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Increased Cytotoxic CD4$^+$ T Cells with Reduced Cytotoxic Gene Profile Expression in Cytomegalovirus Reactivated Kidney Transplant Patients

Yashgin Hassanzadeh[1], Ramin Yaghobi[2], Parviz Pakzad[1], and Bita Geramizadeh[2]

[1] Department of Microbiology, North Tehran Branch, Islamic Azad University, Tehran, Iran
[2] Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Cytotoxic CD4$^+$ T cells eliminate human cytomegalovirus (HCMV)-infected cells through direct cytotoxic granules exocytosis. We aimed to evaluate the functional cytotoxic gene profile of CD4$^+$ T cells alongside the frequency of the cytotoxic phenotype in renal transplant recipients with cytomegalovirus reactivation.

Blood samples were collected from twenty renal recipients with and without HCMV reactivation (HCMV+ and HCMV- groups) and ten healthy adults (control group). CD4$^+$ T cells were isolated to assess the frequency of cytotoxic CD4$^+$ T cells via CD107a surface staining using flow cytometry and to evaluate gene expression of perforin, granzyme B, Runt-related transcription factor 3 (RUNX3), and Eomesodermin (Eomes) by quantitative PCR.

The frequency of CD4$^+$ CD107a$^+$ T cells was higher in the HCMV+ group compared to the HCMV- group and significantly higher than in the control group (22.69 ± 3.47 vs 16.41 ± 2.24 and 11.60 ± 1.17, respectively). Perforin gene levels were reduced in the HCMV+ group compared to the other two groups, while granzyme B gene levels were similar between HCMV+ and HCMV- groups but lower than in the control group (0.63 ± 1.24 vs 0.67 ± 2.27 and 1.00 ± 0.00, respectively).

This study demonstrated an increased frequency of cytotoxic CD4$^+$ T cells with potentially reduced functionality in kidney transplant patients with HCMV infection. It also suggests that these cells might employ other mechanisms, such as death receptor-mediated killing, or the production of other granules.

Keywords: CD107a antigen; Cytomegalovirus; Cytotoxic T lymphocytes; Perforin; RUNX3 protein

INTRODUCTION

Human cytomegalovirus (HCMV) can cause severe morbidity and even mortality in organ transplant recipients, persisting in the host's body for life.[1,2,3,4] CD4$^+$ cytotoxic T lymphocytes (CTLs) play a crucial role in eliminating chronic HCMV infections by releasing cytotoxic granules, such as granzyme B and perforin.[1,5,6,7] Granzyme B is abundant in these granules, while perforin facilitates their entry into target cells.[5,8,9]

The CD107a degranulation marker is used to identify CD4$^+$ CTLs.[10] These cells also upregulate Runt-related transcription factor 3 (RUNX3) for differentiation and Eomesodermin (Eomes) for granzyme B and perforin production.[5,6,10] After the primary HCMV infection resolves, CD4$^+$ CTLs producing perforin and granzyme

Corresponding Author: Ramin Yaghobi, PhD;
Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Postal Code: 7193711351, Shiraz, Iran. Tel/Fax: (+98 71) 3628 1529, Email: rayaviro@yahoo.com

B expand in the host.[1,5,6] CD107a mobilization and granzyme B production are two significant features of these cells in HCMV seropositive healthy and transplanted individuals.[1,5,11]

The quality of CD4+ CTL responses and their ability to home to infected tissues are critical for resolving HCMV infection.[12] Effective responses involve the upregulation of granzyme B and perforin genes, suggesting that a deficiency in cytotoxic granule production may hinder HCMV elimination.[5,12] CD4+ CTLs migrate to peripheral infected tissues, where inflammatory signals and antigens support their differentiation and function.[1,5,10] HCMV can persist latently and reactivate in multiple tissues, causing localized infections such as pneumonitis and colitis. To resolve these infections and prevent systemic spread, circulating CD4+ T cells must migrate from the blood to the infected tissue, perform their effector functions, and establish residency as tissue-resident cells.[13,14]

The lamina propria of various organs (e.g., lung and intestine) hosts CD4+ CTL precursors (CRTAM+ CD4+ T cells), which are predominantly found in murine models of infection.[1,5,10] In the gut mucosa, CD4+ T cells often differentiate into CD4+ CTLs, acquiring RUNX3 expression over ThPOk.[15] The bowel and lung are common sites for HCMV reactivation.[16] Evidence shows that perforin-producing CD4+ CTLs are the predominant cytotoxic T cells in the skin of the elderly, eradicating HCMV-infected fibroblasts.[12]

Cytotoxic CD4+ T cells migrate through the expression of various chemokine receptors.[1,10] In particular, CC chemokine receptor 10 (CCR10) directs T cells to mucosal surfaces and skin in response to chemokines CCL28 and CCL27. The mucosal-trafficking CCR10+ T cells typically reside in the epithelial cells of tissues such as the gut and airways, where they act as their effector sites.[17,18]

To determine an individual's ability to combat HCMV, it is essential to evaluate the functional characteristics of CD4+ CTLs and their migration to mucosal sites, rather than just their frequency. Our study employs a comprehensive assessment that combines these aspects to provide a more precise evaluation of the immune response against HCMV. We simultaneously examined the production of granzyme B and perforin at the molecular level, along with changes in the frequency of CCR10-expressing CD4+ CTLs using real-time PCR and flow cytometry. Furthermore, we analyzed the gene expression of transcription factors RUNX3 and Eomes to identify any differences in the differentiation or functional status of CD4+ CTLs, providing deeper insights into their role in fighting HCMV.

MATERIALS AND METHODS

Study Subjects

To ensure consistency among enrolled participants, criteria including age, donor/recipient serostatus, type of transplantation, and post-transplant duration were considered. Twenty HCMV seropositive adults (≥18 years) who had undergone kidney transplantation at least six months prior and received organs from seropositive donors (D+/R+) at Abu-Ali Sina Hospital, Shiraz, Iran, were enrolled in the study. The ethics committee of Shiraz University of Medical Sciences approved the study, and informed consent was obtained from each participant. Additionally, ten seropositive individuals from the Iranian Blood Transfusion Organization, who had not undergone transplantation and had no infections, were enrolled as a healthy control group.

HCMV DNAemia, assessed by real-time PCR, was used to categorize kidney recipients into two groups. Ten recipients who became HCMV DNAemic, with DNA levels of ≥10,000 copies/mL in their whole blood were categorized as the HCMV+ group. The other ten recipients, who had no detectable HCMV DNAemia during the same period, were classified as the HCMV- group.

Samples from different groups were collected simultaneously, with HCMV DNA assessed immediately afterward. The standard immunosuppressive regimen for kidney recipients included prednisolone (10 mg daily), mycophenolate mofetil (500 mg daily), and tacrolimus (2 mg daily). Neither the HCMV+ nor the HCMV- group received ganciclovir or valganciclovir treatment at the time of sampling. Recipients who were co-infected with other viruses (BK, HCV, HIV, and HBV), those who had undergone a second organ transplant, or those receiving antiviral therapy (ganciclovir or valganciclovir) were excluded from the study

Blood Collection, Processing, and Viremia Detection

Blood samples were collected in EDTA tubes. To determine HCMV viremia, plasma was isolated from the blood by centrifugation, and HCMV DNA was then extracted using the Sinaclon DNP extraction kit (Iran) according to the manufacturer's protocol. For viral load quantification, a mixture consisting of master mix (15 µL), internal control (1 µL), and DNA (5 µL) was

used to target exon four of the IE antigen with the GeneProof Cytomegalovirus PCR Kit (Czechia). A standard curve was generated from a series of standard solutions provided in the kit to quantify the HCMV load.

Real-time PCR was performed using the StepOnePlus instrument (Applied Biosystems, USA) with a two-step program. The protocol included an initial hold step of 2 minutes at 37℃, followed by 10 minutes at 95℃. The amplification step consisted of 45 cycles, each comprising 5 seconds at 95℃, 40 seconds at 60℃, and 20 seconds at 72℃.

The remaining blood was used to isolate peripheral blood mononuclear cells (PBMCs) through density gradient centrifugation using Ficoll (Inno-Train, Germany) according to the manufacturer's instructions. The isolated PBMCs were then cryopreserved in liquid nitrogen using a cryopreservation medium composed of 10% dimethyl sulfoxide (DMSO) (Merck, Germany) and 90% fetal bovine serum (FBS) (Merck, Germany) for subsequent analysis.

Purification of $CD4^+$ T Cells from PBMCs

To assess the differences in the frequency of $CD4^+CD107a^+$ cytotoxic T cells, the Miltenyi $CD4^+$ T cell isolation kit (Bergisch Gladbach, Germany) was used to negatively isolate $CD4^+$ cells from cryopreserved PBMCs using an LS column. The purity of the isolated $CD4^+$ T cells was then evaluated by staining with CD3 (PE, OKT3 clone) and CD4 (PerCp/Cy5.5, OKT4 clone) antibodies (BioLegend, USA). The $CD3^+CD4^+$ T cell populations with a purity greater than 95%, as determined by flow cytometry (FACSCalibur, BD Pharmingen, USA), were considered for further analysis.

Degranulation Assay and Flowcytometry

For CD107a surface protein staining, 2×10^6 isolated $CD4^+$ T cells were suspended in 2 ml of RPMI 1640 medium (Biosera, France) and distributed equally into 2 wells of a 24-well plate, with 1×10^6 cells per well. The RPMI 1640 medium was supplemented with Penicillin/Streptomycin (100 U/mL, Biosera, France), 10% fetal bovine serum, and 2mM L-glutamine (Gibco, USA). To the first well, Phorbol Myristate Acetate (PMA)/Ionomycin (2 µL) (Cell Activation Cocktail, BioLegend, USA) and CD107a antibody (Alexa Fluor 488, H4A3 clone) (BioLegend, USA) were added to stimulate and stain the purified $CD4^+$ T cells. For the unstimulated control, dimethyl sulfoxide (2 µL) and CD107a antibody were added to the second well. The plate was incubated at 37°C with 95% humidity and 5% CO_2 for 6 hours. After one hour of incubation, Monensin (GolgiStop, BD Pharmingen, USA) was added to both wells as a protein transport inhibitor.

After incubation, the cells from both wells were rinsed with RPMI 1640, and their viability was assessed using trypan blue under a microscope. The cells were then distributed into flow cytometry tubes designated for stimulated and unstimulated samples. In both tubes, the cells were stained with a CCR10 antibody (PE, 6588-5 clone) (BioLegend, USA) in flow cytometry buffer (PBS + 1% FBS) and incubated for 15 minutes at 4°C. Finally, the samples were analyzed using a FACSCalibur flow cytometer (BD Pharmingen, USA), and the data were processed with FlowJo software (version 10.5.3, Tree Star Inc., Ashland, USA). Propidium iodide (Sigma, Germany) was used to assess the viability of the isolated $CD4^+$ T cells. Isotype controls, Armenian hamster IgG (PE, HTK888 clone) and mouse IgG2b, κ (Alexa Fluor 488, MPC-11 clone) (BioLegend, USA), were used to validate the gating strategy and antibody specificity.

Transcription Factors and Cytotoxic Granules Expression Profiling

The $CD4^+$ T cells were processed with RiboEx LS total RNA solution (GeneAll, Korea) to extract total RNA according to the manufacturer's instructions. The extracted RNA was then used to synthesize cDNA with the NG dART RT Kit (EURx, Gdańsk, Poland), utilizing a mixture of oligo(dT)20 and random hexamer primers. The cDNA was subsequently used to analyze the expression of *PRF1* (perforin), *GZMB* (granzyme B), *Eomes*, and *RUNX3*. This was achieved using specific primers and the SG qPCR Master Mix (EURx, Gdańsk, Poland) on a StepOnePlus instrument (Applied Biosystems, USA).

The quantitative PCR conditions included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles, each consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Details on the primer sequences and reaction mixture composition are provided in the Supplementary Table. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene for normalizing gene expression. The data were analyzed using the Livak method ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

Data analysis and graph generation were performed using IBM SPSS Statistics (version 27.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 8.4.3, GraphPad Software, San Diego, CA, USA), respectively. To compare the characteristics of the three study groups, the Kruskal-Wallis test was used. For comparisons between the two study groups, the Mann–Whitney U test and Fisher's exact test were employed. The Spearman correlation coefficient was used to evaluate the correlation between two continuous variables. All data are presented as the mean ± SEM (standard error of the mean), with meaningful differences determined by a p value of less than 0.05.

RESULTS

Baseline Characteristics of Study Groups

The baseline clinical characteristics and demographic data of transplanted subjects are summarized in Table 1.

Table 1. Baseline characteristics of kidney recipients.

Variable	HCMV- (n=10)	HCMV+ (n=10)	*p*
Age at the time of sampling (Year) #	38.10 ± 4.80	53.40 ± 4.27	**0.02**
Sex††			
Male	6 (60%)	8 (80%)	0.06
female	4 (40%)	2 (20%)	
Living donor Tx††	3 (30%)	2 (20%)	
Related	1 (10%)	1 (10%)	>0.99
Unrelated	2 (20%)	1 (10%)	
Dialysis prior to Tx††	5 (50%)	7 (70%)	0.64
Types of dialysis††			
HD	4 (40%)	7 (70%)	ND
PD	1 (10%)	0	
Time on dialysis (Months) #	15.20 ± 6.61	24.33 ± 3.76	0.23
ESRD aetiology††			
Hypertension	9 (90%)	7 (70%)	ND
Diabetes mellitus	0	2 (20%)	
Glomerulonephritis	1 (10%)	0	
HCMV viral load (copy/mL)	<50 for all	>10^4 for all	ND

n: number; Tx: transplantation; HD: haemodialysis; PD: peritoneal dialysis; ESRD: end stage renal disease; ND: not determined; P: patients; # Results demonstrated as mean ± SEM; †† Results demonstrated as number (% of patients in the corresponding group); * $p \leq 0.05$.

During the study period, 19 males and 11 females (out of 30 subjects) were categorized into three groups: 10 in the HCMV+ group, 10 in the HCMV- group, and 10 in the control group. All subjects were HCMV IgG seropositive. The transplanted groups (HCMV+ and HCMV-) consisted of 14 males (70%) and 6 females (30%), while the control group comprised 5 males (50%) and 5 females (50%). The mean age at the time of sampling was 45.75 ± 3.58 years for the transplanted subjects and 38.70 ± 3.00 years for the control subjects. Notably, the mean age of subjects in the HCMV+ group was significantly higher than that in both the HCMV- and control groups (53.40 ± 4.27 vs 38.10 ± 4.80; $p = 0.02$ and 38.70 ± 3.00; $p = 0.01$, respectively). All kidney recipients had ABO-compatible transplants, with 25% receiving organs from living donors (10% related and 15% unrelated). In the HCMV+ group, the mean viral load was 697,067.80 ± 420,694.675 copies/mL, based on HCMV DNAemia results.

Frequency of CD4+ CD107a+ T cells

After gating on the CD4+ T lymphocytes (Figure 1A) and verifying their purity (Figure 1B), the population of cytotoxic CD4+ T cells expressing CD107a was evaluated across the different study groups (Figure 1C). The analysis revealed that the percentage of degranulating CD4+ T cells was higher in the HCMV+ group compared

to the HCMV- group, and significantly higher than in the control group (22.69 ± 3.47 vs 16.41 ± 2.24; $p = 0.19$ and 11.60 ± 1.17; $p = 0.01$, respectively). The HCMV- group also showed a higher percentage of degranulating $CD4^+$ T cells compared to the control group (16.41 ± 2.24 vs 11.60 ± 1.17; $p = 0.37$), although this difference was not statistically significant (Figure 1D).

Frequency of Mucosal Tissue Migrating $CD4^+$ $CCR10^+$ CD107a T Cells

Next, we investigated the frequency of the mucosal tissue-migrating subset of cytotoxic $CD4^+$ cells ($CD4^+$ $CCR10^+$ $CD107a^+$ T cells). Cells positive for both CCR10 and CD107a proteins (inside the red dashed line) were evaluated across the different study groups (Figure 2B). The results showed that this population was significantly higher in the HCMV+ group compared to the HCMV- and control groups (10.48 ± 2.01 vs 5.27 ± 1.29; $p = 0.04$ and 3.01 ± 0.65; $p = 0.003$, respectively). Additionally, the HCMV- group had a higher frequency of this cell population compared to the control group (5.27 ± 1.29 vs 3.01 ± 0.65; $p = 0.51$), although this difference did not reach statistical significance (Figure 2C).

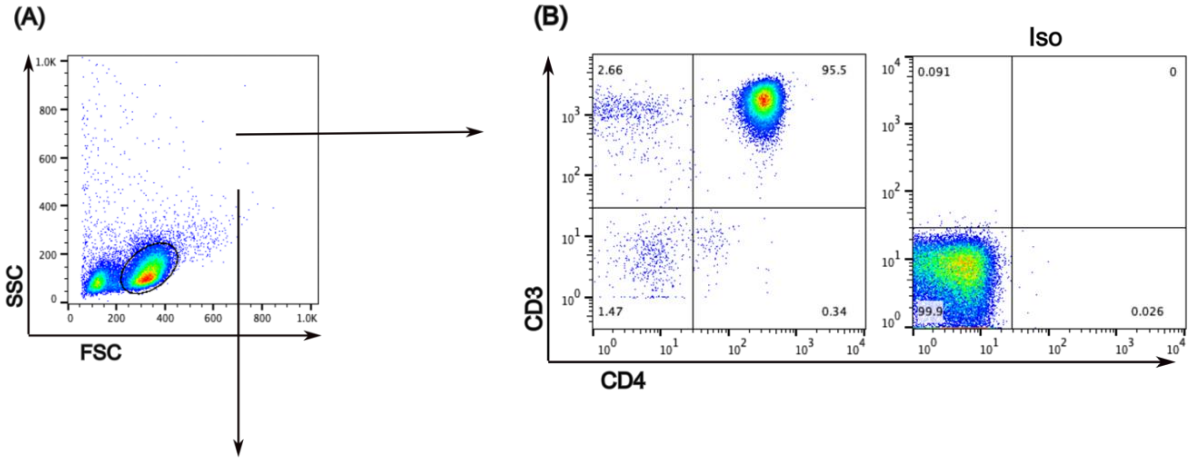
Expression Levels of Cytotoxic Granules and Transcription Factors Genes

We examined the gene expression of granzyme B (*GZMB*) and perforin (*PRF1*) to assess the status of cytotoxic granules at the gene level. The data analysis revealed that the *PRF1* expression level was lower in the HCMV+ group compared to both the HCMV- and control groups, but the differences were not statistically significant (−1.95 ± 1.53 vs −0.60 ± 2.08; $p = 0.79$ and 1.00 ± 0.00; $p = 0.35$, respectively). Additionally, *PRF1* expression was reduced in the HCMV- group relative to the control group (−0.60± 2.08 vs 1.00 ± 0.00; $p = 0.73$). Regarding *GZMB* gene expression, levels were similar between the HCMV+ and HCMV- groups but lower than those in the control group (0.63 ± 1.24 vs 0.67 ± 2.27; $p = 1.00$ and 1.00 ± 0.00; $p = 0.98$, respectively), which was also not statistically significant (Figure 3).

RUNX3 expression levels were comparable between the HCMV+ and HCMV- groups but were lower than in the control group (−4.91 ± 2.76 vs −5.37 ± 4.20; $p = 0.99$ and 1.00 ± 0.00; $p = 0.33$, respectively). Similarly, *RUNX3* levels in the HCMV- group were lower than those in the control group (−5.37 ± 4.20 vs 1.00 ± 0.00; $p = 0.28$), though this difference was not statistically significant. Regarding *Eomes* expression, levels were similar between the HCMV+ group and the control group (0.99 ± 1.41 vs 1.00 ± 0.00; $p = 1.00$, respectively). In contrast, *Eomes* expression was lower in the HCMV- group compared to both the HCMV+ and control groups (0.40 ± 2.09 vs 0.99 ± 1.41; $p = 0.95$ and 1.00 ± 0.00; $p = 0.95$, respectively) (Figure 3).

Correlation Between Cytotoxic Genes

We investigated the expression of cytotoxic genes in transplanted groups to determine potential correlations between them. Our analysis revealed a positive relationship between *GZMB* and *PRF1* gene expression ($r = 0.783$, $p < 0.001$). Both *GZMB* and *PRF1* also showed positive correlations with *Eomes* gene expression (*GZMB* with *Eomes*: $r = 0.643$, $p = 0.002$; *PRF1* with *Eomes*: $r = 0.757$, $p < 0.001$). Additionally, *PRF1* expression was positively correlated with *RUNX3* expression ($r = 0.628$, $p = 0.003$). However, no association was observed between *GZMB* and *RUNX3* ($r = 0.420$, $p = 0.065$), or between *RUNX3* and *Eomes* ($r = 0.439$, $p = 0.053$) (Figure 4).



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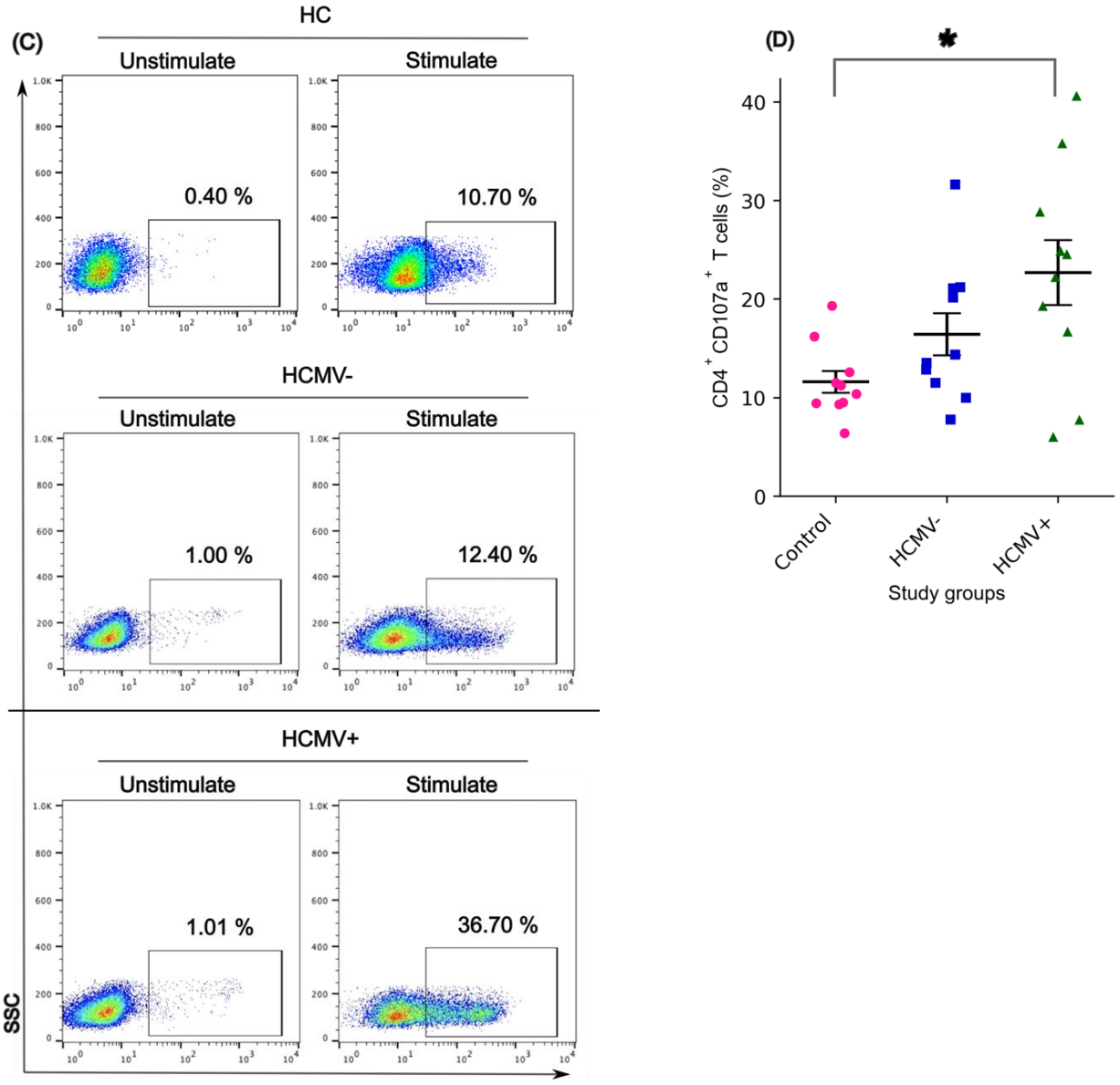


Figure 1. Increased frequency of CD4⁺ T cells with a cytotoxic phenotype in the HCMV+ kidney transplanted group. Flow cytometry analysis was performed to evaluate the frequency differences of CD4⁺ CD107a⁺ T cells among the study groups. A: CD4⁺ T lymphocytes were initially gated using side scatter and forward scatter (black circle). B: CD4⁺ T cell purity was verified by identifying cells positive for both CD3 and CD4 surface proteins. A population with more than 95% CD3⁺CD4⁺ T cells was considered pure CD4⁺ T cells. Isotype controls were used for gating and to ensure the specificity of antibody staining. C: The frequency of degranulating CD4⁺ CD107a⁺ T cells was assessed by evaluating CD107a⁺ cells against side scatter (black rectangle) after stimulation with Phorbol Myristate Acetate (PMA)/Ionomycin. An unstimulated control was used to establish the baseline frequency and validate the stimulation process. D: Statistical comparison of CD4⁺ CD107a⁺ T cell frequencies among the study groups was performed using the Kruskal-Wallis test. The baseline frequency of CD4⁺ CD107a⁺ T cells was subtracted. Horizontal lines on the bars represent the mean, and error bars indicate the standard error of the mean (SEM). The black bracket indicates a significant difference between pairs of the study groups. *p* values: Control vs HCMV- ($p = 0.37$), Control vs HCMV+ ($p = 0.01$), HCMV- vs HCMV+ ($p = 0.19$). SSC: side scatter; FSC: forward scatter; Iso: isotype control; HC: healthy control; HCMV-: kidney recipients without HCMV reactivation; HCMV+: kidney recipients with HCMV reactivation; * statistically significant at $p \leq 0.05$.

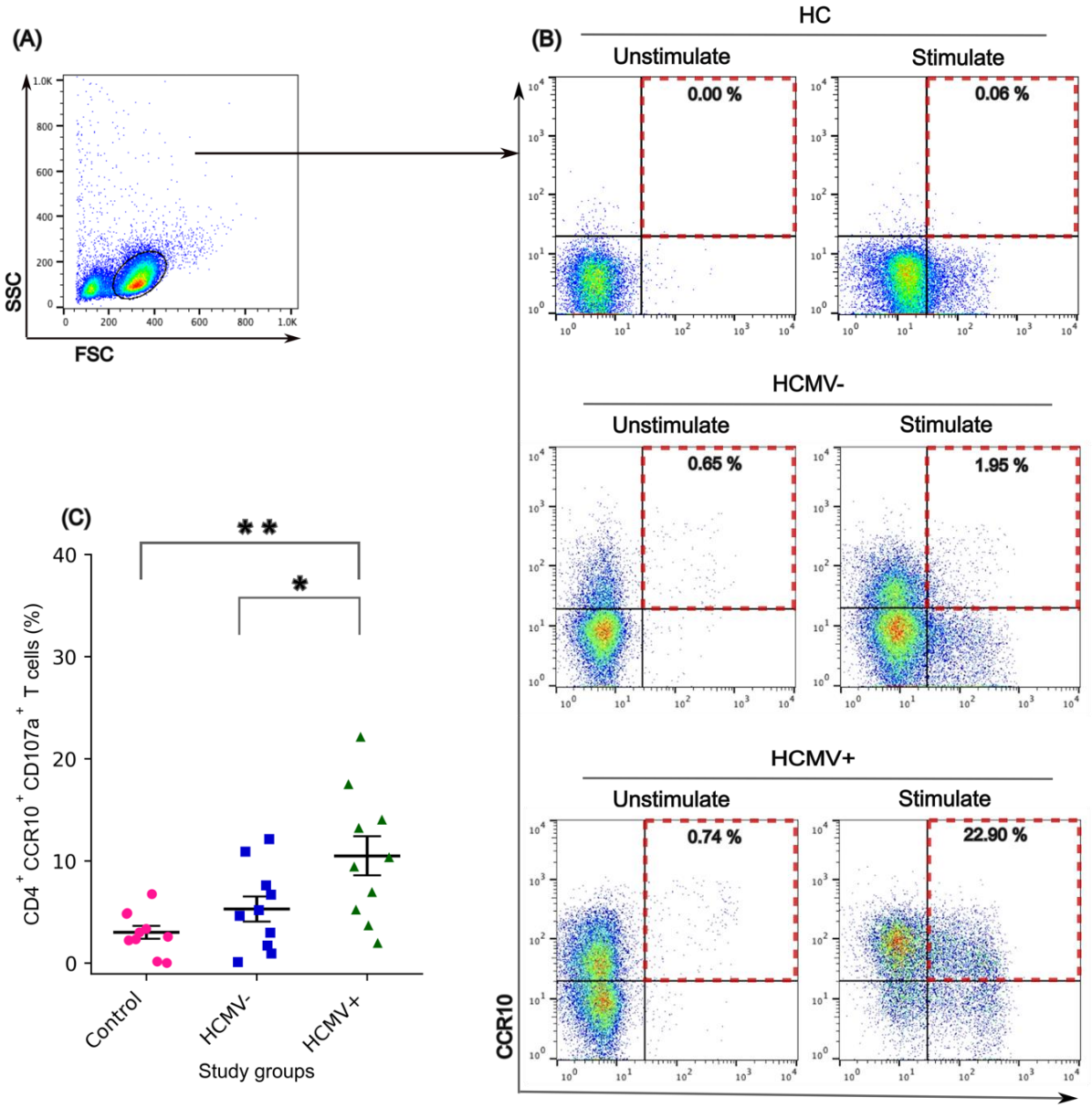


Figure 2. Increased frequency of mucosal tissue-migrating CD4⁺ T cells with a cytotoxic phenotype in the HCMV+ kidney transplanted group. Flow cytometry analysis was conducted to determine the frequency differences of CD4⁺ CCR10⁺ CD107a⁺ T cells among the study groups. A: CD4⁺ T lymphocytes were gated based on side scatter and forward scatter (black circle) to isolate the desired cell population. B: Within the gated CD4⁺ T cells, the frequency of CCR10⁺ CD107a⁺T cells was assessed after stimulation with Phorbol Myristate Acetate (PMA)/Ionomycin (red dashed line). An unstimulated control was used to establish the baseline frequency and ensure the accuracy of the stimulation process. C: The frequency of CD4⁺ CCR10⁺ CD107a⁺ T cells was statistically compared across the different study groups using the Kruskal-Wallis test. The baseline frequency of CD4⁺ CD107a⁺ T cells was subtracted. Horizontal lines on the bars represent the mean, and error bars indicate the standard error of the mean (SEM). Black brackets indicate significant differences between the study groups. *p* values: Control vs HCMV- ($p = 0.51$), Control vs HCMV+ ($p = 0.003$), HCMV- vs HCMV+ ($p = 0.04$). SSC: side scatter; FSC: forward scatter; HC: healthy control; HCMV-: kidney recipients without HCMV reactivation; HCMV+: kidney recipients with HCMV reactivation; * statistically significant at $p \leq 0.05$; ** statistically significant at $p \leq 0.01$.

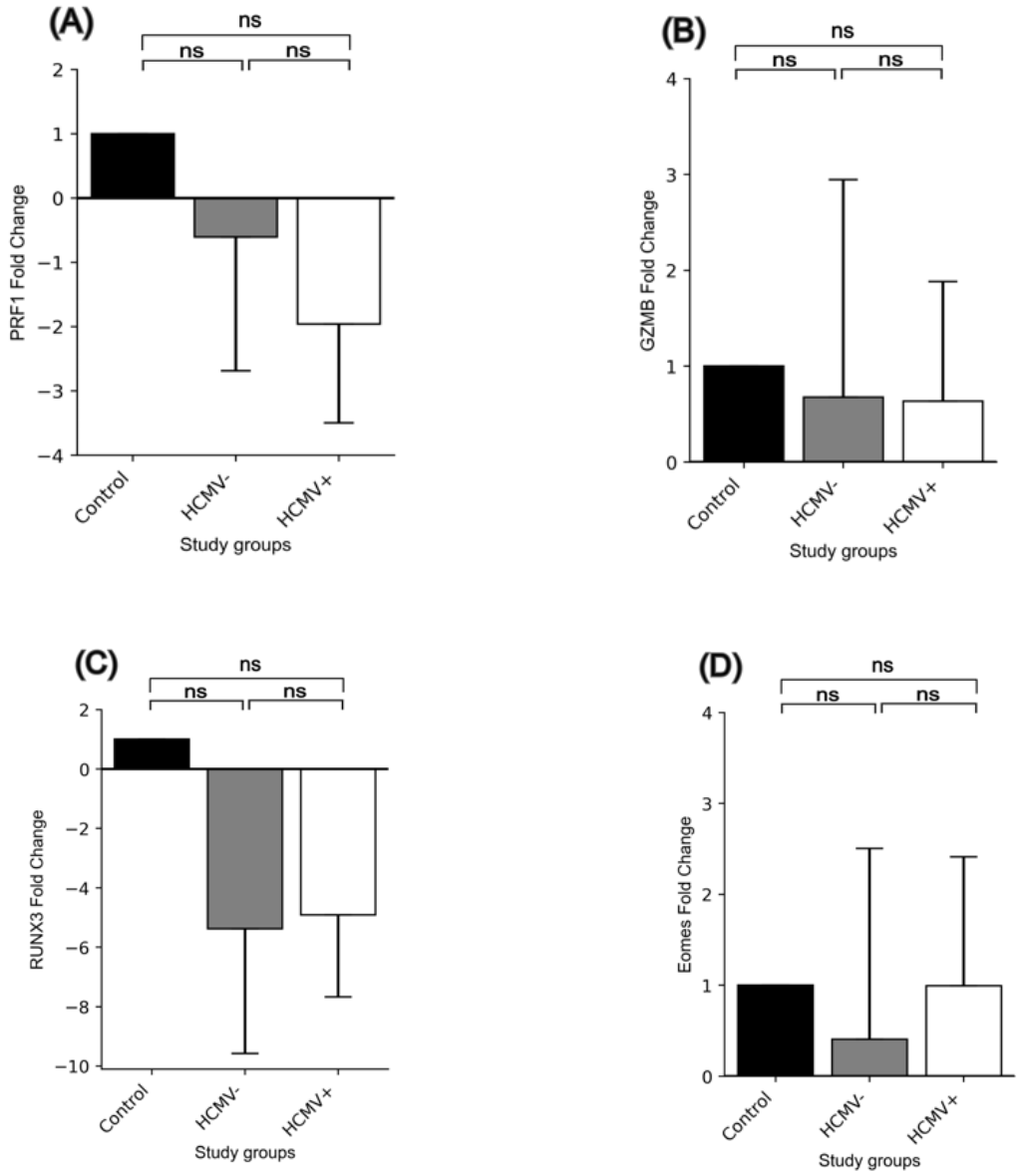
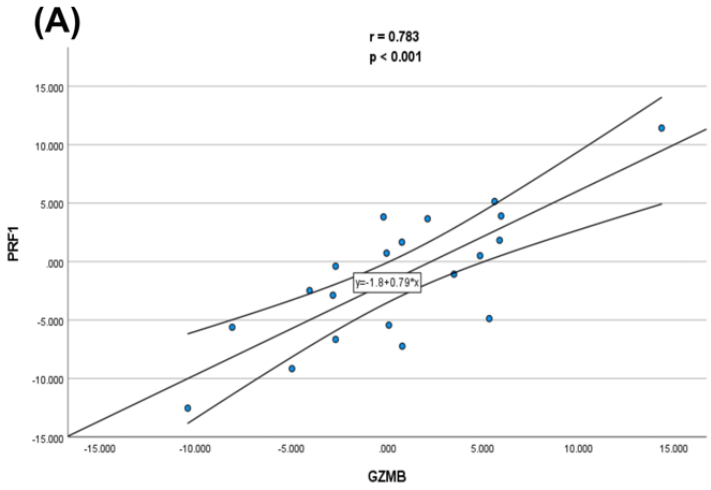
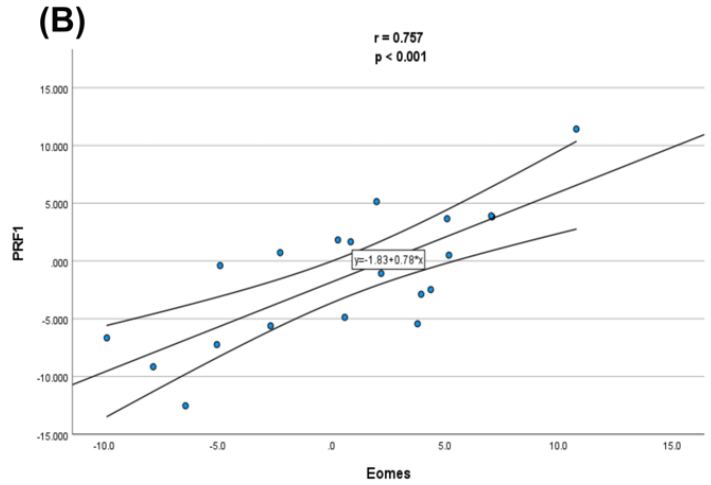


Figure 3. Cytotoxic gene expression profile of CD4+ T cells. Quantitative real-time PCR was used to determine the differences in cytotoxic gene expression in purified CD4+ T cells among the study groups. The results indicate a decreased expression profile of A: perforin (*PRF1*) B: granzyme B (*GZMB*), C: Runt-related transcription factor 3 (*RUNX3*), and D: Eomesodermin (*Eomes*) genes in the transplanted groups compared to the control group; however, the differences were not significant. Horizontal lines on the bars represent the mean, and the error bars indicate the standard error of the mean (SEM). Black brackets indicate statistical differences between study groups. *PRF1 p* values: Control vs HCMV- ($p = 0.73$), Control vs HCMV+ ($p = 0.35$), HCMV- vs HCMV+ ($p = 0.79$); *GZMB p* values: Control vs HCMV- ($p = 0.98$), Control vs HCMV+ ($p = 0.98$), HCMV- vs HCMV+ ($p = 1.00$); *RUNX3 p* values: Control vs HCMV- ($p = 0.28$), Control vs HCMV+ ($P = 0.33$), HCMV- vs HCMV+ ($p = 0.99$); *Eomes p* values: Control vs HCMV- ($p = 0.95$), Control vs HCMV+ ($p = 1.00$), HCMV- vs HCMV+ ($p = 0.95$). ns: not significant; control: healthy individuals; HCMV-: kidney recipients without HCMV reactivation; HCMV+: kidney recipients with HCMV reactivation.


(A)
r = 0.783
p < 0.001
y=-1.8+0.79*x
PRF1
GZMB


(B)
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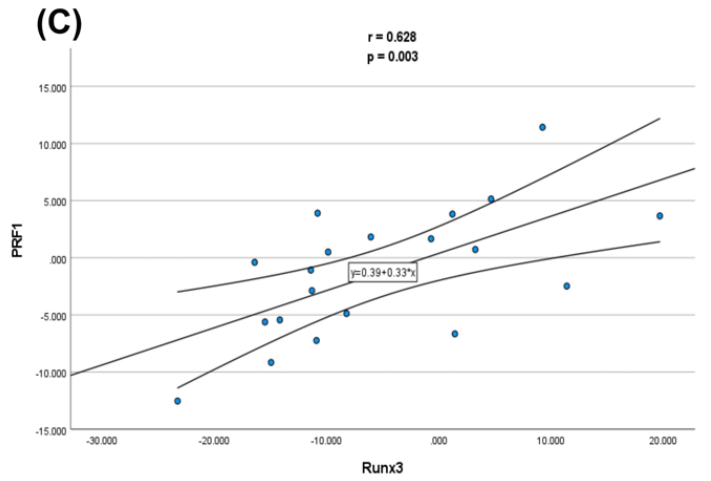
p < 0.001
y=-1.83+0.78*x
PRF1
Eomes


(C)
r = 0.628
p = 0.003
y=0.39+0.33*x
PRF1
Runx3

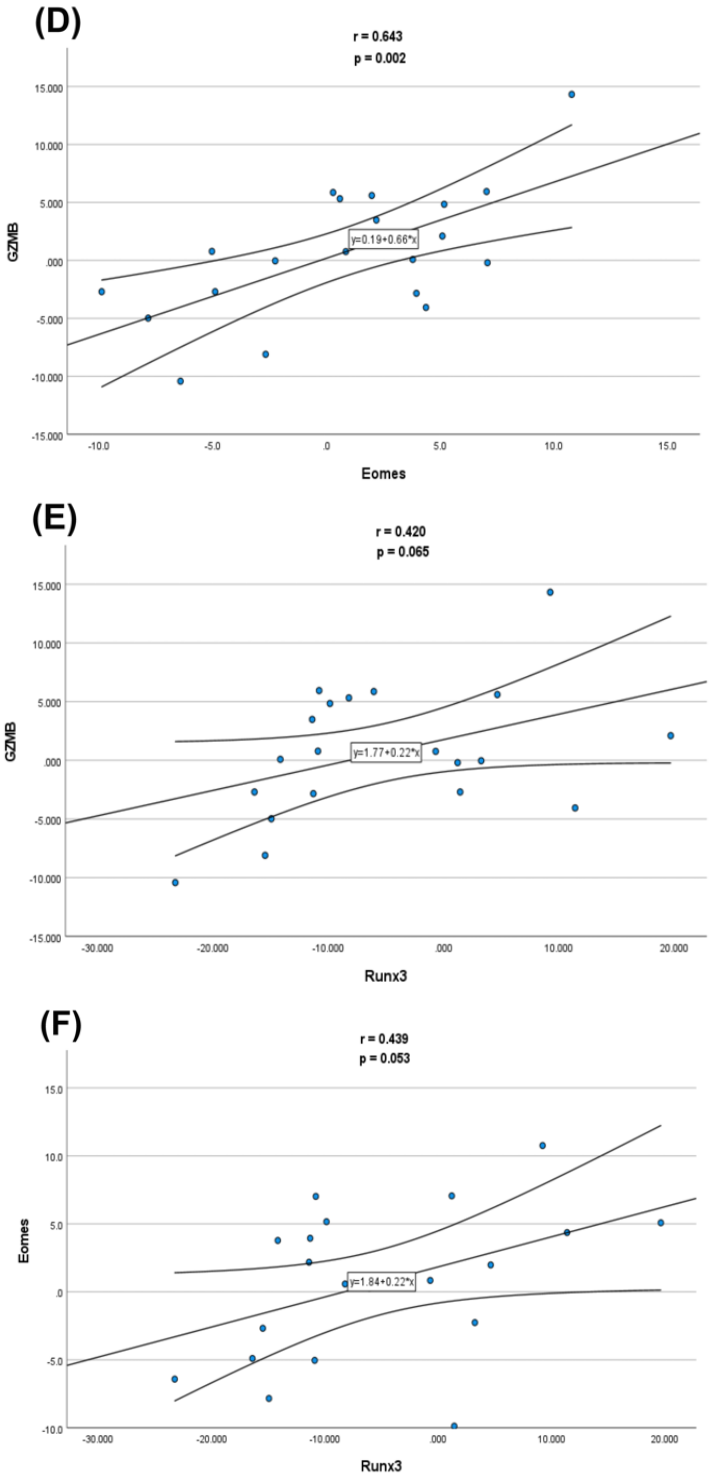


Figure 4. Spearman correlation analysis between cytotoxic genes in kidney transplanted groups. The data analysis revealed a positive correlation between A: *PRF1* and *GZMB* (r=0.783, p<0.001), B: *PRF1* and *Eomes* (r=0.757, p< 0.001), C: *PRF1* and *RUNX3* (r=0.628, p=0.003), D: *GZMB* and *Eomes* (r = 0.643, p = 0.002) genes in cytotoxic CD4+ T cells. No correlation was found between E: *GZMB* and *RUNX3* (r=0.420, p = 0.065) and (F) *Eomes* and *RUNX3* (r = 0.439, p = 0.053). The equations of the best fit line are shown within the rectangles in the middle of each dot plot. *PRF1*: perforin; *GZMB*: granzyme B; *Eomes*: eomesodermin; *RUNX3*: runt-related transcription factor 3.

DISCUSSION

Functional features of immune cells, when combined with frequency analysis, provide a more accurate assessment of the host's response to HCMV infection.[4,19-21] Cytotoxic $CD4^+$ T cells function through the secretion of cytotoxic granules to mount protective immune responses against HCMV.[12] Therefore, we evaluated the functionality of $CD4^+$ CTLs by assessing the gene expression of key cytotoxic granules (granzyme B and perforin) and the transcription factors that induce their production (Eomes and RUNX3) in kidney recipients with HCMV reactivation. Additionally, we examined the frequency of degranulating $CD4^+$ T cells and their migratory subset to mucosal tissues by surface staining for CD107a protein ($CD4^+$ $CD107a^+$ T cells) and the CCR10 chemokine receptor ($CD4^+$ $CCR10^+$ $CD107a^+$ T cells).

Our findings indicate that, despite a higher frequency of $CD4^+$ CTLs in HCMV+ kidney recipients compared to HCMV- recipients and healthy individuals, these cells likely exhibit reduced functionality. Perforin gene expression was lower in HCMV+ patients than in the other two groups, while a slight reduction in granzyme B expression was observed across all transplanted groups. No significant differences were detected in *Eomes* gene expression levels among the study groups. However, *RUNX3* expression levels were reduced in both transplanted groups compared to the control group. Furthermore, the higher frequency of CCR10-expressing $CD4^+$ CTLs in HCMV+ patients suggested their potential to migrate to mucosal tissues and skin. Although the gene expression differences were not statistically significant, discussing the possible reasons for their increase or decrease can guide future research efforts.

HCMV infection typically leads to an upregulation of cytotoxic gene profiles in $CD4^+$ T cells, including granzyme A, B, H, and perforin.[1,22-24] However, there are few reports of high CD107a surface mobilization either on $CD4^+$ T cells or other cell types, concurrent with a defect in cytotoxic granule production in transplanted patients.[11,19,25] The defect in gene expression of granzyme B and perforin in HCMV-specific $CD4^+$ T cells has been reported in seropositive transplanted patients, although these cells maintained a stable cytotoxic phenotype indicated by CD107a surface expression over a year post-transplant.[19] The increased CD107a surface expression, downregulation of perforin, and lack of difference in granzyme B gene expression that we observed in HCMV+ patients align with these studies. Similar results of increased CD107a expression have been reported in transplant patients with HCMV reactivation, with no differences in perforin gene expression observed in NK cells.[11]

Evidence indicates no direct link between CD107a expression, granule production, and direct target cell killing, and CD107a expression may not necessarily indicate granzyme degranulation.[19,26] In fact, cytotoxic $CD4^+$ T cells might produce other granules than perforin and granzyme B.[19] Therefore, according to our findings and existing evidence, we speculate that CD107a surface expression may indicate the degranulation capability of $CD4^+$ CTLs rather than their overall functional ability to kill target cells. To more accurately assess $CD4^+$ T cell cytotoxic function, a better approach could involve measuring cytotoxic granule production followed by killing assays or evaluating other mechanisms such as death receptors (e.g., FAS ligand) and cytokine-mediated killing (e.g., TNF-α or IFN-γ).[19,27]

The observed reduction in *RUNX3* expression in both transplanted groups in our study contradicts the findings of Higdon et al who reported stable *RUNX3* levels in $CD4^+$ T cells over a year post-transplant in seropositive heart and kidney recipients.[19] This reduction may suggest a defect in the acquisition of cytotoxic function by $CD4^+$ T cells. Consequently, our patients might not be generating new cytotoxic $CD4^+$ T cells, and the higher frequency observed in the HCMV+ group could reflect the initial cytotoxic population. Immunosuppressive drugs used in transplantation and HCMV immune evasion strategies may influence RUNX3 regulation, necessitating further research for clarification.

RUNX3 regulates Eomes and T-bet, which are key transcription factors involved in the production of perforin and granzyme B.[10,28-30] Eomes are more commonly associated with $CD4^+$ CTLs, while T-bet is linked to $CD8^+$ T cells.[10,31] Reports on Eomes' role in perforin production and cytotoxicity induction in $CD4^+$ CTLs are inconsistent.[5,6,10,28] Some studies suggest that Eomes promotes cytotoxic functionality in $CD4^+$ T cells, while others indicate a lesser essential role in inducing perforin expression.[28,32,33] One study suggests a crucial cooperation of RUNX3 with T-bet, rather than Eomes, to enable cytotoxic functionality and perforin production in $CD4^+$ T cells.[28]

The positive correlation between *Eomes* and *RUNX3* with perforin in this study, combined with the downregulation of *RUNX3* and perforin genes (but not *Eomes*) in the HCMV+ group, suggests less collaboration between RUNX3 and Eomes and raises the possibility of T-bet's role in perforin induction. Current evidence shows that nullifying Eomes does not influence granzyme B and perforin expression in HCMV-specific $CD4^+$ CTLs while reducing these proteins is affected by T-bet knockdown.[22] Given the observed correlation between Eomes and perforin in our study, we cannot rule out the possibility that Eomes contributes to perforin production. However, it is possible that the $CD4^+$ T cells in our patients did not mature sufficiently, resulting in lower perforin levels and the production of other granzymes than granzyme B, or they may have differentiated from non-Th1 precursors.[34,35] Further research is needed in both cases.

$CD4^+$ T cells are categorized into different tissue-homing subsets based on their expression of various chemokine receptors involved in trafficking. Some chemokine receptors, such as CCR7, direct $CD4^+$ T cells to specific tissues, while others facilitate trafficking between multiple tissues.[29,36] $CD4^+$ CTLs express CCR5, CXCR3, and CX3CR1 for trafficking to peripheral tissues and lack CCR7, which limits their migration to lymph nodes.[1,23,37] The increased frequency of $CD4^+$ $CCR10^+$ $CD107a^+$ CTLs and the progressive proliferation of the virus in the HCMV+ group suggests that these cells are capable of, and are urgently needed for, patrolling and homing into inflamed mucosal and skin tissues where HCMV can reactivate.

This study has several limitations. First, we analyzed gene expression and phenotype frequency in a small population. Expanding the study to include different serological transplanted groups and age- and sex-matched individuals would be beneficial. Second, we examined the general cytotoxic population rather than specific responses to virus-specific antigens. Third, evaluating exhaustion and senescence, characteristics of dysfunctional T cells, in these patients could be valuable. Markers of these phenomena have been reported on the surface of cytotoxic HCMV-specific $CD4^+$ CTLs with low granzyme B and perforin production, and they are more abundant in older individuals than in younger ones.[12,20,26,38,39] Given the higher average age of our HCMV+ kidney recipients, the progressive infection requiring further antiviral therapy, and the downregulation of perforin and granzyme B genes, we speculate that their $CD4^+$ CTLs might exhibit signs of exhaustion or senescence (at least in part), warranting further investigation.

Further research is crucial to determine if $CD4^+$ CTLs can become tissue-resident cells within infected tissues and whether the tissue microenvironment supports RUNX3 expression. Studying the mechanisms regulating RUNX3, T-bet, and Eomes will clarify their roles in cytotoxicity induction. Additionally, exploring other cytotoxic functions will broaden insights into $CD4^+$ CTL functionality during HCMV infection.

In conclusion, during HCMV reactivation in kidney recipients, cytotoxic $CD4^+$ T cell function may decrease due to lower levels of cytotoxic granules, despite increased frequency and enhanced migration ability to mucosal tissues. CD107a surface expression is useful for assessing degranulation ability, but it does not fully reflect overall cytotoxic functionality, and reduced *RUNX3* expression suggests a possible impairment in the transition from helper to cytotoxic cells.

STATEMENT OF ETHICS

All applied methods in this study were in complete accordance with the Helsinki Declaration and its subsequent amendments. The local Ethics Committee of Shiraz University of Medical Sciences approved every step of the study with the relevant code (IR.SUMS.REC.1396.S814). Written informed consent was obtained from each patient after explaining the study objectives.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Data Availability

Data is available from the corresponding author upon reasonable request.

REFERENCES

1. Muraro E, Merlo A, Martorelli D, Cangemi M, Dalla Santa S, Dolcetti R, et al. Fighting viral infections and virus-Driven Tumors with Cytotoxic $CD4^+$ T Cells. Front Immunol. 2017;8:197.
2. Afshari A, Yaghobi R, Golshan M. Cytomegalovirus microRNAs level determination in kidney recipients post transplantation. Virol J. 2022;19(1):147.
3. Soleimanian S, Yaghobi R, Karimi MH, Geramizadeh B, Roozbeh J. The Direct Influence of Cytomegalovirus Lysate on the Natural Killer Cell Receptor Repertoire. Iran J Allergy Asthma Immunol. 2021;20(6):721-33.
4. Hassanzadeh Y, Yaghobi R, Pakzad P, Geramizadeh B. Decreased frequency of Th22 cells and IL- 22 cytokine in kidney transplant patients with active cytomegalovirus infection. BMC Immunol. 2023;24(1):18.
5. Juno JA, van Bockel D, Kent SJ, Kelleher AD, Zaunders JJ, Munier CML. Cytotoxic CD4 T Cells—Friend or Foe during viral infection? Front Immunol. 2017;8:19.
6. Cenerenti M, Saillard M, Romero P, Jandus C. The Era of Cytotoxic CD4 T Cells. Front Immunol. 2022;13:867189.
7. Hassanzadeh Y, Yaghobi R, Pakzad P, Geramizadeh B. Risk assessment of human cytomegalovirus infection in solid organ transplantation: Insight into CD4+ T cell subsets. Scand J Immunol. 2022:96(5):e13212.
8. Cohnen A, Chiang SC, Stojanovic A, Schmidt H, Claus M, Saftig P, et al. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. Blood. 2013;122(8):1411-1418.
9. Verschoor CP, Picard E, Andrew MK, Haynes L, Loeb M, Pawelec G, et al. NK- and T-cell granzyme B and K expression correlates with age, CMV infection and influenza vaccine-induced antibody titres in older adults. Front Aging. 2023;3:1098200.
10. Takeuchi A, Saito T. CD4 CTL, a Cytotoxic Subset of $CD4^+$ T Cells, Their Differentiation and Function. Front Immunol. 2017;8(2):194.
11. Soleimanian S, Yaghobi R, Karimi MH, Geramizadeh B, Roozbeh J, Hossein Aghdaie M, et al. Circulating NKG2C + NK cell expressing CD107a/LAMP-1 subsets at the onset of CMV reactivation in seropositive kidney transplant recipients. Transpl Immunol. 2021;69:101460.
12. Hasegawa T, Oka T, Son HG, Oliver-Garcı´a VS, Azin M, Eisenhaure TM, et al. Cytotoxic CD4+ T cells eliminate senescent cells by targeting cytomegalovirus antigen. Cell. 2023;186(7):1417–31.
13. Gordon CL, Miron M, Thome JJC, Matsuoka N, Weiner J, Rak MA, et al. Tissue reservoirs of antiviral T cell immunity in persistent human CMV infection. J Exp Med. 2017;214(3):651–67
14. Almanan M, Raynor J, Sholl A, Wang M, Chougnet C, Cardin RD, et al. Tissue-specific control of latent CMV reactivation by regulatory T cells. PLoS Pathog. 2017;13(8):e1006507.
15. Verma S, Weiskopf D, Gupta A, McDonald B, Peters B, Sette A, et al. Cytomegalovirus-specific CD4 T cells are cytolytic and mediate vaccine protection. J Virol. 2016;90(2): 650 -8.
16. Perera MR, Greenwood EJD, Crozier TWM, Elder EG, Schmitt J, Crump CM, et al. Human Cytomegalovirus Infection of Epithelial Cells Increases SARS-CoV-2 Superinfection by Upregulating the ACE2 Receptor. J Infect Dis. 2023;227(4):543–53.
17. Xiong N, Fu Y, Hu S, Xia M, Yang J. CCR10 and its ligands in regulation of epithelial immunity and diseases. Protein Cell. 2012;3(8):571–80.
18. Murphy PM. Chemokines and Chemokine Receptors, in: Drs. Robert R. Rich, Thomas A. Fleisher, William T. Shearer, Harry W. Schroeder, A.J. Frew, C.M. Weyand (Eds.), Clinical Immunology (Fifth Edition): Principles and Practice, Elsevier Ltd, 2019, p157-70. https://doi.org/10.1016/C2015-0-00344-6.
19. Higdon LE, Ahmad AA, Schaffert S, Margulies KB, Maltzman JS. CMV-Responsive CD4 T Cells Have a Stable Cytotoxic Phenotype Over the First Year Post-Transplant in Patients Without Evidence of CMV Viremia. Front Immunol. 2022;13:904705.
20. Kallemeijn MJ, Boots AMH, van der Klift MY, Brouwer E, Abdulahad WH, Verhaar JAN, et al. Ageing and latent CMV infection impact on maturation, differentiation and exhaustion profiles of T-cell receptor gammadelta T-cells. Sci Rep. 2017;7(1):5509.
21. Chanouzas D, Sagmeister M, Faustini S, Nightingale P, Richter A, Ferro CJ, et al. Subclinical Reactivation of Cytomegalovirus Drives $CD4^+CD28^{null}$ T-Cell Expansion and Impaired Immune Response to Pneumococcal Vaccination in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis. J Infect Dis. 2019;219(2):234–44.
22. Tamura Y, Yamane K, Kawano Y, Bullinger L, Wirtz T, Weber T, et al. Concomitant Cytotoxic Effector

Differentiation of CD4+ and CD8+ T Cells in Response to EBV-Infected B Cells. Cancers. 2022;14(17):4118.
23. Pachnio A, Ciaurriz M, Begum J, Lal N, Zuo J, Beggs A, et al. Cytomegalovirus Infection Leads to Development of High Frequencies of Cytotoxic Virus-Specific CD4+ T Cells Targeted to Vascular Endothelium. PLoS Pathog. 2016;12(9):e1005832
24. Soleimanian S, Yaghobi R, Karimi MH, Geramizadeh B, Roozbeh J. Altered Signatures of Plasma Inflammatory Proteins and Phonotypic Markers of NK Cells in Kidney Transplant Patients upon CMV Reactivation. Curr Microbiol. 2023;80(1):9.
25. Casazza JP, Betts MR, Price MA, Precopio ML, Ruff LE, Brenchley JM, et al. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J Exp Med. 2006;203(13):2865–77.
26. Luo XH, Meng Q, Rao M, Liu Z, Paraschoudi G, Dodoo E, et al. The impact of inflationary cytomegalovirus-specific memory T cells on anti-tumour immune responses in patients with cancer. Immunology. 2018;155(3):294–308.
27. Malyshkina A, Littwitz-Salomon E, Sutter K, Zelinskyy G, Windmann S, Schimmer S, et al. Fas Ligand-mediated cytotoxicity of CD4+ T cells during chronic retrovirus infection. Sci Rep. 2017;7(8):7785.
28. Serroukh Y, Gu-Trantien C, Hooshiar Kashani B, Defrance M., Manh TPV, Azouz A, et al. The transcription factors Runx3 and ThPOK cross-regulate acquisition of cytotoxic function by human Th1 lymphocytes. eLife. 2018;7:e30496.
29. Hoeks C, van Puijfelik F, Koetzier SC, Rip J, Corsten CEA, Wierenga-Wolf AF, et al. Differential Runx3, Eomes, and T-bet expression subdivides MS-associated CD4+ T cells with brain-homing capacity. Eur J Immunol. 2024;54(2):e2350544.
30. Cachot A, Bilous M, Liu YC, Li X, Saillard M, Cenerenti M, et al. Tumor-specific cytolytic CD4 T cells mediate immunity against human cancer. Sci Adv. 2021;7(9):eabe3348.
31. Eshima K, Misawa K, Ohashi C, Iwabuchi K. Role of T-bet, the master regulator of Th1 cells, in thecytotoxicity of murine CD4+ T cells. Microbiol Immunol. 2018;62(5):348–56.
32. Phetsouphanh C, Aldridge D, Marchi E, Munier ML, Meyerowitz J, Murray L, et al. Maintenance of Functional CD57+ Cytolytic CD4+ T Cells in HIV+ Elite Controllers. Front Immunol. 2019;10:1844.
33. Takeuchi A, El Sherif Gadelhaq Badr M, Miyauchi K, Ishihara C, Onishi R, Guo Z, et al. CRTAM determines the CD4+ cytotoxic T lymphocyte lineage. J Exp Med. 2016;213(1):123–38.
34. Wang Y, Chen Z, Wang T, Guo H, Liu Y, Dang N, et al. A novel CD4+ CTL subtype characterized by chemotaxis and inflammation is involved in the pathogenesis of Graves' orbitopathy. Cell Mol Immunol. 2021;18(3):735–45.
35. Mazzoni A, Maggi L, Siracusa F, Ramazzotti M, Rossi MC, Santarlasci V, et al. Eomes controls the development of Th17-derived (non-classic) Th1 cells during chronic inflammation. Eur J Immunol. 2019;49(1):79–95.
36. Al-Banna NA, Vaci M, Slauenwhite D, Johnston B, Issekutz TB. CCR4 and CXCR3 play different roles in the migration of T cells to inflammation in skin, arthritic joints, and lymph nodes. Eur J Immunol. 2014;44(6):1633–43.
37. Shabir S, Smith H, Kaul B, Pachnio A, Jham S, Kuravi S, et al. Cytomegalovirus-Associated CD4+CD28null Cells in NKG2D-Dependent Glomerular Endothelial Injury and Kidney Allograft Dysfunction. Am J Transplant. 2016;16(4):1113–28.
38. Hassouneh F, Goldeck D, Pera A, van Heemst D, Slagboom PE, Pawelec G, et al. Functional Changes of T-Cell Subsets with Age and CMV Infection. Int J Mol Sci. 2021;22(18):9973.
39. Huygens A, Lecomte S, Tackoen M, Olislagers V, Delmarcelle Y, Burny V, et al. Functional Exhaustion Limits CD4+ and CD8+ T-Cell Responses to Congenital Cytomegalovirus Infection. J Infect Dis. 2015;212(3):484-94.