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RESEARCH ARTICLE

THE ROLES OF IMMUNE MOLECULES IN THE ACUTE POST-TRANSPLANT PERIOD

Aslı Özkızılcık Koçyiğit[1,2], Melek Pehlivan[2,3], Tülay K. Ayna[1,2], Mustafa Soyöz[1,2], Erhan Tatar[4], Mehmet Tanrısev[5], İsmail Sert[6], Zeki Soypaçacı[7], Cem Tuğmen[8], İbrahim Pirim[1,2]

[1]Izmir Katip Celebi University, Faculty of Medicine, Department of Medical Biology, Izmir, Turkey
[2]Izmir Katip Celebi University, Cell, Tissue, Organ Transplantation Research and Application Center, Izmir, Turkey
[3]Izmir Katip Celebi University, Vocational School of Health Services, Medical Laboratory Techniques, İzmir, Turkey
[4]The University of Health Ministry, Bozyaka Education and Research Hospital, Nephrology, Izmir, Turkey
[5]The University of Health Ministry, Tepecik Education and Research Hospital, Nephrology, Izmir, Turkey
[6]The University of Health Ministry, Tepecik Education and Research Hospital, General Surgery, Izmir, Turkey
[7]Izmir Katip Celebi University, Atatürk Education and Research Hospital, Nephrology, Izmir, Turkey
[8]Bakırcay University, Faculty of Medicine, Department of General Surgery, Izmir, Turkey

ORCID: A.O.K. 0000-0002-0015-7070, M.P. 0000-0001-8755-4812, T.K.A. 0000-0001-7993-978X, M.S. 0000-0001-5159-6463, E.T. 0000-0002-5068-4231, M.T. 0000-0002-2886-1166, I.S. 0000-0001-5190-9124, Z.S. 0000-0003-3019-0178, C.T. 0000-0002-2668-5197, İ.P. 0000-0001-8485-3286

Corresponding author: Aslı Özkızılcık Koçyiğit, **E-mail:** asli.ozkizilcik.kocyigit@ikc.edu.tr
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ABSTRACT

Purpose: Renal transplantation is a therapeutic choice that enhances the quality of life for patients suffering from end-stage renal failure. The objective of this study was to ascertain the alterations in the levels of immune molecules following transplantation and to examine the correlation between these changes and the medical records of the patients.

Materials and Methods: The gene expression of an immune molecule panel (FOXP3, TNF-α, IFN-γ, IL-18, IL-6, IL-17a, IL-12a, IL-2, IL-10, and TGF-β) in peripheral blood specimens of 30 kidney transplant patients was determined by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) method with SYBR Green Dye. The serum proteins were quantified using Enzyme-Linked ImmunoSorbent Assay (ELISA).

Results: TGF-β exhibited the most significant alteration in gene expression levels compared to the levels before transplanting ($p<0.05$). A strong association was seen between the change in IFN-γ levels and the estimated glomerular filtration rate (eGFR) values of the patients ($p<0.05$).

Conclusion: The cytokine expression alterations may provide information on patients' clinical condition. Individualized immune scanning after transplantations may contribute to personalized treatment of each patient. The communication between the laboratory and the clinics is important for the accurate consultation of the patients.

Keywords: kidney transplantation, real-time pcr, cytokine, gene expression

INTRODUCTION

Organ transplantation is a vital treatment that saves the lives of people who are suffering from organ failure at an advanced stage, yet its success is often jeopardized by the host immune response, which can lead to graft rejection. The balance between pro-inflammatory and regulatory pathways is pivotal in determining whether the transplanted organ is accepted or rejected. Understanding these immunological changes is critical for improving post-transplant outcomes (1,2).

Cytokines are key mediators in the immune response, and their levels post-transplantation can significantly influence graft survival. Tregs (regulatory T cells) require the transcription factor Forkhead box P3 (FOXP3) for both their growth and operation, which help maintain immune tolerance to the graft. Elevated levels of FOXP3-expressing Tregs are often associated with reduced incidence of graft rejection and improved transplant outcomes (3).

In contrast, Tumor necrosis factor-alpha (TNF-α) and Interpheron-gamma (IFN-γ) are pro-inflammatory cytokines that play a role in the development and propagation of immune responses that might result in graft rejection. TNF-α is known for its role in promoting inflammation and apoptosis, while IFN-γ enhances the immune response by activating macrophages and increasing antigen presentation, thereby exacerbating graft rejection (4,5). Recent studies have highlighted the complex role of Interleukin 17a (IL-17a) in transplant immunology, where it can contribute to both graft rejection and tolerance depending on the context (6).

Additional cytokines like IL-6, IL-18, and IL-12a play a role in the pro-inflammatory response, contributing to the activation and differentiation of T helper cells, which are critical in orchestrating the immune response (7,8). On the other hand, IL-10 and Transforming growth factor-beta (TGF-β) are central to immune regulation, with IL-10 acting as an anti-inflammatory cytokine and TGF-β playing a dual role in promoting both immune tolerance and, paradoxically, fibrosis in the graft (9,10).

Despite advances in immunosuppressive therapies, the challenge of graft rejection persists, highlighting the need for a deeper understanding of the immune dynamics post-transplantation. This study aims to investigate the alterations in the levels of FOXP3, IFN-γ, TNF-α, IL-6, IL-18, IL-17a, IL-12a, IL-2, IL-10, and TGF-β in patients following transplantation. Additionally, it seeks to correlate these changes with the clinical outcomes documented in patients' medical records. By integrating cytokine profiles with clinical data, this research aims to identify potential biomarkers that could predict transplant outcomes and guide personalized therapeutic strategies.

MATERIALS AND METHODS

Sampling

Blood samples of 30 patients were collected from three different hospitals at certain intervals (before transplantation, 1 month and 3 months after transplantation). The ready platform was used to calculate the patients' estimated glomerular filtration rate (eGFR) values at the time of blood sampling using the brief Modification of Diet in Renal Disease (MDRD) formula (1).

Signed informed consents were obtained from all patients. In compliance with the Declaration of Helsinki, our Institutional Non-Interventional Clinical Research Ethics Committee approved the study (Date: 22.02.2019, Decision No: 02).

RNA Isolation and cDNA Synthesis

RNAs were isolated by GeneJet Whole Blood RNA Purification Mini Kit (Thermo Scientific, Vilnius, Lithuania). Prior to isolation, the pellets were produced using 1X concentrated ACK (ammonium-chloride-potassium) buffer for the red blood cell lysis. The purification method was performed according to the manufacturer’s instructions. The quantity and purity of the RNA samples were measured on a Nanodrop (Thermo Scientific, USA) instrument. The absorbance ratios (A260/A280) between 1.9 and 2.1 were accepted as pure and the samples with 5 ng/µl and above concentration were included in the study. Complementary DNA (cDNA) was synthesized from RNA samples by RevertAid First Strand Synthesis Kit (Thermo Scientific, Lithuania). The procedure was performed according to the manufacturer’s instructions. The cDNA samples were stored at -20 °C until their usage.

Quantitative Real-time PCR (qRT-PCR)

IDT Integrated DNA Systems (https://www.idtdna.com/pages) and NCBI primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) online platforms were used to design the primers for the target gene amplification.

Table 1. The primer sequences used for gene expression analysis

Name	Sequence	Length (bp)
β-Aktin [2]	F: 5'-CTTCCTGGGCATGGAGTCCTG-3'	21
	R: 5'-GGAGCAATGATCTTGATCTTC-3	21
IL-2	F: 5'-CTCACCAGGATGCTCACATTTA-3'	22
	R: 5'-CCTCCAGAGGTTTGAGTTCTTC-3'	22
IL-6	F: 5'-GGAGACTTGCCTGGTGAAA-3'	19
	R: 5'-CTGGCTTGTTCCTCACTACTC-	21
IL-10	F: 5'-CCTTGCTGGAGGACTTTAAG-3'	20
	R: 5'-TCTTGGTTCTCAGCTTGGGG-3'	20
IL-12A	F: 5'-GATGTACCAGGTGGAGTTCAAG-3'	22
	R: 5'-GCCTGCATCAGCTCATCAATA-3'	21
IL-17A	F: 5'-CTCATTGGTGTCACTGCTACT-3'	21
	R: 5'-GGGAAGTTCTTGTCCTCAGAAT-3'	22
IL-18	F: 5'-GAAGAGGAAAGGAACCTCAGAC-3'	22
	R: 5'-GGTTCAGCAGCCATCTTTATTC-3'	22
IFN-γ	F: 5'-CTGCCAGGACCCATATGTAAA-3'	21
	R: 5'-GTCACTCTCCTCTTTCCAATTCT-3'	23
TNF-α	F: 5'-CCAGGGACCTCTCTCTAATCA-3'	21
	R: 5'-TCAGCTTGAGGGTTTGCTAC-3'	20
TGF-β	F: 5'-TTGATGTCACCGGAGTTGTG-3'	20
	R: 5'-TCCACTTGCAGTGTGTTATCC-3'	21
FOXP3	F: 5'-CAAGTTCCACAACATGCGAC-3'	20
	R: 5'-ATTGAGTGTCCGCTGCTTCT-3'	20

F: Forward, R: Reverse, IL: interleukin, IFN-γ: Interferon-gamma, TNF-α: tumor necrosis factor-alpha, TGF-β: Tumor growth factor-beta, FOXP3: Forkhead box P3, bp: base pair

Table 2. The demographic features of the patients

Patients		n (%)
Patient average age		46.1
Donor average age		49
Patients Gender	Female	15 (%53.6)
	Male	13 (%46.4)
Donor Gender	Female	11 (%39.3)
	Male	17 (%60.7)
Patient Blood group	A Rh+	15 (%53.6)
	A Rh-	1 (%3.5)
	B Rh+	4 (%14.3)
	0 Rh+	4 (%14.3)
	0 Rh-	4 (%14.3)
Donor Blood group	A Rh+	15 (%53.6)
	A Rh-	1 (%3.5)
	B Rh+	4 (%14.3)
	0 Rh+	4 (%14.3)
	0 Rh-	4 (%14.3)

The primers for the reference β-actin gene were obtained from a study (2). The sequences of the primers were given in Table 1. The qRT-PCR analysis was conducted with the SYBR Green dye (Ampliqon, Odense, Denmark) on the Thermo Scientific PikoReal Real-Time PCR System (Thermo Scientific, Vantaa, Finland). The qRT-PCR protocol was followed according to the manufacturer's instructions. The results were evaluated according to ΔΔCt method.

Enzyme Linked Immunosorbent Assay (ELISA)

The samples with most significant changes in gene expression of IFN-γ, IL-17A, IL-12A, IL18, IL-10, and TGF-β were analyzed for their protein levels in the patients' sera samples. The BT-LAB ELISA kits (Zhejiang, China) were used to perform ELISA tests. The procedure was conducted according to the manufacturer's instructions. The optical density of each well was determined at 450 nm by Thermo Scientific Multiskan go (Thermo Fisher Scientific, Vantaa, Finland).

Table 3. The immunological complications of the patients at the post-operative first and third months

Patient No	Complication	
	1st Month	3rd Month
P3	Urinary infection, DSA+	NC
P4	Urinary infection	NC
P5	TIN	TIN
P6	NC	Incisional hernia
P9	NC	Urinary infection
P12	Urinary infection	NC
P15	NC	Fever, leukopenia
P25	NC	Urinary infection
P28	Acute TIN	TIN

DSA: Donor Specific Antibody, NC: No Complication, ACR: Acute Cellular Rejection, TIN: Tubulointerstitial Nephritis

Statistical Analyses

Demographic and clinical average values were calculated by Microsoft Office Excel 2016 program. The statistical evaluations of the gene expression results were conducted with IBM SPSS Statistics 25 Software Program using paired t test. p values <0.05 were accepted as significant. HeatMap and scatter plot and bar graphs were created by GraphPad Prism 9.3.1 Software Program.

RESULTS

Demographic and clinical features of the patients

In total, 28 patients were evaluated because a patient had a primary non-functional kidney after transplantation and the other had biopsy-proven cellular rejection during the first month. The allografts were removed by nephrectomy in these patients. Because P28 rejected the allograft 15 months after transplantation, the patient was evaluated as without rejection on the post-transplant first and third months. The allograft was removed from P10 by nephrectomy, who lost renal function and returned to hemodialysis due to transplant renal arteria stenosis (TRAS) three months after transplantation. These patients were transplanted from a deceased donor (Figure 1). The demographic information of the patients was given in Table 2. Post-transplant maintenance treatment included Tacrolimus or Cyclosporine, Mycophenolate Mofetil or Azathioprine, and steroids.

Two patients (6.6%) had no alloimmunization (pregnancy, blood transfusion, and previous transplantation status). Prior to transplantation, all patients tested negative for crossmatch and PRA. Some of the patients had complications in the post-operative 1st and 3rd months (Table 3).

The relationship between gene expression profiles and clinical outcomes in patients

The ΔΔCt method was used to calculate the results. Fold change of <1 and >1 was interpreted as a decrease and an increase, respectively.

The post-transplant first and third month mean ΔCt values did not differ significantly from the pre-transplant values ($p>0.05$) (Figure 2a). We discovered a significant difference between pre- and post-transplant expression levels of patients individually. The greatest number of significant differences were observed in P9 when compared to pre-transplantation (in 11 immune molecules, 8.4%). While seven (5.3%) of these results were obtained in the first month, four (3%) were obtained in the third month. In the 15th and 18th patients, there was no statistically significant change in gene expression of the immune molecules ($p>0.05$).

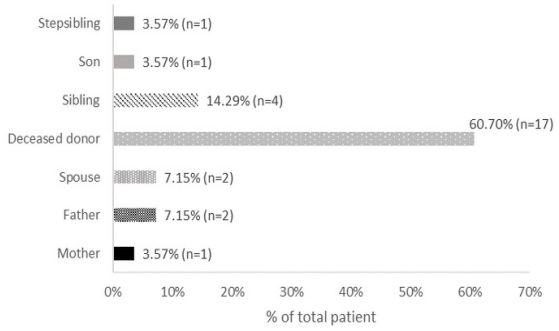

Figure 1. The relationship between the recipients and donors.

TGF-β gene expression levels had the highest number of statistical significance (p<0.05) including 65.5% (n=19) of the patients. Subsequently, the gene expression levels of IFN-γ (n=15, 51.7%) and IL-10 (n=13, 44.8%) had the greatest number of significant differences (p<0.05). IL-2 (n=3, 10.3%) was found to have the least number of statistically significant gene expressions (Figure 2b). The percentage of statistically significant (p<0.05) results was higher in the first month (n=75, 25.9%) than in the third month (n=56, 19.3%).

The relationship between cytokine serum levels, gene expression levels, and the clinical picture of the patients

The serum levels of IL-10, IL12A, IL-17A, IL-18, TGF-β, and IFN-γ were determined by ELISA method. The correlation between serum levels and gene expression levels was positive but not statistically significant (p<0.05). There was a significant difference in IL-10 serum levels between the pre-transplant and first post-transplant month (p=0.025, t=3.148). They were also strongly and positively correlated (r=0.839, p=0.005). When the patients were evaluated individually, however, there were significant differences (Figure 2c).

DISCUSSION

IL-2 is a well-known pro-inflammatory cytokine released from T cells. Kutukculer et al. found a significant increase in post-transplant IL-2 levels compared to pre-transplant by ELISA method in their sera samples (3). However, there was no significant difference in IL-2 gene expression levels like the other investigations (4,5). IL-6 is a pro-inflammatory cytokine involved in the activation of cellular and humoral immune responses. It prevents the development of regulatory T (Treg) cells, whereas it induces acute phase reactions, B-cell maturation and differentiation, and the differentiation of cytotoxic T cell. Omrani et al. compared the serum IL-6 levels of the kidney-transplanted patients with a control group, and found association with allograft rejection (7). In contrast, Waiser et al. reported that IL-6 levels were not an indicator of the rejection (8). Nevertheless, treatment approaches are being considered to prevent the interaction of the cytokine IL-6 and its receptor, IL-6R, to prevent kidney damage following kidney transplantation (6). We determined

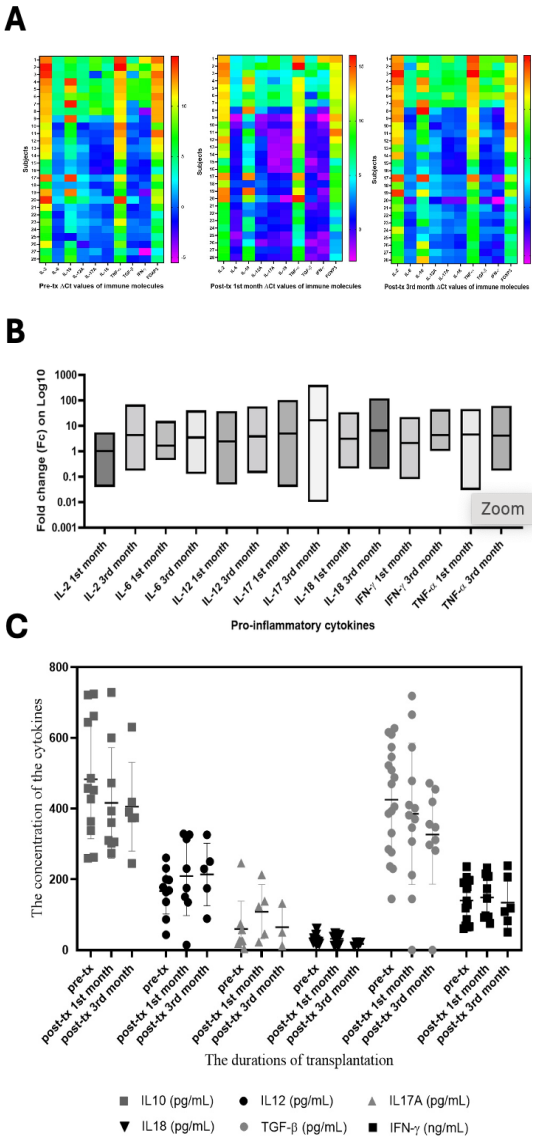

Figure 2. The alterations in gene and protein expression levels A) The alterations in ΔCt values at first and third months after transplantation compared to pre-transplantation were analyzed with HeatMap analysis. The values were normalized to β-actin. The fold changes (Fc) were calculated according to ΔΔCt method using these ΔCt values. Colors turning to red and purple at first and third months indicate the increase and decrease in expression, respectively. B) The fold changes of the immune molecules on the 1st and 3rd month compared to their pre-transplantation expression. Each line in the boxes indicates the mean values. The value above 10 indicates an increase and the value below 10 indicates a decrease. C) The pre-transplant, post-transplant 1st month and 3rd month serum levels of the patients are shown on dot plot graph. Each symbol indicates an individual. IL-10, IL-18, and TGF-β decreased, whereas IL-12 increased compared to pre-transplant values. IL-17A serum level increased in the first month but decreased in the third month after transplantation. The serum levels of IFN-γ were similar to pre-transplant values.

significant changes in gene expression and serum levels compared to pre-transplantation levels. IL-12 is another pro-inflammatory cytokine that activates NK and cytotoxic T cells. Benett et al. reported that IL-12 serum levels of pre- and post-transplant samples were similar in kidney transplanted patients (9). We observed significant alterations in gene and serum levels but did not determine a correlation with the clinics. The most common member of the IL-17 cytokine family is IL-17A. Th17 cells produce this cytokine, which has a pleiotropic effect. In our study, we found significant differences between pre- and post-transplantation gene expression levels. However, we could not find a correlation with the recipients' immune responses. Haouami et al., on the other hand, proposed that elevated IL-17A mRNA and protein levels could predict early acute organ rejection (10). As another pro-inflammatory cytokine IL-18 plays a role in both innate and adaptive immune responses (11). In their animal models, Wyburn et al. discovered that IL-18 expression levels increased significantly during acute organ rejection (12). There were significant differences in gene expression and serum levels of our patients after transplantation. IFN-γ is also a pro-inflammatory cytokine, which plays crucial roles in organ transplantations, viral infections, autoimmune responses, and adaptive immune responses (13). There were significant changes in gene expression and serum levels after transplantation in our study cohort. According to Nazari et al., patients with acute organ rejection episodes had higher levels of IFN-γ gene expression than patients who did not have this episode.14 Spivey et al. reported that patients with antibody-mediated organ rejection produced more IFN-γ than the control group (15). TNF-α is another pro-inflammatory cytokine that contributes to organ rejection following kidney transplants (16). In our study cohort, there were significant decreases and increases in gene expression of patients after transplantation.

IL-10 is an anti-inflammatory cytokine that regulates B and T cell functions and decides the initiation or prevention of the immune response (17). Gao et al. investigated if IL-10 was associated with cardiovascular and/or all-cause mortality after kidney transplantation (18). They found that IL-10 serum levels were independently associated with cardiovascular and all-cause mortality after the transplantations. In our study cohort, there were statistically significant results after transplantation. TGF-β plays important roles in cell proliferation, differentiation, and apoptosis. It is the primary cause of fibrosis in all chronic kidney diseases (19). Hribova et al. observed that TGF-β gene expression was elevated in acute organ rejection patients after examining 174 biopsy samples (20). In our study, we observed significant changes in its expression levels; however, there was not a full rejection condition in our study group. Another study found that the expression levels of TGF-β may be affected using tacrolimus and cyclosporine A as immunosuppressive drugs (21). In our study, there was no significant correlation between the drug usage and the levels of TGF-β.

FOXP3 is an important regulatory transcription factor for the regulatory T cell function. Treg cells play crucial roles in the control of immune response to the allograft and development of transplant tolerance. In a study, FOXP3 gene expression levels were high in stable patients, but low in patients with chronic organ rejection (22).22 In contrast, Bunnag et al. investigated the FOXP3 gene expression levels in 83 kidney biopsy materials and concluded that the levels of FOXP3 were not associated with the kidneys' post-transplant condition. They did believe, however, that the FOXP3+ cells would infiltrate the allograft over time and stabilize the inflammation areas (23). Muthukumar et al. compared FOXP3 gene expression levels in 36 acute rejection patients, 18 chronic kidney nephropathy patients, and 29 stable kidney transplanted patients. They reported that patients with acute organ rejection had lower levels of FOXP3 (24). Hayato et al. found that FOXP3 mRNA levels decreased soon after kidney transplantation and then increased in peripheral blood samples from 272 kidney transplanted patients (25). We determined significant differences between pre- and post-transplant expression levels. The limitations of this study were: 1) There was a small scale of study cohort, 2) We assessed the patients individually, 3) There was no active acute-rejection episode sample.

CONCLUSION

In conclusion, the pro-inflammatory, anti-inflammatory, and regulatory effects of immune molecules in kidney transplantation are significant. We observed significant changes in the expression of all the cytokines compared to pre-transplantation

levels of the patients, although there was no significant association with their clinical symptoms. However, the number of individuals was insufficient for the statistical assessment. It would be beneficial to follow up the patients for their immune responses after transplantation. Hereby, the communication between the laboratories and the clinics becomes even more important to provide the recipients' samples accurately. The expression values or features of the study group may be assessed due to their different immune responses in different people. Accordingly, individualized immunotherapies may be considered as a protective, preventive, and/or therapeutic way for the recipients to improve the transplantation outcomes.

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P. Writing: AÖK, MP, TKA. Critical Review: MP, TKA, MS, İP.

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