DYSFUNCTION OF THE IMMUNE SYSTEM DURING ACUTE KIDNEY INJURY

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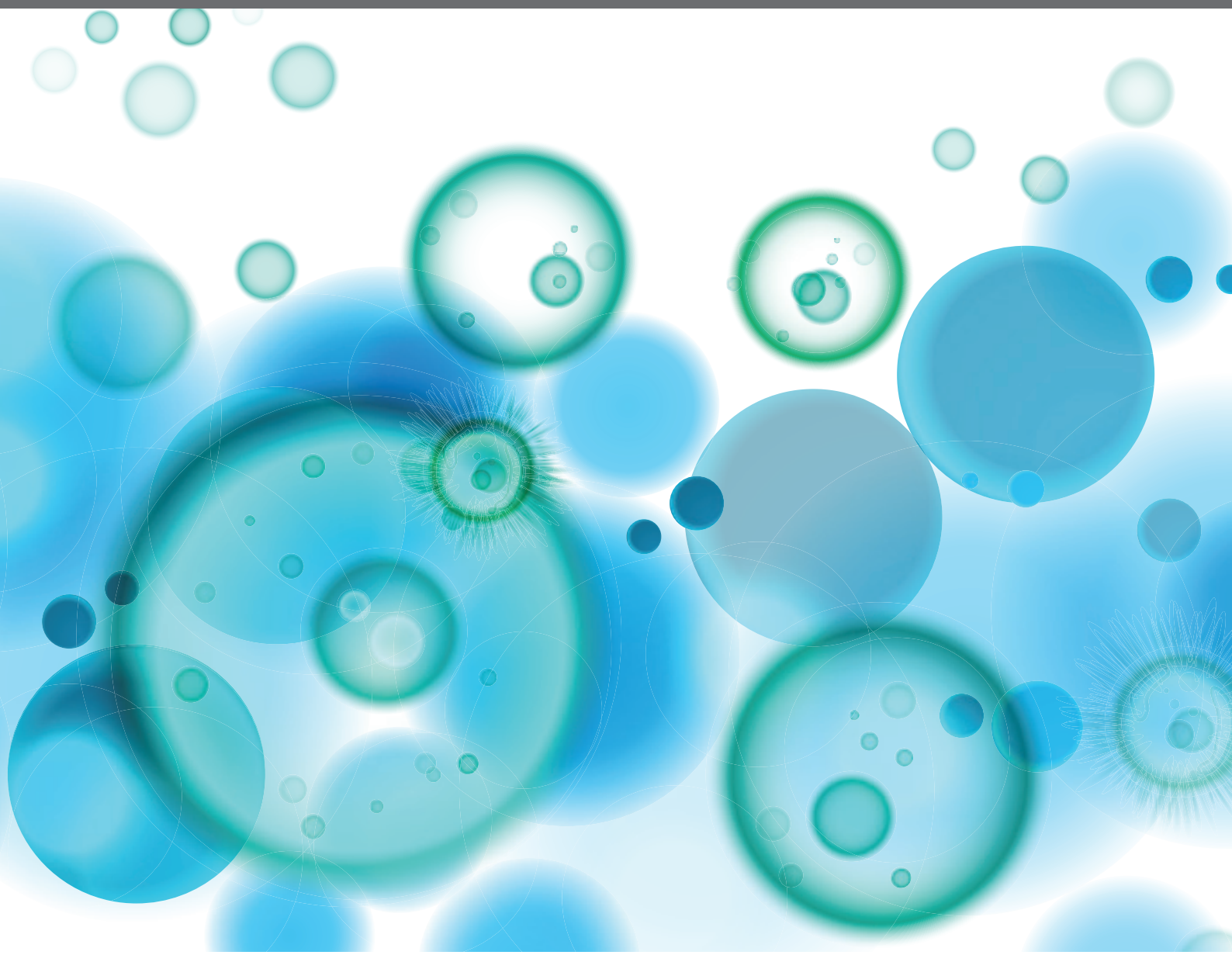

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DYSFUNCTION OF THE IMMUNE SYSTEM DURING ACUTE KIDNEY INJURY

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Low C3 Serum Levels Predict Severe Forms of STEC-HUS With Neurologic Involvement

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Background: The correlation between the severity of hemolytic uremic syndrome related to Shiga toxin–producing *Escherichia coli* (STEC-HUS) and involvement of the complement system has been examined in a small number of studies, with conflicting results. In the present study, we investigated whether serum C3 levels on admission are associated with neurologic involvement.

Methods: To this purpose, 68 consecutive STEC-HUS patients were recruited and main clinical and laboratory variables ad hospital admission were compared between those with or without neurologic involvement.

Results: STEC-HUS patients who developed neurologic involvement (NI) showed significant higher leukocyte count, C-reactive protein and hemoglobin, and lower sodium levels as compared with those without. Interestingly, baseline serum levels of C3 were significantly lower in patients with NI as compared with those without ($p < 0.001$). Moreover, when stratified according to need of Eculizumab rescue therapy due to severe NI, patients treated with this drug showed baseline C3 serum levels significantly lower than those who were not ($p < 0.001$).

Low C3 was independent risk factor for NI in our patients' population when entered as covariate in a multivariate logistic regression analysis including other major variables previously proposed as possible predictors of poor prognosis in STEC-HUS (for instance, leukocyte count, c-reactive protein, sodium levels) (HR 6.401, 95%CI 1.617–25.334, $p = 0.008$ for C3).

To underline the role of complement in the worsening of STEC-HUS patients' clinical conditions and outcomes, all patients were divided into two groups according to the baseline lower vs. normal serum levels of C3 and the main data on care needs were assessed. Interestingly more patients with lower C3 serum levels required renal replacement therapy ($p = 0.024$), anti-hypertensive therapy ($p = 0.011$), Intensive Care Unit admission ($p = 0.009$), and longer hospitalization ($p = 0.003$), thus displaying

significantly more severe disease features as compared with those with normal C3 serum levels.

Conclusions: Our data suggests that children with STEC-HUS with decreased C3 concentrations at admission are more likely to develop neurologic involvement and are at increased risk of having severe clinical complications.

Keywords: hemolytic uremic syndrome, Shiga-toxin *E. coli*, neurologic involvement, complement system, C3 serum levels, Eculizumab

INTRODUCTION

Hemolytic uremic syndrome (HUS) secondary to gastrointestinal infections due to Shiga toxin-producing *Escherichia coli* (STEC) is characterized by micro-angiopathic hemolytic anemia, thrombocytopenia, and renal injury (1). In children, STEC-HUS accounts one of the main causes of acute kidney injury (AKI); death occurs in 1–5% of affected patients while long-term renal sequelae are demonstrated in almost 30% of the survivors (2–4).

STEC-HUS mainly affects the kidney, but extra-renal complications are frequently described (5). The involvement of the central nervous system (CNS) often represents a life-threatening condition and it can result in severe long-term disability in HUS patients who overcome the acute phase of illness (6). For these reasons it's mandatory the early diagnosis of the STEC-HUS might require dedicated surveillance protocols (7); in addition, the discovery of early markers of disease severity is necessary in the attempt to promptly treat the patients and to reduce the risk of long term renal and extra-renal sequelae.

The endothelial damage caused by Shiga toxin (Stx) is more likely to be the culprit pathogenic mechanism of the disease (8); however, there is increasing evidence for complement system activation as a contributing factor involved in organ damage (9). Several reports during last decades have described low plasma C3 concentrations and augmented complement products' degradation in children affected by STEC-HUS (10–12). Recently an *in vitro* study showed that high doses of STX2 are able to induce direct activation of complement alternative pathway (AP) and to bind factor H, decreasing its activity on the cell surface (13). In addition, Morigi et al. demonstrated that alternative pathway activation of complement system by Stx promotes large C3a formation that triggers microvascular thrombosis (14). Moreover, complement activation was also inferred by the detection of circulating micro-vesicles derived from platelets, monocytes, and red blood cells bearing C3 and C9 in STEC-HUS patients (15, 16). More recently, deposits of C5b-9 were detected in renal tissues from STEC-HUS affected patients and additional studies revealed that Stx induces complement-mediated injury in glomerular endothelial cell and podocyte (17–19).

Despite all these relevant findings, only a few clinical studies have correlated the complement system activation with the clinical course. Furthermore, they presented conflicting results and most of them reported small series of patients, or even included patients without microbiological diagnosis (20–26).

Since 2011, we have incorporated serum C3 determination into the initial laboratory profile in patients with STECHUS; thus, we aimed to explore further the association between C3 concentrations on admission and severe neurologic involvement in a large cohort of patients with proven STEC infection.

SUBJECTS AND METHODS

Patients

In this retrospective single-center study, we included 69 consecutive children (33 males, 36 females) affected by STEC-HUS and treated at the Pediatric Nephrology and Dialysis Unit of the Pediatric Hospital "Giovanni XXIII" in Bari between January 2011 and December 2019. All the enrolled patients fulfilled the following criteria: (1) diagnosis of HUS with confirmed STEC infection; (2) age under 18 years old; and (3) C3 levels tested at admission. Patients with history of (1) recurrent or family history of hereditary HUS, (2) HUS associated with systemic diseases such as organ transplantation, systemic lupus erythematosus, pneumococcal infection, or HIV infection, and (3) pre-existing renal disease were excluded, as shown in **Figure 1**.

The main clinical and laboratory parameters were recorded at hospital admission and during the hospital stay in 68/69 children; an 18-month-old boy was excluded from further analysis due to death on the first day of hospitalization for severe gastrointestinal hemorrhage (**Figure 1**). Blood laboratory determinations performed and analyzed at admission were: white blood cell count (WBC), hemoglobin, hematocrit, platelets count, creatinine, c-reactive protein, sodium, potassium, lactic dehydrogenase (LDH), and albumin. Moreover, the C3 and the C4 concentrations were also assessed at hospital admission.

To assess the severity of the disease, the following data on care needs were collected: blood product administration, plasma-exchange, need for dialysis, treatment with antihypertensive drugs, intensive care unit admission, and days of hospitalization.

Among extra-renal complications, clinical manifestations of the central nervous system (CNS) involvement were recorded and assessed according to the Pediatric Neurologic Assessment Score for HUS (PNAS-HUS), previously described by Giordano et al. (27).

Patients with severe CNS involvement were treated with Eculizumab, after their parents signed informed consent for "off-label" use of this drug.

This retrospective single-center study was based on registry data. All procedures performed were in accordance with the 1964 Helsinki declaration and its later amendments. Ethical approval was obtained from the Institutional Review Board of

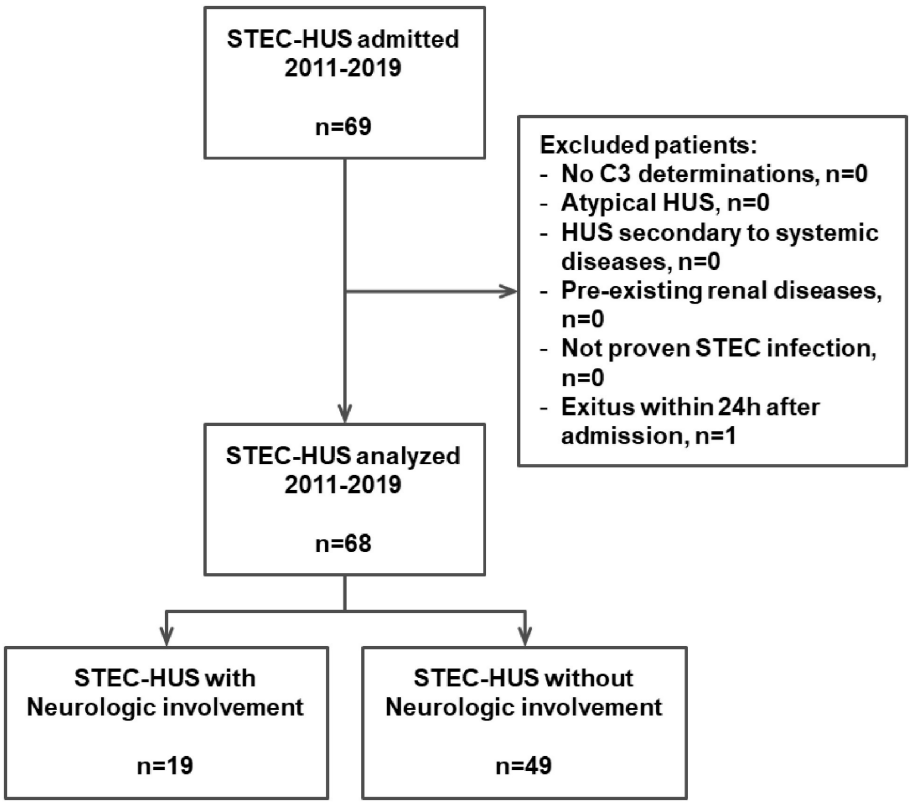

FIGURE 1 | Algorithm of the study. STEC, Shiga toxin-producing *Escherichia coli*; HUS, hemolytic uremic syndrome.

the University Hospital "Policlinico Consorziale" of Bari (Italy) (Prot. 1624/2018). All the minors' legal guardians signed a written informed consent to collect their clinical data at time of hospital access and for the publication of any potentially identifiable images or data included in this article.

Laboratory Methods

The diagnosis of STEC-HUS in children with clinical and laboratory signs of active thrombotic microangiopathy (low platelet count, hemolysis, and kidney damage) was confirmed by specific diagnostic assays: (a) Shiga toxin (Stx)-free fecal examination detection by the Vero cells assay; (b) isolation of Shiga toxin-producing *Escherichia coli* (STEC) with serological typing and PCR detection of the genes coding for virulence factors vtx1, vtx2, and eae; (c) specific anti-lipopolysaccharide serum antibodies (LPS) against the major STEC serotypes mainly related to typical HUS (O26, O157, O103, O111, and O145) (28, 29).

The C3 and the C4 concentrations were assessed by nephelometry (Image® 800 Beckman Coulter, Fullerton, CA, USA) and the normal reference values ranged within 0,79–1,52 g/L and 0,16–0,38 g/L, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS 25.0 software (SPSS Inc., Evanston, IL), as previously described (30–35). Normality of variable distribution was tested using Kolmogorov-Smirnov test. Comparison of variables between the different groups was obtained with Student's *t*-test and Mann-Whitney *U*-test owing to normal or non-parametric distribution. Frequencies were compared among groups by χ^2-test. Correlation between two variables was ascertained by Pearson or Spearman's correlation tests, as appropriate.

A receiver operating characteristic (ROC) curve analysis was performed to validate the association of baseline C3 serum levels with neurologic involvement in STEC-HUS patients, and an operational cut-off level was defined to differentiate STEC-HUS patients at higher risk of CNS involvement. Subsequently all the patients were stratified according to baseline C3 cut-off level with the highest sensitivity and specificity for predicting neurologic involvement during the follow-up.

To test the independent effects of different variables on neurologic involvement onset, univariate and multivariate binary logistic regression analysis was used and partial correlation coefficients were computed and presented as hazard ratio and 95% confidence intervals (HR; 95%CI), as previously described (36, 37). Covariates included in the univariate binary logistic regression model were: age at admission, baseline leukocyte count, and baseline serum levels of C-reactive protein, hemoglobin, sodium, and C3. All the covariates were entered as quartiles, while C3 serum levels were entered as dichotomous variable. The variables were included in the multivariate analyses

TABLE 1 | Main clinical, laboratory, and therapeutic information on STEC-HUS patients with neurologic involvement.

Patient	Gender	Age	*E. coli* strain	Days of hospitalization	Neurologic Involvement						Treatment			Outcome
					Day of onset*	Clinical evaluation**	EEG	NMR	CT scan	PNAS-HUS	Plasma-exchange	RRT	Eculizumab	
1	M	25.1	O26	23	3	Yes	Yes	No	No	3,5	1	1	No	Discharge
2	M	20.4	O26	22	10	Yes	Yes	No	No	4	1	0	No	Discharge
3	M	15.5	O26	21	5	Yes	Yes	No	No	5	0	1	No	Discharge
4	M	18.3	O145	16	3	Yes	Yes	No	No	5	0	0	No	Discharge
5	F	17.0	O111	26	6	Yes	Yes	Yes	No	5,5	0	1	No	Discharge
6	F	26.6	O26	8	9	Yes	Yes	No	Yes	5,5	0	0	No	Discharge
7	F	53.3	O111	29	7	Yes	Yes	Yes	Yes	7,5	1	1	No	Discharge
8	M	13.0	O26	27	5	Yes	Yes	Yes	Yes	9	1	0	No	Discharge
9	M	19.8	O145	31	8	Yes	Yes	Yes	Yes	9	1	1	No	Discharge
10	F	22.8	O111	16	5	Yes	Yes	Yes	Yes	11,5	1	1	Yes	Discharge
11	M	7.7	O26	21	4	Yes	Yes	Yes	No	13	1	1	Yes	Discharge
12	F	17.1	O26	24	4	Yes	Yes	Yes	No	14	0	1	Yes	Discharge
13	F	19.5	O157	18	5	Yes	Yes	Yes	Yes	15	0	1	Yes	Discharge
14	F	16.1	O103	5	2	Yes	Yes	Yes	Yes	18	1	1	Yes	Death
15	M	45.7	O26	22	3	Yes	Yes	Yes	Yes	20	1	1	Yes	Discharge
16	F	109.2	O111	45	7	Yes	Yes	Yes	Yes	20	0	1	Yes	Discharge
17	F	19.8	O26	25	3	Yes	Yes	Yes	Yes	22	0	1	Yes	Discharge
18	F	24.7	O26	20	8	Yes	Yes	Yes	Yes	24	1	1	Yes	Discharge
19	M	33.0	O157	17	4	Yes	Yes	Yes	Yes	25,5	0	1	Yes	Discharge

**Days of NI onset after hospital admission. **Clinical manifestations observed at neurologic examination: alteration of consciousness, epileptic seizures, strabismus/eye fixing, nystagmus, virus disorders/amaurosis, disorders of muscle tone (hypo/hypertone), disorders of communication skills, neurovegetative system (heart rate, hypotension, respiratory rate alterations). CT, computed tomography; EEG, electroencephalogram; NMR, nuclear magnetic resonance; PNAS-HUS, Pediatric Neurologic Assessment Score for HUS; RRT, renal replacement therapy.*

if they had a $p < 0.05$ in the univariate analysis or if they were clinically relevant confounders.

A $p \leq 0.05$ was considered statistically significant. Results are expressed in the text as mean ± standard deviation, unless otherwise stated.

RESULTS

Between January 2011 and December 2019, 69 children (33 males, 36 females) (mean age 35 months, min 5 – max 197) were admitted to the Pediatric Nephrology and Dialysis Unit of the Pediatric Hospital "Giovanni XXIII" in Bari with a diagnosis of STEC-HUS. Due to a severe gastrointestinal hemorrhage, an 18-month-old boy died within the first day of hospitalization and was excluded from further analysis (**Figure 1**). All the 68 STEC-HUS patients fulfilled the inclusion criteria and were analyzed.

Median age of patients was 22 months and 33 (48.5%) were females. Time from first symptoms to STEC-HUS diagnosis was 5 days (2–9); bloody diarrhea was found in 86.8% of cases ($n = 59$). By stool culture and/or anti-lipopolysaccharide antibodies, serogroup O26 accounted for 38 cases, O111 for 13 cases, O145 for 9 case, O103 and O157 for 3 each. The remaining children were positive for O121 (2 cases) and O80 (only 1 case).

In 19/68 cases (27.9%), clinical manifestations of the central nervous system (CNS) involvement were recorded. Neurologic involvement (NI) was diagnosed along a median period of 5 days (minimum 2, maximum 10 days) after hospital admission when the following clinical manifestations were observed: alteration of consciousness, confusional state, disorders of communication skills, strabismus/eye fixing, nystagmus, amaurosis, seizures, hyporeactivity, disorders of muscle tone (hypo/hypertone), and neurovegetative system (heart rate, hypotension, respiratory rate alterations). The patients with NI was analyzed with clinical neurological evaluation, with electrophysiological investigations (EEGs) (19/19 cases), and when needed for severe neurologic sign, with neuroimaging techniques: in detail computer tomography of the brain was performed in 12/19 cases (63.2%), while magnetic resonance of the brain was performed in 15/19 cases (78.9%).

The mean age of children with neurologic involvement (9 males and 10 females) was 20 months, ranging between 8 and 109 months. All these patients were scored with PNAS-HUS assessment and their main clinical, laboratory, and therapeutic features are reported in **Table 1**.

Ten out of 19 showed severe neurologic involvement (PNAS-HUS >9) and, accordingly to previously proposed therapeutic recommendation (27), were effectively treated with Eculizumab, an anti-C5-convertase monoclonal antibody.

The main clinical and laboratory features of all patients after stratification in two groups according to presence or absence of CNS involvement are shown in **Table 2**.

In our study, at hospital admission STEC-HUS patients with neurologic involvement showed significantly higher leukocyte

count (22.7 ± 8.0 vs. 14.6 ± 5.0 × 10^3/μl, $p < 0.001$), C-reactive protein (53.2 ± 17.8 vs. 22.3 ± 4.4 mg/dL, $p = 0.021$), hemoglobin (8.7 ± 1.5 vs. 8.0 ± 1.0 g/dL, $p = 0.023$), and lower Sodium serum levels (131.1 ± 6.0 vs. 135.4 ± 4.6 mEq/L, $p = 0.002$), as compare with those without neurologic involvement (**Table 2**).

TABLE 2 | Clinical and laboratory characteristics of STEC-HUS patients with and without central nervous system (CNS) involvement.

Variables	Total ($n = 68$)	CNS involvement ($n = 19$)	No CNS involvement ($n = 49$)	P*
Age (months)	22 [5–196]	20 [8–109]	23 [5–196]	0.589
Gender (male/female), *n*	33/35	9/10	24/25	0.905
Blood pressure				
Systolic	105 [95–125]	105 [95–115]	108 [103–125]	0.773
Diastolic	65 [60–85]	70 [60–80]	65 [60–85]	0.863
Serum creatinine (mg/dL)	2.98 ± 2.42	2,76 ± 1.4	3.07 ± 2.71	0.637
Leukocyte count (×10^3/μL)	16.9 ± 6.9	22.7 ± 8.0	14.6 ± 5.0	**<0.001**
C reactive protein (mg/dL)	30.9 ± 15.3	53.2 ± 17.8	22.3 ± 4.4	**0.021**
Hemoglobin (g/dL)	8.1 ± 1.2	8.7 ± 1.5	8.0 ± 1.0	**0.023**
Hematocrit (%)	27.0 ± 5.4	27.0 ± 4.4	27.0 ± 5.8	0.964
Platelets (×10^3/μL)	51.2 ± 47.7	48.7 ± 37.5	52.1 ± 51.4	0.764
LDH (UI/mL)	3,068 ± 1,884	3,367 ± 2,076	2,952 ± 1,813	0.450
Sodium (mEq/L)	134.1 ± 5.4	131.1 ± 6.0	135.4 ± 4.6	**0.002**
Potassium (mEq/L)	4.3 ± 0.8	4.4 ± 0.9	4.3 ± 0.7	0.547
Albumin (g/dL)	3.1 ± 0.4	3.0 ± 0.3	3.1 ± 0.5	0.698
C3 (g/L)	0.83 ± 0.24	0.65 ± 0.19	0.90 ± 0.22	**<0.001**
C4 (g/L)	0.15 ± 0.07	0.13 ± 0.02	0.15 ± 0.06	0.313

**CNS involvement vs. No CNS involvement. P-values in bold are statistically significant ($p < 0.05$).*

Interestingly baseline serum levels of C3 fraction, but not C4, were significantly lower in patients with neurologic involvement as compared with those without neurologic involvement (0.65 ± 0.19 vs. 0.90 ± 0.22 g/dL, $p < 0.001$; 0.13 ± 0.02 vs. 0.15 ± 0.06 g/dL, $p = 0.313$, for C3 and C4, respectively; **Table 2** and **Figure 2A**). Moreover, when stratified according to need of Eculizumab rescue therapy due to severe neurologic involvement, patients treated with this drug showed baseline C3 serum levels significantly lower than those who has not be treated (0.50 ± 0.08 vs. 0.82 ± 0.11 g/L, $p < 0.001$; **Figure 2B**). Of note the severity of neurologic involvement in patients with lower C3 levels was underlined by negative correlation between this parameter and the PNAS-HUS score (Pearson's correlation coefficient = 0.993, $r^2 = -0.9181$, $p < 0.001$).

A ROC curve analysis was performed to evaluate the predictive role of baseline serum C3 levels as risk factor for neurologic involvement in STEC-HUS patients. The analysis showed that a C3 serum level of 0.765 g/dL as the best cut-off value associated with the higher risk to develop CNS involvement in STEC-HUS patients with an 73.1% specificity and a 73.5% sensitivity (AUC = 0.804, CI 95% 0.678–0.930, $p < 0.001$; **Figure 3**).

To estimate the relative risk for central nervous system (CNS) involvement in STEC-HUS patients showing serum C3 level above or below the cut-off value (0.765 g/L), a binary logistic regression analysis was performed using onset of neurologic involvement as dependent variable, and patient's age, leukocyte count, C-reactive protein, hemoglobin, sodium, and C3 serum levels at baseline as covariates (**Table 3**).

Univariate analysis showed that the following covariates affected neurologic involvement onset in STEC-HUS: Leukocyte count (HR 2.719, 95%CI 1.478–5.000, $p < 0.001$),

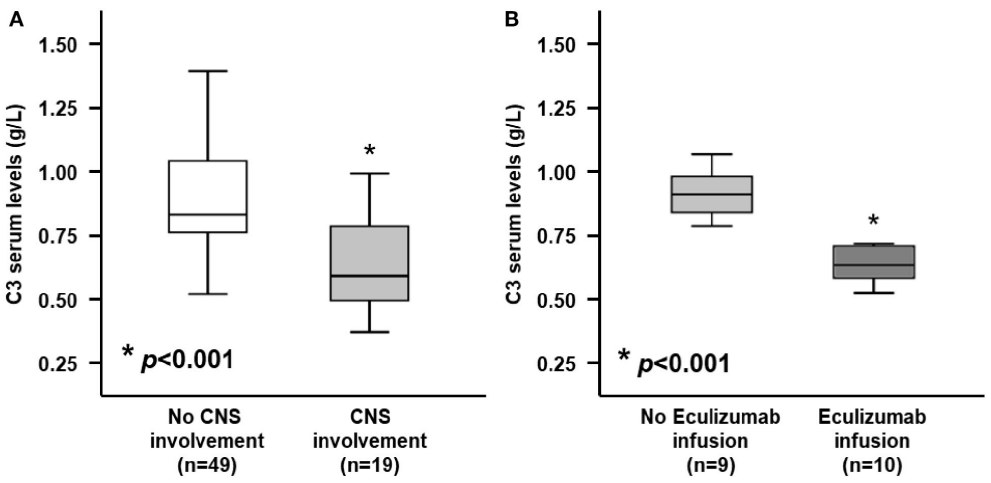

FIGURE 2 | Serum C3 levels at admission between STEC-HUS patients depending on central nervous system (CNS) involvement and Eculizumab infusion. **(A)** At hospital admission, patients who would develop neurologic involvement (light gray box), showed significantly lower serum C3 levels, as compared with those who would not develop neurologic involvement (white box) (0.65 ± 0.19 vs. 0.90 ± 0.22 g/L; $p < 0.001$). **(B)** Among patients who would develop neurologic involvement, those who needed rescue therapy with Eculizumab (dark gray box) showed significantly lower C3 levels, as compared with those who did not need it (light gray box) (0.50 ± 0.08 vs. 0.82 ± 0.11 g/L; $p < 0.001$). Mann-Whitney *U*-test for non-parametric data. Data in the graphs are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. *$p < 0.001$.

C-reactive protein (HR 1.686, 95%CI 1.011–2.811, $p = 0.045$), Hyponatremia (HR 2.227, 95% 1.276–3.888, $p = 0.005$) and C3 serum levels (HR 7.75, 95%CI 2.331–25.796, $p < 0.001$). As shown in **Table 3**, the results of the multivariate analysis confirmed a significant effect on neurologic involvement onset all of C3 levels, Leukocyte count and Hyponatremia at baseline, while the other were not significant (HR 6.401, 95%CI 1.617–25.334, $p = 0.008$ for C3; HR 2.355, 95%CI 1.136–4.882, $p = 0.021$ for Leukocyte count; HR 2.049, 95%CI 1.071–3.918, $p = 0.030$ for Hyponatremia).

Finally, to further evaluate the influence of complement activation on STEC-HUS patient's clinical conditions and outcomes, all patients were assigned to two groups according to the baseline serum levels of C3 above or below the cut-off value found in the whole study group (0.765 g/L). Interestingly patients with lower C3 serum levels required more frequently renal replacement therapy (74.1 vs. 46.3%, $p = 0.024$), anti-hypertensive therapy (51.8 vs. 21.9%, $p = 0.011$), Intensive Care Unit admission (22.2 vs. 2.4%, $p = 0.009$), and longer hospitalization (19.8 ± 8.1 vs. 14.8 ± 5.1 days, $p = 0.003$), as compared with patients with normal C3 serum levels (**Table 4**). Noteworthy, baseline serum levels of C3 in STEC-HUS patient negatively correlated with days of hospital stay (Pearson's correlation coefficient = 0.373, $r^2 = -0.1426$, $p = 0.002$).

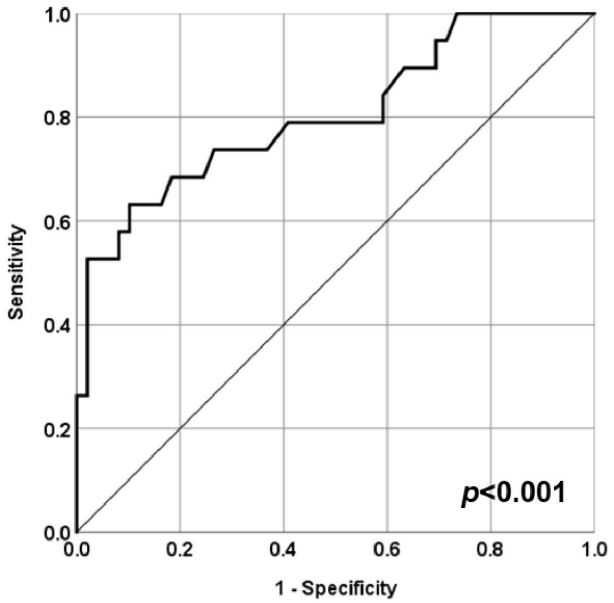

FIGURE 3 | ROC curve for serum C3 levels at admission and central nervous system (CNS) involvement in STEC-HUS patients. ROC curve analysis to evaluate the predictive role of baseline serum C3 levels as risk factor for neurologic involvement in STEC-HUS patients (AUC = 0.804, CI 95% 0.678–0.930, $p < 0.001$).

DISCUSSION

The results of the present study suggest that decreased serum C3 concentrations, assessed at hospital admission, were significantly associated with CNS involvement in STEC-HUS. The patients, who would develop neurologic involvement, at hospital admission showed significant alterations of specific laboratory parameters (higher leukocyte count, higher c-reactive protein, higher hemoglobin, lower sodium) which had been related to poor prognosis or more severe disease (38). Among them, serum levels of C3 were significantly lower in patients with

TABLE 4 | Clinical and laboratory characteristics of STEC-HUS patients with low vs. normal C3 serum levels at baseline.

Variables	Total ($n = 68$)	C3 <0.765 g/L ($n = 27$)	C3 >0.765 g/L ($n = 41$)	P*
RBC/PLT infusion (yes/no)	66/2	27/0	39/2	0.244
Plasma-infusion (yes/no)	59/9	25/2	34/7	0.250
Plasma-exchange (yes/no)	18/50	9/18	9/32	0.298
Renal replacement therapy (yes/no)	39/29	20/7	19/22	**0.024**
Anti-hypertensive drugs (yes/no)	23/45	14/13	9/32	**0.011**
Intensive Care Unit admission (yes/no)	7/61	6/21	1/40	**0.009**
Days of Hospital stay (*n*)	16.8 ± 6.8	19.8 ± 8.1	14.8 ± 5.1	**0.003**

P-values in bold are statistically significant (p < 0.05).

TABLE 3 | Univariate and multivariate binary logistic regression for central nervous system (CNS) involvement in STEC-HUS patients.

Variables	Category	Univariate analysis				Multivariate analysis			
		HR	CI 95%		P	HR	CI 95%		P
			Lower	Higher			Lower	Higher	
Age	Quartiles	0.864	0.536	1.009	0.546	NA	NA	NA	NA
Leukocyte count	Quartiles	2.719	1.478	5.000	**0.001**	2.355	1.136	4.882	**0.021**
C-reactive protein	Quartiles	1.686	1.011	2.811	**0.045**	1.108	0.536	2.293	0.782
Hemoglobin	Quartiles	1.388	0.852	2.263	0.188	NA	NA	NA	NA
Hyponatremia	Quartiles	2.227	1.276	3.888	**0.005**	2.049	1.071	3.918	**0.030**
C3 serum levels	<0.765 vs. >0.765 g/L	7.75	2.331	25.796	**0.001**	6.401	1.617	25.334	**0.008**

CI, confidence interval; HR, hazard ratio. P-values in bold are statistically significant (p < 0.05).

CNS involvement, as compared with those without neurologic signs. More interestingly, patients with neurologic involvement, who required rescue therapy with Eculizumab (an anti-C5-convertase monoclonal antibody), showed even lower levels of C3, as compared with patients with mild neurologic impairment which were not treated with this drug.

Despite the role of complement in STEC-HUS physiopathology had been widely analyzed in recent years, clinical data supporting a key role of complement activation affecting the course of illness are still limited. Moreover, the conclusions of many pediatric studies linking complement activation and disease evolution are conflicting. While some studies showed a relationship between increased activity of complement alternative pathway and poor prognosis, other reports denied such association or just observed trends toward more severe disease, which did not reach statistically significant differences probably due to the small sample size analyzed (20–26).

In our study, low levels of C3 seemed to be significantly related not only with CNS involvement *per se*, but also with severe forms of neurologic involvement. To confirm this potential predictive role, lower baseline C3 serum levels were inversely correlated with the severity of neurological observation, as assessed by PNAS-HUS score (27).

Noteworthy, low C3 was an independent risk factor for neurologic involvement in our patients' population even as entered as covariate in a multivariate logistic regression analysis including other major variables previously proposed as possible predictors of poor prognosis in STEC-HUS (for instance, leukocyte count, c-reactive protein, sodium levels).

In this model, only leukocytosis and hyponatremia also resulted independently correlated with higher risk of CNS manifestations in these patients. Previous studies had shown that leukocytosis was associated with a higher risk of poor prognosis (39, 40) and hyponatremia was even identified as a predictor of death (41). Probably our findings underline that clinical neurologic outcome depends not only by the direct effects of Stx but also by secondary effects due to activation of the complement system (9, 23) and by neuronal cells dehydration due to hyponatremia (41, 42).

In univariate analysis, patients with higher hemoglobin concentration and C-reactive protein were at higher risk of neurologic involvement. These parameters are, respectively, an acute phase protein and a surrogate marker of dehydration and have been repeatedly associated with adverse outcomes in STEC-HUS patients (43–45). In our study, however, these laboratory markers did not significantly affect the development of CNS involvement in the multivariate model, probably due to the key role of alternative pathway activation in the onset of thrombotic microangiopathy onto brain microvascular endothelial cells (46, 47).

An intriguing issue to be addressed includes the different decrease in complement factors C3 and C4 serum levels. In our study baseline serum levels of C3 fraction, but not C4, were significantly lower in patients with neurologic involvement. Although decreased C4 levels have been occasionally reported in some patients (23, 48), its significance remains unclear probably due to the lack of evidence in the activation of classical and/or lectin pathways in STEC-HUS (9, 14, 22, 49).

To underline the possible role of complement C3 levels in the worsening of STEC-HUS patient's clinical conditions and outcomes, all patients were divided into two groups according to the baseline serum levels of C3 above or below the cut-off (0.765 g/L) and the main data of clinical and therapeutic management were evaluated. Interestingly patients with lower C3 serum levels required more frequently renal replacement therapy, anti-hypertensive therapy, Intensive Care Unit admission and longer hospitalization, thus displaying significantly more severe disease phenotypes as compared with those with normal C3 serum levels. These observations are in line with multiple previous reports which underline the key role of alternative pathway activation in the onset of organ damage during STEC-HUS (14, 50).

Taken together, these results suggest the potential role of low C3 serum level as reliable marker of both neurologic involvement onset and diseases severity in STEC-HUS patients.

Although this study encompasses a larger series of children with microbiologically diagnosed STEC-HUS and analyzes the association between C3 concentrations and disease outcomes, some limitations deserve consideration.

The monocentric retrospective analysis and the rather limited number of cases might limit our observations. However, further prospective multicenter studies are warranted to confirm our findings. Moreover, we measured C3 level, but not complement pathway fragments (i.e., soluble C5b9). Furthermore, C3 titration during the follow-up was not performed. However, our data show that C3 determination is a readily available assay in most laboratories and able to predict neurologic involvement onset and diseases severity in STEC-HUS patients.

CONCLUSIONS

Our data suggests that children with STEC-HUS with decreased C3 concentrations at admission are more likely to develop neurologic involvement and are at increased risk of having severe clinical complications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was obtained from the Institutional Review Board of the University Hospital Policlinico Consorziale of Bari (Italy) (prot. 1624/2018). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GN and LS conceived and designed the study, analyzed the data, and drafted the manuscript. LP, GP, DT, VC, and PG collected the clinical data and helped to interpret the results. FS and GC analyzed the data, interpreted results, and prepared the figures. GS, LG, MC, and ER helped to draft the manuscript. MG edited and revised manuscript and approved the final version of manuscript. All authors contributed to the article and approved the submitted version.

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T Cells and Acute Kidney Injury: A Two-Way Relationship

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Acute Kidney Injury (AKI) complicates up to 10% of hospital admissions substantially increasing patient morbidity and mortality. Experimental evidence supports that AKI initiation and maintenance results from immune-mediated damage. Exogenous injury sources directly damage renal cells which produce pro-inflammatory mediators recruiting immune cells and furthering kidney injury. Many AKI studies focus on activation of innate immunity; major components include complement pathways, neutrophils, and monocytes. Recently, growing evidence emphasizes T lymphocytes role in affecting AKI pathogenesis and magnitude. In particular, T helper 17 lymphocytes enhance tissue injury by recruiting neutrophils and other inflammatory cells, while regulatory T cells conversely reduce renal injury and facilitate repair. Intriguingly, evidence supports local parenchymal-T cell interactions as essential to producing T cell phenotypic changes affecting long-term kidney and patient survival. Herein, we review T cells effects on AKI and patient outcomes and discuss related new therapeutic approaches to improve outcomes of affected individuals.

Keywords: AKI, IRI, regulatory T cell, Treg, TH1, TH2, TH17

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Acute kidney injury (AKI) is clinically defined by rapid renal function decline indicated by serum creatine rise $\geq$0.3 mg/dl (or >50% from baseline) and/or urine output $\leq$500 ml/day (1). It is classified as pre-renal, post-renal or parenchymal (also known as intrinsic) depending on the primary site of injury. Pre-renal and post-renal AKI are consequences of altered renal perfusion or urinary tract obstruction, respectively; thus, they represent extrinsic disorders. However, if pre/post-renal injuries persist, AKI will eventually evolve to cellular damage and intrinsic kidney disease. Pathophysiologically, AKI represents complex interactions of exogenous injury and host responses culminating in decreased glomerular filtration.

In the last decade, new approaches focused on more specific nomenclature across types of parenchymal AKI (2). Indeed, while pre- and post-renal AKI are frequently reversible and minimally impactful on patient survival (3), parenchymal AKI is an emerging global health concern, increases patient morbidity/mortality risk, and rose in incidence over the last 30 years (4). In industrialized countries, AKI affects 5–10% of hospitalized patients and 25–50% of those in intensive care units (ICU) (4, 5). A 2013 meta-analysis estimated that mortality rates for hospital-acquired AKI is ∼23% and rises to 50% in subsets requiring dialysis (5). Similarly, a large registry study on >190,000 patients reported 90-day AKI mortality rates of 37% (vs. 7% in non-AKI group). In the same cohort 2 years post-discharge, AKI survivors' combined risk of death, end stage renal disease (ESRD) or chronic kidney disease (CKD) was >30%, more than double of the cohort without AKI (6, 7).

Taken together, clinical data and experimental animal AKI models, concur that AKI associates or contributes to lung, heart, liver, brain, or gut damage (8) that produces long-term sequelae in

affected organs (9). Importantly, immune system function is tightly linked to AKI with bidirectional influence. While sepsis is a recognized leading cause of hospital-associated kidney injury (4), AKI also associates with increased infection risk even after full recovery of renal function (10, 11). The first studies about immune cell activation during AKI focus on innate immune response; more recently research shows adaptive immunity activation during AKI contributing to renal and extra-renal outcomes. Herein, we will review both adaptive immune contributions to AKI and immune function changes related to AKI.

ETIOLOGIES OF HOSPITAL RELATED ACUTE KIDNEY INJURY

AKI encompasses a broad spectrum of renal insults causing decreased filtration. In the last decade, multiple classifications were proposed to identify and study underlying conditions (2). From an epidemiological point of view, an important difference exists between community acquired vs. hospital related AKI. Community-AKI is more likely pre-renal and usually occurs in older or medically compromised patients from dehydration or from drugs that limit glomerular perfusion (e.g., non-steroidal anti-inflammatory drugs or inhibitors of the renin angiotensin aldosterone axis) (3). Conversely, hospital-acquired AKI is more often intrinsic and more likely to be severe. Another classification identifies major clinical syndromes and procedures that have a strong causative link with AKI (e.g., sepsis related-AKI, post-cardiac surgery AKI etc.); the definition of these clinical settings may guide clinicians in the diagnostic and therapeutic approach. From an etiologic point of view, these AKI types share a large part of the underlying mechanisms (2) (**Figure 1**).

Sepsis is a leading cause of in-hospital AKI accounting for 30–50% of cases (4). During sepsis, microbial and released host products act as alarm signals (or alarmins) targeting pattern recognition receptors (PRR) (12). Renal endothelium, tubular epithelial cells (TEC) and immune cells express PRR that sense a wide variety injury related molecular motifs. PRR activation produces pro-inflammatory phenotypes in renal cells which also activate programmed cell death pathways. Immune cells migrate to the site of alarmin release and contribute to local inflammation. In addition to infection consequences, patient courses are further complicated by nephrotoxic drugs (e.g., aminoglycosides) and sepsis-related ischemic injury, discussed in more depth below.

Ischemia-reperfusion injury (IRI) is probably the most studied experimental AKI model. Clinically, it occurs from severe renal hypoperfusion caused by blood loss, septic shock, and other anatomical abnormalities of renal blood supply. Some authors classify post-surgical AKI as a distinct entity due to predictable complicating factors of alarmin release (as consequence of bacterial contamination or tissue damage) or nephrotoxic drug administration (e.g., contrast dye) (13). In animal models, protracted IRI induces acute tubular necrosis (ATN), a lesion characterized by the extensive necrosis of the proximal tubular segments at the corticomedullary junction. However, renal biopsies from patients with ischemic AKI show mild parenchymal damage, despite severe organ dysfunction; thus, since 2000, more studies hypothesize microvascular failure and forms of cellular dysfunction (e.g., loss of polarity, epithelial leaking, organelle injury etc.) being predominant features in humans (14).

Multiple interacting etiologies contribute to cancer-related AKI. Oncologic patients suffer AKI from combinations of tumor lysis syndrome, infections, procedural complications, neoplastic renal invasion, paraproteinemia (mostly related to plasma-cell cancers) and drug toxicity. Traditionally, alkylating agents most frequently caused ATN (e.g., platin compounds). In the last decade new agents, most prominently immune checkpoint blockade inhibitors, are increasingly used. Multiple studies showed how immune checkpoint inhibitors can trigger intra-renal inflammation and autoimmune renal damage (15).

AKI frequently occurs in hospitalized patients but is rarely the cause for admission and frequently occurs from distant organ injury/dysfunction. In hepatorenal syndrome, for example, vasoactive aminic metabolites accumulate in liver failure and produce renal circulatory failure (8). Subtypes of acute cardiorenal syndromes involve renal injury resulting from decreased cardiac output or venous congestion, while others involve cytokine release and neurohormonal changes. Other relevant detrimental cross-talk are mediated by lung (hypoxia, cytokine release), brain (natriuretic peptides, cytokines), or intestine (leaking of bacteria and toxic metabolites) (12). Finally, massive muscle cell lysis seen in crush injuries cause injury. Cell lysis byproducts produce electrolyte unbalance, alarmin release, increased circulating waste products, and myoglobin precipitation in tubular lumen (cast nephropathy) that can combine with dehydration and profoundly injury kidneys (16).

ROLE OF THE INNATE INFLAMMATORY RESPONSE IN AKI PATHOGENESIS

Innate immune cells perpetrate AKI damage directly (e.g., neutrophil degranulation) or by recruiting the adaptive immune cells. At baseline, renal tissue hosts mainly macrophages and dendritic cells (DC), while immature monocytes and neutrophils migrate to the kidney in response to the alarm signal.

Neutrophils

Neutrophils are the most abundant leucocytes infiltrating the kidney immediately after IRI (17) and multiple studies have shown that in-hospital risk of AKI positively correlates with the percentage of circulating neutrophils, suggesting an involvement of these cells in AKI pathogenesis (18). In response to stress, tubular cells acutely release IL-18 which recruits and activates neutrophils (19). Activated renal endothelial cells express E-selectins that bind neutrophil β-integrins and initiate the diapedesis process (20). Neutrophils damage renal tissue by degranulation, IFN-γ release and by recruiting of NK T-cells (21).

Macrophages

Macrophages are the most abundant kidney resident immune cells. During AKI, TEC acutely release IL-34 that activates

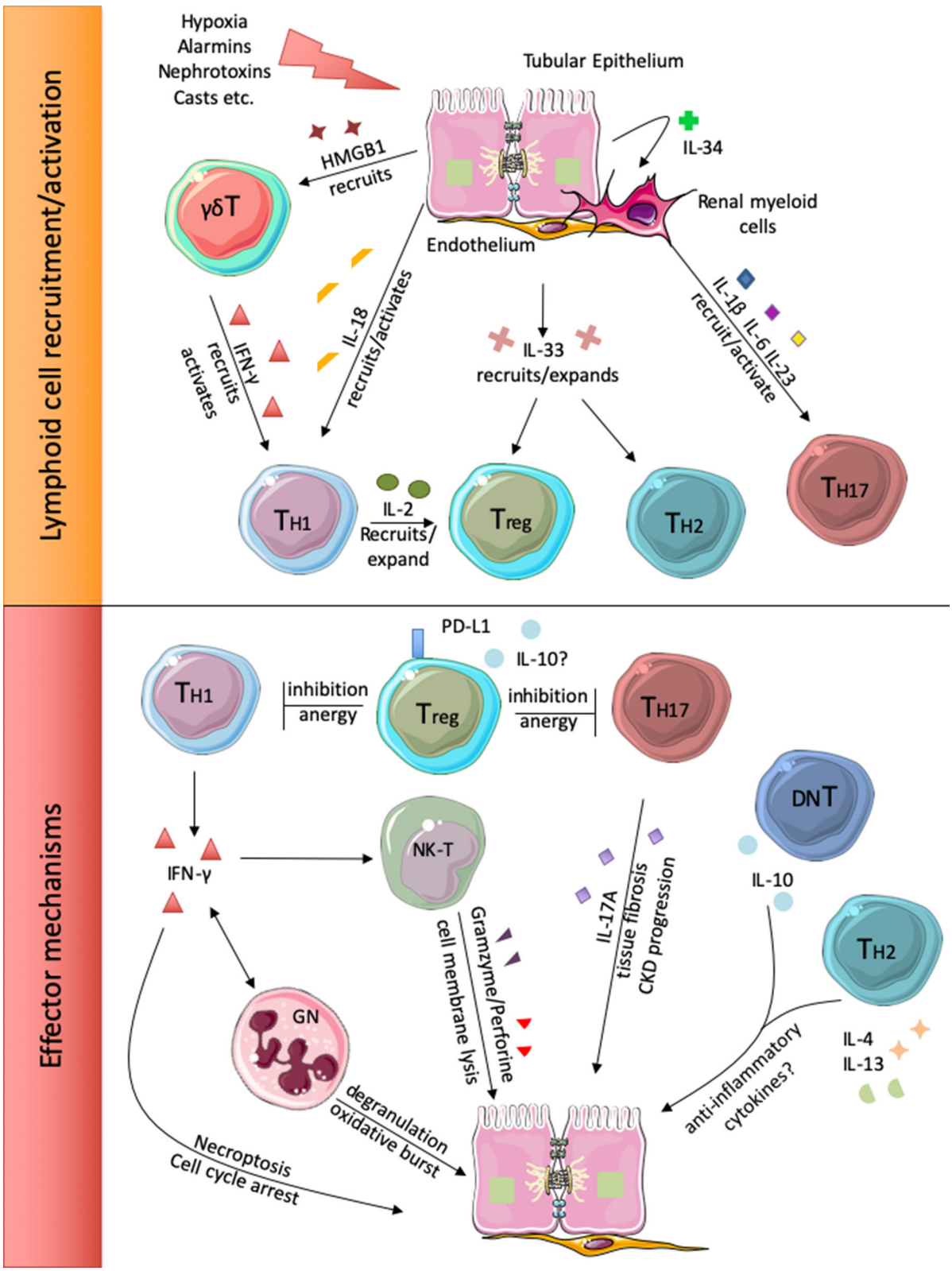

FIGURE 1 | Injured renal cells release different alarm signals that recruit and activate local and circulating lymphoid cells (upper panel). Subsequently, the different lymphocyte subsets participate to renal injury perpetration or inhibition (lower panel).

resident macrophages and promotes tubular injury (22). TEC damage induces also the recruitment and the activation of circulating monocytes (23), which perpetrate the injury by releasing pro-inflammatory cytokines as IL-1β, TNF-α, IL-6, and IL-12 (24). Subsequently, both resident and infiltrating macrophages switch to the regulatory phenotype and promote tissue repair. In particular, resident macrophages develop an embryonic-like gene signature soon after IRI and secrete WNT4, which in turn activates β-catenin pathway in TEC and promotes their proliferation (17, 23).

Dendritic Cells and NK Cells

Resident renal dendritic cells (DC) are the predominant source of TNF-α during AKI and promote T_{H1} activation by secreting IL-12p40 (25, 26); moreover, they release T_{H17} inducing cytokines as IL-1 and IL-23 (27).

Natural killer (NK) cells migrate to the kidney in inflammatory conditions and induce TEC damage by releasing perforin (28). A subset of NK expresses an invariant form of T cell receptor (NKT or invariant NK) and substantially contributes to interferon production after renal IRI (21).

Complement System

Complement cascade is acutely activated during AKI and contributes to renal damage, as indicated by data from C3 knock-out mice that are protected from IRI (29). Human and murine data have shown that complement get activated during AKI trough the alternative (30) and the mannose binding leptin pathways (31). Additionally, C3 can be activated in kidney parenchyma after binding ammonia (amidic-C3) (32). Complement activation injuries perpetrates renal injury trough the generation of membrane attack complex, the recruitment of immune cells and the activation of C3b and C5b receptors on tubular and endothelial cells (33).

ADAPTIVE IMMUNE RESPONSE

Most studies investigating the role of T cells as AKI mediators focus on CD4 (i.e., T helper cells) while CD8 (cytotoxic) involvement is controversial (see below). Depending on inflammatory context, naïve CD4 cells differentiate to T-helper (T_H) 1, 2, 17, or regulatory T cells (T_{reg}). After AKI, TEC primarily release T_{H1} inducing signals and renal myeloid cells polarize toward T_{H17}; T_{H2} activation during AKI is less understood.

T_{H1} CELLS

T_{H1} responses are orchestrated by master regulator transcription factor T-bet and characterized by expression of surface marker CXCR3. T_{H1} are classically associated with IFN-γ secretion and responses to intracellular pathogens. IFN-γ is a cytokine with a ubiquitous receptor promoting MHC expression, autophagy, reducing cell proliferation, and activating inflammatory death pathways (e.g., necroptosis and pyroptosis) (34). T_{H1} differentiation and activity are promoted by IL-12, IL-18, and IFN-γ itself. Upon injury, TEC release IL-18, leading to conversion of naïve CD4 T cells into T_{H1} (35). Rapid intrarenal migration of IFN-γ+ CD4 cells after LPS injection is prevented in IL-18 receptor deficient mice that, in turn, limits kidney injury (36). Cytokines alone cause cell injury, but T_{H1} cells also recruit other immune cells. Li et al. reported T_{H1} rapidly travel to ischemic kidneys and promote neutrophil and NK chemotaxis (that peaks after 3 h) (21). Additionally, IFN-γ alters TEC making them apro-inflammatory via expression of costimulatory molecules ICOS-L and B7-1 (37), preventing TEC proliferation (38), and promoting their death by necroptosis (39).

Although T cells promote inflammatory transformation of TEC, cytokine dependent damage appears predominantly based on T_{H1} responses. Day et al. observed that infiltrating lymphocytes were the main source of IFN-γ, while the cytokine fraction released by TEC was dispensable (40) in experimental models. Human data is corroboratory: in a multicenter prospective study on 1,400 patients undergoing cardiac surgery, post-operatory increase in serum IFN-γ significantly and directly associated with AKI incidence and 1-year mortality (41). Rather than non-specific responses to damage associated molecular patterns (DAMP) experimental evidence points to antigen-specific T cell function. Renal injury was worse after IRI in T-cell depleted mice reconstituted with heterogeneous CD4 cells compared to those given monoclonal ones (42).

T_{H2} CELLS

Little is known regarding T helper 2 (T_{H2}) cells during AKI. T_{H2} constitutively express transcription factor GATA3 and surface marker Crth2 (CCR4 in mice): canonically, these cells orchestrate the anti-parasitic immune response via IL-4 and IL-13 secretion and are associated with asthma and allergic diseases. Yokota and colleagues induced IRI in mice lacking the transcription factors STAT4 or 6; the first is essential for T_{H1} response while STAT6 induces T_{H2} phenotype (43). While STAT4 deficient mice were protected from AKI, STAT6 knock down associated with worse outcomes. Increased tubular injury in IL-4 deficient mice further supports a renoprotective designation for T_{H2} responses (43). Conversely, human data from post-cardiac surgery patients showed direct relationships between IL-4 and IL-13 serum concentrations, AKI incidence, and 1-year mortality (41). Clearly, further experimental and clinical evidence are required to understand how T_{H2} responses affect AKI.

T_{H17} CELLS

T helper 17 cells (T_{H17}) are a subset of CD4 lymphocytes characterized by expression of IL-17A, IL-17F, and the transcription factor ROR-γt. T_{H17} are frequently identified by surface marker CCR6, with variable expression of IL-23R, CCR4, and CCR2 (humans) or CCR7 (mice) (27). In both humans and mice, naïve CD4 experimental polarization is driven by TGF-β, IL-6, IL-21, and IL-23. Other stimuli contribute to mature T_{H17} activation: angiotensin II, salt excess and IL-1β (27, 44). T_{H17} cells are particularly abundant in barrier epithelia (e.g., skin, gut) and respond primarily to fungal and extracellular

bacterial infections by recruiting neutrophils and activating epithelium via IL-17. T_{H17} are linked to various autoimmune diseases; the prototypical T_{H17} disease is psoriasis and it is effectively treated with anti-IL-17 therapies (45).

More recently, T_{H17} cells emerged as main players in AKI pathophysiology. Different groups demonstrated that T_{H17} are the most abundant kidney infiltrating lymphocytes infiltrating following AKI in mice (27, 44). Pindjakova et al. observed that resident dendritic cells and TEC release IL-1 (α and β), IL-23, and IL-6 to promote intrarenal IL-17 migration and activation after AKI from ureteral obstruction. IL-1 signaling dominates the phenomenon and its suppression pushes lymphocytes phenotypes to T_{H1} (27). Mehrota and colleagues demonstrated that T_{H17} cells expressing calcium channel Orai1 are solely responsible for IL-17 production after IRI and, ultimately, for renal injury (46). A 10-fold increase in circulating Orai1$^+$ T_{H17} cells are found in ICU patients with AKI compared to those without (46). *In vivo*, intra-renal expression of Orai1 persisted for days after AKI resolution and its inhibition prevented the transition to chronic kidney disease (CKD) (46). Intriguingly, the kidney also possesses mechanisms to counteract T_{H17} cell activation. Our group observed that erythropoietin (EPO), a kidney produced hormone, prevents T_{H17} induction (47) and ameliorates renal injury in a murine model of Balkan nephropathy. Together with studies demonstrating EPO prevents IRI, current experimental evidence strongly support inhibition of dominant T_{H17} responses are feasible to prevent AKI and related progression to CKD.

T REGULATORY CELLS

CD4 regulatory cells (Treg) are immunosuppressive T cells characterized phenotypically by constitutively high levels of IL-2 receptor (CD25) expression and maintained functionally via transcription factor FOXP3.

Research suggests that Treg attenuate AKI (48). Jaworska and colleagues observed IRI amelioration after Treg transfer, an effect that was dependent on programmed death ligand 1 and 2 (PD-L1/2) expression by Treg. The relevance of PDL to renal inflammation is supported by experimental demonstration of PD-1 expression by tubular cells (49) and clinical observations of renal adverse events in patients treated with immune checkpoint inhibitors targeting PD-1/PD-L1 axis (50).

After injury, renal Treg inhibit inflammation in multiple ways; they release TGFβ and IL-10, halt production of IL-1β, TNF-α, and IFN-γ, and reduce overall CD4 proliferation (51). It is unclear which between intra-renal or circulating Treg represent the active pool during AKI, though evidence exists supporting both central and peripheral sources. Investigators observed that DC heat shock protein 70 (HSP70) production increased splenic Tregs that migrated to the kidney and attenuated IRI (52), while another recent paper pointed out the role of renal resident Treg during IRI (53). The authors observed a progressive increase in CD3$^+$ T cells after ischemia that paralleled the development of tissue fibrosis. Among the most expanded subsets there was a resident Treg population that was characterized by the expression of IL-33 receptor, a marker usually associated with T_{H2} phenotype. The administration of IL2 and IL33 at the time of IRI activated this population, promoted rapid recovery, and prevented tissue fibrosis. Of note, a previous study demonstrated that IL-33 is released by renal endothelium after cisplatin administration (54). Given these conflicting results, the answer may be model dependent.

OTHER T CELLS (GAMMA-DELTA, DOUBLE NEGATIVE, CD8)

Nomenclature of T cells is receptor based: αβ T cells constitute the majority (i.e., T_{H1}, T_2, T_{H17}, and Treg subsets) while γδ T cells, resident in skin and the gut epithelia, constitute <1% of peripheral T cells. Their effector responses, based on still undefined antigens, include release of IL-17, IFN-γ, and TNF-α. In murine IRI, γδ lymphocytes rapidly infiltrate kidney tissue and promote subsequent migration of αβ cells; of note, γδ depletion delays but does not prevent injury, while αβ T cell ablation is protective (55). This suggests γδ T cells affect kinetics of kidney injury but are not necessary for AKI. In a clinical study of 20 patients undergoing abdominal aortic repair, magnitude of γδ T cells disappearance from the circulation was proportional to kidney injury markers (56). The same pattern was seen experimentally in mice where TEC HMGB1 release induced γδ T cell kidney migration, supporting the paradigm that early γδ T cell AKI responses facilitate αβ T cell recruitment.

CD8 T cells role in AKI is less defined, if it exists. A 2001 study found no significant pathogenic role for CD8 cells in IRI (57). A subsequent paper reported that CD8 deficient mice were mildly protected from cisplatin induced AKI, but less than those with CD4 depletion (58). Finally, in a study on acute aristolochic acid nephropathy, authors reported both CD4 and CD8 depletions were *detrimental*. In particular, absence of CD8 cells was associated to higher intra-renal TNF-α production and reduction of anti-inflammatory macrophages (59). More work is required to more clearly define how, and if, CD8 cells affect AKI.

Double negative (DN) T cells represent an early stage of T cell maturation lacking CD4 and CD8 expression. DN T cells are ubiquitous and some authors hypothesize they are an independent differentiated population rather than a maturation stage (60). These lymphocytes constitute more than 30% of kidney T cells at baseline and rapidly proliferate after tubular damage. Martina et al. reported that DN cells secrete IL-10 after IRI thus being anti-inflammatory (61) and, ostensibly, protective.

B CELLS

Experimental B-cells work has not definitively defined their role in AKI. One IRI study with B-cell deficient mice showed decreased AKI. Serum from control mice having restored AKI; these results suggests an antibody-mediated mechanism (62). Renner et al. observed the opposite effect; B-cell deficient mice had less intra-renal IL-10 production and a worse renal outcome (63). Of note, the same study reported a harmful B-cell subset; natural-IgM from peritoneal lymphocytes precipitated on

the glomerular basal membrane and activated the complement alternative pathway. Conversely, Lobo and Okusa reported that infusion of natural-IgM was actually protective from IRI by inducing B-regulatory cells (64). These conflicting studies are emblematic of ongoing conflict regarding B cells and AKI.

Larger consensus has been reached about the B-cell role in post-AKI renal fibrosis (65). It has been shown that fibroblasts increase their collagen production in tissues with a higher B cell signature (66). Consistently, B-cell depleting therapy (anti-CD20) prevented kidney interstitial fibrosis after ureteral obstruction (67).

IMMUNE THERAPIES IN AKI

The important effects adaptive immune responses have in AKI pathogenesis suggest, that immune modulatory therapies might effectively achieve clinically desirable results. Pechman and coworkers observed that mycophenolate mofetil (an immunosuppressive agent that inhibits purine synthesis in lymphocytes) prevented AKI long-term sequalae as renal fibrosis and salt-sensitive hypertension (68). Experimental models further link T cells, MMF, and CKD; a murine study modeling AKI transitions to CKD, showed an inverse association between mycophenolate treatment vs. T_{H17} proliferation and CKD (69). Clinically relevant approaches preventing T_{H17} responses (i.e., EPO receptor agonism and Orai1 inhibition) effectively halted kidney disease in murine models. Moreover, targeting T_{H17} effector molecule, IL-17, prevented calcineurin inhibitor related renal fibrosis (70). Taken together, these findings justify further studies targeting T_{H17} responses to prevent CKD.

A promising approach to treat AKI is the promotion of endogenous Treg expansion. A known strategy promoting Treg expansion involves IL-2 function. The IL-2/anti-IL2 complex is a mixture of IL-2 with an antibody that prevents IL-2 binding to the β-chain of its receptor (CD25); this complex strongly induced Treg proliferation and attenuated IRI by reducing neutrophil and macrophage migration in renal tissue (71). Other authors generated a fusion IL-2/IL-33 cytokine that expanded intra-renal IL-33R$^+$ Treg, halted CD4 effector cell proliferation and prevented 100% of the observed mortality in a murine IRI model (72). Dimethylsphingosine (DMS) promotes CD4 migration to kidneys at baseline and after ischemia; Lai et al. demonstrated that FOXP3+ lymphocytes were proportionally more abundant in the renal tissue after DMS treatment and prevented IRI in mice (73).

Treg adoptive transfer effectively downregulates IRI and other types of renal injury in pre-clinical models (48); however, its clinical application remains challenging. Therapeutic cell products need specific cell factories, are temperature and time-sensitive (thus needing complicated stock and transport procedures), are prone to contamination, may degenerate in neoplastic disease and can trigger the host immune response toward allo-antigens (73).

Stem cell therapies have been proposed for the management of AKI; in particular, mesenchymal stromal cells (MSC) have been successfully used in different preclinical models and are currently under investigation in clinical trials (74). MSC are immune-regulatory; importantly, MSC infusion expands intra-renal Treg after IRI. Consequent reductions in circulating IL-6, TNF-α and IFN-γ levels are Treg dependent (75). Treg strategies, therefore, intersect with other established experimental protocols.

However, cell infusion poses previously mentioned challenges, even if MSC are relatively easy to expand and not immunogenic. An interesting alternative comes from the observation that MSC conditioned medium is as effective as MSC infusion in promoting tissue regeneration (76). Indeed, MSC beneficial effects in AKI are not contact-mediated and MSC do not differentiate in any mature kidney cell. If future efforts identify substances inducing intra-renal Treg expansion therapies would avoid cell infusion complications.

CONCLUSIONS

Inflammation produces AKI via reciprocal interactions between renal parenchyma, resident immune cells, and recruited immune cells. Increasing recent evidence indicates a dominant role of the adaptive immune response, and T cells in particular, as prominent pathogenic elements as well as mitigating factors. Myriad AKI etiologies frequently condense into recurrent identifiable immune patterns associated with tubular injury and T cells. In particular, CD4 and γδ T cells are initial immune effectors migrating to kidneys and orchestrating activation of innate cells. Early injury phases are characterized by a strong IFN-γ response, possibly produced by T_{H1} cells. In later phases, T_{H17} perpetuate injury and tissue fibrosis. Conversely, Treg and possibly T_{H2} exert opposing anti-inflammatory roles and limit or prevent injury. Pre-clinical and observational studies provide strong bases of feasibility for future pharmacological interventions targeting lymphocyte function to prevent and limit AKI as well as subsequent renal fibrosis.

AUTHOR CONTRIBUTIONS

SD wrote the first draft. JL reviewed it. PC had the original idea and reviewed the manuscript. All authors approved the final manuscript.

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Mesenchymal Stromal Cell Uses for Acute Kidney Injury—Current Available Data and Future Perspectives: A Mini-Review

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There is growing evidence concerning the potential use of mesenchymal stromal cells (MSCs) for different tissue injuries. Initially, the intended physiological use of MSCs was due to their ability to differentiate and replace damaged cells. However, MSCs have multiple effects, including being able to significantly modulate immunological responses. MSCs are currently being tested for neurodegenerative diseases, graft vs. host disease, kidney injury, and other chronic unremitting tissue damage. Using MSCs in acute tissue damage is only now being studied. Acute kidney injury (AKI) is a common cause of morbidity and mortality. After the primary insult, overactivation of the immune system culminates in additional secondary potentially permanent kidney damage. MSCs have the potential to ameliorate the secondary damage, and recent studies have shed important light on their mechanisms of action. This article summarizes the basics of MSCs therapy, the newly discovered mechanisms of action, and their potential application in the setting of AKI.

Keywords: acute renal failure, AKI, mesenchymal stem cells, MSC, immune response

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INTRODUCTION

Acute kidney injury (AKI) is a syndrome of rapid renal function deterioration over a period of hours or days (1). AKI is a common cause of morbidity and mortality, complicating 20% of hospitalized patients, half of them needing renal replacement therapy (2). This severe form of AKI is related to a 50% increase in mortality among other devastating long-term consequences, including end-stage renal disease (ESRD), and dialysis dependence (2, 3).

The etiologies of AKI are varied and are usually divided into pre-, intra-, and post-renal categories (4). The most common category is prerenal, secondary to hypoperfusion of the kidney. Hypoperfusion of the kidney can be caused by reduced effective blood volume secondary to hemorrhage, cardiac insufficiency, and/or third spacing of fluids (4). Intrarenal AKI is caused by intrinsic injury to the renal tissue, with the most common injury being acute tubular necrosis secondary to ischemia–reperfusion injury (IRI) (5–7). Other intrinsic renal causes include glomerular pathologies such as glomerulonephritis and interstitial and vascular injury (4). Postrenal AKI occurs from obstruction of the ureters, bladder outlet, or urethra (4). Irrespective of the primary cause, an intrarenal inflammatory cascade is activated following the acute kidney injury, which if not quickly controlled, culminates in additional renal damage and irreversible renal fibrosis (detailed below).

In the last decade, cumulative evidence has shown the significant role that overactivated immune responses play in the development of AKI (8). This understanding paved the way to new therapeutic strategies for this relatively common and life-threatening acute kidney condition. Unfortunately, despite the progress in our understanding of AKI biology, treatment options for AKI in the daily clinical setting are still limited (1, 3, 5). While dialysis can be relatively effective in handling the hazardous electrolytes and volume complications as a supportive therapy, there is a need for a treatment that can counteract the pathological cascade that can culminate in irreversible loss of renal tissue (1, 5).

THE IMMUNE RESPONSE TO ACUTE KIDNEY INJURY

The immune system plays a crucial role in AKI mechanisms with involvement of both the innate and adaptive immune system branches (9). Regarding the innate immune system, cytokines serve as major mediators with both increased production of cytokines and reduced clearance being reported during AKI (10). Interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α are usually elevated and are related to endothelial dysfunction and tubular injury (11). Conversely, IL-10 has an ameliorating effect by promoting immune tolerance (12). In the AKI setting, growth factors also play a role by regulating inflammation and programmed cell death. When administrated early after the acute insult, growth factors such as the epidermal growth factor (EGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF) can promote renal repair and renal function restoration in animal models (13).

The complement system, a part of the innate immune system, also has an important role in the pathogenesis of renal injury and is involved in glomerular, tubulointerstitial, and vascular kidney injuries (14). The final common pathway of the complement system is the membrane attack complex that induces direct cellular damage and causes activation and migration of neutrophils, which further amplifies the injury (15). Suppressing the complement system in AKI has shown promising results in preclinical studies (16).

The cellular response to AKI includes both pro- and anti-inflammatory characteristics. Dendritic cells, monocytes/macrophages, neutrophils, T lymphocytes, and B lymphocytes are all involved in AKI and can be detected as early as 1 h after the acute insult (17). The involvement of these cells can directly and indirectly induce apoptosis of the renal tubular cells (17). Neutrophils recruited to the injured kidney cause vascular congestion that, together with the secreted cytotoxic compounds, including reactive oxygen species, further exacerbate tissue damage (11). M1 macrophages release chemokines, proinflammatory cytokines, and inducible nitric oxide synthase, which form peroxynitrites. These peroxynitrites have a vasoconstrictive effect, which can aggravate the ischemic and inflammatory damage (11). Lymphocytes enhance AKI by releasing IL-17, a proinflammatory cytokine that also increases vascular permeability (11, 17, 18). In contrast, M2 macrophages and regulatory T cells are essential for suppressing the overactivated inflammatory response and for regenerating damaged renal tissue and are detected while recovering from the acute insult (9).

The relation between the different arms of the immune system can either escalate or downgrade the final injury (11, 15). To steer the cells and factors toward a less devasting rout, new treatments are being investigated including the use of stem-cell therapy.

MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs) are fibroblast-like multipotent cells that can differentiate into mesodermal-line cells including adipocytes, chondroblasts, osteoblasts, and renal tubular cells (19, 20). These cells exhibit self-renewal properties, with a potential to replace damaged cells (21). MSCs are defined by three main characteristics: (1) plastic adherent when maintained in standard culture conditions; (2) expression of CD105, CD73, and CD90, with no expression of other CDs that are not mesenchyme related [including CD45, CD34, CD14, or CD11b, CD79-α, or CD19 and human leukocyte antigen (HLA)-DR surface molecules]; and (3) the ability to differentiate into a mature mesoderm related cell line *in vitro* (22). Unlike embryonic stem cells, MSCs are found in many organs even in adults (20, 22, 23).

In the past two decades, MSCs from different origins are being used in different clinical trial settings (24). For example, bone-marrow-derived MSCs are used in children to treat graft-vs.-host disease, autologous marrow MSCs for heart disease (23), and both bone-marrow and adipose-derived MSCs are used in Crohn's-related enterocutaneous fistular disease (25). In the neurodegenerative field, MSCs are being studied in amyotrophic lateral sclerosis, multiple system atrophy, Parkinson's disease, Alzheimer's disease, and multiple sclerosis. While animal studies have been promising, clinical studies have demonstrated conflicting results (26, 27). The encouraging results obtained in the field of degenerative diseases can be related, among others, to the effect that MSCs have on the immune factors in these disease settings (26, 27).

THE BIOLOGY OF MESENCHYMAL STROMAL CELLS

MSCs can affect and be affected by other cells through different immune mediators. Cytokines, chemokines, and transcription factors can influence the differentiation of MSCs. Expression in MSCs of specific transcription factors, including Runx2, Sox9, PPARγ, MyoD, GATA4, and GATA6, promote their differentiation into specific cell lineage (20).

The primary rationale for using MSCs to rejuvenate damaged tissue was initially related to their ability to differentiate into the damaged tissue-related cells. Following IRI, MSCs migrate to the injured site and alleviate the damage (21). Studies have demonstrated that MSCs have beneficial effects even at very early stages after their migration, before any differentiation and proliferation can be expected (28). This observation has led to

the understanding that the MSC's early beneficial effects are related to their paracrine activity in the surrounding tissue (29, 30). Recent studies have demonstrated that MSCs can induce both local and remote anti-inflammatory effects (31). The immunomodulatory effects of MSCs are broad and cover much of the innate and adaptive immune systems (19). For example, MSCs can secrete factors such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), angiopoietin 1, keratinocyte growth factor, and macrophage inflammatory protein 1α. These broad signaling factors are capable of promoting cell proliferation, angiogenesis, and wound healing (30). Paracrine or extracellular vesicle-delivered growth factors, such as hepatocyte growth factor (HGF) or VEGF, represent additional mechanisms by which MSCs exert therapeutic effects on renal injury (13).

MSCs can present both pro- and anti-inflammatory profiles. These different phenotypes are related to their ability to sense the environment and respond to changes in the tissue. The effect is induced by activation of different macrophage populations (19). Macrophages are divided to two main groups: M1 and M2 macrophages. M1 macrophages are considered proinflammatory cells and secrete proinflammatory cytokines including IL-1, IL-6, TNF-α, and interferon-γ. M2 macrophages are anti-inflammatory cells that secret anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β1 (19, 32, 33). Thus, MSCs can induce differentiation of monocytes to one of the macrophage phenotype groups according to the inflammatory status of the damaged tissue (19). MSCs can also affect T-cell activation and differentiation toward T-regulatory cells that have anti-inflammatory properties (34). In addition to the paracrine effects on the immune system, MSCs can transfer mitochondria into the damaged cells, enabling better energy utilization, and restoration of the adenosine triphosphate (ATP) supply, thus promoting cellular recuperation (34). MSCs might also assist in preserving tubular mitochondria, thus preserving the functionality of these cells (35). By improving oxygen metabolism and energy utilization, MSCs reduce the oxidative stress and induce antioxidant activity (36).

To conclude, MSCs can promote tissue regeneration even before differentiating into the damaged cell line of the injured tissue. This influence is related to their early multifaceted paracrine effects.

TREATMENT WITH MESENCHYMAL STROMAL CELLS IN ACUTE KIDNEY INJURY

In the setting of AKI, MSCs promote protective effects on the injured kidney and ameliorate tissue damage (34, 36). The beneficial effects of MSCs are noticeable early after their injection and can be attributed to the following paracrine related mechanisms (**Table 1** and **Figure 1**):

- An increase in the M2 macrophage CD68/CD163 population. As discussed, these M2 macrophages have anti-inflammatory and proregenerative properties (28, 32).
- A shift from the proinflammatory cytokines TNF-α, and IL-1β to the anti-inflammatory cytokine IL-10 with a favorable expression of the homing adhesion molecules intracellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (50).
- An inhibitory effect on the complement system's overactivation and the related cellular damage generated by the membrane attack complex (28).
- Release of proangiogenesis growth factors such as VEGF, and proliferative growth factors (IGF, EGF) that promote cellular repair and promote cell regeneration (13, 51).
- Exosomes—one of the most exciting discoveries in intercellular communication. Exosomes are membrane-bound extracellular vesicles that are produced by most eukaryotic cells. Their size is about 30–120 nm in diameter (around the size of lipoproteins) and contain various molecular constituents of their cell of origin, including proteins, mRNA, and miRNA or double-stranded DNA (52, 53). Recent studies have demonstrated that administration of MSCs-derived exosomes can ameliorate the expected renal damage in the setting of AKI (52).
- Epigenetic effects—a shift in gene expression. Xie et al. (54) demonstrated that overexpression of the Klotho gene, which regulates apoptosis, can reinforce the protective effect of MSCs in the setting of AKI. Chen et al. demonstrated that the protective effect of MSCs in the setting of AKI can be related to TNF-inducible gene 6 protein expression. This protein, in addition to its anti-inflammatory effect, can promote renal tubular epithelial cell proliferation (55).

While there is a growing body of knowledge in preclinical studies, the available clinical data on MSCs in AKI is still scarce. A

TABLE 1 | Immunomodulatory mechanisms of mesenchymal stromal cells in the setting of acute kidney injury.

Immune system component	Mechanism	References
Complement system	Amelioration of complement system activation	(28, 37)
Cytokines	Downregulation of proinflammatory cytokines: IL-1β, IL-6, IL-17, TNF-α, INF-γ, TGF-β	(28, 38–43)
	Upregulation of anti-inflammatory cytokines: IL-10, IL-4, bFGF, and TGF-α	(28, 38, 39, 43–45)
Macrophages	Proliferation and migration of the M2 macrophage population	(28, 43, 46)
	Inhibition of macrophage infiltration	(45)
T-cells	Inhibition of T-cell infiltration	(43, 45)
	Differentiation to T-cell regulatory cells	(40, 43, 47, 48)
Neutrophils	Inhibition of neutrophils infiltration	(43, 49)

IL, interleukin; TNF, tumor necrosis factor; INF, interferon; TGF, transforming growth factor; bFGF, basic fibroblast growth factor.

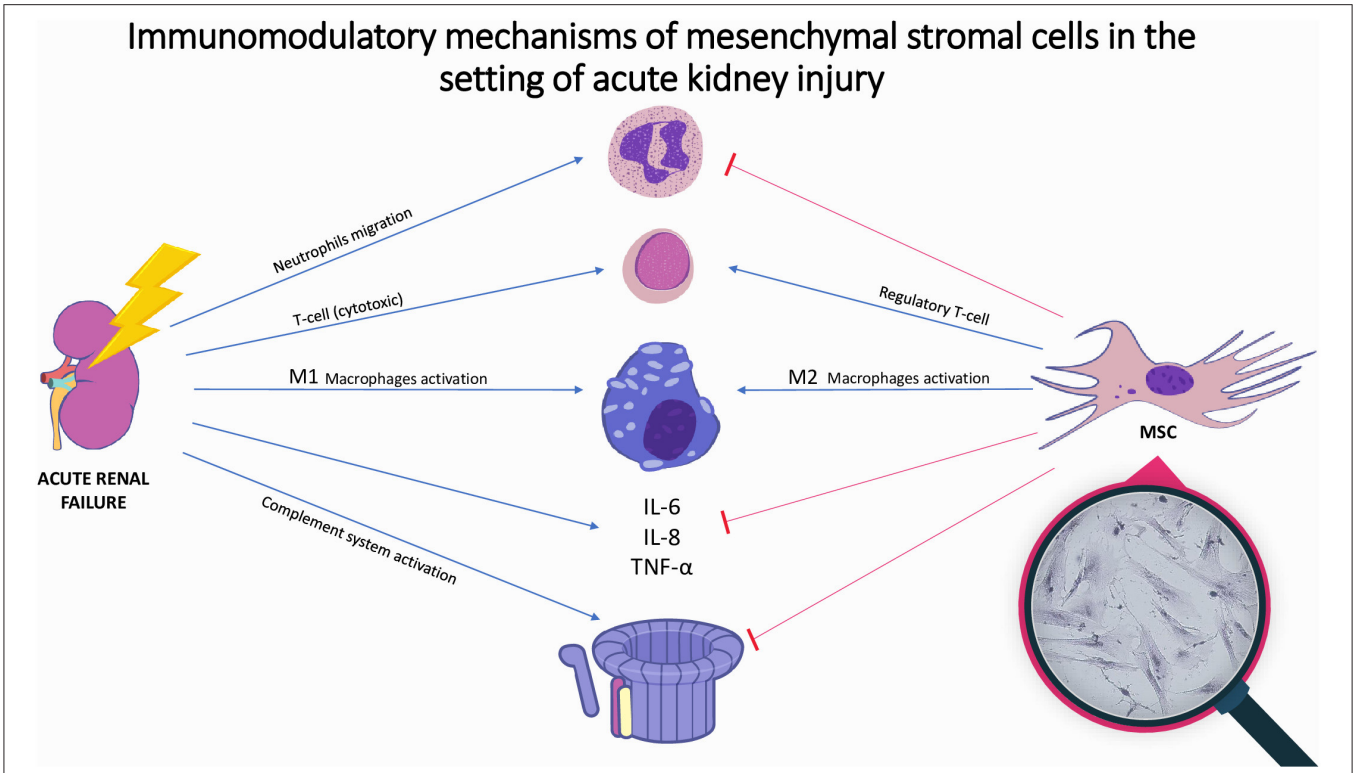

FIGURE 1 | Immunomodulatory mechanisms of mesenchymal stromal cells in the setting of acute kidney injury. Illustration of the immune mechanisms of acute kidney injury and the immunomodulatory effect of mesenchymal stromal cells. Acute kidney injury is accompanied by increase in inflammatory cytokines, complement activation and immune cell activation. Mesenchymal stromal cells inhibit cytokines release, complement system activation, and neutrophils migration, while promoting M2-anti-inflammatory macrophages and regulatory T-cells proliferation; In magnifying glass: microscopic picture of mesenchymal stromal cells-placenta origin; MSC, mesenchymal stromal cell; IL, Interleukin; TNF, tumor necrosis factor.

recent study using MSCs in postcardiac surgery patients did not show beneficial effects regarding postsurgery AKI (56). This unfortunate result can be attributed to time of MSCs administration. The optimal results are obtained when MSCs are administered closest to the initiation of IRI (50). The detection of AKI, based on commonly used blood markers in humans (serum creatinine and urea), is usually late, after AKI and tubular necrosis are well-established (7). In this scenario, when the damage is already well-established, the potential immunological benefits of MSCs are probably negligible. In addition, the MSCs themselves might be injured by an overactivated complement system (57).

In addition to AKI, there is growing evidence of MSC benefits in the setting of chronic kidney disease (CKD). Even though the clinical studies done so far included relatively small numbers of patients, the evidence looks promising regarding the ability of MSCs to prevent the expected kidney function deterioration over time (58–60). In patients suffering from chronic diabetic nephropathy, allogeneic transplantation of MSCs demonstrated renal function improvements compared to placebo (60). The effect can be attributed to the paracrine secretion of VEGF and IGF-1 needed for angiogensis and tissue regeneration, and to the anti-inflammatory effect that controls the overactivated immune response that accompanies most CKD before significant sclerosis develops (59, 61).

One of the relevant clinical settings where MSCs potentially have beneficial effects is in postrenal transplantation patients. In the immediate posttransplantation period, IRI is one of the main reasons for AKI (62). Thanks to the above-discussed immunomodulating effects of MSCs, there are promising results in preclinical trials, and clinical studies are currently ongoing (63).

CURRENT AVAILABLE MSC SAFETY DATA

Several safety concerns are related to the use of MSCs in clinical settings. The first concern is related to the administration technique. When the cells are administered intravenously (IV), most of the cells are found within the lungs (28, 64). When the lung capillaries are blocked with these cells, ventilation, and respiratory difficulties ensue. Therefore, higher dosage with high concentration of MSCs should be avoided. The second concern is related to exposing the immune system to foreign cells when administering donor cells. Luckily, MSCs do not stimulate an intense immune response, since they only express the HLA-DR but lack other HLA typings (29). In a CKD trial, none of the patients developed persistent donor-specific

anti-HLA antibodies (60). In particular, fetal MSCs have very low immunogenicity by nature and can be used to overcome this potential barrier (36). The last concern is related to the proliferation and differentiation of pluripotent cells injected to a living body, with their potential of being transformed into malignant cells. This concern is probably irrelevant, since stromal cells need a special environment and signaling factors to act as stem cells and differentiate, and usually do not survive after administration (20, 64). In any case, to address this scenario, more research with long-term follow-up is needed.

Even though clinical trials with long-term follow-up are still lacking, some preclinical trials have addressed the safety issues. Till now, no serious adverse effects were reported in either preclinical (65, 66) or clinical studies (21, 56, 60, 67).

SUMMARY AND FUTURE PERSPECTIVES

The ongoing cumulative data on the beneficial physiological effects of MSCs open new treatment opportunities for diseases that are currently being managed with only supportively therapy. While other types of stem cells, such as hematopoietic stem cells, are used in the clinical practice, the clinical data on MSCs is still scarce. In the setting of AKI, MSCs by way of their paracrine effects, can modulate the hazardous results of an overactivated inflammatory response. MSCs hold hope for future novel therapies, and a better understanding of the immune-biological effects of these cells will enable development of new treatment strategies.

AUTHOR CONTRIBUTIONS

SZ-I and SE conducted a review of articles and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Recent Advances in the Role of Natural Killer Cells in Acute Kidney Injury

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Growing evidence is revealing a central role for natural killer (NK) cells, cytotoxic cells belonging to the broad family of innate lymphoid cells (ILCs), in acute and chronic forms of renal disease. NK cell effector functions include both the recognition and elimination of virus-infected and tumor cells and the capability of sensing pathogens through Toll-like receptor (TLR) engagement. Notably, they also display immune regulatory properties, exerted thanks to their ability to secrete cytokines/chemokines and to establish interactions with different innate and adaptive immune cells. Therefore, because of their multiple functions, NK cells may have a major pathogenic role in acute kidney injury (AKI), and a better understanding of the molecular mechanisms driving NK cell activation in AKI and their downstream interactions with intrinsic renal cells and infiltrating immune cells could help to identify new potential biomarkers and to select clinically valuable novel therapeutic targets. In this review, we discuss the current literature regarding the potential involvement of NK cells in AKI.

Keywords: inflammation, natural killer (NK) cells, tubular epithelial cells, acute kidney injury, innate immunity

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INTRODUCTION

Acute kidney injury (AKI) is a life-threatening multifactorial clinical condition leading to a rapid deterioration of the renal function associated with high morbidity, mortality (ranging from 25% to more than 50% depending on severity), and healthcare costs. In a large number of patients, AKI may be followed by irreversible and progressive chronic kidney damage (1–3).

Recent efforts have been made to standardize definitions and classification systems for AKI, and in 2004, the Acute Dialysis Quality Initiative first proposed the Risk, Injury, Failure, Loss, and End-Stage Renal Disease (RIFLE) criteria for diagnosis and classification of acute impairments in kidney function, which included five stages ranging from small changes in kidney function or urine output to kidney failure and end-stage renal disease (4). These criteria were subsequently refined into a three-stage system and further disseminated by the Acute Kidney Disease Network in 2007 (5, 6). In 2012, the KDIGO Clinical Practice Guideline for AKI consolidated these criteria into the most recent definition and classification system for AKI (7). The current definition and classification of AKI rely upon functional criteria including changes in serum creatinine (SCr) and urine output (6, 8–10). However, despite the harmonization in clinical definition and staging, identification of the complete biology and pathophysiology of AKI remains a major unmet need.

To this purpose, several research strategies have been undertaken to identify new cellular/biological elements implicated in the AKI-derived organ damage networking. Among them, the immune-inflammatory deregulation in this condition has been emphasized. As largely reported, the activation of immune-mediated mechanisms in AKI patients is a common thread, with immune cells playing a prominent role from initiating injury to promoting tissue repair (11, 12).

Renal epithelial cells, just after the acute offense, increase the expression of damage-associated molecular pattern (DAMP) molecules, Toll-like receptors (TLRs), and other alarmins (13–17) that, all together, facilitate a rapid recruitment to the site of injury of innate immune cells, including neutrophils, activated and resident macrophages, and dendritic cells (DCs) (18–21). Furthermore, in the last years, natural killer (NK) cells, extravasated from the vascular system to the site of injury, have been shown to play a role in the propagation of the immune response and the recruitment of adaptive immune cells (22–24).

In this review, we focus on NK cell populations found in the kidney, and we discuss their role in the induction and progression of AKI.

NATURAL KILLER CELLS

NK cells are cytotoxic cells belonging to the broad family of innate lymphoid cells (ILCs) (25–28). Their effector functions range from the recognition and elimination of virus-infected and tumor cells to the secretion of cytokines/chemokines; importantly, they also display immune regulatory properties, exerted through interactions with different innate, and adaptive immune cells (29–31). In addition, they display the capability of sensing pathogens through TLR engagement and also to develop a kind of immunological memory (32–34). In order to exert these heterogeneous functions, NK cells use a large array of receptors able to sense stimuli from the microenvironment and, consequently, to mediate appropriate responses (35–37).

The recognition and elimination of abnormal cells can be fulfilled through receptor–ligand interactions involving several inhibitory and activating receptors and different types of ligands expressed on target cells. NK cells express multiple inhibitory surface receptors involved in the interaction with major histocompatibility complex (MHC) class I molecules and responsible for the "missing-self" recognition. The "missing-self hypothesis," formulated by Ljunggren and Kärre (38) in the early 90s, postulated that NK cells can detect the absence of self MHC class I molecules on target cells. In humans, human leukocyte antigen (HLA) class I molecules expressed on autologous healthy cells allow the delivery of a negative signal, thus sparing normal cells from NK cell-mediated killing. On the other hand, virus-infected or tumor cells can lose or downregulate HLA class I expression, and the lack or dampening of the inhibitory interaction makes them susceptible to an NK cell-mediated attack. The main HLA class I-specific inhibitory NK cell receptors include killer immunoglobulin (Ig)-like receptors (KIR), leukocyte Ig-like receptor, subfamily B member 1 (LIR1)/Ig-like transcript 2 (ILT2), and the cluster of differentiation 94 (CD94)/NK group 2 member A (NKG2A) heterodimer. Collectively, they can recognize different HLA-A, -B, -C alleles, and non-classical HLA-E molecules, representing an efficient system to detect alterations in HLA class I expression (39–43). Later on, it was shown that recognition and killing of target cells by NK lymphocytes requires additional signals, mainly delivered by activating receptors (37, 44–47).

Besides CD16 (FcγRIII), representing the first characterized activating NK cell receptor and responsible for antibody-dependent cell-mediated cytotoxicity (ADCC), a variety of surface receptors and co-receptors, involved in the so-called natural cytotoxicity, were discovered over the years. The receptors playing a major role in the recognition of abnormal cells are represented by natural cytotoxicity receptors (NCRs, namely, NKp46, NKp30, and NKp44), NKG2D, and DNAX accessory molecule 1 (DNAM-1) (45, 48–55). While the ligands specific for NKG2D and DNAM-1 were identified long time ago and have been extensively characterized, ligands recognized by NCR started to be defined later, and the knowledge about NCR-ligand interactions is still incomplete (49, 56–61).

The best characterized ligands for activating NK receptors include molecules that are scarcely expressed on healthy/normal cells and that can be induced or upregulated following cellular stress, neoplastic transformation, and/or viral infection (52, 53, 55, 62–65). While many of these ligands are surface-expressed molecules, also nuclear proteins have been shown to bind to activating NK receptors following translocation to the target cell surface (66, 67). More recently, the landscape of NK cell receptor ligands has become even more heterogeneous in view of the finding that some NK cell receptors can also bind to secreted soluble factors, circulating molecules belonging to the complement system, or extracellular matrix components (68–70).

Given the multiplicity of receptor–ligand interactions, the engagement of NK cell receptors by specific ligands can result in opposite signals dictating the outcome of NK cell-mediated effector functions. NK cells can also respond to cytokines, including interleukin (IL)-12, IL-15, and IL-18 (mainly produced by myeloid cells upon inflammatory stimuli), and, in turn, release cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CC-chemokine ligand 4 (CCL4) (30, 31, 71, 72).

Finally, the immunoregulatory role of NK cells has been deeply explored, starting from the characterization of NK–DC cross-talk (73–75). In this context, NK cells participate both in DC maturation and in the "DC editing" process through the recognition and killing of immature DCs that lack appropriate levels of MHC class I molecules. On the other hand, DC can favor NK cell proliferation and effector functions (76). More recently, the relevance of NK cells in immune regulation was further investigated, demonstrating the ability of NK cells to establish interactions also with other innate immune cells, i.e., macrophages and granulocytes, as well as with T lymphocytes (77–81).

HETEROGENEITY OF NATURAL KILLER CELLS

A further level of complexity arises from the heterogeneity of NK cells, i.e., the existence of different NK cell subsets characterized by distinct phenotypic and functional features and from their different localizations in the body (30, 72, 82, 83).

Human Natural Killer Cells

In humans, NK cells were initially divided into two populations based on the expression of CD56 and CD16 surface markers (84). $CD56^{bright}CD16^{dim/neg}$ NK cells usually express the inhibitory HLA-E-specific receptor CD94/NKG2A but not KIR and low or undetectable CD16; they are poorly cytotoxic, being characterized by low intracellular levels of perforin and granzymes A and B, but can secrete high amounts of cytokines (primarily IFN-γ and TNF-α) in response to IL-2, IL-12, IL-15, and IL-18 (85, 86). According to their expression of chemokine and homing receptors (i.e., CCR7, CXCR3, CXCR4, and CD62-L), $CD56^{bright}$ NK cells are mainly found in secondary lymphoid organs (SLOs), particularly in lymph nodes and tonsils, and also constitute a detectable fraction of NK cells in different organs and tissues (87). On the other hand, the $CD56^{dim}CD16^{pos}$ NK cell population is the predominant subset in peripheral blood, expresses NKG2A and/or KIR, and displays a high cytolytic potential and cytokine secretion capability following recognition of target cells expressing ligands for triggering NK receptors (88–90). Besides being more abundant in peripheral blood, the $CD56^{dim}$ subset represents a remarkable fraction of NK cells found in spleen, bone marrow, and in certain non-lymphoid organs, such as lungs and kidney. $CD56^{dim}$ NK cells can be further classified in different subsets based on distinct differentiation stages, the terminally differentiated one being represented by a KIR^{pos} $CD57^{pos}$ $CD16^{bright}$ subset which may express the activating HLA-E-specific receptor CD94/NKG2C (30, 91–96).

In addition, in recent years, it has also been discovered that, similar to adaptive T lymphocytes, also NK cells can undergo a process of clonal-like expansion and develop a kind of immunological memory. This concept was initially explored in the context of cytomegalovirus (CMV) infection, which was shown to modify the composition of the total NK cell repertoire and to drive a clonal-like expansion of given NK subsets (32, 97–100). In humans, these "memory" NK cells are distinguished by the expression of self HLA class I-binding KIRs, the terminal differentiation marker CD57, and the activating receptor complex CD94/NKG2C (101–103).

Finally, in the last decade, tissue-resident NK (trNK) cells were characterized as an additional NK cell population, resembling $CD56^{bright}$ NK cells populating secondary lymphoid tissues but displaying markers of tissue residency/retention and mainly localized in non-lymphoid tissues (104–108). In view of these findings, the "traditional" $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, mediating a potent cellular cytotoxicity and able to produce IFN-γ, are often defined as conventional NK (cNK) cells.

The discovery of tissue residency markers, such as CD69, CD49a (α1 integrin), and CD103 (αE integrin), was essential for the characterization of these trNK cells. CD69, which for a long time has been considered an activation marker for T and NK cells, plays an important role in retaining cells in tissues, thus representing a marker of local residency, both in humans and in mice (109–111). In particular, CD69 inhibits sphingosine-1 phosphate receptor 1 (SIP1), specific for sphingosine-1 phosphate (SIP), which normally promotes the egress of lymphocytes from tissues into the blood. NK cells localized in different tissues have been shown to express CD69, while cNK cells derived from peripheral blood generally do not express this marker. CD103 and CD49a play a similar role in retaining cells in tissues, and their expression is induced by transforming growth factor-β (TGF-β) (112). Indeed, CD69, CD103, and CD49a markers allow to distinguish trNK cells from circulating cNK cells that are transiently recruited into tissues. Another possible mechanism related to tissue retention involves chemokines and chemokine receptors. While $trCD56^{bright}$ NK cells found in lymphoid organs and liver are characterized by CXCR6 and CCR5 expression, circulating $CD56^{bright}$ NK cells mainly express CCR7 (24, 107, 113).

Murine Natural Killer Cells

In mice, NK cells are phenotypically characterized by the expression of several surface markers including CD161 (NK1.1), NKp46, the family of MHC class I-specific Ly49 receptors, and CD49b (α2 integrin, DX5) (35, 42, 114–118). While circulating cNK cells are defined as $NKp46^{+}$ $CD49a^{-}$ $CD49b^{+}$, trNK cells display an $NKp46^{+}$ $CD49a^{+}$ $CD49b^{-}$ phenotype (108, 119). Similar to human NK cells, cNK cell maturation in the mouse is a stepwise process, characterized by four stages according to CD11b and CD27 expressions (120, 121), with terminally mature NK cells being $CD27^{-}CD11b^{+}$ and also expressing KLRG1. Recent studies based on single-cell RNA-sequencing approaches have provided a more detailed view of murine NK cell developmental stages (122, 123). Five subsets have been identified on the basis of different genetic signatures, including the least mature NK and most mature NK clusters and three clusters defined as transitional NK subsets, which may represent intermediate steps of maturation or unique NK subsets that diverge late during development.

NATURAL KILLER CELLS AND INNATE LYMPHOID CELLS

NK cells are not the only innate lymphocytes, being included in the family of innate lymphoid cells (ILCs) that are involved in homeostatic functions and in innate immune responses against different classes of pathogens (27, 28, 124, 125).

While cytokine release is a common feature of all ILCs, NK cells are the only cytotoxic cells among the ILCs. Initially, ILCs were divided into three main groups according to the expression of key transcription factors and distinct cytokine profiles. More recently, a greater heterogeneity of ILCs was appreciated, and these cells have been consequently classified into five subsets (NK cells, ILC1, ILC2, ILC3, and LTi cells) based on their development and function (27). NK cells share some features with ILC1,

being characterized by the expression of T-bet transcription factor and the production of type I cytokines, such as IFN-γ and TNF-α. NK cells are the main ILCs found in peripheral blood, spleen, and bone marrow, whereas non-NK ILCs are more abundant in other secondary lymphoid tissues, including mucosa-associated lymphoid tissue (MALT) (126, 127). Notably, co-expression of T-bet and Eomesodermin (Eomes) transcription factors, besides cytotoxic potential, discriminates NK cells from ILC1. A further degree of complexity exists among ILC1 in relation to the heterogeneous expression of several markers. For instance, while ILC1 had been originally described as $CD56^{-}$, the expression of CD56 can identify a subgroup of tonsil and intraepithelial ILC1 (ieILC1) characterized by cytotoxic granule expression and the ability to produce IFN-γ (125, 128, 129). In addition, CD56 can also be expressed by a subset of ILC3 (125, 130). ILC2 express GATA binding protein 3 (GATA3) transcription factor, display the ability to produce T helper type 2 (TH2)-like cytokines (i.e., IL-4, IL-5, and IL-13), and are tissue-resident. ILC3 are characterized by retinoic acid receptor-related orphan nuclear receptor gamma (ROR-γt) expression, produce TH17-like cytokines, and are abundant in the mucosae; based on the expression of NKp44 in humans (and of NKp46 in mice), they can be further divided into two subsets (131). LTi cells share with ILC3 the expression of ROR-γt transcription factor, but they have a distinct developmental path and are involved in the formation of secondary lymphoid structures (132). Since tissue residency is a general hallmark of ILCs, it has to be considered that some old studies analyzing NK cells in tissues should actually be reevaluated in view of recent findings concerning ILCs.

As for other organs, the presence of ILCs has been investigated in the kidney, revealing that group 2 ILCs represent the prevalent ILC population, both in mice and in humans, and can be expanded and activated by the epithelial cell-derived cytokines IL-25 or IL-33 (133–136). Notably, these cells can exert a protective effect in AKI through the induction of alternatively activated (M2) macrophages.

NATURAL KILLER CELLS IN THE KIDNEY

The high heterogeneity of NK cells became more evident especially when their tissue localization was analyzed in different body compartments. It is now well-established that NK cells are found not only as circulating cells in peripheral blood, where they represent about 5–15% of lymphocytes, but also in SLOs as well as in different organs and tissues, in which specific NK cell subsets have been characterized (87, 105, 137).

NK cell trafficking from blood to tissues or lymphoid organs is coordinated by chemokines and their respective receptors, dictating the migration of different NK cell subsets to given compartments or to inflammatory sites (22–24). Notably, depending on the organ or tissue, NK cells can exhibit unique phenotypic characteristics and develop specific functional properties. trNK cells exhibit differences in terms of trafficking and tissue retention. Interestingly, trNK cells residing in different districts share some common features but also peculiar properties that might reflect the influence of the local microenvironment in shaping these cells (104–106, 108, 119). The body districts with the highest prevalence of NK cells are the liver (137–144), lungs (145–147), and uterus (148–151). NK cells have also been found in several other organs including the kidney, intestinal mucosa, breast tissue, synovia, pleural and peritoneal fluids, skin, salivary glands, and adipose tissue (105, 137, 152–154). Notably, the relative distribution of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets is heterogeneous in different tissues; while in most cases, $CD56^{bright}perforin^{low}$ cells (non-cytotoxic) represent the prevalent subset, the lungs contain a higher proportion of $CD56^{dim}$ $perforin^{high}$ NK cells, and the kidney is populated by intermediate levels of these two NK cell populations.

NK cells represent about 25% of lymphocytes in the healthy human kidney, with an enrichment in the $CD56^{bright}$ NK cell subset as compared with peripheral blood (137, 155–157). Until recently, however, information on tissue-resident lymphocyte populations, and in particular on trNK cells in the kidney, had been relatively limited. Although the presence of both innate and adaptive lymphocytes in this district was known for a long time, it was not clear whether these cells displayed features of tissue residency, similar to what was previously observed in other organs and tissues. In recent years, several studies helped to clarify this issue both in mice and in humans. In this context, parabiosis experiments performed in mouse models proved to be very effective in demonstrating the presence of trNK cells in the kidney.

The study by Victorino et al. in the mouse showed that about 15–20% of NK cells in the kidney are represented by a tissue-resident $CD49a^{+}$ $DX5^{-}$ NK cell population reminiscent of trNK cells harbored in other organs, such as the liver and uterus (158). The discrimination between trNK cells and ILC1 residing in non-lymphoid tissues is crucial, in view of the similarities between these two cell populations, including the $CD49a^{+}DX5^{-}$ phenotype. Studies performed in different tissues allowed to establish that most murine ILC1 are $CD127^{+}$ ($IL\text{-}7R\alpha^{+}$) and do not express the Eomes transcription factor, while murine trNK cells are $CD127^{-}$ and depend on Eomes for their development. In addition, CD200R1 surface marker has been associated with ILC1 but not with NK cells (27, 127, 159, 160). Finally, trNK cells display some cytotoxic capability thanks to the expression, albeit at low/moderate levels, of perforin and granzymes, whereas ILC1 (except for ieILC1) are non-cytotoxic cells. Although not all these markers have been analyzed so far in renal NK cells, it is conceivable that trNK cells in the kidney may share several markers with trNK cells that have been better characterized in other tissues.

The murine kidney also harbors a substantial number of $CD49a^{-}$ $DX5^{+}$ NK cells that are considered cNK cells passing through the organ. Contrary to cNK cells, kidney trNK cells do not require NFIL3 and Tbet transcription factors for their development and express lower levels of Asialo-GM1 (AsGM1) as compared to $CD49a^{-}DX5^{+}$ cNK cells; this finding suggests that NK cell depletion by anti-AsGM1 antibodies could be incomplete/inefficient and gives the possibility to investigate trNK cell function in animal models. Indeed, selective (by anti-AsGM1 mAb) or total (by anti-NK1.1 mAb) depletion of NK cells

allowed to assess the predominant role of trNK cells in a model of ischemic AKI (see below).

In addition, NK cells residing in the kidney are very efficient in producing IFN-γ, and this property has been shown to play an important role in progressive tubule-interstitial fibrosis and chronic kidney disease (CKD) (155). IFN-γ can induce the production of profibrotic factors, such as transglutaminase 2 (TG2) and the heparan sulfate proteoglycan syndecan-4 (sdc4) that contribute to the accumulation of extracellular matrix, thus favoring the development of renal fibrosis. This issue has been recently explored in a murine model of aristolochic acid nephropathy (AAN), in which the presence of trNK cells positively correlated with the progression of tubule-interstitial fibrosis (161).

Also in humans, healthy kidney harbors a relevant NK cell compartment: NK cells represent approximately 25% of total lymphocytes; both $CD56^{bright}$ and $CD56^{dim}$ NK cells can be found, with a higher proportion of $CD56^{bright}$ NK cells in the kidney (about 37% of total NK cells) as compared to peripheral blood (>10%) (137). Interestingly, a recent study, analyzing kidney biopsies from patients with different renal diseases, revealed the existence of a $CD56^{bright}$ NK cell population with tissue residency features (CD69 expression) and the ability to release IFN-γ (155).

NATURAL KILLER CELLS IN ACUTE KIDNEY INJURY

AKI is a clinical condition characterized by acute impairment of kidney function and induced by different causes, including ischemia, sepsis, and toxic insults (1, 162–164). In particular, ischemia–reperfusion injury (IRI) is one of the most frequent events leading to severe AKI.

A common hallmark of severe AKI is the occurrence of acute tubular necrosis. In the kidney, different parenchymal cells, including tubular epithelial cells (TECs) and endothelial cells, can respond to DAMPs or to pathogen-associated molecular patterns (PAMPs) through several TLRs and/or inflammasome components and thus contribute to renal inflammation. Indeed, several DAMPs, released as a consequence of tissue damage, or PAMPs expressed by infectious agents, can activate not only innate immune cells but also non-immune cells. Several studies concerning the role of TECs in kidney injury confirmed an active role for these cells both in the induction and in the regulation of inflammatory responses (13–17, 165, 166).

The expression of TLR2 and TLR4 on TECs allows these cells to sense endogenous inducers of inflammation and subsequently to be activated to produce several cytokines and chemokines (167). In particular, TLR2 involvement has been assessed in kidney IRI, where hypoxic conditions can induce tubular necrosis and the consequent release of endogenous TLR ligands, which will act at both autocrine and paracrine levels (i.e., on endothelial cells and on innate immune cells) (13, 16, 168, 169). DAMPs are also recognized by renal DCs, which contribute to the inflammatory response and to neutrophil recruitment by the secretion of inflammatory mediators, cytokines, and chemokines.

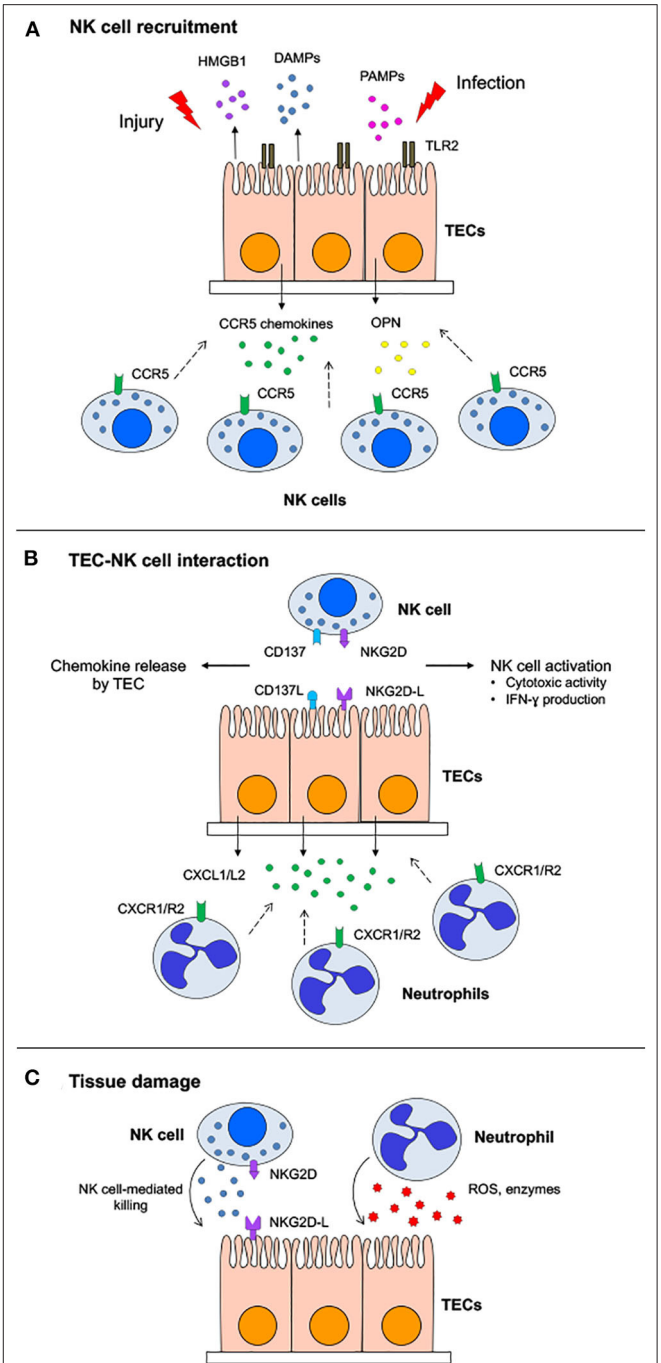

FIGURE 1 | Role of natural killer (NK) cells in acute kidney injury. **(A)** Following acute kidney injury, damage-associated molecular patterns (DAMPs) released by damaged tubular epithelial cells (TECs) or pathogen-associated molecular patterns (PAMPs) derived from infectious agents are recognized by pattern recognition receptors expressed on TECs that in turn release osteopontin (OPN) and CCR5 chemokines able to recruit NK cells. **(B)** TEC–NK cell cross-talk occurs through different receptor–ligand pairs. NKG2D ligands (NKG2D-L), upregulated on TECs, engage NKG2D on NK cells, inducing both cytotoxic activity and interferon (IFN)-γ production. On the other hand, CD137–CD137L interaction stimulates in TECs the secretion of chemokines attracting neutrophils. **(C)** TECs are killed by NK cells through the release of cytotoxic granules, while activated neutrophils are responsible for tissue damage due to reactive oxygen species (ROS) and lytic enzymes.

Tubular Epithelial Cell–Natural Killer Cell Interactions

Of particular interest is the interplay occurring between NK cells and TECs in the context of kidney injury, especially in view of the finding that NK cells are recruited in the earliest stages of IRI, already 4 h after injury (**Figure 1**) (170). The injured TECs release high-mobility group box protein 1 (HMGB1), an endogenous TLR2 ligand released following tissue damage that stimulates CCR5 chemokine production through TLR2 in an autocrine manner. CCR5 chemokines (CCL3, CCL4, and CCL5) in turn mediate recruitment of NK cells that induce TEC to release CXCL1 and CXCL2 chemokines, responsible for the accumulation of neutrophils in the kidney (169). Overall, TECs play a critical role in the induction and orchestration of acute renal inflammation by regulating the sequential migration of NK cells and neutrophils into the kidney during the early phase of IRI.

The involvement of NK cells in IRI was further supported by the finding that, in mice, the expression of ligands for the activating receptor NKG2D (Rae-1, MULT-1, and H60), is increased during kidney IRI and is paralleled by a concomitant rapid NK cell infiltration in injured kidney (171–173). This seems mediated by HMGB1 through engagement of TLR4 and subsequent MyD88-dependent signaling (174). The role of TLR4 was further confirmed by *in vitro* experiments showing RAE-1 and MULT-1 upregulation on isolated TECs following lipopolysaccharide (LPS) exposure (173).

Both in murine and human TECs, the expression of ligands specific for activating NK cell receptors has been demonstrated, suggesting that these receptor–ligand interactions could be involved in the recognition and killing of TECs. Thus, activated spleen-derived murine NK cells were shown to efficiently kill syngeneic TECs *in vitro* mainly through the engagement of NKG2D activating receptor by Rae-1 ligand expressed on TECs and by the use of perforin (172).

Similarly to what was observed in murine models, human NK cells display the *in vitro* ability to kill TECs (HK-2 cell line) exposed to hypoxia, a condition mimicking ischemic AKI, following the interaction of NKG2D receptors with MHC class I chain-related protein A (MICA), whose expression is upregulated in human TECs by hypoxia-inducible factor-1 alpha (HIF-1α) transcription factor (175). One possible mechanism of MICA upregulation in hypoxic conditions involves TGF-β, a cytokine playing multifunctional roles in inflammation, injury, and tissue repair and induced in the kidney and in TECs, following ischemic injury (176, 177). It is of note, however, that TGF-β expression has been shown to correlate with limitation of renal IRI, better TEC survival, and protection against NK cell-mediated killing (177, 178).

These effects can be explained by the fact that TGF-β, besides increasing MICA surface expression on TECs, also induces higher levels of soluble MICA, a well-known mechanism of modulation of NK cell-mediated cytotoxic activity (62, 179). In addition, TGF-β exerts a regulatory role on NK cell function mainly through the downregulation of different activating receptors, including NKG2D and NKp30 (180, 181). In view of these findings, the modulation of surface and soluble MICA expression could represent a useful strategy to reduce renal injury.

Although the mechanisms responsible for NK cell recruitment and activation in renal IRI have not been fully elucidated, a role for osteopontin (OPN) has been demonstrated. OPN is a secreted glycoprotein expressed in different immune cells, including NK cells, and exerting pro-inflammatory functions (182–184). Notably, mRNA and protein OPN expression is increased in the kidney shortly after IRI (185–187), and OPN has been shown to promote ischemic kidney injury (186, 187).

The role of OPN, however, is still debated since a protective effect for OPN both in kidney IRI and in tissue repair was reported (188). Interestingly, it has been shown that TECs display the ability to secrete high levels of OPN, which in turn can induce a rapid NK cell migration with an indirect, still undefined, mechanism, possibly involving the induction of chemokines or other chemotactic factors able to recruit NK cells. In addition,

TABLE 1 | NK cell populations described in human and murine kidney.

Human NK cells		
Phenotype	**Main observations**	**References**
$CD45^{pos}CD3^{neg}CD94^{pos}CD56^{dim}$ $perforin^{high}$ $CD45^{pos}CD3^{neg}CD94^{pos}CD56^{bright}$ $perforin^{low}$	NK cells: 25% of total lymphocytes in the kidney $CD56^{bright}$ subset: 37% of total NK cells	(137)
$CD3^{neg}CD56^{dim}CD16^{pos}$ $CD3^{neg}CD56^{bright}CD16^{neg/low}CD69^{pos}$	$CD56^{bright}$ subset involved in tubulointerstitial fibrosis $CD56^{bright}$ subset: IFN-γ production	(155)
Murine NK cells		
Phenotype	**Main observations**	**References**
$CD45^{pos}CD3^{neg}DX5^{pos}$	NK cells involved in kidney IRI	(172, 187, 189)
$CD45^{pos}CD3^{neg}NK1.1^{pos}$	NK cells involved in kidney IRI	(191)
$NK1.1^{pos}NKp46^{pos}CD49a^{pos}DX5^{neg}AsGM1^{low}$ (trNK) $NK1.1^{pos}NKp46^{pos}CD49a^{neg}DX5^{pos}AsGM1^{high}$ (cNK)	cNK and trNK cells described in the kidney trNK cells involved in kidney IRI	(158)
$CD3^{neg}NKp46^{pos}DX5^{neg}$ (trNK) $CD3^{neg}NKp46^{pos}DX5^{pos}$ (cNK)	trNK cells involved in tubulointerstitial fibrosis trNK cells: accumulation in fibrotic tissue and IFN-γ production	(161)

NK, natural killer; IRI, ischemia–reperfusion injury; cNK, conventional NK cells; trNK, tissue-resident NK cells; AsGM1, Asialo-GM1; IFN-γ, interferon-γ.

OPN can activate NK cells and increase their cytotoxic activity against primary TECs (187).

More recently, the involvement of OPN in renal injury following ischemia–reperfusion was further validated by Cen et al. in an *in vivo* model. This study confirmed an OPN increase following IRI, both at the mRNA and protein levels, and demonstrated that neutralization of OPN by an anti-OPN mAb resulted in a decreased NK cell infiltration in the kidney associated with a reduced severity of renal injury, lower levels of pro-inflammatory cytokines, and decreased neutrophil infiltration (189). Interestingly, high OPN expression was also observed in kidney grafts, and chronic transplant kidney injury was abrogated in OPN-deficient kidney grafts after transplantation, suggesting that OPN could play a role also in kidney allograft injury (190).

The search for additional TEC–NK cell interactions involved in renal IRI led to the characterization of the co-stimulatory CD137–CD137 ligand (CD137L) axis. Previously, several reports had already pointed to a role for CD137–CD137L interaction in inflammation. CD137L expressed on professional APC can co-stimulate TH1 helper T cells through the engagement of CD137; on the other hand, reverse signaling induced on APC can promote cytokine and chemokine secretion.

In the context of renal IRI, CD137 expression on activated NK cells results in the transmission of a "reverse signal" on TECs through the binding to CD137L; in turn, TECs produce high levels of CXC chemokines, such as CXCL1 and CXCL2, responsible for neutrophil recruitment and the subsequent acute inflammatory response (191).

Indeed, in a mouse model of acute IRI, the expression of CD137 on NK cells and CD137L on TECs was required for kidney injury. In addition, NK cell depletion experiments demonstrated the essential role of NK cells in neutrophil recruitment and the resulting renal injury. Depletion of neutrophils abrogated renal IRI as well, suggesting that neutrophils were directly responsible for tissue damage associated with renal IRI, while NK cells were responsible for neutrophil recruitment. In this context, it is of note that NK cells can be rapidly recruited in the kidney, within 4 h after IRI, and upregulate CD137 surface expression, suggesting their important role in the first phases of acute tissue damage.

The role of trNK cells as central mediators of ischemic tissue injury was clearly demonstrated in a model of ischemic AKI (158). The analysis of both cNK and trNK cells at 4 and 24 h after reperfusion revealed that IRI did not modify either the relative distribution or the original phenotype of these two cell subsets. Notably, trNK cells were characterized by a higher expression of several markers, including CD160, CD44, and TRAIL, suggestive of a higher activation state, and by lower levels of KLRG1 and CD244 inhibitory receptors. Based on their tissue residency and activation state, trNK cells can exert a prominent role in the early local response during IRI; it is conceivable that cNK cells recruited into the tissue can further enhance tissue damage.

IRI is an inevitable event associated with kidney transplantation. Being actively involved in the induction of inflammatory responses, TECs play a major role in this process (13, 17, 168, 192). Moreover, in kidney transplant rejection, TECs represent one of the major targets of the alloreactive immune response mediated by $CD8^+$ T lymphocytes and NK cells. The study by Demmers et al. analyzed the *in vitro* susceptibility of primary donor-derived TECs activated by IFN-γ and TNF-α to CTL- and NK cell-mediated killing. While unstimulated allogeneic TECs were efficiently killed by both $CD8^+$ T cells and NK cells of the recipient, cytokine-activated TECs became more resistant to NK cell-mediated killing presumably because of the increased expression levels of HLA class I molecules. This study also evaluated the effect of different immunosuppressive drugs on immune-mediated TEC lysis, showing their limited efficacy *in vitro* and differential inhibitory effects on CTL vs. NK cells (193).

CONCLUDING REMARKS

In recent years, the knowledge about blood-derived and tissue-resident NK cells found in the kidney is improved, revealing once again the complexity and the versatility of this ILC population (**Table 1**). In this context, the role of NK cells has also been addressed in immune-mediated pathologic conditions affecting the kidney. For instance, it is now clear that NK cells are involved in the pathogenesis of AKI, as demonstrated both in animal models and in humans. In particular, the role of NK cells in AKI can occur by distinct mechanisms, including (i) NK cell recruitment and activation mediated by CCR5 chemokines (directly) or OPN (indirectly) secreted by TECs; (ii) secretion of neutrophil-attracting chemokines induced in TECs through the CD137–CD137L axis; iii) NK cell-mediated killing of TECs through NKG2D–NKG2D-L interactions.

Therefore, because of their major involvement in AKI pathogenesis, NK cells could represent a novel target for future strategies for this important clinical condition.

AUTHOR CONTRIBUTIONS

CC and GZ wrote the manuscript. SG, MB, GS, and GC reviewed the manuscript and provided critical input. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Yin and Yang of Alarmins in Regulation of Acute Kidney Injury

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Acute kidney injury (AKI) is a major clinical burden affecting 20 to 50% of hospitalized and intensive care patients. Irrespective of the initiating factors, the immune system plays a major role in amplifying the disease pathogenesis with certain immune cells contributing to renal damage, whereas others offer protection and facilitate recovery. Alarmins are small molecules and proteins that include granulysins, high-mobility group box 1 protein, interleukin (IL)-1α, IL-16, IL-33, heat shock proteins, the Ca^{++} binding S100 proteins, adenosine triphosphate, and uric acid. Alarmins are mostly intracellular molecules, and their release to the extracellular milieu signals cellular stress or damage, generally leading to the recruitment of the cells of the immune system. Early studies indicated a pro-inflammatory role for the alarmins by contributing to immune-system dysregulation and worsening of AKI. However, recent developments demonstrate anti-inflammatory mechanisms of certain alarmins or alarmin-sensing receptors, which may participate in the prevention, resolution, and repair of AKI. This dual function of alarmins is intriguing and has confounded the role of alarmins in AKI. In this study, we review the contribution of various alarmins to the pathogenesis of AKI in experimental and clinical studies. We also analyze the approaches for the therapeutic utilization of alarmins for AKI.

Keywords: alarmins, AKI, inflammation, regeneration, IL-33, T-regulatory cells, Cytokines, DAMP

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INTRODUCTION

Acute kidney injury (AKI) is a global problem associated with high mortality, morbidity, and clinical burden (1). AKI is defined as an abrupt deterioration of kidney function indicated by an increase in circulating levels of creatinine and blood urea nitrogen (BUN) and a decline in urine output and glomerular filtration rate (GFR) (1). Several factors can result in AKI including ischemia/reperfusion injury (IRI), sepsis, hemodynamic changes, systemic inflammation, muscle wasting, and nephrotoxicity (2, 3). The pathophysiology of AKI is multifaceted, exhibiting inflammation, tubular injury, and vascular damage (4), and can cause damages to the brain, heart, and lungs in the long run. There is no approved drug for treating AKI patients, and current clinical care involves renal replacement therapy (RRT) (1).

With the ever-changing definitions of damage-associated molecular patterns (DAMPs) and alarmins, newer criteria were established during the International DAMP & Alarmins meeting held in Japan in November 2019 (5). "Alarmins" are a class of endogenous immunomodulatory molecules released or expressed by living cells upon cell injury, death, stress, or infection that triggers activation of the immune system (5, 6). In February 2006 in an European Molecular Biology Organization

workshop on innate danger signal held in Milano, Italy, Dr. Joost Oppenheim coined the term "alarmin" to designate endogenous molecules that signal tissue and cellular damage (7). Originally proposed by Dr. Polly Matzinger, DAMPs are endogenous molecules released upon non-programmed cell death that triggers inflammatory and immune responses (8), whereas pathogen-associated molecular patterns (PAMPs) are derived from invading microbes, for example, lipopolysaccharides (LPSs) that exhibit distinct biochemical property such that they alert intrusion of the pathogens (9). The PAMPs and DAMPs were shown to trigger specific pattern recognition receptors (PRRs), for example, Toll-like receptors (TLRs) for immune activation (10, 11). Although DAMPs may now be recognized as molecules that are released or secreted from dead cells, and alarmins constitute molecules that are secreted by living cells (5), there is still a lot of overlap and ambiguity in the literature. Nevertheless, to our understanding and for the purpose of this review, all DAMPs are alarmins, but not all alarmins are DAMPs. Several types of alarmins have now been recognized and are classified as nuclear, cytosolic, mitochondrial, extracellular matrix, and secreted (granule-derived) (**Table 1**). Recent evidences suggest that alarmins are pleiotropic factors that promote both inflammatory and regulatory responses (6). Both alarmins and their receptors are emerging as important biomarkers in a variety of disease conditions (6). Here, we review and discuss the inflammatory, regulatory, and regenerative capabilities of alarmin as it relates to AKI (**Figure 1**). Based on the available literary evidence, we classify the "yin" and "yang" of alarmins (**Figure 2**).

TABLE 1 | Classification of alarmins.

Origin	Types	Receptors	Preclinical	Clinical
Nuclear	HMGB1	CXCR4, RAGE, TLR2,4,9 (12)	(13)	(14)
	IL-1α	IL-1R (15)	(15)	(16)
	IL-33	IL-1RL1 (ST2) (17)	(17, 18)	(19)
	Histones	TLR2,4 (20)	(20)	—
Cytosolic	Heat shock proteins	TLR2,4, CD91 (21)	(22)	(23)
	S100 proteins	RAGE, TLR4 (24)	(24)	(25)
	Uric acid	P2X7 (26)	(27)	(28, 29)
	Haptoglobin	CD163 (30)	(31)	(32)
	Heme	TLR4, CD91, CD163 (33)	(34)	(35)
Mitochondrial	Mitochondrial fragments	—	(36)	(37)
	ATP	P1, P2Y2,6,12, P2X1,3,7 (38)	(39)	(40)
	Mitochondrial DNA	cGAS, endosomal TLR9, AIM2, NLRP3 (41)	(42)	(42–44)
	N-formyl peptides	FPR (45)	(46)	(45)
	TFAM	—	(47, 48)	—
	Succinate	GPR91 (49)	(50)	(51)
	Cardiolipin	CD1d (52), NLRP3 (53)	(54)	—
Cell membrane	HAVCR1	—	(55)	(56–58)
	Uromodulin	TLR4	(59)	(60, 61)
Extracellular matrix	Heparin sulfate	FGFRs (62)	(62)	(63)
	Hyaluronan	TLR2,4, NLRP3 (64)	(65)	(66)
	Biglycan	TLR2,4 (67)	(68)	(69)
Secreted/granule-derived	Defensins (α,β)	TLR4, CCR6 (70)	(70)	(71)
	Cathelicidin (LL37/CRAMP)	TLR7,8,9, FPRL1, FPR2, P2X7 (72)	(72)	(72)
	EDG	TLR2 (73)	(74)	(73)
	Granulysin	TLR4 (75)	—	(76, 77)
	TIMP-2	MT1-MMP, integrins, AGTR2 (78)	(78)	(79)
	IGFBP7	IGF1R (80)	(78)	(79)
	TSLP	TSLPR-IL-7Rα (81)	(81)	(81)

This table represents the majority of alarmins and DAMPs that are reportedly involved in AKI for the purpose of this review. For a more extensive understanding of DAMPS outside of AKI purview, refer to Gong et al. (11). AIM2, absent in melanoma 2; ATP, adenosine triphosphate; AGTR2, angiotensin II receptor type 2; CCR6, C-C motif chemokine receptor 6; CXCR4, C-X-C motif chemokine receptor 4; CRAMP, cathelicidin-related antimicrobial peptide; cGAS, cyclic GMP-AMP synthase; EDG, eosinophil-derived granules; FGFRs, fibroblast growth factor receptors; FPR, formyl peptide receptor; FPRL1, formyl peptide receptor like 1; GPR91, G protein–coupled receptor 91; HAVcr-1, hepatitis A virus cellular receptor 1; HMGB1, high mobility group box 1; IGF1R, insulin-like growth factor 1 receptor; IGFBP7, insulin-like growth factor–binding protein 7; IL-1α, interleukin 1α; IL-33, interleukin 33; IL-1R, interleukin 1 receptor; IL-1RL1, interleukin 1 receptor like 1 receptor; MT1-MMP, membrane type 1-matrix metalloproteinase; TFAM, mitochondrial transcription factor A; NFP, N-formyl peptides; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; RAGE, receptor for advanced glycation end-products; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor; TIMP2, TIMP metallopeptidase inhibitor 2; TLR, Toll-like receptor.

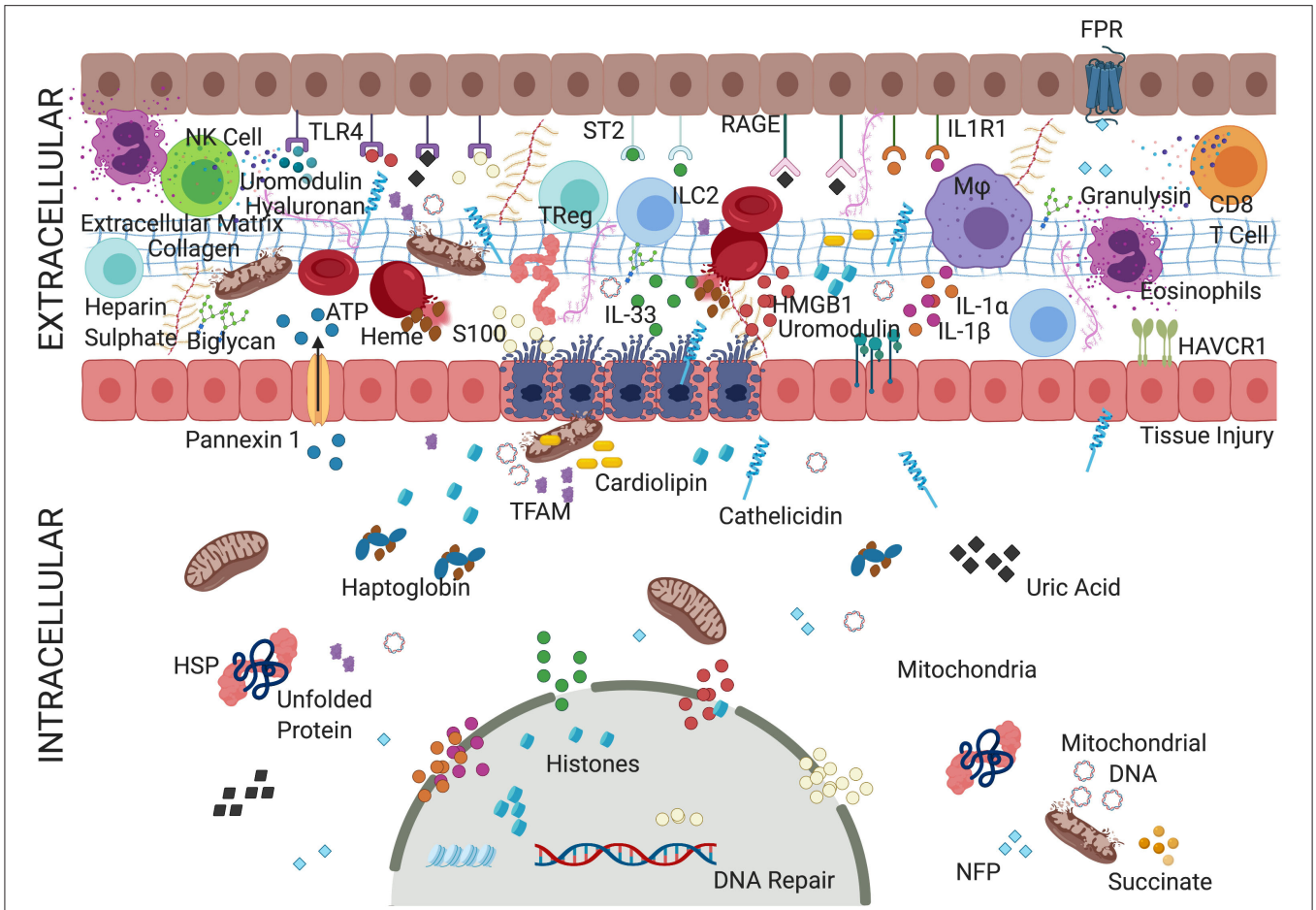

FIGURE 1 | Convoluted mechanism of action of alarmins. An overview depicting complex but critical mechanisms of action of alarmins. During AKI, various biomolecules, termed alarmins, are triggered that include various proteins, non-protein small molecules, metabolites, and cellular organelles. Alarmins have been implicated in both pro-inflammatory activity, promoting inflammatory cells, and mounting anti-inflammatory effects and facilitating repair. The alarmins are classified as nuclear, cytosolic, cell membrane, extracellular matrix, and secreted (granule-derived). Most of the alarmins exert their pathological effects through cell surface receptors such as TLRs, IL-1Rs family, or RAGE triggering activation of various downstream targets such as NF-κB and interferon responsive factors (IRFs). Adenosine triphosphate (ATP), cathelicidins, defensins act through ionotropic, metabolic, and purinergic receptors, which facilitate the organization of NLRP3 inflammasome complex. Alarmins such as heparin sulfate (HS) and insulin-like growth factor–binding protein 7 (IGFBP7) binds to growth factor receptors activating immunomodulatory and prosurvival signals. Fragmented mitochondria released from the damaged cells could trigger inflammatory milieu. Thus, various alarmins activated during cellular injury not only induce inflammatory cells but act as a source of biomarkers and recruit regulatory cells to resolve the inflammation and initiate tissue repair. The specific role of alarmins in tissue injury, inflammation, and repair is underexplored but slowly evolving. For details refer to manuscript text. TLRs, Toll-like receptors; IL-1R, interleukin 1 receptor; IL-1RL1, interleukin-1 receptor-like 1 receptor; RAGE, receptor for advanced glycation end products; interleukin 33 (IL-33); interleukin 1α/β (IL-1α/β); HMGB1, high mobility group box 1; HAVcr-1, hepatitis A virus cellular receptor 1; HSPs, heat shock proteins; NFP, N-formyl peptides.

PRO-INFLAMMATORY ROLE OF ALARMINS IN AKI

Nuclear Alarmins

IL-1 family cytokines consisting of IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Rα, IL-37, IL-38, and IL1Ra are nuclear proteins that are produced as pro-proteins and are matured by proteases (82). Interleukin 1α and IL-1β promote pro-inflammatory cytokine production by multiple immune cells in toxin-induced AKI (83). Interleukin 1α-deficient mice were protected from cisplatin-induced AKI (15). However, there was no difference in inflammatory cell infiltration between wild-type and IL-1α$^{-/-}$ mice. The IL-1 family cytokine IL-33 has emerged as a critical factor in controlling the type 1 cytokine production. IL-33 is a nuclear protein that is typically released from the damaged cell and promotes inflammatory response (84). Increased expression of IL-33 was observed in kidneys of cisplatin and IRI-induced AKI models (17, 18). In the IRI model, IL-33 was postulated to amplify the recruitment of myeloid cells through secretion of chemokines monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 2 by the epithelial cells early after injury and promoted activation of invariant natural killer (NK) T cells in later stages (18). Following

renal transplantation in patients, increased levels of IL-33 were observed in serum and urine and may contribute to renal IRI (19).

High mobility group box 1 (HMGB1) is a nuclear protein that acts as a cotranscription factor and plays an important role in DNA repair, differentiation, and development (12). Upon release from the damaged cells, HMGB1 plays an active role in pro-inflammatory responses. HMGB1 exerts its pathogenic effects on kidneys through receptor for advanced glycation end products (RAGE) and TLRs including TLR2/TLR4/TLR5/TLR9 (12, 85). A cross-section clinical study demonstrated a rise in serum HMGB1 levels in patients with AKI (14). In experimental studies too, the administration of rHMGB1 after IRI exacerbated injury (13). Sepsis-induced AKI in mice with chronic kidney disease (CKD) increased the expression of vascular endothelial growth factor (VEGF) and HMGB1 levels; however, inhibition of HMGB1, but not VEGF, was found to be protective (86).

Mice with a deficiency in TLR4, one of the receptors for HMGB1, were protected against kidney IRI. Moreover, neither the anti-HMGB1 antibody nor rHMGB1 administration affected the renoprotection in TLR4$^{-/-}$ mice (13). The results indicate that HMGB1 might promote kidney injury through TLR4 signaling. Glycyrrhizic acid could also attenuate renal IRI by inhibiting the interactions of HMGB1 with tubular epithelial cells (TECs) (87). Treatment with mycophenolate mofetil (MMF), a commonly used immunosuppressant, resulted in the improvement of renal function in IRI along with reduced levels of plasma creatinine and cytokines, as well as lower TLR4 expression (88). However, there was no change in HMGB1 levels, thus implying that MMF reduces TLR4 expression directly. Interestingly, pretreatment with carbon monoxide-releasing molecule-2 prevented the nuclear histone acetyltransferase activity by inhibiting HMGB1 release (89). This resulted in a reduction in the pathological damage to the kidney and was accompanied by downregulation of TLR4, RAGE, tumor necrosis factor α (TNF-α), IL-1β, IL-6, and MCP-1 and protection from IRI, indicating HMGB1 as one of the mechanisms of MMF treatment. Elevated levels of circulating HMGB1 were found in patients with AKI (14) and were independently associated with leukocyte count and correlated negatively with proteinuria in AKI settings.

Histones are highly basic proteins, rich in arginine and lysine, and highly conserved across species. They provide structural stability to chromatin and regulate gene expression (90). Histones in extracellular space may appear either due to release from damaged cells, by pro-inflammatory cells through active secretion, or as a component of neutrophil extracellular traps from infiltrating neutrophils (91). Extracellular histones released from dying tubular cells were associated with AKI, and were found not only to exhibit direct toxicity to renal cells but to induce pro-inflammatory cytokine and activate the innate immune response in a TLR2/TLR4-dependent manner (20).

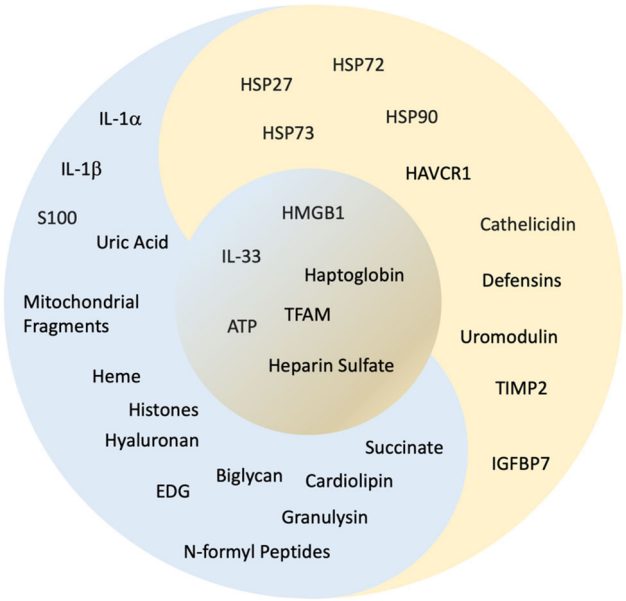

FIGURE 2 | "Yin and yang" classification of alarmins. The concept of yin and yang is dualism. It shows how apparently opposing or contrary powers can really be similar, intertwined, and interdependent in the natural universe and how they can give rise to each other as they are engaged during AKI. Here, based on the available evidence, we have classified the alarmins, which have a negative influence as "yin" as represented in "blue," alarmins with positive influence as "yang" represented in "gold" and alarmins with both "yin"/"yang" qualities are placed in the center represented in contrast between "blue" and "gold." Refer to **Figure 1** and **Table 1** for abbreviations and the text for details.

Cytosolic Alarmins

Heat shock proteins (HSPs) play an important role in a variety of cellular processes such as cryoprotection, intracellular assembly, protein folding, and translocation of oligomeric proteins (23). AKI increases the expression of HSP27, HSP72, and HSP73 in kidney tissues (21, 92–94). HSP27, HSP72, and HSP73 prevent apoptosis by decreasing intracellular reactive oxygen species (ROS) and by targeting mitochondrial caspase-dependent apoptotic pathways (92, 93, 95). They may also help with the stabilization and refolding of aggregated cellular proteins in an adenosine triphosphate (ATP)–dependent fashion (93). HSP90, on the other hand, participates in regeneration and differentiation of injured tubules (96). In a clinical study, the urinary level of HSP72 did not increase significantly in kidney transplant recipients with prerenal AKI, and a small increase in HSP70 level was noted at patients with other factors of AKI, namely, obstructive uropathy, calcineurin inhibitor drug toxicity, recurrence of primary glomerular disease, and non-steroidal anti-inflammatory drug use (97). Additionally, in the pediatric patient group, it was shown that HSP60 could be used as a diagnostic tool for AKI secondary to septic shock (98).

S100 proteins are a family of cytosolic calcium-binding proteins of ∼25 known members that are involved in controlling apoptosis, proliferation, differentiation, migration,

energy metabolism, calcium balance, protein phosphorylation, and inflammation (99). S100A8 and S100A9 are secretory proteins that can form both heterodimers and homodimers. S100A8/A9 derived from neutrophils and monocytes acts as an activator of the innate immune system through TLR4 (24). Based on the observations that levels of S100A8/A9 were proportionally elevated with increasing severity of experimental kidney injury (24), their serum levels were utilized as an early prognostic marker of AKI associated with cardiac surgery in a clinical study (25).

Uric acid crystallization has long been associated with gouty arthritis and kidney stones. However, a strong correlation of serum uric acid and AKI is emerging with multiple chronic conditions including hypertension, CKD, cardiovascular diseases, stroke, diabetic nephropathy, and metabolic syndrome (26). Uric acid acts as an antioxidant in the extracellular environment but exhibits pro-oxidant activity in the intracellular environment (100). Hyperuricemia in AKI results in dilatation of the collecting ducts leading to flattening of the epithelium, and multiple downstream consequences that include intraluminal crystal precipitation, increased intraluminal hydrostatic pressures, decrease in GFR and renal plasma flow, activation of inflammasome and necroptosis, crystal adhesion, granuloma formation, interstitial inflammation, and tubular cell injury (101, 102).

Haptoglobin is a protein produced exclusively in the liver that can bind to hemoglobulin and myoglobulin (103). Interestingly, it was observed that renal cells start expressing haptoglobin in AKI (31). Paradoxically, haptoglobin was reported to participate in both pro-inflammatory and anti-inflammatory responses. On the one hand, haptoglobin could prevent respiratory burst in stimulated neutrophils, blunt endotoxin-stimulated T-lymphocyte proliferation, and modulate macrophage and dendritic cell function; on the other hand, it could also activate TLR signaling and contribute to inflammation. Furthermore, haptoglobin abruptly released from kidneys could also exert adverse pathophysiological effects in acute transplant rejection, which is also caused by AKI (30). An increase in haptoglobin levels in cardiac surgery patients has been associated with postoperative AKI indicating a direct role in ischemic AKI (32).

Heme is an iron-containing, tetrapyrrole ring that is an essential prosthetic group in an array of proteins and influences cellular and metabolic functions (33). Free heme at higher than physiological levels can be cytotoxic because of its bioreactivity and pro-oxidative effects. Higher levels of heme were observed following ischemia-induced AKI (104). Mechanistically, heme contributed toward cellular toxicity by oxidizing lipids, denaturing proteins, cytoskeletal rearrangement, inhibiting enzyme activity, denaturing DNA, and affecting mitochondrial metabolism (105). It also induced pro-inflammatory response by inducing chemokines such as MCP-1 by the action of nuclear factor κB (NF-κB) signaling, increased leukocyte recruitment, and vascular permeability (34). Pigment nephropathy due to rhabdomyolysis and hemolysis accounts for ∼10% of all cases of AKI (35).

Mitochondrial Alarmins

Considered to be the powerhouse of the cell and critical for maintaining the cellular functions, mitochondria are also a source of factors that can induce cell apoptosis (106). Fragmentation of mitochondria is an important early event in the manifestation of AKI of both chemical and ischemic etiology (36). The release of cytochrome C from mitochondria into the cytoplasm is an endogenous signal for the cell to undergo apoptosis. Swollen mitochondria were observed in renal tissues in mice treated with LPS, a model of sepsis-associated AKI (107). These mitochondria stained poorly for cytochrome c oxidase, an indication of underlying reduced electron transport chain activity. The mitochondria are fast becoming a critical target, and mitochondrial DAMPs that include mitochondrial DNA (mtDNA), ATP, N-formyl peptides, TFAM, succinate, and cardiolipin [reviewed in (108, 109)] are also being identified for their pathological roles in renal injury and dysfunction as discussed below.

Mitochondrial DNA (mtDNA), identified as a DAMP, has been suggested to also act as an alarmin that upon release into the cytoplasm triggers an inflammatory response and has been proposed to be used as a potential biomarker for kidney injury (109, 110). Cellular stress results in leakage of mtDNA leading to inflammation, likely through recognition by four innate receptors: cytosolic cyclic GMP-AMP synthase (cGAS), endosomal TLR9, and two inflammasomes: absent in melanoma 2 (AIM2), and NOD, LRR, and pyrin domain-containing protein 3 (NLRP3) (41). Levels of urinary mtDNA were elevated in mice after IRI-induced AKI (42). Clinical studies have indicated an association of urinary mtDNA with initiation and progression of AKI in the surgical intensive care unit (43), cardiac surgery (42), and sepsis (44) patients.

Adenosine triphosphate (ATP) is the vital source of energy for cellular processes, and its intracellular level is regulated by mitochondrial oxidative phosphorylation. However, extracellular ATP is an indication of mechanical stress and cellular damage (38). Binding of extracellular ATP activates the membrane-anchored ionotropic P2X (P2XRs) and metabolic P2Y (P2YRs) purinergic receptors. Activation of these purinergic signals by ATP triggers a variety of biological responses such as inflammation, tissue damage, and cell proliferation in renal diseases (38). Inhibition of purinergic receptors was protective in both ischemic and sepsis-induced AKI (111, 112). ATP and selective agonists of the $P2X_7$ receptor were shown to activate peptidyl arginine deaminase 4 (PAD4) in proximal tubular cells (PTCs) and exacerbate IRI (113). Recently, the $P2X_4$ receptor was shown to exacerbate ischemic AKI through NLRP3 inflammasome signaling in the renal proximal convoluted tubules (PCTs) (114). CD39 and CD73 are two ectonucleotidases that break down ATP to adenosine, which has anti-inflammatory properties (115). The absence of CD73 in mice exacerbated inflammation and worsened AKI outcomes (116), whereas mice transgenic for overexpression of human CD39 were protected against AKI (117). The release of ATP to the extracellular milieu and its intracellular levels is also regulated

by pannexin receptors (39). Panx1 was recently shown to induce ferroptosis in renal IRI and its deletion protected from IRI (118, 119).

Mitochondrial N-formyl peptides (FMIT, mtFPs, NFP, or mitocryptides) are similar to bacterial DAMP peptides. The evidence of the role of FMIT leading to AKI is rather indirect through the progression of sepsis. It was reported that mitochondrial N-formyl peptides induce sepsis-like syndrome, which could further affect organs including kidneys, lungs, and brain (46). It is known that a significant proportion of trauma patients presents sepsis-like syndrome without bacterial infections, and this condition is termed systemic inflammatory response syndrome (SIRS). One of the most common complications of SIRS is AKI, which is triggered by FMIT through formyl peptide receptor activation leading to hypotension and vascular collapse (45).

Mitochondrial transcription factor A (TFAM) is a member of a high mobility group (HMG) box proteins (109). It is an important regulator of the transcription and replication of mtDNA, as well as a key regulator of mitochondrial dynamics and function (47). The development of TFAM-deficient mice has enhanced our understanding of the role of TFAM in renal injury. It was recently reported using this versatile mouse model that mitochondrial damage activates the widely investigated cGAS-STING pathway leading to renal inflammation and fibrosis (47). The role of mitochondrial damage and the cGAS-STING pathway was also recapitulated recently in the cisplatin-induced AKI mouse model (48).

Succinate is an intermediate of the tricarboxylic acid cycle, which reaches extracellular milieu upon injury or ischemic conditions in the tissue (109). Succinate receptor GPR91 expressed in immature DCs and macrophages binds to the extracellular succinate and gets activated, resulting in either initiation or exacerbation of immune response (49). Plasma succinate levels were shown to be upregulated in studies on the changes in the metabolic profiles in murine AKI (50).

Cardiolipin is a class of phospholipids that account for ∼20% of lipids in the inner mitochondrial membrane (120). It is critical for many mitochondrial processes such as protein import, dynamics, respiratory chain functionally, and metabolism. Extracellular cardiolipin release due to mitochondrial stress or injury is sensed by T cells through the presentation on the major histocompatibility complex–like molecule CD1d (52). Cardiolipin can also bind to NLRP3 directly, eliciting, and inflammasome-mediated immune response (53). Peroxidation and loss of cardiolipin have been shown to contribute to pathogenesis in experimental AKI (54).

Extracellular Matrix Associated Alarmins

The epithelial injury and inflammation in AKI also lead to disruption of the glycocalyx, an endothelial surface layer consisting of lectin and proteoglycan (62).

Heparin sulfate (HS) is a major component of glycocalyx that helps in the organization of ∼50% of the glycocalyx. Heparanase is an endoglycosidase enzyme that functions to cleave HS. Increased expression of heparanase has been observed in AKI, suggesting it could be used as an early biomarker (62). Shedding of glycocalyx is accompanied by reduction of endothelial nitric oxide synthase and an increase in inflammation (121). Activation of heparanase was also observed early in the sepsis-induced AKI in mice and correlated with higher pro-inflammatory cytokine levels (122). Detectability of heparanase in the urine also supported its potential as an important biomarker in sepsis–AKI (63). Further, inhibitors of heparanase activation attenuated the renal transcription of the pro-inflammatory mediators (122).

Hyaluronic acid (HA) is also an important component of the extracellular matrix. It is mainly composed of N-acetyl glucosamine and glucuronic acid (64). HA synthesis has been shown to increase during fibrosis and inflammatory conditions. Endothelial cells and TECs express abnormally high levels of CD44 and HA receptor during AKI (64). Further, the uptake of HA by these cells resulted in cellular dysfunction. In a pioneering study, urinary HA was correlated with AKI in patients, also suggesting that it could be used as a biomarker to differentiate AKI from CKD in patients. Additionally, an increase in HA has been attributed to T-cell and macrophage infiltration and formation fibrosis in AKI (65).

Biglycan is expressed as a component of ECM in all organs and belongs to the small leucine-rich proteoglycan (SLRP) family that is released from the extracellular matrix (68). Overexpression of biglycan is a common clinical feature in many renal pathologies. Overexpressing biglycan triggered activation of TLR2 and TLR4 to exacerbate pathophysiology of experimental AKI (67). More recently, it was reported that biglycan activates autophagy in macrophages through a novel CD44–TLR4 signaling axis in the setting of IRI (123). Both preclinical and human studies have identified soluble biglycan as biomarkers in inflammatory renal diseases [detailed specific review in (69)].

Cell Membrane–Bound Alarmins

Hepatitis A virus cellular receptor 1 (HAVCR1), initially identified as a receptor for several viruses, is also known as T-cell immunoglobulin and mucin domain 1 (TIM-1) or kidney injury molecule 1 (KIM-1). KIM-1, although expressed in multiple tissues, is not expressed in normal kidneys; however, it gets rapidly upregulated in PCT of the kidney in AKI (55). KIM-1 was the first non-myeloid phosphatidylserine receptor identified that could transform epithelial cells into "semiprofessional" phagocytes; thus, playing a role in the removal of apoptotic cells and necrotic tissue fragments (124). Recently, KIM-1 has also been attributed to the resolution of kidney inflammation, suggesting additional possible roles for this alarmin molecule or receptor (55). KIM-1 was shown to activate the ERK/MAPK signaling to promote the migration and proliferation of renal TECs (125). KIM-1 is detected in the urine of kidney injury patients and is being evaluated as a prominent biomarkers for AKI [extensively reviewed in (56–58)].

Uromodulin or Tamm–Horsfall protein (THP) is a glycoprotein expressed in the thick ascending limb of the kidney and is the highest excreted protein in the urine following proteolytic cleavage (60). Although the function of uromodulin is not completely understood, it is proposed as a biomarker of kidney injury (60), polycystic kidney disease (126), and acute

transplant rejection (127). Uromodulin was shown to promote immune cell activation via activating TLR4 in experimental studies (128). Clinical studies suggested that uromodulin may also be involved in the progression of CKD with its serum levels positively correlating with serum levels of pro-inflammatory cytokines (129). Paradoxically, uromodulin also has a protective effect in AKI. Uromodulin was shown to exhibit anti-inflammatory effects through reducing TLR4 expression in the thick ascending limb as kidneys from THP-deficient mice exhibited more inflammation and injury in the outer medulla (59). In cardiac surgery–associated AKI, a lower uromodulin-to-creatinine ratio correlated with higher odds of AKI and higher peak serum creatinine levels (130). In another clinical study in acute pancreatitis related AKI, serum uromodulin concentration had a positive correlation with GFR, and patients with AKI had lower serum uromodulin (131). Lower serum uromodulin levels were thus predictors of AKI in pediatric cardiac surgery (132), patients with cirrhosis (61), or renal cancer patients with partial nephrectomy (133).

Secreted/Granule-Derived Alarmins

Many granule-derived alarmins were initially identified as antimicrobial products secreted by cells, but their role in sterile inflammation is now increasingly recognized (134).

Defensins are a class of antimicrobial peptides, present in the granules of many cell types, and have a broad range of antimicrobial activity in both Gram-negative and Gram-positive bacteria (135). Defensins can be categorized into two families, the α-defensins and β-defensins (136). Although Paneth cells in the intestine are the main source of α-defensins in mice, higher levels of defensins were observed in the kidneys in glomerulonephritis and CKD (137). Elevated levels of defensin were detected after AKI and were shown to induce inflammation, injury, and impaired barrier functions in the gut (70). As a result, the delivery of defensins and other pro-inflammatory molecules such as IL-17A from intestinal macrophages to the liver resulted in hepatic inflammation and apoptosis. In turn, overproduction of hepatic IL-6 and TNF-α led to systemic inflammation and enhancement of renal dysfunction in a feed-forward loop (70, 138). Urinary β-defensins were proposed to be a useful biomarker in early prediction of contrast-induced nephropathy, which accounts for ∼10 to 15% of hospital-acquired AKI (71).

Cathelicidins are a family of antimicrobial and immunomodulatory peptides expressed in epithelial and immune cells under homeostasis and inflammation (139). A single cathelicidin is found in humans—hcAP18/LL-37 and rodents—cathelicidin-related antimicrobial peptide (CRAMP) (140). Cathelicidin expression was significantly downregulated in clinical AKI as well as in murine models (72). NLRP3 overactivation was discovered to be one of the major effects of this deficiency in cathelicidin that causes elevated inflammatory responses and apoptosis (141).

Tissue inhibitor of metalloproteinases 2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) have gained recognition as clinical biomarkers of AKI, collectively known as Nephrocheck™ commercially (79). TIMP-2 is a natural inhibitor of matrix metalloproteinases involved in the degradation of the extracellular matrix (142). Under steady state, TIMP-2 is expressed in monocytes, B cells, and T cells (142). Increased levels of TIMP-2 were detected in urine immediately following AKI (78). In the normal kidneys, TIMP-2 is localized in PCT. However, there was an apparent reduction of TIMP-2 signals after AKI and directly correlated to the severity of AKI (78). IGFBP-7 binds to the IGF and regulates its bioavailability in body fluids and tissues. Following AKI, a massive increase in IGFBP7 in urine was observed (78). Similar to TIMP-2 strong cortical proximal tubular staining of IGFBP7 was observed in normal under normal conditions. However, upon AKI, there was a severe reduction of proximal tubular IGFBP7 (143). Insulin-like growth factor–binding protein has been hypothesized to be involved in cellular senescence (78) and immune cell function (80). More detailed mechanistic studies are required to uncover the molecular and cellular basis of IGFBP7 in the context of inflammation.

Thymic stromal lymphopoietin (TSLP) is mainly produced from stromal and epithelial cells, and its function to promote T helper type2 (TH2) cell response has linked it to allergic inflammation (144). The TSLP levels were elevated in sepsis-associated AKI in both humans and rodent models (81). TSLP was associated with NF-κB signaling to elicit the inflammatory response. Other granule-derived peptides such as those produced by eosinophils (73), and granulysins that are secreted by cytotoxic T lymphocytes and NK cells (145), were reported in renal allograft rejection (76, 77), and may also be linked with AKI and mortality (75).

POTENTIAL THERAPEUTIC APPLICATION OF TARGETING ALARMIN SIGNALING

Alarmins were initially identified as acute-phase molecules that cause immune activation and were deemed pro-inflammatory. Consequently, several approaches to inhibit alarmins and their receptors have been explored for intervention in AKI. Interestingly, several alarmins also have dual functions and can promote protective pathways and thus are being explored for therapeutic use. We review these two opposing approaches below in the context of AKI.

Inhibiting Alarmin Signaling

Nuclear Alarmins

Administration of the soluble form of IL-33 receptor ST2 (sST2) was shown to prevent the onset of acute inflammation (84). It is believed that sST2 may act as a decoy receptor and neutralizes the IL-33 activity. Treatment with sST2 in the cisplatin-induced AKI model exhibited fewer CD4-infiltrating T cells, lower serum creatinine, and decreased acute tubular necrosis (ATN) and apoptosis as compared to the untreated controls (17). In contrast, treatment with recombinant IL-33 (rIL-33) exacerbated the AKI with an increase in CD4 T-cell infiltration, serum creatinine, ATN, and apoptosis (17). Interestingly, it was observed that the administration of rIL-33 did not exacerbate AKI in CD4-deficient mice, suggesting a direct effect of IL-33 activity on

CD4 T cells (17). These data indicated that inhibiting the IL-33 signaling has therapeutic potential in treating or preventing AKI. Similarly, treatment with HMGB1 neutralizing antibody after IRI led to attenuation of TNF-α and MCP-1 levels and protected against kidney IRI, as evidenced by lower levels of serum creatinine, tubulointerstitial neutrophil infiltration, and tubular damage compared to the control mice (13). Various IL-1β/IL-1α/IL-1R1–specific inhibitory molecules are currently in different phases of clinical trials (16). Neutralization of histones using targeted neutralizing antibody also led to the attenuating pathogenic effect of histones, thus preventing AKI (20).

Cytosolic Alarmins

HSP90 transduces signals via binding to the transforming growth factor β type I (TGFβI) and type II (TGFβII) receptors (22). Blocking the interaction of HSP90 with TGFβII receptor by using 17-allyamino-17-demethoxygeldanamycin reduced fibrosis by promoting the ubiquitination of TGFβII. S100A8/A9–TLR4–NLRP3 inflammasome pathway was shown to trigger inflammation, apoptosis, and tissue injury during AKI. Inhibition of this pathway through siRNA to TLR4–NLRP3 ameliorated the kidney function in contrast-induced acute kidney injury model (24). Inhibition of TSLP, a TH2-inducing cytokine, with siRNA also resulted in lowering the sepsis-associated organ dysfunction and inflammatory cytokine levels (81).

In a rat model of cisplatin-induced AKI, moderate hyperuricemia was associated with the absence of intrarenal crystals but correlated with greater tubular injury, significant macrophage infiltration, and increased expression of MCP-1 (27). Treatment with rasburicase, a uric acid oxidase, reversed the inflammation and tubular injury (28). Many clinical approaches employed in AKI, including allopurinol, febuxostat, and Renal Replacement Therapy (RRT), may act by decreasing circulating urate to reduce its pro-inflammatory effects (29).

Mitochondrial Alarmins

Mitochondrial fragmentation has been thought to be one of the possible mechanisms contributing to injury in AKI. Inhibition of mitochondrial fragments was observed by blocking fission protein Drp1 along with the reduction in cytochrome c release and apoptosis (36). Similar results were obtained by blocking Drp1 using a new pharmacological inhibitor mdivi-1 (36). Targeting mitochondria by promoting mitochondrial health for therapeutic effects on AKI includes promoting metabolism by augmenting fatty acid oxidation using peroxisome proliferator-activated receptor α (PPARα) overexpression (146) or augmenting ETC using CoQ10 (ubiquinone) (147). Mitochondrial fragmentation induces ROS, which was targeted using MitoQ and SS-31 to attenuate AKI (148). Cyclosporine that is used in transplantation may also counter AKI by regulating mitochondrial membrane permeability by inhibiting cyclophilin D (149). Agents such as temsirolimus (150) function by targeting mitophagy through activating mTOR signaling. Finally, improving mitochondrial biogenesis by enhancing nuclear transcription of mitochondrial proteins using PPARγ-coactivator-1α (PGC1α) (107) or by activating β-adrenergic receptors using formoterol (151) may also contribute to protection from AKI by reducing mitochondrial fragmentation. Compound SS-31, which reenergizes mitochondria by preventing matrix swelling and preserving cristae structure, thus restoring ATP, is being clinically tested. SS-31 selectively binds to cardiolipin, preventing its peroxidation and loss (37).

Depletion of extracellular ATP with apyrase, or blocking of P2XR with pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), has been shown to prevent necrosis-related inflammation (152). In the same study, treatment with A438079, a selective $P2X_7$ receptor inhibitor or knockdown of the $P2X_7$ receptor with siRNA, reduced the apoptosis of PTCs. The use of recombinant alkaline phosphatase has been tested both experimentally (153) and clinically (40) in sepsis-associated AKI. It is believed that the mechanism of action may involve dephosphorylation of LPS for reduced TLR activation (154) and of ATP for conversion to the anti-inflammatory adenosine (155). Binding of adenosine or its synthetic analogs to adenosine receptors protected mice from IRI in an IL-11–dependent manner (156). Adenosine was also shown to induce immune tolerance through dendritic cells (157) and T-regulatory cells (Tregs) (158). Conversely, inhibition of adenosine kinase with a small molecule (ABT-702) to prevent the conversion of adenosine to ATP was protective in cisplatin nephrotoxicity (159). Paradoxically, extracellular nucleotides including ATP released from dying cells were also shown to promote wound repair in renal tubular injury (160).

Secreted and Extracellular Alarmins

Blocking of glycans with doxycycline, a broad-spectrum matrix metalloprotease inhibitor, was shown to restrict the secretion of pro-inflammatory cytokines in cisplatin and IRI-induced AKI (161, 162). Heparanase inhibitors such as PG545 was found protective in experimental ischemic IRI (63) and is currently in clinical testing. Mice receiving a diet containing 4-methylumbelliferone, a potent hyaluronic acid synthesis inhibitor, resulted in attenuation of AKI (66). Pharmacological treatment with a zinc chelator, dithizone, resulted in depletion of Paneth cell granules in adult mice (163) and rats (164). These mice exhibited less leukocyte infiltration, pro-inflammatory cytokine generation, and reduced epithelial necrosis and apoptosis. In contrast, studies have also indicated that a chronic loss of Paneth cell α-defensin expression could also skew toward a more pro-inflammatory phenotype (165). These opposing outcomes warrant additional mechanistic studies to fully understand the role of defensins in AKI.

Direct Application of Alarmins

Nuclear Alarmins

In contrast to the pro-inflammatory reports of IL-33, evidence also suggests that IL-33 is a potential mediator of type 2 immunity and a regulator of the protective immune response (166, 167). We identified that ST2, the receptor for IL-33, is regulated by IL-2 (168) and is expressed on a major subset of Tregs (169). Based on our data that IL-2 and IL-33 by themselves increased Tregs and partially protected from IRI and that these cytokines

synergize to completely protect from AKI, we generated a novel hybrid cytokine (termed IL233) bearing activities of IL-2 and IL-33 in a single molecule (169). Treatment with IL233 robustly increased Tregs and the group 2 innate lymphoid cells (ILC2) and strongly protected kidneys from IRI, as well as cisplatin- and doxorubicin-induced nephrotoxic injuries (169, 170). A similar strategy of using exogenous IL-33 alone was demonstrated to increase ILC2 to protect from IRI in T cell–independent manner (171). Interestingly, reduction or depletion of ILC2 did not affect the severity of IRI in a mouse model, suggesting that ILC2 may be redundant for IRI (172), despite the finding that the adoptive transfer of *ex vivo*–expanded ILC2 was protective in murine IRI (169).

Cytoplasmic Alarmins

Preconditioning the mice with rHMGB1 prior to IRI protects the kidney against IRI was indicated by low serum creatinine, tubular damage, and tubulointerstitial neutrophil and macrophage infiltration (173). Pretreatment with rHMGB1 resulted in the upregulation of Siglec-G, which in turn negatively regulated HMGB1-mediated TLR4 pathway activation. This indicated significant protection from renal IRI from the activation of TLR4-dependent inflammatory response. It was also observed that lentivirus-mediated renal overexpression of HSP27 prevented the loss of renal function and decreased necrosis, inflammation, apoptosis, and F-actin cytoskeleton after IRI injury in mice (174). In a retrospective observational study, it was found that the intraoperative administration of haptoglobin administration was independently associated with a lower risk of AKI incidence after cardiovascular surgery (175).

Studies in 1989 identified heme oxygenase 1 (HO-1) as a protein induced in hypoxic cells. Protective responses of HO have been confirmed in various AKI studies (176). HO-1 participates in the dissipation of heme, thereby protecting the kidneys from inflammation and cellular damage. Induction of HO-1 and ferritin in the kidney protects against heme-induced kidney injury (177). HO-1 induction by granulocyte colony-stimulating factor has been shown to protect against AKI both *in vivo* and *in vitro* (178). Adiponectin, a cytokine produced from white fat, induces HO-1 in renal epithelial cells *in vitro* and prevents AKI following IRI (179). Along with heme, ferrous iron (Fe) that is found in heme also correlated with AKI (180). Administration of the iron-regulating hormone hepcidin reduced inflammation and decreased oxidative stress in mouse models of AKI (181). Further, the administration of a furin inhibitor to induce high levels of hepcidin also reduced AKI in mouse models (182).

Extracellular Matrix and Cell-Surface Alarmins

The use of extracellular matrix-associated alarmins for protection in AKI is largely understudied but is gaining attraction. In an interesting study (183), an HA-curcumin prodrug targeting the HA receptor-CD44 could assist in epithelial cell survival from oxidative stress during AKI. CRAMP-deficient ($Cnlp^{-/-}$) mice exhibited exacerbated renal dysfunction accompanied by aggravated inflammatory response and apoptosis (72). Exogenous treatment with CRAMP markedly attenuated AKI accompanied by reduced NLRP3 orchestrated inflammatory response and apoptosis. In LPS-induced inflammatory settings, it was observed that overexpression of TIMP-2, a major diagnostic marker of AKI, significantly attenuated the production of nitric oxide, TNF-α, IL-1β, and ROS with increased production of anti-inflammatory cytokine (IL-10) (184). Future studies on the use of TIMP-2 are likely to produce interesting results.

Implications of Alarmins in Repair Post-AKI

The renoprotective role of alarmins also suggests their potential in repair after renal injury. Stem cells play an important role in tissue homeostasis, as well as tissue repair following injury (185). Researchers have used exogenous stem cells to improve tissue regeneration using a variety of approaches. However, still, there is a very limited clinical success than anticipated especially for solid organ injuries (185). Alternatively, harnessing the endogenous tissue-resident stem cells for mediating repair could be promising. In a breakthrough study in 1970, it was observed that priming injury at a distant site at the time of, or before the second trauma, resulted in accelerated repair (186, 187). In a recent study, Lee et al. (188) have used the alarmin, HMGB1, to accelerate repair using a bone fracture model. Exogenous treatment with HMGB1 accelerated facture healing through the formation of heterodimer complex between HMGB1 and chemokine, CXCL12 (stromal cell–derived factor1), which then signals through CXCR4 receptor (188). Because remote ischemic preconditioning was accompanied by an upregulation of HMGB1 (189), preconditioning with recombinant HMGB1 was tested and found to be protective in AKI (173). Such an approach may as well be investigated to promote repair in AKI.

Heat shock proteins, although identified as biomarkers for AKI, are now being investigated for their beneficial role in AKI. HSP73 and HSP90 were found to be induced in the injured PTC and loop of Henle early on after injury and then were upregulated again in the regenerating cells, suggesting these HSPs may participate in repair post-IRI, and may be exploited in future studies (94). HSP70 was shown to interact with cytoskeletal elements during the restoration of the cytoskeletal structure and polarity of proximal tubules after ischemic injury, indicating the role of HSP70 in renal repair (190). An interesting concept is that T-cell reactivity to HSP may induce tolerogenic responses, which may be beneficial for the resolution of inflammatory diseases (21, 191, 192). Indeed, a recent study showed that, in a murine model of IRI, heat preconditioning induced the release of HSP-70, which in turn promoted the expansion of Tregs that was renoprotective (193, 194).

A reparative role of Tregs in AKI was initially shown in murine IRI through depletion studies (195). Recently, we demonstrated that treatment with the fusion protein IL233 utilizes the synergy of IL-2 with the IL-33 alarmin in protection when administered after the onset of injury (169). IL233 treatment, initiated 2 weeks after renal injury, induced near-complete restoration of renal structure and function (170). IL233 treatment invoked the proliferation and renal recruitment of Tregs and ILC2s. Antibody-mediated depletion of these cells ameliorated the restoration of renal injury. Further, mobilization of these cells near the site of injury promoted the recruitment of progenitor cells in the kidneys. It remains to be evaluated

whether this may be either a direct effect of these cells or through inducing an anti-inflammatory milieu, which may be conducive for progenitor cells to promote regenerative responses. Treatment with IL233 after the onset of lupus nephritis and diabetic nephropathy in animal models also induced persistent remission, suggestive of a reparative role of IL-33 alarmin in chronic renal injury (170, 196, 197). Current studies in our group are addressing the role of the IL-33/ST2 and IL233 in the repair of renal injury in both an immune-dependent and independent manner.

CONCLUSION

The immunoregulatory potential of alarmins, as well as their predictive value as a biomarker in a host of disease conditions, renders the study of alarmins beneficial for clinical applications. Despite all the advances in the understating of the pathophysiology of kidney diseases, the dearth of treatment strategies for AKI remains a major unmet clinical need. Novel therapeutic options or perhaps a combination of those in a concerted manner is required to solve this problem. Exploring the role of alarmins as diagnostic markers, immunomodulators, and harbingers of repair could be one of the strategies that may lead to therapy of AKI.

AUTHOR CONTRIBUTIONS

RS conceived the idea and performed the final revision. VS performed the bulk of literature search in collaboration with RV and MD. RS, VS, RV, and MD co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Use of Immune Checkpoint Inhibitors in Oncology and the Occurrence of AKI: Where Do We Stand?

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Immune checkpoint inhibitors (ICIs) are a novel class of immunotherapy drugs that have improved the treatment of a broad spectrum of cancers as metastatic melanoma, non-small lung cancer or renal cell carcinoma. These humanized monoclonal antibodies target inhibitory receptors (e.g. CTLA-4, PD-1, LAG-3, TIM-3) and ligands (PD-L1) expressed on T lymphocytes, antigen presenting cells and tumor cells and elicit an anti-tumor response by stimulating immune system. Nevertheless, the improved overall survival is complicated by the manifestation of Immune-related Adverse Effects (irAEs). During treatment with ICIs, the most common adverse kidney effect is represented by the development of acute kidney injury (AKI) with the acute tubulointerstitial nephritis as recurrent histological feature. The mechanisms involved in ICIs-induced AKI include the re-activation of effector T cells previously stimulated by nephrotoxic drugs (i.e. by antibiotics), the loss of tolerance versus self-renal antigens, the increased PD-L1 expression by tubular cells or the establishment of a pro-inflammatory milieu with the release of self-reactive antibodies. For renal transplant recipient treated with ICIs, the increased incidence of rejection is a serious concern. Therefore, the combination of ICIs with mTOR inhibitors represents an emerging strategy. Finally, it is relevant to anticipate which patients under ICIs would experience severe irAEs and from a kidney perspective, to predict patients with higher risk of AKI. Here, we provide a detailed overview of ICIs-related nephrotoxicity and the recently described multicenter studies. Several factors have been reported as biomarkers of ICIs-irAEs, in this review we speculate on potential biomarkers for ICIs-associated AKI.

Keywords: immune checkpoint inhibitors, AKI (acute kidney injury), mTOR inhibitor, CTLA-4, PD-1-PDL-1 axis, immunosenescence and inflammaging, gut microbiome, renal cell cancer (RCC)

INTRODUCTION

Cancer immunotherapy encompasses a number of different treatments aimed at stimulating the immune system in order to promote the recognition and the elimination of tumor cells (1). In the past decade, Immune checkpoints inhibitors (ICIs) have emerged as anticancer agents able to modify, for the better, the natural history of a wide range of malignancies, such as melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), bladder cancers, Hodgkin lymphoma and others (2). Although these agents have dramatically improved the prognosis of many cancer patients, they are critically associated with a broad spectrum of sometimes ill-defined adverse events, caused by the uncontrolled activation of the immune system, due to the lack of physiological brakes (i.e. the immune checkpoints themselves), referred to as immune-related adverse events (irAEs) characterized by clinical manifestations that closely resemble autoimmunity disorders (3, 4). Given the extrarenal clearance of ICIs, the contribution of these agents to kidney toxicity has been neglected and underestimated for several years (5). This was further complicated by the fact too often, renal toxicities from anticancer agents in general, are reported, within oncology clinical trials, just as "creatinine increase", or similar definitions, without any further pathogenic insight. On the contrary, increasing evidence supports the central involvement of ICIs in the development of acute kidney injury (AKI), proteinuria, and renal electrolyte abnormalities. Strikingly, after episodes of ICIs-induced AKI, impaired renal function recovery correlated with increased mortality (6). In this review, we will discuss the molecular pathways modulated by ICIs on T-cell activation, the proposed mechanisms of ICIs-related renal injury, with a particular focus on the development of AKI, as well as recent insights into clinical trials, and biomarkers studies aimed at assessing response to treatment.

IMMUNE CHECKPOINT ON T LYMPHOCYTES

In the tumor microenvironment, cancer cells can evade the immunosurveillance by changing their surface antigens, thus avoiding the detection and destruction by host lymphocytes. A central mechanism of tumor-induced immune suppression is the increased expression of ligands able to bind inhibitory T cell receptors (2, 3, 5). These ligands are known as immune checkpoints and act in physiological conditions to prevent the development of autoimmunity at multiple steps during the immunological response. The main mechanisms involved in the T cell modulation are the suppression of potential autoreactive naïve- T cell (characterized by a TCR directed against self-antigens) at initial stages in lymph nodes, or in later phases the T cell deactivation in peripheral tissues (**Figure 1**). This process is called peripheral tolerance and is exerted mainly by the immune checkpoints cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) pathways. Tumor cells have developed ways to take advantage of peripheral tolerance by inducing a deranged immune checkpoint expression by T cell in order to avoid immune recognition (7, 8).

ICIs represent different classes of monoclonal antibodies that interrupt the delivering of inhibitory signals to T cells, and reprogram adaptive immunity to participate to cancer elimination.

Given that our immune system is ontologically programmed to recognize and eliminate what could ultimately harm our organism, an effective anticancer immune response is achieved through the interaction between the T-cell receptor (TCR) on quiescent T cells, and a tumor associated antigen (TAA) presented by antigen-presenting cells (APCs), mainly but not exclusively represented by dendritic cells, within molecules of the major histocompatibility complex (MHC). In the immune synapse, antigen binding to TCR, and the following T cell activation is functionally dependent on a second signal mediated by the binding of the T cell CD28 transmembrane protein, and APC CD80/86 (also indicated as B7-1/B7-2 ligands). The resulting intracellular pathway mediated by the non-variable CD3 coreceptor, culminates in T cell proliferation, differentiation, and cytokines (e.g. IL-2) secretion. Importantly, the absence of this co-stimulation leads to T cell impaired activation and apoptosis. To prevent an overstimulation, after antigen binding to TCR, the immune checkpoint protein CTLA-4 is shuttled from intracellular vesicles to T cell surface, where it exerts a co-inhibitory signal by competitively binding the same CD80/86 molecules on APCs (9). Since the lack of CD28-mediated second signal in presence of CTLA-4 results in T cell anergy, the inhibition of CTLA-4 receptor (by means of the use of specific monoclonal antibodies) allows T cell activation, thus restoring anti-tumor immunity.

CTLA-4 signaling occurs in the tumor draining lymph nodes (**Figure 1**). This signaling is initiated when an APC migrates from cancer peripheral tissues to T cell-dependent areas and presents a tumor-associated antigen (TAA) to a naïve T -cell. Interestingly, in contrast with CD28, which is constitutively expressed on naïve T cells, CTLA-4 appears to be induced after 48 to 72 h following TCR triggering, and has been showed to replace the CD28 signaling with higher affinity at a lower surface density. The CTLA-4 (CD152) is a type 1 transmembrane glycoprotein belonging to the Ig superfamily. Initially discovered in 1987 by Brunet JF et al. (10), during the screening of a mouse T-cell derived cDNA libraries CTLA-4 has been demonstrated to be expressed not only on activated T cells, but also on regulatory T-cells (Tregs), due to their high levels of FoxP3, which is known to regulate CTLA-4 expression (11). Regarding the signals transduced upon binding, the CTLA-4 cytoplasmic tail has been demonstrated to contain PI3K-like

Abbreviations: AIN, acute interstitial nephritis; AKI, acute kidney injury; APC, antigen-presenting cells; ATIN, acute tubulointerstitial nephritis; CKD, chronic kidney disease; CNI, calcineurin inhibitors; CTLA-4, cytotoxic T-lymphocyte antigen 4; ESRD, end-stage renal disease; ICIs, immune checkpoint inhibitors; irAEs, immune related adverse events; IRI, ischemia/reperfusion injury; mTORi, mTOR Inhibitors; NSAID, non steroidal anti-inflammatory drugs; NSCLC, non-small-cell lung cancer; PD-1, programmed cell death protein 1; PD-L1/2, programmed death-ligand 1; PPI, proton pump inhibitors; TCR, T cell receptor.

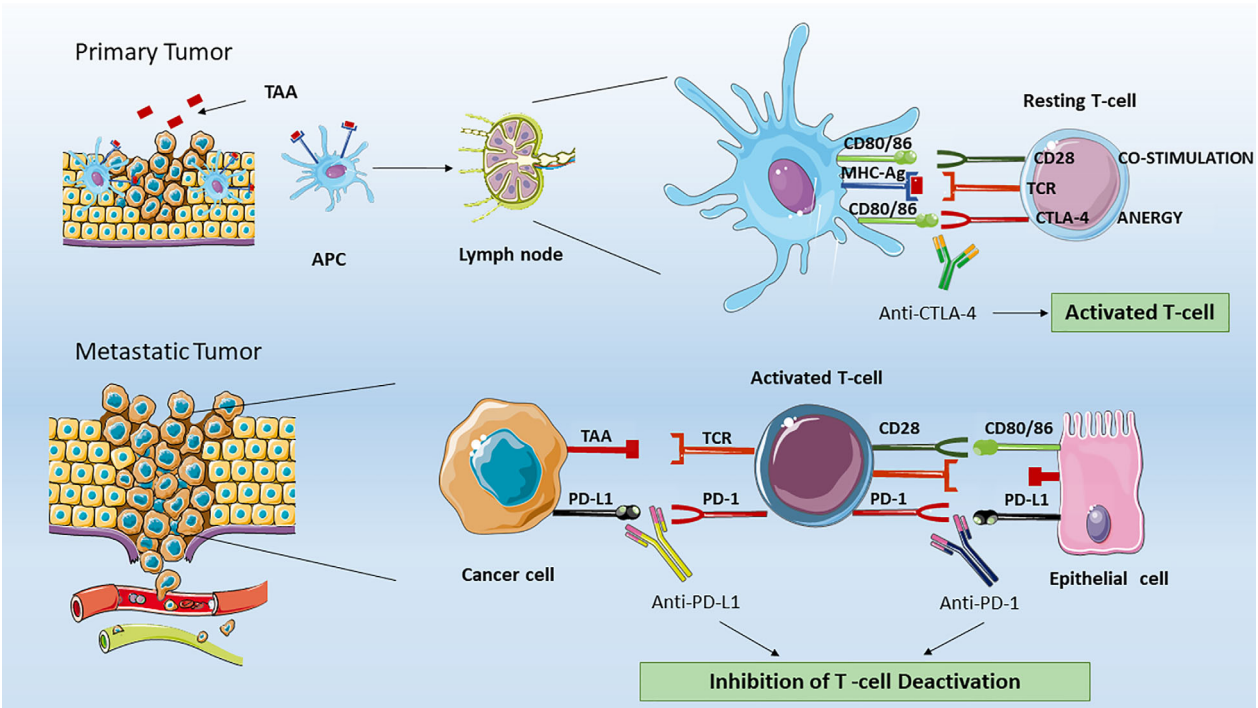

FIGURE 1 | Effect of ICIs on T lymphocytes. In the tumor microenvironment, professional APCs, such as dendritic cells processed specific tumor peptides (TAA) and complexed them to MHC molecules. Then, APC migrated to T cell-dependent areas of tumor draining lymph node and presented TAA to naïve or quiescent T cells. In the immune synapse between resting T cell and APC, the lymphocytes activation is dependent by two signals. The first is mediated by the binding of TAA to T-cell receptor (TCR). The second signal could be activatory in the case of binding of T cell-CD28 to co-stimulatory CD80/CD86 or inhibitory. The latter is mediated by the binding of T cell-CTLA-4 to the same CD80/CD86 APC molecules. Therefore, CTLA-4 and CD28 compete for the binding to CD80/CD86 proteins. The CTLA-4 signaling will lead to T cell anergy by inhibiting the T naïve activation and clonal expansion. The anti-CTLA-4 blocking by monoclonal antibodies as ipilimumab restore CD28 pro-activatory signaling and result in effective anti-tumor T lymphocyte responses. In peripheral tissues, the activated T cell can be de-activated by the binding of PD-L1 (or PD-L2, not shown) expressed on tumor cells, organ cells or other immune cells to effector T cell- PD-1 receptor. The anti-PD-1 or anti-PD-L1 blocking by monoclonal antibodies (as Nivolumab, Pembrolizumab for PD-1 or Atezolizumab for PD-L1) ipilimumab restore CD28 pro-activatory signaling and restore effective anti-tumor T lymphocyte responses. APC, Antigen Presenting Cells; MHC-Ag, Major Histocompatibility Complex with tumor antigen; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1.

motif therefore suggesting an interaction with PI3K, MAPK, and NF-kB pathways.

In addition, beside the structural similarity with CD28, CTLA-4 receptors are capable to sequester CD80/86 from the surface of the APCs, resulting in significant depletion of the ligands on their surface. The role of CTLA-4 as essential "brake" on T cells to restrain immune responses was supported by studies performed in CTLA-4–deficient mice. The latter showed early after birth the development of lymphoproliferative disease, an impressive enlargement of lymphoid organs, and a lethal autoimmune phenotype (12, 13).

The hypothesis that CTLA-4 blockade could improve anti-tumor immune response was confirmed by Allison JP et al. in transplantable murine colon carcinoma, and fibrosarcoma, models (14).

A large body of experimental evidences confirmed the beneficial role of CTLA-4 inhibition in increasing immune recognition and elimination also of poorly immunogenic murine melanoma (15) and prostate cancers (16).

The CTLA-4 immune checkpoint provided the first target for the treatment of advanced melanoma. From initial murine studies and clinical trials, it took 15 years before the US Food and Drug Administration (FDA) approved ipilimumab, the first Ig1 human immunoglobulin monoclonal antibody directed against CTLA-4 (**Table 1**).

PD-1 AND PD-L1

At tissue level and in tumor microenvironment, cancer cells immune escape is mediated by the PD-1 inhibitory signaling (**Figure 1**). Normally, the PD-1 receptor (PDCD1 or CD279) is expressed on effector T cells, B and NK cells, while its ligands PD-L1 and PD-L2 are expressed in various types of self-cells (as tubular epithelial, endothelial cells, fibroblastic reticular cells, pancreatic islet cells, astrocytes, neurons) thus avoiding autoimmunity and host organ injury. PD-L2 expression is limited primarily to APC (17). More importantly, on T cells,

TABLE 1 | Overview of principal ICIs, targeted tumor and clinical trials.

Immune checkpointed inhibited	Drugs	Year of approval	FDA-approved indications	Clinical Trial
CTLA-4	**Ipilimumab**	**2011**	Metastatic melanoma	Non-smal cell lung carcinoma NCT03469960, NCT03351361, NCT02785952, NCT03302234
			Renal cell carcinoma	Mesothelioma NCT02899299
			Colonrectal cancer	Gastric cancer NCT02872116
				Squamous cell lung carcinoma NCT02785952
PD-1	**Nivolumab**	**2015**	Metastatic melanoma	Mesothelioma NCT03063450
			Colonrectal cancer	Non-Hodgkin lymphoma NCT03366272
			Classical Hodgkin's lymphom	Metastatic clear cell renal carcinoma NCT01668784
			Renal cell carcinoma	Head and neck cancer NCT02741570, NCT03342352
			Non-small cell lung carcinoma	Lung cancer NCT03348904
			Head and neck squamous cell carcinoma (HNSCC)	
			Metastatic urothelial carcinoma	
			Hepatocellular carcinoma (HCC)	
			Colorectal cancer with MSI-H	
	Pembrolizumab	**2015**	Metastatic melanoma	Small cell lung cancer NCT03066778
			metastatic NSCLC	Renal cell carcinoma NCT03142334, NCT02853331
			classical Hodgkin's lymphoma,	Gastric adenocarcinoma NCT02370498
			primary mediastinal B-cell lymphoma (PMBCL)	Urothelial carcinoma NCT02853305, NCT03244384, NCT02256436, NCT03374488,
			Head and neck squamous cell carcinoma (HNSCC)	Colorectal cancer NCT02563002
			gastric cancer	Pleural mesothelioma NCT02991482
			solid tumors with MSI-H and MMR aberrations	Esophageal neoplasms NCT03189719, NCT02564263
			metastatic urothelial carcinoma	Multiple myeloma NCT02579863, NCT02576977
			Merkel cell carcinoma	Hodgkin lymphoma NCT02684292
			renal cell carcinoma	Hepatocellular carcinoma NCT02702401, NCT03062358
			Cervical cancer	
			Hepatocellular carcinoma	
	Cemiplimab	**2018**	Metastatic cutaneous squamous cell carcinoma	Cutaneous squamous cell carcinoma NCT04154943
PD-L1	**Atezolizumab**	**2016**	Metastatic urothelial carcinoma	Renal cell cancer NCT02684006
			Metastatic Non-small cell lung carcinoma	Gastric and gastroesophageal junction cancer NCT02625623, NCT02625610
			Metastatic Small cell lung carcinoma	Ovarian cancer, fallopian tube cancer NCT03038100, NCT02839707, NCT02891824
			Metastatic triple negative breast cancer	
	Avelumab	**2017**	Merkel cell carcinoma	Non-small cell lung carcinoma NCT02576574, NCT02395172
			Metastatic urothelial carcinoma	Urothelial cancer NCT02603432
				Diffuse large B-cell lymphoma NCT02951156

(Continued)

TABLE 1 | Continued

Immune checkpointed inhibited	Drugs	Year of approval	FDA-approved indications	Clinical Trial
	Durvalumab	**2018**	Metastatic urothelial carcinoma,	Non-small cell lung carcinoma NCT02273375, NCT02542293, NCT03164616, NCT02125461
			Unresectable stage III Non-small cell lung carcinoma	Squamous cell lung carcinoma NCT02154490, NCT02551159 Urothelial cancer NCT02516241 Advanced solid malignancies NCT03084471
Combination of CTLA-4 and PD-1	**Ipilimumab plus nivolumab**	**2016**	Metastatic melanoma	Non-small cell lung cancer NCT02659059
		2018	Metastatic renal cell carcinoma	Metastatic renal cell carcinoma NCT0223174
		2018	Colorectal cancer with MSI-H	Colorectal cancer with MSI-H NCT02060188
		2020	Hepatocellular carcinoma (HCC)	Hepatocellular carcinoma (HCC) NCT01658878

the PD-1 expression is a feature of "exhausted" lymphocytes that have previously experienced high levels of stimulation. This state of exhaustion is frequently observed during chronic infections and cancer and is characterized by deterioration of T cell function, resulting in inefficient control of infections and tumors (18). On the other hand, cancer cells strongly upregulate PD-L1 ligands, and in metastatic tissues the PD-1 pathway on memory T cell causes T cell deactivation. PD-L1 increased expression has been assessed on cell surface in several types of cancers including melanoma, bladder, lung, kidney, colon, ovary, breast, glioblastoma, multiple myeloma and T-cell lymphoma. The main mechanism associated to enhanced PD-L1 expression on tumor cells have been correlated to PTEN deletion (19), PI3K signaling and persistent high IFNγ levels in the tumor microenvironment (20). Blocking the PD-1, PD-L1, and PD-L2 signaling by monoclonal antibodies allows tumor-infiltrating lymphocytes to be reactivated to identify and destroy malignant cells. Initially discovered from Ihshida Y et al. (21) as an immunoglobulin expressed on dying thymocytes, PD-1 would have been later associated as essential negative regulator of T cell response. In accordance, PD-1–deficient mice were showed to develop autoimmune disorders such as lupus like syndrome, characterized by glomerulonephritis and arthritis, and autoimmune cardiomyopathy (22). The binding of PD-1 is known to induce the phosphorylation of the tyrosine residue located within Immunoreceptor Tyrosin-based Switch Motifs (ITSM) of the cytoplasmic tails, leading to recruitment of phosphatases SHP1 and SHP2, and dephosphorylation of downstream effectors such as Syk, PI3K, and CD3 (17). Currently, several monoclonal anti-antibodies have been approved by the US FDA targeting PD-1 (i.e., pembrolizumab, nivolumab, and cemiplimab) and the ligand PD-L1 (atezolizumab, avelumab, and durvalumab) for the treatment of a number of different malignancies, including NSCLC, metastatic melanoma, bladder cancer, advanced renal cell carcinoma, and others (**Table 1**).

In summary, one CTLA-4 inhibitor and five PD-1/PD-L1 inhibitors have been approved by the FDA and others are undergoing testing within phase 3 clinical trials.

NOVEL TARGET OF ICIs

Apart from CTLA4-4 and the PD-1/PD-L1, novel checkpoints have been discovered, which can be targeted by specific monoclonal antibodies (23). Indeed, ongoing research is focused to the improvement of the clinical management of cancer patients treated with ICIs, in order to reduce the occurrence of immune adverse effect (including nephrotoxicity), and overcome the resistance after prolonged treatments (24). Several experiments led to hypothesize that the blockade of a single immune checkpoint may result into a compensatory enhancement of other checkpoint receptors in the tumor microenvironment (25). For that reason, research moved towards the synergistic effect obtained by the combined blockade of different immune checkpoints, as in the case of the combination of ipilimumab plus nivolumab (**Table 1**).

The next generation of immune checkpoints includes the lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), B and T cell lymphocyte attenuator (BTLA), T cell immunoglobulin and ITIM domain (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), and B7 homolog 3 protein (B7-H3) (26).

LAG-3 (CD223) was first discovered by Triebel F et al. in 1990 as a novel lymphocyte activation gene closely related to CD4 (27). Further analysis of amino acid sequence would have revealed an approximately 20% of identity to CD4. LAG3 is expressed on CD4+ and CD8+ T cells, Tregs, B cells and plasmacytoid dendritic cells.

The LAG-3 signaling plays a negative regulatory role in T helper 1 (Th1) cell activation, proliferation, and cytokine secretion. Even if, given the high structural similarity between LAG-3 and CD4 should support the predominant binding to

MHC-II, other molecules can interact with LAG3 as galectin-3 (28) , LSECtin , and α-synuclein (29).

However, the binding affinity of LAG-3 for MHC-II is 100-fold higher than CD4, thus MHC-II is considered the canonical ligand (26).

In a murine model of ovarian cancer, Huang R-Y et al. (25) explored the effect of combined blockade of LAG-3 and PD-1 pathways. Authors showed that the dual blocking suppressed tumor growth by enhancing CD8+ tumor infiltrating T cells and decreasing Tregs in the tumor microenvironment in synergic manner (26).

Furthermore, Huang R-Y et al. also supported the hypothesis of a compensatory mechanism. Indeed, when they evaluated the level of other inhibitory receptors, they found that in mice treated with anti-PD-1, the levels of LAG-3 and CTLA-4 were increased. In accordance, the anti-LAG-3 administration led to augmented PD-1 levels (25).

The results from Fourcade J et al. (30) and Koyama S et al. (31) provided similar insights also in melanoma and lung cancer.

Besides experimental model, the first single center, phase I trial was run in 2006 in stage IV Renal Cell Carcinoma patients (NCT00351949). The trial tested the monoclonal antibody anti-LAG3 named IMP321 (Eftilagimod alpha), initially proposed as a vaccine adjuvant (32). The trial showed an overall reduction of tumor progression, as well as increased levels of activated CD8+ T cells.

The combination with anti LAG3 and PD-1 has been assessed also in in patients with previously unresectable or metastatic NSCLC and with metastatic squamous cell carcinoma of the head and neck (NCT03625323).

A great repertoire of LAG-3 monoclonal antibodies and blocking agents is under active evaluation within ongoing clinical trials, making compelling results on safety, efficacy and potential nephrotoxicity, not yet available.

More than 20 clinical trials have been registered using the first commercially available monoclonal antibody directed against LAG-3 named Relatlimab (BMS-986016) (33).

In advanced solid tumor (as NSCLC, renal cell carcinoma, bladder cancer, squamous cell carcinoma of the head and neck and melanoma) the trial NCT01968109 is evaluating the efficacy of Relatlimab as a monotherapy or in combination with Nivolumab (an anti-PD-1 antibody).

IMMUNE-RELATED ADVERSE EVENTS (IRAEs)

In the past few years, treatment with ICIs dramatically improved the outcome of a number of solid tumors, extending progression-free and/or overall survival in patients with melanoma, NSCLC, urothelial cancer, renal cell cancer, and many other malignancies (**Table 1**). However, the exuberant activation of immune response generated by treatment with ICIs is complicated by a new class of side effects called immune-related adverse events (irAEs). IrAEs are often serious, characterized by clinical manifestations that closely resemble autoimmune diseases. Almost all organ and system can be affected by irAEs, mainly skin, gastrointestinal tract and liver followed by lungs, nervous system, endocrine organs, joints, heart, pancreas and the kidneys (34). Thus, the most frequent ICIs-induced irAEs are dermatitis, rash, vitiligo, colitis, pneumonitis, hypophysitis, hypothyrodims, and other endocrinopathies (35). The incidence of irAEs is wide, ranging from 15% to 90%, with severe forms ranging from 0.5% to 13% (36).

The manifestations occurred can vary depending on the type of ICIs used, although the frequency and severity are higher with anti-CTLA4 antibodies (especially ipilimumab) (37).

Even more severe (grade III and IV) toxicities may occur in as many as 20% of the patients treated with combined anti–CTLA-4 and anti–PD-1 agents. The timing of the onset of these irAEs varies widely, but appears to be within weeks to months of exposure, and may occur even after ICIs discontinuation (38). In addition, those irAEs that develop with one class ICIs (i.e. anti–CTLA-4) may not necessarily occur with exposure to another class (i.e. anti–PD-1/PD-L1) (34).

An emerging complication of ICIs administration is kidney damage, which includes acute kidney injury (AKI) – possibly evolving towards chronic kidney disease (CKD), proteinuria, and electrolyte abnormalities (5).

Originally, in contrast to extrarenal irAEs, the incidence of adverse effects affecting the kidneys appeared to be less common. Yet, epidemiological data were mainly retrieved by sparse, small case report, and were far from being reliable. In addition, too often oncologist reports these events just as "creatinine increase," without further specifications. The estimated incidence of ICIs-associated AKI (ICIs-AKI) derives from Cortazar FB et al., who used pooled data from all phase 2 and 3 clinical trials published between 2014 and 2015, which enrolled at least 100 patients treated with ICIs (38, 39).

From a total of 3695 patients treated with ICIs monotherapy, overall incidence of AKI was of 2,2%. Regarding severe AKI, defined as an increase in serum creatinine (SCr) more than threefold above baseline, an increase in SCr to 4.0 mg/dl, or the need for renal replacement therapy (RRT), the detected incidence was lower (0.6%) (38, 39).

However, even if incidence of nephrotoxicity with monotherapy with any of the classes of ICIs was moderate, combinations including both an anti-CTLA-4, as well as an anti–PD-1, agent has been shown to be up to 5%. In particular, AKI was more common with combination therapy with ipilimumab/nivolumab combination therapy (4.9%) than with monotherapy with ipilimumab (2%), nivolumab (1.9%), or pembrolizumab (1.4%) alone (5, 35).

Fittingly, a meta-analysis by Manohara S et al., which evaluated 48 clinical trials that included 11,482 patients, reported an estimated incidence of ICIs-AKI of 2% (40). Consistently, Seethapathy H et al. (41) examined the incidence of ICIs-AKI in a setting of 1843 patients treated from May 2011 to December 2016 at the Massachusetts General Hospital. As estimated by the above mentioned meta-analysis, an incidence of 3% was determined. Interestingly, given the increasing use of these agents in a broad spectrum of malignancies (42), the

incidence of relatively new irAEs such as AKI has been theorized to be rising from 9,9 to 29% in a near future (43).

CLINICAL FEATURES OF ICIs-AKI

Previous case reports based the diagnosis of ICIs-induced AKI solely on renal biopsy (39, 44). The major histological features observed were acute tubular interstitial nephritis (ATIN) associated with edema, interstitial inflammation, and infiltration of T-lymphocytes, eosinophils and plasma cells. Urine analysis often displayed sterile pyuria and white blood cell casts (45, 46). In the last years, given the increased use of ICIs in a wide range of cancer, a need to harmonize the definition of AKI has emerged.

The definition and stage of AKI is regulated by the Kidney Disease Improving Global Outcomes (KDIGO) criteria according to relative changes in SCr (47). For instance, AKI stage II is defined as doubling of SCr, while stage III as tripling of SCr, or the need for RRT.

In order to standardize the ICIs-AKI definition across different studies, National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAEs) (48) describes AKI, in part, by comparing changes in SCr to the "upper limit of normal" cut-off parameters. In particular, the NCI-CTCAEs recognize five different grades of renal injury based on creatinine levels (grades 1–3), dialysis requirement (grade 4), and death (grade 5).

However, patients with cancer often have decreased muscle mass and these definitions may therefore be inadequate to detect increases in SCr that would fall within the "normal range". Therefore, the application of NCI-CTCAEs criteria seem to fail in capture the lower grade kidney complications, and to completely ignore several AKI episodes that would have encountered as ICIs complication (5). These observations could explain the difficulty in the precise estimation of AKI in patient treated with ICIs. Additionally, for all definitions of ICIs-AKI, it is critical that the renal injury be directly attributable to the ICIs, and not to an alternative causes.

Another confounding element is the longer latency period between ICIs initiation and AKI development. In contrast with typical drug-induced acute interstitial nephritis (AIN) and to other extrarenal irAEs, the recently evaluated median time from ICIs initiation to AKI occurrence is 14 weeks (49), with several patients developing AKI later (6). As examples, an extrarenal irAE as dermatitis usually occurs within 4 weeks of treatment (50), whereas colitis within 6 weeks from ICIs start (49).

The delayed onset of AKI could be explained by the prolonged longevity of activated T-cells, rather than a direct toxicity of ICIs. Yet, even though all the ICIs have a long half-life of 2 to 3 weeks which allows longer intervals between dosing, the onset of AKI can occur from 8 months to 2 years (51) after treatment start. Thus, could not merely associated to classical drug nephrotoxicity as conventional chemotherapeutics. Furthermore, the pharmacokinetic of ICIs revealed that these drugs are not cleared by the kidney, but are primarily cleaved by proteolytic degradation within the target tissues by lysosomes, after receptor mediated endocytosis (5). For that reason, ICIs do not need dose adjustment for kidney impairment, and has been safely used in patients with end-stage renal disease (ESRD) (52, 53). However, patients with advanced CKD showed an increased risk of ICIs-induced AKI, therefore it is highly recommended that these patients should have a careful evaluation of renal function during treatment with ICIs.

As anticipated, a large body of evidences demonstrated that ATIN represents the most common histological finding in biopsies from patients experiencing ICIs-AKI (39, 45, 54–56).

However, the AIN induced by ICIs is closer to what observed during autoimmune diseases (46), as compared to drug hypersensitivity reactions (57). In a multicentric study that enrolled 138 patients with ICIs-induced AKI, ATIN was found in 93% of biopsied patients (6). This finding confirmed previous results in a series of 13 patients with ICIs-AKI in which the dominant pathologic lesion (observed in 12 patients) was AIN, characterized by diffuse interstitial infiltrates of CD3+ and CD4+ T lymphocytes and associated to granulomas in 3 cases (39). Thrombotic microangiopathy was a defining feature of one patient's pathology in this series, whose comorbid conditions included pre-existing hypertension.

In another smaller series, AIN was found in all 6 patients treated with ICIs who underwent kidney biopsy for AKI (54).

Glomerular lesions are less common, as compared to ATIN; however, in some case reports they have been associated predominately to the use of the anti-PD1 nivolumab. In one study of 16 patients with biopsy-proven ICIs-AKI, ATIN was present in 14 of the 16 cases, but co-occurred with glomerular disease in nine cases, including glomerulonephritis, IgA nephropathy, pauci-immune glomerulonephritis, and thrombotic microangiopathy (45).

Other small series have reported nephrotic syndrome with minimal change disease associated to the treatment with both anti-CTLA-4 (55), and anti–PD-1, antibodies (45, 58).

In a case report, Daanen RA et al. (59) described the occurrence of a severe nephrotic syndrome with AKI secondary to treatment with nivolumab in a patient with papillary renal cell carcinoma. Interestingly, during 8 weeks of nivolumab treatment, the patient showed AKI, hypoalbuminemia and proteinuria, whereas renal biopsy exhibited focal segmental glomerulosclerosis. In another case, Jung K et al. (60) presented the autoimmune glomerulonephritis as well as the tubulointerstitial injury in a patient treated with nivolumab for clear cell carcinoma. Interestingly, an immune complex-mediated glomerulonephritis with cellular crescents and necrosis was observed together with diffuse mesangial deposition of IgA, C3, and kappa and lambda light chains. At electron microscopy, one glomerulus showed several hump-like subepithelial deposits, and no subendothelial deposits and partial podocyte foot process abnormalities. Proximal tubules were flattened with simplified tubular epithelium and shorter microvilli. Pathologic examinations confirmed the final diagnosis of acute toxic-type tubular injury and IgA-dominant acute post-infectious glomerulonephritis (60).

These observations emphasize the heterogeneity of histopathologic features of injury from ICIs, as well as the immune activation seen in patients with ICIs-AKI. A further

mechanism leading to podocyte foot process impairment, as observed for minimal-change disease and focal segmental glomerulosclerosis, could be the persistent, chronic release of inflammatory cytokines by T cells associated to ICIs-induced abrogation of CTLA-4 signaling (61).

Recently, risk factors for the development of ICIs-AKI have been evaluated (6). In the same multicenter study of 138 patients with ICIs-AKI, which included 276 unmatched control patients who received ICIs contemporaneously but did not develop AKI, Cortazar FB et al. identified three independent risk factors for ICIs-AKI: concomitant use of proton pump inhibitors (PPI), the combined treatment with anti–CTLA-4 and anti–PD-1/PD-L1 agents, and a lower baseline eGFR (6, 39, 41). Regarding the use of PPI as risk factor for ICIs-induced AKI, Cortazar FB et al. confirmed previous studies (62) describing an increased susceptibility to ATIN by the PPI in the general population. This event could be explained by the mechanism of reactivation of drug-specific T cell that will be further described. The finding that combination therapy is also associated to ICIs-AKI could be explained by the well-documented enhanced predisposition to irAEs.

The synergistic effect of dual checkpoint blockade was investigated by several researchers (63) mainly in murine model of advanced melanoma. Under the combined inhibition of the inhibitory receptors CTLA-4 and PD-1, tumor-infiltrating T cell numbers increased, a change in the ratio of effector T cell to Tregs was induced, and effector T cell function was improved. Given these promising animal models, Wolchock et al. (64) tested the combination of ipilimumab and nivolumab in metastatic melanoma patients. Although objective clinical responses were found in the range of 40%, more than 53% of patients exhibited grade 3 and 4 toxicities. Combination therapy with nivolumab plus ipilimumab has resulted in a prolonged overall survival also in patients with renal cell carcinoma (65). Nevertheless, the study from Motzer RJ et al. showed an incidence of any-grade irAEs of 93%, despite an objective response rate of 42%.

Finally, in a recent open-label, phase 3 trial involving patients with advanced NSCLC, treatment with nivolumab plus ipilimumab resulted in a longer duration of overall survival (66), while the percentage of patients with grade 3 or 4 treatment-related adverse events was 32.8%.

These results clearly demonstrate the superiority of the combination of ipilimumab and nivolumab over monotherapy, and the heterogeneity in irAEs depending on cancer type. To date, grade 3 and 4 irAEs are frequent, although these effects are usually easily manageable, at least in referral centers where ICIs are commonly administered.

MECHANISMS OF ICIs-INDUCED AKI

Although the mechanisms underlying ICIs-induced AKI are yet to be elucidated, some hypotheses have nevertheless been advanced, based on murine models and commonly observed extrarenal irAEs (**Figure 2**).

First, CTLA-4 and PD-1 inhibition could lead to the development of autoantibodies against self-antigens present on tubular epithelial cells, mesangial cells, or podocytes (56). Relevantly, ipilimumab treatment was associated to a lupus-like glomerulopathy, and to serum circulating levels of anti dsDNA and anti-nuclear antigen antibodies closely resembling the autoimmune lupus nephritis phenotype (4, 67). More importantly, the level of circulating autoantibodies appeared to be restrained by ICIs interruption, and glucocorticoid administration (56, 68) (**Figure 2**).

Second, another mechanism could be the development, the proliferation and the aberrant activation of a clone of self-reactive T-cells. This hypothesis can be supported by the presence of a robust infiltration of effector T-cell in organs not related to the tumor, which presented an impressive high level of similarities in TCR sequence. Intriguingly, Johnson DB et al. reported the cases of patients with melanoma treated with ipilimumab and nivolumab in whom fatal myocarditis developed. Within the tumors of these patients, Authors observed high levels of self-muscle-specific antigens (desmin and troponin) indicating that T cells could be targeting an antigen shared by the melanoma, skeletal muscle, and the heart (69).

It is reasonable to hypothesize that also an intrinsic kidney antigen, initially tolerated but recognized as non-self with the brake of CTLA-4/PD-1 signaling in self-reactive T cells could be responsible for acute tubulointerstitial nephritis (70, 71). It has been reported that some auto-reactive T cells escape negative selection in the thymus and are kept dormant by several mechanisms to prevent autoimmunity. Further studies are required to demonstrate the TCR clonality in tumor and kidney in ICIs-T cells-related nephrotoxicity.

An alternative hypothesis is that renal tubular cells express PD-L1, which protects them from T-cell-mediated autoimmunity. Ding H et al. showed that PD-L1 is constitutively expressed on HK-2 cells, and is dramatically upregulated by IFNγ. In normal kidneys, in situ hybridization and immunohistochemical staining revealed constitutive low expression of PD-L1 on proximal tubules at both mRNA and protein levels. However, PD-L1 higher expression was found in kidneys with type IV lupus nephritis. In vitro, pre-treatment of IFNγ-stimulated HK-2 cells with anti-PD-L1 significantly enhanced IL-2 secretion from co-cultured, mitogen-activated Jurkat or human peripheral blood T cells (72, 73). Therefore, anti-PD-L1 antibodies administrated for cancer immunotherapy could bind other sites than T cell or cancer cells leading to organ-specific injury (74, 75). However, given that ipilimumab is a fully human IgG1 characterized by the lack of antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, the underlying mechanisms of renal injury deserve more investigation (23). Together with PD-L1, renal allograft cells have been shown to upregulate also PD-1 during acute rejection as a protection mechanism of tubular cells from T cell mediated injury. The PD-1 increased level and the consequent enhanced PD-1/PD-L1 on Tregs has been extensively demonstrated to be beneficial during renal ischemia/reperfusion injury (IRI) (76, 77). In a mouse model of IRI, PD-L1 or PD-L2

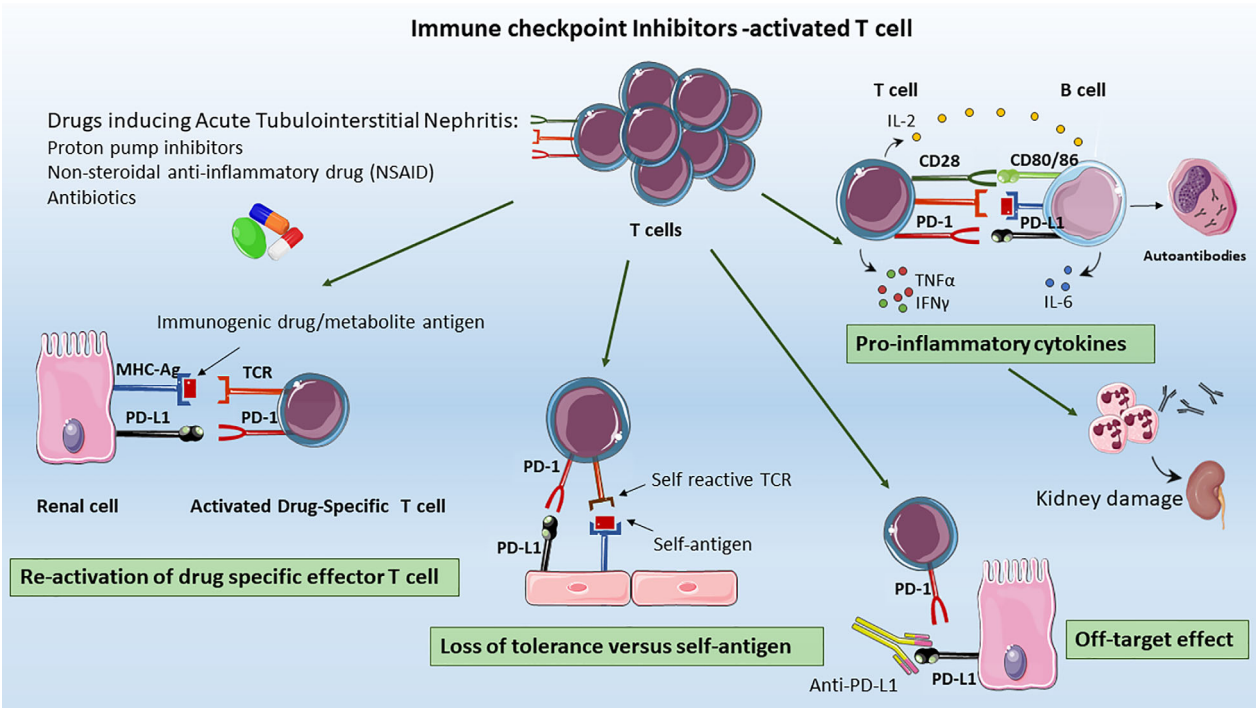

FIGURE 2 | Mechanisms of ICIs-associated AKI. The proposed mechanisms underlying ICIs-induced AKI include: *Re-activation of drug specific T cells:* T cell primed by different drugs (e.g. previous or concomitant antibiotics, PPIs, or NSAIDs) became latent over the time; however they can be re-activated by ICIs, leading to loss of tolerance*; Loss of tolerance versus self-antigens:* the formation, the selection and proliferation of a clone of self-reactive T-cells, the auto-reactive T cell could activated self-reactive B cells leading to auto-antibody release, that to renal injury; *Off Target Effect*: the upregulation of PD-L1 on renal tubular epithelial cells can lead to kidney damage by effector T lymphocytes infiltration resulting in acute tubulointerstitial nephritis, *Pro-inflammatory cytokines:* ICIs promote the migration and activation of effector T cells in renal tissue, the infiltration of other immune cells as B cells together with pro-inflammatory cytokines release as CXCL10, TNFα, IL-6 that contribute to the generation of an inflammatory milieu, leading to renal damage.

blocking by monoclonal antibodies, reduced Treg-mediated protection and significantly exacerbated the loss of kidney function, renal inflammation, and acute tubular necrosis (76) (**Figure 2**).

Thirdly, another explanation for ICIs-induced AKI is the reactivation of drug specific T cell through ICIs loss of tolerance. In the majority of reports, patients received concomitant medications as PPI and nonsteroidal anti-inflammatory drug (NSAID) to treat ATIN. The ATIN-drug exposure, iatrogenic and xenobiotic molecules can trigger an immune response either by itself or after binding to tubular antigens, thus acting as haptens. These T cell primed by drugs administration became latent over the time, however by ICIs they can be reactivated leading to loss of tolerance. Accordingly, in a patient with NSCLC treated with the anti-PD-1 antibody nivolumab who developed kidney injury, circulating lymphocytes appeared to be effectively stimulated by the PPI lansoprazole (78). Strikingly, patient showed rapid improvement in kidney function in 3 days (creatinine decreased from 2.74 to 1.82 mg/dl) upon discontinuation of lansoprazole (78). In summary, patients with ICIs-induced AKI receiving a concomitant drug (e.g. NSAID) showed a greater probability to completely recover renal function after the interruption of the drug (6, 78). This effect may be explained by T cell reactivity to the drug rather than to endogenous autoantigens; therefore, the cessation of the offending NSAID, antibiotics, or PPI would lead to a more rapid attenuation of T cell immunologic activity (38).

Furthermore, in the first multicentric study of 138 patients evaluating the clinical feature of ICIs-AKI, (6) nearly 70% of the patients with ICIs-AKI were receiving an ATIN related medication. In particular, 9% was receiving antibiotics, 22% NSAID and, surprisingly, 54% PPI. The latter recently emerged as the most common causes of drug-induced ATIN. Therefore, PPI should be used with caution in patients receiving ICIs treatment, and should be discontinued in those who develop ICIs-AKI.

In brief, anti–PD-1 antibody treatment can disrupt the peripheral immune tolerance between renal tubular cells, dormant auto-reactive T-cells, and tolerogenic dendritic cells (79). The prevalence of immature and functional defective plasmacytoid dendritic cells could also explain the development of tubulointerstitial nephritis after PD-1 therapy, irrespective of whether re-activated T-cells recognize kidney intrinsic antigens or specific drugs.

Finally, treatment with ICIs promotes the migration and activation of effector T cells in renal tissue and the infiltration of other immune cells together with pro-inflammatory cytokines release. In accordance, patients treated with ICIs exhibit increased serum level of CXCL10, TNFα, IL-6 that contribute

to the generation of an inflammatory milieu, leading to renal damage (74). The importance of the increased cytokines levels has recently emerged in association with cytokine release syndrome (CRS), a serious complication of the switch from immunotherapy to targeted therapies (80). There are limited data regarding the efficacy of treatments in ICIs-AKI. Small case series have shown recovery of renal function with glucocorticoids in the majority of cases (39, 41, 54). A recent multicenter study from Cortazar FB et al. (6) demonstrated that the glucocorticoid treatment in a cohort of ICIs-AKI patients was independently associated with complete renal recovery. However, we are still far beyond the identification of a glucocorticoid regimen to prevent the progression of AKI.

ICIs AND KIDNEY TRANSPLANTATION: THE SWITCH TO mTORi AS A STRATEGY

Kidney transplantation is a life-saving therapy for patients with ESRD leading to improved survival and quality of life (81). Immunosuppression may increase susceptibility to cancer by inhibiting immune surveillance, predisposing to oncogenic viral infections, and reducing the rate of DNA repair (82). The risk of cancer is two to four-fold higher in transplant recipients as compared to age-, sex-, and race-matched individuals from similar geographic areas (82). This higher risk is often associated to prolonged immunosuppression that revert the balance between graft immune-tolerance and anti-tumoral immunity. After a solid transplant, the most common occurring cancers are the carcinomas of the skin, non-Hodgkin's lymphoma, Kaposi sarcoma, lung and cancer of the transplanted organ (i.e. liver or kidney) (83, 84). Besides the occurrence of the *de novo* cancer, patients with a history of cancer before transplantation are more likely to experience early cancer relapse, usually within 2 years after transplantation (85, 86).

The success of the first clinically approved ICIs has created an increased appreciation of immunotherapy also in transplanted patients.

Currently, there are no guidelines for the treatment of ICIs requiring transplanted patients since they have been excluded from all clinical trials of ICIs, and there are no randomized control trials. Conceptually, the crucial core of ICIs is whether stimulates the immune system to destroy the cancer cells, or else suppress immune response to prevent allograft rejection. In order to find a balance between ICIs-mediated T cell stimulation, and anti-rejection immunosuppression, case reports and small case series of transplanted recipients receiving ICIs have slowly appeared. By reviewing case reports, the frequency of rejection with the anti-CTLA-4 ipilimumab monotherapy emerged lower (33%) compared to patients treated with anti–PD-1 monotherapy (52%) (87).

The higher rate of rejection with anti–PD-1 treatment is not surprising. The PD-1/PD-L1 signaling has been described as pivotal in peripheral organ transplant homeostasis (88) and the PD-L1 overexpression in renal tubular cells is a mechanism to modulate T cell activation (72).

Regard the rejection type, acute rejection of transplanted kidney after PD-1 inhibitors occurs mainly through T cell mediated rejection, although antibody mediated rejection has been also observed (89).

Acute T-cell-mediated rejection after administration of PD-1 inhibitors may be explained by the ICIs-induced activation of T-cells against donor allograft antigens. This loss of tolerance leads to graft failure via T-cell infiltration in the renal interstitium, damaging renal tubular epithelial and endothelial cells. These findings are in line with the reported AIN, characterized by infiltration of T-cells and granulocytes in renal tissue, after treatment with ICIs in non-transplanted patients (90). As concerns the antibody-mediated rejection, it may be attributed to the proliferative response of B-cells induced by activated T-cells or activation of memory B-cells expressing PD-1 induced by the reduction in immunosuppressant use during PD-1 inhibitor treatment (91).

The sharp rates of rejection in transplant recipients under ICIs medications have led to the development of strategies before the initiation of ICIs. These approaches includes modification of dosage of immunosuppressive medications, the pre-emptive switch to corticosteroids and, more importantly, the switch from a calcineurin inhibitors (CNI) to mTOR inhibitors (mTORi) before ICIs initiation.

Lowering the dose of immunosuppressant has been considered a crucial management strategy in some cancers, such as post-transplant lymphoproliferative disorders and skin cancer. However, the reduction of immunosuppressive drugs before the initiation of PD-1 inhibitor has been observed to significantly increase the risk of graft failure. Indeed, it is well known that immunosuppressive therapies are vital in regulating acute allograft rejection and inducing long-term transplanted kidney survival (92–94).

In 1999, the main mTORi (sirolimus) obtained FDA approval for use in clinical kidney transplantation (95). Since then, an extensive literature has emerged not only on its effects on graft survival, reduced rejection and mortality but also on other important clinical outcomes, such as malignancy, cardiovascular disease and infection (84, 96). Thus, the majority of studies of mTOR inhibitors involved conversion from CNI either early (2–6 months) or late (>6 months) post-transplantation (97).

A large body of evidences suggest that early conversion from a CNI to an mTORi-based maintenance regimen can reduce the development of malignancies as non-melanoma skin cancer in transplant recipients (98–101).

The mTOR pathway is a key regulator of immune cells metabolism, proliferation and anti-inflammatory reactivity in both innate (dendritic cells and macrophages) and adaptive effectors (T and B lymphocytes) (102). As widely discussed, this pathways is often dysregulated in many types of solid and hematological malignancies (103). Therefore, in this scenario, mTORi are used both as immunosuppressive strategy to prevent graft rejection in transplanted patients and as antitumor therapy, indicating that a careful immunosuppressive dose modification in combination with immunotherapy should be administrated. However, to the best of our knowledge, in transplanted

patients with *de novo* cancer, there is no consensus on immunosuppressive treatment schedule, since early phase clinical trials are still ongoing (CA209-933ISR).

In an elegant study Sabbatini et al., investigated the oscillatory inhibition of mTOR activity in kidney transplant recipients and found that lower level of everolimus were able to induce a robust proliferation of Treg by TCR triggering, a decrease of neutrophils and CD8 T cells and a reduced proinflammatory activity. Authors then hypothesized the possibility that management of mTORi dosage level (lower by six to 10-fold than in the oncology setting) and administration schedule (twice versus once a day in cancer therapy) could target respectively immune tolerance or cancer growth control (104, 105).

The switching to mTORi, together with the dose reduction of other immunosuppressive drugs has been associated to better overall survival of both oncologic patients and graft independently of the stage, type of carcinoma and oncologic treatment. Indeed, in a monocentric cohort of more than 500 kidney and liver allograft recipients with *de novo* cancer, Rousseau B et al, demonstrated that mTOR inhibitor introduction with optimal oncologic treatment significantly improved survival of patients (106). Vanasek TL et al. showed that treatment with mTOR inhibitors and concomitant ICIs could maintain T-cell anergy (107). In addition, mTORi have been demonstrated to stimulate naïve T-cell differentiation into Tregs, especially in the presence of IL-2 (108). Therefore, it is arguable to sustain that treatment with mTORi not only could reduce cancer progression in a broad spectrum of malignancies, but could exert anti-tumor effects (109).

In a case report, a kidney transplant recipient was treated with nivolumab for metastatic duodenal adenocarcinoma. Immunosuppressive regimens included concurrent prednisolone and the mTOR inhibitor sirolimus. Tacrolimus was replaced by sirolimus before anti–PD-1, and serum sirolimus levels were initially maintained at lower levels (4–6 ng/mL) after anti–PD-1, and then increased to regimen values (10–12 ng/mL) 2 weeks after. Intriguingly, the patient maintained renal graft function without tumor progression (110).

In addition, in a recent case report Esfahani K et al. (111) investigated the effect of the combination of mTORi and an ICIs (sirolimus plus pembrolizumab) in a kidney transplanted patient with melanoma. Interestingly, the ICIs-mTORi combination decreased the global CD8+ T cell activation responsible of ICIs-induced kidney allograft rejection. Furthermore, the dual therapy did not reduce the IFN-γ–producing CD4+ T cells that persisted in circulation. Thus, mTORi supported the immune tolerance while potentially adding anti-tumor efficacy to PD-1 blockade in patients with metastatic melanoma.

Finally, the ability of sirolimus to prevent T cells responses against renal allograft was investigated in 64 patients of multicenter trial (101).

Euvrard S et al. randomly assigned transplant recipients who were taking CNI and had cutaneous squamous-cell carcinoma either to receive sirolimus as a substitute for CNI or to maintain their initial treatment. Strikingly, switching from CNI to sirolimus led to longer disease-free survival among kidney-transplant recipients with previous squamous-cell carcinoma (101). Further studies are necessary to assess the potential effect of conversion from CNIs to mTOR inhibitors on both rejection and cancer. However, as the incidence of rejection in patients receiving ICIs therapy is very high, the general recommendation is to frequently monitor patients' kidney function by weekly SCr during all treatment.

BIOMARKERS FOR ICI-BASED IMMUNOTHERAPY AND ICIs-INDUCED AKI

ICIs-based immunotherapies has been shown to improve survival of patients in several types of advanced cancers (112). However, despite the promising results in terms of overall survival, many patients still experience severe irAEs (2, 36). There is a critical need to define biomarkers that can anticipate clinical outcome and the risk of organ toxicity in patients receiving ICIs. Major efforts in biomarker studies are ongoing and ICIs appeared encouraging (113). Several candidates have been proposed including the body composition parameters (e.g., age>75 years and female gender) (114, 115), systemic non-invasive biomarkers, tumor associated molecular features (e.g., PD-L1 expression and tumor mutation burden) (116, 117) and commensal bacteria (118). For the large part of these factors, the validation in independent patient cohorts with large sample size is still required (119). Nevertheless, some factors have been approved by FDA to select patients that would benefit from the treatment (e.g., PDL1 expression as a biomarker for patient selection) (120, 121). Despite promising results, more research is required to identify and validate the exact combination of biomarkers able to predict treatment outcomes and the occurrence of kidney nephrotoxicity (122). Here we will summarize the principal biomarkers associated to ICIs-irAEs and that could correlate with the development of AKI.

SYSTEMIC BIOMARKERS OF IRAEs AND ICI-INDUCED AKI

Systemic biomarkers of irAEs as lymphocytes and eosinophils counts, neutrophil-to-lymphocytes ratio (123, 124), and cytokine circulating level have been assessed due to poor invasive and routine measurements.

Regarding blood cell count, the higher number of eosinophils and T lymphocytes has been demonstrated to correlate with better survival in melanoma patients treated with pembrolizumab (125). Recently, Nakamura Y et al. and Diehl A et al. provided evidence that in melanoma and renal carcinoma eosinophils counts was also associated to the incidence of irAEs (124, 126).

In the plethora of pro-inflammatory cytokines, IL-17 is associated with autoimmune disease like rheumatoid arthritis, psoriasis, and inflammatory bowel disease (127) (i.e. Chron's disease). Therefore, the evaluation of IL-17 levels in ICIs-treated patients seems more than reasonable (128).

IL-17 is mainly released by Th17 CD4+ cells that are potent inducers of autoimmunity and are regulated by CTLA-4. Indeed, CTLA4 blocking by means of tremelimumab has been demonstrated to increase Th17 cells in peripheral blood of patients with metastatic melanoma and to correlate with autoimmune toxicity (129). Tarhini AA et al. reported that higher levels of circulating IL-17 at baseline associated with incidence of irAEs as diarrhea and colitis in melanoma patients treated with ipilimumab. In addition, TGF-β1 and IL-10 levels were associated with clinical outcome (128).

Besides irAEs, IL-17 is also over-released during AKI and associated with poor outcome (130). In a recent study, Maravitsa P et al. showed that IL-17 was the only cytokine highly produced from peripheral blood mononuclear cells (PBMCs) and CD4- lymphocytes of patients with septic shock and AKI, and that was gradually consumed from the kidney (131). Interestingly, a persistent increase in circulating Th17 cells was observed in mice model of renal IRI and correlated with systemic organ damage as pulmonary fibrosis (132). Similar results were found in renal transplanted patients with Delay Graft Function (130). Another promising biomarker of irAEs is CD163, a receptor expressed from M2 macrophages that are largely present in the tumor microenvironment (133). M2 macrophages are characterized by immunosuppressive properties, thus often associated with poor prognosis. The soluble sCD163 obtained by the proteolytic shedding of the receptors is increased in autoimmune disease (134) and fittingly, in melanoma patients during anti-PD1 treatment (135).

Recently, the conversion of pro-inflammatory (M1) to anti-inflammatory (M2) macrophage types has obtained a renewed appreciation particularly during the AKI-to-CKD transition (136).

In a cohort of sepsis patients, the diagnosis value of urine sCD163 levels were evaluated for predicting AKI occurrence, as well as for assessment of patients' prognosis (137). More recently, Sun PP et al. enrolled 205 patients with renal intrinsic AKI revealing a sharp augment of urinary sCD163 in glomerulopathy cases (138). In addition, urinary CD163 showed better diagnostic performance in differentiating disease etiologies compared to traditional urinary biomarkers of AKI (i.e. NGAL and KIM-1). Similar findings were reported in human ATIN biopsies (139). Kim M-G et al. showed a positive correlation between the density of CD68+ macrophages and the severity of AKI, whereas the density of CD163+ M2 macrophages was associated with a lack of renal functional recovery.

Inflammation has an integral role in the pathophysiology of irAEs. The principal pro-inflammatory cytokine IL-6 can promote tumor progression via inhibition of cancer cell apoptosis as well as promotion of angiogenesis. Plasma increased IL-6 levels have been correlated with poor overall survival in melanoma patients treated with ICIs-based immunotherapy (115).

In accordance, IL-6 is commonly elevated in inflammatory arthritis following ICIs therapy (140) as demonstrated in several type of cancer (e.g. malignant melanoma) (141).

From a renal perspective, IL-6 is a well-recognized biomarkers of renal injury (142, 143) and has been evaluated as central tool for predicting the development of AKI in critically ill patients (144, 145) as well as in the recent pandemic COVID-19 disease (146).

The measurement of the serum enzyme lactate dehydrogenase (LDH) is well recognized in the follow-up of patient with metastatic melanoma (147, 148) as it has prognostic value in renal cell carcinoma (149).

LDH is released by rapidly growing tumors characterized by a high cellular turnover. Recently, LDH has emerged as an independent factor for poor prognosis in patients with advanced melanoma (147, 150) treated with ipilimubab (151), nivolumab, and pembrolizumab (152). Similar results were reported also in NSCLC (153).

In the kidney, LDH has been shown to correlate with the principal parameters of kidney impairment (including estimated glomerular filtration rate (eGFR), microalbuminuria and proteinuria) (154, 155); in addition, urinary LDH has been evaluated in the early detection of acute tubular necrosis (156, 157) preceding AKI.

Altogether, these results lead us to speculate that IL-17, sCD163, IL-6, and LDH levels during ICIs treatment may serve as predictive markers for irAEs and evaluated in combination with other urinary markers of AKI (commonly KIM-1, L-FABP, IGFBP7, IL-18) could provide additional information also for the risk of ICIs-induced AKI.

IMMUNOSENESCENCE AND CELL CYCLE ARREST AS BIOMARKERS OF ICIs-INDUCED AKI

Immunosenescence describes the process of progressive deterioration of the immune functions during aging due to a several causes such as: (i) reduced NK cell-mediated cytotoxicity and perforin release, (ii) altered TLRs and NODs activation on monocytes, decreased phagocytosis, ROS generation; increased basal production of proinflammatory cytokines; (iii) less efficient antigen presentation and phagocytosis by dendritic cells; reduced secretion of IFNγ and IL-12, (iv) systemic inflammaging as a state of chronic, low grade inflammation and the (v) thymic involution leading to reduced naïve T cell and increased memory cells in the elderly (158–160). Dysregulated functions associated to immunosenescence can include reduced responses to vaccination, lower antitumor ability of CD4, CD8 T cells and APC, increased systemic inflammation, as well as autoimmunity (79). Aging contributes to a reduced repertoire of naive CD8+ T cells and to an increased pull of memory, senescent or exhausted T cells, hence leading to a decline of adaptive immunity (161, 162).

Several clinical trials have observed the impact of immunosenescence on the effectiveness of ICIs. From a bird eye view, aged patients benefit less from the PD-1 inhibitors and CTLA-4 inhibitors in certain cancers, even though several exceptions have been reported (163).

A possible explanation behind this observation is that ICIs rely on intact immune responses to tumor neoantigens, thus a misbalance in immunocompetent T cells significantly impaired the efficacy of treatments.

However, even if meta analyses suggested the correlation between poor survival benefit for anti-PD-1 agents and age older than 75 years, it should be observed that chronological age does not necessarily reflect biological age of immune system. Besides age, many other conditions can induce immunosenescence since caloric restriction, nutrition or physical activity can delay this process (158) .

Recently, Moreira A et al. analyzed immunosenescence markers from PBMC of patients with newly diagnosed, untreated, metastatic melanoma (164). Regardless to patients' age, the Authors demonstrated that the loss of senescence markers on PBMC is correlated with clinical response to ICIs. These markers included CD27 and CD28, as well as Tim-3 and CD57 (165). The loss of CD27 and CD28 on CD4+ and CD8+ T cells, as well as the expression of the Tim-3 and CD57, all of them senescence markers, correlated with resistance to ICIs. In particular, the mucin domain containing protein T-cell immunoglobulin-3 (Tim-3) is a marker for T-cell exhaustion and combined PD-1/PDL1 and Tim-3 blockade have been proposed to prevent T-cell exhaustion in patients with hematologic malignancies (164).

Latterly, Zaretsky JM et al. performed a whole-exome sequencing in biopsy samples from metastatic melanoma patients treated with anti–PD-1 therapy (166), reporting that resistance to ICIs was associated with defects in the interferon pathway that plays an important role in immunotherapy resistance mechanism since it can induce cell senescence (167, 168). Thus, the disruption of INF-γ-induced cellular senescence could partially explain late acquired resistance to ICIs and disease progression.

In recent clinical trials, cell cycle arrest biomarkers as tissue inhibitor of metalloproteinase 2 (TIMP2) and insulin-like growth factor binding protein 7 (IGFBP7) have been demonstrated to be effective in the early detection of AKI (169–171), and to perform better than other biomarkers such as NGAL, IL-18 (172), L-FABP, and KIM-1 (173, 174). During AKI, in response to tubular injury or DNA damage, IGFBP7 is highly expressed and directly can increase the expression of p53 and p21, whereas TIMP2 promoted the augment in p27. The proteins p53, p21 and p27 together with p16 blocked the cyclin-dependent protein kinase (CDKs) od cell cycle resulting in G1 phase arrest (171). Furthermore, AKI is associated to progressive increased level of other cell cycle arrest markers as p16 and p21 and klotho reduction (175). The premature renal aging has been observed in several model of IRI-induced AKI with p21 augmented amount both at renal (176–178) and at urinary levels (179).

In conclusion, cell cycle arrest biomarkers could represent a step forward toward prediction of ICIs response and recognition of ICIs-induced AKI. Additional validation studies are needed in order to fully characterize their clinical usefulness in combination with other markers, in order to predict survival, occurrence of irAEs, and renal function deterioration (180).

GUT MICROBIOME AS BIOMARKERS OF IRAE AND ICI-INDUCED AKI

The gut microbiome is composed by more than 3.8×10^{13} bacteria able to maintain host physiology and immune homeostasis (181). Recent advances in metagenomic analysis has improved our understanding of microbiota-related effects in health and disease. Alterations in intestinal microbiota dynamics (dysbiosis) has been linked to multiple human diseases, including intestinal disorders and cancers (182). In addition, gut microbiota composition has been associated to ICIs response, ICIs-induced irAEs (as colitis) and AKI (119).

Regarding response to ICIs, through the analysis of fecal samples and gut bacteria identification, several authors showed that bacteria composition correlated with immunotherapy response in the treatment of melanoma (183–185), renal cell carcinoma (118, 186) or NSCLC (118).

In melanoma patients, Chaput N et al. provided evidences that a microbiota enriched with *Faecalibacterium* genus and *Firmicute*, instead of *Bacteroides*, was associated to a better outcome during ipilimumab therapy (183).

Furthermore, Gopalakrishnan et al. by performing a bioinformatics analysis of gut microbiome samples of melanoma patients indicated that higher diversity and abundance of the *Ruminococcaceae* family bacteria was protective before anti-PD treatment (185). However, despite the study from Chaput N et al. and others (183, 185, 187) indicated a better outcome in *Faecalibacterium* and *Firmicute* gut microbioma, they also revealed a higher frequency of ICIs-induced irAEs such as colitis. In the plethora of commensal bacteria, Routy B et al. identified that the *Akkermansia muciniphilia*, one of the most abundant bacteria in the ileum microbiota, was able to strengthen the efficacy of anti-PD1 therapy by reinforcing intestinal barrier integrity and reducing systemic inflammation (118, 184).

Gut microbiota appears to have a central in the progression of renal injury since strongly linked to uremic toxins (188, 189). Several studies the improvement of CKD and ESRD after gut microbiota-directed intervention (190, 191). Bidirectional interaction between gut microbiota and kidney is being recognized as an important modulating factor in AKI (192).

The profile of AKI-microbiota has been recently characterized by metagenomic sequencing. Interestingly, the abundancy of *Erysipelotrichia*, *Lactobacillus salivarius* and *Bacteroides sp* in rodent model of renal IRI and cisplatin-induced AKI was reported (193, 194).

Other data supports the hypothesis that gut microbiota influence kidney function and kidney resident immune cells through Short Chain Fatty Acids (SCFAs). SCFAs (as acetate and propionate) are produced as the end products of the fermentation of dietary fibers by gut microbiota, and are released into the systemic circulation (193, 195). Administration of SCFAs was found to significantly improve renal dysfunction in a model of IRI-induced AKI (195).

It is well known that antibiotics can perturbate the gut microbiota and increase the risk of developing inflammatory bowel disease (196). Interestingly, the transfer of an antibiotic-perturbed microbiota from mouse mother to newborn promoted and accelerated the development of gut inflammation in the offspring (197).

Regarding the combination with antibiotics, Routy B et al. showed that dysbiosis generated by administration of antibiotics

significantly affect antitumor response to ICIs in both mice and humans (118). Similar results were found by Jang HR et al. that demonstrated commensal microbes have a protective role in the pathogenesis of AKI, since they regulate CD8 T cells trafficking and modulated renal inflammation and injury (198). In contrast, depletion of gut microbiota using broad spectrum antibiotics protected from IRI-induced AKI by reducing maturation status of F4/80+ renal resident macrophages and bone marrow monocytes (199).

Finally, gut microbiome has been implicated in the modulation of metabolism and linked to nutrition-related chronic diseases such as obesity and diabetes. This lead to hypothesize that lack of a healthy diet may result in impaired immune function during ICIs treatment. Surprisingly, a study shows that obese patients with metastatic melanoma may acquire more benefit from anti-PD therapy than those with normal body mass index (200). Many possible explanations have been mentioned to elucidate this paradox of obesity in cancer, some of which relate to observational biases and the inadequacy of body mass index as an accurate representation of obesity (201).

In summary, gut microbiota may have important implications for the immune response to ICIs and to subsequent development of AKI. Although we are still far from utilization of gut microbiome as ICIs biomarker, the manipulation of commensal bacteria constitution (i.e. by administration of SCFAs) could offer new therapeutic strategies to reduce ICIs-related irAEs and nephrotoxicity (202).

PD-L1 OVEREXPRESSION

The PD-L1 overexpression is a strategy of tumor cells to evade immune surveillance. The mechanism of escape is exerted by promoting the T cell exhaustion through PD-1 inhibitory signaling.

Patients with high PD-L1 tumor expression seemed more likely to benefit from anti-PD-1 treatment, although responses were seen even in patients with low or no PD-L1 expression (203, 204).

In several retrospective studies, PD-L1 was the first factor shown to correlate with better outcomes as observed by higher response rate and longer overall survival in melanoma (205) and NSCLC (206). Given these promising results, the PDL-1 expression by immunohistochemistry in tumor biopsy has been considered as one most widely used biomarkers for response to ICIs and has been approved by FDA for patients with NSCLC (112, 207).

However, several concerns still remain for accurate measurement of PD-L1 expression, including different protocols used in each laboratory, the tumor heterogeneity that cannot be represented by the small region of the biopsy sample, the methods for PD-L1 quantization that rely on pathologist evaluations (112). For that reason, additional biomarkers of response to ICIs as the high tumor mutational burden (TMB) are currently assessed and reviewed elsewhere (66, 208).

From a renal perspective, PD-L1 overexpression by renal tubular epithelial cells has been reported in mice model of sepsis-induced AKI (209).

Authors demonstrated that overexpression of PD-L1 is a central mechanism to induce immunosuppression during sepsis, leading to T cell apoptosis and impairment of renal vessel permeability. In addition, they speculated that PD-L1 overexpression could be a potential biomarker to diagnose septic AKI and the treatment with anti-PD-L1 might be a beneficial therapy for septic AKI. In other studies, PD-L1 and PD-L2 have been demonstrated to be involved in AKI and inflammation in a model of bilateral IRI (76). The blocking of PD-L1 and PD-L2 by monoclonal antibodies prior kidney IRI significantly exacerbated the loss of renal function, kidney inflammation and ATIN. A possible explanation could be found in the beneficial PD-L1 /PD-L2 on immunosuppressive Treg able to mediated protection against kidney IRI (77).

In conclusion, PD-L1 signaling appeared to have both a detrimental effect in cancers requiring ICIs or during sepsis, and a beneficial effect on Treg after renal ischemic injury.

However, PD-L1 overexpression on tumor and renal tissue appeared to be a useful biomarker of response to ICIs and the assessment of PD-L1 expression on Treg cells could be important to predict nephrotoxicity during ICIs-induced AKI.

AUTHOR CONTRIBUTIONS

RF and ER mainly contributed to the conception, the design, and the writing of the manuscript. GN and FS partly contributed to literature bibliography search. GC and CP critically supported the final draft editing and revised the manuscript. LG and GS revised the final manuscript. RF conceived of all figures. RF took the lead in writing the manuscript, reviewers' revisions, and figure changes. All authors contributed to the article and approved the submitted version.

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Poly (ADP-Ribose) Polymerase Inhibitor Treatment as a Novel Therapy Attenuating Renal Ischemia-Reperfusion Injury

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Intrarenal robust inflammatory response following ischemia-reperfusion injury (IRI) is a major factor in the pathogenesis of renal injury in ischemic acute kidney injury (AKI). Although numerous studies have investigated various agents of immune modulation or suppression for ischemic AKI, few showed reproducible effects. We hypothesized that poly (ADP-ribose) polymerase (PARP) inhibitor may favorably change post-ischemic intrarenal immunologic micromilieu by reducing damage-associated molecular pattern (DAMP) signals and improve renal outcome in ischemic AKI. The effects of JPI-289 (a PARP inhibitor) on early renal injury in a murine IRI model and hypoxic HK-2 cell model were investigated. Bilateral IRI surgery was performed in three groups of 9-week-old male C57BL/6 mice (control, JPI-289 50 mg/kg, and JPI-289 100 mg/kg; n = 9–10 in each group). Saline or JPI-289 was intraperitoneally injected. Renal function deterioration was significantly attenuated in the JPI-289 treatment groups in a dose-dependent manner. Inflammatory cell infiltration and proinflammatory cytokine/chemokine expressions in the post-ischemic kidneys were also attenuated by JPI-289 treatment. JPI-289 treatment at 0.5 and 0.75 μg/ml facilitated the proliferation of hypoxic HK-2 cells. PARP inhibition with JPI-289 treatment showed favorable effects in ischemic AKI by attenuating intrarenal inflammatory cascade in a murine model and facilitating proliferation of hypoxic HK-2 cells.

Keywords: acute kidney injury, Ischemia-reperfusion injury, poly(ADP-ribose) polymerase (PARP) inhibitor, parthanatos, inflammation, translational immunology

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INTRODUCTION

Ischemic acute kidney injury (AKI) caused by renal ischemia-reperfusion injury (IRI) develops in various ischemic conditions of both native and transplanted kidneys. The intrarenal robust inflammatory process that is initiated and facilitated by damage-associated molecular pattern (DAMP) signals following IRI is the most crucial factor in the pathogenesis of renal injury in ischemic AKI (1, 2). Although the long-term renal outcomes of allografts have been improved in both living donor and deceased donor kidney transplantation (KT) (3, 4), the clinical impact of renal IRI is still an important unresolved issue because renal IRI causes delayed graft function (DGF) and increases risk of acute rejection in renal allografts (5, 6). Although numerous reno-protective therapies for renal IRI have been studied in animals, few have demonstrated reproducible effects in patients with ischemic AKI and none of the interventions have been translated into clinical practice (7). Therefore, the development of a novel treatment strategy for ischemic AKI is required.

Poly (ADP-ribose) polymerases (PARPs) are cell signaling enzymes that catalyze the transfer of ADP-ribose units from nicotinamide adenine dinucleotide (NAD^+) to several acceptor proteins. PARP-1, the best characterized member of the PARP family, is an abundant nuclear enzyme implicated in the cellular response to DNA injury provoked by genotoxic stress (8). The earliest functions ascribed to PARP were DNA repair and the maintenance of genomic integrity (9). In response to low levels of DNA damage, PARP promotes cell survival and DNA repair. However, overactivation of PARP facilitates cell death through two distinct pathways: driving cells into an energetic deficit caused by depletion of intracellular NAD^+ and catalyzing the activation of proinflammatory pathways (9, 10). This overactivation of PARP by a wide array of stimuli including reactive oxygen species (ROS), termed parthanatos, is implicated in the pathogenesis of renal IRI (7), suggesting that PARP is a potential treatment target in renal IRI. Parthanatos can be blocked by genetic deletion of PARP-1 or pharmacological inhibition of PARP-1 by PARP inhibitors. PARP-1-deficient mice were protected in renal IRI (11). JPI-289, a recently developed novel PARP-1 inhibitor with strong PARP-1 inhibitory activity, showed beneficial effects in ischemic stroke models (12, 13).

In this study, we hypothesized that pharmacologic PARP-1 inhibition with JPI-289 may favorably change the postischemic intrarenal immunologic micromilieu and mitigate early renal injury following IRI.

Abbreviations: AKI, acute kidney injury; AU, arbitrary unit; BUN, blood urea nitrogen; CCL, CC-chemokine ligand; DAMP, damage-associated molecular pattern; DGF, delayed graft function; HK-2 cell, human kidney-2 cell; IFN-γ, interferon gamma; IL, interleukin; IRI, ischemia-reperfusion injury; KMNC, kidney mononuclear cell; KT, kidney transplantation; NAD^+, nicotinamide adenine dinucleotide; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; SEM, standard error of mean; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

MATERIALS AND METHODS

Study Design

We used an established murine IRI model for *in vivo* study and HK-2 cell (an immortalized proximal tubule epithelial cell line from normal adult human kidney) hypoxia model for *in vitro* study to investigate the effects of JPI-289 on ischemic AKI. In the murine IRI model, measurements of plasma creatinine and blood urea nitrogen (BUN) concentration and histological assessment of renal tissues were performed to assess functional and structural renal outcomes. For comprehensive analysis of the immunologic mechanism, intrarenal leukocyte trafficking, phenotypes of kidney mononuclear cells (KMNCs), and the expressions of intrarenal cytokines/chemokines and proinflammatory signaling pathways were assessed in post-ischemic kidney tissues. Cell proliferation and the expression of inflammatory/apoptotic signaling molecules were measured in the HK-2 cell hypoxia model. We also measured PARP-1 activities in the dose of JPI-289 applied in the hypoxic HK-2 cell model with biotinylated poly (ADP-ribose) loaded samples and the mice postischemic kidney protein extracts to test the PARP-1 suppression activity of JPI-289.

Mice and Renal IRI Model

The Samsung Medical Center Animal Care and Use Committee approved all studies. This study was approved by the institutional review board of Samsung Medical Center. Animal studies are reported in compliance with the ARRIVE guidelines (14, 15). Male C57BL/6 mice (9 weeks old) were purchased from Orient Bio Inc. (Seongnam, Kyoungki-do, Korea). All mice were housed in a specific pathogen-free barrier facility.

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg; Yuhan, Seoul, Korea) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). After an abdominal midline incision, the renal pedicles of both kidneys were carefully isolated and clamped for 27 min with a microvascular clamp (Roboz Surgical Instrument, Gaithersburg, MD). During the surgery, anesthetized mice were placed onto a thermostatically controlled heating table and kept well hydrated with warm sterile saline. After 27 min, microvascular clamps were released from renal pedicles for reperfusion. After being sutured, mice were allowed to recover with free access to chow and water. Sham controls underwent the same surgical procedure except for clamping of the renal pedicles. All mice were sacrificed on day 3 after surgery and kidneys were harvested after exsanguination.

Administration of JPI-289

Renal IRI model mice were randomized into three groups: control (n = 10), JPI-289 50 mg/kg (n = 10), and JPI-289 100 mg/kg (n = 9). JPI-289, 10-ethoxy-8-(morpholinomethyl)-1,2,3,4-tetrahydrobenzo [h] [1,6] naphtyridin-5(6H)-one dihydrochloride dehydrate, was synthesized by Jeil Pharmaceutical Co. Ltd (Seoul, Korea). JPI-289 (50 mg/kg or 100 mg/kg) or saline were administered twice intraperitoneally: right before reperfusion during the IRI surgery and at 24 h after the surgery. The surgery and administration of JPI-289 were performed by different investigators than the following experiments, ensuring that all analyses were performed in a blinded fashion.

Assessment of Renal Function

Plasma creatinine (Arbor Assays, Ann Arbor, MI) and BUN (Fujifilm, Bedford, UK) levels were measured in plasma samples obtained on days 1, 2, and 3 after surgery using colorimetric kits according to the manufacturer's recommended methods. Baseline plasma creatinine and BUN levels were measured at 7 days before surgery.

Measurement of PARP-1 Activity

The PARP-1 inhibitory effect of JPI-289 was assessed using a colorimetric PARP assay kit (R&D Systems, Minneapolis, MN) based on the incorporation of biotinylated ADP-ribose into histone proteins. Briefly, samples were loaded onto a 96-well plate coated with histones and biotinylated poly (ADP-ribose), incubated for 30 min followed by strep-HRP treatment, and read at 450 nm on a spectrophotometer.

Tissue Histological Analysis

After exsanguination, both postischemic kidneys were harvested. Tissue sections were fixed with 10% buffered formalin followed by paraffin embedding and staining with hematoxylin and eosin. A renal pathologist who was blinded to the experimental groups scored renal tubular damage of postischemic kidneys and the percentage of necrotic tubules were compared.

Assessment of Leukocyte Phenotype

KMNCs were isolated according to previously described methods (16). Briefly, decapsulated kidneys were immersed in RPMI buffer (Mediatech, Manassas, VA) containing 5% FBS and disrupted mechanically using a Stomacher 80 Biosmaster (Sweward, Worthing, West Sussex, UK). Samples were strained, washed, and resuspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) followed by gentle overlaying onto 72% Percoll. After centrifugation at 1,000 g for 30 min at room temperature, KMNCs were collected from the interface of 36% and 72% Percoll. KMNCs were then washed twice and counted on a hemocytometer using trypan blue exclusion.

Isolated KMNCs were preincubated with anti-CD16/CD32 Fc receptor blocking antibody for 10 min to minimize nonspecific antibody binding. KMNCs were then incubated with anti-mouse anti-CD3, -CD4, -CD8, -CD19, -CD21/35, -CD25, -CD27, -CD44, -CD45, -CD62L, -CD69, -CD126, -CD138, -F4/80, -FoxP3, -GR1, -TCRβ, and -NK1.1 (all from BD Biosciences, San Jose, CA) for 25 min at 4°C, washed with FACS buffer, and fixed with 1% paraformaldehyde solution. Acquisition and analyses of eight-color immunofluorescence staining were performed using a FACSVerse instrument and FACSuite program (BD Biosciences), respectively. Each assay included at least 10,000 gated events. Detailed gating strategies for flow cytometry analyses are provided in **Supplemental Figures**.

Immunohistochemistry of Renal Tissues With CD45

Formalin-fixed renal tissue sections were stained for CD45 using immunohistochemistry. Sections (4-μm-thick) were deparaffinized with xylene, rehydrated in a graded alcohol series, and then transferred to citrate buffer solution (pH 6.0). Slides were placed in a pressure cooker and heated by microwaving for 10 min to enhance antigen retrieval. After cooling, the kidney sections were immersed in a hydrogen peroxide solution (DAKO, Carpinteria, CA) for 30 min to block endogenous peroxidase activity, followed by overnight incubation at 4°C with serum-free protein block (DAKO). The next day, the slides were incubated with a 1:100 dilution of anti-mouse CD45 monoclonal antibody (BD Biosciences, San Jose, CA) for 1 h at room temperature. After being rinsed, the CD45-stained sections were incubated for 30 min at room temperature with a secondary antibody using a Dako REAL EnVison kit (DAKO). Subsequently, 3,3'-diaminobenzidine tetrahydrochloride (DAKO) was applied to the slides to produce a brown color and then the slides were counterstained with Mayer's hematoxylin solution (DAKO).

To calculate the percentage of CD45-positive cells in kidney samples, whole fields of slides including both cortex and medulla were scanned and analyzed with a TissueFAXS work station (Tissue Gnostics, Vienna, Austria), as described previously (17).

Multiplex Cytokine/Chemokine Assay

A panel of cytokines and chemokines was measured in whole kidney protein extracts using the Milliplex MAP Mouse Cytokine/Chemokine Kit (Luminex, Austin, TX) according to the manufacturer's instructions. This multiplexed, particle-based, flow cytometric assay uses anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct properties of two fluorescent dyes. Our assay was designed to quantify interleukin (IL)-2, IL-4, IL-6, IL-10, interferon (IFN)-γ, CC-chemokine ligand (CCL)-2 (monocyte chemoattractant protein-1), CCL5 (regulated on activation, normal T cell expressed and secreted), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). The limit of detection values of each cytokine/chemokine were as follows: IL-2, 1.0 pg/ml; IL-4, 0.4 pg/ml; IL-6, 1.1 pg/ml; IL-10, 0.8 pg/ml; IFN-γ, 1.1 pg/ml; CCL-2, 6.7 pg/ml; CCL-5, 2.7 pg/ml; TNF-α, 2.3 pg/ml; VEGF 0.3 pg/ml. The concentration (pg/ml) of intrarenal cytokine or chemokine was normalized by dividing the raw protein concentration (mg/ml, measured by Pierce BCA protein assay kit, Thermo Fisher Scientific, Waltham, MA) of whole kidney protein extract. Therefore, the final unit of cytokine/chemokine value was expressed as "pg/mg".

Western Blot Analysis

Toll-like receptor (TLR)-4, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Bax, and Bcl-2 were assayed by western blot analysis. Equal amounts of whole kidney protein extract (30 μg) were separated by electrophoresis on a NuPAGE Bolt mini gel system (Thermo Fisher Scientific) following the manufacturer's instructions. After electrophoresis, the gels were transferred onto a nitrocellulose membrane using an iBot 2 Dry Bottling system (Thermo Fisher Scientific). Membranes were blocked with 5% skim milk tris-buffered saline solution with 0.1% Tween20 for 1 h at room temperature and then incubated overnight at 4°C with one of the following antibodies: mouse monoclonal anti-TLR4 antibody (Novus Biologicals, Centennial, CO), mouse monoclonal anti-NFκB antibody (R&D Systems),

rabbit anti-Bax antibody (Cell Signaling Technology Inc., Danvers, MA), rabbit anti-Bcl-2 antibody (Cell Signaling Technology Inc.). After washing with TBST, the horseradish peroxidase-conjugated secondary antibody was applied for 30 min at room temperature. After washing, the signal was visualized using the Amersham ECL detection system (GE Healthcare, Chicago, IL), according to the manufacturer's instructions. Bands were then densitometrically analyzed using ImageJ 1.52k software (Wayne Rasband, National Institutes of Health, MD). Band density, expressed as an arbitrary unit (AU), was normalized against corresponding β-actin band intensities as an internal control.

HK-2 Cell Hypoxia Model

HK-2 cells were purchased from American Type Culture Collection (CRL-2190, Manassas, VA) and cultured in keratinocyte serum-free media (Thermo Fisher Scientific) supplemented with bovine pituitary extract and human recombinant epidermal growth factor. Cells were incubated at 37°C in humidified atmosphere of 5% CO_2 with media changes every 2 to 3 days.

Hypoxia was induced by exposure to 1% O_2 and 5% CO_2 balanced with nitrogen in a multi-gas incubator (APM-30D, Astec, Fukuoka, Japan) for 48 h. HK-2 cells were divided into 11 groups. The 2 control groups were the normoxia control group (21% O_2) and the hypoxia control group (1% O_2). The single-dose groups were treated with 0.1, 0.25, 0.5, 0.75, or 1.0 μg/ml of JPI-289 on day 0 (the day when cells were taken out from the multi-gas incubator) after hypoxia. The double-dose groups were treated with 0.1, 0.25, 0.5, and 0.75 μg/ml of JPI-289 on day 0 and day 1 after hypoxia.

HK-2 cells under normoxia were also treated with above mentioned dosages of JPI-289 to explore the effect of JPI-289 on normal kidney.

The degree of HK-2 cell proliferation on day 0, day 1, and day 2 was assessed with the Cell Titer96 aqueous one solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions.

To further analyze the effect of JPI-289 on hypoxia-induced cell signaling pathways and apoptosis, TLR-4 and NFκB were measured with western blot, and Bax and Bcl-2 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, MA).

Data and Statistical Analyses

All data were expressed as mean ± standard error of mean (SEM). Group means were compared with Mann-Whitney *U* test, Kruskal-Wallis test followed by Dunn's test, or ANOVA followed by Newman-Keuls *post hoc* analysis using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

RESULTS

JPI-289 Treatment Mitigated Functional and Structural Renal Injury Following IRI

Renal function following the operation was evaluated with daily measurement of BUN and plasma creatinine up to day 3 after the operation. BUN and plasma creatinine were stable in the sham control group but increased following IRI. Both BUN and plasma creatinine were significantly lower in the JPI-289–treated groups compared with the IRI control group in a dose-dependent manner (**Figures 1A, B**). We evaluated the tubular injury of cortex using H&E staining. Postischemic kidneys of the IRI model mice showed marked tubular damage compared to the sham control group on day 3 after operation. The proportion of damaged or necrotic tubules was significantly lower in the cortex of the JPI-289–treated groups compared with the IRI control group (**Figures 1C, D**).

JPI-289 Treatment Reduced Leukocyte Trafficking into Postischemic Kidneys

The trafficking of total leukocytes into postischemic kidneys was evaluated using immunohistochemical staining of CD45. The proportion of total leukocyte expressing CD45 among total nuclei in the whole field of each slide was semiquantitatively calculated with TissueFAXS system. Intrarenal total leukocytes expressing CD45 significantly increased in the postischemic kidneys compared to the kidneys of sham control mice. The percentage of total leukocytes was significantly lower in the JPI-289 treatment groups in a dose-dependent manner (CD45-positive cells among total nucleated cells, sham control vs. IRI control vs. 50 mg/kg vs. 100 mg/kg of JPI-289 (mean ± SEM): 0.22 ± 0.07 vs. 3.05 ± 0.37 vs. 1.82 ± 0.29 vs. 1.54 ± 0.21; **Figure 2**).

JPI-289 Treatment Decreased Intrarenal NK Cells and Macrophages

We analyzed the phenotypes of KMNCs by flow cytometry to assess the effect of JPI-289 on major effector cells of both innate and adaptive immune systems in the postischemic kidneys on day 3 after IRI. The percentages of total T cells, CD4 and CD8 T cells, activated CD4 and CD8 T cells, regulatory T cells, and effector memory CD4 and CD8 T cells were comparable between the control group and the JPI-289 treatment groups (**Table 1**). The percentages of total B cells, subpopulations of B cells, NK T cells were also comparable between groups. Although JPI-289–treated groups showed lower percentages of neutrophils, the differences were not statistically significant. However, intrarenal NK cells and macrophages on day 3 after IRI surgery were decreased by JPI-289 treatment (**Figure 3**).

JPI-289 Treatment Altered Cytokine and Chemokine Expressions in Postischemic Kidneys

To explore whether JPI-289 treatment affects intrarenal expressions of major pro- and anti-inflammatory cytokines/chemokines in postischemic kidneys, we analyzed intrarenal expression of IFN-γ, CCL2, CCL5, TNF-α, IL-2, IL-4, IL-6, IL-10, and VEGF. Comparison of the sham control group and the IRI control group showed that IRI enhanced intrarenal expressions of IFN-γ (P = 0.012), CCL2 (P = 0.009), TNF-α (P = 0.007), and IL2 (P = 0.036), and suppressed the expression of VEGF (P = 0.009). The expressions of proinflammatory

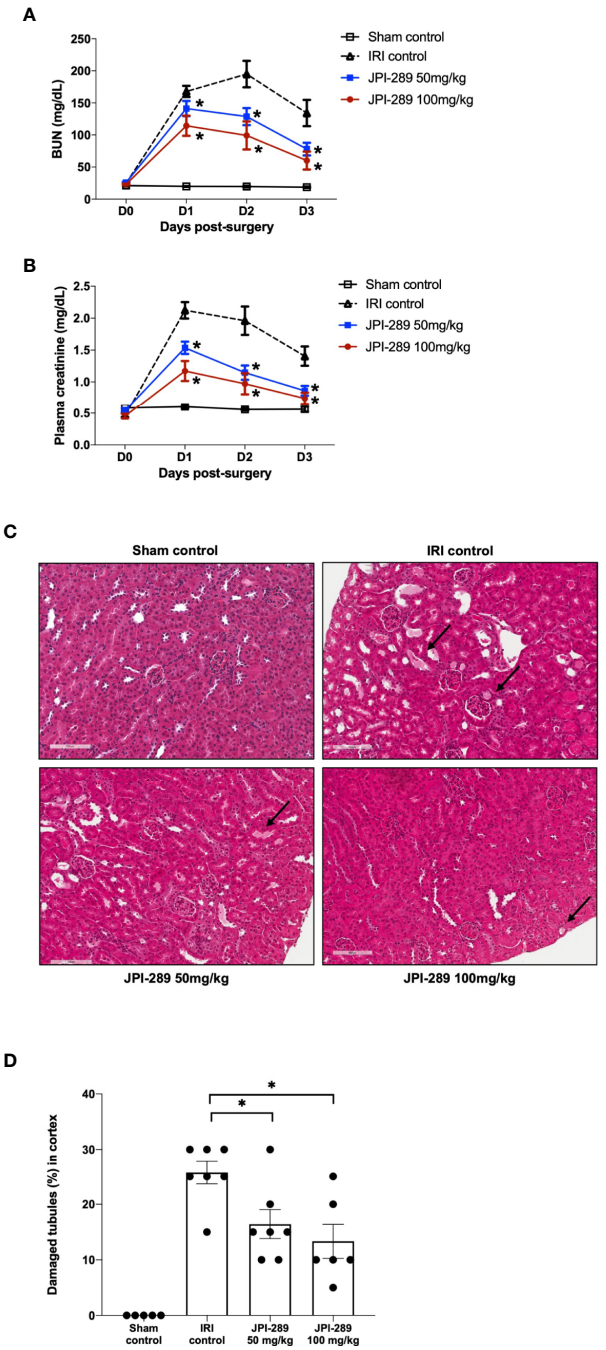

FIGURE 1 | Functional and structural renal injury following IRI. **(A, B)** Blood urea nitrogen (BUN) and plasma creatinine levels were significantly lower in the JPI-289-treated groups compared with the IRI control group in a dose-dependent manner. **(C)** Hematoxylin and eosin staining of the cortex of postischemic kidneys. Arrows indicate damaged or necrotic tubules (×200). **(D)** On day 3 after IRI, the JPI-289-treated groups showed less damaged or necrotic tubules compared to the IRI control group. A total of 10 fields magnified 200× were scored for each mouse by a pathologist blinded to the groups. Data are from three independent experiments. *$P < 0.05$, compared with the IRI control group (n = 6–10 in each IRI group, n = 5 in the sham control group). Statistical analyses were performed with two-way ANOVA test followed by Newman-Keuls test **(A, B)** or the Mann-Whitney U test **(D)**.

cytokines/chemokines including IFN-γ, CCL2, and IL-2 were significantly lower in the JPI-289 treatment groups compared with the IRI controls. In contrast, the intrarenal expression of VEGF was significantly higher in the JPI-289 treatment groups compared with the IRI control group. The expressions of CCL5, TNF-α, IL-4, IL-6, and IL-10 were comparable between IRI groups (**Figure 4**).

JPI-289 Treatment Suppressed TLR4 and NFκB Expression in Postischemic Kidneys

Since TLR4 and NFκB pathways are known to play critical roles in renal IRI by activating series of inflammatory genes and innate immune response, intrarenal expressions of TLR4 and NFκB were evaluated by western blotting of protein samples extracted from postischemic kidneys. The expressions of TLR4 tend to be lower with JPI-289 treatment. The expression of NFκB in the JPI-289 100 mg/kg group was significantly lower compared with the control group (**Figure 5A**).

JPI-289 Inhibited Apoptosis in Postischemic Kidneys

As apoptosis is a central feature of renal IRI, we measured the expression of an antiapoptotic molecule, Bcl-2, and proapoptotic molecule, Bax to examine the molecular responses associated with apoptosis pathway. Western blot assays of Bcl-2 and Bax proteins showed that JPI-289 downregulated the expression of Bax and upregulated the expression of Bcl-2 in the JPI-289 100 mg/kg group compared with controls (P = 0.03 between control group and 100 mg/kg of JPI-289 group, **Figure 5B**).

JPI-289 Treatment Facilitated the Proliferation of Hypoxic HK-2 Cells

To explore the effects of JPI-289 on human postischemic kidney, hypoxic HK-2 cells were treated with JPI-289. **Figure 6A** shows the degree of HK-2 cell proliferation after hypoxic insult according to the dose of JPI-289. Following hypoxic insult, 0.5 or 0.75 μg/ml of JPI-289 treatment facilitated the proliferation of hypoxic HK-2 cells compared with the hypoxia control group. However, lower or higher dosages, such as 0.25 or 1 μg/ml of JPI-289, did not show favorable effects on hypoxic HK-2 cells, which suggest that appropriate dosage may be important for attenuating IRI (**Figure 6A**). Under normoxic condition, JPI-289 treatment did not affect the proliferation of HK-2 cells (**Figure 6B**).

JPI-289 Treatment Suppressed TLR4 and NFκB Signaling Pathway in Hypoxic HK-2 Cells

Considering that JPI-289 facilitated the proliferation of HK-2 cell after hypoxic insult, we investigated whether JPI-289 treatment would suppress proinflammatory signaling pathways in hypoxic HK-2 cells. Western blotting of TLR4 and NFκB in protein extracts of hypoxic HK-2 cells showed that 0.5 or 0.75 μg/ml JPI-289 reduced the expressions of TLR4 and NFκB compared with the hypoxia control group on day 2 after hypoxic insult (**Figure 7A**).

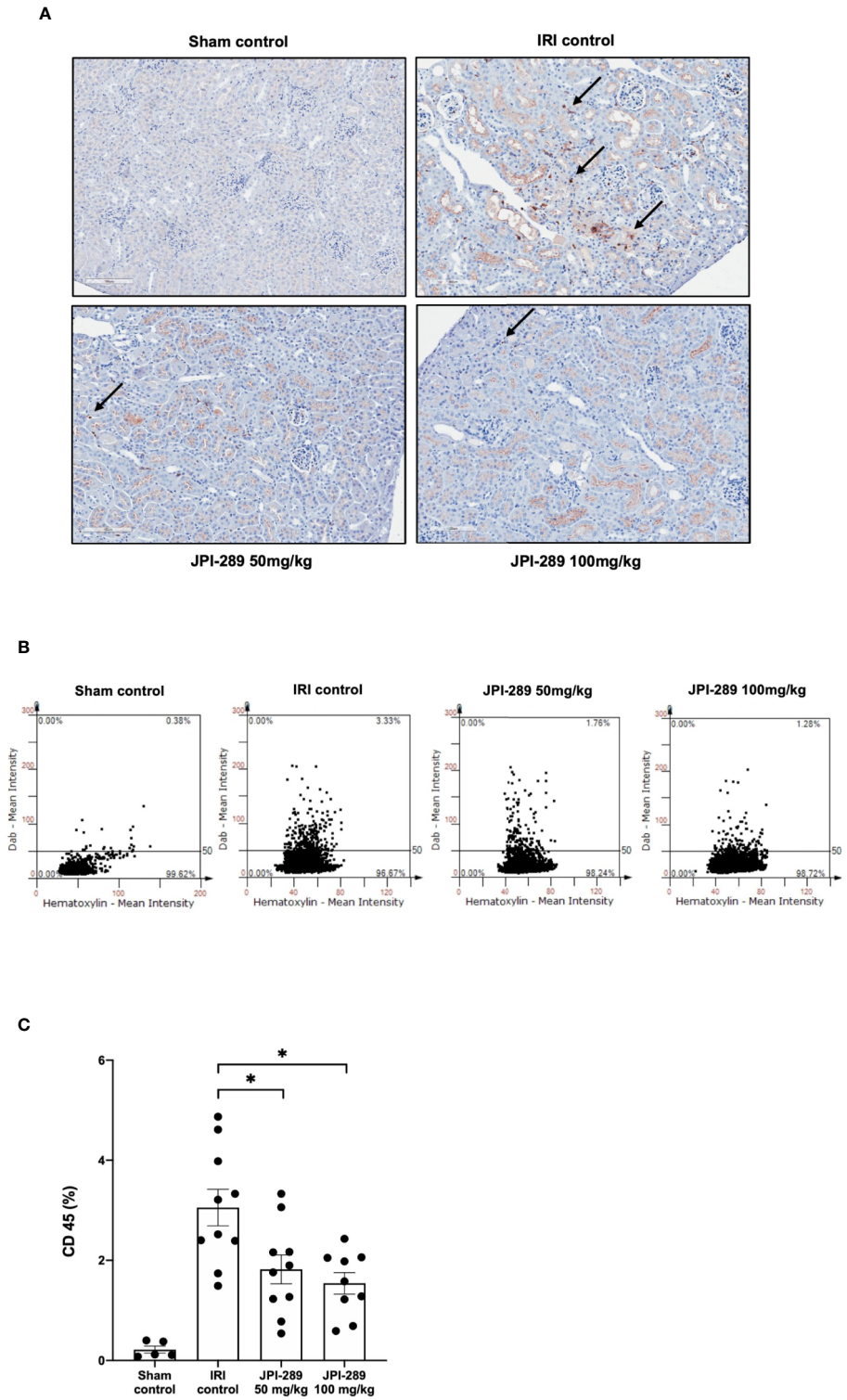

FIGURE 2 | Leukocyte trafficking into the postischemic kidneys. **(A)** There were more pronounced infiltrations of leukocytes into the postischemic kidneys of IRI control mice compared with JPI-289-treated groups. Arrows indicate CD45-positive leukocytes (×200). **(B)** Semiquantitative analysis of CD45-positive leukocytes in the postischemic kidney using automated imaging analysis system (TissueFAXS). The whole fields of slides including both cortex and medulla were evaluated. **(C)** The percentages of total leukocytes expressing CD45 among total nucleated cells were lower in the postischemic kidneys of JPI-289-treated mice compared with those of IRI control mice. Data are from three independent experiments. *$P < 0.05$, compared with the IRI control group (n = 6–10 in each IRI group, n = 5 in the sham control group). Statistical analysis was performed using the Mann-Whitney U test.

TABLE 1 | The leukocyte populations in the postischemic kidney on day 3 after IRI.

Leukocytes' types (% in parent gate)	Control	JPI-289 50 mg/kg	JPI-289 100 mg/kg
Of size and granularity-based gate			
Macrophages, %	39.0 ± 2.6	30.8 ± 2.1*	33.9 ± 2.2*
NK T cells, %	2.5 ± 0.3	1.9 ± 0.2	2.5 ± 0.2
NK cells, %	16.7 ± 2.5	10.4 ± 1.0*	11.4 ± 1.4*
Of size and granularity-based gate			
Neutrophils, %	4.9 ± 0.8	2.7 ± 0.3	3.2 ± 0.3
Among total lymphocytes			
Total T cells, %	25.1 ± 1.7	23.7 ± 1.1	22.3 ± 2.4
Total B cells, %	37.1 ± 2.5	42.1 ± 4.2	42.3 ± 1.9
Among total T cells			
CD4 T cells, %	67.9 ± 2.1	63.6 ± 1.6	65.8 ± 2.0
CD8 T cells, %	12.7 ± 1.6	15.1 ± 1.1	13.0 ± 1.7
Among total CD4 T cells			
Activated CD4 T cells, %	3.2 ± 0.3	3.2 ± 0.4	3.7 ± 0.3
Effector memory CD4 T cells, %	39.5 ± 2.9	39.6 ± 3.6	42.8 ± 4.9
Regulatory T cells, %	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
Among total CD8 T cells			
Activated CD8 T cells, %	2.3 ± 0.6	2.5 ± 0.4	2.1 ± 0.6
Effector memory CD8 T cells, %	14.9 ± 2.6	15.2 ± 3.2	20.3 ± 5.2
Among total B cells			
Activated B cells, %	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1
Mature B cells, %	93.3 ± 0.7	93.5 ± 1.2	94.6 ± 0.5
Memory B cells, %	5.1 ± 0.2	5.5 ± 0.7	6.1 ± 0.4
Plasma cells among KMNCs	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1

*Data (mean ± standard error of mean) are from two independent experiments. *P < 0.05, compared with the control group (n = 6–8 mice in each group).*
*Detailed gating strategies are provided in **Supplemental Materials**.*
IRI, ischemia-reperfusion injury; KMNCs, kidney mononuclear cells.

JPI-289 Inhibited Apoptosis of Hypoxic HK-2 Cells

We also examined the apoptosis pathway in the hypoxic HK-2 cell model. ELISA of Bax and Bcl-2 proteins in protein extracts of HK-2 cells showed that JPI-289 treatment downregulated the expression of the proapoptotic protein Bax and upregulated the expression of the antiapoptotic protein Bcl-2 on day 2 after hypoxic insult (**Figure 7B**).

JPI-289 Treatment Effectively Suppressed PARP-1 Activity

We measured PARP-1 activities depending on the JPI-289 dose applied in the hypoxic HK-2 cell model with biotinylated poly (ADP-ribose) loaded samples. The PARP-1 inhibitory effect was measured by the reduction in the amount of histone ribosylated by PARP-1 after JPI-289 treatment at the following concentrations: 0.25, 0.5, 0.75, and 1 μg/ml. PARP-1 activity was effectively inhibited by JPI-289 in a dose dependent fashion (**Figure 8A**).

To confirm that JPI-289 suppressed PARP-1 activity in the postischemic kidney, PARP-1 activities were measured in the postischemic kidney protein extracts. PARP-1 activities were lower in the postischemic kidney protein extracts of JPI-289-treated mice compared with controls in a dose-dependent manner (**Figure 8B**).

DISCUSSION

This study demonstrates that inhibition of PARP-1 by JPI-289 attenuated renal injury in a murine ischemic AKI model and facilitated the proliferation of hypoxic HK-2 cells. In addition, intrarenal inflammatory cell infiltration and proinflammatory cytokine/chemokine expressions were also mitigated by JPI-289 treatment. JPI-289 treatment during the early injury phase modified the proinflammatory micromilieu of postischemic kidneys to more favorable conditions with less inflammation and apoptosis without altering the proportion of intrarenal lymphocyte subpopulations, subsequently attenuating ongoing renal tissue damage. These results suggest the therapeutic potential of JPI-289 as a novel renoprotective agent without directly affecting adaptive immune system during the early injury phase of ischemic AKI.

Renal IRI is involved in various clinical situations including hypotension, shock from various etiologies, anesthesia, and surgical conditions such as cardiac and aorta surgery. Renal IRI is also an inevitable consequence of kidney procurement for kidney transplantation, which is associated with delayed graft function and rejections (1, 2, 7). Despite significant advances in immunosuppressive strategies and critical care medicine, conservative treatment including fluid therapy and dialysis remain the main treatment of ischemic AKI including DGF (1). Therefore, a novel pharmacological agent ameliorating renal IRI is required. Increased reactive oxygen and nitrogen radicals induce parthanatos, causing DNA fragmentation and pathological overactivation of PARP-1 in the postischemic kidney after IRI (7, 10). Since parthanatos plays a substantial role in the pathogenesis of renal IRI, this pathway may be a potential therapeutic target for the treatment of ischemic AKI (7).

Our results showed that JPI-289 exhibited immunomodulatory effects by suppressing intrarenal infiltration of total leukocytes and the production of proinflammatory cytokines/chemokines without changing the subpopulations of intrarenal lymphocytes. The trafficking of intrarenal total leukocytes was decreased in the postischemic kidneys of JPI-289-treated mice, but the proportions of total T cells, total B cells, and their subpopulations were comparable between the groups. In contrast, the infiltration of NK cells and macrophages was decreased in JPI-289-treated mice. Although the precise role of NK cell recruitment in renal IRI is not fully established (1), NK cells were reported to exert pathogenic effects in the development of AKI (18). The pathogenic roles of intrarenal macrophages during the injury phase after IRI were also well documented in previous studies (19, 20). JPI-289 treatment reduced the expression of intrarenal proinflammatory cytokines such as INF-γ, IL-2, and CCL2 that were reported to contribute to renal injury in postischemic kidneys (18, 21, 22). JPI-289 treatment also increased the expression of intrarenal VEGF, a well-known renoprotective chemokine after IRI (23, 24), whereas levels of IL-10, a well-known renoprotective cytokine (25), were comparable among groups. JPI-289 treatment also effectively suppressed the TLR4 and NFκB signaling pathways and apoptosis. Overall, these results show that JPI-289 treatment reduced DAMP signals in the

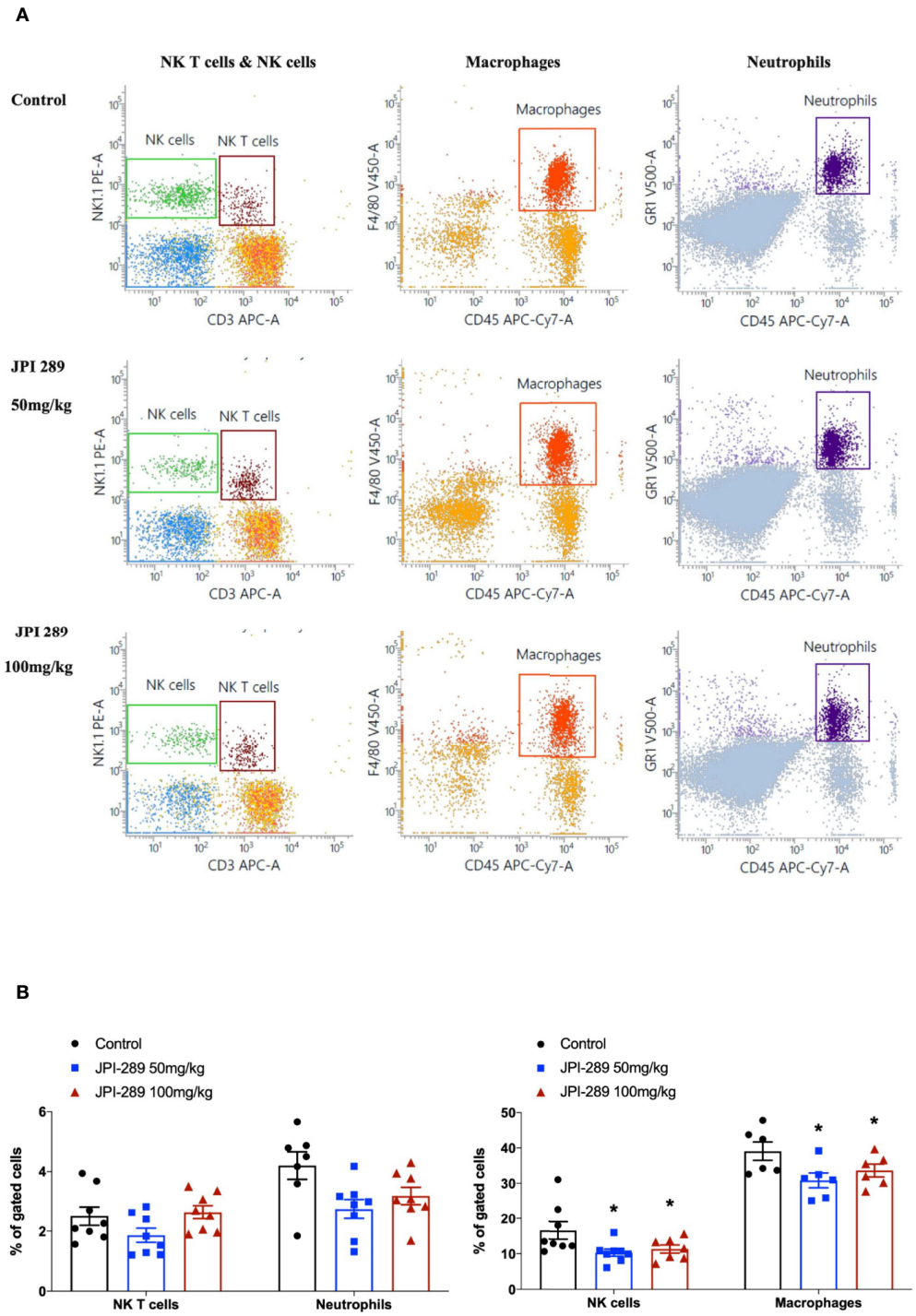

FIGURE 3 | Flow cytometry analyses of KMNCs isolated from postischemic kidneys on day 3 after IRI. **(A, B)** The infiltration of NK T cells were comparable among the 3 groups. The infiltration of neutrophils tended to be lower in the JPI-289 treated group. JPI-289 reduced the infiltration of intrarenal NK cells and macrophages. Data are from two independent experiments. *$P < 0.05$, compared with the control group ($n = 6–8$ in each group). Statistical analysis was performed with the Kruskal-Wallis test followed by Dunn's test. Detailed gating strategies for each population were as follows; Lymphocytes, monocytes, and granulocytes were first identified on the basis of their FSC and SSC. $CD45^+$ cells were gated to identify lymphocytes within the FSC and SSC based lymphocytes population. NK T cells were identified by $CD3^+$ and $NK1.1^+$ gate within lymphocytes. NK cells were identified by $CD3^-$ and $NK1.1^+$ gate within lymphocytes. Macrophages were identified by $CD45^+$ and $F4/80^+$ gate within the FSC and SSC based monocyte population. Neutrophils were identified by $CD45^+$ and $GR1^+$ gate within FSC and SSC based granulocyte population (more details are provided in **Supplemental Figure 3**).

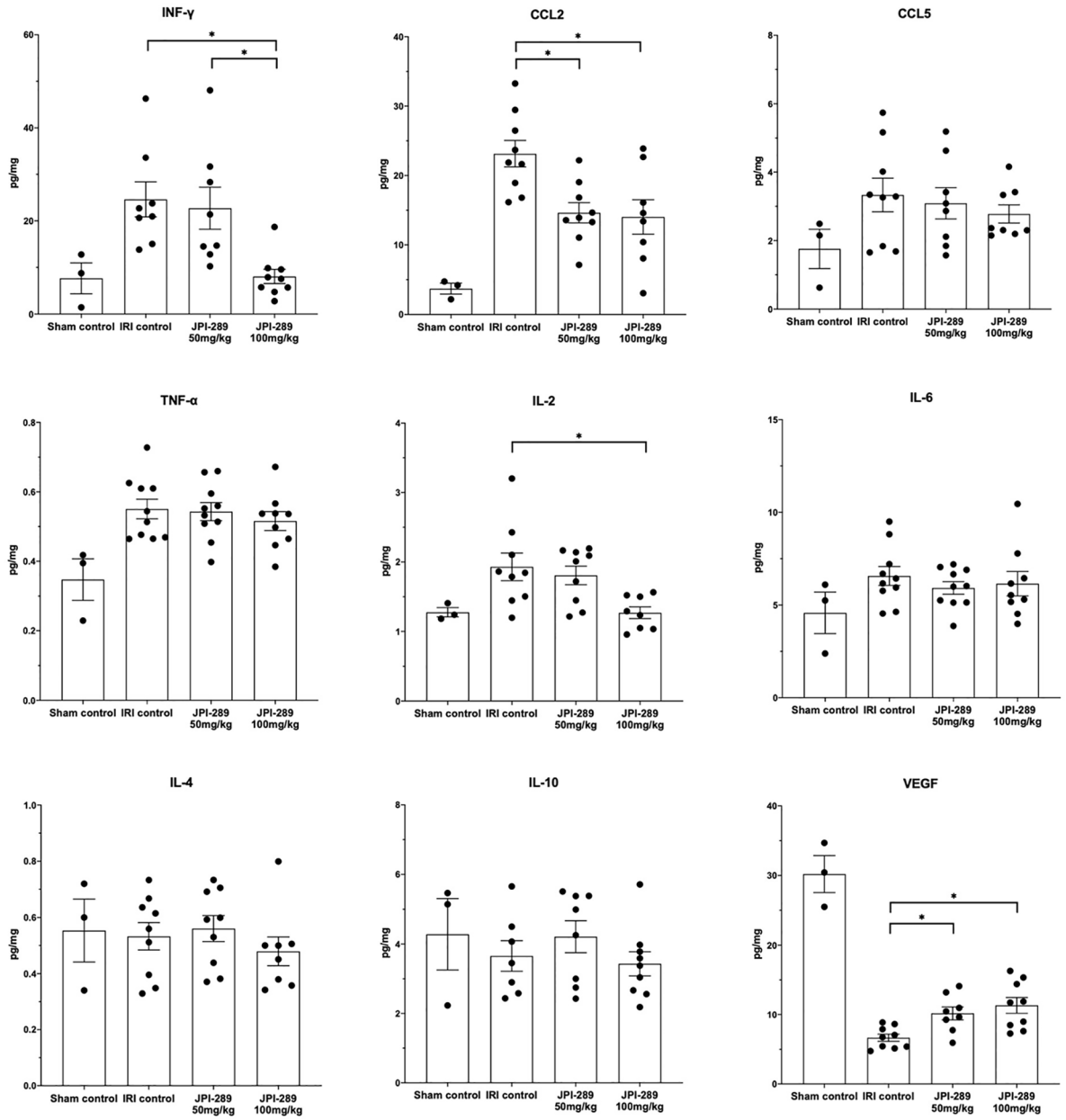

FIGURE 4 | The expression of intrarenal cytokines and chemokines in the postischemic kidneys on day 3 after IRI. JPI-289 treatment significantly decreased the expression of IFN-γ, CCL2, and IL-2 and increased the intrarenal expression of VEGF. *$P < 0.05$, compared with the IRI control group (n = 6–10 in each IRI group, n = 3 in the sham control group). Kidney protein extracts were obtained from three independent experiments. Statistical analysis was performed with the Kruskal-Wallis test followed by Dunn's test.

postischemic kidney, suppressed intrarenal proinflammatory cascades, and subsequently switched the intrarenal immunologic micromilieu following IRI to favorable conditions mitigating renal injury.

We further investigated the effects of JPI-289 in a HK-2 cell hypoxia model and found that JPI-289 treatment reduced proinflammatory signaling pathways and facilitated the cellular proliferation of hypoxic HK-2 cells. This finding suggests that JPI-289 blocked hypoxia-mediated hyperactivation of PARP-1 and subsequently enhanced the proliferation of HK-2 cells even after hypoxic insult. These results are in agreement with previous *in vitro* studies demonstrating the effect of PARP-1 inhibitors on toxin-mediated HK-2 cell death (26) and ROS-mediated rat proximal tubular cell death (27).

Since robust inflammatory responses mediated by both innate and adaptive immune systems induce renal injury following IRI (1), we focused on intrarenal-infiltrated immune cells and the inflammatory micromilieu, highlighting the fact that PARP

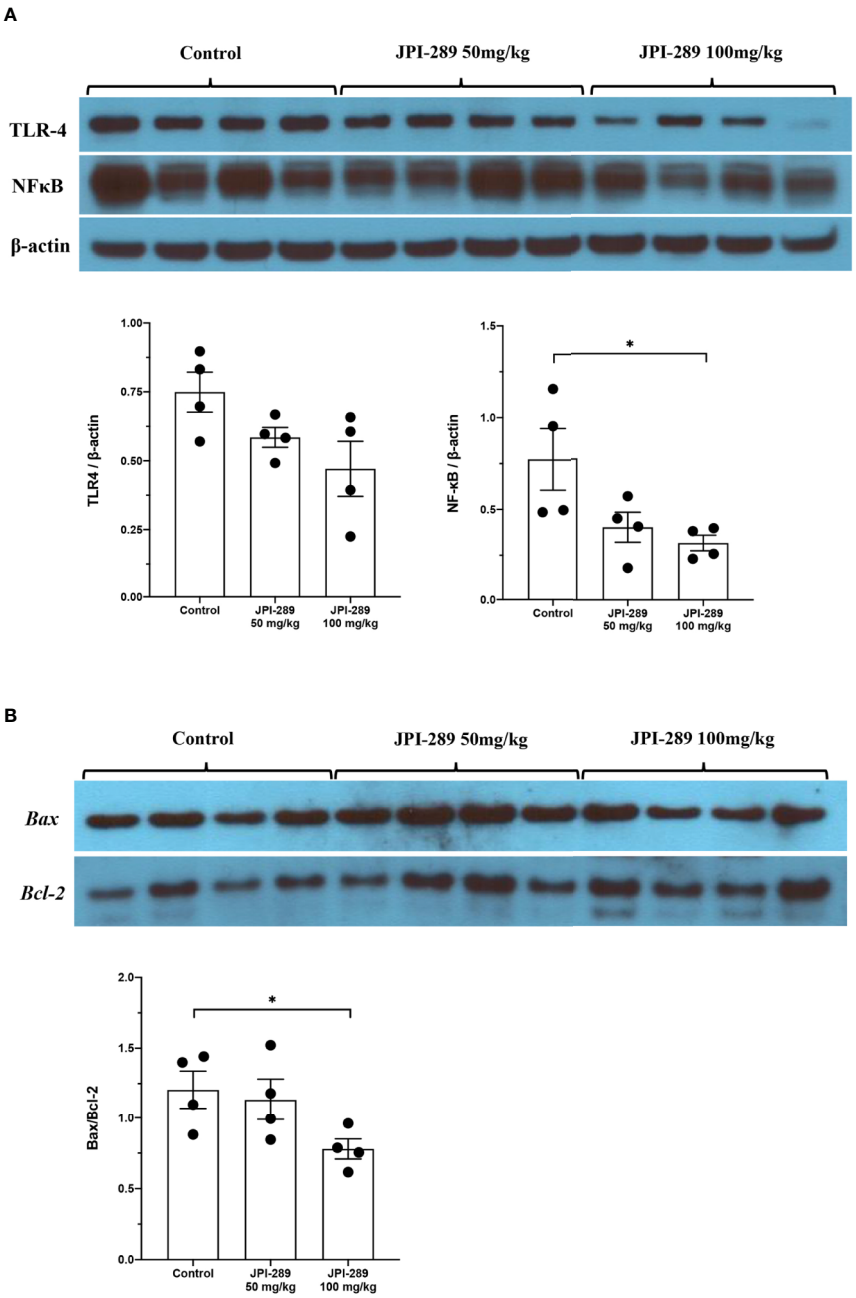

FIGURE 5 | Western blotting of protein samples extracted from postischemic kidneys and densitometry analyses. **(A)** The expression of NFκB was significantly decreased by JPI-289 treatment. The expression of TLR4 tended to be lower with JPI-289 treatment. **(B)** Bax/Bcl-2 ratios were lower in the JPI-289 treatment groups. *$P < 0.05$, compared with the control group. Kidney protein extracts (n = 6–10 in each group) were obtained from two independent experiments. Statistical analysis was performed with the Mann-Whitney U-test.

activation is involved in the development of proinflammatory cascades. Although several previous studies investigated the effects of other PARP inhibitors, such as benzamide and isoquinolone derivatives, in experimental kidney injury models, none of the studies assessed intrarenal immunologic micromilieu with reliable methods (11, 28–33). Unlike previously studied PARP inhibitors, JPI-289 is very potent and readily dissolves in saline or water. Its stability during long-term storage at room temperature and higher temperatures has been established in a previous study (12). In addition, as JPI-289 has high oral bioavailability, it could be used by oral administration in the clinical situation. JPI-289 has also shown promising results in ischemic stroke models (13) and pilot clinical trials are currently underway (NCT01983358).

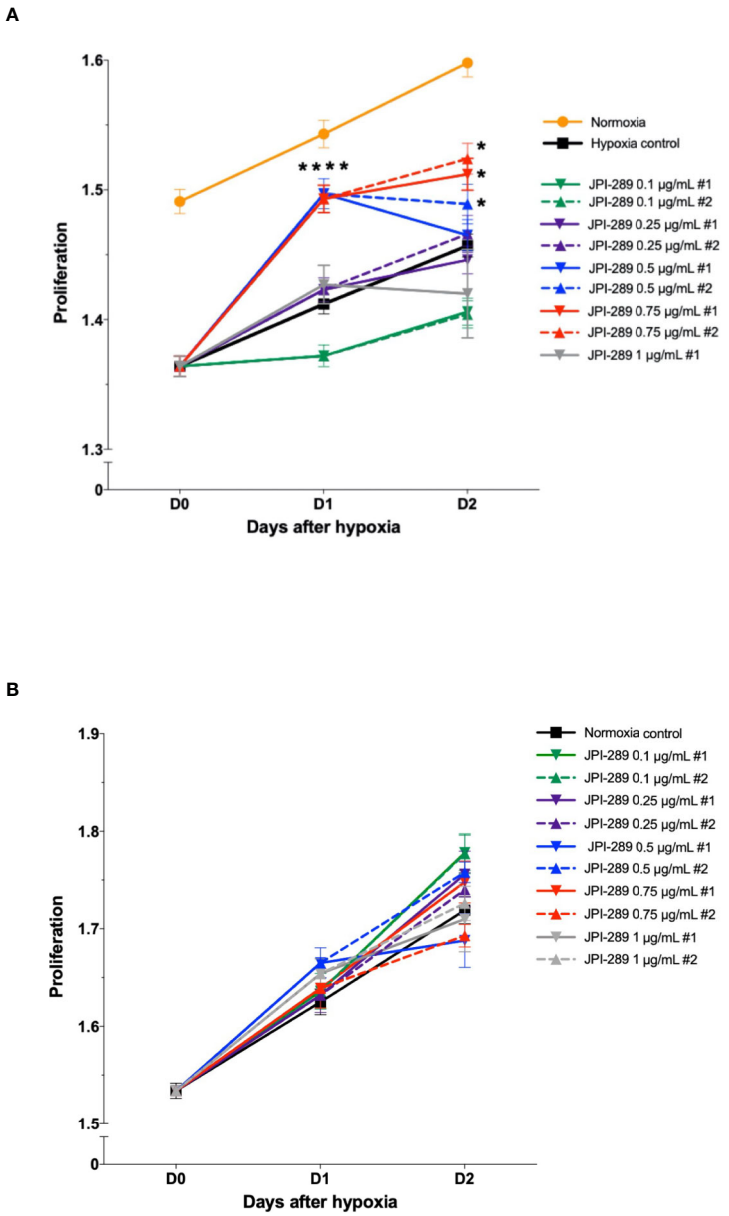

FIGURE 6 | Proliferation of hypoxic HK-2 cells according to the dose of JPI-289. **(A)** JPI-289 treatment at 0.5 or 0.75 μg/ml facilitated proliferation of hypoxic HK-2 cells compared with the hypoxia control group. Day 0 corresponds to the day when HK-2 cells were taken out from the multi-gas incubator after 48 h of hypoxia. Data are from eight independent experiments. *$P < 0.05$, compared with the hypoxia control group. Statistical analysis was performed with the Mann-Whitney *U* test. **(B)** The proliferation of normoxic HK-2 cells treated with JPI-289 was comparable with that of the normoxia control group. Data are from eight independent experiments.

There may be potential concerns with the involved mechanism of the PARP enzyme when considering the clinical application of PARP inhibitors in ischemic AKI. Inhibiting PARP-1 might increase the susceptibility to infection based on the immunostimulatory role of PARP-1. However, IRI following KT were known to enhance immunogenicity and increase the risk of allograft rejection (7). In our study, JPI-289 treatment reduced DAMP signals and suppressed overall proinflammatory cascades without changing the proportion of major effector cells of the adaptive immune system. Therefore, JPI-289 may be used as a novel drug to mitigate renal IRI without significant risk of serious infection. PARP inhibition also involves interference with DNA repair pathways, which may elicit concerns of developing malignancies. Although PARP inhibitors have been widely used in the clinical field as anticancer agents, secondary malignancies have been very rare and their causal relationship was weak (34).

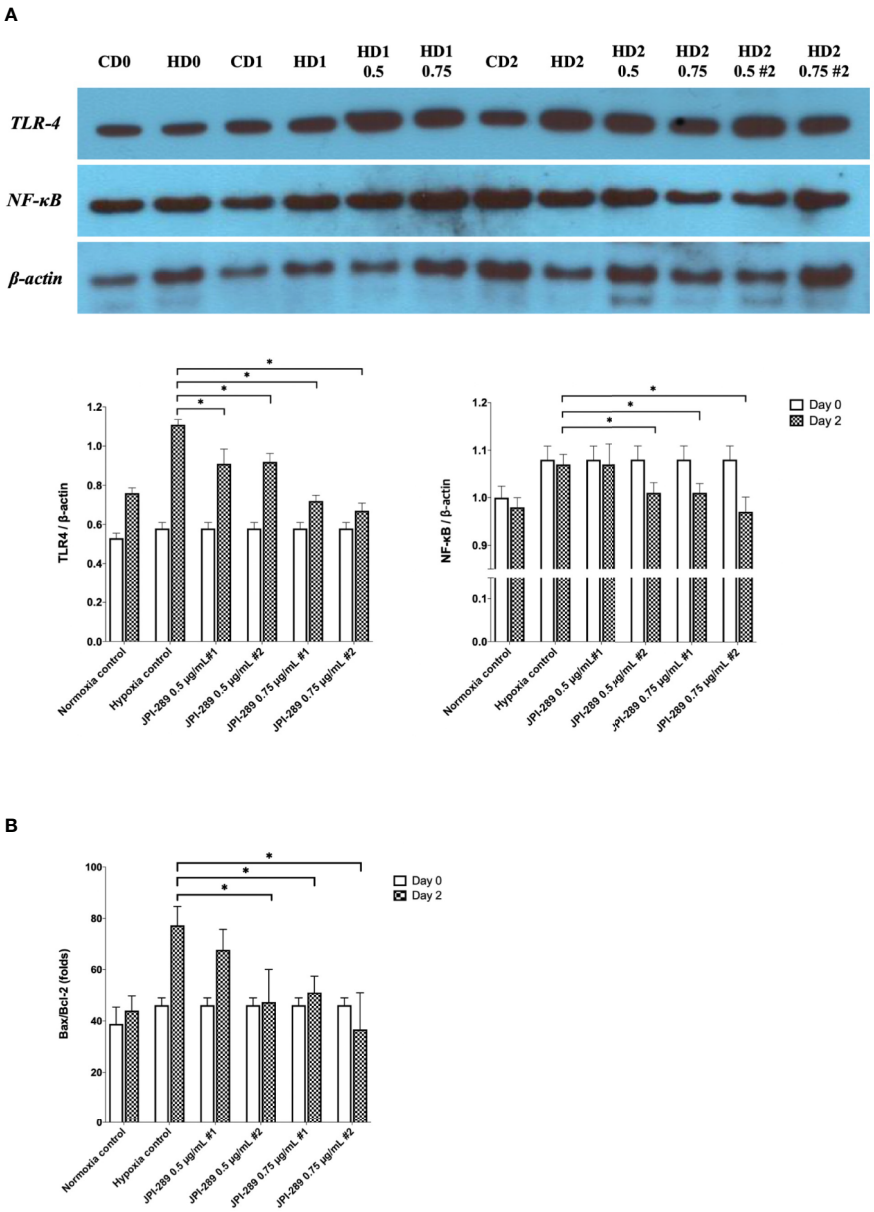

FIGURE 7 | Analyses of cellular proinflammatory signaling pathways and apoptosis of hypoxic HK-2 cells. **(A)** Western blotting of TLR4 and NFκB showed that JPI-289 treatment reduced the expressions of TLR4 and NFκB compared with the hypoxia control group. Data are from six independent experiments. *$P < 0.05$, compared with the hypoxia control group. Statistical analysis was performed with the Mann-Whitney *U*-test. CD0, normoxia control group on day 0; CD1, normoxia control group on day 1; CD2, normoxia control group on day 2; HD0, the hypoxia group on day 0 in normoxia; HD1, the hypoxia group on day 1 in normoxia; HD2, the hypoxia group on day 2 in normoxia. The concentration of JPI-289 (ng/ml) is expressed in the parentheses. **(B)** Enzyme-linked immunosorbent assay of Bax and Bcl-2 showed that JPI-289 treatment reduced Bax/Bcl-2 ratios. Data are from six independent experiments. *$P < 0.05$, compared with the hypoxia control group. Statistical analysis was performed with the Mann-Whitney *U* test.

Furthermore, these risks may be even smaller because PARP blockade would be required only for a few days in the injury phase following IRI after KT or cardiovascular surgery.

There are several limitations in this study. First, the role of PARP in renal IRI has been only reported in animal models to date. The differences in the immune system between human and mice also limit the direct implication of our results to clinical settings. However, we also demonstrated reduced expression of proinflammatory signaling pathways and enhanced cellular proliferation by JPI-289 treatment after hypoxia in the human cellular AKI model using HK-2 cells. Second, the effects of JPI-289 on the postischemic kidney during the early injury phase

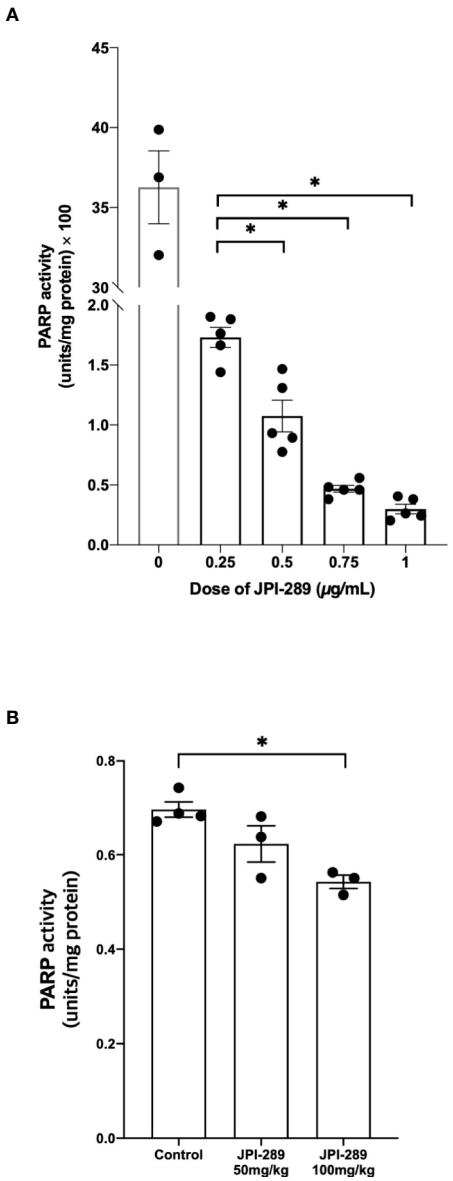

FIGURE 8 | PARP-1 activities in the postischemic kidney protein extracts on day 3 after IRI. **(A)** JPI-289 effectively suppressed PARP-1 activity in a dose dependent fashion. Data are from two independent experiments. *$P < 0.05$, compared with the JPI-289 0.25 μg/ml treated group. Statistical analysis was performed with the Mann-Whitney *U* test. **(B)** Intrarenal PARP-1 activities in postischemic kidney protein extracts were lower in the JPI-289-treated mice. Data are from two independent experiments. *$P < 0.05$, compared with the control group. Statistical analysis was performed with the Mann-Whitney *U* test.

following IRI were investigated in this study. Considering the important role of PARP1 in the DNA repair process, the remote effects of JPI-289 administered in the early injury phase on the postischemic kidney during repair phase need to be investigated. In addition, later time points of administration might result in different outcomes. Future studies to elucidate the most adequate time period for PARP inhibition are also required. Third, JPI-289 was intraperitoneally injected and tested at two different dosages in our study. As JPI-289 is orally available, the effects of oral administration with diverse dosages need to be investigated in future studies.

In conclusion, our study demonstrates that JPI-289 treatment suppressed the inflammatory response by reducing DAMP signals in postischemic kidneys and subsequently attenuated renal injury following IRI. These results indicate that early treatment of JPI-289 may be a novel therapeutic approach for ischemic AKI, especially for high risk patients of DGF. This study provides a rationale for the pharmacological use of PARP-1 inhibitors to reduce renal injury following IRI and accelerate recovery from ischemic AKI.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Samsung Medical Center Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

WH and HRJ designed the study. WH, HRJ, and KL performed experiments, analyzed and interpreted data, and drafted the manuscript. JJ, J-RK, JEL, GYK, Y-GK, DJK, and J-WK analyzed the data and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.564288/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The patent application for therapeutic use of JPI-289 in ischemic AKI was submitted by Samsung Medical Center and JEIL Pharmaceutical Co., LTD. to the Korean Intellectual Property Office.

All authors have no conflict of interest, financial, or otherwise with JEIL Pharmaceutical Co., LTD.

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ORIGINAL RESEARCH
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Endotoxemia-Induced Release of Pro-inflammatory Mediators Are Associated With Increased Glomerular Filtration Rate in Humans *in vivo*

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Introduction: Sepsis is the most prevalent cause of Acute Kidney Injury (AKI). Conversely, in some septic patients the glomerular filtration rate (GFR) is augmented. The role of the inflammatory response and blood pressure to induce this increased GFR is unknown. Herein, we relate inflammatory mediators and blood pressure to the iohexol clearance-derived "true" GFR and kidney injury markers during systemic inflammation in healthy volunteers.

Methods: Twelve healthy subjects underwent experimental endotoxemia (i.v. administration of 2 ng/kg Escherichia coli-derived lipopolysaccharide, LPS). As a gold-standard to determine the GFR, iohexol plasma clearance ($GFR_{iohexol}$) was calculated during a 6-h period on the day before (baseline) as well as 2 and 24 h after LPS administration. Intra-arterial blood pressure was recorded continuously using a radial artery catheter. Circulating inflammatory mediators and urinary excretion of kidney injury markers were serially measured.

Results: Experimental endotoxemia profoundly increased plasma concentrations of inflammatory mediators, including [mean ± SD or median [IQR] peak values (pg/mL) of tumor necrosis factor (TNF)-α: 92 ± 40, interleukin (IL)-6: 1,246 ± 605, IL-8: 374 ± 120, IL-10: 222 ± 119, IL-1 receptor antagonist (RA): 8,955 ± 2,429, macrophage chemoattractant protein (MCP)-1: 2,885 [2,706–3,765], vascular adhesion molecule (VCAM)-1: 296,105 ± 34,822, intercellular adhesion molecule (ICAM)-1: 25,0170 ± 41,764]. Mean arterial pressure decreased with 13 ± 11 mmHg ($p < 0.0001$). No significant increase in the urinary excretion of tubular injury markers was observed following LPS administration. $GFR_{iohexol}$ increased from 97 ± 6 at baseline to 118 ± 10 mL/min/1.73m^2 ($p < 0.0001$) post-LPS administration and returned to baseline levels at 24 h post-LPS (99 ± 9 mL/min/1.73m^2). Peak plasma concentrations of IL-6 ($R^2 = 0.66$, $p = 0.001$) and IL-8 ($R^2 = 0.51$, $p = 0.009$), MCP-1 ($R^2 = 0.38$, $p = 0.03$) and VCAM-1 levels ($R^2 = 0.37$, $p = 0.04$) correlated with the increase in $GFR_{iohexol}$, whereas a trend

was observed for TNF-α ($R^2 = 0.33$, $p = 0.0509$) and IL-1RA ($R^2 = 0.28$, $p = 0.08$). None of the kidney injury markers or changes in blood pressure were associated with $GFR_{iohexol}$. In multiple linear regression analysis, both peak IL-6 ($p = 0.002$) and IL-8 ($p = 0.01$) concentrations remained significantly correlated with $GFR_{iohexol}$, without collinearity.

Discussion: Concentrations of pro-inflammatory cytokines, but not blood pressure, are correlated with the endotoxemia-induced increase in GFR in healthy volunteers. These findings may indicate that inflammatory mediators orchestrate the augmented GFR observed in a subgroup of sepsis patients.

Keywords: augmented renal clearance, sepsis, acute kidney injury, systemic inflammation, iohexol plasma clearance, glomerular filtration rate, endogenous creatinine clearance

INTRODUCTION

Sepsis influences renal function. Naturally, most of the focus is on sepsis-associated deterioration of renal function leading to Acute Kidney Injury (AKI) in sepsis patients. Sepsis is the most commonly observed cause of AKI, and AKI is often severe in this group of patients (1). The inflammatory environment in the kidney may lead to the redistribution of intrarenal perfusion (2) and subsequent deterioration of the renal microcirculation.

On the other hand, renal hyperfiltration, defined as increased creatinine clearance ≥130 mL/min/1.73m^2, is also observed in sepsis patients (3), with a reported prevalence ranging from 40 to 65% (4–6). The high cardiac output often observed in the early phase of sepsis (7, 8), appears to be the most important predictor of increased renal blood flow (7). Furthermore, renal functional reserve is probably necessary for an increase in RBF to induce an increased GFR (4). Importantly, while augmented renal clearance may represent the renal reserve and is the opposite of AKI, it may have detrimental clinical consequences, for instance due to influencing the plasma concentrations of renally excreted agents, such as antibiotics. Accordingly, evidence suggests that renal hyperfiltration is associated with impaired outcome in the general ICU population receiving antibiotic treatment (9). Yet, the mechanisms driving renal clearance in the critically ill remain poorly understood.

The aim of this study was to investigate the relation between systemic inflammation, blood pressure, and kidney function in healthy volunteers challenged with intravenous administered bacterial endotoxin.

MATERIALS AND METHODS

Study Population

Data of healthy volunteers randomized to the placebo group of a previously performed study (10) were used for the analyses described in the present work. The study was approved by the ethics committee CMO Arnhem-Nijmegen (NL56102.091.15; 2015-2231), registered at clinicaltrials.gov (NCT02629874) and conducted according to the ethical principles of the Declaration of Helsinki ICH E6 (R1), the Dutch Medical Research Involving Human Subjects Act and the guidelines of Good Clinical Practice. All subjects provided written informed consent. Quality assurance, monitoring and full data validation was performed by an independent contract research organization.

Healthy male volunteers with a minimum age of 18 years were screened for eligibility. Exclusion criteria consisted, among others, of a body mass index of <18 or >30 kg/m^2, illness in the 2 weeks before start of the study and significant blood loss within 90 days prior to the study. The use of medication, recreational drugs, nicotine, caffeine, and alcohol were prohibited during the duration of the study.

Study Procedures

Study procedures were performed on 3 consecutive days on our intensive care unit: a baseline day, the day of the endotoxin challenge and a follow-up day. A complete overview of the study design is depicted in **Figure 1**. Escherichia coli-derived lipopolysaccharide (LPS, 2 ng/kg, purified [US Standard Reference Endotoxin Escherichia Coli O:113], [NIH, Bethesda, MD, USA]) was administered to induce a transient systemic inflammatory response. To pre-hydrate the subjects, 1.5 L 2.5% glucose/4.5% NaCl was administered intravenously in the hour before LPS administration, or at the same time point on the baseline and follow-up day, according to our standardized endotoxemia protocol (11). Following LPS administration, 250 mL/hour 2.5% glucose/ 0.45% NaCl was administered for 2 hours, followed by 150 mL/hour until discharge, 9 h after endotoxin challenge. Fluid administration was identical on the three study days. Cardiac rhythm was monitored using a 3-lead electrocardiogram and blood pressure was continuously measured using a 20-gauge arterial catheter. Data was recorded using a Philips MP50 patient monitor and an in-house developed data capturing system.

Iohexol-Based GFR Measurements

Iohexol plasma clearance has a very strong correlation with inulin clearance: $r^2 = 0.96$ (12). The water-soluble iodine contrast agent iohexol (OMNIPAQUE 240, containing 518 mg/mL iohexol and 240 mg iodine/mL, GE Healthcare, Eindhoven, the Netherlands) was administrated as a single intravenous bolus of 5 mL (13) at 2 h after LPS administration or at the same time point on the baseline and follow-up day, see **Figure 1**. The blood samples to

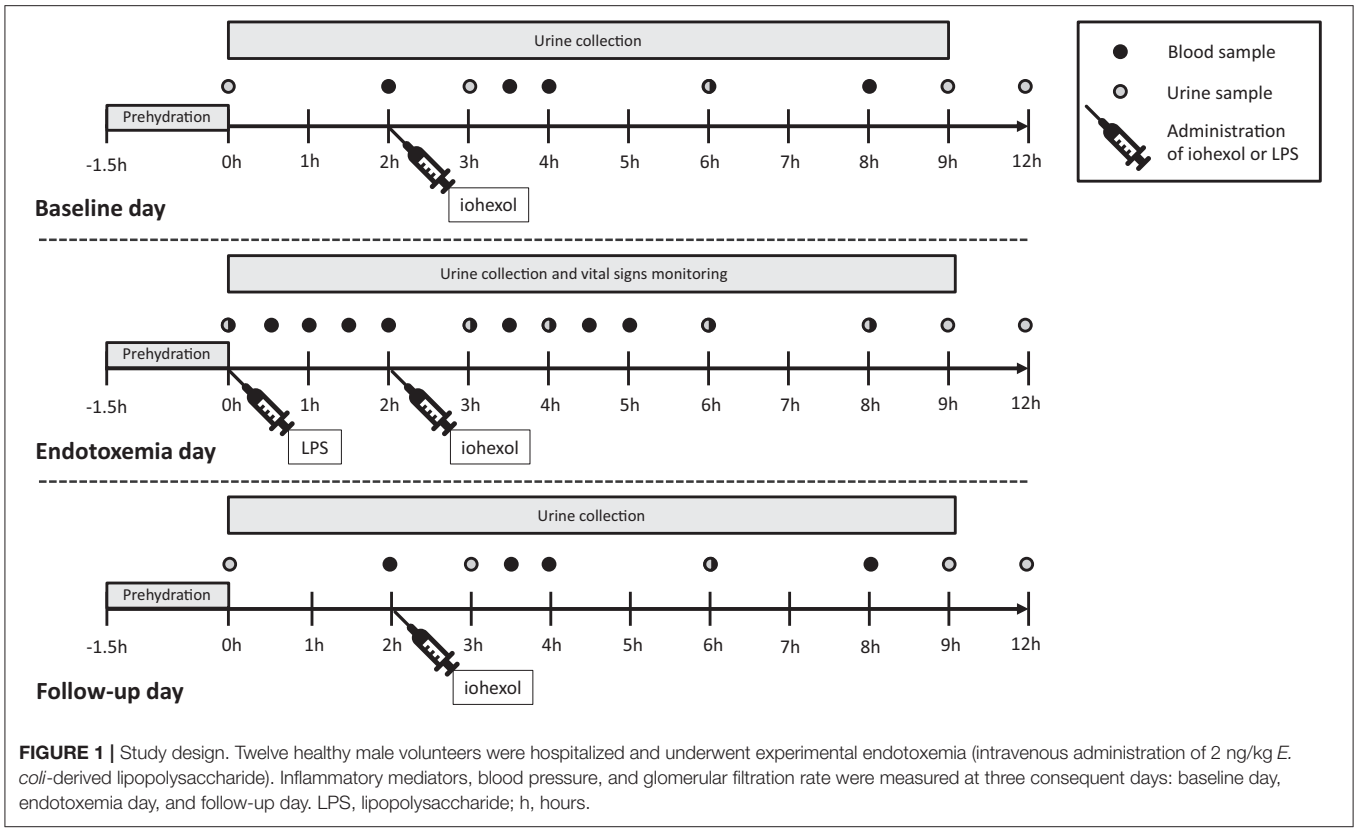

FIGURE 1 | Study design. Twelve healthy male volunteers were hospitalized and underwent experimental endotoxemia (intravenous administration of 2 ng/kg *E. coli*-derived lipopolysaccharide). Inflammatory mediators, blood pressure, and glomerular filtration rate were measured at three consequent days: baseline day, endotoxemia day, and follow-up day. LPS, lipopolysaccharide; h, hours.

TABLE 1 | Demographics of the study population.

	Healthy male volunteer ($n = 12$)
Age (years)	23 ± 3
Length (cm)	183 ± 5
Weight (kg)	77 ± 8
BMI (kg/m^2)	23.2 ± 2.5
BSA (m^2)	2.0 ± 0.1
MAP (mmHg)	95 ± 11
$GFR_{iohexol}$ (mL/min/1.73m^2)	97 ± 6
GFR_{ECC} (mL/min/1.73m^2)	152 ± 16

Data is presented as mean ± standard deviation. BMI, body mass index; BSA, body surface area; MAP, mean arterial pressure; GFR, glomerular filtration rate; ECC, endogenous creatinine clearance.

determine iohexol concentration were obtained at 2, 3.5, 4, 6, and 8 h after LPS administration, centrifuged at 2,000 g for 10 min at 4° Celsius (C) and stored at −80°C until analysis using High Performance Liquid Chromatography (HPLC) at the department of Pharmacology and Toxicology, Radboudumc Nijmegen. The plasma disappearance curve of iohexol was used to calculate the GFR using the slope interception method, as described previously (13). The GFR was corrected using the Brøchner-Mortensen correction and for body surface area using the Mosteller formula (14). $GFR_{iohexol}$ measurements were conducted on all 3 days (**Figure 1**).

Creatinine-Based GFR Assessments

The GFR was also calculated using endogenous creatinine clearance (GFR_{ECC}) on all 3 days. Urine was collected for a period of 9 h and plasma and urine was sampled at the end of the collection period to determine creatinine concentrations according to routine clinical laboratory analysis methods.

Urinary Excretion of Kidney Injury Markers

For determination of kidney injury markers, urine was sampled on the endotoxemia day at 0, 3, 6, 9, and 12 hours following LPS administration or at the same time points on the baseline and follow-up day. Urine was homogenized and samples were stored at −80° C until analysis. Concentrations of neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule (KIM)-1 were measured using enzyme-linked immunosorbent assays (ELISAs, Duoset, R&D systems, McKinley, USA), as were levels of liver-type fatty acid binding protein (L-FABP, CMIC holdings, Tokyo, Japan). Levels were normalized for urinary creatinine concentrations.

Circulating Inflammatory Mediators

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples for measurement of inflammatory parameters were obtained at 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h following LPS administration. Samples were immediately centrifuged at 2,000 g for 10 min at 4°C after which plasma was stored at −80°C until analysis of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10, IL-8, IL-12, IL-1 receptor antagonist (RA), monocyte

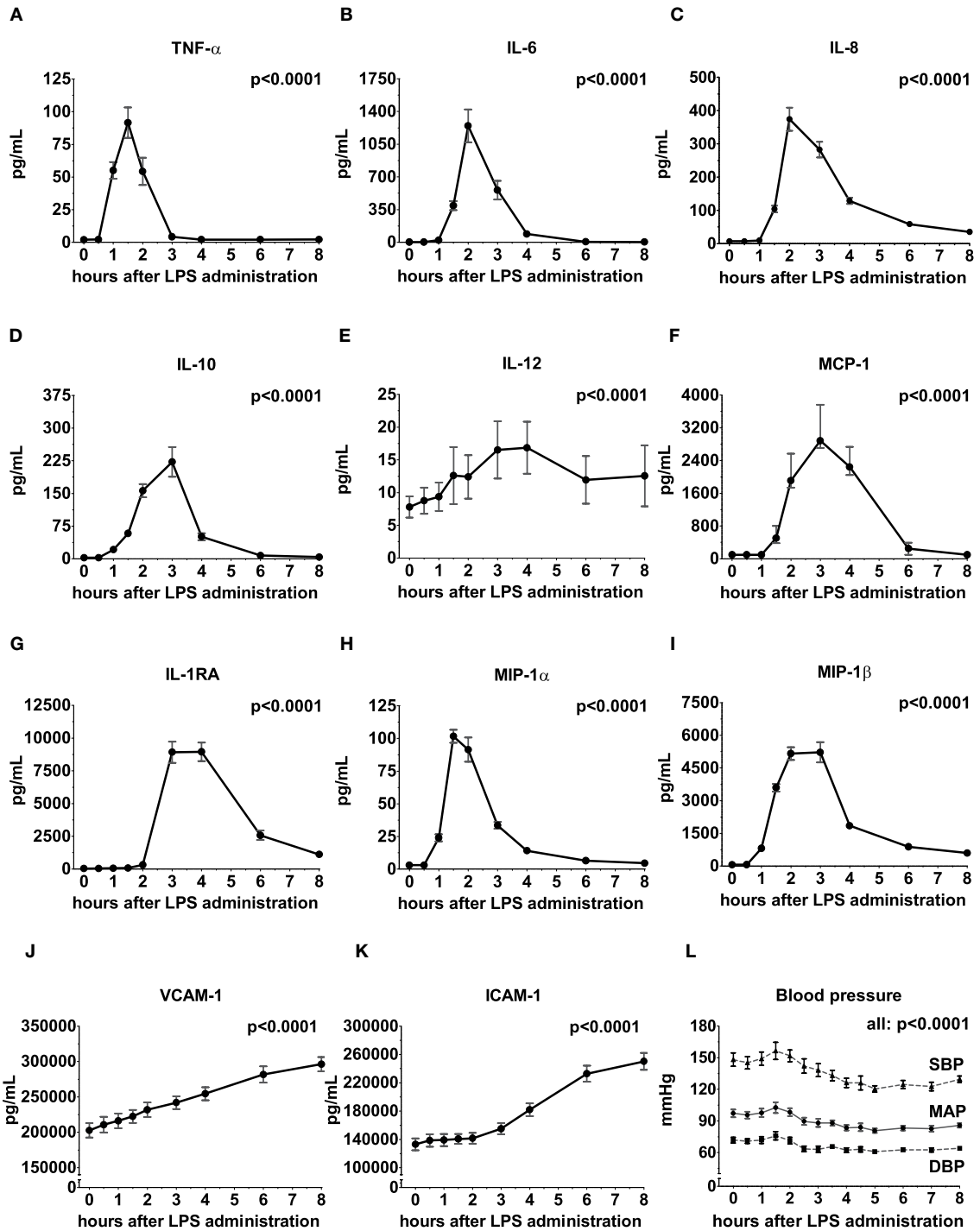

FIGURE 2 | Inflammatory mediators and blood pressure following LPS administration. All variables are depicted over time, relative from LPS administration, starting from 0 h until 8 h. **(A)** Tumor Necrosis Factor-alpha (TNF-α), **(B)** Interleukin (IL)-6, **(C)** IL-8, **(D)** IL-10, **(E)** IL-12, **(F)** Monocyte Chemoattractant Protein-1, **(G)** IL-1 Receptor Antagonist, **(H)** Macrophage Inflammatory Protein-1α, **(I)** MIP-1β, **(J)** Vascular Cell Adhesion Molecule, **(K)** Intercellular Adhesion Molecule, **(L)** Blood pressure. Variance over time tested using a repeated measures one-way ANOVA. SBP, systolic blood pressure; MAP, mean arterial pressure; DBP, diastolic blood pressure.

chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)1-α, MIP1-β, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion protein (VCAM)-1. Levels of IL-6, IL-10, and TNF-α were determined using a validated ISO9001 certified multiplex immunoassay (Luminex, Austin, TX, USA) at the Laboratory of Translational Immunology of the University Medical Center Utrecht, as described elsewhere (15), whereas concentrations of IL-8, MCP-1, IL-1RA, MIP-1α, MIP-1β, ICAM-1, and VCAM-1 were determined using a Luminex assay according to the manufacturer's instructions (ICAM-1 and VCAM-1: Bio-plex, Bio-rad, Hercules, CA, USA; rest: Milliplex; Merck Millipore, Billerica, MA, USA).

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test and presented as mean ± standard deviation (mean ± standard error of the mean in figures), or median [interquartile range]. One-way repeated measures analysis of variance (ANOVA) was used to test serial data. Correlations were calculated using Pearson's correlation coefficient. Multiple linear regressions were performed and collinearity was evaluated. Logarithmic transformation was used if data was not normally distributed. A p-value of <0.05 was considered statistically significant. The data was analyzed with SPSS version 25 (IBM, Armonk, NY, USA), ANOVA analysis and figures were made using GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Twelve healthy male volunteers, aged 23 ± 3 years, were enrolled in the study. The demographic characteristics of the study population are listed in **Table 1**.

Systemic Inflammatory Response Parameters

Following LPS administration, a swift and profound increase of both pro- and anti-inflammatory cytokines, chemokines, and vascular adhesion molecules was observed, illustrated in **Figure 2** (peak values [all in pg/mL] of TNF-α: 92 ± 40 $p < 0.0001$, IL-6: 1,246 ± 605 $p < 0.0001$, IL-8: 374 ± 120 $p < 0.0001$, IL-10: 222 ± 119 $p < 0.0001$, IL-12: 14.5 [9.3 – 20.6] $p < 0.0001$, IL-1RA: 8,955 ± 2,429 $p < 0.0001$, MCP-1: 2885 [2,706 – 3,765] $p < 0.0001$, MIP-1α: 102 ± 17 $p < 0.0001$ and MIP-1β: 5,162 ± 1,016 $p < 0.0001$, VCAM-1: 296,105 ± 34,822 $p < 0.0001$, and ICAM-1: 250,170 ± 41,764 $p < 0.0001$.

Kidney Function and Urinary Excretion of Tubular Injury Markers

Following administration of LPS, a significant increase in $GFR_{iohexol}$ was observed, which returned to baseline levels on the follow-up day (baseline: 97 ± 6; endotoxemia day: 118 ± 10; follow-up day: 99 ± 9 ml/min/1.73m^2, $p < 0.001$, **Figure 3**). GFR_{ECC} showed a similar trend, but changes did not reach statistical significance (baseline: 152 ± 16; endotoxemia day: 164 ± 14; follow-up day: 157 ± 22 mL/min/1.73m^2, $p = 0.15$, **Figure 3**). LPS administration did not result in an increase of the urinary tubular injury markers NGAL and L-FABP, whereas urinary KIM-1 concentrations significantly decreased following LPS administration ($p = 0.0025$, **Figure 4**).

Hemodynamic Parameters

The mean arterial pressure (MAP) initially increased post-LPS administration, peaking at 1.5 h (from 94 ± 10 to 103 ± 18 mmHg), followed by a decrease with a nadir at 5 h after LPS administration (−13 ± 11 mmHg compared to baseline,

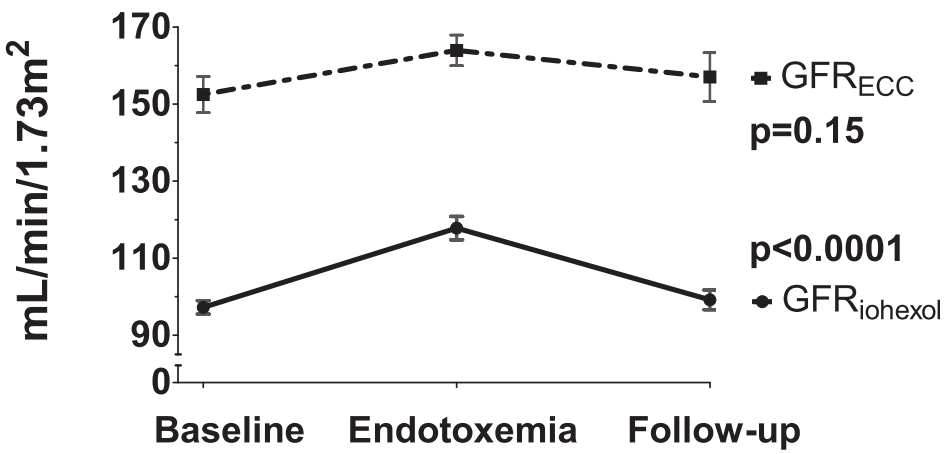

FIGURE 3 | Kidney function changes due to LPS administration. The iohexol-derived GFR and GFR calculated using endogenous creatinine clearance are determined on the three consecutive study days: baseline day prior to LPS administration, endotoxemia day in the hours following LPS administration and the follow-up day after LPS administration. Variance over time tested using a repeated measures one-way ANOVA.

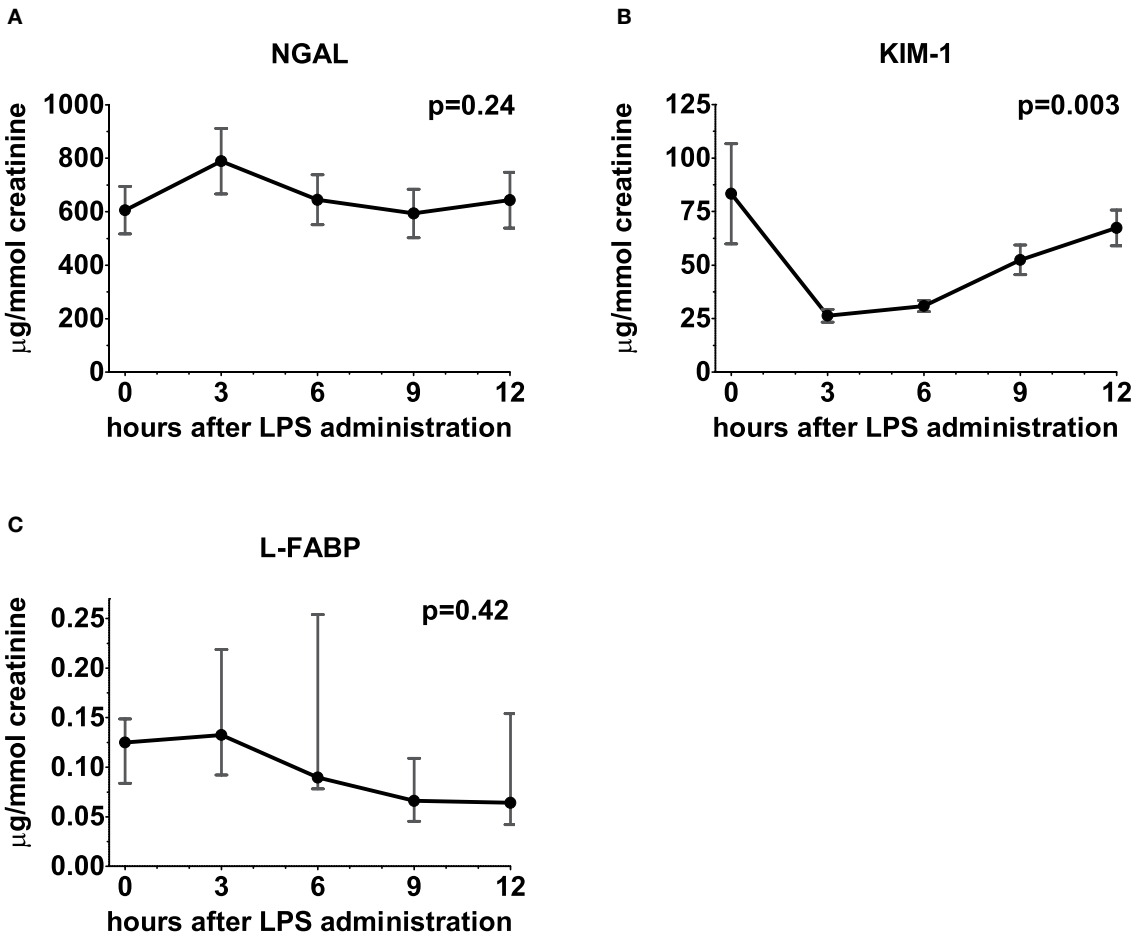

FIGURE 4 | Urinary excretion of tubular injury markers following LPS administration. Concentration of tubular injury markers in urine depicted over time relative from LPS administration, starting from 0 h until 12 h. **(A)** Neutrophil gelatinase-associated lipocalin, **(B)** Kidney injury molecule-1, **(C)** Liver fatty acid-binding protein. Variance over time tested using a repeated measures one-way ANOVA.

Figure 2). The systolic and diastolic blood pressure decreased by 22 ± 17 and 9 ± 9 mmHg compared with baseline, respectively ($p = 0.0003$ and $p = 0.003$) (**Figure 2**).

Relationship Between Inflammatory Markers and Iohexol-Based GFR

Peak plasma concentrations of the pro-inflammatory cytokines IL-6 ($R^2 = 0.66$, $p = 0.001$) and IL-8 ($R^2 = 0.51$, $p = 0.009$), MCP-1 ($R^2 = 0.38$, $p = 0.03$) and the maximum increase in VCAM-1 levels ($R^2 = 0.37$, $p = 0.04$) were significant correlated with the increase in $GFR_{iohexol}$ (**Figure 5** and **Supplementary Table 1**), whereas trends were observed for TNF-α ($R^2 = 0.33$, $p = 0.0509$) and IL-1RA ($R^2 = 0.28$ $p = 0.08$). Peak levels of IL-10, IL-12, MIP-1α, MIP-1β, and ICAM-1 did not correlate with $GFR_{iohexol}$. In a multiple linear regression analysis, with $GFR_{iohexol}$ as dependent variable and the cytokines that correlated with $GFR_{iohexol}$ as independent variables, peak IL-6 ($p = 0.002$) and IL-8 ($p = 0.01$) remained independently associated with the increase in $GFR_{iohexol}$. Peak IL-6 and IL-8 levels showed no collinearity with regard to their association with $GFR_{iohexol}$ (VIF = 1.185, VIF = 1.185). The peak plasma concentrations of the inflammatory mediators did not correlate with the non-significant change in GFR_{ECC} following LPS administration (**Figure 5** and **Supplementary Table 1**).

Relationship Between Blood Pressure and GFR

The peak and nadir of the arterial blood pressures (SBP, DBP, and MAP) following LPS administration did not correlate with the change in $GFR_{iohexol}$ and GFR_{ECC} (**Supplementary Table 2**).

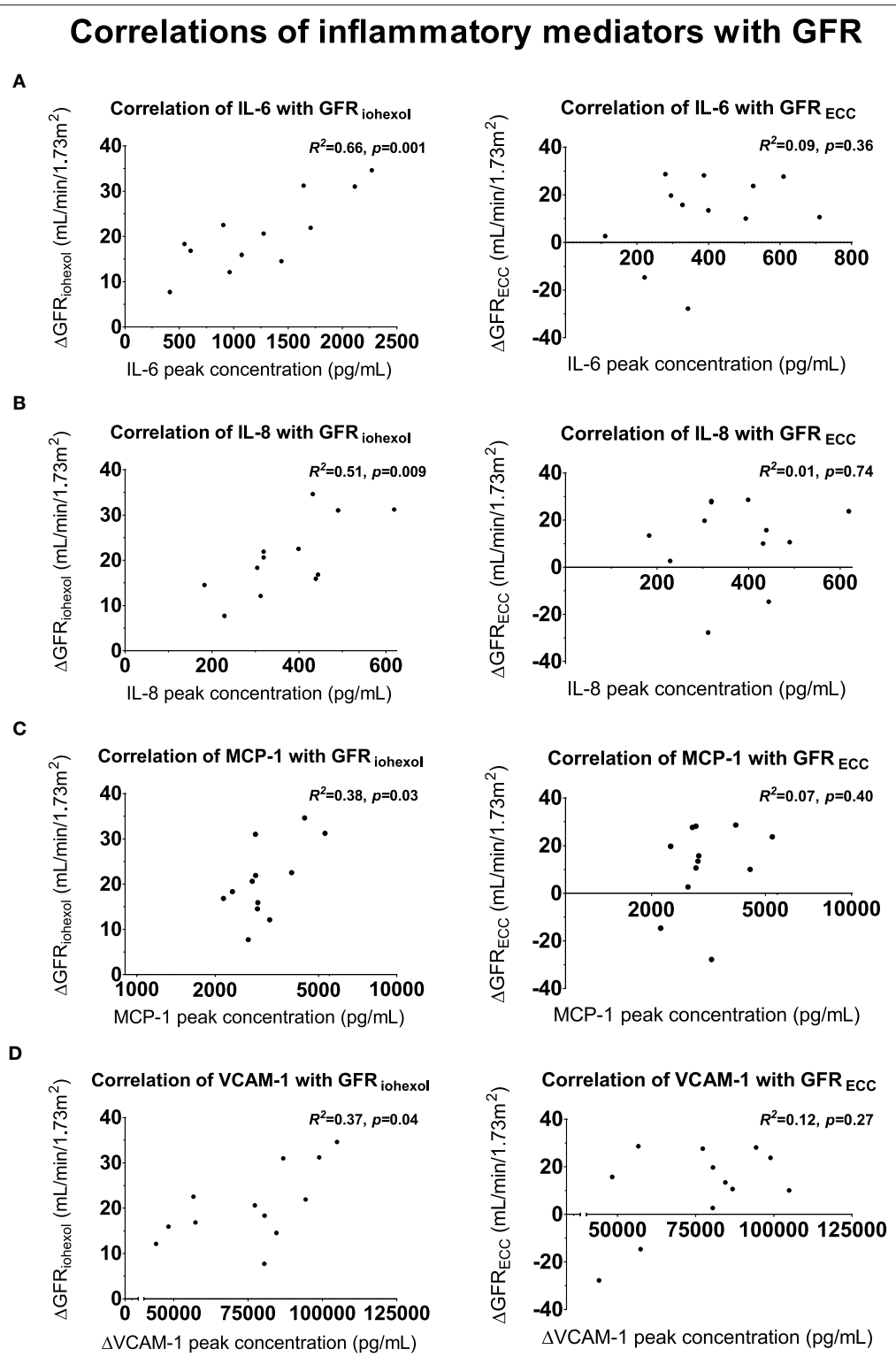

FIGURE 5 | Correlations between inflammatory mediators and GFR. Scatter plots of peak concentrations of inflammatory mediators correlated with the increase in glomerular filtration rate (GFR, iohexol-derived and using endogenous creatinine clearance) on the endotoxemia day compared to the baseline day. **(A)** Proinflammatory cytokine interleukin (IL)-6 with $GFR_{iohexol}$ (left) and GFR_{ECC} (right), **(B)** proinflammatory cytokine IL-8 with $GFR_{iohexol}$ (left) and GFR_{ECC} (right), **(C)** Monocyte Chemoattractant Protein-1 with $GFR_{iohexol}$ (left) and GFR_{ECC} (right), **(D)** Vascular Cell Adhesion Molecule-1 correlated with $GFR_{iohexol}$ (left) and GFR_{ECC} (right). For a complete overview of all correlations with $GFR_{iohexol}$ and GFR_{ECC}, see **Supplementary Table 1**. Correlations were tested using Pearson's correlation coefficient.

DISCUSSION

In this study in healthy volunteers with a systemic immune response elicited by endotoxin administration, increases in inflammatory mediators were significantly associated with the increase in the "true GFR" measured by plasma clearance of iohexol. This is the first report demonstrating a direct relationship between the systemic inflammatory response and increased GFR in humans.

Sepsis influences renal function. On the one hand AKI, defined as a decrease in GFR, clearly recognized in the clinic, but on the other hand also an augmented GFR is possible, a phenomenon that is much less known. Renal hyperfiltration is now increasingly being recognized as a clinical entity (16–18). A highly relevant clinical consequence is the augmented clearance of renally excreted drugs, most notably antibiotics, which is important for daily practice as well as for clinical trials investigating novel therapeutic compounds (19–21). Adjustment of the dosing of these drugs should be considered when GFR is increased, as is already common practice when the GFR is decreased (22). The increase in GFR during early sepsis has up to now mainly been explained from altered hemodynamics. The hyperdynamic circulation observed in these patients is characterized by a high cardiac output which is the most important and independent predictor of renal blood flow (RBF) (7). In addition, redistribution of blood flow through the kidney during systemic inflammation may result in hyperfiltration (4, 7). However, to the best of our knowledge, we here describe for the first time that the increase in GFR may also reflect a direct consequence of the inflammatory process, independent of hemodynamic changes.

Pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-8, are important orchestrators of the innate immune response and are associated with impaired outcome in ICU patients (23, 24). Until now, many studies have focused on the occurrence of AKI and a decrease of GFR due to inflammation (25, 26). This is in contrast with the relation between inflammation and increase in GFR that we demonstrate. It is known that Damage- and Pathogen Associated Molecular Patterns (DAMPs and PAMPs) such as cytokines and LPS interact directly with tubular cells (27) and induce a pro-inflammatory cascade (28). A dysregulated microcirculation in the kidney during systemic inflammation may lead to a prolonged exposure of the inflammatory mediators in regions with low blood flow and sustain the inflammatory response and its consequences. It appears plausible that both the intensity as well as the duration of the inflammatory response is of relevance for the development of AKI. In experimental human endotoxemia, the intensity is limited (as no increase in tubular injury markers was found) and the duration is short-lived (11, 29). Therefore, this model of systemic inflammation in humans is too mild to induce AKI and to decrease GFR, but nevertheless, the correlation of these inflammatory mediators with the increase in GFR is very clear. This can indicate that the inflammation-induced renal response may orchestrate the pathophysiology of augmented renal function observed early in a subgroup of patients with sepsis (4, 30).

A strength of this study is the use of a gold standard method to measure GFR. De discrepancy between the correlations with inflammation between the "true" iohexol-derived GFR and the creatinine clearance-derived GFR advocates the use of accurate methods in studies that test mechanistical or pathophysiological risen hypotheses. However, methods using intravenously administered exogenous compounds such as iohexol are labor-intensive and have higher costs compared to creatinine-based methods. Another strength of this study is the standardized experimental translational design which limits confounders that influence the kidney, hemodynamics or the immune response; the healthy volunteers had a normal kidney function and the standardized LPS-dose and schedule elicits a reproducible and controlled inflammatory response (11). The absence of cardiac output measurements and the possibility to correct for changes in cardiac output is a limitation of the study. It is known that experimental human endotoxemia results in a hyperdynamic circulation with a high cardiac output (31). In septic patients a high cardiac output is an important and independent predictor of increased renal blood flow (RBF) (7). Nevertheless, we are able to conclude that the increase in GFR during experimental human endotoxemia is not dependent of perfusion pressure, as it was observed while blood pressure was significantly lower compared to baseline. In addition, an increase in cardiac output and RBF does not always result in an augmentation of the GFR. For example, concurrent vasodilatation during systemic inflammation may result in a lower glomerular capillary pressure and a low GFR (32). The correlations of the cytokines with the increase in the measured GFR we demonstrated suggest that systemic inflammation is an important determinant of the GFR, even when corrected for blood pressure.

In conclusion, in a highly standardized and controlled experimental endotoxemia study in healthy volunteers, the systemic immune response is significantly associated with the increase in GFR measured using the gold standard iohexol plasma clearance method, independent of hemodynamic effects. This correlation between the inflammatory response and an increase in GFR may explain the augmented GFR sometimes observed early in sepsis patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors on reasonable request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CMO Arnhem-Nijmegen. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PP, LE, MK, and RG designed the study. RG and GL conducted the study. RB, MS, and PP analyzed the data and drafted the manuscript. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.559671/full#supplementary-material

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MINI REVIEW
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Acute Kidney Injury Induces Innate Immune Response and Neutrophil Activation in the Lung

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Complication in acute kidney injury (AKI) is significantly associated with developing acute respiratory failure (ARF), while ARF is one of the most important risks for AKI. These data suggest AKI and ARF may synergistically worsen the outcomes of critically ill patients and these organ injuries may not occur independently. Organ crosstalk between the kidney and the lung has been investigated by using animal models so far. This review will focus on innate immune response and neutrophil activation among the mechanisms that contribute to this organ crosstalk. AKI increased the blood level of an inflammatory mediator in high-mobility group box 1, which induces an innate immune reaction via toll-like receptor 4. The remarkable infiltration of neutrophils to the lung was observed in animal AKI models. IL-6 and IL-8 have been demonstrated to contribute to pulmonary neutrophil activation in AKI. In addition, the formation of a neutrophil extracellular trap was also observed in the lung after the exposure of renal ischemia reperfusion in the animal model. Further investigation is necessary to determine whether targeting innate immune response and neutrophil activation will be useful for developing new therapeutics that could improve multiple organ failure in critically ill patients.

Keywords: neutrophil, IL-6, high mobility group box 1, toll-like receptor, neutrophil extracellular trap

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INTRODUCTION

Acute kidney injury (AKI) is a common complication in critically ill patients treated in the intensive care unit (ICU). Reportedly, AKI occurs in ∼30–60% of patients admitted to the ICU and is associated with significantly poor outcomes including morbidity and mortality (1). ICU patients with AKI will be complicated by a broad range of other organ injuries including heart failure, respiratory failure, liver injury, and immunological abnormalities. Many animal studies demonstrated that AKI induces cardiac, lung, liver, brain, and splenic injury (2). Therefore, AKI should be considered as a systemic disease that can have significant impacts on distant organs.

Though the primary roles are different, lung and kidney functions are intimately related regarding the maintenance of homeostasis. The blood acid-base balance is regulated by carboxy dioxide excretion by the lung and bicarbonate reabsorption by the kidney. A compensative mechanism will activate when one organ fails to keep these levels within a normal range by the other organ. Thus, respiratory complication in AKI patients is particularly important. Clinical studies revealed AKI patients were twice as likely to require invasive mechanical ventilation (3). On the other hand, acute respiratory failure (ARF)/acute respiratory distress syndrome (ARDS) patients, especially those who require invasive mechanical ventilation, are at high risk for developing AKI (4, 5).

In this review, we provide a brief overview regarding (1) the clinical data on AKI and ARF/ARDS and (2) the potential mechanisms by which AKI leads to lung injury. Recently identified mechanisms of the innate immune system including toll-like receptors (TLRs) and damage-associated molecular patterns (DAMPs) were evaluated in the kidney-lung crosstalk using animal models. Neutrophil extracellular traps (NETs) was identified as another player in the innate immune response. NETs were also demonstrated to contribute to AKI-induced lung injury.

CLINICALLY REPORTED ASSOCIATIONS BETWEEN AKI AND ARF/ARDS

Many clinical studies suggested associations between AKI and ARF/ARDS in critically ill patients. AKI often occurs in ARF/ARDS patients, while ARF/ARDS is often observed in AKI patients. First, clinical data about AKI complication in ARF/ARDS are described below. The secondary analysis of a randomized control trial conducted by the ARDS network reported AKI occurred in ∼25% of ARDS patients. The mortality rate of AKI patients was significantly higher than the non-AKI patients (6). Another study reported that AKI was observed in ∼35% of hospitalized patients with community-acquired pneumonia. AKI was significantly associated with the requirement of mechanical ventilation (MV) and mortality (4). A meta-analysis including 31 studies reported a significantly high AKI occurrence when the patients were mechanically ventilated (5). These data suggest that AKI is a common complication and a risk factor for poor outcomes of ARF/ARDS.

Second, ARF/ARDS complication in AKI is described below. Severe ARF requiring MV was observed in more than 70% of AKI patients in a multinational, multicenter prospective observational study. MV requirement was significantly associated with higher mortality (7). Dialysis-requiring AKI (AKI-D) is the most severe form of AKI (defined as AKI stage 3 by the KDIGO criteria). A small retrospective study reported that 44% of AKI-D patients treated in an ICU developed ARDS (8). Therefore, ARF/ARDS is a common complication and a risk factor for poor outcomes of AKI.

Third, AKI and ARF/ARDS are organ failures that can be simultaneously complicated in a critically ill patient. An observational study conducted in 18 French ICUs reported ARDS occurrence as 23%, while AKI occurrence was at 31%. Hospital mortality was 14% in this population and it increased to 42% in patients with both AKI and ARDS (9). In a large cohort of 200,000 admissions for subarachnoid hemorrhage (SAH) from the Nationwide Inpatient Sample database, the incidence of ARDS in SAH patients was ∼35% and renal dysfunction predicted ARDS development (10). A nationwide study including ∼7 million patients with acute ischemic stroke showed AKI patients had a significantly higher risk of requiring MV than non-AKI (11). As described above, both AKI and ARF/ARDS are common organ failures in critically ill patients with other etiologies such as sepsis and the coexistence of these issues will exacerbate the outcomes. However, it should be noted that these clinical observational studies do not indicate causal relationships but only associations between these two types of organ failures.

TABLE 1 | Pathophysiological mechanisms of respiratory failure in AKI.

Non-inflammatory	Fluid overload
	Cardiac dysfunction
Inflammatory	Increased cytokine levels (tumor necrosis factor-α, IL-6, and IL-8)
	Neutrophil activation
	Pulmonary endothelial apoptosis
	Oxidative stress

POSSIBLE MECHANISM OF ORGAN CROSSTALK BETWEEN THE KIDNEY AND THE LUNG

The pathophysiological mechanisms of respiratory failure in patients with AKI can be categorized into inflammatory and non-inflammatory mechanisms (**Table 1**). Non-inflammatory mechanisms include fluid overload and cardiac dysfunction. Rapid progression of lung edema and fluid overload, which do not respond to diuretics, are frequently observed in AKI patients. Although inflammatory mechanisms are not well-recognized in basic clinical research, using animal models demonstrated that increased inflammatory mediators in AKI play a crucial role in lung injury.

Rabb and colleagues first demonstrated increased inflammatory gene expressions in the lung after exposure of renal ischemia reperfusion injury by using microarray analysis (12). Faubel et al. (13) demonstrated the role of IL-6 in AKI-induced lung injury with IL-6 knockout mouse experiments. The reduced clearance of inflammatory mediators including IL-6 in AKI seemed to contribute to lung injury. Furthermore, they reported that IL-6-induced expression of the chemokine CXCL1 in the lung was responsible for recruiting neutrophil into the lung induced by AKI (14). These findings suggest that circulating IL-6 is a pathogenic mediator of lung injury in AKI. IL-10, an anti-inflammatory cytokine, was shown to be produced by spleen CD4-positive T cells in response to IL-6 in AKI. The induction of IL-10 expression by IL-6 appeared to contribute to the suppression of lung injury induced by AKI as a counterbalance (15). Other mediators and inflammatory cells were also reported as possible contributors to lung injury induced by AKI (**Table 2**).

Although these studies suggested that the induction of an inflammatory reaction by AKI is involved in lung injury, other studies demonstrated AKI suppressed inflammatory reactions and subsequently worsened pulmonary infection. Neutrophil function against pneumonia was evaluated in the model of bacterial pneumonia in mice complicated with AKI by folic acid administration or glycerol injection (rhabdomyolysis) (16). Neutrophil migration was rather weakened and the pneumonia was exacerbated in AKI animals. Another study reported that the neutrophil migration ability at microcirculation of the cremaster muscle was decreased in AKI mice (17). AKI-induced neutrophil

TABLE 2 | Mediators of lung injury in AKI.

IL-6	Serum IL-6 can be used as a predictor of AKI
	Serum IL-6 level elevates in the very early stage of AKI
	Higher level of serum IL-6 is associated with the prolonged mechanical ventilation in patients with AKI
	IL-6 antibody-treated mice exhibit less lung inflammation and fewer capillary leaks
	IL-6 deficient mice exhibit less lung inflammation and fewer capillary leaks
	Intravenous IL-6 injection to IL-6 deficient mice restores lung inflammation
	Circulating IL-6 causes lung inflammation in AKI
IL-8	Serum IL-8 levels elevate in the very early stage of AKI
	Higher level of serum IL-8 is associated with the prolonged mechanical ventilation in patients with AKI
	Higher level of serum IL-8 is a predictor of increased mortality in patients with AKI
	IL-8 antibody-treated mice exhibit less lung injury
	IL-8 deficient mice exhibit less lung injury
TNFR1 + caspase-3-mediated apoptosis	Circulating TNF and caspase-3 increase endothelial apoptosis and lead to non-cardiogenic pulmonary edema
	Pan-caspase inhibition reduces pulmonary edema after AKI
	TNF inhibition reduces apoptosis and pulmonary edema after AKI
	TNFR1 deficient mice exhibit less lung caspase-3 and lung apoptosis after AKI
T cells	T cells participate in lung apoptosis via caspase-3 and lead to non-cardiogenic pulmonary edema
	T cells are recruited to the lung within 24 h after AKI
	T cell deficient mice exhibit less caspase-3 and less pulmonary edema

TNFR1, tumor necrosis factor receptor 1.

dysfunction might be caused by an adipokine resistin (18). Resistin is known as a uremic toxin and pro-inflammatory cytokine secreted by monocytes, neutrophils, and epithelial cells (19). It should be noted septic patients in the ICU showed higher blood levels of resistin than non-septic patients (20).

Taken together, increased humoral mediators by reduced clearance and increased expression in AKI seem to contribute to lung injury. On the other hand, few studies revealed the mechanisms of the opposite direction of crosstalk, i.e., ARF/ARDS-induced kidney injury. Slutsky et al. (21) reported increased epithelial cell apoptosis in the kidney in a rabbit ARDS model followed by mechanical ventilation with injurious ventilatory strategies (high tidal volume and high PEEP). An infiltration of lymphocytes was seen in the renal cortex in a pig ARDS model with mechanical ventilation (22). On the other hand, another study found no renal structural and functional alterations in a canine ARDS model when hemodynamics and arterial blood gas tensions are carefully controlled (23), indicating the possible role of a non-inflammatory mechanism for kidney injury in ARDS. Further investigation is necessary for whether inflammatory mediators play any role in ARF/ARDS-induced kidney injury.

INNATE IMMUNE RESPONSE AND NEUTROPHIL ACTIVATION

Neutrophil infiltration is a major finding in lung injury induced by AKI. A remarkable neutrophil infiltration in the lung together with increased neutrophil elastase (NE) activity in blood and lung tissues were observed in a mouse AKI model and specific NE inhibitor reduced lung injury (24). Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs play an important role in innate immunity, and are involved not only in the defense against infection but also in various pathological conditions. An inflammatory mediator, high-mobility group protein B1 (HMGB1) is one of the DAMP TLR4 ligands. We revealed that TLR4 loss-of-function mutant mice (C3H/HeJ) were resistant to lung injury caused by AKI (25). Although blood levels of HMGB1 increased in AKI regardless of TLR4 mutation, anti-HMGB1 neutralizing antibody treatment reduced lung injury only in the wild-type mice, indicating the organ protecting effect was mediated by the HMGB1-TLR4 pathway.

Extracellular histones induce cytotoxicity as DAMPs trigger the inflammatory cascade via toll-like receptors. Extracellular histones are released from neutrophils during the formation of neutrophil extracellular traps (NETs), that comprise DNA studded with histones and proteases, including neutrophil elastases (26). Extracellular histones and NETs were reported to associate with the pathogenesis of acute lung injury in an animal AKI model. Nakazawa et al. (27) showed that the induction of necrosis in renal tubular epithelial cells *in vitro* caused by hypoxia stimulated neutrophils to form NETs by extracellularly released histones. In a mouse intestinal ischemia-reperfusion model, extracellular histone accumulation and NETs formation were observed in the liver rather than the intestine (28). Extracellular histones derived from the intestinal tract were considered to transported to the liver via the portal system. In the mouse renal ischemia-reperfusion injury model, elevated extracellular histones in blood and NETs formation in the lung was observed (27). Human recombinant thrombomodulin (rTM) reportedly trap extracellular histones *in vitro*. Although no renal protection by rTM was observed in the renal ischemia-reperfusion model, significant improvement of lung injury together with NTEs accumulation induced by AKI was observed in rTM-treated animals (29).

These findings above suggest that extracellular histones and NETs formation might be responsible for AKI-induced lung injury, although other pathways of inflammatory mediators such as IL-6 also contribute to AKI-induced lung injury. For the development of new therapeutics against organ crosstalk between the kidney and lung, targeting multiple pathways will be necessary. In addition, extracellular histones and NETs formation

have not been sufficiently evaluated in human ARDS (30) and no clinical study examined this pathway in terms of lung-kidney crosstalk. Further evaluation of these pathways in a clinical setting is absolutely required.

POSSIBLE ORGAN CROSSTALK BETWEEN THE KIDNEY AND THE LUNG IN COVID-19

The novel coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan in December 2019, which has spread around the world. SARS-CoV-2 dominantly affects the respiratory system and recent clinical studies reported AKI as a significant comorbidity in COVID-19 (31). The cell surface protein, angiotensin-converting enzyme 2 (ACE-2), which is used by the virus as an entry receptor, is abundantly expressed not only in lung epithelial cells but in renal tubular epithelial cells (32), indicating SARS-CoV-2 could directly infect the kidneys. We recently observed that the urinary neutrophil gelatinase-associated lipocalin (NGAL) level at ICU admission was significantly elevated among patients who developed AKI. Furthermore, urinary NGAL was correlated with length of mechanical ventilation use (33). Because urinary NGAL is expected to reflect renal tubular epithelial cell damage, elevated NGAL might reflect the burden of viral insult of renal damage directly and lung injury indirectly.

DISCUSSION

Multiple organ failure frequently observed in critically ill patients was previously considered as the "sum" of each organ failure. The sequential organ failure assessment (SOFA) score, which is widely used for evaluating the severity of ICU patients, is calculated by summing up each organ injury score. No consideration for organ crosstalk can be found in these scoring systems. Several clinical studies demonstrated possible organ crosstalk in ICU patients by applying network analysis (34, 35). As described above, basic research revealed several different pathways that contributed to organ crosstalk related to AKI (36). Unfortunately, no clinical evidence is available for organ crosstalk demonstrated by animal experiments. Future studies that could clarify the significance of organ crosstalk in the clinical setting is necessary to develop a novel therapeutic strategy that targets currently unrecognized organ crosstalk in critically ill patients.

This review covers kidney-lung crosstalk mostly regarding lung injury caused by AKI. The opposite pathological mechanism in which ARF/ARDS causes AKI should also be considered. As described above, only a limited number of basic studies reported possible mechanisms in animal experiments. In clinical studies, ARF/ARDS caused a systemic release of pro-inflammatory mediators (plasminogen activator inhibitor-1, IL-6, and soluble TNF receptors-1 and 2), which could induce or worsen AKI (6). AKI can be caused by a reduction in renal blood flow caused by hypoxemia or hypercapnia, and a decreased glomerular filtration rate due to the elevation of central venous pressure (renal congestion). In patients with ARF/ARDS, these mechanisms could initiate or aggravate AKI and cause those patients to fall into a vicious cycle (37).

Mechanical ventilation-induced lung injury has been investigated so far. Low tidal ventilation strategy against ARDS is recommended based on evidence obtained by both basic and clinical studies (38). Does MV have any significant impacts on the kidney injury? Excessive tidal volume leads to proinflammatory mediator release. High positive end-expiratory pressure can cause elevated intrathoracic pressure and systemic venous pressure, leading to a reduced net glomerular filtration rate. Lung protective ventilation based on the volume-pressure curve can achieve lower levels of TNF-α, IL-1b, IL-6, and IL-8 in bronchoalveolar lavage fluid with a lower incidence of AKI in the clinical setting (39).

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AM, NH, and KD contributed to the writing of the manuscript and approved the final version of the manuscript.

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REVIEW
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Glomerulonephritis in AKI: From Pathogenesis to Therapeutic Intervention

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Acute kidney injury (AKI) is increasingly emerging as a global emergency. Sepsis, major surgery, and nephrotoxic drugs are the main causes of AKI in hospitalized patients. However, glomerulonephritis accounts for about 10% of AKI episodes in adults, mainly related to rapidly progressive glomerulonephritis resulting from granulomatous polyangiitis (GPA, Wegener granulomatosis), microscopic polyangiitis (MPA), and anti-glomerular basement membrane (GBM) disease. Also, diffuse proliferative lupus nephritis, immunoglobulin A nephropathy, post-streptococcal glomerulonephritis, mixed cryoglobulinemia, mesangiocapillary glomerulonephritis, membranous nephropathy, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and scleroderma can induce acute renal failure. Early diagnosis of AKI due to glomerulonephritis is crucial for prompt, effective management to improve short- and long-term outcomes. Kidney biopsy is the gold standard for the diagnosis of glomerular disease, but it is not frequently performed in critically ill patients because of their clinical conditions. In this setting, a growing number of diagnostic assays can support the working hypothesis, including antineutrophil cytoplasmic antibodies (ANCAs), anti-double-stranded DNA antibodies, anti-GBM antibodies, antistreptolysin O and anti-DNase B antibodies, cryoglobulins, antiphospholipid antibodies, and complement levels. Therapeutic strategies in AKI patients with glomerulonephritis include high-dose corticosteroids, cyclophosphamide, and plasma exchange. This article reviews the wide spectrum of glomerulopathies associated with AKI, describing the immunological mechanisms underlying glomerular diseases and presenting an overview of the therapeutic options.

Keywords: AKI, glomerulonephritis, antibodies, complement, immunosuppression

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INTRODUCTION

Acute kidney injury (AKI) is a severe medical condition involving up to 10 million people worldwide (1), with an increasing global incidence especially in hospitalized patients (2). AKI affects ∼10–15% of hospital inpatients and more than 50% of patients hospitalized in the intensive care units (ICUs) (2, 3). Renal replacement therapy (RRT) is necessary in 5–6% of critically ill patients and is characterized by an increased risk of progression to chronic kidney disease

(CKD) and end-stage kidney disease (ESKD) (about 10% annually) (4–6). The definition of AKI is based on standard criteria such as serum creatinine and/or urine output (7, 8), although several biomarkers have recently been proposed in this clinical setting (9). Severe short- and long-term consequences are frequently associated with AKI, and the mortality rate in critically ill patients is still significant, ranging from 37% to 60% (4, 10–12). Moreover, patients who survive AKI present a major long-term mortality rate and an increased risk of developing CKD (13) and other chronic comorbidities (14–19).

Glomerulonephritis accounts for about 10% of AKI in adults (20). AKI episodes in glomerular disease are usually due to rapidly progressive glomerulonephritis (RPGN), in which the renal function declines over days or weeks. The most common causes are small-vessel vasculitis and anti-glomerular basement membrane (GBM) disease, although other glomerular diseases may manifest with acute renal impairment, including IgA nephropathy (IgAN), thrombotic microangiopathy (TMA), lupus nephritis, and post-streptococcal glomerulonephritis (16). Furthermore, acute renal failure in glomerulonephritis can also result from non-glomerular conditions such as acute tubular necrosis (ATN) from renal hypoperfusion or the nephrotic syndrome and drug- or radiocontrast agent-induced tubular epithelial cell injury.

Early diagnosis and prompt, effective treatment of glomerular disease may dramatically change the disease course and improve patient outcomes (21). In this scenario, kidney biopsy remains the gold standard for the diagnosis of kidney disease when the patient's clinical condition allows the performance of this procedure (22). This article reviews the main glomerular diseases manifesting with AKI (summarized in **Table 1**), describing the immunological mechanisms underlying glomerular diseases and the potential therapeutic strategies, summarizing the main features.

ANTI-GBM DISEASE

Epidemiology and Disease Pathogenesis

Anti-GBM disease is a rare but severe immunological disorder manifesting with RPGN and pulmonary complications (lung hemorrhage). It accounts for about 5% of all cases of RPGN and affects especially young males and the elderly (23, 24). A genetic predisposition has been reported: anti-GBM disease is strongly associated with class II human leukocyte antigen (HLA), including DRB1*1501 and DR4 alleles, whereas DR1 and DR7 confer strong protection (25). Furthermore, several environmental triggers (smoking, hydrocarbon exposure, and drugs) appear to be important in the disease etiology (24, 26). The pathogenesis of the disease is immunological and related to the formation of antibodies specifically targeting the NC1 domain of the α3-chain of type IV collagen localized in the glomerular and alveolar basement membranes (24, 27, 28): complement activation, phagocyte accumulation, and T-cell recruitment after immunocomplex deposition contribute to the extent of the glomerular damage (29).

Clinical Presentations and Diagnosis

Patients typically present a mild prodromal phase followed by severe clinical features with macroscopic hematuria and/or AKI. Lung involvement is often characterized by hemoptysis and dyspnea that characterize the Goodpasture syndrome. Kidney and respiratory functions decline more rapidly than in any other form of RPGN, and mortality is often due to renal failure requiring RRT or to massive alveolar bleeding (30). The clinical suspicion, based on the simultaneous renal and pulmonary involvement, is a critical step to improve patient outcomes. The rapid renal function decline, the presence of a very active urinary sediment, and the scarce systemic involvement differentiate anti-GBM disease from vasculitis and lupus nephritis. An atypical variant of the disease, with no significant pulmonary involvement and undetectable serum anti-GBM antibodies, has also been described (30). Antineutrophil cytoplasmic antibody (ANCA) can be detected in rare cases showing double antibody positivity. This double-positive variant presents a bad prognosis requiring early and more aggressive treatment (30).

The diagnosis is confirmed by the detection of circulating anti-GBM antibodies, although their levels do not correlate with disease severity (27). Kidney biopsy is essential as it indicates the extent and severity of the renal lesions. The histological picture in anti-GBM disease is usually characterized by diffuse extracapillary proliferation with extensive crescent formation, often with associated fibrinoid necrosis of the glomerular tuft. Different degrees of glomerulosclerosis (as a result of previous proliferative lesions), tubular necrosis, and interstitial inflammation can also be observed. A pathognomonic sign of the disease is the linear deposition of IgG and, often, C3 (75%) along the glomerular and sometimes (10–67%) also distal tubular basement membranes and the alveolar basal membrane in the lung. The extent of glomerular involvement (percentage of crescents and fibrinoid necrosis) is correlated with the prognosis (29, 30).

Treatment

Since the clinical course is rapidly progressive, early treatment is needed, to remove circulating anti-GBM antibodies and reduce renal inflammation. Immunosuppressive therapy should be started immediately, except in patients with minimal renal involvement or irreversible kidney disease without lung involvement (31). The Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines for glomerulonephritis recommend the use of corticosteroids (usually intravenous pulses of methylprednisolone up to 1,000 mg/day for three consecutive days followed by oral prednisone of 1 mg/kg/day tapered to 20 mg/day for 6 weeks), oral cyclophosphamide (CYC, 2 mg/kg/day), and plasmapheresis (daily exchange of 1–1.5 volume of plasma against 5% human albumin for 14 days) in patients affected by anti-GBM disease (31–33). Lung hemorrhage is usually responsive to this treatment, and hemoptysis resolves within a few days. Renal recovery is more variable, and the clinical response to therapy is usually slower in patients requiring RRT and/or with serum creatinine values exceeding 600 μmol/L and in patients with histological evidence of a high proportion of crescents (34, 35).

TABLE 1 | Summary of key features for each disease.

Disease	Specific mechanisms of acute damage	Diagnosis	Kidney biopsy	Treatment	Apheresis	Prognostic markers
Anti- GBM disease	Autoantibody against the NC1 domain of the α3-chain of type IV collagen (46, 47)	Clinical signs Anti-GBM antibody CT Kidney/lung biopsy	Diffuse proliferative GN, crescents, necrosis ± tubular loss IF: Linear deposition of IgG and C3	CYC (2 mg/kg/day) + Methylprednisolone (1 g/day for 3 days followed by tapering with oral prednisone) ± TPE	**TPE (Category III, Grade 2B)** Volume treated: 1–1.5 TPV Frequency: Daily or every other day Duration: at least 10–20 days, or until resolution of evidence of ongoing glomerular or pulmonary injury	Glomerular lesions: - crescents necrosis - Tubular damage Dialysis dependent sCr > 600 μmol/L
Small Vessel Vasculitis	ANCAs (PR3; MPO) and Neutrophils activation	Clinical signs ANCA antibody Radiological investigations Tissues biopsies/Kidney biopsy	Necrotizing and extra-capillary crescentic GN Vascular segmental fibrinoid necrosis, sclerosis, thrombosis	**Induction therapy:** Methylprednisolone (7 mg/Kg/day for 3 days followed by oral prednisolone 1 mg/Kg/day) + CYC (2 mg/kg/day) or, in alternative, RTX (375 mg/m^2 weekly for 4 weeks) **Maintenance therapy:** AZA (1–2 mg/kg/day) or MMF (up to 1 g twice daily) or MTX (0.3 mg/kg/wk, maximum 25 mg/wk) or RTX	**TPE (Category I, Grade 1A)** Volume treated: 1–1.5 TPV Frequency: Daily or every other day Duration: median number is 7, over a median period of 14 days, up to 12 for further improvement of renal function BVAS	Glomerular lesions: - focal - crescents - mixed - sclerotic
SLE: lupus nephritis class IV	Immunocomplexes, complement, leucocytes, TMA	Kidney biopsy	Endocapillary or extracapillary GN, glomerulosclerosis, sub-endothelial immune deposits, tubular atrophy, interstitial fibrosis IF: "full house" pattern	**Induction therapy:** CYC (low-dose: 500 mg every 2 weeks for 3 months or high-dose: 0.5–1 g/m^2 monthly for 6 months)/MMF (2–3 g/daily) + Corticosteroids. In alternative, RTX (375 mg/m^2 weekly for 4 weeks) **Maintenance therapy**: Low dose Prednisone (<10 mg/day) + AZA (1.5–2 mg/kg/day) or MMF (1–2 g/day in two doses) for at least 12 months after complete remission. CNIs (in patients intolerant to MMF or AZA)	**TPE (Category II, Grade 2C)** Volume treated: 1–1.5 TPV Frequency: LN or DAH: daily or every other day; Other severe complications: 1–3 times per week Duration: course of 3–6 TPE	Glomerular lesions: - crescents - necrosis Heavy proteinuria Racial background
IgAN	Massive hematuria; erytro-phagocytosis process, crescents	Kidney biopsy	Mesangial hypercellularity, focal segmental necrotizing GN, crescents, glomerulosclerosis, tubule-interstitial inflammation ± fibrosis	High-dose corticosteroids (Methylprednisolone 1 g/daily for three consecutive days, followed by oral prednisone 0.5 mg/kg/day and subsequent tapering/Oral prednisone 1 mg/kg/day for 2 months followed by tapering) for 6 months + CYC (1–2 mg/kg/day) for 3 months, followed by AZA (1–2 mg/kg/day) for 3 months	**TPE (Category III, Grade 2C)** Volume treated: 1–1.5 TPV Frequency: 4–11 over 21 days Duration: until clinical resolution	High sCr Crescents Duration of hematuria

(Continued)

TABLE 1 | Continued

Disease	Specific mechanisms of acute damage	Diagnosis	Kidney biopsy	Treatment	Apheresis	Prognostic markers
Post-streptococcal GN	Immunocomplexes, complement, leucocytes	Clinical signs Antistreptolysin O and anti–DNase B antibodies	Diffuse endocapillary GN, monocytes and lymphocytes infiltration	No specific immunosuppressive treatment	**TPE (Category III, Grade 2B)** Volume treated: 1–1.5 TPV Frequency: Daily or every other day Duration: 3–6 TPE over 1–2 weeks	Deficiency of complement regulatory proteins Nephrotic proteinuria Age Diabetic nephropathy
TMA	TMA	**TTP:** ADAMTS 13 activity, Anti ADAMTS13 autoantibody **STEC-HUS:** STEC (cultured test) or STX (P.C.R.) **aHUS:** microangiopathic haemolytic anemia, acute kidney injury, ADAMTS13 and STEC-HUS negativity	Intraluminal platelet thrombi, partial or complete obstruction of vessel lumina, myointimal proliferation and reduplication of the lamina densa, sever ischemic change	**TTP:** ADAMTS 13 auto-antibodies: TPE +/prednisone/prednisolone or RTX **Maintenance therapy:** PI: Caplacizumab + TPE + immunosuppressive therapy **STEC-HUS**: i.v 10–15 ml/kg/h of isotonic solution; Eculizumab (900 mg/ weakly for 4 weeks, followed by 1,200 mg every 2 weeks) and/or TPE/PI **aHUS:** Eculizumab (900 mg/ weakly for 4 weeks, followed by 1,200 mg every 2 weeks) **FHAA-mediated aHUS:** TPE + immunosuppressive therapy	**TPE (Category III, Grade 2C)** Volume treated: 1–1.5 TPV Frequency: Daily Duration: No standardized approach, duration and schedule should be made based upon patient response and condition	**TTP:** ADAMTS 13 activity; severe neurological signs; haemolysis **STEC-HUS:** severe neurological signs; haemolysis **aHUS:** genetic background
MPGN and Mixed Cryoglobulinemia	Immunocomplexes	Cryoglobulins, HCV markers; clinical signs/ symptoms	Mesangial and endocapillary proliferation, extracellular matrix expansion, diffuse leucocytes and monocytes infiltration, double-contouring of the GBM, intraluminal thrombi	TPE + RTX(375 mg7m^2/week for 4 weeks)/CYC (2 mg/Kg/day for 2–4 months), + i.v. methylprednisolone (0.5/1 g/Kg/day for three consecutive days followed by oral prednisone), + antiviral therapy	**TPE (Category II, Grade 2B)** Volume treated: 1–1.5 TPV Frequency: Every 1–3 days Duration: 3–8 procedures for acute symptoms, weekly or monthly maintenance treatments to prevent recurrent symptoms	High sCr Nephrotic proteinuria Severe hypertension >50% crescents Marked interstitial fibrosis
Membranous nephropathy	Volume depletion Ischemic tubular damage	Kidney biopsy	Uniform increase in thickness of glomerular capillary walls, double-contouring and "spikes" of the GBM. IF: Diffuse, finely granular deposition of IgG along outer surface of all capillary walls	Volume correction	–	Severe hypoalbuminemia, age, male sex

(Continued)

TABLE 1 | Continued

Disease	Specific mechanisms of acute damage	Diagnosis	Kidney biopsy	Treatment	Apheresis	Prognostic markers
Scleroderma	Ischemia, microangiopathy	Clinical signs; ANA and anti scl-70 antibody	Malignant hypertensions lesions, TMA in small interlobular and arcuate arteries Glomerular ischemic collapse; fibrinoid necrosis ± tubular atrophy and interstitial fibrosis	ACE-i TPE/ECP	**TPE (Category III, Grade 2C)** Volume treated: 1–1.5 TPV Frequency: 1–3 per week Duration: course of 6 TPE over the 2–3 weeks followed by 4 weekly treatments. Long-term TPE protocol (2–3 weekly for 2 weeks, 1 TPE weekly for 3 months, and 1 TPE every other week as a maintenance therapy) was also used. **ECP** Volume treated: Typically, 1.5 L of whole blood processed Frequency and Duration: Two procedures on consecutive days (one series) every 4–6 wk for 6–12 months	Anti-DNA polymerase

GBM, Glomerular Basement Membrane; CT, Computer Tomography; GN, glomerulonephritis; IF, immunofluorescence; CYC, cyclophosphamide; TPE, therapeutic plasma exchange; sCr, serum creatinine; ANCAs, anti-neutrophil cytoplasmic autoantibodies, PR3, proteinase 3; MPO, myeloperosidase; RTX, Rituximab; AZA, azathioprine; MMF, mycophenolate; MTX, methotrexate; BVAS, Birmingham Vasculitis Activity Score; SLE, Systemic Lupus Erythematosus; TMA, Thrombotic Microangiopathy; IgA N, IgA nephropathy; TTP, thrombotic thrombocytopenic purpura; STEC-HUS, Shiga-toxin producing E. coli-associated Hemolytic Uremic Syndrome; STEX, Shiga-toxin; PCR, polymerase chain reaction; aHUS: atypical Hemolytic Uremic Syndrome; PI, Plasma Infusion; i.v., intravenous; FHAA-HUS, Factor H antibody associated Hemolytic Uremic Syndrome; MPGN, Membranoproliferative Glomerulonephritis; ANA, Anti-nuclear antibody; anti scl-70, anti-topoisomerase antibody, ACE-i, ACE inhibitors; ECP, Extracorporeal photopheresis.

Oral prednisone and CYC are generally discontinued after 6 and 3 months, respectively, while plasmapheresis is continued for at least 14 days or until anti-GBM antibodies become undetectable.

SMALL-VESSEL VASCULITIS

Epidemiology and Disease Pathogenesis

Small-vessel vasculitis is a group of inflammatory systemic diseases characterized by a segmental necrotizing polyangiitis of small vessels, including GPA (Wegener granulomatosis), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (Churg–Strauss syndrome), and renal-limited vasculitis (36). The first two diseases account for the majority of cases of RPGN, presenting as an emergency in the critical care setting (37, 38). The diseases can occur at any age, but the prevalence is greatest at ages 50–70 years, with a slight predominance in Caucasians and male gender (36). Small-vessel vasculitis is associated with the production of ANCAs, which have a central role in the disease pathogenesis, since they are directed against specific enzymes found within the cytoplasmic granules of neutrophils and monocytes, proteinase 3 (PR3), and myeloperoxidase (MPO) antigens (39, 40). Genetic background (specific α1-antitrypsin and neutrophil FcRγ111 receptor genotypes) and environmental triggers (in particular, infections and drugs) are reported as potential co-players in disease pathogenesis (41–45); specific HLA polymorphisms are associated with disease predisposition (46). After expression of PR3 and MPO on the surface following trigger conditions (infections), ANCAs bind these antigens, inducing neutrophil degranulation and the release of inflammatory mediators (cytokines, reactive oxygen species, and lytic enzymes), leading to endothelial damage (47, 48). Neutrophils also release PR3 and MPO, which adhere to the endothelium and induce *in situ* immunocomplex formation. Monocytes/macrophages, T cells, and complement system are also involved in the pathogenic process (49–52).

Clinical Presentations and Diagnosis

Wegener granulomatosis is characterized by a wide range of clinical manifestations, including RPGN with the formation of extracapillary crescents, alveolar hemorrhage, episcleritis, rhinitis, sinusitis, hearing loss, purpura, peripheral neuropathy, subglottic tracheal stenosis, and angina abdominis (53). The disease course is often recurrent, with relapses occurring within a few years after disease remission. In patients with MPA, renal involvement is always reported, while respiratory tract diseases are less common; moreover, the frequency of relapses is lower than that in Wegener granulomatosis.

The clinical manifestations drive the diagnosis, which is supported by the detection of circulating ANCA. Anti-PR3 autoantibodies [cytoplasmic ANCA (c-ANCA)] are positive in about 90% of patients with active Wegener granulomatosis, and anti-MPO autoantibodies [perinuclear ANCA (p-ANCA)] are typically detected in about 80% of patients with active MPA (54, 55). Typically, ANCA levels are higher at the onset, and their levels are directly correlated with disease activity; in fact, a significant increase in ANCA levels is reported in relapses. These antibodies are also found in other immunological disorders (inflammatory bowel disease, autoimmune liver disease, and rheumatoid arthritis). A limited percentage of patients (10–20%) affected by small-vessel vasculitis does not show ANCA positivity (56). Thus, ANCA negativity may not exclude the diagnosis in the presence of clinical symptoms. Radiological investigations and tissue biopsies can support the diagnosis.

Although kidney biopsy in these patients is considered to be at high risk, since bleeding complications are frequent due to vessel inflammation, histology can be very useful for predicting the response to treatment and clinical outcome.

ANCA-associated glomerulonephritis is histologically characterized by necrotizing and crescentic glomerulonephritis, with a variable degree of glomerular involvement (57). Granulomas are a specific feature of Wegener granulomatosis (58, 59). Immunofluorescence shows little or no glomerular staining for immunoglobulins or complement, and this feature is typically termed a "pauci-immune" staining pattern. Electron microscopy demonstrates subendothelial edema, microthrombosis, and degranulation of neutrophils, without immune deposits (60). Vascular lesions feature fibrinoid necrosis, sclerosis, and thrombosis. Apart from glomerular and vascular lesions, acute and chronic tubulointerstitial lesions are critical risk factors indicating disease severity and progression. A recent histological classification comprises four categories of glomerular lesions, which correlate the loss of function with increasing degrees of severity: focal, crescentic, mixed, and sclerotic. Kidney survival at 5 years is 93% in the focal, 76% in the crescentic, 61% the mixed, and 50% in the sclerotic class (55). A growing number of studies have investigated the utility of TIMP 1, C–X–C motif chemokine ligand 13, and matrix metalloproteinase-3 as biomarkers of ANCA-associated vasculitis and for treatment assessment (61, 62).

Treatment

A prompt diagnosis and early, appropriate treatment are essential to prevent loss of renal function and progression to CKD. Kidney and patient survival rates range between 65 and 75% at 5 years after appropriate treatment (63). KDIGO guidelines recommend the use of high-dose corticosteroids (intravenous methylprednisolone ar 7 mg/kg/day for 3 days followed by oral prednisone at 1 or 1 mg/kg/day of oral prednisone tapered to 0.25 mg/day for 3 months) plus oral or intravenous CYC (2 mg/kg/day or 0.5 g/m^2 monthly, respectively. Combined treatment induces remission in about 90% of patients at 6 months. When CYC is contraindicated or the disease is resistant to this regime, the anti-CD20 monoclonal antibody rituximab (RTX) (375 mg/m^2 weekly for 4 weeks) plus steroids is recommended (27–29). In this context, the RAVE and RITUXIVAS studies have shown that RTX is equivalent to CYC in terms of efficacy, with a similar risk profile (64, 65).

The addition of plasma exchange is recommended for patients requiring dialysis, with rapid increases of serum creatinine, diffuse pulmonary hemorrhage, and/or with an overlap syndrome of ANCA vasculitis and anti-GBM disease (31–33). Therapeutic plasma exchange (TPE) reduces the

development of ESKD by ∼40% (66), but the true efficacy is still questioned (67).

During maintenance therapy, CYC is replaced by azathioprine (AZA, 1–2 mg/kg/day) or alternatively mycophenolate (MMF, up to 1 g twice daily) or methotrexate (initially 0.3 mg/kg/week, maximum 25 mg/week). An alternative regimen includes the use of CYC for the induction treatment and perhaps RTX for maintenance (32, 33). Patients should be monitored for general symptoms and laboratory data, as well as urinary RBC counts, C-reactive protein, renal function, and ANCA levels. A rapid response to treatment is typical, with a significant improvement of the clinical condition within few weeks. High ANCA levels may persist for several months. The maintenance treatment is carried out for at least 18 months up to 3 years in Wegener granulomatosis and 2 years in MPA if no relapses occur (68), which can often happen during immunosuppression tapering and may require a further short course of high-dose corticosteroids or CYC.

In ANCA-associated pauci-immune glomerulonephritis, the mortality rate within the first year of diagnosis is reported to be about 20% of cases: ESKD occurs in up to 25% of the patients within 4 years after diagnosis (63).

OTHER GLOMERULONEPHRITIS FORMS THAT CAUSE AKI

Systemic Lupus Erythematosus (SLE)

Renal involvement in patients with SLE disease is associated with a worse outcome and higher mortality. Kidney damage is characterized by the deposition of immunocomplexes, complement activation, leukocyte infiltrations, and microangiopathic thrombosis probably driven by the type I interferon signature, involving all renal compartments (glomeruli, vessels, and tubule interstitium) (69). The histological findings in patients with lupus nephritis (LN) may range from initial mesangial immune deposits [class I in the World Health Organization (WHO) classification] to diffuse global glomerulosclerosis (class VI) (70); however, <10% of all patients with LN develop RPGN, which is associated with the diffuse proliferative glomerulonephritis form (Class IV).

Clinically, LN should be suspected in the presence of urinary abnormalities (hematuria) and nephrotic proteinuria; in addition, patients with Class IV LN typically exhibit acute impairment of renal function and hypertension. Low complement levels and positivity for anti-dsDNA antibodies may confirm the clinical suspicion. However, renal biopsy is crucial to assess the extent of renal lesions and to tailor appropriate treatment (21, 71, 72). In class IV LN, more than 50% of the glomeruli exhibit endocapillary hypercellularity and/or cellular crescents associated with other active (i.e., fibrinoid necrosis, karyorrhexis, presence of neutrophils, abundant subendothelial immunocomplex deposits, and interstitial inflammation) and chronic lesions (global and segmental glomerulosclerosis, adhesions, fibrous crescents, interstitial fibrosis, and tubular atrophy). Immunofluorescence shows a typical "full house" pattern, characterized by the evidence of IgG, IgM, IgA, C3, and C1q immune deposits. Electron microscopy reveals abundant electron-dense subendothelial deposits conferring a "wire loop" profile to the capillary walls (70, 73).

In patients with class IV LN, induction therapy can be based on intravenous CYC (low dose: 500 mg every 2 weeks for 3 months or high dose: 0.5–1 g/m^2 monthly for 6 months) or MMF (2–3 g/day) combined with oral glucocorticoids (0.5–1.0 mg/kg/day) with or without three pulses of intravenous methylprednisolone (32, 33). In addition, RTX and other biological agents may be useful in class IV LN and in refractory LN as induction therapy (71). The induction therapy may be associated with TPE, as reported in **Table 1** (67). After the induction treatment, maintenance therapy is required; oral AZA (1.5–2.5 mg/kg/day) or MMF (1–2 g/day in divided doses) is recommended and effective, combined with low-dose oral prednisone (≤10 mg/day) (31–33). The maintenance therapy should be continued for at least 1 year after complete remission is achieved, while a repeat kidney biopsy is required when complete remission has not been achieved. The maintenance phase of the Aspreva Lupus Management Study (ALMS) showed that regardless of the induction therapy, MMF was superior to AZA in maintaining the renal response and preventing relapse of proliferative LN: the overall treatment failure rate in the MMF group was half that observed in the AZA group (16.4 vs. 32.4%), and renal flares were significantly higher in patients treated with AZA (12.9 vs. 23.4%) (74). The superiority of MMF demonstrated in the ALMS was not apparent in the MAINTAIN nephritis trial, published in 2010 (probably due to differences in the study design): however, renal flares occurred in 19% of the MMF group, compared with 25% of the AZA group, suggesting that MMF should be considered the drug of choice (75). Finally, calcineurin inhibitors (CNIs) should be used for maintenance therapy in patients intolerant to MMF and AZA.

IgAN

IgAN is the most common primary glomerulonephritis worldwide; it is characterized by persistent microscopic hematuria, mild proteinuria, and episodes of gross hematuria concurrently with upper respiratory tract infections (76). However, RPGN has been described in one third of patients with IgAN where histology reveals >50% crescents. Crescentic IgAN is a critical condition, leading to CKD and ESKD in a few years and so requiring prompt, efficacious treatment (77, 78). High-dose corticosteroids and CYC are recommended (79, 80). Corticosteroid treatment can be based on methylprednisolone (1 g/day for three consecutive days), followed by oral prednisone 0.5 mg/kg/day and subsequent tapering or, alternatively, on oral prednisone 1 mg/kg/day for 2 months followed by tapering (0.2 mg/kg monthly) within 6 months. CYC [1–2 mg/kg/day, based on the patient's glomerular filtration rate (GFR)] should be administered for 3 months, followed by AZA (1–2 mg/kg/day) for the following 3 months. TPE can be combined with immunosuppressive treatments until clinical improvement is achieved (67).

In addition, AKI episodes in IgAN patients can be due to massive hematuria of glomerular origin (81). In cases of massive hematuria, tubular cell damage has been described as

the consequence of red blood cell cast formation and intratubular obstructions, combined with erythrophagocytosis processes (81). The hemoglobin products released by red blood cells induce oxidative stress, inflammation, podocytes, and tubular cell apoptosis and consequently cell detachment and fibrosis (82). In this setting, adequate and sustained hydration is crucial to prevent renal damage and enhance renal recovery. Renal biopsy should be performed in cases of persistent kidney function impairment despite supportive care and massive hydration.

Post-streptococcal Glomerulonephritis

Post-streptococcal glomerulonephritis typically occurs in children in developing countries at 1–3 weeks after upper respiratory tract infections (83). Clinically, 20% of affected patients develop a classic nephritic syndrome characterized by hematuria, hypertension, oliguria, and worsening renal function. The diagnosis is based on the presence of these symptoms associated with the detection of antistreptolysin O and anti-DNase B antibodies and low C3 (83). In this setting, renal biopsy is not mandatory since the prognosis is usually excellent and no specific treatments are needed (21). However, 1% of patients develop crescentic glomerulonephritis, which is sometimes associated with ANCA (83). The role of immunosuppression in these patients is debated. Although there is no evidence yet from randomized controlled trials (RCTs), the use of high-dose corticosteroids can be considered in patients with extensive glomerular crescents and RPGN.

TMA

TMA is a clinical phenotype which includes multiple diseases leading to thrombosis of arterioles and capillary vessels. Laboratory findings (thrombocytopenia with microangiopathic hemolytic anemia) lead to a suspicion of TMAs and reflect the mechanical disruption of red blood cells and platelets in the microvasculature (84). Thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) are the two classic TMA disorders (85). TTP is mainly characterized by neurological involvement, while only a limited proportion of patients (25%) show renal involvement; TTP patients usually exhibit congenital or acquired ADAMTS 13 deficiency leading to the accumulation of the ultra-large multimers of von Willebrand factors (vWFs) that cause platelet aggregation and consequently microvascular thrombosis (86). Conversely, kidney injury typically occurs in all patients with HUS, as a consequence of the massive thrombotic capillaries' occlusion (87). The histological features comprise microvascular thrombosis, swelling, and detachment of the endothelial cells from the GBM; patchy cortical necrosis; and focal-segmental sclerosis, which is a long-term sequela of acute HUS (88, 89).

HUS includes STEC-HUS due to Shiga toxin-producing *Escherichia coli* infection (90–92), pneumococcal HUS caused by infections with *Streptococcus pneumoniae*, and genetic HUS, also called aHUS (93). STEC-HUS occurs most frequently in pediatric patients (the incidence is five to six children per 10,000 children population per year). Patients typically present bloody diarrhea and gastroenteritis 6–10 days before the development of TMA. STX closely adheres to gut epithelial cells, causing apoptosis and destruction of the brush border of the villi. Then, the toxin enters the circulation: in the kidney, STX is internalized via the Gb3 receptor and releases a protease into the cytoplasm. This protease inhibits protein synthesis and activates inflammatory pathways, inducing cell death (92). The diagnosis is driven by the clinical suspicion and the detection of STEC (cultured test) or STEX (polymerase chain reaction). Prompt intravenous hydration (10–15 ml/kg/h of isotonic solution) is recommended as soon as *E. coli* infection is suspected, in order to reduce the systemic effects of STX (94, 95). The use of antibiotics must be avoided, because they increase STX gene expression and induce massive STX release. Only azithromycin can be administered to a very limited number of children presenting with bacteremia (94). Supportive management of anemia, renal failure, and hypertension is required in these patients. In cases of severe clinical manifestations and neurological involvement, eculizumab and/or plasma exchange can be used as rescue therapy (88). The prognosis in children is generally favorable, and up to 70–85% of patients recover renal function.

aHUS is a multifactorial rare disease linked to an uncontrolled activation of the alternative complement pathway. About 60% of aHUS patients carry genetic variants or risk haplotypes in genes of the alternative pathway (CFH, CFI, CFB, MCP, C3, CFHR1-5, and THBD) and/or in diacylglycerol kinase E (DGKE). Genetic background accounts for different outcomes, as response to therapy and risk of relapses may vary depending on the underlying mutation; in addition, specific environmental factors (pregnancy, drugs, infections, etc.) have been reported as triggers in disease pathogenesis (93). aHUS is a life-threatening disorder requiring immediate diagnosis and treatment, often in the ICU. In addition, 50% of aHUS patients progress to ESKD, and the mortality rate during the acute phase of the disease is significant. The diagnosis of aHUS is made on exclusion of STEC infection and ADAMTS 13 deficiency; moreover, a genetic screening should be recommended, particularly in patients who are candidates for kidney transplantation. Treatment should be promptly started. The use of eculizumab in the aHUS treatment has dramatically changed short- and long-term outcomes in this setting and is currently the first-line treatment. The attack dose is 900 mg weekly for 4 weeks, followed by 1,200 mg at week 5 and then every 2 weeks: however, the duration of treatment is not yet well established. The Food and Drug Administration and the European Medicines Agency have recently approved a long-acting anti-C5 antibody (ravulizumab) for the treatment of aHUS, to be administered every 8 weeks (half-life of approximately 51 days).

It is important to remember that patients with DGKE variants do not respond to eculizumab therapy and thus pose an open challenge for scientists and physicians (96). Finally, 3–8% of aHUS patients present antibodies to complement factor H protein (FHAA), which are associated with a homozygous deficiency of FHR1 (85%) and homozygous deletion of CFHR3-CFHR1 (97). In this setting, plasma exchange alone was associated with disease recurrence (58%), CKD (39%), ESKD (27%), and death (9%) (98). A combination of TPE and immunosuppression reduces the antibody production and improves patient outcomes (99, 100).

Membranoproliferative Glomerulonephritis (MPGN) and Mixed Cryoglobulinemia

In MPGN, the histological pattern includes a group of chronic immune-mediated diseases characterized by GBM thickening and proliferative changes (101). On the basis of the pathophysiological processes, MPG is currently classified as immunocomplex-mediated MPGN and complement-mediated GNs (DDD and C3GN) (102). Among these disorders, mixed cryoglobulinemia is the most prevalent disorder in older adults (103). Mixed cryoglobulinemia is a small-vessel vasculitis characterized by the precipitation of circulating immunocomplexes after cold exposure (polyclonal IgG with or without monoclonal IgM with rheumatoid factor activity), involving several organs such as the skin, the joints, the peripheral nervous system, and the kidneys (103). Hepatitis C virus (HCV) disease is the main cause of mixed cryoglobulinemia. In HCV-positive patients, renal involvement accounts for about 30–40% of all cases (104). Clinical symptoms are weakness, arthralgias, and purpura, followed by a nephritic/nephrotic syndrome and rapidly deteriorating kidney function (104, 105). Kidney histology is characterized by mesangial and endocapillary proliferation, extracellular matrix expansion, diffuse leukocyte and monocyte infiltration, double contouring of the GBM, and the presence of intraluminal PAS-positive thrombi. On electron microscopy, electron-dense deposits (cryo-immunocomplexes) can be detected in subendothelial and mesangial regions (104, 106). The diagnosis is based on specific laboratory findings (detection of serum cryoglobulins and serologic hepatitis C markers) combined with the clinical signs and symptoms (104). In this scenario, KDIGO guidelines suggest the use of TPE combined with immunosuppressive treatment, such as RTX (375 mg/m^2/week for 4 weeks) or CYC (2 mg/kg/day for 2–4 months), combined with intravenous methylprednisolone (0.5/1 g/kg/day for three consecutive days followed by oral prednisone). Importantly, concomitant antiviral therapy should be performed in HCV-positive patients (31–33).

Glomerulonephritis Manifesting With Nephrotic Syndrome

Several forms of glomerulonephritis are associated with a nephrotic syndrome, characterized by heavy proteinuria, severe hypoalbuminemia, edema, weight gain due to fluid retention, and dyslipidemia. Membranous nephropathy is the main cause of nephrotic syndrome in adults (107); in addition, diabetic nephropathy, minimal change disease, focal-segmental glomerulosclerosis, SLE, and amyloidosis usually manifest with nephrotic syndrome. AKI episodes have been in rare cases described as a potential complication of nephrotic syndrome, independently of the underlying pathogenesis (108, 109). The AKI pathogenesis in this setting is multifactorial. Some clinical observations supported the hypothesis that the massive urinary albumin loss induces microvascular injury due to volume depletion (109), leading to a massive expression of endothelin 1, inducing a decrease in blood flow and GFR (110). In addition, ischemic tubular damage and cell necrosis have been reported in some cases. Recent studies have shown that some urine biomarkers (NGAL and alpha 1-microglobulin) are elevated in patients with AKI and nephrotic syndrome (111). Although the pathogenesis still needs to be clarified, AKI in NS is generally reversible. The majority of patients with idiopathic nephrotic syndrome have a complete recovery of kidney function after volume depletion correction. Severe hypoalbuminemia, older age, and male sex are risk factors (109).

Scleroderma

Scleroderma is an autoimmune systemic disorder characterized by uncontrolled expansion of connective tissue in skins and other visceral organs. Kidney involvement is usually characterized by the presence of low-grade proteinuria, but AKI episodes can occur (112, 113). The so-called scleroderma renal crisis is a life-threatening complication of scleroderma and typically presents with the abrupt onset of hypertension and rapid progressive renal insufficiency, followed by encephalopathy, heart failure, and signs and symptoms of TMA (113, 114). The typical histological features are ischemic glomerular changes, malignant hypertension lesions, and TMA in small interlobular and arcuate arteries: glomeruli may show ischemic collapse and fibrin thrombi; various degrees of tubular atrophy and interstitial fibrosis have also been reported (115). Clinical presentation, along with the detection of speckled ANA (positive in 90% of cases) and anti-topoisomerase I antibodies (scl-70) (positive in 20% of cases), supports the diagnosis (113). Renal biopsy should be performed in patients with atypical presentations (increased serum creatinine in normotensive patients or in the presence of active urine sediment or nephrotic range proteinuria). Activation of the renin–angiotensin–aldosterone system (RAAS) is crucial in the pathogenesis of scleroderma renal crisis. Thus, the introduction of angiotensin-converting enzyme (ACE) inhibitors has substantially improved the prognosis of patients with scleroderma crisis, reducing the mortality associated with scleroderma crisis to <10% (113). Calcium channel blockers and, as third line, diuretics and alpha-blockers could be used as additional therapy if blood pressure control remains suboptimal despite maximum doses of ACE inhibitors (116). In addition, recent evidence suggests a role of complement activation and endothelin-1 in the pathogenesis of scleroderma crisis, thus suggesting the use of C5 inhibitors and endothelin receptor antagonists, particularly in refractory cases. Finally, TPE seems to confer some benefits in patients with scleroderma crisis with evidence of TMA or in patients with ACE inhibitor intolerance (116): a typical course of six TPE over 2–3 weeks followed by 4-weekly treatments is a reasonable therapeutic approach, resulting in long-lasting improvements in symptoms (67).

CONCLUSIONS

Several glomerular diseases have been reported to manifest with AKI episodes: early diagnosis is crucial since different conditions have a similar clinical profile but

require different, often aggressive, treatment in order to preserve renal function and delay the onset of ESKD. In this scenario, renal biopsy is essential for an accurate diagnosis and to describe the extent of reversible/irreversible renal lesions.

AUTHOR CONTRIBUTIONS

FPe and ES wrote the manuscript. MR provided histological images. MF, FPi, GS, GC, and LG revised the paper. All authors contributed to the article and approved the submitted version.

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Dietary Modification Alters the Intrarenal Immunologic Micromilieu and Susceptibility to Ischemic Acute Kidney Injury

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The versatility of the intrarenal immunologic micromilieu through dietary modification and the subsequent effects on susceptibility to ischemic acute kidney injury (AKI) are unclear. We investigated the effects of high-salt (HS) or high-fat (HF) diet on intrarenal immunologic micromilieu and development of ischemic AKI using murine ischemic AKI and human kidney-2 (HK-2) cell hypoxia models. Four different diet regimens [control, HF, HS, and high-fat diet with high-salt (HF+HS)] were provided individually to groups of 9-week-old male C57BL/6 mice for 1 or 6 weeks. After a bilateral ischemia-reperfusion injury (BIRI) operation, mice were sacrificed on day 2 and renal injury was assessed with intrarenal leukocyte infiltration. Human kidney-2 cells were treated with NaCl or lipids. The HF diet increased body weight and total cholesterol, whereas the HF+HS did not. Although the HF or HS diet did not change total leukocyte infiltration at 6 weeks, the HF diet and HF+HS diet increased intrarenal CD8 T cells. Plasma cells increased in the HF and HS diet groups. The expression of proinflammatory cytokines including TNF-α, IFN-γ, MCP-1, and RANTES was increased by the HF or HS diet, and intrarenal VEGF decreased in the HS and HF+HS diet groups at 6 weeks. Deterioration of renal function following BIRI tended to be aggravated by the HF or HS diet. High NaCl concentration suppressed proliferation and enhanced expression of TLR-2 in hypoxic HK-2 cells. The HF or HS diet can enhance susceptibility to ischemic AKI by inducing proinflammatory changes to the intrarenal immunologic micromilieu.

Keywords: diet, high-salt diet, high-fat diet, acute kidney injury, immunologic micromilieu, ischemia-reperfusion injury

INTRODUCTION

Ischemic acute kidney injury (AKI) is the most common cause of AKI and frequently contributes to development and progression of chronic kidney disease (CKD) in both native and transplanted kidneys (1, 2). Ischemia-reperfusion injury (IRI) induces graft injury, an inevitable consequence of kidney transplantation (3). Substantial roles of immunologic mechanisms, beyond simple hypoxic injury, in the pathogenesis of ischemic AKI have been demonstrated in many studies (4, 5). In the

post-ischemic kidney following IRI, a robust inflammatory response caused by both innate and adaptive immune systems results in kidney damage (6). In addition to infiltration of circulating immune cells, the intrarenal immunologic micromilieu, including resident intrarenal immune cells (7) and Toll-like receptors (TLRs) on renal tubules (8), contributes significantly to renal injury following IRI (6).

Recent studies have reported a relationship between diet and immune function (9). Changes in dietary composition have the potential to exacerbate or alleviate the severity of diseases in which immune mechanisms play an important role in the pathogenesis, such as hypertension in a murine model of salt-sensitive hypertension or obesity-related kidney damage in a high-fat (HF) diet-induced obesity model (10–12). However, it is yet to be determined whether dietary modification can alter the intrarenal immunologic micromilieu and susceptibility to ischemic AKI, although the role of dietary intervention in CKD has been reported (13). Considering the potential for preventive or therapeutic effects of dietary intervention in ischemic AKI, it is important to investigate the effects of dietary modification in normal kidneys.

In this study, we aimed to reveal the effects of HF or high-salt (HS) diet on normal kidneys and development of ischemic AKI with a focus on the intrarenal immunologic micromilieu.

MATERIALS AND METHODS

Dietary Modification and the Renal IRI Model

This study was approved by the Samsung Medical Center Animal Care and Use Committee and the Institutional Review Board of Samsung Medical Center (IACUC No. 20180314002) and was performed in compliance with the animal research: reporting *in vivo* experiments guidelines (14, 15). Male 9-week-old C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Kyoungki-do, Korea). All mice were housed in a specific pathogen-free barrier facility.

We investigated the effects of HF or HS diet on normal kidney and post-ischemic kidneys in an ischemic AKI model induced by bilateral IRI surgery. In each model, mice were randomly allocated into four diet regimens; normal diet (0.25% NaCl by weight, 10% fat by calories), HF diet (0.25% NaCl by weight, 60% fat by calories), HS diet (8% NaCl by weight, 10% fat by calories), and high-fat diet with high-salt (HF+HS) (8% NaCl by weight, 60% fat by calories). The composition of the normal and HS diets was 20% protein (main source casein), 70% carbohydrate (main source sucrose), and 10% fat (main source soybean oil) (D12450B, Research Diets, New Brunswick, NJ). The composition of the HF diet and the HF+HS diet was 20% protein (main source casein), 20% carbohydrate (main source Lodex 10), and 60% fat (main source lard) (D12492, Research Diets). All diet regimens had the same mineral and vitamin concentrations except for NaCl.

As for normal mice, each group was maintained on the allocated diet for 42 days and then switched to a normal diet for 14 days.

Regarding the ischemic AKI model, we used an established murine IRI model with a laparotomy approach (16, 17). Bilateral IRI was induced after 1-week or 6-week durations of dietary modification. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg; Yuhan, Seoul, Korea) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). After an abdominal midline incision, both renal pedicles were isolated and clamped for 27 min with a microvascular clamp (Roboz Surgical Instrument, Gaithersburg, MD). During the operation, anesthetized mice were kept well-hydrated with warm sterile saline and placed on a thermostatically controlled heating table. After 27 min, the microvascular clamps were released from the renal pedicles for reperfusion. After applying sutures, mice were allowed to recover with free access to the allocated diet and water. All mice were sacrificed on day 2 after the IRI operation, and post-ischemic kidneys were harvested after exsanguination.

Assessment of Renal Function

In normal mice, blood urea nitrogen (BUN; Fujifilm, Bedford, UK) and plasma creatinine (Arbor Assays, Ann Arbor, MI) concentrations were measured in plasma samples collected from tail veins serially on days 0, 7, 14, 28, 42, and 56 after dietary modification. Colorimetric kits were used according to the manufacturer's recommended methods. In the IRI model, plasma samples were measured on days 0, 1, and 2 after the operation using the same methods.

Tissue Histological Analysis

In the IRI model, post-ischemic kidney tissue sections were fixed with 10% buffered formalin and stained with hematoxylin and eosin. A renal pathologist who was blinded to the diet allocation scored renal tubular necrosis in the cortex and outer medulla of post-ischemic kidneys.

CD45 Immunohistochemistry and TissueFAXS Analysis

Formalin-fixed renal tissue sections were immunostained for detection of CD45 as follows. Sections (4-μm-thick) were deparaffinized with xylene, rehydrated in a graded alcohol series, and placed in a citrate buffer solution (pH 6.0). Slides were placed in a pressure cooker and heated for 10 min to enhance antigen retrieval. After cooling, the kidney sections were immersed in a hydrogen peroxide solution (Dako, Carpinteria, CA) for 30 min to block endogenous peroxidase activity, followed by overnight incubation at 4°C with serum-free protein block (Dako). The next day, the slides were incubated with a 1:100 dilution of anti-mouse CD45 monoclonal antibody (BD Biosciences, San Jose, CA) for 1 h at room temperature. After being rinsed, the CD45-stained sections were incubated for 30 min at room temperature with a secondary antibody using a Dako REAL EnVison kit (Dako). Subsequently, 3,3′-diaminobenzidine tetrahydrochloride

Abbreviations: AKI, acute kidney injury; BIRI, bilateral ischemia-reperfusion injury; BUN, blood urea nitrogen; HK-2 cell, human kidney-2 cell; IFN-γ, interferon-γ; IL, interleukin; IRI, ischemia-reperfusion injury; KMNCs, kidney mononuclear cells; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; SEM, standard error of the mean; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

(Dako) was applied to the slides to produce a brown color, and the slides were counterstained with Mayer's hematoxylin solution (Dako).

A TissueFAXS workstation (Tissue Gnostics, Vienna, Austria) was used to analyze and calculate the percentage of CD45-positive cells in kidney samples, as described previously (18).

Flow Cytometric Analysis of Kidney-Infiltrating Mononuclear Cells

Isolation of kidney mononuclear cells (KMNCs) was based on an established protocol (19). Briefly, decapsulated kidneys were immersed in RPMI buffer (Mediatech, Manassas, VA) containing 5% FBS and disrupted mechanically using a Stomacher 80 Biomaster (Seward, Worthing, UK). Samples were strained, washed, and resuspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) followed by gentle overlaying onto 72% Percoll. The samples were centrifuged at 1,000*g* for 30 min at room temperature. KMNCs were collected from the interface of 36% and 72% Percoll.

Isolated KMNCs were resuspended in FACS buffer and pre-incubated with anti-CD16/CD32 antibodies for 10 min to minimize non-specific binding through Fc-receptors. KMNCs were incubated with anti-mouse anti-CD3, CD4, CD8, CD19, CD21, CD25, CD44, CD45, CD62L, CD69, CD126, CD138, Gr-1, F4/80, FoxP3, and NK1.1 (all from BD Biosciences, San Jose, CA) for 25 min at 4°C, washed with FACS buffer, and fixed with 1% paraformaldehyde solution. Samples were acquired using a BD FACSVerse flow cytometer. Data were analyzed using the FACSuite program (BD Biosciences).

Multiplex Cytokine/Chemokine Assay

Multiplex cytokine and chemokine analysis in whole kidney protein extracts was conducted using a Milliplex MAP Mouse Cytokine/Chemokine Kit (Luminex, Austin, TX) following the manufacturer's instructions. Anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct properties of two fluorescent dyes were used in this multiplexed particle-based flow cytometric assay. Our assay was designed to quantify interleukin (IL)-2; IL-4; IL-6; IL-10; interferon (IFN)-γ; monocyte chemoattractant protein (MCP)-1; regulated on activation, normal T cell expressed and secreted (RANTES/CCL5); tumor necrosis factor (TNF)-α; and vascular endothelial growth factor (VEGF). The value of each cytokine or chemokine was normalized by dividing the raw concentration (pg/ml) by the kidney protein concentration (mg/ml, measured by using a Pierce BCA protein assay kit, Thermo Fisher Scientific, Waltham, MA).

Western Blot Analysis of Intrarenal Toll-Like Receptors 2 and 4

Intrarenal Toll-like receptors 2 and 4 (TLR-2 and TLR-4) were analyzed by western blot analysis. According to the manufacturer's instructions, equal amounts of whole kidney protein extract (30 μg) were separated by electrophoresis on a NuPAGE Bolt mini gel system (Thermo Fisher Scientific). The gels were transferred onto a nitrocellulose membrane using an iBlot 2 Dry Blotting System (Thermo Fisher Scientific) after electrophoresis. Membranes were blocked with 5% skim milk tris-buffered saline solution with 0.1% Tween20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with one of the following antibodies: mouse monoclonal anti-TLR2 antibody (MyBioSource, San Diego, CA) or anti-TLR4 antibody (Novus Biologicals, Centennial, CO). The horseradish peroxidase-conjugated secondary antibody was applied for 30 min at room temperature after washing with TBST. The signal was visualized using an Amersham ECL detection system (GE Healthcare, Chicago, IL), following the manufacturer's instructions. Bands were densitometrically analyzed using ImageJ 1.8 software (Wayne Rasband, National Institutes of Health, MD) and normalized against corresponding β-actin band intensity as an internal control.

HK-2 Cell Hypoxia Model and Proliferation Assay

Human kidney-2 (HK-2) cell (an immortalized proximal tubule epithelial cell line from a normal adult human kidney) hypoxia model was used for *in vitro* study to investigate the effects of a HS or HF environment at a cellular level.

Human kidney-2 (HK-2) cells were purchased from the American Type Culture Collection (CRL-2190, Manassas, VA) and cultured in keratinocyte serum-free media (Thermo Fisher Scientific) supplemented with bovine pituitary extract and human recombinant epidermal growth factor. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 with the media changed every 2–3 days. Hypoxia was induced by exposure to 1% O_2 and 5% CO_2 balanced with nitrogen in a multi-gas incubator (APM-30D, Astec, Fukuoka, Japan) for 48 h.

HK-2 cells were divided into four groups. The first and second groups were controls under normoxia (21% O_2) and hypoxia (1% O_2), respectively. The third and fourth groups were treated with additional NaCl 25 mM and 1:250 diluted lipid concentrate (Thermo Fisher Scientific), respectively, before and after hypoxic insult.

The degree of HK-2 cell proliferation on days 0, 1, and 2 after hypoxia was assessed with a Cell Titer96 aqueous one solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions.

For quantification of inflammatory signaling molecule expression in the hypoxic HK-2 cells, TLR-2 and TLR-4 were measured with western blot analysis using mouse monoclonal anti-TLR2 (Santa Cruz Biotechnology, Dallas, TX) and anti-TLR4 (Novus Biologicals, Centennial, CO) antibodies on days 0 and 2. Briefly, the cells were placed in RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 13,000*g* at 4°C for 10 min, and the supernatant was subjected to the aforementioned western blotting procedures.

Statistical Analyses

All data were expressed as mean ± standard error of the mean (SEM). Differences between groups or time points were analyzed using the Mann-Whitney *U*-test or two-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* analysis. All statistical analyses were conducted using GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA). P values <0.05 were considered statistically significant.

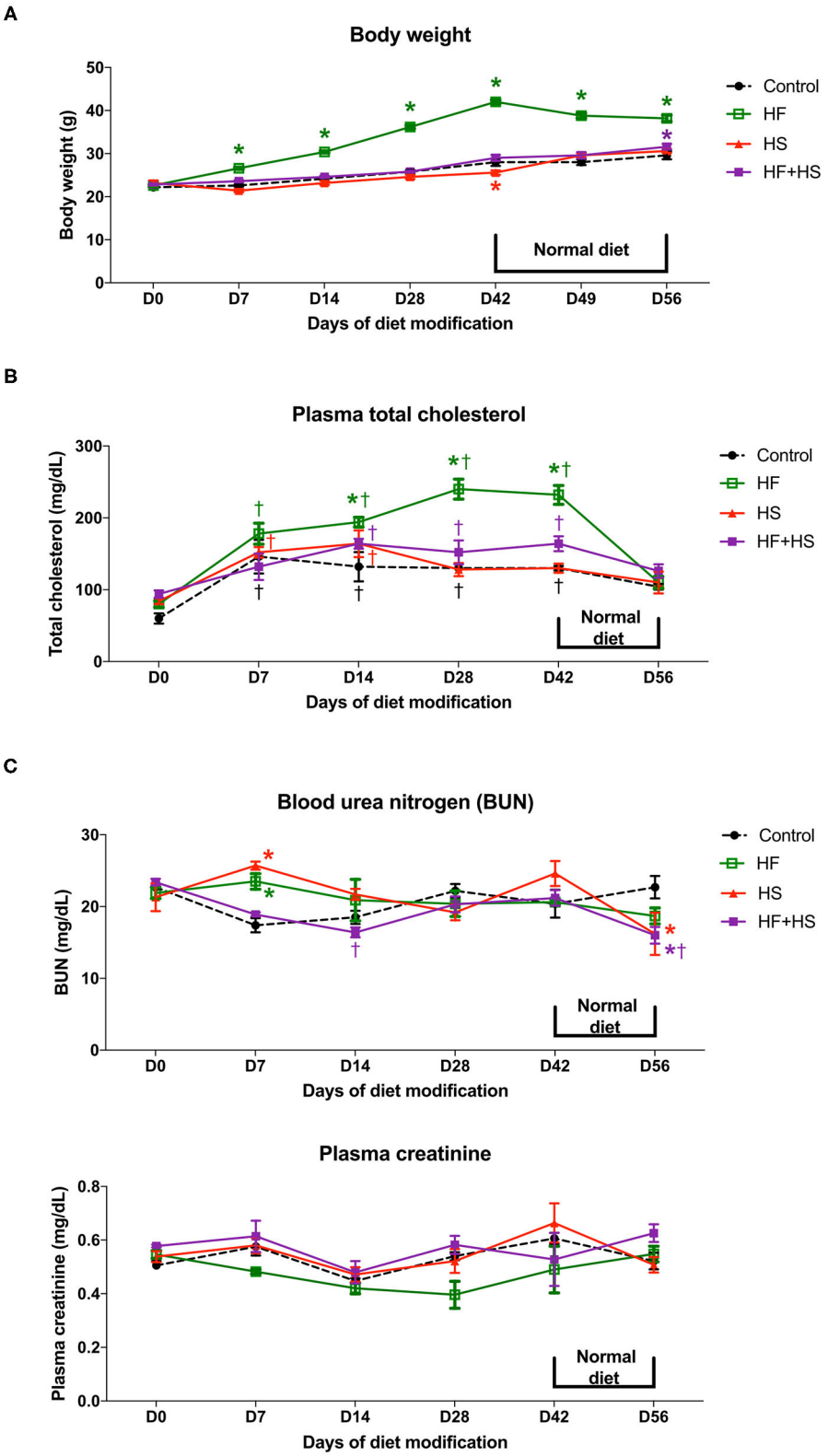

FIGURE 1 | Effects of dietary modification on physiologic changes of normal mice. **(A)** HF diet group gained weight significantly from day 7 after starting the HF diet and showed stationary body weight after switching to a normal diet. The HS diet group had a lower body weight than the control group on day 42 and returned to a body weight comparable with that of the control group 1 week after switching to a normal diet. **(B)** HF diet significantly increased plasma total cholesterol concentration

(Continued)

FIGURE 1 | from day 14 after dietary modification. The total cholesterol level of the HF diet group returned to a comparable concentration after switching to a normal diet**. (C)** BUN level on day 7 was significantly higher in the HF diet and HS diet groups. Overall renal function measured by plasma creatinine was comparable among groups for the whole study period.*$P < 0.05$, compared with the control group at each time point. †$P < 0.05$, compared with day 7 in the same group ($n = 5$ for each group at each time point). Statistical analyses were performed with two-way ANOVA test followed by Tukey's test. HF, high-fat; HS, high-salt; HF+HS, high-fat with high-salt.

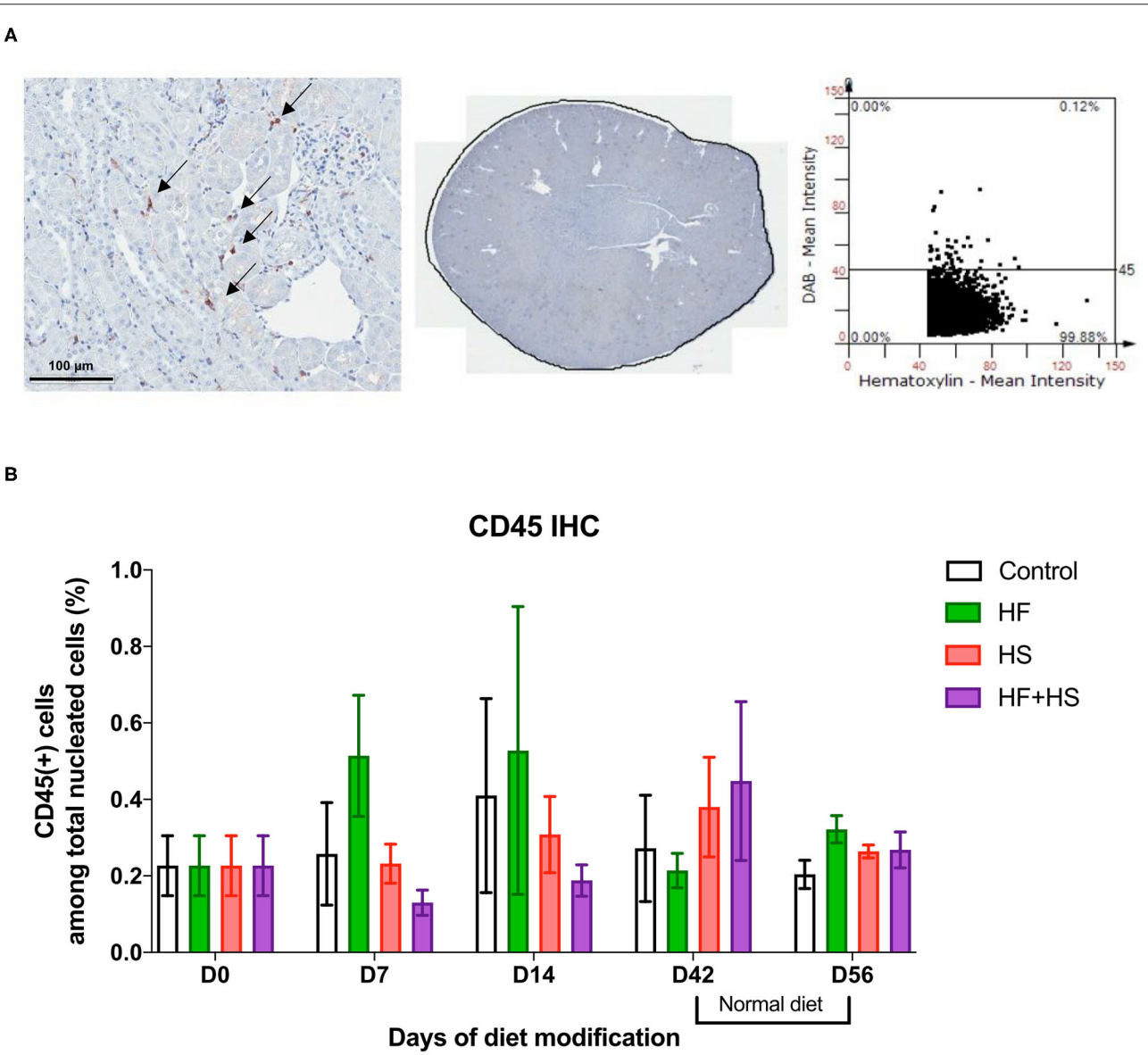

FIGURE 2 | Effects of dietary modification on intrarenal leukocytes of normal mice. Resident leukocytes were analyzed with immunohistochemistry of CD45 and flow cytometry on normal renal tissue. **(A)** Representative immunohistochemistry findings and semiquantitative analysis of CD45-positive leukocytes in normal kidney of the control group on day 0. Arrows indicate CD45-positive leukocytes (×200). **(B)** The percentages of total leukocytes expressing CD45 among total nucleated cells were comparable between diet-fed groups.

RESULTS

Effects of Dietary Modification on Physiologic Changes in Normal Mice

To investigate the physiologic changes caused by dietary modification in normal mice, body weight, plasma total cholesterol, creatinine, and BUN concentration were measured serially. The total amount of dietary intake in the HF, HF+HS, and control groups was similar, while the HS group tended to consume slightly more chow (**Supplemental Figure 1**). The body weight of the HF diet group significantly increased from day 7 after starting the HF diet. The HS diet group had a lower

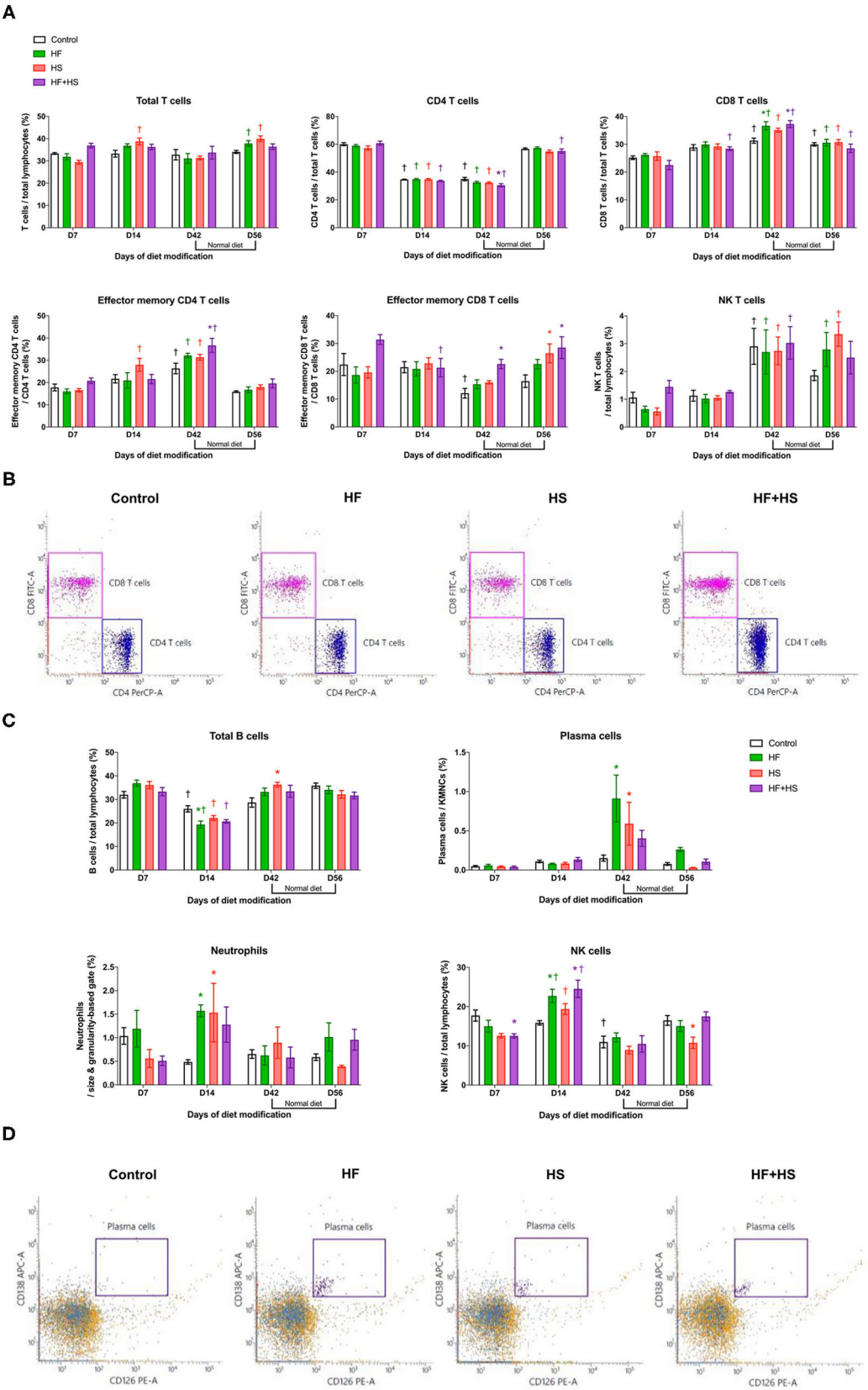

FIGURE 3 | *(Continued)*

FIGURE 3 | Flow cytometry analyses of KMNCs isolated from kidneys of normal mice. **(A)** Changes in the intrarenal T cell subpopulation by dietary modification. HF and HF+HS diets increased the proportion of total CD8 T cells among total T cells on day 42 after dietary modification. HF+HS diet also increased the proportion of effector memory CD4 and CD8 T cells. **(B)** The representative dot plots analyzing T cells on day 42. Gated cells indicate CD4 T cells and CD8 T cells among total T cells. **(C)** HF diet and HS diet increased the infiltration of plasma cells and neutrophils on days 42 and 14 after dietary modification, respectively. HF diet and HF+HS diet increased the infiltration of activated mature B cells and NK cells on day 14 after dietary modification. **(D)** Representative dot plots analyzing plasma cells on day 42. Gated cells indicate plasma cells expressing CD138 and CD126 among total kidney mononuclear cells. $^{*}P < 0.05$, compared with the control group at each time point. $^{\dagger}P < 0.05$, compared with day 7 in the same group ($n = 5$ for each group at each time point). Statistical analyses were performed with two-way ANOVA test followed by Tukey's test. HF, high-fat; HS, high-salt; HF+HS, high-fat with high-salt.

body weight than the control group on day 42 and comparable body weight to that of the control group from 1 week after switching to a normal diet (**Figure 1A**). Plasma total cholesterol level of the HF diet group was significantly higher compared to that of the control group on day 14 and became comparable to that of the control group at 1 week after switching to a normal diet. Plasma cholesterol level in the control, HS diet, and HF+HS diet groups was comparable during the 6 weeks of dietary modification (**Figure 1B**). BUN was higher in the HF diet group and the HF+HS diet group on day 7. Overall renal function was comparable among the groups for 6 weeks of dietary modification (**Figure 1C**).

Effects of Dietary Modification on the Intrarenal Leukocytes of Normal Mice

Trafficking of total leukocytes into normal kidneys was evaluated by immunohistochemical staining of CD45, followed by semiquantitative analysis with TissueFAXS (**Figure 2A**). The proportion of intrarenal total leukocytes among total nucleated cells was comparable between groups at each time point (**Figure 2B**).

Major effector cells of both innate and adaptive immune systems trafficked into the kidneys after dietary modification were analyzed with flow cytometry. Regarding T cell subtype, total CD8 T cells, effector memory CD4 T cells, and NK T cells increased over time during the diet modification, whereas total CD4 T cells decreased. In terms of intergroup differences at each time point, the HF group and the HF+HS group showed a larger proportion of CD8 T cells among total T cells on day 42 after dietary modification compared to the control diet group. The HF+HS group also showed larger proportions of the effector memory subsets of CD4 and CD8 T cells and activated CD4 and CD8 T cells (**Figures 3A,B**, **Supplemental Figure 2**). Regarding non-T cell populations, total B cells decreased and NK cells increased on day 14 compared to the day 7 after diet modification. Plasma cells reached peak numbers in the HF diet and HS diet groups on day 42 and decreased to levels comparable with those of the control group after a normal diet for 2 weeks. The proportion of NK cells and activated mature B cells among total B cells were significantly higher in the HF diet and the HF+HS diet groups on day 14 after dietary modification. The HF diet and HS diet groups showed higher intrarenal infiltration of neutrophils on day 14 after dietary modification (**Figures 3C,D**, **Supplemental Figure 2**).

Effects of Dietary Modification on Intrarenal Cytokines/Chemokines of Normal Mice

Compared to the normal diet, the HS diet or the HF+HS diet enhanced the intrarenal expression of proinflammatory cytokines/chemokines including TNF-α, INF-γ, MCP-1, and RANTES (**Figure 4A**). Expression of IL-6 was higher in the HF+HS diet group on day 7 (**Supplemental Figure 3**). Conversely, the intrarenal expression of VEGF in mice fed the HS diet and the HF+HS diet was significantly lower than that of the control group (**Figure 4B**).

Dietary Modification Affects Susceptibility to Ischemic AKI

To investigate the effects of dietary modification on post-ischemic kidneys, bilateral IRI was performed 1 or 6 weeks after dietary modification. Overall, deterioration of renal function following IRI was more prominent in the mice receiving the HS-based diet modification for both 1 week and 6 weeks compared to the control group. Both BUN and plasma creatinine concentrations were significantly higher in the mice fed the HS or HF+HS diet for 1 week compared to the control group on day 2 after IRI (**Figure 5A**). Plasma creatinine concentration in the mice fed the HF+HS diet for 6 weeks was significantly higher than that in the control group on days 1 and 2 after IRI (**Figure 5B**).

The proportions of necrotic tubules tended to be higher in the mice fed the HS or HF diet compared to the control group (**Figure 6**). The group fed an HF+HS diet for 1 week showed a significantly larger proportion of necrotic tubules than the control group (**Figure 6C**).

The percentage of total leukocytes expressing CD45 among total nuclei in the post-ischemic kidneys was significantly higher in the group fed an HF+HS diet for both 1 week and 6 weeks (**Figures 7A,B**). The group fed an HF diet for 6 weeks also showed a larger proportion of CD45-positive cells than did the control group (**Figure 7C**).

Intrarenal expression of TLR-2 and TLR-4 on day 2 following IRI was evaluated with western blotting of protein samples extracted from post-ischemic kidneys. The expression of both TLR-2 and TLR-4 tended to increase in mice fed HF or HS diet for 1 week (**Figures 8A,C**). Mice fed an HF+HS diet for 6 weeks showed significantly increased expression of both TLR-2 and TLR-4 (**Figures 8B,C**).

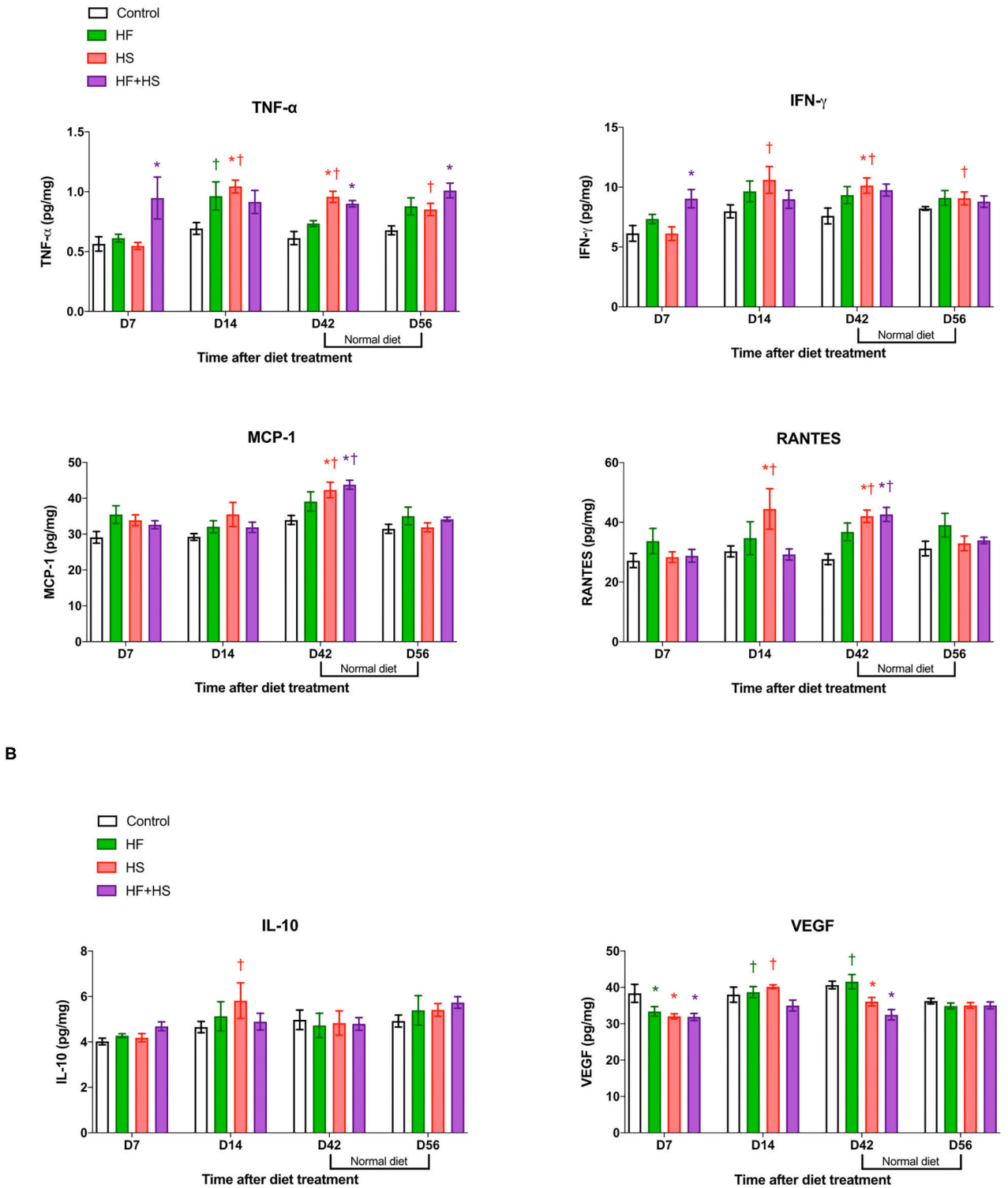

FIGURE 4 | Effects of dietary modification on intrarenal cytokines and chemokines of normal mice. HS and HF+HS diets increased intrarenal expressions of TNF-α, INF-γ, MCP-1, and RANTES on day 42. The HF+HS diet also increased the expression of IL-6 on day 7 after dietary modification. Although there were no significant differences in the expression of IL-10, the intrarenal expression of VEGF was lower in the HS diet group and the HF+HS diet group on day 7 and day 42 after dietary modification. $^{*}P < 0.05$, compared with the control group at each time point. $^{\dagger}P < 0.05$, compared with day 7 in the same group ($n = 5$ for each group at each time point). Statistical analyses were performed with two-way ANOVA test followed by Tukey's test. HF, high-fat; HS, high-salt; HF+HS, high-fat with high-salt.

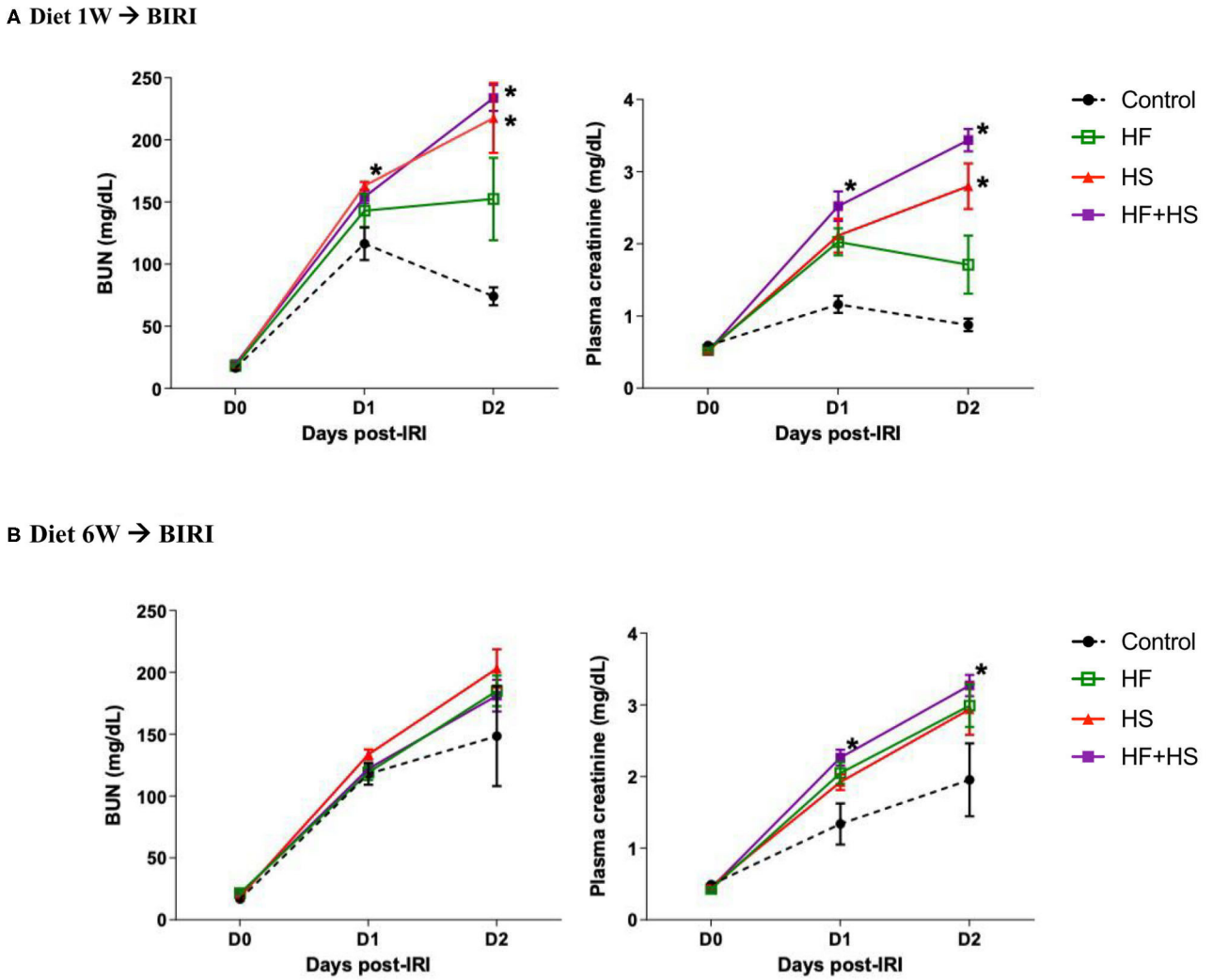

FIGURE 5 | Effects of dietary modification on development of acute kidney injury (AKI) after bilateral IRI surgery. **(A,B)** The renal functional changes following IRI in each diet group. Deterioration of renal function following IRI tended to be aggravated by HF or a HS diet fed for both 1 week and 6 weeks. BUN and plasma creatinine levels were significantly higher in the HS and HF+HS diet groups fed for 1 week after IRI. Plasma creatinine level in the HF+HS diet group fed for 6 weeks was significantly higher than that of the control group on day 2 after IRI. *$P < 0.05$, compared with the control group at each time point ($n = 5$–10 for each group). Statistical analysis was performed using the Mann-Whitney *U*-test. HF, high-fat; HS, high-salt; HF + HS, high-fat with high-salt.

Effects of High Sodium and Lipid on Hypoxic HK-2 Cells

Figure 9A shows the degree of HK-cell proliferation after hypoxic insult depending on additional NaCl and lipid treatment. Day 0, the end of hypoxia, was the day when HK-2 cells were removed from the multi-gas incubator after 48 h of hypoxia. Treatment with high salt (additional NaCl 25 mM) suppressed the proliferation of hypoxic HK-2 cells. Conversely, lipid treatment facilitated the proliferation of hypoxic HK-2 cells.

Western blot analysis of TLR-2 and TLR-4 in protein extracts of hypoxic HK-2 cells showed that lipid treatment reduced the expression of TLR-2 and enhanced the expression of TLR-4, compared with the hypoxia control group on day 2 after hypoxic insult (**Figures 9B,C**). The additional NaCl treatment did not significantly change the expression of TLR-2 orTLR-4 compared to those of the hypoxia control group.

DISCUSSION

In this study, we investigated the effects of an HF or HS diet on normal kidneys and the development of ischemic AKI focusing on the intrarenal immunologic micromilieu in murine ischemic AKI and HK-2 cell hypoxia models. A HF or HS diet increased intrarenal CD8 T cells and plasma cells as well as changed intrarenal lymphocytes to more activated and mature phenotypes in normal kidneys. An HS diet increased intrarenal proinflammatory proinflammatory cytokines and decreased intrarenal anti-inflammatory cytokines. We also found

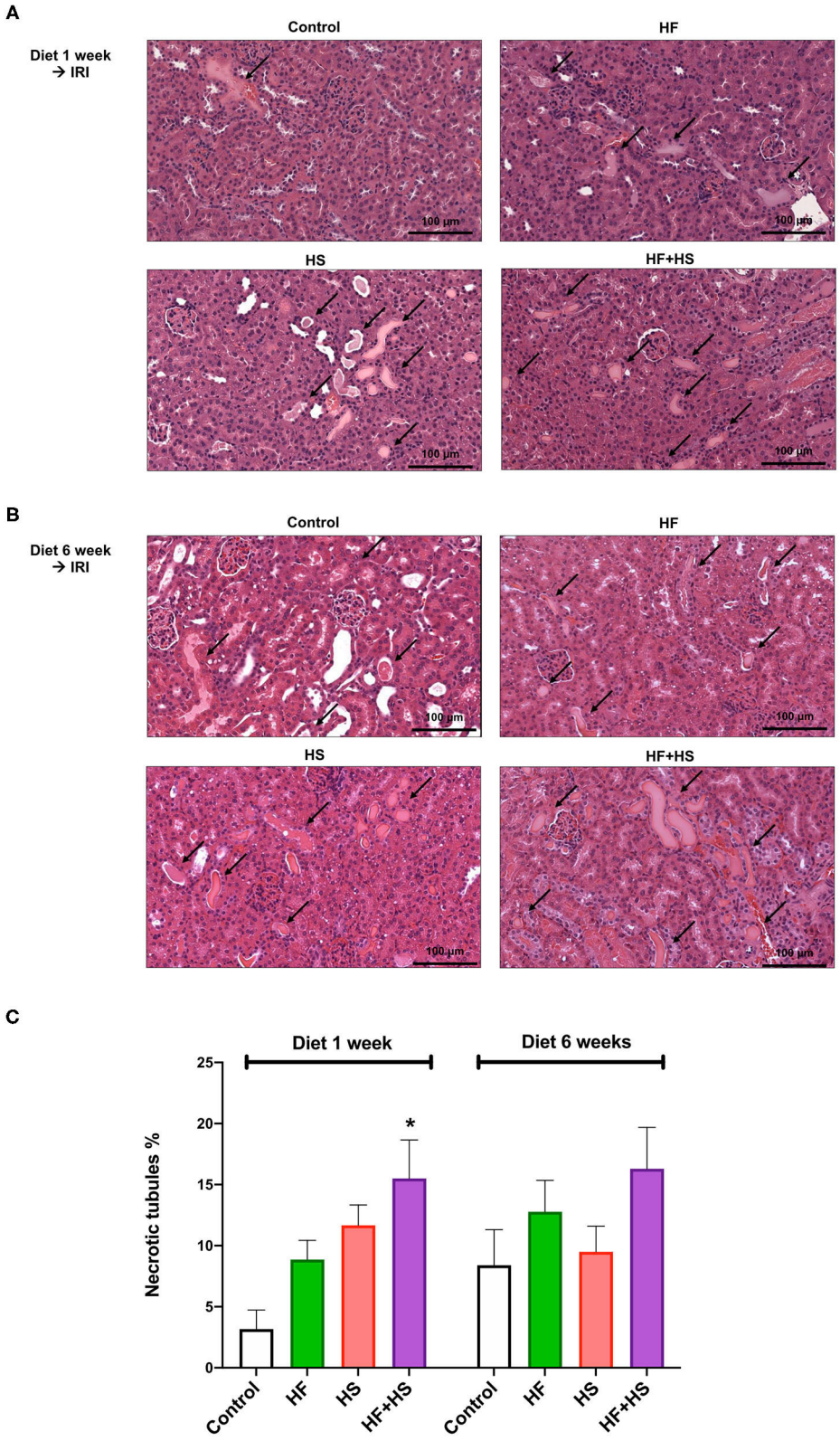

FIGURE 6 | Effects of dietary modification on structural injury following IRI surgery. **(A,B)** Hematoxylin and eosin staining of post-ischemic kidneys on day 2 after IRI. Arrows indicate damaged or necrotic tubules (×200). **(C)** The percentages of necrotic tubules tended to be higher in the mice fed an HF or HS diet compared to the control group. The HF+HS diet group fed for 1 week showed a significantly higher percentage of necrotic tubules than the control group. *$P < 0.05$, compared with the control group ($n = 5$–10 for each group). Statistical analysis was performed using the Mann-Whitney *U*-test. HF, high-fat; HS, high-salt; HF + HS, high-fat with high-salt.

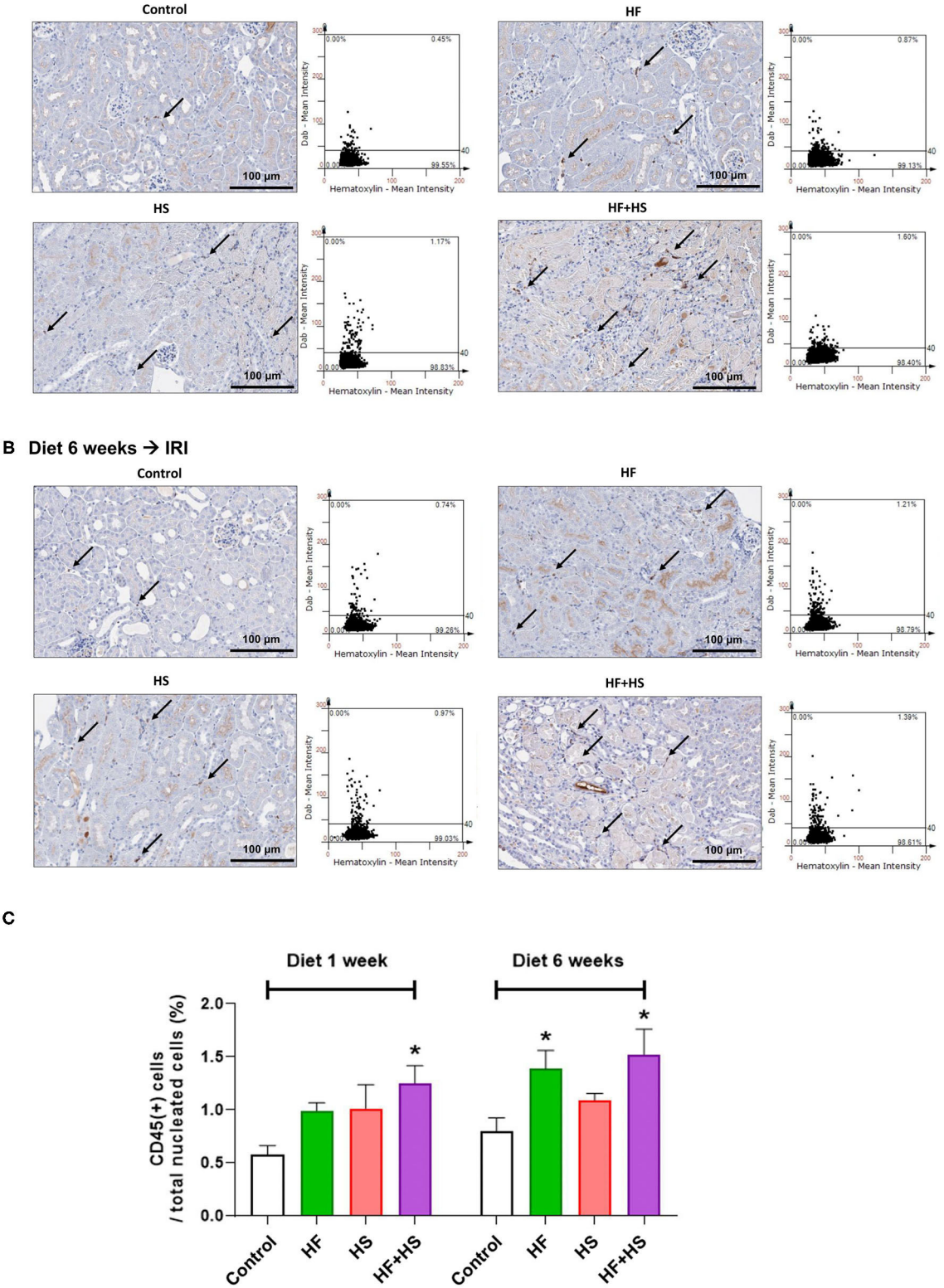

FIGURE 7 | Effects of dietary modification on leukocyte trafficking into the post-ischemic kidney. **(A,B)** Representative immunohistochemistry findings and semiquantitative analyses of CD45-positive leukocytes in the post-ischemic kidneys on day 2 after IRI. There were more pronounced infiltrations of leukocytes into the

(Continued)

FIGURE 7 | post-ischemic kidneys of mice fed with an HF or HS diet for both 1 week and 6 weeks. Arrows indicate CD45-positive leukocytes (×200). **(C)** The percentages of total leukocytes expressing CD45 among total nucleated cells were higher in the post-ischemic kidneys of HF-or HS-fed mice compared with that of mice fed a normal diet. *$P < 0.05$, compared with the control group ($n = 5$–10 for each group). Statistical analysis was performed using the Mann-Whitney *U*-test. HF, high-fat; HS, high-salt; HF+HS, high-fat with high-salt.

variability in the intrarenal immunologic micromilieu by dietary modification in a diet switching experiment. However, effector memory CD8 T cells and TNF-α remained increased after returning to a normal diet. Therefore, an HF or HS diet seemed to induce a proinflammatory intrarenal immunologic micromilieu that was not completely reversible even after returning to a normal diet for at least 2 weeks. The deterioration of renal function following IRI was more prominent in the mice receiving a HS-based dietary modification before IRI. Dietary modification *per se* did not induce clinically apparent renal dysfunction. However, the proinflammatory changes in the intrarenal immunologic micromilieu caused by HF or HS diet seem to significantly increase susceptibility of renal injury following ischemic insult.

Inflammation plays an important role in the pathogenic mechanisms of AKI and CKD (6, 20–22). In addition to recruitment of circulating immune cells to kidneys, renal parenchymal cells and immune cells residing in the normal renal tissue comprise the intrarenal immunologic micromilieu (7, 22). Previous studies have demonstrated that environmental factors, such as commensal microbes or dietary composition, affect the immune system (9, 11, 12, 23). The effects of high levels of salt or fat on the intrarenal immune system have been studied in specific immune cells such as TH17 cells and regulatory T cells (24, 25), a salt-sensitive hypertensive model (11), and an HF diet-induced obesity model (12, 26). However, these studies have focused on only a few cellular components of the immune system or disease-specific experimental models and have provided a limited view of the complex effects of dietary modification. We evaluated the effects of HF or HS diet on the overall changes to the immunologic micromilieu of the normal kidney and reversibility after returning to a normal diet. Considering that there is no preventive management or effective treatment for AKI and transition of AKI to CKD, our study investigating the effects of dietary modification on susceptibility to ischemic AKI is important clinically.

Our study showed that dietary modification increased CD8 T cells and switched intrarenal T cells into effector memory subtypes, some of which persisted even after a normal diet for 2 weeks. These changes were most prominent in the group fed an HF+HS diet. T cells, especially CD4 T cells, play an important role in the early phase of ischemic AKI (23, 27). On the other hand, CD8 T cells did not appear to play a major role in the pathophysiology of AKI compared with CD4 T cells (28). Although the role of CD8 T cell has not been fully determined, production of IFN-γ by CD8 T cells increases in murine renal IRI and glomerulonephritis models, suggesting that CD8 T cells substantially contribute to the intrarenal inflammatory response in these diseases (29, 30).

During dietary modification, activated mature B cells increased at 2 weeks, and plasma cells increased at 6 weeks followed by decrement to level comparable with that of the control group after returning to a normal diet. These results suggest that an HF or HS diet can facilitate B cell activation and differentiation into plasma cells in normal kidneys. Furthermore, neutrophil and NK cells increased at 2 weeks after dietary modification and then decreased. As phenotype changes in T cells were observed mainly at 6 weeks, it can be hypothesized that HF and HS diets first activate the innate immune system and then activate the adaptive immune system. Previous studies have reported that B cells and plasma cells have pathogenic roles in post-ischemic kidneys (16, 31). Increased levels of intrarenal plasma cells by HF or HS diet seemed to promote renal injury after IRI in our study.

HF or HS diet also induced overall changes in intrarenal cytokines and chemokines of normal kidneys to proinflammatory conditions. IRI is known to stimulate the synthesis of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-6 (32, 33) and chemokines such as MCP-1 (34, 35). Our results suggest that HF or HS diet can switch the intrarenal immunological micromilieu to a proinflammatory direction and subsequently enhance renal injury in ischemic AKI.

An HF or HS diet before IRI increased total CD45 T cells and TLR2 and TLR4 expression following IRI and induced more severe renal dysfunction during the early injury phase of IRI. This suggests that HF or HS diet enhances the innate immune response and aggravate IRI. It is worth mentioning that the increases in TLR4 expression and plasma creatinine in the group fed HF+HS diet were more remarkable compared with other groups, suggesting that HF and HS diets have additive effects on renal injury after IRI. In the HK-2 cell hypoxia model, HS treatment suppressed proliferation, but HS or high-lipid treatment did not show consistent effects on TLR expression. These differences of TLR expression in the *in vivo* and *in vitro* models suggest that the effects of dietary modification on the intrarenal immunologic micromilieu are complex responses based on the interaction between hypoxic tubular epithelial cells and intrarenal immune cells. The expression of TLR-2 and TLR-4 on renal tubular epithelial cells is enhanced in ischemic AKI (8), and TLR-2 contributes to renal injury after IRI as an important initiator of inflammatory responses (36). Therefore, our results further support more severe renal damage in ischemic AKI due to changes in the intrarenal immunologic micromilieu induced by dietary modification containing HF or HS compared to normal diet.

Interestingly, changes in the HF or HS diet were not consistent and some changes were offset, although the precise mechanisms of the different changes between the HF diet and the HS diet are unclear. For example, despite comparable amounts of dietary

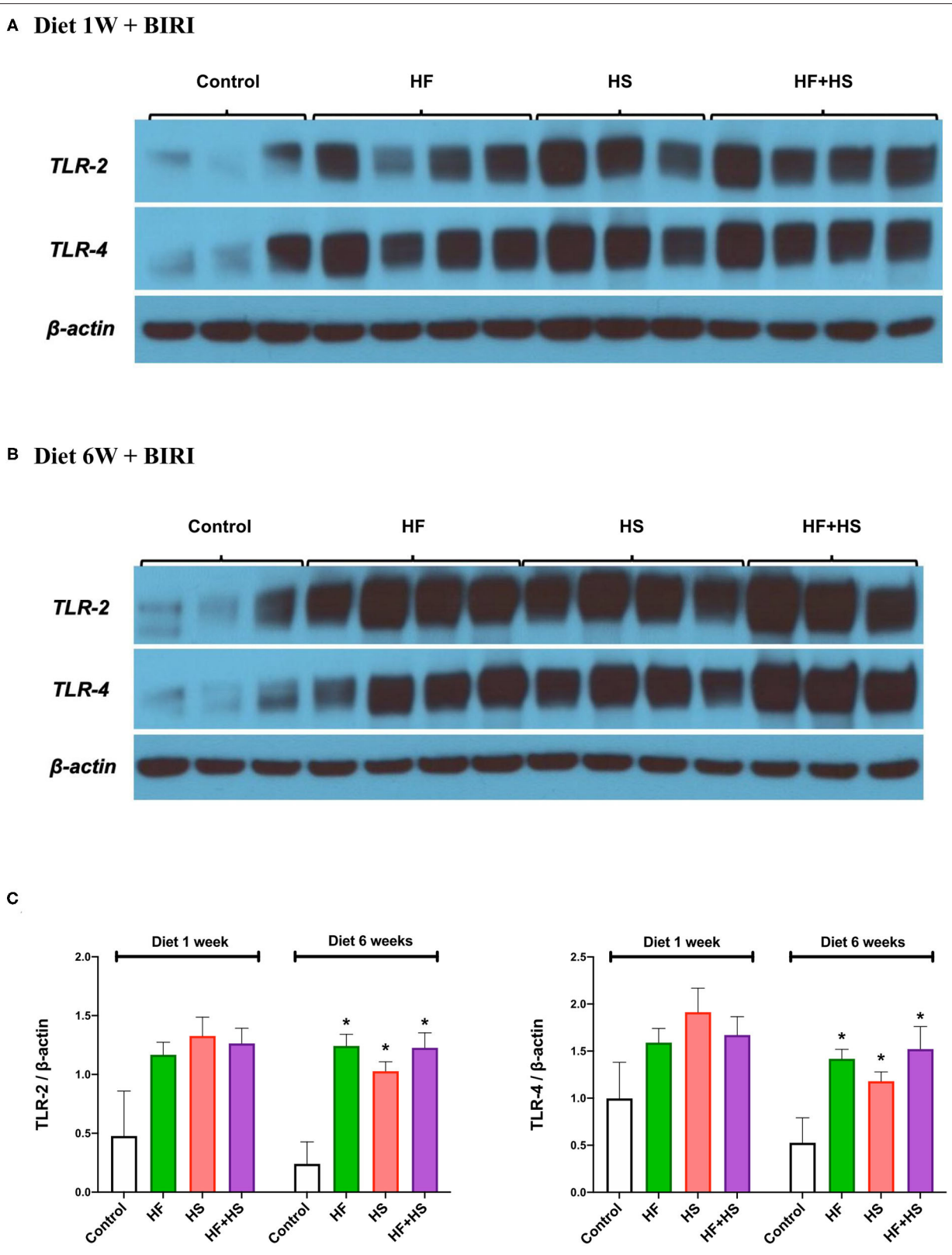

FIGURE 8 | Western blotting of protein samples extracted from post-ischemic kidneys on day 2 after IRI. **(A)** Overall expression of TLR2 and TLR4 tended to be higher in post-ischemic kidneys of mice fed an HF or HS diet for 1 week compared to those of the mice fed a normal diet. **(B,C)** The expression of TLR-2 and TLR-4 was significantly higher in the HF, HS, and HF+HS diet groups fed for 6 weeks compared to the control group. *$P < 0.05$, compared with the control group ($n = 3$–4 for each group). Statistical analysis was performed using the Mann-Whitney *U*-test. HF, high-fat; HS, high-salt; HF+HS, high-fat with high-salt.

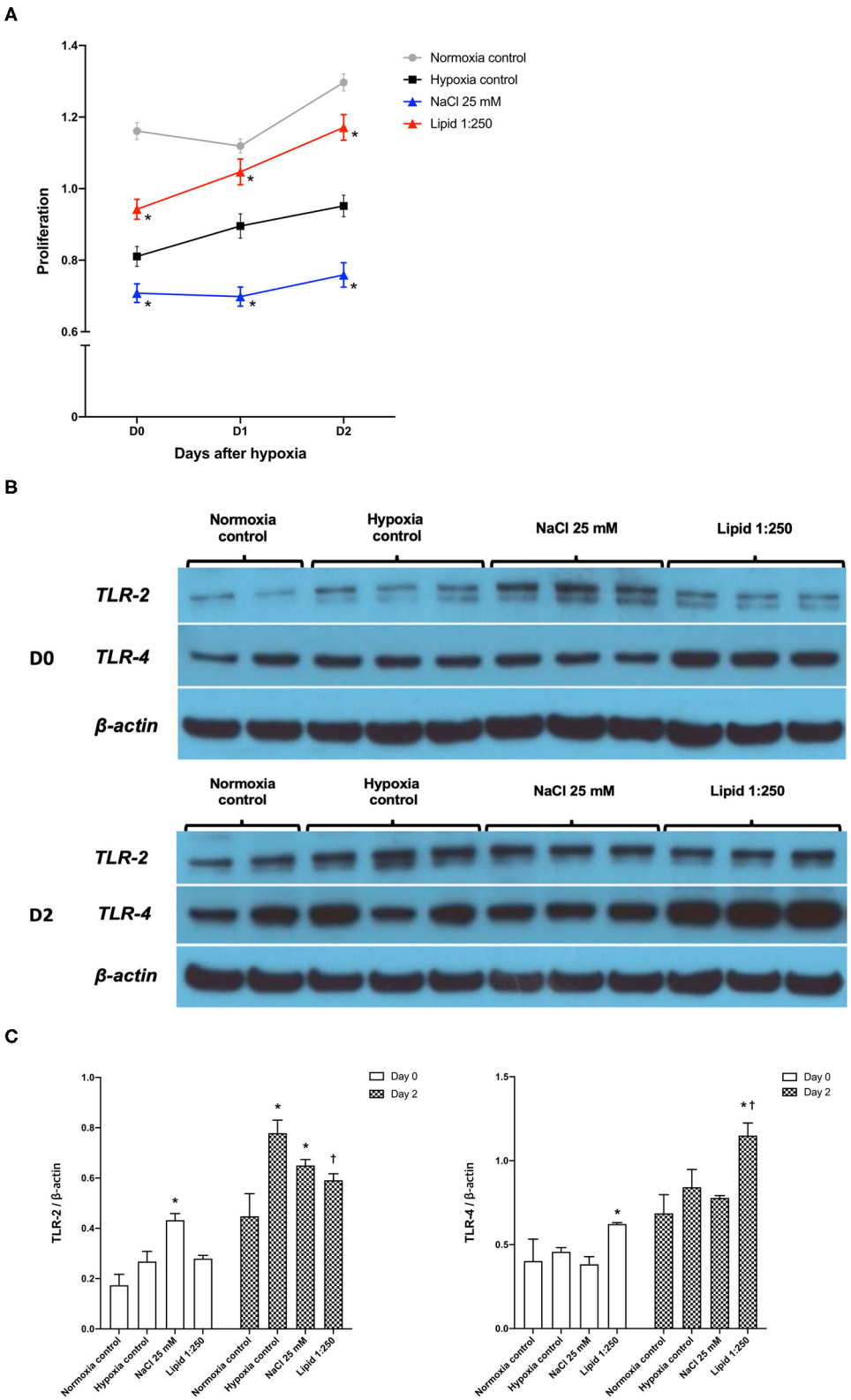

FIGURE 9 | Effects of sodium and lipid treatment on hypoxic HK-2 cells. **(A)** Additional NaCl treatment inhibited the proliferation of HK-2 cells after hypoxic insult compared with the hypoxia control group. Conversely, additional lipid treatment facilitated the proliferation of HK-2 cells after hypoxic insult. **(B,C)** Western blotting of TLR-2 and TLR-4 showed that lipid treatment reduced the expressions of TLR-2 and enhanced the expression of TLR-4 compared with the hypoxia control group on day 2 after hypoxic insult. Day 0: Day when HK-2 cells were removed from the multi-gas incubator 48 h after hypoxia. $^{*}P < 0.05$, compared with the normoxia control group. $^{\dagger}P < 0.05$, compared with the hypoxia control group. Statistical analysis was performed using the Mann-Whitney *U*-test.

intake in the HF diet, HF+HD diet, and the normal diet group, body weight gain was more prominent in the HF diet group compared to the normal diet group, whereas HF+HS diet did not show this effect. The weight gain effect of the high fat component in the HF+HS group seemed to be offset by the high salt component. HS diet was previously reported to have an additive effect on the obesity induced by HF diet (37). However, weight gain of HF diet-fed mice was attenuated by HS diet in recent studies, which is consistent with the results of our study (38–40). One study suggested impaired digestive efficiency in the HF+HS group due to changes in renin-angiotensin system activity by HS (39), but another study did not find a difference in digestive efficiency between HF diet and HF + HS diet (40). Difference in cholesterol levels between the HF group and the HF+HS group showed a similar pattern. In addition, both the HF and HS groups promote proinflammatory immunologic micromilieu, but their effects on individual inflammatory cell or cytokine were slightly different. Although the molecular mechanisms of HS and HF on the immunologic micromilieu are not fully elucidated, different mechanisms of HF and HS diet, such as altered renal lipid metabolism and increased expression of sterol regulatory element-binding proteins in HF diet (41) and increased activity of intrarenal renin-angiotensin system in HS diet (25), have been reported to contribute to enhanced intrarenal inflammation. Therefore, the different molecular mechanisms of HS and HF diet induce different complex changes in intrarenal immunologic micromilieu. The HF diet and HS diet seem to have different effects on both physiologic changes and the intrarenal immunologic micromilieu. As shown in body weight changes in the HF+HS group, some immunologic effects including cellular and humoral components affected by HF or HS diet alone seemed to be counterbalanced by the other in the HF+HS group. Further study including comprehensive analysis of the effects of HF or HS diet on gut microbiome is required to reveal more precise effects of HF or HS diet on intrarenal immunologic changes and the underlying mechanisms of the interaction between HF diet and HS diet.

In our study, longer dietary modification did not show a greater negative effect on plasma creatinine concentration or renal pathologic findings. These were unexpected results since some intrarenal inflammatory cells and the expression of proinflammatory cytokines and TLR2/TLR4 were further increased after 6 weeks compared to 2 weeks of dietary modification. Physiologic adaptation limiting the negative effects of dietary modification might be a main reason for these findings, similar to the aldosterone escape phenomenon occurring in hyperaldosteronism (42). In addition, the effect of dietary modification in normal kidney was not constant over time. Some intrarenal inflammatory cells, such as activated mature B cells, neutrophils, and NK cells, increased at 2 weeks but decreased thereafter, and regulatory T cells increased at 6 weeks. It can be hypothesized that complex immunologic adaptation occurs, and different immunologic processes seem to be activated over time. It is well-known that the intensity of immunologic response can be decreased under hypo-responsive adaptation by low-level repeated stimulation, such as "LPS tolerance" (43). Our study suggested that even short-term dietary modification can adversely affect the course of AKI. Further studies are required to reveal more precise mechanisms of time-dependent intrarenal immunologic changes by dietary modification.

There are some limitations to this study. First, the precise mechanisms of time-dependent changes in some intrarenal immune cell populations in the control group were not fully elucidated. These changes might be caused by renal senescence since we previously reported that an NK T cell population tended to increase with aging (17). Second, the amount of salt used in the HS diet group was exceptionally large compared to human HS diet. Although the effects of HS diet depending on quantity and duration might be different, our study adequately simulated the long-term effects of moderate salt intake in humans using previously reported HS diet in murine models (44). Third, there was some discrepancy in TLR expression between the *in vivo* model and *in vitro* model. Further studies are required to investigate the complex interaction between intrarenal immune cells and injured tubular cells in post-ischemic kidneys.

In conclusion, this study demonstrated that dietary modification including HF or HS diet altered intrarenal immunologic micromilieu to proinflammatory condition and induced more severe renal injury following IRI. Our data can be used as the basis for recommendations to avoid HF or HS diet before surgery or procedure in kidney donors or patients who are at risk of ischemic AKI, such as heart failure patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Samsung Medical Center Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HJ conceived and designed research. HJ, JJ, and KL performed experiments, analyzed data, interpreted results of experiments, prepared figures, and drafted manuscript. KY analyzed data. JL, GK, WH, DK, and Y-GK analyzed data, interpreted results of experiments, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.621176/full#supplementary-material

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PMMA-Based Continuous Hemofiltration Modulated Complement Activation and Renal Dysfunction in LPS-Induced Acute Kidney Injury

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Sepsis-induced acute kidney injury (AKI) is a frequent complication in critically ill patients, refractory to conventional treatments. Aberrant activation of innate immune system may affect organ damage with poor prognosis for septic patients. Here, we investigated the efficacy of polymethyl methacrylate membrane (PMMA)-based continuous hemofiltration (CVVH) in modulating systemic and tissue immune activation in a swine model of LPS-induced AKI. After 3 h from LPS infusion, animals underwent to PMMA-CVVH or polysulfone (PS)-CVVH. Renal deposition of terminal complement mediator C5b-9 and of Pentraxin-3 (PTX3) deposits were evaluated on biopsies whereas systemic Complement activation was assessed by ELISA assay. Gene expression profile was performed from isolated peripheral blood mononuclear cells (PBMC) by microarrays and the results validated by Real-time PCR. Endotoxemic pigs presented oliguric AKI with increased tubulo-interstitial infiltrate, extensive collagen deposition, and glomerular thrombi; local PTX-3 and C5b-9 renal deposits and increased serum activation of classical and alternative Complement pathways were found in endotoxemic animals. PMMA-CVVH treatment significantly reduced tissue and systemic Complement activation limiting renal damage and fibrosis. By microarray analysis, we identified 711 and 913 differentially expressed genes with a fold change >2 and a false discovery rate <0.05 in endotoxemic pigs and PMMA-CVVH treated-animals, respectively. The most modulated

genes were Granzyme B, Complement Factor B, Complement Component 4 Binding Protein Alpha, IL-12, and SERPINB-1 that were closely related to sepsis-induced immunological process. Our data suggest that PMMA-based CVVH can efficiently modulate immunological dysfunction in LPS-induced AKI.

Keywords: LPS-induced AKI, PMMA-CVVH treatment, immunological dysfunction, complement modulation, gene expression profile

INTRODUCTION

Sepsis represents a very relevant problem in critical ill patients without specific and efficient therapies (1, 2). Clinical and experimental studies describe sepsis as a systemic disease characterized by an early overwhelmed inflammatory response to a primary insult leading to multiple organ failure and death (2). This systemic response is accompanied by excessive activation of the Complement and coagulation system with cytokine storm and endothelial dysfunction triggered both by the pathogen itself and by damaged tissue (2, 3).

One of the severely affected organs is the kidney and sepsis-associated acute kidney injury (AKI) has become a widespread and important problem in the ICU (1, 4). The pathophysiology of AKI during sepsis is complex and is associated with permanent alterations of renal resident cells that may promote chronic renal failure (5–7). A broad range of mediators have been identified as responsible for sepsis-related tissue injury, with cellular and soluble components of innate immunity. Among them, Complement system play a critical role since the uncontrolled Complement activation can affect organ damage with poor prognosis for septic patients (2, 3).

Recently, it has been shown that the pathogenetic mechanisms of COVID-19 disease reflects the maladaptive host immune response observed in sepsis (8). Indeed, SARS-CoV-2 infection and the damaged tissues lead to an uncontrolled systemic inflammatory response with increased complement activation (9), abnormal coagulation, endothelial dysfunction, and subsequent multi-organ damage (10). Like sepsis, the occurrence of AKI was considered the most commonly reported co-morbidity of COVID-19 disease (11–13). Therefore it is reasonable that advances in septic field could have a great impact in the pathophysiology and mechanisms of COVID-19 disease (14).

Gram-negative infections are a common variant of sepsis that occurs in 15% of diagnosed patients frequently inducing circulatory collapse and death within hours (2). This is mainly due to the release of their outer wall component, named lipopolysaccharide (LPS) or endotoxin that can activate a wide variety of cells through interaction with specific pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) (15). In the kidney, LPS binds TLR4 and activates tubular, endothelial cells (16) and pericytes (17). Endotoxin requires a carrier protein, known as LPS-binding protein (LBP) that brings LPS to TLR4 and maximizes the activation of the downstream intracellular signaling (5, 6, 18).

During the last three decades numerous clinical trials based on the employment of anti-inflammatory mediators, specific inhibitors of LPS–TLR4 signaling (19) have failed to improve outcome in septic subjects (20). Although these approaches may in part modify the systemic inflammatory response, the protracted immunosuppression may lead to immunoparesis with the acquisition of a secondary infection in patients (20, 21). Interestingly, therapeutic strategies based on blood purification have been developed for the treatment of sepsis-induced AKI *via* an extracorporeal circuit through a device (sorbent), modulating the disequilibrium between pro and anti-inflammatory mediators (22). The use of polymethyl methacrylate (PMMA) membrane hemofilter could improve septic patients outcome, due to excellent biocompatibility and adsorptive properties of middle- to high molecular weight substances (23, 24). Here we investigate the capacity of PMMA-based-Continuous Veno-Venous Hemofiltration (PMMA-CVVH) in modulating innate immune activation in a swine model of LPS-induced AKI.

MATERIALS AND METHODS

Animal Model

Animal study was performed in domestic swine, after approval by the ethical committee of the Italian Ministry of Education, University and Research (MIUR) (Prot. n 823/2016-PR). Briefly, LPS-induced AKI was induced by intravenous infusion of a saline solution containing 300 μg/kg of LPS (lipopolysaccharide membrane of *Escherichia coli*), as previously described (25).

The animals were randomized into four groups: healthy (healthy pigs, n = 7), LPS (endotoxemic pigs, n = 7), PS-CVVH (Polysulfone CVVH-treated endotoxemic pigs, n = 7; TORAYLIGHT NS-S 1.8, Toray Medical Co, Japan), and PMMA-CVVH (Polymethylmethacrylate CVVH-treated endotoxemic pigs, n = 7; HEMOFEEL CH 1.8W-Toray Medical Co, Japan).

HEMOFEEL CH-1.8W is a non-ionic PMMA membrane with an effective surface area of 1.8 square meters, an internal hollow fiber diameter of 240 μm and a wall thickness of 30 μm. This new membrane recently obtained the CE mark and is available for the clinical use in Europe. An ultrafiltration rate (UFR) of 30 ml/h/mmHg was measured in standard experimental setting condition with a Qb = 200 ml/min. The treatments in PMMA-CVVH group (n = 7) or PS-CVVH group (n = 7) were performed with a CVVH heparin-based treatment in pre-dilution modality using a dedicated machine

EQUAsmart® (Medica S.p.A, Medolla, Italy). The Blood Flow rate was set at 150 ml/min with an ultrafiltration rate set at 2,000 ml/h using PrismaSol® solution (Baxter Renal Products, Inc, USA) as replacement fluid. The anticipated net weight loss was set at zero and neither plasma water nor albumin compensation were necessary at the end of 7 h of treatments.

In both PS and PMMA group, the CVVH treatments started after 3 h from LPS infusion with a duration of 7 h. Hemodynamic parameters included mean arterial pressure (mmHg) were automatically registered and recorded every 30 s (25).

Animals were sacrificed after 24 h from LPS/saline infusion with an overdose of IV propofol, immediately followed by a 10-ml IV bolus of an oversaturated solution of potassium chloride (2 mEq/ml, Galenica Senese, srl, Italy).

In total, twenty-eight animals were examined in this study. Three animals (two animals of the LPS group and one animal of the PS group) died because of severe low blood pressure before completing the study protocol. The data of these animals were included until the last useful measurement (LPS animals: 6 and 8 h after LPS injection; PS animal: 12 h after LPS injection). Therefore, Healthy group and PMMA group, n = 7 for all time points; LPS group, T0-T3 n = 7 and T10-T24 n = 5; PS group, T0-T3-T10 n = 7 and T24 n = 6.

Collection of Samples

At sacrifice, kidneys were collected from all animals and processed by using standard procedures as previously described (6). Urine samples were collected from all animals and urinary output was measured and recorded every hour. Swine sera were collected at baseline, at intermediate time points and at 24 h from an arterial blood catheter. Renal function was assessed by serum creatinine and monitoring urinary output.

Histologic Analysis

Renal tissues were processed for histologic staining [hematoxylin and eosin (HE) and Masson's trichrome, (Millipore Sigma)]. Then, digital slides were analyzed and acquired by the AperioScanScope CS2 device (Aperio, Vista, CA, USA) with 20× or 40× magnification as previously described (25). HE and Masson trichrome staining were performed to evaluate histological injury and fibrosis. Tubular and glomerular injury was scored semi quantitatively by two blinded observers who examined at least 20 visual field of each kidney section using criteria of previously published studies (26–30). The tubular damage was defined by evident signs of tubular dilation, tubular atrophy, tubular epithelial cell necrosis, and cast formation. The score of tubular lesions was assessed by the following score: 0 = normal kidney without tubular injury; 1 = <10% of tubules injured; Score 2: 10–25% of tubules injured; Score 3: 25–50% of tubules injured; Score 4: 50–74% of tubules injured; Score 5: >75% of tubules injured (26). The glomerular lesion score: 0 = normal glomeruli; 1 = mild deposition of fibrin and reduced number of capillaries in a few glomeruli; 2 = moderate fibrin deposition and reduced number of capillaries in numerous glomeruli; 3 = marked fibrin deposition and reduced number of capillaries in numerous glomeruli; 4 = marked fibrin deposition and reduced number of capillaries in numerous glomeruli and glomerulosclerosis; 5 = glomerulosclerosis (26). The score index in each animal was expressed as a mean value of all scores obtained. Both tubular and glomerular pathological score of each group was expressed as median ± IQR (Healthy and PMMA T24 group, n = 7, LPS T24 n = 5, PS T24 n = 6). Tubulointerstitial fibrosis was assessed by Masson's trichrome staining and the green-stained area was calculated using Adobe Photoshop software (Adobe, San Jose, CA, USA) and expressed it as positive pixel/total pixel (25).

Confocal Laser Scanning Microscopy

Swine paraffin-embedded renal sections were stained for PTX3 (clone MNB4, rat, Exira Life Sciences In., Larsen, Switzerland) and C5b-9 (mouse, Abcam, Cambridge, UK). Tissue sections were deparaffinized through xylene and alcohol and underwent epitope retrieval through three microwave (750 W) cycles of 5 min in citrate buffer (pH = 6). Then, they were incubated with specific blocking solution, primary antibodies (anti-PTX3 1:100 and anti-C5b-9 1:50) and the corresponding secondary antibodies (Alexa Flour 488, Molecular Probes, Eugene, OR, USA). All sections were counterstained with TO-PRO-3 (Molecular Probes) or DAPI (Thermo Fisher, MA, USA) and mounted with Fluor mount. Negative controls were prepared omitting the primary antibody. Image acquisition was performed by confocal microscope Leica TCS SP8 (Leica, Wetzlar, Germany). Both PTX3 and C5b-9 fluorescence signals were quantified by confocal microscope Leica TCS SP2 software and expressed as area fraction (percentage).

LBP, sCD40, and sCD40L Serum ELISA

Serum LBP levels were measured by ELISA kit from HycultBiotech (Uden, Netherlands) as well as sCD40 (LifeSpan Biosciences Inc, Seattle, WA, USA) and CD40 ligand (MyBioSource, San Diego, USA).

Assessment of the Activity of Classical, Lectin, and Alternative Complement Pathways

Swine sera were analyzed using the Wieslab kit (WIESLAB Complement System Screen COMPL 300, Euro-Diagnostica) as previously described (31).

PBMC Isolation and RNA Extraction

Thirty ml of whole blood was harvested at baseline (T0, before LPS infusion) and at the end of the study (T24). PBMCs were isolated by density separation over a Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Total RNA was extracted automatically and qualitatively and quantitatively analyzed through Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with good quality characterized by RIN >8 were used in the microarray experiment.

Microarray Experiment

For transcriptomic profiling, labeled cRNA was obtained using the Low Input Quick Amp Labeling Kit (Agilent Technologies)

from RNA samples of pigs at baseline (respectively five T0 LPS group and three PMMA T0 group), four endotoxemic animals (T24 LPS group), and four PMMA-CVVH treated-endotoxemic pigs (T24 PMMA group). However, one sample of LPS T24 group was excluded due to technical problem. Gene expression data were obtained using Agilent Feature Extraction software (v.10.7.3). The Agilent microarray data are Minimum Information About a Microarray Experiment (MIAME) compliant, and raw data have been deposited in the database of the European Bioinformatics Institute (EMBL-EBI) and are accessible through Experiment ArrayExpress accession number E-MTAB-9145. Gene expression analysis was performed by Genespring GX 7.2 (Agilent, Santa Clara, CA, USA), using a false discovery rate (FDR) <0.05 and a fold change (FC) >2.

Real-Time PCR Analysis

Real-time PCR was performed to validate microarray gene expression data of five differentially modulated genes [Granzyme B (GZMB), Complement factor B (CFB), Complement Component 4 Binding Protein Alpha (C4BPA), SERPIN B1, and IL-12]. These experiments were performed on the same samples used in the microarray experiments and extended to the other samples of each group not used in microarray analysis. Total RNA (500 ng) was used in a reverse transcription reaction by using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR was performed on the Light Cycler@96 (Roche) by using GZMB, CFB, C4BPA, SERPINB1, and IL-12 primers (validated primers for swine genome: GZMB, Assay ID: qSscCED0016345; CFB, Assay ID: qSscCED0017919; C4BPA, Assay ID: qSscCID0003068; SERPINB1, Assay ID: qSscCID0012570; IL-12B, Assay ID: qSscCED0008922; Bio-Rad) in combination with SsoAdvanced™ Universal SYBR® Green Supermix (Biorad). The relative amounts of mRNA were normalized to β-actin mRNA as the housekeeping gene. Data were analyzed using the $\Delta\Delta C_t$ method.

Statistical Analysis

Identification of genes differentially expressed between LPS and PMMA treatments was carried out with FDR method of Benjamini-Hochberg (32) and gene probe sets were filtered on the basis of the false discovery rate, FDR (adjusted-P value with multiple testing on 1,000 permutations), and fold-change (28). Fold change filter was set to 2-fold in each comparison. Only genes that were significantly modulated (adjusted-P value <0.05 and fold-change >2) were considered for further analysis. For real-time PCR, data were presented as mean ± standard deviation (SD) (Healthy and PMMA group, T0 and T24 n = 7; LPS group, T0 n = 7 and T24 n = 5; PS group, T0 n = 7 and T24 n = 6) and compared by paired or unpaired Student t-test as appropriate.

In the other analyses, the results were expressed as median ± interquartile range (IQR) (Healthy and PMMA group, n = 7 for all time points; LPS group, T0-T3 n = 7 and T10-T24 n = 5; PS group, T0-T3-T10 n = 7 and T24 n = 6) and compared with a Mann–Whitney U test. All data (results) were analyzed using GraphPad Prism 5.0 (GraphPad software, Inc., San Diego, CA, USA). A *p-value* <0.05 was considered statistically significant.

RESULTS

Improvement of LPS-Induced AKI by PMMA-Based CVVH Treatment

We first analyzed animal survival in 24 h by Kaplan-Meier curve (**Figure 1A**). No significant statistical difference was observed by log-rank, in the four groups. Three animals (two animals of the LPS group and one animal of the PS group) died because of severe low blood pressure before completing the study protocol. Next, we found that AKI was successfully induced in LPS and treated animals (**Figures 1B–D**). As previously showed (25), LPS injection led to a time-dependent increase of serum creatinine, with significant reduction in urinary output (ml/kg/h) (**Figures 1B, C**) and hypotension (**Figure 1D**) compared to healthy group. The presence of oliguria (<0.5 ml/kg/h), rise in serum creatinine (>1.2 mg/dl), and hypotension (<70 mm/Hg) well characterized renal damage in endotoxemic animals.

Interestingly, PMMA-CVVH significantly improved 24-h creatinine level, 24-h urine output, and arterial blood pressure (**Figures 1B–D**) compared to both LPS and PS-treated group. On the contrary, we observed very limited effects by PS-CVVH treatment. Indeed, PS-CVVH was no effective to recover creatine level and urine output.

Following 24 h from LPS infusion, we observed significant morphological changes in renal parenchymal, including tubular vacuolization, epithelial flattening, necrosis, infiltration of inflammatory cells, and marked fibrin deposition with reduced number of capillaries in numerous glomeruli (**Figure 2A**, T24 LPS).

The pathological scores of renal tubular and glomerular injury in PS-CVVH group was significantly higher than those observed in healthy group (**Figures 2B, C**). The treatment by PS-CVVH was less capable to limit renal tissue damage (**Figure 2A**, T24 PS-CVVH).

Comparing with both septic and PS-CVVH pigs (**Figures 2A–C**, T24 LPS and T24 PS-CVVH), PMMA-CVVH significantly reduced tubular and glomerular damage and hampered the recruitment of inflammatory infiltrate (**Figure 2**, T24 PMMA-CVVH).

PMMA-CVVH Treatment Reduced Collagen Deposition and Renal Fibrosis in Endotoxemic Animals

The presence of glomerular thrombi and collagen deposits are considered hallmark of sepsis-induced AKI (5, 6). Compared to healthy pigs, collagen deposits significantly increased after 24 h from LPS injection thus indicating the acute development of fibrosis (**Figure 3A**, T24 LPS); PS-CVVH was not effective in counteracting the development of renal fibrosis and glomerular thrombi and injury (**Figure 2A**, T24 PS-CVVH). On the contrary, PMMA-CVVH treatment preserved renal parenchyma significantly decreasing glomerular damage and

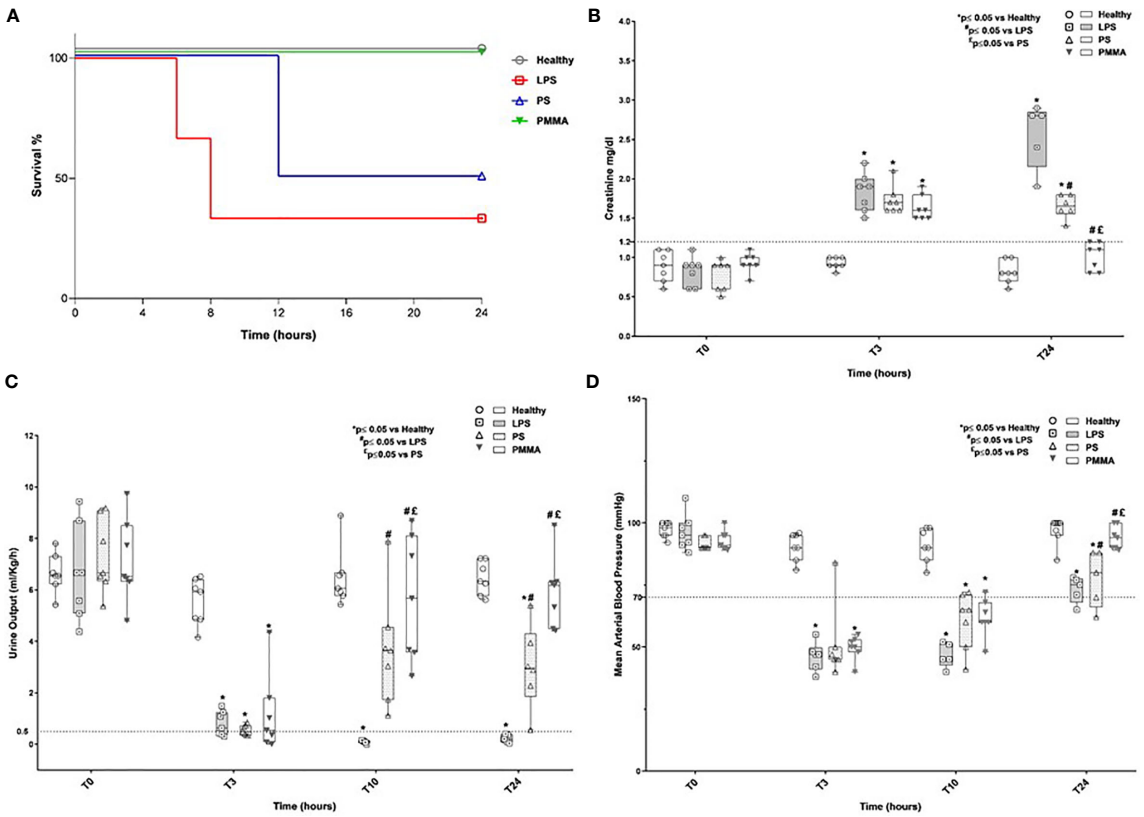

FIGURE 1 | Survival rate, renal function, oligo-anuria, and hypotension in a swine model of LPS-induced AKI. **(A)** Three animals (two animals of the LPS group and one animal of the PS group) died because of severe low blood pressure before completing the study protocol. **(B, C)** Endotoxemic pigs developed AKI with time-dependently increased creatinine serum levels and reduced urinary output. PMMA-CVVH treatment significantly reversed LPS effects ($^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS). **(D)** After LPS infusion, animals presented hypotension and PMMA-CVVH treatment recovered MAP levels more than PS-CVVH treatment. **(B–D)** PS-CVVH treatment was no effective to recover creatine level, urine output, and arterial blood pressure to baseline median of healthy group. Data were obtained as described in the *Methods* section and expressed as median ± IQR of at least five pigs for each group (Healthy group and PMMA group, n = 7 for all time points; LPS group, T0-T3 n = 7, T10-T24 n = 5; PS group, T0-T3-T10 n = 7 and T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test (*$p < 0.05$ *vs* Healthy, $^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS).

collagen deposits respect to both LPS and PS group (**Figures 3A, B**).

Effects of PMMA-CVVH Treatment on LPS-Induced PTX-3 Deposits and Complement Activation

PTX3 has been implicated in septic shock (33) and it can activate the classical pathway of Complement (34, 35) through C1q binding, amplifying the severity of the disease. We then analyzed PTX3 localization and distribution in our model of sepsis-induced AKI. We observed very limited PTX3 deposits in healthy pigs (**Figures 4A, B**); after 24 h from LPS infusion, PTX-3 deposits significantly increased in peritubular and glomerular capillaries. Interestingly, we found a partial reduction in PTX-3 deposits by PS-CVVH, that were virtually absent in PMMA-CVVH treated animals (**Figures 4A, B**).

Next to PTX3, we investigated the tissue activation of Complement in order to make comparison with human data (3). We evaluated the deposition of the terminal Complement complex C5b-9, using an antibody directed against the C9-neo-epitope (**Figures 4C, D**). In control animals, C5b-9 deposits were rarely present, while, in endotoxemic pigs, we found significant C5b-9 activation, mainly localized at tubule-interstitial level. Renal C5b-9 staining was still detectable in PS-CVVH group but completely absent in PMMA-CVVH treatment (**Figures 4C, D**).

We then examined systemic Complement activation by analyzing serum from septic and treated pigs (**Figure 4E**); we found that serum from LPS pigs induced a significant increase in the activation of classical and alternative pathways, with no effects on the lectin pathway, after 3 and 24 h from LPS infusion. Interestingly, PMMA-CVVH treatment was able to significantly inhibit both the classical and alternative pathways at 24 h compared to LPS and PS group. Regard to PS-CVVH, we did not detect significant differences in Complement activation compared to LPS group.

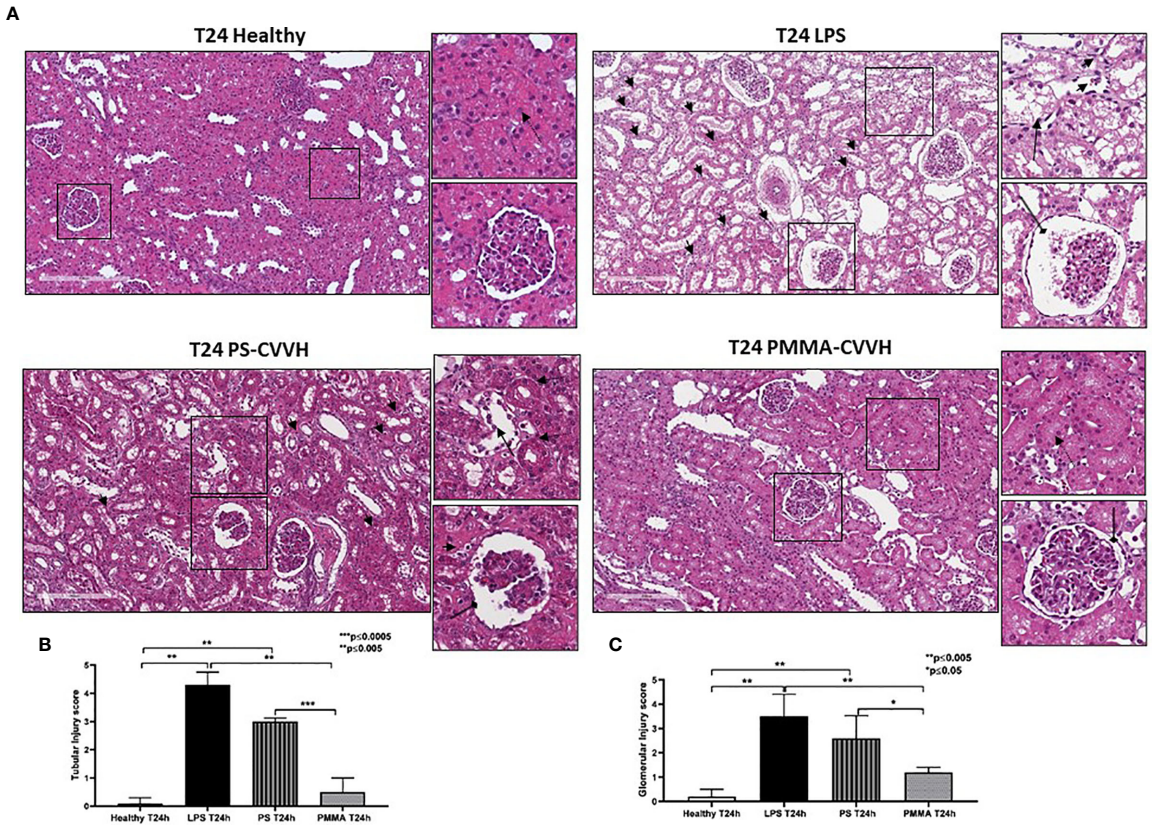

FIGURE 2 | Recovery of parenchymal damage in endotoxemic animals. **(A)** H&E staining showed significant desquamation of proximal tubular epithelial cells (zoomed image, black arrow), marked fibrin deposition and loss of capillaries in numerous glomeruli, Bowman's capsule expansion (zoomed image, black arrow with rounded tip), and interstitial inflammatory infiltrate (T24 LPS and zoomed image, small black arrows) after 24 h from LPS infusion compared to control (T24 Healthy). PS-CVVH treatment reduced inflammatory infiltrate (small black arrows) but not preserved glomerular (loss of capillaries and Bowman's capsule expansion; zoomed image, black arrow with rounded tip) and tubular compartment from LPS injury (zoomed image, black arrow). In PS-CVVH group, few tubules did not show signs of damage and vacuolization (zoomed image, dotted arrows). Renal biopsies of endotoxemic animals after PMMA-CVVH treatment showed recovery of tubular damage (zoomed image, dotted arrow) and reduced pathological changes at glomerular level (zoomed image, black arrow with rounded tip) and inflammatory infiltrate (zoomed image, black arrows). **(B, C)** Tubular and glomerular pathological score was obtained as described in the *Methods* section and expressed as median ± IQR of at least five pigs for each group (Healthy and PMMA T24 group, n = 7, LPS T24 n = 5, PS T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test (*p < 0.05; **p < 0.005; ***p < 0.0005).

Immunomodulatory Effects of PMMA-CVVH on Circulating Pro-inflammatory Factors

We then measured LBP serum levels in endotoxemic pigs, and we observed a significant increase after 3 and 24 h from LPS infusion. Interestingly, PMMA-CVVH treatment significantly reduced LBP levels in the circulation thereby counteracting LPS signaling (**Figure 5A**). On the contrary, we found no significant difference between the PS-CVVH group and the LPS group.

In addition, we also evaluated the sCD40/CD40L axis that has been associated with development of AKI (36) (**Figures 5B, C**). sCD40L has been considered as a marker of inflammation (37) and thrombosis (38), and it is significantly increased in patients with severe sepsis (39). We found high levels of sCD40L in endotoxemic pigs after 3 and 24 h from LPS infusion. PMMA-CVVH treatment reversed LPS effects, significantly reducing sCD40L serum level compared to both endotoxemic and PS-treated animals; on the contrary, PS-CVVH treatment did not exert significant effects in sCD40L removal (**Figure 5B**). In accordance, PMMA-CVVH treatment induced a significant downregulation of sCD40 serum level (**Figure 5C**), demonstrating a protective effect of PMMA membrane in sustaining immune competence. On the contrary PS-CVVH treatment was not able to reduce sCD40 level at baseline.

PMMA-CVVH Modulated Expression of Pro-inflammatory Genes in Swine PBMC

In order to identify genes specifically modulated after 24 h from LPS infusion, we compared the whole-genome gene expression profiles of swine PBMC after 24 h from LPS infusion (T24) and before LPS infusion (T0). We identified 711 genes significantly

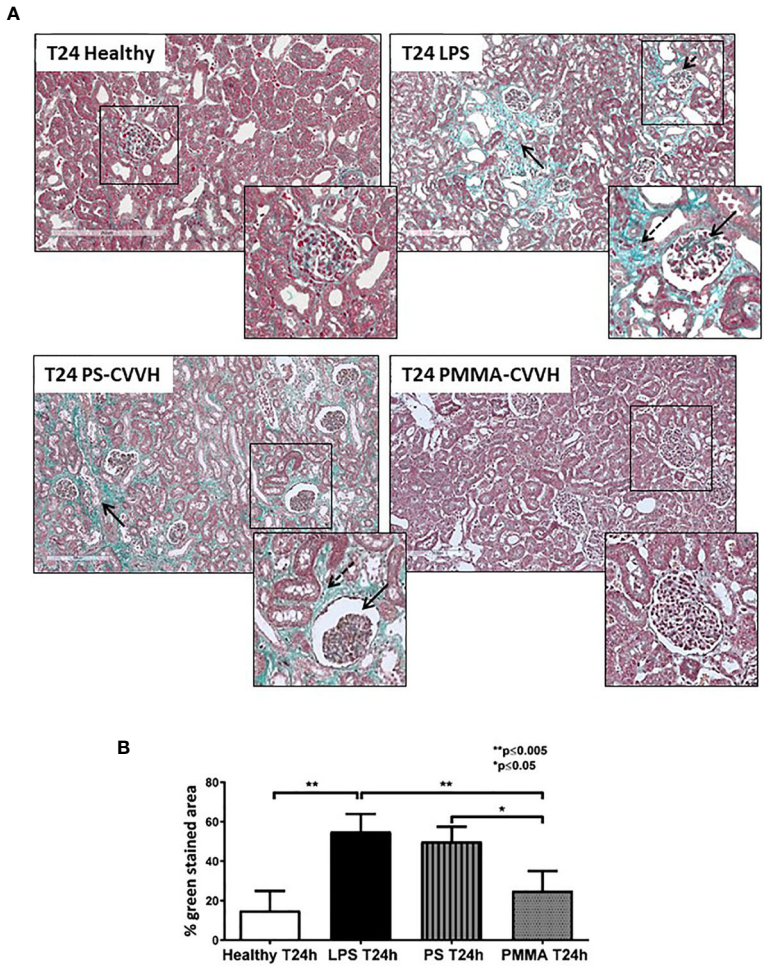

FIGURE 3 | PMMA-CVVH treatment prevented early fibrosis in endotoxemic pigs. Masson trichrome staining **(A)** revealed extensive collagen deposition at the interstitial level, along capillaries (zoomed image, black arrow), and diffuse glomerular thrombi (zoomed image, dotted black arrow) in endotoxemic pigs compared with T24 of the control group (T24 Healthy). Renal biopsies after PS-CVVH treatment did not reduce collagen deposits (black arrows). Otherwise, PMMA-CVVH treatment strongly decreased collagen deposits (zoomed image, black arrows) and renal damage. **(B)**The quantitative analysis was obtained as described in the Methods section and expressed as median ± IQR of at least five pigs for each group (Healthy and PMMA T24 group, n = 7, LPS T24 n = 5, PS T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test (*$p < 0.05$; **$p < 0.005$).

modulated with an FDR <0.05 and a FC>2 (**Figure 6A**, **Supplementary Table 1**); 310 genes were upregulated and 401 genes were downregulated. Then, we compared the PBMC gene expression profiles of PMMA-treated endotoxemic pigs (PMMA T24 group) with that of endotoxemic animals at T24 (LPS T24 group). We identified 913 genes significantly modulated with an FDR <0.05 and a FC >2 (**Figure 6B**, **Supplementary Table 2**); 640 genes were upregulated and 273 genes were downregulated. We found that the Principal Component Analysis (PCA, **Figure 6C**) could well discriminated LPS and PMMA-CVVH treated pigs from baseline gene expression (LPS T0 and PMMA T0).

Finally, we crossed the two analyses to identify genes specifically modulated by the PMMA-CVVH treatment. We found that 581 genes were modulated exclusively by LPS; interestingly, the treatment by PMMA-CVVH could modulate 783 genes after LPS infusion. However, 130 genes were modulated by LPS even in presence of PMMA treatment (**Figure 6D** and **Supplementary Tables 3–5**). Among these modulated genes, we focused on those involved in sepsis-induced immunological activation. We found two genes, GZMB and CFB, that were significantly upregulated in LPS T24 group compared to basal condition (LPS T0). GZMB belong to the family of serine proteases and is constitutively expressed in several immune cells, including cytotoxic T lymphocytes, natural killer cells, NKT cells, and γδ T cells and its augmented expression is correlated with disease severity. CFB is a necessary component of the Complement alternative pathway and an important downstream effector of TLR signaling in sepsis.

Interestingly, we observed that PMMA-CVVH treatment induced a down-regulation of GZMB and the upregulation of

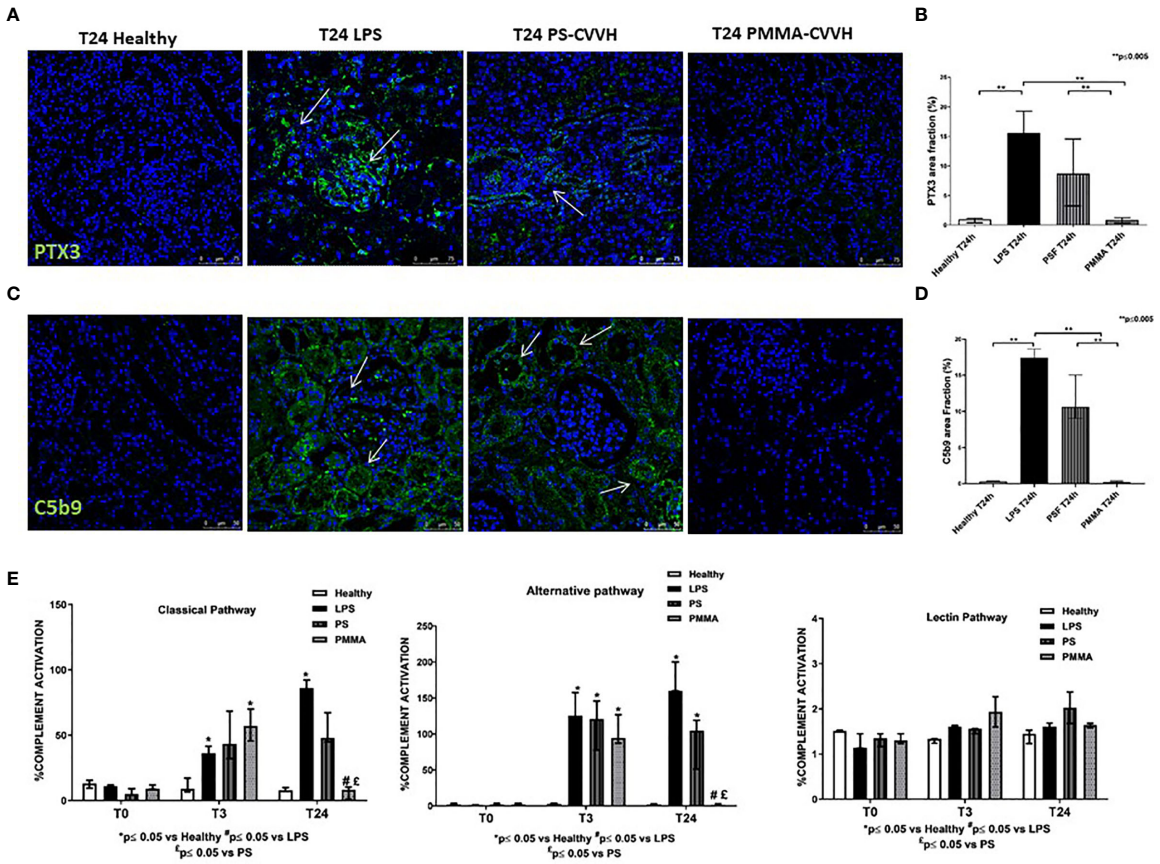

FIGURE 4 | PMMA-CVVH treatment reduced PTX3 deposits and local and systemic Complement activation. **(A)** In healthy pigs, no PTX3 deposits (green) were found. A significant increase in PTX3 deposits were observed at peritubular and glomerular level (white arrows) in LPS group. PS-CVVH reduced PTX3 deposits. PMMA-CVVH treatment significantly reversed LPS effects, respect to both LPS and PS-CVVH group. Magnification 630×. The fluorescent dye To-pro 3 was used to counterstain nuclei (blue). **(B)** C5b9 deposits (green) were observed at tubular and glomerular level in endotoxemic pigs (white arrows). Renal C5b-9 staining was still detected in PS-CVVH group (white arrows). A significant decrease of C5b-9 deposits was observed after PMMA-CVVH treatment. Magnification 630×. The fluorescent dye TOPRO was used to counterstain nuclei (blue) The quantitative analyses of PTX3 **(C)** and C5b-9 staining **(D)** were obtained as described in the Methods section and expressed as median ± IQR of at least five pigs for each group (Healthy and PMMA T24 group, n = 7, LPS T24 n = 5, PS T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test (**p < 0.005). **(E)** Wieslab assay revealed systemic activation of complement classical and alternative pathway in endotoxemic swine sera after 3 and 24 h from LPS injection. PMMA-CVVH treatment significantly decreased both CP and AP ($^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS). PS-CVVH treatment did not reduce systemic complement activation. Results represent the median ± IQR of at least five pigs for each group (Healthy group and PMMA group, n = 7 for all time points; LPS group, T0-T3 n = 7, T10 -T24 n = 5; PS group, T0-T3-T10 n = 7 and T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test (*p < 0.05 *vs* Healthy, $^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS).

Complement Component 4 Binding Protein Alpha (C4BPA), SERPIN B1, and IL-12 (**Supplementary Table 2**). C4BPA is regulator of Complement activation and provides an alternative pathway for activating B cells and T cells through the CD40 receptor. SERPIN B1 and IL-12 are critical factors involved in the regulation of the immune responses.

Gene Signature Validation

In order to validate the microarray experiments, we performed qRT-PCR analysis of the five genes in all pig groups (**Figure 7**). The qRT-PCR findings were broadly consistent with our microarray data (**Figure 6**). We observed that GZMB expression was significantly increased in LPS T24 group compared to LPS T0. This alteration trend in gene expression was also observed for the transcription of CFB gene. Next, we validated these genes also in PMMA group at T0 and T24. Interestingly, PMMA-CVVH treatment reversed LPS effects, significantly decreasing GZMB and CFB expression. On the contrary these genes were not downregulated by PS-CVVH treatment.

Then, we analyzed the effects of PMMA on C4BPA, SERPIN B1, and IL-12 gene expression. The expression of these genes was significantly reduced in endotoxemic animals respect to basal condition (LPS T0). In line with microarray results, PMMA-CVVH treatment (PMMA T24) significantly increased the expression of these genes, compared with LPS T24 group. Finally, the expression levels of these genes did not increase after PS-CVVH treatment and no significant difference was found between PS-CVVH and LPS group.

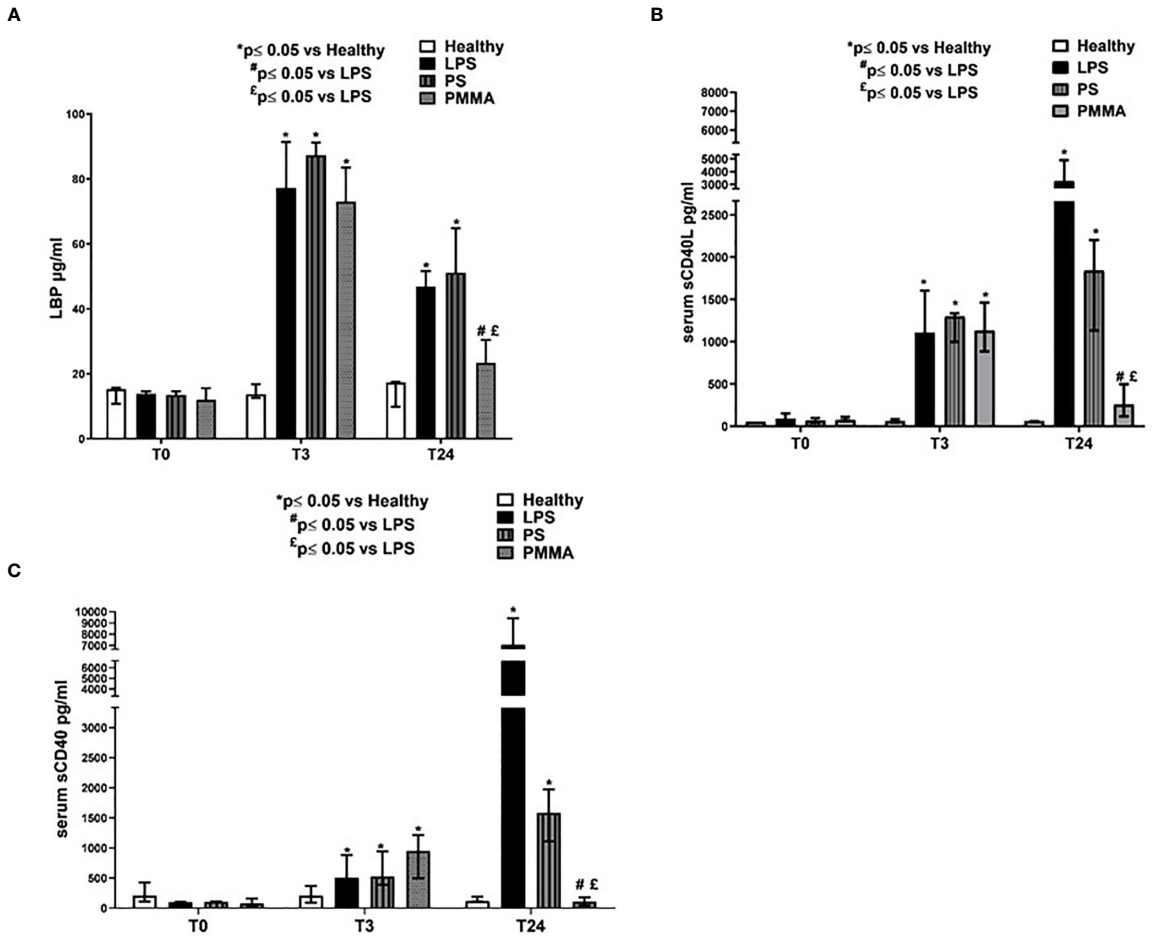

FIGURE 5 | PMMA-CVVH treatment modulated TLR-4 signaling and reduced sCD40L and sCD40 sera levels. **(A)** ELISA assay showed significantly increased level of serum LBP in endotoxemic pigs after 3 and 24 h from LPS infusion respect to healthy group. A strong reduction in serum LBP levels was found after PMMA-CVVH treatment ($^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS). PS-CVVH did not modulate LBP sera levels. **(B, C)** ELISA assay showed significantly increased level of serum sCD40L **(B)** and sCD40 **(C)** in endotoxemic pigs after 3 and 24 h from LPS infusion respect to healthy group. A significant strong decrease in serum sCD40L and sCD40 levels was found after PMMA-CVVH treatment ($^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS). PS-CVVH reduced sCD40L and sCD40 sera levels but it did not reach the basal condition as with PMMA treatment. **(A–C)** The histograms represent the median ± IQR of at least five animals for each group (Healthy group and PMMA group, n = 7 for all time points; LPS group, T0-T3 n = 7, T10 -T24 n = 5; PS group, T0-T3-T10 n = 7 and T24 n = 6). Statistically significant differences were assessed by the Mann–Whitney test ($^{*}p < 0.05$ *vs* Healthy, $^{\#}p < 0.05$ *vs* LPS, and $^{£}p < 0.05$ *vs* PS).

DISCUSSION

In this paper, we demonstrated for the first time the efficacy of PMMA-based CVVH treatment in reducing Complement activation and renal injury in a swine model of sepsis-induced AKI. Our results shed more light on the mechanism by which PMMA-based CVVH might modulate cytokine storm by preserving immunological homeostasis.

Sepsis-induced AKI is a growing health care problem and it is characterized by an overwhelmed immune response against a primary insult that become responsible for organ dysfunction and poor outcome (40). The inflammatory mediators including pathogen- and damage-associated molecular patterns bind the pattern recognition receptors (PRR), such as Toll-like receptors (TLR), expressed on the surface of immune cells, promoting a downstream cascade of signals that will amplify the immune response (1, 40, 41). Moreover, renal resident cells also expressed TLR, in particular TLR2 and TLR4, and actively participate in sepsis-induced AKI (40). During Gram-negative sepsis or endotoxin based sepsis, LPS is considered the main PAMP and is specifically recognized by TLR-4 (15) that induces the activation of pro-inflammatory and pro-fibrotic pathways in renal resident cells, contributing to fibroblasts accumulation and local inflammatory process (17, 25, 42–45). Accordingly, our data showed that the activation of TLR-4 signaling enhanced by LPS infusion, induced collagen deposition, and increased the infiltration of inflammatory cells in renal parenchyma.

The pathophysiology of sepsis is complex and involves many systems, such Complement cascade triggered both by the pathogen itself and by damaged tissue (1, 46). PTX3 plays

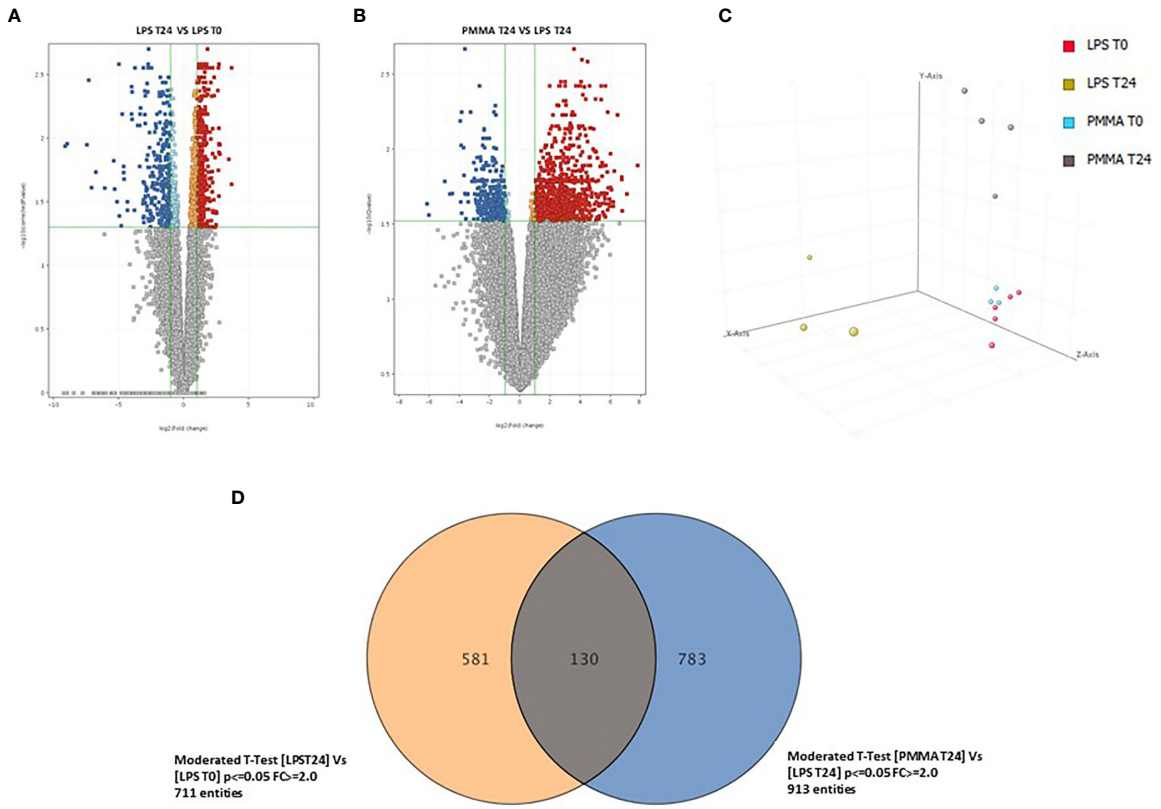

FIGURE 6 | Comparison of the whole-genome gene expression profiles of swine PBMC. **(A)** Volcano plot show 711 genes significantly modulated with a FDR < 0.05 and a FC > 2; 310 genes were upregulated and 401 genes were downregulated (LPS 24 *VS* LPS T0). **(B)** Volcano plot show 913 genes significantly modulated with an FDR < 0.05 and a FC > 2; 640 genes were upregulated and 273 genes were downregulated (PMMA T24 *VS* LPS T24). **(C)** Principal component analysis 3-D diagram showed different spatial distribution of the LPS T24h and PMMA T24h pigs from the healthy cohort (LPS T0 and PMMA T0). **(D)** Venn diagram showing shared and specific genes for LPS T24 and PMMA group in swine PBMCs.

essential role in innate immunity and Complement activation (47) and its serum level correlates with the severity of sepsis disease (33). Moreover, the end product of Complement activation, the C5b-9 complex, is associated to the development of multiple organ failure (46). Our data are in line with these findings and support the involvement of local PTX3 and Complement system in this setting.

Emerging evidences suggest that Complement activation is critical in the pathogenesis of the novel Coronavirus, SARS-CoV-2 (48). Both in bacterial and viral infections, complement system is a key player of protective immunity against pathogens, together with other innate immune system components. However, the exacerbated Complement activation induced by the pathogen, or secondarily *via* damaged host tissues become detrimental and could contribute to the maladaptive inflammatory response observed in patients with severe COVID-19 disease (9, 48). Indeed, COVID-19 patients met the diagnostic criteria for sepsis and septic shock according to the Sepsis-3 International Consensus, and the terms "viral sepsis "are more accurate to describe this complex disease (8). Therefore, it is highly plausible that the efficacy of immunomodulatory therapies in sepsis disease should be assessed in COVID-19 field.

Despite the advances in the understanding the pathogenesis of sepsis-induced AKI, the complexity of this disease might explain the failure of therapeutic intervention targeting just one of the many systems involved (49). The Surviving Sepsis Campaign Guidelines (SSCG) (50), international guidelines for diagnosis and treatment of severe sepsis/septic shock, include little description on the treatment of AKI. About renal replacement therapy (RRT), the SSCG contains weak recommendations for the choice of intermittent hemodialysis and continuous renal replacement therapy (CRRT) (4). Therefore, the hypothesis that blood purification could improve the outcome of septic AKI has attracted much attention (51). The use of PMMA membrane hemofilter in CHDF modality showed excellent removal capacity of cytokines and then it has been widely accepted in the field of chronic maintenance dialysis (52, 53). Moreover, clinical studies showed that the use of PMMA membrane hemofilter in CHDF modality reduced systemic inflammation, improved blood pressure and urine output in critically ill patients (54).

In our study, PMMA treatment induced significant modulation of hypotension, with preservation of renal function and urine output in endotoxemic animals. These data were

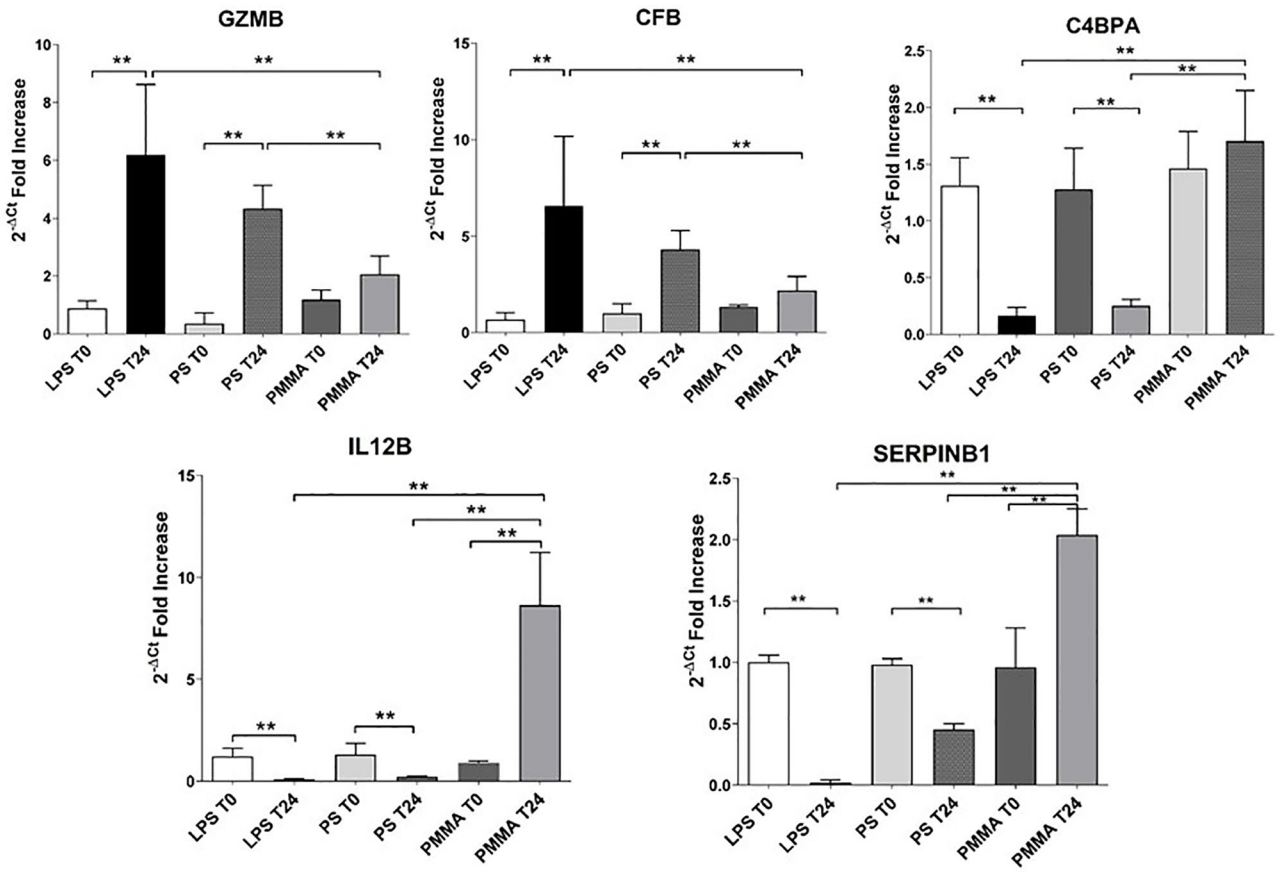

FIGURE 7 | Validation of targeted genes. Real-time PCR of six genes (GZMB, CFB, C4BPA, and SERPINB1) differentially expressed in PBMC of LPS T0, LPS T24, PMMA T0, PMMA T24, PS T0, and PS T24 groups. Data are expressed as mean ± SD; **$p < 0.005$.

associated with a decrease of glomerular and tubular pathological changes, inflammatory infiltrate, and early fibrosis. In addition, we observed that PMMA treatment was critical in reducing systemic Complement activation, and the acute phase protein, LBP, that it is crucial in enhancing and amplifying cellular response to endotoxin (5, 6, 18, 43).

Since Complement plays a central role in leucocyte activation (3) we investigated the modulation of Complement system in swine PBMC. Our analyses demonstrated the expression of GZMB and CFB in endotoxemic animals. These results are in agreement with those of several authors who reported increased level of GZMB and sustained Complement activation in septic patients with higher mortality and organ dysfunction (3, 55–57). When we analyzed the differentially expressed genes in PBMC of PMMA-CVVH pigs, we found that PMMA-CVVH treatment down-regulated both GZMB and CFB expression. On the contrary, these genes were not modulated by PS-CVVH treatment.

Interestingly, we also identify C4BPA, a circulating inhibitor of the classical and the lectin pathway. C4BPA provides an alternative mechanism for activating B and T cells through the CD40 receptor in a manner similar to CD40L, establishing a novel interface between B and T cell activation (58). In our animal model, we found that LPS completely downregulated C4BPA, thereby amplifying Complement system activation (59); otherwise, PMMA-CVVH was able to preserve C4BPA expression in circulating leucocytes. In addition, we observed that PMMA treatment modulate B and T cell response through the removal of the soluble CD40 (sCD40). It has been shown experimentally that sCD40 reduces immunoglobulin production and T cell activation as an immunosuppressive factor of lymphocytes activation (60). Accordingly to our results, PMMA-based filters for intermittent hemodialysis have been shown to effectively reduce the levels of sCD40 (36).

Moreover, we also observed increased serum levels of sCD40L in endotoxemic pigs and a significant reduction after PMMA treatment. As well known, sCD40L is pivotal marker of inflammation and thrombosis in several inflammatory diseases including sepsis (61, 62) and in in renal pathology (63). Therefore, PMMA was able to modulate the sCD40L/sCD40 axis, removing those mediators that are present in large number during cytokine storm and restoring homeostatic balance to assure better immune competence in septic patients. We also found increased expression of IL-12 in PBMCs of PMMA-CVVH pigs. This result is similar to the data reported by

Stanilova et al. (64), who showed significant differences in the amounts of IL-12 synthesis in LPS-stimulated PBMCs between survivors and non-survivors with severe sepsis. The principal immunological function of IL-12 is to control infection increasing cellular immunity and phagocytic functions. Indeed, IL-12 induces native T-lymphocyte differentiation to type 1 T helper (Th1) cells. Th1 cells synthetize interferon gamma, which regulates macrophage and NK cell activation, induces immunoglobulin secretion by B cells, and promotes Th1 cell differentiation. Thus, increased IL-12 production in patients with severe sepsis could prevent the development of immune paralysis reducing the risk of lethal outcome (64). Accordingly, Weighardt et al. reported that impaired IL-12 synthesis augments the risk of infection after surgery and should be considered a predictive factor for the lethal outcome of postoperative sepsis (65, 66). In a prospective observational study, Chuang et al. analyzed the different mechanisms of immune dysfunction in survivors and no survivor septic patients. They observed that augmented levels of IL-12 are associated with improved general conditions in most of recruited patients and favorable outcome (67).

Another interesting gene that was upregulated in PMMA-CVVH group was SERPINB1. A recent study revealed that SERPINB1 deficiency enhances the inflammatory response to LPS, thereby exacerbating mortality and morbidity (68). Therefore, the modulation of SERPINB1 expression represents an important mechanism to minimize host damage from exacerbated inflammatory response against infection.

Finally, the gene expression analysis by qRT-PCR in PBMCs of PS-CVVH did not show any difference respect to endotoxemic animals. In conclusion, our results demonstrated that PMMA-CVVH treatment prevented the development of tubulointerstitial fibrosis, tubular damage, and inflammatory process by interfering with the activation of renal resident cells and circulating leucocytes. Indeed, PMMA showed excellent removal capacity of soluble components involved in inflammation and in immune system and reduced local and systemic Complement activation, recovering the balance between the pro- and anti-inflammatory mediators. Finally, PMMA treatment modulated gene expression in circulating leucocytes, thereby controlling complement system and innate immunity and preserving B and T cells response against infection, limiting immunological dysfunction and renal damage.

Despite the lack of a comparison of gene expression pattern between the two treatments, our results highlighted for the first time the efficacy of a new PMMA membrane in CVVH modality as a possible future therapeutic strategy with a significant impact on short- and long-term outcomes for patients with systemic inflammatory syndrome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the Italian Ministry of Education, University and Research (MIUR) (Prot. n 823/2016-PR).

AUTHOR CONTRIBUTIONS

AS and GC planned the research, coordinated the study, performed all the experiments and statistical analyses, and wrote the manuscript. RF participated in the design of the study, performed most experiments with AS, analyzed the respective data, and contributed to draft the manuscript. CD participated in the coordination of the study and in the immunolabeling and confocal microscopy of renal sections. FSa performed microarray analysis and assisted in manuscript preparation. CC and AP assisted *in vitro* and *in vivo* experiments and in manuscript preparation. PP participated in the design of the study and helped to draft the manuscript. FSt, LL, and AC performed the animal model and helped to revise the manuscript. VC, DM, CR, MD, AL, and MZ supervised all the research. GP, GS, and LG participated in the coordination of the study and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.605212/full#supplementary-material

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Immunopathology of Acute Kidney Injury in Severe Malaria

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Acute kidney injury (AKI) is a common feature of severe malaria, and an independent risk factor for death. Previous research has suggested that an overactivation of the host inflammatory response is at least partly involved in mediating the kidney damage observed in *P. falciparum* patients with AKI, however the exact pathophysiology of AKI in severe malaria remains unknown. The purpose of this mini-review is to describe how different aspects of malaria pathology, including parasite sequestration, microvascular obstruction and extensive intravascular hemolysis, may interact with each other and contribute to the development of AKI in severe malaria, by amplifying the damaging effects of the host inflammatory response. Here, we highlight the importance of considering how the systemic effects and multi-organ involvement of malaria are intertwined with the localized effects on the kidney.

Keywords: malaria, inflammation, acute kidney injury (AKI), immune response, hemolysis, *P. falciparum*, kidney injury

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INTRODUCTION

Malaria is one of the most common infectious diseases in the world and one of the most significant challenges faced by public health systems in many developing countries. An estimated 229 million cases of malaria and 409,000 malaria deaths were reported worldwide in 2019, with the overwhelming majority of cases being reported in Africa, and children accounting for approximately 67% of worldwide deaths (1). The COVID-19 pandemic has posed a worrying threat to already fragile malaria control programs, by further burdening the healthcare systems of malaria-endemic countries (2).

Malaria is transmitted through the bite of a *Plasmodium* spp.-infected female *Anopheles* mosquito, which inadvertently provides the parasite with access into the human host (3). The clinical spectrum of infection with these parasites can range from asymptomatic, to uncomplicated malaria, characterized by non-specific paroxysmal symptoms such as fever, headache and muscle ache, to severe malaria (SM), characterized by life-threatening symptoms, including severe anemia, coma, acute kidney injury and metabolic acidosis (4).

Acute kidney injury (AKI) is defined as an abrupt and rapid deterioration in kidney function, and it represents one of the most serious complications of severe *P. falciparum* infection in humans, having been repeatedly associated with higher patient mortality. The KDIGO (Kidney Disease: Improving Global Outcomes) classification currently defines AKI as either: 1) an increase in serum creatinine by at least 0.3 mg/ld. within 48 hours, or 2) an increase in serum creatinine to at least 1.5 times the baseline level within the previous 7 days, or 3) a decrease in urine output to less than 0.5 mL/kg/h for 6 hours (5). AKI is a multifactorial condition with many potential risk factors, while the causes of AKI are usually

categorized as prerenal (including hypovolemia and obstruction of blood flow), postrenal (obstruction of urinary flow) and renal (including nephrotoxins, infection, and inflammation) (6). Prior to the use of the KDIGO consensus guidelines, the prevalence of AKI in malaria had been significantly underrepresented in both adults and children (7, 8). Recent studies using the KDIGO classification, have reported that the prevalence of AKI ranges from 20% to 40% among adults and children with SM, while some studies have reported an AKI incidence of as high as 59% for children (9–14). Despite the similar frequency of AKI between child and adult SM patients, the overall burden of malaria-associated AKI cases is likely to be greater in children, since the vast majority of SM patients in endemic countries are children (15). Importantly, kidney injury has been found to be an independent predictor of mortality in children with SM, though it is not yet known if and how AKI directly contributes to death (16). At the same time, a study examining long-term clinical outcomes of malaria-related AKI patients, showed that 5% of patients developed chronic kidney disease, further highlighting the significance of AKI as a complication of SM (17).

Typical histopathology features in malaria-related AKI include acute tubular necrosis (ATN) and, less commonly, interstitial nephritis and glomerulonephritis (18, 19). Examination of kidney tissues from autopsies of Southeast Asian adult SM patients revealed the presence of sequestered pRBCs within glomerular and tubulo-interstitial vessels, as well as the accumulation of host monocytes within glomerular and peritubular capillaries (20). Increased glomerular cell proliferation and decreased expression of zonula occludens-1 protein (ZO-1) has also been observed in kidney tissue from malaria-related AKI patients compared to non-AKI SM patients (21).

The pathogenic mechanisms of AKI in malaria are not yet fully defined, although there are multiple pathological processes which may converge on the kidney, including parasite sequestration, endothelial dysfunction, oxidative stress and immune-mediated damage. One of the hallmarks of malaria infection is intravascular hemolysis, primarily of *Plasmodium*-infected red blood cells (pRBCs), which leads to the release of cell-free heme, as well as both host and parasite-derived molecules that potently trigger inflammatory responses (4). Some degree of intravascular hemolysis occurs with all parasite species, but the most extensive hemolysis occurs in *P. falciparum* infection, as a consequence of the higher parasite densities typically occurring in the blood with this parasite species (22). The particular virulence of *P. falciparum* is also attributed to expression of parasite proteins on the surface of pRBCs, which allows their adherence to the endothelium of blood vessels, through binding to endothelial receptors (e.g. ICAM-1, EPCR) (23). Sequestered pRBCs evade splenic clearance, contributing to high parasite load, obstruct small blood vessels, leading to tissue hypoxia, and activate vascular endothelial cells (4). Endothelial activation is likely a central pathological event, resulting in impairment of its barrier function, dysregulation of blood flow and coagulation cascades, and secretion of proinflammatory cytokines, further amplifying the host inflammatory response (3).

The synergistic occurrence of the unique features of SM pathology, including parasite sequestration, microvascular dysfunction, endothelial activation, extensive intravascular hemolysis and hemodynamic instabilities, culminating in the exacerbation of a vigorous systemic inflammatory response on the kidneys, could represent the predominant mechanism through which SM leads to the development of AKI (**Figure 1**). Understanding the role of the inflammatory response and the extent to which it contributes to malaria-associated AKI, could lead to the development of novel immunomodulatory therapies that significantly decrease the number of deaths caused by both malaria-related and other causes of AKI.

IMMUNE AND INFLAMMATORY RESPONSES IN MALARIA

The host immune response has been implicated as a detrimental factor in the pathogenesis of SM, wherein an exaggerated proinflammatory response against the parasite is thought to contribute to the pathology of the disease (3). The intravascular hemolysis of both pRBCs and non-infected RBCs leads to the release of parasite and host-derived molecules that act as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively (24). These PAMPs and DAMPs interact with pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), which activate multiple downstream transcriptional programs related to the host immune and inflammatory response. Glycosylphosphatidylinositol (GPI) anchors are one of the main *Plasmodium* PAMPs that act through the activation of TLRs, inducing the production and secretion of proinflammatory cytokines such as tumor necrosis factor (TNF) and the expression of cell-adhesion molecules (e.g. VCAM-1) (25). Another major PAMP is hemozoin, a molecule produced by the *Plasmodium* parasite within RBCs during the detoxification of heme. Hemozoin crystals elicit the production of TNF and IL-1β by monocytes and macrophages, while hemozoin complexed with *Plasmodium* nucleic acids can further amplify the inflammatory response by interacting with cytosolic DNA sensors, leading to the activation of inflammasomes (e.g. NLRP3, AIM2) (26). Host-derived DAMPs are also released during intravascular hemolysis, with cell-free hemoglobin and heme considered to be the predominant ones. Although the activation of these PRRs is vital for the mounting of a protective immune response against the parasite, the constant and excessive activation that occurs during malaria due to continuous release of PAMPs and DAMPs leads to a state of systemic hyperinflammation, which may contribute to disease pathology.

SM patients have been found to produce greater levels of proinflammatory cytokines (e.g. IL-1β, IL-6 and TNF), than patients with uncomplicated malaria, while evidence has accumulated over the years implicating the upregulation of the inflammatory response as a contributory factor to SM pathogenesis, and malaria-associated AKI in particular (27, 28). For instance, macrophage infiltrates have been observed in the glomeruli of *P. berghei* ANKA-infected mice, which were accompanied by a strong upregulation in the expression of proinflammatory cytokines in kidney tissue, including TNF

and IL-6 (29). Both TNF and IL-6 signaling can potently propagate the inflammatory response and induce activation of the endothelium, while TNF has also been shown to cause changes to the glomerular endothelial permeability (30, 31). Endothelial activation induces an upregulated expression of cell adhesion molecules on the surface of endothelial cells (e.g. VCAM-1, ICAM-1, E-selectin), which could in turn lead to increased sequestration of pRBCs and infiltration of leukocytes into the kidneys, thus contributing to AKI in malaria. Multiple studies have concluded that markers of endothelial activation and microvascular obstruction are associated with malaria-related AKI in both children and adults with SM (32–34).

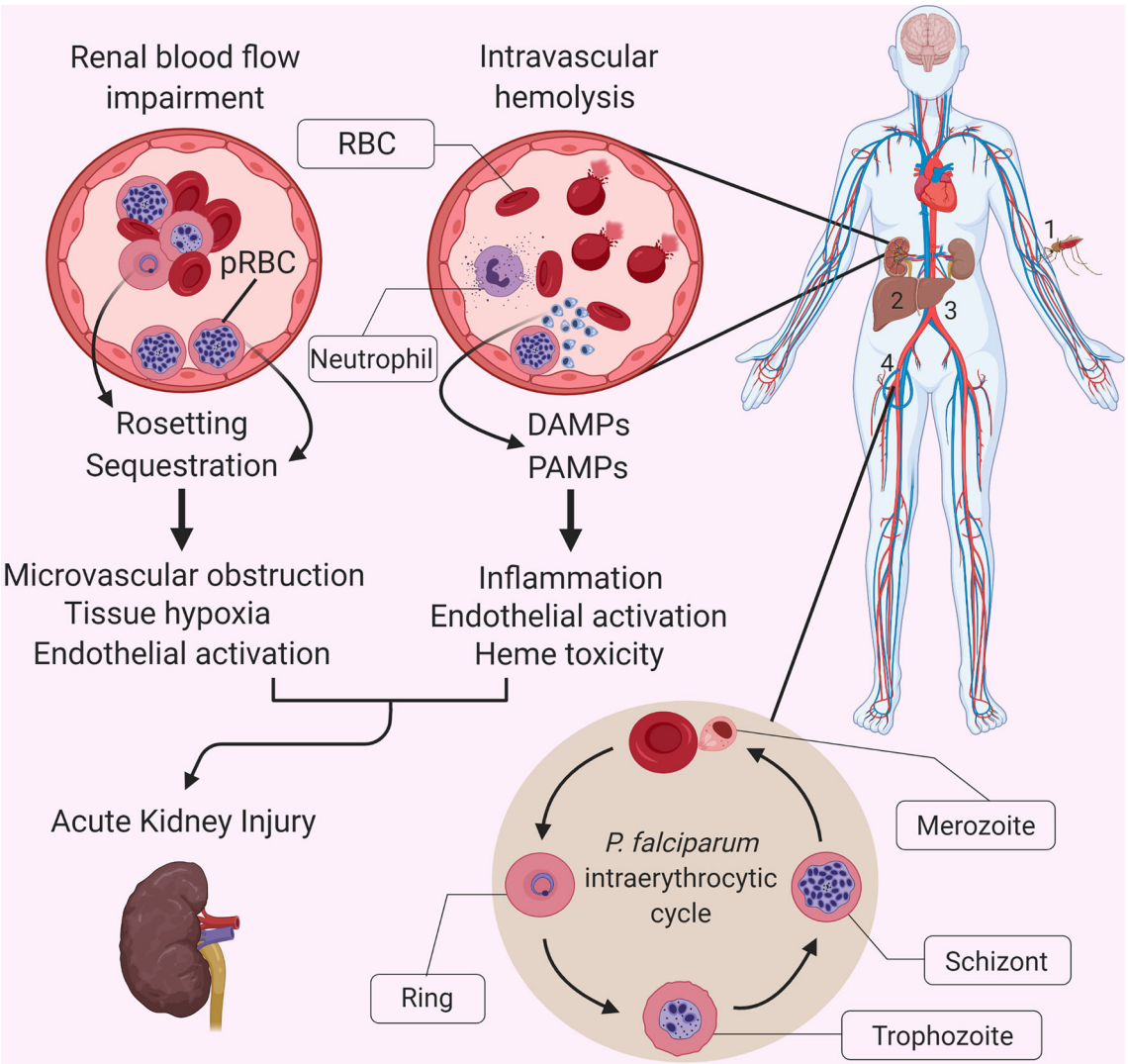

FIGURE 1 | Interactions between *P. falciparum* malaria-specific pathology and the systemic inflammatory response in malaria-related acute kidney injury. The causative agents of malaria are protozoan parasites belonging to the Plasmodium genus, which includes multiple species, five of which regularly infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (4). A bite by a *Plasmodium* spp.-infected mosquito (1) leads to the injection of motile sporozoites within the host, which travel through the lymphatics and blood until they reach the liver and invade hepatocytes (2). Once inside the hepatocyte, each sporozoite replicates and gives rise to thousands of merozoites, which are released into the bloodstream (3) when the infected hepatocyte bursts. Free merozoites then proceed to infect red blood cells (RBCs) within the bloodstream (4) and enter their asexual reproductive cycle, also referred to as the intraerythrocytic cycle. This developmental stage is characterized by a replication cycle that typically lasts approximately 48 hours and which culminates in the simultaneous rupturing of the parasitized RBCs (pRBCs), and the release of a massive number of merozoites into the bloodstream, which go on to infect further RBCs. Malaria is associated with a vigorous inflammatory response, which shares some features with other infectious diseases, but it is accompanied by unique aspects of pathophysiology that exacerbate the impact of systemic inflammation on individual organs. These unique aspects include the cytoadherence of parasite-infected red blood cells (pRBCs) to the microvascular endothelium, and the extensive release of cell free hemoglobin and heme during hemolysis. Although the exact pathophysiology of AKI in severe malaria remains unknown, we propose that the sequestration of parasitized red blood cells, rosetting and accumulation of parasite products in the kidney, resulting in endothelial activation and microvascular obstruction, promote the already damaging effects of an exuberant inflammatory response and heme-mediated oxidative damage. DAMP, Damage-associated molecular pattern; PAMP, Pathogen-associated molecular pattern.

Activation of the complement system has also been suggested to contribute to kidney damage in cases of intravascular hemolysis (35). The liberation of hemoglobin and its breakdown products potently activates the alternative complement pathway, which then induces opsonization of both infected and uninfected RBCs, further exacerbating the intravascular hemolysis that characterizes malaria (36, 37). Activation of the alternative complement pathway has been repeatedly shown to contribute to tubular injury and kidney function deterioration in mouse models of ischemia/reperfusion injury-related AKI, while selective inhibition of the pathway significantly reduced degree of kidney injury (38, 39). Importantly, neutrophils activated by proinflammatory cytokines such as TNF, are known to be potent inducers of the alternative complement pathway (40). At the same time, activated complement goes on to further propagate the activation of neutrophils, thus creating a positive feedback loop that amplifies both the neutrophil proinflammatory response and the activation of the complement system, which can be detrimental for the kidneys (40, 41).

HYPOVOLEMIA AND OBSTRUCTION OF KIDNEY BLOOD FLOW

The kidneys are especially susceptible to the damaging effects of ischemic events and hemodynamic instabilities, and obstruction of blood flow into the kidneys represents one of the primary causes of AKI (5). Significant hypovolemia is a common phenomenon in SM, often leading to kidney hypoperfusion, decreased GFR, secretion of vasoactive mediators and the activation of inflammatory processes, all of which could contribute to the development of kidney injury (28, 42). Recent evidence implicating hypovolemia-related inflammation in playing a role in the kidney damage associated with SM came from a study investigating the activation of the Angiotensin II (Ang II)/AT1 receptor pathway in the *P. berghei* ANKA malaria mouse model (28). Activation of this pathway has been found to play a central role in the occurrence of kidney injuries in multiple AKI mouse models, through its ability to increase the expression of proinflammatory cytokines and induce immune cell infiltration into tissues (28, 43). Importantly, inhibition of the Ang II/AT1 pathway in the *P. berghei* ANKA mouse model led to a significant downregulation of the inflammatory response and a substantial mitigation of kidney damage (28).

In addition to hypovolemia, kidney hypoperfusion in SM might result from obstruction of blood flow within the kidneys. During SM, clumps of both infected and uninfected RBCs, also referred to as 'rosettes', accumulate in the lumen of small blood vessels and obstruct blood flow, leading to microvascular dysfunction and tissue hypoxia, while also contributing to the sequestration of greater parasite biomass within tissues (3). High parasitemia and extensive sequestration of pRBCs in tissues and vital organs has been correlated with poorer outcomes in SM patients (44). Cerebral malaria (CM) has been associated with a high parasite burden in the brain and retinal tissue of patients (45). Similarly, total body parasite burden and immune activation were found to be associated with the incidence of AKI in Bangladeshi patients with SM, while both factors increased with AKI severity (46). Extensive sequestration of parasites within the kidney could elicit a stronger local inflammatory response, which may in turn exacerbate the degree of kidney injury.

EXTENSIVE INTRAVASCULAR HEMOLYSIS AND HEME-MEDIATED TOXICITY

Both cell-free hemoglobin and cell-free heme released during intravascular hemolysis can be particularly harmful, mainly due to oxidative stress and by acting as DAMPs that stimulate the immune system and lead to an upregulation of the inflammatory response (47). The body possesses natural scavenging systems in order to clear cell-free hemoglobin and cell-free heme, but these systems can be quickly overwhelmed by extensive hemolysis such as that observed during SM (35). Furthermore, the kidneys are especially vulnerable to the damaging effects of cell-free heme and hemoglobin, since they are the primary route for clearance of these molecules when the scavenging systems become saturated (35).

Both infectious and non-infectious causes of extensive intravascular hemolysis have been associated with the development of AKI, while increased levels of plasma and urine hemoglobin have also been strongly associated with AKI in SM patients (35, 48). Higher levels of cell-free hemoglobin and markers of lipid peroxidation have been observed in SM patients with AKI compared to those without AKI (48). A recent clinical trial showed that SM patients receiving acetaminophen, a drug which can inhibit hemoprotein-mediated lipid peroxidation, had a lower risk of developing AKI than patients in the control group. The effect of acetaminophen was most prominent in patients with greatest intravascular hemolysis, further strengthening the association of cell-free hemoglobin and oxidative stress with AKI (49).

Past studies have investigated the effect of sterile intravascular hemolysis on the kidneys by treating mice with phenylhydrazine (PHZ), a chemical compound that induces massive intravascular hemolysis (47). One of these studies found that kidney endothelial cells from mice treated with PHZ had increased expression of cell adhesion molecules (e.g. E-selectin and ICAM-1), while they also detected markers and ultrastructural signs of tubular damage (47). In the context of SM, this upregulation of adhesion molecules by the endothelium could significantly worsen the extent of parasite sequestration within tissues, as well as increase the infiltration of inflammatory immune cells into the kidneys, as previously discussed (47). Interestingly, the same study showed that the injection of heme alone did not recapitulate this effect of tubular injury and vascular inflammation, supporting the notion that it is a combination of factors and mechanisms acting in concert that lead to the development of both malaria-related and non-related AKI, rather than one individual mechanism (47).

Kidney endothelial cells exposed to cell-free heme *in vivo* have been found to upregulate their secretion of

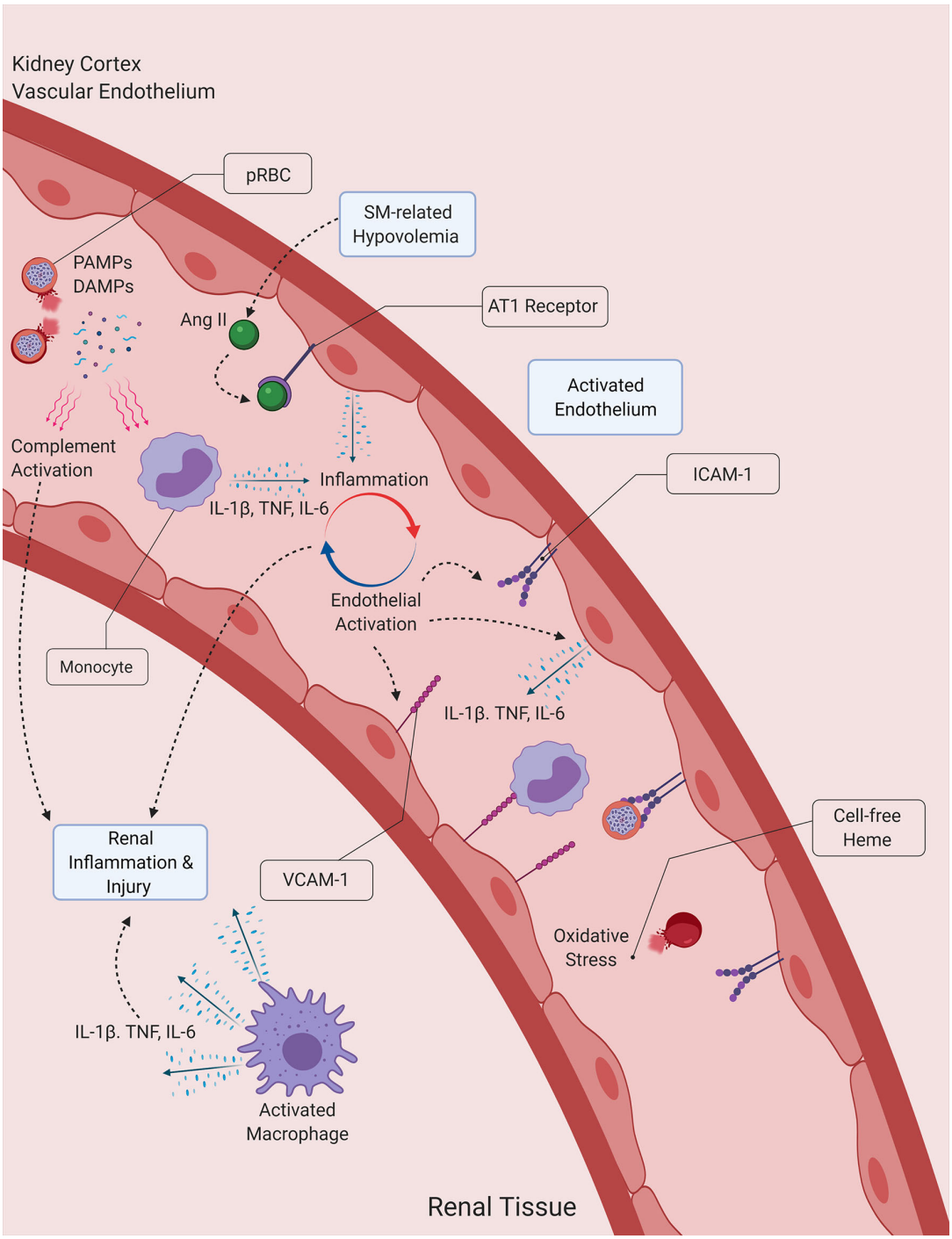

FIGURE 2 | Proposed interactions between inflammation, hemolysis and hypovolemia in malaria-induced kidney injury. Malaria PAMPs and DAMPs released during the lysis of *Plasmodium*-infected RBCs (pRBCs) stimulate the immune and inflammatory responses, leading to the secretion of proinflammatory cytokines, which subsequently induce the activation of the endothelium and the propagation of the inflammatory process. Activated endothelial cells contribute to the secretion of proinflammatory cytokines, and express surface receptors that facilitate the infiltration of leukocytes into kidney tissue. Cell-free heme acts a source of oxidative stress for the vascular endothelium and the tubular epithelial cells of the kidney, while also inducing the formation of NETs. The activation of the angiotensin (Ang) II/AT1 receptor pathway due to malaria-induced hypovolemia also contributes to the amplification of the host inflammatory response, by further inducing the secretion of proinflammatory cytokines by endothelial cells. DAMP, Damage-associated molecular pattern ICAM-1, Intercellular adhesion molecule 1, IL-1β, Interleukin-1β; IL-6, Interleukin-6; NET, Neutrophil extracellular trap; PAMP, Pathogen-associated molecular pattern; SM, Severe malaria, TNF, Tumor necrosis factor; VCAM-1, Vascular cell adhesion molecule 1.

proinflammatory chemokines, such as monocyte-chemoattractant protein 1 (MCP-1), through the activation of the NFκB transcription factor by heme (50, 51). Intriguingly, a recent study showed that a mouse model of malaria with a renal proximal tubule-specific knockout of heme oxygenase-1 (HO-1), a heme detoxifying enzyme, was more susceptible to AKI, further suggesting the involvement of heme-mediated damage in the propagation of AKI in SM (52). Additionally, the same effect was found when the researchers knocked out ferritin, which is responsible for sequestering the labile iron released after the detoxification of heme by HO-1, strengthening the association of oxidative stress with kidney injury (52). Multiple pieces of evidence have indicated the involvement of heme oxygenase-1 (HO-1) and the by-product of heme degradation, carbon monoxide (CO), to be intricately involved in the establishment of disease tolerance to SM complications, through their antioxidant and anti-inflammatory properties (52). Carbon monoxide therapy, administered as carbon monoxide-releasing molecules, has been reported to confer full protection against experimental cerebral malaria and acute lung injury in a mouse model when used in conjunction with an antimalarial drug (53). Since the beneficial impact of CO is thought to be thanks to its ability to prevent the further release of heme from hemoglobin and to induce the expression of HO-1, it is possible that there would be a therapeutic potential against malaria-associated kidney damage.

Finally, cell-free heme is known to trigger neutrophil activation, inducing the release of neutrophil extracellular traps (NETs). In a study using *P. chabaudi*-infected mice, heme-induced NET generation was found to be necessary for the development of liver and lung pathology and also increased the extent of parasite sequestration in the organs of the mice (54). If the same phenomenon of heme-induced NET release were to occur in the kidneys, it would provide another mechanism through which intravascular hemolysis might lead to immune-mediated kidney damage. NETs have also been implicated as a contributing factor in non-malaria related causes of AKI, including systemic lupus erythematosus (SLE) and ANCA-associated vasculitis (55). Furthermore, the release of histone proteins from necrotic kidney tubular cells during ischemic AKI is known to induce the formation of NETs, and in a study utilizing an ischemia-reperfusion injury mouse model of AKI, pre-treatment of the mice with an inhibitor of NET formation led to a mitigation of kidney injury (56). NETs might also be involved in further obstructing microvascular blood flow in cases of ischemic AKI, by creating thrombus-like structures made up of trapped cells and cellular debris (57). In addition, the formation of NETs may represent a proinflammatory stimulus by itself, since the histone proteins and DNA that are released during the process can act as DAMPs that further activate the immune response (57). Immunofluorescence studies to detect neutrophil infiltration and the presence of NETs in kidney tissue from mouse models of malaria could prove useful in the future, to determine whether NETs are also involved in mediating kidney pathology during malaria-related AKI.

DISCUSSION

Multiple studies have identified malaria as one of the most frequent etiologies of pediatric AKI cases in developing countries (58). Importantly, both adult and pediatric studies have shown that when AKI occurs in the context of malaria, there is an increase in patient mortality (59, 60). One of the key questions that remains to be answered is whether AKI plays a causal role in SM deaths, or if it is a consequence of pathological processes which lead to death through their effects on other organs.

Here, we have described the factors and pathways plausibly involved in the pathophysiology of AKI in SM. It is hypothesized that a vigorous host inflammatory response which is amplified by features of malaria pathology appears to be a central aspect of kidney injury in SM. Hypovolemia and obstruction of kidney blood flow lead to tissue hypoxia and endothelial activation, which could promote the secretion of proinflammatory cytokines and leukocyte infiltration into the kidneys. At the same time, the cell-free heme that is liberated due to intravascular hemolysis may also contribute to kidney damage, not just by acting as a source of oxidative stress, but also by triggering the activation of the complement system, the formation of NETs and by further amplifying the proinflammatory response (**Figure 2**).

In light of the pivotal role that the inflammatory response is hypothesized to play in mediating kidney injury, it is imperative that we obtain a greater understanding of the immunopathology of AKI in malaria, which will hopefully lead to the development of more effective treatments and a decrease in the mortality of the disease.

AUTHOR CONTRIBUTIONS

AC and AG conceived the idea for this review. OK wrote the original draft and designed the figures of the manuscript. AC and AG provided critical feedback and reviewed the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Chess Not Checkers: Complexities Within the Myeloid Response to the Acute Kidney Injury Syndrome

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Immune dysregulation in acute kidney injury (AKI) is an area of intense interest which promises to enhance our understanding of the disease and how to manage it. Macrophages are a heterogeneous and dynamic population of immune cells that carry out multiple functions in tissue, ranging from maintenance to inflammation. As key sentinels of their environment and the major immune population in the uninjured kidney, macrophages are poised to play an important role in the establishment and pathogenesis of AKI. These cells have a profound capacity to orchestrate downstream immune responses and likely participate in skewing the kidney environment toward either pathogenic inflammation or injury resolution. A clear understanding of macrophage and myeloid cell dynamics in the development of AKI will provide valuable insight into disease pathogenesis and options for intervention. This review considers evidence in the literature that speaks to the role and regulation of macrophages and myeloid cells in AKI. We also highlight barriers or knowledge gaps that need to be addressed as the field advances.

Keywords: macrophages, monocytes, F4/80, neutrophils, acute kidney injury, cell death, inflammation

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INTRODUCTION

Acute kidney injury (AKI) is exemplified by a disruption in renal homeostasis that leads to a rapid decrease in kidney function. This is a devastating condition that continues to lack effective therapies. The combination of severe health impairment and high prevalence creates a significant burden for both patients and health systems. Patients with AKI have higher hospitalization costs, longer hospital stays, decreased quality of life, and an increased risk of death compared to non-AKI patients (1–4). A 2013 meta-analysis concluded that 1 in 5 adults and 1 in 3 children develop AKI during a hospital stay (5); currently, it is estimated that ∼500,000 hospitalized individuals are affected by AKI in the U.S. alone (1). This is clearly a widespread and pervasive threat to human health that must be addressed.

Cell death following injury is a key event in AKI initiation (2, 6, 7). Tissue damage and cellular stress leads to the release of molecules and byproducts with a wide range of potential effects on surrounding cells. It is therefore important to understand how specific, well-defined cell populations respond to cues within the microenvironment and participate in AKI pathogenesis, since such knowledge can inform and improve intervention strategies.

Cellular death can occur via multiple pathways, including regulated mechanisms (e.g. apoptosis, necroptosis, pyroptosis, ferroptosis) or unregulated, accidental necrosis (7–11). The extent and type of cell death within injured tissue has important consequences for downstream responses, the content of the microenvironment, and ultimate outcome (12). Generally, apoptosis is considered a

well-contained event that minimally affects surrounding cells and promotes homeostasis. Other forms of cell death, however, can trigger immune cell activation and inflammatory pathways, leading to extension of the original injury (7–14).

Inflammatory events downstream of cellular death in AKI is an area of ongoing investigation, but fully understanding these cascades will be instrumental for improving therapy (6). While the decline in kidney function during AKI stems from damage to epithelial cells and loss of tubule function, immune cells play important roles in early and late phases of AKI (2, 6, 15–21). The increased presence of myeloid cells has been widely reported and is considered a key event in the pathogenesis of AKI (2, 6, 15–21). Myeloid cells (e.g., macrophages, monocytes, neutrophils) can disrupt the structural integrity of tissue and produce molecules that are toxic to surrounding cells, such as cytokines, reactive oxygen or nitrogen species, and purines. These activities can extend the initial injury and potentially exacerbate the severity of AKI. Later in the course of AKI, myeloid cells are also capable of delaying resolution and recovery by sustaining inflammation and vascular impairment (10, 16, 22).

Macrophages, in particular, are equipped with an extensive array of danger and cytokine sensing receptors for surveying their surroundings (23, 24). They are exquisitely sensitive to changes in the microenvironment and can adopt a spectrum of activation states in response to environmental cues (25–27). Macrophages are the major immune population present in healthy kidneys and have the capacity to participate in many aspects of AKI pathogenesis (2, 6, 15, 16, 20, 21, 28). They have diverse functions in tissue which include engulfment of debris and damaged cells, detection of danger and damage via pattern recognition and cytokine receptors, production of cytokines and oxygen/nitrogen species, destruction and deposition of tissue matrix, and recruitment of additional immune cells (29). Important concerns regarding the general complexity of macrophage differentiation and activation states are beyond the scope of this discussion, but have been nicely addressed elsewhere (25, 27, 30, 31). Due to the controversy surrounding this topic and M1/M2 nomenclature, we will focus on the regulation of macrophages and myeloid cell populations during AKI rather than classification of activation states.

The role of macrophages as sentinels of the tissue environment and the fact that they are the pre dominant immune population pre-injury makes them prime candidates for further study in the context of AKI pathogenesis. A clearer understanding of the relationships between tissue damage, macrophage activation, inflammation, myeloid accumulation, and injury progression holds promise for the development of novel therapies for AKI. Here we will discuss the potential involvement of macrophages and key myeloid populations in AKI as well as some barriers that have generated confusion in the context of the kidney.

CELL DEATH AND MACROPHAGES

Cell death is a key element in the initiation of injury and reduced renal function in AKI (2, 6, 7). As stated above, cell death can manifest via multiple mechanisms including apoptosis, necrosis, necroptosis, pyroptosis, and ferroptosis (8, 9). Broadly, apoptosis is considered an immunologically silent process that does not result in inflammation, although this may not always be the case (8). Currently, there is some controversy surrounding the extent of the role of apoptosis in AKI (7). While heightened apoptotic death of tubule cells can be an important element of initial injury and loss of renal function, current knowledge suggests it is unlikely that this death mechanism contributes extensively to downstream inflammatory responses in AKI. On the other hand, cell death stemming from necrosis, necroptosis, pyroptosis, or ferroptosis can function as an important initiator of macrophage and myeloid cell activation, representing a potential bridge from initial injury to pathogenic inflammation (9, 10, 13, 32).

Cell Death Mechanisms and Inflammation

Apoptosis is a programmed form of cell death that occurs during homeostatic turnover, accumulation of cellular stress and damage, or during immune recognition of infected or improperly functioning cells. This process hinges on the ultimate activation of executioner caspases 3 and 7 and multiple intrinsic and extrinsic pathways can trigger this outcome (8). Generally, apoptosis proceeds after controlled permeabilization of the outer membrane occurs without fully disrupting its integrity. Following initiation of apoptosis, the cell undergoes controlled dismantling and fragments into membrane-encapsulated apoptotic bodies. This prevents mass-escape of cellular contents into the surrounding environment and allows the apoptotic material to be removed without inducing excessive local inflammation. The clearance of apoptotic cells and the apoptotic bodies they produce is referred to as efferocytosis.

Inflammatory forms of cell death lead to the release of intracellular components and activation of extracellular molecules that can be interpreted as danger signals and potentially shift macrophages from a maintenance phenotype to an inflammatory phenotype (9, 24, 26, 33). Un-programmed, accidental necrosis can occur when cells are suddenly and irreparably damaged and can no longer maintain their structural integrity. Induction of necrosis can result from direct tissue trauma or drastic changes in the environment, such as severe hypoxia, removal of growth factors, or depletion of cellular ATP (8, 12). Necrosis can also occur downstream of apoptosis. If apoptotic cells cannot be cleared and processed and are unable to return to a homeostatic state, they will progress to secondary necrosis (12). This mechanism could be at play in injured kidneys if the degree of apoptosis surpasses the efferocytic capacity of the tissue. Necrotic cell death is characterized by a loss of cell membrane integrity leading to swelling and eventual rupture and the unregulated release of cellular contents.

While necrosis is the only described form of unregulated cell death, the necrotic pattern of cell death (i.e., the release of cellular components) can also manifest as the result of programmed cellular execution mechanisms. Each of these mechanisms culminates in destabilization of the cell membrane and the release of toxic or inflammatory cellular contents. During necroptosis, association of RIPK1 and RIPK3 leads to activation of the executioner protein mixed lineage kinase domain-like (MLKL) (8, 9, 12, 32, 34). MLKL goes on to form pores in

the membrane and precipitate necrotic death. In pyroptosis, the executioner gasdermin proteins (gasdermin-D is the most commonly studied to date) are cleaved into their active form by caspases. The active fragment of gasdermins, similar to the role of MLKL, oligomerizes and forms pores in the membrane (8, 12, 32). Ferroptosis is a distinct form of cell death that is dependent on iron availability and occurs as the result of increased lipid oxidation (8, 9, 12, 32, 35). Iron promotes lipid oxidation either directly via Fenton reactions or indirectly as a component of enzymes, such as lipoxygenases (LOX) (8, 36, 37). Since lipids are a major component of cell membranes, unrestricted lipid oxidation within cells is extremely damaging to their structural integrity and leads to rapid necrotic death (8, 32, 35, 36, 38). All of these mechanisms are capable of creating an environment that favors inflammation over resolution of injury.

Cell Death as the Bridge Between Injury and Inflammation

While the identity and activity of pro-inflammatory mediators produced during cell death are still under investigation, the list of culprits includes DNA and DNA-chromatin complexes, heat shock proteins (HSP), high-mobility group box 1 (HMGB1), uric acid, galectins, purines (e.g., ATP), extracellular matrix components, and complement system activation (13, 39). In addition, cytokines in the IL-1 family can act as inflammatory danger signals during cell death, including IL-1β and IL-33 (40). The bioactivity of these cytokines is generally proteolytically regulated. Caspase activity can variably increase or dampen the ability of these cytokines to stimulate inflammation, so the array of cytokine activity will depend on the mechanism of cell death. For example, full-length IL-1β does not bind the IL-1 receptor and requires cleavage by caspase-1 (or potentially other enzymes) to exert its activity. Conversely, active IL-33 is sequestered within cells and is inactivated by caspases 3 and 7 (40). Caspase-independent cell death can therefore bypass this inactivation and lead to the release of active, pro-inflammatory IL-33 that acts as an alarmin and induces inflammation (40). Macrophages express receptors that can detect a majority of these damage-associated molecules and are prime candidates for shaping the immediate response following initial injury (**Figure 1**). Macrophage receptor machinery and responses to cell death have been nicely reviewed elsewhere (9, 13, 23, 24, 41), but these interactions will be briefly explored here.

In addition to the above-mentioned forms of cell death, NETosis can also play a role in AKI. This is a form of death specific to neutrophils upon the release of neutrophil extracellular traps (NETs) (32, 42, 43). NETosis occurs when neutrophils are triggered to release mesh-like structures of their intracellular components comprised of DNA, histones, and granule proteins (32, 42, 43). While this process is specific to neutrophils and NETosis is not a form of death that occurs in tissue cells, the release of these intracellular components forms a milieu reminiscent of other forms of necrotic cell death (32, 42, 43). Thus, NETosis can also participate in cell death-induced inflammation and thrombosis during AKI.

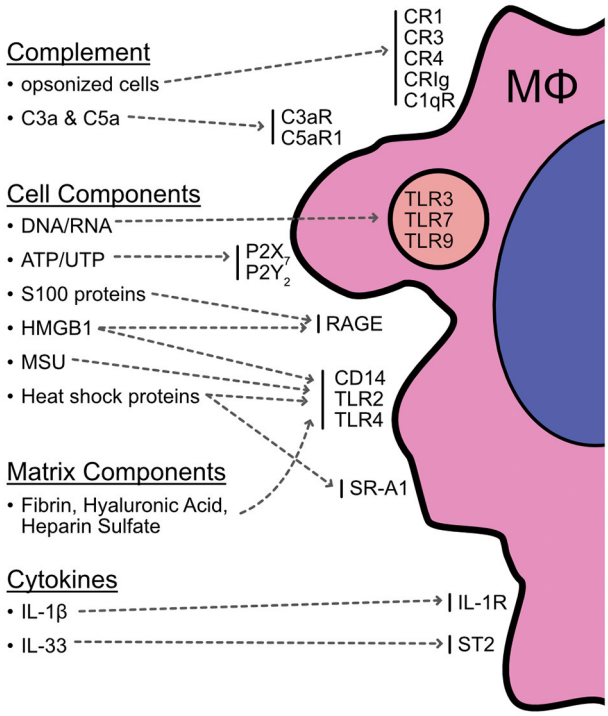

FIGURE 1 | Summary of potential interactions between macrophages and cell death. Macrophages express a variety of machinery for detecting cell death and products released from cells or the surrounding matrix as a result of necrotic death. These include receptors for complement opsonized cells (CR1, CR3, CR4, CRIg, C1qR); complement cascade components (C3aR, C5aR); DNA/RNA (endosomal TLRs 3, 7, & 9); purines ($P2X_7$, $P2Y_2$); monosodium urate (CD14/TLRs); S100 proteins (RAGE); HMGB1 (RAGE, TLRs); heat shock proteins (TLRs, SR-A1); the matrix components fibrin, hyaluronic acid, and heparin sulfate (TLRs); and cytokine alarmins such as IL-1β and IL-33 (IL-1R, ST2). This list is not exhaustive and additional interactions are continuously being described and investigated. It is still unclear to what extent these interactions are involved in AKI and if macrophage stimulation is a critical element for bridging injury and pathogenic inflammation, but it is clear there are multiple avenues for macrophages to engage with cell death events in a tissue setting.

Complement can be deposited on the surface of dead/dying cells and apoptotic bodies to facilitate recognition and uptake by macrophages via complement receptors (CR). These include CR1 (CD35), CR3 (CD11b/CD18), CR4 (CD11c/CD18), CRIg, and C1qR (CD93) (24, 44). Clearance of dead/dying material is generally beneficial during injury and appears more suppressive than stimulatory for macrophages (13, 24, 41). Thus, this may be a means of attempting to limit inflammation in the face of cell death. However, in addition to this interaction with dead/dying cells, macrophages can also detect the C3a and C5a products of the complement cascade via C3aR and C5aR1 (CD88) (44). While detection of these components activates macrophages and can stimulate the production of proinflammatory cytokines (45), there is also evidence that they can suppress macrophage functions and promote tumor growth or metastasis (46–50). Given this, the impact of complement on macrophages is likely

context dependent and dictated by the extent and type of cell death as well as additional stimuli experienced by macrophages. Interestingly, a study by Peng et al. has shown that C3aR and C5aR deficiency can protect mice from renal ischemia reperfusion injury (IRI) (51), which shows these receptors play a role in pathogenesis. However, since the genetic deficiency was not restricted to macrophages, it is still unclear if the impact of complement signaling was due specifically to macrophages sensing these molecules. The relationship between macrophages and complement during tissue injury is clearly complex and is further obfuscated by the fact that a wide variety of cells respond to complement components. It will require careful study to dissect the importance of complement for macrophage function relative to other cells during AKI pathogenesis.

Once necrotic death has occurred and cell contents have been released into the environment, macrophages sense these components through a variety of receptor machinery. The toll-like receptor (TLR) family of receptors is expressed extensively by macrophages and may play an important role in the macrophage response to cell death, similar to its role in pathogen detection (13, 24, 52, 53). Members of the TLR family (TLR2 and TLR4) can bind HSP, HMGB1, fibrin, hyaluronic acid, and heparan sulfate (13, 24). These molecules are released from the dead cells themselves (HSP, HMGB1) or generated by the breakdown of extracellular components (fibrin, hyaluronic acid, heparin sulfate) by enzymes released from dead/dying cells (13). TLR can also recognize DNA and RNA released from dead cells (TLR3, TLR7, TLR9) (13, 24). There is also evidence for the stimulation of TLR2/TLR4 by uric acid (also referred to as monosodium urate; MSU), the final cellular product from the breakdown of purines (13, 54). CD14 is another pattern recognition receptor that associates with TLR2 and TLR4 and has been experimentally shown to directly bind MSU, indicating a potential means of MSU recognition, internalization, and cellular activation via CD14/TLR cooperation (13, 55). However, there is still controversy surrounding the stimulatory mechanisms of MSU and its crystal form since others have observed a response to MSU independent of TLR (13, 56).

In addition to TLR and TLR-associated proteins, other macrophage receptors are capable of engaging with products of cell death. The scavenger receptors RAGE and SR-A1 detect HMGB1, S100 proteins, and HSP (13, 24). $P2X_7$ and $P2Y_2$ receptors bind extracellular ATP and UTP and can trigger macrophage activation (13, 24, 57, 58). Macrophages also express the IL-1 receptor and ST2 and can be stimulated by IL-1β and IL-33 (respectively) (24, 59, 60). This is not a comprehensive list of the interactions that can occur between macrophages and the products of cell death and there are likely additional mechanisms yet to be described, but it is clear there is ample opportunity for engagement and stimulation of macrophages in an environment of widespread tissue damage and cell death.

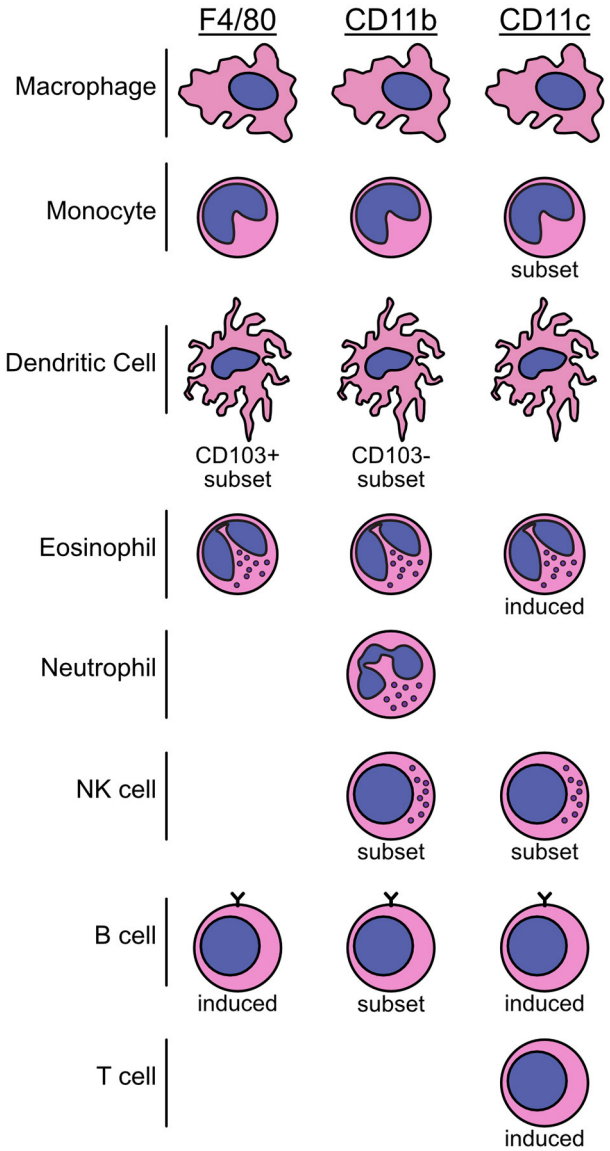

FIGURE 2 | Summary schematic of immune cell populations in mice that express the F4/80, CD11b, and CD11c markers. The use of F4/80, CD11b, and CD11c markers to specifically detect or deplete macrophages has led to a lack of clarity in the AKI field. These molecules are not restricted to macrophages, monocytes, or DC and can be expressed by a variety of immune cell populations and subsets. Further, they can be expressed basally or be induced on specific cell types. Over-reliance on these three molecules without additional characterization can lead to uncertainty about which cells are responsible for an experimental outcome, hindering the ability to draw precise conclusions. The overlapping, diverse expression patterns of these markers is illustrated schematically here.

THE AMBIGUITY OF F4/80

While F4/80 has traditionally been used as a lineage marker for macrophages, expression of this molecule is far from definitive or exclusive. In reality, many cells express the F4/80 antigen, either basally or as an induced marker. This list includes macrophages, monocytes, DC, eosinophils, and potentially some B cell populations (**Figure 2**) (61–68). Such non-selectivity can be problematic when attempting to make conclusions about renal macrophages based solely on F4/80 staining in tissue sections, a common occurrence in older literature.

Recent work has shed light on more refined strategies for separating mononuclear phagocyte cell (MPC) populations within the kidney and shows promise for enhancing our sophistication and accuracy when investigating these cells in the context of AKI. In addition to F4/80, flow cytometry studies have shown that CD11b, CD11c, CX3CR1, Ly6C, CD64, CD14, CD16, MHC II, and CD103 are also useful markers for delineating myeloid populations within the mouse kidney (**Table 1**) (21, 69–80). A study from Lee et al. used several of these markers to identify a novel MPC population in the kidney that is also CD45-intermediate ($CD45^{int}$ $CD11b^{int}$ $F4/80^{+}$ $MHCII^{+}$ $CX3CR1^{+}$ $Ly6C^{-}$). This population is more sensitive to depletion with clodronate than other kidney MPC, but their precise role in kidney injury is still unclear (81). While progress has been made in defining myeloid subsets, the populations identified using these markers still represent macrophages, monocytes, and dendritic cells to varying degrees and each of these cell types contain additional subpopulations. Care must be taken to properly classify these subsets and their functions as the field progresses. Investigators should always be deliberate when describing the populations they are studying and explicit when conveying conclusions that can or cannot be drawn from their work.

TABLE 1 | Potential surface markers for classifying renal MPC populations in mice.

	Macrophages	Monocytes	Dendritic cells
CD11[a,b]	++/+++	+++	+/+++ (**)
CD11c	+/++	++ (subset)	+++
CD14	+++	++	++
CD16	++	–/+	++/+++
CD64	++	–/+	–
CD68	+++	++	–
CD103	–	–	+++ (subset)
CD115 (*)	–	–/++	–
MHC II	+/++	++	+++
F4/80	+++	++	–/+ (subset)
CX3CR1	++	++	++
Ly6C	–	+++ (subset)	–
Ly6G	–	–	–

[a] *Marker expression compiled from sources cited in the text and personal experience.*
[b] *-, not detectable; +, low expression; ++, intermediate expression; + + +, high expression; –/+, range of expression from negative to low; +/++, range from low to intermediate; ++/+ + +, range from intermediate to high.*
(*) CD115 expression may be variable between blood and tissues and is susceptible to loss in some conditions, so may not be consistently reliable.
(**) The two identified DC subsets in kidney have differential CD11b expression. CD103+ DC have low expression while the other subset exhibits high expression.
Subset means the expression is found on a defined sub-population of the cell type and not on the cell population as a whole.

COLLATERAL DAMAGE IN DEPLETION STRATEGIES

Depletion studies are a powerful tool for attributing phenotypes to specific cell populations. For investigating myeloid cells in AKI, the most common depletion models encountered in the literature are injection of liposome encapsulated clodronate to deplete phagocytic cells or injection of diphtheria toxin (DT) to deplete cells that specifically express the diphtheria toxin receptor (DTR) under control of CD11b or CD11c regulatory elements. However, these models often contain pitfalls that may not be fully accounted for in all studies. A particular concern when investigating myeloid populations stems from the large degree of marker and functional overlap within the myeloid compartment (82–84). Thus, results from depletion studies can compound confusion generated by imprecise cell type classifications. In addition, different depletion models or the same depletion strategy used in different disease models may produce conflicting results. This is readily evident in AKI investigations and has hindered the ability to draw clear conclusions in some cases.

Clodronate is a bisphosphonate compound that is converted into a toxic ATP analog within cells. The toxic product of clodronate metabolism interferes with processes critical to cell survival and triggers subsequent apoptosis (85). Encapsulation of clodronate in liposomes targets its uptake to phagocytic cells. While this is touted as an efficient means of depleting macrophages, macrophages are not the only phagocytic cells. Despite its ability to remove macrophages, clodronate also efficiently depletes DC and monocytes (86–88). Whenever a depletion strategy is used, best practice should be to show the impact of the treatment on additional cell populations, not just the cells of interest. Generally, clodronate experiments should be supported by additional evidence and/or additional depletion models to specifically implicate macrophages, DC, or monocytes.

The DTR has been identified as the heparin-binding EGF-like growth factor (HB-EGF) precursor (89). Although mice express this molecule, key sequence differences greatly impair or prevent the binding of DT and render normal murine cells insensitive to DT-induced death and depletion. This dichotomy allows the engineering of mice that transgenically express the human or simian form of the DTR in specific cell populations (90, 91). This can be done with a DTR transgene under direct transcriptional control of a specific promoter or via a Cre-lox system. In the Cre-lox version, expression of the Cre recombinase enzyme is under control of a specific gene promoter (e.g., CD11b or CD11c promoters) that restricts its activity to a specific cell population. Cre activity in these cells excises a lox-p-flanked STOP codon preceding the DTR gene, allowing transcription, and expression of the DTR to proceed (92).

Similar to the clodronate depletion model, CD11b- or CD11c-dependent DTR expression is not sufficient to attribute outcomes specifically to macrophages or DC (93). CD11b is widely expressed within the myeloid compartment and is variably expressed by tissue macrophage populations. Monocytes, neutrophils, and eosinophils all express high levels of CD11b and some natural killer (NK) cell and DC subsets express this marker as well (**Figure 2**) (21, 94–97). Lung macrophages express low levels of CD11b while splenic and renal macrophages express low-mid levels and peritoneal macrophages express high levels of the marker (98, 99). CD11c is a ubiquitous DC marker, but it is also expressed by macrophage populations, monocyte subsets, some NK cells, and can be induced in many others (**Figure 2**) (96, 97, 100–105). The non-specificity of CD11b and CD11c

models should lead to a reduction in their use in favor of more specific depletion models in the future.

The complexity of these depletion strategies is readily evident in the AKI literature. Multiple studies have shown that pretreatment with liposome encapsulated clodronate can lessen initial injury in ischemic AKI models (106–109). However, this protection is not observed when depletion is performed with CD11b-DTR or CD11c-DTR models (107, 108). This was nicely shown by Ferenbach et al. (107) and Lu et al. (108) in studies where they compared the effects of clodronate and CD11b-DTR depletion. These investigations noted multiple interesting differences between the models. Clodronate treatment resulted in substantial depletion of kidney F4/80+ cells and blood monocytes, but DT treatment resulted in more complete depletion of both (107). Clodronate also depleted F4/80+ cells in the spleen and liver while DT treatment left these populations essentially intact (107). Pretreatment with clodronate lessened the increase in serum creatinine and tubule injury after AKI, but DT pretreatment did not exhibit the same benefit (107, 108). Further, when clodronate and DT were given in combination, the protection seen with clodronate alone was negated and injury restored to the level seen in control mice (107). The implication here is that the myeloid compartment contains both beneficial and detrimental populations that are active during AKI. While clodronate appears to remove an injury-promoting population, the more extensive depletion from CD11b-DTR also removes cells that are involved in ameliorating injury. This is promising data that requires additional investigation to determine the relevant cells and their functional responses. The impacts of CD11c-DTR depletion, however, are somewhat unclear as conflicting results have been observed showing either minimal impact or some degree of protection in the IRI setting (108, 110).

As stated above, the $CD45^{int}$ MPC population identified by Lee et al. was more sensitive to clodronate depletion than other kidney MPC populations, making these cells an interesting candidate for a pathogenic population (81). At steady state, they were more phagocytic than their $CD45^{hi}$ counterparts but this profile flipped during IRI with $CD45^{hi}$ MPC exhibiting stronger phagocytic activity (81). The $CD45^{int}$ MPC were not significant producers of cytokines during IRI, but this may require additional studies to confirm (81). The contributions of this population at steady state and during injury are still enigmatic and warrant additional study, especially given that they were identified in human samples as well as mice.

While clodronate treatment prior to injury appears beneficial in ischemic AKI, this treatment offers minimal protection in other AKI models such as cisplatin or DT-induced death of DTR-expressing tubule cells (111–114). In fact, macrophage depletion in these models is sometimes associated with worse or prolonged disease. CD11c-DTR depletion in particular exacerbates disease during cisplatin-induced injury (114). It is unknown what causes the divergent effects of depletions in different AKI models, but differences in the mechanism of injury initiation could play a role (inflammation-mediated cell death vs. direct nephrotoxicity). The environment generated by complete ischemia is very different from the environment during targeted nephrotoxicity, and thus the involvement of myeloid cells may vary between models and stages of pathogenesis.

THE PATHOGENIC IMPORTANCE OF MYELOID INFILTRATION OF INJURED KIDNEYS

The ability of macrophages to recruit additional immune cells is a key area of interest in AKI. The increased representation of neutrophils and F4/80+ cells in the kidney is frequently associated with disease progression and pathogenic inflammation (16, 28). However, the exact contribution of these infiltrating cells to disease is an unsettled issue. Studies that investigate these phenomena often report conflicting results and this has hindered the ability to draw clear conclusions and develop therapeutics.

Neutrophils

Neutrophils have been investigated in multiple AKI models with varying results, but IRI studies are most prevalent in the literature. Early studies with rats and rabbits indicated that neutrophil depletion strategies produced no significant relief from kidney IRI, aside from reducing the degree of leakage observed in renal tubules (115–117). Another study in rats, however, observed that inducing neutropenia did indeed result in lower creatinine levels and injury scores post-IRI (118). Despite these incongruous results, the fact remains that neutrophils are recruited early and in large numbers during kidney injury and interest in their involvement has persisted.

A variety of mouse studies have now been performed that also speak to the role of neutrophils in AKI. A 2002 study reported that, while pan-caspase inhibition was capable of preventing ischemic injury by reducing cell death, neutrophil depletion produced only a mild benefit to serum creatinine levels and no improvement in acute tubule necrosis (ATN) scores (119). However, several others have shown that interfering with molecules that regulate trafficking and tissue infiltration (ICAM-1, P-selectin, E-selectin, Slp76, ADAP) can limit neutrophil recruitment to the kidney and lessen injury severity (120–122). Interrupting the inflammatory response during injury via adenosine receptor agonism, preventing NKT cell activation, or disrupting an IL-17/INFγ signaling axis can also restrict the influx of neutrophils during IRI and limit injury (18, 19, 123). The biggest issue with these observations is that these types of interventions affect immune cell function in a relatively broad manner and do not specifically target neutrophils. However, several of these studies bolstered their results by including neutrophil depletions that also exhibited reduced injury (120, 121, 123). It remains to be seen if differences between previous work and more recent mouse studies are due to species or procedural differences, but the preponderance of evidence indicates a role for neutrophil involvement in ischemic AKI.

Other AKI models have also reported a benefit to neutrophil depletion. In endotoxemia/sepsis-induced AKI, several studies have reported that neutrophil depletion can limit creatinine increases and kidney injury markers (124–126). Neutrophil depletion in the context of mercuric chloride-induced kidney

injury also prevented increases in BUN to a large degree (127). When cholesterol crystals were used to induce renal infarcts and kidney injury, neutrophil depletion improved all injury measurements except glomerular filtration rate (GFR) (128). This is an interesting outcome since GFR is the most relevant measurement for kidney function; however, the reduced GFR was due to obstruction caused by the cholesterol crystal clots, an aspect in which neutrophils may have a minimal or redundant role. Thus, this could be interpreted as neutrophil depletion preventing additional inflammation and injury that is secondary to the initial injury caused by the obstructions.

Cisplatin-induced AKI, however, stands apart from other injury models. Neutrophil depletion in this context has repeatedly failed to produce a benefit. One study noted an association of increased IL-1β, IL-18, IL-6, and neutrophil infiltration with cisplatin-induced injury. However, when the activity of the cytokines was inhibited or removed or neutrophils were depleted, there was no reduction in kidney injury (129). Another study also observed that neutrophil depletion failed to prevent the increases in serum creatinine and BUN associated with cisplatin-induced injury (130). Interestingly, this study also investigated the role of neutrophils in the context of enhanced injury. The authors had previously reported that depletion of CD11c+ cells worsens cisplatin-associated injury (114). Thus, they performed a double-depletion of CD11c+ cells and neutrophils to determine if neutrophils were responsible for the additional level of injury. Again, though, they saw that neutrophil depletion provided no benefit.

An additional study that may provide evidence against a role for neutrophils in ischemic injury investigated the role of the NLRP3 inflammasome. NLRP3 is a major player in organizing the inflammatory response in a variety of conditions and helps coordinate the processing and production of molecules like IL-1β and IL-18. NLRP3 also has a role in promoting pyroptosis (131). While NLRP3-deficient mice had no protection from cisplatin-induced AKI, they were protected to some degree from ischemia (132). This protection from IRI was observed despite no change in neutrophil recruitment to the kidney. This observation merits further investigation to explore nuances in AKI pathogenesis. If neutrophils are indeed involved in the development of AKI in certain settings, it is possible that NLRP3 does not impact neutrophil trafficking but plays a role in disease-promoting properties of neutrophils or directly protects kidney cells from death via inhibiting pyroptosis. In short, while there is evidence against the involvement of neutrophils in AKI, there is also an array of data that indicates they play a role in multiple settings. It is clear our knowledge in this arena is still incomplete and there is a need for further, careful investigation.

F4/80+ Myeloid Cells: Macrophage, Monocyte, or Dendritic Cell?

The importance of infiltrating F4/80+ cells to AKI pathogenesis has proven a challenging element to resolve due to difficulties distinguishing between recruited monocytic cells and resident macrophage or DC populations. The distinction between macrophages and monocytes is often not clearly made and many studies conflate these populations. Further, there is frequent ambiguity about the delineation between DC, monocytes, and macrophages in the literature. This is a major source of confusion within the AKI field and has hindered our ability to draw precise conclusions.

Many methods for investigating the role of macrophages in disease also impact the monocytic or DC compartment in some fashion. As stated above, clodronate liposome, CD11b-DTR, and CD11c-DTR depletion models all have the capacity to deplete monocyte and DC populations, among others. Studies that manipulate trafficking signals such as integrins and selectins may also impair monocyte trafficking and other leukocytes in addition to the target cells of a given study.

Recent advances and development of new tools will hopefully allow clearer descriptions of distinct MPC populations' contributions during AKI. For example, it is now known that classical dendritic cells and their precursors express the ZBTB46 transcription factor while other myeloid lineages do not (94). Exploiting this discovery revealed that, indeed, only a small proportion of kidney-resident immune cells are DC (74, 94). This discovery has also led to the generation of ZBTB46-DTR mice for the specific depletion of DC (133), but this tool has yet to be used in models of AKI. The distinctions between monocytes and macrophages are still poorly defined since monocytes can transition into macrophages within tissue. Shared markers and closely related differentiation pathways continue to make it difficult to separate the biological contributions of macrophages and monocytes in living systems. Thus, limited means for specifically depleting or sequestering monocytes and macrophages has hindered investigations of these cells and their respective roles in AKI. Continued progress in this area will likely require the use of creative experimental systems, such as parabiotic models in which the skin of two mice is sutured together to allow for shared vasculature and circulating cells. Studies by Park et al. (79) and Lever et al. (71) have recently used this model and differential replacement kinetics following depletion to nicely investigate the role resident macrophages and monocyte/monocyte-derived macrophages in AKI. Their work showed that resident macrophages are minimally renewed by circulating cells, specifically express the V-domain Ig suppressor of T cell activation (VISTA) marker, and play an important role in recovery and repair following ischemic injury. These are powerful tools for distinguishing between resident and infiltrating cells in a given tissue. Use of additional markers to classify populations and subpopulations will also be beneficial for assessing myeloid cells present during kidney injury and some progress has been made in this regard.

An investigation of Tamm-Horsfall Protein's (THP) impact on macrophage regulation assessed myeloid subpopulations by sub-setting based on CD11b and MHC II (134). THP-deficient mice had lower proportions of $CD11b^{hi}$ myeloid cells at baseline while a $CD11b^{mid}$ MHC II^{hi} population was unchanged. The authors referred to the $CD11b^{hi}$ cells as macrophages, but they more likely represent monocytes or monocyte-derived macrophages that have infiltrated the kidney (73, 74). Conversely, the $CD11b^{mid}$ population most likely represents resident macrophages. Thus, there appeared to be a

specific defect in monocytic cells in the absence of THP while resident cells remained constant.

IRI in the THP-deficient setting revealed that THP-deficient mice had greater increases in kidney neutrophils but reduced accumulation of the CD11b^{hi} Ly6G^{lo} cells (presumably monocytic lineage) cells. THP-deficient mice also showed greater neutrophil presence in steady-state kidneys prior to IRI. Interestingly, macrophages in THP-KO mice were less phagocytic, as demonstrated by impaired uptake of liposomes. Logically, it is possible that impaired macrophage phagocytic activity could result in impaired clearance of debris and damaged cells and lead to continuous, low-level inflammation and neutrophil recruitment, but this will require further investigation. It has been shown, though, that lack of the EPO receptor on macrophages leads to impaired clearance of apoptotic cells and age-dependent immune cell infiltration and kidney disease (135); an observation that could support this hypothesis. Another striking aspect of the THP-deficient mice was a notable lack of colony stimulation factor-1 (CSF-1, also referred to as M-CSF) after AKI. CSF-1 is an important growth factor for the differentiation of macrophages from precursors and monocytes. This may in part explain the preferential accumulation of neutrophils over monocyte-derived macrophages.

AKI was not extensively assessed in this model, but serum creatinine steadily increased in THP-deficient mice post-IRI through the 72-h analysis period. Reconstituting THP-deficient mice with a bolus of exogenous THP at 24 h post-IRI produced a transient reduction in serum creatinine which then began to increase again by 72 h, presumably as the effect of the bolus diminished. This indicates a role for THP in dampening injury by potentially supporting monocyte differentiation and macrophage phagocytic function. However, it is also interesting to note that diminished monocyte recruitment in the absence of THP did not prevent increases in creatinine. This raises questions about the role of F4/80+ cell accumulation in AKI pathogenesis.

Studies investigating the role of heme oxygenase-1 (HO-1) in AKI have also used additional markers to assess myeloid populations (136, 137). HO-1 is an enzyme that is induced in response to the accumulation of free heme. Heme is an iron containing compound that normally associates with multiple proteins to form hemoproteins that regulate a wide variety of biological processes. In contrast to its beneficial role in hemoproteins, free heme that is not bound to a functional protein can catalyze the formation of reactive oxygen species, a major source of cell stress and damage during IRI (138). HO-1 is the rate-limiting enzyme for the conversion of free heme into biliverdin, carbon monoxide, and free iron (Fe^{2+}) and thus plays crucial cytoprotective roles in multiple tissue-injury settings (139, 140). Reperfusion following a period of ischemia can lead to an excess of free heme in the reperfused tissue and is a likely source of cellular toxicity during AKI.

A study performed with global HO-1-deficient mice employed the CD11b, MHC II, and F4/80 markers to assess myeloid subpopulations in greater detail (136). Mild ischemia (bilateral IRI, 10 min of ischemia) in global HO-1-deficient mice resulted in 60% mortality by day 2 post-IRI but was entirely sublethal in control mice, which highlights the important protective effects of HO-1. In the HO-1-deficient mice, kidney injury observed at day 1 post-ischemia was associated with a large increase in renal neutrophil representation (CD11b^{hi} MHC-II^{lo} Ly6G^{hi}). The actual numbers of total monocytes/macrophages (CD11b+ MHC II+) were not closely examined and the overwhelming predominance of the neutrophil population made it difficult to evaluate potential increases or decreases in this population based on percentages alone. However, within the CD11b+ MHC II+ population, there were two readily distinguishable subpopulations: CD11b^{hi} F4/80^{mid} and CD11b^{mid} F4/80^{hi}. The authors referred to the F4/80^{hi} population as DC, but the majority of this population has been shown to represent macrophages (74, 94). As referred to in Table 1, only a subset of DC expresses the F4/80 marker and this subset's expression level is lower than that of macrophages. The CD11b^{hi} cells are most likely monocyte-lineage cells that infiltrate and accumulate post-injury. Again, cell numbers of these 2 populations pre- and post-injury were not assessed, but HO-1-deficient mice exhibited an obvious skewing toward the CD11b^{hi} population when compared to WT mice. Thus, the absence of HO-1 leads to dramatic increases in neutrophilic inflammation and disruption of the macrophage compartment, even after mild ischemia.

The differences between global HO-1-deficient and WT mice and the importance of the observed disturbances in the myeloid compartments merit further investigation. HO-1 deficiency has dramatic impacts on tissue macrophage populations and the impacts of this during AKI are not yet understood. For example, macrophages in the spleen and liver are essentially depleted in HO-1-deficient mice and renal macrophages basally express high levels of the haptoglobin receptor CD163 (141). The skew toward CD11b^{hi} F4/80^{mid} cells could indicate basal fragility of resident macrophages and continual replacement by monocytes. It is still not fully known if adult monocyte-derived macrophages have phenotypic and functional alterations compared to true tissue-resident populations. Thus, if the majority of macrophages within the HO-1-deficient kidney are monocyte-derived, there is a possibility that their behavior during disease progression is altered. More detailed analysis of the resident cells in HO-1-deficient and WT mice will provide further insight into the phenotypic properties of tissue macrophages and their potential for promoting beneficial or detrimental processes.

Another study examined the role of HO-1 specifically in myeloid cells using a LysM-Cre conditional HO-1-knockout (137). These mice lack HO-1 expression specifically in LysM-expressing populations (predominantly macrophages, neutrophils, and monocytes) and do not suffer from the same degree of sensitivity to ischemia as global HO-1-deficiency. Thus, the mice were able to tolerate 26 min of IRI. While this ischemic time was not lethal, myeloid deletion of HO-1 was sufficient to cause increased levels of plasma creatinine and tubular injury in response to IRI when compared to littermate controls. Myeloid populations were broken into populations based on CD11b and F4/80 expression. Three populations were identified: CD11b^{hi} F4/80^{lo} (classified as neutrophils due to high Ly6G expression), CD11b^{hi} F4/80^{mid} (likely monocytes/monocyte-derived cells), and CD11b^{mid} F4/80^{hi} (mostly resident macrophages). Similar to

global HO-1 deletion, IRI led to dramatic increases in neutrophils at 24 h post-injury with essentially no changes detected in the other populations. However, HO-1 expression was significantly upregulated in the non-neutrophil populations. Thus, HO-1 expression in macrophage/monocyte populations constitutes an important protective response during kidney injury.

The authors investigated this further using a hemin pretreatment protocol. The hemin molecule is nearly identical to heme, with the major difference being hemin contains ferric iron (Fe^{3+}) rather than ferrous iron (Fe^{2+}). Hemin is a potent inducer of HO-1 and the authors exploited this ability to increase HO-1 levels prior to inducing injury. Pretreatment with hemin prevented increases in plasma creatinine and specifically increased HO-1 expression in the $CD11b^{hi}$ $F4/80^{mid}$ population following IRI. Interestingly, accumulation of the $CD11b^{hi}$ $F4/80^{mid}$ population in kidneys 24 h after IRI was also enhanced by the hemin treatment. Therefore, HO-1 induction, specifically in monocytic populations, may be an important anti-inflammatory element of renal protection during injury. This is also interesting due to the fact that HO-1 is induced in myeloid cells by reno-protective cholinergic stimulation (142, 143).

Several questions regarding HO-1 and myeloid cells still remain. Since global HO-1-KO mice are extraordinarily susceptible to injury and death and have basally disrupted macrophage populations, this extreme model may not be ideal for investigating nuanced questions in the future. It will be interesting to know how heme release and HO-1 expression evolve during IRI in WT mice that progress to lethal disease. Are heme processing and scavenging systems overwhelmed in the setting of severe or sustained injury? In addition, the monocyte compartment contains multiple subpopulations. These studies did not investigate if HO-1 is equally expressed by these subsets or the cells they may differentiate into within tissue. Additionally, the ability of HO-1 to modulate iron species could imply a connection with ferroptosis. Perhaps HO-1 provides support to the anti-ferroptotic activity of GPX4 during injury and helps lessen disease severity. This is an interesting line of inquiry with potential to yield increased understanding of AKI pathogenesis and promising therapies.

Previous work showed that manipulating chemokine receptors known to be involved in monocyte trafficking (CCR2, CX3CR1, CXCR4) can limit the increase in renal F4/80+ cells and provide protection during AKI (21, 144, 145). One of the studies on CCR2 and CX3CR1 included a nice assessment of surface marker expression to distinguish between resident and monocyte-derived populations and revealed that the drop in F4/80+ cell accumulation was due to prevention of monocyte infiltration (21). This work showed that removing the ability of cells to respond to either CCR2 or CX3CR1 was sufficient to prevent IRI-associated increases in serum creatinine within 24 h, thus implicating monocyte-derived cells in AKI and supporting a previous observation that the lack of CCR2 signaling was able to reduce ischemic kidney injury (145). The work with CXCR4 is interesting since this cytokine receptor provides homing and retention signals. Signaling through CXCR4 retains mature cells and precursors in the bone marrow and prevents their release into circulation (146–149). This study used a CXCR4 antagonist compound to interfere with this signal and found that, although circulating leukocytes were increased, myeloid infiltration of the kidney was decreased and injury was ameliorated (144). Thus, while blocking CXCR4 signals reduces bone marrow retention, it also limits homing to and infiltration of the kidney by inflammatory cells.

However, the role of monocytic cells in AKI remains unclear. The THP and HO-1 studies above seem to indicate that injury can progress without substantial monocyte infiltration and that monocytic cells may even contribute to protection. The trafficking studies, on the other hand, suggest that the prevention of homing to the kidney is an effective means to reduce injury. The absence of a tool for specifically depleting monocytes makes sorting out the impact of these cells a difficult and complex task which will require creative solutions. It should be noted, however, that neutrophils can also express CCR2, CX3CR1, and CXCR4 so any benefit attributed to reduced monocyte trafficking in studies involving these molecules could be due to additional effects on neutrophils (144, 150–153).

Overall, development of kidney injury is a heterogeneous process with multiple routes to disease. Different immune populations may play larger or smaller roles depending on the AKI-initiating event and this may be a source of confusion within the field. The involvement of neutrophils vs. monocytes requires further careful investigation to refine our knowledge base. The role of macrophages in recruiting these cells is still unsettled due to the inability of many models to specifically manipulate macrophages over other related cell types. There is likely a link between cell death and activation of resident populations, but there is much work to be done to prove this connection and define the role of additional myeloid populations that may be recruited via macrophage activation.

REGULATION THROUGH CSF1R DURING AKI

The CSF-1 receptor (also known as Csf1R, CD115, and c-Fms) is critical for macrophage development. Disruption of the *Csf1r* gene results in a near complete lack of F4/80+ cells in adult tissues (154). These mice are also osteopetrotic due to the lack of oscteoclasts, which creates complications for assessing the impact of CSF1R on bone marrow-derived monocytes. Egress of these cells into circulation is diminished in *Csf1r*-deficient mice. However, blocking CSF1R later in life with antibody treatment allows analysis of this receptor's impact without interfering with development. This approach has revealed that CSF1R activity is not required for the production of monocytes but does regulate their subsequent differentiation (155, 156). As monocytes mature, they transition from a $Ly6C^{hi}$ $CX3CR1^{lo}$ phenotype to a $Ly6C^{lo}$ CX3CR1+ phenotype (157). Blocking CSF1R leads to severe reductions in the $Ly6C^{lo}$ subset in circulation and tissue. Blockade also prevents the reconstitution of tissue macrophages by peripheral monocytes. Thus, the CSF1R appears to govern the differentiation pathways of $Ly6C^{hi}$ monocytes and the ability of monocytes to transition into macrophages.

The CSF1R has two distinct ligands that may differentially impact outcome during kidney injury. Knockout of one of

these ligands, IL-34, resulted in lesser F4/80+ cells in the kidneys following IRI (158). In IL-34-sufficient mice, F4/80+ cells were slightly increased by day 1 post-IRI and further accumulated at days 3 and 5. IL-34-deficient mice showed a similar pattern of accumulation but to a lesser overall degree at all time points. Reduced infiltration of F4/80+ cells was accompanied by lower levels of kidney injury molecule-1 (KIM-1), serum neutrophil gelatinase-associated lipocalin (NGAL), and urine albumin during early injury. At 20 days post-IRI and later, IL-34-deficient mice exhibited less severe fibrosis and better preservation of kidney architecture. Thus, IL-34 signaling appears to promote an inflammatory environment that favors worse injury, potentially through its action on macrophages and monocytes. Although absence of IL-34 does not completely prevent the accumulation of F4/80+ cells, lack of this cytokine was still sufficient to improve outcome. This supports the concept that while increases in F4/80+ cells is an indicator of disease, the molecules and signals the cells experience in the kidney are also important for determining their contribution.

A role for colony stimulating factor-1 (CSF-1), the second CSF1R ligand, was investigated in IRI and DT models of kidney injury (111). As referred to above, the DT model of disease relies on expression of the human DTR under control of a proximal tubule specific promoter (*Ggt1* promoter in this case). Since murine cells are insensitive to DT, injection of DT results in toxicity specifically to tubule cells which results in injury and decreased kidney function.

CSF-1 knockout also limited the accumulation of F4/80+ cells in the kidney following injury, reminiscent of the IL-34 deficient mice. Although the IL-34- and CSF-1-deficient models cannot be directly compared here, the degree of residual F4/80+ accumulation appeared greater in IL-34-deficient mice. Despite the potentially more profound lack to F4/80+ accumulation, the absence of CSF-1 signaling worsened early injury and delayed recovery. This directly contrasts with the protection observed with IL-34-deficiency. This study also probed the involvement of macrophage/dendritic cell (DC) populations during injury by performing depletions with clodronate and

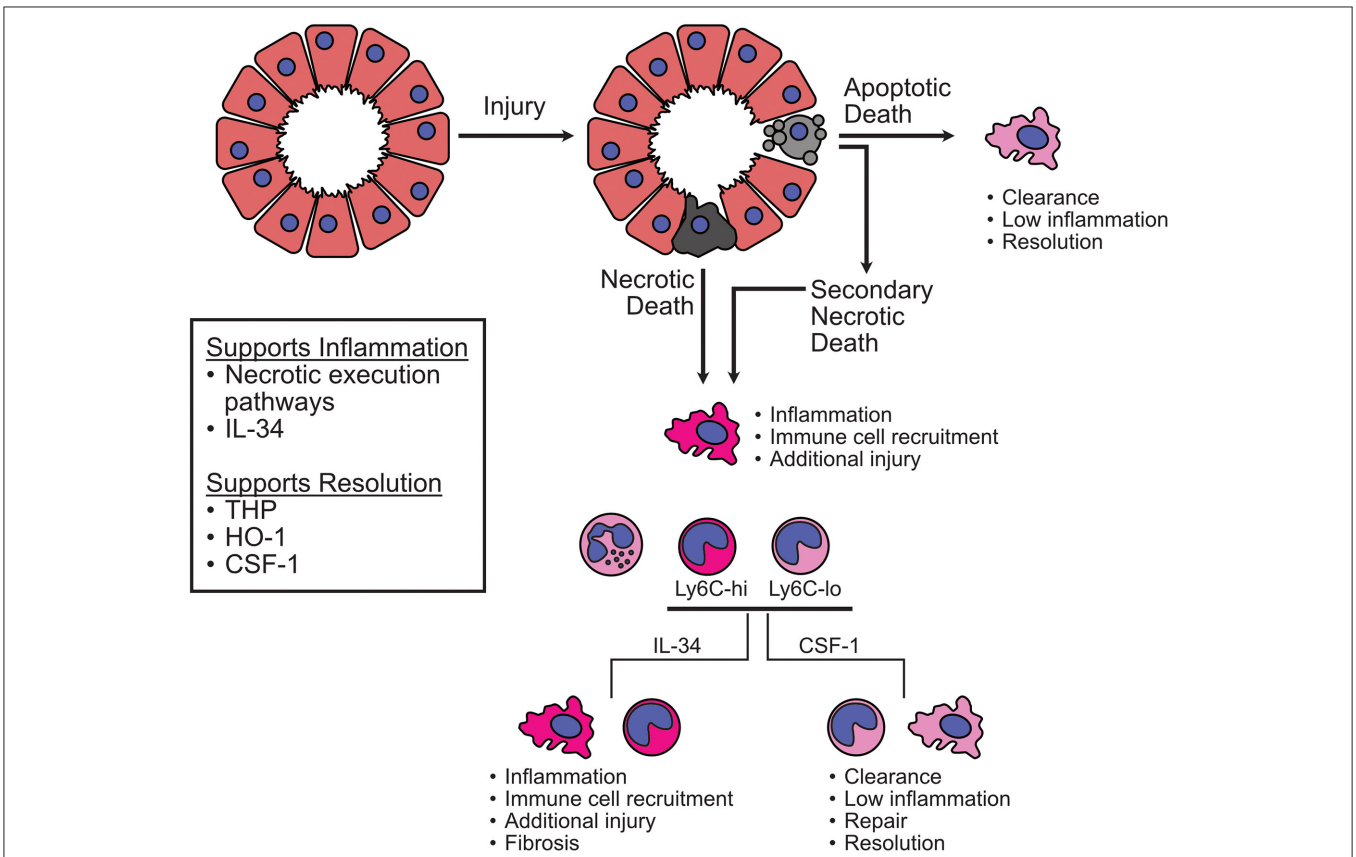

FIGURE 3 | Potential macrophage/monocyte responses to initial injury and regulation via CSF1R during AKI. After initial injury, cell death can occur via multiple pathways. Apoptotic cells can be cleared by efferocytosis and results in relatively low inflammation. If clearance is insufficient to resolve the injury, more inflammatory forms of cell death may pre-dominate, such as necroptosis or necrosis. Based on the evidence discussed in this review, inflammatory cell death can activate resident macrophages to produce proinflammatory molecules and recruit additional cells. This leads to an influx of neutrophils and monocytic cells. Monocytes can transition into additional macrophage populations whose activity is determined by signals from the environment. According to the studies discussed here, IL-34 supports more proinflammatory activity while CSF-1 supports more reparative activity. Interfering with the necroptotic pathway of cell death lessens injury-induced inflammation and promotes recovery. THP appears capable of enhancing the phagocytic function of macrophages, which supports clearance and resolution. HO-1 allows for the breakdown of toxic free heme to lessen injury and inflammation. Overall, a balance must be struck between interrupting progressive inflammation and injury while supporting clearance of damaged material and repair.

CD11c-DTR models. In both depletion strategies, the lack of macrophage/DC prevented overall F4/80+ cell accumulation in the kidney, but also worsened injury. Altogether, the data from these studies point to complex and opposing functions of monocyte/macrophage populations during kidney injury. The phagocytic and debris clearing properties of macrophages may be important for ameliorating the extent of initial injury. Macrophage-mediated inflammation in response to death of tissue cells, on the other hand, appears to drive the accumulation of additional F4/80+ cells (and likely neutrophils). The recruited myeloid populations may then contribute to either injury or recovery, depending on the signals they receive. According to the data currently available, IL-34 promotes proinflammatory activity and delayed recovery while CSF-1 skews cells toward tissue support and injury resolution phenotypes. Interestingly, tubules are a major source of CSF-1 in the kidney (112). This begs the question of whether tubule injury and death reduces the overall concentrations of CSF-1 by removing a cellular source of the cytokine and skews the environment toward IL-34 signaling—thereby limiting the beneficial functions of macrophages and promoting inflammation. These interesting findings merit additional, detailed studies to directly compare the impact of these molecules on monocyte/macrophage dynamics, phenotypic profile, and function in various models of kidney injury.

DISCUSSION

Clearly, the involvement of cell death, macrophage activation, inflammation, and immune cell infiltration in AKI disease is complex. Our understanding is steadily improving, but there are still key unknowns that need to be addressed. There is strong evidence for the importance of necrotic death pathways in promoting injury and disease, but different modes of death may have greater or lesser roles in specific disease models and may even work in concert during the development of injury. The impact of these death pathways on resident macrophages is an important knowledge gap that must be resolved, but anecdotal evidence suggests a connection between inflammatory death, immune recruitment, and disease severity which could center around macrophage activation.

However, depletion models also indicate macrophages may be involved to varying degrees depending on the injury model used. This divergence could also relate to the death pathways at play and we should work toward a unified understanding of injury models, death pathways, and macrophage/myeloid involvement. This information would be invaluable for properly assessing AKI patients and developing adaptive/adaptable treatment strategies. Given the diverse data that has been published in this field, it is important to remember that AKI represents a syndrome that can manifest from distinct insults that may produce variations on a theme of defining characteristics. It is likely that different manifestations will require more tailored therapies to address root causes and key differences.

Further investigation of myeloid populations is also warranted. The relevance of neutrophils to disease is still not clear despite many studies investigating their involvement. Again, injury model and cell death contexts should be carefully considered when evaluating the impact of neutrophils. Data shows that macrophages/monocytes are relevant to processes that contribute to both tissue damage and support. The dynamic nature of these cells and their ability to integrate environmental stimuli does not preclude contradictory roles, but defining the critical elements that dictate their impact during AKI will be a delicate endeavor requiring sophisticated experimental design. As the data stands now, beneficial processes appear to revolve around proper debris/dead cell clearance and prevention of excessive cell death. For example, the ability of THP to enhance macrophage phagocytic activity, HO-1 to limit heme-mediated ROS production and iron availability, and RIPK deficiency/blockade to reduce inflammatory cell death (**Figure 3**). Detrimental elements appear to stem from unrestrained inflammation in the context of sterile cell death. This is illustrated by the continuous accumulation of myeloid cells from early injury through later stages of disease. From this point of view, it is possible that IL-34 activity is designed to induce anti-pathogen activity in monocytes/macrophages and induction of this cytokine in response to danger signals results in the accumulation of an inappropriate cell type that causes additional damage in the absence of a pathogen to attack (**Figure 3**). Overall, additional studies employing sophisticated means of delineating the specific impacts of macrophages, monocytes, and DC are needed. The rise of high-dimensional single cell analysis techniques is making headway toward this goal and should yield interesting, informative data in the near future (70, 159, 160). We have entered a new era with exciting tools emerging that will help address these issues and provide much needed answers. More detailed analysis of important death pathways and myeloid populations in the context of AKI will enable pursuit of innovative and targeted treatment options for AKI patients. Ultimately, we must continue to ensure that our strategies for research analysis and therapy development are sufficiently advanced to address the complexity inherent to the AKI syndrome. This is chess, not checkers.

AUTHOR CONTRIBUTIONS

WN wrote and prepared the manuscript, table, and figures with input and revision from MO.

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Association Between Syndecan-1, Fluid Overload, and Progressive Acute Kidney Injury After Adult Cardiac Surgery

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Background: Acute kidney injury (AKI) is a common complication after cardiac surgery and the prognosis of AKI worsens with the increase in AKI severity. Syndecan-1(SDC-1) is a biomarker of endothelial glycocalyx degradation. Fluid overload (FO) is associated with poor outcomes in AKI patients and may be related to the damage of endothelial function. This study aimed at demonstrating the association between elevated SDC-1, FO, and AKI progression.

Methods: In this prospective study, we screened patients who underwent cardiac surgery and enrolled patients who experienced an AKI within 48 h after surgery from December 1, 2018 to January 31, 2019. Blood and urine samples were collected at the time of AKI diagnosis for plasma SDC-1 (pSDC-1) and urine SDC-1 (uSDC-1) measurements. Fluid balance (FB) = accumulated [fluid intake (L) - fluid output (L)]/body weight (kg) × 100%. FO was defined as FB > 5%. The primary endpoint was progressive AKI, defined as AKI progression from a lower to a higher stage. The patients were divided into progressive AKI group vs. non-progressive AKI group.

Results: The quartiles of pSDC-1 concentration (117.3 [67.4, 242.3] ng/mL) showed a graded association with the incidence of progressive AKI, ranging from 5.0, 11.9, 32.6 to 52.4% (p for trend <0.001). Multivariate logistic regression showed that increased pSDC-1 was an independent risk factor for progressive AKI. The AUC-ROC area of pSDC-1 concentration in predicting AKI progression was 0.847. Linear regression showed a positive correlation between FB and pSDC-1 concentration (R^2 = 0.384, p < 0.001). In patients with FO, the progressive AKI incidence was significantly higher in the high pSDC-1 (≥117.3 ng/mL) subgroup than in the low pSDC-1 subgroup (58.3 vs. 17.6%, OR = 9.167, P = 0.005). In patients without FO, the progressive AKI incidence was also significantly higher in the high pSDC-1 subgroup with a lower odds ratio (30.4 vs. 7.4%, OR = 6.714, P = 0.002).

Conclusion: Elevated pSDC-1 concentration was associated with progressive AKI after cardiac surgery and showed good predictive ability for progressive AKI. FB was related to the increase of pSDC-1. The interaction between pSDC-1 and FB may further aggravate the progression of AKI.

Keywords: syndecan-1, fluid overload, acute kidney injury, cardiac surgery, risk factor

INTRODUCTION

The incidence of cardiac surgery-associated acute kidney injury (CSA-AKI) varies from 20 to 40% depending on its definition, and the mortality was reported to be between 15 and 30% (1, 2). The prognosis of AKI worsens with the increase in AKI severity. When AKI progresses to stage 3 or renal replacement therapy (RRT), the mortality is as high as 50–80%, and has a higher risk of long-term end-stage renal disease and death (1, 3). Therefore, it is necessary to explore early biomarkers for the prediction of progressive AKI.

Syndecan-1 (SDC-1), a member of the syndecan family, is a protective layer for covering the endothelium (4). It has been demonstrated that SDC-1 is a biomarker for endothelial glycocalyx degradation (5, 6). Rehm et al. provided the first evidence in humans to the shedding of the endothelial glycocalyx during global or regional ischemia/reperfusion (I/R) procedures in major vascular surgery (7). Studies have shown that elevated serum and urinary SDC-1 is a potential biomarker in predicting renal dysfunction and mortality in patients with AKI after cardiac surgery (8, 9).

Fluid overload has been demonstrated as an important risk factor for AKI development and is associated with poor outcomes in critical patients (10, 11). In our previous study, we found that 30-day mortality was significantly higher in the fluid overload group in AKI-RRT patients after cardiac surgery (12). Moreover, it was revealed that in patients who underwent elective surgery and those with severe sepsis, hypervolemia could cause intravascular changes in volume and pressure, thereby destroying the endothelial glycocalyx, with a significant elevation of serum and urinary syndecan-1 (13, 14).

In brief, it is known that SDC-1 can predict AKI occurrence, but little or no studies have demonstrated the association between SDC-1 and AKI progression. It is also unknown whether fluid overload has an effect on this. The aim of this study therefore was to investigate the relationship between glycocalyx degradation (measured as SDC-1), fluid overload, and progressive AKI in adult patients following cardiac surgery, thereby identifying new strategies for the prevention of CSA-AKI.

METHODS

Patient Selection

This was a single-center, prospective study. We screened 721 patients who underwent cardiac surgery in our hospital from December 1, 2018, to January 31, 2019, and collected data of patients who developed AKI during this period. The exclusion criteria and study flowchart are shown in **Figure 1**. The Ethical Committee of our hospital approved this study (No. B2017-039).

Definitions

AKI was defined and graded based on the Kidney Disease Improvement Global Outcomes (KDIGO) 2012 guidelines (15) within 7 days after surgery. Progressive AKI was defined as the worsening of an established AKI stage (16, 17): from AKI stage 1 to stage 2 or 3 or from AKI stage 2–3 during hospitalization. We defined fluid balance (FB) using the equation,

$$FB = (fluid\ intake\ (L) - fluid\ output\ (L))/admission\ weight\ (kg) \times 100\%$$

Fluid overload (FO) was defined as FB > 5% (18).

SCr measured in the ICU was adjusted by the following formula (19–21):

$$\begin{aligned} Adjusted\ SCr &= SCr \times correction\ factor, \\ \text{Where, } Correction\ factor &= [admission\ weight\ (kg) \times 0.6 \\ &\quad + cumulative\ (fluid\ intake\ (L) - fluid\ output\ (L)]/admission\ weight \times 0.6. \end{aligned}$$

Complete renal recovery was defined as the ADQI criteria (22): SCr at discharge returning to 50% above baseline SCr.

Study Design

All patients were admitted to the Intensive Care Unit (ICU) regularly after anesthesia resuscitation. The urine output was recorded every 6 h from the urine collection bag, and fluid intake and output (including fluid removed by drain tube, blood lost from hemorrhage, and urine output) were recorded every day until they were discharged from the ICU. The SCr was monitored every morning. We screened each patient every morning within 48 h after surgery, and AKI was diagnosed according to the KDIGO criteria. Blood and urine samples of the AKI patients were collected at the time of AKI diagnosis for the SDC-1 measurements, and the cumulative fluid balance was calculated from the time of operation to the time of AKI diagnosis.

The main endpoint was progressive AKI. The secondary endpoints were RRT rate, complete renal recovery rate, 28-d mortality, in-hospital mortality, length of ICU stay and hospital stay.

Measurement of Syndecan-1

Fresh blood and urine samples were centrifuged at 3,000 rpm for 10 min, after which the separate plasma and urine supernatant were collected and stored at −80°C. The pSDC-1

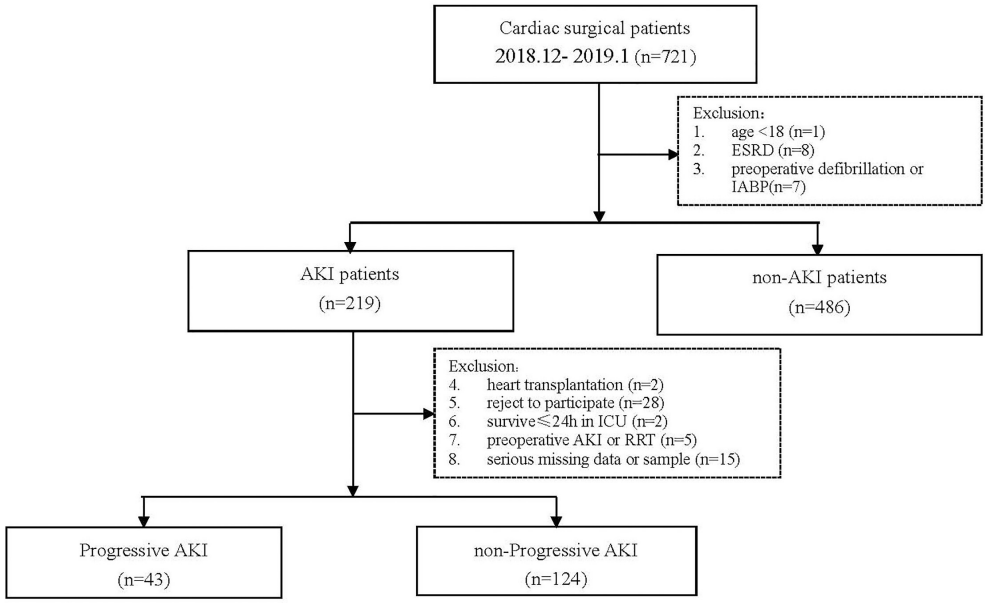

FIGURE 1 | Study flowchart.

TABLE 1 | Baseline characteristics in progressive AKI vs. non-progressive AKI groups.

	All ***n* = 167**	**Non-progressive AKI group** ***n* = 124**	**Progressive AKI group** ***n* = 43**	***P***
Preoperative				
Male [*n* (%)]	117 (70.1%)	89 (71.8%)	28 (65.1%)	0.411
Age	60 ± 12	59 ± 12	61 ± 13	0.358
Weight (kg)	69.3 ± 13.5	69.9 ± 14.0	67.5 ± 11.9	0.316
BMI(kg/m^2)	24.8 ± 3.9	25.1 ± 4.0	23.8 ± 3.4	0.058
Hypertension [*n* (%)]	81 (48.5%)	56 (45.2%)	25 (58.1%)	0.142
Diabetes [*n* (%)]	15 (9.0%)	11 (8.9%)	4 (9.3%)	0.932
History of cardiac surgery [*n* (%)]	17 (10.2%)	14 (11.3%)	3 (7.0%)	0.420
NYHA>II [*n* (%)]	80 (47.9%)	61 (49.2%)	19 (44.2%)	0.571
Coronary angiography[*n* (%)]	102 (61.1%)	72 (58.1%)	30 (69.8%)	0.175
Contrast medium (ml)	57 ± 19	53 ± 14	69 ± 27	0.002
BUN(mmol/L)	7.8 ± 3.1	7.9 ± 3.3	7.6 ± 2.6	0.589
SCr(μmol/L)	96.6 ± 30.2	94.8 ± 28.3	102.4 ± 34.9	0.179
eGFR[ml/(min/1.73 m^2)]	77.6 ± 20.6	79.6 ± 20.7	70.5 ± 17.9	0.009
eGFR < 60 ml [*n* (%)]	33 (19.8%)	19 (15.3%)	13 (30.2%)	0.032
Albumin (g/L)	39.6 ± 5.7	39.4 ± 6.1	40.3 ± 4.2	0.372
Proteinuria [*n* (%)]	22 (13.2%)	14 (11.3%)	8 (18.6%)	0.221
Intra-operative				
Type of surgery				0.262
- Valve	81 (48.5%)	64 (51.6%)	18 (41.9%)	
- coronary artery bypass graft	29 (17.4%)	23 (18.5%)	6 (13.9%)	
- large vessels	36 (21.6%)	24 (19.4%)	12 (27.9%)	
- Others	21 (12.6%)	13 (10.5%)	8 (18.6%)	
Use of CPB [*n* (%)]	145 (86.8%)	104 (83.9%)	41 (95.3%)	0.055
CPB duration (min)	128 ± 75	112 ± 65	175 ± 84	<0.001
Aortic clamping duration (min)	71 ± 38	66 ± 37	87 ± 38	0.002
Ultrafiltration volume (ml)	2,628 ± 1,492	2,413 ± 1,436	3,247 ± 1,492	0.001

BMI, body mass index; NYHA, New York Heart Association; BUN, blood urea nitrogen; SCr, serum creatinine; eGFR, estimated glomerular filtration rate; CPB, cardiopulmonary bypass; FB, fluid balance.

and uSDC-1 concentrations were measured within 6 months after surgery, using sCD138 ELISA kits (Human Syndecan 1, Abcam) according to the manufacturer's instructions (13, 23, 24). SDC-1 is also known as CD138, and soluble CD138 (sCD138) means SDC-1 in the circulation or body fluid. The detection range was 8–256 ng/mL, and the coefficient of variation was 6.2%.

Statistical Analysis

To estimate the sample size for this study, we applied our unpublished data from a pre-test of 20 AKI patients for the pSDC-1 concentration showing a 2-fold increase in patients with progressive AKI compared with those who did not progress. The coefficient of variation of pSDC-1 was 0.800. Based on these parameters and taking into account the design effect of consecutive sampling (1.5-fold), a minimum of 35 patients was needed to achieve a 90% power and significance level (alpha) of 0.05 in detecting a 2-fold increase in pSDC-1 concentration in progressive AKI patients.

Continuous variables were expressed as mean ± SD or median and interquartile range, and were compared using independent sample *t*-test or Mann-Whitney test. Categorical variables were expressed as proportions and compared using the chi-square or Fisher exact test. $P < 0.05$ were considered statistically significant. The correlation between fluid balance and syndecan-1 concentrations was assessed using linear regression. We included the variables with a $p < 0.10$ from the baseline characteristics table into the univariate regression analyses to identify risk factors for AKI progression, and those with $p < 0.05$ were further included in the multivariate analysis. The area under the curve receiver operating characteristic (AUC-ROC) was used to analyze the predictive value of syndecan-1 for AKI progression. SPSS 22.0 software (IBM Corporation, Armonk, NY, USA) was used for overall statistical analysis.

RESULTS

Baseline Characteristics

Finally, 167 AKI patients were enrolled in the analysis, including 43 (25.7%) in the progressive AKI group and 124 (74.3%) in the non-progressive AKI group. The preoperative and intraoperative conditions of the two groups are shown in **Table 1**. There were no significant differences in gender, age, or weight between the two groups. The body mass index (BMI) in the progressive

TABLE 2 | Postoperative variables and shot-term outcomes in progressive AKI vs. non-progressive AKI groups.

	All *n* = 167	Non-progressive AKI group *n* = 124	Progressive AKI group *n* = 43	*P*
Postoperative (ICU admission)				
APACHE II score	11.4 ± 5.5	10.2 ± 4.9	15.0 ± 5.7	<0.001
Euro Score	4.7 ± 2.7	4.5 ± 2.6	5.3 ± 2.8	0.091
Postoperative (at AKI diagnosis)				
AKI stage (initial)				0.467
- Stage 1	135 (80.8%)	102 (82.3%)	33 (76.7%)	
- Stage 2	24 (14.4%)	16 (12.9%)	8 (18.6%)	
- Stage 3	8 (4.8%)	6 (4.8%)	2 (4.7%)	
pSDC-1(ng/ml)	117.3 [67.4, 242.3]	96.6 [51.4, 150.6]	245.2 [78.9, 290.5]	<0.001
uSDC-1(ng/ml)	54.2 [34.9, 110.2]	52.1 [22.3, 108.9]	86.8 [42.3, 148.7]	0.193
FB(%)	3.4 ± 2.8	2.5 ± 1.8	6.0 ± 3.6	<0.001
Fluid overload [n(%)]	53 (31.7%)	28 (22.6%)	25 (58.1%)	<0.001
Lactic acid (mmol/L)	4.3 ± 3.5	3.5 ± 2.4	6.6 ± 4.8	<0.001
PCT (ng/ml)	1.3 [0.5, 4.9]	1.1 [0.4, 4.2]	3.9 [2.7, 7.8]	0.006
Outcomes				
AKI stage (worst)				<0.001
- Stage 1	102 (61.1%)	102 (82.3%)	0	
- Stage 2	47 (28.1%)	16 (12.9%)	31 (72.1%)	
- Stage 3	18 (10.8%)	6 (4.8%)	12 (27.9%)	
RRT [*n*(%)]	17 (10.2%)	6 (4.8%)	11 (25.6%)	<0.001
Complete renal recovery [*n*(%)]	133 (79.6%)	104 (83.9%)	29 (67.4%)	0.021
Length of hospital stay (d)	17 ± 8	15 ± 7	21 ± 9	<0.001
Length of ICU stay (d)	4 [2, 6]	3 [2, 4]	5 [4, 16]	<0.001
Mechanical ventilation days	2 [1, 3]	2 [1, 3]	3 [2, 10]	0.003
In-hospital mortality [*n* (%)]	6 (3.6%)	1 (0.8%)	5 (11.6%)	0.001
28-d mortality [*n* (%)]	4 (2.4%)	1 (0.8%)	3 (7.0%)	0.022

APACHE, acute physiology and chronic health evaluation; AKI, acute kidney injury; FB, fluid balance; PCT, procalcitonin; RRT, renal replacement treatment; ICU, intensive care unit.

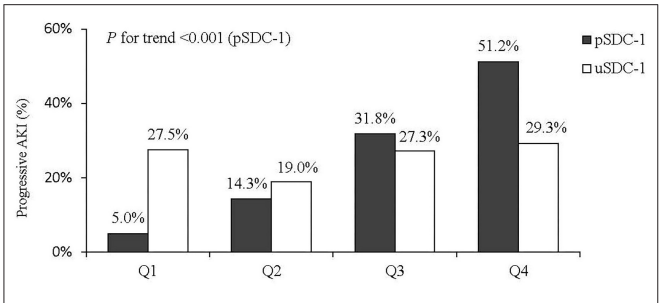

FIGURE 2 | Quartiles of pSDC-1 and uSDC-1 concentration and AKI progression.

TABLE 3 | Logistic regression analyze the risk factors for AKI progression.

	OR (*95%CI*)	*P*
Univariate		
BMI	0.908 (0.787–1.047)	0.183
Contrast medium	1.025 (0.997–1.054)	0.086
Baseline eGFR < 60 ml/(min/1.73 m^2)	1.886 (0.941–3.944)	0.016
CPB duration	1.018 (1.007–1.030)	0.002
Aortic clamping duration	1.033 (1.012–1.054)	0.002
Intraoperative ultrafiltration volume	1.001 (1.000–1.001)	0.039
APACHE II score	1.209 (1.083–1.350)	0.001
Euro score	1.067 (0.891–1.277)	0.480
pSDC-1	1.012 (1.006–1.018)	<0.001
Fluid overload (Y/N)	4.200 (1.350–13.065)	0.013
Lactic acid (mmol/L)	1.274 (1.087–1.493)	0.003
PCT (ng/ml)	1.064 (0.998–1.133)	0.056
Multivariate		
CPB duration	1.014 (1.002–1.046)	0.026
APACHE II on ICU admission	1.201 (1.013–1.437)	0.046
pSDC-1	1.030 (1.001–1.068)	0.020

BMI, body mass index; NYHA, New York Heart Association; eGFR, estimated glomerular filtration rate; APACHE, Acute Physiology and Chronic Health Evaluation; PCT, procalcitonin.

AKI group was significantly lower than that in the non-progressive AKI group. The estimated glomerular filtration rate (eGFR) in the progressive AKI group was significantly lower than that in the non-progressive AKI group. The type of surgery was comparable between the two groups. The CPB duration and aortic clamping duration in the progressive AKI group were significantly longer than in the non-progressive AKI group.

Postoperative Variables and Short-Term Outcomes

The interquartile pSDC-1 and uSDC-1 concentrations in all patients were 117.3 [67.4, 242.3] ng/mL and 54.2 [34.9, 110.2] ng/mL, respectively. The pSDC-1 concentration in the progressive AKI group was significantly higher than that in the non-progressive AKI group (245.2[78.9, 290.5] vs. 96.6[51.4, 150.6] ng/mL, $p < 0.001$). There were no significant differences in uSDC-1 concentration between the two groups (86.8 [42.3, 148.7] vs. 52.1 [22.3, 108.9] ng/mL, $p = 0.193$). The accumulated FB in the progressive AKI group was significantly higher (6.0 ± 3.6 vs. 2.5 ± 1.8, $p < 0.001$). The lactic acid and procalcitonin (PCT) levels in the progressive AKI group were significantly higher as well **(Table 2)**.

In the progressive AKI group, there were 8 patients with stage 2 progressed to stage 3, and 31 patients with stage 1 progressed to stage 2, and 2 patients with stage 1 progressed to stage 3. Significantly more patients received RRT treatment in the progressive AKI group (25.6 vs. 4.8%, $p < 0.001$). The length of hospital and ICU stay, and mechanical ventilation duration in the progressive AKI group were significantly longer than in the non-progressive AKI group. The 28-d mortality and in-hospital mortality was significantly higher in the progressive AKI group than in the non-progressive AKI group (7.0 vs. 0.8%, $p = 0.022$; 11.6 vs. 0.8%, $p = 0.001$) **(Table 2)**.

Syndecan-1 and Progressive AKI

Associations between progressive AKI and the pSDC-1 and uSDC-1 concentrations were categorized into quartiles **(Figure 2)**. The quartiles of pSDC-1 concentration (117.3 [67.4, 242.3] ng/mL) showed a graded association with the incidence of progressive AKI, ranging from 5.0, 11.9, 32.6 to 52.4% (*P* for trend < 0.001). We divided patients into high pSDC-1 group ($n = 82$) and low pSDC-1 group ($n = 85$) according to the median (117.3 ng/mL). The progressive AKI incidence in the high pSDC-1 group was greater than that in the low pSDC-1 group (42.7 vs. 9.4%, $p < 0.001$).

Logistic Regression Analysis for the Risk Factors of Progressive AKI

The univariate logistic regression showed that baseline eGFR < 60 mL/min/1.73 m^2, increased CPB duration, aortic clamping duration, intraoperative ultrafiltration volume, APACHE II score, Euro Score, pSDC-1 concentration, fluid overload, lactic acid levels, and PCT were risk factors for progressive AKI **(Table 3)**. In multivariate logistic regression, the independent risk factors for progressive AKI included increased CPB duration (OR = 1.014, 95% CI: 1.002–1.046), increased APACHE II on ICU admission (OR = 1.201, 95% CI: 1.013–1.437), and increased pSDC-1 concentration (OR = 1.030, 95% CI: 1.001–1.068) **(Table 3)**.

The AUC-ROC areas for increased pSDC-1 concentration to predict AKI progression and 28-d mortality were 0.847 and 0.892. The cut-off values were 123.8 ng/ml (with the sensitivity 0.897 and the specificity 0.652) and 277.2 ng/ml (with the sensitivity 0.667 and the specificity 0.883), respectively.

Syndecan-1 and Fluid Overload

Linear regression showed a positive correlation between FB and pSDC-1 concentration in all AKI patients ($R^2 = 0.358$, $p < 0.001$) and in the FO group ($R^2 = 0.360$, $p < 0.001$). In the non-FO group, there was no significant

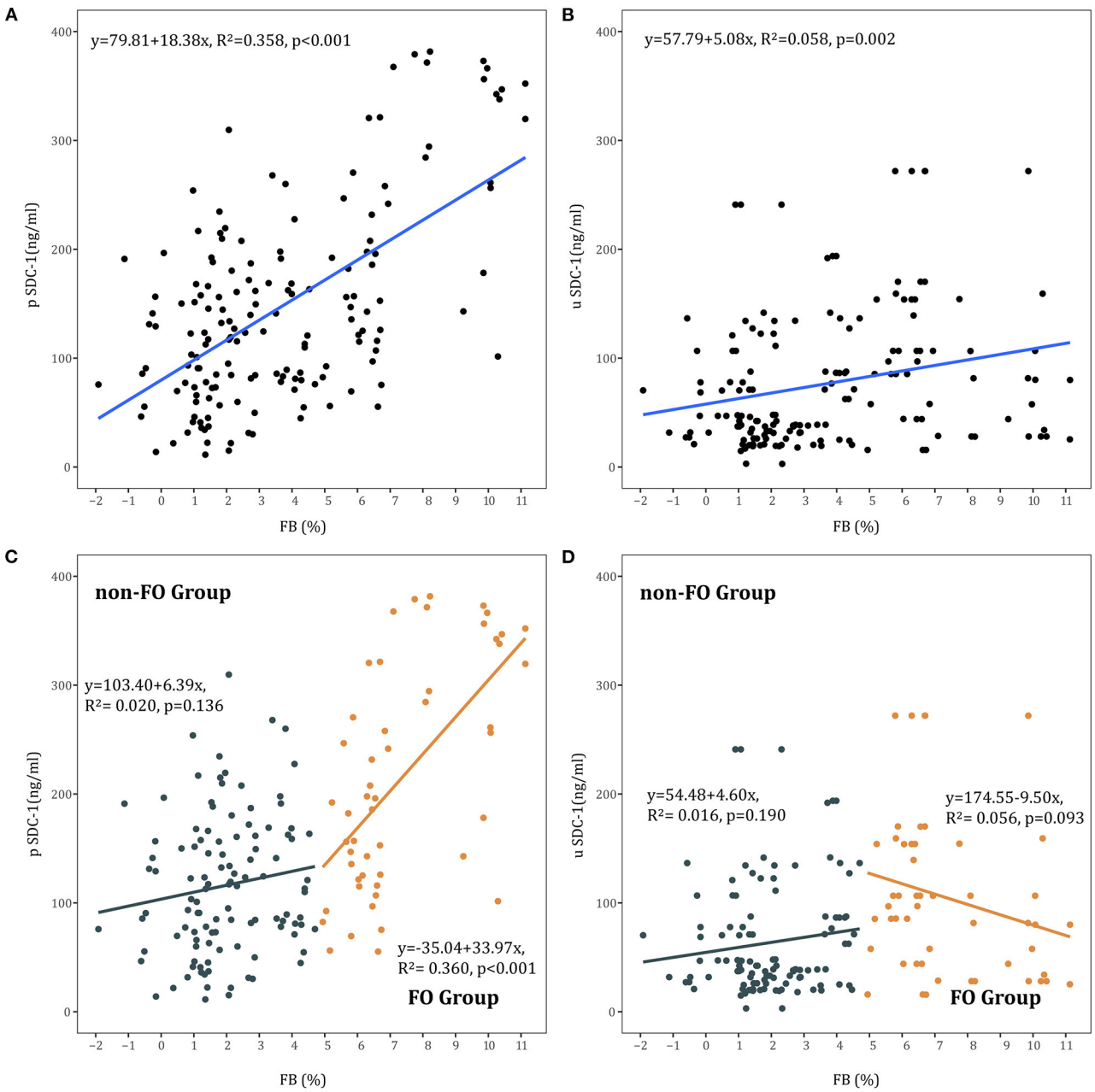

FIGURE 3 | (A) Association between FB and pSDC-1 in all AKI patients. **(B)** Association between FB and uSDC-1 in all AKI patients. **(C)** Association between FB and pSDC-1 in non-FO group and FO groups. **(D)** Association between FB and uSDC-1 in non-FO group and FO groups.

correlation between FB and pSDC-1 concentration ($R^2 = 0.020$, $p = 0.136$) (**Figures 3A,C**).

Moreover, linear regression showed a positive correlation between FB and uSDC-1 concentration in all AKI patients ($R^2 = 0.058$, $p = 0.002$). There was no correlation between FB and uSDC-1 concentration in the FO and non-FO groups, respectively (**Figures 3B,D**).

We further performed a logistic regression analysis to understand the potential risk factors for high pSDC-1 (≥117.3 ng/mL). It was showed that FB was the only risk factor for high pSDC-1 (OR = 1.531, 95% CI: 1.141–2.054) (**Table 4**).

Syndecan-1, Fluid Overload, and Progressive AKI

The progressive AKI incidence was highest in the FO & high pSDC-1 group (58.3%, $n = 21$), followed by the non-FO & high pSDC-1 group (30.4%, $n = 14$), FO & low pSDC-1 group (17.6%, $n = 3$), and non-FO & low pSDC-1 group (7.4%, $n = 5$) (**Figure 4**).

TABLE 4 | Logistic regression analyze the risk factors for High pSDC-1 (≥117.3 ng/ml).

	OR (95%CI)	P
Univariate		
Baseline eGFR [ml/(min/1.73 m^2)]	0.996 (0.984–1.021)	0.531
CPB duration	1.012 (1.004–1.020)	0.004
Aortic clamping duration	1.009 (0.997–1.016)	0.125
APACHE II score	1.124 (1.026–1.232)	0.012
Euro score	1.160 (0.979–1.376)	0.087
FB (%)	1.586 (1.258–2.010)	<0.001
Lactic acid (mmol/L)	1.174 (1.005–1.371)	0.043
PCT (ng/ml)	1.112 (1.006–1.254)	0.048
Multivariate		
FB (%)	1.531 (1.141–2.054)	0.005

eGFR, estimated glomerular filtration rate; APACHE, Acute Physiology and Chronic Health Evaluation; FB, fluid balance; PCT, procalcitonin.

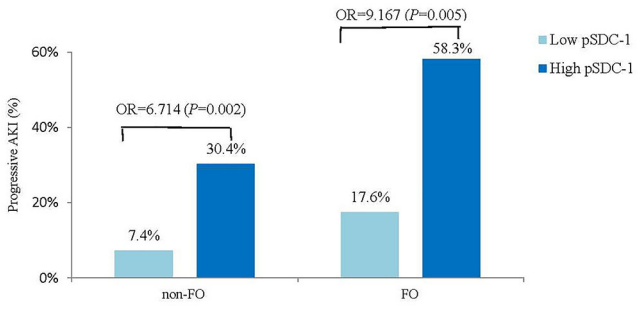

FIGURE 4 | Compare the progressive AKI incidence between high vs. low pSDC-1 in non-FO vs. FO groups.

In the FO group, the progressive AKI incidence was significantly higher in the high pSDC-1 subgroup than in the low pSDC-1 subgroup (58.3 vs. 17.6%, OR = 9.167, $p = 0.005$). In the non-FO group, the progressive AKI incidence was also significantly higher in the high pSDC-1 subgroup with a lower odds ratio (30.4 vs. 7.4%, OR = 6.714, $p = 0.002$) (**Figure 4**).

DISCUSSION

In this study, we found that elevated pSDC-1 concentration measured at AKI diagnosis was associated with progressive AKI after cardiac surgery. Moreover, pSDC-1 alone showed a good predictive ability for progressive AKI with an AUC-ROC of 0.847. We also found a positive correlation between FB and pSDC-1 concentration, and the interaction between pSDC-1 and FB may further aggravate AKI, as the odds ratio of elevated pSDC-1 for progressive AKI was higher in the FO group than in the non-FO group.

Our findings had two possible explanations. First, cardiac surgery-induced I/R injury results in damage to the endothelial glycocalyx and increased SDC-1 (25), and it has been demonstrated that there is a relationship between the severity of I/R injury and the extent of endothelial activation (26). In our previous findings in mice, I/R induced shedding of SDC-1 from the kidney resulted in the elevation of serum SDC-1 level (27). Therefore, our assumption was that for AKI patients, persistent I/R injury may continue to increase the pSDC-1 concentration. This may be why the pSDC-1 concentration in the progressive AKI group was significantly higher than that in the non-progressive AKI group. Inflammation also plays an important role. Our study found in the univariate logistic regression (**Table 4**) that PCT concentration was one of the risk factors for elevated pSDC-1. CPB induces a systemic inflammatory reaction, and inflammatory cytokines (IL-6, IL-8, and IL-10) were associated with glycocalyx degradation, measured as plasma syndecan-1 concentrations (28). As endothelial cells serve as the first barrier to prevent inflammation, SDC-1 degradation results in increased vessel wall permeability, enhanced adhesion of leukocytes, and increased perivascular inflammation (4, 5, 29). Thus, the interaction between inflammation and SDC-1 may lead to a vicious cycle, which aggravates the pathological process in the renal.

This confirms that positive fluid balance is associated with worse outcomes (10–12). Patients are prone to receive fluid resuscitation after cardiac surgeries due to hemodynamic instability or low cardiac output syndrome. In the present study, we found a positive correlation between fluid balance, and pSDC-1 and uSDC-1 in AKI patients. SDC-1 contributed to maintaining cell shape and structure by regulating the integrin and tight junction proteins (Occludin and ZO-1) (30, 31). Hypervolemia may stretch the vascular wall and worsen vascular permeability, possibly by atrial natriuretic peptide-induced damage to the glycocalyx (32). Chappel (13) and Puskarich (14) found that in patients who received elective surgery and those with severe sepsis, hypervolemia increased syndecan-1. Furthermore, in our multivariate logistic regression, FB was the only risk factor for elevated pSDC-1, which surpassed the CPB duration and PCT.

Another important result was that, although the progressive AKI incidence was significantly higher in the high pSDC-1 subgroup than in the low pSDC-1 subgroup in both FO and non-FO groups, the odds ratio was higher in the FO group. This means that the elevated pSDC-1 combined with fluid overload may further aggravate AKI. It is not new that fluid overload leads to AKI progression and worse outcomes. The present study showed that the number of patients with fluid overload in the progressive AKI group was significantly higher (58.1 vs. 22.6%, $p < 0.001$). Our previous study found that both excessively negative and positive accumulative 48-h FBs after cardiac surgery increased the risk of AKI progression (33). Patients with fluid overload tend to have more severe endothelial dysfunction with shedding of the glycocalyx and subsequent capillary leakage (13). This may cause intravascular hypovolemia, thereby prompting the need for more fluid administration. Furthermore, there has been an interconnected relationship between AKI and FO (34). It is very important to understand this vicious circle between AKI and fluid and endothelial dysfunction.

We chose 5% as the definition of fluid overload because fluid administration is relatively strict in our center. Our previous study showed that the cumulative fluid balance within 24 h after surgery was 0.7% (−0.4 to 1.9%) in all surgery patients (33). In the present study, the FB from surgery to AKI diagnosis was 3.4 ± 2.8%, and there were only 1/3 of patients with FB > 5%. Furthermore, studies have demonstrated that FB > 10% is associated with adverse outcomes, the variables were more severe by receiving RRT and the adverse event referred to mortality (35, 36), while our study was AKI progression.

Shed SDC-1 in blood can be filtered by glomeruli and detected in urine (37). However, in our present study, only the quartiles of pSDC-1 concentration showed a graded association with the incidence of progressive AKI, ranging from 5.0 to 52.4%, while this trend was not observed for uSDC-1. It's very interesting that the difference between plasma and urine SDC-1 seems to predict progressive AKI (as in **Figure 2**, pSDC - uSDC <0 in Q1 and Q2, but >0 in Q3 and Q4). This seems likely due to shifting relationship between the gradient for filtration and the GFR, as with mild AKI, GFR is intact and high pSDC is filtered and excreted, but with loss of GFR at high pSDC, the uSDC drops as it is no longer filtered or excreted.

Our study has some limitations. First, although it was a prospective study, it was also a single-center study with small samples, which needs further confirmation. Second, the samples were evaluated at the time of AKI diagnosis which was highly variable. Although we tried the best to make the diagnosis as early as possible (e.g., the urine output was recorded every 6 h after surgery, and the SCr was monitored at least once every day), it is still hard to diagnosis at the earliest time for each patient. Third, we adjusted the SCr for fluid balance in all samples, and fluid overload may not the only reason for artifactually low creatinines after cardiac surgery. In some centers and some patients ultrafiltration is performed during CPB, and this results in reduced creatinine. Fourth, although ELISA tests have previously been used to measure syndecan-1 concentration in urine samples (13, 24), the measurement of urinary glycocalyx is still not very clear, thereby prompting more studies for more accurate measurement modalities.

CONCLUSION

Elevated pSDC-1 concentration measured at AKI diagnosis was associated with progressive AKI after cardiac surgery, and pSDC-1 can predict progressive AKI well. The interaction between pSDC-1 and FO may further aggravate AKI. This may represent a potential biomarker to identify the risk of AKI progression after cardiac surgery and highlights the risk of fluid overload in the treatment of CSA-AKI.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and was approved by the Ethical Committee of Zhongshan Hospital affiliated to Fudan University (Approval No. B2018-175, Principal Investigator: XD).

AUTHOR CONTRIBUTIONS

JX did the study design, collected samples, extracted data, performed the analysis and drafted the manuscript. WJ and YL extracted data, performed the analysis, and revised the manuscript. JT, XX, and XD conceived the idea, did the study design and participated in manuscript revision. XG and JH performed the experiment and helped to generate figures. HL and XC collected samples and data and performed the literature research. JH, BS, YF, and YW helped to do the study design and revise the manuscript. CW, GT, and ZL provided patients and participated in manuscript revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.648397/full#supplementary-material

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Complement Activation Is Associated With Crescents in IgA Nephropathy

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Introduction: Crescents, especially those found at a percentage greater than 50%, are often associated with rapid progression of kidney disease in IgA nephropathy (IgAN). The mechanism of crescents forming in IgAN is still unclear. In this study, we aimed to evaluate whether excess complement activation participates in the formation of crescents in IgAN.

Methods: One hundred IgAN patients with various proportions of crescents—24 with 1%–24%, 27 with 25%–49%, 21 with 50%–74% 12 with more than 75%, and 16 without crescents—were included. Urinary concentrations of mannose-binding lectin (MBL), Bb, C4d, C3a, C5a, and soluble C5b-9 (sC5b-9) were measured at the time of biopsy. Receiver operating characteristic (ROC) curves were performed to evaluate predictive ability of renal survival for urine complement activation. In addition, historical C4d, C5b-9, and C3d were stained by immunohistochemistry.

Results: IgAN patients with more than 50% crescent formation showed higher complement activation levels than the other patients (urinary C3a/creatinine (C3a/Cr): 6.7295 ng/mg, interquartile range (IQR) 1.4652–62.1086 ng/mg vs. 0.1055 ng/mg, IQR 0–1.4089 ng/mg; urinary C5a/Cr: 15.6202 ng/mg, 4.3127–66.7347 ng/mg vs. 0.3280 ng/mg, IQR 0.0859–2.4439 ng/mg; urinary sC5b-9/Cr: 98.6357 ng/mg, 8.8058–1,087.4578 ng/mg vs. 1.4262 ng/mg, 0.0916–11.0858 ng/mg, all p-values <0.001). The levels of urinary MBL and C4d representing lectin complement pathway showed a linear association with the proportion of crescents (r = 0.457 and 0.562, respectively, both p-values <0.001). Combined urine complement products could increase the predictive ability compared with crescents alone from 0.904 to 0.944 (p = 0.062) with borderline significance. Moreover, the glomerular C4d deposition rate elevated with the increase of proportions of crescents.

Conclusion: Excess complement activation may be involved in the formation of crescents, especially diffuse crescent formation, in patients with IgAN. Urinary C4d correlated with the proportion of crescents and was a potential biomarker for disease monitoring in crescentic IgAN.

Keywords: immunoglobulin A nephropathy (IgAN), crescent, complement, lectin pathway, urinary C4d

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INTRODUCTION

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide and is characterized by mesangial IgA deposition (1, 2). The clinical course of IgAN ranges from isolated hematuria to rapidly progressive renal failure, and the kidney biopsy findings vary from mild mesangial proliferation to diffuse crescent formation (3). Crescent formation on kidney biopsy is regarded as a prognostic indicator of poor outcomes. Those with high levels of crescents usually show rapidly progressive kidney failure and need aggressive immunosuppressive therapy (4, 5). However, to date, the pathogenesis of crescent formation in IgAN is still unclear.

Complement activation is involved in the development or progression of IgAN. Glomerular deposits of C3, properdin, C4d, mannose-binding lectin (MBL), and C5b-9 but limited C1q indicate that the alternative and lectin pathways are primarily involved in this disease (6). Complement activation can occur directly on IgA1-containing immune complexes in circulation and/or after their deposition in the mesangium, thus playing an important role in the development of IgAN (7). Glomerular deposition of C4d and plasma levels of iC3b-d were associated with the severity of the histologic lesions or clinical features (8, 9). However, to date, the roles of complement activation in the development of diffuse crescent formation in IgAN are unclear. In this study, we aimed to evaluate the extent of complement activation and its association with crescent formation.

PATIENTS AND METHODS

Study Participants

One hundred adult IgAN patients with different proportions of crescents who were diagnosed by renal biopsy from 2004 to 2019 at Peking University First Hospital were enrolled this study: 24 patients with a proportion of crescents of 1%–24%, 27 patients with 25%–49%, 21 patients with 50%–74%, 12 patients with more than 75%, and 16 patients without crescent formation. Plasma samples were obtained from 29 of 100 patients. An additional 41 patients from whom plasma samples were collected in the same period were enrolled for the plasma complement analysis (**Supplementary Table 1**). IgAN was diagnosed by immunofluorescence showing the presence of at least 1+ (range, 0–3) IgA mesangial deposits as the dominant or codominant immunoglobulin in the mesangial deposits and the deposition of electron-dense materials in the mesangium on ultrastructural examination. Renal biopsies were scored by two pathologists blinded to the clinical data, with any differences in grading resolved by viewing the slide together under a two-headed microscope. Patients with serological or clinical evidence of other renal damage, such as systemic lupus erythematosus, vasculitis, anti-glomerular basement membrane (anti-GBM) disease, and Henoch–Schönlein purpura, were excluded. Patients with other autoimmune diseases or pregnancies were also excluded. A crescent was defined as extracapillary proliferation of more than two cell layers of any size; a cellular crescent was defined by >50% of the lesion occupied by cells; a fibrocellular crescent was defined by ≤50% of the lesion occupied by cells and <90% occupied by matrix; a fibrous crescent was defined by ≥90% matrix composition. Clinical characteristics, including age, sex, serum creatinine, 24-h protein excretion, blood pressure, and complement C3 and C4 levels, were collected at the time of renal biopsy.

The study was approved by the ethics committee at the Peking University First Hospital. Informed written consent was obtained from all participants.

Detection of Activated Complement Components

Urine and plasma samples were collected from the patients before renal biopsy, and samples from the healthy controls were stored at −80°C until analysis. Activated complement components, including Bb, C4d, C3a, C5a, and SC5b-9, were detected by ELISA kits (Quidel, USA). Urinary levels of MBL were detected by ELISAs using a commercial MBL Oligomer ELISA kit (Bioporto, Hellerup, Denmark). All assays were conducted according to the manufacturer's instructions. Urine and plasma samples had only one freeze/thaw cycle before analysis. Frozen specimens were thawed rapidly at 37°C and immediately moved to ice to prevent complement activation prior to dilution. After dilution, the samples were loaded into the microassay wells as rapidly as possible.

Immunohistochemistry for C4d, C3d, and C5b-9

To study the deposition of complement activation products in kidneys of IgAN patients with different proportions of crescents, complement activation products C4d, C3d, and C5b-9 were detected by immunohistochemistry. Ten patients with 0%–24% crescents, 10 patients with 25%–49% crescents, and 10 patients with over 50% crescents were included. C4d, C5b-9, and C3d immunohistochemical staining was performed on 4-μm deparaffinized and rehydrated sections of formaldehyde-fixed renal tissue, using polyclonal rabbit anti-human C4d (Biomedica, Vienna, Austria) (1:50), polyclonal rabbit anti-human C3d (DAKO, Denmark) (1:1,000), and monoclonal mouse anti-human C5b-9 (DAKO, Denmark) (1:50) as the antibodies, respectively. The sections for C3d and C4d staining were treated with 0.4% pepsin (Zhongshan Golden Bridge Biotechnology, Beijing, China) 30 min for antigen retrieval. The sections for staining C5b-9 were treated with 0.5 mg/ml of proteinase K for antigen retrieval. Sections of IgAN patients previously proven C4d, C5b-9, and C3d positive and a section with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis served as the positive and negative controls, respectively. The sections were examined by light microscopy. Patients were classified as positive when >25% of the non-sclerotic glomeruli were positive for complement products.

Prognosis End Point

For each patient, the date of renal biopsy was recorded as the baseline point. Follow-up time was considered as the interval

between renal biopsy and the last outpatient visit. The primary end point of the study was the cumulative percentage of patients who developed end-stage kidney disease (ESKD) (defined by 12 or more continuous weeks of renal replacement therapy or by kidney transplantation). No patient died before the time of ESKD.

Statistical Analysis

Non-normally distributed and normally distributed quantitative data are expressed as medians and interquartile ranges (IQRs) and means ± standard deviations, respectively. Categorical data are shown as frequencies or ratios. Student's t-test for normally distributed data, or non-parametric test (Mann–Whitney U test) for non-normally distributed data, was used for the differences of quantitative parameters between groups. Spearman's correlation was performed between urinary complement levels and other variables. The Kaplan–Meier curves were performed to evaluate the effect of different proportions of crescent formation on renal survival. Package "pROC" was used to create receiver operating characteristic (ROC) curves to determine the different models in prediction of renal survival. A two-tailed p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0, and graphs were generated using R version 4.0.3.

RESULTS

Baseline Demographic, Clinical, and Pathological Characteristics

Overall, 100 IgAN patients with different proportions of crescents were enrolled in this study: 16 patients without crescents (C0), 24 with 0%–24% crescents (C1), 27 with 25%–49% (C2), 21 with 50%–74%, and 12 with more than 75%. There were 68 (68%) males and 32 (32%) females with a mean age at the time of kidney biopsy of 36.2 ± 12.5 years. As shown in **Table 1**, a higher proportion of crescents was associated with higher levels of plasma creatinine or proteinuria, especially in those patients with crescent more than 50% (**Table 1**).

Association of the Levels of Urinary Complement Activation Products and Crescents

As shown in **Figure 1**, urinary C3a and C5a, representing the common pathway of complement activation, remained at low levels in the patients with a proportion less than 50%, while they increased significantly when the proportion of crescents reached 50% ($p < 0.001$). A similar trend was observed in the levels of Bb, representing the alternative pathway. The patients with a percentage of crescents >50% showed higher complement activation levels than the other patients (urinary C3a/Cr: 6.7295 ng/mg, IQR 1.4652–62.1086 ng/mg vs. 0.1055 ng/mg, IQR 0–1.4089 ng/mg; urinary C5a/Cr: 15.6202 ng/mg, 4.3127–66.7347 ng/mg vs. 0.3280 ng/mg, IQR 0.0859–2.4439 ng/mg and urinary Bb/Cr: median 0.0224 μg/mg, IQR 0.0125–0.0484 μg/mg vs. 0.2684 μg/mg, 0.0476–4.007 μg/mg, all p-values <0.001).

The levels of urinary C4d and MBL, representing the lectin pathway, and those of C5b-9, the terminal complement activation products, were positively correlated with the proportion of crescents in IgAN (all p-values for the trend <0.001). This association was most obvious in urinary C4d. The median urinary C4d/Cr value among the patients with proportions of 0%, 1%–24%, 25%–49%, 50%–74%, and >75% were 0 ng/mg (range 0–0.637), 1.9651 ng/mg (0–93.6551), 35.3148 ng/mg (0–317.9359), 552.6448 ng/mg (17.6306–2,112.9755), and 1,403.1766 (66.5081–3,724.6789, respectively (p for trend <0.001). The levels of urinary C4d showed a linear association with the proportion of crescents ($r = 0.562$, $p < 0.001$) (**Figure 2**).

Association of Urinary Complement Activation Products and Clinical Characteristics

As shown in **Table 2**, the levels of urinary complement activation products, including urine C3a, C5a, Bb, MBL, C4d, and C5b-9,

TABLE 1 | Baseline characteristics of IgAN patients with various crescents forming.

Characteristics	0% (N = 16)	0%–24% (N = 24)	25%–49% (N = 27)	50%–74% (N = 21)	75%–100% (N = 12)	Total (N = 100)	*P*-value
Male (%)	9 (56.3%)	17 (70.8%)	17 (63.0%)	17 (81.0%)	8 (66.7%)	68 (68.0%)	0.550
Age	31.8 ± 7.8	40.1 ± 11.6	39.1 ± 14.0	32.5 ± 12.0	34.2 ± 15.4	36.2 ± 12.5	0.096
SBP (mmHg)	127.4 ± 15.3	133.6 ± 21.9	133.4 ± 24.2	147.0 ± 18.0	136.3 ± 15.2	135.7 ± 20.8	0.051
DBP (mmHg)	80.9 ± 11.5	86.0 ± 13.7	82.0 ± 16.5	90.8 ± 16.6	80.6 ± 10.8	84.5 ± 14.8	0.159
Scr (μmol/L)	89.0 (71.3–136.1)	122.7 (88.1–166.6)	182.1 (94.9–333.5)	398 (289.9–511.6)	292.5 (201.8–686.8)	163.5 (97.3–375.9)	<0.001
Proteinuria (g/day)	0.9 (0.5–3.7)	1.8 (1.1–3.3)	2.4 (1.4–5.3)	5.5 (4.2–7.4)	5.4 (3.5–8.4)	3.4 (1.4–5.6)	<0.001
Albumin (g/L)	39.9 ± 3.8	36.6 ± 5.1	34.2 ± 5.5	31.7 ± 5.1	28.1 ± 7.2	34.4 ± 6.3	<0.001
IgG (g/L)	9.9 ± 3.1	10.4 ± 2.5	9.6 ± 2.7	7.5 ± 2.5	9.2 ± 5.5	9.4 ± 3.2	0.036
IgA (g/L)	3.1 ± 1.4	2.8 ± 0.8	3.4 ± 1.1	2.9 ± 1.2	2.7 ± 0.9	3.1 ± 1.1	0.267
C3 (g/L)	1.01 ± 0.31	1.01 ± 0.21	0.96 ± 0.20	0.95 ± 0.19	1.00 ± 0.24	0.98 ± 0.22	0.837
C4 (g/L)	0.21 ± 0.06	0.27 ± 0.08	0.26 ± 0.77	0.27 ± 0.53	0.27 ± 0.52	0.26 ± 0.69	0.128
Total crescents (%)	0% (0–0)	15.1 (9.5–20.5)	39.1 (30.0–52.6)	64.3 (54.8–74.0)	82.3 (78.7–86.6)	35.1 (11.8–64.3)	<0.001
Cellular+fibrocellular crescents (%)	0 (0–0)	12.1 (8.8–15.9)	33.3 (30.0–40.0)	60.0 (52.4–66.7)	81.5 (78.0–86.5)	32.5 (10.5–57.4)	<0.001
Fibrous crescents (%)	0 (0–0)	0 (0–0)	0 (0–9.10)	0 (0–6.8)	0 (0–0)	0 (0–0)	0.283

SBP, systolic blood pressure; DBP, diastolic blood pressure; Scr, serum creatinine; IgAN, IgA nephropathy.

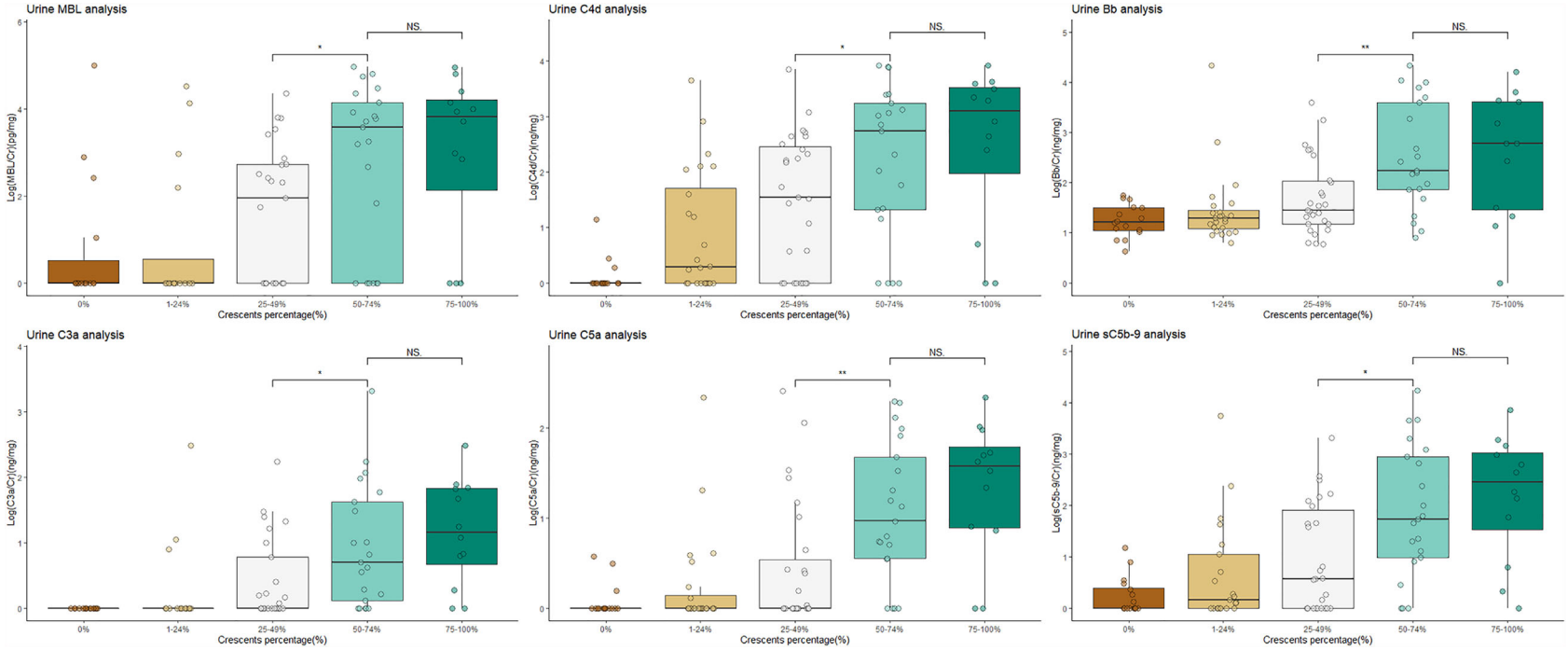

FIGURE 1 | Patients with crescentic IgAN had higher levels of activated complement products in urine than non-crescentic IgAN patients. NS, not significant; IgAN, IgA nephropathy. The urine values were corrected by urinary creatinine. All the data were log10-transformed.

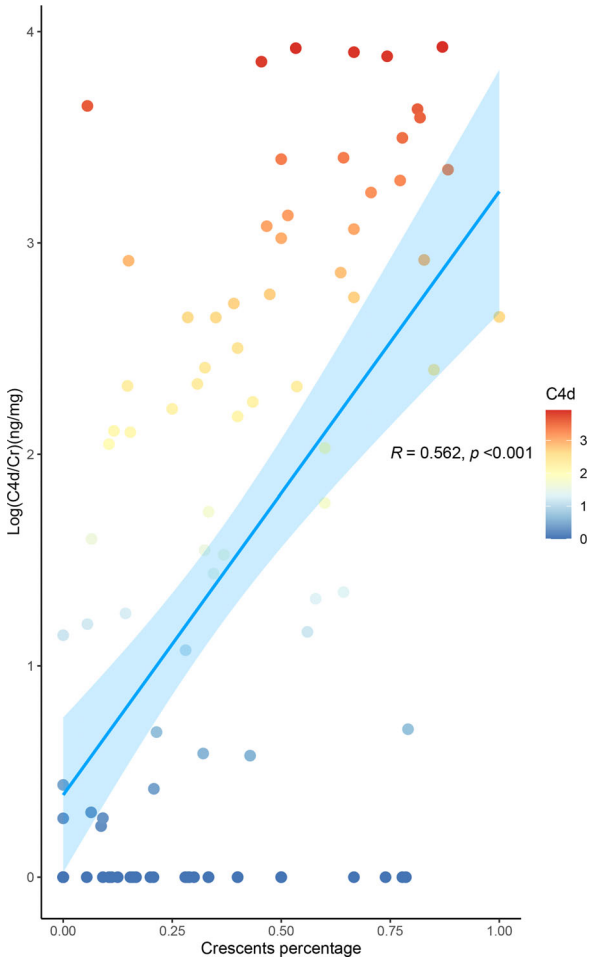

FIGURE 2 | Level of urinary C4d is associated with the proportion of crescents in IgA nephropathy. The urine values were corrected by urinary creatinine. All the data were log10-transformed.

TABLE 2 | Association of urinary activated complement products and baseline characteristics in patients with IgA nephropathy.

Urine complement products	Proteinuria (g/day)		Serum creatinine (μmol/L)	
	r	p	r	p
MBL/Cr	0.487	<0.001	0.408	<0.001
C4d/Cr	0.547	<0.001	0.583	<0.001
Bb/Cr	0.552	<0.001	0.557	<0.001
sC5b-9/Cr	0.599	<0.001	0.599	<0.001
C3a/Cr	0.652	<0.001	0.677	<0.001
C5a/Cr	0.719	<0.001	0.655	<0.001

Cr, creatinine; IgAN, IgA nephropathy; MBL, mannose-binding lectin.

were positively correlated with serum creatinine (the values of r were 0.677, 0.655, 0.557, 0.408, 0.583, and 0.599, respectively) and proteinuria (r values were 0.652, 0.719, 0.552, 0.487, 0.547, and 0.599, respectively) (all *p*-values <0.001).

Immunohistochemistry for C4d, C3d, and C5b-9

A total of 30 participants including patients with crescents of 0%–24% (n = 10), 25%–49% (n = 10), and over 50% crescent formation (n = 10) were included for immunohistochemistry. Glomerular staining for C4d (>25% of glomeruli) were observed in two (20%) cases in group with crescents of 0%–24%, seven (70%) cases in 25%–49%, and all 10 cases in the groups with crescents over 50%. Positive glomerular staining was seen predominantly in the mesangial area, very often within the crescents and the sclerotic lesions. Glomerular C5b-9 deposition was observed in seven (70%) cases in the group with crescents 0%–24%, while all positive in groups with crescents >25% or >50%. Positive C3d staining was observed in all 30 patients (**Figure 3**).

Prognosis of Patients With Different Proportions of Crescents

To evaluate the effect of crescent formation on kidney progression in IgAN, the Kaplan–Meier method was used to assess cumulative incidence of ESKD among patients with different percentage of crescents. According to the percentage of glomeruli involved with crescents, the patients were divided into four groups: group 1, absence of crescents; group 2, crescents less than 25%; group 3, 25%–49%; and group 4, more than 50%. During a mean follow-up of 29.5 months, 29 patients progressed to ESKD. No patient died before the time of ESKD. ESKD occurred in zero of 13 patients in group 1, in two of 24 (8.3%) in group 2, in six of 26 (23.1%) in group 3, and in 21 of 26 (80.8%) (log-rank, $p < 0.001$) (**Figure 4**).

Assessment of the Prognostic Values of Urine Complement Activation

To explore the prognostic values of urine complement products, different clinical models were evaluated for their ability to predict renal survival by performing ROC curve analysis. Combined urine-activated complement products showed good performance in predicting prognosis. The area under the curve (AUC) for combined urine complement activation products (Model 1) was 0.904 (95% confidence interval [CI], 0.825–0.984). Combined urine complement products could increase the predictive ability compared with crescents alone from 0.904 to 0.944 ($p = 0.062$) with borderline significance. The model fitted best when combined with clinical or pathological parameters (including serum creatinine, urine proteinuria, and crescents) and urine complement products activation (Model 4), whose AUC was 0.973 (CI, 0.933–1) (**Figure 5**).

DISCUSSION

High levels of crescent formation are often associated with rapid progression of kidney disease and clinically severe proteinuria or hematuria in patients with IgAN. To date, the pathogenesis of crescent formation in IgAN is unclear. In this study, we found a

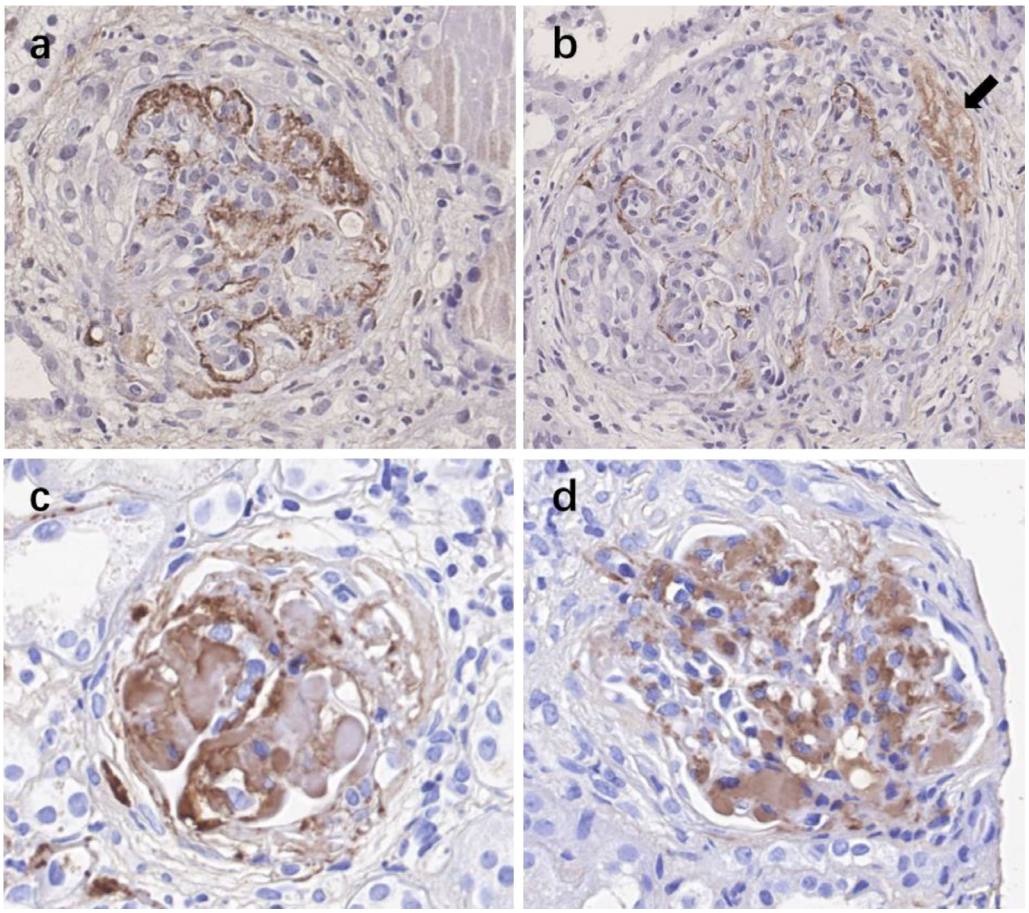

FIGURE 3 | Immunohistochemistry staining for complement activation products C4d, C3d, and C5b-9 in kidney specimens of IgAN patients with various crescent formations. **(A)** Positive staining of C4d in the mesangial area of the glomerulus. **(B)** Positive staining of C4d both in the mesangial area of the glomerulus and within the crescent (black arrow). **(C)** Positive staining of C5b-9. **(D)** Positive staining of C3d. IgAN, IgA nephropathy.

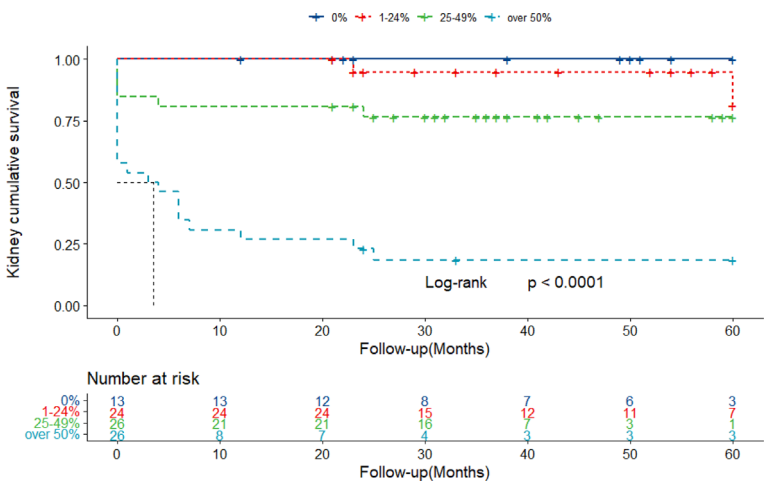

FIGURE 4 | Kaplan–Meier curves of renal survival in IgAN patients with different proportions of crescents. Patients were retrospectively followed up with a mean of 29.5 months. Twenty-nine ESKD cases were collected throughout the follow-up. IgAN, IgA nephropathy; ESKD, end-stage kidney disease.

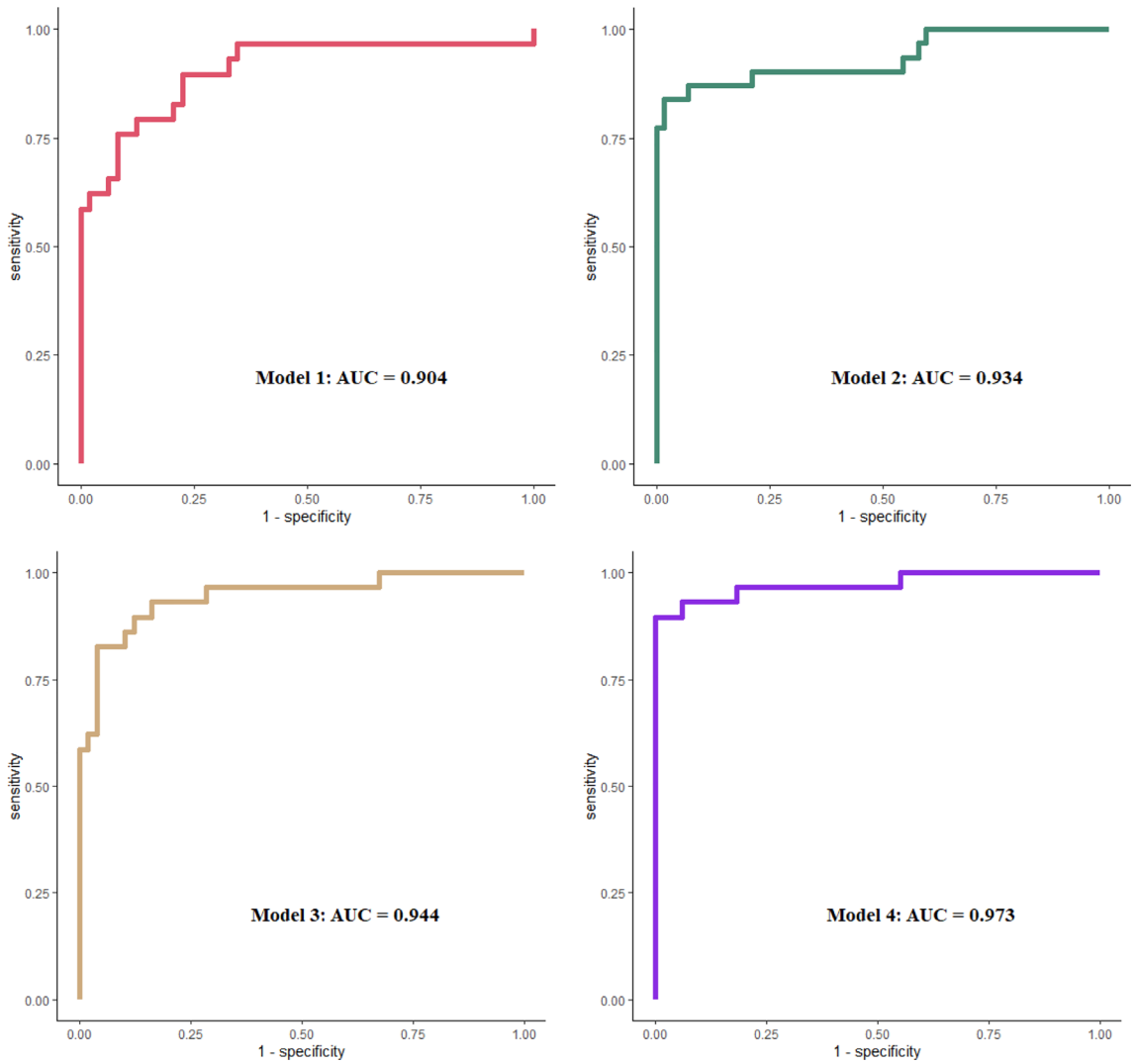

FIGURE 5 | ROC curves in evaluation different models to predict ESKD. Model 1 included urinary MBL, Bb, C4d, C3a, C5a, and sC5b-9, all corrected by urine creatinine. Model 2 included Scr, UTP, and crescent percentage. Model 3 included covariates in model 1 plus crescent percentage. Model 4 included covariates in model 1 plus model 2. All covariates were analyzed as continuous data. ROC, receiver operating characteristic; ESKD, end-stage kidney disease; MBL, mannose-binding lectin; Scr, serum creatinine.

strong association between urinary complement activation products and crescents on kidney biopsy, especially in those with more than 50% crescents. Additionally, the levels of urinary C4d or glomerular C4d deposition representing the lectin pathway in IgAN were strongly associated with the proportion of crescents in the kidney. These findings indicate that excess complement activation is involved crescent formation in IgAN. Urinary C4d might be a potential biomarker for monitoring crescent formation in this disease.

Based on the frequent codeposition of C3 with IgA in the glomerular mesangium, complement activation has been speculated to be an important player in the pathogenesis of IgAN since the 1980s (10, 11). Codeposition of alterative pathway components such as properdin (75–100%), factor H (30–90%), and C3 (>90%) with IgA was reported [11]. Complement activation by the lectin pathway is also involved in the progression of IgAN. MBL was found to be codeposited with IgA in the mesangial area in 17%–25% of IgAN biopsies (12, 13), and deposition of MBL or C4d by the lectin pathway correlated with the severity of the disease and could predict renal outcome (9, 14, 15). In the current study, we found that the patients with a proportion of crescents greater than 50% had much higher (10–50 times) urinary levels of activated complement products in both the alternative and lectin pathways than the other IgAN patients. In addition, these urinary complement products were associated with severe proteinuria or a low estimated glomerular filtration rate (GFR). These findings suggest that diffuse crescent formation with

rapidly progressive kidney failure was associated with overactivation of both the alternative and lectin pathways. Crescent formation is partially related to lectin pathway activation. In this study, we did not find that circulating complement products were associated with crescents or clinical parameters (**Supplementary Figure 1**). These results indicate that urinary complement levels, but not circulating complement levels, represent renal complement activation. However, only a small number of patients in this study had both serum sample and urine sample, which limited the findings. Nevertheless, we found that the urinary C4d level, representing the lectin pathway, showed a linear relationship with the proportion of crescents. In addition, histological C4d-positive rate synchronously increased with the crescent percentage elevation, indicating C4d as a potential useful biomarker for monitoring crescent formation in this group of patients.

Extensive crescent formation in IgAN is often associated with rapidly progressive kidney failure and other pathologies. The KDIGO guidelines recommend aggressive immunosuppressive therapy; however, even with this approach, our prior cohort study showed that patients with more than 50% crescent formation demonstrated poor outcomes, with more than two-thirds of patients developing ESKD. To date, specific therapy for crescentic IgAN is lacking. In this study, we demonstrated that excess complement activation might involve diffuse crescent formation in IgAN, especially through the lectin pathway. This finding suggests that approaches that target complement activation (such as the recently available mannan-associated lectin-binding serine protease-2 (MASP-2) antibodies) may represent a promising option for the treatment of crescentic IgAN. To date, two case reports have shown that complement inhibition using the humanized anti-C5 monoclonal antibody eculizumab may be beneficial to crescentic IgAN (16, 17).

The definition of crescentic IgAN is still controversial. In the 2012 KDIGO guidelines, crescentic IgAN was defined as IgAN with crescents in more than 50% of glomeruli in the renal biopsy with rapidly progressive renal deterioration. In this study, we included patients with different proportions of crescent formation. We found that urinary complement activation products, including C3a, C5a, and Bb, increased significantly when the proportion of crescents reached 50% or more, while they remained at low levels and showed no difference among the different groups with a proportion of crescents less than 50%. These results indicated that crescentic IgAN with a proportion of 50% or more represents a specific phenotype.

In conclusion, in this study, we found that IgAN patients with large numbers of crescents, especially those with 50% crescent formation, demonstrated much higher levels of both alternative and lectin pathway complement activation products in urine. These findings indicate that excess complement activation is involved in crescent formation in IgAN. Thus, approaches that target complement activation may represent a promising option for the treatment of crescentic IgAN. Furthermore, urinary C4d levels were associated with the proportion of crescents on biopsy and disease severity and may be a useful biomarker for disease monitoring.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZW and XX designed and conducted the study, analyzed the results, and prepared the manuscript. JYL, XZ, JH, and MW helped with the experiment. JCL and HZ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.676919/full#supplementary-material

Supplementary Table 1 | Baseline characteristics of IgAN patients with various crescents enrolled for plasma complement tests. Scr, serum creatinine.

Supplementary Figure 1 | Plasma activated complement products are not always associate with the proportion of crescents in IgA nephropathy. NS., Not significant.

Supplementary Figure 2 | Year distribution of urine samples recruitment.

Supplementary Figure 3 | Year distribution of blood samples recruitment.

Supplementary Figure 4 | Year distribution of histological samples recruitment.

Supplementary Figure 5 | ROC curve of crescent in evaluation of predicting ESKD.

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REVIEW
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Hantavirus Induced Kidney Disease

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Hantavirus induced hemorrhagic fever with renal syndrome (HFRS) is an emerging viral zoonosis affecting up to 200,000 humans annually worldwide. This review article is focused on recent advances in the mechanism, epidemiology, diagnosis, and treatment of hantavirus induced HFRS. The importance of interactions between viral and host factors in the design of therapeutic strategies is discussed. Hantavirus induced HFRS is characterized by thrombocytopenia and proteinuria of varying severities. The mechanism of kidney injury appears immunopathological with characteristic deterioration of endothelial cell function and compromised barrier functions of the vasculature. Although multidisciplinary research efforts have provided insights about the loss of cellular contact in the endothelium leading to increased permeability, the details of the molecular mechanisms remain poorly understood. The epidemiology of hantavirus induced renal failure is associated with viral species and the geographical location of the natural host of the virus. The development of vaccine and antiviral therapeutics is necessary to avoid potentially severe outbreaks of this zoonotic illness in the future. The recent groundbreaking approach to the SARS-CoV-2 mRNA vaccine has revolutionized the general field of vaccinology and has provided new directions for the use of this promising platform for widespread vaccine development, including the development of hantavirus mRNA vaccine. The combinational therapies specifically targeted to inhibit hantavirus replication and vascular permeability in infected patients will likely improve the disease outcome.

Keywords: hantavirus, HFRS, acute kidney injury, bunyavirus, hemorrhagic fever

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INTRODUCTION

Viral hemorrhagic fever refers to a multisystem syndrome triggered by severe damage to the vascular system by the viruses from six distinct families: *Filoviridae*, *Arenaviridae, Hantaviridae, Nairoviridae, Phenuiviridae,* and *Flaviviridae* (**Table 1**). The disease symptoms are accompanied by fever and hemorrhage (bleeding), although the bleeding by itself is hardly ever life-threatening. These enveloped RNA viruses are carried by animal or arthropod vectors in nature. Humans are infected by contact with infected hosts or their contaminated body fluids such as saliva, feces, or blood. The mode of transmission and severity of the disease depends upon virus species, although each can cause hemorrhagic fever. Outbreaks of viral hemorrhagic fever are sporadic and their occurrences are not easily predictable. Based on certain characteristics such as morbidity and mortality, the possibility of person-to-person transmission, aerosolic dissemination, availability of vaccine or therapeutic treatments, stability in the environment, and potential for large scale production etc, some of the hemorrhagic fever viruses have been classified as potential bio-warfare agents. These viruses include Ebola, Marburg, Lassa fever, Machupo, Junin, Guanarito, Sabia, Rift valley fever, yellow fever, Omsk hemorrhagic fever, and Kyasanur forest disease (1). Among other

TABLE 1 | Hemorrhagic fever virus.

Family	Virus	Disease
Filoviridae	Ebola[1], Marburg	Ebola HF, Marburg HF
Arenaviridae	Lassa, NW Arenaviruses[2]	Lassa fever, NW2 hemorrhagic fever.
Nairoviridae	CCHFV[3]	CCHF hemorrhagic fever
Phenuiviridae	RVFV[4]	Rift valley fever
Hantaviridae	New word hantavirus	Hantavirus cardiopulmonary syndrome
	Old world hantavirus	**Hemorrhagic fever with renal syndrome**
Flaviviridae	Dengue	Dengue fever, Dengue HF, Dengue SY[5]
	YFV[6]	Yellow fever
	Omsk HFV[7]	Omsk hemorrhagic fever
	Kyasanur FDV[8]	Kyasanur forest disease

[1]*There are four subtypes of Ebola (Zaire, Sudan, Ivory Coast and Reston), Ebola HF stands for Ebola hemorrhagic fever and Marburg HF stands for Marburg hemorrhagic fever.* [2]*Stands for New word ArenavirusesThe new word Arena viruses include (Machupo, Junin, Guanarito, Sabia).* [3]*Crimean Congo Hemorrhagic Fever Virus.* [4]*Rift Valley Fever Virus.* [5]*Dengue shock syndrome.* [6]*Yellow Fever Virus.* [7]*Osmak Hemorrhagic Fever Virus.* [8]*Kyasanur Forest Disease Virus. The information presented in this table was obtained from (1).*

TABLE 2 | Old world hantaviruses species.

Virus	Host	Geographical distribution/reference
Hantaan (HTNV)	Striped field mouse	Asia (10)
Seoul (SEOV)	Rat worldwide	Worldwide (11)
Dobrava (DOBV)	Yellow-necked mouse	Europe (9)
Saaremaa (SAAV)	Striped field mouse	Europe (12)
Thailand (THAIV)	Bandicoot rat	Thailand (13)
Amur (AMRV)	Korean field mouse	Asia (14)
Puumala (PUUV)	Bank vole	Europe (15)
Hokkaido (HOKV)	Red bank vole	Asia (16)
Tula (TULV)	European common vole	Europe/Russia (17)
Prospect Hill (PHV)	Meadow vole	North America (18)
Bloodland Lake (BLLV)	Prairie vole	North America (19)
Isla Vista (ISLAV)	Californian vole	North America (20)
Khabarovsk (KHAV)	Reed vole	Asia/East Russia (21)
Topografov (TOPV)	Lemming	Siberia/Russia (22)
Thottapalayam (TPMV)	Shrew	Asia/India (23)

hemorrhagic fever viruses (**Table 1**), the old-world hantaviruses are known to cause hemorrhagic fever with renal syndrome (HFRS), a group of clinically similar illnesses targeting the kidney.

Hantaviruses are emerging negative strand RNA viruses and members of the *Hantaviridae* family (2–4). They are carried by rodents, and humans get infected by the inhalation of aerosolized excreta such as saliva and urine droppings of infected rodent hosts (5–8). Their infection causes a significant impact on human health (8, 9). Hantavirus species such as Puumala virus (PUUV), Seoul virus (SEOV), Dobrava Belgrade virus (DOBV), and hantaan virus (HTNV) are predominantly found in Asia and Europe and are referred to as old world hantaviruses (**Table 2**). The hantavirus species such as Sin Nombre virus (SNV) and Andes virus (ANDV) are mostly found in America are referred to as new world hantaviruses. Old and new world hantaviruses have distinct pathologies. Old world hantaviruses infect the highly specialized and differentiated endothelial cells of the kidney, causing acute renal failure with tubular and glomerular involvement, which is referred to as hemorrhagic fever with renal syndrome (HFRS). The new world hantaviruses cause hantavirus cardiopulmonary syndrome (HCPS) (24), a fibril illness characterized by respiratory failure and cardiac dysfunction. The mortality rates of HFRS and HCPS can go as high as 15 and 50%, respectively, in certain outbreaks (25, 26). Annually, 150,000 to 200,000 cases of hantavirus infection are reported worldwide (27), and more than 50,000 reported cases are found in China alone. There is no FDA approved vaccine or an antiviral therapeutic against hantavirus infections.

Under an electron microscope hantavirus particles appear spherical in shape (28). The three hantaviral genomic RNA segments: S, M, and L encode viral nucleocapsid protein (N-protein), glycoprotein precursor (GPC), and viral RNA dependent RNA polymerase (RdRp), respectively. The GPC precursor is post-translationally cleaved in the middle generating two glycoprotein Gn and Gc that are incorporated in the virus envelop (29). Hantaviruses primarily target endothelial cells with the receptor (β3 integrin) for virus attachment and entry. Hantaviruses use surface glycoproteins to attach to the cell surface receptor of the target cell (**Figure 1**). The endothelial cells make the internal linings of blood vessel walls, making the body's vascular system susceptible to viral infection. Hantavirus replication occurs exclusively in the host cell cytoplasm (**Figure 1**). Immediately after entering into the host cell, viral uncoating occurs and viral RdRp initiates transcription by a unique cap snatching mechanism to generate 5' capped viral mRNAs (30–32). The multifunctional N-protein plays diverse roles in the virus replication cycle. It is involved in viral transcription initiation in conjunction with viral RdRp, facilitates mRNA translation, and encapsidates the viral genome (33–36). Since this article is mainly focused on hantavirus induced HFRS that leads to AKI, a brief overview of AKI is presented below, followed by discussion of hantavirus induced HFRS leading to AKI. Consistent with the objectives of this journal, the review article provides a link between basic research and clinical practice, with special emphasis on studies that are directly relevant to patient care.

Acute Kidney Injury (AKI)

AKI refers to the rapid decrease in the renal filtration function of the kidney. The condition is primarily observed by increased levels of blood urea nitrogen (BUN) and creatinine. AKI is a general healthcare problem affecting up to 40% of patients admitted to critical care hospital units (37). Apart from predisposition risk factors, the degenerative processes affecting renal epithelium and vasculature play an important role in AKI (38). Moreover, innate and adaptive immune responses impacting renal epithelium and vasculature functions also contribute to AKI (38). Apoptosis and necrosis of tabular

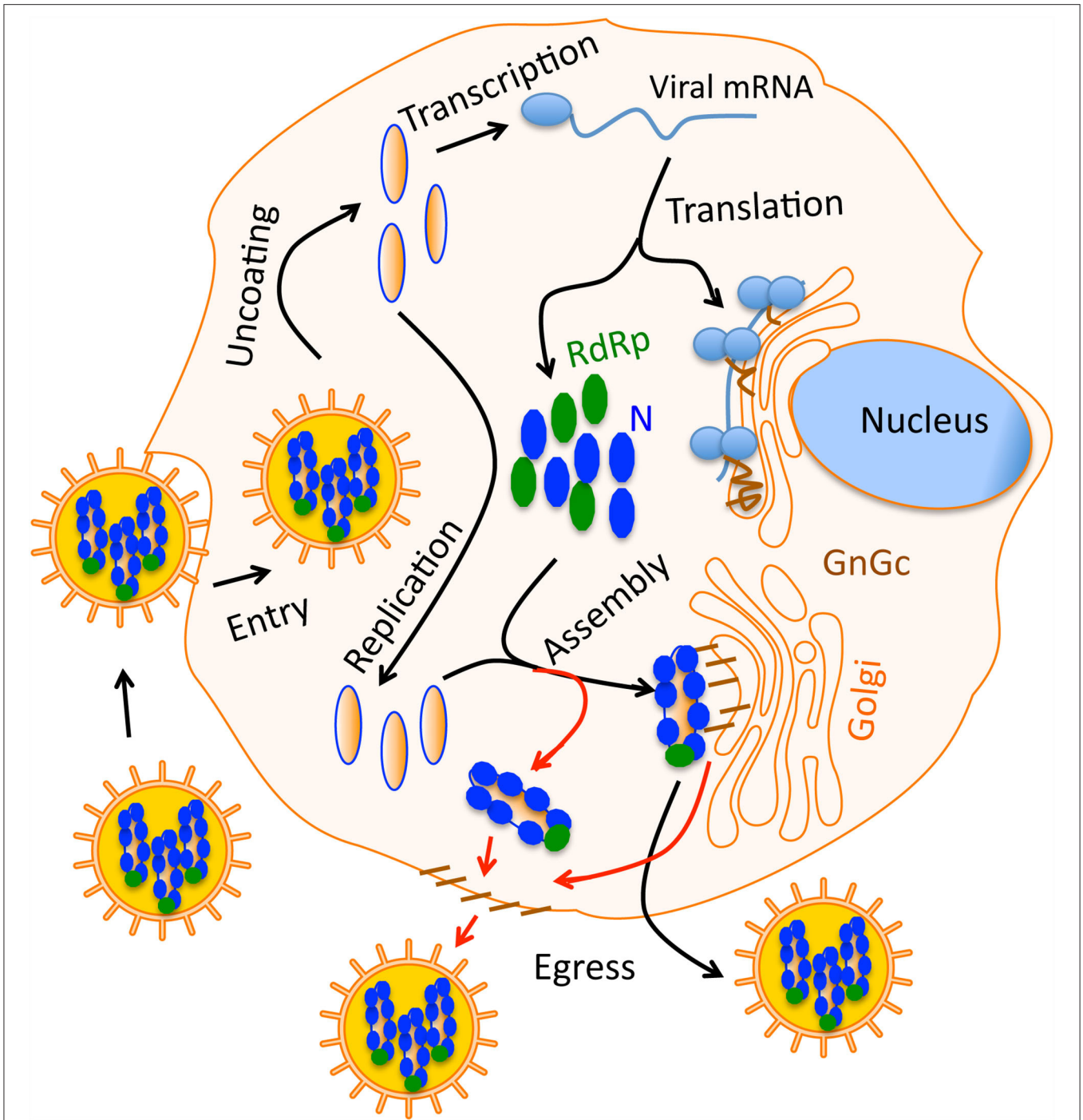

FIGURE 1 | A simple graphical sketch of the hantavirus replication cycle. Hantavirus particles harboring the three nucleocapsids (blue) bind the host cell surface receptor. After entry virus uncoating takes place and capped viral mRNAs are synthesized by transcription. Viral RdRp replicates the viral genome. Viral proteins are synthesized by the host translation machinery. Glycoprotein Gn and Gc are transported to Golgi. During virus assembly, the nucleocapsids meet the glycoprotein on the Golgi surface and new virus particles are born inside the Golgi, which then egress the cell through secretary mechanisms. In some hantaviruses, the assembly occurs on the host cell membrane (red line). In this case, nucleocapsids meet the glycoprotein on the cell membrane that are transported through Golgi.

epithelium can lead to nephron loss accompanied by the activation of the immune response, resulting in the decline of the kidney's filtration capacity (39). The increased chemokine and cytokine expression along with elevated innate and adaptive immune cell response are observed during renal ischemia, another major cause of AKI (39). However, the T-regulatory cell

(Treg) response prevents kidney tissue damage by suppressing the inflammatory response to self-antigens (40). Oxidative stress is another leading cause of AKI. Mitochondrial dysfunction due to renal ischemia may lead to increased production of reactive oxygen species (ROS), promoting AKI due to acute tubular necrosis (40). The use of mitochondria specific ROS scavenger (Mito-TEMPO) (41) and stimulation of mitochondria biogenesis by formoterol has been reported to improve AKI in animal models (42). Thus, selective improvement in mitochondrial function can reduce kidney injury and ultimately reverse AKI. Endoplasmic reticulum (ER) stress, occurring due to the accumulation of misfolded proteins in the ER can also lead to AKI. The stress can be relieved by the expression of molecular chaperons such as, heat shock proteins that transiently bind the misfolded target proteins and help them to refold correctly and attain proper biological function. The unrelieved ER stress has been shown to generate reactive oxygen species (ROS) that ultimately lead to AKI by apoptosis or acute tubular necrosis (43). Nephrotoxic drugs such as tuncamycin have been reported to induce ER stress due to protein misfolding (44). Induction of heat shock protein expression in AKI rodent models has been reported to improve AKI by preventing tubular necrosis (45). The induced expression of pro-apoptotic mediators CHOP/GADD153 due to severe ER stress is consistent with the observed loss of nephron epithelial cells by apoptosis during AKI (46, 47). The use of a chemical chaperone 4-phenylbutyrate reduced both the CHOP/GADD153 protein expression and tubular necrosis in nephrotoxin induced AKI mouse models (44). ER stress inhibitors such as 4-phenylbutyrate have demonstrated efficacy in reducing AKI in preclinical trials (44, 48). The endothelium regulates the blood flow to the local tissue beds and modulates numerous processes related to coagulation, inflammation and vascular permeability (38). The severe impact on endothelium due to AKI leads to microvasculature dysfunction, causing further injury and complications in renal function (49, 50). Due to limited regenerative power of per-tubular capillaries, the endothelial damage due to AKI leads to their rarefaction, causing interstitial fibrosis and increased risk of chronic kidney disease (CKD) (39, 49–51).

HANTAVIRUS INDUCED HFRS LEADS TO AKI

The old-world hantaviruses primarily target the kidney, explaining why the hantavirus disease was initially called "nephropathia epedemica (NE)" in the western world. The kidney tropism and molecular mechanism of NE remain poorly understood. Later WHO started to refer to the old world hantavirus disease as HFRS, although the term HFRS is most popular in Asia and eastern Russia where the disease due to Hantaan virus (HTNV) species is more severe compared to Puumala virus (PUUV) induced NE in Europe and western Russia (52). Hantavirus induced HFRS is listed as one of the fifteen major factors leading to acute kidney injury (AKI) in the Western world (53, 54). Both HCPS and HFRS patients present non-specific flue-like symptoms such as fever, headache, abdominal pain, malaise, and nausea to the clinic. This early febrile phase may last for 2–3 days and is followed by a hypotensive phase in which patients present severe thrombocytopenia, elevated levels of lactate dehydrogenase, C-reactive protein, increased vascular leakage, and leukocytosis (5). Thrombocytopenia was observed in 80% of documented PUUV infections and is even more frequent in other HFRS causing viruses such as HTNV, DOBV, and SEOV. In the 1996 NE outbreak in Belgium, the platelet level at the time of patient's admission to the clinic was reported below 150,000/ml in 79% of 217 infected patients (55). After the hypotensive phase, the oliguric phase begins during which viral infection manifests in different organs. In HCP patients, cardiopulmonary involvement is predominantly observed although renal symptoms cannot be completely ruled out. However, HFRS and NE selectively impact the kidney. The laboratory examination of HFRS and NE patient samples shows proteinuria and high serum creatinine concentrations. The urinalysis shows hematuria and albuminuria (5). Proteinuria is a constant sign in all HFRS and HCPS patients, even though HCPS does not primarily target the kidney (56). Proteinuria has been reported in 100% of HCPS cases. The proteinuria in HFRS can be as high as 29 g/L (56), and some severely ill patients may require dialysis. The severe kidney injury by DOBV infection prompted dialysis in 30% of infected patients in an outbreak in Greece (57). Due to their high frequency, a case presentation without early thrombocytopenia and proteinuria is likely not a hantavirus case, even in HCP patients (56). Acute renal failure (ARF) in the oliguric phase is observed in 90–95% of HFRS patients infected with old world hantaviruses, although the ARF due to PUUV induced NE can be mild (5). An examination of 217 patients in PUUV induced NE outbreak in 1996 in Belgium revealed that 70% of infected patients developed ARF with serum creatinine levels ranging between 1.6 to 20.72 mg/dl (55). Acute myopia is another most common presenting sign in about 25% of NE cases (58). This early transient ophthalmic sign is very specific for old world hantavirus infections due to its absence in other acute infections mimicking HFRS (56). The oliguric phase is followed by the diuretic phase in which high proteinuria rapidly starts to decrease and renal function gradually improves. The proteinuria lasting for years due to hantavirus infection has never been previously demonstrated convincingly (59). However, a recent follow-up study (7–35 months) on 456 PUUV infected patients in Germany revealed hematuria, hypertension, and proteinuria in 25, 23, and 7% patients, respectively (60). NE-associated hypertension and proteinuria do not appear to be concerning in the long run, but NE-associated hematuria might (60). During convalescent phase patients completely recover. Due to a favorable prognosis, the mortality rate of PUUV induced NE is $<1\%$ (61), although long term hypertension and hematuria due to PUUV infection are being discussed (62). The mortality rate of 5–15% in HFRS is likely due to several complications including renal insufficiency, edema, hemorrhages, encephalopathy, and shock (5). Although the predisposition factors may impact the hantavirus disease outcome, the severity of illness mostly depends upon the hantavirus species causing the infection (11).

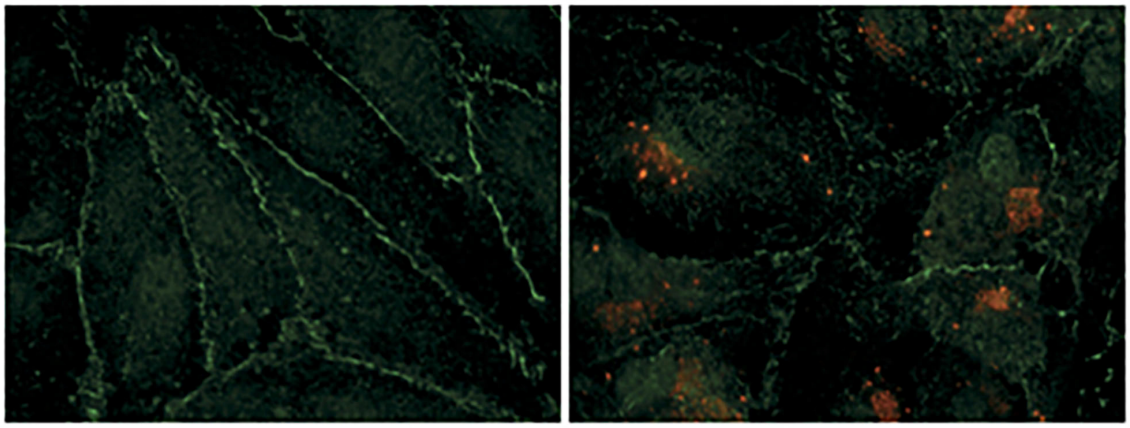

FIGURE 2 | Hantavirus infection damages the contacts between endothelial cells. Human renal glomerular endothelial cells were infected with puumala hantavirus. Cells were examined by immunofluorescence microscopy. Hantavirus nucleocapsid protein is shown by red color and the tight junction marker protein (ZO-1) is shown by green color. The uninfected cells on the left show well-organized cell-to-cell contacts evident from continuous peripheral staining of ZO-1. The uninfected cells form an intact monolayer. The virus-infected cells on the right display discontinue ZO-1 staining, demonstrating the breakdown of endothelial barrier function. This picture was borrowed from (5) and is reused with permission from the Nature publishing group.

Mechanism of Hantavirus Induced AKI

The clinical description of HFRS is an acute renal failure with significantly elevated proteinuria caused by tubular and glomerular involvement. The interdisciplinary research approaches from molecular virology, epidemiology, and nephrology have provided crucial insights into the pathogenesis of hantavirus infection. The mechanism of kidney injury appears immunopathological, characterized by deterioration of endothelial cell function and compromised barrier functions of the vasculature, likely due to cytokine storm in infected patients during the virus infection (**Figure 2**). Infection of human renal cells critical for barriers functions of the kidney such as podocytes, tubular epithelial and glomerular endothelial cells revealed disturbances in structure and integrity of cell to cell contacts, observed by redistribution and reduction of the light junction protein ZO-1 along with decreased transepithelial resistance in infected epithelial monolayers (63) (**Figure 4**). The in-depth molecular details of hantavirus induced AKI remain poorly understood. As the human leukocyte antigen (HLA) haplotypes were found to play a role in the outcome of hantavirus disease (70–72), the severity of hantavirus infection in certain endemic areas may likely be influenced by the genetic susceptibility due to the prevalence of certain HLA genes in the inhabitant population (5). The relationship between HLA alleles and disease severity suggests the involvement of T-cell mediated immune response in hantavirus infection. This is supported by the observations of elevated CD8+ cell count in HCPS and HFRS patients (73, 74). The characteristic feature of hantavirus induced AKI is the increased vascular permeability without apoptotic damage to the capillary endothelium, suggesting the likely breakdown of endothelium due to cytokine release (**Figure 3**). This immunological rather than anatomical insult to the endothelium is reflected by the scarcity of renal lesions on kidney biopsies (56). The observed lesions are largely normal except interstitial edema sometimes accompanied with patchy monocellular infiltrate can be noted. The lesions with interstitial microhemorrhages are very rare and exceptional (52, 56). The primary function of the endothelium is to regulate vascular permeability. However, upon hantavirus infection, the endothelial cells up-regulate certain signaling pathways and induce the expression of proinflammatory cytokines, thereby manifesting the amplified immune response for the rapid recruitment of immune cells at the site of inflammation (**Figure 4**). The vigorous immune response activates the compliment system and triggers the release of proinflammatory cytokines that interfere in endothelial cell function and likely induce vascular permeability. Although numerous cytokines are released in humans (75), the identification of cytokines mediating the vascular leakage could provide new directions for therapeutic strategies of hantavirus induced AKI. T-regulatory cells (TRegs) on the other hand are known to prevent kidney injury by suppressing the proinflammatory response. Interestingly, the TReg response is down-regulated in humans during hantavirus infection (76–78), which may likely contribute to the inflammation-mediated AKI in hantavirus infected patients. In contrast, the up-regulated Treg response promotes hantavirus persistence in infected rodent hosts (79). Although the elevated levels of T cells and cytokine producing cells in hantavirus infected patients support the cytokine induced vascular leakage during hantavirus AKI (80), a recent study demonstrated that depletion of T cells did not impact the outcome of hantavirus disease in a Syrian hamster model (81).

Another hypothesis of increased vascular leakage during hantavirus induced AKI stems from the observations that over-expressed vascular endothelial growth factor (VEGF) could impact vascular permeability by promoting the degradation of VE-cadherin (81–84). VE-cadherin is an important adhesion molecule facilitating intracellular contacts between endothelial

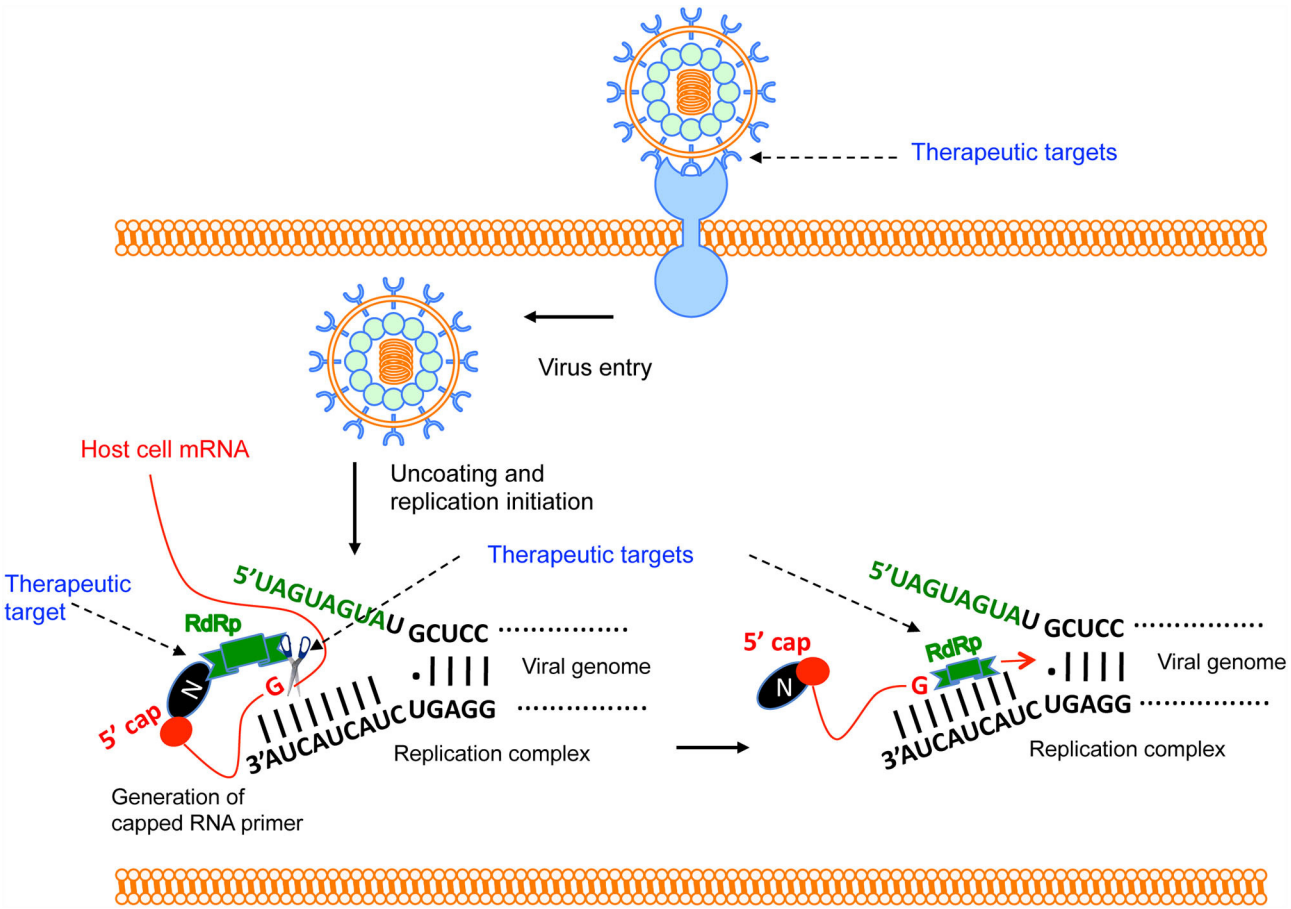

FIGURE 3 | Brief overview of Hantavirus replication cycle and therapeutic targets. The virus binds to the host cell's receptor. After entry, virus uncoating takes place and virus replication is initiated. N protein binds to the host mRNA caps (36). RdRp binds to the N protein through C-terminus (33). The N-terminal endonuclease domain of RdRp cleaves the host cell mRNA at a "G" residue 14 nucleotides downstream of the 5′ cap to generate the capped RNA primer (30). The primer anneals with the 3′ terminus of the viral genome and transcription is initiated by the prime and re-align mechanism. Potential therapeutic targets are shown by the arrow.

cells and regulating vascular permeability (85). One more *in vitro* study has suggested the role of the kallikrein-kinin system (KKS) in the vascular leakage of hantavirus infected patients (65). Activation of this system results in the release of a nine amino acid long peptide called bradykinin (BK) (86). The BK is an extremely potent inflammatory molecule that plays an active role in the vasodilation and increased permeability of the vasculature (87–89). The *in vitro* finding of increased KKS activation, clinical studies demonstrating activation of prekallikrein (an intermediate in the KKS cascade), and successful treatment of PUUV infected HFRS patients using BK antagonists suggest that KKS activation and release of BK might play a role in the hantavirus induced AKI (65, 90).

Epidemiology of Hantavirus Induced Kidney Injury

The epidemiology of hantavirus induced kidney injury is related to the hantavirus species and the geographical distribution of the natural host carrying the virus (**Table 2**). Mostly, the hantavirus induced AKI is caused by old world hantaviruses born out of *Myodes*, *Rattus*, and *Apodemus* rodents. Hantavirus infections in other animals such as shrews, bats, and moles are considered spillover infections and there is little information about their transmission and severity of disease in humans (**Table 2**) (91). The chances of acquiring the disease are based on the exposure of humans to rodents or their infected excreta in the endemic zones of the disease. The human-human transmission has not been reported in old world hantaviruses. Moreover, the human - rodent contacts are influenced by numerous factors such as climate changes and disturbances in rodent habitats by deforestation may favor the migration of rodents to human dwellings [discussed in detail in (27)].

Hantan virus (HTNV) and Seoul virus (SEOV) infections are mostly found in Eurasia, especially in China, south Korea, east Russia, and northern Europe. China has the highest HFRS case load in the world, accounting for more than 90% of the total number of HFRS cases worldwide (92). From 2006 to 2012 a total number of 77,558 HFRS cases and 866 deaths were reported in China alone. More than 90% of these

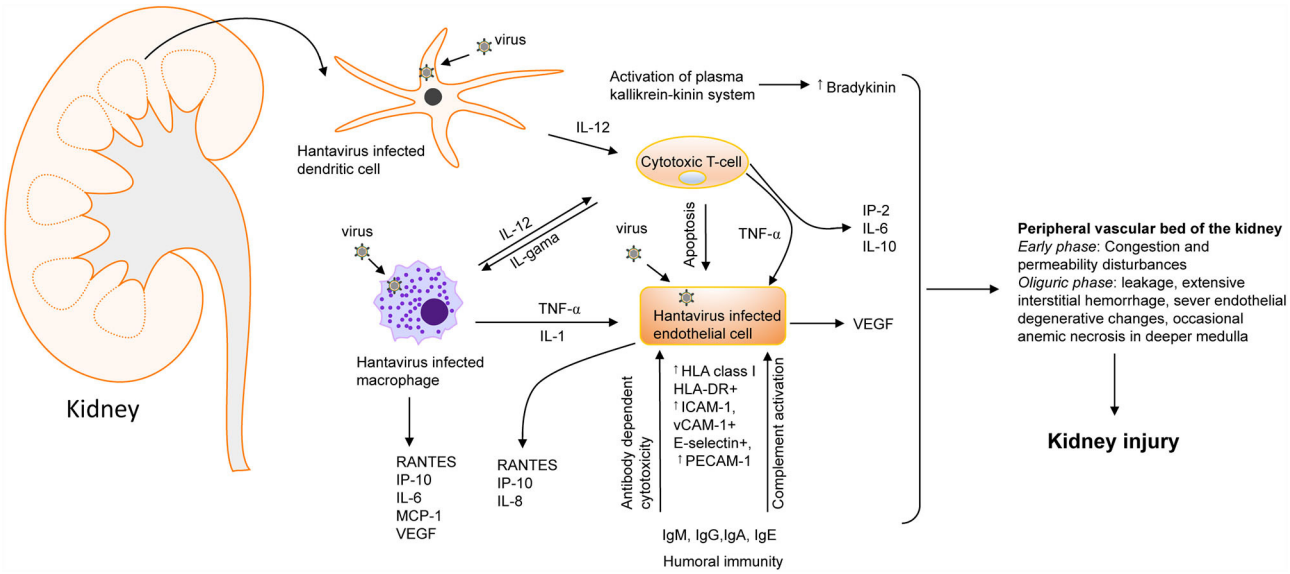

FIGURE 4 | Hantavirus induced kidney injury. A flow chart showing the involvement of cytokines [IL-1, IL-2, IL-6, IL-10, IL-12, TNF-, INF-, and vascular endothelial growth factor (VEGF) and chemokines] [RANTES, monocyte chemoattractant protein-1 (MCP-1), IP-10, and IL-8. ICAM-1, intercellular adhesion molecule-1; PECAM-1, platelet-endothelial cell adhesion molecule-1; VCAM, vascular cell adhesion molecule-1] in hantavirus induced kidney injury (64). Increased bradykinin levels can also trigger cytokine storms during hantavirus infection (65). the most severe vascular affection includes congestion and permeability disturbances during the early phases, followed by severe blood stasis accompanied by leakage, extensive interstitial hemorrhage, severe endothelial degenerative changes, and occasionally anemic necrosis in the deeper medulla that culminate into kidney injury (64, 66–69).

cases were clustered in nine provinces and mainly reported in the spring and autumn seasons (93). Observed SEOV infection in urbanized cities has put an end to the earlier thoughts that hantavirus infection is a rural disease (63, 94–96). PUUV associated AKI is found throughout the European content within the range of *Myedous Glareolus* habitat. In Europe, 35,424 cases of PUUV were reported by the end of 2006, although most of these cases were reported to have an origin from Finland (63, 97, 98). Other countries having significant cases of PUUV kidney injury include Sweden, Belgium, France, Germany, and Norway (97). The DOBV infections are most common in the Balkan region, although both PUUV and DOBV seroprevalence is reported in different Balkan countries including Bosnia, Greece, Slovenia (99, 100). Hantavirus induced HFRS likely occur in other Asian countries as the hantavirus antibodies have been found in rodents and humans in Thailand (101, 102), Indonesia (103, 104), and India (105). The epidemiological studies have revealed that males are more prone to hantavirus infection as compared to females. The male: female disease ratios vary from 2–5:1. However, the fatality rates of infected females are higher compared to males (106–108). Apart from gender, the clinical parameters play a role in the prediction of hantavirus disease severity. For example, patients with low blood platelet count (<60G/l) usually suffer from severe acute renal failure characterized by high creatinine levels in the serum (>620 μM/l) (5, 109). The discovery of 23 hantavirus species and their broad host ranges have potentially elevated the future risks of broad-spectrum epidemics among populations.

Diagnosis of Hantavirus Infection

The serologic tests detecting IgM and/or IgG antibodies to hantavirus antigen are most commonly used for the diagnosis of HFRS and HCPS in suspected patients. The IgG and IgM antibodies against hantavirus N protein can be detected in all most all acute HFRS and HCPS cases upon the onset of symptoms. The recombinant N protein purified from numerous expression systems such as E.coli (110, 111), baculovirus (112), saccharomyces (113, 114), plant (115, 116)and mammalian systems (117) is used as antigen for serologic testing. All three structural proteins (N protein, glycoproteins Gn and Gc) can trigger IgM response at the onset of symptoms (15, 118, 119), however, the IgG response to glycoproteins may be delayed (120). The most common serologic method for hantavirus diagnosis is the rapid IgM capture ELISA method developed by the U.S Army Medical Research Institute of Infectious Diseases and Centers for Disease Control and Prevention (CDC) (121). The test can be completed in 4–6 h (27). The rapid IgM immunochromatography strip test is commercially available for diagnosis of hantavirus infection.

Very specific and rapid diagnostic tests have been developed based on the identification of viral genome in the infected patient samples such as blood, serum, or tissue samples. This sensitive assay can detect the hantavirus infection from day one after the onset of symptoms. However, there are reports that identification of viral genome in infected patient samples can be detected before the first day of the onset of symptoms (122, 123). At this stage, the viral genome can be detected before the appearance of IgM against hantavirus antigens (124). The assay involves

the reverse transcription of the viral genome from the patient samples and PCR amplification of the required viral sequence using the appropriate primer set. Due to low levels of viral RNA in infected patient samples, a nested PCR may be required, using primers targeted to the genomic regions of high homology. The nested PCR approach as a diagnostic method has been developed for HTNV (125), SNV (126), and PUUV (124). Development of multiplex PCR based diagnostic approaches focused on the identification of numerous infectious pathogens from a single patient sample in a short turnaround time is required for quick diagnostic answers and initiation of counter measures to improve disease prognosis. The rapid IgM immunochromatography strip test is commercially available for the diagnosis of hantavirus infection (127). In addition, the rapid HFRS IgG/IgM combo test is also available that simultaneously detects both IgG and IgM antibodies in the serum. since patients develop higher titers of IgM antibody at the time of clinical presentation, the rapid IgM test is more reliable for the detection of acute infection.

Differential Diagnosis

It is important to include leptospirosis and hantavirus infection in the differential diagnosis of acute renal failure (128). Both leptospirosis and HFRS present with classical flu-like symptoms and may be complicated by thrombotic microangiopathy with hemorrhagic phenomena and hepatic and pulmonary involvement (128). However, Jaundice should alert the physician to icteric leptospirosis (128). In high-risk areas, HFRS should be included in the differential diagnosis of acute renal failure of uncertain cause associated with febrile illness, hemorrhagic phenomenon, renal or hepatic dysfunction (129). In addition, the differential diagnosis of hantavirus induced HFRS should include spotted fevers, murine typhus, malaria, hepatitis (non-A, non-B), Colorado tick fever, septicemia, heat shock, leptospirosis, hemolytic uremic syndrome, acute abdominal disease and acute kidney injury (129).

Vaccines Against Hantaviruses

In the United States and Europe, there is no FDA approved vaccine or antiviral therapeutic available for any of the hemorrhagic fever viruses including hantaviruses causing HFRS or NE or HCPS. Thus, except for supportive care, there is no treatment for hantavirus infection at present. However, in Korea, an inactivated hantaan virus vaccine (Hantavax^{TM}) was developed that was put into commercial production in 1990 (130). Although a three dose schedule of this inactivated vaccine showed 90.14% seroconversion in phase III clinical trial, there was no statistically significant protective effect on HFRS patients (131). In China, a bivalent inactivated vaccine against the Hantaan virus and Seoul virus was produced in 1994 that was approved by the Pharmacopeia of China in 2005 (132). Under the expanded immunization program against HFRS by the government of China, approximately 2 million doses of HFRS inactivated bivalent vaccine are used annually (132, 133). HFRS cases have dropped in China after the introduction of an inactivated bivalent vaccine, suggesting the induction of effective humoral immunity that can be maintained up to 33 months after vaccination (132, 133).

The previous research focus was to develop a DNA vaccine against HFRS and HCPS (134). The focus was to express the hantavirus M protein from a plasmid harboring the M gene. Plasmid DNA based vaccines have advantages as they can't replicate or restore virulence and can't spread to the environment (93, 134). Numerous plasmids expressing the M protein from several hantavirus species were developed by the Hopper's group and tested for the development of neutralizing antibody response in Syrian hamsters [Reviewed (93)]. During vaccination, the plasmid DNAs were introduced into the host by a gene gun approach (93, 134). The M gene was cloned in the expression vector WRG 7077 and the resulting plasmids were introduced into hamster and non-human primate models, followed by the evaluation of antibody response (135). Interestingly the expression of Hantaan virus M gene was protective against Hantaan, Seoul and Dobrava virus infections in the hamster model (136). The Rhesus monkeys inoculated with plasmid (pWRG/ANDV-M) expressing the Andes virus M gene, using a gene gun approach, developed higher levels of neutralizing antibodies, and the resulting monkey serum protected 100% of infected hamsters from the fatal hantavirus disease (137). Hoppers's group has used different combinations of plasmids to determine whether simultaneous expression of M gene from different hantavirus species generates a broad immune response protective against multiple hantavirus species. Interestingly, a mixture of plasmids targeting a total of four HCPS and HFRS viruses triggered neutralizing antibodies against all four of them (138). Thus, the plasmid DNA vaccine technology against hantaviruses has created hope for the development of FDA approved vaccine against hantaviruses. The Andes virus DNA vaccine entered clinical trains in 2019. The DNA vaccine trials against HTNV are under way (139).

mRNA Vaccine for HFRS

The groundbreaking new approach to produce mRNA vaccine against SARS-CoV-2 by biopharmaceutical industries (Pfizer and Moderna) in 2020 has given a new direction to the general field of vaccinology and have created new hope for the rapid production of vaccines using this technologically advanced approach. The mRNA vaccines have multiple advantages compared to traditional subunit vaccines, killed and live attenuated viruses, as well as DNA-based vaccines. These advantages include safety, efficacy, and rapid production (140). The mRNA is a non-infectious, non-integrating platform, there is no potential risk of infection or insertional mutagenesis (141). The mRNA is degraded by the host RNA degradation machinery and thus the half-life of synthetic mRNA can be regulated by the chemical modification of constituent nucleotides and the modification of the delivery system used (140–142). The high efficacy of the mRNA vaccine is achieved by various modifications of the synthetic mRNA, increasing its stability and translatability. Due to the high yield of *in vitro* transcription reactions, the mRNA vaccines have the potential for rapid and inexpensive scalable manufacturing. The Conventional mRNA-based vaccines, such as Pfizer and Moderna mRNA vaccine for SARS-CoV-2, encode the antigen of interest and contain 5′ and 3′ untranslated regions (UTRs), a 5′ cap and a 3′ poly A tail (143–145).

TABLE 3 | Some of the therapeutic countermeasures against hantavirus induced HFRS, tested in cell culture or animal models.

Therapeutic type	Target	Mechanism of action	Virus	Model used
Human MAbs (Fab fragments)	Viral Gc	Blocks viral entry	PUUV	Cell culture (151)
Goose PAbs (Igγ/∧Fc)	Viral GP	Blocks viral entry	ANDV	Syrian Hamsters (152)
Rat PAbs (serum)	Viral GP	Blocks viral entry	SEOV	New born rats (153)
Mice MABs	Gc/NP	Blocks viral entry	HTNV	Mice/cell culture (154)
Lactoferin	Viral GP/host	Blocks viral entry	SEOV	Cell culture/mice (155)
Peptides (stem III)	Viral Gc	Blocks viral entry	ANDV/PUUV	Cell culture (156)
Peptidomimetic compounds	Host Receptor	Blocks viral entry	ANDV/HTNV	Cell culture (157)
Nucleoside analogs (Ribavirin)	RdRp	Virus replication	PUUV/HTNV	Mice (158–160)
Nucleoside analogs (ETAR)	RdRp	Virus replication	HTNV	Cell culture (161)
Small molecule inhibitors (K31)	NP	Virus replication	ANDV	Cell culture (162)
Small molecule inhibitors (Arbidol)	Unknown	Virus replication	HTNV	Cell culture (163)
siRNA	Viral genome	Virus replication	HTNV	Cell culture (164)
Ang-1 and S1P	Host	Improves vascular functions	HTNV/ANDV	Cell culture (165)
Corticoids or methylprednisolone	Host	Hormone (immunotherapy)	HTNV	Clinical trial (166, 167)

The mRNA is synthesized *in vitro*, followed by purification by chromatographic methods such as reverse-phase fast protein liquid chromatography (FPLC) or high-performance liquid chromatography (HPLC) (140). The purified mRNA can be administered with or without a career using a proper delivery approach to enhance the efficacy (140). Since the hantavirus M gene encoding the surface glycoproteins has been the focus of the vaccine development for hantaviruses (93). It is possible to transcribe the M gene encoding the glycoprotein by an *in vitro* transcription system. The mRNA can be engineered to harbor 5′ and 3′ UTRs, known to increase the mRNA translation, along with a 5′ cap and a 3′ poly A tail of appropriate length. The mRNA can be codon optimized, chemically modified by incorporating modified nucleotides during synthesis, followed by chromatographic purification to remove the double strand RNA contaminants. Strikingly, purification by fast protein liquid chromatography (FPLC) has been shown to increase protein production from *in vitro* transcribed mRNA by up to 1,000.fold in primary human DCs (146). The purified mRNA can be tested for immunological response in animal models, followed by optimization until the appropriate efficacy is achieved. Vaccination seems to be a viable approach to prevent this zoonotic infection in at least endemic areas or individuals with a higher risk for hantavirus exposure. The current vaccination efforts focused on glycoproteins (139), which elicit a protective neutralization response (137, 147–150), have created hope for the development of the hantavirus vaccine.

Vaccination Strategy

Hantavirus vaccine development must also be viewed from a geographical perspective. A universal hantavirus vaccine will have to consist of several antigenic components to cover for all pathogenic hantaviruses. After testing in animal models, human clinical trials should be carried out in areas with a higher prevalence of hantavirus infection. Once a safe vaccine is developed, its distribution among the population might be a challenge, people may remain less interested in vaccination due to the relatively low incidence of hantavirus infection worldwide. However, the vaccination strategy should consider priorities based on disease susceptibility, age, immunity, and chances for higher virus exposure such as populations living in rural areas or health care professionals working in hospital settings.

Therapeutics for Hantavirus Infection

Hantaviruses primarily infect the endothelial cells of various body organs especially the kidney and lungs. The basic pathological feature of HFRS is the increased vascular permeability whose pathogenesis involves high viral load and excessive immune response of the host. Excessive capillary leakage can lead to hypotensive shock during HFRS. There are no FDA approved post-exposure therapeutic interventions for HFRS. However, several anti-viral drug development strategies have focused to interrupt the virus attachment to the host cell or disrupt the post entry steps of the viral replication cycle (**Figure 4**). Although some of these countermeasures (**Table 3**) have shown protective effects *in vitro*, none of these countermeasures are approved by FDA in the United States for clinical use. In addition, the countermeasure targeting the host system is designed to improve vasculature functions and rebuild immune homeostasis. Ribavarin, a nucleoside analog, has shown antiviral activity in both *in vitro* and *in vivo* studies against the members of Bunyavirales (27). Studies on hantavirus infected patients in China, suffering from acute kidney injury, has revealed that ribavarin therapy starting before the end of the first week of illness reduces the chances of death by seven fold (168, 169). However, ribavarin therapy on HCPS patients did not show any promising results. It was observed that 71% of HCPS patients receiving intravenous ribavarin became anemic and 19% underwent transfusion, suggesting that the efficacy of ribavarin for the treatment of HCP is questionable (170–172). The efficacy of ribavarin as a treatment for hantavirus induced AKI may depend upon the severity of the disease at the time of first administration (27). This is supported by recent observations that early intravenous treatment of ribavarin in hantavirus infected

patients reduced the occurrence of oliguria and severity of renal insufficiency (173). Recently a high throughput screen identified lead compounds targeting the hantavirus N protein (162). Identification of these compounds has created new possibilities for the development of anti-hantaviral therapeutics. The passive transfer of monoclonal antibodies or polyclonal sera to HTNV or PUUV in hamsters, rats, and primates have protected these animals from hantavirus challenges (137, 174–177). A recent study suggested that a DNA vaccine /goose platform can be used to produce an antiviral biological product capable of preventing hantavirus disease when administered post-exposure (152). These observations suggest that a post-hantavirus prophylaxis treatment regime may be effective (178). New treatment strategies focused on the inhibition of virus replication and rapid prevention of vascular leakage in infected patients are urgently needed to prevent the high fatality rates in HCPS and HFRS patients. Elucidation of molecular mechanism and identification of viral and host factors involved in hantavirus induced endothelial cell dysfunction and increased vascular permeability will reveal novel targets for the design of therapeutic molecules to prevent hantavirus induced vascular leakage. Similar approaches to identify host and viral factors playing key roles in the virus replication cycle will provide avenues for the development of antiviral therapeutic agents (**Figure 3**). Some of the well characterized therapeutic targets, such as, the interaction between hantavirus glycoprotein and the host cell receptor, the interaction between N protein and viral genomic RNA, the interaction between N protein and RdRp, the cap snatching endonuclease and polymerase activities of the RdRp (**Figure 3**) can be used for the development of antiviral therapeutics. Nonetheless, the combined therapies targeting both virus replication and vascular leakage will likely improve the prognosis of this zoonotic illness. Finally, the control of animal reservoirs and the advice to populations living in endemic areas to limit the risk of exposure will significantly contribute to the preventive measures of this viral illness.

Kidney Injury by Non-hemorrhagic Fever Viruses Might Provide Insight Into the Hantavirus Induced HFRS That Leads to AKI

Non-hemorrhagic fever viruses such as HIV are known to induce kidney disease. Although retroviral therapies have improved the outcome of HIV infection, the patients living with HIV remain at higher risk for chronic kidney disease due to frequent exposure to nephrotoxins. The kidney biopsies of patients with HIV associated nephropathy (HIVAN) reveal focal glomerulosclerosis and tubular cyst formation with tubulointerstitial inflammation, although such phenotypes may be more severe in patients having widespread use of combination antiretroviral therapy (cART) (179, 180). Such distinct pathologies have not been reported in hantavirus induced AKI. Numerous studies carried out in *in vitro* systems (181–183) and transgenic animal models (179, 184) have demonstrated that HIV can infect glomerular and tubular epithelial cells, and renal expression of HIV genes plays a key role in HIVAN pathogenesis. The expression of HIV transgene lacking *gag* and *pol* genes have been reported to develop kidney disease in rats and mice, showing clinical and pathological resemblance with HIVAN. Since *gag* and *pol* play crucial roles in virus replication, these studies suggest that virus replication is not necessary for HIVAN pathogenesis (184, 185). Further studies in transgenic mice showed that expression of HIV genes *vpr* and *nef* in podocytes induce glomerular disease resembling HIVAN (179, 186). The mechanism by which *vpr* induces podocyte injury remains unclear. However, *nef* is known to induce podocyte differential and proliferation by activating MAPK1,2 and Stat3 signaling pathways (187). The knockout out of Stat3 in podocytes has been reported to ameliorate the HIVAN phenotype in HIV transgenic mice (188). Similarly, *in vitro* studies have revealed that HIV *tat* gene expression induces podocye injury (189). Recent studies have demonstrated the role of Notch signaling and renin angiotensin system in podocyte injury and progression of kidney disease in HIVAN (190–192). This is supported by the amelioration of the HIVAN phenotype in animal models using chemical inhibitors targeted to these pathways (193, 194). In comparison to HIV, it is still unclear whether hantavirus replication or the expression of individual hantaviral genes is sufficient to induce AKI. Inflammatory responses have also been reported to play a role in HIVAN. The noticeable up-regulation of Kappa-B regulated proinflammatory mediators in HIV infected tubular epithelial cells and podocyte in HIVAN models suggested Kappa-B as a target molecule for therapeutic intervention of HIVAN (195, 196). Interestingly, the use of Kappa-B inhibitors ameliorated the HIVAN phenotype in HIV transgenic mice (197, 198). The molecular mechanism by which these viral factors induce kidney injury in HIV patients will help to identify targets for therapeutic intervention of HIVAN. HIV positive people harboring two copies of the APOL1-risk allele are at more risk of developing HIVAN without the use of retroviral inhibitors as compared to HIV positive people having zero or one risk alleles (199). Thus, genetic susceptibility plays a role in kidney injury induced by both HIV and hantavirus infections. Antiretroviral therapies especially nucleoside and nucleotide analogs targeting reverse transcriptase such as tenofovir, adefovir, cidofovir are all capable of inducing renal tubular injury (200, 201). AKI due to acute tebular obstruction and chronic tubulointerstital nephrits by indinavir has limited its use as an antiretroviral drug (202). Thus, while developing antivirals for hantaviruses, it is necessary to pay attention to the possible kidney injury resulting from the use of antivirals, which might worsen kidney disease.

CONCLUSION

Multidisciplinary research studies have provided insights about host mechanisms such as inflammatory responses, endothelial dysfunction, oxidative and ER stress in kidney injury. Virus infection alters the host gene expression and disturbs numerous molecular pathways that may collectively contribute to kidney injury in infected hosts. Although the overwhelming immune response plays a major role in hantavirus disease (**Figure 4**), it is still difficult to draw a detailed mechanistic picture for the pathogenesis of hantavirus induced AKI. Identification of viral

and host factors such as gender, HLA haplotype, viral load, and inflammatory response have helped physicians to predict the clinical outcome of the disease. Analysis of vascular leakage has revealed the breakdown of the endothelial cell barrier by the impairment of cell-to-cell contact. The loss of cellular contact in the endothelium may be due to disturbances in signaling pathways involving vascular endothelial growth factor, E-cadherin, and kallikrein-kinin system (**Figure 4**). Identification and characterization of host factors mediating the vascular leakage during hantavirus infection will provide crucial insights for the development of therapeutic strategies to prevent vascular leakage and improve the prognosis of hantavirus disease. Combinational therapeutic approaches aimed at inhibiting both virus replication and vascular leakage would likely have a better outcome. AKI induced by old word hantaviruses has a good prognosis at present, both in the long and short term. However, hantaviruses are continuously evolving due to mutations in the genome by RdRp, which is deficient in proof-reading activity. The emergence of future virulent strains with the potential to cause severe AKI with a bad prognosis cannot be ruled out. This is supported by the emergence of hantavirus cases in Asia and Europe with clinical manifestations resembling new world hantaviruses and vice versa (203, 204). Thus, the development of potential vaccines and antiviral therapeutics is necessary to keep this zoonotic illness under control. Due to the lack of vaccine and antiviral therapies, preventive measurements such as closer attention of endemic areas, control of mice inside and outside of homes, and prevention of contact with contaminated aerosols is the only way to reduce hantavirus disease mortalities.

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The Icarus Flight of Perinatal Stem and Renal Progenitor Cells Within Immune System

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Our immune system actively fights bacteria and viruses, and it must strike a delicate balance between over- and under-reaction, just like Daedalus and Icarus in Greek mythology, who could not escape their imprisonment by flying too high or too low. Both human amniotic epithelial and mesenchymal stromal cells and the conditioned medium generated from their culture exert multiple immunosuppressive activities. They have strong immunomodulatory properties that are influenced by the types and intensity of inflammatory stimuli present in the microenvironment. Notably, very recently, the immunomodulatory activity of human adult renal stem/progenitor cells (ARPCs) has been discovered. ARPCs cause a decrease in Tregs and $CD3^+$ $CD4^-$ $CD8^-$ (DN) T cells in the early stages of inflammation, encouraging inflammation, and an increase in the late stages of inflammation, favoring inflammation quenching. If the inflammatory trigger continues, however, ARPCs cause a further increase in DN T cells to avoid the development of a harmful inflammatory state. As in the flight of Daedalus and Icarus, who could not fly too high or too low to not destroy their wings by the heat of the sun or the humidity of the sea, in response to an inflammatory environment, stem cells seem to behave by paying attention to regulating T cells in the balance between immune tolerance and autoimmunity. Recognizing the existence of both suppressive and stimulatory properties, and the mechanisms that underpin the duality of immune reaction, will aid in the development of active immunotherapeutic approaches that manipulate the immune system to achieve therapeutic benefit.

Keywords: immune system, stem cells, renal stem cells, perinatal stem cells, immunomodulation, T cells, inflammatory response

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IMMUNE RESPONSE IN HOMEOSTASIS OR HEALING PROCESS

Human beings are regularly exposed to millions of potential pathogen events and organisms, through contact, inhalation or ingestion. Our innate ability to prevent or respond to pathogens is not specific, the adaptive immune response is. Innate immune response is common among vertebrates and invertebrates, and also plants, shielding and preserving them. Approximately 500 million years ago,

we have pieces of evidence supporting the rise of adaptive immune system in the evolution, confinely to vertebrates, in response to complex systems and growing risks associated with elaborate activities and metabolism. The adaptive immune response is slow, particularly for the first antigen encounter. It may take days when not weeks to activate and expand specific clones of B and T cells, and we rely on such long-term memory to react against previous encountered pathogens (1).

Both innate and adaptive immune components are daily involved in preventing bacterial and viral infections, striking a delicate balance between over- and under-reaction to such stimuli. Such delicate and strict activity bears resemblance to the Greek myth of "Daedalus and Icarus", two mythological figures whose restrained and regulated flight, neither too high nor too low, led to successful imprisonment break. At the same manner, when the human immune system is out of balance, not only does it fail to defend the body, but it can even over-react attacking itself. A deranged, rampant immune response may mistake "self" cells for invading pathogens, leading to devastating auto-immune disorders, like lupus and rheumatoid arthritis. At the same manner, when the immune system misidentifies a harmless particle (such as pollen) as an invading parasite, allergies can develop and escalate to life-threating disease. For a long time, immunologists and cell biologists have been struggling to identify molecular pathways involved in such balance and constrain or maneuver immune response towards favorable counteroffensive.

In addition to infective prevention, the innate and adaptive immune response maintains homeostasis and supports innate healing process. Homeostasis is the essential process aimed to preserve normal, healthy ranges for critical factors as energy intake, body temperature and growth. Such articulated and entangled system has the well-known ability to react to external stimuli. In the "Cellular Immunology" book, Barnet offered an important reflection on clonal selection hypothesis, suggesting as "self-defense" was originally concerned not with defense against infection but with the preservation of cellular integrity of the body (2).

When a body is under attack, infected by micro-organisms, it needs to invest a lot of energy to fight invaders back, through immune cellular response. Nature developed an intriguing mechanism, privileging immune reaction over homeostasis (such as preserving hydration or temperature levels), in order to ensure survival of the whole body in such critical event. Nevertheless, homeostasis needs to be reassured as soon as possible, and immune response is suddenly restored once the insult is contained and the risk removed.

Therefore, scientists immediately recognized the importance in understanding the molecular aspects of such critical response, and they have been making huge efforts to understand and develop a method to control and eventually enhance (innate and/or adaptive) immune system.

In this delicate balance also some types of stem cells play a role. Mesenchymal stromal cells (MSC) can move towards inflammatory areas and exert immunomodulatory and anti-inflammatory effects *via* cell-to-cell contacts with lymphocytes or *via* the generation of bioactive molecules, such as cytokines, growth factors, and chemokines, that have autocrine/paracrine effects (3). During the past decades, embryonic and perinatal stem cells (derived from extra-embryonal and gestational tissues) have attracted interest and endeavor. In particular, perinatal cells have been recently gained recognition as advanced therapy medical products proposed in regenerative applications (4).

Recently, it has been discovered that also human adult renal stem/progenitor cells (ARPC) have a potent immunomodulatory capacity and may contribute locally to limit tissue damage and inflammation (5). Researchers are considering new ways to treat autoimmune disorders, sepsis, and transplant surgery, exploiting these cells (6).

HUMAN AMNIOTIC EPITHELIAL CELLS AND THEIR IMMUNE-MODULATORY MOLECULES

Placenta-derived perinatal stem cells are characterized by steady immunomodulatory capacity to nourish protection from maternal immune system. During a 9-month incubation, the (semi)allogeneic fetus requires maternal immune adaption and acquisition of tolerance at the maternal–fetal interface. Although decidua-resident T cells and macrophages, characterized by regulatory properties, have been recently described playing a central role in promoting fetal tolerance, stromal cells have been largely implicated in modulatory and tolerogenic effects (7). The inner layer of human placenta, amnion-chorionic layer and amnion membrane in particular, represents a protective barrier against maternal recognition and fetal rejection. Several groups have recently collected and described evidence in support of restraining effects granted by human amniotic epithelial cells (hAEC) towards immune effector cells. Recent reports describe altered maturation of antigen-presenting or dendritic cells, inhibitory effect on natural killer (NK) cell, decreased circulatory mononuclear cell proliferation or activation, and switch toward regulatory phenotype in B and T cells or macrophages (8–11). All these immune-regulatory properties, rather than immune-suppressive effects, have successfully bridged the embryo during a 9-month development and may convey towards new, innovative cellular treatments for regenerative purposes.

Embryonic and perinatal tissues possess potent immune-protective properties, not fully understood or characterized. Constitutive expression of surface proteins and enzymes, couple with secreted forms of modulatory mediators have been recently described and reported critical in modulatory processes. Initially, peculiar expression of tolerogenic human leukocyte antigen G (HLA-G), both at mRNA and protein levels, has been reported in embryonic tissues (12, 13), and lately identified and described in fetal and maternal layers of human placenta (11, 14). Conversely, to ubiquitously expressed polymorphic HLA class Ia antigens, HLA-1b molecules display a restricted pattern of expression in selected tissues (as thymus,

cornea, proximal nail matrix and erythroblasts) in healthy individuals (15–17). HLA-G protein was controversy described also in MSC (18), lately restricted to soluble forms (19). Soluble form of HLA-G (sHLA-G) has been measured in the serum or plasma, secreted by monocytes or T cells in pathological conditions, such as inflammatory diseases or viral infections, or in neoplastic and autoimmune disorders, but also in response to solid organ transplantation (20, 21).

On the contrary, trophoblast cells and epithelial cells lining the umbilical cord or amnion membrane have recently described to expressed both membrane-bound molecules and release soluble forms of HLA-G and HLA-E (both HLA-1b molecules) (14, 22, 23). The non-polymorphic HLA-1b expression has been correlated to other paracrine factors (i.e., chemo- or cyto-kines as IFN-gamma, TGF-beta, IDO, GM-CSF, pro-inflammatory or anti-inflammatory interleukins) (24–27) or to oxygen tension (hypoxic or near-hypoxic conditions) (28).

Among immunomodulatory molecules, adenosine (ADO) has recently gained an important role in different patho-physiological settings and in innovative cell-based therapies. Extracellular ATP is the primary substrate for ADO typically hydrolyzed by membrane-bound ectoenzymes as CD73 (5′-nucleotidase). CD73, even if expressed by several cells, has been for long time recognized as identity marker for an efficient and rapid identification of MSC products (29). CD73 represents the final ecto-enzyme to complete the adenosinergic loop (30). A membrane-bound ecto-nucleoside hydrolase (CD39), present on the same cell or another adjacent element, is responsible for hydrolyzation into mono- or di-phosphate nucleotide offered to CD73 in the so called "canonical" pathway (31). However, such "canonical" way is not the sole molecular pathway responsible for T cell modulation. Another "alternative" pathway has been recently described where nicotinamide adenine dinucleotide (NAD^+) rather than ATP is the initiating factor for modulation for T lymphocytes (32). Nicotinamide adenine dinucleotide can be actively secreted across cell membrane and trigger a cascade of extracellular signals (27). CD38, another membrane ecto-nucleotidase, lends ADPR over to CD203a, a surface nucleotide pyrophosphatase/phosphodiesterase 1 that generates mono-phospate adenosine (AMP) (**Figure 1**). Similarly to "classical" pathway, AMP generated in the "alternative" cascade is the converted into ADO by CD73 (31).

Several aforementioned immunomodulatory pathways were initially identified and ascribed limitedly to MSC. In the position manuscript published in 2006 by the International Society for Cell and Gene Therapy (ISCT) experts, the ecto-nucleotidase enzyme CD73 was exclusively described on MSC, leading to an efficient identity marker to qualify stromal multipotent cells (29). The limited but constitutive expression of CD73 on human MSC has been lengthy maintained in quality assurance assays for MSC therapies. Such membrane-bound enzyme has also been detected, in combination or close proximity to all the aforementioned ectoenzymes, on different cell populations, namely, immune effector cells (33) or tumor cells (34). A complete loop achieved by interaction of different cells or eventually on a single cell has been proved to confer immune-protection. Recently, confirmation of constitutive, high-density presence of CD73 on the surface of hAEC has been reported. Interestingly, dichotomic effects has been measured and described in hAEC when exposed to immune cells: while T and NK cells are significantly inhibited in proliferation and activation by hAEC direct contact (10) or close proximity (23). B cell expansion *in vitro* has been surprisingly increased by few amnion cells in the compartment (10). Furthermore, ATP, NAD^+ and ADO released into the extracellular space, have been recently described having an important role in the promotion of T and B regulatory cells and M2 macrophages (10). The same group reported for the first time the constitutive presence of all five plasma membrane nucleotidases on a single non-neoplastic cell (hAEC), suggested as such ecto-enzymatic activity plays an important role in addition to the non-polymorphic HLA-G mediation.

The most recent paradigm is that hAEC or perinatal stem cell in general, do not necessarily need to mature into adult somatic cell types (multipotency), but these fetal-derived stem cells can exert important immuno-modulatory activities *via* indirect paracrine mediators. Such mediators (collectively known as secretome) include soluble proteins (cytokines, chemokines, growth factors and proteases) (35) and extracellular vesicles (EVs) of micro- and nano-size (23). Notably, the expression of both membrane-bound HLA-G and soluble form of non-polymorphic HLA-1b molecules (sHLA-G and sHLA-E) in every primary hAEC effects on immune cells generated by surface molecules. To support the moiety in soluble molecules, an impressive amount of small and large size vesicles were released by full term hAEC, mediating anti-inflammatory, anti-fibrotic, anti-microbial, anti-apoptotic, pro-regenerative and immune-modulatory properties sufficient to restore normal architecture and function to damaged organs.

FAS receptor expression has also been observed at high levels in embryonic tissues (36, 37). Amniotic cells and their conditioned medium have been suggested for the treatment of chronic inflammation and immune alterations due to their broad immunosuppressive properties (38).

ADULT RENAL STEM/PROGENITOR CELLS: NEW PERSPECTIVE FOR THE T CELL MODULATION

Notably, very recently the immunomodulatory activity of adult renal stem/progenitor cells (ARPCs) has been reported. Tissue-specific cells, expressing CD133 and CD24 antigens on their surface, have been described as progenitors of tubular cells and podocytes during human kidney formation (39–46). Renal progenitors are abundant in the kidneys at 8–9 weeks of gestation, when the kidney is largely made up of immature metanephric mesenchyme-derived structures; the percentage of progenitor cells decreases over the time, until they account for around 2% of renal cells in adult human kidneys (47).

ARPCs can help in kidney regeneration in two ways: by directly differentiating and by secreting reparative molecules.

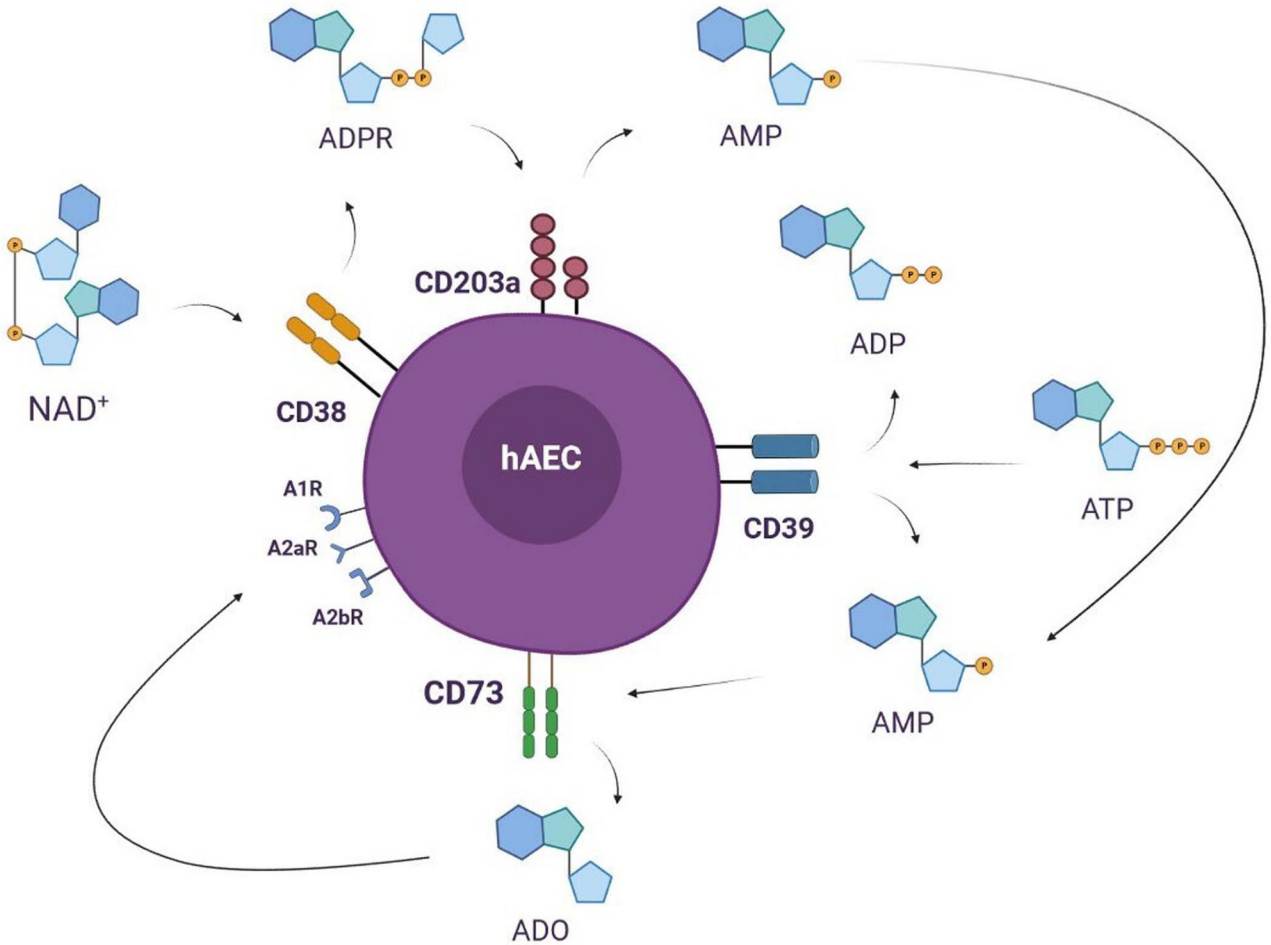

FIGURE 1 | The canonical pathway of ADO production initiates with CD39, an ecto- diphosphohydrolase responsible for hydrolyzation into mono- or di-phosphate nucleotide which are suddenly converted into soluble ADO by CD73. CD39 and CD73 can be present on the same cell or in close proximity when two cellular elements are adjacent. Interestingly, hAEC has been recently proved to constitutively expressed both canonical ecto-enzymes on membrane surface. However, such "canonical" way is not the sole molecular pathway responsible extracellular ADO production. Another "alternative" pathway has been described where nicotinamide adenine dinucleotide (NAD^+), rather than ATP, is the initiating factor for ADO production (and relative immune cell modulation). NAD can be actively secreted across cell membrane and trigger a cascade of extracellular signals, starting with CD38 resulting in soluble ADPR. CD38, another membrane ecto-nucleotidase, lends ADPR over to CD203a, a surface nucleotide pyrophosphatase/phosphodiesterase 1, that generate mono-phosphate adenosine (AMP). Similarly to "classical" pathway, AMP generated in the "alternative" cascade is converted into ADO by CD73, the point of contact for the two canonical and alternative pathways.

They can differentiate into epithelial, endothelial, osteogenic, and adipogenic cells, among others (43, 48, 49). ARPCs have been shown to be able to regenerate lengthy segments of renal tubules and missing podocytes in cortical nephrons after acute kidney injury (AKI) (50, 51). Furthermore, Toll-Like Receptor 2 (TLR2) ligands activate $CD133^+$ renal progenitors, which can secrete reparative factors that can repair renal tubular cells damaged by chemical agents like cisplatin (46).

TLR2 can act as a damage sensor, and its activation can result in stem cell proliferation and differentiation, among other things. Following TLR2 stimulation, cytokines and inflammatory chemokines such as C-3 and MCP-1, IL-6, and IL-8 are released (43, 44, 52, 53). Renal progenitors can repair both physical and chemical damage, such as a wound in epithelial tissue caused by cisplatin, a widely used chemotherapeutic drug that can cause nephrotoxicity side effects. Following renal tubular cell injury, ARPCs released inhibin-A and decorin, which were directly involved in the cell regeneration process (43).

In addition, following LPS exposure, ARPCs have the ability to prevent endothelial dysfunction and protect the endothelium compartment, facilitating kidney healing. The antifibrotic activity of ARPCs is mediated by the release of antiseptic molecules CXCL6, SAA4, and BPIFA2 (54).

Recently, it has been reported, for the first time, that human ARPCs also possess immunomodulatory capabilities (5). Specifically, ARPCs have shown modulatory features towards $CD3^+$ $CD4^-$ $CD8^-$ (double negative; DN) T cells and promotive capacity for regulatory T cells (Tregs). However, renal progenitors

need to be triggered to achieve full potential immunomodulative effect. Toll Like Receptor 2 (TLR2) agonists, such as lipoteichoic acid (LTA), a major constituent of the protective wall in gram-positive bacteria, are required to stimulate innate immune responses to gram-positive bacteria (55), and activate ARPCs (46, 56). Activated ARPCs have been proved to inhibit peripheral blood mononuclear cell (PBMC) proliferation, limitedly to Treg and DN T cells. When ARPCs detected inflammation (by LTA binding on TLR2), Treg generation was inhibited both in the short- (5 days) and long-term (15 days) (5, 43). If ARPCs were not activated by LTA, a partial Treg inhibition was observed, limitedly to short-term effect. Such effect was reverted resulting in increased Treg generation in the longer term. Renal progenitor cells reach out immune cells through paracrine mediators. Soluble factors as PAI1, CXCL1/GRO-α, GM-CSF, MCP1, IL-6, IL-8, and MIF have been detected in ARPC secretome analysis when co-cultured with T cells. Interestingly, PBMC stimulation by combination of PAI1, CXCL1, MCP1, and GMCSF chemokines, had an immunomodulatory effect specifically on Treg and DN T cells.

The ability of stem/progenitor cells to affect Tregs is not new. MSC can also modulate T cell sub-populations (57, 58). Instead, what is novel is the evidence supporting stem/progenitor cell ability to modulate the recently discovered population of DN T cells. In both mice and humans, DN T cells have been described as powerful suppressor cells. They are antigen-specific suppressor cells and use trogocytosis to regulate T cells with the same antigen specificity. This is a distinguishing trait that makes them intriguing for cellular treatment, transplantation, and autoimmunity. Moreover, to control tissue immune response, proliferative DN T cells released potent anti-inflammatory cytokines such as IL-27 and IL-10 (59).

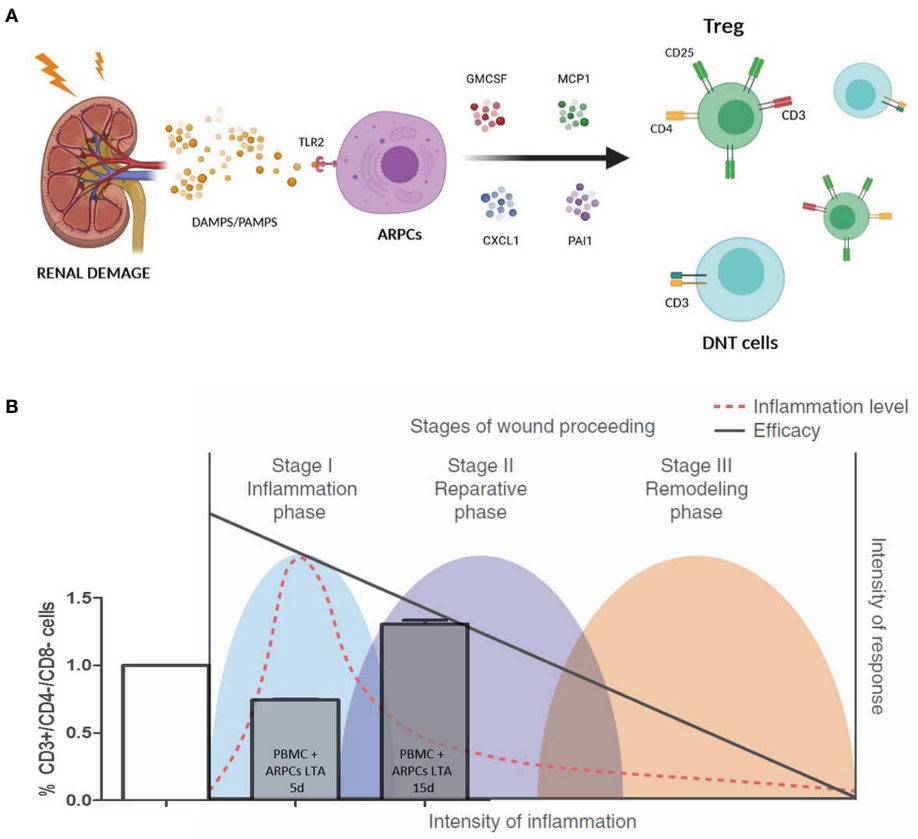

FIGURE 2 | **(A)** Following a renal damage ARPCs detect the insult by the binding of Pathogen Associated Molecular Patterns (PAMPs), such as lipoteichoic acid (LTA), or damage-associated molecular patterns (DAMPs) on the Toll-like receptor 2 (TLR2). ARPCs then inhibhit the Treg and the double negative (DN) T cells through paracrine mediators as PAI1, CXCL1, GM-CSF, and MCP1 to favor the initial phase of inflammation. **(B)** ARPCs can mediate immunomodulation and affect inflammatory state. The physiological response to tissue damage can be divided into three phases: inflammatory, reparative, and remodeling. During this process, inflammatory status (defined as the types and concentrations of cytokines and cells of the immune system present) changes considerably: proinflammatory influences (red dashed line) are dominant in the inflammatory, infection-fighting phase and diminish in the reparative and remodeling phases that follow, which allows wound healing. In the context of the intensity of the immune response (right vertical axis), the inflammatory response (red dashed line) fluctuates during the wound-healing process. Such changes in inflammation substantially alter the effects of mesenchymal stem cells (MSC)-meditated immunomodulation, which results in a variable correlation between the intensity of inflammation and efficacy of MSC treatment (solid black line). Such changes in inflammation are also affected by ARPC-meditated immunomodulation. ARPCs cause DN T cell decrease at the initial phase, promoting inflammation, and DN T cell increase in the late inflammation stages, favoring the inflammation quenching.

Allograft rejection, GVHD, and auto-immune diabetes can all be prevented or reversed by DN T and Treg cells (60). Their homeostatic role is attained by suppressing excessive host immune responses (61, 62). Furthermore, recent reports suggest that DN T cells in turn can regulate B cells, DCs, and NK cells (60).

The physiological response to tissue damage usually follows a three-step pattern: inflammatory, reparative, and remodeling. Inflammation status (expressed as the types and quantity of immune system cytokines and cells present) may significantly vary. Inflammation intensity is high in the infection-fighting stage, while decreases in the subsequent reparative and remodeling stages that enable wound healing (63). As a result, ARPCs reduce Tregs and DN T cells in the early stages of a tissue damage, encouraging inflammation, whereas they increase these regulatory cells in the late stages of the tissue repair process, favoring inflammation quenching. In chronic inflammatory event, ARPCs further support DN T cell generation to prevent escalation and related risks (**Figure 2**). The ARPCs therefore reveals useful also in the setting of AKI in which the role of innate immunity in acute tissue injury is well established, with engagement of complement, cytokines, and leucocytes (64).

Different stimuli may modulate the immunomodulatory activity of ARPCs. Both IFN-α or IFN-β can suppress renal progenitor differentiation into mature podocytes (65) whereas no data are present on IFN-γ stimulation on ARPCs. However, tubular epithelial cells express PD-L1, an inducible antigen that negatively regulates T-cell responses elicited by IFN-γ (66). Further studies are needed to deep this recently discovered ARPC ability to regulate T cells.

DEADALUS AND ICARUS: THE IMPORTANCE OF STRIKING BALANCE

Still more than two thousand years later, Greek mythology passes an invaluable heritage of knowledge in the form of tales where general concepts can help and lead to a more general level of knowledge. We find in Daedalus and Icarus flight a perfect analogy with the immune balance. Daedalus, to revert his own fate and fly away from their imprisonment, wore wings made of feathers and wax. But the wise father warned him on the importance of a balanced and a fine-tuned control: an unbalanced flight would have eventually resulted in wing consumption by the heat of the sun or the humidity of the sea, leading to fatal loss. Icarus, the boisterous son, disattended the recommendations of his father, paying such uncontrolled, heighten flight with his own life. Such ancient allegoric tale perfectly resembles our modern view of immune system response. Human immune system over- or under-reaction to external or internal stimuli leads to an uncontrolled cascade of effects, eventually leading to death. A closer look to renal progenitor cells in response to an inflammatory environment leads to a close similarity between ARPC and the wise Daedalus, where a tweaked and calibrated regulation in Tregs and DN T cells conveys towards efficient balance between immune tolerance and autoimmunity.

Moving from the awareness that many renal diseases are characterized by inflammatory infiltrating T cells, and the recent identification of prevalence in DN T cells, further research into the role of ARPCs in immune system modulation may pave the road to new therapeutic interventions or adjunct cell-based therapies for both acute and chronic kidney disorders. It is in such direction, that new proposed approaches based on allogenic perinatal stem cells offering an immune privileged locale to the kidney stem/progenitor cells of the recipient can convoy towards innovative therapies, where immune-modulation, rather than immune-suppression, will support regenerative capacity, abetting normal immune response towards eventual infective agents (such as SARS viruses and COVID-19).

Innovative stem cell strategies are promising new cellular tool for advanced medical treatments, currently evaluated and tested by different groups worldwide, aimed to refine and handle balance between immune-repression and immune-stimulation. Recognizing the existence of both suppressive and stimulatory properties, and the mechanisms that underpin the duality of immune reaction, will eventually aid in the development of active immunotherapeutic approaches that manipulate the immune system to achieve therapeutic benefit.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FS and RG made the concept and design of the article. AP, CC, KK, and AS drafted the article and contributed to literature search. GC, GBP, and LG critically revised the article. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Stem Cell-Derived Extracellular Vesicles as Potential Therapeutic Approach for Acute Kidney Injury

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Acute kidney injury is a frequent complication of hospitalized patients and significantly increases morbidity and mortality, worsening costs and length of hospital stay. Despite this impact on healthcare system, treatment still remains only supportive (dialysis). Stem cell-derived extracellular vesicles are a promising option as they recapitulate stem cells properties, overcoming safety issues related to risks or rejection or aberrant differentiation. A growing body of evidence based on pre-clinical studies suggests that extracellular vesicles may be effective to treat acute kidney injury and to limit fibrosis through direct interference with pathogenic mechanisms of vascular and tubular epithelial cell damage. We herein analyze the state-of-the-art knowledge of therapeutic approaches with stem cell-derived extracellular vesicles for different forms of acute kidney injury (toxic, ischemic or septic) dissecting their cytoprotective, regenerative and immunomodulatory properties. We also analyze the potential impact of extracellular vesicles on the mechanisms of transition from acute kidney injury to chronic kidney disease, with a focus on the pivotal role of the inhibition of complement cascade in this setting. Despite some technical limits, nowadays the development of therapies based on stem cell-derived extracellular vesicles holds promise as a new frontier to limit acute kidney injury onset and progression.

Keywords: acute kidney injury, acute tubular necrosis, ischemia-reperfusion injury, sepsis, chronic kidney disease, stem cell, extracellular vesicles, regenerative medicine

INTRODUCTION: DEFINITION AND CLASSIFICATION OF ACUTE KIDNEY INJURY

The term Acute kidney injury (AKI) indicates a sudden worsening of renal function due to acute renal damage, with consequent accumulation of nitrogenous waste products and alteration of hydrosaline and acid-base homeostasis. In the past decades, several criteria have been proposed in order to uniform the definition of AKI: the recommendation statements of 2012 Kidney Disease

Improving Global Outcomes (KDIGO) Clinical Practice Guideline define AKI as a rise in the serum creatinine (sCr) level by 0.3 mg/dl within 48 hours, or a 1.5-fold increase from baseline within prior 7 days; or oliguria (<0.5 ml/kg/h for 6 hours). In addition, AKI is staged for severity – from mild stage 1 to most severe stage 3 – according to serum creatinine values and urine output, usually classifying patients requiring renal replacement therapy (RRT) as stage 3 KDIGO. Several causes may induce AKI in patients with or without underlying chronic kidney disease (CKD); a potential classification is based on pathophysiological mechanisms of renal injury such as kidney hypoperfusion (pre-renal AKI), parenchymal kidney diseases (intra-renal or parenchymal AKI, which includes acute tubular necrosis, ATN) and obstruction of the urinary tract (post-renal AKI). Incomplete recovery of an AKI event due to persistence of renal pathophysiologic process can lead to AKI-CKD transition, especially in patient with some degree of pre-existent CKD (**Figure 1**). As for AKI epidemiology, incidence and prevalence are not well defined due to different AKI definitions. Despite these limitations, a metanalysis of Susantitaphong et al. (1) observed that in-hospital AKI incidence was 22% – using 2012 KDIGO AKI definition – while it reached 57% in intensive care units (ICU) according to the multinational AKI-EPI study (2). Overall AKI incidence seems to be rising in the United States and it is associated with higher health care costs, greater long-term care, increased risk of CKD and hospital mortality (3, 4). This increase especially affects Afro-American population, due to genetic factors which also condition a reduced number of nephrons. A large metanalysis (5) and recent studies (6) confirmed that Black race is an independent risk factor for AKI.

Similar to CKD, other factors associated to the rise of AKI incidence are older age, increasing burden of comorbidities (e.g. hypertension, diabetes mellitus, CKD, heart failure, sepsis, cancer), improved clinician's awareness (leading to inclusion of less severe forms), growing use of nephrotoxic drugs and increasing frequency of surgical and angiographic procedures (7, 8). Among these, older age is strongly associated with AKI incidence through multifactorial mechanisms. Renal senescence reduces nephron number and functional reserve, predisposing to relapsing AKI episodes and also to maladaptive repair, incomplete recovery and AKI-CKD transition. This process can be considered as a form of accelerated renal senescence and will be analysed in a specific Section (9, 10).

Different causes related to ischemia-reperfusion injury due to hypovolemia and/or hypoperfusion, administration of nephrotoxic drugs and the presence of sepsis/septic shock have been identified as hallmarks for AKI development. Recent studies have also provided new insights into complex AKI pathophysiology, with remarkable progress especially in the field of sepsis-associated AKI (s-AKI).

Sepsis is a dysregulated immune response to infection that causes multiple organ dysfunction: the immune response after a septic insult is characterized by unbalanced hyperinflammation and immune suppression (11). Sepsis-associated excessive inflammation is sustained by several cell types including leukocytes (neutrophils, macrophages, natural killer cells), endothelial cells (EC), cytokines, complement products, and the coagulation system (12). The widely used concept of "cytokine storm" refers to the release of proinflammatory cytokines such as TNF-alpha, IL-1b, IL-12, and IL-18 that may contribute to organ injury (12). However, a complex network of several mediators is embedded in sepsis-associated multiple organ dysfunction such as the release of extracellular traps by neutrophils (NETs) (13), the complement activation resulting in the release of the anaphylatoxins C3a and C5a, the immunothrombosis in the microvasculature mainly triggered by tissue factor that initiates blood coagulation by forming a complex with clotting factor (F) VIIa, thereby inciting blood coagulation by activating FX and FIX (14). These alterations have been also recently described in COVID-19-associated AKI (15).

Sepsis represents nowadays the most frequent cause of AKI in critically ill patients admitted to Intensive Care Units (ICU) with pathogenic mechanisms similar to those described above for immune dysfunction. Indeed, experimental and clinical studies aimed to evaluate kidney perfusion clearly demonstrated that during sepsis, AKI can develop in the presence of a normal or even increased renal blood flow: this finding suggested the presence of other key mechanisms of tissue injury such as microvascular derangement, endothelial dysfunction, inflammation, metabolic reprogramming and sub-lethal injury of tubular epithelial cells, overthrowing the simplistic old paradigm based on kidney hypoperfusion (16, 17). Recent works have further shed light on these etiopathogenetic mechanisms: for example, interaction of damage-associated molecular pattern (DAMPs) and pathogen- associated molecular pattern (PAMPs) molecules with Toll-like Receptors (TLRs) expressed on renal tubular epithelial cells (RTEC) and other renal resident cells can activate several pro-inflammatory pathways (18) and mitochondrial DNA (mDNA) damage due to oxidative stress also plays a key role in inducing RTEC dysfunction (19). Basically, PAMPs and DAMPs can be freely filtered by glomeruli, a process favored by sepsis-induced increased permeability, thus reaching the tubular lumen and inducing functional alterations of RTECs; moreover, microvascular damage of ECs located in the peritubular capillaries may enhance metabolic alterations of RTECs (20).

Innate and adaptive immunity activation during AKI have been increasingly recognized to play an important role in AKI. Cross-talk between RTECs and immune cells through a network of cytokines seems to be essential to induce T cell phenotype changes which affect AKI evolution (21). In this setting an important role of IL-15 has been delineated. RTECs express IL 15 and its receptors and intrarenal IL 15 levels inversely correlate with AKI severity in experimental models. Inhibition of RTECs apoptosis during pathological stress appears to explain IL 15 protective effects (22) Furthermore, IL 15 can reduce extracellular matrix (ECM) synthesis by myofibroblasts and monocyte chemoattractant protein (MCP-1) release by macrophages and also inhibits Transforming Growth Factor-β

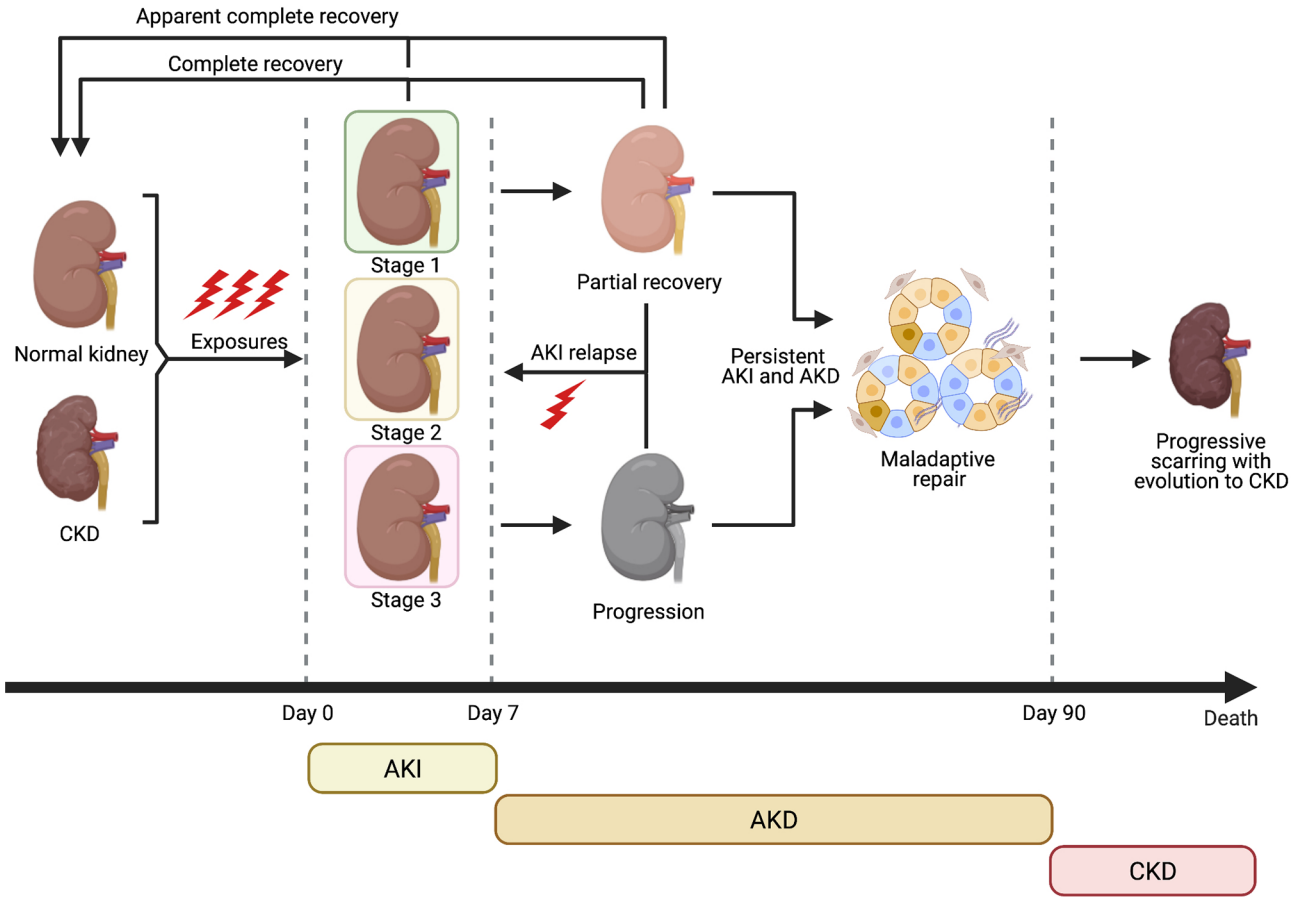

FIGURE 1 | Clinical course of AKI and evolution towards CKD (Created with BioRender.com).

(TGF-β) 1-induced RTEC epithelial-mesenchymal transition (EMT): these multiple anti-fibrotic effects suggest a role in the prevention of AKI-CKD transition (23, 24).

Despite progress in understanding AKI pathophysiology, however, efforts to develop targeted therapies has not led to robust results yet (16) and treatment is still mainly supportive (e.g. hemodynamic stabilization, dose adjustment or discontinuation of nephrotoxic drugs, antibiotic therapy in s-AKI) (25–28). Also in s-AKI, neither pharmacological approaches nor extracorporeal blood purification therapies aimed at removing PAMPs and DAMPs have led to a significant improvement of in-hospital AKI incidence and mortality (16).

In this setting, stem-cell (SC) therapy and SC-derived extracellular vesicles (EVs) represents a new frontier in the treatment of acute and chronic kidney disorders, because of their anti-inflammatory, immunomodulatory and regenerative properties. Recent studies observed the beneficial therapeutic properties of Mesenchymal Stromal Cells (MSCs) in ischemic AKI, renal transplantation, lupus nephritis and diabetic nephropathy (29, 30). These actions are mainly paracrine and mostly mediated by transfer of EVs containing microRNAs, mRNAs, and proteins that reprogram cell functions *via* immunomodulatory and regenerative effects (31), as detailed in the following section.

In this review, we summarize the state-of-the-art knowledge on EVs derived from different types of stem cells (SCs) as therapy of AKI, focusing on impact of EVs on different pathophysiological mechanisms underlying toxic, ischemic and s-AKI (29–31) and AKI-CKD transition.

GENERAL FEATURES OF STEM CELLS AND EXTRACELLULAR VESICLES (EVs)

SCs are unspecialized cells with self-renewal capacity, which can potentially differentiate into any cell type of organism. Their therapeutic potential is revolutionizing regenerative medicine and is providing promising applications also in the field of Nephrology (32).

Among progenitor cells, MSCs represent the most studied type over the last decades: MSCs are adult multipotent stromal cells with a high proliferative potential, derived from non-hematopoietic precursors. They can differentiate into

mesenchymal (osteocytes, adipocytes and chondroblasts) and non-mesenchymal lineages (33). Initially found in bone marrow (BM) (34), they were subsequently isolated from multiple fetal and adult tissues such as adipose tissue (AD-MSCs), umbilical cord blood (UC-MSCs), fetal membrane (FM-MSCs) and human placenta (hP-MSCs) (35–37). This makes them one of the most accessible SC type and an attractive candidate source to develop products for cell therapies (38). Due to the ease of preparation, MSCs remain the most common option among cellular therapies and have already proven to be safe and effective in reducing AKI in experimental models and clinical trials, displaying cytoprotective, regenerative and immunomodulatory properties (39, 40).

Other types of SCs have been recently investigated as a therapy for AKI, including inducible Pluripotent Stem Cells (iPSCs). These are derived from differentiated adult cells (e.g. keratinocytes, fibroblasts) which are induced into pluripotency by exposing them to specific reprogramming factors through viral vectors and directed towards renal lineages such as podocyte progenitors (41). Administration of iPSC has proved to be effective in a rat model of AKI, reducing oxidative stress and inflammation (42).

Spermatogonial stem cells (SCCs) have also been shown to be capable of differentiating into pluripotent stem cell lines, converting into embryonic-like SCs and differentiating into renal tubular-like cells. Also, this type of SC has shown promise in restoring kidney function after AKI (43).

Endothelial progenitor cells (EPCs), a BM-derived progenitor type able to circulate in the bloodstream, play a major role in vascular integrity by protecting ECs and promoting angiogenesis and recovery also in AKI models (44).

Of interest, the beneficial effects of all these SC types are predominantly mediated by paracrine/endocrine actions, *via* secretion of growth factors and especially EVs. The latter represent crucial components of cellular secretome and mediate intercellular communication through transfer of bioactive molecules between originator and recipient cells, especially mRNAs and microRNAs (miRNAs), modifying phenotype and function of target cells (45, 46). As their parental cells, EVs are not immunogenic and may successfully activate regenerative processes in injured cells and tissues (47, 48).

Recently, EVs released from renal cells themselves have been investigated as therapy for AKI: kidney-derived MSCs (49), glomerular and tubular renal progenitors (50), renal tubular epithelial cells (RTEC) (51) and even urinary EVs (uEVs) from healthy subjects have shown initial promising results in this setting (52). The efficacy of SC-derived EVs has been demonstrated in different settings including numerous studies showing an interesting cross-kingdom communication: EVs from different eukaryotic and prokaryotic kingdoms are involved in many processes including host-pathogen interactions and modulation of cellular functions (53). Of note, some studies showed that exogenous dietary RNAs of plant and animal origin are protected from food processing and gut microenvironment through encapsulation within EVs. EV-carried RNAs (in particular miRNAs) are able to exert biological activities between the host and gut microbiota influencing organ function in the recipient after ingestion (54). Our group has already demonstrated the horizontal transfer of mRNAs and miRNAs from SC-derived EVs of human origin in rat cells: indeed, in a rat model of anti-Thy1.1-induced mesangioproliferative glomerulonephritis, EVs released from human Endothelial Progenitor Cells (EPCs) horizontally transferred to rat mesangial cells distinct mRNAs coding for Factor H, CD55 and CD59, thus inhibiting complement-induced apoptosis and C5b-9/C3 mesangial cell deposition (55). Moreover, the same type of EVs protected the kidney from ischemia-reperfusion injury in rats by delivering their RNA content, the miRNA cargo of which was shown to contribute to reprogramming hypoxic renal endothelial and tubular epithelial cells to a regenerative program (56).

EV-based therapeutic approach has some advantages compared to cell-therapy. First, EVs exhibit a superior efficacy profile as they pass through the blood-tissue barriers and efficiently reach injured cells (57). Second, no adverse immune responses have been reported in patients undergoing allogeneic administration of SC-EVs and no evidence of oncogenic potential of SC-EVs has been reported. In fact, they can inhibit tumor growth by interfering with cell cycle and inducing apoptosis and/or necrosis of cancer cells (58, 59). Thus, EVs represent a feasible, cell-free therapeutic alternative and their role has recently been investigated in several renal diseases (45, 46), especially in AKI. This is the setting in which therapeutic properties of SC-EVs and the rationale for their employment has been better defined and represents the main focus of this Review.

RATIONALE OF THERAPY WITH EVs IN AKI

A growing body of pre-clinical studies indicates a potential therapeutic role of EVs derived from MSCs and other progenitor cell types especially in IRI-induced AKI. EVs can shuttle miRNAs and other genetic material into injured renal cells – such as RTEC and ECs – and epigenetically re-programme them. This leads to activation of signaling pathways, which exerts multiple beneficial effects within three main areas (60, 61) (**Figure 2**).

- renal protection: inhibition of apoptosis/necrosis, oxidative stress, senescence and fibrogenesis; promotion of autophagy (62).
- renal regeneration: stimulation of cell proliferation, migration, tubular dedifferentiation, angiogenesis (63).
- immunomodulation: anti-inflammatory and immunosuppressive effects, mainly through induction of M2 macrophages and T-regulatory cells (Treg) (64) and modulation of NK cell activity (65).

The combination of these effects can heal injured RTECs and ECs, thus promoting regression of AKI (66). Of note, pre-

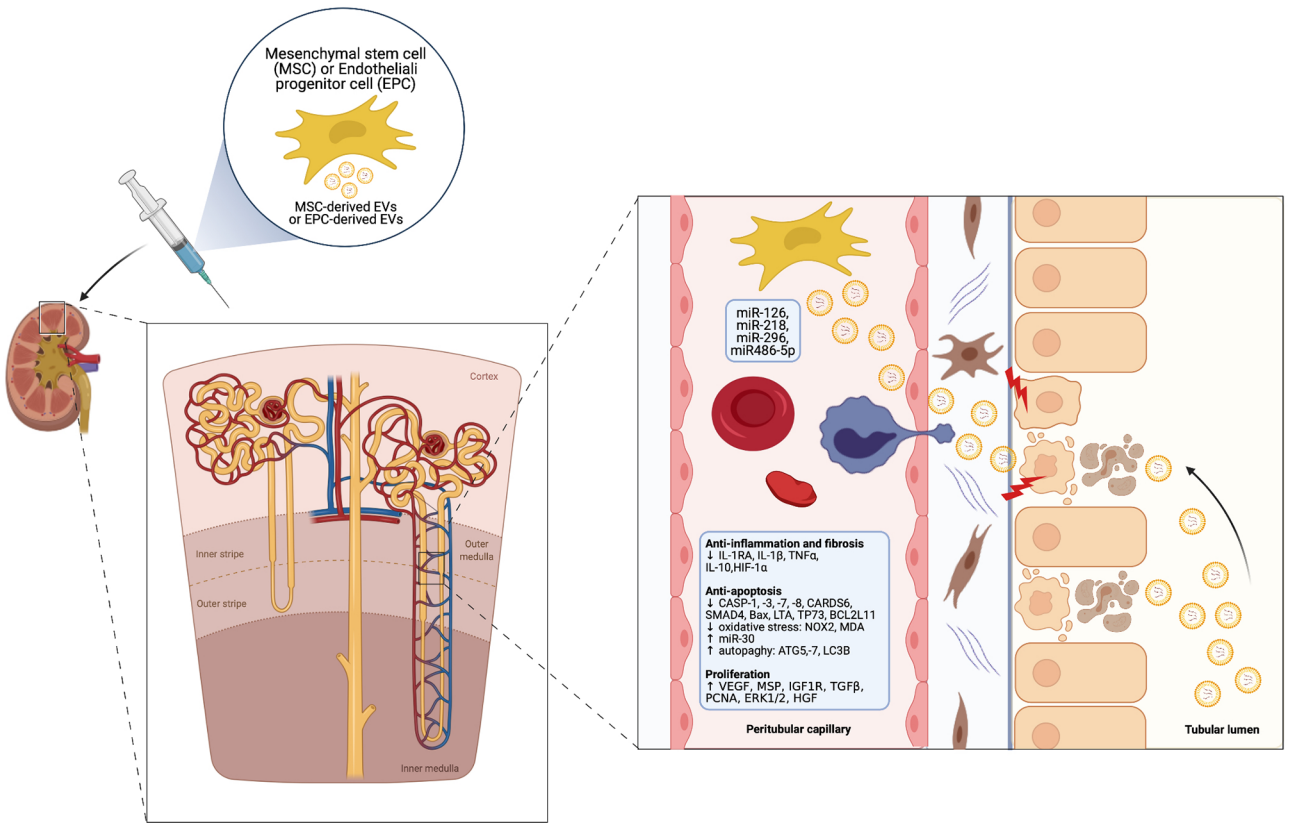

FIGURE 2 | Cytoprotective, regenerative and immunomodulatory effects of MSC-derived EVs in the setting of AKI (Created with BioRender.com).

treatment with RNAase consistently abolished these effects, indicating that mRNA and miRNA transfer from SCs to injured renal cells is crucial in this healing process (67). Ferguson et al. identified 23 top-miRNAs which account for over 79% of total miRNAs in MSC-derived exosomes and seem to mediate the predominant effects, targeting 5481 genes (68). A comprehensive review of miRNA repertoire carried by SC-derived EVs for AKI treatment has been recently published and it is beyond the scope of this work (69).

THERAPY WITH SC-DERIVED EVs IN DIFFERENT FORMS OF AKI

As previously mentioned, while most studies were performed with EVs derived from BM-MSCs, UC-MSCs and AD-MSC, other EV sources have been recently employed, including kidney resident populations (50, 70) and uEVs (71). Potential mechanisms of actions and main mediators, with a focus on miRNAs, will be briefly analyzed. A summary of the main studies on EVs as therapy of AKI is outlined in **Table 1**, in which they are categorised according to type of originating cell type (49–51, 56, 65–67, 72–96).

Nephrotoxic AKI

Several cellular sources of SCs have been tested in models of toxic AKI. Bruno et al. first demonstrated that BM-MSC-derived EVs facilitated morphological and functional renal recovery in a model of glycerol-induced AKI mimicking rhabdomyolysis-associated tubular damage (66). Human liver stem cells (HLSCs) cells were then tested in the same experimental model with similar results. A single intravenous injection of HLSCs facilitated histological and functional renal recovery through the induction of RTEC proliferation and inhibition of apoptosis. Of interest, EVs were the main component of HLSC-derived conditioned medium capable of promoting regeneration (72).

Furthermore, uEVs have been recently employed in the same mouse model of glycerol-induced toxic AKI. Intravenously injected uEVs stimulated RTEC proliferation, reduced expression of inflammatory markers and restored endogenous Klotho. Interestingly, murine uEVs derived from Klotho-null mice lost these reno-protective effects, suggesting a key-role of Klotho in mediating uEVs beneficial effects on RTECs (52).

EVs obtained from BM-MSCs were also tested in a lethal cisplatin-induced AKI model, in which they stimulated RTEC proliferation and conferred them resistance to apoptosis *in vitro*, resulting in improvement of renal function and morphology in an *in vivo* SCID mouse model (73). Similar results were reported

TABLE 1 | EVs derived from MSCs or other cell types as therapy of AKI.

Source of EVs	Type of experiments	Mechanisms of action and mediators	References
Human BM-MSC	Animal model (mice) *In-vitro*	Increased proliferation of RTECs	(66)
Human Liver Stem cells (HLSC)	Animal model (mice) *In-vitro*	Increased proliferation and reduced apoptosis of RTECs	(72)
Human BM-MSC	Animal model (mice) *In-vitro*	Increased proliferation and reduced apoptosis of RTECs; anti-inflammation (upregulation of genes involved in metabolic pathways and downregulation of genes involved in inflammation)	(73)
Human UC-MSC	Animal model (rat) *In-vitro*	Increased proliferation and reduced apoptosis of RTECs	(74)
Human BM-MSC	Animal model (rat)	Increased proliferation and reduced apoptosis of RTECs; protection against chronic kidney injury	(67)
Human BM-MSC	*In-vitro*	Reduced apoptosis (EVs transfer miR-148b-3p, miR-410, miR-495, miR-548c-5p and miR-886-3p to RTECs)	(75)
Human UC-MSC	Animal model (rat) *In-vitro*	RTECs dedifferentiation and proliferation (increased ERK1/2 and HGF expression)	(76)
BM-MSC	Animal model (mice) *In-vitro*	Reduced RTEC apoptosis (inhibited NLRP3 expression through miR-223)	(77)
Mouse kidney resident glomerular progenitors (GI-MSC)	Animal model (mice) *In-vitro*	Increased RTECs proliferation	(50)
Human UC-MSC	Animal model (rat) *In-vitro*	Increased RTECs proliferation (releasing from G2/M cell cycle arrest).	(78)
Human BM-MSC	Animal model (mice) *In-vitro*	Inhibition of RTECs apoptosis (downregulation of Sema3A expression and activation of AKT/ERK pathways through miR-199a-3p); inhibition of NK.	(79)
Rat BM-MSC	Animal model (rat) *In-vitro*	Anti-inflammation (reduced IL1β and TNFα)	(80)
Human BM-MSC	Animal model (rat) *In-vitro*	Immunosuppression (NK cells inhibition)	(65)
Human UC-MSC	Animal model (rat) *In-vitro*	Anti-oxidation (decreased expression of NOX2 and activation of Nrf2/ARE)	(81, 82)
Human UC-MSC	Animal model (rat)	Inhibition of mitochondrial fission (miR-30) and reduced RTECs apoptosis	(83)
Human BM-MSC	Animal model (mice) *In-vitro*	Suppression of ER stress (through miR-199a-5p)	(84)
Mouse kidney resident MSC	Animal model (mice) *In-vitro*	Increased angiogenesis; increased proliferation and reduced apoptosis	(49)
EPC	Animal model (rat) *In-vitro*	Increased angiogenesis	(56)
ECFC	Animal model (mice) *In-vitro*	Increased angiogenesis (transfer of miR-486-5p to EC inhibits apoptosis and Endo-MT)	(85, 86)
RAVPCs	Animal model (mice) *In-vitro*	Increased angiogenesis, increased ECs migration (transfer of miR-218)	(87)
Human BM-MSC	*In-vitro*	Increased angiogenesis (transfer of miR-125a)	(88)
MSC-EVs	Animal model (rat) *In-vitro*	Increased angiogenesis	(89)
Human BM-MSC	Animal model (mice) *In-vitro*	Increased angiogenesis (transfer of miR-199a-3p)	(79)
Human UC-MSC	Animal model (rat) *In-vitro*	Anti-inflammation (downregulation of CX3CL1, reduction in CD68+ macrophages infiltration) and decreased renal fibrosis (reduction of α-SMA). Downregulated CX3CL1 was associated with specific miRNAs in EVs (miR-15a, miR-15b, mi-R16).	(90)
BM-MSCs	Animal model (mice) *In-vitro*	Anti-inflammation (EVs enriched in CCR-2 suppress macrophage functions)	(91)
Rat AD-MSC	Animal model (rat)	Anti-oxidation, inhibition of apoptosis and renal fibrosis	(92)
Human UC-MSC	Animal model (rat)	Inhibition of apoptosis, increased proliferation of RTECs; Anti-inflammation (reduced CD68+macrophages infiltration); Anti-fibrosis (decreased expression of α-SMA and TGFβ; Increased expression of HGF)	(93)
HPC-Human RTECs	Animal model (rat) *In-vitro*	Hypoxia can enhance and differentiate EVs regenerative properties compared with EVs released under normal oxygenation.	(51, 94)

(Continued)

TABLE 1 | Continued

Source of EVs	Type of experiments	Mechanisms of action and mediators	References
		HPC-Human RTECs can release EVs with anti-oxidant properties and the potential to induce SCs differentiation normal RTEC	
AD-MSC	Animal model (mice) *In-vitro*	Increased proliferation of RTECs; Inhibition of AKI-to-CKD transition (activation of Sox 9)	(95, 96)

EC, Endothelial Cells; ER, endoplasmatic reticulum; HLSC, Human Liver Stem Cell; HGF, Hepatocyte Growth Factor; ECFC, Endothelial Colony-forming Cell; EPC, Endothelial Progenitor Cells; RAVPC, Renal Artery Vascular Progenitor Cells; GI-MSC, Glomerular progenitor MSC; NLRP3, NLR family-pyrin domain containing 3.

employing UC-MSCs-derived EVs in the same experimental setting (74).

Last, BM-derived MSC repaired but did not prevent gentamycin-induced AKI (97).

Ischemic AKI

SC-derived EVs have also been studied in several models of ischemia/reperfusion injury (IRI)-associated AKI, as outlined in **Table 1**. In all of these experimental models, administration of EVs derived from different cell types after IRI accelerated recovery of renal function and/or decreased histological tubular injury through multiple mechanisms (61).

Gatti S et al. demonstrated a beneficial effect of BM-MSC-derived EVs in favouring recovery from ischemic AKI and the need for multiple injections to achieve renal function normalization. This effect was mainly due to upregulation of anti-apoptotic genes in injured RETCs and prevented transition from AKI to CKD (67).

Of interest is the fact that i.v. administered human MSC-derived EVs were effective in alleviating renal damage even in rats which had received kidney transplant after cardiac death, a setting characterized by severe IRI (93).

Another study highlighted the role of specific miRNAs within the cargo of MSC-derived EVs (miR-148b-3p, miR-410, miR-495, miR-548c-5p, miR-886-3p) in protecting RTECs from IRI in an *in vitro* model induced by ATP-depletion. Down-regulation of miRNAs involved in apoptosis, hypoxia and cytoskeletal reorganization mediated this effect (75).

Other studies brought out a wide spectrum of beneficial actions of BM-MSC-derived EVs in ischemic AKI: they can induce RTEC dedifferentiation and growth *via* hepatocyte growth factor (HGF) induction (76) and prevent apoptosis through transfer of miR-21 (98) and miR-223 (77); they inhibit CXC3CL1, blunting evolution towards fibrosis (90)**;** finally, they are enriched in chemokine receptor type 2 (CCR-2), which enables them to buffer extracellular free chemokine ligand 2 (CCL-2), suppressing macrophage functions (91).

Of note, several EVs biological activities are specifically related to aerobic metabolism; for example, MSC-derived EVs can carry respiratory complexes, supporting an independent aerobic metabolism when mitochondrial respiratory capacity is impaired (99); they can inhibit mitochondrial fission through miR-30 transfer (83) and re-establish adequate intracellular ATP levels, with beneficial epigenetic changes such as reversion of histone H2 and H2B up-regulation, a typical feature of apoptotic cells (100); they can attenuate mitochondrial damage in RTECs by stabilising mitochondrial DNA (mDNA) previously depleted by oxidative stress (101).

Furthermore, they might contribute to the anti-oxidant potential of injured cells, for example down-regulating calnexin, a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase 4 (NOX4)-interacting protein (102).

Similarly, Zhang et al. reported that human Warton Jelly (hWG) MSC-derived EVs can reduce expression of NADPH-oxidase 2 (NOX2) and stimulate the nuclear factor eryhroid 2-related factor 2 (NRF-2) and anti-oxidant response element pathway (81, 82). All these actions result in an overall reduction of reactive oxygen species (ROS) formation and could limit tubular cell death and senescence after re-oxygenation.

EVs derived from iPSC (103) have also been shown to protect mitochondrial function and regulate several genes associated with oxidative stress. Interestingly, iPSC-EVs showed a higher efficiency in renal protection than AD-EVs (104, 105).

Another aspect which contributes to reparative effects of EVs is their capacity to promote angiogenesis, counteracting renal hypoxia. EPCs administration determined increased tubular proliferation and reduction in capillary rarefaction, glomerulosclerosis and tubulointerstitial fibrosis in a rat model of IRI-associated AKI, suggesting protection against post-IRI fibrosis. In this study, pro-angiogenic miR-126 and miR-296 shuttled by EPC-derived EVs to ECs located in peritubular and glomerular capillaries accounted for this effect, as treatment with RNAase or specific antagomiRs abolished it (56). Other studies subsequently proved that similar pro-angiogenetic effects were mediated by EVs released from other human SC types, such as kidney-derived MSCs (49, 50), endothelial colony-forming cells (ECFCs) (85, 86), vascular progenitor cells derived from renal arteries (87) and BM-MSCs (79, 88, 89).

Interestingly, the study of Zou X et al. showed that hWJ-MSC-derived EVs upregulated vascular endothelial growth factor (VEGF) and downregulated hypoxia-inducible factor 1 (HIF-1α) in a rat model of IRI and that VEGF was through directly transferred by EVs (89).

Recent studies have shown that hypoxia preconditioning (HPC) can improve and differentiate EVs regenerative properties compared with EVs released under normal oxygenation, as secreted EVs convey the metabolic state of originating cell, including trophic factors protecting against hypoxia (106).

In the study by Collino et al. peculiar anti-apoptotic, anti-oxidative, mitochondrial energy-supply and pro-angiogenic

pathways were activated by hypoxic AD-MSC derived EVs and induced a distinct proteomic pattern is in RTECs (107). Four effects were specifically enhanced in hypoxic EVs: downregulation of fibroblast growth factor receptor 1 (FGFR-1) and reduction of Transforming Growth Factor β-1 (TGFβ-1)-induced epithelial-to-mesenchimal transition (EMT) (108); promotion of angiogenesis through vascular endothelial growth factor (VEGF), blunting renal microvasculature rarefaction (109); translocation of Nrf-2 into the nucleus, activating antioxidant genes including Heme-oxigenase-1 (HO-1) (110); downregulation of IL6, reducing macrophage infiltration and polarization towards a M2 phenotype (111).

Hypoxic conditions have also been shown to promote angiogenic potential of iPSC-derived EVs (112).

An interesting aspect is that injured RTECs can themselves, especially when treated with HPC (113, 114), release EVs with anti-oxidant properties (94) and have the potential to modulate phenotypic and functional features of SCs, stimulating them to differentiate into normal RTEC. This effect may be ascribed to the release of a specific EV phenotype by de-differentiated tubular cells (57).

In another study, Dominguez JM et al. harvested hypoxic human RTECs and derived EVs from kidneys declined for transplantation and demonstrated that, after injecting them into nude rats exposed to bilateral renal ischemia, they both preserved renal function; however, EVs proved superior in maintaining renal vascular and epithelial networks, preventing oxidative stress and blunting pro-inflammatory and fibrogenic pathways. Proteomic analysis demonstrated broad ischemia-induced alterations at all cell levels and prevention of major drift in transcriptome by EV infusion (377 out of 628 altered proteins were "corrected" by EVs). This resulted in a reduced risk of evolution towards fibrosis and CKD (51).

On this basis, association of normoxic and hypoxic EVs has been proposed with the rationale of integrating respective peculiar effects (92, 115).

Finally, a recent study by Liu et al. showed that encapsulation of EVs isolated from human placenta (hP)-derived MSCs in a collagen matrix improved their retention in an AKI murine model, remarkably enhancing their therapeutic effects (inhibition of RTEC proliferation and of endoplasmic reticulum stress, stimulation of angiogenesis) compared with hP-MSC-derived EVs alone (116).

Taken together, all these studies indicate that SC-derived EVs have a multi-level therapeutic potential and that different type of EVs may specifically target pathophysiological aspects involved in ischemic AKI, either preventing it or accelerating its recovery.

Sepsis-Associated AKI (s-AKI)

As described above, sepsis is a common and life-threatening systemic disorder often leading to AKI in a clinical scenario of multiple organ failure due to the maladaptive host response to infection. S-AKI is not merely a consequence of ischemic damage due to hypoperfusion (renal overflow is in fact often normal or even increased) but recognizes a more complex pathogenesis. This includes microvascular damage and intrarenal redistribution of renal blood flow, activation of immune cells and complement with massive release of inflammatory molecules causing RTEC dysfunction (autophagy and mitophagy; arrest of cell cycle; dedifferentiation), endocrine dysregulation (16).

Transfer of miRNAs, mRNAs and proteins from activated immune and ECs through EVs may play a pivotal pathogenetic role in these processes but, on the other hand, it may also represent a therapeutic option for the use of SC-derived EVs (117). For example, EPC-derived EVs carrying mi-RNA-93 5p conferred renal protection in a LPS-induced mouse model of S-AKI, also alleviating multiple organ injury and vascular leakage (118) and blunted LPS-induced HK2 cell injury in another study (119).

During infections, MSC-derived EVs have been shown to eliminate pathogens and to regulate immune response through the secretion of antimicrobial factors, both inhibiting the replication of pathogens and activating the phagocytic function of macrophages (120). In a mouse model in which Escherichia Coli-derived outer membrane vesicles were intraperitoneally injected to establish sepsis, MSC-derived EVs significantly suppressed cytokine release into the systemic circulation, as well as PMN and monocyte infiltration in the peritoneum, by upregulating IL-10 production (121). In experimental models of sepsis obtained by LPS administration or by cecal ligation and puncture (CLP) MSC-derived EVs inhibited the development of disease by downregulating JMJD3 and inactivating the NF-κB signaling pathway through the selected transfer of miR-27b (122). EVs isolated from AD-MSCs have been shown to attenuate inflammation and protect from organ dysfunction by regulating the Notch-miR148a-3p signaling axis and decreasing macrophage polarization to M1 (123). In experimental s-AKI, the administration of EVs isolated from AD-MSCs exerted a renal protective effect through SIRT1 signaling pathway (124). In another CLP model of s-AKI, EVs obtained from UC-MSCs decreased IRAK1 expression through the up-regulation of miR-146b level, inhibited NF-κB activity and limited AKI and mortality (125). These results suggest an important immunomodulatory effect induced by MSC-EV administration in sepsis and a specific protective effect from AKI.

Last, EVs derived from mice pre-treated with remote ischemic preconditioning, elicited by brief periods of IRI in femoral arteries, appears to protect against s-AKI through miR-21, which integrates into RTECs and targets the downstream PDCD4/NF-κB and PTEN/AKT pathway (126).

THE PROCESS OF AKI TO CKD TRANSITION

Recent studies have demonstrated that maladaptive repair after an AKI episode can predispose to evolution towards CKD and end-stage renal disease (ESRD) (127–129). Different factors appear to contribute to maladaptive repair during AKI, including oxidative stress, DNA damage, microvascular rarefaction, tubular loss, early fibrosis induced by endothelial-to-mesenchymal transition (EndMT) and pericyte-to-mesenchymal transition (PMT), lymphocyte infiltrates, inflammatory cytokine storm (128, 129). Pathophysiology of

AKI-CKD transition has been the focus of research and complement system activation is increasingly recognised as having an essential role in this inflammatory scenario, which is closely related to renal senescence (130). These aspects and the potential of EVs as a tool to treat AKI and prevent AKI-CKD transition will be analysed in the following sections.

The Role of Complement in Renal Senescence and AKI-CKD Transition

In addition to liver synthesis, complement components can derive from renal cells (131) and complement C3 can be expressed by proximal RTECs, ECs, glomerular epithelial and mesangial cells after IRI (132, 133). Recent evidence indicates a crucial role of complement activation in renal tubules and vessels in AKI secondary to rhabdomyolysis (134) and trauma (135).

An aberrant complement activation and a high number of senescent cells also characterise both renal senescence and AKI-CKD transition, which is currently viewed as an accelerated form of kidney aging (136–138) and shares the same pathway and intracellular mediators (139, 140). Thus, there is actually a tight relationship between AKI itself and mechanism of senescence activation (129). From a molecular point of view, senescence refers to a well-defined program associated with cell cycle arrest, apoptosis inhibition and a pro-inflammatory "senescence-associated secretory phenotype" (SASP) (141). The SASP secretome relies on the production of a wide range of pro-inflammatory cytokines, chemokines, growth factors and matrix degrading factors promoting spread of senescence and fibrosis (142). Chronic accumulation of SASP cells leads to "inflammaging", a persistent, low-grade inflammatory state which causes tissue deterioration (143, 144). Renal senescent cells can be detected by several markers, including loss of key nephroprotective factors such as Klotho. This transmembrane protein, expressed mainly on proximal RTECs where it interacts with the fibroblast growth factor receptor (FGF-23), regulates phosphate homeostasis (145) and exerts anti-fibrotic and anti-inflammatory actions through its 65-kDa soluble form, released into the bloodstream and urine (146). Disruption of Klotho gene determines shortened life span due to premature arteriosclerosis in mice (147) and AKI-induced Klotho deficiency accelerates renal fibrogenesis, retards renal tissue regeneration and promotes AKI-CKD transition (148, 149). Aberrant complement activation during AKI triggers inflammaging and represents an important link between AKI and CKD. Complement activation is involved in two key processes leading to CKD lesions: endothelial-to-mesenchymal transition (EndMT) and pericyte-to-myofibroblast transition (PMT) (130). In EndMT, complement drives ECs to acquire a myofibroblast phenotype, contributing to vascular damage and early fibrosis (150), as demonstrated in preclinical models of AKI induced by LPS and I/R (151). Similarly, complement also promotes PMT and enhances renal fibrogenesis. The loss of pericytes, which play a key role in angiogenesis and vascular homeostasis, is a hallmark of AKI and correlates with the decline of kidney function (152–154). Another interesting aspect is the role of C3 and C1q complement components in macrophages polarization, an essential factor in AKI evolution. While classically activated M1 macrophages contribute to initial injury, conversion to M2 anti-inflammatory macrophages during the recovery phase is critical in resolving inflammation and restoring tubular function. Strikingly, their differentiation into the M1 or M2 phenotype is regulated by C3 and C1q (155, 156). Overall, available evidence supports a critical role of complement in accelerating the process of premature aging which characterizes AKI-CKD transition. This phenomenon is more marked in the elderly, increasing susceptibility to accumulate chronic irreversible lesions after AKI events (157).

Potential Therapeutic Role of EVs in AKI-CKD Transition

Can EVs play a therapeutic role in reducing the risk of AKI-CKD transition? Although no study has specifically focused on this endpoint, there is some evidence from some of the previously mentioned studies that EV pleiotropic actions (**Table 1**) could inhibit AKI-CKD transition (51, 67). Activation of specific mediators such as Sox 9 (95) and transfer of miRNAs such as mi-R29b, which modulates Angiotensin 2-induced EMT of RTECs (158), are examples of effects which could be exploited to inhibit mechanisms leading to irreversible renal damage. Furthermore, as already mentioned, uEVs from healthy subjects can carry Klotho protein and transfer it to RTECs, restoring normal intra-tubular levels with beneficial effects on recovery from AKI (70).

Another interesting approach to prevent fibrosis after AKI is through complement blockade (159). A few studies suggest that EVs may exert an anti-complement activity through transfer of specific complement inhibitors. Cantaluppi et al. demonstrated that EPC-derived EVs could protect from complement mediated injury in experimental anti-Thy1.1 glomerulonephritis by transferring mRNAs coding for Factor H, CD55 and CD59 and related proteins to mesangial cell, thus inhibiting antibody/complement-induced apoptosis and C5b-9/C3 mesangial cell deposition (55). Similarly, EPC-derived EVs were able to preserve glomerular EC and podocyte integrity from complement-induced damage in a co-culture model mimicking the glomerular filtration barrier (160).

Although this setting is completely different from AKI, it is tempting to speculate that this mechanism of action might also explain some of the beneficial effects of EVs in AKI-CKD transition. Initial evidence indicates that human MSCs can ameliorate complement-induced inflammatory cascade and improve renal function at very early stages in experimental ischemic AKI, suggesting an immunomodulatory capacity possibly mediated by EVs (161). Finally, preliminary results from our group showed that EPC-derived EVs may limit ischemic AKI through complement inhibition (data not shown). Further studies are needed to investigate the potential of EVs as anti-complement therapy in order to prevent AKI-CKD transition. However, the potential of EV therapy to limit AKI development and AKI-CKD progression based on the horizontal transfer of proteins, receptors, bioactive lipids and

different types of RNAs represents a great incentive for future research in this field.

LIMITS AND PERSPECTIVES OF EV-BASED THERAPY

The use of SC-derived EVs as a therapeutic tool to deliver growth factors, proteins and genetic material to injured renal resident cells is promising in different AKI fields: however, several obstacles still limit their translation to clinic and have been recently reviewed (162, 163).

Biochemical composition is not defined and can vary depending on parental cell but also on surrounding milieu (e.g. inflammation, hypoxia). EVs released from the same cell-type may even have contradictory effects: for example, hypoxic RTECs have proved beneficial in alleviating tubular damage and fibrosis but injured RTECs can also release EVs which contribute to amplify inflammation (164). Moreover, focusing on SC-derived EVs, a different phenotype may depend on donor characteristics (autologous vs. heterologous, age, gender, presence of comorbidities or transient inflammatory states, etc.)

Furthermore, EV production or uptake mechanisms by kidney resident cells or infiltrating inflammatory cells are not completely defined and an intact glomerular filtration barrier could prevent EVs from reaching podocytes and tubular cells (165).

Finally, lack of good manufacturing practice standards and high-scale EV production hinder clinical application. At the moment, the clinical use of EVs is not classified as cell therapy and their mechanisms of action look like more to administration of a drug rather than a real cell therapy. Moreover, despite the development of new isolation procedures, cGMP production of SC-derived EVs for clinical application seems still to depend on an ultracentrifugation step to be performed within a cell factory.

Despite these limits, EV-based therapy has many strengths and is opening new therapeutic perspectives for a condition currently treated only with supportive therapy.

In general, EV lipid and surface protein composition (e.g.CD47) limits phagocytosis by circulating monocytes and prolongs blood half-life if compared to liposomes or other nanoparticles employed to carry drugs. EVs usually express integrins and adhesion molecules which allow to enhance their homing to inflamed or injured tissue: moreover, EVs protect RNA from degradation after their intravenous administration and at tissue level.

Technologies such as tangential flow filtration (TFF) appears to allow large-scale production of high-quality, reproducible EVs from AD-MSCs, paving the way for potential widespread clinical application in AKI (166). Other technological advances may potentiate EV qualitative therapeutic properties. For example, EV encapsulation could make their therapeutic content (e.g. miRNAs, mRNAs, proteins) more protected and stable. Nanomedicine techniques may help engineering EV features (size, shape, surface charge) in order to enable them to pass specific biological barriers, including glomeruli. Decoy exosomes have been proposed to antagonize inflammatory mediators (167). Another therapeutic approach is that of transfecting MSCs with specific miRNA mimics in order to enrich them with selected miRNAs. These enriched EVs proved to be more effective than naïve ones, potentially allowing the use of a lower amount of them (168). Combination therapy of pulsed focused ultrasound (pFUS) and EVs has proved more effective than either approach alone in reversing AKI-related inflammation through suppression of heat shock protein 70 – mediated NLPR3 inflammasome (169).

An interesting tool to increase therapeutic potential of MSCs is the adoption of three-dimensional (3D) culture of human placental MSC (hPMSCs), which proved to be more effective than two-dimension (2D) culture in preventing renal damage when injected in a mouse model of IRI-induced AKI (170). MSC 3D spheroid structures enable increased cell-cell interactions and enhance MSC trophic and immunomodulatory functions, with more reproducible clinical outcomes in many preclinical models (171) including cisplatin-induced AKI (172).

We have already discussed the potential of hypoxia and collagen matrix encapsulation to enhance EV protective effects, paving the way to new possibilities of therapeutic manipulation (106, 107, 116). Of note, the use of EVs avoid the possible adverse effects associated with whole cell therapies such as pulmonary embolism, vascular thrombosis, maldifferentiation and tumorigenesis (173).

CONCLUSIONS

A growing body of evidence based on pre-clinical studies suggests that EVs released from MSCs of different origin and from other SC types could be effective to treat toxic, ischemic and septic AKI through direct interference with multiple etiopathogenetic mechanisms of tubular and endothelial damage. A network of cytoprotective, regenerative and immunomodulatory EV properties is being defined. EV-based therapy could prevent renal fibrosis and AKI-CKD transition, also through inhibition of complement-mediated processes such as EndMT and PMT. Hypoxia-conditioned and engineered EVs with enhanced therapeutic properties are promising new tools. Even though some technological hurdles must still be overcome before widespread clinical application, EV-based therapies may become a cornerstone for the treatment of the most common forms of AKI in the near future.

AUTHORS CONTRIBUTIONS

MQ and VC designed and wrote the initial manuscript. GM and AC designed Figures (Created with BioRender.com) and Tables and arranged References. SB, AS, RF, EG, and VF reviewed the article focusing on experimental models. GC wrote the paragraph on AKI-CKD transition and all authors critically revised the whole article.

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GLOSSARY

AKI	acute kidney injury
AD	adipose tissue
ATN	acute tubular necrosis
BM	bone marrow
CCL-2	chemokine ligand 2
CCR-2	chemokine receptor type 2
C1-INH	C1-Inhibitor
CKD	chronic kidney disease
CLP	cecal ligation and puncture
CM	contrast medium
DAMP	damage associated molecular pattern
DC	dendritic cell
EC	endothelial cell
ECFC	endothelial colony-forming cells
ECM	extracellular matrix
EMT	epithelial-to-mesenchimal transition
EndMT	endothelial-to-mesenchymal transition
EPC	endothelial progenitor cell
EV	extracellular vesicle
FGFR-1	fibroblast growth factor receptor 1
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor 1
HLSCs	human liver stem cells
HO	heme-oxigenase
hP	human placenta
HPC	hypoxia pre-conditioning
ICU	intensive care unit
iPSC	induced pluripotent stem cells
IRI	ischemia-reperfusion injury
LPS	lipopolysaccharide
mDNA	mitochondrial DNA
miRNA	microRNA
MCP-1	monocyte chemoattractant protein 1
MSC	mesenchymal stromal cell
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NOX	nicotinamide adenine dinucleotide phosphate oxidase
NRF-2	nuclear factor eryhroid 2-related factor 2
rIPC	remote ischemic pre-conditioning
PAMP	pathogen associated molecular pattern
PMN	polymorphonuclear cell
PMT	pericyte-to-mesenchymal transition
pFUS	pulsed focused ultrasound
ROS	reactive oxygen species
RRT	renal replacement therapy
RTEC	renal tubular epithelial cells
s-AKI	sepsis-associated AKI
SASP	senescence-associated secretory phenotype
SC	stem cell
TGFβ-1	Transforming Growth Factor β-1
TLR	Toll-like receptors
UC	umbilical cord
uEVs	urinary extracellular vesicles
VEGF	vascular endothelial growth factor
WJ	Warton Jelly

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Pre-Transplant Expression of CCR-2 in Kidney Transplant Recipients Is Associated With the Development of Delayed Graft Function

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Background: Delayed graft function (DGF) leads to a reduced graft survival. Donors' features have been always considered as key pathogenic factors in this setting. The aim of our study was to evaluate the recipients' characteristics in the development of DGF.

Methods: We enrolled 932 kidney graft recipients from 466 donors; 226 recipients experienced DGF. In 290 donors, both recipients presented with early graft function (EGF, group A), in 50 both recipients experienced DGF (group B), and in 126 one recipient presented with DGF and the other with EGF (group C). In group C, we selected 7 couples of DGF/EGF recipients and we evaluated the transcriptomic profile by microarray on circulating mononuclear cells harvested before transplantation. Results were validated by qPCR in an independent group of 25 EGF/DGF couples.

Findings: In the whole study group, DGF was associated with clinical characteristics related to both donors and recipient. In group C, DGF was significantly associated with body mass index, hemodialysis, and number of mismatches. In the same group, we identified 411 genes differently expressed before transplantation between recipients discordant for the transplant outcome. Those genes were involved in immune dysfunction and inflammation. In particular, we observed a significant increase in DGF patients in the expression of C–C chemokine receptor type 2 (CCR2), the monocyte chemoattractant protein-1 (MCP-1) receptor. CCR-2 upregulation was confirmed in an independent cohort of patients.

Conclusions: Our results suggest that recipients' clinical/immunological features, potentially modulated by dialysis, are associated with the development of DGF independently of donors' features.

Keywords: kidney transplantation, delayed graft function, gene expression, peripheral blood mononuclear cells, CCR-2

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INTRODUCTION

Delayed graft function (DGF) is a form of post-transplant acute kidney injury, commonly defined as the requirement for dialysis in the recipient within 7 days after renal transplantation (1). Although the incidence depends on its definition and by donor type, DGF is reported in up to 50% of renal allografts (2, 3).

An increasing body of evidence suggests that DGF might adversely affect short- and long-term transplant outcomes, increasing the risk of acute rejection and reducing both graft and patient survival with a consequent increase in healthcare costs (4–6).

Several immunological and non-immunological factors can influence DGF onset and graft loss, and those factors can be referred to donors and recipients features or, in alternative, to the transplant procedure itself (7). The use of expanded criterion donors and prolonged warm or cold ischemia time are significantly associated with the development of DGF (8). Indeed, ischemia-reperfusion damage, with the subsequent activation of pro-inflammatory and pro-fibrotic pathways, and donors' features have been always considered as the pivotal pathogenic factors in this setting (9, 10).

Several predictive biomarkers (11, 12) and predictive models have been proposed to quantify the risk of DGF using different methods such as logistic regression or machine learning (13). These methods comprise a combination of donor risk factors, including age, body weight, and kidney function, variables related to the surgical procedure (for example, cold ischemia time), and recipient risk factors, such as obesity, diabetes, and dialysis-related variables (2, 14). However, the importance of the recipients' characteristics, in particular their immune-phenotype, in the development of DGF is not yet clearly defined, since there is a lack of knowledge of the recipient characteristics that could be mechanistically involved in the development of DGF.

Thus, the aim of our study was to evaluate the role of recipients' characteristics, especially the immune phenotype by the analysis of gene expression profiling in peripheral blood mononuclear cells (PBMC), that could be mechanistically involved in the pathogenesis and development of DGF.

MATERIALS AND METHODS

Patients and Ethics

This is a single-center, observational, prospective, cohort study performed in a University Hospital. We enrolled 932 uremic patients receiving a kidney transplant from 466 deceased donors with brain death in the University of Bari Kidney Transplant Center from January 1, 1999, to December 31, 2011. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul. The study was approved by the Local Ethical Committee (Prot. N. 670/CE-2017) and was in accordance with the Declaration of Helsinki. We excluded from the study patients receiving a double kidney transplant, graft recipients with primary non-function, and patients who received a single kidney from donors whose other kidney was transplanted in a different center. The main demographic and clinical data of the patients included in the study are summarized in **Table 1**.

None of the grafts underwent machine perfusion. All patients included in the study received anti-CD25 monoclonal antibodies at day 0 and day 4 after transplantation as induction therapy. Maintenance immunosuppression was represented by calcineurin inhibitors, either cyclosporine or tacrolimus, mycophenolic acid, and corticosteroids. A small group of patients received mTOR inhibitors.

To exclude patients who were dialyzed for reasons other than impaired graft function (post-transplant hyperkalemia, fluid retention, etc.), DGF was defined as the need of more than one session of dialysis in the first week after transplantation (15). The patients who did not present these features were included in the EGF groups.

Among the 466 donors, in 290 couples of recipients both patients presented with early graft function (EGF, group A), in 50 couples both recipients experienced DGF (group B), and in 126 one recipient presented DGF and the other promptly recovered graft function (group C).

PBMC Isolation, RNA Extraction, and Microarray

Twenty ml of whole blood was harvested from patients included in the study at the time of transplantation, before the administration of induction therapy. PBMCs were isolated by density separation over a Ficoll-Paque™ (GE Healthcare, Uppsala, Sweden). In group C, we randomly selected seven EGF/DGF couples for microarray analysis (training group). To validate the microarray results, we randomly chose in the same group C further 25 kidney graft recipients with EGF and 25 with DGF (testing group). The main demographic characteristics of patients included in the microarray cohort and in the testing group are reported in **Table 2**.

Total RNA was extracted in the selected patients, using the RNeasy Mini Kit (Qiagen AG, Basel, Switzerland), and qualitatively and quantitatively analyzed through Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with good quality, as indicated by a RIN > 8, were used in the microarray experiment.

For the microarray experiments, we used the GeneChip® Human Genome U133A oligonucleotide microarray (Affymetrix, Santa Clara, CA) which contains 22,283 gene probe sets, representing 12,357 human genes, plus approximately 3,800 expressed sequence tag clones (ESTs), according to the manufacturer's instructions. We used the default settings of Affymetrix Microarray Suite software version 5 (MAS 5.0; Affymetrix) to calculate scaled gene expression values.

Results of the microarray experiments are available in the database of the European Bioinformatics Institute (EMBL-EBI) and are accessible through Experiment ArrayExpress accession E-MTAB-10747.

Abbreviations: CCR2, C–C chemokine receptor type 2; DGF, delayed graft function; EGF, early graft function; MCP-1, monocyte chemoattractant protein-1; PBMC, peripheral blood mononuclear cells.

TABLE 1 | The main demographic and clinical data of the patients included in the study.

	All (%)	EGF (%)	DGF (%)	p
n.	932	706 (75.7)	226 (24.3)	
Age (years)	45.8 ± 11.2	44.9 ± 11.3	48.5 ± 10.7	<0.0001
Gender (M/F)	595 (63.8)/337 (36.2)	444 (62.9)/262 (37.1)	150 (66.4)/76 (33.6)	0.4
Dialysis (HD/PD)	851 (91.3)/81 (8.7)	631 (89.4)/75 (10.6)	218 (96.5)/8 (3.5)	0.005
Dialysis vintage (years)	6.2 ± 4.4	5.8 ± 4.3	7.4 ± 4.7	<0.0001
HCV+ (n,%)	156, 16.7	98, 13.9	58, 25.4	<0.0001
Body mass index (BMI) (kg/m^2)	24.0 ± 3.8	23.7 ± 3.8	24.9 ± 3.8	0.0001
Panel reactive antibodies (%)	7.5 ± 5.2	7.4 ± 4.9	7.6 ± 5.7	0.8
Cold ischemia time (hours)	12.7 ± 5.0	13.0 ± 5.2	12.2 ± 6.5	0.4
Mismatches (n)	3.2 ± 0.9	3.1 ± 0.9	3.3 ± 0.9	0.1
Donor age (years)	45.1 ± 17.5	43.3 ± 17.3	50.5 ± 16.7	<0.0001
Donor serum creatinine (mg/dl)	1.28 ± 0.75	1.23 ± 0.6	1.44 ± 1.0	0.04
Cause of death (trauma/vascular)	304 (32.6)/628 (67.4)	289 (40.9)/417 (59.1)	75 (33.2)/151 (66.8)	0.02
Cyclosporine (n)	408 (43.7)	307 (43.5)	101 (44.7)	0.6
Tacrolimus (n)	479 (51.4)	368 (52.1)	111 (49.1)	0.8
mTOR inhibitors (n)	80 (4.9)	76 (4.4)	4 (6.2)	<0.0001

Real-Time PCR

We validated the results gathered by microarray in the testing group by quantitative real time-PCR. Reverse transcription of total RNA (500 ng) was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. Relative quantification was obtained as previously described (16) by a comparative Ct method using 18s rRNA as a stably expressed endogenous reference gene. The following TaqMan Gene Expression Assays (Applied Biosystems) were employed: Hs00704702_s1 (CCR2), Hs00234140_m1 (MCP1), and Hs01060665_g1 (ACTB). The qPCR was carried out with the Roche Light-Cycler Real-Time PCR system with 5 µl TaqMan Universal PCR Master Mix in a 10-µl-reaction volume.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) or median and interquartile range and compared by ANOVA or Wilcoxon test, as appropriate. The multivariate logistic regression model was used to identify the variables independently associated with DGF. The risk is expressed as odds ratio (OR) - 95% confidence interval (CI). In the multivariate analysis, all the variables that at the univariate analysis presented $p \leq 0.1$ were included. $p < 0.05$ was considered statistically significant. Statistical analysis was performed with the Statistical Package for Social Sciences version 23.0 (SPSS, Inc., Chicago, IL).

The differentially expressed genes obtained by microarray experiments were identified by applying a fold change ≥1.5 and p value <0.05 after comparison of the two groups by t-test (moderate t-test). Permutation analysis was applied to reduce the false discovery rate. Results were statistically analyzed using the software GeneSpring GX 12.5 in order to identify genes differentially expressed, and functionally analyzed using the Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com) as previously reported (16, 17).

RESULTS

Donors' and Recipients' Characteristics Predict DGF in the Whole Cohort

We prospectively enrolled 932 kidney graft recipients from 466 donors for both kidneys, 226 of whom experienced DGF.

TABLE 2 | The main demographic and clinical characteristics of patients included in the microarray group and in the testing group.

	Training (microarray) group		Testing group	
	EGF (%)	DGF (%)	EGF (%)	DGF (%)
n.	7	7	25	25
Age (years)	46.8 ± 11.0	47.8 ± 9.3	46.8 ± 11.0	47.8 ± 9.3
Gender (M/F)	4 (57.1)/3 (42.9)	5 (71.4)/2 (28.6)	16 (64)/9 (36)	17 (68)/8 (32)
Dialysis (HD/PD)	5 (71.4)/2 (28.6)	6 (85.7)/1 (14.3)	21 (84)/4 (16)	22 (88)/3 (12)
Dialysis vintage (years)	5.8 ± 5.0	7.3 ± 4.9	6.0 ± 4.9	7.5 ± 5.3
HCV+ (n)	1 (14.3)	1 (14.3)	2 (8)	3 (12)
Body mass index (BMI) (kg/m^2)	22.8 ± 4.3	24.9 ± 4.8	23.7 ± 5.3	25.0 ± 5.9
Panel reactive antibodies (%)	6.5 ± 6.9	7.0 ± 8.7	7.7 ± 6.2	7.5 ± 6.9
Cold ischemia time (hours)	12.9 ± 6.4	13.1 ± 6.5	13.0 ± 6.3	13.2 ± 6.6
Mismatches (n)	3.4 ± 1.1	3.5 ± 1.2	3.3 ± 1.3	3.5 ± 1.0
Cyclosporine (n)	2 (28.6)	2 (28.6)	4 (16)	4 (16)
Tacrolimus (n)	4 (57.1)	5 (71.4)	21 (84)	21 (84)
mTOR inhibitors (n)	1 (14.3)	0	0	1

Patients with DGF were significantly older, more frequently on renal replacement therapy with hemodialysis, with significantly longer dialysis vintage, higher prevalence of HCV infection, and higher BMI than patients with EGF. Donors of patients with DGF were older, with a worse renal function, and more frequently with a cerebrovascular cause of death (**Table 1**). The multivariate analysis revealed that donors' age and renal function as well as recipients' features, including dialysis vintage, BMI, and HCV infection, were independently associated with the development of DGF (**Table 3**).

Interestingly, among the 466 donors included in the present study, in the 62% of the cases (290) both recipients experienced a prompt recovery of graft function (group A). Only in 10.7% (50 cases) did both recipients develop DGF (group B), whereas in 27% (126 cases) the two recipients presented a discordant recovery of graft function (group C). The three groups significantly differ for donors' features. In particular, donors of group A presented the most favorable characteristics in terms of age, cause of death, and renal function, whereas donors of group C presented with a worse clinical profile (**Table 4**). This observation underlines the relevance of recipients' risk factors in group C in addition to the ones related to donors.

The Relevance of Recipients' Clinical Features in the Incidence of DGF

We, then, analyzed the clinical data of the 252 recipients of groups C, where from each donor we have one recipient who developed DGF and one who did not (**Table 5**). The univariate analysis showed that patients with DGF presented a significantly longer time on dialysis and higher BMI and were more frequently on hemodialysis than on peritoneal dialysis compared with patients with EGF (**Table 6**). In addition, HCV prevalence and a higher number of mismatches were associated with DGF, although this association did not reach statistical significance. The frequency of right versus left kidney was equally distributed between the two groups (66 left and 60 right kidneys in the EGF group and 60 left and 66 right kidneys in the DGF group). At the multivariate logistic regression, the variables that remain significantly and independently associated with DGF were BMI, number of mismatches, and renal replacement therapy with hemodialysis (**Table 6**).

Recipients Who Developed DGF Showed Transcriptomic Profiles Featuring a Specific Immune Signature in Peripheral Blood Mononuclear Cells

In order to identify recipients' transcriptomic patterns associated with DGF, we analyzed gene expression profiling of PBMCs at the time of transplantation in 7 recipients who developed DGF and 7 who had EGF after transplantation paired from the same donor, randomly selected from group C, since patients belonging to this group exclude all the bias linked to donor. The analysis of the transcriptomic profiles, applying a FC ≥1.5, showed that 411 genes were differentially expressed in PBMC before transplantation in graft recipients who experienced a DGF compared to those who had a normal functional recovery of the graft. Principal component analysis (PCA) revealed a complete separation of the two patient groups based on the expression profiles of the 411 differentially regulated genes (**Figure 1A**).

The analysis by IPA of the main biological and pathological functions in which the differentially expressed genes (**Figure 1B**) were included indicated inflammatory disease (p range = 1.3E-03-1.5E-2, 33 genes) and inflammatory response (p range = 2.65E-03-1.07E-02, 49 genes), two functional patterns featuring the immunological profile of hemodialysis patients.

IPA analysis also revealed the presence of 5 upstream regulators (liraglutide, EOGT, PKA, CCL2, NPS), which are molecules that can affect the expression, transcription, or phosphorylation of other molecules included in the dataset according to interactions described in literature. Interestingly, among this there was CCL-2 with an activation z-score of 1.206 (the z-score algorithm is used to make predictions and is designed to reduce the possibility that random data will generate significant predictions) (18) and a p-value of 8.06E-04. Among the 9 molecules interacting with CCL-2 and included in the dataset (IFNAR1, CCR2, FOS, FOLR2, CD40, NCF1, IL23A, SCARB1, BIRC5) identified by IPA software, there was CCR2, with an expression fold change in DGF vs. EGF of 1.869. The main functional networks in which the differentially expressed genes were included demonstrated the existence of several networks potentially modulated in patients who developed DGF. CCR-2 was included among the highest-ranked network (IPA score = 20) associated with inflammatory response and cell-to-cell signaling and interaction (**Figure 1C**).

TABLE 3 | The univariate and multivariate analyses of donors' and recipients' features associated with DGF.

	Univariate			Multivariate		
	OR	95% CI	p	OR	95% CI	p
Recipient age (years)	1.030	1.016–1.044	<0.0001	0.978	0.942–1.016	0.2
Dialysis vintage (years)	1.075	1.035–1.117	0.0002	1.096	1.013–1.185	0.02
Body mass index (BMI) (kg/m^2)	1.080	1.038–1.125	0.0002	1.136	1.035–1.248	0.007
Recipient's HCV (positive vs. negative)	2.110	1.462–3.044	<0.0001	2.829	1.242–6.446	0.01
HD (vs. PD)	3.249	1.537–6.869	0.002	1.317	0.396–4.385	0.6
Donor's age (years)	1.025	1.015–1.034	<0.0001	1.032	1.006–1.058	0.01
Donor' serum creatinine (mg/dl)	1.384	1.007–1.932	0.04	1.645	1.098–2.464	0.01
Cause of death (cerebrovascular vs. trauma)	1.479	1.059–2.065	0.02	1.013	0.455–2.258	0.9

TABLE 4 | The main clinical and demographic characteristics of Groups A, B, and C.

	Group A (%)	Group B (%)	Group C (%)	p
n.	580	100	252	
Age (years)	44.6 ± 11.4	49.0 ± 12.0	47.3 ± 10.1	<0.0001
Gender (M/F)	365 (62.9)/215 (37.1)	69 (69)/31 (31)	161 (63.9)/91 (36.1)	0.5
Dialysis (HD/PD)	523 (90.1)/57 (9.9)	95 (95)/5 (5)	232 (92.1)/20 (7.9)	0.2
Dialysis vintage (years)	5.8 ± 4.3	7.0 ± 4.5	6.8 ± 4.5	0.01
HCV+ (n,%)	76, 13.1	24, 24.0	56, 22.2	0.0006
Body mass index (BMI) (kg/m2)	23.8 ± 3.8	24.8 ± 3.8	24.2 ± 3.6	0.03
Panel reactive antibodies (%)	7.1 ± 4.3	7.3 ± 4.4	7.0 ± 5.1	0.9
Cold ischemia time (hours)	13.2 ± 5.0	10.6 ± 3.2	13.1 ± 5.4	<0.0001
Mismatches (n)	3.1 ± 0.9	3.0 ± 0.8	3.4 ± 0.9	<0.0001
Donor age (years)	42.3 ± 17.3	53.0 ± 15.9	48.5 ± 16.9	<0.0001
Donor serum creatinine (mg/dl)	1.18 ± 0.61	1.69 ± 1.16	1.37 ± 0.84	0.002
Cause of death (trauma/vascular)	211 (36.4)/304 (63.6)	24 (24)/66 (66)	81 (32.1)/150 (67.9)	0.02
Cyclosporine (n)	300 (51.7)	93 (93)	15 (5.9)	<0.0001
Tacrolimus (n)	262 (45.2)	3 (3)	214 (84.9)	<0.0001
mTOR inhibitors (n)	74 (3.1)	4 (4)	2 (0.8)	<0.0001

We decided to focus on this molecule, since our group already demonstrated that an increased CCR2 gene and protein expression on uremic peripheral blood mononuclear cells may contribute to chronic micro-inflammation related to dialysis (19). Thus, we aimed to investigate whether CCR-2-increased expression might also influence DGF occurrence. CCR2 or CD192 is a seven-transmembrane G-protein-coupled receptor that interacts with several ligands, in particular monocyte chemoattractant protein 1 (MCP-1), a chemokine which specifically mediates the chemotaxis of monocytes/macrophages.

CCR2 Might Represent a Predictive Marker of DGF

To confirm the microarray data and to evaluate whether the identified genes were specific for recipients who developed DGF after kidney transplantation, quantitative real-time PCR was used to compare the expression levels of both CCR-2 and MCP-1 in an independent group of PBMC from recipients who developed DGF (n = 25) and those who presented EGF after transplantation (n = 25). We confirmed that before transplantation, recipients who developed DGF had higher levels of both CCR2 and MCP-1 (**Figure 1D**). In addition, we observed a significantly higher CCR2 expression in HD compared with PD patients (HD 1.66 ± 0.83 vs. PD 0.71 ± 0.32; p = 0.003) and a significant and direct correlation of CCR2 gene expression with dialysis vintage (r2 = 0.117, p = 0.2).

Finally, we applied a ROC curve analysis to evaluate the sensitivity and specificity of CCR2 as a predictive marker of DGF. The AUC was 0.732, and the value of relative expression of CCR2 of 1.38 allowed discriminating patients who developed DGF with a specificity of 68% and a sensitivity of 74% (**Figure 1E**).

DISCUSSION

The present study provides for the first time an integrated overview of the recipients' characteristics that may influence DGF occurrence after kidney transplantation and describe a molecular signature based on whole-genome PBMC gene expression profiles of those recipients who will develop DGF compared to those who will have a normal graft function recovery after kidney transplantation.

Doshi et al., examining recipients' pairs from the UNOS database, who shared a common deceased donor and were discordant in DGF occurrence, identified recipients' factors

TABLE 5 | The main clinical and demographic features of Group C: EGF and DGF patients.

	Group C (%)	EGF (%)	DGF (%)	P
n.	252	126	126	
Age (years)	47.3 ± 10.1	46.8 ± 11.0	47.8 ± 9.3	0.4
Gender (M/F)	161 (63.9)/91 (36.1)	80 (63.5)/46 (36.5)	81 (64.3)/45 (35.7)	0.9
Dialysis (HD/PD)	232 (92.1)/20 (7.9)	109 (86.5)/17 (13.5)	123 (97.6)/3 (2.4)	0.0006
Dialysis vintage (years)	6.8 ± 4.5	5.9 ± 4.1	7.7 ± 4.8	0.008
HCV+ (n,%)	56, 22.2	22, 17.4	34, 26.9	0.07
Body mass index (BMI) (kg/m2)	24.2 ± 3.6	23.5 ± 3.3	24.8 ± 3.9	0.007
Panel reactive antibodies (%)	7.0 ± 5.1	7.5 ± 5.9	7.3 ± 6.7	0.9
Cold ischemia time (hours)	13.1 ± 5.4	13.0 ± 5.3	13.2 ± 5.5	0.8
Mismatches (n)	3.4 ± 0.9	3.3 ± 0.9	3.5 ± 0.9	0.1
Cyclosporine (n)	15 (5.9)	19 (15.1)	19 (15.1)	1
Tacrolimus (n)	214 (84.9)	107 (84.9)	107 (84.9)	1
mTOR inhibitors (n)	2 (0.8)	2 (1.5)	2 (1.5)	1

TABLE 6 | Univariate and multivariate analyses of the features of Group C associated with DGF.

	Univariate			Multivariate		
	OR	95% CI	p	OR	95% CI	p
Dialysis vintage (years)	1.094	1.021–1.172	0.01	1.047	0.967–1.132	0.2
Body mass index (BMI) (kg/m^2)	1.102	1.025–1.184	0.008	1.154	1.051–1.268	0.002
Recipient's HCV (positive vs. negative)	1.747	0.954–3.200	0.07	1.530	0.697–3.356	0.3
HD (vs. PD)	6.394	1.824–22.415	0.003	4.699	1.169–18.886	0.03
Mismatches (n)	1.206	0.917–1.588	0.1	1.661	1.153–2.392	0.006

including gender, race, diabetes, and obesity that were strongly associated with DGF risk (20). In line with these results, we observed that BMI, number of mismatches, and hemodialysis were independent predictors of DGF in pairs of recipients from the same donor. It is well known that ischemia reperfusion injury may induce a strong recipient immune response (21, 22) and can represent an important factor for long-term allograft failure. However, compared to Doshi's dataset (20), our patients' population presented a limited cold ischemia time, due to the regional allocation policy currently in use in Italy. Thus, it is conceivable that the short cold ischemia time featuring our study reduced the role of this variable on DGF incidence rate. In addition, Doshi et al. (20) performed their analysis on a population of patients identified based on the definition of DGF as the need of dialysis during the first week after transplantation and on a smaller group where DGF was identified using the definition that we adopted in the present study. The definition of DGF as the need of dialysis during the first week after transplantation might result in the inclusion of patients with a normal recovery of graft function that may

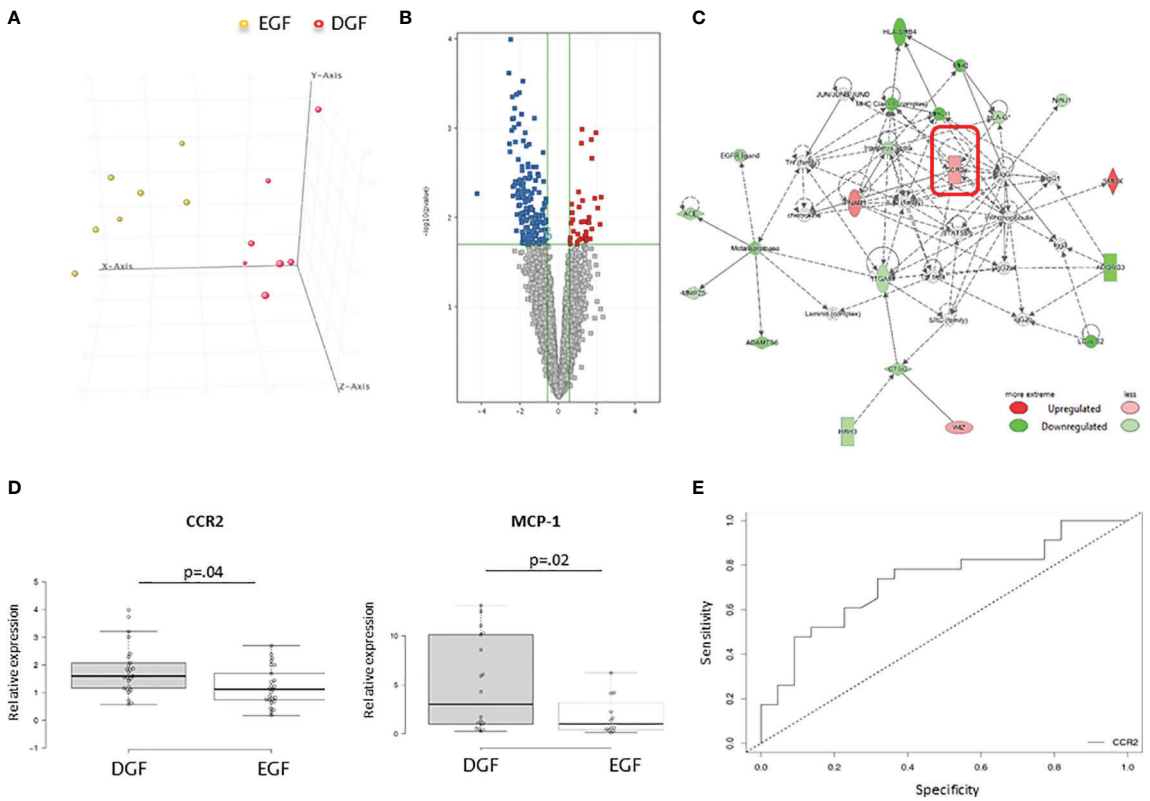

FIGURE 1 | **(A)** Principal component analysis (PCA) of mRNAs discriminating DGF and EGF subjects. DGF (n = 7) and EGF (n = 7) patients are indicated in red and yellow, respectively. **(B)** Volcano plot of the 411 differentially expressed genes among DGF patients and EGF patients with a FDR <0.05 and a FC > 1.5. **(C)** Functional network of the differentially expressed genes among DGF patients and EGF controls. The most significant network is displayed graphically as nodes (genes) and edges (biological relationship between nodes). The node color intensity indicates the fold change of that gene's expression. Shaded nodes are those genes identified by our microarray analysis, and empty nodes represent genes automatically included by IPA. The shapes of nodes indicate the functional class of the gene product, and the lines indicate the type of interaction. **(D)** Validation by quantitative real-time PCR of CCR2 and MCP-1 in PBMCs isolated from an independent group of 25 DGF and 25 EGF patients for CCR-2 gene expression and 11 DGF and 11 EGF for MCP-1 gene expression. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. **(E)** ROC curve analysis to evaluate the sensitivity and specificity of CCR2 as a predictive marker of DGF.

undergo a dialysis session for a transient volume overload immediately after transplantation or for the appearance of a significant hyperkalemia.

The recipients' immunological features, possibly modulated by dialysis modality (hemodialysis vs. peritoneal dialysis), are significantly associated with the development of DGF. A lower number of recipients' pre-transplant circulating regulatory T cells were predictive of delayed or slow graft function in kidney graft recipients from deceased donors (23). In order to understand the role of the recipients' immune system in the pathogenesis and development of DGF, we analyzed specific gene expression profiles of circulating immune cells. Early transcriptomic biomarkers identified with this approach might represent valuable mechanistic information, suggesting a possible molecular mechanism explaining the association of DGF with recipients' clinical features and might help clinicians to identify patients with higher risk and suggest potential novel therapeutic approaches.

In the present study, we report that DGF occurrence was associated with specific recipient gene expression profiles of PBMC at the time of transplantation, mainly involving the inflammatory pathway. This molecular observation might fit with the clinical data suggesting hemodialysis as an independent risk factor for DGF when compared with peritoneal dialysis. Indeed, we have demonstrated by a similar microarray-based approach that the expression profiles of circulating lymphomonocytes of hemodialysis patients were characterized by the upregulation of the inflammatory pathway when compared with peritoneal dialysis patients that resembles the one observed in graft recipient with DGF (24). Peritoneal dialysis has been often recognized as a protective factor for DGF, although the cause of the potential beneficial effect of this dialysis modality was identified in the frequent overhydration of peritoneal dialysis patients facilitating the recovery of diuresis after transplantation.

Among the genes characterizing the inflammatory signature predicting DGF, CCR2 expression might represent an interesting molecular candidate. Chemokine production is a characteristic feature occurring after ischemia-reperfusion injury and is associated with an increased risk of acute and chronic allograft rejection (25). Increased MCP-1 expression at the tissue and urinary level is a predictor of acute kidney injury and is associated with an adverse outcome after graft rejection (26, 27). Moreover, several inflammatory cytokines such as G-CSF, IL-6, IL-9, IL-16, and MCP-1 are released by the kidney from brain dead donors, thus initiating an inflammatory state of the graft and massive inflammatory cytokine release upon reperfusion (28). High levels of these cytokines could therefore promote the recall of monocyte/macrophages in the graft, thus representing a promoting factor for the development of DGF. This evidence strongly supports our results of an increased expression of CCR2 before transplantation in the PBMCs of DGF patients compared to graft recipients presenting with an early graft function.

Patients with chronic renal failure undergoing hemodialysis treatment have elevated serum levels of MCP-1 and an increased expression of its CCR2 receptor on circulating monocytes (29). This process is secondary to both uremia (30) and the activation of the coagulation cascade induced by the contact between the blood and the dialysis membranes (19). Indeed, we have previously demonstrated that the activation of the coagulation cascade during hemodialysis may induce CCR2 gene and protein expression in circulating lymphomonocytes. Interestingly, this increased expression was significantly reduced by an approach that limited coagulation cascade priming on the dialyzer (19). Moreover, Liu et al. (31) have already demonstrated that ischemia reperfusion injury promotes production of IL-18 from endothelial cells and expands a T-cell population (CD4 +CD45RO+PD-1hiICOS+CCR2+CXCR5-) displaying features of recently described T peripheral helper cells with increased CCR2 expression.

In accordance with this evidence, in our predictive model DGF occurrence was strictly associated with the number of mismatches and hemodialysis and could depend on the specific expression of the MCP-1/CCL2 receptor CCR2 on PBMC, whose increased levels could promote the recruitment of recipient inflammatory cells in the graft, thus influencing the onset of DGF.

Our data would suggest that the measurement of CCR2 expression before transplantation on the recipients alongside the evaluation of the clinical characteristics of the donor and recipient (32) could be used to develop novel predictive models of DGF, although this hypothesis should be formally confirmed in larger prospective studies. The association between an increased CCR2 gene expression and DGF might represent valuable mechanistic information, suggesting a possible molecular mechanism explaining the association of DGF with recipients' clinical features and, in particular, with previous hemodialysis treatment. This approach would allow implementing new therapeutic strategies in patients at higher risk of DGF such as the allocation of organs with a reduced ischemia time or from optimal donors to patients at risk of DGF; the use of reperfusion machines after organ harvesting; and the inhibition of inflammation both in the donor and in the recipient. In addition to these therapeutic approaches, our data would suggest that also MCP-1 antagonists might represent an interesting alternative in the attempt to prevent DGF and improve long-term graft outcomes.

The main limitation of our investigation is its being a single-center study, being well known that single-center studies result in a selection bias. However, we tried to overcome this limit in the transcriptomic study by randomly selecting the patients included in the testing and validation groups. In addition, a strength of our study is represented by the prospective analysis.

In conclusion, our data suggest that recipients' clinical and immunological features, possibly modulated by dialysis, independently of donors' characteristics, are significantly associated with the development of DGF. In addition, we identified potential transcriptomic biomarkers for DGF that might be introduced in clinical practice to define the risk for DGF before kidney transplantation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the following: https://www.ebi.ac.uk/arrayexpress/, E-MTAB-10747.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Policlinico di Bari Ethical Committee (Prot. N. 670/CE-2017) and were in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PP and SS conceived and performed the experiments and clinical analysis, designed and coordinated the study, analyzed the data, and drafted the manuscript. FR and FC carried out the experiments, analyzed the data, and revised the manuscript. FP collected the clinical data, helped to interpret the results, and revised the manuscript. FS supported the analysis of microarray data. BI, GC, MF, GZ, and GS enrolled the patients, participated in the performance of the research, and revised the manuscript. AG, MB, and PD participated in the research design and revised the manuscript. LG and GG designed, coordinated, and supervised the study and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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The Role of the Superior Cervical Sympathetic Ganglion in Ischemia Reperfusion-Induced Acute Kidney Injury in Rats

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Acute kidney injury (AKI) has been found to be a serious clinical problem with high morbidity and mortality, and is associated with acute inflammatory response and sympathetic activation that subsequently play an important role in the development of AKI. It is well known that the sympathetic nervous system (SNS) and immune system intensely interact and mutually control each other in order to maintain homeostasis in response to stress or injury. Evidence has shown that the superior cervical sympathetic ganglion (SCG) participates in the bidirectional network between the immune and the SNS, and that the superior cervical ganglionectomy has protective effect on myocardial infarction, however, the role of the SCG in the setting of renal ischemic reperfusion injury has not been studied. Here, we sought to determine whether or not the SCG modulates renal ischemic reperfusion (IR) injury in rats. Our results showed that bilateral superior cervical ganglionectomy (SCGx) 14 days before IR injury markedly reduced the norepinephrine (NE) in plasma, and down-regulated the increased expression of tyrosine hydroxylase (TH) in the kidney and hypothalamus. Sympathetic denervation by SCGx in the AKI group increased the level of blood urea nitrogen (BUN) and kidney injury molecule-1 (KIM-1), and exacerbated renal pathological damage. Sympathetic denervation by SCGx in the AKI group enhanced the expression of pro-inflammatory cytokines in plasma, kidney and hypothalamus, and increased levels of Bax in denervated rats with IR injury. In addition, the levels of purinergic receptors, P2X3R and P2X7R, in the spinal cord were up-regulated in the denervated rats of the IR group. In conclusion, these results demonstrate that the sympathetic denervation by SCGx aggravated IR-induced AKI in rats via enhancing the inflammatory response, thus, the activated purinergic signaling in the spinal cord might be the potential mechanism in the aggravated renal injury.

Keywords: cervical sympathetic ganglion, superior cervical ganglionectomy, sympathetic nervous system, immune system, renal ischemic reperfusion injury, acute kidney injury

INTRODUCTION

Acute kidney injury (AKI), is a frequent clinical syndrome primarily caused by renal ischemic-reperfusion (IR) injury in situations such as during major surgery, septic shock, cardiogenic shock, hypovolemia, and nephrotoxic drugs (1). AKI has a high risk of morbidity and mortality, as well as progression to chronic kidney disease (CKD) and end-stage renal disease (ESRD), leading to increased resource utilization and social burden (2, 3). The mechanism underlying IR-induced AKI is not fully understood, however, it has been reported that inflammation, apoptosis, oxidative stress and other factors contribute to the pathogenesis of this renal injury (1, 4). A wide array of studies have demonstrated that the kidney is innervated by both afferent sensory nerve fibers and efferent sympathetic nerve fibers, which are activated in renal IR injury (5, 6). It is also well established that a kidney-central nervous system crosstalk exists in the acute setting that renal sympathetic nerve activity arises from and provides context to the central integration with incoming sensory information from the somatosensory and viscerosensory systems that regulate fluid volume and blood pressure (5, 7). A "reno-renal" reflex is formed in which tissue damage associated with ischemia results in activation of primary afferent nerve fiber terminals in addition to the release of the mediators of calcitonin gene-related peptide (CGRP), substance P (SP) and other neuropeptides from the afferent nerve fiber terminals. These transfer signals to the central nervous system followed by improved neural activity of the hypothalamus, a central site for the integration of sympathetic activity, which results in increased sympathetic outflow to the kidney (4, 8). And the renal sympathetic nervous system and circuiting catecholamines are considered to be involved in the development of ischemic acute kidney injury (9).

Over the last few decades, a body of data has continually emerged implicating an inextricable link between the nervous and immune systems. Evidence has demonstrated that sympathetic overactivity aggravates IR-induced renal damage via pro-inflammation mechanisms (10, 11). Activation of the pro-inflammation response is characterized by the so called "cytokine storm," which is represented by excessive cytokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 (12). In turn, in the early course of AKI, the various pro-inflammatory cytokines released into the arterial inflow and venous outflow of the kidney (13) can pass through the brain-blood barrier in order to stimulate the paraventricular nucleus (PVN), the rostral ventrolateral medulla (RVLM) and the solitary tract (NTS), which augment sympathetic outflow to the peripheral (14–16). Anti-inflammation actions of the sympathetic nervous system (SNS) also have been demonstrated in several systems (17, 18). The interaction between the SNS and the immune system in AKI-induced by renal IR injury is unclear and is a subject worth in-depth study.

The superior cervical ganglion (SCG) is one of the important parts of the sympathetic nervous system with the most traffic branches and special distribution positions. Bilateral sympathectomy of SCG has been shown to lead to degeneration of the tyrosine hydroxylase (TH) nerve fiber, the rate-limiting enzyme in the biosynthesis of catecholamine and a marker of SNS (19), in dura (20), and major cerebral arteries (21), and the regulation of cerebral blood flow (CBF) (22, 23). Likewise, SCG partly provides noradrenergic innervation of the hypothalamus (24, 25). Removal of the superior cervical ganglion significantly decreased NE uptake in the medial basal hypothalamus (MBH) and the result could possibly be explained by the peripheral sympathetic neurons or fibers from SCG projection to the MBH and/or the changed neuroendocrine activity brought by SCGx (26). Previous studies have demonstrated that after SCGx, there were changes in various hormones synthesized and/or released at hypothalamic nuclei (27–29), some of which have the ability to regulate cardiovascular function such as vasopressin. In fact, suppression of the cervical sympathetic innervation could affect not only the median cerebral structure and hypothalamic-pituitary axis, but also generally alter numerous neuroendocrine system (22, 30).

An earlier study demonstrated that the cervical sympathetic trunk as a relay of the bidirectional communication network between the nervous and immune system, and bilateral ganglionectomy of SCG, notably reduced the pulmonary inflammation potentially mediated by tissues or organs innervated by SCG (31). However, there exist some ambiguity concerning the effect, with a recent study revealed that lipopolysaccharide-induced inflammation was increased after sympathetic denervation (32). Sympathetic hypofunction might lead to an increase in parasympathetic activation and vice versa. The influence of parasympathetic nervous system on immune response has been sought in the light of studies showing that vagus nerve stimulation attenuated inflammation via activating the sensory efferent vagus nerve that suppresses monocyte and/or macrophage production of pro-inflammation cytokines such as TNF-α and IL-6 (33–35). The latest research shows that superior cervical ganglionectomy attenuated myocardial inflammation and cardiac disfunction after myocardial infarction (36). Hence, the role of SCG in the immune system is ambiguous and controversial, and needs further investigation.

In the light of the above research findings, the significance of the superior cervical ganglion in regulating acute renal injury is vague. The sympathetic neuron derived from SCG could modulate the stress-induced hypothalamus neurotransmitter, norepinephrine (NE), which plays a central role in the neuroendocrine response to stress. Thus, we hypothesized whether the superior cervical ganglion plays a role in AKI induced by renal IR injury through adjusting the sympathetic outflow from the hypothalamus and regulating the immune response. Our objective was to test the hypothesis that the "reno-brain axis" interacts via changes in renal afferent and efferent sympathetic nerve activity that contribute to the renal and brain inflammation and to the progression of ischemic AKI.

MATERIALS AND METHODS

Animals

Male Sprague Dawley (SD) rats (180–200 g; specific pathogen-free grade) were purchased from the Laboratory Animal Center

of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China). Animals were individually housed in a climate controlled room (temperate of 25 ± 1°C, relative humidity of 50 ± 10%, and a 12 h light/dark cycle) with *ad libitum* food and water. All experiments were strictly carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The present experimental protocol was approved by the Institutional Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) (TJH-202102002).

Experimental Design

After 1 week of acclimation, all rats were randomly divided into the following four groups:(1) Sham group (sham, $n = 6$); (2) IR group (IR, $n = 6$); (3) superior cervical ganglionectomy + sham group (SCGx, $n = 7$); (4) IR + superior cervical ganglionectomy group (IR + SCGx, $n = 7$). All surgical procedures were conducted with sterile instruments. For all surgeries, rats were anaesthetized with 40 mg/kg sodium pentobarbital intraperitoneal injection and placed on a heating pad to maintain body temperature. The SCGx surgery was performed as previously described (37). After induction of anesthesia and disinfection, a 2 cm vertical incision was performed at the neck region and then salivary glands were exposed and carefully dissected to expose the underlying muscles. With blunt forceps, the cranial portion of the sternomastoid muscle (SMM) and the omohyoid muscle (OMH) were transected for clear visibility of the common carotid artery (CCA). Next the CCA was bluntly dissected cranially to locate the carotid bifurcation into the external and internal carotid arteries (ECA and ICA), the SCG was identified behind the carotid bifurcation. Finally, the cell body of the ganglion was gently pulled until its full avulsion from the sympathetic chain and the SCG tissue were collected. Complete superior cervical ganglionectomy was achieved by the additional removal of the SCG on the contralateral side. After SCG removal, the incision was closed with 4–0 sutures and compound lidocaine cream was applied to the wound locally to alleviate incision pain. In the sham group, only exposure of the superior cervical ganglion was performed. After surgery, rats were placed back on heated pads for recovery and then returned to their own cages with free access to food and water. The palpebral ptosis was used as an indicator of the successful removal of the SCG. Fourteen days after the intervention, acute kidney ischemic reperfusion (IR) injury was performed according to previous published protocol (38). A midline laparotomy was made to expose the bilateral renal pedicles and the renal IR were induced by clamping both renal pedicles for 45 min with a non-traumatic vascular clamp. The ischemic was confirmed by visual inspection of the kidney from bright red to purple-black. To reduce abdominal air, 1 ml warm normal saline was given intraperitoneally before abdominal closure. In the sham operation group, a similar surgical procedure was performed, except for renal pedicle clamping. All rats were euthanized 24 h after reperfusion, and blood and tissue samples (kidney, T8-T12 spinal cord and hypothalamus) were collected for experimental studies.

Analysis of Renal Function and Histology

Renal function was detected by measuring serum creatinine (Scr) and blood urea nitrogen (BUN) in plasma at the Department of Clinical Laboratories of Tongji Hospital. For histology, excised left kidney were processed for light microscopic observation, according to the standard procedure. The kidney specimens were first fixed with 10% formalin solution and embedded with paraffin. Then, 3 um thick renal tissue sections were cut and stained with hematoxylin and eosin. Histopathological changes were analyzed and graded as follows: (1) tubular epithelial smoothness or tubular expansion: score 1, (2) loss of brush-like edge: score 1or 2, (3) obstruction of tubular lumen: score 1 or 2, (4) cytoplasmic vacuolization: score1, and (5) cell necrosis: score 1. The evaluation of histological data was performed by two independent observers blinded to the experimental groups.

Western Blot

Protein from kidney samples was extracted by RIPA lysis containing freshly added phosphatase and protease inhibitors. Protein concentrations were determined using the BCA Protein Assay Kit (Wuhan Boster Biological Technology, Ltd., China). Equal amounts of total protein (30–50 μg/lane) were separated on 10% SDS-PAGE gels and subsequently transferred to PVDF membranes. The membranes were blocked in 5% skim milk in TBST for 2 h at room temperature and then incubated with primary antibody overnight at 4°C. Primary antibodies were applied as follows: rabbit anti-TNF-α (1:1000; Cat No. 17590-1-AP; Proteintech Group Co., Ltd., China), rabbit anti-IL-6 (1:1000; Cat No. A0286; Abclonal Technology Co., Ltd., China), rabbit anti-TH (1:1,000; #58844S; Cell Signaling Technology, United States), rabbit anti-Bcl2 (1:1,000; Cat No. A11313; Abclonal Technology Co., Ltd., China), rabbit anti-BAX (1:1,000; Cat No. A0207; Abclonal Technology Co., Ltd., China), rabbit anti-Caspase-3 (1:1,000; Cat No. A19664; Abclonal Technology Co., Ltd., China), rabbit anti-P2X3R (1:1,000; Cat No. A12965; Abclonal Technology Co., Ltd., China), rabbit anti-P2X7R (1:1,000; Cat No. A10511; Abclonal Technology Co., Ltd., China), rabbit anti-GAPDH (1:10,000; Cat No. BM1623; Wuhan Boster Biological Technology, Ltd., China), rabbit anti-β-actin (1:100,000; Cat No. AC026; Abclonal Technology Co., Ltd., China). After washing, the membrane was incubated with HRP-conjugated goat-anti-rabbit IgG (1:5,000; Cat No. BA1065; Wuhan Boster Biological Technology, Ltd., China) for 2 h at room temperature, bands were visualized using Super-Lumia ECL Plus HRP Substrate Kit (K22030; Abbkine Scientific Co., Ltd., China) and detected using a computerized image analysis system (ChemiDoc XRS1, Bio-Rad, Hercules, CA). The mean intensities of selected areas were normalized to values of the internal control (GAPGH and β-actin) and the areas of these images were calculated using the image lab software (Bio-Rad).

TABLE 1 | Primers used for real-time PCR.

Primer name	Forward primer (5′→3′)	Reverse primer (3′→5′)
TNF-α	AAAGGACACCATGAGCACGGAAAG	CGCCACGAGCAGGAATGAGAAG
IL-6	ACTTCCAGCCAGTTGCCTTCTTG	TGGTCTGTTGTGGGTGGTATCCTC
KIM-1	ATAGTGGTCTGTATTGTTGCCGAGTG	TGTGGTTGTGGGTCTTGTAGTTGTG
NGAL	TTGACAACTGAACAGACGGTGAGC	GAAAGATGGAGCGGCAGACAGAC
TH	TTGACCCTGATCTGGACCTGGAC	ATTGGTTCACCGTGCTTGTACTGG
P2X3R	AAAGAGATGTGGGAGAGGGAGAGTC	GTGGGCAAGCAGAGGGAAGAAAG
P2X7R	ATTAACCAGACTAGAAGCCATCGCATC	AGCAGTCACTTAGAACCATAGCATAGC

TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; TH, tyrosine hydroxylase; P2X3R, purinergic receptors P2X3; P2X7R, purinergic receptors P2X7.

Immunofluorescence Staining

After removal, the SCG was fixed with PBS in 4% paraformaldehyde overnight at 4°C. The ganglia were washed three times in PBS for 5 min per wash, dehydrated in 20% sucrose/PBS for 24 h and 30% sucrose/PBS for 48 h and embedded in OCT. 20 μm thick serial sections of the ganglia were cut with a Leica cryostat. These sections underwent three 10-min washes in PBS and permeabilized with PBST (0.3% TritonX-100 in PBS) for 30 min. After washing, the tissue slices were incubated with blocking solution (5% bovine serum albumin) for 1 h at room temperature. Then sections were incubated at 4°C overnight with the rabbit anti-TH antibody (1:100; #58844S; Cell Signaling Technology, United States). After three washes in PBS for 10 min per wash, sections were incubated with secondary antibody conjugated with Alexa Fluor 488 (Goat Anti-Rabbit IgG, 1:100; Protomer) for 2 h at room temperature followed by counterstaining with DAPI (Wuhan Boster Biological Technology, Ltd., China). The sections were examined using fluorescence microscope (DM2500; Lecia).

Enzyme-Linked Immunosorbent Assay

Blood samples were collected (1.5 ml) from the postcava into heparinized tube and then centrifuged at 3,000 rpm for 10 min at 4°C. The supernatants were collected, and the levels of plasma IL-6, TNF-α, NE were determined using the Rat IL-6R ELISA kit (Cat No. E-EL-R0896c; Elabscience Biotechnology Co., Ltd., China), Rat TNF-α ELISA kit (Cat No. E-EL-R2856c; Elabscience Biotechnology Co., Ltd., China) and Noradrenaline/Norepinephrine ELISA kit (Cat No. E-EL-0047c; Elabscience Biotechnology Co., Ltd., China). The procedures were according to the manufacturer's instructions, and the concentration was presented as pg/ml.

Real-Time PCR

Total RNA from the kidney, hypothalamus or spinal cord (T8-T12) was extracted by Trizol (Takara, Japan), and then the RNA concentration was quantified by a spectrophotometer (Eppendorf, Germany). RNA reverse transcription was conducted using PrimerScript^{TM} reagent kit (Takara, Japan) according to the manufacture's instruction. TNF-α, IL-6, NGAL, Kim-1, TH, P2X3R, P2X7R were amplified using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotechnology Co., Ltd., China) on the ABI7900 (Illumina). The relative expression level of mRNA was quantified by normalization to β-actin of the Sham group and analyzed with $2^{-\Delta\Delta CT}$ method. The sequences of the primers are listed in **Table 1**.

Statistical Analyses

All data are present as mean ± SEM, P-value < 0.05 was considered statistically significant. We used one-way ANOVA followed by the Bonferroni test for statistical analysis with the GraphPad Prism 8.0 software.

RESULTS

Sympathetic Denervation by Superior Cervical Ganglionectomy Effectively Reduced Levels of Norepinephrine in Plasma and the Expression of Tyrosine Hydroxylase in the Kidney and Hypothalamus After Ischemic Reperfusion Injury

The experimental outline is depicted in **Figure 1A**. Fourteen days prior to renal IR surgery, superior cervical ganglionectomy (SCGx) was conducted, and the animals were randomly assigned into the following four groups, i.e., a sham group (sham), a sympathetic denervation group (SCGx), a renal ischemic reperfusion group (IR) and a renal ischemic reperfusion group with sympathetic denervation group (IR + SCGx). Palpebral ptosis is usually used as an indicator to assess the effectiveness of the SCGx and the rat with bilateral blepharoptosis was apparent after surgery (**Figure 1B**). In addition, we stained cryosection with antibodies against TH, a marker for SNS neurons (19), in order to validate that the removed tissue indeed contained the ganglionic sympathetic neurons ($n = 6$). All of the removed structure stained positive for TH with no signal detectable in the absence of the primary antibody (**Figure 1C**). We then sought to determine the effect of sympathetic denervation on the SNS and examine the expression of TH in the kidney and hypothalamus. As shown in **Figure 1D**, marked reduction of NE concentration was observed in the IR + SCGx group in plasma, compared with IR rats (1.235 ± 0.08399 ng/ml vs. 1.641 ± 0.08838 ng/ml, $P<0.001$). The protein levels of TH were significantly increased in the kidney after IR injury, as compared with the

sham control (**Figure 1E**). However, sympathetic denervation markedly decreased TH levels after IR injury (2.877 ± 0.3784 vs. 1.3 ± 0.1356, $P < 0.0001$, **Figure 1E**). Consistent with the results in the kidney, the mRNA and protein levels of TH in the hypothalamus were also determined and remarkedly increased in renal IR animals, compared with the sham-operated rats (**Figures 1F,G**). However, levels were significantly reduced in the denervated rats with renal IR-induced AKI (mRNA 2.155 ± 0.3414 vs. 5.204 ± 1.434, $P < 0.05$, **Figure 1F**; protein 0.9622 ± 0.1333 vs. 1.495 ± 0.1441, $P < 0.05$, **Figure 1G**). Taken together, the results above demonstrate that sympathetic denervation by SCGx resulted in a drastic reduction in NE content of plasma and reduced expression of TH in the kidney and hypothalamus after IR injury.

Sympathetic Denervation Contributed to Renal Functional and Structural Impairment After Ischemic Reperfusion Injury

Compared with sham-operated rats, the renal function of rats allocated to the IR group showed a marked deterioration with significant increase in Scr and BUN concentration (**Figures 2A,B**). In addition, the increased BUN induced by renal IR injury was significantly enhanced by sympathetic denervation in ischemic acute kidney injury rats (36.49 ± 1.031 mmlo/l vs. 45.74 ± 2.398 mmol/l, $P < 0.001$, **Figure 2B**). However, there was no difference in Scr concentration between the IR group and IR + SCGx group (330 ± 19.14 μmol/L vs. 328.3 ± 32.12 μmol/L, $P > 0.05$, **Figure 2A**). To confirm the renal injury further, we also detected the Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (KIM-1) in the kidney and found a notable enhancement in rats from the IR group, compared with sham-operated rats. Rats with AKI induced by IR injury treated with sympathetic denervation had higher levels of KIM-1 (339.2 ± 69.8 vs. 165.2 ± 40.98, $P < 0.05$, **Figure 2C**) compared to the non-denervated rats of IR injury; however, the difference in NGAL between the two groups was not statistically significant (106.1 ± 24.2 vs. 61.98 ± 14.53, $P > 0.05$, **Figure 2D**). Histopathological examination of kidney tissue by H&E staining revealed severe lesion in rats both from IR group and IR + SCGx group. As shown in **Figure 2E**, the IR group showed severe tubular lysis, loss of brush border, inflammatory cell infiltration, and sloughed debris in the tubular lumen space, as compared with the sham group. SCGx to ischemic acute kidney injury further promoted the development of all these lesions (**Figures 2E,F**). Results indicate that sympathetic denervation by SCGx aggravates AKI induced by IR injury.

Sympathetic Denervation Enhanced Renal Ischemic Reperfusion-Induce Inflammation Response and Expression of Pro-apoptosis Protein After Ischemic Reperfusion Injury

Inflammation and apoptosis are key factors in the development of renal IR injury, we, therefore, decided to test whether they are affected by sympathetic denervation. According to Enzyme-Linked Immunosorbent Assay (ELISA) results, the inflammatory cytokine levels of TNF-α and IL-6 were significantly higher in the IR + SCGx group than the IR group (TNF-α 171.2 ± 20.74 vs. 105.5 ± 18.17, $P < 0.05$, **Figure 3A**; IL-6 80.2 ± 8.689 vs. 51.74 ± 8.969, $P < 0.05$, **Figure 3B**). Likewise, the mRNA levels of pro-inflammation cytokines were found to be clearly increased in IR rats with sympathetic denervation in the hypothalamus, compared with the non-denervated IR rats (TNF-α 20.57 ± 3.969 vs. 2.887 ± 0.6566, $P < 0.0001$, **Figure 3C**; IL-6 3.538 ± 1.076 vs. 1.082 ± 0.2916, $P < 0.05$, **Figure 3D**). In addition, the mRNA and protein levels of TNF-α and IL-6 in the kidney were also determined and remarkedly increased in IR rats with sympathetic denervation (mRNA TNF-α 17.84 ± 6.476 vs. 5.409 ± 0.9479, $P < 0.05$, **Figure 3E**; IL-6 5.918 ± 1.455 vs. 2.697 ± 0.5214, $P < 0.05$, **Figure 3F**; protein TNF-α 1.878 ± 0.08540 vs. 1.608 ± 0.03856, $P < 0.05$, **Figure 3G**; IL-6 1.932 ± 0.3039 vs. 1.767 ± 0.3091, $P > 0.05$, **Figure 3H**). In the subsequent experiment, we examined the expression levels of apoptosis-related proteins, such as Bax, Bcl-2 and Caspase-3, and found that sympathetic denervation also increased levels of Bax in the kidney after IR injury, in spite of no statistical significance in caspase-3, whereas the down-regulated expression of Bcl-2 induced by IR was not affected, compared with the IR group (Bax 2.221 ± 0.09131 vs. 1.562 ± 0.1927, $P < 0.01$; Caspase-3 1.962 ± 0.1487 vs. 1.689 ± 0.08954, $P > 0.05$; Bcl-2 0.6831 ± 0.08026 vs. 0.6666 ± 0.07026, $P > 0.05$, **Figures 3I–L**).

Sympathetic Denervation Enhanced the Expression of P2X3R and P2X7R in the Spinal Cord After Renal Ischemic Reperfusion Injury

In the superior cervical sympathetic ganglion, P2X3 and P2X7 receptors mediated the sympathoexcitatory reflex induced by myocardial ischemic injury (39–41). T_{8-12} spinal cord segments are primarily involved in the sympathetic regulation of renal function (42). To demonstrate whether these purinergic receptors are also involved in the pathophysiology of the adverse effect of sympathetic denervation on renal IR injury, we examined the expression of P2X3R and P2X7R at both mRNA and protein levels. The expression of P2X3R and P2X7R were significantly increased in denervated rats of the IR-induced AKI, compared with non-denervated IR rats (P2X3R mRNA 49.03 ± 14.89 vs. 9.229 ± 2.771, $P < 0.01$; protein 1.87 ± 0.1658 vs. 1.353 ± 0.1131, $P < 0.05$; P2X7R mRNA 10.9 ± 2.994 vs. 1.353 ± 0.1131, $P < 0.01$; protein 1.788 ± 0.1931 vs. 1.282 ± 0.08897, $P < 0.05$, **Figure 4**). However, the protein levels of P2X3R and P2X7R in kidney showed no significantly difference in IR+SCGx group and IR group (**Supplementary Figure S1**).

DISCUSSION

The major results of the present study are as follows: (1) Increased sympathetic activity in the kidney and hypothalamus induced by renal IR injury were suppressed following the induction of

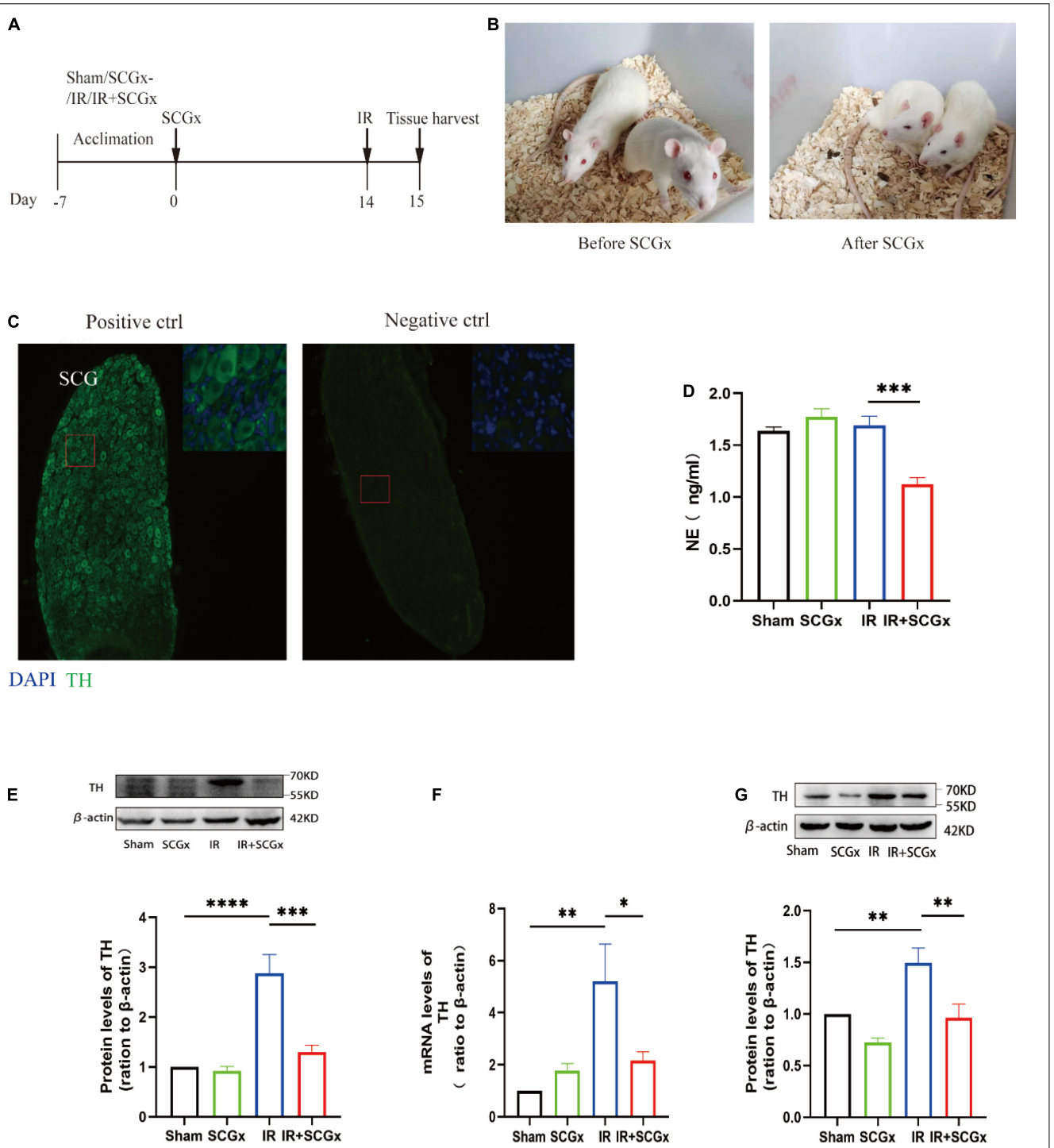

FIGURE 1 | Surgical removal of SCGs in IR rats reduced sympathetic activity. **(A)** Experimental scheme. The intervention group included Sham, SCGx (bilateral removal of the SCG), IR (bilateral renal ischemic for 45 min and reperfusion for 24 h), and IR + SCGx (bilateral removal of the SCG followed by renal ischemic reperfusion surgery). Tissue harvesting was performed after 24 h reperfusion. **(B)** Bilateral blepharoptosis after bilateral superior cervical ganglionectomy. **(C)** Immunofluorescence staining of surgically removed SCG. The same staining procedure served as a negative control, except that the primary antibody was omitted. Tyrosine hydroxylase (TH). Scale bar: 50 μm. **(D)** The NE concentration in plasma by ELISA. **(E)** The protein levels of TH in the kidney. β-actin served as an internal control. **(F)** The mRNA levels of TH in the hypothalamus. β-actin served as an internal control. **(G)** The protein levels of TH in the hypothalamus. β-actin served as an internal control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, each group contains at least 4 rats.

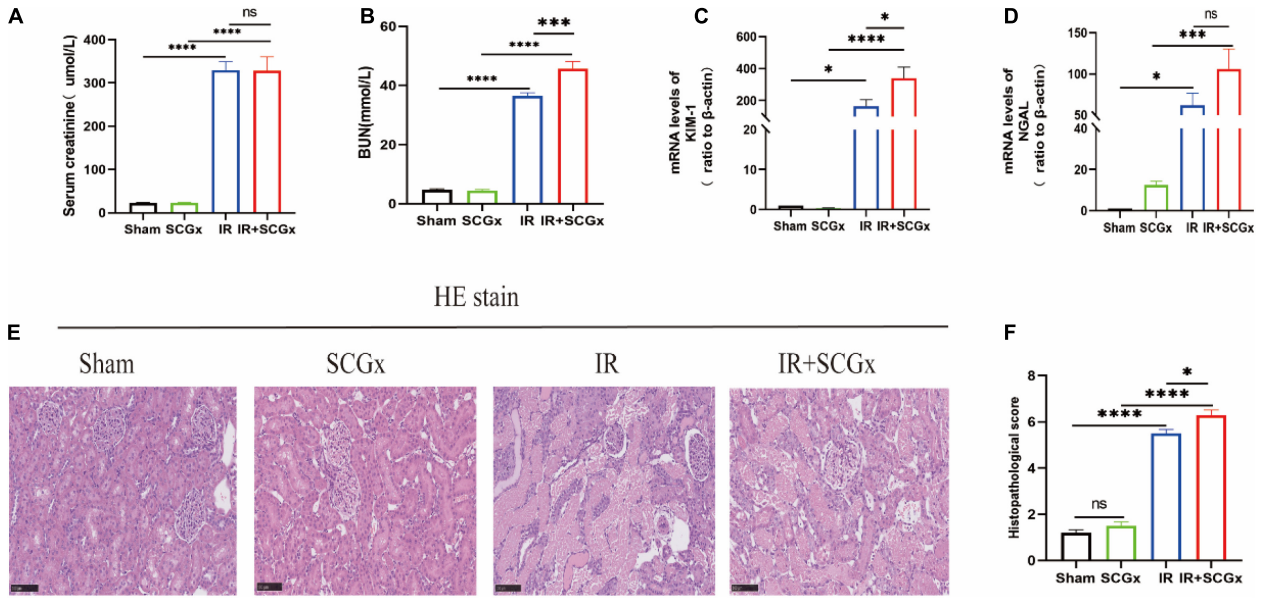

FIGURE 2 | Sympathetic denervation aggravated renal IR injury. **(A)** The Scr levels. **(B)** The BUN levels. **(C,D)** The mRNA levels of KIM-1 and NGAL in kidney. β-actin used as internal control. **(E)** Representative images of H&E staining in rat kidneys. **(F)** Histological score from H&E samples (n = 10). Scale bar = 100 μm. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$, each group contains at least 4 rats.

sympathetic denervation by SCGx in IR rats. (2) The functional and structural damage in IR rats was aggravated by SCGx. (3) Excessive expression of pro-inflammation cytokines and apoptotic protein were observed in denervated rats of AKI induced by IR injury. (4) Those effects brought by the SCGx may have resulted from the activated P2X3R and P2X7R in the spinal cord.

The renal nerves consist of both efferent and afferent nerve fibers. Tissue damage associated with ischemia results in the activation of primary afferent nerve fiber terminals and the transference of signals to the central nervous system, followed by the efferent nerve fiber activated and responsible for modulating renal hemodynamics and renal function. The efferent renal innervation contains postganglionic sympathetic fibers that typically exert their effects through release of NE onto the postsynaptic adrenoceptor (43). Moreover, the majority of experimental research support the evidence that the SNS activity is elevated in response to acute IR (11, 44, 45). The activation of renal sympathetic nerves occurs in acute kidney injury and has been identified as a key pathophysiologic factor contributing to disease progression (46, 47). Here, we carried out sympathectomy by bilateral ganglionectomy of SCG that could suppress the augmented sympathetic activity after renal IR injury and regulate the renal dysfunction and tissue injury during the early post-ischemic phase of renal IR injury.

Most previous studies have supported that the renal venous plasma NE concentration and TH expression is elevated in response to the reperfusion of the ischemic kidney (44, 45), as well as increased sympathetic activity in the cerebral sympathetic regulatory regions (48). Consistent with the above findings, our data also confirmed the presence of sympathetic overactivation after IR injury. In the present study, we obtained evidence that the expression of TH, the rate-limiting enzyme in the biosynthesis of catecholamine, was significantly elevated in the kidney and hypothalamus after IR injury, although the plasma NE concentration by ELISA was not changed. We also found that increases in TH were suppressed by SCGx. Interestingly, NE content notably decreased followed by sympathetic denervation in postischemic rats.

It has long been considered that, inhibition of the SNS is the target of a wide variety of therapeutic interventions in a number of clinical conditions. Current therapeutic interventions [such as renal denervation (49), ganglionic blockade (14) and centrally acting sympatholytic drugs (50)] have been shown to attenuate renal dysfunction and histological damage in experimental IR rats, mice and rabbits. Salman et al. reported that renal denervation before ischemia attenuated the deteriorated renal hemodynamic and excretory functions during the early post-ischemic phase of renal IR injury (49). Notably, an earlier study reported that GABA content increased after chronic SCGx in the mediobasal and anterior hypothalamic (51), while GABA was demonstrated to have protective effect on IR-induced renal injury in rats (52). In contrast, with regard to the effect of sympathetic denervation by SCGx on the progression of IR-induced AKI injury, our study showed aggravated renal morphological and functional changes in denervated rats with renal IR injury, manifested by the elevated biochemical markers such as BUN, KIM-1 and NGAL, as well as severe histopathological alteration.

Evidence also suggests, that there is a neuroimmune bidirectional network between the immune and SNS (53). Inflammation response resulting from acute kidney injury/acute stress is associated with suppressed parasympathetic nervous

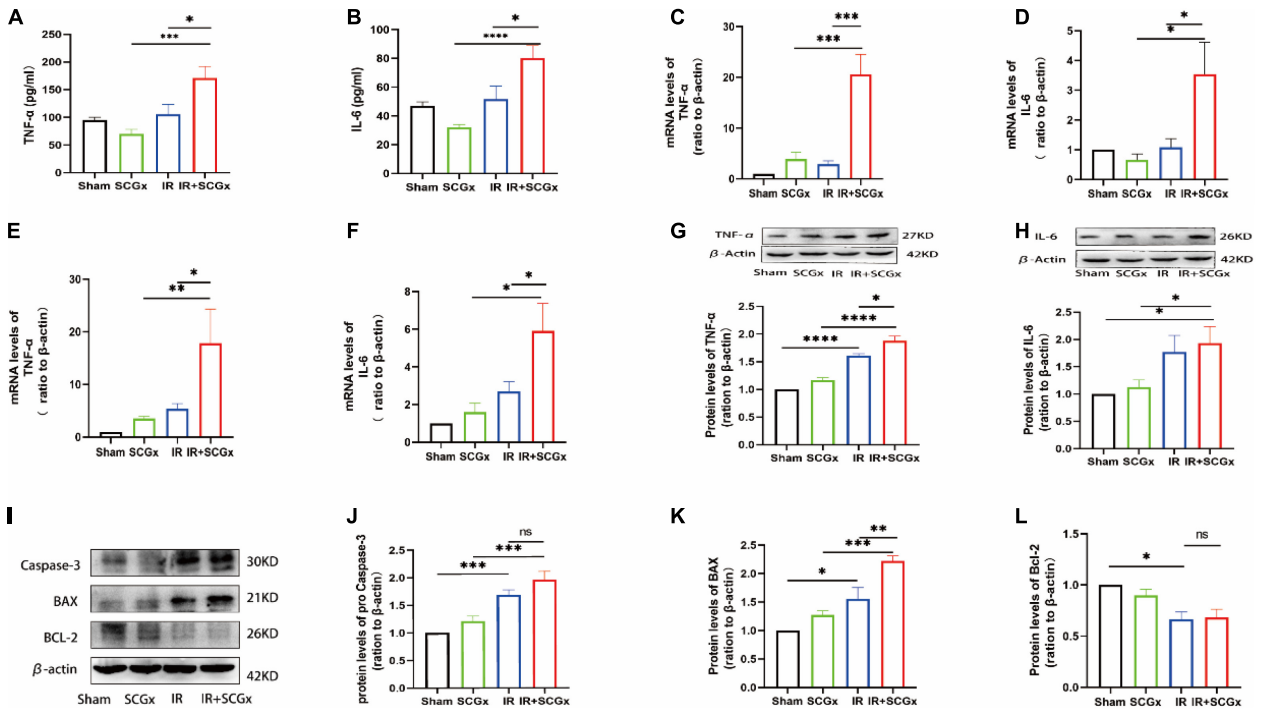

FIGURE 3 | Enhanced inflammation response and expression of pro-apoptosis protein upon sympathetic denervation. **(A,B)** The TNF-α and IL-6 are concentrated in plasma by ELISA. **(C,D)** Real-Time PCR for TNF-α and IL-6 in the hypothalamus. β-actin used as internal control. **(E,F)** Real-Time PCR for TNF-α and IL-6 in the kidney. β-actin used as internal control. **(G,H)** The protein levels of TNF-α and IL-6 in the kidney. β-actin served as an internal control. **(I)** Representative images of protein levels of Bax, Bcl-2 and pro Caspase-3. **(J–L)** Expression levels of Bax, Bcl-2 and Caspase-3 in the kidney were normalized to β-actin levels within the same sample. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, each group contains at least 4 rats.

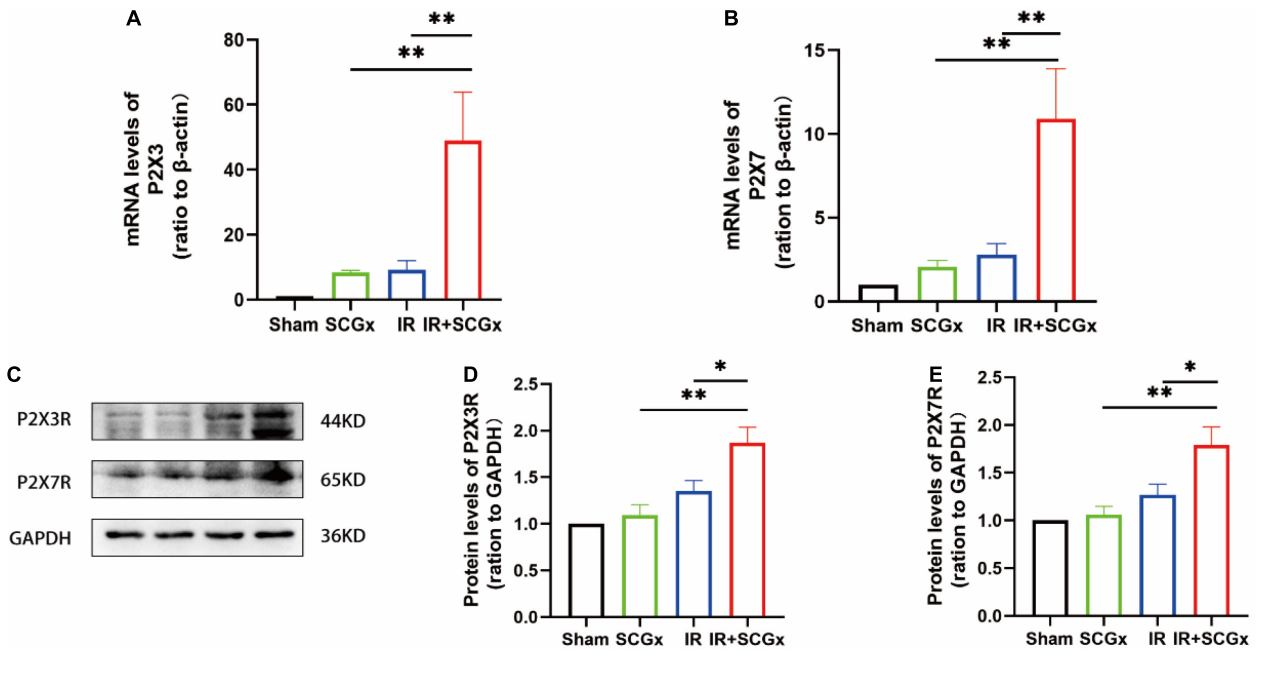

FIGURE 4 | The activated purinergic P2X receptor in the spinal cord upon sympathetic denervation. **(A,B)** Real-Time PCR for P2X3R and P2X7R in the spinal cord. β-actin used as internal control. **(C)** Representative images of protein levels of P2X3R and P2X7R. **(D,E)** The expression levels of P2X3R and P2X7R in the spinal cord were normalized to GAPDH within the same sample. $^{*}P < 0.05$; $^{**}P < 0.01$, each group contains at least 4 rats.

system activity and prevalence of the activation of the SNS. Furthermore, systemic and tissue release of NE, the primary neurotransmitter of the SNS coupled with the specific receptor expression in immune cells, can exert marked effects on the inflammation/immune response, such as modulation of cytokine production (54). A previous study showed augmented TNF-α secretion by peritoneal macrophages observed in sympathectomized mice (55). Similar exacerbation of TNF-α production after liver injury in chemically sympathectomized mice was reported by other investigators (56). Electrical stimulation of sympathetic nerves is also known to directly inhibit LPS-induced TNF-α secretion (57). However, Zhou et al. showed that NE upregulated TNF-α production *in vitro* experiments (58). Taken together, conclusions from the above studies seem to be contradictory, probably because of the differential effects mediated by the specific adrenergic receptor subtype. It has been suggested that the anti-inflammation effects of NE appear to be mediated via β_2-adrenergic receptors, in which NE inhibits proinflammatory cytokines and stimulates the production of anti-inflammatory cytokines through β_2-adrenergic receptor-cAMP-protein kinase A pathway (59). Whereas, α_2-adrenoceptro activation was demonstrated to have pro-inflammation effects (60). On the other hand, electrical stimulation of the vagus nerve inhibited synthesis of TNF-α in the liver, spleen and heart, and attenuated serum concentration of TNF during endotoxemia by means of the so-called "cholinergic anti-inflammation pathway" (61). The disparate actions of the SNS may depend on the degree and duration of its activation with short activation periods having anti-inflammatory effects and sustained activation showing pro-inflammatory effects (14). Therefore, the role of the sympathetic system involved in regulating the immune system needs to be further studied.

To investigate the specific mechanism, we examined the expression levels of associated pro-inflammatory factors. Evidence from research has indicated that sympathetic overactivity aggravates IR-induced renal damage via pro-inflammation mechanisms (10, 11). And several lines of other evidence suggest that peripheral production of pro-inflammation cytokines can signal the brain and alter neural signaling in the hypothalamus (62–64). In the current study, AKI did result in significant increase in levels of TNF-α and IL-6 in the blood, kidney and hypothalamus at 24 h after ischemia. Furthermore, our data obtained with sympathetic denervation by SCGx showed enhanced inflammation response after renal IR injury. Consistent with our findings, a pervious study by Grigoryev et al. demonstrated that IR-induced AKI leads to vigorous inflammation response in blood and lungs (65). Another study reported that ischemic AKI markedly increased brain inflammation evidenced by increased pro-inflammation cytokines, glial activation, and disrupted blood-brain barrier (66). The central nervous system receives sensory input from the immune system and responds to increased levels of circulating TNF-α, and activation of the hypothalamus-pituitary response to renal IR injury. Over and above, Martín et al. concluded that peripheral sympathetic nerve terminals originating in the SCG may modulate acute stress responses (67), which may help to explain the effect of SCGx on renal IR injury. Apoptosis, likewise, is a marker of tissue injury in AKI. In the present study, we also investigated apoptosis activity and found the expression of the pro-apoptosis protein to be increased, such as Bax, which provide greater insights of the deleterious effect of SCGx against renal IR injury.

Renal epithelial cells contain abundant ATP, and during acute renal ischemia, the visceral spinal afferent neurons including nociceptors can be activated by the ATP released from the damage kidney (68). The biological actions of extracellular ATP are mediated by purinergic receptors, including P2XRs and P2YRs. The P2X receptors, consist of seven subtypes (P2X1-7), which are involved in a variety of biological responses, but mainly associated with inflammation, tissue damage and cell proliferation (69). With respect to the P2X3 receptor, it is highly and selectively expressed in nociceptive sensory neurons, and could be activated by chemical mediators released from damaged tissue such as CGPR, thus, plays a crucial role in the processing of sensory inputs in the spinal cord (70). The association of the P2X7R with inflammation is long-standing. The P2X7R promotes the release of IL-6 and TNF from mouse microglia (71), which are implicated in tubular fibrosis and apoptosis in response to ureteral obstruction in mice (72). A recent report suggests that P2X7R activation accelerates the development of AKI by potentiating renal tubular cell death and the inflammation response (73). In our data, sympathetic denervation by SCGx markedly upregulated the expression of P2X3R and P2X7R at both mRNA and protein in T_{8-12} spinal cord segments after renal IR injury. This is consistent with other studies that demonstrated that P2X3R and P2X7R in the superior cervical ganglion are involved with the increased sympathoexcitatory reflex induced by myocardial ischemia (39–41). Thus, our results indicated that sympathetic denervation by SCGx regulated sympathoexcitatory reflex in renal ischemic reperfusion probably through purinergic signaling.

CONCLUSION

In summary, our study clearly indicates that surgical removal of SCG resulted in decreased norepinephrine overflow and reduced sympathetic hyperactivation in the kidney and hypothalamus after renal IR injury. Although such sympathetic denervation process aggravates IR-induced AKI in rats, the underlying mechanism may involve the modulation of inflammation and the activated purinergic signaling in the spinal cord segments that dominate the kidney.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.792000/full#supplementary-material

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