Heterogeneity of peripheral immune cell landscape in systemic lupus erythematosus patients after belimumab treatment

W. Sun[1], C. Yang[1], J. Zhang[2], Z. Qu[3], X. Hou[1], N. Liu[1], J. Yang[4], J. Sun[1], L. Wang[5], H. Wang[5], Z. Wang[6], D. Xu[1,2]

[1]Medical Research Center, Weifang People's Hospital, Shandong Second Medical University, Weifang, China; [2]Department of Rheumatology and Immunology, Weifang People's Hospital, Shandong Second Medical University, Weifang, China; [3]Department of Physiology, Basic Medical School, Shandong Second Medical University, Weifang, China; [4]Department of Rheumatology and Immunology, Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University, Nanjing, Jiangsu, China; [5]Department of Clinical Laboratory, Weifang People's Hospital, Shandong Second Medical University, Weifang, China; [6]Department of Gynaecology and Obstetrics, Weifang Maternal and Child Health Hospital, Shandong Second Medical University, Weifang, China.

Abstract

Objective

The diversity and heterogeneity of circulating immune cells have been extensively investigated in systemic lupus erythematosus (SLE). However, little is known about the influence of belimumab, an anti-BAFF (B cell-activating factor) monoclonal antibody, on the heterogeneity of peripheral immune cell landscape in SLE patients. In this study, we aimed to investigate the altering effect of belimumab on autoimmunity in SLE.

Methods

The single-cell RNA sequence revealed a total of 11 cell clusters by comparing the transcriptome profiles of 24.869 peripheral blood mononuclear cells (PBMCs) from normal controls (NC) and SLE patients with/without belimumab treatment. Flow cytometry was conducted to further confirm the diversity and heterogeneity of peripheral immune cell landscape. The disease-specific T cell, B cell, monocyte (M), and NK cell subpopulations in SLE patients treated with/without belimumab were identified.

Results

Compared to the NC group, SLE patients exhibited a significant upregulation of $CD3^+$T, $CD8^+$T, $CD3^+PD\text{-}1^+$T, and $CD8^+PD\text{-}1^+$T cells, while the proportions of $CD16^+CD56^+$NK cell, $CD14^+CD206^+$ monocyte and $CD14^+CD163^+$ monocyte, and the ratio of $CD3^+CD4^+$T/$CD3^+CD8^+$T were significantly reduced in SLE. After belimumab treatment, the proportions of $CD19^+$B and $CD3^+PD\text{-}1^+$T cells were significantly decreased in the peripheral blood of SLE patients.

Results

Our study has implicated the substantial heterogeneity and disease-specific immune cell subsets in belimumab-treated and non-belimumab-treated SLE patients. Belimumab treatment may exert therapeutic effects in SLE patients probably by regulating the proliferation, phenotypes and functions of $CD19^+$B cells and $CD3^+PD\text{-}1^+$T cells, which warrants further investigation in the future particularly regarding their potential roles and molecular mechanisms.

Key words

systemic lupus erythematosus, single cell RNA sequencing, immune cells, autoimmunity, belimumab

*Wenchang Sun, PhD**
*Chunjuan Yang**
*Jin Zhang**
*Zhuojian Qu**
Xinyue Hou
Na Liu
Jinghan Yang
Jiamei Sun, PhD
Lin Wang
Honggang Wang, MD, Prof.
Zengfang Wang, MD, Prof.
Donghua Xu, MD, PhD, Prof.

**Contributed equally.*

Please address correspondence to:
Donghua Xu
Department of Medical Research Center, Weifang People's Hospital, Shandong Second Medical University, Weifang, Shandong 261000, China.
E-mail: xudh@sdsmu.edu.cn

and to:
Honggang Wang
Dept. of Central Laboratory, Weifang People's Hospital, Shandong Second Medical University, Weifang, Shandong 261000, China.
E-mail: rmyywf661188@sdsmu.edu.cn

Zengfang Wang
Dept. of Gynaecology and Obstetrics, Weifang Maternal and Child Health Hospital, Shandong Second Medical University, Weifang, Shandong 261000, China.
E-mail: wangzf123@yeah.net

Received on September 3, 2024; accepted in revised form on February 21, 2025

Funding: this work was supported by funds from the National Natural Science Foundation, China (82201925 and 82171790), Natural Science Foundation, Shandong Province, China (ZR2022QH203 and ZR2024MH079), Medical and Health Science and Technology Development Plan, Shandong Province, China (202403110363), and Weifang City Health Commission Plan, Weifang, Shandong Province, China (WFWSJK-2021-220).
We were also supported by the Shandong Provincial Health Science and Innovation Team Construction Project.

Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is a common autoimmune disease defined by the aberrant activation of disease-specific immune cells and long-lasting immune and inflammatory responses, leading to impairment of key organs, such as the skin, joints, and kidneys (1-3). The diversity and unpredictability of clinical symptoms in SLE impede the progress in treatment. SLE is caused by various intricate elements, including genetic predisposition, environmental triggers, immunological factors, and hormonal dysregulation (4, 5). Autoimmune disorders due to abnormal proliferation or overactivation of immune cells or immunological evasion of the body's immune system contribute to SLE (5-7). Besides, the excessive removal of dying cell debris can lead to aberrant activation of T cells and B cells through activating type I interferon (IFN) and Toll-like receptor (TLR) signalling pathways, resulting in the subsequent amplification of immune and inflammatory responses (7-9). Furthermore, the aberrant activation of B cells and overproduction of autoantibodies enhance the effective presentation of specific antigens by activated B cells to T cells, thereby facilitating T cell activation in SLE (10-12). In addition to the overabundance of self-reactive B cells, dysregulation of T cell subsets, such as $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells, plays a critical role in the pathogenesis of autoimmune diseases (13, 14). Increasing evidence has supported that the disruption of immune balance due to the dysregulated immunometabolism is also a pivotal factor for the development of SLE (15, 16). Understanding the atypical alterations in SLE-specific immune cell subpopulations is crucial for assessing the efficacy of current treatment approaches and identifying novel therapeutic targets.

Belimumab, a monoclonal antibody against B cell-activating factor (BAFF) also known as B lymphocyte stimulating factor (BLyS), has gained approval for the treatment of SLE as a targeted biological drug. Belimumab hinders the attachment of soluble BlyS to its receptors on B cells, therefore inhibiting the survival of B cells including self-reactive B cells and decreasing their transformation into Ig-producing plasma cells (17). At present, belimumab is extensively utilised in the clinic, despite the ongoing debate on its efficacy. Most randomised clinical trials (RCTs) have consistently demonstrated that belimumab is efficacious in improving disease activity in patients with lupus nephritis (LN) (18). However, the retrospective observational data from a Swedish study has implicated that belimumab treatment increased the risk of new LN among non-renal SLE patients (19, 20). Moreover, the underlying molecular mechanism of belimumab in regulating innate or adaptive immunity in SLE is not fully understood. Hence, it is valuable to investigate how belimumab regulates the proliferation, differentiation and function of immune cell subsets, providing valuable insight into the biological treatment of SLE.

In this study, we aimed to investigate the altering effect of belimumab on autoimmunity in SLE by the single-cell RNA sequencing (scRNA seq) examining 24.869 peripheral blood mononuclear cells (PBMCs) from 3 healthy controls, 3 originally-diagnosed and untreated SLE patients, and 3 SLE patients treated with belimumab. The disease-specific immune subsets were further validated using flow cytometry technology by a larger cohort study of 105 healthy controls, 136 belimumab-untreated SLE patients, and 47 SLE patients treated with belimumab. The findings of this study will elucidate the disease-specific immune cell subpopulations in SLE and the influence of belimumab on immune cell landscape primarily including T cells, B cells, NK cells and monocytes, offering novel perspectives and implications on the anti-BAFF therapy for individuals with SLE.

Material and methods

Study population

This study included a total of 105 healthy individuals as controls without any medical conditions, 136 firstly diagnosed and untreated SLE patients, and 47 patients with SLE who had been treated with belimumab (Benlysta, GSK Plc, UK). All patients were recruited from the Weifang People's Hospital,

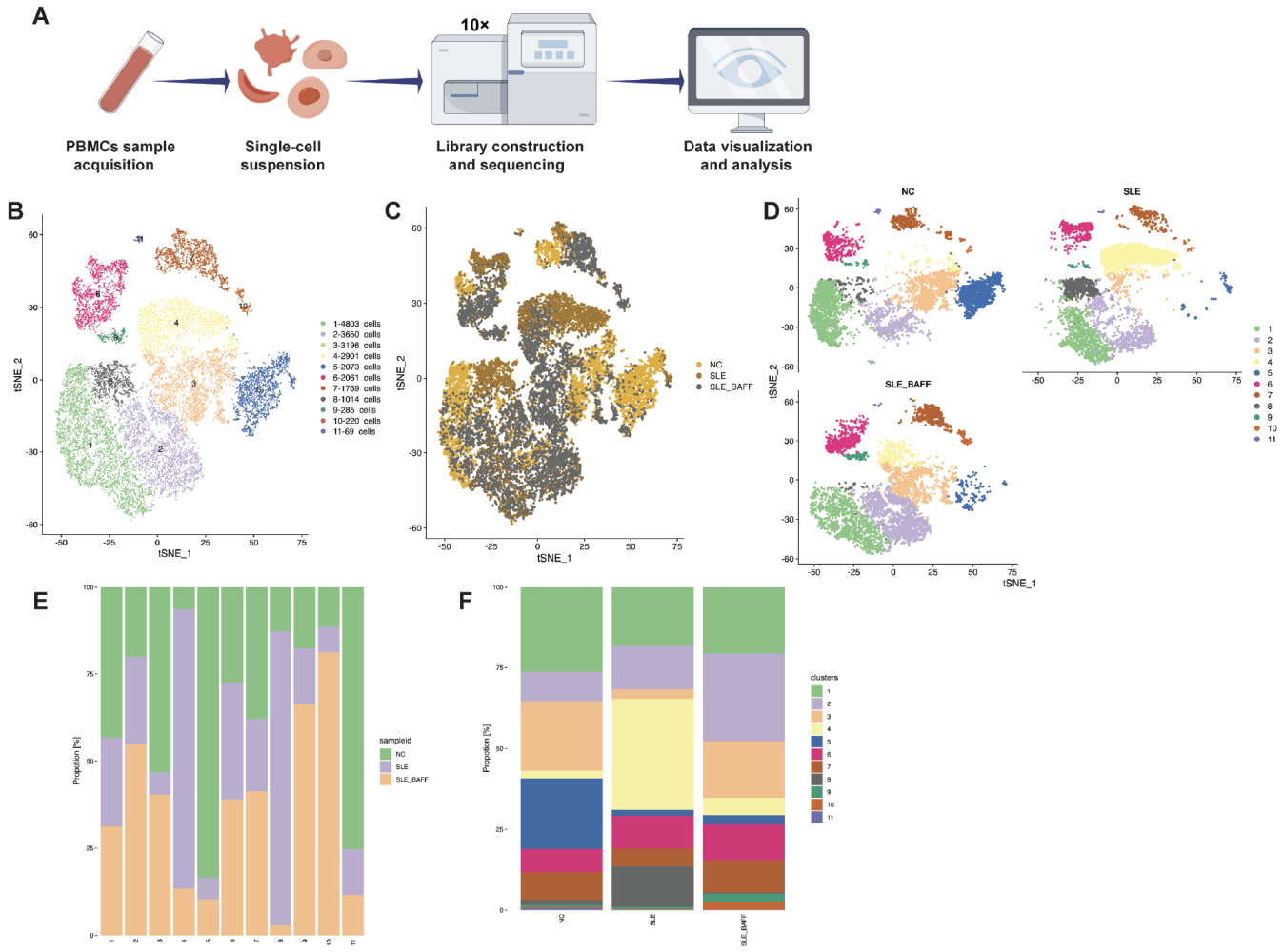

Fig. 1. Flowchart and t-SNE clustering for scRNA sequence.
A: Schematic diagram of single-cell sequencing process (completed by Figdraw online tool, ID: RAOSW0033a). **B**: Scatter plot of t-SNE dimensionality reduction clustering results (each point represents a cell). **C**: Scatter plot showing the total t-SNE grouping of samples from NC group, SLE group, and SLE-BAFF group. **D**: Scatter plot of t-SNE grouping between NC group, SLE group, and SLE-BAFF group samples. **E**: The distribution bar chart of clusters among the NC group, SLE group, and SLE-BAFF group samples. **F**: The distribution of each cluster within the three sample groups NC group, SLE group, and SLE-BAFF group forms a bar chart.

Shandong Second Medical University, between November, 2019 to October, 2023. All patients receiving belimumab treatment must have undergone at least five treatments before blood can be collected for following determinations. Normal control (NC) group was individuals who completed physical examinations during the same admission period in the same hospital. The Institutional Medical Ethics Committee of the Weifang People's Hospital, Shandong Second Medical University granted this study (no. KYLL2022YX73). All participants were required to approve and sign a written informed consent form. Additionally, the research was adhered to the ethical guidelines outlined in the Helsinki Declaration. SLE patients were diagnosed according to the EULAR/ACR classification criteria established by the European Union Against Rheumatology (EULAR) and the American Society of Rheumatology (ACR) in 2019 (21). Patients with any other immunological problems, such as malignant tumours or rheumatoid arthritis, were excluded. During the trial, patients were categorised into two groups depending on whether they were administered to belimumab treatment, namely Belimumab-treated SLE patients and belimumab-untreated SLE patients. The clinical data obtained from SLE patients include the following parameters: erythrocyte sedimentation rate (ESR), concentrations of complement 3 (C3) and complement 4 (C4), red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), urinary microalbumin (mALB), titres of anti-nuclear antibodies (ANA), and titres of anti-double-stranded DNA (dsDNA) antibodies.

Isolation of PBMCs

3–10ml of fresh peripheral blood were obtained from individuals who are in NC, individuals with SLE, and those with SLE who have been treated with belimumab (SLE-BAFF). PBMCs were isolated by the Ficoll Paque gradient centrifugation for 30 minutes at 2000 rpm. Cells in the intermediate white membrane were extracted and washed twice with phosphate-buffered saline (PBS), which were then centrifuged at

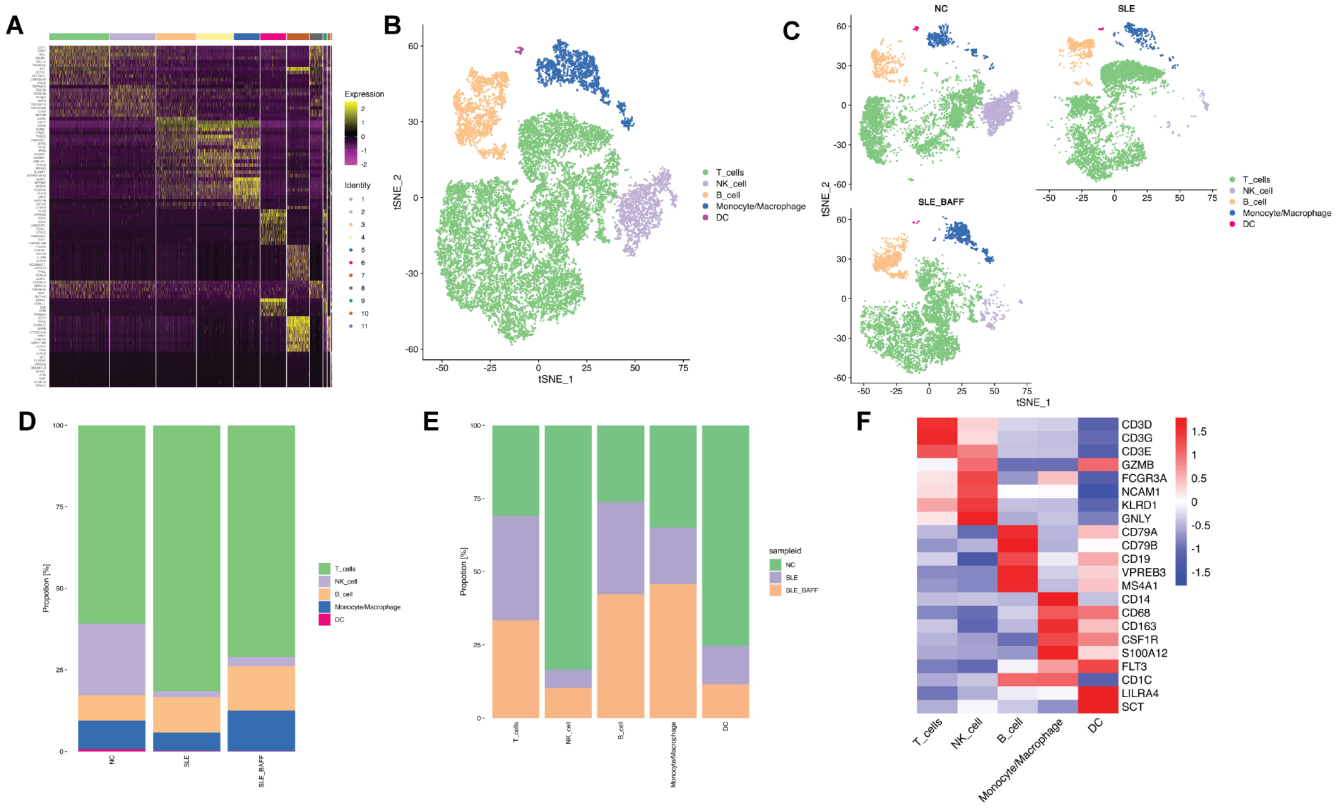

Fig. 2. Identification of major immune cell populations between groups.
A: Topmarker gene expression heatmap. **B**: Scatter plot of t-SNE for cell type identification. **C**: Scatter plot of t-SNE for cell type identification of NC group, SLE group, and SLE-BAFF group samples. **D**: The proportion of different cell subpopulations in the NC group, SLE group, and SLE-BAFF group samples. **E**: The proportion of different cell types in the NC group, SLE group, and SLE-BAFF group samples. **F**: Heat map related to cell type identification.

1500 rpm for 5 minutes. At last, PBMCs were precipitated and used for the flow cytometry analyses of T cells, B cells, NK cells. In addition, 100 µl of whole blood sample was used for flow cytometry analyses of monocytes according to the protocols.

Single-cell RNA sequencing (scRNA-seq) analysis

This study collected PBMCs from fresh peripheral blood samples of three healthy controls (NC group), three newly diagnosed and untreated SLE patients (SLE group), and three SLE-BAFF patients who received belimumab treatment (SLE-BAFF group) for scRNA-seq, with technological assistance from Shanghai Ouyi Company (Shanghai, China). Concisely, PBMCs were promptly and fully thawed in a water bath at a temperature of 37°C, followed by two washes with preheated culture medium (IMDM with 10% FBS). PBMCs were obtained by centrifugation at 4°C, 300 rcf, for 5 minutes, and then suspended in 1 x PBS with 0.04% bovine serum albumin (BSA, Thermo Fisher, USA). Cell viability was assessed after cell counting using Thermo Fisher (USA) staining. Next, the 10 × Genomics Chromium Controller was employed to introduce the cell suspension into the droplets, resulting in the creation of individual cell gel beads within the lotion. The complementary DNA (cDNA) library by utilising the Single Cell 3 'Reagent v3 Kits was applied for sequencing using the Agilent 2100 Bioanalyzer (Agilent, USA) on the Illumina sequencing platform (Illumina, Inc., USA). Raw data can be acquired from the GEO database located at https:/ww.ncbinlm.nih. gov/geo/ (serial no.: GSE266852, GSE270215). The scRNA-seq data was analysed using the Cell Ranger software (v. 3.1.0, USA) by 10 × Genomics. The R package Seurat (22) (v. 3.0) was utilised to manage the counting matrix of unique molecular identifiers. The method outlined by Macosko *et al* (23) was used to identify the top-level variable genes across single cells. Cells were visualised using t-distribution random neighbourhood embedding (t-SNE) for clustering. We use the R package SingleR (24) for unbiased cell type recognition of scRNA seq. DEGs were determined using Seurat (22). $p<0.05$ and | log2 fold change |>1 (or | log2 fold change |>0.58) were set as thresholds for differential expression. GO functional enrichment and KEGG signalling pathway enrichment analyses were performed for the prediction analysis of the selected DEGs.

Flow cytometry

According to the protocol of the specific fluorescent labelled antibodies, the antibodies were incubated with whole blood samples and then detected using flow cytometry using Agilent NovoCell FCS (Agilent Technologies, Inc., USA). The specific antibodies tested for in the peripheral blood lymphocyte subsets included CD3-FITC, CD8-PE, CD4-APC, CD45 PerCP, $CD16^{+}CD56$-PE, CD19-APC, PD-1-PE, CD4-FITC, CD25-APC, and CD127-PE (Agilent Tech-

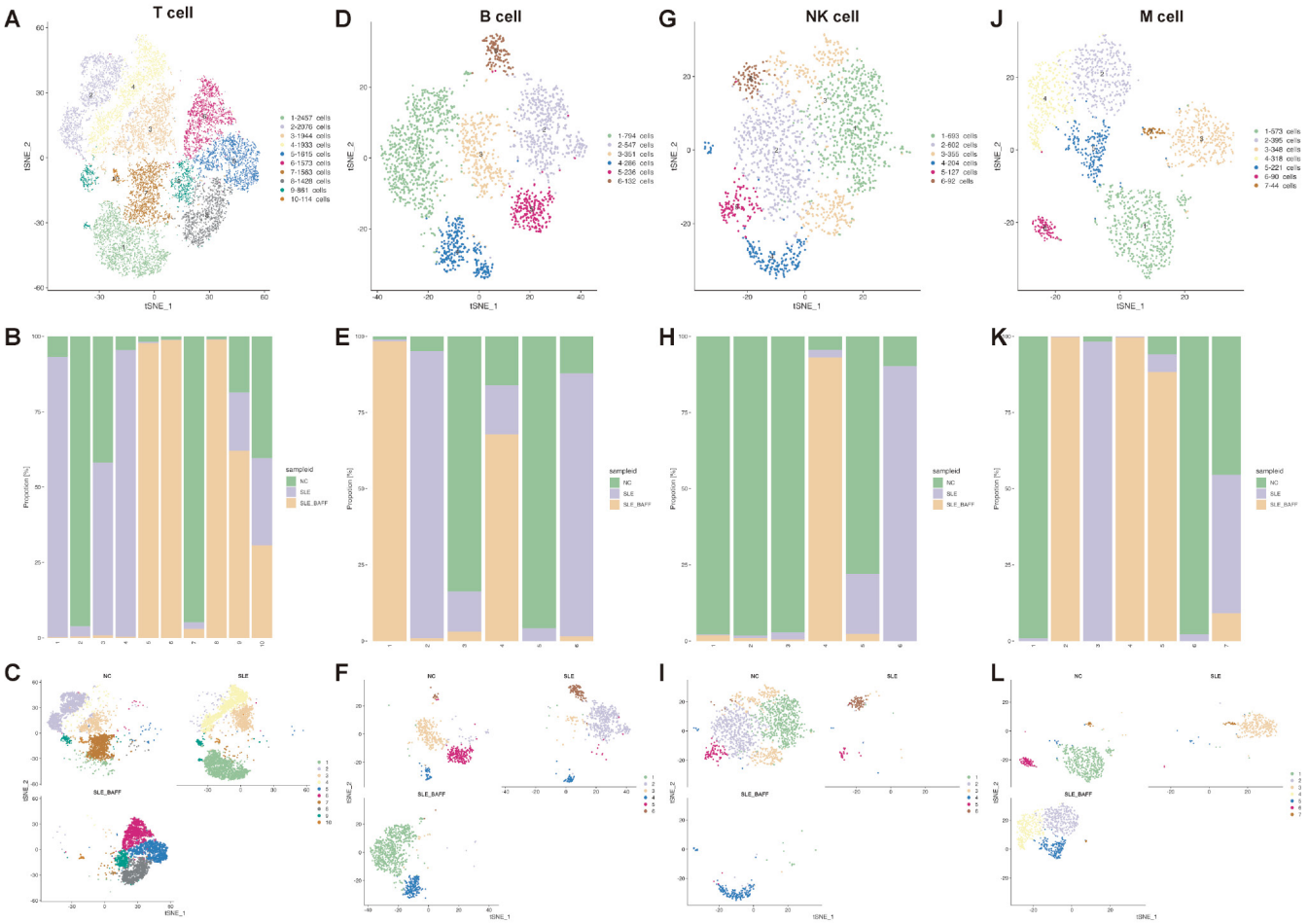

Fig. 3. Cluster analyses of T, B, NK, and M cells.
A: Scatter plot of T-cell t-SNE clustering. **B**: The distribution bar chart of T cells in clusters among the NC group, SLE group, and SLE-BAFF group samples. **C**: Scatter plot of t-SNE classification of T cells in NC group, SLE group, and SLE-BAFF group samples. **D**: Scatter plot of t-SNE clustering in B cells. **E**: Bar chart of cluster distribution of B cells among NC group, SLE group, and SLE-BAFF group samples. **F**: Scatter plot of t-SNE classification of B cells in NC group, SLE group, and SLE-BAFF group samples. **G**: Scatter plot of NK cell t-SNE clustering. **H**: Histogram of cluster distribution of NK cells among NC group, SLE group, and SLE-BAFF group samples. **I**: Scatter plot of t-SNE classification of NK cells in NC group, SLE group, and SLE-BAFF group samples. **J**: Scatter plot of t-SNE clustering in M cells. **K**: Cluster distribution bar chart of M cells in NC group, SLE group, and SLE-BAFF group samples. **L**: Scatter plot of t-SNE classification of M cells in NC group, SLE group, and SLE-BAFF group samples.

nologies Inc., USA). Monocyte-specific antibodies used for detection include CD14-FITC, HLA-DR-PE, CD206-PE, CD163-APC, CD64-FITC, and CD161-PE antibodies (Bio Legend, USA).

Statistical analysis

The statistical analyses were conducted using SPSS 17.0. Data was given as the mean value plus or minus the standard deviation (SD). Three sets of samples were subjected to one-way analysis of variance (ANOVA), while two sets of samples were subjected to paired sample t-tests, Wilcoxon tests, or Kruskal-Wallis tests, depending on the peculiarities of the data. The difference was considered statistically significant when the *p*-value was less than 0.05.

Results

ScRNA seq cell clustering

We utilised the 10 × Genomics Chromium platform to conduct scRNA sequence of 24.869 PBMCs, among which 8.899 cells from the NC group, 7.810 cells from the newly diagnosed and untreated SLE group, and 8.160 cells from the SLE-BAFF group undergoing belimumab treatment. The precise procedures were outlined in Figure 1A. The t-SNE clustering was used to visually single cells with comparable expression profiles in a two-dimensional space. The analysis revealed a total of 11 distinct cell clusters (Fig. 1B). The disease-specific cell populations between groups were shown in Figure 1C.

In brief, clusters 1, 3, 5, and 11 showed a higher level of enrichment in the NC group, while cluster 4 and 8 exhibited a higher level of enrichment in the SLE group. However, clusters 3, 5, 7, 9, 10, and 11 showed a relatively lower level of enrichment in the SLE group. In the SLE-BAFF group, clusters 2, 9, and 10 are enriched, while clusters 4, 5, 8, and 11 showed a relatively lower level of enrichment (Fig. 1D-F). Additionally, there are notable differences of clusters 3, 4, 5, 8, and 11 between the SLE group and NC group, and clusters 2, 3, 4, 7, 8, 9, and 11 between the SLE group and SLE-BAFF group, as well as cluster 2, 5, 8, 9, 10, and 11 between the NC group and SLE-BAFF group (Fig. 1D-F).

These findings implicated a notable immune cell landscape in the peripheral

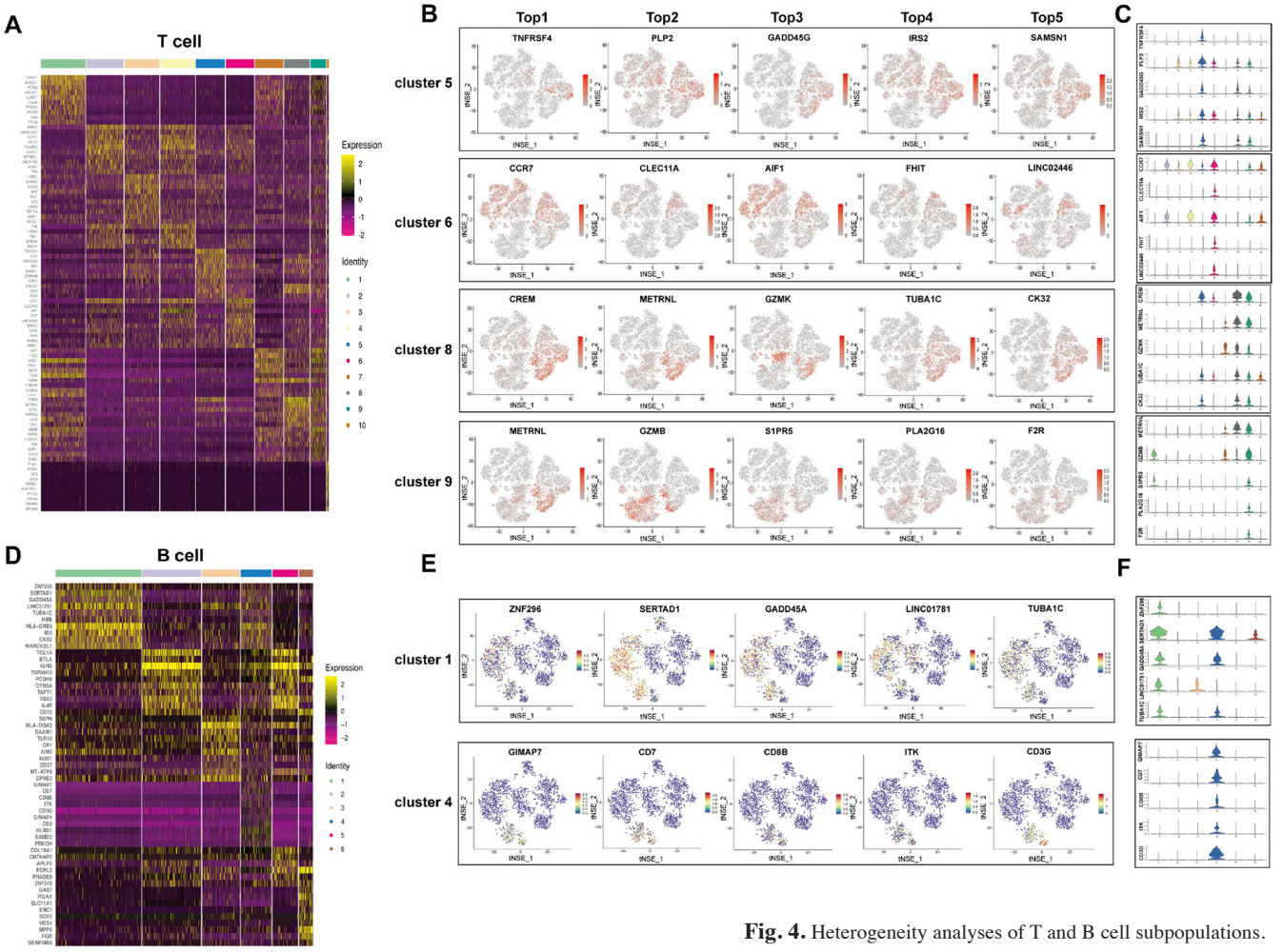

Fig. 4. Heterogeneity analyses of T and B cell subpopulations.

blood of patients with SLE compared to healthy individuals. Furthermore, SLE patients who received belimumab treatment exhibited distinct alterations in the immune cell populations in the peripheral blood, particularly regarding clusters of 2, 3, 4, and 8.

Identification of marker genes and major cell types

According to the t-SNE clustering analysis, we have identified the top 10 Topmarker genes specifically for each cluster (Fig. 2A). We categorised the 11 clusters into 5 immune cell groups based on the specific cell markers, including T cells (clusters 1, 2, 3, 4, 8), NK cells (cluster 5), monocyte cells (M) (clusters 7, 10), B cells (clusters 6, 9), and DC cells (cluster 11) (Fig. 2B). Besides, the comparison of major immune cell populations between groups revealed that the abundance of T cell populations in the SLE group was greater than that in the NC group and SLE-BAFF group (Fig. 2. C-E). Furthermore, the B cell population exhibited a substantial increase in both the SLE group and SLE-BAFF group in contrast to the NC group. Conversely, the NK cell population was much lower in the SLE group and SLE-BAFF group compared to the NC group. In comparison to the NC group, the M cell group exhibited an obvious reduction in the SLE group and a greater enrichment in the SLE-BAFF group (Fig. 2C-E).

These findings had implicated that belimumab treatment might have a significant impact on the differentiation and function of B cells and monocytes. The heatmap in Figure 2. F displayed distinct markers of the immune cell group. The T cell clusters exhibited strong expression of specific markers, such as CD3 and KLRD1, while the expression of specific markers was lower, such as LILRA4, VPREB3, and FLT3. The B cell clusters showed higher expression of CD79, CD19, VPREB, MS4A1, and CD1C, but lower expression of functional markers of GZMB, CSF1R, and S100A12. NK cells had higher expression of FCGR3A (CD16), NCAM1 (CD56), GZMB, KLRD1, and GNLY but lower expression of FLT3 and MS4A1. Monocytes displayed higher expression of CD14, CD68, CD163, S100A12, CSF1R, and CD1C, but lower expression of GZMB and SCT.

Cluster analysis of T, B, NK, and M cells

We performed an in-depth t-SNE clustering analysis on the characteristics of the four immune cell populations, primarily including T, B, NK, and M, utilising the unique marker genes. Figure 3. A-C displayed the division of the T cell population into 10 cell distinct clusters. The B cell population were grouped into 6 clusters (Fig. 3. D-F). As shown in Figure 3. G-I, the NK cell group into 6 clusters. The M cell population was categorised into 7 clusters

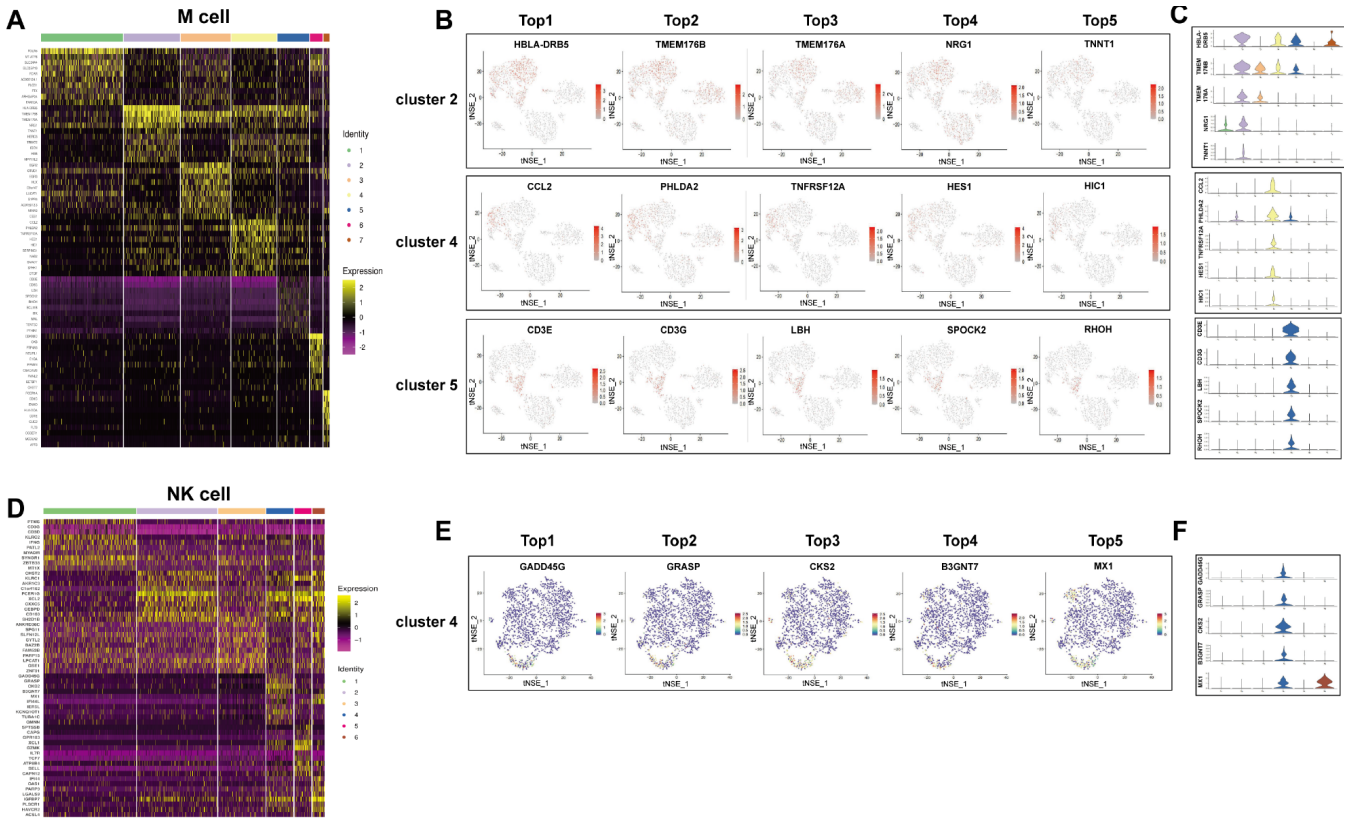

Fig. 5. Heterogeneity analyses of M and NK cell subpopulations.
A: Topmarker hotspot map of M cells. **B**: Scatter plot of differences in cluster Top5 gene characteristics among M cell subpopulations. **C**: Cluster Top5 gene violin plot of M cell subpopulation differences. **D**: Topmarker hotspot map of NK cells. **E**: Scatter plot of cluster Top5 gene characteristics for NK cell subpopulations. **F**: Cluster Top5 gene violin plot of NK cell subpopulation differences.

(Fig. 3J-L). In addition, T cell clusters 1, 2, 4, 5, 6, 8, and 9 in the peripheral blood were different between SLE patients and the NC group (Fig. 3A-C). Interestingly, belimumab treatment resulted in a significant decrease of clusters 1, 2, and 4 but a significant increase of clusters 5, 6, 8, and 9 in the SLE-BAFF group (Fig 3B-C). Regarding B cell population, the clusters of 1, 2, 3, 5, and 6 were different between SLE patients and the NC group (Fig. 3D-F). Belimumab treatment induced a significant upregulation of B cell clusters 1 and 4, but an obvious reduction in B cell clusters of 2, 3, 5, and 6 in the SLE-BAFF group (Fig. 3E-F). Clusters 1, 2, 3, 5, and 6 were disease-specific NK cell subpopulations in SLE (Fig. 3G-I). In the group of SLE patients treated with belimumab, there was a considerable rise in the enrichment of NK cell cluster 4, whereas NK cell clusters 5 and 6 were dramatically decreased (Fig. 3H-I). Nevertheless, the alterations in clusters 1, 2, and 3 were not significant. The clusters of 1, 2, 3, 4, 5, and 6 were disease-specific monocytes in SLE (Fig. 3J-L). Additionally, SLE patients treated with belimumab exhibited a significant higher enrichment of M cell clusters 2, 4, and 5, but less enrichment of M cell clusters 1, 3, 6, and 7 in the peripheral blood (Fig. 3K-L).

Taken together, the scRNA-seq had identified SLE-specific T cell, B cell, M cell, and NK cell populations in the peripheral circulation. In particular, SLE patients treated by belimumab displayed specific alterations in common immune cell subpopulations, including T cell, B cell, M cell, and NK cell. In following experiments, we conducted a more in-depth analysis of the diversity and specificity of main immune cell subpopulations in SLE patients and BAFF-treated SLE patients.

Table I. Characteristics of study populations.

	NC	SLE untreated with Belimumab	SLE treated with Belimumab
Sample size	105	136	47
Age (mean±SD)	32.28±8.204	37.71±12.16****	31.70±11.87▲▲▲▲
Male	3	6	5
Female	102	130	42

Compared with NC group, **** $p<0.0001$ (Kruskal-Wallis test). Compared with SLE untreated with belimumab group, ▲▲▲▲ $p<0.0001$ (Kruskal-Wallis test). $p<0.05$ was considered to be significant.

Heterogeneity analyses of T, B, M, and NK cell subpopulations

Using the clustering results depicted in Fig. 3, we used the Seurat software to identify the T, B, M, and NK cell populations. We then chose the Top 5 marker genes of the cell cluster that had a notable influence on the diversity of immune cells following belimumab administration. Within the T cell subgroup, the SLE-BAFF group that received be-

Table II. Characteristics of SLE patients treated and untreated with belimumab.

	ESR (mm/h)	C3 (g/L)	C4 (g/L)	WBC (×10^9/L)	RBC (×10^12/L)	PLT (×10^9/L)	mALB (mg/L)	ANA (au/mL)	Anti-dsDNA antibody (u/MI)
SLE untreated with belimumab (n=136)	24.94±22.54	0.72±0.23	0.16±0.08	8.34±25.66	4.27±0.63	215.8±76.25	354.369±987.57	121.9±118.7	69.0±68.53
SLE treated with belimumab (n=47)	20.68±18.31	0.74±0.19	0.19±0.11	5.99±2.25	4.46±1.08	242.95±80.65*	322.193.1±412.55	177.4±121.4	64.80±84.93

Compared with SLE untreated with belimumab group, * $p<0.05$(Wilcoxon test).
ESR: erythrocyte sedimentation rate (0-20mm/h); C3: serum concentrations of complement factors 3 (0.79-1.52g/L); C4: serum concentrations of complement factors 4(0.16-0.38g/L); WBC: white blood cell count (4.0-10.0×10^9/L); RBC: erythrocyte count (3.5-5×10^12/L); PLT: platelet count (100.00-300.00×10^9/L); mALB: micro-albminuria (0-30mg/L); ANA: antinuclear antibody (0-32au/mL); Anti-dsDNA antibody(0-25u/MI).

Table III. Characteristics of SLE patients before and after treatment with belimumab.

	ESR (mm/h)	C3 (g/L)	C4 (g/L)	WBC (×10^9/L)	RBC (×10^12/L)	PLT (×10^9/L)	mALB (mg/L)	ANA (au/mL)	Anti-dsDNA antibody (u/MI)
SLE before belimumab treatment (n=22)	25.53±18.52	0.60±0.25	0.17±0.23	6.54±2.41	4.30±0.96	215.76±74.27	628.26±773.43	160.2±114.2	72.77±63.38
SLE after belimumab treatment (n=22)	20.10±18.34▲	0.74±0.19▲▲▲	0.17±0.09▲	5.92±2.10	4.35±0.66	254.55±75.91▲▲	292.89±451.47▲▲	195.3±141.0	62.56±86.20

Compared with SLE before belimumab treatment group, ▲$p<0.05$ (Wilcoxon test).▲▲$p<0.01$ (Wilcoxon test); ▲▲▲$p<0.001$ (Wilcoxon test). $p<0.05$ was considered to be significant.
ESR: erythrocyte sedimentation rate (0-20mm/h); C3: serum concentrations of complement factors 3 (0.79-1.52g/L); C4: serum concentrations of complement factors 4 (0.16-0.38g/L); WBC: white blood cell count(4.0-10.0×10^9/L); RBC: erythrocyte count (3.5-5×10^12/L); PLT: platelet count (100.00-300.00×10^9/L); mALB, micro-albminuria (0-30mg/L); ANA: antinuclear antibody (0-32au/mL); Anti-dsDNA antibody (0-25u/MI).

limumab treatment had a noteworthy rise in clusters 5, 6, 8, and 9. The top 5 marker genes expressed in cluster 5 were TNFRSF4, PLP2, GADD45G, IRS2, and SAMSN1. The top 5 marker genes expressed in cluster 6 were CCR7, CLEC11A, AIF1, FHIT, and LINC02446. Cell cluster 8 had the top 5 marker genes expressed as CREM, METRNL, GZnK, TUBA1C, and CKS2. The five most significant marker genes expressed in cell cluster 9 were METRNL, GZMB, S1PR5, PLA2G16, and F2R (Fig. 4A-C).

Within the B cell subgroup, the SLE-BAFF group that received belimumab treatment exhibited a significant augmentation in clusters 1 and 4. The top 5 marker genes expressed in cluster 1 were ZNF296, SERTAD1, GADD45A, LIN01781, and TUBA1C. In cluster 4, the top 5 marker genes were GIMAP7, CD7, CD8B, ITK, and CD3G (Fig. 4D-F).

The M cell subpopulation of the SLE-BAFF group exhibited a notable augmentation in clusters 2, 4, and 5. The top 5 marker genes expressed in cluster 2 were HLA-DRB5, TMEM176B, TMEM176A, NRG1, and TNNT1. In cluster 4, the top 5 marker genes were CCL2, PHLDA2, TNFRSF12A, HES1, and HIC1. In cluster 5, the top 5 marker genes were CD3E, CD3G, LBH, SPOCK2, and RHOH (Fig. 5A-C).

Belimumab therapy led to a significant enrichment of NK cell cluster 4 in the SLE-BAFF group. The top 5 marker genes expressed in the cluster 4 were GADD45G, GRASP, CKS2, B3GNT7, and MX1 (Fig. 5. D-F).

The effect of belimumab treatment on the heterogeneity of peripheral blood immune cell subsets in SLE patients

- Impact of belimumab treatment on the general clinical characteristics of SLE patients

Table I presented the characteristics of the study population enrolled in this study, such as age and gender. The mean age of the NC group differed from the belimumab-untreated SLE group ($p<0.001$). There was no statistically significance between the NC group and the Belimumab-treated SLE group regarding the status of age ($p=0.374$). The age disparity between the treatment group and the untreated group exhibited statistical significance ($p=0.002$). Table II and Table III showed a summary of the characteristics of ESR, C3, C4, RBC, WBC, PLT, mALB, ANA, and anti dsDNA antibodies. Following administration of belimumab, a minor elevation in the level of mALB of SLE patients was observed ($p<0.05$), while no other significant differences were observed with regard to other clinical characteristics. A paired comparison analysis was performed on the clinical indicators of 22 SLE patients before and after belimumab therapy. It was shown that belimumab treatment resulted in a large increase in serum levels of C3 and C4 ($p<0.001$) and a significant reduction in the inflammatory index ESR ($p<0.05$) among SLE patients. No statistical significance was found regarding other clinical indexes, including WBC, RBC, ANA, and anti-dsDNA antibody.

- Effect of belimumab treatment on the heterogeneity of peripheral blood lymphocyte subsets in SLE patients

The scRNA seq analysis had demonstrated notable diversity and heterogeneity of main lymphocyte subsets in SLE with or without belimumab treatment, primarily including $CD3^+$T cells, $CD3^+CD4^+$T cells, $CD3^+CD8^+$T cells, $CD19^+$B cells, $CD3^+PD\text{-}1^+$T

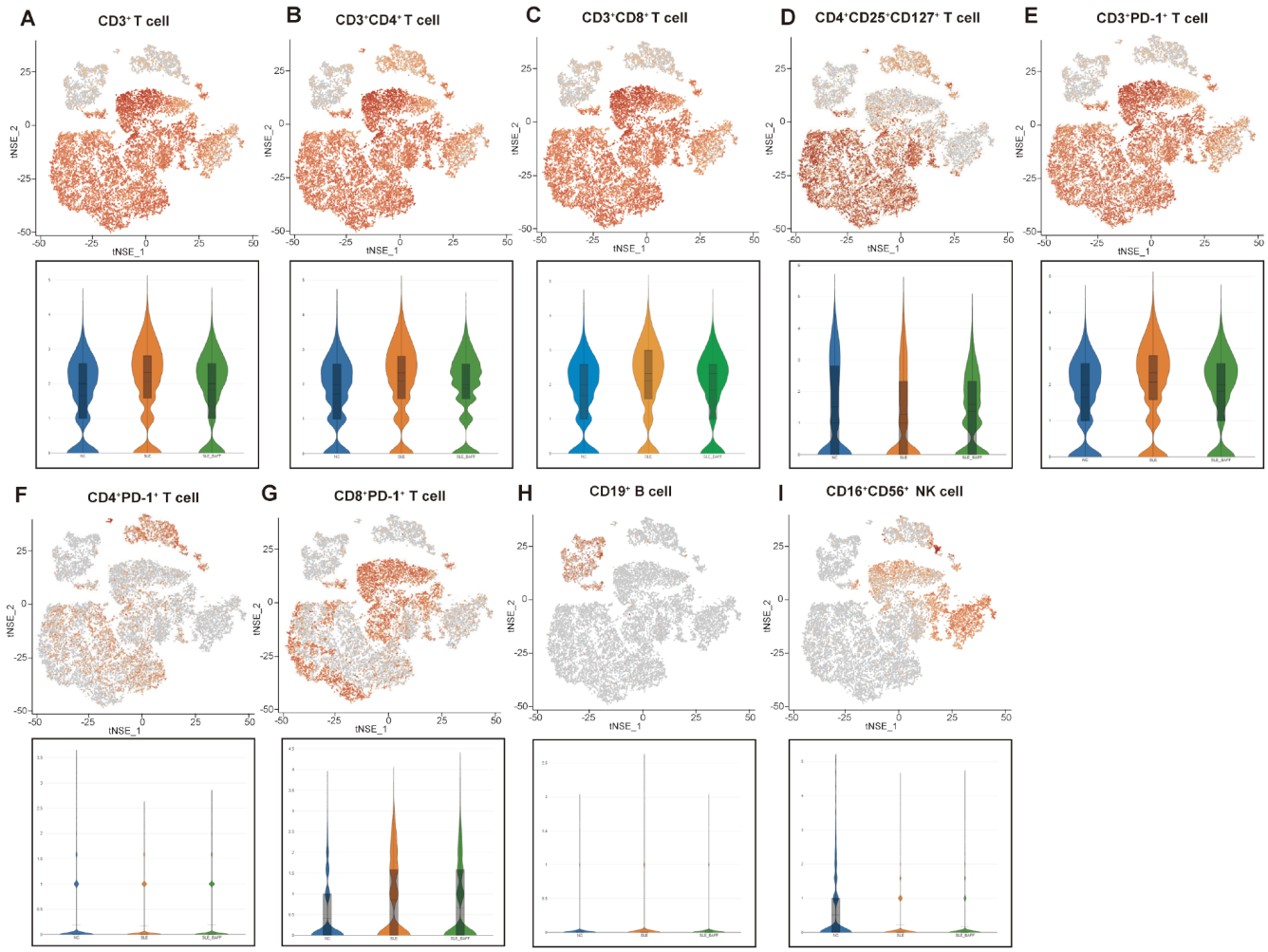

Fig. 6. Heterogeneity of peripheral blood lymphocyte subsets analysed by single-cell sequencing.

cells, $CD4^+PD\text{-}1^+$T cells, $CD8^+PD\text{-}1^+$T cells, $CD4^+CD25^+CD127^+$T cells, and $CD16^+CD56^+$NK cells (Fig. 6A-E). We further performed flow cytometry to validate the alterations in the peripheral lymphocyte subsets in SLE. Table IV displayed the flow cytometry analysis of peripheral blood lymphocyte subsets distributions in a total of 288 individuals, including 105 healthy controls, 136 SLE patients untreated with belimumab, and 47 SLE patients treated with belimumab. It has been found the peak age of SLE patients in China ranges from 30 and 49 (25). To further assure whether the confounding factor of age could affect the results obtained, we re-analysed the data of the age-overlapping population. The findings were consistent with the original data in Table IV, implicating that age has no significant effects on the lymphocyte subsets distributions between groups (Supplementary Tables S1 and S2). However, belimumab treatment has significantly influenced the differentiation of peripheral blood lymphocytes in SLE patients. Besides, the peripheral blood samples from 10 healthy individuals, 10 SLE patients who were not treated with belimumab, and 10 SLE patients treated with belimumab were detected to analyse the alterations of T lymphocyte subsets, namely $CD4^+CD25^+CD127^+$T cells, $CD3^+PD\text{-}1^+$T cells, $CD4^+PD\text{-}1^+$T cells, and $CD8^+PD\text{-}1^+$T cells (Table V). The flow cytometry results were consistent with the data of scRNA sequencing. In comparison to the NC group, SLE patients untreated with belimumab exhibited significantly higher percentages

Table IV. Heterogeneity of peripheral lymphocyte subsets in SLE patients treated/untreated with belimumab.

	NC (n=105)	SLE untreated with belimumab (n=136)	SLE treated with belimumab (n=47)
$CD3^+$ T (%)	71.25±6.53	81.83±7.25****	81.52±7.68 ▲▲▲▲
$CD3^+CD4^+$T (%)	36.40±5.82	37.12±7.36	33.30±10.18
$CD3^+CD8^+$T (%)	29.46±5.96	41.30±11.32 ****	38.21±6.89 ▲▲▲▲
$CD3^+CD4^+$T/ $CD3^+CD8^+$T (%)	1.29±0.41	1.03±0.44 ****	0.93±0.36▲▲▲▲
$CD19^+$B	12.13±3.70	12.65±7.37	5.74±2.64 ▲▲▲▲, ΔΔΔΔ
$CD16^+CD56^+$NK (%)	15.08±6.56	8.37±6.07****	8.45±6.39▲▲▲▲

Compared with NC group, ****p<0.0001 (Kruskal-Wallis test); Compared with NC group, ▲▲▲▲p<0.0001 (Kruskal-Wallis test); Compared with SLE un-treated with belimumab group, ΔΔΔΔ p<0.0001 (Kruskal-Wallis test).

Table V. Heterogeneity of $CD4^+CD25^+CD127^+$T and PD-1^+T lymphocytes in SLE patients treated/untreated with belimumab.

	NC (n=10)	SLE untreated with belimumab (n=10)	SLE treated with belimumab (n=10)
$CD4^+CD25^+CD127^+$T (%)	6.82±2.14	9.97±4.21	8.11±2.72
$CD3^+$PD-1^+T (%)	13.3±13.24	27.09±14.00▲▲	16.33±7.70 Δ
$CD4^+$PD-1^+T (%)	16.53±7.50	27.99±13.05	16.69±9.66
$CD8^+$PD-1^+T (%)	15.52±8.05	29.25±13.52▲	21.04±15.02

Compared with NC group, ▲p<0.05 (Kruskal-Wallis test); ▲▲p<0.01, (Kruskal-Wallis test).
Compared with SLE un-treated with Belimumab group, Δ p<0.05.

Table VI. Heterogeneity of peripheral lymphocyte subsets in SLE patients before and after belimumab treatment.

	SLE before belimumab treatment (n=22)	SLE after belimumab treatment (n=22)
$CD3^+$T (%)	80.86±7.93	84.70±9.12 *
$CD3^+CD4^+$T (%)	35.36±10.60	36.60±11.64
$CD3^+CD8^+$T (%)	42.28±9.54	44.80±13.50
$CD3^+CD4^+$T/$CD3^+CD8^+$T (%)	0.92±0.46	0.93±0.45
$CD19^+$B (%)	8.51±4.45	7.69±16.45 **
$CD16^+CD56^+$NK (%)	8.56±6.59	9.08±8.25

Compared with SLE pre-treated with Belimumab group, * p<0.05 (self-paired Wilcoxon test).
** p<0.01 (self-paired Wilcoxon test). p<0.05 was considered to be significant.

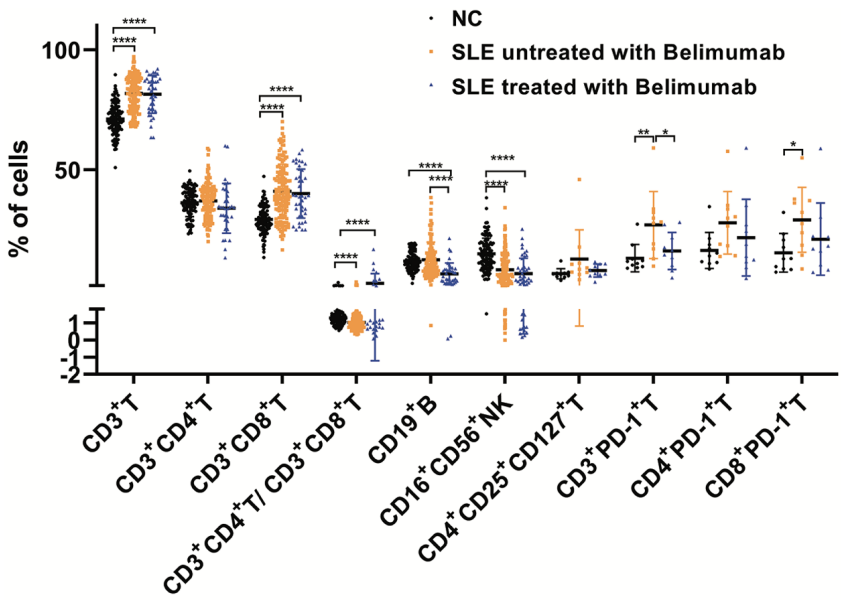

Fig. 7. Heterogeneity of lymphocyte subpopulations in belimumab-treated and non-belimumab-treated SLE patients.
*p<0.05 (Kruskal-Wallis test); ** p<0.01 (Kruskal-Wallis test); ****, p<0.0001 (Kruskal-Wallis test). A p-value of 0.05 was considered to be significant.
For $CD3^+$ T, $CD3^+$ $CD4^+$T, $CD3^+$ $CD8^+$T, $CD3^+$ $CD4^+$T/$CD3^+$ $CD8^+$T, $CD19^+$B and $CD16^+$ $CD56^+$NK, NC group, n=105, SLE untreated with belimumab group, n=136, SLE treated with belimumab group, n=47; for $CD4^+CD25^+CD127^+$T, $CD3^+$PD-1^+T, $CD4^+$PD-1^+T, $CD8^+$PD-1^+T, n=10 for each group.

of $CD3^+$T, $CD8^+$T, $CD3^+$PD-1^+T, and $CD8^+$PD-1^+T cells in the peripheral blood. Besides, significantly decreased percentage of $CD16^+CD56^+$NK cell was observed in SLE group compared to NC group (Table IV). Reduced ratio of $CD3^+CD4^+$T/$CD3^+CD8^+$T was also found in SLE (Table IV). There was no significant difference in the percentages of $CD4^+$T, $CD19^+$B, $CD4^+CD25^+CD127^+$T, $CD4^+$PD-1^+T cells, and NK cells between SLE and NC groups (Table IV, Table V and Fig. 7). Moreover, the proportions of

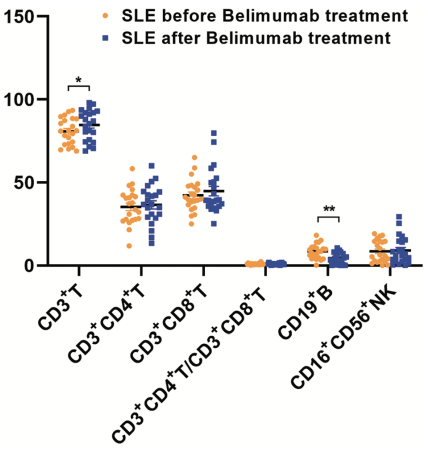

Fig. 8. Heterogeneity of peripheral blood immune cell subsets in SLE patients before and after treatment with belimumab.
*p<0.05 (Kruskal-Wallis test); **p<0.01 (Kruskal-Wallis test). p<0.05 was considered to be significant. n=22.

$CD19^+$B and $CD3^+$PD-1^+T cells were significantly lower in the peripheral blood of SLE patients treated with Belimumab compared to belimumab-untreated SLE patients (Table IV, Table V and Fig. 7). These results suggested that belimumab, functioning as a BLyS inhibitor, exerted its therapeutic effect on SLE mainly by regulating B cells-mediated immune and inflammatory responses. $CD3^+$PD-1^+T cell could also be potentially influenced by belimumab. More future studies are warranted to investigate the precise effects and mechanism of belimumab in regulating the differentiation and function of $CD19^+$B and $CD3^+$PD-1^+T cells in SLE, which might help to guide and improve the therapeutic efficacy of belimumab in SLE.

- Heterogeneity of peripheral blood immune cells in SLE patients before and after treatment with belimumab

Here, we focused on examining the heterogeneity of immune cells in the peripheral blood before and after treatment by belimumab. A total of 22 patients with SLE treated with belimumab were enrolled in this analysis. As shown in Table VI and Figure 8, belimumab treatment significantly increased the level of total $CD3^+$T cells in the peripheral blood of SLE patients (p<0.05), while significantly reduced $CD19^+$B cells were induced by belimumab treatment (p<0.01). Here, the

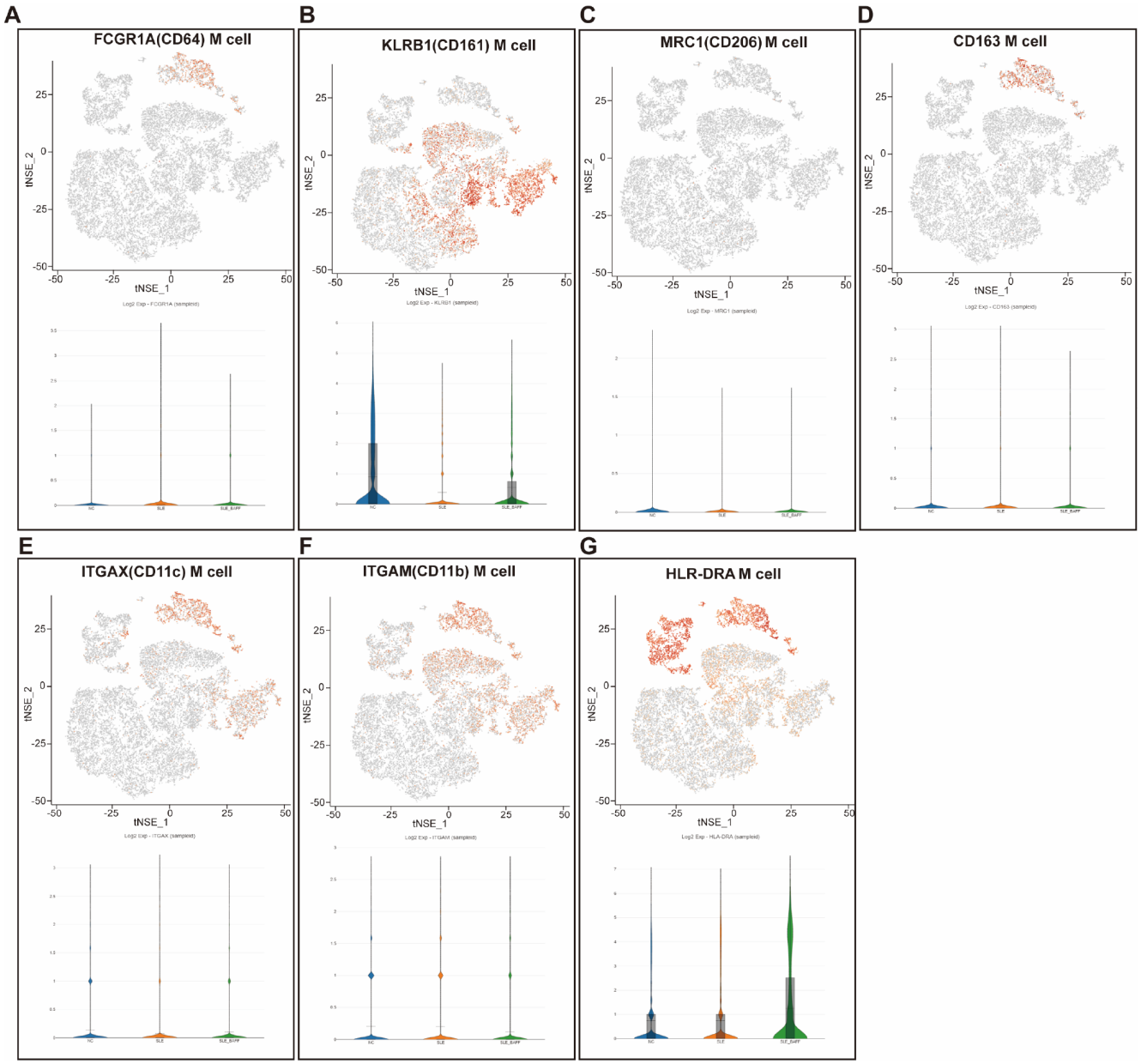

Fig. 9. Heterogeneity of peripheral blood monocyte subsets analysed by scRNA-seq.

alterations of total $CD3^+$T and $CD19^+$B cells were similar to findings in previous study in all SLE patients treated by belimumab. Accordingly, belimumab therapy can effectively suppress $CD19^+$B cells but promote $CD3^+$T cells in the peripheral circulation of SLE patients. However, the function and activation of those cells need to be elucidated in the future.

- The effect of belimumab treatment on the heterogeneity of peripheral blood monocyte subsets in SLE

Initially, we found some disease-specific subpopulations of monocytes using scRNA-seq, including monocytes specifically expressing FCGR1A (CD64), KLRB1 (CD161), MRC1 (CD206), CD163, ITGAX (CD11c), ITGAM (CD11b), and HLA-DRA (Fig. 5). Those cells exhibited notable heterogeneity in SLE. Subsequently, we performed flow cytometry analysis to determine the proportions of different monocyte subsets in the peripheral blood between the three groups of NC group (14 cases), SLE patients untreated with belimumab (15 cases), and SLE patients treated with belimumab (21 cases), including $CD14^+$HLA-DR^+, $CD14^+CD206^+$, and $CD14^+CD163^+$ monocytes. Besides, we included an additional 10 SLE patients in the NC group, 10 SLE patients untreated with belimumab, and 10 SLE patients treated with belimumab to examine the effect of belimumab treatment on the peripheral blood $CD64^+CD161^+$ monocyte in SLE. As shown in Figure 9, the scRNA-seq data had implicated obvious heterogeneity of peripheral blood monocyte subsets in SLE patients treated with or without belimumab, mainly including $CD14^+CD206^+$ monocyte, $CD14^+CD163^+$ monocyte, $CD64^+$ monocyte, and $CD161^+$ monocyte. We further performed flow

cytometry to confirmed the disease-specific monocyte subsets in SLE. As presented in Table VII and Fig. 10, the percentages of $CD14^+CD206^+$ monocyte and $CD14^+CD163^+$ monocyte were significantly decreased in the peripheral blood of SLE patients. Belimumab treatment did not significantly influence the differentiation of $CD14^+CD206^+$ and $CD14^+CD163^+$ monocytes. Taken together, there was significant heterogeneity of peripheral blood monocyte subsets in SLE, characterised by reduced M2-type monocytes ($CD14^+CD206^+$ and $CD14^+CD163^+$ cells) in SLE. Nonetheless, belimumab did not have notable impacts on the heterogeneity of peripheral blood monocyte subsets in SLE.

Table VII. Heterogeneity of peripheral monocyte subsets in SLE patients treated/untreated with belimumab.

	NC (n=14)	SLE untreated with belimumab (n=15)	SLE treated with belimumab (n=21)
$CD14^+$HLA-DR^+ monocyte (%)	97.80±3.03	95.65±4.43	95.64±3.86
$CD14^+CD206^+$ monocyte (%)	22.06±11.29	11.82±17.65▲	4.76±8.73****
$CD14^+CD163^+$ monocyte (%)	98.34±2.52	85.22±9.31▲▲▲	83.83±6.98****
$CD64^+CD161^+$ monocyte (%) (n=10)	37.67±7.90	32.26±13.85	27.83±11.60

Compared with NC group, ▲ $p<0.05$ (Kruskal-Wallis test); ▲▲▲ $p<0.001$ (Kruskal-Wallis test).
Compared with NC group, **** $p<0.0001$ (Kruskal-Wallis test). $p<0.05$ was considered to be significant.

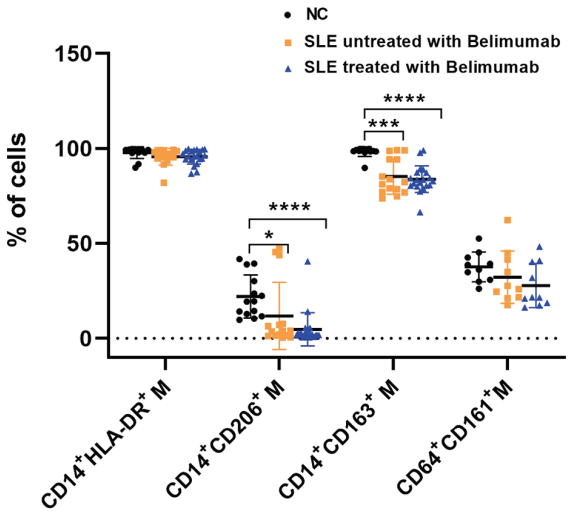

Fig. 10. Distribution bar chart of peripheral blood monocyte subsets in patients with SLE. **$p<0.001$ (Kruskal-Wallis test); ****$p<0.0001$ (Kruskal-Wallis test). $p<0.05$ was considered to be significant.
For $CD14^+$HLA-DR^+ monocyte, $CD14^+CD206^+$ monocyte, $CD14^+CD163^+$ monocyte, n=14 (NC group), n=15 (SLE untreated with belimumab group), n=21 (SLE treated with belimumab group); $CD64^+CD161^+$ monocyte, n=10.

Differential expression genes and functional enrichment prediction analysis

We performed GO and KEGG enrichment analyses of all differential expressed genes between the NC group, SLE group, and SLE-BAFF groups. During the GO enrichment analysis, we specifically chose GO Level 2 and filtered out GO entries that had more than 2 differential gene counts in the biological process, cellular component, and molecular function categories. Fig. 11. A demonstrated that GO terms of biological adhesion, reproduction, reproductive process, cell junction, synapse, synapse part, enzyme regulator activity, protein binding transcription factor activity, protein tag, receiver regulator activity, and translation regulator activity were enriched in SLE group compared to the NC group, while GO terms of cell killing, catalytic activity, and transporter activity were significantly downregulated in the SLE group. In comparison to the NC group, the SLE-BAFF treatment group exhibited some notable GO terms, such as extracellular matrix, nucleoid, protein tag, antioxidant activity, receiver activity, structural motor activity, and channel regulator activity. However, the GO term for molecular transmitter activity was downregulated in the SLE-BAFF treatment group (Fig. 11B). In comparison to the SLE group, the SLE-BAFF treatment group showed significant enrichment of some GO terms, such as extracellular matrix, nucleoid, protein tag, receiver regulator activity, structural motor activity, and translation regulator activity. However, the GO term of molecular transmitter activity was downregulated in the SLE-BAFF treatment group (Fig. 11C).

Regarding KEGG enrichment analysis, we chose pathway entries based on more than 2 differentially expressed genes, which were arranged in descending order based on the $-\log_{10}p$-value associated with each entry. Figure 12 displayed the gene bubble diagram featuring the top 20 genes. In comparison to the NC group, the SLE group showed significant enrichment of a number of KEGG pathways, such as allograft rejection, graft *versus* host disease, antigen processing and presentation, cell adhesion molecules, Th1 and Th2 cell differentiation, and Th17 cell differentiation (Fig. 12A). However, only the pathway related to natural killer cells mediated cytotoxicity showed downregulation in the SLE group (Fig. 12B). In comparison to the NC group, the SLE-BAFF group showed considerable enrichment of ribosome, ferroptosis, and other KEGG pathways (Fig. 12C). Similarly, KEGG pathways of apoptosis, natural killer cell mediated cytotoxicity, regulation of active cytotoxicity showed a significant decrease in the SLE-BAFF group (Fig. 12D). In comparison to the SLE group, the SLE-BAFF group showed a significant enrichment of the ribosome pathway (Fig. 12E). Additionally, KEGG pathways of cell adhesion molecules, haematopoietic cell lineage, Th17 cell differentiation, Th1 and Th2 cell differentiation, were significantly downregulated in the SLE-BAFF group (Fig. 12F). The KEGG Level2 horizontal distribution map showed the differentially expressed genes (DEGs) that were enriched in various KEGG pathways (Fig. 13).

The GO and KEGG enrichment analyses of all DEGs between groups might play critical roles in SLE through intricate biological functions and signal transduction mechanisms mentioned above. BAFF treatment might exert regulatory effects on these DEGs and mediate immune and inflammatory re-

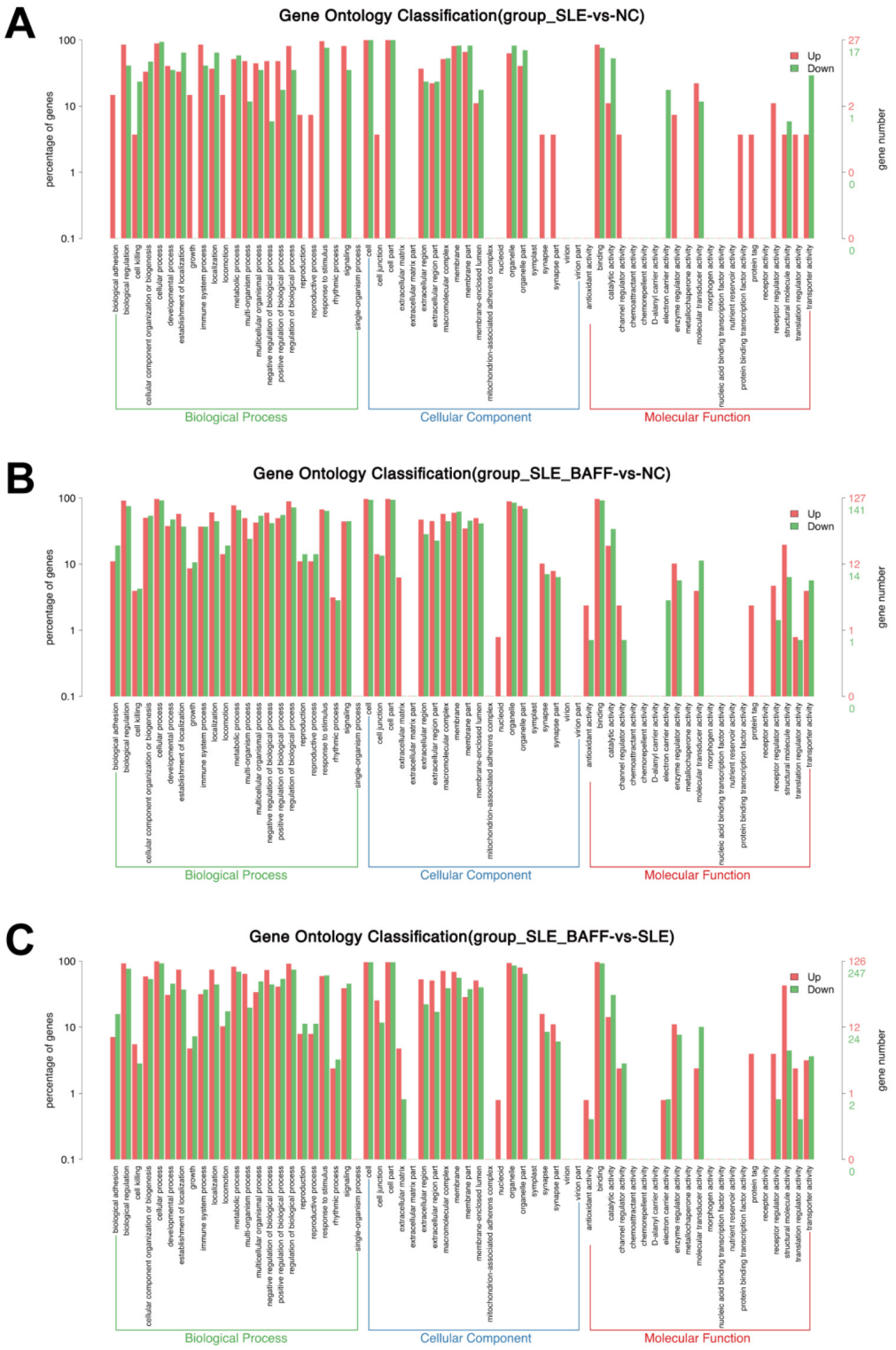

Fig. 11. GO enrichment analyses of all DEGs between groups at the GO Level 2 level.
A: Comparison chart of differentially expressed genes (GO) between NC group and SLE group.
B: Comparison of GO genes between NC group and SLE-BAFF group.
C: Comparison of GO genes between SLE group and SLE-BAFF group.

sponses in SLE, which warranted further investigations in future studies.

Discussion

BAFF or BLyS, a key activator of B lymphocytes, is critical for the survival, differentiation, and activation of B cells, thereby maintaining reactive B cells and preventing their depletion (26-28). The upregulation of BAFF has been shown to enhance B cell activation, with the interaction between BAFF and its receptor BAFF-R facilitating B cell maturation and plasma cell survival (29). In animal studies, the introduction of transgenic mice expressing BAFF can induce a SLE-like phenotype, whereas the use of BAFF antagonists has been demonstrated to relieve disease activity (30). Elevated levels of circulating BAFF are demonstrated in mice genetically predisposed to spontaneous SLE, such as NZBWF1 and MRL/*MpJ-Fas*^{LPR} mice, while treatment with BAFF antagonists

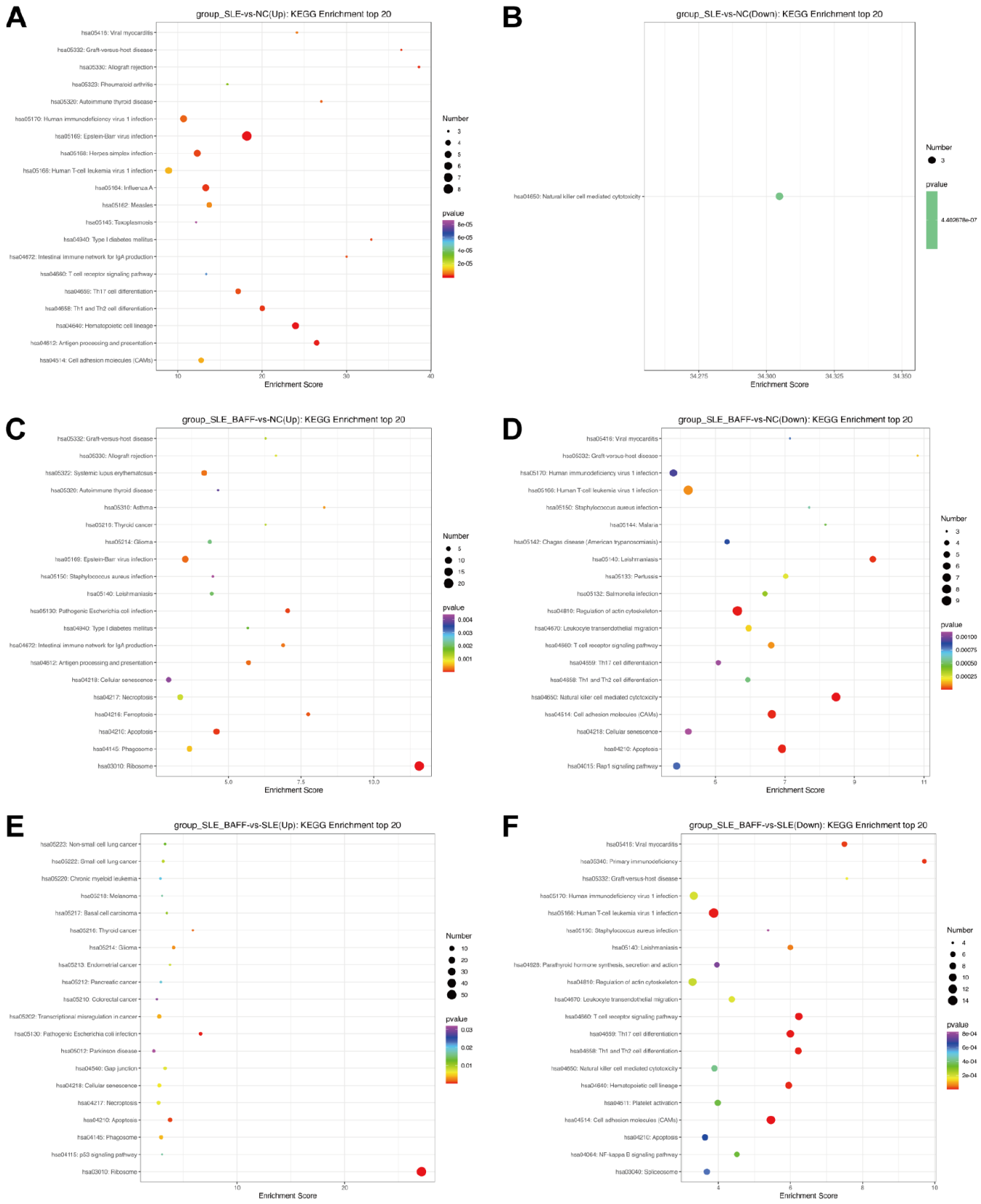

Fig. 12. Bubble diagram of Top 20 upregulated and downregulated KEGG enrichment pathways.
A: The NC group and SLE group showed an upregulation of the top 20 pathway bubble chart. **B**: Bubble plots of the top 20 downregulated pathways in the NC and SLE groups. **C**: The NC group and SLE-BAFF group showed an upregulation of the top 20 pathway bubble chart. **D**: Bubble plots of the top 20 downregulated pathways in the NC group and SLE-BAFF group. **E**: The SLE group and SLE-BAFF group showed an upregulation of the top 20 pathway bubble chart. **F**: The top 20 downregulated pathways in the SLE group and SLE-BAFF group are shown in the bubble chart.

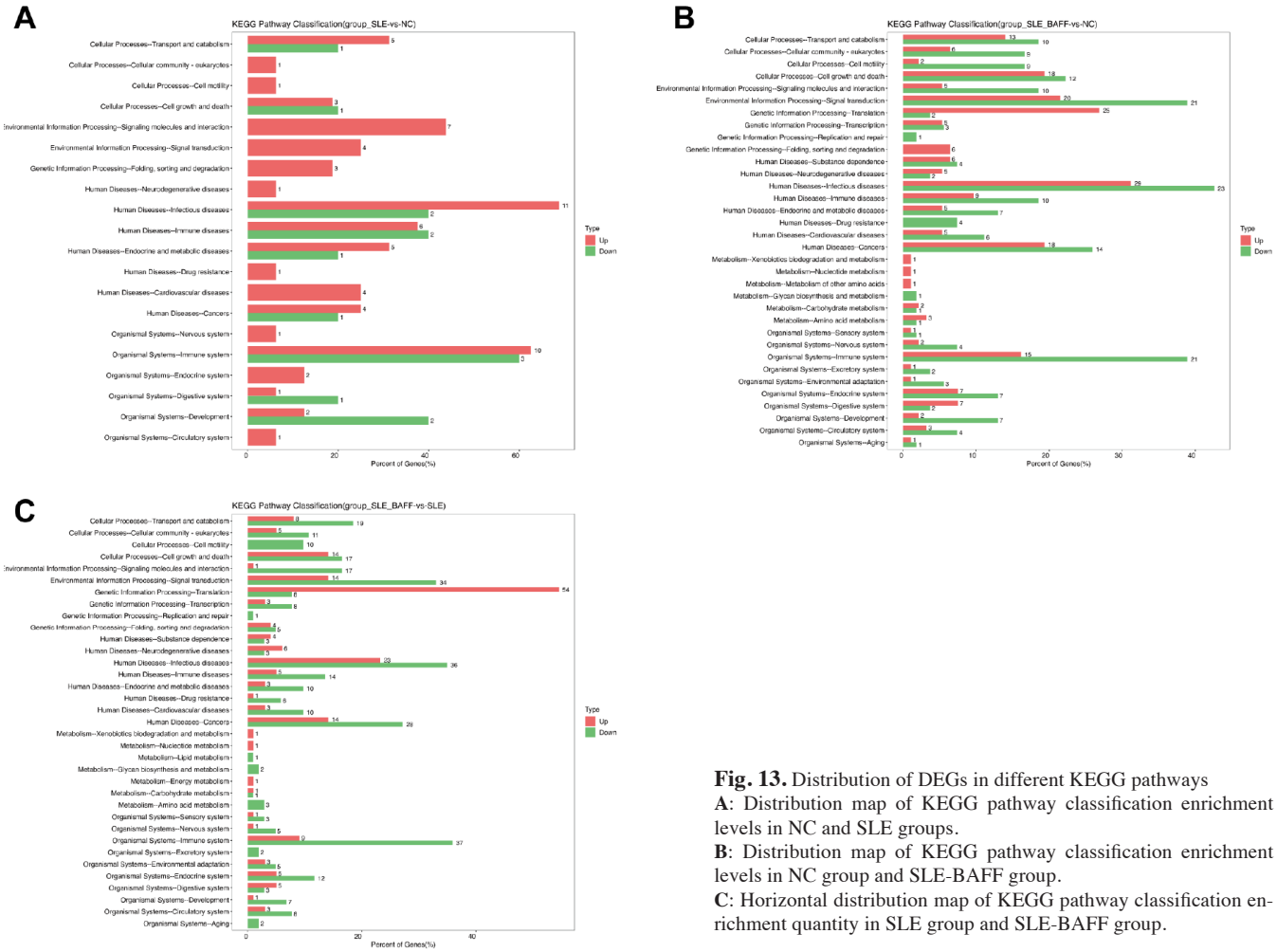

Fig. 13. Distribution of DEGs in different KEGG pathways
A: Distribution map of KEGG pathway classification enrichment levels in NC and SLE groups.
B: Distribution map of KEGG pathway classification enrichment levels in NC group and SLE-BAFF group.
C: Horizontal distribution map of KEGG pathway classification enrichment quantity in SLE group and SLE-BAFF group.

has shown promise in improving disease progression in these lupus mice models (29). In human populations, it has been well documented that more than half of SLE patients exhibited an increased level of BAFF, which was correlated with high level of anti-dsDNA antibodies and enhanced disease activity (29). Furthermore, BAFF has been shown to modulate B-cell homeostasis by activating the PI3K/Akt/mTOR and NF-κB signalling pathways through BAFF/BAFF-R interaction (31, 32). These findings have well supported the critical role of BAFF in SLE pathogenesis and the promising application of BAFF antagonism in the treatment of this disease. This study is aimed to investigate SLE-specific immune cell subsets and the influence of belimumab treatment on the immune cell landscape in SLE by a series experiments including scRNA-seq, offering novel perspectives and implications on the anti-BAFF therapy for SLE patients.

The balance of the immune system is crucial for maintaining human body health. Accumulated studies have demonstrated that the dysregulation of immune cell differentiation, polarisation and function, specifically T, B, NK lymphocytes and monocytes, plays a significant role in the development and progression of SLE (15, 33). As a prevalent autoimmune disorder, SLE is distinguished by an imbalance in the differentiation of lymphocyte and monocyte subsets, along with impaired cellular function (22, 23). The pathogenesis of SLE is primarily driven by abnormal B cell activation, the generation of excessive autoantibodies, immune complex formation, and complement system activation (30). The disease activity of SLE is closely linked to heightened B cell activation. Autoantibodies in SLE patients are predominantly derived from autoallergic B cells expressing high levels of CD27 and CD38 (34). It has been demonstrated that B cells, specifically plasma cells and long-lived plasma cells, are the primary sources of autoantibodies, contributing to the maintenance of long-sustained inflammation and immune disorders in the body through the generation of pro-inflammatory factors and immune complexes (35). Wang *et al*. (36) found a significant increase in $CD11c^{hi}T$ bet B cells in patients with SLE, which have the potentials to differentiate into autoreactive plasma cells and play a crucial role in the pathogenesis of SLE. The involvement of B cells in SLE is multifaceted. A previous study has implicated the autoantibody-secreting B cell played a significant role in the progression of SLE (35). Therefore, targeting B cell differentiation, activation, and plasma cell generation is essential for exploring the effective SLE therapy. The current study has shown similar findings by scRNA-seq and flow cytometry analyses. We have found that the total percentage of $CD19^{+}$B cell in

the peripheral blood of SLE patients was slightly increased compared to NC group but without statistical significance. However, the proportion of $CD19^+$B cell was significantly lower in the peripheral blood of SLE patients treated with belimumab compared to Belimumab-untreated SLE patients, suggesting the pivotal role of B cell in SLE pathogenesis and the response to belimumab treatment. This trend was also observed in SLE patients after belimumab therapy compared to the same patients before belimumab therapy, demonstrating the significantly inhibitory effect of belimumab on $CD19^+$B cell in SLE. These findings are consistent with previous publications, suggesting that belimumab primarily functions by suppressing B cell proliferation and activation in the treatment of SLE (29, 37, 38).

In addition to B cells, the dysregulation of T cells is recognised as a pivotal factor in the SLE pathogenesis (29). Previous research has demonstrated that elevated levels of OX40L in B cells could contribute to the proliferation of T follicular helper (Tfh) cells, thereby promoting B cell activation and the onset of SLE (31). Tfh cells are implicated in the regulation of B cell activation and function (39, 40). Furthermore, B cells have the capability to initiate a cascade of inflammatory processes in SLE by activating T cells (21, 41). Therefore, T cell response and the interaction between B cells and T cells are closely related to SLE. In this study, the SLE-specific T cell subpopulations in the peripheral circulation of patients with SLE have been well documented, notably characterised by a reduction in $CD3^+CD4^+$T cells and a marked elevation in $CD3^+$T and $CD8^+$T cells in SLE. Besides, a notable increase in the percentages of $CD3^+PD\text{-}1^+$T, and $CD8^+PD\text{-}1^+$T cells in the peripheral blood of SLE patients compared to healthy controls, suggesting $CD3^+PD\text{-}1^+$T and $CD8^+PD\text{-}1^+$T cells as disease-specific T cell subsets in SLE. These T cell subsets might confer important effects in the development and progression of SLE. Following the administration of belimumab, a notable rise in the peripheral blood of $CD3^+$T cells was observed in SLE patients after belimumab treatment, suggesting that belimumab might exert therapeutic effects in SLE by expanding $CD3^+$T cells. Furthermore, a significant reduction in the proportion of $CD3^+PD\text{-}1^+$T cells in the peripheral blood was observed SLE patients treated with belimumab compared to belimumab-untreated SLE patients. Accordingly, as a BLyS inhibitor, belimumab plays a crucial role in the regulation of $CD3^+PD\text{-}1^+$T cell response, thereby exerting its therapeutic effect in SLE. Nonetheless, more future studies are warranted to elucidate the precise molecular mechanisms through which belimumab regulates $CD3^+$T cell and $CD3^+PD\text{-}1^+$T cell in SLE, in order to better guide and improve the therapeutic efficacy of belimumab.

Increasing evidence has suggested that the dysregulated activation and function of monocytes plays an important role in the pathogenesis of autoimmune diseases (42). The aberrant differentiation and phenotypic polarisation of monocytes/macrophages has been demonstrated in SLE (43). Expansion and abnormal activation of monocytes/macrophages is distinctive in SLE-prone mice, with monocytes comprising approximately more than 50% of white blood cells in elderly BXSB male mice than healthy mice (42, 44). Monocytes have the potential to differentiate into pro-inflammatory M1 cells or anti-inflammatory/immunosuppressive M2 cells under specific immune microenvironment (45). Currently available studies have demonstrated an imbalanced polarisation of monocytes/macrophages in SLE (45, 46). Moreover, the inadequate clearance of apoptotic cells due to abnormal phagocytic and efferocytotic functions of monocytes/macrophages also contributes to the development of SLE (47-49). In the NZB/W lupus mouse model, the TLR2/1 agonist PAM3 has been shown to improve the outcome of SLE by promoting the differentiation of M2 macrophages (50), implicating that the vital role of M1/M2 balance in maintaining homeostasis. In the present study, the scRNA-seq has revealed significant alterations in monocyte subpopulations in the peripheral circulation of SLE patients, particularly in patients following treatment with belimumab. The peripheral blood M2-type subpopulations of $CD14^+CD206^+$ and $CD14^+CD163^+$ monocytes were obviously reduced in SLE, suggesting the important role of M1/M2 macrophage imbalance in SLE pathogenesis. However, the administration of belimumab did not yield a significant impact on the differentiation of $CD14^+CD206^+$ and $CD14^+CD163^+$ monocytes in SLE. Although there is significant heterogeneity of peripheral blood monocytes/macrophages subsets in SLE, characterised by reduced M2-type monocytes, more studies are needed to clarify the regulatory roles of $CD14^+CD206^+$ and $CD14^+CD163^+$ monocytes in SLE onset and progression. Furthermore, it can be concluded that belimumab has no significant impacts on the heterogeneity of peripheral blood monocyte subsets in SLE.

As a critical cell in the innate immune system, NK cell plays an important role in the regulation of immune response and the maintenance of immune balance (51). It has been shown that restoring the normal cellular function of NK cells via targeting SLAMF7 and CD38 led to a reduction in plasma cell level and the improvement in SLE (52). Our research has found a significant decrease in $CD16^+CD56^+$NK cell in the peripheral blood of SLE patients, whereas the belimumab therapy did not affect the proportion of this NK cell subpopulation. However, a previous study has shown the evidence that decreased NK cell in blood and salivary was related to a better response to belimumab treatment in primary Sjögren's syndrome (53). Despite mounting evidence suggesting the significance of NK cells in SLE, the existing data remains insufficient to conclusively support their representative importance in the pathogenesis of the disease, as well as the influence of belimumab treatment on NK cell proliferation and activity.

There are several limitations needing to be considered when interpreting the data in the present study. The first limitation of this study is the restricted sample size in the scRNA-seq and flow cytometry analyses illustrating the influence of belimumab treatment on the M1/M2 imbalance in SLE. Besides, age bias exists in this study, which should

be thought about in this investigation. However, the findings by eliminating the effect of age are still consistent with the original data in this study, suggesting the significant effect of belimumab treatment on lymphocytes differentiation in SLE. Lastly, the potential effects and regulatory mechanisms of belimumab on SLE-specific immune cells validated in this study, warrant to be investigated in a series of cellular and animal experiments *in vitro* and *in vivo*.

Conclusion

Belimumab, an approved drug for clinical treatment over the past six decades, has been validated in certain clinical trials for its efficacy and safety in managing SLE. Nevertheless, the impact of belimumab on the differentiation and function of immune cells in SLE remains poorly understood. Our study reveals the significant heterogeneity of immune cells in the peripheral circulation of patients with SLE, which underscores the necessity for additional investigations into the molecular pathways of belimumab in regulating inflammation and autoimmunity in SLE. Belimumab therapy is a promising approach for SLE patients, particularly those resistant to achieving clinical remission. In addition to the suppressive effect on $CD19^+$B cells, belimumab may participate into regulating the proliferation, phenotypes and functions of $CD3^+PD\text{-}1^+$T cells, thereby exerting therapeutic effects in SLE. Further comprehensive investigations are warranted to elucidate the precise regulatory mechanisms of belimumab on SLE-specific immune cells to better guide the clinical use of the anti-BAFF reagent in SLE treatment.

References

1. SIEGEL CH, SAMMARITANO LR: Systemic lupus erythematosus: a review. *JAMA* 2024; 331(17): 1480-91. https://doi.org/10.1001/jama.2024.2315
2. PISETSKY DS: Unique interplay between antinuclear antibodies and nuclear molecules in the pathogenesis of systemic lupus erythematosus. *Arthritis Rheumatol* 2024; 76(9): 1334-43. https://doi.org/10.1002/art.42863
3. HOI A, IGEL T, MOK CC, ARNAUD L: Systemic lupus erythematosus. *Lancet* 2024; 403(10441): 2326-38. https://doi.org/10.1016/s0140-6736(24)00398-2
4. FAVA A, PETRI M: Systemic lupus erythematosus: diagnosis and clinical management. *J Autoimmun* 2019; 96: 1-13. https://doi.org/10.1016/j.jaut.2018.11.001
5. FANOURIAKIS A, TZIOLOS N, BERTSIAS G, BOUMPAS DT: Update on the diagnosis and management of systemic lupus erythematosus. *Ann Rheum Dis* 2021; 80(1): 14-25. https://doi.org/10.1136/annrheumdis-2020-218272
6. WANG S, YANG N, ZHANG H: Metabolic dysregulation of lymphocytes in autoimmune diseases. *Trends Endocrinol Metab* 2024; 35(7): 624-37. https://doi.org/10.1016/j.tem.2024.01.005
7. HERRADA AA, ESCOBEDO N, IRURETAGOYENA M *et al*.: Innate immune cells' contribution to systemic lupus erythematosus. *Front Immunol* 2019; 10: 772. https://doi.org/10.3389/fimmu.2019.00772
8. DAI D, GU S, HAN X *et al*.: The transcription factor ZEB2 drives the formation of age-associated B cells. *Science* 2024; 383(6681): 413-21. https://doi.org/10.1126/science.adf8531
9. CROW MK: Pathogenesis of systemic lupus erythematosus: risks, mechanisms and therapeutic targets. *Ann Rheum Dis* 2023; 82(8): 999-1014. https://doi.org/10.1136/ard-2022-223741
10. BARNAS JL, LOONEY RJ, ANOLIK JH: B cell targeted therapies in autoimmune disease. *Curr Opin Immunol* 2019; 61: 92-9. https://doi.org/10.1016/j.coi.2019.09.004
11. HAMILTON JA, HSU HC, MOUNTZ JD: Autoreactive B cells in SLE, villains or innocent bystanders? *Immunol Rev* 2019; 292(1): 120-38. https://doi.org/10.1111/imr.12815
12. FILLATREAU S, MANFROI B, DöRNER T: Toll-like receptor signalling in B cells during systemic lupus erythematosus. *Nat Rev Rheumatol* 2021; 17(2): 98-108. https://doi.org/10.1038/s41584-020-00544-4
13. LI H, BOULOUGOURA A, ENDO Y, TSOKOS GC: Abnormalities of T cells in systemic lupus erythematosus: new insights in pathogenesis and therapeutic strategies. *J Autoimmun* 2022; 132: 102870. https://doi.org/10.1016/j.jaut.2022.102870
14. CRISPIN JC, HEDRICH CM, SUÁREZ-FUEYO A, COMTE D, TSOKOS GC: SLE-associated defects promote altered T cell function. *Crit Rev Immunol* 2017; 37(1): 39-58. https://doi.org/10.1615/critrevimmunol.2018025213
15. ROBINSON GA, WILKINSON MGL, WINCUP C: The role of immunometabolism in the pathogenesis of systemic lupus erythematosus. *Front Immunol* 2021; 12: 806560. https://doi.org/10.3389/fimmu.2021.806560
16. XIAO ZX, MILLER JS, ZHENG SG: An updated advance of autoantibodies in autoimmune diseases. *Autoimmun Rev* 2021; 20(2): 102743. https://doi.org/10.1016/j.autrev.2020.102743
17. BLAIR HA, DUGGAN ST: Belimumab: a review in systemic lupus erythematosus. *Drugs* 2018; 78(3): 355-66. https://doi.org/10.1007/s40265-018-0872-z
18. FURIE R, ROVIN BH, HOUSSIAU F *et al*.: Two-year, randomized, controlled trial of belimumab in lupus nephritis. *N Engl J Med* 2020; 383(12): 1117-28. https://doi.org/10.1056/nejmoa2001180
19. BASTA F, FASOLA F, TRIANTAFYLLIAS K, SCHWARTING A: Systemic lupus erythematosus (SLE) therapy: the old and the new. *Rheumatol Ther* 2020; 7(3): 433-46. https://doi.org/10.1007/s40744-020-00212-9
20. PARODIS I, VITAL EM, HASSAN SU *et al*.: De novo lupus nephritis during treatment with belimumab. *Rheumatology* (Oxford) 2021; 60(9): 4348-54. https://doi.org/10.1093/rheumatology/keaa796
21. ARINGER M, COSTENBADER K, DAIKH D *et al*.: 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2019; 71(9): 1400-12. https://doi.org/10.1002/art.40930
22. BUTLER A, HOFFMAN P, SMIBERT P, PAPALEXI E, SATIJA R: Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* 2018; 36(5): 411-20. https://doi.org/10.1038/nbt.4096
23. MACOSKO EZ, BASU A, SATIJA R *et al*.: Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* 2015; 161(5): 1202-14. https://doi.org/10.1016/j.cell.2015.05.002
24. ARAN D, LOONEY AP, LIU L *et al*.: Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol* 2019; 20(2): 163-72. https://doi.org/10.1038/s41590-018-0276-y
25. LI M, LI C, CAO M *et al*.: Incidence and prevalence of systemic lupus erythematosus in urban China, 2013-2017: A nationwide population-based study. *Sci Bull (Beijing)* 2024; 69(19): 3089-97. https://doi.org/10.1016/j.scib.2024.04.075
26. GUPTA K, KESHARWANI A, RUA S *et al*.: BAFF blockade in experimental autoimmune encephalomyelitis reduces inflammation in the meninges and synaptic and neuronal loss in adjacent brain regions. *J Neuroinflammation* 2023; 20(1): 229. https://doi.org/10.1186/s12974-023-02922-7
27. ALFARO R, RODRíGUEZ-AGUILAR L, LLORENTE S *et al*.: Early cytomegalovirus reactivation in renal recipients is associated with high levels of B cell maturation antigen transcript expression prior to transplantation. *Int J Mol Sci* 2023; 24(13). https://ddoi.org/10.3390/ijms241310491
28. PATTARABANJIRD T, LI C, MCNAMARA C: B cells in atherosclerosis: mechanisms and potential clinical applications. *JACC Basic Transl Sci* 2021; 6(6): 546-63. https://doi.org/10.1016/j.jacbts.2021.01.006
29. MÖCKEL T, BASTA F, WEINMANN-MENKE J, SCHWARTING A: B cell activating factor (BAFF): Structure, functions, autoimmunity and clinical implications in systemic lupus erythematosus (SLE). *Autoimmun Rev* 2021; 20(2): 102736. https://doi.org/10.1016/j.autrev.2020.102736
30. KRUSTEV E, CLARKE AE, BARBER MRW: B cell depletion and inhibition in systemic lupus erythematosus. *Expert Rev Clin Immunol* 2023; 19(1): 55-70. https://doi.org/10.1080/1744666x.2023.2145281
31. MCALLISTER E, JELLUSOVA J: BAFF signaling in B cell metabolism. *Curr Opin Immunol* 2021; 71: 69-74.

https://doi.org/10.1016/j.coi.2021.05.011
32. QI W, TIAN J, WANG G *et al*.: Advances in cellular and molecular pathways of salivary gland damage in Sjögren's syndrome. *Front Immunol* 2024; 15: 1405126. https://doi.org/10.3389/fimmu.2024.1405126
33. PSARRAS A, CLARKE A: A cellular overview of immunometabolism in systemic lupus erythematosus. *Oxf Open Immunol* 2023; 4(1): iqad005. https://doi.org/10.1093/oxfimm/iqad005
34. SUURMOND J, ATISHA-FREGOSO Y, MARASCO E *et al*.: Loss of an IgG plasma cell checkpoint in patients with lupus. *J Allergy Clin Immunol* 2019; 143(4): 1586-97. https://doi.org/10.1016/j.jaci.2018.10.041
35. PARODIS I, GATTO M, SJöWALL C: B cells in systemic lupus erythematosus: Targets of new therapies and surveillance tools. *Front Med* (Lausanne) 2022; 9: 952304. https://doi.org/10.3389/fmed.2022.952304
36. WANG S, WANG J, KUMAR V *et al*.: IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c(hi)T-bet(+) B cells in SLE. *Nat Commun* 2018; 9(1): 1758. https://doi.org/10.1038/s41467-018-03750-7
37. WANG J, JU B, ZHU L *et al*.: The rapid inhibition of B-cell activation markers by belimumab was associated with disease control in systemic lupus erythematosus patients. *Front Pharmacol* 2023; 14: 1080730. https://doi.org/10.3389/fphar.2023.1080730
38. ARBITMAN L, FURIE R, VASHISTHA H: B cell-targeted therapies in systemic lupus erythematosus. *J Autoimmun* 2022; 132: 102873. https://doi.org/10.1016/j.jaut.2022.102873
39. WEI X, NIU X: T follicular helper cells in autoimmune diseases. *J Autoimmun* 2023; 134: 102976. https://doi.org/10.1016/j.jaut.2022.102976
40. BOCHARNIKOV AV, KEEGAN J, WACLECHEVS *et al*.: PD-1hiCXCR5- T peripheral helper cells promote B cell responses in lupus via MAF and IL-21. *JCI Insight* 2019; 4(20). https://doi.org/10.1172/jci.insight.130062
41. XUE J, XU L, ZHONG H *et al*.: Impaired regulatory function of granzyme B-producing B cells against T cell inflammatory responses in lupus mice. *Lupus Sci Med* 2023; 10(2). https://doi.org/10.1136/lupus-2023-000974
42. HIROSE S, LIN Q, OHTSUJI M, NISHIMURA H, VERBEEK JS: Monocyte subsets involved in the development of systemic lupus erythematosus and rheumatoid arthritis. *Int Immunol* 2019; 31(11): 687-96. https://doi.org/10.1093/intimm/dxz036
43. JIN Z, FAN W, JENSEN MA *et al*.: Single-cell gene expression patterns in lupus monocytes independently indicate disease activity, interferon and therapy. *Lupus Sci Med* 2017; 4(1): e000202. https://doi.org/10.1136/lupus-2016-000202
44. SANTIAGO-RABER ML, AMANO H, AMANO E *et al*.: Fcgamma receptor-dependent expansion of a hyperactive monocyte subset in lupus-prone mice. *Arthritis Rheum* 2009; 60(8): 2408-17. https://doi.org/10.1002/art.24787
45. KADOMOTO S, IZUMI K, MIZOKAMI A: Macrophage polarity and disease control. *Int J Mol Sci* 2021; 23(1). https://doi.org/10.3390/ijms23010144
46. MA C, XIA Y, YANG Q, ZHAO Y: The contribution of macrophages to systemic lupus erythematosus. *Clin Immunol* 2019; 207: 1-9. https://doi.org/10.1016/j.clim.2019.06.009
47. KWANT LE, VEGTING Y, TSANG ASMWP *et al*.: Macrophages in lupus nephritis: exploring a potential new therapeutic avenue. *Autoimmun Rev* 2022; 21(12): 103211. https://doi.org/10.1016/j.autrev.2022.103211
48. LIU Y, LUO S, ZHAN Y *et al*.: Increased expression of PPAR-γ modulates monocytes Into a M2-like phenotype in SLE patients: an implicative protective mechanism and potential therapeutic strategy of systemic lupus erythematosus. *Front Immunol* 2020; 11: 579372. https://doi.org/10.3389/fimmu.2020.579372
49. FERRETé-BONASTRE AG, MARTíNEZ-GALLO M, MORANTE-PALACIOS O *et al*.: Disease activity drives divergent epigenetic and transcriptomic reprogramming of monocyte subpopulations in systemic lupus erythematosus. *Ann Rheum Dis* 2024; 83(7): 865-78. https://doi.org/10.1136/ard-2023-225433
50. HORULUOGLU B, BAYIK D, KAYRAKLIOGLU N, GOGUET E, KAPLAN MJ, KLINMAN DM: PAM3 supports the generation of M2-like macrophages from lupus patient monocytes and improves disease outcome in murine lupus. *J Autoimmun* 2019; 99: 24-32. https://doi.org/10.1016/j.jaut.2019.01.004
51. HOJJATIPOUR T, ASLANI S, SALIMIFARD S *et al*.: NK cells - Dr. Jekyll and Mr. Hyde in autoimmune rheumatic diseases. *Int Immunopharmacol* 2022; 107: 108682. https://doi.org/10.1016/j.intimp.2022.108682
52. HUMBEL M, BELLANGER F, FLUDER N *et al*.: Restoration of NK cell cytotoxic function with elotuzumab and daratumumab promotes elimination of circulating plasma cells in patients with SLE. *Front Immunol* 2021; 12: 645478. https://doi.org/10.3389/fimmu.2021.645478
53. SEROR R, NOCTURNE G, LAZURE T *et al*.: Low numbers of blood and salivary natural killer cells are associated with a better response to belimumab in primary Sjögren's syndrome: results of the BELISS study. *Arthritis Res Ther* 2015; 17(1): 241. https://doi.org/10.1186/s13075-015-0750-y