Characterisation of T and B cell receptor repertoire in patients with systemic lupus erythematosus

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Abstract

Objective

Systemic lupus erythematosus (SLE) is an autoimmune disease with extreme heterogeneity, marked clinically by multi-systemic inflammatory involvement. However, the molecular mechanism of breakdown of self-tolerance is still unclear. T cell/B cell-mediated immune disorders may play a vital role in the pathogenesis of SLE.

Methods

In this context, we used a combination of multiplex-PCR, Illumina sequencing and IMGT/HighV-QUEST for a standardised analysis of the T cell receptor β-chain (TCRβ) and B cell receptor H-chain (BCR-H) repertoire of peripheral blood mononuclear cells in SLE patients compared with healthy volunteers.

Results

The results showed that there was an obvious reduction in BCR-H repertoire diversity and BCR-H CDR3 length in SLE patients. Notably, the pre-selection BCR-H CDR3s in SLE patients also displayed abnormal shortening, which suggests that early events in bone marrow B cell development and repertoire generation were abnormal in SLE patients. However, there was no obvious change of T cell repertoire in SLE patients, including repertoire diversity and CDR3 length. In addition, there was skewed usage of V genes and CDR3 sequences in SLE patients, which might be the result of physiological responses to environmental antigens or pathogens.

Conclusion

In conclusion, our data revealed the specific changes of the TCR and BCR repertoires in SLE patients, which may provide new ideas for its prevention and treatment.

Key words

systemic lupus erythematosus, T cell receptor, B cell receptor, next generation sequencing

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Received on November 7, 2022; accepted in revised form on March 24, 2023.

Funding: this work was supported by grants from the National Natural Science Foundation of China (82101877), Guangxi Natural Science Foundation (2019GXNSFBA245032), Central Government Guided Local Science and Technology Development Project (Gui Ke AD20238021), China Postdoctoral Science Foundation (2021M691239), Innovation Project of Guangxi Graduate Education (JGY2021140), Guilin Science Research and Technology Development Project (20210218-2, 20190218-5-5)

Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterised by the loss of self-tolerance and formation of antinuclear antibodies and immune complexes resulting in inflammation of multiple organs (1). Although the pathogenesis of SLE is not well understood, several factors are suspected to contribute to the aetiology, including environmental factors, hormones, immunomodulatory factors, and genes associated with disease susceptibility (2). Additionally, many studies have demonstrated that autoimmune diseases are caused by aberrant immune responses. Aside from abnormal B-cell responses that induce autoantibodies, T-cell abnormalities play a vital role in inducing autoimmunity and ensuing organ damage. B and T cells respond to antigens through their receptors (TCR/BCR) on their surface, which are specific to antigens. The hypervariable CDR3 of the receptor β chain/H chain determines the antigen specificity of each TCR/BCR, which is generated by somatic recombination of the variable (V), diversity (D), and joining (J) gene segments and the deletion and insertion of nucleotides at the V(D)J junctions (2). Recombination events at TCR/BCR loci can produce non-functional (out-of-frame) TCRs/BCRs with frameshifts or stop codons. T cells in this case try to arrange the second allele, and if successful (in-frame) TCR formation occurs, there is a functional TCR gene as well as a non-functional TCR gene on the T cell. Although the non-functional TCR genes do not translate into functional TCRβ chains, the corresponding RNA is still present in the T cell at some concentration (4-6). Since non-functional TCRs/BCRs are not subject to functional selection (positive or negative selection), they can be representative of the pre-selection TCR repertoire (7, 8). Additionally, functional TCRs can be used to investigate post-selection TCR repertoires.

To probe human TCR diversity, several strategies have been developed over the past two decades, including FACS, PCR, and Sanger sequencing technique (9). Unfortunately, this approach has a keyhole perspective that has important drawbacks, such as they are low input, and do not give a full-repertoire perspective. Nowadays, with the advent of next generation sequencing (NGS) technologies, as a routine experiment, millions of receptor clones representing the entire immune repertoire can now be sequenced (10). NGS has provided us with a large amount of data for analysing the TCR and BCR. The aim of this study was to determine whether the SLE disease affected TCR and BCR repertoires in specific ways, which may be helpful to clarify the mechanisms underpinning autoantigen responses with this information. This study can help us search for potential autoreactive clones and autoantigens, which might help in offering important new information for the monitoring and classification of SLE diseases, and developing strategies to induce tolerance selectively against autoantigens (1). Additionally, these findings may lead to the development of new diagnostic, therapeutic, or preventive strategies.

Materials and methods

Patients and controls

A total of 10 healthy individuals and 10 patients with SLE were recruited; collecting the peripheral blood was collected from the subjects, and the peripheral blood mononuclear cells (PBMCs) were separated (1). The diagnostic criteria of SLE were according to the 1982 revised criteria. All of the 10 SLE patients were female, with a mean age of 34.12±11.33 years, ranging from 20 to 54 years. Ten healthy subjects matched for age, sex and ethnicity served as controls. This study was conducted in accordance with the tenets of the Helsinki Declaration on ethical principles for medical research involving human subjects. All the participating individuals provided their written informed consent.

DNA extraction, library construction and high-throughput sequencing

In accordance with the manufacturer's instructions, DNA was extracted from PBMCs using GenFIND DNA (Agencourt, Beckman Coulter, Brea, CA,

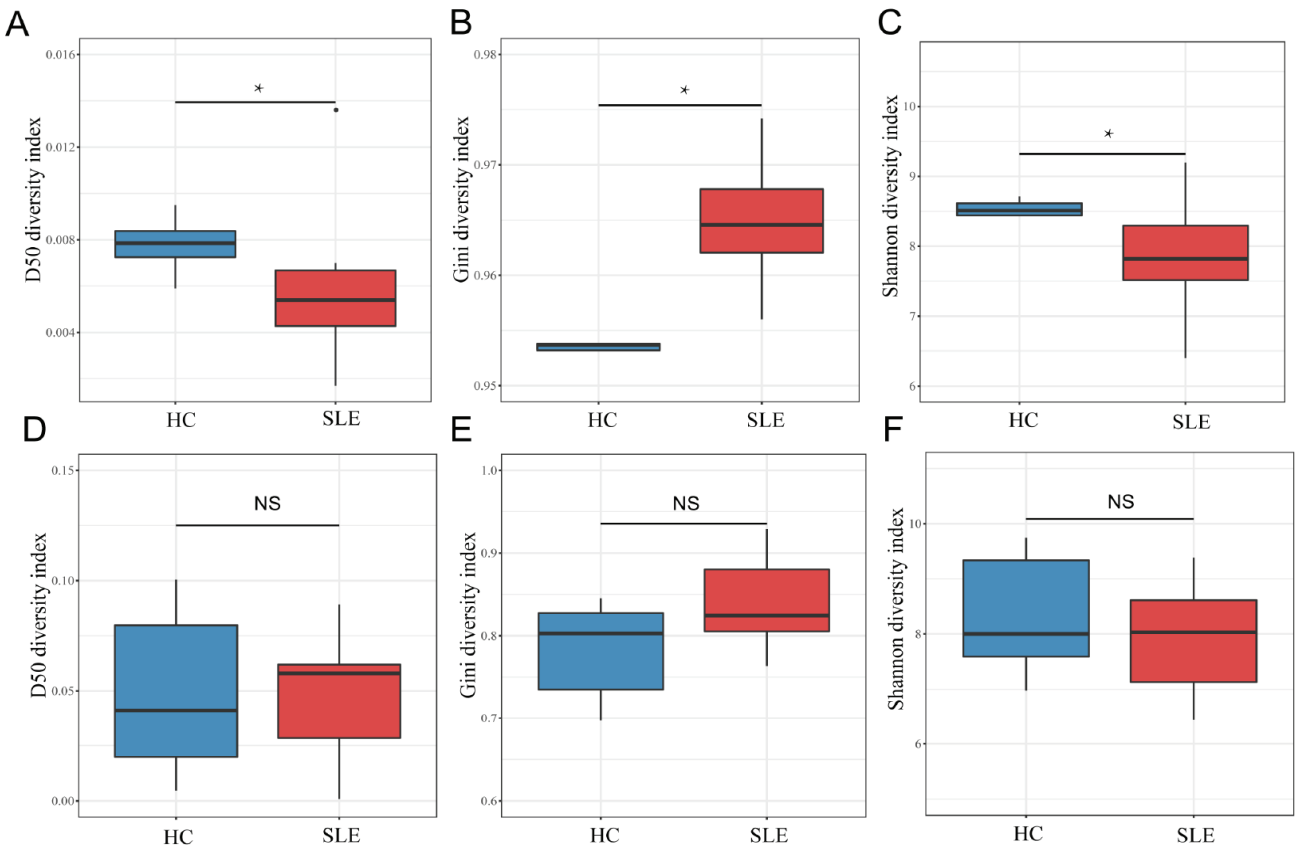

Fig. 1. Comparative of the diversity of BCR-H/TCR-β repertoires in patients with SLE and healthy controls.
A-**C**: The diversity of the BCR-H repertoire measured by D50 index (**A**), Gini index (**B**), and Shannon index (**C**). The diversity of the TCR-β repertoire measured by D50 index (**D**), Gini index (**E**), and Shannon index (**F**). Data were compared using unpaired *t*-test, and Bonferroni-Holm correction for multiple comparisons. SLE: systemic lupus erythematosus; HC: healthy control; *p<0.05.

USA) extraction kits. Subsequently, a relative conserved region upstream of CDR3 (in frame region 3) was selected as a potential forward primer region. Similarly, reverse primers were designed corresponding to the J gene family. Supplementary Tables S1-S2 show the primer sequences for TCR-β CDR3 and BCR-H CDR3 region. The following reaction conditions were used: 1× Qiagen Multiplex PCR Master Mix, 0.2 μm Vβ F pool, 0.2 μm Jβ R pool, and 0.5× Q solution. Cycle conditions were as follows: 95 °C for 15 min, followed by 30 cycles at 94°C for 30s, 60°C for 90s, 72 °C for 30s, plus a final extension of 72°C for 5 min (11). After that, to purify the PCR products, a QIA quick PCR Purification Kit was used. The Agilent 2100 Bioanalyzer was then used to quantify the final library. cBot was used to amplify libraries to generate clusters on the flow cell, HiSeq2000 was used to pair-end sequence the amplified flow cell, with a read length of 100 bp generally (11).

Data processing and bioinformatic analyses

We filtered the raw data, including adapter contamination, after receiving the data from the sequencer. The reads with an average quality score below 15 (Illumina 0-41 quality system) will be removed. If the proportion of N bases exceeds 5%, it was been removed. We trimmed the last few bases with low quality (less than 10). After trimming, the quality score should be over 15, and the remaining sequence length should be greater than 60 nt. Pair-end (PE) read pairs were merged into one contig sequence after filtering. In the merging process, there were two steps: 1, aligning the tail parts of both sequences, analysing their identity. It was required that 10 bases overlap and that 90% of the bases in the overlapped section match. 2, as different primers might isolate different length sequences, In some cases, they might be very short (less than 100bp) and might cover all bases in the sequence (11). By aligning the sequence head, such reads were merged. Subsequently, we merged contig sequences in fasta and fastq formats, along with a length distribution plot. Nucleotide sequences of the CDR3 regions that ranged from conserved cysteine at position 104 (Cys104) of IMGT nomenclature to conserved phenylalanine or tryptophan at position 118 (Phe118 or Trp118) were translated to deduced amino acid sequences. For B cell receptor: Contigs were aligned with IMGT database for V, D, and J reference using BLASTn software. Based on the blast alignment, we selected the best match. For T cell receptor: alignment was performed using miTCR, developed by milaboratory: http://mitcr.milaboratory.com/downloads/. The programme had an automated mechanism for adjusting errors introduced by sequencing, PCR, etc. The alignment statistics information, such as CDR3 expression and INDEL were provided (11). The expression frequency of each distinct DNA sequence, V-J combination, and amino

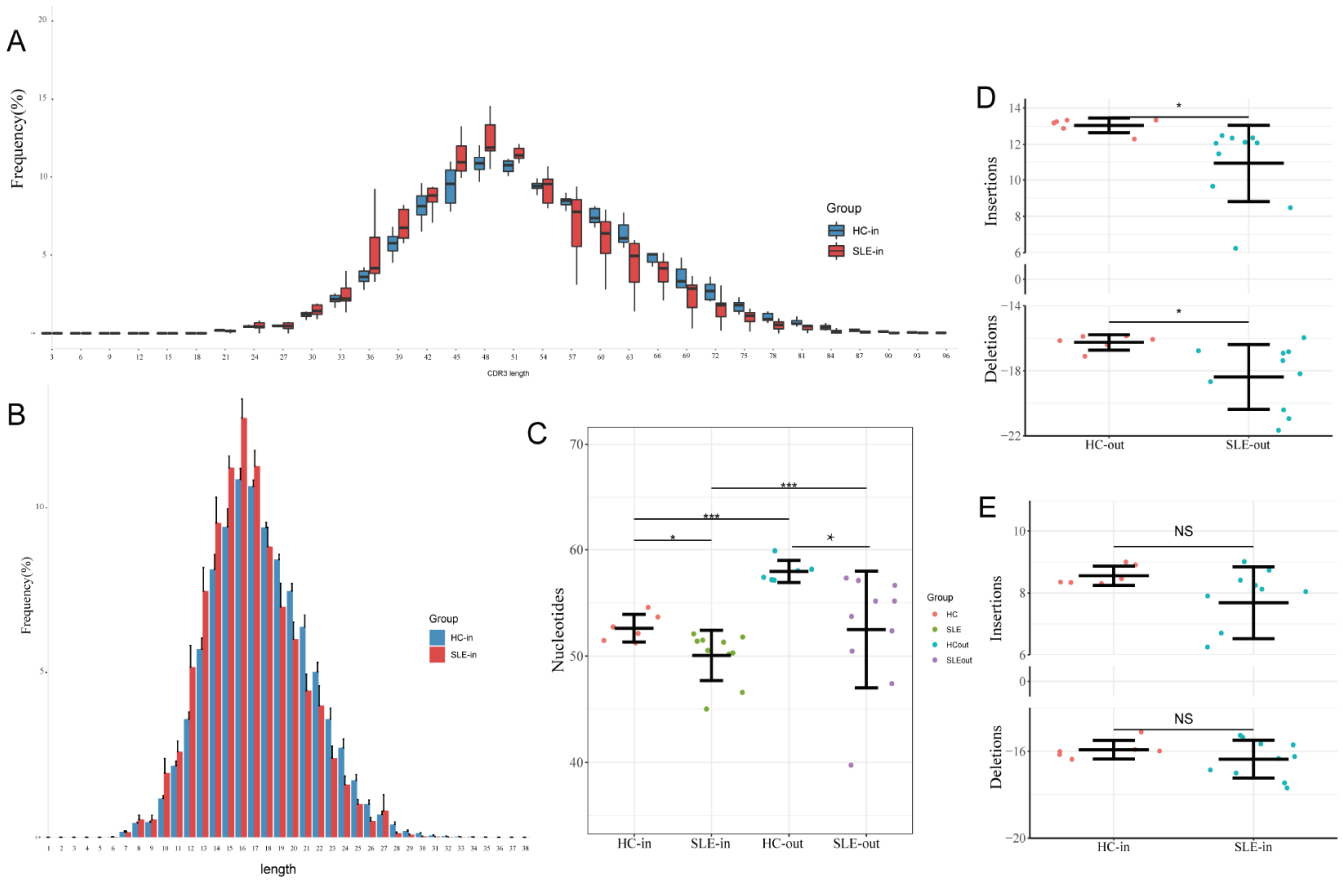

Fig. 2. Shorter BCR-H CDR3s with abnormal length of insertion and deletion in SLE patients. **A**-**B**: Comparison of BCR-H CDR3 nucleotide length (**A**) and amino acid sequence length (**B**) between SLE patients and healthy controls. BCR-H CDR3s were shorter in SLE patients, represented by higher frequencies of short TCRβ CDR3s (and lower frequencies of long ones). **C**: A significant reduction in average BCR-H CDR3 nucleotide length was observed in SLE patients *vs*. healthy controls, no matter in pre-selection repertoires (out, out of frame) or post-selection repertoires (in, in frame).
D-**E**: Comparison of the mean length of the nucleotide insertions or deletions of the Pre-selection BCR-H repertoire (**D**) and post-selection BCR-H repertoires (**E**) between SLE patients and healthy controls. Data were presented as the mean ± SD values and compared using an unpaired *t*-test.
$*p<0.05$, $**p<0.01$, $***p<0.001$ (two-tailed).

acid sequence was determined following alignment. Based on previous publications, the TCR/BCR repertoire diversity was assessed using inverse Simpson index, D50 index, Simpson index, and Shannon index (12, 13).

Statistical analysis

Unpaired *t*-tests were used to analyse the data. *p*-values under 0.05 were considered statistically significant. In addition, Holm-Bonferroni's adjustment for multiple comparisons was performed when calculating all diversity and sharing indexes to avoid the significant difference between groups caused by false discovery rate (FDR) in statistics.

Results

Decreased of IgH repertoire diversity in SLE patients

We investigated the TCRβ and BCR-H diversity of SLE patients and healthy controls using several evaluation methods, which including D50 index, Gini index, and Shannon diversity index. For Gini index, the smaller the index value, the greater the CDR3 diversity. For D50 index and Shannon index, the greater the index value, the greater the CDR3 diversity. As shown in Figure 1, the BCR-H diversity was significantly decreased in SLE patients (Fig. 1A-C), however, there was no significant difference in TCRβ diversity between SLE patients and healthy controls (Fig. 1D-F).

CDR3s display abnormal shortening in IgH repertoire of SLE patients

Different rearrangements may lead to variable CDR3 lengths, and the distribution in the CDR3 sequence lengths is another feature that provides an overall view of the repertoire composition (14). We found that the BCR-H CDR3 length distribution was significantly shorter in SLE patients than that in healthy controls, no matter in nucleotide level or amino acid level (Fig. 2A-B). It was worth noting that the length of the BCR-H CDR3 sequences displayed abnormal shortening in SLE patients, no matter in Pre-selection or Post-selection repertoires (Fig. 2C), which suggested that the early BCR-H repertoire was abnormal in SLE patients. To understand the molecular basis of these features, we analysed the recombination events (nucleotides inserted or deleted), since a higher frequency of a short BCR-H CDR3s length in SLE may arise from decreased nucleotide insertions or increased nucleotide deletions during the BCR-H rearrangement process. Our results also showed

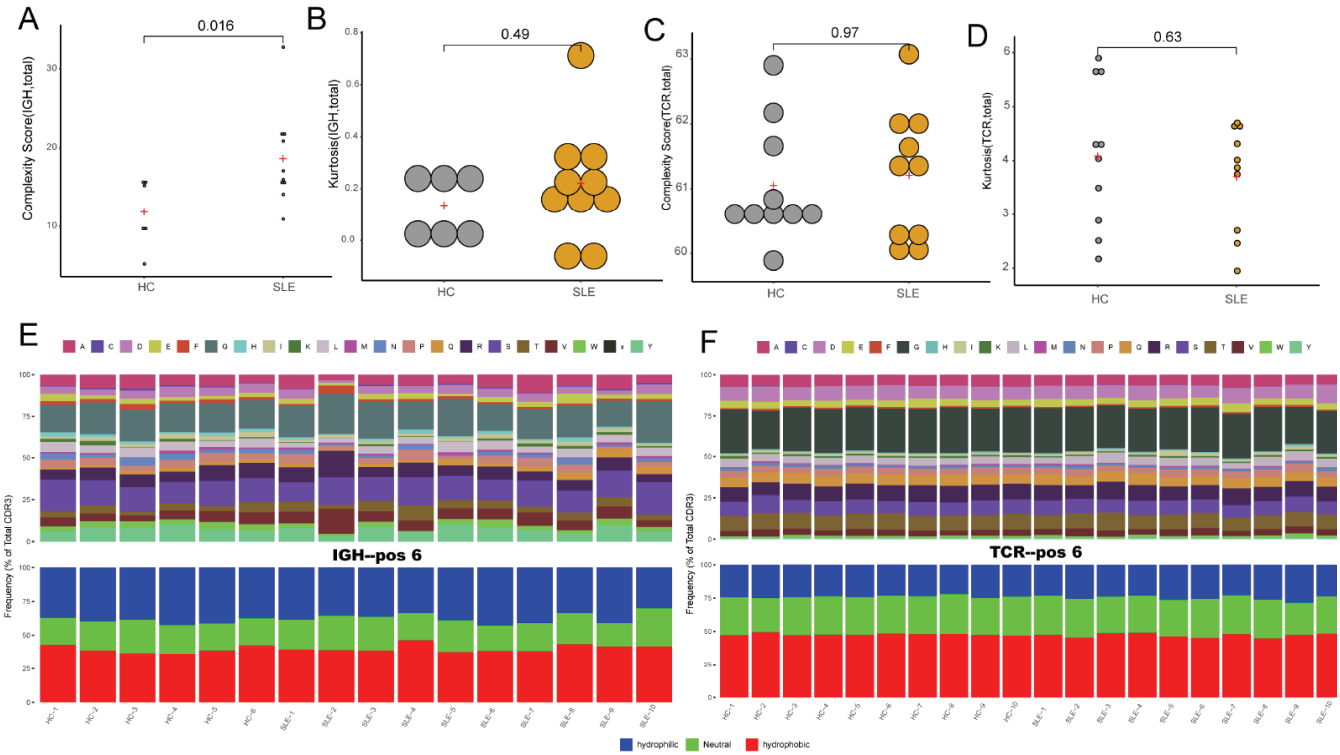

Fig. 3. Analysis of CDR3 length and amino acid composition in SLE patients. Complexity scores, kurtosis of the BCR-H (**A**-**B**) and TCR-β (**C**-**D**) CDR3 total sequences in patients with SLE and healthy controls. Amino acid composition of CDR3 in SLE patients and healthy controls for amino acid positions 6 of the 13-amino acid-long BCR-H CDR3s (**E**) and TCR-β CDR3s (**F**). Data were presented as the mean±SD values and compared using an unpaired *t*-test.

that the mean length of the nucleotide insertions or deletions was significantly decreased in the Pre-selection BCR-H repertoire of SLE patients compared with healthy controls (Fig. 2D), however, there was no significant difference in Post-selection BCR-H repertoires (Fig. 2E). Moreover, we found that the TCRβ CDR3 length distribution was no significantly difference in SLE patients than that in healthy controls, no matter in nucleotide level or amino acid level (Suppl. Fig. S1).

The CDR3 complexity score and its kurtosis were calculated in order to better identify abnormalities of the CDR3 length distribution (15). Particularly, the complexity score is calculated based on the number of major peaks of CDR3 length (defined as those with amplitudes of at least 10% of the sum of all peak heights) and their height contribution to the sum of all peak heights. As a measure of peakiness, kurtosis determines the number of events in the central part of the CDR3 distribution as opposed to the tails (16). Our experimental results showed that there were significant differences on the complexity score of the CDR3 length profiles of BCR-H repertoire between the total sequences from SLE patients and that from healthy controls (Fig. 3A). Whereas the CDR3 length profiles of TCRβ repertoire was no significant differences between SLE patients and healthy controls (Fig. 3C). In addition, no significant differences were observed for the kurtosis, no matter in BCR-H repertoire (Fig. 3B) and TCRβ repertoire (Fig. 3D).

The amino acid composition in BCR-H/TCRβ repertoire of SLE patients

Recent data indicate that the 13-amino acid-long CDR3 containing hydrophobic amino acids at positions 6 and 7 has been found to promote T-cell self-reactivity (16, 17). We also conducted a similar analysis, however, we found that the amino acid composition at positions 6 (Fig. 3E-F) and positions 7 (Suppl. Fig. S2) was very conserved in SLE patients and healthy controls, and there was no significant differences between SLE patients and healthy controls, no matter in TCRβ repertoire or BCR-H repertoire.

Skewed usage of V genes and CDR3 sequences in SLE patients

To determine whether pathogenic factor of SLE alter targeting of individual V genes, we compared the usage of V genes in total BCR-H and TCRβ sequences from healthy controls and SLE patients. The usage frequency of IGHV3-49, IGHV2-70, IGHV2-5 gene were significant differences in SLE patients compared with healthy controls (Suppl. Fig. S3A). For the TCRβ repertoire, the usage frequency of many TRBV genes (such as TRBV6-5, TRBV11-3, TRBV6-3, TRBV6-1, TRBV27, TRBV28, etc.) showed significant differences between SLE patients and healthy controls (Fig. 4A). Notably, PCA of individual IGHV and TRBV gene usage clearly segregated SLE patients from controls (Suppl. Fig. S3B, Fig. 4B). In addition, we compared the abundance of BCR-H CDR3 sequences (Suppl. Fig. S3C) and TCRβ CDR3 sequences (Fig. 4C) between SLE patients and healthy controls, and identified some CDR3 sequences emerged clonal expansion in SLE patients.

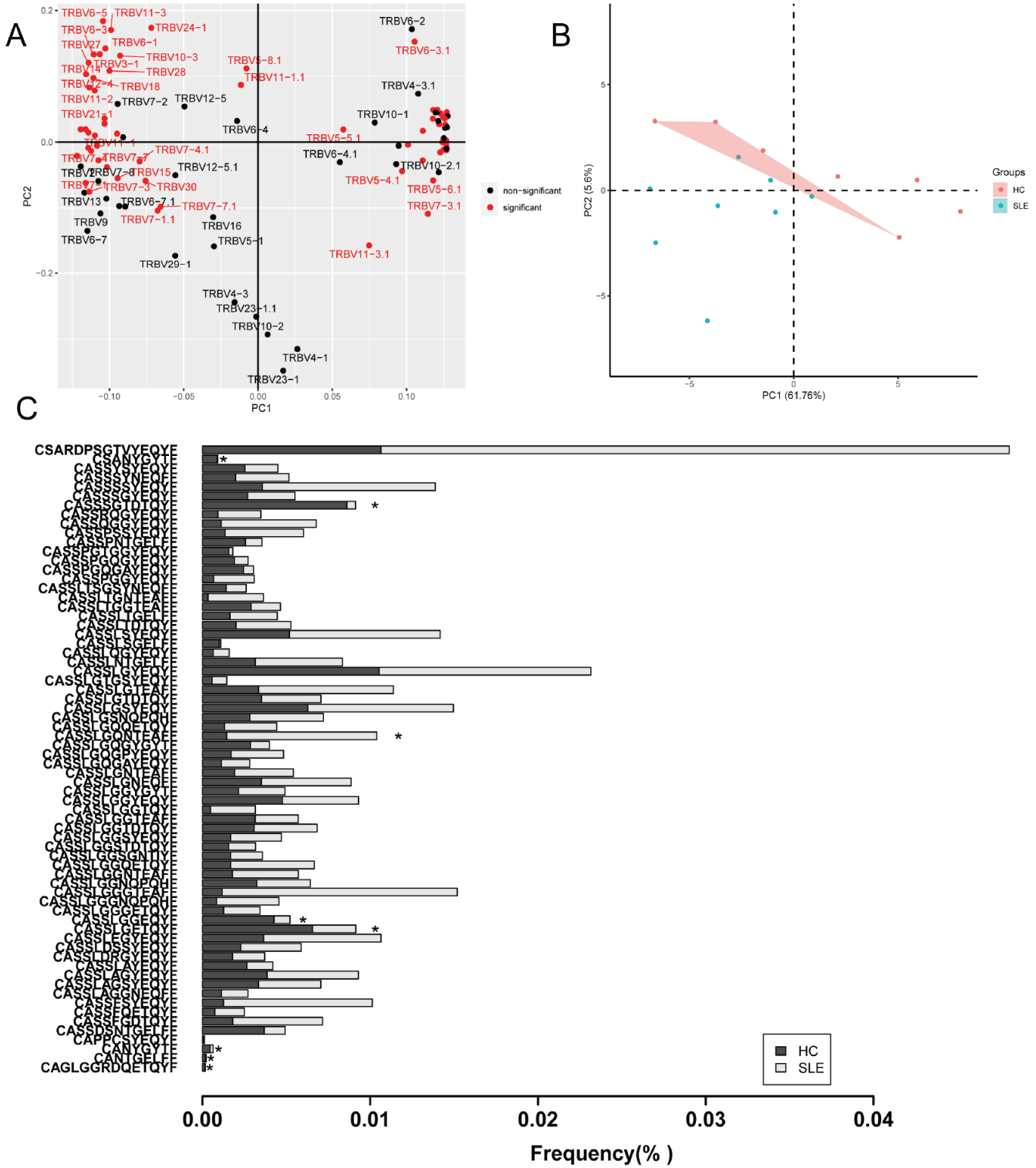

Fig. 4. Differential usage of TRBV gene and abnormal abundance of TCR-β CDR3 amino acid sequences in SLE patients.
A-**B**: Differential usage of TRBV genes, segregating normal controls and SLE patients and the various genes according to PC1 and PC2, was shown as variable plots (**A**) and sample plots (**B**).
C: Comparing of the abundance of TCR-β CDR3 sequences between SLE patients and healthy controls.

Discussion

SLE is a systemic autoimmune disease characterised by aberrant activity of the immune system, leading to variable clinical symptoms (18). The repertoire features of immune cells, including T cells and B cells, play an important role in the pathogenesis of SLE (19, 20). In the present study, we used next-generation sequencing to comprehensively characterise the BCR-H repertoire and TCRβ repertoire of peripheral B cell and T cells in SLE patients and healthy volunteers. We found that SLE patients showed an obvious reduction in

BCR-H repertoire diversity and BCR-H CDR3 length. It was noteworthy that the pre-selection BCR-H CDR3s in SLE patients also display abnormal shortening. Thus, early events in bone marrow B cell development and repertoire generation are abnormal in SLE patients. Relevant to this investigation, the results from Zhang *et al*. (2020) had also drawn similar conclusions. They applied immune repertoire sequencing technology for parallel analysis of the CDR3s of B cell receptors in patients with immunoglobulin A nephropathy (IgAN) and healthy individuals. In their experimental results, a significant decrease in CDR3 length was also observed in the IgAN group (21). We further analysed and found that the mean length of the nucleotide insertions or deletions was significantly decreased in the Pre-selection BCR-H repertoire of SLE patients, which might be due to the reduced activity of BCR-H rearrangement related enzymes. It is widely known that recombination activation gene 1 (RAG1) and RAG2 are crucial for V(D)J recombination. V(D)J recombination is initiated by the RAG1 and RAG2 proteins, which comprise an endonuclease that recognises specific recombination signal sequences (RSSs) adjacent to each V, D, and J segment (22). The RAG1/2 endonuclease induces DNA double-strand breaks (DSBs) between the RSSs and coding segments. Joining of the RAG1/2-generated DSBs requires the terminal deoxynucleotidyl transferase (TdT) and the ubiquitously expressed non-homologous endjoining (NHEJ) DNA repair factors (23). Therefore, we speculate that due to some unknown factors, the activity of RAG and Tdt enzyme decrease. Our study demonstrated the length of the nucleotide insertions or deletions was decreased in the Pre-selection BCR-H repertoire of SLE patients. Thus, the abnormal shortening CDR3 sequences might be not result from the decreased insertions and deletions. Our previous research has found that the mean length of the CDR3 nucleotide sequences is positive correlation with the length of the original germline sequences, and don't significantly correlate with the mean length of the inserted nucleotides in both the pre-selection or post-selection repertoires (14).

As for the repertoire diversity and CDR3 length, there was no significant difference in TCRβ repertoire between SLE patients and healthy controls. As for the complexity score of the CDR3 length profiles, there was significant difference in BCR-H repertoire, and no significant difference in TCRβ repertoire of SLE patients. In addition, the amino acid composition at positions 6 and 7 was very conserved in SLE patients and healthy controls, which is inconsistent with the results of previous studies (16, 17), and its reason waits for further studying. Moreover, the skewed usage of V genes and CDR3 sequences in SLE patients may be the result of physiological responses to environmental antigens or pathogens, which may be used as highly specific or sensitive disease markers. This was confirmed in another study, also describing there is a skewed and restricted VDJ segment usage in autoimmune disease, such as rheumatoid arthritis (24), primary biliary cholangitis (12), and primary Sjögren's syndrome (25).

In conclusion, we provide a comprehensive immune profile of peripheral T cells and B cells in patients with SLE. The main limitation of our study is the current sample size, hence the size of our sample is small, and this could lead to sample bias, thus these findings need to be confirmed by other studies including larger cohorts. Moreover, future work should focus on the molecular mechanisms in order to eventually develop a strategy for TCR/BCR-specific immunotherapy of SLE.

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