Immune repertoire analysis of normal Chinese donors at different ages

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Abstract

Objectives: This study investigated the characteristics of the immune repertoire in normal Chinese individuals of different ages.

Materials and Methods: In this study, all seven receptor chains from both B and T cells in peripheral blood of 16 normal Chinese individuals from two age groups were analyzed using high-throughput sequencing and dimer-avoided multiplex PCR amplification. Normal in this study is defined as no chronic, infectious or autoimmune disease within 6 months prior to blood draw.

Results: We found that compared with the younger group, the clonal expression of T-cell receptor repertoire increased in the older group, while diversity decreased. In addition, we found that the T-cell receptor repertoire was more significantly affected by age than the B-cell receptor repertoire, including significant differences in the use of the unique TCR-alpha and TCR-beta V-J gene combinations, in the two groups of normal participants. We further analyzed the degree of complementarity determining region 3 sequence sharing between the two groups, and found shared TCR-alpha, TCR-gamma, immunoglobulin-kappa and immunoglobulin-lambda chain complementarity determining region 3 sequences in all subjects.

Conclusion: Taken together, our study gives us a better understanding of the immune repertoire of different normal Chinese people, and these results can be applied to the treatment of age-related diseases. Immune repertoire analysis also allows us to observe participant's wellness, aiding in early-stage diagnosis.

1 | INTRODUCTION

The Adaptome is a collection of all the structural and functional diversity of T cell receptors (TCRs) and B cell receptors (BCRs) of an individual at any given time, which accurately and comprehensively reflects the dynamic changes of the immune system.\(^1\) Within the...
Adaptome, the repertoire consists of all seven chains from TCR and BCRs and their clonotypes.\textsuperscript{2–4} Immune repertoire high throughput sequencing (IR-HTS) is the study of the full-length receptor or complementarity determining region 3 (CDR3) region from both TCRs and BCRs to assess immune system diversity and immune system relationship to disease.\textsuperscript{5} Each peptide chain of TCR and BCR includes a variable (V), diversity (D), joining (J) and constant (C) region.\textsuperscript{6} The V region includes three CDRs (CDR1, CDR2, and CDR3) with CDR3 having the greatest variability.\textsuperscript{7} IR diversity is due to the V (D) J gene rearrangement and addition or deletion of nucleotides in the CDR3 region, as well as somatic hypermutation (SHM) in B cells.\textsuperscript{8,9} With the rapid development of HTS technology, it is increasingly applied to complex structures and large-scale IR analysis.\textsuperscript{10,11} An accurate and comprehensive understanding of the diversity of TCRs and BCRs in different age groups will facilitate the development of new immunotherapy measures for diseases\textsuperscript{12,13} and the provision of personalized therapies for patients.\textsuperscript{14–16}

In recent years, with the rapid development of HTS technology, IR has been widely used in disease-related research.\textsuperscript{17–23} One study found that there were differences in the use of V and J genes in both \( \beta \) and IGH chains in patients with non-small cell lung cancer (NSCLC). It was found that the clonality and diversity of the TCR repertoire in the early recurrence patients were increased and decreased, respectively, compared to the early non-recurrence patients.\textsuperscript{24} Wang’s team used HTS to study TCR diversity in the peripheral blood and bone marrow of patients with acute lymphoblastic leukaemia before and after treatment, and found that the diversity of TCRs in peripheral blood and bone marrow decreased and the clonality increased after treatment with chimeric antigen receptor T cells (CAR-T).\textsuperscript{25} One study reported a significant increase in TCR \( \beta \) chain CDR3 diversity in normal participants after the second hepatitis B vaccination, while a significant decrease in BCR IgG Heavy chain CDR3 diversity.\textsuperscript{26} Another study shows in patients with coronavirus disease 2019 (COVID-19), the TCR repertoire diversity was reduced, and the TCR \( \beta \) clone is expanded. This study also showed that the CDR3 sequence length of TCR is longer in COVID-19 patients that were co-infected with pneumonia compared with patients without pneumonia.\textsuperscript{27} The above studies indicate that the diversity of IR is related to the occurrence and development of diseases.\textsuperscript{28–30} At the same time, the above studies have also enabled us to have a better understanding of the IR, but our understanding of the IR of normal Chinese people of different ages is still very limited. Therefore, the study of IR in normal Chinese subjects of different ages has tremendous implications for the treatment of age-related diseases.\textsuperscript{31–34}

Dimer-avoided multiplex PCR (Dam-PCR) can be used to amplify all seven chains of the IR in the same PCR reaction system under the same conditions, with unique molecular IDs (UMIs), which can reduce the amplification bias and directly compare the gene expression level quantitatively. In this study, 16 normal Chinese participants were divided into two groups based on age: Group 1 (ages 19–30) and Group 2 (ages 50–67), with eight participants in each group. Peripheral blood samples from the participants were collected, RNA was extracted and Dam-PCR combined with HTS was used to analyze the IR of each participant. The results showed that with the increase of age, the diversity of peripheral blood TCR repertoire decreased, and the average length of TRA CDR3 sequence increased significantly. In addition, we found that V and J genes existed independently in both groups, and the expression difference of TRB V-J gene combination between the two groups was the most significant. Shared CDR3 sequences from TRA, TRG, IGL, and IGL were found among all individuals. Our results were designed to reveal the diversity of the IR in Chinese normal subjects of different ages.

\section{MATERIALS AND METHODS}

\subsection{Sample collection}

Sixteen normal participants were included in the study. The experiment was divided into two groups: Group 1, ages 19–30, and Group 2, ages 50–67, with eight participants in each age group. The participants were excluded from neurological, infectious, autoimmune and other serious cardiovascular and cerebrovascular diseases. Peripheral blood samples were collected from participants using BD PAXgene Blood RNA Tube (Ca# 762165, BD, US). The study was approved by the NanJing XiaoZhuang University Ethics Committee (lot number 2021NO.01) and the informed consent of all normal participants was obtained.

\subsection{RNA extraction}

The PAXgene Blood RNA Kit (Ca# 762174, PreAnalytix, US) was used to extract RNA from peripheral blood from all 16 participants. The concentration and purity of RNA were measured by NanoDrop One UV spectrophotometer.

\subsection{IR amplification and sequencing}

iRepertoire’s automated iR-RepSeq-plus 7-Chain Cassette (Ca# iR + 7chain-HLRI-C, iRepertoire, US) was used to construct a 7-chain library for each sample. Each cassette contains all reagents needed to perform Dam-PCR. 1200 ng of RNA was used as template per cassette. The iR-RepSeq-plus cassette performs four rounds of PCR: first strand synthesis (FSS), second strand synthesis (SSS), and two rounds of enrichment. FSS contains a gene specific reverse primer mix for reverse transcription and first strand cDNA synthesis. Following FSS, unused primers are removed and a gene specific forward primer mix performs second strand cDNA synthesis. Residual primers are removed after SSS and a 2-step enrichment PCR with Illumina communal adaptor sequences is performed for amplification and enrichment. Detailed library construction procedures are available in the literature\textsuperscript{35} and on iRepertoire’s website. SPRSelect magnetic beads were used to clean the libraries. Each sample was
individually barcoded, pooled and multiplexed in one sequencing run and de-multiplexed in downstream data analysis. The multiplexed library was sequenced on a 500-cycle kit on the Illumina NovaSeq platform.

### 2.4 Statistical analysis

The iRmap program was used to analyze the raw data. GraphPad Prism 8.0 was used for statistical analysis. NS indicates no statistical difference, *$p < 0.05$ indicated a statistical difference, while **$p < 0.01$ indicated a significant difference.

### 3 RESULTS

#### 3.1 Research participant metadata

The purpose of this study was to study the characteristics of all seven chains from peripheral blood IR in Chinese normal subjects of different ages. Sixteen normal participants were coded from 1 to 16. Table 1 describes participant data from the 16 normal participants and sequencing results of seven chains. By analyzing the total RNA receptor count and the unique CDR3 (uCDR3) obtained by sequencing, we found that total receptor RNA in Group 1 averaged about 180,000 total RNA and an average of 60,000 total uCDR3. In Group 2, total receptor RNA averaged about 240,000 and total uCDR3 about 80,000. uCDR3 discovery for both groups were similar, around 30% uCDR3 from total reads. The above results show that the sequencing results of all samples are successful and can be used for subsequent analysis.

| Sample ID | Sex (M/F) | Race | Ethnicity | Age | Age group | Total receptor RNA | Total uCDR3 |
|-----------|-----------|------|-----------|-----|-----------|-------------------|-------------|
| 1         | F         | Asian | Han       | 20  | 19–30     | 176735            | 72300       |
| 2         | F         | Asian | Han       | 22  |           | 316526            | 91389       |
| 3         | M         | Asian | Han       | 27  |           | 110693            | 53826       |
| 4         | F         | Asian | Han       | 29  |           | 139996            | 79333       |
| 5         | F         | Asian | Han       | 30  |           | 272024            | 79185       |
| 6         | F         | Asian | Han       | 24  |           | 112408            | 27594       |
| 7         | M         | Asian | Han       | 20  |           | 160304            | 64425       |
| 8         | F         | Asian | Han       | 19  |           | 208671            | 69678       |
| 9         | F         | Asian | Han       | 50  |           | 416159            | 119173      |
| 10        | F         | Asian | Han       | 52  | 50–67     | 211901            | 74161       |
| 11        | M         | Asian | Han       | 60  |           | 184716            | 79518       |
| 12        | F         | Asian | Han       | 60  |           | 275400            | 114450      |
| 13        | M         | Asian | Han       | 60  |           | 379226            | 91829       |
| 14        | F         | Asian | Han       | 60  |           | 89974             | 38232       |
| 15        | M         | Asian | Han       | 65  |           | 192929            | 68031       |
| 16        | F         | Asian | Han       | 67  |           | 193569            | 88172       |

#### 3.2 Adaptome analysis

Adaptome refers to all B and T cell receptor genes expressed in a sample. Differences were found in the seven chains repertoire expression between the two groups (Figure 1A). It can be seen that the TCR and BCR repertoires each accounted for about half of the IR in Group 1, while TCR repertoire expression increased in Group 2 (Figure 1C, D). It was also found in both groups that the expression levels of TRA and TRB were significantly higher than TRD and TRG, while IGK expression levels were significantly higher than IGH and IGL. The mean expression levels of TRA, TRB, IGK, and IGL in Group 2 were higher compared with Group 1, while the mean expression level of IGH was slightly lower (Figure 1B).

#### 3.3 CDR3 region sequence length analysis

The CDR3 is of particular interest because it is the most variable portion of the antigen-binding site, and is associated with antigen-specificity. The longer the length of CDR3, the more complex the peptide chain folding structure and the higher the specificity of the receptor. CDR3 length from both T and B lymphocytes was compared between groups. TCR and BCR CDR3 regions had the greatest variability within the seven chains repertoire. Significant differences in CDR3 nucleotide length were found among the participants (Figure 2A). It was found that the morphology of the CDR3 region sequence length distribution maps of TRB, IGK, and IGL chains in the two groups of normal participants was similar (Figure 2B). However, the sequence length distribution maps of CDR3 region of TRD and TRG showed significant morphological differences (Figure 2B). Further analysis of the average CDR3 sequence length showed that with an increase in age,
the average CDR3 sequence length of TRA, TRD, IGH, and IGK increased significantly, while the average CDR3 sequence length of TRB, TRG, and IGL decreased, but there was no statistical significance (Figure 2C).

3.4 | Treemap and D50 analysis

Treemap, developed by iRepertoire, can visually describe the diversity of immune repertoire in a given sample. The Treemap reads from left to right and top to bottom as TRA, TRB, TRD, TRG, IGH, IGK, and IGL. Each rounded rectangle represents a unique CDR3, where the size of the spot represents the relative frequency. The different sizes are related to clonal expansion within the immune repertoire. The more uCDR3, or spots, generally represents a higher diversity in the given immune repertoire. Treemaps from two individuals in Group 1 and two individuals in Group 2, can be seen in Figure 3A,B, respectively. As can be seen from Figure 3A,B, TRA, TRB, and IGK occupied a large area in the IR of both groups. Compared with Group 2, the color blocks in the TCR Treemap of Group 1 contained less clonal expansion, thus having a higher diversity.

D50 is a numerical value developed by iRepertoire to quantitatively describe the diversity of the immune database. D50 is the percentage of the total number of unique CDR3 that account for the cumulative 50% of the total CDR3s in a given sample, and the closer it is to 50, the more diverse a library. Shannon, Simpson, and Pielou evenness indexes were also used to describe the diversity and evenness of the IR. The diversity index analysis of the seven chains in the
FIGURE 2
Legend on next page.
IR of the two groups is shown in Figure 3C. As can be seen from Figure 3C, compared with Group 1, the TCR D50 values in Group 2 were significantly reduced. The D50 values of the BCR repertoire between the groups were not significantly different. Shannon index of TRD in Group 2 showed a significant decrease compared to Group 1. Both Shannon and Pielou evenness indices showed a significant decrease in TRD and TRG diversity and homogeneity in Group 2.

3.5 | V and J gene analysis

The diversity of both TCR and BCR CDR3 regions is caused by recombination of V(D)J genes and addition or deletion of nucleotides. The CDR3 region was amplified and sequenced for both TCRs and BCRs to reveal the use of V and J genes in all seven chains. GraphPad Prism software was used to make statistics on the use frequency data of V and J genes and draw a 2-dimensional histogram for comparison. Figure 4 shows the expression of V and J genes of all TCR and BCR chains in different age groups. Although most V/J genes were expressed similarly between the two groups, significant differences were found in the use of certain V/J genes. Overall, most of the genes that were high expressed in Group 1 were also observed in the Group 2. TRA was the only chain that showed a significant difference in gene expression between groups (Figure 4A). Within Group 2, significant increases were discovered in TRAV18, TRAV20, TRAJ21, TRAJ38, TRAJ54, and TRAJ25, while significant decreases were discovered in TRAV1-2, TRAV19, TRAV21, TRAJ33, TRAJ48, and TRAJ49 genes (Figure 4A). Surprisingly, TRA V/J gene use differed the most between the two groups of normal participants (17 TRA V genes and 18 TRA J genes were expressed differently), with some genes being found only in Group 2, such as TRAV18, TRAV39, and TRAJ25. TRAV38-2DV8, TRAV4, and TRAV6 genes were found only in Group 1 (Figure 4A). These results can indicate that the increase of age has a great influence on TRA gene use in normal Chinese people.

3.6 | V-J gene usage

In order to observe the use of V-J gene combination of seven chain receptors of the two groups, we amplified and sequenced the CDR3 region sequence length in IR from peripheral blood. (A) Seven chains CDR3 sequence length distribution of IR in each participant. Each column represents a study subject, #1 is the numbered 1 subject, #16 is the numbered 16 subject, from left to right is 1–16. The same color block represents the same length of CDR3, and its size represents the frequency. (B) Seven chains CDR3 region sequence length distribution between groups. (C) Comparison of mean sequence length of seven chains CDR3 region between groups of normal participants. (C) was analyzed by t test. Mean ± SEM was shown. ns indicates no statistical difference and * represents a statistical difference.
region from all seven chains, and visually displayed the frequency of V-J gene usage using 2D map. The results show that the TRA, TRD, TRG, IGH, IGK, and IGL V-J gene combinations were similarly expressed in both groups (Figure 5B and Supplementary material Figure S1A–E). TRAV1-2/TRAJ33, TRDV36DV7/TRDJ3, TRGV1/TRGJP2, IGHV3-75/IGHJ2, IGKV3-34/IGKJ4, and IGLV3-16/IGLJ2 gene combinations were expressed the most of the two groups. These results suggest that the highly expressed combination of V-J genes may play an important role in human health. In addition to the highly expressed TRA V/J genes, significant differences were also found in

FIGURE 5 V-J gene usage. (A) V-J gene usage of TRB between the two groups. (B) V-J gene usage of IGH between the two groups. Different colored arcs in the outer circle represent V/J gene types, and the size of the arc represents the frequency. The area of the inner circle V-J gene linkage represents the combined V-J gene expression frequency. The longer the outer circle arc and the larger the area of the inner circle V-J gene linkage represents the higher frequency of V/J gene and V-J gene combination use.

FIGURE 4 Seven chains V and J genes usage between groups. (A) TRA V and J genes usage between groups. (B) TRB V and J genes usage between groups. (C) TRD V and J genes usage between groups. (D) TRG V and J genes usage between groups. (E) IGH V and J genes usage between groups. (F) IGK V and J genes usage between groups. (G) IGL V and J genes usage between groups. Blue represents the Group 1, yellow represents the Group 2, the horizontal coordinate is the V/J gene type, and the vertical coordinate represents the V/J gene frequency.
TRB V-J gene combination expression between the two groups (Figure 5A). TRBV5-2/TRBJ1-5, TRBV10-2/TRBJ1-5, TRBV21-1/TRBJ2-1, and TRBV30/TRBJ1-5 were highly expressed in Group 1. TRBV28/TRBJ2-7, TRBV20-1/TRBJ2-7, TRBV19/TRBJ2-1, TRBV29-1/TRBJ2-7, and TRBV20-1/TRBJ2-1 were highly expressed in Group 2, while the expression of TRBV5-2/TRBJ1-5, TRBV10-2/TRBJ1-5, TRBV21-1/TRBJ2-1, and TRBV30/TRBJ1-5 decreased. These results indicated that the expression of TRB V-J gene combination was different in Chinese normal subjects of different ages.

### 3.7 CDR3 sharing

To assess the prevalence of CDR3 sharing among both groups, we combined all sequencing data from the participants for analysis. It was found that almost all CDR3 sequences were unique in each participant, and only a few CDR3 sequences were shared among all participants (Figure 6A). Shared seven chain CDR3 sequences are expressed at different levels between groups. Using the Mann–Whitney U test, the expression level of uCDR3 sequences and representative sequences were plotted, showing significantly different expression between younger and older subjects (p < 0.05) (Figure 6B and Supplementary material S2). Each column represents an object, and a cell in each row represents the expression frequency of the CDR, (lowest as blue, highest as red).

We failed to detect TRB, TRD, and IGH CDR3 sequences shared among all participants in our study. However, we found shared TRA, TRG, IGK, and IGL CDR3 sequences in all participants (Figure 6A). Interestingly, we found a high number of TRA and IGK CDR3 sequences (100 and 274 sequences, respectively) shared among all participants (Figure 6A). Some shared sequences were highly expressed in all participants, such as CATWDGGYVKLF and CATWDGYLYKLF (Figure 6A). These sequences are highly similar and may have similar functions, suggesting that these sequences may be related to maintaining human life and health. In addition, we found that older normal participants shared more TRA and IGL CDR3 sequences, while younger normal subjects shared more TRG and IGK CDR3 sequences (Figure 6B and Supplementary material S2).

### 4 DISCUSSION

Aging plays a big factor on the immune system. As age increases, the potential ability of the immune system to resist newly encountered pathogens decreases, resulting in a decreased vaccination effect and an increased risk of age-related diseases such as cancer.
osteoporosis and diabetes.\textsuperscript{39–44} The reduced naïve T-cell repertoire in elderly COVID-19 patients significantly reduces the body’s ability to mount an adaptive immune response to the neoantigen SARS-CoV-2.\textsuperscript{45} Thevarajan et al. found rapid production and significant increase in activated CD8+ T cells in young COVID-19 patients with good prognosis on day 7–9 after SARS-CoV-2 infection,\textsuperscript{46} while the number of CD4+ and CD8+ T cells was significantly reduced in elderly and critically ill patients.\textsuperscript{47} The above study showed a positive correlation between total T cells, CD4+ and CD8+ T cells and patient survival. It has been reported that the diversity of TRA and TRB in the elderly is lower than that in the young,\textsuperscript{48} our findings are consistent with this. However, it is still unclear how age affects the state of the immune system and the characteristics of the IR of normal individuals of different ages. This study allowed us to comprehensively analyse the full seven chains receptor from B and T cells in Chinese normal participants by using HTS and dam-PCR amplification technology. Our results showed that the clonal expression of the TCR repertoire increased in Group 2, the older population, while the diversity of TCR repertoire decreased, suggesting that the increase of age may lead to the clonal expansion of some autoimmune T cells. TCR repertoire diversity was significantly different between different age groups and the degree of immune repertoire diversity is not dependent on gender.\textsuperscript{49}

The recombination of V(D)J gene and the addition or deletion of nucleotides in CDR3 region leads to the diversity of the IR. Our study showed that there were differences in V and J gene use in different age groups, and the increase of age had a significant impact on TRA and TRB. We observed that TRAV18, TRAV39, and TRAV25 genes were uniquely expressed in Group 2, the older population, while TRAV38-2DV8, TRAV4, and TRAV6 genes were independently expressed in the Group 1, the younger population. At the same time, we observed significant differences in the expression of TRB V-J gene combinations between the two groups of normal participants. The V-J gene combination that was highly expressed in Group 1 was decreased in Group 2. These results showed that there may be a correlation between the expression of V and J genes and age. In addition, we observed some highly expressed shared CDR3 sequences in TRA, TRG, IGK, and IGL of all participants. T and B cells achieve antigen specificity through V(D)J gene rearrangement to produce more efficient and accurate clonal amplification, which is essential for a successful immune response.\textsuperscript{50} Peng et al. found increased use of some V gene segments and V-J gene segment pairings in PBMC from Avian Influenza Virus Infection (AIV) donors compared to healthy donors, such as TRBV12-3 and TRBV30/TRBJ1-2, which could provide additional information for developing more effective treatment measures.\textsuperscript{50} Lian et al. found that TRB CDR3 amino acid sequences were most highly expressed between 21 and 30 years of age, and our results showed the presence of highly expressed shared TRB CDR3 amino acid sequences between Group 2, which were largely unexpressed in Group 1 (Figure 6B).\textsuperscript{49} We constructed CDR3 sequence models of the two age groups and can use this to compare CDR3 sequence characteristics of patients with corresponding ages, and how differentially expressed CDR3 sequences might be associated with disease. Heather et al. found that public TCR sequences were absent in HIV patients compared to healthy individuals, and that patients undergoing short-term antiretroviral therapy were accompanied by rapid changes in the abundance of many individual TCR sequences, a reduction in the abundance of the most common sequences, and a reduction in most HIV-associated CDR3 sequences.\textsuperscript{51}

Our study shows that the IR of normal Chinese participants of different ages is individual specific. Our results provide us with a better understanding of the characteristics of the immune pool in normal Chinese people and a method which provides a foundation to study age-related diseases and monitor treatments, thus improving future treatments. This study shows the importance of conducting larger studies of repertoire analysis from different ages, populations, and health status, as this can be used as disease markers thus aiding in early diagnosis\textsuperscript{52–55} and personalized treatment.\textsuperscript{56–60}

**AUTHOR CONTRIBUTIONS**

Calling Song performed the sample preparation, data analysis and wrote the manuscript. Wenjing Pan analyzed the data and revised the manuscript for important intellectual content. Houao Chen and Nan Peng collected the data and assisted in the data analysis. Congli Tang and Yunqi Huang assisted in the nucleic acid extraction and library construction. Brittany Brown, Zhe Wang, Daniel Weber, and Miranda Byrne-Steele contributed reagents and analysis tools, Haijing Wu, Hongna Liu, Yan Deng, and Nongyue He provided revisions. Song Li initiated the project research, designed the experimental protocol, revised the manuscript, and provided funding. All authors have read and approved the manuscript for publication.

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**CONFLICT OF INTEREST**

Author Wenjing Pan and Congli Tang are employed by Nanjing ARP Biotechnology Co. Ltd, Nanjing, China. Author Brittany Brown, Daniel Weber, Miranda Byrne-Steele are employed by iRepertoire Inc., Huntsville, USA. The remaining authors declare there is no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The dataset used in this study has been uploaded to the online repository: https://ngdc.cncb.ac.cn/gsa-human/, HRA002445. Further enquiries for all data and figures used in this study can be made to the corresponding authors.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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