

Immunomodulatory Role of Combined BK Polyomavirus Small and Large T Antigens on CD4⁺ T Cell Subset Function and Differentiation in Kidney Transplant Recipients

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ABSTRACT

BK polyomavirus (BKPyV) reactivation is a major cause of allograft dysfunction and BKPyV-associated nephropathy (BKPyVAN) in kidney transplant recipients (KTRs). Although CD4⁺ T-cell responses are considered important for viral control, the immunomodulatory effects of BKPyV small T antigen (ST-Ag) remain poorly understood. This study investigated the effects of ST-Ag alone and combined large T antigen (LT-Ag)/ST-Ag stimulation on CD4⁺ T-cell differentiation and function in KTRs.

Peripheral blood mononuclear cells (PBMCs) from 10 KTRs and five healthy controls were stimulated with ST-Ag alone or a combined LT-Ag/ST-Ag peptide pool. Naive, central memory, and effector memory CD4⁺ T-cell subsets were analyzed by flow cytometry using CCR7, CD45RO, CD107a, and granzyme B. Expression of *T-bet*, *GATA3*, *STAT3*, *STAT6*, and *perforin* was quantified by real-time PCR.

Combined LT-Ag/ST-Ag stimulation was associated with a higher frequency of naive CD4⁺ T cells and lower frequencies of central and effector memory subsets in BKPyV-reactivated KTRs compared with BKPyV-negative recipients. Expression of *T-bet*, *GATA3*, *STAT3*, and *STAT6* was significantly reduced, whereas *perforin* expression was increased. These findings are consistent with altered CD4⁺ T-cell differentiation in BKPyV-reactivated recipients.

BKPyV ST-Ag may contribute to altered CD4⁺ T-cell differentiation and immune dysregulation during viral reactivation. These findings provide preliminary insight into immune alterations associated with BKPyV reactivation and warrant validation in larger, independent cohorts.

Keywords: BK virus; CD4-positive T-lymphocytes; Kidney transplantation; Viral large T antigen; Viral small T antigen; Viral tumor antigen

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INTRODUCTION

BK polyomavirus (BKPyV) is one of the smallest double-stranded circular DNA viruses in the

Polyomaviridae family and lacks an envelope and infects humans around the world.¹⁻³ The viral genome is divided into three regions:⁴ the early region contains viral tumor antigens (LT-Ag and ST-Ag), which are responsible for modulating the cell cycle of the host and participate in the replication of the virus, tumor generation, and cell conversion.^{5,6} The late region comprises the genes responsible for the structural proteins, including Vp1-3 and an agnoprotein.^{6,7} Finally, the non-coding control region (NCCR) is located between two other regions and consists of the transcription control region (TCR) and the viral replication origin.^{8,9} Approximately 90% of the world's population is BKPyV seropositive.^{10,11} The epidemiological burden of BKPyV infection is particularly notable in the context of kidney transplantation in Iran, where the prevalence of BKPyV viremia and viremia among kidney transplant recipients (KTRs) has been reported to range from approximately 20% to over 50%, depending on the cohort characteristics and the sensitivity of the diagnostic assay employed.¹² BKPyV-associated nephropathy (BKPyVAN) has been documented as a clinically significant complication in Iranian KTRs, contributing to allograft dysfunction and graft loss in a subset of affected patients. Given the relatively high rate of immunosuppression-related viral reactivation in this population, characterization of the host T-cell immune response to BKPyV antigens is of particular clinical relevance in the Iranian transplant setting. BKPyV generally leads to complications after kidney transplantation, which might result in BKPyVAN in KTRs.¹³⁻¹⁶ The occurrence rate of BKPyVAN during the first year after graft transplantation is 10%, and it may result in loss of a transplanted graft in 30-80% of graft recipients because of delayed recognition of viral reactivation.^{13,17} Immunosuppressive treatment in KTRs facilitates viral reactivation.^{5,18} Reducing immunosuppressive drug doses facilitates T-cell immune reconstitution, which plays a crucial role in viral infection.¹⁸ Studying T-cell responses to BKPyV LT-Ag and ST-Ag may help assess BKPyV-specific T-cell function and inform the development of new approaches to treating BKPyV-associated disease. BKPyV ST-Ag is a multifunctional regulatory protein that modulates host signaling pathways through mechanisms distinct from LT-Ag. Unlike LT-Ag, which primarily promotes viral replication by inactivating p53 and pRb, ST-Ag exerts its effects mainly through

interaction with the PP2A complex, altering the phosphorylation of proteins involved in cell proliferation, apoptosis, and immune signaling.^{5,19} Because PP2A regulates transcription factors (TFs) such as STAT3, STAT6, and NFAT that are essential for CD4⁺ T-cell differentiation, ST-Ag-mediated PP2A inhibition may disrupt CD4⁺ T-cell effector and memory responses. Furthermore, studies of related polyomaviruses, including MCPyV and SV40, have shown that ST-Ag contributes to immune evasion by suppressing antigen presentation and antiviral T-cell responses. Given the structural and functional similarity of BKPyV ST-Ag to these proteins, it is plausible that BKPyV ST-Ag similarly impairs CD4⁺ T-cell immunity in KTRs, thereby promoting viral persistence and BKPyVAN.¹⁹

The present investigation aims to assess the responses of CD4⁺ T cells to the BKPyV tumor antigens in healthy individuals, BKPyV⁺, and BKPyV⁻ according to their surface markers (CD45RO and CCR7). Healthy individuals were included as a reference group to define baseline CD4⁺ T-cell phenotypes rather than as a direct comparator for BKPyV-specific effects. In addition, CD4⁺ T-cell cytotoxic function was evaluated by analyzing the expression of cytotoxicity-associated markers such as granzyme B and CD107a to evaluate the effects of BKPyV tumor antigens on CD4⁺ T-cell differentiation. Although CD107a and granzyme B are classically associated with CD8⁺ cytotoxic T cells, accumulating evidence indicates that a subset of CD4⁺ T cells can acquire cytotoxic properties under chronic antigenic stimulation, including persistent viral infections.²⁰

The study evaluated ST-Ag alone and the LT-Ag/ST-Ag combination because it was designed as an extension of our previous work,² which had already characterized the effects of LT-Ag alone on CD4⁺ T-cell subsets. The combined antigen condition was included to better reflect the physiological co-expression of both early antigens during BKPyV infection. Therefore, an LT-Ag-only group was intentionally omitted to avoid redundant experiments without providing additional mechanistic insight.

Accordingly, this investigation evaluated the expression levels of TFs, including signal transducer and activator of transcription 3 and 6 (*STAT3* and *STAT6*), T-box transcription factor TBX21 (*T-bet*), GATA3 binding protein 3 (*GATA3*), and *perforin* to compare their expression among the study groups.

MATERIALS AND METHODS

Study Subjects

This study included 10 KTRs, comprising five BKPvV-reactivated recipients (60% male; mean age, 46 years) and five recipients without active BKPvV infection (60% male; mean age, 38.4 years), who underwent deceased-donor kidney transplantation (except a recipient without active BKPvV infection who receive the graft from living donor) at Abu Ali Sina Transplant Hospital, Shiraz, Iran, between 2020 and 2022. Five healthy volunteers (20% male; mean age, 31 years) were enrolled as the control group. Blood samples from BKPvV⁻ and BKPvV⁺ KTRs were collected 9±2.55 and 9.2±2.28 months after transplantation, respectively. BKPvV DNA was quantified by real-time PCR, and recipients with a viral load >10 000 copies/mL were classified as the BKPvV-reactivated group. All participants were BKPvV-seropositive adults (18–60 years). Individuals with a history of allograft rejection or loss, or active co-infection with cytomegalovirus (CMV), adenovirus, hepatitis B virus (HBV), hepatitis C virus (HCV), or human immunodeficiency virus (HIV), were excluded. All KTRs received a standard immunosuppressive regimen consisting of mycophenolate mofetil, cyclosporine, and prednisolone.

The study was designed as an exploratory pilot investigation; therefore, no formal a priori sample size calculation was performed. The sample size was determined by the availability of eligible participants who met the predefined inclusion criteria during the study period. The relatively low frequency of BKPvV reactivation, together with the strict eligibility criteria, limited the number of participants available for enrollment. The study was approved by the Institutional Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (Ethics Approval Code: IR.SUMS.REC.1399.723). All procedures were conducted in accordance with the Declaration of Helsinki and its subsequent amendments. Written informed consent was obtained from all KTRs and healthy volunteers before enrollment.

Sampling

Peripheral blood mononuclear cells (PBMCs) were separated from 10 mL of blood treated with K3-EDTA from each healthy control and KTR with or without active viral infection using a Ficoll (Inotrain, Germany) density gradient. After washing three times with

phosphate-buffered saline (PBS), debris and platelets were removed.²

BKPvV Assessment

Following sampling, each KTR was tested to confirm the presence of BKPvV using a TaqMan real-time PCR assay (ABI, Step One Plus, USA). Following the manufacturer's instructions for the CinnaGen DNA extraction kit, all samples underwent DNA extraction. A 200-μL aliquot of each PBMC suspension was used. The concentration and purity of the extracted DNA were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) by measuring the optical density at 260/280 nm. To assess BKPvV, the TaqMan real-time PCR assay was performed using a real-time PCR instrument. For this purpose, a TaqMan Real-Time PCR kit (Gene Proof, Korea) was used according to the manufacturer's protocol.

Stimulation of PBMCs with BKPvV Tumor Antigens

As in our previous study, isolated PBMCs were prepared for stimulation in complete RPMI (RPMI (Gibco, United States) supplemented with 10% FBS (Biosera, Europe), 0.5 mL of glutamine (Gibco, United States), 0.5 mL of penicillin-streptomycin antibiotics (Pen/strep)); 2 μL of BKPvV ST-Ag overlapping peptide pool/10⁶ cells and/or a mix of 2 μL LT-Ag and 2 μL of ST-Ag overlapping peptide pool/10⁶ cells (LT-Ag Pepmix, JPT, Germany and ST-Ag Pepmix, JPT, Germany) were added.² To validate the responsiveness of PBMCs and the effectiveness of the stimulation conditions, both positive and negative controls were included. As a positive control, cells were treated with a combination of phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 μg/mL), which robustly activates T cells through protein kinase C and calcium influx. Unstimulated PBMCs served as the negative control to establish baseline activity levels. For the presentation of viral tumor antigen peptides to immune cells via antigen-presenting cells (APCs), stimulated PBMCs were incubated at 37 °C for 1 hour. In the third step, to clear the residual antigens, incubated PBMCs were centrifuged (1500 RPM, 5 minutes, 25 °C). Then, the cell pellet was resuspended in 1 mL complete RPMI and divided among the U-bottom 96-well plate wells (250 000 cells/well) (SPL Life Sciences, Korea). In the last step, the plate was incubated in a 37 °C CO₂ incubator for 48 hours.² While the PBMCs were stimulated with overlapping peptide pools of BKPvV

LT-Ag and ST-Ag, we were unable to include activation-induced markers (e.g., OX40, CD25) to specifically identify antigen-reactive CD4⁺ T cells, due to reagent limitations.

Stimulated PBMC Preparation for Flow Cytometry

After 48 hours, the U-bottom 96-well plate was removed from the 37 °C, CO₂ incubator. Stimulated PBMCs were collected and transferred into 5 mL specific tubes for flow cytometry. To assess cell viability, we used propidium iodide (PI), and FITC Annexin-V impermeable dyes. Compensation for spectral overlap was performed prior to data acquisition using single-color compensation controls prepared from antibody-capture beads matched to each fluorochrome. Fluorescence-minus-one (FMO) controls were prepared for all markers and used to establish gating boundaries for each fluorochrome, particularly for markers with continuous expression distributions such as CD45RO and CCR7. Unstained cells were used to define baseline autofluorescence. The sequential gating strategy applied was as follows: (i) lymphocyte identification based on forward scatter (FSC) and side scatter (SSC) characteristics; (ii) singlet discrimination using FSC-height versus FSC-area; (iii) viability exclusion using propidium iodide and FITC-Annexin V; (iv) CD4⁺ T-cell gating based on FSC-area and PE-CD4 expression; (v) identification of naive (CD45RO⁻CCR7⁺), central memory (CD45RO⁺CCR7⁺), and effector memory (CD45RO⁺CCR7⁻) subsets; and (vi) intracellular marker analysis (Granzyme B and CD107a) within gated CD4⁺ populations. All subset frequencies are expressed as

percentages of total gated CD4⁺ T cells. Representative gating plots with population percentages are provided in Figures 1–4. Inter-sample variability was assessed by examining within-group coefficient of variation (CV) for each subset, and values are noted in the supplementary materials. To investigate different subtypes of CD4 T cells, PE anti-human CD4 antibody (Clone RPA-T4), PerCP anti-human CD45RO antibody (Clone UCHL1), and FITC anti-human CD197 (CCR7) antibody (Clone G043H7) were used (Table 1). Intracellular staining for PerCP/Cyanine5.5 anti-human/mouse Granzyme B antibody (Clone QA18A28) was performed following fixation and permeabilization using saponin, while Alexa Fluor 488 anti-human CD107a (LAMP-1) antibody (Clone H4A3) was stained during stimulation. Antibody titration and optimization were performed to obtain the optimal concentration. We also used PE mouse IgG2b, κ Isotype Ctrl Antibody (Clone MPC-11), PerCP mouse IgG2a, κ Isotype Ctrl Antibody (Clone MOPC-173), FITC mouse IgG2a, κ Isotype Ctrl (FC) Antibody (Clone MOPC173), Alexa Fluor 488 mouse IgG1, κ Isotype Ctrl (FC) Antibody (MOPC-21), PerCP/Cyanine5.5 Mouse IgG1, κ Isotype Ctrl Antibody (Clone MOPC-21) to account for the nonspecific antibody binding. All antibodies and their isotypes were obtained from BioLegend, USA. The cell suspension was stained in a FACS buffer (FACS buffer consisted of phosphate-buffered saline containing 2% FBS), and a total of 2×10⁴ cells were evaluated per tube using a FACS Calibur Flow Cytometer (Becton, Dickinson, and Company; BD).²

Table 1. Studied CD4⁺ T-cell subgroups and their specific surface markers

Surface marker	Naive T cell	Central memory T cell	Effector memory T cell
CD4	+	+	+
CD45RO	-	+	+
CCR7	+	+	-

CCR7: C-C chemokine receptor type 7; CD4: cluster of differentiation 4; CD45RO: cluster of differentiation 45RO.

Flow Cytometry Gating Strategy

The flow cytometry gating strategy employed in this study is illustrated in Figure 1 (CD4⁺ T-cell subset identification) and Figure 2 (cytotoxic marker analysis), which are presented as methodological reference figures. Briefly, lymphocytes were first identified based on FSC and SSC parameters. Following singlet discrimination and viability exclusion using propidium

iodide and FITC-Annexin V, CD4⁺ T cells were gated based on PE-CD4 expression relative to FSC. Within the CD4⁺ gate, naive T cells were identified as CD45RO⁻CCR7⁺, central memory T cells (Tcm) as CD45RO⁺CCR7⁺, and effector memory T cells (Tem) as CD45RO⁺CCR7⁻, using PerCP-CD45RO and FITC-CCR7 (CD197) markers. For cytotoxic marker analysis, CD4⁺ T-cell subsets were further classified as

BKPyV T Antigens Modulate CD4⁺ T Cell Function

CD107a⁺GranzymeB⁻, CD107a⁺GranzymeB⁺, and CD107a⁻GranzymeB⁺ based on Alexa Fluor 488-CD107a and PerCP/Cy5.5-Granzyme B staining.

Representative gating plots demonstrating this sequential strategy, including isotype controls and FMO gates, are shown in Figures 1 and 2.

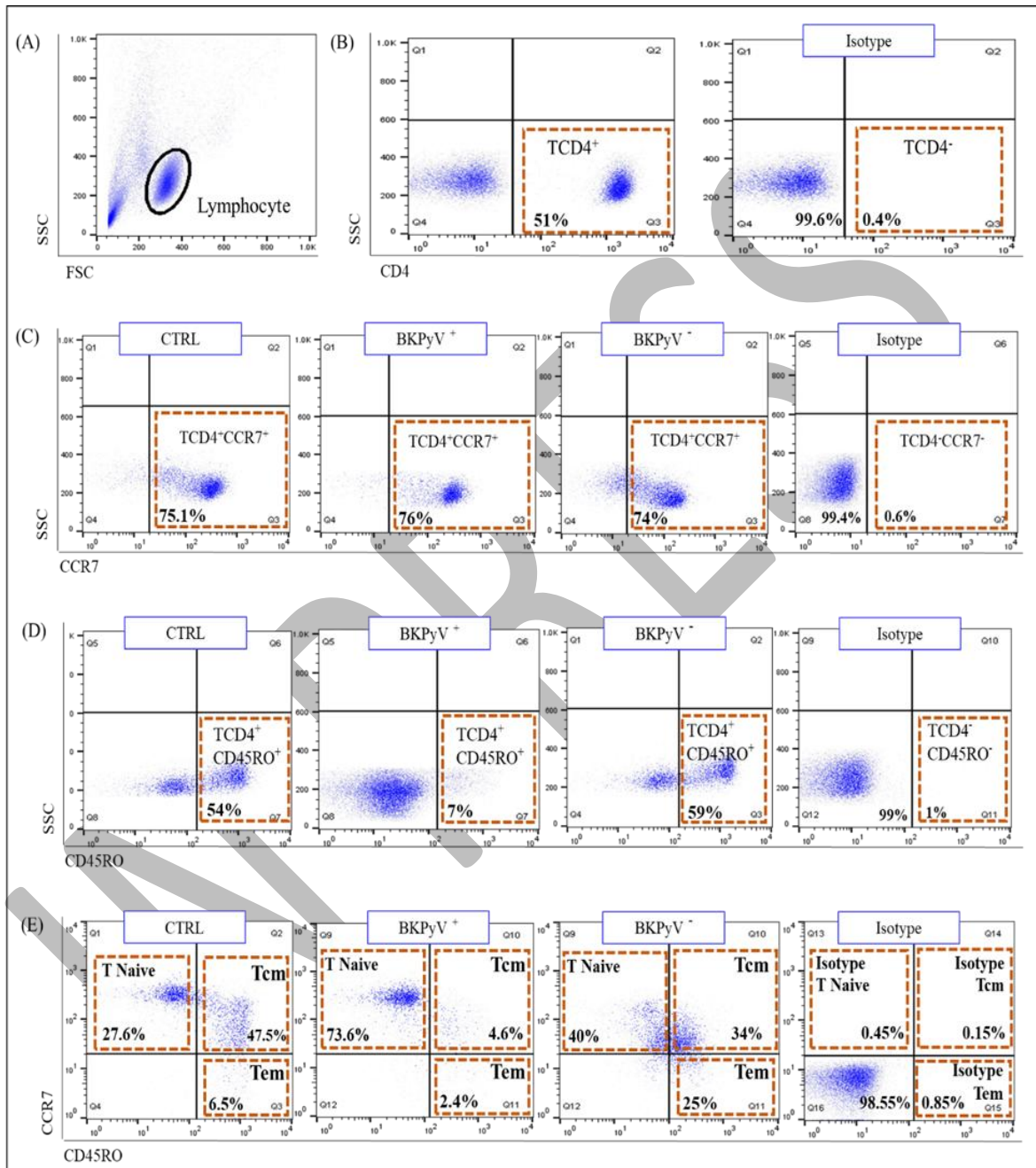


Figure 1. The gating strategy to detect the mentioned subsets of CD4⁺ T cell. A. Gating of lymphocytes was done considering FSC and SSC. B. Gating strategy of CD4⁺ T cells regarding FSC and CD4. C. CD4⁺ CCR7⁺ T cells were gated in KTRs with and without active BKPyV infection and the healthy control ones. D. Gating strategy of CD4⁺ T cells with CD45RO markers on their surfaces in studied groups. E. CD4⁺ T cell subsets gating strategy in all studied groups contain naive, Tcm, and Tem cells. On the right side of each row, the isotype of each used antibody and how to gate them are shown. Abbreviations: BKPyV: BK polyomavirus; CD: cluster of differentiation; CCR7: C-C chemokine receptor type 7; CTRL: control; FSC: forward scatter; KTR: kidney transplant recipient; SSC: side scatter; Tcm: central memory T cell; Tem: effector memory T cell.

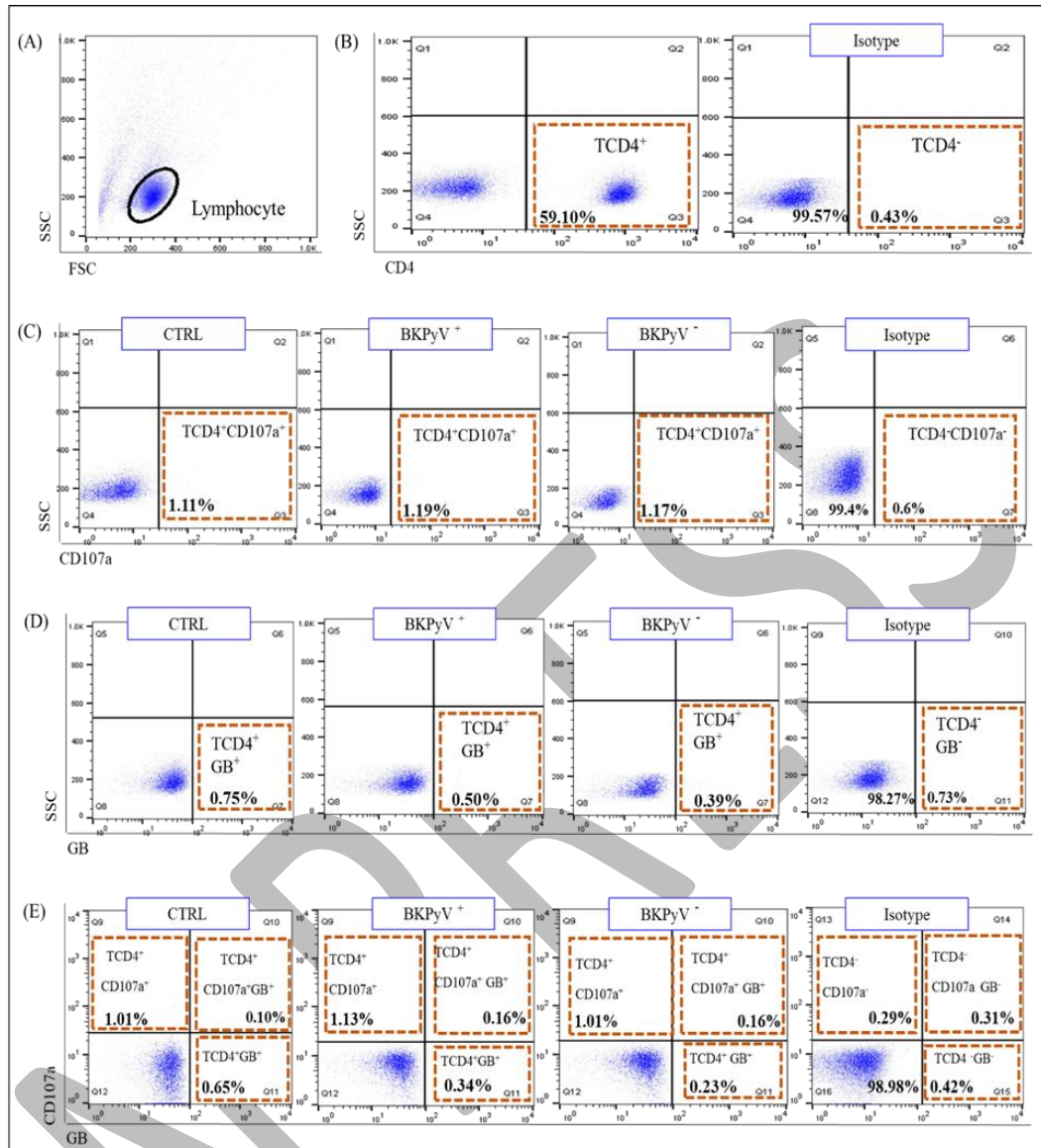


Figure 2. The gating strategy for different subsets of CD4⁺ T cells. **A.** According to FSC and SSC, gating of lymphocytes was done. **B.** Based on FSC and CD4 scatter, T cells with CD4 on their surfaces were gated. **C.** The gating method for CD4⁺CD107a⁺ T cells in KTRs with and without viral active infection and healthy controls are shown. **D.** CD4⁺Granzyme B⁺ T cells gating approach in studied groups. **E.** CD4⁺ T cell various phenotypes in BKPyV⁺, BKPyV⁻, and healthy control groups. On the right side of each row, the isotype of each used antibody and how to gate them are shown. Abbreviations: BKPyV: BK polyomavirus; CD: cluster of differentiation; CTRL: control; FSC: forward scatter; GB: granzyme B; KTR: kidney transplant recipient; SSC: side scatter.

Gene Expression

RNA Extraction and cDNA Synthesis

RNA was extracted from PBMCs using TRIzol reagent. A Nanodrop spectrophotometer device (Thermo Fisher Scientific, USA) was used to evaluate the purity and concentration of each RNA sample by optical density at 260/280 nm. cDNA synthesis was then

performed with a cDNA synthesis kit (Eurox, UK) according to the manufacturer's instructions.

SYBR Green real-time PCR

The expression levels of the studied TFs and *perforin* were quantified by SYBR Green real-time PCR. As an internal control, glyceraldehyde 3-phosphate

BKPyV T Antigens Modulate CD4⁺ T Cell Function

dehydrogenase (GAPDH) gene was used. The real-time PCR reactions were performed in a final volume of 20 μ L using a SYBR-Green PCR master mix (Eurox, UK). The ABI Step One Plus instrument (Applied Biosystems Step One Plus™) was used to run the reactions. The primer sequences for the studied genes, the program of

SYBR Green Real-Time PCR, and the mixture of reactions are listed in Table 2. The analysis of melting curves was performed to confirm the specificity of the amplification reaction. PCR efficiency for each gene was confirmed to ensure comparable amplification efficiency.

Table 2. Sequences of studied primers and real-time PCR conditions

Gene name	Primer sequence, 5' to 3'	Annealing temperature, °C	PCR condition	Reaction mixture
<i>Perforin</i>	F-GAGTGCCGCTTCTACAGTTT R-GGTGCCGTAGTTGGAGATA3AG	57	1 cycle: 95°C/5 min 40 cycles: 95°C/30 s; annealing/20 s; 72°C/30 s Followed by melt curve	10 μ L SYBR Green Master Mix; 0.2 μ L ROX solution; 1 μ L each forward and reverse primer (5 pm); 2 μ L cDNA template; water to 20 μ L
<i>T-bet</i>	F-TGACCCAGATGATTGTGCTCC R-CTCTCCGTCGTTACCTCAA	58	As above	As above
<i>GATA3</i>	F-CCTGTGGGCTCTACTACA R-GGTTTCTGGTCTGGATGC	56	As above	As above
<i>STAT3</i>	F-TTGTGATGCTTCCCTGAT R-GGTCTCTAGGTCAATCTTG	55	As above	As above
<i>STAT6</i>	F-CCAGATTTGGAAACGGCAGC R-CACCAGGCTTTCACACCTCT	58	As above	As above
<i>GAPDH</i>	F-GGACTCATGACCACAGTCCA R-CCAGTAGAGGCAGGGATGAT	58	As above	As above

cDNA: complementary DNA; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *GATA3*: GATA binding protein 3; PCR: polymerase chain reaction; ROX: carboxy-X-rhodamine; *STAT3*: signal transducer and activator of transcription 3; *STAT6*: signal transducer and activator of transcription 6; *T-bet*: T-box transcription factor TBX21.

Statistical Analysis

Receiver operating characteristic (ROC) curve analysis was performed using GraphPad Prism version 9.2.5 (GraphPad Software, Inc., San Diego, CA, USA) to evaluate the discriminatory performance of the studied TFs and *perforin* for distinguishing BKPyV-reactivated from non-reactivated KTRs. The area under the ROC curve (AUC) was calculated as a measure of classifier performance. However, given the very small sample size ($n = 5$ per group), the ROC analysis is highly susceptible to overfitting, and the reported AUC values, including those of 1.0, should be interpreted with considerable caution. In small datasets, ROC estimates are inherently unstable, and near-perfect AUC values are

likely to reflect sample-specific overfitting rather than true biomarker performance. The 95% confidence intervals illustrate the uncertainty associated with these estimates. Accordingly, the present findings should not be interpreted as evidence of validated clinical biomarker utility, and independent validation in larger, adequately powered prospective cohorts is required before any clinical conclusions can be drawn.

Data are presented as mean $\pm$ standard deviation (SD). Relative gene expression levels were calculated using the Livak ($2^{-\Delta\Delta CT}$) method. Flow cytometry data were analyzed using FlowJo version 10 (Tree Star Inc., Ashland, OR, USA).²

The selection of statistical tests was guided by the distributional characteristics of the data and the small sample size ($n = 5$ per group). Because normality could not be reliably confirmed in such small groups, non-parametric tests were considered the primary analytical approach. Accordingly, the Mann-Whitney U test was used for comparisons between two independent groups, whereas the Kruskal-Wallis test followed by Dunn's multiple-comparison test was used for comparisons among three groups. Parametric tests (Student's t-test and one-way ANOVA with appropriate post-hoc analysis) were applied only when distributional assumptions were considered acceptable. Spearman's rank correlation coefficient was used for all correlation analyses.

Due to the small sample size ($n=5$ per group), statistical tests had limited power, and results should be interpreted cautiously. As an exploratory pilot study, the analyses were intended to identify trends and generate hypotheses rather than provide definitive conclusions.

RESULTS

It is important to note that, in the present study, antigen-specific CD4⁺ T-cell identification was not achievable through the use of activation-induced markers such as OX40 (CD134) or CD25, due to technical and reagent limitations at the time of experimentation. Accordingly, all flow cytometry-based analyses reflect the behavior of total CD4⁺ T-cell populations following peptide stimulation, rather than confirmed BKPyV-specific T-cell responses. Therefore, the observed differences in naive, central memory, and effector memory CD4⁺ T-cell subsets between groups should be interpreted as stimulation-associated immunophenotypic changes rather than direct evidence of antigen-specific reactivity. This distinction is maintained throughout the presentation of results and is discussed in the context of experimental limitations. Table 3 shows the demographic data of the studied groups. The average of BKPyV load in the BKPyV reactivated group (BKPyV⁺) was 1522618 copies/mL.

CD4⁺ T Cell Subgroups' Evaluation and Analysis

After PBMC stimulation with the ST-Ag overlapping peptide pool, no significant difference was observed in T-cell subset frequencies between healthy

controls and BKPyV⁺ KTRs. The frequency of naive T cells ($p=0.67$) and central memory T cells ($p>0.9$) in BKPyV⁺ KTRs were the same as in healthy control individuals, and the frequency of effector memory T cells in BKPyV⁺ KTRs was lower than in healthy controls. Although a lower frequency was observed, the difference did not reach statistical significance ($p=0.33$; Figure 3). After stimulating the extracted PBMCs with a combined BKPyV tumor antigen overlapping peptide pool, a reduction was observed in the frequency of naive T cells (CD4⁺ CD45RO⁻CCR7⁺) in both KTRs with and without BKPyV activation compared with the control group ($p=0.14$; 95% CI=-13.94 to 63.02) and ($p=0.02$; 95% CI=10.44 to 41.96), respectively (Figure 5A). Similarly, the central memory T cells (CD4⁺ CD45RO⁺CCR7⁺) displayed a reduction in both KTRs with and without BKPyV activation groups compared with healthy individuals ($p=0.06$; 95% CI=-0.5125 to 37.19), and ($p=0.99$; 95% CI=-9.677 to 22.64), respectively (Figure 5B). In effector memory T cells (CD4⁺ CCR7⁻CD45RO⁺), a higher frequency was observed in KTRs without active viral infection compared with KTRs with active viral infection and the control group ($p=0.19$; 95% CI=-7.979 to 54.66) and ($p=0.99$; 95% CI=-28.68 to 13.96), respectively (Figure 5C). Analysis showed that the naive T cells with CD4⁺ CD45RO⁻ CCR7⁺ phenotype, and CD4⁺ T cells expressing CD107a and granzyme B were the most frequent subsets in KTRs with active BKPyV infection (Table 4).

BKPyV T Antigens Modulate CD4⁺ T Cell Function

Table 3. Studied groups' descriptive and demographic characteristics

Factor/group	Control	BKPyV ⁻	BKPyV ⁺	<i>p</i>
Number of participants	5	5	5	1
Gender of participants, N (%)				
Male	1 (20%)	3 (60%)	3 (60%)	1
Female	4 (80%)	2 (40%)	2 (40%)	1
Age, mean, y	30	38.4	46.2	0.97
Blood group, N (%)				
O	AB+, 4 (80%)	O-, 1 (20%)	O+, 3 (60%)	0.45
B	A+, 1 (20%)	B+, 3 (60%)	B+, 1 (20%)	0.45
AB	NA	AB+, 1 (20%)	AB-, 1 (20%)	1
Type of transplantation				
Cadaver donor	NA	5 (100%)	4 (80%)	0.37
Living donor	NA	0	1 (20%)	1
Underlying disease				
Hypertension	NA	5 (100%)	3 (60%)	0.36
Diabetes mellitus	NA	1 (20%)	1 (20%)	1
Other diseases	NA	2 (40%)	2 (40%)	1
Time since transplantation, mean±SD, mo	NA	9±2.55	9.2±2.28	0.9

BKPyV: BK polyomavirus; N: number; SD: standard deviation.

Table 4. Percentages of CD4⁺ T-cell subsets in BKPyV⁺ kidney transplant recipients

CD4 ⁺ T-cell phenotype	Percentage of each CD4 ⁺ T-cell subset
CCR7 ⁺ CD45RO ⁻	36.43±66.62
CCR7 ⁺ CD45RO ⁺	12.65±14.62
CCR7 ⁻ CD45RO ⁺	25.31±24
CD107a ⁺ Granzyme B ⁻	7.02±10.96
CD107a ⁺ Granzyme B ⁺	4.59±4.41
CD107a ⁻ Granzyme B ⁺	1.17±1.37

BKPyV: BK polyomavirus; CCR7: C-C chemokine receptor type 7; CD4: cluster of differentiation 4; CD45RO: cluster of differentiation 45RO; CD107a: cluster of differentiation 107a; KTR: kidney transplant recipient.

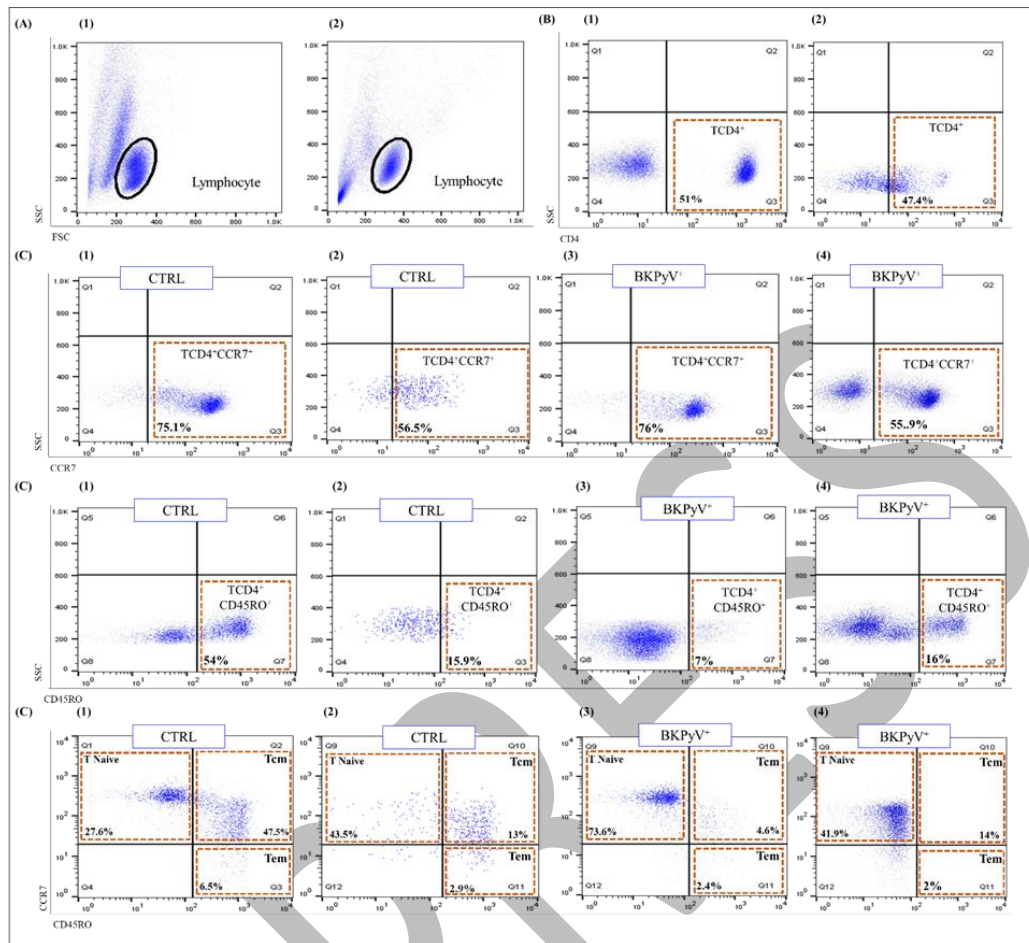


Figure 3. The gating strategy to detect CD4⁺ T cell subsets. **A.** Gating of lymphocytes was done according to FSC and SSC. **A. 1** showed the alive lymphocytes after stimulation with a mix of BKPyV tumor antigens, and **A. 2** showed the lymphocytes after stimulation with BKPyV ST-Ag. **B. 1** displays the gating of CD4⁺ T cells regarding FSC and CD4 in PBMCs stimulated with a mix of BKPyV tumor antigens and **B. 2** also showed the gating of CD4⁺ T cells regarding FSC and CD4 in PBMCs stimulated with BKPyV ST-Ag. **C.** The strategy of gating CD4⁺ CCR7⁺ T cells in KTRs with active BKPyV infection and healthy controls. **D.** Gating of CD4⁺ CD45RO⁺ T cells in BKPyV⁺ and control groups. **E.** CD4⁺ T cell subsets gating strategy in BKPyV⁺ KTRs and healthy control, including naive, Tcm, and Tem cells. (In all rows, figures 1 and 3 show the frequency after stimulation with a mix of BKPyV tumor antigens, and figures 2 and 4 show the cell frequency after stimulation with BKPyV ST-Ag overlapping peptide pool). BKPyV: BK polyomavirus; CD: cluster of differentiation; CCR7: C-C chemokine receptor type 7; CTRL: control; FSC: forward scatter; KTR: kidney transplant recipient; PBMC: peripheral blood mononuclear cell; SSC: side scatter; Tcm: central memory T cell; Tem: effector memory T cell.

Analysis of Secretion of CD107a and Granzyme B from CD4⁺ T Cells

After PBMC stimulation with the ST-Ag peptide pool, cytotoxicity-associated marker analysis showed a higher frequency of CD4⁺CD107a⁺GB⁻ cells in KTRs with active viral infection in comparison with healthy individuals ($p=0.9$), while lower frequencies were observed in these recipients for CD4⁺ CD107a⁺ GB⁺ T cells ($p=0.33$) and CD4⁺ CD107a⁻ GB⁺ T cells ($p=0.33$)

(Figure 4). Following PBMC stimulation with the combined BKPyV tumor antigens peptide pool, an increase was observed in the frequency of CD4⁺ T cells expressing CD107a (CD4⁺ GB⁻ CD107a⁺) in KTRs without active viral infection compared to control participants and KTRs with active viral infection ($p=0.99$; 95% CI=-39.70 to 13.78) and ($p=0.99$; 95% CI= -15.57 to 37.13), respectively (Figure 5D). The CD4⁺ T cells expressing granzyme B with CD4⁺ GB⁺

BKPyV T Antigens Modulate CD4⁺ T Cell Function

CD107a⁻ phenotype demonstrated a reduction in KTRs with BKPyV active infection and control group in comparison with KTRs without active infection ($p=0.99$; 95% CI= -6.390 to 13.93) and ($p=0.47$; 95% CI=-14.88 to 5.974) respectively (Figure 5E). In the subset of CD4⁺ T cells expressing both GB and CD107a with the CD4⁺GB⁺CD107a⁺ phenotype, a higher frequency was observed in BKPyV⁻ KTRs in comparison with KTRs with active viral infection and control cases ($p=0.99$; 95% CI= -2.510 to 7.902) and ($p=0.99$; 95% CI= -8.694 to 1.446) respectively (Figure 5F). Analysis showed that CD4⁺ T cells expressing CD107a (CD4⁺GB⁺CD107a⁺) were more frequent in KTRs with active viral infection than in KTRs without active viral infection (Table 4).

Analysis of the Levels of Studied Transcription Factors and *Perforin* Expression in PBMCs Isolated from the Studied Participants

The expression level of *T-bet* was significantly higher in KTRs without active infection compared to KTRs with active viral infection ($p=0.009$) (Figure 6A). A notable reduction was seen in the expression level of *GATA3* mRNA in BKPyV⁺ KTRs compared to KTRs without active BKPyV infection ($p=0.028$) (Figure 6B). Figures 6C and 6-D show a significant decrease in the expression levels of *STAT3* and *STAT6* in KTRs with BKPyV active infection in comparison with KTRs without active viral infection ($p=0.009$ in both of them). Also, the level of *perforin* expression has shown an increase in KTRs with active BKPyV infection compared to KTRs without active BKPyV infection (Figure 6E). Correlation at different levels was detected by analysis for studied mRNA expression. The expression level of *GATA3* shows a significant correlation with *STAT3* and *perforin*, and the *STAT3* expression level shows a significant relation to *STAT6* and *perforin* in KTRs with active BKPyV infection (Figure 7).

The Receiver Operating Characteristic (ROC) Curve for KTRs with and without BKPyV Active Infection

The studied genes' ROC curves were designed to display the categorized model performance at all thresholds of categories. Calculating the entire 2D region under the ROC curves was done by the area under the ROC curve (AUC). All details regarding the studied

gene's ROC curves are displayed in Figure 8 and Table 5, respectively. The consequences showed that *T-bet*, *STAT3*, and 6 ($p=0.009$, AUC =1) can have a rather crucial figure amongst other studied genes.

The Relation of Viral DNA Load and Studied Transcription Factors

Exploring the correlation between viral DNA load and the level of expression of studied genes was accomplished by using the Spearman test. These examinations displayed a considerable positive and direct correlation between the enhancement of all studied genes' expression (except for *T-bet*) and viral DNA load (Figure 9). Spearman rank correlation analysis demonstrated significant positive correlations between BKPyV DNA load and the expression of *GATA3* ($\rho=0.9$, $p=0.037$), *STAT3* ($\rho=1$, $p=0.001$), and *STAT6* ($\rho=0.9$, $p=0.037$), whereas *T-bet* showed no significant association ($\rho=0.3$, $p=0.624$). Given the five simultaneous comparisons, these findings should be interpreted cautiously due to potential type I error inflation; after Bonferroni correction ($p<0.01$), only the *STAT3* correlation remained statistically significant. The apparent discrepancy between the reduced overall expression of *STAT3* and *STAT6* in BKPyV-reactivated KTRs and their positive correlation with viral load likely reflects differences between between-group and within-group analyses. Specifically, among BKPyV-reactivated KTRs, increasing viral load may be associated with a compensatory upregulation of these TFs in response to greater antigen burden. However, this interpretation is speculative and warrants confirmation in larger cohorts.

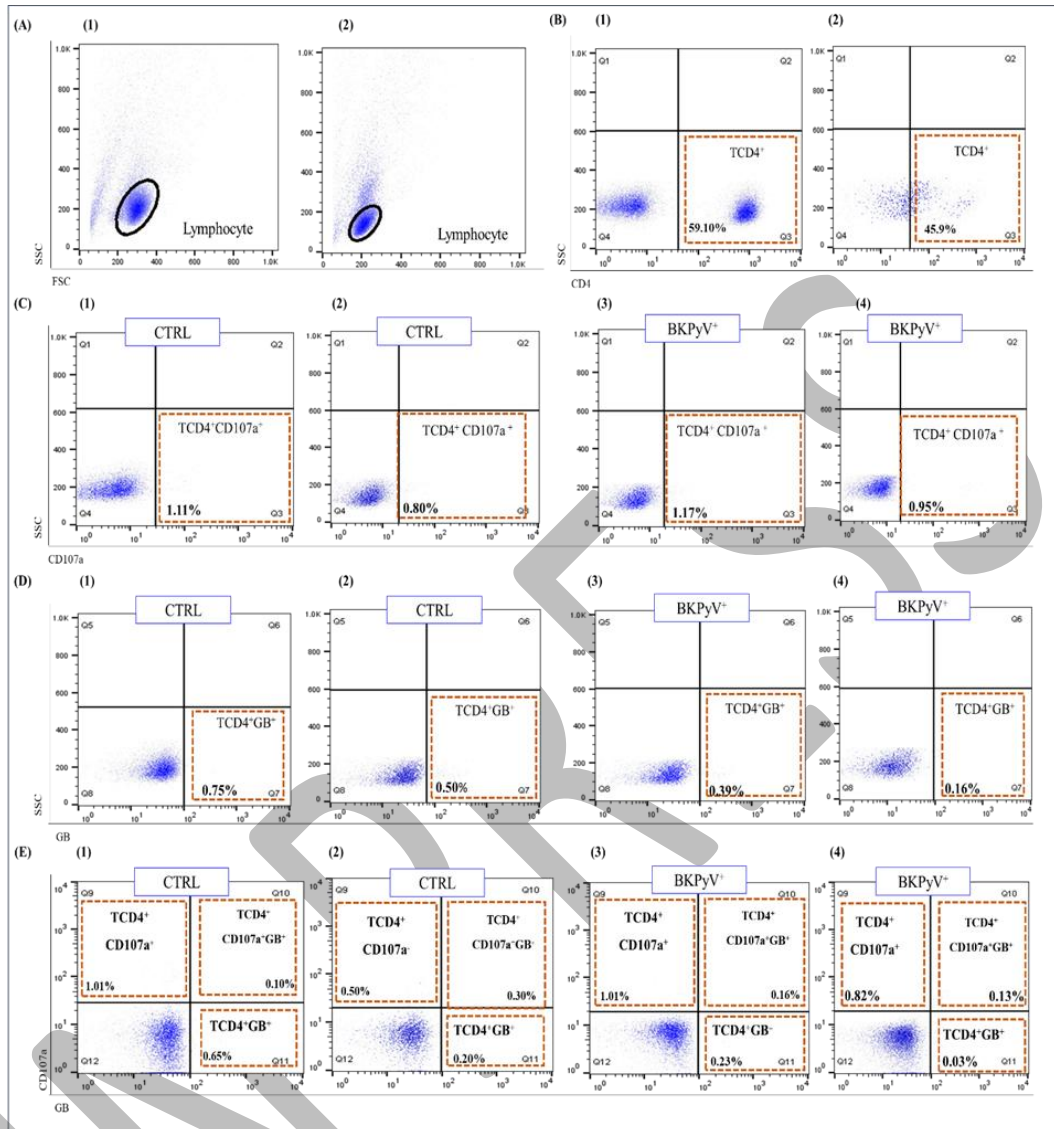


Figure 4. The gating strategy of different subsets of CD4⁺ T cells. **A.** Gating of lymphocytes was done regarding FSC and SSC. **B.** CD4⁺ T cells were gated based on FSC and CD4 scatter. **C.** The gating method evaluated the subset with CD4⁺ CD107a⁺ phenotype in KTRs with viral active infection and healthy controls. **D.** CD4⁺ Granzyme B⁺ T cells gating approach in BKPyV⁺ group and healthy individuals. **E.** Various phenotypes of CD4⁺ T cells in BKPyV⁺ and control ones. (In all rows, figures 1 and 3 show the frequency after stimulation with a mix of BKPyV tumor antigens, and figures 2 and 4 show the cell frequency after stimulation with BKPyV ST-Ag overlapping peptide pool). BKPyV: BK polyomavirus; CD: cluster of differentiation; CTRL: control; FSC: forward scatter; GB: granzyme B; KTR: kidney transplant recipient; SSC: side scatter.

BKPyV T Antigens Modulate CD4⁺ T Cell Function

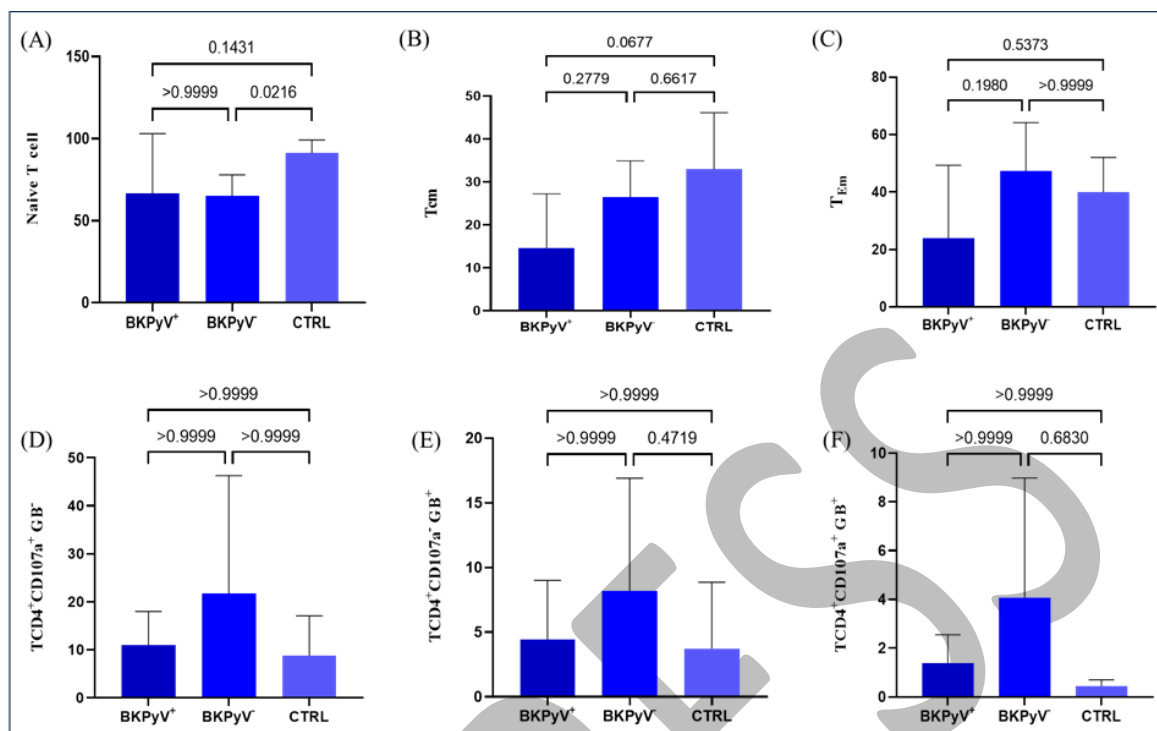


Figure 5. This graph demonstrates the mean difference of CD4⁺ T cells surface markers and also secretion of cytotoxic markers (CD107a and Granzyme B) $\pm$ SD between the studied groups (n=5). A. The frequency of Naive T cells (mean $\pm$ SD) in BKPyV⁺ and BKPyV⁻ KTRs and healthy controls. B. T_{cm} cell frequency (mean $\pm$ SD) in studied groups. C. Compared to the healthy control group, the T_{em} cell frequency (mean $\pm$ SD) in BKPyV⁺ and BKPyV⁻ KTRs. D. CD4⁺ GB⁻ CD107a⁺ T cells frequency (mean $\pm$ SD) in BKPyV⁺ and BKPyV⁻, and healthy group. E. The CD4⁺ CD107a⁺ GB⁺ T cells frequency (mean $\pm$ SD) in different studied groups. F. CD4⁺ GB⁺ CD107⁺ T cells' frequency (mean $\pm$ SD) in renal BKPyV⁺ and BKPyV⁻ transplant recipients and healthy individuals. BKPyV: BK polyomavirus; CD: cluster of differentiation; GB: granzyme B; KTR: kidney transplant recipient; SD: standard deviation; T_{cm}: central memory T cell; T_{em}: effector memory T cell.

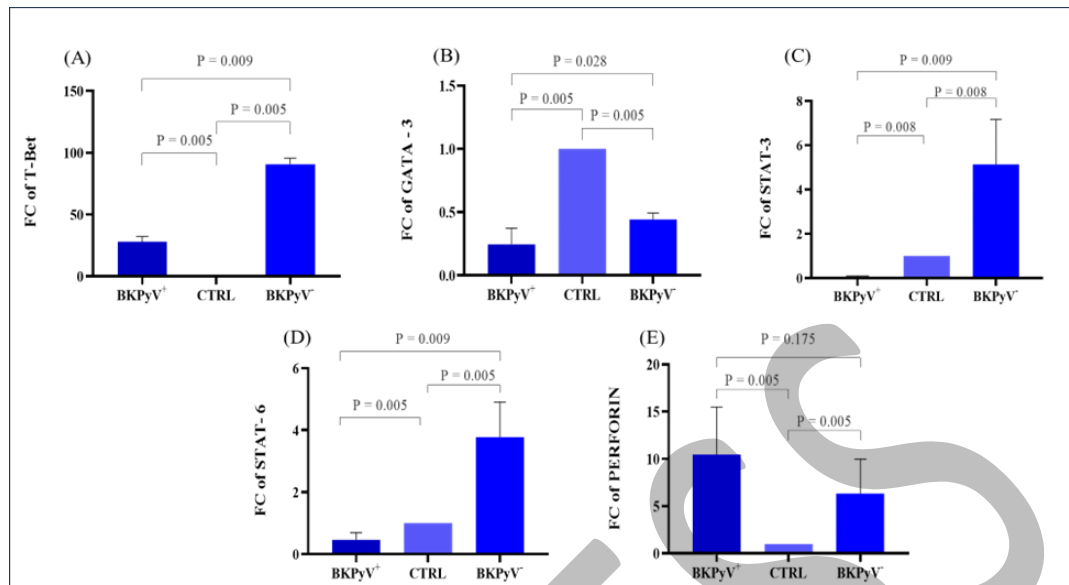


Figure 6. The levels of studied TF expression. **A.** The expression level of *T-bet* significantly increases in KTRs without active viral infection more than in KTRs with active BKPyV infection, and healthy controls. **B.** The expression level of the TF *GATA3* in control individuals is remarkably higher than in KTRs with and without active BKPyV infection. **C.** and **D.** Expression levels of *STAT3* and *STAT6* in KTRs without active BKPyV are notably more than BKPyV⁺ and healthy ones. **E.** In comparison with healthy healthy controls, the expression level of *perforin* in KTRs with active BKPyV infection is more than in KTRs without active BKPyV infection. The results indicate the mean $\pm$ SD in the studied groups. BKPyV⁺: BKPyV reactivated KTRs; CTRL: control group; BKPyV⁻: BKPyV non-reactivated KTRs. Abbreviations: BKPyV: BK polyomavirus; CTRL: control; KTR: kidney transplant recipient; SD: standard deviation; *STAT*: signal transducer and activator of transcription; TF: transcription factor.

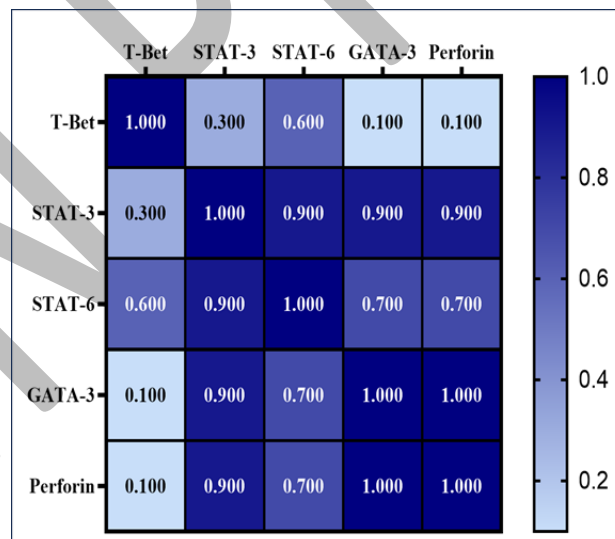


Figure 7. The figure indicates a matrix heatmap using the Spearman rank correlation test for expressed genes in KTRs with and without BKPyV infection PBMCs. The highest correlations are shown in dark blue, and the lowest are in bright blue. Dark blue cells are related to self-to-self gene expression correlations. The range of correlation coefficients is from 0.1 to 1. This heatmap displays *STAT3* and *STAT6*, *GATA3* and *perforin* with a 0.9 coefficient range have an excellent correlation. Abbreviations: BKPyV: BK polyomavirus; *GATA3*: GATA binding protein 3; KTR: kidney transplant recipient; PBMC: peripheral blood mononuclear cell; *STAT*: signal transducer and activator of transcription.

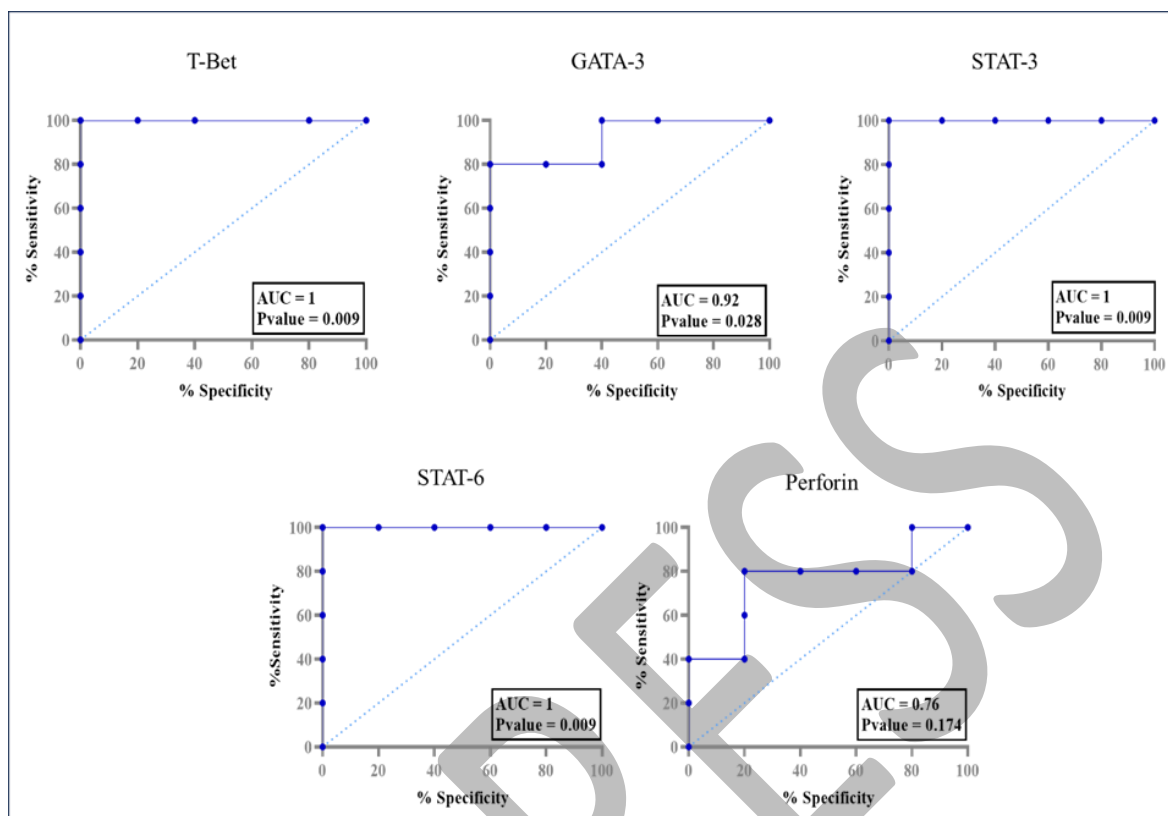


Figure 8. Studied genes ROC curve: An AUC=1 is shown for *T-bet*, *STAT3*, and *STAT6* genes ($p=0.009$), while for the other two genes, the quantity of the AUC declined to 0.92 for *GATA3* ($p=0.028$) and 0.76 for *perforin* ($p=0.174$), respectively. AUC: area under the receiver operating characteristic curve; *GATA3*: GATA binding protein 3; ROC: receiver operating characteristic; *STAT*: signal transducer and activator of transcription.

Table 5. Area under the curve, p values, cutoff values, sensitivity, and specificity of the studied genes

Gene name	AUC (95% CI)	p	Cutoff value	Sensitivity (95% CI)	Specificity (95% CI)
<i>T-bet</i>	1 (1–1)	0.009	<58	100 (56.55–100)	100 (56.55–100)
<i>GATA3</i>	0.92 (0.73–1)	0.028	<0.33	80 (37.55–98.97)	100 (56.55–100)
<i>STAT3</i>	1 (1–1)	0.009	<1.382	100 (56.55–100)	100 (56.55–100)
<i>STAT6</i>	1 (1–1)	0.009	<1.798	100 (56.55–100)	100 (56.55–100)
<i>Perforin</i>	0.76 (0.43–1)	0.174	>6.37	80 (37.55–98.97)	80 (37.55–98.97)

AUC: area under the receiver operating characteristic curve; CI: confidence interval; *GATA3*: GATA binding protein 3; KTR: kidney transplant recipient; ROC: receiver operating characteristic; *STAT3*: signal transducer and activator of transcription 3; *STAT6*: signal transducer and activator of transcription 6; *T-bet*: T-box transcription factor TBX21.

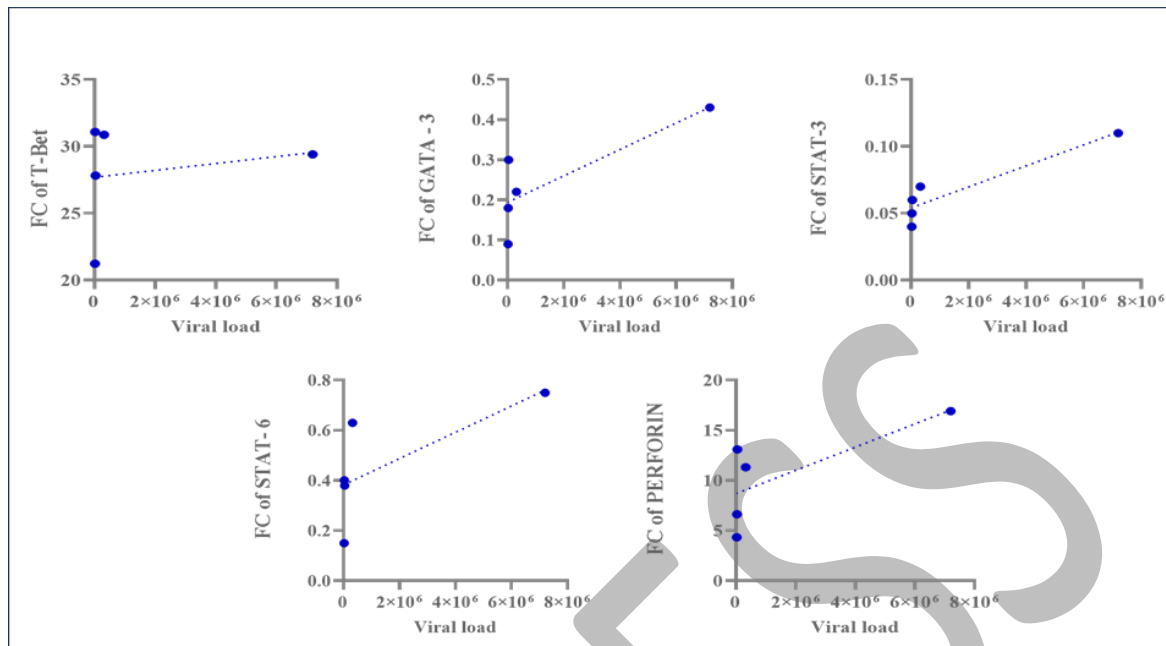


Figure 9. The correlation between the viral load of KTRs with BKPyV infection and the levels of studied gene expression. A notable positive correlation was found between all genes and viral load (Except for *T-bet*). Abbreviations: BKPyV: BK polyomavirus; KTR: kidney transplant recipient; *STAT*: signal transducer and activator of transcription.

DISCUSSION

To contextualize the present findings, it is informative to compare them with our previous investigation evaluating BKPyV LT-Ag alone.² In that study, LT-Ag stimulation was associated with impaired CD4⁺ T-cell differentiation in BKPyV-reactivated KTRs, characterized by an increased proportion of naïve CD4⁺ T cells accompanied by reduced central and effector memory subsets. A broadly similar pattern was observed in the present study following combined LT-Ag/ST-Ag stimulation, suggesting that the addition of ST-Ag did not markedly alter the overall distribution of CD4⁺ T-cell subsets. In contrast, stimulation with ST-Ag alone produced only modest, statistically non-significant changes in naïve, central memory, and effector memory CD4⁺ T-cell frequencies. These observations suggest that the immunomodulatory effects observed after combined stimulation may largely reflect LT-Ag activity, although additive or modulatory contributions of ST-Ag cannot be excluded. Because LT-Ag alone was not included as a parallel experimental condition in the current study, it remains uncertain whether the combined response reflects true antigen synergy or predominantly LT-Ag-driven effects. Future studies incorporating independent LT-Ag, ST-Ag, and

combined stimulation conditions will be required to clarify the relative contribution of each viral antigen.

Following stimulation with the combined BKPyV tumor antigen peptide pool, BKPyV-reactivated KTRs exhibited a higher frequency of naïve CD4⁺ T cells and reduced central and effector memory subsets compared with BKPyV-negative recipients. Consistent with these findings, *STAT3*, *STAT6*, *GATA3*, and *T-bet* expression was significantly decreased, while *perforin* showed a non-significant upward trend, collectively suggesting altered CD4⁺ T-cell differentiation during BKPyV reactivation. These results are consistent with previous studies demonstrating the importance of BKPyV-responsive CD4⁺ T-cell immunity in controlling viral replication after kidney transplantation,^{6,18,21–23} as well as reports linking expansion of BKPyV-specific T-cell populations following immunosuppression reduction with improved viral control.^{1,24–28} In addition, both CD4⁺ and CD8⁺ T cells have been shown to respond to BKPyV tumor antigens.^{5,29,30} However, because antigen-specific T cells were not directly identified in the present study, these findings should be interpreted with caution.

An important methodological consideration in the interpretation of these findings pertains to the distinction between total CD4⁺ T-cell responses and BKPyV-specific CD4⁺ T-cell responses. Given the absence of

activation-induced marker analysis (including OX40 and CD25, which are standard tools for identifying antigen-reactive T cells) it should be noted that the immunophenotypic differences observed following peptide stimulation cannot be attributed exclusively to BKPyV-specific CD4⁺ T-cell responses. The responses measured represent stimulation-associated changes in total CD4⁺ T-cell compartments. Although the use of overlapping peptide pools derived from BKPyV LT-Ag and ST-Ag is expected to enrich for antigen-responsive cells, definitive conclusions regarding true antigen specificity cannot be drawn from the present data alone. Consequently, the findings should be interpreted as reflecting peptide-induced changes within total CD4⁺ T-cell populations rather than definitively BKPyV-specific CD4⁺ T-cell responses.

The current study assessed CD4⁺ T-cell differentiation following stimulation with a pool of BKPyV LT-Ag and ST-Ag by evaluating the surface markers of CD4⁺ T cells such as CD45RO and CCR7. The results demonstrated that the naive CD4⁺ T-cell subset was more prevalent in BKPyV⁺ renal recipients, which may contribute to altered antiviral immune responses and potentially promote viral persistence. Conversely, the other CD4⁺ T-cell subsets, such as central and effector memory T cells, were more prevalent in KTRs without active BKPyV infection. To estimate the cytotoxicity of CD4⁺ T cells, cytotoxicity-associated markers such as CD107a and granzyme B were measured. The interpretation of CD107a and Granzyme B expression as evidence of CD4⁺ T-cell cytotoxicity requires caution. Although CD107a is widely used as a marker of degranulation, it may also be upregulated during non-cytolytic activation, while Granzyme B can be expressed by activated CD4⁺ T-cell subsets independent of direct cytotoxic function. Accordingly, CD107a and Granzyme B were used as surrogate markers of cytotoxic potential rather than definitive evidence of cytotoxicity. Direct functional assays, including target cell killing, ⁵¹Cr-release, or live-cell cytotoxicity assays, were not performed. In addition, IFN- γ , TNF- α , and IL-2 production was not assessed, limiting comprehensive evaluation of CD4⁺ T-cell effector function. Therefore, the identified cells are described throughout the manuscript as exhibiting a cytotoxic-associated phenotype rather than confirmed cytotoxic CD4⁺ T cells. The results demonstrated that CD4⁺CD107a⁺Granzyme B⁻ T cells were more frequent in BKPyV-negative KTRs than in the other CD4⁺ T-cell

subsets. Similarly, Ambalathingal et al reported that BKPyV-specific CD4⁺ and CD8⁺ T-cell activation in response to LT-Ag and ST-Ag declines with increasing viral load compared with healthy controls.¹³ Consistent with these findings, stimulation of PBMCs from BKPyV-reactivated recipients with BKPyV early antigens in the present study showed a predominance of naïve CD4⁺ T cells. A cytotoxicity-associated phenotype was assessed using CD107a and granzyme B expression, with CD4⁺CD107a⁺Granzyme B⁻ cells representing the predominant subset in recipients without active BKPyV infection. Furthermore, Zhou et al demonstrated that most healthy seropositive individuals harbor BKPyV-specific CD4⁺ and CD8⁺ T cells capable of expressing CD40L, TNF, IFN- γ , Granzyme B, and CD107a following antigen stimulation, suggesting an important role for virus-specific CD4⁺ T cells in maintaining memory responses and antiviral immunity.³⁰ TFs are critical regulators of CD4⁺ T-cell differentiation into memory and effector subsets.³¹ An important methodological caveat in the interpretation of transcription factor gene expression data presented herein relates to the cellular source of the measured transcripts. *STAT3*, *STAT6*, *GATA3* and *T-bet* mRNA expression levels were quantified in total PBMC preparations rather than in fluorescence-activated cell-sorted CD4⁺ T-cell fractions. Consequently, the observed differences in TF expression between groups cannot be attributed exclusively to CD4⁺ T cells, as other immune cell populations present within PBMCs including CD8⁺ T cells, natural killer (NK) cells, B cells, monocytes, and innate lymphoid cells may express these TFs and contribute to the bulk mRNA signal. This limitation precludes direct mechanistic inference regarding the relationship between TF expression and CD4⁺ T-cell subset differentiation as measured by flow cytometry. While the pattern of reduced *STAT3*, *STAT6*, *GATA3*, and *T-bet* expression in BKPyV-reactivated KTRs is consistent with impaired T-cell differentiation, this remains an associative observation. Future studies employing cell sorting or single-cell transcriptomic approaches would be necessary to establish cell-type-specific TF expression profiles and to confirm the direct contribution of these regulators to the CD4⁺ T-cell phenotypes observed in this study. In the present study, the expression of *STAT3*, *STAT6*, *GATA3*, and *T-bet* was evaluated in PBMCs from all study groups. Previous studies have reported reduced expression of these transcriptional regulators in virus-specific CD4⁺ T cells,

although BKPyV stimulation may increase *T-bet* expression ex vivo.¹⁰ *STAT6* has been shown to inhibit Th1 differentiation by suppressing IL-12 and *STAT4* signaling,³² whereas *GATA3* directs naïve CD4⁺ T-cell differentiation toward the Th2 lineage.³³ Co-expression of *GATA3* and *T-bet* has also been implicated in maintaining T-cell effector plasticity in lymphocytic choriomeningitis virus infection.^{34–36} Consistent with these observations, the present study demonstrated significantly reduced expression of *T-bet*, *GATA3*, *STAT3*, and *STAT6* in KTRs with active BKPyV infection compared with the other study groups. Previous studies have further highlighted roles for *STAT3* in ischemia/reperfusion-associated acute kidney injury and post-transplant immune regulation.^{37,38}

Inflammatory activity was assessed by *perforin* expression, which was higher in BKPyV-reactivated KTRs than in BKPyV-negative recipients. This finding is consistent with previous studies reporting reduced *T-bet* expression accompanied by increased granzyme and *perforin* expression in BKPyV-reactivated KTRs.¹³ Correlation analysis demonstrated significant associations between BKPyV DNA load and the expression of all investigated genes except *T-bet*. Previous studies have shown that persistent BKPyV replication may influence *T-bet* expression, although no significant correlation with viral load has been consistently observed.^{39,40} Similarly, while *GATA3* expression has been reported to increase following BKPyV reactivation, its correlation with viral load remains inconsistent.^{41,42} *STAT3* activation has also been implicated in viral reactivation and may be associated with BKPyV load,^{13,43} whereas *STAT6* is activated in response to viral DNA and promotes chemokine expression.⁴⁴ Although BKPyV infection has been linked to increased *perforin* expression, no significant association between *perforin* levels and viral DNA load was observed in the present study, consistent with previous reports.⁴⁵ ROC curve analysis yielded AUC values of 1.0 for *T-bet*, *STAT3*, and *STAT6*, and 0.92 for *GATA3*, within the present sample. While these results suggest discriminatory potential, the perfect or near-perfect AUC values should be interpreted conservatively given the very small sample size ($n = 5$ per group). ROC analysis with such limited sample sizes is highly susceptible to overfitting, and the reported AUC values almost certainly overestimate true potential biomarker performance in the general transplant population. The 95% confidence intervals provided in

Table 5 reflect the wide uncertainty surrounding these point estimates. Accordingly, the present findings are considered preliminary and should not be construed as evidence of validated clinical utility. Independent validation in larger, prospectively designed cohorts with appropriate correction for multiple testing is a prerequisite before these TFs can be proposed as biomarkers for high-risk BKPyV infection.

The conclusion that BKPyV ST-Ag promotes immune evasion should be regarded as a working hypothesis rather than a mechanistically demonstrated finding within the scope of the present study. The observed immunophenotypic patterns including the relative accumulation of naïve CD4⁺ T cells, the reduced frequency of memory subsets, and the decreased expression of key differentiation TFs in BKPyV-reactivated KTRs are consistent with, but do not directly demonstrate, ST-Ag-mediated immune evasion. Several alternative explanations must be considered: (A) Chronic antigen exposure and T-cell exhaustion: persistent high-level BKPyV replication may lead to chronic TCR stimulation, resulting in T-cell exhaustion characterized by reduced effector differentiation and functional impairment, independent of a direct immunomodulatory effect of ST-Ag; (B) Immunosuppressive therapy effects: all KTRs in the present study received a standardized triple immunosuppression regimen including mycophenolate mofetil, cyclosporine, and prednisolone. These agents are known to broadly suppress T-cell proliferation, differentiation, and cytokine production, and their confounding contribution to the observed CD4⁺ T-cell phenotypes cannot be excluded; (C) Host immune variability: individual differences in HLA haplotype, pre-existing BKPyV-specific immunity, and baseline immune status may markedly influence CD4⁺ T-cell responses independently of viral antigen effects; (D) Bystander immune activation: stimulation with overlapping peptide pools may induce non-specific T-cell activation in addition to, or instead of, antigen-directed responses, given the absence of AIM-based specificity confirmation. In light of these alternative explanations, mechanistic claims regarding ST-Ag-driven immune evasion are not made directly, and the relevant conclusions are framed as working hypotheses to guide future experimental investigation.

Limitations

The primary limitation of this study is the small sample size ($n = 5$ per group), which limits statistical power and the generalizability of the findings. Accordingly, both non-significant and significant results should be interpreted cautiously and require validation in larger, independent cohorts. In addition, the absence of antigen-specific activation markers (e.g., OX40, CD25, and CD69) prevented the precise identification of BKPyV-specific CD4⁺ T cells, and virus-unrelated peptide controls (e.g., CMV or EBV) were not included to account for background immune responses. Furthermore, logistical constraints precluded validation of TFs such as *T-bet*, *STAT3*, and *STAT6* as biomarkers in an independent cohort. Despite these limitations, the findings provide a preliminary framework for understanding the effects of BKPyV tumor antigens on CD4⁺ T-cell differentiation and function in KTRs and support further investigation in larger, well-controlled studies.

The present study provides preliminary evidence that BKPyV ST-Ag, alone or in combination with LT-Ag, may alter CD4⁺ T-cell differentiation during active BKPyV reactivation in KTRs. Stimulation with BKPyV tumor antigens was associated with a relative accumulation of naïve CD4⁺ T cells and a reduction in central and effector memory subsets, suggesting impaired antigen-driven T-cell maturation. This phenotype was accompanied by reduced expression of *T-bet*, *GATA3*, *STAT3*, and *STAT6* in PBMCs from BKPyV-reactivated recipients, supporting the concept of a dysregulated T-cell differentiation program during viral reactivation. These findings indicate that CD4⁺ T-cell subset distribution and the expression of selected transcription factors may warrant further investigation as complementary immunological markers in BKPyV surveillance, in conjunction with established virological parameters such as viral load. In addition, the observed impairment of responses following ST-Ag stimulation supports further investigation of ST-Ag as a potential target for future BKPyV-specific immunotherapeutic strategies, including adoptive T-cell therapy and peptide-based vaccine approaches. Given the exploratory design and limited sample size, these results should be interpreted cautiously. Validation in larger prospective cohorts using antigen-specific T-cell identification and functional assays will be required before clinical application. Nevertheless, this study provides a foundation for future hypothesis-driven

investigations into how BKPyV tumor antigens shape CD4⁺ T-cell immunity and contribute to immune evasion in BKPyVAN.

STATEMENT OF ETHICS

The studies involving human participants were reviewed and approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1399.723). The participants provided their written informed consent to participate in this study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Microsoft Copilot was used solely to assist with language editing, proofreading, and improvement of manuscript clarity. The authors reviewed and verified all AI-assisted content and take full responsibility for the accuracy and integrity of the final manuscript.

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BKPyV T Antigens Modulate CD4⁺ T Cell Function

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