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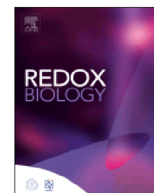
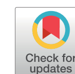


Research Paper

iRhom2 loss alleviates renal injury in long-term PM2.5-exposed mice by suppression of inflammation and oxidative stress



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ABSTRACT

Particulate matter (PM2.5) is a risk factor for organ injury and disease progression, such as lung, brain and liver. However, its effects on renal injury and the underlying molecular mechanism have not been understood. The inactive rhomboid protein 2 (iRhom2), also known as rhomboid family member 2 (Rhbdf2), is a necessary modulator for shedding of tumor necrosis factor-α (TNF-α) in immune cells, and has been explored in the pathogenesis of chronic renal diseases. In the present study, we found that compared to the wild type (iRhom2$^{+/+}$) mice, iRhom2 knockout (iRhom2$^{-/-}$) protected $PM_{2.5}$-exposed mice from developing severe renal injury, accompanied with improved renal pathological changes and functions. iRhom2$^{-/-}$ mice exhibited reduced inflammatory response, as evidenced by the reduction of interleukin 1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and IL-18 in kidney samples, which might be, at least partly, through inactivating TNF-α converting enzyme/TNF-α receptors (TACE/TNFRs) and inhibitor of α/nuclear factor κ B (IκBα/NF-κB) signaling pathways. In addition, oxidative stress was also restrained by iRhom2$^{-/-}$ in kidney of $PM_{2.5}$-exposed mice by enhancing heme oxygenase/nuclear factor erythroid 2-related factor 2 (HO-1/Nrf-2) expressions, and reducing phosphorylated c-Jun N-terminal kinase (JNK). In vitro, blockage of HO-1 or Nrf-2 rescued the inflammatory response and oxidative stress that were reduced by iRhom2 knockdown in $PM_{2.5}$-incubated RAW264.7 cells. Similar results were observed in JNK activator-treated cells. Taken together, our findings indicated that iRhom2 played an essential role in regulating $PM_{2.5}$-induced chronic renal damage, thus revealing a potential target for preventing chronic kidney diseases development.

1. Introduction

Exposure to fine particulate matter ($PM_{2.5}$) is related to the development of cardiovascular health impacts, including elevated risk of irregular heartbeat and pulmonary embolism [1]. A new longitudinal study supplies early evidence that $PM_{2.5}$ exposure is associated with reduced kidney function, and an elevated rate of kidney function decline over time [2,3]. Kidney injury molecule-1 (KIM-1), a recently discovered transmembrane protein, is undetectable in normal kidneys, but it is significantly induced in renal injury including acute kidney injury (AKI) and chronic kidney disease (CKD) [4–6]. Many studies indicate that KIM-1 is a sensitive and specific marker of kidney injury as well as a predictor of prognosis [7,8]. Blood urea nitrogen (BUN) and creatinine (Cr) are essential parameters associated with the loss of renal functions [9,10]. Proteinuria in patients with kidney injury is common [11]. Proteinuria promote the infiltration of renal macrophages, leading to the generation of proinflammatory cytokines and ROS, contributing to renal damage [12,13]. However, the relationship between $PM_{2.5}$ and renal function, an independent cardiovascular risk factor, as well as the underlying molecular mechanism, is poorly understood.

$PM_{2.5}$ is a significant promoter of systemic inflammation and enhances circulating levels of inflammatory cytokines [14]. After

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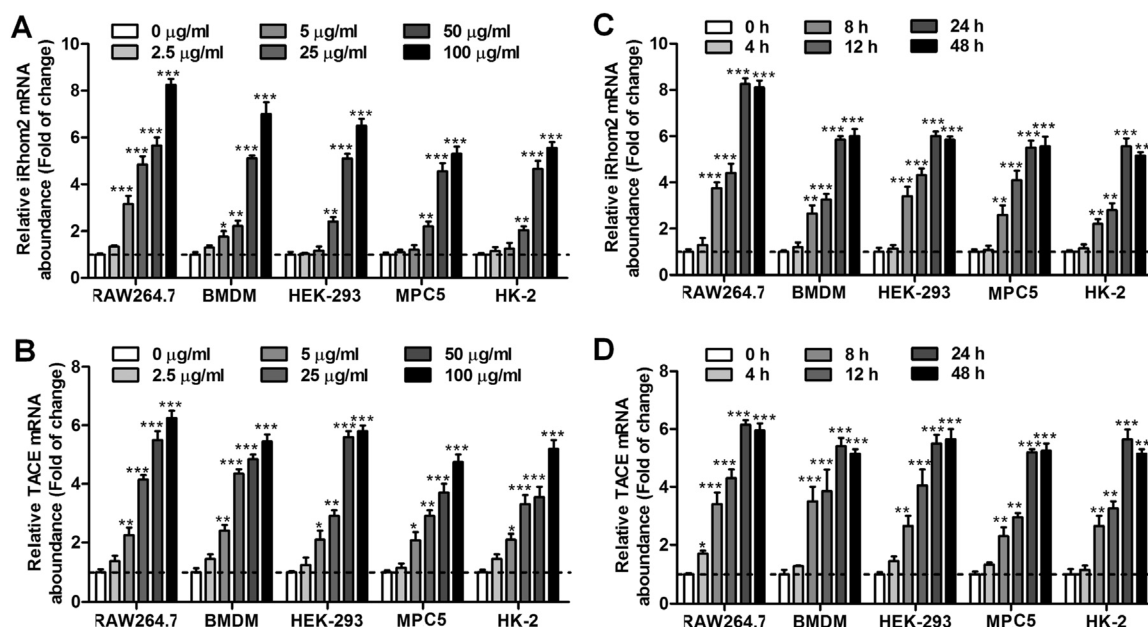

Fig. 1. The expression profiles of iRhom2 in $PM_{2.5}$-incubated different cell lines. Different cell lines, including RAW264.7, BMDM, HEK-293, MPC5 and HK-2, were treated with $PM_{2.5}$ at the described concentrations (0, 2.5, 5, 25, 50 and 100 μg/ml) for 24 h, followed by RT-qPCR analysis of (A) iRhom2 and (B) TACE. Different cell lines of RAW264.7, BMDM, HEK-293, MPC5 and HK-2 were treated with 100 μg/ml $PM_{2.5}$ for the exhibited time (0, 4, 8, 12, 24 and 48 h), followed by RT-qPCR analysis of (C) iRhom2 and (D) TACE. Data are represented as mean ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the Con group.

inhalation and deposition in the epithelium of respiratory tract and pulmonary tissues, particles could move into interstitial spaces between cells, followed by induction of various pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, which leads to endothelial dysfunction [15]. Our previous study has demonstrated that long-term $PM_{2.5}$ exposure results in inflammation in brain and liver tissues [16,17]. In addition, $PM_{2.5}$-induced oxidative stress is considered as a key molecular mechanism of $PM_{2.5}$-regulated toxicity [16,18]. Oxidative stress is caused by an imbalance between generation of reactive oxygen species (ROS) and activity of antioxidant [19]. $PM_{2.5}$-induced oxidative stress in central nervous system (CNS) has been reported [16,20]. Therefore, long-term exposure to $PM_{2.5}$ could result in central and periferal damage mainly through inducing inflammation and ROS generation; however, its effects on renal function have not yet been well studied.

iRhom2, also known as Rhbdf2, is a proteolytically inactive member of the seven transmembrane family of Rhomboid serine protease [21]. iRhom2 could control activation and trafficking of TACE, also known as ADAM17, from the endoplasmic reticulum to the Golgi and subsequently to the cell surface [22]. TACE is a TNF-α converting enzyme. TACE is a membrane-anchored metalloprotease with a wide range of substrates, including TNF-α, TNFR, growth factors and adhesion proteins [23]. As we previously reported, iRhom2 was involved in hepatic injury induced by $PM_{2.5}$ through modulating inflammatory response, and loss of iRhom2 significantly attenuated $PM_{2.5}$-triggered inflammation in liver tissue samples, along with remarkable reduction of TACE/TNFRs [17]. Recently, we reported that reducing iRhom2 could alleviate acute hepatic injury by blocking inflammatory response [24]. Therefore, we hypothesized that iRhom2 might be also involved in the regulation of renal function.

In the present study, iRhom2$^{+/+}$ and iRhom2$^{-/-}$ mice were subjected to long-term exposure of $PM_{2.5}$ to explore the effects of iRhom2 on renal function or injury. The results indicated that $PM_{2.5}$ exposure led to lower renal function and severe kidney damage; however, iRhom2$^{-/-}$ protected mice from severe renal injury induced by $PM_{2.5}$. Mechanically, iRhom2 deficiency restrained TACE/TNFRs and IκBα/NF-κB signaling pathways, leading to the reduction of pro-inflammatory cytokines. Further, oxidative stress was repressed in iRhom2$^{-/-}$ mice with $PM_{2.5}$ exposure, accompanied with the enhanced anti-oxidants, while the reduced oxidants and JNK activation. Significantly, blocking the expression of anti-oxidants or activating JNK rescued inflammation and oxidative stress in iRhom2-knockdown RAW264.7 cells exposed to $PM_{2.5}$. Together, our data demonstrated that iRhom2 and its regulated pathways play a critical role in modulating renal inflammation and oxidative stress induced by long-term $PM_{2.5}$ exposure.

2. Materials and methods

2.1. Animals and culture

Male, 6 to 8 week old wild type mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The iRhom2-knockout (iRhom2$^{-/-}$) based on C57BL/6 background mice weighed 20–25 g were used in this study. They were housed in a specific pathogen-free (SPF), temperature and humidity-controlled environment (25 ± 2 °C, 50 ± 5% humidity) with a standard 12 h light/12 h dark cycle with food and water in cages. After adaptation, mice were exposed to concentrated $PM_{2.5}$ (150.1 ± 2.5 μg/m^3, flow rate of 65 L/min) or filtered air (Con) for 6 h/day, 5 times a week in a mobile exposure system-automatic nose and mouth type inhalation exposure system [16]. The components of $PM_{2.5}$ were shown in Supplementary table 1. After $PM_{2.5}$ exposure for 24 weeks, all mice were sacrificed for blood collection. The renal tissue was isolated from mice for further study. All procedures were performed in accordance the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China. The Institutional Animal Care and Use Committee at Chongqing Key Laboratory of Medicinal Resources in the Three Gorges Reservoir Region of Chongqing University of Education (Chongqing, China) approved the animal study protocols.

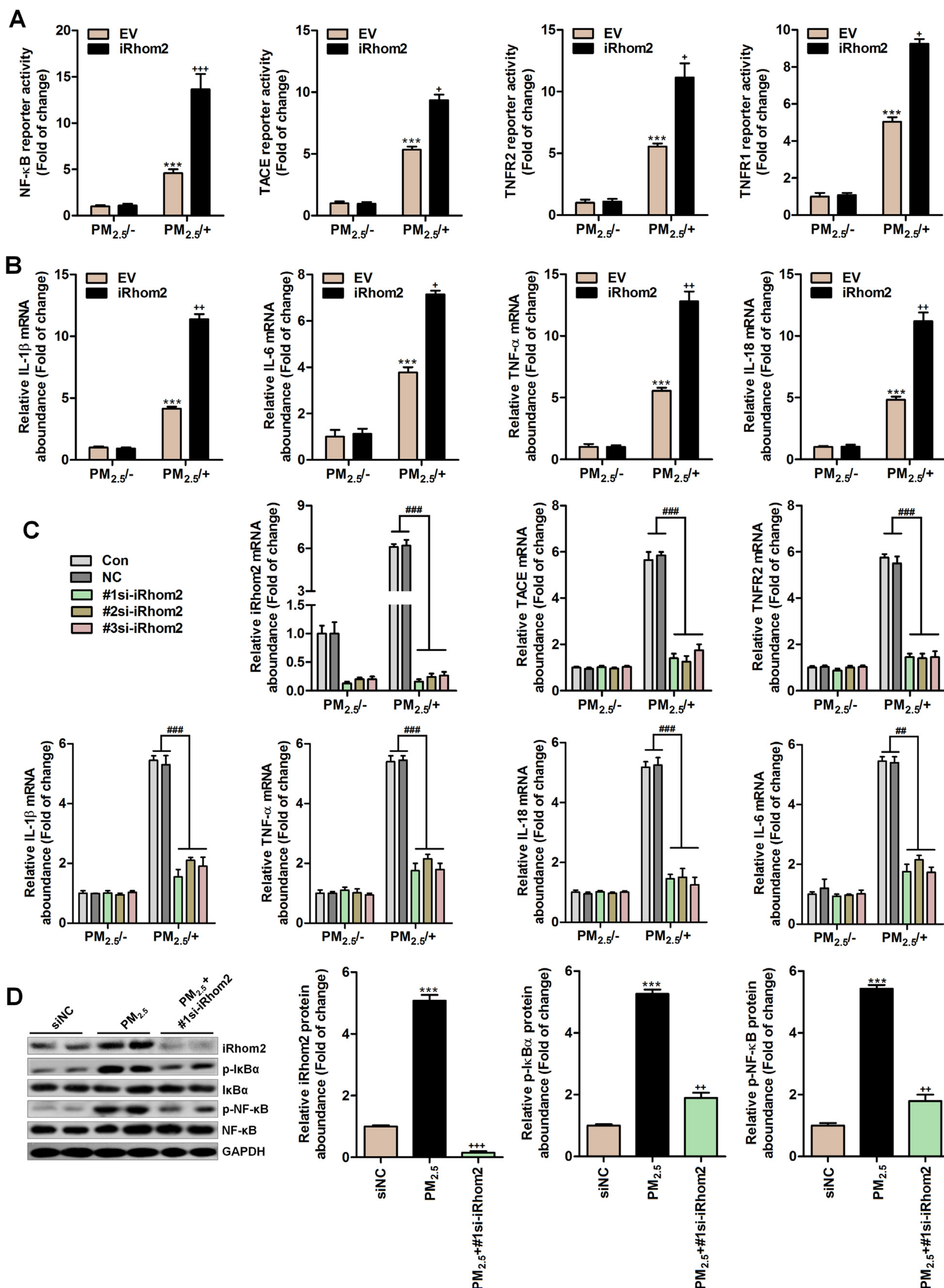

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Fig. 2. iRhom2 suppression negatively regulates inflammatory response in RAW264.7 cells. (A) Luciferase reporter analysis with mouse RAW264.7 cells that were co-transfected with the indicated reporter plasmids plus empty vector (EV) or iRhom2 and then left untreated or treated with 100 μg/ml $PM_{2.5}$ for 24 h. (B) RT-qPCR analysis of IL-1β, IL-6, TNF-α and IL-18 in mouse RAW264.7 cells that were co-transfected with the indicated reporter plasmids plus EV or iRhom2 and then treated with or not 100 μg/ml $PM_{2.5}$ for 24 h. $^{***}p < 0.001$ versus the EV/$PM_{2.5}$/- group; $^{+}p < 0.05$ and $^{++}p < 0.01$ versus the EV/$PM_{2.5}$/+ group. Mouse RAW264.7 cells that were co-transfected with the indicated reporter plasmids plus EV or iRhom2 and then left untreated or treated with 100 μg/ml $PM_{2.5}$ for 24 h. (C) RT-qPCR analysis of the indicated inflammation-related genes in mouse RAW264.7 cells transfected with negative control (NC) siRNA or iRhom2 siRNA for 24 h before exposure to 100 μg/ml $PM_{2.5}$ for 24 h. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$. (D) WB analysis of iRhom2, p-IκBα and p-NF-κB in mouse RAW264.7 cells transfected with NC or iRhom2 siRNAs for 24 h before incubation with 100 μg/ml $PM_{2.5}$ for 24 h. $^{***}p < 0.001$ versus the NC group; $^{++}p < 0.01$ and $^{+++}p < 0.001$ versus the $PM_{2.5}$ group. Data are represented as mean ± SEM (n = 8).

2.2. Cells and culture

Human Embryonic Kidney 293 (HEK293) cells, mouse RAW264.7 macrophages, human proximal tubule epithelial cell line (HK-2), were purchased from American Type Culture Collection (ATCC, Manassas, VA). Mouse podocytes (MPC5) were purchased from Rantai Company (Shanghai, China). Bone marrow derived macrophages (BMDM) cells were isolated according to the procedures as described previously [25,26]. All cells were incubated in DMEM or RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone) and 100 U/ml penicillinstreptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Control and iRhom2- and Nrf-2-specific siRNAs were synthesized by Shanghai Generay Biotech (Shanghai, China) and transfected into cells using Lipofectamine® 2000 (Invitrogen, USA) following the manufacturer's protocol. HO-1 inhibitor, tin protoporphyrin (SnPP), was purchased from Porphyrin Products (Logan, UT). Anisomycin (ANI), JNK activator, was purchased from Santa Cruz Biotechnology (USA).

2.3. Plasmids transfection

The iRhom2 plasmid and empty vector (EV), as well as NF-κB, TACE, TNFR2 and TNFR1 luciferase reporter plasmids were constructed as previously described by standard molecular biology techniques [27–29]. All constructs were confirmed by DNA sequencing. Plasmids were transfected into mouse RAW264.7 cells with a mouse macrophage nucleofector kit (Lonza) following the instructions of the manufacturer.

2.4. Real time-quantitative PCR analysis (RT-qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen) in accordance with its instructions. First strand cDNA was synthesized using Reverse EasyScript One Step gDNA Removal and cDNA Synthesis SuperMix (TAKALA, Dalian, China). The RNA expression levels were determined using SYBR® Green mixture (Qiagen) reagent on ABI PRISM 7900HT detection systems (Applied Biosystems, USA). The primer sequences were provided in Supplementary table 2 and 3. GAPDH was taken as an internal control and the gene expressions were assessed using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot analysis (WB)

Total protein was extracted renal tissue samples or cells using RIPA lysis buffer (Solarbio, Beijing, China). Then, the final liquid supernatants were harvested by centrifugation at 13,500 rpm for 30 min. Protein concentrations were calculated using Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo, USA). Next, the protein samples were subjected to 10% or 12% SDS-PAGE and transferred into PVDF membranes (Millipore, USA). The membranes were blocked in 5% non-fat milk, then incubated with specific primary antibodies at 4 °C overnight (Supplementary table 4). The membranes were incubated with HRP-conjugated secondary antibody. After washing, protein bands were visualized using Super ECL Detection Reagent (Yeasen Biotech Co., Ltd., Shanghai, China) and exposed to Kodak (Eastman Kodak Company, USA) X-ray film. Corresponding protein expression will be determined as grey value (ImageJ, Version 1.4.2b, National Institutes of Health, USA) and standardized to housekeeping gene (GAPDH) and expressed as a fold of control.

2.6. Biochemical measurements

Serum blood urea nitrogen (BUN) and kidney injury molecule 1 (KIM1) levels were detected using corresponding kits that were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Proteinuria was determined by evaluating the urine albumin/creatinine ratio with Albuwell M Test Kit and Creatinine Companion Kit (Exocell, Philadelphia, PA). ELISA detection for TNF-α (catalog MTA00B), TNFR1 (catalog MRT10) and TNFR2 (catalog MRT20) levels in serum were tested according to the manufacturer's introductions. NADH oxidase (NOX), ROS in kidney, superoxide dismutase (SOD), xanthine oxidase (XO), nitric oxide synthase (NOS), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and total antioxidant capacity (TAC) levels were detected using corresponding kits from commercially available kits (Beyotime Institute of Biotechnology, Haimen, China) or Nanjing Jiancheng Bioengineering Institute. Total ROS generation in cell was also determined using the fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA, KeyGEN BioTECH, nanjing, China).

2.7. Immunohistochemistry analysis (IHC)

Kidney samples from each group of mice were fixed in 10% v/v formalin/ PBS, and then embedded in paraffin and sectioned at 4 μm for staining with hematoxylin and eosin (H&E) and Masson trichrome staining. Images were obtained using a microscope. All sections were detected by 3 histologists without knowledge of the treatment procedure. IHC analysis for phosphorylated JNK (ab131499, 1:200, Abcam, USA), phosphorylated NF-κB (ab86299, 1:200, Abcam) and TACE (ab2051, 1:200, Abcam) were performed as previously described [30]. In brief, renal tissue sections were incubated in 3% H_2O_2 to block endogenous peroxidase activity for 10 min 5% bovine serum albumin (BSA, Shanghai Boao Biotechnology Co., Ltd., Shanghai, China) was used for blocking non-specific binding for 1 h. Then, tissues were incubated with primary antibodies. After immunostaining, tissue sections were counterstained with hematoxylin.

2.8. Data analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad PRISM (version 6.0; GraphPad Software, USA) by analysis of variance with Dunnet's least significant difference post hoc tests. A p value < 0.05 was considered significant.

3. Results

3.1. Suppression of iRhom2 negatively regulates inflammatory response in mouse macrophages RAW264.7 cells

As shown in Fig. 1A-D, $PM_{2.5}$ dose- and time-dependently up-regulated iRhom2 and TACE mRNA levels in macrophages or renal cell lines, including mouse RAW264.7, BMDM, HEK-293, MPC5 and HK-2.

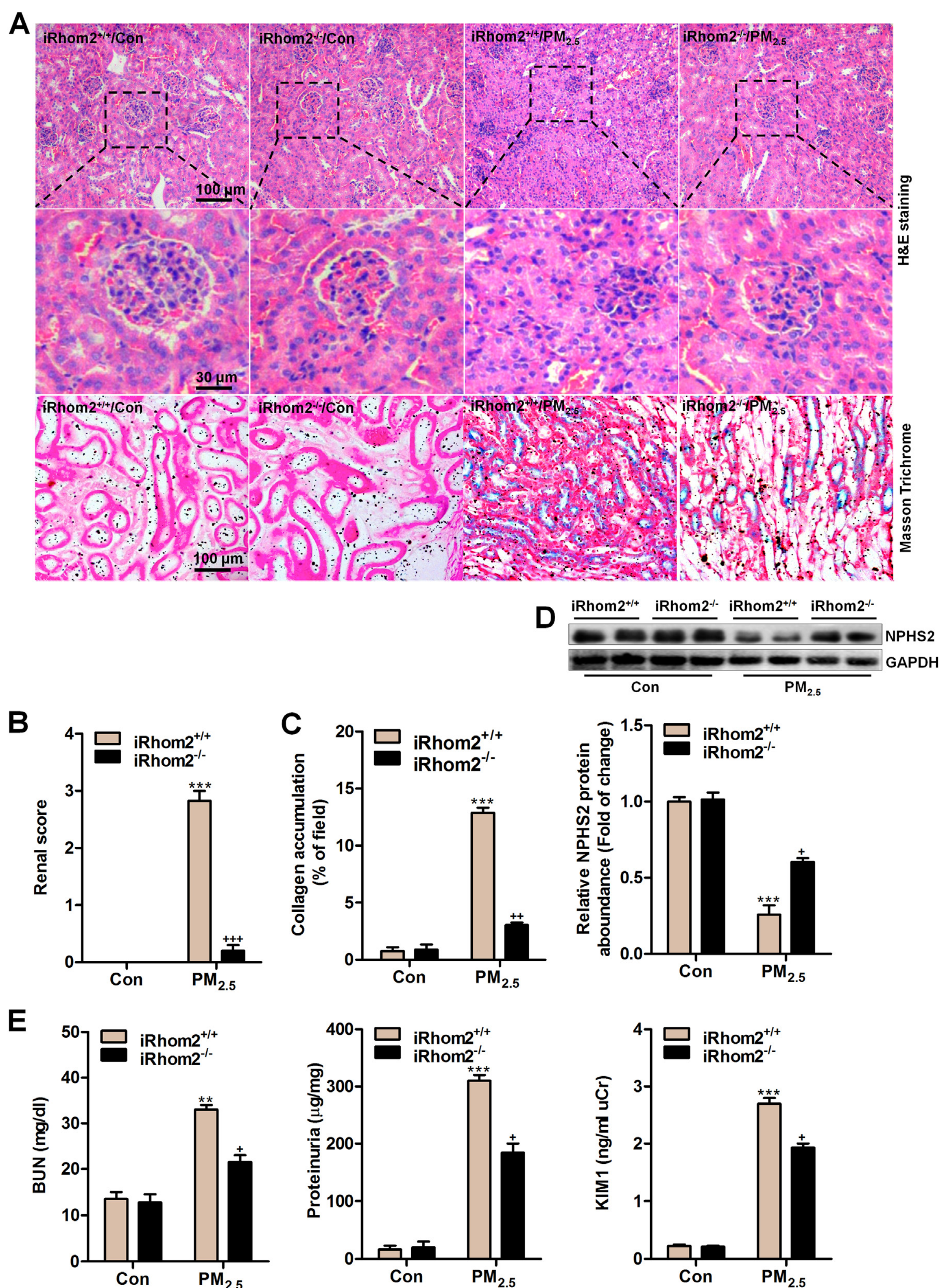

Fig. 3. iRhom2 deficiency alleviates $PM_{2.5}$-induced renal dysfunction. (A) H&E, and Masson Trichrome staining of renal tissue sections from the indicated groups of mice. Quantification of (B) renal score and (C) collagen contents based on histological staining. (D) WB analysis of NPSH2. (E) Determination of serum BUN, proteinuria and KIM1. Data are represented as mean ± SEM (n = 8). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the iRhom2$^{+/+}$/Con group. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ versus the iRhom2$^{+/+}$/ $PM_{2.5}$ group.

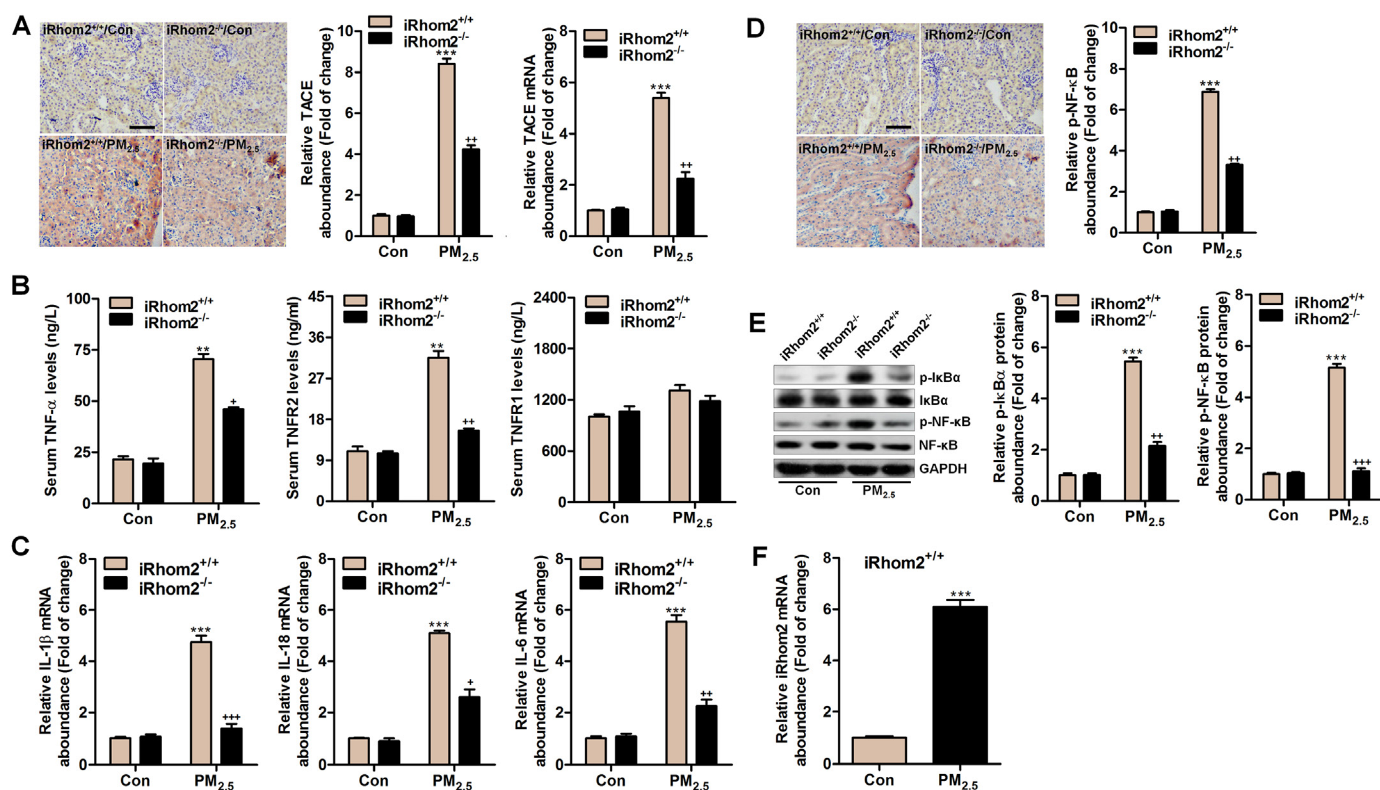

Fig. 4. iRhom2 deficiency alleviates $PM_{2.5}$-induced inflammation. (A) Measurements of TACE using IHC and RT-qPCR analysis. (B) Calculation of serum TNF-α, TNFR2 and TNFR1 using ELISA analysis. (C) RT-qPCR analysis of IL-1β, IL-6, and IL-18 in kidney. (D) IHC analysis of renal p-NF-κB. (E) WB analysis of p-IκBα and p-NF-κB in kidney. (F) WB analysis of iRhom2 mRNA levels in kidney of $iRhom2^{+/+}$ mice. Data are represented as mean ± SEM (n = 8). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the $iRhom2^{+/+}$/Con group. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ versus the $iRhom2^{+/+}$/ $PM_{2.5}$ group.

The in vitro results indicated the potential role of iRhom2 in regulating renal injury. Further, over-expressing iRhom2 enhanced $PM_{2.5}$-induced NF-κB, TACE, TNFR2 and TNFR1, as well as the mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-18) in mouse RAW264.7 cells (Fig. 2A and B). Inversely, iRhom2 knockdown inhibited $PM_{2.5}$-induced transcription of iRhom2, TACE, TNFR2, IL-1β, IL-6, TNF-α and IL-18 in RAW264.7 cells (Fig. 2C). Similarly, iRhom2 silence reduced the expression levels of iRhom2, phosphorylated IκBα and NF-κB in $PM_{2.5}$-treated cells (Fig. 2D). The findings demonstrated that iRhom2 played an essential role in $PM_{2.5}$-induced inflammatory response in mouse macrophages.

3.2. iRhom2 deficiency alleviates $PM_{2.5}$-induced renal injury by reducing inflammatory infiltration

Histological analysis indicated that compared to the control group of $iRhom2^{+/+}$ mice, long term $PM_{2.5}$-exposure led to pathological alterations in renal tissue sections, accompanied with significant collagen accumulation. Conversely, in the $iRhom2^{-/-}$ mice, these histologic changes were markedly alleviated in renal tissue samples from mice after $PM_{2.5}$ challenge (Fig. 3A-C). Western blot analysis demonstrated that $iRhom2^{-/-}$ improved the decrease of NPHS2 in kidney of $PM_{2.5}$-treated mice, along with the reduction of serum BUN, proteinuria and KIM1, indicating the rescued renal functions (Fig. 3D and E). IHC staining and qPCR analysis showed the increased level of TACE in kidney of $iRhom2^{+/+}$ mice exposed to long-term $PM_{2.5}$, which were, however, decreased in kidney of mice lacking iRhom2 (Fig. 4A). Compared to $iRhom2^{+/+}$/$PM_{2.5}$ group, $PM_{2.5}$-induced higher levels of TNF-α and TNFR2 in serum of mice were down-regulated by iRhom2-shortage, while no significant difference was observed in the change of TNFR1 (Fig. 4B). Consistently, $iRhom2^{-/-}$/$PM_{2.5}$ mice exhibited lower mRNA levels of IL-1β, IL-6, TNF-α and IL-18 in kidney than that of $iRhom2^{+/+}$/$PM_{2.5}$ mice (Fig. 4C). We observed a significant reduction of phosphorylated NF-κB and IκBα in renal tissue samples of $PM_{2.5}$-treated mice with iRhom2 deficiency, which was comparable to the $iRhom2^{+/+}$/$PM_{2.5}$ group (Fig. 4D and E). Finally, $PM_{2.5}$ exposure markedly elevated iRhom2 mRNA expressions in kidney of $iRhom2^{+/+}$ mice (Fig. 4F).

3.3. iRhom2 inhibition reduces oxidative stress and JNK activation in $PM_{2.5}$-induced renal injury in vitro and in vivo

Exposure of $PM_{2.5}$ led to oxidative stress in mouse RAW264.7 cells, evidenced by the up-regulated ROS production, H_2O_2, MDA, iNOS, and XO levels, while the down-regulated SOD and TAC contents; however, the process was abolished by the knockdown of iRhom2 (Fig. 5A and B). WB analysis showed a significant reduction of HO-1 and Nrf-2 in RAW264.7 cells exposed to $PM_{2.5}$, and a remarkable elevation of the two proteins was observed in iRhom2-knockdown cells. Conversely, iRhom2-silence markedly decreased Keap-1, XO and phosphorylated JNK expressions in $PM_{2.5}$-stimulated cells (Fig. 5C-E). In vivo, we also found that long-term exposure of $PM_{2.5}$ caused an obvious promotion of total ROS, MDA, iNOS, XO and H_2O_2 levels in renal tissue samples, whereas being reduced by iRhom2-deficiency. SOD activity, however, exhibited an inversed trend (Fig. 6A and B). Moreover, $PM_{2.5}$-induced decrease of HO-1 and Nrf-2 was restored by iRhom2-knockout. In contrast, over-expression of Keap-1, XO and phosphorylated JNK induced by $PM_{2.5}$was markedly decreased in $iRhom2^{-/-}$ mice (Fig. 6C-E).

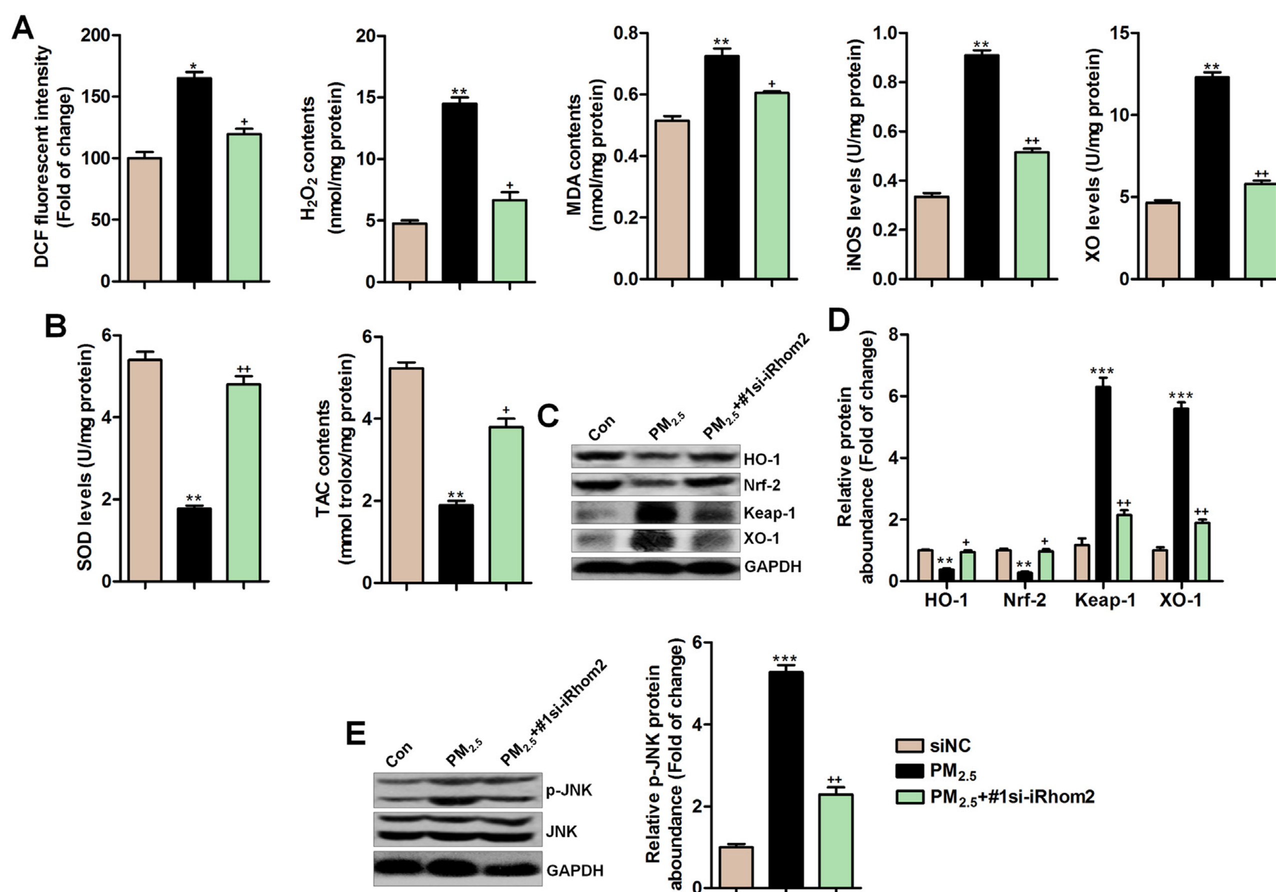

Fig. 5. iRhom2 knockdown reduces oxidative stress and JNK activation in $PM_{2.5}$-induced RAW264.7 cells in vitro. Mouse RAW264.7 cells were transfected with NC or iRhom2 siRNAs for 24 h before incubation with 100 μg/ml $PM_{2.5}$ for 24 h. (A) Oxidative stress in cells were calculated through assessing cellular total ROS using DCF analysis, H_2O_2, MDA, XO, (B) SOD, and TAC levels. (C,D) WB analysis of HO-1, Nrf-2, Keap-1 and XO-1, as well as (E) phosphorylated JNK. Data are represented as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the NC group; $^{+}p < 0.05$ and $^{++}p < 0.01$ versus the $PM_{2.5}$ group.

3.4. $PM_{2.5}$-induced renal injury via iRhom2-regulated oxidative stress and inflammation

The findings above illustrated that iRhom2 could regulate $PM_{2.5}$-induced renal injury via meditating inflammation and oxidative stress. Here, HO-1 was markedly inhibited by using its suppressor of SnPP. Nrf-2 expression was successfully knock down by Nrf-2 siRNA (Fig. 7A and B). Also, JNK phosphorylation was elevated by ANI, a JNK activator (Fig. 7C). We found that reducing HO-1, Nrf-2 or promoting JNK activation markedly recovered TACE, TNFR2 and TNFR1 expressions in $PM_{2.5}$-exposed RAW264.7 cells with iRhom2 knockdown (Fig. 7D). Consistently, the mRNA levels of pro-inflammatory cytokines reduced by iRhom2 suppression in $PM_{2.5}$-treated cells were significantly regained by restraining HO-1 or Nrf-2 expression, or by activating JNK (Fig. 7E). Finally, we also observed that iRhom2 inhibition-triggered decrease of oxidative stress was also rescued by the treatment of SnPP, Nrf-2 siRNA or ANI in RAW264.7 cells challenged with $PM_{2.5}$ (Fig. 7F). Collectively, the findings above indicated that iRhom2 inhibition-ameliorated renal injury could be abrogated by ROS production.

4. Discussion

In the present study, long-term exposure of $PM_{2.5}$ led to kidney damage in murine animals. We established a pivotal role of iRhom2, an essential regulator of TACE, in the pathogenesis of renal injury. We found that $PM_{2.5}$-exposed mice lacking iRhom2 were protected from progressive kidney damage induced by $PM_{2.5}$, as evidenced by the reduced pathological alterations and the rescued NPHS2 expressions in renal tissue sections. NPHS2 plays a critical role in the regulation of glomerular permeability and functions probably as a linker between the plasma membrane and the cytoskeleton [31]. The attenuated processes were also accompanied with reduced BUN, proteinuria and KIM1 levels. KIM1 is a biomarker of acute and chronic kidney injury and renal cell carcinoma [4–8,32]. Further, iRhom2-knockout resulted in the blockage of TACE/TNFRs and IκBα/NF-κB signaling pathways, which led to the suppression of pro-inflammatory cytokines. Moreover, iRhom2$^{-/-}$/$PM_{2.5}$ mice showed attenuated oxidative stress, associated with the increase of HO-1/Nrf-2 and decrease of JNK activation. Notably, suppressing HO-1, Nrf-2 or enhancing JNK activity restored $PM_{2.5}$-triggered inflammatory response and oxidative stress in mouse RAW264.7 cells with iRhom2 knockdown. Therefore, we for the first time supplied the proof that iRhom2 could be a promising target to prevent renal function or injury.

Exposure to $PM_{2.5}$ could lead to chronic systematic inflammation [3,14–17]. It has been wildly demonstrated that $PM_{2.5}$ is associated with inflammatory cytokines whereby it stimulates the over-expression of various transcription factor genes and inflammation-related cytokine genes, contributing to inflammatory injury [33–35]. Recently, we reported that long-term exposure to $PM_{2.5}$ resulted in severe inflammation in brain and liver, along with over-release of pro-inflammatory

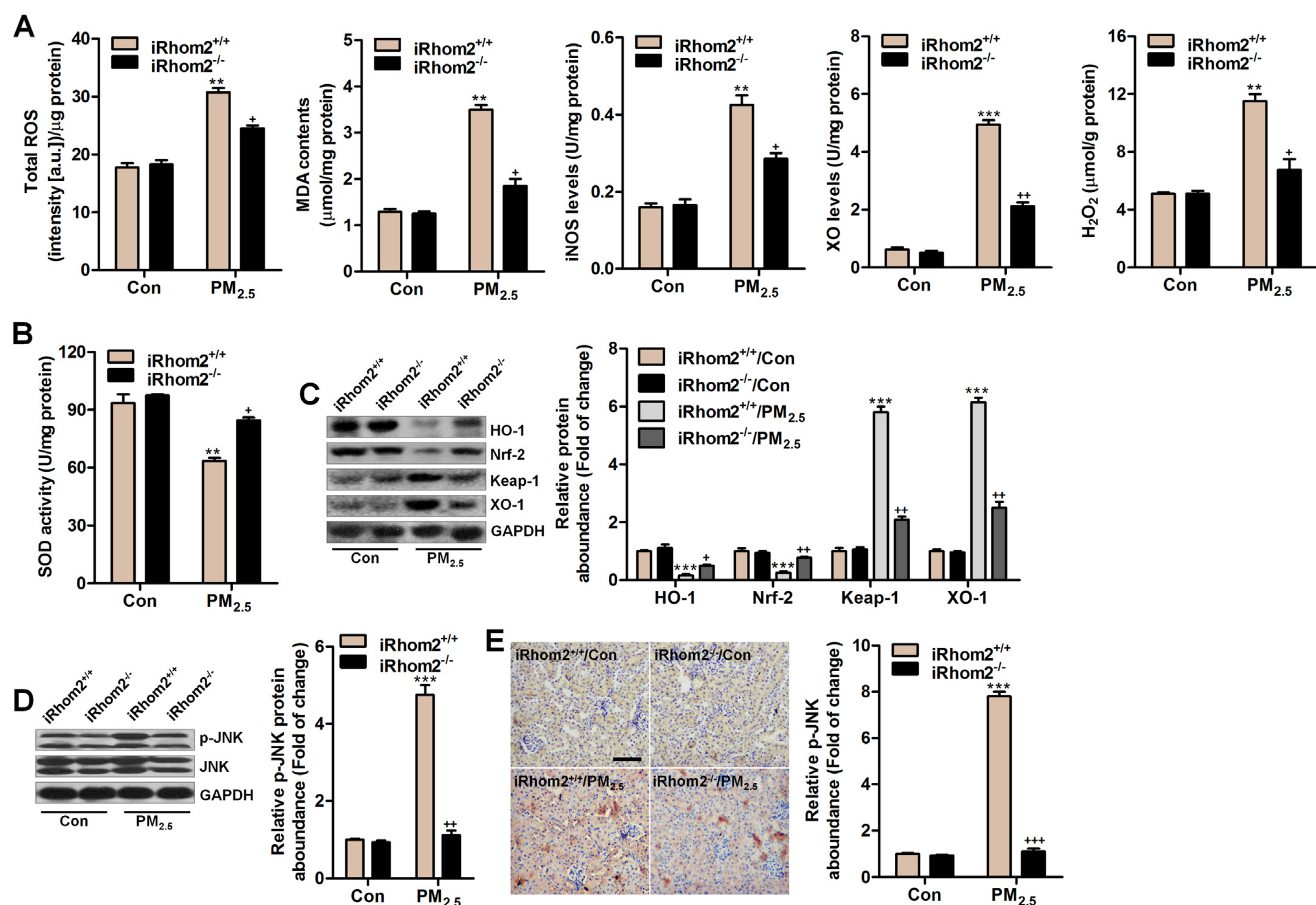

Fig. 6. iRhom2 inhibition reduces oxidative stress and JNK activation in $PM_{2.5}$-induced renal injury in vivo. (A) Determination of oxidative tress-associated indexes, including total ROS, H_2O_2, MDA, and XO, as well as (B) anti-oxidant of SOD in renal of mice from the indicated groups. (C) WB analysis of kidney HO-1, Nrf-2, Keap-1, XO-1, and (D) phosphorylated JNK. (E) IHC analysis of phosphorylated JNK in renal tissue sections. Data are represented as mean ± SEM (n = 8). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the iRhom2$^{+/+}$/Con group. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ versus the iRhom2$^{+/+}$/ $PM_{2.5}$ group.

cytokines, such as IL-1β, IL-6, and TNF-α [16,17]. TACE is an important enzyme responsible for TNF-α release and is needed for the cleavage of other ligands [36]. TACE could cleave TNFR to dissociate the TNFR-binding complex and terminates the TNFR-regulated signal transduction [23]. As reported before, ectodomain shedding of TNFR by TACE leads to termination of cellular response to TNF-α [37,38]. Consistently, in the present study, $PM_{2.5}$ exposure caused circulating and renal inflammation, as evidenced by the enhanced expression of TNF-α, TNFR2, IL-1β, IL-6 and IL-18 in serum or renal tissue samples. Also, activated IκBα/NF-κB signaling pathway was observed in kidney of mice with $PM_{2.5}$-exposure. Accordingly, blockade of iRhom2 has the advantage of specifically suppressing production of soluble TNF-α and thus primarily targeting the pro-inflammatory pathway [39]. Here, we found that iRhom2$^{-/-}$ attenuated renal inflammatory response, which was, at least partly, through the suppression of TACE/TNFRs and IκBα/NF-κB signaling pathways, consequently leading to the reduction of the secretion of pro-inflammatory cytokines. Similar results were observed in mouse RAW264.7 cells incubated with $PM_{2.5}$ that knockdown of iRhom2 restrained inflammatory response via inactivating TACE/TNFRs and IκBα/NF-κB pathways. Therefore, we hypothesized iRhom2$^{-/-}$ -alleviated kidney damage was attributed to the inhibition of inflammation, which was in line with our previous study.

Earlier studies indicated that the free radicals, metal and the organic components of $PM_{2.5}$ could induce free radical production to oxidize lung cells, which may be the primary cause of body injury [40,41]. Oxidative stress is definitely caused by an imbalance between production of various ROS and antioxidant defense. $PM_{2.5}$ could cause excessive production of free radicals or ROS and reduce the antioxidant capacity of cells, contributing to the peroxidation of lipids on the cell membrane and the enhancement of intracellular Ca^{2+} concentrations, which result in inflammation or cell damage [42,43]. Oxidative stress is the result of an imbalance in the pro-oxidant/antioxidant homeostasis [44]. Recently, our previous study has indicated that $PM_{2.5}$-induced CNS injury was partly attributed to oxidative stress, associated with the increase of oxidants and the decrease of anti-oxidants. SOD1, HO-1, and Nrf-2 reduction was involved in $PM_{2.5}$-triggered oxidative stress in CNS [16]. Here, both in vitro and in vivo studies demonstrated that $PM_{2.5}$ exposure caused ROS generation, along with the reduction of HO-1/Nrf-2. JNK could be activated by oxidative stress, which modulates cellular functions. JNK MAPK has been indicated to be of great importance in regulating oxidative stress [45–47]. iRhom2 was suggested to regulate MAPKs (ERK1/2) in kidney damage [48–50]. Here, JNK was markedly activated by $PM_{2.5}$. Intriguingly, iRhom2 suppression reduced JNK phosphorylation, whereas enhanced HO-1/Nrf-2 expressions in $PM_{2.5}$-treated animals or cells. Importantly, the in vitro results indicated that blocking HO-1 or Nrf-2 expression, or promoting JNK

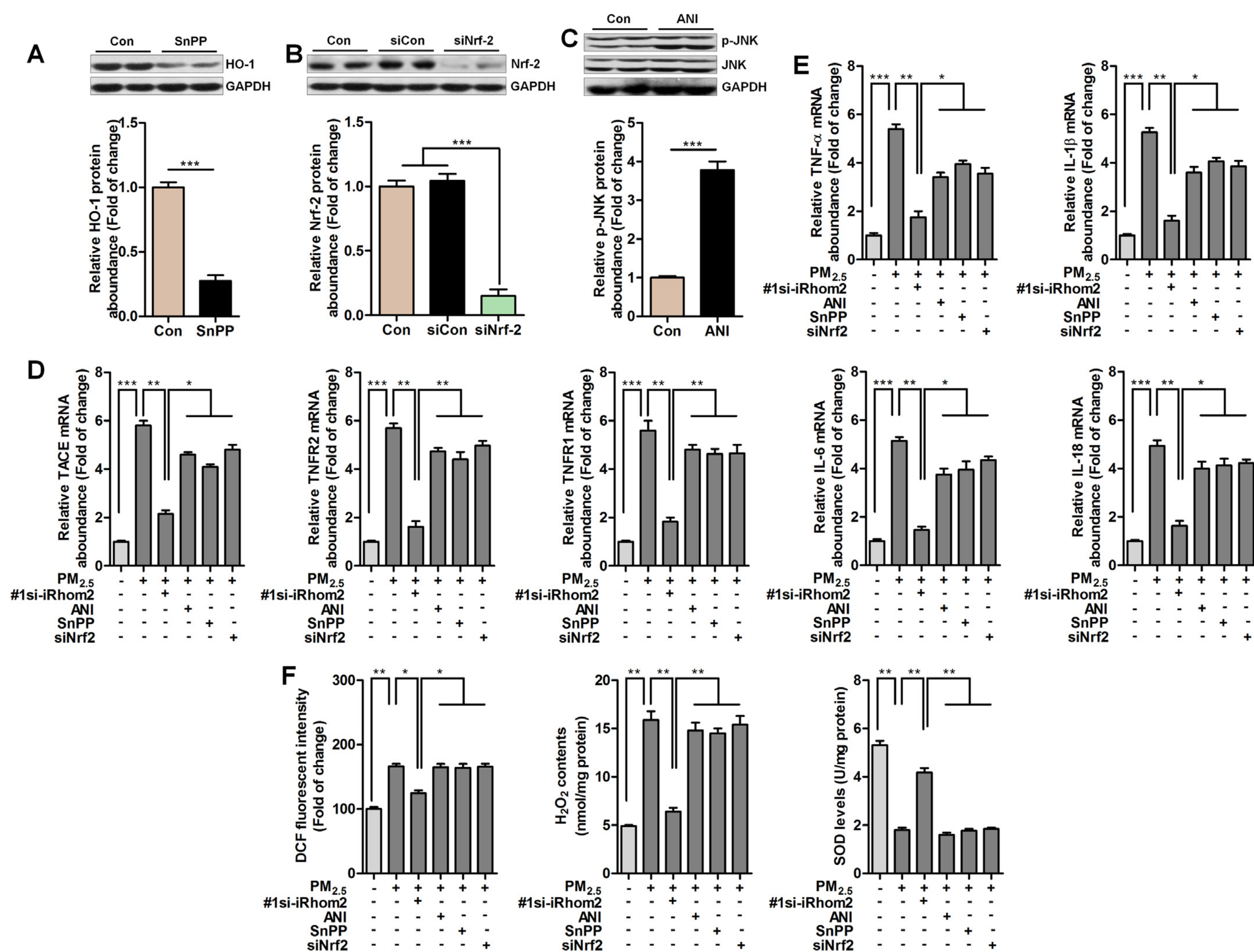

Fig. 7. $PM_{2.5}$-induced renal injury via iRhom2-regulated oxidative stress and inflammation. (A) WB analysis of HO-1 in mouse RAW264.7 cells after 5 μM SnPP pre-treatment for 3 h. (B) WB analysis of Nrf-2 in mouse RAW264.7 cells after transfected with NC or Nrf-2 siRNA for 24 h. (C) WB analysis of phosphorylated JNK in mouse RAW264.7 cells after 5 μM ANI pre-treatment for 3 h. Mouse RAW264.7 cells were pre-treated with SnPP or ANS for 3 h, or with Nrf-2 siRNA for 24 h, and then were subjected to si-iRhom2 transfection for 24 h. Finally, all cells were incubated with or without 100 μg/ml $PM_{2.5}$ for 24 h. (D) RT-qPCR analysis of TACE, TNFR2, and TNFR1. (E) RT-qPCR analysis of inflammatory cytokines. (F) Assessments of DCF fluorescent intensity, H_2O_2 and SOD levels in cells. Data are represented as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

activation apparently rescued inflammation and oxidative stress in $PM_{2.5}$-incubated cells lacking of iRhom2 expressions. Therefore, we supposed that iRhom2 deficiency-attenuated chronic renal injury was largely attributed to the repression of oxidative stress.

Collectively, for the first time we provided evidence that iRhom2 was crucial for the progression of renal injury induced by $PM_{2.5}$ exposure, which was most likely through activating TACE/TNFRs and IκBα/NF-κB signaling pathways. Moreover, iRhom2 blocked HO-1/Nrf-2 pathway, and activated JNK MAPK to promote oxidative stress in renal tissues of mice or cells with $PM_{2.5}$ exposure (Fig. 8). Therefore, blockage of iRhom2 supplied a potential therapeutic approach to chronic renal injury. However, further study is still necessary in future to comprehensively explore the effects of iRhom2 on kidney injury, including its influence on fibrosis formation.

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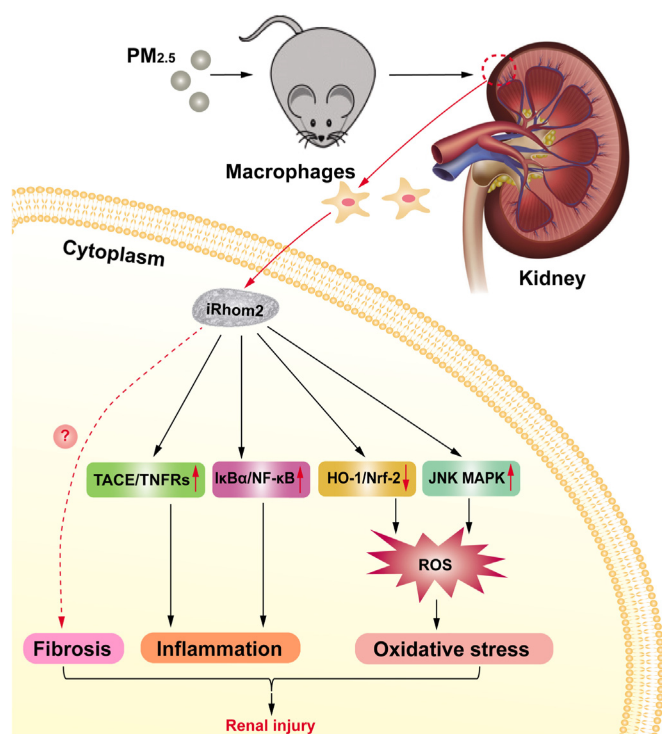

Fig. 8. Model showing the role of iRhom2 as a positive regulator of $PM_{2.5}$-induced renal injury. iRhom2 played an essential role in regulating the progression of renal injury in $PM_{2.5}$-exposed mice, most likely through activating TACE/TNFRs and IκBα/NF-κB signaling pathways to promote inflammation. In addition, iRhom2 inactivated HO-1/Nrf-2 pathway, whereas activated JNK expression to enhance oxidative stress, thus exacerbating renal injury.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.08.009.

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