



Role of the IL-33/ST2 pathway in renal allograft rejection

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ABSTRACT

The interleukin-33 (IL-33)/suppression of tumorigenicity 2 (ST2) pathway modulates immune response and inflammation, associated with allograft dysfunction and rejection. We hypothesized that IL-33/ST2 is a marker of renal allograft rejection and IL-33/ST2 expression may differ according to rejection type. IL-33/ST2 expression was measured in sera and kidney tissues from recipients with acute antibody-mediated rejection (AAMR), acute cell-mediated rejection (ACMR), chronic antibody-mediated rejection (CAMR), and healthy controls. The soluble ST2 and IL-33/ST2 expression levels were higher in participants with all three rejection types than in controls. Although the expression levels in recipients with AAMR and ACMR were significantly higher than those with CAMR, there was no significant difference between the expression levels in AAMR and ACMR. Although IL-33, IL-8, and fibronectin expression were significantly increased after the addition of the recipients' serum in primary cultured human renal proximal tubular epithelial cells, the levels decreased after treatment with an anti-ST2 antibody. Furthermore, the anti-ST2 antibody specifically suppressed the upregulation of the mixed lymphocyte reaction. Boyden chamber assays demonstrated that anti-ST2 antibody abrogated chemotaxis induced by recombinant IL-33. Thus, IL-33 and ST2 are potent mediators of rejection. Treatment with an anti-ST2 antibody ameliorates rejection and could be a potential therapeutic strategy for renal allograft rejection.

1. Introduction

In kidney transplantation, the accurate and rapid diagnosis of rejection is important for clinical management. Kidney core biopsy is the gold standard diagnostic test for allograft rejection. However, it is an invasive procedure, which, in severe cases with uncontrolled bleeding, can lead to graft loss. Moreover, there is a risk of bleeding, which can be life threatening. A non-invasive diagnostic method for allograft rejection

is needed and has been the subject of several studies [1–5].

Treatment and prognosis vary according to whether the rejection is chronic or acute and cellular or antibody mediated. Currently, there are no established treatment guidelines for chronic rejection. In comparison with chronic rejection, acute rejection has a better prognosis with treatment based on the rejection type. Different types of acute rejection, namely acute antibody-mediated rejection (AAMR) and acute cell-mediated rejection (ACMR) are treated differently. Therefore,

Abbreviations: AAMR, acute antibody-mediated rejection; ACMR, acute cell-mediated rejection; AKI, acute kidney injury; CAMR, chronic antibody-mediated rejection; eGFR, estimated glomerular filtration rate; ELISA, enzyme linked immunosorbent assay; HC, healthy control; hPTECs, human proximal tubular epithelial cells; IL, interleukin; mST2, membrane-bound suppression of tumorigenicity 2; MLR, mixed lymphocyte reaction; mPTECs, mouse proximal tubular epithelial cells; PCR, polymerase chain reaction; SEM, standard error of the mean; sST2, soluble suppression of tumorigenicity 2; ST2, suppression of tumorigenicity 2; Th, T helper.

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distinguishing AAMR and ACMR is clinically significant. Detection of donor-specific antibodies could assist in the diagnosis of antibody-mediated rejection. This is not feasible when donor information, such as in overseas transplantations, is unknown or when the donor data is lost. C4d staining can also be used to confirm antibody-mediated rejection, however, C4d negative antibody-mediated rejection may also exist [6].

Interleukin (IL)-33 is a member of the IL-1 family of cytokines and has dual functions: The promotion of inflammation and the regulation of the immune response [7]. IL-33 is expressed in epithelial cells, endothelial cells, and fibroblasts, and functions according to the target tissue and disease signaling pathway. IL-33 binds to the suppression of tumorigenicity 2 (ST2) receptor and facilitates the activation of these pathways. ST2 is divided into soluble ST2 (sST2) and membrane-bound ST2 (mST2), and these contain the same binding domains. IL-33 binds both sST2 and mST2. In several studies, IL-33/ST2 has been regarded as a modulator of acute myocardial infarction, chronic heart failure, or allergic airway inflammation [8,9]. Tissue injuries, such as trauma, infection, or allergen stimulation, causes nuclear IL-33 to be released, which can result in kidney disease progression by stimulating ST2⁺ target cells, such as neutrophils, invariant natural killer T cells, T cells, endothelial cells, and macrophages [10–12]. IL-33/ST2 were identified as a prognostic biomarker found to be elevated in recipients with cardiovascular event [13], recurrence GN [14], and graft-versus-host disease (GVHD) [15,16]. Recently, transplantation studies have shown that chronic allograft dysfunction and acute rejection are associated with higher ST2 or IL-33 expression [7,17–21].

We hypothesized that IL-33/ST2 is a marker of renal allograft rejection and IL-33/ST2 expression may vary according to rejection type (acute vs chronic, or AAMR vs ACMR). Depending on the type of T cells involved in the rejection, the expression level of IL-33/ST2 may differ. To test our hypothesis, we measured the expression of IL-33 and ST2 in different rejection types of renal allograft rejection samples. In addition, we tested whether the upregulation of the IL-33/ST2 pathway is blocked by the addition of a specific anti-ST2 antibody.

2. Materials and methods

2.1. Patients and sample collection

A retrospective cohort study was conducted with patients who had received kidney transplants at Seoul National University Hospital between January 1, 2000, and December 31, 2017. Patients who did not agree to receive multi-transplantation and re-transplantation were excluded. All enrolled patients agreed to provide blood and kidney tissue samples, collected at the time of rejection, by submitting an informed consent form. Among these, we selected kidney transplant recipients with AAMR, ACMR, or chronic antibody-mediated rejection (CAMR). We defined three groups via biopsy-proven kidney rejection based on the Banff 2013 classification [22]. All recipients received induction therapy (either basiliximab or thymoglobulin), a calcineurin inhibitor (either cyclosporine or tacrolimus), a secondary agent (either mycophenolate mofetil or enteric-coated mycophenolate sodium), and steroid as the immunosuppressive treatment. Renal function was assessed by measuring the estimated glomerular filtration rate (eGFR) according to the simplified diet modification in renal disease [23]. Among the kidney biopsy samples obtained during the study, those with normal histological findings and normal kidney function were used as healthy controls (HC). In order to confirm whether sST2 can be used as rejection indicator, the serum of kidney recipient controls without rejection (RC) was obtained. The patients' clinical and laboratory findings at the time of kidney biopsy were retrospectively obtained by reviewing their medical charts. The study protocol complied with the Declaration of Helsinki and was approved by the Institutional Review Board of our institute (approval no. 1804-113-939).

2.2. Anti-ST2 antibody

The anti-ST2 antibody (AF1004 for mouse and AF523 for human) was purchased from R&D Systems (Minneapolis, MN, USA). The anti-ST2 Antibody was manufactured using full sequence proteins corresponding to immunogens rather than to capture a specific site, employing the Ser27-Arg332 (Mouse Accession #P14719), segment of mouse ILR-1 or Lys19-Phe328 (Human Accession # BAA02233) of human ST-2. The anti-ST2 antibody can block both mST2 and sST2.

2.3. Isolation of renal tubular epithelial cells

An *in vitro* model of transplantation was established using primary cultured human proximal tubular epithelial cells (hPTECs). Primary cultured hPTECs were isolated from unaffected kidney specimens from nephrectomized patients diagnosed with renal cell carcinoma from the Seoul National University Hospital (Seoul, Korea). Kidney cortices were mechanically dissected. Glomerular cells were sieved using optimized media [24], and isolated using flow cytometry for cytokeratin-18-positive (1:200; cat. no. IC3165G; R&D Systems, USA) and CD90 negative (1:200; cat. no. 12-0909-42; eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) populations. The purity of the sorted cells was >98%. Subsequent gating analyses were conducted using FlowJo software (version 10; FlowJo LLC, Ashland, OR, USA). After 3 d, cells were detached from the petri dishes using a 3 mM ethylenediaminetetraacetic acid (EDTA) solution and a minimal amount of trypsin. For *in vitro* stimulation, cells were maintained at a concentration of 1×10^5 cells per well in 6-well plates, starved for 24 h, and then washed twice with phosphate-buffered saline. The anti-ST2 antibody was added under various conditions. Primary cultured mouse proximal tubular epithelial cells (mPTECs) were isolated from B6 kidneys as previously described [24].

2.4. Enzyme linked immunosorbent assay (ELISA)

Serum sST2 and IL-8 levels were measured using ELISA (DuoSet; R&D Systems, USA) according to the manufacturer's instructions. The lower limit of detection for sST2 was 31.3 pg/mL. The concentration was estimated from a standard curve and expressed as the mean $\pm$ standard error of the mean (SEM).

2.5. Histological analysis and immunohistochemistry

Kidney sections were deparaffinized and rehydrated with xylene and ethanol. Endogenous streptavidin activity was blocked using 0.3% hydrogen peroxide. Tissue sections were examined by a renal pathologist who was blinded to sample classification. Antigens were retrieved by heating paraffin-embedded sections in 10% citrate buffer in a microwave oven, five times at 5-min intervals. Sections were probed with primary antibodies, followed by counterstaining with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). The following primary antibodies were used: anti-IL-33 antibody (1:200; Sigma-Aldrich, USA); anti-ST2 antibody (1:200; Abcam, USA). The degree of non-specific staining was assessed using secondary antibodies and isotype control IgGs. Stained slides were photographed using an Olympus inverted microscope (Olympus Imaging America, Center Valley, PA, USA). For each sample, five fields (magnification: $\times 100$) were randomly selected, and brown stained areas (immunohistochemistry) were quantified using computer-based morphometric analysis (Qwin 3; Leica). Scoring was performed in a blinded manner using the mean values of the positive areas (%).

2.6. Western blot analysis

To induce the immune response, we harvested primary cultured hPTECs 48 h after serum (500 μ g/mL) treatment with or without the

anti-ST2 antibody (1.0 µg/mL, 1 h before; R&D Systems, USA). The serum used was selected from patients with concentrations closest to the mean value of sST2 in the control, AAMR, ACMR, and CAMR groups. The hPTECs were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.3; 150 mM NaCl, 0.1 mM EDTA, 1% (vol/vol) sodium deoxycholate, 1% Triton X-100, 0.2% NaF, and 100 µM Na₃VO₄) [25] supplemented with complete protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). The protein concentration of the supernatant was determined using the Bradford method. After protein extraction, equal amounts of protein (40 µg) were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto Immobilon-FL 0.4 µM polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Tris-buffered saline containing 0.1% Tween-20 was used as the washing buffer. Primary antibodies targeting fibronectin (1:200; Santa Cruz Biotechnology Inc, TX), IL-33 (1:200; Abcam, USA), ST2 (1:200; Abcam, USA), and β-actin (1:5000; Sigma-Aldrich, USA) were used. Anti-mouse IgG (1:5000; Cell Signaling, MA, USA) and anti-rabbit IgG (1:5000; Cell Signaling, USA) were used as the secondary antibodies. The target molecule expression levels were normalized to β-actin expression. Densitometry was performed using the gel analysis procedure in ImageJ (NIH, Bethesda, MD, USA).

2.7. Mixed lymphocyte reaction (MLR) assay in mouse transplant model

Lethally irradiated splenocytes (2500 rad, 5×10^5 /well) from DBA/2 mice were cultured with allogeneic lymphocytes from C57BL/6 mice (2.5×10^5 /well) in 200 µL RPMI-1640 culture medium containing 10% FBS (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (Gibco, USA), and β2-mercaptoethanol (Gibco, USA) in U-bottom 96-well plates (Nunc, Roskilde, Denmark). After 4 d of culture, cells were pulsed with ³H-TdR (1 µCi per well; Amersham, UK) for 24 h. Subsequently, cells were harvested using a multi-well harvester, and ³H-TdR incorporation was determined in a liquid scintillation counter. Cells were cultured with or without anti-ST2 antibody (0.5, 1.0 µg/mL, 1 h before; R&D Systems, USA). The results were expressed as the mean counts per minute ± SEM from triplicate cultures.

2.8. Chemotaxis in mouse transplant model

A Boyden chamber assay with the wells separated by a polycarbonate filter and pores small enough to allow only active passage from cells (8-µm porous membrane; Cell Biolabs Inc., San Diego, CA, USA) was used for an *in vitro* chemotactic migration assay [26,27]. Twelve h before the migration assay, fresh DMEM/F12 (Lonza, Walkersville Inc, Maryland, USA) medium containing 15% FBS (Gibco, USA) was added. A chamber assay with a fibronectin-coated membrane (0.1% solution, 1 mg/mL, Sigma-Aldrich, USA) was used for approximately 4–5 h after drying the membrane at room temperature. The primary cultured mPTECs were then cut and counted, and stabilized cells were placed in 1.0×10^6 /mL 15% complete media for 1 h at 37 °C. The primary cultured mPTECs of wild-type mice were seeded at the bottom. Upon dissection of the cortices, fresh specimens were minced and dissolved with Hank's balanced salt solution containing 3 mg/mL collagenase (Sigma-Aldrich). Following centrifugation for 5 min at 500×g, primary cultured mPTECs were isolated. After 30 min, recombinant IL-33 (0.5 µg/mL, R&D Systems, USA) and anti-ST2 antibody were added (0, 0.5, 1.0 µM, R&D Systems, USA). After 12 h of incubation at 37 °C, the cells were inverted and stained with 4' 6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA). The membrane was then washed in distilled water, and the chamber membrane was turned, attached to a glass cell, and the cells were counted [26,27]. Cell migration was quantified by counting the cells on the lower surface using a phase-contrast microscope (Eclipse TS100; Nikon, Japan) at 100 × magnification. The migration index was used to quantify the migrated cells by manual counting and calculated using the following formula:

migration index = cells migrated at 12 h/cells migrated at 0 h

2.9. Quantitative real-time polymerase chain reaction (PCR)

After incubating primary cultured mPTECs with recombinant IL-33 with or without anti-ST2 antibody, total RNA was isolated from cells using the RNeasy Kit (Qiagen, Hilden, Germany), and mRNA levels were analyzed using real-time PCR. Briefly, 1 µg total RNA was reverse transcribed using oligo (dT) primers and AMV-RT Taq polymerase (Promega, Madison, WI, USA). Real-time PCR was performed using Assay-on-Demand TaqMan probes for kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), IL-8, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems PRISM 7500 Sequence Detection System. Thermocycler conditions for both methods were as follows: 95 °C for 10 min followed by 40 cycles of dissociation (95 °C for 10 s); annealing (55 °C for 30 s); elongation (72 °C for 30 s). Relative quantification was performed using the ΔΔCt method. GAPDH was used as a reference gene. A two-tailed Student's t-test was used to compare fold-changes.

2.10. Statistical analysis

Data are expressed as the mean ± SEM. The Kruskal-Wallis test was used to compare the demographic and clinical features of the study groups. The difference between two groups for each parameter was compared using non-parametric Mann-Whitney U test. Data pre- and post-treatment with anti-ST2 antibody were analyzed using the Wilcoxon signed rank test. The performance of sST2 was determined using receiver operating characteristic (ROC) curve analysis. Differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using SPSS statistical software version 26.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Clinical characteristics of the patients and controls

The demographic and clinical data of the patients, including sex, age, follow-up duration after transplantation, serum creatinine levels, and eGFR are presented in Tables 1 and 2. Table 1 presents the patients in the HC, AAMR, ACMR, and CAMR groups in which serum IL-33 and sST2 levels were measured. Table 2 presents the patients for whom the renal expression of IL-33 and ST2 was estimated. No differences were observed with sex between the HC and rejection groups. Among the patients whose sera was analyzed, the CAMR group was the youngest. Among the patients whose kidneys were investigated, the HC group was youngest. As expected, the follow-up duration after kidney transplantation was longer in the chronic rejection group than in the acute rejection groups. Significant differences were observed in serum creatinine and eGFR levels among the four groups. Serum creatinine level was the lowest in the HC groups and the highest in the CAMR group.

3.2. Upregulation of serum sST2 in rejection groups

Fig. 1 exhibited the abundance of sST2 in serum of rejection groups. The mean ± SEM (%) of sST2 (pg/mL) was 6871 ± 827.2 , 9655 ± 931.4 , $17,054 \pm 2,412$, $16,668 \pm 2,629$, and $18,277 \pm 2949$ in HC, RC, AAMR, ACMR and CAMR groups, respectively. Serum sST2 levels were significantly higher in rejection groups than in HC group. All mean values of serum sST2 were higher in rejection groups than in than RC groups, although this difference achieved statistical significance only between RC and CAMR groups. The differences of sST2 level were not

Table 1

Demographic and clinical features of subjects in whom serum IL-33 and ST2 levels were measured.

	Control group			Rejection group		P value ^a
	HC (N = 7)	RC (N = 9)	AAMR (N = 12)	ACMR (N = 12)	CAMR (N = 7)	
Age (years)	46.0 (27–52)	47.0 (30–60)	49.0 (28–63)	39.0 (26–55)	27.0 (22–42)	0.162
Sex, Male (%)	5 (50)	4 (44)	6 (50)	9 (75)	5 (71.4)	0.478
Follow-up (days)	–	61 (30–220)	41 (10–372)	235 (84–358)	4229 (3001–5404)	<0.001
sCr (mg/dL)	0.7 (0.6–0.8)	1.0 (0.85–1.40)	1.4 (0.9–2.1)	1.5 (1.3–2.3)	2.6 (1.5–3.6)	<0.001
eGFR (mL/min)	109.1 (96.2–125.6)	67.4 (49.6–82.1)	53.2 (30.4–81.7)	61.2 (47.2–77.1)	34.9 (32.0–59.6)	0.001

AAMR, acute antibody-mediated rejection; ACMR, acute cell-mediated rejection; CAMR, chronic antibody-mediated rejection; eGFR, estimated glomerular filtration rate; HC, healthy control; RC, recipient without rejection control; IL, Interleukin; N, number; sCr, serum creatinine; ST2, suppression of tumorigenicity 2.

^a Comparisons were made using Kruskal-Wallis test.

Table 2

Demographic and clinical features of subjects in whom renal IL-33 and ST2 levels were investigated.

	Healthy control group	Rejection group			P value ^a
	(N = 4)	AAMR (N = 9)	ACMR (N = 10)	CAMR (N = 9)	
Age (years)	19 (18–20)	42 (33–56)	35 (17–46)	48 (37–58)	0.034
Sex, male (%)	4 (100)	7 (77.8)	7 (70)	6 (66.7)	0.607
Follow-up (days)	–	11 (9–1729)	32 (9–623)	3620 (2188–5302)	0.044
sCr (mg/dL)	0.79 (0.74–0.91)	1.30 (0.84–1.71)	1.60 (1.28–2.10)	1.66 (1.45–2.42)	0.008
eGFR (mL/min)	149.9 (121.7–177.4)	71.0 (57.3–79.5)	47.3 (35.0–57.5)	45.0 (37.4–58.3)	0.002

AAMR, acute antibody-mediated rejection; ACMR, acute cell-mediated rejection; CAMR, chronic antibody-mediated rejection; eGFR, estimated glomerular filtration rate; IL, Interleukin; sCr, serum creatinine; ST2, suppression of tumorigenicity 2.

^a Comparisons were made using the Kruskal-Wallis test.

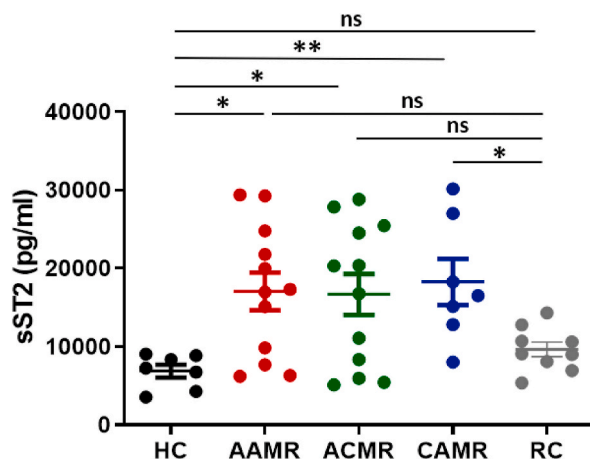


Fig. 1. Serum sST2 levels were higher in recipients in rejection groups than in the HC group. Recipient without rejection had serum higher sST2 compared to CAMR group. There were no statistical differences between the rejection groups. The means of sST2 levels (pg/ml) were 6,871, 9,655, 17,054, 16,668, and 18,277 in HC (N = 7), RC (N = 9), AAMR (N = 12), ACMR (N = 12), and CAMR (N = 9) groups, respectively. Kruskal-Wallis test, $P = 0.014$. * $P < 0.05$. ** $P < 0.005$. P -values were calculated using the Mann-Whitney test. ACMR, acute cell-mediated rejection; CAMR, chronic antibody-mediated rejection; HC, healthy control; N, number; RC, recipient without rejection; sST2, soluble suppression of tumorigenicity 2.

significantly different among the rejection groups. The ROC curve analysis revealed that sST2 could indicate rejection. Compared to recipients without rejection, sST2 levels varied between recipients without rejection and rejection groups with high sensitivity (64.5%) and specificity (100.0%). Their area under the curve of ROC was 0.756 (95% CI: 0.611–0.901; $P = 0.021$) and the optimal cutoff level was 14713.198 pg/ml. The serum IL-33 levels were too low to be analyzed.

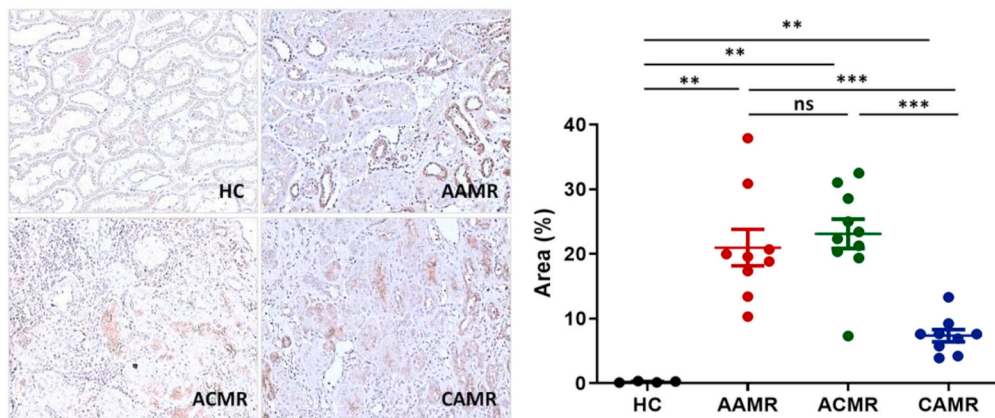
3.3. Upregulation of IL-33 and ST2 expression in kidney tissue of rejection groups

Immunohistochemical analysis demonstrated that IL-33 ($P < 0.001$ by Kruskal-Wallis test, Fig. 2A) and ST2 ($P < 0.001$ by Kruskal-Wallis test, Fig. 2B) expressions were higher in the kidney tissue of the rejection groups than in the HC group. IL-33 and ST2 positive staining was localized to the renal tubules and interstitium in the rejection groups. The mean $\pm$ SEM (%) of IL-33 and ST2 expression was 0.23 ± 0.05 and 1.25 ± 0.21 in the HC group; 21.00 ± 2.83 and 19.81 ± 1.96 in the AAMR group; 23.13 ± 2.26 and 17.54 ± 1.41 in the ACMR group; and 7.37 ± 0.94 and 6.71 ± 0.88 in the CAMR group. Increased IL-33 and ST2 staining was more visible in the acute rejection group than in the chronic rejection group ($P < 0.001$ by Mann-Whitney U test between AAMR and CAMR, ACMR and CAMR). However, the expression levels of IL-33 and ST2 were not significantly different between the AAMR and ACMR groups ($P = 0.077$ by Mann-Whitney U test).

3.4. IL-8, IL-33, and fibronectin production is upregulated in primary cultured human proximal tubular epithelial cells when stimulated with serum of patients from rejection groups

Fig. 3A shows the results of IL-8 release from hPTECs stimulated with sera (500 μ g/mL) of a representative patient from each group incubated for 48 h. IL-8 was upregulated in the rejection groups (HC, 161.4 ± 32.33 pg/mL; AAMR, 1905 ± 66.13 pg/mL; ACMR, 1564 ± 155.7 pg/mL; CAMR, 871.9 ± 63.52 pg/mL), especially in the acute rejection groups ($P = 0.008$ by Mann-Whitney U test between HC and AAMR, HC and ACMR, HC and CAMR, respectively). The expression of IL-33 (0.98 ± 0.03 vs 1.71 ± 0.02 , vs 1.38 ± 0.02 , vs 1.25 ± 0.01 for HC vs AAMR, vs ACMR, vs CAMR, respectively; $P = 0.008$ by Mann-Whitney U test) and ST2 (1.33 ± 0.03 vs 2.05 ± 0.06 , vs 2.49 ± 0.09 , vs 2.05 ± 0.04 for HC vs AAMR, vs ACMR, vs CAMR, respectively; $P = 0.008$ by Mann-Whitney U test) was upregulated in hPTECs stimulated by serum from patients in the rejection groups compared with those from the HC group (Fig. 3B and C). A statistically significant increase in fibronectin expression was observed only in the CAMR group (2.10 ± 0.05 vs 2.06 ± 0.05 , vs 1.72 ± 0.04 , vs 2.28 ± 0.02 ; $P = 0.589$, $P = 0.002$, $P = 0.004$, in HC vs AAMR, vs

A.



B.

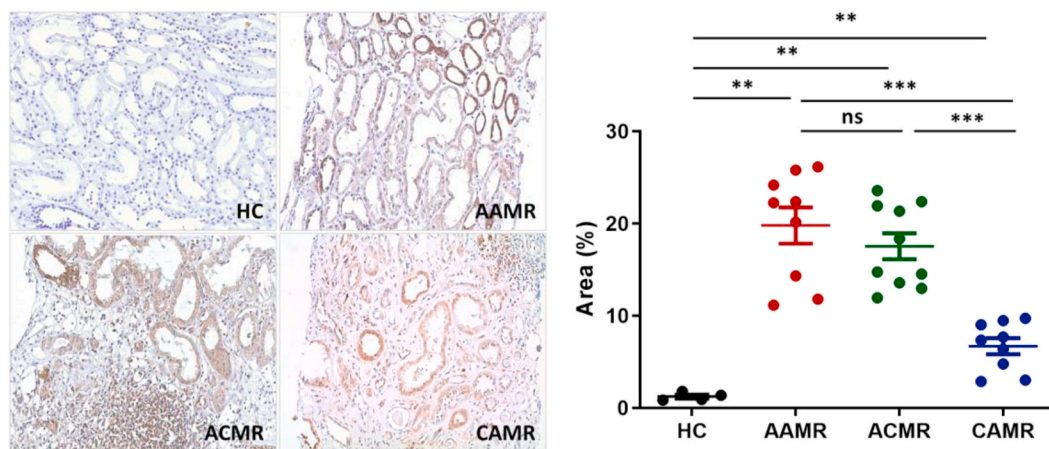


Fig. 2. Rejection upregulated IL-33 and ST2 expression in kidney tissue. A. IL-33 (brown) expressed in kidney tissues of HC (N = 4), patients with AAMR (N = 9), ACMR (N = 10), and CAMR (N = 9) (Left, $\times 100$). Quantification of IL-33 expression in tissues is shown on the right. Kruskal-Wallis test, $P < 0.001$. B. ST2 (brown) expressed in kidney tissues of HC (N = 4), patients with AAMR (N = 9), ACMR (N = 10), and CAMR (N = 9) (Left, $\times 200$). Quantification of ST2 expression in tissues is shown on the right. The areas with increased IL-33 and ST2 expression were larger in the rejection groups than in the HC group. The number of areas was higher in the acute rejection groups than in the chronic rejection groups, but there was no significant difference between the AAMR and ACMR. Kruskal-Wallis test, $P < 0.001$. * $P < 0.05$. ** $P < 0.005$. *** $P < 0.001$. P -values were calculated using the Mann-Whitney test. HC, healthy control; ACMR, acute cell-mediated rejection; CAMR, chronic antibody-mediated rejection; AAMR, acute antibody-mediated rejection.

ACMR, vs CAMR, respectively, Fig. 3B and C).

3.5. Anti-ST2 antibody reduced the levels of IL-8, IL-33, and fibronectin, which are promoted by recipients' serum in primary cultured human proximal tubular epithelial cells

In response to anti-ST2 antibody treatment, the primary cultured hPTECs that had been stimulated by the recipients' serum exhibited a decrease in levels of IL-8, a proinflammatory cytokine ($P < 0.001$, Fig. 3A). The mean level of IL-8 extracted *in vitro* from incubated hPTECs decreased from 1905 ± 66.13 pg/mL to 1482 ± 81.81 pg/mL in the AAMR group ($P = 0.043$), from 1564 ± 155.7 pg/mL to 976.6 ± 81.78 pg/mL in the ACMR group ($P = 0.043$), and from 871.9 ± 63.52 pg/mL to 491.4 ± 40.25 pg/mL in the CAMR group ($P = 0.043$). Fibronectin, IL-33, and ST2 levels also decreased after anti-ST2 antibody treatment in all samples ($P < 0.001$, Fig. 3B and C). Even when each group was analyzed separately, fibronectin (AAMR, ACMR, CAMR; $P = 0.028$, $P = 0.028$, $P = 0.028$, respectively), IL-33 (AAMR, ACMR, CAMR; $P = 0.043$, $P = 0.043$, $P = 0.043$, respectively) and ST2 (AAMR, ACMR, CAMR; $P = 0.043$, $P = 0.043$, $P = 0.043$, respectively) levels decreased after anti-ST2

antibody treatment. The Wilcoxon signed rank test was used for pre- and post-comparison within the groups.

3.6. Anti-ST2 antibody decreased the MLR and chemotaxis in an in-vitro mouse transplant model

Treatment with the anti-ST2 antibody downregulated the MLR (Fig. 4A) and IL-8 levels (Fig. 4B) in a dose-dependent manner *in vitro*, ranging from 0 μ g/mL to 1 μ g/mL. The decrease in MLR ($P = 0.043$) and IL-8 ($P = 0.043$) levels was statistically significant at high concentrations (1 μ g/mL). In addition, the anti-ST2 antibody significantly abrogated recombinant IL-33-induced chemotaxis in an in-vitro mouse transplant model ($P = 0.028$, Fig. 5A). High dose anti-ST2 antibody treatment also led to decreased expression of IL-33 ($P = 0.028$, Fig. 5B), and tubular injury markers, KIM1 and NGAL ($P = 0.028$ and $P = 0.028$, Fig. 5C). Treatment with 1 μ g/mL of anti-ST2 antibody increased mRNA expression of IL-8 and E-cadherin, a cell junction marker ($P = 0.028$ and $P = 0.028$, Fig. 5C). The effect of anti-ST2 antibody was statistically significant at high concentrations. The Wilcoxon signed rank test was used for pre- and post-comparison within the groups.

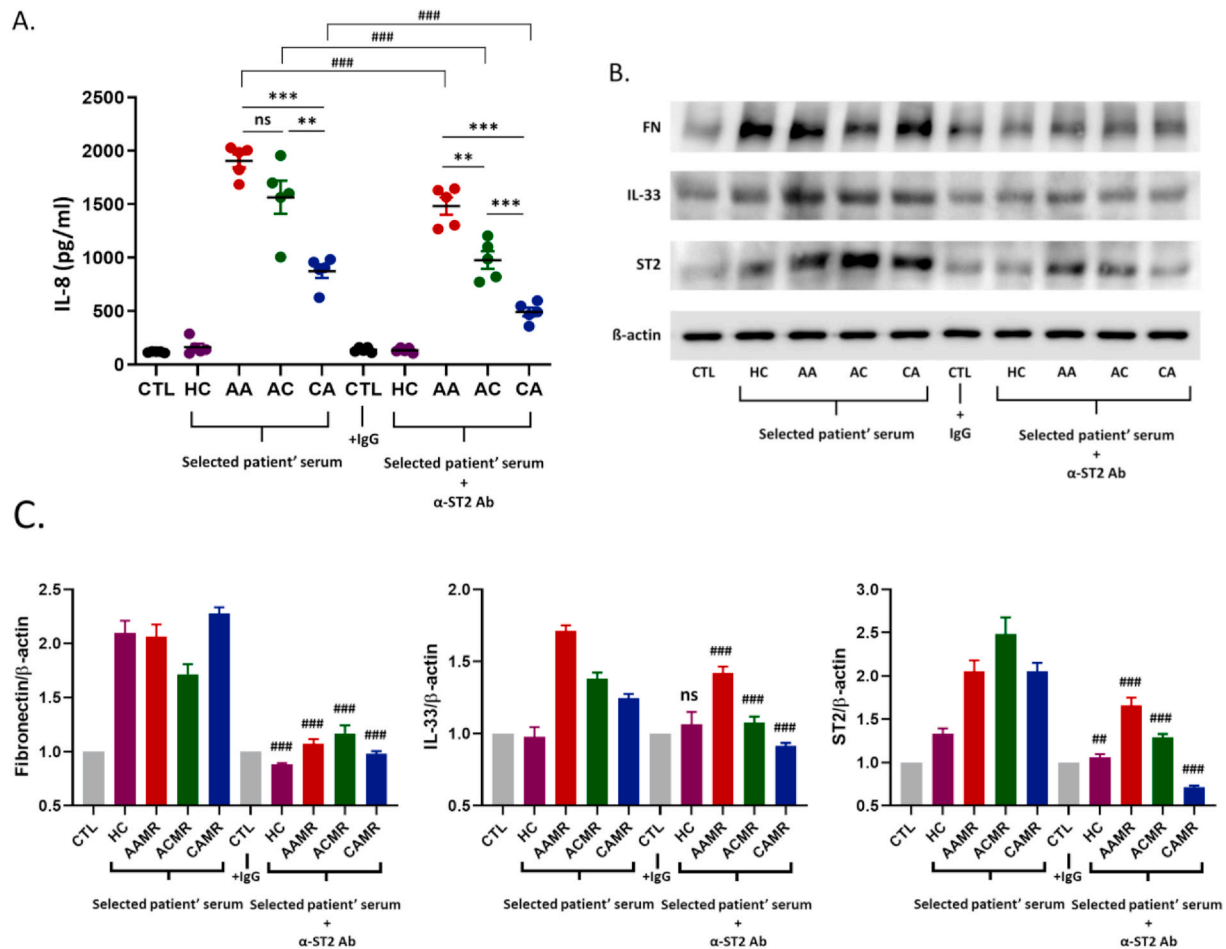


Fig. 3. Anti-ST2 monoclonal antibody reduced fibrosis and IL-33 expression in hPTECs. A. hPTECs were incubated for 48 h with the serum of HC, patients with AAMR, ACMR, and CAMR followed by an analysis of cytokine IL-8 expression in the media. Cytokine IL-8 expression was increased in hPTECs (N = 5 in each group) stimulated with serums of AAMR, ACMR, CAMR groups compared to in those with serum of HC group. Following anti-ST2 antibody treatment, the mean level (pg/mL) of IL-8 decreased from 1905 to 1482 in the AAMR group, from 1564 to 977 in the ACMR group, and from 872 to 491 in the CAMR group. B. Densitometric analysis of Western blot analysis data of hPTECs under the same conditions. The expressions of ST2, IL-33, and fibronectin were significantly elevated in the AAMR, ACMR, and CAMR groups compared with that in the healthy control group and were significantly attenuated upon anti-ST2 antibody treatment. C. Quantifications of fibronectin/β-actin (N = 6 in each group), IL-33/β-actin (N = 5 in each group), and ST2/β-actin (N = 5 in each group) in Western blot analysis were decreased in the AAMR, ACMR, and CAMR groups upon anti-ST2 antibody treatment. *P < 0.05. **P < 0.005. ***P < 0.001. P-values were calculated using Mann-Whitney test. #P < 0.05. ##P < 0.005. ###P < 0.001. P-values were calculated using wilcoxon signed rank test between each pair of value. HC, healthy control; ACMR, acute cell-mediated rejection; CAMR, chronic antibody-mediated rejection; AAMR, acute antibody-mediated rejection; hPTECs, human proximal tubular epithelial cells.

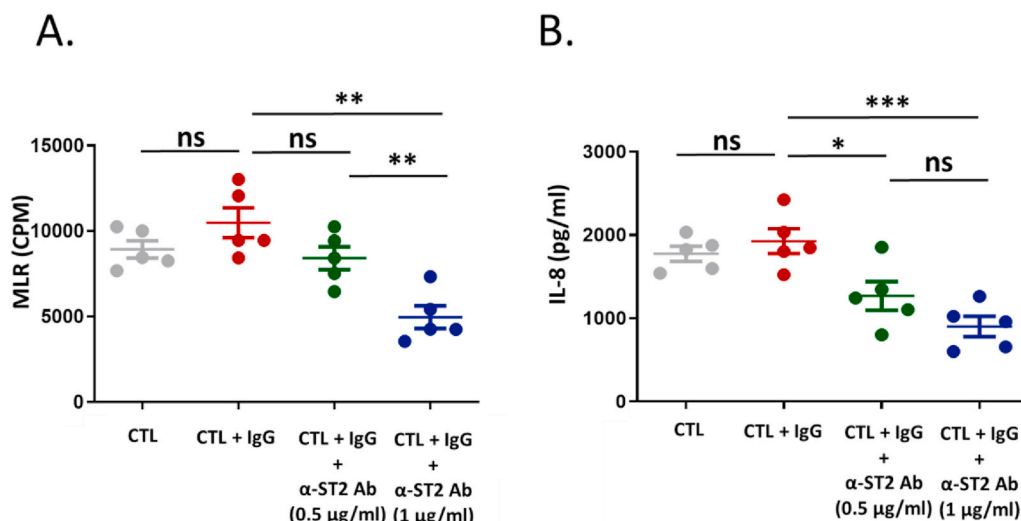


Fig. 4. Anti-ST2 antibody caused a statistically significant decrease in lymphocyte proliferation and inflammation at high concentrations. A. In mixed lymphocyte reaction, the mean ± SEM (CPM) was 10,481 ± 873.9 without the anti-ST2 antibody, 8413 ± 670.9 with 0.5 μg/mL of the anti-ST2 antibody, and 4958 ± 664.0 with 1.0 μg/mL of the anti-ST2 antibody (N = 5). B. The expression (mean ± SEM) of IL-8 was 1927 ± 148.7 pg/mL without anti-ST2 antibody, 1269 ± 172.6 pg/mL with 0.5 μg/mL of anti-ST2 antibody, and 900.9 ± 122.5 with 1.0 μg/mL of anti-ST2 antibody (N = 5). *P < 0.05. **P < 0.005. ***P < 0.001. P-values were calculated by wilcoxon signed rank test.

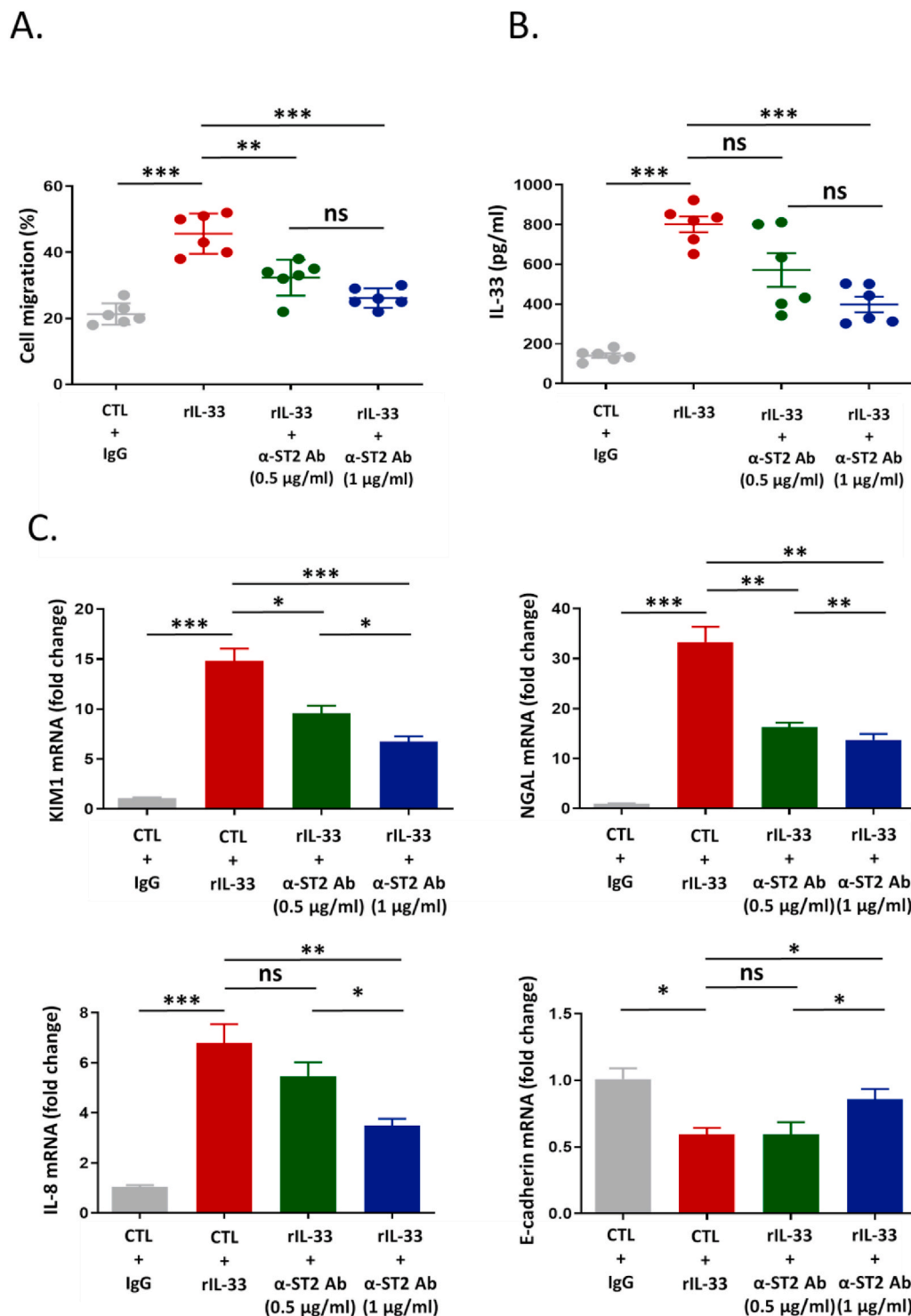


Fig. 5. The anti-ST2 antibody significantly decreased chemotaxis and tubular epithelial cell injury induced by rIL-33 at 1 μ g/mL. A. Cell migration was significantly decreased at all concentrations. B. IL-33 with a mean cytotoxic event was significantly reduced at 1 μ g/mL of the anti-ST2 antibody. C. Tubular injury markers KIM1 and NGAL, and IL-8 with a mean cytotoxic event, were significantly suppressed, while cell junction marker E-cadherin was enhanced, upon treatment with anti-ST2 antibody (N = 6 per marker). Tubular cell injury recovered with a low-dose anti-ST2 antibody. *P < 0.05. **P < 0.005. ***P < 0.001. P-values were calculated by wilcoxon signed rank test.

4. Discussion

In this study, we found that IL-33 and ST2 levels were upregulated during renal allograft rejection. In addition, the expression levels of IL-33 and ST2 in kidney tissue were higher in acute rejection compared to those in chronic rejection; however, there was no difference between ACMR and AAMR. Proinflammatory cytokine IL-8 was also activated in renal allograft rejection. This was effectively reversed upon anti-ST2 antibody treatment.

Kidney transplantation is the best available treatment option for end-

stage renal disease. Despite marked improvements in grafts and recipient survival, the long-term success rate of kidney transplantation is limited by acute or chronic allograft rejection due to patient immune response [28]. Allograft rejection after transplantation is primarily mediated by T helper (Th) cells, and the Th1/Th2 balance is regarded as the major mechanism of rejection [29]. Therefore, the development of immunosuppressants has focused on the pathways targeting Th1 and Th2 [30]. IL-33 has gained attention as a potential target to modulate immune response when it was shown to participate in the development of Th2-mediated response [31,32]. It is now widely accepted that IL-33

contributes to Th1-driven pathologies [33,34].

Recent studies have shown that IL-33 and its receptor, ST2, are biomarkers in allograft rejection. In 2011, Pascual-Figal et al. [19] showed that high level of ST2 is indicative of acute cardiac allograft rejection. Other studies have shown that the ST2 level correlates with acute rejection or inflammation in pediatric heart and small bowel transplantation [18,21]. On the other hands, Li et al. suggested that graft IL-33 protects against chronic rejection by regulating macrophages in heart transplantation [35]. However, only a few studies have investigated the role of IL-33/ST2 in kidney transplantation. Renal ischemic injury is one of the most important risks during kidney transplantation. Thierry et al. [36] demonstrated that both serum and urine IL-33 levels are elevated after renal transplantation and that invariant natural killer T cells are potential targets of IL-33 in renal ischemic injury. Chronic allograft dysfunction results in long-term graft failure. A study of 64 renal transplant recipients revealed that serum IL-33 levels are significantly higher and correlated with elevated production of Th2 cytokines and CD4⁺ T cells in patients with chronic allograft dysfunction compared to recipients with stable allograft function [20]. In patients experiencing chronic kidney allograft dysfunction, elevated IL-33 levels promote the epithelial-to-mesenchymal transition of human kidney cells [7].

To our knowledge, our study is the first to demonstrate that IL-33 and ST2 levels in kidney tissue are increased during renal allograft rejection. ST2 expression was significantly increased in the rejection group compared to the control groups, and the addition of serum from recipients with rejection to hPTECs induced an increased inflammatory response. The degree of response was particularly apparent in cases of acute rejection compared to chronic rejection. We obtained the same results not only when using the sera of recipients with rejection but also when using pure recombinant IL-33 in primary cultured hPTECs. Unlike serum sST2 level was significantly higher in CAMR than in control groups, sST2 level was not statistically different between acute rejection group and RC. Serum IL-33 level was too low to analyze in all group. Recent study proposed the role of IL-33 in homeostasis and inflammation [37]. IL-33/ST2 act locally in a cell- and tissue-specific manner in the early homeostasis stage, but IL-33 may exceed sST2 capacity to suppress systemic IL-33 effect in chronic damage and lead to systemic effects. Therefore, the reason for the large variation in individual serum sST2 concentrations during acute rejection is may be related to the timeline of IL-33/ST2 pathway.

The IL-33/ST2 pathway was found to dominantly stimulate a type 2 response by modulating ST2⁺ cells, such as ILC2 or Th cells. Therefore, it is more closely related to cell-mediated rejection than antibody-mediated rejection [38]. However, ST2 expression levels in this study exhibited no significant difference between the AAMR and ACMR groups. IL-33 is involved in the immune response targeting not only T cells but also B cells and antigen-presenting cells, among others [38]. Therefore, IL-33/ST2 expression could be as highly correlated with AAMR as ACMR. Another possibility is that it might be difficult to distinguish between AAMR and severe ACMR; the two processes might also coexist. In addition, in up to 25% of the cases in which allograft dysfunction was attributed, at least in part, to AAMR, the histological findings suggest only cellular-mediated rejection or acute tubular injury [39].

The present study revealed that in hPTECs, the inflammatory response was stimulated upon recombinant IL-33 and reduced upon anti-ST2 antibody treatment. In vitro studies validated that anti-ST2 antibody treatment relieves the MLR, as a process of T cell activation and proliferation, and chemotaxis [40]. Together, our findings indicate an influential role for the IL-33/ST2 pathway in allograft rejection. This emerging concept can be applied for the development of novel therapeutic strategies for renal allograft rejection, which is a major risk factor for graft failure. Our findings also indicated the therapeutic potential of anti-ST2 antibody treatment in reducing acute rejection.

The role of IL-33 in certain autoimmune, allergic, and cardiovascular

diseases has been confirmed. However, its effect can be either pro- or anti-inflammatory, depending on the disease and the disease model [31, 33,41–43]. Soluble ST2 acts as a decoy receptor for IL-33 [44], attenuating the Th2 inflammatory response by preventing IL-33 from binding to mST2, which is involved in several T cell-mediated diseases [45]. In our study, serum sST2 and tissue ST2 levels were elevated in recipients with rejection. However, it was not possible to determine whether sST2 or mST2 levels were elevated in the kidney tissue with rejection, or to distinguish which type of ST2 was blocked by the anti-ST2 antibody.

Increased levels of the proinflammatory cytokines IL-33 and ST2 were previously found in both ACMR and ABMR [18]. We found that the expression of the proinflammatory cytokine IL-8 was associated with IL-33 and ST2 levels. IL-8 is produced in CD4⁺ T cells and is a proinflammatory and cytotoxic cytokine. IL-8 is a diagnostic marker in renal allograft rejection [46–48]. In this study, IL-8 was stimulated by the serum of recipients with rejection, and *in vivo* experiments revealed that IL-8 levels decreased in a dose-dependent manner after treatment with the anti-ST2 antibody. Thus, alternations in the levels of IL-33/ST2 were associated with the IL-8 response. Further studies on the relationship between IL-8 and the IL-33/ST2 pathway in renal allograft rejection are required.

NGAL, a protein belonging to the lipocalin superfamily, initially found in activated neutrophils, is expressed in several cell types, including kidney tubules [49]. An increase in NGAL production, and its subsequent release from tubular cells in response to insults, has been used to predict acute kidney injury (AKI). In the context of renal transplants, delayed graft function, defined as the need for dialysis during the first-week post-transplant, can be viewed as a form of AKI following kidney transplantation, and is associated with increased susceptibility to acute and chronic rejection and a poor long-term outcome [50,51]. Recent studies have shown that urinary NGAL and plasma NGAL may be markers of graft function recovery in the early post-transplantation period [52–54]. NGAL is involved in cellular immunity, where it induces immune tolerance by upregulating human leukocyte antigen-G expression and expansion of T-regulatory cells in healthy donors. This provides the basis for further studies to evaluate its possible role in immunomodulation and tolerance induction in transplant recipients [55].

5. Conclusion

In conclusion, we demonstrated that the expression of IL-33/ST2 in the kidney proximal tubule epithelial cells can be robustly activated in response to renal allograft rejection and hence acts as a biomarker for acute graft rejection. IL-33 and its receptor, ST2, are potent mediators of rejection and increase proinflammatory cytokine production after kidney transplantation. Treatment with an anti-ST2 antibody ameliorates acute responses and could be a potential therapeutic strategy for renal allograft rejection. Further studies using different ST2 subsets blocked by the anti-ST2 antibody are needed to improve our understanding of the relationship between IL-33/ST2 and renal allograft rejection.

Author contributions

Mi-Yeon Yu, Formal analysis, Investigation, Writing – original draft. Soie Kwon, Revision analysis. Jong Joo Moon, Investigation. Yong-Chul Kim, Formal analysis, Investigation. Eun Young Song, Resources. Hajeong Lee, Conceptualization, Writing – original draft. Kyung Chul Moon, Resources, Visualization. Jongwon Ha, Resources. Dong Ki Kim, Conceptualization, Writing – original draft. Sang-Woong Han, Conceptualization, Writing – original draft. Gheun-Ho Kim, Conceptualization, Writing – original draft. Yon Su Kim, Supervision. Seung Hee Yang, Investigation, Writing – original draft.

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Data statement

The data that support the findings of this study are available from the corresponding author, SH Yang, upon reasonable request.

Declaration of competing interest

The authors of this manuscript have no conflicts of interest.

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References

- [1] M. Eikmans, E.M. Gielis, K.J. Ledeganc, J. Yang, D. Abramowicz, F.F.J. Claas, Non-invasive biomarkers of acute rejection in kidney transplantation: novel targets and strategies, *Front. Med.* 5 (2019) 358, <https://doi.org/10.3389/fmed.2018.00358>.
- [2] M. Suthanthiran, J.E. Schwartz, R. Ding, M. Abecassis, D. Dadhania, B. Samstein, S. J. Knecht, J. Friedewald, Y.T. Becker, V.K. Sharma, N.M. Williams, C.S. Chang, C. Hoang, T. Muthukumar, P. August, K.S. Keslar, R.L. Fairchild, D.E. Hricik, P. S. Heeger, L. Han, J. Liu, M. Riggs, D.N. Ikle, N.D. Bridges, A. Shaked, Clinical Trials in Organ Transplantation 04 (CTOT-04) Study Investigators. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts, *N. Engl. J. Med.* 369 (2013) 20–31, <https://doi.org/10.1056/NEJMoa1215555>.
- [3] S. Roedder, T. Sigdel, N. Salomonis, S. Hsieh, H. Dai, O. Bestard, D. Metes, A. Zeevi, A. Gritsch, J. Cheeseman, C. Macedo, R. Peddy, M. Medeiros, F. Vincenti, N. Asher, O. Salvatierra, R. Shapiro, A. Kirk, E.F. Reed, M.M. Sarwal, The kSORT assay to detect renal transplant patients at high risk for acute rejection: results of the multicenter AART study, *PLoS Med.* 11 (2014), e1001759, <https://doi.org/10.1371/journal.pmed.1001759>.
- [4] D. Hricik, P. Nickerson, R. Formica, E.D. Poggio, D. Rush, K.A. Newell, J. Goebel, I. W. Gibson, R.L. Fairchild, M. Riggs, K. Spain, D. Ikle, N.D. Bridges, P.S. Heeger, CTOT-01 consortium. Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury, *Am. J. Transplant.* 13 (2013) 2634–2644, <https://doi.org/10.1111/ajt.12426>.
- [5] M. Rabant, L. Amrouche, X. Lebreton, F. Aulagnon, A. Benon, V. Sauvaet, R. Bonifay, L. Morin, A. Scemla, M. Delville, F. Martinez, M.O. Timsit, J.P.D. Van Huyen, C. Legendre, F. Terzi, D. Anglicheau, Urinary C-X-C motif chemokine 10 independently improves the noninvasive diagnosis of antibody-mediated kidney allograft rejection, *J. Am. Soc. Nephrol.* 26 (2015) 2840–2851, <https://doi.org/10.1681/ASN.2014080797>.
- [6] B.J. Orandi, N. Alachkar, E.S. Kraus, F. Naqvi, B.E. Lonze, L. Lees, K.J. Van Arendonk, C. Wickliffe, S.M. Bagnasco, A.A. Zachary, D.L. Segev, R. A. Montgomery, Presentation and outcomes of C4d-negative antibody-mediated rejection after kidney transplantation, *Am. J. Transplant.* 16 (2016) 213–220, <https://doi.org/10.1111/ajt.13434>.
- [7] Z. Xu, C. Zhao, Z. Wang, J. Tao, Z. Han, W. Zhang, R. Tan, M. Gu, Interleukin-33 levels are elevated in chronic allograft dysfunction of kidney transplant recipients and promotes epithelial to mesenchymal transition of human kidney (HK-2) cells, *Gene* 644 (2018) 113–121, <https://doi.org/10.1016/j.gene.2017.11.010>.
- [8] H. Hayakawa, M. Hayakawa, A. Kume, S.I. Tominaga, Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation, *J. Biol. Chem.* 282 (2007) 26369–26380, <https://doi.org/10.1074/jbc.M704916200>.
- [9] S. Sanada, D. Hakuno, L.J. Higgins, E.R. Schreiter, A.N.J. McKenzie, R.T. Lee, IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system, *J. Clin. Invest.* 117 (2007) 1538–1549, <https://doi.org/10.1172/JCI30634>.
- [10] W.Y. Chen, L.C. Li, J.L. Yang, Emerging roles of IL-33/ST2 axis in renal diseases, *Int. J. Mol. Sci.* 18 (2017) 783, <https://doi.org/10.3390/ijms18040783>.
- [11] J.H. Riedel, M. Becker, K. Kopp, M. Düster, S.R. Brix, C. Meyer-Schwesinger, L. A. Kluth, A.C. Gnirck, M. Attar, S. Krohn, B. Fehse, R.A.K. Stahl, U. Panzer, J. E. Turner, IL-33-mediated expansion of type 2 innate lymphoid cells protects from progressive glomerulosclerosis, *J. Am. Soc. Nephrol.* 28 (2017) 2068–2080, <https://doi.org/10.1681/ASN.2016080877>.
- [12] T.R. Shelite, Y. Liang, H. Wang, N.L. Mendell, B.J. Trent, J. Sun, B. Gong, G. Xu, H. Hu, D.H. Bouyer, L. Soong, IL-33-dependent endothelial activation contributes to apoptosis and renal injury in *Orizantia tsutsugamushi*-infected mice, *PLoS Negl. Trop. Dis.* 10 (2016), e0004467, <https://doi.org/10.1371/journal.pntd.0004467>.
- [13] H. Mansell, M. Soliman, H. Elmoselhi, A. Shoker, Elevated circulating interleukin 33 levels in stable renal transplant recipients at high risk for cardiovascular events, *PLoS One* 10 (2015), e0142141, <https://doi.org/10.1371/journal.pone.0142141>.
- [14] S. Bruneau, L. Le Berre, C. Hervé, A. Valancité, M. Kamal, J. Naulet, L. Tesson, Y. Foucher, J.P. Soullou, D. Sahali, J. Dantal, Potential role of soluble ST2 protein in idiopathic nephrotic syndrome recurrence following kidney transplantation, *Am. J. Kidney Dis.* 54 (2009) 522–532, <https://doi.org/10.1053/j.ajkd.2009.03.021>.
- [15] J. Zhang, A.M. Ramadan, B. Griesenauer, W. Li, M.J. Turner, C. Liu, R. Kapur, H. Hanenberg, B.R. Blazar, I. Tawara, S. Paczesny, ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease, *Sci. Transl. Med.* 7 (2015), <https://doi.org/10.1126/scitranslmed.aab0166>, 308ra160.
- [16] A.M. Ramadan, W. Daguindau, J.C. Rech, K. Chinnaswamy, J. Zhang, G. L. Hura, B. Griesenauer, Z. Bolten, A. Robida, M. Larsen, J.A. Stuckey, C.Y. Yang, S. Paczesny, From proteomics to discovery of first-in-class ST2 inhibitors active in vivo, *JCI Insight* 3 (2018), e99208, <https://doi.org/10.1172/jci.insight.99208>.
- [17] G.Y. Lee, J.O. Choi, E.S. Ju, Y.J. Lee, E.S. Jeon, Role of soluble ST2 as a marker for rejection after heart transplant, *Korean Circ. J.* 46 (2016) 811–820, <https://doi.org/10.4070/kcj.2016.46.6.811>.
- [18] L.R. Mathews, Pro-inflammatory Cytokine Increased IL-33 and ST2 Indicate Pediatric Allograft Rejection, Unpublished master's Thesis, University of Pittsburgh, Pittsburgh, PA, 2014.
- [19] D.A. Pascual-Figal, I.P. Garrido, R. Blanco, A. Minguela, A. Lax, J. Ordoñez-Llanos, A. Bayes-Genis, M. Valdés, S.A. Moore, J.L. Januzzi, Soluble ST2 is a marker for acute cardiac allograft rejection, *Ann. Thorac. Surg.* 92 (2011) 2118–2124, <https://doi.org/10.1016/j.athoracsur.2011.07.048>.
- [20] J. Zhang, Z. Wang, Z. Xu, Z. Han, J. Tao, P. Lei, Z. Huang, W. Zhou, C. Zhao, R. Tan, M. Gu, The potential role of IL-33 in renal transplant recipients with chronic allograft dysfunction, *Ann. Transplant.* 21 (2016) 611–618, <https://doi.org/10.12659/aot.899263>.
- [21] L. Mathews, J. Lott, K. Isse, A. Lesniak, D. Landsittel, A.J. Demetris, Y. Sun, D. F. Mercer, S.A. Webber, A. Zeevi, R.T. Fischer, B. Feingold, H.R. Turnquist, Elevated ST2 distinguishes incidences of pediatric heart and small bowel transplant rejection, *Am. J. Transplant.* 16 (2016) 938–950, <https://doi.org/10.1111/ajt.13542>.
- [22] M. Haas, B. Sis, L.C. Racusen, K. Solez, D. Glotz, R.B. Colvin, M.C.R. Castro, D.S. R. David, E. David-Neto, S.M. Bagnasco, L.C. Cendales, L.D. Cornell, A.J. Demetris, C.B. Drachenberg, C.F. Farver, A.B. Farris 3rd, I.W. Gibson, E. Kraus, H. Lapis, A. Loupy, V. Nickleleit, P. Randhawa, E.R. Rodriguez, D. Rush, R.N. Smith, C. D. Tan, W.D. Wallace, M. Mengel, Banff meeting report writing committee. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions, *Am. J. Transplant.* 14 (2014) 272–283, <https://doi.org/10.1111/ajt.12590>.
- [23] A.S. Levey, J. Coresh, T. Greene, L.A. Stevens, Y.L. Zhang, S. Hendriksen, J. W. Kusek, F. Van Lente, Chronic Kidney Disease Epidemiology Collaboration. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate, *Ann. Intern. Med.* 145 (2006) 247–254, <https://doi.org/10.7326/0003-4819-145-4-200608150-00004>.
- [24] P. Mundel, J. Reiser, W. Kriz, Induction of differentiation in cultured rat and human podocytes, *J. Am. Soc. Nephrol.* 8 (1997) 697–705.
- [25] S.H. Yang, J.Z. Jin, S.H. Lee, H. Park, C.H. Kim, D.S. Lee, S. Kim, N.H. Chung, Y. S. Kim, Role of NKT cells in allogeneic islet graft survival, *Clin. Immunol.* 124 (2007) 258–266, <https://doi.org/10.1016/j.clim.2007.06.003>.
- [26] C.Y. Chen, K.C. Hsiao, C.A. Chung, Measurement of chondrocyte chemotaxis using a Boyden chamber: a model of receptor-mediated cell migration combined with cell sedimentation, *Math. Med. Biol.* 30 (2013) 213–239, <https://doi.org/10.1093/imammb/dqs022>.
- [27] R. Thomsen, A.L. Nielsen, A. Boyden chamber-based method for characterization of astrocyte protrusion localized RNA and protein, *Glia* 59 (2011) 1782–1792, <https://doi.org/10.1002/glia.21223>.
- [28] P.F. Halloran, Immunosuppressive drugs for kidney transplantation, *N. Engl. J. Med.* 351 (2004) 2715–2729, <https://doi.org/10.1056/NEJMra033540>.
- [29] Z. Liu, H. Fan, S. Jiang, CD4(+) T-cell subsets in transplantation, *Immunol. Rev.* 252 (2013) 183–191, <https://doi.org/10.1111/imr.12038>.
- [30] A. Sanchez-Fueyo, T.B. Strom, Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs, *Gastroenterology* 140 (2011) 51–64, <https://doi.org/10.1053/j.gastro.2010.10.059>.
- [31] L.H. Ho, T. Ohno, K. Oboki, N. Kajiwara, H. Suto, M. Iikura, Y. Okayama, S. Akira, H. Saito, S.J. Galli, S. Nakae, IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcεR1 signals, *J. Leukoc. Biol.* 82 (2007) 1481–1490, <https://doi.org/10.1189/jlb.0407200>.
- [32] J.L. Barlow, S. Peel, J. Fox, V. Panova, C.S. Hardman, A. Camelo, C. Bucks, X. Wu, C.M. Kane, D.R. Neill, R.J. Flynn, I. Sayers, I.P. Hall, A.N.J. McKenzie, IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction, *J. Allergy Clin. Immunol.* 132 (2013) 933–941, <https://doi.org/10.1016/j.jaci.2013.05.012>.
- [33] G. Palmer, D. Talbot-Ayer, C. Lamacchia, D. Toy, C.A. Seemayer, S. Viatte, A. Finckh, D.E. Smith, C. Gabay, Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis, *Arthritis Rheum.* 60 (2009) 738–749, <https://doi.org/10.1002/art.24305>.
- [34] D. Xu, H.R. Jiang, P. Kewin, Y. Li, R. Mu, A.R. Fraser, N. Pitman, M. Kurowska-Stolarska, A.N.J. McKenzie, I.B. McInnes, F.Y. Liew, IL-33 exacerbates antigen-induced arthritis by activating mast cells, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 10913–10918, <https://doi.org/10.1073/pnas.0801898105>.

- [35] T. Li, Z. Zhang, J.G. Bartolacci, G.K. Dwyer, Q. Liu, L.R. Mathews, M. Velayutham, A.S. Roessing, Y.C. Lee, H. Dai, S. Shiva, M.H. Oberbarnscheidt, J.L. Dziki, S. J. Mullet, S.G. Wendell, J.D. Wilkinson, S.A. Webber, M. Wood-Trageser, S. C. Watkins, A.J. Demetris, G. S Hussey, S.f. Badylak, H.R. Turnquist, Graft IL-33 regulates infiltrating macrophages to protect against chronic rejection, *J. Clin. Invest.* 130 (2020) 5397–5412, <https://doi.org/10.1172/JCI133008>.
- [36] A. Thierry, S. Giraud, A. Robin, A. Barra, F. Bridoux, V. Ametieu, T. Hauet, J. P. Girard, G. Touchard, J.M. Gombert, A. Herbelin, The alarmin concept applied to human renal transplantation: evidence for a differential implication of HMGB1 and IL-33, *PLoS One* 9 (2014), e88742, <https://doi.org/10.1371/journal.pone.0088742>.
- [37] A.B. Molofsky, A.K. Savage, R.M. Locksley, Interleukin-33 in tissue homeostasis, injury, and inflammation, *Immunity* 42 (2015) 1005–1019, <https://doi.org/10.1016/j.immuni.2015.06.006>.
- [38] T. Gajardo Carrasco, R.A. Morales, F. Perez, C. Terraza, L. Yáñez, M. Campos-Mora, K. Pino-Lagos Alarmin' Immunologists, IL-33 as a putative target for modulating T cell-dependent responses, *Front. Immunol.* 6 (2015) 232, <https://doi.org/10.3389/fimmu.2015.00232>.
- [39] S. Mauiyyedi, M. Crespo, A.B. Collins, E.E. Schneeberger, M.A. Pascual, S. L. Saidman, N.E. Tolkoff-Rubin, W.W. Williams, F.L. Delmonico, A.B. Cosimi, R. B. Colvin, Acute humoral rejection in kidney transplantation: II. Morphology, immunopathology, and pathologic classification, *J. Am. Soc. Nephrol.* 13 (2002) 779–787.
- [40] E. O'flaherty, W.K. Wong, S. Pettit, K. Seymour, S. Ali, J.A. Kirby, Regulation of T-cell apoptosis: a mixed lymphocyte reaction model, *Immunology* 100 (2000) 289–299, <https://doi.org/10.1046/j.1365-2567.2000.00048.x>.
- [41] J. Schmitz, A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, R.A. Kastelein, IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines, *Immunity* 23 (2005) 479–490, <https://doi.org/10.1016/j.immuni.2005.09.015>.
- [42] K. Oboki, T. Ohno, N. Kajiwara, H. Saito, S. Nakae, IL-33 and IL-33 receptors in host defense and diseases, *Allergol. Int.* 59 (2010) 143–160, <https://doi.org/10.2332/allergolint.10-RAI-0186>.
- [43] J. Lin, L. Zhang, G. Zhao, Z. Su, R. Deng, S.C. Pflugfelder, D.Q. Li, A novel interleukin 33/ST2 signaling regulates inflammatory response in human corneal epithelium, *PLoS One* 8 (2013), e60963, <https://doi.org/10.1371/journal.pone.0060963>.
- [44] K. Oshikawa, K. Kuroiwa, K. Tago, H. Iwahana, K. Yanagisawa, S. Ohno, S. I. Tominaga, Y. Sugiyama, Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation, *Am. J. Respir. Crit. Care Med.* 164 (2001) 277–281, <https://doi.org/10.1164/ajrcm.164.2.2008120>.
- [45] R.K. Kumar, P.S. Foster, ST2: marker, activator and regulator of Th2 immunity? *Clin. Exp. Allergy* 32 (2002) 1394–1396, <https://doi.org/10.1046/j.1365-2745.2002.01517.x>.
- [46] M. Matz, C. Lorkowski, K. Fabritius, K. Wu, B. Rudolph, S. Frischbutter, S. Brakemeier, J. Gaedeke, H.H. Neumayer, M.F. Mashreghi, K. Budde, The selective biomarker IL-8 identifies IFTA after kidney transplantation in blood cells, *Transpl. Immunol.* 39 (2016) 18–24, <https://doi.org/10.1016/j.trim.2016.09.003>.
- [47] T. Wada, N. Tomosugi, K. Kobayashi, I. Ishikawa, A. Shinoda, R. Tsugawa, N. Mukaida, K. Matsushima, Appearance of interleukin-8 in the urine predicts acute rejection of renal transplantation, *Nephrol. Dial. Transplant.* 9 (1994) 874–875.
- [48] T. Tefik, H.S. Çiftçi, M.S. Karadeniz, H. Yazici, T. Oktar, T. Kocak, O. Ziyilan, A. Turkmen, F.S. Oğuz, I. Nane, Predictive value of interleukin 2 and interleukin 8 on early rejection in living related kidney transplant recipients, *Transplant. Proc.* 51 (2019) 1078–1081, <https://doi.org/10.1016/j.transproceed.2019.02.015>.
- [49] J.B. Cowland, N. Borregaard, Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans, *Genomics* 45 (1997) 17–23, <https://doi.org/10.1006/geno.1997.4896>.
- [50] W.K. Wu, O. Famure, Y. Li, S.J. Kim, Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation, *Kidney Int.* 88 (2015) 851–858, <https://doi.org/10.1038/ki.2015.190>.
- [51] M. Scolari, M. Cappuccilli, N. Lanci, G.L. Manna, G. Comai, E. Persici, P. Todeschini, A. Faenza, S. Stefoni, Predictive factors in chronic allograft nephropathy, *Transplant. Proc.* 37 (2005) 2482–2484, <https://doi.org/10.1016/j.transproceed.2005.06.092>.
- [52] I. Fonseca, J.C. Oliveira, M. Almeida, M. Cruz, A. Malho, L.S. Martins, L. Dias, S. Pedrosa, J. Santos, L. Lobato, A.C. Henriques, D. Mendonça, Neutrophil gelatinase-associated lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function, *J. Transplant.* 2013 (2013) 650123, <https://doi.org/10.1155/2013/650123>.
- [53] A. Pezeshgi, S.A. Azar, H. Ghasemi, K. Kamali, A. Esmailzadeh, B. Hajsalimi, S. Pour-Azhar, M.R. Behmanesh, M. Kiafar, Role of plasma neutrophil gelatinase-associated lipocalin as an emerging biomarker of acute renal failure following kidney transplantation and its correlation with plasma creatinine, *J. Ren. Inj. Prev.* 5 (2016) 98–103, <https://doi.org/10.1517/jrip.2016.21>.
- [54] V. Cantaluppi, S. Dellepiane, M. Tamagnone, D. Medica, F. Figliolini, M. Messina, A.M. Manzione, M. Gai, G. Tognarelli, A. Ranghino, C. Dolla, S. Ferrario, C. Tetta, G.P. Segoloni, G. Camussi, L. Biancone, Neutrophil gelatinase associated lipocalin is an early and accurate biomarker of graft function and tissue regeneration in kidney transplantation from extended criteria donors, *PLoS One* 10 (2015), e0129279, <https://doi.org/10.1371/journal.pone.0129279>.
- [55] G. La Manna, G. Ghinatti, P.L. Tazzari, F. Alviano, F. Ricci, I. Capelli, V. Cuna, P. Todeschini, E. Brunocilla, P. Pagliaro, L. Bonsi, S. Stefoni, Neutrophil gelatinase-associated lipocalin increases HLA-G+/FoxP3+ T-regulatory cell population in an in vitro model of PBMC, *PLoS One* 9 (2014), e89497, <https://doi.org/10.1371/journal.pone.0089497>.