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Abnormally high expression of D1-like dopamine receptors on lupus CD4$^+$ T cells promotes Tfh cell differentiation

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ABSTRACT

Objective SLE is a chronic autoimmune disease that places a great burden on human society. T follicular helper (Tfh) cells play a critical role in the pathological process of SLE. Therefore, elucidating the mechanism of Tfh cell differentiation will contribute to SLE treatment. Dopamine receptors (DRDs) are members of the family of G protein-coupled receptors and are primarily divided into D1-like and D2-like receptors. Previous studies have found that DRDs can regulate differentiation of immune cells. However, there is currently a lack of research on DRDs and Tfh cells. We here explore the relationship between DRDs and Tfh cells, and analyse the relationship between DRD expression on Tfh cells and the course of SLE.

Methods We first detected plasma catecholamine concentrations in patients with SLE and healthy controls by mass spectrometry, followed by reverse transcription-quantitative PCR (RT-qPCR) to detect DRD messenger RNA (mRNA) expression in peripheral blood mononuclear cells (PBMCs) and CD4$^+$ T cells, and flow cytometry to detect DRD expression in Tfh cells. Finally, in vitro experiments and RNA sequencing (RNA-seq) were used to explore the possible pathway by which DRDs regulate Tfh cell differentiation.

Results The plasma dopamine concentration in patients with SLE was significantly increased, and abnormal mRNA expression of DRDs was observed in both PBMCs and CD4$^+$ T cells. The results of flow cytometry showed that D1-like receptors were highly expressed in Tfh cells of patients with SLE and associated with disease activity. In vitro induction experiments showed that differentiation of naïve T cells into Tfh cells was accompanied by an increase in D1-like receptor expression. RNA-seq and RT-qPCR results indicate that D1-like receptors might promote Tfh cell differentiation through the Phosphatidylinositol3-kinase (PI3K)/protein kinase B (AKT)/Forkhead box protein O1 (FOXO1)/Kruppel-like factor 2 (Klf2) pathway.

Conclusion Tfh cells in patients with SLE highly express D1-like receptors, which correlate with disease activity. D1-like receptors may promote Tfh cell differentiation through the PI3K/AKT/FOXO1/Klf2 pathway.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ SLE is a chronic inflammatory autoimmune disease that is closely related to follicular helper T (Tfh) cells; therefore, knowledge of the mechanism of Tfh cell differentiation may contribute to treatment of SLE.
⇒ Currently, there are still many gaps to be filled regarding the differentiation mechanism of Tfh cells.
⇒ Dopamine receptors (DRDs) are an important class of neurotransmitters that are mainly divided into D1-like and D2-like receptors.
⇒ DRDs have been proven to be expressed in T cells and can regulate T-cell differentiation.
⇒ The expression of DRDs varies greatly depending on the cell type, and the type of DRDs can also affect their regulatory function on T cells; therefore, it is necessary to conduct specific analysis for different cell types.
⇒ There is currently a lack of research on the relationship between DRDs and Tfh cells.

WHAT THIS STUDY ADDS

⇒ Our research shows that D1-like DRDs might promote differentiation of Tfh cells through the PI3K/AKT/FOXO1/Klf2 pathway, and that expression of D1-like receptors in Tfh cells of patients with SLE correlates positively with disease activity.
⇒ Our results fill a gap in the regulatory mechanism of Tfh cells and reveal the close relationship between D1-like receptors and the course of SLE.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our research suggests that D1-like receptors in Tfh cells may serve as effective therapeutic targets for SLE, providing new ideas for the treatment of SLE.

INTRODUCTION

SLE is a chronic autoimmune disease characterised by the presence of autoantibodies. These autoantibodies bind to autoantigens and form immune complexes, which accumulate in the whole body through the bloodstream and activate complement and cytokines, leading to multiorgan inflammation and tissue damage.[1 2] As the disease progresses, patients often have multiple complications, among which neuropathy and kidney damage are the most common.[3 4] Activation of the SLE immune system features over-reaction of B and T cells. In other words, there is a general breakdown in the immune

tolerance of B and T cells in SLE, causing activation of antigen-mediated changes, which manifests as amplification of early signal events and generation of a stronger immune response.[5] According to data from the US lupus registry, the number of patients with SLE in North America reaches 23.2/100 000, and the prevalence of SLE is higher among women and non-white individuals due to genetic and environmental factors.[6–8] The estimated prevalence rate of the Chinese population is approximately 50–100/100 000.[9] In addition, China has a large population, and SLE has become an important disease burden affecting the health of the Chinese people.

An important part of SLE pathogenesis is production of autoantibodies. The B cells that secrete autoantibodies are mainly located in germinal centres (GCs), which are the major site where B cells mature and differentiate into plasma cells.[10] Tfh cells are another major cell type in GCs and are a subset of $CD4^+$ helper T cells (Th). Tfh cells express high levels of chemokine receptor 5 (CXCR5), B-cell lymphoma 6 (Bcl-6) and programmed cell death protein 1 (PD-1), which play a critical role in high-affinity B-cell selection and help B-cell differentiation.[11] Therefore, Tfh cells may participate in the course of SLE by promoting the GC B-cell response. In patients with SLE, Tfh cell frequency is significantly increased and correlates with disease activity. In addition, inhibition of Tfh cell frequency can significantly relieve SLE.[12–14] However, GCs are transient microanatomical structures formed in secondary lymphoid organs (SLOs). Since SLOs are difficult to obtain in humans, circulating Tfh (cTfh) cells are usually used for studies.[15]

Other organs mainly regulate the immune system via interactions with surface receptors of immune cells. Previous studies have shown that neurotransmitters and their respective receptors could serve as common mediators in the neuroendocrine-immune network, causing the body respond appropriately to changes in the internal and external environment.[16] Catecholamines are important neurotransmitters, including dopamine and its downstream norepinephrine as well as epinephrine. Dopamine is a pivotal component among the transmitters that participate in neuroimmunity and exerts physiological function by combining with the corresponding receptors. Dopamine receptors (DRDs) are members of the G protein-coupled receptor family and are mainly divided into D1-like and D2-like receptors. D1-like receptors include DRD1 and DRD5, and DRD2, DRD3 and DRD4 are categorised as D2-like receptors.[17] Flow cytometry results show that DRDs are expressed in T cells.[18] This may indicate the crucial role of DRDs in immune regulation. Recently, Jafari *et al* found that expression of DRDs in T cells of patients with SLE was significantly abnormal.[19] In addition, some studies have shown that DRDs can regulate activation and differentiation of several subsets of T cells, such as T helper cell 17 (Th17) and regulatory T cells (Tregs), and participate in the course of autoimmune diseases in this way.[20 21] However, whether DRDs can regulate Tfh cell differentiation and the relationship between DRDs and SLE remain unclear.

Based on the above evidence, we sought to explore the relationship between DRDs and SLE and whether DRDs are involved in regulation of Tfh cells.

MATERIALS AND METHODS

Study subjects

In this study, 95 patients with SLE from the Second Xiangya Hospital of Central South University were recruited. We excluded patients with other autoimmune, infectious or inflammatory diseases. Disease activity was assessed by the SLE Disease Activity Index (SLEDAI) score in patients with SLE. Seventy-nine sex-matched and age-matched healthy controls (HCs) were recruited from hospital health administration centres. Patients with active SLE are defined as SLEDAI scores >4, while patients with inactive SLE are defined as SLEDAI scores ≤4. Detailed information can be found in online supplemental table 1,2.

Reverse transcription-quantitative PCR

After washing the cells twice with phosphate-buffered saline (PBS), RNA was extracted by TRIzol (AG, cat. 21102) according to the reagent manufacturer's instructions. The concentration of RNA was measured by a NanoDrop 2000c spectrophotometer (ND-2000, Thermo). The reverse transcription (RT) process was performed following the protocol (AG, cat. 11728). Quantitative PCR (qPCR) was performed by using SYBR Green Premix Ex Taq (AG, cat. 11701) and a thermocycler (Bio-Rad CFX Connect). Relative expression levels of target genes are expressed as a ratio of GAPDH values. The oligonucleotide primer sequences used for specific amplification are shown in online supplemental table 3.

Mass spectrometry

All of the serum catecholamines were determined by mass spectrometry at KingMed Diagnostics, Changsha, Hunan, China. A Waters Xevo TQ-S ultrahigh-performance liquid chromatography-tandem mass spectrometer with an Agilent Pursuit 3 PFP chromatographic column were used. Mobile phase A consisted of 0.2% formic acid water and mobile phase B of 0.2% formic acid methanol. The column temperature was 30°C and the flow rate 0.3 mL/min. Sample preparation included 200 µL serum added to 200 µL mixed isotope internal standard solution, followed by vortex mixing and centrifugation. The supernatant was added to a Waters WCX 96-well plate, and after solid-phase extraction, the target compound was eluted into the 96-well plate, dried with nitrogen at room temperature and redissolved with 200 µL 0.2% formic acid aqueous solution. Finally, sample injection analysis was performed.

Cell culture and drug treatment

Human peripheral blood mononuclear cells

After diluting whole blood with PBS in a 1:1 ratio, human peripheral blood mononuclear cells (PBMCs) were separated in Ficoll-Paque Premium medium (GE HealthCare, cat. 17-5442-03).

Human $CD4^+$ T cells

Human $CD4^+$ T cells were separated by immunomagnetic beads (Miltenyi Biotec, cat. 130-045-101). The procedure is as follows. PBMCs in human whole blood were separated by the Ficoll separation method and then centrifuged and resuspended in buffer. Buffer and anti-$CD4^+$ T microbeads (Miltenyi Biotec, cat. 130-045-101) were added at a proportion of 4:1, incubated on ice in the dark for 15 min and passed through the column. After the cell suspension had passed through the column, the sample was rinsed with buffer three times. Finally, 2 mL of buffer was added to the column, and the $CD4^+$ T cells were eluted. A total of 1.5×10^6 $CD4^+$ T cells per well were seeded in a 24-well plate precoated with anti-hCD3 antibody (Calbiochem, 2 µg/mL) and then treated with anti-hCD28 antibody (Calbiochem, 1 µg/mL). For the experimental group, the D1-like receptor agonist SKF38393 (TargetMol, 5 µM) or inhibitor SCH23390 (TargetMol, 50 µM) was added; an equal concentration of dimethyl sulfoxide (DMSO) was used as a control. The $CD4^+$ T cells were collected after 48 hours of culture, and the messenger RNA (mRNA) and protein expression levels of D1-like receptors were analysed.

Human $CD4^+$-naïve T-cell sorting and Tfh cell differentiation

Human $CD4^+$-naïve T cells were also separated by microbeads (Miltenyi Biotec, cat. 130-094-131). The procedure was as follows. PBMCs in human whole blood were separated by the Ficoll separation method and centrifuged and resuspended in buffer. Subsequently, anti-$CD4^+$-naïve T microbeads were added to isolate $CD4^+$-naïve T cells in a negative selection manner. A total of 1.5×10^6 $CD4^+$-naïve T cells per well were seeded in a 24-well plate precoated with anti-hCD3 antibody (Calbiochem, 2 µg/mL), and then treated with anti-hCD28 antibody (Calbiochem, 1 µg/mL) and several cytokines (Chamot Biotechnology), including interleukin (IL)-6 (20 ng/mL), transforming growth factor-β (5 ng/mL), IL-12 (10 ng/mL) and IL-21 (20 ng/mL). $CD4^+$-naïve T cells were collected after 72 hours and 120 hours of stimulation, and the messenger RNA (mRNA) and protein expression levels of D1-like receptors in $CD4^+$-naïve T cells were analysed.

Flow cytometry analysis

The PBMCs or sorted total $CD4^+$ T or differentiated Tfh cells were resuspended in PBS and incubated with surface-labelled antibodies on ice for 45 min in the dark. Experimental data were analysed by FlowJo (V.10.5.3). The antibodies used were as follows: FITC anti-hCD4 (eBioscience, clone SK3, cat. 11-0047-42), APCcy7 anti-hCXCR5 (Biolegend, clone J252D4, cat. 356926), PE-Cy7 anti-hPD-1 (Biolegend, clone EH12.2H7, cat. 329917), PerCP-Cy5.5 anti-hCD45RA (eBioscience, clone HI100, cat. 45-0458-42), PE anti-hDRD1 (Biolegend, clone L205G1, cat. 366404) and APC anti-hDRD5 (R&D Systems). All samples were examined using a BD FACSCanto II Flow Cytometer (USA, BD Biosciences).

RNA sequencing and bioinformatics analysis

RNA sequencing

RNA sequencing (RNA-seq) of all samples was performed by Novo Gene, Tianjin, China. The first strand of complementary DNA (cDNA) was synthesised using reverse transcriptase and random primers, and residual RNA was synthesised using RNA enzymes. Then, DNA polymerase and dNTP were used to synthesise second-strand cDNA. The 3′ end of the synthesised double-stranded DNA fragment was adenosylated and then connected via splice hybridisation. Finally, PCR amplification was performed, and the product was purified with AMPure XP beads to obtain a library.

Different databases were collected according to the target data volume, and an Illumina NovaSeq 6000 was used for sequential analysis, generating a 150 bp paired-end read. Four fluorescently labelled dNTP, DNA polymerase and splicing primers were added, followed by amplification. When the measured sequence extends the complementary chain, each fluorescently labelled dNTP releases corresponding fluorescence. The sequence information of the segment to be measured is obtained by capturing the fluorescence information and converting the optical signal to the sequencing peak through software.

Bioinformatics analysis

To identify differentially expressed genes, using R limma (V.4.2.2) and edge package for sequencing of the expression of matrix differential expression analysis, set $|\log FC|>1$, p value <0.05. Then, the enrichment analysis of differentially expressed genes was performed according to clusterProfiler. Finally, ggplot2 and pheatmap were used for visualisation.

Statistical analysis

All data were statistically analysed by SPSS V.24.0 and are expressed as the mean±SEM. The statistical significance of each index was calculated by a two-tailed unpaired t-test or paired t-test between two groups. When the data were not normally distributed, a two-tailed Mann-Whitney U test was used. Pearson's r test was used to analyse the correlation between normally distributed data, and Spearman's r test was used to analyse the correlation between non-normal data.

RESULTS

Aberrant expression of DRDs in patients with SLE

First, we detected catecholamine in the serum of patients with SLE and HCs. Compared with HCs, serum dopamine and its downstream metabolites norepinephrine in

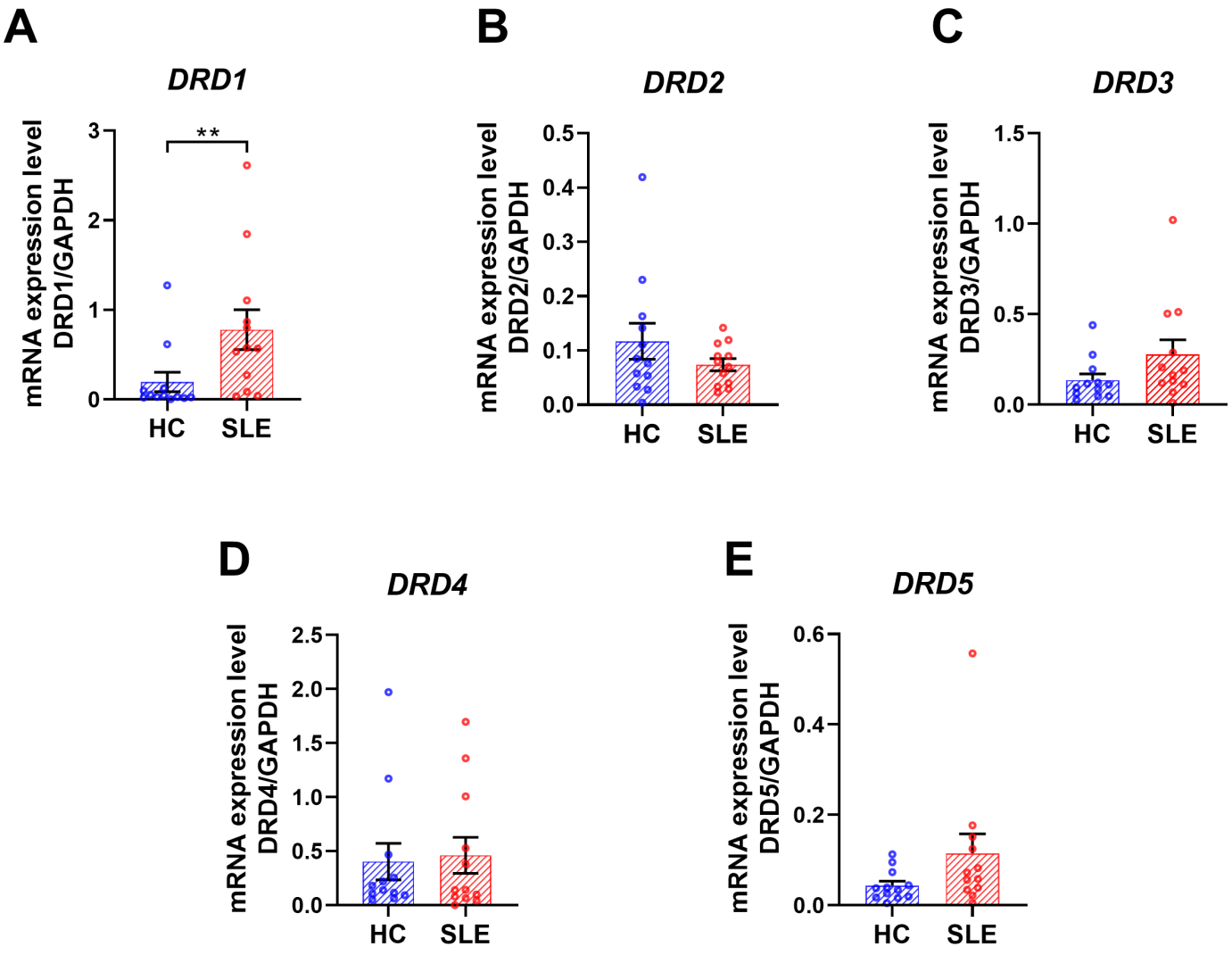

Figure 1 Higher messenger RNA (mRNA) expression levels of dopamine receptors (DRDs) in CD4$^+$ T cells from patients with SLE than healthy controls (HCs). The mRNA expression level of (A) DRD1, (B) DRD2, (C) DRD3, (D) DRD4 and (E) DRD5 in CD4$^+$ T cells from patients with SLE (n=12) and HCs (n=12) (**p<0.01).

patients with SLE were increased (online supplemental figure 1). Subsequently, we measured DRD expression in the PBMCs of patients with SLE and HCs. Compared with HCs, DRD2, DRD3 and DRD5 mRNA expression in the of patients with SLE was significantly decreased (online supplemental figure 2).

In consideration of the various influencing factors in PBMCs, we measured DRD mRNA expression in CD4$^+$ T cells. We observed that mRNA expression of DRD1 in patients with SLE was significantly increased, and that of DRD2 exhibited a downward trend. Moreover, the mRNA expression of DRD3 and DRD5 tended to increase, but that of DRD4 showed no significant difference (figure 1).

Abnormal expression of D1-like receptors in CD4$^+$ Tfh cells was related to disease activity

Considering expression of DRDs in PBMCs and CD4$^+$ T cells, the disorder of D1-like receptors in patients with SLE was more significant than that of D2-like receptors. Therefore, we analysed D1-like receptor expression in CD4$^+$ Tfh cells from patients with SLE and HCs. The flow cytometry loop gate strategy applied is shown in figure 2A. We first detected the frequency of Tfh cells (including CD4$^+$ Tfh (total Tfh) and CD4$^+$ CD45RA$^-$ Tfh (activated Tfh) cells). Compared with HCs, there was no significant change in the frequency of Tfh cells in patients with SLE. After dividing patients with SLE into active and inactive groups based on disease activity score SLEDAI, compared with HC, the frequency of Tfh cells in patients with active SLE is increased (figure 2B). In addition, D1-like receptor expression in all CD4$^+$ T-cell subsets (including total CD4$^+$, CD4$^+$ CD45RA$^+$, CD4$^+$ CD45RA$^-$, CD4$^+$ Tfh and CD4$^+$ CD45RA$^-$ Tfh cells) was increased in patients with SLE, with the increase in Tfh cells being most prominent (figure 2C,D). In addition, after dividing patients with SLE into active and inactive groups based on SLEDAI score, expression of D1-like receptors in Tfh cells in the active group showed an upward trend compared with that in the inactive group (figure 3A,B). Therefore, we conducted correlation analysis between the SLEDAI score and D1-like receptor expression in Tfh cells, which

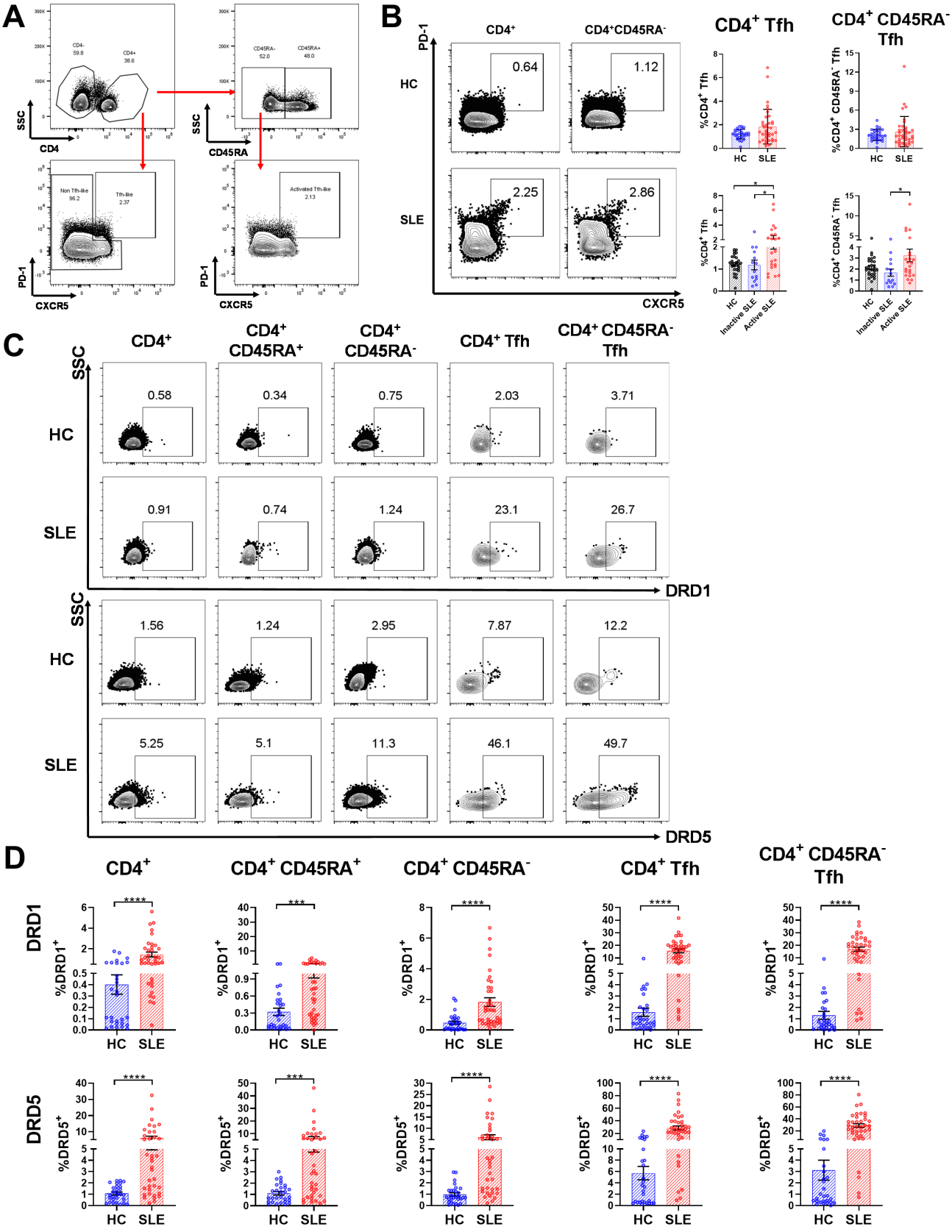

Figure 2 Enhanced expression of D1-like receptors in CD4⁺ T subsets of peripheral blood mononuclear cells from patients with SLE compared with healthy controls (HCs). (A) Flow cytometry gating strategy. (B) Representative flow cytometry diagrams and statistical analysis of T follicular helper (Tfh) cell frequency. (C) Representative flow cytometry diagrams of D1-like receptors in CD4⁺ T cell subsets from patients with SLE and HCs. (D) Statistical analysis of expression of D1-like receptors in CD4⁺ T cell subsets from patients with SLE (n=37) and HCs (n=29) (*p<0.05, ***p<0.005, ****p<0.0001). DRD, dopamine receptor; PD-1, programmed cell death protein 1; SSC, side scatter.

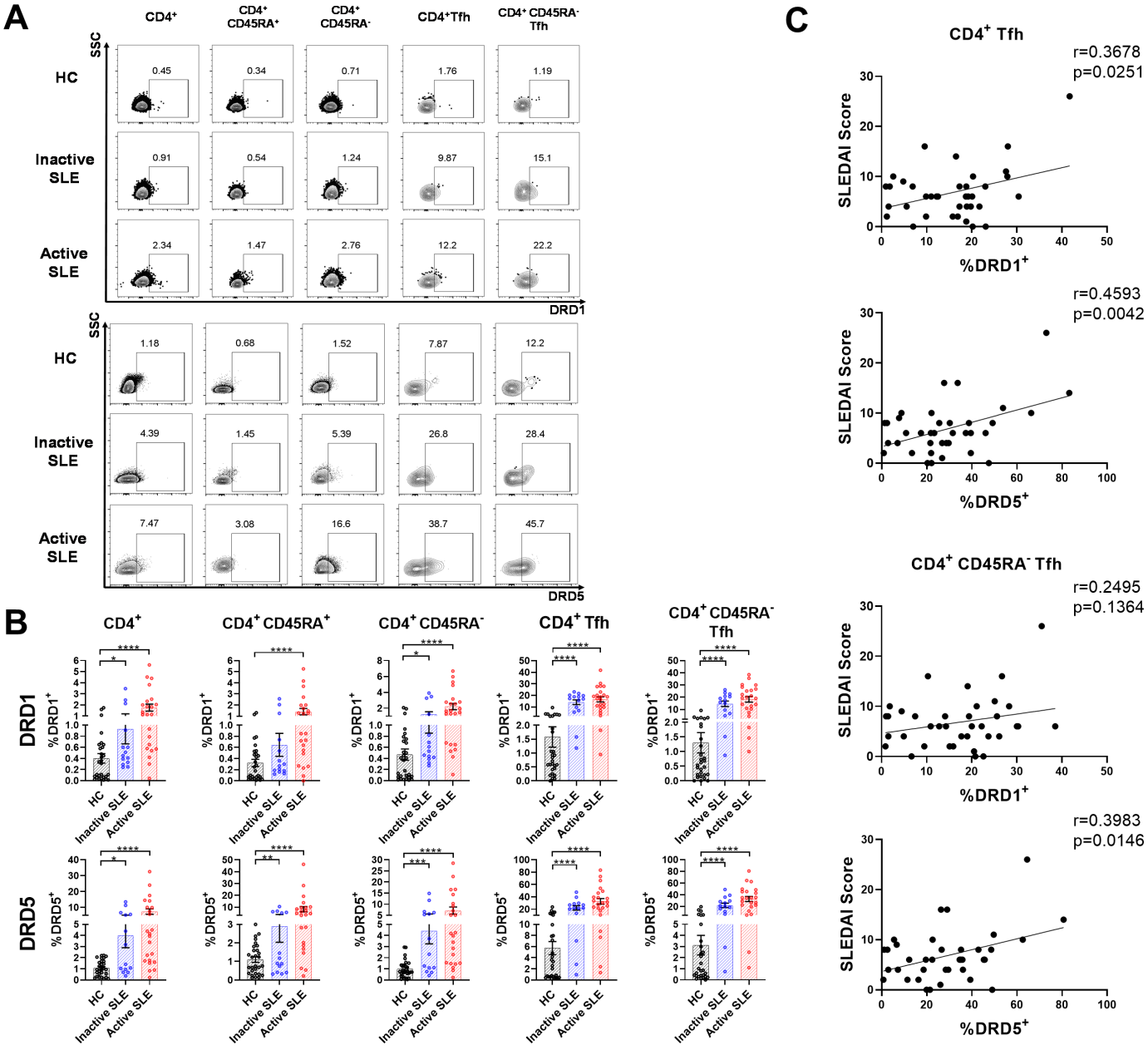

Figure 3 Upregulated D1-like receptors in T follicular helper (Tfh) cells from patients with SLE correlated positively with disease activity. (A) Representative flow diagram of gating for $CD4^+$ T cells, $CD4^+$ $CD45RA^+$ T cells, $CD4^+$ $CD45RA^-$ T cells, $CD4^+$ Tfh cells and $CD4^+$ $CD45RA^-$ T cells. (B) Statistical analysis of the frequency of $CD4^+$ T cells from patients with SLE (n=37) and HCs (n=29). (C) Correlation analysis of the SLEDAI score and expression of D1-like receptors in Tfh and $CD4^+$ $CD45RA^-$ Tfh cells (*p<0.05, **p<0.01, ***p<0.005, ****p<0.0001). DRD, dopamine receptor; HC healthy control; SLEDAI, SLE Disease Activity Index; SSC, side scatter.

showed that D1-like receptor expression in $CD4^+$ Tfh cells and DRD5 expression in $CD4^+$ $CD45RA^-$ Tfh cells increased with SLEDAI score (figure 3C).

The activity of SLE is related to inflammation and immune abnormalities in the body. Therefore, we analysed the correlation between expression of D1-like receptor in Tfh cells of patients with SLE and the inflammatory indicators C reactive protein and the erythrocyte sedimentation rate (ESR), immune indicators complement C3 and C4. The results showed that expression of DRD5 in Tfh cells correlated positively with ESR; however, there was no significant correlation with the other indicators (online supplemental figure 3,4). In addition, the disease heterogeneity of SLE is partly due to various autoantibodies.[22] Therefore, patients with SLE were grouped and compared according to different autoantibody detection results. The results showed that there was no significant relationship between autoantibodies and the expression of D1-like receptors (online supplemental figure 5).

It is worth noting that we conducted a horizontal comparison of D1-like receptor expression on $CD4^+$ $CD45RA^+$ ($CD4^+$-naïve T), $CD4^+$ Tfh (total Tfh) and $CD4^+$ $CD45RA^-$ Tfh (activated Tfh) cells of HCs and patients with SLE. The results showed that D1-like receptor

expression in Tfh cells were higher than that in CD4$^+$-naïve T cells in both HCs and patients with SLE (online supplemental figure 6A,B). The same trend was observed in patients with both active and inactive SLE. Additionally, compared with the inactive group, D1-like receptor expression in T-cell subsets of the active disease group tended to be higher (online supplemental figure 6C,D). We also performed an in vitro experiment to induce differentiation of CD4$^+$-naïve T cells into Tfh cells for further verification. The results showed that during differentiation of CD4$^+$-naïve T cells into Tfh cells, expression of D1-like receptors in Tfh cells increased, which further suggested the relationship between D1-like receptors and Tfh cell differentiation (online supplemental figure 7). However, we have not been able to determine the causal relationship between them.

D1-like receptors promote the differentiation of Tfh cells

According to the above results, differentiation of Tfh cells is related to D1-like receptors. Therefore, we selected the D1-like receptor agonist SKF38393 and inhibitor SCH23390 to clarify the effect of D1-like receptors on Tfh cell differentiation. First, we performed an induced differentiation assay of CD4$^+$ T cells. The gating strategy employed is shown in figure 4A. The frequency of Tfh cells in the SKF38393 group was significantly increased, whereas the frequency of Tfh cells in the SCH23390 group was decreased (figure 4B). However, expression of D1-like receptors in Tfh cells did not change significantly after use of D1-like agonists or inhibitors (figure 4C). These results explain the relationship between D1-like receptors and Tfh cell differentiation, suggesting that D1-like receptor activation promotes Tfh cell differentiation.

D1-like receptors may regulate Tfh cell differentiation through PI3K/AKT/FOXO1/Klf2 pathway

We have shown that D1-like receptors affect Tfh cell differentiation, although their specific molecular mechanism remains unclear. To explore the mechanism by

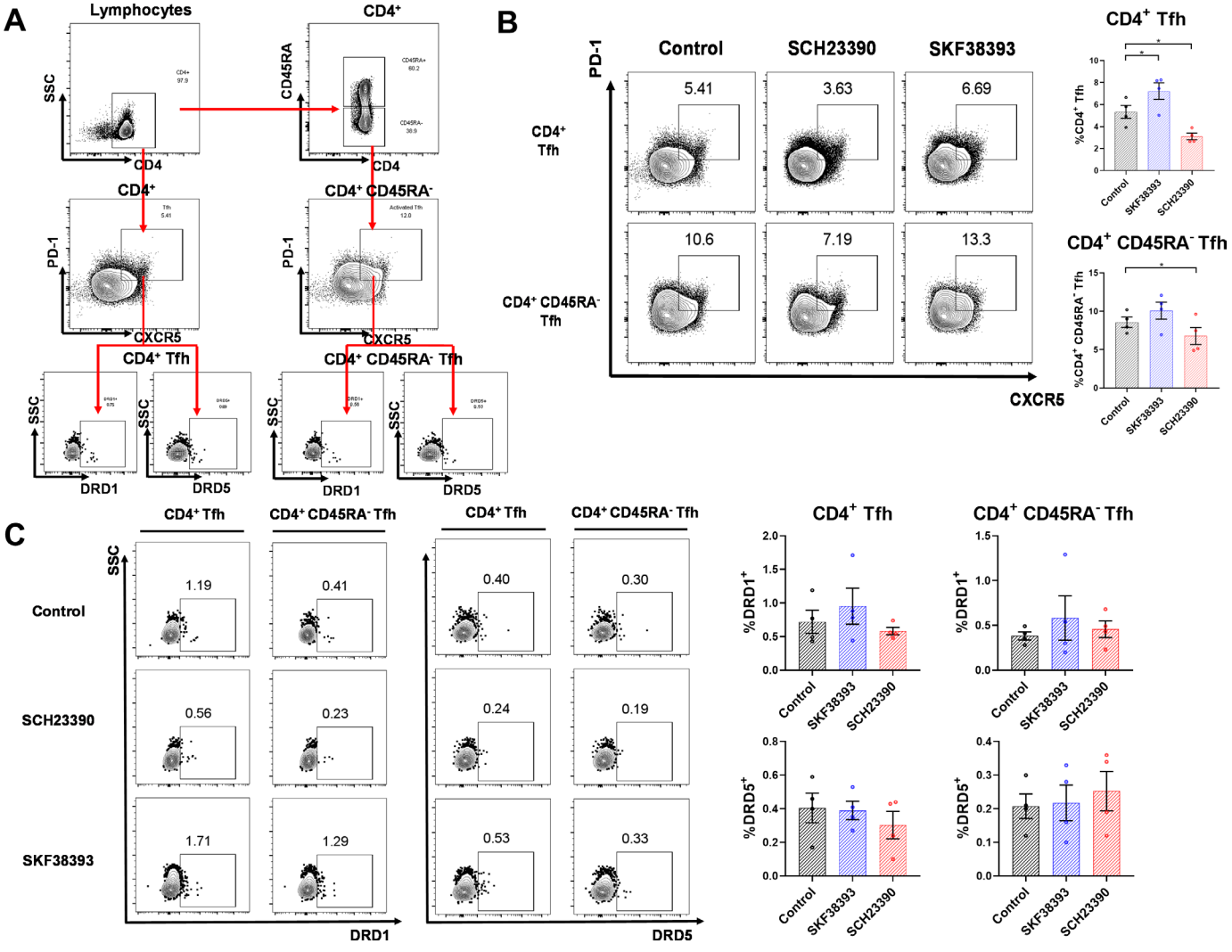

Figure 4 D1-like drug treatment influences differentiation of T follicular helper (Tfh) cells. (A) Flow cytometry gating strategy. (B) Statistical analysis of frequencies of Tfh and CD4$^+$ CD45RA$^-$ Tfh cells after D1-like drug treatment. (C) Representative flow cytometry diagrams and statistical analysis of expression of D1-like receptors in Tfh cells (*p<0.05). DRD, dopamine receptor; PD-1, programmed cell death protein 1; SSC, side scatter.

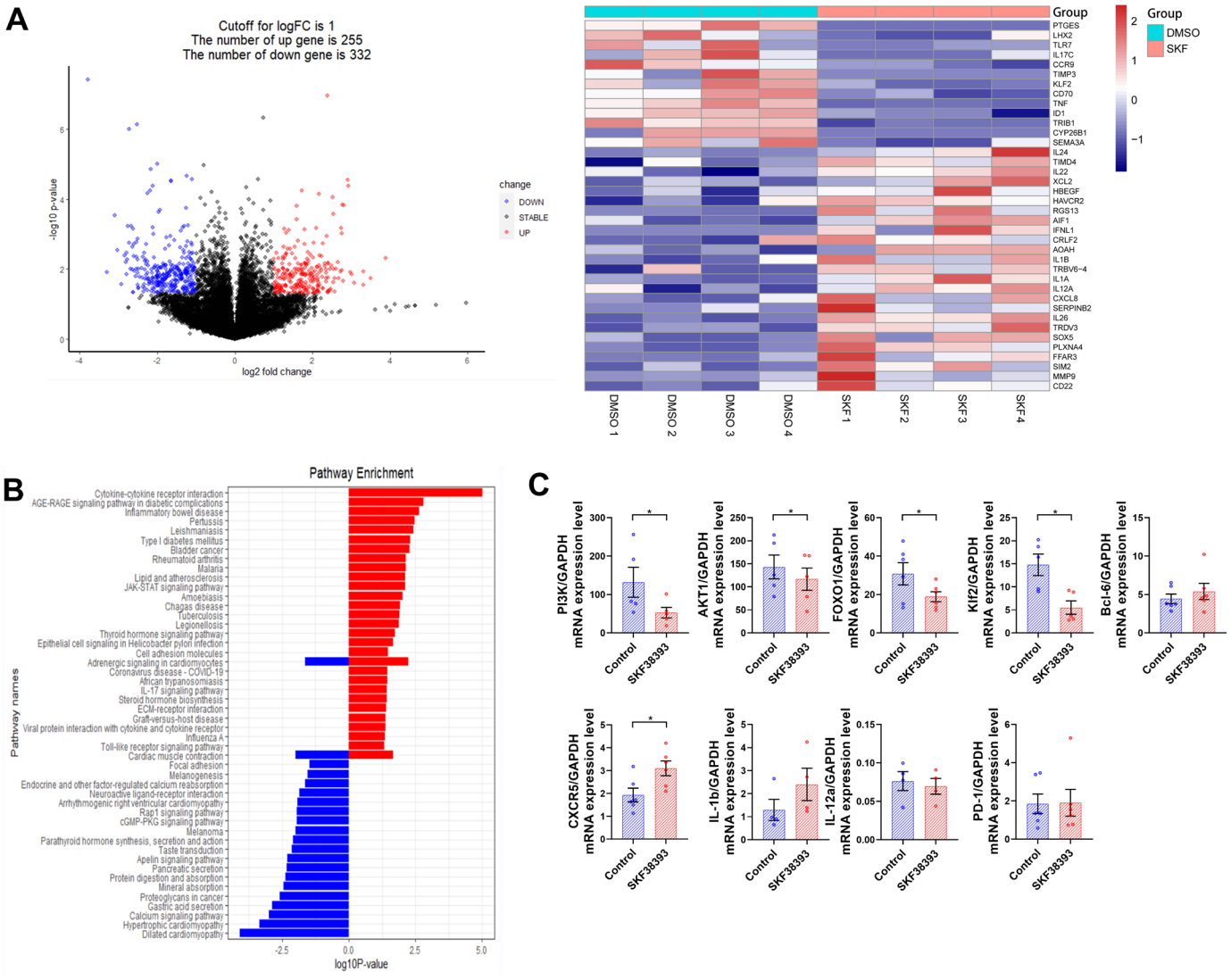

Figure 5 Biological information analysis and genetic authentication of SKF38393 group and dimethyl sulfoxide (DMSO) group. (A) Volcano plot and heatmap of datasets of CD4$^+$ T cells in DMSO and SKF38393 groups based on RNA sequencing (RNA-seq). Blue plots represent expression of genes with p 1. Grey plots represent genes normally expressed in messenger RNA (mRNA). (B) KEGG pathway analysis of differentially expressed genes (DEGs). Red represents pathways with increased differentially expressed genes (DEGs) in the SKF38393 group, and blue represents pathways with decreased DEGs in the SKF38393 group. (C) mRNA expression levels of DEGs associated with T follicular helper (Tfh) cells (*p<0.05). KEGG, Kyoto Encyclopedia of Genes and Genomes.

which D1-like receptors regulate differentiation of Tfh cells, we conducted RNA-seq of CD4$^+$ T cells treated with a D1-like small-molecule agonist or inhibitor and then performed bioinformatics analysis. Compared with the control group (DMSO group), in the SKF38393 group, 255 upregulated genes and 332 downregulated genes were identified (log2 fold change (LFC) ≥1, p<0.05). There were several differentially expressed genes (DEGs) that are directly related to Tfh differentiation, including interleukin (IL)-12, IL-1β and Kruppel-like factor 2 (Klf2) (figure 5A). Among them, IL-12 and IL-1β, which promote Tfh cell differentiation,[23 24] were upregulated in the SKF38393 group. In contrast, Klf2, an inhibitor of Tfh cell differentiation,[25] was downregulated in the SKF38393 group.

The results of Gene Ontology (GO) analysis suggested that the DEGs are mainly associated with activity of receptors and cytokines (online supplemental figure 8). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Set Enrichment Analyses (GSEA) showed that the DEGs are mainly associated with immune and metabolic regulation pathways, such as inflammatory bowel disease, rheumatoid arthritis and cytokine-cytokine receptor interaction (figure 5B, online supplemental figure 9). We also found that Klf2 of the KEGG pathway results is involved in the Apelin pathway of the SKF38393 group. However, we checked the upstream and downstream molecules of Klf2 on the KEGG website (https://www.kegg.jp), and the results showed that the Apelin pathway does not participate in regulation of the

immune response. Therefore, we expanded the range of LFC values (LFC ≥0) and conducted KEGG analysis again. The results showed that Klf2 is also involved in the FOXO pathway (online supplemental figure 10) and that its family member forkhead box protein O1 (FOXO1) is a vital transcription factor in regulating Klf2.[25] Then, we examined the upstream genes of FOXO1, mainly including phosphatidylinositol3-kinase (PI3K) and protein kinase B (AKT), which positively regulate the expression of FOXO1.[26 27]

One of the known functions of Klf2 is to inhibit Bcl-6 expression, thereby negatively regulating Tfh cell differentiation.[25] We hypothesised that D1-like receptor activation might negatively regulate the PI3K/AKT/FOXO1/Klf2 pathway, promoting Bcl-6 expression and Tfh cell differentiation. To our surprise, Bcl-6 expression was almost unchanged in the SKF38393 group compared with the DMSO group (figure 5C). Therefore, we explored other ways in which Klf2 may affect Tfh cell differentiation. Recently, Stone *et al* showed that overexpression of Klf2 can cause Tfh cells to lose the CXCR5/PD-1 phenotype.[28] Hence, we assessed these two molecules, and RT-qPCR results showed that compared with the control group, mRNA expression levels of PI3K, AKT, FOXO1 and Klf2 in the SKF38393 group were significantly downregulated, accompanied by significant upregulation of CXCR5. However, expression of PD-1 did not change significantly (figure 5C).

We also performed RNA-seq and bioinformatics analysis for the SCH23390 and DMSO groups. A total of 709 upregulated genes and 702 downregulated genes were identified (LFC ≥1, p≤0.05). In the SCH23390 group, the DEGs included several genes directly associated with Tfh cell differentiation, such as NR2F6, EIF4EBP1 and TNFRSF14 (HVEM), which are all inhibitory molecules of Tfh cell differentiation (online supplemental figure 11A). GO analysis showed that the DEGs are mainly related to binding of signalling molecules (online supplemental figure 12). KEGG and GSEA analysis showed that the DEGs are mainly associated with metabolism and immune disorders, such as regulation of lipolysis in adipocytes and SLE (online supplemental figure 11B,13). TNFRSF14 and NR2F6 were reported to inhibit Tfh cell differentiation by negatively regulating IL-21.[29 30] Accordingly, we verified expression of IL-21. Compared with that in the DMSO group, expression of TNFRSF14 in the SCH23390 group was significantly upregulated, although that of IL-21 remained unchanged (online supplemental figure 11C).

DISCUSSION

SLE usually affects multiple organs throughout the body, and among the complications of SLE, the most common are kidney disease and neuropathy.[4 31] Our results show significant abnormalities in dopamine levels and DRD expression in patients with SLE. Since dopamine mainly exists in the central nervous system, disorder of dopamine and DRDs may be associated with neuropathy in patients with SLE. Since the difficulty in obtaining GC Tfh cells, cTfh cells are commonly used for research in human subjects.[15] Previous studies showed that GC Tfh cells could secrete dopamine.[32] However, without GC microenvironment, cTfh cells and GC Tfh cells have many different characteristics, such as low expression of Bcl-6 and increased secretion of IL-21 in cTfh cells.[33] Therefore, it is still unclear whether cTfh can secrete dopamine. It is worth noting that we found in the RNA-seq results that the SLCA6C9 gene, which promotes dopamine synthesis, was downregulated in the SCH23390 group. This information provides the basis for the secretion of dopamine by cTfh cells, which we will verify in further experiments.

Previous research has shown significant dysregulation of DRD expression in patients with SLE, including decreased DRD2 and elevated DRD4.[19] DRD2 effectively regulates activation and differentiation of naïve $CD4^+$ T cells by promoting polarisation of Tregs. Thus, abnormal expression of DRD2 partially explains the decrease in the number of Tregs in patients with SLE.[19] Moreover, in an animal model of SLE, combined treatment with oestrogen and bromocriptine (a dopaminergic drug) prevented lupus-like syndrome in BALB/c mice. Bromocriptine inhibits maturation and activation of DNA-reactive B cells.[34] Overall, the dopamine system is closely associated with SLE.

In our study, dopamine and its downstream metabolites norepinephrine showed abnormalities. This is basically consistent with a previous case report of patients with SLE.[35] Dopamine exerts its physiological function by binding with DRDs.[36] We detected expression of DRDs in PBMCs and found that all had different degrees of abnormal expression, except for DRD1 and DRD4. However, due to the multiple influencing factors in PBMCs, we isolated $CD4^+$ T cells for detection and found that compared with HCs, patients with SLE only showed an increase in DRD1 expression. This is not entirely consistent with the research results of Jafari *et al*.[19] The reason may be differences in the cell types studied. The study by Jafari *et al* mainly focused on total T cells, and there is evidence that there are significant differences in expression of DRDs in different types of immune cells.[18] Comparison between our results and those of Jafari *et al* also confirms the high heterogeneity of DRD expression in immune cells. Even in different subtypes of the same type of immune cells, there are significant differences in DRD expression, which may be closely related to the function of immune cells.

Tfh cells promote progression of SLE by enhancing GC formation and B-cell differentiation.[37] Therefore, elucidating the differentiation mechanism of Tfh cells may be helpful for treatment of SLE. In recent years, the regulatory effects of DRDs on various immune cells have been confirmed.[38 39] However, few studies have focused on DRDs and Tfh cells. In this study, we demonstrated for the first time that Tfh cells from patients with SLE overexpress D1-like receptors and correlate positively

with disease activity. As the correlation between disease activity and the course of patients with SLE is extremely close, we attempted to further explore the relationship between D1-like receptor expression in Tfh cells of patients with SLE and the course of the disease. As SLE is an inflammatory autoimmune disease, some inflammatory and immune indicators are commonly used to reflect the severity of the disease.[40 41] Correlation analysis shows that expression of D1-like receptors on Tfh cells in patients with SLE correlate positively with ESR, indicating that expression of D1-like receptors in Tfh cells is associated with inflammation in patients. In addition, we further demonstrated that D1-like receptor activation can promote Tfh cell differentiation. The observed high expression of D1-like receptors in Tfh cells may be one of the reasons for the significant increase in Tfh cell frequency in patients with SLE.

Through bioinformatics analysis, we also found other molecules related to Tfh cell differentiation, such as IL-1β and IL-12α in the SKF38393 group and EIF4EBP1 in the SCH23390 group. We also assessed mRNA expression of these molecules. However, compared with the control group, expression of IL-1β and IL-12α in the SKF38393 group did not change significantly (figure 5C), but EIF4EBP1 expression in the SCH23390 group was increased. EIF4EBP1 inhibits differentiation of Tfh cells by reducing Bcl-6.[42] However, the mRNA expression level of Bcl-6 in the SCH23390 group showed an increasing trend (online supplemental figure 11C). This phenomenon has been discussed in previous studies. Although high expression of EIF4EBP promotes a reduction in Bcl-6 protein levels, its mRNA expression level is elevated, which might be caused by disruption of negative regulation of Bcl-6.[42] We did not measure the protein expression level of Bcl-6 in this study. Thus, whether increased expression of EIF4EBP1 caused by inhibition of the D1-like receptor affects the protein level of Bcl-6 remains to be confirmed. TNFRSF14 and N2RF6 inhibit Tfh cell differentiation by decreasing IL-21 expression.[29 30] However, as our results show, there was no significant change in IL-21 expression in the SCH23390 group compared with the control group. Therefore, the molecular pathway that antagonises D1-like receptors to inhibit Tfh cell differentiation needs to be further explored.

CONCLUSION

In summary, we demonstrate that D1-like receptors may promote Tfh cell differentiation through the PI3K/AKT/FOXO1/Klf2 pathway. Moreover, abnormal expression of D1-like receptors in patients with SLE may be an important cause of the disturbance of Tfh cell frequency, which may affect the course of SLE.

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