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Klotho restrain RIG-1/NF-κB signaling activation and monocyte inflammatory factor release under uremic condition

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ABSTRACT

Aims: Systemic inflammation is a main hallmark of chronic kidney disease (CKD), but the underlying mechanisms of pathogenesis of CKD-associated systemic inflammation is unclear. Current study was designed to investigate the relationship between indoxyl sulphate (IS) and CKD-associated systemic inflammation along with the protective effects of Klotho in CKD.

Methods: IS serum levels from patients were detected by high-performance liquid chromatography (HPLC), and Serum Klotho, IL-6 and TNF-α were measured separately by ELISA and Real-Time PCR analysis. Monocytes were incubated with or without Klotho, while the expressions of retinoic acid-inducible gene I (RIG-I) and NF-κB were analyzed through Western blot assay. Heterozygous *kl/kl (kl/+)* mice or WT mice were treated with 5/6 renal damage. Thereafter, the CKD mice were intraperitoneally injected with recombinant Klotho protein or PBS.

Key findings: It shows that in 286 CKD patients, the serum levels of inflammatory factors were positively related with IS, but negatively related with Klotho. Klotho significantly inhibited IS-induced RIG-I/NF-κB activation and productions of both IL-6 and TNF-α in cultured monocytes. *In vivo*, along with the increase of IS and decrease of Klotho in the serum, the activation of RIG-I/NF-κB signaling was observed in peripheral blood monocytes in both CKD mice and patients. Notably, higher levels of IL-6 and TNF-α were detected in *kl*+/− mice given CKD. Klotho administration has evidently attenuated RIG-I/NF-κB activation in monocytes and systemic inflammation in CKD mice.

Significance: The findings suggest that Klotho can suppress CKD-associated systemic inflammation through inhibiting IS-induced RIG-1/NF-κB activation and monocyte inflammatory factor release.

1. Introduction

Chronic kidney disease (CKD) significantly reduces the quality of life for the patients along with certain serious complications which may aid the risk of death [1,2]. It is reported that the patients with end-stage renal disease (ESRD) having higher mortalities are often linked with systemic inflammation [3]. Studies demonstrated that inflammatory responses are involved in the pathogenesis of cardiovascular disease (CVD) [3], a major cause of death in patients with ESRD. Clinical studies shows that the inflamed uremic phenotype was closely linked with heart failure, atherosclerosis, and coronary artery calcification in CKD patients [4–9]. The systemic inflammation usually results in severe CKD, hence this urges the exploration of effective therapy for controlling systemic inflammation in CKD patients [10,11].

The accumulation of uremic toxins, especially protein-bound toxins such as indoxyl sulfate (IS) and p-cresol (PCS), plays a critical role in the proinflammatory milieu in CKD patients [12]. A clinical study indicated that serum IS are independently associated with serum levels of certain inflammatory cytokines in patients with CKD during stage 3–4 [13]. Certain studies reported that the administration of an oral adsorbent for reducing uremic toxins AST-120 significantly attenuated inflammation in CKD mice [14]. Furthermore, IS can induce vascular inflammation, thereby accelerating the development of atherosclerosis in CKD mice [15,16], but the underlying mechanisms of IS-induced systemic inflammation in CKD remains largely unknown.

Klotho is originally known as an anti-aging protein highly expressed

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in the kidneys [17]. There exist an alternative transcriptional termination, hence-generated two transcripts of the Klotho gene which respectively translated into a membrane form and a secreted form of the protein [18]. The former transmembrane Klotho is a co-receptor for fibroblast growth factor 23 and acts as a regulator in the phosphorus metabolism [19]. While the latter secreted Klotho protein mimics like a hormone that protects cells and tissues against the oxidative stress and certain pathological injuries through regulating of multiple signaling pathway, such as insulin/-like growth factor-1, NADPH oxidase and Wingless/INT-1 (Wnt) [20–22]. Huge studies showed that the reduced Klotho expression in kidney and serum is correlated with the progression of CKD and its associated cardiovascular complications [22–24]. Interestingly, a significantly higher level of serum IL-6 in Klotho-deficient *(kl/kl)* mice has been reported compared to wild type mice [25]. Moreover, Klotho depletion contributes to the increased inflammation in the kidney in diabetic mice [26]. These studies suggested that Klotho may act as an anti-inflammatory modulator for both local and systemic inflammation. But until now, the direct association between Klotho and systemic inflammation in CKD remained unclear.

In current study, initially relationships between the increases in inflammatory cytokines and IS, and decrease in Klotho in the serum in CKD patients was observed. Then the underlying mechanisms of the effect of IS on monocyte activation, as well as the protective effect of Klotho against IS-induced monocyte activation were studied. The results showed that IS can induce monocytes to produce inflammatory cytokines by RIG-I/NF-κB activation, and Klotho potentially suppresses the effect of IS on monocyte activation.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and trypsin EDTA solution were obtained from Hyclone (Logan, UT). Fetal calf serum (FCS) was purchased from Gibco (Carlsbad, CA). Anti-RIG-1, pNF-κB, NF-κB and β-actin antibodies were obtained from Abcam (Cambridge, UK). IS and PDTC were from Sigma-Aldrich (St. Louis, MO). Reverse Transcription Kit was purchased from Promega (WI, USA). Mouse or human ELISA Quantification kits for IL-6, TNF-α and Klotho were obtained from Cusabio (Cologne, Germany), and the creatinine test kit was ordered from njjcbio (Nanjing, China). The recombinant mouse Klotho protein was purchased from R&D Systems (Minneapolis, MN).

2.2. Selection of patients

A total of 286 CKD stage 2–5 patients with primary chronic renal diseases were recruited from the Department of Nephrology of Xinqiao Hospital (Chongqing, China). Patients with secondary nephropathy including diabetic nephropathy, hypertensive nephropathy, purpuric nephritis, systemic lupus erythematosus nephritis, *etc.*, and pregnancy and accompanied with infections were excluded. The eGFR was estimated according to the Modification of Diet in Renal Disease equation, which includes four variables: eGFR (ml/min/ $1.73\,m^2$) = 175 × (serum creatinine) − 1.154 × (Age) − 0.203 × (0.742 if female) × (1.212 if African-American) (conventional units). The patients were then classified into five CKD stages: > 90 ml/min/$1.73\,m^2$ (CKD stage 1), from 90 to 60 ml/min/$1.73\,m^2$ (CKD stage 2), from 60 to 30 ml/min/$1.73\,m^2$ (CKD stage 3), from 30 to 15 ml/min/$1.73\,m^2$ (CKD stage 4), and < 15 ml/min/$1.73\,m^2$ (CKD stage 5). Serum samples were obtained from these patients for subsequent IS, Klotho and inflammatory cytokines measurements.

2.3. Biochemical examination

Data concerning concentration of creatinine, uric acid, albumin and C-reactive protein were obtained for the patients from the hospital records.

2.4. IS measurement

IS serum levels of patients and mice were examined by high-performance liquid chromatography (HPLC) following previous method [22].

2.5. ELISA assay of serum Klotho, IL-6 and TNF-α

The blood samples were collected from patients and mice, and then centrifuged at 3000 rpm for 10 min. The serum was stored at −80 °C for later use. Serum Klotho, IL-6 and TNF-α were measured separately using a human or mouse Klotho, IL-6 and TNF-α ELISA kit according to the manufacturer's instructions (Cusabio, Cologne, Germany).

2.6. Human samples research

The study protocol was approved by the Ethics Committee of Xinqiao Hospital, and experiments were carried out in accordance with the Declaration of Helsinki. The approved protocol no. by Ethics Committee in M&M section is 2012050–1. 5 ml of peripheral blood were drawn from 286 patients with primary CKD. After centrifugation at 3000 rpm for 10 min, serum samples were collected for the subsequent IS, Klotho and inflammatory factors measurements. In another experiment, 60 ml of peripheral blood was collected from five healthy volunteers and five CKD stage 5 patients (eGFR < 15 ml/min/$1.73\,m^2$) with non-dialysis. Serum and monocytes samples were isolated from the peripheral blood of each individual for the subsequent IS measurements along with NF-κB and RIG-1 protein analyses.

2.7. Magnetic separation assay

Human peripheral blood samples were collected and isolated using the SepMate™ tubes and EasySep™ Human CD14 Positive Selection Kit (Stemcell, Vancouver, Canada) following the manufacturer's instructions as previous described [27]. The peripheral blood was diluted with PBS and added to lymphocyte separation solution in the SepMate™ tube, centrifuged at 1200 *g* for 10 min and harvested the PBMCs by pouring into the fresh tubes. Washed the enriched cells twice and incubated with EasySep ™ selection cocktail for 5 min at room temperature, then EasySep magnetic nanoparticles were added to the antibody-labeled cell suspension for 10 min. Following the incubation, the cell suspension was adjusted to 2.5 ml by adding PBS buffer. Subsequently, the cell suspension was placed in a magnetic field for 5 min, and washed twice to increase the purity. Finally we got 99.6% pure mononuclear cells, then cultured the cells in RPMI1640 medium supplemented with 10% heat-inactivated FCS for further use.

2.8. Cell culture and transfections

THP-1, human monocyte-like cells, were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

The THP-1 were transfected with control siRNA or RIG-1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using siRNA Transfection Reagent in siRNA Transfection Medium for 6 h and serum-containing medium for 24 h. Cells were cultured with fresh medium for 24 h.

2.9. Western blotting

The proteins isolated from cultured and treated cells were separated by 8% SDS polyacrylamide gel electrophoresis, transferred onto PVDF membrane (Millipore, Billerica, MA), and incubated overnight at 4 °C

Table 1
The basic information of 286 CKD patients.

CKD stage	CKD 2(n = 36)	CKD 3(n = 51)	CKD 4(n = 55)	CKD 5(n = 144)	P
Age, years	39.86 ± 11.93	49.88 ± 13.88	47.27 ± 14.89	46.29 ± 14.18	< 0.05
Male gender, n(%)	19(53)	22(43)	34(62)	60(42)	< 0.001
eGFR(ml/min/1.73m^2)	71.16(60.06,95.10)	42.79(30.09,59.90)	19.71(15.01,29.09)	8.14(3.92,14.93)	< 0.001
BMI(kg/m^2)	24.06 ± 2.89	24.50 ± 3.50	23.23 ± 2.99	22.34 ± 3.43	< 0.001
ALB(g/L)	41.50(13.00,51.30)	39.00(15.60,46.70)	40.40(19.50,50.20)	38.85(22.30,51.30)	> 0.05
Uric acid(μmol/L)	434.50 ± 90.46	429.24 ± 94.02	500.60 ± 113.53	539.04 ± 123.22	< 0.001
TNF-α (pg/ml)	24.75(6.16,31.17)	27.61(2.24,37.61)	31.79(15.22,37.54)	33.47(21.82,38.73)	< 0.001

with the primary antibodies against RIG-1, pNF-κB, NF-κB and β-actin (RIG-1, 1:1000; pNF-κB, 1:1000; NF-κB, 1:1000; β-actin, 1:1000). After three washes with PBS containing 0.1% Tween 20, the membranes were incubated with the secondary antibodies. The signals were developed using an enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). The densitometry were analyzed using ImageJ Software (US National Institutes of Health, Bethesda, MD, USA).

2.10. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using reverse transcription System Kit (Promega). The quantitative real time PCR (qPCR) was performed in triplicate with SYBRII qPCR master mix (Takara, Dalian, China) according to the manufacturer's protocol, taking β-actin as an internal control [28]. The relative mRNA levels of the target genes were calculated with $2 - \Delta\Delta Ct$ method. The primers sets for qPCR are listed in Table S1.

2.11. Animals trials

All procedures followed were in compliance with the institutional animal care guidelines established by the Institutional Animal Care and Use Committee of the Army Medical University. Male Balb/c mice were obtained from Beijing HFK Biologic Technology (Beijing, China); Klotho deficient mice (*kl/kl)* were kindly provided by Professor Jun Gu (State Key Laboratory of Protein and Plant Gene Research, College of Life Science, Peking University, Beijing, China), and the mice were backcrossed to background mice for over six generations to achieve congenic background.

Heterozygous *kl/kl* ($kl/^{+}$) *mice* or WT mice at 8 weeks of age were treated with 5/6 renal damage to establish a mouse model of chronic kidney disease as previously described [22]. Briefly, the mice were first inflicted with 2/3 electrocoagulation of the right renal cortex and then received left total nephrectomy 2 weeks later. After left kidney nephrectomy, the mice were separated into four groups *viz.* WT, CKD, $kl/^{+}$ and $kl/^{+}$ + CKD. The mice were euthanized, and the blood samples were obtained for further experiments after 4 weeks. Blood urea nitrogen (BUN) and serum creatinine were also recorded. In other *in vivo* experiments, male Balb/c mice at 8 weeks of age were treated with 5/6 renal damage. The mice were separated into four groups *i.e.*, control (mice received a sham operation that included decapsulation of both kidneys), CKD, CKD + PBS (0.01 mol/L PBS was administered through intraperitoneal injection), and CKD + Klotho (0.01 mg/kg Klotho protein dissolved in 0.01 mol/L PBS was administered through intraperitoneal injection). The intraperitoneal injections with Klotho protein were repeated every 48 h for 4 weeks before the blood samples were collected.

2.12. Flow cytometry for isolating mononuclear cells

Mononuclear cells were isolated according to EuroFlow Standard Operating Procedure. Briefly, fresh peripheral blood of mice was initially incubated in erythrocyte lysate for 10 min and then centrifuged 1200 rpm for 5 min, and then washed twice with PBS. Each sample was incubated with antigen CD14 for another 20 min and then subjected to flow cytometer analysis to detect surface antigen positivities of CD14 antigen to isolate mononuclear cells. Flow cytometry analysis was performed using Moflo XDP (Beckman coulter).

2.13. Statistical analysis

In vivo and *in vitro* experimental, continues variables were examined for normality of distribution using the Shapiro-Wilks test. Normally distributed data are expressed by using Mean ± SDs and non-normally distributed data are expressed by using median and min-max values. Statistical analysis were performed using unpaired, two-tailed *t*-test and one-way analysis of variance (ANOVA) with Tukey multiple comparison test. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The correlation was analyzed by spearman correlation. The correlation between Klotho and inflammatory factors adjusted for serum albumin and uric acid was analyzed by partial spearman correlation. This data was analyzed using SPSS 19.0 (IBM SPSS Office, Chicago, USA). A value of $P < 0.05$ for the difference was statistically significant.

3. Results

3.1. Serum levels of IS and Klotho have a close correlation with systemic inflammation in patients with CKD

To determine the relationship between IS accumulation and CKD-associated systemic inflammation, we examined the concentrations of IS and inflammatory factors in the serum samples from 286 patients with primary CKD. The basic information of all the patients has been summarized in Table 1.

As shown in Fig. 1, the serum CRP, IL-6 and TNF-α levels markedly increased with decline of eGFR (Fig. 1A–C) accompanied by a significant decline in serum level of Klotho (Fig. 1D). Besides, the serum level of IS markedly increased with the progression of CKD (Fig. 1E).

Further analysis showed that serum levels of CRP, IL-6 and TNF-α were negatively correlated with reduction of eGFR (Fig. 2A–C), while the serum levels of inflammatory factors were positively correlated with serum level of IS (Fig. 2D–F). Further adjustment for uric acid, BMI and age were resulted in slight attenuation of the association between serum Klotho levels and inflammatory makers, but Klotho levels was still significantly and negatively correlated with inflammatory makers (Fig. 2G–I).

3.2. IS promotes systemic inflammation by activation of RIG-I/NF-κB signaling in monocytes

It is well known that monocyte activation that leads to the production and release of a large amount of inflammatory factors plays a central role in systemic inflammation. To determine the effect of IS on monocyte activation, the cultured THP-1 cells were treated with different concentrations of IS (0, 2, 10 or 50 mg/L) for 24 h. The optimum

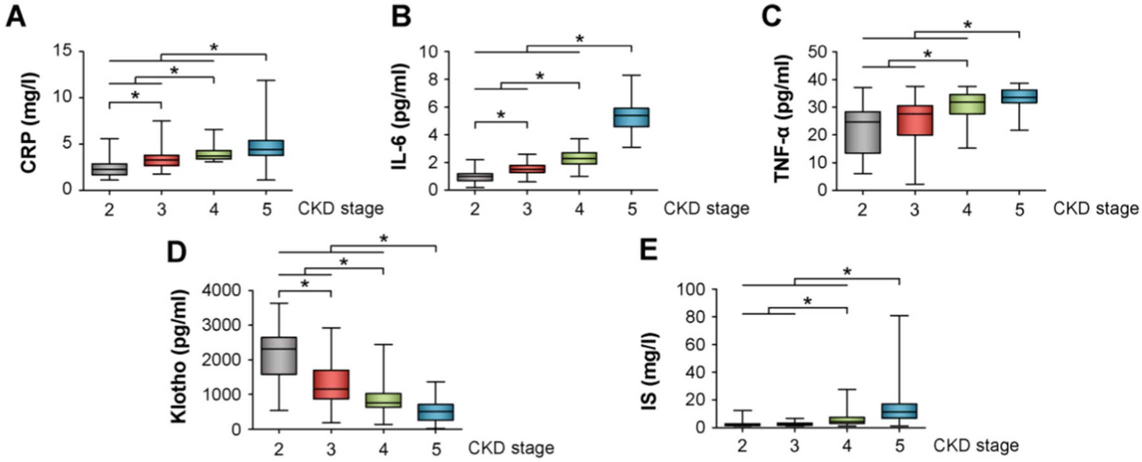

Fig. 1. Serum levels of inflammatory factors, Klotho and IS in at each stage (1–5) of CKD patients. (A-C) The serum levels of inflammatory factors including CRP, IL-6 and TNF-α markedly increased with decline of eGFR in CKD patients. (D) The serum Klotho level significantly decreased with decline of eGFR. (E) The serum IS level increased accompanied by a decline of eGFR in CKD patients. Values are expressed as means ± SD from at least three independent experiments. *$P < 0.05$.

concentrations of IS used in current experiments was according to a recent study [29]. It was found that IS could dose dependently promoted the expression and release of IL-6 and TNF-α, two representative inflammatory cytokines, from THP-1 cells (Fig. 3A and B). We then found that NF-κB, an important transcription factor, regulated the production of inflammatory cytokines [30], was significantly activated in THP-1 cells following IS treatment for 24 h (Fig. 3C), while pre-incubation with 20 μmol/L PDTC, a selective pNF-κB inhibitor, for 1 h dramatically inhibited IS-induced pNF-κB upregulation (Fig. 3D) and the release of IL-6 and TNF-α (Fig. 3E and F).

To further reveal the mechanism underlying the effect of IS on NF-κB activation, the signal changes upstream of NF-κB were measured. Interestingly, it was found that similar to NF-κB, the expression of NF-κB activator RIG-I, binds to the caspase-recruitment domain-like region at its N-terminus to activate the signaling cascade [31], remained much higher in the monocytes of CKD stage 5 patients but not in the healthy

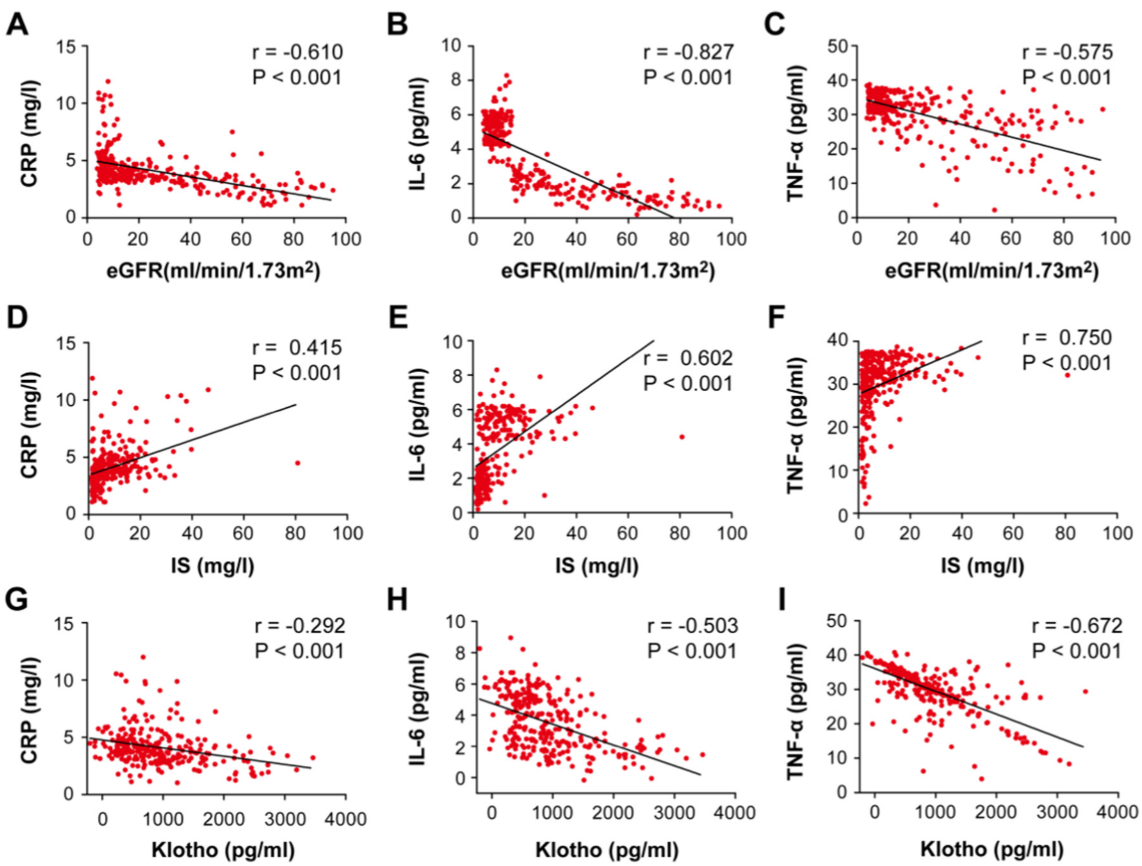

Fig. 2. The correlation of serum levels of CRP, IL-6 and TNF-α with eGFR, serum Klotho and IS levels in CKD patients. (A, B and C) The correlation of serum levels of CRP, IL-6 and TNF-α with eGFR. (D, E and F) The correlation of serum levels of CRP, IL-6 and TNF-α with serum IS level. (G, H and I) The correlation of serum levels of CRP, IL-6, TNF-α, with serum Klotho level. The correlation was analyzed by spearman correlation. The correlation between Klotho and inflammatory factors adjusted for uric acid, BMI and age was analyzed by partial spearman correlation.

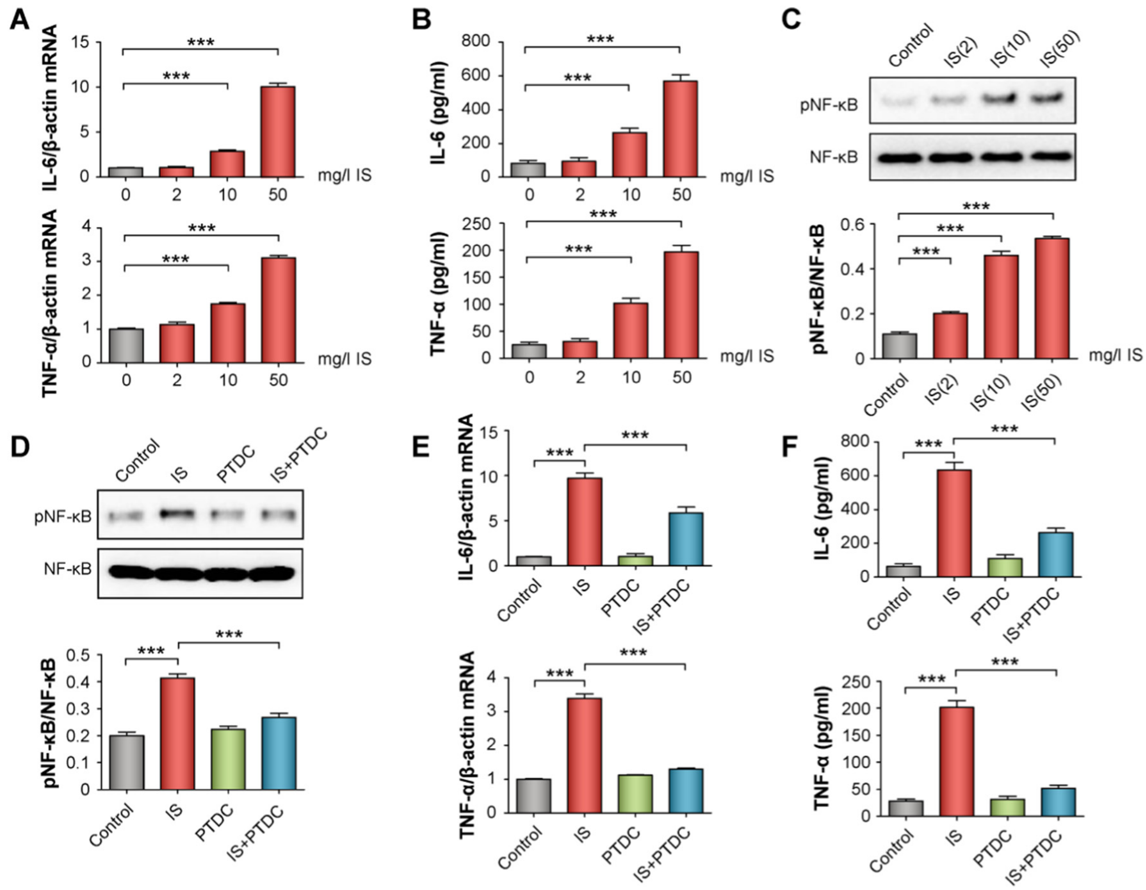

Fig. 3. IS induces systemic inflammation by activation of the NF-κB signaling in monocytes. (A) In THP-1 cells, the mRNA expression of IL-6 and TNF-α was increased in a dose-dependent manner after treatment with IS (2, 10, and 50 mg/L) for 24 h, as detected by qRT-PCR. (B) Serum levels of IL-6 and TNF-α were also elevated in a dose-dependent manner after exposure to IS, as measured by ELISA. (C) IS dose dependently promoted the expression of pNF-κB in THP-1 cells, as assessed by Western blot assay. (D) Pretreatment with PDTC (20 μmol/L) significantly inhibited IS-induced pNF-κB upregulation. (E and F) Pretreatment with PDTC suppressed IS-induced release of IL-6 and TNF-α in THP-1 cells. Values are expressed as means ± SD from at least three independent experiments. *** $P < 0.001$.

volunteers (Fig. 4A and B). Accordingly, the serum levels of IS in these CKD patients were assessed and found higher than the control group (Fig. 4C). Meanwhile, it was found that the expression of RIG-1 was dramatically upregulated in THP-1 cells after treatment with IS (Fig. 4D). In contrast, RIG-1 inactivation by siRNA (Fig. 4E) not only suppressed IS-induced pNF-κB upregulation in THP-1 cells Fig. 4F), but also blocked the release of inflammatory factors (Fig. 4G and H). All these data indicate that IS has a strong ability to induce monocytes activation through the activation of RIG-1/NF-κB signaling pathway.

3.3. Klotho inhibits IS-induced monocyte activation by restraining RIG-1/NF-κB signaling

Interestingly, Klotho was reported to function as a negative regulator of RIG-1/NF-κB signaling [25]. The effects of Klotho on RIG-1/NF-κB signaling activation was assessed that was induced by IS in primary monocytes. Similarly, treatment with 400 pmol/L Klotho significantly inhibited the increase in IS-induced expressions of RIG-1 and pNF-κB in primary monocytes (Fig. 5A and B). The dosage of Klotho protein was optimized following a recent study [23]. Consequently, pretreatment with Klotho markedly abrogated IS-induced upregulation and release of IL-6 and TNF-α by primary monocytes (Fig. 5C and D). This data indicate that Klotho has a potent protective property against IS-induced monocyte activation by restraining RIG-1/NF-κB signaling.

3.4. Klotho deficiency aggravates systemic inflammation in CKD mice

The influence of Klotho reduction on systemic inflammation in CKD was evaluated, and compared the differences of serum levels of Klotho and inflammatory factors between WT CKD mice and heterozygous Klotho-deficient (*kl/+*) CKD mice. As shown in Fig. 6A, the serum Klotho level were decreased remarkably in both WT CKD mice and *kl/+* CKD mice, and it was much lower in *kl/+* CKD mice in comparison with that in WT CKD mice. On the contrary, the serum levels of IL-6 and TNF-α significantly increased in both WT CKD mice and *kl/+* CKD mice, and the increases of IL-6 and TNF-α were much greater in *kl/+* CKD mice (Fig. 6B and C). Meanwhile, it was found that the expression level of RIG-I in peripheral blood monocytes in *kl/+* CKD mice was much higher than that in WT CKD mice (Fig. 6D). These results suggested that Klotho reduction may aggravate CKD-associated systemic inflammation due to its decreased ability to restrain IS-induced monocytes activation.

3.5. Exogenous Klotho administration attenuates systemic inflammation in CKD mice

Finally, the therapeutic effects of Klotho supplementation on CKD-associated systemic inflammation were assessed. The CKD mice were intraperitoneally injected with recombinant Klotho protein (0.01 mg/kg Klotho protein dissolved in 0.01 mol/L PBS) or PBS (0.01 mol/L) every 48 h for 4 weeks. The dosage of Klotho protein administration in current experiments was according to a previous study [22]. Notably,

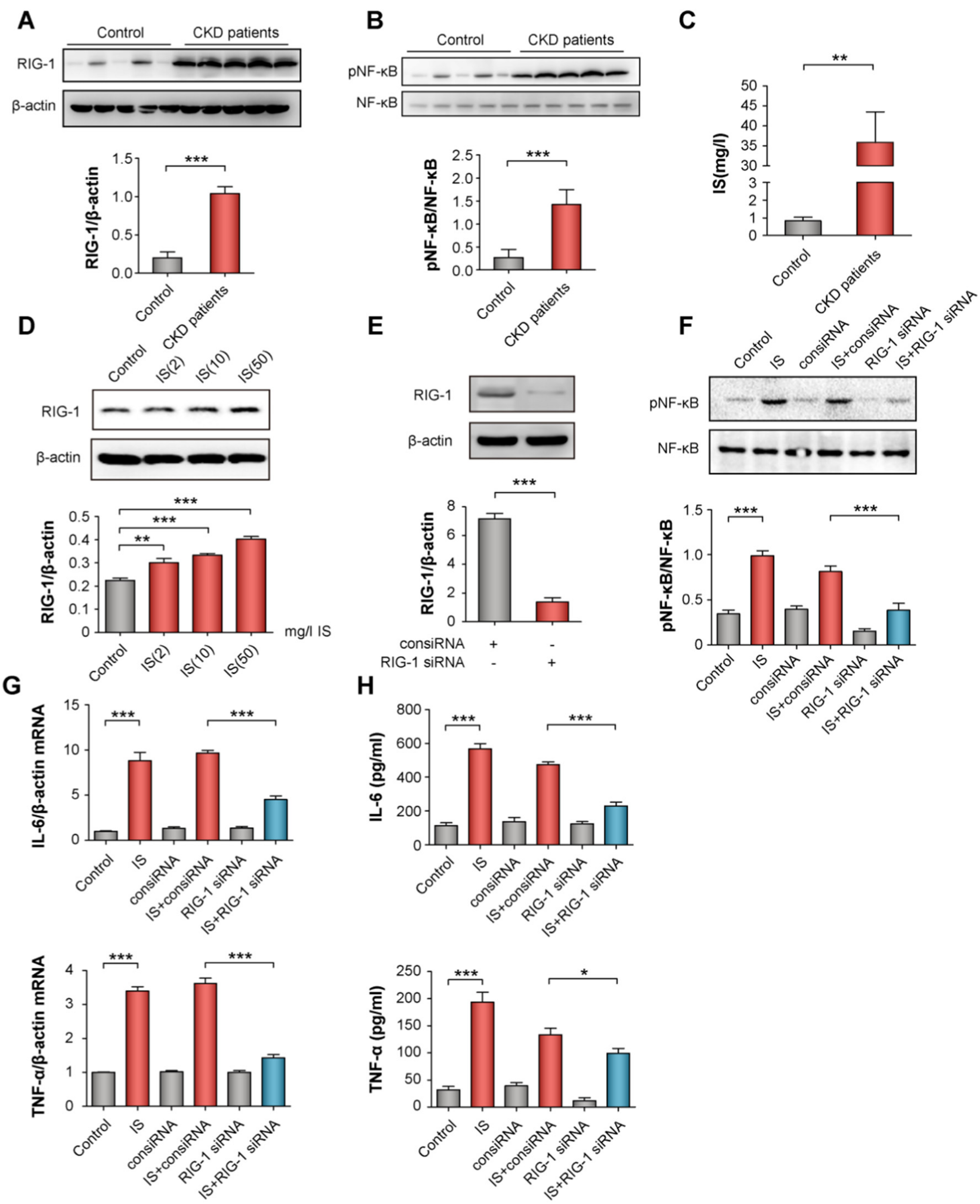

Fig. 4. IS promotes monocytes activation through the activation of RIG-1/NF-κB signaling pathway. (A) Significant increased in protein expression of RIG-1 was observed in the monocytes of CKD stage 5 patients, compared with healthy volunteers. (B) The protein expression of pNF-κB was also increased in the monocytes of CKD patients, compared with healthy volunteers. (C) The serum IS levels in CKD patients was higher than healthy volunteers. (D) In THP-1 cells, the expression of RIG-1 was upregulated in a dose-dependent manner followning treatment with IS. (E) Depletion RIG-1by RIG-1 siRIG-1 suppressed RIG-1 protein expression compared with scrambled control in THP-1 cells (F) Transfection with siRIG-1 suppressed IS-induced pNF-κB expression. (G and H) Transfection with siRIG-1 inhibited IS-induced release of IL-6 and TNF-α in THP-1 cells. Values are expressed as means ± SD from at least three independent experiments. * $P < 0.05$, *** $P < 0.001$.

treatment with exogenous Klotho protein led to an increased expression of serum Klotho (Fig. 7A) while a decreased expression of RIG-1 in peripheral blood monocytes, compared with PBS control group (Fig. 7B). Consequently, the serum levels of IL-6 and TNF-α dropped significantly after Klotho treatment (Fig. 7C and D), demonstrating that exogenous Klotho supplementation can attenuate CKD-associated systemic inflammation by inhibiting monocyte activation.

4. Discussion

Clinical and experimental studies showed that CKD is tightly associated with immune system damage and proinflammatory milieu. For instance, uremia sera can impair the maturation and endocytosis of monocytes and monocyte-derived dendritic cells in patients with hemodialysis [32]. Meanwhile, the expansion of $CD14^+CD16^+$ monocyte

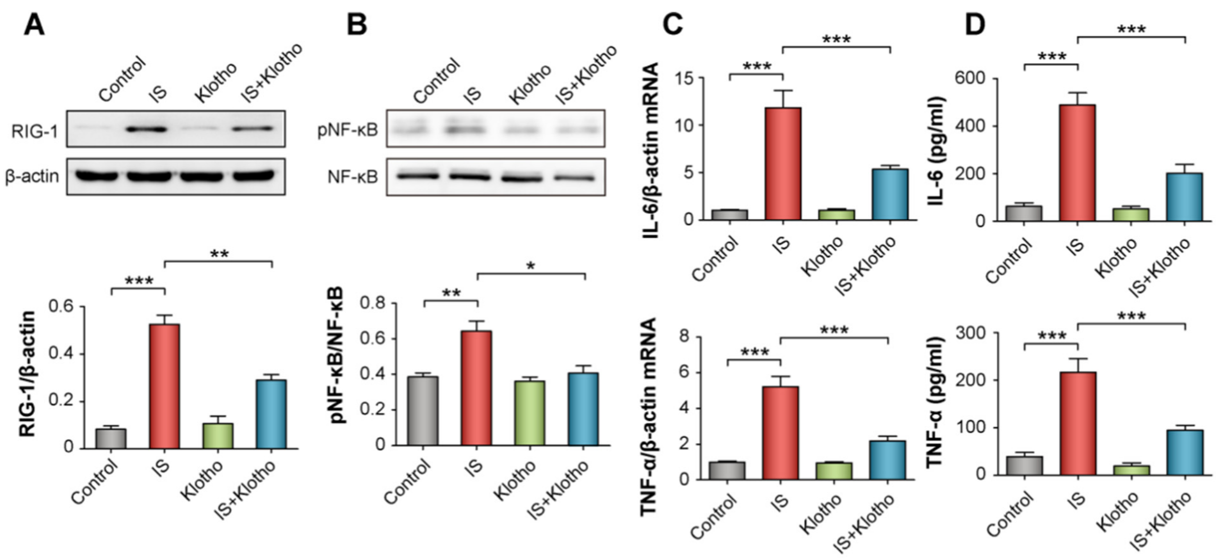

Fig. 5. Klotho suppressed IS-induced monocyte activation by inhibiting RIG-1/NF-κB signaling. (A and B) In primary monocytes, pretreatment with Klotho significantly inhibited the increase in the expressions of RIG-1 and pNF-κB induced by IS. (C and D) Klotho also obviously inhibited IS-mediated release of IL-6 and TNF-α. Values are expressed as means ± SD from at least three independent experiments. *** $P < 0.001$.

subpopulation and cytolytic $CD4^{+}CD28^{-}$ T cells are frequently observed in ESRD patients [33,34]. On contrary, numerous clinical studies reported that increased levels of inflammatory biomarkers including c-reactive protein (CRP), TNF-α and IL-6 were inversely correlated with the decline of renal function [35–37]. In addition, the high morbidity and mortality of CKD-associated cardiovascular disease (CVD) are at least in part ascribed to the uremic proinflammatory milieu [3]. In fact, the increased level of inflammatory markers is also an independent risk factor for CVD development in CKD patients [38–40]. It has been shown that uremic toxins, especially protein-bound uremic toxins, are quiet hard to efficiently remove with hemodialysis compared with non-protein-bound uremic toxins, and reported to have distinct properties of inducing immune dysfunction and systemic inflammation [41]. Moreover, the retention of protein-bound uremic toxins mediates a vicious cycle between oxidative stress and persistent inflammation, which aids adversity in cardiovascular systems [42]. Although the inflammatory properties of protein-bound uremic toxins have been recognized, but the exact the underlying mechanisms are not fully known yet and hardly any effective avenues reported that control protein-bound uremic toxins-induced systemic inflammation in CKD patients.

Current clinic data is consistent with the previous reports [13], and showed that serum levels of IS and inflammatory factors increased gradually with the declining of eGFR in CKD patients, with a positive correlation among the serum IS level and serum CRP, IL-6 and TNF-α levels in patients with CKD stage 2–5. Monocytes are originated from monoblasts differentiated from hematopoietic stem cells in the bone marrow. Normally, monocytes circulate in the bloodstream for 1–3 days and then differentiate into macrophages and dendritic cells after moving within the tissues. Monocytes can directly be activated by a variety of stimulating factors that generate inflammatory responses, includes TNF-α, IL-1, IL-6 and IL-12. Previous study revealed that IS enhanced macrophage activation in response to lipopolysaccharide from *E. coli* (LPS) accompanied by increased TNF-α and IL-6 levels [43]. In current study, it is confirmed that IS has a distinct ability to promote IL-6 and TNF-α secretion from monocytes cultured *in vitro*. It is well known that CRP is synthesized in the liver and its expression get increased following IL-6 secretion from monocytes and adipocytes [44]. Therefore, our study supports that the accumulation of IS in the serum may be an important contributor to systemic inflammation by activation of peripheral blood monocytes in CKD patients. In addition, different uremic substances particularl p-cresol (PCS) has also been reported to induce systemic inflammation in CKD patients [13]. Consistent to previous studies, current results showed that IS has significant correlation with serum levels of IFN-γ, IL-6 and TNF-α, whereas PCS was only correlated with the serum level of IL-6 in CKD patients [13]. This further suggests that IS may be an independent factor in CKD-associated systemic inflammation. Nevertheless, the observations can't exclude various other uremic toxins effect on CKD-associated inflammation, which possibly has little impact.

Recently the studies shown that IS could prompt monocytes

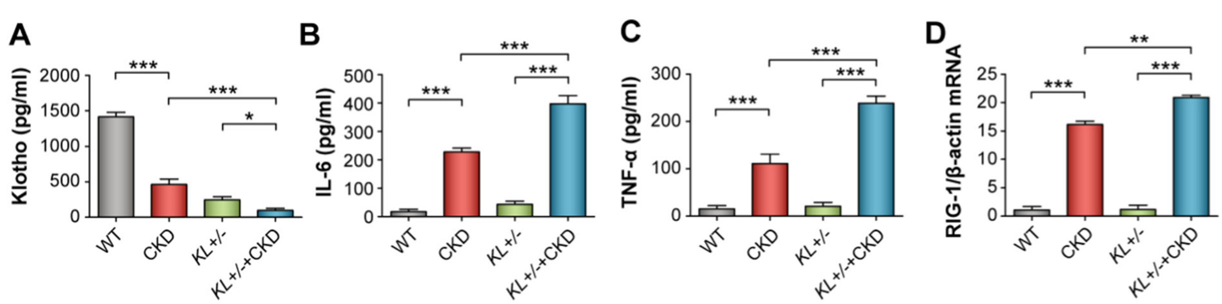

Fig. 6. Klotho reduction aggravates systemic inflammation in CKD mice. (A) The serum Klotho level was significantly decreased in WT CKD mice and kl/+ CKD mice, with a much lower serum Klotho level in kl/+ CKD mice, compared with WT CKD mice. (B and C) The serum levels of IL-6 and TNF-α obviously increased in WT CKD mice and kl/+ CKD mice, and much higher serum levels of IL-6 and TNF-α were observed in kl/+ CKD mice. (D) the expression of RIG-I in peripheral blood monocytes in kl/+ CKD mice was much greater than that in WT CKD mice, which was revealed by RT-PCR. Values are expressed as means ± SD from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 8$ per group.

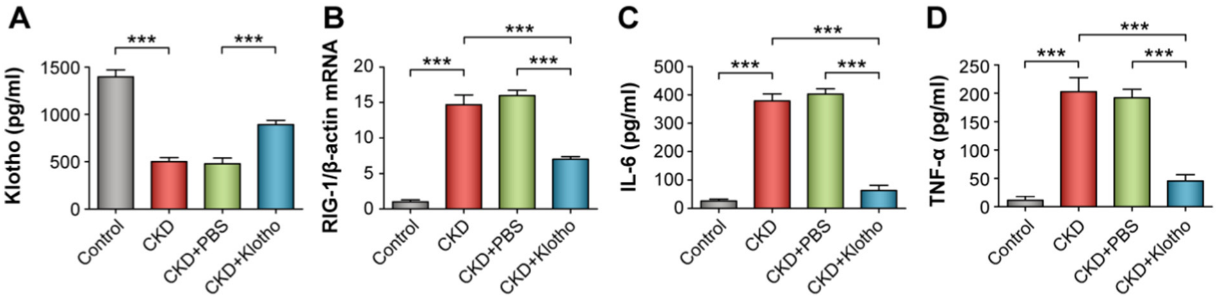

Fig. 7. Klotho treatment ameliorates systemic inflammation in CKD mice. (A) The RT-PCR assay showed that Klotho treatment (0.01 mg/kg Klotho protein dissolved in 0.01 mol/L PBS) increased the expression of serum Klotho while (B) reduced the expression of RIG-1 in peripheral blood monocytes, compared with PBS control group. (C and D) Klotho treatment also inhibited the elevation of serum IL-6 and TNF-α levels in CKD mice, compared with PBS control group assessed by ELISA. Values are expressed as means ± SD from at least three independent experiments. *** $P < 0.001$. $n = 8$ per group.

transition into macrophages by the activation of Aryl hydrocarbon Receptor (AhR)/Nrf2 [45], and potentially able for direct induce the expression of Mac-1 and ROS production in THP-1 cells that lead to an increased number of Mac-1 positive stained peripheral blood monocytes in IS-treated mice [14,45]. On the other hand, it was found that the effect of IS-mediated monocytes activation was evidently inhibited by p38MAPK inhibitor, and the oral charcoal adsorbent AST-120 significantly suppressed the enhanced expression of Mac-1 and ROS production in monocytes by reducing IS in CKD mice [14]. Results of current study showed that IS induced NF-κB activation in a dose-dependent manner in monocytes, and inhibition of NF-κB by PDTC could block IS-induced IL-6 and TNF-α production. As known, NF-κB is an important transcription factor regulating the transcriptions of various inflammatory factors and it can be activated by Toll-like receptors and certain kinases [30]. RIG-I is part of the RIG-I-like receptor family, which activates transcription factors such as interferon-regulatory factor and NF-κB through the caspase-recruitment domain-like region at its N-terminus, leading to the production of type I interferon and inflammatory factors [31]. Herin, it is found that IS induced NF-κB activation and inflammatory factors secretion through the upregulation of RIG-I expression in cultured monocytes. Moreover, the activation of RIG-I/NF-κB signaling was also observed in peripheral blood monocytes of CKD stage 5 patients. Nevertheless, we noticed that even with a higher concentration of IS in cell experiments, RIG-1 induction was relatively low, compared with primary monocytes in CKD patients. Due to the complex situation *in vivo*, it could not be excluded the possibility that other uremic substances were also involved in increasing RIG-1 in CKD patients. However, for the disparity of RIG-1 induction between *in vitro* and *in vivo* experiments, one possible reason is the continuous exposure to IS in real life allows solutes more time to reach the intracellular compartments of monocytes where biologic activity is exerted. Thus a higher concentration of IS should be considered to compensate for short exposure *in vitro*, whereas possibly has lesser impact [46]. On the contrary, knockdown of RIG-I significantly inhibited IS-induced NF-κB activation and the production of inflammatory factors in monocytes. Therefore, current findings not only demonstrates that IS contributes to CKD-associated systemic inflammation, but also provides a new insight into the molecular mechanism of IS-induced monocyte activation. Interestingly, it has also reported that indoxyl sulfate induced the TNF-α expression *via* AhR in human monocytes [47]. Bioinformatics tools [48] predicted that there are four putative binding sites of AhR in RIG-1 gene promoter region (the forecast score is above 85 points), and AhR could directly bind to one of the RIG-1 regions. However, whether AhR has the ability to directly regulate RIG-1 gene expression needs to be explored, and studies are under the pipelines.

Notably, a negative correlation between serum Klotho level and serum levels of inflammation factors in CKD patients was also observed. Previously, Klotho was reported to inhibit TNFα-induced up-regulation of intercellular adhesion molecule-1 (ADAM-1), vascular cell adhesion molecule-1 (VCAM), and NF-κB activation in endothelial cells [49]. In addition, it was reported that high salt intake-caused hypertension and renal structure injury in Klotho-deficient mice was at least in part ascribed to monocyte chemotactic protein-1specific cell surface receptor (CCR2)-mediated inflammation [50]. Thus, it is speculated that Klotho may be an endogenous protector against IS-mediated systemic inflammation.

Current study further indicated that Klotho could significantly inhibit IS-induced RIG-I/NF-κB activation and the production of IL-6 and TNFα in monocytes. As the serum levels of inflammatory factors were even higher in $KL^{+/-}$ CKD mice than those in WT CKD mice, reflecting that Klotho reduction may aggravate CKD-associated systemic inflammation. In fact, it is found that exogenous Klotho administration evidently attenuated the activation of RIG-I/NF-κB signaling in monocytes and decreased the serum levels of inflammatory factors in CKD mice. A recent study reported that intracellular overexpression of Klotho was able to inhibit RIG-I-induced IL-6 production in HEK293 cells [25]. The findings of present study showed that extracellular Klotho also has the ability to inhibit IS-induced activation of RIG-I/NF-κB signaling in monocytes, thereby extending our understanding of the action of Klotho. However, administering klotho is still very far from clinical routine. Other therapeutic options, such as probiotic measures, which have the ability to reduce excessive uremic toxins including IS by influencing gut microbiota alteration [51], also need to be explored in the future.

5. Conclusions

Present study reveals the relationship between IS and Klotho and their implications in CKD-associated systemic inflammation. Furthermore, current findings suggests that exogenous supplementation with Klotho may be a potential therapeutic approach to target uremic inflammatory milieu in patients with CKD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116570.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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