

Adaptive human immunity drives remyelination in a mouse model of demyelination

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One major challenge in multiple sclerosis is to understand the cellular and molecular mechanisms leading to disease severity progression. The recently demonstrated correlation between disease severity and remyelination emphasizes the importance of identifying factors leading to a favourable outcome. Why remyelination fails or succeeds in multiple sclerosis patients remains largely unknown, mainly because remyelination has never been studied within a humanized pathological context that would recapitulate major events in plaque formation such as infiltration of inflammatory cells. Therefore, we developed a new paradigm by grafting healthy donor or multiple sclerosis patient lymphocytes in the demyelinated lesion of nude mice spinal cord. We show that lymphocytes play a major role in remyelination whose efficacy is significantly decreased in mice grafted with multiple sclerosis lymphocytes compared to those grafted with healthy donors lymphocytes. Mechanistically, we demonstrated *in vitro* that lymphocyte-derived mediators influenced differentiation of oligodendrocyte precursor cells through a crosstalk with microglial cells. Among mice grafted with lymphocytes from different patients, we observed diverse remyelination patterns reproducing for the first time the heterogeneity observed in multiple sclerosis patients. Comparing lymphocyte secretory profile from patients exhibiting high and low remyelination ability, we identified novel molecules involved in oligodendrocyte precursor cell differentiation and validated CCL19 as a target to improve remyelination. Specifically, exogenous CCL19 abolished oligodendrocyte precursor cell differentiation observed in patients with high remyelination pattern. Multiple sclerosis lymphocytes exhibit intrinsic capacities to coordinate myelin repair and further investigation on patients with high remyelination capacities will provide new pro-regenerative strategies.

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Abbreviations: CNPase = 2',3'-cyclic-nucleotide 3'-phosphodiesterase; EAE = experimental autoimmune encephalomyelitis; GalC = galactocerebroside; IGF-1 = insulin growth factor 1; MIG = microglial cell; OPC = oligodendrocyte precursor cell

Introduction

Multiple sclerosis is an autoimmune disease of the CNS in which the myelin formed by oligodendrocytes is destroyed by successive inflammatory attacks. An endogenous myelin repair occurs in some patients (Bramow *et al.*, 2010) but remyelination extent and efficacy is highly variable among patients and this variability is not linked to age, disease duration, or clinical forms of the disease (Patani *et al.*, 2007). A recent longitudinal follow-up of newly formed demyelinated lesions—using PET and MRI combined with myelin specific markers—documented a large interindividual heterogeneity of remyelination (Bodini *et al.*, 2016). Interestingly, the remyelination index was inversely correlated with clinical disability, a result confirming that myelin repair is essential to slower disease evolution. It is therefore of utmost importance to define the key players of a successful remyelination in multiple sclerosis conditions to slow down disability progression.

Inflammatory events exert a key role in driving remyelination (Arnett *et al.*, 2003; Foote and Blakemore, 2005; Miron and Franklin, 2014; Döring *et al.*, 2015; Moore *et al.*, 2015). Indeed, depletion of innate and adaptive immune cells (Kotter *et al.*, 2001; Bieber *et al.*, 2003) leads to a decrease of remyelination efficacy in animal models of focal demyelination. More specifically, myelin repair is dependent of the state of activation of the invading macrophages and resident microglial cells (MIGs). At least two main activated states exist: (i) classically activated (or M1) after exposition to the pro-inflammatory cytokines IFN- γ ; and (ii) alternatively activated (or M2) after exposure to IL-4 (Butovsky *et al.*, 2006). M1 cells appear to influence the first steps of remyelination by stimulating proliferation and migration of oligodendrocytes precursors cells (OPCs) towards the lesion site while M2 MIGs foster OPC differentiation into myelinating oligodendrocytes (Butovsky *et al.*, 2006; Miron *et al.*, 2013). These results were further strengthened by the observation that intraventricular (Butovsky, 2006) or intranasal (Zhang *et al.*, 2014) injection of M2 MIGs led to a decrease of symptom severity in the multiple sclerosis animal model of experimental autoimmune encephalomyelitis (EAE), by modulating inflammation and increasing remyelination. All these data suggest that the control of the local inflammatory environment is a key component of a successful remyelination.

As these studies were performed using animal models, it remains challenging to extrapolate to human pathological conditions the prerequisites for efficient myelin repair. Therefore, in order to examine this regenerative process within multiple sclerosis pathological conditions, we established new *in vitro* and *in vivo* experimental paradigms to

investigate how human immune cells influence endogenous remyelination. We show, for the first time, that multiple sclerosis patient lymphocytes impede remyelination *in vivo* when grafted in demyelinated lesions of nude mouse spinal cord. To decipher the underlying mechanism, we used *in vitro* experiments demonstrating that, upon stimulation, multiple sclerosis lymphocytes directed MIGs more toward a pro-inflammatory phenotype compared to healthy donor lymphocytes, leading to impairment of OPC maturation. Moreover, we found that patient lymphocytes influenced differentially the remyelination process, some lymphocytes showing a beneficial and some a deleterious effect, a pattern mimicking what is observed in multiple sclerosis patients. Finally, by comparing these two subgroups of patients using elaborated biostatistical analysis, we identified five molecular targets and validated *in vitro* CCL19 as a potential target.

Materials and methods

Study design

The aim of the study was to define the molecular and cellular elements leading to a proper remyelination in a humanized context. The number of individual patients or independent replicate is indicated in all figure legends. Data were analysed in a blinded fashion (third party concealment). All statistics were performed under the supervision of the biostatistics platform. Alpha < 0.05 was considered as statistically significant.

Recruitment of multiple sclerosis patients and healthy donors

Collection of blood and cells for the study was approved by the French Ethics committee and the French ministry of research (DC-2012-1535 and AC-2012-1536). Written informed consent was obtained from all study participants. All patients fulfilled diagnostic criteria for multiple sclerosis, and individuals (multiple sclerosis patients and healthy donors) with any other inflammatory or neurological disorders were excluded from the study.

Mice

Nude (RjOrl:NMRI-Foxn1^{nu}/Foxn1^{nu}) and wild-type (C57BL/6J) mice were purchased from Janvier (France). All animal protocols were performed in accordance with the guidelines published in the National Institute of Health Guide for the Care and Use of Laboratory Animals, EU regulations (agreement n° A75-1319) and the local 'Charles Darwin' ethics committee.

Collection and activation of peripheral blood mononuclear cells

Blood (30 ml) was collected from multiple sclerosis patients (from the BRC-REFGENSEP cohort) and healthy donors (from the French Blood Organization EFS) in acid citrate dextrose (ACD) tubes. Peripheral blood mononuclear cells (PBMCs) were purified through centrifugation (2200 rpm, 20 min) on a Ficoll gradient and several washings in 10% foetal calf serum (FCS) RPMI. Cells were resuspended at 2×10^6 cells/ml in RPMI 10% FCS and activated using anti-CD2/anti-CD3/anti-CD28 antibodies conjugated to beads (Miltenyi) at a ratio of one bead per two cells in 24-well plates (TPP). After 72 h, cells were collected for grafting and supernatants were harvested and used for MIG culture experiments and cytokines/chemokines analysis by Luminex®.

Flow cytometry

To test human lymphocyte survival after grafting, 50–200 μ l of blood were sampled retro-orbitally 8–12 days after surgery. Cells were fixed with 1-step Fix/Lyse solution (Affymetrix) for 15 min, washed, resuspended in FACS buffer and stained with antibodies (Supplementary Table 1). The percentages of hCD4⁺ and HLA⁺ cells, identified by forward and side scatter, were calculated on total leucocytes.

For lymphocyte characterization, PBMCs were activated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and GolgiPlug™ (1 μ g/ 10^6 cells; BD Biosciences) over 4 h. Cells were then washed in staining buffer [phosphate-buffered saline (PBS) 1% FCS and 0.1% sodium azide] and stained with antibodies (Supplementary Table 1).

Data were acquired on a FACSVerse (BD Biosciences) and analysed using FlowJo software (Tree Star).

Secretion profile measurement

The Luminex®-based multiplexed immunoassays with fluorescent microspheres Milliplex Map assays (HCYTMA60K-PX41, HCYP3MAG-63K-11 and HCP2MAG-62K-PX23, Merck Millipore) were used to measure 72 cytokines/chemokines (five were not detectable).

Lysophosphatidylcholine injection and peripheral blood mononuclear cell graft

Eight-week-old nude mice were anaesthetized with a solution of ketamine (100 mg/ml) and xylazine (10 mg/ml). Demyelination was performed by stereotaxic injection of 1 μ l of lysophosphatidylcholine (1%, Sigma) in the dorsal horn of the spinal cord. After 48 h, lymphocytes from multiple sclerosis patients or healthy controls [10^5 cells in 1 μ l of X-VIVO™ 15 medium (Lonza)] were engrafted within the lesion site previously marked by charcoal. Three to five mice were grafted per individual.

Perfusion and tissue processing

Grafted mice were euthanized by over-anaesthesia of ketamine/xylazine and perfused intracardially with a solution of PBS 4% paraformaldehyde (PFA) for immunohistochemistry and 4% PFA, 5% glutaraldehyde for electron microscopy. Spinal cords were removed and post-fixed for 1 h. For immunohistochemistry, tissues were cryoprotected overnight in a 20% sucrose solution, frozen in cooled isopentane and cryosected longitudinally (12- μ m slices). For electron microscopy, spinal cords were cut into 1 mm sections and contrasted first with 2% osmium tetroxide and with 5% uranyl acetate before several steps of dehydration. Spinal cord sections were embedded in Epon™ before being cut into semithin and ultrathin sections for analysis.

Microglial and oligodendrocyte precursor cell culture

MIGs and OPCs were obtained using mixed cultures of glial cells. Hemispheres from C57BL/6 P0/P1 newborn mice were isolated, mechanically dissociated and put in culture in polyornithine (Sigma) coated flasks (TPP) in Dulbecco's modified Eagle medium supplemented with 10% horse serum and 10% FCS. After 12–14 days, MIGs were collected in the floating fraction of the culture after 2 h of shaking at 100 rpm. OPCs were collected following a shaking period of 18 h at 250 rpm. OPCs were purified after two differential adhesions on low adhesion petri dishes (TPP).

Microglial cell pretreatment, oligodendrocyte precursor cell proliferation and differentiation test

MIGs were plated on 4-well dishes (Gibco) at 10^5 cells per well or in 96-well plates (TPP) at 4×10^4 cells per well in DMEM/F12 media with N2 (1%), B27 (0.5%), insulin (25 μ g/ml), glucose (6 mg/ml), and supplemented with 2.5% FCS (differentiation test) or not (proliferation test). After adhesion, MIGs were pretreated over 24 h with lymphocyte supernatant from multiple sclerosis patients or healthy control subjects. The MIG-conditioned media was harvested and cells fixed using 4% PFA. OPCs were plated in 4-well dishes or 96-well plates (10^5 and 4×10^4 cells per well, respectively) coated with polylysine. After adhesion, MIG-conditioned media or lymphocyte supernatant was added to OPC cultures. After 24 h (proliferation test) or 72 h (differentiation test), cells were fixed using 4% PFA. To test the influence of CCL19, recombinant human CCL19 (1 ng/ml, R&D systems) was added to lymphocyte supernatant.

Immunocytochemistry and immunohistochemistry

Cells and tissues were permeabilized, saturated and stained with antibodies described in Supplementary Table 1. All primary antibodies were incubated overnight at 4°C, and secondary antibodies for 45 min at room temperature. Nuclei were counterstained with Hoechst (Sigma, 33342).

Image acquisition

Pictures were taken using fluoromicroscopes DMRB (Leica) and ApoTome.2 (Zeiss), plate scanner CellInsight CX5 (ThermoFisher) or slide scanners Axioscan.Z1 (Zeiss) and NanoZoomer-XR Digital slide scanner C12000 (Hamamatsu) and converted to TIFF for quantification when needed.

Photographic image quantification

In vitro

To assess MIG polarization, the proportion of inducible nitric oxide synthase (iNOS⁺) and insulin growth factor 1 (IGF-1⁺) cells on the total number of cells was evaluated using the imageJ software. The same quantification method was used for the evaluation of O4⁺, galactocerebroside (GalC⁺) and CNPase⁺ (2',3'-cyclic-nucleotide 3'-phosphodiesterase) cells. Proliferative Olig2⁺/Ki67⁺ oligodendroglial cells were quantified automatically using HCS studio.

In vivo

The number of Olig2⁺ and Olig2⁺/CC1⁺ in immunohistochemistry, the area of remyelination in electron microscopy and the number of HLA⁺ cells in spinal cords were quantified using ImageJ software. The graph represents the mean of three to five mice per individual with a good reproducibility among the mice grafted with the lymphocytes of the same individual.

Statistical analysis

Errors bars on graphs represent mean \pm SEM. Statistical tests were run by Prism GraphPad software (GraphPad Prism version 5.00 for Windows). For the analysis of two groups, an unpaired two-tailed Student *t*-test or a Mann-Whitney test were performed. For more than two group analyses, a one-way ANOVA with Tukey's multiple comparison test or a Kruskal-Wallis with Dunn's multiple comparisons test were performed. Parametric or non-parametric tests were applied according to Gaussian or non-Gaussian distributions of the residuals evaluated by Shapiro Wilk test for all datasets and d'Agostino and Pearson when the number of residuals was sufficient.

Multivariate analysis

All the coding for the multivariate analysis has been run with the R software. Barycentric discriminant analysis (BADA) is a robust form of discriminant analysis that creates (linear) combinations of variables—called factors or dimensions—that best discriminate between *a priori* defined groups of observations (Abdi *et al.*, 2012). BADA evaluates the quality of the group separations by using a non-parametric Bootstrap resampling scheme to derive confidence intervals that are drawn around the points representing the groups on the factor maps. A ranking procedure was applied on the cytokine data block to avoid any bias introduced by 0s corresponding to undetectable levels of cytokine secretion.

To assess the contributions of each block of data to the remyelination process, we applied a hierarchical partial least square regression (PLS) (Fig. 6B), to evaluate their relative link to the percentage of CNPase⁺ cells. Network inference was performed by computing partial correlations and assessing

their significance with GeneNet (Schaefer and Strimmer, 2005), which is particularly adapted to high dimensional data.

Results

In vivo evaluation of human lymphocyte effect on remyelination

In nude mice, spontaneous remyelination occurs after focal demyelination from lysophosphatidylcholine (LPC) injection and is completed within 4 weeks (Jeffery and Blakemore, 1995). To evaluate how lymphocytes influence remyelination, we combined LPC-induced demyelination in the spinal cord of nude mice and the graft within the lesion 48 h after demyelination of 10⁵ activated lymphocytes isolated from blood samples of healthy control subjects and patients with multiple sclerosis (Table 1, Supplementary Table 2 and Fig. 1A).

To follow lymphocyte fate, we detected in mice blood samples of grafted mice the presence, among mice leucocytes, of HLA⁺ (1.13% \pm 0.25) and human cluster of differentiation 4⁺ (hCD4⁺) (0.56% \pm 0.06) cells by flow cytometry (Supplementary Fig. 1A–C) and we confirmed the presence of HLA⁺ cells within the lesion by immunohistochemistry 21 days post-grafting (Supplementary Fig. 1D–L). Lymphocytes were preferentially located in the lesion centres and borders, a very low number were found in the intact white matter (Supplementary Fig. 1D–F). The numbers of cells in the lesions were similar when healthy control or multiple sclerosis patient lymphocytes were grafted (Supplementary Fig. 1F). Moreover, lymphocytes were usually associated with blood vessels present in the lesion (Supplementary Fig. 1G–L).

The effect of lymphocyte grafting on OPC recruitment and differentiation was evaluated using the combination of Olig2 (expressed throughout the oligodendroglial lineage) and adenomatous polyposis coli/clone CC1 (expressed in mature stages of the lineage) immunolabelling (Fig. 1B–E) in the lesion border underlined by a strong staining of astrocytic marker glial fibrillary acidic protein (GFAP) (Fig. 1D–E). While no difference in the total number of Olig2⁺ cells was observed, the proportion of Olig2⁺CC1⁺ cells, representing OPC engaged in differentiation, was significantly reduced in multiple sclerosis lymphocyte grafted mice compared to healthy donor lymphocyte grafted mice and non-grafted (NG) mice (Fig. 1F). These results demonstrate that multiple sclerosis lymphocytes did not interfere with OPC recruitment, but impeded OPC differentiation.

Our data revealed that some patient lymphocytes have a deleterious effect while lymphocytes from other patients did not disturb OPC differentiation. Therefore, we defined two subgroups of multiple sclerosis patients based on their lymphocyte effect on OPC differentiation (Fig. 1N): multiple sclerosis patients with a high Olig2⁺CC1⁺ (Patients 1–5; Fig. 1N) and low Olig2⁺CC1⁺ profile (Patients 7–9; Fig. 1N). To investigate this heterogeneity, we performed electron microscopy on a second set of mice grafted with

Table 1 Multiple sclerosis patients and healthy donor cohort description

Characteristic	All multiple sclerosis	RRMS	SPMS	PPMS	Healthy donors
<i>n</i>	27	17	7	3	14
Female/male	19/8	13/4	4/3	2/1	6/8
Mean age (range) [IQR]	46.9 (25–72) [15.5]	43.2 (27–57) [12]	47.9 (25–67) [11.5]	65.7 (59–72) [6.5]	46.6 (28–65) [11.75]
Mean age at disease onset (range) [IQR]	30.4 (17–57) [13]	29.3 (17–44) [12]	23.9 (20–31) [5.5]	51.7 (48–57) [4.5]	
Mean disease duration (range) [IQR]	16.4 (4–47) [13]	14.0 (4–29) [11]	24.0 (5–47) [11.5]	12.7 (7–16) [0.5]	
Mean EDSS (range) [IQR]	3.6 (0–7) [3.75]	2.6 (0–6.5) [2]	5.8 (4–7) [1.75]	4.0 (3.5–4.5) [0.5]	
Mean MSSS (range) [IQR]	4.0 (0.04–8.83) [4.355]	3.0 (0.04–8.5) [2.88]	6.2 (1.69–8.83) [1.855]	4.8 (4.14–5.75) [0.805]	
Under treatment/treatment-free	18/9	16/2	3/4	0/3	

EDSS = Expanded Disability Status Scale; IQR = interquartile range; MSSS (EDSS related to disease duration) = Multiple Sclerosis Severity Score; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

lymphocytes of healthy donors (Fig. 1G and J), of the low subgroup (Fig. 1H and K) and of the high subgroup (Fig. 1I and L). The lesion area was calculated on semithin sections (Fig. 1G–I). Ultrathin sections (Fig. 1J–L) revealed axons remyelinated by oligodendrocytes (green), Schwann cells (blue) or non-remyelinated (yellow). The percentage of remyelinated area revealed a decrease of oligodendrocyte remyelination in mice grafted with low subgroup lymphocytes, which was significantly different both from the mice grafted with lymphocytes from healthy donors or the high subgroup (Fig. 1M).

Overall, multiple sclerosis lymphocyte presence within the lesion is sufficient to disturb oligodendrocyte remyelination and different patterns of remyelination were observed among the mice grafted with multiple sclerosis lymphocytes.

In vitro assessment of lymphocyte effect on microglial cell activation and oligodendrocyte precursor cell proliferation/differentiation

We explored *in vitro* the underlying cellular mechanisms of the decreased remyelination *in vivo*. As applying lymphocyte supernatant directly on OPC did not have a significant effect on OPC differentiation (Supplementary Fig. 2), we investigated whether lymphocytes affected OPC behaviour through their influence on MIGs.

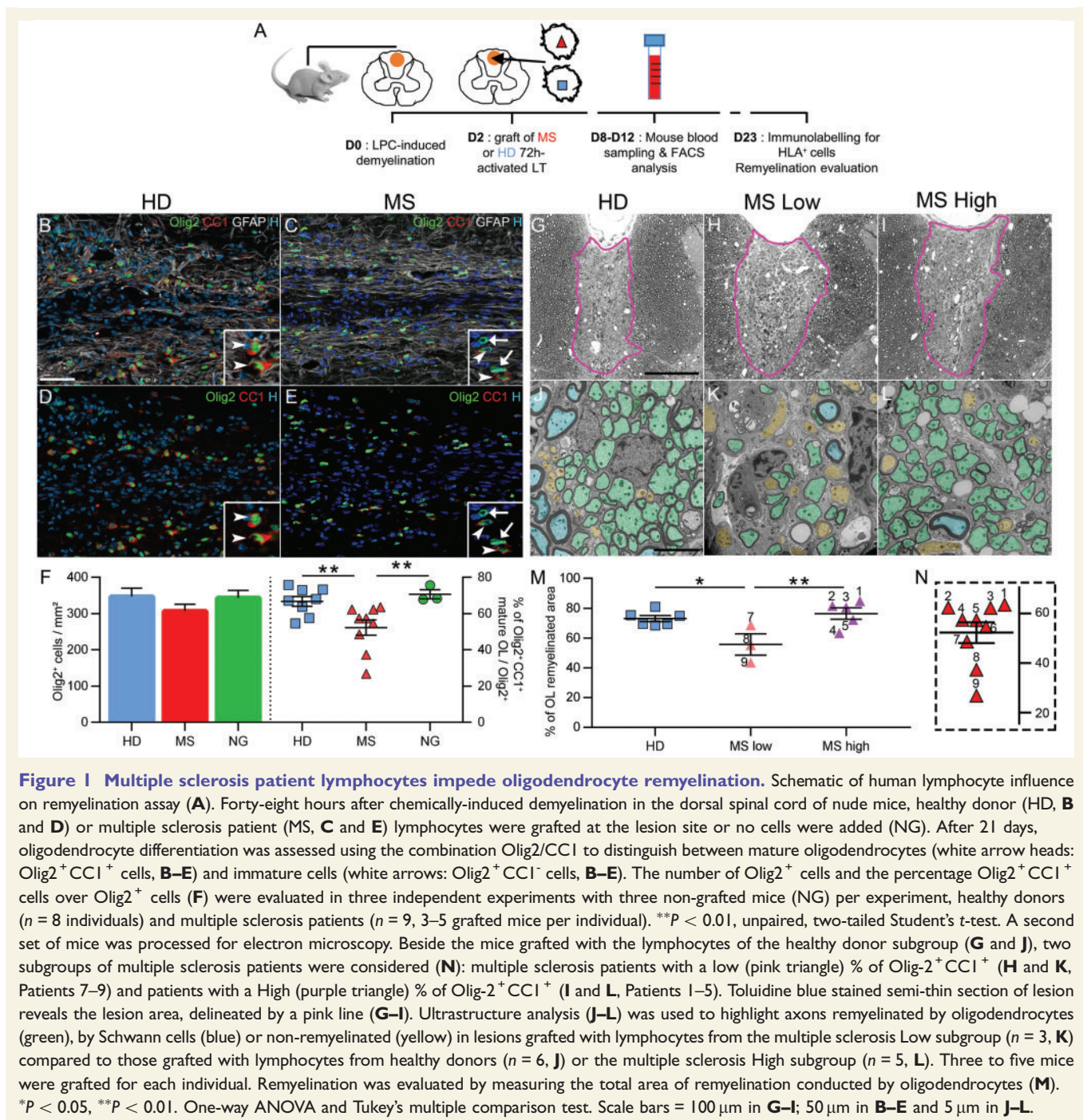
Lymphocyte supernatants from multiple sclerosis patients, healthy donors or fresh culture media [control condition (CT)] were added to MIGs (Fig. 2A). After 24 h, cells were fixed and stained for two markers of MIG activation: iNOS, which labels the M1 state (Fig. 2B–D, H–J), and IGF-1, a marker of the M2 state (Fig. 2B–G). We evaluated the ratio of iNOS⁺ cells to IGF-1⁺ (Fig. 2K) and showed that healthy donor lymphocyte supernatants did not change the M1/M2 ratio compared to control conditions, while multiple sclerosis lymphocyte supernatants induced a significant shift towards an M1 phenotype leading to an increase of the M1/M2 ratio. These results were further confirmed using arginase-1 (Arg-1), another M2 marker (Supplementary Fig. 3).

The consequences of MIG differential activation on OPC behaviour were next assessed. The conditioned media of MIGs pre-exposed to culture media (CT), healthy donors, or multiple sclerosis lymphocyte supernatants were placed in a primary culture of mouse OPCs (Fig. 3A). After 24 h, OPCs were fixed and labelled for Olig2 combined with Ki67, a marker of proliferation (Fig. 3B–D). The proportion of proliferating Olig2⁺Ki67⁺ cells was evaluated (Fig. 3E). We observed, in multiple sclerosis conditions, an increase of OPC proliferation compared to the control condition. Furthermore, MIG-conditioned media pre-exposed to multiple sclerosis patient lymphocyte supernatants induced a significant increase of OPC proliferation compared to healthy donor conditions. To analyse OPC differentiation, OPCs were fixed 72 h after exposure to the different MIG-conditioned media (Fig. 3A) and stained for three markers expressed at different stages of OPC differentiation: O4 for immature oligodendrocytes (Fig. 3F–H), GalC for pre-oligodendrocytes (Fig. 3J–L), and CNPase for mature oligodendrocytes (Fig. 3N–P). The proportion of positive cells for each marker was evaluated (Fig. 3I, M and Q). MIGs activated by multiple sclerosis patient lymphocyte supernatants appear to disturb OPC differentiation, as demonstrated by a significant reduction in the proportion of O4⁺ cells (Fig. 3I), GalC⁺ pre-oligodendrocytes (Fig. 3M) and CNPase⁺ mature oligodendrocytes (Fig. 3Q) in multiple sclerosis conditions compared to control or healthy donor conditions.

Thus, the preferential polarization of MIGs to the pro-inflammatory M1 phenotype, induced by multiple sclerosis patient lymphocyte supernatants, results in an increase of OPC proliferation and impairment of OPC maturation.

Identification of cellular and molecular cues leading to oligodendrocyte precursor cell differentiation

To assess potential differences between healthy donors and multiple sclerosis patients, we characterized immune cell composition by flow cytometry and evaluated their



secretory profiles by a Luminex-based multiplex assay. While no substantial difference in the subtypes of T helper or B cells between multiple sclerosis patients and healthy donors was observed (Supplementary Table 3), we found that, among the 72 molecules analysed (Fig. 4A), three were statistically differentially expressed between multiple sclerosis patients and healthy donors: IL-7 and IL-20 were increased while CCL19 was downregulated in multiple sclerosis patients versus healthy donor conditions (Fig. 4B). Thus, even if lymphocyte subtype proportions do not differ between multiple sclerosis patients and healthy donors, their lymphocytes have a different intrinsic capacity to respond to stimulation.

Our *in vivo* and *in vitro* results reveal a heterogeneity among multiple sclerosis patients (Supplementary Fig. 4): some patients exhibit a remyelination pattern close to healthy donors whereas other patients exhibit a low remyelination pattern. To test whether individual features of the patients or healthy donors might reflect this heterogeneity, we evaluated the effect of age (Fig. 5A and B) and sex (Fig. 5C and D), but no correlation was found with MIG activation (Fig. 5A and C) or OPC differentiation (Fig. 5B and D). Patients were next clustered according to their disease form (Fig. 5E and H), disease duration (Fig. 5F and I) and treatment status (Fig. 5G and J), but none of these clinical

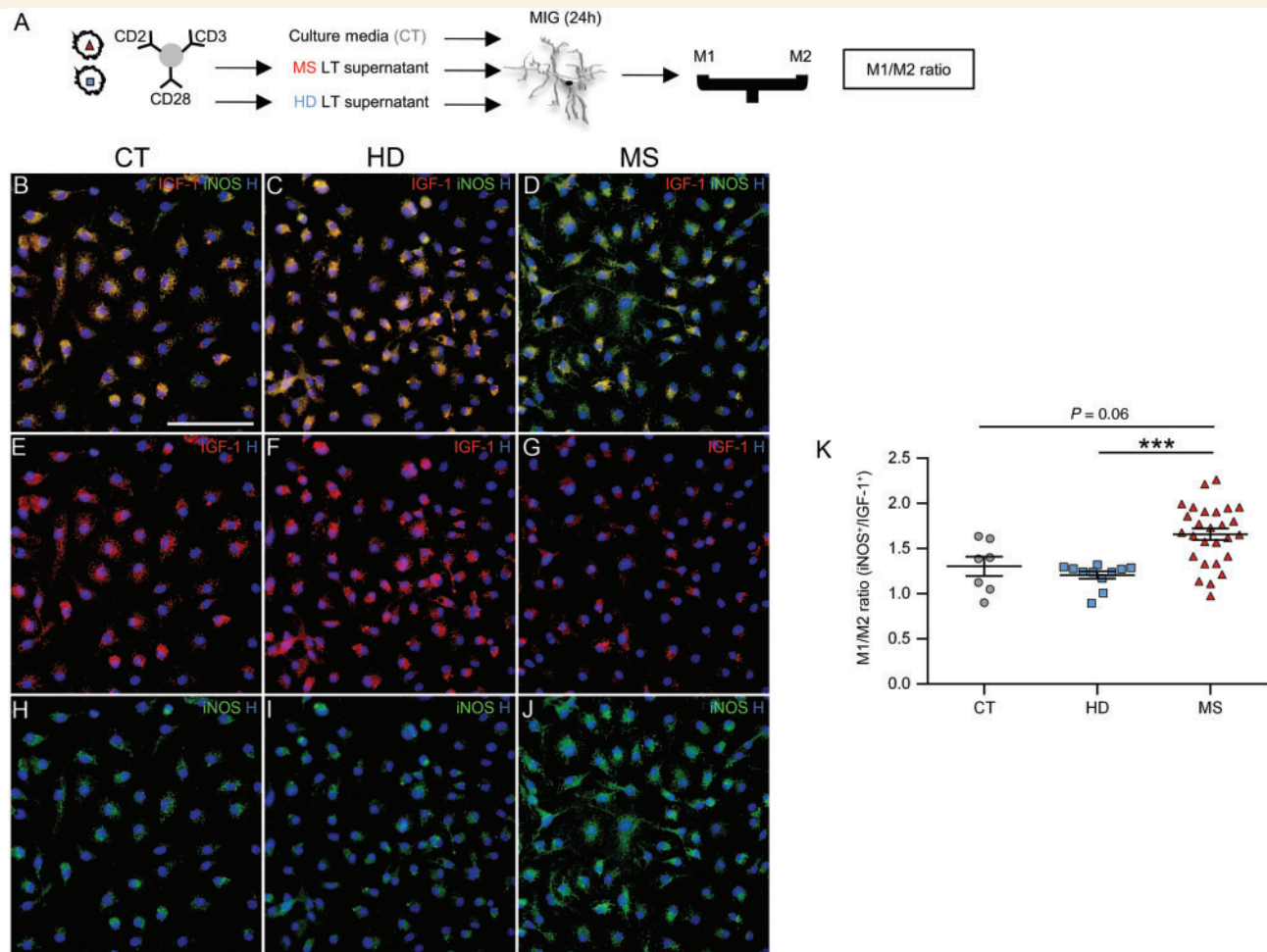


Figure 2 Multiple sclerosis patient lymphocyte supernatants induce an increase in the M1/M2 ratio of microglia. Schematic of the MIG activation assay in response to lymphocyte supernatants (A). Healthy donor (HD) or multiple sclerosis (MS) lymphocytes were activated during 72 h with anti CD2/CD3/CD28 antibodies and the supernatants were collected. Murine microglia cells were next exposed to culture media (CT) (B, E and H), healthy donor (C, F and I) or multiple sclerosis patient (D, G and J) lymphocyte supernatants over 24 h. M1 and M2 cells were labelled using iNOS (B–D, H–J) and IGF-1 (B–G), respectively. The iNOS⁺ IGF-1⁺ cells ratio was calculated for the CT (n = 7), healthy donors (n = 11) and multiple sclerosis (n = 27) groups (K). Each experiments was performed in triplicate. ***P < 0.001, Kruskal-Wallis and Dunn's multiple comparison test. Scale bar = 100 μ m.

features could explain the heterogeneous pattern observed. To further exclude an effect of a disease-modifying treatment on the heterogeneous remyelination pattern observed, we replicated our *in vitro* results in a second independent treatment-free cohort (Supplementary Table 3 and Supplementary Fig. 5A and B).

To characterize the cellular and molecular players inducing OPC differentiation heterogeneity, multiple sclerosis patients were clustered into two subgroups for further analysis: those inducing a high percentage of CNPase⁺ cells *in vitro* (HIGH) and those inducing a low percentage of CNPase⁺ cells (LOW). To detect the influence of combinations of variables (Schaefer and Strimmer, 2005) on OPC differentiation, we performed a multivariate analysis on our *in vitro* data using BADA with ranked data on the three subgroups (HIGH, LOW and healthy donors) based on lymphocyte secretion profiles. As expected, the healthy

donor profiles best discriminated from the two multiple sclerosis groups as indicated by their respective position on the first dimension (Fig. 6A). The second dimension highlighted the difference between the HIGH and LOW groups. Focusing on the differences among multiple sclerosis patients, we performed a partial least squares regression analysis (Vinzi *et al.*, 2010) (PLS) to find the best combination of lymphocyte composition, lymphocyte cytokine secretion, and MIG activation that could predict high levels of CNPase (Fig. 6B). We found that both MIG activation ($P < 0.001$) and lymphocyte cytokine secretion ($P < 0.05$) significantly predicted the percentage of CNPase (Fig. 6C). Bootstrap ratio analysis identified the cytokines/chemokines that strongly correlated with the percentage of CNPase⁺ cells. Leukaemia inhibitory factor (LIF), TNF-related apoptosis-inducing ligand (TRAIL), and IL-15 were positively correlated with the percentage

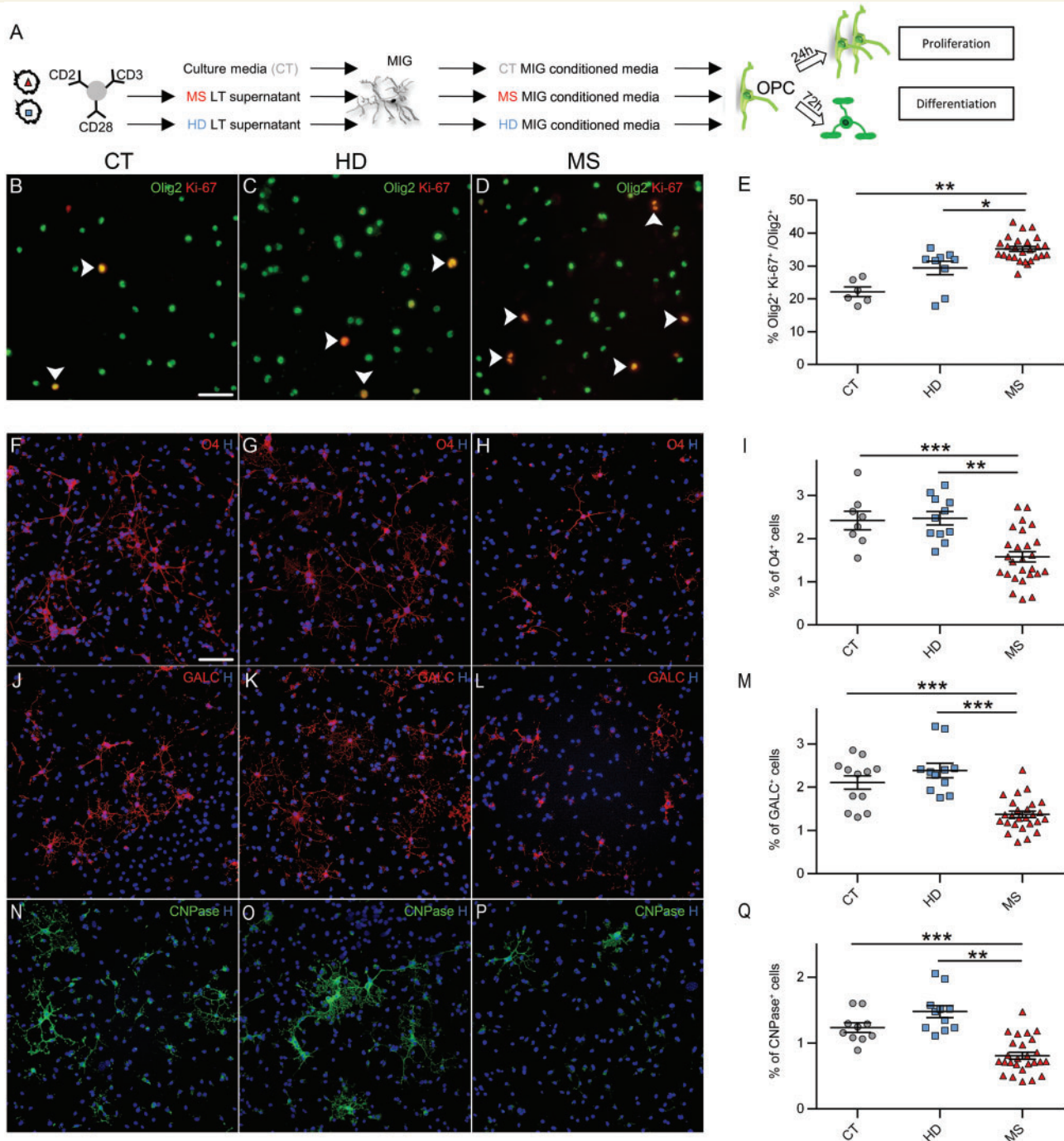


Figure 3 The conditioned media of MIG pre-exposed to multiple sclerosis patient lymphocyte supernatants induces an increase in OPC proliferation and a decrease in differentiation. Schematic of the OPC proliferation and differentiation assay in response to MIG-conditioned media (A). Murine OPCs were exposed over 24 h (proliferation) or 72 h (differentiation) to the MIG-conditioned media that were incubated with fresh culture media (CT) (B, F, J and N), to healthy donor (C, G, K and O) or to multiple sclerosis patient (D, H, L and P) lymphocyte supernatants. Proliferating OPCs were highlighted using Olig2⁺ and Ki67 (B–D). The percentage of Olig2⁺/Ki67⁺ proliferating OPCs (white arrowheads) among the total oligodendroglial cells Olig2⁺ was calculated in the culture media (n = 6), healthy donor (n = 9) and multiple sclerosis (n = 27) conditions (E). Each experiment was performed in quadruplicate. For the differentiation assay, the state of maturation of oligodendrocytes was analysed using chronologically expressed markers: O4 (F–H), GalC (J–L) and CNPase (N–P). The percentage of positive cells for each of the markers was calculated in CT (n = 8–12), healthy donor (n = 11) and multiple sclerosis (n = 27) conditions (I, M and Q). Each experiment was performed in triplicate. *P < 0.05, **P < 0.01. ***P < 0.001, Kruskal-Wallis and Dunn's multiple comparison test (E) and one-way ANOVA and Tukey's multiple comparison test (I, M and Q). Scale bar = 100 μm.

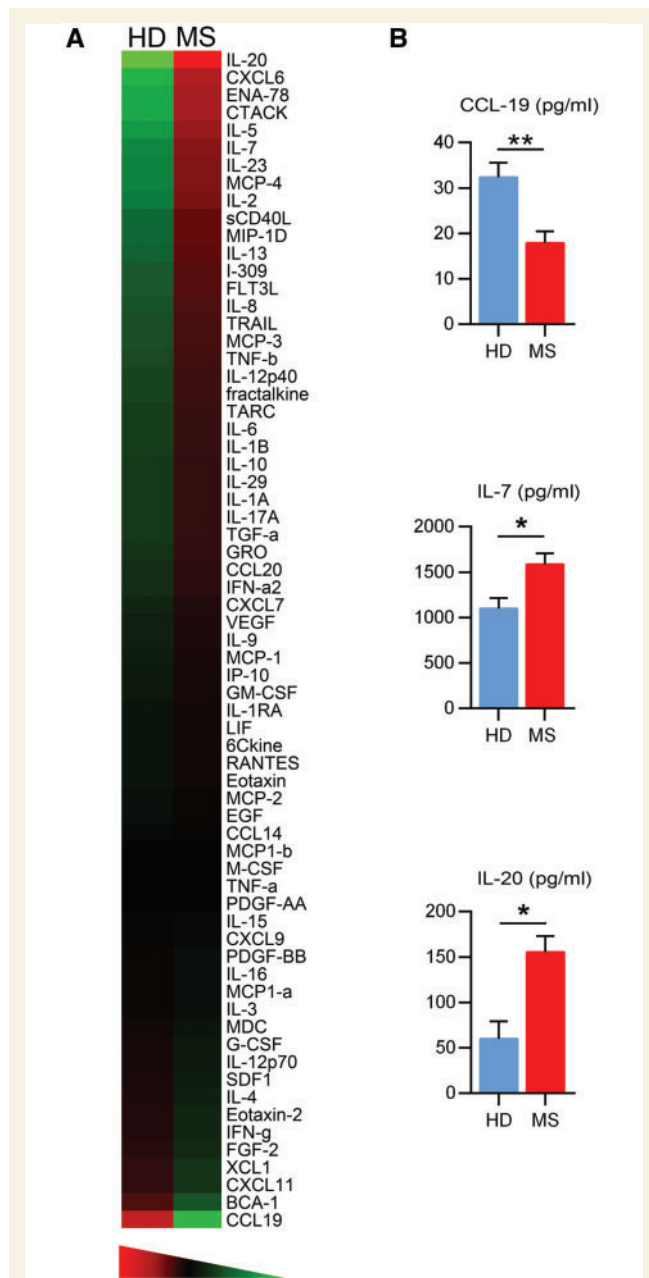


Figure 4 Multiple sclerosis patient and healthy donor lymphocytes have a different secretory pattern. After *in vitro* activation of multiple sclerosis patient (MS, $n = 27$) or healthy donor (HD, $n = 12$) lymphocytes with anti-CD2/CD3/CD28 antibodies over 72 h, supernatants were collected. The level of expression of 72 cytokines was evaluated by Luminex (67 cytokines were detectable). Mean values for each tested cytokine were calculated. A heatmap was generated with colour coding representing the lower values in green, the higher values in red, and in grey the values close to the mean for each cytokine in healthy donor and multiple sclerosis conditions (A). Three cytokines were evidenced as differentially expressed between multiple sclerosis patient ($n = 27$) and healthy donor ($n = 8$) lymphocytes (B). * $P < 0.05$, ** $P < 0.01$, Mann Whitney's test. See also Supplementary Table 3.

of CNPase, whereas stromal cell derived factor 1 (SDF-1/CXCL12), epithelial neutrophil-activating protein 78 (ENA-78/CXCL5) and CCL19 were negatively correlated with the percentage of CNPase (Fig. 6D).

To validate molecular candidates, we added CCL19 to lymphocyte supernatants and studied its effect on MIG activation and OPC differentiation. The addition of CCL19 in the lymphocyte supernatant of LOW patients did not have any significant effect on M1/M2 ratio (Fig. 6E, F and I) or OPC differentiation (Fig. 6J, K and N). However, when considering the HIGH group, adding CCL19 was enough to increase the M1/M2 ratio (Fig. 6G–I) and, as a consequence, decrease the percentage of CNPase⁺ (Fig. 6L–N) cells to values close to the LOW group. This deleterious action of CCL19 was replicated in an independent cohort of treatment-free patients (Supplementary Fig. 5C and D).

Discussion

In a comprehensive investigation, we demonstrated for the first time that multiple sclerosis patient lymphocytes control the local inflammatory environment and influence the remyelination process (Fig. 7). Our findings show that multiple sclerosis patient lymphocytes have a specific molecular signature that directs and/or maintains MIGs in a pro-inflammatory state, resulting in a remyelination defect. Capitalizing on both patient clinical information and a novel experimental model, we investigated factors that may account for the differential ability of multiple sclerosis patient lymphocytes to efficiently instruct repair. This new model is clinically relevant for multiple sclerosis as genetic studies of the diseases have shown the importance of genes encoding molecules of the immune system as the primary component in multiple sclerosis pathophysiology (The International Multiple Sclerosis Genetics Consortium *et al.*, 2012). Because remyelination seems to be efficient at early stages of the disease when inflammation manifests by successive acute attacks and remission periods, our model may be useful to understand reasons why remyelination may fail or succeed. As expected from what is known about the natural history of multiple sclerosis, the effects of multiple sclerosis patient lymphocytes were heterogeneous. Based on our original observations, we propose a new concept to account for the differential ability of patients to appropriately remyelinate, revealing the intrinsic capacity of each individual to coordinate the repair process based on their specific lymphocyte secretion profile.

An appropriate immune response controlled in time, space, and intensity is critical for remyelination, but its mechanisms in pathological and humanized context have remained speculative (Schwartz *et al.*, 1999). Both macrophages/MIGs depletion or inhibition (Kotter *et al.*, 2001, 2005) and lymphocyte depletion (Bieber *et al.*, 2003) led to impaired remyelination. The role of MIGs and lymphocytes are likely interlinked, as differential activation states in

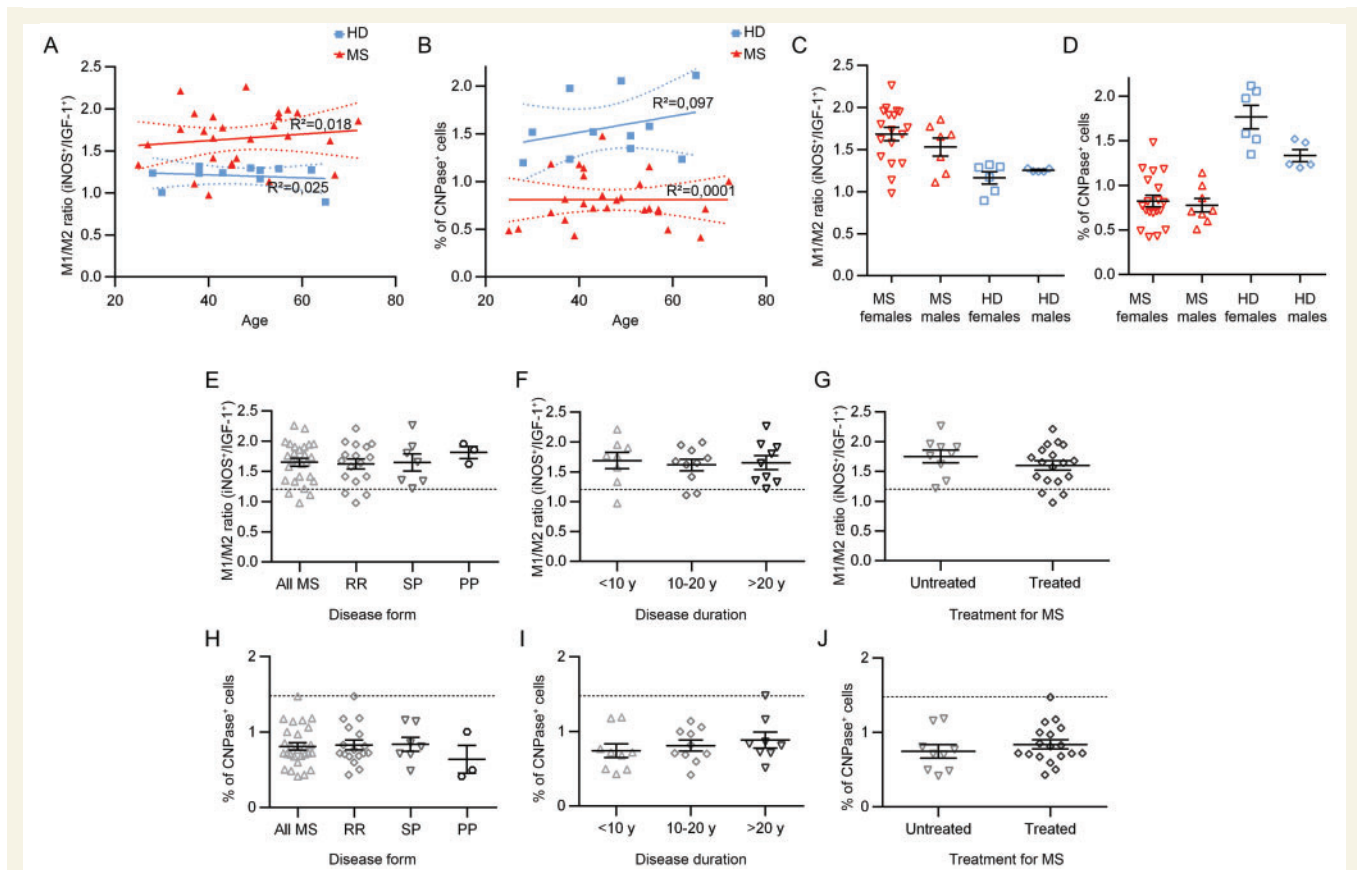


Figure 5 The capacity of multiple sclerosis patients to induce a proper inflammatory context for remyelination is not dependent on the age, sex, treatment status, disease form or duration. The M1/M2 ratio (A, C and E–G) and the percentage of CNPase⁺ cells (B, D and H–J) were calculated as previously described (Figs 2 and 3). Correlations between the age of individuals (patients or healthy donors) and the M1/M2 ratio (A) or the percentage of CNPase (B) that their lymphocyte supernatants were inducing were calculated. Patients (females $n = 18$, males $n = 8$) and healthy donors (females $n = 6$, males $n = 5$) were also stratified according to their sex (C and D). To analyse the influence of disease characteristics on the M1/M2 ratio or the percentage of CNPase⁺ cells, patients were next stratified according to their disease form (E and H): relapsing-remitting (RR, $n = 17$), secondary progressive (SP, $n = 7$), or primary progressive (PP, $n = 3$); to their disease duration (F and I): under 10 years ($n = 9$), between 10 and 20 years ($n = 10$), and >20 years ($n = 8$); or to their treatment status (G and J): either treated ($n = 18$), or not ($n = 9$). In graphs E–J, the mean value in healthy donor conditions is represented by a dashed line. Two-tailed unpaired student's t-test or one-way ANOVA and Tukey's multiple comparison test.

MIGs can be obtained through exposure to lymphocyte-derived cytokines. Th1-associated pro-inflammatory cytokine IFN- γ and Th2-associated cytokine IL-4 elicit activation states at the far ends of the spectrum: MIGs with a rather pro-inflammatory profile (M1) and MIGs endowed with more trophic properties (M2), respectively (Butovsky *et al.*, 2006). The injection of IL-4 treated MIGs in the CSF of EAE animal induced an increase of oligodendrogenesis in the spinal cord (Butovsky, 2006). In contrast, Th1 supernatants inhibit OPC differentiation *in vitro* (Moore *et al.*, 2015) and transfer of enriched myelin-reactive Th17 cells impedes remyelination in a cuprizone model (Baxi *et al.*, 2015). However, because cells were injected in the systemic compartment and not in the CNS parenchyma, the observed effects in the modulation of the remyelination process could be indirect.

To dissect precisely how the inflammatory environment shapes the success or failure of remyelination, we developed a new *in vivo* model, grafting human lymphocytes in the

demyelinated lesion induced in nude mouse spinal cord. As nude mice are athymic, the only lymphocytes present in the lesion are the grafted ones, and because remyelination is spontaneous and is well-characterized in this model (Jeffery and Blakemore, 1995) we could easily assess the remyelination efficacy. Healthy donor lymphocytes did not perturb the remyelination process compared to non-grafted animals but we observed a global deleterious effect on oligodendrocyte differentiation after multiple sclerosis lymphocyte grafting. In the last decade, the cause of the failure of remyelination in some patients has been debated: whether it results from a defect of OPC recruitment (Wolswijk, 2002; Boyd *et al.*, 2013), a decrease of OPC differentiation (Chang *et al.*, 2002), or both (Sim *et al.*, 2002). In our model, OPC recruitment was overall not disturbed but OPC differentiation was impeded, arguing in favour of a defect in OPC differentiation at the site of injury.

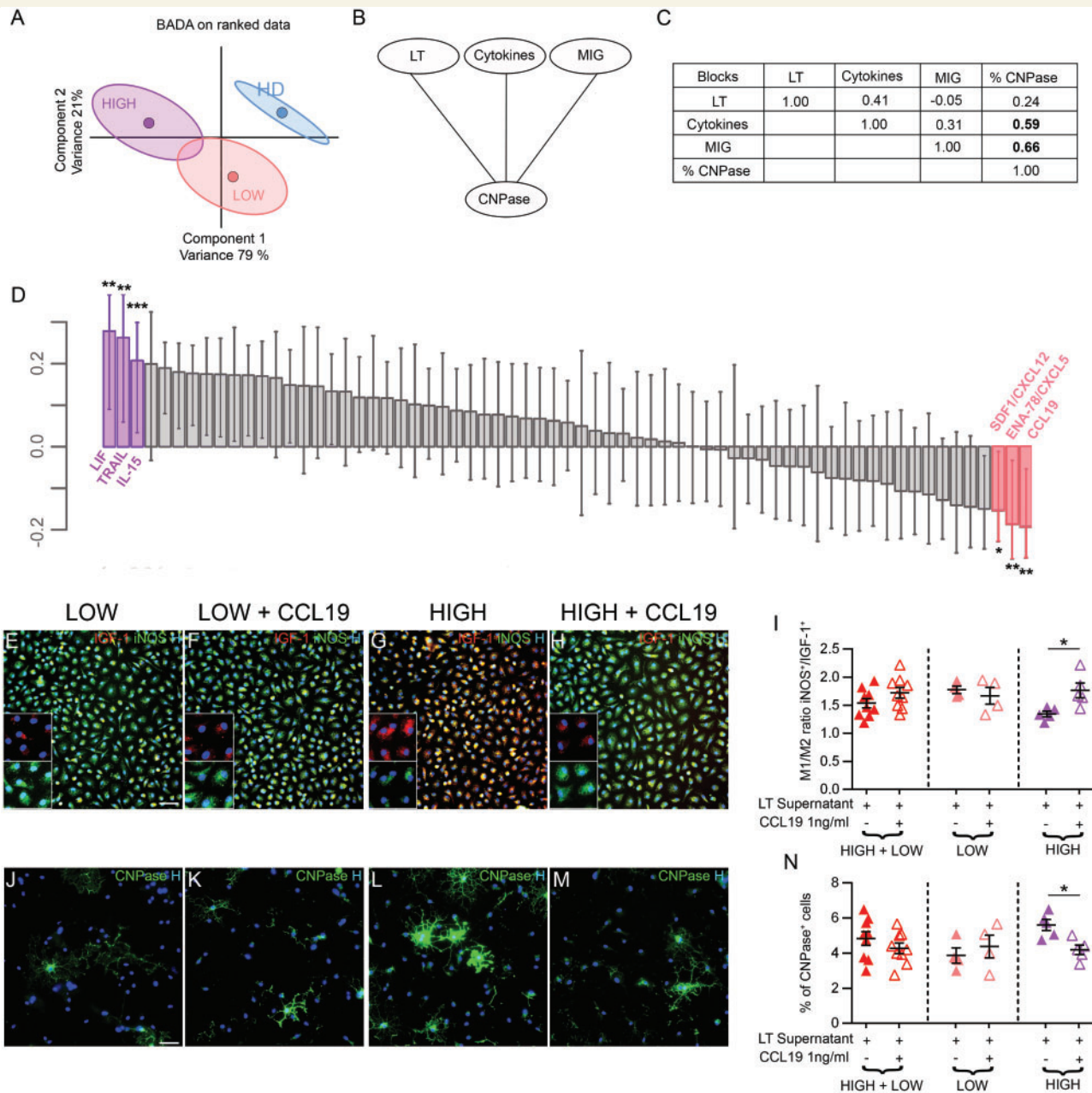


Figure 6 Multiple sclerosis patient lymphocyte secretory pattern analysis revealed the cytokines correlated to OPC differentiation. All individuals used for the study were clustered in three subgroups: healthy donors and multiple sclerosis patients inducing an *in vitro* CNPase expression either above (HIGH, $n = 13$) or below (LOW, $n = 13$) the median value. A barycentric discriminant analysis (BADA) was performed (A) to define which variables were implementing variability in our dataset. A partial least square regression was performed on the dataset of patients according to a linear model (B) to determine which of the three blocks (lymphocyte composition, cytokine expression level and MIG activation) were correlated and could predict the value of the percentage of CNPase⁺ cells. Correlations between the different blocks and the percentage of CNPase⁺ cells were calculated (C). Bootstrap ratios were calculated to evaluate the significance of each variable of each block allowing us to highlight cytokines that were not correlated (grey bars), significantly positively (purple bars) or significantly negatively (pink bars) correlated with the percentage of CNPase⁺ cells (D). The effect of CCL19 on OPC differentiation was evaluated in patients: MIG were exposed to lymphocyte (LT) supernatants (as described in Fig. 2) from LOW (E and F, $n = 4$) or HIGH (G and H, $n = 5$) with (F and H) or without (E and G) human recombinant CCL19 at 1 ng/ml. The M1/M2 ratio was calculated (I). Microglia-conditioned media pre-exposed to lymphocyte supernatants from LOW (J and K) or HIGH (L and M) subgroups, which were supplemented (K and M) or not (J and L) with CCL19 at 1 ng/ml were put on OPC (as described in Fig. 3). The proportion of CNPase⁺ cells was evaluated 72 h later (N). Each experiment was performed in quadruplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Bootstrap ratio (PLS) or paired two-tailed student's t-test.

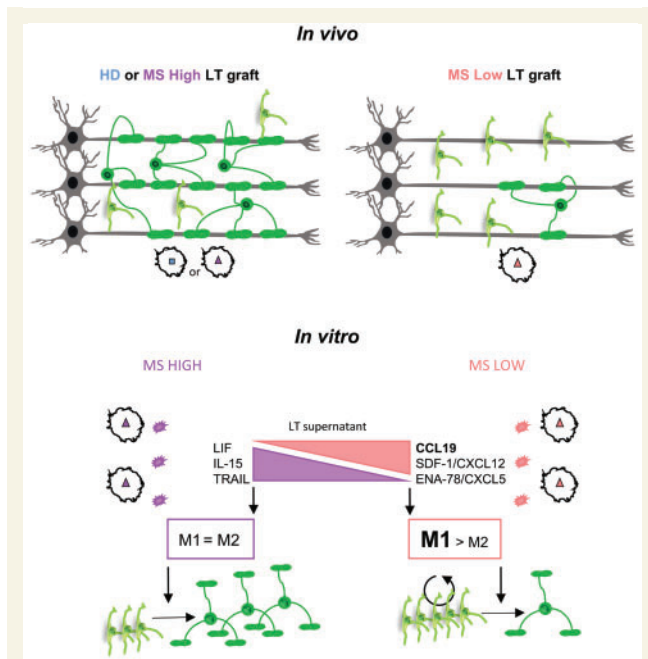


Figure 7 Schematic representation of multiple sclerosis patients and healthy donor lymphocyte influence on MIG activation, OPC behaviour and remyelination. *In vivo* OPC remyelination (but not OPC recruitment) were impeded after graft of multiple sclerosis patients (MS) with Low repair capacities compared to healthy donors (HD) and multiple sclerosis patients with High repair capacities. *In vitro*, the overexpression of CCL19, SDF-1(CXCL12), and ENA-78(CXCL5) in the lymphocyte (LT) supernatant of patients with LOW OPC differentiation capacities was strongly correlated with pro-inflammatory MIG activation and a decreased OPC differentiation.

To characterize the cellular and molecular mechanisms leading to remyelination failure *in vivo*, we analysed the effects of multiple sclerosis patient lymphocyte supernatants on MIG polarization/activation state. We showed that multiple sclerosis patient lymphocytes directed preferentially MIGs toward a M1 activation compared to healthy donor lymphocytes. This result confirmed post-mortem tissue studies on multiple sclerosis plaques, which reported that a majority of MIGs and macrophages present in chronically activated lesions display a pro-inflammatory phenotype (Koning *et al.*, 2007; Vogel *et al.*, 2013). Furthermore, OPC proliferation was increased *in vitro* and their differentiation impeded in multiple sclerosis patient conditions compared to CT and healthy donor conditions. The observed effect in multiple sclerosis patient conditions is detected early on in oligodendrocyte lineage, with a decrease of early marker of differentiation O4 expression and an even more striking effect with the mature markers GalC and CNPase. These results indicate that in multiple sclerosis conditions, even though normal OPC recruitment occurs, the pro-inflammatory context subsisting in the lesion mediated by M1 MIGs and/or the lack of M2 MIGs secreting trophic factors prevents efficient OPCs

differentiation. This concurs with the observations that in demyelinated multiple sclerosis lesions a high number of OPCs are present, a pattern underlining a failure of differentiation rather than recruitment (Wolswijk, 1998; Kuhlmann *et al.*, 2008).

To understand what drives the dissimilarity between healthy donors and multiple sclerosis patients, we investigated the difference between multiple sclerosis and healthy donor lymphocytes. We found the same proportion of different lymphocyte subtypes, indicating that there is no major over-representation of certain circulating lymphocytes between multiple sclerosis patients and healthy donors. However, we found that the major difference between healthy donors and multiple sclerosis lymphocytes resides in a differential functional response to activation with a specific secretory profile defining the two subgroups. We identified three differentially expressed cytokines/chemokines between healthy donors and multiple sclerosis patients: IL-7, IL-20 and CCL19. Even though the dysregulation of IL-7 and IL-20 have been identified as key players in various autoimmune diseases (Lundström *et al.*, 2012; Finch *et al.*, 2013; Rutz *et al.*, 2014), none of these molecules are known to have a major role either in MIG activation or in remyelination.

In addition, we also observed a strong heterogeneity in the effects of multiple sclerosis patient lymphocytes on MIG activation, *in vitro* OPC differentiation and *in vivo* remyelination. While lymphocytes from some of the patients induced a proregenerative MIG activation that led to proper OPC differentiation and successful remyelination *in vivo* (high group), other patients' lymphocytes induced a hostile environment for remyelination (low group), a pattern suggesting that the intrinsic capacity to induce remyelination is specific to each patient. This theory was previously suggested by the observations in post-mortem tissue that a subset of multiple sclerosis patients exhibited more than 80% of their lesions remyelinated (Patrikios *et al.*, 2006). Recently, the concept of good or bad 'remyelinator' was also supported by the dynamic imaging of remyelination patterns among multiple sclerosis patients (Bodini *et al.*, 2016).

Gathering all our datasets, we performed a multivariate analysis to define which factors drive heterogeneity between HIGH and LOW multiple sclerosis patients and identified LIF, SDF-1 (CXCL12), ENA78 (CXCL5), TRAIL, IL-15 and CCL19 as discriminating factors between HIGH and LOW multiple sclerosis patients. We confirmed the effect of known inhibitors such as SDF-1 (CXCL12) (Banisadr *et al.*, 2011; Williams *et al.*, 2014) or positive regulators such as LIF on remyelination (Fischer *et al.*, 2014). We also identified new interesting molecular players such as TRAIL, IL-15, ENA78 (CXCL5) and CCL19. While previous studies suggested an ambivalent role of TRAIL and IL15 in multiple sclerosis and its animal models (Diehl *et al.*, 2004; Hoffmann *et al.*, 2009; Schneider *et al.*, 2011; Rentzos and Rombos, 2012; Broux *et al.*, 2015), these two cytokines were highly correlated to both M2 MIG polarization

and OPC differentiation and are of great interest for therapeutic applications.

CCL19 is a T cell attractive chemokine and has a major role in balancing immunity and tolerance through its receptor CCR7. Indeed, increased expression of CCR7 is also correlated to MIG M1 phenotype (Förster *et al.*, 2008). We found CCL19 to be negatively correlated with MIG M2 phenotype and OPC maturation when comparing ‘LOW’ versus ‘HIGH’ subgroups. Intriguingly, CCL19 is downregulated in multiple sclerosis lymphocytes compared to healthy donor supernatants. The BADA analysis indicated that healthy donor profiles and multiple sclerosis patient ‘HIGH’ versus ‘LOW’ profiles do not seem to involve the same molecular players. Thus, while we could have expected CCL19 to have a positive effect when considering its high expression in healthy donor conditions, the addition of CCL19 to the ‘HIGH’ subgroup lymphocyte supernatant was sufficient to elicit an M1/M2 ratio and CNPase levels close to those observed with the ‘LOW’ subgroup. This suggests that the molecular players in remyelination are really specific in pathological conditions compared to healthy donor conditions. We are therefore considering using different approaches based on CCL19 antagonism to correct the negative effect observed in the ‘LOW’ group.

Taking advantage of our innovative *in vivo* model to study the role of human lymphocytes in remyelination, we demonstrated a strong implication of adaptive immune cells in this repair process. In particular, multiple sclerosis patient lymphocytes induce detrimental environment for the repair process notably by directing MIGs toward a pro-inflammatory M1 phenotype. Strikingly, the molecular cues needed for a successful remyelination were different when considering multiple sclerosis patient and healthy donor lymphocytes and this difference suggests a new concept in myelin repair. Additionally, we reproduce individual intrinsic capacities for remyelination among multiple sclerosis patients. Further investigation of the specific features of multiple sclerosis patients with high repair capacities needs to be conducted but our results pave the way to new strategies to identify molecular targets particular to pathological conditions.

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Supplementary material

Supplementary material is available at *Brain* online.

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