AIM2 Inflammasome Contributes to Aldosterone-induced Renal Injury via Endoplasmic Reticulum Stress

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Abstract

Inflammatory response and renal fibrosis are the hallmarks of chronic kidney disease (CKD). However, the specific mechanism of aldosterone-induced renal injury in the progress of CKD requires elucidation. Emerging evidence has demonstrated that absent in melanoma 2 (AIM2)-mediated inflammasome activation and endoplasmic reticulum stress (ERS) play a pivotal role in the renal fibrosis. Here, we investigated whether overexpression or deficiency of AIM2 affects ERS and fibrosis in aldosterone-infused renal injury. Interestingly, we found that AIM2 was markedly expressed in the diseased proximal tubules from human and experimental chronic kidney disease. Mechanically, overactivation of AIM2 aggravated aldosterone-induced ERS and fibrotic changes in vitro while knockdown of AIM2 blunted these effects in vivo and vitro. By contrast, AIM2 deficiency ameliorated renal structure and function deterioration, decreased proteinuria levels and lower systolic blood pressure in vivo; silencing of AIM2 blocked inflammasome-mediated signaling pathway, relieved ERS and fibrotic changes in vivo. Furthermore, mineralocorticoid receptor antagonist eplerenone and ERS inhibitor tauroursodeoxycholic acid (TUDCA) had nephroprotective effects on the basis of AIM2 overactivation in vitro while they failed to produce a more remarkable reno-protective effect on the treatment of AIM2 silence in vitro. Notably, the combination of TUDCA with AIM2 knockdown significantly reduced proteinuria levels in vivo. Additionally, immunofluorescence assay identified that apoptosis-associated speck-like protein (ASC) recruitment and Gasdermin-D (GSDMD) cleavage respectively occurred in the glomeruli and tubules in vivo. These findings establish a crucial role for AIM2 inflammasome in aldosterone-induced renal injury, which may provide a novel therapeutic target for the pathogenesis of CKD.
Introduction

Chronic kidney disease (CKD) is characterized by the irreversible damage of renal function and renal fibrosis, but the pathogenetic mechanisms of CKD still have not been completely elucidated[1]. As the major component in the renin-angiotensin-aldosterone system (RAAS), aldosterone activates the mineralocorticoid receptor (MR) and plays a critical role in the pathogenesis of chronic kidney disease[2, 3]. It not only regulates salt and water homestasis, but also induces glomerulosclerosis and tubular epithelial cells apoptosis, and even aggravates the progression of end-stage renal disease (ESRD)[4-7].

Absent in melanoma 2 (AIM2), as one of the major members in the absent in melanoma-2-like receptor (ALRs) family, is a novel inflammatory factor of interferon (IFN)-inducible multiprotein complex located in the cytoplasm[8]. In general, AIM2 contains a N-terminal PYD and C-terminal HIN domains, and the interaction between the PYD and HIN domains causes AIM2 molecule to be in a self-inhibiting state[9]. However, in response to “dangerous signal” or “injury signal”, AIM2 senses and binds cytoplasmic double stranded DNA (dsDNA) from necrotic cells, then recruits Caspase 1 and apoptosis-associated speck-like protein (ASC) to reach the assembly of polycomplex inflammasome[10]. Subsequently, AIM2 inflammasome activation would trigger the maturation and release of proinflammatory cytokine, such as IL-1β and IL-18. Furthermore, it also fosters the cell membrane perforation to achieve the cleavage of Gasdermin-D (GSDMD), eventually resulting in cells pyroptosis[11, 12].

Emerging research progress has indicated that AIM2 inflammasome is strongly linked to the pathogenesis of renal diseases. To date, serval studies have been proved that AIM2 expression is closely associated with the pathological grade of lupus nephritis[13, 14]. Moreover, increased AIM2 expression was related to the high titer level of anti-dsDNA in plasma of systemic lupus erythematosus (SLE) mice, and knockdown of AIM2 also significantly inhibited macrophage activation and inflammation, thus relieving the symptoms of SLE[15]. More strikingly, Komada and colleagues reported that AIM2 inflammasome aggravated renal fibrosis and triggered inflammation in unilateral ureteral obstruction (UOO) mice model through macrophage uptake of necrotic cells dsDNA[16]. Nevertheless, increasing
literature demonstrates that inflammasome activation could trigger reactive oxygen species (ROS) synthesis, endoplasmic reticulum stress and mitochondrial dysfunction in the course of innate immune response[17]. Previously, we reported that NLRP3 inflammasome mediated mitochondrial ROS, leading to aldosterone-infused renal injury, and knockout NLRP3 gene significantly attenuates aldosterone-induced renal fibrosis[18]. Endoplasmic reticulum stress (ERS), which is featured with misfolded proteins accumulation and disorder in the endoplasmic reticulum in response to pathological injury signal, plays an important role in the progression of chronic kidney disease[19]. Also, our previous findings demonstrated that aldosterone contributed to podocytes injury and tubular epithelial cells apoptosis via ERS both in vivo and in vitro [5, 20, 21]. As a distinctive inflammatory mediator, AIM2 inflammasome has not been extensively studied in chronic kidney disease. However, it remains elusive whether and how AIM2 inflammasome mediates aldosterone-infused renal injury via ERS. In addition, the effects of combining AIM2 inflammasome overactivation or knockdown with MR antagonist eplerenone or ERS inhibitor TUDCA on the aldosterone-infused injury model still remains unclear.

The current study intends to explore the role of AIM2 inflammasome and ERS in aldosterone-induced renal injury and its related mechanism, and we are supposed to provide the new perspective and target in the progression and treatment of chronic kidney disease.

Methods and Materials
Animal experiments
The animal protocols were approved by the Ethics Committee for Experimental Research at Fudan University (No.2020 Huashan Hospital JS-507) and all animal experiments were conducted in the Department of Laboratory Animal Science of Fudan University. Male C57BL/6 mice (4-5 weeks old, 14-17g) were used for intrarenal adeno-associated virus (AAV) delivery. Firstly, all mice were anesthetized by the intraperitoneal injection of 1% amobarbital sodium. Then a microsyringe was used to deliver 100ul of a recombinant adeno-associated viral (AAV) vector carrying the AIM2 gene (HBAAV2/9-m-AIM2-NULL, 1×10^{12} vg/ml, Hanbio,
shanghai, CHN) or a vehicle control (HBAV2/9-NULL, 1×10^{12} vg/ml, Hanbio, shanghai, CHN) into the mouse left kidneys. Then, after 4 weeks, all mice (8-9 weeks old, 20-25g) underwent a right uninephrectomy with anesthesia. After 14 days of recovery, all mice were given drinking water containing 1% NaCl and randomly divided into six groups for 4 weeks: (1) sham+vehicle (0.5% ethanol subcutaneously; saline vehicle i.p., n=6); (2) Aldo+vehicle (Aldo 0.75ug/h subcutaneously, Sigma-Aldrich, USA; saline vehicle i.p., n=6); (3) Aldo+TUDCA (Aldo 0.75ug/h subcutaneously; TUDCA 250mg/kg/d, Sigma-Aldrich, USA, i.p., n=6); (4) Aldo+AAV-vehicle (Aldo 0.75ug/h subcutaneously; saline vehicle i.p., n=6); (5) Aldo+AAV-AIM2 (Aldo 0.75ug/h subcutaneously; saline vehicle i.p., n=6); (6) Aldo+AAV-AIM2+TUDCA (Aldo 0.75ug/h subcutaneously; TUDCA 250mg/kg/d i.p., n=6). At the end of experiment, the caudal artery systolic blood pressure was measured by BP-2000 blood pressure machine (Visitech System, USA), and 24-hour urine samples were collected in the metabolic cages. Finally, all mice were anesthetized by injecting amobarbital sodium and killed, and the serum and kidney were respectively collected for further analysis.

**Human renal biopsy samples**

Excessive aldosterone induces the hyperactivation of MR, which exacerbates glomerulosclerosis and renal interstitial fibrosis, and aggravates the progression of chronic kidney disease[22, 19]. The animal aldosterone-infused injury models were also established to induce renal fibrotic changes [2, 7]. Accordingly, to investigate the potential role of AIM2 in renal fibrosis, we screened and collected human renal biopsy specimens of CKD patients with Stage G1-G4 (n=12) and normal nephrectomy samples adjacent to renal carcinoma (n=8) at Department of Nephrology, Huashan Hospital, Fudan University from January 2018 to December 2020. Kidney sections were stained with MR and AIM2 by immunohistochemistry staining, respectively. The cellular location of AIM2 in the kidney was detected by immunofluorescence staining under the Olympus FV1000 confocal microscope. This study was approved by the Ethics Committee on Human Research of Huashan Hospital, Fudan University and was conducted in accordance with the World Medical Association Declaration of Helsinki.
All participants have accepted and signed the informed consent documents before their enrollment.

**Biochemical Measurements**

The serum creatinine, blood urea nitrogen and 24-hour urine protein quantitation levels of mice were measured by an automatic chemistry analyzer (Roche c702, KingMed Diagnostics, Shanghai, China).

**Cell culture and treatment**

Mouse proximal tubular epithelial cells (mTEC) were kindly provided by Dr. Sujuan Xu (Department of Nephrology, Zhongshan Hospital, Fudan University) and cultured in DMEM medium supplemented with 10% fetal bovine serum (16000044, Thermo, USA) at 37°C in a humidified atmosphere with 5% CO2; mouse glomerular mesangial cells (SV40MES13) were purchased from National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in DMEM: DMEM/F12 medium (2:1) supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO2. Both mTEC and SV40MES13 cells were exposed to aldosterone at different concentrations and different time treatment. Then the optimal concentration of aldosterone for SV40MES13 cells and mTEC cells was 10^{-6} M and 10^{-7} M, respectively; the optimal time of aldosterone on both cells was 36 h. For cell transfection, poly (dA: dT) (1ug/ml, invivogen) and shRNA (50nM, Hanbio Company, Shanghai, China) were applied to respectively upregulate or silence AIM2 gene of both SV40MES13 and mTEC cells according to the manufacturer’s instructions. The top strand of AIM2 shRNA were GATCCGCAGAGTTAGCTGACCACCTGATTCATTCAAGAGATGATCAGGTGGTCAGCTAACTCTGTTTTTTG; the bottom strand of AIM2 shRNA were AAT TCAAAAAACAGAGTTAGCTGACCACCTGATTCATCCTTGAATGATCAGGTGGTC AGCTAACTCTGCG. Regarding overactivation of AIM2, the detailed cells experimental groups were as follows: (1) control;(2) poly(dA:dT);(3) poly(dA:dT)+aldosterone; (4)
poly(dA:dT)+aldosterone+10^{-6}nM Eplerenone(Sigma-Aldrich, USA); (5) poly(dA:dT)+aldosterone+20nM Tauroursodeoxycholic acid (TUDCA, Sigma-Aldrich, USA).

For silencing of AIM2, the specific cells treatments were divided into the following groups: (1) control; (2) aldosterone; (3) sh-vehicle+aldosterone; (4) sh-AIM2+aldosterone; (5) sh-AIM2+aldosterone+10^{-6}nM Eplerenone; (6) AIM2+aldosterone+20nM TUDCA.

**Western blotting**

The total protein from kidney issues and cells were lysed with RIPA buffer (P0013B, Beyotime) containing 1% PMSF (ST506, Beyotime) and 1% protease inhibitor cocktail (HY-K0010, MedChemExpress). Primary antibodies against the proteins were as follows: anti-AIM2(#63660, Cell Signaling Technology), anti-cleaved-Caspase 1 (#89332, Cell Signaling Technology), anti-IL-1β (#63124, Cell Signaling Technology), anti-α-SMA(#19245, Cell Signaling Technology), anti-Collagen I(AF7001,Affinity), anti-GRP78(#3177,Cell Signaling Technology), anti-Fibronectin(15613-1-AP,Proteintech), anti-GAPDH(AC002, Abclone), anti-β-actin(AF0003, Beyotime), and anti-TGF-β 1(AF1027, Affinity). Western blots were imaged by using the Odyssey Fc Imaging System (LI-COR Bioscience) and band intensities were quantified by the image J software.

**RNA extraction and real-time PCR**

The total mRNA from kidneys and cells were extracted by using Trizol Reagent (Thermo Scientific), and then were reversed transcribed into cDNA according to the manufacturer’s protocol (TAKARA, Dalian, China). ASC and CHOP transcripts were quantified by using SYBR Green I reagents (TAKARA). The gene expression levels were normalized to the housekeeping gene GAPDH. The sequence of these primer pairs above are demonstrated in the Supplementary material (Table S1).

**Renal histologic analysis**
The mouse kidneys were fixed in 4% paraformaldehyde and made into paraffin, and then were cut at 3-μm thickness. The glomerulus sclerosis was estimated by periodic acid-schiff (PAS) staining, which was indicated based on the percentage of glomerular sclerosis area to the total area. The glomerulus sclerosis score was categorized as the following 5 grades: 0; 1,10%; 2, 10%–25%; 3, 26%–50%; score 4, 51%–75%; score 5, >75%.

**Immunohistochemistry and immunofluorescence staining**

The kidney sections were deparaffinized, rehydrated, subsequently subjected to antigen retrieval in EDTA by microwaving, and blocked with 5% bovine serum albumin. Then these sections were incubated with anti-AIM2(sc-515514, Santa Cruz; #DF3514, Affinity), anti-MR(#DF13302, Affinity), anti-ASC(sc-514414, Santa Cruz), anti-GSDMD(sc-393581, Santa Cruz), anti-α-SMA(#AF1032, Affinity), and anti-TGF-β1(#AF1027, Affinity) overnight at 4 °C. The slides were incubated with second antibodies for 1 hour and quantified on a microscope (Leica).

**Cytokine measurement by ELISA**

The serum IL-18 levels were quantified by the mouse IL-18 ELISA kit (70-EK218-96, Lianke biotech, Hangzhou, China). A standard curve was drawn according to the manufacturer’s protocol. Each value was expressed as pg/ml.

**Statistical analysis**

The data are shown as the mean ±SEM and were analyzed by SPSS version 23.0 (IBM Corp.). Column graphs were drawn by using GraphPad Prism version 8.0 (San Diego, CA). Comparisons between groups were conducted by the Student t-test (2 groups) and one-way analysis of variance with post hoc analysis using Tukey’s test as appropriate. P < 0.05 was considered statistically significant.
Results

AIM2 is upregulated predominantly in the renal biopsy of CKD patients.

To determine the involvement of AIM2 in the pathogenesis of CKD-induced renal fibrosis, we first examined AIM2 distribution and expression in human kidney issues from normal healthy control group and CKD patients. We screened the patients with CKD Stage G1-G4 whose renal biopsies were pathologically diagnosed as IgA nephropathy with glomerulosclerosis index more than 50% according to the KIDGO guidelines. A total of 12 CKD patients were enrolled in this study, and normal nephrectomy samples adjacent to renal carcinoma (n=8) served as normal control group. All patients’ clinical and laboratory data were also displayed in the Table 1. As shown in the Figure 1, the contents of MR protein were significantly increased in the kidney of CKD patients. Compared with normal control group, both renal cortex and medulla staining expression of AIM2 was upregulated predominantly among CKD group (Figure 2a-d). Then, we used double immunofluorescence staining with proximal tubule biomarker Megalin to detect the specific cellular location of AIM2 in the human kidney issues. Strikingly, AIM2 was mainly found in the diseased proximal tubules area in CKD patients compared with normal control group (Figure 3). Together, these results provide the novel clinical evidence that AIM2 is closely correlated to aldosterone-induced chronic kidney disease.

AIM2 knockdown ameliorates the deterioration of renal pathological structure and function, decreases proteinuria levels and lowers systolic blood pressure in aldosterone-infused injury mice model.

To further investigate the critical role of AIM2 gene silencing in the aldosterone-infused injury mice model, we established the AIM2 gene knockdown mice by delivering the recombinant adeno-associated viral (AAV) vector into renal parenchyma. The detailed animal experiments in vivo were illustrated in the Figure 4a-c. Compared with sham group, kidney weight/body weight ratio was increased significantly in both Aldo group and AAV-Vehicle+Aldo group; TUDCA markedly decreased this proportion while there were not significant changes among both AAV-AIM2+Aldo group and AAV-AIM2+Aldo+TUDCA group (Figure 4d). Notably, as
showed in the Figure 4e, both AAV-AIM2+Aldo group and AAV-AIM2+Aldo+TUDCA group had significant lower levels of serum creatine than that of AAV-Vehicle+Aldo group (15.10±5.63 μmol/L vs 35.99±4.31 μmol/L, p< 0.001; 17.78±3.58 μmol/L vs 35.99±4.31 μmol/L, p< 0.01). However, just AAV-AIM2+Aldo+TUDCA group had significant lower levels of blood urea nitrogen (BUN) than that of AAV-Vehicle+Aldo group (14.60±1.88mmol/L vs 18.78 ±1.67mmol/L, p< 0.05) (Figure 4f). Additionally, compared with AAV+vehicle+Aldo group, AIM2 gene silencing significantly decreased the levels of 24-hour urine protein quantification (0.88±0.20 mg vs 2.55±0.51mg, p< 0.001), as well as systolic blood pressure of caudal artery (127.50 ± 9.33mmHg vs 164.60 ±14.15 mmHg, p< 0.01) (Figure 4g, h). Moreover, PAS staining indicated that aldosterone infusion markedly increased extracellular matrix deposition, exacerbated inflammatory cell infiltration, and aggravated the glomerular sclerosis; however, AIM2 knockdown and TUDCA attenuated these effects (Figure 4i, j). Taken together, these findings illustrate that knockdown of AIM2 ameliorates the damage of renal pathology and renal function, reduces proteinuria levels and lowers systolic blood pressure in aldosterone-infused injury mice.

AIM2 knockdown blocks inflammasome-mediated signaling pathway, relieves ERS and fibrotic changes in aldosterone-induced injury mice model.

We next investigated the mechanism whereby AIM2 knockdown in the aldosterone-induced renal fibrosis in vivo. As shown in the Figure 5a, b, e, AIM2 knockdown significantly decreased the inflammasome-mediated cytokines expression, including cleaved-Caspase 1 protein (p20) and ASC mRNA recruitment (Figure S1a), IL-1β release and IL-18 excretion, inhibited ERS marker GRP78 protein and CHOP mRNA levels (Figure S1b), and reduced fibrotic changes, such as α-SMA and Fibronectin. Similar to the immunoblotting results, immunohistochemistry staining findings also indicated that AIM2 knockdown and TUDCA remarkably attenuated renal fibrotic changes, such as α-SMA and TGF-β1 (Figure 5c,d). Meanwhile, immunofluorescence assay showed that increased fluorescent signal of ASC was significantly
localized in the glomerulus of aldosterone-infused mice while that of GSDMD was predominantly expressed in the renal tubule; in contrast, fluorescence signal intensities of both ASC and GSDMD were significantly weakened by AIM2 knockdown and TUDCA (Figure 6a-d). These data indicate that deficiency of AIM2 is inhibitory to inflammasome-mediated inflammation, ERS and renal fibrosis

**ERS inhibitor TUDCA has nephroprotective effects on the aldosterone-infused injury mice model.**

To determine whether both AIM2 knockdown and ERS inhibitor TUDCA exert a combined nephroprotective effect on the aldosterone-infused injury mice model, we additionally used TUDCA treatment for AAV-AIM2+Aldo mice. As shown in the Figure 4g, TUDCA significantly decreased the levels of 24-hour proteinuria on the basis of AIM2 gene knockdown (0.38±0.20mg vs 0.88±0.20mg) while there was not significant decrease in the serum creatine, BUN and systolic blood pressure among these groups. Moreover, TUDCA markedly inhibited ERS and inflammatory response, but failed to generate a more remarkable protective impact compared with the AAV-AIM2+Aldo group (Figure 5a-e).

**AIM2 is concentration-dependently and time-dependently induced by aldosterone in cultured both glomerular mesangial cells and tubular epithelial cells.**

To explore the specific role of AIM2 in the aldosterone-induced glomeruli and tubules injury, our findings indicated that the expression of AIM2 protein was markedly induced by the different concentrations of aldosterone in SV40MES13 and mTEC (Figure 7a-d). Subsequently, we respectively selected the optimal concentration of aldosterone to simulate SV40MES13 and mTEC within different time. Similarly, AIM2 induction was significantly upregulated by aldosterone with the increase of time (Figure 7a-d). These results demonstrate that aldosterone induces AIM2 protein expression in a concentration-dependent and time-dependent manner.
Overactivation of AIM2 aggravates aldosterone-induced ERS and fibrosis changes in both glomerular mesangial cells and tubular epithelial cells.

To investigate the effects of AIM2 overactivation on both glomerular mesangial cells and tubular epithelial cells, we transfected poly(dA: dT) to achieve the overexpression of AIM2 inflammasome. The immunoblotting results showed that the combination of both aldosterone and poly(dA: dT) markedly activated AIM2, cleaved-Caspase1, IL-1β as well as ERS marker GRP78, and also significantly increased fibrosis marker protein expression, including α-SMA and collagen I (Figure 8a-d). These findings indicate that overactivation of AIM2 further triggers the release of inflammatory mediators and induced ERS, which aggravates aldosterone-induced renal fibrosis.

Deficiency of AIM2 attenuates aldosterone-induced ERS and fibrosis changes in both glomerular mesangial cells and tubular epithelial cells.

To examine the critical effects of AIM2 knockdown, we next tested whether silencing of AIM2 by shRNA affects aldosterone-induced ERS and renal fibrosis level. The immunoblotting findings showed that sh-AIM2 remarkably decreased the protein expression of cleaved-Caspase-1, IL-1β, GRP78, α-SMA and collagen I (Figure 9a-h). These results suggest that AIM2 deficiency suppresses aldosterone-induced inflammation and ERS, as well as attenuating renal fibrosis.

MR antagonist eplerenone and ERS inhibitor TUDCA inhibits aldosterone-induced ERS and inflammation in both glomerular mesangial cells and tubular epithelial cells.

To investigate the further insights of nephroprotective mechanism, both SV40MES13 and mTEC were treated additively with MR antagonist eplerenone and ERS inhibitor TUDCA in the presence of AIM2 overactivation. As expected, eplerenone and TUDCA significantly reduced the protein levels of AIM2, cleaved-Caspase 1, IL-1β, GRP78, α-SMA and Collagen I (Figure 8a-d). Furthermore, to identify the combined reno-protective impacts, both
SV40MES13 and mTEC were treated additively with eplerenone and TUDCA in the presence of AIM2 knockdown. However, eplerenone and TUDCA had not remarkable superimposable protective effects on aldosterone-induced renal injury (Figure 9c, d, g, h). These data indicate that eplerenone and TUDCA alleviate aldosterone-induced ERS and inhibit inflammation caused by the excessive activation of AIM2 inflammasome while they fail to cooperatively have a more protective effect on the basis of AIM2 gene silencing.

Discussion

Persistent inflammation and renal fibrosis are the prominent feature and common pathway leading to chronic kidney disease. As the indispensable mineralocorticoid hormone, aldosterone acts on various renal cells and vessels, including promoting epithelial mesenchymal transformation, increasing extracellular matrix and collagen deposition, and aggravating vascular remodeling, which incurs issue inflammation injury, and eventually exacerbates glomerulosclerosis and tubulointerstitial fibrosis[7]. AIM2 inflammasome, the essential mediator of innate immune response, recognizes pathogen-associated molecular patterns or damage-associated molecular patterns, and triggers the maturation and release of proinflammatory cytokines, such as IL-1β, IL-18 and GSDMD, thus inducing inflammation and even pyroptosis[23]. Notably, we identified that AIM2 inflammasome, a multiprotein complex with PYD and HIN domains, played a crucial role in the pathogenesis of chronic kidney disease. In this study, we provide the new evidence that AIM2 inflammasome activation mediates aldosterone-induced ERS and exacerbates renal fibrosis in vitro and in vivo.

It has been reported that AIM2 was distributed constitutively in glomerulus and to a lesser extent in tubular epithelial cells in normal healthy human group; however, the protein expression of AIM2 was significantly upregulated in tubular epithelium and in infiltrating leukocytes in the renal biopsy of CKD patients diagnosed as hypertensive nephropathy and diabetic nephropathy[16]. Strikingly, we also found that AIM2 was markedly increased among CKD patients of Stage G1-G4 pathologically verified as IgA nephropathy, especially in the
renal cortex and medulla diseased tubular epithelium. In line with this, our animal experiments also identified that there were significant inducible AIM2 expression of renal tubules in aldosterone-induced mice models by immunohistochemistry. However, Zhen et al. detected that there were remarkable AIM2 expression in glomerular mesangial cells and endothelial cells among the patients with hepatitis B virus-related glomerulonephritis[24]. Huang et al. also found that increased AIM2 was significantly expressed in the glomerulus of the patients with lupus nephritis of type II[14]. Nevertheless, the expression level of AIM2 in other different pathological types of CKD has not been fully evaluated, and the specific pathological association between AIM2 and CKD still requires further investigations to be clarified.

Previous reports have suggested that AIM2 deficiency blunts inflammation activation, attenuates tubular injury and fibrosis observed in UUO mouse model[16]. Interestingly, our vitro experiment results identified that AIM2 was concentration-dependently and time-dependently induced by exogenous aldosterone stimulation in cultured both mouse glomerular mesangial cells and tubular epithelial cells. Notably, we detected that AIM2 inflammasome mediated aldosterone-stimulated inflammation and fibrotic changes by affecting Caspase 1 activation, ASC recruitment, IL-1β release, IL-18 excretion, α-SMA induction and collagen I synthesis in both mTEC and SV40MES13 cells. Although there was some discrepancy of AIM2 distribution and induction by aldosterone in vitro and vivo experiments, we finally observed that AIM2 knockdown significantly attenuated aldosterone-infused glomerulosclerosis and tubulointerstitial fibrosis, suggesting that AIM2 deficiency has reno-protective effects on the aldosterone-induced kidney injury. Thus, the blockage of AIM2 inflammasome may offer a new strategy for alleviating the progress of chronic kidney disease. However, the immune system activation is now recognized as a dispensable contributor in aldosterone-induced cardiovascular and cardiorenal injury[25, 26]. It has been reported that AIM2 serves as the promoter of innate immunology, playing a crucial role in some autoimmune-related renal diseases and cancers, especially SLE and renal carcinoma. Due to strong sense abilities of serum dsDNA levels, hyperactivation of AIM2 inflammasome in macrophages would exacerbate the severity of lupus
nephritis[15, 27]. Notably, Chai and colleagues found that overactivation of AIM2 promotes hepatocellular carcinoma metastasis to inhibit the invasion of renal carcinoma[28], and they subsequently designed H1/pAIM2 nanoparticles to provide a novel therapeutic target for renal carcinoma[29, 30]. Recently, they further found that AIM2 exerted significant anti-tumour effects in treatment of renal cancer[31], whereas M1 macrophages activated by AIM2 would foster the rejection of renal carcinoma[32]. Hence, these results provide adequate evidence for the immunological mechanism of AIM2 involvement in renal diseases or cancers. Thus, we cannot rule out the significance that aldosterone mediates AIM2 inflammasome activation in other renal immune cells or inflammatory cells, such as T cells, B cells or resident macrophages in the kidney. Indeed, it is essential to acknowledge the role of AIM2 inflammasome in other different types of renal cells to get a comprehensive understanding of aldosterone-induced injury mechanism.

AIM2 inflammasome-mediated signals are mainly categorized into two approaches: canonical and non-canonical functions[33]. Initially, AIM2 is composed of PYI and HIN domains, senses pathogen signals or host-derived dsDNA, and then recruits Caspase 1 to form the canonical inflammasome complex requiring ASC specks as a binding bridge [11, 34, 35]. Interestingly, we observed that ASC specks were significantly localized in the glomerulus of aldosterone-infused mice model while GSDMD was predominantly expressed in the renal tubule though immunofluorescence assay. In our opinion, we speculated that their distribution and expression discrepancy may be due to AIM2 inflammasome-mediated different inflammation signaling pathways in the mouse glomeruli and tubules. Additionally, during the non-canonical inflammatory response, both AIM2 inflammasome and NLRP3 inflammasome modulate the activation of Caspase 4, Caspase 5 and Caspase 11, thus leading to GSDMD cleavage and pyroptosis[33]. Moreover, Miao et al. has confirmed that Caspase11-dependent GSDMD cleavage signaling pathway induces tubular epithelial cell pyroptosis and triggers urinary IL-18 excretion in cisplatin-induced mouse acute kidney injury (AKI) model[36]. Hence, we cannot ignore the important role of other inflammasomes in the pathogenesis of acute or chronic renal injury, such as NLRP3. Previous studies have demonstrated that NLRP3
deficiency attenuates apoptosis through the activation of mitochondrial oxidation and autophagy in contrast-induced mouse AKI model[37]. Our previous researches also have indicated that knockout of NLRP3 gene alleviated mitochondrial impairment and reversed renal fibrosis in mouse UUO model of CKD[18]. In fact, Komada and colleagues have identified that no matter single geen deficiency (AIM2 -/- or NLRP3 -/-) or double geen deficiency (AIM2/-NLRP3-/-) has remarkable protective effects on the UUO-induced renal fibrosis. Of note, the double deficient mice (AIM2/-NLRP3-/-) failed to confer a more significant nephroprotective benefit than single AIM2 deficient (AIM2-/-) and single NLRP3 deficient (NLRP3-/-) mice[16]. These findings suggest that both AIM2 and NLRP3 inflamasomes play an indispensable role in the renal inflammation and fibrosis injury, whereas the protective effect of double AIM2 and NLRP3 knockout is approximately equivalent to single AIM2 or NLRP3 deficiency. Since the AIM2 or NLRP3 inflamasome activation-mediated innate immunology also matters in response to injury signal, both AIM2 and NLRP3 deficiency may alleviate the inflammatory response, but it might cause a lack of innate immunity, which may explain why it fails to generate a more significant renal protective effect. Therefore, further investigations are needed to elucidate the specific mechanism of discrepancy between AIM2 and NLRP3 inflamasome in aldosterone-induced renal fibrosis.

Eplerenone is the classical MR antagonist, which has been widely applied into clinical treatment of CKD and aldosterone-infused injury animal models. In addition to significant anti-inflammatory and anti-fibrotic effects, we also observed that eplerenone attenuated aldosterone-induced ERS on the basis of AIM2 overactivation in vitro while it failed to produce a superimposed nephroprotective effect when combined with AIM2 silence. Mounting evidence has suggested that ERS plays an essential role in the pathogenesis of CKD, and aggravates the progression of ESRD[38, 20, 39, 40]. Our previous findings have identified that ERS inhibitor TUDCA is able to relieve inflammatory response and renal fibrosis in the aldosterone-infused mouse model[41], and also have significant cardioprotective effects on the uremic rat model[42]. For the sake of investigating the combined reno-protection, we applicated TUDCA on the basis of AIM2 knockdown in aldosterone-infused injury mice. However, except for the
significant proteinuria decrease, the combination of TUDCA and AIM2 knockdown exerted similar effects on ERS, inflammation and renal fibrosis compared with the single AIM2 knockdown mice. However, we found that TUDCA blocked AIM2-mediated renal inflammatory response and fibrosis changes in the aldosterone-infused injury mice, suggesting that ERS also in turn induced AIM2 inflammasome activation. To our knowledge, growing evidence has demonstrated that the activation of ERS exacerbates NLRP3 inflammasome-mediated inflammation signaling cascades, which initiates and aggravates chronic injury [47, 48], including atrial fibrillation [49], cerebral venous sinus thrombosis [50], and diabetes [51]. However, Zhang et al. found that knockout of NLRP3 ameliorates angiotensin II-induced mouse renal injury by relieving ERS and mitochondria damage [47], suggesting that NLRP3 was also the upstream of ERS. Indeed, the distinctive interplay between ERS and different inflammasome activation in renal diseases has been not completely elucidated. Based on these data, ongoing researches are required to investigate the distinct cross-talk between ERS and AIM2 inflammasome.

In conclusion, our findings demonstrate that AIM2 inflammasome mediates inflammation activation and ERS in the process of aldosterone-induced renal injury. Overexpression of AIM2 significantly aggravates aldosterone-infused ERS and fibrotic changes in vitro; by contrast, silencing of AIM2 effectively ameliorates aldosterone-induced ERS and renal fibrosis in vivo and in vitro. Hence, AIM2 inflammasome may provide a novel therapeutic target to prevent chronic kidney disease.

Clinical perspectives

(i) Chronic kidney disease (CKD) is characterized by persistent inflammation and renal fibrosis; AIM2 inflammasome activation and endoplasmic reticulum stress (ERS) play a pivotal role in the renal diseases. However, the specific mechanism of aldosterone-induced renal injury in the progress of CKD needs elucidation.

(ii) AIM2 markedly increases in the renal biopsy of CKD patients. AIM2 inflammasome
mediates inflammation activation and ERS in the process of aldosterone-induced renal injury both in vivo and in vitro experiments.

(iii) The distinctive interaction between AIM2 inflammasome and ERS may provide the novel perspective and target in the progression and clinical treatment of CKD.

**Data availability**

Data that support the findings of this study are available upon request from the corresponding author.

**Conflicts of interest**

All authors declare no conflicts of interest.

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**Author contributions**

YW, HY, SJX and MC performed the experiments. SJX, JG and WCZ provided guidance on experimental technology and statistical analysis. SJL and MC collected data in human kidney samples of CKD patients. YW analyzed the data and drafted the article. MMZ and YW designed this research, and MMZ revised the paper critically for important intellectual content. All authors have read and approved the final version of the manuscript.

**Ethic statement**

The present study was approved by the Ethics Committee on Human Research of Huashan Hospital, Fudan University. All participants have accepted and signed the informed consent documents before their enrollment.
Supplementary material

Table S1. The mouse gene primer pairs and sequences for RT-PCR are listed.

Figure S1. The relative mRNA levels of ASC and CHOP in the kidney issue of aldosterone-infused injury mice model.

Figure S2. Representative original images of Western blotting in Figure 3b.

Figure S3. Representative original images of Western blotting in Figure 4a.

Figure S4. Representative original images of Western blotting in Figure 6a&c.

Figure S5. Representative original images of Western blotting in Figure 7a.

Figure S6. Representative original images of Western blotting in Figure 7c.

Figure S7. Representative original images of Western blotting in Figure 8a&c.

Figure S8. Representative original images of Western blotting in Figure 8c.

Figure S9. Representative original images of Western blotting in Figure 8g.

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Figures legends

Figure 1 MR expression in the kidney of normal control group and CKD patients.
(a) Representative micrographs of MR expression in the renal biopsies among both normal control group (n=8) and CKD patients (n=12). (b) Quantitative determination of MR expression. ×200, Bar=100μm. All values were presented as mean±SEM (n=6). ***p < 0.001 compared with normal control group.

Figure 2 AIM2 markedly increases in the renal biopsy of patients with CKD.
Representative micrographs of AIM2 expression in the renal cortex (a) and medulla (c) area among both normal healthy group (n=8) and CKD patients (n=12). Quantitative determination of AIM2 expression in both renal cortex (b) and medulla (d). ×400, bar=50μm. All values were presented as mean±SEM (n=6). ***p < 0.001 compared with normal healthy group.

Figure 3 Cellular location of AIM2 in the kidney of CKD patients and normal healthy group.
Megalin was the specific biomarker of proximal tubules. The confocal microscopy identified AIM2 (red) expression was located in the diseased proximal renal tubules (Megalin, green; DAPI, blue) among CKD patients. ×200, bar=100μm; ×400, bar=50μm.

Figure 4 AIM2 gene knockdown ameliorates renal damage and dysfunction, decreases proteinuria levels and lowers systolic blood pressure in aldosterone-infused injury mice model.
(a) Diagrammatic representation of aldosterone-infused injury mouse models and treatment in the following groups: 1) WT: sham, Aldo, Aldo+TUDCA; 2) AAV: AAV-Vehicle+Aldo, AAV-AIM2+Aldo, AAV-AIM2+Aldo+TUDCA (n=6/each group). (b and c) Western blotting analysis and verification of kidney-specific deletion of AIM2 in AAV mice. Data were presented as mean±SEM (n=3). ***p < 0.01 v.s. WT group. (d) The ratio of kidney weight / body weight was calculated in the different groups. Data were presented as mean±SEM (n=3-6). (e-g) The renal function was evaluated by serum creatine, blood urea nitrogen and 24-hour urine protein quantification. Data were presented as mean±SEM (n=3-6). (h) The caudal artery systolic blood pressure was measured by BP-2000 machine. Data were presented as mean±SEM (n=3-6). (i and j) The renal histopathologic changes and glomerulosclerosis index were assessed by PAS staining. ×400, bar=50μm. Data were presented as mean±SEM (n=3-4). *p < 0.05 v.s. sham group, **p < 0.01 v.s. sham group, ***p < 0.001 v.s. sham group; #p < 0.05 v.s. Aldo group, ##p < 0.01 v.s. Aldo group, ###p < 0.01 v.s. Aldo group; $ p < 0.05 v.s. AAV-Vehicle+Aldo group, $$ p < 0.01 v.s. AAV-
Vehicle+Aldo group, $$$ p < 0.001 \text{ v.s. AAV-vehicle+Aldo group}; @@ p < 0.01 \text{ v.s. AAV-AIM2+Aldo group. WT, wild type; AAV, adeno-associated virus; UNx, Unilateral nephrectomy; Aldo, aldosterone; TUDCA, Tauroursodeoxycholic acid; SBP, systolic blood pressure.}

**Figure 5** Knockdown of AIM2 inhibits inflammasome signaling pathway, relieves ERS and renal fibrosis in aldosterone-induced injury mice model.

(a) Western blotting for AIM2, cleaved-Caspase 1, IL-1β, GRP78, α-SMA and Fibronectin in kidneys from aldosterone-infused WT and AAV mice at 28 days. (b) Quantitative determination of AIM2, cleaved-Caspase 1, IL-1β, GRP78, α-SMA and Fibronectin. Data were presented as mean±SEM (n=3). (c) Representative immunohistochemical micrographs from aldosterone-infused WT and AAV mice kidneys stained with AIM2, α-SMA and TGF-β 1. ×400, bar=50μm. (d) Semiquantitative determination of AIM2, α-SMA and TGF-β 1 protein expression. Data were presented as mean±SEM (n=3-4). (e) Quantitative analysis of serum IL-18 levels in aldosterone-infused WT and AAV mice model. Data were presented as mean±SEM (n=3-4). *p < 0.05 v.s. sham group, **p < 0.01 v.s. sham group, ***p < 0.001 v.s. sham group; #p < 0.05 v.s. Aldo group, ##p < 0.01 v.s. Aldo group, ###p < 0.01 v.s. Aldo group; $ p < 0.05 v.s. AA-V-vehicle+Aldo group, $$ p < 0.01 v.s. AA-V-vehicle+Aldo group, $$$ p < 0.001 v.s. AA-V-vehicle+Aldo group; @@ p < 0.01 v.s. AAV-AIM2+Aldo group. WT, wild type; AAV, adeno-associated virus.

**Figure 6** Silence of AIM2 suppresses inflammasome-mediated ASC recruitment and GSDMD cleavage in aldosterone-induced injury mice model.

Representative immunofluorescence micrographs from aldosterone-infused WT and AAV mice kidneys stained with ASC (a) and GSDMD(c). ×400, bar=50μm. Semiquantitative determination of ASC (b) and GSDMD (d) protein expression. All data were presented as mean±SEM (n=3). *p < 0.05, **p < 0.01, ***p < 0.001. WT, wild type; AAV, adeno-associated virus.

**Figure 7** AIM2 is concentration-independently and time-dependently induced by aldosterone in cultured both SV40MES13 and mTEC cells.

(a and b) Immunoblotting analysis and quantification of AIM2 in SV40MES13 cells with different concentration aldosterone for 24h and with 10⁻⁶M aldosterone at different time. (c and d) Immunoblotting analysis and quantification of AIM2 in mTEC cells with different concentration aldosterone for 24h and with 10⁻⁷M aldosterone at different time. All data were presented as mean±SEM (n=3). *p < 0.05 v.s. control group, **p < 0.01 v.s. control group, ***p < 0.001 v.s. control group. Ctrl, control.

**Figure 8** Excessive activation of AIM2 aggravates aldosterone-induced ERS and fibrosis changes in both SV40MES13 and mTEC cells; Eplerenone and TUDCA inhibited aldosterone-induced AIM2 inflammasome activation in both SV40MES13 and mTEC cells.

(a and b) Western blotting analysis and quantification of AIM2, cleaved-Caspase 1, IL-1β, GRP78 and α-SMA in SV40MES13 cells with poly(dA:dT) (1μg/ml), 10⁻⁶M Aldo and 10⁻⁶ Epl or 20nM TUDCA for 36h. (c and d) Western blotting analysis and quantification of AIM2, cleaved-Caspase 1, IL-1β, GRP78 and Collagen I in mTEC cells with poly(dA:dT) (1μg/ml), 10⁻⁷M Aldo and 10⁻⁶ M Epl or 20nM TUDCA for 36h. All Data were presented as mean±SEM (n=3). *p < 0.05 v.s. sham group, **p < 0.01 v.s. sham group; #p < 0.05 v.s. poly+Aldo group, ##p < 0.01 v.s. poly+Aldo group.
Figure 9 Knockdown of AIM2 attenuates aldosterone-induced ERS and fibrosis changes in both SV40MES13 and mTEC cells; Eplerenone and TUDCA inhibited aldosterone-induced AIM2 inflammasome activation in both SV40MES13 and mTEC cells.

(a and b) Verification for AIM2 knockdown in SV40MES13 cells with sh-vehicle and sh-AIM2. **p < 0.01 v.s. control group. (c and d) Immunoblotting analysis and quantification of AIM2, cleaved-Caspase 1, IL-1β, GRP78 and α-SMA in SV40MES13 cells with sh-vehicle or sh-AIM2, 10^{-6}M Aldo and 10^{-6} Epl or 20nM TUDCA for 36h. (e and f) Verification for AIM2 knockdown in mTEC cells with sh-vehicle and sh-AIM2. *p < 0.05 v.s. control group. (g and h) Immunoblotting analysis and quantification of AIM2, cleaved-Caspase 1, IL-1β, GRP78 and Collagen I in mTEC cells with sh-vehicle or sh-AIM2, 10^{-7}M Aldo and 10^{-6} Epl or 20nM TUDCA for 36h. All data were presented as mean±SEM (n=3). *p < 0.05 v.s. sham group, **p < 0.01 v.s. sham group, ***p < 0.001 v.s. sham group; #p < 0.05 v.s. Aldo group, ##p < 0.01 v.s. Aldo group, ###p < 0.01 v.s. Aldo group; $$ p < 0.01 v.s. AAV-Vehicle+ Aldo group, $$$ p < 0.001 v.s. AAV-Vehicle+ Aldo group. Ctrl, control; Aldo, aldosterone; Epl, eplerenone; TUDCA, tauroursodeoxycholic acid.
a) Western blot images showing protein levels of AIM2, cleaved-Caspase 1, IL-1β, GRP78, α-SMA, Fibronectin, and GAPDH.

b) Graphs showing relative levels of AIM2, cleaved-Caspase 1, IL-1β, GRP78, and Fibronectin with comparisons of different treatments.

c) Immunohistochemistry images of WT, AAV-Vehicle, and AAV-AIM2 conditions with stains for AIM2, α-SMA, and TGF-β1.

d) Bar charts showing positive area percentages for AIM2, α-SMA, and TGF-β1 with comparisons of different treatments.

e) Serum IL-18 level graphs comparing different treatments.
Table 1. Clinical characteristics of CKD patients and normal healthy controls.

| Mark | Gender | Age (Years) | SCr (μmol/L) | BUN (mmol/L) | Cys-C (mg/L) | eGFR (ml/min/1.73m²) | Stage | Urine protein (g/24h) | BP (mmHg) | Pathological diagnosis |
|------|--------|-------------|--------------|--------------|--------------|-----------------------|-------|-----------------------|----------|-----------------------|
| CKD 1 | Male | 65 | 181 | 10.7 | 2.52 | 34.9 | G3 | 0.11 | 158/93 | IgAN |
| CKD 2 | Female | 24 | 75 | 6.1 | 0.80 | 96.1 | G1 | 1.02 | 126/78 | IgAN; FSGS |
| CKD 3 | Female | 50 | 70 | 6.4 | 1.25 | 87 | G2 | 2.86 | 155/104 | IgAN; FSGS |
| CKD 4 | Male | 28 | 127 | 7.7 | 1.57 | 65.8 | G2 | 1.62 | 124/79 | IgAN |
| CKD 5 | Male | 39 | 179 | 10.0 | 1.90 | 40.2 | G3 | 1.75 | 140/81 | IgAN |
| CKD 6 | Female | 39 | 181 | 9.9 | 1.66 | 29.8 | G4 | 3.26 | 117/75 | IgAN |
| CKD 7 | Female | 24 | 79 | 7.2 | 1.13 | 90.3 | G1 | 5.06 | 133/90 | IgAN; FSGS |
| CKD 8 | Male | 32 | 117 | 8.4 | 1.80 | 70.7 | G2 | 1.17 | 118/85 | IgAN |
| CKD 9 | Male | 36 | 162 | 15.1 | 2.23 | 46.7 | G3 | 2.39 | 174/98 | DN; IgAN |
| CKD 10 | Female | 31 | 282 | 17.6 | 2.59 | 18.5 | G4 | 0.86 | 120/77 | IgAN |
| CKD 11 | Male | 47 | 135 | 10.6 | 1.76 | 53.5 | G3 | 7.92 | 176/113 | IgAN |
| CKD 12 | Male | 31 | 165 | 8.1 | 2.46 | 47 | G3 | 5.78 | 158/89 | IgAN |

| Mark | Gender | Age (Years) | SCr (μmol/L) | BUN (mmol/L) | Cys-C (mg/L) | eGFR (ml/min/1.73m²) | BP (mmHg) |
|------|--------|-------------|--------------|--------------|--------------|-----------------------|-----------|
| NC 1 | Female | 53 | 54 | 3.8 | 0.59 | 103.5 | 120/90 |
| NC 2 | Female | 37 | 68 | 5.0 | 0.76 | 98.8 | 108/83 |
| NC 3 | Male | 52 | 81 | 6.9 | 0.78 | 95.8 | 134/70 |
| NC 4 | Female | 43 | 46 | 7.3 | 0.65 | 117.4 | 132/77 |
| NC 5 | Female | 65 | 67 | 6.1 | 0.90 | 82.9 | 132/77 |
| NC 6 | Male | 55 | 67 | 7.5 | 0.93 | 102.8 | 132/76 |
| NC 7 | | | | | | |
|---|---|---|---|---|---|---|
| Female | 47 | 46 | 4.1 | 0.84 | 114.1 | 117/67 |
| NC 8 | Male | 37 | 59 | 4.6 | 0.70 | 122.9 | 120/76 |

Note: SCr, serum creatinine; BUN, blood urea nitrogen; Cys-C, cystatin C; eGFR, estimated glomerular filtration rate; BP, blood pressure; CKD, chronic kidney disease; IgAN, IgA nephropathy; FSGS, focal segmental glomerulosclerosis; DN, diabetic nephropathy; NC, normal control.