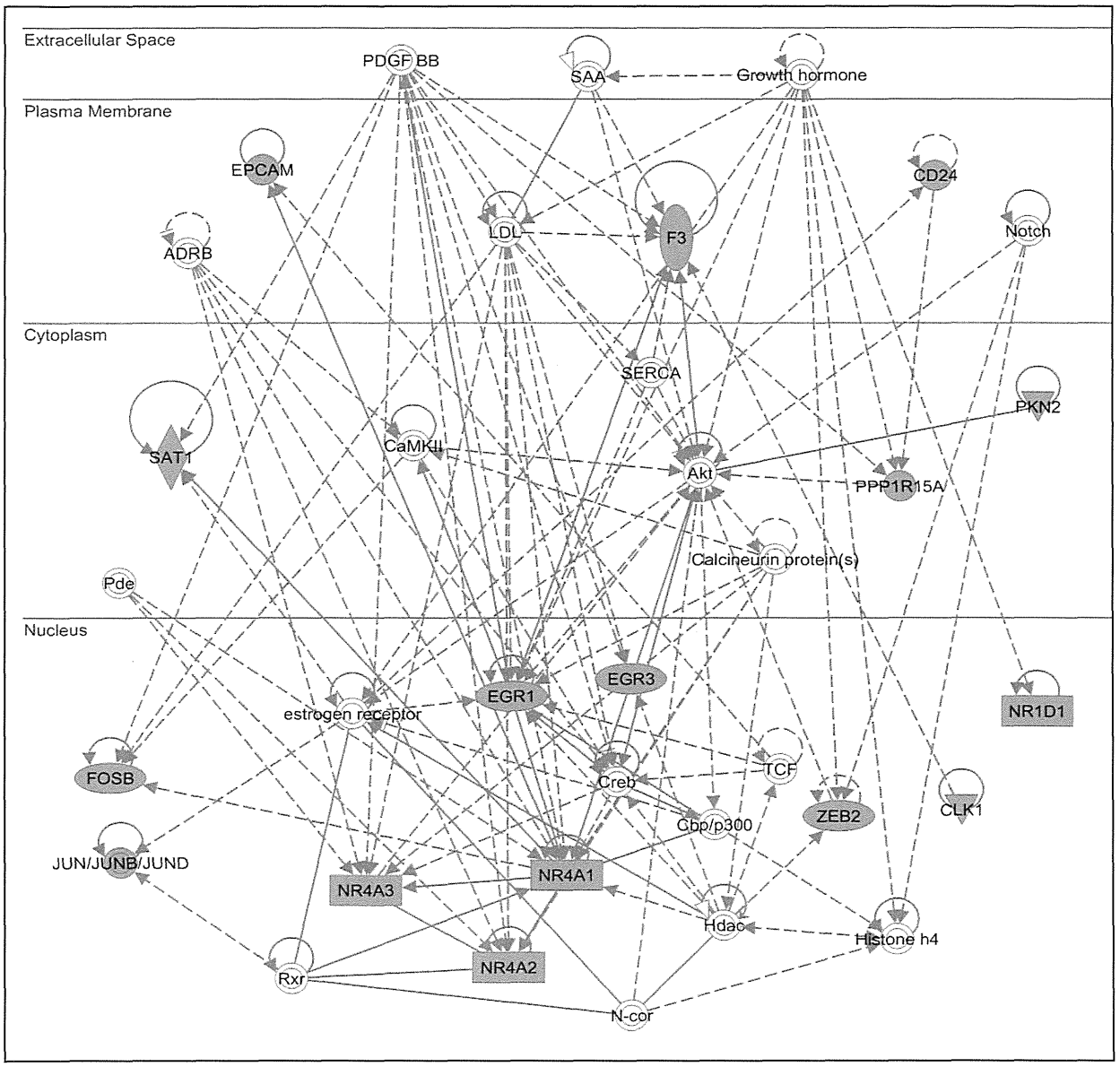

図 2. NLRP3 インフラマゾーム活性化反応性遺伝子群の分子ネットワーク.

Entrez Gene IDs corresponding to the set of 83 NLRP3 inflammasome activation-responsive genes in THP-1 cells (Table 1) were imported into the core analysis tool of IPA. The functional network defined as “Cell Cycle, Cellular Development, Cell Death and Survival” is shown. Red nodes indicate NLRP3 inflammasome activation-responsive genes.

表 1. NLRP3 インフラマゾーム活性化で誘導される遺伝子群.

Table 1. Top 30 up-regulated genes in THP-1 monocytes following activation of NLRP3 inflammasome

Rank	FC Related to Signal 1	FC Related to Signal 2	Entrez Gene ID	Gene Symbol	Gene Name
1	1.06819645	18.61247501	8013	NR4A3	nuclear receptor subfamily 4, group A, member 3
2	1.942378012	12.91651537	6348	CCL3	chemokine (C-C motif) ligand 3
3	1.63109973	11.69111	414062	CCL3L3	chemokine (C-C motif) ligand 3-like 3
4	1.100615838	11.24166642	9308	CD83	CD83 molecule
5	1.819566773	10.85127008	3576	IL8	interleukin 8
6	1.292541852	7.633454043	1960	EGR3	early growth response 3
7	0.948867136	6.576691539	4929	NR4A2	nuclear receptor subfamily 4, group A, member 2
8	1.116320272	5.51767318	3164	NR4A1	nuclear receptor subfamily 4, group A, member 1
9	1.842348508	5.271896351	64332	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
10	1.268131184	4.992502002	643616	MOP-1	MOP-1
11	1.222058201	4.99018398	1959	EGR2	early growth response 2
12	1.716614387	4.456895103	5734	PTGER4	prostaglandin E receptor 4 (subtype EP4)
13	1.067764134	4.401932449	10746	MAP3K2	mitogen-activated protein kinase kinase kinase 2
14	1.076240121	4.353030131	2920	CXCL2	chemokine (C-X-C motif) ligand 2
15	1.443866138	4.329651804	6364	CCL20	chemokine (C-C motif) ligand 20
16	1.506881527	4.037790353	5743	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
17	1.143021068	3.908082725	153020	RASGEF1B	RasGEF domain family, member 1B
18	1.00701348	3.793627448	1958	EGR1	early growth response 1
19	1.188818931	3.318906546	23645	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A
20	0.978133301	3.154899408	65125	WNK1	WNK lysine deficient protein kinase 1
21	1.116953399	3.113268501	84807	NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
22	1.431860551	3.025219884	51561	IL23A	interleukin 23, alpha subunit p19
23	0.654486344	2.985745104	645188	LOC645188	hypothetical LOC645188
24	1.082721348	2.867304268	1843	DUSP1	dual specificity phosphatase 1
25	1.877501415	2.813972064	8870	IER3	immediate early response 3
26	1.458901009	2.788511085	9021	SOCS3	suppressor of cytokine signaling 3
27	0.930381294	2.730662487	728715	LOC728715	ovostatin homolog 2-like
28	1.251031395	2.703465614	2353	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog

To activate NLRP3 inflammasome, THP-1 cells were initially exposed to 0.2 μg/ml LPS for 3 hours (Signal 1). They were then washed by PBS and exposed to 10 μM nigericin for 2 hours (Signal 2 after Signal 1). At 5 hours after initiation of the treatment, total RNA was isolated and processed for gene expression profilong on a Human Gene 1.0 ST Array. The set of 83 genes that satisfy fold change (FC) related to Signal 1 (LPS + versus LPS -) smaller than 2-fold and FC related to Signal 2 (nigericin + versus nigericin -) greater than 2-fold are shown with FC, Entrez Gene ID, Gene Symbol, and Gene Name.

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（平成26年度）

書籍

	著者氏名	論文タイトル名	書籍全体の編集者名 書籍名	出版社名 出版地	出版年 ページ
1		該当なし			
2					

研究成果の刊行に関する一覧表（平成26年度）

雑誌

	発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
1	Manabu Araki, Takako Matsuoka, Katsuichi Miyamoto, Susumu Kusunoki, Tomoko Okamoto, Miho Murata, Sachiko Miyake, Toshimasa Aranami, Takashi Yamamura.	Efficacy of the anti-IL-6 receptor antibody tocilizumab in neuromyelitis optica	Neurology	82 (15)	1302-1306	2014
2	Masakazu Nakamura, Takako Matsuoka, Norio Chihara, Sachiko Miyake, Wakiro Sato, Manabu Araki, Tomoko Okamoto, Youwei Lin, Masafumi Ogawa, Miho Murata, Toshimasa Aranami, Takashi Yamamura	Differential effects of fingolimod on B-cell populations in multiple sclerosis.	Multiple Sclerosis J	20 (10)	1371-1380	2014
3	Yamamura, T	How do T cells mediate central nervous system inflammation?	Clin. Exp. Neuroimmunol.	5	107	2014
4	佐藤和貴郎, 山村　隆	免疫動態	Clinical Neuroscience	32	1218-1221	2014
5	Kamachi F, Harada N, Usui Y, Sakanishi T, Ishii N, Okumura K, Miyake S, Akiba H.	OX40 ligand regulates splenic CD8(-) dendritic cell-induced Th2 responses in vivo.	Biochem Biophys Res Commun.	444	235-240	2014
6	Miyamoto K, Tanaka N, Moriguchi K, Ueno R, Kadomatsu K, Kitagawa H, Kusunoki S	Chondroitin-6-O-sulfate ameliorates experimental autoimmune encephalomyelitis.	Glycobiology	24	469-475	2014
7	Satoh J, Motohashi N, Kino Y, Ishida T, Yagishita S, Jinnai K, Arai N, Nakamagoe K, Tamaoka A, Saito Y, Arima K.	LC3, an autophagosome marker, is expressed on oligodendrocytes in Nasu-Hakola disease brains.	Orphanet Journal of Rare Diseases	9	e68	2014
8	Choi SS, Lee HJ, Lim I, Satoh J, Kim SU.	Human astrocytes: Secretome profiles of cytokines and chemokines.	PLoS One	9 (4)	e92325	2014
9	Kawana N, Yamamoto Y, Kino Y, Satoh J.	Molecular network of NLRP3 inflammasome activation-responsive genes in a human monocyte cell line.	Austin Journal of Clinical Immunology	1 (4)	e1071	2014
10	Satoh J, Kino Y, Kawana N, Yamamoto Y, Ishida T, Saito Y, Arima K.	TMEM106B expression is reduced in Alzheimer's disease brains.	Alzheimer's Research and Therapy	6 (2)	e17	2014
11	Satoh J, Asahina N, Kitano S, Kino Y.	A comprehensive profile of ChIP-Seq-based PU.1/Spi1 target genes in microglia.	Gene Regulation and Systems Biology	8	127-139	2014

研究成果の刊行物・別刷

Efficacy of the anti–IL-6 receptor antibody tocilizumab in neuromyelitis optica

A pilot study

OPEN

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ABSTRACT

Objective: To evaluate the safety and efficacy of a humanized anti-interleukin-6 receptor antibody, tocilizumab (TCZ), in patients with neuromyelitis optica (NMO).

Methods: Seven patients with anti-aquaporin-4 antibody (AQP4-Ab)-positive NMO or NMO spectrum disorders were recruited on the basis of their limited responsiveness to their current treatment. They were given a monthly injection of TCZ (8 mg/kg) with their current therapy for a year. We evaluated the annualized relapse rate, the Expanded Disability Status Scale score, and numerical rating scales for neurogenic pain and fatigue. Serum levels of anti-AQP4-Ab were measured with AQP4-transfected cells.

Results: Six females and one male with NMO were enrolled. After a year of TCZ treatment, the annualized relapse rate decreased from 2.9 ± 1.1 to 0.4 ± 0.8 ($p < 0.005$). The Expanded Disability Status Scale score, neuropathic pain, and general fatigue also declined significantly. The ameliorating effects on intractable pain exceeded expectations.

Conclusion: Interleukin-6 receptor blockade is a promising therapeutic option for NMO.

Classification of evidence: This study provides Class IV evidence that in patients with NMO, TCZ reduces relapse rate, neuropathic pain, and fatigue. *Neurology*® 2014;82:1302–1306

GLOSSARY

Ab = antibody; **AQP4** = aquaporin-4; **AZA** = azathioprine; **EDSS** = Expanded Disability Status Scale; **IL** = interleukin; **IL-6R** = interleukin-6 receptor; **NMO** = neuromyelitis optica; **PB** = plasmablasts; **PSL** = prednisolone; **TCZ** = tocilizumab.

Neuromyelitis optica (NMO) is a relatively rare autoimmune disease that predominantly affects the spinal cord and optic nerve. Anti–aquaporin-4 antibody (AQP4-Ab), which is a disease marker of NMO, has an important role in causing the destruction of astrocytes that express AQP4.[1] Empirically, the use of disease-modifying drugs for multiple sclerosis, including interferon β, is not recommended for NMO,[2] which is consistent with the distinct pathogenesis of NMO and multiple sclerosis. We have recently described that plasmablasts (PB), which are a subpopulation of B cells, increased in the peripheral blood of patients with NMO and that PB are a major source of anti-AQP4-Ab among peripheral blood B cells.[3] In addition, we observed that exogenous interleukin (IL)-6 promotes the survival of PB and their production of anti-AQP4-Ab in vitro. Given the increased levels of IL-6 in the serum and CSF during relapses of NMO,[1,3] we postulated that blocking IL-6 receptor (IL-6R) pathways might reduce the disease activity of NMO by inactivating the effector functions of PB. A humanized anti–IL-6R monoclonal antibody, tocilizumab (TCZ) (Actemra/RoActemra), has been approved in more than 100 countries for use in the treatment of rheumatoid arthritis.[4] Herein, we describe our clinical study that aimed to explore the efficacy of TCZ in NMO.

Editorial, page 1294

From the Multiple Sclerosis Center (M.A., T.O., S.M., T.A., T.Y.) and Department of Neurology (T.O., M.M.), National Center Hospital, and Department of Immunology, National Institute of Neuroscience (T.M., S.M., T.A., T.Y.), National Center of Neurology and Psychiatry, Tokyo; Department of Neurology (K.M., S.K.), Kinki University School of Medicine, Osaka; and Department of Pediatrics (T.M.), Graduate School of Medicine, University of Tokyo, Japan.

Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

Table Demographics of the patients

	Patient						
	1	2	3	4	5	6	7
Age, y/sex	37/F	38/F	26/F	31/M	55/F	62/F	23/F
Age at onset, y	23	27	21	12	38	60	21
Anti-AQP4-Ab	+	+	+	+	+	+	+
Myelitis	+	+	+	+	+	+	–
Optic neuritis	+	+	+	+	+	+	+
EDSS score	3.5	6.5	3.5	6.0	6.5	6.5	3.0
Total no. of relapses	20	9	6	16	20	3	7
ARR before TCZ	3	2	2	2	3	3	5
Immunotherapies for exacerbations	IVMP, PLEX	IVMP, PLEX	IVMP, PLEX	IVMP, OBP, PLEX	IVMP, PLEX	IVMP, PLEX	IVMP, PLEX
Past immunotherapies	IFNβ, IVIg	IFNβ	—	IFNβ, MITX	IFNβ, AZA	—	AZA
Present immunotherapies	PSL, AZA	AZA	PSL	PSL, AZA	PSL, CyA	PSL, CyA	PSL, tacrolimus
Neuropathic pain (e.g., girdle pain), NRS	4	4	2	4	4	3	0
General fatigue, NRS	5	8	6	7	5	3	9
Pain and antispasticity medication	GBP, CZP, NTP, NSAID	CZP, mexiletine, NTP, tizanidine, NSAID	—	CBZ, baclofen, NSAID	CBZ	PGB	—

Abbreviations: AQP4-Ab = aquaporin-4 antibody; ARR = annualized relapse rate; AZA = azathioprine; CBZ = carbamazepine; CZP = clonazepam; CyA = cyclosporine; EDSS = Expanded Disability Status Scale; GBP = gabapentin; IFNβ = interferon β; IVIg = IV immunoglobulin; IVMP = IV methylprednisolone; MITX = mitoxantrone; NRS = numerical rating scale; NSAID = nonsteroidal anti-inflammatory drug; NTP = Neurotropin (an extract from the inflamed skin of *vaccinia virus*-inoculated rabbits); OBP = oral betamethasone pulse therapy; PGB = pregabalin; PLEX = plasma exchange; PSL = prednisolone; TCZ = tocilizumab.

METHODS **Level of evidence.** The aim of this Class IV evidence study was to evaluate the effect and safety of a monthly injection of TCZ (8 mg/kg) with their current therapy in patients with NMO. We evaluated the adverse events based on Common Terminology Criteria for Adverse Events, version 4.0.

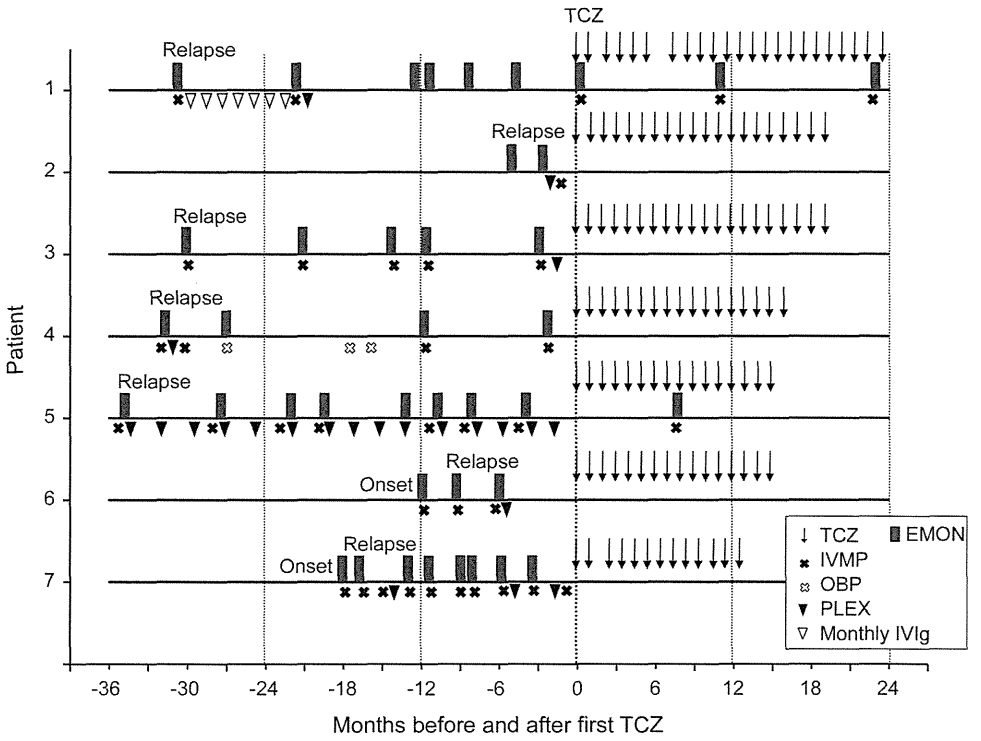
Standard protocol approvals, registrations, and patient consents. All patients gave written informed consent before the first treatment with TCZ. The institutional ethical standards committee on human experimentation approved this clinical study. The study is registered with University Hospital Medical Information Network Clinical Trials Registry, numbers UMIN000005889 and UMIN000007866.

Patients and treatment. Seven patients who met the diagnostic criteria of NMO in 2006 were enrolled after providing informed consent (table). Results of chest x-rays, interferon γ release assays, and plasma 1,3-β-D-glucan measurement excluded latent tuberculosis and fungal infection. All of the patients had been treated with combinations of oral prednisolone (PSL) and immunosuppressants, including azathioprine (AZA). Nevertheless, they had at least 2 relapses during the year before enrollment (figure 1). Among their past immunomodulatory medications, interferon β had been prescribed in 4 patients before the anti-AQP4-Ab assay became available. Although symptomatic treatments had been provided, the patients experienced general fatigue and intractable pain in their trunk and limbs. There were no abnormalities in their routine laboratory blood tests. Neither pleocytosis nor increased levels of IL-6 were observed in the CSF. MRI revealed high-intensity signals in the optic nerves and longitudinally extensive lesions in the spinal cord. All patients except one had scattered brain lesions. A monthly dose (8 mg/kg) of TCZ was added to the patients' oral corticosteroid and immunosuppressive drug regimen.

Clinical and laboratory assessment. As clinical outcome measures, we evaluated alterations in the number of relapses, Expanded Disability Status Scale (EDSS) scores, and pain and fatigue severity scores (numerical rating scales). A relapse was defined as an objective exacerbation in neurologic findings that lasted for longer than 24 hours with an increase in the EDSS score of more than 0.5. Brain and spinal cord MRI scans were examined every 4 or 6 months. CSF examinations, sensory-evoked potentials, and visual-evoked potentials were also evaluated at the time of entry into the study and 12 months later. We measured serum anti-AQP4-Ab levels by evaluating the binding of serum immunoglobulin G to AQP4 transfectants, as previously described.[5] All outcome measures were analyzed with nonparametric Wilcoxon rank-sum tests, with the use of 2-tailed statistical tests at a significance level of 0.05.

RESULTS After starting TCZ treatment, the total number of annual relapses in the patients significantly reduced (figures 1 and 2). Notably, 5 of the 7 patients were relapse-free after starting TCZ. The relapses observed in patients 1 and 5 were mild and their symptoms recovered after IV methylprednisolone. On average, the annualized relapse rate reduced from 2.9 ± 1.1 (range, 2–5) during the year before study to 0.4 ± 0.8 (range, 0–2) during the year after

Figure 1 Clinical course of the patients before and after tocilizumab treatment



The zero on the x-axis represents the first administration of tocilizumab (TCZ). Dark gray bars: exacerbations of myelitis or optic neuritis (EMON); downward arrow: TCZ treatment; black X: IV methylprednisolone (IVMP); white X: oral betamethasone pulse (OBP) therapy; black triangle: plasma exchange (PLEX); white triangle: IV immunoglobulin (IVIg). After receiving 12 injections, all patients continued treatment with TCZ by entering an extension study that evaluates the long-term safety and efficacy of TCZ. We showed the clinical status after completion of the 1-year study to indicate the continuation of remission.

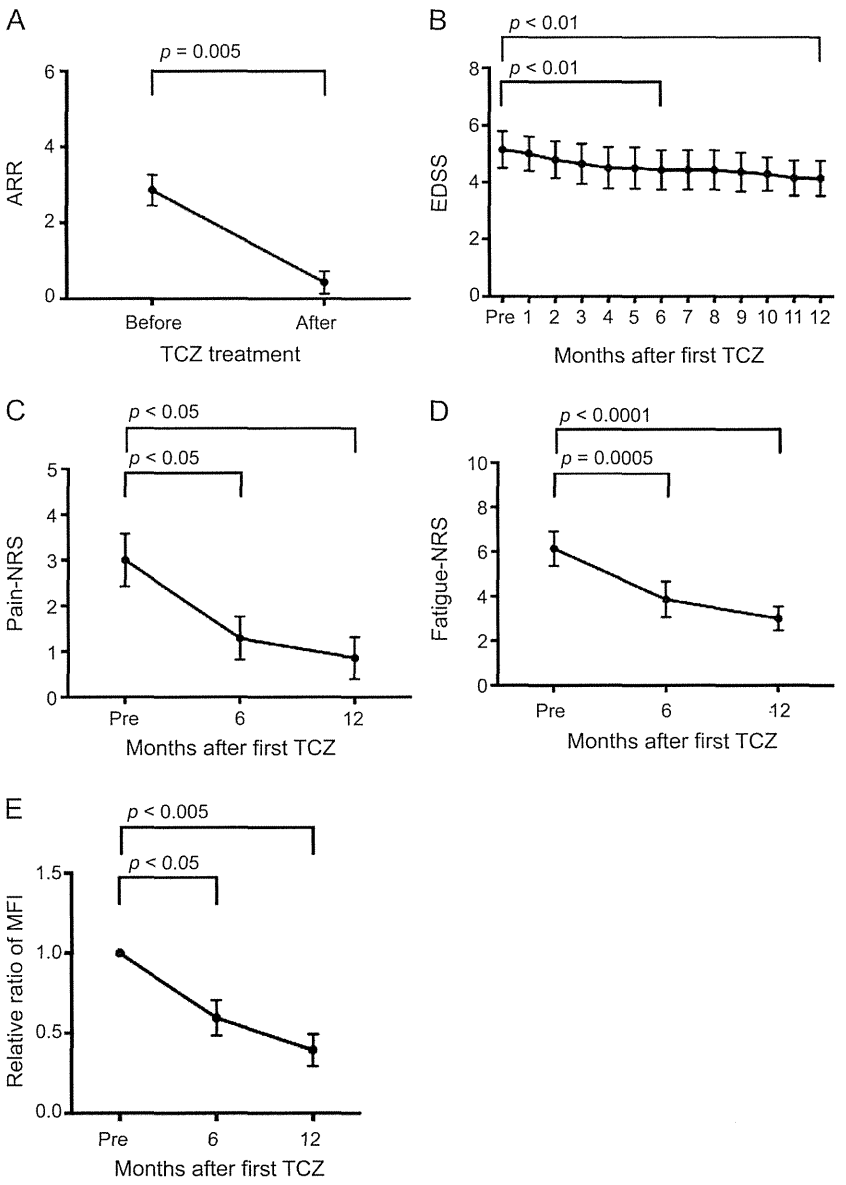
starting TCZ (figure 2). The EDSS score decreased modestly but significantly from 5.1 ± 1.7 (range, 3.0–6.5) to 4.1 ± 1.6 (range, 2.0–6.0) at 12 months. The chronic neurogenic pain in their trunk and extremities, which is characteristic of NMO[6,7] (table), gradually lessened after the patients started TCZ. Consequently, the numerical rating scale for pain reduced from 3.0 ± 1.5 upon study entry to 1.3 ± 1.3 after 6 months and 0.9 ± 1.2 after 12 months. General fatigue also improved from 6.1 ± 2.0 to 3.9 ± 2.1 at 6 months and 3.0 ± 1.4 at 12 months. The MRI scans, sensory- and visual-evoked potentials, and CSF observations did not show any interval changes. Serum anti-AQP4-Ab levels represented by the relative mean fluorescence intensity were significantly reduced (figure 2E).

Adverse events included upper respiratory infections (patients 1 and 7), acute enterocolitis (patients 1 and 4), acute pyelonephritis (patient 1), leukocytopenia and/or lymphocytopenia (patients 1, 4, and 7), anemia (patients 3 and 7), and a slight decline in systolic blood pressure (patient 1). However, none of the events was severe. Oral PSL and AZA were tapered in patients 1, 3, 4, and 7, resulting in a reduction of the mean doses (PSL from 19.5 ± 7.6 to 8.8 ± 5.6 mg/d [average of patients 1, 3, 4, and 7], AZA from 37.5 to 5.4 mg/d [average of patients 1 and 4]).

DISCUSSION Pain management is a difficult problem in patients with NMO. In fact, a retrospective study of 29 patients with NMO who experienced pain has documented that 22 of the 29 patients were taking pain medications, but none of them rated their current pain as 0 out of 10 on a 10-point scale.[6] In the present study, the intractable pain reduced gradually after the patients started TCZ treatment. After 6 or 12 months of therapy, 3 of the 6 patients with pain were completely free of pain. These results suggested a role of IL-6 in NMO pain and the possible merits of the use of TCZ in clinical practice as a pain reliever.

The pathophysiology of neurogenic pain is now understood in the context of interactions between the immune and nervous systems,[8] which involve proinflammatory cytokines such as IL-6 as well as immune cells, activated glia cells, and neurons. Supportive for the role of IL-6 in pain, recent work in

Figure 2 **Effects of tocilizumab on clinical and immunologic parameters**



(A) Annualized relapse rate (ARR) before and after tocilizumab (TCZ) treatment. (B) Expanded Disability Status Scale (EDSS) score during the 1-year study period. Pain severity (numerical rating scale [NRS]) (C) and fatigue severity (D) scores before, 6 months after, and 12 months after the start of TCZ treatment. The dots and I bars indicate means ± SEM. We analyzed only data obtained during the first year of TCZ treatment. (E) The alterations in the serum anti-aquaporin-4 antibody (AQP4-Ab) were evaluated by the relative ratio of the mean fluorescence intensity (MFI), which was based on the MFI before TCZ treatment. Serum anti-AQP4-Ab detection assay was performed as described previously[3,5] with minor modifications. In brief, optimally diluted serum was added to human AQP4-expressing Chinese hamster ovary (CHO) cells. CHO cell-bound anti-AQP4-Ab was detected using fluorescein isothiocyanate-anti-human immunoglobulin G antibody by flow cytometry. For comparison, the MFI of each sample was divided by the MFI of the sample before the start of TCZ treatment.

rodents showed that gp130 expressed by nociceptive neurons might have a key role in pathologic pain.[9] Although expression of membrane-bound IL-6R is restricted to hepatocytes, neutrophils, and subsets of T cells, the gp130, ubiquitously expressed in cellular membranes, can transduce IL-6R signaling via binding to the IL-6/soluble IL-6R complex.[4] This indicates that IL-6 trans-signaling via the soluble IL-6R could be pivotal in causing pain in NMO, although alternative possibilities cannot be excluded.

TCZ treatment recently showed efficacy for patients with aggressive NMO who were refractory to the anti-CD20 antibody rituximab.[10] The efficacy of TCZ could result from its effect on IL-6–dependent inflammatory

processes, involving CD20-negative PB, pathogenic T cells, and regulatory T cells. This work, however, does not restrict the use of TCZ in serious NMO. Although the need for monitoring latent infection and adverse events is obvious, we propose that the use of TCZ may be considered at an early stage of NMO before disability or a lower quality of life becomes evident.

AUTHOR CONTRIBUTIONS

T.Y., S.M., S.K., M.M., and M.A.: design and conceptualization of the study. M.A., K.M., T.O., and T.Y.: analysis and internalization of the data. T.M. and T.A.: flow cytometry analysis and anti-AQP4-Ab assay. M.A. and T.Y.: drafting and revising of the manuscript. T.Y.: supervising the entire project.

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M. Araki has received honoraria from Novartis. T. Matsuoka reports no disclosures relevant to the manuscript. K. Miyamoto has received honoraria from Novartis, Bayer, and Biogen Idec. S. Kusunoki serves as an editorial board member of *Experimental Neurology*, *Journal of Neuroimmunology*, and *Neurology & Clinical Neuroscience* (associate editor). He received honoraria from Teijin Pharma, Nihon Pharmaceuticals, Japan Blood Products Organization, Novartis Pharma, Dainippon Sumitomo Pharma, Kyowa Kirin, Asahi Kasei, Bayer, Sanofi, and GlaxoSmithKline. He is funded by research grants from the Ministry of Health, Labour and Welfare, Japan, and grants from the Japan Science and Technology Agency and the Ministry of Education, Culture, Sports, Science and Technology, Japan. He received research support from Novartis, GlaxoSmithKline, Dainippon Sumitomo Pharma, Teijin Pharma, Astellas, Sanofi, Japan Blood Products Organization, and Nihon Pharmaceuticals. T. Okamoto reports no disclosures relevant to the manuscript. M. Murata received honoraria for consulting and/or lecturing from GlaxoSmithKline Co., Ltd., Boehringer Ingelheim Co., Ltd., Dainippon Sumitomo Pharma Co., Ltd., Novartis Pharma, and Hisamitsu Pharma. S. Miyake has received speaker honoraria from Biogen Idec, Pfizer Inc., and Novartis Pharma. T. Aranami reports no disclosures relevant to the manuscript. T. Yamamura has served on scientific advisory boards for Biogen Idec and Chugai Pharmaceutical Co., Ltd.; has received research support from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Teva Pharmaceutical K.K., Mitsubishi Tanabe Pharma Corporation, and Asahi Kasei Kuraray Medical Co., Ltd.; has received speaker honoraria from Novartis Pharma, Nihon Pharmaceutical Co., Ltd., Santen Pharmaceutical Co., Ltd., Abbott Japan Co., Ltd./Eisai Co., Ltd., Biogen Idec, Dainippon Sumitomo Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Bayer Holding Ltd., and Astellas Pharma Inc. Go to Neurology.org for full disclosures.

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Efficacy of the anti–IL-6 receptor antibody tocilizumab in neuromyelitis optica: A pilot study

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Research Paper

MULTIPLE SCLEROSIS JOURNAL | MSJ

Differential effects of fingolimod on B-cell populations in multiple sclerosis

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Abstract

Background: Fingolimod is an oral drug approved for multiple sclerosis (MS) with an ability to trap central memory T cells in secondary lymphoid tissues; however, its variable effectiveness in individual patients indicates the need to evaluate its effects on other lymphoid cells.

Objective: To clarify the effects of fingolimod on B-cell populations in patients with MS.

Methods: We analysed blood samples from 9 fingolimod-treated and 19 control patients with MS by flow cytometry, to determine the frequencies and activation states of naive B cells, memory B cells, and plasmablasts.

Results: The frequencies of each B-cell population in peripheral blood mononuclear cells (PBMC) were greatly reduced 2 weeks after starting fingolimod treatment. Detailed analysis revealed a significant reduction in activated memory B cells ($CD38^{int\text{-}high}$), particularly those expressing Ki-67, a marker of cell proliferation. Also, we noted an increased proportion of activated plasmablasts ($CD138^{+}$) among whole plasmablasts, in the patients treated with fingolimod.

Conclusions: The marked reduction of Ki-67^{+} memory B cells may be directly linked with the effectiveness of fingolimod in treating MS. In contrast, the relative resistance of $CD138^{+}$ plasmablasts to fingolimod may be of relevance for understanding the differential effectiveness of fingolimod in individual patients.

Keywords

B cells, CD38, CD138, fingolimod, memory B cell, multiple sclerosis, plasmablast, proliferation, resistance, sphingosine 1-phosphate receptor 1

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Introduction

It is currently assumed that a large proportion of autoreactive T cells in multiple sclerosis (MS) is derived from a pool of $CCR7^{+}$ central memory T cells that are passing through the secondary lymphoid tissues (SLT).[1] Accordingly, egress of the T cells from the SLT represents a key process in MS pathogenesis. This process follows a rule of chemotaxis, in which the sphingosine 1-phosphate (S1P) receptor 1 (S1P1) expressed by lymphocytes is critically involved.[2] Fingolimod, an oral drug for treating relapsing–remitting MS (RRMS), serves as a functional antagonist for S1P1: Fingolimod induces internalisation and degradation of S1P1 in lymphocytes, causing the lymphocytes to lose the ability to respond to S1P and consequently, to become trapped in the SLT.[3] Analysis of large cohorts of patients with RRMS demonstrate the overall effectiveness of fingolimod in reducing the annualised relapse rate (ARR), as well as the appearance of new brain lesions in the patients' magnetic resonance imaging (MRI) scans.[4,5]

The number of central memory interleukin 17-producing $CD4^{+}$ T cells (Th17 cells) is reduced in the peripheral blood of fingolimod-treated patients. This is now being interpreted as a major mechanism of drug action;[6] however, fingolimod is not able to prevent relapses nor exhibit

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Table 1. Clinical data of the patients in this study.

Patient	Gender	Age (years)	Duration (years)	Relapse frequency (last 2 yrs)	EDSS	DMT before initiation of fingolimod	Complications
1	M	34	7	5	1.5	IFNβ1a + PSL	Asthma
2	M	43	6	2	2.5	PSL	Graves' disease
3	M	39	5	1	3.5	None	Depression
4	M	41	13	1	3.5	IFNβ1b	None
5	M	29	2	3	2.0	IFNβ1b	Pectus excavatum
6	F	41	24	6	3.5	IFNβ1b → GA → Dex	Depression
7	M	56	16	2	5.5	IFNβ1b → IFNβ1b + PSL → IFNβ1a + AZP	Osteoporosis
8	M	41	9	2	4.0	IFNβ1b → IFNβ1a	Depression
9	M	60	20	1	3.5	AZP → MZR → IFNβ1b	None
mean ± SD		42.7 ± 9.8	11.3 ± 7.4	2.5 ± 1.8	3.3 ± 1.2		

AZP: Azathioprine; Dex: dexamethasone; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; F: female; GA: glatiramer acetate; IFN: interferon; M: male; MZR: mizoribine; PSL: prednisolone.

appreciable effectiveness in all patients. In fact, recent case reports document the presence of fingolimod-treated MS patients who have developed tumefactive brain lesions, after receiving fingolimod.[7–10] Moreover, clinical worsening accompanied by large brain lesions is described in patients with neuromyelitis optica (NMO), within months of starting fingolimod.[11,12] Our current understanding of fingolimod-related biology therefore remains incomplete, particularly regarding differential effectiveness in individual patients.

Not only the presence of clonally-expanded B cells in the central nervous system (CNS),[13,14] but the efficacy of the anti-CD20 monoclonal antibody (mAb) rituximab[15] rationally indicates the involvement of B cells in the pathogenesis of MS. Therefore, B-cell migration can serve as a therapeutic target in MS, so we were prompted to investigate whether inhibition of B-cell migration may explain the differential effectiveness of fingolimod. Because the effects of fingolimod on B cells in MS have not been fully characterised,[16] we analysed the alterations of B-cell populations in fingolimod-treated RRMS patients by flow cytometry, measuring the frequencies and activation states of their peripheral blood B-cell populations.

Materials and methods

Patients and sample collection

The following subjects were enrolled in the Multiple Sclerosis Clinic of the National Centre of Neurology and Psychiatry (NCNP) in Japan:

(a) Fingolimod-naïve patients with RRMS ($n = 9$);
(b) RRMS patients who were treated with other disease-modifying treatments (DMTs) or corticosteroids ($n = 19$); and
(c) Healthy donors ($n = 3$).

All MS patients fulfilled the revised McDonald criteria.[17] Fingolimod (0.5 mg once/day) was administered to nine fingolimod-naïve patients. These patient's blood samples were collected before and 2 weeks after initiating fingolimod therapy. Most of these patients discontinued other DMTs at least 2 weeks before entry into the study, due to non-responsiveness to their DMT treatment or due to adverse events. The absence of serum anti-aquaporin 4 (AQP4)-Ab was confirmed by cell-based assays.[18,19] Upon MRI, no patient showed longitudinally-extensive spinal cord lesions extending over three or more vertebrae. The clinical data of these nine patients are summarised in Table 1.

Control blood samples were collected from 19 patients with RRMS (mean age ± SD: 41.8 ± 13.8 years; female:male ratio: 15:4) who had not been exposed to fingolimod before nor during the study. The three healthy donors were males (mean age ± SD: 40.0 ± 3.6 years). This study was approved by the Ethics Committee of the NCNP. We obtained written informed consent from all subjects.

Reagents

The following fluorescence- or biotin-labelled mAbs were used: anti-CD19-allophycocyanin (APC)-cyanine 7 (Cy7), anti-CD27-V500 and anti-CD27-phycoerythrin (PE)-Cy7 (BD Biosciences, San Jose, CA, USA); anti-CD180-PE and anti-CCR7-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Jose, CA, USA); anti-CD38-FITC, anti-CD3-FITC and mouse IgG1-FITC (Beckman Coulter, Brea, CA, USA); anti-CD138-APC, mouse IgG1κ-APC, anti-HLA-DR-Pacific Blue, mouse IgG2Aκ-Pacific Blue, anti-CD183 (CXCR3)-peridinin-chlorophyll-protein (PerCp)-cyanine 5.5 (Cy5.5), mouse IgG1κ-PerCp-Cy5.5, anti-CD38-APC, anti-CD38-PerCp-Cy5.5, anti-CD14-Pacific Blue, anti-Ki-67-Brilliant Violet, mouse IgG1κ-Brilliant Violet and streptavidin-PE-Cy7 (BioLegend, San

Diego, CA, USA); and anti-CXCR4-biotin and mouse IgG2A-biotin (R&D Systems, Minneapolis, MN, USA).

Cell preparation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Ficoll–Paque Plus (GE Healthcare Bioscience, Oakville, ON, Canada). B-cell populations were defined in reference to our previous paper,[19] as follows: total B cells, $CD19^+$; naïve B cells (nBs), $CD19^+CD27^-$; memory B cells (mBs), $CD19^+CD27^+CD180^+$; and plasmablasts (PBs), $CD19^+CD27^+CD180^-CD38^{high}$.

To evaluate the frequency and activation state of each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-V500, anti-CD38-FITC, anti-CD180-PE, anti-CD138-APC, anti-CXCR3-PerCp-Cy5.5, anti-CXCR4-biotin, streptavidin-PE-Cy7 and anti-HLA-DR-Pacific Blue. To assess the expression of CCR7 in each B cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC, anti-CD180-PE and anti-CCR7-FITC.

For examining Ki-67 expression in each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-PerCp-Cy5.5, anti-CD180-PE and anti-CD138-APC, then fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and permeabilised with 0.1% saponin. Subsequently, these cells were stained with anti-Ki-67-Brilliant Violet. We used the appropriate isotype control antibodies as negative controls for each staining. At the end of the incubation, the cells were washed and resuspended in PBS supplemented with 0.5% bovine serum albumin (BSA) and analysed by FACS Canto II (BD Biosciences), according to the manufacturer's instructions.

Cell sorting

PBMC were labelled with CD3 and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated into positive and negative fractions by AutoMACS (Miltenyi Biotec). The positive fraction was stained with anti-CD3-FITC and anti-CD14-Pacific Blue, whereas the negative fraction was stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC and anti-CD180-PE. Each positive and negative fraction was sorted into $CD3^+$ T cells and $CD14^+$ monocytes, or into nBs, mBs and PBs by a FACS Aria II cell sorter (BD Biosciences). The purity of the sorted cells was > 95%.

Quantitative real-time PCR

Messenger ribonucleic acid (mRNA) was prepared from the sorted cells using the RNeasy Kit (Qiagen, Tokyo, Japan), further treated with DNase using the RNase-Free DNase Set (Qiagen), and reverse-transcribed to complementary DNA (cDNA) using the cDNA Synthesis Kit (Takara Bio, Shiga, Japan). We performed polymerase chain reaction (PCR) using iQ SYBR Green Supermix (Takara Bio) on a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). RNA levels were normalised to endogenous β-actin (ACTB) for each sample. The following primers were used: S1P1 forward, CGAGAGCACTACGCAGTCAG; and S1P1 reverse, AGAGCCTTCACTGGCTTCAG.

Data analysis and statistics

We used Diva software (BD Biosciences) to analyse our flow cytometry data. We performed the statistical analysis with Prism software (GraphPad Software, San Diego, CA, USA). Paired or unpaired *t*-tests were used once the normality of the data was confirmed by the Kolmogorov-Smirnov test. Otherwise, the Wilcoxon signed-rank test or the Mann-Whitney *U*-test was used, as appropriate. One-way analysis of variance (ANOVA) was used to compare data from more than two groups. If the one-way ANOVA was significant, we performed *post hoc* pairwise comparisons using Tukey's test. A p value < 0.05 was considered statistically significant.

Results

B-cell populations express S1P1 mRNA

First, we used flow cytometry to examine S1P1 expression on the surfaces of the B-cell populations; however, surface S1P1 was hardly detected (data not shown). This is probably because of its internalisation following S1P binding. In support of this, it is known that S1P is abundantly present in peripheral blood.[2] Thus, we measured S1P1 mRNA in purified lymphocyte populations from the PBMCs of three healthy donors. Each B-cell population was identified by flow cytometry, as shown in Figure 1(a). We found that comparable levels of S1P1 mRNA were expressed in T cells, nBs and mBs. In comparison, PBs expressed a significantly lower level of S1P1, and S1P1 expression in monocytes was virtually absent (Figure 1(b)). Of note, a lower S1P1 expression by PBs, as compared with other B cell populations, is also described in mice.[20,21] These S1P1 mRNA expression profiles suggested that not only T cells, but B-cell migration, could also be influenced by fingolimod.

Next, we measured the frequencies of the B-cell populations in the PBMCs from nine patients with RRMS, before and 2 weeks after starting fingolimod. Results of flow cytometry showed that the frequencies of nBs, mBs and PBs among PBMCs were significantly decreased after initiating fingolimod treatment (Figure 1(c)). We confirmed that the absolute numbers of each population in the peripheral blood were also significantly decreased after starting fingolimod (Figure 1(d)). The mean decrease rate ± SD of each cell population was calculated based on the absolute cell number, giving the following results: total B cells, 87.6 ± 5.8%; nBs, 88.1 ± 6.0%; mBs, 85.4 ± 9.1% and PBs, 89.8

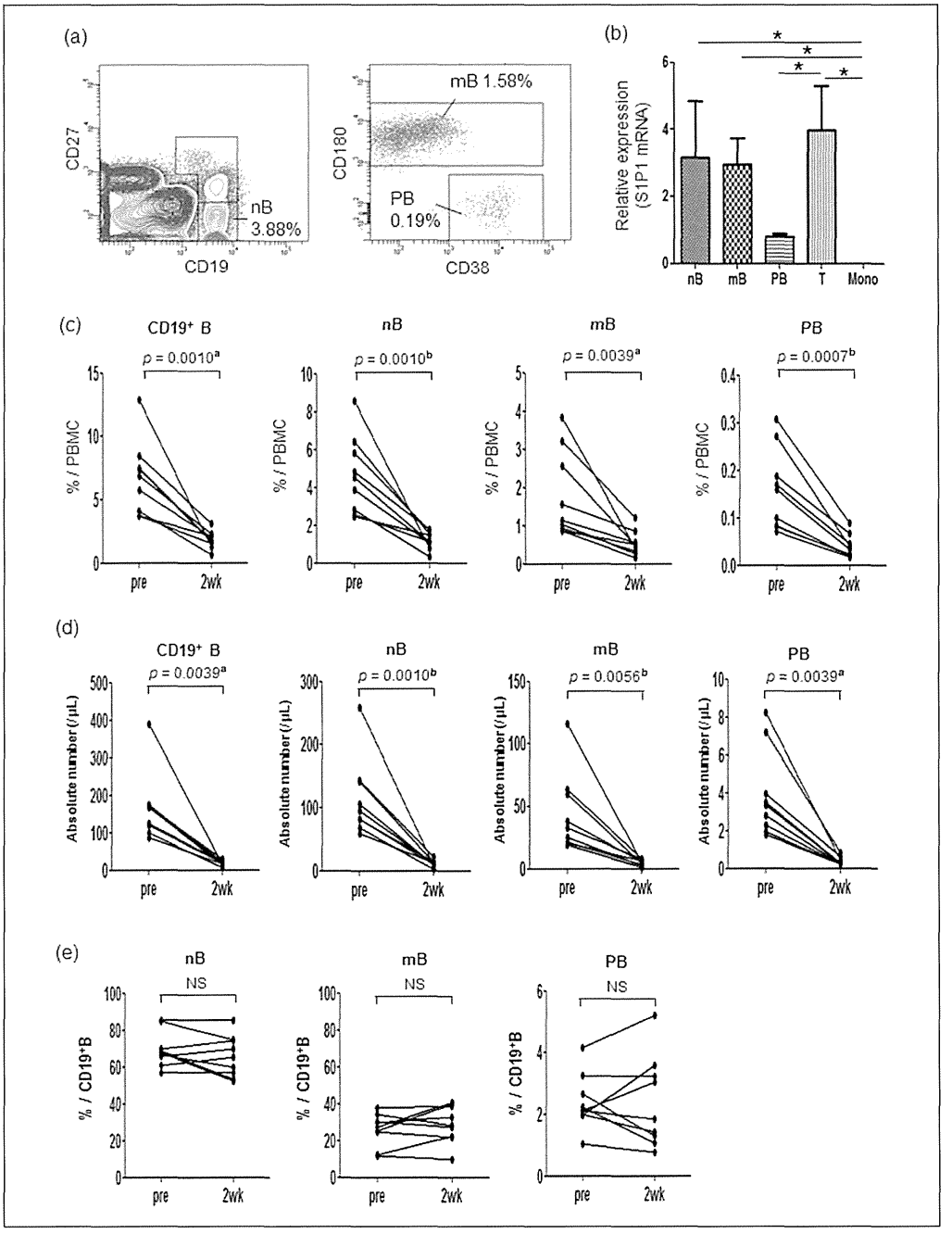

Figure 1. Frequency and absolute number of each B-cell population found in peripheral blood from MS patients.
(a)Representative flow cytometry scheme to analyse B-cell populations in PBMC. The PBMC were simultaneously stained with fluorescence-conjugated anti-CD19, -CD27, -CD38 and -CD180 mAbs. The gate for $CD19^+CD27^-$ nBs is shown in the left panel. The $CD19^+CD27^+$ fraction partitioned in the left panel was analysed for CD180 and CD38 expression to specify $CD180^+$ cells (mBs), and for $CD180^-CD38^{high}$ cells (PBs) in the right panel. Values represent frequencies of B-cell populations in PBMC. Total $CD19^+$ B cell counts were calculated by summing the frequencies of the partitioned populations in the left panel. (b) Each B-cell population, $CD3^+$ T cells and $CD14^+$ monocytes in PBMCs from three healthy donors were sorted by FACS, and S1P1 mRNA expression levels were determined by quantitative RT-PCR. Data were normalised to the amount of ACTB for each sample. Data are represented as mean relative expression ± SD. *$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c), (d), and (e) Data shown are the frequencies of B-cell populations in PBMC (c), the absolute numbers of B cell populations in peripheral blood (d) and the frequencies of B-cell populations in $CD19^+$ B cells (e) from nine patients with MS before (pre) and 2 weeks after (2 wk) initiating fingolimod. Data from the same patients are connected with lines.
$p^a < 0.05$ by Wilcoxon signed-rank test.
$p^b < 0.05$ by paired *t*-test.
ACTB: endogenous beta actin; ANOVA: analysis of variance; FACS: Fluorescence-activated cell sorting; mAbs: monoclonal antibodies; mBs: memory B cells; mono: monocytes; mRNA: messenger ribonucleic acid; MS: multiple sclerosis; nBs: naïve B cells; NS: not statistically significant; PBMC: peripheral blood mononuclear cells; PBs: plasmablasts; pre: before treatment; RT-PCR: reverse transcriptase - polymer chain reaction; S1P1: sphingosine 1 phosphate receptor 1; T: T cells; 2 wk: 2 weeks after treatment initiation.

± 3.3%. Thus, all B-cell populations decreased at similar rates, regardless of their S1P1 expression levels. We also noticed that reduction of the B-cell populations did not correlate with CCR7 expression (a large proportion of nBs and mBs expresses CCR7, whereas only a small percentage of PBs expresses CCR7 (Supplementary Figure 1)). Consistently, the frequency of each B-cell population within $CD19^+$ B cells was not significantly altered in the fingolimod-treated patients (Figure 1(e)).

$CD38^{int}$- and $CD38^{high}$-activated memory B cells are preferentially decreased in fingolimod-treated patients

We next assessed mBs, which are assumed to play an important role in MS.[22,23] To evaluate the effects of fingolimod on the activation state of mBs, we first analysed CD38 expression of mBs in the nine patients, before and after initiating fingolimod. CD38 is a marker that is upregulated upon B-cell activation.[24] We found that mBs could be classified into three subpopulations according to CD38 expression levels ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$). Notably, frequencies of $CD38^{int}$ and $CD38^{high}$ mBs were significantly decreased 2 weeks after initiating fingolimod, whereas the frequency of the $CD38^{low}$ subpopulation became significantly increased (Figure 2(a) and (b)).

We further examined the expression of another activation marker, HLA-DR, within the $CD38^{low}$, $CD38^{int}$ and $CD38^{high}$ mB subpopulations. We found that the $CD38^{high}$ subpopulation expressed a significantly higher level of HLA-DR, compared with the $CD38^{low}$ mB population, as assessed by mean fluorescence intensities (MFIs) (Figure 2(c) and (d)). Although not statistically significant, HLA-DR expression in the $CD38^{int}$ subpopulation was intermediate, compared with that in the $CD38^{low}$ mB subpopulation. We also found that the MFIs of forward scatter (FSC), which reflects cell size, were significantly higher in the $CD38^{high}$ subpopulation, compared with the $CD38^{low}$ and $CD38^{int}$ subpopulations (Figure 2(c) and (d)). These findings suggest that $CD38^{high}$ mBs may contain a larger number of recently-activated blastic cells.

Fingolimod reduced Ki-67^+ recently-activated memory B cells in peripheral blood

The nuclear antigen Ki-67 is exclusively expressed in the active stages of the cell cycle (G1, S, G2 and M phases),[25] and Ki-67^+ circulating immune cells are considered to be recently activated cells that have just egressed from the SLT. To clarify whether $CD38^{high}$ and $CD38^{int}$ mB subpopulations are enriched for recently-activated cells, we examined the frequency of Ki-67^+ cells in each mB subpopulation, in the six MS patients who were not treated with fingolimod. This analysis revealed that $CD38^{high}$ mBs contained a significantly higher frequency of Ki-67^+ cells than did $CD38^{low}$ and $CD38^{int}$ mBs, and that $CD38^{int}$ mBs were likely to contain a higher frequency of Ki-67^+ cells than the $CD38^{low}$ mBs (Figure 3(a) and (b)). In addition, we compared the frequency of Ki-67^+ cells in each mB subpopulation, between fingolimod-treated (n = 5) and -untreated control patients (n = 6), and found that $CD38^{int}$ and $CD38^{high}$ mBs of the fingolimod-treated patients contained a significantly lower percentage of Ki-67^+ cells compared with those of the untreated patients (Figure 3(c)). These findings suggest that recently activated mBs are enriched in $CD38^{int}$ and $CD38^{high}$ subpopulations and that fingolimod efficiently blocks the egress of these cells from the SLT into the peripheral circulation.

The $CD138^+$ subpopulation in plasmablasts is relatively resistant to fingolimod

Finally, we analysed alterations of PBs by fingolimod in more detail. As PBs serve as migratory B cells that produce pathogenic autoantibody directed against AQP4,[19] their role in the antibody-mediated pathology is being considered also in the pathogenesis of MS. Notably, CD138 expression appears to separate PB subpopulations that could become differentially altered during the inflammatory process. In fact, $CD138^+$ PBs have a higher potential to migrate to inflamed tissues than $CD138^-$ PBs.[26] Moreover, as has recently been reported by us, $CD138^+HLA\text{-}DR^+$ PBs are selectively enriched in the cerebrospinal fluid (CSF) during relapse of NMO, and the $CD138^+HLA\text{-}DR^+$ PBs migrating to the CSF express CXCR3.[27] Therefore, we compared the frequencies of $CD138^+$ cells in PBs, as well as their expression of HLA-DR and CXCR3, before and after fingolimod treatment.

We found that the frequencies of $CD138^+$ PBs among total PBs were significantly increased after fingolimod initiation (Figure 4(a) and (b)); however, the absolute numbers of both subpopulations decreased, implying that $CD138^+$ PBs are relatively resistant to fingolimod, compared with $CD138^-$ PBs (Supplementary Figure 2(a) and (b)). After initiating fingolimod, $CD138^-$ PBs showed lower expression of HLA-DR, whereas the percentages of $CXCR3^+$ cells remained unchanged (Figure 4(c) – (e)). In contrast, fingolimod treatment did not significantly reduce the expression level of HLA-DR among $CD138^+$ PBs. More interestingly, $CD138^+$ PBs became more enriched with $CXCR3^+$ cells after initiating fingolimod (Figure 4(c) – (e)). The definition of PBs as $CD19^+CD27^+CD180^-CD38^{high}$ cells in this study was modified to efficiently specify autoantibody-producing cells;[19] however, adopting a more commonly used definition of PBs as $CD19^+CD27^+CD38^{high}$ cells did not alter the results (Supplementary Figure 3(a) – (e)).

Discussion

Previous studies show that fingolimod markedly decreases the number of T and B cells in the peripheral blood, without

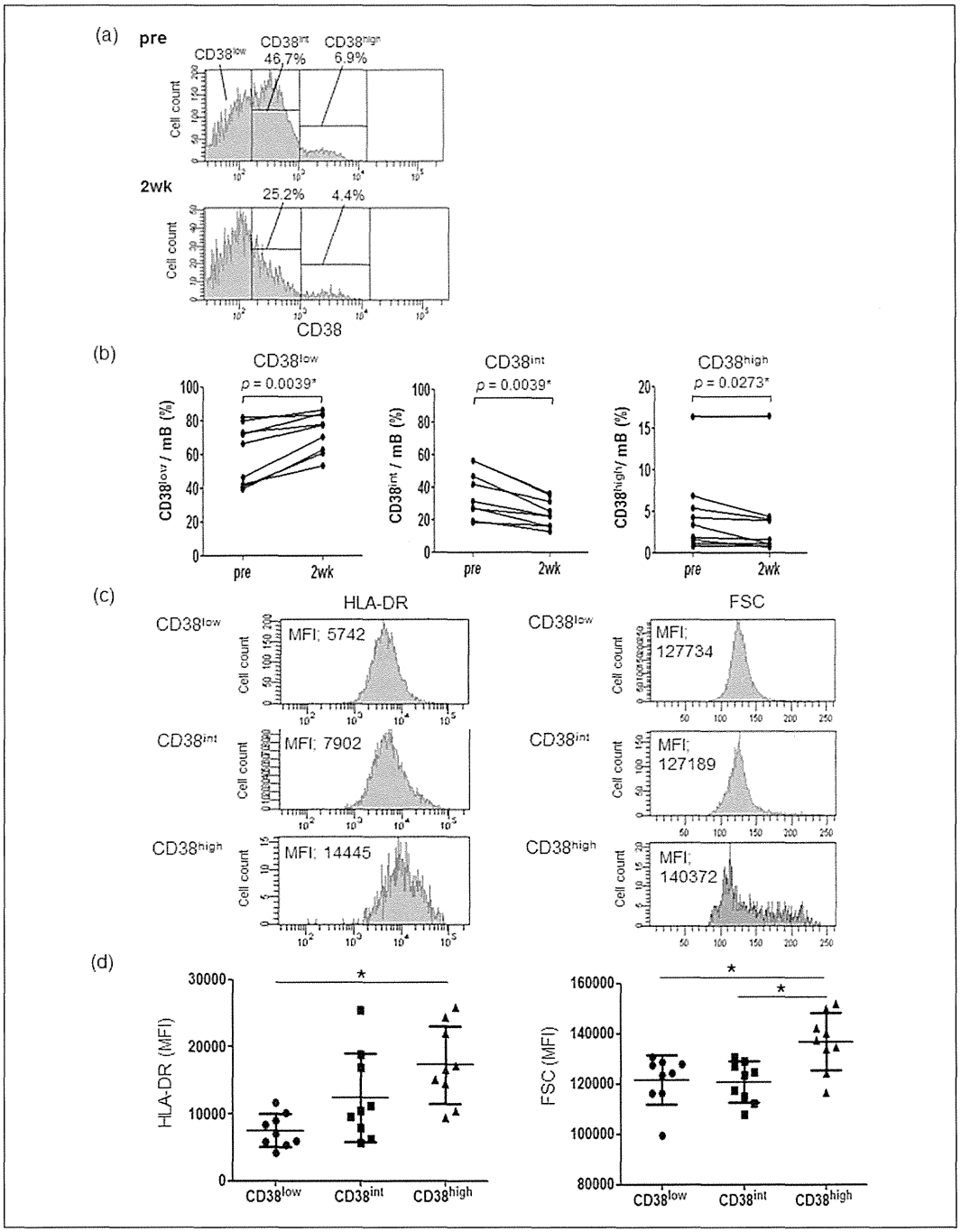

Figure 2. Frequency and activation state of each mB subpopulation in the peripheral blood of MS patients.
(a)Representative histograms of CD38 expression in mB of peripheral blood from a fingolimod-treated patient. Upper (pre) and lower (2wk) panels show the histograms before and 2 weeks after fingolimod initiation, respectively. The two values above each histogram indicate frequencies of the mB subpopulations with intermediate ($CD38^{int}$, left) and high ($CD38^{high}$, right) CD38 expression. (b) Data shown are frequencies of mB subpopulations, classified by CD38 expression levels ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel) and $CD38^{high}$ (right panel)), in the peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test. (c) Representative histograms of HLA-DR (left column) and FSC (right column) expression in each mB subpopulation ($CD38^{low}$ (upper row), $CD38^{int}$ (middle row) and $CD38^{high}$ (lower row)) of peripheral blood from a patient with MS, before fingolimod initiation. Values represent MFIs of HLA-DR and FSC. (d) Data shown are MFI of HLA-DR (left panel) and FSC (right panel) in mB subpopulations ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$) of peripheral blood from nine patients with MS, before fingolimod treatment. Data are represented as mean ± SD.
*$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test.
ANOVA: analysis of variance; FSC: forward scatter; HLA: human leukocyte antigen; mB: memory B cells; MFI: mean fluorescence intensity; MS: multiple sclerosis; pre: before treatment; 2wk: 2 weeks after treatment initiation.

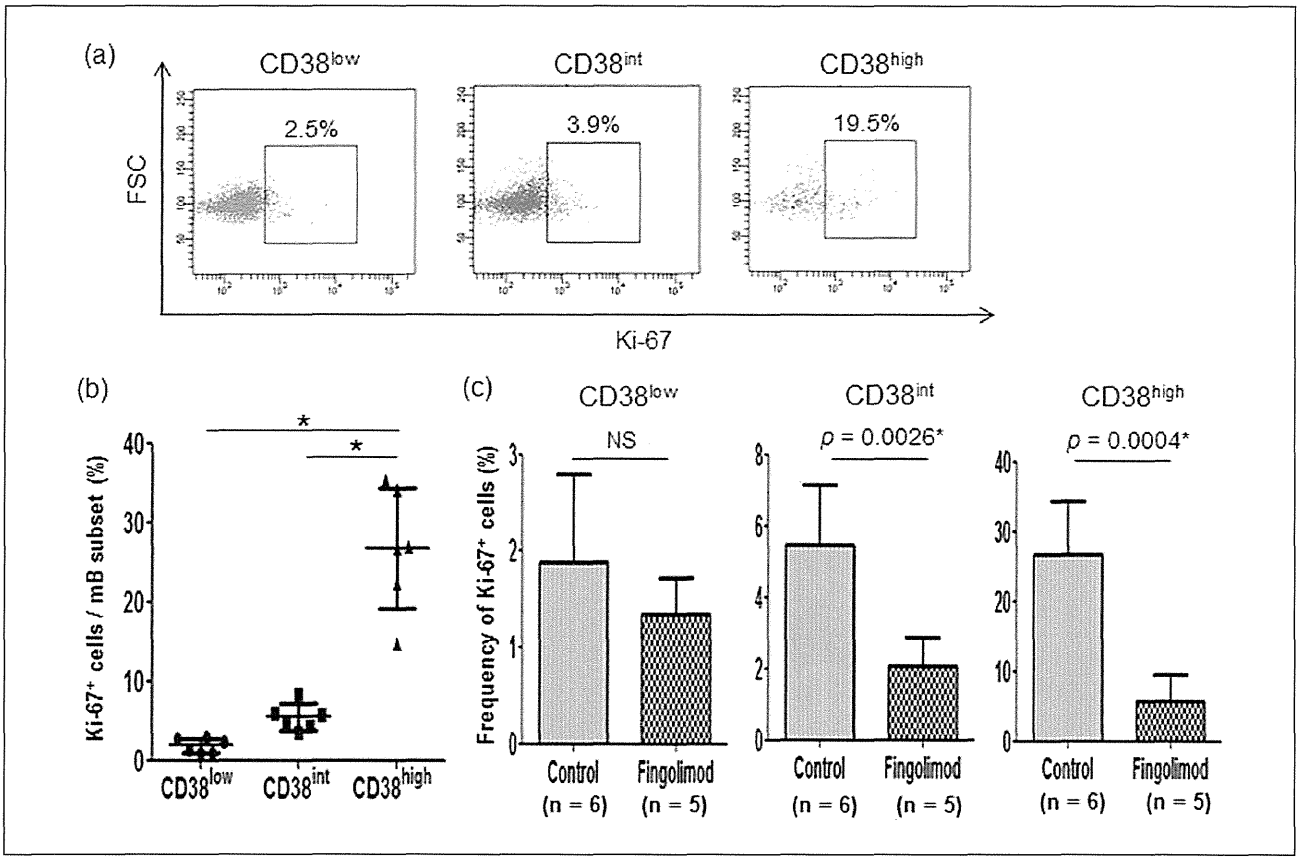

Figure 3. Ki-67 expression in mB subpopulations of peripheral blood from MS patients.
(a)Representative flow cytometry analyses of intracellular Ki-67 expression in mB subpopulations ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) of peripheral blood from an untreated patient with MS. Each mB subpopulation was analysed for FSC and Ki-67 expression. Values in each plot represent frequency of $Ki\text{-}67^+$ cells in each mB subpopulation. (b) Frequency of $Ki\text{-}67^+$ cells in each mB subpopulation of peripheral blood from six untreated patients with MS. Data are represented as mean ± SD. *$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c) Frequency of the $Ki\text{-}67^+$ population in each mB subpopulation ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) is compared between untreated patients with MS (control; $n = 6$) and fingolimod-treated patients with MS (Fingolimod; $n = 5$). Mean duration with fingolimod treatment ± SD is 15.8 ± 8.8 (6 to 30) weeks. Data are represented as mean ± SD.
*$p < 0.05$ by unpaired *t*-test.
FSC: forward scatter; Ki-67: a marker present only during cell growth or proliferation; mB: memory B cells; MS: multiple sclerosis; NS: not statistically significant.

affecting the total numbers of monocytes and natural killer (NK) cells.[16,28,29] Furthermore, in MS, fingolimod selectively reduces naïve T cells, as well as $CD4^+$ central memory T cells that are enriched for Th17 cells.[6,30] In addition, fingolimod treatment may induce a relative increase in $CD27^-CD28^-$ $CD8^+$ T cells[31] and a decrease in $CD56^{bright}CD62L^+CCR7^+$ NK cells.[32]

The role of autoreactive $CD4^+$ T cells in MS pathogenesis has been emphasised over decades.[33] In contrast, B-cell involvement in MS was highlighted lately, after the clinical effectiveness of rituximab was demonstrated in RRMS patients. Rituximab's effectiveness in MS may result from the depletion of autoantibody-producing B cells, but it can also be explained by depletion of B cells that are able to induce or support activation of autoreactive T cells.[15] In fact, B cells exhibit the ability to present antigen to T cells, and mBs are more capable than nBs of supporting the proliferation of neuroantigen-specific $CD4^+$ T cells, in vitro.[23] The presence of oligoclonal bands in the CSF suggests local production of antibodies within the CNS.[34] Consistent with this, brain lesions[13] and CSF[14] of patients with MS contain clonally-expanded B cells. These results collectively support the postulate that mBs can potentially trigger the inflammation of MS, either via autoantibody production or via autoantigen presentation to autoreactive T cells.

The focus of this study is to investigate the alterations of peripheral blood B-cell types in fingolimod-treated patients with RRMS. We showed that activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were highly susceptible to

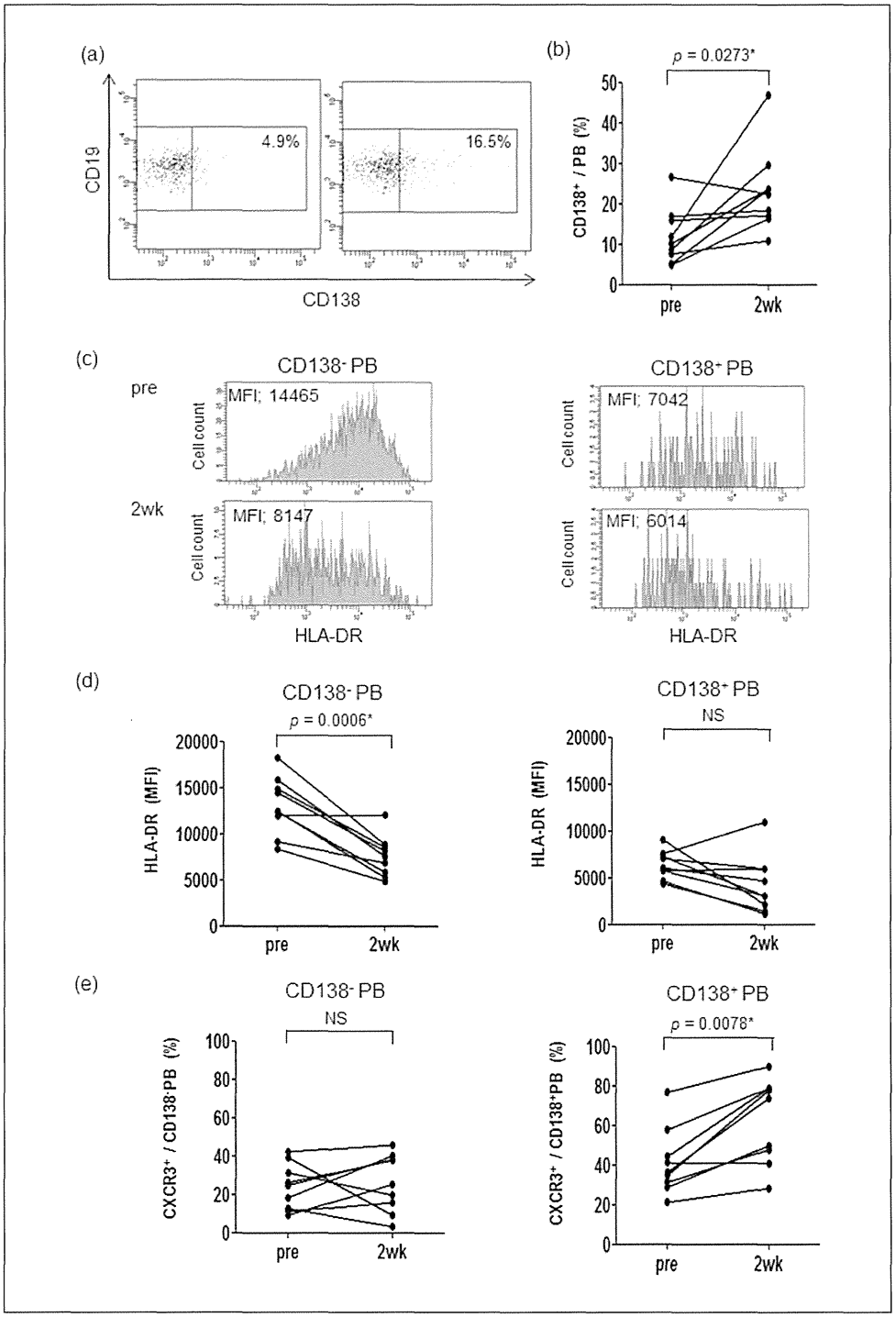

Figure 4. Phenotypic alteration of the remaining PBs in peripheral blood following fingolimod treatment.
(a)Representative dot plots of $CD19^+CD27^+CD180^-CD38^{high}$ PB, analysed for CD19 and CD138 expression before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent frequencies of the $CD138^+$ subpopulation in total PB. (b) Data are frequencies of the $CD138^+$ subpopulation in total PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test. (c) Data are representative histograms of HLA-DR expression in $CD138^-$ and $CD138^+$ PB of peripheral blood, from a patient with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent MFI of HLA-DR. (d) Data are MFI of HLA-DR in $CD138^-$ and $CD138^+$ PB of peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by paired *t*-test. (e) Data are frequencies of $CXCR3^+$ cells in $CD138^-$ PB and $CD138^+$ PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test.
MFI: mean fluorescence intensity; MS: multiple sclerosis; NS: not statistically significant; PB: plasmablast; pre: before treatment; 2wk: after 2 weeks of treatment.