Gut microbiota-dependent $\text{CCR9}^+ \text{CD4}^+$ T cells are altered in secondary progressive multiple sclerosis

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The mechanism underlying the progression of relapsing-remitting multiple sclerosis to secondary progressive multiple sclerosis (SPMS), characterized by accumulating fixed disability, is yet to be fully understood. Although alterations in the gut microbiota have recently been highlighted in multiple sclerosis pathogenesis, the mechanism linking the altered gut environment with the remote CNS pathology remains unclear. Here, we analyse human CD4$^+$ memory T cells expressing the gut-homing chemokine receptor CCR9 and found a reduced frequency of CCR9$^+$ memory T cells in the peripheral blood of patients with SPMS relative to healthy controls. The reduction in the proportion of CCR9$^+$ cells among CD4$^+$ memory T cells ($\%\text{CCR9}$) in SPMS did not correlate with age, disease duration or expanded disability status scale score, although $\%\text{CCR9}$ decreased linearly with age in healthy controls. During the clinical relapse of both, relapsing-remitting multiple sclerosis and neuromyelitis optica, a high proportion of cells expressing the lymphocyte activating 3 gene ($\text{LAG3}$) was detected among CCR9$^+$ memory T cells isolated from the CSF, similar to that observed for mouse regulatory intraepithelial lymphocytes. In healthy individuals, CCR9$^+$ memory T cells expressed higher levels of CCR6, a CNS-homing chemokine receptor, and exhibited a regulatory profile characterized by both the expression of C-MAF and the production of IL-4 and IL-10. However, in CCR9$^+$ memory T cells, the expression of ROR$\gamma$t was specifically upregulated, and the production of IL-17A and IFN$\gamma$ was high in patients with SPMS, indicating a loss of regulatory function. The evaluation of other cytokines supported the finding that CCR9$^+$ memory T cells acquire a more inflammatory profile in SPMS, reporting similar aspects to CCR9$^+$ memory T cells of the elderly healthy controls. CCR9$^+$ memory T cell frequency decreased in germ-free mice, whereas antibiotic treatment increased their number in specific pathogen-free conditions. Here, we also demonstrate that CCR9$^+$ memory T cells preferentially infiltrate into the inflamed CNS resulting from the initial phase and that they express LAG3 in the late phase in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis. Antibiotic treatment reduced experimental autoimmune encephalomyelitis symptoms and was accompanied by an increase in CCR9$^+$ memory T cells in the peripheral blood. Antibodies against mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), which is capable of blocking cell migration to the gut, also ameliorated experimental autoimmune encephalomyelitis. Overall, we postulate that the alterations in CCR9$^+$ memory T cells observed, caused by either the gut microbiota changes or ageing, may lead to the development of SPMS.

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Introduction

Multiple sclerosis is a demyelinating autoimmune disease of the CNS. Previous studies using the experimental autoimmune encephalomyelitis (EAE) animal model of multiple sclerosis, genome-wide association studies of patients, and the success of T-cell targeted therapies in clinical settings have collectively indicated that helper (CD4+) T cells play a major role in multiple sclerosis pathogenesis. Although patients with multiple sclerosis initially present a relapsing and remitting disease course, 30–40% could transition into a secondary progressive phase, characterized by the steady accumulation of fixed disability, within 10 years from disease onset (Rovaris et al., 2006; Stankoff et al., 2007; Mahad et al., 2015). The immunological and neurodegenerative aspects of the secondary progressive multiple sclerosis (SPMS) pathology are reportedly influenced by both age and disease duration. Notably, age at onset is known to be a predictor of SPMS progression (Rovaris et al., 2006; Stankoff et al., 2007; Mahad et al., 2015). However, the environmental factors and peripheral immunological changes associated with SPMS progression are still largely unknown.

Results from both EAE and arthritis animal models showed the alterations in the gut environment and microbiota to lead to augmented or suppressed development of autoimmune inflammation (Yokote et al., 2008; Berer et al., 2011; Haghikia et al., 2015). Recent analysis of the gut microbiota composition in patients with multiple sclerosis revealed the reduction in potentially beneficial bacteria and the increase in proinflammatory bacteria to be involved in autoimmunity regulation (Miyake et al., 2015; Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017). Besides multiple sclerosis, the gut microbiota may also be implicated in neuromyelitis optica pathogenesis (Varrin-Doyer et al., 2012; Zamvil et al., 2018). Nonetheless, the immune mechanisms that connect the gut environment to systemic immune responses are largely unclear.

Here, we hypothesized that gut-derived CD4+ T cells shape the gut-systemic immune axis. The gut microbiota is known to provide cross-reactive antigens that can activate self-antigen–specific CD4+ T cells, presumably via molecular mimicry (Horai et al., 2015; Kadowaki et al., 2016), which may gain regulatory properties. We recently reported the abundance of CD4+ intraepithelial lymphocytes (IELs) in MOG-specific TCR transgenic mice and their protective role against EAE, through the lymphocyte activating 3 gene (Lag3), which is upregulated after the entry of CD4+ IELs in the CNS (Kadowaki et al., 2016). Since gut CD4+ T cells, including lamina propria CD4+ T cells and CD4+ IELs, were suggested to recirculate in the blood (Suzuki et al., 1998; Morton et al., 2014; Yang et al., 2014), they likely play a role in the physiological regulation of immune responses in the systemic compartment.

Interactions between T-cell C-C chemokine receptor type 9 (CCR9) and its ligand CCL25 in the small intestinal epithelium are crucial for the migration of T cells to the gut. The latter also depends on the expression of integrin α4β7, whose ligand (mucosal vascular addressin cell adhesion molecule 1, MADCAM1) is expressed in the gut lamina propria venules. Therefore, CCR9 and α4β7, expressed by T cells, are designated gut-tropic molecules (Guy-Grand et al., 2013) and are upregulated by conventional CD4+ T cells after activation in gut-associated lymphoid tissues, such as Peyer’s patches and mesenteric lymph nodes. They subsequently reach the thoracic duct and recirculate through the peripheral blood to the gut wall, by interacting with CCL25 and MADCAM1. Blockade of the CCR9–CCL25 interaction inhibits recirculation of these cells, leading to a reduction in the number of IELs (Svensson et al., 2002; Guy-Grand et al., 2013), which provides indirect evidence of the inclusion of CD4+ IELs precursors in the peripheral blood CCR9+ CD4+ T cells, which can regulate autoimmune inflammation (Guy-Grand et al., 2013; Kadowaki et al., 2016). Since all human IELs express CCR9 (Zabel et al., 1999), peripheral blood CCR9+ T cells may also include recirculating CD4+ IELs, as mentioned above. Furthermore, it is worth noting that CD8+ T cells that are abundant in chronic multiple sclerosis lesions (Machado-Santos et al., 2018) are CD8αα T cells, not CD8αβ, which are abundantly observed in gut IELs that originate from gut-homing CD4+ and double-negative T cells (Cheroutre et al., 2011). Therefore, CD8αα T cells found in multiple sclerosis lesions may be related to CCR9+ CD4+ T cells. We then hypothesized that patients with either relapsing remitting multiple sclerosis (RRMS) or SPMS may have an altered induction of CCR9+ CD4+ T cells in peripheral blood, which contributes to disease pathogenesis.

In this study, we demonstrate the potential of human CCR9+ CD4+ memory T cells (Tm) in the regulation of the acute autoimmune inflammation associated with RRMS or neuromyelitis optica. However, CCR9+ CD4+ Tm cells display reduced frequency in patients with SPMS and are functionally biased in a proinflammatory direction. Furthermore, CCR9+ CD4+ Tm cells were found to naturally decrease with age, while the corresponding CCR9+ Tm cells were dependent on the gut microbiota status in mice. We postulate that the alterations in CCR9+ Tm cells may represent a disturbance in the gut-systemic immune axis caused by dysbiosis and underlie SPMS pathogenesis.
Materials and methods

Participants

This study included 22 healthy controls, 12 elderly healthy controls (age: >60 years), 33 patients with RRMS, 34 with SPMS, 13 with neuromyelitis optica, and 17 with non-inflammatory non-immunological neurodegenerative diseases (NINDs), comprising nine patients with Parkinson’s disease and eight patients with NINDs other than Parkinson’s disease (non-Parkinson’s disease). The demographic features of each group are presented in Table 1 and the detailed profile of RRMS and SPMS patients are displayed in Supplementary Table 1. In all cases, the diagnosis was made by board-certified neurologists and the differential diagnoses of RRMS, SPMS, and neuromyelitis optica were established according to either the McDonald or Wingerchuk diagnostic criteria (Polman et al., 2011; Wingerchuk et al., 2015). Specifically, SPMS diagnosis is based on a history of gradual worsening of the disease after an initial relapse (Lublin et al., 2014). Disease activity was evaluated by clinical relapse or MRI within 1 month prior to sampling. The Ethics Committee of the National Center of Neurology and Psychiatry (NCNP) approved the current study and written informed consent was obtained from all participants. CSF samples from psychiatric patients were collected as part of the research on the development of biomarkers for psychiatric diseases in NCNP (Hidese et al., 2017). The approval numbers for CSF collection and biobanking are A2014–141 and A2012–091, respectively.

Cell preparation, flow-cytometry analysis, and sorting of human lymphocytes

Fresh peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare Bioscience) and stained for cell surface antigens employing the following fluorescently labelled monoclonal and isotype control antibodies: CD45RA (HI100), CD3 (UCHT1, OKT3), CD4 (OKT4), CD8α (HIT8a, RDA-T8), CCR9 (L053E8), CCR6 (G034E3), CCR7 (G043H7), β1 (TS2/16), β7 (FIB504) (BioLegend), α4 (9F10) (BD), CXCR3 (49801) (R&D). Dead cells were stained with the 7-aminoactinomycin D (7-AAD) viability staining solution (BioLegend). In some experiments, intracellular staining was performed with antibodies against ROR$^\gamma$ (AFKJS-9), T-BET (eBio4B10), and C-MAF (sym0F1) (eBioscience) using the Cytofix or Cytoperm™ Kit (Becton Dickinson). Mouse or rat serum (eBioscience) was used for blocking. Intracellular FOXP3 was stained using the PE anti-human FOXP3 staining kit (eBioscience). Cells were analysed or sorted by BD FACSCanto II or FACSAria II.

Quantitative RT-PCR

Cells were lysed in RLT buffer (Qiagen) containing 1% 2-mercaptoethanol and subsequently homogenized using the QIAshredder (Qiagen). The total RNA was obtained with the

Table 1 Demographic characteristics of the subjects analysed

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<td>N/A</td>
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<td>3 SCD</td>
<td>1 MSA-C</td>
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ALS = amyotrophic lateral sclerosis; BMI = body mass index; HC = healthy control; MSA-C = multiple system atrophy cerebellar type; N/A = not applicable; NMO = neuromyelitis optica; Non-PD = non-Parkinson’s disease; SCD = spinocerebellar degeneration.
RNA was generated using a SuperScript™ VILO cDNA Synthesis Kit (Invitrogen) and used as a template for quantitative RT-PCR performed with the Fast Start Essential DNA Green Master kit (Roche). Primers for TBX21, GATA3, RORC, FOXP3, MAF, and AHR were purchased from Qiagen. A primer for ACTB was designed as follows: forward 5’-CCTCTTCCAGCCTTCCTTCC-3’, reverse 5’-GATAAGCGTCCTTGCGGATG-3’.

**Analysis of cell proliferation and cytokine production**

For proliferation assays and cytokine measurements, cells were suspended in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin-streptomycin, and 50 µM 2-mercaptoethanol (Gibco). Cells (5.0 x 10^6) were stimulated with immobilized anti-CD3 (OKT3, 4.0 µg/ml) and anti-CD28 (CD28.2, 2.0 µg/ml) for 3 days in 96-well flat-bottom plates and then incubated with 3H-thymidine (1 µCi per well) for the final 8 h of culture. Radioactivity incorporation was analysed using a scintillation counter and was expressed as counts per minute. Supernatants were collected, and cytokines were measured using the Bio-Plex® cytokine assays (Bio-Rad).

**Mice**

C57BL/6J mice were purchased from theCLEA Laboratory Animal Corp and maintained in specific pathogen-free conditions (SPF) in accordance with the institutional guidelines. Furthermore, age- and sex-matched germ-free and SPF C57BL/6N mice were also purchased. This study was approved by the Committee for Small Animal Research and Animal Welfare of NCNP.

**EAE induction**

For EAE induction, mice were injected subcutaneously with 100 µg MOG (35–55) peptide (Toray Research Center) and 1 mg heat-killed Mycobacterium tuberculosis H37RA emulsified in complete Freund’s adjuvant (Difco). On Days 0 and 2 after immunization, 200 µg of pertussis toxin (List Biological Laboratories) were injected intraperitoneally. EAE clinical symptoms were scored (0, no clinical signs; 1, weak tail; 2, flaccid tail; 3, partially weak hind limb; 4, total hind limb paralysis; and 5, hind and fore leg paralysis).

**Mouse antibiotic treatment**

Mice were orally treated with a mixture of kanamycin sulphate (10 mg), colistin sulphate (2.6 mg), and vancomycin hydrochloride (3 mg) dissolved in 200 µl of distilled water every day through a gavage needle.

**In vivo treatment with the anti-MADCAM1 antibody**

For in vivo neutralization of MADCAM1, 500 µg of anti-MADCAM1 monoclonal antibody (MECA-367; BioLegend) or purified rat IgG (Invitrogen) in phosphate-buffered saline (PBS) were injected intraperitoneally on Days −1 and +2 of EAE induction.

**Mouse cell preparations, staining, and flow-cytometry analysis**

To obtain PBMCs from mice, blood was withdrawn by left ventricle cardiac puncture. Mononuclear cells were isolated through density gradient centrifugation using Lymphosepar II (Ficoll–Conray solution; Immuno-Biological Laboratories), according to the manufacturer’s protocol. Small intestinal IELs and spleen cells were isolated as described previously (Kadowaki et al., 2016). CNS-infiltrating mononuclear cells were isolated from spinal cords and brains as follows: CNS tissues were cut into small pieces and digested in a RPMI medium containing 25 µg/ml of Liberase™ Blendzyme 3 (Roche) and 100 µg/ml of DNase I (Roche) at 37 °C for 30 min. The resulting tissue homogenates were passed through a 70-µm strainer, suspended in 30% Percoll®, and centrifuged for 30 min at 970g at 4 °C.

Cells at the interface were CNS mononuclear cells. Non-specific staining was inhibited through incubation with anti-MCD16/32 (BioLegend). Cells were then stained with fluorescence-labelled antibodies, whereas dead cells were stained by 7-AAD. Antibodies against TCR Vα4, CD4 (RM4–5, 1:140), CD8ε (53–6.7), CCR9 (eBioCW-1.2), CD44 (IM7) and LAG3 (C9B7W) were purchased from BioLegend. Cells were analysed or sorted by BD FACS Aria II.

**Statistical analysis**

Differences between groups were analysed with one-way analysis of variance, Wilcoxon signed-rank test, or Student’s t-test as indicated. P < 0.05 was considered significant or otherwise indicated.

**Data availability**

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

**Results**

**Peripheral blood CCR9⁺ CD4⁺ Tm cells frequency is reduced in SPMS**

To investigate the involvement of gut-derived CD4⁺ T cells in multiple sclerosis pathogenesis in human peripheral blood, we examined the expression of the gut-homing chemokine receptor CCR9. As the β7 integrin molecule can pair with α4 to form the α4β7 integrin, which binds MADCAM1, CCR9⁺ or β7⁺ cells frequency among CD4⁺ or CD8⁺ T cells, gated as CD45RA⁻CD3⁺ cells, was measured. CCR9⁺ T cells comprised ~5% of CD4⁺ Tm cells in healthy controls, which highly co-expressed β7 integrin (Supplementary Fig. 1A and B). In contrast, few CD8⁺ Tm cells expressed CCR9.

Successively, we evaluated CCR9⁺ cells frequency among CD4⁺ Tm cells (%CCR9) in RRMS, SPMS, neuromyelitis optica and age-matched healthy controls. The %CCR9 value in neuromyelitis optica was similar to that in healthy controls,
although a reducing trend was found in RRMS, whereas it was significantly reduced in SPMS relative to healthy controls (Fig. 1A). Additionally, the %CCR9 was not significantly different between NINDs and younger healthy controls, although a trend was reported (Table 1 and Supplementary Table 2). Elderly healthy controls’ %CCR9 was significantly lower than that of younger healthy controls. Ages were not significantly different among younger healthy controls, RRMS, SPMS, and neuromyelitis optica, whereas they were higher in elderly healthy controls and NINDs (Supplementary Fig. 3A). As the SPMS group included two %CCR9 subsets (Fig. 1A), we compared the demographic feature of the 10 higher (%CCR9 > 4.2) and lower %CCR9 patients with SPMS (Supplementary Fig. 2). No significantly different feature was observed.

**Figure 1** Proportion of CCR9⁺ cells in peripheral blood memory CD4⁺ T cells. (A) The proportion of CCR9⁺ cells in CD4⁺ memory T cells (%CCR9) from healthy controls, elderly healthy controls (HC), and patients with RRMS, SPMS, neuromyelitis optica (NMO), non-inflammatory neurodegenerative diseases (NIND) including Parkinson’s disease (PD) and other neurodegenerative diseases (non-PD) were analysed by flow cytometry. Box and whisker plots are shown. Whiskers are drawn from minimum to maximum points. Each individual value is plotted as a dot superimposed. **P < 0.01 by one-way ANOVA with Dunnett’s multiple comparison test. (B) Correlation analysis of %CCR9 in CD4⁺ Tm cells and age in healthy controls and elderly healthy controls combined (Control), RRMS and SPMS group was performed and linear regression lines (solid line) are displayed (Control: black, SPMS: red). R² and P-values were calculated. Patients with neuromyelitis optica and non-inflammatory neurodegenerative diseases are depicted in the same graph for comparison. (C) %CCR9 of elderly healthy controls, Parkinson’s disease, and non-Parkinson’s disease are compared (right). *P < 0.05 by one-way ANOVA with Bonferroni’s post-test. (D) Correlation analysis of %CCR9 in CD4⁺ Tm cells and disease duration (left) or Expanded Disability Status Scale (EDSS, right) in RRMS and SPMS group were performed and linear regression lines (solid line) were displayed (RRMS: blue, SPMS: red). R² and P-values were calculated.
Subsequently, given the significant difference of %CCR9 values in younger and older healthy controls, we analysed the correlation between age and %CCR9 in the healthy control and patients groups. A strong negative correlation with age was found in healthy controls (Fig. 1B), whereas it was weak in RRMS and even weaker in SPMS; most plots were below the healthy control regression line (Fig. 1B). Therefore, we assumed that the reduction in %CCR9 in SPMS could not be merely attributed to age. We confirmed that the %CCR9 values were neither influenced by gender using age-matching or body mass index (Supplementary Fig. 3B and C). To match the ages more closely between healthy controls and SPMS, patients between 35 and 55 years of age were selected [healthy control 43.7 ± 1.81 SPMS 45.7 ± 0.84 years old, mean ± standard error of the mean (SEM); not significant by unpaired t-test]. The %CCR9 was still significantly reduced in SPMS patients (Supplementary Fig. 3D).

Thereafter, we examined the effect of treatment on %CCR9 alteration. RRMS, neuromyelitis optica, and SPMS patients were classified based on their treatment (Supplementary Table 1) and their %CCR9 was compared with that of healthy controls. Fingolimod-treated SPMS patients had significantly lower %CCR9 (Supplementary Fig. 4A), although it was statistically comparable to other drug-treated subgroups or non-treated groups (Supplementary Fig. 4B). Besides SPMS, no specific treatment significantly reduced %CCR9 among RRMS and neuromyelitis optica, suggesting that the %CCR9 reduction observed in SPMS cannot be explained by therapies.

Subsequently, we analysed whether a correlation between %CCR9 and disease duration or Expanded Disability Status Scale existed in the RRMS and SPMS groups (Fig. 1D), however, it was not found. Since gastrointestinal symptoms and altered gut microbiota characterized by small intestinal bacterial overgrowth are considered hallmarks of Parkinson’s disease (Fasano et al., 2013; Keshavarzian et al., 2015), we examined whether %CCR9 is altered in Parkinson’s disease. A sub-analysis of elderly healthy controls and the Parkinson’s disease and non-Parkinson’s disease groups found increased %CCR9 in Parkinson’s disease compared to elderly healthy controls, while it remained unaltered in the non-Parkinson’s disease group (Fig. 1C).

These results indicate that CCR9+ Tm cells reduction may be related to SPMS pathology, that it may correlate with age and that other factors, including the gut microbiota, may also be involved in the %CCR9 changes seen in SPMS.

**CCR9+ Tm cells infiltrate into the CNS and express LAG3 during multiple sclerosis and neuromyelitis optica relapses**

We examined the possible migration of CCR9+ Tm cells from the peripheral blood to the CNS during RRMS and neuromyelitis optica relapses to protect or augment CNS inflammation. The expression of CCR6 and CXCR3 on CCR9+ Tm cells from the peripheral blood was investigated, as T cells expressing these chemokine receptors preferentially traffic into inflamed CNS tissues (Reboldi et al., 2009; Sporici and Issekutz, 2010). A higher expression of CCR6 in CCR9+ Tm cells, when compared to that in CCR9− Tm cells, was observed in all groups (Fig. 2A). In contrast, CXCR3 expression was similar between CCR9+ and CCR9− Tm cells in both groups, although it was higher in the CCR9+ Tm cells of the neuromyelitis optica patient group. Additionally, we examined surface integrin expression on CCR9+ Tm cells and found that most CCR9− Tm cells, and lower portions of CCR9+ Tm cells, expressed α4 integrin (Fig. 2B). Further, α4 and β1 constitute VLA-4, which binds the vascular cell adhesion protein 1 expressed on vascular endothelial cells of inflamed tissues. The proportions of α4−β1+ cells in CCR9+ Tm cells were low, although they were much higher in both healthy controls and SPMS (Fig. 2B). A slight, however, significant (P < 0.05 by unpaired t-test), increase in α4−β1+ cells was observed in CCR9+ Tm cells, but not in CCR9− cells, of SPMS patients relative to healthy controls. Furthermore, expression of CCR7, which facilitates the trafficking to the secondary lymphoid organs and may also play a critical role in invading the CNS (Kivisakk et al., 2004) were comparable between CCR9+ and CCR9− Tm cells among the examined healthy controls and SPMS patients (Supplementary Fig. 5). The collective data indicate that CCR9+ Tm cells have the potential to migrate into the inflamed CNS via a CCR6-dependent mechanism.

Next, we investigated whether CCR9+ Tm cells could infiltrate into the CNS. To this end, we examined the expression of CCR9 in CSF lymphocytes obtained from the healthy control and patient groups (Fig. 2C). Most of the control samples were acquired from psychiatric patients (Supplementary Table 2). The %CCR9 in CSF Tm cells was significantly higher in the neuromyelitis optica group when compared to healthy controls, indicating that CCR9+ Tm cells could infiltrate into the inflamed CNS tissue. Consistent with this hypothesis, %CCR9 values tended to increase in the RMS group, although they did not reach statistical significance. In contrast, %CCR9 was not increased in the SPMS group, although the ratio of %CCR9 in the CSF to the peripheral blood Tm cells showed a significant increase.

Given that CCR9+ Tm cells are thought to include regulatory CD4+ IELs precursors that would upregulate LG3 in inflamed tissues (Svensson et al., 2002; Gug-Grand et al., 2013; Kadowaki et al., 2016), we examined whether CCR9+ Tm cells might express LG3 in the CSF. Furthermore, LG3 is considered a marker of type 1 regulatory T (Tr1) cells (Gagliani et al., 2013). We analysed LG3 expression in CCR9+ or CCR9− Tm cells in the paired PBMCs and CSF samples from patients during RRMS or neuromyelitis optica relapse and a substantial
Figure 2 Analysis of the homing capacity of CCR9\(^+\) CD4\(^+\) memory T cells and their infiltration into the CSF. (A) CCR6 expression in CCR9\(^-\) (9\(--\)) or CCR9\(^+\) (9\(+\)) CD4\(^+\) memory T cells of PBMCs derived from randomly selected individuals [10 healthy control (HC), seven RRMS, 11 neuromyelitis optica (NMO), and eight SPMS]. CXCR3 expression was analysed in six healthy controls, 13 RRMS, five SPMS, and six neuromyelitis optica. \(^{**}P<0.01\), \(^{*}P<0.05\) by Wilcoxon signed-rank test (one-sided). (B) Per cent expression of integrin \(\alpha4+\beta1+\) and \(\alpha4+\beta7+\) in CCR9\(^+\) (9\(+\)) and CCR9\(^-\) (9\(--\)) of PBMC CD4\(^+\) memory T cells derived from three healthy controls and three patients with SPMS (mean and SEM). Whiskers are drawn from minimum to maximum points. Each individual value is plotted as a dot superimposed. \(^{*}P<0.05\) by unpaired t-test. (C) %CCR9 in CD4\(^+\) memory T cells in the CSF of 11 controls (Supplementary Table 2), eight RRMS, six SPMS, and eight neuromyelitis optica (left). The gating strategies were the same as PBMC (Supplementary Fig. 1). Among them, in seven controls, eight RRMS, six SPMS, and six neuromyelitis optica whose PBMCs were also available, %CCR9 of the PBMCs were also analysed and ratios (%CCR9 in CSF/ %CCR9 in PBMCs) were calculated for each sample. Whiskers are drawn from minimum to maximum points. Each individual value is plotted as a superimposed symbol. \(^{**}P<0.01\), \(^{*}P<0.05\) by one-way ANOVA with Dunnet’s multiple comparison tests. (D) Expression of surface LAG3 assessed in CCR9\(^-\) (9\(--\)) or CCR9\(^+\) (9\(+\)) CD4\(^+\) Tm cells in PBMCs (P) or CSF (C). Data from individual patients are connected by a solid line, except for the data from whom only the CSF samples are available, which are connected by a tinted line. Per cent expression was measured in comparison with isotype-matched fluorescently-labelled antibodies tested in each sample. \(^{**}P<0.01\) by one-way ANOVA with Tukey’s multiple comparison tests.
CCR9+ Tm cells portion was found to express LAG3 in the CSF, but not in the peripheral blood (Fig. 2D). This indicates that CCR9+ Tm cells actively migrate to the CNS during multiple sclerosis or neuromyelitis optica lesions and likely exert an immune function, at least partially, via LAG3. LAG3 expression was seen in one SPMS CCR9+ Tm cells (Supplementary Fig. 6), although more samples are needed to validate such a conclusion.

CCR9+ Tm cells constitutively express C-MAF and are biased toward Th17 in SPMS

To investigate whether CCR9+ Tm cells are functionally altered in multiple sclerosis patients, we analysed the expression of key transcriptional factors involved in the differentiation of the CD4+ T cell subtypes in physiological or inflammatory settings. We sorted CCR9+ and CCR9+ Tm cells from healthy controls, RRMS and SPMS, and performed quantitative RT-PCR using mRNA of TBX21, GATA3, RORC, FOXP3, MAF, and AHR to screen whether the phenotypes had been altered toward the Th1, Th2, Th17, Treg, or Tr1 directions. The Th1 transcriptional factor TBX21 was lower in CCR9+ than in CCR9+ Tm cells in both healthy controls and SPMS, although it was comparable in patients with RRMS (Fig. 3A). Further, Th2 transcriptional factor GATA3 expression was not significantly different between CCR9+ and CCR9+ Tm cells in healthy controls and RRMS. In contrast, GATA3 expression was significantly higher in CCR9+ than CCR9+ Tm cells from patients with SPMS. RORC expression, a Th17 transcriptional factor, was significantly lower in healthy controls CCR9+ than in CCR9+ Tm cells. Surprisingly, CCR9+ Tm cells derived from some SPMS patients expressed exceptionally high levels of RORC (5/16, 31%) by quantitative RT-PCR. Furthermore, RRMS patients with CCR9+ Tm cells expressing high levels of RORC (2/13, 15%) were also found. Patients with high RORC expression had similar %CCR9 and demographic features to patients with low RORC expression (Supplementary Fig. 7). FOXP3 was comparable between CCR9+ and CCR9+ Tm cells in the healthy controls, RRMS, and SPMS groups. In both healthy controls and SPMS patients, CCR9+ Tm cells expressed higher levels of MAF compared to CCR9+ Tm cells (Fig. 3A), also seen in some patients with RRMS. AHR was comparable between CCR9+ and CCR9+ Tm cells of healthy controls, RRMS, and SPMS patients, suggesting that CCR9+ Tm cells are not Tr1 cells (Gagliani et al., 2013). These results implicated that transcription factors expression in CCR9+ Tm cells is differentially altered in patients with SPMS compared to healthy controls and RRMS patients.

To detect these changes at the protein level and validate the screening results, we performed intracellular staining of transcriptional factors. In the healthy controls, RRMS and SPMS groups, C-MAF, encoded by the MAF gene, was significantly increased in CCR9+ Tm cells compared with CCR9+ Tm cells, although its expression levels in CCR9+ Tm cells did not differ between the groups. In healthy controls, CCR9+ Tm cells expressed lower levels of RORyt, encoded by RORyt, than CCR9+ Tm cells (Fig. 3B). Notably, the expression of RORyt in CCR9+ Tm cells was significantly higher in the SPMS group than in the healthy controls, although RORyt expression in CCR9- Tm cells did not differ between the healthy controls and the RRMS/SPMS groups. T-BET expression, a protein encoded by TBX21, was also reduced in CCR9+ Tm cells compared to CCR9- Tm cells in all groups (Fig. 3B). Altogether, the data indicated that CCR9+ Tm cells are significantly biased toward a Th17 phenotype in SPMS, providing a possible link to SPMS pathogenesis.

CCR9+ Tm cells in SPMS have an inflammatory phenotype

To examine the cytokine profile of CCR9+ Tm cells and determine whether it deviated toward the Th17 phenotype in SPMS, we sorted CCR9+ and CCR9+ Tm cells, stimulated them, and measured IL-4, IL-9, IL-10, IL-17A, GM-CSF, IFNy, and TNFα levels, which are known to modulate CNS inflammation. For this analysis, we age-matched healthy controls to RRMS and SPMS patients and included elderly healthy controls to examine the alteration in CCR9+ Tm cell function with ageing (Supplementary Table 2). An absence of significant differences was observed in CCR9+ and CCR9+ Tm cells proliferative capacity (Supplementary Fig. 8A). Healthy control CCR9+ Tm cells produced higher levels of IL-4 and IL-10 than CCR9+ Tm cells, which is consistent with the higher C-MAF expression in CCR9+ Tm cells (Fig. 4A) (Ouyang et al., 2000; Apetoh et al., 2010).

Successively, Tm cells cytokine production was compared across groups and prominent variations in the absolute cytokine production value among samples were seen (Supplementary Fig. 8B), rendering the comparison challenging. This was likely caused by the genetic background, prescription drugs, and experimental conditions. To account for these variables and evaluate whether CCR9+ Tm cells exhibit population-specific alterations in cytokine production during SPMS, we calculated the ratio of cell proliferation:production in each CCR9+ Tm-derived cytokine to cytokines from CCR9- Tm cells in each individual. The CCR9+ :CCR9- ratios regarding cell proliferative responses were not different between the healthy controls, RRMS and SPMS, and elderly healthy controls groups (Fig. 4B). In contrast, we reported altered CCR9+ :CCR9- cytokine production ratio among these groups (Fig. 4C). For example, the CCR9+ :CCR9- ratio for IL-4 (IL-4 ratio) tended to decrease in RRMS, SPMS and elderly healthy controls when compared to younger healthy controls, although this finding was not statistically significant.
Figure 3 Analysis of the transcriptional factors of CCR9\(^+\) CD4\(^+\) memory T cells. (A) Gene expression of the indicated transcriptional factors of PBMCs of 14 healthy controls (HC), 13 RRMS and 16 SPMS. Messenger RNA was obtained from CCR9\(^+\) or CCR9\(^-\) CD4\(^+\) Tm cells and analysed by quantitative RT-PCR. Expression levels are normalized to Actb (\(\beta\)-actin). \(*\) \(p < 0.05\), \(*\) \(p < 0.01\), \(*\) \(p < 0.001\), n.s. (not significant) by Wilcoxon signed-rank tests (one-sided). Box and whisker plots are shown. Whiskers are drawn from minimum to maximum points. Each individual value is plotted as a dot superimposed. (B) Intracellular staining of the transcriptional factors was performed in PBMCs from six healthy controls, six RRMS and 10 SPMS. Proportion of transcriptional factor-positive cells in CCR9\(^+\) or CCR9\(^-\) CD4\(^+\) Tm cells are displayed. \(*\) \(p < 0.05\), n.s. (not significant) by one-way ANOVA with Dunnet’s multiple comparison tests, comparing HC-CCR9\(^+\) CD4\(^+\) Tm cells with RRMS-, SPMS-CCR9\(^+\) CD4\(^+\) and HC-CCR9\(^-\) Tm cells, or comparing HC-CCR9\(^-\) CD4\(^+\) Tm cells with RRMS-, and SPMS-CCR9\(^-\) Tm cells.
Further, the IL-10 ratio was comparable between healthy controls, RRMS, SPMS, although a decreasing trend was seen in elderly healthy controls. However, the IL-17A ratio was elevated in the SPMS group, while elderly healthy controls reported an increasing trend compared with healthy controls and the RRMS group. The IFNγ ratio was

![Figure 4](image-url)
significantly higher in SPMS patients and, surprisingly, also in elderly healthy controls. Finally, the IL-9 ratio tended to increase in SPMS patients, whereas GM-CSF and TNFα were comparable among healthy controls and RRMS and SPMS patients, although it tended to decrease in elderly healthy controls. These results suggest that CCR9+ Tm cells of patients with SPMS are specifically deviated to the inflammatory phenotype and that similar alterations could be seen in elderly healthy controls.

As both TGFβ1 and TGFβ3 from CD4+ T cells are known to limit the development of immunopathology (Li et al., 2007; Okamura et al., 2009), we additively examined these cytokines. TGFβ1/β3 production from CCR9+ and CCR9- Tm cells was comparable in healthy controls (Supplementary Fig. 8C). Furthermore, TGFβ1/TGFβ3 ratios were comparable between the healthy controls, RRMS, SPMS, and elderly healthy control groups.

Overall, these data indicate CCR9+ Tm cells bias toward bearing an inflammatory phenotype in SPMS, resembling aged CCR9+ Tm cells.

**CCR9+ Tm cells are influenced by the gut microbiota**

CCR9 expression by CD4+ T cells is induced by retinoic acid, which is produced by intestinal dendritic cells (Iwata, 2009). As determining whether the CCR9+ Tm cells are influenced by the gut microbiota is of great interest, the numbers of mouse CD4+ Tm cells in the peripheral blood of germ-free and SPF (Fig. 5) mice were analysed. The frequency of CCR9+ cells in CD4+CD44hiTCRβ+ Tm cells, which resemble human CCR9+ Tm cells, was similar between SPF and germ-free mice. Furthermore, %CCR9 for peripheral blood Tm cells was much higher in mice than in humans (Figs 1A and 5A), indicating the dominant role of the gut in shaping the CD4+ Tm-cell repertoire in mice. Notably, %CCR9 was reduced in germ-free mice compared to SPF mice, suggesting a role for the gut microbiota in CCR9+ Tm-cell induction. The number of small intestinal CD4+ intraepithelial lymphocytes was greatly reduced in germ-free mice.

To evaluate the influence of the gut microbiota on CCR9+ Tm cells, we subsequently treated wild-type SPF mice with a mixture of antibiotics capable of modulating their gut microbiota. Thereafter, the frequency of CD44hi memory cells in CD4+ T cells tended to increase, although this finding was not statistically significant (Fig. 5B). Moreover, the antibiotic treatment induced a higher %CCR9 in SPF mice in contrast to germ-free mice.

To understand the involvement of CCR9+ Tm cells in EAE further, we examined whether CCR9+ Tm cells could infiltrate the CNS and a secondary lymphoid organ of EAE mice. Most of the infiltrating CD4+CD44hi Tm cells in the CNS were CCR9+CD44+ T cells in EAE early time points and gradually decreased during the disease course (Fig. 5C). These data support the preferential migratory capacity of CCR9+ Tm cells to the CNS. CCR9+ Tm cells are present in the spleen during EAE (Supplementary Fig. 9). Furthermore, CCR9+ T cells were found to express LAG3 in the CNS only in its late phase (Fig. 5D), whereas CCR9+CD44+ T cells did not express LAG3 in the spleen (Supplementary Fig. 9). These findings suggest an alteration in CCR9+ T cell phenotype during EAE disease course and a significant potential for CCR9+ T cells in the modulation of CNS inflammation, similar to regulatory IELs (Kadowaki et al., 2016). Next, we induced EAE in wild-type mice and treated them with short-term antibiotic therapies, which induced an increase in %CCR9+ in CD44hi cells (Fig. 5B) and led to a significant reduction in EAE severity (Fig. 5E). Furthermore, EAE mice were treated with blocking antibodies against MADCAM1, which are known to block CCR9+CD4+ T cells migration to the gut, increasing %CCR9+ T cells outside the gut and in secondary lymphoid organs (Cassani et al., 2011). MADCAM1 blocking antibodies injected around the day of EAE-induction significantly ameliorated EAE severity until ~Day 18 (Fig. 5F). These data suggest that the accumulated CCR9+CD4+ Tm cells in the peripheral circulation may ameliorate the development of acute CNS autoimmune inflammation.

**Discussion**

In the present study, we provide evidence that gut tropic CCR9+ Tm cells represent a key regulatory lymphocyte involved in SPMS pathogenesis. We demonstrated a reduction in gut-tropic CCR9+ Tm cells in human peripheral blood and the deviation of their phenotype to an inflammatory Th17 phenotype in patients with SPMS. CCR9+ Tm cells, which upregulated LAG3 in the CSF, expressed high levels of C-MAF and produced disease-protective cytokines. Therefore, gut tropic CCR9+ Tm cells may play a critical role in the natural regulation of CNS autoimmunity; their reduced frequency and altered phenotype may (at least partly) account for the non-remitting nature of SPMS. We also showed the alterations of CCR9+ Tm cell frequency or function by ageing and changes in the gut microbiota.

We showed that %CCR9 decreased linearly with age in healthy individuals. The naïve T cell compartment is significantly diminished in elderly individuals given the involution of the thymus (Montecino-Rodriguez et al., 2013). Since CCR9+ Tm cells are mainly induced by activation of naïve T cells in gut-associated lymphoid tissues, the decreased number of CCR9+ Tm cells in the elderly resulted likely from the reduction of naïve T cells. Conversely, as the core microbiota of elderly individuals is distinct from that of younger adults (Claesson et al., 2011), an alternative possibility is that the alteration of the gut microbiota composition with age may explain the decrease of %CCR9 in elderly people. Surprisingly, we also found a similar deviation of CCR9+ Tm cells in elderly people inflammatory phenotype and SPMS. It is tempting
Figure 5 Alteration of CCR9+ CD4+ memory T cells by changing the gut microbiota and their immunoregulatory potential.

(A) The proportions (%) of CD44hi cells in CD4+ TCRβ+ cells and the proportions (%) of CCR9 in CD4+ TCRβ+ CD44hi cells were analysed in CCR9+ CD4+ T cells in SPMS BRAIN 2019: 142; 916–931 | 927

(continued)
to hypothesize that the reduction in regulatory cells implies immune system ageing and that it can facilitate the transition of multiple sclerosis to a progressive phase with brain ageing (Mahad et al., 2015). Although %CCR9 tended to be lower in RRMS, it was more remarkably reduced in SPMS. Previously, we reported that patients with multiple sclerosis can be characterized by a lower abundance of bacteria species belonging to the Clostridiala cluster XIVa and IV, which produce short-chain fatty acids (SCFA) and exert anti-inflammatory roles (Miyake et al., 2015). Since a decreasing trend of %CCR9 in RRMS was present, it is possible that such bacterial species producing SCFAs could be even less abundant in SPMS.

In contrast to SPMS, patients with Parkinson’s disease had higher frequencies of CCR9+ Tm cells in the peripheral blood relative to age-matched healthy controls. Contrary to what has been reported for inflammatory bowel disease and multiple sclerosis (Furusawa et al., 2013; Haghikia et al., 2015), SCFAs reportedly activate microglia and reduce motor symptoms in a mouse model of Parkinson’s disease (Sampson et al., 2016). Therefore, the SCFAs produced by the multiple sclerosis and Parkinson’s disease gut microbiota may be significantly different. The intestine is one of the first affected organs in Parkinson’s disease and both small intestinal bacterial overgrowth (SIBO) and dysbiosis were linked to Parkinson’s disease clinical symptoms, which may contribute to the induction of high frequency CCR9+ Tm cells in Parkinson’s disease (Fasano et al., 2013; Keshavarzian et al., 2015; Sampson et al., 2016).

CCR9+ Tm cells were found to infiltrate into the CSF in neuromyelitis optica and less efficiently in RRMS, SPMS, and healthy controls. The high CSF to peripheral blood ratio of %CCR9 was seen in SPMS. The vast majority of CCR9+ Tm cells expressed CXCR3, constitutive CCR6, and CXCR3, chemokine receptors which facilitate the migration into the inflamed CNS. In contrast, lower proportions of CCR9+ Tm cells expressed VLA-4 (α4β1), an integrin important for the transmigration of CD4+ T cells from the periphery into the CNS (Bornsen et al., 2012). Since prominent breakdown of the blood–brain barrier is the hallmark of neuromyelitis optica (Shimizu et al., 2017), CCR9+ Tm cells lacking VLA-4 may migrate to the CNS via CCR6 and CXCR3. Furthermore, as the blood–brain barrier disruption is associated with SPMS (Shimizu et al., 2014), the high CSF to peripheral blood ratio of %CCR9 found in SPMS was expected. The significant increased expression of α4 and β1 on CCR9+ Tm cells in SPMS patients should enhance CCR9+ Tm cells migration into the CNS.

Although neuromyelitis optica is characterized by aquaporin-4 (AQP4)-specific antibodies and considered to be primarily a humoral autoimmune disease, CD4+ T cells are also thought to be involved in its pathogenesis (Pohl et al., 2011). Interestingly, AQP4-specific CD4+ T cells in neuromyelitis optica showed Th17 bias and recognized the Clostridium perfringens adenosine triphosphate-binding cassette transporter permease, which indicates the role of gut-derived CD4+ T cells in neuromyelitis optica pathogenesis (Varrin-Doyer et al., 2012).

A previous report found the proportion of CCR9 to be comparable between CD4+ Tm cells in the peripheral blood (8.0 ± 2.8%) and the CSF of non-inflammatory neurological diseases (5.1 ± 4.3%) (Kivisakk et al., 2006). In contrast, a prominent reduction in CCR9-positive Tm cells was seen in our control CSF samples when compared to the paired PBMCs which were isolated from healthy controls and psychiatric patients. This discrepancy may result from the differences in the background disease status among the control groups.

Both CCR9+ Tm cells in the CSF from RRMS and neuromyelitis optica during relapses and those isolated from EAE lesions, expressed LAG3, which plays an immunoregulatory role in autoimmune diseases (Huang et al., 2004; Liang et al., 2008; Bettini et al., 2011). As CD4+ IELs suppress the symptoms of EAE via LAG3 (Kadowaki et al., 2016), we speculate a role for LAG3 expression in CSF-infiltrated CCR9+ Tm cells. CNS antigen-presenting cells, including ependymal cells and meningial, choroid plexus, and perivascular macrophages (Prinz and Priller, 2017), may both present cognate antigens to CCR9+ Tm cells and facilitate LAG3 expression (Anderson et al., 2016).
Blood analysis from healthy subjects showed CCR9+ Tm cells to have a Th2/Tr1-like phenotype characterized by high C-MAF expression and production of IL-4 and IL-10, two anti-inflammatory cytokines. Furthermore, IL-4 exerts direct neuroprotective effects on neurons, astrocytes, and microglial cells (Gadani et al., 2012). Therefore, CCR9+ Tm cells could exhibit anti-inflammatory and neuroprotective effects. However, CCR9+ Tm cells upregulated RORγt and produced elevated levels of IL-17 and IFNγ in SPMS. Huber et al. (2014) reported the frequencies of IL-17 and IFNγ-producing MBP-specific T cells to be higher in SPMS when compared to healthy subjects and emphasized the role of Th17 cells in SPMS immunopathogenesis (Huber et al., 2014). In this regard, a role of C-MAF is possible, as this molecule not only controls IL-4 and IL-10 production, but also acts as one of the activators of RORγt and IL-17 production (Ouyang et al., 2000; Apetoh et al., 2010; Tanaka et al., 2014). We assume that CCR9+ Tm cells expressing elevated levels of C-MAF are prone to produce high levels of IL-17 when they are exposed to Th17 cell-promoting signals, including cytokines IL-6, IL-23, IL-1β (Zielinski et al., 2012).

Th17-promotive commensals [i.e. the segmented filamentous bacteria (SFB)] would colonize and induce RORγt+ Th17 cells in the ileum, for which local serum amyloid A plays a critical role (Sano et al., 2015). It is of note that bacterial strains analogous to SFB were also demonstrated in humans (Atarashi et al., 2015). Upr egulation of RORγt in CCR9+ Tm cells of patients with SPMS, accompanied by the induction of Th17 cells in the gut, may result from an increase in SFB-analogous bacteria.

Although the frequencies of CCR9+ Tm cells in peripheral blood were reduced in germ-free mice, they were increased in antibiotic-treated mice. It may be relevant to note that upon antibiotic treatment, small intestinal CX3CR1+ dendritic cells migrate to the mesenteric lymph nodes (MLN) and exhibit stronger gut-bacteria-specific T-cell response in the MLN, leading to an increased faecal IgG and IgA production (Diehl et al., 2013). In contrast, germ-free mice are known to have a poor ability to produce gut microbiota-specific faecal IgG and IgA (Zeng et al., Immunity 2016). These published results are helpful in interpreting our results on the differences between antibiotic-treated mice and germ-free mice.

As for the EAE experiments, we used the acute C57BL6 model as it was established to be related to the gut microbiota (Yokote et al., 2008; Lee et al., 2011; Cekanaviciute et al., 2017), although further studies using the NOD model, which includes the progressive phase (Simmons et al., 2013), may be informative.

A potential weakness of this study is that the functional roles of CCR9+ Tm cells in human patients in vivo could not be evaluated, which could be overcome with further mechanistic investigations using mice. Moreover, although the significant reduction in CCR9+ Tm cells in SPMS could not be explained by specific treatments, this issue should be re-addressed in a larger cohort.

To conclude, we propose the progression of RRMS to SPMS to be (at least partially) attenuated by CCR9+ Tm cells. When CCR9+ Tm cells are reduced and their phenotype is altered by factors such as age or modified gut microbiota, the CNS inflammation is dysregulated and SPMS pathology is promoted. Therefore, although further studies are required, CCR9+ Tm cells may be a potential diagnostic marker for SPMS and may be also employed as a potential therapeutic target for multiple sclerosis and other diseases related to the dysregulation of the gut-systemic immune axis in the future.

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A.K. conceived the work, designed the study, performed the experiments, analyzed the data and drafted and revised the manuscript. R.S. performed the experiments and analyzed the data. W.S., T.Y. and Y.L. offered samples. T.Y. revised the manuscript. All the authors offered discussion.

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Competing interests

The authors declare no competing financial interests.

Supplementary material

Supplementary material is available at Brain online.

References


