



## Expression and Significance of RANTES and MCP-1 in Renal Tissue With Chronic Renal Allograft Dysfunction

Q. Yan<sup>a,\*</sup>, H. Jiang<sup>b</sup>, B. Wang<sup>a</sup>, W. Sui<sup>a</sup>, H. Zhou<sup>a</sup>, and G. Zou<sup>a</sup>

From the <sup>a</sup>Nephrology Department of Guilin 181st Hospital, Guangxi Key Laboratory of Metabolic Diseases Research, Guangxi, China; and <sup>b</sup>Department of Urology, The Fifth Affiliated Hospital of Zunyi Medical College, Zhuhai, China

### ABSTRACT

**Background.** To investigate the expression of RANTES (regulated upon activation, normal T-cell-expressed and -secreted) and monocyte chemoattractant protein-1 (MCP-1) in renal allografts with chronic renal allograft dysfunction (CRAD), and explore its relationship with interstitial fibrosis and tubular atrophy (IF/TA).

**Methods.** An immunohistochemical assay and computer-assisted, genuine colored image analysis system were used to detect the expression of RANTES and MCP-1 in renal allografts with CRAD. The relationship among the expression level of MCP-1, RANTES, and the grade of inflammatory cell infiltration, interstitial fibrosis, and tubular atrophy in renal allografts were analyzed. Ten specimens of healthy renal tissue were used as controls.

**Results.** Compared to the normal tissues, the expressions of RANTES and MCP-1 were significantly higher in the renal tissues with CRAD ( $P < .001$ ), and the expressions tended to increase along with the pathological grade of IF/TA. The expression of RANTES and MCP-1 were positively correlated with the pathological grades of IF/TA ( $r = 0.940$  and  $0.954$  respectively,  $P < .001$  for both).

**Conclusion.** In renal allograft tissue with CRAD, the up-regulated expressions of RANTES and MCP-1 may be related to the progression of chronic renal allograft dysfunction and allograft fibrosis.

**C**URRENTLY, kidney transplantation is the optimal treatment for patients with end-stage renal disease (ESRD) and is widely regarded as the gold standard in renal replacement therapy. The short-term survival rate of renal transplantation has increased significantly, due to the use of novel immunosuppressants and the improvements in surgical techniques and postoperative care. In the past 20 years, the 1-year allograft survival rate has increased from 50% to almost 90% after kidney transplantation. However, the long-term survival rate is still lower in the current decade, as the long-term renal allografts survival is still 68% in living donor and 51% in cadaveric donor [1] transplants.

The main factors influencing renal allograft survival are cardiovascular disease and chronic renal allograft dysfunction (CRAD). There is less reported about RANTES (regulated upon activation, normal T-cell-expressed and -secreted) and monocyte chemoattractant protein-1 (MCP-1) in patients with CRAD. Therefore, in this study, we focused mainly on detecting the expression of RANTES, MCP-1, and collagen

IV in the tissue of renal allografts, and analyzed their relationship with interstitial fibrosis and tubular atrophy (IF/TA), for exploring the role of RANTES and MCP-1 in the development of chronic renal allograft dysfunction.

### METHODS

#### Clinical Data

Renal biopsy samples were collected from renal transplant patients with elevated serum creatinine levels and proteinuria from January

This work was supported by Natural Science Foundation of Guangxi (No: 2014GXNSFAA118179), Self-funded Research Projects of Guangxi Health Department (No. Z2014533), and Science and Technology Planning Project of Guilin (No. 20130121-6).

\*Address correspondence to Qiang Yan, Nephrology Department of Guilin 181st Hospital, Guangxi Key Laboratory of Metabolic Diseases Research, 541002 Guilin, Guangxi, China. E-mail: [yanqiang1967@sohu.com](mailto:yanqiang1967@sohu.com)

0041-1345/16

<http://dx.doi.org/10.1016/j.transproceed.2016.05.007>

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

360 Park Avenue South, New York, NY 10010-1710

2008 to January 2013 in Guilin No. 181 Hospital of Southern Medical University of China. Among the 40 patients with clinical diagnoses of chronic renal allograft dysfunction (CRAD), 23 were male (aged  $44 \pm 11$  years) and 17 were female (aged  $47 \pm 5$  years). The duration between sample collection and kidney transplantation was 0.5–12 years. The patients' mean level of serum creatinine was  $302 \pm 206 \mu\text{mol/L}$ . The triple immunosuppressant protocols were tacrolimus + mycophenolate mofetil + prednisone in 15 patients, and cyclosporine + mycophenolate mofetil + prednisone in the remaining 25 patients. Before renal biopsy, serum drug concentration test and color Doppler ultrasound detection of renal allografts were performed to exclude acute rejection, obstruction/reflux of bladder-ureter, nephrotoxicity of immunosuppressant, thrombosis or embolism in renal arteries or veins, and other diseases [2]. According to the Banff 2013 criteria [3] pathological classification criteria for renal allografts, the patients were diagnosed with CRAD. The donor and recipient were matched in regard to ABO blood type, and two or more human leukocyte antigens (HLA) were matched. The results of lymphocytotoxicity test were less than 10%, and the results of panel reaction antibody (PRA) were negative. The renal samples of 10 control cases were collected from routine donor kidney biopsy samples before transplantation, the histological manifestation of which was normal. Informed consent was obtained from all patients who were involved in the study. Our study was performed under the supervision of Institutional Review Board of Southern Medical University, and abided by the principles of the World Medical Association Declaration of Helsinki.

### Histological Examination

The paraffin-embedded kidney sections were sliced into 3- $\mu\text{m}$ -thick tissue sections, which were deparaffinized through xylene and hydrated through graded ethanol (100%, 96%, 90%, and 70%) and distilled water. Standard histological procedures were used to stain the sections, including hematoxylin and eosin stain, Masson trichrome, periodic-acid Schiff (PAS) staining, and periodic acid-silver methenamine (PASM) staining. Hematoxylin and eosin staining was used to assess the class of interstitial cellular infiltration and tubular atrophy, Masson and PASM were used to evaluate the interstitial fibrosis and basement membrane disorder, and PAS reaction was used to evaluate the arterio- and glomerulosclerosis.

### Pathological Diagnosis

According to the Banff 2013 [3] pathological classification criteria for renal allografts, the degree of interstitial fibrosis and tubular atrophy was defined as: IF/TA-I, mild interstitial fibrosis and tubular atrophy (<25% of cortical area); IF/TA-II, moderate interstitial fibrosis and tubular atrophy (26%–50% of cortical area); and IF/TA-III, severe interstitial fibrosis and tubular atrophy/loss (>51 of cortical area).

### Immunohistochemistry Examination

Immunohistochemistry with Envision was used to detect the expression of RANTES, MCP-1, and C4d in the renal allograft tissue. Paraffin sections with 3- $\mu\text{m}$  thickness underwent routine baking and were dewaxed step by step. A 3% hydrogen dioxide solution was adopted to clear endogenous hydrogen peroxidase. Before immunohistochemical staining, the antigens were microwaved for 15 minutes. Then the first antibody (RANTES, rabbit anti-human monoclonal antibody, Wuhan Boshide Company product, No. BA-1383, 1:200 diluted; MCP-1, rabbit anti-human monoclonal antibody, Wuhan Boster Biological Technology

product, No. BA-1254, 1:200 diluted; C4d, rabbit anti-human polyclonal antibody, working solution Santa Cruz product, No. ZA-0415) was added and incubated over night at 4°C. After washing with phosphate-buffered saline solution (PBS), rabbit anti-mouse antibody (Fuzhou Maixin Company) was added, and the sections were incubated for 30 minutes at 37°C. PBS washing was followed by DAB coloration and then hematoxylin counterstaining.

At the same time, immunohistochemistry tests for IgA, IgG, IgM, Clq, C4c, and C3 were also performed in the same way. Immunohistochemical staining of each group was compared with the control group, with PBS as a substitute for the first antibody.

Positive results were defined as pale yellow, brownish yellow, and yellowish-brown particles appeared in tubular epithelial cell and interstitial cell cytoplasm.

### Image Semi-quantitative Analysis

Semi-quantitative analysis was performed by means of the Leica DMR-X+Q550 renal color patho-image analysis system. Ten discontinuous tubulointerstitial fields (400 $\times$ ) per kidney were randomly selected in different regions including the renal cortex, cortex-medulla juncture, and medullary interstitium (not including glomeruli and veins and arteries). More than 60 tubules were observed in each part. Image-Pro Plus (IPP) was used to calculate the average optical density (the ratio of the positive to the total area excluding the area of tubular lumens). The relative expression of each kind of molecules in kidney tubulo-interstitium was represented by the ratios.

### Statistic Analysis

All experimental results were expressed as mean  $\pm$  standard deviation (SD). SPSS 13.0 statistical software was performed to analyze the experiment data. Measurement data was analyzed by single factor analysis. The correlation between two variables was performed with Pearson linear correlation. Statistical significance was set at the level of  $P < .05$ .

## RESULTS

### Histological Examination

The main histological disorders of renal allograft tissue with hematoxylin and eosin staining were expressed as follows: the glomerular mesangial matrix became broader, and diffuse inflammatory cells including lymphocytes and monocytes were found in the renal interstitium. Under Masson and PASM staining, there were increased mesangial matrix and extracellular matrix, thickening of glomerular basement membranes, and false double track sign. There were focal segmental glomerulosclerosis, interstitial fibrosis, and tubular atrophy accompanied by infiltration of inflammatory cells including monocytes and lymphocytes, and thickening of Bowman's capsule.

None of the manifestations of histological examination coincided with presentation of nephrotoxicity of immunosuppressant, acute rejection, chronic glomerulonephritis, or obstructive uropathy/reflux nephropathy.

### RANTES Expression

There was either no or only rare expression of RANTES in the normal renal tissue. However, in the renal allograft

**Table 1. RANTES and Monocyte Chemoattractant Protein-1 (MCP-1) Expression in Renal Allografts of Patients With Chronic Renal Allograft Dysfunction and Various Degrees of Interstitial Fibrosis and Tubular Atrophy**

Group	n	RANTES (%)	MCP-1 (%)	Collagen IV (%)
Normal controls	10	1.83 ± 0.77	4.05 ± 1.33	10.14 ± 3.39
IF/TA-I group	17	18.53 ± 4.93*	29.20 ± 5.89*	37.11 ± 6.60*
IF/TA-II group	14	30.98 ± 8.68 <sup>*,†</sup>	48.36 ± 9.17 <sup>*,†</sup>	52.91 ± 6.75 <sup>*,†</sup>
IF/TA-III group	9	52.60 ± 4.77 <sup>*,†,‡</sup>	67.78 ± 6.82 <sup>†,‡</sup>	67.18 ± 4.75 <sup>*,†,‡</sup>

Data are mean ± standard deviation.

Abbreviation: IF/TA, interstitial fibrosis and tubular atrophy.

\**P* < .001 vs control group.

<sup>†</sup>*P* < .001 vs IF/TA-I group.

<sup>‡</sup>*P* < .001 vs IF/TA-II group.

tissue with CRAD, a large amount of RANTES was expressed in the tubular epithelia and mesangial area, along with significant inflammatory cell infiltration in the renal interstitium. The stronger expression of RANTES in

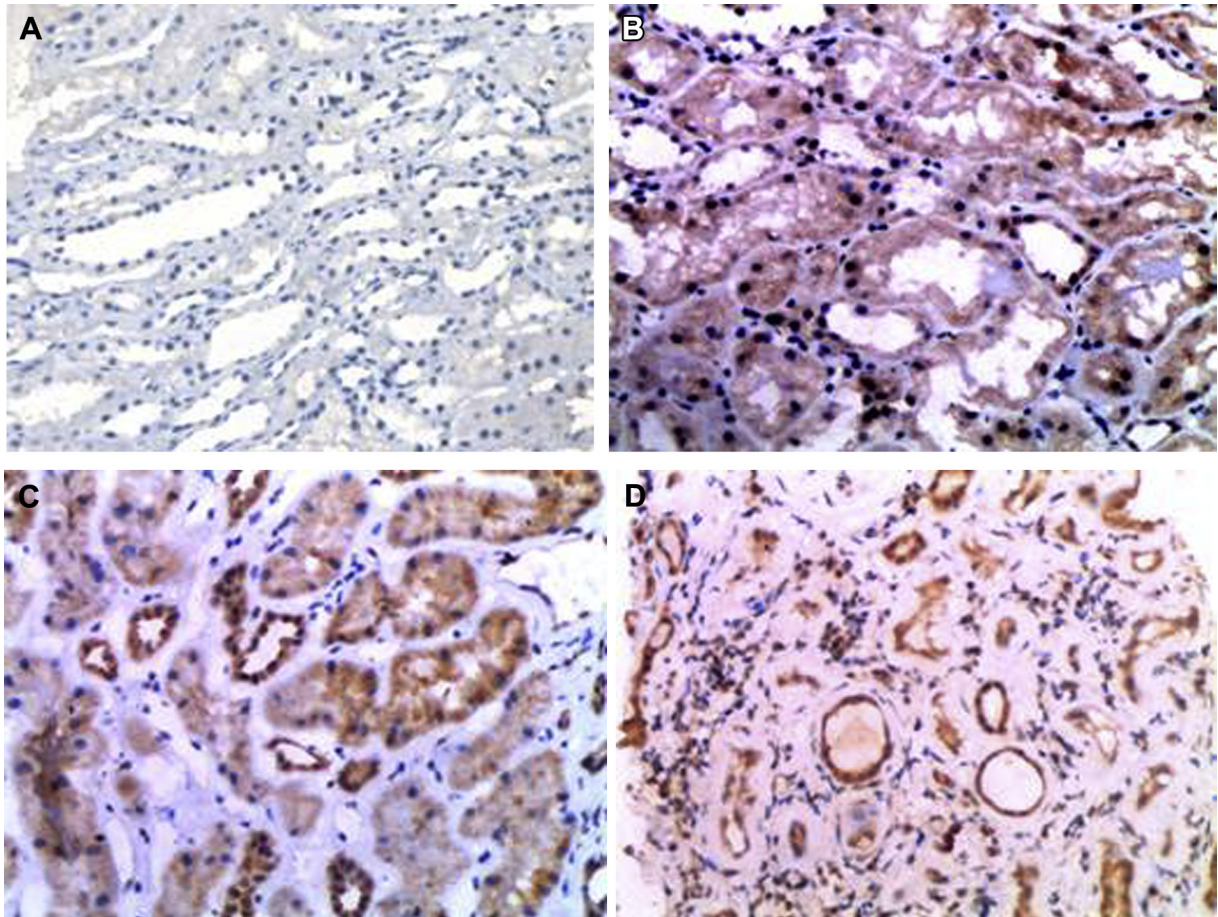
allograft tissue was accompanied by more monocyte and lymphocyte infiltration and by severe tubulointerstitial disorders. The expression of RANTES increased along with the increase in IF/TA classification ( $r = 0.940$ ,  $P < .001$ ) (Table 1 and Fig 1).

#### MCP-1 Expression

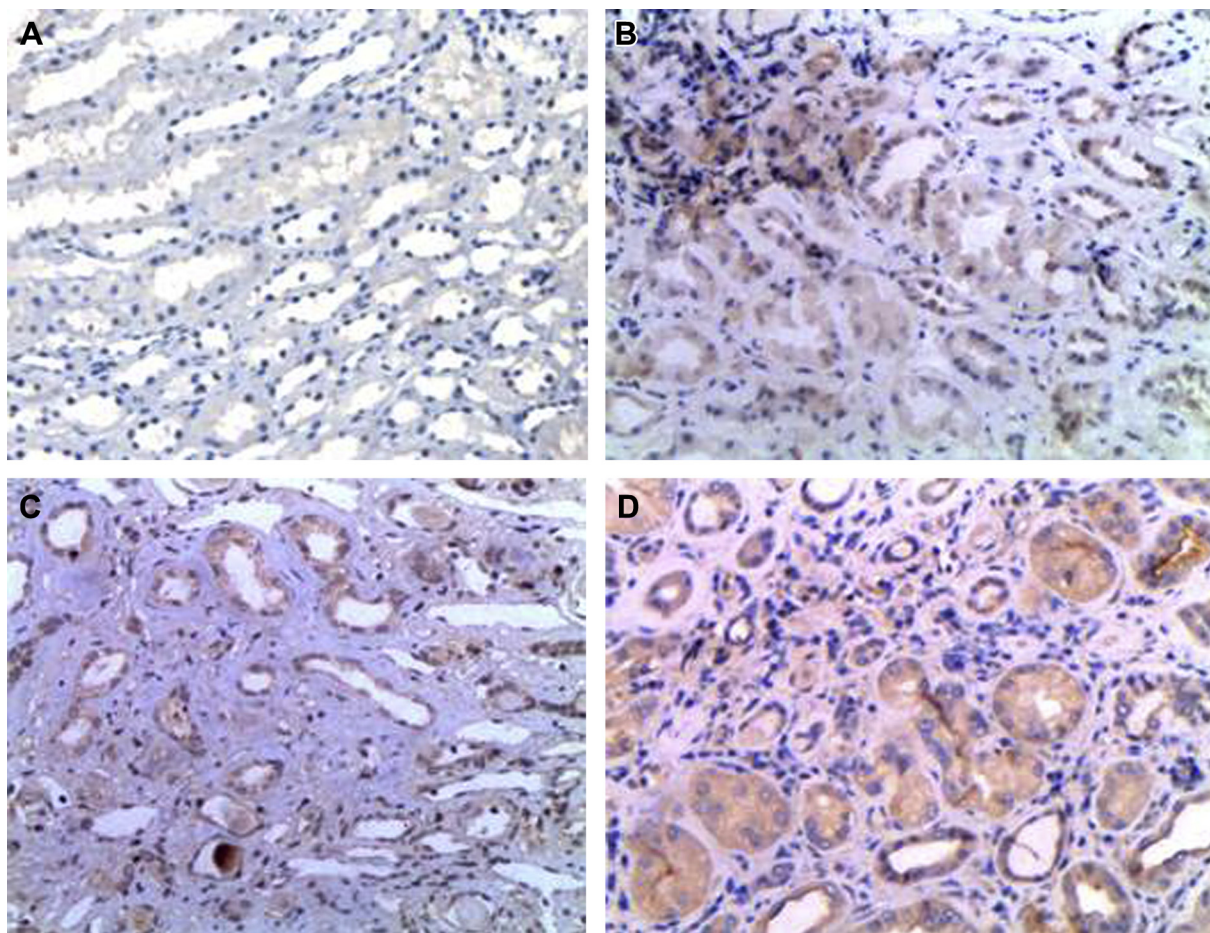
Weak MCP-1 expression was observed in renal tissue of controls. However, in the renal allograft tissue with CRAD, MCP-1 expression was significantly shown in the cytoplasm of interstitial cells, mesangial area, and endothelial side of glomerular capillary loops. The expression of MCP-1 increased along with the increase in IF/TA pathological grade ( $r = 0.954$ ,  $P < .001$ ) (Table 1 and Fig 2).

#### DISCUSSION

Renal transplantation results in improved quality of life and survival time of patients with end-stage kidney disease. However, the long-term renal allograft survival rate remains extremely low. Inflammatory cell infiltration plays an important role in chronic inflammation and interstitial



**Fig 1.** Expressions of RANTES in kidney allograft tissues with chronic renal allograft dysfunction (CRAD). (A) Normal controls. (B) Group IF/TA-I. (C) Group IF/TA-II. (D) Group IF/TA-III (EnVision assay, original magnification ×400).



**Fig 2.** Expressions of monocyte chemoattractant protein-1 (MCP-1) in kidney allograft tissues with CRAD. (A) Normal controls. (B) Group IF/TA-I. (C) Group IF/TA-II. (D) Group IF/TA-III (EnVision assay, original magnification  $\times 400$ ).

fibrosis of the transplanted kidney; inflammation is the initial culprit in the development of renal fibrosis [4]. In the early stage of CRAD, the infiltration of monocytes is the primary pathological manifestation. As is well known, the infiltration, accumulation, and activation of inflammatory cells require chemokines to participate in the pathogenesis. However, the mechanism has not been fully explored, as there was lack of research on its pathological mechanism in previous literature. In our present study, we first report that RANTES and MCP-1 are involved in the pathogenesis of CRAD, and their high expression is closely related to inflammatory cell infiltration, interstitial fibrosis, and tubular atrophy, which were induced by CRAD.

Numerous research articles over the past decade have provided compelling evidence to suggest that macrophages could cause chronic progressive renal damage. Pilmore et al [5] found that the degree of myofibroblast proliferation and macrophage infiltration in early biopsy specimens predicted the subsequent development of CAN. There is also evidence to suggest that the degree of macrophage

infiltration positively correlated with the degree of renal fibrosis in an *in vivo* study of rats, and the degree of renal fibrosis was alleviated when knocking out the macrophage gene or inhibiting the gene encoding the generation of macrophages [6]. Liposome-clodronate can selectively deplete macrophage infiltration from unilateral ureteral obstruction (UUO), and can markedly suppress the progression of renal interstitial fibrosis [7]. Monocytes are recruited to the interstitium of renal allograft by local production of chemokines and cytokines including RANTES and MCP-1 [8,9]. RANTES and MCP-1 are two important members of CC-chemokine family. They play important roles in inflammatory cell infiltration, activation, and development of fibrosis in the transplanted kidney, and a recently study has demonstrated that blockade of MCP-1 can have profound effects on islet allograft survival [10]. In addition, RANTES and MCP-1 not only contribute to the accumulation and activation of lymphocytes and monocytes/macrophages in the renal interstitium, but also are secreted by those inflammatory cells; thus, a positive feedback loop could be formed. This pathological process has been found

to aggravate glomerular and tubulointerstitial damage as well as inflammatory cells influx and activation [11,12].

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is an important effect factor that is necessary for the synthesis and degradation of extracellular matrix (ECM) [13]. Collagen IV is the major component of collagens in the kidney, and it has been demonstrated that the overexpression of ECM (especially collagen IV) is a significant pathological event in the development of renal fibrosis [14]. It has been found in our previous study in CRAD patients that collagen IV deposit in the kidney interstitium could be an important process in the renal allograft fibrosis [15], and that macrophage not only secretes several inflammatory cytokines that could induce persistent injury of mesangial cells and tubular epithelial cells, but also generates a large number of pro-fibrogenic factors, such as TGF- $\beta$ 1 and platelet-derived growth factor (PDGF), resulting in overgeneration of extracellular matrix, collagen IV, and renal fibrosis [16]. Furthermore, in our present study, it is suggested that the high expression of RANTES and MCP-1 significantly correlated with IF/TA processes in the pathological mechanism of CRAD.

In a study of diabetic nephropathy contributing to interstitial fibrosis, it was shown that MCP-1 played an important role in macrophage recruitment and migration, and that the macrophages could secrete interleukin-1 $\beta$  that acts as a growth factor in renal mesangial cells to increase the generation of TGF- $\beta$ , PDGF, and collagen IV in diabetic nephropathy, eventually leading to renal fibrosis [17]. In a rat model of nephrosis, Wastson et al [18] found that RANTES and MCP-1 DNA vaccines can protect against chronic renal disease by reducing the recruitment of T cells and macrophages. Evidently, upregulation of RANTES and MCP-1 may play a vital role in the pathological and molecular mechanisms of progressive chronic renal allograft dysfunction. In our present study we also found that the expression of RANTES and MCP-1 were much higher than in normal renal tissue, and become stronger along with the increasing IF/TA pathological grade. This is consistent with our previous study of CRAD in a rat model [19].

It was shown in our previous study that in vivo blockade of chemokine receptors by Met-RANTES, an antagonist of CCL5/RANTES chemokine receptors, could decrease early infiltration and activation of monocytes in renal allograft tissue, as well as markedly improve IF/TA, and thereby prolong renal allograft survival time [20]. Other authors have found that CCR1 antagonist BX 471 could markedly reduce the mRNA expression of fibrogenic cytokine plasminogen activator inhibitor-1 (PAI-1) and TGF- $\beta$ 1, and weaken collagen deposition in renal grafts; in addition, the development and progression of chronic allograft dysfunction was significantly inhibited by BX471 [21]. The results of the above studies suggest that CC chemokine MCP-1, RANTES, and their receptors play major roles in chronic allograft damage.

Renal allograft fibrosis is a complex pathophysiological processes that is mediated by a number of inflammatory cells, including macrophages and T cells. It requires an

orchestrated series of molecular events including the participation of TGF- $\beta$ 1, PDGF, PAI-1, and TIMP-1, and also needs the participation of chemotactic proteins such as RANTES and MCP-1. It is demonstrated in our study that RANTES and MCP-1 play important roles in the initial recruitment and activation of inflammatory cells and the production of pro-fibrogenic factors such as TGF- $\beta$ 1, PDGF, PAI-1, and TIMP-1, thereby leading to renal allograft interstitial fibrosis and tubular atrophy.

Our present work has assessed the functional role of chemokines, RANTES, and MCP-1, which play key roles in the process of CRAD, and therefore suggest the potential efficacy of therapies targeting RANTES and MCP-1 or their receptors in inhibiting inflammatory response and thus treating CRAD.

#### ACKNOWLEDGMENTS

We thank Dr. Hequn Zou for his help in critical reading and editing the manuscript, and Dr. Guimian Zou for his generous research gift and help.

#### REFERENCES

- [1] Cecka JM. The UNOS renal transplant registry. *Clin Transpl* 2001;1-18.
- [2] Fletcher JT, Nankivell BJ, Alexander SI. Chronic allograft nephropathy. *Pediatr Nephrol* 2009;24:1465-71.
- [3] Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of C4D-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant* 2014;14:272-83.
- [4] Filiopoulos V, Vlassopoulos D. Inflammatory syndrome in chronic kidney disease: pathogenesis and influence on outcomes. *Inflamm Allergy Drug Targets* 2009;8:369-82.
- [5] Pilmore HL, Painter DM, Bishop GA, et al. Early up-regulation of macrophages and myofibroblasts: a new marker for development of chronic renal allograft rejection. *Transplantation* 2000;69:2658-62.
- [6] Sung SA, Jo SK, Cho WY, et al. Reduction of renal fibrosis as a result of liposome encapsulated clodronate induced macrophage depletion after unilateral ureteral obstruction in rats. *Nephron Exp Nephrol* 2007;105:e1-9.
- [7] Kitamoto K, Machida Y, Uchida J, et al. Effects of liposome clodronate on renal leukocyte populations and renal fibrosis in murine obstructive nephropathy. *J Pharmacol Sci* 2009;111:285-92.
- [8] Pattison J, Nelson PJ, Huie P, et al. RANTES chemokine expression in cell-mediated transplant rejection of the kidney. *Lancet* 1994;343:209-11.
- [9] Grandaliano G, Gesualdo L, Ranieri E, et al. Monocyte chemoattractant peptide-1 expression and monocyte infiltration in acute renal transplant rejection. *Transplantation* 1997;63:414-20.
- [10] Lee I, Wang L, Wells AD, et al. Blocking the monocyte chemoattractant protein-1/CCR2 chemokine pathway induces permanent survival of islet allografts through a programmed death-1 ligand-1-dependent mechanism. *J Immunol* 2003;171:6929-35.
- [11] Wada T, Furuichi K, Sakai N, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int* 2000;58:1492-9.
- [12] Zoja C, Donadelli R, Colleoni S, et al. Protein overload stimulates RANTES production by proximal tubular cells depending on NF-kappa B activation. *Kidney Int* 1998;53:1608-15.
- [13] Border WA, Noble NA. TGF-beta in kidney fibrosis: a target for gene therapy. *Kidney Int* 1997;51:1388-96.

- [14] Zeisberg M, Bonner G, Maeshima Y, et al. Renal fibrosis: collagen composition and assembly regulates epithelial-mesenchymal transdifferentiation. *Am J Pathol* 2001;59:1313–21.
- [15] Yan Q, Sui W, Xie S, et al. Expression and role of integrin-linked kinase and collagen IV in human renal allografts with interstitial fibrosis and tubular atrophy. *Transpl Immunol* 2010;23:1–5.
- [16] Yang N, Wu LL, Nikolic-Paterson DJ, et al. Local macrophage and myofibroblast proliferation in progressive renal injury in the rat remnant kidney. *Nephrol Dial Transplant* 1998;13:1967–74.
- [17] Sassy-Prigent C, Heudes D, Mandet C, et al. Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. *Diabetes* 2000;49:466–75.
- [18] Watson D, Zheng G, Wu H, et al. CCL2 DNA vaccine to treat renal disease. *Int J Biochem Cell Biol* 2009;41:729–32.
- [19] Wang G, Zou H, Yao J, et al. Expression and significance of MCP-1 and RANTES in renal tissue of chronic allograft nephropathy. *Chin J Organ Transplant* 2005;26:157–9.
- [20] Song E, Zou H, Yao Y, et al. Early application of Met-RANTES ameliorates chronic allograft nephropathy. *Kidney Int* 2002;61:676–85.
- [21] Bedke J, Kiss E, Schaefer L, et al. Beneficial effects of CCR1 blockade on the progression of chronic renal allograft damage. *Am J Transplant* 2007;7:527–37.