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

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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

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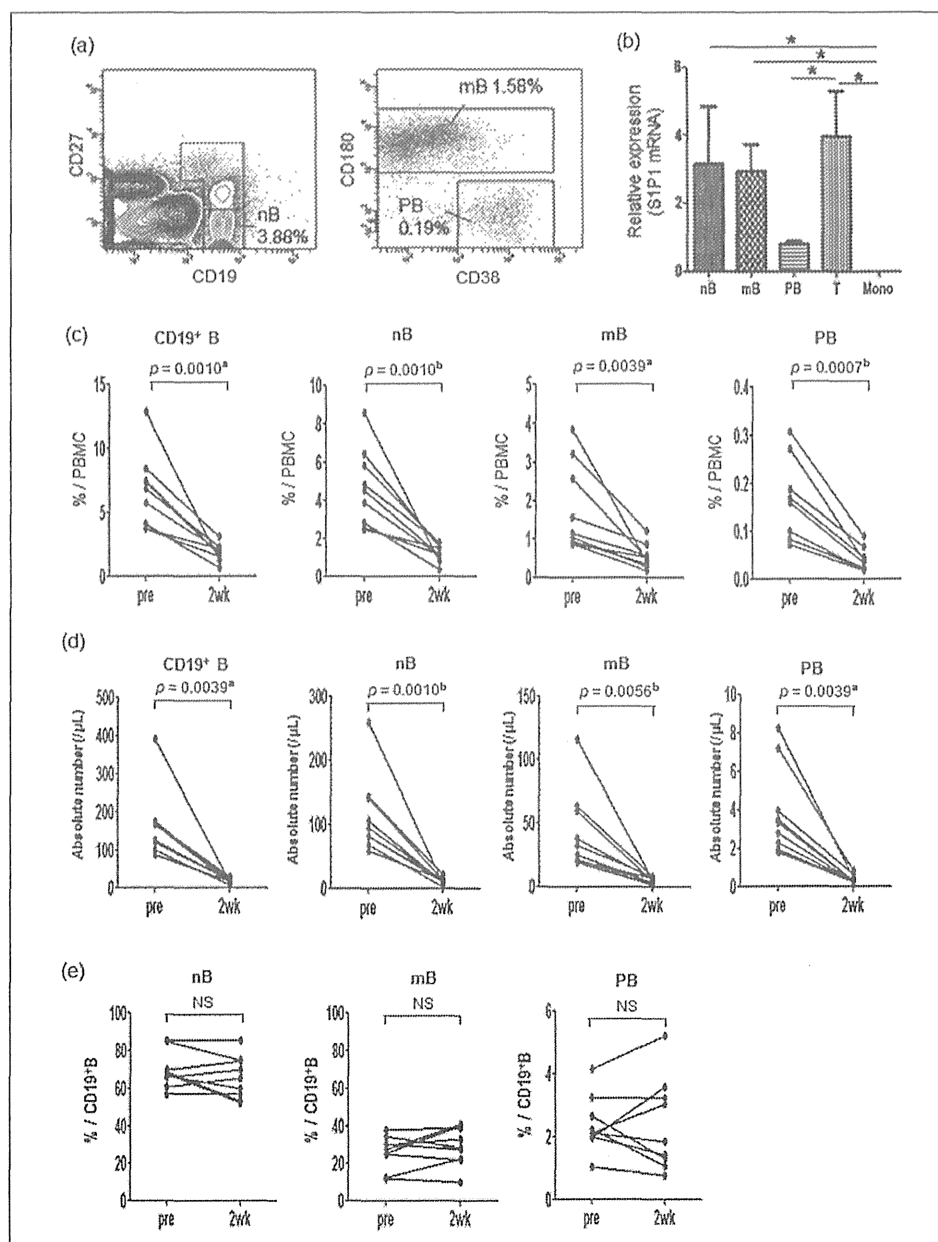

Figure 1. Frequency and absolute number of each B-cell population found in peripheral blood from MS patients.
(a)Representative flow cytometry scheme to analyse B-cell populations in PBMC. The PBMC were simultaneously stained with fluorescence-conjugated anti-CD19, -CD27, -CD38 and -CD180 mAbs. The gate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

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± 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 $Ki\text{-}67^+$ 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 $Ki\text{-}67^+$ 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 $Ki\text{-}67^+$ 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 $Ki\text{-}67^+$ cells than did $CD38^{low}$ and $CD38^{int}$ mBs, and that $CD38^{int}$ mBs were likely to contain a higher frequency of $Ki\text{-}67^+$ cells than the $CD38^{low}$ mBs (Figure 3(a) and (b)). In addition, we compared the frequency of $Ki\text{-}67^+$ 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 $Ki\text{-}67^+$ 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

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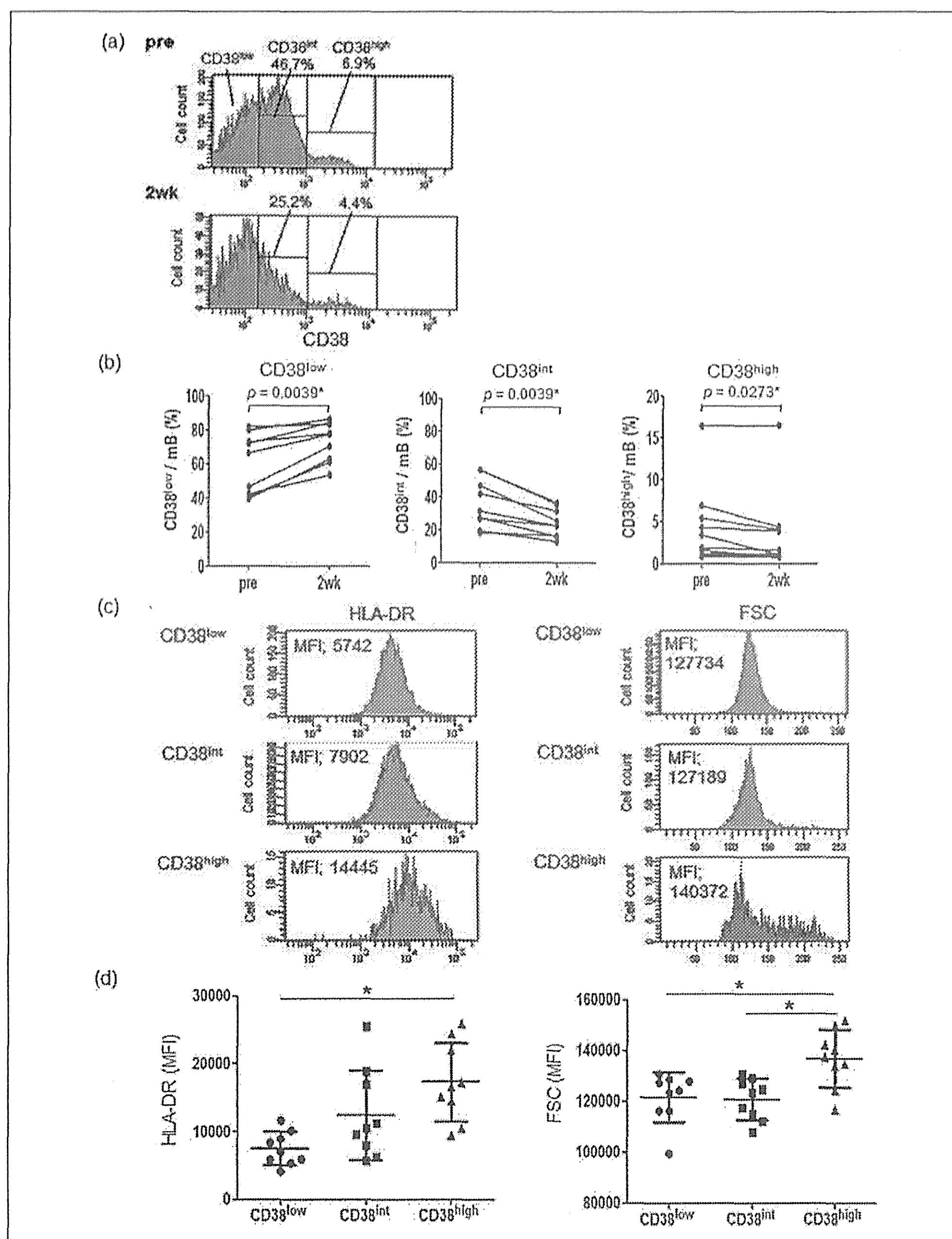

Figure 2. Frequency and activation state of each mB subpopulation in the peripheral blood of MS patients.
(a)Representative histograms of CD38 expression in mB of peripheral blood from a fingolimod-treated patient. Upper (pre) and lower (2wk) panels show the histograms before and 2 weeks after fingolimod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

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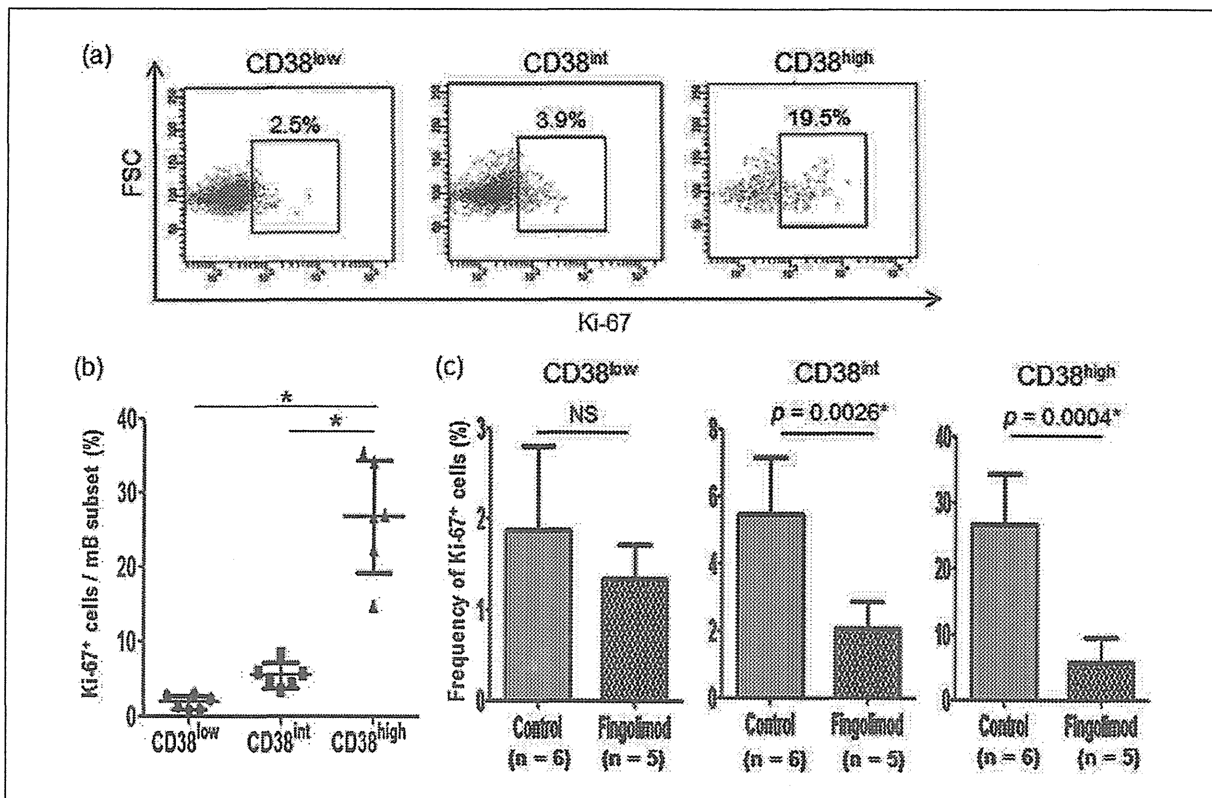

Figure 3. Ki-67 expression in mB subpopulations of peripheral blood from MS patients.
(a)Representative flow cytometry analyses of intracellular Ki-67 expression in mB subpopulations (CD38^low^ (left panel), CD38^int^ (middle panel), and CD38^high^ (right panel)) of peripheral blood from an untreated patient with MS. Each mB subpopulation was analysed for FSC and Ki-67 expression. Values in each plot represent frequency of Ki-67+ cells in each mB subpopulation. (b) Frequency of Ki-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-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

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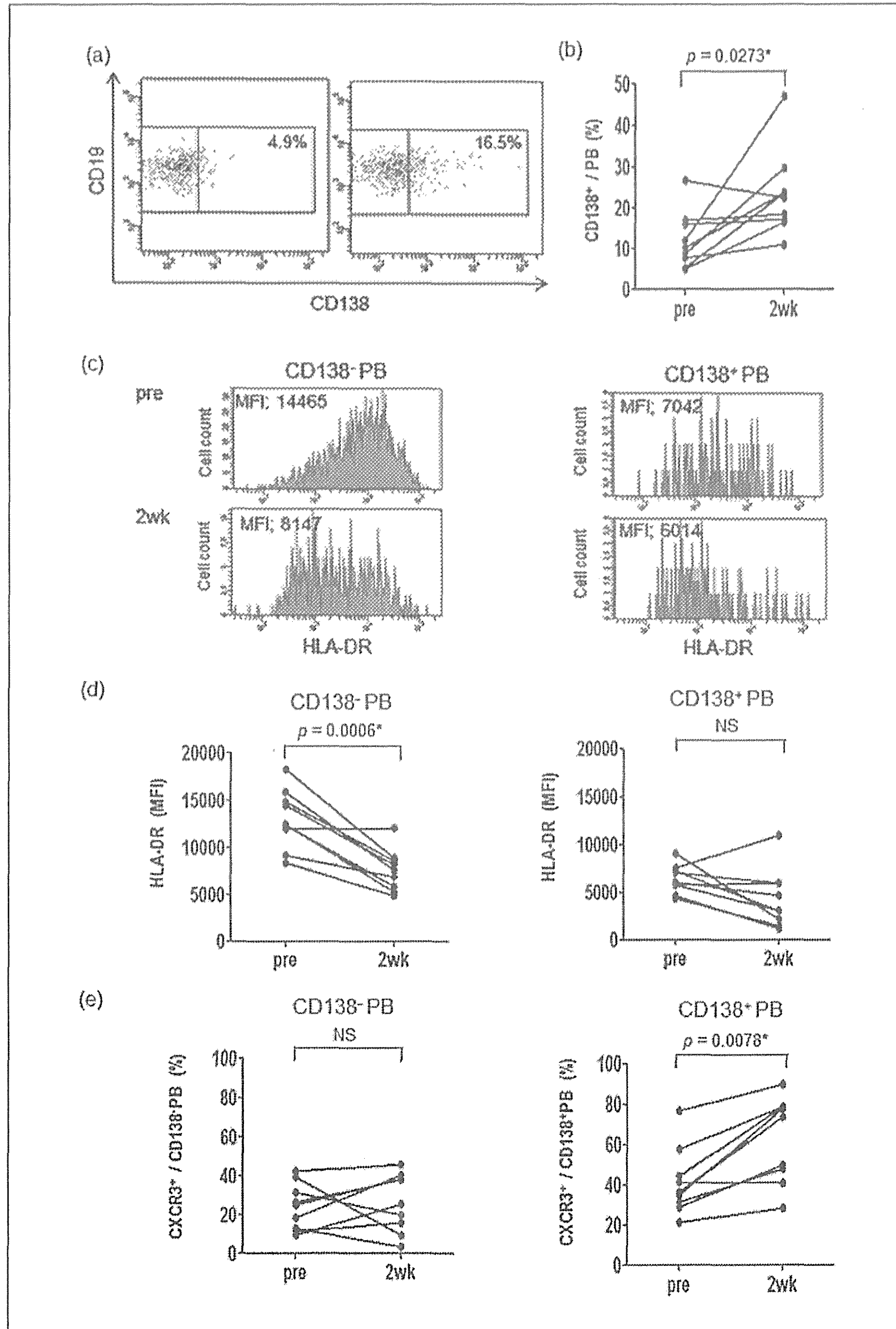

Figure 4. Phenotypic alteration of the remaining PBs in peripheral blood following fingolimod treatment.
(a)Representative dot plots of CD19⁺CD27⁺CD180⁻CD38^{high} PB, analysed for CD19 and CD138 expression before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent frequencies of the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

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fingolimod, as indicated by their reduction in the peripheral blood following fingolimod treatment. It is demonstrated in mice that surface expression levels of S1P1 on B cells in the SLT are controlled by transcription levels and CD69-mediated internalisation of S1P1. Stimulation of B-cell receptors induces not only a cessation of S1P1 transcription, but also an upregulation of CD69. Both of these changes reduce the expression levels of surface S1P1 in the SLT to some extent.[2]

Although we were not able to directly analyse B cells in the SLT of the patients, we speculated that surface S1P1 expression on mBs within the SLT in human may also decrease greatly, following antigen activation and exposure to fingolimod, which would result in these B lymphocytes having a reduced responsiveness to S1P. In fact, the activated mB subpopulations that we isolated from the patients' peripheral blood, in particular CD38^{high} mB, were found to contain a substantial proportion of Ki-67^{+} cells (Figure 3(a) and (b)). We confirmed that the proportions of Ki-67^{+} cells in the activated CD38^{int} and CD38^{high} mB subpopulations were significantly decreased following fingolimod treatment, suggesting that recently-activated cells were selectively trapped in the SLT following fingolimod treatment. Because activation of autoreactive mBs in the SLT followed by their migration to the CNS could trigger a relapse of RRMS,[35] we assumed that inhibition of activated mB cell egress from the SLT was at least partly involved in the reduced relapses of RRMS after fingolimod treatment.

We also identified a PB subpopulation that is relatively resistant to fingolimod as being CD138^{+} PBs. The frequency of the CD138^{+} subpopulation in the total PBs, and that of CXCR3^{+} cells in CD138^{+} PBs, was significantly increased by fingolimod treatment. Of note, the CD138^{+}CXCR3^{+} PBs are enriched in the CSF of NMO during relapse,[27] and fingolimod could induce exacerbation of NMO, accompanied by the appearance of large brain lesions.[11,12] Although knowledge on the biology of PBs is limited, the percentages of CCR7^{+} cells are much lower as compared with nBs or mBs, indicating that fingolimod may differentially alter the in vivo migration of PBs and other B cells.

It is of relevance to note that despite reductions of circulating lymphocytes, RRMS patients receiving fingolimod may develop clinical relapses. These relapses are not always mild, but could be serious and accompany huge brain lesions.[7–10] Although the trapping of regulatory lymphocytes in the SLT[8,9] or the enrichment for CD45RO^{-}CCR7^{-}CD8^{+} T cells in the CSF[7] is proposed as a possible mechanism for formation of tumefactive brain lesions, we were very curious to know if the increased proportion of CD138^{+} PBs over other lymphocytes in the peripheral blood might influence the character of the CNS pathology and induce large demyelinating lesions. In fact, it was recently reported that CD45^{+}CD19^{+}CD138^{+} PBs are relatively enriched in the CSF of fingolimod-treated MS patients,[16] raising the possibility that the dominance of CD138^{+} PBs in the peripheral blood is preserved or even promoted in the CNS of patients with MS who develop tumefactive brain lesions[7–10] and NMO patients who deteriorate[11,12] after being treated with fingolimod. Therefore, resistance of activated PBs in fingolimod-treated patients with MS or NMO may give us a clue to understanding the individual patients' differences regarding the effectiveness of fingolimod therapy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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EDITORIAL

How do T cells mediate central nervous system inflammation?

In the history of experimental multiple sclerosis (MS) research, the real breakthrough came around 1970, when experimental autoimmune encephalomyelitis (EAE), a classical animal model of MS, was successfully reproduced in syngeneic rodents, which were transferred with sensitized T cells. In contrast, EAE transfer by immune serum failed without exception. Along with the development of basic immunology and of supportive technology, T cell lines and clones reactive to myelin antigen were established *in vitro*, and the works using these cloned T cells soon gained popularity and flourished in the research community. Currently, the T cells used for EAE transfer are not derived from the *in vitro*-generated T cell clones, but are from T cell receptor transgenic mice lymphocytes. However, characterization of pathogenic T cells has stayed in the mainstream of EAE/MS research for almost three decades.

The basic research of CD4+ T cells, recognizing myelin peptide associated with major histocompatibility complex class II molecules, actually brought us a deeper understanding of central nervous system inflammation and led us to design a reasonable strategy to combat MS. In fact, most current MS drugs, including fligolimod and anti-Vα4 integrin antibody natalizumab, were tested in EAE for their efficacy, and showed efficacy by influencing the functions or behavior of autoreactive T cells *in vivo*. Although potential target molecules in MS are not restricted to those expressed by T cells, but extend to B cell markers and others, it is still believed that basic research of T cell biology will continuously give us correct answers to key questions and provide us new ideas on how to discover a cure for MS.

In this special issue of *Clinical and Experimental Neuroimmunology*, five review articles were contributed by experts, given the theme entitled "New mechanistic insights into the pathogenesis of multiple sclerosis – from a T cell point of view". As known to senior scientists over the age of 40 years, the classical paradigm of Th1/Th2 balance prevailed for several years, until Th17 cells were discovered approximately one decade ago. Reflecting the shorter history of research, there remain many questions about the biology of Th17 cells regarding their identity and plasticity. Furthermore, the presence of Th17 cells in the lesions of MS is still a matter of controversy, as reviewed by Thomas Korn,[1] although the clinical relevance of Th17 pathogenic T cells in neuromyelitis optica (NMO) has substantial support. However, elevation of interleukin-17 mRNA was shown in lesions of MS,[2] and Th17 cell-associated molecule, NR4A2, was also increased in the peripheral blood of MS patients,[3] showing the role of Th17 cells in MS. Therefore, Th17-associated molecules, NR4A2[3] and Tob1,[4] are potential targets of therapy in MS and NMO.

For future perspectives, the authors[5,6] and the Editor recognize that more research is required into human T cells in conditions of health and disease. It will be important to re-analyze the human T cell populations without holding any prevailing dogma. Supporting this, $CCR2^+CCR5^+$ Th1 cells, which have not been given attention, unexpectedly increased in the cerebrospinal fluid of patients with MS in relapse, and showed pathogenic potentials.[5] Readers of this special issue might share the opinion with us that application of new technologies will bring the next breakthrough within years, and immunology textbooks could require major revision.

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REVIEW ARTICLE

Towards understanding the role of orphan nuclear receptor NR4A2 in Th17 cell-mediated central nervous system autoimmunity: An experimental approach using an animal model of multiple sclerosis

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Abstract

Although details of its pathogenesis remain elusive, multiple sclerosis (MS) is now widely accepted as an autoimmune disease of the central nervous system (CNS) in which autoreactive helper T cells play a pivotal role in triggering pathogenic cascades. Recently developed drugs and ongoing clinical trials clearly reflect the significance of targeting pathogenic immune cells, such as T helper 17 (Th17) cells, for MS treatment. Through comprehensive gene expression profiling analysis, we previously showed that the orphan nuclear receptor, NR4A2, is selectively upregulated in peripheral blood T cells from relapsing–remitting MS patients. Furthermore, using experimental autoimmune encephalomyelitis, an animal model of MS, we have shown that NR4A2 is selectively upregulated in peripheral blood T cells and T cells from inflamed CNS tissues. T cells expressing NR4A2 *in vivo* were induced only when immunized with self-peptide, not with irrelevant exogenous peptides. Accordingly, interleukin-17 (IL-17)-producing helper T cells exclusively express NR4A2, whether or not they secrete interferon (IFN)-γ, suggesting that NR4A2-expressing T cells represent a pathogenic Th17 subset during autoimmunity. Therefore, NR4A2 could be a useful biomarker to estimate pathogenic Th17 cell behavior in MS patients. In addition, a blockade of NR4A2 expression in differentiating Th17 cells with small interfering RNA not only abolished IL-17 secretion, but also Th17-related genes, such as IL-21, c-Maf and IL-23 receptor. Finally, *in vivo* administration of NR4A2-specific small interfering RNA significantly ameliorated experimental autoimmune encephalomyelitis, implying that NR4A2 is essential for triggering MS/experimental autoimmune encephalomyelitis, and could serve as a novel therapeutic target of the diseases. (Clin. Exp. Neuroimmunol. doi: 10.1111/cen3.12128, May 2014)

Introduction

Multiple sclerosis (MS) is a complex disease of the central nervous system (CNS) in which inflammatory and neurodegenerative processes cause intermittent neurological disorder and subsequent progression of debilitating symptoms. In general, MS is categorized into several major disease forms, including the most common form, relapsing–remitting MS (RR-MS), which sometimes exacerbates into secondary progressive MS (SP-MS), and the less frequent form primary progressive MS (PP-MS).[1] Although the etiology and specific causes of MS are not well understood, the susceptibility for individuals to develop MS could be attributed to both genetic (e.g. disease susceptibility genes) and environmental (e.g. external and internal environmental microorganisms) factors. Recent genome-wide association studies (GWAS) have identified many potential risk loci and multiple variants that might have a key role

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in disease susceptibility.[2,3] Of note, these studies highlight a number of immunologically relevant genes, particularly those linked to helper T cell differentiation and function, suggesting the intrinsic participation of T cell components for MS pathogenesis.[4] As such, interest in interventions against autoreactive T cells to treat MS has grown, resulting in recently-developed therapies and ongoing clinical trials, such as those using natalizumab (humanized anti-α 4 integrin monoclonal antibody), fingolimod (FTY720) and glatiramer acetate, all of which are aimed to mitigate excessive immune responses of autoreactive T cells.

Organ-specific autoimmune diseases, including MS, emerge when autoreactive T cells primed in the periphery infiltrate into the CNS where reactivation of those T cells *in situ* initiates local damage and drives the recruitment of other inflammatory components (macrophages, B cells, granulocytes etc.). In early studies of MS, the major cause of organ-specific autoimmunity was believed to be the induction of immune responses by Th1 cells secreting interferon (IFN)-γ. However, recent studies have shown that autoimmune responses mediated by T helper 17 (Th17) cells secreting interleukin (IL)-17 might play key roles in the induction of autoimmune diseases. Accordingly, the role of Th17 cells in MS has been highlighted in recent years, and a large body of research has shown that such T cell responses could potentiate the pathogenesis of CNS-specific autoimmune inflammation; for example, elevated IL-17 responses and increased IL-17-secreting T cell numbers have been detected in MS patients, and correlate with active MS relapses.[5,6] Furthermore, Th17 responses have also been observed in the case of experimental autoimmune encephalomyelitis (EAE). Th17 responses appear to be critical for the induction of EAE as the severity of EAE was greatly reduced in mice lacking IL-23, IL-23R, IL-17 or IL-17R.[7–10] Interestingly, the CNS milieu in established EAE provides signals that preferentially drive Th17 responses.[11] As diverse sites, such as secondary lymphoid organs and peripheral circulation, as well as tertiary lymphoid-like structures, particularly those in the target organ (s), are involved in the development of organ-specific autoimmunity, the dynamics of the process by which pathogenic Th17 cells develop in EAE and MS have not yet been fully elucidated.

Through comprehensive gene expression profiling analysis, we previously showed that NR4A2, an orphan nuclear receptor that plays a versatile role in many aspects of biological and pathological responses, is selectively upregulated in the peripheral blood T cells of RR-MS patients in remission compared with healthy subjects.[12] Using EAE, an animal model of MS, we have further shown that NR4A2 is selectively upregulated in both T cells isolated from peripheral blood and those infiltrating into the CNS, but not from T cells in secondary lymphoid organs, such as the spleen and draining lymph nodes.[13,14] Here, the possible link between Th17 cells expressing NR4A2 and their pathogenic properties for CNS autoimmunity is summarized, the molecular mechanism of NR4A2-mediated Th17 cell differentiation is discussed, and the potential clinical application of NR4A2 as a novel therapeutic target for CNS autoimmunity with experimental data using small interfering RNA (siRNA) targeting the NR4A2 gene is suggested.

Th17 cells and EAE, an animal model of MS

Naive CD4+ T cells differentiate into Th1 cells on antigenic exposure in the presence of IL-12, and, once differentiated, Th1 cells maintain their phenotype even in different cytokine milieus, suggesting the relative robustness of the Th1 phenotype. In contrast, inflammatory processes crucial for Th17 differentiation are currently less understood. Originally, Th17 differentiation *in vitro* was observed after stimulation of naive T cells in the presence of transforming growth factor (TGF)-β in combination with IL-6.[15,16] However, IL-21 has also been reported to act as a differentiation factor by supporting the expansion of developing Th17 cells in an autocrine manner, and as an inducer of IL-23R expression on Th17 cells.[17] In that case, IL-23 could act as a critical factor for the stabilization and maturation of the phenotype of Th17 cells that express IL-23R on their cell surface.[18] In addition, a combination of different cytokines, such as IL-1β, IL-6 and IL-23, in the absence of TGF-β induces differentiation of IL-17-producing cells that apparently have an increased pathogenicity compared with conventional Th17 cells obtained by cultures with TGF-β and IL-6.[19] Therefore, it is not necessarily clear which differentiation pathway is critical for the physiological emergence of pathogenic T cells secreting IL-17 *in vivo*, and all of the factors described here could contribute to the differentiation of those cells to a greater or lesser extent.

It is well known that Th17 cells express the master transcriptional regulator retinoic acid-related orphan receptor γt (RORγt), and the deletion of the RORγt gene leads to impaired Th17 cell differentiation and reduces the severity of EAE development.[16] Pheno-

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typically, Th17 cells show a greater degree of context-dependent plasticity[20,21] associated with a higher *in vivo* survival and self-renewal capacity.[22] Using fate-reporter animals, IFN-γ producing T cells that appeared in the CNS of EAE mice were shown to be almost exclusively derived from cells that formerly produced IL-17.[23] The conversion of IL-17-producing T cells into IFN-γ producers during EAE appears to confer an increased pathogenic phenotype to those T cells, accompanied by downregulation of RORγt and upregulation of T-bet. This suggests that the tracking of RORγt expression in T cells is not necessarily enough to identify ongoing pathogenic responses resulting from Th17 cells in CNS autoimmunity. In addition, the development of conventional Th17 cells and distinct T cell subsets producing IL-17 cells requires RORγt. Deletion of RORγt results in impaired differentiation of all types of IL-17-producing T cell subsets, as well as the impaired development of other newly-identified immune cell subsets, lymphoid tissue inducer (LTi) cells and a part of type 3 innate lymphoid cells (ILC3) that also express RORγt.[24,25] It is conceivable that not all of those RORγt-positive T cells producing IL-17 are necessarily involved in pathogenesis of CNS autoimmunity. Therefore, identification of novel molecular marker(s) that exclusively represent pathogenic IL-17-producing T cells is highly desirable.

EAE is a prototype autoimmune disease model that has greatly contributed to elucidating the pathogenesis of MS.[26] EAE can be induced in laboratory animals by active immunization with myelin antigens or by passive transfer of myelin antigen-reactive T cells. Th1 cells reactive to myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) are capable of inducing clinical and pathological manifestations of EAE after transfer into naive mice; thus, Th1 cells producing IFN-γ have long been believed to play a central role in the pathogenesis of MS. However, the "Th1 disease" dogma has been challenged by contradicting results showing that gene-targeted mice deficient in IFN-γ[27,28] or IFN-γ receptor, and mice deficient for IL-12 signaling, are still susceptible to EAE. Subsequently, it was shown that IL-23, and not IL-12, is essential for the development of EAE,[7] resulting in the identification of pathogenic IL-23-dependent Th17 cells that produce the unique inflammatory cytokine, IL-17.[29] Currently, it is widely accepted that Th17 cells play an important role in the development of inflammatory autoimmune diseases either independently or collaboratively with Th1 cells.

What is NR4A2?

NR4A2, also known as Nurr1, is a nuclear receptor family member that, to date has no known endogenous ligand (Fig. 1). The members of the NR4A subfamily (NR4A1/Nur77, NR4A2/Nurr1 and NR4A3/NOR-1) are mostly expressed at very low levels in a wide variety of metabolically-demanding and energy-dependent tissues, such as skeletal muscle, adipose tissue, heart, kidney, liver and brain.[30] On particular stimulation, high levels of NR4A expression are induced in these tissues, reminiscent of immediate early genes. The diversity of signals that lead to this expression suggests that NR4A2 functions in a manner highly dependent on cell type and context. NR4A2 is primarily expressed in the CNS, particularly in the cortex, ventral midbrain, brain stem and the spinal cord, and it appears to have important functions in both the development and specific responses of dopaminergic neurons.[31,32] Therefore, many studies regarding NR4A2 have focused on the functional analysis of NR4A2 and its relevance to the pathology of Parkinson's disease. Indeed, mutations in the NR4A2 gene are well known to be associated with familial Parkinson's disease, reflecting the essential role for NR4A2 in the development and survival of neuronal organization of substantia nigra.[33,34] In contrast, much less attention has been paid to the functional role of NR4A2 in T cells. More than a decade ago, NR4A1 and NR4A3 were shown to mediate apoptotic processes of mature and immature T cells.[35–37] However, these studies do not provide insight into the functional implications of upregulated expression of NR4A2 in T cells. Recently, NR4A2 has come into the spotlight as a pivotal pathogenic component for modification of inflammatory milieus of rheumatoid arthritis, atherosclerosis and cancer, which will be discussed later in more detail.[38,39] Conversely, NR4A2 expression has also been implicated in reducing immune responses, including a potential role in neuroprotection from inflammation and repression of matrix metalloproteinases in joint inflammation, suggesting diverse roles for this transcription factor that are altered in a cell-type and context-dependent manner.[40,41]

Nuclear receptors are composed of several conserved functional domains including DNA-binding domain (DBD) with two zinc fingers in the N-terminal region of the molecule and the ligand-binding domain (LBD) in the C-terminal region with a less conserved structure. In the absence of specific ligands, most of the nuclear receptors are inactive by

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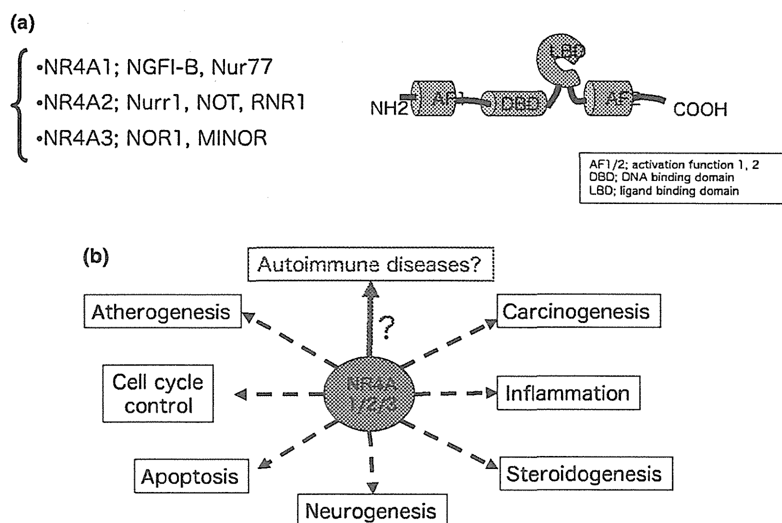

Figure 1 Versatile function of NR4A2 in a variety of biological and pathological responses. (a) Members of the NR4A family of nuclear receptors and their typical molecular structure. (b) Schematic summary of the organ and tissue-specific biological and pathological roles of the NR4A2.

interactions with co-repressor proteins. On ligand binding to a hydrophobic cleft in the LBD, a conformational repositioning occurs at the C-terminal amphipathic α-helix (H12) of the LBD that provides a well-defined surface (activation-function 2 [AF-2]) recognized by co-activator proteins, leading to the formation of multiprotein complex mediating gene activation, such as histone acetylation and chromatin modifications. However, NR4A2 encodes unusual and atypical LBD that lack canonical ligand binding properties.[42] Therefore, NR4A2 is believed to be a ligand-independent and constitutively active receptor, and its activity is tightly controlled at the level of transcription, post-transcriptional modification and multivalent complex formation with other molecules. The DNA-binding motif for the NR4A family members is the octanucleotide 5′-A/TAAAGGTCA (NGFI-B response element [NBRE]), where NR4A2 binds as monomers and homodimers. The pro-opiomelanocortin gene promoter contains another class of transcriptional targets for homodimers: Nur-responsive element (NurRE), with an inverted repeat, the NBRE-related octanucleatide, AAAT(G/A)(C/T)CA. NR4A1 and NR4A2 also bind as heterodimers with the retinoid X receptor (RXR) and bind a motif called DR-5. In addition, multivalent complex formation of NR4A2 with other transcription factors enables it to show non-canonical DNA binding.[43,44]

NR4A2-deficent neonates typically die at birth as a result of a severe defect in respiratory function despite having intact NR4A1/3 genes, suggesting a unique functional property for NR4A2.[45,46] Because of the selective expression of NR4A2 in the CNS, most of the target genes of NR4A2 known to date are limited to its role in this region. For example, NR4A2 is shown to play a role in the transcriptional activation of tyrosine hydroxylase involved in the synthesis of dopamine.[47,48] Another group of NR4A2 target genes reside in those relevant to bone formation, such as osteopontin and osteocalcin.[49,50] It is suggested that NR4A1 and NR4A3 are expressed in the thymus and mediate T cell receptor-mediated T cell apoptosis, but the distribution and function of NR4A2 in immune cells has not been extensively studied. Accordingly, a recent report showed that NR4A family proteins have essential roles in regulatory T (Treg) cell development by inducing promoter activity of the forkhead box P3 (Foxp3) gene;[51] however, NR4A2 itself was shown to be much less effective for transcription of the Foxp3 gene and subsequent Treg differentiation than other NR4A family members.[52] Meanwhile, a series of reports have suggested pivotal roles for NR4A family members, especially the NR4A2 subtype, in inflammatory responses, and they are aberrantly expressed in inflamed synovial tissue of patients with rheumatoid arthritis, psoriatic skin and atherosclerotic lesions. Therefore, NR4A receptors might contribute to the cellular processes that control inflammatory disorders including autoimmunity.

NR4A2 in MS

MS has an autoimmune pathology that is initiated by the development of autoimmune T cells reactive to myelin antigens, such as MBP, MOG and PLP. Immunologically, naïve T cells differentiate into

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encephalitogenic T cells on encountering those with myelin autoantigens. Such encephalitogenic T cells must be preactivated in the periphery before they are able to penetrate into the CNS parenchyma.[53,54] Then, expansion of inflammatory processes within the CNS is triggered by pro-inflammatory cytokines and chemokines secreted by infiltrating autoreactive T cells after recognizing self-antigens in a major histocompatibility complex (MHC) class II-restricted manner in the CNS. Encephalitogenic T cells that generate the development of MS can be composed of both Th1 and Th17 cells, and the relative contributions of either of these distinct helper T cell populations might help explain the diversity of clinical and pathological manifestations, as well as the varying responses to therapy.[4] However, little is known about the helper T cell population responsible for the development of MS partly because of a lack of appropriate methodology to discriminate those encephalitogenic T cells. For example, although RORγt is a good marker to identify Th17 cells, the aforementioned complex behavior of RORγt expression in T cells, in addition to the fact that RORγt is also involved in lymphoid organogenesis, means that it is necessary to find new specific marker(s) that enable identification of pathogenic T cells in MS. Through comprehensive gene expression profiling analysis of RR-MS patients in remission, we previously showed that NR4A2 is selectively upregulated in peripheral blood T cells of MS patients.[12] Quantitative reverse transcription polymerase chain reaction analysis further revealed that NR4A2 expression in T cells from MS patients showed an approximately fivefold increase compared with healthy donors. We then applied an animal model of MS to further assess the role of the novel orphan nuclear receptor gene on T cell function.

NR4A2 in EAE

In EAE induced in C57BL/6 mice by immunization with a MOG_{35-55} peptide, NR4A2 was selectively upregulated in T cells of the peripheral circulation and those infiltrating into the CNS, but NR4A2 expression was not observed in T cells from secondary lymphoid organs, such as the spleen or draining lymph nodes.[13,14] In a kinetic analysis using reverse transcription polymerase chain reaction, we observed that NR4A2 expression in peripheral circulating T cells reached a maximum value 21 days after EAE induction, and the entire expression pattern of NR4A2 in peripheral blood T cells was well correlated with the clinical severity of EAE. Meanwhile, significant expression of NR4A2 was observed in the CNS-infiltrating T cells from day 9, when early signs of EAE become evident. These results suggest that NR4A2 expression was induced in T cells on induction of EAE, but the kinetics of expression significantly differs between peripheral blood T cells and CNS-infiltrating T cells. Recent studies have shown that autoimmune Th17 cells producing IL-17 play a central role in causing autoimmune inflammation,[55] and analysis of fate-reporter animals showed that those Th17 cells have a tendency to change their phenotype to become IFN-γ producing T cells in the CNS milieu of EAE mice.[23] Therefore, T cells accumulating in the CNS are characterized by massive production of those inflammatory cytokines along with significant expression of NR4A2. Accordingly, retroviral transduction of NR4A2 cDNA into splenic CD4+ T cells *in vitro* augmented production of IL-17 and IFN-γ on restimulation. Furthermore, NR4A2-expressing T cells in the CNS of EAE animals were accumulated in those producing IL-17 regardless of their secretion of IFN-γ (Fig. 2).[14] Therefore, NR4A2 might be considered as a useful marker to identify pathogenic Th17 cells in target organs or peripheral circulation. In addition, T cell expression of NR4A2 seems to have a strong link with exposure to a certain autoantigen, as *in vivo* induction of NR4A2 in T cells is observed only when immunized with self-peptide and not with a peptide of exogenous origin, such as ovalbumin. Interestingly, forced subcutaneous inflammation by intradermal injection of IL-23 causes upregulation of NR4A2 in peripheral blood T cells.[14] Therefore, T cell upregulation of NR4A2 emerges only after recognition of self-antigen that induces autoreactive IL-17-producing cells *in situ*.

It is noteworthy to point out that despite an apparent requirement for NR4A2 in Th17 differentiation in target organs, little NR4A2 expression was detected in secondary lymphoid tissues that are the proposed sites for T cell priming on encounter with self-antigens and the subsequent acquisition of a Th17 phenotype.[14] In addition, NR4A2 expression by CD4+ T cells was first observed in the target organ, much earlier than in peripheral blood T cells. Intriguingly, when autoreactive cells were induced by immunization with self-antigens in the absence of pertussis toxin administration, NR4A2 expression was not upregulated, possibly because of impaired access to target organs. Thus, the target organs, the CNS in the case of EAE, might represent the site of NR4A2 upregulation where pathogenic Th17 differentiation occurs *in vivo*, rather than during initial

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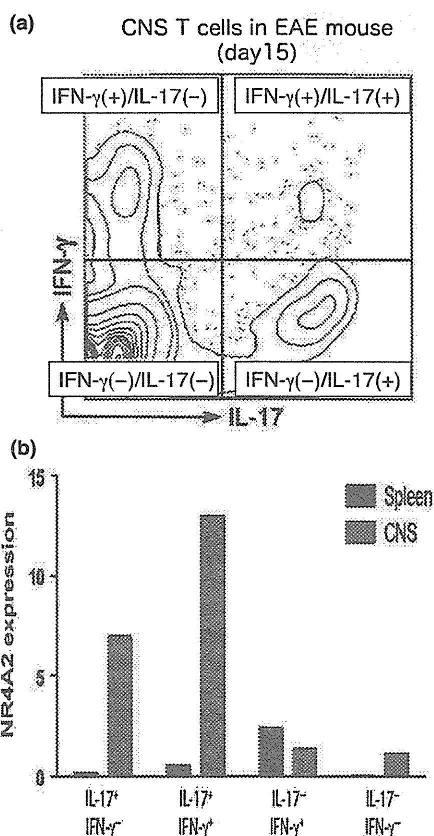

Figure 2 Distribution of cytokine-producing T cells in the central nervous system (CNS) of experimental autoimmune encephalomyelitis (EAE) mice and their expression of NR4A2. (a) There are distinct subsets of T cells accumulated in the CNS of EAE mice, composed of interleukin (IL)-17-producing T cells, interferon (IFN)-γ-producing T cells and double producers. (b) NR4A2 expression by subsets of cytokine-producing CD4+ T cells was analyzed using quantitative polymerase chain reaction at day 15 post-EAE induction for spleen and CNS-infiltrating cells.

T cell priming in the secondary lymphoid tissues. Cognate antigen interactions *in situ* are required to permit primed T cells moving from peripheral circulation to the CNS parenchyma.[53,54] Accordingly, primed encephalitogenic CD4+ effector T cells transferred into naive animals rapidly infiltrated CNS tissue, requiring MHC class II-dependent antigen presentation, whereas effector T cells specific for an irrelevant antigen did not enter CNS lesions.[54] Therefore, our data could suggest that active infiltration of encephalitogenic T cells primed in the periphery is not sufficient for generation of CNS autoimmunity despite the inflammatory potential of autoimmune responses and the potential T cell pathogenicity, and instead suggests that clinical induction of autoimmune disease is dependent on local reactivation of infiltrating T cells.[56] Furthermore, the differentiation processes of pathogenic T cells in the target organ under autoimmune conditions might enable the upregulation of NR4A2 in CD4+ T cells after reactivation by interactions with target organ antigen-presenting cells expressing CNS antigens, and the NR4A2-expressing T cells could fully represent activated pathogenic Th17 cells. Indeed, when NR4A2 expression is prevented by administration of NR4A2-specific siRNA *in vivo*, CNS-infiltrating T cells are still observed, albeit at lower numbers, but the Th17 responses in the target organ are markedly reduced with a reduction in clinical EAE. Again, although the importance of the activation of particular local responses has been previously suggested, our data shows that pathogenic Th17 responses in EAE result from a critical differentiation in the target organ.[11] The subsequent appearance of NR4A2 expression in the peripheral blood might represent T cells trafficking from, rather than to, the target organ and perhaps it is these T cells that are later reactivated after returning to the target organ and triggering disease relapses. Thus, NR4A2 might provide a cell marker identifying T cells, both in the target organ and circulatory systems, which have been reactivated during pathogenic inflammatory responses in the CNS. In addition, measurement and manipulation of NR4A2 could prove to be useful in clinical settings. As NR4A2 expression by peripheral blood CD4+ T cells is only observed after the initiation of inflammatory Th17 responses in the target organ, the presence of NR4A2 expression in the blood might be used to indicate when such responses have developed or that they are ongoing. It is conceivable that the status of immune activation in a target organ might be determined by measuring NR4A2 expression in a patient's blood. Thus, the use of NR4A2 as a biomarker for MS could indicate whether T cell infiltration into the target organ has been recently established, giving valuable insight into disease status.

Regulation of IL-21 by NR4A2 in autoimmunity

IL-21 is a pleiotropic cytokine primarily produced by activated T cells.[57] IL-21 plays a pivotal role in CD4+ T cell differentiation, the survival of both CD4+ and CD8+ T cells, and the effector function of cytotoxic T cells.[58–68] In addition, IL-21 is crucial for B cell survival and differentiation, leading to proper development of antibody-producing cells secreting mature immunoglobulins.[61] Intriguingly, IL-21 is shown to have a strong link to inflammation and autoimmune

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diseases,[62] such as patients with systemic lupus erythematosus (SLE),[63,64] inflammatory bowel diseases (IBD)[65,66] and type 1 diabetes (T1D),[67,68] or in animal models for SLE,[69] IBD,[66] T1D[70] and rheumatoid arthritis (RA).[71] Although follicular helper T cells are known as professional IL-21-producing CD4+ T cells, IL-21 is also produced by Th17 cells.[72] The primary role for IL-21 in mouse Th17 cells is the expansion of developing Th17 cells.[17] IL-6 can induce the production of IL-21 by Th17 cells, and in turn, IL-21 acts in an autocrine manner to induce the expression of IL-23R on Th17 cells and further stabilize the Th17 phenotype.[73] The requirement of IL-21 for Th17 cell development *in vivo* is still controversial, as some reports show that Th17 cells can develop in the absence of IL-21,[74,75] whereas other reports show that the generation of Th17 cells is impaired in the absence of IL-21.[73,76,77] This is partly due to the redundant role of IL-6 and IL-21, in which Th17 cell differentiation *in vivo* under a strong inflammatory milieu with massive IL-6 production would conceal the effects of IL-21.[73,77] Interestingly, the amount of IL-21 production induced by Th17 differentiation is strongly reduced by transfection of NR4A2-specific siRNA into differentiating Th17 cells *in vitro*. We further showed that the sequential upregulation of IL-21 and c-Maf, followed by the induction of IL-23R and IL-17 transcripts during Th17 differentiation, was abolished when NR4A2 expression was prevented by siRNA treatment. Therefore, one consequence of preventing NR4A2 upregulation during Th17 differentiation *in vitro* is an absence of IL-21 secretion. The essential role of NR4A2 expression in Th17 differentiation through regulation of IL-21 is shown by the fact that the addition of exogenous IL-21 led to IL-23R upregulation, subsequently yielding normal IL-17 secretion. The precise mechanisms of the NR4A2-mediated regulation of IL-21 in Th17 cell development *in vivo* have not yet been elucidated, but it is postulated that NR4A2 might regulate T cell production of IL-17 *in vivo* by controlling signaling pathways intrinsic for effective Th17 cell differentiation (Fig. 3).

One obvious mechanism by which IL-21 drives autoimmunity is by supporting the expansion, promotion and survival of pathogenic helper T cell subsets. Accordingly, it is well known that autoimmune-prone mice produce more IL-21 compared with resistant strains,[59,68] and that the level of IL-21 production is well correlated to the progression of autoimmune diseases.[60] As aforementioned, IL-21 has a strong link to inflammation and organ-specific or systemic autoimmune diseases including SLE, IBD, T1D, and RA. Regarding the possible link of the

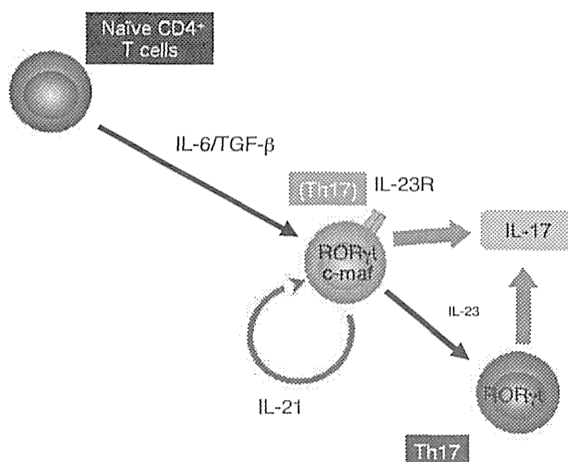

Figure 3 Multistep differentiation processes of T helper 17 (Th17) cells. T cell receptor stimulation of naive T cells in the presence of transforming growth factor (TGF)-β and interleukin (IL)-6 triggers initial Th17 cell differentiation. Th17 cells acquiring *c-Maf* expression produce IL-21, which augments Th17 cell amplification in an autocrine manner. IL-21 induces expression of the IL-23 receptor on the surface of differentiating Th17 cells that renders them responsive to IL-23. Exogenous IL-23 stabilizes the Th17 phenotype to secrete IL-17 and confers its effector function. RORγt, retinoic acid-related orphan receptor γt.

IL-21–Th17 axis to human CNS autoimmunity, such as MS, the proportion of memory Th17 cells and the IL-17 level are both shown to be much higher in patients with MS and neuromyelitis optica (NMO).[78] Accordingly, CNS-infiltrating cells expressing IL-21 were observed in both acute and chronic active white matter MS lesions in which IL-21 expression was restricted to CD4+ helper T cells.[6] Furthermore, therapeutic treatment with alemtuzumab causes secondary autoimmunity in a subset of MS patients who selectively show higher levels of serum IL-21, possibly through excessive T cells apoptosis and cell cycling after alemtuzumab-mediated lymphocyte depletion.[79] Interestingly, there are a couple of reports suggesting a significant correlation between IL-21 and NMO. First, production of IL-6 and IL-21 by CD4+ T cells *ex vivo* is shown to be directly associated with neurological disability in NMO patients.[80] In addition, higher concentrations of serum IL-21 were observed in NMO patients,[81] and concentrations of IL-21 protein in cerebrospinal fluid were significantly elevated in NMO patients, suggesting a positive correlation with humoral immunity.[82] Therefore, regulation of the IL-21–Th17 cell axis through NR4A2-mediated intervention holds considerable significance not only for MS, but also for NMO and other related neuroimmunological diseases.

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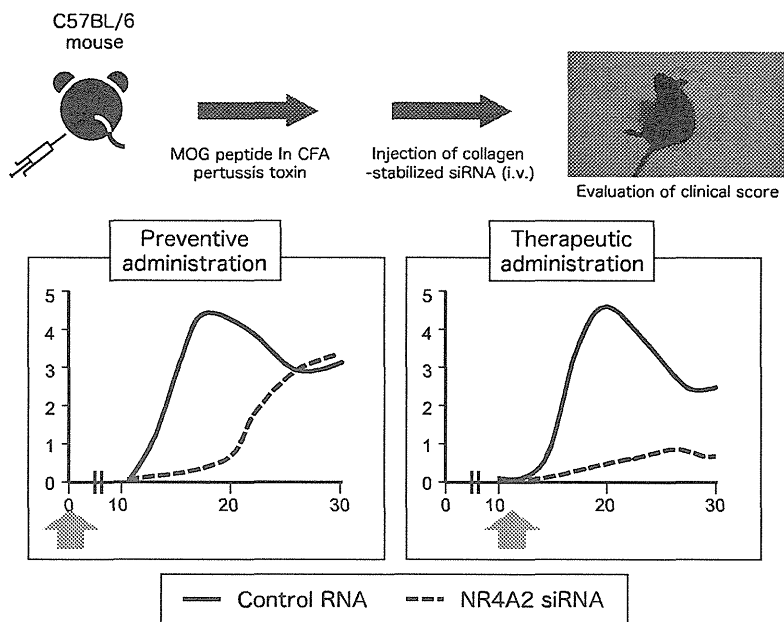

Figure 4 Effect of systemic administration of NR4A2-specific small interfering RNA (siRNA) on experimental autoimmune encephalomyelitis (EAE). NR4A2-specific or control siRNA was stabilized in a collagen matrix and administered intravenously to EAE mice either at the time of (preventive administration) or 10 days after (therapeutic administration) myelin oligodendrocyte glycoprotein (MOG) immunization. CFA, complete Freund's adjuvant.

NR4A2 as a possible target for MS therapy

NR4A2 expression is associated with the generation of autoimmune Th17 responses, and NR4A2 appears to control Th17 differentiation. We then aimed to prevent NR4A2 upregulation *in vivo* by systemically administering NR4A2-specific siRNA stabilized in a collagen matrix.[14] Both clinical EAE and NR4A2 expression in T cells were significantly reduced, with peak disease delayed by 10 days (Fig. 4). In addition, IL-17 production, but not IFN-γ production by CD4+ T cells infiltrating the CNS, was also reduced. NR4A2 siRNA-treatment prevented the accumulation of CD4+ T cells, particularly those secreting IL-17 into the CNS. Furthermore, equivalent numbers of IL-17-producing T cells in the CNS were observed between the control and the NR4A2 siRNA-treated mice at the delayed onset of clinical EAE. The late onset of EAE could be attributed to degradation of the siRNA, as administration of the siRNA at the onset of EAE significantly prevented the induction of clinical EAE. These findings suggest that the absence of NR4A2 expression during active autoimmune disease reduces clinical symptoms of EAE and Th17 responses. Therefore, NR4A2 might prove to be a potent therapeutic target for the treatment of MS and other Th17-mediated autoimmune diseases.

It is well known that methotrexate significantly suppresses expression of NR4A2 in patients with active psoriatic arthritis.[83] Accordingly, the expression level of NR4A2 after treatment with methotrexate is well-correlated to the disease activity score. Therefore, intervention of NR4A2 activity with chemical compounds might provide a potential strategy for future treatment of MS. In addition, the fact that NR4A2 mutations are associated with familial Parkinson's disease has led to significant interest in the identification of selective low-molecular-weight modulators that are helpful for analyzing the mode of action of the NR4A subfamily.[43] A growing number of NR4A2 modulators with unique chemical structures have also been described.[84–87] Furthermore, the antineoplastic and anti-inflammatory drug, 6-mercaptopurine, has been shown to activate NR4A2 through modulation of the cellular content of purine nucleotides.[88] A number of typical and atypical antipsychotic drugs, such as haloperidol, chlorpromazine, clozapine and so on, induce the transcription of NR4A2, even though they are all developed to augment NR4A2 activity.[89,90] Therefore, therapeutic application of NR4A2 inhibitors or NR4A2 modifiers converted from those NR4A2 activators through modification of chemical structure might be considered for possible future treatment of RR-MS.

Future perspective

EAE is a versatile experimental model useful for analyzing the immunopathological, neuropathological and therapeutic aspects of MS, including inflam-

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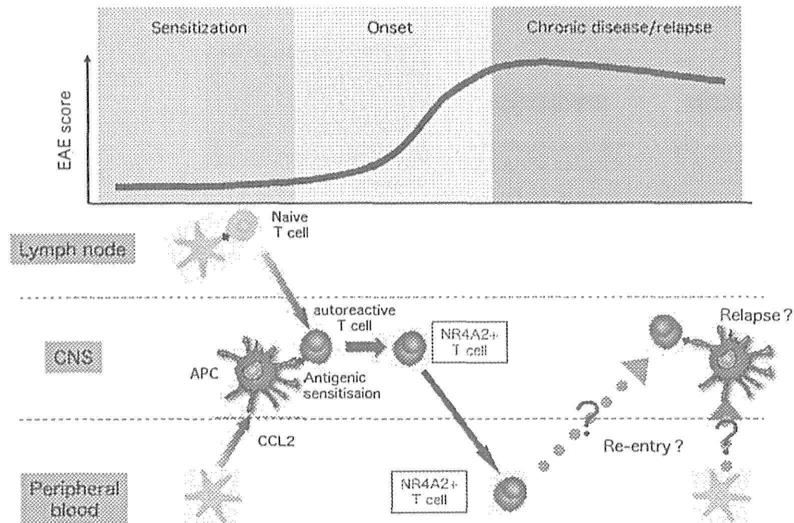

Figure 5 Possible behavior of NR4A2-expressing T helper 17 (Th17) cells during the course of multiple sclerosis/experimental autoimmune encephalomyelitis (EAE). On encountering the myelin antigen, naive T cells are primed to differentiate into effector T cells, such as Th17 cells in secondary lymphoid tissue. Then, those effector T cells are recruited to the central nervous system (CNS) and restimulated with antigen presented by local antigen presenting cells, resulting in upregulation of NR4A2 expression in pathogenic Th17 cells. Th17 cell-mediated local inflammation causes recruitment of inflammatory effector cells to the CNS, leading to immunopathogenic symptoms of multiple sclerosis/EAE. The subsequent appearance of NR4A2 expression in the peripheral blood could represent T cells trafficking from the target organ. At the later phase of EAE, T cells might egress the target organ to peripheral circulation and perhaps it is these T cells that are reactivated thereafter in the target organ triggering disease relapses. Therefore, NR4A2 could provide a good biomarker for identifying pathogenic T cells in both the target organ and in circulation. APC, antigen-presenting cells; CCL2, chemokine (C-C motif) ligand 2.

mation, demyelination, axonal damage, and after gliosis, the resolution of inflammation, remyelination and drug screening. Given the data showing that NR4A2 is selectively upregulated in the peripheral blood T cells of RR-MS patients, we have shown a strong link between NR4A2-expressing Th17 cells and their pathogenic role in CNS autoimmune inflammation through the analysis of EAE (Fig. 5), suggesting that NR4A2 represents a promising therapeutic target for MS and other autoimmune diseases. In addition, there are other inflammatory CNS diseases with distinct, but overlapping with the RR-MS, phenotype, such as NMO, progressive forms of MS and related demyelinating diseases.[91] Therefore, further analysis of RR-MS as an NR4A2-expressing Th17-mediated autoimmune disease will provide helpful clues for understanding the pathogenesis of CNS autoimmunity.

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Conflict of interest

None.

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