


Induction of Brain-Infiltrating T-bet-Expressing B Cells in Multiple Sclerosis

Jamie van Langelaar ^{1,*}, Liza Rijvers,^{1,*} Malou Janssen,^{1,2} Annet F. Wierenga-Wolf,¹ Marie-José Melief,¹ Theodora A. Siepman, MD,² Helga E. de Vries, PhD,³ Peter-Paul A. Unger,⁴ S. Marieke van Ham, PhD,^{4,5} Rogier Q. Hintzen, MD, PhD,^{1,2†} and Marvin M. van Luijn, PhD¹

Objective: Results from anti-CD20 therapies demonstrate that B- and T-cell interaction is a major driver of multiple sclerosis (MS). The local presence of B-cell follicle-like structures and oligoclonal bands in MS patients indicates that certain B cells infiltrate the central nervous system (CNS) to mediate pathology. Which peripheral triggers underlie the development of CNS-infiltrating B cells is not fully understood.

Methods: Ex vivo flow cytometry was used to assess chemokine receptor profiles of B cells in blood, cerebrospinal fluid, meningeal, and brain tissues of MS patients (n = 10). Similar analyses were performed for distinct memory subsets in the blood of untreated and natalizumab-treated MS patients (n = 38). To assess T-bet(CXCR3)⁺ B-cell differentiation, we cultured B cells from MS patients (n = 21) and healthy individuals (n = 34) under T helper 1- and TLR9-inducing conditions. Their CNS transmigration capacity was confirmed using brain endothelial monolayers.

Results: CXCR3 chemokine receptor 3 (CXCR3)-expressing B cells were enriched in different CNS compartments of MS patients. Treatment with the clinically effective drug natalizumab prevented the recruitment of CXCR3^{high} IgG1⁺ subsets, corresponding to their increased ability to cross CNS barriers in vitro. Blocking of interferon- γ (IFN γ) reduced the transmigration potential and antigen-presenting function of these cells. IFN γ -induced B cells from MS patients showed increased T-bet expression and plasmablast development. Additional TLR9 triggering further upregulated T-bet and CXCR3, and was essential for IgG1 switching.

Interpretation: This study demonstrates that T-bet^{high} IgG1⁺ B cells are triggered by IFN γ and TLR9 signals, likely contributing to enhanced CXCR3-mediated recruitment and local reactivity in the CNS of MS patients.

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B cells are one of the main contributors to chronic autoimmune pathology in multiple sclerosis (MS), as supported by results from large genome-wide association studies.¹ B-cell repertoires in the central nervous system (CNS) and the periphery are closely connected, suggesting that disease-relevant B-cell networks interact at both sides

of the blood–brain barrier.^{2–5} There is evidence that the beneficial effects of anti-CD20 monoclonal antibody therapy are related to the ablation of functional B cells interacting with T cells.^{6,7} The meninges of MS patients contain tertiary lymphoid structures that are filled with B and T cells, close to cortical lesions.⁸ This strongly suggests that B- and T-cell

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Address correspondence to Dr van Luijn, Erasmus MC, University Medical Center, MS Center ErasMS, Department of Immunology, Wytemaweg 80, 3015 CN, Rotterdam, the Netherlands. E-mail: m.vanluijn@erasmusmc.nl

*J.v.L. and L.R. contributed equally.

[†]Deceased.

From the ¹Department of Immunology and ²Neurology, MS Center ErasMS, Erasmus Medical Center, University Medical Center, Rotterdam; ³Department of Molecular Cell Biology and Immunology, Amsterdam University Medical Center, MS Center Amsterdam, Amsterdam Neuroscience, Amsterdam; ⁴Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Center, University of Amsterdam, Amsterdam; and ⁵Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands.

interaction is a major event in triggering and sustaining inflammation in the CNS.

In MS, autoreactive (naive) B cells escape peripheral selection and probably receive specific signals from CD4⁺ T cells within secondary lymphoid organs to differentiate into memory populations before entering the CNS.^{5,9,10} The presence of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) of MS patients implies that these memory B cells undergo local reactivation (with the help of CD4⁺ T cells) to further develop into immunoglobulin (Ig)-producing plasmablasts and plasma cells.^{8,11} Although memory B cells have been recently shown to promote the differentiation of CNS-infiltrating CD4⁺ T cells in MS, little is known about how and which functional B-cell subsets are triggered in the periphery to infiltrate the CNS and contribute to MS pathology.

In mice, the T helper 1 (Th1) cytokine interferon- γ (IFN γ) induces the interaction between autoreactive B cells and CD4⁺ T cells to form tertiary lymphoid structures and promote systemic autoimmune diseases such as systemic lupus erythematosus (SLE).¹² In these cases, IFN γ induces the expression of the T-box transcription factor T-bet, resulting in enhanced Ig class switching and CXC chemokine receptor 3 (CXCR3) expression in murine B cells.^{13,14} Interestingly, B-cell-intrinsic T-bet expression associates with increased pathogenic responses^{14,15} and is induced by systemic infections,¹⁶ a major environmental trigger in MS.¹⁷ Toll-like receptor 9 (TLR9), which binds to pathogen-related CpG-DNA, integrates with the B-cell receptor (BCR), CD40, and cytokine signals to stimulate T-bet⁺ B-cell development.^{18,19} Additionally, B cells from MS patients were previously reported to exhibit an enhanced proinflammatory phenotype when activated with IFN γ and TLR9 ligand CpG-DNA.⁷

Here, we aimed to explore the CNS transmigration capacity of T-bet(CXCR3)-expressing B cells and which peripheral triggers are involved in the development of such populations in MS patients. We explored the recruitment of human CXCR3⁺ B cells to the CNS both *ex vivo* and *in vitro*. Furthermore, the susceptibility of blood-derived B cells from MS patients and healthy individuals to T-bet-inducing stimuli and how this influences their differentiation into CXCR3⁺ memory subsets was determined using different T-cell-based culture systems.

Subjects and Methods

Patients

Postmortem CSF, meninges, brain tissues, and blood samples were freshly obtained from MS brain donors (Netherlands Brain Bank, Amsterdam, the Netherlands). The main cause of death was legally granted euthanasia (8 of 10 donors). The 2 other donors died from pneumonia or MS. These tissues had a very short postmortem delay

of 8.92 hours (interquartile range [IQR] = 8.50 – 9.50 hours), and pH of the CSF was 6.59 (IQR = 6.44–6.87). All other MS patients and healthy controls were included at Erasmus Medical Center (Rotterdam, the Netherlands), which is a national tertiary referral center for MS patients (Multiple Sclerosis Center ErasMS). Patients and controls were age- and gender-matched per study group. Patient characteristics are summarized in Table 1. Primary material was obtained between 2007 and 2018. All patients gave written informed consent, and study protocols were approved by the medical ethics committee of the Erasmus Medical Center (Rotterdam) and VU University Medical Center (Amsterdam, the Netherlands).

Mononuclear Cell Isolation from Blood and CNS Compartments

Peripheral blood mononuclear cells (PBMCs) were isolated according to the manufacturer's instructions from blood of MS patients and matched controls with the use of vacutainer CPT[®] tubes containing sodium heparin (BD Biosciences, Erembodegem, Belgium). PBMCs were frozen and stored in liquid nitrogen until use as previously described.²⁰ Mononuclear cells were isolated from buffy coats using Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany) and density gradient centrifugation. Blood and CSF samples from MS brain donors were acquired postmortem through heart puncture and ventricle drainage, respectively.²⁰ Heart blood mononuclear cells were isolated as described for buffy coat material. Collection tubes with CSF were centrifuged for 10 minutes at 500 $\times g$. CSF and blood mononuclear cell fractions were resuspended in RPMI 1640 (Lonza, Verviers, Belgium) containing 10% heat-inactivated human AB serum (Sanquin, Rotterdam, the Netherlands) and 1% penicillin/streptomycin (Lonza) and left to rest at 37°C until further use. Meninges were washed in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) 3 times, cut into pieces, and incubated with Liberase (Roche Applied Science, Penzberg, Germany) for 1 hour at 37°C, after which the meninges were filtered through a cell strainer (45 μ m) and cells were washed using Ficoll-Paque Plus (GE Healthcare). Single-cell suspensions from the meninges were resuspended in PBS containing 0.1% BSA until further use. Brain tissue samples were processed and single-cell suspensions were obtained as previously reported.²¹

Antibodies and Flow Cytometry

Multicolor flow cytometric analysis was performed using fluorochrome-labeled monoclonal anti-human antibodies (mAbs; Table 2). PBMCs were stained extracellularly for 30 minutes at 4°C. Cultured B cells were stained with a fixable viability stain (FVS 700) for 15 minutes at 4°C and subsequently stained for either extracellular only or both extracellular and intracellular markers. For intracellular staining, cells were fixed with 2% paraformaldehyde (Merck, Schiphol-Rijk, the Netherlands) and permeabilized with PBS pH7.4 containing 0.3% BSA and 0.5% saponin (Sigma-Aldrich, St Louis, MO) and stained with T-bet for 60 minutes at 4°C. All measurements were conducted with an LSRII-Fortessa flow cytometer and analyzed using FACS Diva software, version 8.0.1 (both BD Biosciences).

TABLE 1. Characteristics of Patients and Controls Used in This Study

Cohorts	Subject, n	Gender, Female, n (%)	Age, Median yr (IQR) ^a	Disease Duration, Median mo (IQR) ^b
Ex vivo B cells, CNS vs blood				
MS	10	9 (90%)	52 (50–65)	NA
Ex vivo B cells, blood subsets				
HC	10	7 (70%)	47 (32–54)	NA
MS, no Tx	10	7 (70%)	45 (43–53)	48 (24–120)
MS, NAT Tx				
First cohort	10	7 (70%)	40 (29–46) ^c	90 (31–124)
Second cohort	9	6 (66%)	36 (26–43) ^c	46 (41–130)
Third cohort ^d	9	5 (56%)	28 (21–43) ^c	28 (19–41)
In vitro–stimulated B cells				
HC				
Total	10	8 (80%)	44 (32–56)	NA
Naive	8	5 (63%)	39 (27–50)	NA
MS, no Tx				
Total	9	7 (80%)	41 (34–56)	36 (36–73)
Naive	12	8 (67%)	38 (28–42)	4 (3–15)

^aAt time of sampling.
^bTime from MS diagnosis to sampling.
^cAt time of pretreatment sampling.
^dIgG subclass instead of total IgG analysis.
CNS = central nervous system; HC = healthy controls; IQR = interquartile range; MS = multiple sclerosis; NA = not applicable; NAT = natalizumab; Tx = treatment.

Ex vivo Th17.1 (IFN γ ^{high}IL-17^{low}) and Th17 (IFN γ ^{neg}) cells in blood were defined as CCR6⁺CXCR3⁺CCR4⁻ (Th17.1) and CCR6⁺CXCR3⁻CCR4⁺ (Th17), described recently.²⁰

Human B-Cell Migration Assays

Flow-activated cell sorting (FACS)-sorted CD27⁻ and CD27⁺ memory CD19⁺ B cells from buffy coat–derived PBMCs were placed on 96 permeable transwell plates with a 0.3 μ m pore size (2×10^5 cells/well; Corning, Amsterdam, the Netherlands). B-cell migration toward medium or CXC chemokine ligand (CXCL)10 (900ng/ml; R&D Systems, Abingdon, UK) was analyzed after 3 hours at 37°C. In addition, $2.5\text{--}5 \times 10^5$ memory B cells were placed on confluent monolayers of human brain endothelial cells (hCMEC/D3) on 5 μ m pore size transwell plates (Corning) coated with collagen, and migration was analyzed after 5 hours.²² Percentages of memory B-cell subsets were compared before and after transmigration using flow cytometry.

Antigen-Primed Autologous B- and Th-Cell Cocultures

BCR-mediated uptake and presentation of *Salmonella typhimurium* SL1344 was used as a model for antigen presentation, as previously

demonstrated.²³ mAb anti-human IgG (MH16-1; Sanquin, Amsterdam, the Netherlands) was mixed with mAb against *S. typhimurium* lipopolysaccharide (LPS; 1E6; Invitrogen, Paisley, UK) and rat anti-mouse IgG1 antibody (RM161.1, Sanquin) to generate BCR-LPS tetrameric antibody complexes. Exponentially grown bacteria were washed twice with PBS, incubated with BCR-LPS tetrameric antibody complexes for 30 minutes at room temperature, and washed twice to remove unbound antibodies. B cells were incubated with viable anti-IgG–coated *S. typhimurium*²³ at 20 bacteria per cell for 45 minutes at 37°C without antibiotics. Next, cells were washed 3 times and cultured for 60 minutes in medium containing 100 μ g/ml gentamicin (Invitrogen) to eliminate nonphagocytosed bacteria. B cells were cocultured in RPMI supplemented with 5% fetal calf serum, 1% (100U/ml) penicillin, 1% (100 μ g/ml) streptomycin (Lonza), 1% (2mM) UltraGlutamine (Lonza), 0.1% (50 μ M) beta-mercaptoethanol (Sigma-Aldrich), 0.1% (20 μ g/ml) apotransferrin (depleted for human IgG with protein-G sepharose; Sigma-Aldrich; further referred to as B-cell medium), and 10 μ g/ml gentamicin together with autologous CD4⁺ T cells (magnetic activated cell sorted). B cells (1×10^5) and T cells (0.5×10^5) were cultured in 200 μ l at 37°C in the presence of 5%

TABLE 2. Monoclonal Antihuman Antibodies Used for Flow-Activated Cell Sorting

Antibody Marker	Fluorochrome	Clone	Company
CD3	AF700	SK7	BioLegend ^a
CD3	BV785	SK7	BD Biosciences ^b
CD4	BV510	OKT4	BioLegend
CD19	BV785	HIB19	BD Biosciences
CD20	AF700	2H7	BD Biosciences
CD21	BV711	B-Ly4	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD38	PE-Cy7 and PerCP-Cy5.5	HIT2	BioLegend
CD49d (VLA-4)	APC	9F10	BD Biosciences
CCR4	PE-Cy7	L291H4	BioLegend
CCR6	PE	G024E3	BioLegend
CXCR3	BV605 and APC	G025H7	BioLegend
CXCR5	APC-R700	RF8B2	BD Biosciences
IgA	FITC	IS11-8E10	Miltenyi Biotec ^c
IgD	PE and PE-CF594	IA6-2	BD Biosciences
IgG	ACP-H7	G18-145	BD Biosciences
IgG1	PE	HP6001	Southern Biotech ^d
IgG2	AF488	HP6002	Southern Biotech
IgM	BV510	MHM-88	BioLegend
T-bet	PE-Cy7	4B10	BioLegend
Fixable viability dye (FVS)	AF700		BD Biosciences

^aBioLegend, London, UK.^bBD Biosciences, Erembodegem, Belgium.^cMiltenyi Biotec, Leiden, the Netherlands.^dSouthern Biotech via ITK Diagnostics, Uithoorn, the Netherlands.

CO₂ in 96-well round-bottom plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) for 6 days. Cultures were performed in the presence of recombinant interleukin (IL)-21 (50ng/ml; Thermo Fisher Scientific, Landsmeer, the Netherlands) and recombinant IL-2 (50IU/ml, Miltenyi Biotec, Bergisch Gladbach, Germany), and with or without an anti-IFN γ blocking antibody (MD-1, 10 μ g/ml; U-CyTech Biosciences, Utrecht, the Netherlands).

IL-21/3T3-CD40L Assay for Human B-Cell Differentiation

To mimic B-cell differentiation in vitro, murine NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L)²³ were irradiated at 30Gy using a RS320 X-ray (Beckhoff, Eindhoven, the Netherlands), taken up in B-cell medium, and seeded on flat-bottom 96-well plates (10 \times 10³ cells per well; Greiner Bio-One) to allow adherence overnight. CD19⁺ (total) B cells were isolated from buffy coat-derived PBMCs using CD19 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec). Total, naive (CD38^{-dim}CD27⁻IgG⁻IgA⁻) and memory (CD38^{-dim}CD27⁺IgG⁺) B cells were isolated from healthy and MS blood using a BD FACSAria III cell sorter. These fractions were resuspended in B-cell medium, and 20–25 \times 10³ cells were cocultured with irradiated 3T3-CD40L cells and stimulated with a combination of IL-21 (50ng/ml; Thermo Fisher Scientific), IFN γ (50ng/ml; Peprotech/Bio-Connect, Huissen, the Netherlands), and CpG-ODN (2006-G5; 10 μ g/ml; InvivoGen/Bio-Connect) at 37°C and 5% CO₂. After 6 and 11 days of culture, supernatants were collected and stored at –80°C until use for enzyme-linked immunosorbent assay (ELISA). The cells were stained and assessed using flow cytometry as described above.

IgG1 ELISA

Nunc MaxiSorp plates (Sanbio, Uden, the Netherlands) were coated overnight with antihuman IgG1 monoclonal capture antibody (1 μ g/ml; clone MH161-1; Sanquin, Amsterdam, the Netherlands) in PBS. After washing with PBS–0.02% Tween-20, the supernatants from in vitro B-cell cultures (described above) were diluted in high-performance ELISA buffer (HPE, Sanquin) and incubated for 60 minutes. Subsequently, plates were washed and incubated for 60 minutes with anti-human IgG conjugated with horseradish peroxidase, a monoclonal detection antibody (1 μ g/ml; clone MH16-1, Sanquin). After washing, the ELISA was developed with MQ containing 0.11M sodium-acetate (pH 5.5), 100 μ g/ml tetramethylbenzidine, and 0.003% (vol/vol) H₂O₂ (all from Merck). The reaction was stopped by addition of 2M H₂SO₄ (Merck). Optical densities at 450nm were measured with a BioTek (Winooski, VT) Synergy 2. Background readings at 540nm were subtracted. Results were related to a titration curve of a serum sample of a healthy donor in each plate.

Statistical Analyses

All datasets were analyzed with GraphPad Prism Software, version 7 (GraphPad Software, San Diego, CA) and compared using 2-sided Mann–Whitney *U* tests, Wilcoxon matched-pairs signed rank test, 1- or 2-way analysis of variance with Tukey post hoc

test, Friedman paired test with Dunn post hoc test, and Spearman correlation coefficients (as indicated in each figure legend). Experimental data are depicted as the mean \pm standard error of the mean. Prior to statistical analyses, datasets were tested for normal distribution. Probability values <0.05 were considered significant.

Results

CXCR3-Expressing B Cells Are Selectively Enriched in Distinct CNS Compartments of MS Patients

Enhanced chemotaxis is one of the key mechanisms by which B cells can enter distinct CNS compartments of MS patients.¹¹ Production of the chemoattractants CXCL10, CXCL13, and CCL20 in the CNS has been associated with B-cell recruitment, distribution, and reactivity in MS.^{24–26} We compared the presence of B cells that express the chemokine receptors that correspond to these ligands, CXCR3⁺ (CXCL10), CXCR5⁺ (CXCL13), and CCR6⁺ (CCL20), between paired blood, CSF, and meningeal and brain tissues from 10 MS patients (see Table 1). To realize this, single-cell suspensions were obtained from autopsied brain compartments using a standardized protocol.²¹ From these fractions, viable CD45⁺CD3⁻CD19⁺ B cells were gated and analyzed for chemokine receptor expression using flow cytometry (Fig 1A). We were able to measure sufficient numbers of viable B cells from each compartment for all donors (mean [range]: blood, 21,509 [610–98,562]; CSF, 12,629 [50–59,499]; meninges, 13,819 [91–36,644]; brain tissue, 2,889 [26–18,050]). The frequency of CXCR3⁺ but not CXCR5⁺ or CCR6⁺ B cells was strongly increased in ex vivo cell suspensions from MS brain tissues ($p < 0.0001$), meninges ($p = 0.0003$), and CSF ($p < 0.0001$) compared to blood (See Fig 1B). CXCR3-expressing T cells, including Th17.1, were also enriched in the CNS compartments of these donors (data not shown), supporting our recent observations.²⁰

Reduced Frequencies of CXCR3⁺IgG(1)⁺ B Cells in the Blood of MS Patients Are Abrogated after Natalizumab Treatment

To determine how CXCR3 is involved in the local attraction of different B-cell populations in MS, we assessed the proportion of CXCR3-expressing naive (CD27⁻IgM⁺; IgM_{naive}) and both IgM⁺ memory (CD27⁺IgM⁺; IgM_{mem}) and IgG⁺ memory (CD27⁺IgG⁺) B cells in the peripheral blood of untreated MS patients ($n = 10$) and age- and gender-matched healthy controls (HCs; $n = 10$; see Table 1 and Fig 2A). CXCR3-expressing IgG⁺ cells were reduced ($p = 0.007$), whereas no differences were seen for IgM_{naive} and IgM_{mem} cells in MS versus HC blood (See Fig 2B).

To address this potential migration of CXCR3⁺IgG⁺ memory B cells into the CNS, we analyzed the distribution

of these B-cell subsets in the blood of MS patients treated with the anti- $\alpha 4\beta 1$ integrin (VLA-4) antibody natalizumab (see Table 1), a drug that effectively reduces MS disease activity by blocking lymphocyte recruitment to the CNS.²⁷ VLA-4 was most abundantly expressed on blood IgG⁺ B cells from MS patients prior to natalizumab treatment (see Fig 2). Elevated frequencies of both IgM_{mem} (pretreatment vs 6 months post-treatment $p = 0.042$ and 12 months post-treatment $p = 0.011$) and IgG⁺ (pretreatment vs 6 months post-treatment $p = 0.022$ and 12 months post-treatment $p < 0.001$) B cells were found in the blood of MS patients both 6 and 12 months after versus before treatment. However, only IgG⁺ and not IgM_{naive} or IgM_{mem} B cells from MS patients treated with natalizumab for 12 months showed increased expression levels of CXCR3 ($p < 0.01$), and not CXCR5 or CCR6. These findings were validated in a second cohort of 9 MS patients treated with natalizumab (see Table 1; data not shown). Notably, CD20 expression levels were increased on IgG⁺ B cells and higher on CXCR3⁺ compared to CXCR3⁻ counterparts in the blood of natalizumab-treated MS patients.

Because intrathecally synthesized OCBs are restricted to the IgG1 subclass in the CSF of MS patients,²⁸ we also analyzed IgG1⁺ B cells for their frequencies and CXCR3 expression in the blood of a third cohort of natalizumab-treated MS patients (see Fig 2). IgG1⁺ B cells not only expressed higher levels of CXCR3 ($p = 0.007$) but also showed increased frequencies in post-treatment samples ($p = 0.027$) compared to IgG2⁺ B cells. The selective accumulation of CXCR3^{high} IgG(1)⁺ B cells in the blood of natalizumab-treated patients underlines the potency of this subset to transmigrate into the CNS to mediate MS disease activity.

CXCR3⁺IgG1⁺ B Cells Have an Enhanced Capacity to Transmigrate Across the Blood–Brain Barrier In Vitro

To functionally test the transmigration potential of CXCR3⁺IgG1⁺ B cells into the CNS, we sorted memory B cells from the blood and assessed in vitro migration of subsets toward CXCL10. Fractions of CXCR3-expressing IgM_{mem}, IgG1⁺, and IgG2⁺ B cells were assessed within the total memory pool before and after migration through transwell filters. In contrast to IgM_{mem} and IgG2⁺ populations, IgG1⁺ B cells showed prominent recruitment to CXCL10 ($p < 0.0001$ before vs after migration; Fig 3). This was not seen using medium only. To mimic B-cell transmigration across the blood–CNS barrier, these experiments were repeated using cultured confluent monolayers of human brain endothelial cells.²² We found a similar CXCL10-mediated migratory advantage of IgG1⁺ B cells ($p < 0.0001$), which is consistent with the abundance of CXCR3 on IgG1⁺ compared to IgM_{mem} and IgG2⁺ B cells

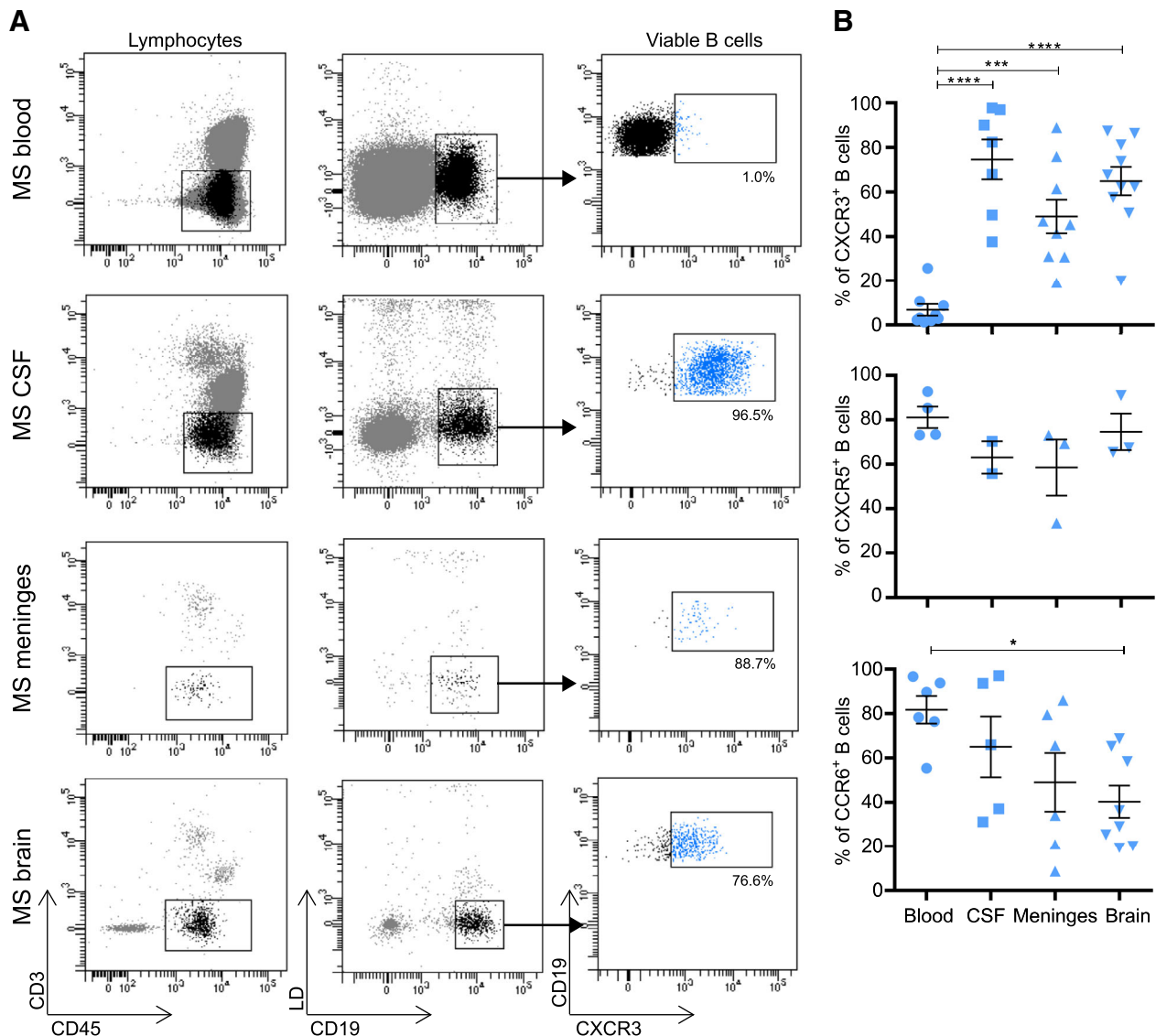


FIGURE 1: CXCR3⁺ B cells are abundant in the central nervous system compared to blood of multiple sclerosis (MS) patients. (A) Representative flow-activated cell sorting (FACS) plots and gating of CXCR3-expressing CD19⁺ B cells within viable CD45⁺CD3⁻ lymphocyte fractions derived from the blood, cerebrospinal fluid (CSF), meninges, and brain tissue of an MS patient. (B) Frequencies of CXCR3⁺, CXCR5⁺, and CCR6⁺ B cells in distinct paired compartments from MS patients. For blood, CSF, and meningeal samples each dot represents a different patient. A total of 10 brain tissues from 7 different MS patients were used for the analysis of CXCR3⁺ B cells. Any samples with <25 viable B cells were excluded from these analyses. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. The p values for B were calculated by a 1-way analysis of variance test. LD = live/dead (for detection of viable cells).

in MS patients (see Fig 2G–I). These data demonstrate that CXCR3^{high} IgG1⁺ B cells in the blood have a heightened ability to infiltrate the CNS, probably accounting for the local IgG1 subclass restriction of OCBs in MS patients.²⁸

IFN γ Promotes CXCR3 Expression and CD4⁺ T-Cell Activation by Human T-bet⁺ B Cells under T Follicular Helper-like Culture Conditions

In MS blood, the proportion of CXCR3⁺IgG⁺ B cells correlated to Th17.1 (IFN γ ^{high}IL-17^{low}; $r = 0.566$, $p = 0.0003$) and not to Th17 (IFN γ ^{neg}) cells (Fig 4).²⁰ Th-cell-derived

IFN γ is known as a central driver of autoreactive B cells in mice¹⁰ and also induces CXCR3 expression on human memory B cells.²⁹ Therefore, we aimed to better understand how the differentiation and function of human CXCR3⁺ memory B cells is influenced by IFN γ before entering the CNS. To address this, we mimicked the effects of IFN γ -producing T follicular helper (Tfh) cells on B-cell subsets in vitro. First, an IL-21-based human B- and T-cell coculture system was used to assess whether Th1-derived IFN γ influenced CXCR3 expression on IgG⁺ B cells in an antigen-specific manner. Because antigen uptake by B cells is

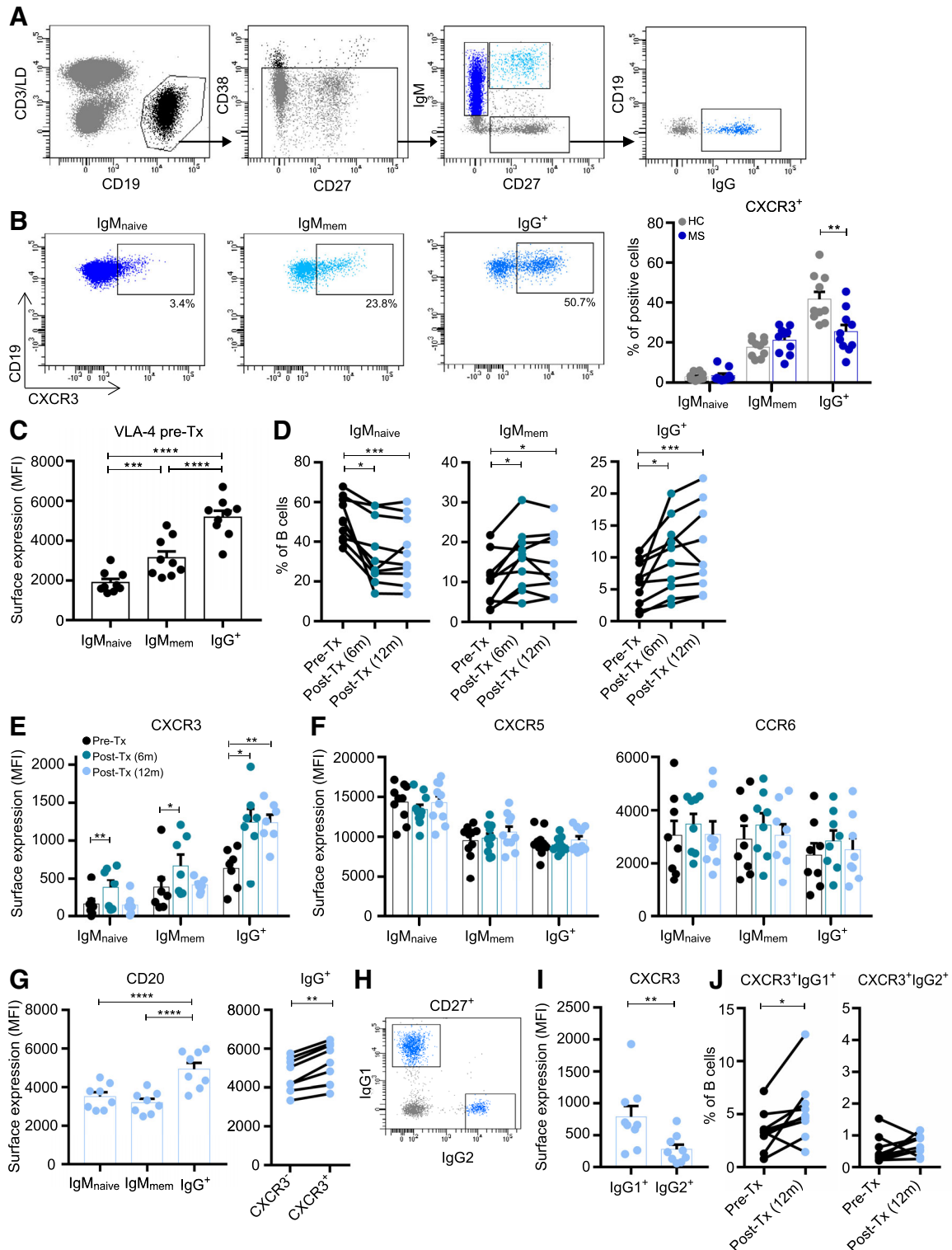


FIGURE 2: Reduced frequencies and natalizumab-mediated accumulation of CXCR3⁺IgG(1)⁺ B cells in multiple sclerosis (MS) blood. (A) FACS gating strategy used to define IgM_{naive} (CD27⁻IgM⁺), IgM_{mem} (CD27⁺IgM⁺), and IgG⁺ (CD27⁺IgG⁺) B-cell subsets. (B) Gating and quantification of CXCR3-expressing IgM_{naive}, IgM_{mem}, and IgG⁺ B-cell frequencies in the blood of untreated MS patients (n = 10; dark blue dots) and both age- and gender-matched healthy controls (HC; n = 10; gray dots, see Table 1). (C) VLA-4 surface expression on IgM_{naive}, IgM_{mem}, and IgG⁺ B cells from blood of MS patients before natalizumab treatment (n = 9). (D) The percentage of IgM_{naive}, IgM_{mem}, and IgG⁺ B cells in MS blood before (black dots) and both 6 months (marine blue dots) and 12 months (light blue dots) after natalizumab treatment (paired samples; n = 10; see Table 1). (E, F) Surface expression levels of CXCR3 (E), CXCR5 and CCR6 (F) on IgM_{naive}, IgM_{mem}, and IgG⁺ B cells in MS patient blood before and after natalizumab treatment (n = 7–10). (G) CD20 expression on IgM_{naive}, IgM_{mem}, and IgG⁺ B cells as well as paired CXCR3⁻ and CXCR3⁺ IgG⁺ populations in blood of MS patients treated with natalizumab for 12 months (n = 8). (H–J) Gating example and quantifications of IgG1⁺ and IgG2⁺ B cells expressing CXCR3 in MS patients treated with natalizumab for 12 months (n = 9). Data are presented as the mean ± standard error of the mean. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The p values were calculated by Mann–Whitney U (B), 2-way analysis of variance (C, G), Friedman paired (D–F), and Wilcoxon matched-pairs signed rank (G, I, and J) tests. MFI = mean fluorescence intensity; Tx = treatment; LD = live/dead (for detection of viable cells).

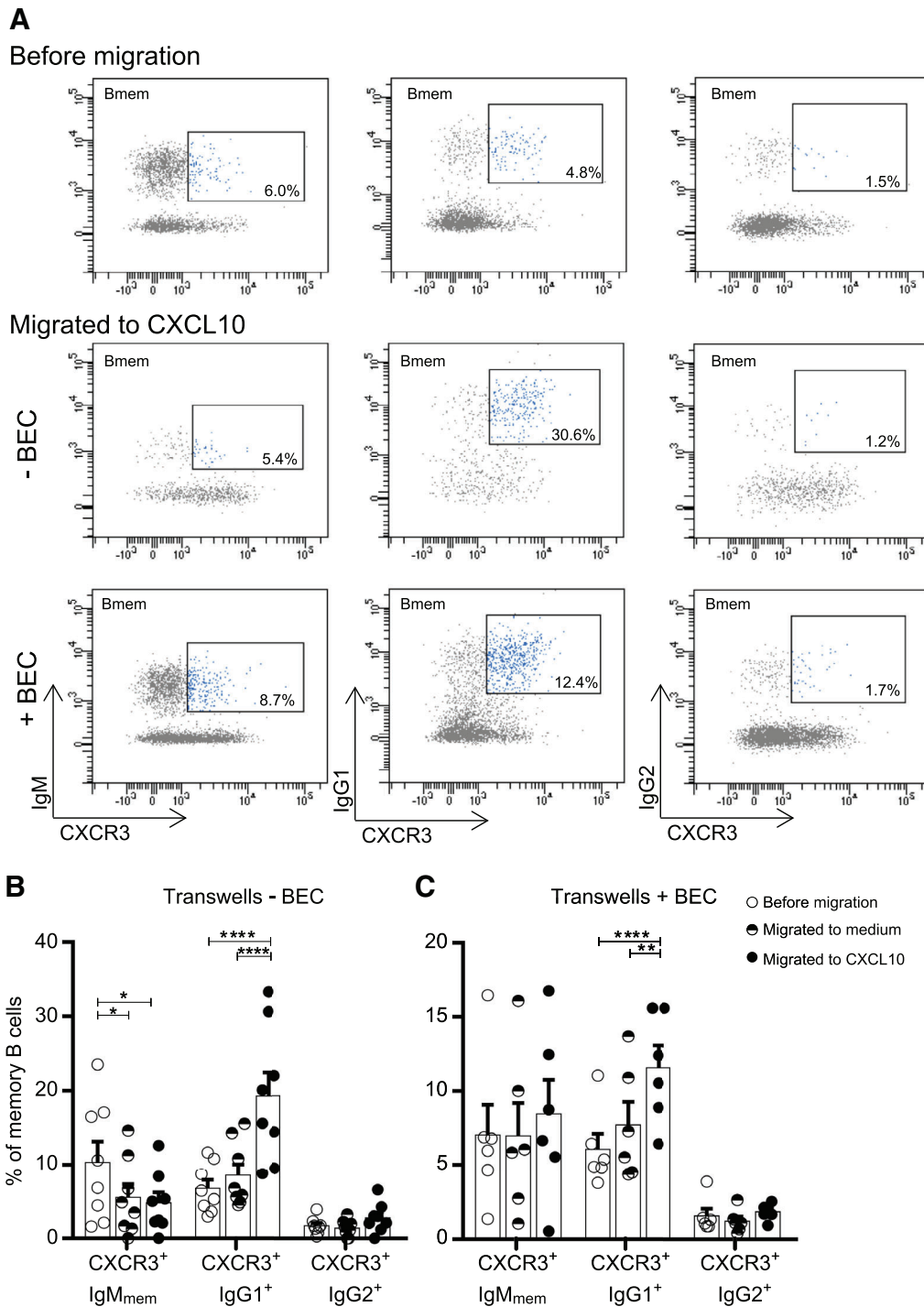


FIGURE 3: Enhanced migration of CXCR3⁺IgG1⁺ B cells across transwell filters and human brain endothelial monolayers in vitro. Sorted memory B cells from healthy donor blood were assessed for selective in vitro transmigration toward CXCL10. (A) Representative FACS plots and (B, C) quantifications of viable CXCR3-expressing IgM_{mem}, IgG1⁺ and IgG2⁺ B cells migrating across transwell filters with and without confluent monolayers of human brain endothelial cells (BEC). Percentages of subsets within the total memory pool were compared before and after migration, both to medium and to CXCL10 (–BEC, n = 8; +BEC, n = 6). These experiments were performed in duplicate for each donor for which the average is shown. Data are presented as the mean ± standard error of the mean. *p < 0.05, **p < 0.01, ****p < 0.0001. The p values were calculated by 2-way analysis of variance (B, C).

limited by the selectivity of the BCR, surface IgG was crosslinked with Th1-associated pathogen *S. typhimurium* for efficient internalization, processing, and presentation to autologous Th cells.^{23,30} After 6 days of coculture, we

found that the induced expression of CXCR3 on *Salmonella*-containing IgG⁺ and not IgM_{naive} or IgM_{mem} B cells was abrogated by the addition of an IFN γ -blocking antibody (p < 0.0001). Furthermore, blocking of IFN γ

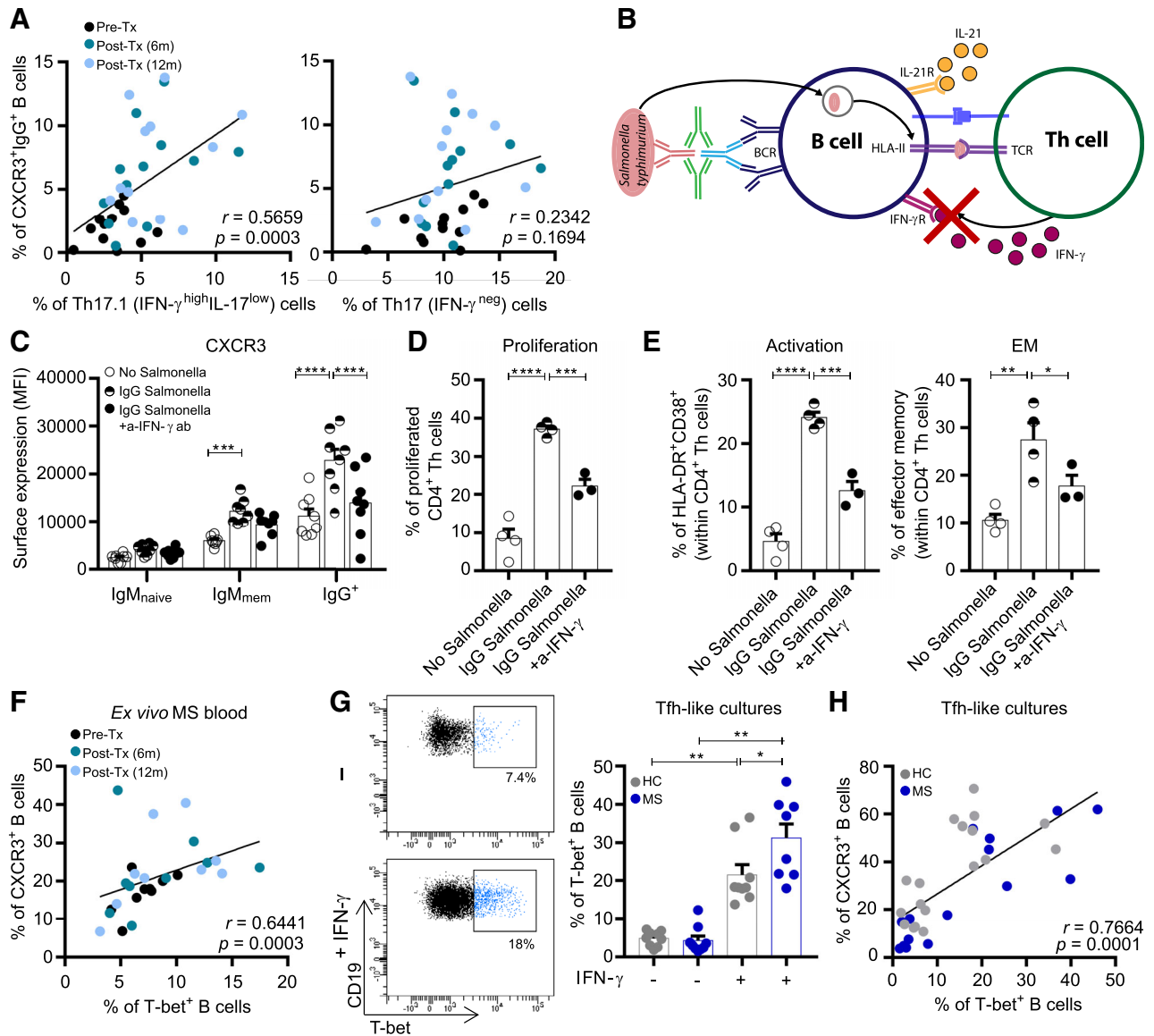


FIGURE 4: T helper (Th) 1 cytokine interferon- γ (IFN γ) is a major trigger of CXCR3⁺(T-bet⁺) B-cell differentiation in multiple sclerosis (MS). (A) Correlation of ex vivo CXCR3⁺IgG⁺ B cells with Th17.1 (IFN γ ^{high}IL-17^{low}) and Th17 (IFN γ ^{neg}) cells in MS blood before and after natalizumab treatment (pre-Tx and post-Tx; n = 12). (B) Experimental model of *Salmonella*-primed autologous B- and T-cell cocultures. (C–E) B cells from healthy donor blood were primed with *S. typhimurium* through B-cell receptor (BCR) crosslinking using a tetrameric antibody complex, as described in Subjects and Methods. This allows BCR-mediated *Salmonella* uptake, processing, and presentation on MHC II molecules to Th cells. IL-21 was added with and without an IFN γ blocking antibody to analyze the effects on CXCR3 expression by B cells (C), and on the proliferation, activation, and effector memory (EM) phenotype of Th cells (D, E). These experiments were performed in 2 independent experiments and in duplicate for (C) 4 and (D, E) 2 different blood donors. (F) Correlation of surface CXCR3 and intracellular T-bet expression in ex vivo B cells of MS patients before and after natalizumab treatment (pre-Tx and post-Tx; n = 9). (G, H) Total B cells from the blood of MS patients (n = 9) and both age- and gender-matched healthy controls (HC; n = 10) were cultured in vitro under T follicular helper (Tfh)-like conditions with IL-21 and 3T3-CD40L cells, and with or without IFN γ for 11 days. Representative FACS plots and quantification of in vitro-induced Tbet⁺ B cells (G) and correlation of CXCR3 and Tbet expression in these cultured B cells (H) are shown. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The p values were calculated by 2-way analysis of variance (ANOVA; C), 1-way ANOVA (D, E), and Wilcoxon matched-pairs signed rank (G) tests. The correlation coefficients and p values for A, F, H were calculated by Spearman rank. m = months; MFI = mean fluorescence intensity; TCR = T-cell receptor.

impaired B-cell-induced T-cell proliferation, activation, and effector memory formation. This implies that IFN γ additionally stimulates the antigen-presenting function of CXCR3⁺IgG⁺ B cells, in parallel with previous findings in mice.³¹

CXCR3 expression is under the direct control of IFN γ -inducible transcription factor Tbet, a critical regulator of memory B-cell differentiation in mice.^{13,14} Consistent with this, intracellular Tbet positively correlated to

surface CXCR3 expression in ex vivo B cells ($r = 0.644$, $p = 0.0003$) and showed a similar association with Th17.1 (IFN γ^{high} IL-17 $^{\text{low}}$) cells in MS patients (see Fig 4 and data not shown).²⁰ To further explore the susceptibility of B cells to IFN γ in MS,⁷ we compared B cells from MS and matched healthy control blood for IFN γ -mediated T-bet induction under Tfh-like culture conditions. After 11 days of stimulation with 3T3-CD40L cells, IL-21, and IFN γ , T-bet was predominantly upregulated in B cells of MS patients ($p = 0.021$), whereas conditions without IFN γ did not show this. This in vitro-induced T-bet was coexpressed with surface CXCR3 ($r = 0.766$, $p = 0.0001$), in line with our ex vivo results. These findings reveal that Th-cell-derived IFN γ is a major trigger of peripheral CXCR3(T-bet)⁺ B cells in MS.

IFN γ Stimulates Plasmablast Formation and Synergizes with CpG-DNA to Establish IgG1 Switching during Human Tfh-like B-Cell Cultures

Besides IFN γ , TLR9 ligand CpG-DNA has also been reported to induce T-bet in murine B cells^{14,18} and promote proinflammatory cytokine responses of B cells from MS patients.⁷ To assess how TLR9 signals integrate with IFN γ to regulate human T-bet⁺ B-cell development, we first determined whether naive or memory B cells are more prone to this type of coactivation. Naive (CD27⁻IgG⁻) and memory (CD27⁺IgG⁺) B cells were sorted from healthy donor blood and stimulated with 3T3-CD40L cells, IL-21, IFN γ , and/or CpG-DNA. After 11 days of naive B-cell cultures, both T-bet and CXCR3 expression was induced by IFN γ , and further enhanced after addition of both IFN γ and CpG-DNA ($p = 0.001$ and $p = 0.021$; Fig 5A). This additional effect of CpG-DNA was not found when using sorted memory B cells (see Fig 5B). Both IFN γ -induced and CpG-DNA-induced T-bet(CXCR3)⁺ B cells also showed strongly reduced CD21 expression (data not shown), a typical feature seen for T-bet-expressing B cells.³¹

During a germinal center response, naive B cells can either differentiate into plasmablast or memory populations, depending on the local inflammatory environment.^{10,32} We investigated the effects of IFN γ and CpG-DNA on plasmablast formation and IgG subclass switching during IL-21-/CD40L-induced naive B-cell differentiation. After 11 days of culture, sorted naive B cells from healthy donors developed into plasmablasts under IFN γ stimulatory conditions only ($p = 0.034$; Fig 5). IFN γ and CpG-DNA together did not induce plasmablast formation, but instead triggered IgG1 and not IgG2 expression on differentiating B cells (IFN γ only vs IFN γ + CpG-DNA: $p = 0.002$). Interestingly, this in vitro-induced IgG1 switching was subjected to differentiation of sorted naive and not memory (data

not shown) B cells. CpG-DNA alone did not upregulate CXCR3, T-bet, and IgG1 in differentiating naive B cells (data not shown), indicating that both IFN γ and TLR9 signaling are required for enhanced expression of these markers. To verify that B-cell intrinsic expression also corresponds with secretion of IgG1, we performed ELISAs on culture supernatants of these B cells. The percentage of IgG1⁺ B cells positively correlated to IgG1 secretion ($r = 0.640$, $p = 0.016$).

Finally, to address how this is regulated in MS, we isolated naive B cells from MS patients and performed similar culture experiments. IFN γ -mediated plasmablast formation was more induced after 11 days of culture compared to matched controls ($p = 0.031$; see Fig 5F). This was not seen after stimulation with both IFN γ and CpG-DNA. Instead, this type of triggering resulted in a robust induction of CXCR3⁺IgG1⁺, and not CXCR3⁺IgG2⁺ subsets in MS (see Fig 5G, H). Collectively, these data demonstrate that TLR9 signaling potentiates IFN γ -induced T-bet and CXCR3 expression during naive B-cell differentiation in vitro, and that this is important for IFN γ -mediated formation of IgG1⁺ memory B cells rather than plasmablasts under Tfh-like circumstances in MS.

Discussion

Evidence has accumulated that at least in the periphery, antibody-independent roles of B cells are driving the pathology of MS.⁷ However, local production of autoantibodies should not be ruled out as an underlying B-cell mechanism in this disease.³³ Although autoreactive naive B cells are highly active in MS blood,⁹ the vast majority of B cells identified in the MS brain have undergone further maturation into antibody-producing cells.^{34,35} It has also been demonstrated that memory B cells of MS patients are the most potent antigen-presenting cells and likely have specific proinflammatory propensities, including the capacity to express enhanced levels of immune-activating molecules.³⁵ This is of special interest considering the presence of meningeal B-cell-rich follicle-like structures in MS and adjacent subpial cortical demyelinating injury,⁸ which probably contributes to progressive loss of neurological function in patients with MS. Thus, identification of the particular B-cell subsets that can preferentially migrate into the CNS and clarification of how they may contribute to propagating local injury responses are of considerable interest in such an organ-specific disease. In this study, we demonstrate that integrating IFN γ and pathogen-associated TLR9 signals is critical for the development of human T-bet⁺ memory B cells, probably underlying their selective recruitment to the CNS of MS patients.

Recent studies have shown that in MS identical B-cell clones are present in both the periphery and CNS.²⁻⁴

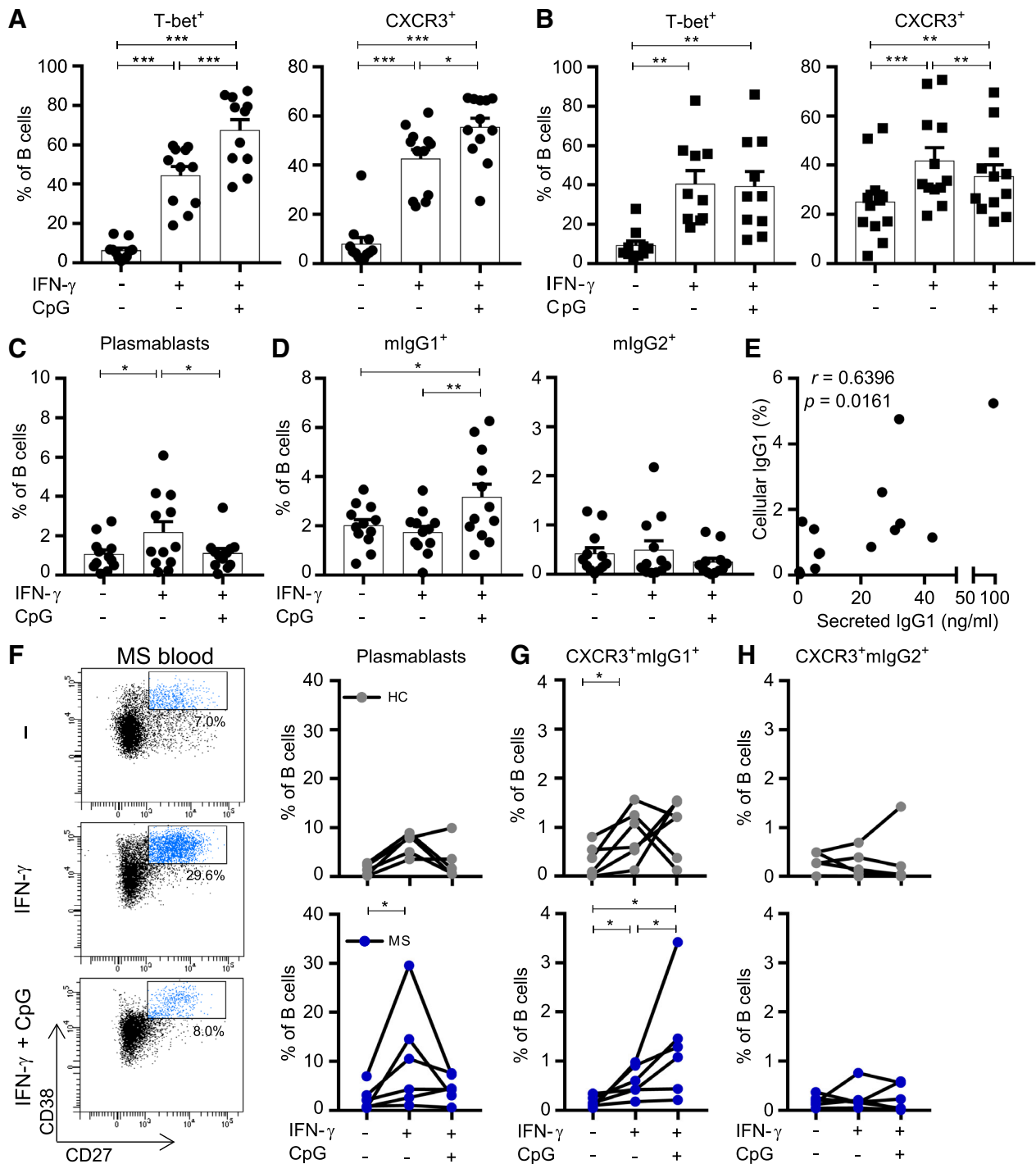


FIGURE 5: Interferon- γ (IFN γ) induces plasmablast differentiation, whereas both IFN γ and CpG-DNA further upregulate T-bet and trigger IgG1 switching in B cells of multiple sclerosis (MS) patients. (A–D) Naive (IgG⁻CD27⁻; dots) and memory (IgG⁺CD27⁺; squares) B cells were sorted from peripheral blood of healthy donors and were cultured under T follicular helper (Tfh)-like conditions with IL-21 and 3T3-CD40L cells, with or without IFN γ and/or CpG-DNA. Frequencies of T-bet⁺ and CXCR3⁺ B cells after 11 days of culture using (A) naive B cells (n = 12) and 6 days of culture using (B) memory B cells (n = 10–12) are shown. The frequencies of (C) plasmablasts (CD38^{high}CD27⁺; n = 12) and (D) membrane-bound (m) mIgG1⁺ and mIgG2⁺ B cells were analyzed after culturing naive populations for 11 days (n = 12). (E) Correlation between cellular expression and secretion of IgG1 was determined by FACS and enzyme-linked immunosorbent assay (pooled stimulation conditions for 5 donors). (F–H) Naive B cells from the blood of MS patients (n = 6; dark blue dots) and healthy controls (HC; n = 6; gray dots) were cultured under the same Tfh-like conditions and analyzed for (F) plasmablast (CD38^{high}CD27⁺) and (G, H) CXCR3⁺mIgG1⁺ and CXCR3⁺mIgG2⁺ B-cell differentiation after 11 days of culture. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The p values for A–D and F–H were calculated by the Wilcoxon matched-pairs signed rank test. The correlation coefficient and p value for E were calculated by Spearman rank correlation.

That these B-cell populations further undergo somatic hypermutation in the brain implies the presence of functional germinal centers within the CNS. Such structures have been identified in the meninges of MS patients and are obvious localizations that play a role here.⁸ The enrichment that we observe of CXCR3⁺ B cells in paired CSF, meninges, and brain tissue compartments compared to blood of MS patients is in line with studies that show higher levels of CXCR3 ligand CXCL10 in the CSF of MS patients.²⁴ These results are also consistent with our previous findings that CXCR3⁺ T cells, including Th17.1, are abundant within the CNS,^{20,24} suggesting a common CXCR3-driven lymphocyte recruitment pathway in MS.^{24,36,37} Other studies have also put forward CXCR5 and its ligand CXCL13 as important contributors to B-cell recruitment to the CNS.^{3,38} We did not find differences in CXCR5-expressing B cells between CNS tissues and paired blood. Hence, the CXCR5/CXCL13 axis is probably related to local organization rather than recruitment of pathogenic B (and T) cells,^{39,40} in a process similar to that in secondary lymphoid organs.⁴¹ Although these studies indicate a role for germinal center B cells within the CNS, little is known about which peripheral mechanisms underlie their development and local recruitment. In mice, it has been shown that in an autoimmune setting, IFN γ , likely produced by activated Tfh cells, induces germinal centers,¹⁰ which can be found in meningeal follicle-like structures.⁸ In these situations, IFN γ induced B-cell-intrinsic expression of T-bet, possibly resulting in enhanced Ig class switching and CXCR3 expression.^{13,14} This points to a central role of IFN γ -associated CXCR3⁺ B-cell subsets in the meningeal process.^{10,12}

While the inducing effects of peripheral B cells on autoreactive Th1 cells are currently being elucidated,³⁷ far less is known about the impact of Th1 cells on peripheral B-cell differentiation and function in MS patients. Therefore, we were interested in the signals needed for B cells to differentiate into T-bet⁺ cells and postulated that IFN γ - and IL-21-producing Tfh1 cells in germinal centers can trigger development of such B cells. In MS patients, B cells were found to express higher T-bet levels under Tfh1-like culture conditions. Furthermore, IL-21-based B- and T-cell cocultures revealed that CXCR3-expressing IgG⁺ memory B cells were less induced after blocking of IFN γ , which corresponds to studies that show IFN γ regulates CXCR3 expression in human B cells.²⁹ Th-cell proliferation, activation, and effector memory formation were also affected in these cultures. In line with our findings, a recent study demonstrated that memory B cells induce proliferation of CNS-infiltrating Th1 cells in MS, which was inhibited after IFN γ abrogation.³⁷ Therefore, in MS patients, peripheral interaction of CXCR3 (T-bet)⁺ B and IFN γ -producing Th cells probably generates a feedforward loop, in which IFN γ enhances the potency of

B cells as antigen-presenting cells, resulting in the activation of (IFN γ -producing) pathogenic Th cells.

Furthermore, we found that naive B cells from MS patients developed into plasmablasts rather than IgG1-switched memory B cells under IFN γ -only conditions. Because T-bet mediates class-switching in murine B cells,¹³ we expected that an additional signal would be required for triggering such a mechanism in human B cells. Besides Tfh1 cells, innate TLR signaling is also critical for naive B-cell differentiation.⁴² Especially pathogen-associated TLR9 and its ligand CpG-DNA have been shown to promote the development of T-bet⁺ B cells in mice.^{19,31,42} Correspondingly, we found that the induction of MS-blood-derived naive B cells with both IFN γ and CpG-DNA resulted in the development of IgG1-switched, T-bet^{high} B cells during Tfh-like cultures. Likewise, CXCR3 surface expression was more enhanced under these conditions, reflecting the high CXCR3 levels on ex vivo IgG1⁺ B cells. This additional effect of TLR9 signaling on human T-bet⁺ B cells in MS links to the role of TLR9 in driving neuroinflammatory responses, including increased production of chemokines in the CNS.⁴³ Moreover, CXCR3⁺IgG1⁺ B cells showed an enhanced transmigration potential over brain endothelial layers, and selectively accumulated in MS blood after natalizumab therapy. The importance of pathogenic immune cells in contributing to MS disease progression, such as CXCR3⁺ memory B cells, has been put forward by the recurrence of often-fatal clinical relapses in MS patients when discontinuing the use of natalizumab.^{27,44,45} During these rebounds, Epstein-Barr virus-infected memory B cells that have accumulated in the blood show massive influx into brain tissues of MS patients.⁴⁵ Furthermore, persistent viral infections are suggested to sustain the development of T-bet-expressing B cells,¹⁵ which further supports the enhanced differentiation and local recruitment of CXCR3(T-bet)^{high} memory B cells in an organ-specific autoimmune disease such as MS (Fig 6).

Although the exact role of (local) autoantibody production in MS is not clear, the question of whether and how T-bet⁺ B cells are involved in this process deserves further attention. In SLE, T-bet⁺ B cells have autoreactive BCRs and are prone to differentiate into IgG autoantibody-producing plasmablasts.⁴⁶ Inappropriate T-bet expression in B cells also impaired CXCR3-mediated plasmablast differentiation within germinal centers¹⁴ and autoantibody production.¹³ In our study, IFN γ -induced and CpG-DNA-induced human CXCR3(T-bet)^{high} B cells showed increased IgG1 expression and secretion. This strongly suggests that after preferential recruitment and reactivation in the CNS, CXCR3(T-bet)^{high}IgG1⁺ B cells are responsible for local production of IgG1 in MS (see Fig 6).²⁸ Although B cells within the CNS of MS

MS patient

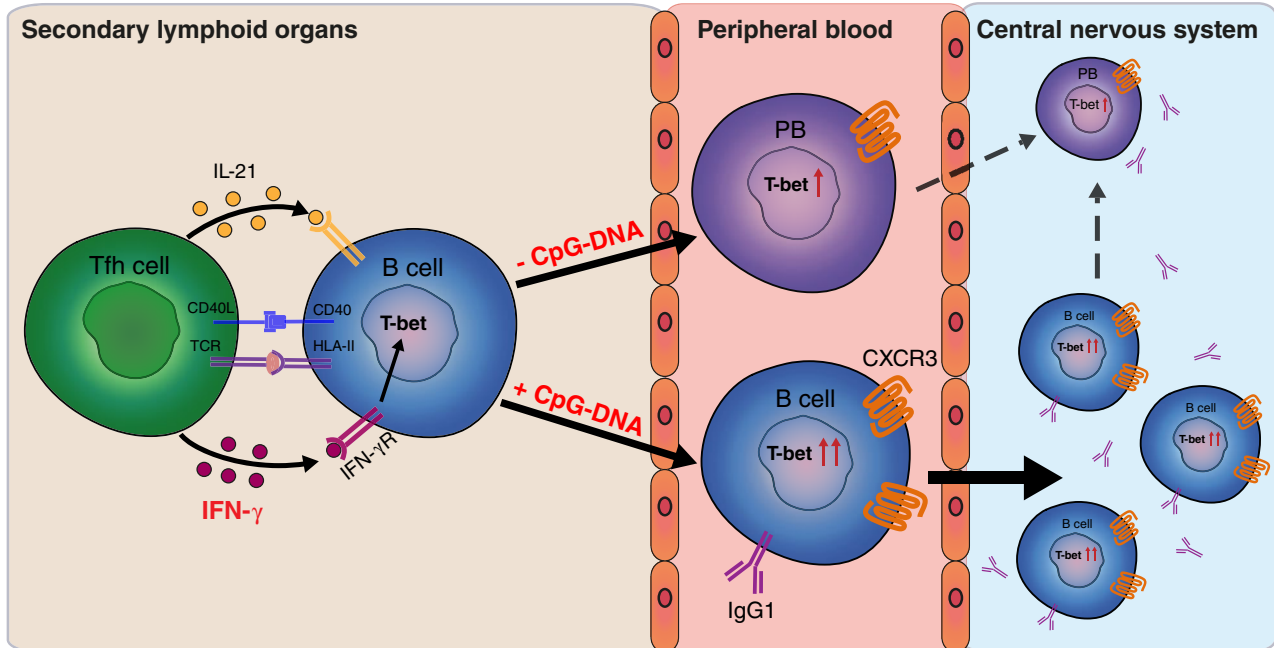


FIGURE 6: Interferon- γ (IFN γ) and Toll-like receptor 9 (TLR9) signaling upregulate Tbet in peripheral B cells, likely driving CXCR3-mediated recruitment and IgG1 production in the central nervous system of multiple sclerosis (MS) patients. Our findings suggest that in the secondary lymphoid organs of MS patients, IFN γ triggers naive B cells to differentiate into Tbet-expressing populations in a T follicular helper (Tfh)-dependent manner. Human Tbet⁺ B cells can either develop into plasmablasts or undergo further differentiation into IgG1⁺ memory B cells mediated by TLR9 ligation. The enhanced CXCR3 expression on both IFN γ - and TLR9-induced IgG1⁺ B cells makes these subsets highly capable of transmigrating across the blood-brain barrier and mediate local pathology in MS. TCR = T-cell receptor.

patients show characteristics of an antigen-driven response, the specific antigens driving this response remain unknown. MS disease heterogeneity is reflected by the identification of several candidate target antigens, including nonmyelin proteins such as neurofilament light and RAS guanyl releasing protein 2 (RASGRP2).^{33,37} In addition to this, increased Epstein-Barr nuclear antigen 1 (EBNA1)-specific IgG1 titers have been found in active MS, which may be explained by the interaction of B cells with pathogen-associated TLR ligands and EBNA1-specific, IFN γ -producing T cells that cross-recognize myelin antigens.⁴⁷ Therefore, we propose that the relevant antigen specificity of B cells in MS can be found within this subset, which should be further explored in the near future.

Taken together, not only a disrupted blood-brain barrier, but also peripheral Tbet-mediated differentiation and transmigration of IgG1⁺ memory populations could explain how B cells are eventually able to mediate CNS pathology in MS patients (see Fig 6). The relevance of T- and B-cell interaction in tolerance breakthrough is stressed by the finding that antigen-specific B cells are potentially 1,000 to 10,000 times better presenters of autologous peptides to T cells than nonspecific B cells.⁴⁸ We here reveal that human CXCR3(Tbet)⁺ B cells are a product of T- and B-cell interaction. Similar to SLE, such populations probably

serve as potent antigen-presenting cells in CNS-specific autoimmune diseases such as MS.³¹ Anti-CD20 therapy exerts immediate effects and is assumed to predominantly affect this function of B cells in MS patients.³⁵ The potential role of CXCR3(Tbet)⁺ B cells as prime targets of this therapy is further supported by their abundant CD20 expression, as shown in the current study. The development of new targeted strategies to inhibit Tbet function have the potential to become a double-edged sword in MS by suppressing pathogenic, IFN γ -producing T (Th17.1) cells together with their counterpart CXCR3(Tbet)⁺ B cells. Small molecule inhibitors of IFN γ signaling (jakinibs)⁴⁹ and the TLR/myD88 pathway⁵⁰ are already used in clinics for other inflammatory diseases, and are promising candidates for combined suppression of IFN γ and TLR signals to control pathogenic Tbet⁺ B cells in autoimmune diseases such as MS.

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Author Contributions

J.v.L., L.R., M.M.v.L., R.Q.H., P.P.A.U., S.M.v.H., and H.E.d.V. contributed to the study concept and design. J.v.L., L.R., A.F.W.-W., M.-J.M., M.J., P.-P.A.U., T.A.S., and M.M.v.L. acquired and analyzed data. J.v.L., M.M.v.L., L.R., and R.Q.H. drafted the manuscript and figures.

Potential Conflicts of Interest

Nothing to report.

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