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Single-Cell RNA Sequencing Reveals Transcriptional Landscape of Neutrophils and Highlights the Role of TREM-1 in EAE

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Abstract

Background and Objectives

Neutrophils, underestimated in multiple sclerosis (MS), are gaining increased attention for their significant functions in patients with MS and the experimental autoimmune encephalomyelitis (EAE) animal model. However, the precise role of neutrophils in cervical lymph nodes (CLNs), the primary CNS-draining lymph nodes where the autoimmune response is initiated during the progression of EAE, remains poorly understood.

Methods

Applying single-cell RNA sequencing (scRNA-seq), we constructed a comprehensive immune cell atlas of CLNs during development of EAE. Through this atlas, we concentrated on and uncovered the transcriptional landscape, phenotypic and functional heterogeneity of neutrophils, and their crosstalk with immune cells within CLNs in the neuroinflammatory processes in EAE.

Results

Notably, we observed a substantial increase in the neutrophil population in EAE mice, with a particular emphasis on the significant rise within the CLNs. Neutrophils in CLNs were categorized into 3 subtypes, and we explored the specific roles and developmental trajectories of each distinct neutrophil subtype. Neutrophils were found to engage in extensive interactions with other immune cells, playing crucial roles in T-cell activation. Moreover, our findings highlighted the strong migratory ability of neutrophils to CLNs, partly regulated by triggering the receptor expressed on myeloid cells 1 (TREM-1). Inhibiting TREM1 with LR12 prevents neutrophil migration both in vivo and in vitro. In addition, in patients with MS, we confirmed an increase in peripheral neutrophils with an upregulation of TREM-1.

Discussion

Our research provides a comprehensive and precise single-cell atlas of CLNs in EAE, highlighting the role of neutrophils in regulating the periphery immune response. In addition, TREM-1 emerged as an essential regulator of neutrophil migration to CLNs, holding promise as a potential therapeutic target in MS.

Introduction

Multiple sclerosis (MS), a chronic inflammatory and degenerative autoimmune disease characterized by infiltration of inflammatory cells into the CNS, leads to demyelination and neuronal injury.[1,2] MS occurs in early to mid-adulthood and is 2–3 times more common in women.[3.] Approximately 80% of patients with MS demonstrate a relapsing-remitting course

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Glossary

CLN = cervical lymph node; **CSI** = connection specificity index; **DLN** = draining lymph node; **EAE** = experimental autoimmune encephalomyelitis; **FBS** = fetal bovine serum; **ILC** = innate lymphoid cells; **JSD** = Jensen-Shannon divergence; **LWR** = lymphocyte-to-WBC ratio; **MACS** = magnetic-activated cell sorting; **MS** = multiple sclerosis; **NKs** = natural killer cells; **NKTs** = natural killer T cells; **NLR** = neutrophil-to-lymphocyte ratio; **NWR** = neutrophil-to-WBC ratio; **ODs** = optical densities; **ROS** = reactive oxygen species; **RRMS** = relapsing-remitting course; **RSS** = regulon specificity score; **scRNA-Seq** = single-cell RNA sequencing; **TF** = transcription factor; **TREM-1** = triggering receptor expressed on myeloid cells 1; **WBC** = white blood cell.

(RRMS).[3] Pathology and inflammatory response of MS has been extensively explored using the most commonly used animal model experimental autoimmune encephalomyelitis (EAE).[4] Myelin oligodendrocyte glycoprotein peptide $MOG_{35–55}$, established as encephalitogenic epitope, can induce full-scale clinical EAE with acute and chronic stages.

Most studies focus on the role of T cells; however, studies on MS and EAE have shown that other types of immune cells also contribute to pathogenesis.[1] Neutrophils are the most abundant leukocytes in human peripheral blood, providing a first line of defense against bacterial pathogens. Recent studies in both patients with EAE and MS suggest a vital role for neutrophils in disease pathogenesis,[2] which are underestimated players in CNS autoimmune diseases.[3] Neutrophils are primed in MS and display an activated phenotype.[4] The ratio of $CD15^+$ neutrophils to $CD45^+$ immune cells in circulating blood can be used for robust discrimination between MS types.[3] An elevated neutrophil-to-lymphocyte ratio (NLR) is associated with disease progression, neurologic disability, and brain atrophy.[5,6] In EAE mice, neutrophils are more numerous in the periphery blood and CNS in the preonset and acute stages.[7,8] Depletion[7] or inhibition of neutrophil migration[9] significantly alleviated EAE onset and severity. Neutrophils exhibit a broad array of mechanisms relevant for MS/EAE pathogenesis, including cytokine production, blood-brain barrier breakdown, reactive oxygen species (ROS), neutrophil extracellular traps, antigen presentation, and T-cell activation.[2] However, neutrophils are not a pronounced pathologic feature in MS CNS tissue sections,[2] and CSF neutrophils decrease with disease duration, although they are recruited in CNS early in the inflammatory process before disease onset.[7]

Immunologic episodes in MS are initiated in CNS-draining lymph nodes, wherein immune cells first activated and have been reported to continuously traffic between the CNS and its draining LNs in EAE.[10] CNS-draining LNs, cervical lymph nodes (CLNs), are believed to be the key sites for the activation and sensitization of immune cells responsible for MS. Recent research has shown that neutrophils exhibit their significant effects in CNS-draining lymph tissue,[11-14] where they quantitatively regulate the expansion of antigen-specific $CD4^+$ T cells.[14] However, at the resolution of single cell, the specific role of neutrophils in CLNs still needs to be explored. Moreover, the mechanism of neutrophil migration to CLNs, quantity of subtypes included in CLN neutrophils, the particular functions executed by each subtype, and the interactions between neutrophils and other immune cells in CLNs remain uncertain.

Single-cell RNA sequencing (scRNA-Seq) is a powerful and well-established method for exploring transcriptomic variation, uncovering novel cell types, and providing insights into developmental processes and transcriptional heterogeneity.[15] In this study, we firstly certificated that the neutrophils were expanded in both patients with MS and EAE mice and then constructed a comprehensive immune cell atlas of CLNs during development of EAE. Through our analysis, we investigated the cellular crosstalk, heterogeneity, functional versatility, and developmental characteristics of CLN neutrophils. Our findings revealed that CLN neutrophils exhibit a robust migratory ability, indicating neutrophils might migrate to CLNs during EAE development and subsequently play their critical proinflammatory role. In addition, we observed that the migration process was partially regulated by TREM-1. Notably, the administration of LR12, a TREM-1 inhibitor suppresses neutrophil migration both in vivo and in vitro.

Methods

See eMethods.

Standard Protocol Approvals, Registrations, and Participant Consents

The experiment was approved by the Experimental Ethics Committee of the Second Hospital of Hebei Medical University. Informed consent was obtained from all participants.

Data Availability

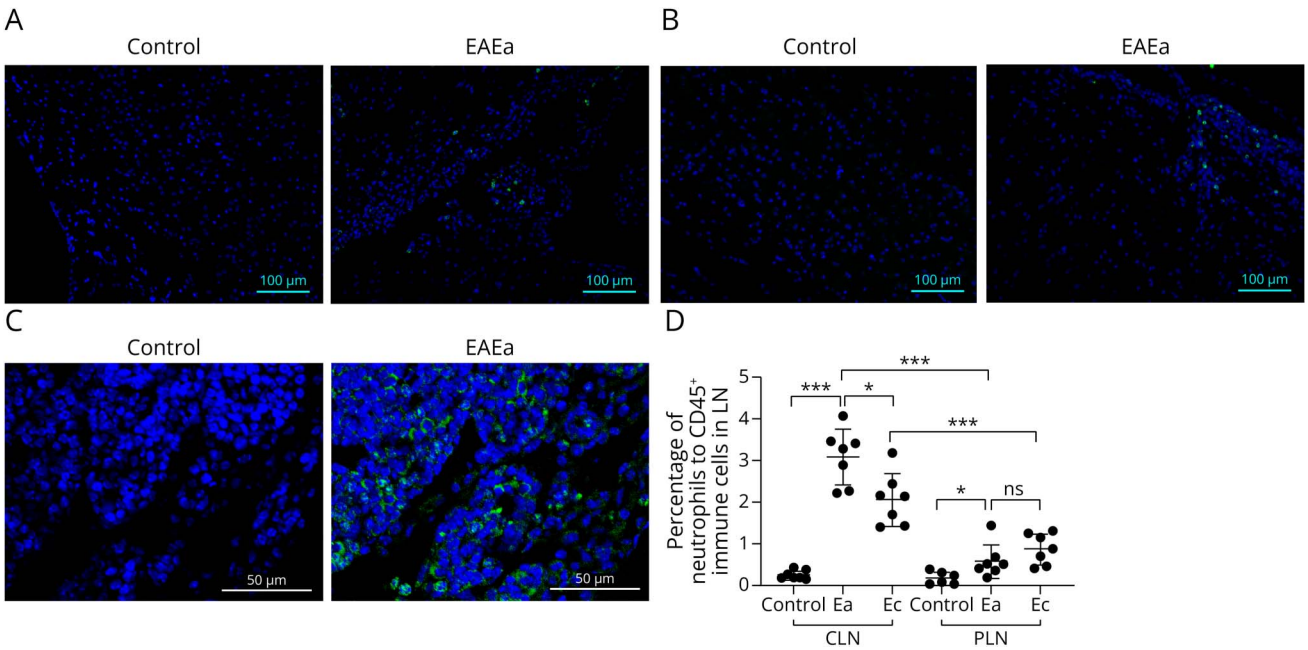
The data sets used and/or analyzed during this study are available from the first author on reasonable request.

Results

Neutrophil Expansion in MS and EAE

In EAE mice, the percentage of neutrophils in peripheral blood was higher in both acute-stage and chronic-stage EAE (eFigure 1, A and B). Moreover, neutrophils infiltrated into the brain (Figure 1A) and spinal cord (Figure 1B) in the EAE

Figure 1 Neutrophil Expansion in EAE Mice



(A–C) Immunofluorescent analysis of anti-ly6G–stained neutrophils (green fluorescence) in brain tissue (A), lumbar enlargement (B), and CLNs (C). (D) The percentage of neutrophils to CD45$^+$ immune cells in CLNs and PLNs evaluated by flow cytometry (n = 6 or 7). Data are mean ± SD (error bars) based on 6 or 7 independent biological replicates. *p < 0.05, ***p < 0.001, NS means no statistical difference. CLN = cervical lymph node; EAE = experimental autoimmune encephalomyelitis.

acute stage. Besides CNS, we also examined the proportion of neutrophils in lymph nodes (eFigure 1, C–D, and Figure 1, C and D). Immunohistochemistry (Figure 1C) and flow cytometry (eFigure 1D) revealed minimal neutrophil presence in control mice CLNs, while an increase in neutrophils was observed during EAE. Furthermore, the dynamic ratio of neutrophils in cervical (CNS-draining) and inguinal (non–CNS-draining) lymph nodes during different stages of EAE was validated using flow cytometry (eFigure 1, C and D). The percentage of neutrophils was higher in both acute and chronic EAE stages in cervical and inguinal lymph nodes with the highest levels observed in the acute stage (Figure 1D). Regardless of the stage (acute or chronic), the neutrophil ratio in CLNs was consistently higher than that in inguinal lymph nodes (Figure 1D), suggesting a potentially role of neutrophils in CNS-draining lymph nodes. As a result, we focus on neutrophils in CLNs by applying single-cell RNA sequencing.

Single-Cell Immune Atlas in EAE CLNs

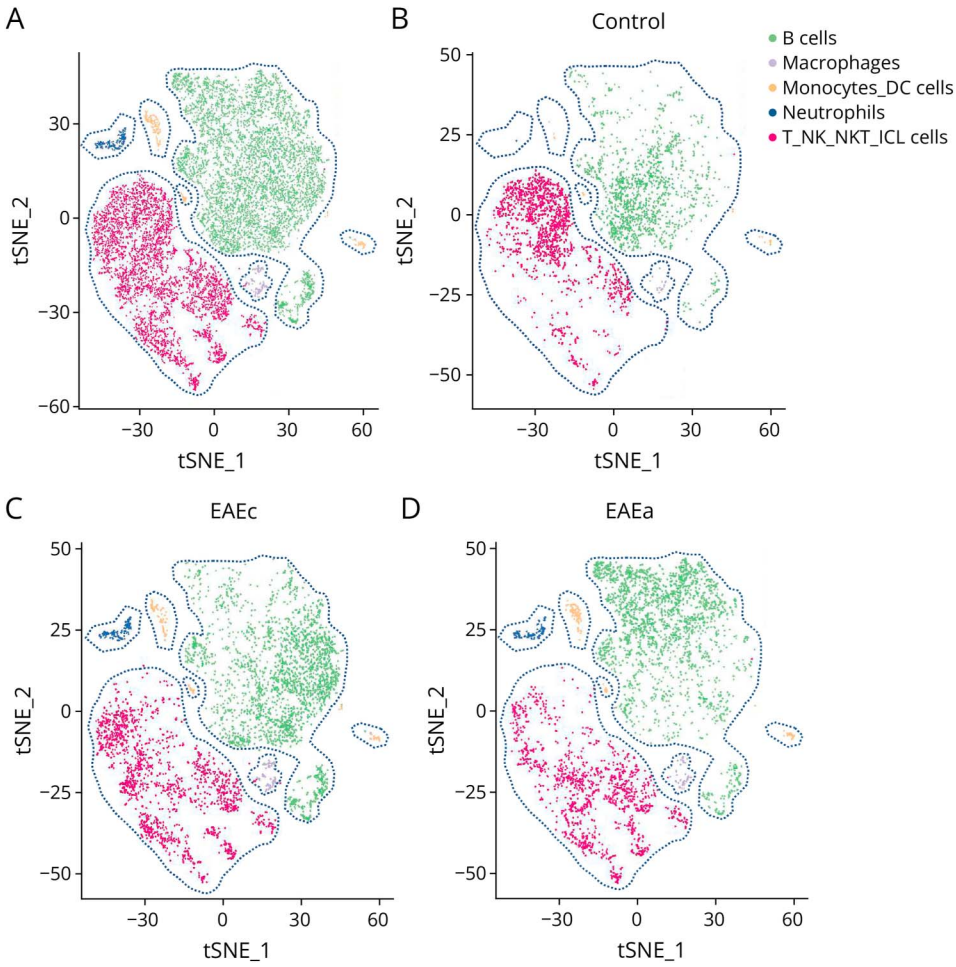
We used scRNA-Seq for gene expression analysis with the expectation that genomic information from individual cell types would help elucidate the pathophysiologic mechanisms of immune cells, especially neutrophils, in EAE that might be concealed in bulk RNA analysis. Considering the progression characteristics of MOG-induced EAE, we selected the acute and chronic stages to encompass the key phases of peripheral immune cell development. To delve into the pathogenesis of the disease, we augmented our analysis with samples from control mice. Subsequently, we conducted scRNA-Seq on CD45$^+$ immune cells isolated from CLNs through MACS. These cells underwent barcoding, library preparation, and sequencing processes[16] (eFigure 2A). After application of quality control filters, 3,159 cells (control), 4,938 cells (EAE acute stage), and 4,454 cells (EAE chronic stage) were included in the subsequent scRNA-Seq analysis.

Major immune cell populations were identified using a graph-based clustering approach and t-distributed stochastic neighbor-embedding dimensionality reduction.[17,18] The unsupervised Seurat-based clustering method identified the presence of 12 distinct clusters (eFigure 2B). Cell identity was determined based on the expression of classic genes (eFigure 2, C and D), and the identified immune cell types mainly included monocytes/dendritic cells (Mo-DCs), resident macrophages, neutrophils, B cells, and T cells/natural killer T cells (NKTs)/natural killer cells (NKs)/innate lymphoid cells (ILCs) (Figure 2A). Among all types of lymphoid and myeloid immune cells, notably we found nearly no neutrophils in control CLNs (Figure 2B), and there was a striking increase in neutrophil numbers in acute and chronic EAE mice (Figure 2, C and D). We further investigated the function of neutrophils in the subsequent analysis.

Possible Function of CLN Neutrophils in EAE

Neutrophils exhibit a wide range of effector mechanisms that may be relevant for MS and EAE pathogenesis, including cytokine production, breakdown of blood-brain barrier, release of neutrophil extracellular traps, antigen presentation, and T-cell

Figure 2 Single-Cell Analysis Revealed the Dynamic Changes of Immune Cells in CLNs During EAE Development



(A) Annotation of cell types, (B) t-SNE plot of immune cells in CLNs from the control mouse, (C) t-SNE plot of immune cells in CLNs from the mouse in EAE chronic stage, and (D) t-SNE plot of immune cells in CLNs from the mouse in EAE acute stage. CLN = cervical lymph node; EAE = experimental autoimmune encephalomyelitis.

activation.[2,4] However, the specific neutrophil functions contributing to the development and progression of CNS inflammatory disorders in CLNs are still not well understood. Therefore, we conducted an analysis of the potential functions of neutrophils in CLNs that could be involved in EAE pathogenesis.

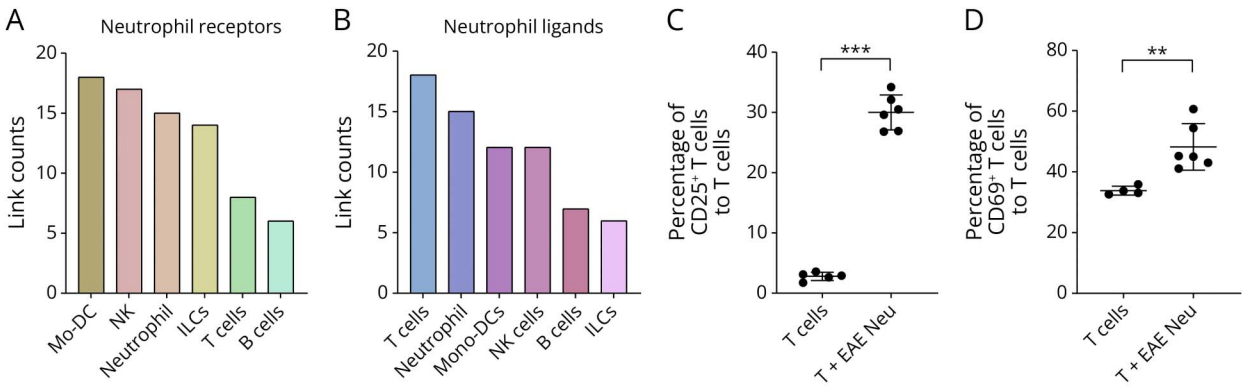
Initially, the top 500 highly expressed genes in neutrophils recognized by sc-RNA sequencing were identified, and GO enrichment analysis was performed (eFigure 3A). Notably, the top 5 biological process terms, namely, "T-cell differentiation involved in immune response," "regulation of neutrophil migration," "regulation of mast cell activation," "immune system process," and "inflammatory response," were found to be enriched in neutrophils (eFigure 3A). This observation indicated that neutrophils migrate to CLNs, actively participating in T-cell function, immune cell activation, and modulation of immune response.

To further explore the role of neutrophils in cellular communication, we conducted cell chat analysis. eFigure 3, B and C illustrates the interaction strengths between immune cell types, highlighting extensive communication between cells. Notably, the neutrophil emerged as the cell type with the strongest contribution in ligand interactions, indicating their key role in cellular communication as the primary ligand cell type.

Subsequently, we quantified the usage of ligands and receptors in neutrophil interactions with other cell types (Figure 3, A and B). Among the putative receptors identified in neutrophils, monocytes/DCs displayed the largest number of cell-cell links, confirming the role of monocytes/DCs in neutrophil regulation (Figure 3A). In addition, T cells exhibited the highest link counts and strong strength with neutrophil ligands, indicating a robust intercellular communication between neutrophils and T cells (Figure 3B and eFigure 3, B and C). For the analysis of the interaction between neutrophils and T cells, neutrophils from EAE mice were cocultured with pan-naïve T cells. After coculturing, we found an upregulation of markers associated with T-cell activation, namely, CD25 and CD69, indicating that neutrophils derived from EAE promote T-cell activation (Figure 3, C and D and eFigure 3, D and E).

Based on the putative ligand-receptor pairs, we observed that neutrophils regulate T cells primarily through the MHC-I, GALECTIN, and SELPLG pathways (eFigure 3F and

Figure 3 Neutrophils Crosstalk With Other Immune Cells and Stimulate T-Cell Activation



(A and B) Quantification of receptor (A) and ligand (B) count usage in neutrophils in their interaction with other cell types. (C and D) Flow cytometry analysis of $CD25^+$ and $CD69^+$ T cells. Shown are quantitative analysis of activated T cells (n = 4–6). Data are mean ± SD based on 4 to 6 independent biological replicates. $^{**}p < 0.01$, $^{***}p < 0.001$.

eAppendix 1). Furthermore, we identified $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Through CellChat analysis of neutrophils, $CD4^+$ helper T cells, and $CD8^+$ cytotoxic T cells, we aimed to explore potential communication relationships among them. The results revealed that neutrophils, acting as ligand cells, primarily interacted with $CD8^+$ cytotoxic T cells (eFigure 3G). Neutrophils engaged in significant interactions with $CD8^+$ cytotoxic T cells through the MHC-I signaling pathway (eFigure 3H), contributing notably to the ligand-receptor relationships, particularly in the context of H2-d1-Cd8b1 and H2-k1-Cd8b1 (eFigure 3I). In addition, SELPLG, which plays a critical role in leukocyte trafficking during inflammation by tethering leukocytes, acts as a ligand gene in neutrophils targeting $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells through SELPLG-SELL (eFigure 3J). The results of this section indicate that, compared with $CD4^+$ T cells, $CD8^+$ T cells are the main recipients of neutrophil communication relationships through the MHC-I signaling pathway.

Moreover, we noted that neutrophils also regulate themselves through secreted signaling or cell-cell contact. Several significant pathways, including the IL-1, CXCL, and ANNEXIN signaling pathways, were influenced by other immune cells, particularly monocytes/DCs (eFigure 3, K–M). In more detail, monocytes/DCs could affect the IL-1 pathway, which involves contributions from IL-1b/IL-1r2 and IL-18/(IL-8r1+IL-8rap) (eFigure 3K). Both ILCs and monocytes/DCs may also influence the CXCL signaling pathway, involving CXCL2/CXCR2 and CXCL16/CXCR6 interactions (eFigure 3L). Furthermore, AnxA1/Fpr2 and AnxA1/Fpr1 contribute to the ANNEXIN signaling pathway (eFigure 3N), where neutrophils themselves play a significant role (eFigure 3M).

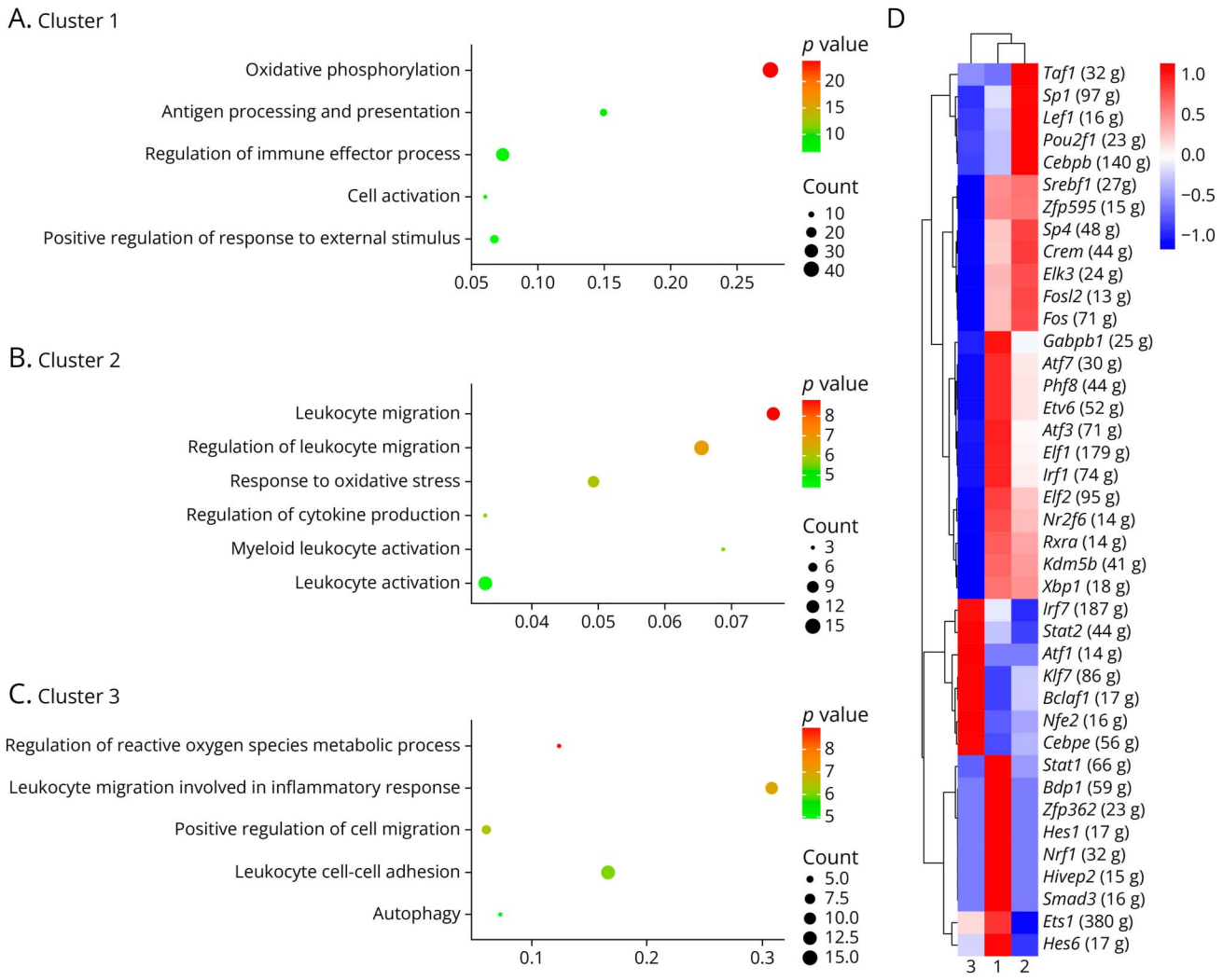
Neutrophil Subtypes and Function in CLNs in EAE

Increasing evidence has revealed unexpected phenotypic heterogeneity and functional versatility among neutrophils.[19] However, the extent of this landscape remains uncharacterized, particularly in CLNs of EAE mice. Through scRNA-Seq analysis of 245 neutrophils, we uncovered transcriptionally distinct clusters in the 2 EAE stages. To dissect this heterogeneity, we performed unsupervised subclustering on neutrophils, identifying 3 distinct subclusters (eFigure 4A) annotated by specific markers (eFigure 4, B and C), with their distributions in each sample shown in eFigure 4D. Compared with acute-stage EAE, the proportion of cluster 2 was decreased and clusters 1 and 3 were increased in acute-stage EAE (eFigure 4E left). Most cells in cluster 2 were present in the acute stage, whereas most cells in cluster 3 were predominantly found in the chronic stage (eFigure 4E right).

Previous studies have revealed a variety of distinct neutrophil subpopulations emerging during differentiation and maturation.[20,21] In our analysis, Cluster 1 was identified as a "G5b/c subset"[22] in peripheral lymph tissue, as indicated by the highly expressed marker genes *dusp1*, *zfp36*, and *slc7a11* (eFigure 4F). This subset also displayed high expression of *s100a8*, *s100a9*, *wfdc21*, *mmp8*, *lgals3*, and *lyz2*, which were similarly highly expressed in cluster 3 (eFigure 4G). The expression of marker genes *gm5483*, *timp2*, and *rdh12* (eFigure 4H) further assisted in identifying cluster 2 as a "G5a subset."[22] Neutrophil cluster 3 expressed marker genes *camp*, *ngp*, *ltf*, *chil3*, *lcn2*, *ifitm6*, *cd177*, and *serpinb1a*, indicating its identity as segmented neutrophils (eFigure 4I), which are the most mature neutrophils in bone marrow before they migrate to peripheral blood and immune tissue.[22]

To elucidate the specific function of each neutrophil subtype, we performed GO analysis within the neutrophil subclassification, revealing the top 20 biological processes enriched for each subcluster (eAppendices 2–4). Notably, cluster 1 exhibited enrichment in processes such as "oxidative phosphorylation," "antigen processing and presentation," "regulation of immune effector process," "cell activation," and "positive regulation of

Figure 4 GO and SCENIC Analysis Revealed the Different Function of Neutrophil Subtypes in the CLNs of EAE Mice



(A) Biological process terms in GO analysis through top 500 deferentially expressed genes (DEGs) in subcluster 1 of neutrophils. (B) Biological process terms in GO analysis through top 500 DEGs in subcluster 2 of neutrophils. (C) Biological process terms in GO analysis through top 500 DEGs in subcluster 3 of neutrophils. (D) Inferred TFs across 3 neutrophil subclusters through SCENIC analysis. Numbers in brackets indicate the regulons for respective transcription factors. CLN = cervical lymph node; EAE = experimental autoimmune encephalomyelitis.

response to external stimulus" (Figure 4A). In cluster 2, enrichments were observed in "leukocyte migration," "regulation of leukocyte migration," "response to oxidative stress," "regulation of cytokine production," "myeloid leukocyte activation," and "leukocyte activation" (Figure 4B). Cluster 3 showed enrichment in "regulation of reactive oxygen species metabolic process," "leukocyte migration involved in inflammatory response," and "positive regulation of cell migration" (Figure 4C).

We proceeded to characterize subtype-specific TFs using SCENIC analysis[23] (Figure 4D). The regulons of ATF3 and HES1 were specifically expressed in cluster 1, indicating chemokine CXCL1 production.[24,25] In cluster 2, we detected TFs including SP1 and FOS, which regulate inflammation and chemokines such as IL-1beta and CXCL1.[26,27] Within cluster 3, we identified CEBPE, a TF involved in neutrophil development and differentiation.[28,29] In addition, NFE2 was identified in cluster 3, which correlates with immune infiltration of neutrophils.[30]

To obtain insight into the transcriptional changes occurring in neutrophils during EAE progression, we subjected these cells to pseudotime analysis using Monocle2 (eFigure 4J). Neutrophils from different EAE stages were organized in a transcriptional pseudotrajectory that can predict neutrophil dynamics. The pseudotemporal progression initiated with cluster 3, followed by cluster 2 and culminated with cluster 1 (eFigure 4J).

A set of high variable genes (HVGs) were identified across 4 modules showcasing dynamic expression changes along the trajectory (eFigure 4K), with the most predominantly changes being visualized in eAppendix 5. To probe into the gene functions in each cluster, metascape analysis was

performed. The genes specific to module 1 were enriched in "lymphocyte differentiation," while the specific gene set in module 2 characterized by "regulation of reactive oxygen species metabolic process, mononuclear cell migration, leukocyte migration, cellular response to oxidative stress, and positive regulation of apoptotic processes" (eFigure 4L). Moreover, genes associated with module 3 revealed terms such as "regulation of cytokine production, regulation of leukocyte activation, regulation of toll-like receptor signaling pathway, and antigen processing and presentation of peptide antigens" (eFigure 4L). Module 4 featured enriched terms including "regulation of cytokine production, negative regulation of immune system process, regulation of leukocyte activation, regulation of toll-like receptor signaling pathway, antigen processing and presentation of peptide antigen" (eFigure 4L). The reconstructed hierarchical trajectory further revealed functions and development of neutrophils at the single-cell level.

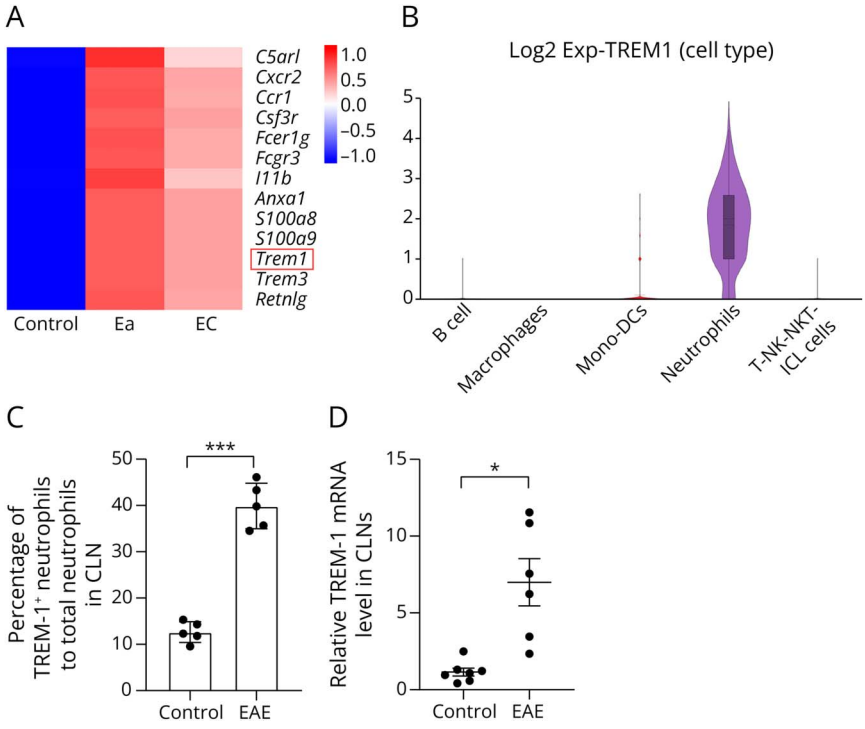
This trajectory aligns with the GO analysis of DEGs across different neutrophil clusters. Based on the GO analysis of DEGs from module 1 and module 3, it is evident that Cluster 1, positioned at the trajectory's endpoint, is primarily associated with the regulation of lymphocyte differentiation and antigen presentation. Remarkably, cluster 1 also demonstrated potential involvement in the negative regulation of immune system processes. Cluster 2 genes, activated midway through the trajectory, play roles in regulating cytokine production, as well as controlling leukocyte activation and migration, as indicated by the GO analysis of DEGs from module 4. In the GO analysis of DEGs from module 2, genes within cluster 3 were prominently implicated in migration and the production of ROS.

Myelin Oligodendrocyte Glycoprotein-Induced Inflammatory Activation in CD45$^+$ Immune Cells Revealed by Bulk RNA-Seq

To determine the dynamics of DEGs, we performed bulk RNA-Seq on MACS-purified CD45$^+$ cells from CLNs of control, acute-stage, and chronic-stage EAE mice. We identified 708 upregulated and 222 downregulated DEGs in the acute stage compared with controls (eFigure 5A) and 199 upregulated and 294 downregulated genes in comparison with the chronic stage (eFigure 5B).

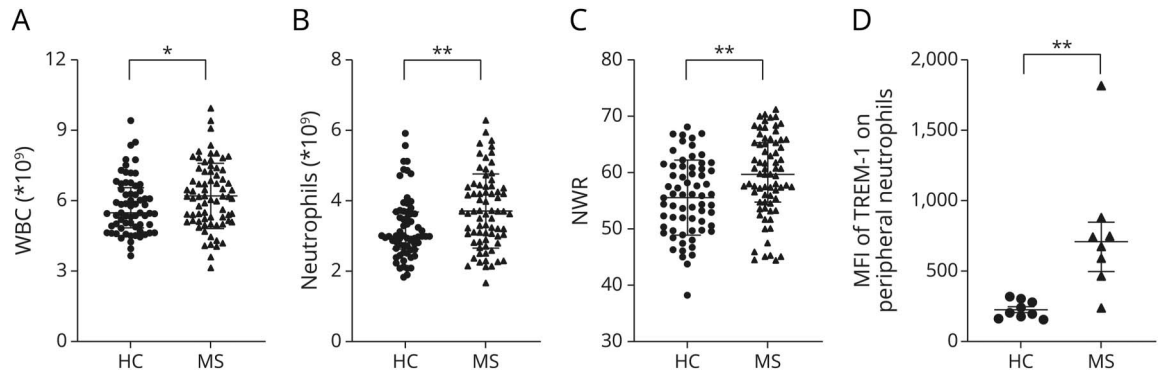
Neutrophils were scarcely present in control CLNs, significantly more prevalent in the acute stage, and relatively reduced during the chronic stage. In our search for genes exhibiting similar expression dynamics, we identified 423 genes displaying an increase in the acute stage followed by a decrease in the chronic stage (eFigure 5, C–D, and eAppendix 6), with 55 of these genes also falling with the top 100 neutrophil marker genes identified by scRNA-Seq (eFigure 5E and eAppendix 6). GO analysis conducted on these 55 genes (eFigure 5F) revealed their involvement in various processes, including leukocyte chemotaxis (*C5ar1, Cxcr2, Ccr1, Csf3r, Fcer1g, Fcgr3, Il1b, Anxa1, S100a8, S100a9, Trem1, Trem3, Retnlg*) (Figure 5A), regulation of cytokine production, cytokine-mediated signaling pathways, defense

Figure 5 TREM-1 Is Upregulated in CLNs of EAE Mice



(A) Heatmap displayed TREM-1 and other 12 other genes involved in leukocyte chemotaxis. (B) The violin plot revealed that among all immune cells, TREM-1 is exclusively and specifically expressed on neutrophils. (C) Flow cytometric analysis of the percentage of TREM-1 expression on CLN neutrophils in controls and EAE mice. Data are mean ± SD (error bars) based on 5 independent biological replicates. (D) The relative levels of TREM-1 mRNA transcripts in CLNs of individual mice. Data are mean ± SEM (error bars) based on 6 or 7 independent biological replicates. *p < 0.05, ***p < 0.001. CLN = cervical lymph node; EAE = experimental autoimmune encephalomyelitis.

Figure 6 Neutrophil Expansion and TREM-1 Upregulation in Peripheral Blood of Patients With MS



(A–C) Laboratory data (WBC, neutrophils count, NWR) of healthy control (n = 65) and patients (n = 72) during the acute attack of MS. (D) Flow cytometry analysis of the MFI of TREM-1 on neutrophils in periphery blood in healthy controls (HCs) and patients with MS. Data are mean ± SEM (error bars) based on 7 or 8 independent biological replicates. *$p < 0.05$, **$p < 0.01$. TREM-1 = triggering receptor expressed on myeloid cells 1.

responses to bacteria, regulation of leukocyte migration, myeloid leukocyte activation, and acute inflammatory responses. Further gene scoring based on sc-RNA sequencing revealed higher cell migration scores in neutrophils when compared with other immune cells (eFigure 5 G and eAppendix 7).

A prominent neutrophil marker gene, *Trem1*, exhibited nearly exclusive expression in neutrophils (Figure 5B) and showed a remarkably high genediff value (eAppendix 8) (genediff is commonly used in single-cell sequencing to measure the specificity of marker genes and is defined as genediff = pct.1/pct.2, where pct.1 represents the percentage of expressing cells in the target cell population and pct.2 represents the percentage of expressing cells for the gene in the remaining cells). TREM-1 also played a role in both leukocyte chemotaxis and migration as well as acute inflammatory responses (eAppendix 9). Immunofluorescence substantiated the expression of TREM-1 in CLN neutrophils from EAE mice (eFigure 5H). Furthermore, we validated TREM-1 at both protein and transcriptional levels. The expression of TREM-1 on neutrophils (Figure 5C and eFigure 5I) and the relative levels of TREM-1 mRNA transcripts in CLNs (Figure 5D) were found to be elevated for EAE compared with the control group.

Neutrophil Expansion and TREM-1 Upregulation in Peripheral Blood of Patients With MS

Of the 72 patients with MS, 50 (70.77%) were women. The mean age ± SD was 33.39 ± 12.00 years. The HC group (N = 65) consisted of 46 (69.44%) women, with a mean age of 35.94 years. There was no significant difference ($p = 0.149$) between the groups. The demographic characteristics and EDSS of the patients with MS are summarized in eTable 1. Peripheral WBC and neutrophil counts, as well as NLR and NWR were higher in RRMS than controls (Figure 6, A–C and eTable 1). However, there is no difference in lymphocyte counts between the 2 groups (eTable 1).

Furthermore, individuals with MS displayed significantly higher mean fluorescence intensities of TREM-1 in $CD16^+$ blood neutrophils than healthy controls (Figure 6D). This also demonstrates a correlation between the TREM-1 on neutrophils and the pathogenesis of MS.

Inhibiting TREM1 With LR12 Prevents Neutrophil Migration

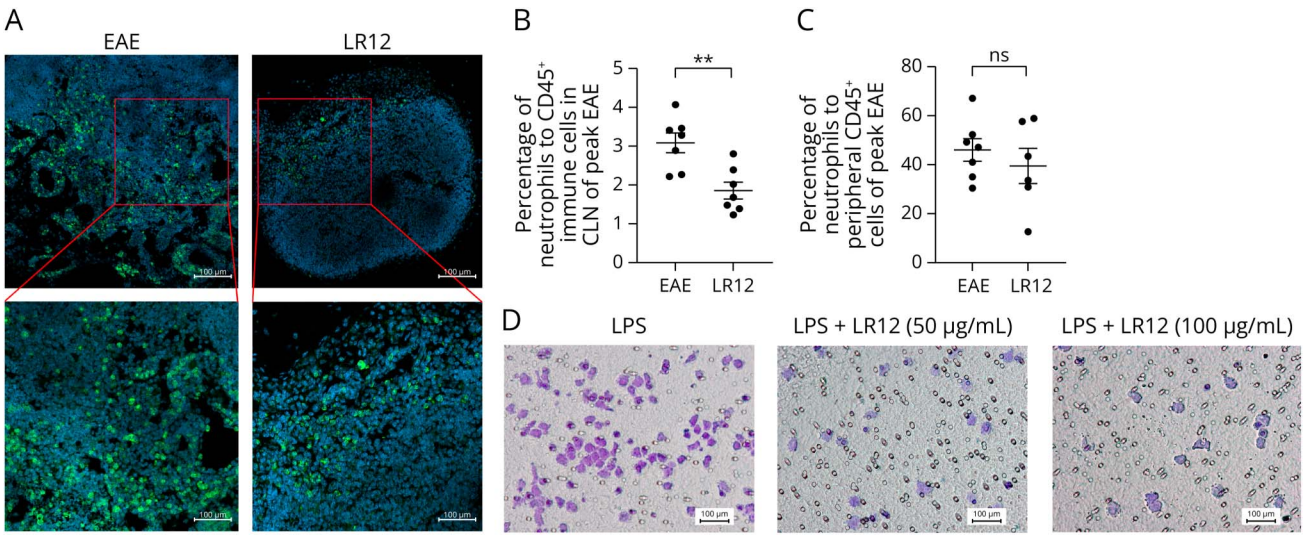
Immunofluorescent analysis demonstrated that LR12 treatment resulted in a reduced population of the anti-ly6G-stained neutrophils within the CLNs of EAE mice (Figure 7A). Furthermore, flow cytometry analysis corroborated this finding, indicating a significantly lower proportion of neutrophils to $CD45^+$ immune cells in the CLNs of LR12-treated mice when compared with the EAE mice during the acute stage (Figure 7B and eFigure 6A). There is a declining trend, but no statistically significant difference was observed in the ratio of neutrophils to $CD45^+$ immune cells in peripheral blood between the groups (Figure 7C and eFigure 6B). It could be attributed to the relatively diminished representation of migrating neutrophils within the peripheral blood neutrophil population, thus contributing to the lack of pronounced statistical significance in the results. Based on these results, we postulated that LR12 might influence neutrophil migration to the CLNs. To delve deeper into this potential role, we conducted further investigations using the neutrophil cell line HL-60.

Subsequent investigations using the Transwell migration assay revealed a significant decrease in the number of HL-60 cells treated with LR12 (50–100 μg/mL) that migrated to the lower chamber in comparison with the control group (Figure 7D and eFigure 6C).

Discussion

MS is considered an inflammatory demyelinating and neurodegenerative disorder of the CNS. EAE is indeed the most

Figure 7 LR12 Prevents Neutrophil Migration Both In Vivo and In Vitro



(A) Immunofluorescent analysis of anti-ly6G–stained (green fluorescence) neutrophils in the CLN tissue sections from vehicle-treated and LR12-treated EAE mice (n = 3). (B) Flow cytometry analysis of the percentage of neutrophils in CLNs from the vehicle-treated and LR12-treated EAE mice (n ≥ 6). (C) Flow cytometry analysis of the percentage of neutrophils in periphery blood from vehicle-treated and LR12-treated EAE mice. (D) Representative picture of migratory HL-60 cells attached in the bottom chamber (n = 6). $^{**}p < 0.01$, NS means no statistical difference. CLN = cervical lymph node; EAE = experimental autoimmune encephalomyelitis.

extensively used model for investigating MS. Nonetheless, it does have certain limitations and significant differences when compared with the human condition, notably that it is artificially induced through passive immunization and mainly mediated by $CD4^+$ T cells.[31] The animal model of EAE continues to play a key role as a front-line model in exploring the significant pathogenesis and developing new therapeutic approaches.[31]

Recent research has increasingly highlighted the significance of neutrophils, the most abundant circulating innate myeloid cells that act as first-responders, as significant contributors to the pathogenesis of MS and EAE.[3] In peripheral blood of individuals with MS, both the count of neutrophil and biomarkers indicating neutrophil activity increase during MS relapses.[8,32] Patients with RRMS have a higher blood NLR compared with healthy controls, which is associated with disease activity.[5] Our finding is in line with these observations. Specifically, at our medical center, peripheral NWR and NLR as well as the counts of neutrophils are elevated in patients with RRMS during the acute stage. Moreover, we observed an increase in neutrophils in peripheral blood of EAE mice from our research.

Several studies have highlighted the involvement of neutrophils in breaching the BBB and infiltrating the spinal cord parenchyma, subsequently accessing the CNS where they undertake various pathologic roles.[8,33] Within the confines of the CNS, neutrophils secrete a diverse range of cytokines and chemokines, thereby attracting and activating numerous immune cells. In addition, the release of ROS by neutrophils contributes to neuronal damage.[34,35] It is important to note, however, that the inflammation associated with MS and EAE is not confined solely to the CNS.

CNS-draining lymph node (DLNs), where the CNS autoimmune cascade initiates,[10] holds a pivotal position in the pathogenesis of MS and EAE. Interventions such as cervical lymphadenectomy or targeted hyperthermia directed by focused ultrasound can reduce the number and activity of lymphocytes within CLNs, resulting in alleviation of EAE symptoms.[36,37] Recently, research has shed light on the significant role of neutrophil accumulation in dLNs as a key amplifier of localized expression of autoreactive $CD4^+$ T cells and subsequent exacerbation of EAE severity.[14] Correspondingly, amelioration of EAE severity has been achieved by the reduction of activated neutrophil populations in secondary lymphoid organs.[38] Indeed, these findings underscore the substantial involvement of neutrophils and inflammation mechanisms within DLNs, providing compelling evidence of peripheral immune dysregulation.[39] However, the precise and specific role of neutrophils within CLNs remains inadequately understood.

Consequently, to gain further insight, we embarked on an exploration of the single-cell RNA landscape with mouse CLNs, served as major CNS-draining lymph nodes,[12,13] using scRNA-Seq. This endeavor unveiled the presence of 5 predominant immune cell types, encompassing T/NK/NKT/ILC cells, B cells, monocytes/DCs, macrophages, and neutrophils, and each distinctly characterized by representative genes. Intriguingly, we noted a scarcity of neutrophils in control CLNs, which stood in stark contrast to the significantly elevated neutrophil percentages observed in EAE. This observation implies their active participation in this disease.

Subsequently, we determined possible neutrophil functions in CNS-dLN using GO analysis, indicating that neutrophils have strong migration ability and play an important role in T-cell differentiation, immune cell activation, and inflammatory responses. Neutrophils contribute to the regulation of both innate and adaptive immunity, establishing connections with other immune cells within lymph nodes.[40,41] Further exploration delved into the intricate interplay between neutrophils and other immune cell populations within CLNs. Through coculture experiments, we further confirmed the activating effect of neutrophils on T cells in EAE. Remarkably, it was observed that the strength of putative ligand-receptor pairs between neutrophils and T cells was particularly noteworthy. Specifically, compared with $CD4^+$ T cells, $CD8^+$ T cells are the main recipients of neutrophil communication relationships through the MHC-I signaling pathway. This revelation underscores the dynamic and multifaceted communication network that underlies the functional relationships between neutrophils and T cells within the lymph node microenvironment.

Among the immune cell types, monocytes/DCs exhibited the highest number of interactions with identified neutrophil receptors. Remarkably, a distinctive ligand-receptor pair, Ccl9-Ccr1, was identified between them. A recent study has reported that polymorphonuclear neutrophils might be recruited to lymph nodes as a response to CCL9 secretion by Mφs/Mons, triggered through interaction between CCR1 and CCL9.[42] This intriguing finding raises the possibility that CCL9, secreted by monocytes/DCs, could similarly contribute to the migration of neutrophils to lymph nodes by engaging with CCR1 in EAE. Moreover, previous research has suggested that neutrophils exert their inflammatory effects in part by promoting the maturation of antigen-presenting cells, including DCs.[43] Our results indicated that DCs might play a role in fostering an inflammatory response during neutrophil migration. This finding suggests a potential feedback loop where DCs and neutrophils mutually influence and exacerbate the inflammatory milieu within the CLNs, thereby potentially contributing to the progression of EAE.

In addition to crosstalk with other immune cells, neutrophils also possess a regulatory capacity over their own functions through IL-1, CXCL, and ANNEXIN signaling pathways. Notably, the persistent activation of the IL-1/IL-1R1 pathway has been implicated in pathogenesis of MS,[44] and therapeutic agents targeting the IL-1 pathway have exhibited promising potential in preclinical studies on MS.[45] In our results, we found that neutrophils primarily secrete IL-1 and subsequently activate their own IL-1 receptors. This autocrine mechanism suggests that the pathogenic role of the IL-1/IL-1R1 pathway could potentially be attributed to the actions of neutrophils. Furthermore, the CXCL signaling pathway, particularly involving CXCL2/CXCR2, plays a significant role. Our investigation confirmed that neutrophils are the main source of the CXCR2 ligand CXCL2. CXCR2, expressed on neutrophils, plays a pivotal role in mediating neutrophil migration and has been implicated in the pathogenesis of MS and EAE.[34,46,47] Intriguingly, studies using picomolar antibodies or selective Cxcr2 deletion have demonstrated protection against CNS neurodegeneration by inhibiting neutrophil activation, leading to alleviation of EAE symptoms.[34,48] Another important signaling pathway was the ANNEXIN pathway, encompassing ANXA-FPR1 and ANXA-FPR2. Previous studies have reported that release of IL-1β was impaired in $AnxA1^{-/-}$ neutrophils illustrating that AnxA1 participates in cytokine release and the inflammatory response.[49] Conversely, AnxA1-FPR2 exerts anti-inflammatory effects by attenuation of leukocyte infiltration and suppression of IL-6.[50] As a result, the ANNEXIN pathway could potentially assume multiple roles in neutrophil function and progression of EAE. Taken together, our findings suggest that the autocrine role of neutrophils could potentially serve as a pathogenic mechanism in EAE, highlighting the intricate web of interactions and signaling pathways that contribute to the complex immune responses underlying EAE progression.

Neutrophils have been shown to possess unexpected phenotypic heterogeneity and functional versatility,[51] indicating their potential significance in therapeutic interventions. Targeting specific neutrophil subsets strategically, without compromising the antimicrobial functions of other subtypes, could present an effective and safe therapeutic approach. Consequently, it becomes imperative to ascertain the various neutrophil subtypes and delineate their distinct functions.

New neutrophil subtypes under both healthy and inflammatory conditions are currently being explored.[19] However, the absence of widely accepted and explicit markers for defining neutrophil subtypes has led to variations in their neutrophils.[51] A recent study[22] used single-cell transcriptome profiling to uncover neutrophil heterogeneity in bone marrow, peripheral blood, and the spleen, identifying 8 distinct populations (G0–4, G5a–c) according to distinct molecular signatures. G5 cells (G5a–c), comprising the majority of peripheral tissue neutrophils, were identified as the most mature neutrophils, while G4 cells showed the highest maturation scores among bone marrow samples.[22] In our study, we identified 3 clusters within CLNs of EAE mice. Cluster 1 corresponded to G5b/c (marked by high expression of Dusp1, Zfp36, Slc7a11), while cluster 2 was confirmed as G5a (characterized by high expression of s100a8, s100a9, wfdc21, mmp8, lyz2, lgals3, gm5483, timp2, rdh12). Cluster 3 was recognized as G4 cells (expressing high levels of camp, ngp, ltf, chil3, lcn2, ifitm6, cd177, serpinb1a), constituting a minor proportion of CLN neutrophils. The distribution of these 3 subclusters varied across different stages of EAE. During the chronic stage, the proportions of clusters 1 and 3 increased, whereas cluster 2 decreased. This dynamic shift in cluster proportions implies that cluster 2 likely plays a crucial role in EAE progression, while cluster 1 might contribute to the chronic stage of EAE. These findings underscore the intricate diversity of neutrophil subtypes and their potential roles in the context of EAE, emphasizing the need for a comprehensive understanding of neutrophil heterogeneity to develop targeted therapeutic strategies.

Using a pseudotrajectory-based transcriptional analysis, we endeavored to glean insights into the differentiation of neutrophils during the progression of EAE. Our observations indicated a sequential progression initiated by cluster 3, followed by cluster 2 and finally cluster 1. Within this context, we identified a set of highly variable genes (HVGs) that belonged to 4 distinct modules exhibiting varying expression patterns. Subsequently, we delved into the functional roles of these neutrophil subpopulations.

The GO analysis of DEGs associated with the subclusters and 4 modules revealed intriguing insights. Cluster 3 appeared to be primarily involved in the regulation of immune cell migration, cell death, and the generation of ROS. The generation of ROS has been implicated in axon degeneration in the context of MS/EAE, and previous studies in a rhesus monkey EAE model have suggested that neutrophils contribute to demyelination through ROS generation.[52] Thus, it seems that cluster 3, which emerges as neutrophils migrate to CLNs, may primarily contribute to EAE progression through oxidative injury. Cluster 2 was inferred to regulate leukocyte migration, activation, and cytokine production. Neutrophils exhibit distinct transcriptional profiles, hinting at their potential to secrete immunostimulatory factors.[53] As for cluster 1, its development seemed to be associated with antigen presentation and the promotion of T-cell differentiation.

Of interest, SCENIC analysis offered further insights into the functions of neutrophil subclusters. For instance, NFE2, found in cluster 3, was implicated in regulating neutrophil infiltration, while SP1 and FOS, identified in cluster 2, appeared to regulate inflammatory chemokines. These findings provided additional support for the roles proposed by the GO analysis. However, an intriguing twist emerged as we discovered ATF3 and HES1 in cluster 1. These factors might exert protective effects by inhibiting CXCL1. This could suggest a regulatory role for cluster 1 in mitigating EAE progression, potentially explaining its increase during the chronic stage which there is a partial alleviation of the severity of EAE. This intricate interplay highlights the multifaceted nature of neutrophil functions and their potential contribution to both the progression and attenuation of EAE.

In a EAE mouse model of MOG antigen immunization, neutrophils demonstrated their capacity to infiltrate lymph nodes.[54] Our study further validated that neutrophils indeed migrate to and develop within the CLNs of EAE mice. Within this microenvironment, neutrophils engage in interactions with other immune cells, thereby facilitating their migration and activation, fostering the production of inflammatory cytokines, and participating in antigen presentation. Consequently, inhibiting the migration of neutrophils to CLNs emerges as a promising therapeutic strategy for MS.

We observed that the majority of neutrophil markers (55 of 100) exhibited an initial increase in expression during the acute stage of EAE, followed by a decrease in the chronic stage. This pattern corresponds to the changing frequency of neutrophils in CLNs at different stages of EAE. To gain further insight into these expression trends, we conducted Gene Ontology (GO) analysis on genes exhibiting similar patterns. Notably, some of these genes, including *Trem-1* (which is primarily expressed in CLN neutrophils), were associated with processes like leukocyte chemotaxis.

TREM-1, initially identified in human neutrophils and monocytes, serves as a significant amplifier of immune signaling.[55] Although the initial understanding of TREM-1 primarily linked it to sepsis,[56] recent research has shed light on its involvement in autoimmune diseases and their corresponding animal models, including chronic inflammatory bowel disease and rheumatoid arthritis.[57,58] Inhibition or knockout of TREM-1 has been shown to yield significant alleviation of autoimmune conditions, characterized by reduced inflammatory infiltration and diminished expression of proinflammatory cytokines.[57,58] Our findings revealed higher expression of TREM-1 on neutrophils in the peripheral blood of patients with MS compared with healthy controls. This observation suggests that TREM-1 might contribute to highlighting the pathogenic effects of neutrophils in MS. However, the specific role of TREM-1 in the pathophysiology of MS and EAE remains to be fully elucidated.

Studies have demonstrated that TREM-1 exerts a central role in modulating inflammatory signaling and facilitating neutrophil transepithelial migration into diverse settings such as the alveolar airspace, uterus, and uterine glands.[59,60] We put forth the hypothesis that TREM-1 facilitates the migration of neutrophils to CLNs, where neutrophils exert diverse effects, and inhibiting TREM-1 could potentially curtail neutrophil migration. As anticipated, LR12 exhibited the capacity to curtail neutrophil infiltration into CLNs during the acute stage of EAE and impede HL-60 cell migration.

In conclusion, our study has presented a comprehensive and precise single-cell atlas of CLNs in EAE. Through this atlas, we have successfully dissected the intricate crosstalk between neutrophils and other immune cell populations within CLNs, shedding light on the putative functions of CLN neutrophils. Furthermore, we delved into the specific roles and developmental trajectories of distinct neutrophil subtypes. We identified migration as a pivotal capability of neutrophils, with its regulation partly mediated by TREM-1. Notably, the inhibitor of TREM-1, LR12, attenuates neutrophil migration to CLNs. Our data set not only unveils a promising target for ameliorating EAE and MS but also provides a valuable resource for comprehensive assessments of CLN cellular compositions and illuminate the multifaceted roles of neutrophils in MS and EAE.

Indeed, to further establish TREM-1 as a potential therapeutic target for MS, we need to explore the effect of knocking down neutrophils' TREM-1 on the progression and pathology of EAE. Future research should address these limitations while further elucidating the comprehensive mechanisms of LR12 in EAE.

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