Serum levels of CXCR3 ligands predict T cell-mediated acute rejection after kidney transplantation

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Abstract. The early diagnosis of acute rejection is crucial for graft survival after kidney transplantation. The interferon-γ (IFNγ)-CXCR3-chemokine-dependent inflammatory loop plays a pivotal role in the recruitment of T lymphocytes during acute rejection. Previously published studies have typically focused on the CXCR3 receptor rather than on its ligands. In the present study, we used Luminex assays to detect the levels of CXCR3 ligands, monokine induced by IFNγ (MIG), IFN-induced protein 10 (IP-10) and IFN-induced T-cell chemoattractant (I-TAC), in the serum of renal allograft recipients. According to a renal biopsy performed one month after kidney transplantation, 32 recipients were diagnosed with T cell-mediated acute rejection and 38 patients were evaluated as stable. Serum was collected after the diagnosis of acute rejection or one month after transplantation. The concentrations of MIG (median, 4,271 pg/ml), IP-10 (median, 686.7 pg/ml) and I-TAC (median, 44.32 pg/ml) in the serum during an acute rejection episode were significantly higher compared with those of the stable patients (MIG, P=0.0002; IP-10, P=0.0001; I-TAC, P=0.0103; vs. the stable function group, P<0.05). Based on the receiver-operating characteristic (ROC) curve, the joint detection of MIG, IP-10 and I-TAC in the serum using Luminex analysis may constitute a non-invasive and efficient method for the early prediction of T cell-mediated acute rejection following kidney transplantation.

Introduction

Despite the significant advances in immunosuppression, acute rejection remains a crucial barrier to long-term survival following kidney transplantation (1,2). An early diagnosis of acute rejection is critical for graft survival. Renal biopsy is currently the primary method to monitor the dynamic changes of graft rejection; however, this technique is invasive and graft damage is detected at a late stage. Although there have been efforts to identify non-invasive biomarkers for the early diagnosis of acute graft rejection (3-5), current acute rejection diagnostic methods are not specific or sensitive enough. Certain chemokines and chemokine receptor pathways have been shown to be critical in acute allograft rejection (6-8). The interferon-γ (IFNγ)-CXCR3-chemokine-dependent inflammatory loop is crucial in recruiting T lymphocytes during acute rejection following renal transplantation (9-11). Monokine induced by IFNγ (MIG, CXCL9), IFN-induced protein 10 (IP-10, CXCL10) and IFN-induced T-cell chemoattractant (I-TAC, CXCL11) are CXCR3-specific ligands induced by IFNγ in a wide variety of cell types. These ligands direct migration and stimulate the adhesion of activated Th1 cells and cytoxic T lymphocytes (CTLs) via the IFNγ-CXCR3-chemokine loop (12-14). Multiple chemokines act as proinflammatory cytokines and produce signals for the dynamic trafficking and recruitment of leukocytes, which leads to an inflammatory response (12,15).

We hypothesized that determining MIG, IP-10 and I-TAC levels using Luminex assays may offer a non-invasive means to diagnose T cell-mediated acute rejection in renal allograft recipients. The Luminex method is a high-throughput tool, which detects numerous chemokines and cytokines simultaneously using only 25 μl of serum. It is a more efficient and practical method compared with ELISA. In the present study, we collected the serum of patients who either had biopsy-confirmed T cell-mediated acute renal allograft rejections during the first month after transplantation or were diagnosed as stable kidney transplant recipients. Using the Luminex method, the levels of MIG, IP-10 and I-TAC in the serum of patients were detected. It was concluded that the concentrations of MIG, IP-10 and I-TAC in the serum during an acute rejection episode were significantly higher compared with those of stable patients. The joint detection of MIG, IP-10 and I-TAC in the serum using Luminex analysis may constitute a non-invasive and efficient method for the early prediction of T cell-mediated acute rejection following kidney transplantation.

Materials and methods

Study population. The study protocol was approved by the Ethics Committee of the Chinese PLA 309th Hospital

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(Beijing, China) and informed consent was obtained from each patient. Seventy patients undergoing kidney transplantation were included in this retrospective study. All the patients had no other co-morbidities. Thirty-two patients had biopsy-confirmed T cell-mediated acute renal allograft rejection during the first month after transplantation (Fig. 1). The indications of renal needle biopsy were serum creatinine increase, hypourocrinia and hardened texture of the renal graft and ultrasonography revealed the increase of renal vascular resistance after transplantation. Thirty-eight patients with stable function underwent protocol biopsies one month post-transplantation and were diagnosed as stable kidney transplant recipients. All the patients were administered triple therapy based on a combination of calcineurin inhibitors (CNIs), mycophenolate mofetil (MMF) and steroids [8-10 mg/kg/day methylprednisolone (MP) for 3 days and prednisone gradually reduced to 10 mg/day] for the maintenance of immunosuppression. When acute rejection was diagnosed, the patients were typically treated with high-dose corticosteroids for 3 days. Patient demographic information and the parameters of kidney transplantation are shown in Table I.

Renal needle biopsy of the renal graft. Patients were in the supine position and were kept in this position for <8 h. Color Doppler ultrasound was used as a guide to ensure blood vessels and the renal pelvis were avoided and the inferior pole of the kidney was obliquely punctured with a BARD biopsy needle (Bard Biopsy Systems, Tempe, AZ, USA). The depth of needle penetration was ~2.2 cm and two punctures were carried out at separate sites. Following the needle biopsy, the puncture site and local area were covered and appropriately compressed with a pressure dressing. Hemostasis and anti-infection treatment were administered. The blood pressure, heart rate, urine output and urine color of patients were closely monitored.

Pathological examinations. Graft biopsy specimens were immediately immersed in formaldehyde solution, followed by careful identification of the specimen to determine whether it was renal tissue, perirenal fat or a different tissue type. An additional renal needle biopsy at a different puncture site was performed when necessary, in order to improve the success rate of the biopsy. Pathological examinations of the biopsy specimens were performed immediately, including paraffin embedding, sectioning and hematoxylin and eosin (H&E), Periodic acid-Schiff (PAS) and Masson staining. Pathological changes were observed using a light microscope. In specimens with suspected rejection, C4d immunohistochemical staining was also performed.

Immunohistochemical analysis. All the renal allograft specimen sections were deparaffinized and rehydrated through a series of xylene and graded alcohols. Endogenous peroxidase was blocked in 3% H_2O_2 for 5 min. Antigen retrieval was carried out by placing the slides in a Black & Decker vegetable steamer in Maixin-Bio retrieval solution (pH 6.1; Maixin Biotechnology Company, Shanghai, China). The primary polyclonal rabbit anti-human CXCR3 antibody (cat. no. PA1-32503; Thermo Fisher Scientific, Inc., Rockford IL, USA) was applied at a dilution of 1:100 at 4˚C overnight. The IP-10 staining procedure was performed using the Elivision™ Plus kit (cat. no. kit-9901; Maixin Biotechnology Company). Following primary antibody incubation, a polymer enhancer from the kit was added at room temperature for 20 min. Polymerized HRP-Anti Ms/Rb IgG from the kit was also applied at room temperature for 30 min. A detection kit (DAB-0031; Maixin Biotechnology Company) was used with DAB as a chromogen. The slides were counterstained with hematoxylin (CTS-1099; Maixin Biotechnology Company), dehydrated through graded alcohols, cleared in xylene and coverslipped with CytoSeal.

Histopathological evaluation and immunohistochemical quantification. Samples from each organ were studied using hematoxylin and eosin staining of the paraffin-embedded sections. Histopathological evaluation was performed by two pathologists who specialized in rejection diagnosis according to the Banff '05 classification (16,17). The number of infiltrating cells was measured in at least 20 randomly selected high-power fields (hpf; x400) by two independent observers. The final count was calculated as the mean of the two measures. The inter-observer variability was not >15% at any point.

Preparation of serum samples and chemokine bead arrays. Serum samples were collected from the peripheral blood (10 ml) and drawn into additive-free Vacutainer tubes (Insepack, Beijing, China) following the acute rejection diagnosis in the rejection group and one month following transplantation in the stable patients. Serum was separated by centrifugation, aliquoted into Axygen cryovial tubes and stored at -80˚C. The Luminex assays were performed on single-thawed serum samples.

The serum concentrations of MIG, IP-10 and I-TAC in 25 μl were assayed simultaneously using a human cytokine/chemokine bead kit (Milliplex, Billerica, MA, USA) and a Luminex-200 array assay reader (Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. Serum samples were randomly assigned to the plates to avoid assay bias and inter- and intra-assay reproducibility was confirmed. The data were analyzed with a five-parameter curve fit using the Milliplex® Analyst software (Milliplex). The concentration of each chemokine was detected as the mean fluorescence intensity (MFI) and was subsequently converted to pg/ml of chemokine using a simultaneously generated standard curve (18).

Statistical analysis. A Mann-Whitney U test was used to compare the MIG, IP-10 and I-TAC levels between the acute rejection and stable groups. $P<0.05$ was considered to indicate a statistically significant difference.

A logistic model of multiple chemokine synergy was established by logistic regression. Based on the logistic model, the receiver-operating characteristic (ROC) curves were generated to determine the highest diagnostic accuracy in distinguishing patients with acute renal allograft rejection from control groups. The area under the curve (AUC) was calculated. An AUC of 1.0 would indicate a perfect test, whereas a test that is no better than expected by chance would have an AUC of 0.5 (19).

Table I. Patient demographic information and parameters of kidney transplantation (mean ± SD).

Parameters	Acute rejection patients	Stable function patients
Patient no.	32	38[a]
Male gender, n (%)	24 (75)	18 (47)[a]
Age (years)	39.0±11.0	40.5±12.1[a]
HLA mismatch	1.7±0.8	1.5±0.8[a]
PRA (%)	2.6±0.7	2.5±0.8[a]
Warm ischemia times (min)	5.2±2.0	4.4±2.4[a]
Cold ischemia times (min)	472.4±276.1	447.8±285.1[a]
Serum creatinine (μM)	219.39±74.15	74.29±15.72[b]
Immunosuppression protocol, n (%)		
Corticosteroids +MMF + CsA	16 (50)	18 (47)[a]
Corticosteroids + MMF + FK506	15 (47)	20 (53)[a]
Corticosteroids + AZA + CsA	1 (3)	0[a]

[a]Not significant; [b]P<0.0001. HLA, human leukocyte antigen; PRA, panel reactive antibody; MMF, mycophenolate; CsA, cyclosporine A; AZA, azathioprine; FK506, tacrolimus.

The expression of CXCR3 in different groups was assessed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Serum chemokine levels. The median concentrations of MIG, IP-10 and I-TAC were 4,271, 686.7 and 44.32 pg/ml, respectively. The Mann-Whitney U test indicated that the serum concentrations of MIG, IP-10 and I-TAC measured during an episode of T cell-mediated acute rejection were significantly increased compared with those of the stable patients (MIG, P<0.0001; IP-10, P=0.0002; I-TAC, P=0.0103; Table II and Fig. 2).

ROC curve analysis of chemokine levels. The ROC curves show the true-positive (sensitivity) and false-positive fractions (1 - specificity) for detecting each chemokine alone and the synergy of multiple chemokines (Fig. 3). The AUC in the different assays is shown in Table III. Thus, joint detection of MIG, IP-10 and I-TAC is the best method to predict acute rejection following kidney transplantation.

CXCR3 protein expression following transplantation in renal biopsy patients with acute rejection and stable renal allograft function. CXCR3$^+$ cells were barely detectable in biopsies with stable graft function. A significant increase in the number of CXCR3$^+$ cells in graft biopsies with ACR was observed (P=0.015; Fig. 1).

Table II. Levels of chemokines (median, pg/ml) in the serum of patients following transplantation.

Type of chemokine	Patients		P-value
	Acute rejection	Stable function	
MIG	4,271	1,148	<0.0001
IP-10	686.7	332.2	0.0002
I-TAC	44.32	22.92	0.0103

Monokine induced by interferon-γ (IFNγ); IP-10, interferon-induced protein 10; I-TAC, interferon-induced T-cell chemoattractant.

Table III. The calculated area under the curve (AUC) for chemokine levels.

Type of chemokine	AUC	SD	P-value	95% CI
MIG	0.877	0.043	<0.0001	0.794-0.961
IP-10	0.760	0.061	<0.0001	0.641-0.879
I-TAC	0.679	0.064	0.010	0.553-0.806
MIG + IP-10	0.876	0.042	<0.0001	0.793-0.959
MIG + I-TAC	0.875	0.042	<0.0001	0.793-0.957
IP-10 + I-TAC	0.765	0.060	<0.0001	0.648-0.882
MIG + IP-10 + I-TAC	0.878	0.041	<0.0001	0.797-0.959

MIG, monokine induced by interferon-γ (IFNγ); IP-10, interferon-induced protein 10; I-TAC, interferon-induced T-cell chemoattractant.

Discussion

CD4$^+$ T cells and effector CD8$^+$ T cells play a key role in acute rejection episodes (20). During the course of acute rejection, CD4$^+$ and CD8$^+$ T cells differentiate into Th1 cells and CTLs, respectively, due to the effect of specific cytokines (15). The induction of Th1 and CTLs is closely linked to

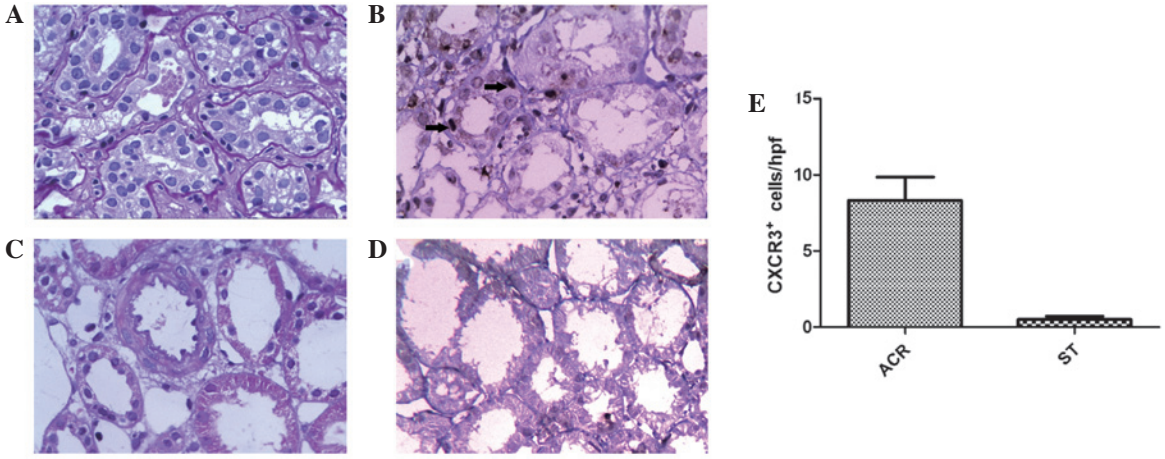

Figure 1. (A and C) Periodic acid-Schiff (PAS) staining in graft kidney biopsies with (A) acute cellular rejection (ACR) and (C) stable function (ST). (B and D) CXCR3 expression in graft kidney biopsies with (B) ACR (n=20) and (D) ST (n=10). (E) The number of CXCR3$^+$ infiltrating cells was measured as described in Materials and methods and was significantly increased in ACR patients compared with patients who had stable renal allograft function (P=0.015). For each group, all images are from a single patient and are representative of the whole group of patients. Results are expressed as the mean ± SD of the number of cells/high–power field (hpf). Magnification, x400. Arrows show CXCR3$^+$ cells.

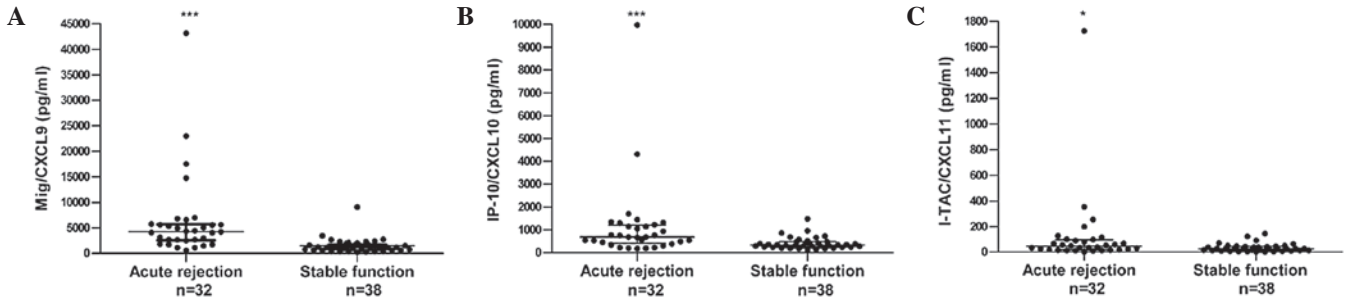

Figure 2. Concentrations of serum chemokines (bars, median with interquartile range). *P<0.05; ***P<0.0001. MIG, monokine induced by interferon-γ (IFNγ); IP-10, interferon-induced protein 10; I-TAC, interferon-induced T-cell chemoattractant.

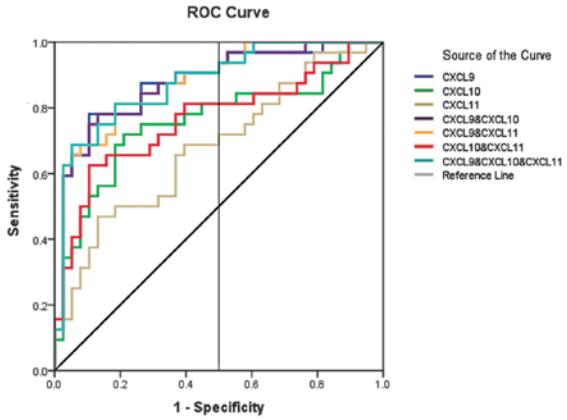

Figure 3. Receiver-operating characteristic (ROC) curve of chemokine levels. The fraction of true-positive (sensitivity) and false-positive results (1 - specificity) for MIG, IP-10 and I-TAC levels as markers of acute rejection are shown. A value of 0.5 is what would be expected by chance and a value of 1.0 reflects a perfect indicator. MIG, monokine induced by interferon-γ (IFNγ); IP-10, interferon-induced protein 10; I-TAC, interferon-induced T-cell chemoattractant.

the upregulation of the chemokine receptor CXCR3. CXCR3 binds three chemokines, MIG (CXCL9), IP-10 (CXCL10) and I-TAC (CXCL11), to induce activated T-cell migration *in vitro* and *in vivo*. As suggested by their original names, all three of the CXCR3 ligands are induced by IFNγ, which is secreted by CXCR3$^+$ effector cells. The increased secretion of CXCR3 ligands promotes the additional recruitment of CXCR3$^+$ effector cells. Subsequently, these effectors locally secrete IFNγ, which further amplifies the infiltration of effector cells. This inflammatory loop allows CXCR3 and its ligands to coordinate T-cell responses in the inflamed periphery (21,22). Previously published studies have demonstrated that competing for binding with IP-10, I-TAC and MIG or inhibiting CXCR3 disrupts CXCR3$^+$ Th1 cell trafficking and may be a novel anti-inflammatory strategy (23,24).

The expression of MIG, IP-10 and I-TAC has been revealed to play a role, not only in various autoimmune and infectious diseases that are associated with an increased expression of IFNγ (Th1-type diseases) (21,25-27), but also in hypoxia-induced inflammation associated with solid organ transplantation, including heart and lung transplants (6,8,28). A number of chemokines and their receptors in human renal transplantation have shown an increased expression in allograft acute rejection, including IP-10, MIG and CXCR3 (29,30).

In the present study, we hypothesized that CXCR3 and its ligands, MIG, IP-10 and I-TAC, may be reliable biomarkers for T cell-mediated acute rejection. The detection of MIG, IP-10

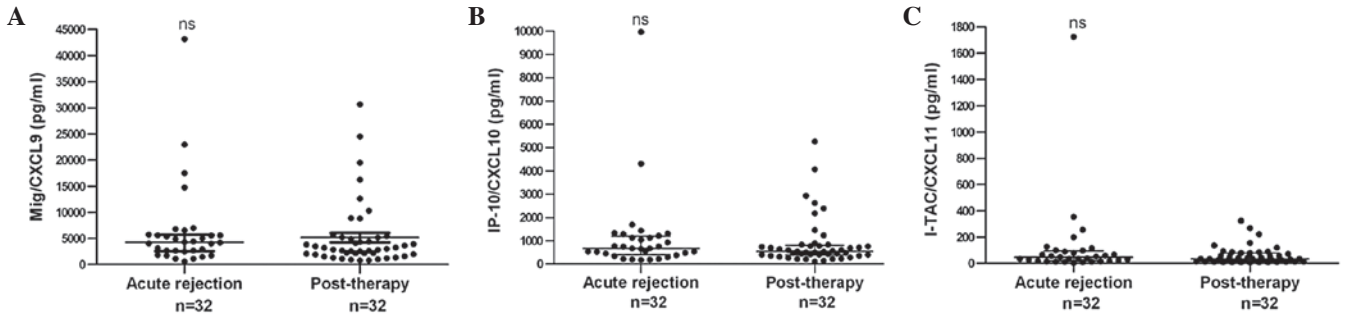

Figure 4. CXCR3 ligands level before and after anti-rejection therapy. ns, not significant; MIG, monokine induced by interferon-γ (IFNγ); IP-10, interferon-induced protein 10; I-TAC, interferon-induced T-cell chemoattractant.

and I-TAC in the serum is easier and more efficient compared with the determination of CXCR3 on the cell surface. Therefore, it is suggested that the detection of MIG, IP-10 and I-TAC in the serum of recipients may constitute a method for diagnosing acute rejection episodes after kidney transplantation. This study has shown that the serum levels of MIG, IP-10 and I-TAC in T cell-mediated acute rejection patients were significantly higher compared with those in stable patients. Immunohistochemical analysis was also performed to confirm that the increased CXCR3 ligands trigger an additional recruitment of $CXCR3^+$ effector cells in allografts.

Although the concept of an IFNγ-CXCR3-chemokine-dependent inflammatory loop has been firmly established, the differential induction of these CXCR3 ligands may be due to different cellular sources. In the cerebral malaria model, Campanella *et al* (21) demonstrated that endothelial cells predominantly expressed MIG and that neurons predominantly expressed IP-10. This finding may explain the non-overlapping roles of the two CXCR3 ligands in the pathogenesis of cerebral malaria (21). As compared with the remaining two CXCR3 ligands, MIG is more dependent on and strongly induced by IFNγ (22). The present study also suggests that MIG is a better indicator of acute rejection compared with the other two chemokines. According to the study mentioned above, initial innate challenges activate the endothelial cells to secrete MIG, which recruits Th1 cells and CTLs to the target tissue in T cell-mediated acute rejection after kidney transplantation. Th1 cells and CTLs produce large amounts of IFNγ, which further induces resident tissue cells to produce more MIG and additional CXCR3 ligands. Based on the ROC curve, the joint detection of MIG, IP-10 and I-TAC is the best method to more specifically and effectively predict T cell-mediated acute rejection.

Increased chemokine release amplifies inflammation, leading to further recruitment of CXCR3-expressing Th1 T cells and CTLs. Acute rejection becomes more serious via the IFNγ-CXCR3-chemokine-dependent inflammatory loop.

Thus, blocking the IFNγ-CXCR3-chemokine-dependent inflammatory loop may be an effective immunosuppressive therapy and has the potential to be applied as a therapeutic method for allograft rejection, and should be investigated further..

In the present study, all the patients were separately administered three different therapy protocols for the maintenance of immunosuppression. However, previous studies have shown that cyclosporine A affects the recruitment of chemokines (31,32). In the present study, chemokine levels in the serum of patients who were treated with three different therapy regimens were not significantly different (Table I).

The one-year follow-up of all the patients indicated that three patients were diagnosed with acute rejection after renal transplantation. Following steroid pulse therapy, 2 cases became chronic allograft rejections and 1 had graft loss. Several of the remaining patients had complications, however, their graft function was stable. No significant correlation was identified between CXCR3 ligands and graft survival or rejection one year after transplantation.

To detect the alterations in chemokine levels following anti-rejection therapy, the serum of patients who were administered routine anti-rejection therapy (4-6 mg/kg MP) for 3-5 days was also collected. It was demonstrated that serum creatinine was returned to normal levels. There was no significant difference in the CXCR3 ligand level prior to and following anti-rejection therapy (Fig. 4). This may indicate that the recovery of chemokine levels require more time compared with the recovery of kidney function. Future studies investigating this further should be conducted.

During the last decade, despite abundant improvements in the investigation of the molecular basis of allograft rejections, renal biopsy remains the primary method to monitor the dynamic changes of graft rejection. However, the detection and screening of chemokines using immunohistochemistry in allograft biopsies is inefficient and invasive. Luminex analysis, as a high-throughput and non-invasive tool for the measurement of different cell products, is increasingly facilitated.

In the present study, Luminex analysis was applied to detect the chemokine levels in serum and to demonstrate that higher concentrations of MIG, IP-10 and I-TAC in the serum of recipients after kidney transplantation constitutes a risk factor for T cell-mediated acute rejection episodes. The joint detection of MIG, IP-10 and I-TAC in the serum using Luminex analysis may be a non-invasive and efficient method for the early-stage prediction of T cell-mediated acute rejection.

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