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Impaired CD4$^+$ Cytotoxic T Lymphocyte Activity in Polyomavirus BK Infected Kidney Transplant Recipients

Nasrin Noshadi[1,2], Ramin Yaghobi[3], Afsoon Afshari[4], Mohsen Forouzanfar[1], and Saeede Soleimanian[5]

[1] Department of Biology, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran
[2] Department of Biology, Fars Science and Research Branch, Islamic Azad University, Marvdasht, Iran
[3] Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4] Shiraz Nephro-Urology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[5] Allergy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

The reactivation of polyomavirus BK (BKPyV) contributes to increased morbidity and mortality rates of transplant patients, especially kidney transplant recipients (KTRs). CD4$^+$ T cells are important immune cells active during BKPyV infection in KTRs. This research tried to examine the phenotype of CD4$^+$ T cells in the stage of BKPyV activation in KTRs.

The recipients were separated into 2 groups of BKPyV-active and nonactive KTRs (10 patients in each group) and were compared with 10 healthy control subjects. The viral load was evaluated by Taq-man quantitative real-time PCR. The frequency of different CD4$^+$ T cell subsets was determined by analyzing markers such as CD45RO, CCR7, CD27, CD107a, perforin, and granzyme B using flow cytometry. The gene expression levels of transcription factors, including TBX21, GATA3, STAT3, and STAT6, contributing to CD4$^+$ T cell activation, were also assessed.

A significantly higher proportion in CCR7$^+$CD27$^+$CD45RO$^-$CD4$^+$ T cell (naive Tcell) subsets was detected in BKPyV-active KTRs compared to nonactive ones. A significant increase was detected in the frequency of CD107a$^+$, perforin$^+$, and granzyme B$^+$ CD4$^+$ T cells in the BKPyV-active group compared to the nonactive group. In CD4$^+$ T cells of KTRs, the mRNA expression of TBX21 and GATA3 was significantly increased in KTRs without BKPyV reactivation compared to BKPyV-active ones.

This investigation focused on the CD4$^+$ T cell as an immunodominant T cell type with potential cytotoxicity. Based on these results, BKPyV may have a direct influence on the repertoire of CD4$^+$ T cell subsets. Particularly, cytotoxic CD4$^+$ T cells need further investigation to be considered as a therapeutic approach for BKPyV infection.

Keywords: CD4 positive T lymphocytes; Cytotoxic; Human polyomavirus BK; Kidney transplantations; T-lymphocytes

Corresponding Author: Ramin Yaghobi, PhD;
Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran Tel/Fax: (+98 71) 3647 3954, E-mail: rayaviro@yahoo.com

INTRODUCTION

Polyomavirus BK (BKPyV) is a globally prevalent, circular, double-stranded DNA virus that infects

humans. It is a small, nonenveloped virus.[1,2] BKPyV is considered an opportunistic infection that poses a significant challenge for kidney transplant recipients (KTRs) and is one of the key factors contributing to late renal graft loss and failure.[3]

The genome of BKPyV consists of 3 functional genetic components: coding regions, including large T antigen (T-Ag), small t antigen (t-Ag), and 4 late genes (capsid proteins VP1, VP2, VP3, agnoprotein [agno], and a pre-microRNA resulting in miRNA-5p and -3p). Among these, VP1 is the major capsid protein, prominently displayed on the virus surface, and contains a small groove that potentially binds to host cell molecules serving as receptors.[4,5] Additionally, the noncoding control region encompasses the origin of viral DNA replication, ori.[5,6] Research on cellular immunity has established that VP1 is the primary target for $CD4^+$ T cells and BKPyV-specific cytotoxic T lymphocytes.[7,8]

Previous research has established that BKPyV infection poses a substantial challenge for KTRs,[9,10] and BKPyV-associated nephropathy (PVAN) can potentially lead to graft rejection.[11,12] Currently, the adjustment of immunosuppressive drugs is the recommended treatment approach for PVAN since there is currently no proven effective antiviral drug available for the treatment of these patients.[8]

The development of BK virus nephropathy (BKVN) is significantly influenced by the adaptive immune response. In healthy individuals, both subtypes of $CD4^+$ T lymphocytes, including cytotoxic and helper T cells, play a crucial role in effectively combating BKPyV infection.[13,14] However, BKPyV-specific $CD8^+$ T cells are scarcely detected in cases of BK virus infection.[15] Therefore, further exploration of $CD4^+$ T cells in the context of BKPyV patients is warranted.

Weist et al. have described the critical role of BKPyV-specific cytotoxic and helper $CD4^+$ T cells as important immune cells in KTRs, leading to the prevention of viral infection development.[9] cytotoxic $CD4^+$ T lymphocytes exert a suppressive effect on BKPyV replication, which is mediated through various proinflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α).[16,17]

Moreover, the assessment of cytotoxic markers, such as CD107a, perforin, and granzyme B, on $CD4^+$ T cells is crucial for evaluating their functional capacity. [18] Therefore, investigating the frequency of different subsets of $CD4^+$ T cells can be informative in detecting immune effector mechanisms against BKPyV infection.[19] When exposed to antigens and cytokine signaling, $CD4^+$ helper T cells differentiate into specific effector subtypes, providing tailored responses to different infectious agents. This differentiation process is regulated by transcription factors, including T-box transcription factor TBX21 (T-bet), signal transducer, and activator of transcription 3 (STAT3), STAT6, and GATA binding protein 3 (GATA3).[20] To gain further insight into the impact of BK infection on the development of $CD4^+$ T cells, the expressions of TBX21, STAT3, STAT6, and GATA3 were assessed in helper T cell development.

In this study, we examined the functional characteristics of $CD4^+$ T cell subsets in KTRs with BKPyV infection. We specifically evaluated the frequency of cytotoxic effector markers, including CD107a, perforin, and granzyme B. Our research aimed to determine whether the functional subtypes of $CD4^+$ T cells alone could serve as indicators of cytolytic effector activity in patients undergoing intensive immunosuppressive treatments.

MATERIALS AND METHODS

Donor Cells and Preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated from 20 mL of ethylenediaminetetraacetic acid (EDTA)-treated blood obtained from 10 adult BKPyV active KTRs, 10 KTRs without BKPyV reactivation, and 10 healthy controls with no history of other infectious diseases such as hepatitis C virus, hepatitis B virus, cytomegalovirus, human immunodeficiency virus (HIV), or adenovirus. The samples were collected between March 2018 and December 2020 at Abu Ali Sina Transplant Hospital in Shiraz, Iran.

The KTRs included in this study were admitted to the hospital with elevated serum creatinine levels and blood urea nitrogen levels and exhibited clinical manifestations such as diarrhea, fever, abdominal pain, and fatigue. BKPyV active infection was determined using Taq-man real-time polymerase chain reaction (PCR), with a viral load of more than 10,000 copies/mL considered a positive result for viral infection.

The standard treatment regimen for KTRs consisted of cyclosporine, initially administered at a dose of 5 mg/kg, followed by a maintenance dose of 3.0 mg/kg per day. Prednisolone was administered at an initial dose of

120 mg/day, which was then reduced to a maintenance dose of 10 mg/day. Additionally, mycophenolate mofetil capsules were administered twice daily as per the recommended dose. The blood samples for research were collected after discontinuing the BKPyV prevention and treatment regimen for at least 6 months. Participants who experienced rejection during the sampling process were excluded from the study.

Determining Antibody Titer Against BKPyV

The plasma of the samples was used to determine the BKPyV serostatus by specific immunoglobulin (Ig) G serum antibody titers against BKPyV using direct enzyme-linked immunosorbent assay (ELISA; MyBioSource, United States), according to the manufacturer's instructions.

$CD4^+$ T Cell Isolation from PBMC

PBMCs and plasma were isolated from KTRs and healthy controls to collect $CD4^+$ T cells. In brief, 3 mL of fresh Ficoll Paque (Lymphodex, inno-train, Spain) was slowly added to a 50-mL centrifuge sterile tube, followed by adding 11 mL of the diluted blood sample. All samples were centrifuged for 20 minutes at 22°C and 350*g*. The enriched mononuclear cells in the interface between the Ficoll and plasma layer were harvested by careful pipetting into a new tube. The cells were washed twice in a sterile phosphate-buffered saline solution.

$CD4^+$ T cells were separated from collected PBMCs by using a commercial column-based method called magnetic cell sorting protocols based on negative selection ($CD4^+$ T Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) to acquire highly purified $CD4^+$ T cell subsets. The harvested cells were collected, washed, and counted with a conventional hemocytometer and then added to the culture media. The purpose of using cell culture was to keep the cells fresh before flow cytometry and immunotyping and to increase the percentage of cell viability. The culture medium was compromised of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA), and 1% penicillin and streptomycin (5 mg/mL).

Purity Assessment of Isolated Samples

The purified T cells were treated with a cocktail containing antihuman monoclonal antibodies specific for CD3 and antihuman monoclonal antibodies specific for CD4 to determine the proportion of T cells representing $CD8^+$ T cell contamination. Forward and side scatter (FSC and SSC) gating were utilized to detect differences in lymphocyte cell debris counts.

Antibodies and Immunofluorescence Detection

The cell surface staining was performed with the following panel of mouse monoclonal antibodies against: PerCP/Cyanine5.5 antihuman CD3 Antibody (300429, Biolegend, USA), PE antihuman CD4 Antibody (317409, Biolegend, USA), PE antihuman CD27 Antibody (356405, Biolegend, USA), PerCPCY antihuman CD45RO Antibody (304221, Biolegend, USA), FITC antihuman CCR7 Antibody (353215, Biolegend, USA), and to detect the cytotoxicity of cells, including PE antihuman Perforin Antibody (353303, Biolegend, USA), FITC antihuman/mouse Granzyme B Recombinant Antibody (372205, Biolegend, USA), and PerCP/Cyanine5.5 antihuman CD107a (LAMP-1) Antibody (328615, Biolegend, USA) were used. The blocking step was done for nonspecific antibody binding with 10% heat-inactivated bovine serum albumin.

Isotype control antibodies were used as a negative control to characterize the antibody's nonspecific binding, including PerCP/Cyanine5.5 Mouse IgG2a, kappa Isotype Ctrl (400251, Biolegend, USA), PE Mouse IgG1, kappa Isotype Ctrl (400113, Biolegend, USA), PE Mouse IgG2b, kappa Isotype Ctrl (400313, Biolegend, USA), PerCP/Cyanine5.5 Mouse IgG1, kappa Isotype Ctrl (400149, Biolegend, USA), IgG2a, κ-FITC Isotype Ctrl (400209, Biolegend, USA), FITC Mouse IGg1 kappa Isotype Ctrl (400137, Biolegend, USA) The cell suspension was stained in a staining buffer, and 20,000 events were obtained per sample using a BD fluorescence-activated cell sorting (FACS) flow cytometer.

Fluorochrome Standard Beads

The standard fluorescent microspheres were colored beads with conjugated antibodies to either PE, FITC, or PerCP/Cyanine5.5. After staining, the bead dilution buffer was provided with the beads in a buffer before assay on BD FACSCalibur (per tube obtaining a single-color standard). This approach supports establishing spectral overlap and exact compensation corrections of any combination of fluorochrome-conjugated antibodies.

Gene Expression

The relative mRNA levels of the STAT6, STAT3, GATA3, and TBX21 were evaluated in the isolated $CD4^+$T cells using SYBR Green real-time PCR protocols. A TRIzol reagent was used to extract total human RNA from $CD4^+$ T cells. The PrimeScript Reverse Transcription Reagent Kit (Takara, Japan) was used to perform first-strand cDNA synthesis.[21] PCR reactions were prepared in triplicates at a final volume of 20 µL using ABI StepOnePlus (Applied Biosystems, USA). Protocol for the SYBR Green real-time PCR program, reaction mixtures. Primer sequences can be found in Supplementary Table.

Statistical and Analysis

The data description was presented as the mean±SEM. The student's independent t-test, Kruskal-Wallis, and analysis of variance (ANOVA) tests were used for analysis and according to the bivariate normality assumption confirmed by the Pearson and D'Agostino omnibus tests. GraphPad Prism software version 7.0 was used for statistical analysis and visualization. Significant differences in the means between the groups are represented by $*p<0.05$, $**p<0.01$, and $***p<0.001$.

RESULTS

The demographic data and clinical characteristics of both KTRs and healthy individuals are briefly summarized in Table 1. BKPyV active KTRs had a mean peak of viral load value above 10,000 copies per mL.

Table 1. Demographic and descriptive characteristics of KTRs and healthy controls

	BKPyV active KTRs	KTRs without BKPyV reactivation	Healthy control
Total number of patients	10	10	10
Gender	Male = 5	Male = 5	Male = 7
	Female = 5	Female = 5	Female = 3
Mean age (years)	53.4	48.9	38.1
Blood groups	O^+ = 4	O^+ = 4	O^+ = 3
	A^+ = 3	A^+ = 3	A^+ = 5
	AB^+ = 1	B^+ = 3	B^+ = 2
	B^+ = 2		
Type of transplantation			
Cadaver donor	100%	100%	
Living donor	0%	0%	
Underlying disease			
Hypertension and diabetes	**5**	**7**	
Only hypertension	**2**	**1**	
Others	**3**	**3**	

KTRs: kidney transplant recipients

The gating strategy was used on isolated $CD4^+$ T cells (based on live, SSC, and FSC gating). The following gating strategy was used to evaluate the percentage of $CCR7^+$ and $CD27^+$ T cells expressing $CD4^+$ with the presence or absence of the CD45RO surface marker.

Different subpopulations of $CD4^+$ T cells were determined as follows: $CCR7^+CD27^+CD45RO^-CD4^+$ T cells (naive T cells), $CCR7^+CD27^+CD45RO^+CD4^+$ T central memory cells (TCM), and $CCR7^-$, $CD27^-$ $CD45RO^+$ $CD4^+$ T effector memory cells (TEM) (Figures 1A, 1B, and 1C). Cytotoxic $CD4^+$ T cells were evaluated based on intracellular markers, including CD107a, perforin, and granzyme B (Figures 1D, 1E, and 1F).

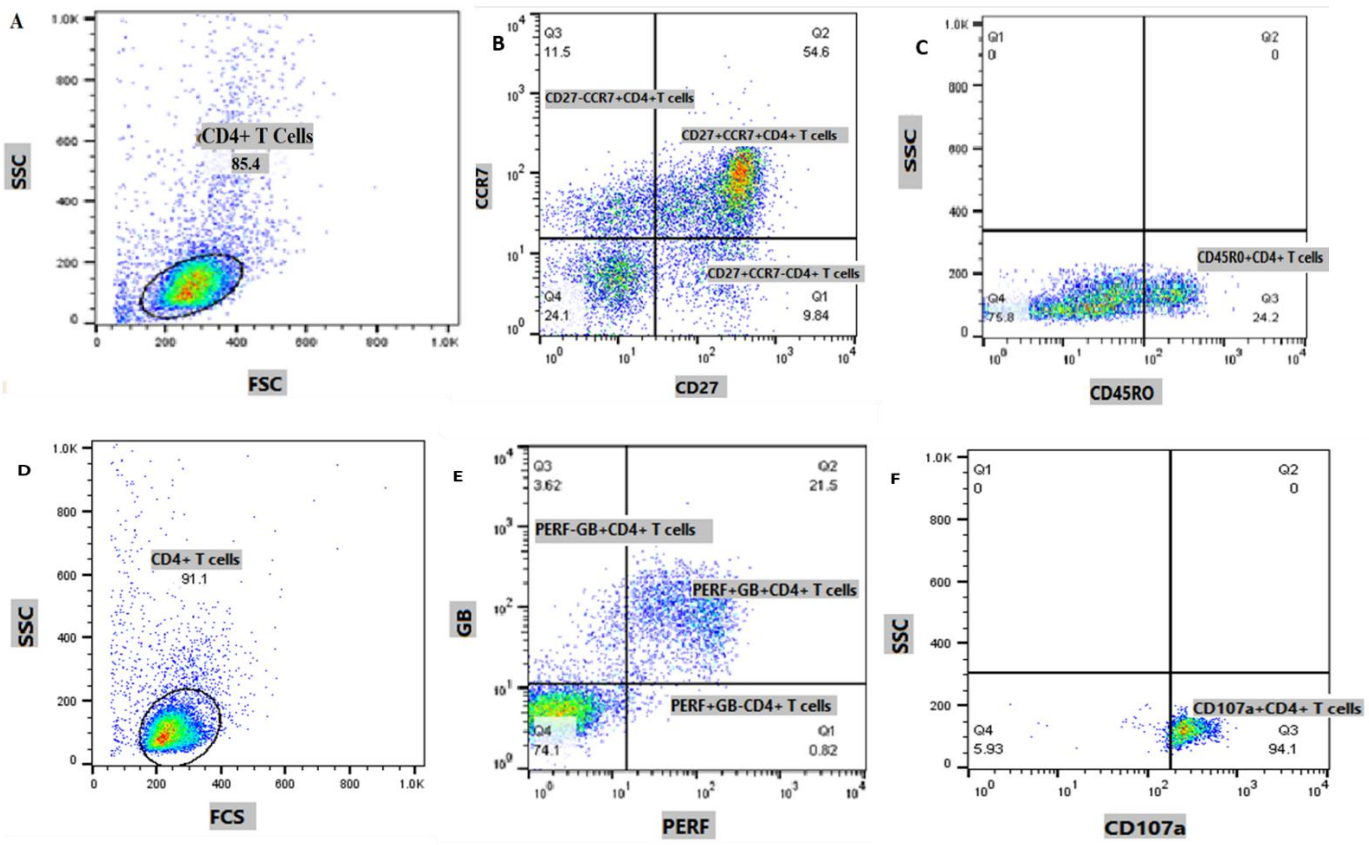

Figure 1. The gating strategy used for CD4⁺ T cells. A and D: CD4⁺ T cells were gated according to FSC/SSC. B: CCR7/CD27 were gated on the CD4⁺ T cells dot plot. C: CD45RO/SSC cells were gated based on the CCR7 and CD27 dot plot. E: Perforin and granzyme B were gated on the CD4⁺ T cells dot plot. F: CD107a⁺ cells were gated on perforin and granzyme B dot plot. FSC: forward scatter; SSC: side scatter.

Analysis of Naive T Cells, TCM, and CD4⁺ TEM Cells

CD4⁺ T cells coexpressing CCR7, CD27, and CD45RO were counted to determine the frequency of 3 phenotypically different subpopulations: naive T cells, TCM, and TEM CD4⁺ T cells (Figure 2).

The percentage of $CCR7^{+}CD27^{+}CD45RO^{-}CD4^{+}$ T cell (naive T cell) subpopulation was nonsignificantly increased in patients with active BKPyV compared to KTRs without BKPyV reactivation. Moreover, BKPyV-active KTRs had similar levels of naive T cells as healthy controls, while KTRs without BKPyV reactivation had significantly lower levels (p=0.0186; mean difference=15.52) (Figure 2A).

After analyzing the $CCR7^{+}CD27^{+}CD45RO^{+}CD4^{+}$ T cell subset (TCM), we found that BKPyV-active KTRs had a nonsignificantly decreased frequency compared to healthy controls, and BKPyV-active KTRs had a lower frequency than Individuals KTRs without BKPyV reactivation. KTRs without BKPyV reactivation exhibited a relatively increased TCM frequency compared to healthy controls; however, this disparity did not reach statistical significance (Figure 2B).

BKPyV-active KTRs had a higher percentage of TEM ($CCR7^{-}CD27^{-}CD45RO^{+}CD4^{+}$ T cells) than both KTRs without BKPyV reactivation and healthy controls, but the difference was not significant. Similarly, there was no significant difference in TEM frequency between KTRs without BKPyV reactivation and healthy controls (Figure 2C).

Analysis of CD107a/LAMP-1, Perforin, and Granzyme B in CD4⁺ T Cells

When the cytotoxic activity was assessed in BKPyV active subjects, the percentage of CD107A/LAMP-1⁺, perforin⁺ granzyme B⁺ CD4⁺ T cells was significantly

less than KTRs without BKPyV reactivation (p=0.0262, mean difference=15.95%; Figure 2D). In healthy people, the number of the CD107A/LAMP-1$^+$ Perforin$^+$ Granzyme B$^+$ CD4$^+$ T cell subset was significantly lower than KTRs without BKPyV reactivation (p=0.0177; mean difference=10.94%; Figure 2D). Furthermore, there were no statistically significant differences between the percentage of CD107A/LAMP-1$^+$ perforin$^+$ granzyme B$^+$ CD4$^+$ T cells in BKPyV-active KTRs compared to healthy individuals.

Analysis of mRNA Expression Levels of Studied Genes in Isolated CD4$^+$ T Cells

We found that BKPyV-active KTRs had lower levels of TBX21 mRNA, a gene that regulates immune cells, than healthy people (p=0.0216, mean difference=5.24%) (Figure 4A). Moreover, BKPyV-active KTRs had much lower TBX21 mRNA levels than KTRs who did not have BKPyV reactivation (p=0.0003, mean difference=7.25%).

GATA3 mRNA expression level was significantly decreased in BKPyV-active KTRs compared to both KTRs without BKPyV reactivation ones and healthy controls, respectively (p=0.0097, mean difference=1.9%; p=0.0136, mean difference=4.97%; Figure 4B). As presented in Figures 4A and 4B, the analysis of TBX21 and GATA3 mRNA demonstrated significantly reduced expression levels of these genes in BKPyV-active KTRs in comparison with KTRs without BKPyV reactivation; however, this finding resembles the healthy group.

A significant correlation was detected between GATA3 expression with both STAT6 and STAT3 mRNA levels (with 0.743 and 0.675 coefficient ranges, respectively). Furthermore, there is a significant correlation between STAT3 and STAT6 with a 0.655 coefficient range among BKPyV-active KTRs (Figure 3).

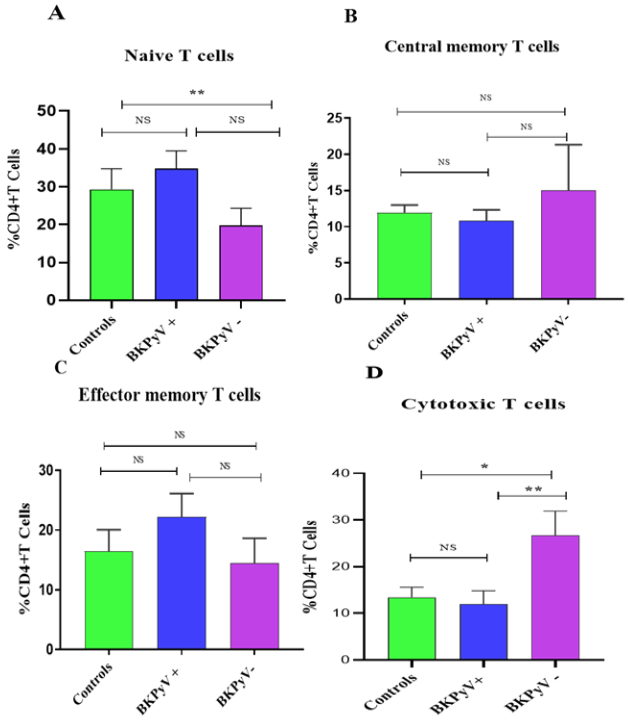

Figure 2. Analyzing the expression patterns of distinct CD4$^+$ T cell subsets within 3 different experimental categories: 10 BKPyV-active KTRs, 10 KTRs without BKPyV reactivation, and 10 healthy controls; A: CCR7$^+$CD27$^+$CD45RO$^-$CD4$^+$ T cells (naive T cell); B: CD27$^+$CD45RO$^+$CD4$^+$ T Cells (central memory cells); C: CCR7$^-$CD27$^-$CD45RO$^+$CD4$^+$ T cells (Effector memory cells); D: CD107a$^+$perforin$^+$granzyme B$^+$CD4$^+$ T cells (cytotoxic activity). The mean frequency of all subpopulations of CD4+ T cells among BKPyV-active and KTRs without BKPyV reactivation has been analyzed. Significant consequences: *p<0.05; **p<0.01; ***p<0.001. The data are expressed as means±SEM.

KTRs: kidney transplant recipients, BKPyV+: BKPyV-active KTRs, BKPyV-: KTRs without BKPyV reactivation.

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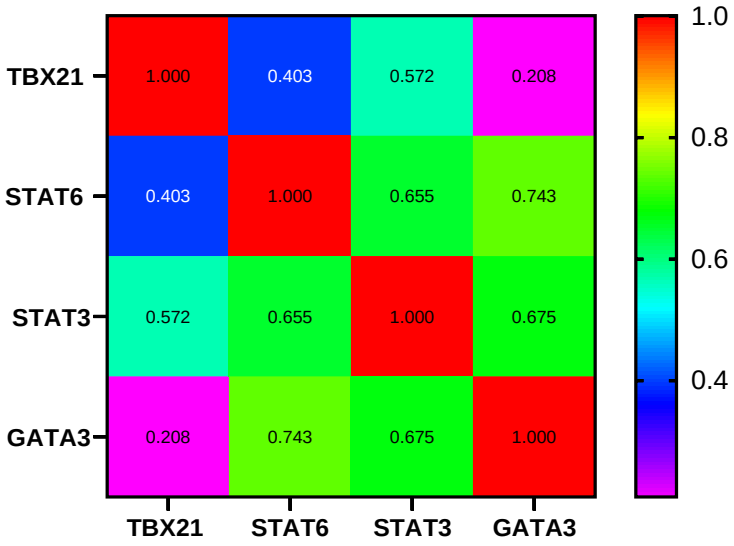

Figure 3. Matrix heatmap using Spearman rank correlation analysis between gene expression levels of CD4⁺ T cells among BKPyV-active KTRs. Significant positive (red) and negative (blue) correlations are shown in the figure. Dark red segments displayed self-to-self correlation analysis for data on monitoring gene expression. The correlation coefficients range from +1 to −1.

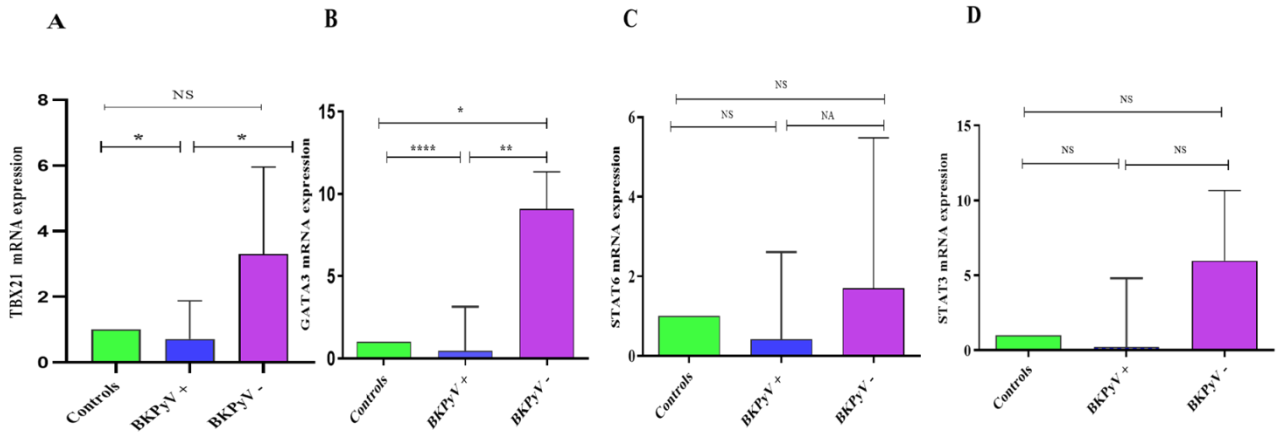

Figure 4. The charts depicting CD4+ T cell behavior in BKPyV-infected KTRs highlight variations in the levels of TBX21, GATA3, STAT3, and STAT6 mRNA expression. A: Outcomes presented a significantly higher frequency of TBX21 mRNA expression in KTRs without BKPyV reactivation compared to active ones; however, this level of mRNA expression is very similar to healthy controls. B: The mRNA expression level of *GATA3* in BKPyV-active KTRs is significantly less than healthy controls and KTRs without BKPyV reactivation. Healthy controls have significant upregulation of TBX21 and *GATA3* expression compared to both groups of KTRs. C and D: The results of *STAT3* and *STAT6* gene expression levels are not significant among BKPyV-active KTRs, KTRs without BKPyV reactivation, and healthy controls. The healthy control group is used for normalizing the studied genes. The results are displayed as mean±SEM of 3 independent cultures (*$p < 0.05$; **$p < 0.01$ and ****$p < 0.0001$).

KTRs: kidney transplant recipients, BKPyV+:BKPyV-active KTRs, BKPyV-: KTRs without BKPyV reactivation.

DISCUSSION

Cellular immunity plays a crucial role in preventing BKPyV replication, which can ultimately lead to allograft failure. [15,22] Previous studies have highlighted the significance of T cells in protecting against BKPyV infection.[9,21] Despite significant progress in the management of BKPyV infection, the clearance of viremia varies among patients, ranging from a few weeks to several years. [23]

The available evidence suggests that $CD4^+$ T cells have a substantial role in eliminating BKPyV-infected cells in KTRs. However, this function is compromised by the administration of prednisolone and calcineurin inhibitors. Additionally, studies have demonstrated the importance of cytotoxic $CD4^+$ T cells in other infections, such as Epstein-Barr virus and HIV.[24,25]

BK virus-specific $CD4^+$ T cell lines exhibit a helper effector cell phenotype in vitro, which can be rapidly stimulated to exert cytotoxicity through the secretion of granzyme B.[9] Recent studies have demonstrated that $CD4^+$ T cells, similar to $CD8^+$ T cells, possess cytotoxic potential by exhibiting the degranulation marker CD107a and releasing cytotoxic effector molecules such as granzyme B.[8] In the present investigation, a significant decrease in the expression of CD107a/LAMP-1, perforin, and granzyme B was observed in $CD4^+$ T cells from BKPyV-active KTRs compared to KTRs without BKPyV reactivation. Conversely, $CD4^+$ T cells from KTRs without BKPyV reactivation exhibited stronger cytotoxic responses against BKPyV, as evidenced by the upregulation of CD107a/LAMP-1, perforin, and granzyme B, which potentially contributes to the control of BKPyV reactivation. It is noteworthy that in BKPyV-infected KTRs, a significant proportion of the $CD4^+$ T-cell population is involved in lysing infected cells through the secretion of perforin and granzyme B, particularly in high-risk immunocompromised individuals such as patients with chronic kidney disease.[26]

This finding suggests that the granzyme B/perforin pathway, which leads to the lysis of target cells, is a crucial immunological mechanism employed by BKPyV-specific $CD4^+$ T lymphocytes for the direct killing of infected cells. [27] Additionally, the activation of cytotoxic memory $CD4^+$ T cells also plays an important role in reducing viral replication.[26]

It is important to note that the induction of adaptive immune responses is influenced by $CD4^+$ T cell signals. These signals enable antigen-presenting cells to stimulate naive $CD8^+$ T cells, leading to the generation of a large population of cytotoxic T lymphocytes. Furthermore, $CD4^+$ T cells assist in the differentiation of B cells into plasma cells that secrete immunoglobulins. Therefore, the generation of cytolytic $CD8^+$ T cells and antibody-secreting plasma cells for protection against chronic viral infections relies on the assistance of $CD4^+$ T cells. A high count of $CD4^+$ T cells contributes to the development of memory $CD8^+$ T cells and facilitates their independent recognition of target cells.[28,29]

We observed a nonsignificant increase in the T naive subset of $CD4^+$ cells in BKPyV active- KTRs compared to KTRs without BKPyV reactivation. However, this difference would likely become significant with a larger sample size. Furthermore, TCM $CD4^+$ T cells showed a nonsignificant decrease in BKPyV-active KTRs compared to KTRs without BKPyV reactivation. Espada et al. reported that the majority of $CD4^+$ T cells in BKPyV-infected KTRs were TCM and T naive cells. Conversely, very few $CD8^+$ cells with naive or TCM phenotypes were found during BKPyV activation following allogeneic hematopoietic cell transplantation.[15]

The observed results align with logical expectations, as an increase in the naive phenotype and a decrease in the TCM phenotype were observed in the BKPyV-active population. Conversely, the KTRs without BKPyV reactivation exhibited the opposite pattern. This finding suggests that TCM cells play a crucial role in preventing BKPyV active infection. Additionally, TEM $CD4^+$ T cells showed a nonsignificant enhancement in BKPyV-active KTRs compared to both the KTRs without BKPyV reactivation and the healthy control groups. This could indicate a delayed recovery of TEM $CD4^+$ T cells, which may contribute to the increased susceptibility to BKPyV activation. A study by Menter et al. observed that BKPyV-specific $CD4^+$ helper T lymphocytes predominantly consisted of TEM cells. Furthermore, the TCM cell population, which constituted a minority of memory T cells, significantly increased after the clearance of severe BKPyV infection.[30]

Transcription factors also play a significant role in this context, as studies have demonstrated the critical involvement of transcription factors such as TBX21, STAT3, STAT6, and GATA3 in the differentiation of various memory and effector subsets.[31-35] In vitro studies have revealed that BKPyV-specific T cells from KTR

patients often exhibit reduced expression levels of key transcriptional regulators, including TBX21. However, ex vivo stimulation of T cells with BKPyV epitopes selectively increased the expression of TBX21 .[19] Consistent with these findings, our results showed significant downregulation of TBX21 in BKPyV-active KTRs compared to both KTRs without BKPyV reactivation and the control group.

Yoshida et al. demonstrated that BKPyV-specific T cells expressing STAT3 are crucial in generating an immune response in renal transplant recipients.[36] In our study, we observed lower expressions of STAT3 in $CD4^+$ T cells from patients with active BKPyV compared to KTRs without BKPyV reactivation and healthy controls, although the difference was not statistically significant. Besides STAT3, STAT6 plays a vital role in renal fibrosis and has a cell-protective effect against kidney cell apoptosis.[37] Although not statistically significant, we also observed decreased mRNA levels of STAT6 in BKPyV active KTRs compared to KTRs without BKPyV reactivation and healthy controls.

Another crucial transcription factor is GATA3, which plays a regulatory role in the differentiation of naive $CD4^+$ T cells into the T helper type 2 (Th2) cell lineages.[38] In vitro, the coexpression of GATA3 and TBX21 has been observed in Th1 cells, suggesting their involvement in the plasticity of effector T-cell subtypes.[39-41]

Our findings demonstrated a significant reduction in GATA3 expression in BKPyV-active KTRs compared to the other groups studied. This observation indicates that the expression of both TBX21 and GATA3 markers decreases in $CD4^+$ T cells following viral activation. Consequently, the deficiency of TBX21 and GATA3 expression may contribute to BKPyV activation.

These results highlight the importance of efficient $CD4^+$ T cell activation as a protective factor against BKPyV infection and its impact on defining the clinical course of viral proliferation and infection. These significant findings may have implications for the monitoring and treatment of BKPyV infection and nephropathy, as well as the development of immune cell therapy targeted against human BKPyV infection. We should take into consideration the limitations of our study, specifically the lack of interval times between our assessments due to the unavailability of our study groups.

Based on the findings of this study, we suggest that monitoring the levels of perforin, CD107a, and granzyme B as indicators of cytotoxic activation could serve as important markers with high sensitivity for assessing the functionality of $CD4^+$ T cells in BKPyV-infected KTRs. Consequently, cytotoxic $CD4^+$ T cells may be considered a potential target for therapeutic strategies in the management of BKVAN.

STATEMENT OF ETHICS

The Research Ethics Committee of Shiraz University of Medical Sciences approved the project (Reference number: IR.SUMS.REC. 1396.S835).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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