ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
In press.

Phenotyping of T_H9 Cells in Cytomegalovirus-reactivated Kidney Transplant Recipients

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Received: 9 June 2025; Received in revised form: 28 October 2025; Accepted: 9 November 2025

ABSTRACT

Human cytomegalovirus (HCMV) is a frequent complication in kidney transplant recipients (KTRs), often impacting immune regulation. T_H9 cells, a subset of $CD4^+$ T cells, are characterized by their secretion of interleukin-9 (IL-9). This study aimed to analyze the phenotypic profile of T_H9 cells and their associated cytokine expression in KTRs, and to evaluate mRNA levels of IL-4 and transforming growth factor-β (TGF-β), key cytokines involved in T_H9 differentiation.

Ten $HCMV^+$ and 10 $HCMV^-$ KTRs, along with 10 age- and sex-matched healthy controls, were enrolled. HCMV viral load was quantified using TaqMan real-time polymerase chain reaction. Flow cytometry was used to assess surface markers ($CCR6^+CCR4^-IL\text{-}4R\alpha^+CD4^+$) and intracellular IL-9 expression in T_H9 cells. Gene expression levels of *IL-4* and *TGF-β* were also assessed.

Surface staining revealed a significantly higher frequency of $CD4^+CCR4^-$ and $CD4^+CCR6^+$ T cells in $HCMV^-$ KTRs compared to $HCMV^+$ patients. Conversely, the overall frequency of $CD4^+$ T_H9 cells was elevated in $HCMV^+$ KTRs. Intracellular staining demonstrated a significant increase in $CD4^+IL\text{-}9^+$ and $CD4^+IL\text{-}4R\alpha^+$ T cells in the $HCMV^+$ group. Additionally, mRNA expression levels of *IL-4* and *TGF-β* were markedly higher in $HCMV^+$ KTRs than in $HCMV^-$ counterparts.

These findings suggest a potential role for T_H9 cells and their signature cytokine IL-9 in the antiviral immune response in KTRs. While T_H9 cells may contribute to HCMV-related immune modulation, further research is needed to fully elucidate their protective mechanisms against viral infections.

Keywords: Cytomegalovirus; Interleukin-9; Kidney transplant; T-helper9

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the β-herpesvirus subfamily, can establish a latent infection with periodic or sporadic reactivation after the initial infection.[1,2] This infection frequently produces complications post-renal transplantation, especially when the recipient is seropositive. After transplantation, HCMV might present as 2 clinical spectra, typical viral syndrome or invasive disease.[3] The impact of HCMV is important in the context of transplantation outcomes, and multiple studies indicate HCMV seropositivity of the recipient and/or the donor is related to the adverse

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prognosis and the reduced overall survival posttransplantation.[4] Although different strategies have been considered for treating this infection, recurrence of HCMV infection is still a source of many complications in kidney transplant recipients (KTRs).[5,6]

T-cell–mediated immune response (TCMI) has a major role in protection against HCMV infection in transplant recipients and has already been studied.[7-9] T cells comprise a heterogeneous cell population and various immune cell subsets originate from these cells, which are responsible for various cell-mediated immunity functions. Activated $CD4^+$ T helper (T_H) cells differentiate into distinct lineages with characteristic patterns.[10-13] After encountering pathogens, $CD4^+$ subsets differentiate and create pathogen-specific signatures. The functional and compositional quality of these $CD4^+$ T_H cells can be studied during various conditions such as viral infections, using flow cytometry.[14,15] Currently, some $CD4^+$ subsets are detected to be potentially valuable during HCMV infection and a number of them still require further research.[16] Additionally, studies revealed that as the immune system of the host improves, the danger of HCMV viremia diminishes in seropositive KTRs or seronegative healthy controls and when the incidence of $CD4^+$ T cells specific for HCMV falls into about lower than 0.25%, viral proliferation would no longer be controlled.[17]

Based on the quality and quantity of environmental stimuli, such as cytokines or ligand interactions, $CD4^+$ T helper subsets differentiate into several lineages, among which T_H9 cells are a recent addition.[18] As part of a varied group of $CD4^+$ helper T cells, the production of interleukin-9 (IL-9) by T_H9 cells is regulated by a combination of interleukin-4 (IL-4) and transforming growth factor-beta (TGF-β).[19] Different mechanisms may be employed by T_H cells to reduce pathogen burden. The functional heterogeneity of T_H9 cells renders the production of diverse arrangements in IL-9 cytokine production, various compositions of $CD4^+CCR4^-CCR6^+$ surface markers and different expression levels of related transcription factors engaged in immune regulation which are important characteristics for identifying T_H9 cells.[10-12,20]

Finally, while subsets such as T_H1, T_H2, T_H17, and regulatory T (Treg) cells have been extensively studied in transplant immunology, particularly in relation to graft rejection, tolerance, and viral defense, the role of T_H9 cells remains largely unexplored. T_H1 cells mediate antiviral responses via interferon-γ (IFN-γ), and T_H17 cells contribute to inflammation through IL-17. In contrast, T_H9 cells produce IL-9, a pleiotropic cytokine implicated in immunity, inflammation, and tissue remodeling. Despite emerging evidence of T_H9 involvement in allergic and autoimmune conditions, their contribution to antiviral immunity in transplant recipients, especially during HCMV reactivation, is poorly understood. This study addresses a critical gap by characterizing T_H9 cell phenotypes and cytokine profiles in KTRs with and without active HCMV infection, potentially revealing novel immunological pathways relevant to infection control and graft outcomes.

MATERIALS AND METHODS

Study Strategy and Patient Characteristics

This descriptive cross-sectional study was conducted between 2019 and 2021, using clinical and laboratory data retrieved from the patient registry of Abu-Ali-Sina Hospital. Thirty individuals were enrolled, including 10 KTRs with active HCMV infection ($HCMV^+$), 10 KTRs without active infection ($HCMV^-$), and 10 healthy controls (HCs). The $HCMV^+$ group was defined by a viral load exceeding 10 000 copies/mL, confirmed via quantitative real-time polymerase chain reaction (PCR). Healthy controls were matched to the patient groups based on age (±5 years), sex, and absence of chronic comorbidities such as hypertension, diabetes mellitus, or renal disease. All controls were HCMV-seronegative and had no history of organ transplantation or immunosuppressive therapy.

Eligible participants were adults aged 18 years or older who had undergone kidney transplantation at least 6 months prior and were no longer receiving antiviral prophylaxis. Exclusion criteria included coinfection with hepatitis viruses, BK polyomavirus, adenovirus, or HIV; history of multiorgan transplantation; and current HCMV preemptive therapy. Written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC. 1396. 814) and conducted in accordance with the Declaration of Helsinki.

HCMV IgG Enzyme-Linked Immunosorbent Assay

Commonly, IgM class antibodies are quite reliable for indicating acute infection. However, these antibodies cannot differentiate between HCMV primary infection

and reactivation. In cases of detecting positive IgM findings in samples, checking the avidity of pathogen-specific IgG antibodies is a helpful method for differentiating between primary infection and reactivation. The existence of anti-HCMV IgG antibodies was studied in the plasma samples of KTRs by an enzyme-linked immunosorbent assay (ELISA) kit (DIA.PRO, Milano, Italy).

Quantitative PCR for Detection of HCMV-DNA

HCMV reactivation in KTRs was evaluated using quantitative PCR. According to the manufacturer's instructions, the viral load of HCMV-DNA was calculated by using a quantitative real-time PCR kit (Primer Design Ltd kit, United Kingdom). Finally, the status of HCMV reactivation was determined using a quantitative HCMV PCR assay.[21]

Isolation of Mononuclear Cells and $CD4^+$ T Cells

Twelve milliliters of blood samples were gathered in EDTA tubes from all participants according to institutionally approved protocols. Plasma samples were cryopreserved at the time of transplantation. Fresh blood samples were used to ensure the high viability of isolated lymphocytes. Samples were prepared at room temperature (18–20 °C). An equal volume of phosphate-buffered saline (PBS) was added to 10 mL blood and mixed by drawing the blood and the buffer in and out by a Pasteur pipette. Centrifugation was carried out at 24 °C for 20 minutes at 450*g*. A clean Pasteur pipette was used to remove the upper layer. The mononuclear cells located at the interface were collected, washed twice with PBS, and utilized for the isolation of $CD4^+$ T cells. $CD4^+$ T cells from peripheral blood mononuclear cells (PBMCs) were enriched through negative selection using a $CD4^+$ T cells Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Specifically, 20 µL of $CD4^+$ T Cell Micro Bead Cocktail per 10^7 total cells was added to the mixture. Subsequently, an LS column was positioned in the magnetic field of an appropriate MACS separator and the column was primed by rinsing with 3 mL of buffer. The cell suspension was then applied onto the column, with the flow-through cells collected representing the enriched $CD4^+$ T cells.

Estimating the Purity Percent of $CD4^+$ T Cells

The $CD4^+$ T cells were separated from PBMCs, using the $CD4^+$ T Cell Isolation Kit (Miltenyi-Biotec, Bergisch Gladbach, Germany) based on the manufacturer's guidelines. The flow cytometry was used to evaluate the purity of native $CD4^+CD3^+$ T cells, which was 95%. The cells were fluorescently stained with CD3-PE (# 130-091-374) and CD4-PerCP.Cy5.5 (# 130-094-965) (BioLegend, San Diego, California, USA). Scatter signals and propidium iodide fluorescence were monitored for exclusion of the cell debris and dead cells.

Flow Cytometry Control and Standardization Beads

Collecting accurate flow cytometry data is dependent on proper flow cytometer maintenance and experimental setup. Using quality control beads minimizes variation in instrument alignment, allows for more precision across longitudinal studies and contributes to more reliable data comparisons from site-to-site and user-to-user. Using bead controls and standards helps ensure that your instrument is operating properly and that your data is both reproducible and reliable. Antibodies were conjugated to either FITC, PE, or PERCPCY5.5 and used for staining the fluorophore standard beads that were diluted in a buffer previous to analysis that performed by a BD FACSCalibur (every tube contained a single-color standard). This process helped establish precise compensation corrections for spectral overlap of combinations of fluorochrome-labeled antibodies.

Immunofluorescent Staining: The Fluorescent Antibody Method

T_H9 Cells Viability and Phenotypic Analysis by Flow Cytometry

The purity of the $CD4^+$ cells was assessed with anti-human monoclonal antibodies (mAb) that were specifically designed for CD3 and CD4, on the basis of these 2 surface markers expression. The forward scatter (FSC) and side scatter (SSC) were used to calculate and differentiate cell debris from lymphocytes. The viability of the cells was assessed using propidium iodide (Cat no. P4170, Sigma, San Diego, California, USA), and any samples containing 5% or more dead cells were omitted from the study. Some fluorophore-labeled mouse anti-human mAbs which were used in this study are composed of anti CD3-PE (Clone OKT3, Biolegend, San Diego, California, USA), anti CD4-PerCPcy5.5 (Clone OKT4, Biolegend, San Diego, California, USA). To analyze the T_H9 phenotype through surface staining, $CD4^+$ T cells were stained with anti-CD194 (CCR4)-PerCPcy5.5 (clone L291H4, Biolegend, San Diego,

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California, USA), anti-CD124 (IL-4Rα)-PE (clone G077F6, Biolegend), and anti-CD196 (CCR6)-Alexa (clone G034E3, Biolegend, San Diego, California, USA) in a staining buffer (PBS + 1% fetal bovine serum [FBS]) for 10 minutes in the dark at 4 °C. Subsequently, the incubated cells were washed with 1 mL of staining buffer and then analyzed using an FACSCalibur analyzer (BD Pharmingen, USA).

Intracellular Cytokine Staining and Cell Stimulation

To perform intracellular cytokine staining, cells at a density of 1 × 106 cells/mL were diluted in complete RPMI 1640 medium (Biosera, France) supplemented with 10% FBS, 100 U/mL penicillin-streptomycin (Biosera, France), and 2 mM L-glutamine (Cat.no., 11539876) (Gibco). For cell stimulation, 2 µL of cell activation cocktail containing phorbol myristate acetate (PMA)/ionomycin (cat. no., 423301, Biolegend, USA) was added to the medium, followed by incubation under 5% CO2 at 37 °C and 95% humidity for 6 hours. Brefeldin A (cat. no., 555029, Golgi Plug, BD Pharmingen, USA) was added for the final 5 hours of incubation. To determine TH9 cytokine profiles, cells were stained with anti-IL-9-PerCPcy5.5 (clone MH9A4, Biolegend, San Diego, California, USA). Isotype controls, including IgG1, κ-PerCPcy5.5 (clone MOPC-21, Biolegend, San Diego, California, USA), IgG2a, κ-PE (clone MOPC-173, Biolegend, San Diego, California, USA), IgG1-PerCPcy5.5 (clone MOPC-21, Biolegend, San Diego, California, USA), IgG2a, κ-PE (clone MOPC-173, Biolegend, San Diego, California, USA), and IgG2b, κ-PerCPcy5.5 (clone MPC-11, Biolegend, San Diego, California, USA), were used to assess nonspecific antibody binding. Flow cytometry data was collected using FACSCalibur Software (BD Biosciences, USA), and data analysis was performed using FlowJo version 10 software (Tree Star Inc, Ashland, USA).

RNA Extraction, cDNA Synthesis, and Gene Expression Study

The mRNA expression levels of *TGF-β* and *IL-4* genes were assessed in the isolated $CD4^+$ T cells using an in-house SYBR Green real-time PCR method. Total RNA was extracted from the isolated $CD4^+$ T cells using TRIzol reagent, and cDNA synthesis was carried out using the Prime Script RT Reagent Kit (Takara, Japan), following the manufacturer's recommendations. Real-time PCR reactions were conducted using the ABI Step One Plus instrument (Applied Biosystems Step One plus™, USA). Details of the SYBR Green real-time PCR program, primer sequences, and reaction mixtures can be found in SupplementaryTable 1.

RESULTS

The demographic data of HCs and both KTRs with and without active viral infection are summarized in Table 1. The age range of participants was 23 to 69 (mean = 54.3 years). The $HCMV^+$ KTRs consisted of 2 (20%) females and 8 (80%) males. Although age-matching was attempted, the $HCMV^+$ group exhibited a higher mean age than the other groups, which may influence immune parameters and should be considered when interpreting results.

HCMV-DNA Viral Load Relationship with Clinical Parameters

In $HCMV^+$ KTRs, the laboratory tests were completed on the sampling day. The correlations between clinical data and viral load are reported in Table 2. Only a significant correlation was detected between viral load and Hb level (p=0.050).

Gating Strategy for the Surface Staining of $CCR6^+CCR4^-IL\text{-}4R\alpha^+CD4^+$ T_H9 Cells in Studied Groups

In order to assess the proportion of $CD4^+$ T cells, a gating strategy was performed to evaluate the expression of T_H9 cells with absence of the $CCR4^-$ surface marker and presence of markers, including $CCR6^+$ and $IL\text{-}4R\alpha^+$ (Figure 1). In this analysis, the frequency of $CCR6^+CCR4^-IL\text{-}4R\alpha^+$ T_H9 cell phenotypes was evaluated by surface staining.

Table 1. The demographic data of HCs and both HCMV+ and HCMV- KTRs

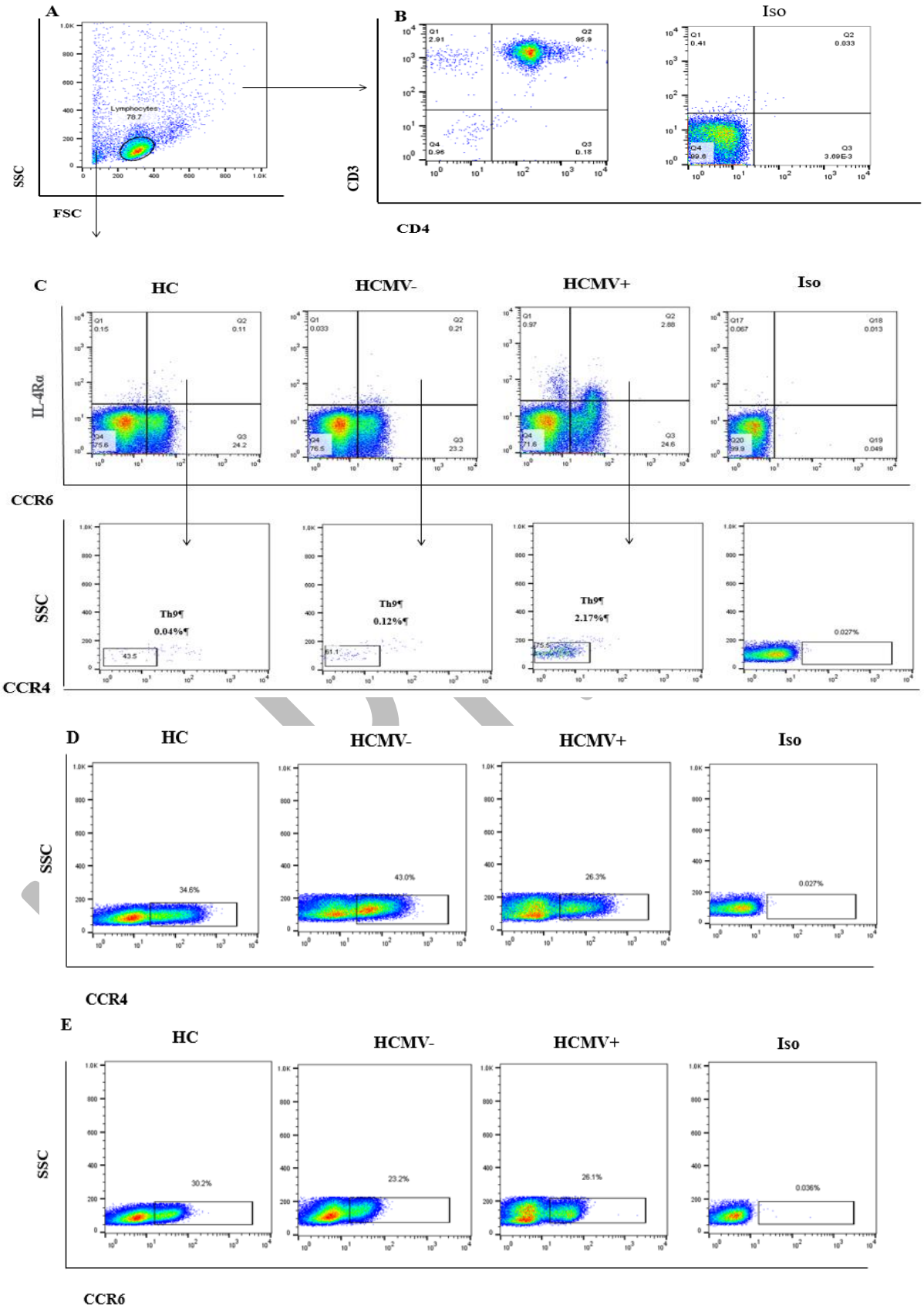
Factor/Group	HCMV+	HCMV-	HC	*p*
Number of participants	10	10	10	-
Gender of participants, N (%)				0.109
Male	8 (80%)	6 (60%)	5 (50%)	
Female	2 (20%)	4 (40%)	5 (50%)	
Age of participants, y (mean)	54.3	36.9	37.9	1.000
Type of transplantation				0.011
CD	6 (60%)	10 (100%)	-	
LD	4 (40%)	0	-	
Underlying disease				0.011
HTN	8 (80%)	10 (100%)	-	
DM	3 (30%)	1 (10%)	-	
Two-year renal graft outcome (graft survival)	10 (100%)	10 (100%)	-	-

CD: cadaver donor; DM: diabetes mellitus; HC: healthy control; HCMV: human cytomegalovirus; HTN: hypertension; KTRs: kidney transplant recipients; LD: living donor.

Table 2. Correlations between the HCMV DNA load and laboratory indices in HCMV+ KTRs

Lab Parameter' Characteristics	Lab Parameters' Mean (±SEM) and HCMV DNA Load correlation	Spearman's Rho	*p*
Creatinine, mg/dL	3.67±0.615	0.046	0.894
BUN, mg/dL	42.63±4.300	0.377	0.253
K, mEq/L	3.75±0.27	0.227	0.503
Na, mEq/L	135.72±1.34	-0.2	0.556
FBS mg/dL	106.40±11.46	-0.205	0.544
Uric-A mg/dL	6.70±0.31	0.078	0.820
Hb g/dL	9.40±0.57	-0.598	0.050
Mg mg/dL	1.65±0.13	-0.002	0.995

BUN: blood urea nitrogen; DNA: deoxyribonucleic acid; FBS: fasting blood sugar; Hb: hemoglobin; HCMV: human cytomegalovirus; K: potassium; KTRs: kidney transplant recipients; Mg: magnesium; Na: sodium; SEM: standard error of the mean; Uric-A: uric acid.


A
Lymphocytes
78.7
SSC
FSC
B
Iso
CD3
CD4
C
HC
HCMV-
HCMV+
Iso
IL-4Rα
CCR6
SSC
Th9¶
0.04%¶
Th9¶
0.12%¶
Th9¶
2.17%¶
0.027%
CCR4
D
HC
HCMV-
HCMV+
Iso
SSC
34.6%
43.0%
26.3%
0.027%
CCR4
E
HC
HCMV-
HCMV+
Iso
SSC
30.2%
23.2%
26.1%
0.036%
CCR6

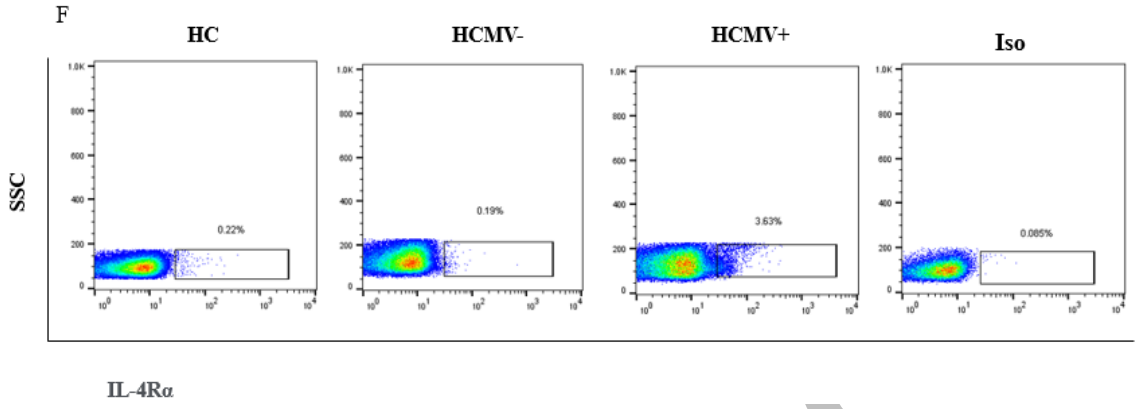


Figure 1. The frequency of CCR6$^+$CCR4$^-$IL-4Rα$^+$ T_H9 cells was assessed in kidney transplant patients with active HCMV infection (HCMV$^+$), without infection (HCMV$^-$), healthy controls (HC). CD4$^+$ T cells were isolated and gated based on side scatter (SSC) and forward scatter (FSC) to identify the lymphocyte population (A). Purity of isolated cells was confirmed by flow cytometry, with ≥95% CD3$^+$CD4$^+$ double-positive cells considered a pure population (B). To evaluate the T_H9 phenotype (CCR6$^+$CCR4$^-$IL-4Rα$^+$), CCR6$^+$IL-4Rα$^+$ double-positive cells were gated first, followed by CCR4$^-$ gating against SSC. Isotype controls were used to set gates (C). Individual surface marker frequencies were also assessed: CCR4$^-$ (D), CCR6$^+$ (E), and IL-4Rα$^+$ (F), each gated against SSC across all three study groups using isotype controls.

Analysis of CCR6$^+$CCR4$^-$IL-4Rα$^+$CD4$^+$ T_H9 Cells Among HCMV$^+$ and HCMV$^-$ KTRs and Healthy Controls

To evaluate the phenotypic distribution of T_H9 cells, CD4$^+$ T cells were gated for CCR6$^+$, CCR4$^-$, and IL-4Rα$^+$ expression. The frequency of CD4$^+$CCR4$^-$ T cells was significantly higher in HCMV$^-$ KTRs (18.4% ± 2.1%) compared to HCMV$^+$ KTRs (11.2% ± 1.7%, p=0.004), and also higher than in healthy controls (14.6% ± 1.9%, p=0.035) (Figure 2A).

For CD4$^+$IL-4Rα$^+$ T cells, no significant difference was observed between HCMV$^+$ (16.9% ± 2.3%) and HCMV$^-$ KTRs (15.7% ± 2.0%, p>0.05). However, both transplant groups showed elevated frequencies compared to healthy controls (10.2% ± 1.6%), with HCMV$^-$ vs HC (p=0.029) and HCMV$^+$ vs HC (p=0.024) (Figure 2B).

The frequency of CD4$^+$CCR6$^+$ T cells was significantly higher in HCMV$^-$ KTRs (20.1% ± 2.5%) than in HCMV$^+$ KTRs (13.3% ± 1.8%, p=0.026) and healthy controls (12.5% ± 1.6%, p=0.030) (Figure 2C).

Finally, the overall frequency of CCR6$^+$CCR4$^-$IL-4Rα$^+$CD4$^+$ T_H9 cells was elevated in HCMV$^+$ KTRs (9.8% ± 1.4%) compared to HCMV$^-$ KTRs (7.1% ± 1.2%, p>0.05), and significantly higher than in healthy controls (4.3% ± 0.9%, p=0.04). Additionally, HCMV$^-$ KTRs also showed a significant increase in T_H9 cell frequency compared to healthy controls (p=0.007) (Figure 2D).

Gating Strategy for Intracellular Staining CCR6$^+$IL-4Rα$^+$IL-9$^+$CD4$^+$ T_H9 Cells in Studied Groups

To assess the proportion of CD4$^+$ T_H9 cells coexpressing the surface markers CCR6$^+$ and IL-4Rα$^+$ along with the intracellular cytokine IL-9, a detailed gating strategy was employed (Figure 3). Intracellular cytokine staining was utilized to identify IL-9$^+$ cells within the CCR6$^+$IL-4Rα$^+$CD4$^+$ T cell population. Panels 3D–F illustrate the IL-9 staining profiles, displaying both IL-9$^+$ and IL-9$^-$ subsets for comparative analysis. These plots represent the full distribution of IL-9 expression rather than selectively gating only IL-9$^+$ cells, providing a comprehensive overview of cytokine expression across the study groups.

Additionally, it is worth mentioning that a slight background signal observed in the isotype controls for CCR6 and IL-9 likely reflects minimal nonspecific binding or autofluorescence, remaining well within the acceptable threshold (<1%). Crucially, all gating thresholds were established using isotype controls to ensure accurate discrimination between true positive populations and background noise. As a result, the reported frequencies of CCR6$^+$IL-4Rα$^+$IL-9$^+$CD4$^+$ T_H9 cells are not influenced by this background staining and reflect genuine marker expression.

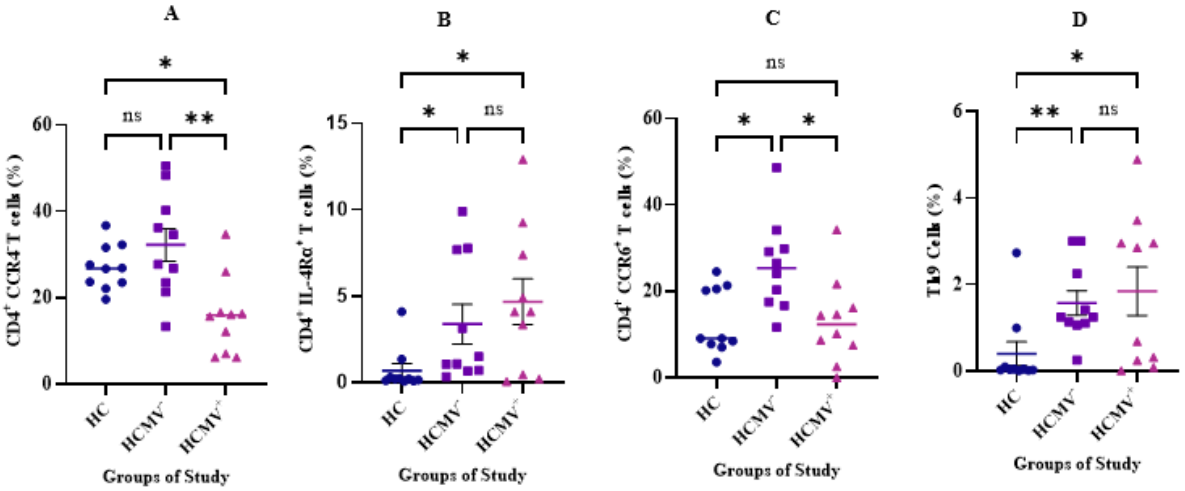
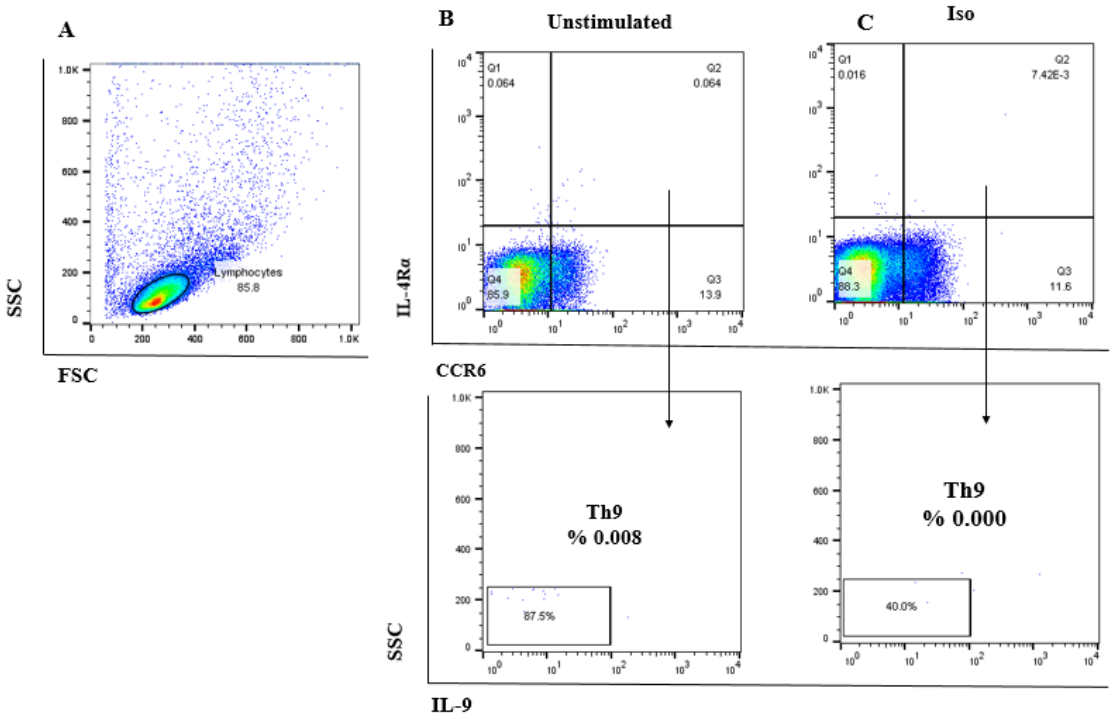
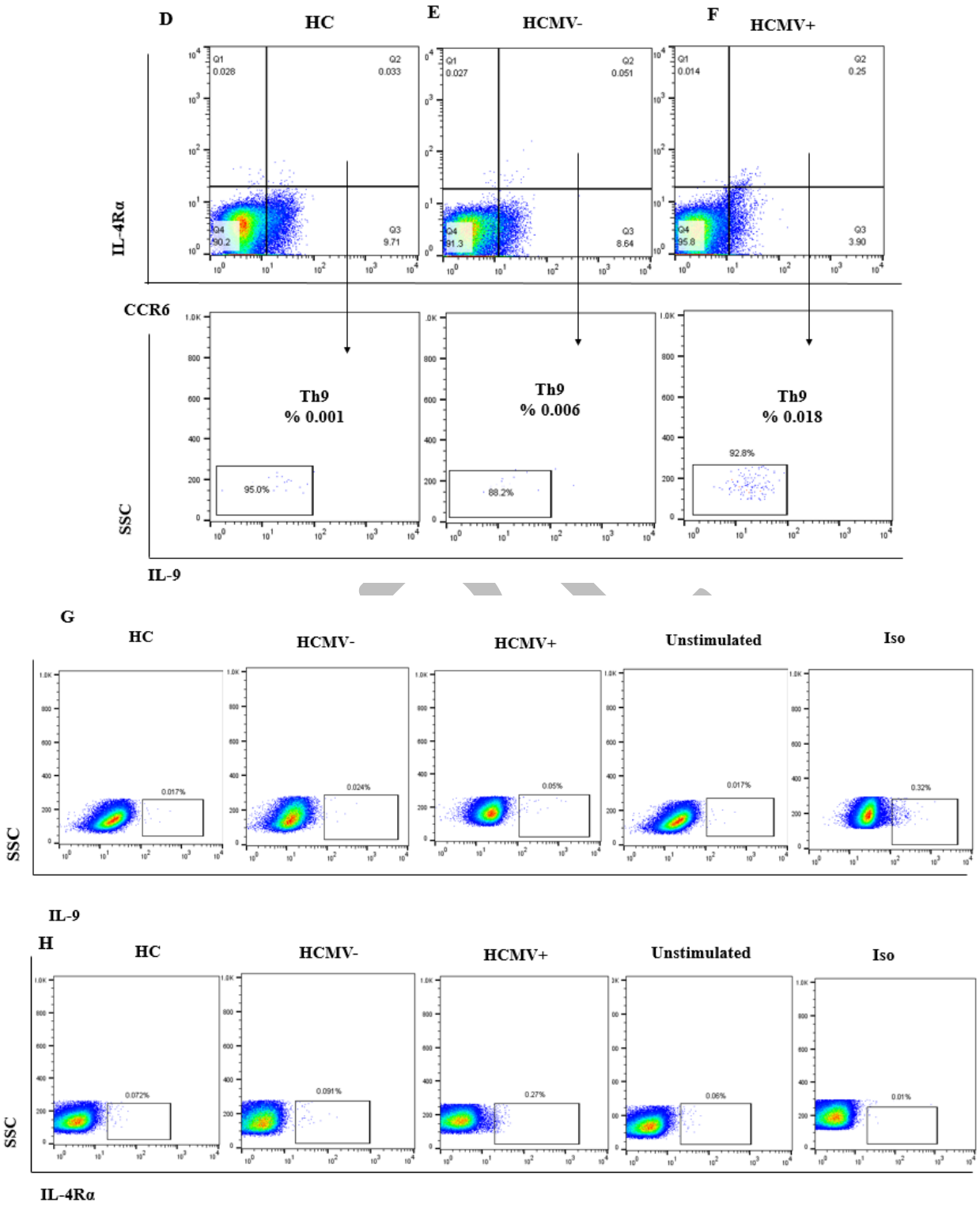


Figure 2. The data presented depict the mean ± SEM among the studied groups, which include HCMV+ and HCMV- KTRs, as well as HC subjects in CCR6+CCR4- IL-4Rα+ CD4+ T Cell. Sections A, B, and C illustrate the statistical comparison of CCR6+, CCR4-, and IL-4Rα+ frequency in the various study groups. Section D provides the statistical comparison of T_H9 cells across different study groups. The abbreviations used in the figure are SSC (side scatter), FSC (forward scatter), Iso (isotype control), HC (healthy control), HCMV- (kidney transplant patients without active infection), and HCMV+ (kidney transplant patients with active infection); *p<0.05; **p<0.01; ***p<0.001.





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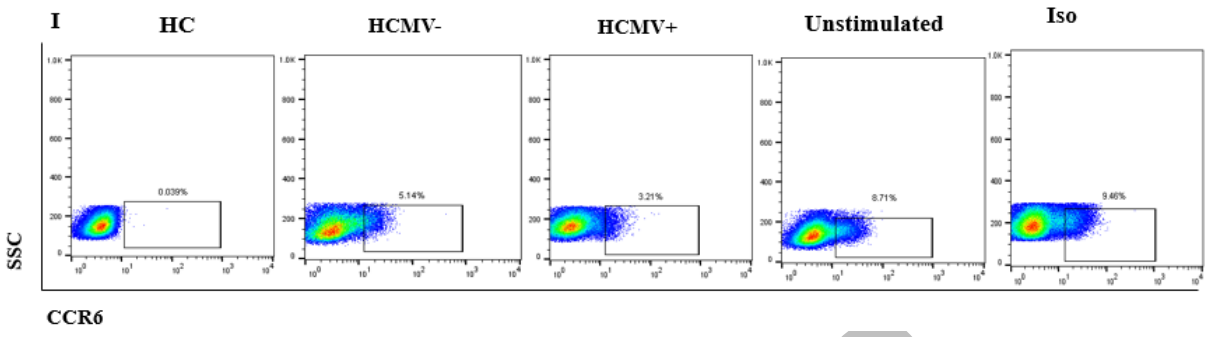

Figure 3. The phenotype frequency of CCR6$^+$IL-4Rα$^+$IL-9$^+$CD4$^+$ T_H9 cells was analyzed in kidney transplant patients with active HCMV infection (HCMV$^+$), without infection (HCMV$^-$), and healthy controls (HC). (A) Gating of isolated CD4$^+$ T cells based on forward scatter (FSC) and side scatter (SSC) to identify the lymphocyte population. (B–F) Gating strategy for CCR6$^+$IL-4Rα$^+$IL-9$^+$CD4$^+$ T_H9 cells. IL-4Rα$^+$CCR6$^+$ double-positive cells were first gated, followed by IL-9$^+$ evaluation against SSC. Isotype controls and unstimulated samples were used to define gating parameters. (G–I) Frequency analysis of IL-9$^+$, IL-4Rα$^+$, and CCR6$^+$ expression against SSC across the three study groups.

Analysis of CCR6$^+$IL-9$^+$IL-4Rα$^+$ T_H9 Cells among HCMV$^+$ and HCMV$^-$ KTRs and Healthy Controls

To assess the intracellular cytokine profile of T_H9 cells, CD4$^+$ T cells were gated for CCR6$^+$, IL-9$^+$, and IL-4Rα$^+$ expression. The frequency of CD4$^+$CCR6$^+$ T cells was highest in HCMV$^-$ KTRs (21.3% ± 2.4%) compared to HCMV$^+$ KTRs (13.9% ± 1.9%) and healthy controls (12.7% ± 1.6%). However, no statistically significant differences were observed among the three groups (p>0.05) (Figure 4A).

The frequency of CD4$^+$IL-9$^+$ T cells was significantly elevated in HCMV$^+$ KTRs (17.6% ± 2.1%) compared to HCMV$^-$ KTRs (11.2% ± 1.8%, p=0.016) and healthy controls (7.4% ± 1.3%, p=0.0001) (Figure 4B).

For CD4$^+$IL-4Rα$^+$ T cells, HCMV$^+$ KTRs showed a significantly higher frequency (19.8% ± 2.2%) than both HCMV$^-$ KTRs (14.1% ± 1.9%, p=0.013) and healthy controls (9.6% ± 1.5%, p=0.001) (Figure 4C).

Finally, the overall frequency of CCR6$^+$IL-9$^+$IL-4Rα$^+$CD4$^+$ T_H9 cells was significantly increased in HCMV$^+$ KTRs (10.7% ± 1.6%) compared to HCMV$^-$ KTRs (6.9% ± 1.3%, p=0.010) and healthy controls (4.1% ± 0.8%, p=0.002) (Figure 4D).

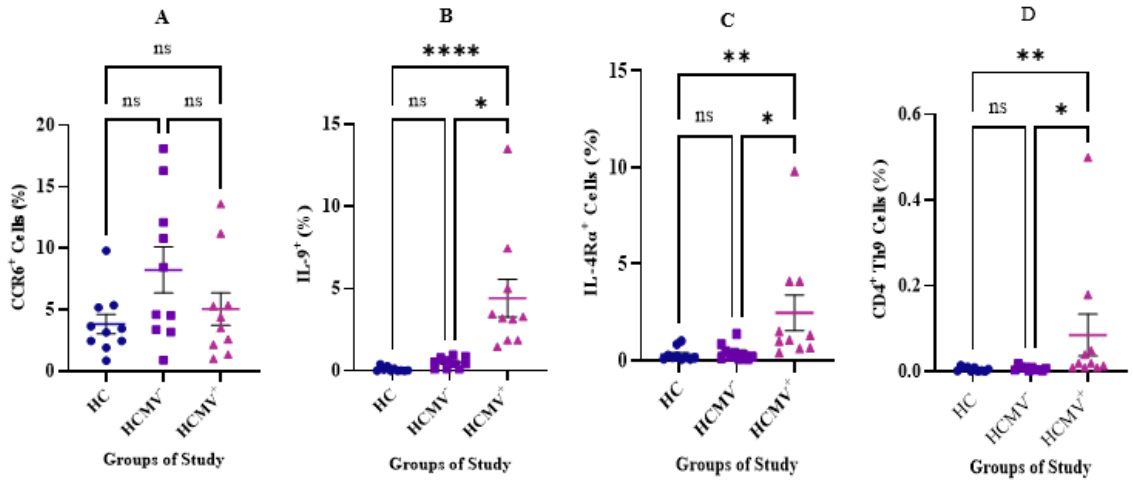

Figure 4. Analysis of CCR6+IL-9+IL-4Rα+ T_H9 cells; the frequency of CD4+ CCR6+ T cells was evaluated in three study groups (A); the frequency of CD4+ IL-9+ T cells was evaluated against side scatter in three study groups (B); the frequency of CD4+ IL-4Rα+ T cells was evaluated against side scatter in three study groups (C); the frequency of CD4+ T_H9 cells was evaluated against side scatter in three study groups (D). HC, healthy control; HCMV-, kidney transplant patients without active infection; HCMV+, kidney transplant patients with active infection; *p<0.05; **p<0.01; ***p<0.001.

Analysis of mRNA Expression of *TGF-β* and *IL-4* Genes in Isolated CD4$^+$ T Cells

The mRNA expression levels of *IL-4* and *TGF-β* were assessed in CD4$^+$ T cells isolated from all study groups. *IL-4* expression was significantly elevated in HCMV$^+$ KTRs (32.7 ± 3.4) compared to HCMV$^-$ KTRs (21.5 ± 2.8, p<0.001) and healthy controls (18.2 ± 2.6, p=0.002) (Figure 5A).

Similarly, *TGF-β* mRNA levels were significantly higher in HCMV$^+$ KTRs (2.03 ± 0.27) than in HCMV$^-$ KTRs (1.48 ± 0.21, p=0.023) and healthy controls (1.12 ± 0.19), with significant differences observed between both transplant groups and the control group (p=0.014 and p=0.012, respectively) (Figure 5B).

These findings suggest that elevated *IL-4* and *TGF-β* expression may contribute to T_H9 cell differentiation and cytokine production in the context of HCMV reactivation.

The Receiver Operating Characteristic Curve Analysis of Studied Genes Between HCMV$^+$ and HCMV$^-$ KTRs

The receiver operating characteristic (ROC) curve analysis and area under the ROC curve (AUC), used for studying the function of a classification model in all studied genes (Figure 6 and Table 4), respectively. These results showed that *IL-4* (p=0.049, AUC=0.760), *TGFβ* (p=0.021, AUC=0.805) have significant roles in discriminating studied groups.

Evaluating the Relationships Between HCMV-DNA Load and Studied Genes in HCMV$^+$ KTRs

The correlation between HCMV-DNA load and the expression levels of *IL-4* and *TGF-β* in HCMV$^+$ kidney transplant recipients was assessed using Spearman's rank correlation method, as summarized in Figure 7. The analysis revealed no statistically significant associations between viral load and the expression levels of the studied genes.

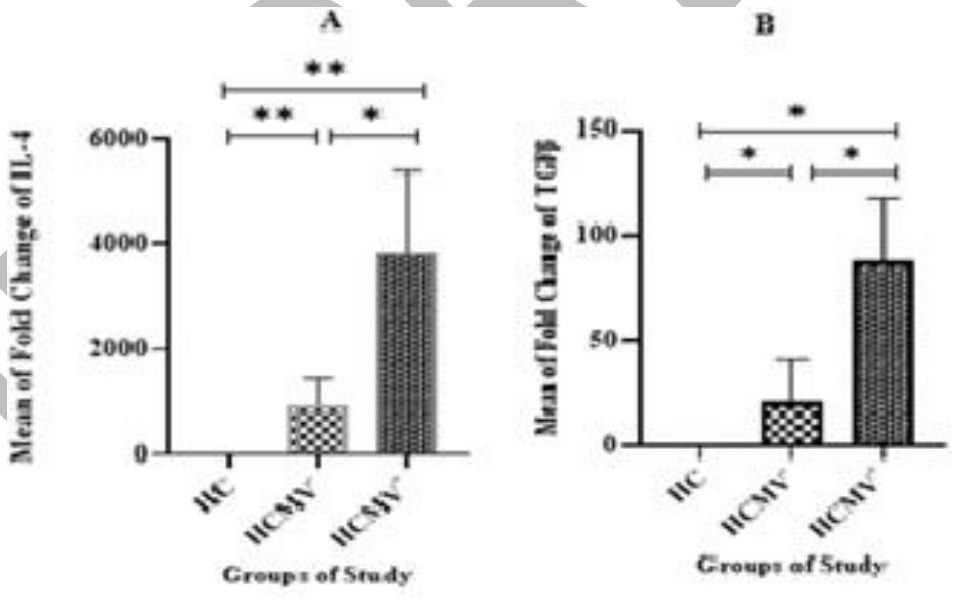

Figure 5. The mRNA expression of *IL-4* and *TGFβ* genes; *IL-4* mRNA expression level in the HCMV+ KTRs is significantly higher than in HC groups and HCMV- KTRs (Figure 6A). The mRNA expression level of *TGFβ* in HCMV+ KTRs is significantly higher than in HCMV- ones (Figure 6B). The results show the mean ± SE in three independent experiments (*: p<0.05; **: p<0.01 and ****: p<0.0001). HC, healthy control; HCMV-, kidney transplant patients without active infection; HCMV+, kidney transplant patients with active infection.

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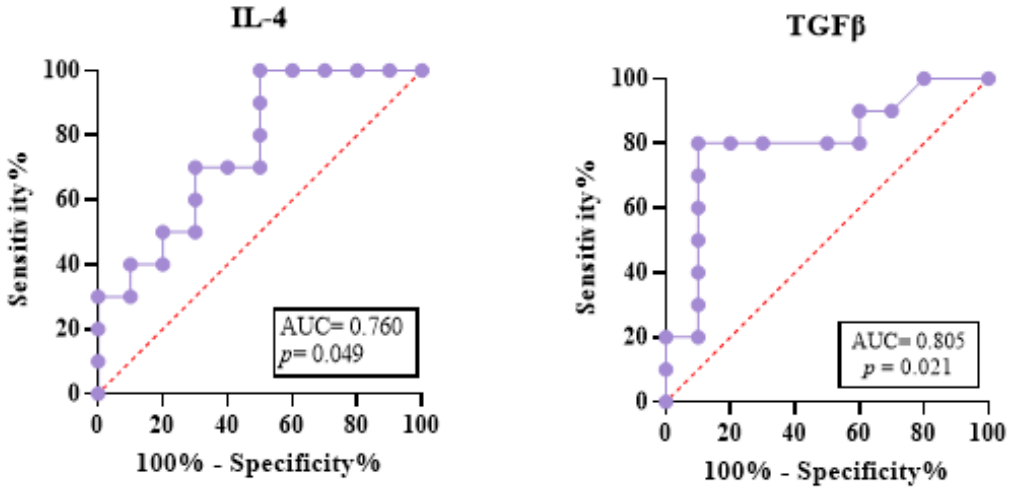

Figure 6. The ROC curve of *IL-4* and *TGFβ* genes.

Table 4. The AUC, p, cut-off value, sensitivity and specificity of IL-4 and TGFβ

Gene name	AUC (95% CI)	*p*	Cut off value	Sensitivity (95% CI)	Specificity (95% CI)
IL-4	0.760 (0.547-0.9729)	≤0.0494	29.93	100% (72.25%-100.0%)	50% (23.66%-76.34%)
TGFβ	0.805 (0.5971-1.000)	≤0.021	<1.520	80% (49.02%-96.45%)	90% (59.58%-99.49%)

AUC: area under the curve; CI: confidence interval; IL: interleukin; TGF: transforming growth factor.

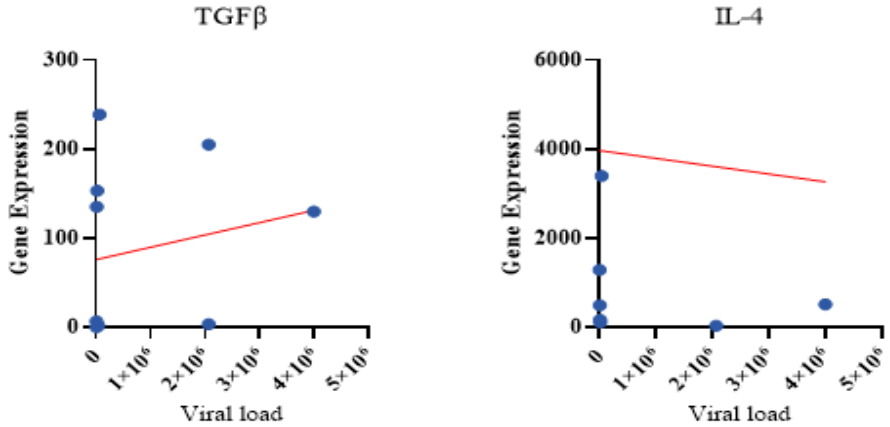

Figure 7. Correlation between viral load and gene expression level in HCMV+; no significant correlation was detected between these genes.

DISCUSSION

HCMV infection continues to pose a significant challenge in kidney transplantation, not only due to its direct cytopathic effects but also because of its profound influence on immune homeostasis, which may contribute to chronic graft dysfunction and reduced long-term outcomes.[22] Despite extensive research into HCMV immunobiology, the precise contributions of $CD4^+$ T cell subsets in regulating viral reactivation and shaping transplant immunity remain incompletely elucidated.[23] This study advances the field by delineating the phenotypic and transcriptional characteristics of T_H9 cells, a relatively novel $CD4^+$ subset, in KTRs with and without active HCMV infection.

T_H9 cells are differentiated under the influence of IL-4 and TGF-β, and are defined by their secretion of IL-9, a pleiotropic cytokine with both proinflammatory and immunomodulatory properties.[24-26] Although IL-9 has been extensively studied in the context of allergic and autoimmune diseases,[27] its role in viral infections, particularly in immunosuppressed transplant recipients, remains poorly characterized. Prior investigations have demonstrated that T_H9 cells can amplify inflammatory responses by promoting mast cell proliferation and chemokine release.[28,29] Conversely, they may also suppress antiviral T_H1 responses and enhance Treg activity, thereby contributing to immune tolerance or facilitating viral persistence depending on the immunologic milieu.[39,40,46]

Our findings revealed a significant elevation in $CD4^+IL\text{-}9^+$ T_H9 cells in $HCMV^+$ KTRs compared to $HCMV^-$ patients and healthy controls. This increase was paralleled by upregulated mRNA expression of *IL-4* and *TGF-β*, suggesting an active T_H9 differentiation axis during HCMV reactivation. Notably, no direct correlation was observed between T_H9 cell frequency and HCMV DNA load, although a modest association with hemoglobin levels was detected, potentially reflecting indirect hematologic effects of viral activity.

These observations align with findings from other viral contexts. For example, Ali et al reported a positive correlation between T_H9 cell frequency and disease severity in HCV-infected individuals, including associations with elevated liver enzymes and viral load.[30] Abdelhamid et al further linked increased IL-9 levels to poor therapeutic outcomes and progression to hepatocellular carcinoma in HCV patients.[31] Similar patterns have been documented in respiratory syncytial virus (RSV) infections, where IL-9 levels were elevated during severe disease,[32] and in HCV cases where IL-9 and IL-6 upregulation predicted treatment failure.[33] IL-9 has also been implicated in T_H17 expansion and hepatic inflammation,[34-38] and its capacity to inhibit T_H1 responses may contribute to viral persistence.[39,40]

In contrast, studies in chronic HBV infection have demonstrated reduced T_H9 cell frequencies and a negative correlation with HBV DNA levels,[41] underscoring the context-dependent nature of T_H9-mediated immunity. Liu et al reported elevated IL-9, IL-4, and TGF-β levels in wheezing infants, reinforcing the cytokine-driven differentiation of T_H9 cells.[42] Collectively, these findings suggest that T_H9 cells may exert dual functions, either promoting inflammation or facilitating immune regulation, depending on the viral pathogen and host environment.[43-45]

In our cohort, the upregulation of *IL-4* and *TGF-β* in $HCMV^+$ KTRs supports the hypothesis of active T_H9 differentiation. Previous studies have shown that HCMV DNA and viral proteins can induce TGF-β expression in renal tissues, contributing to vascular remodeling and graft injury.[51,52] TGF-β is also known to promote Treg development and suppress antiviral immunity in chronic hepatitis settings.[53,54] IL-4, meanwhile, has been associated with impaired $CD8^+$ T cell function and sustained viral replication in both HBV and HCV infections.[55-60] Elevated IL-4 levels have been reported in patients with severe hepatitis C and chronic HCV compared to those with milder disease, further highlighting its role in immune modulation.[61,62]

Taken together, our data suggest that T_H9 cells and their signature cytokine IL-9 may contribute to the immunological landscape of HCMV reactivation in KTRs. While their proinflammatory potential is well recognized, the immunoregulatory properties of IL-9, particularly its ability to suppress T_H1 responses and enhance Treg activity, may represent a novel mechanism for tolerance induction in transplant settings.[43,46,47] Nevertheless, the precise role of T_H9 cells in transplantation remains to be fully defined. Limitations in current literature, including small sample sizes, heterogeneous transplant populations, and methodological variability, hinder definitive conclusions. Emerging evidence, however, suggests that IL-9–producing Tregs may recruit mast cells and exert immunosuppressive effects that favor graft survival,[43,64] warranting further investigation into their therapeutic potential.

Additionally, an apparent discrepancy in T_H9 cell frequencies between surface and intracellular staining analyses reflects the distinction between phenotypic presence and functional activity. Surface marker-defined T_H9 cells ($CCR6^+CCR4^-IL\text{-}4R\alpha^+$) were more frequent in $HCMV^-$ patients, whereas intracellular $IL\text{-}9^+$ T_H9 cells were enriched in $HCMV^+$ patients. This suggests that while the surface phenotype may be broadly present, active IL-9 production is more prominent during viral reactivation. These findings highlight the importance of using both surface and intracellular markers to fully capture T_H9 cell dynamics and functional relevance in transplant immunology.

One notable limitation of this study is the significant age difference between the $HCMV^+$ group and both the $HCMV^-$ and healthy control groups. Aging is known to influence immune cell composition and cytokine profiles through mechanisms such as immunosenescence and inflammaging. These age-related changes may affect T_H9 cell differentiation, IL-9 production, and the expression of IL-4 and TGF-β, potentially confounding the observed associations with HCMV status. Although our findings suggest a role for T_H9 cells in HCMV reactivation, future studies with age-matched cohorts and multivariate analyses are needed.

Our findings reveal a significant elevation of T_H9 cells and IL-9 levels in $HCMV^+$ KTRs, underscoring their potential role in the immunopathogenesis of HCMV reactivation. These results suggest that T_H9/IL-9 dynamics may serve as valuable biomarkers for monitoring HCMV-related complications and guiding clinical decision-making. Moreover, the immunomodulatory properties of T_H9 cells position them as promising candidates for targeted immunotherapy in HCMV-infected KTRs. Future studies should explore T_H9-mediated mechanisms across diverse infectious contexts to validate their broader diagnostic and therapeutic potential.

STATEMENT OF ETHICS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the local ethics committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396. 814) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

FUNDING

This study was supported by Shiraz University of Medical Sciences (Grant No. 12980). Funding sources had no influence on study design, data collection, analysis or interpretation or in manuscript preparation and submission.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors wish to thank Shiraz University of Medical Sciences for the funding support.

DATA AVAILABILITY

All data generated or analysed during this study are included in this published article.

AI ASSISTANCE DISCLOSURE

Not applicable.

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