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Original Article

Pharmacological modulation of inflammatory oligodendrocyte progenitor cells using three multiple sclerosis disease modifying therapies *in vitro*

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ABSTRACT

Preclinical studies of pro-remyelinating therapies for multiple sclerosis tend to neglect the effect of the disease-relevant inflammatory milieu. Interferon-gamma (IFN-γ) is known to suppress oligodendrocyte progenitor cell (OPC) differentiation and induce a recently described immune OPC (iOPC) phenotype characterized by expression of major histocompatibility complex (MHC) molecules. We tested the effects of cladribine (CDB), dimethylfumarate (DMF), and interferon-beta (IFN-β), existing anti-inflammatory therapies for MS, on the IFN-γ-induced iOPC formation and OPC differentiation block. In line with previous reports, we demonstrate that IFN-β and DMF inhibit OPC proliferation, while CDB had no effect. None of the drugs exhibited cytotoxic effects at the physiological concentrations tested *in vitro*. In a differentiation assay, none of the drugs were able to promote differentiation, under inflammatory or basal conditions. To study drug effects on iOPCs, we monitored MHC expression *in vitro* with live cell imaging using cells isolated from MHC reporter mice. IFN-β suppressed induction of MHC class II, and DMF led to suppression of both class I and II. CDB had no effect on MHC induction. We conclude that promoting proliferation and differentiation and suppressing iOPC induction under inflammatory conditions may require separate therapeutic strategies and must be balanced for maximal repair. Our *in vitro* MHC screening assay can be leveraged across cell types to test the effects of drug candidates and disease-related stimuli.

Introduction

Failed remyelination in multiple sclerosis (MS) is thought to promote degeneration by leaving denuded axons vulnerable to metabolic stress and damage, leading to progressive disability accumulation [1]. Thus, identifying pro-remyelinating therapies has become a major focus of MS research. However, most preclinical studies have tested putative remyelinating therapies on oligodendrocyte progenitor cells (OPCs) under basal conditions which likely do not mimic a disease-relevant environment [2]. A screen of drugs known to promote OPC differentiation showed that they lost pro-differentiating effects in the presence of fibrinogen, a blood component that infiltrates the parenchyma in MS [3], illustrating the importance of testing pro-myelinating compounds in the presence of disease-relevant stimuli.

Another relevant inhibitory factor is the pro-inflammatory cytokine interferon gamma (IFN-γ), which plays a critical role in MS pathophysiology. IFN-γ inhibits OPC differentiation [4] and more recently, our group and others have shown that IFNγ also induces OPCs to take on an immune profile (iOPC) characterized by expression of major histocompatibility complex (MHC) class I and II molecules [5,6]. iOPCs are present in MS patient tissue and can process and present antigen to both $CD4^+$ and $CD8^+$ T cells *in vitro* [5–8]. Given these inhibitory effects of IFN-γ on remyelination, it is possible that traditional anti-inflammatory drugs currently approved for MS have additional direct effects on OPCs. While IFN-γ triggers both iOPC induction and impairs differentiation, it is unclear whether these outcomes are related and if targeting one effect of IFN-γ exposure can ameliorate the other. We recently developed MHC reporter mice to detect iOPCs *in vivo* [9]. Here, we leveraged these mice to study the direct effects of three approved MS disease-modifying anti-inflammatory therapies (DMTs) on OPCs in the presence of IFN-γ *in vitro*.

Cladribine is a chemotherapeutic agent used to treat relapsing-remitting MS that inhibits DNA repair in proliferating lymphocytes, leading to apoptosis. Cladribine is activated intracellularly by

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deoxycytidine kinase (DCK) and broken down by 5′-nucleotidase (5′NT) [10]. While B and T cells express the highest levels of DCK and 5′NT in the body, conferring the drug's specificity, OPCs and other OL lineage cells also express these enzymes [11]. Cladribine can cross the blood-brain-barrier [12], thus raising the possibility that it could have deleterious effects on OPCs and remyelination. However, no studies to date have investigated the direct effects of cladribine on OPCs.

Dimethylfumarate (DMF) is an immunomodulatory drug approved for the treatment of MS and psoriasis. While limited, previously published studies have hinted at direct effects on OPCs. Under basal conditions, DMF promoted the differentiation of neural progenitor cells to the oligodendrocyte lineage [13]. In the Oli-neu cell line, DMF suppressed proliferation and promoted differentiation [14], in line with studies showing anti-proliferative effects of DMF in tumor cells and lymphocytes [15,16]. It should be noted that the clinical benefits of DMF have long been attributed to its metabolite monomethylfumarate (MMF), as DMF is rapidly converted to MMF *in vivo* [17]. However, DMF is detected in the CNS [18] and DMF rather than MMF is responsible for key anti-inflammatory effects [19–21]. For this reason, we used DMF rather than MMF for our experiments and hypothesize that DMF may have anti-inflammatory and pro-differentiation effects on OPCs in the presence of IFN-γ.

Lastly, interferon beta (IFN-β) was the first drug approved for MS. Previous studies showed that IFN-β had no direct effect on OPCs but did inhibit OPC differentiation when cells were cultured with astrocytes and microglia [22]. Additionally, IFN-β has been shown to have effects on MHC expression in other cell types with conflicting results. IFN-β leads to increased MHC class I expression in mice that received a sciatic nerve crush [23] and in human astrocytes *in vitro* [24]. However, in human fetal astrocytes and microglia, IFN-β reduced IFN-γ-induced MHC class II expression and antigen presentation to T cells [25]. We therefore hypothesized that IFN-β may have direct effects on iOPC induction and differentiation in an inflammatory context.

Methods

Isolation of OPCs

All animal procedures were performed in accordance with protocols approved by the Johns Hopkins Animal Care and Use Committee. On the day prior to the isolation, three 15 cm uncoated petri dishes were pre-incubated overnight at 4 °C, two with 6 μg/mL goat anti-rat IgG (Jackson 112-005-167) and one with 2.5 μg/mL BSL1 (Vector L1100), all in Tris-HCl pH 9.5. On the day of isolation, secondary antibody plates were washed with HBSS and incubated with 1 μg/mL rat anti-mouse CD11b (Thermo Scientific 14-0112-85) or 1 μg/mL rat anti-mouse PDGFRα (BD Pharmingen 558774) in 0.2% bovine serum albumin (BSA) at room temperature for at least 2 h while the BSL1 plate was equilibrated at room temperature. 4–5 neonatal forebrains from postnatal day 6–9 C57BL/6J mouse pups of both sexes were dissected and enzymatically dissociated by incubation at 37° in 2.11 mg/mL papain (Worthington LS003120) with 100 U/mL DNase I (Millipore Sigma 10104159001) in papain buffer (22.5 mM D(+)-glucose, 0.5 mM EDTA, 2.2 g/L $NaHCO_3$, 5.5 mM L-cysteine in EBSS, pH 7.4) for 3 rounds of 10 min followed by mechanical trituration. Cells were then resuspended in 0.2% BSA in HBSS and filtered through a 70-μm filter. Panning plates were washed with HBSS and cell suspension was added to the BSL1 plate (negative selection for endothelial cells and microglia) for 10 min. The unbound suspension was transferred to the CD11b plate (negative selection for microglia) for 20 min and then the PDGFRα plate (positive selection for oligodendrocyte progenitors) for 90 min. To harvest bound cells, the PDGFRα plate was washed with HBSS and 0.0625% trypsin in HBSS was added for 10 min at 37 °C. Fetal bovine serum was used to inactivate trypsin and cells were harvested by pipetting with a 5 mL serological pipet. Cells were spun for 10 min at 300 g and plated in plates coated with 50 μg poly-D-lysine (Sigma Aldrich P6407) at densities of 30,000 cells/well for 96-well plates, 100,000 cells/well for 12-well plates, or 7000 cells/glass coverslip. Cells were maintained in OPC basal media containing: Dulbecco's Modified Eagle Medium high glucose (Thermo Fisher 31053028) containing 100U/100 μg/ml penicillin/streptomycin (Fisher Scientific 15-140-122), 5 μg/ml N-acetyl-L-cysteine, 1xSATO (1 μg/ml human apotransferrin, 1 μg/ml BSA, 0.16 μg/ml putrescine, 0.6 mg/ml progesterone, 0.4 ng/ml sodium selenite), 1xB27 (ThermoFisher 17504044), 1xTrace Elements B (Cellgro 99-175-C), 5 μg/ml insulin, 10 ng/ml d-biotin, 4.2 μg/ml forskolin, 1 mM sodium pyruvate (Millipore Sigma S8638), 4 mM L-glutamine (Thermo Fisher 25-030-081). The media was supplemented with growth factors: 40 ng/ml PDGFAA (PeproTech 100-13A), 1 ng/ml NT-3 (PeproTech 450-03), 10 ng/ml CNTF (PeproTech 450-13).

After 24–48 h when cells reached appropriate confluence, half of the media was replaced with fresh growth factors and drugs: 20 ng/ml PDGFAA, 1 ng/ml NT-3, 10 ng/ml CNTF, 50 ng/mL IFN-γ (Peprotech 315-05), 2.5–1000 IU/mL IFN-β (R&D Systems 12400-1), 5–1000 nM cladribine (Cayman Chemicals 12085), 0.125–50 μM DMF (Cayman Chemicals 14714), and/or 12.5 nM Cytotox Green (Sartorius 4632) as indicated on the figures for Incucyte assays.

Incucyte assays

B2M-TdT and CD74-TdT cells were kept in a Sartorius Incucyte live cell imager and imaged every 4 h. At the conclusion of the experiments, Incucyte software was used to measure percent confluency, number of Cytotox + cells, and total integrated intensity of tdTomato.

Differentiation assays

OPCs were plated on coverslips coated with poly-D-lysine (Sigma Aldrich P6407) at a density of 7000 cells/well. After 24–48 h, when cells reached appropriate confluence, three-fourths of the media was replaced with differentiation media and the drugs (100 nM CDB, 10 μM DMF and 1000 IU/mL IFN-β) with or without IFN-γ. Differentiation media was prepared from OPC basal media supplemented with 10 nM T3 (Sigma Aldrich T2877) and 10 ng/ml CNTF (PeproTech 450-13). After 72 h, the cells were washed with PBS and fixed for immunocytochemistry or harvested for RNA isolations.

Immunocytochemistry

Cells were fixed in ice-cold 4% paraformaldehyde (Sigma Aldrich P6148) for 10 min, washed 3 times for 5 min in PBS, permeabilized in PBS + 0.5% Triton (PBST) for 5 min, and blocked for 1 h in 5% normal donkey serum in PBST at room temperature. Cells were then incubated overnight at 4° in primary antibodies in PBST with serum (rabbit Olig2 (Millipore AB9610), mouse CC1 (Calbiochem OP80), rat MBP (Millipore MAB386)). Cells were washed with PBST for 5 min and incubated in secondary antibodies (donkey anti-rabbit AlexaFluor 488 (Jackson ImmunoResearch 711-545-152), donkey anti-mouse AlexaFluor 555 (Invitrogen A-31570), donkey anti-rat AlexaFluor 647 (Jackson ImmunoResearch 712-605-153)) in 5% normal donkey serum in PBST for 1 h at room temperature. Cells were washed for 5 min in PBS, incubated in PBS + 0.5 μg/mL DAPI (Invitrogen 21306) for 10 min, washed 3 more times in PBS, and mounted with Prolong Glass Antifade Mountant (Thermo Scientific P36984).

Imaging and quantification

The coverslips were imaged on Zeiss Axio Observer Z1 with 20× objective with multi-focal Z point supported tiling. The number of Olig2+ and CC1+ cells were counted using the automated counter on ImageJ: in brief, scans of the entire coverslip were subset into 8 × 8 subset images which were then converted to grayscale. A threshold was set to create a binary image (keeping marker thresholds the same within

Table 1
qPCR primer sequences.

Target	Forward	Reverse
β-actin	ACCTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG
Hprt	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT
Cd74	AAGTACGGCAACATGAACC	ATCTTCCAGTTCACGCCATC
H2-dma	TCCTAGAGAATGCCCTGTGT	TGTGCTGTTCCAAGATCTCC
B2m	CAGCTCTCAGCACTGGATCA	CACCGTGCACCATCTTACAT
Tap2	TCGTGTAATTGACATCCTGGG	CTGGACATGGTGAAGAGGAAG
H2-k1	AGACACAGAAAGCCAAGGG	CACTTCACAGCCAGAGATCAC
Psmb8	CAGTCCTGAAGAGGCCTACG	CACTTTCACCCAACCGTCTT
Mbp	TACCTGGCCACAGCAAGTAC	GTCACAATGTTCTTGAAG

each experiment). Then cells were automatically counted based on size and circularity. Integrated density was also measured using ImageJ. Where indicated, the integrated density of MBP was normalized for differences in proliferation (normalized integrated density = integrated density x (Olig2 $count_{max}$/Olig2 $count_{current\ coverslip}$)).

Quantitative polymerase chain reaction

RNA was extracted using the RNeasy Micro Plus kit (Qiagen). 500 ng of RNA were used to make cDNA using iSCRIPT cDNA Synthesis Kit (Bio-Rad). qPCR was performed using iQ SYBR Green reagent (Bio-Rad) on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Delta-delta Ct was performed by normalizing to the average of *β-actin* and *Hprt*. Primer sequences are listed in Table 1.

Statistics

Statistics were performed using Graphpad Prism software. For differentiation assays and qPCR data, one-way ANOVA and Dunnett's post hoc test were used (with the exception of the qPCR data in Supplemental Fig C-D, where we performed t-tests). For Incucyte assays, multiple t-tests were performed to compare each condition to IFNγ-treated and the Holm-Šídák method was used to adjust for multiple comparisons. A p-value less than 0.05 was considered significant.

Results

Concentrations of CDB, DMF, and IFNβ used in these experiments were based on a combination of previously published studies on relevant cell types *in vitro* and available data on drug concentrations in the cerebral spinal fluid (CSF) of patients, as shown in Table 2. In the live imaging screening assays the lowest dose tested represented the concentrations observed in the CSF of patients. The CSF concentrations indicated for CDB and IFN-β were obtained from healthy controls, but are likely to be higher in patients with blood-brain-barrier compromise [26]. Two other higher doses tested were based on *in vitro* studies. When significant effects were observed in the screening assays at these moderate to high doses,

Table 2
Justification for selected drug concentrations.

Drug	Peak human CSF concentration	*In vitro* concentration	Concentration range selected
IFNβ	6 IU/mL in healthy controls [42]	10–1000 IU/mL (OPCs) [22]	2.5–1000 IU/mL
Cladribine	19–25 nM in healthy controls [43]	20 nM (Neuroblastoma cells) [44] 10–10000 μM (microglia) [43]	5–1000 nM
DMF/MMF	~0.45 μM in PMS patients [45]	25 μM (Oli-neu cell line) [14] 10–100 μM (endothelial cells) [46]	0.125-50 uM

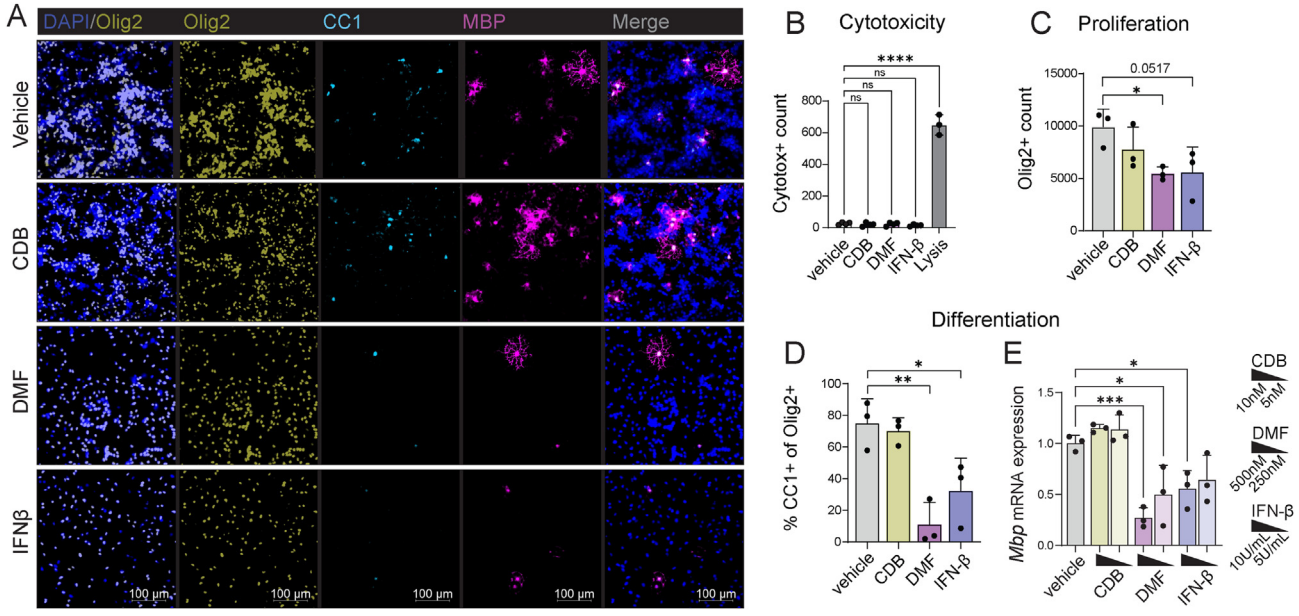
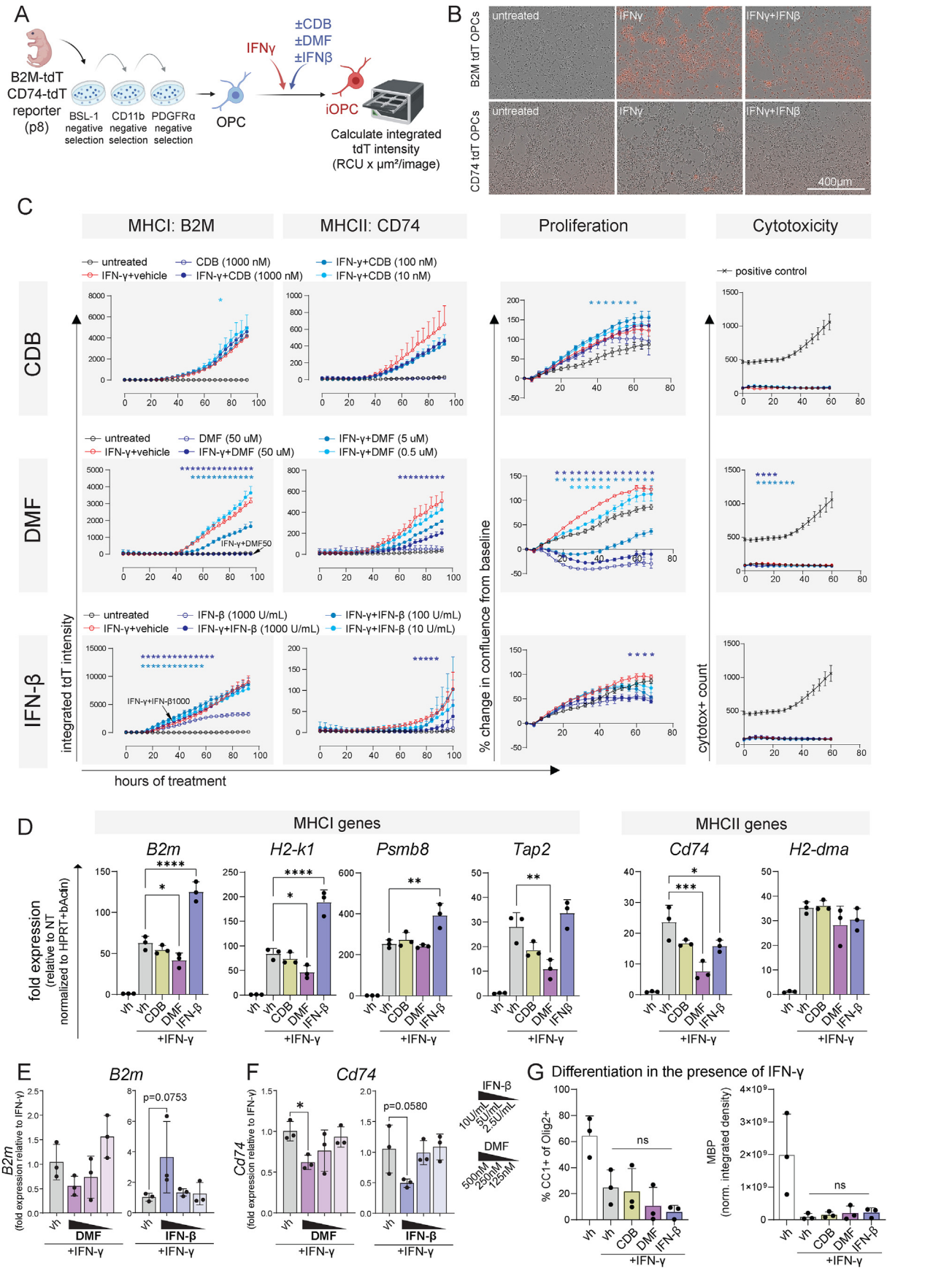


Fig. 1. IFN-β and dimethyl fumarate (DMF) reduce OPC differentiation and proliferation. (A) Representative images of OPCs differentiated for 72 h in the presence of 100 nM cladribine (CDB), 5 μM DMF or 100 IU/mL IFN-β and stained for an oligodendrocyte lineage marker (Olig2), a marker for cell maturity (CC1) and myelin (MBP). (B) Quantification of cell death measured by Cytotox + cells normalized to starting confluency at the end of the experiment (n = 4 technical replicates; error bars: SD; ****p < 0.0001). Quantification of (C) the number of oligodendrocytes (Olig2+ cells; *p < 0.05) and (D) their differentiation state assessed by the % mature oligodendrocytes (n = 3 independent biological replicates; error bars: SD; % CC1+ of Olig2+; *p < 0.05; **p < 0.005). (E) Fold induction of *Mbp* mRNA in OPCs differentiated in the presence of physiological drug concentrations determined by qPCR (n = 3 technical replicates; error bars: SD; *p < 0.05; **p < 0.001).



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experiments were repeated with two additional lower CSF-relevant doses to confirm the results at physiological concentrations.

Effect of DMTs on OPC differentiation

To determine whether CDB, DMF, and IFN-β influence OPC differentiation, we treated OPCs under pro-differentiating conditions with the intermediate doses of the drugs (100 nM CDB, 5 μM DMF, 100 IU/mL IFN-β) for 72 h. We then fixed and stained cells and quantified cell density by Olig2+ cell counts (Fig. 1C) and maturity by CC1 and MBP staining (Fig. 1D and Supplemental Fig 1B) and assessed MBP transcript levels by qPCR (Fig. 1E).

None of the drugs tested exhibited an effect on cell viability (Fig. 1B). DMF led to a significantly decreased density of Olig2+ cells compared to vehicle-treated cells, in line with previous reports, while IFN-β trended toward reducing Olig2+ density. CDB had no significant effect on cell density (Fig. 1C). At lower doses, DMF again reduced OPC confluency assessed with live cell imaging, and IFN-β similarly trended toward reduced confluency (Supplemental Fig 1A).

Despite published literature showing that DMF promotes OPC differentiation, we observed that it caused a significant decrease in CC1+ and MBP + cells compared to controls, as did IFN-β (Fig. 1D, Supplemental Fig 1). CDB had no significant effect on differentiation (Fig. 1D). At lower doses, DMF and IFN-β also significantly reduced *Mbp* transcript levels, while CDB had no effect (Fig. 1E). Thus, none of the drugs tested promoted OPC differentiation under basal conditions.

Effect of DMTs on iOPC induction

We next sought to determine the effects of these DMTs on OPCs under inflammatory conditions. We leveraged our previously described MHC reporter mice, in which cells expressing β-2-microglobulin (B2M) or cluster of differentiation 74 (CD74), class II invariant chain, also express tdTomato (tdT) fluorescent protein as an indicator of MHC class I and II expression, respectively [9]. We isolated OPCs from reporter mice and treated them with IFN-γ and three doses of CDB, DMF, or IFN-β (Fig. 2A). tdT expression was monitored in real time over 100 h using the Incucyte (Fig. 2B).

In this experimental set up, we replicated the findings from Fig. 1 under differentiating conditions that the drugs do not affect cell viability (Fig. 2C). Real-time monitoring of changes in cell confluence showed effects of all 3 drugs on cell proliferation. CDB increased cell confluence at intermediate doses, DMF led to decreased confluence, and IFN-β also led to slight reductions in confluence (Fig. 2C).

CDB did not have any effects on B2M-tdT or CD74-tdT intensity at any dose, nor did it affect MHC-related transcripts (Fig. 2C).

DMF was tested at 50, 5, and 0.5 μM. At 50 μM, DMF led to a significant reduction in both B2M-tdT and CD74-tdT signal (Fig. 2C). Because the tdT intensity data was normalized to confluency, we conclude that the effect of the drug on the MHC intensity was not solely driven by its effect on cell number. This was confirmed by qPCR where 5 μM DMF also significantly reduced levels of *B2m*, *H2-k1*, and *Tap2*, and *Cd74* (Fig. 2D). We also performed qPCR for lower CSF-relevant DMF concentrations (0.125–0.5 μM). 0.5 μM DMF led to a significant decrease in *Cd74* transcript levels (Fig. 2F and G). Thus, we conclude that DMF has concentration-dependent effects on MHC induction in OPCs.

Interestingly, IFN-β had opposing effects on B2M-tdT and CD74-tdT intensity. At early time points, both 1000 and 100 U/mL significantly increased B2M-tdT intensity relative to cell treated with IFN-γ alone. Additionally, 1000 U/mL of IFN-β alone induced B2M-tdT expression. In contrast, the high dose of IFNβ suppressed CD74-tdT expression. This pattern is also observed at the transcript level. 24 h after treatment, cells treated with both IFN-γ and IFN-β had higher levels of MHC class I-related transcripts (*B2m, H2-k1, Psmb8*) and lower levels of an MHC class II-related transcript (*Cd74*) compared to IFN-γ alone (Fig. 2C). At concentrations at or below that seen in patient CSF (2.5–10 U/mL), the same trends hold but the effects are no longer significant (Fig. 2F and G).

Effect of DMTs on OPC differentiation under inflammatory conditions

Finally, we tested our hypothesis that the effects of IFN-γ on iOPC induction and cell differentiation are related by repeating the differentiation assay experiments in the presence of IFN-γ. If these two outcomes are linked, we would expect that IFN-β and DMF would be able to overcome suppressed differentiation, while CDB cannot, as only these DMTs showed an effect on MHC induction. However, none were able to overcome the suppressive effect of IFN-γ on OPC differentiation, as measured by CC1 and MBP staining (Fig. 2G).

Discussion

The effects of each drug on OPC proliferation, differentiation, and MHC induction under basal and inflammatory conditions are summarized in Fig. 3. We showed that none of the drugs studied cause OPC toxicity at the doses tested, and both DMF and IFN-β suppressed OPC proliferation. While DMF and IFN-β affected MHC induction, they were not able to overcome the suppressive effects of IFNγ on OPC differentiation. Our study addresses two weaknesses of previous studies of MS DMTs: failure to study direct effects of drugs on OPCs and a lack of consideration for the effects of the inflammatory milieu on drug efficacy.

We hypothesized that drugs that were able to suppress IFN-γ's induction of MHC in OPCs would also be able to overcome the inhibitory effect of the cytokine on OPC differentiation. However, this was not supported by our data. None of the drugs tested promoted differentiation under basal conditions nor were they able to overcome the differentiation block under inflammatory conditions, even though IFN-β and DMF exhibited effects on MHC expression. This indicates that the effects of IFN-γ on OPC differentiation and immune gene induction are distinct and may require different therapeutic strategies. Under basal conditions, we saw a decrease in OPC differentiation in cells treated with DMF or IFN-β. In the differentiation assay with IFN-γ, there was no significant differences in differentiation in drug treated compared to vehicle treated cells. This reinforces

Fig. 2. MHC class I (CD74) and II (B2M) reporter OPC screening assays show that IFN-β and dimethyl fumarate (DMF) affect IFN-γ-induced iOPC formation. (A) Schematic of the high throughput *in vitro* MHC reporter OPC screening assay made with BioRender (B) Representative Incucyte images of B2M- and CD74-tdT signal in unstimulated, IFN-γ-stimulated, and drug-treated cells. (C) Dynamic measurement of tdT intensity, cytotoxicity dye (cytotox) cell count, and confluency in CD74-tDT- or B2M-tdT-reporter OPCs stimulated with IFN-γ and the drugs starting from the first image taken after the addition of drugs and reagents (n = 4 technical replicates are representative of 3 experiments for CD74-TdT cells and B2M-Tdt cells; error bars: SD; tdT and cytotox measurements are normalized to confluency). Asterisks indicate significance ($p < 0.05$) at the corresponding time point compared to the IFN-γ condition and the asterisks' colors represent the concentration at which the significant effects are observed. Significantly changed conditions overlapping with other conditions are additionally labeled with arrows. For simplicity, significance of drug only and untreated conditions is not shown on the graph. (D) Fold induction of MHCI and MHCII pathway transcripts in IFN-γ only or IFN-γ plus drugs-treated OPCs determined by qPCR (n = 3 technical replicate representative of 3 experiments; error bars: SD; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0005$; ****$p < 0.0001$). Fold induction of (E) *B2m* and (F) *Cd74* mRNA as assessed by qPCR in IFN-γ only or IFN-γ plus drugs-treated OPCs at physiological drug concentrations as indicated (n = 3 technical replicate representative of 2 experiments; error bars: SD; *$p < 0.05$). (G) Quantification of the degree of differentiation assessed by the % of mature oligodendrocytes and by myelin production in oligodendrocytes differentiated in the presence of IFN-γ and the drugs for 72 h (n = 3 independent biological replicates; error bars: SD).

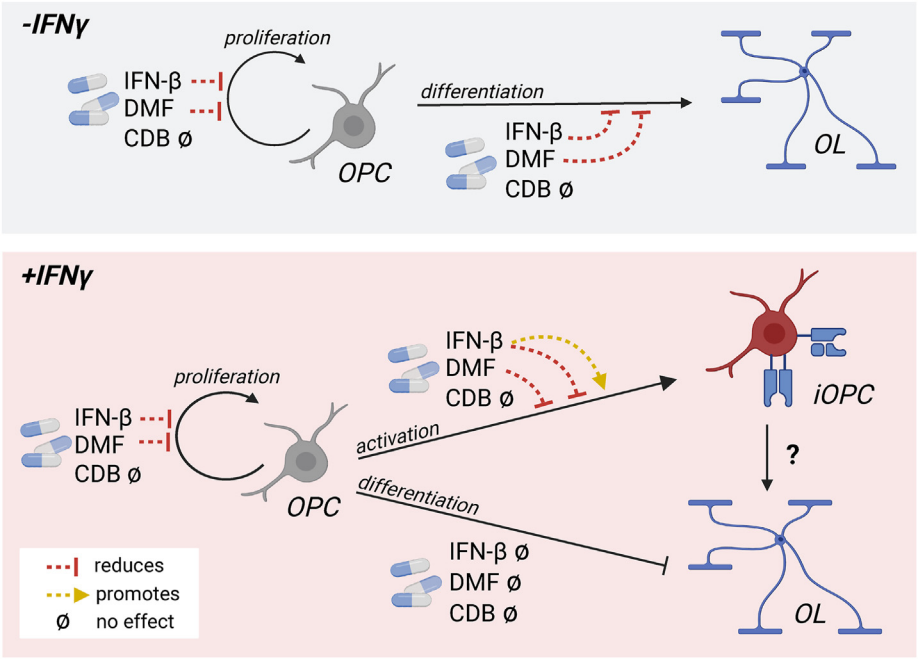

Fig. 3. Effects of IFN-β, dimethyl fumarate, and cladribine on OPC proliferation, differentiation, and MHC induction in basal and inflamed contexts. Schematic rendering of the observed effects of each drug on OPCs without (top) and with (bottom) IFN-γ. Under basal conditions, IFN-β and DMF suppress both OPC proliferation (DMF more strongly than IFN-β) and OPC differentiation, while CDB has no effect. In the presence of IFN-γ, IFN-β and DMF still suppress OPC proliferation. IFN-β has opposing effects on MHC induction, promoting MHC class I while reducing MHC class II, and DMF suppresses both class I and II. None of the drugs is able to overcome the inhibitory effect of IFN-γ on differentiation. It remains unclear if OPCs that have taken on an immune phenotype can later differentiate into mature oligodendrocytes. Figure made with BioRender.

the importance of testing putative pro-remyelinating drugs in the presence of inflammatory stimuli, as their effects may be blunted under inflammatory conditions, which are likely more physiologically relevant.

By testing the direct effects of DMF, IFN-β, and CDB on OPCs, we showed none of these drugs affect cell viability, though the drugs had an effect on OPC proliferation. This is of particular importance for CDB, which induces cell death when phosphorylated intracellularly [10]. Our study suggests that DCK and 5′NT levels in OPCs might not allow for cladribine activation, explaining why no off-target deleterious effects on myelination have been reported for CDB. Instead, CDB had a mild proliferative effect in the live cell imaging studies, though this effect was not detectable in our differentiation assays – possibly due to a lower sensitivity of the immunocytochemistry approach. DMF, on the other hand, inhibits OPC proliferation, as has been reported in other cell types [14–16]. However, we were unable to replicate reports that DMF promotes OPC differentiation, though this could be explained by the use of primary OPCs in this experiment versus the Oli-neu cell line in published data [14]. Similarly, to DMF, IFN-β also reduced OPC proliferation and differentiation. This too is in line with known anti-proliferative effects of IFN-β on other cell types [27–31]. Our additional differentiation assays in the presence of IFN-γ are likely more physiologically relevant for MS.

Testing the effects of DMTs in the presence of IFN-γ allowed us to identify effects of these anti-inflammatory drugs on the iOPC phenotype, which has not been an aspect of OPC biology previously considered in the design or testing of MS therapeutics. IFN-β and DMF both showed an effect on MHC class I induction. Interestingly, we found that IFN-β also leads to a transient increase in IFN-γ-induced MHC class I. It has been reported that IFN-β can cause initial worsening of RRMS symptoms at the onset of treatment, although overall it reduces exacerbations [32]. Symptom worsening could be related to the transient increase in MHC class I we observed in OPCs we observed with IFN-β. In contrast to IFN-β, DMF reduced MHC class I induction. DMF/MMF effects on MHC class II, discussed in more detail below, have been more widely reported. Additionally, MMF treatment was shown to reduce expression of MHC class I molecules in human dendritic cells, associated with a more immature cell phenotype [33].

However, more research into the function and fate of MHC class I-expressing OPCs *in vivo* is needed to know whether suppression would be therapeutic or detrimental. On the one hand, MHC class I expression could lead to CD8-mediated cell death and thus reducing expression, as with DMF, would be desirable [5]. On the other, MHC class I can protect cells from NK-mediated death [34], and therefore transiently increasing its expression, as with IFN-β, would be protective. It is also unclear if iOPCs represent a terminal cell fate, in which case clearance of iOPCs by immune cells might be necessary in order to promote remyelination.

In contrast to MHC class I, both IFN-β and DMF suppressed MHC class II induction. In the case of DMF, high doses were required to see this effect on a protein level while effects on MHC class II transcripts were already detected at lower concentration, suggesting an overall weaker effect of DMF on MHC class II induction compared to IFN-β at CNS relevant concentrations. Our findings on IFN-β align with previous reports in other cells types. IFN-β suppresses IFN-γ-induced MHC class II in numerous cells including both human and murine glia [27,35,36]. DMF's suppressive effects on IFN-γ-induced MHC class II have primarily been studied in dendritic cells [37]. Functional studies on the role of MHC class II in OPCs have been limited, but *in vitro*, MHC class II-expressing OPCs can activate memory $CD4^+$ T cells [6]. In our previous work, we demonstrated that MHC class II-expressing oligodendroglia are specific to the CNS of mouse models of MS, while MHC class I-expressing cells are observed in the naïve CNS [9]. This suggests that MHC class I-expressing OPCs may have a homeostatic role, but MHC class II-expressing cells are specific to disease. Therefore, suppression of MHC class II induction in iOPCs by IFN-β and DMF may contribute to its therapeutic effects in MS.

We chose an experimental paradigm in which cells were simultaneously treated with IFN-γ and drug. However, in patients, cells might have already been exposed to inflammatory cues before the initiation of treatment or might be exposed to the drug before inflammatory activation. In addition, the *in vitro* environment is lacking pro-differentiation and anti-inflammatory cues likely to be present in the CNS that may influence OPC differentiation and MHC induction [38], such as BDNF, N-CAM, and osteopontin. Therefore, the physiological ability of these DMTs to affect proliferation, differentiation, and iOPC induction may diverge from the *in vitro* effects seen here. Finally, our results do not provide insight into the effects of these DMTs on mature oligodendrocytes. In contrast to its effect on OPCs, IFN-γ exerts protective effects on mature oligodendrocytes via activation of the integrated stress response [39,40], and thus, the effects of these drugs on myelinating cells under inflammatory conditions might vary from those observed here in OPCs. These limitations may explain, for example, why we detected negative effects of IFN-β on OPCs here, but imaging studies have shown that

patients taking IFN-β reduces the formation of persistent black holes, areas of chronic demyelination and axon damage, on MRI [41]. Therefore, while our findings provide insight into the direct effects of these DMTs on OPCs under inflammatory conditions, these must be balanced with the known beneficial effects they are known to exert on MS pathophysiology.

In sum, our data illustrate the importance of considering the immune roles of oligodendroglia and the inflammatory environment they are exposed to in MS when developing pro-myelinating drugs. In addition, our findings highlight the challenge for pro-remyelinating therapies to balance OPC proliferation, differentiation, and suppression of inflammation. More research is needed to determine which factor is the most important in promoting repair and should therefore be prioritized. Likely, a combination of approaches will be needed to ensure maximal remyelination. More broadly, the live imaging assay established here can be used to screen novel compounds or disease-relevant stimuli for their ability to suppress or promote MHC induction in OPCs or any other cell type of interest using MHC reporter mice.

Authors' Contributions

PAC supervised the work and contributed to its conception. LJ, RBC, VM, and DG conducted experiments and contributed to data acquisition. LJ and RBC carried out the analyses. PAC, LJ, and RBC contributed to the interpretation of the data. RBC and LJ significantly contributed to preparing the manuscript and figures. All authors contributed to editing the final manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Riley Catenacci reports financial support was provided by National Science Foundation. Larissa Jank reports financial support was provided by Multiple Sclerosis Society. Peter Calabresi reports financial support was provided by National Institute of Neurological Disorders and Stroke. Peter Calabresi reports a relationship with Myelin Repair Foundation that includes: funding grants. Peter Calabresi reports a relationship with US Department of Defense that includes: funding grants. Peter Calabresi reports a relationship with National Multiple Sclerosis Society that includes: funding grants. Peter Calabresi reports a relationship with Genentech that includes: funding grants. Peter Calabresi reports a relationship with Idorsia Pharmaceuticals Ltd that includes: consulting or advisory. Peter Calabresi reports a relationship with Eli Lilly and Company that includes: consulting or advisory. Peter Calabresi reports a relationship with Project Efflux that includes: board membership. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurot.2024.e00379.

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