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High-throughput T cell receptor sequencing reveals the effect of Human Cytomegalovirus IE2 immediate early protein on mouse immunohistochemistry

Xiaoke Liu, Dongmeng Qian, Xianjuan Zhang, Ziying Qin, Ting Liu, Bin Wang*

Department of Pathogenic Biology, Qingdao University Medical College, Qingdao, China, 266071

Abstract: **As an important immune cell, T lymphocytes can recognize the antigenic peptide through the specific T cell receptor on the cell surface, and activate the human acquired immune response. The diversity of T cell antigen recognition receptors determines the host immune response and disease prognosis. It has been reported that congenital Human cytomegalovirus (HCMV) infection causes the immune system to be suppressed in immunodeficient individuals. However, as an important protein of HCMV, whether IE2 plays a key role during HCMV latent infection and whether IE2 will change the TCR library is still unknown. The purpose of this study is discussed the effects of IE2 on TCR library and its diversity. First, UL122 genetically modified mice models that can steadily and continuously express IE2 protein were established. Then, we divided the transgenic mice into two groups, the experimental group (positive mice identified) and control group (negative mice, n=8 in each group). The establishment of UL122 genetically modified mice was identified by PCR technology. We used immunohistochemistry to detect the expression of IE2 in both groups. Flow cytometry was measured the proportion of CD4+ and CD8+T cells. High-throughput T cell receptor sequencing was performed to detect the differences of Vβ and Jβ gene segments and the change of TCR diversity. We found that IE2 was widely expressed in immune organs of experimental mice, and the expression of IE2 affects the distribution of CD4+ and CD8+ T cell. In addition, we first determined the profile of the TCRβ immune repertoire during HCMV latent infection, indicating that the expression of IE2 resulted in remarkably lower TCRβ diversity in peripheral blood T lymphocytes. Taken together, our data suggest that the expression of IE2 gives a rise to aberrant immune microenvironment that affects immune response, and the low diversity of TCR caused by IE2 expression may be a mechanism of HCMV immunosuppression.**

Keywords: IE2; T cell receptor; Immune evasion; Immune repertoire; High-throughput sequencing

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*Corresponding Author: Bin Wang, superiority5822@163.com

1. Introduction

Human cytomegalovirus (HCMV) belongs to the herpesvirus subfamily and the genome is a linear double-stranded DNA[1]. It can infect up to 90% of individuals depending on the population studied[2]. In healthy people with normal immune function, HCMV establishes lifelong asymptomatic latent infection, in which reactivation of the virus can be successfully controlled by the immune system[3]. However, in individuals lacking adequate immune regulation, the immune system is inhibited, resulting in uncontrolled viral replication and ultimately morbidity and mortality[2, 3]. Therefore, HCMV infection seriously affects the health of individuals, especially those who lack immune control.

The HCMV genome can selectively splicing transcription to produce two major immediate early gene products, IE1 and IE2. Previous studies have shown that IE2 not only negatively regulates the promoter of MIE, but also transactivates the early promoter of the virus[4]. Owning to species specificity, HCMV can only infect human and not be infectious in other creatures[5]. Therefore, we established the ul122 genetically modified mice model in which IE2 can be expressed persistently and steadily under an organismic internal environment. The establishment of the ul122 genetically modified mice model overcome the species specificity will contribute to expound the mechanism of IE2 inducing diseases, as well as efforts to provide theoretical basis for the prevention and treatment of disease.

Both humoral and cell-mediated immune responses are involved in the host's immune defense against HCMV infection, but cellular immunity is particularly important for controlling infection[2, 6]. CD4+ T helper cells and CD8+ cytotoxic T cells (CTL), as part of important cellular immunity, are involved in the production and maintenance of immune responses against HCMV[7-9]. TCR is a specific receptor that T cells recognize and bind to foreign antigens which can be expressed on the surface of all mature T cells[10]. More than 90% of TCR consist of α and β heterodimeric peptide chains. Among them, the random combination of V, diversity (D) and J gene segments in α and β chains makes the diversity of

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TCR[11]. Random insertions and deletions of non-template nucleotides at the junctions of V(D)J gene segments further increase the diversity of TCRs, and up to 1018 TCR types have been reported[12]. TCR can recognize antigen molecules which were processed by antigen presenting cells (APCs) and linked to major histocompatibility complex (MHC) molecules and form antigen peptide-MHC- molecular complexes, thereby activating T cells[13].

A recent investigation of the mechanism of HCMV immune evasion indicated that HCMV can evade immune recognition of CD8+ and CD4+ T cells by down-regulating the expression of MHC class I and class II on infected cells[14, 15]. Moreover, TCR diversity is closely associated with host immune response and immune evasion[16]. However, the diversity of TCRs during HCMV latent infection is still not clear. This study aim to figure out the effects of IE2 on the diversity and stability of TCR during the latent infection of HCMV.

2. Materials and methods

2.1. Animals Preparation

Eight mice ul122 genetically modified mice (two months old) were obtained from Laboratory of pathogenic biology of Qingdao University. After the DNA extracted analysis of the tails of half a month old mice, the ul122 positive mice were left for the experimental group and the negative as the control. All animal experiments were authorized by the Animal Experiments Committee of Qingdao University.

2.2. Isolation of peripheral blood lymphocytes

The lymphocytes of mice were separated from whole blood by Ficoll-Paque density gradient separation (Solarbio Life Sciences, Beijing, China) and then suspended in Hank's balanced salt solution (HBSS, Solarbio). After centrifuging it at 250 × g for 30 min at 20℃, we removed the supernatant and repeat and wash again with Hank's balanced salt solution (HBSS, Solarbio). The cell pellets was suspended with 1 ml PBS to the further experiment.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from peripheral blood lymphocytes using an RNAprep Pure Cell/Bacteria Kit (TIANGEN BIOTECH, Beijing, China) according to the manufacturer's instruction. RNA concentrations were quantified using an Eppendorf BioPhotometer Plus (Eppendorf, Hamburg, Germany), and 200 ng of total RNA was converted into cDNA by reverse transcription using a Transciptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol on a T100TM Thermal Cycler (Bio-Rad Inc., CA, USA).

2.4. TCR library preparation

Two-round nested amplicon arm-PCR was performed for TCRβ CDR3 library preparation using specific primers against each variable and constant gene (the multiplex primers in Table S1) with a Multiplex PCR Assay Kit Ver.2 (TaKaRa, Dalian, China). The first round of PCR was performed using cDNA templates in a 50 μl reaction with the following program: one cycle of 94°C for 60 sec, 30 cycles of denaturation at 94°C for 30 sec and annealing at 60°C for 60 sec, and a final extension for 10 min at 72°C. For the second round of PCR amplification, a 5 μl sample of the first round PCR product was used, and the same PCR protocol was followed. PCR products were purified by agarose gel electrophoresis and amplified using Illumina sequencing primers with different sample barcodes. Then, the purified PCR product was subjected to high-throughput sequencing using the Illumina HiSeq X Ten platform with a read length of 2 × 150 bp.

2.5. Immunohistochemistry

The Lymph node tissue and spleen tissue excised from positive mice and negative mice were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded tissue sections (5 μm thick) were deparaffinized, heated in citrate buffer for antigen retrieval and an appropriate amount of endogenous peroxidase blocker was added to inactivate endogenous catalase. After washed with distilled water and PBS, sections were incubated with anti-IE2 antibody (Millipore, USA) 1 hour at 37℃ and with secondary antibodies for 40 min at 37℃ (PowerVision ™ Two-Step Histostaining Reagent，ZSGB-BIO，Beijing, China). The tissue sections were then exposed to DAB for 1 min.

2.6. Flow cytometry

Mouse peripheral blood lymphocytes were incubated with fluorescently conjugated antibodies directed against mouse CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7) and TCRβ (H57-597) from Biolegend (San Diego, CA) and eBioscience (Vienna, Austria) for half an hour. Cell counting was performed on a BD FACSAria II (BD Biosciences, Mountain View, CA, USA) and analysed with FlowJo Version 10 Software (TreeStar, Ashland, Oregon, United States).

2.7. Analysis of high-throughput sequencing data

The raw image data files obtained using the Illumina HiSeq sequencing platform were converted to the original sequencing sequence by base recognition and the results were stored in FASTQ format[10]. The V, J and CDR3 regions of TCRβ consensus sequences were identified using BLAST Plus in the international ImMunoGeneTics (IMGT) information system by a

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standard algorithm[17]. The diversity of TCRβ was measured by Gini coefficient, Simpson index and normalized Shannon diversity entropy, which have been widely used for assessing the richness and diversity of TCR as previously described [18-20].

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 Package (GraphPad Software, La Jolla, CA). Data are presented as the mean ± standard error of the mean (SEM). An unpaired t-test was used to compare samples between positive and negative mice groups. For immune repertoire data, statistical significance between two groups was assessed using a Student's t test. Two-sided p-values < 0.05 were considered statistically significant.

3. Results

3.1. Extraction of DNA and PCR results of mouse

We extracted DNA from the tail of IE2 genetically modified mice and detected the expression of IE2 genes by PCR (Figure S1). According to the existence of IE2 genes, we divided the mice into two groups (experiment group and control group). The IE2 positive were treated as the experiment group and the negative as the control group.

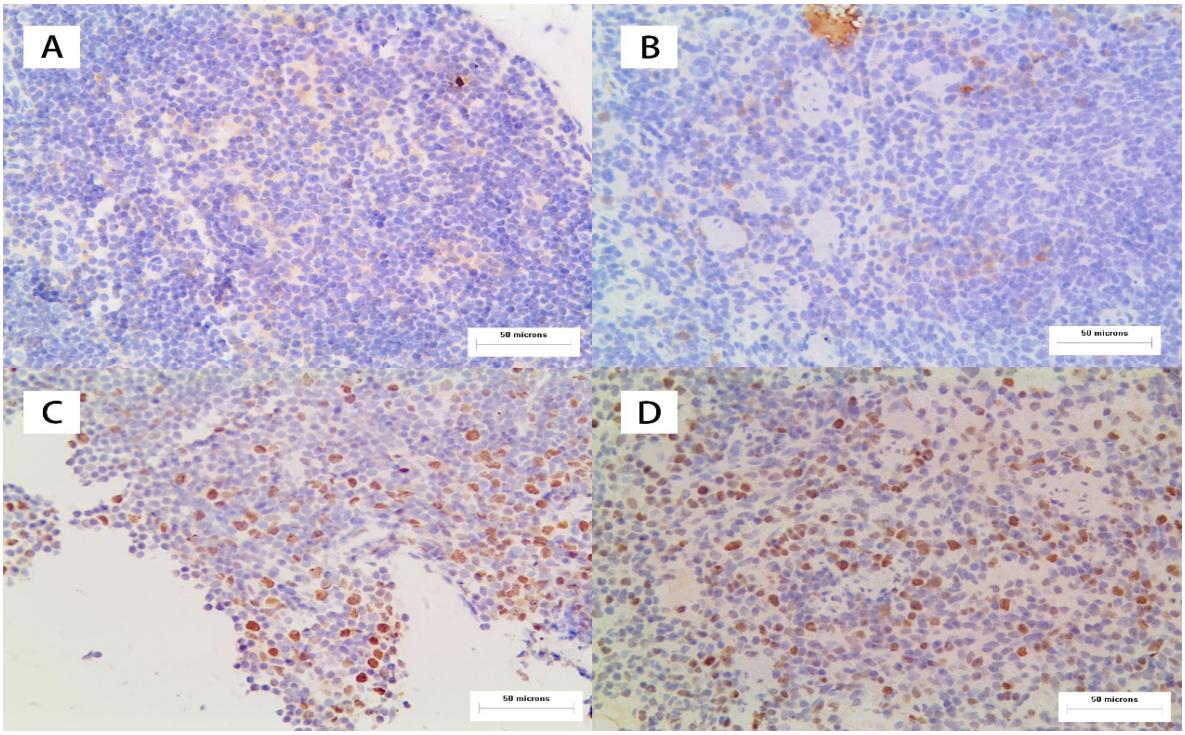


Figure 1. IE2 immunohistochemistry in immune organ of positive group and the negative group mice. (A) Immunohistochemistry for IE2 in lymph nodes tissue of negative mice. (B) Immunohistochemistry for IE2 in spleen tissue of negative mice. (C) Immunohistochemistry for IE2 in lymph nodes tissue of positive mice. (D) Immunohistochemistry for IE2 in spleen tissue of positive mice. In the positive group, nuclear staining was strongly positive (C, D).

3.2. The expression of IE2 in immune organs of IE2 transgenic mice

We observed the expression of IE2 by immunohistochemistry in lymph nodes and spleens of transgenic mice. The result shows that the expression of IE2 in the nucleus was cannot be seen in the negative group (Figure 1A, B). However, as shown by immunohistochemistry, nuclear staining was strongly positive both in lymph nodes and spleens in the positive group (Figure 1C, D). According to the results of immunohistochemistry, the expression of IE2 can be found in lymph nodes and spleen, the most important immune organ. These indicated that IE2 has the ability to affect the function of the host immune system to some degree.

3.3. The proportion of CD4+ and CD8+T cells in peripheral blood lymphocytes

Due to the above results illuminating the IE2 has express in mice immune system, we wondered whether the proportion of immune cells that perform major functions in the immune system has changed. After flow cytometry analysis, the results showed that the proportion of CD4+ T cells in lymphocytes in

peripheral blood of IE2-positive transgenic mice was decreased. Conversely, a robustly elevated CD8+ T cell number was observed in positive group (Figure 2A, B). The difference is statistically significant ($P<0.05$).

All the results indicated that IE2 changes the proportion of CD4+ and CD8+T cells in peripheral blood lymphocytes and affects the function of cellular immune.

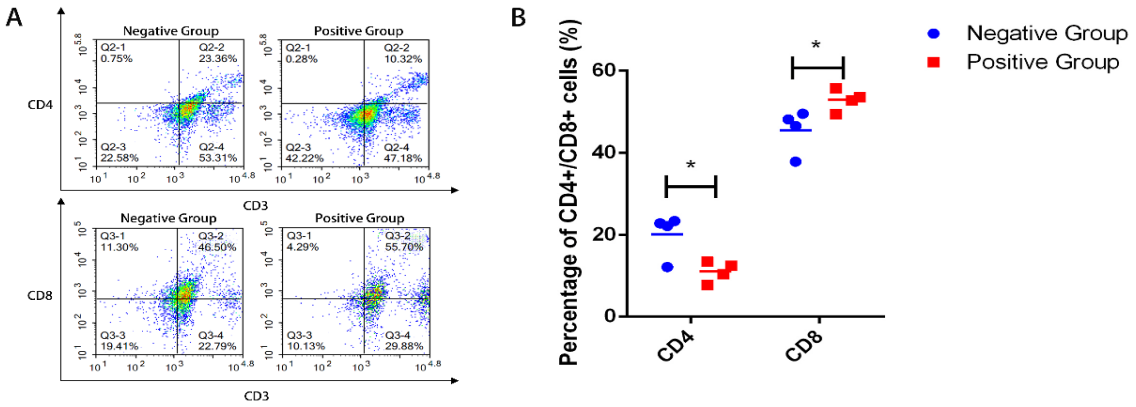


Figure 2. Flow cytometry analysis result showed the proportion of CD4+ and CD8+T cells in peripheral blood lymphocytes. (A) Flow cytometry analysis of CD4+ T cells and CD8+ T cells in IE2-positive and negative transgenic mice. (B) The proportion of CD4+ T cells were decreased and CD8+ T cells were increased in positive mice.

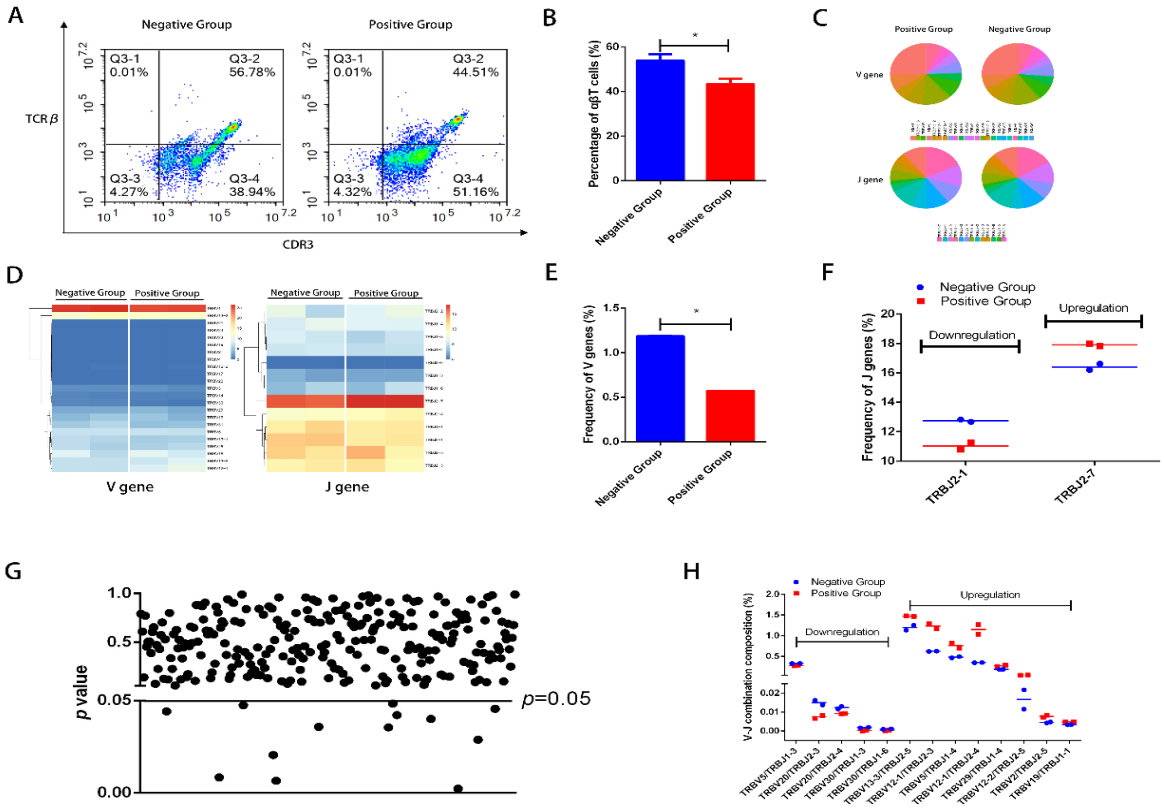


Figure 3. The distribution and abundance of V and J genes and V-J gene combinations in TCRβ. (A) Flow cytometry of intrahepatic TCRβ T lymphocytes from both groups. (B) The percentage of αβ T cells was measured by flow cytometry analysis. (C) The mean distribution of all identified V and J gene segment usage. (D) The heat map of VJ gene segments in positive and negative groups. (E) The frequency of V gene segment usage that had significant differences (P<0.05). (F) The frequency of J gene segment usage that had significant differences (P<0.05). (G) The abundance discrepancy of V-J gene combinations in two groups. (H) The frequency of V-J gene combinations that had significant differences (P<0.05).

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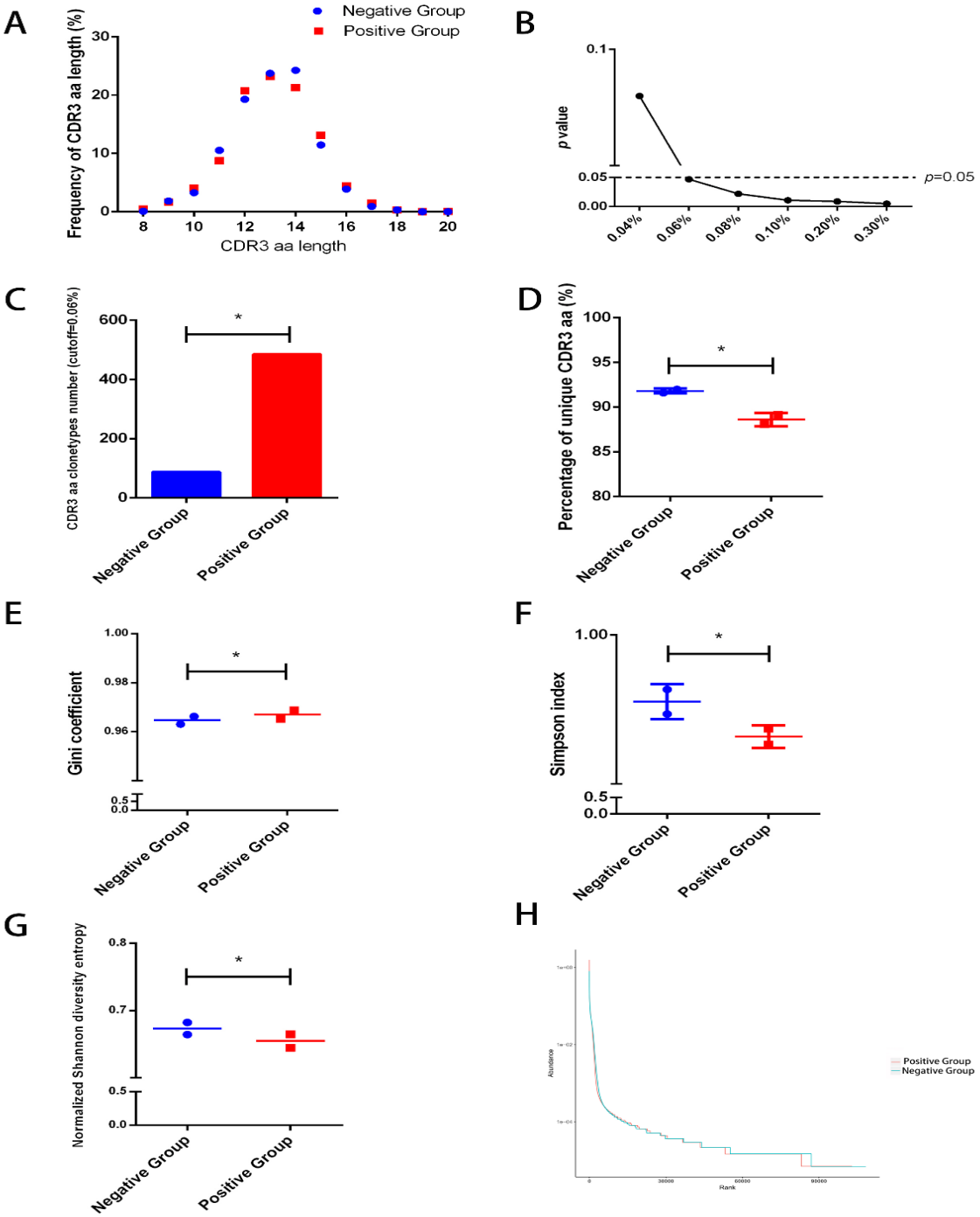


Figure 4. Analysis of CDR3 aa clonotypes in liver regeneration. (A) The distribution of CDR3 aa length in the TCRβ chain. (B) Comparison of CDR3 aa clonal frequency exceeding a certain threshold in positive and negative group. (C) Comparison of CDR3 aa clonotype numbers (clonal frequency cut-off threshold = 0.06%). (D) Comparison of the percentage of unique CDR3 aa. (E-H) Comparison of CDR3 aa diversity by the Gini coefficient, Simpson index, normalized Shannon diversity entropy and Rank-abundance. Data are representative of 2 independent experiments with 2 mice per group in each. P<0.05.

3.4. Profiling of Vβ and Jβ gene segments in positive and negative mice

To assess the variation of the TCRβ immune repertoire in blood αβ T cells, we first measured this population in both positive and negative mice. The number of blood αβ T cells decreased sharply in positive group (Figure 3A, B). Subsequently, four paired blood αβ T cells were prepared as a PCR sample and two paired were subjected to high-throughput

sequencing (Figure S2).

Our study was consisted of two positive mice and two negative mice. The total number of reads was 33,377,722, with an average of 8,344,431 reads per sample. The results of high-throughput sequencing show that a total of 23 distinct Vβ gene segments and 13 distinct Jβ gene segments were identified from all samples. The most frequent Vβ gene segments in both groups were TRBV1 (24.95% in positive group, 25.94% in negative group) and TRBV13-3 (12.45% in positive group, 12.58% in negative group). The most frequent Jβ gene segments in positive and negative group was TRBJ2-7 (17.91% in positive group, 16.42% in negative group) (Figure 3C).

According to the heat map of VJ gene segments which was generated from the usage frequency of Vβ and Jβ gene segments (Figure 3D), the usage patterns of Vβ and Jβ gene segments were similar between positive and negative group, whereas some Vβ and Jβ gene segments exhibited significantly usage differences in positive group, such as TRBV1, TRBV15, TRBV30 and TRBJ2-7, TRBJ2-1, and TRBJ2-4 (Figure S3). The comparison of all the usage frequency Vβ gene segments between positive and negative group had shown that only 1 Vβ gene segment (all p-values were <0.05, unpaired t-test, Figure S4), TRBV30, has significant difference (Figure 3E). Then, we also analyzed the usage frequency of Jβ gene segments in two groups, and two Jβ gene segments (TRBJ2-1 and TRBJ2-7) that have noticeable differences were observed (all p-values were <0.05, unpaired t-test, Figure S5). Among them, TRBJ2-1 was used more frequently in negative group, and TRBJ2-7 exhibited increased frequency in positive group (Figure 3F).

In addition, we detected the composition of V-J gene combinations to further analyzed the results and found a total of 288 distinct V-J gene combinations. Compared with the negative group, the general usage patterns of V-J combinations were similar with positive group (Figure S6), and there were 13 VJ gene combinations (4.5%) exhibited significant usage differences between IE2 positive group and negative group (all p-values were <0.05, unpaired t-test, Figure 3G). Among them, 5 V-J gene combinations (38.5%) were used more frequently in negative group, and 8 V-J gene combinations (61.5%) exhibited increased frequency in positive group (Figure 3H).

3.5. Lower diversity of CDR3 amino acids (aa) was showed in positive mice

In our study, 358590 distinct CDR3 amino acid (aa) clonotypes were identified in all simples. CDR3 aa clonotypes, including a conserved cysteine in the Vβ region and a conserved phenylalanine in the Jβ region, determine the diversity of the TCRβ repertoire. We analyzed CDR3 aa length at first and the TRB CDR3 aa length in most samples were 10-40bp, with an average length of 25 ± 2bp (mean$\pm$SD). We found that CDR3 with the length of 13 and 14 aa positive group showed a lower usage frequency than that in negative group (Figure 4A). Although the total CDR3 aa clonotypes number were nearly identical in both groups, the number of CDR3 aa clonotypes showed a significant difference when the cut-off threshold was 0.06%. Obvious increments in high-frequency CDR3 aa clonotypes was observed in the positive mice group (cut-off threshold $>0.06\%$, $P<0.05$) (Figure 4B, C), in which IE2 was expressed persistently and steadily. However, the percentage of unique CDR3 aa showed clear reductions in positive group (Figure 4D). These results suggest that the positive group might have potentially lower CDR3 aa diversity. Furthermore, the Gini coefficient, Simpson index, normalized Shannon diversity entropy (NSDE) and Rank-abundance were used to compare TCR CDR3 aa diversity in positive and negative mice (Figure 4E-H). All results suggested a remarkably lower TCR diversity in positive group compared with negative group. These date all indicated that the expression of IE2 changes the diversity of TCRβ CDR3.

4. Discussion

Human cytomegalovirus (HCMV) usually acquired early in life, the infection can be transferred through all bodily fluids[3]. The IE2 encoded by ul122 in HCMV plays an important role in viral replication and has been indicated in the pathogenesis of some diseases[21]. Using ul122 genetically modified mice model to study the influence of IE2 on immune response not only overcomes the highly species-specific of HCMV, but also provides the possibility to study the influence of IE2 on immune response in vivo.

HCMV establishes a latent infection that generally remains asymptomatic in immune-competent hosts for decades but can cause serious disease in immune-compromised individuals. Increasing evidence suggests that HCMV has developed multiple immune evasion strategies to establish latency and co-exist with its host. Meanwhile, the infection adversely affects both innate and adaptive immune responses[2]. In past few decades, more attention has focused on a single parameter of the immune system, especially in the innate immune system. However, the interaction between immune cells and the correlation between the innate immune system and adaptive immune system remains unclear.

Immunohistochemistry results revealed IE2 positive signals in the spleen and lymph nodes of transgenic mice, which suggested HCMV successfully infect these areas. Based on the above results, we observed the distribution of immune cells in transgenic mice and found CD4+ T cells remained low and CD8+ T cells maintained high levels during HCMV latent infection.

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This result is consistent with previous studies reporting that HCMV particles have ability to inhibit CD4+ T cell proliferation and render them unresponsive to stimulation signals in vitro experiment[7]. Meanwhile, the proliferation of CD8+ T cells may be related to the rapid expansion of CD8 during HCMV infection during acute active infection[22, 23]. Previous studies have shown that HCMV has ability to escape immune recognition by CD8+ and CD4+ T cells by downregulating expression of major histocompatibility complex (MHC) class I and II on infected cells[14, 24]. However, αβ T cells mainly exert immune surveillance function by using its surface TCRs to recognize peptides bound to an MHC molecule on infected or otherwise altered cells. Due to previous research and the above results, we observed that HCMV infection can not only change the host immune microenvironment, but also cause a variety of diseases in individuals with immune dysfunction such as perinatal infections, neonatal diseases, and congenital malformations. In particular, primary infection or latent reactivation of HCMV can cause very serious and even life-threatening consequences in allogeneic stem cells and solid organ transplants, or AIDS patients. Therefore, it is meaningful to analyze the TCR diversity in IE2 positive and negative group to aid the analysis of immune response and immune escape mechanism in HCMV infection.

Our results are the first to reveal an intact profile of Vβ, Jβ gene segments and CDR3 clonotypes during HCMV latent infection. The Vβ and Jβ gene segments are the main components of the TCR repertoire. In this study, no noticeably difference in the distribution of Vβ, Jβ gene and V-J combinations were found between two groups. However, the percentage of unique CDR3 aa clonotypes was higher in negative group, despite the positive group had higher ratio of high-frequency CDR3 aa clonotypes. These result are consistent with the Gini coefficirnt, Simpson index, NSDE and Rank-abundance and suggest that TCR diversity is lower in positive transgenic mice compared with the negative group. Our observation of lower clonality and diversity of αβ T cells in IE2-positive mice provides evidences of HCMV-associated immune response in the adaptive immune system. This finding may offer an new insights into the HCMV immune escape mechanism and the immune regulation function of the adaptive immune system, which is synergistic with the innate immune system.

Some researchers have indicated that TCR diversity is related to some disease prognosis. Jia et al. reported that high NSDE of the mucosal T cell repertoire in gastric cancer correlates with patient short-term and overall survival time[25]. Muraro et al. reported that CD4CT and CD8CT TCR diversity in response patients was increased compared with non-response patients before and after haematopoietic stem cell transplantation[11]. Chen et al. reported that TCR diversity is higher in tumors compared with adjacent non-tumor tissues in HBV-associated HCC[26]. These results suggest that TCR diversity might be a new biomarker for the prediction of the prognosis of different diseases. However, as our study is a cross-sectional study, Whether TCR diversity can predict HCMV-associated disease prognosis must be further analyzed in a large, longitudinal study.

5. Conclusion

Our work for the first time exhibits that IE2 can widely expressed in lymphoid organs (lymph nodes and spleen) and changed greatly the number of T lymphocytes. In addition, the TCR library of the ul122 genetically modified mice exhibits low diversity, which may result from the expression of IE2. All experimental results indicated that IE2 not only can affects immune response at the cellular degree, but also changes the diversity of TCRβ, which is the important and characteristic structure to recognize antigen peptides on the T cell surfaces at the genetic level. Synthesis of previous research found that, hosts are subject to different immunosuppressions in multiple processes of immune response during the HCMV latent infection. The research of TCRβ library provides novel orientation and insights into the mechanism of Human cytomegalovirus immune evasion.

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Competing Interests Statement

The authors declare no conflict of interest

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