<0.05$ was considered statistically significant.

Declarations

Acknowledgements

This work was supported by the Research Grants Council of Hong Kong (Collaborative Research Fund, grant no. C7018-16G), Hong Kong Society of Nephrology/HK Kidney Foundation Research Grant 2019, and philanthropic donations from Dr. Rita T Liu SBS of L & T Charitable Foundation Ltd. & Bingei Family of Indo Café, Mr. Winston Leung, Mr. K.K. Chan, Ms. Siu Suet Lau, Dr. Y.Y. Cheung and an Endowment Fund established at the University of Hong Kong for the *Yu* Professorship in Nephrology awarded to SCWT. XR received the HKU Postgraduate Fellowships in Integrative Medicine from 2018 – 2022. The funders played no role in the design or writing of this manuscript. The authors thank Dr. Arthur Tang in assisting extraction of clinical data of control subjects in the cross-sectional cohort study.

Conflict of interest

All authors report no competing interests related to this study.

g>
All authors report no competing interests related to this study.

Acknowledgements

This work was supported by the Research Grants Council of Hong Kong (Collaborative Research Fund, grant no. C7018-16G), Hong Kong Society of Nephrology/HK Kidney Foundation Research Grant 2019, and philanthropic donations from Dr. Rita T Liu SBS of L & T Charitable Foundation Ltd. & Bingei Family of Indo Café, Mr. Winston Leung, Mr. K.K. Chan, Ms. Siu Suet Lau, Dr. Y.Y. Cheung and an Endowment Fund established at the University of Hong Kong for the *Yu* Professorship in Nephrology awarded to SCWT. XR received the HKU Postgraduate Fellowships in Integrative Medicine from 2018–2022. The funders played no role in the design or writing of this manuscript. The authors thank Dr. Arthur Tang in assisting extraction of clinical data of control subjects in the cross-sectional cohort study.

References

1. Vijayan, A. Tackling AKI: prevention, timing of dialysis and follow-up. *Nat Rev Nephrol* **17**, 87-88 (2021).

2. Chan, K.W., Yu, K.Y., Lee, P.W., Lai, K.N. & Tang, S.C. Global REnal Involvement of CORonavirus Disease 2019 (RECORD): A Systematic Review and Meta-Analysis of Incidence, Risk Factors, and Clinical Outcomes. *Front Med (Lausanne)* **8**, 678200 (2021).

3. Peerapornratana, S., Manrique-Caballero, C.L., Gomez, H. & Kellum, J.A. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. *Kidney Int* **96**, 1083-1099 (2019).

4. Arshad, A.*, et al.* Progression of Acute Kidney Injury to Chronic Kidney Disease in Sepsis Survivors: 1-Year Follow-Up Study. *J Intensive Care Med* **36**, 1366-1370 (2021).

5. Ronco, C., Bellomo, R. & Kellum, J.A. Acute kidney injury. *Lancet* **394**, 1949-1964 (2019).

6. Legrand, M.*, et al.* Pathophysiology of COVID-19-associated acute kidney injury. *Nat Rev Nephrol* **17**, 751-764 (2021).

7. Schmitt, A.M. & Chang, H.Y. Long Noncoding RNAs in Cancer Pathways. *Cancer Cell* **29**, 452-463 (2016).

8. Kopp, F. & Mendell, J.T. Functional Classification and Experimental Dissection of Long Noncoding RNAs. *Cell* **172**, 393-407 (2018).

9. Yamazaki, T.*, et al.* Functional Domains of NEAT1 Architectural lncRNA Induce Paraspeckle Assembly through Phase Separation. *Mol Cell* **70**, 1038-1053 e1037 (2018).

10. Wang, Y.*, et al.* Genome-wide screening of NEAT1 regulators reveals cross-regulation between paraspeckles and mitochondria. *Nat Cell Biol* **20**, 1145-1158 (2018).

11. Wen, S.*, et al.* Long non-coding RNA NEAT1 promotes bone metastasis of prostate cancer through N6-methyladenosine. *Mol Cancer* **19**, 171 (2020).

12. Yang, Y.L.*, et al.* Long noncoding RNA NEAT1 is involved in the protective effect of Klotho on renal tubular epithelial cells in diabetic kidney disease through the ERK1/2 signaling pathway. *Exp Mol Med* **52**, 266-280 (2020).

13. Zhang, P., Cao, L., Zhou, R., Yang, X. & Wu, M. The lncRNA Neat1 promotes activation of inflammasomes in macrophages. *Nat Commun* **10**, 1495 (2019).

14. Mangan, M.S.J.*, et al.* Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov* **17**, 588-606 (2018).

15. He, Y., Hara, H. & Nunez, G. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem Sci* **41**, 1012-1021 (2016).

16. Jo, E.K., Kim, J.K., Shin, D.M. & Sasakawa, C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol* **13**, 148-159 (2016).

17. Wang, L. & Hauenstein, A.V. The NLRP3 inflammasome: Mechanism of action, role in disease and therapies. *Mol Aspects Med* **76**, 100889 (2020).

18. Elliott, E.I. & Sutterwala, F.S. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol Rev* **265**, 35-52 (2015).

19. Rathinam, V.A.K., Zhao, Y. & Shao, F. Innate immunity to intracellular LPS. *Nat Immunol* **20**, 527-533 (2019).

20. Pan, L.J.*, et al.* Upregulation and clinicopathological significance of long non-coding NEAT1 RNA in NSCLC tissues. *Asian Pac J Cancer Prev* **16**, 2851-2855 (2015).

21. Wu, Y.*, et al.* Nuclear-enriched abundant transcript 1 as a diagnostic and prognostic biomarker in colorectal cancer. *Mol Cancer* **14**, 191 (2015).

22. Chakravarty, D.*, et al.* The oestrogen receptor alpha-regulated lncRNA NEAT1 is a critical modulator of prostate cancer. *Nat Commun* **5**, 5383 (2014).

23. Bu, F.T.*, et al.* LncRNA NEAT1: Shedding light on mechanisms and opportunities in liver diseases. *Liver Int* **40**, 2612-2626 (2020).

24. Wang, Y.*, et al.* LncRNA NEAT1 Targets Fibroblast-Like Synoviocytes in Rheumatoid Arthritis via the miR-410-3p/YY1 Axis. *Front Immunol* **11**, 1975 (2020).

25. Gast, M.*, et al.* Long noncoding RNA NEAT1 modulates immune cell functions and is suppressed in early onset myocardial infarction patients. *Cardiovasc Res* **115**, 1886-1906 (2019).

26. Kinsey, G.R., Sharma, R. & Okusa, M.D. Regulatory T cells in AKI. *J Am Soc Nephrol* **24**, 1720-1726 (2013).

27. Linkermann, A.*, et al.* Regulated cell death in AKI. *J Am Soc Nephrol* **25**, 2689-2701 (2014).

28. Kayagaki, N.*, et al.* Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246-1249 (2013).

29. Liu, T., Zhang, L., Joo, D. & Sun, S.C. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* **2**(2017).

30. Lin, M.*, et al.* Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. *J Am Soc Nephrol* **23**, 86-102 (2012).

31. Fu, D., Ju, Y., Zhu, C., Pan, Y. & Zhang, S. LncRNA NEAT1 Promotes TLR4 Expression to Regulate Lipopolysaccharide-Induced Trophoblastic Cell Pyroptosis as a Molecular Sponge of miR-302b-3p. *Mol Biotechnol* (2022).

32. Nong, W. Long non-coding RNA NEAT1/miR-193a-3p regulates LPS-induced apoptosis and inflammatory injury in WI-38 cells through TLR4/NF-kappaB signaling. *Am J Transl Res* **11**, 5944-5955 (2019).

33. Zhou, W.*, et al.* Galectin-3 activates TLR4/NF-kappaB signaling to promote lung adenocarcinoma cell proliferation through activating lncRNA-NEAT1 expression. *BMC Cancer* **18**, 580 (2018).

34. Zhang, X.*, et al.* Mechanisms and Functions of Long Non-Coding RNAs at Multiple Regulatory Levels. *Int J Mol Sci* **20**(2019).

35. Zhuang, L.*, et al.* Exosomal LncRNA-NEAT1 derived from MIF-treated mesenchymal stem cells protected against doxorubicin-induced cardiac senescence through sponging miR-221-3p. *J Nanobiotechnology* **18**, 157 (2020).

36. Chen, Q.*, et al.* Long Noncoding RNA NEAT1, Regulated by the EGFR Pathway, Contributes to Glioblastoma Progression Through the WNT/beta-Catenin Pathway by Scaffolding EZH2. *Clin Cancer Res* **24**, 684-695 (2018).

37. Zhang, M.*, et al.* The lncRNA NEAT1 activates Wnt/beta-catenin signaling and promotes colorectal cancer progression via interacting with DDX5. *J Hematol Oncol* **11**, 113 (2018).

38. Li, W.*, et al.* The FOXN3-NEAT1-SIN3A repressor complex promotes progression of hormonally responsive breast cancer. *J Clin Invest* **127**, 3421-3440 (2017).

39. Komada, T. & Muruve, D.A. The role of inflammasomes in kidney disease. *Nat Rev Nephrol* **15**, 501-520 (2019).

40. Li, N., Wang, Y., Wang, X., Sun, N. & Gong, Y.H. Pathway network of pyroptosis and its potential inhibitors in acute kidney injury. *Pharmacol Res* **175**, 106033 (2021).

41. Wang, K.C. & Chang, H.Y. Molecular mechanisms of long noncoding RNAs. *Mol Cell* **43**, 904-914 (2011).

42. Jiang, L.*, et al.* NEAT1 scaffolds RNA-binding proteins and the Microprocessor to globally enhance pri-miRNA processing. *Nat Struct Mol Biol* **24**, 816-824 (2017).

43. Wang, S.*, et al.* Long noncoding RNA Neat1 modulates myogenesis by recruiting Ezh2. *Cell Death Dis* **10**, 505 (2019).

44. Duan, Y.*, et al.* RACK1 Mediates NLRP3 Inflammasome Activation by Promoting NLRP3 Active Conformation and Inflammasome Assembly. *Cell Rep* **33**, 108405 (2020).

45. Imamura, K.*, et al.* Long noncoding RNA NEAT1-dependent SFPQ relocation from promoter region to paraspeckle mediates IL8 expression upon immune stimuli. *Mol Cell***53**, 393-406 (2014).

Tables

Tables 1 and 2 are available in the supplementary files section.

Figures

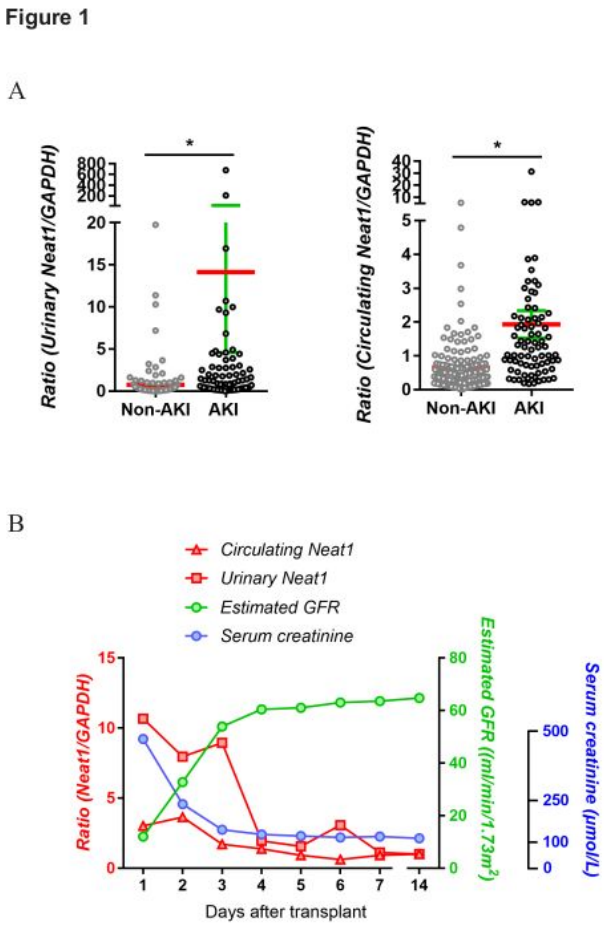

Figure 1

Changes in urinary and circulating Neat1 in patients with AKI. (**A**) Cross-sectional cohort among 66 hospitalized patients with AKI versus 152 control subjects without AKI from a primary care clinic. (**B**)

Longitudinal cohort (n=6) showing temporal changes in Neat1 levels in relation to kidney function after kidney transplantation. *P<0.05.

Figure 2

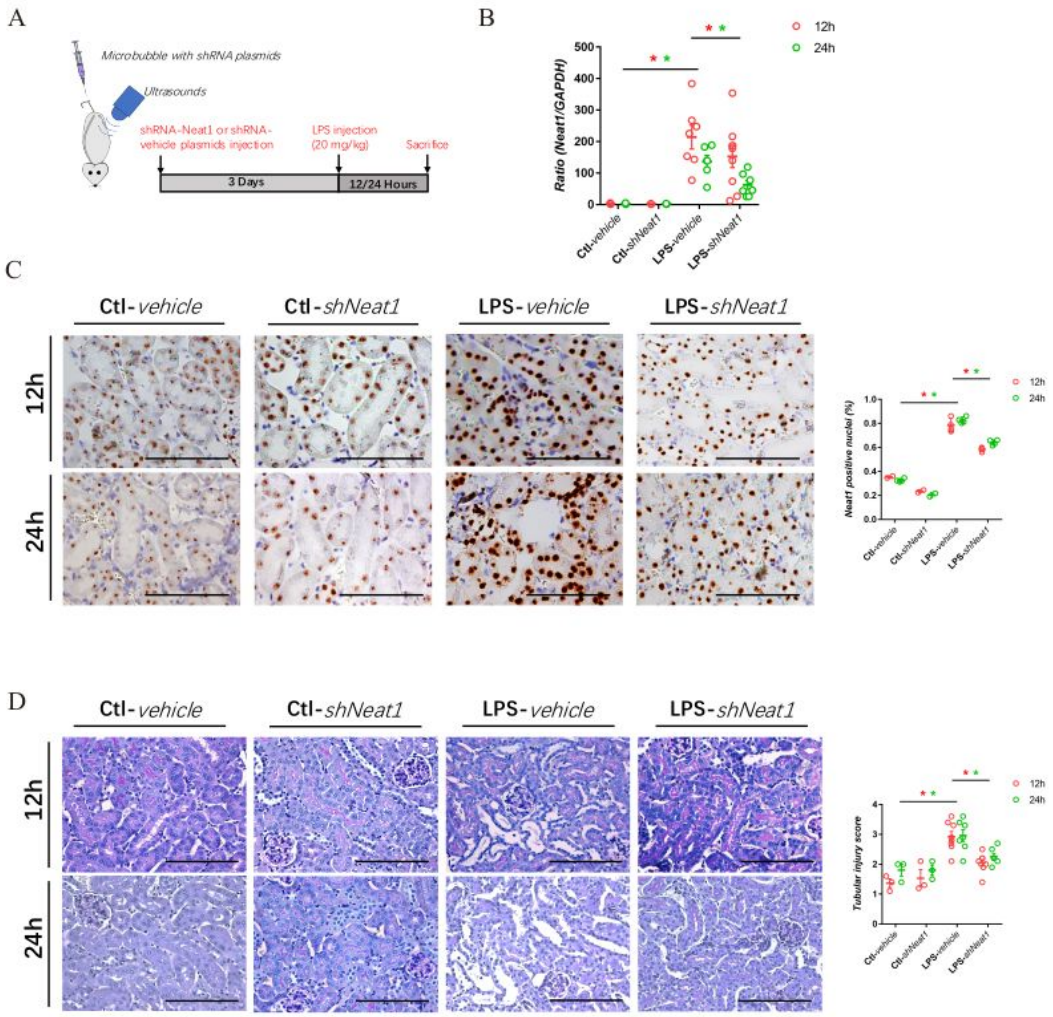

Figure 2

shRNA-mediated Neat1 knockdown in the kidney ameliorated LPS-induced AKI in mice. (**A**) Experimental design. Three days before LPS injection, shRNA plasmid targeting Neat1 (shNeat1) or empty plasmid

(vehicle) was delivered into the kidney of C57BL/6J mice via tail vein injection, followed by ultrasound microbubble-mediated gene transfer. (**B**) Relative Neat1 expression in the kidney at 12 h and 24 h after LPS injection. (**C**) Representative *in situ* hybridization of Neat1 expression in the kidney and the corresponding quantitative analyses. (**D**) Representative kidney morphology and the corresponding quantitative analyses. Scale bar=100 μm. *P<0.05.

Figure 4

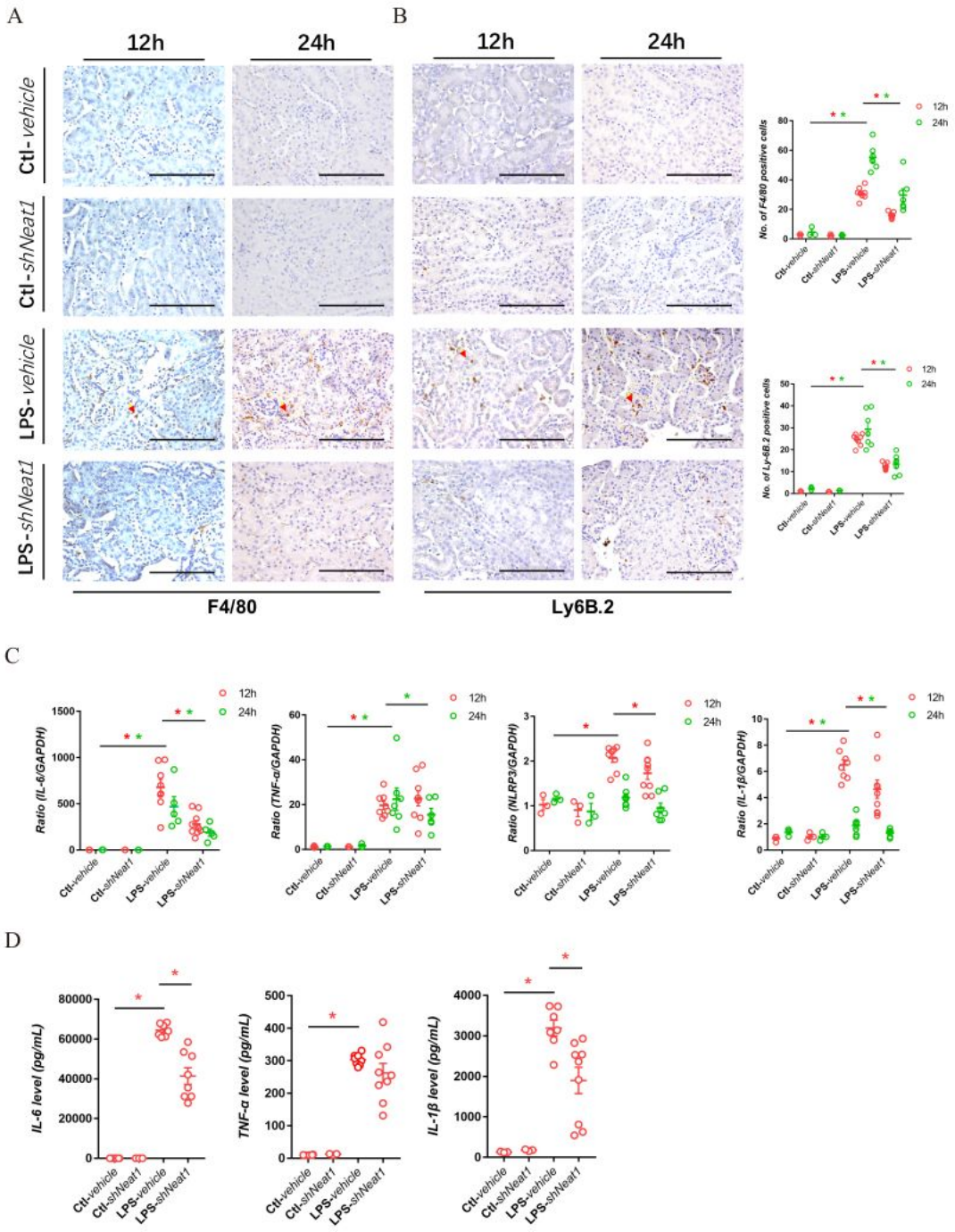

Figure 4

Knockdown of Neat1 reduced kidney inflammation in LPS-induced AKI mice. Representative immunohistochemical staining of (**A**) F4/80 and (**B**) Ly6B.2 in the kidney interstitium at 12 h and 24 h (red arrowheads indicate stain-positive cells) and the corresponding quantitative analyses. (**C**) Relative mRNA expression of IL-6, TNF-α, NLRP3 and IL-1β in the kidney. (**D**) Serum IL-6, TNF-α and IL-1β levels at 12 h after LPS injection by ELISA. Scale bar=100 μm. *P<0.05.

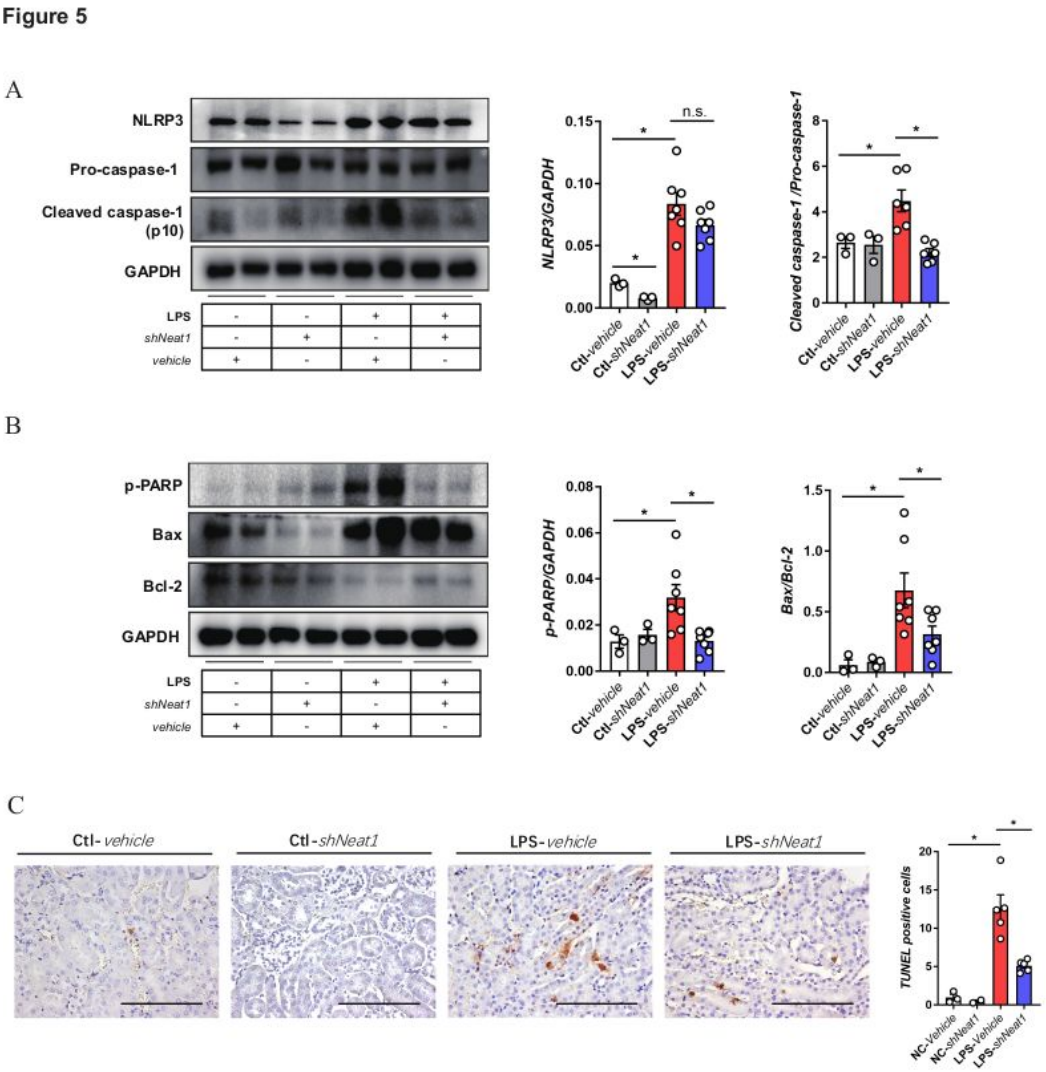

Figure 5

Knockdown of Neat1 attenuated apoptosis by suppressing NLRP3 inflammasome activation in LPS-induced AKI mice. Representative Western blot analyses of the expression levels of (**A**) NLRP3, pro-caspase-1 and cleaved caspase-1 (p10) in the kidney at 12 h and the corresponding quantitative analyses, and (**B**) p-PARP, Bax and Bcl-2 in the kidney at 24 h and the corresponding quantitative analyses. (**C**) Representative TUNEL staining of apoptotic cells in the kidney at 24 h and the corresponding quantitative analyses. Scale bar=100 μm. *P<0.05.

Figure 6

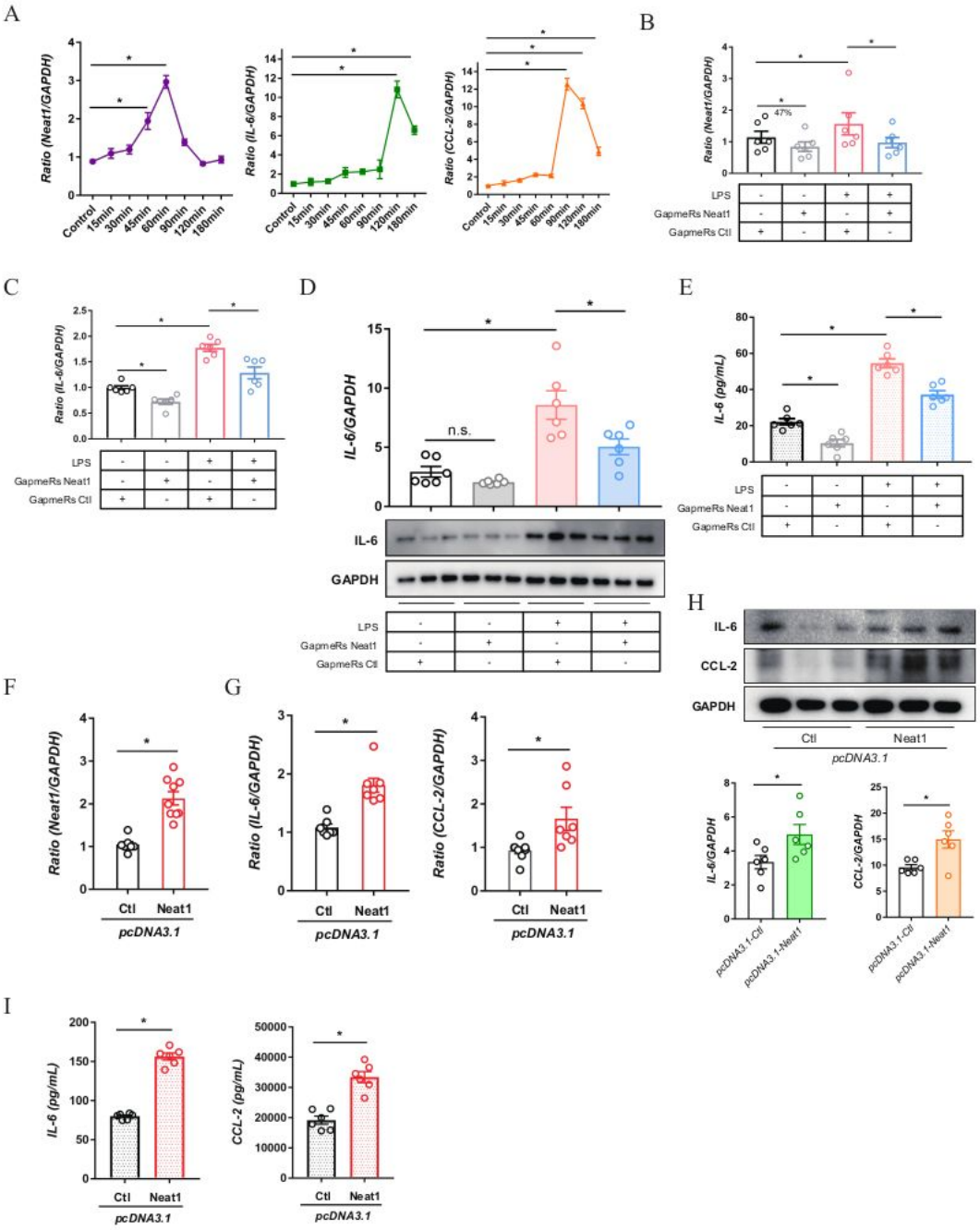

Figure 6

Neat1 mediated inflammation in C1.1 mouse tubular cells (**A**) Relative expression of Neat1, IL-6, and CCL-2 at different times after LPS stimulation by qRT-PCR. (**B**) Efficiency of Neat1 knockdown by Neat1 antisense LNA GapmeR compared with negative control (Ctl) with or without LPS stimulation by qRT-PCR. Relative expression of IL-6 in cells transfected with Ctl or GapmeRs Neat1 under LPS stimulation as determined by (**C**) qRT-PCR, (**D**) Western blotting and (**E**) ELISA. (**F**) Neat1 overexpression after transfection with pcDNA3.1 Neat1 plasmids by qRT-PCR. Relative expression of IL-6 and CCL-2 in cells transfected with Ctl or Neat1 plasmids as determined by (**G**) qRT-PCR, (**H**) Western blotting and (**I**) ELISA. *P<0.05.

Figure 7

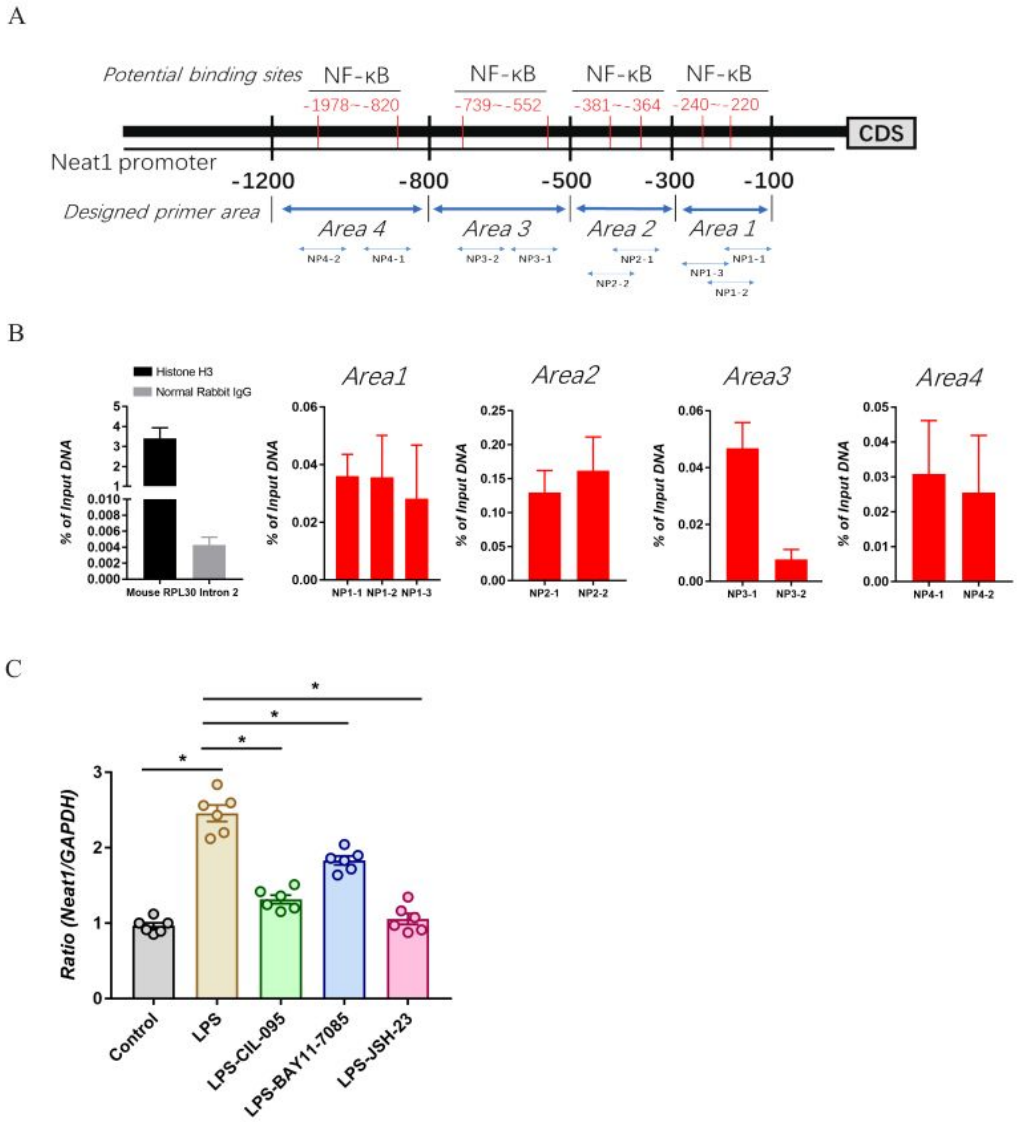

Figure 7

Induction of Neat1 expression was regulated by TLR/NF-κB signaling in C1.1 cells (**A**) Schematic diagram of Neat1's promoter region on mouse chromosome 19. Primers were designed for the four putative p65/NFκB binding sites (Areas 1–4). (**B**) Enrichment of p65/NFκB binding fragment presented as percentage relative to input DNA. Anti-histone H3 antibody and normal rabbit IgG were used as

positive and negative controls, respectively. (**C**) Relative expression of Neat1 under LPS stimulation with pretreatment of TLR4 inhibitor (CIL-095) or NF-κB inhibitors (BAY11-7085, JSH-23) by qRT-PCR. *P<0.05.

Figure 8

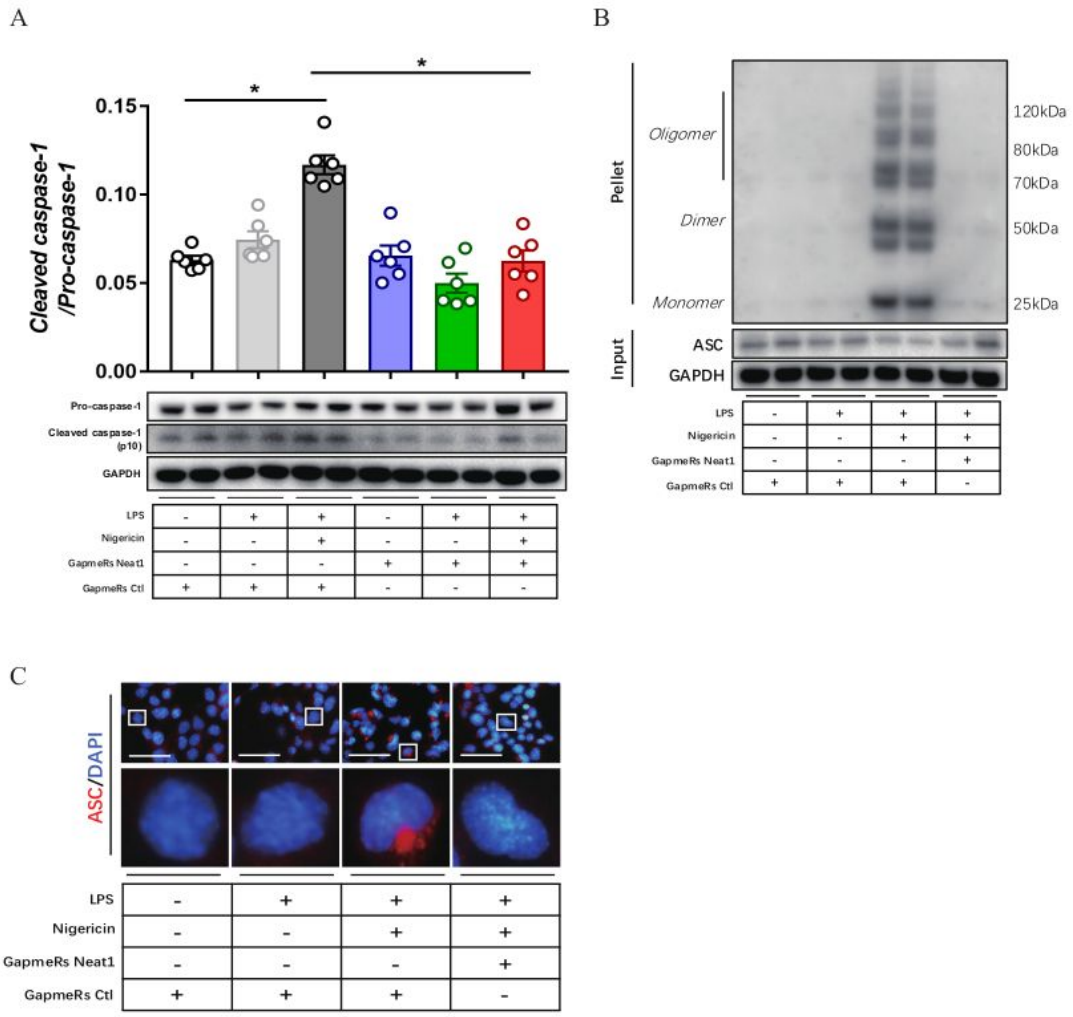

Figure 8

Knockdown of Neat1 attenuated NLRP3 inflammasome activation in C1.1 cells. (**A**) Representative Western blot analysis of expression levels of pro-caspase-1 and cleaved caspase-1 (p10) in LPS-primed,

nigericin-induced cells with Ctl or GapmeR Neat1 and the corresponding quantitative analyses. (**B**) Western blotting showing the effect of Neat1 knockdown on ASC oligomerization in cell pellets from LPS-primed, nigericin-induced cells. Soluble cell lysates were used as input. (**C**) Representative immunofluorescence staining of endogenous ASC speck (red) and DAPI (blue) in LPS-primed, nigericin-induced cells with Ctl or GapmeR Neat1. Scale bar=100 μm.*P<0.05.

Figure 9

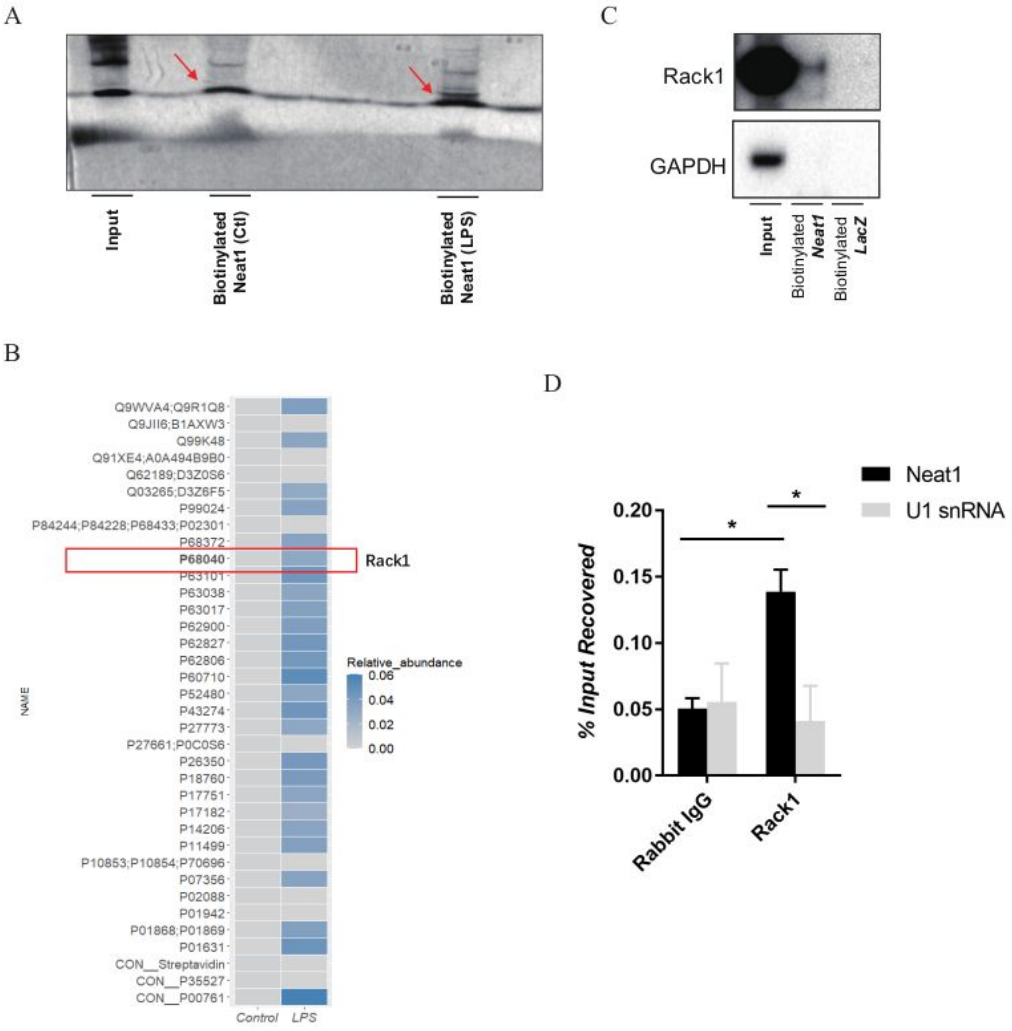

Figure 9

Rack1 is identified as a Neat1 binding partner. (**A**) Proteins binding to biotinylated Neat1 probe in unstimulated (Ctl) and LPS-stimulated (LPS) C1.1 cells from RNA pull-down assay were separated in SDS-PAGE and silver stained. Bands indicated by red arrows were excised for mass spectrometry. (**B**) Relative abundance of the protein candidates from mass spectrometry analysis. (**C**) Detection of Rack1 by Western blotting in RNA pull-down sample using biotinylated Neat1 probe in LPS-stimulated C1.1 cells. LacZ probe was used as negative control. (**D**) RIP using antibody against Rack1 or normal rabbit IgG (negative control) in LPS-stimulated C1.1 cell extracts followed by qRT-PCR with specific primers to Neat1 or U1 snRNA (negative control). *P<0.05.

Figure 10

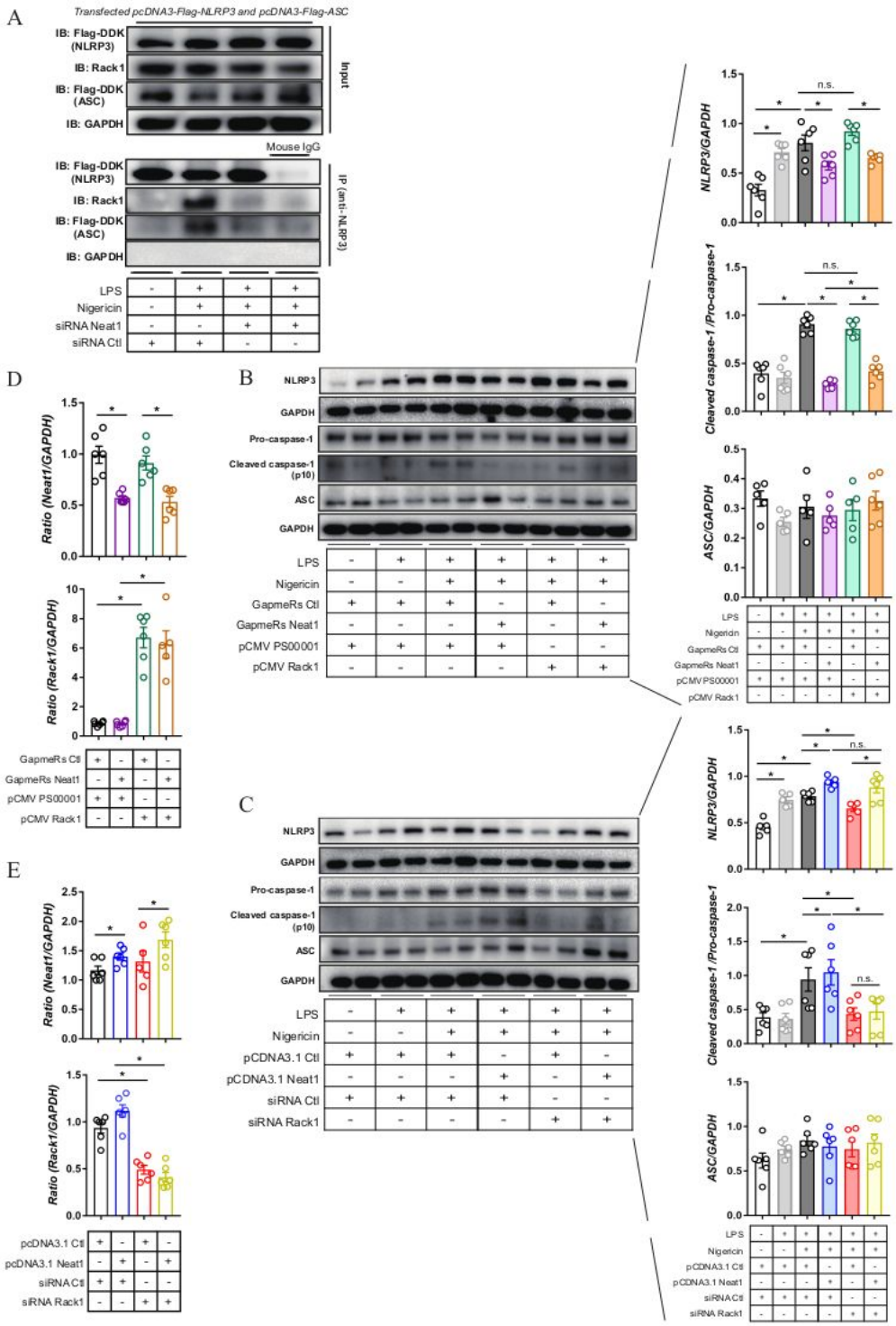

Figure 10

Neat1 interacts with Rack1 and regulates NLRP3 inflammasome. (**A**) Co- immunoprecipitation with anti-NLRP3 antibody on LPS-primed, nigericin-induced HK293T cells co-transfected with pcDNA3-Flag-NLRP3 and pcDNA3-Flag-ASC plasmids with control (Ctl) or Neat1 siRNA. Normal mouse IgG was used as negative control. (**B**) Representative Western blot analysis of the effect of Neat1 knockdown and Rack1 overexpression and (**C**) of the effect of Neat1 overexpression and Rack1 knockdown on expression of

NLRP3, pro-caspase-1, cleaved caspase-1 (p10) and ASC in LPS-primed, nigericin-induced C1.1 cells and quantification. (**D and E**) The relative expression of Neat1 and Rack1 by qRT-PCR in C1.1 cells. *P<0.05.

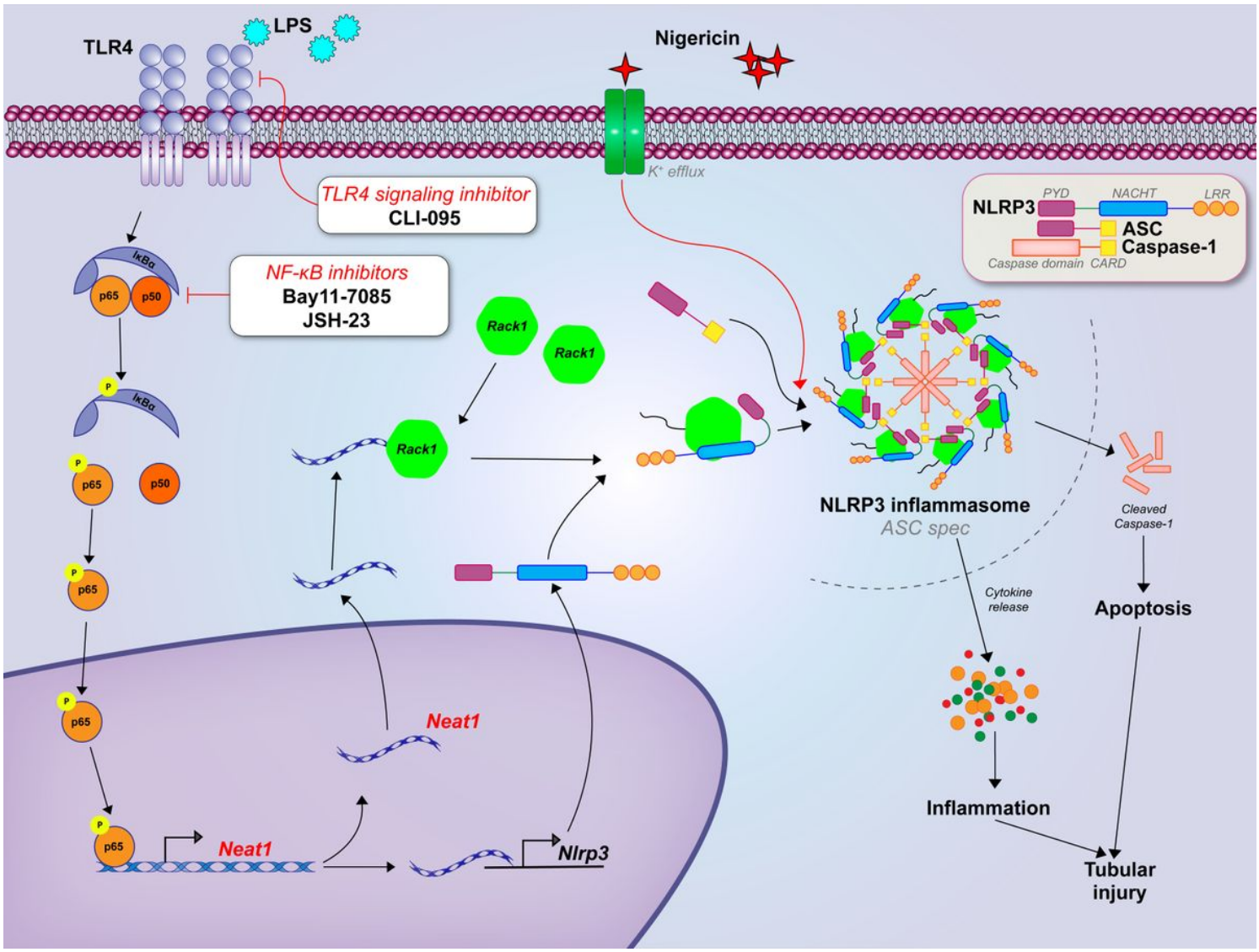

Figure 11

Schematic diagram showing Neat1's role in NLRP3 inflammasome assembly triggered by LPS/nigericin. LPS stimulates TLR4/NF-κB signaling and causes the expression of Neat1, which in turn binds to its protein partner Rack1 to further activate the assembly of NLRP3 inflammasomes in the presence of nigericin, resulting in kidney tubular cell inflammation, apoptosis and eventually acute kidney injury.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- R8SupplementaryMaterials.pdf
- Table1.pdf

- Table2.pdf