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Immunologic Monitoring of T-Lymphocyte Subsets and Hla-Dr-Positive Monocytes in Kidney Transplant Recipients

A Prospective, Observational Cohort Study

Jang-Hee Cho, MD, PhD, Young-Deuk Yoon, MD, Hye Min Jang, PhD, Eugene Kwon, MD,
Hee-Yeon Jung, MD, Ji-Young Choi, MD, PhD, Sun-Hee Park, MD, PhD,
Yong-Lim Kim, MD, PhD, Hyung-Kee Kim, MD, PhD, Seung Huh, MD, PhD,
Dong-Il Won, MD, PhD, and Chan-Duck Kim, MD, PhD

Abstract: The clinical significance of circulating T-lymphocyte subsets and human leukocyte antigen (HLA)-DR-positive monocytes in the peripheral blood of kidney transplant recipients (KTRs) remains unclear. We examined the efficacy of enumerating these cells for the immunologic monitoring of KTRs.

Blood samples were obtained before transplantation, 2 weeks after transplantation and at diagnosis, and 2 weeks after treating biopsy-proven acute cellular rejection and cytomegalovirus (CMV) infection. Serial flow cytometric analysis was performed using peripheral blood obtained from 123 patients to identify the frequencies of HLA-DR^{+}, CD3^{+}, CD4^{+}, CD8^{+}, and CD25^{+} T-lymphocytes and HLA-DR-positive monocytes.

Frequencies of CD4^{+}CD25^{+}/CD4^{+} T cells, CD8^{+}CD25^{+}/CD8^{+} T cells, and HLA-DR-positive monocytes were significantly lower at 2 weeks after transplantation than before transplantation (all $P < 0.001$). This decrease was not correlated with clinical parameters. The frequency of CD4^{+}CD25^{+}/CD4^{+} T cells was significantly higher in KTRs with acute rejection than in KTRs at 2 weeks after transplantation (9.10% [range 4.30–25.6%] vs 5.10% [range 0.10–33.3%]; $P = 0.024$). However, no significant differences were observed between stable KTRs and KTRs with CMV infection. Analysis of the receiver operating characteristic curve adjusted by covariates showed that acute rejection could be predicted with 75.0% sensitivity and 68.4% specificity by setting the cutoff value of CD4^{+}CD25^{+}/CD4^{+} T cell frequency as 5.8%.

Circulating T-lymphocyte and monocyte subsets showed significant and consistent changes in their frequencies after immunosuppression. Of the various immune cells examined, circulating levels of CD4^{+}CD25^{+} T cells might be a useful noninvasive immunologic indicator for detecting acute rejection.

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Abbreviations: CGN = chronic glomerulonephritis, CMV = cytomegalovirus, CNI = calcineurin inhibitor, CTLA4 = cytotoxic T lymphocyte antigen 4, FOXP3 = forkhead box P3, GITR = glucocorticoid-induced TNF-receptor-related protein, HLA = human leukocyte antigen, HTN = hypertension, IL-2R = interleukin-2 receptor, KTR = kidney transplant recipients, MFI = mean fluorescence intensity, MHC = major histocompatibility complex, MIP = macrophage inflammatory protein, RANTES = regulated on activation, normal T cell expressed and secreted, ROC = receiver-operating characteristic, TCR = T cell receptor, TNF = tumor necrosis factors.

INTRODUCTION

K idney transplantation is a curative treatment for patients with end-stage renal disease (ESRD). Outcomes of kidney transplant recipients (KTRs) have improved with the introduction of more potent immunosuppressive agents. However, patient and allograft survival after kidney transplantation are greatly affected by acute rejection and opportunistic infections.[1] Optimal dosage of immunosuppressive agents must be prescribed to prevent the occurrence of these adverse events. In addition, a clinically applicable and relevant immunologic monitoring test is needed for diagnosing these events in the earliest phase.

T-lymphocytes play immunomodulatory roles in kidney transplantation. T-lymphocytes interact with antigen-presenting cells and modulate T cells through various signals. The most important signal is derived from the contact of T cell receptor–CD3 with a major histocompatibility complex (MHC)–peptide complex. However, various other signaling molecules expressed on T cells also influence the activation and differentiation of T cells into effector or regulatory cells.[2] These activated T cells participate in the initiation and maintenance of allograft rejection.[3]

Previous immunohistologic analyses have shown that many monocyte-derived chemotactic transcripts such as tumor necrosis factors (TNF)-α, macrophage inflammatory protein-1α, and regulated on activation, normal T cell expressed and secreted are amplified,[4] and human leukocyte antigen (HLA)-DR expression is increased in allograft biopsies from patients with acute rejection.[5–7] Intensity of monocyte infiltration into the allograft

and HLA-DR expression are correlated with the degree of allograft dysfunction during a rejection episode in KTRs.[8] In addition, transplant patients with decreased HLA-DR expression on monocytes are at a high risk of various infections such as cytomegalovirus (CMV) infection.[9]

Although a few studies on transplant models have reported an association of activated T cells[10–12] and monocytes[13,14] with alloimmune state, the clinical significance of these cells in the peripheral blood remains unclear. The present study evaluated the changes in T cell and monocyte subsets in various post-transplant situations, including acute rejection and CMV infection, and validated the efficacy of measuring these cells for the immunologic monitoring of KTRs.

MATERIALS AND METHODS

Study Population

This study included adult patients aged ≥20 years who underwent ABO-compatible single-organ kidney transplantation from a living or deceased donor. Patients receiving immunosuppressive therapy within the preceding month; patients with a history of malignant disorders besides nonmelanotic skin cancer within the preceding 5 years; patients who experienced a serious injury or who underwent a major surgery within the preceding month; patients with an active infection, including HIV and viral hepatitis; and patients with autoimmune or hematologic disorders were excluded from the study. All the patients provided written informed consent before enrollment. The study protocol was reviewed and approved by the institutional review board of Kyungpook National University Hospital (Daegu, Korea).

Immunosuppressive Treatment

All the patients received an induction treatment with anti-interleukin-2 receptor (IL-2R) antibody. Immunosuppressive treatment after kidney transplantation was based on standard triple therapy, including corticosteroid, calcineurin inhibitor (CNI), and mycophenolate mofetil. Corticosteroid treatment included 500 mg intravenous methylprednisolone at the time of surgery; this was tapered to 5 mg/day oral prednisolone after 6 months. Tacrolimus trough level was adjusted to 8 to 12 ng/mL in the first 3 months and to 5 to 8 ng/mL thereafter. Cyclosporine trough level was adjusted to 250 to 300 ng/mL in the first 3 months and 100 to 150 ng/mL thereafter. Mycophenolate mofetil was given daily at a fixed dose of $1.0 \sim 2.0$ g.

Study Design

This prospective, observational, and single-center study evaluated the efficacy of immunologic monitoring for diagnosing acute cellular rejection and CMV infection. Peripheral blood samples of living donor kidney transplantation were obtained 1 week before transplantation and 2 weeks after transplantation. In case of deceased donor kidney transplantation, blood samples were collected on the day of transplantation before administering immunosuppressive agents and 2 weeks after transplantation. During the follow-up period, additional blood samples were taken at diagnosis and 2 weeks after treating the clinical end points, that is, biopsy-proven acute cellular rejection and CMV infection. The diagnosis of acute cellular rejection was confirmed by allograft biopsy with at least grade 1 rejection by Banff classification.[15] Patients showing acute cellular rejection were treated using pulsed intravenous steroid therapy (0.5 g methylprednisolone daily for 3 days), which was later tapered to

a maintenance dose. CMV infection was diagnosed by directly detecting CMV proteins (pp65) in peripheral blood leukocytes or tissue biopsy. Patients with CMV infection were treated with ganciclovir. Doses of ganciclovir were reduced according to renal function. Clinical data were collected during the course of each clinical end point. Changes in T-lymphocyte subsets and HLA-DR-positive monocytes were evaluated 2 weeks after transplantation. Results of flow cytometric analysis at the time of acute cellular rejection and CMV infection were compared with those after transplantation.

Flow Cytometric Analysis of T Cell and Monocyte Subsets

$CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, and HLA-DR$^+$ T-lymphocytes and HLA-DR-positive monocytes in the peripheral blood were analyzed by performing flow cytometry. Whole blood samples were stained with fluorochrome-conjugated monoclonal antibodies in the dark for 20 minutes at room temperature. The following antibodies were used: anti-CD25-FITC (clone M-A251), anti-CD8-PE-Cy5 (clone HIT8a), anti-CD3-PE (clone UCHT1), anti-CD4-PE-Cy5 (clone RPA-T4), anti-CD14-PE (clone MφP9), and anti-HLA-DR-FITC (clone TU36) antibodies (BD Biosciences, San Jose, CA). Values were expressed as percentage and mean fluorescence intensity (MFI). After incubation, RBCs were lysed in a lysis solution (DN RBC Lysis Buffer; DiNonA Inc., Seoul, Korea). The cells were washed once with phosphate-buffered saline, resuspended, examined using FACSCalibur flow cytometer, and analyzed using CellQuest software (all from BD Biosciences).

Statistical Analysis

The sample size of the study was calculated from the previous data of $CD4^+CD25^+$ T cells in which mean $CD4^+CD25^+$ T cell (standard deviation) frequencies were 1.64% (0.84%) and 14.2% (8.8%) in the no rejection and rejection groups, respectively.[16] Six patients in each group were required to detect a difference of $CD4^+CD25^+$ T cell frequency with a power of 0.8 and type I error of 0.05 using the 2-sided Mann–Whitney U test (G∗Power program version 3.1.9.2; Franz Paul, Kiel, Germany). Assuming that the incidence of acute rejection was about 6% in our center and drop-out rate was 10%, we enrolled at least 120 KTRs for the present study.

Data were described as median (range) or frequency (percentage). Differences in T lymphocyte subsets and monocyte percentages between groups were compared using the Mann–Whitney U test for 2 groups and Kruskal–Wallis test for more than 3 groups, as all parameters were not distributed normally. Wilcoxon signed rank test was used to compare T lymphocyte subsets and monocyte percentages before and after kidney transplantation. Receiver operating characteristic (ROC) curve was used for analyzing the optimal percentage of T cell subsets for determining cutoff points that yielded the highest sensitivity and specificity to distinguish an episode of acute cellular rejection. The ROC curve analysis was then adjusted by sex, age, donor type, underlying kidney disease, type of calcineurin inhibitor, and mismatch number.[17] SPSS version 19.0 (SPSS, Chicago, IL) and SAS system for Windows, version 9.2 (SAS Institute Inc., Cary, NC) were used in performance of statistical analyses. A P value less than 0.05 was considered statistically significant.

RESULTS

The present study included 123 consecutive KTRs (76 men and 47 women; median age of 46 [range 24–67] years). The

TABLE 1. Demographic Characteristics of Kidney Transplant Recipients

Variable	Number (%) or Median Value (Range) (n = 123)
Male sex, %	76 (61.8)
Age at transplantation, y	46 (24–67)
Living donor transplantation, %	92 (74.8)
Underlying kidney disease, %	
CGN	76 (61.8)
Diabetes	27 (22.0)
HTN nephrosclerosis	8 (6.5)
Cystic disease	3 (2.4)
Others	9 (7.3)
CNI, %	
Tacrolimus	115 (93.5)
Cyclosporine	8 (6.5)
Mismatch number	
HLA	3 (0–6)
DR	1 (0–2)

CGN = chronic glomerulonephritis, CNI = calcineurin inhibitor, HLA = human leukocyte antigen, HTN = hypertension.

most common cause of ESRD among these patients was chronic glomerulonephritis (61.8%). All the patients had received their first transplant, except 1 patient who had undergone a second transplant. The median follow-up period after transplantation was 55.0 (range 3.4–93.8) months. In all, 115 (93.5%) patients received triple-therapy regimen with tacrolimus and 8 (6.5%) patients received triple-therapy regimen with cyclosporine. A summary of the demographic characteristics of the enrolled KTRs is provided in Table 1.

Baseline frequencies of T-lymphocyte subsets and HLA-DR-positive monocytes are summarized in Table 2. Comparisons of the frequencies of these cells according to recipient sex, recipient age, donor type, and cause of ESRD showed no

significant difference between the groups. Frequencies of $CD4^+CD25^+/CD4^+$ T cells, $CD8^+CD25^+/CD8^+$ T cells, and HLA-DR-positive monocytes were significantly lower at 2 weeks after transplantation than before transplantation (11.95% [range 0.50–29.40%] vs 5.10% [range 0.10–33.3%], 0.90% [range 0.00–5.80%] vs 0.60% [range 0.00–7.70%], and 99.0% [range 81.0–100%] vs 98.0% [range 87.0–100%], respectively; all $P < 0.001$). Median MFI of monocytes was also significantly decreased after transplantation (313.5 [range 70.0–666.0] vs 198.0 [range 66.0–520.0], $P < 0.001$). However, no significant change was observed in the percentages of $CD4^+DR^+/CD4^+$ and $CD8^+DR^+/CD8^+$ T cells after transplantation (Fig. 1).

We investigated the association of the clinical parameters of the KTRs with the frequencies of immune cells after kidney transplantation. We observed that recipient sex, recipient age, donor type, number of HLA mismatches, and immunosuppressive agent were not correlated with the frequencies of T-lymphocyte subsets and HLA-DR-positive monocytes (Table 3).

During the follow-up period after transplantation, 12 patients were diagnosed with acute cellular rejection, 4 patients with CNI toxicity, and 5 patients with no specific pathology by allograft biopsy. A comparison of the baseline characteristics of the patients is summarized in Table 2. No significant differences were observed between patients with acute rejection and those without acute rejection. Median creatinine level at the diagnosis of acute rejection was 3.06 (range 1.71–8.50) mg/dL. The frequency of $CD4^+CD25^+/CD4^+$ T cells was significantly higher in KTRs with acute rejection than in KTRs at 2 weeks after transplantation (9.10% [range 4.30–25.6%] vs 5.10% [range 0.10–33.3%]; $P = 0.024$). In addition, the frequency of $CD4^+CD25^+/CD4^+$ T cells was significantly higher in KTRs with acute rejection than in KTRs with CNI toxicity and without specific pathology (3.95% [range 1.80–5.60%] and 3.80% [range 1.60–5.60%]; $P = 0.045$ and $P = 0.006$, respectively). All the patients with acute rejection were treated using steroid pulse therapy, except 1 patient who was refractory to steroid treatment and was treated using anti-thymoglobulin antibody. The frequency of $CD4^+CD25^+/CD4^+$ T cells was lower at 2 weeks after steroid pulse therapy than at the diagnosis of acute

TABLE 2. Comparison of the Baseline Phenotypes of T-Lymphocyte Subsets and HLA-DR-Positive Monocytes Before Kidney Transplantation

Variable (n)	$CD4^+CD25^+/CD4^+$ T Cells (%)	$CD8^+CD25^+/CD8^+$ T Cells (%)	$CD4^+DR^+/CD4^+$ .T Cells (%)	$CD8^+DR^+/CD8^+$ T Cells (%)	DR^+ Monocyte (%)	DR^+ Monocyte.(MFI)
Sex						
Male (76)	12.1 (0.50–0.90)	0.90 (0.0–5.80)	16.8 (5.40–58.0)	40.9 (7.30–70.8)	99.0 (87.0–100)	297.5 (99.0–666.0)
Female (47)	11.8 (0.90–28.4)	0.90 (0.0–5.40)	13.7 (2.60–36.8)	36.4 (8.50–76.6)	99.0 (81.0–100)	337.0 (70.0–592.0)
Age at transplantation (yrs)						
Age <45 (59)	9.80 (0.50–25.7)	0.60 (0.0–5.4)	18.0 (7.50–31.1)	37.5 (15.5–61.5)	100 (81.0–100)	337.0 (70.0–666.0)
Age ≥45 (64)	12.6 (2.40–29.4)	1.30 (0.10–5.8)	18.6 (2.60–58.0)	48.4 (7.30–76.6)	99.0 (87.0–100)	301.0 (99.0–534.0)
Type of transplantation						
Living donor (92)	11.6 (0.90–28.4)	0.90 (0.0–5.80)	14.7 (2.60–58.0)	40.0 (7.30–76.6)	100 (87.0–100)	335.0 (99.0–666.0)
Deceased donor (31)	15.5 (0.50–29.4)	1.60 (0.0–5.40)	16.4 (7.0–36.8)	40.7 (17.6–48.7)	98.0 (81.0–100)	289.0 (70.0–556.0)
Underlying kidney disease						
CGN (76)	9.90 (0.50–29.4)	0.70 (0.0–4.80)	13.7 (2.60–36.8)	34.2 (7.30–76.6)	100 (81.0–100)	323.0 (70.0–666.0)
Diabetes (27)	13.7 (4.30–25.7)	1.45 (0.20–5.80)	17.7 (7.0–58.0)	47.1 (10.1–61.3)	99.0 (87.0–100)	294.0 (99.0–556.0)
HTN nephrosclerosis (8)	11.5 (3.00–28.4)	1.40 (0.10–2.60)	17.4 (7.50–48.3)	58.4 (15.5–70.8)	100 (99.0–100)	346.0 (321.0–460.0)
Others (12)	10.7 (7.10–16.2)	0.80 (0.30–2.00)	12.9 (10.6–34.8)	28.6 (19.7–73.6)	98.0 (97.0–99.0)	231.0 (168.0–284.0)

Data are expressed as median value (range). CGN = chronic glomerulonephritis, HTN = hypertension, MFI = mean fluorescence intensity.
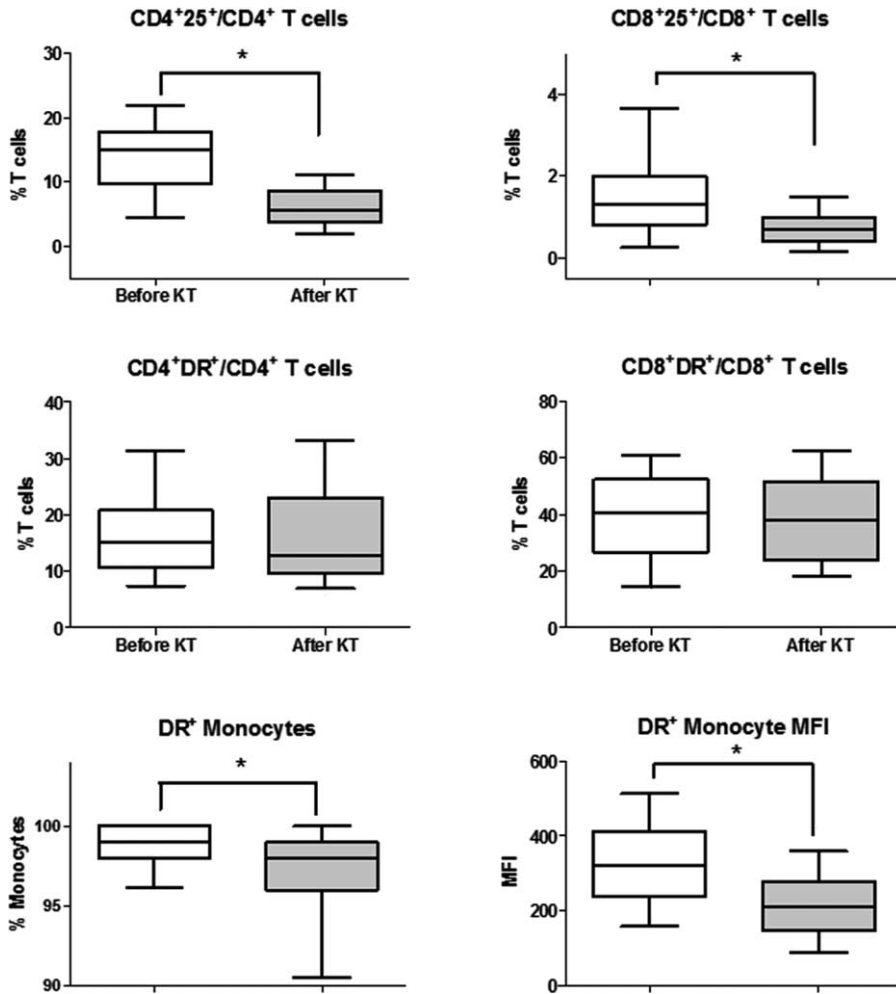
FIGURE 1. Changes in T-lymphocyte subsets and HLA-DR-positive monocytes before and after transplantation. Median, interquartile range (boxes), and range (whiskers) are shown. Frequencies of CD4$^+$CD25$^+$/CD4$^+$ T cells, CD8$^+$CD25$^+$/CD8$^+$ T cells, and HLA-DR-positive monocytes and MFI of monocytes were significantly lower at 2 weeks after transplantation than before transplantation. However, the percentages of CD4$^+$DR$^+$/CD4$^+$ and CD8$^+$DR$^+$/CD8$^+$ T cells were not significant different before and after transplantation; $^*P < 0.001$ compared with percentages before transplantation. KT = kidney transplantation.

rejection; however, the difference was not statistically significant (7.4% [range 1.80−12.2%] vs 9.10% [range 4.30−25.6%], $P = 0.320$; Table 4). There were 9 acute cellular rejections with Banff grade 1A, 1 rejection with grade 1B, and 2 rejections with grade 2A. However, the frequency of CD4$^+$CD25$^+$ T cell was not associated with the severity of acute cellular rejection (data not shown).

Analysis using the ROC curve showed that acute rejection could be predicted with 75.0% sensitivity and 43.0% specificity by setting the cutoff value of CD4$^+$CD25$^+$/CD4$^+$ T cell frequency at 4.8%. ROC analysis adjusted by confounding variables showed comparable predictability, with 75.0% sensitivity and 68.4% specificity using a cutoff value of 5.8% frequency of CD4$^+$CD25$^+$/CD4$^+$ T cells. The areas under the curves of CD4$^+$CD25$^+$/CD4$^+$ T cell frequency for predicting acute rejection were 0.704 and 0.764, respectively, demonstrating that CD4$^+$CD25$^+$/CD4$^+$ T cell frequency could be considered as a fair predictor of acute rejection (Fig. 2).[18]

Five patients developed CMV infection (as defined above) after transplantation. Of these 5 patients, 3 had CMV colitis, 1

had CMV gastritis, and 1 had CMV pneumonitis. Before transplantation, the serostatus of all recipients and donors showed positivity for anti-CMV immunoglobulin G. Intravenous ganciclovir was administered to all patients for a median of 15 (range 5−30) days. Significant differences were not observed in the frequencies of T-lymphocytes and HLA-DR-positive monocytes between KTRs at 2 weeks after transplantation and those with CMV infection. The frequency of CD4$^+$CD25$^+$/CD4$^+$ T cells also did not show a significant interval change before and after ganciclovir treatment (data not shown) Table 5.

DISCUSSION

In this prospective study, we assessed the changes of lymphocyte and monocyte subsets in the peripheral blood of KTRs. The frequencies of CD4$^+$CD25$^+$/CD4$^+$ T cells, CD8$^+$CD25$^+$/CD8$^+$ T cells, and HLA-DR-positive monocytes significantly decreased after immunosuppression. These changes were not affected by baseline demographics or other immunologic factors. The frequency of CD4$^+$CD25$^+$ T cells

TABLE 3. Association of Clinical Parameters With the Phenotypes of T-Lymphocyte Subsets and HLA-DR-Positive Monocytes After Kidney Transplantation

Variable (n)	CD4$^+$CD25$^+$/CD4$^+$ T Cells (%)	CD8$^+$CD25$^+$/CD8$^+$ T Cells (%)	CD4$^+$DR$^+$/CD4$^+$ T Cells (%)	CD8$^+$DR$^+$/CD8$^+$ T Cells (%)	DR$^+$ Monocyte (%)	DR$^+$ Monocyte (MFI)
Sex						
Male (76)	5.80 (0.60–28.3)	0.70 (0.0–3.20)	12.7 (2.10–43.2)	37.9 (7.50–73.5)	98.0 (87.0–100)	207.0 (74.4–520.0)
Female (47)	4.70 (0.10–33.3)	0.60 (0.0–7.70)	13.8 (5.60–66.7)	38.5 (17.1–79.6)	98.0 (90.0–100)	157.0 (66.0–489.0)
Age at transplantation (y)						
Age <45 (59)	5.70 (0.20–28.3)	0.55 (0.0–2.60)	11.1 (2.10–66.7)	37.3 (7.50–79.6)	99.0 (89.0–100)	198.0 (93.0–520.0)
Age ≥45 (64)	4.80 (0.10–33.3)	0.60 (0.0–7.70)	15.2 (7.0–45.8)	44.0 (12.0–63.7)	98.0 (87.0–100)	181.0 (66.0–489.0)
Type of transplantation						
Living donor (92)	5.70 (0.10–33.3)	0.70 (0.0–7.70)	12.6 (2.10–66.7)	38.2 (7.50–79.6)	98.0 (87.0–100)	207.5 (66.0–520.0)
Deceased donor (31)	3.80 (0.20–16.6)	0.50 (0.0–3.20)	15.2 (5.60–42.7)	36.8 (20.8–62.7)	98.0 (89.0–100)	158.0 (79.0–370.0)
Mismatch number						
Mismatch ≤3 (43)	5.40 (0.10–33.3)	0.60 (0.0–3.20)	12.5 (5.60–66.7)	34.9 (9.10–79.6)	98.0 (87.0–100)	187.0 (66.0–420.0)
Mismatch >3 (80)	5.0 (0.50–25.5)	0.60 (0.0–7.70)	14.8 (2.10–45.8)	43.0 (7.50–63.7)	98.0 (90.0–100)	168.0 (74.0–520.0)
CNI						
Tacrolimus (115)	5.0 (0.10–33.3)	0.60 (0.0–7.70)	12.8 (5.60–66.7)	37.8 (9.10–79.6)	98.0 (87.0–100)	194.0 (66.0–520.0)
Cyclosporine (8)	5.40 (1.80–11.2)	0.70 (0.60–1.90)	39.3 (2.10–42.7)	41.1 (7.50–61.5)	99.0 (99.0–99.0)	232.0 (188.0–276.0)

Data are expressed as median value (range). CNI = calcineurin inhibitor, MFI = mean fluorescence intensity.

increased at the diagnosis of acute cellular rejection and decreased after steroid pulse therapy. In ROC analysis, the frequency of CD4$^+$CD25$^+$/CD4$^+$ T cells showed fair predictability for the diagnosis of acute rejection, even after adjustment of covariates. These findings suggested that circulating CD4$^+$CD25$^+$ T cells were a useful noninvasive immunologic indicator of acute rejection after transplantation.
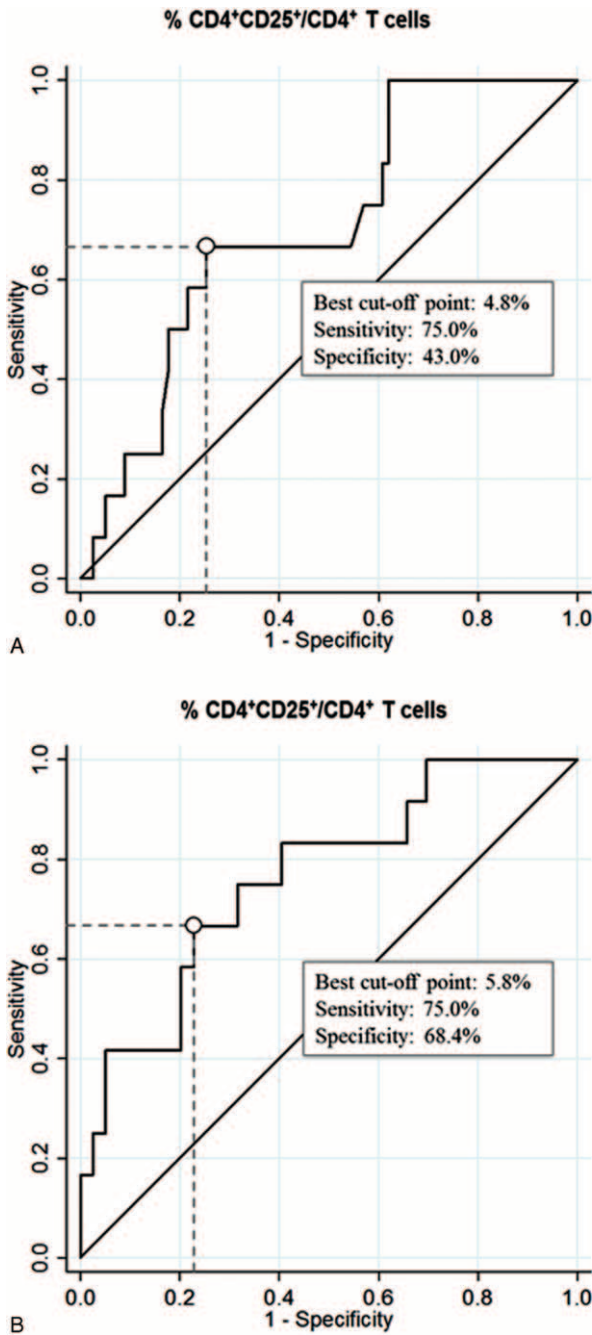
The present study revealed that the frequencies of CD8$^+$CD25$^+$/CD8$^+$ T cells as well as CD4$^+$CD25$^+$/CD4$^+$ T cells decreased after CNI-based regimen. The proportion of circulating CD4$^+$CD25$^+$ cells in patients with ESRD was comparable to that in healthy individuals.[19] After transplantation, anti-IL-2R antibody and CNIs prevent the acquisition of CD25 molecules and suppress the expansion of CD25$^+$ cells by inhibiting the generation of IL-2.[20] IL-2Rα chain (also known as CD25) is a surface marker of activated T cells, which is a surviving factor for regulatory T cells.[21,22] Therefore, patients receiving CNIs or anti-IL-2R antibody show significantly fewer CD4$^+$CD25$^+$ cells than patients receiving sirolimus.[19] The results of the present study showed that CD4$^+$CD25$^+$ cells were not affected by demographic factors (such as sex, age, and cause of ESRD) and immunologic factors (such as donor type, number of HLA mismatches, and immunosuppressive agent). These findings are in accordance with those of a recent study that reported no significant association between clinical parameters and the frequency of CD4$^+$CD25$^+$ T cells after kidney transplantation. However, the frequency of CD4$^+$CD25$^+$ T cells is higher in patients with chronic liver disease than in patients with ESRD.[23] In addition, the frequencies of circulating CD4$^+$CD25$^+$ T cells differ according to the cause of chronic liver disease. Hepatitis B and alcoholic cirrhosis are associated with a higher frequency of CD4$^+$CD25$^+$ T cells than other conditions.[24,25] Therefore, consistent frequency of CD4$^+$CD25$^+$ T cells in patients with ESRD suggested that

TABLE 4. Comparisons of Clinical Characteristics in Patients With Acute Rejection and CMV Infection

Variable	Without Acute Rejection (n = 111)	Acute Rejection (n = 12)	P	Without CMV Infection (n = 118)	CMV Infection (n = 5)	P
Sex, %			0.128			0.648
Male	66 (59.5)	10 (83.3)		72 (61.0)	4 (80.0)	
Female	45 (41.5)	2 (16.7)		46 (39.0)	1 (20.0)	
Age at transplantation, y	46 (24–67)	43.5 (33–66)	0.950	46 (24–67)	33 (28–52)	0.275
Type of transplantation, %			0.495			0.599
Living donor	84 (75.7)	8 (66.7)		89 (75.4)	3 (60.0)	
Deceased donor	27 (24.3)	4 (33.3)		29 (24.6)	2 (40.0)	
Calcineurin inhibitor, %			0.176			0.290
Tacrolimus	105 (94.6)	10 (83.3)		111 (94.1)	4 (80.0)	
Cyclosporine	6 (5.4)	2 (16.7)		7 (5.9)	1 (20.0)	
Mismatch number						
HLA	3 (0–6)	3 (0–5)	0.896	3 (0–6)	2.5 (0–6)	0.864
DR	1 (0–2)	1 (0–2)	0.326	1 (0–2)	0.5 (0–2)	0.684

Data are expressed as number (%) or median value (range). CMV = cytomegalovirus, HLA = human leukocyte antigen.

% CD4⁺CD25⁺/CD4⁺ T cells

Best cut-off point: 4.8%
Sensitivity: 75.0%
Specificity: 43.0%

A

% CD4⁺CD25⁺/CD4⁺ T cells

Best cut-off point: 5.8%
Sensitivity: 75.0%
Specificity: 68.4%

B

FIGURE 2. Analysis using the receiver-operating characteristic curve (ROC). The calculated area under the curve (AUC) was 0.704 for CD4⁺CD25⁺/CD4⁺ T cells. Acute rejection could be predicted with a sensitivity of 75.0% and a specificity of 43.0% using a cutoff value of 4.8% frequency of CD4⁺CD25⁺/CD4⁺ T cells (A). ROC analysis adjusted by confounding variables revealed the comparable predictability with a sensitivity of 75.0% and a specificity of 68.4% using a cutoff value of 5.8% frequency of CD4⁺CD25⁺/CD4⁺ T cells (AUC = 0.764) (B).

measurement of these cells was a promising monitoring tool, especially in the field of kidney transplantation.

In experimental organ transplant models, CD4⁺CD25⁺ T cells inhibit allograft rejection by modulating allogeneic

TABLE 5. Comparison of the Frequency of Circulating CD4⁺CD25⁺/CD4⁺ T Cells Among Different Groups

Group (n)	% CD4⁺CD25⁺/CD4⁺ T Cells	P*
Acute cellular rejection (12)	9.10 (4.30–25.6)	
2 weeks after transplantation (123)	5.10 (0.10–33.3)	0.024
After rejection treatment (9)	7.40 (1.80–12.2)	0.320
CNI toxicity (4)	3.95 (1.80–5.60)	0.045
No specific pathology (5)	3.80 (1.60–5.10)	0.006

Data are expressed as median value (range). CNI = calcineurin inhibitor.
* Significance compared with acute cellular rejection group.

immune response.[10] Further, transfer of CD4⁺CD25⁺ T cells from a tolerant animal to an allograft may induce donor-specific transplant tolerance in the naive animal.[11,12] However, the clinical significance of CD4⁺CD25⁺ T cells in acute rejection is controversial. In the present study, the frequency of circulating CD4⁺CD25⁺ T cells showed a significant association with acute cellular rejection. A cross-sectional study by Beik et al[16] also reported that the percentages of CD4⁺CD25⁺ and CD8⁺HLA/DR⁺ T cells were higher in patients with rejection than in patients without rejection. Because CD4⁺CD25⁺ T cells perform an immunosuppressive role, increased frequency of these cells might be a host defense mechanism to ameliorate cellular rejection and to prevent further injury. In contrast, Kim et al[23] reported an association between low frequency of CD4⁺CD25⁺ T cells and subclinical rejection diagnosed using protocol biopsy at 14 days after transplantation. This suggested that CD4⁺CD25⁺ T cells have different frequencies or perform different functions during subclinical rejection in the early post-transplantation period. Because protocol biopsy was not performed in the present study, the frequency of CD4⁺CD25⁺ T cells during subclinical rejection could not be determined. Therefore, further studies should be performed to measure the frequencies of CD4⁺CD25⁺ T cells in diverse clinical settings and post-transplantation periods to determine the optimal frequency of regulatory T cells in each condition. Activated CD4⁺CD25⁺ T cells do not always indicate regulatory or effector T cells. Several biomarkers have been introduced for identifying regulatory T cells, such as forkhead box P3 (FOXP3), cytotoxic T-lymphocyte antigen 4 (CTLA4), glucocorticoid-induced TNF receptor-related protein (GITR), and CD25.[26] Some surface markers are useful for identifying regulatory T cells in animal models.[27] However, human regulatory T cells are phenotypically heterogeneous and show activation-induced modulation of specific cell surface molecules,[28] indicating that a single surface marker cannot be used as a diagnostic tool. Although the present study evaluated only CD25 and HLA-DR as surface makers on activated cells, flow cytometric analysis showed that the immunologic monitoring of KTRs could serve as an important tool for diagnosing acute cellular rejection. Compared with conventional allograft biopsy, flow cytometry is a noninvasive, convenient, and rapid diagnostic technique that allows us to perform repeated measurements to estimate treatment response. However, whether the simple and inexpensive flow cytometry can be routinely used for monitoring rejection should be investigated in future studies involving larger number of patients. In addition, further studies with more specific surface markers for

regulatory and effector T cells are needed to characterize the increased CD4$^+$CD25$^+$ T cells in acute rejection.

Immunosuppressive therapy decreased HLA-DR expression after transplantation[29] as shown in the present study. Patients with CMV infection showed significantly decreased monocytic HLA-DR expression, which indicated a very high risk of subsequent bacterial or fungal superinfections.[9,30,31] Findings of the present study showed that the percentage and MFI of HLA-DR-positive monocytes decreased after transplantation. Our study was designed to monitor acute rejection and CMV infection simultaneously, which stand for under-and over-immunosuppressive states, respectively, with only a single immunologic test in KTRs. However, the changes in HLA-DR expression were not associated with acute rejection or CMV infection. Unlike the frequency of monocytes infiltrating allografts with acute rejection, the frequency of circulating HLA-DR-positive monocytes might not be a useful marker for detecting rejection. This might be related that the severity of CMV infection showed wide variation, as observed with the large deviation in treatment duration and the number of patients with CMV infection was relatively small, suggesting that the chance that this change was consistent was low.

To the best of our knowledge, this is the first prospective study to compare the changes in T-lymphocyte and monocyte subsets and to validate the diagnostic value of immune cell monitoring in KTRs with acute rejection. However, our study has a limitation, in that CD4$^+$CD25$^+$ T cell infiltration in renal allografts was not investigated in patients with acute cellular rejection. Infiltration of regulatory T cells such as FOXP3-positive T cells has been detected in renal allografts.[32,33] However, allografts without any clinically detectable phenotype also showed T cell infiltration.[34,35] In addition, T cell infiltration was observed in allografts with normal function for a long period after rejection therapy.[36] Therefore, T cell infiltration is not an absolute indicator of acute cellular rejection. In addition, tissue infiltration of CD25$^+$ T cells might not be a useful indicator of acute rejection because monitoring of tissue infiltration is not a clinically feasible tool compared with flow cytometric analysis.

In conclusion, circulating T-lymphocyte and monocyte subsets showed significant and consistent changes in their frequencies after immunosuppression. Of the various immune cells examined, increased frequency of CD4$^+$CD25$^+$ T cells was associated with acute cellular rejection. Furthermore, CD4$^+$CD25$^+$/CD4$^+$ T cell frequency was a fair predictor of acute rejection. Our results suggested that noninvasive immunologic monitoring of circulating CD4$^+$CD25$^+$ T cells was a useful method for diagnosing acute rejection in KTRs. However, a further study involving larger number of patients should be performed to confirm the diagnostic value of noninvasive monitoring and the potential role of evaluating treatment response to rejection therapy.

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