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Insights into cytomegalovirus-associated T cell receptors in recipients following allogeneic hematopoietic stem cell transplantation

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Abstract

Background Cytomegalovirus (CMV) reactivation is a serious problem in recipients of allogeneic hematopoietic stem cell transplantation. Long-term latency depends on specific T cell immune reconstitution, which identifies various pathogens by T cell receptors (TCRs). However, the mechanisms underlying the selection of CMV-specific TCRs in recipients after transplantation remain unclear.

Methods Using high-throughput sequencing and bioinformatics analysis, the T cell immune repertoire of seven CMV reactivated recipients (CRRs) were analyzed and compared to those of seven CMV non-activated recipients (CNRs) at an early stage after transplant.

Results The counts of unique complementarity-determining region 3 (CDR3) were significantly higher in CNRs than in CRRs. The CDR3 clones in the CNRs exhibit higher homogeneity compared to the CRRs. With regard to T cell receptor β-chain variable region (TRBV) and joint region (TRBJ) genotypes, significant differences were observed in the frequencies of TRBV6, BV23, and BV7–8 between the two groups. In addition to TRBV29–1/BJ1–2, TRBV2/BJ2–2, and TRBV12–4/BJ1–5, 11 V-J combinations had significantly different expression levels between CRRs and CNRs.

Conclusions The differences in TCR diversity, TRBV segments, and TRBV-BJ combinations observed between CNRs and CRRs might be associated with post-transplant CMV reactivation and could serve as a foundation for further research.

Keywords Cytomegalovirus, Allogeneic hematopoietic stem cell transplantation, T cell receptor, Complementarity-determining region 3, High-throughput sequencing

Background

Cytomegalovirus (CMV) is a ubiquitous pathogen worldwide and its global mean seroprevalence is 86% [1]. Although CMV infection is usually asymptomatic in immunocompetent individuals, reactivations are common among immunodeficient individuals (e.g., allograft recipients), which leads to CMV-related diseases, graft failure, and even life-threatening consequences [2–4]. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a key component of the treatment of many hematological diseases, such as myelodysplastic syndrome, myeloid leukemia, and aggressive T-cell

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lymphoma [5–7]. Therefore, it is necessary to identify the protective factors of CMV reactivation in allo-HSCT recipients.

T cells play important roles in the response against CMV reactivation in allo-HSCT recipients [8,9]. T cell receptors (TCRs) on the cell surface are responsible for the diverse T cell responses to pathogens. TCRs bind to peptide/major histocompatibility complex (MHC) via the complementarity-determining region 3 (CDR3) encoded by variable (V), diversity (D), and joint (J), as well as their rearrangement, splicing, and insertion, which forms the molecular basis of CMV reactivation suppression by CMV-specific T cells. Due to its high variability, CDR3 plays a crucial role in determining the specificity and diversity of TCRs in recognizing viral antigens, such as those presented by CMV. Studies have already demonstrated interactions between CDR3 and CMV, including specific T cell responses triggered by direct stimulation with CMV peptides, the development of antigen peptide vaccines, and analyses of the complementarity between CDR3 and CMV [10–12]. Therefore, investigating the role of CDR3 in CMV reactivation is both important and holds significant potential for further exploration. CMV reactivation and specific T cell clones influence the clonality of TCR repertoire after transplant [13]. Previous studies mostly focused on patients with specific human leukocyte antigen (HLA), and few selected particular patients and time points. The mechanism of CDR3 clones related to CMV reactivation after allo-HSCT is still unclear.

In the present study, we used high-throughput next-generation sequencing and bioinformatics analysis to characterize and compare the TCR β-chain repertoire of seven CMV reactivated recipients (CRRs) and seven CMV non-activated recipients (CNRs) at 1 month after the transplant. The results provide more data of CDR3 immune repertoire associated with CMV reactivation.

Methods

Participants and allo-HSCT protocols

Between July 2020 and March 2021, 179 allo-HSCT recipients were prospectively enrolled from the First Affiliated Hospital, College of Medicine, Zhejiang University, China. The study excluded individuals aged < 18 years and those with tumors, comorbid diseases, infusion of chimeric antigen receptor T cells, autologous HSCT, retransplantation, or history of infection with hepatitis B virus, treponema pallidum, mycobacterium tuberculosis, or human immunodeficiency virus. The grafts were from CMV-seropositive donors. Conditioning regimen, graft versus host disease (GVHD) prophylaxis, and antiviral treatment were administered according to protocols of the bone marrow transplant center.

The study participants were followed for at least 9 months and categorized into CRRs, i.e., CMV-seropositive recipients who experienced significant CMV DNAemia, (peak load > 1000 copies/mL) for > 3 weeks; CNRs, i.e., CMV-seropositive recipients with no evidence of CMV DNAemia during follow-up; and other recipients (ORs), i.e., those who had low CMV DNA load or short duration of CMV DNAemia.

Isolation and processing of peripheral blood mononuclear cells (PBMCs)

At day 30 post-HSCT, PBMCs were obtained from the participants using Ficoll density gradient centrifugation and cryopreserved in freezing medium (90% fetal bovine serum + 10% dimethyl sulfoxide). The process of PBMCs isolation was as follows. Whole blood was diluted with an equal volume of phosphate-buffered saline (PBS). The diluted blood was carefully layered over Lymphoprep (StemCell Technologies, Melbourne, Australia) in a 15 mL conical tube. The tubes were centrifuged at 2500 rpm for 20 min at room temperature with no brake applied. After centrifugation, the PBMC layer, located at the interface, was carefully collected. The collected PBMCs were washed twice with PBS and resuspended in the freezing medium for cryopreservation.

Monitoring of CMV DNA load

Ethylenediaminetetraacetic acid-anticoagulant peripheral blood samples were obtained every week during the first 3 months after transplantation and monthly or bimonthly thereafter.

CMV DNA was extracted from fresh whole blood samples and detected using nuclei acid detection kits (Daan, Zhongshan, China) after grafting. According to the manufacturer's instructions, load ≥ 500 copies/mL was considered CMV DNAemia.

RNA extraction and evaluation

Total RNA was extracted from cryopreserved PBMCs in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA concentration was determined via spectrophotometry (Nano Drop 2000; ThermoFisher Scientific, Waltham, MA, USA) and the RNA integrity was evaluated via agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The TCR profiling system was used for the construction of the sequencing library and TCR β chain sequencing (ImmuQuad Biotech, Hangzhou, China) as described previously [14–17]. Briefly, a 5' rapid amplification of cDNA ends (RACE) unbiased amplification protocol was used. Two rounds of PCR were performed under suitable conditions. Final PCR products were purified using

Agencourt AMPure XP beads and analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to confirm the concentrations required for the sequencing samples.

High throughput sequencing (HTS) and bioinformatics analysis

Sequencing was performed using an Illumina NovaSeq® system with PE150 mode. Raw data were processed by basic filtration, screening, quality control, and merging. Then the sequence was compared to the international ImMunoGeneTics (IMGT) database (http://www.imgt.org).

Finally, gene annotation and bioinformatics analyses were performed. We calculated the quantities and frequencies of various CDR3 gene and amino acid sequences; corresponding formations of V, D, J, and constant (C) segments; the length distribution of the CDR3 region.

Statistical analysis

GraphPad Prism (version 7.0; GraphPad Software Inc., San Diego, CA, USA) and R (version 3.6.2; R Foundation for Statistical Computing, Vienna, Austria) were used to analyze and visualize the results. Continuous variables were compared using the t-test, corrected t' test, or non-parametric Mann–Whitney U test based on data distribution and homogeneity. Categorical variables were compared using the chi-square test or Fisher's exact test as appropriate. The level of statistical significance was set at $p < 0.05$.

Results

Detailed information about selected recipients

Seven CRRs and seven CNRs were selected based on the demographic and follow-up information. Table 1 and Table S1 present the characteristics of study participants involved in the sequencing analyses.

Samples sequencing data

The read counts and CDR3 clone counts from study participants' samples are presented in Table S2. By analyzing these parameters, the study aims to assess differences in the overall abundance of TCR β sequences, the breadth of CDR3 region diversity, and the functional variation in T cell responses. These comparisons provide insights into the potential differences in immune response capabilities, particularly in relation to CMV recognition and reactivity, between the patient groups.

Notably, the counts of unique CDR3 genes and amino acid sequences were significantly higher in CNRs compared to CRRs, with p-values of 0.0307 and 0.0298, respectively (Fig. 1). Additionally, the overlap of shared CDR3 sequences between the two groups was minimal (Fig. S1).

Clonal overlap rate of TCR CDR3 between CRRs and CNRs

The clonal overlap rate between the two groups was calculated using the Baroni–Urbani and Buser (BUB) index, as described in the Supplementary Method. By evaluating the clonal overlap, the study aims to determine whether there are similarities or differences in the T cell repertoires of CRRs and CNRs, which could provide insights into the commonality or uniqueness of their immune responses, particularly in the context of CMV reactivation. The resulting heatmap visually illustrates these overlap rates, highlighting significant differences in clonal overlap between CRRs and CNRs (Fig. 2).

Frequency of T cell receptor β-chain variable region (TRBV) and joint region (TRBJ) use

By analyzing the usage patterns of TRBV and TRBJ regions, the study aims to identify whether certain segments are more commonly associated with either CRRs or CNRs. This could provide insights into how these segments contribute to the immune response, particularly in relation to CMV reactivation and the effectiveness of T cell-mediated immunity in these patient groups.

A total of 61 TRBV segments were identified and grouped into 27 families. Among the 26 TRBV families shared between the two groups, TRBV6 and TRBV23 were significantly more frequently used in CRRs compared to CNRs ($p = 0.0212$ and 0.0262, respectively; Fig. 3A). Additionally, we analyzed the usage of individual TRBV segments and found that the TRBV7–8 segment was significantly more prevalent in CRRs than in CNRs ($p = 0.0023$; Fig. S2). In contrast, the analysis of TRBJ segments identified 13 segments (6 from the TRBJ1 family and 7 from the TRBJ2 family), with no significant differences in usage between the two groups, except for TRBJ2–2P, which was present in one CNR (Fig. 3B).

Differences in V-J combinations

V and J segments are the most variable components of TCRs and play a crucial role in targeting a wide range of pathogenic microorganisms. As a result, the combination of V and J segments has been the primary focus of many TCR-related studies. This comparison could reveal how particular V-J pairings contribute to the recognition and response to CMV, and help explain differences in clinical outcomes or immune effectiveness between the groups.

The preferred V/J genes and their combinations are illustrated as Circos plots in Fig. S3. The stacked bar graph below displays the frequencies of differentially selected V-J combinations between the two subject groups (Fig. 4), with detailed data provided in

Table 1 Characteristics and clinical outcomes of enrolled subjects

Characteristics	CRR	CNR
Number of subjects	7	7
Age, median (IQR), y	40 (37–49)	41 (35.5–48.5)
Gender (male/female)	2/5	1/6
(D)onor/(R)ecipient CMV serostatus		
D + R +	7	7
Underlying disease		
Acute myeloid leukaemia	4	3
Acute lymphoblastic leukaemia	2	1
myelodysplastic syndrome	1	0
Lymphoma	0	2
Other	0	1
Stem cell source		
Peripheral blood	7	7
Type of donor		
Identical related	0	3
Haplo-identical	5	4
Matched Unrelated	2	0
Conditioning regimen		
AraC + BuCy + MECCNU + ATG	5	4
BuCy + MECCNU + ATG	1	1
Flu + BU + ATG	1	0
AraC + VP-16 + BuCy + MECCNU	0	1
Bucy + MeCCNU	0	1
Immunosuppressive agents		
MTX + CsA + MMF	7	7
Steroid usage[1]	7	6
Time to engraftment, median (IQR), d		
Neutrophil	15 (12–16.5)	13 (11–15)
Platelet	13 (12–23)	13 (11.5–15)
Initial DNA (copies/mL)	17,200 (9225–23,100)	/
Peak DNA (copies/mL)	87,700 (17,600–139000)	/
Follow-up, median (IQR), d	366 (339–442.5)	353 (292.5–505.5)
Viral complications	0	0
aGVHD	1	0

Data are shown as absolute number or median (25th–75th percentile)

IQR, interquartile range; AraC, cytosine arabinoside; BuCy, busulfan and cyclophosphamide; MECCNU, methyl cyclohexyl nitrosourea; ATG, anti-human thymocyte globulin; VP-16, etoposide; Flu, fludarabine; BU, busulfan; MTX, methotrexate; CsA, cyclosporin A; MMF, mycophenolate mofetil; ACV, acyclovir; GCV, ganciclovir; FOS, Foscarnet; aGVHD, acute graft-versus-host disease

[1] Recipients received methylprednisolone injection within 30 days posttransplan

Table S3. Notably, the TRBV16/BJ1–6 and TRBV11–3/BJ1–5 combinations were absent in CRRs but present in most or all CNRs, leading to a significant difference in expression between the groups according to Fisher's exact tests ($p = 0.021$ and 0.001, respectively; Table 2). Additionally, nine V-J combinations showed significantly different expression levels between the CRR and CNR groups (Table 2). Specifically, the frequencies of TRBV2/BJ2–2, TRBV14/BJ2–2, TRBV29–1/BJ1–2, TRBV12–4/BJ1–5, and TRBV5–4/BJ1–4 were significantly higher in CNRs than in CRRs. However, this difference did not account for the higher frequency of certain CDR3 gene sequences in the CNR group; instead, it reflected the significantly greater diversity of CDR3 gene sequences in CNRs compared to CRRs (Fig. S4).

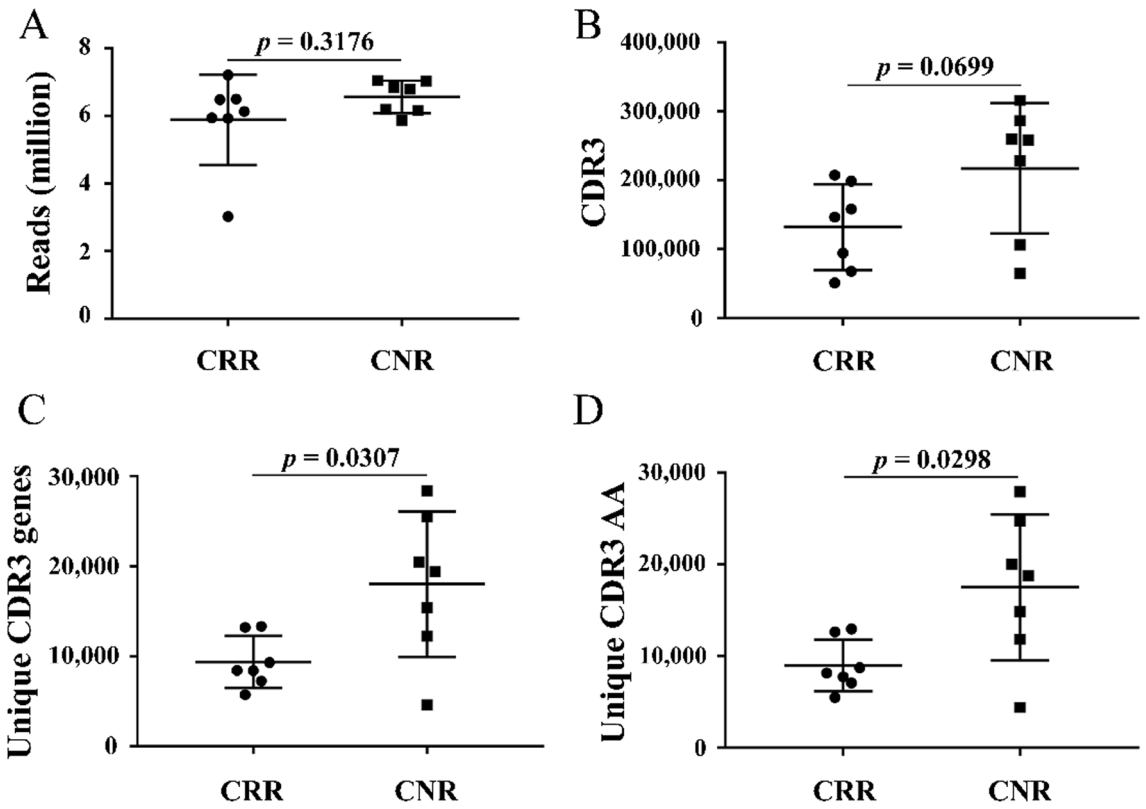

Fig. 1 Comparison of TCR gene amplification between CRR and CNR groups. **A** The number of total reads. **B** The number of total CDR3. **C** The number of unique CDR3 genes. **D** The number of unique CDR3 amino acids. AA, amino acid. Mean and SD are indicated

CDR3 length distribution

CDR3 length is a critical factor in determining the specificity and binding properties of TCRs. Analysis revealed that the CDR3 length distribution pattern deviated from a Gaussian distribution to varying degrees in both CRRs and CNRs (Fig. S5). The majority of CDR3 lengths ranged from 30 to 60 base pairs (bp) in both groups. There was no significant difference in the average CDR3 length between CRRs and CNRs.

Discussion

CMV reactivation is a serious problem in allo-HSCT recipients [18], which necessitates antiviral treatment with the associated side effects and limitations [19,20]. Therefore, the mechanism underlying anti-CMV immune responses should be elucidated to improve the diagnosis and treatment of CMV reactivation. T cells are the main protection against CMV. The CDR3 region of the β chain of TCRs is formed by rearrangement, insertion, mutation, and deletion of the V, D, and J gene segments, which generated T cells targeting specific pathogens. HTS can determine the CDR3 sequences of TCRs at the molecular level with higher resolution. In the present study, we compared TCR sequences between allo-HSCT recipients with disparate CMV outcomes at an early stage after allo-HSCT and characterized the TCR repertoire, which will provide more meaningful data contributing to the diagnosis and treatment of CMV reactivation.

The diversity of the overall TCR repertoire is positively correlated with the reservoir capacity and homeostatic stability of the immune system, which resists the invasion of diverse pathogens [21,22]. The absolute numbers of clones of unique CDR3 genes and AAs were significantly higher in the CNR group than in the CRR group, consistent with previous studies [23,24].

To analyze the difference in the CDR3 immune repertoire between CRRs and CNRs, we compared the BUB index of CRRs and CNRs. The clone overlap rate was significantly higher in the CNR group than in the CRR group; therefore, individuals with resistance against CMV reactivation had high CDR3 homology.

Previous studies have reported the skewed preferences of BV and BJ in CMV-specific T cells, which are influenced by antigen specificity [25,26]. In the present study, TRBV6, TRBV23, and TRBV7–8 had higher expression levels in CRRs than in CNRs, which suggests that the BV segments were associated with subsequent CMV outcomes and may be promising biomarkers for the early

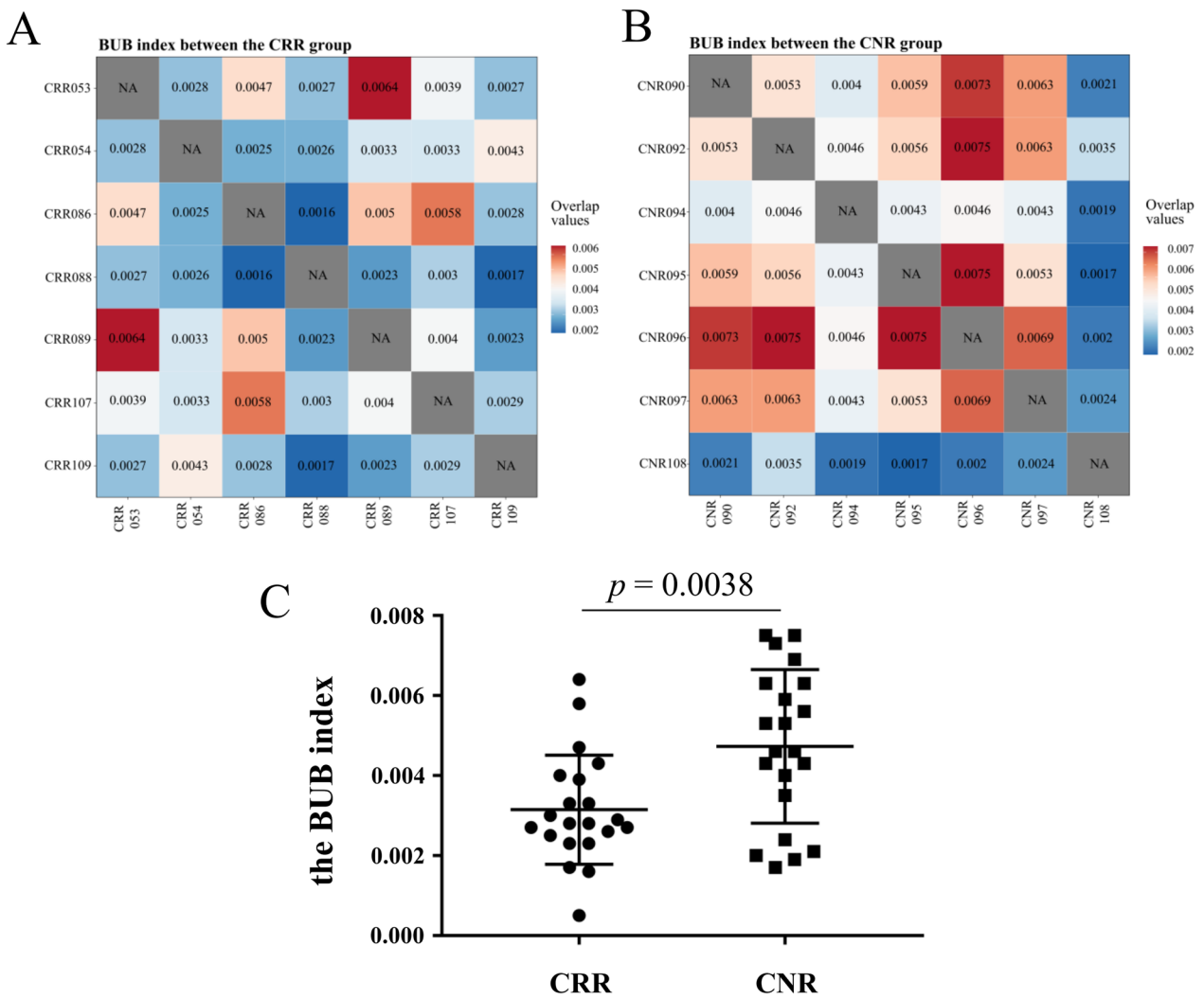

Fig. 2 Heatmap of clonal overlap rate of CDR3 for samples in each group. BUB index between samples in (**A**) CRR and (**B**) CNR groups, there was (**C**) significant difference of BUB index between the two groups. Mean and SD are indicated

identification of recipients at risk of CMV reactivation. However, additional studies are needed to verify these results.

The V-J gene combination, which contributes to CDR3 diversity and resistance against various pathogens, is one of the most important properties of the TCR repertoire. In a large cohort of 666 individuals with different HLA alleles, Emerson et al. [27] found that 164 clones were significantly enriched in CMV-positive individuals compared to CMV-negative individuals. Consistent with this finding, three V-J combinations (TRBV29–1/BJ1–2, TRBV2/BJ2–2, and TRBV12–4/BJ1–5) were highly expressed in CNRs compared to CRRs. These combinations were also found in pp65 peptides of HLA-A02-restricted CMV-specific T cells in previous studies [28,29]. In addition, TRBV14/BJ2–2 and TRBV5–4/BJ1–4 expression levels were higher in CNRs compared to CRRs, which has not previously been reported. We further investigated the gene sequences of these five V-J combinations and found higher diversity in CNRs than in CRRs. Moreover, the TRBV16/BJ1–6 and TRBV11–3/BJ1–5 gene combinations were detected in most or all CNRs (albeit at low frequencies), but they were observed in few or no CRRs. Our results provide more evidence that TRBV29–1/BJ1–2, TRBV2/BJ2–2, and TRBV12–4/BJ1–5 probably play vital roles in protecting against CMV infection and reactivation, and the newly discovered V-J gene combinations warrant further investigations. Meanwhile, future research needs to pay more attention to the diversity of these V-J combination genes.

Considering the influence of HLA alleles on antigen presentation and TCR recognition, previous studies [30,31] have mainly focused on individuals with one or several types of HLA allele and corresponding

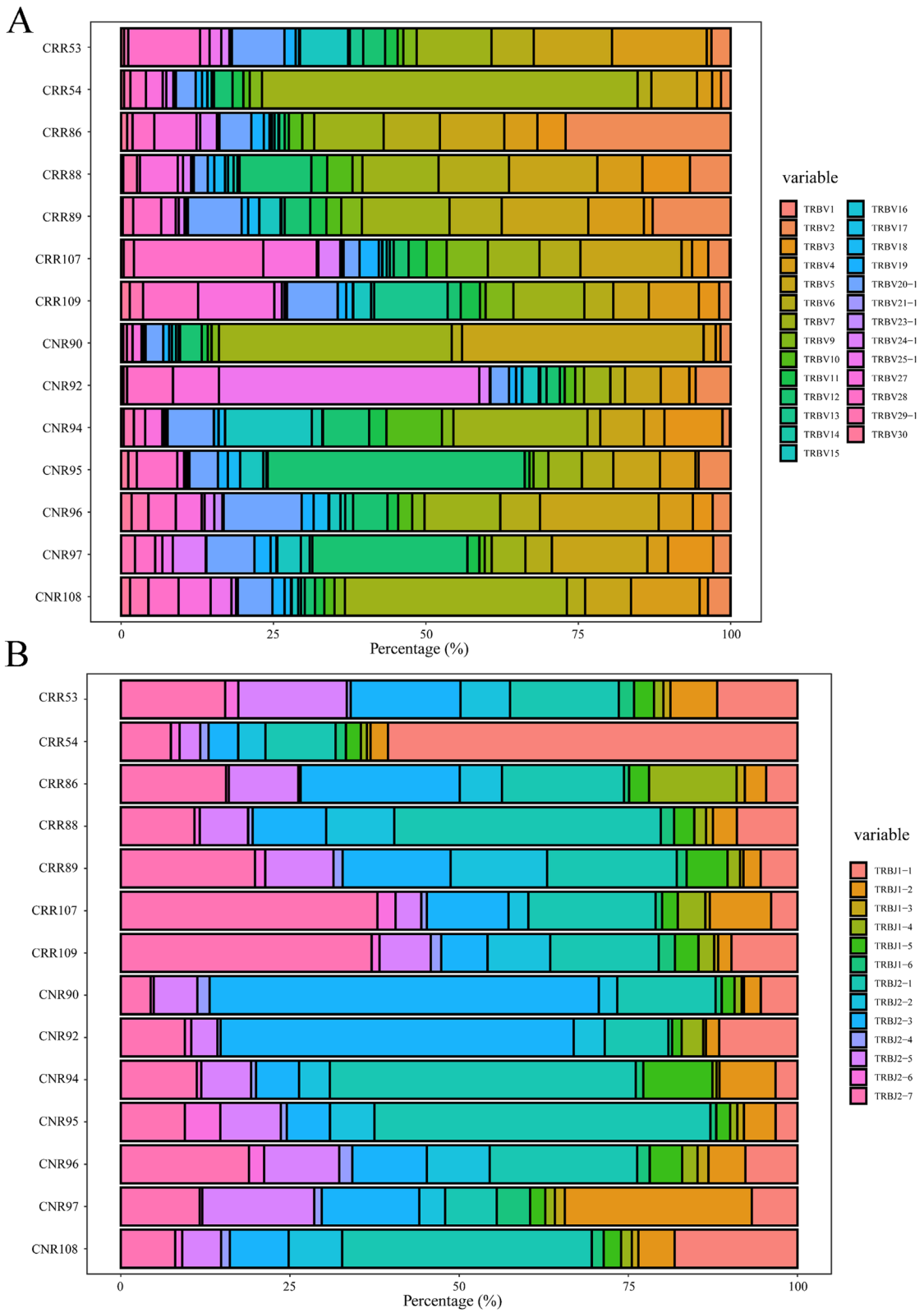

Fig. 3 The percentages of TRBV and BJ family and segment usage in each subject of CRRs and CNRs. **A** TRBV family usage in PBMC from each subject. **B** TRBJ family usage in PBMC from each subject

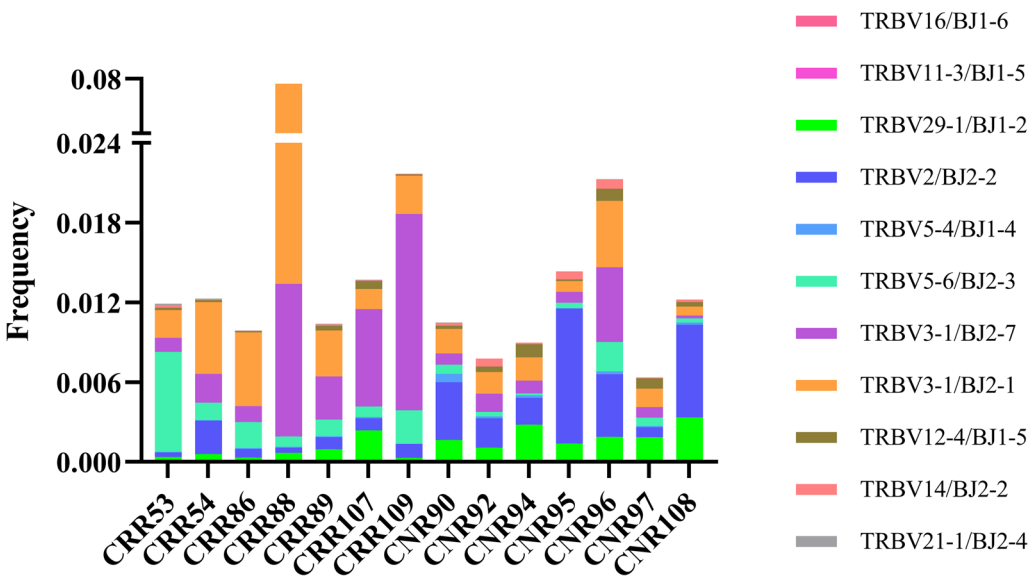

Fig.4 The stacked bar chart illustrates the frequency distribution of differentially selected V-J combinations between two subject groups. Each color within the bars represents one of the 11 distinct V-J combinations. The data are presented as frequencies, reflecting the relative abundance of each V-J combination within the subjects' TCR repertoire

Table 2 V-J gene combinations with significant differences between CRR and CNR

V-J combinations	Test method	P value	Mean value comparing
TRBV16/BJ1-6	Fisher's exact test[a]	0.021	CRR < CNR
TRBV11-3/BJ1-5		0.001	CRR < CNR
TRBV29-1/BJ1-2	Mann–Whitney U test	0.0111	CRR < CNR
TRBV2/BJ2-2		0.0175	CRR < CNR
TRBV5-4/BJ1-4		0.0221[b]	CRR < CNR
TRBV5-6/BJ2-3		0.0111	CRR > CNR
TRBV3-1/BJ2-7		0.0175	CRR > CNR
TRBV3-1/BJ2-1		0.0262	CRR > CNR
TRBV12-4/BJ1-5	t test	0.0477	CRR < CNR
TRBV14/BJ2-2	Corrected t' test	0.0319	CRR < CNR
TRBV21-1/BJ2-4		0.0376[b]	CRR > CNR

[a] Comparison of categorical variables. There was no usage of these two V-J combinations in all CRRs, while they were used in most or all CNRs

[b] Exclusion of one subject in CRR and CNR group respectively because of no usage of corresponding V-J combinations

restricted peptides. However, CMV reactivation occur in recipients with different HLA alleles. In addition, the previously identified CDR3 sequences are usually observed in specific MHC-restricted populations. These findings suggest that the MHC-restricted CDR3 sequences do not indicate the underlying immune mechanism of anti-CMV T cells in all recipients. In the present study, we identified three V-J gene combinations with significant differences between the two groups. Although these combinations have previously been reported, the CDR3 gene sequences identified in the present study differ from those reported previously. Meanwhile, the V-J gene combinations were identified in an overwhelming majority of recipients, irrespective of the MHC. Therefore, we need further analysis of the TCR CDR3 AA or gene sequences related to CMV reactivation in the future.

The activity of TCRs against specific antigens is not solely determined by CDR3 genes but is also influenced by other physical and chemical properties (e.g., CDR3 length) [32]. In the present study, we respectively analyzed the CDR3 length distribution and mean length in CNRs and CRRs, which can impact the folding of the TCR loop and its affinity to the peptide–MHC complex, and even result in the changes in TCRs [33]. Previous studies have indicated that CDR3 length is normally distributed in the healthy population [32,34]. However, in our study, we observed varying degrees of skewness in CDR3 length, which could be attributed to immune reconstitution and CMV reactivation. Nakasone et al. reported that the preferred AA length in CMV-specific cytotoxic T cells was almost 15 [35]. Consistent with this finding, the most frequently observed CDR3 lengths in our study participants were 45 bp (equivalent to 15 amino acids), which significantly differed from those in influenza A-specific cytotoxic T cells [25]. Additionally, certain diseases are characterized by short CDR3 lengths [36,37]. The mean CDR3 lengths were not different between the two groups, suggesting

that recipients in both groups of the present study were exposed to a similar CMV level.

There were several noteworthy features in our study. Firstly, sequencing was performed on a small number of participants. Although we prospectively studied hundreds of recipients, only 14 were included to minimize potential factors that could disrupt the TCR repertoire, such as drug application and lymphocyte infusion. The CMV serostatus of both donors and recipients also affects TCR diversity; however, in this study, all donors and recipients were positive, as CMV infection rates are high in the Chinese population. Secondly, we obtained the sequencing data of total lymphocytes to comprehensively evaluate the data from all T cells, instead of specific subsets, such as cytotoxic CD8 + T cells or CMV antigen tetramer + T cells restricted by HLA alleles [35,38]. Each sequencing method has its advantages. The former method may be more directly related to the immune response against CMV, while it has limitations in cell sorting and the results may not be comprehensive. Additionally, previous studies have reported the significance of T cells in preventing CMV reactivation and infection, regardless of specific HLA types [27,39]. Despite the limitations in sequencing PBMCs, three V-J combinations described in this study were consistent with those from previous reports on CMV-specific T cells, which further support the credibility and value of our results. Thirdly, we focused solely on the β chain of TCRs. However, the α chain also play an important role in the recognition of antigen-MHC complexes, and the binding affinity of TCR α and β chains may also influence the immune responses [40]. Hence, future studies should analyze the TCR α chain as well. Finally, although the TCR repertoire after allo-HSCT is associated with risks of relapse and GVHD [41,42], no relapse was observed in our study participants and only one recipient had acute GVHD.

Conclusion

In conclusion, the early characteristics of the post-transplant TCR repertoire of recipients correlate with CMV outcomes. Our results enhance understanding of the mechanisms underlying the T cell resistance against CMV reactivation.

Abbreviations

CMV	Cytomegalovirus
TCR	T cell receptor
CRR	CMV reactivated recipient
CNR	CMV non-activated recipient
OR	Other recipient
allo-HSCT	Allogeneic hematopoietic stem cell transplantation
MHC	Major histocompatibility complex
CDR3	Complementarity-determining region 3
TRBV	T cell receptor β-chain variable region
D	Diversity
TRBJ	T cell receptor β-chain joint region
C	Constant
HLA	Human leukocyte antigen
GVHD	Graft versus host disease
5′RACE	5′Rapid amplification of cDNA ends
HTS	High throughput sequencing
BUB	Baroni–Urbani and Buser
PBS	Phosphate-buffered saline

Supplementary Information

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The online version contains supplementary material available at https://doi.org/10.1186/s12985-024-02511-x. Table S1. Comprehensive information about the study subjects. Table S2. Overview of samples sequencing data. Table S3. Frequencies of differentially selected V-J combinations between CRR and CNR. Fig. S1. Shared CDR3 genes and amino acids between the CRR and CNR groups. Fig. S2. The percentages of TRBV segment usage in each subject within the CRR and CNR groups. Fig. S3. Usage of V-J combinations by each subject. Fig. S4. Comparison of the gene sequences of five V-J combinations. Fig. S5. Distribution of CDR3 lengths for each subject. Supplementary Method: Introduction to the BUB index.

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Not applicable.

Author contributions

JTX, YJX and JF contributed to the conception and design of the study, SNG and GYG performed the CMV DNA detection. JTX, HQW and XJL performed the remaining assays. RY and JTX performed the statistical analyses. JTX wrote the first draft of the manuscript and JF revised the manuscript. All authors reviewed the manuscript and approved the final version for publication.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University under protocol number 2019-484 on 25 February 2019. Consent was obtained in writing after providing participants with detailed information about the study's purpose, procedures, and potential risks.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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