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Single-Cell Transcriptome Reveals the Heterogeneity of T Cells in Mice with Systemic Lupus Erythematosus and Neuronal Inflammation

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Introduction: Systemic lupus erythematosus is a heterogeneous autoimmune disease. A burst of autoimmune reactions in various systems can lead to severe clinical conditions closely associated with mortality. T cells serve as mediators that drive the occurrence and maintenance of inflammatory processes.

Methods: In this work, we employed single-cell transcriptome sequencing (scRNA-seq) involving 27704 cells from the brain and spleen tissues of MRL/lpr mice and 25355 healthy controls from BALB/c mice to explore the heterogeneity of T cells and their migration from the spleen to the brain.

Results: We identified a distinct group of double-negative T cells in systemic lupus erythematosus (SLE) mice that significantly expressed *Eomes* and other specific markers. We used the analysis of pseudotime trajectory and enrichment to show that double-negative T cells in SLE mice are strongly associated with cellular senescence and exhaustion. Additionally, we focused on the interactions among DNT, astrocytes, and microglia in the mice brain. We observed greater expression of MDK-related ligand–receptor pairs between astrocytes and double-negative T cells, indicating that MDK may be a therapeutic target for treating neuroinflammation in SLE.

Discussion: This research sheds light on the intricate dynamics of immune responses in mice with SLE, specifically highlighting the role of double-negative T cells and their connection to cellular senescence. The exploration of interactions between T cells, astrocytes, and microglia in the mice brain unveils potential avenues for therapeutic intervention, particularly in addressing neuronal inflammation in SLE.

Keywords: single-cell transcriptome, T cell, SLE, neuroinflammation

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease driven by abnormalities in nuclear autoantibodies leading to the formation of immune complexes. SLE manifests a wide spectrum of clinical presentations involving multiple organs, directly impacting quality of life and, in severe cases, contributing to increased mortality rates.[1] Neuropsychiatric systemic lupus erythematosus (NPSLE) involves the central nervous system (CNS) based on the occurrence of SLE. NPSLE presents nonspecific symptoms, ranging from slight headache to severe stroke, due to complicated immunoreactions and inflammatory mechanisms. Emotional changes and cognitive disorders may also be indicative of CNS involvement in SLE.[2] At the core of the intricate pathogenic mechanism of SLE are autoantibodies and their original producers, B cells. However, T cells are more likely to play a pivotal role in the initiation and maintenance of immune dysregulation and the inflammatory cascade. Research has shown that abnormal T cells in SLE are related to cell metabolism,[3] phenotypic alterations, and cell activation.[4]

In recent studies, exhausted Tregs and double-negative T cells have been found to be dysregulated in patients with SLE,[5,6] suggesting that reversing the exhaustion process or compensating for exhausted T cells may alleviate SLE. Because of the multisystemic impact of SLE, the management of SLE is currently a challenging task in the clinic, as

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accessible treatments often fall short of achieving complete remission. Considering the adverse effects and tolerance issues associated with long-term therapy, studies on these drugs have focused more on T cells, B cells and their related cytokines and inflammatory signaling pathways.[7] Biomedications targeting B cells and their related pathways are applied in the clinic. Targeting T cells in SLE may be another option for possible treatment and a potential key to SLE treatment in the future. Therefore, our study contributes to the understanding of the role of T cells in the pathogenesis of SLE and provides additional details through the use of single-cell RNA sequencing (scRNA-seq) techniques.

As we delve into the molecular intricacies of SLE, the recognition of T-cell heterogeneity has emerged as a critical focal point in understanding the diverse clinical manifestations of this disease. Single-cell transcriptome sequencing (scRNA-seq) is a cutting-edge technique that provides a high-resolution landscape of individual cells. Mapping of SLE patients, initially performed by using peripheral blood mononuclear cells (PBMCs) with single-cell RNA sequencing (scRNA-seq), involves a general review of cell heterogeneity, biomarkers, and regulation of expressed genes.[8] Recently, scRNA-seq has helped researchers discover the heterogeneity of T cells. Type I interferon-induced functional exhaustion of Tregs[5] and elevated levels of IFN-stimulated genes (ISGs)[9,10] are involved in the heterogeneity of SLE. A reduction in the number of naïve CD4+ T cells related to ISGs and an increase in the number of repertoire-restricted *GZMH*+ CD8+ T cells were also measured via scRNA-seq.[11] Considering the previous attention given to T cells and the significant results obtained, we also focused on T cells in our study. Brain cells from murine models provide a direct and accessible means to observe the heterogeneity of T cells involved in nervous system diseases. To comprehensively assess T cell heterogeneity in the brain in systemic lupus erythematosus (SLE), we also included the spleen—an upstream organ where

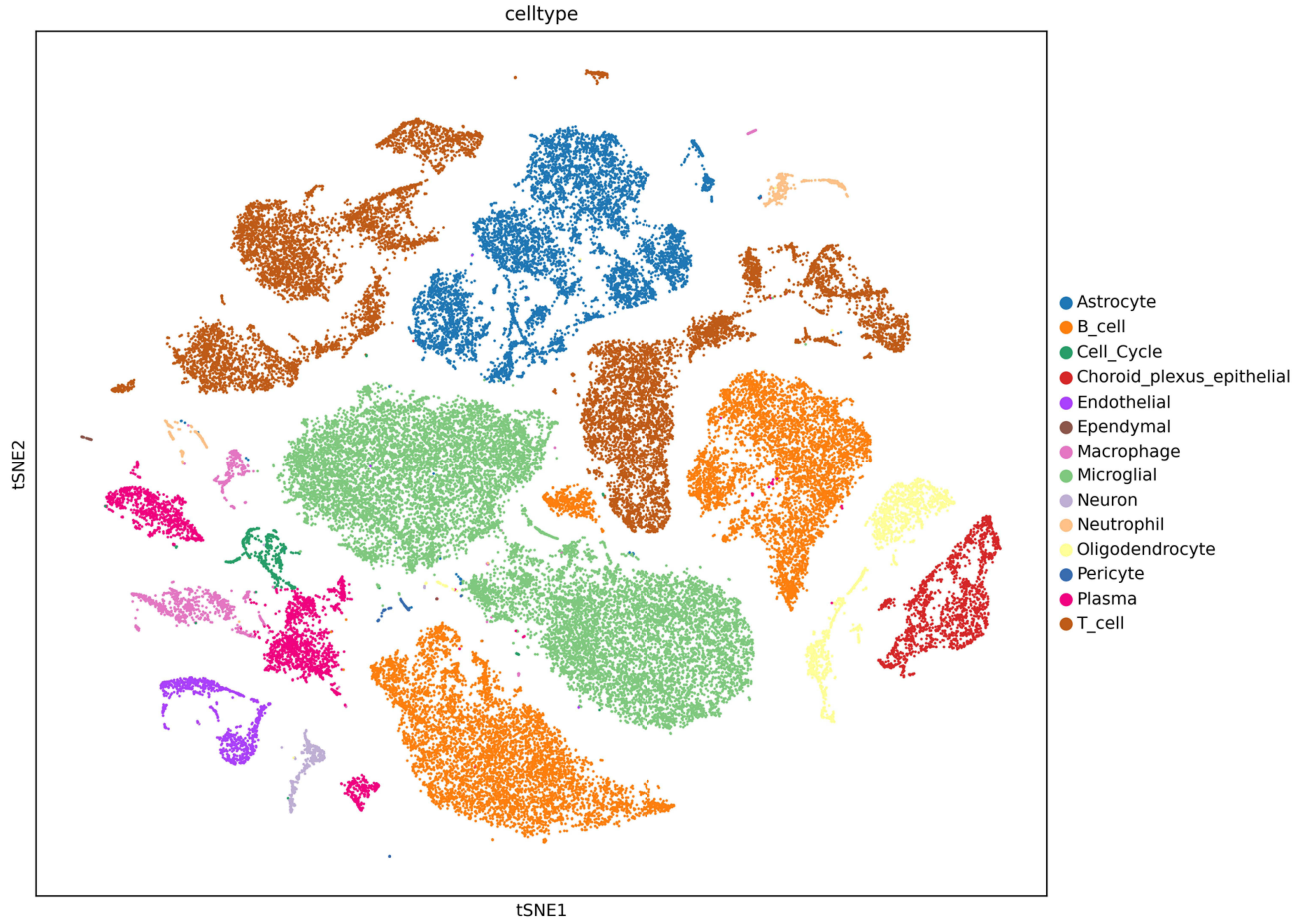


Figure 1 T-SNE plot of clustering all cells from the spleen and brain samples. Each colour represents a certain cluster of the corresponding cell type labelled on the right.

T cells differentiate and proliferate before migrating to the periphery via the bloodstream. Both the similarities and differences between the spleen and brain may explain the landscape of T cells in SLE.

By analyzing the transcriptomes of cells isolated from the brain and spleen tissues of MRL/lpr mice, a well-established murine model of SLE, and comparing them with cells from the same tissue in healthy BALB/c mice, our study aimed to elucidate the nuanced cell profiles of T cells that contribute not only to the complexity of SLE pathogenesis but also to neuron-related inflammation networks. We performed scRNA-seq based on the Chromium Controller (10X Genomics) and further identified a distinct subset of double-negative T cells (DNTs) in both the spleen and brain samples from a murine model of SLE. Pseudotime trajectory analysis and enrichment studies revealed strong associations between DNT cells in SLE mice and processes of cellular senescence and exhaustion. This new understanding of DNT cells provides us with a foundation for targeted therapeutic interventions aimed at modulating these processes in SLE. To expand our understanding of the central nervous system, we explored the interactions among DNT cells, astrocytes, and microglia in the brain—a critical nexus in autoimmune neuroinflammation. The interplay between the immune system and the nervous system in SLE is an emerging frontier.[12] This study may help us to attain a growing body of knowledge surrounding autoimmune disorders and holds promise for the development of targeted therapeutics addressing the complex landscape of SLE.

Materials and Methods

Sample Collection and Preparation

Murine Sample Preparation

Brain and spleen samples were collected from 3 MRL/lpr mice and 3 BALB/c mice that had been raised for 20 weeks. The spleen tissue was further routinely collected and processed via dissection, homogenization, digestion, filtration and adjustment. The brain tissue was processed with a mouse brain dissociation kit provided by Miltenyi Biotec. Single-cell suspensions of each sample were prepared for further steps of scRNA-seq.

Table 1 Corresponding Marker Genes of Each Cell Type in the Annotation

Cell Type	Gene Name
T cell	*Cd3d, Cd3e, Cd3g, Cd4, Cd8a, Trac, Trbc1, Trbc2, Trdc*
B cell	*Cd79a, Cd79b, Ms4a1, Cd19*
Plasma	*Cd79a, Jchain, Tnfrsf17*
Macrophage	*Csf1r,C1qa,C1qb*
Neutrophil	*S100a8, S100a9, Csf3r*
Endothelial	*Pecam1, Vwf, Cldn5, Flt1*
Astrocyte	*Aqp4,Gja1,Slc1a2, Gfap*
Oligodendrocyte	*Mbp, Opalin, Mog, Olig1*
Microglial	*Crybb1, Ctss, Cst3, P2ry12*
Pericyte	*Cspg4, Kcnj8, Rgs5, Mcam*
Ependymal	*Ccdc153, Tmem212, Ccdc153, Enkur, Foxj1*
Neuron	*Snhg11, Gad1, Gap43, Stmn3*
Choroid_plexus_epithelial	*Slc22a17, Ttr*
Cell Cycle	*Top2a, Mki67*

Cell Preparation Section

For the quality check and counting of single-cell suspensions, the cell survival rate was generally greater than 80%. The cells that passed the test were washed and resuspended to prepare a suitable cell concentration of 700~1200 cells/μL for 10x Genomics ChromiumTM. The system is operated on the machine.

Steps in GEM Creation and Thermal Cycling

GEMs (Gel Beads in Emulsion) were constructed for single-cell separation according to the number of cells harvested. After GEMs were normally formed, the GEMs were collected for reverse transcription in a PCR machine for labelling.

Postcycling Cleanup and cDNA Amplification

The GEMs were oil-treated, and the amplified cDNA was purified by magnetic beads and then subjected to cDNA amplification and quality inspection.

Library Preparation and Quantification

The 3′ Gene Expression Library was constructed with the quality-qualified cDNA. After fragmentation, adaptor ligation, sample index PCR, etc., the library was quantitatively examined.

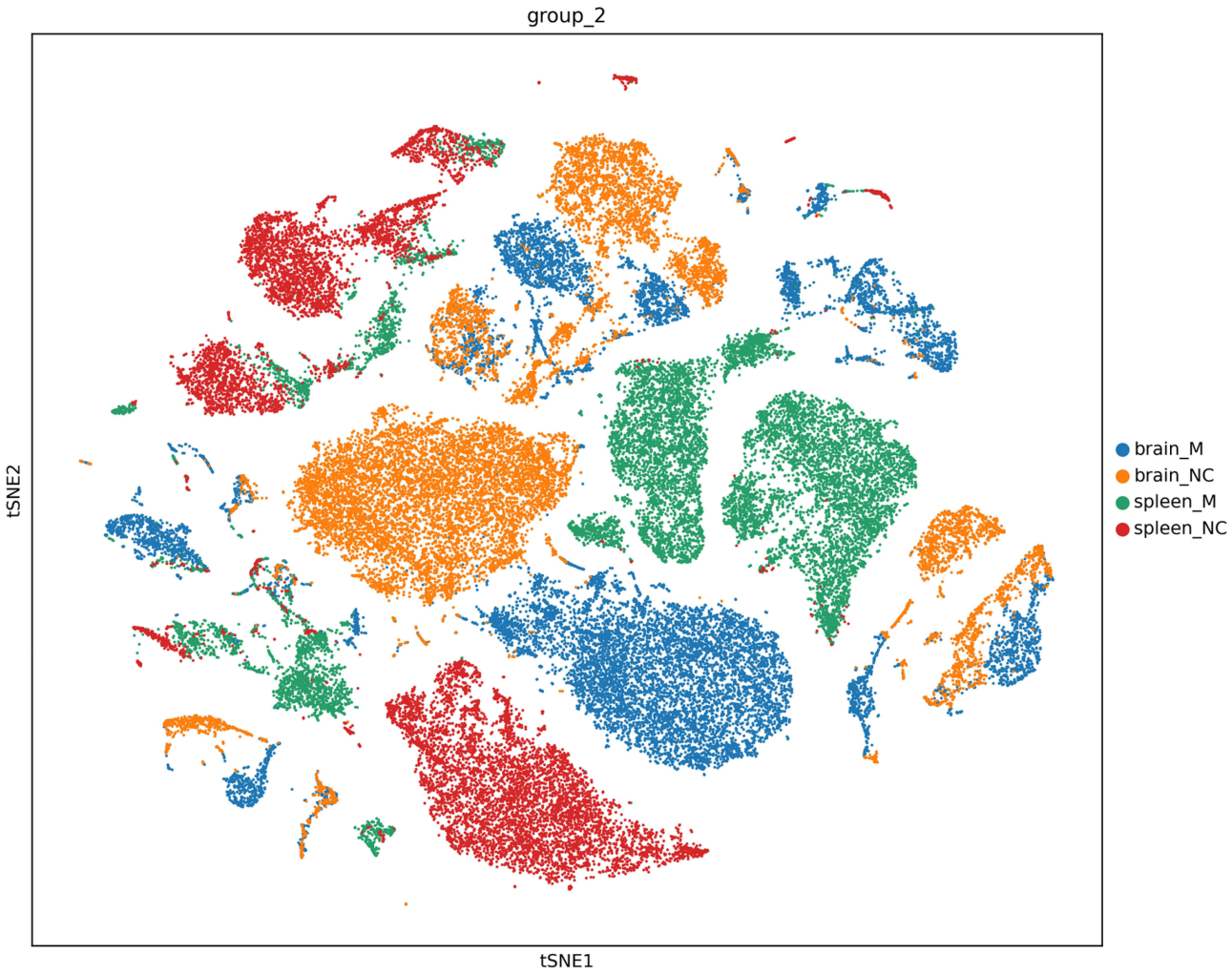


Figure 2 The tSNE plot of clustering all cells from the spleen and brain samples. Each colour represents a certain cluster of sample resources labelled on the right. M for the MPL/lpr group, NC for the control group.

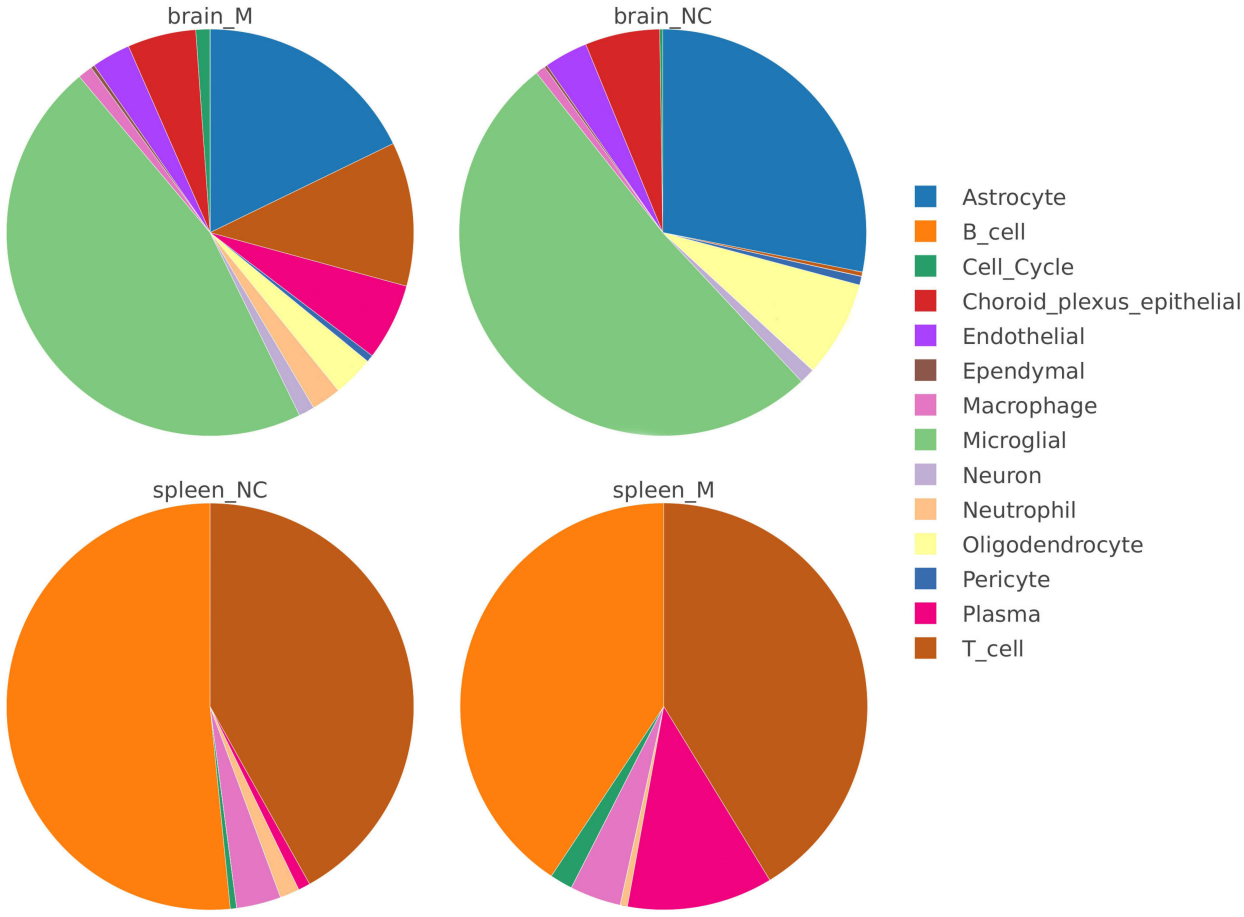
Sequencing

The final library pool was sequenced on an Illumina NovaSeq 6000 instrument using 150-base-pair paired-end reads.

Methods and Steps of Single-Cell RNA Sequencing

Single-Cell RNA Statistical Analysis

The cells contained 200–7000 expressed genes, and when the mitochondrial UMI rate was less than 10%, the cells were filtered, and mitochondrial genes were removed from the expression table The Seurat package (version: 4 https://satijalab.org/seurat/) was used for cell normalization and regression. PCA was constructed based on the scaled data with the top 2000 highly variable genes, and the top 50 principal components were used for tSNE construction and UMAP construction (Detailed see supplementary material 1).



Sample	Astrocyte	B_cell	Cell_Cycle	Choroid_plexus_epithelial	Endothelial	Ependymal	Macrophage	Microglial	Neuron	Neutrophil	Oligodendrocyte	Pericyte	Plasma	T_cell
brain_M	17.79%	0.01%	1.12%	5.44%	3.08%	0.30%	1.16%	46.14%	1.26%	2.35%	3.24%	0.56%	6.11%	11.46%
brain_NC	28.11%	0.00%	0.25%	5.93%	3.47%	0.21%	0.79%	51.29%	1.22%	0.03%	7.66%	0.68%	0.02%	0.34%
spleen_NC	0.00%	51.58%	0.50%	0.00%	0.00%	0.00%	3.54%	0.00%	0.00%	1.55%	0.00%	0.00%	0.95%	41.87%
spleen_M	0.00%	40.69%	1.81%	0.00%	0.00%	0.00%	4.05%	0.01%	0.00%	0.58%	0.00%	0.00%	11.57%	41.28%

Figure 3 Pie plot of the differential cell composition of six samples. The percentage of the proportion is marked in the corresponding part, while each colour represents a certain cell type.

GO Analysis and KEGG Enrichment

Gene Ontology (GO) enrichment analysis of marker genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. We used the clusterProfiler R package to test the statistical enrichment of marker genes in KEGG pathways as well.

Pseudotime Trajectory Analysis

We utilized Monocle2 for pseudotime analysis. Monocle2 employs the technique of reversed graph embedding to learn the main structure of cell trajectories and subsequently sorts cells along these trajectories. This approach allows for an accurate interpretation of complex biological processes.

Cell Communication CellPhoneDB

CellPhoneDB has constructed a signalling database based on UniProt, Ensemble, PDB, IUPHAR, and other resources. CellPhoneDB uses the expression levels of these signals to predict interactions between different cells, thereby inferring the communication relationships between cells.

Results

Cell Compositions of the Mouse Cells Profiled by scRNA-Seq

To explore cell heterogeneity in an SLE mouse model, we used scRNA-seq to profile a sample of 27704 cells from MRL/lpr mice and 25355 healthy controls from BALB/c mice. The samples were obtained from three MRL/lpr mice and three BALB/c mice at 20 weeks of age. Brain and spleen cells were individually fully enzymatically digested to obtain single-cell suspensions. After infiltrating low-quality cells, the remaining cells were counted and loaded onto a Chromium Controller (10X Genomics) for recovery of more than 50k single cells. Samples were processed following the manufacturer's protocol, and sequencing was performed on an Illumina Nova6000 platform with 150-base-pair paired-end reads. The cells were clustered via t-SNE, revealing the overall compositions of the brain and spleen samples. The cells were first divided into 46 clusters, and the differential gene expression of each cell was analysed. After the clustering process, groups of cells with similar marker gene expression can be further annotated with different cell types due to the existing gene lists.(Figure 1 and Table 1) In this study, the clusters were distinctly annotated into 14 cell types or states: Astrocyte (Cluster 7,11,13,16,23,36, 6466 cells), B-cell (Cluster 1,3,10,15,18,30, 10963 cells), Cell cycle (Cluster 30, 491 cells), Choroid plexus epithelial (Cluster 8, 1652 cells), Endothelial (Cluster 21, 951 cells), Ependymal (Cluster 45, 74 cells), Macrophage (Cluster 26,33,41, 1199 cells), Microglial (Cluster 0,4,5,17,25,44, 14158 cells), Neuron (Cluster 31, 361 cells), Neutrophil (Cluster 32, 37, 599 cells), Oligodendrocyte (Cluster 19, 27, 1573 cells), Pericyte (Cluster 39,180 cells), Plasma (Cluster 14,22,35,38, 2504 cells), and T-cell (Cluster 2,6,9,12,20,24,28,29,34,40,42,43,46, 11708 cells). The original source of the cells and the proportions of every cell type from the six samples were also determined with tSNE and pie plots, enabling the observation of differential cell compositions. (Figures 2 and 3). Further differential analysis revealed significant differences in cell proportions between the disease group and the control group. In brain samples, the proportions of astrocytes ($P<0.0001$), neutrophils ($P<0.0001$), oligodendrocytes ($P<0.0001$), plasma cells ($P<0.0001$), T cells ($P<0.0001$), cells in the cell cycle ($P<0.05$), and microglia ($P<0.05$) significantly differed. Conversely, spleen samples exhibited notable differences only in B cells ($P<0.0001$) and plasma ($P<0.0001$). These findings not only confirm the proliferation of B cells in SLE, particularly in the peripheral context but also provide valuable information on potential interactions among T cells, astrocytes and microglia during neuroinflammation in SLE.

Subclustering and Annotating T Cells Reveal the T-Cell Types and a Group of "Unknown" T Cells

We directed our attention towards understanding the potential role of T cells in both peripheral immunoreactions and neuroinflammation in mice with SLE. Initially, T cells were classified into 13 clusters, and we further subclustered

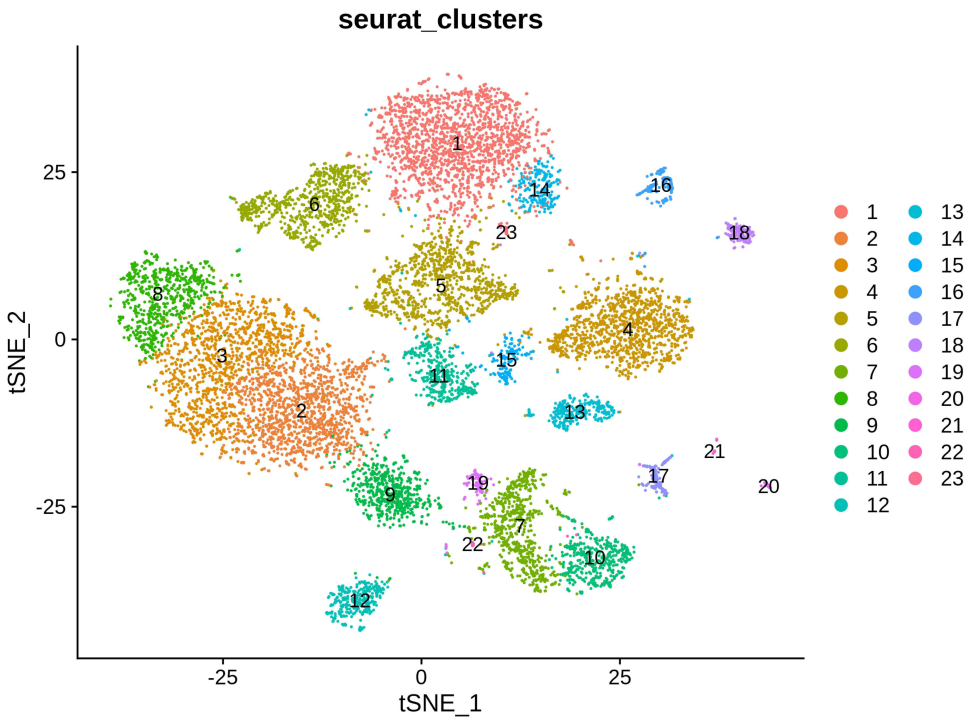


Figure 4 T-SNE plot of the T-cell subclusters. Each colour represents a certain cluster identified by specific marker genes.

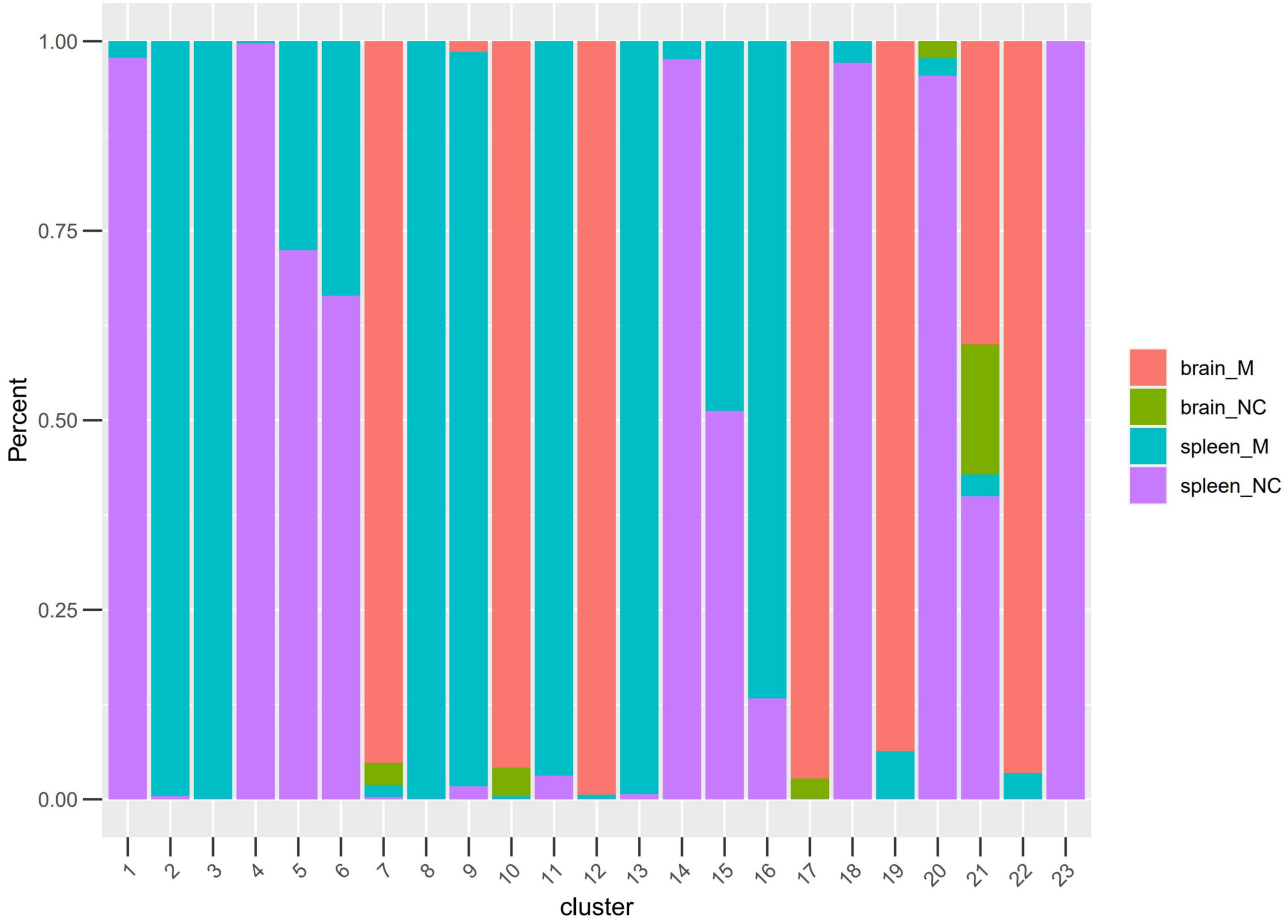


Figure 5 Percentage of the original identity in each subcluster.

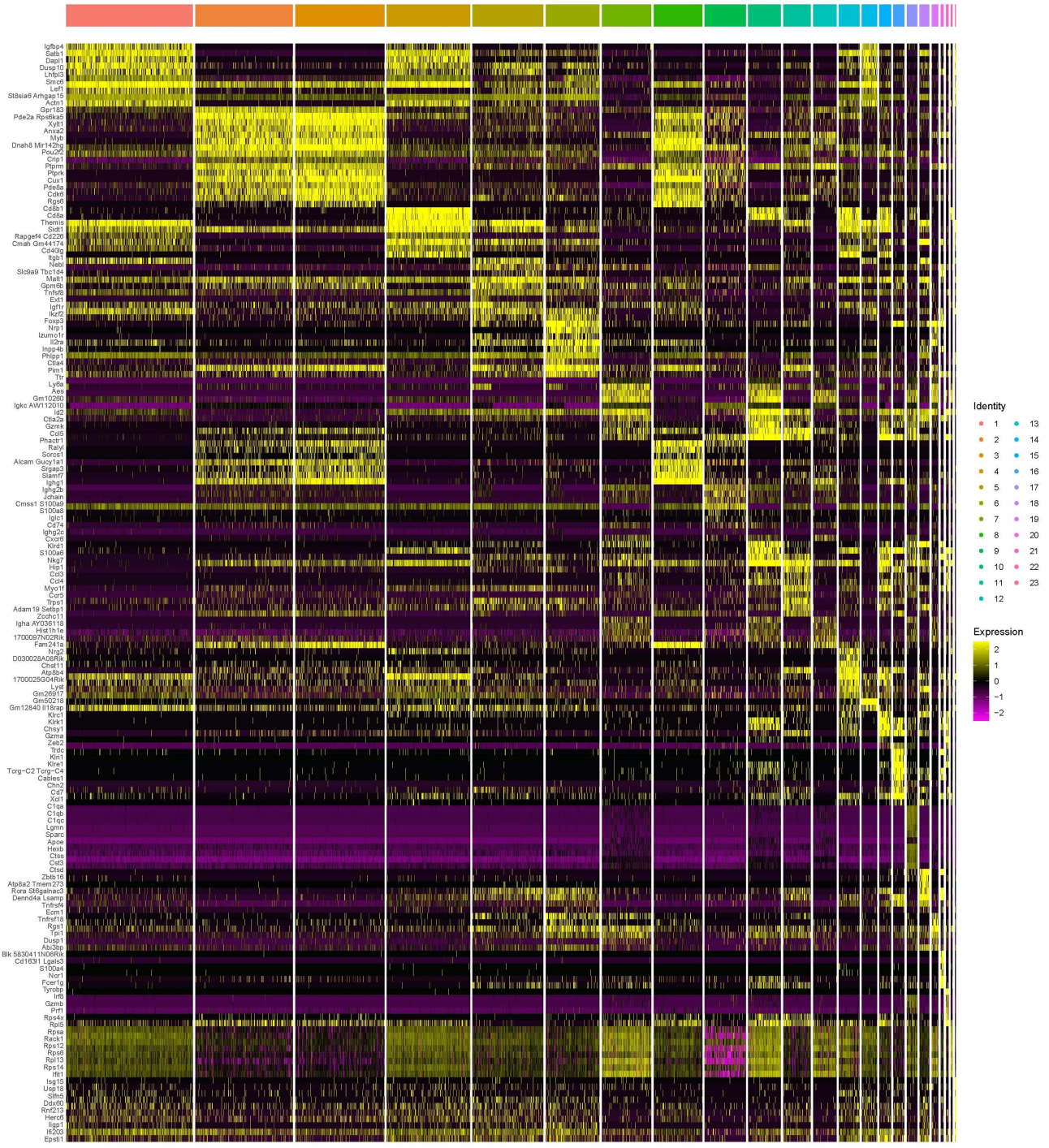


Figure 6 Heatmap of the top 10 markers of T cells in 23 subclusters.

these T cells into 23 subclusters using Seurat, each of which was identified by specific marker genes (Figure 4). The percentage of the original identity in each subcluster (Figure 5) may indicate the specific subcluster of T cells from each sample.

In the spleen control group, subclusters 1, 4, 14, 18, 20, and 23 were predominant, collectively representing 95% of the population. Conversely, subclusters 2, 3, 8, 9, 11, and 13 were specific to the disease group (spleen), with 95%

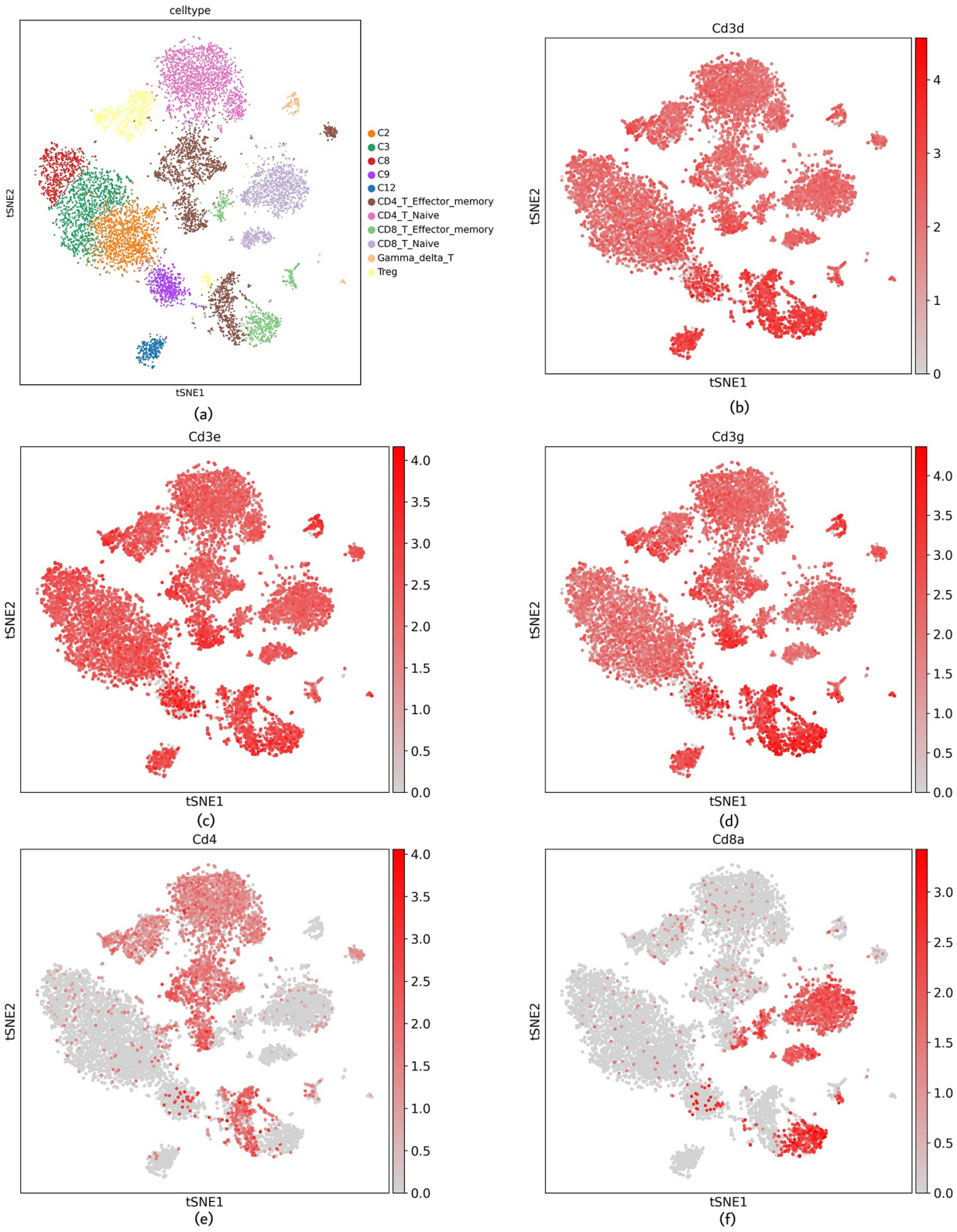


Figure 7 (**a**) t-SNE plot of cell types in T-cell subclusters. C2, C3, C8, C9, and C12 are five subclusters that are not categorized as any common type of T cell. (**b–f**). The expression levels of CD3d, CD3e, CD3g, CD4 and CD8 in "unknown" T cells.

representation. T cells in subclusters 5, 6, 15, and 16 were mostly collected from spleen samples, while brain-originating T cells were commonly found in subclusters 7, 10, 12, 17, 19, and 22. Control brain T cells were epitomized in subcluster 21 with a representation of less than 20%.

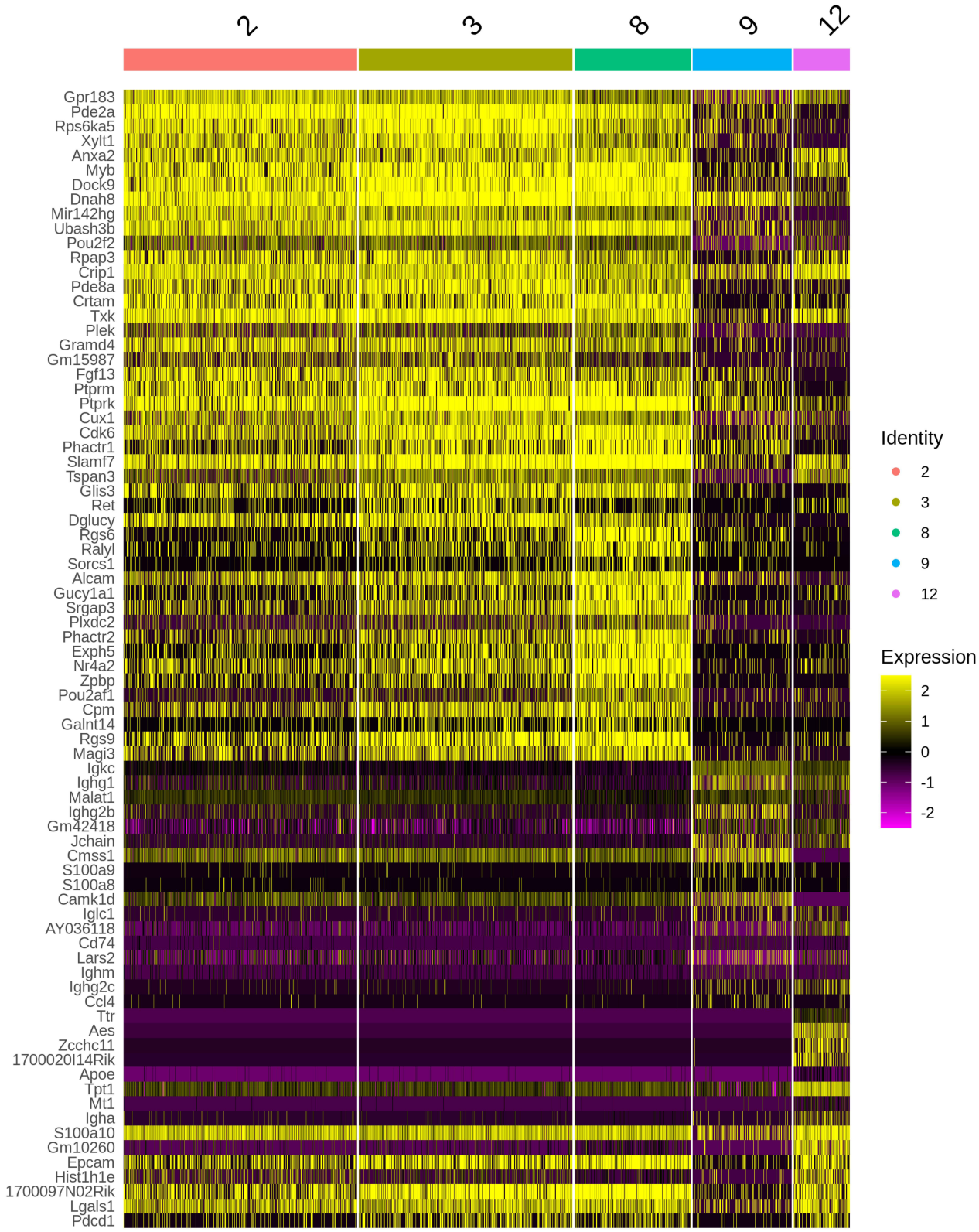


Figure 8 Heatmap of the cluster's top 20 expressed marker genes in T cell subclusters C2, C3, C8, C9, and C12.

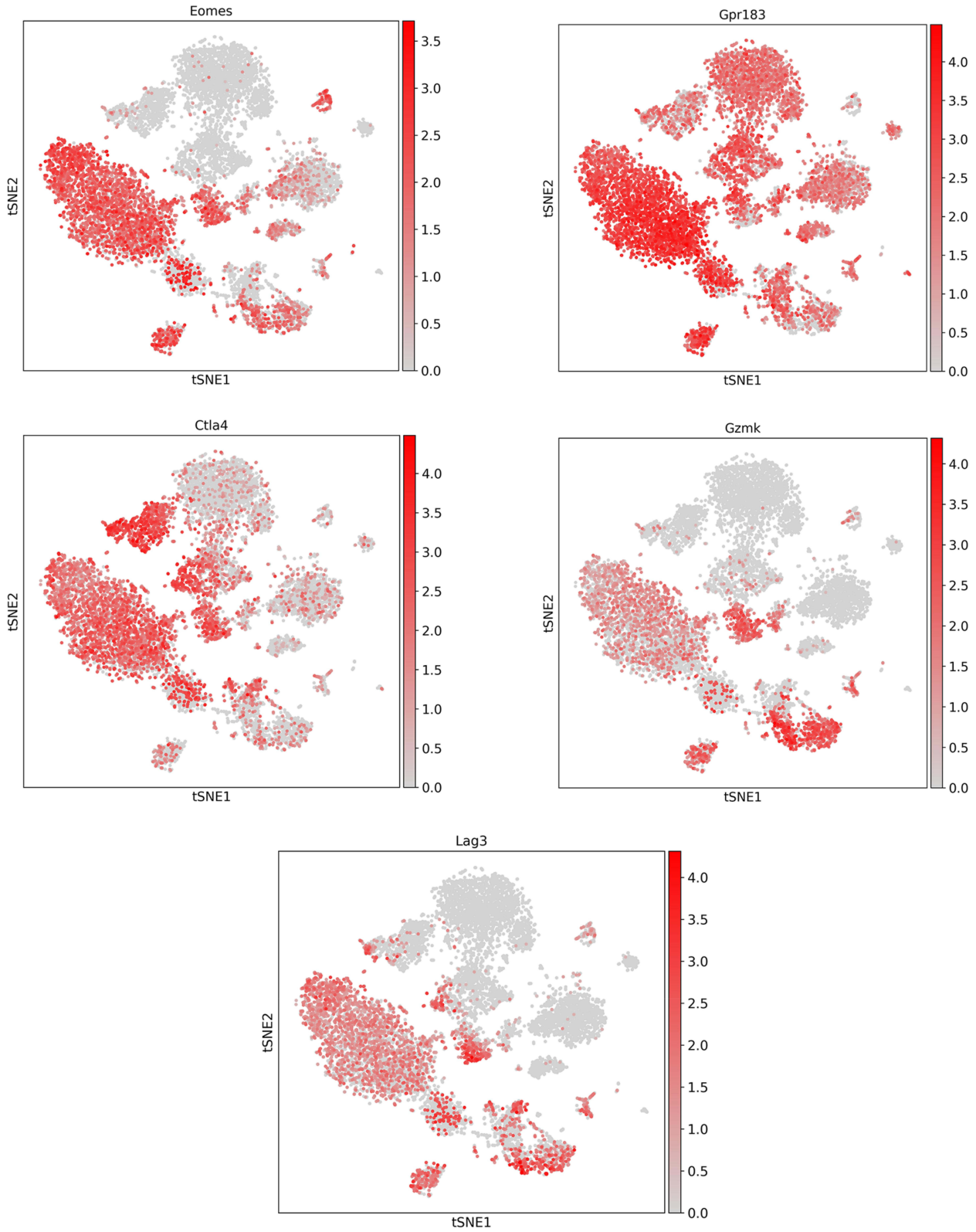


Figure 9 T-SNE plot of the expression levels of the biomarkers *Eomes*, *Gpr183*, *Ctla4*, *Gzmk*, and *Lag3* in DNT cells.

By analysing the heatmap and known markers of T cells (Figure 6), the 23 subclusters were annotated to match their known markers. The T-cell compartment consisted of effector/memory T cells that positively expressed *Il7r* but not *Sell* or *Ccr7*; γδT cells that positively expressed *Trdc* or *Trdv1*; naïve T cells that expressed *Sell, Lef1, Tcf7, Ccr7, Il7r*; and Tregs that expressed Ctla4 and Foxp3. Naïve T cells and effector/memory T cells are further divided according to CD4+ or CD8+ T-cell identity. The final annotations are shown in Figure 7 with CD4+ T naïve cells (Clusters 1, 14, and 23), CD4+ effector/memory cells (Clusters 5, 7, 11, and 18), CD8+ T naïve cells (Clusters 4, 13, and 22), CD8+ T effector/memory cells (Clusters 10, 15, and 17), Tregs (Clusters 6 and 19), γδ T cells (Clusters 16, 20, and 21), and a group of undefined cells (Clusters 2, 3, 8, 9, and 12). A deeper analysis revealed that the group of cells rarely expressed *Cd8a* or *Cd4* but only with *Cd3g, Cd3d* or *Cd3e*. Based on previous studies,[6] this group of T cells was annotated as 'double-negative T cells' (Figure 7).

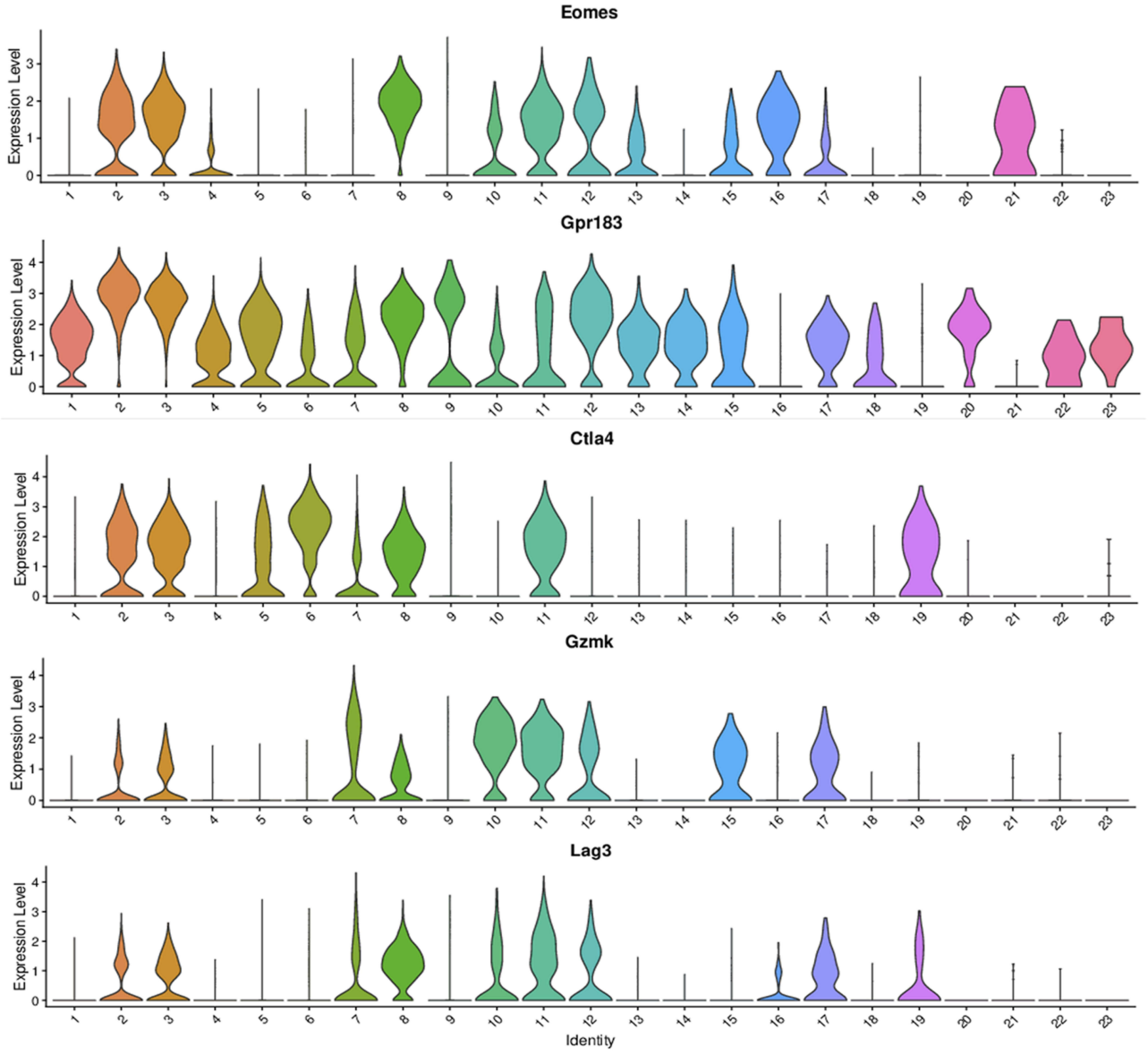


Figure 10 Violin plot of the expression levels of the biomarkers *Eomes, Gpr183, Ctla4, Gzmk*, and *Lag3* in DNT cells.

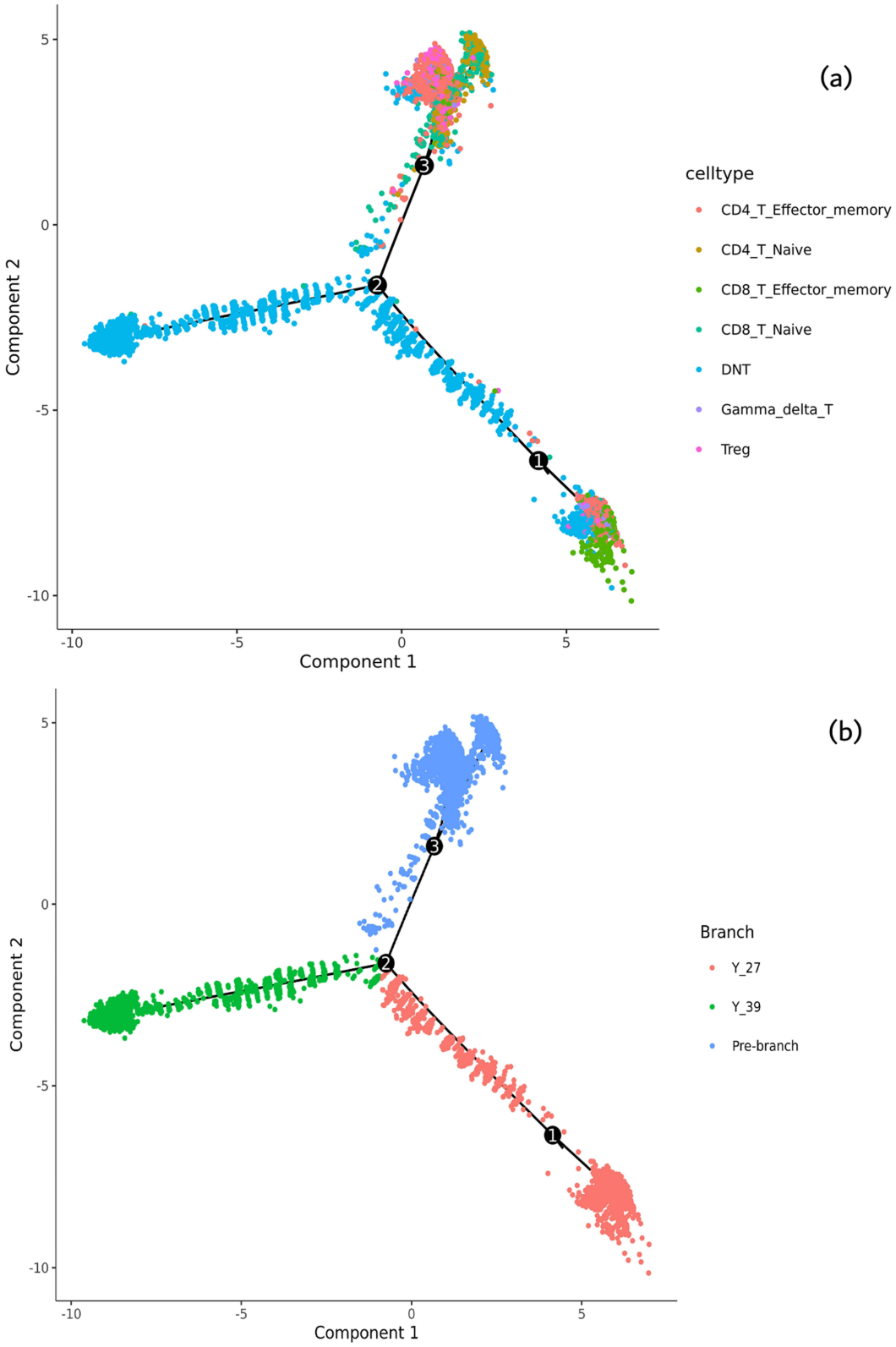


Figure 11 Results of the pseudotime trajectory analysis. Each dot represents a single cell. The black lines indicate the main differentiation trajectories of the cells, and the black circles with numbers are the important nodes on the differentiation trajectories. (**a**) Each colour represents a certain type of T cell. (**b**) Each colour represents a certain branch of the trajectory.

Eomes and Other Biomarkers are Highly Expressed in Double-Negative T Cells in an SLE Mouse Model

Given that previous analyses suggested that subclusters 2, 3, 8, 9, and 12 are essentially double-negative T cells, the tSNE plot of origin identity (Figure 5) further revealed the heterogeneity of the T cells in the SLE mouse model. More in-depth

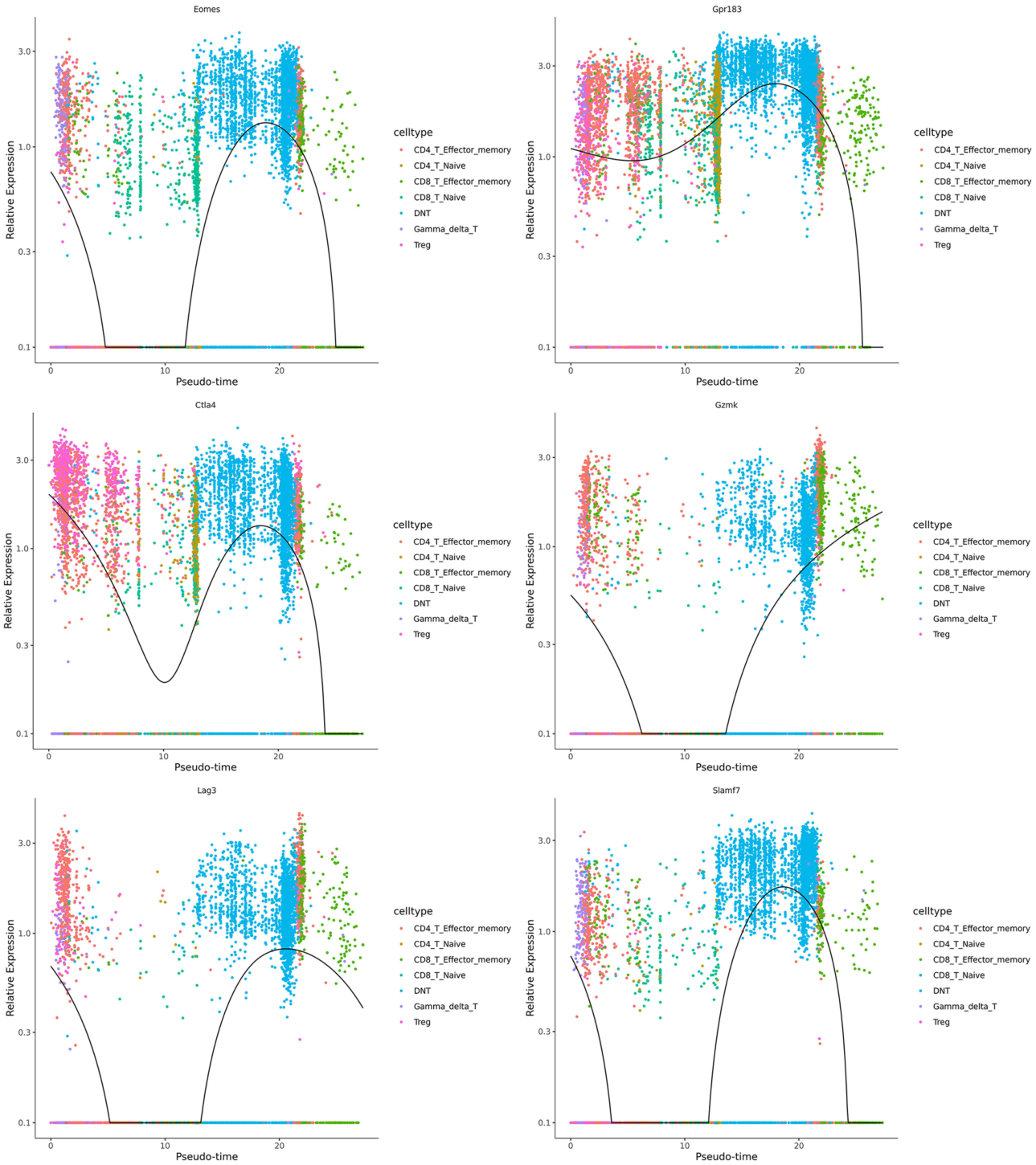


Figure 12 The expression levels of *Eomes*, *Gpr183*, *Ctla4*, *Gzmk*, and *Slamf7* according to pseudotime analysis. Each dot represents a single cell. The colour of the dot indicates the cell type, the abscissa is the pseudotime, the ordinate is the expression level of the specified gene in the cell, and the black curve indicates the trend of the gene between different cells with pseudotime.

studies of double-negative T cells and their biomarkers, including subclusters 2, 3, 8, 9, and 12, are needed. We examined each cluster's top 20 expressed marker genes (Figure 8), as well as their differential expression levels compared with those of other non-DNT subclusters. Combined with recent relevant research, we found that biomarkers such as *Eomes, Gpr183, Ctla4, Gzmk*, and *Lag3* were uniquely and partially more highly expressed in DNT-related subclusters (Figures 9 and 10). Recent studies have shown that *Eomes* plays a crucial role in regulating T-cell differentiation. Liu et al[13] conducted a study using qPCR, demonstrating that the mRNA expression levels of *Eomes* were significantly higher in SLE patients compared to healthy controls. Additionally, in the context of research on exhausted T cells in SLE, the high expression of *Eomes* in DNTs may be implicated in this disease.[14]

Pseudotime Trajectory Analysis Revealed That the DNT to Constitutes One End Stage or the Middle Part of the Trajectory of T Cells

Given that DNT may represent a state of T-cell exhaustion in the inflammatory process of the disease,[14] pseudotime trajectory analysis was conducted to validate this conclusion. The analysis using Monocle2 with the selected DNT revealed that naïve T cells were positioned at the initial part of the trajectory. CD4+ T effector/memory cells were mostly located in the initial stage, while CD8+ T effector/memory cells were predominantly located at one end stage of the trajectory. Tregs and γδT cells were distributed in both the initial stage and one end stage, where most CD8+ T cells are effector/memory cells. However, DNT exhibited a unique state with another end stage of the trajectory. In the process of pseudotime, following branch point 2, two different trajectories emerged, with one group of DNTs located in the middle and end stages alongside other T cells and another group of DNTs dominating the other lineage and the end stage (Figure 11). This finding provides strong evidence that DNT may be a marker of T-cell exhaustion in the context of SLE.

Pseudotime trajectory analysis also offers insights into key genes related to the growth or differentiation of DNT. In the previous t-SNE analysis, the potential roles and increased expression of *Eomes, Gpr183, Ctla4, Gzmk*, and *Lag3* in DNT were identified. After examining the varying expression levels of these genes over pseudotime, we determined that their trends of upregulation tended to coincide with the aggregation of DNT cells. Additionally, the gene marker *Slamf7* was identified as another potential target gene in DNT (Figure 12).

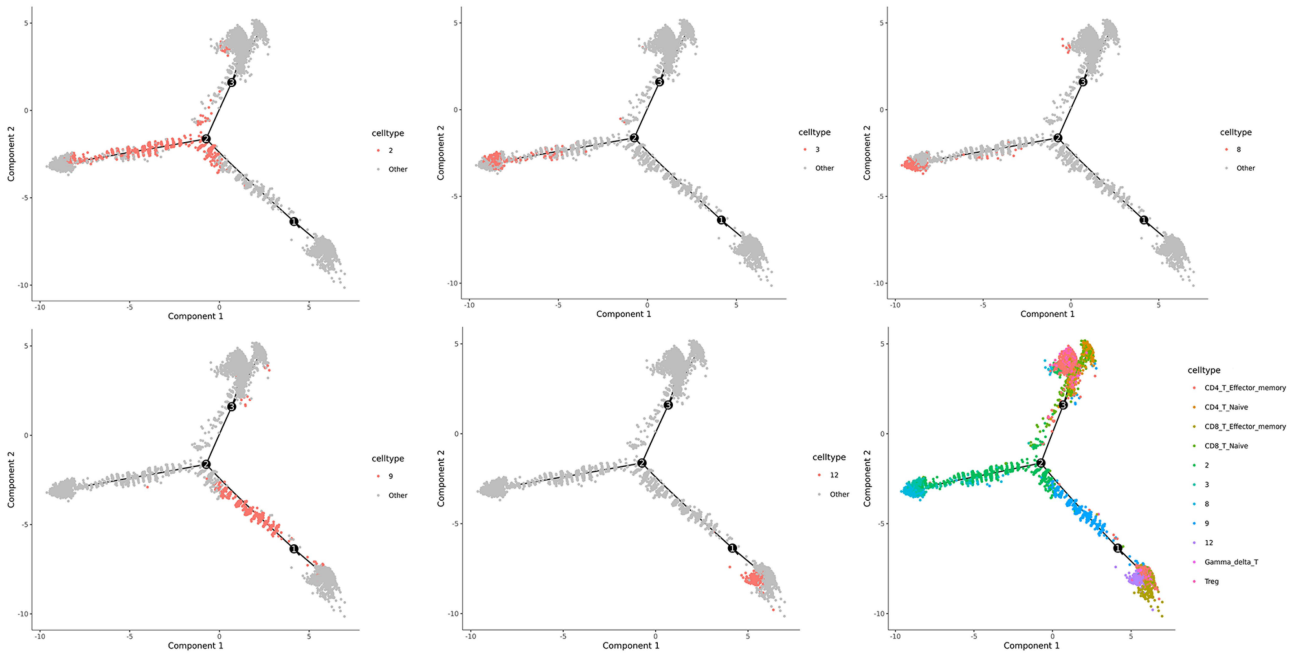


Figure 13 Results of the pseudotime trajectory analysis of DNT in both the spleen and brain. Each dot represents a single cell. The colour of the dot represents the different subclusters or cell types. The black lines indicate the main differentiation trajectories of the cells, and the black circles with numbers are the important nodes on the differentiation trajectories.

Then, we separated the five clusters of DNTs, with Clusters 2, 3, 8, and 9 representing spleen DNTs and Cluster 12 representing brain DNTs. According to the trajectory analysis (Figure 13), it is easy to classify Clusters 2, 3, and 8 into one group of DNTs and Clusters 9 and 12 into another. The latter finding may indicate that some DNTs in the spleen can eventually migrate to the brain through the blood through different biomarkers and compensate for the specific DNTs in the brain. This result supports our introduction of T cells from both the spleen and brain into our study.

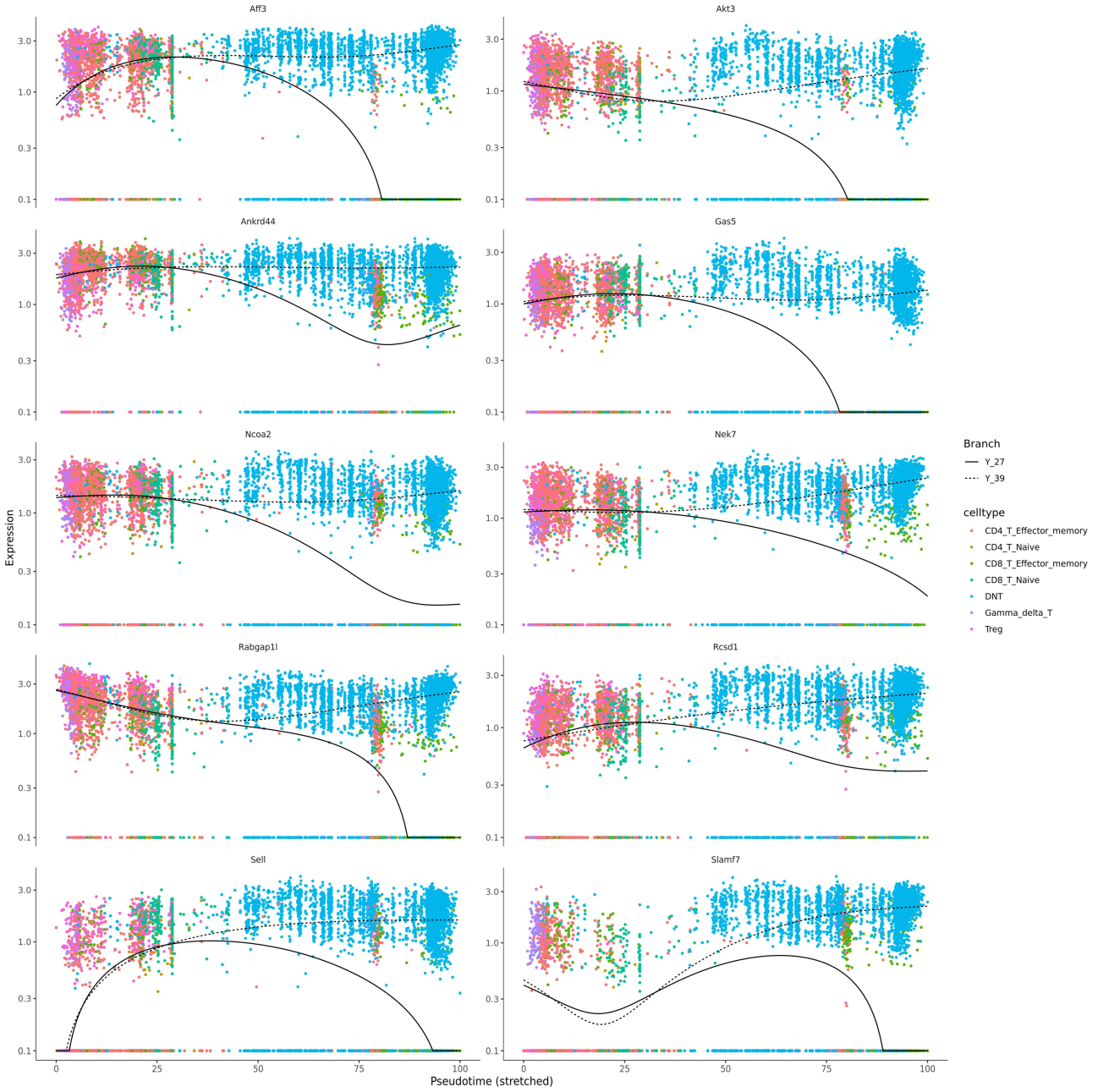


Figure 14 The expression levels of genes according to pseudotime on different branches. Each dot represents a cell, the colour of the dot represents the pseudotime, the abscissa is the pseudotime, the ordinate is the expression level of the specified gene in the cell, and the solid and dashed lines in black represent the trend of the gene with pseudotime between cells in different branches.

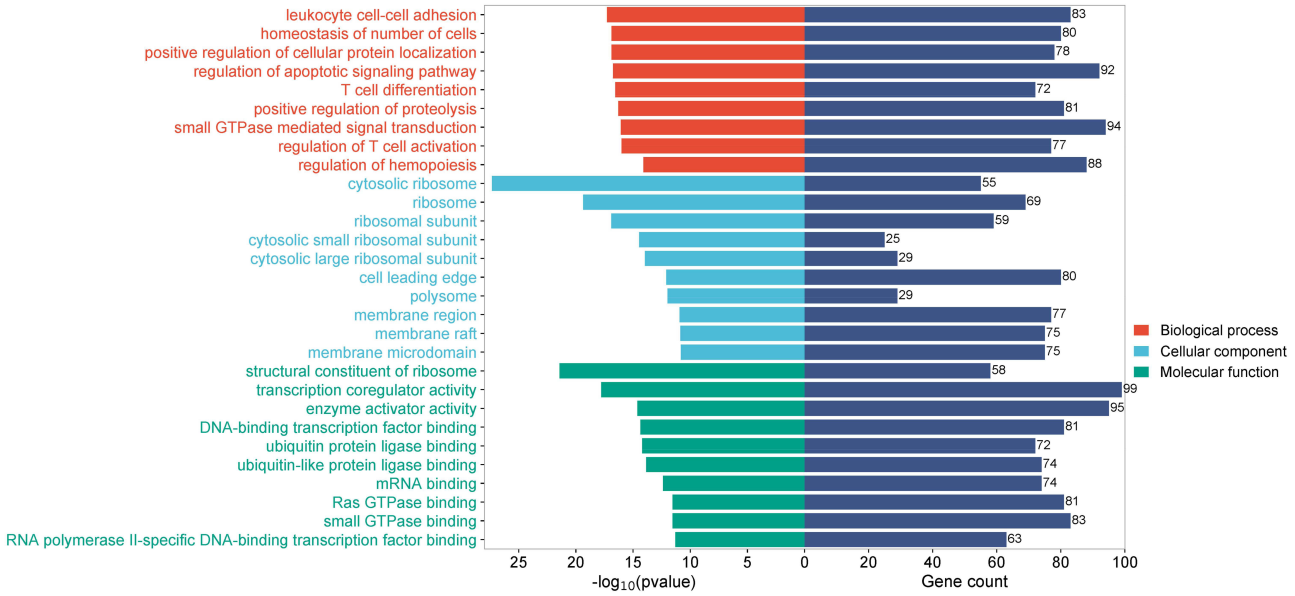


Figure 15 The histogram of GO of DNT.

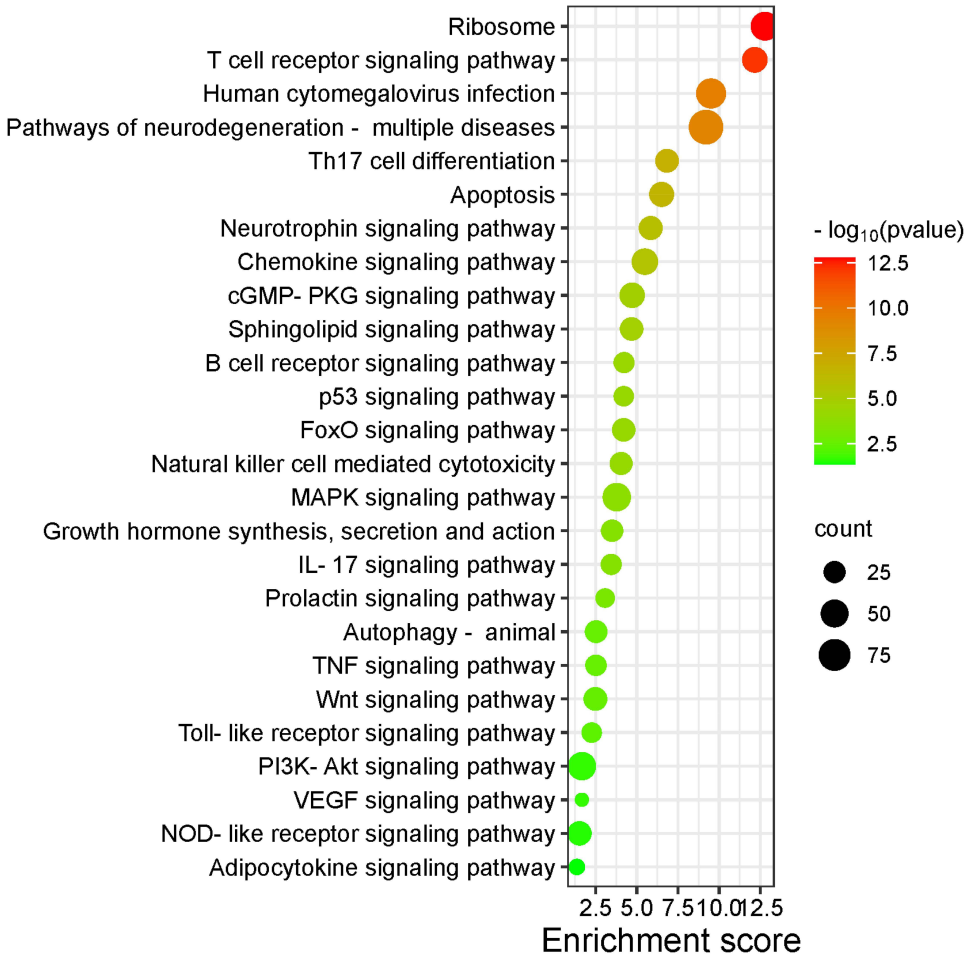


Figure 16 Bubble plot of the DNT KEGG database.

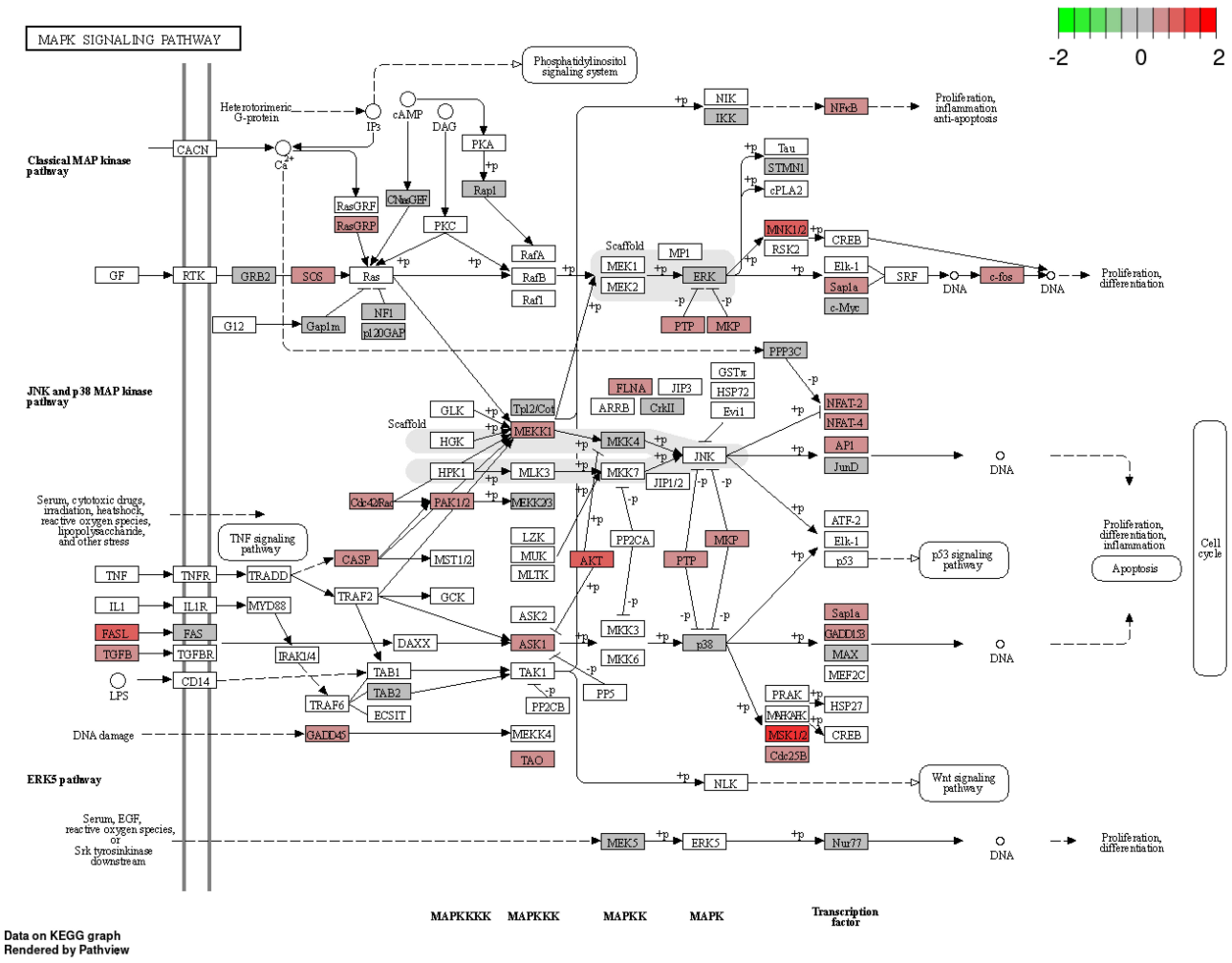


Figure 17 The MAPK signalling pathway KEGG plot of pathways associated with DNT.

The branch point is a crucial aspect of trajectory analysis, which enables us to identify additional underlying key biomarkers in DNT. We designated branch point 2 as the key point and divided the cells into three branches, including one prebranch, according to the pseudotime. The remaining two branches, known as the postbranches, were labelled Y27 and Y39 by Monocle2 (Figure 11). Subsequently, a branch-dependent gene analysis revealed several genes that exhibited a significant tendency to be expressed in Y39, which had been previously identified as being dominated by DNT. These genes displayed varying expression levels throughout the pseudotime, with some maintaining high expression in Y39, such as *Aff3, Akt3, Ankrd44, Gas5, Ncoa2*, and *Rabgap1l*, or exhibiting slightly increased expression, such as in *Nek7* and *Rcsd1*. *Sell* rapidly increased during pseudotime, and *Slamf7* exhibited unique high expression in the Y39 branch (Figure 14). Recent studies have strongly correlated *Slamf7* with T-cell exhaustion[15] and autoimmunity in the central nervous system.[16]

Cellular Senescence or Exhaustion-Related Signalling Pathways are Expressed in Double-Negative T Cells in SLE

Gene Ontology (GO) analysis and KEGG pathway are commonly employed tools in scRNA-seq analysis, which involves a large-scale database of functional genes and their related pathways. Based on the differential expression of double-negative T cells in the SLE mouse model, noteworthy results from the GO and KEGG enrichment analyses identified the occurrence of cellular senescence and exhaustion. The GO analysis revealed three levels of gene function, namely,

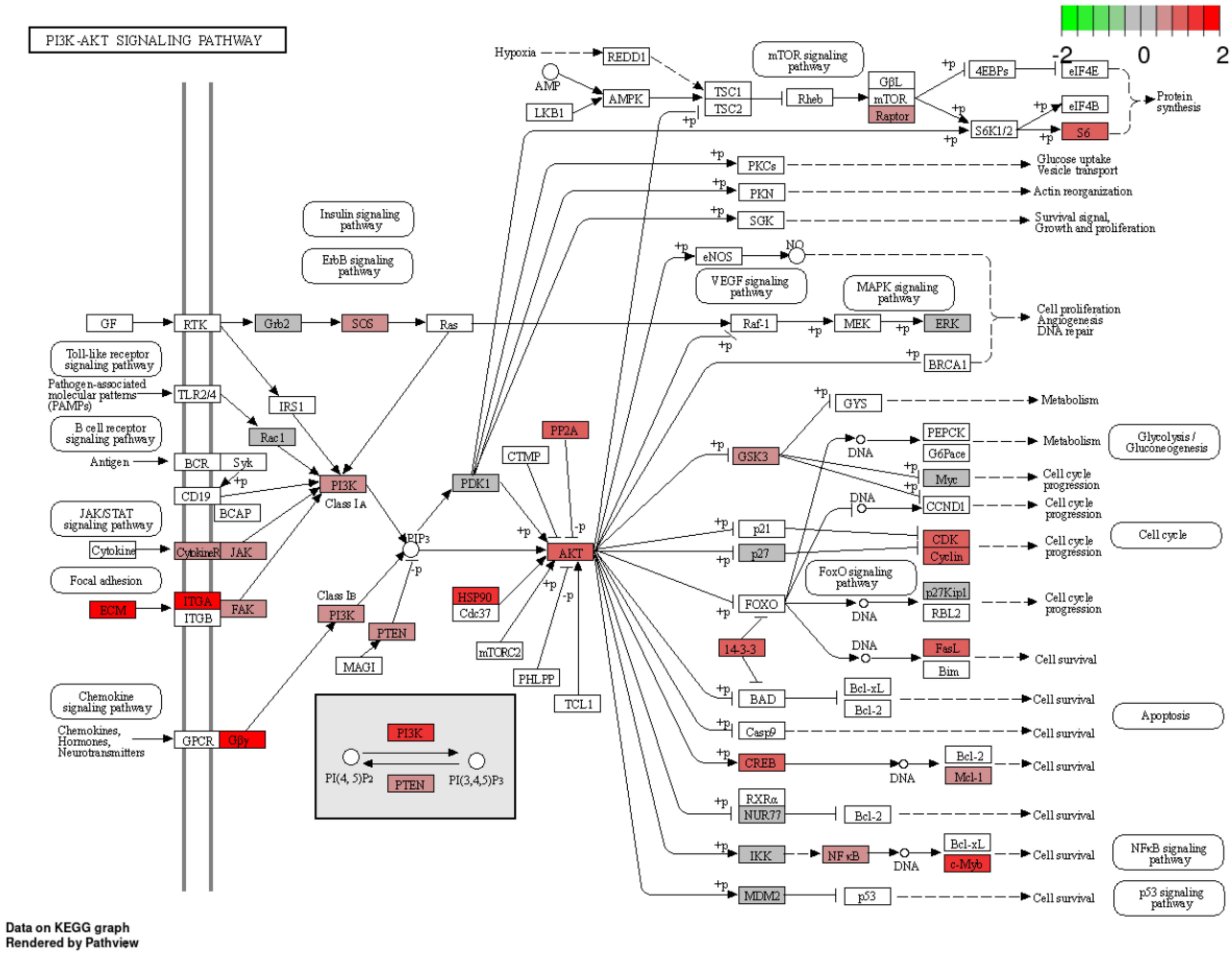


Figure 18 The PI3K-Akt signalling pathway KEGG plot of pathways associated with DNT.

cellular component (CC), molecular function (MF), and biological process (BP). The plot of GO (Figure 15) and KEGG (Figure 16) enrichment data helps to illustrate the distinct characteristics of double-negative T cells corresponding to subclusters.

As shown in the KEGG plots, pathways such as cellular senescence were identified in multiple clusters (Clusters 2 and 3). Additionally, the MAPK signalling pathway (Cluster 8) (Figure 17), PI3K-Akt signalling pathway (Cluster 8) (Figure 18), and NOD-like receptor signalling pathway were detected in double-negative T cells. Notably, the NOD-like receptor pathway has been confirmed to be closely related to autoimmune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and SLE[17] (Figure 19). In the GO plot, features such as negative regulation of cell population proliferation (Clusters 2, 3, 8, and 9), positive regulation of the apoptotic process (Clusters 2 and 3), and negative regulation of cell migration (Cluster 8) demonstrated the ageing and exhaustion of double-negative T cells. Pathway-related characteristics, such as positive regulation of GTPase activity (Cluster 9) and the ERK1/ERK2 cascade (Cluster 8), were also implicated (Figure 20).

Cluster 12 included double-negative T cells from the diseased brain. According to the results, the GO plot did not show any remarkable results except for the high expression of translation- and ribosome-related genes. Moreover, KEGG analysis revealed potential pathways related to neurodegeneration, along with diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease. This finding demonstrates the heterogeneity of brain T cells in SLE mice (Figures 21 and 22).

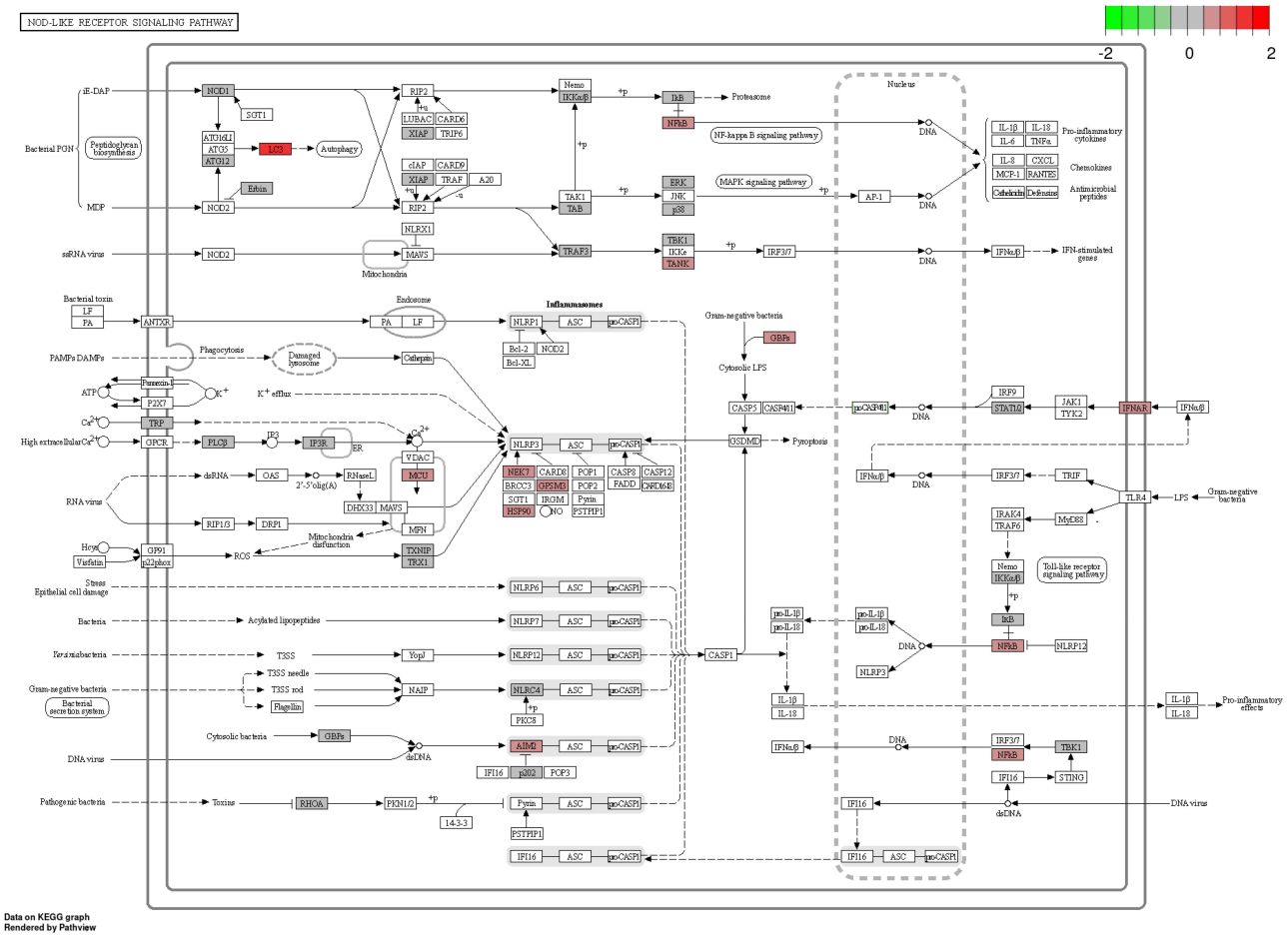


Figure 19 The NOD-like receptor signalling pathway KEGG plot of pathways associated with DNT.

Potential Interactions Between DNT and Astrocytes and Microglia in Neuroinflammation in Mice with SLE

Our focus extends beyond peripheral T cells and includes the intricate interactions between DNT and both astrocytes and microglia within the central nervous system. To explore the divergence of functional genes during the disease state of the brain, we performed group-based differential expression analysis and functional enrichment with two brain cell samples from the control and disease groups. Despite the initial lack of conclusive results in astrocytes and microglia, we remained committed to elucidating the complex molecular dynamics underlying neuroinflammation, emphasizing DNT.

Next, we used a signal dataset, CellPhoneDB, to predict the interactions between different kinds of cells, revealing possible cell communication between these cells. Strikingly, our analysis revealed evidence of cell interactions (Figure 23) and potential ligand–receptor pairs (Figure 24) involving DNT, astrocytes, and microglia. Subsequently, we delved deeper into the interaction network of these three cell types using the same database, with a particular emphasis on DNT acting as both a ligand and a receptor. The dot plots provide us with an intuitive view of the different states of cell signalling in SLE (Figure 25). Remarkably, *MDK-SORL1* and *BMP7-PTPRK*, which act as receptors in the DNT signalling pathway, changed from astrocytes to DNT cells, and *LGALS9-PTPRK* and *GRN-SORT1*, which act as receptors from microglia to T cells, were slightly upregulated. As a ligand, *SELL-SELPLG* demonstrates an unupgraded pathway from DNT to microglia. Midkine, also known as MDK, is a heparin-binding growth factor implicated in various systems and diseases, including cancers,[18] infections,[19] and autoimmune inflammation. Previous studies have established its potential connection, in some cases as dysfunctions, to T cells.[20] The elevated levels of MDK in serum identified in

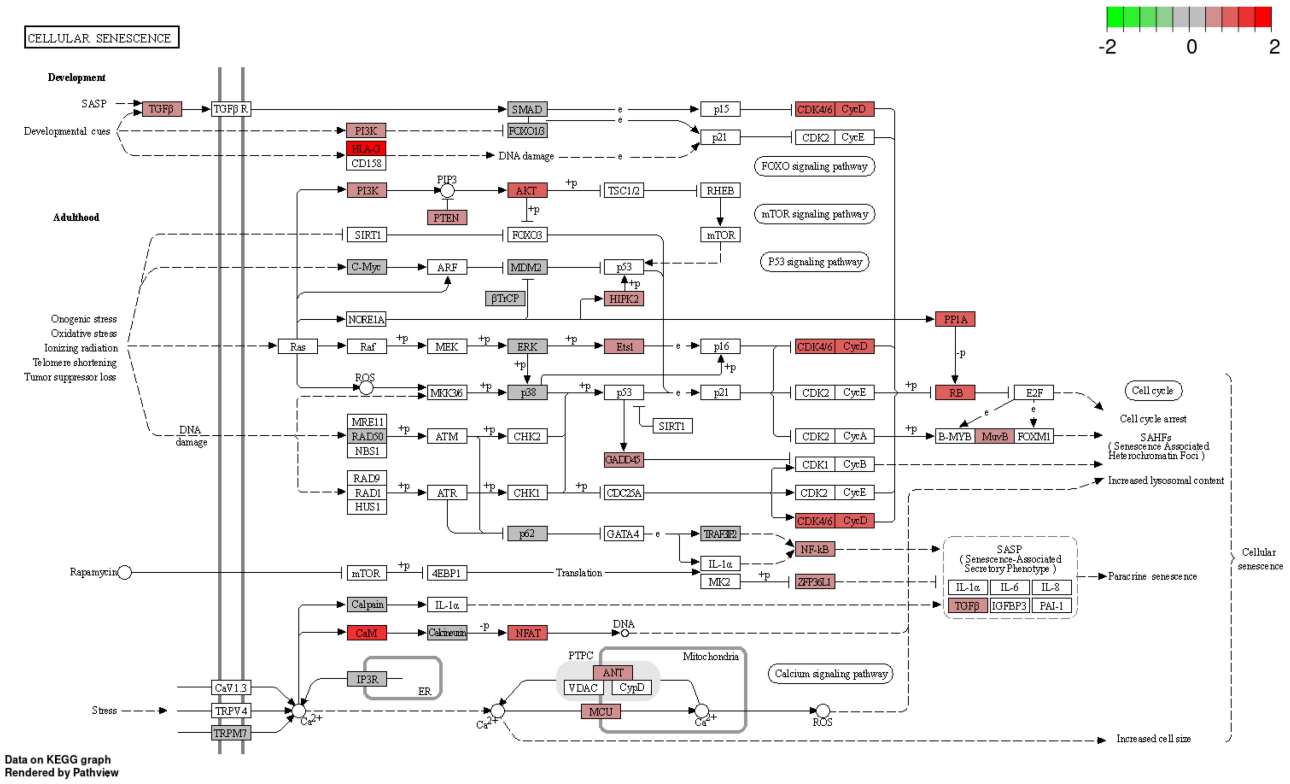


Figure 20 The Cellular senescence KEGG plot of pathways associated with DNT.

SLE studies[21] underscore its potential role as a key mediator in the intricate interplay between midkine and T cells within the context of SLE-associated neuroinflammation.

Discussion

Systemic lupus erythematosus is a globally prevalent autoimmune disease affecting multiple organs.[22] Despite ongoing research, the diagnosis and treatment of SLE remain challenging due to its intricate pathways.[23] For SLE patients with CNS involvement, so-called NPSLE, makes understanding the disease much more complex.[2] NPSLE shares immune changes with SLE but differs in cell types and complicated environments in the CNS. As one of the comorbidities of SLE, NPSLE is inherently connected to SLE, necessitating a combined approach to depict a comprehensive landscape.

In our study, we utilized single-cell transcriptome sequencing (scRNA-seq) to gain a nuanced understanding of T-cell heterogeneity in mice with SLE, shedding light on the complex interplay between T-cell heterogeneity and neuroinflammation. Our exploration began with profiling cells from both brain and spleen tissues in MRL/lpr mice compared with those from healthy BALB/c mice. T cells, migrate from the spleen, via the blood, to the brain to realize their peripheral function. The comprehensive analysis of clustering identified diverse cell types in the brain and spleen, which provides a general review of cell composition and its differences between disease mice and healthy controls. We are delighted to find that brain samples exhibit great heterogeneity, especially in terms of astrocytes, microglia, and T cells. This finding encouraged further investigations into T cells, as supported by previous studies.[24,25] Considering recent studies highlighting the role of microglia as a factor in the onset of inflammation in NPSLE patients,[26,27] we analysed microglia, astrocytes, and DNT cells using cell communication techniques.

The identification of a distinct subset of double-negative T cells (DNTs) in SLE mice draws attention to the potential role of this subset in the inflammatory processes of the disease. DNTs are currently a focus of research in SLE. Abnormal proliferation of DNTs has been observed not only in the MRL/lpr mouse model but also in SLE

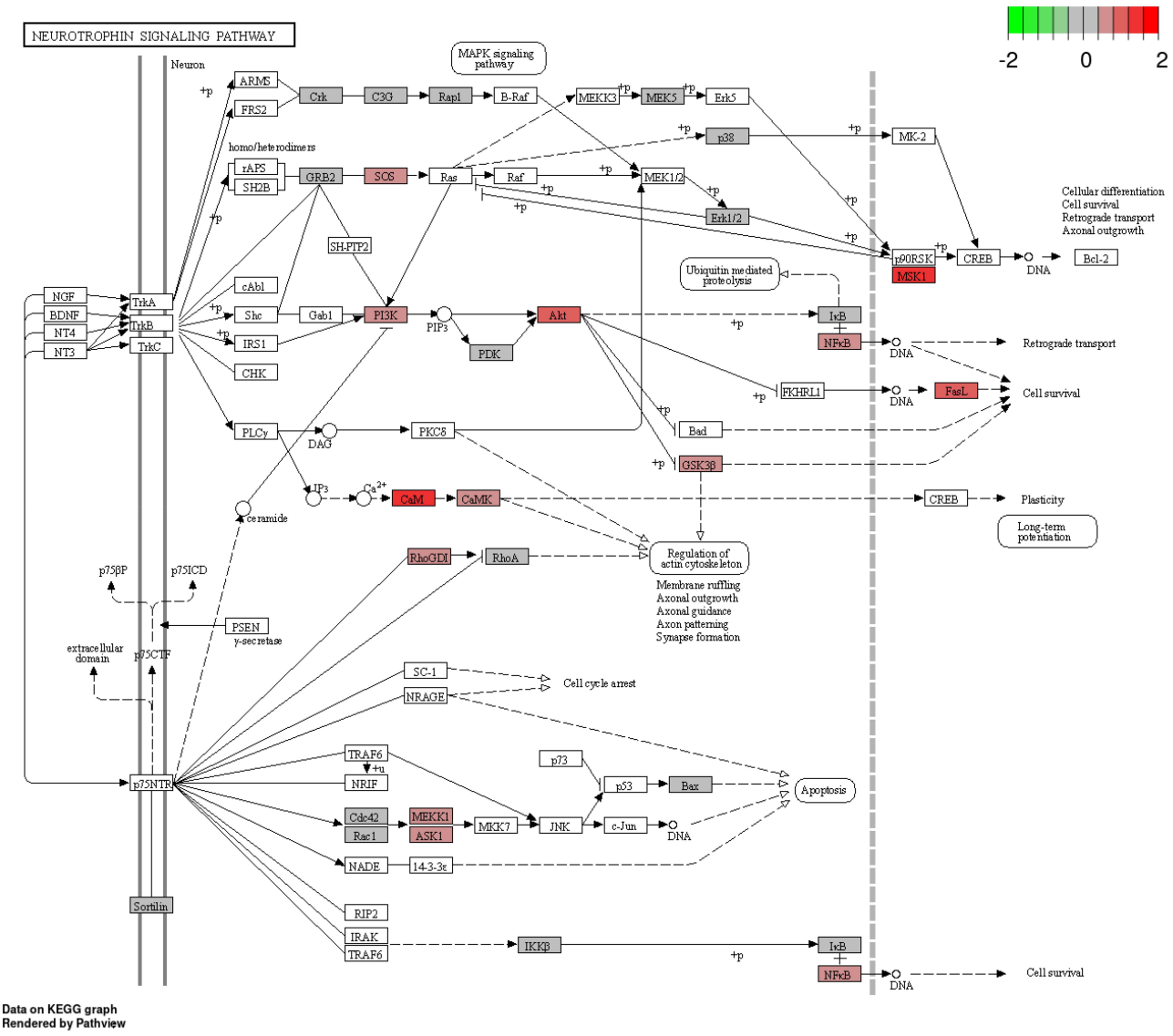


Figure 21 Neurotrophin signalling pathway KEGG plot of pathways associated with DNT.

patients. DNTs are known to increase the secretion of inflammatory cytokines such as IL-4 and IL-17 and are highly expressed in patients with active SLE rather than those in remission.[28] Recent studies have identified a post-translational modification known as neddylation that may enhance DNT accumulation in both SLE murine models and patients. Inhibiting neddylation can induce apoptosis in DNTs, thereby attenuating lupus progression. Consequently, DNTs have emerged as a promising therapeutic target for SLE.[29] The expression of *Eomes, Gpr183, Ctla4, Gzmk*, and *Lag3* in DNTs indicates their involvement in the pathogenesis of SLE. In particular, the elevated expression of *Eomes* in DNTs aligns with previous findings linking this transcription factor to T-cell exhaustion in autoimmune conditions. DNT cells are a subset of unique T cells with CD3+CD4-CD8- expression. The existence and elevated levels of DNTs contribute to the pathogenesis of SLE and are correlated with disease activity.[30] Although DNTs were originally found in peripheral blood, our study revealed a subset of DNTs inside the brain during the onset of NPSLE. *Eomes*, also known as eomesodermin, was identified in a recent study using single-cell RNA sequencing (scRNA-seq) of DNTs,[31] as was T-cell exhaustion in patients with SLE remission.[14]

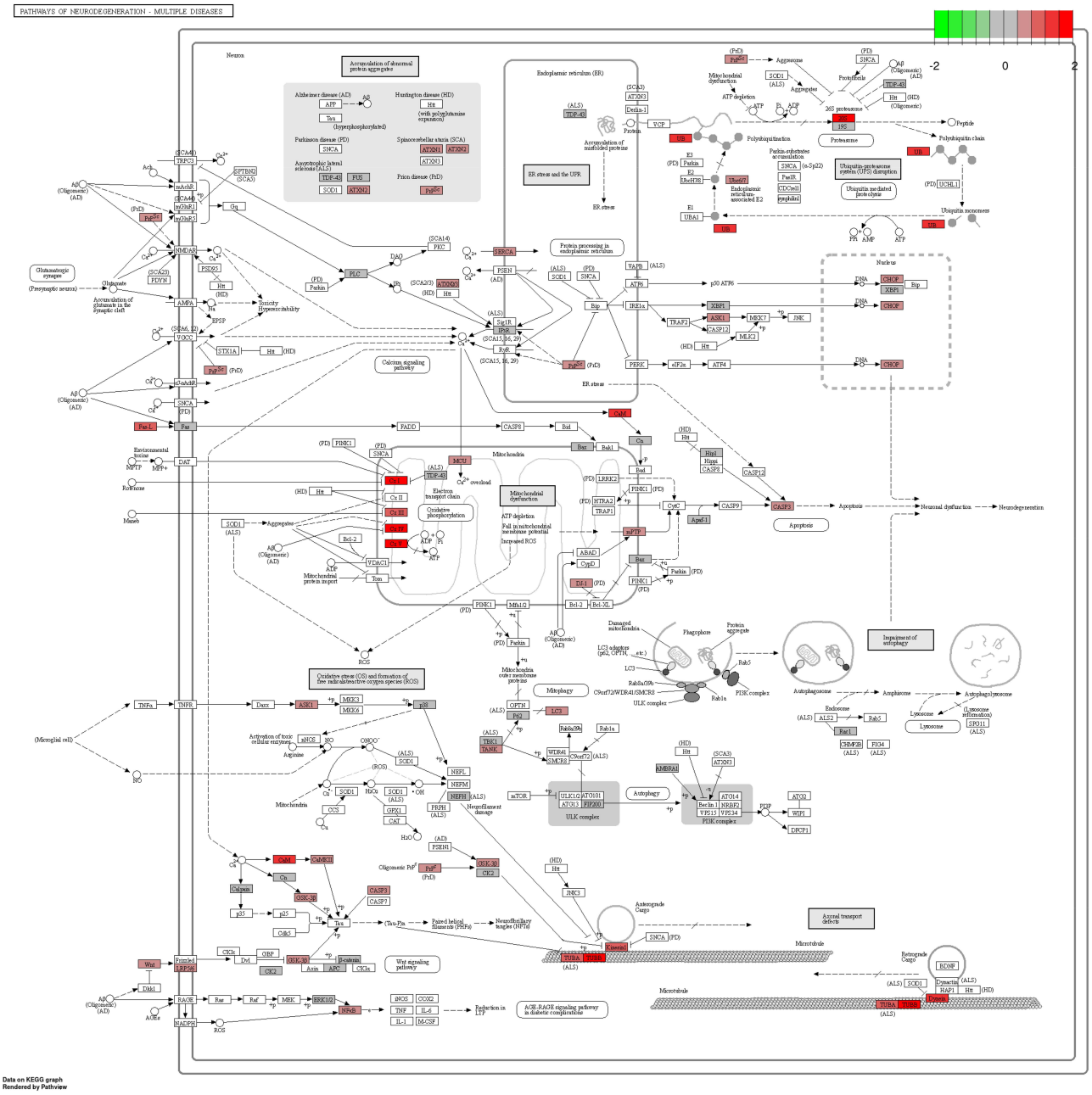


Figure 22 Pathways associated with multiple neurodegenerative diseases.

Several studies on the use of *Eomes* as a biomarker for SLE have shown that its expression is increased. Oncologists are more likely to focus on *Eomes* because of its antitumour function in T-cell exhaustion.[32] GO and KEGG pathway analyses further revealed evidence of cellular senescence, exhaustion, and neurodegenerative processes associated with DNTs in SLE, as well as suggestive pathways.

Pseudotime trajectory analysis in this study not only confirmed that DNT is a distinctive end stage or intermediate phase and that DNT in the brain may migrate from the spleen through the blood but also provided a temporal framework for understanding the progression of DNTs. The unique trajectory of DNTs, diverging from that of other T-cell subsets, indicates a potential role in the chronicity of the immune response in SLE. Branch-dependent gene analysis revealed that *Slamf7* is a notable marker associated with T-cell exhaustion[15] and autoimmunity in the inflammatory

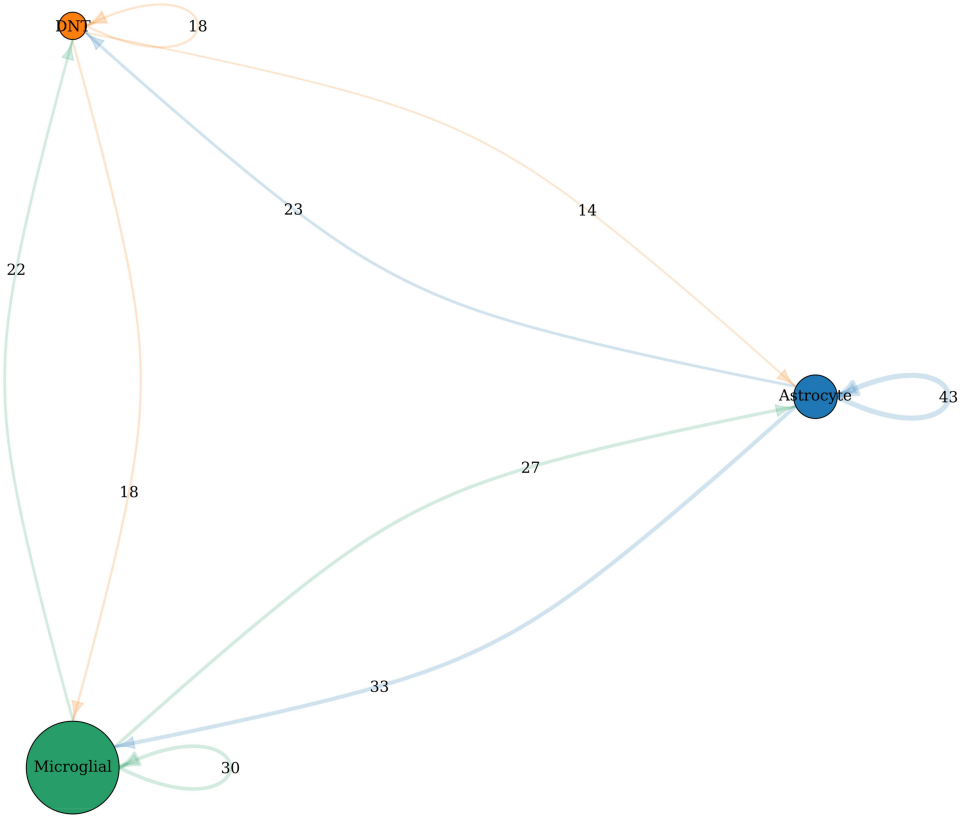


Figure 23 Cell communication network among DNTs, microglia and astrocytes in the brain. The different coloured circles represent different cell populations, the size of the circles is proportional to the number of cells in the cell population, the thickness of the connection represents the number of significant interactions between cells, and the number above represents the specific value. The thicker the connection line is, the stronger the communication. The connecting line arrow points from the signalling cell to the signalling cell, and the colour matches the type of cell from which the signal is emitted.

microenvironment[33] as well as in the central nervous system,[16] providing a potential target for therapeutic intervention. We further constructed an interaction network for SLAMF7 with other proteins using STRING analysis and identified coexpression with Fcgr3 and Itgam, which drives an inflammatory signaling cascade that activates macrophages. Additionally, interactions with CD48 and other members of the SLAM family regulate NK cell responses to infection and T-cell generation. These interactions have emerged as promising targets for immunotherapy in autoimmune diseases.

We expanded our focus to the central nervous system and scrutinized interactions among DNT cells, astrocytes, and microglia. Although conclusive results in astrocytes and microglia are lacking, the application of the CellPhoneDB dataset revealed compelling similarities in cell interactions and potential ligand–receptor pairs between these cell types in the disease and nondisease groups. The identification of DNT involvement in the *MDK-SORL1* and *BMP7-PTPRK* signalling pathways in astrocytes and *LGALS9-PTPRK* and *GRN-SORT1* signalling in microglia highlights potential molecular mediators of SLE-associated neuroinflammation. An increase in the *SELL-SELPLG* pathway from DNT to microglia is also a notable finding. MDK has emerged as a key player in the interplay between midkine and T cells during SLE-associated neuroinflammation. Previous studies have implicated MDK in various diseases, including cancers and autoimmune inflammation, establishing its potential connection, sometimes as a dysfunction, to T cells.[20,21] The elevated levels of MDK in the serum of mice with SLE further underscore its potential role as a mediator of the intricate crosstalk between midkine and T cells within the context of neuroinflammation. However, it needs further investigation to prove its possible functional target and underlying cellular interactions in neuroinflammation of SLE.

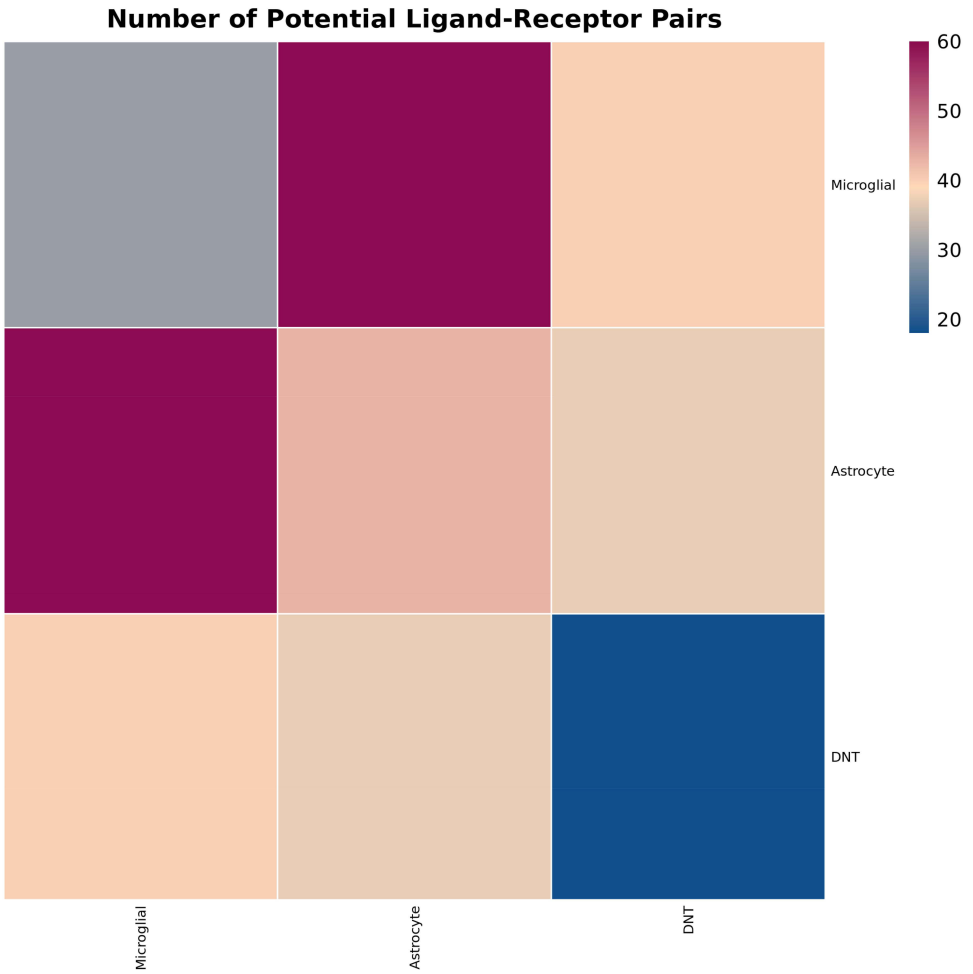


Figure 24 Heatmap of cell communication. The horizontal and vertical axes represent the cell type, and the colours correspond to the number of interactions, which gradually increases as the colour patch changes from dark blue to light blue to dark violet.

In conclusion, our study, employing advanced scRNA-seq techniques, provides a better understanding of T-cell heterogeneity in mice with SLE and its implications for neuroinflammation. The identification of DNTs as a potential exhausted state, the elucidation of key genes and pathways, and the exploration of interactions within the central nervous system have contributed to the growing knowledge of autoimmune disorders. These findings offer valuable insights into the complex landscape of SLE and potential targets for precise therapeutic interventions. Moving forwards, further investigations into the molecular intricacies of T-cell heterogeneity and its interplay with neuroinflammation will open new avenues for therapeutic development in the management of SLE.

Due to sample accessibility limitations, our study used a murine model instead of clinical patients. Ethical considerations are paramount for future studies on T-cell heterogeneity in humans. However, our study provides strong evidence for the role of T cells, especially DNTs, not only in SLE but also in CNS involvement in NPSLE. With the increasing number of studies on DNT and T-cell exhaustion contributing to various diseases, the body of knowledge surrounding autoimmune disorders may lead to the development of targeted therapeutics addressing more than just SLE. Additionally, the relationships of brain-specific cells such as astrocytes and microglia with T cells are another essential topic for exploration. There may be additional findings beyond those in our study that remain to be revealed during scRNA-seq-related analyses.

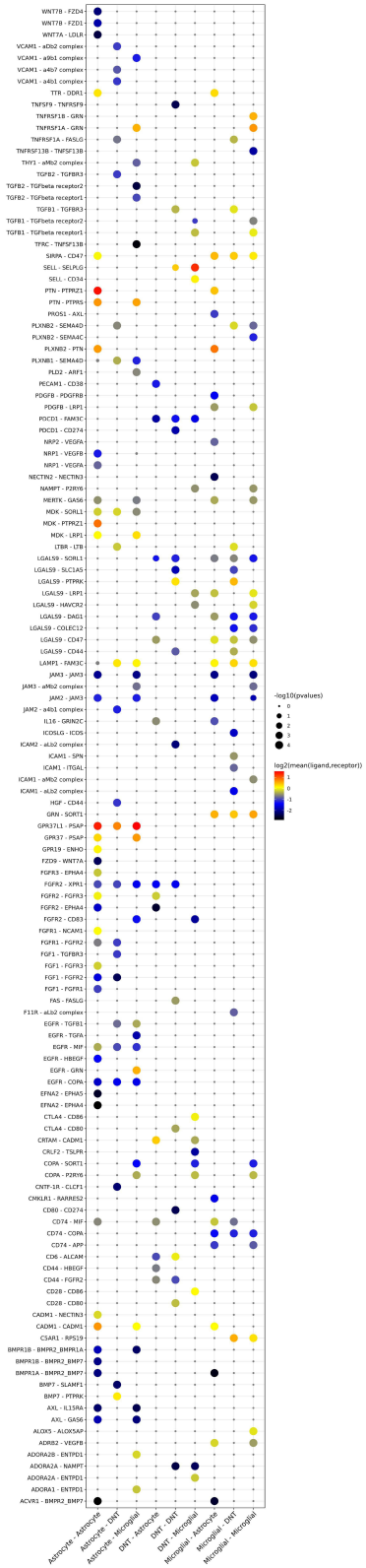


Figure 25 Dot plot of cell communication. The horizontal axis represents the two cell types involved in communication. The left side of the en-dash represents the cell type of the ligands, and the right side represents the cell type of the receptors. The vertical axis represents cell signalling. The size of the circle represents the P value after -log10 transformation, so the larger the circle is, the greater the confidence there is in this interaction. The colour represents the average expression of the ligands and the receptors.

Ethics

The study was reviewed and approved by the Animal Welfare and Ethics Committee of Department of Laboratory Animal Science Fudan University (Approved No. of ethic committee: 2022JS Huashan Hospital-213), followed by the guideline of Laboratory animals—General code of animal welfare (GB/T 42011-2022), National Standard of People's Republic of China.

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Disclosure

The authors declare no conflict of interest.

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