Intrathecal CD8^+CD20^+ T Cells in Primary Progressive Multiple Sclerosis

Marina Rode von Essen, PhD, Jacob Talbot, MD, Rikke Holm Holm Hansen, MSc, Helene Højsgaard Chow, MD, PhD, Henrik Lundell, PhD, Hartwig Roman Siebner, MD, PhD, and Finn Sellebjerg, MD, PhD

Neurological Neuroimmunology Neuroinflammation 2023;10:e200140. doi:10.1212/NXI.00000000000200140

Abstract

Background and Objective
Despite accumulating evidence of intrathecal inflammation in patients with primary progressive multiple sclerosis (PPMS), immunomodulatory and suppressive treatment strategies have proven unsuccessful. With this study, we investigated the involvement of CD20^+ T cells and the effect of dimethyl fumarate on CD20^+ T cells in PPMS.

Methods
The main outcomes in this observational, case-control study were flow cytometry assessments of blood and CSF CD20^+ T cells and ELISA measurements of myelin basic protein and neurofilament light chain in untreated patients with PPMS and patients treated for 48 weeks with dimethyl fumarate or placebo. MRI measures included new and enlarging T2-weighted lesions over 48 weeks and lesion, normal-appearing white matter, cortical, and thalamic volume.

Results
Assessing CD20^+ T cells in patients with PPMS and controls showed an increased percentage of CD20^+ T cells in the blood of untreated patients and a strong enrichment in the CSF. In addition, a higher frequency of CD8^+CD20^+ T cells in the CSF correlated with a higher concentration of myelin basic protein and T2-weighted lesion volume and with a lower normal-appearing white matter and thalamus volume. Furthermore, CD8^+CD20^+ T cells were associated with the development of new T2 lesions. After 48 weeks of treatment with dimethyl fumarate, total T cells in CSF were reduced; however, CD20^+ T cells were unaffected.

Discussion
This study shows an association between intrathecal CD8^+CD20^+ T cells, white matter injury, and thalamic atrophy in PPMS, suggesting a role of CD8^+CD20^+ T cells in the immunopathogenesis of PPMS. The results also suggest that limited efficacy of dimethyl fumarate in PPMS may, at least partly, be a consequence of failure to suppress CD8^+CD20^+ T cells in CSF.
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CD20+ T cells in the pathogenesis of PPMS.

Study Protocol Approvals, Registrations, and Patient Consents

All participants gave informed, written consent to participation. The study was approved by the regional scientific ethics commit-

Blood and CSF Samples

Venous blood was collected, and peripheral blood mono-

Flow Cytometry Analysis

From freshly isolated PBMCs and within minutes of CSF cell separation, cells were incubated in the FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) to prevent nonspecific Ab binding and thereafter stained in PBS/2% FBS/0.02% NaN3 with a combination of fluorochrome-conjugated antibodies against (conjugate; clone) CD3 (FITC, APC/Cy7; UCHT1), CD4 (PerCP/Cy5.5, APC/ Cy7; RPA-T4), CD8 (APC/Cy7, PerCP/Cy5.5; RPA-T8), CD20 (PE/Cy7, BV421; 2H7), CXCR3 (PE/Cy7, BV421; G025H7), CCR6 (PerCP/Cy5.5, BV605; G034E3), and CCR5 (AF647; HEK/1/85a), all from BioLegend (San Diego, CA). Isotype matched controls were used to correct for nonspecific Ab binding and spectral overlap, where appropriate. 

Methods

Study Population

In this observational case-control study, samples from 34 healthy controls (mean 42 years; range 25–69) and 37 untreated patients with PPMS (mean 55 years; range 39–65) were included; a difference in age between groups was observed (p < 0.0001). In addition, a smaller replication cohort of 16 untreated patients with PPMS was included (mean 55 years; range 44–61). Healthy controls had no autoimmune, neurologic, or other chronic illnesses. Patients with PPMS participated in a placebo-controlled randomized treatment trial with DMF for 48 weeks at the Danish Multiple Sclerosis Center, Rigshospitalet, Denmark. Main inclusion criteria were a PPMS diagnosis, age between 18 and 65 years, no immunosuppressive or immunomodulatory treatment within 6 months, and no steroid treatment for at least 3 months before inclusion. In addition to baseline samples, we obtained samples after 48 weeks from 24 placebo-treated patients (mean 56 years; range 46–66) and 21 DMF-treated patients (mean 57 years; range 43–66); no difference in age or sex was observed (p = 0.82; p = 0.78).

Study Protocol Approvals, Registrations, and Patient Consents

All participants gave informed, written consent to participation. The study was approved by the regional scientific ethics committee (protocol number KF-01314009 and H-16047666). The trial is registered at ClinicalTrials.gov (NCT02959658).

Flow Cytometry Analysis

From freshly isolated PBMCs and within minutes of CSF cell separation, cells were incubated in the FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) to prevent nonspecific Ab binding and thereafter stained in PBS/2% FBS/0.02% NaN3 with a combination of fluorochrome-conjugated antibodies against (conjugate; clone) CD3 (FITC, APC/Cy7; UCHT1), CD4 (PerCP/Cy5.5, APC/Cy7; RPA-T4), CD8 (APC/Cy7, PerCP/Cy5.5; RPA-T8), CD20 (PE/Cy7, BV421; 2H7), CXCR3 (PE/Cy7, BV421; G025H7), CCR6 (PerCP/Cy5.5, BV605; G034E3), and CCR5 (AF647; HEK/1/85a), all from BioLegend (San Diego, CA). Isotype matched controls were used to correct for nonspecific Ab binding and spectral overlap, where appropriate. TruCount staining of whole blood to measure absolute cell count was performed using the BD Multitest 6-color TBNK Reagent according to the manufacturer (BD Biosciences, San Jose, CA). Data were acquired on a FACS Canto II flow cytometer (BD Biosciences) and data analyses performed using the software FlowJo (TreeStar, Ashland, OR). Analyses of data were performed blinded.
Cytokine Analysis

For cytokine analysis, a modified protocol of a previously described procedure was used. Cryopreserved PBMCs from 5 untreated patients with PPMS were thawed and T cells negatively isolated using the Human T-cell Isolation kit from StemCell Technologies (Vancouver, Canada). T cells were hereafter stained with antibodies targeting αβ TCR (BV605; IP26) and CD20 (PE/Cy7; 2H7) both from BioLegend and sorted into CD20− and CD20+ T cells on a BD FACSMelody BRV 9 color (BD) by fluorescence-activated cell sorting. The purity of CD20− T cells was 100% and 98%–100% for CD20+ T cells. 40–80,000 CD20− and CD20+ T cells were stimulated with 10 ng/mL PMA and 0.5 μg/mL ionomycin for 30 minutes after which 5 μg/mL brefeldin A was added to the cell culture and the cells were further incubated for 4 hours at 5% CO2, 37°C (all from Sigma-Aldrich, MO); a total duration of stimulation of 4:30 hours. Cells were then stained with Live/Dead stain (Life Technologies) and fluorochrome-conjugated Ab against CD3 (BV785; UCHT1; BioLegend), CD4 (AF488; OKT4; Biolegend), and CD8 (APC/Cy7; HIT8a, Biologic). Cells were then fixed and permeabilized using Fixation Buffer and Permeabilization Wash Buffer from BioLegend, according to the manufacturer’s protocol. Finally, cells were intracellularly stained with fluorochrome-conjugated Ab against IFN-γ (PerCP-Cy5.5; B27), TNF-α (APC; Mab11), GM-CSF (PE; BVD2-21C11), IL-17 (BV421; BL168), IL-4 (APC; MP4-25D2), all from BioLegend, IL-10 (BV421; JES3-9D7) from BD, or corresponding isotype controls. Data were acquired on a BD FACSsymphony A3 (BD) flow cytometer and data analysis performed using the software FlowJo.

Biomarker and MRI Outcomes

Soluble myelin basic protein (MBP) and neurofilament light (NFL) chain in the CSF of patients with PPMS were measured by ELISA (R&D Systems, Minneapolis, MN; UMAN Diagnostics, Umeå, Sweden) as previously described. All patients included in the treatment trial underwent MRI of the brain at Copenhagen University Hospital, Hvidovre. MRI measures included a number of new and enlarging T2 lesions from baseline to week 48; and baseline measures of white matter lesion volume, magnetization transfer ratio (MTR) in lesions, fractional anisotropy (FA) in NAWM, thalamus, cortical gray matter (CGM), and NAWM volume. Details regarding the MRI data acquisition and analysis can be found in the supplementary material of our earlier work.

Statistical Analysis

For analysis of sex differences between groups, a chi² test was performed, and for analysis of age differences between groups, a Mann–Whitney U test was applied. To compare cell frequencies between healthy controls and patients with PPMS, logarithmically transformed data were analyzed in a linear model with correction for age. To compare cell frequencies and absolute number of cells between placebo and DMF-treated patients with PPMS, a Mann–Whitney U test was performed. In addition, correlations of CD20+ T cells from untreated patients with MBP, NFL, and baseline MRI measures were assessed by Spearman rank correlation analysis. Finally, correlation between CD8+CD20+ T cells of treated patients with PPMS and white matter lesion activity was assessed by a linear model to correct for the treatment applied. To compare cytokine production in CD8+CD20+ T cells and CD8+CD20− T cells from untreated patients with PPMS, a paired t test was performed. A significance level p < 0.05 was considered statistically significant for all analyses.

STROBE Guidelines

For this manuscript, the STROBE reporting guidelines for observational studies were used.

Data Availability

Data are available in anonymized form and can be shared by request from any qualified investigator. Sharing requires approval of a data transfer agreement in accordance with GDPR and Danish data protection regulation.
Results

Increased Frequency of CD20+ T Cells in the Blood of Patients With PPMS

To investigate a possible implication of CD20+ T cells in the pathogenesis of PPMS, we measured the percentage of CD20+ T cells in the blood from 34 healthy controls and 37 untreated patients with PPMS by flow cytometry; a gating example is shown in eFigure 1A (links.lww.com/NXI/A879). This analysis was performed blinded, and a strict gating strategy excluding doublet cells and CD20+ B cells was applied. Despite an equal frequency of peripheral CD4+ T cells (p = 0.84) and CD8+ T cells (p = 0.83) in healthy controls and patients with PPMS, we found an increased frequency of circulating CD20+ T cells in patients with PPMS, both within the CD4 and CD8 T-cell compartment (p < 0.0001; p = 0.009), Figure 1, A and B.

Enrichment of CD20+ T Cells in the CSF of Patients With PPMS

In parallel with the measurement of CD20+ T cells in the blood, an analysis of CD20+ T-cell frequency in the CSF was performed in 25 of the untreated patients with PPMS; a gating example is shown in eFigure 1B (links.lww.com/NXI/A879). This demonstrated an enrichment of CD20+ T cells in the CSF of patients with PPMS, both in the CD4 and CD8 T-cell compartment (p < 0.0001), Figure 1, C and D. The enrichment of CD4+ and CD8+ CD20+ T cells in the CSF of patients with PPMS was validated in a smaller replication cohort of 16 untreated patients with PPMS (p < 0.0001 for both populations).

Correlation Between Intrathecal CD8+CD20+ T Cells, Demyelination, and Neurodegeneration

To investigate a possible role of CD20+ T cells in demyelination in patients with PPMS, the association between intrathecal CD20+ T cells and MBP in the CSF was assessed by Spearman rank correlation analysis. To strengthen the analysis, the sample size was increased by pooling the primary cohort and the replication cohort (n = 41). This showed a positive correlation between the prevalence of CD8+CD20+ T cells in CSF and the concentration of MBP (p = 0.0005; rs = 0.53), Figure 2A; no correlation between CD4+ CD20+ T cells and MBP was found (p = 0.51; rs = 0.11), eFigure 2A (links.lww.com/NXI/A879). In addition, no significant correlation between total CD4+ T cells (p = 0.12; rs = 0.25) or CD8+ T cells (p = 0.13; rs = 0.25) in the CSF and MBP was found.

(A–D) Correlation analysis between CD8+CD20+ T cells in CSF and myelin basic protein (MBP) (A), lesion volume (B), normal-appearing white matter (NAWM) volume (C), and thalamus volume (D). The exploratory cohort is depicted by circles and the replication cohort by hexagons.

Figure 2 Correlation Between Intrathecal CD8+CD20+ T Cells, Demyelination, and Neurodegeneration
Release of NFL is a biomarker of neuroaxonal damage; however, we found no significant correlation between CSF concentrations of NFL and frequencies of CD4+CD20+ T cells (p = 0.33; rs = 0.17) or CD8+CD20+ T cells (p = 0.057; rs = 0.32).

All patients with PPMS participating in the treatment trial underwent brain MRI. Correlating MRI measures with the percentage of CD20+ T cells in CSF showed a positive correlation between the frequency of intrathecal CD8+CD20+ T cells and white matter lesion volume (p = 0.0092; rs = 0.43), Figure 2B; by contrast, there was no significant correlation for CD4+CD20+ T cells (p = 0.27; rs = 0.19), eFigure 2B (links.lww.com/NXI/A879). In addition, a negative correlation between NAWM volume and intrathecal CD8+CD20+ T cells (p = 0.021; rs = −0.39), Figure 2C, and CD4+CD20+ T cells (p = 0.044; rs = −0.34), efigure 2C, was found. No correlations between CGM volume (CD4+CD20+: p = 0.62, rs = −0.09; CD8+CD20+: p = 0.28, rs = −0.19, Figure 2D), MTR in lesions (CD4+CD20+: p = 0.79, rs = −0.05; CD8+CD20+: p = 0.99, rs = −0.01) or FA in NAWM (CD4+CD20+: p = 0.49, rs = −0.12; CD8+CD20+: p = 0.057, rs = −0.33), and intrathecal CD20+ T cells were found.

**DMF Treatment Depletes CD20+ T Cells From the Periphery But Not Intrathecally**

We recently performed a placebo-controlled randomized trial investigating DMF treatment in patients with PPMS.10 From freshly drawn blood and CSF collected after 48 weeks of treatment with placebo (blood n = 24; CSF n = 14) or DMF (blood n = 21; CSF n = 15), we measured the levels of CD20+ T cells; flow cytometry dot plot examples are shown in eFigure 3, A and B (links.lww.com/NXI/A879). In the blood, both frequency and absolute number of T cells (eFigure 3, C and D) as well as CD4+CD20+ and CD8+CD20+ T cells (Figure 3, A–D) were decreased after 48 weeks of treatment with DMF compared with placebo (p < 0.0001 for all analysis). By contrast, we found that despite a decrease in

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**Figure 3** DMF Treatment Depletes CD20+ T Cells From the Blood in Patients With PPMS

(a and b) Percent CD4+CD20+ T cells (a) and CD8+CD20+ T cells (B) in the blood of placebo and dimethyl fumarate (DMF)-treated patients with primary progressive multiple sclerosis (PPMS). (C and D) Absolute number of CD4+CD20+ T cells (C) and CD8+CD20+ T cells (D) in the blood of placebo and DMF-treated patients with PPMS. The median value is shown for all groups analyzed.

**Figure 4** DMF Treatment Does Not Deplete CD20+ T Cells Intrathecally in Patients With PPMS

(a and b) Percent CD4+CD20+ T cells (a) and CD8+CD20+ T cells (B) in the CSF of placebo and dimethyl fumarate (DMF)-treated patients with primary progressive multiple sclerosis (PPMS). (C and D) Absolute number of CD4+CD20+ T cells (C) and CD8+CD20+ T cells (D) in the CSF of placebo and DMF-treated patients with PPMS. The median value is shown for all groups analyzed.
the frequency of intrathecal T cells after DMF treatment \( (p = 0.0051; \text{eFigure 3E}) \), the frequency of intrathecal CD4+CD20+ and CD8+CD20+ T cells was significantly higher in the DMF treatment group \( (p < 0.0001 \text{ and } p = 0.0004) \), Figure 4, A and B. Calculating the absolute cell count of T cells in the CSF of DMF-treated patients confirmed a decrease in the overall T-cell population \( (p = 0.020) \), eFigure 3F. By contrast, the number of CD4+CD20+ T cells in CSF was unaffected by DMF, and the number of CD8+CD20+ T cells in patients treated with DMF was even increased compared with placebo-treated patients \( (p = 0.002) \), Figure 4, C and D.

**Frequency of Intrathecal CD8+CD20+ T Cells Is Associated With T2 Lesion Development in Patients With PPMS**

We next investigated a possible association between intrathecal CD8+CD20+ T cells at baseline and new or enlarging T2 MRI lesions after 48 weeks. Using a general linear model, the percentage of CD8+CD20+ T cells in CSF at baseline positively correlated with the number of new T2 lesions after 48 weeks \( (p = 0.001; \text{Figure 5A}) \) but not with the number of enlarging lesions (Figure 5B); the number of new T2 lesions was independent of the treatment applied \( (p = 0.432) \). No correlation with CD4+CD20+ T cells was found.

**Phenotypic Characteristics of CD8+CD20+ T Cells in Patients With PPMS**

To investigate the phenotype of intrathecal CD8+CD20+ T cells, freshly drawn CSF cell samples were stained with fluorochrome-conjugated antibodies specific for various chemokine receptors and analyzed by flow cytometry. This showed that 84% (mean; range 63%–94%) of CD8+CD20+ T cells were CXCR3+CCR6− indicative of a Tc1-like phenotype and 91% (mean; range 72%–100%) were CCR5+ (Figure 6A). This CCR5+ Tc1-phenotype was confirmed in a replication cohort (Figure 6B). The cytokine profile of CD8+CD20+ T cells in patients with PPMS was evaluated using cryopreserved PBMCs that was thawed and sorted into CD20+ and CD20− T cells by fluorescence-activated cell sorting and thereafter stimulated with PMA and ionomycin and their production of cytokines measured by flow cytometry. Sorting of CD20+ and CD20− T cells before stimulation was performed because a possible PMA/ionomycin-induced downregulation of surface-expressed CD20 would make identification of CD20− and CD20+ T cells difficult. The analysis showed that most of the CD8+CD20+ T cells produced IFNγ (86%), TNFα (95%), and GM-CSF (64%), and to a far greater degree than observed for CD8+CD20− T cells \( (p < 0.0001; p = 0.0006; p = 0.0004, \text{respectively}) \), Figure 4, C and D. We also analyzed IL-17, IL-4, and IL-10 production; however, these cytokines were mainly produced by CD4+ T cells.

**Discussion**

Despite accumulating evidence of systemic and intrathecal inflammation in patients with PPMS, deciphering the contribution of inflammatory processes to disease development and progression remains a considerable challenge. Clinical trials of disease-modifying therapies in PPMS have been disappointing, suggesting that the inflammatory-driven demyelination and neuroaxonal injury seen in PPMS may be induced/maintained by astrocytes and microglia as well as CNS-resident immune cells trapped in earlier disease stages rather than by infiltrating peripheral immune cells.

Recently, a clinical trial with the anti-CD20 antibody ocrelizumab showed some efficacy in slowing disability progression in PPMS. Ocrelizumab is a humanized monoclonal antibody targeting peripheral immune cells expressing CD20. This includes circulating B cells but also a small population of T cells expressing low levels of CD20 on their cellular surface. CD20+ T cells are proinflammatory cells highly

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**Figure 5 Frequency of Intrathecal CD8+CD20+ T Cells Is Associated With T2 Lesion Development in Patients With PPMS**

A. Frequency of intrathecal CD8+CD20+ T cells at baseline (W0) in patients with primary progressive multiple sclerosis (PPMS) and new lesions after 48 weeks. B. Frequency of intrathecal CD8+CD20+ T cells at baseline (W0) in patients with PPMS and enlarged lesions after 48 weeks. Placebo-treated patients are depicted by circles and dimethyl fumarate (DMF)-treated patients by hexagons. The median value is shown for all groups.
reactive to myelin antigens and likely implicated in various autoimmune and inflammatory diseases, including RRMS. With this study, we therefore investigated a possible role of CD20+ T cells in the pathogenesis of PPMS.

We first measured CD20+ T cells in the blood of untreated patients with PPMS and found that the frequency of CD4+CD20+ and CD8+CD20+ T cells was increased compared with healthy controls, despite an equal frequency of T cells, CD4+ and CD8+ T cells. This indicates a change in the dynamics of the peripheral CD20+ T-cell populations in patients with PPMS, similar to what was previously observed in patients with RRMS. Furthermore, we observed that both CD4+CD20+ and CD8+CD20+ T cells were strongly enriched in the CSF of patients with PPMS compared with blood, with CD8+CD20+ T cells comprising approximately 30% of the CD8+ T-cell population in CSF. Recent studies have shown that white matter lesions of patients with secondary progressive MS and PPMS are populated by CD8+CD20+ T cells and myelin-specific CD8+ T cells in MS patients are enriched in CD8+CD20+ T cells, substantiating a possible role of CD8+CD20+ T cells in nervous tissue damage in PPMS. We have previously shown in RRMS that CD20+ T cells are associated with demyelination as assessed by the CSF concentration of MBP but not with neuroaxonal injury as assessed by the CSF concentration of NFL. Similarly, in this study of PPMS, we found a positive correlation between intrathecal CD8+CD20+ T cells and the CSF concentration of MBP but not with the CSF concentration of NFL. We also found a positive correlation between intrathecal CD8+CD20+ T cells and white matter lesion volume and the number of new T2 lesions after 48 weeks of follow-up, and further a negative correlation with NAWM volume. By contrast, there was no relationship between CD8+CD20+ T cells and enlarging T2 lesions after 48 weeks. This is consistent with the notion that whereas classic active demyelinated lesions are associated with profound T-cell and B-cell infiltration, slowly expanding lesion may depend more on activated microglia and other innate immune mechanisms. In addition to a negative correlation with NAWM volume, we observed a negative correlation with thalamus volume for both CD4+ and CD8+CD20+ T cells, indicating an association with brain atrophy.

Overall, this shows that a higher frequency of intrathecal CD8+CD20+ T cells is associated with demyelination and T2 lesion load and development, whereas both CD4+ and CD8+CD20+ T cells are associated with NAWM and thalamic...
volume loss. This is consistent with the notion that whereas CD8⁺ T cells are prominent in the parenchyma in progressive MS, CD4⁺ T cells may be more important in the initiation of the immune response in MS.⁵

Finally, we analyzed the effect of treatment with DMF on CD20⁺ T cells in PPMS. In patients treated for 48 weeks with DMF, the frequency and absolute number of circulating T cells were decreased, particularly CD20⁺ T cells. In the CSF, the number of T cells was also decreased, but both intrathecal CD4⁺CD20⁺ and CD8⁺CD20⁺ T cells were either unaffected or increased in DMF-treated patients. As we found no significant decrease in the CSF concentrations of MBP in patients treated with DMF,¹⁰ we hypothesize this may reflect the lack of an effect of treatment on CD20⁺ T cells in CSF.

The decrease in total T-cell counts in CSF observed in DMF-treated patients may be a consequence of the reduced circulating T-cell pool and thereby reduced CNS recruitment.¹¹ The unaffected populations of CD20⁺ T cells in the CSF, on the other hand, may indicate that CD20⁺ T cells are part of the CNS-compartmentalized immune response observed in progressive MS. Indeed, tissue resident memory CD20⁺ T cells have been detected in the perivascular space from controls and were found to accumulate in MS lesions.¹⁴ It cannot be excluded, however, that a longer treatment duration could have led to a reduction in the intrathecal population of CD20⁺ T cells because of a continued diminished renewal of cells from the blood. Nevertheless, our observations suggest that the main CD8⁺CD20⁺ T-cell population involved in the pathogenesis of PPMS are CNS-resident cells rather than migrated peripheral cells.

In conclusion, we suggest that CNS-resident myelin-specific CD8⁺CD20⁺ T cells may serve as central effector cells in the demyelination process in PPMS, a hypothesis that awaits further investigation.

Acknowledgment
The authors acknowledge Lisbeth Stolpe at the Danish Multiple Sclerosis Center for her excellent technical assistance. The authors also thank Hanne Schmidt and Sascha Gude at the Reader Centre, DRCMR, for manual delineation of lesions and QA of MRI.

Study Funding
This work was supported by the Aase and Ejnar Danielsen Foundation, Læge Sofus Carl Emil Friis and Wife Olga Friis’ Grant, the Jascha Foundation, the Toyota Foundation, the A.P. Møller Foundation, the Foundation for Research in Neurology, and the Research Board at Copenhagen University Hospital, Rigshospitalet. Henrik Lundell has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant Agreement No. 804746). Hartwig R. Siebner holds a 5-year professorship in precision medicine at the Faculty of Health Sciences and Medicine, University of Copenhagen which is sponsored by the Lundbeck Foundation (Grant No. R186-2015-2138). Finn Sellebjerg holds a professorship at University of Copenhagen sponsored by the Danish Multiple Sclerosis Society.

Disclosure
F. Sellebjerg has served on scientific advisory boards for, served as a consultant for, received support for congress participation or received speaker honoraria from Alexion, Biogen, Merck, Novartis, Roche, and Sanofi Genzyme. His laboratory has received research support from Biogen, Merck, Novartis, Roche, and Sanofi Genzyme; J. Talbot reports non-financial support from Biogen, non-financial support from Sanofi Genzyme outside the submitted work; H. Hojsgaard Chow reports non-financial support from Merck, non-financial support from Teva, non-financial support from Biogen, non-financial support from Roche, outside the submitted work; H.R. Siebner has received honoraria as a speaker from Sanofi Genzyme, Denmark; Lundbeck AS, Denmark; and Novartis, Denmark, as a consultant from Sanofi Genzyme, Denmark; Lophora, Denmark; and Lundbeck AS, Denmark, and as an editor-in-chief (NeuroImage Clinical) and a senior editor (NeuroImage) from Elsevier Publishers, Amsterdam, The Netherlands. He has received royalties as a book editor from Springer Publishers, Stuttgart, Germany, and from Gyldendal Publishers, Copenhagen, Denmark; M.R. von Essen, R.H. Hansen, and H. Lundell report no disclosures relevant to the manuscript. Go to Neurology.org/NN for full disclosure.

Publication History
Received by Neurology: Neuroimmunology & Neuroinflammation November 16, 2022. Accepted in final form May 15, 2023. Submitted and externally peer reviewed. The handling editor was Deputy Editor Scott S. Zamvil, MD, PhD, FAAN.

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### References


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*Neurol Neuroimmunol Neuroinflamm* 2023;10;
DOI 10.1212/NXI.00000000000200140

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