Monitoring of blood immunosuppressant concentrations and lymphocyte activation for predicting viral infections following kidney transplantation
A pilot study

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Abstract

The current standard pharmacokinetic monitoring of immunosuppressive therapy does not consider inter- and intra-individual differences in the biological response to multidrug immunosuppressive therapy. The authors evaluated the blood levels of the immunosuppressive drugs IL-2 and IFN-γ in circulating lymphocytes as surrogate indicators of the development of viral infections after living kidney transplantation. This single-center prospective study included 20 kidney transplant recipients who underwent living-donor transplantation at the Mie University Hospital. All the study participants received tacrolimus, mycophenolic acid, methylprednisolone, and basiliximab. The area under the concentration curves (AUCs) of blood tacrolimus and serum mycophenolic acid were measured 1 day prior to transplantation and on post-transplantation days (PTD) for up to 5 months. IL-2 and IFN-γ levels in circulating lymphocytes were measured simultaneously. One recipient experienced an acute graft rejection. Although the AUC of tacrolimus at PTD 7 was significantly higher in the virus-infected group than that in the non-infected group, the AUC of mycophenolic acid did not differ significantly between the 2 groups. The expression levels of IFN-γ$^+$ NK, IFN-γ$^+$ CD4$^+$ T, and CD8$^+$ T cells in the infected group also tended to be higher than those in the noninfected group. During the study period, there was a clear difference in the expression of IFN-γ$^+$ CD8$^+$ T cells, which increased significantly during or after infection. Circulating IFN-γ$^+$ CD8$^+$ T cell counts may serve as promising biomarkers for predicting opportunistic viral infections early after kidney transplantation.

Abbreviations: AUC = area under the concentration curve, BKV = BK virus, CMV = cytomegalovirus, PD = pharmacodynamic, PK = pharmacokinetic, PTD = pre- or post-transplantation days.

Keywords: biomarker, immunosuppressant, kidney transplantation, lymphocyte, viral infection

1. Introduction

Advances in immunosuppressive therapies using calcineurin inhibitors, mycophenolic acids, basiliximab, and corticosteroids have led to considerable improvements in kidney transplantation outcomes. However, opportunistic viral infections, including those of the BK virus (BKV) and cytomegalovirus (CMV), often occur following kidney transplantation, and prevention of these events is of particular importance in preserving graft kidney function.[1] It has been reported that BK viremia occur in 10% to 30% of kidney transplant patients, and BKV-associated nephropathy occurs in 1% to 10% of patients.[2,3] BKV nephropathy and allograft rejection are 2 serious post-transplant complications that represent the opposite ends of the immune response spectrum, namely, excessive and insufficient immunosuppression.[4]

Although dosing adjustments based on pharmacokinetic (PK) monitoring may enable the individualization of immunosuppressive therapy,[5,6] such monitoring does not consider inter- and intra-subject physiological differences in immune reactivity and responses to immunosuppressive drugs. Additionally, although PK monitoring of immunosuppressive drugs can be used to predict response to individual drugs, it does not necessarily reflect the effects of concomitant drug therapy. Compared to PK monitoring, pharmacodynamic (PD) monitoring may reflect the clinical effects of immunosuppressive drugs. Monitoring

cell-mediated immune function has been proposed as a promising approach to reduce the incidence of post-transplant infections caused by individualized immunosuppressive therapy.[7] Given that T cells serve as the primary target for most immunosuppressive drugs used for the treatment of solid organ transplantations, and that the effector function of B cells is facilitated by T cells, T cell bioreactivity has attracted considerable interest as a potential PD marker for immunosuppressive drugs. In this regard, non-donor-derived antigen-specific assays that assess the functional state of T cells using polyclonal stimulants are more likely to be applied in clinical settings. With regard to viral infections, reductions in CD4^{+} and/or CD8^{+} T cell counts and T cell function have been reported to be associated with the occurrence of opportunistic infections,[8–11] whereas BK viral infection can be accompanied by elevated levels of activated T cells, including CD8^{+} polyfunctional T cells expressing multiple cytokines, in kidney transplantation.[12,13] In our institution, the Cylex immune cell function assay (Immuknow), which is used to measure intracellular adenosine triphosphate in stimulated CD4^{+} T cells, has been shown to be a useful test for screening high-risk patients for viral infections late in the posttransplantation period (>120 days). To date, there is no established method for monitoring patients during the early (initial 50 days) post-transplantation phase.[14]

In this study, the authors evaluated the blood concentrations of immunosuppressive drugs, along with the levels of IL-2 and IFN-γ in T cells, as surrogate markers for opportunistic viral infections during the early period after living-donor kidney transplantation.

2. Methods

2.1. Patient selection and drug administration

This was a single-institute prospective study of kidney transplant recipients who underwent living-donor kidney transplantation at the Mie University Hospital. All study participants received prolonged-release tacrolimus (Graceptor Capsules; Astellas Pharma Inc.), mycophenolic acid (Cellcept Capsules; Chugai Pharmaceutical Co., Ltd.), methylprednisolone, and basiliximab (Simulect IV injection; Novartis Pharma K.K.). Rituximab (Rituxan Intravenous Infusion; Zenyaku Kogyo Co., Ltd.) was administered to recipients with donor-specific antibodies or blood-type incompatibility. CMV infection was determined by detecting neutrophils phagocytosed by the initial structural antigen (pp65) in peripheral blood using a monoclonal antibody against pp65 of CMV (C7-HRP method. BML, INC Tokyo, Japan). In the C7-HRP method, more than 1/50,000 cells are considered positive. When CMV antigenemia was confirmed, valganciclovir was initiated and immunosuppressive drugs were reduced. Antiviral prophylaxis was not administered to all recipients. Organ rejection, viral infections, bacterial infection, changes in serum creatinine levels, and changes in lymphocyte numbers were evaluated for up to 5 months after kidney transplantation.

This study was approved by the Ethics Committee of Mie University (confirmation no. 2596) and written informed consent was obtained from each participant between October 2013 and August 2018.

2.2. Blood sample collection and drug concentration measurements

The area under the concentration curve (AUC) measurements for blood tacrolimus and serum mycophenolic acid were obtained on the day prior to transplantation (PTD -1) and on post-transplantation days (PTD) 7, 14, 60–90, and 120–150. Tacrolimus blood sampling was performed immediately before administration and at 0.5, 1, 2, 3, 4, 6, and 8. At 12 and 24 hours after drug administration, mycophenolic acid analysis was performed immediately before drug administration and at 0.5, 1, 2, 3, 4, 6, 8, and 12 hours after drug administration. Blood tacrolimus and serum mycophenolic acid concentrations were measured using ARCHITECT i1000SR (Abbott Japan, Tokyo, Japan) and Dimension Xpand Plus (Siemens Healthineers) analyzers, respectively. The AUCs of these 2 drugs were calculated using the trapezoidal method. Target trough concentrations and AUC for tacrolimus at the time of kidney transplantation were predetermined in the protocol: 6–8 ng/mL and 250 ng·h/mL (0–24 hours) for Days 1–28, and 5 ng/mL and 200 ng·h/mL (0–24 hours) after Day 29, respectively.

2.3. Assessment of activated T cells

To activate lymphocytes, peripheral blood mononuclear cells isolated from patient blood samples were incubated with phorbol 12-myristate 13-acetate and calcium ionophore A23187 for 5 hours. Cell pellets were incubated for 15 minutes with PE-Cy7-labeled anti-CD3 antibodies (Beckman Coulter, Tokyo, Japan), APC-labeled anti-IL2R antibodies (Biolegend, Tokyo, Japan), and PE-labeled anti-CD4, anti-CD8, or anti-CD56 antibodies (Biolegend). The cell pellets were treated with a cell permeation reagent for 20 minutes and reacted with Pacific blue-labeled anti-IFN-γ antibodies and FITC-labeled IL-2 (BioLegend). After rinsing, NK, CD4^{+} T, and CD8^{+} T cells were fractionated from CD3 negative and CD56 positive gates, CD3 positive and CD4 positive gates, and CD3 positive and CD8 positive gates, respectively. The expression levels of IL-2R in CD4^{+} T cells and IL-2 and IFN-γ in T and NK cells were measured. Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo 7.6.5 software (BD Biosciences). Flow cytometric quantification of lymphocyte subsets. A typical flow cytometric quantification of the percentage of lymphocyte subsets is shown in Figure 1.

2.4. PK/PD analysis and statistical analysis

The blood concentrations of immunosuppressive drugs and PD changes in NK and T cells in the blood were evaluated with respect to their association with the events of organ rejection and viral infection. The non-parametric Mann–Whitney *U* test and either Fisher's exact test or chi-square test were used to assess the quantitative and categorical differences between the data from the 2 groups. Statistical analyses were performed using GraphPad Prism (version 9.3.1; GraphPad Software, CA). The reported *P* values were 2-sided and considered significant at $P < .05$.

3. Results

3.1. Enrolment of patients

Twenty transplant recipients (10 men and 10 women with a median age of 49.0 years) were enrolled in this study, among whom 8 had diabetic nephropathy, 4 had IgA nephropathy, 3 had glomerulonephritis, and 5 had other disorders. In terms of genotype, 1, 11, and 8 recipients had the *CYP3A5 *1/*1*, **1/*3*, and **3/*3* genotypes, respectively. Seven recipients received blood-type incompatible transplantation and 4 received donor-specific antibodies. In total, 7 recipients were administered rituximab. The median start date for the administration of the immunosuppressive drugs tacrolimus and mycophenolic acid was 5 days prior to transplantation. The median (minimum–maximum) follow-up days for the transplant recipient was 139.5 (120–150) days.
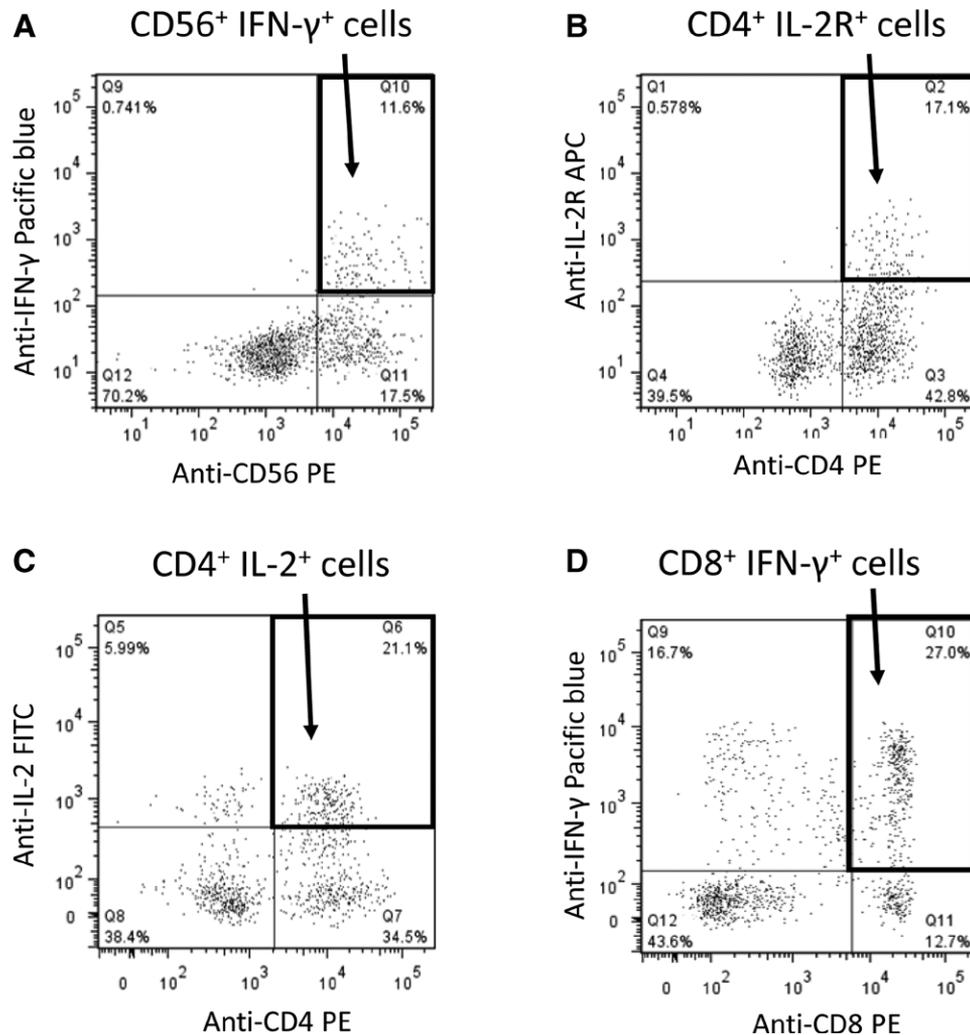
Figure 1. Flow cytometry quantification of the proportions of lymphocyte subsets.

3.2. Incidence of acute rejection

One recipient who received an ABO-incompatible transplant developed an acute allograft rejection. In this recipient, the tacrolimus AUC_{0-24} on PTD -1 (142.8 ng·h/mL) was found to be considerably lower than the target of 200 to 250 ng·h/mL, whereas mycophenolic acid AUC_{0-12} was within the target range (58.7 µg·h/mL). Inhibition of the IL-2 receptor by basiliximab, as assessed by IL-2R levels in CD4+ T cells, was successful in this recipient. In addition, the expression levels of the T cell activation markers IFN- and IL-2 in this recipient did not differ significantly from the median levels recorded in all recipients. This recipient diagnosed with acute antibody-mediated rejection on PTD 3 and additional steroid pulse, plasmapheresis, rituximab, and γ-globulin therapies were administered starting from PTD 4. After treatment, renal blood flow was gradually improved, and increase in urine volume was observed.

3.3. Incidence of viral infection and its association with immune biomarkers

The symptoms of the 5 patients who developed CMV infections were mild, and none developed interstitial pneumonia or gastroduodenal ulcer. The 5 patients who developed BKV infections also had mild symptoms, and none developed ureteral stricture or hemorrhagic cystitis. The symptoms of the

HCV-infected patients were oropharyngeal inflammation, which was treated with a dose reduction to mycophenolic acid 500 mg/d and acyclovir 250 mg ×2/d intravenous infusion for 7 days.

Total of 84 sets of blood samples were collected for the AUC measurements of tacrolimus and mycophenolic acid. Additionally, 120 blood samples were collected for the longitudinal monitoring of T and NK cells. As shown in Table 1, opportunistic viral infections developed in 11 recipients (55%), among whom 5 were infected with BKV, 5 with CMV, and 1 with herpes simplex virus. The median (minimum–maximum) days of BKV, CMV, and herpes simplex viral infection onset after kidney transplantation were 48 (22–91), 62 (35–116) and 82, respectively. When BKV was detected in blood, only mycophenolic acid was discontinued (n = 3). For 1 patient, mycophenolic acid was discontinued because the urinary BKV was >9 × 109 copies/mL by PCR. For the other patient, BKV was detected by PCR at 4 × 109 copies/mL, but was not as high, and therefore mycophenolic acid was not discontinued or reduced.

Compared with non-infected recipients, these infection-positive recipients tended to have high serum creatinine levels during the study period (Table 1). On PTD 7, the AUC of tacrolimus in the infected group was significantly higher than that in the non-infected group (248.4 vs 199.2, $P < .05$), whereas there was no significant difference in the AUC of mycophenolic acid between the 2 groups during the study period (Fig. 2). Similarly, the expression of IFN-γ+ NK cells (PTD -1), CD8+ IFN-γ+ T cells

Table 1

Comparison of the characteristics of recipients with and without viral infection.

	Infected group[†] (n = 11)	Non-infected group[†] (n = 9)	P value
Gender (M/F) (n)	6:5	4:5	1.00
Age (yr)*	54 (34–64)	44 (18–55)	0.14
*CYP3A5*3/*3* (n)	6	2	0.20
Cytomegalovirus antibody pattern			
Donor/recipient			
Positive/positive	10	7	
Positive/negative	1	0	
Negative/positive	0	1	
Negative/negative	0	1	
Viral infection onset date after transplantation (d)	62 (22–116)	-	
Rituximab administration (n)	3	4	0.65
Blood type compatibility			
Incompatible (n)	3	4	0.65
Donor-specific antibodies			
Positive (n)	2	2	1.00
Start date of immunosuppressants*	-5 (-21 – -4)	-5 (-11 – -5)	0.73
Serum creatinine levels (mg/dL)			
Day −1	10.41 (5.59–18.44)	6.66 (4.60–9.88)	<.05
Day 7	1.36 (0.73–2.94)	0.99 (0.60–4.50)	.13
Day 14	1.23 (0.67–2.37)	0.92 (0.78–1.65)	.37
Day 60	1.19 (0.66–1.86)	0.97 (0.93–1.59)	.06
Day 90	1.18 (0.70–1.97)	1.06 (0.88–1.46)	.08
Day 150	1.25 (0.67–2.00)	1.05 (0.89–1.46)	.32
Peripheral blood lymphocytes (n/µL)			
Day −1	1755 (365–4010)	1410 (1040–1870)	.45
Day 7	936 (130–2400)	1397 (490–2520)	.45
Day 14	1660 (330–3170)	1550 (950–2960)	.59
Day 60	1740 (551–2500)	1560 (1150–2090)	.68
Day 90	1320 (680–2280)	1401 (1120–2640)	.40
Day 150	1500 (249–2180)	1290 (774–2320)	1.00

*Data are expressed as mean values (minimum−maximum).

[†]Viral infections were confirmed during the initial 6 mo after transplantation.

(PTD -1, 60 and 120–150, $P < .05$; PTD 90, $P < .01$), and CD4$^+$ IFN-γ$^+$ T cells (PTD -1, $P < .01$; PTD 90, $P < .05$) in the infected group was significantly higher than that in the non-infected group (Figs. 3 and 4).

Furthermore, there was a visible difference in the expression of CD8$^+$ IFN-γ$^+$ T cells in viral infection recipients during the study period (Figure S1, Supplemental Digital Content, http://links.lww.com/MD/H932), and the expression of these cells was significantly elevated, coinciding with or following viral infection events ($P < .01$, Figure S2, Supplemental Digital Content, http://links.lww.com/MD/H933).

4. Discussion

In this study, the authors evaluated the relationship between organ rejection and viral infection in kidney transplant recipients by analyzing the immune response of circulating NK cells and effector T cells induced by polyclonal stimulants as well as the PKs of immunosuppressive drugs. Among the 11 patients who developed viral infections, the tacrolimus AUC within 3 months post-transplantation tended to be higher than that in patients who did not develop viral infections. In particular, there was a significant difference in tacrolimus AUC between the 2 groups at PTD 7. Notably, 6 of these 11 patients were found to have a *CYP3A5 3/*3* genotype, which is considered to be associated with poor metabolism of tacrolimus; thus, we speculate that this may be a factor contributing to the high blood levels of tacrolimus. The authors also established that peripheral blood lymphocyte counts tend to be lower in patients with viral infectious diseases. In this regard, the findings of a recent retrospective study indicated that a peripheral blood lymphocyte count of less than 1900 cells/mL is associated with a higher risk of BK viral infection.[8] Given that 5 of the 11 patients experienced BK

viral infection, it is plausible that a relatively high blood tacrolimus AUC during the early post-transplantation period may have contributed to a reduction in lymphocyte number, thereby rendering the recipients more susceptible to viral infection. In the relation to the onset of viral infections, the AUC of tacrolimus near the onset of viral infection was 265.9 ng-h/mL (data not shown), which was higher than the target AUC of 200 ng-h/mL after PTD 29. The median serum creatinine level near the onset of viral infection was 1.17 mg/mL (data not shown), which was higher than that of the transition in those who did not develop viral infections (Table 1). Thus, high blood tacrolimus levels are risk for viral infections, and high serum creatinine levels may be related not only to renal dysfunction caused by BKV but also to a decrease in renal function caused by tacrolimus.

Although tacrolimus has the pharmacological effect of suppressing the production of T cell-derived cytokines, such as IL-2 and IFN-γ,[15,16] no reduction in the production of IL-2 and IFN-γ in T cells or NK cells in recipients with viral infection was detected. In contrast, the authors found that the expression of IFN-γ in CD4$^+$ and CD8$^+$ T cells and NK cells tended to be higher in recipients who developed viral infections, and an increase in IFN-γ expression in CD8$^+$ T cells was detected almost simultaneously with the onset of viral infection. These observations are consistent with the findings of a recent study, which revealed that the levels of the membrane proteins CD137 and CD154, as well as TNF-α and IFN-γ, were increased in CD4$^+$ and CD8$^+$ T cells when peripheral blood mononuclear cells were co-cultured with BKV in *ex vivo* experiments and that the peak in alloreactive T cell levels was observed prior to or simultaneously with the onset of BK viral infection.[17] In addition, the results of immunostaining analyses performed in a further study revealed a significant increase in the number of CD4$^+$ and CD8$^+$ cells in BKV-infected allografts compared to stable allografts.[18]
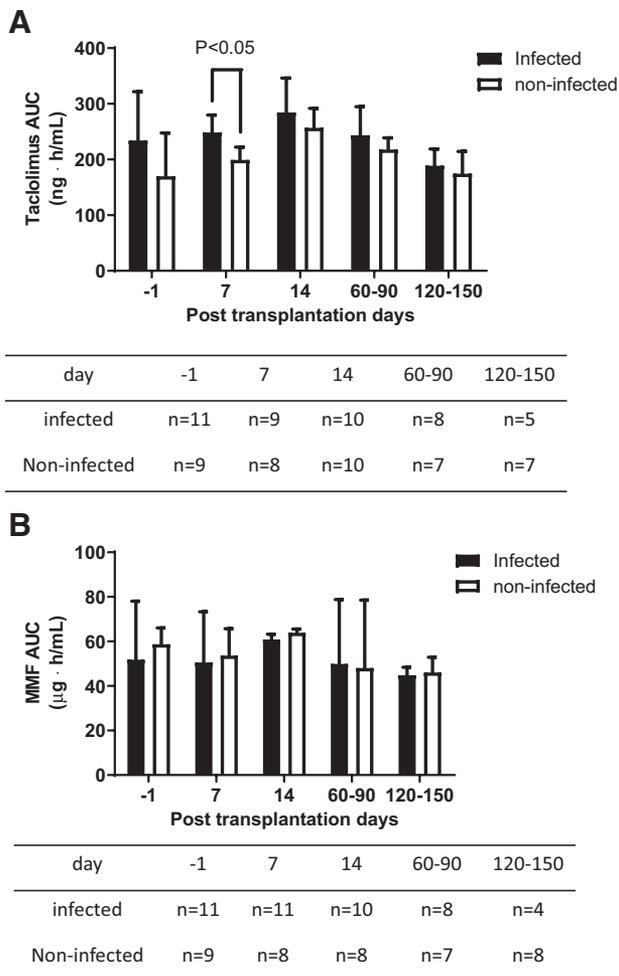
Figure 2. Trends in the AUC of tacrolimus (A) and mycophenolic acid (B) in recipients with and without viral infections. AUC = area under the concentration curve.

Figure 3. Trends in the ratio of (A) CD56^{+} IFN-γ^{+} cells in CD3^{+} cells, (B) CD8^{+} IL-2^{+} cells in CD3^{+} cells and (C) CD8^{+} IFN-γ^{+} cells in CD3^{+} cells after transplantation in recipients with and without viral infections. Statistical analysis was performed using the non-parametric Mann–Whitney U test.

In the present study, we monitored the activation of circulating lymphocytes during the initial 5 months post-transplantation and obtained results similar to those obtained in the aforementioned *ex vivo* experiment with regard to lymphocyte profiles, particularly CD8^{+} T cells, in recipients who developed viral infections. To the best of our knowledge, this is the first report on the relationship between viral infection development and increased IFN-γ expression in CD8^{+} T cells in kidney transplant recipients. Accordingly, we believe that the activation of CD8^{+} T cells in response to polyclonal stimulants following kidney transplantation may serve as a promising biomarker for early detection of viral infection in transplant recipients.

It has also been reported that BKV replication in infected kidney transplant recipients is associated with significantly lower levels of CD3^{+}, CD4^{+}, and CD8^{+} T cells and IFN-γ following transplantation, although with significantly higher alloreactive T cells.[19] The authors speculated that the activation of CD8^{+} T cells observed in the present study may have contributed to progressive immune-mediated graft injury associated with the deterioration of kidney function in transplant recipients with BKV infections. In this regard, it has been demonstrated that in addition to PK monitoring, tailoring immunosuppressive therapy based on viral-specific T cell levels can contribute to the development of safe and personalized immunosuppressive therapy by lowering exposure to immunosuppressive drugs.[20] In the study, patients in the intervention group had significantly lower trough levels of everolimus and cyclosporin A than those
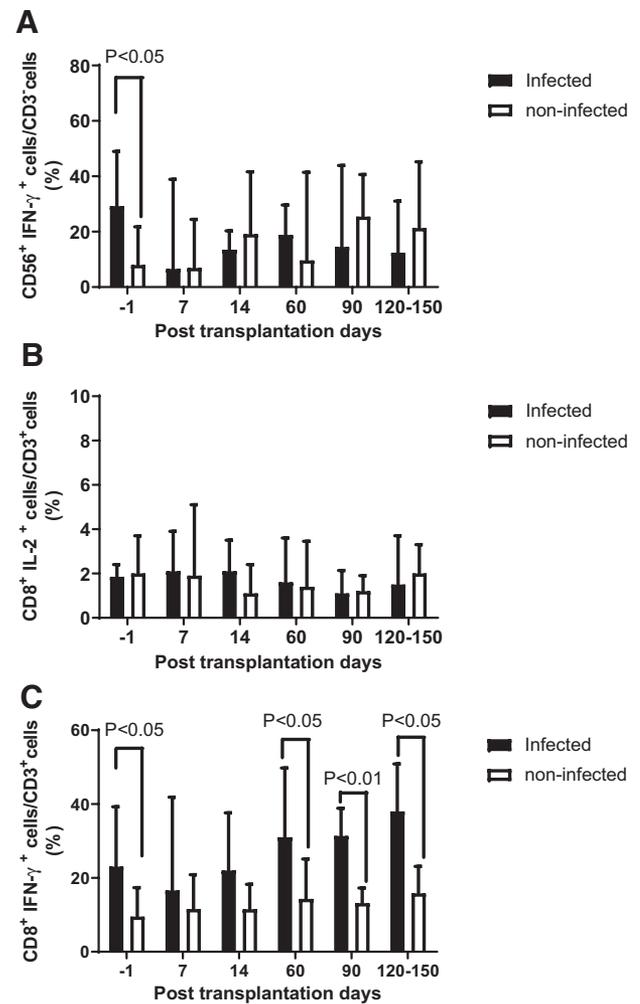
in the control group, whereas both groups experienced similar numbers of serious adverse events. Functional immune monitoring of BKV using an IFN-γ ELISPOT assay, which is primarily based on circulating CD8^{+} T cell-derived IFN-γ, has also been reported to be useful in determining treatment strategies after kidney transplantation.[21] Although the authors were unable to evaluate viral-specific T cell levels in the present study, they believe that the promotion of CD8^{+} T cell activation using polyclonal stimulants would contribute to determining the onset of viral infection following kidney transplantation. In this study, there were 4 recipients with higher than 40% in IFN-γ^{+} CD8^{+} T cell expression at the timing of viral infections; 3 (49.1%, 65.7%, 75.2%) had CMV infections and 1 (58.6%) had BKV infections. Although a matter of speculation, it is possible that CD8^{+} T-cell reactivity to polyclonal stimulation is more likely to be elevated in CMV-infected patients.

With regard to CD4^{+} T cells, a significantly higher median percentage of IFN-γ^{+} CD4^{+} T cells stimulated with polyomavirus simian viral 40 large T antigen was observed 3 months after an adjustment for immunosuppression than at the time of BKV infection diagnosis.[22] However, it has been reported that the exhaustion status of BKV-specific CD4^{+} T cells, as indicated by the co-expression of PD1 and TIM3 in cells, is strongly associated with a prolonged clearance time of BKV reactivation.[23] This phenomenon was not observed in BKV-specific
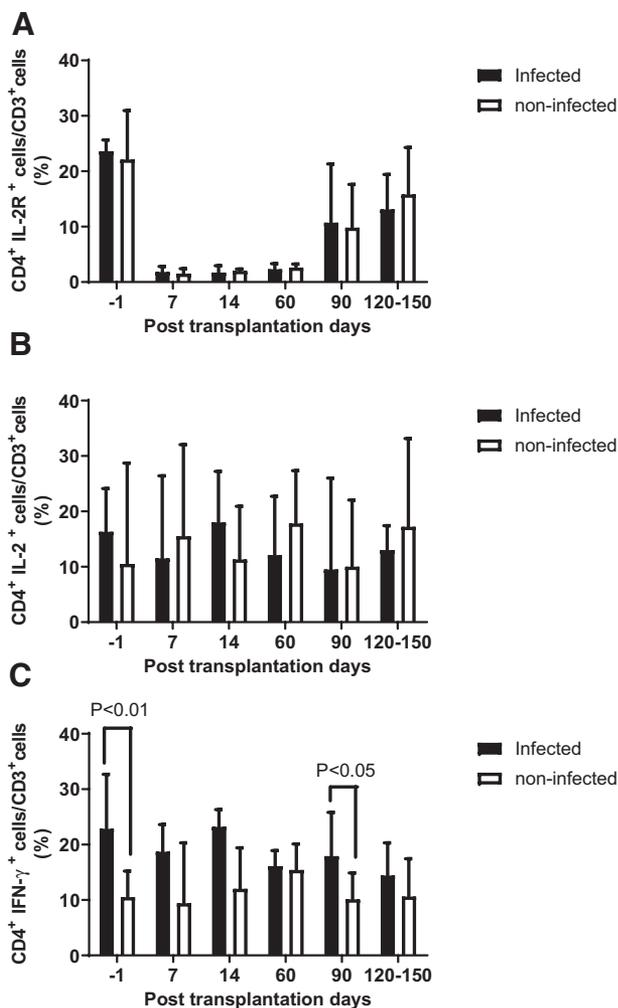
Figure 4. Trends in the ratios of (A) CD4+ IL-2R+ cells in CD3+ cells, (B) CD4+ IL-2+ cells in CD3+ cells, and (C) CD4+ IFN-γ+ cells in CD3+ cells after transplantation in the recipients with and without viral infections. Statistical analysis was performed using the non-parametric Mann–Whitney U test.

CD8+ T-cells. The kinetics of circulating IFN-γ+ CD4+ T cells in response to viral infection has been established to be dependent on the balance between the elevation and exhaustion of activated cells. Consequently, it is plausible that IFN-γ+ CD4+ T cells were not significantly elevated, coinciding with or subsequent to the onset of viral infection, as observed for IFN-γ+ CD8+ T cells.

The recipients who did not receive rituximab were 8 in the viral-infected group and 5 in the non-infected group, and the results of the reanalysis are shown in Figure S3, Supplemental Digital Content, http://links.lww.com/MD/H934. The expression of IFN-γ +NK cells (PTD -1, $P < .01$), CD8+ IFN-γ+ T cells (PTD 90, $P < .05$), and CD4+ IFN-γ+ T cells (PTD -1, $P < .05$) in the infected group was significantly higher than those in the non-infected group. The expression of IFN-γ+ levels in NK and CD4+ T cells just before kidney transplantation will be a good biomarker for the prediction of viral infection. This analysis also supports the fact that increased IFN-γ+ expression in CD8+ T cells in response to polyclonal stimulants may reflect opportunistic viral infections after kidney transplantation.

Only one of the 20 recipients assessed in this study experienced acute rejection, which was confirmed on PTD 7. This patient had received a blood group-incompatible transplant and was consequently at a heightened risk of acute rejection. Moreover, the area under the curve of tacrolimus on the day before transplantation did not reach the target concentration. It

is well established that the *CYP3A5* genotype is associated with variability in blood tacrolimus concentrations, and the authors have also reported correlations between genetic polymorphisms and blood tacrolimus levels in individuals who have undergone kidney, liver, or hematopoietic stem cell transplantation.[24–26] The patient who experienced acute rejection was found to have the *CYP3A5* 1/*3 genotype, which is not generally associated with markedly elevated blood tacrolimus levels. However, there were no appreciable changes in the AUC of mycophenolic acid, inhibition of the IL-2 receptor by basiliximab in CD4+ T cells, or the expression levels of IL-2 and IFN-γ in effector T cells. Consequently, it can be speculated that low blood tacrolimus levels during the early post-transplantation phase may have been one of the factors contributing to acute rejection.

This study has certain limitations. Notably, given the small number of recipients enrolled, the findings of this pilot study are limited. Furthermore, the authors focused on the early phase (initial 5 months) following kidney transplantation, which tended to limit the observational period for viral infection events. Since most of the viral infected patients in this study had mild symptoms, the association between severity of illness and biomarkers is a topic for further study. However, we believe that this study has particular value with respect to our assessment of blood levels of immunosuppressive drugs, immune cell reactivity of peripheral blood over time, and the associated development of viral infections.

In conclusion, this pilot study demonstrated a relationship between peripheral blood immune cell reactivity and viral infection following kidney transplantation. Patients with viral infections tended to have a higher AUC for tacrolimus and lower peripheral blood total lymphocyte count. Additionally, increased IFN-γ expression in CD8+ T cells in response to polyclonal stimulants may reflect opportunistic viral infections after kidney transplantation. We believe that these findings will be of considerable interest to healthcare providers involved in the safe management of immunosuppressive therapy in organ transplant recipients.

Acknowledgments

Author contributions

Both authors contributed substantially to the conception and design of the study and the acquisition, analysis, or interpretation of the data associated with the study. Both authors have approved their submission to the journal.
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Methodology: Takuya Iwamoto.
Supervision: Kohei Nishikawa.
Writing – original draft: Takuya Iwamoto.
Writing – review & editing: Takuya Iwamoto, Kohei Nishikawa.

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