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Original Article

Basic science

Landscape of immune cells in systematic lupus erythematosus patients with Epstein–Barr virus infection: assessed by single-cell sequencing

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Abstract

Objectives: To analyse the immune cell and B-cell receptor (BCR) profiles of patients with SLE, with or without EBV infection, and identify the differences between them.

Methods: We included two patients with SLE and positive detection of EBV infections (SLE-EBV+), four with SLE with negative detection of EBV infections (SLE-EBV–) and two healthy controls. Single-cell RNA sequencing was used to investigate the heterogeneity of cell populations by combining the transcriptomic profiles and BCR repertoires.

Results: A total of 83 478 cells were obtained and divided into 31 subtypes. The proportion of CD8+ proliferation T cells was higher in the SLE-EBV+ group than in the SLE-EBV– group. The IFN-α/β pathways were upregulated in most T cells, monocytes and B cells in the SLE-EBV+ group, compared with the SLE-EBV– group. Moreover, T-cell trajectory indicated CD4+ Tregs may play crucial roles in SLE combined with EBV infection. In the BCR heavy chain, the *IGHV3* and *IGHV4* gene families were frequently present in all groups. Additionally, IgM was the largest component of five Ig isotypes, but its proportion was significantly decreased in the SLE-EBV+ group.

Conclusion: This study provides a comprehensive characterization of the immune cell profiles and BCR repertoires of patients with SLE, both with and without concurrent EBV infections, contributing to a better understanding of the mechanism underlying the immune response to EBV infection in patients with SLE.

Keywords: systemic lupus erythematosus and autoimmunity, viruses, lymphocytes, B cells, T cells.

Rheumatology key messages

- Landscape of immune cells in patients with SLE and EBV infection.
- CD4+ Tregs may play crucial roles in SLE combined with EBV infection.
- Changes in B-cell receptor repertoire between SLE-EBV+, SLE-EBV– and healthy controls.

Introduction

SLE is a disease that involves multiple systems and vital organs such as the brain, blood and kidney in most patients. Although significant advancements have been made in the treatment of SLE in recent years, increased organ damage is associated with a poorer prognosis in some patients. Various factors, including genetic, epigenetic, environmental, hormonal, immune regulation and others, contribute to the loss of self-tolerance in patients with SLE [1, 2]. Additionally, infections, particularly viral infections, have been linked to both the onset and exacerbation of SLE [3].

Among these pathogens, EBV is of particular interest. EBV is frequently found in humans, and a large percentage of the population carries antibodies against this virus. It is found even more frequently in patients with SLE than in healthy individuals and is considered to be the key agent that triggers SLE [4, 5]. EBV is a potent activator of autoreactive B cells, and serves as a superantigen that stimulates T cells [6]. A previous study of ours showed that EBV B-cell epitopes can trigger SLE, and their indirect levels may serve as potential biomarkers for SLE diagnosis and disease severity [7].

However, while previous single-cell RNA sequencing (scRNA-seq) studies have provided insights into immune cell diversity in SLE, none has specifically focused on patients with SLE and comorbid EBV infection [8–10]. Nowadays, scRNA-seq has emerged as a powerful tool for investigating

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the heterogeneity of complex cell populations, by combining information on such elements as transcriptomic profile, and the repertoires of both T-cell receptors and B-cell receptors (BCRs) [11]. Therefore, the aim of this study was to conduct scRNA-seq analysis in patients with SLE and comorbid EBV infection to elucidate the immune cell landscape.

Our study represents the first exploration of immune cell diversity in patients with SLE and comorbid EBV infection. It has unveiled distinct cell populations, subgroups and pathways, providing valuable insights for future investigations into the mechanisms of SLE in the presence of EBV infection. Furthermore, these findings hold the potential to enhance the efficacy of personalized therapeutic approaches for improved treatment outcomes.

Methods

Study design

Blood samples were obtained from patients fulfilling the diagnosis of SLE according to the criteria established by the ACR. Patients with SLE and comorbid EBV infection were diagnosed according to a positive result in EBV-VCA-IGM ELISA (Fine Biotech, Wuhan, P.R. China). Healthy controls were visiting the clinic either for reasons not related to autoimmunity or for surgery not associated with any inflammatory diseases. The clinical data for the six patients are shown in Supplementary Table S1, available at *Rheumatology* online. In accordance with previous studies, the dilution used for ANA screenings in our laboratory was 1:100 [12, 13]. The Local Ethics Committee of the first affiliated hospital of Wenzhou Medical University reviewed the study protocol and approved the study. All participants gave written informed consent. This study complies with the Declaration of Helsinki.

Blood preparation for scRNA-seq

The freezing medium was 10% DMSO + 90% FBS. Peripheral blood mononuclear cells (PBMCs) were thawed quickly at 37°C and resuspended in DMEM supplemented with 10% FBS. Cells exhibiting a viability rate <70% were excluded. Cells were centrifuged at 400 g, for 10 min. Cells were washed once with 1× PBS supplemented with 0.04% BSA and finally resuspended in 1× PBS with 0.04% BSA. Viability was determined using trypan blue staining and measured on a Countess FLII. Single-cell RNA sequencing and subsequent analysis in detail are shown in the Supplementary Data S1, available at *Rheumatology* online.

Results

Single-cell transcriptional profiling of peripheral immune cells in patients with SLE and EBV infection

We performed scRNA-seq to study the transcriptomic profiles of PBMCs from six patients with SLE and two healthy controls (HCs; Fig.1A). The six patients with SLE were classified into two groups: EBV-IgM-positive (SLE-EBV+, $n=2$) and EBV-IgM-negative (SLE-EBV–, $n=4$). The clinical characteristics, laboratory findings, and medications used by the patients are presented in Supplementary Table S1, available at *Rheumatology* online. The SLEDAI showed no significant difference between the two patient groups. After filtering, 83 478 cells were included in our subsequent analysis, and the median gene number was 1678 per cell. Of the total cells, 17 308 (20.7%) were SLE-EBV+, 34 034 (40.8%) were SLE-EBV– and 32 136 (38.5%) were HCs.

We captured the transcriptomes of eight major cell types or subtypes, according to the expression of canonical gene markers. These cells were divided into B cells, CD4+ T cells, CD8+ T cells, other T cells, NK cells, monocytes, dendritic cells and other cells (Fig. 1B). Clusters were differentially represented in the individuals (Fig. 1C); however, there was no significant difference in the proportion of these cells between the two patient groups (Fig. 1D).

Differences in cell compositions between patients with SLE, with or without EBV infection

The cells were further divided into 31 subtypes based on their expressions of gene markers (Fig. 2A and B). This analysis identified single clusters of mucosal-associated invariant T cells, Tregs, double-negative T (dnT) cells, γδ T cells, innate lymphoid cells, doublets, platelets, hematopoietic stem progenitor cells and erythroid cells. In addition, four dendritic cells, three NK cell clusters, four B-cell clusters, monocytes (CD14+ and CD16+), five CD4+ T-cell clusters and four CD8+ T-cell clusters were identified (Fig. 2B).

To reveal the differences in cell compositions across patients with SLE and comorbid EBV infections, we calculated the relative percentages of the 31 major cell types in each group (Fig. 2C) and found that the proportions of CD8+ naïve T cells and CD8+ proliferation T cells were higher in SLE-EBV+. In addition, the percentages of many other cell clusters varied considerably with EBV infection; however, there was no statistically significant difference between the two groups (Fig. 2D). Taken together, these results show that immune cells, particularly T cells, account for a higher proportion of cells in patients with EBV infection.

Previous studies on SLE have indicated a primary role of type I IFN (IFN-I) signalling in SLE immunological pathogenesis [14]. Therefore, we determined the IFN-I scores for all the cell clusters and found that the IFN-I score increased significantly in most, including NK, CD8+ effector memory T cell (TEM), B memory, B naïve, dnT, CD4+ cytotoxic T lymphocyte (CTL), CD4+ TEM, Treg, CD8+ naïve, CD8+ central memory T cell (TCM), CD4+ proliferation and CD4+ naïve cell clusters (Fig. 2E and F).

Changes in T-cell trajectories in patients with SLE and EBV infection

T cell is an important cell subtype in SLE. To further explore the characteristic changes in T cells that occur in patients with SLE and EBV infection, we sub-clustered T cells from PBMCs and obtained 17 subsets, according to the expression levels of certain T-cell markers (Fig. 3A and B). This analysis identified single clusters of γδ T cells, innate lymphoid cells, mucosal-associated invariant T (MAIT) and proliferation T cell, as well as five CD4+ and CD8+ T-cell clusters, and three NK-cell clusters. Of the 17 T cell subtypes, we defined the proliferation T subtype according to the state of the G2M or S period.

As T cells are known for their capacity to transition between different functional and differentiation states, we used pseudotemporal inference to understand the relationship between the cell states we identified (Fig. 3A). The CD4+ Treg cells seemed to be in a separate state in the SLE-EBV– group, whereas in the SLE-EBV+ group they occurred along the entire CD4+ T-cell

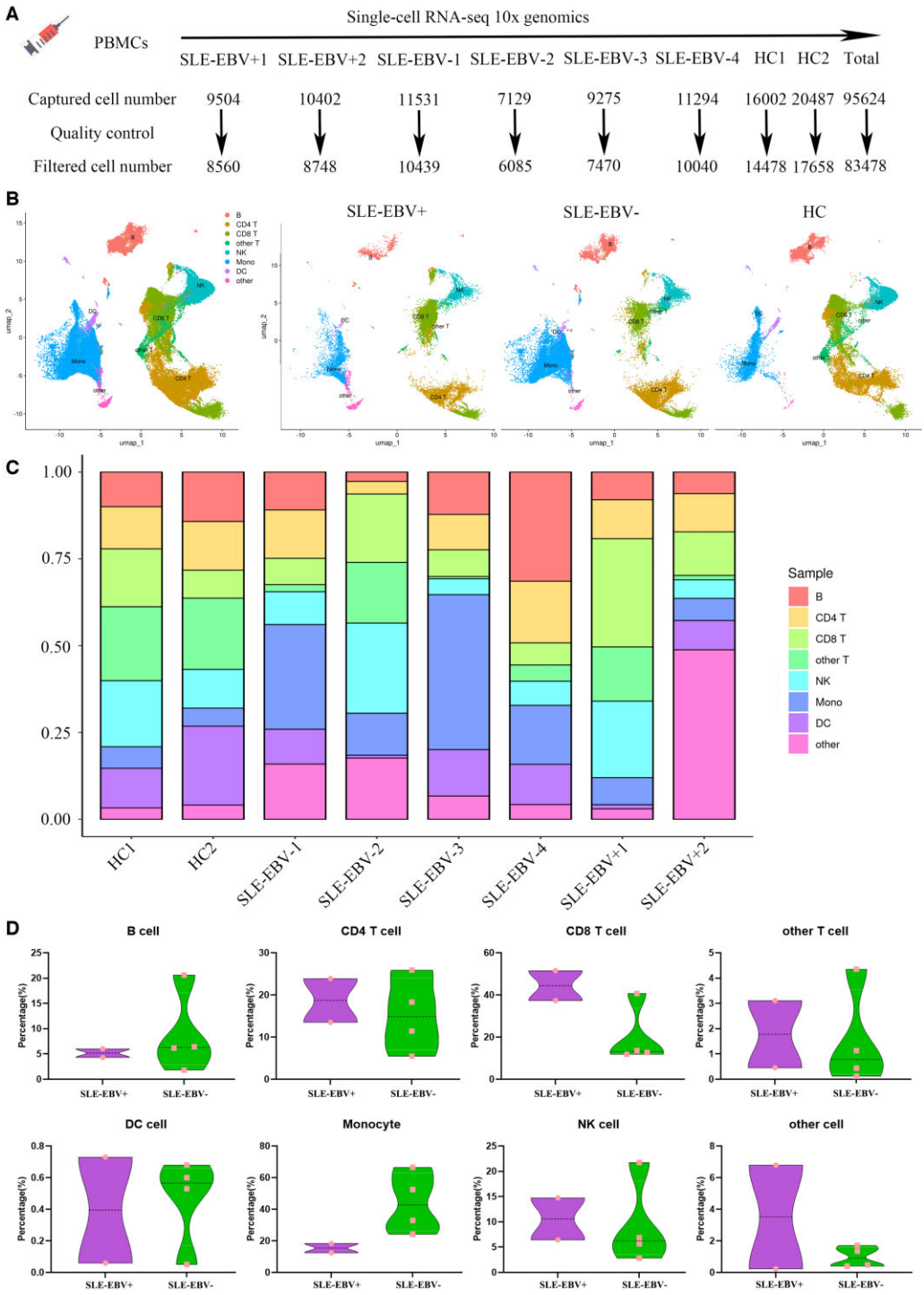

Figure 1. Study design and single-cell transcriptional profiling of PBMCs from HCs and patients with SLE, with or without concurrent EBV infection. (**A**) Study design schematic. (**B**) Cellular populations identified from two SLE-EBV+, four SLE-EBV– and two HCs, forming eight clusters with their respective labels. Each dot corresponds to a single cell, coloured according to cell type. (**C**) Bar plot representing cell abundance in each cluster ($n = 8$) across individuals. (**D**) Violin plots comparing the proportion of each cluster between SLE-EBV+ ($n = 2$) and SLE-EBV– ($n = 4$). SLE-EBV+ are shown in purple and SLE-EBV– in green, with each dot representing one sample. *P*-values were calculated using a Wilcoxon test. PBMC: peripheral blood mononuclear cell; SLE-EBV+: patients with SLE and positive detection of EBV infection; SLE-EBV–: patients with SLE and negative detection of EBV infection; HC: healthy control

trajectory. Additionally, this trajectory suggested that NK-cells states were completely different between the two groups. The differentiation hierarchy of the three NK subgroups in the SLE-EBV+ group was in the following sequence: natural killer T cells (NKT)–NK-cyto–NK-rest. Conversely, in the SLE-EBV– group, the differentiation hierarchy was as follows: NKT–NK-rest–NK-cyto. Subsequently, we analysed the checkpoints of NK cells and found that KIR3DL1, KIR2DL1 and KIR3DL2 were significantly decreased in NK T cells in the SLE-EBV+ group (Supplementary Fig. S1, available at *Rheumatology* online).

We also calculated the relative percentages of the 17 cell types in each individual and group (Fig. 3C and D), and found

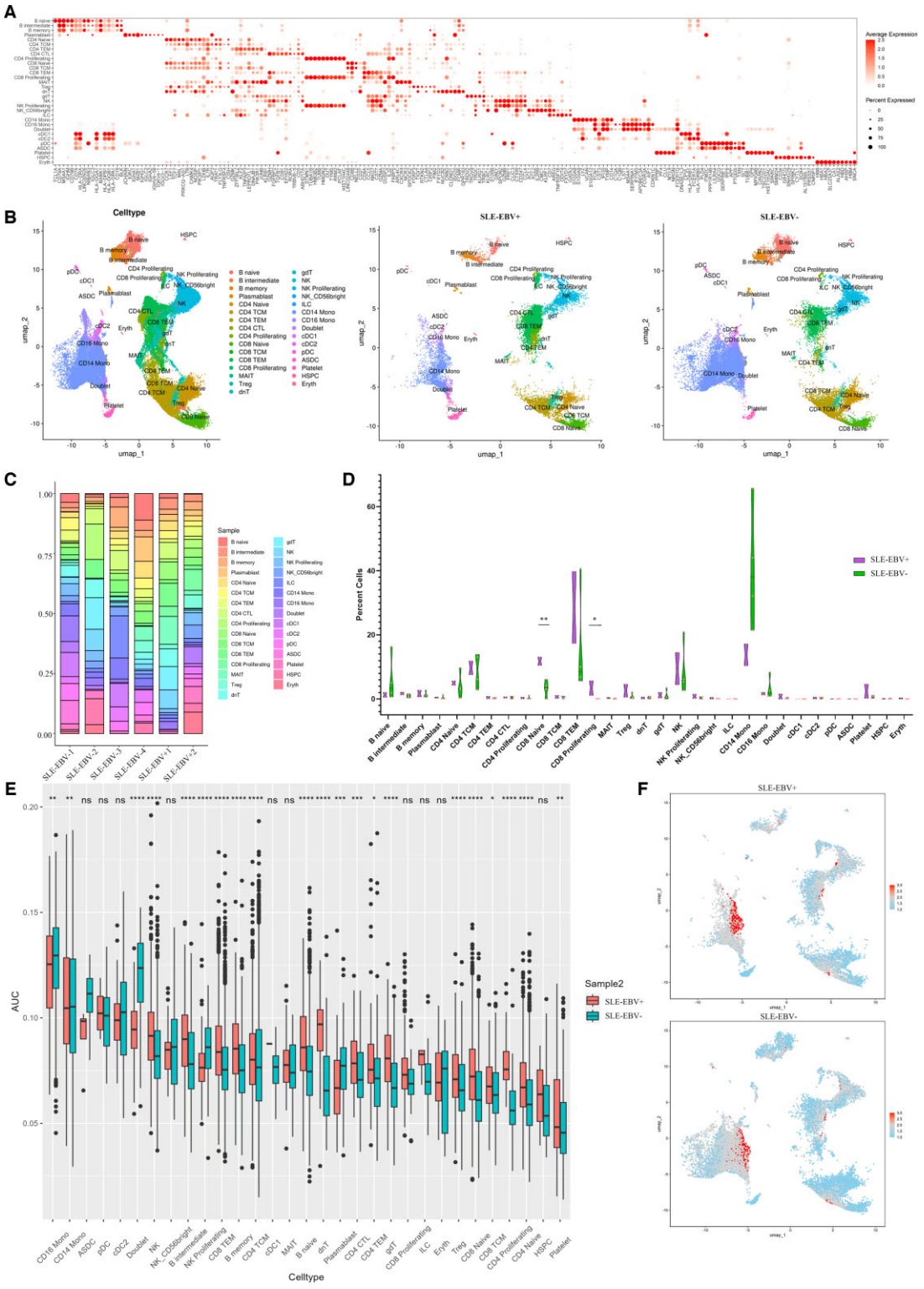

Figure 2. Sub-clustering analysis of PBMCs from patients with SLE, with or without EBV. (**A**) Dot plot representing the expression values of selected genes (*x*-axis) across each cluster (*y*-axis). Dot size represents the percentage of cells expressing the marker of interest. Colour intensity indicates mean expression within expressing cells. (**B**) U-MAP plot with their respective labels from two SLE-EBV+ and four SLE-EBV– samples. Each dot corresponds to a single cell. (**C**) Bar plot representing cell abundances in each cluster ($n = 31$) across individuals. (**D**) Violin plot comparing the proportion of each cluster ($n = 31$) across the groups. SLE-EBV+ are shown in purple and SLE-EBV– in green. *P*-values were calculated using a Wilcoxon test. (**E**) Bar plot representing IFN-I signalling score in the cell clusters. (**F**) U-MAP plot representing IFN-I signalling scores in each cluster. Colour intensity indicates the mean expression within the expressing cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$: $****P < 0.0001$. PBMC: peripheral blood mononuclear cell; SLE-EBV+: patients with SLE and positive detection of EBV infection; SLE-EBV–: patients with SLE and negative detection of EBV infection

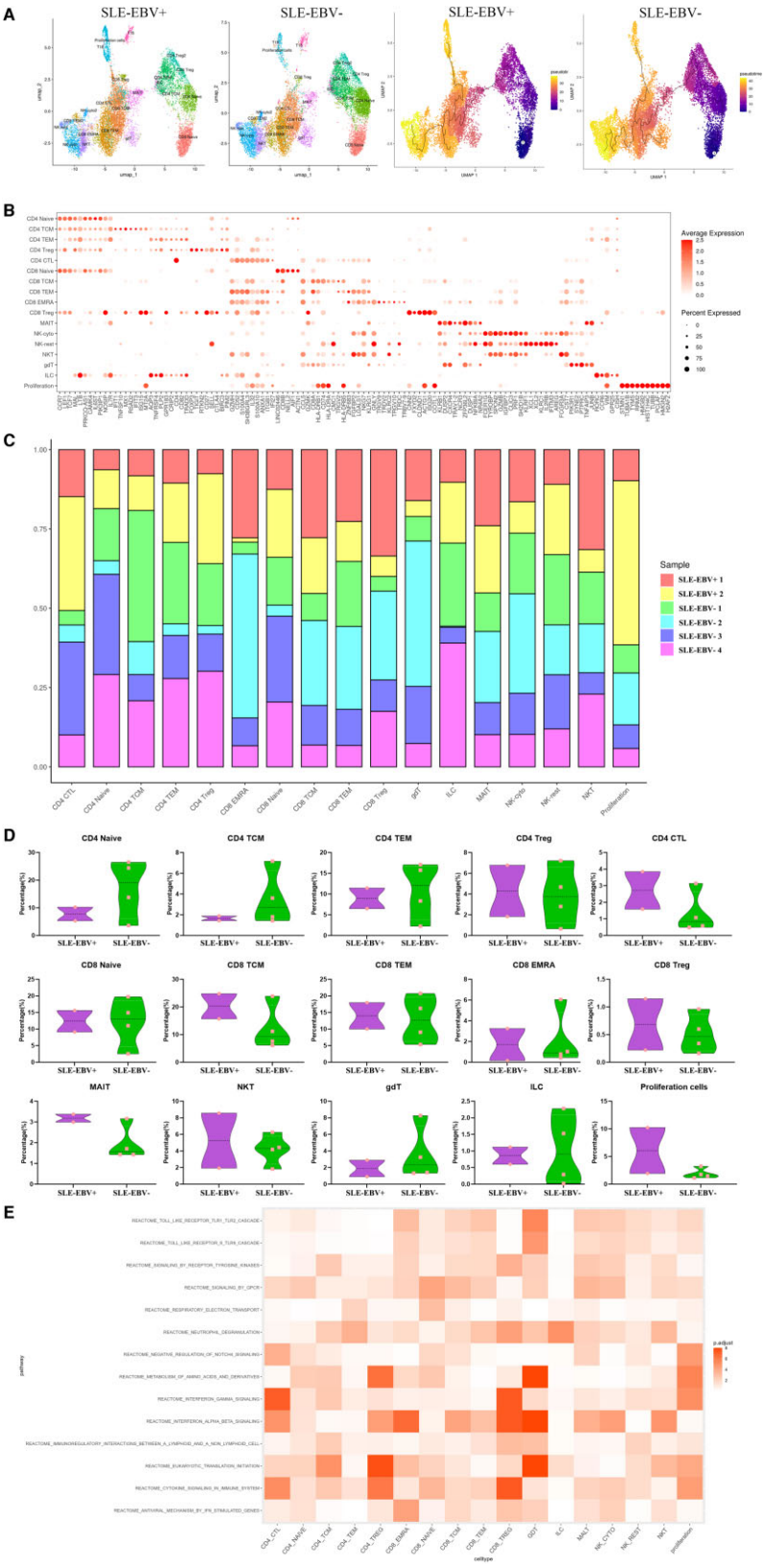

Figure 3. Sub-clustering analysis of T cells identifies major T-cell subclusters in PBMCs from patients with SLE, with or without EBV. (**A**) U-MAP plot representing 17 clusters and pseudotemporal trajectory inference analysis of T cells, with respective labels from two SLE-EBV+ and four SLE-EBV– samples. Each dot corresponds to a single cell. (**B**) Dot plot representing the expression values of selected genes (*x*-axis) across each cluster (*y*-axis). Dot size represents the percentage of cells expressing the marker of interest. Colour intensity indicates mean expression within expressing cells. (**C**) Bar plot representing cell abundances in each cluster ($n = 17$) across individuals. (**D**) Violin plot comparing the proportion of each cluster across the groups. SLE-EBV+ are shown in purple, and SLE-EBV– in green. *P*-values were calculated using a Wilcoxon test. (**E**) Heat map representing the mean expression of signalling across the clusters. Colour indicates the mean expression within each cluster. PBMC: peripheral blood mononuclear cell; SLE-EBV+: patients with SLE and positive detection of EBV infection; SLE-EBV–: patients with SLE and negative detection of EBV infection

no significant differences between them. Regarding signalling pathways, SLE-EBV+ cells upregulated the IFN-α/β pathways in most cells, including CD4+ CTL, CD4+ Treg, CD8+ EMRA, CD8+ TCM, CD8+ TEM, CD8+ Treg, γδ T, MAIT and NK-cyto cells. In addition, eukaryotic translation initiation, cytokine signalling, and antiviral mechanism by IFN stimulation signallings were upregulated in most T cell clusters.

Characteristics of BCRs and Ig isotypes analysis in EBV-IgM-positive SLE

EBV targets B cells and achieves latent infection [15]. Therefore, we analysed a subpopulation of B cells and differentially expressed genes using scRNA-seq to explore this mechanism. Four distinct B-cell clusters were identified in the PBMC samples, including B naïve, B intermediate, B memory and plasmablasts (Figs 2A and B, and 4A). There were no differences in the percentages of these B-cell clusters in the PBMCs between the two patient groups (Fig. 2D).

Single-cell V(D)J sequencing was also performed on a total of eight samples. For the V gene segments in the heavy chain, the *IGHV3* and *IGHV4* gene families were frequently present in all three groups, particularly the *IGHV3* family. However, the frequencies of the gene segments in each IGHV family were different among the three groups (Fig. 4B). Among these genes, *IGHV4-34*, strongly related to autoreactivity, exhibited higher

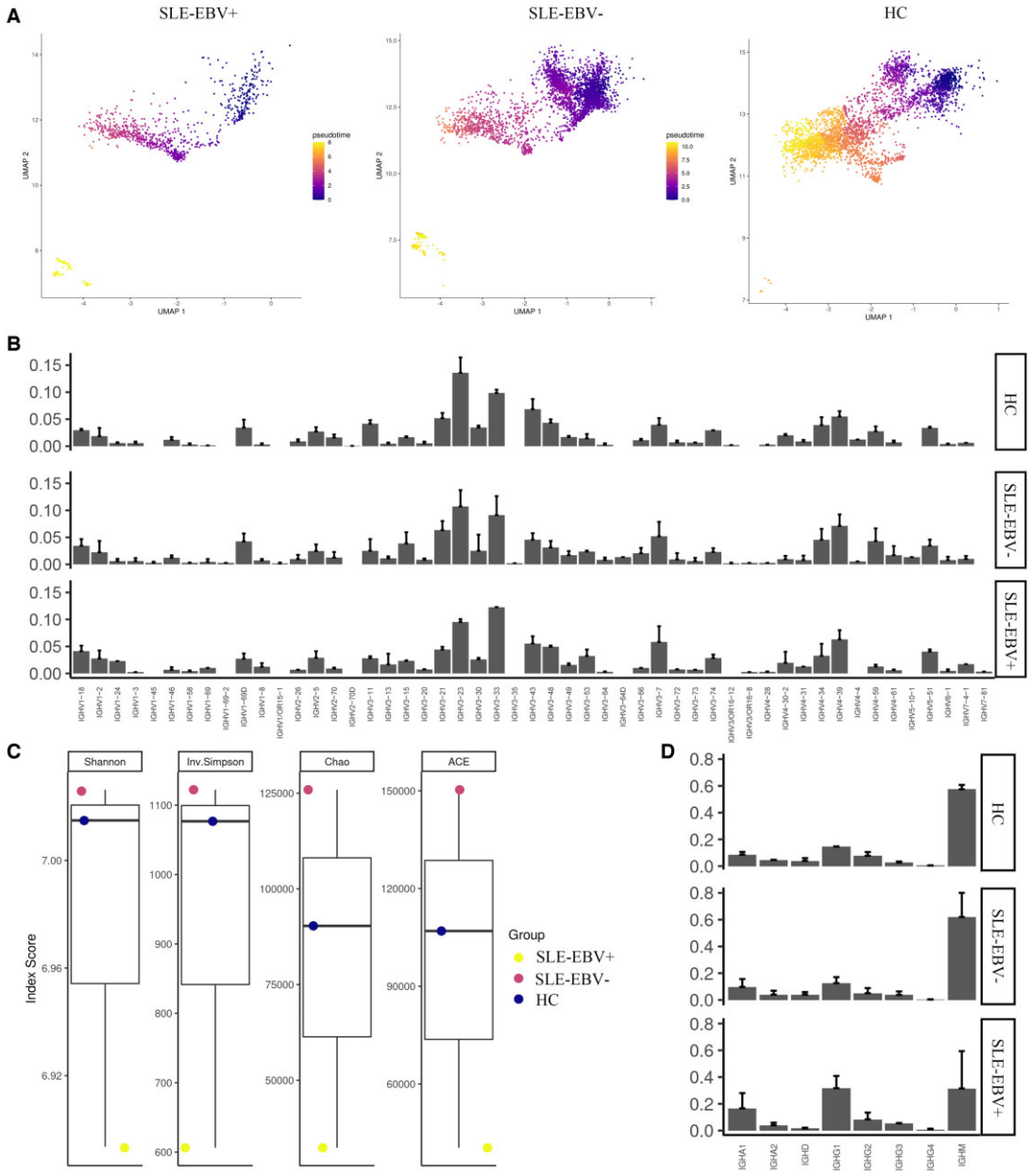

Figure 4. Comparison of B cell single-cell landscapes and BCRs between SLE-EBV+, SLE-EBV–, and HC samples. (**A**) U-MAP plots representing four B-cell clusters with their respective labels. (**B**) Comparison of variable (V) gene usage in BCR heavy chain between the three groups. IGHV genes usage is calculated as the percentage of the total analysed sequences. (**C**) The clonal diversity of SLE-EBV+ ($n=2$), SLE-EBV– ($n=4$) and HCs ($n=2$) samples. (**D**) Analysis of Ig isotypes in the three groups. BCR: B-cell receptor; SLE-EBV+: patients with SLE and positive detection of EBV infection; SLE-EBV–: patients with SLE and negative detection of EBV infection; HC: healthy control

expression in the SLE-EBV– group than in the HC group. Conversely, its expression was decreased in the SLE-EBV+ group compared with that in the SLE-EBV– group, although this difference was not statistically significant.

Additionally, analysis of the BCR clonotypes showed that the clonal diversity of patients with SLE and EBV infection was significantly lower than that of patients with SLE and without EBV infection and HCs (Fig. 4C). We also compared the frequencies of Ig isotypes between these groups. IgM was the largest component of Ig, but its proportion was significantly decreased in patients with SLE and EBV infection. However, the proportions of IgG1, IgG3 and IgA1 were significantly increased in the SLE-EBV+ group compared with the HC or SLE-EBV– groups (Fig. 4D).

Discussion

Although recent scRNA-seq studies of SLE PBMCs revealed the heterogeneity of immune cell subsets [8, 16], our study represents the first application of scRNA-seq to unveil the heterogeneity of peripheral blood immune cells in SLE with EBV infection. With increasing attention being paid to treat-to-target approaches for SLE, a critical issue that has been raised is the identification of immunological pathways and precise target cell clusters related to prognosis.

In this study, we used scRNA-seq to uncover the cell populations or pathways that give rise to immune cell changes in patients with SLE and EBV infection. A total of 83 478 PBMCs from six patients with SLE, including two with EBV infection and four without EBV infection, as well as two HCs, were included in the analysis. Based on the transcriptional data of these patients, as well as concurrent single-cell BCR sequencing, we present a model for the differentiation and relationship of immune cells in patients with SLE who have EBV infection.

An imbalance of Th17/Treg cells and impaired functions of EBV-specific T cells were observed in patients with SLE and EBV infections in previous research [17, 18]. Our study is the first to investigate all immune cell subsets, and we found that the proportions of CD8+ naïve and CD8+ proliferation T cells were higher in SLE with EBV infection than that in SLE without EBV infection. However, in the analysis of T cell subclusters, we found that there were no significant differences in the proportion of CD8+ naïve T cells between the two groups, while the percentage of proliferation T cells was still higher in patients with SLE and EBV infection, despite no statistical difference. The cause of the alterations in proliferation T cells might be that the immune system was overactivated in patients with SLE and EBV infection, eventually leading to T-cell proliferation, which indicates the strong interactions between T-cell subsets [19]. However, further in-depth research with larger sample sizes must be conducted to verify the precise proportions of CD8+ naïve and CD8+ proliferation T cells.

Based on transcriptional data from six patients with SLE, we analysed the differentiation trajectory of T cells in the two SLE groups, and found differences in CD4+ Tregs and NK cells. The position of CD4+ Tregs on our pseudotemporal trajectory was at the initial stage of cell differentiation, indicating that CD4+ Treg cells may play crucial roles in SLE combined with EBV infection. Previous studies have shown that CD4+ Tregs and EBV contribute to the pathogenesis of many diseases, including infectious mononucleosis, and acute B-lymphoblastic leukaemia, as well as SLE [17, 20, 21]. It has been demonstrated that CD4+ Treg cells can control the proliferative and cytokine responses of virus-specific CD4+ and CD8+ effector cells [22, 23]. Therefore, we speculate that the CD4+ Tregs play significant roles in SLE with EBV infection by regulating other T-cell subpopulations. NK cells, as innate lymphocytes endowed with potent cytotoxic activity, can clear EBV-infected cells [24]. Additionally, it has been established that Treg can inhibit NK-cell functions, and even kill NK cells, while, simultaneously, NK/Treg interactions are bidirectional [25]. However, in this study, the position of these cells in the trajectory indicated that the Tregs may be the origin of the abnormalities of T cell subclusters in patients with SLE and EBV infection. Therefore, appropriate immunomodulatory therapy for CD4+ Treg cells may be crucial for patients with SLE and EBV infection.

In our study, NK-rest represents quiescent NK cells, while NK-cyto represents those with enhanced cytotoxic functions against tumours or viruses. NK cells remain in a quiescent state without stimulation, but when exposed to inflammatory signals, cytotoxic NK-cell responses involve vigorous proliferation, synthesis of proinflammatory cytokines and the development of cytotoxic machinery. After the inflammation subsides, NK cells reduce their activity and return to a quiescent state [26]. Pseudotemporal analysis revealed differing positions of NK-rest and NK-cyto, indicating distinct underlying biological processes. The capacity of cells to generate energy through metabolic processes has emerged as a critical factor in supporting immune cell effector functions [26–28]. Therefore, variations in pseudotemporal changes may signify biological distinctions and differing metabolic profiles between the NK subpopulations in the SLE-EBV+ and SLE-EBV– groups.

Meanwhile, we observed a significant reduction in the expression of NK-cell checkpoints, particularly KIR3DL1, KIR2DL1 and KIR3DL2, in NKT of patients with SLE and EBV infection. Additionally, we found decreased expression of KIR2DL1 and increased expression of KIR2DL3 in NK-cyto cells. Killer cell immunoglobulin-like receptors (KIRs), a subset of the Ig superfamily, are transmembrane proteins that interact with classical human leucocyte antigens A, B and C [29]. Remarkably, KIR3DL1, KIR2DL1, KIR3DL2, KIR2DL1 and KIR2DL3 all function as inhibitory receptors. However, their expression levels exhibit variability, suggesting heterogeneity among different patients. Consequently, a more extensive sample should be examined to elucidate the checkpoint profiles and actual functions of NK cells. This endeavour holds the potential to serve as an effective therapeutic strategy for patients with SLE and EBV infection.

The BCR consists of constant and variable regions, and its diverse repertoire arises from V(D)J recombination, where V, D and J gene segments are assembled in various combinations [30, 31]. Our analysis revealed that the BCR profiles of patients exhibited convergence in different IGHV3 and IGHV4 rearrangements. Particularly, the *IGHV4-34* gene, known for its strong association with autoreactivity, was increased in SLE-EBV– patients compared with HCs, aligning with the findings from numerous studies on BCR repertoires in SLE patients [31, 32]. However, in SLE-EBV+ patients, the usage of IGHV4-34 appeared to decrease, albeit not significantly. This observation suggests that EBV infection may potentially influence BCR rearrangements, which may contribute to the analysis of antibody function and the early

diagnosis of EBV-related diseases. There are five human Ig types: IgM, IgD, IgG, IgA and IgE. In our study, IgG1 and IgA1 were increased significantly in SLE-EBV+ patients compared with SLE-EBV– patients. Notably, in SLE, IgG antibodies are more strongly associated with SLE activity status and tissue damage than other types, such as anti-dsDNA IgG and anti-C1q IgG [33]. In EBV infection, IgG antibodies, particularly EBV-specific IgG antibodies, typically play a crucial role in viral neutralization and long-term protection [34]. However, the dominant IgG subtype remains unclear in both SLE and EBV infection. Our study shows that the IgG1 subtype predominates, indicating its key role in clearing EBV infection in patients with SLE. IgA plays an important role in mucosal immunity [35]. EBV was reported to be associated with gastric cancer, mucocutaneous ulceration and other mucosal diseases [36, 37]. Our analysis of isotypes revealed an increased proportion of IgG and IgA and a decreased proportion of IgM in SLE-EBV+ compared with SLE-EBV–. This is consistent with the results of other isotype analyses in the anti-infective process, suggesting a strong humoral immune response to clear viruses in the blood and mucosa [38]. Our investigation, focusing on isotype-resolved BCR repertoire sequencing, aids in the identification of distinct clones that have undergone class-switch recombination and related processes. This knowledge holds potential significance for tailoring individualized treatments for patients infected with EBV.

This study had several limitations. First, the most significant limitation was the small number of samples due to difficulty in finding patients with SLE who also had active EBV infections, thus suggesting a need for cautious generalization of the results. We plan to include more cases in future work to validate these results using flow cytometry. Second, the cross-sectional nature of the analyses also limited the study. Larger and longitudinal studies will pave the way towards both a better understanding of pathogenesis and heterogeneity of SLE concurrent with EBV infection, hopefully allowing for the implementation of more effective personalized therapeutic approaches in the future.

In this study, we used scRNA-seq to characterize the heterogeneity of immune-cell populations in patients with SLE, both with and without EBV infection. We thus provide a comprehensive characterization of the immune-cell profiles and BCR repertoires of patients with SLE, both with and without concurrent EBV infection. We expect these findings to have an impact on the understanding of the pathogenesis of EBV infection in SLE, which will contribute to the design and development of prophylactic and therapeutic strategies for infections in patients with SLE.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Contribution statement

L.S. designed the study and revised the manuscript. L.Z.H. and J.X.T. performed the analysis and drafted the manuscript. J.J.G. and M.Y.F. collected data. All authors approved the submitted version.

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