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Multiple Sclerosis CSF Is Enriched With Follicular T Cells Displaying a Th1/Eomes Signature

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Abstract

Background and Objectives

Tertiary lymphoid structures and aggregates are reported in the meninges of patients with multiple sclerosis (MS), especially at the progressive stage, and are strongly associated with cortical lesions and disability. Besides B cells, these structures comprise follicular helper T (Tfh) cells that are crucial to support B-cell differentiation. Tfh cells play a pivotal role in amplifying autoreactive B cells and promoting autoantibody production in several autoimmune diseases, but very few are known in MS. In this study, we examined the phenotype, frequency, and transcriptome of circulating cTfh cells in the blood and CSF of patients with relapsing-remitting MS (RRMS).

Methods

The phenotype and frequency of cTfh cells were analyzed in the blood of 39 healthy controls and 41 untreated patients with RRMS and in the CSF and paired blood of 10 patients with drug-naive RRMS at diagnosis by flow cytometry. Using an in vitro model of blood-brain barrier, we assessed the transendothelial migratory abilities of the different cTfh-cell subsets. Finally, we performed an RNA sequencing analysis of paired CSF cTfh cells and blood cTfh cells in 8 patients sampled at their first demyelinating event.

Results

The blood phenotype and frequency of cTfh cells were not significantly modified in patients with RRMS. In the CSF, we found an important infiltration of Tfh1 cells, with a high proportion of activated $PD1^+$ cells. We demonstrated that the specific subset of Tfh1 cells presents increased migration abilities to cross an in vitro model of blood-brain barrier. Of interest, even at the first demyelinating event, cTfh cells in the CSF display specific characteristics with upregulation of *EOMES* gene and proinflammatory/cytotoxic transcriptomic signature able to efficiently distinguish cTfh cells from the CSF and blood. Finally, interactome analysis revealed potential strong cross talk between pathogenic B cells and CSF cTfh cells, pointing out the CSF as opportune supportive compartment and highlighting the very early implication of B-cell helper T cells in MS pathogenesis.

Discussion

Overall, CSF enrichment in activated Tfh1 as soon as disease diagnosis, associated with high expression of EOMES, and a predicted high propensity to interact with CSF B cells suggest that these cells probably contribute to disease onset and/or activity.

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Glossary

cDNA = complementary DNA; **CIS** = clinically isolated syndrome; **EAE** = experimental autoimmune encephalomyelitis; **FDC** = follicular dendritic cell; **GEO** = Gene Expression Omnibus; **HC** = healthy control; **IQR** = interquartile range; **MS** = multiple sclerosis; **NIND** = noninflammatory neurologic disease; **PBMC** = peripheral blood mononuclear cell; **RRMS** = relapsing-remitting multiple sclerosis; **Tfh** = follicular helper T; **Tfr** = follicular regulatory T; **TLS** = tertiary lymphoid structures.

Multiple sclerosis (MS) is an autoimmune chronic disease of the CNS. Owing to the decisive role of T cells in mouse models of experimental autoimmune encephalomyelitis (EAE) and their predominance in CNS infiltrates, these immune cells have long been considered as the key drivers in MS pathophysiology. This paradigm has been challenged by clinical trials demonstrating a remarkable efficacy of B-cell depleting therapies in relapsing-remitting MS (RRMS)[1,2] with also a more controversial effect on disease progression.[3] Besides the intrathecal immunoglobulin synthesis, several studies reported B-cell follicles in meningeal areas, mainly in patients with secondary progressive MS.[4-8] The presence of organized B-cell follicles belonging to tertiary lymphoid structures (TLS) were found to be strongly associated with cortical lesions and disability,[4-8] suggesting their predominant role in the progressive phase of the disease. The discovery of meningeal aggregates as soon as the RRMS stage leads to believe that TLS start to build up early during disease development.[9]

Follicular helper T (Tfh) cells play a pivotal role in amplifying autoreactive B cells and promoting autoantibody production in several autoimmune diseases.[10] In humans, circulating $CXCR5^+$ memory $CD4^+$ T cells, named cTfh cells, represent the circulating counterpart of germinal center Tfh cells.[11] cTfh cells gather distinct subsets characterized by their differential expression patterns of the 2 chemokine receptors CXCR3 and CCR6 and different B-helper abilities.[11] To date, only very few publications about Tfh cells in patients with MS have been published.[12-18] Nevertheless, a recent study suggests the existence of $CXCR5^+CD4^+$ T cells inside TLS of patients with progressive MS.[19]

In this study, we compared the cell frequency, phenotype, and transcriptome of cTfh cells from paired CSF and blood samples of patients with RRMS at diagnosis and found an important CSF infiltration of activated $PD1^+$cTfh1 cells. In addition, infiltrating CSF cTfh cells present a specific signature including an upregulation of *EOMES* and cytotoxic genes, suggesting a possible role in the maintenance of MS lesions.

Methods

Patients and Controls

Samples from patients with MS, healthy controls (HCs), noninflammatory neurologic disease (NIND) controls, and patients with clinically isolated syndrome (CIS) were obtained from MS expert centers of Nantes and Rennes (France). Patients with MS were diagnosed according to the McDonald 2017 Criteria.[20] CIS was defined as a first inflammatory demyelinating event that can evolve to MS.[20]

Patients had no disease-modifying drugs for a period of at least 3 months before sampling. Demographic and clinical characteristics of patients and HCs are detailed in eTable 1 (links.lww.com/NXI/A748).

Standard Protocol Approvals, Registrations, and Patient Consents

All donors provided written informed consent in compliance with the Declaration of Helsinki. This study was approved by our institutional ethical committee (ethical committee no. 19.131- bis).

Mononuclear Cell Isolation and Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll density centrifugation (Sigma-Aldrich, Burlington, MA). $CD4^+$ T cells were purified from blood using negative selection on magnetic columns (CD4 T-cell isolation kit, human; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained using the following antihuman antibodies: CXCR5-BV605, CD45RA-FITC, CCR7-PE, CXCR3-BV711, CCR6- PercpCy5.5, PD-1-BUV737, CD25-BV786, and CD127-BUV395 (BD Biosciences, Franklin Lakes, NJ). For CSF samples and transmigration assays, CCR7-PE was replaced by a CD4-PE (Beckman Coulter, Brea, CA). Cells were analyzed by flow cytometry (FACS Celesta, BD Biosciences). Dead cells were excluded from the analysis using live/dead Amcyan staining (Invitrogen, Waltham, MA).

Luminex Analysis

Concentrations of IL-6, IL-12p40, CXCL10, CXCL12a, and CXCL13 were measured in the CSF of patients with RRMS and NIND controls using an immune-assay multiplex technique based on the Luminex technology (Thermofisher, Waltham, MA).

Transmigration Assays

Transmigration was assessed using the hCMEC/D3 cell line as an in vitro model of human blood-brain barrier (Merck Millipore). The day before migration experiments, 3×10^5 endothelial cells were seeded on a collagen-coated 3 μm pore size transwell (Falcon; Corning, Corning, NY) in EndoGRO medium (Merck Millipore, Burlington, MA) as previously described.[21] PBMCs were stained with the following antihuman antibodies: CD4-PE, CD8-FITC (Beckman Coulter), and CD3-APC/Fire 750 (Biolegend, San Diego, CA). $CD3^+CD4^+CD8^-$ T cells were sorted using an FACS Aria II (BD Biosciences). Then, 5×10^5 $CD4^+$ T cells in complete Roswell Park Memorial

Institute medium supplemented with 10% of fetal bovine serum were added in the upper chamber of a transwell. After 20 hours at 37°C, migrated and nonmigrated cells were collected. For both compartments, cells were stained as described earlier and analyzed by flow cytometry (FACS Celesta; BD Biosciences).

Cell Sorting and RNA Purification

$CD4^+$ T cells from paired CSF and blood samples were stained using these following antihuman antibodies: CD4-APC, CD45RA-FITC (BD Biosciences), and CXCR5-PeVio615 (Miltenyi Biotec). cTfh cells and memory non-Tfh cells were defined as $CD4^+CD45RA^-CXCR5^+$ and $CD4^+CD45RA^-CXCR5^-$ T cells, respectively. Both groups of cells were sorted using an FACSAria Fusion (BD Biosciences). At least 2,000 cells were necessary to obtain sufficient amount of RNA. RNA was isolated from sorted cTfh cells and memory non-Tfh cells using NucleoSpin RNA Plus XS kit (Macherey-Nagel, Düren, Germany). Total RNA concentration and integrity were assessed using the Agilent 2100 Bioanalyzer RNA Pico chip (Agilent Technologies, Santa Clara, CA).

RNA Sequencing Analysis

Complementary DNA (cDNA) libraries were obtained using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, Kusatsu, Japan) and were purified using solid phase reversible immobilization select beads (Beckman Coulter). cDNA concentration and quality were assessed using the Agilent 2100 Bioanalyzer DNA high-sensitivity chip (Agilent Technologies). Library preparation and sequencing were performed by Next-Generation Sequencing Platform (Institut Curie Genomics, Paris, France). In brief, libraries were obtained using KAPA HyperPlus kit (Roche, Basel, Switzerland) and sequenced with NovaSeq 6000 (Illumina, San Diego, CA). Quality of sequencing data was monitored by FastQC. Reads were mapped to the human genome (GRCh37). Differential gene expression was performed on filtered genes (HTSfilter) using DESeq2 package (R software, version 3.6.3). Genes were declared differentially expressed with a false discovery rate <5% and fold change ≥1.5 in CSF cTfh cells compared with that in blood cTfh cells. Because CXCR5 was used to discriminate cTfh from memory non-Tfh cells, we validated sorting efficiency by the exclusive CXCR5 expression in cTfh populations. Hierarchical clustering was generated using the Ward method. Pathway analysis was performed using Metascape software.[22]

Interactome Analysis

Expressed genes in cTfh cells were defined based on a cutoff applied to RNAseq data, and genes with a log2 mean expression higher than 7.5 were considered as expressed. Raw single-cell RNA-seq data from B-cell transcriptome in active MS were downloaded from NCBI Gene Expression Omnibus (GEO) database (GEO accession no. GSE133028) and reanalyzed according to original instructions.[23] B-cell cluster was isolated according to canonical gene markers (*MS4A1*, *CD19*, *CD79A*, and *CD79B*), and differentially expressed genes between B cells from the CSF and blood were computed with the FindMarkers function from the Seurat package (v3.1.3).[24] For each of these compartments, genes detected in more than 10% of B cells were considered as expressed. NicheNet R package (v1.0.0) was used on the expressed gene lists and gene sets of interest (differentially expressed in CSF B cells compared with that in blood B cells) by following author's recommendations to identify top ligand-receptor interactions between cTfh cells and B cells. Additional bootstrapping with randomized gene sets (1000 iterations) was used to estimate p value of each predicted ligand activity. Ligand-receptor interactions were represented through Circos visualizations (R circlize package v0.4.9)[25] with the best upstream ligands, and mean expression values of major ligands were compared in blood and CSF compartments. In addition, Pearson score of best upstream ligand were represented through a heatmap with the package pheatmap (v1.0.12).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism software, version 8.4. A 2-tailed Mann-Whitney test or a Kruskal-Wallis test with Dunn multiple comparisons test was performed to compare 2 independent groups or more than 2 independent groups, respectively. In addition, a Wilcoxon matched-pair signed rank test or a Friedman test with Dunn multiple comparisons test was performed to compare 2 paired samples or more than 2 paired samples, respectively. p values ≤ 0.05 were considered significant.

Data Availability

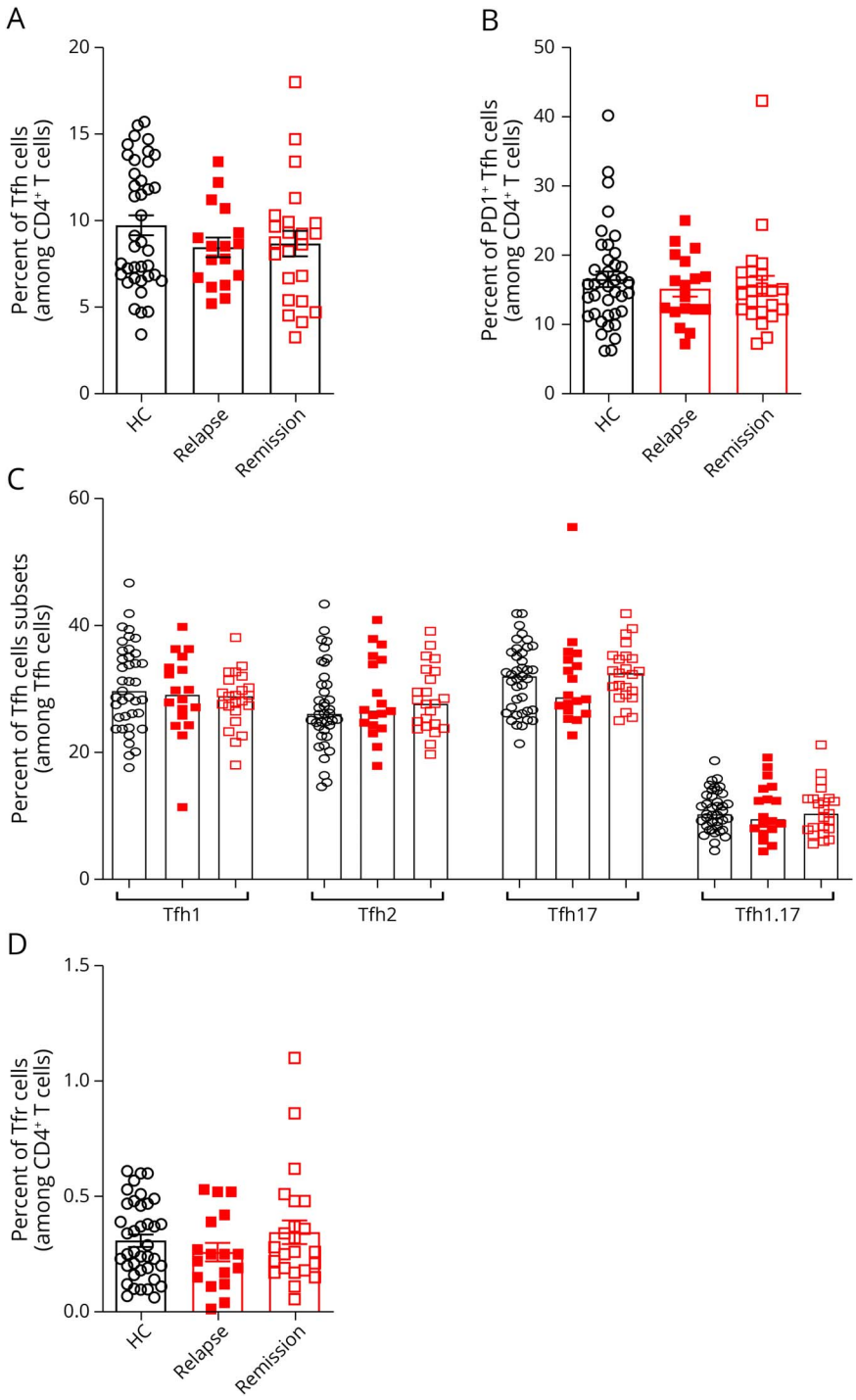
RNAseq dataset of cTfh cells in the CSF and paired blood from patients with CIS has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE173084.

Results

No Significant Alterations for Blood Follicular T-Cell Frequencies of Patients With Untreated RRMS

We analyzed the phenotype of cTfh cells of patients with untreated RRMS sampled during relapse (n = 18) or remission (n = 23), compared with HCs (n = 39). cTfh cells gather several populations with unique phenotype and function. Differential expression of CXCR3 and CCR6 by Tfh cells allowed us to define Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$), Tfh17 ($CXCR3^-CCR6^+$), and Tfh1.17 ($CXCR3^+CCR6^+$) cells.[10] PD-1 is a well-known marker of fully functional Tfh cells in follicles of lymphoid structures. This molecule has been shown to be further expressed in an activated part of cTfh cells in blood.[26] Comparing blood samples of patients with RRMS in relapse or remission with those of HCs, we did not find any statistical difference considering $CD4^+$ cell, cTfh cell frequencies, nor cTfh1, cTfh2, cTfh17, cTfh1.17, and activated $PD1^+$ cTfh cell subset frequencies (Figure 1, A–C). In addition to cTfh cell subsets, circulating follicular regulatory T (cTfr) cells were identified using membrane markers as $CD45RA^-CXCR5^+CD25^{hi}CD127^{low}$. Again, we did not find any statistical difference considering cTfr cell frequencies of blood samples of patients with RRMS or HCs (Figure 1D). Altogether, these data demonstrated no quantitative

Figure 1 Tfh and Tfr Cell Frequencies in Patients With RRMS and HCs



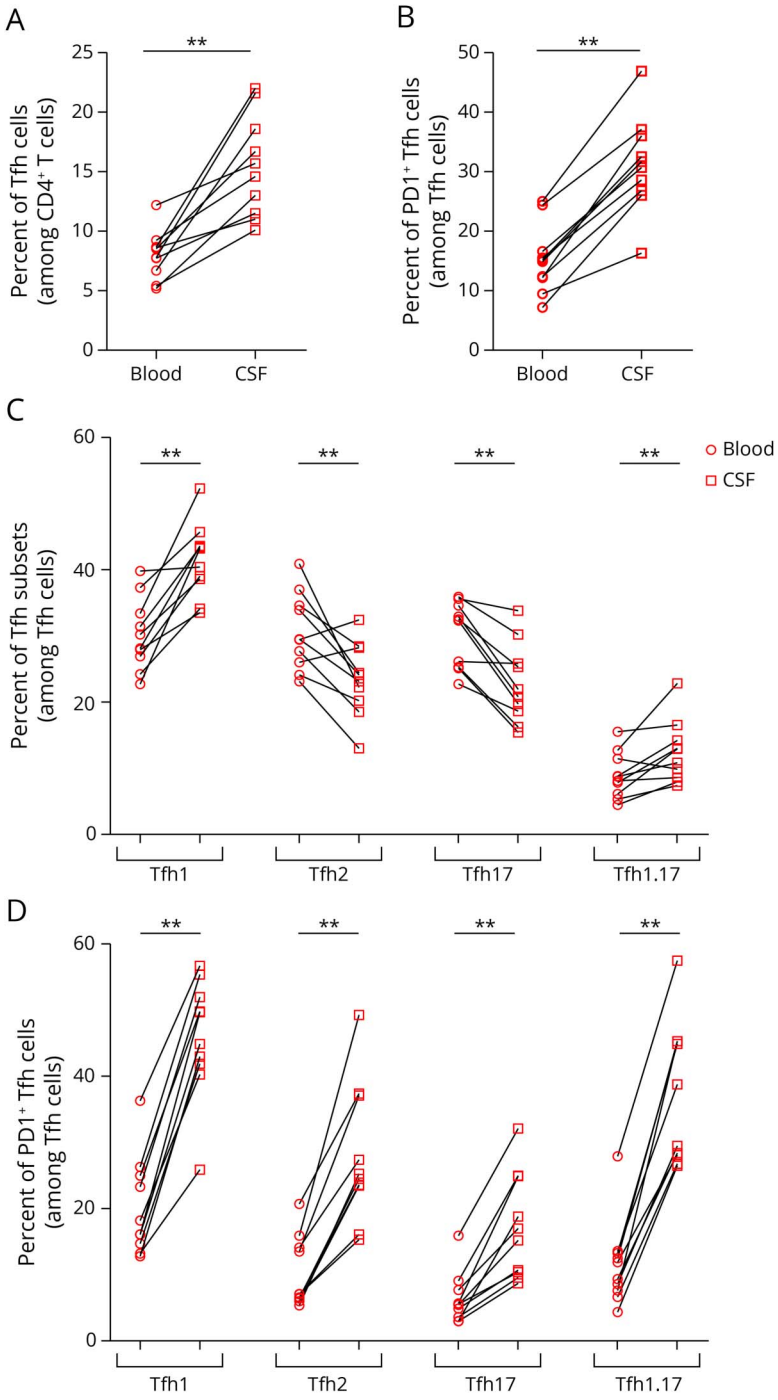
(A-B) Frequencies of Tfh cells (A) and PD-1$^+$ Tfh cells (B) among CD4$^+$ T cells from the blood of patients with untreated RRMS in relapse (n = 18), RRMS patients in remission (n = 23), and HCs (n = 39). (C) Frequencies of Tfh1, Tfh2, Tfh17, and Tfh1.17 subsets among Tfh cells from patients with MS and HCs. (D) Frequencies of Tfr cells among CD4$^+$ T cells from the blood of RRMS patients in relapse and remission and HCs. Black circles represent HCs, solid red squares represent RRMS patients in relapse, and empty red squares represent RRMS patients in remission. Statistical analysis was realized using the nonparametric Kruskal-Wallis test with Dunn multiple comparisons test. Bars indicate median ± IQR. HCs = healthy controls; MS = multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; Tfh = follicular helper T; Tfr = follicular regulatory T.

perturbation of B-cell helper T cells in the blood of patients with untreated RRMS.

Activated PD-1$^+$ cTfh1 Cells Infiltrate the CSF of Patients With RRMS at Diagnosis

We next investigated the frequency and phenotype of cTfh cells in paired CSF and blood samples of 10 patients with untreated RRMS sampled at diagnosis. We found an increased proportion of cTfh cells among CD4$^+$ T cells of the CSF compared with that of paired blood (median: 15.15% vs 8.16% of CSF and blood CD4$^+$ T cells, respectively. p = 0.002; Figure 2A), whereas no significant difference was found in cTfr cell frequency (data not shown). Frequency of activated PD1$^+$ cTfh cells was increased in the CSF (median: 31.20% vs 15.05% of CSF and blood cTfh cells, respectively. p = 0.002, Figure 2B). Increased frequencies of cTfh1 and cTfh1.17 cell subsets were also highlighted in the CSF

Figure 2 Frequencies of Tfh Cells in the CSF of Patients With RRMS



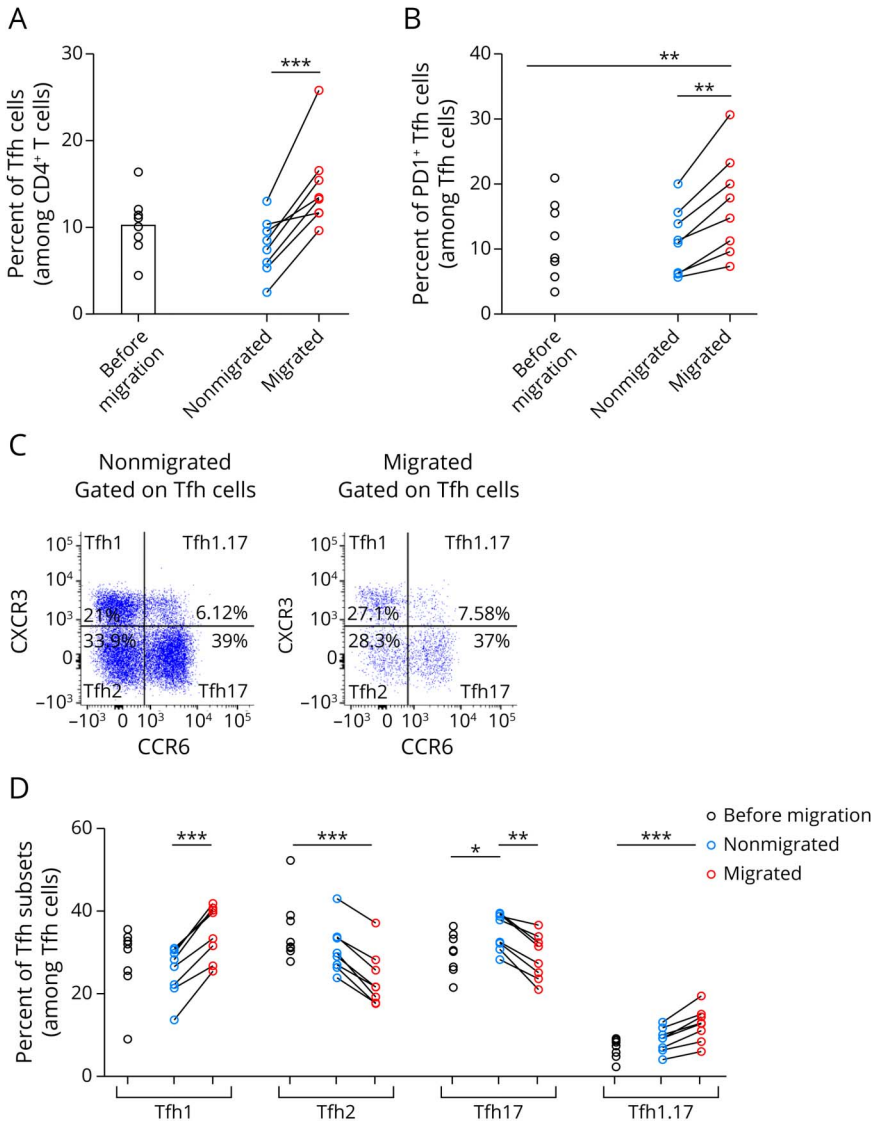
(A) and (B) Frequencies of Tfh (A) and PD-1$^+$ Tfh cells (B) in paired CSF and blood samples of patients with untreated RRMS at the onset of the disease (n = 10). (C) and (D) Frequencies of Tfh cell subsets among Tfh cells (C), and PD-1$^+$ cells among Tfh cell subsets (D) in paired CSF and blood samples. All *p* values were calculated using the nonparametric Wilcoxon matched-pair signed rank test. *$p < 0.05$; **$p < 0.01$. Tfh = follicular helper T.

of patients with RRMS (median cTfh1: 41.8% vs 29.15% of CSF and blood cTfh cells, respectively; $p = 0.002$/median cTfh1.17: 11.85% vs 8.4% of CSF and blood cTfh cells, respectively; $p = 0.0098$, Figure 2C), whereas cTfh2 and cTh17 cell frequencies were decreased. Of note, the proportion of activated PD1$^+$ cells was increased in all CSF cTfh cell subsets (i.e., Tfh1, Tfh2, Tfh17, and Tfh1.17) compared with that in blood subsets (Figure 2D). No significant correlations were found between CSF cTfh frequency and CSF IgG index ([CSF IgG/serum IgG]/[CSF albumin/serum albumin]), IgG, IgM, and IgA levels (data not shown).

Among T Cells, Tfh1 Cells Preferentially Migrate Across a Model of Blood-Brain Barrier

To investigate the differential ability of CD4$^+$ T-cell subsets to migrate into the CNS, we analyzed the trafficking of blood CD4$^+$ T cells from 8 HCs across an in vitro model of blood-brain barrier (hCMEC/D3 cell line).

Figure 3 Migration of Tfh Cells Across an In Vitro Model of Blood-Brain Barrier

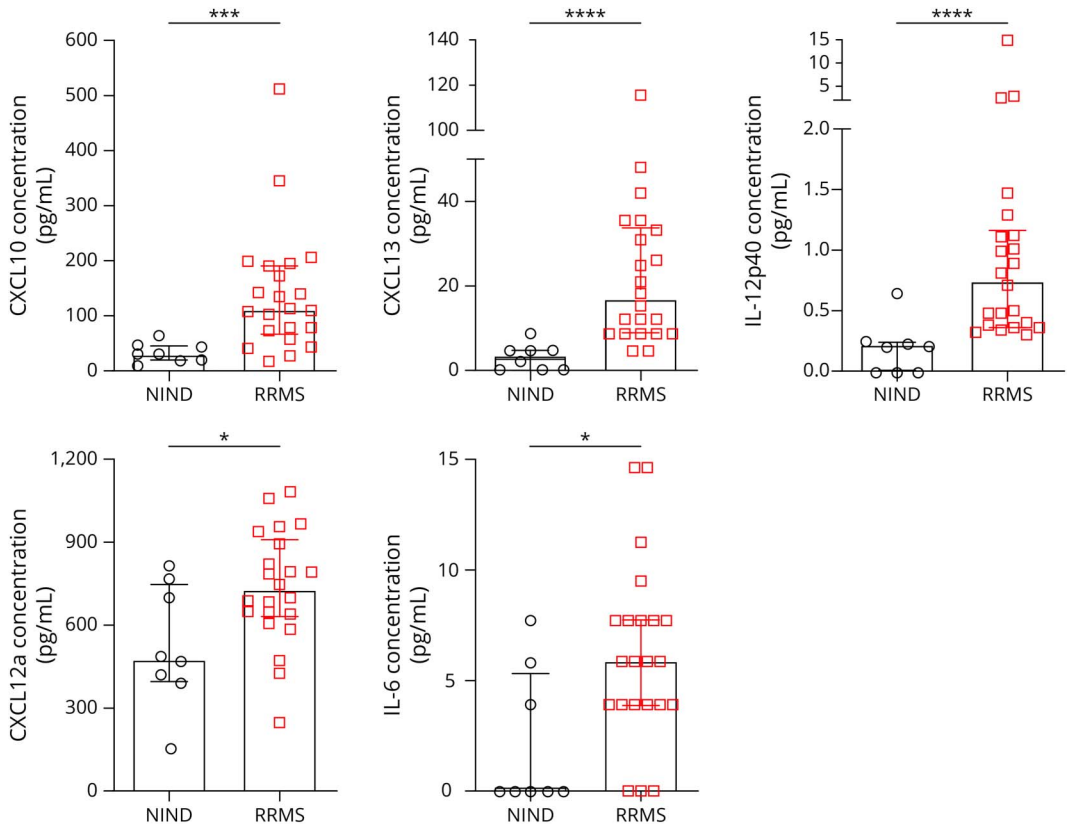


(A) and (B) Frequencies of Tfh (A) and PD-1$^+$ Tfh cells (B) before and after migration (migrated and nonmigrated compartments) of blood CD4$^+$ T cells (n = 8 HCs). (C) Representative dot plots of Tfh1 (CXC3$^+$CCR6$^-$), Tfh2 (CXCR3$^-$CCR6$^-$), Tfh17 (CXCR3$^-$CCR6$^+$), and Tfh1.17. (CXCR3$^+$CCR6$^+$) cells in migrated and nonmigrated compartments. (D) Frequencies of Tfh1, Tfh2, Tfh17, and Tfh1.17 cells among Tfh cells in migrated compartment, nonmigrated compartment, and before the migration assay (n = 8 HCs). *p* values were calculated using the nonparametric Friedman test with Dunn multiple comparisons test. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. HCs = healthy control; Tfh = follicular helper T.

First, migrated cells were enriched in cTfh cells (median: 13.35% in migrated cells vs 7.95% in nonmigrated cells, $p = 0.0002$; Figure 3A) compared with the nonmigrated compartment after 20 hours in culture, with a higher proportion of PD-1 expressing cTfh cells (median; 16.60% in migrated cells vs 11.13% in nonmigrated cells, $p = 0.0081$; Figure 3B). Then, looking at the different cTfh cell subsets, we found a significant increased frequency of cTfh1 cells associated with a decreased frequency of cTfh17 cells in migrated cells compared with that in nonmigrated cells (median: 36.48% in migrated cells vs 27.4% in nonmigrated cells for cTfh1 cells, $p = 0.0005$, and 29.38% in migrated cells vs 35.23% in nonmigrated cells for cTfh17 cells, $p = 0.0035$; Figure 3, C and D). Although nonsignificant, we also observed a trend to an increase in cTfh1.17 cell frequency and to a decrease in Tfh2 cell frequency in migrated cells (Figure 3, C and D).

Using Luminex assays, we measured a high amount of CXCL10 (CXCR3 ligand), CXCL13 (CXCR5 ligand), and IL-12p40, a cytokine important for Th1 cell differentiation and TLS development, in the CSF of patients with RRMS (median CXCL10: 111.5 pg/mL in the CSF of patients with MS [n = 22] vs 32.79 pg/mL in the CSF of patients with NIND [n = 8], $p = 0.0006$; median CXCL13: 16.75 pg/mL in MS vs 3.3 pg/mL in NIND, $p < 0.0001$; median IL-12p40: 0.76 pg/mL in MS vs 0.22 pg/mL in NIND, $p < 0.0001$; Figure 4), and thereby, that inflamed CNS is attractive for cTfh1 cell migration. We also observed high concentrations of CXCL12a and IL-6 in the CSF of patients with RRMS (median CXCL12a: 722.8 pg/mL in MS vs 480 pg/mL in NIND, $p = 0.0395$; median IL-6: 5.86 pg/mL in MS vs undetectable in NIND, $p = 0.0198$; Figure 4), cytokines involved in Tfh cell recruitment and support, respectively. Altogether, these data suggest that inflamed CSF is attractive for cTfh cells and that

Figure 4 Chemokine and Cytokine Quantification in the CSF of Patients With MS and NIND



Measurement by Luminex assay of different cytokines (IL-12p40, IL-6) and chemokines (CXCL10, CXCL13, and CXCL12) in the CSF of 22 patients with MS and 8 patients with NIND. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. Bars indicate the medians ± IQR. MS = multiple sclerosis; NIND = noninflammatory neurologic disease; RRMS = relapsing-remitting multiple sclerosis.

the cTfh1 subset has an increased ability to cross the blood-brain barrier.

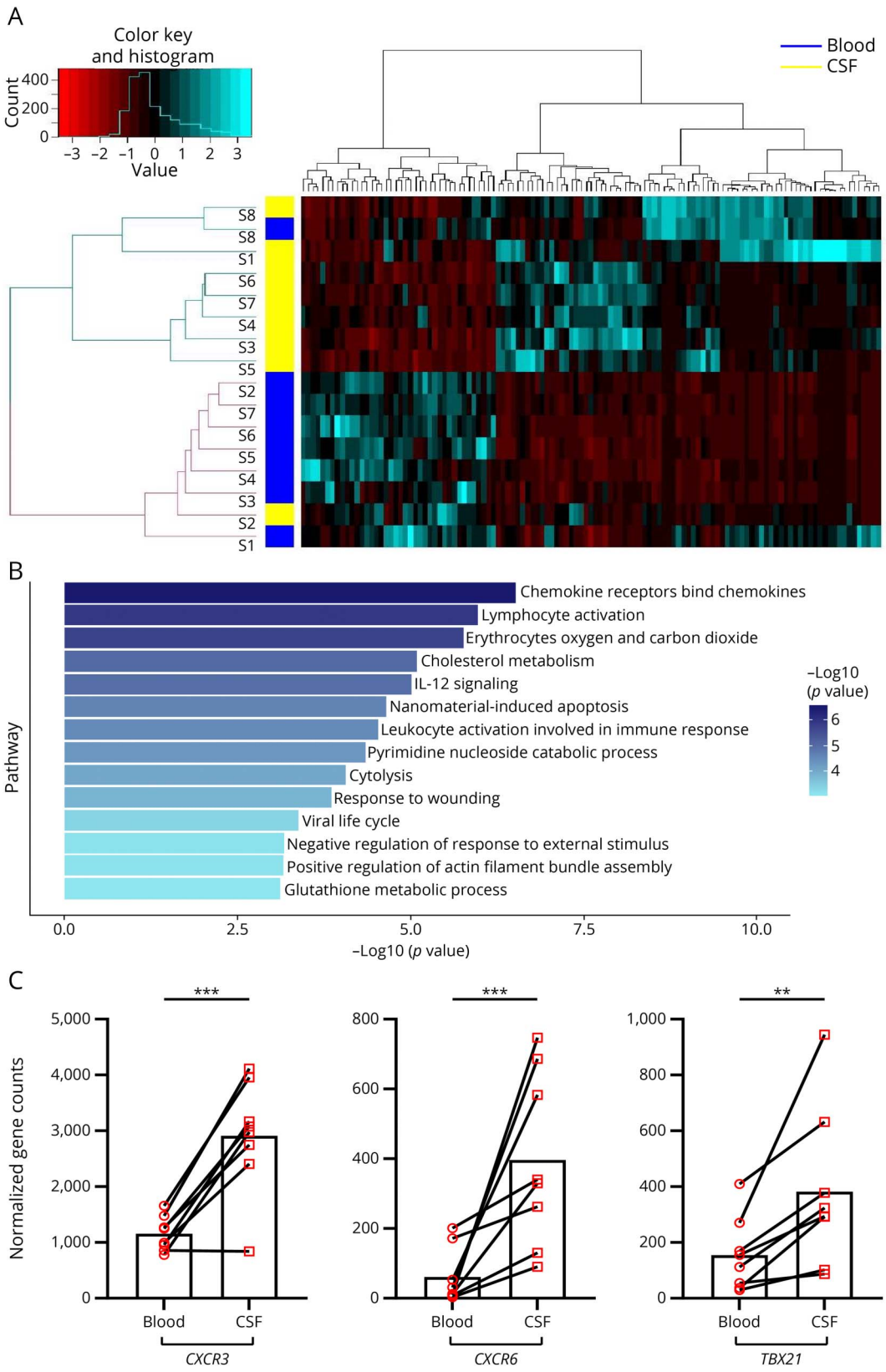
RNA Sequencing Analysis Reveals a Proinflammatory Tfh1 Signature in the CSF at the First Demyelinating Event

We hypothesize that Tfh cells could play a role at very early stage of MS pathogenesis. To that purpose, we performed an RNA sequencing analysis of sorted cTfh cells and memory non-Tfh cells from paired CSF and blood samples of 8 patients with CIS. At the last follow-up, 6 of them converted to RRMS according to McDonald 2017 criteria and 2 remained CIS, but with a high risk of conversion (at least 2 CNS lesions in typical areas) (eTable 1, links.lww.com/NXI/A748).

We found 119 genes differentially expressed in CSF cTfh cells compared with blood counterparts (fold change ≥1.5, adjusted $p < 0.05$). Using a hierarchical unsupervised clustering analysis, we were able to discriminate cTfh cells according to their in vivo compartment except for 2 samples (Figure 5A), suggesting a specific signature of CSF cTfh cells in patients with CIS. Gene enrichment analysis performed using Metascape software revealed significant enrichment in CSF cTfh cells of pathways including "Chemokine receptors bind chemokines" (Reactome Gene Set R-HSA-380108), "Lymphocyte Activation pathway" (GO:0,046,046649), and the canonical pathway "IL-12 signaling" (M54) (Figure 5B). Differentially expressed genes implicated in lymphocyte activation pathway and canonical pathway IL-12 are listed in eTable 2 (links.lww.com/NXI/A748). Among the differentially overexpressed genes in CSF cTfh cells, specific Tfh1 genes were found, especially *CXCR3* (fold-change 2.16), *CXCR6* (fold-change 3.26), and the transcription factor *TBX21* (coding for Tbet protein, fold-change 2.2) (Figure 5C). Comparing the gene expression profile of memory non-Tfh cells from paired CSF and blood samples, only 22 genes were differentially expressed (eFigure 1A, links.lww.com/NXI/A748).

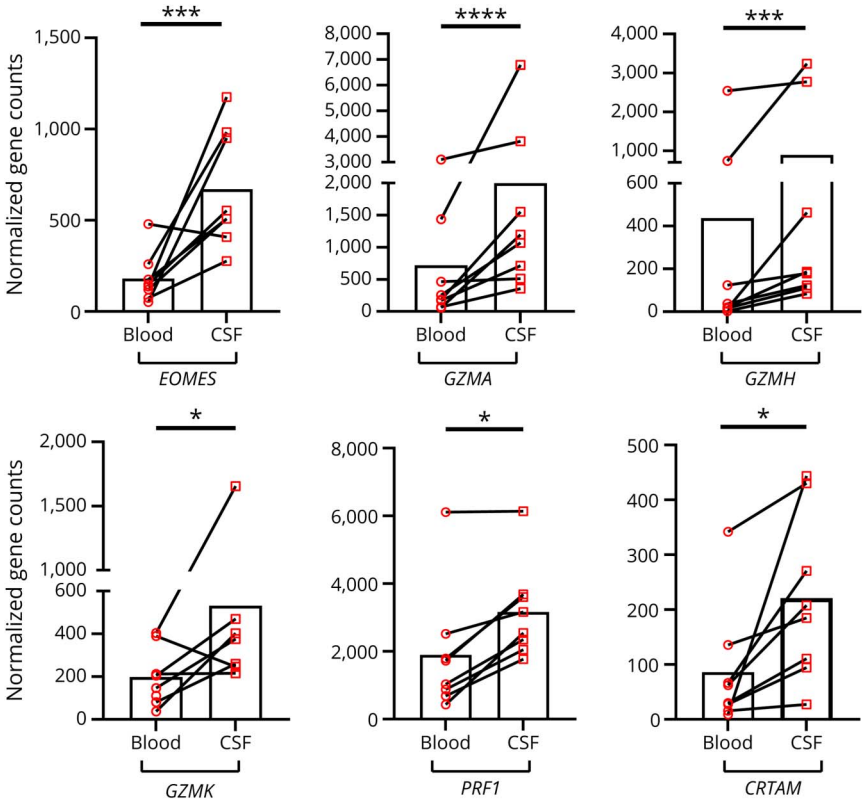
These results suggest that CSF cTfh cells of patients with CIS present a specific activated Tfh1 signature, with potential important implications in B-cell activation in the context of neuroinflammation. In addition to a Tfh1 signature, an increased expression of *EOMES* (Eomesodermin) gene (fold-change 2.65) was found in CSF cTfh cells compared with that in paired blood samples. Owing to EOMES expression by cytotoxic cells (such as cytotoxic $CD8^+$ T cells and natural killer cells), we investigated several cytotoxic gene expressions and revealed a higher expression of *GZMA* (fold-change 2.54), *GZMH* (fold-change 2.39), *GZMK* (fold-change 1.95),

Figure 5 RNA Sequencing Analysis of CSF Tfh Cells From Patients With CIS



(A) Clustering analysis of differentially expressed genes between Tfh cells from paired CSF and blood samples of patients with clinically isolated syndrome. Red and blue represent low and high expression levels, respectively. Patients are indicated in the left of the heatmap by the numbers "S1"–"S8" to visualize paired samples. (B) Pathways implicated in CSF Tfh cells. Analysis was performed using Metascape software. (C) Representative plots of normalized gene counts for specific genes of Tfh1 cells (*CXCR3*, *CXCR6*, and *TBX21*). *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. Bars indicate the mean. Tfh = follicular helper T.

Figure 6 Expression of *EOMES* and Cytotoxic Genes in cTfh of Paired CSF and Blood



Representative plots of normalized gene counts for *EOMES* and genes implicated in cytotoxicity (*GZMA*, *GZMH*, *GZMK*, *PRF1*, and *CRTAM*) in follicular helper T-CSF cells compared with paired blood. *p* values were calculated using the DESeq2 script. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$. Bars indicate the mean.

PRF1 (fold-change 1.86), and *CRTAM* (fold-change 2.00) in CSF cTfh cells, compared with that in paired blood cTfh cells (Figure 6). Similar to cTfh cells, a trend of *EOMES*, *GZMK*, *PRF1*, and *CRTAM* overexpression was observed in CSF memory non-Tfh cells compared with that in paired blood counterparts, but without statistical validation (eFigure 1, links.lww.com/NXI/A748).

CSF cTfh Cells Are More Prone to Interact With B Cells Than Blood cTfh Cells

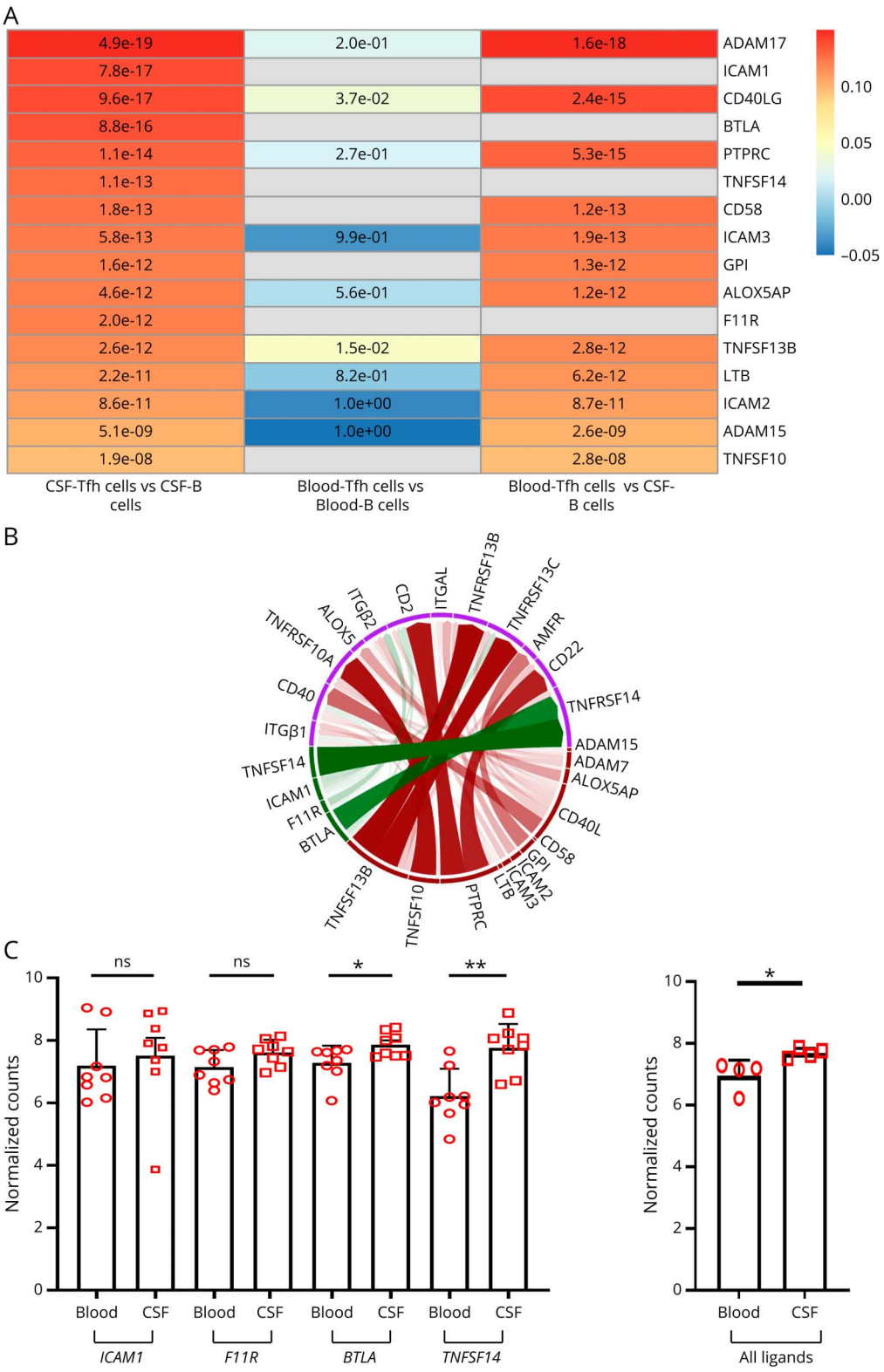
To evaluate the capacity of CSF cTfh cells to interact specifically with CSF B cells of patients with MS, we studied their putative interactions using RNA-seq data of purified CSF and blood cTfh cells from our cohort of patients with CIS and published transcriptomic data of CSF B cells from patients with MS.[23]

To better identify specific interactions between cTfh cells and CSF B cells, we applied the NicheNet computational method using the list of genes upregulated in MS CSF B cells compared with blood B cells. NicheNet algorithm is based on ligand-receptor interactions, on one hand, and on already described subsequent intracellular signaling and target gene regulation, on the other hand. It helps to estimate how sender cell ligands affect target genes in receiver cells. First, we defined ligand activity with Tfh cells as sender cells and B cells as receiver cells in both blood and CSF compartments and represented this activity as a heatmap with bootstrapping with randomized genes (1,000 iterations) to estimate the *p* value of each NicheNet-predicted active ligand (Figure 7A). This analysis showed that CSF cTfh cells, even at the first demyelinating event, are prone to interact with pathogenic B cells rather than in peripheral compartment. In addition, we observed significantly stronger predicted ligand receptor interactions between CSF cTfh cells/CSF B cells than blood cTfh cells/CSF B cells (Figure 7B). Among these interactions, TNFSF14-TNFRSR14, BTLA-TNFRSF14, ICAM1-ITGB2, and F11R-ITGB2 seemed to be stronger for CSF cTfh cells/CSF B cells than blood cTfh cells/blood B cells. Expression of *ICAM1* and *F11R* was not significantly increased in CSF cTfh cells compared with that in blood cTfh cells, but CSF cTfh cells overexpressed *BTLA* and *TNFSF14* compared with blood cTfh cells (Figure 7C) ($p = 0.0156$ and $p = 0.0078$, respectively). When we looked for the expression of all ligands together, the expression was significantly higher in CSF cTfh cells than in blood cTfh cells (Figure 7C). These data suggest that CSF cTfh cells are more prone to interact with CSF B cells compared with blood cTfh cells.

Discussion

Tfh cells are extensively studied in many autoimmune diseases due to their major role in helping B-cell differentiation into antibody secreting cells (ASCs) and in TLS formation. Tfh cells are associated with a wide range of autoimmune diseases and have a clear causal role in disease progression in

Figure 7 Interactions Between CSF Tfh Cells and B Cells



(A) Heatmap representation of ligand activity of CSF-Tfh versus CSF-B cells, blood-Tfh versus blood-B cells and blood-Tfh versus CSF-B cells. In boxes, *p* value of the specificity of the ligand activity, assessed by random permutation. In gray, not available (NA) value. (B) Circos plot showing predicted interactions between CSF or Blood Tfh cells and CSF B cells. Common ligands are represented in red, and specific CSF Tfh cells ligands are represented in green. CSF B-cell receptors are represented in purple. (C) Representative plot of expression of the 4 major ligands (ICAM-1, F11R, BTLA, and TNFSF14) identified by interactome analysis in CSF Tfh compared with blood Tfh (left). The mean expression value of all these ligands in CSF Tfh compared with blood Tfh (right). *p* values were calculated using nonparametric Wilcoxon test. *$p < 0.05$; **$p < 0.01$. Bars indicate the mean. Tfh = follicular helper T.

mice with autoantibody-associated autoimmunity as in the sanroque systemic lupus erythematosus model[27] or other related models.[28] In this prospective study, we reported a significant infiltration of active cTfh1 cells in the CSF of patients with RRMS, correlating with the important ability of this subset to migrate across a model of blood-brain barrier. Of interest, even at the first demyelinating event, cTfh cells in the CSF display specific characteristics with an upregulation of *EOMES* gene and a proinflammatory/cytotoxic transcriptomic signature able to efficiently distinguish cTfh cells isolated from the CSF and blood. Finally, interactome analysis revealed potential strong cross talk between pathogenic B cells and CSF cTfh cells as soon as the first demyelinating event, pointing out the CSF as an opportune supportive compartment for TLS development and highlighting the important and very early implication of B-cell helper T cells in MS pathogenesis.

Circulating Tfh cells include several populations with unique phenotype and functions. Blood cTfh2 and cTfh17 cells, unlike cTfh1 cells, have been demonstrated to support naïve B-cell maturation into ASCs and induce class switch recombination through IL-21 secretion.[10] In our study, we did not observe any significant modifications of the frequency of blood cTfh in our cohort of patients with untreated RRMS compared with HCs, which is in line with many studies[12-14,16] and is now well established. Controversial results are nevertheless published, with 2 studies[12,18] reporting an increase of cTfh1.17 cells in RRMS, whereas another study reports a decrease of cTfh1 cells with no alterations of cTfh1.17.[15] These differences could not only be due to the heterogeneity of the cohorts including in some studies both treated and untreated patients, both patients with RRMS and progressive MS, but also be due to the combination of markers used to define cTfh-cell populations that often differ among the laboratories.

In MS, even if there are arguments to plead for a pathogenic role of CSF Igs,[14,15] their role and specificity in MS development remain ambiguous. Our data indicate that infiltrating cTfh1 likely play a critical role as soon as the beginning of the disease to support pathogenic B cells and MS pathogenesis. In fact, $ICOS^+PD1^+$-activated cTfh1 cells can induce memory B cells to differentiate into ASCs.[29] In addition, cTfh1 frequency was recently correlated with an expansion of T-bet–expressing B cells,[30] a population linked to autoimmune diseases and MS,[31] which can be recruited into CNS.[32] Enhanced predicted ligand-receptor interactions between CSF cTfh and MS CSF B cells support also the hypothesis of a strong pathogenic B-cell support of cTfh in inflamed CSF.

Tfh cells have other functions unrelated to antibody production per se by facilitating the formation or maintenance of ectopic follicles, which serve as niche favoring interactions with other cells that may be pathogenic in MS. TLS are locally inducible leukocyte aggregates that form in chronically inflamed non-lymphoid tissues. Such structures were reported by 2 studies[4,5] in the brain of 40% of patients with secondary progressive MS and correspond to meningeal B cell aggregates comprising notably $CXCL13^+$ follicular dendritic cells (FDC) and B cells. Of importance, the presence of TLS or meningeal inflammation in patients with MS was directly related to cortical pathology.[6,33] And these meningeal inflammations/aggregates can also be found in cases with acute MS.[9,34] Furthermore, recent articles demonstrated local B-cell somatic hypermutation in both lymphoid aggregates of EAE mice[35] and human CSF,[23] pointing toward a potential key role of these structures in disease maintenance/progression within the CNS.

Different mice models and settings support the critical role of Tfh in TLS formation. In EAE and in MS, Tfh and B cells are found in CNS lymphoid aggregates.[19,36] EAE mice treatment with laquinimod reduces Tfh expansion, IL-21 production, myelin oligodendrocyte glycoprotein–specific IgG production, and germinal center induction, resulting in the inhibition of spontaneous meningeal aggregate formation and a decreased disability progression.[37] Tfh1 cells that we found more frequent in the CSF may alternatively contribute to the spontaneous formation of TLS through IFNg production. Actually, IFNgR on B cells have been demonstrated critical in TLS induction and in the expansion of B cells producing autoantibodies[38]; thus, Tfh1-related IFNg may contribute to this mechanism in MS. In a mouse model of ovarian sarcoma, Chaurio et al.[39] demonstrated the direct correlation between the presence of Tfh and the formation of lymphoid structures in the tumor tissue. Interestingly in their model, TLS formation was abrogated by blocking CXCL13 signaling, a cytokine found highly enriched in MS CSF patients,[40] as in our cohort. CXCL13 is classically described as expressed by FDC, a component of lymphoid structures promoting the recruitment of CXCR5 expressing cells as Tfh and B cells and therefore orchestrating germinal center reaction. In line, CXCL13 was found expressed by FDC within B-cell meningeal aggregates of EAE mice,[41] but monocytic/macrophages and microglial cells can also be sources of CXCL13 in specific context.[42,43] The use of CXCL13 KO animals confirmed its role in EAE maintenance, and its blockade results in a milder disease, an attenuated inflammation, and a better recovery, potentially through defective Tfh/B-cell recruitment and TLS induction.[44] CXCL13 expression is detected as early as disease onset in both EAE mice and CSF of patients with MS[33,41,45] and is correlated with increased cortical pathology in patients.[33]

These data together with the enhanced CSF Tfh–cell/B-cell interaction predicted by our interactome analysis point our findings toward a propensity to mount TLS early in the disease course and that Tfh infiltration in the CSF may have a critical role in it.

In this study, we found a cytotoxic signature expressed by CSF cTfh cells. Although not significant, a similar signature is observed concerning the CSF memory non-Tfh cells, indicating that this process may not be restricted to cTfh cells. These data are in line with results obtained from 2 recently published articles highlighting the enrichment of $CD4^+$

T cells in the CSF of patients with RRMS displaying both cTfh1/Th1 (*CXCR3* and *CCR5*) and cytotoxic (*GZM* gene family, *PRF1*) characteristics.[17,46] Furthermore, both articles found a significant upregulation of *EOMES*, a key transcription factor regulating the expression of *IFNG*, *CSF2*, and major cytotoxic genes. Of interest, $EOMES^{+}CD4^{+}$ T cells have been proven pathogenic in EAE[47-49] and are induced by prolactin secretion by APC.[48] Whether such $EOMES^{+}$ $CD4^{+}$ T cells display functional cytotoxic abilities remains unknown and requires more investigations. In fact, *EOMES* expression in $CD4^{+}$ T cells of CSF CIS patients may not be related to cytotoxic activity while being reflective of a specific context. Actually, EOMES expression has also been described as expressed by resident memory T cells in association with high levels of CCR5, CXCR3, GZMK, and GZMA.[50] The restriction of EOMES profile to the CSF compartment in our data suggests that this population has a role in disease activity and is either selected, expanded, or differentiated in the inflamed CSF. Whether CSF cTfh differentially expressed genes correspond to the acquisition of resident memory T cell traits is still unknown as the specific contribution of EOMES expressing CD4 in RRMS remains to be determined.

Overall, even if the function of these activated $EOMES^{high}$ cTfh1 cells remains elusive, their predicted higher propensity to interact with CSF B cells associated with their inflammatory pattern suggest that these cells probably contribute to disease onset and/or disease activity by promoting TLS development and reactivation of pathogenic memory B cells.

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