Loss of CCR7 Expression on CD57 + CD56/ CD16 + NK Cells Correlates with Viral Load in CMV Reactivated Kidney Transplant Recipients

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Original Paper

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Keywords. Cytomegalovirus, Kidney transplantation, NK cells, CC Chemokine Receptor 7, Load, Viral, Reactivation, Infection

Introduction. Despite developing strategies for antiviral treatment, cytomegalovirus (CMV) infection remains one of the most common challenges in kidney transplant recipients (KTRs). The evaluation of CMV viral load is still the most practical main clinical approach for CMV assessment and guides decision-making in recipient antiviral treatment. However, there is not a specific viral load cut off for initiating treatment yet. On the other hand, the cellular immune system and the innate immune response prove their roles in diagnosing CMV reinfection and monitoring the therapeutic regime to control CMV. Interactions among the components of cellular immunity encounter CMV reactivation provide a strong treatment management plan for clinical decisions about antiviral therapy against CMV. Natural killer (NK) cells, as essential effector cells, present potentially antiviral activity through distinct subpopulations. CCR7expressing NK cells were identified by high cytotoxicity and functionality among NK cell subsets. Here, we explored the correlation between CCR7+ expressing NK cells with viral load in CMV reactivated-kidney transplant recipients.
Materials and Methods. A cross-sectional study was conducted among ten CMV reactivated KTRs. The CMV DNA copy number was evaluated utilizing real-time PCR.NK cell phenotypic profiling was done using flow cytometry.
Results. Increasing of CMV viral load in CMV reactivated KTRs had a negative correlation with CCR7+CD57+ CD56/CD16+ NK cell ($P < .05$ r = -0.7) after CMV reactivation. Significantly increased level of CCR7-CD57- CD56/CD16+ NK cell was associated with CMV viral load within CMV reactivated KTRs ($P < .05$, r = 0.68).
Conclusion. CCR7 expression is associated with CMV reactivation, which offers a new aspect of CMV-associated immunity within the NK cell compartment.

IJKD 2022;16:52-62
www.ijkd.org
DOI: 10.52547/ijkd.6721

INTRODUCTION

Despite treatment strategies, recurrence of cytomegalovirus(CMV) infection in kidney transplant recipients (KTRs) remains a common complication [1]. While immunosuppressive regimens cause compromised antiviral-specific T-cell immune response after transplantation [2], natural killer (NK) cell activity seems to be unaffected by such

treatment and could thus contribute to antiviral immunity in the context of transplantation [3, 4]. Recently, NK-mediated immune responses to CMV infection have been considered extensively [5,6]. These cells are members of large granular lymphocytes described by a lack of T-cell receptor (CD3) and the surface expression of CD56 and CD16 (the low-affinity IgG-Fc receptor III; FcγRIII). There are two types of NK cells, based on the expression of CD56 and CD16: CD56dimCD16+ cells (~90% of circulating NK cells), which have cytotoxic activity, and CD56brightCD16- cells, which account for 10% of the NK cells by regulatory functions *via* cytokine production field[7, 8]. CD56bright NK cell subset seems to be a precursor of the terminally differentiated CD56dimCD16+ NK cells. Throughout the maturation of NK cells from CD56 bright to CD56 dim, receptors expression patterns are gradually altered, such as expression changes in CD94/NKG2 receptor family, resulting in the lost expression of NKG2A, sequentially acquire activating receptors NKG2C and NKG2D[9], characterizing the memory-like NK cells. In this regard, several evidence lines demonstrated that compared to the conventional NK cells, more cytotoxicity and effector activity have been identified through these adaptive NK cells[10, 11]. Many studies have mainly focused on CD56 +NKG2C+ NKG2A-CD57+NK cell subsets and their features and functionality during CMV infection [12]. However, according to pathological conditions, NK cells are heterogeneous with this phenotype [13], maturation stage, and functional capabilities [14, 15], .Moreover, and also the influence of CMV-infection on CD56bright NK cell subsets is less well understood. Furthermore, CD56 bright NK cells express different chemokine receptors [16]. Among the chemokine markers, C-C chemokine receptor type 7 (CCR7) characterize CD56bright NK cell populations, considering a vital chemokine receptor in both innate and adaptive immunity [17, 18]. A study found the correlation between $CD56^{bright}$ NK cells lacking CCR7 and HIV viral load in HIV-infected individuals, described by enhanced cytotoxicity and functionality[20]. In monitoring CMV reactivation, it is appreciated to know about the role of CD56 bright NK cells by expressing the CCR7 receptor in the context of clinical transplantation. Notably, to what extent NK cells relate related to CMV DNA copy number is still unknown in recipients. We, therefore, set out to analyze the correlation between CCR7 expressing CD56 bright NK cells and CMV copy numbers at the onset of CMV reactivation among a cohort of KTRs.

METHOD

Study Design and Patient Population

Same as the method applied in our previous According to our previous study report[21], PBMCs were isolated from 20 mL, EDTA fresh blood of ten CMV reactivated KTRs with more than 18 years age up to 18, with and no history of other infections, including; Hepatitis B Virus, Hepatitis C Virus, polyomavirus BK, adenovirus, or and Human Immunodeficiency Virus. The samples of eligible patients were collected from March 2019 to November 2020 in the Abu Ali Sina Transplant Hospital, Shiraz (Shiraz, Iran). These patients had been referred Kidney transplant recipients were referred to the hospital with raised BUN and creatinine and clinical manifestations such as fever, diarrhea, fatigue, and abdominal pain. We determined CMV reactivation by Real-Time quantitative PCR based on CMV-DNA > 10000 copies/mL. The samples of KTRs were taken at the time of CMV diagnosis of CMV infection just before starting antiviral treatment. for patients. The sampling of eligible patients was collected at the Abu Ali Sina Transplant Hospital, Shiraz (Shiraz, Iran) from March 2019 to November 2020. The medication history included; cyclosporine: 5 mg/kg as initial therapy followed by a maintenance dose of 2–2.5 mg/kg, prednisolone 120 mg/day as initial therapy tapering to 10 mg/day, mycophenolate mofetil 360 mg twice a day, and prophylactic Valganciclovir 900 mg/day for six months. All transplant recipients were given cyclosporine: 5 mg/kg as initial therapy following a maintenance dose of 2–2.5 mg/kg, and prednisolone:120 mg/day as initial therapy and then 10 mg/day routinely, and they received mycophenolate mofetil twice daily. Prophylactic Val ganciclovir 900 mg/day was given to all recipients just six months after transplantation, and s Sample collection was done at least six months after stopping using the cytomegalovirus prophylaxis regime. in participants. Those recipients who experienced rejection at the time of sampling were excluded. The demographic data of studied recipients are depicted in Table 1.

Table 1. The Demographical Data of CMV-reactivated KTRs

Factor	Summery
Total number of patients: CMV reactivated KTRs (n = 10)	
Gender of Recipients, %: Male = 6 (60%), Female = 4 (40%)	
Mean Age of Recipients: 51.6	
Blood Group, n (%): A+ (40%), B+ (40%), O+ (20%)	
CMV IgG Status: D+/R+: 98% , D+/R-: 0% , D-/R+: 2% , D-/R-: 0%	
Type of Transplantation CD (Cadaver Donor): 98% LD (Living Donor): 2%	
Cause of Transplantation	
HUS*	1 (10%)
Diabetes Mellitus Type II	4 (40%)
CKD*	1 (10%)
Autoimmune	1 (10%)
HTN*	1 (10%)
ESRD*	2 (20%)
Reason for Referral	
Proteinuria	1 (10%)
High CR and BUN	5 (50%)
PAD*	1 (10%)
UTI*	3 (30%)

Enzyme-Linked Immunosorbent Assay for CMV-IgG

The presence of anti-CMV IgG antibodies in the plasma KTR group was detected by ELISA kit (DIA.PRO, Milano, Italy).

Quantitative PCR for CMV-DNA Detection

CMV reactivation in kidney transplant recipients was performed by quantitative PCR. According to the manufacturer's instruction, the CMV viral load was measured using a quantitative real-time PCR kit (Primer Design Ltd TM kit, United Kingdom) [23]. Viral reactivation has been determined through a quantitative CMV PCR assay. It is determinative for CMV reactivation diagnosis in our center.

CMV Antigenemia Test

Immunofluorescence staining by CMV Brite Turbo kit (IQ Products, Groningen, Netherlands) as a qualitative detection was used to assess CMV lower matrix protein pp65 in KTRs. Besides real-time PCR in some cases, we used the antigenemia test as a complementary test. The procedure was followed according to manufacturer instruction as previously mentioned described in [24, 25]. After staining about 2*105 peripheral blood leukocytes by a cocktail of fluoresceine, the fluorescence microscope was used to detect positive results. The CMV antigen-positive cells were measured, and at least one positive cell per 50,000 WBCs was reported as a positive case.

Fluorochrome Standard Beads

The fluorophore standard beads were dyed with antibodies conjugated to either FITC, PE, PERCPCY5.5. After staining, the beads were diluted in a buffer before analysis on BD FACS caliber (each tube containing a single-color standard). This method support establishing accurate compensation corrections for spectral overlap of any combination of fluorochrome-labeled antibodies.

Isolation of Mononuclear Cells and NK cells

Isolation of PBMCs was done by Ficoll Paque (Lymphodex. Inno-Train-Spain) density gradient centrifugation. For cryopreservation, we used cold freezing solution composed of 90% FBS and 10% of DMSO. Then, the cells were stored in a total volume of 1.8 ml for each cryovial, containing 2 * 10^6 viable PBMC/mL. Finally, they were stored at -196°C (liquid nitrogen) until processed. Thawing of PBMCs was performed in the culture medium, including RPMI 1640 (Gibco Laboratories, Grand Island, NY), 10% fetal bovine serum (FBS) (Gibco Laboratories), L-glutamine (2 mM), and 1% antibiotic mixture (penicillin-streptomycin 5 mg mL−1). Isolation of NK cells from PBMC was done through magnetic bead separation assay using NK Cell Isolation Kit (Miltenyi-Biotec, Bergisch Gladbach, Germany).

Phenotypic Analysis of NK Cells by Flow Cytometry

In this study, Annexin V/PI double staining was used (Figure 1A). NK-cell purity was evaluated with anti-human mAb specific for CD56, and CD3 (to determine T cells contamination"), based on the expression of CD56 and lack of CD3 surface marker (Figure 1B). The differentiation of cell debris from lymphocytes was done through forward scatter(FSC) and side scatters (SSC).A list of fluorophore-labelled mouse anti-human mAbs were employed in this study including: anti CD56mAb (clone: HCD56) PE (Biolegend, USA) ,anti-CD3mAb (clone UCHT1) PerCPcy5.5(Biolegend) , anti-NKG2A mAb (clone: REA110) PE (Miltenyi-Biotec, , Germany) , anti-NKG2D mAb (clone 1D11) PerCPcy5.5 (Biolegend), anti-CD57 mAb (clone HNK-1) PerCPcy5.5(Biolegend) , anti-CD16 mAb

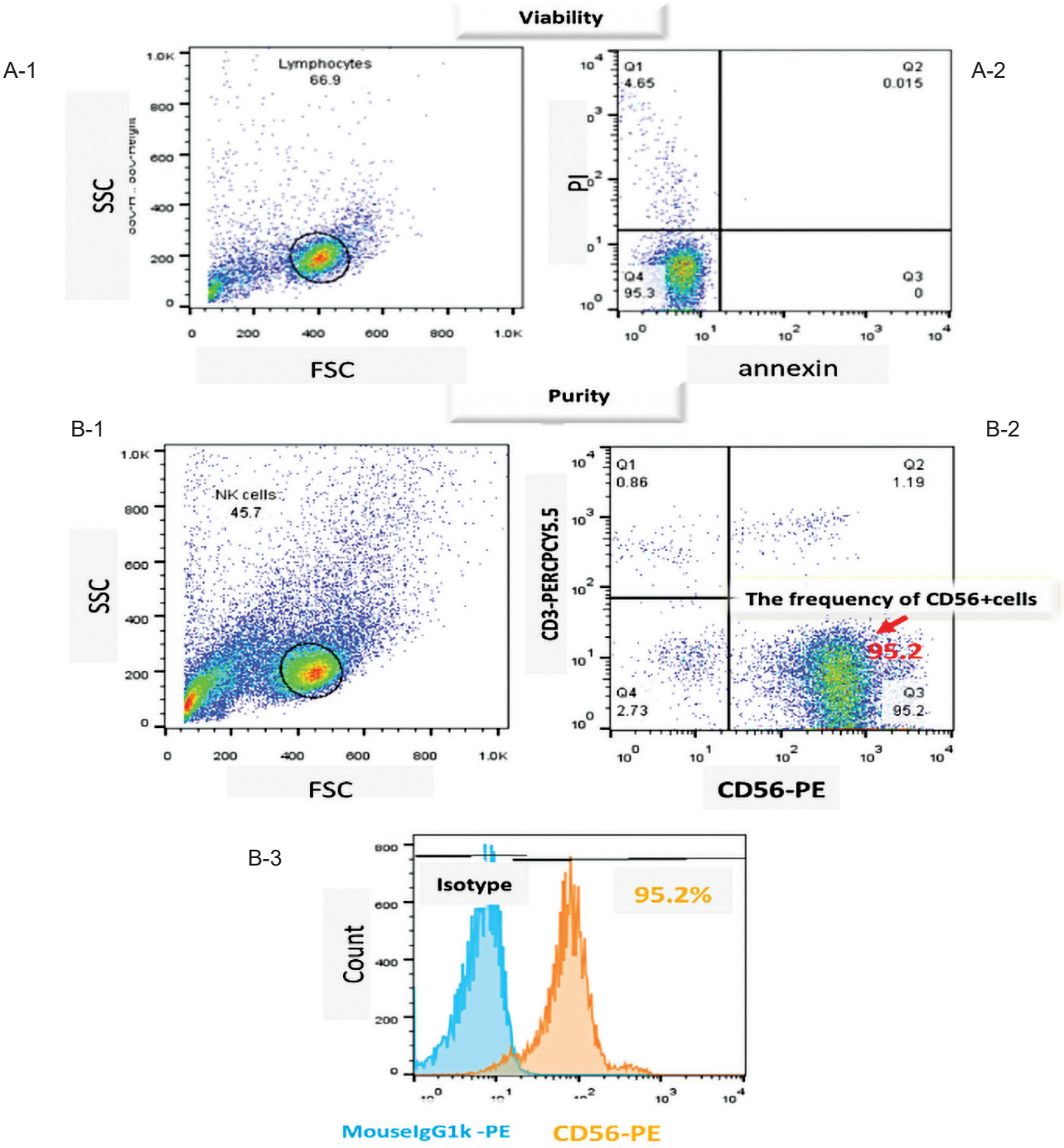

Figure 1. Viability and Purity of isolated NK cells (A and B). (A-1); Forward scatter (FSC) versus side scatter (SSC) dot plot of isolated NK cells. (A-2); The viability of NK cells was assessed by Annexin V/ propidium iodide (PI) as an apoptosis assay. (B-1); Forward scatter (FSC) versus side scatter (SSC) dot plot of isolated NK cells. (B-2); Isolated NK cells were stained with antibodies (CD3 and CD56). (B-3); The purity percentage of isolated NK cells is shown in each sample as mean ± SE (n = 10). B-4; Histogram of CD56 positive cells, the Purity of these cells was more than 85% (specific antibodies: orange histograms; isotype control antibodies: blue histograms).

(clone B73.1) PE (Biolegend) , and anti-CCR7 mAb (clone G043H) FITC (Biolegend) .Isotype controls determined nonspecific antibody binding (Figure 1. B-3), including; REA controls from Miltenyi-Biotec CO, and Iso IgG1k mAb- PE, IgM1k mAb- PerCPcy5.5,andIgG1k mAb- PerCPcy5.5 from Biolegend CO. We used fluorescence minus one control (FMO) and unstained controls for setting

compensation and separation of positive/negative borders. Flow cytometric data acquisition was done by FACS caliber Software (BD Biosciences), and Flowjo version 10 software (TreeStar Inc) was used for data analysis.

Isolated NK cells were evaluated for purification as indicated in Figure 1, based on anti-CD3 negative and CD56 positive gating. Following gating strategy was applied to assess the proportion of CD56+ NKG2C+ NK cells expressing NKG2D without the NKG2A surface marker and the frequency of CD56+ CD16 + NK cells expressing CCR7 and CD57 surface marker (Figure 2).

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analyses. Accordingly, the determination of the relationship between variables was assessed through a two-sided Spearman correlation analysis. The normality test (Kolmogorov-Smirnov test) was done to analyze the normal and non-normal distribution data. The data were represented as mean ± SE and median ± IQR. Differences between variables are characterized by $P > .05$, $P < .05$ and $P < .001$.

RESULTS

Association of CMV DNA viral load with clinical parameters

The age range of participants was 26–70, with a mean of 51.6 years. The CMV reactivated KTRs consisted of 6 (60%) females and 4 (40%) males. Laboratory tests were also performed on the day of sampling in CMV-reactivated KTRs. The analysis of the correlation between clinical data and viral load was done and presented in Table 2, and there were no significant differences in correlation between CMV DNA load and lab parameters; however, a significant correlation was observed between viral load and the count of NK cells measured by flowcytometry in our patients, r = - 0.7, $P < .05$. (Table 2) (r = - 0.7, $P < .05$), (Table 2).

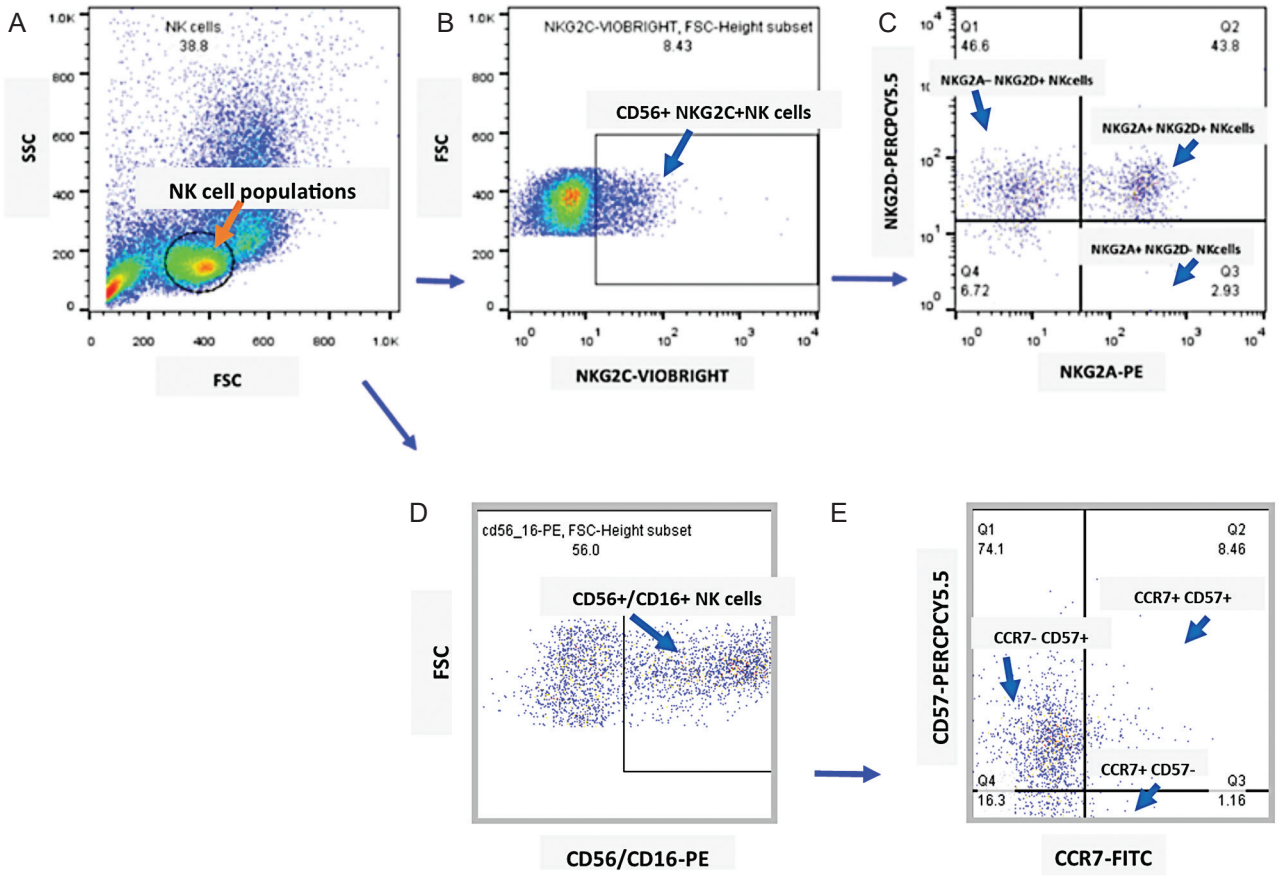

Figure 2. Gating strategy for determining CD56+ NK cell subsets expressing NKG2C, NKG2D, NKG2A, CCR7, and CD57. (A): NK cells were gated according to FSC/SSC. (B, D): NKG2C-FSC and CD56/CD16-FSC dot plot. (C): NKG2C+ NK cells were gated in the NKG2A-PE/NKG2D-PERCPCY5.5 dot plot. (E): CD56+/CD16+ NK cells were gated in the CCR7-FITC /CD57-PERCPCY5.5 dot plot.

Table 2. Analysis of the Correlations Between the CMV DNA Load and Laboratory Indices in CMV Reactivated KTRs

Characteristics	Association Between Lab Parameters and CMV DNA Load		
Lab Parameter	**Mean (± SEM)**	**Spearman's Rho**	***P****
Creatinine, mg/dL	1.844 ± 0.2662	-0.1506	> .05
BUN, mg/dL	46.11 ± 6.244	-0.4000	> .05
Albumin, g/dL	3.833 ± 0.2619	0.3667	> .05
RBCs, 10^6/μL	3.547 ± 0.3883	-0.4000	> .05
Neutrophils, 10^6/L	75.96 ± 5.559	-0.2333	> .05
Platelet cells, 10^6/L	121.1 ± 7.91	0.1088	> .05
Lymphocytes, 10^6/L	13.56 ± 3.653	0.1167	> .05
NK Cells, 10^6/L	11.85 ± 3.039	-0.7167	< .05

*Correlation analysis between the CMV viral DNA load and laboratory parameters indices in CMV reactivated KTRs, statistical tests: Spearman's rho.
A significant correlation is indicated in the redline.

NK cell subpopulations redistribution during CMV reactivation among CMV reactivated KTRs

Analyzing two important subsets of CD56+ NK cells indicates that CD56+ NK cells expressing NKG2D and NKG2C without NKG2A and CD56+CD16+ CD57+NK cells without CCR7 have the most frequency between recipients whom CMV reactivated (Table 3. A and B and Figure 3).

Correlation of CMV viral DNA load and different subsets of isolated NK cells in CMV reactivated KTRs

For identification of an association between CMV viral load and different CD56+NK cell subsets, Spearman's rank-order correlation was done in CMV reactivated KTRs. As indicated in Table 3. B, a positive correlation was found between viral DNA load and CD56+CD16+ CD57-CCR7- NK cells, which was statistically significant (r = 0.68, $P < .05$), and in parallel, a significant negative correlation between CMVDNA load and CD56+CD16+ CD57+CCR7+ NK cells with r = -0.71 and $P < .05$ (Figure 4). Nevertheless, this study found no significant association between CD56+NKG2C+NK cell expressing NKG2A/D subsets and CMV viral load. (Table 3. A)

As indicated in Figure 5, the significant correlation between increased frequencies of CD56+/CD16+ CD57-CCR7- NK cell subset and conversely diminished numbers of CD56+/CD16+ CD57+CCR7+ NK cell subset with higher viral DNA loads in transplant patients.

Table 3. (A) Proportion of CD56+NKG2C+NK Cells Expressing NKG2A/D, and the Correlations Between this Subset and CMV DNA Load Indices in the Blood of CMV Reactivated KTRs

NK Cell Phenotype	% of CD56+ NKG2C+NK cells (mean ± SE)	Spearman's Rho	*P**
NKG2A– NKG2D-	21 ± 2.9	0.31	> .05
NKG2A+ NKG2D+	21.0 ± 3.0	0.32	> .05
NKG2A+ NKG2D-	2.6 ± 0.9	0.28	> .05
NKG2A- NKG2D+	69.0 ± 4.6	- 0.36	> .05

Table 3. (B) Proportion of CD56+CD16+ NK Cells Expressing CCR7/CD57, and the Correlations Between this Subset and CMV DNA Load Indices in the Blood of CMV Reactivated KTRs

NK Cell Phenotype	% of CD56+ CD16+NK Cells (mean ± SE)	Spearman's Rho	*P**
CCR7– CD57-	17.5 ± 2.8	0.68	< .05
CCR7+ CD57+	10.98 ± 2.8	- 0.71	< .05
CCR7+ CD57-	3.2 ± 1.3	- 0.25	> .05
CCR7– CD57+	67.8 ± 2.6	0.31	> .05

*Correlation analysis between the CMV viral DNA load and NK cell subsets indices in CMV reactivated KTRs, statistical tests: Spearman's rho.
A significant correlation is indicated in the redline.

DISCUSSION

CMV infection remains an obstacle after kidney transplantation[26]. The evaluation of CMV viral

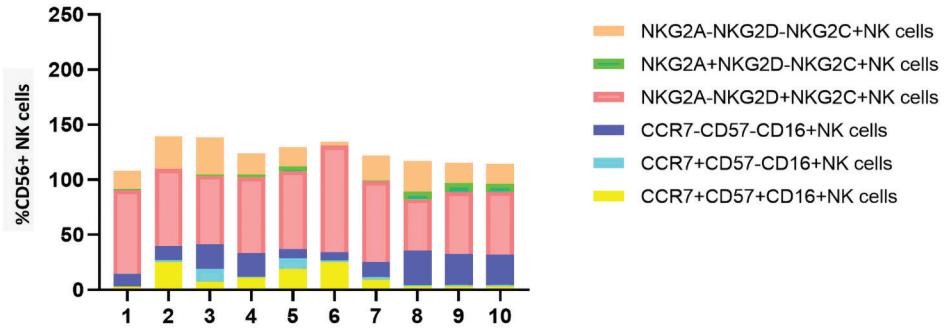

Figure 3. The graphs indicate the distribution of different subpopulations of CD56+ NK cells in each CMV reactivated KTRs.

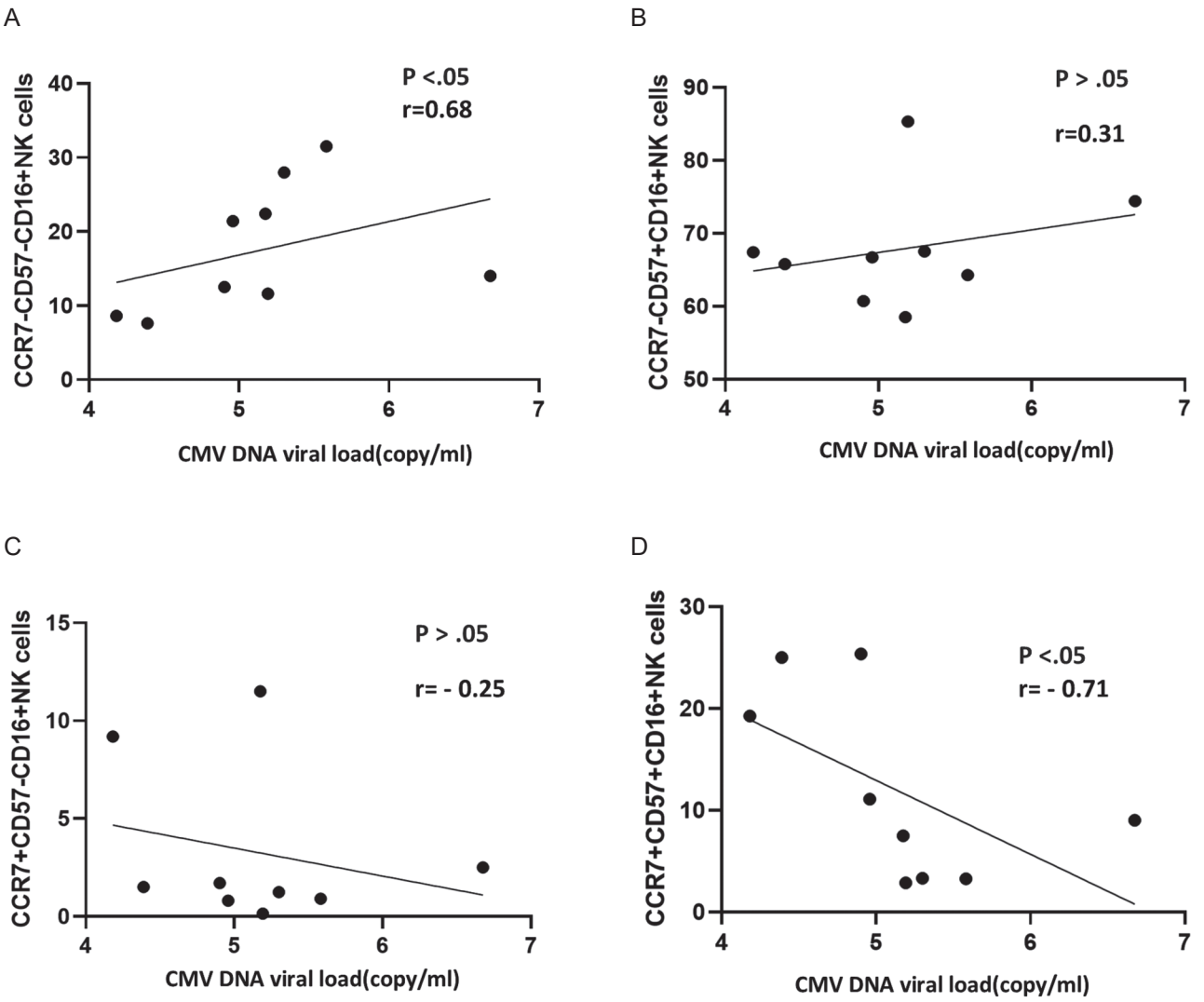

Figure 4. Scatter plots of correlation between CMV viral DNA load and CD56+/CD16+CD57+NK cell expressing CCR7. A: Positive correlation between CD56+/CD16+CD57-CCR7-NK cell and CMV viral load (r = 0.68, *P* < .05). B: No significant correlation between CD56+/CD16+CD57+ CCR7-NK cell CMV viral load .C: No significant correlation between CD56+/CD16+CD57- CCR7+NK cell and CMV viral load. D: Negative correlation between CD56+/CD16+CD57+CCR7+NK cell and CMV viral load (r = -0.71, *P* < .05).

load is still the main clinical approach for CMV assessment and guides decision-making in the antiviral treatment of recipients[27]. Although, there is no specific viral load cut off for using the antiviral therapy treatment for CMV reinfection yet[28]. Hence, further diagnostic strategies for the prevention of CMV reactivation prevention are needed. Based on the evidence, the cellular immune system could help monitor CMV reinfection[29, 30]. There is a long-lasting stable imprint that CMV infection leaves overtime over time on the NK cell population in the context of transplantation [31], initiating alteration in the differentiation and the expansion of NK cells, characterized by NKG2C+ CD57+ NKG2A- CD56 dim NK cell subset with memory features [32]. Recently several studies have indicated distinct NK cell subsets in the context of CMV infection, describing memory-like NK cells with different activation capacities compared to conventional NK cell subsets[32-34]. However, not much is known about the distinct subset of NK cells and CMV viral load in transplant recipients. This study provides the correlation between distinct subsets of NK cells and CMV copy numbers in CMV reactivated recipients.

Regarding, o One of the significant chemokines is CCR7[35]. Indeed, the CCR7 receptor plays a crucial role in migrating NK cells to lymph nodes, leading to homing of CD56bright NK cells [36]. However, recent studies found that the chemokine

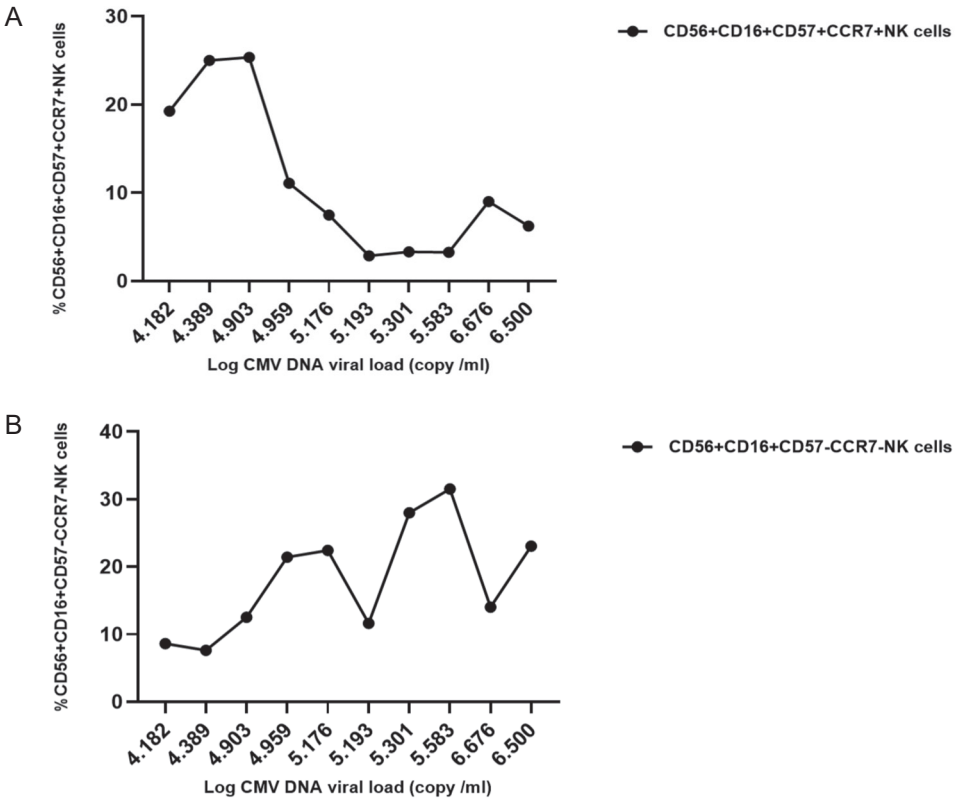

Figure 5. The correlation between CMV viral DNA load and CD56+/CD16+ NK cell with and without expressing both CD57 and CCR7 in each sample of CMV-reactivated KTRs. Increasing CMV copy number is associated with a lower mean frequency of NK cells expressing CD57 and CCR7(A), but a higher mean frequency of NK cells lacking CD57 and CCR7(B).

receptors are identified on both NKCD56bright and NKCD56dim [37]. Notably, one of the hallmarks of CCR7 is the acquisition of this receptor on NK cells that results in homing to lymph node and thus is a therapeutic strategy to kill antigen-presenting cells in recipients in the hematopoietic stem-cell transplantation (HSCT) setting[36, 38]. This approach could be helpful in the treatment of different diseases, including viral infections and autoimmune diseases [39]. Hence, we investigated a correlation between CMV viral load and CCR7 expressing NK cell subset in KTRs.

We first investigated clinical parameters among our transplant subjects to test the association between clinical parameters and viral load at the onset of CMV reactivation. It was revealed that there is a significant correlation between the lower number of total NK cells and CMV DNA copy number upon CMV reactivation in KTRs. Moreover, this study investigated the correlation between the frequency of CCR7 expressing NK cells, and also NKG2C+ NKG2D+ NKG2A- NK cells with CMV DNA load. No significant correlation was found between CMV copy number and the frequency of NKG2D, NKG2Cexpressing CD56+NKG2A- NK cells in CMV reactivated KTRs (Table 3. A). But, we explored a negative correlation between CD56/CD16+ CD57 + NK cells expressing CCR7 and the viral load of CMV in CMV-reactivated KTRs. Conversely, we observed an association between the lack of CCR7 receptors on NK cells and the increase of CMV DNA copy number in these infected recipients. In favor of our finding, a study by Henoch S. Hong revealed that the loss of CCR7 expression on CD56bright NK cells is correlated with HIV viral load[19]. These data are in accordance with a previous study by R. Keith Reeves *et al.* that found the down-regulation of CCR7 on NK cells in Simian immunodeficiency virus (SIV) -infected macaques, indicating that SIV infection drives changes in NK-cell function identified by reduced cytokine production, increased cytotoxicity[40, 41]. Remarkably, it was identified that in the HIV infection CCR7-CD56bright NK cell populations indicated the increased ability of activity and cytotoxicity with a high differentiated phenotype.

Consistent with this concept, we explored more degranulation potential and cytotoxicity through CD107a production and developed IFN-γ expression, respectively on NK cells in CMV reactivated KTRs in comparison to non –reactivated ones[21]. It seems that this NK cell subset has indicated several properties of CD56dimCD16+ NK cells. Various NK cell subpopulations have the capacity for differential programs, which likely impacts NK cells' activity in immune response [42]. In CMV infection investigations, NK cell subsets based on chemokine receptor is less considered. Like CMV, hepatitis viruses such as hepatitis C virus (HCV) and hepatitis B virus (HBV) drive chronic infection, thus constantly inducing the immune system through their antigens[43]. Therefore, it will thus be valuable to see how such chronic viral infections can influence NK cell activation and phenotype feature. However, it should be noted that the impact of CMV pathogenesis on NK cell differentiation seems to exceed other known chronic viral infections.

According to finding in figure 3 (from A to D), increased the frequency of CD56+/CD16+CD57-CCR7-NK cell and CD56+/CD16+CD57+CCR7-NK cell subpopulations are associated with increased CMV viral DNA load, and in contrast to this positive correlation, reduced frequency of CD56+/CD16+CD57-CCR7+ and CD56+/CD16+CD57+CCR7+ NK cell subsets are related to augmented CMV viral DNA. In addition, an increasing trend of viral load correlating with the decreased frequency of NK cells expressing CCR7 is observable (Figure 4). This finding suggests that the acquisition of CCR7 could serve as an increased chemokine involved in the pathogenesis of CMV infection. Our results identified a negative correlation between CCR7 expressing NK cell subpopulation and copy number of CMV at the onset of CMV reinfection in recipients. Indeed future studies will also be interesting to see whether the expression level of other chemokines, including CXCL9, CXCL10, and CXCL11on NK cells with more sample size and more interval times, correlates with CMV infection. In conclusion, the present finding could prompt us to believe that our recipients are prone to be infected with CMV if they have a higher CD56+/CD16+ CD57-CCR7- NK cell subset, providing probably more efficient therapeutic management of the treatment of CMV infection.

ETHICS STATEMENT

This study was approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC. 1396.S289). The contributors provided their written informed consent to participate in this project.

ACKNOWLEDGMENTS

We thank all participants from the Abu Ali Sina Hospital, Shiraz, Iran. This work was financially supported by grants from Shiraz University of Medical Sciences (grants No.13843).

DISCLOSURES

The authors declare that they have no competing financial interests.

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Highlights

- The rapid expansion of CCR7+CD57+ CD56/CD16+ NK cell subset at the onset of CMV reactivation.
- The correlation between lack of expression of CCR7 and increased CMV viral load among CMV –reactivated KTRs.

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Received August 2021
Revised October 2021
Accepted December 2021