ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2024; 23(3):272-287.
DOI: 10.18502/ijaai.v23i3.15637

Polyomavirus BK-Specific CD4$^+$ T Cells Response to VP1 Stimulation in Kidney Transplant Recipients

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Received: 25 December 2023; Received in revised form: 17 March 2024; Accepted: 28 March 2024

ABSTRACT

Reactivation of Polyomavirus BK (BKPyV) is related to reduction of T cells response in kidney transplant recipients (KTRs). Here, we examined the differentiation of CD4$^+$ T cells subsets in response to BKPyV KTRs, using the BKPyV VP1 (viral capsid protein 1) as a stimulator.

We categorized our samples into three distinct groups: 1. Reactive BKPyV (BKPyV$^+$), 2. non-reactive (BKPyV$^-$) KTRs and 3. Healthy controls. BKPyV$^-$ KTRs and healthy controls stimulated with VP1 and BKPyV+ unstimulated with VP1. The human CD4$^+$ T cells was stimulation with VP1-Ag. The proportion of CD4$^+$ T lymphocytes and their various subsets, including naive T cells, central memory T cells (TCM), and effector memory T cells (TEM) was measured using flowcytometry.

BKPyV$^-$ KTRs VP1$^+$ indicated significantly lower TCM CD4$^+$ T cells in contrast with both BKPyV$^+$ KTRs VP1$^-$, and healthy controls VP1$^+$. This indicates that VP1 stimulation may reduce TCM cell levels in these patients. The percentage of TEM in the BKPyV$^-$ KTRs VP1$^+$ group was significantly less prevalent than the BKPyV$^+$ KTRs VP1$^-$ group. The percentage of TEM cells in BKPyV$^+$ KTRs VP1$^-$ was significantly lower than the healthy controls VP1$^+$. Stimulation with VP1 protein significantly increased the frequency of cytotoxic CD4$^+$ T cells in BKPyV$^-$ KTRs VP1$^+$ compared to BKPyV$^+$ KTRs VP1$^-$.

The present research has shown that the VP1 stimulation of CD4$^+$ T cells can induce cytotoxic CD4$^+$ T cells responses that may help overcome BKPyV infection in KTRs. However, VP1 stimulation may also differentially affect TCM and TEM CD4$^+$ T cells subsets.

Keywords: CD4$^+$ T cell; Kidney transplant; *Polyomavirus BK*; VP1 protein

INTRODUCTION

Polyomavirus BK (BKPyV) is a small circular double-stranded DNA genomes approximately 5 kb, leading to opportunistic infections in kidney transplant recipients (KTRs) and is the predominant cause of acute kidney failure in these patients.[1] Maternal antibodies against BKPyV offer protection against newborns

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during the initial months of life, and infections can occur after the disappearance of antibodies, which has been observed in 10-30% of infants,[2] and 65-90% of 5-to 10-year- old children.[3-6]

The three structural proteins that comprise the viral capsid; VP1, VP2, and VP3[7,8] that are responsible for shielding the viral genome and facilitating viral entry into the host cells by binding to cell surface receptors.[9,10] Among viral capsid, VP1 is the most antigenic capsid protein. Antibodies generated against VP1 are capable of blocking viral entry and preventing viral infection,[11] developing effective treatments and vaccinations targeting the BK virus requires an understanding of the structure and function of VP1.

In KTRs, reactivation of BKPyV can cause various clinical conditions, such as tubulointerstitial nephritis, hemorrhagic cystitis, ureteric stenosis, allograft failure, and BKPyV-associated nephropathy (BKVAN).[12-14] Up to 10% of KTRs may develop BKVAN, with 45% of KTRs eventually having irreversible kidney transplant rejection.[15-17] After kidney transplantation, the main issue that causes BKVAN is virus reactivation.[18] In addition, immunosuppressive therapy in solid-organ transplant recipients can lead to viral reactivation and subsequent clinical diseases.[19,20]

Considering the fact that there is no approved drug for treatment of BKPyV infection in KTRs, and most of the scientists believe that the only standard therapy is reducing the amount of immunosuppressive drug regimens. Using other therapeutic approaches, such as cytotoxic T cell (CTL)-based immunotherapy has been emerged as an attractive approved treatment strategy. The adoptive transfer of donor's activated virus-specific T cells is a potential immunotherapeutic strategy to restore viral immunity in patients with donor-derived memory VSTs (Virus-Specific T-Cell). This strategy has definitely the potential to restore baseline kidney function and reduce acute rejection (AR) post-transplant complications.[21,22]

Polyfunctional BKPyV-specific cell responses are crucial for preventing BKPyV viremia and viruria, according to studies in KTRs with BKPyV reactivation. However, it appears that $CD4^+$ T cells are more effective in response to BKPyV infection.[13] Additionally, it has been found that the capacity of $CD4^+$ T cells has direct regulatory abilities via generation of proinflammatory cytokines including IL-1α, IL-1β, IL-6 and IL-12. Notably, these activities are possible even in the absence of immunity from $CD8^+$ T cells.[23]

Based on their phenotype and activity, $CD4^+$ T cells can be classified into naive, central memory (TCM), and effector memory (TEM).[24] Overall, $CD4^+$ T cells can recognize and respond to BKPyV antigens, including VP1, leading to a particular immune response against the virus infection.[25]

Cytotoxic $CD4^+$ T lymphocytes are a subset that has the ability to directly kill the infected cells.[26] Cytotoxic molecules including granzyme B, perforin, and CD107a (LAMP-1) are expressed in these cells. CD107a (LAMP-1) is as a marker for degranulating lymphocytes, which is the process by which cytotoxic T cells release their cytotoxic molecules.[27] The primary method by which cytotoxic lymphocytes destroy virus-infected or altered cells is perforin/granzyme B-induced apoptosis.[28] In the present investigation, our aim was to examine the BKPyV-Specific CD4+ T Cells Response to VP1 Stimulation in KTRs. Additional research is necessary to fully find out the complex function of cytotoxic $CD4^+$ T cells in BK virus reactivation.

MATERIALS AND METHODS

Study Design and Patient Population

Every sample was taken from patients who were admitted to Abu Ali Sina Hospital and the Shiraz Organ Transplant Center, from December 2017 to November 2021. Overall, 10 patients with BKPyV reactivated KTRs (BKPyV-DNA levels>10,000 copies/ml) ($BKPyV^+$ KTRs) and 10 patients without active ones ($BKPyV^-$ KTRs) were included, all donors were cadavers, and 10 healthy controls as control during the same period with no history of other infectious diseases such as hepatitis C virus, hepatitis B virus, cytomegalovirus, human immunodeficiency virus (HIV), or adenovirus from the Iranian Blood Transfusion Organization, Shiraz, Fras, Iran. In this study $BKPyV^-$ KTRs and healthy controls stimulated with VP1 and $BKPyV^+$ unstimulated with VP1. Real-time qPCR was used to assess BKPyV-DNAemia.

All of the individuals who participated in the study were adult people (more than 18 years). KTRs were without the history of allograft failure and other viral infections including adenoviruses, human immunodeficiency virus, cytomegalovirus, hepatitis B, and C were excluded from this investigation. Written informed consent was given by each participant. Immunosuppressive regimen was administered to all patients as follows: initial dose of 5 mg/kg cyclosporine;

a maintenance starting dose of 2.5 mg/kg; initial doses of prednisolone with 120 mg/day followed by 10 mg/day; and twice daily mycophenolate mofetil. Table 1 shows the study participants' demographic information.

Detecting Quantitative Real-time PCR for BKPyV DNA

Reactivation of BKPyV in renal transplant patients was achieved by quantitative PCR. BKPyV viral load DNA was measured using a qRT-PCR kit (Gene Proof, Czech Republic) by the manufacturer's instructions. Quantitative analysis of the BKPyV PCR amplification identified the viral reactivation.

Isolating $CD4^+$ Cells

The whole blood was separated into PBMCs using a density gradient centrifugation technique over Ficoll-Hypaque. (Lymphodex. Inno-Train-Spain). We detected cryopreservation in serum-free freezing solution supplemented by 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS) (Gibco Laboratories, NY, USA). Consequently, we performed freezing protocol for each cryovial; a total amount of 1.8 ml was required to freeze peripheral blood cells, consisting of $2*10^6$ PBMC per vial, which yielded sufficient viable cells for this assay. Lastly, the cells were frozen suspensions at -196°C in liquid nitrogen until they were treated. In the cryopreservation media, containing RPMI 1640 complete medium (supplemented with L-glutamine (2 mM), 10% FBS, and 1% antibiotic mixture (penicillin-streptomycin 5 mg/ mL) for cell lines at 37°C±0.5°C and 5% CO2, the PBMCs were fully thawed for 2h before each experiment. Isolated $CD4^+$ T cell differentiation was performed using these cells.

From PBMCs, $CD4^+$ cells were separated by commercial magnet bead kits and collected (CD4 Cell Isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain a highly enriched population of human $CD4^+$ cells. For enriching $CD4^+$T cells from PBMCs, negative selection protocol is used for $CD4^+$ cells purification, which is indirectly performed using a cocktail of biotinylated antibodies (Cat no. 130-096-533, Miltenyi Biotech). Negative fractions were obtained and collected after being PBS-washed with ice, and live cells counting was performed using a hemocytometer.

Stimulating $CD4^+$ T Cells through VP1 Peptide

A peptide mixture (PepMix) consisting of different 15-mer BK viral peptides that overlapped with 11 amino acids of the main capsid protein VP1 of the BK virus (JPT Peptide Technologies, Berlin, Germany) was used (Swiss-Prot ID: P14996). For each peptide, 1.5 mM (2.5 g/l) DMSO and phosphate-buffered saline (PBS) were used to reconstitute the peptide mix. The overlapping peptide pool of the BK virus was treated with PBMCs ($0.5\text{-}1\times10^7$) 1 g of peptide per milliliter for 16 hours at 37ºC with 5% CO2.

Activating the Isolated $CD4^+$ T Cells with the LCL

Autologous LCL was used to stimulate $CD4^+$ T cells as antigen-presenting cells for 16 h at a ratio of 10:1 with BKPyV -PPM (5 µg/mL). The monoclonal antibody purified fraction was stained with antihuman monoclonal antibodies (mAb) specific for $CD4^+$ and $CD3^+$T cells (to measure the T cell percentage, i.e., "T cells Contamination"). Cell debris differentiation from $CD4^+$ T cells was detected using side and forward scatter (SSC and FSC).

Flow Cytometric Analysis

The cell surface staining was done using the panels of mouse anti-human neutralizing Mabs as previously described[7]; also, we used the degranulation marker for cells, including PE anti human perforin Antibody (353303, Biolegend, USA), FITC anti human/mouse granzyme B Recombinant Antibody (372205, Biolegend, USA), and PerCP/Cyanine5.5 anti-human CD107a (LAMP-1) Antibody (328615, Biolegend, USA). In addition, 2 µg/ml monensin (Rumensin, Eli Lilly & Co.Canada) was added to VP1 of the BK virus.

The solution of cells was combined with the antibody solution over a 20-minute incubation period of 4 h in the dark. Antibody titration was carried out to obtain their optimal antibody dosage. Nonspecific antibody binding was blocked by preincubating the mediums containing 10% heat-inactivated of fetal bovine serum (56 degrees C for 40 minutes). Unstained controls and fluorescence minus one (FMO) control were used to determine the cutoff point between the background, proof of nonspecific binding, and set negative/positive borders. Isotype controls are primary antibodies used to account for helping differentiate nonspecific background signals as previously described[7]. A BD FACSCalibur device was used to collect data for 20,000 events while cells were suspended in a staining solution. FlowJo-v10 software (TreeStar) was utilized to evaluate the expression ratio of the studied $CD4^+$ T cell markers.

Fluorochrome Standard Beads

FITC, PE, or PERCPCY5.5-conjugated antibodies were used to color the fluorophore standard beads. The beads were stained, diluted in a buffer, and then analyzed using a BD FACS caliber, with each tube containing a standard of a single color. This technique enables precise spectrum overlap compensation and correction for any combination of fluorochrome-labeled antibodies.

Statistical and Analysis

The mean and SEM were used to represent the data. The two-variable normality distribution, which was supported by the Pearson correlation and D'Agostino omnibus tests, was applied to the analysis of the independent-samples t-test, the non-parametric Mann-Whitney U-test, and the ANOVA stands for analysis of Variance. GraphPad Prism Mac 7.0e (GraphPad Software, La Jolla, CA, USA) was performed to conduct the statistical tests. Significant differences among the samples are shown by *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Association of BKPyV DNA Viral Load with Clinical Parameters

The research sample consisted of 44% females and 56% males. Table 1 presents the correlation results between clinical data and viral load. In Table 1, the demographic, clinical and laboratory data for both KTRs and the healthy group are briefly presented. BKPyV-activated infection KTRs showed a mean BKPyV viral load suppression of 10,000 cps/mL.

Table 1. Demographic and clinical features of KTRs and healthy group

	BKPyV reactive KTRs	BKPyV non-reactive KTRs	Healthy group
Total number of patients	10	10	10
Gender	Male = 5 Female = 5	Male = 6 Female = 4	Male = 7 Female = 3
Mean age (years)	53.4	51.6	38.1
Blood groups	O^+ = 4 A^+ = 3 AB^+ = 1 B^+ = 2	O^+ = 4 A^+ = 3 AB^+=1 B^+ =2	O^+ = 3 A^+ = 5 B^+ = 2
Type of transplantation			
CD	100%	100%	0%
LD	0%	0%	0%
Couse of Transplantation			
Diabetes	**4**	**4**	**0**
Tumor kidney	**1**	**0**	**0**
Kidney failure	**2**	**0**	**0**
lupus	**1**	**0**	**0**
HTN	**7**	**0**	**0**
ESRD	**2**	**0**	**0**
Reason for referral			
Proteinuria	**2**	**0**	**0**
High CN and BUN	**5**	**5**	**0**
UTI	**0**	**4**	**0**
High loud of BKPyV	**2**	**0**	**0**
Nephropathy	**2**	**0**	**0**
Pulmonary infection	**1**	**1**	**0**
Kidney stone	**0**	**1**	**0**
Hematuria	**0**	**2**	**0**

HTN: Hypertension, ESRD: End-Stage Renal Disease, UTI: Urinary tract infection, CD: Cadaver Donor, LD: Living Donor

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Cells were excluded by staining with the fixable viability dye, gating on $CD4^+$ cells, and gating on the lymphocyte population (morphology based on FSC and SSC). Overall, $CCR7^+$ and $CD27^+$ T cells were gated. The following gating strategy was utilized to determine the amount of $CD45R0^+$ $CD4^+$ T cells in surface markers and intracellular markers expression, such as CD107a, perforin, and granzyme B. (Figure 1).

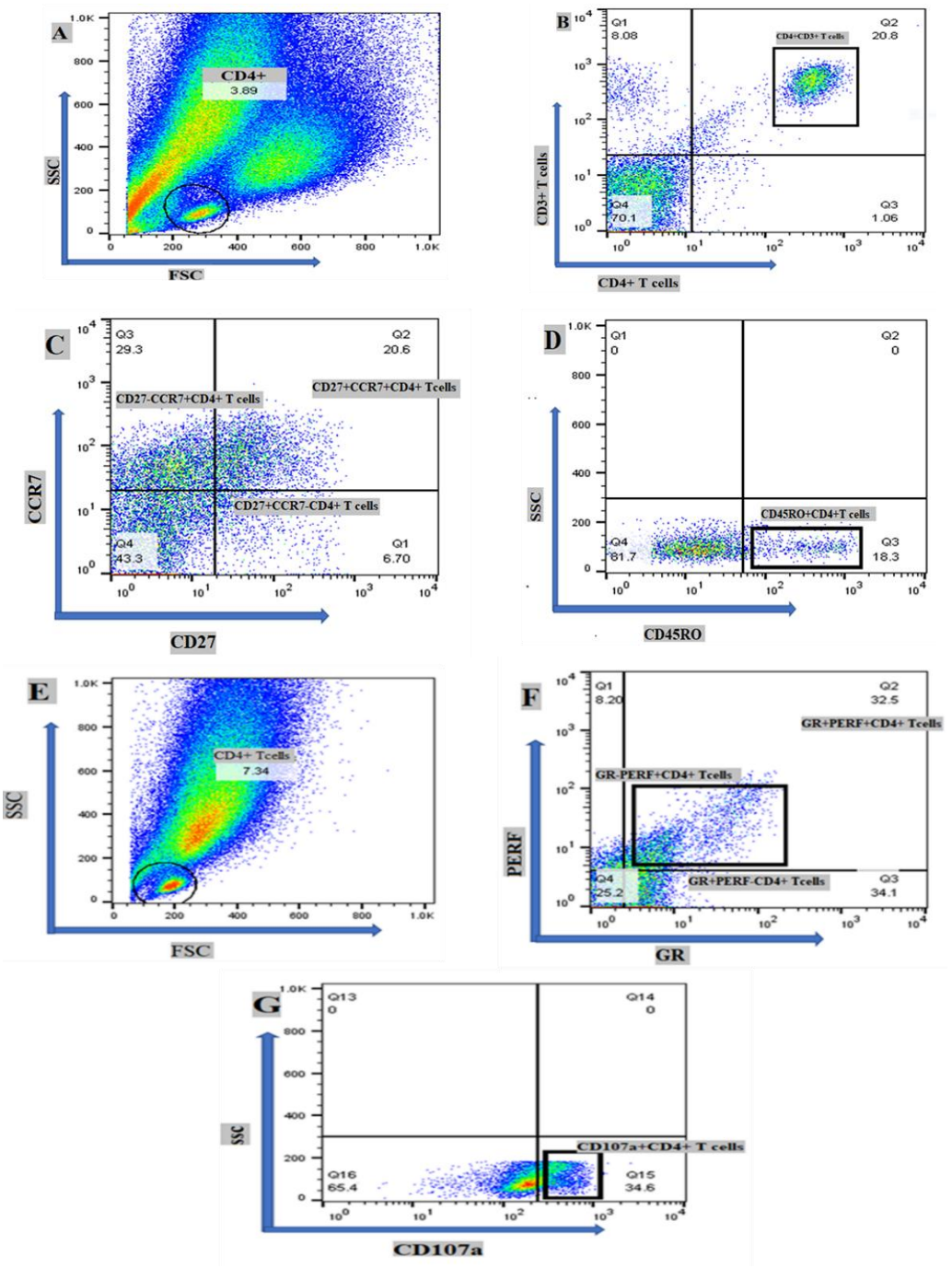

Figure 1. The cytokine secretion gating approach for $CD4^+$ T cells (A, E): $CD4^+$ T cells were gated based on the FSC/SSC intracellular and surface markers. (B): CD4/CD3 dot plot (C): CCR7/CD27 dot plot. (D): CD45RO/SSC cells were gated in the $CD4^+$ T cells dot plot. (F): perforin/granzyme B $CD4^+$ T cells (G): CD107a/ SSC cells were gated in the $CD4^+$ T cells dot plot. FSC/SSC: forward scatter/side scatter.

Expansion of Purified CD3+CD4+ T Cells among the BKPyV- KTRs VP1+, and Healthy Group

We observed significantly reduced specific CD3+CD4+ T cells in the non-reactivated BKPyV infected in comparison to the healthy group (p=0.0317; mean difference; 24.8). (Figure 2)

Analysis Subsets of CD4+ T cells among BKPyV- KTRs

We observed an increase in CCR7+ CD27+ CD45RO- CD4+ (naive T cell) proliferation, but not significantly in the BKPyV- KTRs VP1+ comparison to the BKPyV- KTRs without stimulation VP1 (before stimulation) (p=0.841; mean difference=15.52) and naive T cells decreased, but not significantly, in the BKPyV- KTRs VP1+ in comparison to BKPyV- KTRs to PMA/Ionomycin (PMA/ION) (positive control, some patients were stimulated with PMA/ionomycin) (p=0.09 mean difference=2.2) (Figure 3A).

In BKPyV- KTRs VP1+, the total number of central memory T cells was not significantly higher than in BKPyV- KTRs without stimulation VP1 (p=0.1548; mean difference=0.86), and the abundance of central memory T cells was not significantly reduced in BKPyV- KTRs VP1+ in comparison to BKPyV- KTRs to PMA/ION (p=0.5; mean difference=0.5) (Figure 3B).

It did not show a significant rise in the amount of CCR7- CD27-CD45RO+ CD4+ effector memory T cells in the BKPyV- KTRs VP1+ infected compared to the BKPyV- KTRs without stimulation VP1 (p=0.5; mean difference=0.07) and in comparison, to PMA/ION (p=0.42; mean difference=0.06) KTRs people (Figure 3C).

Analysis of Distinct Subpopulations of CD4+ T Cells among the Healthy Group

It was discovered that the amount of naive T cells in the healthy controls VP1+ was non-significantly higher than that in the healthy controls without stimulation VP1 (before stimulation). (p=0.442; mean difference=3.7), and percentages of the healthy controls VP1+also had a non-significantly lower percentage than the healthy controls to PMA/ION (positive control) (p=0.685; mean difference=5.59) (Figure 4A).

The proportion of central memory T cells in the healthy controls VP1+ was reduced compared to the healthy controls without stimulation VP1 (p=0.5; mean difference=0.89). Slight increases in the central memory T cells were also observed in the healthy controls VP1+ compared to the healthy controls to PMA/ION (p=0.44; mean difference=1.25), but the differences were not significant (Figure 4B).

The outcomes indicated that the proportion of effector memory T cells in the healthy controls VP1+ was decreased compared to the healthy controls without stimulation VP1 p=0.34; mean difference=2.29). The healthy controls VP1+ also had an increased frequency compared to the healthy controls to PMA/ION (p=0.44; mean difference; 1.09).

In summary, while there were some trends in T cell subsets with VP1 stimulation, the differences compared to unstimulated or PMA/ION-stimulated cells were relatively small and not statistically significant. This suggests that VP1 stimulation may not significantly change the distribution of T cell subsets in healthy people.

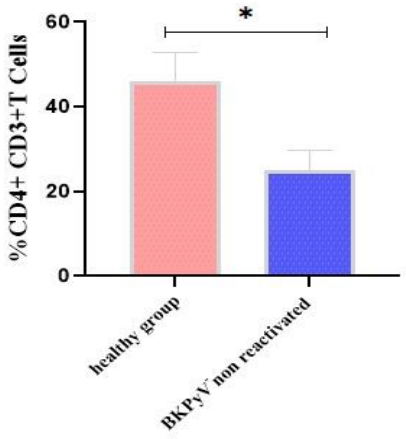

Figure 2. Data sets and graphs included the difference between three separate groups means± SEM containing: BKPyV- KTRs in CD3+CD4+ T cells for purification. *p<0.05. BKPyV- KTRs: Non-reactive BKPyV kidney transplant recipient

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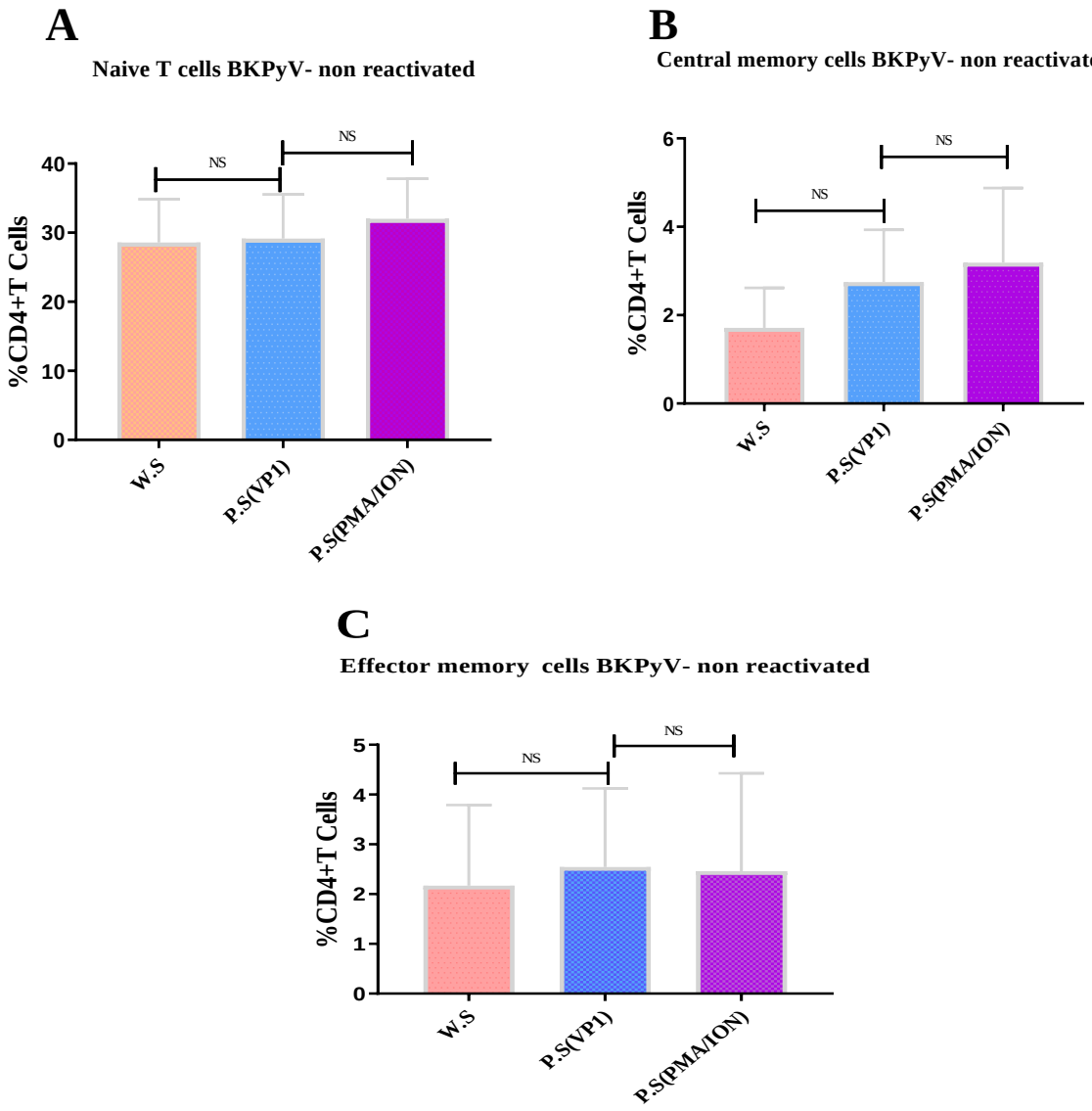

Figure 3. Data sets and graphs included the difference between three separate groups means± SEM containing: BKPyV$^-$ KTRs without stimulation VP1, BKPyV$^-$ KTRs VP1$^+$ and BKPyV$^-$ KTRs to PMA/Ionomycin. A: CCR7$^+$ CD27$^+$ CD45RO$^-$ CD4$^+$ T cells (naive T cells), B: CCR7$^+$ CD27$^+$ CD45RO$^+$ CD4$^+$ T cells (central memory T cells) and C: CCR7$^-$ CD27$^-$CD45RO$^+$ CD4$^+$ T cells (effector memory T cells). The prevalence of CCR7$^+$ CD27$^+$ CD45RO$^-$ CD4$^+$ T cells from the BKPyV$^-$ KTRs VP1$^+$ subjects have examined the expression of naive T cells, central memory T cells and effector memory T cells proportions. BKPyV$^-$ KTRs: Non-reactive BKPyV- kidney transplant recipients. W.S: without stimulation., P.S: post-stimulation, PMA/ION: PMA/Ionomycin, BKPyV$^-$ KTRs: Non-reactive BKPyV kidney transplant recipients

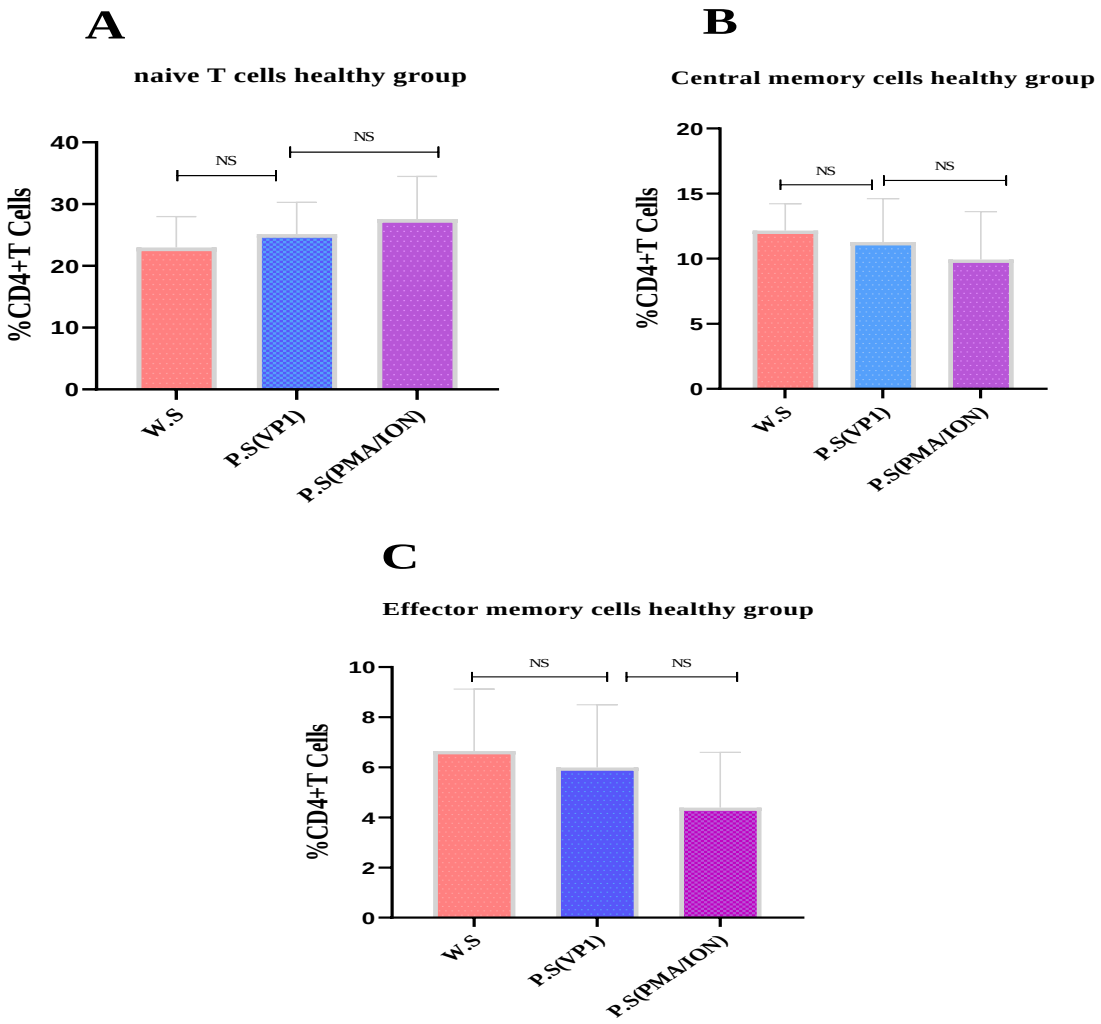

Figure 4. Data sets and graphs included the difference between three separate groups means± SEM containing: the healthy group without stimulation VP1 (W.S), healthy group VP1$^+$, and the healthy group to PMA/Ionomycin (P.S PMA/Ionomycin), and in A: CCR7$^+$, CD27$^+$, and CD45RO$^-$ CD4$^+$ T cells for expression of naive T cells; B: CD27+ CD45RO+ CD4$^+$ T Cells for expression of central memory T cells (TCM) and C: CCR7$^-$ CD27$^-$CD45RO$^+$ CD4$^+$ T cells for expression of effector memory T cells (TEM). The mean frequency of CCR7$^+$, CD27$^+$, and CD45RO$^-$ CD4$^+$ T cells from the healthy group subjects were used to analyze the expression of naive T cells, TCM and TEM proportions. W.S: without stimulation., P.S: post stimulation., PMA/ION: PMA/Ionomycin.

Analysis of Cytotoxic CD4$^+$ T Cells in the BKPyV$^-$ KTRs and Healthy Group

CD107a/LAMP-1, perforin and granzyme B are markers of cytotoxic T cell activity. The results show that in BKPyV$^-$ KTRs VP1$^+$, the expression of the cytotoxic markers (CD107a, perforin, granzyme B) on their CD4$^+$ T cells was not significantly higher than the BKPyV$^-$ KTRs without stimulation VP1 (p=0.5; mean difference=1.69), and assessment of cytotoxic activity in BKPyV$^-$ KTRs VP1$^+$ was lower than in BKPyV$^-$ to PMA/ION (p=0.5; mean difference=1.26) (Figure 5A).

There was a non-significant rise in cytotoxic CD4$^+$ T cells subsets in healthy controls VP1$^+$in comparison to healthy controls without stimulation VP1 (p=0.342, mean difference 8.45). The percentages of cytotoxic CD4$^+$ T cell markers in healthy controls VP1$^+$ compared to healthy controls to PMA/ION did not differ significantly (p=0.442; mean difference;11.05). (Figure 5B)

Analysis of naive CD4$^+$ T Cells Upon BKPyV Activation

The percentage of naive T cells were non-significantly increased in the BKPyV$^-$ KTRs VP1$^+$ group in comparison to the healthy controls VP1$^+$ (p=0.1518, mean difference=6.65). A non-significant decrease in the percentage of naive T cell subsets was observed in BKPyV$^-$ KTRs VP1$^+$ compared with BKPyV$^+$ KTRs VP1$^-$ (p;0.4296; mean difference; 0.44); in comparison, to the healthy controls VP1$^+$, there was a non-significant rise in BKPyV$^+$ KTRs VP1$^-$ (p=0.3651; mean difference=7.09) (Figure 6).

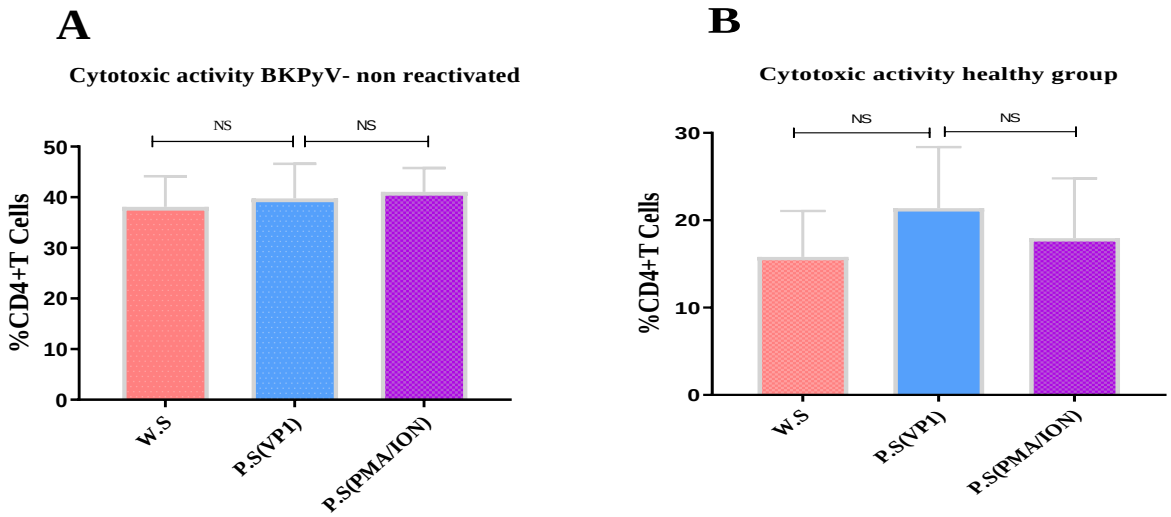

Figure 5. The graphs show the differences between three separate groups means± SEM containing: A: BKPyV$^-$ without stimulation VP1 (W.S), the BKPyV$^-$ KTRs VP1$^+$ (P.S (VP1)) infection and the stimulated BKPyV$^-$ KTRs to PMA/Ionomycin (P.S (PMA/Ionomycin)). B: the healthy controls without stimulation VP1 (W.S), the healthy controls VP1$^+$, and the healthy controls to PMA/Ionomycin (P.S(PMA/Ionomycin)) in CD107a/LAMP-1, perforin, granzyme B CD4$^+$ T cells for expression of assessment of cytotoxic activity (CD107a/LAMP-1$^+$perforin$^+$granzyme B$^+$ CD4$^+$ T Cells); W.S: without stimulation., P.S: post stimulation and PMA/ION: PMA/Ionomycin., BKPyV$^-$ KTRs: Non-reactive BKPyV kidney transplant recipients.

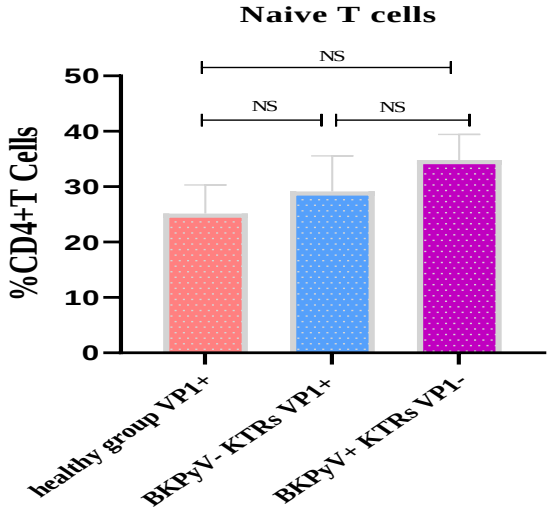

Figure 6. Data sets and graphs included the difference between three separate groups means± SEM containing: the BKPyV$^-$ KTRs VP1$^+$, BKPyV$^+$ KTRs VP1$^-$, and the healthy controls VP1$^+$group in CCR7$^+$, CD27$^+$, CD45RO$^-$ CD4$^+$ T cells for naive T cells. $^-$KTRs: kidney transplant recipients., BKPyV$^-$: Non-reactive BKPyV., BKPyV$^+$: reactive BKPyV.

Analysis of Central Memory CD4+ T Cells Upon BKPyV Activation

The central memory T cells in BKPyV- KTRs VP1+ was significantly less than in BKPyV+ KTRs VP1- (p=0.0013; mean difference=8.96), and central memory T cells in the BKPyV- KTRs VP1+ group was significantly lesser than the healthy controls VP1+(p=0.159; mean difference=8.915); also, the healthy controls VP1+ was increased compared to the BKPyV+ KTRs VP1- although this variation was not statistically significant. (p=0.4845; mean difference=0.04). (Figure 7).

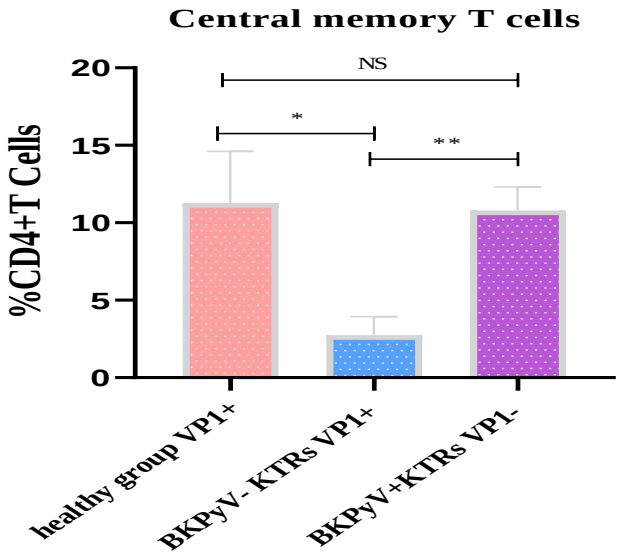

Figure7. Data sets and graphs included the difference between three separate groups means± SEM containing: the BKPyV- KTRs VP1+, BKPyV+ KTRsVP1- , and the healthy controls VP1+in the cells; CD27+ CD45RO+ CD4+ T cells for expression of central memory T cells (TCM). Significant consequences: *p<0.05; **p<0.01. KTRs: kidney transplant recipients, BKPyV: Non-reactive BKPyV, BKPyV+: reactive BKPyV.

Analysis of Effector Memory CD4+ T cells Upon BKPyV Activation

The rate of effector memory T cells in the BKPyV- KTRs VP1+ was non-significantly decreased compared to the healthy controls VP1+ (p value = 01429; mean difference = 4.3), and the percentage of effector memory T cells in BKPyV- KTRs VP1+ was significantly lower than the BKPyV+ KTRs VP1- (p=0.0007; mean difference=17), and BKPyV+ KTRs VP1- was significantly more than the healthy controls VP1+. (p=0.12; mean difference=13.52). (Figure 8)

Analysis of CD4+ Different Subset (naive, TCM and TEM) Cells Upon BKPyV Activation

Among the BKPyV KTRs and the healthy group, naive T cells were the most prevalent type when examining T cells obtained during both the VP1 stimulation and the unstimulated time points. Central and effector memory T cells were lower in stimulated BKPyV non-reactivated KTRs. The maturation phenotype analysis, which was conducted independently of BK virus specificity, demonstrated that native CD4+ T cells were more prevalent in both healthy group and BKPyV KTRs. High frequency of central memory was in BKPyV non-reactivated KTRs and most rate of effector memory T cells in BKPyV reactivated KTRs (Figure 9).

Analysis of Cytotoxic CD4+ T Cells Upon BKPyV Activation

Different rates of cytotoxic CD4+ T cells were observed in the BKPyV- KTRs VP1+ which showed a non-significant enhancement in comparison to the healthy controls VP1+ (p=0.952; mean difference=19.5). The frequency of cytotoxic CD4+ T cells in BKPyV- KTRs VP1+ was significantly increased compared to

BKPyV$^+$ KTRs VP1$^-$ (p=0.0013; mean difference=35.7). BKPyV$^+$ KTRs VP1$^-$ was non-significantly depleted compared to the healthy controls VP1$^+$ (p =0.151; mean difference=16.23) (Figure 10).

This suggests that VP1 can induce a cytotoxic CD4$^+$ T cell response in non-reactivated BKPyV patients, which may help control infection, while BKPyV reactivation was associated with decreased cytotoxic activity

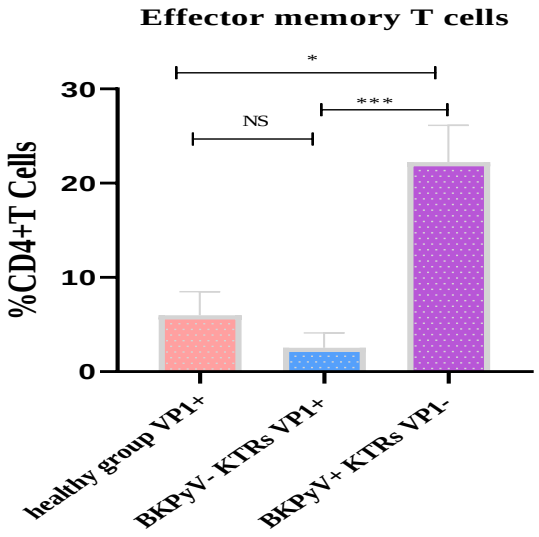

Figure 8. Data sets and graphs included the difference between three separate groups means± SEM containing: the BKPyV$^-$ KTRs VP1$^+$, BKPyV$^+$ KTRs VP1$^-$, and the healthy controls VP1$^+$ in CCR7$^-$ CD27$^-$CD45RO$^+$ CD4$^+$ T cells for expression of effector memory cells (TEM). Significant consequences: *p<0.05; ***p<0.001. KTRs: kidney transplant recipients, BKPyV$^-$: Non-reactive BKPyV, BKPyV$^+$: reactive BKPyV.

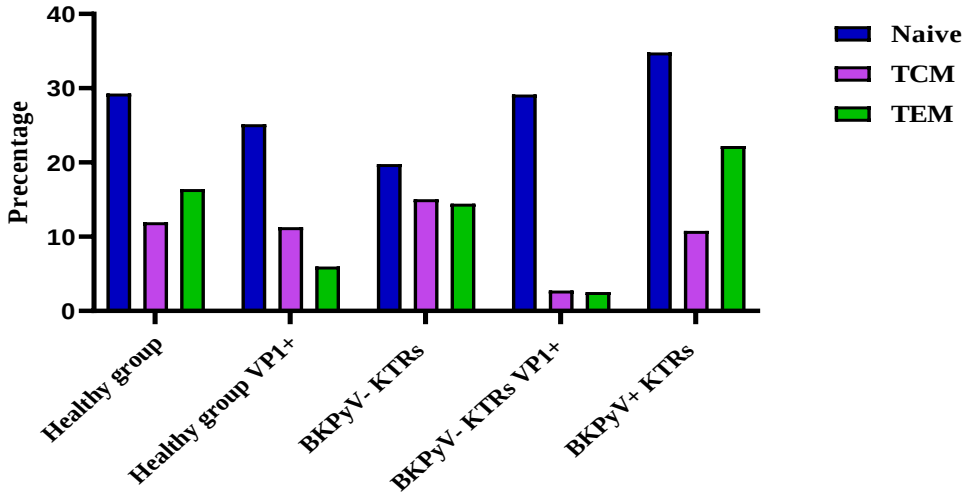

Figure 9. BKPyV-specific CD4$^+$ T cells and healthy group -specific CD4$^+$ T cells have a memory phenotype. The graphs show the differences between the group with condition means± SEM in five separate groups containing: healthy controls, healthy controls VP1$^+$, BKPyV$^-$ KTRs, BKPyV$^-$ KTRs VP1$^+$, BKPyV$^+$ KTRs in CCR7$^+$, CD27$^+$, CD45RO$^-$ CD4$^+$ T cells for naive T cells, CD27$^+$ CD45RO$^+$ CD4$^+$ T cells for expression of central memory T cells (TCM), and CCR7$^-$ CD27$^-$CD45RO$^+$ CD4$^+$ T cells for expression of effector memory cells (TEM). KTRs: kidney transplant recipients, BKPyV$^-$: Non-reactive BKPyV, BKPyV$^+$: reactive BKPyV.

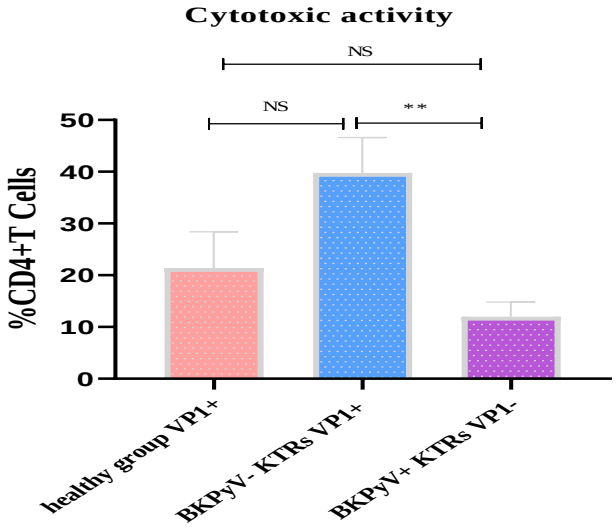

Figure 10. The graphs show the differences between the group with condition means± SEM between three separate groups containing: the BKPyV- KTRs VP1+, BKPyV+ KTRs VP1- , and the healthy group VP1+in CD107a/LAMP-1, perforin, granzyme B CD4+ T cells for expression of assessment of cytotoxic activity (CD107a+perforin+granzyme B+ CD4+T Cells); Significant consequences: **p<0.01. KTRs: kidney transplant recipients., BKPyV-: Non-reactive BKPyV, BKPyV+: reactive BKPyV.

DISCUSSION

BKPyV infections pose a serious challenge for patients after kidney transplantation, resulting in significant mortality and allograft failure.[29] Reactivation of BKPyV in KTRs following immunosuppression can commonly cause allograft dysfunction since 10% to 80% of KTRs experiencing BKPyVAN.[30] The importance of multifunctional CD4+ T cells in preventing BKPyV infections has been demonstrated in previous investigations.[31,32] The contribution of CD4+ T cells is critical for immune response against BKPyV especially effector memory CD4+ T cells.[33,23] It remains uncertain whether BKPyV immune responses are intermediated, especially by CD4+ T-lymphocytes.[34,35]

Yu Fan et al, described that VP1 and VP3 exhibit significantly higher immunogenicity compared to VP2.[36] Brumin et al. reported that VP1 is more prevalent than related CD4+ T cells in comparison to LT-Ag[37] and for this reason, we chose VP1 to stimulate CD4 T cells. BKPyV VP1-specific CD4+ T cells secrete cytokines that activate and support BKPyV -specific CD8+ T cells and B cells.[35,38] Stimulation of CD4+ T cells with VP1 protein can induce cytotoxic CD4+ T cell responses that may help control BKPyV infection in KTRs.[39] We found that stimulation with VP1 elicited differences in BKPyV-specific immune responses compared to non-stimulation condition. However, in the current study we demonstrated a higher frequency of BKPyV-specific CD4+ T cells in naive T cell subsets in BKPyV+ KTRs. Another study has demonstrated that the number of naive CD4+ T cells increases in recipients with early CMV reactivation, compared to recipients without CMV reactivation.[40] These findings align with the results of our own study. Naive CD4+ T cell frequencies in BKPyV- were not significantly associated with the effects of VP1 stimulation which could play a role viral evasion of the immune system during immune suppression. These results suggest that VP1-induced immune responses involve more proportions of the effector and memory T cells than naive CD4+ T cells. In summary, while there were trends of small differences in naive CD4+ T cell percentages between the groups, the variations were not large enough to be statistically significant. However, given the relatively small sample sizes and non-significant p-values, large-scale studies are necessary to confirm the results of this research.

In recipients with BKPyVAN, development of the TEM CD4+T cell population was found. These results show that mechanisms of effector differentiation may also be implicated in the control of BKPyV infection.[34] According to the research of van Aalderen et al., when

granzyme increase in patient TEM of BKPyV-specific $CD8^+$ T cells in patients with BKPyV is impaired and decreased. Circulating BKPyV VP1-specific$CD8^+$ T cells in healthy individuals were predominantly observed in TCM ($CD45RA^-CCR7^+CD27^+$) and TEM cells ($CD45RA^-CCR7^-CD27^+$).[16,33] In their study, Foroudi et al. observed a reduction in the frequency of naive T cells (defined as $CCR7^+$ $CD45RO^-$ $CD4^+$) in $BKPyV^-$ KTRs when compared to both healthy controls and $BKPyV^+$ individuals. Additionally, the TCM cells, characterized by ($CD4^+CCR7^+CD45RO^+$) exhibited a decline in $BKPyV^-$ KTRs compared to healthy controls.[9] A study of TEM found that when compared to the control group, $BKPyV^+$ had more urine and PBMC $CD4^+$ T cells as well as PBMC $CD8^+$ T cells.[41] Combined analysis of phenotypic marker expression showed that KTRs had higher levels of $BKPyV^+$ specific TCM $CD4^+$ T cells ($CD45RA^+CCR7^+CD27^+$ $CD28^+$) than did the healthy population.[42] In a recent publication, The T cell subpopulation distributions in seropositive and seronegative CMV were compared, and it was discovered that CMV seropositivity was associated with an increased frequency of TEM CD4+ T cells.[43] Zhang et al. According to reports, they used PBMCs from CMV seropositive and CMV seronegative subjects as an initial point to investigate the heterogeneity of $CD4^+$ T cells in CMV-infected individuals. Individuals with CMV seropositivity demonstrated a higher frequency compared to CMV seronegative individuals, despite the fact that the $CD45RA^-CCR7^-$effector memory had similar frequencies,[43] the results above support the findings of our research.

According to our recent findings, the distributions proportion of multifunctional naive, TCM and TEM cells specific to $BKPyV^-$ $VP1^+$ study group in comparison to $BKPyV^-$ KTRs-PMA/ION and the $BKPyV^-$ before stimulation groups did not demonstrate a significant correlation (Figure 3). It was found that in order to stimulate the $CD4^+$ cell subtype in $BKPyV^-$, VP1 must be combined with other capsid proteins. Moreover, in $BKPyV^-$ $VP1^+$ specific $CD4^+$ T cells we observed that naive T cells, TCM, and finally TEM cells were sequentially decreased which the most prevalent detected population was naive T cells (Figure 9). Additionally, the results showed that one polyfunctional protein, VP1, was identified in TCM and TEM in $BKPyV^-$ KTRs $VP1^+$; it is significantly lower than $BKPyV^+$ KTRs VP1.

VP1-specific $CD4^+$ T cells produced Inflammatory cytokines and chemokines can activate other immune cells to the sites of BKPyV infection.[45] VP1-specific $CD4^+$ T cells help control and clear BKPyV infection, including direct cytotoxic activity and differentiation into memory T cells that provide lasting immunity.[46] According to an investigation carried out healthy controls have higher frequencies of cytotoxic $CD4^+$ T cells than KTRs without BKPyV reactivation, but it was not statistically significant.[39] Also, in the current research, the frequencies of cytotoxic $CD4^+$ T cells assessed by CD107a, perforin, and granzyme B expression were non-significantly greater in healthy controls compared to $BKPyV^-$ KTRs $VP1^+$. Also, Eduardo Espada demonstrated that following hematopoietic cell transplantation (HCT), the fraction of patients with $CD4^+$ T cells specific to the cytolytic BK virus declined in comparison to those without BKV disease, and there were no significant differences (stimulated by overlapping 15-mer peptides made of the VP1 and LT proteins of the BK virus).[47] In individuals with severe BKPyV reactivation, Schaenman et al. previously observed reduced $CD8^+$ and $CD4^+$ T cell responses specific for BKPyV. This group found that cytotoxic activity BKPyV-specific $CD8^+$ T cells with VP1 and LT-Ag protein produced CD107a frequently.[47] However, van Aalderen et al. recently demonstrated different findings.[33] According to the findings of another study, there was an increase in $BKPyV^-$ KTRs as compared to $BKPyV^+$ KTRs and healthy controls, in $CD4^+$ T cells stimulated with LTA that release both CD107a and GB ($CD4^+CD107a^+GB^+$).[9] In this study, also BKPyV VP1-Ag significantly got larger amounts of cytotoxic $CD4^+$ T cells in $BKPyV^-$ $VP1^+$ recipients compared to $BKPyV^+$ KTRs VP1. These findings demonstrate that the emergence of $BKPyV^-$specific cellular immune responses contributes to the regulation of viral replication and this suggests that VP1 stimulation can induce cytotoxic $CD4^+$ T cell responses that maybe helpful in controlling BKPyV infection.

The specific critical function of $CD4^+$ T cells is direct suppression of viral infections in the recipients and it is needed to understand the impacts of the various protocols for producing BKPyV specific $CD4^+$ T cells and in this project, we aimed to discover whether BKPyV specific $CD4^+$ T cells could directly use cytotoxic measures and the underlying processes for this inducer function. Consequently, we found out the

perfect conditions for producing $CD4^+$ T cells with LCL from the low-incidence BKPyV specific $CD4^+$T cells.

According to these findings, $CD4^+$ T cell subsets are important for BKPyV clearance. The ability of killing and cytotoxic $CD4^+$ T cells indicates the reactivation of BKPyV in KTRs even in the absence of $CD8^+$ T cell immunity. TCM $CD4^+$T cells can be stimulated to differentiate by BKPyV VP1-Ag, and VP1-Ag significantly increased the frequency of cytotoxic $CD4^+$ T cells in $BKPyV^-$ KTRs compared to $BKPyV^-$ KTRs $VP1^+$. We recommend that the use of CD107a, perforin, and granzyme B should be performed more frequently as reliable markers to assess the cytotoxic role to control BKPyV infection.

STATEMENT OF ETHICS

The study was approved by the Research Ethics Committee (RECs) of SUMS (Reference number: IR.SUMS.REC. 1396.S835). All adult subjects provided informed consent for participation in this research.

FUNDING

This study was financially supported by SUMS (Grants No. 12968).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank the Abu Ali Sina Hospital, Shiraz, Iran, and the participants for the sample collection.

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