CCR2$^+$CCR5$^+$ T Cells Produce Matrix Metalloproteinase-9 and Osteopontin in the Pathogenesis of Multiple Sclerosis

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Multiple sclerosis (MS) is a demyelinating disease of the CNS that is presumably mediated by CD4$^+$ autoimmune T cells. Although both Th1 and Th17 cells have the potential to cause inflammatory CNS pathology in rodents, the identity of pathogenic T cells remains unclear in human MS. Given that each Th cell subset preferentially expresses specific chemokine receptors, we were interested to know whether T cells defined by a particular chemokine receptor profile play an active role in the pathogenesis of MS. In this article, we report that CCR2$^+$CCR5$^+$ T cells constitute a unique population selectively enriched in the cerebrospinal fluid of MS patients during relapse but not in patients with other neurologic diseases. After polyclonal stimulation, the CCR2$^+$CCR5$^+$ T cells exhibited a distinct ability to produce matrix metalloproteinase-9 and osteopontin, which are involved in the CNS pathology of MS. Furthermore, after TCR stimulation, the CCR2$^+$CCR5$^+$ T cells showed a higher invasive potential across an in vitro blood–brain barrier model compared with other T cells. Of note, the CCR2$^+$CCR5$^+$ T cells from MS patients in relapse are reactive to myelin basic protein, as assessed by production of IFN-γ. We also demonstrated that the CCR6$^-$, but not the CCR6$^+$, population within CCR2$^+$CCR5$^+$ T cells was highly enriched in the cerebrospinal fluid during MS relapse ($p < 0.0005$) and expressed higher levels of IFN-γ and matrix metalloproteinase-9. Taken together, we propose that autoimmune CCR2$^+$CCR5$^+$ CCR6$^-$ Th1 cells play a crucial role in the pathogenesis of MS. *The Journal of Immunology*, **2012, 189: 5057–5065.**

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS that is presumably mediated by CD4$^+$ T cells reactive to myelin Ag, such as myelin basic protein (MBP) (1). Approximately two thirds of patients with MS have relapsing-remitting MS (RR-MS), which is characterized by acute episodes of exacerbations followed by partial or complete recovery. Although there are periods of remission in the RR-MS stage, a proportion of patients enters a stage of secondary progressive MS decades after the onset of MS. There are no real periods of remission in secondary progressive MS, in which neurodegeneration can be the major cause of irreversible neurologic disability (2).

It is proposed that an initiation of relapse in RR-MS is preceded by activation of autoimmune CD4$^+$ T cells in the peripheral lymphoid organs. These T cells that are potentially reactive to myelin Ag could be activated in response to cross-reactive Ag that are generated by microbial infections (3) or following exposure to proinflammatory factors, such as osteopontin (OPN) (4), thereby acquiring the ability to migrate and infiltrate into the CNS (5, 6). The study performed in experimental autoimmune encephalomyelitis (EAE) showed that activated MBP-specific T cells first reach subarachnoid spaces filled with the cerebrospinal fluid (CSF) after crossing the endothelial barrier. After encountering perivascular APC presenting myelin Ag, the autoimmune T cells are reactivated and produce proinflammatory cytokines, such as IFN-γ and IL-17, as well as proteases, including matrix metalloproteinase (MMP)-9 (7). The proteases degrade components of the basement membranes, leading to the disruption of the blood–brain barrier (BBB). The T cells may invade into the parenchyma through the disrupted area of the BBB and cause CNS inflammation (8).

Research on EAE demonstrated that both IFN-γ–producing Th1 and IL-17–producing Th17 cells could cause inflammatory pathology in the CNS (9, 10). Although characterization of pathogenic T cells in EAE has ignited a search for similar cells in humans, the identity of pathogenic T cells in MS has not been established (10). Recent studies showed the involvement of Th17 cells (11) and of T cells producing both IFN-γ and IL-17 in the pathology of MS (12). However, because the administration of

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Abbreviations used in this article: BBB, blood–brain barrier; CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; ECD, energy-coupled dye; HS, healthy subject; MBP, myelin basic protein; MMP, matrix metalloproteinase; MS, multiple sclerosis; NHA, normal human astrocyte; NIND, noninflammatory neurologic disease; OIND, other inflammatory neurologic disease; OPN, osteopontin; PB, peripheral blood; RR-MS, relapsing-remitting multiple sclerosis.

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IFN-γ worsened MS in a previous clinical trial (13), the role of Th1 cells in MS needs to be analyzed further. In addition, increasing evidence suggest a pathogenic role for cytotoxic effector T cells in MS (14, 15). Moreover, a recent clinical trial of anti–IL-12p40 Ab to block IL-12/IL-23 signaling failed to modulate MS (16), making it difficult to portray a complete picture of MS (9).

Chemokines are a family of secreted proteins that function as key regulators of cell migration via interaction with a subset of seven-transmembrane, G protein-coupled receptors (17, 18). Chemokines are known to be highly efficient and potent chemoattractants for inflammatory cells in EAE (19). In the Th cell-differentiation process, $CD4^+$ T cells acquire the ability to produce sets of cytokines and to express chemokine receptors. Although Th1 cells preferentially express CCR5 and CXCR3, Th2 cells express CCR4 and CRTh2 (20, 21). The chemokine receptor expression pattern would confer to each Th subset a unique characteristic of migration to corresponding ligand chemokines (22). It was recently reported that human Th17 cells are enriched in $CCR4^+CCR6^+$, $CCR2^+CCR5^-$, and $CCR6^+$ populations (23–25).

The present study using multicolor flow cytometry was initiated to address whether Th17 cells bearing Th17 phenotypes ($CCR4^+CCR6^+$, $CCR2^+CCR5^-$, or $CCR6^+$) are increased in the CSF of patients with MS compared with the peripheral blood (PB). In contrast to our expectations, none of these populations was increased in the CSF of MS. Instead, we found that T cells expressing both CCR2 and CCR5 were selectively enriched in the CSF of patients with exacerbated MS but not in patients with other neurologic diseases. The $CCR2^+CCR5^+$ memory $CD4^+$ T cells were shown to produce IFN-γ (24). Comparison with other memory T cell subpopulations revealed that the $CCR2^+CCR5^+$ T cells possessed a distinct ability to produce MMP-9 and OPN, which are critical for initiating and perpetuating the inflammatory pathology in the CNS (4, 7). Consistent with the increased production of MMP-9, which is capable of degrading basement membranes, the $CCR2^+CCR5^+$ T cells showed a greater potential to invade across an in vitro model of the glia limitans, the physiological barrier separating CSF from the CNS parenchyma. Furthermore, the $CCR2^+CCR5^+$ T cells in the PB of active MS contained MBP-reactive T cells producing IFN-γ. We further demonstrated that $CCR6^-$, but not $CCR6^+$, cells within $CCR2^+CCR5^+$ T cells were enriched in the CSF of patients with MS during relapse and expressed high levels of IFN-γ and MMP-9. These results suggest that $CCR2^+CCR5^+CCR6^-$ Th1 cells play a crucial role in the pathogenesis of MS.

Materials and Methods

Subjects

Thirty-four RR-MS patients were examined for the expression of chemokine receptors on T cells. As controls for MS, 11 sex- and age-matched healthy subjects (HS), 6 patients with noninflammatory neurologic disease (NIND), and 4 patients with other inflammatory neurologic disease (OIND) were enrolled in this study. All of the MS patients fulfilled the diagnostic criteria of McDonald et al. (26). Patients with serum aquaporin 4 Abs or with longitudinally extensive spinal cord lesions on the magnetic resonance imaging scan were excluded from this study. In this article, we define "MS in remission" as patients who have been clinically stable without i.v. corticosteroid pulse therapy for >1 mo; "MS in relapse" is defined as patients who have developed an apparent exacerbation within an interval of 1 wk. The detailed demographic characteristics of the cohorts are summarized in Table I. None of the above patients had received IFN-β, i.v. corticosteroids, other immunomodulatory drugs, plasma exchange, or i.v. Ig for ≥1 mo before blood sampling.

CSF and PB pairs were obtained from 12 MS patients in relapse, 6 NIND patients, and 4 OIND patients (Table I). Although NIND patients were significantly older than the MS patient cohort, we confirmed that there was no correlation between age and the frequency of T cell subsets in the CSF of NIND patients. All MS patients were recruited from the National Center Hospital, National Center of Neurology and Psychiatry. OIND patients were recruited from the Yokohama City University Graduate School of Medicine. Written informed consent was obtained from all of the subjects. The National Center of Neurology and Psychiatry Ethics Committee approved this study.

Reagents

Anti–CCR2-biotin, anti–CCR5-FITC, anti–CCR6-FITC, and anti–CCR7-FITC mAb were purchased from R&D Systems (Minneapolis, MN). Streptavidin-PE, streptavidin–energy-coupled dye (ECD), anti–CD45RA-ECD, and mouse IgG1-FITC mAb were purchased from Beckman Coulter (Brea, CA). Anti–CD4-PerCP-Cy5.5, anti–CCR4-PE-Cy7, anti–CCR5-allophycocyanin, anti–CCR4-PE, and anti–CCR6-biotin mAb were purchased from BD Biosciences (San Jose, CA). Human MBP was prepared as described previously (27). For cell culture medium, we used RPMI 1640 (Invitrogen, La Jolla, CA) supplemented with 0.05 mM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% FBS.

Cell preparation

PBMC were freshly isolated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Oakville, ON, Canada). We used a Memory $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) to purify memory $CD4^+$ T cells from PBMC. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAb directed against nonmemory $CD4^+$ T cells and then reacted with magnetic microbead-conjugated anti-biotin mAb. The magnetically labeled nonmemory $CD4^+$ T cells were depleted with auto-MACS (Miltenyi Biotec), which yielded >80% purity of memory $CD4^+$ T cells, as assessed by flow cytometry.

To further separate memory $CD4^+$ T cells according to CCR2, CCR5, CCR4, and CCR6 expression, the cells were labeled with anti–CCR2-biotin, anti–CCR5-allophycocyanin, anti–CCR4-PE-Cy7, and anti–CCR6-FITC mAb and streptavidin-PE, in addition to CD4-PerCP-Cy5.5 and CD45RA-ECD. The stained cells were separated by a flow cytometric cell sorter (FACSAria; BD Biosciences). To measure Ag-specific responses, memory $CD4^+$ T cells were separated into $CCR2^+CCR5^+$ T cells and those depleted of $CCR2^+CCR5^+$ T cells by the cell sorter FACSAria II (BD Biosciences). To prepare APC, PBMC depleted of memory $CD4^+$ T cells were stained with anti–CD3-allophycocyanin-Cy7 and anti–CD56-PE mAb. Subsequently, $CD3^-CD56^-$ cells were sorted by FACSAria II and used as APC. This procedure yielded >95% purity of the cells.

Flow cytometric analysis of chemokine receptors

To evaluate expression of chemokine receptors on memory $CD4^+$ T cells, PBMC were first labeled with magnetic microbead-conjugated anti-CD14 mAb, and the labeled $CD14^+$ cells were depleted with auto-MACS, which yielded >95% purity of non-$CD14^+$ PBMC. $CD14^+$ cell-depleted PBMC were stained with anti–CD4-PerCP-Cy5.5, anti–CD45RA-ECD, anti–CCR2-biotin, anti–CCR5-allophycocyanin, anti–CCR4-PE-Cy7, and anti–CCR6-FITC mAb, as well as streptavidin-PE. anti–CCR7-FITC, anti–CCR4-PE, and anti–CCR6-biotin mAb and streptavidin-ECD were used for the staining of CCR7. CSF cells were stained directly with the above-mentioned Abs without depleting $CD14^+$ cells. An isotype control of each Ab was used as a negative control. At the end of the incubation, cells were washed and resuspended in PBS supplemented with 0.5% BSA and immediately analyzed by FACSAria.

Cell culture and cytokine measurements by ELISA

Purified memory $CD4^+$ T cell subsets were suspended at 5×10^5 cells/ml and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in 96-well U-bottom plates for 24 h. The concentrations of IFN-γ, IL-17, and OPN in the supernatants were measured by Human IFN-γ ELISA Set (BD Biosciences), Human IL-17 DuoSet (R&D Systems), and Human Osteopontin DuoSet (R&D Systems). The procedures were performed according to the manufacturers' instructions.

Intracellular cytokine staining of IL-17 and IFN-γ

Purified memory $CD4^+CCR2^+CCR5^+$ and $CD4^+CCR2^-CCR5^+$ T cells were stimulated with PMA and ionomycin in the presence of monensin for 18 h, fixed in PBS containing 2% paraformaldehyde, and permeabilized with 0.1% saponin. Subsequently, the cells were stained with anti–IL-17-Alexa Fluor 488 and anti–IFN-γ-PE-Cy7 mAb (eBioscience, San Diego, CA). Mouse IgG1-Alexa Fluor 488 and Mouse IgG1-PE-Cy7 were used as isotype control Abs.

T cell stimulation with MBP

To assess the presence of memory MBP-reactive T cells in the purified T cell subsets, FACS-sorted T cell subsets (2×10^4 cells/well) were cocultured

with the irradiated (3500 rad) APC (2×10^5 cells/well), in the presence or absence of MBP (10 μg/ml) or OVA (10 μg/ml), in 96-well flat-bottom plates for 5 d. rIL-2 (20 IU/ml) was added to support the growth of T cells. Cytokine concentrations in the culture supernatants were measured by ELISA.

Real-time RT-PCR

FACS-sorted cells were stimulated with PMA and ionomycin for 12 h, as described above. Total RNA was extracted from cultured cells with an RNeasy Mini Kit (QIAGEN, Tokyo, Japan), according to the manufacturer's instructions. cDNA was synthesized with a PrimeScript RT-PCR kit using oligo-dT Primers (Takara Bio, Otsu, Shiga, Japan). Gene expression was quantified by LightCycler (Roche Diagnostics, Indianapolis, IN) with SYBR Premix Ex Taq (Takara Bio). All procedures were performed according to the manufacturers' protocols. mRNA levels were normalized to endogenous β-actin (ACTB) in each sample. The specific primers used in this study are listed in Table III.

Zymography

MMP-9 activity was determined as previously reported (28). Briefly, SDS-polyacrylamide gels were copolymerized with 1 mg/ml type A gelatin derived from porcine skin (Sigma-Aldrich, St. Louis, MO). $CCR2^+CCR5^+$ T cells and $CD4^+$ T cells depleted of $CCR2^+CCR5^+$ T cells were stimulated with PMA and ionomycin, and 20 μl the culture supernatant and recombinant MMP-9 were electrophoresed. The gels were washed twice in 2% Triton X-100 for 30 min and incubated for 18 h at 37°C in buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, and 0.02% NaN_3, [pH 7.5]). After fixing with methanol containing acetic acid, the gels were stained with 0.1% Coomassie blue R-250 (Nakarai Tesque, Kyoto, Japan). The gels were scanned with a UV transilluminator (BioDoc-It Imaging System, UVP, Upland, CA) in grayscale mode, and the image was inverted by Adobe Photoshop (Adobe Systems, Mountain View, CA). Recombinant MMP-9 (GE Healthcare) was used as a positive control.

Migration assay

Migration assays were performed with 24-well Transwell membrane inserts (Corning, Wilkes-Barre, PA). The upper sides of Transwell membrane inserts (8 μm; Corning) were coated with 10 μg/ml laminin-1 (Sigma) or 20 μg/ml laminin-2 (Bio Lamina, Stockholm, Sweden). After aspirating the laminin solutions, the membrane inserts were turned upside down, and normal human astrocytes (NHA; Takara Bio) were seeded on the lower sides of the membrane inserts (2×10^5/well). After 18 h, astrocytes formed a confluent monolayer, as confirmed by Diff-Quick staining. Then the membranes were washed twice with RPMI 1640 medium supplemented with 10% FBS and settled in a 24-well plate. PBMC from HS were sorted into memory $CD4^+CCR2^+CCR5^+$ T cells, memory $CD4^+$ T cells depleted of $CCR2^+CCR5^+$ T cells, and memory $CD4^+$ T cells by flow cytometry. These T cells were stimulated with plate-bound anti-CD3/CD28 mAb for 60 h. Then the cells were harvested, suspended in the fresh medium, and seeded onto the upper chambers at 1×10^5 cells/ well, and 600 μl the medium was added to the lower chambers. After 8 h, 500 μl cell suspension was collected from the lower chambers after careful pipetting, and absolute numbers of migrated cells were counted by flow cytometry using Trucount tubes (BD Bioscience).

Statistics

A one-way ANOVA test was used to compare the frequency of chemokine receptor expression within each group of patients or HS. A paired Student *t* test was used to evaluate the difference in the percentage inhibition of migration and in the frequency of chemokine receptor expression between PB and CSF from the same patients. For statistical analysis of other data, an unpaired Student *t* test or one-way ANOVA was used. The *p* values < 0.05 were considered statistically significant.

Results

$CCR2^+CCR5^+$ T cells are enriched in the CSF of MS patients in relapse

First, we analyzed the chemokine receptor-expression profile of memory $CD4^+$ T cells in the PB of MS patients (Table I) compared with HS and those with NIND. Multicolor flow cytometric analysis was performed on PBMC after staining with differentially labeled anti-CCR2, -CCR4, -CCR5, and -CCR6 mAb. Patterns of coexpression for four chemokine receptors are summarized in Supplemental Fig. 1 and Supplemental Table I. When the memory T cells in PB were grouped based on CCR2 versus CCR5 or CCR4 versus CCR6 expression (Fig. 1A), no particular population was found to be altered in MS patients compared with HS or NIND patients, irrespective of whether the MS patients were in relapse or in remission (Fig. 1B). We next analyzed sets of CSF and PB samples from individual patients with MS, OIND, or NIND. As shown in Fig. 1C, $CCR2^-CCR5^+$ T cells formed the predominant T cell population in the CSF of patients with NIND or MS during relapse, suggesting that this population, which was previously shown to be enriched for Th1 cells (24), is allowed to enter the CSF spaces in the patients with MS and NIND. It was reported that human IL-17–producing T cells or Th17 cells are enriched in $CCR2^+CCR5^-$, $CCR4^+CCR6^+$, or $CCR6^+$ cells (23–25). We were initially interested in knowing whether examination of the chemokine receptor profile could reveal an increase in Th17 cells in the CSF of MS patients. However, the frequencies of $CCR2^+CCR5^-$, $CCR4^+CCR6^+$, and $CCR6^+$ cells were lower, rather than higher, in the CSF compared with the PB of patients with MS or NIND. In contrast, the frequency of $CCR2^+CCR5^+$ T cells in the CSF of patients with MS was significantly higher than in the PB (Fig. 1C). Of note, this increase was specific for MS and was not found in the patients with other neurologic diseases, indicating that cells of this subset are selectively recruited to autoimmune inflammatory lesions or would expand in the CSF during relapse of MS. In addition, if we separate the MS patients by disease duration (<10 y [$n = 8$] or >10 y [$n = 4$]), the higher frequency of $CCR2^+CCR5^+$ T cells in CSF compared with PB was evident ($p < 0.0005$) in those with the shorter history of MS (<10 y) (Fig. 2, Table II), but not in those with longer history (data not shown). We also noted that enrichment for $CCR2^-CCR5^+$ T cells in the CSF was not detected in the patients with the shorter history of MS. In contrast, the proportion of $CCR4^+CCR6^+$ T cells was significantly lower ($p < 0.005$) in CSF compared with PB of these

Table I. Patient summary

PB Analysis	MS in Remission	MS in Relapse	HS	NIND[a]
Males/females (*n*)	3/8	5/6	5/6	2/4
Age (y; mean ± SD)	44 ± 12	42 ± 13	39 ± 5	64 ± 13
PB/CSF Analysis	**MS in Relapse[b]**	**NIND**	**OIND[c]**	
Males/females (*n*)	5/7	2/4	2/2	
Age (y; mean ± SD)	46 ± 15	64 ± 13	44 ± 14	

[a]NIND includes one patient with Parkinson's disease, one patients with myasthenia gravis, three patients with normal pressure hydrocephalus, and one patient with multiple system atrophy.

[b]Five MS patients were being treated with immunomodulatory drugs (one with IFN-β, two with oral corticosteroids, and two with an immunosuppressive drug) before their relapses.

[c]OIND includes one patient with mumps meningitis, one patient with herpes encephalitis, and two patients with undiagnosed viral meningitis in acute phase.

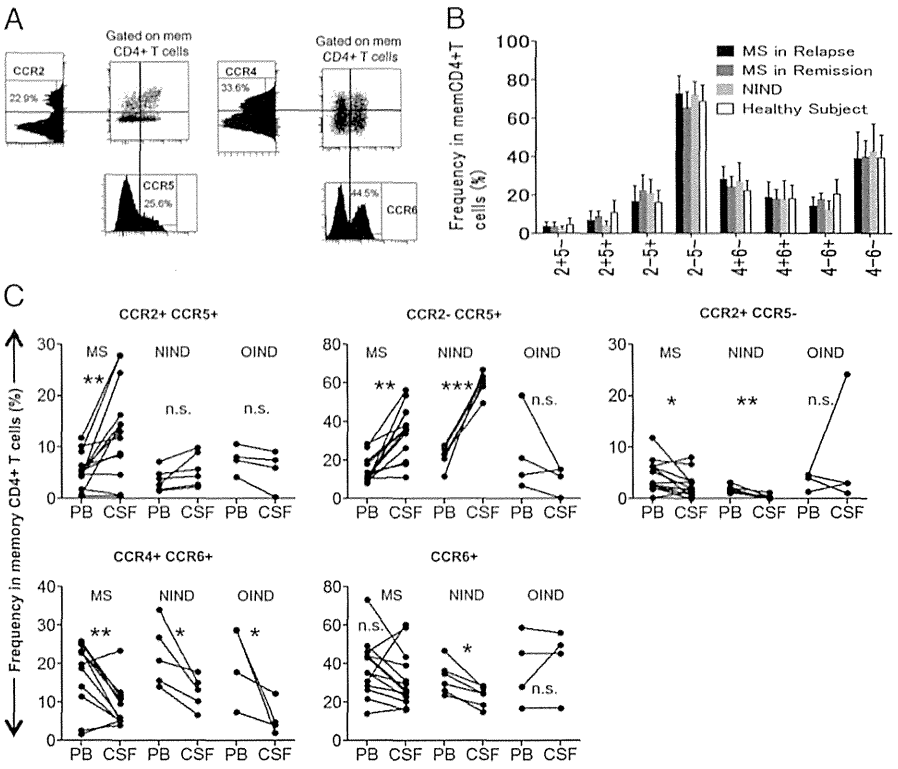

FIGURE 1. $CCR2^+CCR5^+$ T cells are enriched in the CSF of MS patients in relapse. (**A**) PBMC depleted of $CD14^+$ cells were stained with differentially labeled anti-CD4, -CD45RA, -CCR2, -CCR5, -CCR4, and -CCR6 mAb simultaneously. The $CD4^+CD45RA^-$ population was analyzed for expression of CCR2 and CCR5 (*left panels*) or CCR4 and CCR6 (*right panels*). Graphs of the corresponding parameters are also shown. Numbers (%) indicate the percentage of the positive population in the graphs. (**B**) Cells were stained, as described in (A), and frequencies of T cell subsets in memory $CD4^+$ T cells of 11 MS patients in relapse, 11 MS patients in remission, 6 NIND patients, and 11 HS were calculated. For brevity, "CCR" is omitted from the figure (e.g., 2+5− represents $CCR2^+CCR5^-$). (**C**) Comparison of the frequencies of the T cell subsets in the CSF and PB from 12 MS patients in relapse, 6 patients with NIND, and 4 patients with OIND. Lines connect data for paired CSF and PB samples from the same patients. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. n.s., Not significant.

MS patients. These results indicate that selective enrichment of $CCR2^+CCR5^+$ T cells in the CSF is detected in relatively early stages of MS.

$CCR2^+CCR5^+$ T cells in the PB contain both central and effector memory cells and produce both IFN-γ and IL-17

Memory $CD4^+$ T cells are divided into $CCR7^+$ central memory and $CCR7^-$ effector memory subsets, which are differentially endowed with effector functions (29). The staining of CCR7, together with CCR2/5 or CCR4/6, revealed a higher effector memory/central memory ratio in $CCR2^+CCR5^+$ T cells and $CCR2^-CCR5^+$ T cells (Supplemental Fig. 2). We next analyzed cytokine production by each T cell population bearing a distinct chemokine receptor profile. The cells of interest were separated from PB of HS and were stimulated with PMA and ionomycin. Compared with unfractionated memory $CD4^+$ T cells, $CCR2^+CCR5^+$ and $CCR2^-CCR5^+$ T cells produced a larger quantity of IFN-γ (Fig. 3A). Although $CCR2^+CCR5^+$ T cells produced a significant amount of IL-17, production of IL-17 from $CCR2^-CCR5^+$ T cells was only marginal. $CCR2^+CCR5^-$ T cells and $CCR4^+CCR6^+$ T cells selectively produced IL-17, whereas $CCR4^-CCR6^-$ T cells selectively produced IFN-γ. These results were consistent with the results of previous studies (23, 24). Because T cells expressing both IFN-γ and IL-17 are reportedly present in highly infiltrated lesions of MS brain sections (12), it was of interest to know whether similar T cells producing both IFN-γ and IL-17 are present in $CCR2^+CCR5^+$ T cells. By conducting intracellular cytokine staining, we revealed that the $CCR2^+CCR5^+$ T cells, as well as $CCR2^-CCR5^+$ T cells, are composed of IFN-γ^+IL-17^- cells, IFN-γ^+IL-17^+ cells,

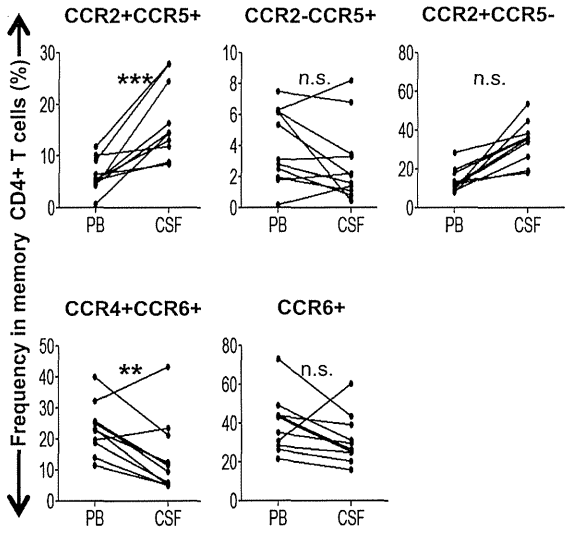

FIGURE 2. Frequencies of the T cell subsets in the CSF and PB from eight MS patients with disease duration <10 y. $^{**}p < 0.005$, $^{***}p < 0.0005$. n.s., Not significant.

Table II. $CCR2^+CCR5^+$ T cells are involved in early stages of MS

Clinical Parameter	Disease History <10 y	Disease History >10 y
Disease duration (y; mean ± SD)	4.8 ± 3.8	15.5 ± 4.4
No. of patients	8[a]	4[b]
Age (y; mean ± SD)	42 ± 14	54 ± 15
Relapse rate (times/y; mean ± SD)	1.8 ± 0.9	2.0 ± 14
EDSS (mean ± SD)	3.1 ± 0.7	4.0 ± 1.2
No. of patients with more $CCR2^+CCR5^+$ T cells in CSF than in PB	8*	0

[a]Three patients were treated with IFN-β ($n = 1$), oral steroid ($n = 1$), or immunosuppressant ($n = 1$).
[b]Two patients were treated with oral steroid.
$^*p < 0.0005$.

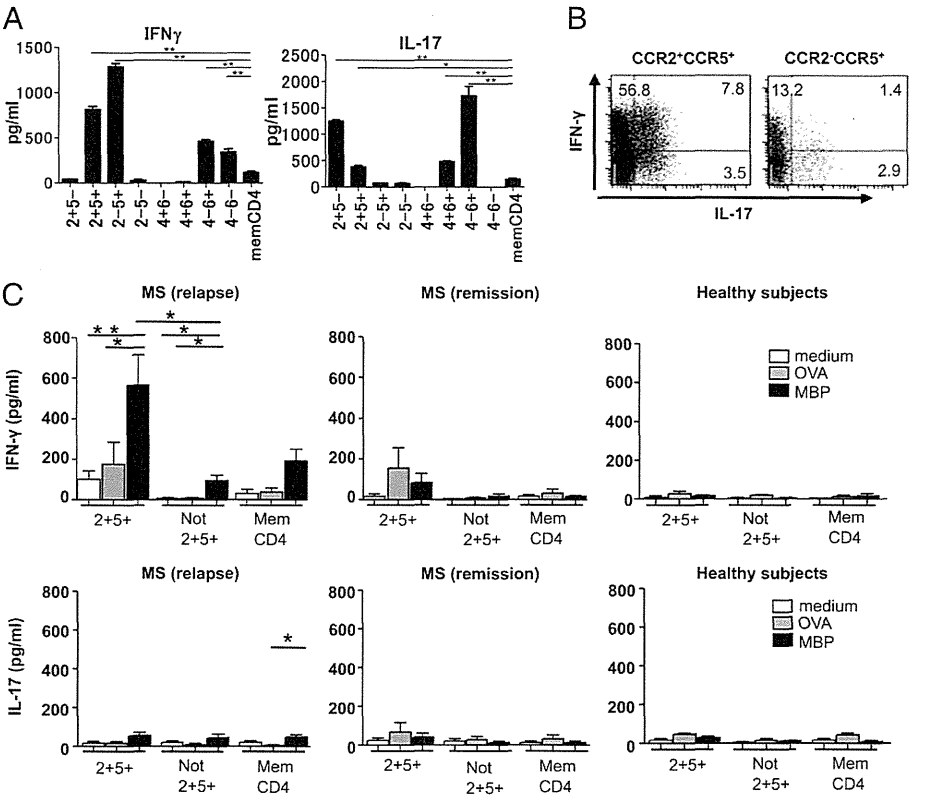

FIGURE 3. Cytokine production and reactivity to MBP by $CCR2^+CCR5^+$ T cells in the PB. (**A**) Memory $CD4^+$ T cell subsets purified from PBMC of HS by flow cytometry were stimulated with PMA and ionomycin. Concentrations of IFN-γ and IL-17 in the supernatants were measured by ELISA. Data represent mean ± SD of three HS. (**B**) Purified $CCR2^+CCR5^+$ T cells (*left panel*) and $CCR2^-CCR5^+$ T cells (*right panel*) were stimulated with PMA and ionomycin for 18 h, and the production of IL-17 and IFN-γ was assessed by intracellular cytokine staining. Numbers indicate the frequency (%) of cells in each quadrant. One representative experiment from three independent experiments with PBMC from HS is shown. (**C**) Purified memory $CD4^+$ T cell subsets were cultured in duplicate with irradiated APC in the presence of MBP (10 μg/ml) or OVA (100 μg/ml) for 5 d. Concentrations of IFN-γ and IL-17 in the supernatants were measured by ELISA. Data represent mean ± SD of six MS patients in relapse, three MS patients in remission, and three HS. $*p < 0.05$, $**p < 0.005$.

and IFN-γ^-IL-17^+ cells (Fig. 3B). In both T cell populations, IFN-γ^+IL-17^- cells were a major subset of IFN-γ production.

$CCR2^+CCR5^+$ T cells in the PB from MS patients during relapse are reactive to MBP

Given that the $CCR2^+CCR5^+$ T cells are proportionally higher in CSF than PB of MS during relapse, we were interested to know whether the $CCR2^+CCR5^+$ T cells are enriched in autoimmune, pathogenic T cells. We therefore examined if the $CCR2^+CCR5^+$ T cells might react to MBP, a putative autoantigen for MS. We isolated memory $CD4^+CCR2^+CCR5^+$ T cells and memory $CD4^+$ T cells depleted of $CCR2^+CCR5^+$ T cells from the PB of MS in relapse, MS in remission and HS. We stimulated these cells with MBP or OVA in the presence of autologous APC and measured the levels of IFN-γ and IL-17 in the supernatants after culture (Fig. 3C). The T cell populations separated from HS did not show any significant response to MBP or OVA in this assay. A marginal IFN-γ response to MBP and OVA was noted in $CCR2^+CCR5^+$ T cells from MS in remission. Strikingly, the $CCR2^+CCR5^+$ T cells from MS in relapse selectively and significantly responded to MBP by producing a large amount of IFN-γ, whereas those depleted of $CCR2^+CCR5^+$ T cells or total memory $CD4^+$ T cells showed a much smaller response. These results suggest that MBP-specific IFN-γ-producing cells might be enriched in $CCR2^+$ $CCR5^+$ T cells during relapse of MS.

$CCR2^+CCR5^+$ T cells in the PB produce MMP-9 and OPN

Lymphocyte migration/infiltration is a critical step for the development of autoimmune pathology in the CNS, and two physical barriers protect the CNS parenchyma from entry of the immune cells: the vascular endothelium barrier and the glia limitans barrier made up of extending astrocyte foot processes (8, 30). Each barrier possesses its own basement membrane, and the CSF circulates in the perivascular space between the two membranes. Thus, initiation of CNS inflammation requires immune cells that are capable of disrupting these physical barriers. Because type IV collagenase MMP-9 is selectively elevated in the CSF in MS, MMP-9 is assumed to play a role in disrupting the BBB in MS (28, 31, 32). Speculating that $CCR2^+CCR5^+$ T cells may have a distinct ability to initiate the processes of CNS inflammation, we examined whether $CCR2^+CCR5^+$ T cells are able to produce MMP-9. Strikingly, quantitative RT-PCR analysis of whole and CCR2/CCR5 fractions of memory T cells from HS and MS showed that expression of MMP-9 was mainly restricted to $CCR2^+CCR5^+$ T cells (Fig. 4A, 4B, Table III). The expression of MMP-1 and MMP-19, which also possess the potential to degrade the basement membrane, was highest in $CCR2^+CCR5^+$ T cells, whereas all T cell populations similarly expressed MMP-10 and MMP-28 (Supplemental Fig. 3). We also measured MMP-2, -7, -14, -15, -23, and -25, but none of these was detected. Using zymography, we further examined MMP-9 enzymatic activity in the culture supernatants of activated $CCR2^+CCR5^+$ T cells. As shown in Fig. 4C, supernatants from $CCR2^+CCR5^+$ T cells exhibited MMP-9 activity, but those from T cells depleted of the $CCR2^+CCR5^+$ population did not.

Recent studies suggested that OPN, which is also expressed by T cells (33, 34), might be involved in the pathogenesis of MS. Although OPN-deficient mice were resistant to relapse of EAE (4, 33), administration of recombinant OPN to OPN-deficient mice reversed the ongoing remission of the disease and induced progressive exacerbation of the clinical symptoms (4). These findings prompted us to examine whether OPN is overexpressed in $CCR2^+$ $CCR5^+$ T cells after stimulation with PMA and ionomycin. As shown in Fig. 4D and 4E, $CCR2^+CCR5^+$ T cells expressed a much higher level of OPN than did the other memory T cell populations at both the mRNA and the protein levels.

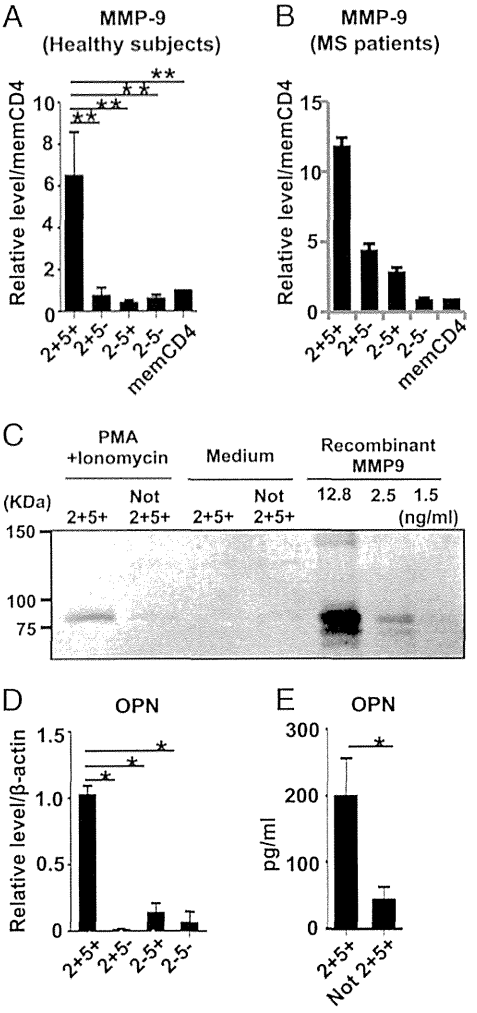

FIGURE 4. CCR2+CCR5+ T cells in the PB have the potential to produce MMP-9 and OPN. Each memory CD4+ T cell subset was isolated from the PBMC of HS (**A**) or MS (**B**) and was stimulated with PMA and ionomycin. Expression levels of MMP-9 mRNA were determined by quantitative RT-PCR. Results were normalized based on the values in unfractionated memory CD4+ T cells. Data represent mean ± SD of four HS or three MS patients. (**C**) CCR2+CCR5+ T cells and CD4+ T cells depleted of CCR2+CCR5+ T cells from HS were stimulated with PMA and ionomycin, and 20 μl of the culture supernatant and recombinant MMP-9 were electrophoresed. Shown are CCR2+CCR5+ T cells activated with PMA and ionomycin (*lane 1*), memory CD4+ T cells depleted of CCR2+ CCR5+ T cells activated with PMA and ionomycin (*lane 2*), CCR2+CCR5+ T cells without activation (*lane 3*), memory CD4+ T cells depleted of CCR2+CCR5+ T cells without activation (*lane 4*), and serial dilution of recombinant MMP-9 (*lanes 5–7*). The results shown are representative of three independent experiments. (**D**) Purified memory CD4+ T cell subsets from HS were stimulated with PMA and ionomycin. Expression levels of OPN mRNA were determined by quantitative RT-PCR. Data were normalized to the amount of β-actin mRNA. Data represent mean ± SD of four independent experiments. (**E**) Purified memory CD4+ T cell subsets were stimulated with PMA and ionomycin. OPN concentrations in the supernatants were measured by ELISA. Data represent mean ± SD of four different HS. $*p < 0.05$, $**p < 0.005$.

CCR2+CCR5+ T cells are superior to other T cells in the ability to invade the CNS

Although activated T cells are able to cross the endothelial barrier and enter the CSF compartment relatively easily, the parenchymal basement membrane and the glia limitans would hamper the further entry of the T cells into the CNS parenchyma. Although the endothelial cell basement membrane contains laminin-8 and -10, the parenchymal basal lamina is composed of laminin-1 or -2 (35). It was suggested that leukocyte penetration through the glia limitans requires MMP, such as MMP-2 and MMP-9 (36). After demonstrating that CCR2+CCR5+ T cells have the potential to produce MMP-9, we explored whether CCR2+CCR5+ T cells efficiently invade the CNS parenchyma across the basal lamina and glia limitans. To recapitulate the glia limitans layered with parenchymal basal lamina experimentally, we coated the upper sides of Transwell membrane inserts with laminin-1 or -2 and seeded NHA on the lower sides of the membrane inserts, as described in *Materials and Methods*. When we applied activated T cells to the upper chamber, their migration across the NHA layered with laminin-1 or -2 was less efficient compared with the migration across the untreated membrane or the membrane treated with laminin alone, as assessed by the number of migrated activated T cells collected from the lower chamber (Fig. 5A). Therefore, we assumed that this model would exhibit barrier functions against the penetration of activated T cells. Moreover, we applied CCR2+ CCR5+ T cells and memory CD4+ T cells depleted of CCR2+ CCR5+ T cells and showed that CCR2+CCR5+ T cells more efficiently penetrated and migrated to the lower chamber compared with the other T cells (Fig. 5B). These results indicate that CCR2+ CCR5+ T cells capable of producing MMP-9 and OPN have a greater potential to invade the brain parenchyma.

Table III. Primers used in this study

Primer	5′–3′
MMP1 forward	GATGAAGCAGCCCAGATGTGG
MMP1 reverse	GGAGAGTTGTCCCGATGATC
MMP9 forward	AGCGAGGTGGACCGGATGTTCC
MMP9 reverse	GAGCCCTAGTCCTCAGGGCA
MMP10 forward	GTGTGGAGTTCCTGACGTTGG
MMP10 reverse	GCATCTCTTGGCAAATCTGG
MMP19 forward	CAAGATGTCTCCTGGCTTCC
MMP19 reverse	CGGAGCCCTTAAAGAGGAACAC
MMP28 forward	TGCAGCTGCTACTGTGGGGCCA
MMP28 reverse	TCCAACACGCCGCTGACAGGTAGC
OPN forward	GGCAACGGGGATGGCCTTGT
OPN reverse	TTTTCCACGGACCTGCCAGCAAC
β-actin forward	CACTCTTCCAGCCTTCCTTCC
β-actin reverse	GCGTACAGGTCTTTGCGGATG
IFN-γ forward	CAGGTCATTCAGATGTAGCG
IFN-γ reverse	GCTTTTCGAAGTCATCTCG
IL-17 forward	CCAGGATGCCCAAATTCTGAGGAC
IL-17 reverse	CAAGGTGAGGTGGATCGGTTGTAG
RORC forward	AGAAGGACAGGGAGCCAAGG
RORC reverse	GTGATAACCCCGTAGTGGATC
T-bet forward	TCAGGGAAAGGACTCACCTG
T-bet reverse	AATAGCCTCCCCCATTCAAA

We noticed that CCR2+CCR5+ T cells consist of CCR6+ and CCR6− subset (Supplemental Fig. 1). Because Th17 cells appear to be enriched in CCR6+ T cells, we were interested to know whether CCR2+CCR5+ T cells could be functionally divided based on the expression of CCR6. When we compared the frequency of CCR2+CCR5+CCR6+ and CCR2+CCR5+CCR6− T cells between PB and CSF, the frequency of CCR6− subset was much higher in the CSF of MS in relapse than in PB ($p < 0.0005$), whereas the CCR6+ subset was not (Fig. 6A). Further analyses revealed that expression levels of IFN-γ in the CCR6− subset were higher than those in the CCR6+ subset, as assessed by RT-PCR (Fig. 6B). In contrast, the CCR6+ subset expressed much

CCR2+CCR5+CCR6− subset producing IFN-γ and MMP-9 is selectively enriched in the CSF in MS

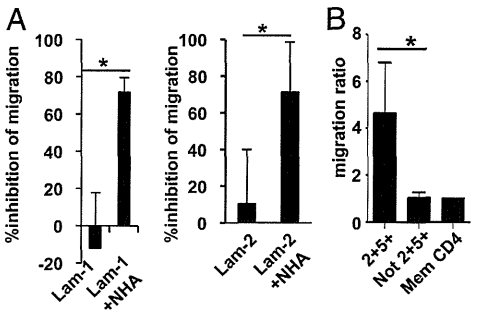

FIGURE 5. T cell migration across an in vitro glia limitans model. (**A**) The upper sides of Transwell membrane inserts were coated with laminin-1 (Lam-1; *left panel*) or laminin-2 (Lam-2; *right panel*), and NHA were seeded on the lower sides of the membrane inserts, as described in *Materials and Methods*. Unfractionated T cells isolated from PBMC were stimulated with PMA and ionomycin for 18 h and seeded onto the upper chambers. Eight hours later, absolute numbers of migrated cells were counted by flow cytometry. Data shown are the percentage inhibition of the migration, calculated as follows: [(migrated cell number through uncoated membrane) − (migrated cell number through membrane coated with laminin alone or laminin and NHA)] × 100/(migrated cell number through uncoated membrane). Data represent mean ± SD of four independent experiments. (**B**) PBMC from HS were sorted into memory $CD4^+CCR2^+CCR5^+$ T cells (2+5+), memory $CD4^+$ T cells depleted of $CCR2^+CCR5^+$ T cells (Not 2+5+), and unfractionated memory $CD4^+$ T cells (Mem CD4) by flow cytometry. The cells were stimulated with plate-bound anti-CD3/CD28 mAb for 60 h and seeded onto the upper chambers, whose membranes were coated with laminin-2 and NHA, as described in (A). Eight hours later, absolute numbers of migrated cells were counted by flow cytometry. To normalize individual variance, data are expressed as migration ratio of the number of migrated cells/number of migrated unfractionated memory $CD4^+$ T cells. Data represent mean ± SD of four independent experiments. $^*p < 0.05$.

higher levels of IL-17 and RORC compared with the $CCR6^-$ subset. We also measured expression levels of MMP-9 mRNA; a much higher level of MMP-9 mRNA was found in the $CCR6^-$ subset (individual relative expression level from two samples = 1.0257 and 0.1127306) compared with the $CCR6^+$ subset (individual relative expression level = 0.0185 and 0.00345). Taken together, we postulate that $CCR2^+CCR5^+CCR6^-$ T cells producing IFN-γ, but not $CCR2^+CCR5^+CCR6^+$ T cells, play a crucial role in triggering the relapse of MS and expand in the CSF during relapse.

Discussion

Chemokines are a family of small chemotactic cytokines, which is a key to understanding the immune homeostasis, self-defense, and inflammation. Interactions between chemokines and their receptors are crucial for the migration of lymphocyte populations, such as T cells, macrophages, dendritic cells, and neutrophils, in autoimmune diseases, allergy, and cancer (37). Although chemokine receptor expression by the CSF lymphocytes or by brain-infiltrating T cells has been repeatedly investigated with regard to the pathogenesis of MS (38), most of the previous studies did not analyze the proportional changes of T cells simultaneously expressing more than two chemokine receptors. We showed that memory $CD4^+$ T cells expressing both CCR2 and CCR5 are selectively enriched in the CSF in MS during relapse but not in NIND or OIND.

Both CCR2 and CCR5 belong to the CC family of chemokines, which have two adjacent cysteines close to their N terminus. CCR2 binds CCL2 (MCP-1), CCL7 (MCP-3), CCL11 (eotaxin), CCL13 (MCP-4), and CCL16 (LEC), whereas CCR5 binds CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL8, CCL11, CCL13, and CCL14. Among these chemokines, CCL5 was increased in the CSF in MS during acute relapses (39), and overexpression of CCL3 was detected in the brain tissues from MS (40). In contrast, CCL2 was decreased in the CSF in MS during relapses (38, 41), and this could be the result of consumption by $CCR2^+$ cells (42). Moreover, the presence of CCL2 and CCL5 was recently demonstrated in endothelial cells in brain samples from MS (43). CCL2 and CCL5 appear to play a critical role in adhesions of the encephalitogenic T cells to brain endothelial cells in a model of EAE (44). More recently, CCL2–CCR2 pairs were shown to play a critical role in the transendothelial migration of effector $CD4^+$ T cells (45), suggesting the importance of CCR2 expression for BBB transmigration. Taking these into consideration, we postulate that the chemokine gradient would facilitate the adherence of $CCR2^+CCR5^+$ T cells to the endothelial cells, as well as T cell entry into the CNS parenchyma during relapses of MS. Interestingly, $CCR2^-CCR5^+$ T cells, which produce a large quantity of IFN-γ, were also enriched in the CSF in MS. However, it was not specific for MS but was also present in the patients with NIND (Fig. 1C), indicating that only $CCR2^+CCR5^+$ T cells are specifically involved in the autoimmune pathology of MS. We subsequently found that the $CCR2^+CCR5^+$ T cells have an exceptional ability to produce MMP-9, an enzyme that is capable of disrupting the glia limitans, which led us to speculate that they have the

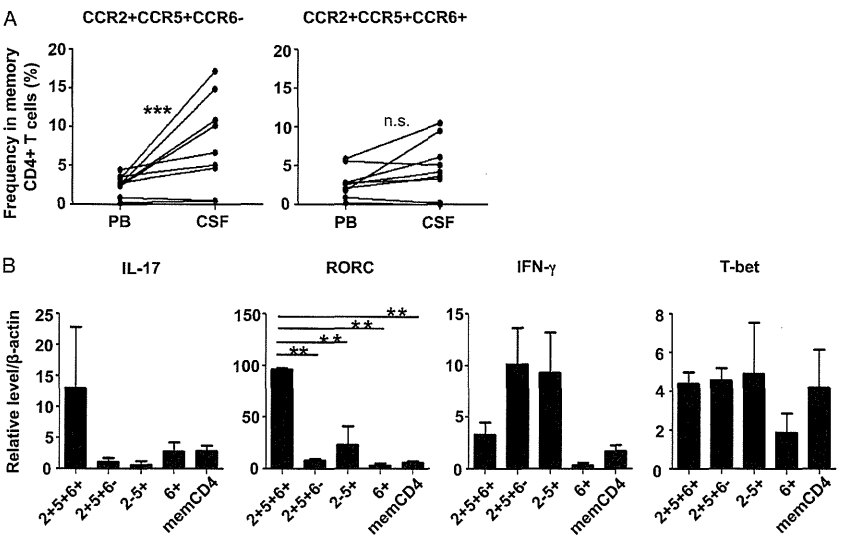

FIGURE 6. $CCR2^+CCR5^+CCR6^-$ T cells, but not $CCR2^+CCR5^+CCR6^+$ T cells, were enriched in the CSF of MS in relapse. (**A**) Cells were stained, as described in Fig. 1C, and frequencies of $CCR2^+CCR5^+CCR6^-$ and $CCR2^+CCR5^+CCR6^-$ T cells in the CSF and PB from nine MS patients in relapse were examined. Lines connect data from paired CSF and PB samples from the same patients. (**B**) Each memory $CD4^+$ T cell subset was isolated from the PBMC of HS and stimulated with PMA and ionomycin for 18 h. Expression levels of IL-17, IFN-γ, RORC, and T-bet mRNA were determined by quantitative RT-PCR. Data represent mean ± SD of three independent experiments. $^{**}p < 0.005$, $^{***}p < 0.0005$. n.s., Not significant.

potential to destroy the integrity of the BBB and trigger the cascade of inflammatory pathology. The MMP-9–producing CCR2+ CCR5+ T cells were indeed superior to the other T cells in their ability to cross the in vitro model of glia limitans layered by laminin-1 or -2.

MMP-9 appears to play a major role in EAE by disrupting the glia limitans, and a specific substrate of MMP-9 was shown to be dystroglycan, anchoring astrocyte end feet to parenchymal basement membrane via interaction with laminin-1 and -2 (36). Laminin-1 and -2 constitute the major laminin isoforms present in the CNS parenchymal basal lamina (35). Taken together, we postulate that the distinguished ability to produce MMP-9 would license the CCR2+CCR5+ T cells to serve as early invaders into the CNS parenchyma during relapses of MS. The CCR2+CCR5+ T cells also produce a large amount of OPN, an integrin-binding protein abundantly expressed in active MS lesions (33). OPN is a pleiotropic protein that interacts with various integrins. In addition to its function as an adhesion molecule, OPN promotes the survival of activated T cells and the production of proinflammatory cytokines by APC (46). It is very likely that paracrine OPN produced by the CCR2+CCR5+ T cells would promote the survival of these MMP-9–producing T cells in the CNS, which leads to further enrichment of the CCR2+CCR5+ T cells in the CSF.

Seeing the specific increase in the CCR2+CCR5+ T cells in the CSF in MS, we were very curious to know whether this T cell population is enriched with autoimmune T cells critical for the initiation of MS pathology. By stimulating the PB CCR2+CCR5+ T cells with MBP, we showed that, in patients with MS relapse, this T cell subset produces a large quantity of IFN-γ and some IL-17 in response to MBP, a representative autoantigen for MS (Fig. 3C). In contrast, the cells from MS in remission or from healthy controls did not respond significantly. Although we did not examine the CSF T cells' response to MBP because of a technical difficulty, it is likely that the CCR2+CCR5+ T cells in the CSF in MS during relapse are enriched with MBP-reactive autoimmune T cells as well.

Of note, Zhang et al. (47) recently reported that CCR2+CCR5+ cells are highly differentiated, yet stable, effector memory CD4+ T cells equipped for provoking rapid recall response. They showed evidence that CCR2+CCR5+ T cells should have undergone reactivation and subsequent proliferation more often than other memory T cell subsets and are resistant to apoptosis. Thus, it is likely that autoreactive T cells are enriched in CCR2+CCR5+ T cells that have survived following repeated reactivation over a long period of time. We assume that once autoreactive T cells differentiate into stable effector memory T cells expressing CCR2 and CCR5, they might persist and trigger relapse repeatedly. We further revealed that the CCR6−, but not the CCR6+, subset of CCR2+CCR5+ T cells was significantly enriched in the CSF of MS patients during relapse.

Reboldi et al. (48) reported that CCR6+ T cells are more enriched in CSF than in the PB of clinically isolated syndrome (CIS). Diagnosis of CIS can be made when patients developed a single attack of neurologic disability that is consistent with demyelinating pathology and that may turn out to be the first episode of MS (49). However, in our Japanese patients having clinically definite MS, we did not detect enrichment of CCR6+ T cells in the CSF. Rather, T cells bearing Th17 phenotypes appeared to be prohibited from entry into the CNS in MS. The difference between the results in CIS and MS could be explained by the premise that autoimmune pathology may be premature at the CIS stage. In contrast, the increase in CCR2+CCR5+ T cells in the CSF was not detected in the patients who had MS for >10 y. These observations are in accordance with the postulate that acquired and innate immune components, as well as neurodegenerative components, differentially contribute to the different stages of MS (50).

In summary, we identified a unique CCR2+CCR5+CCR6− T cell population that is enriched in the CSF of patients with exacerbated MS. Our data suggest that targeting this population may be a novel therapeutic approach for MS.

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Disclosures

The authors have no financial conflicts of interest.

References

1. McFarland, H. F., and R. Martin. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nat. Immunol.* 8: 913–919.
2. Trapp, B. D., and K. A. Nave. 2008. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu. Rev. Neurosci.* 31: 247–269.
3. Wucherpfennig, K. W., and J. L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80: 695–705.
4. Hur, E. M., S. Youssef, M. E. Haws, S. Y. Zhang, R. A. Sobel, and L. Steinman. 2007. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat. Immunol.* 8: 74–83.
5. Ransohoff, R. M., P. Kivisäkk, and G. Kidd. 2003. Three or more routes for leukocyte migration into the central nervous system. *Nat. Rev. Immunol.* 3: 569–581.
6. Bartholomäus, I., N. Kawakami, F. Odoardi, C. Schläger, D. Miljkovic, J. W. Ellwart, W. E. Klinkert, C. Flügel-Koch, T. B. Issekutz, H. Wekerle, and A. Flügel. 2009. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* 462: 94–98.
7. Gijbels, K., R. E. Galardy, and L. Steinman. 1994. Reversal of experimental autoimmune encephalomyelitis with a hydroxamate inhibitor of matrix metalloproteases. *J. Clin. Invest.* 94: 2177–2182.
8. Engelhardt, B. 2010. T cell migration into the central nervous system during health and disease: Different molecular keys allow access to different central nervous system compartments. *Clin. Exp. Neuroimmunol.* 1: 79–93.
9. Steinman, L. 2009. Shifting therapeutic attention in MS to osteopontin, type 1 and type 2 IFN. *Eur. J. Immunol.* 39: 2358–2360.
10. Aranami, T., and T. Yamamura. 2008. Th17 Cells and autoimmune encephalomyelitis (EAE/MS). *Allergol. Int.* 57: 115–120.
11. Tzartos, J. S., M. A. Friese, M. J. Craner, J. Palace, J. Newcombe, M. M. Esiri, and L. Fugger. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am. J. Pathol.* 172: 146–155.
12. Kebir, H., I. Ifergan, J. I. Alvarez, M. Bernard, J. Poirier, N. Arbour, P. Duquette, and A. Prat. 2009. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann. Neurol.* 66: 390–402.
13. Panitch, H. S., R. L. Hirsch, A. S. Haley, and K. P. Johnson. 1987. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet* 1: 893–895.
14. Broux, B., K. Pannemans, X. Zhang, S. Markovic-Plese, T. Broekmans, B. O. Eijnde, B. Van Wijmeersch, V. Somers, P. Geusens, S. van der Pol, et al. 2012. CX(3)CR1 drives cytotoxic CD4(+)CD28(−) T cells into the brain of multiple sclerosis patients. *J. Autoimmun.* 38: 10–19.
15. Saxena, A., G. Martin-Blondel, L. T. Mars, and R. S. Liblau. 2011. Role of CD8 T cell subsets in the pathogenesis of multiple sclerosis. *FEBS Lett.* 585: 3758–3763.
16. Segal, B. M., C. S. Constantinescu, A. Raychaudhuri, L. Kim, R. Fidelus-Gort, and L. H. Kasper; Ustekinumab MS Investigators. 2008. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol.* 7: 796–804.
17. Mackay, C. R. 2001. Chemokines: immunology's high impact factors. *Nat. Immunol.* 2: 95–101.
18. Proudfoot, A. E. 2002. Chemokine receptors: multifaceted therapeutic targets. *Nat. Rev. Immunol.* 2: 106–115.
19. Izikson, L., R. S. Klein, I. F. Charo, H. L. Weiner, and A. D. Luster. 2000. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J. Exp. Med.* 192: 1075–1080.
20. Sallusto, F., and A. Lanzavecchia. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* 177: 134–140.
21. Nagata, K., K. Tanaka, K. Ogawa, K. Kemmotsu, T. Imai, O. Yoshie, H. Abe, K. Tada, M. Nakamura, K. Sugamura, and S. Takano. 1999. Selective expression

of a novel surface molecule by human Th2 cells in vivo. *J. Immunol.* 162: 1278–1286.
22. Ransohoff, R. M. 2009. Chemokines and chemokine receptors: standing at the crossroads of immunobiology and neurobiology. *Immunity* 31: 711–721.
23. Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8: 639–646.
24. Sato, W., T. Aranami, and T. Yamamura. 2007. Cutting edge: Human Th17 cells are identified as bearing CCR2+CCR5− phenotype. *J. Immunol.* 178: 7525–7529.
25. Singh, S. P., H. H. Zhang, J. F. Foley, M. N. Hedrick, and J. M. Farber. 2008. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J. Immunol.* 180: 214–221.
26. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, et al. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50: 121–127.
27. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.
28. Leppert, D., J. Ford, G. Stabler, C. Grygar, C. Lienert, S. Huber, K. M. Miller, S. L. Hauser, and L. Kappos. 1998. Matrix metalloproteinase-9 (gelatinase B) is selectively elevated in CSF during relapses and stable phases of multiple sclerosis. *Brain* 121: 2327–2334.
29. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745–763.
30. Toft-Hansen, H., R. Buist, X. J. Sun, A. Schellenberg, J. Peeling, and T. Owens. 2006. Metalloproteinases control brain inflammation induced by pertussis toxin in mice overexpressing the chemokine CCL2 in the central nervous system. *J. Immunol.* 177: 7242–7249.
31. Stüve, O., N. P. Dooley, J. H. Uhm, J. P. Antel, G. S. Francis, G. Williams, and V. W. Yong. 1996. Interferon beta-1b decreases the migration of T lymphocytes in vitro: effects on matrix metalloproteinase-9. *Ann. Neurol.* 40: 853–863.
32. Bar-Or, A., R. K. Nuttall, M. Duddy, A. Alter, H. J. Kim, I. Ifergan, C. J. Pennington, P. Bourgoin, D. R. Edwards, and V. W. Yong. 2003. Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis. *Brain* 126: 2738–2749.
33. Chabas, D., S. E. Baranzini, D. Mitchell, C. C. Bernard, S. R. Rittling, D. T. Denhardt, R. A. Sobel, C. Lock, M. Karpuj, R. Pedotti, et al. 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* 294: 1731–1735.
34. Shinohara, M. L., M. Jansson, E. S. Hwang, M. B. Werneck, L. H. Glimcher, and H. Cantor. 2005. T-bet-dependent expression of osteopontin contributes to T cell polarization. *Proc. Natl. Acad. Sci. USA* 102: 17101–17106.
35. Sixt, M., B. Engelhardt, F. Pausch, R. Hallmann, O. Wendler, and L. M. Sorokin. 2001. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. *J. Cell Biol.* 153: 933–946.
36. Agrawal, S., P. Anderson, M. Durbeej, N. van Rooijen, F. Ivars, G. Opdenakker, and L. M. Sorokin. 2006. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J. Exp. Med.* 203: 1007–1019.
37. Moser, B., and P. Loetscher. 2001. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2: 123–128.
38. Sørensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, et al. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest.* 103: 807–815.
39. Kivisäkk, P., C. Trebst, Z. Liu, B. H. Tucky, T. L. Sørensen, R. A. Rudick, M. Mack, and R. M. Ransohoff. 2002. T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking. *Clin. Exp. Immunol.* 129: 510–518.
40. Balashov, K. E., J. B. Rottman, H. L. Weiner, and W. W. Hancock. 1999. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. *Proc. Natl. Acad. Sci. USA* 96: 6873–6878.
41. Franciotta, D., G. Martino, E. Zardini, R. Furlan, R. Bergamaschi, L. Andreoni, and V. Cosi. 2001. Serum and CSF levels of MCP-1 and IP-10 in multiple sclerosis patients with acute and stable disease and undergoing immunomodulatory therapies. *J. Neuroimmunol.* 115: 192–198.
42. Mahad, D., M. K. Callahan, K. A. Williams, E. E. Ubogu, P. Kivisäkk, B. Tucky, G. Kidd, G. A. Kingsbury, A. Chang, R. J. Fox, et al. 2006. Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. *Brain* 129: 212–223.
43. Subileau, E. A., P. Rezaie, H. A. Davies, F. M. Colyer, J. Greenwood, D. K. Male, and I. A. Romero. 2009. Expression of chemokines and their receptors by human brain endothelium: implications for multiple sclerosis. *J. Neuropathol. Exp. Neurol.* 68: 227–240.
44. dos Santos, A. C., M. M. Barsante, R. M. Arantes, C. C. Bernard, M. M. Teixeira, and J. Carvalho-Tavares. 2005. CCL2 and CCL5 mediate leukocyte adhesion in experimental autoimmune encephalomyelitis—an intravital microscopy study. *J. Neuroimmunol.* 162: 122–129.
45. Shulman, Z., S. J. Cohen, B. Roediger, V. Kalchenko, R. Jain, V. Grabovsky, E. Klein, V. Shinder, L. Stoler-Barak, S. W. Feigelson, et al. 2012. Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots. *Nat. Immunol.* 13: 67–76.
46. Denhardt, D. T., M. Noda, A. W. O'Regan, D. Pavlin, and J. S. Berman. 2001. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J. Clin. Invest.* 107: 1055–1061.
47. Zhang, H. H., K. Song, R. L. Rabin, B. J. Hill, S. P. Perfetto, M. Roederer, D. C. Douek, R. M. Siegel, and J. M. Farber. 2010. CCR2 identifies a stable population of human effector memory CD4+ T cells equipped for rapid recall response. *J. Immunol.* 185: 6646–6663.
48. Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat. Immunol.* 10: 514–523.
49. Miller, D., F. Barkhof, X. Montalban, A. Thompson, and M. Filippi. 2005. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *Lancet Neurol.* 4: 281–288.
50. Weiner, H. L. 2008. A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis. *J. Neurol.* 255(Suppl. 1): 3–11.

□ CASE REPORT □

New-Onset Type 1 Diabetes Mellitus and Anti-Aquaporin-4 Antibody Positive Optic Neuritis Associated with Type 1 Interferon Therapy for Chronic Hepatitis C

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Abstract

A 60-year-old woman developed type 1 diabetes mellitus and anti-aquaporin-4 antibody positive optic neuritis during type 1 interferon therapies for chronic hepatitis C. The diabetes mellitus was elicited by interferon-α plus ribavirin therapy, while the optic neuritis was induced after interferon-β treatment, followed by interferon-α and ribavirin therapy. It is possible that type 1 interferons lead to the onset of the two autoimmune diseases by inducing disease-specific autoantibodies. Autoimmune disease is an infrequent complication of type 1 interferon treatment; however, once it has occurred, it may result in severe impairments. Patients undergoing type 1 interferon therapy should therefore be carefully monitored for any manifestations of autoimmune diseases.

Key words: aquaporin-4, neuromyelitis optica, chronic hepatitis C, type 1 diabetes mellitus, type 1 interferon

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Introduction

Type 1 interferon (IFN) is widely used to treat patients with chronic viral hepatitis and malignant neoplasms. Approximately two million people in Japan are infected with the hepatitis C virus (HCV). Combination therapy with type 1 IFN and ribavirin (RBV) is used in 50,000-100,000 patients annually. Since type 1 IFN has not only antiviral and antiproliferative effects, but also immunomodulatory effects, it can occasionally induce various autoimmune diseases (1). The onset of autoimmune diseases can be attributed to the overproduction of disease-specific antibodies. We herein present the case of a patient who developed type 1 diabetes mellitus (T1DM) and severe optic neuritis with anti-aquaporin-4 (AQP-4) antibodies during treatment with combinations including IFN-α and IFN-β for chronic hepatitis C.

Case Report

A 60-year-old Japanese woman was diagnosed with hepatitis C (type 1b) in 1994 at the age of 42 years. Since the diagnosis, she had received various types of IFN therapy: natural IFN-α, recombinant IFN-α-2b and RBV, recombinant IFN-αcon-1, and pegylated IFN (PEG-IFN)-α-2b and RBV (Fig. 1). From 1994 to 2008, all of the above-mentioned IFN therapies resulted in a transient reduction in HCV-RNA to undetectable levels, but a sustained virologic response (SVR) was not obtained. While undergoing PEG-IFN/RBV treatment, the patient was noted to have hyperglycemia, and she was diagnosed with T1DM in 2008 (Fig. 1). She was found to be positive for anti-glutamic acid decarboxylase (GAD) antibodies, with a titer of 3,440x. The titers of anti-GAD antibodies were decreased to 128x two years after the initiation of insulin treatment.

In January 2009, the patient underwent combination therapy for virus eradication by double-filtration plasmapheresis

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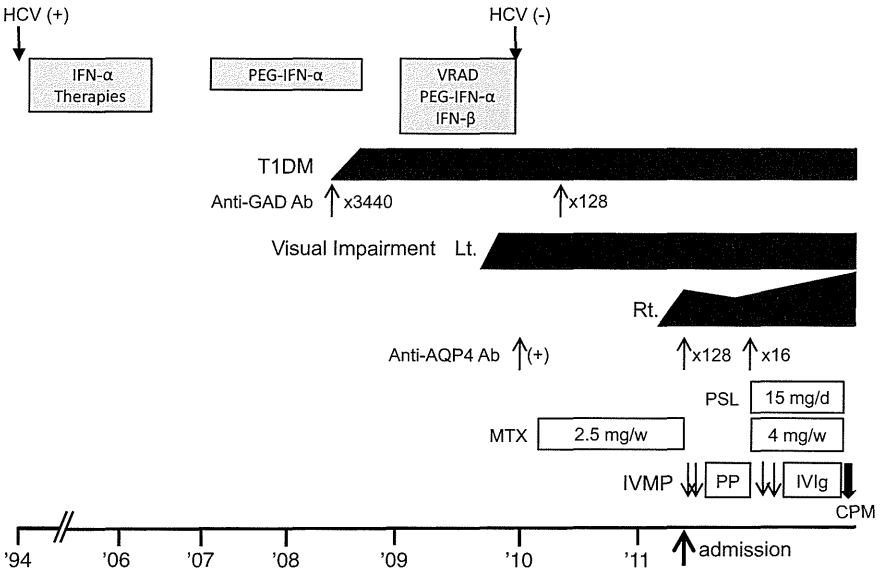

Figure 1. The clinical course of the present case. Anti-AQP-4 Ab: anti-aquaporin-4 antibody, Anti-GAD antibody: Anti-glutamic acid decarboxylase antibody, CPM: cyclophosphamide, HCV: hepatitis C virus, IFN-a therapies: IFN-α for 24 weeks from 1994 to 1995, IFN-α-2b/Ribavirin (RBV) for 24 weeks in 2002, IFN-αcon-1 for 12 weeks in 2004, and PEG-IFN-α-2b/RBV for 48 weeks from 2005 to 2006, IVIg: intravenous immunoglobulin, IVMP: intravenous methylprednisolone, MTX: methotrexate, PEG-IFN: pegylated IFN-α-2b and RBV, PP: plasmapheresis, PSL: prednisolone, T1DM: type 1 diabetes mellitus, VRAD: virus removal and eradication by double-filtration plasmapheresis (DFPP)

(VRAD), intravenous natural IFN-β for 14 days, and PEG-IFN-α-2a plus RBV for 36 weeks to achieve HCV-RNA seronegativity. A SVR was finally achieved with these intensive combination therapies (Fig. 1).

In November 2009, the patient experienced pain when moving her left eye. Her left visual acuity deteriorated to light perception within two weeks. She was diagnosed with left optic neuritis. The IFN therapy was terminated, and triamcinolone was injected locally into the subtenon of the affected side, which was not effective. Serological tests demonstrated that she was positive for AQP-4 antibodies in January 2010, and hence a clinical diagnosis of neuromyelitis optica spectrum disorder (NMOsd) was made (Fig. 1).

To prevent relapse and progression of the optic neuritis, immunosuppressant drug therapy was initiated, with weekly oral methotrexate (MTX) administration at a dose of 2.5 mg. In June 2011, right optic neuritis occurred and the right visual acuity was decreased from normal to finger counting within two weeks. She received two courses of high-dose intravenous methylprednisolone (IVMP) therapy, which were not effective. She was admitted to our hospital for further treatment (Fig. 1).

On admission, her neurological findings were normal, except for the severe visual impairment of 0.02 (20/1,000) in both eyes. The visual field defects were detected by Goldmann perimetry (Fig. 2A). Ophthalmoscopy showed no impairment of the retinal blood vessels. The visual evoked potential indicated no response. The cerebrospinal fluid was normal, with a cell count of less than 1/μL with all mononuclear cells, and a protein concentration of 37 mg/dL. Oligoclonal banding was negative, and the myelin basic protein level was within the normal range. The serum blood sugar level was 196 mg/dL (normal range 70-110), glycosylated hemoglobin was 6.7% (normal range 4.3-5.8), and the anti-GAD antibodies were detected with a value of 9.9 U/mL. The patient's serum was also found to be positive for anti-AQP-4 antibodies, with a titer of 128x. Anti-nuclear antibodies, anti-SS-A/SS-B antibodies, anti-neutrophil cytoplasmic antibodies, and anti-thyroid antibodies were not detected.

Magnetic resonance imaging (MRI) showed a high signal intensity of the left optic nerve on T2-weighted and fluid-attenuated inversion recovery, and T1-weighted imaging with contrast enhancement, whereas the right optic nerve showed no particular findings (Fig. 2B, C). Brain MRI (Fig. 2D, E) showed a small number of high-intensity spots in the cerebral white matter. No obvious abnormality was observed in the spinal cord MRI.

The patient was treated with eight courses of plasmapheresis. During the treatment, her visual acuity slightly improved and she could read a few written characters. The titer of the anti-AQP-4 antibodies was decreased to 16x. However, the patient's visual field defect gradually worsened again soon after the discontinuation of plasmapheresis, so we initiated two courses of IVMP therapy, an additional two courses of plasmapheresis, high-dose intravenous immu-

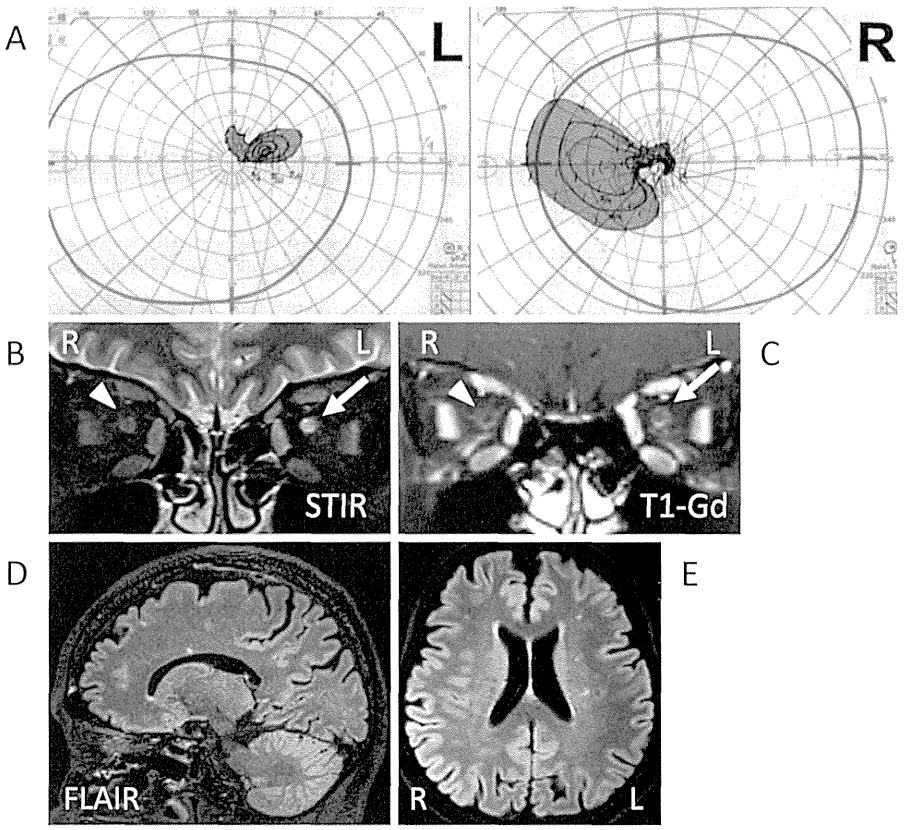

Figure 2. **The visual impairment and the magnetic resonance images of the present case. The Goldmann visual fields on admission are highlighted (A). The left optic nerve showed high signal intensity on the STIR coronal image (white arrow, B) with marginal contrast enhancement (white arrow, C). The right optic nerve showed no remarkable findings (arrow head, B and C). The brain showed no particular findings except for the optic nerve on FLAIR saggital (D) and axial (E) images. FLAIR: fluid-attenuated inversion recovery, STIR: short inversion time inversion-recovery, T1-Gd: gadolinium enhanced T1**

noglobulin (400 mg/kg/day for five days), and high-dose cyclophosphamide (CPM) (500 mg/day for one day). The exacerbation of the visual impairment was halted by this treatment. In September 2011, the patient was discharged from our hospital with a plan to undergo monthly CPM therapy.

Discussion

The present patient developed T1DM during IFN-α therapy and anti-AQP4 antibody positive optic neuritis after IFN-β, followed by IFN-α, therapy. Her severe visual impairment persisted despite the use of intensive immunotherapy. Several reasons for the intractable disease course can be proposed. For example, the type 1 IFNs or HCV infection may have served as a potent activator of autoimmunity, or the involvement of vasculitis as an extrahepatic manifestation of HCV infection (2) could lead to the clinical deterioration.

The first case of T1DM development during IFN-α therapy for chronic hepatitis C was reported in 1992 (3). New-onset DM among IFN-treated patients has been documented to occur in 0.7% of patients in Japan (4). The mechanism underlying immune-mediated pancreatic β-cell destruction can be attributed to genetic and environmental causes thus leading to the generation of islet cell autoantibodies, i.e., anti-GAD autoantibodies. IFN-α may act as an initiator of the autoimmunity directed against β cells, thus leading to the pathogenesis of T1DM. Likewise, IFN-α can be considered to play a critical role in the pathogenesis of systemic lupus erythematosus.

To date, ten cases of new-onset optic neuritis, multiple sclerosis (MS), MS-like disease, or NMOsd associated with IFN-α therapy for chronic viral hepatitis or malignant neoplasms, have been reported (5-11). There were two cases with seropositivity for anti-AQP4 antibodies (Table); one patient with optic-spinal MS (OSMS) after IFN-α2b and RBV (10), and another patient with NMOsd after PEG-IFN-α and RBV (11). In the remaining eight cases, the presence of anti-AQP-4 antibodies was not examined because they had been reported before the discovery of NMO-Immunoglobulin G (IgG) and anti-AQP-4 (12) antibodies.

IFN-β therapy can also play a role as an initiator of autoimmune diseases involving the central nervous system. A case with new-onset optic neuritis after IFN-β therapy for

Table. The Reported Cases of Newly-onset Anti-AQP-4 Antibody Positive OSMS, and NMOsd Provoked by Type 1 IFN Therapy

Patient age, sex	Disease	IFN	ON	SC	B	AQP4-Ab	Duration	References
47, F	Hepatitis C	α-2b/RBV	+	+	+	+	1Y	Kajiyama, et al. 2007[10]
65, F	Hepatitis C	α/RBV	+	-	+	+	2Y10M	Yamasaki, et al. 2012[11]
60, F	Hepatitis C	α, β, α/RBV	+	-	-	+	α: 15Y β: 9M	Present Case 2012

RBV: ribavirin, ON: optic neuritis, SC: spinal cord lesion, B: brain lesion. Duration: duration between the initiation of type 1 IFN therapy and the onset of OSMS or NMOsd

kidney cancer has been reported (13). In addition, a number of exacerbated cases of relapsing-remitting MS (RRMS) have been reported in Japan in patients receiving IFN-β (14). Differentiating between NMO and MS can be achieved based on seropositivity for the anti-AQP-4 antibodies, longitudinally extensive spinal cord lesions, and brain MRI findings not meeting the diagnostic criteria for MS (15). However, before the discovery of this autoantibody, it was difficult to distinguish NMO from MS, especially OSMS, which is common in Asian countries. In 2000, IFN-β therapy was approved in Japan for the prevention of relapse and progression of RRMS, in which patients with OSMS were also included. Consequently, exacerbation of the disease or ineffectiveness of IFN-β was reported among patients with OSMS who underwent IFN-β therapy (14, 16). These cases were later found to be positive for anti-AQP-4 antibodies. Recent articles described that IFN-β treatment was not effective in preventing relapses in NMO patients (17, 18), while strictly defined OSMS showed a response to IFN-β treatment in terms of the prevention of relapses and functional worsening (19).

The mechanism underlying the onset and exacerbation of NMO/NMOsd has not been well understood, but the induction of B-cell activation factors of the tumor necrosis factor (TNF) family by IFN-β is considered to facilitate the production of anti-AQP-4 antibodies (20). For example, Chihara et al. have shown that IL-6-dependent B-cell subpopulations of plasmablasts are involved in the production of anti-AQP-4 antibodies (21). Loss of AQP-4, mediated by immunoglobulins and complements, has been shown in inflammatory lesions of patients with NMO (22). These results indicate that the anti-AQP-4 antibody plays a crucial role in the pathogenesis of NMO, unlike in cases of MS. As another mechanism underlying the development of type 1 IFN-induced NMO/NMOsd, it has been suggested that IFN-β treatment leads to the overproduction of IL-17 from T helper 17 (Th17) cells (23), which is thought to be associated with the pathological feature of NMO.

Type 1 IFN has reciprocal characteristics, with both pathogenic and protective roles in autoimmunity. In general, IFN-β exerts its therapeutic effect on MS by producing anti-inflammatory cytokines and suppressing the proliferation of autoreactive T cells. Both IFN-α and IFN-β bind to a single heterodimeric receptor composed of IFNAR1 and IFNAR2, which can cause similar immunomodulatory effects (24). Hence, it is likely that IFN-α has a similar effect on autoimmunity as does IFN-β, as indicated by the fact that IFN-α has also been developed as a candidate therapeutic agent for MS (25).

Type 1 IFNs served as pathogenic mediators in the present case, inducing T1DM and NMO/NMOsd. Since various types of IFN-α treatment had been carried out intermittently for more than ten years after the onset of chronic hepatitis C, the onset of T1DM was clearly influenced by IFN-α treatment. However, it remains unclear which type of IFN was involved in the induction of NMOsd. We speculate that the combination therapy with IFN-α and IFN-β may have produced synergistic effects to trigger NMOsd in the present case.

The authors state that they have no Conflict of Interest (COI).

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Contributor TK, MA, and MW undertook the clinical management of the patient. MW referred the patient to NCNP and performed the ophthalmological examination. Each of the authors was significantly involved in clinical assessments of the patient.

TK and MA equally contributed to this work.

References

1. Burdick LM, Somani N, Somani AK. Type I IFNs and their role in the development of autoimmune diseases. Expert Opin Drug Saf **8**: 459-472, 2009.
2. Cacoub P, Poynard T, Ghillani P, et al. Extrahepatic manifestations of chronic hepatitis C. Arthritis Rheum **42**: 2204-2212, 1999.
3. Fabris P, Betterle C, Floreani A, et al. Development of type 1 dia-

betes mellitus during interferon alfa therapy for chronic HCV hepatitis. Lancet **340**: 548, 1992.
4. Okanoue T, Sakamoto S, Itoh Y, et al. Side effects of high-dose interferon therapy for chronic hepatitis C. J Hepatol **25**: 283-291, 1996.
5. Manesis EK, Petrou C, Brouzas D, Hadziyannis S. Optic tract neuropathy complicating low-dose interferon treatment. J Hepatol **21**: 474-477, 1994.
6. Isler M, Akhan G, Bardak Y, Akkaya A. Dry cough and optic neuritis: two rare complications of interferon alpha treatment in chronic viral hepatitis. Am J Gastroenterol **96**: 1303-1304, 2001.
7. Matsuo T, Takabatake R. Multiple sclerosis-like disease secondary to alpha interferon. Ocul Immunol Inflamm **10**: 299-304, 2002.
8. Kataoka I, Shinagawa K, Shiro Y, et al. Multiple sclerosis associated with interferon-alpha therapy for chronic myelogenous leukemia. Am J Hematol **70**: 149-153, 2002.
9. Höftberger R, Garzuly F, Dienes HP, et al. Fulminant central nervous system demyelination associated with interferon-alpha therapy and hepatitis C virus infection. Mult Scler **13**: 1100-1106, 2007.
10. Kajiyama K, Tsuda K, Takeda M, Yoshikawa H, Tanaka K. Multiple sclerosis with positive anti-aquaporin-4 antibody, manifested after interferon-a-2b/ribavirin therapy for chronic hepatitis C. A case report. Shinkeinaika (Neurol Med) **66**: 180-184, 2007 (in Japanese, Abstract in English).
11. Yamasaki M, Matsumoto K, Takahashi Y, Nakanishi H, Kawai Y, Miyamura M. A case of NMO (Neuromyelitis optica) spectrum disorder triggered by interferon alpha, which involved extensively pyramidal tract lesion of the brain. Rinshou Shinkeigaku (Clin Neurol) **52**: 19-24, 2012.
12. Lennon VA, Wingerchuk DM, Kryzer TJ, et al. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet **364**: 2106-2112, 2004.
13. Okuma H, Kawamura Y, Ohnuki Y, Takagi S. Optic neuritis caused by interferon-beta administration. Intern Med **47**: 1759, 2008.
14. Shimizu J, Hatanaka Y, Hasegawa M, et al. IFNβ-1b may severely exacerbate Japanese optic-spinal MS in neuromyelitis optica spectrum. Neurology **75**: 1423-1427, 2010.
15. Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG. Revised diagnostic criteria for neuromyelitis optica. Neurology **66**: 1485-1489, 2006.
16. Matsuoka T, Matsushita T, Kawano Y, et al. Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. Brain **130**: 1206-1223, 2007.
17. Tanaka M, Tanaka K, Komori M. Interferon-beta(1b) treatment in neuromyelitis optica. Eur Neurol **62**: 167-170, 2009.
18. Uzawa A, Mori M, Hayakawa S, Masuda S, Kuwabara S. Different responses to interferon beta-1b treatment in patients with neuromyelitis optica and multiple sclerosis. Eur J Neurol **17**: 672-676, 2010.
19. Shimizu Y, Fujihara K, Kubo S, et al. Therapeutic efficacy of interferon b-1b in Japanese patients with optic-spinal multiple sclerosis. Tohoku J Exp Med **223**: 211-214, 2011.
20. Krumbholz M, Faber H, Steinmeyer F, et al. Interferon-beta increases BAFF levels in multiple sclerosis: implications for B cell autoimmunity. Brain **131**: 1455-1463, 2008.
21. Chihara N, Aranami T, Sato W, et al. Interleukin 6 signaling promotes anti-aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica. Proc Natl Acad Sci U S A **108**: 3701-3706, 2011.
22. Misu T, Fujihara K, Kakita A, et al. Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. Brain **130**: 1224-1234, 2007.
23. Axtell RC, Raman C, Steinman L. Interferon-b exacerbates Th17-mediated inflammatory disease. Trends Immunol **32**: 272-277, 2011.
24. Crow MK. Type 1 interferon in organ-targeted autoimmune and inflammatory diseases. Arthritis Res Ther **12**(Suppl 1): S5-S14, 2010.
25. Knobler RL, Panitch HS, Braheny SL, et al. Systemic alpha-interferon therapy of multiple sclerosis. Neurology **34**: 1273-1279, 1984.

B-cell-directed therapy: which B cells should be targeted and how?

"...it is arguable that CD20⁻ B cells may produce pathogenic autoantibodies or play other critical roles in pathogenesis."

KEYWORDS: anti-CD19 ▪ anti-CD20 ▪ neuromyelitis optica ▪ plasmablast ▪ rituximab

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As B-lineage cells and antibodies are thought to play an active role in autoimmune pathogenesis, B-cell populations are an attractive target of therapy in the combat against intractable human autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus and multiple sclerosis (MS). In fact, the B-cell-depleting antibody rituximab has been proven efficacious for RA [1], vasculitis [1,2], idiopathic thrombocytopenic purpura [3], MS [4] and neuromyelitis optica (NMO) [5]. Rituximab is a chimeric monoclonal antibody specific for the B-cell surface antigen CD20, and is able to deplete mature B cells *in vivo* through ADCC. By contrast, its therapeutic effects have not yet been proven in lupus glomerulonephritis, a condition associated with an increase of anti-DNA antibodies and a reduction in circulating complement. Although the anti-CD20 therapy greatly reduced levels of anti-DNA antibodies [6], no significant clinical benefit was seen in the patients treated with rituximab. The complexity in the outcome of B-cell-directed therapy is not fully understood yet; however, emerging results offer interesting ideas regarding the pathogenesis of disease and possibilities for alternative options of therapy.

If rituximab fails to control a certain autoimmune disease condition despite its ability to deplete B cells, it is arguable that CD20⁻ B cells may produce pathogenic autoantibodies or play other critical roles in pathogenesis. In this regard, which B-cell populations rituximab depletes needs to be verified further. B-cell populations highly expressing the CD20 molecule include mature B cells and memory B cells. By contrast, either B cells at very early stage prior to antigen receptor selection (pre-B cells) or terminally differentiated antibody-producing cells (long-lived plasma cells) do not express CD20 on their surface. As such, an anti-CD20 antibody could not wipe out the antibody-producing cells from the repertoire. An antibody against another B-cell marker, CD19, appears to remove a broader range of B-cell populations, indicating that anti-CD19 antibody therapy may be more effective in some diseases [7]. However, the anti-CD19 antibody does not deplete plasma cells in the bone marrow. Immature plasma cells or plasmablasts capable of producing antibodies are CD19⁺ but heterogeneous in the level of CD20 expression. The role of rituximab-resistant, CD20⁻ plasmablasts in autoimmunity is of our current interest.

In the research of antibody-mediated diseases, we have recently seen major breakthroughs in the autoimmune CNS disease NMO [8]. NMO is a severe inflammatory disease characterized by recurrent optic neuritis and episodic myelitis. The clinical picture of NMO sometimes looks very similar to the most common demyelinating disease MS. For example, MS may also develop optic neuritis and myelitis in addition to brain inflammation. Although diagnostic tests to distinguish NMO from MS were not available, Lennon and her colleagues showed in 2004 that autoantibodies directed against the AQP4 water channel protein are increased in the sera of NMO patients and an anti-AQP4 antibody can be regarded as a disease-specific marker in NMO [9]. Subsequent studies showed that the anti-AQP4 antibodies are pathogenic *in vivo*, and would bind to AQP4 expressed by astrocytes in the CNS, leading to complement-dependent destruction of astrocytes.

While acute exacerbations of NMO and MS are responsive to corticosteroid treatment, efficacy of plasma exchange in NMO appears to be more significant than in MS. Long-term treatment with type 1 interferons is effective for MS but not for NMO [10]. Similarly, the anti-α4 integrin antibody natalizumab, an

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approved drug for MS, fails to prevent relapses in NMO [11]. NMO accompanies other autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome more often than MS. Reflecting the lower prevalence of MS in Asia, the ratio of NMO to MS is much higher in Asia than western countries [12].

Recent works have shown that repeated therapy with rituximab effectively reduced relapses of NMO in nine out of ten patients [5]. In this prospective long-term cohort study, two patients developed concomitant infections (e.g., herpes zoster infection), although the safety profile was judged to be acceptable. When additional rituximab was not given before B cells reappeared in the blood, clinical relapses occurred in association with reappearance of B cells. Therefore, B-cell depletion is thought to be a mechanism of rituximab action in NMO. However, it is interesting to note that immunoglobulin isotype levels decreased only in one of the ten patients treated with rituximab, and titers of anti-AQP4 antibodies did not change significantly during the treatment [5], indicating that the anti-CD20 antibody does not efficiently deplete anti-AQP4 antibody-producing cells. Regarding this, experts speculate that rituximab shows efficacy for NMO by deleting $CD20^+$ B cells serving as APCs and/or producing inflammatory cytokines. Other possible mechanisms include inhibition of B-cell–T-cell interactions, increase of Tregs [13] and modulation of the T-cell compartment [14]. A point that cannot be overlooked is that monocyte reactivity to lipopolysaccharide was rather enhanced in patients with NMO treated with rituximab in another study [15]. This proinflammatory effect of the anti-CD20 antibody is not welcome in the clinic owing to its risk of triggering an exacerbation of the disease. If this monocyte activation results from the depletion of IL-10-producing $CD20^+$ regulatory B cells [16], a B-cell-depleting therapy that does not reduce such regulatory B cells should be developed in the future.

We have recently reported that plasmablasts that do not bear CD20 on their surface are significantly increased in the peripheral blood of NMO, particularly during relapse [17]. The $CD20^-$ plasmablasts in the blood of NMO patients displayed $CD19^{int}CD180^-CD27^+CD38^+$ phenotypes and exhibited both morphological and molecular profiles of immature plasma cells. Interestingly, these plasmablasts produced anti-AQP4 antibodies following stimulation *in vitro*, whereas other B cells, including $CD20^+$ memory B cells from NMO, did not. This is consistent with the results that rituximab treatment did not significantly reduce anti-AQP4 antibody titers in NMO [5], and leaves an open question as to whether treatment options targeting the $CD20^-$ plasmablasts are efficacious for NMO.

Among the molecules expressed by plasmablasts, CD19 may be an attractive target considering the availability of anti-CD19 antibodies. However, because the level of CD19 expression on the plasmablasts is relatively weak, depletion of the $CD19^{int}$ plasmablasts by the antibody might be more difficult than that of other $CD19^+$ cells. We are more interested in IL-6 receptor blockade since IL-6 levels are increased in the serum and cerebrospinal fluid of active NMO, and IL-6 was found to enhance production of anti-AQP4 antibodies and promote the survival of plasmablasts. We showed that the anti-IL-6 receptor antibody tocilizumab inhibits anti-AQP4 antibody production and survival of plasmablasts *in vitro*. Tocilizumab, an approved drug for RA, is efficacious for various autoimmune conditions including Castleman's disease [18].

A more recent study indicates that $CD20^-$ plasmablasts may also play a critical role in a proportion of patients with RA, who do not respond to anti-CD20 antibodies. The study by Owczarczyk *et al.* actually showed that amounts of *IgJ* mRNA, a marker for antibody-secreting plasmablasts, are increased in such anti-CD20 nonresponders [19]. It would be of interest to find out whether the $CD19^{int}CD180^-CD27^+CD38^+$ plasmablasts expanded in NMO are involved in the pathogenesis of rituximab-resistant RA.

In general, targeting a broader B-cell repertoire may be more efficacious in inhibiting autoimmune diseases such as NMO. However, we cannot over look potential problems in the long-term safety profile that often accompany efficacious immunosuppressive drugs. Which B cells should be targeted and how? This is obviously a central question when we treat patients with B-cell-mediated autoimmune diseases.

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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References

1 Cohen SB, Emery P, Greenwald MW *et al.* Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy. *Arthritis Rheum.* 54(9), 2793–2806 (2006).

2 Stone JH, Merkel PA, Spiera R *et al.* Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N. Engl. J. Med.* 363(3), 221–232 (2010).

3 Braendstrup P, Bjerrum OW, Nielsen OJ *et al.* Rituximab chimeric anti-CD20 monoclonal antibody treatment for adult refractory idiopathic thrombocytopenic purpura. *Am. J. Hematol.* 78(4), 275–280 (2005).

4 Hauser SL, Waubant E, Arnold DL *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N. Engl. J. Med.* 358(7), 676–688 (2008).

5 Pellkofer HL, Krumbholz M, Berthele A *et al.* Long-term follow-up of patients with neuromyelitis optica after repeated therapy with rituximab. *Neurology* 76(15), 1310–1315 (2011).

6 Pisetsky DS, Grammer AC, Ning TC, Lipsky PE. Are autoantibodies the targets of B-cell-directed therapy? *Nat. Rev. Rheumatol.* 7(9), 551–556 (2011).

7 Tedder TF. CD19: a promising B cell target for rheumatoid arthritis. *Nat. Rev. Rheumatol.* 5(10), 572–577 (2009).

8 Jarius S, Paul F, Franciotta D *et al.* Mechanisms of disease: aquaporin-4 antibodies in neuromyelitis optica. *Nat. Clin. Prac. Neurol.* 4(4), 202–214 (2008).

9 Lennon VA, Wingerchuk DM, Kryzer TJ *et al.* Serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 364(9451), 2106–2112 (2004).

10 Okamoto T, Ogawa M, Lin Y *et al.* Review: treatment of neuromyelitis optica: current debate. *Ther. Adv. Neurol. Disord.* 1(1), 43–52 (2008).

11 Kleiter I, Hellwig K, Berthele A *et al.* Failure of natalizumab to prevent relapses in neuromyelitis optica. *Arch. Neurol.* 69(2), 239–245 (2012).

12 Osoegawa M, Kira J, Fukazawa T *et al.*; Research Committee of Neuroimmunological Diseases. Temporal changes and geographical differences in multiple sclerosis phenotypes in Japanese: nationwide survey results over 30 years. *Mult. Scler.* 15(2), 159–173 (2009).

13 Wilk E, Witte T, Marquardt N *et al.* Depletion of functionally active CD20$^+$ T cells by rituximab treatment. *Arthritis Rheum.* 60(12), 3563–3571 (2009).

14 Stasi R, Del PG, Stipa F *et al.* Response to B-cell depletion therapy with rituximab reverts the abnormalities of T-cell subsets in patients with idiopathic thrombocytopenic purpura. *Blood* 110(8), 2924–2930 (2007).

15 Lehmann-Horn K, Shleich E, Hertzenberg D *et al.* Anti-CD20 B-cell depletion enhances monocyte reactivity in neuroimmunological disorders. *J. Neuroinflammation* 8, 146 (2011).

16 Iwata Y, Matsushita T, Horikawa M *et al.* Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B cells. *Blood* 117(2), 530–541 (2011).

17 Chihara N, Aranami T, Sato W *et al.* Interleukin 6 signaling promotes anti-aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica. *Proc. Natl Acad. Sci. USA* 108(9), 3701–3706 (2011).

18 Tanaka T, Narazaki M, Kishimoto T. Therapeutic targeting of the interleukin-6 receptor. *Annu. Rev. Pharmacol. Toxicol.* 52, 199–219 (2012).

19 Owczarczyk K, Lal P, Abbas AR *et al.* A plasmablast biomarker for nonresponse to antibody therapy to CD20 in rheumatoid arthritis. *Sci. Transl Med.* 3(101), 101ra92 (2011).

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Synthesis and biological evaluation of truncated α-galactosylceramide derivatives focusing on cytokine induction profile

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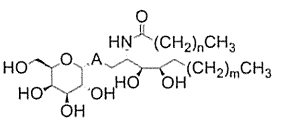
ABSTRACT

A series of truncated analogs of α-galactosylceramide with altered ceramide moiety was prepared, and evaluated for Th2-biased response in the context of IL-4/IFN-γ ratio. Phytosphingosine-modified analogs including cyclic, aromatic and ethereal compounds as well as the C-glycoside analog of OCH (**2**) with their cytokine inducing profile are disclosed.

1. Introduction

Natural killer T (NKT) cells are potent producers of immunoregulatory cytokines, and are restricted to glycolipid antigens presented by CD1d, a glycoprotein structurally and functionally related to non-classical major histocompatibility complex (MHC) class I.[1] Several natural glycolipids of bacterial[2] and mammalian[3] origin, and quite a few synthetic ligands of CD1d are identified and reported to date.[1b,4] Among them, synthetic α-galactosylceramide KRN7000 (**1**)[5] (Fig. 1) is the most extensively studied, for its strong activation of NKT cells as well as its effectiveness in in vivo animal disease models.[6] Compound **1** is known to induce various cytokines including proinflammatory Th1 cytokine interferon-γ (IFN-γ) and immunomodulatory Th2 cytokine interleukin-4 (IL-4), which oppose each other's response and may in part result in its marginal effect. Some studies are reported which aim to increase the selectivity of Th1 or Th2 cytokine induction. The majority are directed towards increased Th1 activity, and not few utilize the derivatives of the acyl chain and/or the sugar moiety which are relatively easy to prepare from a synthetic point of view. One of the most potent compounds reported to date is that with 8-(4-fluorophenyl)octanoyl chain as the acyl tail, which binds two orders of magnitude stronger with CD1d than **1**.[7] Another impressing finding was the conversion of **1** to its C-glycoside analog **3**, which leads to striking enhancement of activity in in vivo animal models of malaria and lung cancer.[8]



1 (**KRN7000**; A = O, m = 12, n = 24)
2 (**OCH**; A = O, m = 3, n = 22)
3 (A = CH_2, m = 12, n = 24)
4 (A = CH_2, m = 3, n = 22)

Figure 1. Structures of KRN7000 (**1**), OCH (**2**) and their C-glycoside analogs **3**, **4**.

An altered analog of **1** termed OCH (**2**) possessing a shorter phytosphingosine side chain[9] has been identified as NKT cell ligand which predominantly induces IL-4 over IFN-γ. Only compound **2** but not **1** is significantly effective in animal models of Th1-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA), which makes it an attractive lead for potential therapeutic application.[9,10]

Complete occupation of the binding groove of CD1d by **1** contributes to the sustained stimulation of NKT cells to induce robust immunological response, as indicated by several examples of X-ray crystallographic structures of compound **1**/CD1d complex.[11,12] Altered analogs such as **2** with short phytosphingosine chain is considered to result in short duration of stimulation and

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cause differential polarization of NKT cells.[9d] Instability of the short-chain analogs to form binary and ternary complexes is shown by molecular dynamics simulation study[13] and more directly by using the surface plasmon resonance (SPR) technique.[14] It was also shown that truncation in the phytosphingosine and not the acyl chain will affect the NKT cell activation profile.[14]

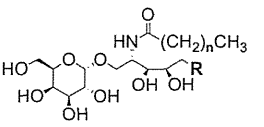
As part of our efforts to obtain more potent compounds for the enhancement of Th2 response, a series of analogs based on **2** with altered ceramide moiety was prepared and evaluated in vitro, some of which are the first to be reported. In this report, the structure–activity relationship in the context of IL-4/IFN-γ ratio is described. In the course of our study, the C-glycoside of **2** was prepared for the first time and its cytokine-inducing profile in vitro and in vivo are also described.

2. Results and discussion

2.1. Chemistry

The analogs were prepared by the versatile method developed by our group (Scheme 1).[15] The phytosphingosine side chain substituents R shown in Tables 1 and 2 were introduced to the known epoxide **5** by means of nucleophilic addition. In addition to the nucleophiles reported earlier utilizing alkyl or aryl lithium reagents or corresponding magnesium bromides,[16] alkoxides and phenoxide were also efficiently introduced. Liquid alcohols were reacted as a solvent, while dioxane was used as a solvent for solid hydroxyls such as phenol. Various nucleophiles, including short or long primary alkyl, secondary alkyl, aryl, alkoxy and aryloxy groups were successfully incorporated via this route. After regioselective mesylation of the more reactive axial hydroxyl group,[17] compound **6** was subjected to benzylidene cleavage and azidation, after which secondary hydroxyl groups were protected to provide isopropylidene acetal **7**. The order of de-benzylidene reaction and azidation could be reversed, but azidation first of the axial mesyloxy group of **6** needed higher temperature, longer time and gave lower yield presumably for its steric demand. On the other hand, azidation later to the deprotected **6** yielded small portions of regio- and stereoisomers as side products along with major product **7**, assumed to have formed via epoxide through nucleophilic addition of the vicinal hydroxyl groups. Generally, deprotection first of **6** gave higher yield in total. Glycosidation with tetra-*O*-benzyl-α-D-galactosyl fluoride in the presence of BF_3·OEt_2, or with tetra-*O*-benzyl-α-D-galactosyl bromide or chloride in the presence of tetra-*n*-butylammonium bromide gave selectively the α-glycoside **9**. The selectivity over the β-isomer was improved in the latter protocol, to a ratio typically greater than 10:1.[15,18] The azido group in **9** was reduced to an amine and acylated with suitable carboxylic acids to give **10**. Finally, all the protective groups were removed to give the desired analogs.

Table 1
Dependency of cytokine induction on alkyl chain lengths[a]



Compound	R	*n*	IL-4[b] (%)	IFN-γ[b] (%)
11a	$-CH_2CH_3$	22	105	96
11b	$-(CH_2)_2CH_3$	21	97	148
11c	$-(CH_2)_2CH_3$	22	115	112
11d	$-(CH_2)_3CH_3$	18	6	9
11e	$-(CH_2)_3CH_3$	20	51	46
11f	$-(CH_2)_3CH_3$	21	103	93
2	$-(CH_2)_3CH_3$	22	100	100
11g	$-(CH_2)_3CH_3$	23	154	103
11h	$-(CH_2)_3CH_3$	24	129	504
11i	$-(CH_2)_3CH_3$	26	178	761
11j	$-(CH_2)_4CH_3$	21	97	113
1	$-(CH_2)_{12}CH_3$	24	128	569

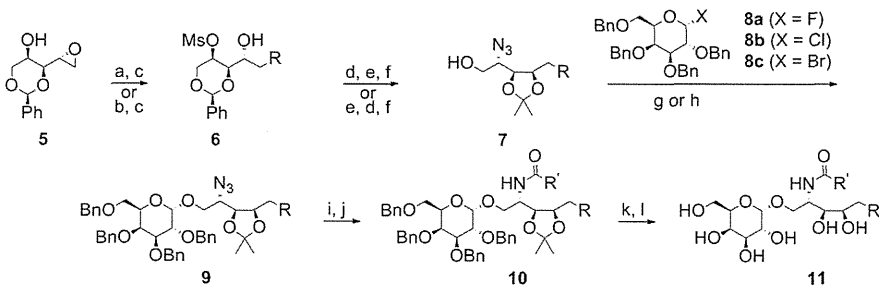
[a] At 100 ng/ml.
[b] Normalized to **2** at 100 ng/ml.

It is worthy of note that alkoxy derivatives (e.g., R = *n*-PrO) or aryl derivatives (e.g., R = Ph) with R at this position are not directly accessible via the Wittig reaction of stereo-fixed, sugar-based starting materials (e.g., D-lyxose).[5a,19]

C-Glycoside **4** was synthesized by short and efficient route as depicted in Scheme 2.[20] Known α-ethynylgalactose derivative **12**[21] and octanal derivative **13** synthesized from L-arabinose were coupled in a chelation-controlled manner to give a 1.6:1 mixture of **14a** and **14b**. Compounds **14a** and **14b** were easily separated by column chromatography over silica gel, and the stereochemistry of the newly formed diastereomeric center was determined for the major isomer **14a** applying modified Mosher's protocol[22] to have the *R*-configuration.[20] The acetylenic bond in **14a** was selectively and efficiently reduced by diimide reduction, after which the hydroxyl group was mesylated to give **15**. The synthesis of **4** was completed in a straightforward manner, after substitution by azido group, reduction, acylation and global deprotection.

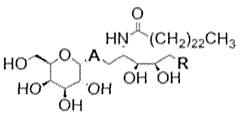
2.2. Biological evaluation

The analogs were evaluated in vitro for their ability to induce IL-4 and IFN-γ relative to **2**. IL-4 and IFN-γ secretion were assessed with spleen cells prepared from C57BL/6 mice, which were



Scheme 1. Synthesis of O-glycosides. Reagents and conditions: (a) RLi or RMgBr, CuI or CuOTf, THF, −40 °C, 52–98%; (b) alcohol or phenol, NaH, (dioxane), rt–80 °C, 83–88%; (c) MsCl, pyridine, −40 °C–rt, 34–93%; (d) H_2, $Pd(OH)_2$/C, EtOH, rt, or 6 N HCl, MeOH, rt, 68–100%; (e) NaN_3, DMF, 95–110 °C, 20–66%; (f) cat. *p*-TsOH, 2,2-dimethoxypropane, rt, 26–75%; (g) **8a**, BF_3·OEt_2, MS 4 Å, $CHCl_3$, −50 °C, 13–73%; (h) **8b** or **8c**, *n*-Bu_4NBr, MS 4 Å, DMF–toluene, rt, 22–68%; (i) H_2, Lindlar catalyst, EtOH, rt; (j) R'CO_2H, EDCI·HCl, HOBt or HOAt, *i*-Pr_2NEt, DMF–CH_2Cl_2, 40 °C, 22–100% (two steps); (k) HCl–dioxane, MeOH–CH_2Cl_2, rt, or 80% AcOH, 80 °C; (l) H_2, $Pd(OH)_2$/C, MeOH–$CHCl_3$, rt–40 °C, 41–91% (two steps).

Table 2
Cytokine induction profile of the phytosphingosine-altered derivatives and the C-glycoside of OCH[a]



Compound	A	R	IL-4[b] (%)	IFN-γ[b] (%)
2	O	$-(CH_2)_3CH_3$	100	100
11k	O	-*c*-Pent	98	74
11l	O	-Ph	211	284
11m	O	$-CH_2Ph$	57	35
11n	O	-*p*-Tol	78	76
11o	O	$-OCH_3$	86	64
11p	O	$-O(CH_2)_2CH_3$	103	161
11q	O	$-O(CH_2)_{11}CH_3$	78	227
11r	O	-OPh	99	80
4	CH_2	$-(CH_2)_3CH_3$	1	0

[a] At 100 ng/ml.
[b] Normalized to **2** at 100 ng/ml.

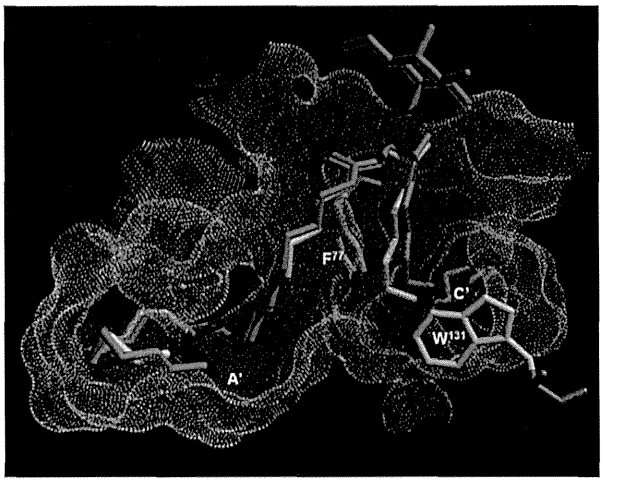


Figure 2. Side view of the optimized structure of **2** (blue)/hCD1d complex. X-ray structure of **1** (red) is superimposed.

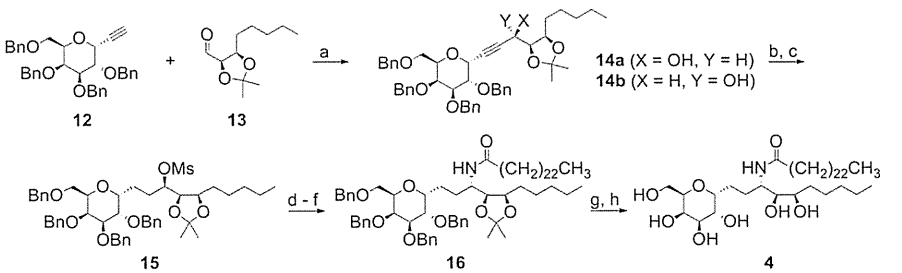
incubated with 100 ng/ml of glycolipids for 72 h. The cytokines in the culture supernatant were measured by ELISA.

Influence of the chain lengths was first examined (Table 1). When the phytosphingosine chain was fixed to that of **2** and acyl chain length altered (compounds **2** and **11d–11i**), the chain length proximal to **2** showed similar cytokine production. As the acyl chain became longer the cytokine release increased, and for chains longer than hexacosanoic acid there was a marked increase in IFN-γ production that dominated IL-4 (**11h**, **11i**), which was comparable to **1**. On the other hand, as the chain became shorter the induction of both cytokines decreased rather drastically, and icosanoyl derivative **11d** showed negligible efficacy.

When the acyl chain length was next fixed to that of **2** and sphingosine base altered, in our hands cytokine producing profile did not change for given derivatives (**11a**, **11c** and **2**; $n = 22$). Compounds **11b**, **11f** and **11j** which have tricosanoyl chain ($n = 21$) also showed similar profile. Taking above results together, we concluded that very close modification of **2** both in acyl and sphingosine chain are tolerated and shows similar profiles, and that further shortening of the acyl chain in aim for more Th2-biased response seems inappropriate.

Our interest was next focused on the phytosphingosine moiety, where it makes **2** a completely different switch of the NKT cell signal. Analogs bearing aliphatic ring (**11k**) or aromatic ring (**11l–11n**) were also prepared. To our knowledge, these are the first examples of non-linear hydrocarbon chain analogs of the phytosphingosine moiety that show similar cytokine inducing ratio to **2**.[23] Compound **11l** with an aromatic ring appears to show slight increase in both cytokines. This is suggestive of aromatic interaction(s) with residues such as Phe77 and Trp131 in CD1d (Fig. 2). Aromatic derivatives with one methylene unit longer (**11m**) or one additional methyl in the *para*-position (**11n**) showed reduced activity, which is indicative of the appropriate length and flexibility in reference to pocket depth, as well as for possible interaction with aromatic residues. We have next prepared analogs bearing ether linkage in the phytosphingosine chain. There is only one report for α-galactosylceramide derivatives with aliphatic ether chain, which assessed the release of IL-2, a Th1 cytokine.[24] Our compounds (**11o–11r**) including oxa- analog of **2** showed similar profiles to corresponding methylene derivatives, which is in line with one example of oxa- analog of **1** shown in the previous report.[24] The oxa- analogs were also shown for the first time to be comparable in the context of IL-4/IFN-γ ratio to the corresponding methylene analogs.

The binding groove of the CD1d consists of two hydrophobic channels A′ and C′ that accommodate two lipid chains of α-glycosyl ceramides. As can be seen from the X-ray structure of **1**/hCD1d complex,[12] the acyl chain occupies A′ pocket and the bent phytosphingosine chain enters the narrow C′ pocket beyond the length of **2** (Fig. 2). We assume the phytosphingosine-altered analogs in Tables 1 and 2 which in length do not reach the C′ pocket showed similar profiles to **2** owing to weak interaction with CD1d. There is a wide space before entering the C′ pocket which allows cycloalkyl and aromatic substituents, and as mentioned earlier the aromatic side chain might interact with aromatic residues such as Phe77 and Trp131. Molecular modeling of **2**/hCD1d complex based on



Scheme 2. Synthesis of C-glycoside **4**. Reagents and conditions: (a) *n*-BuLi, THF, −48 to −30 °C, 47% for **14a**, 30% for **14b** (BRSM); (b) $TsNHNH_2$, DME, NaOAc aq, reflux, 91%; (c) MsCl, pyridine, CH_2Cl_2, 0 °C–rt, 94%; (d) NaN_3, DMF, 90 °C; (e) H_2, Lindlar catalyst, EtOH, rt; (f) Lignoceric acid, EDCI·HCl, HOAt, Et_3N, DMF–CH_2Cl_2, rt, 48% (three steps); (g) 80% AcOH, 60 °C, 88%; (h) H_2, $Pd(OH)_2/C$, MeOH–CH_2Cl_2, rt, quant.