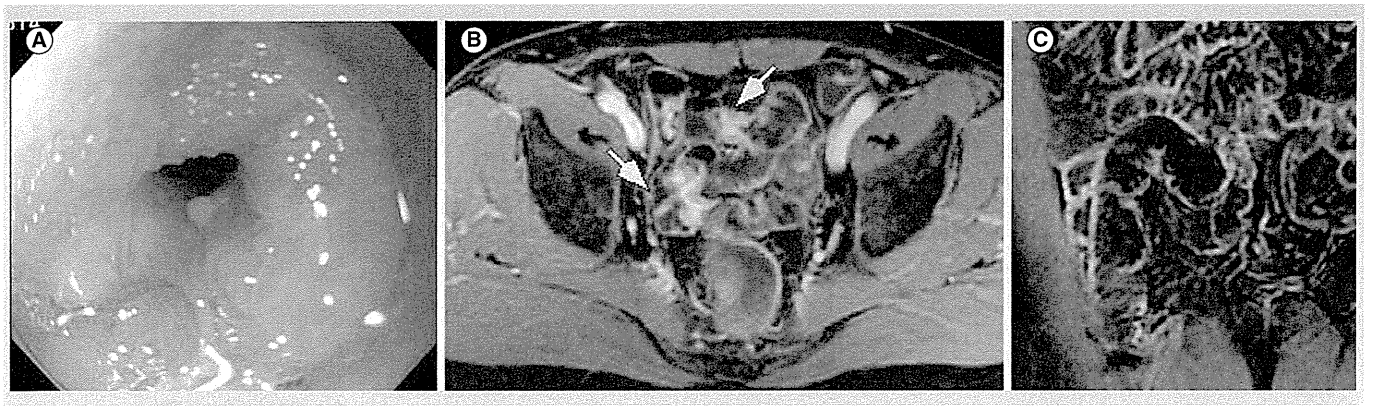

Figure 3. Ileal stricture in patients with Crohn's disease. (A) A moderate stricture was found at the proximal ileum, and balloon-assisted enteroscopy did not pass through the stenosis. **(B)** Ileal lesions with mural enhancement and **(C)** severe strictures with bowel distention were observed on the T1-weighted image.

Extraintestinal complications, such as abscesses, could also be detected on MRE. It is important to assess fistulas and abscesses because these lesions are frequently associated with medical treatment failure, including secondary loss of responsiveness for anti-TNF-α agents [16].

MRE could be used as an observer-independent diagnostic device for the evaluation of CD lesions, although there have been few studies to assess the interobserver agreement in MRE for diagnostic assessments in patients with CD. A recent study indicated that high interobserver agreements for mural inflammation and lymphadenopathy were found in patients with active CD lesions [17]. Another study indicated that wall thickness, the presence of edema, enhancement patterns and length of the disease in each segment showed good interobserver variability between all investigators [18].

The diagnostic accuracy of MRE

Previous reports regarding the diagnostic accuracy of MRE are summarized in Table 1 [12,14,19–28]. MRE has been shown to have excellent sensitivity and specificity for CD lesions. In most studies, the diagnostic accuracies of MRE were assessed by comparing the findings on ileocolonoscopy and the findings on MRE. A recent systematic review showed that the sensitivity and specificity of MRE for the diagnosis of suspected CD was 78 and 85%, respectively [29]. For the extension of CD lesions, the sensitivity and specificity of MRE for small bowel lesions were 74 and 91%, respectively [29]. The diagnostic accuracy for the extension and disease severity of MRE was comparable to the accuracy of CTE [20–23,25,27,28]. No significant differences were observed between MRE and CTE in detecting fistulas [30]. MRE might be preferred to CTE to assess the location and severity of the disease because of the reduced radiation exposure for young patients.

Ultrasonography (US) and MRE are safe and non-invasive devices; however, US is less expensive compared with MRE and could be performed at the bedside. Recently, a diagnostic study for patients with suspected CD was performed to compare the accuracy of MRE and US [25]. For the detection of small intestinal lesions of CD, the sensitivity, specificity, positive predictive value and negative predictive value for MRE and US were high (94–97%). However, US was less accurate than MRE in defining CD extension. A recent systematic review indicated that US, computed tomography and MRE have high accuracy for detecting fistulas, abscesses and strictures, whereas US has high false positives for abscesses [29]; MRE is preferred to US for deep-seated fistulas [31].

The advantages of CE and BAE are that they can directly observe mucosal inflammation, whereas MRE is particularly useful for detecting transmural inflammation, stenosis and extraintestinal lesions, including abscesses and fistulas [32]. MRE is useful when evaluating small and large intestinal lesions even in cases with severe strictures, where full evaluation of the small

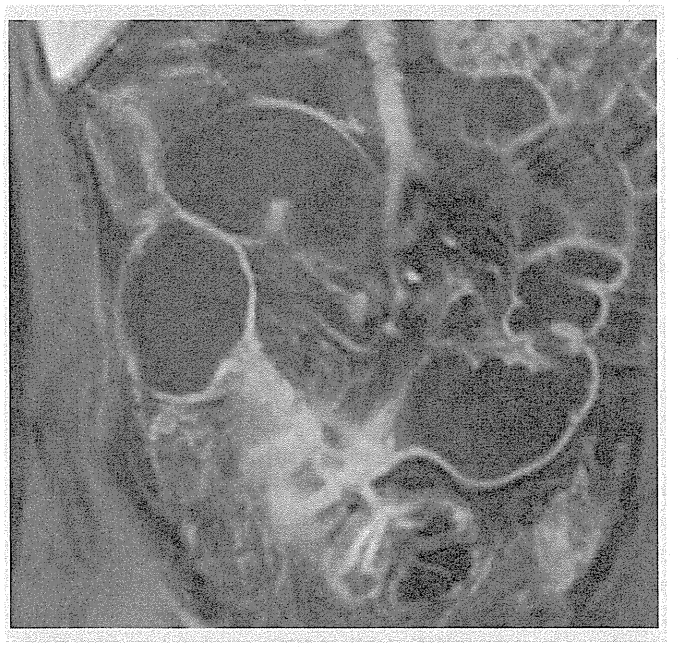

Figure 4. Coronal true imaging with T1-weighted images revealed an ileoileal fistula with inflammation.

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Table 1. Summary of the accuracy of magnetic resonance enterography for Crohn's disease lesions by comparing the findings of conventional diagnostic devices.

Study (year)	Diagnostic devices as gold standard	Number of cases	Location of assessment on MRE	Accuracy of MRE for CD lesions (%)	Ref.
Schreyer *et al.* (2005)	Ileocolonoscopy	30	Colon, TI	Sen 55, Spe 98	[19]
Lee *et al.* (2009)	Ileocolonoscopy	30	TI	Sen 83, Spe 100	[20]
Rimola *et al.* (2009)	Ileocolonoscopy	50	Colon, TI	Sen 81, Spe 89	[14]
Siddiki *et al.* (2009)	Ileocolonoscopy	44	TI	Sen 91, Spe 67	[21]
Fiorino *et al.* (2011)	Ileocolonoscopy	44	TI, colon	For TI Sen 81, Spe 93 (MRE) Sen 81Spe 91 (CTE)	[22]
Grand *et al.* (2012)	Ileocolonoscopy	310	TI, colon	Sen 85, Spe 80	[12]
Grand *et al.* (2012)	Histological findings	310	TI	Sen 87, Spe 88	[12]
Jensen *et al.* (2011)	Ileocolonoscopy/surgery	50	SI	Sen 74, Spe 80 (MRE) Sen 83, Spe 70 (CTE)	[23]
Hyun *et al.* (2011)	Ileocolonoscopy/BAE	30	SI, colon	For SI lesions Sen 62, Spe 93 For colonic lesions Sen 86, Spe 95	[24]
Castiglione *et al.* (2013)	Ileocolonoscopy	249	SI	Sen 96, Spe 94 (MRE) Sen 94, Spe 97 (bowel sonography)	[25]
Takenaka *et al.* (2014)	BAE	100	SI	Ulcerative lesions: Sen 82, Spe 88 Major stricture Sen 59, Spe 90	[26]
Quencer *et al.* (2013)	Histological findings	23	SI	Sen 88, Spe 79 (MRE) Sen 100, Spe 62 (CTE)	[27]
Dillman *et al.* (2011)	Histological findings	22	SI, colon	Sen 66, Spe 90	[28]

BAE: Balloon-assisted enteroscopy; CTE: Computed tomographic enterography; MRE: Magnetic resonance enterography; SI: Small intestine; Sen: Sensitivity; Spe: Specificity; TI: Terminal ileum.

bowel would be virtually impossible to achieve using CE or even BAE. Thus, these technologies appear to complement each other. The diagnostic accuracy of CE and MRE has been compared in the recent studies [33–38]. Recent meta-analysis demonstrated that the diagnostic yield for CE and MRE were comparable [33]. Albert *et al.* investigated the detection rate of small intestinal lesions on CE, MRI and double-contrast fluoroscopy in patients with suspected CD [34]. CE was not accomplished in approximately one-third of patients (14/41) due to bowel strictures in this study. CE was slightly more sensitive than MRI, whereas MRE detected inflammatory conglomerates and enteric fistulae in five cases [34]. In another study, CE was found to be superior to MRE in detecting mucosal lesions in patients with CD; however, MRE could detect severe inflammatory lesions within the bowel wall [33]. These results suggested that CE is capable of detecting mucosal lesions that may be missed by MRE, while MRE is helpful in identifying transmural CD and extraluminal lesions.

Takenaka *et al.* recently compared the diagnostic accuracy of MRE with BAE [26]. In this prospective study, MRE and single BAE were performed in 100 patients. Ulcerative lesions, mucosal lesions and intestinal damage were evaluated. MRE detected ulcerative lesions in the small intestine with a sensitivity of 82%. The specificity for ulcerative lesions and mucosal lesions were 88 and 95%, respectively. MRE detected major stenosis (stricture that the scope could not pass) with a sensitivity of 59% and a specificity of 90%, and strictures were detected with a sensitivity of 41% and a specificity of 94%. MRE is useful for detecting active lesions in the small intestine. However, MR imaging is less sensitive for detecting strictures, which single BAE is able to detect. BAE is preferred for identifying intestinal damage.

When the lesions identified by MRE were compared with the surgical findings in patients with CD, MRE could identify small bowel CD lesions, such as fistulas and abscesses; the results showed that 27 of 30 ileoenteric or ileocolonic fistulas

and 8 of 9 abscesses were detected using MRE in patients who required surgery [39]. Discrete proximal small intestinal lesions might not be detected in all cases because of insufficient bowel distension at the proximal small bowel. Another study using surgical pathological inflammatory grading was significantly associated with MRI findings such as the wall thickness, degree of wall enhancement on delayed phase, pattern of enhancement on both parenchymatous, T2 relative hypersignal wall, blurred wall enhancement, comb sign, fistula, and abscess [40]. The contrast enhancement ratio of abnormal distal ileal segments with inflammation was higher than that with fibrosis only [39], suggesting that strictures with inflammation could be distinguished from fibrotic strictures. However, these studies were not confirmed by another study, which indicated that fibrosis was not associated with wall thickness or with T2 hypersignal [41]. Another study showed that fibrotic lesions alone were also associated with wall thickness, T2 wall hypersignal, comb sign, fistula and abscess [40]. Inflammation with fibrotic changes is frequently observed at the strictures and it is not easy to distinguish inflammatory strictures from fibrotic strictures; recent consensus guidelines from the European Crohn's and Colitis Organisation stated that no validated criteria have been established to determine the fibrotic lesions on MRE [31].

For pediatric CD, MRE is more useful in detecting small intestinal lesions than it is in adolescents because MRE is less complicated than colonoscopy and could be performed without radiation exposure. In some cases of CD, frequent assessment of disease severity and extension of the disease is necessary; therefore, examinations without radiation exposure are important especially for children. A recent systematic review indicated that the pooled sensitivity and specificity for MRE detection at the terminal ileum of CD were 84 and 97%, respectively, in pediatric CD patients [42]. In all studies, ileocolonoscopy was used as the reference test. The diagnostic accuracy for barium enteroclysis was assessed and compared with that of colonoscopy in 3 of the 11 studies in this systematic review. The sensitivity and specificity (72 and 73%) for barium enteroclysis was lower than that for MRE in pediatric CD patients [43–45]. Even if a patient is younger than 10 years old, MRE could be performed without sedation [46]. For children, it is important to perform the MRE procedure in a short time. To reduce the number of MRE sequences, the performance of contrast-enhanced T1-weighted MR alone in the evaluation of CD lesions was compared with the performance of all MRE imaging sequences [47]. This study indicated that the sensitivity, specificity and diagnostic accuracy for detecting active inflammation using contrast-enhanced T1-weighted MR alone were 83, 87 and 85%, which were inferior compared with using all MRE sequences. A higher false-positive rate of abscesses was observed from contrast-enhanced imaging alone, and the absence of abscesses were confirmed when non-fat-suppressed Half fourier Acquision Single shot Turbo spin Echo (HASTE) was conducted in addition to the contrast-enhanced imaging. It is important to reduce the time of the MRE procedure (reduce the number of MR sequences), especially for pediatric patients. However, non-fat-suppressed HASTE imaging might be needed to maintain diagnostic accuracy of MRE in patients with CD.

Comparison of MRE findings with biomarkers

It is important to assess the disease activity of CD for appropriate management of the disease. Clinical symptoms and clinical activity indexes such as the Crohn's disease activity index and Harvey–Bradshaw index are important markers for evaluating the severity of CD. However, these indexes are subjective and are affected by psychological factors. It is necessary to assess biological markers, such as C-reactive protein, white blood cell counts and fecal calprotectin; these biological markers might be elevated in infectious diseases and other chronic inflammatory diseases. MRE enables the simultaneous assessment of the extension and severity of CD. The findings on MRE are closely correlated to clinical severity and serological markers with the previously described reference standards. The wall thickness, T2 signal intensity, T1 enhancement and presence of lymph nodes on MRE are related to Crohn's disease activity index, endoscopic severity (Crohn's disease endoscopic index of severity [CDEIS], simple endoscopic score for Crohn's disease) and histological examinations from biopsy or surgical specimens [48] and to Inflammatory Bowel Disease Questionnaire. MRE could also assess the Montreal classification-based disease behavior in patients with CD [49]. However, assessments using imaging such as endoscopy and CT/MR enterography in addition to clinical symptoms and biological markers are necessary for the management of CD to guide therapeutic decisions.

Assessment of disease severity using scores on MRE

Many investigators have scored disease severity on MRE in patients with CD. More recently, the magnetic resonance index of activity score (MaRIA) was developed by comparing the MRE findings with the CDEIS [14]. After assessment with logistic regression, MRE findings, such as the wall thickness, relative contrast enhancement, edema and ulceration, were selected as independent factors for the prediction of endoscopic severity. The MaRIA score was constructed using these four factors. The MaRIA score had a significant correlation with the CDEIS, C-reactive protein and clinical activity score [14,50]. Macarini *et al.* developed the MRE score by assessing disease severity in 100 patients with CD [51]. The MRE score is composed of multiple MRE parameters, such as wall thickness, wall enhancement, enhancement pattern (transmural, layer), fibrofatty proliferation and local complications [45]. The MRE score was correlated to the Crohn's disease activity index and Inflammatory Bowel Disease Questionnaire. Steward *et al.* developed the Crohn's Disease Activity score, which was derived with reference standards of histological scores in surgical specimens [52]. They demonstrated that the mural thickness and T2 signal were the best predictive factors for histological severity.

Using the scoring system on MRE, disease severity and responsiveness for medical treatments could be objectively assessed (Table 2). Ordás *et al.* demonstrated a significant decrease in the mean MaRIA score at 12 weeks after treatment with anti-TNF-α

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Table 2. Responsiveness of MRI lesions or indexes to medical treatment.

Study (year)	Medical treatment	Assessment of activity index for MRE	Change of MRE parameter over time during medical treatment	Ref.
Ordás *et al.* (2014)	Corticosteroids (n = 14) Adalimumab (n = 34)	MaRIA score (wall thickness, RCE, edema, ulcers)	Mean MaRIA score (baseline → at 12 weeks) Endoscopic healed 18.9 → 8.7 Non-healed 22.1 → 20.8	[53]
Assche *et al.* (2013)	Infliximab (n = 20)	MICD (transmural inflammation, extramural lesion, sign of obstruction)	Median MICD (baseline → 2 weeks → 26 weeks) 7.0 → 6.5 → 5.0	[54]
Tielbeek *et al.* (2013)	Adalimumab (n = 30) Infliximab (n = 20)	Inflammatory score (mural thickness, mural T2 signal, perimural T2 signal T1 contrast)	Mean inflammatory score (first MRE → second MRE) Clinical responder 5.19 → 3.12 Non-responder 5.55 → 5.92	[55]

MICD: MRE score of severity in ileal Crohn's disease; MRE: Magnetic resonance enterography.

agents or steroids compared with the score at baseline if endoscopic healing was obtained; the mean score was not significantly changed among patients without endoscopic healing [53]. The magnitude of change in the CDEIS scores correlated to those in the MaRIA scores. Another study indicated that the clinical effects of infliximab (IFX) could be assessed by MRE in patients with ileal CD. In this study, the MRE index improved in 44% at 2 weeks and in 80% at 26 weeks after treatment with IFX, and the improvement of ileal lesions on MRE correlated to clinical responses after 2 weeks of IFX treatment [54]. Although the median inflammatory score significantly decreased from 7.0 to 5.0, the obstructive score did not change (3.0 → 3.0) after treatment with IFX. Tielbeek *et al.* demonstrated the usefulness of MRE in examining the treatment effect in patients with CD who were treated with IFX/adalimumab [55]. The mean Crohn's disease activity score in anti-TNF responders significantly improved, whereas the scores did not change significantly in non-responders. Thus, MRE could be used as a complementary approach to measure transmural inflammation in patients with CD and could guide the optimal use of TNF antagonists in daily clinical practice [55]. MRE could evaluate the effects of medical treatments at ileocolonic lesions and at the proximal ileum, where conventional ileocolonoscopy is unable to reach (Figure 5).

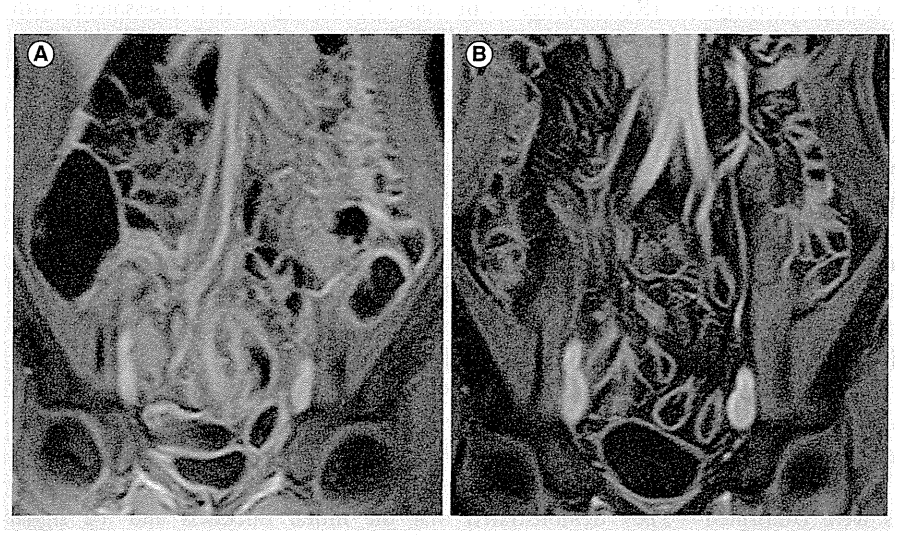

Figure 5. A case of clinical improvement by infliximab in patients with Crohn's disease. (**A**) Prior to the administration of infliximab, magnetic resonance enterocolonography could detect deep mucosal lesions in the terminal ileum and the middle ileum. (**B**) Magnetic resonance enterocolonography revealed that inflammation was improved in the colon and in the small intestine.

Could MRE assess mucosal healing in patients with CD?

Recent studies have indicated that endoscopic improvement after medical treatment reduced hospitalizations and surgeries [56,57]. Endoscopy has been the key device for predicting the long-term prognosis of CD. Because MRE could be repeatedly performed to confirm the effects of medical treatment, a question is raised regarding whether MRE could predict endoscopic remission or ulcer healing.

A recent study demonstrated that the MaRIA score had high sensitivity (85%), specificity (78%) and accuracy (83%) for the diagnosis of mucosal healing with a cutoff of 7. A MaRIA score <11 has a high sensitivity for ulcer healing (90%); however, the specificity is moderate [53]. Another study showed that the CDEIS had a sensitivity of 87% and specificity of 70% for predicting acute inflammation with a cutoff of 3 [52]. These results suggest that MRE might predict endoscopic remission in patients with CD.

Expert commentary & five-year view

A recent study indicated that the diagnostic ability of MRE was comparable to the diagnostic ability of other devices, such as ileocolonoscopy and CTE. The advantage of MRE is the ability to detect small and large intestinal lesions at the same time

without radiation exposure. MRE is also useful for the simultaneous evaluation of luminal inflammation, transmural lesions and extraintestinal complications in CD. For these reasons, the European Crohn's and Colitis Organisation guidelines recommend MRE as the imaging technique with the highest diagnostic accuracy for the detection of intestinal involvement in CD, including extramural complication [31]. Nevertheless, MRE has not been widely used in actual clinical settings because MRE might be an expensive device and it requires increased time for all sequences to be performed compared with other devices. Moreover, there are few experts who evaluate CD lesions on MRE. If the concerns for expertise and cost could be solved, MRE would be widely used for CD patients in the near future.

Conventional ileocolonoscopy has been performed in most medical institutions; however, only colonic lesions could be assessed with this, and additional diagnostic tools might be required to evaluate the lesions in other parts of the small intestine. BAE could be used to simultaneously assess lesions in the small and large intestines. The advantage of BAE is being able to obtain biopsy specimens of small intestine and to treat for intestinal strictures by endoscopic dilation. BAE can directly observe small intestinal lesions, such as erosions and small ulcerations. BAE is preferred for identifying intestinal damage and strictures [58], although this technique might be operator dependent. Severe adhesions, strictures and fistulas are frequently observed in CD patients and result in technical difficulties of observing the entire small intestine when BAE is performed. MRE is less invasive and is not dependent on the operators' technique. MRE is useful for assessing therapeutic efficacy in the small and large intestine simultaneously. A recent study suggested that endoscopic remission might be predicted by MRE. In our experiment regarding MRE, a transmural lesion with increased wall thickness and hyperintensity could be observed even though endoscopic remission was obtained at the same area. The significance of the discrepancy between endoscopic healing and transmural lesions on MRE [59] should be clarified in a future study.

The question of when MRE should be performed in the clinical setting remains. At the time of diagnosis, conventional colonoscopy is needed, especially for pathological diagnosis. CT procedures provide information regarding extraintestinal complications, such as abscesses or fistulas. In actual clinical setting, conventional CT is more widely used than MRI because CT is less expensive and it does not requires long time for all sequences. CT is useful to detect CD lesion for patients with moderate-to-severe disease because it can be performed on the same day when patients visit the hospital. MRE could be performed repeatedly because there is no radiation exposure. Therefore, MRE is useful as a method of follow-up for younger patients with established CD. MRE is also useful for evaluating the efficacy of medical treatments, such as biologics. MRE could also detect small intestinal lesions even if stenosis is observed. To obtain detailed information of the mucosa in the small intestine, BAE or CE for small intestinal lesions might be more helpful. For each situation, the appropriate diagnostic tools should be selected to ensure the detection of CD lesions in the small intestine.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

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Key issues

- The advantage of magnetic resonance enterography (MRE) for Crohn's disease (CD) patients is that it can evaluate both luminal inflammation and extraintestinal complications of CD in the small intestine.
- Diagnostic accuracy of MRE is comparable with accuracy of ileocolonoscopy and computed tomographic enterography for detecting intestinal lesions of CD.
- MRE is useful as a method of follow-up for younger patients with established CD because of no radiation exposure.
- MRE is also useful for evaluating the efficacy of medical treatments and may predict endoscopic healing in patients with CD.
- The concerns for expertise and cost regarding MRE should be solved to be widely used for CD patients in the near future.

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

A pilot study

OPEN

Manabu Araki, MD, PhD
Takako Matsuoka, MD
Katsuichi Miyamoto, MD, PhD
Susumu Kusunoki, MD, PhD
Tomoko Okamoto, MD, PhD
Miho Murata, MD, PhD
Sachiko Miyake, MD, PhD
Toshimasa Aranami, MD, PhD
Takashi Yamamura, MD, PhD

Correspondence to Dr. Yamamura: yamamura@ncnp.go.jp

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.

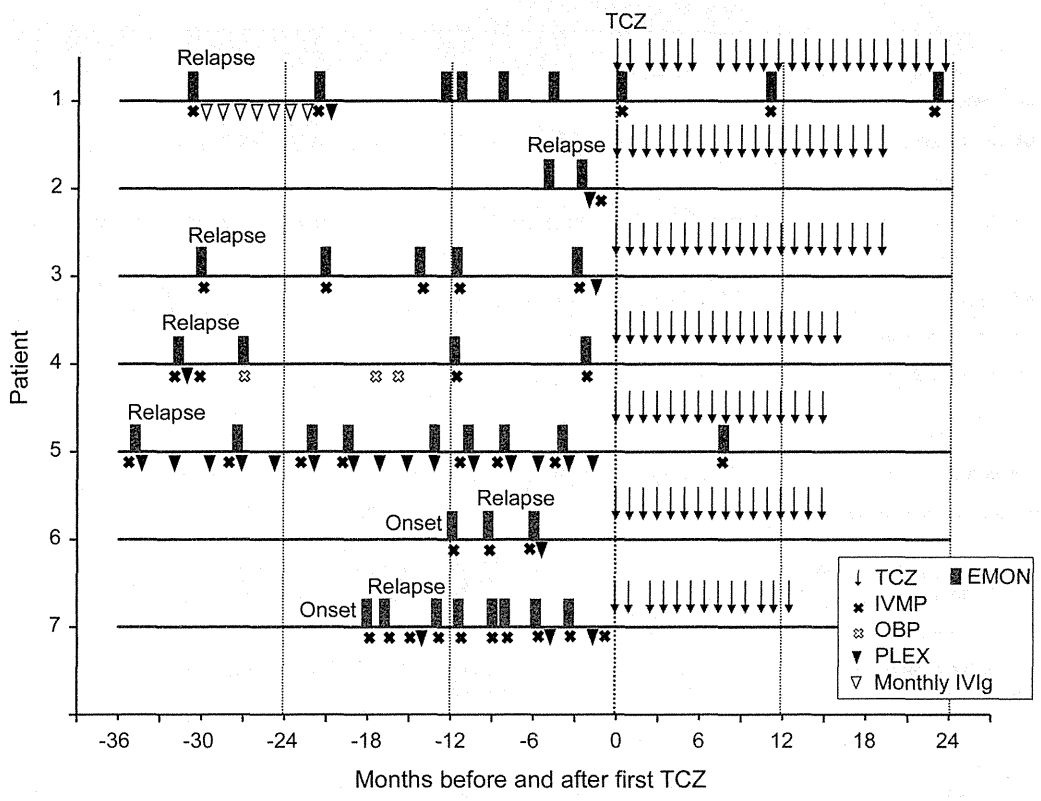
Patients and treatment. Seven patients who met the diagnostic criteria of NMO in 2006 were enrolled after providing informed consent (table). Results of chest x-rays, interferon γ release assays, and plasma 1,3-β-D-glucan measurement excluded latent tuberculosis and fungal infection. All of the patients 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.

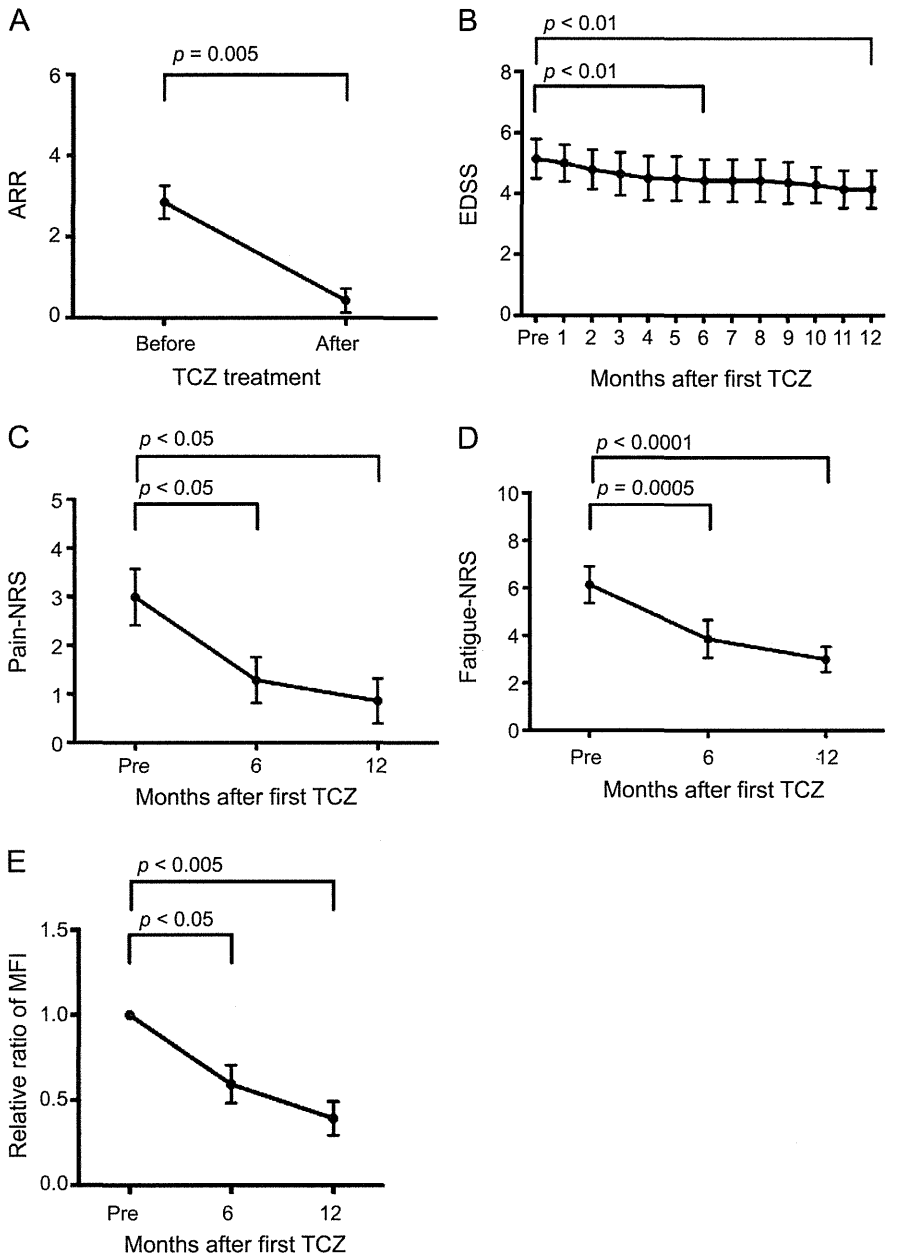
starting TCZ (figure 2). The EDSS score decreased modestly but significantly from 5.1 ± 1.7 (range, 3.0–6.5) to 4.1 ± 1.6 (range, 2.0–6.0) at 12 months. The chronic neurogenic pain in their trunk and extremities, which is characteristic of NMO[6,7] (table), gradually lessened after the patients started 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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Research Paper

MULTIPLE SCLEROSIS JOURNAL | MSJ

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

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Masakazu Nakamura[1,2], Takako Matsuoka[1], Norio Chihara[1], Sachiko Miyake[1,3], Wakiro Sato[3,4], Manabu Araki[3], Tomoko Okamoto[3,4], Youwei Lin[1,3,4], Masafumi Ogawa[3,4], Miho Murata[4], Toshimasa Aranami[1,3] and Takashi Yamamura[1,3]

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 $Ki\text{-}67^{+}$ 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

[1]Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry (NCNP), Tokyo, Japan.
[2]Department of Neurology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[3]Multiple Sclerosis Centre, National Centre Hospital, NCNP, Tokyo, Japan.
[4]Department of Neurology, National Centre Hospital, NCNP, Tokyo, Japan.

Corresponding author:
Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.
Email: yamamura@ncnp.go.jp

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

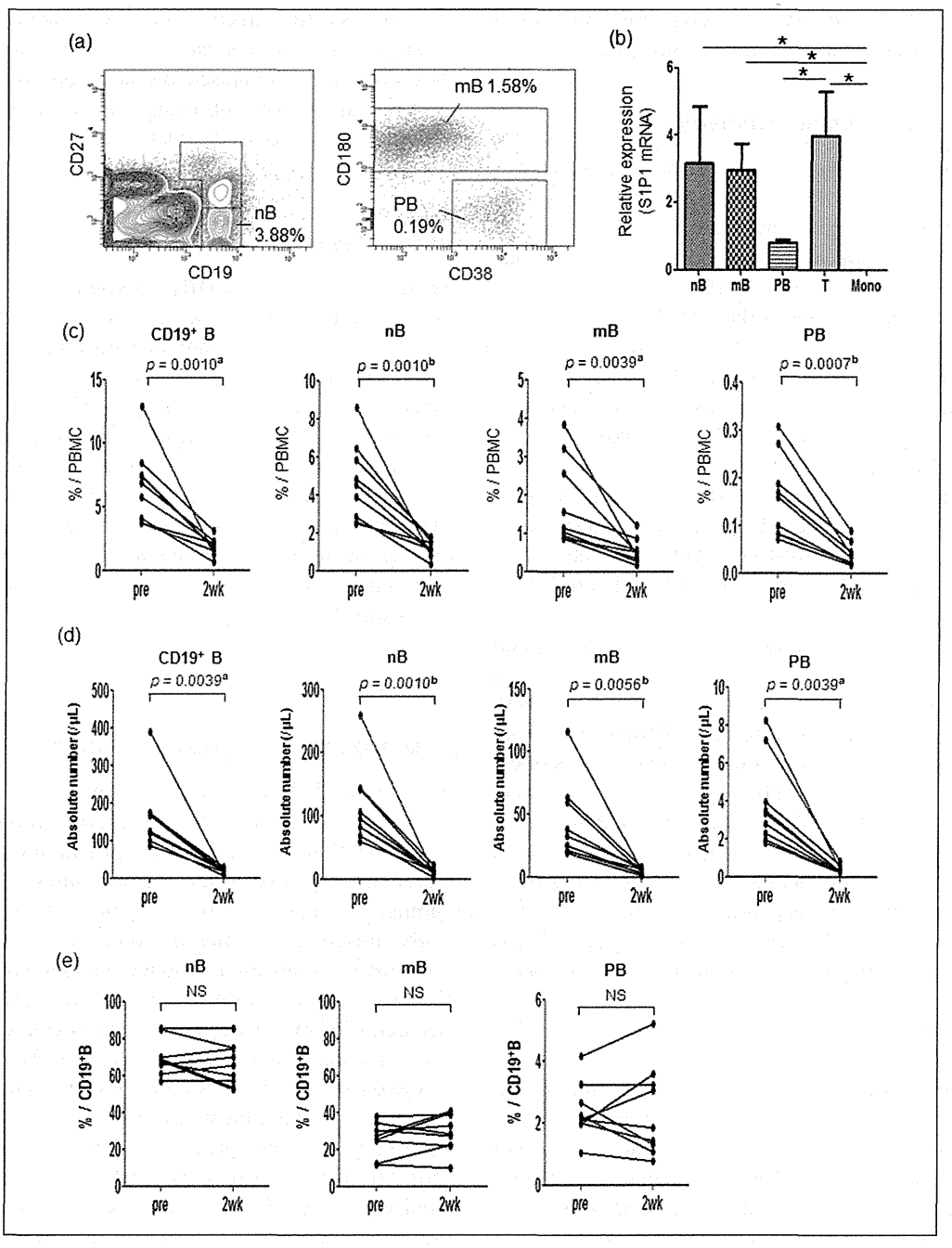

Figure 1. Frequency and absolute number of each B-cell population found in peripheral blood from MS patients.
(a)Representative flow cytometry scheme to analyse B-cell populations in PBMC. The PBMC were simultaneously stained with fluorescence-conjugated anti-CD19, -CD27, -CD38 and -CD180 mAbs. The gate 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

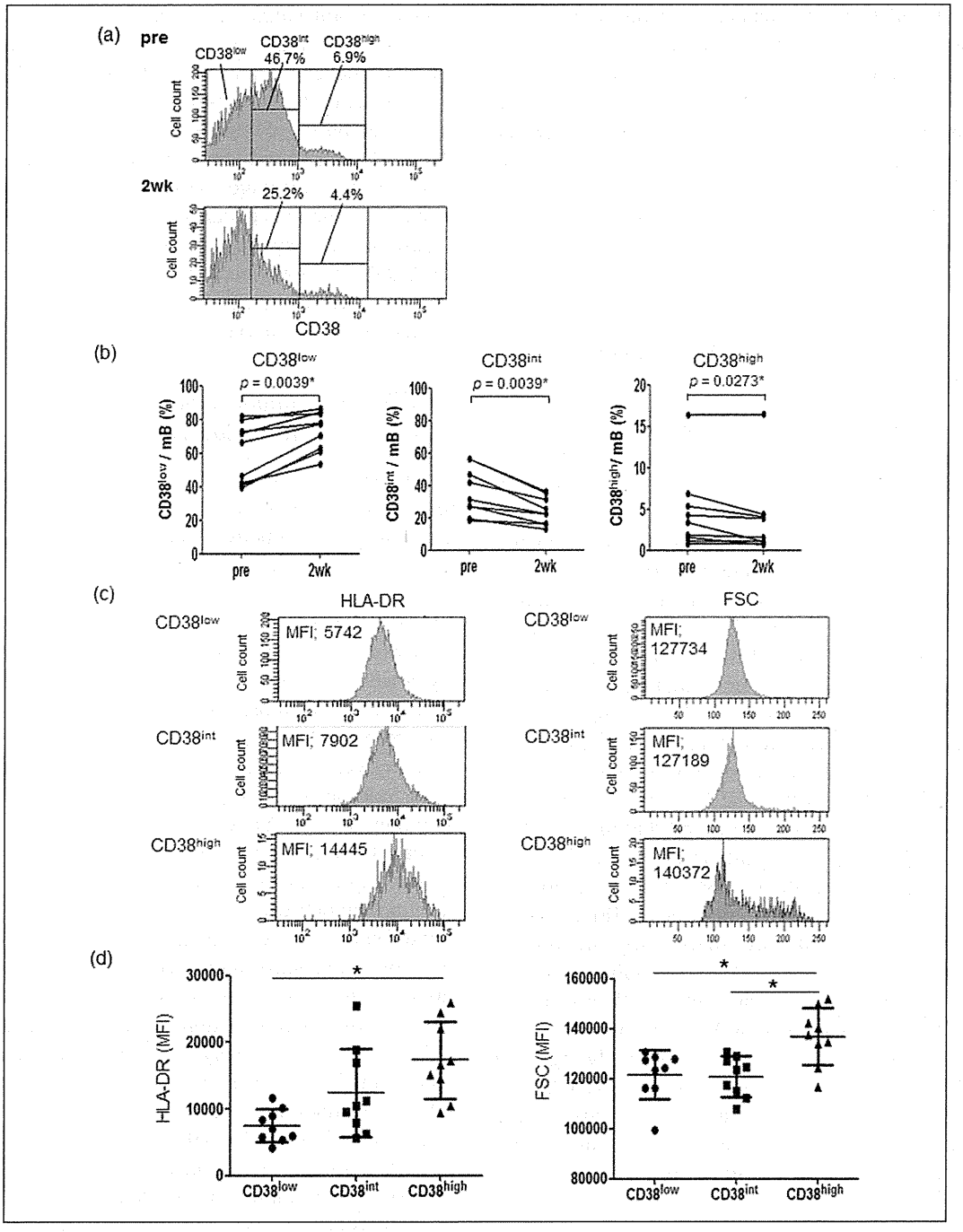

Figure 2. Frequency and activation state of each mB subpopulation in the peripheral blood of MS patients.
(a)Representative histograms of CD38 expression in mB of peripheral blood from a fingolimod-treated patient. Upper (pre) and lower (2wk) panels show the histograms before and 2 weeks after fingolimod 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.

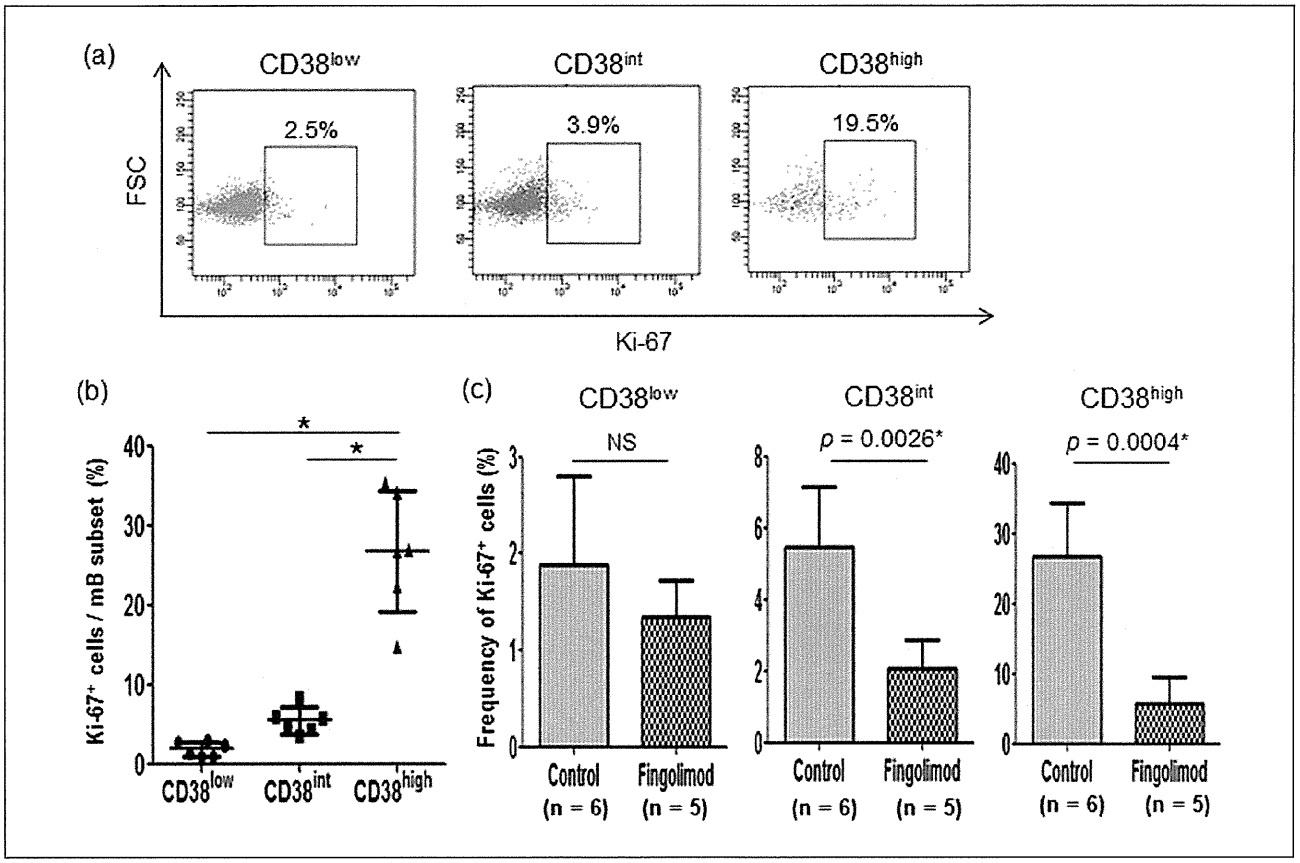

Figure 3. Ki-67 expression in mB subpopulations of peripheral blood from MS patients.
(a)Representative flow cytometry analyses of intracellular Ki-67 expression in mB subpopulations ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) of peripheral blood from an 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

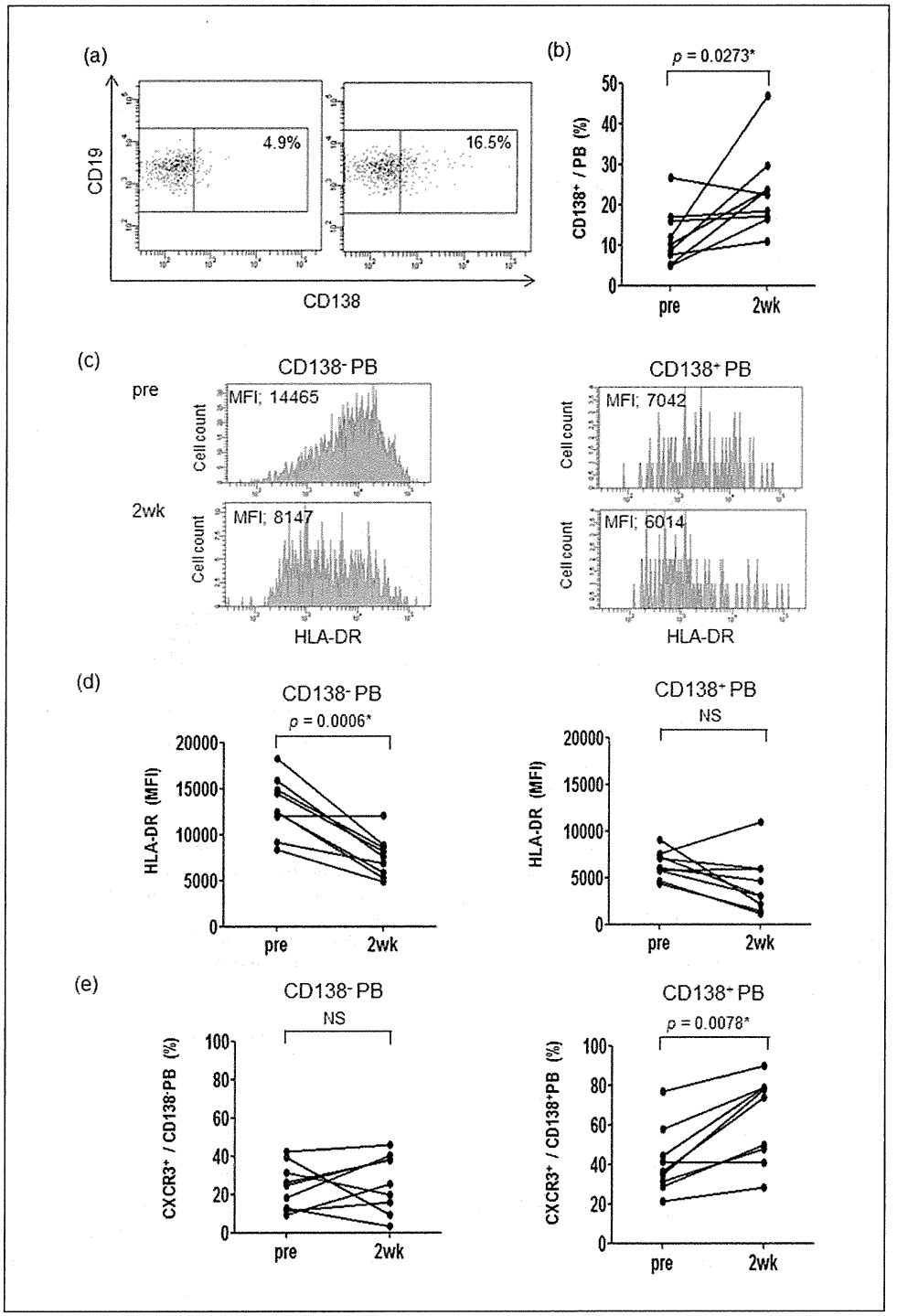

Figure 4. Phenotypic alteration of the remaining PBs in peripheral blood following fingolimod treatment.
(a)Representative dot plots of $CD19^+CD27^+CD180^-CD38^{high}$ PB, analysed for CD19 and CD138 expression before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent frequencies 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.