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Correlation of increased Th17/Treg cell ratio with endoplasmic reticulum stress in chronic kidney disease

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Abstract

To investigate the relationship between the regulatory immune network and endoplasmic reticulum stress (ERS) in patients with different stages of chronic kidney disease (CKD).

A total of 91 patients diagnosed with CKD were divided into different groups according to the stage of disease and treatment with hemodialysis (HD) or peritoneal dialysis (PD). Routine blood and biochemical tests were performed in patients in the different CKD groups and in healthy controls (n = 20). The frequencies of T helper type 17 (Th17) and regulatory T (Treg) cells in the overall T cell population were measured by flow cytometric analysis. Levels of Th17 cell (IL-17) and Treg cell (IL-10) cytokines and the ERS markers CCAAT-enhancer-binding protein homologous protein (CHOP) and glucose-regulated protein 78 (GRP78) were measured by enzyme-linked immunosorbent assay in serum samples collected from controls and patients. Correlations between each parameter and serum creatinine were analyzed by Spearman rank correlation and regression test.

CKD stage showed a positive correlation with serum creatinine level, and increased and decreased percentages of Th17 and Treg cells, respectively, reflected in an increased Th17/Treg cell ratio. Consistent with this, CKD stage was positively correlated with serum concentrations of IL-17 and negatively correlated with serum IL-10 levels. Moreover, serum levels of CHOP and GRP78 increased with advancing CKD stage. These correlations were most pronounced in patients in the CKD5 group, who also had the poorest response to HD and PD treatment, compared with CKD5 patients in the nondialysis group. Correlation analysis showed that serum levels of CHOP and GRP78 were independently and positively correlated with the ratio of Th17/Treg cells.

We have found that an increased Th17/Treg cell ratio and increased serum levels of ERS markers correlate with the progression of CKD. Our results indicate that the interplay between regulation of the immune network and management of ERS is closely associated with the pathogenesis of CKD. Although HD and PD treatment manage chronic kidney conditions and prevent further deterioration of renal function, they have limited effects on improving the immune disorder and relieving ERS. Our study suggests a potential new direction for development of therapeutic strategies in CKD.

Abbreviations: CHOP = CCAAT-enhancer-binding protein homologous protein, CKD = chronic kidney disease, ELISA = enzyme-linked immunosorbent assay, ERS = endoplasmic reticulum stress, GFR = glomerular filtration rate, GRP78 = glucose-regulated protein 78, HD = hemodialysis, MDRD = modification of diet in renal disease, PBMCs = peripheral blood mononuclear cells, PD = peritoneal dialysis, UPR = unfolded-protein response.

Keywords: chronic kidney disease, endoplasmic reticulum stress, Th17 cell, Treg cell

1. Introduction

Chronic kidney disease (CKD) is characterized by progressive loss of kidney function, and has developed into a major global health problem. The incidence and mortality of CKD have increased over the past few decades,[1] with 1 prediction of the prevalence of individuals in the United States with end-stage CKD at over 700,000 by 2015.[2] Moreover, a 2012 Chinese survey found that the overall prevalence of CKD in adults was approximately 10.8%, placing a huge burden on healthcare resources.[3] The absence of an effective treatment to retard or reverse the progression of CKD highlights the need for a better understanding of the mechanistic basis of its development and progression, and of designing novel therapeutic strategies in CKD.

Immune imbalance has been proposed as a critical contributing factor in pathogenesis of CKD, with early studies implicating T helper type 1 (Th1) and type 2 (Th2) cells.[4,5] The subsequent discovery of novel T helper subpopulations such as Th17 and regulatory T (Treg) cells, heralded the concept of T cell-mediated immunity.[6] The ratio of Th17 and Treg cells is important for maintaining the immune balance, and a solid body of evidence has demonstrated the role of imbalances in this ratio in tissue inflammation, autoimmunity, and a wide variety of diseases,[7–10] including uremic and end-stage CKD.[11,12]

Endoplasmic reticulum (ER) is a vital organelle in eukaryotes that is responsible for the production and proper folding of proteins. Consonant with this, ER stress (ERS) and the

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subsequent unfolded-protein response (UPR) have been shown to be among the earliest events occurring in the initiation of many diseases.[13] The recent discovery of crosstalk between ERS pathways and immune signaling suggests the possibility of their cooperation in modulating the immune responses.[14] For example, defective MHC class I molecule expression has been associated with induction of UPR,[15] and protein kinase C-mediated T cell activation has been linked to induction of ERS.[16] Moreover, macrophages have been shown to respond to ERS and UPR via synergistic production of interferon beta.[17] Collectively these studies suggest that the ERS response may be a mechanism of immune alteration. Here, we set out to investigate the association between Th17/Treg imbalance and ERS in different stages of CKD, with the ultimate goal of improving our understanding CKD pathophysiology and the prospects of novel therapies in this disease.

2. Materials and methods

2.1. Patients

All study subjects were recruited from the Dialysis Management and Blood Purification Center in the Second Affiliated Hospital of Xi'an Jiaotong University. The primary disease for all patients was chronic glomerulonephritis, and the inclusion criteria were as follows: stable blood pressure, absence of complicated infection and heart failure within the last 3 months, no blood transfusion or adjuvant therapies received within the past month, no hepatitis or connective tissue diseases, not pregnant or breast feeding, absence of severe cardiovascular, cerebrovascular or hematopoietic complications and other primary diseases, and no severe mental disorders. The patients were not taking any immunosuppressive agents or other drugs which might affect the results of measurement in the study.

A total of 91 patients were enrolled and divided into 3 groups (CKD3, CKD4, and CKD5) according to the CKD stages calculated from measured glomerular filtration rate (GFR). Using the abbreviated modification of diet in renal disease (MDRD) equation to calculate the estimated GFR:GFR (mL/min per 1.73 m^2) = 186 × (creatinine/88.4)$^{-1.154}$ × (age)$^{-0.203}$ × (0.742 if female). CKD3 (30 < GFR < 59), CKD 4 (15 < GFR < 29), and CKD5 (GFR < 15). Patients in CKD5 group were further divided into 3 subgroups based on the dialysis treatment: nondialysis uremic group (n = 14), hemodialysis group (HD, n = 18), and peritoneal dialysis group (PD, n = 18). The patients in the HD group had undergone regular HD treatment (4-h treatment given 3 times a week) for more than 3 months using a sugar-free low-calcium dialysis solution with low-molecular weight heparin as anticoagulant. The dialysis membrane was polysulfone with an area of 1.2 to 1.5 m^2, and the blood flow was set to 200 to 250 mL/min, dialysate flow 500 mL/min. The patients in the PD group had undergone regular PD treatment for more than 3 months using low-calcium PD solution. The catheters placed in patients were adopted from the standard Tenckhoff PD catheter, and the daily volume of replacement fluid was 8000 mL.

Twenty healthy volunteers (12 males and 8 females) were recruited to the control group. They had no medical history of hypertension, diabetes, hepatitis, tuberculosis and related immune disorders, and no preceding infection.

This study was approved by the Ethics Committee in the Second Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from all patients and healthy volunteers participating in the study.

2.2. Sample preparation

Fasting hemospasia was performed in all participants. Blood samples from the patients in the HD group were collected prior to HD on the day of treatment. Routine blood test that included a complete blood count and routine clinical chemistry tests were performed. Peripheral blood (3 mL) was loaded into heparin anticoagulant containing tubes (Sigma, Santa Clara, CA) and peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation. Another 3 mL of peripheral blood was centrifuged at 3000 rpm for 5 min, and the supernatant (serum) was collected and stored at −80°C until further use.

PBMCs were cultured at a density of 2×10^6 cells/mL in RPMI 1640 complete medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD). Cells were treated with 2 μL/mL leukocyte activation cocktail with monensin and GolgiPlug (Brefeldin A) (BD Biosciences, Franklin Lakes, NJ) and incubated for 4 h in a 5% CO_2 incubator at 37°C. Cells were collected by centrifugation at 250g for 5 min and resuspended in Stain Buffer (BD Biosciences) at a density of 1×10^7/mL for flow cytometric analysis of Th17 and Treg cell numbers.

2.3. Flow cytometry

Approximately 1×10^6 cells were fixed in 2 mL 1× Human FoxP3 Buffer A (BD Biosciences) for 10 to 20 min at room temperature (RT) and protected from light. Fixed cells were washed with 2 mL Stain Buffer followed by centrifugation at 500g for 5 min. Cells were permeabilized in 0.5 mL 1× Human FoxP3 Buffer C (BD Biosciences) for 30 min at RT and protected from light. Cells were washed with Stain Buffer and resuspended to 1×10^7 cells/mL in 100 μL aliquots. Antihuman antibodies (PerCP-Cy5.5-CD4, phycoerythrin (PE)-IL-17A, and Alexa Fluor647-FoxP3) or isotype controls (PerCP-Cy5.5-, PE-, and PEAlexa Fluor 647-conjugated mouse IgG1 κ isotype controls) were added to the cells (20 μL/test) and incubated for 40 min at RT protected from light. After staining, cells were washed twice with Stain Buffer and analyzed immediately using BD FACSCalibur flow cytometer (BD Biosciences). Approximately 20,000 to 30,000 cells were acquired and results were analyzed by FlowJo Data Analysis software (FlowJo LLC, Ashland, OR).

2.4. Enzyme-linked immunosorbent assay

All serum samples were stored at −80°C and processed all at once. Serum levels of IL-10, IL-17, glucose-regulated protein 78 (GRP78), and CCAAT-enhancer-binding protein homologous protein (CHOP) were determined by enzyme-linked immunosorbent assay (ELISA) using specific detection reagents following the manufacturer's instruction (Westang, Shanghai, China). Each assay was performed in triplicates and the experiments were repeated once.

2.5. Statistical analysis

Data were analyzed using SPSS 16.0 Statistical Analysis software (SPSS Inc, Chicago, IL). Measurement data are expressed as mean ± standard deviation. Comparison of means between groups was made by 1-way analysis of variance, and comparison between 2 groups was made by Fisher least significant difference test. Spearman rank-order correlation was used for bivariate correlation analysis. A P value < .05 was considered statistically significant, and $P < .01$ indicated a more significant difference.

Table 1

Clinical characteristics of all participants.

Item	Control (n=20)	CKD3 (n=22)	CKD4 (n=19)	CKD5 (n=50)		
				ND (n=14)	HD (n=18)	PD (n=18)
Age, y	48.9±11.4	58.9±19.0	58.3±13.1	54.0±20.1	48.3±17.5	46.6±15.0
Gender (male/female)	12/8	12/10	10/9	8/6	9/9	10/8
WBC, 10^{12}/L	6.17±1.67	7.06±3.21	5.57±1.57†	5.65±1.63	5.64±1.28	6.07±2.10
RBC, 10^{12}/L	4.62±0.57	3.46±0.82*	2.81±0.65*,†	2.55±0.68*,†	3.30±0.72*,§	2.89±0.69*,†
PLT, 10^{9}/L	235±51.7	161±58.5*	167±61.4*	145±60.7*	157±46.3*	164±94.0*
Hb, g/L	135±15.5	104±26.0*	82.8±18.1*	77.5±12.7*,†	101±21.3*,‡,§	85.3±20.0*,†
FPG, mmol/L	5.10±0.74	5.10±0.98	4.75±0.52	4.86±2.09	4.28±0.76‖	5.47±2.56
Scr, μmol/L	69.6±11.6	267.2±112.3*	556.6±80.3*,†	983.4±372.7*,†,‡	897.3±242.2*,†,‡	851.6±218*,†,‡
BUN, mmol/L	4.30±0.93	14.5±7.01*	20.0±5.94*,†	25.8±9.50*,†,‡	26.1±7.64*,†,‡	20.7±8.12*,†
Cystre-C, mg/L	0.77±0.16	3.42±1.90*	5.31±1.86*,†	5.84±1.94*,†	8.22±4.10*,†,‡,§,‖	6.06±1.09*,†
UA, μmol/L	276.0±89.4	400.7±136.4*	370.7±108.1*	412.2±158.4*	400.2±83.8*	434.2±119.8*
PTH, pg/mL	38.0±14.8	129.3±131.7*	203.8±87.1*	274.7±129.2*,†	397.3±349.5*,†,‡	319.5±136.9*,†,‡
ALT, IUl/L	25.8±10.8	18.3±15.3	17.9±12.8	20.7±9.6	12.8±6.1	13.4±7.1
AST, IUl/L	19.4±3.8	23.7±17.8	19.8±8.9	23.5±7.2	14.1±5.1	15.8±6.2
TP, g/L	71.6±3.26	64.4±9.68*	66.9±9.0	62.1±7.82*	65.3±4.64*	54.5±5.17*,†,‡,§,¶
ALB, g/L	45.3±2.73	38.4±6.43*	38.9±6.69*	37.7±5.93*	39.5±4.39*	29.9±4.76*,†,‡,§,¶
Ca, mmol/L	2.01±1.09	2.03±0.34	1.95±0.29	1.87±0.22	1.92±0.29	1.67±0.23*,†,‡,¶
K, mmol/L	4.32±0.44	4.55±0.79	4.55±0.96	5.07±0.65*	5.28±0.76*,†,‡	3.90±1.16†,‡,§,¶
CRP, mg/L	6.8±0.66	12.6±0.78*	15.7±0.58*	18.2±0.46*,†	19.4±0.57*,†	15.7±0.61*
TC, mmol/L	4.45±0.97	4.50±1.13	4.40±1.34	3.94±0.48	3.72±0.67*,†	4.14±0.95
TG, mmol/L	1.85±1.39	1.99±1.24	1.34±0.94	1.28±0.31†	1.13±0.44†	1.12±0.59†

Values are expressed as mean ± standard deviation.

ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = urea nitrogen, CKD = chronic kidney disease, CRP = C-reaction protein, ERS = endoplasmic reticulum stress, FPG = fasting plasma glucose, Hb = hemoglobin, HD = hemodialysis, ND = nondialysis, PD = peritoneal dialysis, PLT = platelet, PTH = parathyroid hormone, RBC = erythrocyte, Scr = serum creatinine, TC = total cholesterol, TG = triglyceride, TP = total protein, UA = uric acid, WBC = leukocyte.

* $P < .05$ vs. control.

† $P < .05$ vs. CKD3.

‡ $P < .05$ vs. CKD4.

§ $P < .05$ vs. NHD.

¶ $P < .05$ vs. PD.

‖ $P < .05$ vs. HD.

3. Results

3.1. Clinical characteristics of CKD patients

A total of 91 patients with primary chronic glomerulonephritis were classified according to the following CKD stages: stage 3 CKD (30 < GFR < 59, 12 males and 10 females, mean age: 58.9 ± 19.0 years), stage 4 CKD (15 < GFR < 29, 10 males and 9 females, mean age: 58.3 ± 13.1 years), and stage 5 CKD (GFR < 15). Stage 5 CKD was further divided into NHD group (8 males and 6 females, mean age: 54 ± 20.1 years), HD group (9 males and 9 females, mean age: 48.3 ± 17.5 years), and PD group (9 males and 9 females, mean age: 46.6 ± 15.0 years). Healthy volunteers (n=20) were recruited to the control group, including 12 males and 8 females with a mean age of 48.9 ± 11.4 years.

Routine blood and clinical chemistry tests were performed using the blood samples obtained from all participants. Complete blood count was measured, and liver and renal functions were assessed. Results were analyzed for each group and comparison was made between groups (Table 1). Significant differences between the healthy controls and CKD patients were observed in parameters, such as serum creatinine, urea nitrogen, uric acid, parathyroid hormone, albumin, and C-reaction protein, indicating the presence of chronic renal disorders and impaired kidney function in CKD patients (Table 1). There were no significant differences in age and sex distributions between the healthy controls and patients in different CKD stages (Table 1).

3.2. Increasing Th17/Treg cell ratio is positively correlated with CKD stage

The percentage of Th17 ($CD4^+IL\text{-}17A^+$) cells was significantly increased in CKD patients compared with controls ($P < .05$), and the percentage of Th17 cells increased with advancing CKD stage (Table 2, Fig. 1A and B). By contrast, the percentage of Treg ($CD4^+FoxP3^+$) cells was lower in CKD3 and CKD4 patients than in healthy controls ($P < .05$), with the lowest percentage of Treg cells observed in CKD5 patients without dialysis treatment ($P < .05$; Table 2, Fig. 1A and C). The changes in the frequencies of Th17 and Treg cells were reflected in an increase in the ratio of Th17/Treg cells in CKD patients that was positively correlated with disease stage (Fig. 1D).

3.3. Correlation of Th17/Treg cell ratio with serum creatinine level in CKD patients

Serum creatinine is among the most accurate and reliable indicators of kidney function. We next investigated the correlation between the peripheral blood frequencies of Th17 and Treg cells and serum creatinine level. Serum creatinine levels in both controls and CKD patients were found to be positively correlated with the frequency of Th17 cells and the ratio of Th17/Treg cells ($r = 0.546$, $P < .01$ and $r = 0.659$, $P < .01$, respectively), and negatively correlated with the frequency of Treg cells ($r = -0.413$, $P < .01$) (Fig. 2). Th17/Treg ratio was more robustly correlated with serum creatinine level ($r = 0.659$) than with the

Table 2

Flow cytometric analysis of Th17 and Treg cells ($\bar{x} \pm s$).

Item	Control (n = 20)	CKD3 (n = 22)	CKD4 (n = 19)	CKD5 (n = 50)		
				ND (n = 14)	HD (n = 18)	PD (n = 18)
4+, %	50.1 ± 5.36	44.9 ± 4.22*	40.5 ± 5.12*	33.8 ± 3.83*,†	36.8 ± 5.22*	38.3 ± 4.44*
Th17, %	0.91 ± 0.31	1.95 ± 1.30*	2.50 ± 1.13*	2.80 ± 0.73*	2.88 ± 1.39*,†	2.35 ± 1.45*
Treg, %	4.51 ± 1.62	3.53 ± 1.54	3.59 ± 1.58	2.06 ± 1.26*,†,‡	2.84 ± 2.34*	2.83 ± 1.53*
Th17/Treg, %/%	0.23 ± 0.12	0.64 ± 0.50	0.89 ± 0.77*	1.44 ± 0.83*,†,‡	1.42 ± 0.75*,†,‡	1.10 ± 0.78*,†

CKD = chronic kidney disease, HD = hemodialysis, ND = nondialysis, PD = peritoneal dialysis.
* $P < .05$ vs. control.
† $P < .05$ vs. CKD3.
‡ $P < .05$ vs. CKD4.

frequency of Th17 ($r = 0.546$) or Treg ($r = -0.413$) cells indicated that Th17/Treg cell ratio is a potential indicator for the degree of renal damage and progression of kidney disease.

3.4. Serum levels of Th17 and Treg cytokines are correlated with creatinine level in CKD patients

The proinflammatory and regulatory functions of Th17 and Treg cells are mediated by IL-17 and IL-10, respectively, serum levels of which were determined by ELISA in CKD patients and healthy controls. The concentration of IL-17 was significantly higher in the CKD patients and increased with the progression of CKD (Fig. 3A). By contrast, the concentration of IL-10 was lower in all CKD groups than in the control group ($P < .05$), but not between different CKD groups (Fig. 3B) (Table 3). Moreover, serum creatinine levels in serum CKD patients correlated positively with serum IL-17 levels ($r = 0.666$, $P < .01$), and negatively with serum IL-10 levels ($r = -0.346$, $P = .01$; Fig. 3C and D). Dialysis

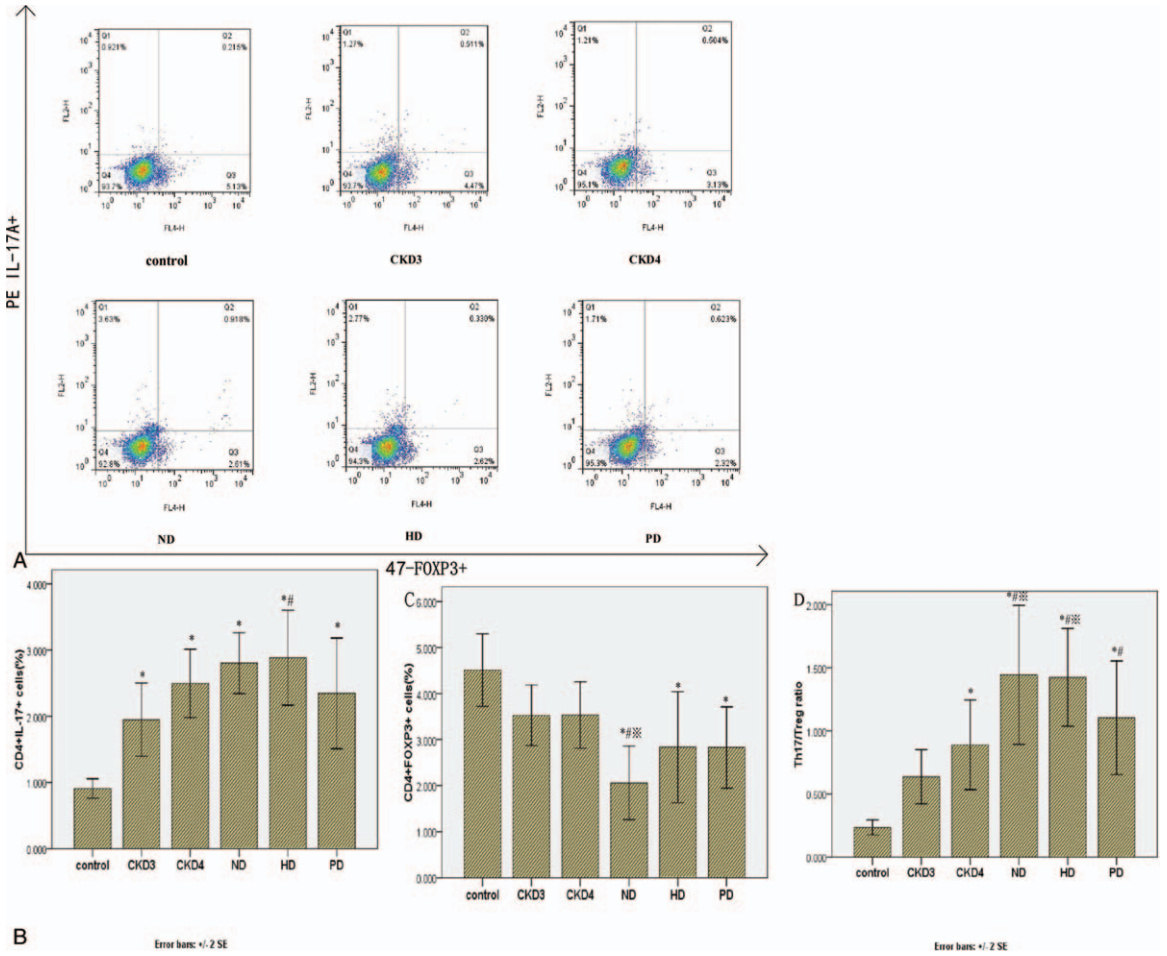

Figure 1. Percentage of Th17 and Treg cells in CKD patients and healthy controls. (A) Representative flow cytometric analysis from a single patient in each group. PBMCs were stained with PerCP-Cy5.5-conjugated CD4 antibodies and PE-conjugated IL-17A and Alexa Fluor 647-conjugated FoxP3 antibodies to measure the percentages of Th17 cells ($CD4^+IL\text{-}17^+$) and Treg cells ($CD4^+FoxP3^+$). (B) The percentage of Th17 cells averaged from all patients within the group. (C) The percentage of Treg cells averaged from all patients within the group. (D) The ratio of Th17/Treg cells in each group. CKD = chronic kidney disease, PBMCs = peripheral blood mononuclear cells.

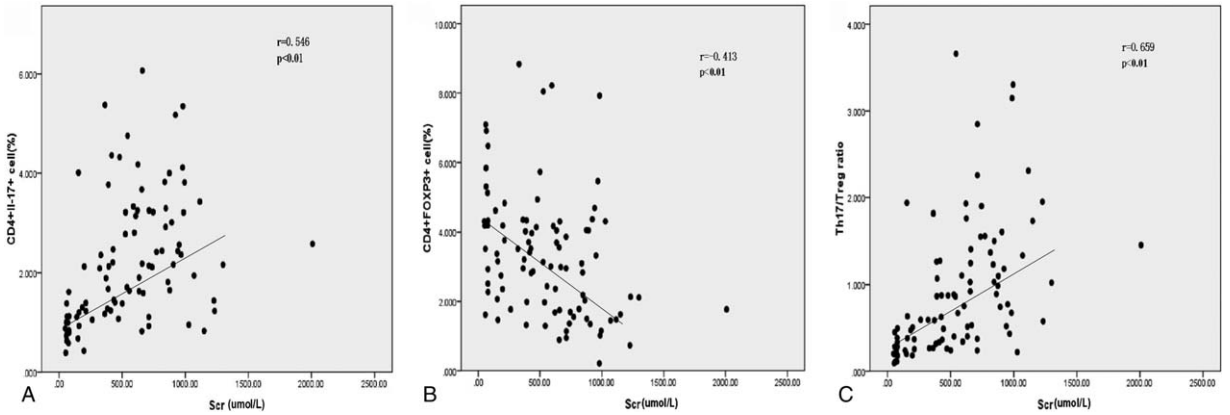

Figure 2. Correlations of the frequency of Th17, frequency of Treg cells, and ratio of Th17/Treg cells with serum creatinine level in controls and CKD patients. Serum creatinine level was measured in all study participates and its correlation with the frequency of Th17 cells (A) and Treg cells (B) and the ratio of Th17/Treg cells (C) was analyzed by Spearman rank correlation and regression test. The *P* value and *r* value are indicated in the graphs. CKD = chronic kidney disease.

treatment had no significant effect on the level of IL-17 and IL-10 in CKD5 patients.

3.5. Correlation of serum levels of the ERS markers CHOP and GRP78 with creatinine level in CKD patients

Historical evidence has linked ERS to the pathophysiology of kidney disease. We next evaluated the correlation of serum levels of the ERS markers CHOP and GRP78 with creatinine levels in CKD patients. Serum levels of CHOP (Fig. 4A) and GRP78 (Fig. 4C) were significantly higher in CKD patients than in controls ($P < .05$) and positively correlated with CKD stage ($P < .05$) (Table 4). By contrast, serum levels of neither CHOP nor GRP78 were altered by dialysis treatment. In addition, serum creatinine level in CKD patients were positively correlated with serum levels of CHOP ($r = 0.603$, $P < .01$ and GRP78 ($r = 0.802$, $P < .01$) (Fig. 4B and D), reflecting an association between the extent of ERS and the severity of renal damage.

3.6. Correlation of the serum Th17/Treg cell ratio with serum ERS marker levels in CKD patients

Having established that the Th17/Treg cell ratio and serum levels of ERS markers were independently correlated with creatinine levels in CKD patients, we next asked whether these parameters were correlated with each other. We found that the ratio of Th17/Treg cells was positively correlated with serum CHOP and GRP78 levels ($r = 0.465$, $P < .01$ and $r = 0.498$, $P < .01$, respectively) (Fig. 5), indicating that ERS and imbalances in immune components might interact to influence the progression of CKD.

Table 3

Serum concentrations of cytokines (IL-17 and IL-10) in CKD patients and normal control subjects ($\bar{x} \pm s$, pg/mL).

Item	Control (n = 20)	CKD3 (n = 22)	CKD4 (n = 19)	CKD5 (n = 50)		
				ND (n = 14)	HD (n = 18)	PD (n = 18)
IL-17	9.29 ± 7.95	27.69 ± 17.09*	36.23 ± 18.93*	59.26 ± 20.35*,†,‡	60.48 ± 24.29*,†,‡	43.1 ± 22.30*,†
IL-10	77.55 ± 22.18	46.25 ± 22.16*	49.42 ± 26.09*	36.97 ± 23.65*	48.20 ± 26.44*	42.90 ± 20.34*

CKD = chronic kidney disease, HD = hemodialysis, ND = nondialysis, PD = peritoneal dialysis.
* $P < .05$ vs. control.
† $P < .05$ vs. CKD3.
‡ $P < .05$ vs. CKD4.

Table 4

Serum concentrations of ERS marks in CKD patients and normal control subjects ($\bar{x} \pm s$, pg/mL).

Item	Control (n = 20)	CKD3 (n = 22)	CKD4 (n = 19)	CKD5 (n = 50)		
				ND (n = 14)	HD (n = 18)	PD (n = 18)
CHOP	54.4 ± 24.1	338.3 ± 178.7*	379.1 ± 158.9*	512.3 ± 209.2*,†	454.9 ± 241.8*,†	450.7 ± 172.7*
GRP78	35.6 ± 23.2	78.2 ± 76.9*	181.1 ± 141.5*,†	348.2 ± 154.9*,†,‡	370.0 ± 197.5*,†,‡	331.9 ± 96.8*,†,‡

CHOP = CCAAT-enhancer-binding protein homologous protein, CKD = chronic kidney disease, ERS = endoplasmic reticulum stress, GRP78 = glucose-regulated protein 78, HD = hemodialysis, ND = nondialysis, PD = peritoneal dialysis.
* $P < .05$ vs. control.
† $P < .05$ vs. CKD3.
‡ $P < .05$ vs. CKD4.

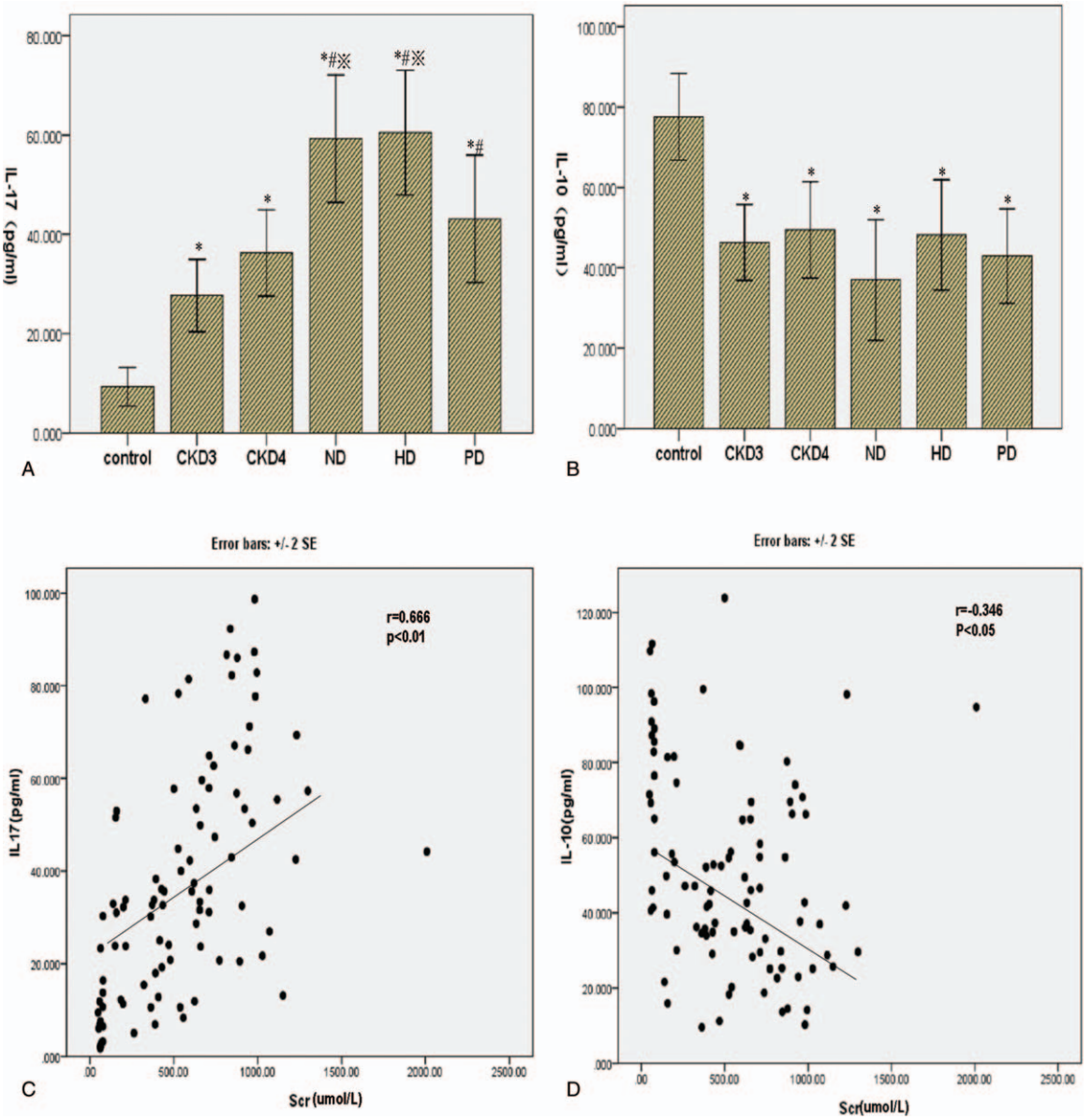

Figure 3. Correlations of IL-17 and IL-10 with serum creatinine level. (A) The serum concentration of IL-17 was measured by ELISA in the control and CKD groups. (B) The serum concentration of IL-10 was measured by ELISA in the control and CKD groups. (C) The correlation between serum IL-17 concentration and creatinine level was analyzed by Spearman rank correlation and regression test. (D) The correlation between serum IL-10 concentration and creatinine level was analyzed similarly. The *P* value and *r* value are indicated in the graphs. CKD = chronic kidney disease, ELISA = enzyme-linked immunosorbent assay.

4. Discussion

In the present study, we investigated the changes in Th17 and Treg cell populations and the levels of specific cytokines and ERS markers in patients with differing CKD stages. In addition, we assessed the impact of different dialysis treatments on restoration of immune balance. We found that compared with controls CKD patients had an increased percentage of Th17 cells and a decreased percentage of Treg cells, reflected in an increased Th17/Treg cell ratio that was positively correlated with CKD stage. Corresponding differences in serum levels of Th17 (IL-17) and Treg (IL-10)-specific cytokines were observed in CKD patients. Moreover, the percentage of Th17 cells, serum IL-17 level, and Th17/Treg ratio were all positively correlated with the severity of kidney disease, as determined by serum creatinine levels. By contrast, the percentage of Treg cells was negatively correlated with serum creatinine level. Furthermore, serum levels of the ERS markers CHOP and GRP78 were positively correlated with CKD stage and serum creatinine levels. Interestingly, while the ratio of Th17/Treg cells showed positive correlation with the levels of CHOP and GRP78, dialysis treatment did not appreciably affect immune balance or amelioration of ERS. Our data suggest a possible mechanism of CKD pathogenesis and disease progression, and indicate a rationale for development of new therapeutic strategies that target the interplay between the immune network and ERS responses.

Risk factors for CKD include metabolism, hemodynamics, and genetic predisposition. With recent advances in studies of CKD pathophysiology, inflammation, and dysregulated immune responses have emerged as important factors contributing to the gradual loss of renal function in CKD patients.[18] Moreover,

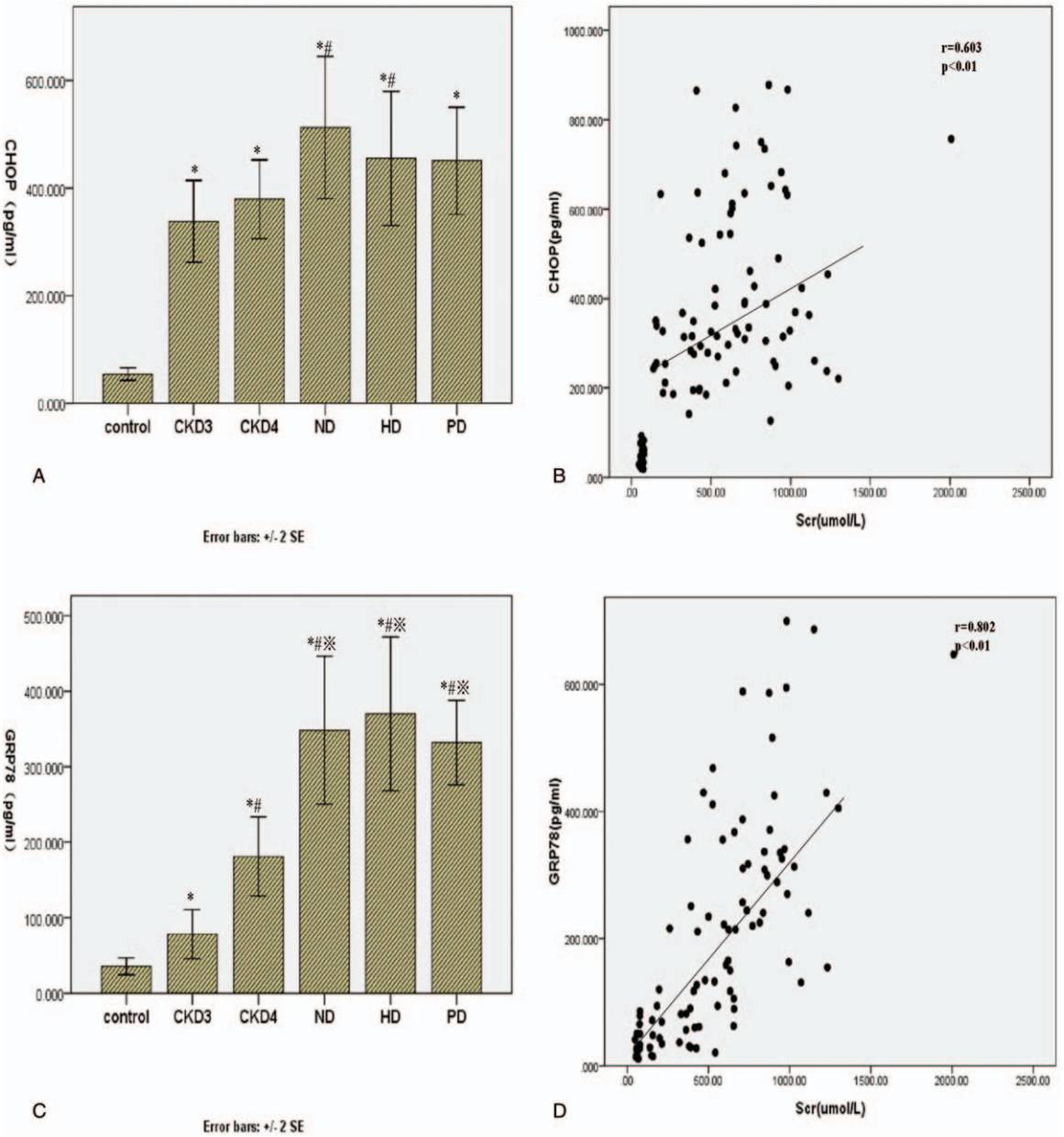

Figure 4. Correlation between ERS markers and serum creatinine level. Serum concentrations of CHOP (A) and GRP78 (B) were measured by ELISA in the control group and different CKD groups. The correlation between serum creatinine level and the CHOP (C) and GRP78 (D) concentrations was analyzed by Spearman rank correlation and regression test. The *P* value and *r* value are indicated in the graphs. CHOP = CCAAT-enhancer-binding protein homologous protein, CKD = chronic kidney disease, ELISA = enzyme-linked immunosorbent assay, ERS = endoplasmic reticulum stress, GRP78 = glucose-regulated protein 78.

the risk of developing heart disease and cancer increases markedly in CKD patients.[19,20] Th17 cells and their major effector cytokine IL-17 play important roles in tissue inflammation and are involved in various immune disorders and human malignancies, including rheumatoid arthritis, systemic lupus, multiple myeloma, and cancer.[21–25] However, the role of Th17 cells in CKD pathogenesis and disease progression remains inconclusive. On the other hand, Treg cells generate immune mediators (IL-10) or immunosuppressive cytokines (TGF-beta) to modulate immune responses and autoimmunity in immune tolerance.[26,27] Th17 and Treg cells function in an interrelated and antagonistic manner, and the balance between the ratio of 2 is important in the maintenance of human health. Although IL-17 is considered a specific Th17 cytokine, IL-17-producing Treg cells have been identified during inflammation or upon activation.[28,29] The balancing mechanism of Th17 and Treg cells is therefore extremely complicated and has not yet been fully elucidated. That said, the role of Th17/Treg imbalance in the development of various diseases, including autoimmune conditions and cancer, has been well established.[12,30,31]

In the present study, the frequency of Th17 cells was markedly increased in CKD3 patients; however, the frequency of Treg cells was significantly decreased in CKD5 patients. It is possible that the decrease of Treg cell frequency was following the increase of Th17 cell frequency. As a result, the Th17/Treg cell ratio was significantly increased with the progression of CKD. These results suggest that in early stage of CKD, the proinflammatory Th17 cells are antagonized by large numbers of immunosuppressive

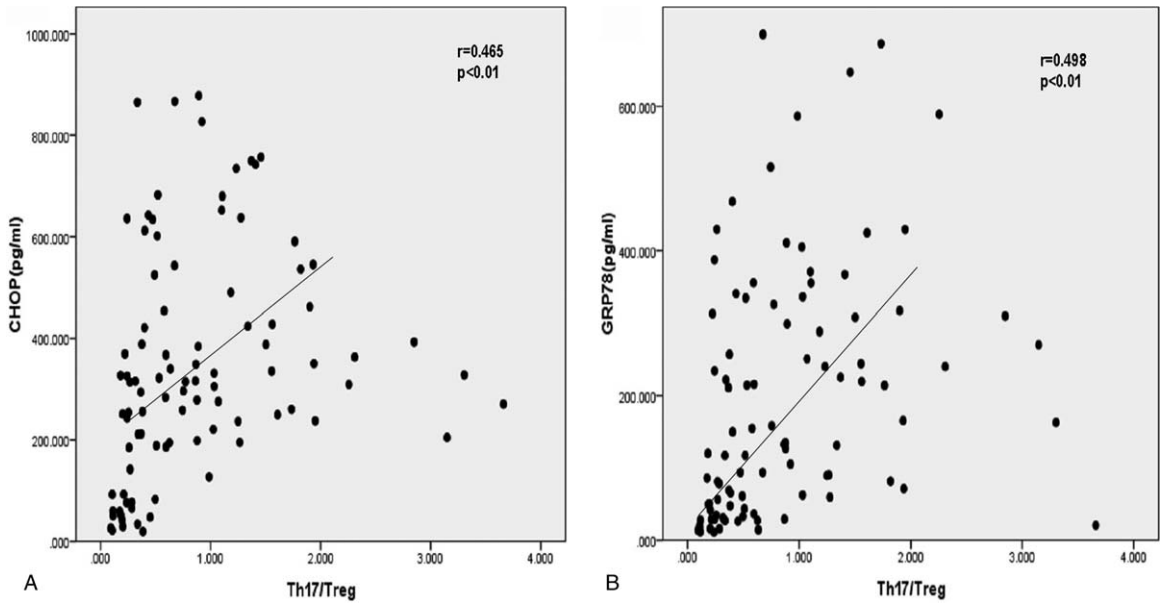

Figure 5. Correlation between serum concentration of ERS markers and ratio of Th17/Treg cells in CKD patients. (A) Correlation between the concentration of CHOP and ratio of Th17/Treg was analyzed by Spearman rank correlation and regression test. (B) Correlation between the concentration of GRP78 and ratio of Th17/Treg was analyzed similarly. The *P* value and *r* value are indicated in the graphs. CHOP = CCAAT-enhancer-binding protein homologous protein, CKD = chronic kidney disease, ERS = endoplasmic reticulum stress, GRP78 = glucose-regulated protein 78.

Treg cells. However, with the progressing of CKD, the Treg cells are consumed in late stage, which causes an increase in Th17 cells and creates an imbalance between Th17 and Treg cells. That explains the increased incidence of complications in late CKD stage. In addition, we demonstrated that the serum creatinine level, an indicator of renal function, was positively correlated with the frequency of Th17 cells, but was negatively correlated with the frequency of Treg cells. An even stronger positive correlation was observed between serum creatinine levels and the Th17/Treg cell ratio. These results clearly indicate that the imbalance between proinflammatory and immunosuppressive cells plays a critical role in CKD progression. What remains unclear is whether the fluctuations in the Th17/Treg ratio are a cause or a result of renal failure.

Advanced stage CKD often requires dialysis treatment, including HD and PD. In our study, although dialysis treatment improved the survival of CKD patients, it had a limited effect on restoring the balance between Th17 and Treg cells, a result that is consistent with a previous study in end-stage renal disease patients.[32] Dialysis augments reduced renal function by removing metabolic waste products and toxins and maintaining the electrolyte balance and acid–base equilibrium. However, due to the possible risks present in HD, such as severe anemia due to blood loss during treatment, hemodynamic instability, aluminum intoxication,[33] and dialyzer biocompatibility,[34] immune disorders may be worsened by dialysis. Although PD has advantages over HD in these respects,[35] problems such as increased protein loss, excessive glucose intake, and triglyceride abnormalities make it ineffective in restoring immune balance in CKD patients. Further investigation is required to identify new therapeutic agents or strategies to improve the patient's immune microenvironment.

ERS is a physiological and pathological disorder of ER characterized by impaired protein and lipid synthesis, and the disturbance of intracellular calcium homeostasis. Markers for ERS include GRP78 as a marker for activation of ERS response, and CHOP as a primary marker for induction of apoptotic pathway.[36,37] Numerous studies have indicated the involvement of ERS in the pathophysiology of kidney diseases by causing damage to glomerular mesangial cells, podocytes, and tubular epithelial cells.[38–40] Consistent with their previously established association with ERS, we found that serum levels of GRP78 and CHOP were positively correlated with serum creatinine levels in CKD patients. The results reiterate that the severity of CKD correlates with the degree of ERS. ERS response is a normal physiological response and has protective effects in patients with adequate renal compensation. However, as the renal function deteriorates, ERS response might impair renal function and the extent of renal damage could conceivably increase during progression of CKD.

Immune disorder and ERS have long been reported as 2 independent causes/consequences of CKD. CHOP and GRP78 in circulation can be contributed by any cells beyond T cells. Although ERS-induced inflammation has been described in previous studies,[41,42] the relationship between ERS and immune regulation is largely unknown. Given the profound impact of oxidative stress on T cell activation and function,[43] ERS—a subtype of oxidative stress—could potentially contribute to immune dysregulation. Our study identified a positive correlation between the serum levels of the ERS markers CHOP and GRP78 and the ratio of Th17/Treg cells in CKD patients, indicating a crosstalk between these 2 components. Because inflammation is a common downstream event of ERS response and Th17/Treg imbalance, we hypothesize that inflammation is a major mediator of ERS and immune dysfunction that contributes significantly to disease progression.

In conclusion, our study is to our knowledge the first to demonstrate the relationship between the ratio of Th17/Treg cells and changes in ERS marker levels and CKD stage. The antioxidant effects of alpha lipoic acid[44] and the flavonoid quercetin[45] suggest the potential of novel therapeutic approaches in CKD. Future studies will focus on the identification of the antioxidant that targets crosstalk between ERS and immune imbalance in order to protect the kidney from inflammation-induced tissue damage.

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