

BRAIN COMMUNICATIONS

Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses

Malou Jansen,^{1,2}  Arlette L. Bruijstens,^{2*} Jamie van Langelaar,^{1*} YuYi Wong,²
Annet F. Wierenga-Wolf,¹ Marie-José Melief,¹  Liza Rijvers,¹ E. Daniëlle van Pelt,²
 Joost Smolders,^{1,2} Beatrijs H. Wokke² and Marvin M. van Luijn¹

*Shared second author.

Neuromyelitis optica spectrum disorders are a group of rare, but severe autoimmune diseases characterized by inflammation of the optic nerve(s) and/or spinal cord. Although naive B cells are considered key players by escaping central tolerance checkpoints, it remains unclear how their composition and outgrowth differ in patients with neuromyelitis optica spectrum disorders. Under complete treatment-naïve circumstances, we found that naive mature/transitional B-cell ratios were reduced in the blood of 10 patients with aquaporin-4 immunoglobulin G-positive disease (neuromyelitis optica spectrum disorders) as compared to 11 both age- and gender-matched healthy controls, eight patients with myelin oligodendrocyte glycoprotein-immunoglobulin G-associated disorders and 10 patients with multiple sclerosis. This was the result of increased proportions of transitional B cells, which were the highest in patients with neuromyelitis optica spectrum disorders with relapses and strongly diminished in a separate group of nine patients with neuromyelitis optica spectrum disorders and myelin oligodendrocyte glycoprotein-immunoglobulin G-associated disorders who received corticosteroid treatment. These findings need to be confirmed in longitudinal studies. For purified naive mature B cells of seven patients with neuromyelitis optica spectrum disorders and myelin oligodendrocyte glycoprotein-immunoglobulin G-associated disorders with relapses, Toll-like receptor 9 ligand synergized with interferon- γ to enhance plasmablast formation during germinal centre-like cultures. This was not seen for 11 patients without relapses and nine healthy controls. In the neuromyelitis optica spectrum disorders group, *in vitro* plasmablast formation corresponded to total and anti-aquaporin-4 immunoglobulin G secretion, of which the latter was found only for relapsing cases. These data indicate that naive B-cell homeostasis is different and selectively targeted by corticosteroids in patients with neuromyelitis optica spectrum disorders. This also supports further exploration of naive B cells for their use in Toll-like receptor 9-dependent *in vitro* platforms in order to predict the activity of neuromyelitis optica spectrum disorders.

1 Department of Immunology, MS Center ErasMS, Erasmus MC, Rotterdam, 3015 GE, The Netherlands

2 Department of Neurology, MS Center ErasMS, Erasmus MC, Rotterdam, 3015 GD, The Netherlands

Correspondence to: Marvin M. van Luijn, PhD, Erasmus MC, MS Center ErasMS, Department of Immunology, Wytemaweg 80, 3015 GE, Rotterdam, The Netherlands
E-mail: m.vanluijn@erasmusmc.nl

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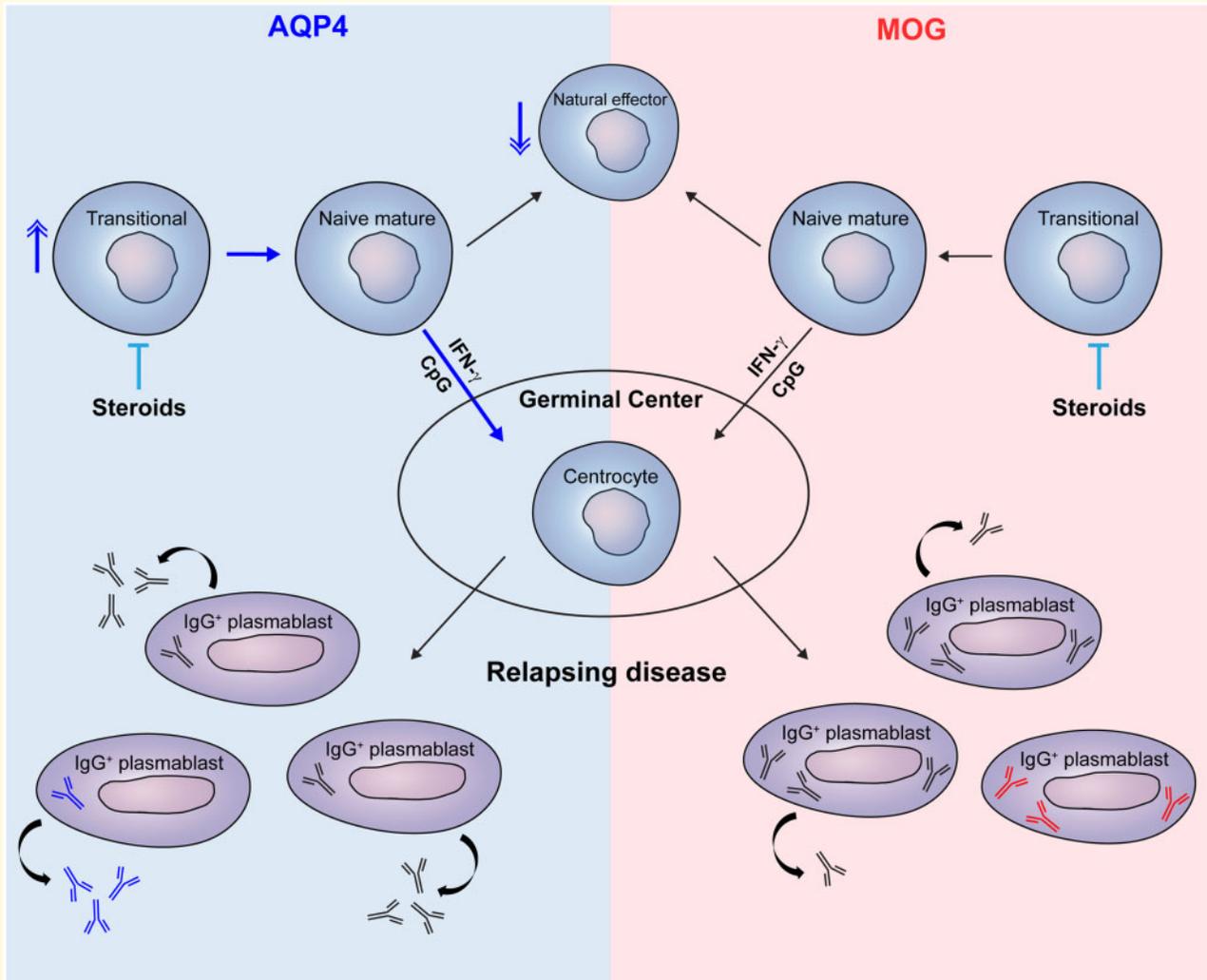
Abbreviations: AQP4 = aquaporin-4; CD40L = CD40 ligand; FACS = fluorescence-activated cell sorting; HEK = human embryonic kidney; IFN- γ = interferon-gamma; Ig = immunoglobulin; IL = interleukin; MOG = myelin oligodendrocyte glycoprotein; MOGAD = MOG-IgG-associated disorder; MS = multiple sclerosis; NMOSD = neuromyelitis optica spectrum disorder; T-bet = T-box transcription factor; Th = T helper; TLR9 = Toll-like receptor 9

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Graphical Abstract



Introduction

Neuromyelitis optica spectrum disorders (NMOSD) are rare and convey a range of severe clinical presentations caused by inflammation with preferential involvement of the optic nerves and spinal cord (Wingerchuk *et al.*, 2007). Although the exact cause of these divergent presentations remains poorly understood, the dominant role of the B-cell lineage is undisputed (Sabatino *et al.*, 2019). In approximately 75% of NMOSD patients, immunoglobulin G (IgG) antibodies are found that target the neuronal water channel protein aquaporin-4 (AQP4; Saadoun and Papadopoulos, 2010). Furthermore, 30–40% of AQP4-IgG-negative NMOSD patients test positive for antibodies against myelin oligodendrocyte glycoprotein (MOG; Pelt van *et al.*, 2016a; Hamid *et al.*, 2017), which are associated with a distinct entity termed MOG-IgG-associated disorders (MOGAD).

There are significant differences in clinical features between AQP4-IgG-positive NMOSD and MOGAD (Kitley *et al.*, 2012; Pelt van *et al.*, 2016b; Jurynczyk *et al.*, 2017), including a higher frequency and worse recovery from relapses in AQP4-IgG-positive NMOSD. Relapses are commonly treated with corticosteroids in both entities. To prevent relapses, AQP4-IgG-positive NMOSD patients and relapsing patients with MOGAD are usually treated with maintenance therapy. Currently, no biomarkers are available to accurately predict relapses and guide treatment decisions. This could be due to the fact that previous studies on the immunopathogenesis of NMOSD primarily used patients treated with corticosteroids or other maintenance therapy, and hence possibly obliterating disease-relevant B-cell subsets.

Recent findings reveal that AQP4-specific B cells are already present in naive populations that escape early tolerance checkpoints (Wilson *et al.*, 2018; Cotzomi *et al.*, 2019). Normally, self-reactive clones are counterselected

during early B-cell development in the bone marrow (central tolerance) and during subsequent maturation of transitional into naive mature B cells after entering the circulation (peripheral tolerance). In patients with AQP4-IgG-positive NMOSD, naive mature B cells escape both these checkpoints and likely develop into antibody-secreting cells in a germinal centre-dependent manner (Kowarik *et al.*, 2017; Wilson *et al.*, 2018; Cotzomi *et al.*, 2019). In systemic autoimmune disease, which coexists in ~20% of AQP4-IgG-positive NMOSD patients (Shahmohammadi *et al.*, 2019), interleukin (IL)-21, interferon (IFN)- γ and Toll-like receptor (TLR9)-ligand CpG-ODN serve as key triggers of auto-reactive germinal centre B cells expressing T-box transcription factor T-bet (Sindhava *et al.*, 2017; Wang *et al.*, 2018).

In this study, we aimed to define the impact of AQP4-IgG serostatus, steroid treatment and relapse occurrence on naive B-cell development in NMOSD. The composition of the naive B-cell pool within the blood was compared between NMOSD, MOGAD and multiple sclerosis groups with and without corticosteroid treatment, as well as matched healthy controls. Furthermore, naive B-cell outgrowth into (anti-AQP4 or -MOG) IgG-secreting plasmablasts was explored *in vitro* for patients with and without relapses during T-bet-inducing, germinal centre-like cultures.

Materials and methods

Participants

We included 10 treatment-naive AQP4-IgG-positive NMOSD patients (Wingerchuk *et al.*, 2015) and eight treatment-naive patients with MOGAD (all with optic neuritis and/or transverse myelitis). These patients did not get immune suppressive therapy before blood sampling; no steroids within 1 month and no other maintenance treatment within 3 months. Additionally, five patients with AQP4-IgG-positive NMOSD and four patients with MOGAD were included who received corticosteroids (i.e. oral prednisone or intravenous methylprednisolone) within 1 month prior to sampling. In the corticosteroid-treated MOGAD group, two patients had an NMOSD phenotype, one patient presented with acute disseminated encephalomyelitis and one patient was diagnosed with encephalitis. None of the patients included in this study received therapy with prolonged immune suppressive activity, such as anti-CD20 or any other B-cell-directed monoclonal antibodies before sampling. Patient characteristics are summarized in Table 1.

Ex vivo B-cell subset frequencies were compared to age- and gender-matched treatment-naive multiple sclerosis patients ($n=10$) as well as healthy controls ($n=20$). As a reference group for corticosteroid-treated patients, we included eight clinically isolated syndrome patients treated with methylprednisolone within 1 month before

sampling and diagnosed according to the McDonald 2017 criteria. For the NMOSD and MOGAD groups, serum was collected at the same time as peripheral blood mononuclear cells. An NMOSD relapse was defined as a new episode of disease activity at least 3 months separated from the previous disease episode. All patients gave written informed consent, and the study was approved by the medical ethics committee of Erasmus MC.

Total immunoglobulin G ELISA

Immunoglobulin G concentrations in supernatants were determined by ELISA using flat-bottom 96-well half-area plates (Corning, Tewksbury, USA) coated overnight at 4°C with goat anti-human Ig (1 mg/ml; Southern Biotech, Birmingham, USA). Plates were washed with PBS/0.05% Tween-20 to remove unbound antibody and blocked with PBS/5% FCS for 2 h at room temperature. Sample and a human IgG standard (Sigma-Aldrich/Merck, Darmstadt, Germany) were added for 1.5 h at room temperature. Subsequently, plates were washed with PBS/0.05% Tween-20 and bound IgG was detected by peroxidase-conjugated goat anti-human IgG (Thermo Fisher Scientific, Landsmeer, The Netherlands). TMB Substrate (Thermo Fisher Scientific) was used to reveal peroxidase activity. The reaction was stopped with H₂SO₄ and optical density was measured at 450 nm.

AQP4- and MOG-IgG cell-based assays

For the determination of AQP4- and MOG-IgG levels in sera and culture supernatants, standardized cell-based assays were used as described previously (Ketelslegers *et al.*, 2011; Pelt van *et al.*, 2016a,b). In short, either HEK293T transfectants with EGFP-tagged AQP4-M23 or 1:1 mixtures of LN18 cells transfected with and without full-length human MOG were incubated with the sample and stained with goat anti-human secondary antibody (IgG labelled with APC; Jackson ImmunoResearch, Amsterdam, The Netherlands). Our AQP4-IgG cell-based assays showed a mean transfection efficiency (GFP⁺) of ~35%. Mean fluorescence intensity (MFI) representing the amount of AQP4- or MOG-IgG bound to the cell surface was compared between transfected (GFP⁺) and untransfected (GFP⁻) cells within the same experiment using flow cytometry.

Cell isolation, antibodies and flow cytometry

Peripheral blood mononuclear cells were collected using Vacutainer CPT[®] tubes containing sodium heparin according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). After centrifugation, cells were taken up in RPMI 1640 (Lonza, Basel, Switzerland) containing 40% of fetal calf serum (Lonza)

Table 1 Patient characteristics

Cohort	Age ^a (years)/ Gender	Disease location onset + location relapse	Treatment ^{a,b}	Time since first event ^a (months)	Relapse	Serum AQP4-IgG/ MOG-IgG level (Δ MFI) ^{a,c}	Ex vivo subgroup	Used in vitro
AQP4-IgG								
Patient 1	41/F	ON + ON	None	7	Yes	7644	NMOSD	Yes
Patient 2	39/F	TM + 2×TM	None	78	Yes	13 365	NMOSD	Yes
Patient 3	56/F	ON bilateral + ON/BS	None	6	Yes	13 736	NMOSD	Yes
Patient 4	61/F	TM	None	5	No	7469	NMOSD	Yes
Patient 5	46/M	ON bilateral	None	15	No	545	NMOSD	Yes
Patient 6	47/F	ON	None	2	No	15 437	NMOSD	Yes
Patient 7	27/F	TM	None	46	No	7874	NMOSD	Yes
Patient 8	26/F	BS	None	122	No	7262	NMOSD	Yes
Patient 9	36/F	ON	None	0	No	ND	NMOSD	Yes
Patient 10	46/F	TM	None	4	No	20 533	NMOSD	No
Patient 11	34/F	TM + TM	P	5	Yes	82	CS-treated	No
Patient 12	50/F	TM + 2×TM	MP	13	Yes	17 061	CS-treated	No
Patient 13	19/F	BS + TM	MP + P	6	Yes	14 055	CS-treated	No
Patient 14	58/F	TM/ON bilateral	MP + P	2	No	15 618	CS-treated	No
Patient 15	37/F	BS/ON bilateral	MP + P	4	No	16 701	CS-treated	No
MOG-IgG								
Patient 1	38/F	ON + 5×ON	None	95	Yes	24 237	MOGAD	Yes
Patient 2	26/M	ON + ON	None	25	Yes	5099	MOGAD	Yes
Patient 3	25/F	TM + 2×ON	None	17	Yes	424	MOGAD	Yes
Patient 4	32/M	ADEM + TM	MP	326	Yes	1681	CS-treated	Yes
Patient 5	52/M	ON	None	10	No	136	MOGAD	Yes
Patient 6	40/F	ON bilateral	None	10	No	1234	MOGAD	Yes
Patient 7	56/F	TM	None	18	No	696	MOGAD	Yes
Patient 8	37/M	TM/ON	MP	1	No	6495	CS-treated	Yes
Patient 9	26/F	ON bilateral	MP + P	1	No	2058	CS-treated	Yes
Patient 10	25/F	ON bilateral + 3×ON	None	201	Yes	6617	MOGAD	No
Patient 11	32/M	E + E	MP	181	Yes	21	CS-treated	No
Patient 12	29/M	TM	None	51	No	1200	MOGAD	No

ADEM, acute disseminated encephalomyelitis; BS, brainstem (area postrema or cranial nerves); CS, corticosteroid; E, encephalitis; MFI, mean fluorescence intensity; MP, methylprednisolone (intravenous); ND, not determined; ON, optic neuritis; bilateral, both eyes; P, prednisone (oral); TM, transverse myelitis.

^aAt time of sample collection.

^bSteroid treatment within 1 month before sampling (all patients did not receive maintenance treatment for at least 3 months).

^cAll AQP4-IgG and MOG-IgG serum titres were measured within the same experiment using Δ MFI.

and 20% of dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen until further use. *Ex vivo* naive mature (CD19⁺CD38^{dim/}CD27⁻IgG⁻IgA⁻) B cells were purified for *in vitro* cultures using a BD FACSAria III cell sorter. For immunophenotyping, cells were incubated with Fixable Viability Stain 700 (BD Biosciences) for 15 min and monoclonal antibodies for 30 min at 4°C. The following FACS antibodies were used: CD24 (BV605, ML5), CD27 (BV421, M-T271), IgD (PE-CF594, IA6), IgG (APC-H7, G18-145; BD Biosciences), CD19 (BV785, HIB19), CD38 (PE-Cy7, HIT2), IgM (BV510, MHM-88), T-bet (PE-Cy7, 4B10; Biolegend, London, UK) and IgA (FITC, IS11-8E10; Miltenyi Biotec, Bergisch Gladbach, Germany). For intracellular T-bet staining, cells were fixed with 2% paraformaldehyde (Merck, Schiphol-Rijk, The Netherlands) and permeabilized using PBS (pH=7.4) containing 0.3% BSA and 0.5% saponin (Sigma-Aldrich). All measurements were calculated with an LSRII-Fortessa flow cytometer and analyzed using FACS Diva software, version 8.0.1 (BD Biosciences). For both *ex vivo* and *in vitro* analyses, we first gated on viable CD19⁺ B cells.

Germinal centre-like B-cell differentiation assay

Germinal centre-like B-cell cultures were performed as described recently (van Langelaar et al., 2019). In short, irradiated murine 3T3 fibroblasts expressing human CD40L were co-cultured with sorted naive mature (CD27⁻CD38^{dim/}-IgG⁻IgA⁻) B cells in the presence of IL-21 (50 ng/ml; Thermo Fisher Scientific) with and without IFN- γ (50 ng/ml; Peprotech, Huissen, The Netherlands) and CpG-ODN (10 μ g/ml; InvivoGen, San Diego, CA, USA). After 11 days of culturing, cells were stained for flow cytometry and supernatants were stored and analyzed for the presence of AQP4- and MOG-IgG.

Statistical analysis

Statistical analysis was performed using Graphpad Prism Software, version 5.04. Kruskal-Wallis and Dunn's *post-hoc* tests were performed for comparing multiple groups. Mann-Whitney *U*-tests were used for comparing two groups. Paired data sets were assessed using Wilcoxon

signed-rank tests. Correlations between variables were tested using the Spearman rank or Pearson coefficients, depending on the results of the D'Agostino and Pearson omnibus normality test. Percentages and MFI were displayed as the mean. *P*-values of <0.05 were considered statistically significant.

Data availability

The raw data that support the here-described findings are available from the corresponding author upon reasonable request.

Results

Naive mature/transitional B-cell ratios are reduced in the blood of treatment-naive AQP4-IgG-positive NMOSD patients

First, we assessed the proportions of naive and memory B-cell subsets in the blood of NMOSD patients without any form of previous immune-suppressive treatment. Because the female-to-male ratio is higher in AQP4- than MOG-IgG-associated disease (Table 1; Pelt van *et al.*, 2016a), we selected healthy controls who were age- and gender-matched either to treatment-naive patients with NMOSD ($n = 11$) or MOGAD ($n = 9$). The proportion of transitional B cells (CD38⁺⁺CD27⁻) and not naive mature (CD38^{dim/-}CD27⁻IgM⁺) B cells (Timmermans *et al.*, 2016; Heeringa *et al.*, 2018) was elevated in NMOSD versus healthy controls (mean 16.2% versus 5.6% for transitional B cells and 47.3% versus 41.5% for naive mature B cells, respectively), which resulted in lowered naive mature/transitional B-cell ratios (Fig. 1A and B). This classification of naive B cells was confirmed using IgD and CD24 (Supplementary Fig. 1). No differences were found between the MOGAD and the healthy control group (Fig. 1C and D). Two out of three NMOSD patients with relapses showed extremely high frequencies of transitional B cells (35% and 44% of the total B-cell pool, Fig. 1B). Naive mature/transitional B-cell ratios were lower in NMOSD compared to MOGAD ($P < 0.01$; Fig. 1F).

Germinal centre-independent natural effector (CD38^{dim/-}CD27⁺IgM⁺IgD⁺) memory B cells (Berkowska *et al.*, 2011) were significantly reduced in the NMOSD versus healthy control group (lowest in two relapsing cases with the highest percentage of transitional B cells; Supplementary Fig. 2A and B). A similar trend was found in the MOGAD group (Supplementary Fig. 2B). The proportions of germinal centre-dependent IgM-only B cells (CD38^{dim/-}CD27⁺IgM⁺IgD⁻) and IgG⁺ (both CD27⁺ and CD27⁻) B cells (Berkowska *et al.*, 2011) (Supplementary Fig. 2B) or plasmablasts

(CD38⁺⁺CD27⁺⁺; Supplementary Fig. 3A) did not differ between groups. None of the populations and ratios correlated to AQP4- or MOG-IgG serum levels (Table 1) in the NMOSD groups (data not shown).

Corticosteroid treatment corresponds to increased naive mature/transitional B-cell ratios in AQP4- and MOG-IgG-positive disease

To study the impact of corticosteroids as a standard treatment of acute relapses, we compared our results to B-cell subsets from the blood of nine AQP4-IgG or MOG-IgG-positive patients who received only corticosteroids and no other forms of immunosuppressive treatment. In this group, naive mature/transitional B-cell ratios were significantly elevated (fold change versus NMOSD: 14.8, $P < 0.0001$; Fig. 1E and F), which correlated positively with time since start of corticosteroid treatment ($r = 0.86$ and $P = 0.002$, Fig. 1G). These elevated ratios were the result of an almost complete absence of transitional B cells (Fig. 1E, Supplementary Fig. 4). The association of corticosteroid treatment with transitional and not naive mature B cells was confirmed in an additional cohort of patients with a clinically isolated syndrome (Supplementary Fig. 5). In the corticosteroid-treated NMOSD and MOGAD group, the proportion of natural effector B cells was similar to healthy controls and correlated to time since start of treatment ($r = 0.71$, $P = 0.02$; Supplementary Fig. 2C and D). Corticosteroid treatment did not affect plasmablast, IgM-only and IgG⁺ (CD27⁻/CD27⁺) B-cell frequencies (Supplementary Fig. 3B and data not shown).

Toll-like receptor 9 ligand synergizes with IFN- γ to promote naive mature B cell to plasmablast formation only for AQP4- or MOG-IgG-positive patients with relapses

To assess how the B-cell germinal centre-like differentiation pathway is regulated in NMOSD, we purified naive mature B cells from the peripheral blood of nine AQP4-IgG-positive patients, nine MOG-IgG-positive patients and nine healthy controls and cultured these populations in the presence of IL-21 and CD40L-3T3 cells with and without IFN- γ and TLR9 ligand CpG-ODN. The percentage of viable CD38⁺⁺CD27⁺⁺ plasmablasts was analysed after 11 days using flow cytometry (Fig. 2A). To further substantiate the clinical relevance of this model, we explored the functional association of *in vitro*-generated plasmablasts with disease activity within the continuum of relapse risk.

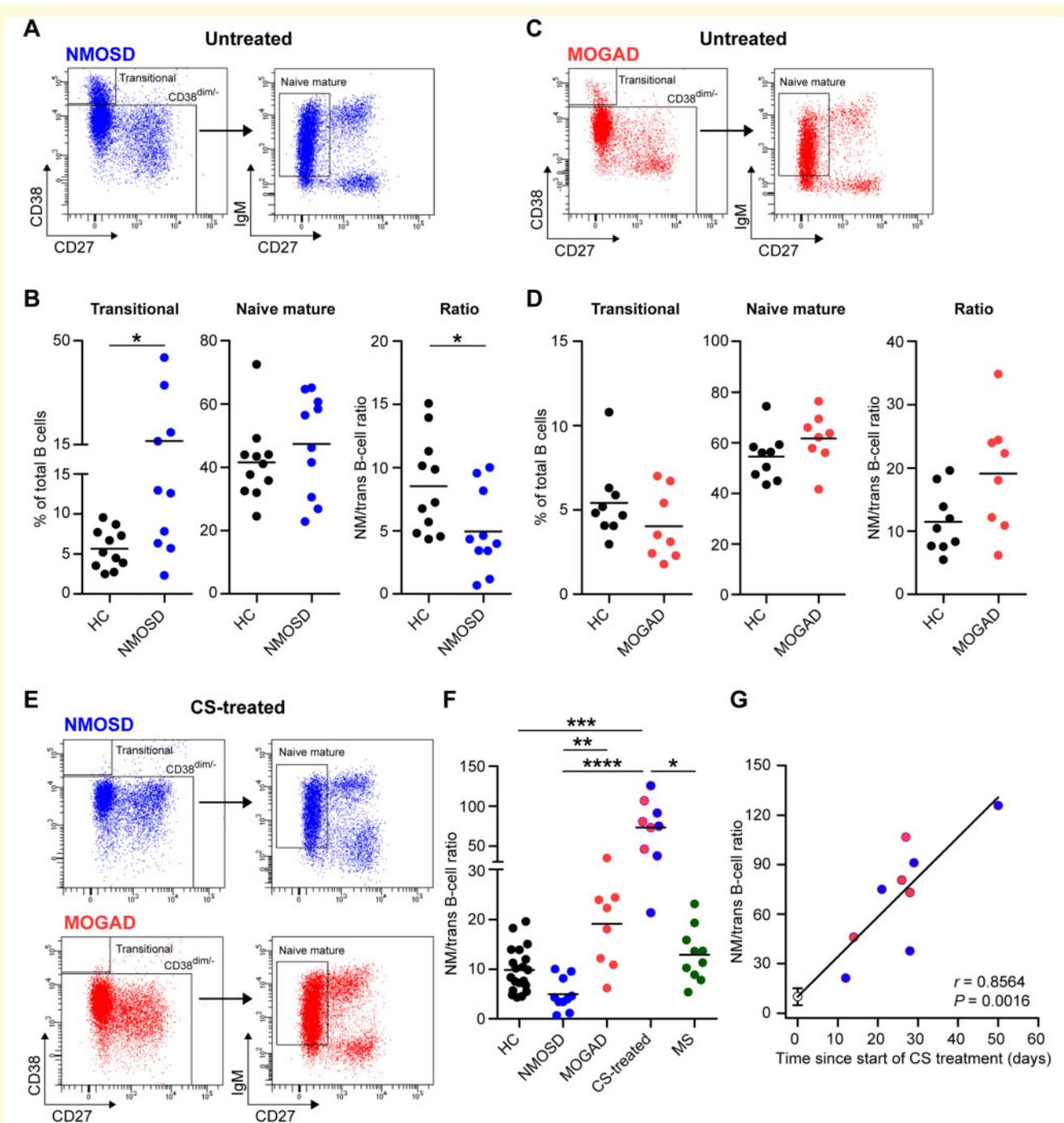


Figure 1 Transitional and naive mature B-cell frequencies in the blood of different NMOSD, MOGAD, multiple sclerosis and healthy control groups. Representative gating, proportions and ratios of transitional ($CD38^{++}CD27^{-}$) and naive mature ($CD38^{dim/-}CD27^{-}$) B cells from blood of treatment-naïve patients with NMOSD ($n = 10$; **A, B**) or MOGAD ($n = 8$; **C, D**). The fractions of transitional and naive mature B cells and their ratios were compared to a separate age- and gender-matched healthy control group (for NMOSD, $n = 11$; for MOGAD, $n = 9$). **(E)** Gating example for the detection of transitional and naive mature B cells in the blood from corticosteroid (CS)-treated patients with NMOSD or MOGAD. **(F)** Naive mature/transitional B-cell ratios in the blood of treatment-naïve NMOSD or MOGAD, CS-treated NMOSD or MOGAD ($n = 9$), treatment-naïve multiple sclerosis (MS; $n = 10$) and healthy control (HC; $n = 20$) groups. **(G)** Correlation of naive mature/transitional B-cell ratios to time since start of CS treatment in patients with NMOSD and MOGAD.

For all tested subjects, $IFN-\gamma$ induced the development of plasmablasts (Fig. 2B), which was similar between the groups. However, in both NMOSD and MOGAD

patients with relapses ($n = 7$), the addition of CpG-ODN to $IFN-\gamma$ -containing cultures significantly increased plasmablast formation (Fig. 2B; mean, $IFN-\gamma$: 19.8%, $IFN-$

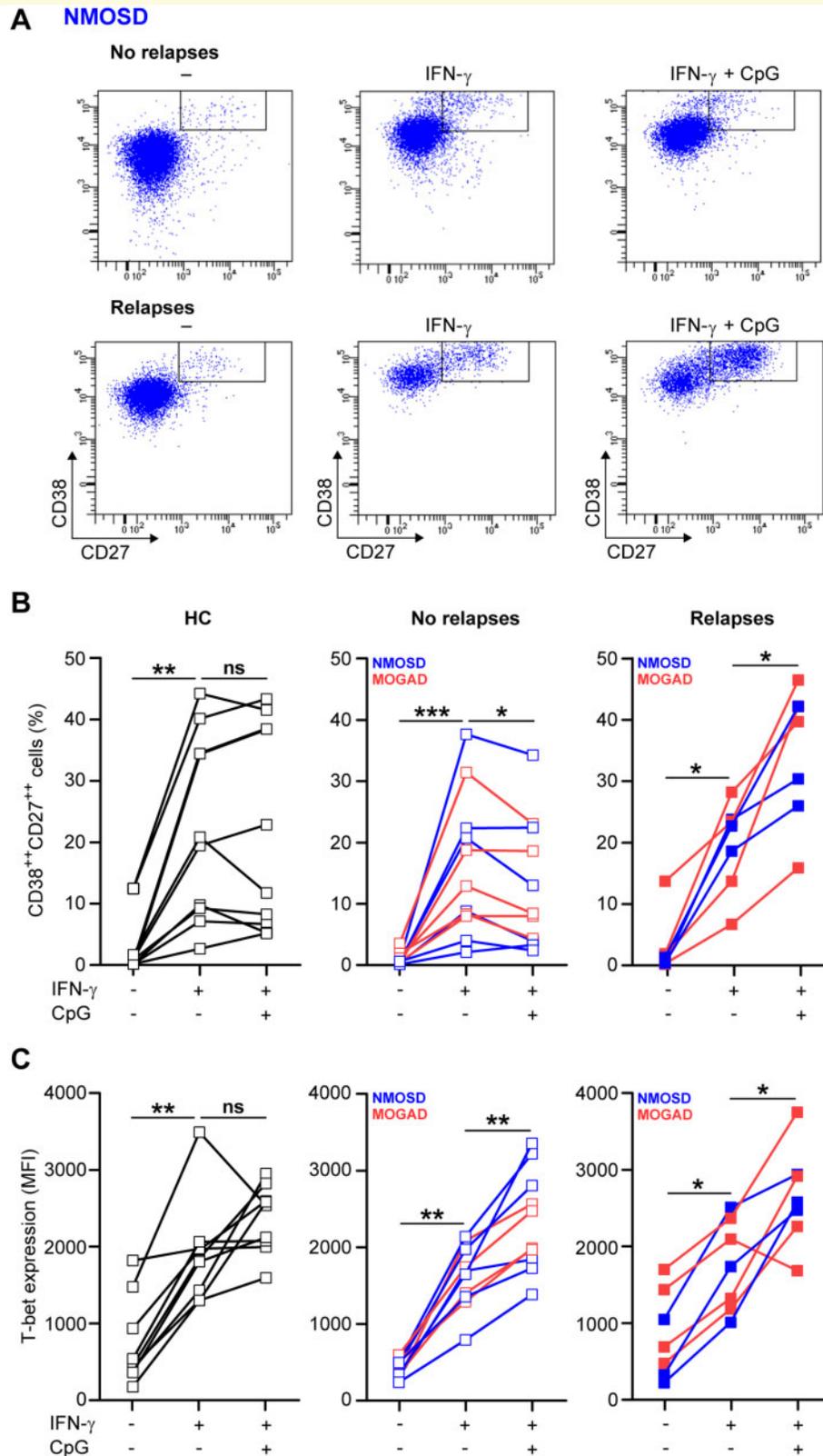


Figure 2 *In vitro* plasmablast outgrowth for naive mature B cells from subgroups with and without relapses under different germinal centre-like conditions. **(A)** Representative gating of viable plasmablasts ($CD38^{++}CD27^{++}$) cultured from naive mature B cells of an NMOSD patient with and without relapses. Cells were triggered with CD40L-3T3, IL-21, IFN- γ and/or TLR9 ligand CpG-ODN for 11 days. Both the percentage of *in vitro*-generated plasmablasts **(B)** and the intracellular T-bet expression **(C)** were determined for cultured naive mature B cells from the blood of NMOSD or MOGAD subgroups with ($n = 7$) or without relapses ($n = 11$), as well as healthy controls (HC; $n = 9$). For one patient with MOGAD, we obtained only sufficient cell numbers to analyze plasmablast frequencies and not T-bet expression.

γ +CpG: 34.9%, $P=0.0156$). The opposite was found in patients without relapses ($n=11$; IFN- γ : 15.8%; IFN- γ +CpG: 12.8%, $P=0.0137$). Although disease duration was longer for relapsing compared to non-relapsing patients (median 43 versus 17 months, respectively), relapsing patients experienced their first relapse within a median time of 10 months since onset. In six out of seven relapsing patients, immune-suppressive treatment was initiated after the first relapse, whereas six out of 11 monophasic patients were treated from onset onwards. For naive mature B cells of healthy controls, these frequencies were identical for both conditions (IFN- γ : 20.9%, IFN- γ +CpG: 20.4%). Intracellular T-bet levels were up-regulated by IFN- γ and further induced by CpG-ODN (Fig. 2C). Since this was comparable between the groups, other factors probably mediate the observed effect of CpG-ODN on *in vitro* plasmablast formation. The proportions of *ex vivo* B-cell subsets and plasmablasts did not differ between the groups with or without relapses (Supplementary Fig. 6).

In both the NMOSD and the MOGAD group, *in vitro* secretion of total IgG was significantly increased after the addition of CpG-ODN (Fig. 3A and Supplementary Fig. 7). For the NMOSD group, this positively correlated with *in vitro* plasmablast formation (Fig. 3B). The increase in both plasmablast formation and IgG secretion was the most pronounced in the two NMOSD patients with relapses (Fig. 3B). We detected anti-AQP4 IgG in naive mature B-cell culture supernatants of all three relapsing but in none of six non-relapsing patients with NMOSD (Fig. 3C). Anti-AQP4 IgG secretion was enhanced by CpG-ODN for two out of three relapsing patients; for one relapsing case with very high levels in culture (Δ MFI, IFN- γ : 24 442, IFN- γ +CpG: 34 725, negative control: 33) and one relapsing case with very low levels in culture (Δ MFI, IFN- γ : 57, IFN- γ +CpG: 94, negative control: 54). In culture supernatants of naive mature B cells of four relapsing and four non-relapsing patients with MOGAD, anti-MOG IgG could not be detected (Fig. 3C; Δ MFI, positive control: 7322). In this group, total IgG levels did not correlate with *in vitro* plasmablast formation (Fig. 3B).

Discussion

Aquaporin-4-IgG production by peripheral B cells is an important driver of NMOSD (Hauser et al., 2008; Bar-Or et al., 2010; Kim et al., 2011). Since central B-cell tolerance mechanisms are defective in NMOSD (Kinnunen et al., 2013; Cotzomi et al., 2019), the selection of AQP4-specific B cells is already disturbed at the earliest stage in the bone marrow. We now show that the composition and functional outgrowth of circulating naive B cells is different in patients with NMOSD, which seems to be linked to corticosteroid treatment, relapse occurrence and AQP4-IgG secretion.

The selective enrichment of transitional B cells in the blood of AQP4-IgG-positive NMOSD patients is likely caused by higher fractions of poly- and auto-reactive clones that escaped selection within the bone marrow (Cotzomi et al., 2019). We now find that transitional B cells are almost completely absent in the blood of corticosteroid-treated patients. Since the patients described by Cotzomi et al. (2019) received immunotherapy including corticosteroids, AQP4-specific B cells and other unique clones may have been missed in their analysis. This effect of steroids on transitional B cells is probably explained by their lack of multidrug-resistance receptor 1 (Wirhth and Lanzavecchia, 2005), a glycoprotein that pumps a wide range of substances including corticosteroids out of the cell (Brinkmann and Eichelbaum, 2001). Transitional B cells were abrogated by corticosteroid treatment in patients with NMOSD and MOGAD. This correlated to time since start of treatment in clinically isolated syndrome patients as well, implicating that corticosteroids have a generic impact on these early B-cell emigrants.

In a previous study, the proportion of circulating CD27⁺ memory B cells was found to be reduced in patients with NMOSD (Kowarik et al., 2017). We find that frequencies of germinal centre-independent CD27⁺IgM⁺IgD⁺ memory B cells are lower in patients with NMOSD and recovered after corticosteroid treatment. These data suggest that AQP4-specific naive mature B cells preferentially enter the germinal centre to undergo proliferation and somatic hypermutation. Consistent with this, under germinal centre-like conditions *in vitro*, AQP4-specific plasmablast development occurred more for naive than memory B cells and did not require antigen (Wilson et al., 2018), and somatic hypermutation was shown to be essential for generating AQP4-specific antibodies (Cotzomi et al., 2019). We did not find any increase in circulating memory B-cell subsets, including those lacking CD27 expression (Kowarik et al., 2017). This implies that within germinal centres, AQP4-specific naive B cells develop into plasmablasts rather than memory B cells. Indeed, some studies showed that *ex vivo* circulating plasmablasts are expanded in NMOSD, which seemed to be irrespective of steroid usage (Chihara et al., 2011; Kowarik et al., 2017). In this study, no differences in *ex vivo* CD38⁺⁺CD27⁺⁺ plasmablasts were found, similar to the observations by Wilson et al. (2018). The vulnerability of plasmablasts for freeze-thaw cycles (Kyu et al., 2009) can be a confounding factor although this was similar for all patients in this study. Another factor is that plasmablasts probably further mature and reside within the bone marrow or inflamed tissues to produce AQP4-specific antibodies (Bennett et al., 2015; Wilson et al., 2018).

Although the ability of *ex vivo* plasmablasts to produce AQP4-IgG is highly controversial (Chihara et al., 2011; Wilson et al., 2018), the development of naive B cells into antibody-secreting cells has become widely accepted. In the study by Cotzomi et al. (2019), none of the

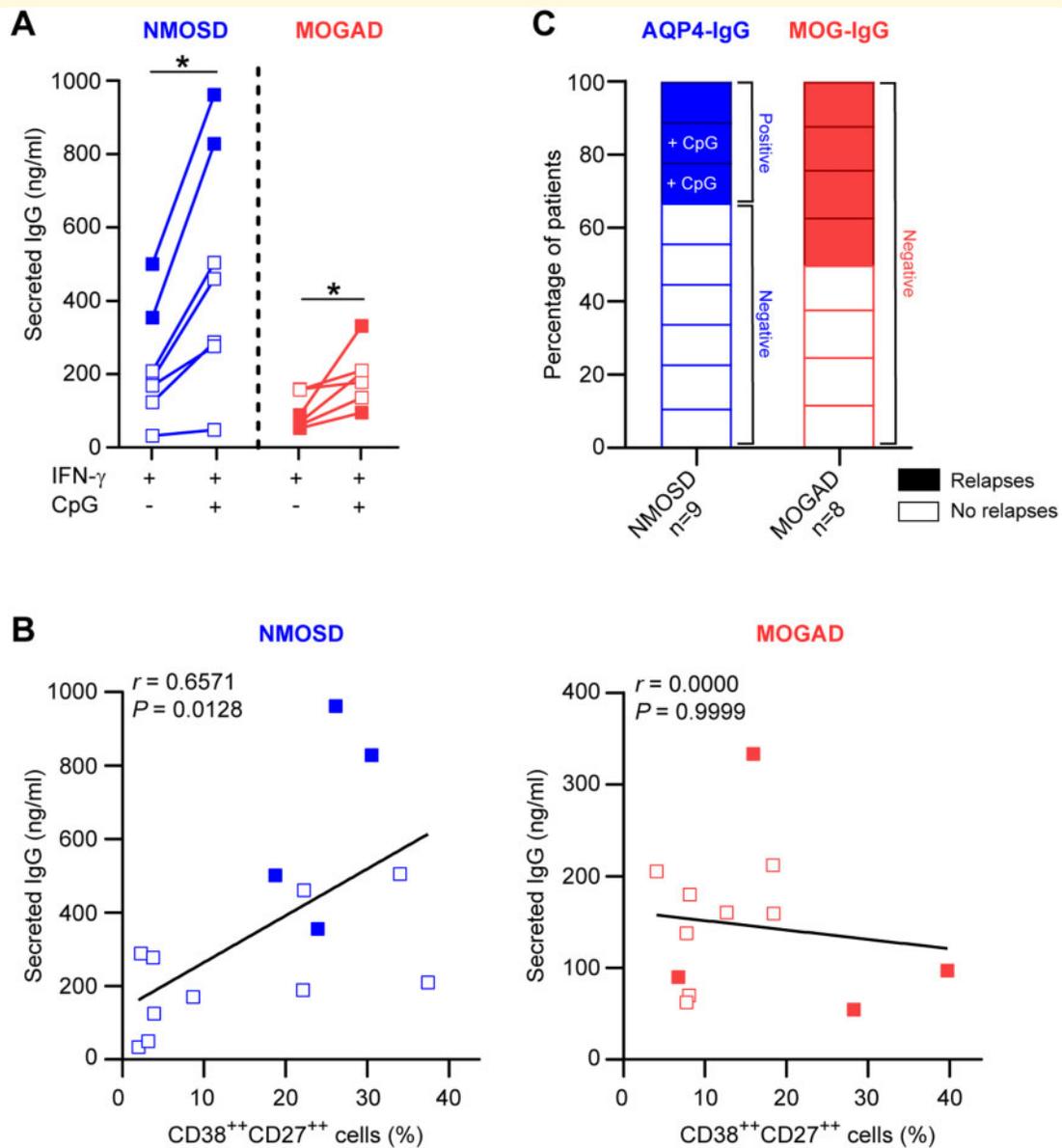


Figure 3 IgG secretion by *in vitro*-generated plasmablasts using naive mature B cells from subgroups with and without relapses. **(A, B)** Total IgG secretion was measured in germinal centre-like cultures with naive mature B cells from patients with NMOSD ($n = 7$) or MOGAD ($n = 6$; 11 days). In each subgroup, two patients had relapsing disease (solid boxes). Data were compared between IFN- γ - and both IFN- γ - and CpG-ODN-inducing conditions **(A)** and correlated to *in vitro* plasmablast ($CD38^{++}CD27^{++}$) formation **(B)**. **(C)** Detection of anti-AQP4-IgG and -MOG-IgG in naive mature B-cell culture supernatants (11 days) for patients with NMOSD or MOGAD. Aquaporin-4-IgG levels were elevated in IFN- γ -containing cultures with CpG-ODN for two NMOSD patients with relapses.

recombinant anti-AQP4 IgGs reverted back to unmutated precursors were able to bind to AQP4, indicating that naive mature B cells need to enter germinal centres to develop into anti-AQP4 IgG producers. Recently, we found that IFN- γ induces naive mature B cells to develop into plasmablasts during germinal centre-like cultures (van Langelaar *et al.*, 2019). The same is true for naive mature B cells of patients with NMOSD (this study). However, in contrast to patients with multiple sclerosis (van Langelaar *et al.*, 2019), the addition of CpG-ODN

had an inducing effect on *in vitro* plasmablast outgrowth for NMOSD patients with recorded relapses. This difference in *in vitro* naive mature B-cell outgrowth is likely related to the impaired central B-cell tolerance found in NMOSD and not in multiple sclerosis (Cotzomi *et al.*, 2019). Based on the data in this study, it is tempting to speculate that the elevated frequencies of (auto-reactive) transitional B cells in patients with a high relapse risk causes preferential development of naive mature B cells into AQP4-IgG-secreting plasmablasts within secondary

lymphoid organs. However, our findings should be interpreted cautiously due to low patient numbers and inherent differences in the clinical course between NMOSD, MOGAD and multiple sclerosis.

CpG-ODN alone is known to suppress (Jackson *et al.*, 2014), but synergizes with IFN- γ to potentiate auto-reactive T-bet⁺ B cells (Rivera-Correa *et al.*, 2017). T-bet expression does not seem to mediate the difference in IFN- γ - and CpG-ODN-induced plasmablast induction *in vitro* between relapsing and non-relapsing NMOSD groups. An intriguing scenario may be that IFN- γ enhances IL-6 production by B cells (Arkatkar *et al.*, 2017), leading to TLR9 up-regulation (Carvalho *et al.*, 2011). Toll-like receptor ligation has been previously associated with the activation of transcription factor X-box-binding protein 1 (Martinon *et al.*, 2010), which enhances the secretion of IgG (Iwakoshi *et al.*, 2003) and links to the IL-6-driven survival of AQP4-IgG-secreting plasmablasts (Chihara *et al.*, 2011).

A limitation of this study is the relatively low sample size. The results obtained here need to be confirmed in longitudinal studies and in additional cohorts. However, to our knowledge, this is the first study that assessed the phenotype and responsiveness of naive B cells of NMOSD patients without immunomodulatory treatment and compared these to MOGAD, multiple sclerosis and healthy control groups. The impact of steroids on transitional but not on naive mature B cells could be a mechanistic explanation why NMOSD relapses recur after discontinuation of steroid treatment. Our findings also provide a rationale for exploring the underlying mechanisms of naive B-cell development in patients with active or stable disease. Further assessment of CpG-ODN-mediated outgrowth of naive B cells under germinal centre-like, IFN- γ -containing *in vitro* conditions may be a new approach to predict NMOSD relapses.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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