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RESEARCH ARTICLE

Cerebrospinal fluid of progressive multiple sclerosis patients reduces differentiation and immune functions of oligodendrocyte progenitor cells

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Abstract

Oligodendrocyte progenitor cells (OPCs) are responsible for remyelination in the central nervous system (CNS) in health and disease. For patients with multiple sclerosis (MS), remyelination is not always successful, and the mechanisms differentiating successful from failed remyelination are not well-known. Growing evidence suggests an immune role for OPCs, in addition to their regenerative role; however, it is not clear if this helps or hinders the regenerative process. We studied the effect of cerebrospinal fluid (CSF) from relapsing MS (rMS) and progressive MS (pMS) patients on primary OPC differentiation and immune gene expression and function. We observed that CSF from either rMS or pMS patients has a differential effect on the ability of mice OPCs to differentiate into mature oligodendrocytes and to express immune functions. CSF of pMS patients impaired differentiation into mature oligodendrocytes. In addition, it led to decreased major histocompatibility complex class (MHC)-II expression, tumor necrosis factor (TNF)-α secretion, nuclear factor kappa-B (NFκB) activation, and less activation and proliferation of T cells. Our findings suggest that OPCs are not only responsible for remyelination, but they may also play an active role as innate immune cells in the CNS.

KEYWORDS
CSF, immunity, multiple sclerosis, neuroimmunology, oligodendrocyte, OPC, remyelination

1 | INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system (CNS), characterized by neuroinflammation, extensive demyelination, gliosis, and axonal damage. MS is a notably heterogeneous and can be broadly categorized into two subtypes: relapsing–remitting MS (RRMS; rMS) and progressive MS (pMS), depending on the clinical course (Goldenberg, 2012; Lublin et al., 2014; Reich et al., 2018).

Although rMS and pMS have been highly investigated and characterized, the mechanisms responsible for the pathogenesis of each course of the disease remain elusive. One of the most described

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differences between rMS and pMS is the involvement of the peripheral versus the central immune system. While rMS is characterized by infiltration of peripheral immune cells across the blood–brain barrier (BBB) (Dendrou et al., 2015; Lassmann et al., 2012; Torkildsen et al., 2016), less CNS infiltration is seen in pMS, in which the BBB is significantly less permeable, and the immune pathogenesis is compartmentalized within the CNS (Lassmann et al., 2012; Meinl et al., 2008; Torkildsen et al., 2016). The mechanisms responsible for these varying outcomes are unclear, and whether there are innate differences in the ability to repair are not fully understood.

One aspect of MS that has recently come into focus for new therapeutics is the aim to repair demyelination and prevent axonal injury (Kornek et al., 2000; Plemel et al., 2017). Differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes is crucial for facilitating the regeneration of myelin following injury or disease (Chang et al., 2000; Hughes et al., 2013). While remyelination successfully occurs in rMS, remyelination failure is considered one of the key features of pMS (Bodini et al., 2016; Bramow et al., 2010; Miron et al., 2011; Plemel et al., 2017).

For many years, oligodendrocytes were known only for their myelinating properties, and were considered as a passive target of the immune system in MS. However, accumulating evidences suggest that OPCs may have immunological properties as well: they survey their microenvironment (Bergles & Richardson, 2016; Hughes et al., 2013), express cytokine receptors (Arnett et al., 2001; Chew et al., 2005; Wang et al., 2017), migrate to injury site and respond to inflammatory cues (Akay et al., 2021; Dombrowski et al., 2017; Kang et al., 2013; Kirby & Castelo-Branco, 2020; Meijer et al., 2020; Wang et al., 2017). These data suggest that OPCs may act as active players in the inflammatory pathogenesis of MS. Whether these immune properties perpetuate an inflammatory response leading to direct damage in MS, or contribute to a regenerative process is still unclear.

Pathological studies have suggested that soluble mediators facilitate the development of cortical and subcortical MS lesions (Baraczka et al., 2004; Krumbholz et al., 2006; Magliozzi et al., 2018; Poirion et al., 2021). The cerebrospinal fluid (CSF) is in contact with brain parenchyma and has the ability to impact the cellular physiology of OPCs (Haines et al., 2015). Moreover, the inflammatory mediators and myelin degradation products within the CSF are known to influence MS inflammatory cortical activity, activation of CNS innate immune cells, and OPC remyelination ability (Magliozzi et al., 2020; Petzold et al., 2016; Sharief & Hentges, 1991).

Here, we examined in vitro OPCs' differentiation and immunological properties upon exposure to CSF from MS patients at different disease stages. We hypothesized that CSF from rMS patients would have a different effect on OPC differentiation and immunomodulatory properties as compared with exposure to CSF from pMS patients. The ability to identify OPCs' functions in different clinical courses is of interest, as this may lead to the development of novel therapies in MS patients.

2 | MATERIALS AND METHODS

2.1 | Approval

The Hadassah Medical Organization Ethics Committee approved this study. All subjects provided written informed consent (0298-17-HMO). The research reported in this study complied with all relevant ethical regulations for animal testing and research and was approved by the Hebrew University Institutional Animal Care and Use Committee (MD-20-16227-1).

2.2 | Subjects and CSF samples

We included MS patients who had been diagnosed in 2000–2011 according to McDonald criteria (McDonald et al., 2001). All patients were followed at the Neurology clinic in the Neurology Department of Hadassah Medical Center, Jerusalem, Israel. As part of the diagnostic procedure, CSF samples were collected by nontraumatic lumbar puncture (LP). After LP, CSF was centrifuged (1300 rpm, 10 min) to remove cellular elements and stored in small aliquot volumes at -80°C. CSF collection and storage are in line with the Consensus Guidelines for CSF and Blood Biobanking (Teunissen et al., 2009). The inclusion criteria were confirmed diagnosis of MS with at least 10 years of follow-up post-diagnosis; CSF sample obtained at diagnosis; no immunoactive drugs were administrated before the LP; and recorded expanded disability status scale (EDSS) at diagnosis (baseline EDSS) and at the beginning of this study (final EDSS). Patients who did not meet the inclusion criteria were excluded. The healthy control (HC) cohort included patients diagnosed with a benign headache at a similar age, in whom CSF was obtained and stored in similar conditions. HCs had no white blood cells (WBCs) in their CSF and had a negative CSF culture.

The participants' characteristics were obtained from medical files from the Neurology clinic (Tables 1 and S1). CSF samples were obtained 14.01 ± 4.33 years prior to this study, enabling classification of the patients into two subgroups according to disease clinical course: rMS ($n = 17$; 12 females; 5 males; age at diagnosis 33.6 ± 7.2 years; baseline EDSS 1.29 ± 0.91; 10-year follow-up EDSS 1.85 ± 1.15) and pMS ($n = 19$; 12 females; 7 males; age at diagnosis 37.8 ± 9.01 years; baseline EDSS 3.57 ± 1.27; 10-year follow-up EDSS 6.6 ± 1.1). All MS CSFs were obtained from patients during the active phase of the disease as part of the initial diagnostic procedure.

2.3 | Primary OPC cultures

Primary OPC cultures were isolated from naïve P0 to P1 neonatal C57/BL6 mice cortices as previously described by Chen et al. (2007), with minor modifications (Barateiro & Fernandes, 2014). Briefly, a mixed glial culture isolated from neonatal mice was grown for 8 days in Dulbecco's modified Eagle's medium (DMEM) low glucose (Biological Industries, Israel) supplemented with 5% fetal bovine serum (Biological Industries, Israel), 1 mM sodium pyruvate (Biological

TABLE 1 Clinical feature of patients

Clinical feature	Healthy controls mean ± *SD*	Relapsing MS mean ± *SD*	Progressive MS mean ± *SD*	*p*-value[a]
No. of patients	18	17	19	NA
Female/male	13 / 5	12 / 5	12 / 7	.8268
Age at diagnosis (years)	35.9 ± 10.0	33.6 ± 7.2	37.8 ± 9.01	.3589
Disease duration (years)	14.8 ± 4.4	13.51 ± 3.83	13.2 ± 4.7	.6763
EDSS at diagnosis	NA	1.29 ± 0.91	3.57 ± 1.27	<.0001
EDSS at the beginning of this study	NA	1.85 ± 1.15	6.61 ± 1.1	<.0001
Positive OCB	NA	5	12	.0441

Abbreviations: CSF, cerebrospinal fluid; EDSS, expanded disability status scale; MRI, magnetic resonance imaging; MS, multiple sclerosis; OCB, oligoclonal bands.

[a]All features estimated using ANOVA, except for MS specific features (EDSS at diagnosis, EDSS at the beginning of this study, and Positive OCB) which were estimated only for progressive MS patients versus relapsing MS patients using independent Student's *t* test.

Industries, Israel), 1 mM L-glutamine (Sigma-Aldrich, Israel) and 0.6% Gentamycin Sulfate (Biological Industries, Israel). Culture medium was replaced every 2 days. After 8 days, microglia were detached by 30 min shaking at 140 rpm using an orbital shaker. After medium was removal, a new fresh culture medium was added, and OPCs at the top of the astrocyte monolayer were detached by shaking for 18 h at 200 rpm. Cells were plated in DMEM/F-12 (Rhenium, Israel) supplemented with 1% B27 supplement (Rhenium, Israel) and 0.6% Gentamycin Sulfate (Biological Industries, Israel). Fibroblast growth factor-2 (FGF-2; 20 ng/ml; R&D systems) and Platelet-derived growth factor (PDGF-AA; 20 ng/ml; R&D systems) were added to cultures daily unless otherwise specified. Timeline is depicted in Figure S1a.

2.4 | Lymph node cells isolation

Lymph nodes were harvested from naïve 8- to 12-week-old C57/BL6 mice, as described previously (Einstein et al., 2003). Briefly, a single-cell suspension was prepared from lymph nodes. Lymph node cells (LNCs) were cultured in vitro for 72 h in RPMI medium (Biological Industries, Israel) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 1% of MEM Non-Essential Amino Acids Solution (Biological Industries, Israel), 1 mM sodium pyruvate, 1 mM L-glutamine, β-mercaptoethanol and 1% antibiotics (penicillin and streptomycin).

2.5 | Differentiation assays

Mice Primary OPCs were supplemented with PDFG-AA and FGF-2 for the first 48 h after shaking. Then, media was replaced, and combinations of Triiodothyronine (T3; 60 ng/ml; Sigma Aldrich) and either a single CSF sample or a mix of three human CSF samples from three patients with similar clinical manifestations (20% of media) were added into the OPC media for 7–20 days. Each assay was repeated independently at least three times. Assessments of oligodendroglial lineage marker Olig2 and astrocytic marker GFAP revealed that all experimental groups uniformly highly expressed Olig2 and low levels of GFAP, and no significant difference was observed among the groups (Olig2: 90.9 ± 2.5%, $p = .9358$; GFAP: 8.6 ± 2.5%, $p = .1112$, Figure S2).

2.6 | MHC-II expression assays

Mixed human CSF (20% of media) and interferon gamma (IFNγ) (10 ng/ml; PeproTech Inc, Rocky Hill, NJ) were supplemented into mice primary OPC media for 72 h to allow transcription and translation of major histocompatibility complex class (MHC)-II pathway mediators and permit for expression time, as described previously (Kirby et al., 2019) (graphical time course can be seen in Figure 7c). We conducted separate assays for MHC-II evaluation: using a single sample of CSF for each culture and using a mix of human CSF of three patients with similar disease outcomes. Each assay was repeated independently at least three times.

2.7 | OPC-LNC coculture

Mice Primary OPCs were cultured with mixed human CSF (20% of media) and IFNγ (10 ng/ml; PeproTech Inc, Rocky Hill, NJ) for 72 h (see above). Following 72 h in culture, cultures were washed and freshly isolated LNCs were added to the OPCs at different ratios (specified for each experiment in the results section) in RPMI medium for 72 h. LNCs were stimulated with 2.5 μg/ml Concanavalin A (ConA; C5275, Sigma-Aldrich, Israel) for 72 h unless otherwise specified (full graphical experiment time course can be found in Figure 8a). Non-activated LNCs and mitogen-stimulated LNCs with OPCs that were not exposed to CSF were used as controls. The proliferation of LNCs after incubation in vitro was evaluated by a Bromodeoxyuridine (BrdU) incorporation assay as previously described (Einstein et al., 2007). Each assay was repeated independently at least three times.

2.8 | Total RNA extraction, library preparation and sequencing

Mice primary OPCs were cultured with human CSF (10% of media) for 24 h. Each culture was exposed to a different CSF sample from a patient with a different clinical outcome (2 rMS and 3 pMS). Total RNA was extracted from cultured OPCs using the RNeasy Micro Kit (74004, Qiagen, Valencia, CA) from two groups: two rMS and three pMS. cDNA synthesis, library construction, and rRNA depletion were performed on 5 ng total RNA using the Trio RNA-Seq System (0507-96, NuGEN). The

resulting single index libraries were validated using the Standard Sensitivity NGS Fragment Analysis Kit (DNF-473-0500, Agilent formerly AATI) for size confirmation and quantified using the Quant-iT dsDNA Assay Kit, high sensitivity (Q33120, Thermo Fisher). Samples were diluted to equimolar concentrations (2 nM), pooled, and denatured according to the manufacturer's protocol. The final library dilution of 1.3 pM was sequenced on a NextSeq500 using the High Output v2 kit (FC-404-2002, Illumina) for paired-end (2 × 75) sequencing of ~40 million reads per sample. All experimental groups highly expressed OPCs signature genes (Zhang et al., 2014) (Figure S3a), expressed homeostasis-related genes (Avet-Rochex et al., 2014; Cheng et al., 2012; Trevaskis et al., 2005), and lower levels of genes associated with unhealthy cell state (Elkon et al., 2005; Olbryt et al., 2006; Tabunoki et al., 2012; Valencia-Cruz et al., 2013) with no significant difference among the three experimental groups (Figure S3b).

2.9 | Bioinformatics analysis

The NextSeq basecalls files were converted to fastq files using the bcl2fastq (v2.18.0.12) program with default parameters. Raw reads (fastq files) were inspected for quality issues with FastQC (v0.11.7). Normalization and differential expression analysis were performed with the DESeq2 package (v1.22.1). Differential expression was calculated with default parameters. Significance threshold was taken as *p*adj<.1. Functional enrichment gene ontology (GO) analysis for up- and downregulated genes was performed using the Broad Institute's Gene Set Enrichment Analysis (GSEA; http://www.broadinstitute.org/gsea, RRID:SCR_003199) tool (Subramanian et al., 2005). Values for GSEA analyses are represented as normalized enrichment score (NES) and false discovery rate (FDR). Differentially expressed genes and signature genes of CNS cells were enriched and analyzed using the Broad Institute's Morpheus tool (https://software.broadinstitute.org/morpheus, RRID:SCR_014975). Nuclear factor kappa-B (NFκB)-related genes were assessed using tumor necrosis factor (TNF-α) signaling via NFκB gene set (M5890; http://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_TNFA_SIGNALING_VIA_NFKB.html). Differently expressed genes are presented as a scatter plot, *y*-axis: $\log_2$ non-adjusted *p*-value for rMS- and pMS-cultured OPCs and *x*-axis: $\log_2$ fold change (rMS/pMS). When analyzing the expression of known signature immune genes, the genes were marked on the "differently expressed" volcano plot. STRING (Szklarczyk et al., 2019) database was used to study protein interactions between significant differential genes (http://string.embl.de, RRID:SCR_005223). The database for annotation, visualization and integrated discovery (DAVID) (Huang et al., 2009) was used to study shared biological processes of significant differential genes (https://david.ncifcrf.gov, RRID:SCR_001881).

2.10 | RNA isolation and reverse transcription

Mice primary OPCs were cultured with 10% human CSF for 24 h. RNA was extracted from cultured OPCs using Tri-reagent (Sigma-Aldrich, Rehovot, Israel) as previously described (Rio et al., 2010). cDNA was synthesized from 250 ng total RNA using the qScript cDNA Synthesis Kit (Quanta Biosciences). Quantitative polymerase chain reaction (PCR) was performed using PerfeCTa SYBR Green FastMix Rox (Quanta Biosciences). Gene amplification was carried out using the StepOnePlus real-time PCR system (Applied Biosystems). The threshold cycle value ($2 - \Delta CT$) was used for statistical analysis. All target mRNAs were normalized to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) reference gene. At least three independent experiments were performed; expression of each gene was evaluated in triplicate and is presented as mean mRNA relative quantification ± *SD*. For each gene, we used at least seven different CSF samples for each group (HCs, rMS, and pMS).

Primers used (Agentek):

HPRT F: 5′ CATGGACTGATTATGGACGGAC R: 5′ ACAGAGGGCCACAATGTGATG.

Cluster of differentiation (CD)74F: 5′ TCCATGGATAACAT GCTCCT R: 5′ GGGAGTTCTTGCTCATCTCA.

Vav Guanine Nucleotide Exchange Factor 1 (Vav1) F: 5′ GTGCAGATGAAGCTGGAGGAA R: 5′ CAGCACCCGCTGCATAGGTA.

TNFaip2 F: 5′ GCGCAGTTCGAGTTGTGTG R: 5′ TCGGGCTGTTGAGAATATCATTG.

CD72 F: 5′ CATCTAACCATCTAGGACAGGAC R: 5′ TCCTGGGACTGGAGACACT.

C motif chemokine ligand (CCL)2 F: 5′ GGCTCAGCCAGA-TGCAGT R: 5′ GCTCTCCAGCCTACTCATTGG.

SPARC Like 1 (SPARCL-1) F: 5′ CCATCCCAGTGACAAGGCT R: 5′ TGTCCTGCTCTGTTGACTGTTC.

2.11 | Protein concentration measurements

Cytometric bead array (CBA) was used according to manufacturer's instructions (mouse inflammation CBA kit; Cat# 552364, RRID: AB_2868960, BD Biosciences, San Diego, CA) to measure cytokine secretion in culture superannuants. Concentrations of interleukin (IL)-6, IL-10, CCL2, IFNγ, TNF-α, and IL-12p70 were measured in mice OPC supernatants. Cytokine concentrations were determined by flow cytometry (BD LSRFortessa, BD bioscience, San Diego, CA) and analyzed using FACSDIVA software. Results were calculated to account for the total protein concentration of the supernatant and are expressed as pg/ml. Intra-assay variability was 2%, and inter-assay variability was 5%. Analyses of human CSF by the above CBA kit found no cytokines, confirming that all cytokines detected in mouse primary OPCs' supernatants had been secreted by them. Plots show all measured proteins with an average concentration above 1 pg/ml (for at least one experimental group).

2.12 | Immunostaining

For cell surface markers, staining was performed on living cells followed by fixation. Anti-Olig2 (Cat# AF2418, RRID:AB_2157554, R&D System, Inc., Minneapolis, MN, 1:50) was used to identify oligodendroglial lineage,

anti-NG2 (Cat# AB5320, RRID:AB_91789, Millipore, Billerica, MA, 1:100) was used to identify OPCs, anti-O1 (Cat# MAB1327, RRID:AB_357618, R&D System, Inc., Minneapolis, MN, 1:100) for mature oligodendrocytes, anti-MBP (Cat# sc-271,524, RRID:AB_10655672, Santa Cruz Biotechnology, Inc., Dallas, TX, 1:100) was used as differentiation marker, anti-IBL-5/22 (Cat# sc-59,322, RRID:AB_831551, Santa Cruz Biotechnology, Inc., Dallas, TX, 1:100) for evaluation of MHC-II expression, anti-nuclear factor kappa B (NFκB)-p65 (Cat# sc-8008, RRID:AB_628017, Santa Cruz Biotechnology, Inc., Dallas, TX, 1:100) for evaluation of immune activation, anti-Glial fibrillary acidic protein (GFAP; Cat# Z0334, RRID:AB_10013382, Agilent, Santa Clara, CA, 1:50) was used to identify astrocytes. Goat anti-rabbit Alexa Fluor 488 (Cat# A-11034, RRID:AB_2576217, Invitrogen, Thermo Fisher Scientific, 1:200), goat anti-rat Alexa Fluor 555 (Cat# A-21434, RRID:AB_141733, Invitrogen, Thermo Fisher Scientific, 1:200), goat anti-mouse Alexa Fluor 488 (Cat# A-11001, RRID:AB_2534069, Invitrogen, Thermo Fisher Scientific, 1:200), goat anti-mouse Alexa Fluor 555 (Cat# A28180, RRID:AB_2536164, Invitrogen, Thermo Fisher Scientific, 1:200) and donkey anti-mouse Alexa Fluor 488 (Cat# A21202, RRID:AB_141607, Invitrogen, Thermo Fisher Scientific, 1:200) were used as secondary antibodies appropriately. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Cat# H-1200, RRID:AB_2336790, Vector Laboratories, Burlingame, CA). Quantification was performed using ImageJ software (NIH, public domain software) by measuring positively stained cells relative to total DAPI. Quantifications are represented as mean percentages from total DAPI+ cells ±*SD* and are from at least 15 random fields captured in three or more independent experiments.

2.13 | Assessment of oligodendrocyte morphological complexity by fractal dimension and skeleton analyses

Fractal dimension (FD) analysis was performed to evaluate morphological complexity of O1+ oligodendrocytes following differentiation essays, as described previously (Behar, 2001; Dyck et al., 2019; Kataria et al., 2018). In this analysis, a numerical value close to 1 represents cells with low morphological complexity (essentially bipolar cells), whereas an FD value near two identifies those with high complexity (highly branched cells or with a bi-dimensional planar structure). FD was calculated using the ImageJ software (https://imagej.nih.gov/ij/, RRID:SCR_003070). Briefly, each image from O1+ oligodendrocytes was converted to an 8-bit grayscale image. Then, using the "Crop" tool, an individual cell was cropped, and the "threshold" was adjusted to select the whole cell. Next, the cell was outlined using the "Outline" tool. Then, in "Analyze," the "Tools" command was chosen. Next, the "Fractal box count" was selected, and the corresponding FD value was obtained. For branch length and end points count each image was further converted into "skeleton" and analyzed using the Analyze Skeleton (2D/3D) (Arganda-Carreras et al., 2010). This procedure was performed for >25 cells per condition for each independent experiment.

2.14 | Flow cytometry analysis

T cell activation was assessed by Phycoerythrin (PE)-anti-CD25 (Cat# 553075, RRID:AB_394605, BD Pharmingen, San Diego, CA) for IL2-Receptor α (IL2-Rα) in combination with Allophycocynanin (APC)-anti-CD3e (Cat# 565643, RRID:AB_2739319, BD Pharmingen, San Diego, CA) for T cells. BrdU incorporation was determined using fluorescein isothiocyanate (FITC) anti-BrdU (Cat# 347583, RRID:AB_400327, BD Pharmingen, San Diego, CA). For T cell characterization, we used FITC-anti-CD4 (Cat# 100407, RRID:AB_312692, BioLegend, San Diego, CA) and FITC-anti-CD8 (Cat# 100707, RRID:AB_312746, BioLegend, San Diego, CA). All fluorescence-activated cell sorting (FACS) samples were analyzed in a Beckman coulter FC500 apparatus using the CXP software.

2.15 | Statistical analyses

Unpaired two-tailed Student's *t* test, one-way ANOVA with Tukey's multiple comparisons post hoc test, and two-way ANOVA with Tukey's multiple comparisons post hoc test was performed. Specific tests are noted in figure legends with significance level annotations. Values are provided as mean ± *SD*, or as described for each figure. All error bars represent *SD*.

3 | RESULTS

3.1 | CSF From progressive MS patients impedes OPC differentiation

We first studied whether the CSF of patients with different clinical forms of MS affects OPCs' differentiation capability. Mice primary OPCs (mean of 87.4% NG2+ cells, Figure S1b,c) were cultured with T3 (60 ng/ml) (Tosic et al., 1992) and with human CSF (20% of media) from three groups (HC, rMS, and pMS; Figure 1a). The differentiation was assayed using mature oligodendrocyte markers O1 and MBP staining (Barateiro & Fernandes, 2014). Seven days after induction, CSF from pMS patients significantly decreased OPC differentiation to mature oligodendrocytes, represented by significantly lower O1 and MBP expression, compared with OPCs exposed to CSF of rMS, HC, or T3 alone that retained their ability to differentiate into mature oligodendrocytes (O1: pMS: 13.7 ± 4.33%, rMS: 58.0 ± 6.9%, HC: 59.2 ± 5.7%, and T3: 72.5 ± 5.7%, $p < .0001$; MBP: pMS: 11.1 ± 2.7%, rMS: 56.7 ± 3.7%, HC: 60.1 ± 2.3%, and T3: 68.0 ± 3.6%, $p < .0001$; Figures 1b,c and S4a). Similar significant results were obtained for O1 staining using a CSF sample from single individuals (pMS: 12.8 ± 2.1%, $n = 4$; rMS: 56.8 ± 4.1%, $n = 7$; HC: 58.6 ± 4.1%, $n = 4$; T3: 71.8 ± 2.3%, $n = 7$, $p < .0001$, Figure S4b). Measurements conducted 20 days after induction resulted in similarly significant findings (pMS: 16.8 ± 1.9%, rMS: 59.7 ± 6.6%, HC: 66.9 ± 5.9%; T3: 76.5 ± 6.4%, $p < .0001$, Figure 1d).

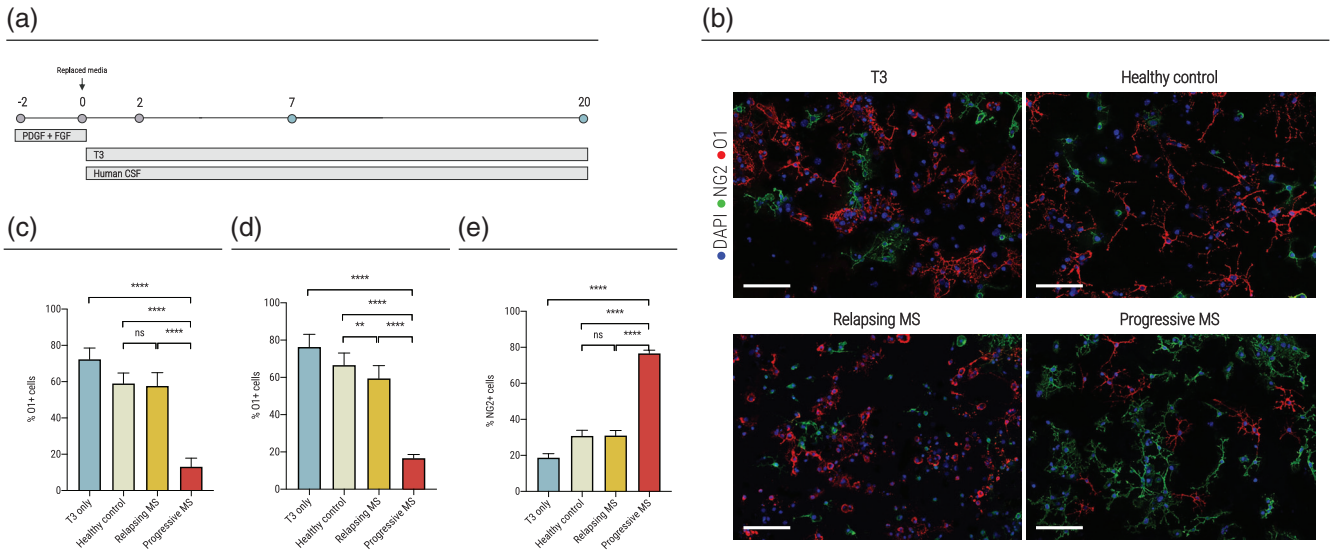

FIGURE 1 Cerebrospinal fluid (CSF) from progressive MS patients impedes OPC differentiation. (a) Time-course of diffreantiation experiments. OPCs were cultured under PDGF and FGF conditions for 48h and then supplemented with thyroid hormone (T3). Each culture was exposed to mixed human CSF from patients with similar disease outcomes (20% of media) for two time-points and evaluated using mature oligodendrocyte marker O1 staining, (b) Representative immunofluorescence analysis of mature oligodendrocyte marker O1 and progenitor marker NG2 on day seven following exposure to CSF of the three groups (scale bar = 50 μm), (c) OPCs cultured with mixed human CSF from HC or rMS induced more differentiation into mature oligodendrocytes as quantified by O1 marker (out of DAPI+ cells) after 7 days in culture (59.2 ± 5.7% and 58.0 ± 6.9%, respectively) in comparison to pMS (13.7 ± 4.3%). Data are presented as means±*SD* ($n = 12$ for each group), (d) OPCs cultured with human CSF from HC or rMS induced more differentiation into mature oligodendrocytes as quantified by O1 marker (out of DAPI+ cells) after 20 days in culture (66.9 ± 5.9% and 59.7 ± 6.6%, respectively) in comparison to pMS (16.8 ± 1.9%). Data are presented as means±*SD* ($n = 12$ for each group), (e) OPCs cultured with human CSF from HC or rMS express lower levels of progenitor marker NG2 (out of DAPI+ cells) after 7 days in culture (30.9 ± 3.0% and 31.1 ± 2.7%, respectively) in comparison to pMS (76.6 ± 1.8%). Data are presented as means±*SD* ($n = 6$ for each group). Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p * ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant)

Analysis using NG2, an oligodendrocyte progenitor marker, revealed significantly higher expression in the pMS-CSF group (76.6 ± 1.83%), compared with OPCs exposed to CSF of rMS, HC, or T3 alone (31.1 ± 2.7%, 30.9 ± 3.0%, and 18.9 ± 2.1%, respectively, $p < .0001$, Figure 1e).

3.1.1 | Lower morphological complexity of O1+ oligodendrocytes upon culture with CSF from rMS patients

Using fractal dimension and skeleton analyses, we performed combined quantification of the morphological pattern of O1+ oligodendrocytes upon culture with CSF for 7 days (Figure 2a–d). Oligodendrocytes cultured with CSF from rMS patients had lower morphological complexity, represented by significantly lower FD index (1.29 ± 0.1), compared with OPCs exposed to pMS CSF (1.45 ± 0.1, $p < .0001$) or HC CSF (1.35 ± 0.05, $p = .0418$; Figure 2e). Oligodendrocytes exposed to rMS CSF also had lower end points/cell index and branches length/cell compared with those exposed to pMS CSF (Figure 2f,g).

3.2 | Immune-like transcriptome of OPCs upon exposure to CSF of MS patients

To obtain a more comprehensive understanding of the effect of CSF from patients with different clinical manifestations of MS on OPCs, we performed RNA sequencing (RNA-seq) analysis. Mice primary OPCs were cultured with human CSF (10% of media) of patients with different clinical outcomes (rMS and pMS) for 24h.

We identified 91 genes that differentiated significantly (*p*adj<.1) between the two MS sub-groups: rMS and pMS (Figure 3a). Among these genes, 89 were downregulated and 2 were upregulated in the pMS-cultured cells as compared with the rMS-cultured cells. Functional and enrichment analyses of the differentially expressed genes were performed using DAVID (Huang et al., 2009). Eight out of the 10 top significant pathways were related to the immune system and inflammatory response ($p < .001$, Table S2). A set of known hallmark inflammatory response genes (including CXCL10, IL1β, and NFκB) (Megan Kong et al., 2011; Messina et al., 2004; Morelandy et al., 2009) were highly enriched in OPCs cultured with CSF from rMS patients compared with those cultured with CSF from pMS patients (NES 2.06, FDR 0.0, Figure 3b). By comparing the profile of our differentially expressed genes

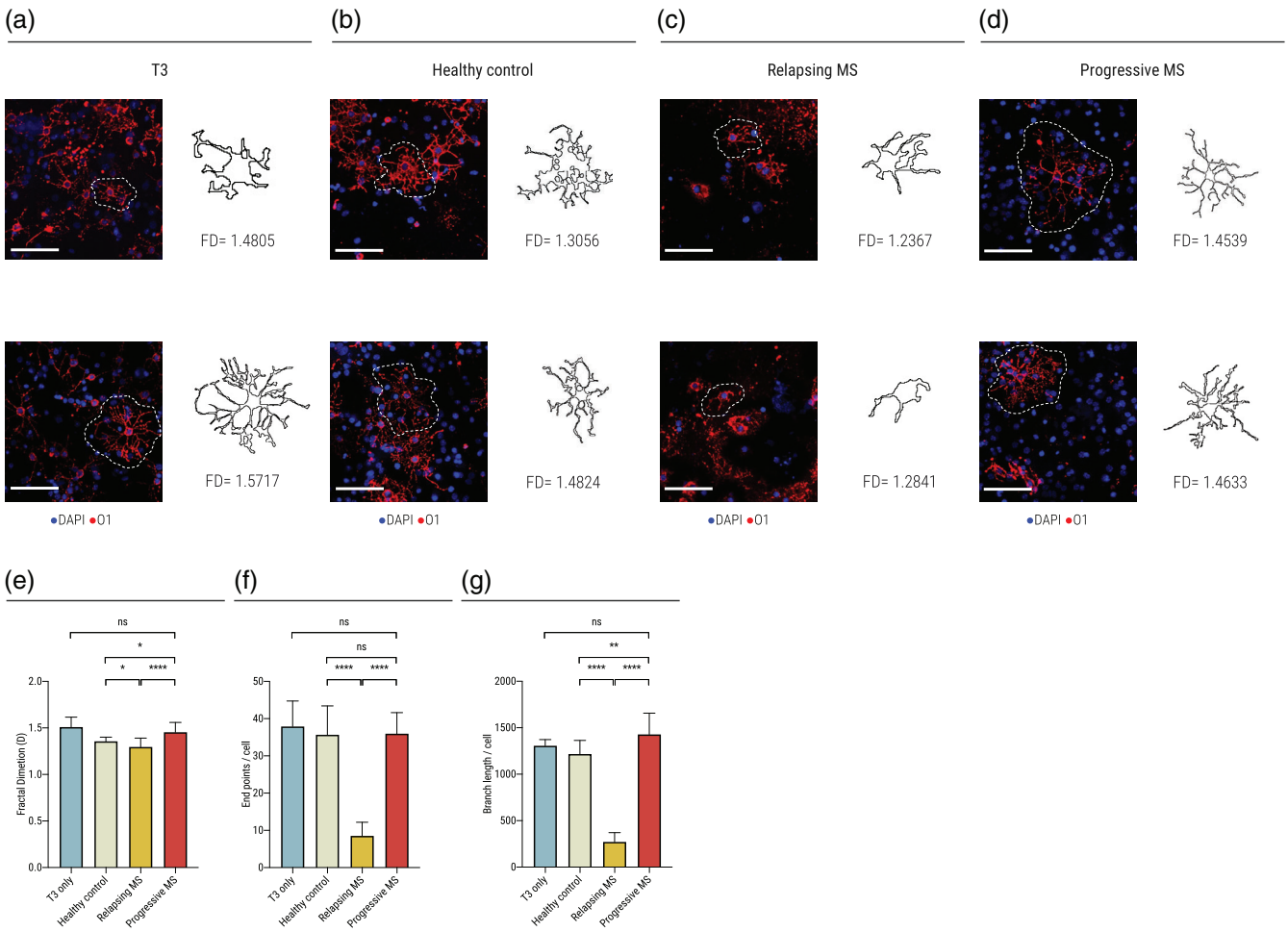

FIGURE 2 Low morphological complexity among OPCs exposed to CSF from relapsing MS patients. O1+ oligodendrocytes were characterized upon 7 days in culture with T3 and CSF from HC, rMS, and pMS patients or T3 alone. (a–d) Representative images of O1+ oligodendrocytes and cell outline with the appropriate fractal dimension (FD) value. (e) Higher levels of morphological complexity among cells exposed to pMS CSF (1.45 ± 0.1) compared with CSF from rMS (1.29 ± 0.1, $p < .0001$) or HC patients (1.35 ± 0.05, $p = .01$) (scale bar = 30 μm), (f) Skeleton analysis resulted in lower endpoints/cell among cells cultures with rMS CSF (7.9 ± 4.0) compared with pMS (33.4 ± 10.7, $p < .0001$) of HC (35.7 ± 7.7, $p < .0001$), (g) Skeleton analysis resulted in lower Branches length/cell among cells cultures with rMS CSF (271.5 ± 99.5) compared with pMS (1428.1 ± 229.1, $p < .0001$) of HC (1216.5 ± 146.8, $p < .0001$). Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test ($p^* \leq .05$, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant). Error bars in all graphs represent *SD*

to immune transcriptomic signatures (Megan Kong et al., 2011; Messina et al., 2004; Morelandy et al., 2009), we found that the majority (57%) of the 91 significantly differentiating genes are implicated in immune processes (Figure 3c). Those genes were enriched for the GO terms related to immune response, inflammatory response, leukocyte activation, and phagocytosis (Figure 3d).

3.3 | Exposure of OPCs to CSF from pMS patients impedes pro-inflammatory activity

In order to validate the finding of differentially expressed immune genes in OPCs cultured with CSF from MS patients at different disease stages, we studied representative genes of three immune pathways that were found to differ significantly between the groups. TNF-α-inducible protein 2 (TNFaip-2) represents the T cell receptor signaling pathway, CD72 represents the B cell receptor signaling pathway, and CCL2 represents the cytokine and chemokine signaling pathways. All three genes were significantly downregulated in OPCs cultured with CSF from pMS compared with rMS in the RNA-seq analysis (TNFaip-2: 48.4 ± 11.5 vs. 164.4 ± 12.8, CD72: 81.9 ± 5.4 vs. 241.0 ± 63.0; and CCL2: 8.5 ± 2.3 vs. 52.5 ± 43.6, Figure 3a). In a validation performed using reverse transcription (rt)-PCR analysis in a larger group of patients, we found that the expression levels of TNFaip-2 and CD72 were significantly decreased in OPCs cultured with pMS CSF compared with rMS and HCs (TNFaip-2: 0.29 ± 0.07 vs. 0.92 ± 0.75, $p < .05$; and CD72: 0.26 ± 0.18 vs. 1.25 ± 0.68, $p = .0008$, Figure 4a,b). CCL2 expression was also downregulated in OPCs upon exposure to pMS CSF compared with rMS CSF (0.8 ± 0.62 vs. 1.28 ± 0.8); however, this difference did not reach statistical significance ($p = .2308$, Figure 4c).

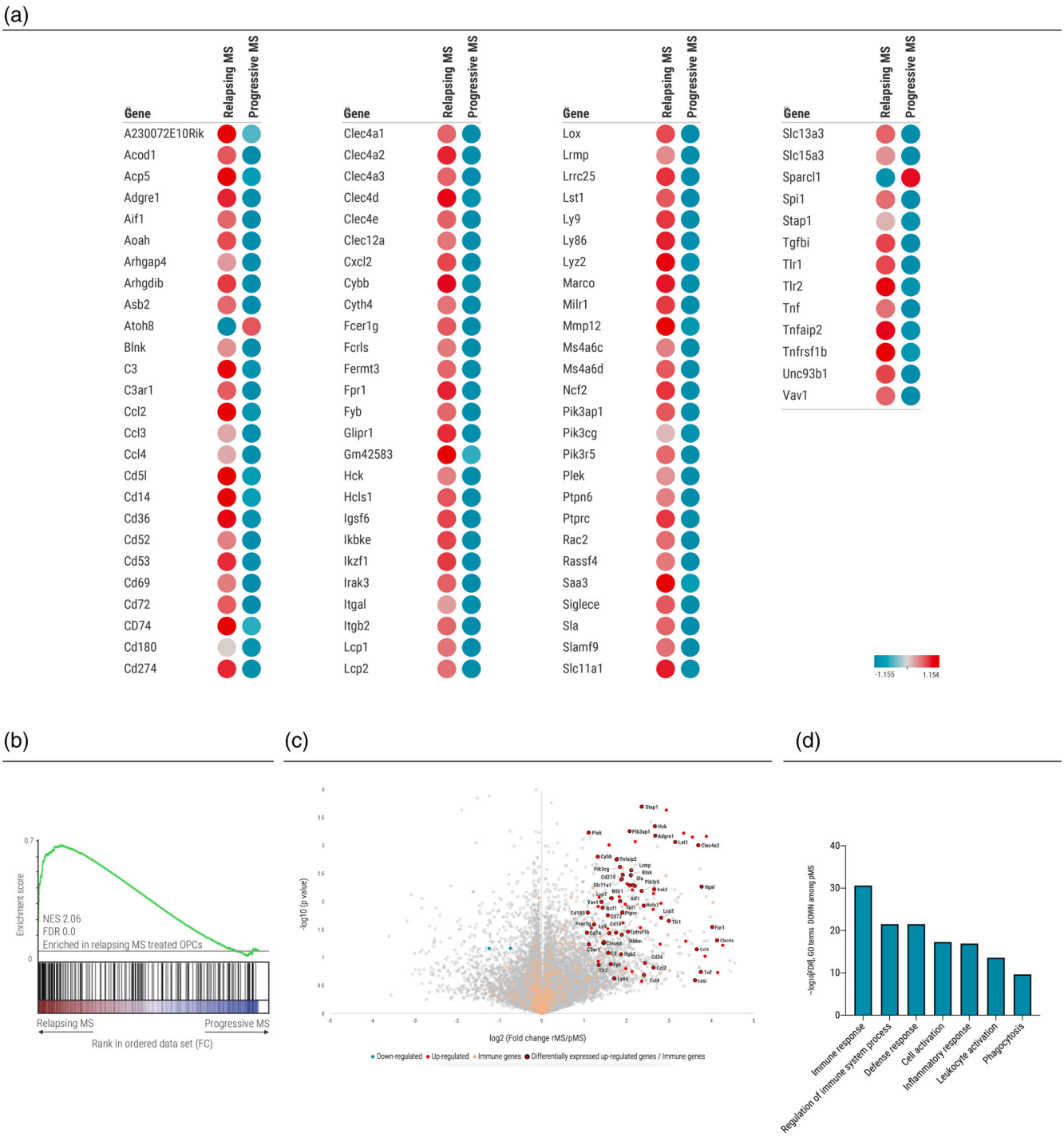

FIGURE 3 Immune-like transcriptome of OPCs upon exposure to CSF of MS patients. RNA-seq analysis of mice primary OPCs cultured with human CSF from rMS ($n = 2$), and pMS ($n = 3$) obtained at disease onset. (a) Dot plot illustrating top 91 differentially expressed genes: blue denotes low expression, red denotes high expression ($p$adj<.1). (b) Gene set enrichment analysis (GSEA) of an inflammatory response signature in mice OPC populations upon culture with CSF. NES, normalized enrichment score; FDR, false discovery rate; FC, fold change. (c) Volcano plot shows in gray all mapped genes with an average expression above 0 (for at least one experimental group). Ninety-one differentially expressed genes were identified ($p$adj<.1). Blue dots indicate downregulated differentially expressed genes among rMS, red dots indicate upregulated differentially expressed genes among rMS, orange dots indicate immune genes, and red dots with black outline indicate immune genes out of differentially expressed genes. Key genes associated with immunological or inflammatory processes are pointed out. (d) Analysis of universally downregulated genes in pMS CSF cultured OPCs versus rMS CSF cultured OPCs. Plot of the top enriched gene ontology (GO) terms (focus on "function" in GOrilla), sorted by −log10[P] in the genes universally downregulated (DOWN) in pMS cultured OPCs versus rMS cultured OPCs

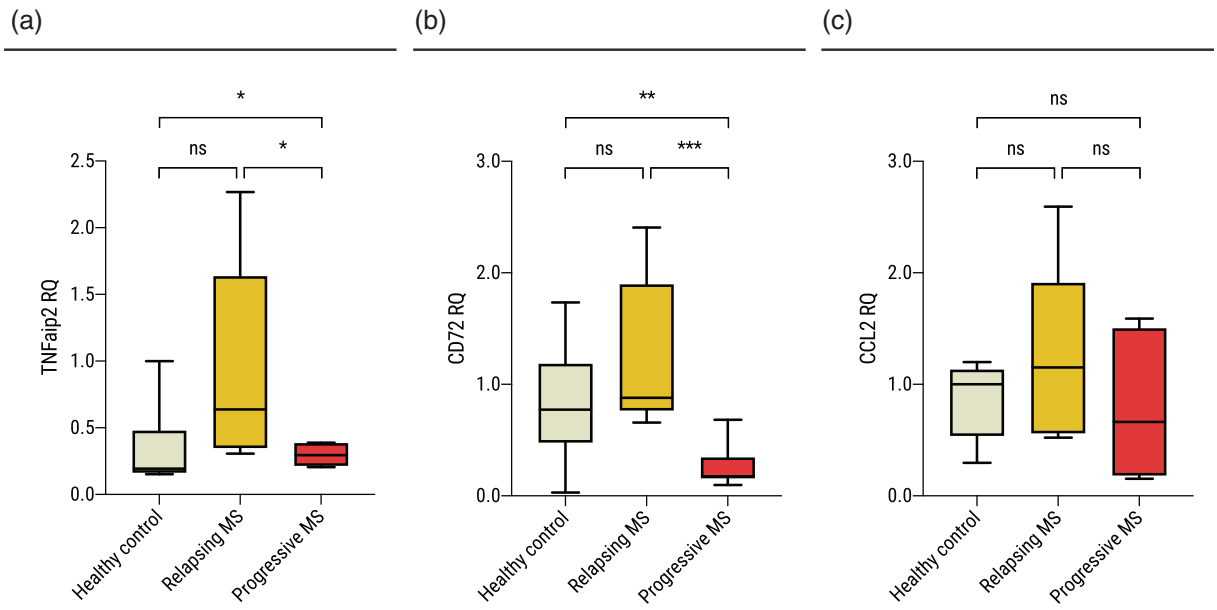

FIGURE 4 Exposure of OPCs to CSF from pMS patients impedes pro-inflammatory activity. rt-PCR gene expression analysis of representative genes of three immune pathways: (a) The expression of TNFaip2 decreased significantly in the pMS cultured OPCs (0.29 ± 0.07, $n = 7$) versus 0.92 ± 0.75 ($n = 8$) of the rMS CSF cultured OPCs and HC (0.34 ± 0.29, $n = 8$), (b) The expression of CD72 decreased significantly in the pMS cultured OPCs (0.26 ± 0.18, $n = 9$) versus 1.25 ± 0.68 ($n = 8$) of the rMS CSF cultured OPCs and HC (0.82 ± 0.56, $n = 8$), (c) The expression of CCL2 decreased in the pMS cultured OPCs (0.8 ± 0.62, $n = 8$) versus 1.28 ± 0.8 ($n = 8$) of the rMS CSF cultured OPCs and HC (0.87 ± 0.35, $n = 7$). Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p * ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant). Error bars in all graphs represent *SD*

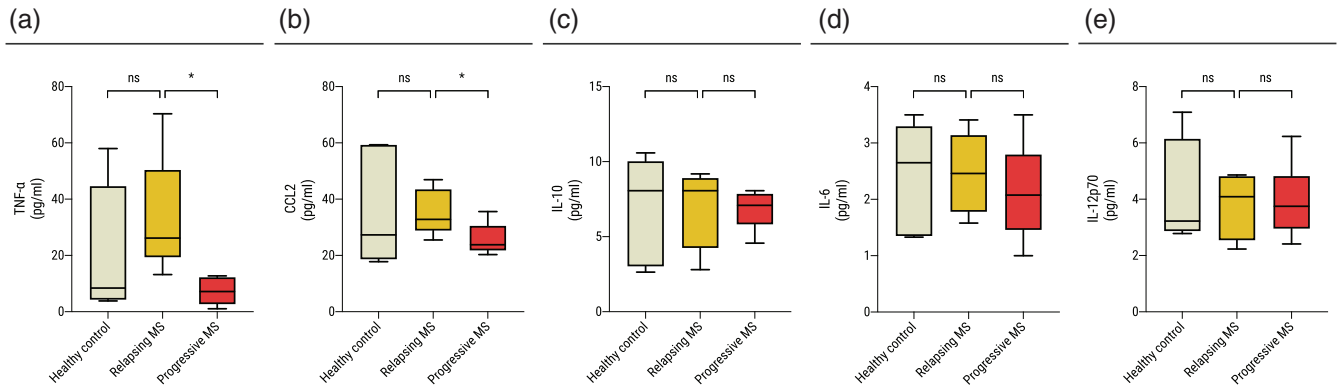

FIGURE 5 Increased TNF-α and CCL2 secretion upon exposure of OPCs to CSF from rMS but not pMS patients. Using CBA analysis, we studied the secretion of six different inflammatory cytokines in the supernatant of OPCs following culture with CSF samples (10% of media) of each group (HCs, rMS, and pMS) for 24h. (a) TNF: HCs 21.2 ± 23.4 pg/ml; rMS cultured OPCs 33.1 ± 21.8 pg/ml; pMS cultured OPCs 7.2 ± 5.1 pg/ml, (b) CCL2: HCs 36.7 ± 20.9 pg/ml; rMS cultured OPCs 35.5 ± 8.2 pg/ml; pMS cultured OPCs 25.77 ± 5.6 pg/ml, (c) IL-10: HCs 6.8 ± 3.6 pg/ml; rMS cultured OPCs 6.9 ± 2.6 pg/ml; pMS cultured OPCs 5.7 ± 3.1 pg/ml, (d) IL-6: HCs 2.4 ± 1.0 pg/ml; rMS cultured OPCs 2.46 ± 0.7 pg/ml; pMS cultured OPCs 2.1 ± 0.9 pg/ml, (e) IL-12p70: HCs 4.1 ± 2.0 pg/ml; rMS cultured OPCs 3.8 ± 1.2 pg/ml; pMS cultured OPCs 3.9 ± 1.3 pg/ml. Data are presented as means±*SD* of HC ($n = 5$), rMS ($n = 5$), and pMS ($n = 6$). IFNγ levels were undetected among all groups. Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p* ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant)

SPARCL-1 is one of two significantly upregulated genes among OPCs cultured with pMS CSF compared with rMS (8382.5 ± 1150.5 and 4464.9 ± 835.5, respectively, $p = .0268$, Figure 3a). SPARCL-1 is known to play an important role in neurogenesis during development (Gongidi et al., 2004) and after acute injury (Lively et al., 2011; Lively & Brown, 2008). OPCs have been shown to express SPARCL-1 after demyelination (Moyon et al., 2015), and in experimental autoimmune encephalomyelitis (EAE), its spinal cord mRNA levels were reported to correlate with paralysis severity (Blakely et al., 2015). In a validation using rt-PCR on a broader group of patients, a similarly

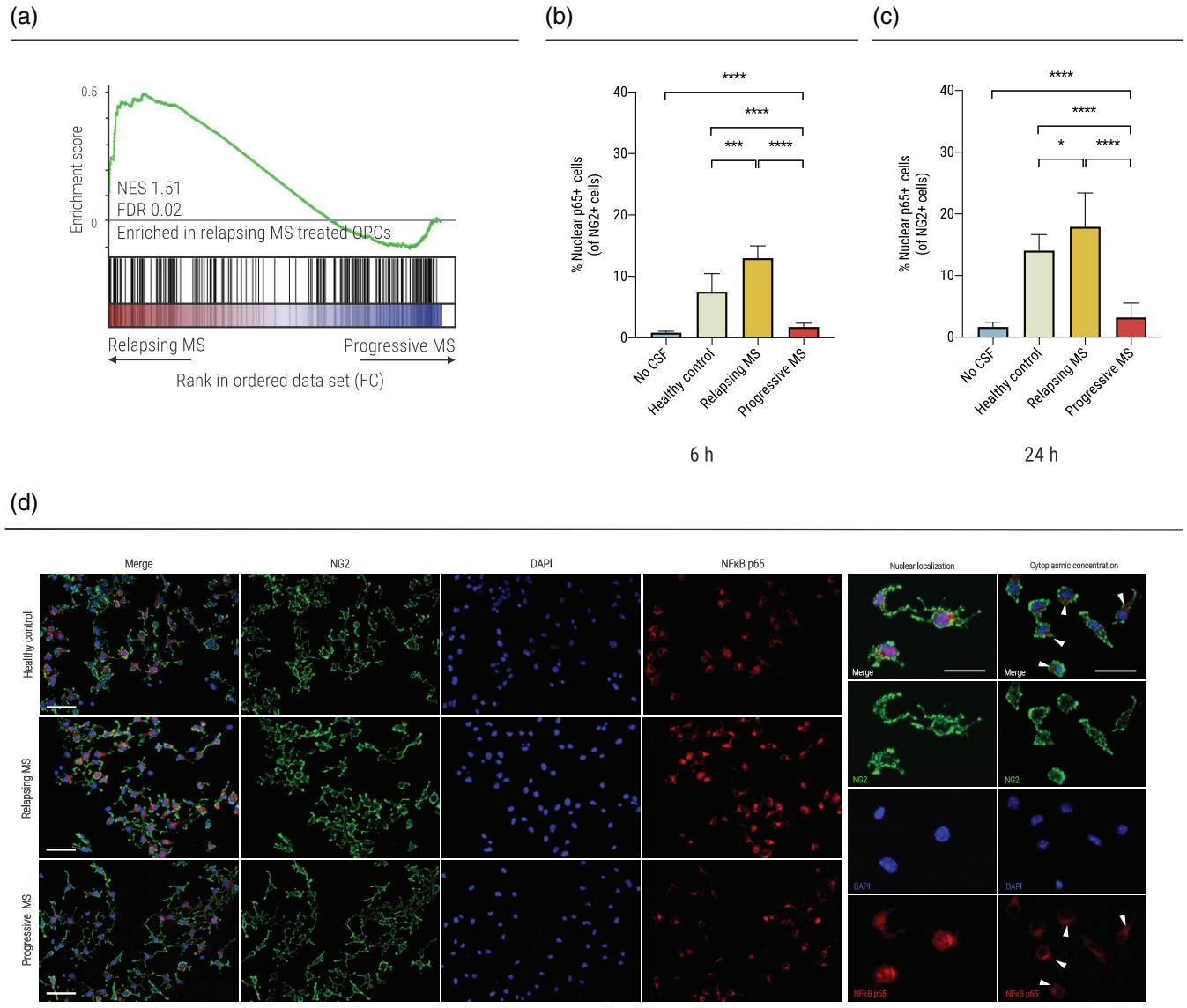

FIGURE 6 CSF from rMS patients increased OPCs NFκB activation. (a) Gene set enrichment analysis, testing a set of TNFα signaling via NFκB on CSF cultured OPCs. NES, normalized enrichment score; FDR, false discovery rate; FC, fold change. (b) Nuclear expression of p65 subunit of NFκB was determined after culture of 6 h: HC: 7.8 ± 2.3%; rMS: 13.1 ± 1.8%; pMS: 1.8 ± 0.5%. Exposure of OPCs to IFNγ alone resulted in 0.8 ± 2.1% expression. Data are means±*SD* ($n = 9$ for each group). (c) Nuclear expression of p65 subunit of NFκB was determined after culture of 24 h: HC 14.2 ± 2.4%; rMS: 18.5 ± 4.7%; pMS: 3.6 ± 1.6%. Exposure of OPCs to IFNγ alone resulted in 1.8 ± 0.7% expression. Data are means ±*SD* ($n = 9$ for each group). (d) Representative immunofluorescence analysis of primary OPCs cultured under PDGF and FGF conditions (daily dose of 20 ng/ml) for 48 h, then IFNγ (10 ng/ml) and CSF (HC, rMS, or pMS) were added for 24 h (scale bar = 50 μm). Activation was assessed by nuclear staining of p65 subunit out of NG2+ cells (scale bar on right pane = 25 μm). Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p * ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant). Error bars in all graphs represent *SD*

significant difference was observed (pMS: 2.16 ± 0.66, rMS: 1.6 ± 0.32, $p = .0191$, Figure S5).

The finding that OPCs express immune-related genes and that the immune-related pathways are affected by CSF of MS patients suggests that OPCs may play a role as immune modulators in the pathogenesis of different clinical manifestations of MS.

3.3.1 | Decreased TNF-α and CCL2 secretion, and NFκB activation upon exposure of OPCs to CSF from pMS patients

To better understand OPCs' inflammatory activity, we studied cytokine secretion of mice primary OPCs cultured with CSF from patients

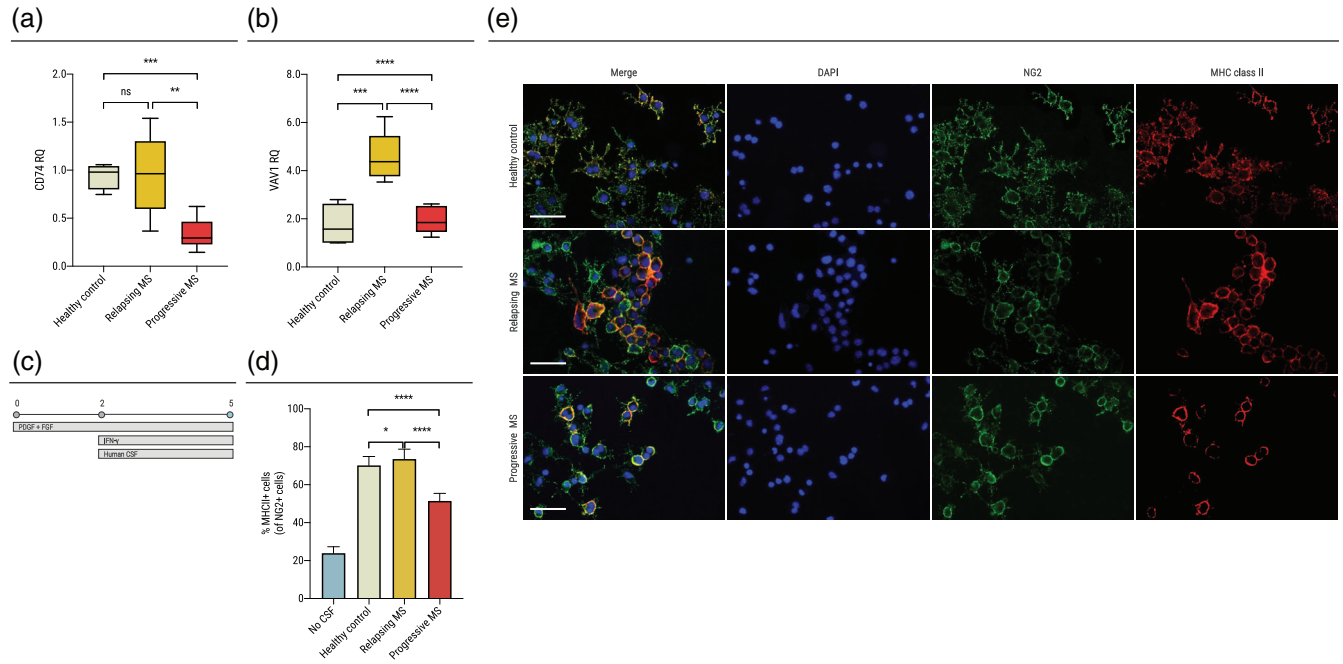

FIGURE 7 CSF from pMS patients reduces OPC MHC-II expression. (a, b) qPCR gene expression analysis of CD74 and Vav-1, which are known to function as regulators of antigen presentation: (a) The expression of CD74 decreased significantly in the pMS CSF cultured OPCs (0.3335 ± 0.16, $n = 8$) versus 0.9532 ± 0.4 ($n = 8$) of the rMS CSF cultured OPCs and HC (0.94 ± 0.14, $n = 7$). (b) The expression of Vav1 decreased significantly in the pMS CSF cultured OPCs (1.91 ± 0.51, $n = 7$) versus 4.64 ± 0.98 ($n = 7$) among the rMS CSF cultured OPCs and HCs (1.73 ± 0.88, $n = 7$). (c) Time-course experiments of MHC-II expression. Primary OPCs were cultured under PDGF and FGF conditions (daily dose of 20 ng/ml) for 48 h. Then, cells were cultured with IFNγ (10 ng/ml) and with human CSF (20% of media) for 72 h, followed by evaluation of MHC-II expression out of NG+ cells using immunofluorescence staining. (d) MHC-II expression was determined upon exposure to both IFNγ and mixed human CSF samples for 72 h: HC: 70.3 ± 4.6% ($n = 8$); rMS: 73.7 ± 5.0% ($n = 8$); pMS: 51.6 ± 3.9% ($n = 8$). Exposure to IFNγ alone without CSF resulted in 24.1 ± 3.0%. (e) Representative immunofluorescence analysis of MHC-II expression among OPCs upon culture with human CSF of patients with different clinical manifestations of MS (scale bar = 40 μm). Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p * ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant). Error bars in all graphs represent *SD*

with different clinical manifestations of MS. Using CBA analysis, we studied the secretion of six different inflammatory cytokines in the supernatant of OPCs following culture with CSF samples (10% of media) of each group (HCs, rMS, and pMS) for 24 h. TNF-α and CCL2 levels were significantly decreased among OPCs cultured with CSF of pMS compared with rMS (TNF-α: 7.24 ± 5.12 pg/ml vs. 33.1 ± 21.8 pg/ml, $p = .0192$; and CCL2: 25.77 ± 5.64 pg/ml vs. 35.5 ± 8.2 pg/ml, $p = .00449$, Figure 5a,b). No statistically significant difference was detected in IL-10, IL-6, and IL-12p70 levels (IL-10: 5.7 ± 3.1 pg/ml vs. 6.9 ± 2.6 pg/ml; IL-6: 2.1 ± 0.9 pg/ml vs. 2.46 ± 0.7 pg/ml; and IL-12p70: 3.9 ± 1.3 pg/ml vs. 3.8 ± 1.2 pg/ml, Figure 5c–e, respectively). IFNγ levels were undetected among all groups.

To further explore the differences in immune properties of OPCs, we studied NFκB activation upon exposure to CSF of MS patients. RNA-seq analyses revealed that a set of known genes related to TNF-α activation via NFκB (Daly et al., 2015) was differently expressed between OPCs cultured with CSF of rMS compared with pMS patients (NES 1.51, FDR 0.02, Figure 6a). Additionally, NFκB activation was assessed by measuring the percentage of nuclear p65 subunit staining among CSF-cultured OPCs. Primary OPCs were cultured for 6 and 24 h with CSF (20% of media) of patients with different clinical manifestations and stimulated using IFNγ (10 ng/ml). We found significantly lower NFκB activation in OPCs exposed to CSF from pMS (1.8 ± 0.5% and 3.6 ± 1.6% after 6 and 24h, respectively) than rMS-cultured OPCs (6 h: 13.1 ± 1.8%; 24h: 18.5 ± 4.7%), HC-cultured OPCs or IFNγ alone (HC: 6h: 7.8 ± 2.3%; 24h: 14.2 ± 2.4, IFNγ: 6h: 0.8 ± 2.1%; 24h: 1.8 ± 0.7%; $p < .0001$, Figure 6b–d). These findings support the possibility of immunomodulatory functions for OPCs.

3.3.2 | Lower expression of MHC-II upon exposure of OPCs to CSF from pMS patients

Next, we aimed to investigate the effect of CSF from the two clinical MS forms on OPCs' ability to present antigens.

CD74 and Vav-1 are known to function as regulators of antigen presentation. In RNA-seq analysis both genes were significantly downregulated in OPCs cultured with CSF of pMS patients compared with rMS patients (CD74: 2.8 ± 0.4 vs. 5.9 ± 1.5, $p = .036$; and Vav-1: 52.1 ± 9.0 vs. 138.4 ± 25.1, $p = .01$; Figure 3a). These findings were further validated using rt-PCR analysis in a larger group of patients

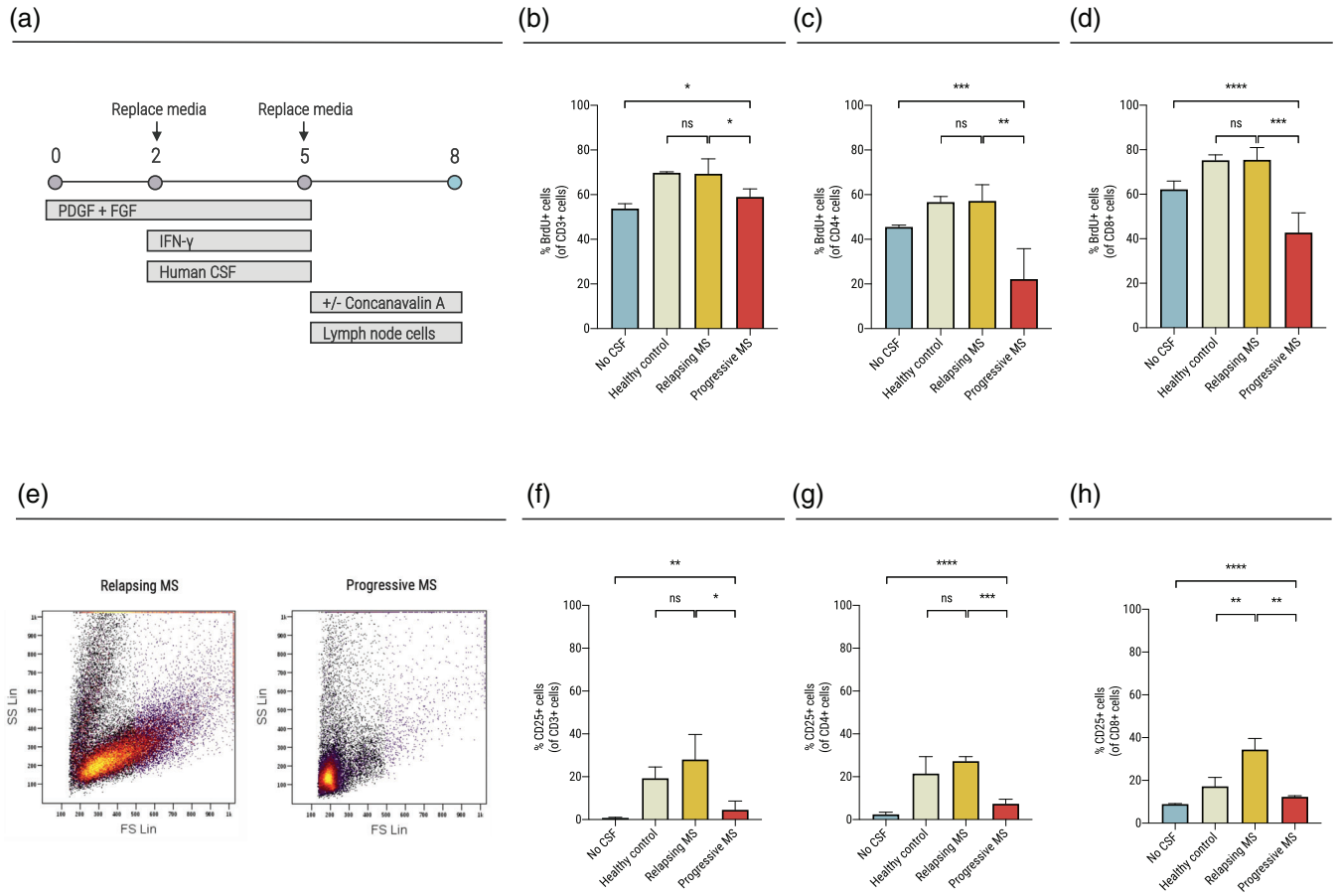

FIGURE 8 Reduced T cell proliferation and activation upon culture with OPCs exposed to CSF from pMS patients. (a) Time course experiments of Lymph node cells (LNCs) activation. OPCs were cultured under PDGF and FGF conditions. After 48 h, cells were cultured with IFNγ (10 ng/ml) and mixed human CSF (20% of media) for 72 h. Then, media was washed and freshly isolated LNCs were added to the culture in different ratios (1:2 and 1:4), (b–e) Evaluation of proliferating LNCs, all LNCs were stimulated with ConA (2.5ug/ml). (b) Evaluation of BrdU +/CD3+ cells using flow cytometry. No CSF: 53.8 ± 2.1% ($n = 3$), HC: 69.8 ± 0.4% ($n = 3$), rMS: 69.5 ± 6.4% ($n = 4$), and pMS: 59.1 ± 3.5% ($n = 4$). (c) Evaluation of BrdU+/CD4+ cells using flow cytometry. No CSF: 45.5 ± 0.8% (n = 3), HC: 56.7 ± 2.5% ($n = 3$), rMS: 57.5 ± 6.8% ($n = 4$), and pMS: 24.2 ± 11.4% ($n = 4$). (d) Evaluation of BrdU+/CD8+ cells using flow cytometry. OPCs:LNCs were cultured in a ratio of 1:2. No CSF: 62.3 ± 3.6% ($n = 3$), HC: 75.3 ± 2.3% ($n = 3$), rMS: 75.6 ± 5.5% ($n = 4$), and pMS: 43.3 ± 7.4% ($n = 4$). (e) Representative flow cytometry analysis of proliferating T cells upon culture with OPCs cultured with CSF from rMS and pMS patients. (f–h) Evaluation of activated LNCs. (f) Evaluation of CD25+/CD3+ cells using flow cytometry. OPCs:LNCs were cultured at a ratio of 1:4. No CSF:0.8 ± 0.3% ($n = 3$), HC: 19.7 ± 4.8% ($n = 3$), rMS: 29.2 ± 10.0% ($n = 3$), and pMS: 5.1 ± 2.8% ($n = 3$). (g) Evaluation of CD25+/CD4+ cells using flow cytometry. OPCs:LNCs were cultured at a ratio of 1:4. No CSF: 2.5 ± 0.8% ($n = 3$), HC: 22.2 ± 6.4% ($n = 3$), rMS: 27.3 ± 2.1% ($n = 3$), and pMS: 7.5 ± 1.8% ($n = 3$). (h) Evaluation of CD25+/CD8+ cells using flow cytometry. OPCs:LNCs were cultured at a ratio of 1:4. No CSF: 8.9 ± 0.2% ($n = 3$), HC: 17.5 ± 4.0% ($n = 3$), rMS: 34.6 ± 4.7% ($n = 3$), and pMS: 12.3 ± 0.5% ($n = 3$). Each assay was repeated independently at least three times. Significance was determined by one-way ANOVA analysis followed by Tukey's multiple comparison analysis and by unpaired two-tailed student's *t* test (p * ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001, ns = not significant). Error bars in all graphs represent *SD*

(CD74: 0.3335 ± 0.16 vs. 0.9532 ± 0.4, $p = .0008$; and Vav-1: 1.91 ± 0.51 vs. 4.64 ± 0.98, $p < .0001$, Figure 7a,b).

To address antigen presentation in OPCs following exposure to CSF, we examined MHC-II expression (I-A/E regions in C57/BL6 mice). Immunofluorescence staining was performed following exposure to human CSF and IFNγ for 72 h (Figure 7c). Exposure of OPCs to CSF of pMS patients (51.6 ± 3.9%) resulted in significantly lower MHC-II expression compared with the exposure to CSF of HC or rMS patients (70.3 ± 4.6% and 73.7 ± 5.0%, respectively, $p < .0001$, Figure 7d,e). Similar significant results were obtained using a CSF sample from single individuals (HC: 59.9 ± 4.5%,; rMS: 61.1 ± 4.6%; pMS: 40.5 ± 4.0%, $n = 4$ for each group; $p < .0001$, Figure S6).

3.4 | Reduced T cell proliferation and activation upon culture with OPCs exposed to CSF from pMS patients

To further explore the mechanisms that support OPC involvement in MS pathogenesis, we aimed to see whether OPC immune functions differ in MS patients with different disease courses.

OPCs were cultured with human CSF (20% of media) and IFNγ (10 ng/ml) for 72 h. Then, cultures were washed, and freshly isolated LNCs were added to the cultures at ratios of 1:2 or 1:4 OPCs:LNCs. Following 72 h of coculture, we evaluated LNC proliferation and activation using flow cytometry (Figure 8a).

For proliferation analyses, LNCs were stimulated using ConA. The proportion of proliferating T cells (BrdU+/CD3+) upon culture with OPCs following exposure to CSF of pMS patients was significantly lower (59.1 ± 3.5%) in comparison to those cultured with CSF of HC or rMS patients (69.8 ± 0.4% and 69.5 ± 6.4%, respectively, $p = .0131$, Figure 8b). On analyses of proliferation among CD4+ T cells (pMS: 24.2 ± 11.4%, rMS: 57.5 ± 6.8%, $p = .0007$) and CD8+ T cells (pMS: 43.3 ± 7.4%, rMS: 75.6 ± 5.5%, $p < .0001$), the differences of proliferating cells were significantly more profound (Figure 8c–e).

The percentages of activated T cells (CD25+/CD3+) upon culture with OPCs following exposure to CSF from pMS patients (5.1 ± 2.8%) also decreased significantly in comparison to those that were cultured with CSF of HC or rMS patients (19.7 ± 4.8% and 29.2 ± 10.0%, respectively, $p = .001$, Figure 8f). In addition, the percentages of activated cells out of CD4+ T cells (pMS: 7.5 ± 1.8%, rMS: 27.3 ± 2.1%, $p = .0002$) or CD8+ T cells (pMS: 12.3 ± 0.5%, rMS: 34.6 ± 4.7%, $p = .0012$) cultured with OPCs following culture with CSF of pMS patients were significantly lower than those that were cultured with CSF of HC or rMS patients (Figure 8g,h).

These data indicate that CSF of pMS patients reduces OPC ability to enhance proliferation and activation of T cells.

4 | DISCUSSION

In the current study, we found that CSF from relapsing and progressive MS patients has a differential effect on OPCs' ability to differentiate into mature oligodendrocytes and to express immune functions. CSF from pMS patients impaired OPC differentiation and immune gene expression, including a decrease in MHC-II expression, TNF-α secretion, and NFκB pathway activation. Along with the differences in immune properties, there are also functional differences such as decreased activation and proliferation of T cells following interaction with OPCs exposed to CSF from pMS patients. These findings suggest that OPCs have a role not only in remyelination but also in immune modulation.

Although MS has been studied extensively for decades, it is still not entirely clear why some of the patients experience a more stable disease while others decline progressively (Goldenberg, 2012). One of the differences between rMS and pMS, is the presence of remyelination, which is associated with lower EDSS scores and slower disease progression (Bodini et al., 2016; Bramow et al., 2010). While remyelination successfully occurs in rMS (Blakemore & Franklin, 2008; Franklin et al., 2013), remyelination failure is considered one of the key features of pMS (Goldschmidt et al., 2009; Morales Pantoja et al., 2020).

Adequate remyelination requires the proliferation, migration, and differentiation of OPCs into mature myelinating oligodendrocytes (Franklin & Ffrench-Constant, 2008). Failure of remyelination is in many cases associated with a presence of OPCs that fail to differentiate into mature oligodendrocytes (Chang et al., 2000; Kuhlmann et al., 2008; Wolswijk, 1998). We observed that the CSF from pMS patients inhibited OPC differentiation, as opposed to exposure to CSF from rMS or HCs that did not influence OPCs' differentiation capacity. It is suggested that differences in the OPC ability to differentiate may contribute to disease heterogeneity (Chang et al., 2002; Franklin, 2002; Kuhlmann et al., 2008; Wolswijk, 1998). Recent work has shown that induced pluripotent stem cells (IPSC) from pMS donors exhibit lower efficiency in differentiating into mature oligodendrocytes than those from rMS and HC donors (Morales Pantoja et al., 2020). In addition, we found decreased morphological complexity among oligodendrocytes exposed to rMS CSF compared with CSF from pMS patients.

Pursuant to our finding of impaired differentiation capability among OPCs cultured with pMS CSF, we proceeded to study the differential effect of CSF from different MS forms on OPCs. We performed genome-wide RNA-seq analyses of OPCs exposed to CSF from pMS and rMS patients. The main differentiating finding was the different immune signature of OPCs cultured with rMS CSF compared with pMS CSF. For many years OPCs were considered solely as the regenerative milieu of myelin in the CNS and thus were seen as a passive target of the immune system in MS (Chang et al., 2000; Hughes et al., 2013). However, several studies have suggested that OPCs may also have immunomodulatory functions and may play a prominent role as part of the immune milieu of the CNS (Akay et al., 2021; Arnett et al., 2001; Chew et al., 2005; Falcão et al., 2018; Jakubzick et al., 2017; Kirby & Castelo-Branco, 2020; Psenicka et al., 2021; Wang et al., 2017; Zeis et al., 2016). Castelo-Branco's group demonstrated that OPCs have phagocytic functions and can present antigen via MHC-I and -II. They also found that MHC-II-expressing OPCs can activate memory and effector CD4+ T cells (Falcão et al., 2018). These findings were re-established by Kirby et al., who showed that OPCs are capable of presenting antigen (Kirby et al., 2019). Another study suggested that oligodendroglia are already primed at the chromatin level and can rapidly activate an immune program in the context of disease (Meijer et al., 2020). Upon injury, OPCs secrete IL1β and CCL2, which enhance their mobilization and thus contribute to the post-injury inflammatory milieu (Duncan et al., 2020; Moyon et al., 2015; Ramesh et al., 2012). These works propose that OPCs undergo phenotypic changes and can adopt features commonly associated with immune cells, such as antigen processing, presentation, and cytokine secretion.

A single-nucleus RNA sequencing from white matter areas of postmortem MS patients' brains identified several subclusters of oligodendroglia that may indicate different functional states (Jäkel et al., 2019). An immune oligodendroglial subcluster, characterized by CD74 and APOe, was enriched in MS patients compared with HCs (Jäkel et al., 2019). CD74 is associated with the MHC-II complex and is known to be upregulated in oligodendrocytes in EAE, the animal model for MS (Falcão et al., 2018; Kirby et al., 2019). In line with these works we found decreased CD74 and MHC-II expression among

OPCs cultured with pMS CSF compared with those cultured with rMS or HC CSF. In addition, exposure of OPCs to CSF of rMS patients activated and increased lymphocyte proliferation in vitro, to significantly higher levels compared with OPCs exposed to CSF of pMS patients. These immune functions are attributed mainly to classic antigen-presenting cells of the hematopoietic system, such as dendritic cells.

In order to further understand the immunological functions of OPCs, we studied the activation of the NFκB pathway in OPCs following exposure to CSF of MS patients. We showed increased NFκB activation in OPCs exposed to CSF of rMS patients compared with pMS. TNF-α, one of the main cytokines that activate the NFκB pathway, was also significantly increased in OPCs exposed to CSF of rMS patients compared with pMS patients. Conflicting results have indicated either an exacerbating or an ameliorating role of TNF-α during CNS pathological conditions, including inflammation (Madsen et al., 2020; Su et al., 2011). A couple of works described that the lack of TNF-α led to a significant delay in remyelination (Arnett et al., 2001; Cunha et al., 2020), and neural precursors proliferated in vivo after injections of TNF-α (Arnett et al., 2001). These data further demonstrate that OPCs can acquire immunological functions and play a role in the inflammation process (Falcão et al., 2018; Jäkel et al., 2019), highlighting the role of the immune system and inflammatory response in remyelination.

Accumulating evidence suggests that the environment may also play an essential role in the ability of OPCs to perform successful remyelination (Magliozzi et al., 2018; Poirion et al., 2021). While rMS is characterized by the infiltration of peripheral immune cells and elevated inflammatory proteins (such as CXCL13, CXCL12, IFNγ, and TNF-α) (Dendrou et al., 2015; Magliozzi et al., 2019), the inflammation in pMS is limited and compartmentalized behind a relatively closed BBB (Hughes et al., 2014; Lassmann et al., 2012). Several regulators of OPC differentiation such as PDGF-AA, FGF2, insulin-like growth factor I (IGF-I), transforming growth factor-β (TGF-β), and IL-1β are increased in acute inflammation and associated with early stages of lesion formation (Arnett et al., 2001; Hinks & Franklin, 1999; Mason et al., 2001; Mason et al., 2003), but are absent in a chronic inflammatory environment (Foote & Blakemore, 2005; Franklin & Ffrench-Constant, 2008). OPCs and ongoing remyelination are found in active inflammatory MS lesions but are rarely observed in immunologically inactive plaques (Confavreux et al., 2000; Khademi et al., 2013; Patani et al., 2007; Wolswijk, 2002). In animal models of chronic demyelination, OPCs were able to achieve effective remyelination only by induction of acute inflammation (Foote & Blakemore, 2005; Setzu et al., 2006). Behi et al. found recently that a pro-inflammatory environment results in increased OPC differentiation through crosstalk with microglial cells (El Behi et al., 2017). Furthermore, they observed heterogeneity in the remyelination pattern in MS patients; high remyelination ability was found in correlation to microglial activation and lymphocyte cytokine secretion (El Behi et al., 2017). Accordingly, it is believed that remyelination depends on an appropriate immune response controlled in time, space, and intensity, although the specific mechanisms are still unclear (Baaklini et al., 2019; Schwartz & Kipnis, 2002).

The inflammatory surrounding appears to affect oligodendrocytes morphology as well. Early observations described that OPCs near inflammatory lesions in EAE undergo morphological changes, including shortening and thickening of processes reminiscent of microglial or astrocytic activation (Nishiyama et al., 1999). Marisca et al. showed that oligodendrocytes with a simpler process network expressed genes of differentiation and proliferation and had a rapid dynamic remodeling ability (Marisca et al., 2020). We observed decreased morphological complexity among oligodendrocytes exposed to rMS CSF compared with CSF from pMS and HC, with intact differentiation. A plausible explanation may be that the pro-inflammatory environment found in the CSF of rMS patients results in the bipolar shape of the cells and preserved differentiation capability. These highlight the importance of inflammation-differentiation balance, suggesting that an inflammatory environment may induce remyelination, depending on the surrounding environment.

Further evidence suggests that the inflammation process contributes both to the myelin damage and repair processes (Jiang et al., 2014). Although the pathology of MS is immune-mediated, the innate immune response to demyelination is important for creating an encouraging environment for remyelination (McMurran et al., 2016). Immune cells are responsible for the clearance of myelin debris, which contains proteins that inhibit OPC differentiation (Baer et al., 2009; Kotter et al., 2006; Rosenberg et al., 2008; Ruckh et al., 2012; Syed et al., 2008; Zhao et al., 2006). Previous works have shown that experimental depletion of macrophages (Kotter et al., 2001), B or T cells (Bieber et al., 2003; Dombrowski et al., 2017) lead to impairment of remyelination. Additionally, steroid administration delayed CNS remyelination in vivo (Chari et al., 2006). We also found that OPCs exposed to CSF of rMS patients had a higher capability of T cells activation and proliferation compared with those exposed to CSF of pMS patients. These observations suggest an active immune role for OPCs in MS pathogenesis. This emphasizes the importance of inflammation in MS pathogenesis and highlights the growing understanding that inflammation in MS might not always be harmful. Understanding the relationships between the immune response and remyelination may help develop new strategies promoting OPC repopulation and differentiation as pro-remyelination therapies in MS.

In conclusion, we showed that following exposure to CSF from MS patients, OPCs become immune activated, inter alia, by NFκB activation, processing and presenting antigens to T cells, and secreting anti- or pro-inflammatory cytokines. Our data enhanced the current understanding of the roles of OPCs, highlighting their specific role in inflammation in different clinical manifestations of MS. These findings may provide new avenues for therapeutic intervention as well as furnishing a better understanding of disease pathogenesis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

OZ contributed to the design, acquisition, analysis, interpretation of the data and drafting the work. NF contributed to the design, acquisition, and the analysis of the data. AR, NH, TG, and IL contributed to the acquisition of data. LB contributed to the conception, design, acquisition, analysis, interpretation of the data and drafting of the work. AV-D contributed to the conception, design, interpretation of the data and drafting of the work. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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