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Validation Study of an Operational Tolerance Signature in Korean Kidney Transplant Recipients

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ABSTRACT

Operational tolerance (OT), defined as maintaining stable graft function without immunosuppression after transplant surgery, is an ideal goal for kidney transplant recipients (KTRs). Recent investigations have demonstrated the distinctive features of B cells, T cells, and dendritic cell-related gene signatures and the distributions of circulating lymphocytes in these patients; nonetheless, substantial heterogeneities exist across studies. This study was conducted to determine whether previously reported candidate gene biomarkers and the profiles of lymphocyte subsets of OT could be applied in Korean KTRs. Peripheral blood samples were collected from 153 patients, including 7 operationally tolerant patients. Quantitative real-time PCR and flow cytometry were performed to evaluate gene expression and lymphocyte subsets, respectively. Patients with OT showed significantly higher levels of B cell-related gene signatures (*IGKV1D-13* and *IGKV4-1*), while T cell-related genes (*TOAG-1*) and dendritic cell-related genes (*BNC2*, *KLF6*, and *CYP1B1*) were not differentially expressed across groups. Lymphocyte subset analyses also revealed a higher proportion of immature B cells in this group. In contrast, the distributions of CD4+ T cells, CD8+ T cells, mature B cells, and memory B cells showed no differences across diagnostic groups. An OT signature, generated by the integration of *IGKV1D-13*, IGKV4-1, and immature B cells, effectively discriminated patients with OT from those in other diagnostic groups. Finally, the OT signature was observed among 5.6% of patients who had stable graft function for more than 10 years while on immunosuppression. In conclusion, we validated an association of B cells and their related signature with OT in Korean KTRs.

Keywords: Operational tolerance; Kidney transplantation; Biomarkers; mRNA; B-lymphocytes

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Abbreviations
AR, acute rejection; AUC, area under the curve; CR, chronic rejection; eGFR, estimated glomerular filtration rate; IOT, Indices of Tolerance; ITN, Immune Tolerance Network; KT, kidney transplantation; KTR, kidney transplant recipient; LTGS, long term graft survival; NP, normal pathology; OT, operational tolerance; IRB, institutional review board; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative real-time PCR; ROC, receiver operating characteristic

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INTRODUCTION

Kidney transplantation (KT) is the treatment of choice for patients with end-stage renal disease. Graft survival has been increasing gradually, and the 1 year graft survival rate is now 96% (1). Most patients require life-long immunosuppressive medications to avoid renal allograft rejection, which could dramatically reduce graft survival and quality of life. However, these drugs frequently cause adverse effects, such as life-threatening infection, metabolic complications, malignancy, and paradoxical allograft dysfunction induced by long-standing use of calcineurin inhibitors (2). Therefore, minimizing or, if possible, stopping immunosuppressive drugs is an ideal goal for all kidney transplant recipients (KTRs), but this is difficult to accomplish due to the substantial risks of rejection in clinical practice.

Operational tolerance (OT) in KT, commonly defined as maintaining stable graft function without any immunosuppressive drugs for more than 1 year, was first reported in 1975 (3). Epidemiologic data showed that KTRs rarely achieve this goal in the real world, with an estimated incidence of 0.03% (4), which is lower than the rate for liver transplant recipients, approximately 20% of whom taper off immunosuppression successfully (5,6). Moreover, patients' non-compliance or medical conditions, rather than an exhaustive withdrawal plan, account for the majority of reasons for the cessation of immunosuppressive drugs (7,8). Indeed, a previous study found that a gene expression profile of OT was detected in 3.5% of a cohort of stable KTRs taking a low-dose immune suppressant (9). Therefore, the development of tolerance markers is needed for clinicians to provide objective evidence for reducing immunosuppression and to identify candidates for OT among KTRs.

Due to rapid advances in high-throughput technologies, transcriptomic approaches in peripheral blood have been performed to discover novel biomarkers of OT during the last decade (7-16). Two seminal studies, the Immune Tolerance Network (ITN) and Indices of Tolerance (IOT), both of which included patients from large nationwide cohort groups, showed that renal allograft tolerance was associated with distinctive features of B cells and their related genes (7,8). However, other studies showed inconsistent findings, revealing that T cell- and dendritic cell-related genes were implicated in the development of OT (12,14,16). Similarly, analyses of circulating immune cells showed varying results, especially within B cell subsets (7,8,13,16-20). One important concern regarding the discovery of biomarkers is that the expression of mRNA and cell phenotypes could be changed by non-pathologic circumstances, such as genetic variations (21). We previously reported that rejection-specific genes were differentially expressed between KTRs in the United States and those in Korea (22). Therefore, it is reasonable to test the applicability of potential biomarkers before their clinical use, especially in patients with different genetic backgrounds. This study was conducted to validate known candidate biomarkers of OT in Korean KTRs and to evaluate the distributions of lymphocyte subsets in the peripheral blood.

MATERIALS AND METHODS

Study design and patient selection
We recruited 153 patients from the Assessment of Immunological Risk and Tolerance in Kidney Transplantation study, which was a cross-sectional sample collection study for Korean KTRs at 6 different hospitals (Kyung Hee University Hospital at Gangdong, Kyung Hee Medical Center, Kyungpook National University Hospital, Samsung Medical Center, Seoul St. Mary's Hospital of the Catholic University of Korea, and Inje University Busan Paik

Hospital) from August 2012 to June 2016. Patients were classified into 5 different diagnostic groups according to their clinical status and pathologic findings: OT was defined as patients maintaining stable kidney function (serum creatinine levels less than 1.2 mg/dl) without any immunosuppressive drugs for more than 1 year; long-term graft survival (LTGS) was defined as patients maintaining stable kidney function while on maintenance immunosuppression more than 10 years after KT; normal pathology (NP) was defined as patients maintaining stable kidney function with histologically confirmed absence of graft injury such as rejection, acute tubular necrosis, calcineurin inhibitor toxicity, BK virus-associated nephropathy, or glomerulonephritis; acute rejection (AR) was defined as patients exhibiting impaired graft function (30% or greater increase in their baseline creatinine level) with pathologic evidence of either acute T cell-mediated rejection or acute antibody-mediated rejection; and chronic rejection (CR) was defined as patients exhibiting impaired graft function and pathologic evidence of chronic active antibody-mediated rejection according to the Banff classification (23).

Information regarding age, sex, immunologic status, medications, total white blood cell count, and serum creatinine levels was obtained from each patient at the time of their visit to the outpatient clinic for patients with OT or LTGS or at the time of graft biopsy for those with NP, AR, or CR. Kidney function was assessed via the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration formula (24). The Institutional Review Coard (IRB) of each hospital approved this study (IRB No. 2012-01-030 for Kyung Hee Neo Medical Center, KC13TNMI0701 for Seoul St. Mary's Hospital), and informed consent was obtained from all patients.

Quantitative real-time PCR (qRT-PCR) of target genes

Peripheral blood was collected in 2.5 ml PAXgene Blood RNA Tubes (PreAnalytiX; Qiagen, Hilden, Germany) for the mRNA analysis. Total RNA was extracted using a PAXgene Blood RNA Kit (PreAnalytiX) according to the manufacturer's instructions and was measured for quantity and purity using a NanoDrop® ND-2000 UV spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed with 500 ng of total RNA using an M-MLV Reverse Transcriptase system (200 U/µl; Mbiotech, Inc., Seoul, Korea), and cDNA was stored at −80°C until use. TaqMan gene expression assays (StepOnePlus™; Applied Biosystems, Foster City, CA, USA) were performed under the standard TaqMan protocol (10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C) in a 96-well microplate. We used primers and probes purchased commercially from Integrated DNA Technologies (Coralville, IA, USA) for PCR assays. As an endogenous control, 18S ribosomal RNA and the commercially available Universal Human Reference RNA (Agilent Technologies, Santa Clara, CA, USA), to reduce the batch effect, were used to calculate gene expression levels using the comparative C_T method. The results were then $\log_{10}$-transformed to reduce the deviation from the normalized data in the qRT-PCR system prior to statistical analysis.

Flow cytometric analyses of lymphocytes and their subsets

Peripheral blood mononuclear cells (PBMCs) (2×10^5 cells/well) were prepared from heparinized blood by Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA, USA) density-gradient centrifugation. In brief, a cell suspension of 1×10^6 cells/ml was prepared in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. For the flow cytometric analyses, PBMCs were surface-stained with different combinations of the following monoclonal antibodies: CD4-PE/Cy7 (RPA-T4, IgG1; BioLegend, Sang Diego, CA, USA), CD8-APC (SK1, IgG1, κ; Pharmingen, San Diego, CA, USA), CD38-PerCP cy5.5 (HIT2, IgG1,κ; PharMingen), CD19–FITC (SJ25-C1, IgG1; SouthernBiotech, Birmingham, AL, USA), and

CD24–PE (ML5, IgG2a,κ; PharMingen). The appropriate isotype controls were used for gating purposes. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

All statistical analyses were performed with SPSS for Windows, version 20.0 (SPSS, Chicago, IL, USA). Baseline clinical data are expressed as the mean±standard deviation or as the numbers of patients and percentages. ANOVA, χ^2 tests and linear-by-linear association analyses were used to compare baseline characteristics and laboratory findings, as appropriate. Comparisons of the levels of mRNA and the percentages of lymphocyte subsets were performed by ANOVA with Scheffé *post hoc* analysis. Receiver operating characteristic (ROC) curves and corresponding area under the curve (AUC) values were generated to assess the predictive power of the OT signature to distinguish operationally tolerant patients from other diagnostic groups. Finally, the probability scores of OT were calculated using a binary regression procedure. The p-values less than 0.05 were considered statistically significant.

RESULTS

Baseline demographics and clinical parameters of enrolled patients

Table 1 summarizes the baseline clinical characteristics of the enrolled patients according to the diagnostic groups. Patients with OT and LTGS were older, had a longer duration after KT, and received kidneys less frequently from deceased donors than those with NP, AR, or CR. The human leukocyte antigen mismatching number was significantly lower in these groups, in accordance with previous studies (7,8). No patients in OT underwent ABO-incompatible KT. The mean eGFR was lower in patients with AR and CR (80.7±7.8 vs. 77.5±14.7 vs. 77.2±20.6 vs. 35.8±18.9 vs. 34.5±18.1 ml/min/1.73 m², OT vs. LTGS vs. NP vs. AR vs. CR; p<0.001).

Table 1. Baseline demographics and clinical parameters of enrolled patients

Characteristics	OT (n=7)	LTGS (n=38)	NP (n=30)	AR (n=63)	CR (n=15)	p-value
Age (yr)	56.1±10.6	57.7±9.4[‖,¶]	46.3±11.7[‖]	47.0±9.9[¶]	51.8±9.6	<0.001
Sex (male)	6 (85.7)	15 (39.5)	19 (63.3)	43 (68.3)	8 (53.3)	0.192
Duration after KT (mon)	598.9±634.0[*,†,‡,§]	198.1±79.3[*,‖,¶]	7.2±13.7[†,‖]	22.8±37.5[‡,¶]	91.7±59.9[§]	<0.001
Deceased donor KT	1 (14.3)	6 (15.8)	11 (36.7)	27 (42.9)	7 (46.7)	0.037
ABO incompatible KT	0 (0)	0 (0)	4 (13.3)	15 (23.8)	1 (6.7)	0.007
HLA mismatching	1.0±1.7	2.4±1.2[¶]	3.6±1.8	3.7±2.1	3.1±1.5	0.004
eGFR (mL/min/1.73m²)	69.2±27.6[‡,§]	77.5±14.7[‖,**]	77.2±20.6[††,‡‡]	35.8±18.9[‡,¶,††]	34.5±18.1[§,**,‡‡]	<0.001
WBC (×10³ cells/mm³)	5.7±1.5	6.1±3.8	7.8±3.9	6.1±3.8	7.2±2.3	0.118
Indication for biopsy	N/A	N/A				N/A
Protocol biopsy			27 (90.0)	9 (14.3)	0 (0)	
Elevated creatinine levels			3 (10.0)	50 (79.4)	12 (80.0)	
Proteinuria			0 (0)	4 (6.3)	3 (20.0)	
Induction immunosuppression						0.820
Basiliximab	5 (71.4)	28 (75.7)	24 (80.0)	44 (69.8)	12 (80.0)	
Anti-thymocyte globulin	2 (28.6)	9 (24.3)	6 (20.0)	19 (30.2)	3 (20.0)	
Maintenance immunosuppression						
Steroid	0 (0)	17 (44.7)	28 (93.3)	54 (85.7)	13 (86.7)	<0.001
Tacrolimus	0 (0)	14 (36.8)	30 (100)	40 (64.5)	8 (53.3)	<0.001
Cyclosporine	0 (0)	21 (55.3)	0 (0)	16 (25.8)	4 (26.7)	<0.001
Mycophenolate mofetil	0 (0)	19 (50.0)	30 (100)	44 (69.8)	10 (66.7)	<0.001
mTOR inhibitor	0 (0)	2 (5.4)	1 (3.3)	6 (9.7)	3 (20.0)	0.560

Values are presented as number (%).
HLA, human leukocyte antigen; WBC, white blood cell; mTOR, mammalian target of rapamycin; N/A, not applicable.
[*]p<0.05, OT vs. LTGS; [†]p<0.05, OT vs. NP; [‡]p<0.05, OT vs. AR; [§]p<0.05, OT vs. CR; [‖]p<0.05, LTGS vs. NP; [¶]p<0.05, LTGS vs. AR; [**]p<0.05, LTGS vs. CR; [††]p<0.05, NP vs. AR; [‡‡]p<0.05, NP vs. CR.

The prescription patterns of induction of immunosuppression did not differ among the 5 groups. With respect to maintenance immunosuppression, a significantly lower proportion of patients with LTGS were taking immunosuppressive drugs, including steroids, tacrolimus, and mycophenolate mofetil, whereas cyclosporine was more frequently prescribed in this group. The prescription rate of mTOR inhibitors was not different among the groups. As defined, operationally tolerant patients were not taking any medications.

Expression of candidate genes for OT in peripheral blood according to renal allograft status

We selected the representative genes of each immune cell known to be associated with OT based on a review of the literature: *IGKV1D-13*, *IGKV4-1*, and *IGLL1* for the B cell signature (7); *BNC2*, *CYP1B1*, and *KLF6* for the dendritic cell signature (16); and *TOAG-1* for the T cell signature (12,14). *IGLL1* was excluded from the analysis due to inadequate amplification in most samples; hence, the levels of the remaining 6 candidate mRNAs were compared across the diagnostic groups (**Fig. 1**). Patients with OT showed significantly higher levels of *IGKV1D-13* and IGKV4-1 than those with NP and AR (**Fig. 1A and B**). However, no difference was found between OT and LTGS patients. The levels of *IGKV4-1*, but not *IGKV1D-13*, were elevated in patients with OT compared with those in patients with CR. The expression of *BNC2* was slightly higher in operationally tolerant patients than in other groups, but the difference was not statistically significant (**Fig. 1C**). Finally, no significant differences were observed in the levels of the remaining 3 genes, namely, *TOAG-1*, *KLF6*, and *CYP1B1* (**Fig. 1D-F**, respectively). Taken together, our data demonstrated that *IGKV1D-13* and *IGKV4-1*, representatives of B cell-associated genes, were highly expressed in operationally tolerant Korean KTRs, whereas dendritic cell-related and T cell-related genes were not highly expressed.
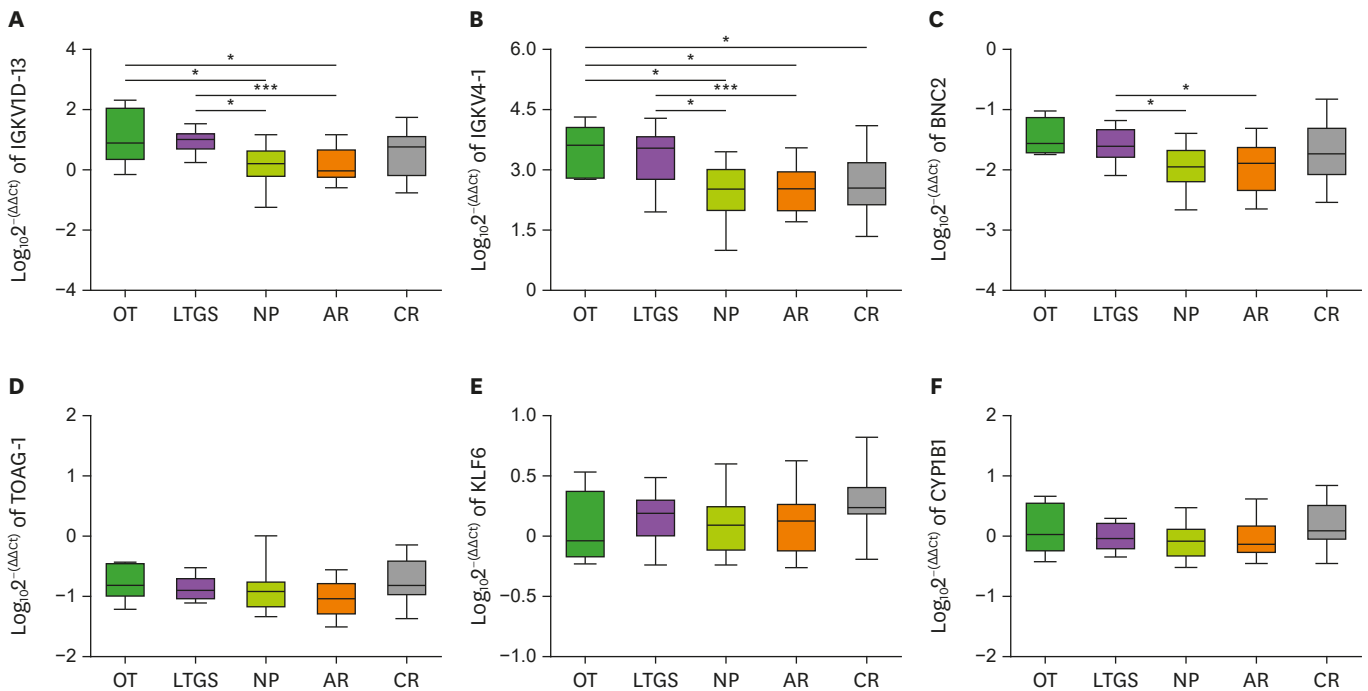

Figure 1. The levels of candidate mRNAs of operational tolerance in peripheral blood according to renal allograft status. (A) *IGKV1D-13*, (B) IGKV4-1, (C) *BNC2*, (D) *TOAG-1*, (E) *KLF6*, and (F) *CYP1B1*.
*p<0.05; ***p<0.001.

Peripheral blood lymphocyte subsets according to renal allograft status

Next, flow cytometric analyses of PBMCs were performed to determine the distributions of lymphocytes, especially B cell subsets (**Figs. 2A** and **3A**). The total white blood cell count was not different among the groups (**Table 1**), whereas the total lymphocyte count was higher in operationally tolerant patients (**Fig. 2B**). The percentages of total B cells, CD4+ T cells, and CD8+ T cells were not different among the diagnostic groups (**Fig. 2C-E**). Further subset analyses of B cells using various surface antigen markers revealed that patients with OT showed marked elevation in the percentages of immature B cells, which was defined as high expression of both CD24 and CD38 at the cell surface (**Fig. 3B**). Notably, this difference was also obvious in a comparison between operationally tolerant patients and those with LTGS, which was not observed in the gene analysis. The distributions of mature B cells and memory B cells showed no differences across diagnostic groups (**Fig. 3C and D**).
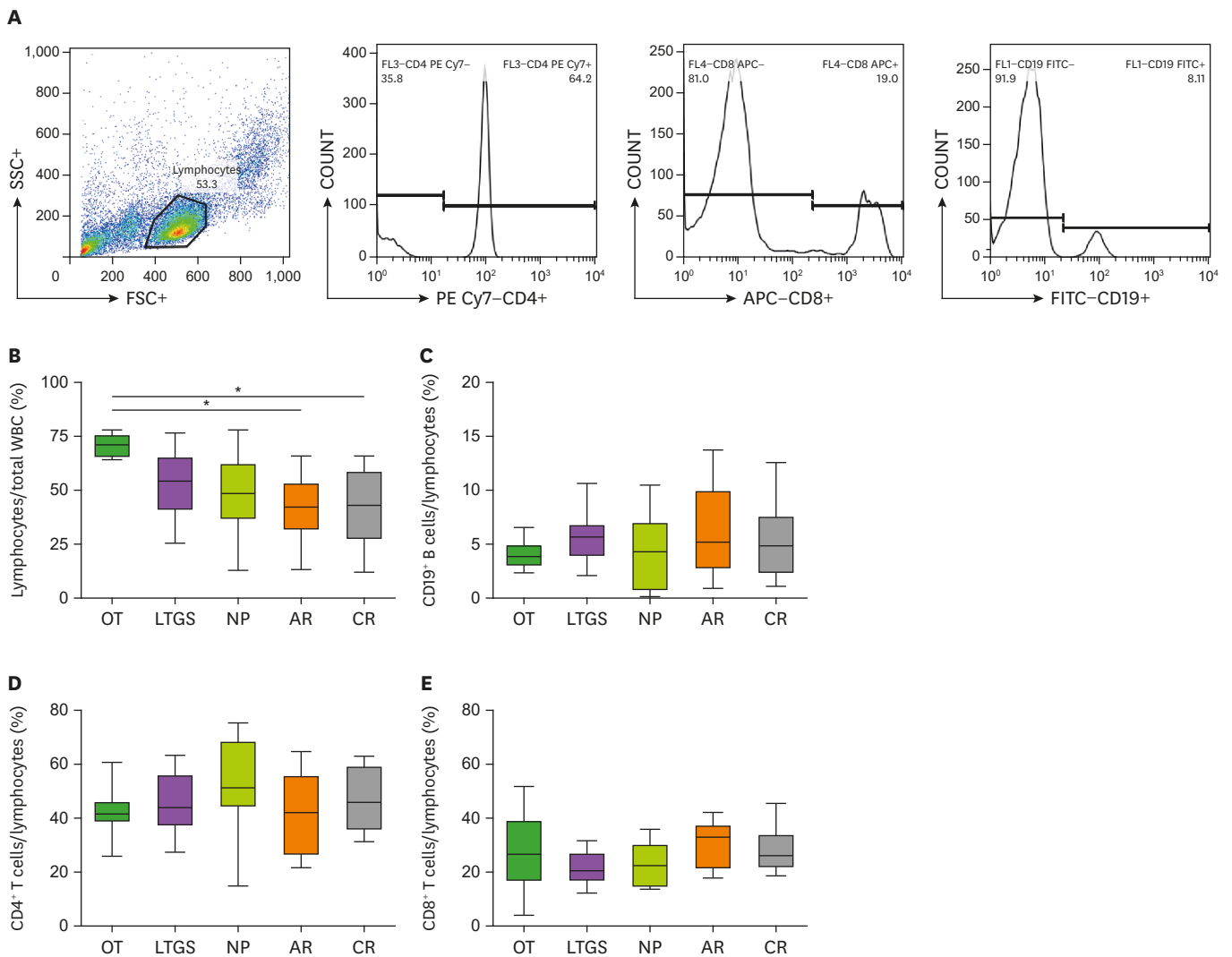

Figure 2. The distribution of peripheral blood lymphocyte subsets in KTRs. (A) Representative flow cytometric analysis of CD4+ T, CD8+ T, and CD19+ B cells. (B-E) Percentages of total lymphocytes, CD19+ B cells, CD4+ T cells, and CD8+ T cells, respectively.
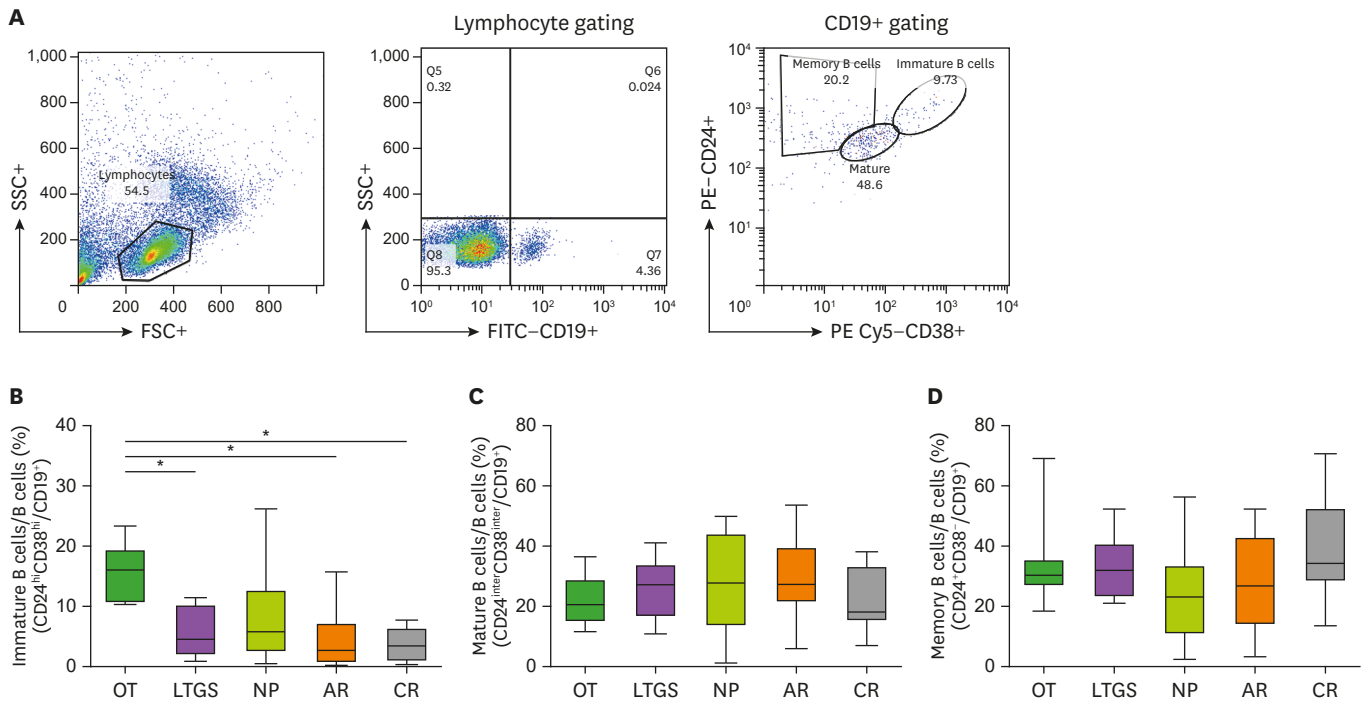*p<0.05.

Figure 3. The distribution of B cell subsets in KTRs. (A) Representative flow cytometric analysis of B cell subsets. (B-D) Percentages of immature, mature, and memory B cells, respectively.
*p<0.05.

ROC curve analyses to distinguish OT from other diagnostic groups

ROC curve analyses were performed to evaluate the diagnostic power of the OT signature. A single gene, either *IGKV1D-13* alone or *IGKV4-1* alone, was ineffective in the differential diagnosis of OT (AUC values of 0.696 and 0.704, respectively; **Supplementary Fig. 1A and B**). The gene signature, composed of *IGKV1D-13* and *IGKV4-1*, showed a fair discriminative power in distinguishing operationally tolerant patients from other groups (AUC value of 0.785, p=0.011; **Fig. 4A**). However, the capacity of this signature to discriminate between OT and LTGS was poor, with an AUC value of 0.632 (**Supplementary Fig. 1E-H**). Immature B cells, in contrast, revealed good discriminative power in distinguishing patients with OT from others (AUC value of 0.825; **Supplementary Fig. 1C**). However, the distinction between OT and NP was difficult based on the percentage of immature B cells alone (AUC value of 0.679; **Supplementary Fig. 1D**). To overcome this limitation, a cross-platform OT signature was generated by integrating gene data and lymphocyte subsets; the signature, created by the combination of the *IGKV1D-13* and *IGKV4-1* levels and the percentage of immature B cells, improved the discriminative power in diagnosing OT (AUC value of 0.891, p=0.001; **Fig. 4B**). The capacity to differentiate OT from LTGS was also improved significantly by applying this cross-platform OT signature, with an AUC value of 0.857 (**Supplementary Fig. 1I-L**).

OT signature profiles among KTRs with long transplant vintage

Finally, the probabilities of KTRs having tolerance profiles were evaluated based on the scores of a cross-platform OT signature (**Table 2**). Patients with long transplant vintage (OT, LTGS, and CR) were selected and analyzed to reduce the bias resulting from the difference in the duration after KT. We found that the OT signature was observed in 5.6% (2/36) of patients with LTGS, similar to a previous study (9). A tolerance signature was detected in one renal allograft recipient among the CR group (1/12); the patient was a 52-year-old female with
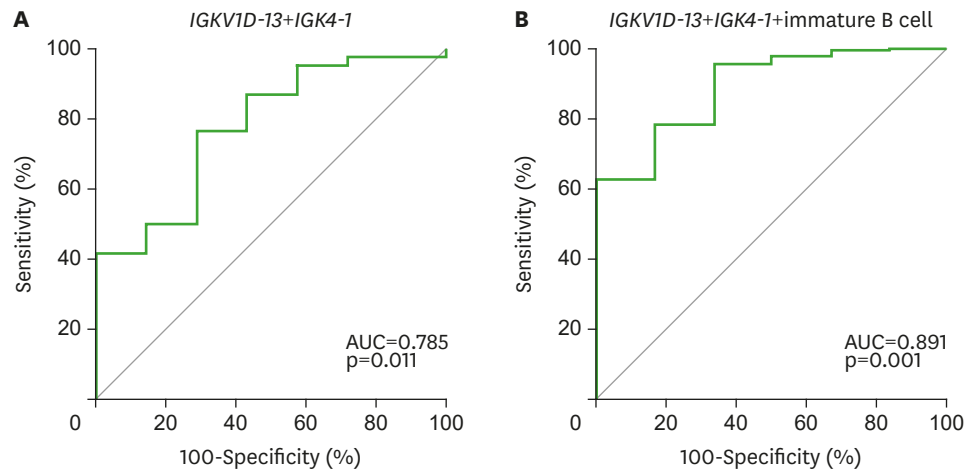
Figure 4. ROC curves for the evaluation of the discriminative power of an OT signature, generated by the combination of (A) the levels of *IGKV1D-13* and *IGKV4-1* and (B) the levels of *IGKV1D-13* and *IGKV4-1* and the percentages of immature B cells.

Table 2. OT signature profiles among KTRs with long post-transplant time

Characteristics	OT signature* (+)	OT signature (−)
OT (n=7)	7 (100)	0 (0)
LTGS (n=36)	2 (5.6)	34 (94.4)
CR (n=12)	1 (8.3)	11 (91.7)

Values are presented as number (%).
*OT signatures were created by the combination of the *IGKV1D-13* and *IGKV4-1* levels and the percentage of immature B cells.

typical pathologic features of severe chronic active antibody-mediated rejection. Intriguingly, disproportionate to the severity of the pathologic findings, this patient had been maintaining stable renal function (eGFR >50 ml/min/1.73 m^2) for 5 years since the diagnosis of CR.

DISCUSSION

The present study examined whether the gene signature and circulating immune cell phenotypes of OT could be validated in Korean KTRs. Despite the need for monitoring techniques for immune quiescence, the development of reliable biomarkers of OT has been hampered by the small number of patients. This issue has been overcome, at least in part, by the organization of large research consortia such as the ITN, IOT, Reprogramming the Immune System for the Establishment of Tolerance, Genetic Analysis and Monitoring of Biomarkers of Immunological Tolerance, and the ONE Study [25]. Nonetheless, molecular analyses have revealed substantial heterogeneity across studies. This inconsistency might result from several confounding factors, such as differences in ethnicity, genetic background, study design, past medical history, or sample collection method [26-30]. Furthermore, there are obstacles to the generalization of molecular analysis results, especially to KTRs whose ethnicities and genetic backgrounds differ from those of the subjects studied. Our findings indicate that upregulated B cell-related gene signatures and an immature B cell population are the main features of operationally tolerant patients in Korea, reinforcing the roles of B cells in the development of OT after KT.

The main results of our study were the increased *IGKV1D-13* and *IGKV4-1* levels in patients with OT among KTRs. However, the differences were significant only when these patients were

compared with those with NP and AR, and a gene signature consisting of *IGKV1D-13* and *IGKV4-1* was not sufficient to discriminate between patients with OT and LTGS. These observations are inconsistent with a previous investigation performed by Newell et al. (7); they reported that the expression levels of both *IGKV1D-13* and *IGKV4-1* were significantly higher in operationally tolerant patients than in those with stable graft function while on immunosuppression, which is identical to the definition of LTGS in our study. One possible explanation for the discrepancies includes the fact that the patients with OT in our study showed slightly worse baseline kidney function than those with LTGS (mean serum creatinine levels of 1.0 vs. 0.9 mg/dl, OT vs. LTGS), whereas the results were inverse in the abovementioned study (mean serum creatinine levels of 1.0 vs. 1.4 mg/dl, OT vs. stable graft function while on immunosuppression). Moreover, it was impossible to compare the prescription patterns of immunosuppressive drugs in patients with LTGS, which might affect the gene expression related to immune cells. A recently published study demonstrated that the expression levels of *IGKV1D-13* and *IGKV4-1* were increased by tacrolimus and decreased by steroids, mycophenolate mofetil, or thymoglobulin treatment (31), suggesting the importance of the effects of drugs in the interpretation of transcriptomic data. We could not exclude the possibility that the molecular and phenotypic features of patients with OT simply originated from the lack of immunosuppressive agents, rather than specific immunological characteristics of tolerance. To address this question, additional investigations should be performed to obtain data that can provide us with the sequential changes of mRNA expression and lymphocyte subsets before and after the adjustment of immunosuppressive medication.

Since the introduction of *IGKV1D-13* and *IGKV4-1* as biomarkers of OT, further studies have evaluated the clinical roles of these genes in KTRs (31,32). Both genes are known to be expressed during the differentiation of B cells, especially phases between pre- and mature B cells, indicating possible links to the elevated proportion of immature B cells in our study. Moreso et al. (32) reported that the expression of these genes was increased in a time-dependent manner in cyclosporine-treated KTRs as opposed to azathioprine-treated KTRs. The usefulness of this tolerance signature was also suggested in another prospective cohort study that demonstrated that KTRs with this signature exhibited a gradual improvement in eGFR for more than 6 years after KT (31). Taken together, elevated expression levels of *IGKV1D-13* and *IGKV4-1* could serve as surrogate biomarkers for OT and could be useful predictors of better long-term graft outcomes in KTRs. Whether this signature could be used as an indicator for adjusting the dose of immunosuppressive drugs should be elucidated in further studies.

Another important finding of this study was the increased number of immature B cells in the OT group compared with those in other clinical groups; this finding was consistent with previous studies (7,8). Previously, B cells with a phenotype of CD19+CD24hiCD38hi expression were identified as immature transitional B cells in humans (33). Of note, this cell type is known to have regulatory capacity; it significantly inhibited the differentiation of proinflammatory cytokine-expressing CD4+ T cells in a dose- and contact-dependent manner (34). Therefore, a significant decrease in the percentage of immature B cells after transplantation might suggest defective immune regulation and could result in allograft rejection (35).

Additionally, in contrast to the results of gene transcript markers, the proportion of immature B cells was different between the OT group and other clinically stable groups (LTGS or NP). Previously, we found that the microenvironment, such as uremic toxins or

immune suppressants, can induce a decrease in the proportion of immature B cells (36,37). Therefore, it is possible that the increase in immature B cells in the OT group compared with the numbers in the LTGS or NP groups may be secondary to the superior allograft function or immunosuppression-free state, suggesting that even though some patients with LTGS may have tolerance at the gene transcript level, uremic toxins or calcineurin inhibitors can induce the dysregulation of immature B cells in those patients. Therefore, it is possible that patients with LTGS having transcriptional tolerance signatures might also show an increase in the proportion of immature B cells when they are weaned off immunosuppressants. Further investigation, including prospective monitoring of immature B cells with gradual reduction of immunosuppressants, may be required to clarify this issue.

Several limitations should be mentioned regarding our study. First, we selected candidate genes of OT based on previous literature without performing omics-based biomarker discovery using techniques such as microarray analysis or next-generation sequencing. As a result, many genes associated with OT may have been missed in our cohort. Furthermore, we could not obtain lymphocyte subset data in a small portion of the enrolled patients. Nonetheless, we speculate that the overall trend of B cell subsets was less likely to be changed because the absolute number of missed samples was relatively small (5.2% [2/38] in the LTGS group and 20% [3/15] in the CR group). Finally, although the OT signature profiles among KTRs with long transplant vintage (**Table 2**) display interesting results, the numbers of patients are small, and this is essentially a description of sensitivity determination.

In conclusion, this study validated the association of the B cell signature with OT in Korean KTRs. Our results suggest that monitoring B cell transcript markers and immature B cells could be a useful biomarker for the detection of OT irrespective of ethnic background. We expect that prospective trials will confirm whether the B cell signature can provide guidelines regarding evidence-based weaning of immunosuppression after KT without an increased risk of allograft rejection.

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SUPPLEMENTARY MATERIAL

Supplementary Figure 1

ROC curves to determine the diagnostic power of *IGKV1D-13*, *IGKV4-1*, and the percentage of immature B cells. (A-C) ROC curves generated by a single parameter to discriminate operationally tolerant patients from other groups; (A) *IGKV1D-13*, (B) *IGKV4-1*, and (C) the percentage of immature B cells. (D) ROC curve generated by the percentage of immature B cells to distinguish patients with OT from those with NP. (E-L) ROC curves generated by (E-H) the combination of the levels of *IGKV1D-13* and *IGKV4-1*, and (I-L) the combination of *IGKV1D-13, IGKV4-1*, and the percentages of immature B cells.

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