Upregulation of RIP3 promotes necroptosis via a ROS-dependent NF-κB pathway to induce chronic inflammation in HK-2 cells

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Abstract. Tubular atrophy/interstitial fibrosis (TA/IF) is a major cause of late allograft loss, and inflammation within areas of TA/IF is associated with adverse outcomes in kidney transplantation. However, there is currently no satisfactory method to suppress this inflammation to improve TA/IF. The present study aimed to determine the proinflammatory role of receptor-interacting protein 3 (RIP3) in TA/IF to discover a novel therapeutic target. Reverse transcription-quantitative PCR and western blotting were performed to detect the expression of RIP3 and inflammation-associated factors. Lactate dehydrogenase release assay was used to determine necroptosis. Fluorescent 2,7-dichlorodihydrofluorescein diacetate was used to detect the levels of reactive oxygen species (ROS). The results demonstrated that patients with chronic TA/IF exhibited upregulated receptor-interacting protein 3 (RIP3) expression compared with the patients who had a favorable recovery after renal transplant. Therefore, the current study used normal renal tubular epithelial cells HK-2 to establish a cellular model with a high expression level of RIP3 in order to investigate the effect of RIP3 on renal epithelial cells after transplantation. The western blotting results demonstrated that overexpression of RIP3 could significantly increase the phosphorylation level of the necroptosis executive molecule mixed lineage kinase domain-like protein. Lactate dehydrogenase release, a key feature of necroptosis, was also markedly improved by RIP3 overexpression. Moreover, a higher inflammatory response was detected in HK-2 cells with RIP3 overexpression, and this elevated inflammation could be restored by the necroptosis inhibitor necrosulfonamide. Of note, it was found that overexpression of RIP3 activated the NF-κB signaling pathway via the excessive accumulation of ROS to induce necroptosis, which ultimately led to inflammation. Collectively, these findings indicated that overexpression of RIP3 promoted necroptosis via a ROS-dependent NF-κB pathway to induce chronic inflammation, suggesting that RIP3 may have the potential to be a therapeutic target against inflammation in TA/IF.

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Introduction

Tubular atrophy/interstitial fibrosis (TA/IF), the histological characteristic of kidney allograft destruction over time, is a major cause of late allograft loss (1,2). TA/IF is a chronic, progressive, non-specific and irreversible histopathological entity that occurs in the early post-transplantation period (3), and is associated with significant morbidity and mortality in patients (4). Currently, the major challenge faced is the incomplete understanding of the factors that promote the development of TA/IF. Therefore, it is of great significance to examine the identifiable causes of chronic allograft TA/IF and to develop cause-specific treatment strategies.

Inflammation is a physiological defense mechanism against adverse stimuli, such as tissue damage and infection (5). A timely and powerful inflammatory response can effectively resist these harmful stimuli, while weak and continuous inflammation will aggravate the situation (6). Kidney allograft survival in patients with inflammation detected in the fibrotic areas of indication biopsies has been reported to be significantly worse compared with patients with non-inflammatory interstitial fibrosis (7-9), which suggested the close association between inflammation and TA/IF. Therefore, we hypothesized that effective inhibition of chronic inflammation may suppress TA/IF.

Receptor-interacting protein 3 (RIP3) is a cytosolic serine/threonine kinase that consists of an active kinase domain at the amino terminus (10), and it serves an important role in the process of necroptosis, a programmed form of necrotic cell death (11,12). RIP3 associates with RIP1 to form a signaling complex known as the necrosome, and then mixed lineage kinase domain-like protein (MLKL) causes a change in its conformation, leading to its translocation to the plasma membrane and subsequent membrane disruption (13,14).

Necroptosis is a highly inflammatory type of cell death caused as a result of the release of intracellular immunogenic contents, which stimulates innate immune cells and subsequently inflammation (15). However, there are few reports regarding the role of necroptosis in kidney transplantation.

Reactive oxygen species (ROS) have long been considered as a driving force for necroptosis (16). For example, it has been reported that TNF can induce mitochondrial ROS, and ROS can enhance necrosome formation (17,18). It has been also been revealed that RIP1 can sense ROS via the modification of three crucial cysteine residues, and its autophosphorylation on S161 is subsequently induced. This phosphorylation event allows for the efficient recruitment of RIP3 to RIP1 to form a functional necrosome (19); however, research examining the relationship between ROS and RIP3 has been inconclusive.

In the present study, the differences in expression of RIP3 in patients with chronic TA/IF and patients who had a good recovery after renal transplant were tested. Then, normal renal tubular epithelial cells HK-2 were used to establish a cellular model with RIP3 overexpression in order to examine the effect of RIP3 on renal epithelial cells after transplantation. Once the importance of RIP3 is determined, novel treatment strategies could be developed that suppress inflammation to improve TA/IF.

Materials and methods

Tissue samples. Patients who underwent kidney transplant and did not have chronic diseases such as diabetes, hypertension or fatty liver disease were enrolled in the present experiment between March 2018 and October 2019. Puncture specimens from all patients with kidney transplant were obtained from the Department of Renal Transplantation, Ningbo Urology and Nephrology Hospital (Ningbo, China). A total of 45 puncture specimens were collected, of which 16 had good recovery [normal transplant (NT) group] and the other 29 samples had chronic TA/IF. The study protocol was approved by the Ethical Committee of Ningbo Urology and Nephrology Hospital (approval no. YZRY2017120034), and informed written consent was obtained from all the subjects. The clinicopathological characteristics of the included patients are summarized in Table I.

Cell culture. The cell line, HK-2, used in the present study was obtained from the American Type Culture Collection, and cells were cultured in DMEM (HyClone; Cytiva), supplemented with 10% heat-inactivated FBS (Shanghai ExCell Biology, Inc.). Cells were maintained at 37˚C with 5% CO_2 in a humid environment.

Generation of stable cell lines. RIP3-expressing lentivirus with green fluorescent protein and puromycin resistance markers were purchased from Shanghai GeneChem Co., Ltd. RIP3-expressing lentivirus packaging used three plasmids: GV492 plasmid carrying RIP3 CDS region, helper plasmids pHelper 1.0 and pHelper 2.0 (all Shanghai GeneChem Co., Ltd.). A 3rd generation lentivirus packaging system was used. A total of 20 GV492, 15 pHelper 1.0 and 10 μg pHelper 2.0 were mixed with 25 μl transfection reagent (cat. no. LPK001; Shanghai GeneChem Co., Ltd.), adjusted to a total volume of 1 ml and added to 293 cells (American Type Culture Collection) after standing for 15 min. The 293 cells were cultured in a 37˚C, 5% CO_2 incubator for 6 h. Next, fresh medium containing 10% serum was added before culturing for 48-72 h. Supernatant was collected, centrifuged at 4,000 x g for 10 min at 4˚C to remove cell debris, then filtered with a 0.45-μM filter. Stable cell lines were established according to the manufacturer's protocol. Briefly, $7.5x10^4$ HK-2 cells were seeded onto a 6-well plate and transfected with lentivirus vectors (MOI=10), and the cell culture medium was replaced with complete medium after 24 h. Lentivirus-transfected HK-2 cells were selected using complete medium containing 4 μg/ml puromycin after 72 h. At 1 week after screening, the cells were used for subsequent experiments. Subsequently, the cells were maintained with 4 μg/ml puromycin. RIP3 overexpression was confirmed via western blotting. Cells overexpressing RIP3 were referred to as the RIP3-OE group. Cells with control lentivirus were used as the negative control (NC) group.

Cell treatment. To inhibit MLKL, HK-2 cells were treated with 5 μM necrosulfonamide (NSA; cat. no. 480073; Sigma-Aldrich; Merck KGaA) and cultured in a 37˚C cell incubator for 3 h, then the cells were collected for subsequent experiments. For NF-κB inhibitor (BAY 11-7085) treatment, HK-2 cells were treated with 5 μM BAY 11-7085 (cat. no. HY-10257; MedChemExpress) and cultured in a 37˚C cell incubator for 3 h, then the cells were collected for subsequent experiments.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from tissue and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was conducted using the PrimeScript RT Master Mix kit according to the manufacturer's protocol. (Takara Biotechnology Co., Ltd.). cDNAs were amplified via RT-qPCR using SYBR Green PCR Master mix (Roche Diagnostics) on a LightCycler® 480 system (Roche Diagnostics) as follows: 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec, 45 cycles in total. The relative expression levels were normalized against the expression level of the endogenous control, GAPDH using the $2^{-\Delta\Delta Cq}$ method (20). The PCR primers are presented in Table II.

Western blot analysis. RIPA kit (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) was used to extract cellular protein. Protein concentrations were determined with BCA kit (cat. no. P0010; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Proteins (30 μg per lane) were separated via 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.) and blocked with TBS with 0.1% Tween-20 (TBST) containing 5% non-fat dry milk at room temperature for 90 min. Anti-RIP3 (1:1,000; cat. no. ab56164; Abcam), anti-phosphorylated (p)-RIP3 (1:1,000; cat. no. ab209384; Abcam), anti-MLKL (1:1,000; cat. no. ab183770; Abcam), anti-p-MLKL (1:1,000; cat. no. ab187091; Abcam), anti-RIP1 (1:1,000; cat. no. 4926; Cell Signaling Technology, Inc.), anti-p-RIP1 (1:1,000; cat. no. 44590; Cell Signaling Technology, Inc.), anti-NF-κB (1:2,000; cat. no. 06-418; EMD Millipore) and anti-GAPDH (1:1,000; cat. no. ab181602; Abcam) antibodies were diluted in TBST containing 3% non-fat dry milk and incubated with the membranes overnight at 4˚C. Membranes were then washed

Table I. Clinicopathological characteristics of the patients included in the present study.

Clinicopathological characteristics	Normal group	TA/IF group
Total number, n	16	29
Age, years, mean ± SEM	43±8	45±12
Sex		
Male, n	9	18
Female, n	7	11
Puncture time, months[a]		
<12	2	3
≥12	14	26
Creatinine, μmol/l, mean ± SEM	97±42	212±98
Hemoglobin, g/l, mean ± SEM	95±14	102±19

[a]Time to kidney transplant /month. TA/IF, tubular atrophy and/or interstitial fibrosis.

Table II. Oligonucleotide sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Primer sequences (5'→3')
RIP3	F: CTGAGTGGCTAAACAAACTGAATC
	R: AGGTAGGGCTGGGCATCTG
IL-8	F: ACTCCAAACCTTTCCACCCC
	R: TTCTCAGCCCTCTTCAAAAACT
IL-1β	F: CAGAAGTACCTGAGCTCGCC
	R: AGATTCGTAGCTGGATGCCG
IL-33	F: TTATGAAGCTCCGCTCTGGC
	R: CCAAAGGCAAAGCACTCCAC
NLRP3	F: AGAACTTTCTGTGTGGACCGA
	R: AGCCCTTCTGGGGAGGATAG
BMF	F: TGGAAACAATACCGCACCGT
	R: ACTCGATTGGGAAGGAGGGA
BNIP3	F: CTGGAGTCTGACTTGGTTCGT
	R: CCACCCCAGGATCTAACAGC
GAPDH	F: AATGGGCAGCCGTTAGGAAA
	R: GCGCCCAATACGACCAAATC

F, forward; R, reverse; RIP3, receptor-interacting protein 3; NLRP3, NLR family pyrin domain containing 3; BMF, Bcl-2-modifying factor; BNIP3, Bcl-2-interacting protein 3.

with TBST and incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:5,000; cat. no. sc2030; Santa Cruz Biotechnology, Inc.). Subsequently, membranes were washed with TBST and developed using an ECL-Plus reagent (Cytiva). ImageJ software version 1.48 (National Institutes of Health) was used to evaluate the gray value of the western blots.

Measuring ROS. In order to detect the level of ROS and exclude the interference of GFP, HK-2 cells transfected with a RIP3-expressing lentivirus that did not contain GFP but had puromycin resistance was also constructed as aforementioned. RIP3-expressing lentivirus packaging used three plasmids: GV341 plasmid carrying RIP3 CDS region, helper plasmids pHelper 1.0 and pHelper 2.0 (all Shanghai GeneChem Co., Ltd.) A total of $3x10^5$ cells suspended in 2 ml fresh media were plated in each well of a 6-well plate and incubated overnight. Then, the cells were treated with or without 10 μM N-acetylcysteine (NAC; Beyotime Institute of Biotechnology) at 37˚C for 6 h. The cells were incubated with 10 μmol/l 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37˚C for 30 min to assess the ROS-mediated oxidation of DCFH-DA to the fluorescent compound DCF. The images showing the green fluorescence of DCF in the cells were acquired using a Nikon Ti-U fluorescence microscope (Nikon Corporation). Next, the cells were harvested, and the pellets were suspended in 1 ml PBS. Samples were analyzed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a FACScan flow cytometer (Becton, Dickinson and Company). Data were analyzed with FlowJo software version 10 (Becton, Dickinson and Company).

Necroptosis assay determining lactate dehydrogenase (LDH) release. RIP3-OE and NC cells ($1x10^4$/well) were seeded onto a 96-well microplate and cultured for 24 h. The LDH cytotoxicity assay kit (cat. no. C0017; Beyotime Institute of Biotechnology) was used to detect cell death after the overexpression of RIP3, according to the manufacturer's instructions. Briefly, cells (~$1x10^5$ cell/well) were added into a 96-well cell culture plate (the cell density did not exceed 80-90% when it was tested), and then each culture well was divided into the following groups: i) Cell-free culture medium wells (background blank control wells); ii) control cell wells without treatment (sample control wells); iii) cell wells that were not treated for subsequent lysis (sample maximum enzyme activity control wells); and iv) cell wells with treatment (experimental sample wells). Then, 1 h before the scheduled detection time, the cell culture plate was removed and the LDH release reagent (10% of the original culture fluid volume) provided in the kit was added to the 'sample maximum enzyme activity control well', mixed several times and incubated for 30 min. Subsequently, the cell culture plate with a multi-well plate was centrifuged at 400 x g, 4˚C for 5 min. A total of 120 μl supernatant was

removed from each well and added to the corresponding wells of a new 96-well plate, and then sample determination was conducted. The absorbance value of each sample was read at 490 nm (iMark™ Microplate Reader; Bio-Rad Laboratories, Inc.).

Statistical analysis. The data are presented as the mean ± SEM of three independent experiments (n=3). GraphPad Prism 5 software (GraphPad Software, Inc.) was used for analysis. All data in this study conformed to a normal distribution. First, a Shapiro-Wilk test was used to analyze whether the data followed a normal distribution. Then, one-way ANOVA was performed followed by post hoc Bonferroni's correction to compare the differences between the groups. To compare differences between two groups, an F-test was first used to compare variances, following which unpaired t-test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of RIP3 can induce necroptosis in renal tubular epithelial cells. To detect the expression level of RIP3 in patients with renal transplant, a total of 45 puncture specimens were collected and RT-qPCR was performed using samples from the NT and chronic TA/IF groups. Compared with the NT group, the patients with chronic TA/AF were found to have upregulated RIP3 expression (Fig. 1A).

To further evaluate the relationship between RIP3 and TA/AF, a lentivirus vector-mediated RIP3 overexpressing stable HK-2 cell line was established and the transfection efficiency of the virus is presented in Fig. 1B. RT-qPCR and western blotting were then performed. Compared with the NC group, the expression of RIP3 was significantly higher in the RIP3-OE group (Fig. 1C and D). Since RIP3 is a key regulatory gene of necroptosis (11,12), the changes in cell morphology were analyzed via microscopy (Fig. 1E). Cells began to round out and cytoplasmic vacuolation appeared following RIP3-OE. The phosphorylation level of RIP3, as well as two key genes RIP1 and MLKL located upstream and downstream, were examined in the RIP3-OE group. As presented in Fig. 1F, the phosphorylation levels of RIP3 and MLKL were significantly increased, while the phosphorylation level of RIP1 was not changed.

Differing from apoptotic cell death, necroptosis does not result in chromatin condensation, shrinkage of the cell body or a fragmented genome, but is characterized by the damaged integrity of the cytoplasm membrane, which can be confirmed by LDH leakage (21,22). The results demonstrated that LDH leakage, which can indicate the degree of necroptosis, was significantly increased in the RIP3-OE group compared with the NC (Fig. 1G). At the molecular level, necroptotic cells usually have higher expression levels of the genes that are associated with the RIP cascades, such as Bcl-2-modifying factor (BMF) and Bcl-2-interacting protein 3 (BNIP3) (21,23). Therefore, the expression levels of BMF and BNIP3 were detected in the RIP3-OE group, and it was found that both were increased (Fig. 1H). These results indicated that RIP3 overexpression can induce necroptosis in renal tubular epithelial cells.

Overexpression of RIP3 causes inflammation in HK-2 cells. Kidney allograft survival in patients with inflammation detected in the fibrotic areas of indication biopsies has been reported to be significantly worse compared with patients with non-inflammatory interstitial fibrosis (7). Thus, the expression levels of the pro-inflammatory cytokines IL-1β, IL-8 and the IL-33 were measured. The results demonstrated a significant increase in the transcript levels of all cytokines in HK-2 cells in the RIP3-OE group (Fig. 2A-C). Moreover, the expression of NLR family pyrin domain containing 3 (NLRP3), which is a well-characterized inflammasome (24), was examined. It was found that the NLRP3 transcript was significantly increased in the RIP3-OE group compared with the NC group (Fig. 2D). Taken together, these data suggested that the overexpression of RIP3 caused inflammation in HK-2 cells.

Necroptosis induced by RIP3 is involved in inflammation in HK-2 cells. Necrotic cell death is a highly inflammatory type of cell death due to the release of intracellular immunogenic contents that stimulate innate immune cells and subsequently inflammation (15). In order to verify whether PIR3-induced necroptosis was the cause of inflammation, NSA was used to detect inflammatory-related indicators. First, it was identified that NSA could effectively inhibit the phosphorylation level of MLKL (Fig. 3A), and significantly reduce the number of necroptotic cells (Fig. 3B). Furthermore, after NSA treatment, the expression levels of IL-1β, IL-8, IL-33 and NLRP3 were significantly lower compared with those of the RIP3-OE group (Fig. 3C-F). These data revealed that necroptosis induced by RIP3 was involved in inflammation in HK-2 cells.

ROS production is a mediator of necroptosis-induced inflammation in HK-2 cells. Necrosomes have been reported to impair mitochondria energy metabolism by disturbing ROS homeostasis, leading to ATP depletion, mitochondrial depolarization and eventually cell death (25). It is well-known that ROS are important mediators to induce oxidative stress, which is a common mechanism of injury in chronic allograft TA/IF (26). Therefore, it was suggested that ROS production may be a mediator of necroptosis-induced inflammation. To confirm this, the fluorescent dye DCFH-DA was utilized to detect ROS levels. From the fluorescence image in Fig. 4A, it was observed that ROS levels were increased in the RIP3-OE group. Moreover, flow cytometry was used for the semi-quantitative analysis of ROS production, and consistent results are presented in Fig. 4B. These results indicated that overexpression of RIP3 caused increased ROS levels in HK-2 cells.

To address whether ROS production was a mediator of necroptosis-induced inflammation, the effects of the antioxidant NAC on the necroptosis induced by the overexpression of RIP3 were evaluated. As shown in Fig. 4A and B, the generation of ROS in the RIP3-OE + NAC group was significantly suppressed. At the same time, it was found that, with the decrease of ROS, the phosphorylation levels of RIP3 and MLKL (Fig. 4C) and the release of LDH (Fig. 4D) were significantly reduced in the RIP3-OE + NAC group, which indicated that necroptosis was inhibited. Furthermore, the expression of NLRP3 was significantly reduced in the RIP3-OE + NAC group (Fig. 4E), indicating that inflammation had been markedly improved with the reduction of ROS.

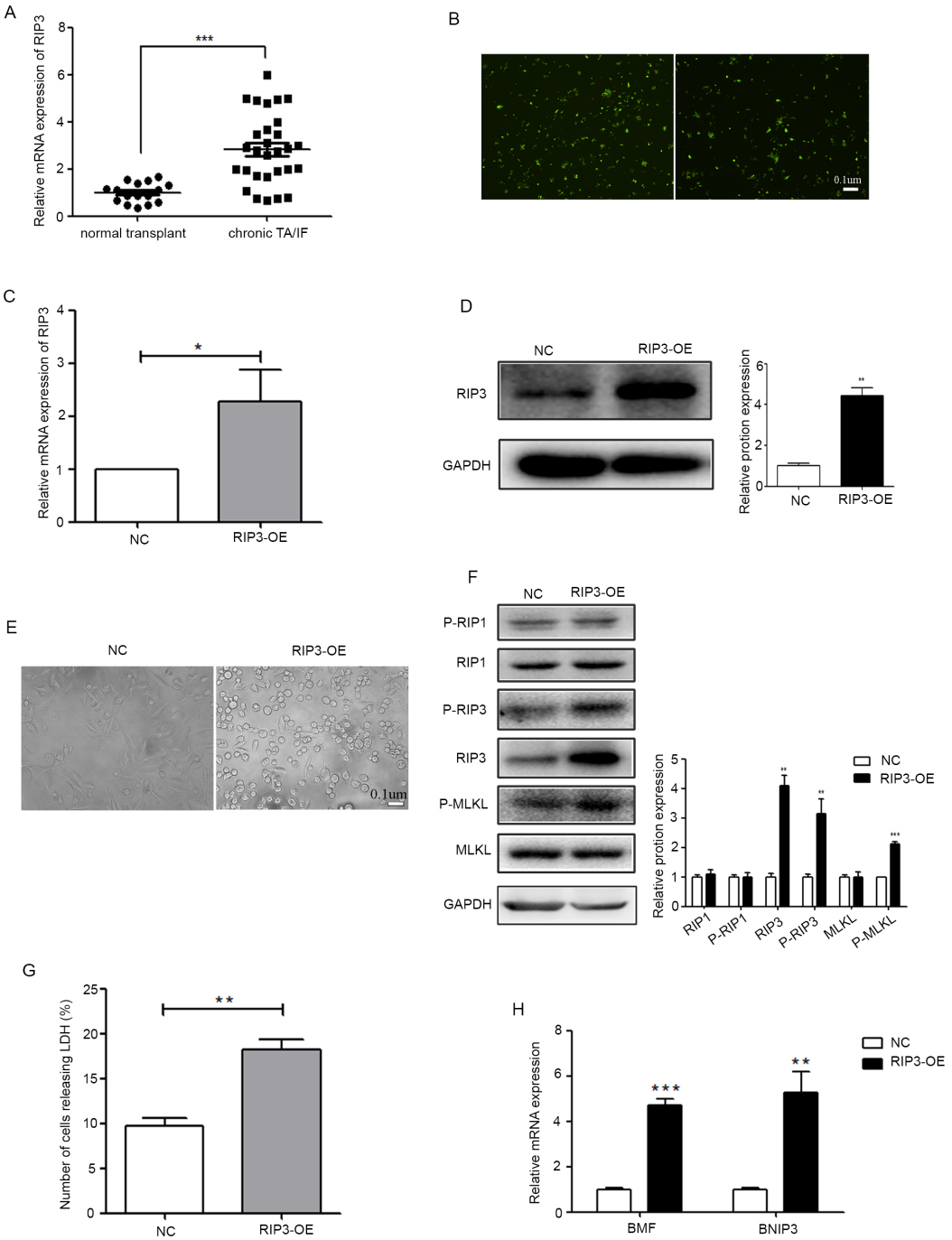

Figure 1. RIP3 overexpression can induce necroptosis in renal tubular epithelial cells. (A) mRNA expression level of RIP3 was analyzed in 16 patients with NT and in 29 patients with chronic TA/IF using RT-qPCR. ***P<0.001 vs. NT group. (B) A lentivirus vector-mediated RIP3 overexpressing stable HK-2 cell line was established and the transfection efficiency of the virus was determined (scale bar, 0.1 μm). (C) RT-qPCR was performed to detect the expression level of RIP3 in HK-2 cells. (D) Western blotting was performed to examine the expression level of RIP3. The lentivirus RIP3-OE vector significantly increased the expression level of RIP3 in HK-2 cells. (E) The influence of RIP3 on the morphological changes of HK-2 cells (scale bar, 0.1 μm). (F) Protein expression levels of RIP1, RIP3 and MLKL and their phosphorylation levels were examined via western blotting in HK-2 cells. (G) LDH release assay results identified that the level of cell death was increased in the RIP3-OE group compared with the NC. (H) RT-qPCR was performed to detect the expression levels of BMF and BNIP3 in HK-2 cells. *P<0.05, **P<0.01, ***P<0.001 vs. NC. RT-qPCR, reverse transcription-quantitative PCR; RIP, receptor-interacting protein; NT, normal transplant; TA/IF, tubular atrophy and/or interstitial fibrosis; OE, overexpression group; NC, negative control; MLKL, mixed lineage kinase domain-like protein; BMF, Bcl-2-modifying factor; BNIP3, Bcl-2-interacting protein 3; LDH, lactate dehydrogenase; p-, phosphorylated.

Collectively, these experimental results suggested that ROS production was a mediator of necroptosis-induced inflammation.

Necroptosis induces inflammation via a ROS-dependent NF-κB pathway. NF-κB consists of a family of transcription factors that serve a central role in the expression levels of inflammatory-related factors (27). Therefore, the current study further investigated the relationship between necroptosis, NF-κB and inflammation. In the RIP3-OE group, it was found that overexpression of RIP3 could activate NF-κB, and reducing the levels of ROS in the RIP3-OE + NAC group

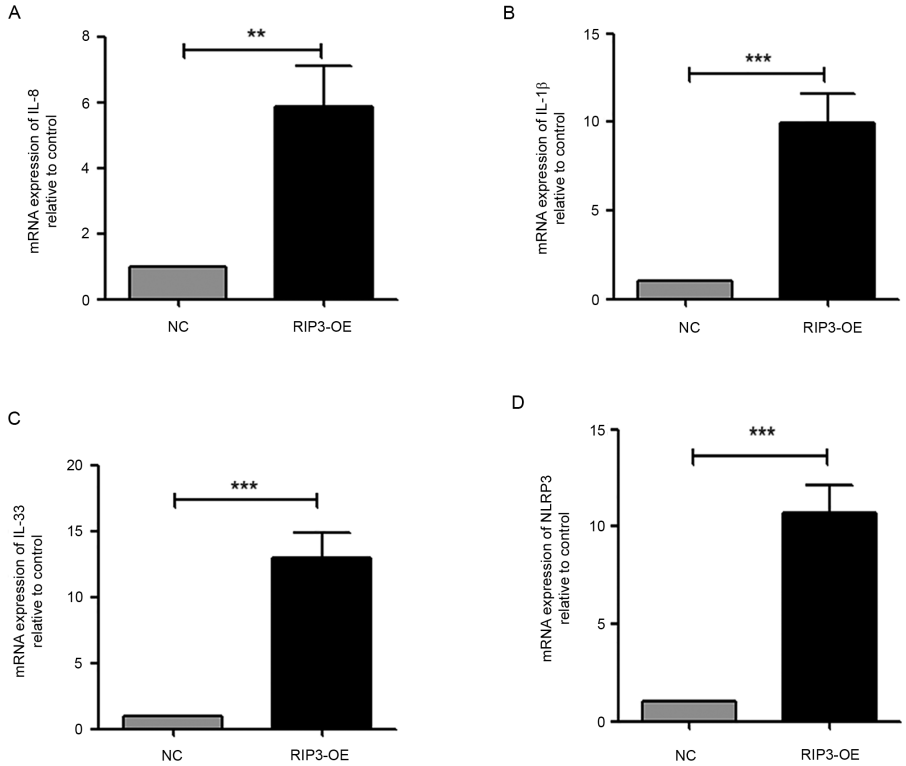

Figure 2. Overexpression of RIP3 causes inflammation in HK-2 cells. Reverse transcription-quantitative PCR analysis of the mRNA expression levels of (A) IL-8, (B) IL-1β, (C) IL-33 and (D) NLRP3 in HK-2 cells following transfection with the RIP3-OE vector. **P<0.01, ***P<0.001 vs. NC. NLRP3, NLR family pyrin domain containing 3; RIP3, receptor-interacting protein 3; OE, overexpression group; NC, negative control.

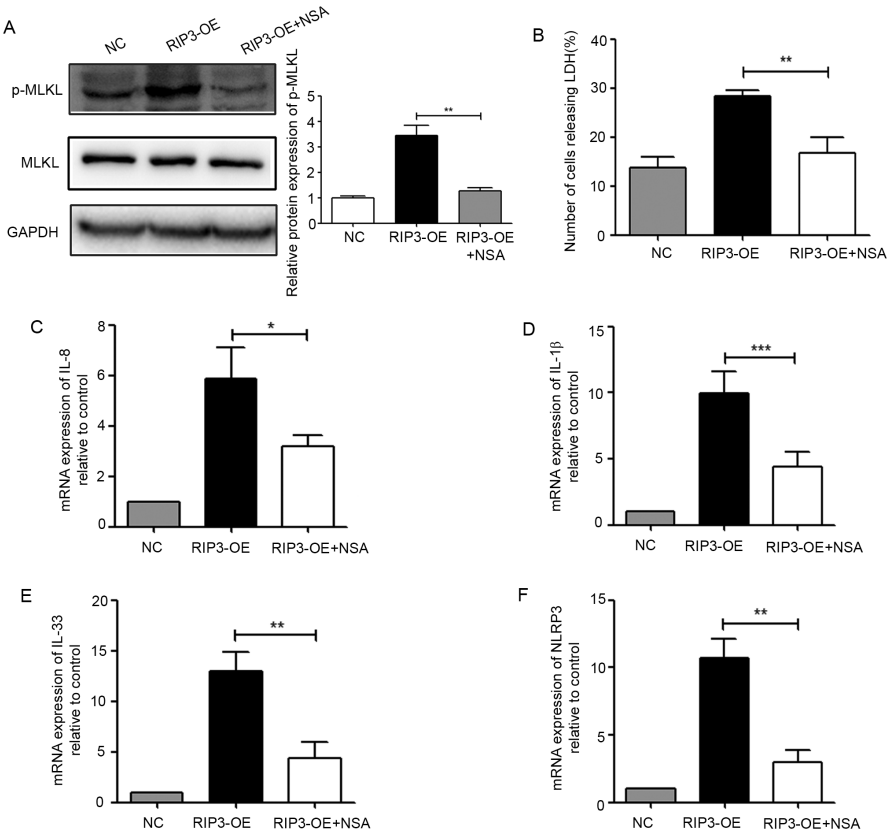

Figure 3. Necroptosis induced by RIP3 is involved in inflammation in HK-2 cells. (A) Western blot analysis of p-MLKL expression in HK-2 cells after treatment with the MLKL inhibitor NSA (5 μM). (B) LDH release was detected in HK-2 cells after treatment with the MLKL inhibitor NSA (5 μM). Reverse transcription-quantitative PCR analysis of the mRNA expression levels of (C) IL-8, (D) IL-1β, (E) IL-33 and (F) NLRP3 in HK-2 cells after treatment with the MLKL inhibitor NSA (5 μM). *P<0.05, **P<0.01, ***P<0.001 vs. RIP3-OE group. NLRP3, NLR family pyrin domain containing 3; RIP3, receptor-interacting protein 3; MLKL, mixed lineage kinase domain-like protein; p-, phosphorylated; LDH, lactate dehydrogenase; NSA, necrosulfonamide; OE, overexpression group; NC, negative control.

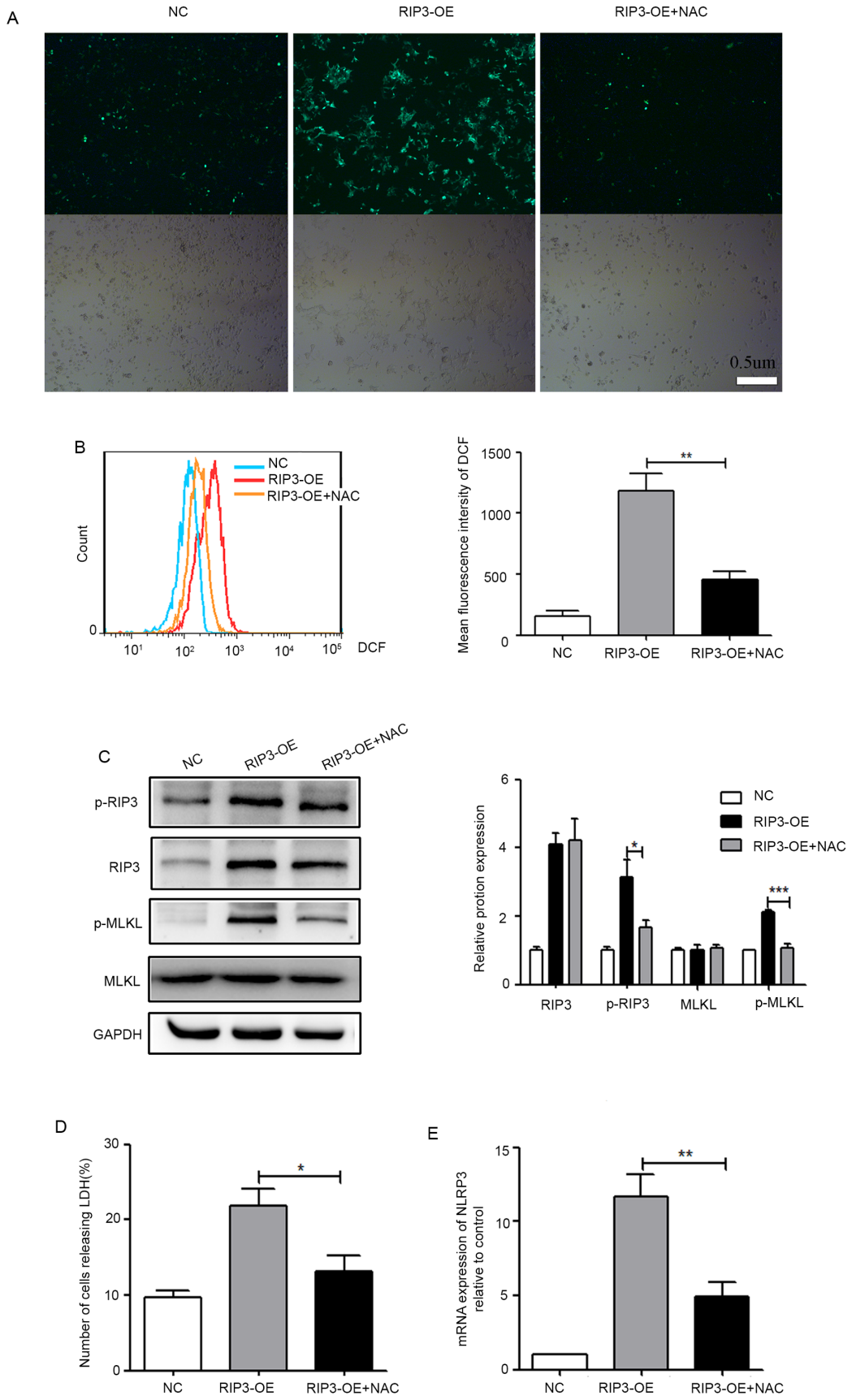

Figure 4. ROS production is a mediator of necroptosis-induced inflammation in HK-2 cells. (A) HK-2 cells were pretreated with/without 10 mM NAC for 6 h, then fluorescence microscopy of cells was conducted following 10 μM DCFH-DA staining for 30 min (scale bar, 0.5 μm). (B) Flow cytometry was used for the semi-quantitative analysis of (A). (C) Western blot analysis of p-RIP3 and p-MLKL expression in HK-2 cells after treatment with NAC to decrease ROS levels. (D) LDH release was detected in HK-2 cells after treatment with NAC. (E) Reverse transcription-quantitative PCR analysis of the mRNA expression level of NLRP3 in HK-2 cells after treatment with NAC. *P<0.05, **P<0.01, ***P<0.001 vs. RIP3-OE group. NLRP3, NLR family pyrin domain containing 3; RIP3, receptor-interacting protein 3; MLKL, mixed lineage kinase domain-like protein; p-, phosphorylated; ROS, reactive oxygen species; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; OE, overexpression group; NC, negative control.

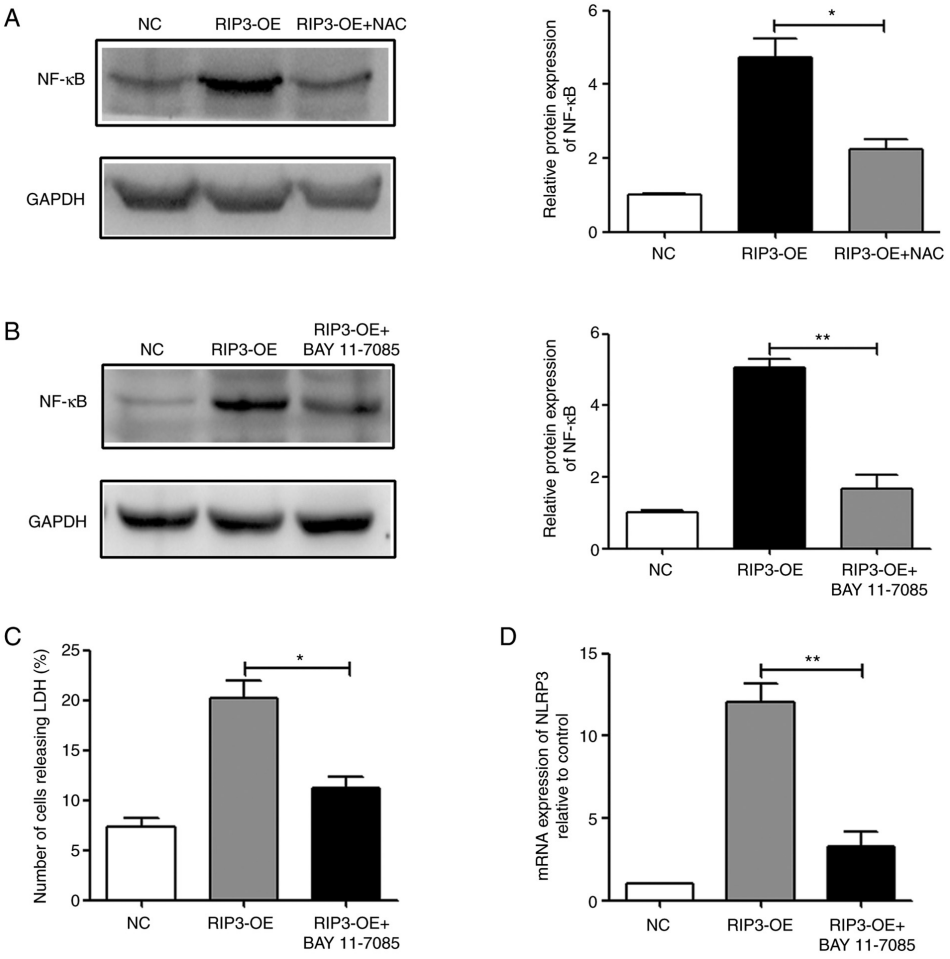

Figure 5. Necroptosis induces inflammation via a ROS-dependent NF-κB pathway. (A) Western blot analysis of NF-κB expression in RIP3-OE cells after treatment with 10 mM NAC for 6 h. (B) Western blot analysis of NF-κB expression in RIP3-OE cells after treatment with 5 μM BAY 11-7085 for 3 h. (C) LDH release was detected in RIP3-OE cells after treatment with 5 μM BAY 11-7085 for 3 h. (D) Reverse transcription-quantitative PCR analysis of the mRNA expression level of NLRP3 in RIP3-OE cells after treatment with 5 μM BAY 11-7085 for 3 h. *P<0.05, **P<0.01 vs. RIP3-OE group. ROS, reactive oxygen species; NAC, N-acetylcysteine; LDH, lactate dehydrogenase; RIP3, receptor-interacting protein 3; NLRP3, NLR family pyrin domain containing 3; OE, overexpression group; NC, negative control; BAY 11-7085, NF-κB inhibitor.

could partially reverse this effect (Fig. 5A). Therefore, it was suggested that overexpression of RIP3 could activate the NF-κB signaling pathway via ROS.

Next, BAY 11-7085 was used to treat RIP3-OE HK-2 cells. The results demonstrated that treatment with BAY 11-7085 could effectively suppress the activity of NF-κB (Fig. 5B). Then, necroptosis and inflammation were examined, and it was identified that in the RIP3-OE + BAY 11-7085 group, LDH release and NLRP3 expression levels were significantly reduced compared with the RIP3-OE group (Fig. 5C and D). Thus, indicating that inhibition of NF-κB activity could effectively reduce the occurrence of necroptosis and the appearance of inflammation caused by RIP3 overexpression. Taken together, the results suggested that necroptosis induced inflammation via a ROS-dependent NF-κB pathway.

Discussion

During clinical renal transplantation, multiple renal allografts are lost in the long-term due to chronic renal dysfunction associated with the development of TA/IF (1,2). Inflammation within the areas of interstitial fibrosis and tubular atrophy are associated with accelerated TA/IF, arterial fibrointimal hyperplasia and chronic glomerulopathy, as well as with reduced renal function (7,28). However, the underlying mechanisms and relationships between inflammation and TA/IF require further investigation. In the present study, patients with chronic TA/IF were found to have upregulated RIP3 expression compared with patients who had good recovery after renal transplant. Therefore, normal renal tubular epithelial cells HK-2 were used to establish a cellular model with RIP3 overexpression in order to investigate the effect of RIP3 on renal epithelial cells after transplantation.

RIP3 is a cytosolic serine/threonine kinase that consists of an active kinase domain at the amino terminus. RIP3 functions as an essential adaptor for necroptosis, which is a type of regulated necrosis controlled by RIP3 and its downstream effector MLKL (11). Thus, in the current study, after the overexpression of RIP3, relevant indicators of necroptosis were detected, and it was found that the phosphorylation levels of RIP3 and MLKL were significantly increased, and LDH release, which is a key feature of necroptosis, was elevated. These findings indicated that the overexpression of RIP3 can induce cell necroptosis.

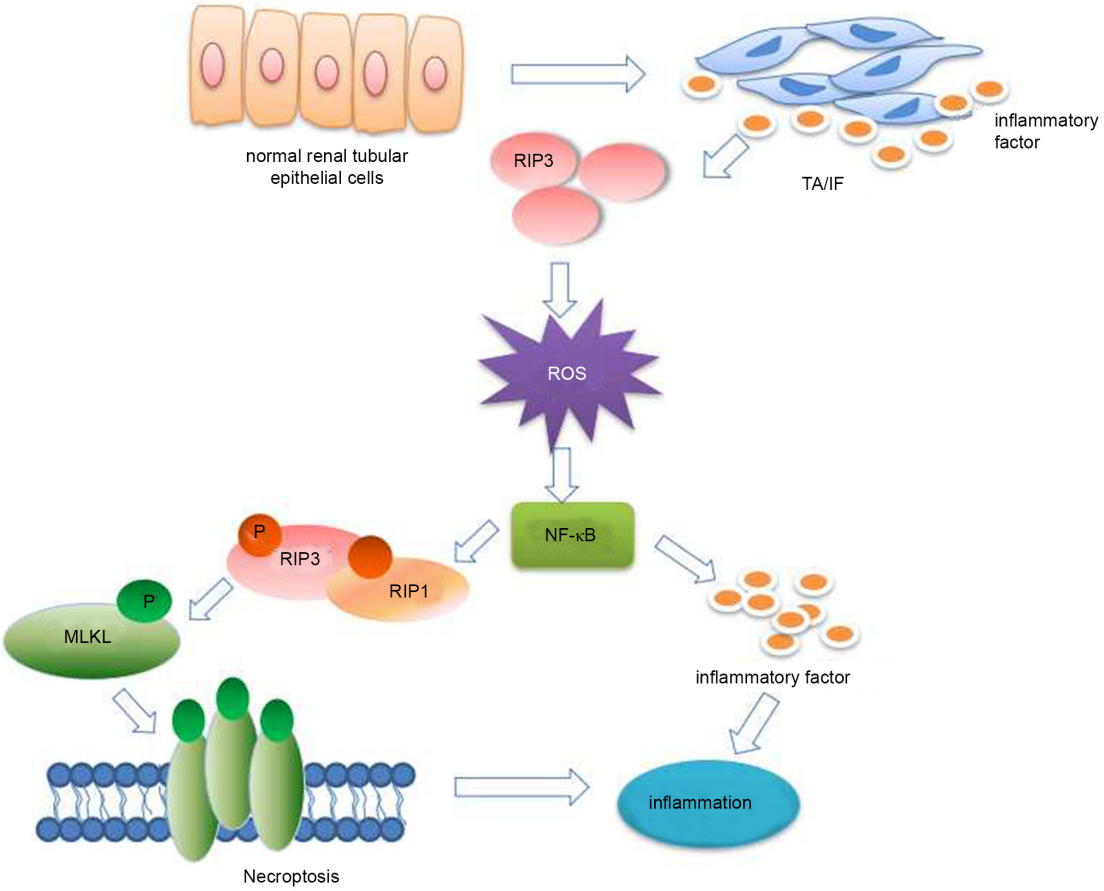

Figure 6. Schematic representation of signal transduction of necroptosis and inflammation caused by the overexpression of RIP3 in HK-2 cells. RIP3 was upregulated in patients with chronic TA/IF. This upregulation of RIP3 can produce excessive ROS and activate the NF-κB signaling pathway to induce necroptosis, and ultimately lead to inflammation. ROS, reactive oxygen species; RIP3, receptor-interacting protein 3; TA/IF, tubular atrophy and/or interstitial fibrosis; MLKL, mixed lineage kinase domain-like protein; p-, phosphorylated.

During necroptosis, cells can release damage-associated molecular patterns molecules that can initiate an inflammatory response in the absence of infection, which suggests that necroptosis is pro-inflammatory (29). Therefore, the present study detected the expression levels of the pro-inflammatory cytokines IL-1β, IL-8 and IL-33, and the well-characterized inflammasome NLRP3, after the overexpression of RIP3. The current study identified a significant increase in the aforementioned indicators, suggesting that overexpression of RIP3 caused inflammation in HK-2 cells. In order to further verify that the inflammatory response caused by the overexpression of RIP3 was induced via necroptosis, the present study used the inhibitor NSA to block the activity of the final executive molecule of necroptosis, MLKL. It was found that after necroptosis was inhibited, the inflammatory response was also markedly suppressed.

Next, the mechanism via which RIP3 induces necroptosis and ultimately leads to inflammation was determined. Reactive species, which mainly include ROS, are products generated as a consequence of metabolic reactions in the mitochondria of eukaryotic cells (30). It is well-known that ROS are important mediators of cellular damage, and lipid peroxidation is the most important cause of ROS-induced oxidative stress (31). The harmful role of ROS in transplanted organs has been revealed both experimentally and clinically in kidney transplantation (26). Of note, the present study demonstrated that, after RIP3 overexpression, the production of ROS was increased significantly. After removing the excessive ROS using NAC, both necroptosis and inflammation could be restored to a large extent. These findings indicated that ROS was closely associated with necroptosis and inflammation.

NF-κB is a transcription factor that regulates the expression levels of multiple genes (32), and governs various cellular functions, including inflammation and necroptotic signaling (33). In addition, NF-κB has been proposed to be a sensor for oxidative stress that can be activated by ROS (34). The present study first detected that overexpression of RIP3 could effectively activate the NF-κB signaling pathway, and this activation could be markedly inhibited by the ROS scavenger NAC, which indicated that ROS are important mediators in the NF-κB pathway. Subsequently, the cells were treated with NF-κB inhibitors, and it was identified that necroptosis and the inflammatory response induced by RIP3 were also inhibited. Collectively, these results suggested that necroptosis induced inflammation via a ROS-dependent NF-κB pathway.

Although the current study identified the importance of the necroptosis signaling pathway at the cellular level, there are some limitations of this study. First, an animal model of

kidney transplantation with TA/IF was not established for *in vivo* studies, and corresponding cells from animals will be obtained for further research. Once the findings have been confirmed both *in vivo* and *in vitro*, RIP3 may be a therapeutic target against inflammation in TA/IF.

In recent years drug repositioning has emerged as a promising alternative to develop drug-like compounds. Accumulating evidence has shown that multi-targeting kinase inhibitors used for the treatment of cancer also display anti-necroptotic activity. For example, dabrafenib and vemurafenib have been reported to block RIP3 (35). Moreover, sorafenib has been revealed to block the kinase activity of both RIP1 and RIP3 (36), while ponatinib directly targets RIP3 (37,38). These therapeutic drugs that have been used clinically to treat cancer provide a foundation for the subsequent development of therapies for TA/IF.

In conclusion, the present study demonstrated that RIP3 expression was upregulated in patients with chronic TA/IF. This overexpression of RIP3 can produce excessive ROS and activate the NF-κB signaling pathway to induce necroptosis, and ultimately lead to inflammation (Fig. 6). These potential mechanisms suggest that RIP3 may be a therapeutic target against inflammation in TA/IF.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SZ and GW conceived the present study. JW and LC designed the experiments. JW, DW, LT, ZX and WC carried out the experiments and collected the data. JW and LC wrote and edited the manuscript. SZ, GW and JW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethical Committee of Ningbo Urology and Nephrology Hospital (Ningbo, China). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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