Single-cell RNA-seq analysis of human CSF microglia and myeloid cells in neuroinflammation

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Abstract

Objective
To identify and characterize myeloid cell populations within the CSF of patients with MS and anti-myelin oligodendrocyte glycoprotein (MOG) disorder by high-resolution single-cell gene expression analysis.

Methods
Single-cell RNA sequencing (scRNA-seq) was used to profile individual cells of CSF and blood from 2 subjects with relapsing-remitting MS (RRMS) and one with anti-MOG disorder. Publicly available scRNA-seq data from the blood and CSF of 2 subjects with HIV were also analyzed. An informatics pipeline was used to cluster cell populations by transcriptomic profiling. Based on gene expression by CSF myeloid cells, a flow cytometry panel was devised to examine myeloid cell populations from the CSF of 11 additional subjects, including individuals with RRMS, anti-MOG disorder, and control subjects without inflammatory demyelination.

Results
Common myeloid populations were identified within the CSF of subjects with RRMS, anti-MOG disorder, and HIV. These included monocytes, conventional and plasmacytoid dendritic cells, and cells with a transcriptomic signature matching microglia. Microglia could be discriminated from other myeloid cell populations in the CSF by flow cytometry.

Conclusions
High-resolution single-cell gene expression analysis clearly distinguishes distinct myeloid cell types present within the CSF of subjects with neuroinflammation. A population of microglia exists within the human CSF, which is detectable by surface protein expression. The function of these cells during immunity and disease requires further investigation.

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Glossary

AD = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **BAM** = border-associated macrophage; **CCA** = canonical correlation analysis; **cDC** = conventional DC; **DAM** = disease-associated microglia; **DC** = dendritic cell; **HC** = healthy control; **HLA-DR** = human leukocyte antigen DR; **IIH** = idiopathic intracranial hypertension; **MOG** = myelin oligodendrocyte glycoprotein; **NMO** = neuromyelitis optica; **PBMC** = peripheral blood mononuclear cell; **PCA** = principal component analysis; **pDC** = plasmacytoid DC; **RRMS** = relapsing-remitting MS; **scRNA-seq** = single-cell RNA sequencing; **UMAP** = Uniform Manifold Approximation and Projection.

CSF evaluation is used to aid in the diagnosis and differentiation of CNS disorders. In inflammatory CNS diseases, the CSF is typically used to assess the immunopathophysiologic processes because biopsy of CNS tissue carries significant potential for harm.[1] However, relatively few cells are obtained from CSF, usually on the order of 1–5 cells/μL. Recent refinements in next-generation sequencing have enabled the efficient determination of individual cell gene expression within biospecimens with relatively sparse cell populations, such as the CSF. Patterns identified using single-cell RNA sequencing (scRNA-seq) can uncover distinct cell types present at low levels within cellular communities and tissues.[2] scRNA-seq was used to assess inflammatory changes within the CSF of subjects with HIV infection, identifying the presence of a "microglial-like" cell,[3] and more recently to explore the clonal expansion of CSF lymphocytes in MS-discordant monozygotic twin pairs.[4] scRNA-seq has also been used to address the issue of microglial heterogeneity within the human brain.[5–7] In addition, using the primary animal model of MS, experimental autoimmune encephalomyelitis, scRNA-seq has been used to identify several populations of myeloid cells, both endogenous to the CNS and from peripheral blood.[8] New methods for characterization of myeloid populations within the CNS during disease offer the opportunity to dissect the origin, function, and pathogenicity of each cell type with much greater resolution than previous methods.

MS is the most common inflammatory demyelinating disease of the CNS, affecting over 600,000 people in the United States.[9] Anti-myelin oligodendrocyte glycoprotein (MOG) disorder is a newly described CNS demyelinating disease that shares clinical and pathologic characteristics with MS.[10,11] MS and anti-MOG disorder appear to be distinct from one another and from aquaporin 4 antibody-positive neuromyelitis optica (NMO).[10,12] We have applied scRNA-seq to examine the CSF and mononuclear cells of the peripheral blood of subjects with relapsing-remitting MS (RRMS) and anti-MOG disorder. Individual spinal fluid samples from 2 subjects with RRMS and 1 subject with anti-MOG disorder were analyzed by using scRNA-seq. In all 3 subjects, we uncovered CSF populations of immune cells including microglial cells, monocytes, and dendritic cells (DCs) based on gene expression. Using CSF and blood from 7 additional subjects with RRMS, another subject with anti-MOG disorder, and 3 control subjects, we designed and tested a flow cytometry strategy that confirmed the presence in CSF of these cell types.

Methods

Subjects

Eleven subjects with inflammatory demyelinating disease (9 with RRMS and 2 with anti-MOG disorder) and 3 control subjects (1 with amyotrophic lateral sclerosis [ALS], 1 with idiopathic intracranial hypertension [IIH], and 1 healthy control [HC]) were recruited for a study to assess the characteristics of CSF and blood cells (table). The institutional review board of Washington University in St. Louis approved study protocols, and each subject provided informed consent. Nine subjects had RRMS based on the current diagnostic criteria.[13] Two additional subjects were diagnosed with anti-MOG disorder: one presented with optic neuritis and the other with partial transverse myelitis. Anti-MOG disorder was diagnosed based on the 6-month sustained positive cell-based assays for MOG IgG1 antibody, absence of antibodies to aquaporin 4 (NMO-IgG), and absence of CSF-restricted oligoclonal bands.[11]

To prevent alterations because of disease-modifying therapies, we selected subjects either on no therapy or remote from therapy. Seven subjects with RRMS were naive to therapy, including corticosteroids. Of the remaining 2 subjects with RRMS, 1 (subject 7) had received fingolimod, stopping 9 months before CSF testing, and the other (subject 10) was treated with glatiramer acetate for 5 months and had received IV steroids 2 months before CSF analysis. The subject with transverse myelitis due to anti-MOG disorder (subject 8) had received mycophenolate and then azathioprine but had discontinued these 6 months before CSF analysis and had received a course of corticosteroids 4 months before CSF examination. Subject 1 with anti-MOG disorder had received no treatment before the examination of CSF. Control specimens were obtained from a subject with IIH who presented with visual disturbances, a patient diagnosed with ALS, and a HC. Each contributed blood and CSF cells for scRNA-seq or flow cytometry studies (table and figure e-1, links.lww.com/NXI/A244).

Standard protocol approvals, registrations, and patient consents

Informed consent was obtained from all participants and approved by the Human Research Protection Office of Washington University in St. Louis.

CSF and peripheral blood mononuclear cell preparation

CSF was collected in a 50-mL conical tube on ice and centrifuged for 10 minutes at 400*g*. Supernatant was removed

Table Demographic and clinical characteristics of subjects

Subject	Age, y	Sex	Race	Diagnosis	Presentation at onset	Time from symptom onset to LP	CSF OCB	Analysis
1	56	F	Caucasian	Anti-MOG disorder	ON OD and paresthesias	8 mo	0	scRNA-seq
2	38	F	Caucasian	RRMS	ON OD	6 wk	3	scRNA-seq
3	53	F	Caucasian	RRMS	Paresthesias	3 y	13	Flow cytometry
4	23	F	Caucasian	RRMS	ON OD	4 mo	7	Flow cytometry
5	34	F	Caucasian	RRMS	ON OD	2 y	11	scRNA-seq
6	40	F	Caucasian	RRMS	Paresthesias and tremor	15 mo	5	Flow cytometry
7	41	F	Caucasian	RRMS	ON OS	12 y	9	Flow cytometry
8	22	M	Caucasian	Anti-MOG disorder	Paresthesias and leg weakness	2 y	0	Flow cytometry
9	45	M	Caucasian	RRMS	Lhermitte sign	6 mo	7	Flow cytometry
10	38	F	Caucasian	RRMS	ON OS	6 mo	10	Flow cytometry
11	38	F	Caucasian	IIH	TVO	1 y	0	Flow cytometry
12	70	M	Caucasian	ALS	Weakness	4 y	N/D	Flow cytometry
13	45	M	Caucasian	HC	N/A	N/A	N/D	Flow cytometry
14	40	M	Caucasian	RRMS	Right-sided weakness	3 mo	4	Flow cytometry

Abbreviations: ALS = amyotrophic lateral sclerosis; HC = healthy control; IIH = idiopathic intracranial hypertension; OD = oculus dextra (right eye); ON = optic neuritis; OS = oculus sinistra (left eye); LP = lumbar puncture with CSF analysis; MOG = myelin oligodendrocyte glycoprotein; N/A = not applicable; N/D = not done; OCB = oligoclonal band; RRMS = relapsing-remitting MS; scRNA-seq = single-cell RNA sequencing; TVO = transient visual obscuration.

without disrupting the cell pellet, which was resuspended in 400 μL of PBS containing 0.04% bovine serum albumin. The CSF cells were counted with a hemocytometer and resuspended in a volume of PBS/0.04% bovine serum albumin to achieve a cell concentration of 700–1,000 cells/μL for scRNA-seq. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation and then processed alongside CSF cells for scRNA-seq or flow cytometry.

Flow cytometry

Flow cytometry analysis was performed using antihuman antibodies to BDCA-2 (clone 201A; BioLegend, San Diego, CA), CD33 (clone P67.6; BioLegend), CD14 (clone 61D3; eBioscience, San Diego, CA), Lyve-1 (clone 537028; R&D Systems, Minneapolis, MN), CD3 (clone SK7; BioLegend), CD19 (clone HIB19; eBioscience), CD16 (clone B73.1; BioLegend), CD1c (clone L161; BioLegend), Lox-1 (clone 15C4; BioLegend), and human leukocyte antigen DR (HLA-DR) (clone Immu-357; Beckman Coulter, Brea, CA). One million PBMCs and between 2×10^4 and 40×10^4 CSF cells were incubated with human-Fc block (BD Biosciences, San Jose, CA) for 10 minutes at RT and then with antibodies for 20 minutes at 4°C. Subsequently, the samples were washed with PBS + 2% FBS (flow buffer) for 5′, spun at 500g, and resuspended in 200 μL of flow buffer. The samples were run on a Gallios flow cytometer (Beckman Coulter Life Sciences) on the same day of the collection. The cells were analyzed using FlowJo software (Tree Star, Ashland, OR).

RNA sequencing and analysis

On the day of cell collection using fresh cells, droplet-based 3′ (for anti-MOG disorder subject 1) and 5′ (for subjects 2 and 5 with RRMS) libraries were prepared using Chromium Single Cell 3′ v2 or 5′ Reagent Kits according to the manufacturer's protocol from 10× Genomics. The generated scRNA libraries were sequenced using an Illumina HiSeq4000 or Novaseq sequencer. Cell Ranger Single-Cell Software 2.2 was used to perform sample demultiplexing, barcode processing, and single-cell 3′ and 5′ counting. Afterward, fastq files for each sample were processed with a cellranger count, which was used to align the samples to GRCh38 genome, filter, and quantify reads. For each sample, the recovered-cells parameter was specified as 10,000 cells that we expected to recover for each individual library.

To reanalyze scRNA-seq data performed with SeqWell publicly available from GSE117397, we downloaded count tables from 2 subjects with HIV for whom both CSF and blood samples were available from GEO DataSets (GSM3293822, HIV1_Bld; GSM3293823, HIV1_CSF; GSM3293824, HIV2_Bld; GSM3293825, HIV2_CSF). To combine data from all samples together, we used the canonical correlation analysis (CCA) algorithm from Seurat 3.[14] Before applying CCA, we removed cells from the 3′ and 5′ data sets that contain more than 10% of mitochondrial RNA. Cells from SeqWell data sets with less than 20% of RNA from mitochondrial genes and more than 500 but less than 2,500 expressed genes were considered viable cells.

After CCA data were scaled, principal component analysis (PCA) was performed with RunPCA function. A Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction was performed on the scaled matrix using the first 20 PCA components to obtain a 2-dimensional representation of the cell states.[15] For clustering, we used FindNeighbours and FindClusters functions on 20 PCA components with a resolution of 0.5. FindAllMarkers function was used to characterize clusters. For heatmap representation, the mean expression of markers inside each cluster was used. Heatmaps were built with Phantasus software (artyomovlab.wustl.edu/phantasus/).

Data availability

Anonymized scRNA-seq is available to qualified investigators at synapse.org (syn21904732).

Results

Discrete immune cell populations can be identified in the CSF and blood of subjects with neuroinflammatory diseases using scRNA-seq

To explore the extent, diversity, and phenotype of leukocyte infiltration into the CNS compartment during CNS demyelination, we performed scRNA-seq of CSF cells and PBMCs from 1 subject with anti-MOG disorder (subject 1) and 2 subjects with RRMS (subjects 2 and 5). During the course of our data collection, scRNA-seq of immune cells isolated from the blood and CSF of subjects with HIV was reported.[3] Given the small number of data sets on CSF, a difficulty to access tissue, we sought to optimally identify populations of immune cells reflective of neuroinflammation using a recently described computational method, CCA, that allows integration across different scRNA-seq technologies.[14] Applying this method, data from a total of 23,363 PBMCs and 14,179 CSF cells were combined and visualized using the UMAP technique[15] and unsupervised clustering.[16,17]

Twenty clusters representing distinct cell populations were identified (figure 1A). We used the characteristic marker gene expression to assign cellular identities to each cluster (figure 1B). Several distinct lymphoid cell clusters, including CD8^{+} T cells, CD4^{+} T cells, γδ T cells, NK cells, and B cells, could be discriminated in both PBMCs and CSF. Myeloid populations were also identified, including microglia, CD14^{+} and CD16^{+} monocytes, and 2 distinct groups of DCs—plasmacytoid (pDCs) and conventional (cDCs). Examining each subject individually, there were no meaningful disparities in cell clusters between the subjects (figure e-2, links.lww.com/NXI/A244).

We also compared the proportional abundance of specific cell clusters within either the CSF or blood (figure 1C). We identified a cluster of cells that exhibited features of cell stress, including expression of the mitochondrial genes *MTRNR2L8* and *MTRNRL12*, which are indicative of low quality or dying cells (labeled "Dead" in figure 1).[7] These dead cells, as well as

red blood cells and platelets, were found almost solely within the blood. Similarly, we observed one population of NK cells (NK_1) that was exclusively present in the blood. This population shows a gene expression profile matching the recently defined blood NK1 cells, which align with previously described CD56^{dim} NK cells (figure e-3, links.lww.com/NXI/A244).[18]

Among the T and B cell clusters, several exist in higher abundance in the blood (naive CD8, CD8_2, CD8_3, naive CD4, γδ T cells, and B_cells_1). CD14^{+} and CD16^{+} monocytes were more prevalent in blood than CSF, with the latter being entirely absent from the CSF. cDCs were a smaller fraction among PBMC relative to CSF cells, whereas pDCs were found in both compartments in similar proportions. Microglia were exclusively found in the CSF. Given the complexity of cells with myeloid characteristics in neuroinflammatory diseases and the historic difficulty of studying these cells in CSF by conventional methods because of limited cell numbers, we dedicated subsequent analyses of our data to CSF myeloid cell populations.

CSF microglia and other myeloid cell types in neuroinflammation

Analyzing the genes expressed by the cluster of CSF cells that we term microglia revealed high expression of genes from the microglial homeostatic gene signature, including *CX3CR1*, *CSF1R*, *SLC2A5*, *MARCKS*, and *P2RY13* (figure 2A).[7,19,20] Recent scRNA-seq analyses of human brain microglia have defined heterogeneity among microglia.[6,7] Sankowski et al.[7] defined 8 clusters of human brain microglia (termed C1–C3, C5–C9) with differential gene expression among these clusters. Expression of these microglia cluster-specific genes across the myeloid cell populations from our scRNA-seq composite data set was next examined. Many of these genes were found to be strikingly microglia-specific, including *APOC1*, *APOE*, *LYVE1*, *TREM2*, *C1QB*, *GPR34*, *OLR1*, and *C3* (figure 2B). Sankowski et al. also created a classifier tool to discriminate microglial clusters, which they successfully applied to publicly available scRNA-seq data sets of brain microglia. When we applied this classifier to the 394 CSF cells in our data set that were grouped as microglia, 88% of these were classified as belonging to C6, with 10% belonging to C7. C6 and C7 were characterized by Sankowski et al. as having high expression of genes in the Gene Ontology pathway 0048002 ("antigen processing and presentation of peptide antigen"). In sum, these data strongly support our conclusion that the myeloid cell populations we find exclusively in the CSF are microglial cells and suggest that they may be involved in antigen presentation.

Two previous studies using scRNA-seq to classify CSF immune cells, each identified a discrete cell population that was categorized as either "microglia-like" cells[3] or "monocytes."[4] Each study produced a list of genes that discriminated these differently named clusters from other CSF immune cells. When we visualized these gene sets among the clusters of CSF cells in our composite data set,
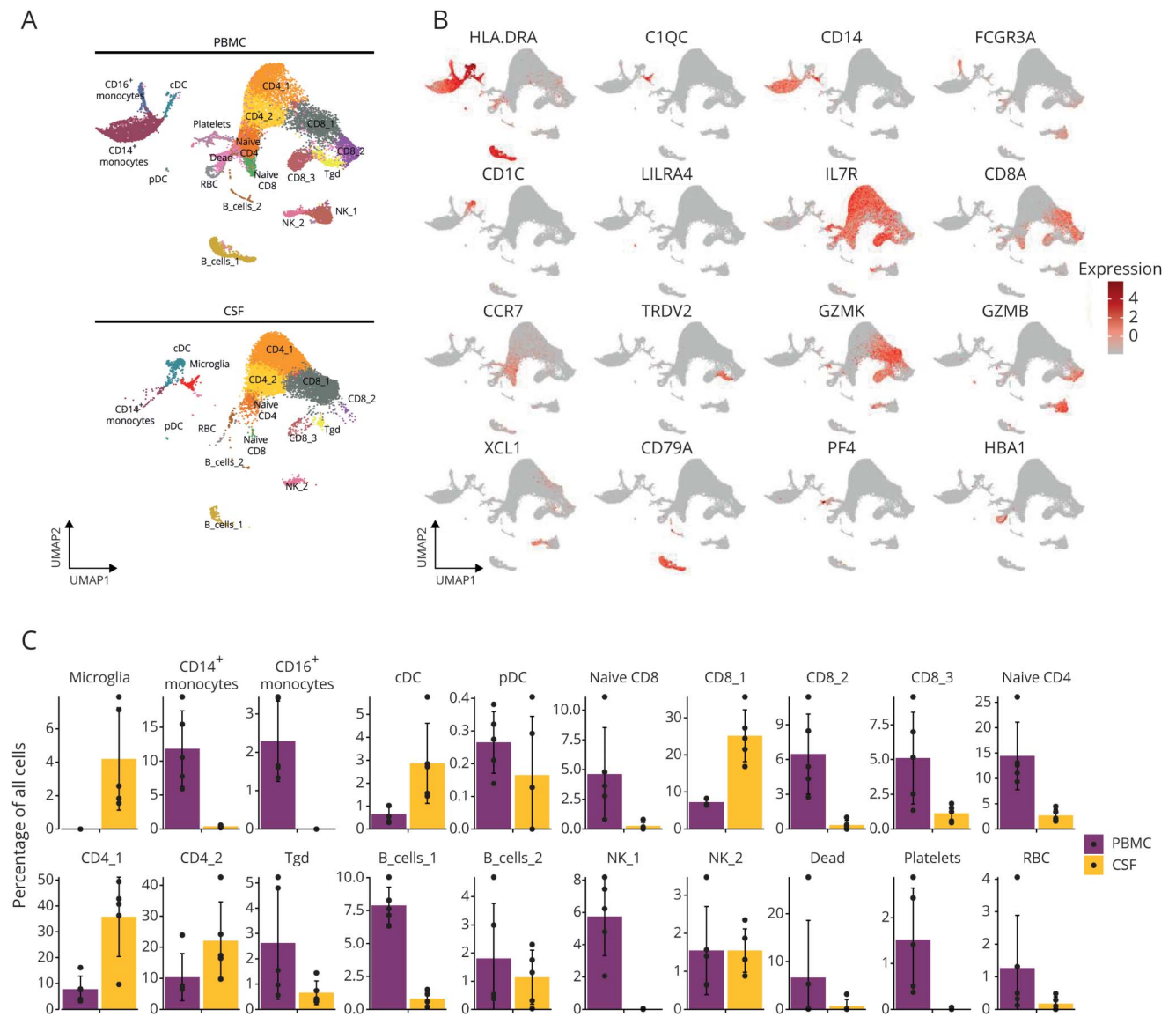
(A) UMAP of immune cell clusters from the blood (top) and CSF (bottom) of all subjects merged. (B) Characteristic marker gene expression assigned to each cluster displayed by UMAP. (C) Proportional abundance of 20 cell clusters within the blood (purple) and CSF (yellow). Y axis represents the percentage of all cells. cDC = conventional DC; PBMC = peripheral blood mononuclear cell; pDC = plasmacytoid DC; RBC = red blood cell; Tgd = γδ T cells; UMAP = Uniform Manifold Approximation and Projection.

which includes a reanalysis of the cells from 2 subjects included in the work of Farhadian et al., we found markedly enriched expression in the cluster that we term microglia (figure 2C). With this, we mean that all 3 studies are in fact referring to the same subset of CSF cells, and given their expression of core microglial genes, we prefer to classify these as microglia.

Other myeloid populations could also be distinctly classified by their transcriptional profiles. Figure 3 is a heatmap of the expression of 10 genes per cell type, which discriminated each myeloid population. Some of these genes were known to discriminate myeloid cell types, yet others are new and could be revealing different classifiers. Notably, expression of major

histocompatibility complex class II genes was higher in CSF cDCs than blood cDCs, possibly indicating a compartmental influence on the antigen presentation function of these cells. These transcriptomic data establish the presence of one monocyte subset, 2 types of DCs, and microglia within the CSF during neuroinflammation.

Flow cytometric characterization of CSF cells validates scRNA-seq identification of microglia and myeloid cell types

We sought to create a flow cytometric-based strategy for the quantification of various CSF myeloid cell types, including microglia, using commercially available antibodies specific for surface markers identified by our gene expression studies

(figure e-4, links.lww.com/NXI/A244) and the literature.[21] PBMCs and CSF cells were stained for CD3 and CD19 to gate out T and B cells. Within the remaining cells in both CSF and blood, pDCs were identified as being HLA-DR^{+} and BDCA-2^{+} (encoded by *CLEC4C*), whereas cDCs were identified as HLA-DR^{+}BDCA-2^{−}CD1c^{+} cells (figure 4A). In the CSF, non-DCs could be seen to resolve as 2 distinct cell types based on the HLA-DR and CD33 levels. PBMCs only contained HLA-DR[mid]CD33^{high} cells, which were defined as monocytes. The CSF additionally contained a population of HLA-DR^{high}CD33[mid] cells, which we interpreted as microglia, further confirmed by their expression of Lyve-1 and Lox-1 (encoded by *OLR1*). CSF from subjects with other neurologic conditions (anti-MOG disorder, ALS, and IIH) and a HC subject also contained similarly staining populations of pDCs, cDCs, monocytes, and microglia (figure 4B). The staining intensity of HLA-DR, CD33, Lyve-1, and Lox-1 discriminated CD14^{+} monocytes from microglia in the CSF of all subjects (figure 4C). Overall, flow cytometric characterization of myeloid cells demonstrated the protein expression of various microglial genes identified by transcriptomic profiling, confirmed the presence of microglia within the CSF, and offered a ready means for identifying this cell type.

Discussion

We performed scRNA-seq to identify the cell types present within the CSF of 2 subjects with RRMS and 1 person with anti-MOG disorder, with comparative analysis to paired PBMCs. These and subsequent subjects were not taking disease-modifying therapies or corticosteroids. Subsequently, we integrated publicly available data on 2 subjects with HIV on whom scRNA-seq was performed on paired PBMCs and CSF immune cells. We then focused on CSF myeloid cell types because less work has been carried out to characterize these populations in part because of their lower abundance. We found that (1) scRNA-seq identifies a diverse set of myeloid cell types within the CSF, including microglia; (2) flow cytometry is a feasible technique for the discrimination of these populations of CSF myeloid cells; and (3) scRNA-seq data can be integrated across technologies to allow for optimal discrimination of cell populations.

Because the data sets we analyzed were derived from different technologies, we used a recently described technique, CCA, to integrate scRNA-seq data sets. This allowed for an increased number of cells used in the analysis, facilitating the optimal discrimination of cell clusters. All CSF samples in the combined analysis contained cells within each cluster, suggesting that among the diseases examined herein, a common set of leukocytes inhabit this critical immune space within the CNS. One limitation of CCA involves the exclusion of some genes from downstream analysis. Nevertheless, in our case, 2,000 genes were still available for use in our assessment of PBMCs and CSF cells from 5 individuals. Our results demonstrate the feasibility of using CCA to integrate the growing number of

scRNA-seq data sets that will continue to be made publicly available using different platforms.

Our scRNA-seq analysis identified microglia in human CSF with a gene expression pattern overwhelmingly similar to that described for parenchymal microglia.[7,19,22,23] We used genes from a recent study describing the transcriptional profile of human tissue-resident microglia as a comparison for the CSF microglia identified in our subjects.[7] The top genes identified in tissue-resident microglia were also highly abundant in our CSF microglia populations (figure 2). Owing to their low abundance in the CSF, this subset of cells has apparently been overlooked in most previous CSF studies using less powerful techniques. One previous report using a different scRNA-seq platform, SeqWell, also identified this population of cells in human CSF and termed them "microglia-like."[3] Our reanalysis of the published data from the latter study confirmed the presence of cells expressing hallmark microglial genes and lacking expression of genes that identify other myeloid cell types. A more recently published study involving patients with MS also identified several distinct myeloid cell types within the CSF.[24] Based on the analysis of our data, we believe this group used the term "Mono2" in reference to a population encompassing CSF microglia. Indeed, they describe a subset of monocytes that expressed microglial-associated genes, including *TREM2*, *TMEM119*, and *GPR34*, and concluded that these cells share features with both border-associated macrophages (BAMs), which have been ascribed immunoregulatory functions within the CNS,[22] and homeostatic microglia. In the future, it will be important to devise nomenclature which prudently encompasses the appropriate degree of segregation among various myeloid cell populations. In particular, BAMs share substantial transcriptional overlap with microglia,[25] prompting the consideration of how CSF microglia differ in ontogeny and function from BAMs that will need to be resolved with further investigation into the ontogeny and function of each cell type.

Microglia are long-lived, self-replicating, yolk sac-derived immune cells of the CNS parenchyma involved in CNS development and neuropathology.[26,27] Disruption of the blood-brain barrier can allow the replacement of microglia by blood-derived progenitors, with the latter eventually taking on the near-complete gene expression profile of fetal-derived microglia.[28] Our studies of CSF microglia cannot determine their ontogeny or how they gained access to the CSF. Microglia from the CNS parenchyma might traverse the pia mater or choroid plexus or traffic into CSF through perivascular spaces or via lymphatic drainage.[29,30] Migration of parenchymal microglia or blood-derived progenitor cells that become microglia may occur in response to chemokines, which is supported by our finding of chemokine receptors on these cells, including CCR1, CCR5, CXCR4, and CX3CR1. It is also possible that a unique microglial subset exists that develops and permanently resides in the CSF. CSF microglia also may not be singular in origin.[31]

Figure 2 Relative expression of myeloid population-defining genes in neuroinflammation
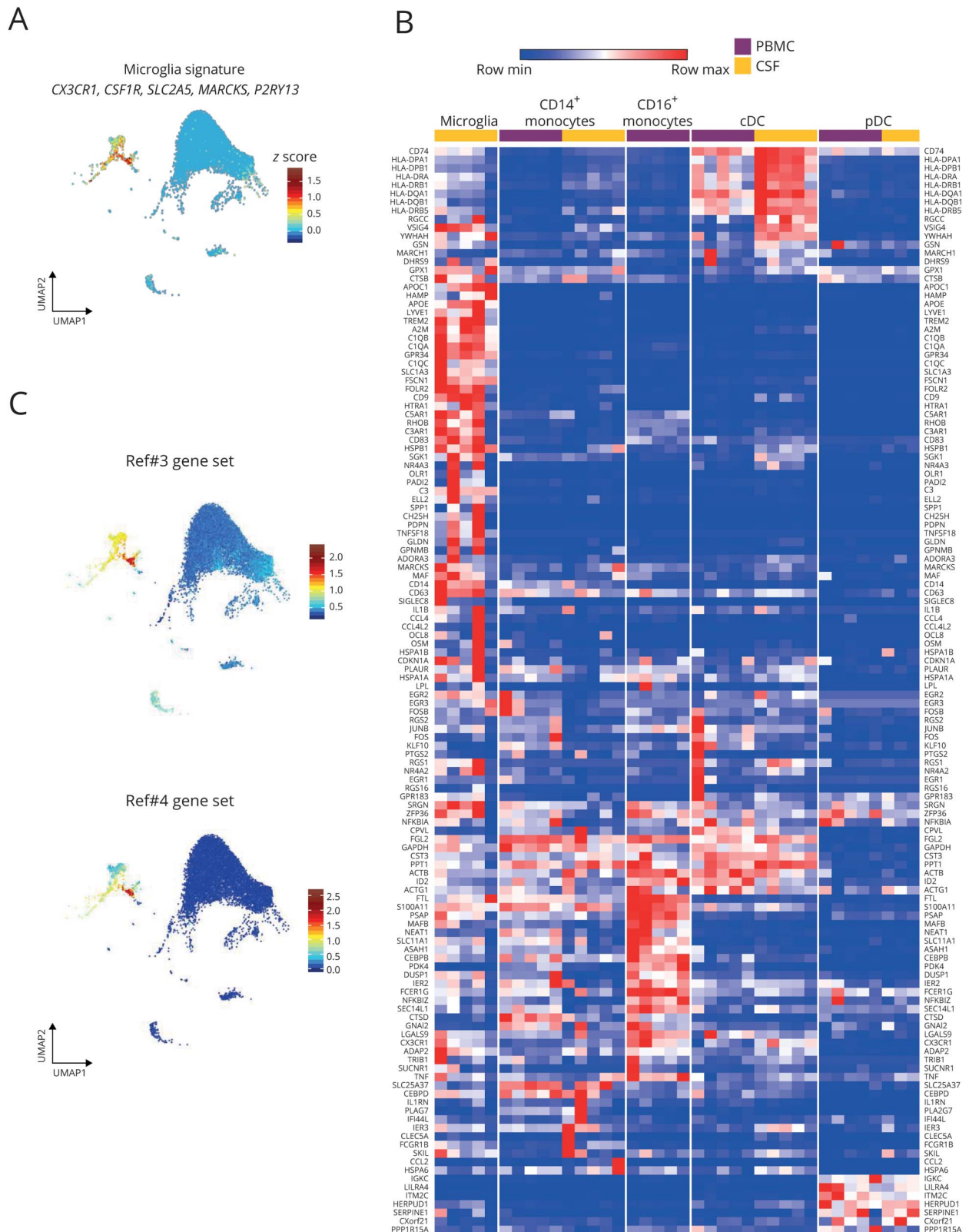
(A) Expression of canonical microglial genes within the UMAP of CSF immune cell clusters. (B) Heatmap representation of gene expression in blood (purple) and CSF (yellow) myeloid populations. Gene list derived from Sankowski et al.[7] (C) Top genes reported by Farhadian et al.[3] as specific for "microglia-like cells" (top) and Beltran et al.[4] as specific for "monocytes" (bottom) displayed in the UMAP of immune cell clusters in the CSF of all subjects. cDC = conventional DC; PBMC = peripheral blood mononuclear cell; pDC = plasmacytoid DC; UMAP = Uniform Manifold Approximation and Projection.

**Figure 3** Discrimination of myeloid cell populations in neuroinflammation by gene expression
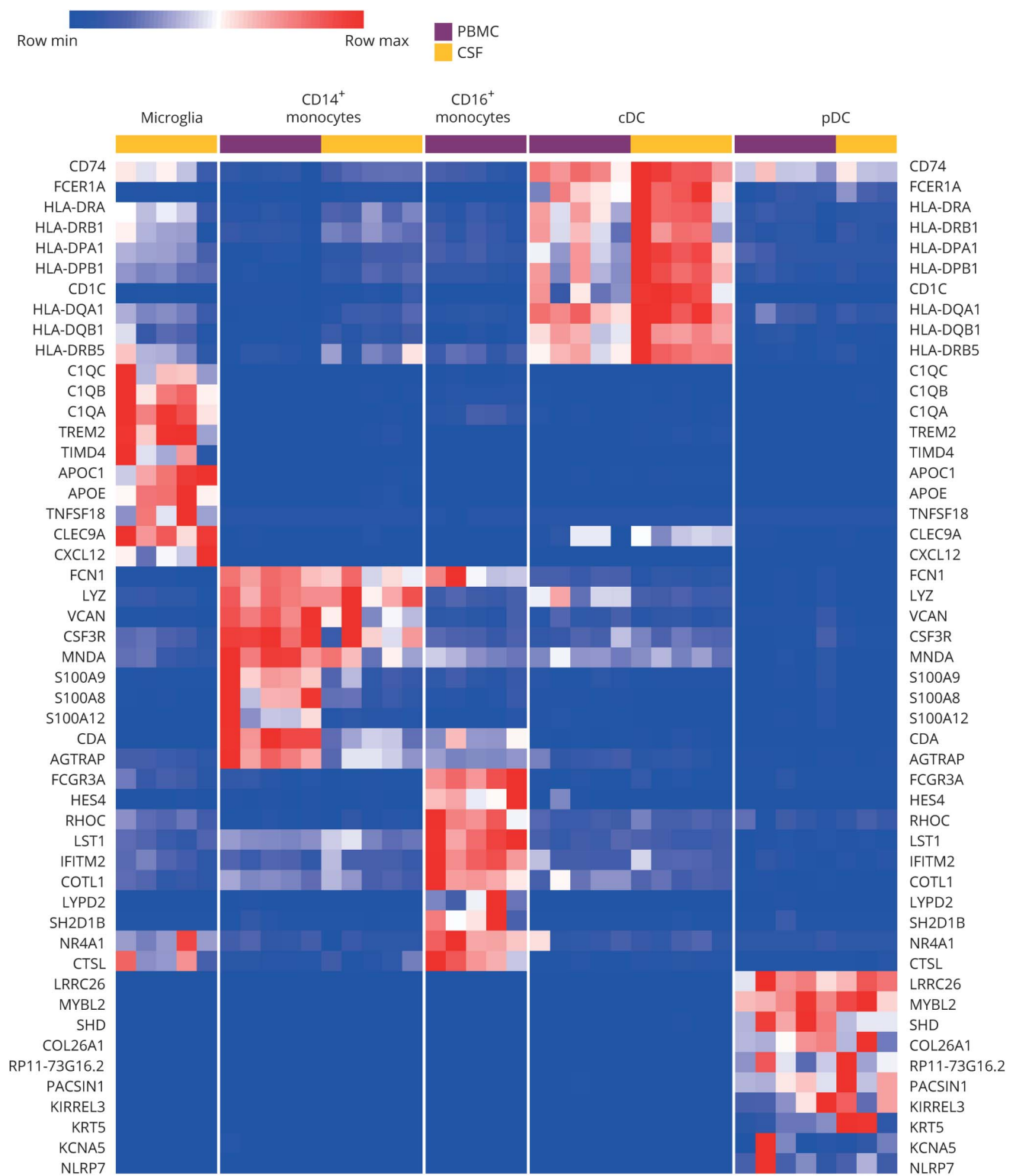
Heatmap of 10 genes that discriminate myeloid cell types within the blood (purple) and CSF (yellow). Columns represent individual subjects. cDC = conventional DC; PBMC = peripheral blood mononuclear cell; pDC = plasmacytoid DC.

The source of CSF microglia may have functionally relevant implications. Microglia are known to be critical modulators of CNS developmental processes, such as synaptic pruning.[32,33]

Recent data reveal the subsets of microglia with distinct functions. In the scRNA-seq analysis of rodent CNS, BAMs and other myeloid populations have been identified within
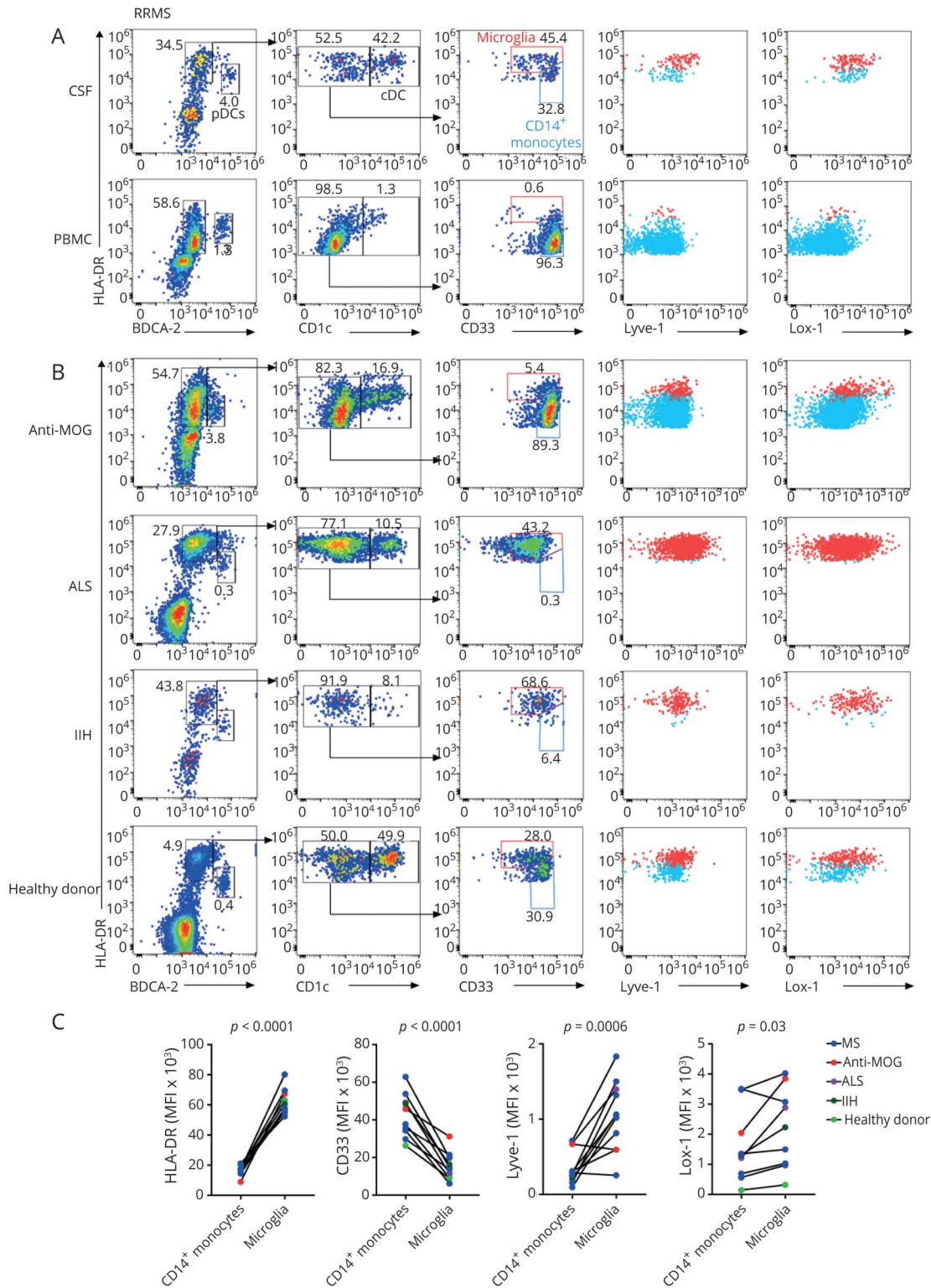
(A and B) Plots were obtained by gating on CD3⁻CD19⁻ singlets. HLA-DR⁺BDCA-2ʰⁱ cells represent pDCs. cDCs are represented as HLA-DR⁺BDCA-2⁻CD1cʰⁱ. Red gates in the third column represent microglia, whereas blue gates represent monocytes. The fourth column shows HLA-DR and Lyve-1 staining of microglia (red) and monocytes (blue). The fifth column shows HLA-DR and Lox-1 staining of microglia (red) and monocytes (blue). (A) Flow cytometry on CSF and blood samples from a subject with RRMS. (B) Flow cytometry on CSF from subjects with anti-MOG disorder (anti-MOG), ALS, IIH, and a healthy control. (C) Mean fluorescence intensity (MFI) of HLA-DR, CD33, Lyve-1, and Lox-1 of CSF monocytes and microglia from subjects. Each pair of connected circles represents one CSF sample. Statistical significance was determined using a paired Student t test. ALS = amyotrophic lateral sclerosis; cDC = conventional DC; IIH = idiopathic intracranial hypertension; MOG = myelin oligodendrocyte glycoprotein; PBMC = peripheral blood mononuclear cell; pDC = plasmacytoid DC.

brain, choroid plexus, and leptomeningeal tissue at steady-state and in the experimental autoimmune encephalomyelitis model of MS.[8] Studies using animal models for Alzheimer disease (AD) have identified a phagocytic subset of microglia termed "disease-associated microglia" (DAM).[23] DAM appear to reduce disease severity in the model and express genes associated with AD such as *Apoe* and *Trem2*. In addition, homeostatic microglia and a subpopulation transitioning between homeostatic microglia and DAM were identified in mouse AD models. Experiments such as these in mice highlight not only the high resolution of CNS myeloid cell subtypes that can be obtained using scRNA-seq but also the apparent dynamic nature of myeloid populations.

Using a flow cytometric panel with commercially available reagents, our data demonstrated the protein expression of some of the microglial genes identified by RNA and indicate that microglia range from 0.2% to 5.5% of CSF mononuclear cells. Overall, the presence of microglia within the CSF was not disease-dependent or disease-activity dependent because they were detected in the CSF of a healthy subject as well as in subjects with noninflammatory neurologic diseases (IIH, ALS) and in 2 distinct neuroinflammatory diseases (MS and anti-MOG disorder). Comparison of CSF microglial frequencies between diseases could not be performed because of low cell numbers. It is possible that subtle differences in the numbers or subtypes of CSF microglia could reflect the state of disease in MS or other diseases. Neither of the subjects with RRMS nor the subject with anti-MOG disorder analyzed for our study was undergoing acute relapse, which may have influenced the presence or number of CSF microglia. Our data demonstrate the feasibility of using standard flow cytometry in the future to address the association of CSF myeloid populations, including CSF microglia, with the clinical state of inflammatory demyelination within the CNS.

cDCs and pDCs were each identified in CSF by both scRNA-seq and flow cytometry. Previous studies have identified these cell types in CSF using lower resolution flow cytometry with fewer markers for discrimination of these myeloid populations and have reported an increased number of pDCs in the CSF during MS relapses.[21,34] Recent scRNA-seq analysis examined DCs and monocytes in human blood, identifying 6 discrete subsets of blood DCs, including 4 subsets of cDCs, a subset of classical pDCs, and a new subset termed AXL+SIGLEC6+ (AS) DCs.[35] ScRNA-seq analysis of the samples in our study did not result in discrete clusters of DC subsets in the blood or CSF, perhaps owing to their low abundance. A dedicated scRNA-seq study focused on sorted CSF DCs would likely reveal additional heterogeneity in these populations. More importantly, our analyses of differentially expressed genes between cDCs in the CSF compared with cDCs in the blood reveal that these populations, although still fundamentally cDCs, may alter some of their gene expression based on their microenvironment. Interestingly, those genes that most discriminated CSF cDCs were more highly expressed by CSF microglia, whereas those that discriminated blood cDCs were more highly expressed by blood monocytes. These results suggest that environmental signals may result in shared tissue-specific gene expression patterns across myeloid cell populations.

Using gene expression-based methods to categorize cell types, we identified microglia, monocytes, cDCs, and pDCs within the CSF. The recognition that microglia are present in CSF may have important clinical implications. Abnormally functioning microglia are believed to contribute to the pathogenesis and/or regulation of several diseases, including AD and MS. Our results indicate that it may be possible to analyze microglial abnormalities directly from the CSF without requiring biopsies.

Disclosure

E. Esaulova, C. Cantoni, I. Shchukina, and K. Zaitsev report no disclosures. R.C. Bucelli received compensation from MT Pharma for advisory board and private funding for Parsonage-Turner syndrome for research; equity in NeuroQuestions, LLC. G.F. Wu received honoraria for consulting for Novartis and Genentech; received research funding from Biogen, EMD Serono, and Roche. M.N. Artyomov reports no disclosures. A.H. Cross received honoraria for consulting for Biogen, Celgene, EMD Serono, Genentech, Novartis, Roche, and TG Therapeutics; received compensation for speaking for Genentech. B.T. Edelson reports no disclosures. Go to Neurology.org/NN for full disclosures.

Appendix Authors

Name	Location	Contribution
Ekaterina Esaulova, MS	Washington University in St. Louis	Data acquisition and analysis; drafting of text and figures; revised the manuscript for intellectual content
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Irina Shchukina, MS	Washington University in St. Louis	Data acquisition and analysis
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