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# Gut microbiota-dependent CCR9$^+$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 (%CCR9) in SPMS did not correlate with age, disease duration or expanded disability status scale score, although %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 (*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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**Abbreviations:** EAE = experimental autoimmune encephalomyelitis; IEL = intraepithelial lymphocyte; PBMC = peripheral blood mononuclear cell; RRMS = relapsing remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; Tm cell = memory T cell

# 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 conversion (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 abundancy 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 $\alpha4\beta7$, whose ligand (mucosal vascular addressin cell adhesion molecule 1, MADCAM1) is expressed in the gut lamina propria venules. Therefore, CCR9 and $\alpha4\beta7$, 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 $CD8\alpha\alpha$ T cells, not $CD8\alpha\beta$, which are abundantly observed in gut IELs that originate from gut-homing $CD4^+$ and double-negative T cells (Cheroutre *et al.*, 2011). Therefore, $CD8\alpha\alpha$ 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 RORγt (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**

| | HC | Elderly HC | RRMS | SPMS | NMO |
|---|---|---|---|---|---|
| *n* | 22 | 11 | 33 | 34 | 13 |
| Male : Female | 10 : 12 | 4 : 7 | 10 : 23 | 14 : 20 | 2 : 11 |
| Age | 41.6 ± 1.78 | 70.4 ± 1.50 | 42.1 ± 2.35 | 48.1 ± 1.35 | 45.8 ± 4.50 |
| BMI | 21.7 ± 0.60 | 21.6 ± 0.48 | 22.2 ± 0.54 | 20.9 ± 0.82 | 21.9 ± 1.24 |
| Disease duration | N/A | N/A | 9.75 ± 1.47 | 16.7 ± 1.32 | 7.85 ± 2.36 |
| EDSS | N/A | N/A | 2.88 ± 0.27 | 6.03 ± 0.25 | 4.34 ± 2.46 |
| Medication for MS/NMO | N/A | N/A | 13 steroid<br>6 immunosuppressant<br>7 interferon-β<br>6 glatiramer acetate<br>4 dimethyl fumarate | 23 steroid<br>15 immunosuppressant<br>5 interferon-β<br>3 glatiramer acetate<br>4 fingolimod<br>1 natalizumab | 11 steroid<br>6 immunosuppressant |
| | **NIND** | **PD** | **Non-PD** | | |
| *n* | 17 | 9 | 8 | | |
| Male : Female | 12 : 5 | 5 : 4 | 7 : 1 | | |
| Age | 69.5 ± 2.92 | 73.1 ± 2.52 | 64.9 ± 5.32 | | |
| BMI | 21.2 ± 0.98 | 21.8 ± 1.16 | 20.4 ± 1.82 | | |
| Diagnosis | N/A | N/A | 3 ALS<br>3 SCD<br>1 MSA-C<br>1 dementia | | |

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.

RNeasy® mini/micro kit (Qiagen), whereas the complementary DNA 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′-CACTCTTCCAGCCTTCCTTCC-3′, reverse 5′-GCATACAG GTCTTTGCGGATG-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 ($5.0 \times 10^4$) 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 $^3$H-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/6 J mice were purchased from the CLEA 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 ng 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®, overlaid on 80% Percoll®, and centrifuged for 30 min at 970*g* at 4°C. Cells at the interface were CNS mononuclear cells. Non-specific staining was inhibited through incubation with anti-CD16/32 (BioLegend). Cells were then stained with fluorescence-labelled antibodies, whereas dead cells were stained by 7-AAD. Antibodies against TCRβ (H57–597), CD4 (RM4–5, GK1.5), CD8α (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.

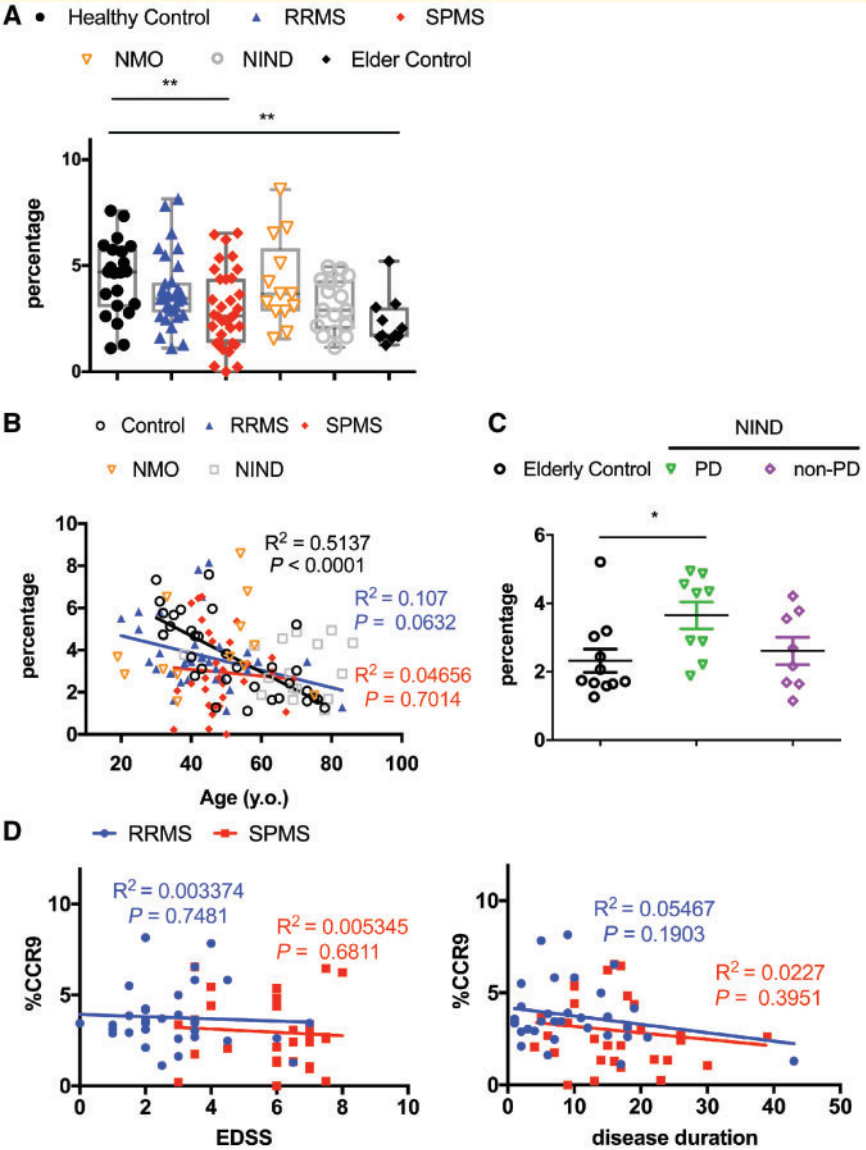


**Figure 1 Proportion of CCR9$^+$ cells in peripheral blood memory CD4$^+$T cells.** (**A**) The proportion of CCR9$^+$ cells in CD4$^+$ memory T cells (%CCR9) from healthy controls, elderly healthy controls (HC), and patients with RRMS, SPMS, neuromyelitis optica (NMO), non-inflammatory neurodegenerative 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). $R^2$ 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). $R^2$ 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 $43.7 \pm 1.81$ SPMS $45.7 \pm 0.84$ years old, mean $\pm$ 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 $\alpha 4$ integrin (Fig. 2B). Further, $\alpha 4$ and $\beta 1$ constitute VLA-4, which binds the vascular cell adhesion protein 1 expressed on vascular endothelial cells of inflamed tissues. The proportions of $\alpha 4^+\beta 1^+$ 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 $\alpha 4^+\beta 1^+$ 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 RRMS 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 LAG3 in inflamed tissues (Svensson *et al.*, 2002; Guy-Grand *et al.*, 2013; Kadowaki *et al.*, 2016), we examined whether $CCR9^+$ Tm cells might express LAG3 in the CSF. Furthermore, LAG3 is considered a marker of type 1 regulatory T (Tr1) cells (Gagliani *et al.*, 2013). We analysed LAG3 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

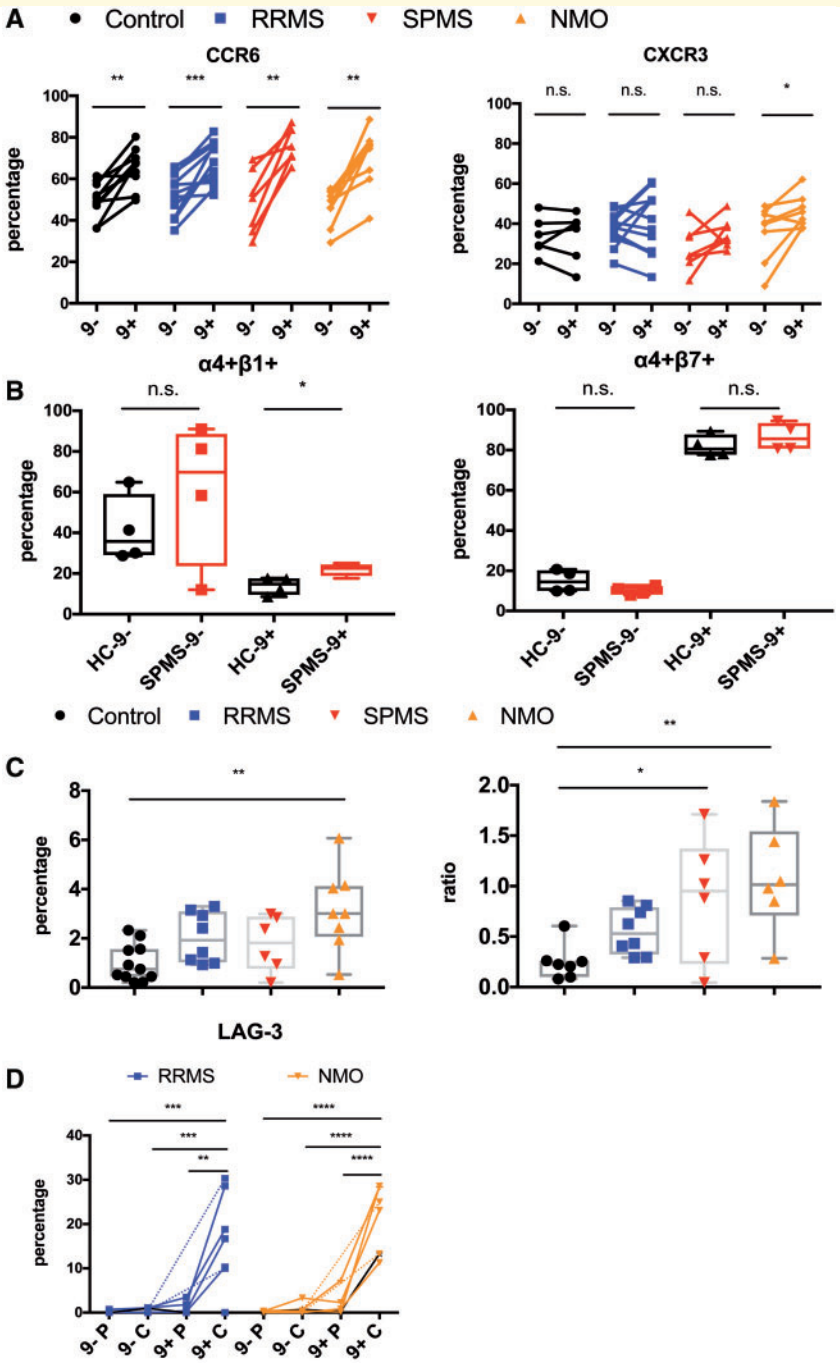


**Figure 2 Analysis of the homing capacity of CCR9$^+$ CD4$^+$ memory T cells and their infiltration into the CSF.** (**A**) CCR6 expression in CCR9$^-$ (9−) or CCR9$^+$ (9+) CD4$^+$ memory T cells of PBMCs derived from randomly selected individuals [10 healthy control (HC), seven RRMS, 11 neuromyelitis 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 ROR$\gamma$t, encoded by *RORC,* than CCR9$^-$ Tm cells (Fig. 3B). Notably, the expression of ROR$\gamma t$ in CCR9$^+$ Tm cells was significantly higher in the SPMS group than in the healthy controls, although ROR$\gamma t$ 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, IFN$\gamma$, and TNF$\alpha$ 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.

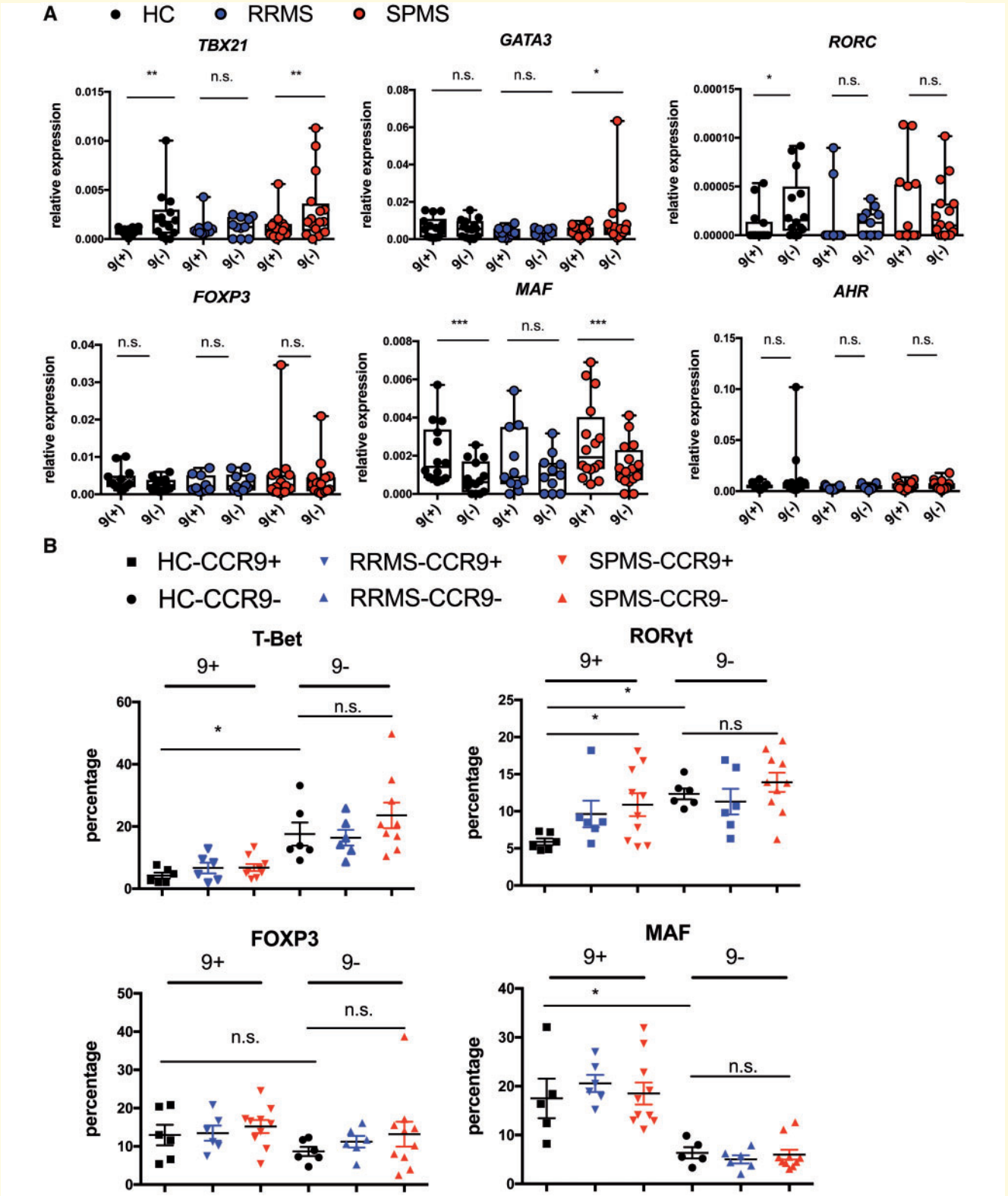


**Figure 3 Analysis of the transcriptional factors of CCR9$^+$ CD4$^+$ memory T cells.** (**A**) Gene expression of the indicated transcriptional factors of PBMCs of 14 healthy controls (HC), 13 RRMS and 16 SPMS. Messenger RNA was obtained from CCR9$^+$ or CCR9$^-$ CD4$^+$ Tm cells and analysed by quantitative RT-PCR. Expression levels are normalized to *Actb* (β-actin). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, 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.

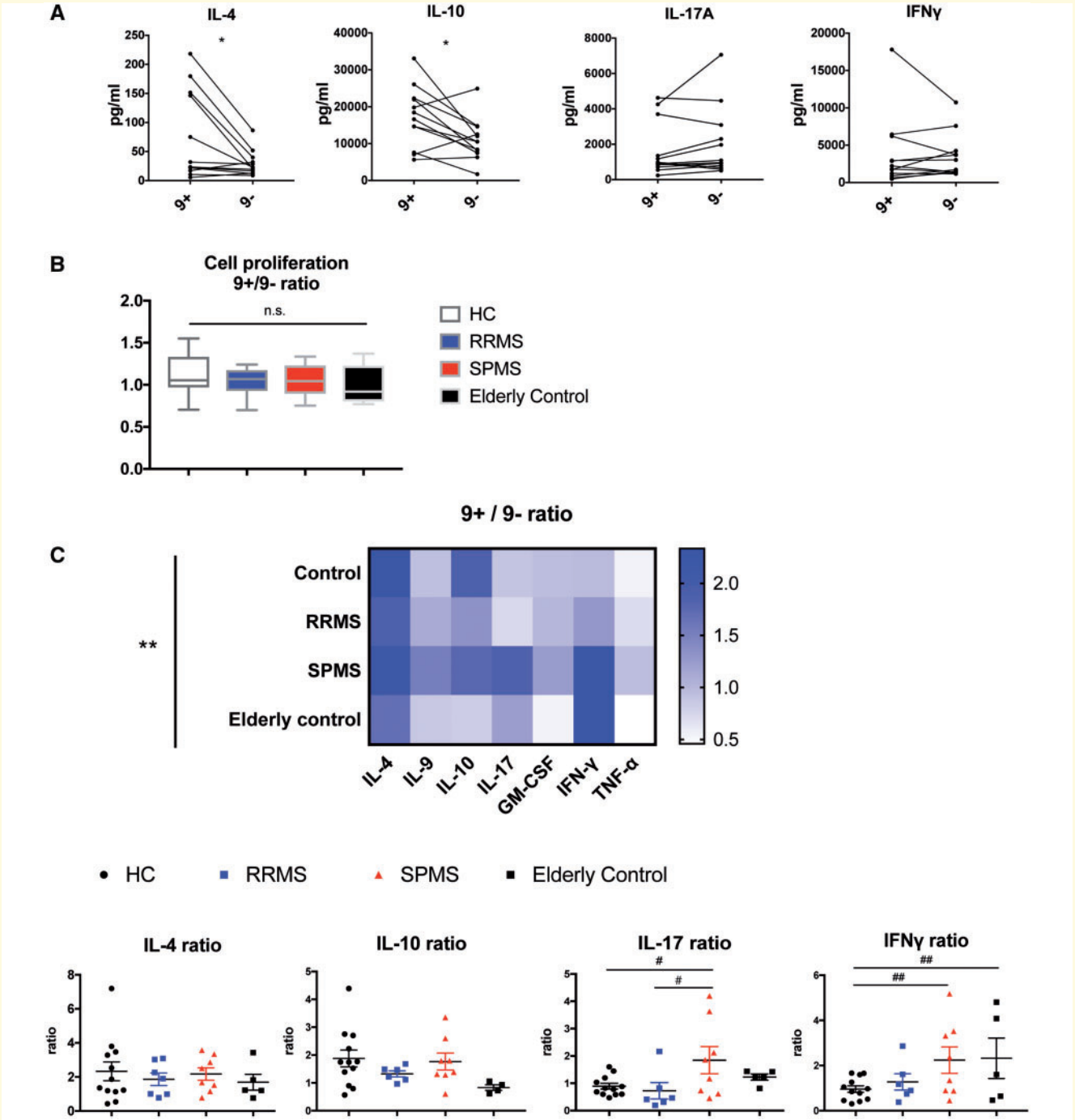


**Figure 4 Analysis of the cytokine production of CCR9$^+$ CD4$^+$ memory T cells.** (**A**) Cytokine production from CCR9$^+$ (9+) or CCR9$^-$ (9−) CD4$^+$ T cells in healthy controls (HC) were measured (mean and SEM). *$P < 0.05$, n.s. (not significant) by Wilcoxon signed-rank test (two-sided). (**B**) CCR9+ or CCR9− Tm cells were analysed from 11 healthy controls, six RRMS, six SPMS, and five elderly healthy control subjects among Supplementary Table 2, and cell proliferation was analysed by incorporating 3H-thymidine (c.p.m.) (Supplementary Fig. 5). Ratios were calculated by (c.p.m of CCR9+ Tm cells)/(c.p.m. of CCR9− Tm cells). Box and whiskers are shown. Whiskers are drawn between 2.5–97th percentile. Not significant (n.s.) by one-way ANOVA. (**C**) Ratio of the cytokine production levels from 9+ to 9− CD4+ T cells (9+/9−) were calculated in each healthy control, RRMS, SPMS, and elderly healthy control sample from Supplementary Table 2. Outliers are determined by ROUT analysis and removed. Mean ratios are represented in a heatmap and dot plots of the 9+/9− ratio of some of the cytokines evaluated are displayed below (mean and SEM). *$P < 0.05$ by two-way ANOVA., $^{\#}P < 0.1$, $^{\#\#}P < 0.05$ by Tukey's multiple comparison post-tests.

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 IFN$\gamma$ ratio was

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$^+$CD44$^{hi}$TCRβ$^+$ 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 CD44$^{hi}$ 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$^+$CD44$^{hi}$ Tm cells in the CNS were CCR9$^+$CD4$^+$ 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$^+$CD4$^+$ T cells did not express LAG3 in the spleen (Supplementary Fig. 9). These findings suggests 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 CD44$^{hi}$ 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

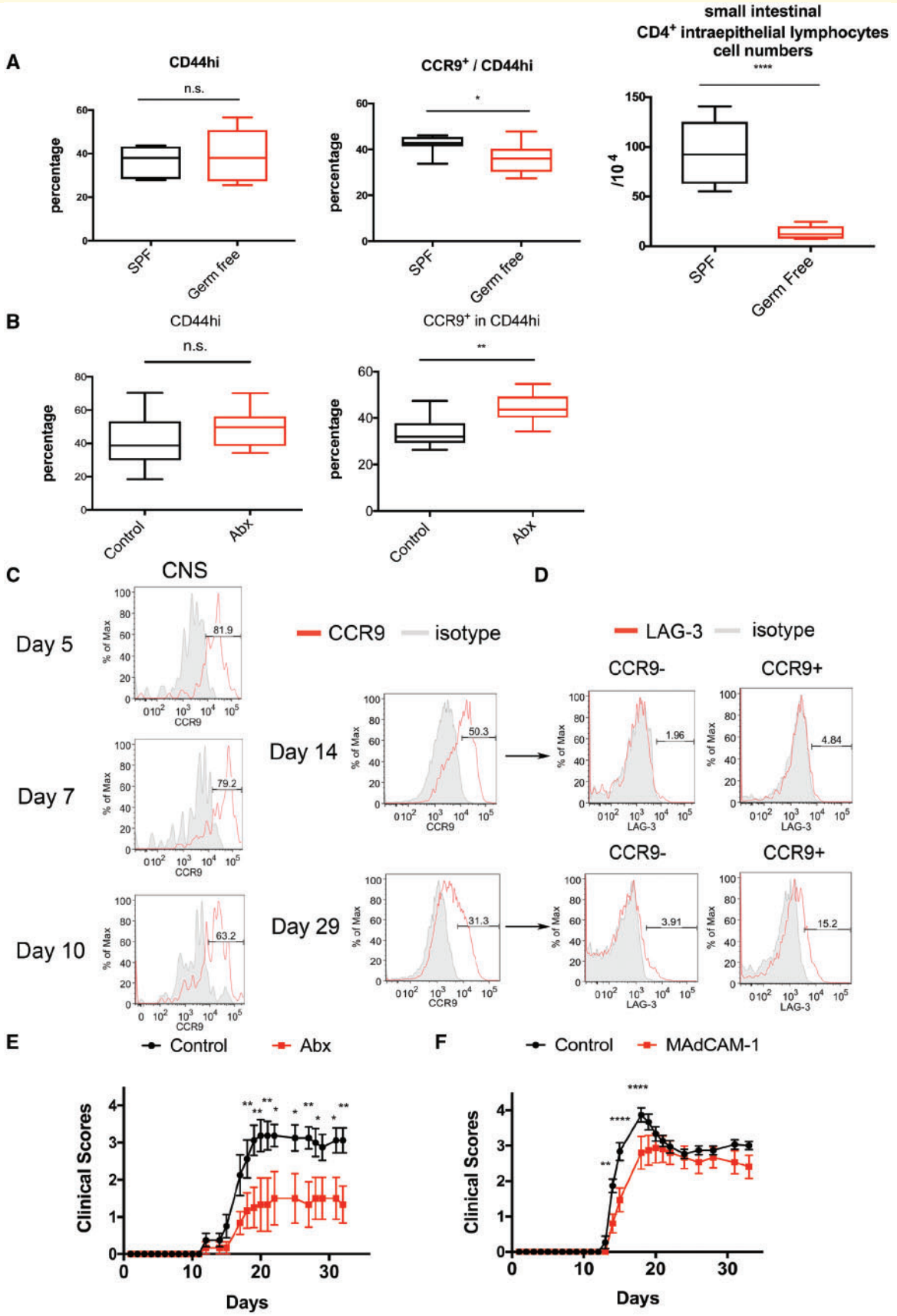


**Figure 5 Alteration of CCR9$^+$ CD4$^+$ memory T cells by changing the gut microbiota and their immunoregulatory potential.** (**A**) The proportions (%) of CD44$^{hi}$ cells in CD4$^+$TCRβ$^+$ cells and the proportions (%) of CCR9 in CD4$^+$TCRβ$^+$CD44$^{hi}$ cells were analysed in

(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 *Clostridia* 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 CCR6 and constitutive CXCR3, chemokine receptors which facilitate the migration into the inflamed CNS. In contrast, lower proportions of CCR9$^+$ Tm cells expressed VLA-4 ($\alpha$4$\beta$1), an integrin important for the transmigration of CD4$^+$ 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 $\alpha$4 and $\beta$1 on CCR9$^+$ 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 ($8.0 \pm 2.8\%$) and the CSF of non-inflammatory neurological diseases ($5.1 \pm 4.3\%$) (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 epiplexus cells and meningeal, 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).

**Figure 5** Continued

eight age-matched specific pathogen-free mice (SPF) and seven germ-free mice. IELs were collected at the same time and absolute cell numbers of CD4$^+$ TCR$\beta^+$IEL were counted. The data are a combination of two experiments. Box and whisker plots are depicted. (**B**) Mice were treated with distilled water (control; $n = 17$) or a mixture of antibiotics (Abx; $n = 11$) for 8 days and peripheral blood was obtained. The proportions (%) of CD44$^{hi}$ cells in CD4$^+$TCR$\beta^+$ cells and the proportions (%) of CCR9 in CD4$^+$TCR$\beta^+$CD44$^{hi}$ cells were analysed. The data are a combination of three experiments. (**A** and **B**) Box and whisker plots are depicted. Whiskers were drawn between the 2.5 and 97th percentile. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, n.s. (not significant) by unpaired *t*-test (two-sided). (**C**) EAE was induced and mononuclear cells of the CNS were collected on Days 5, 7, 10, 14, and 29. Expression of CCR9 in CD44$^{hi}$CD4$^+$TCR$\beta^+$ Tm cells was analysed. Data are pooled from three to five mice. (**D**) Expression of LAG3 in CCR9$^+$ (*right*) or CCR9$^-$ Tm cells (*left*) infiltrated in the CNS was assessed on Days 14 and 29. Bars represent per cent positive cells. (**C** and **D**) Representative of two experiments are shown. (**E**) EAE was induced (Day 0) and mice were orally treated with distilled water (control; $n = 8$) or a mixture of antibiotics (Abx; $n = 6$) from Days 0 to 7 as in **B** and clinical scores were assessed (mean and SEM). The data are a combination of two experiments. (**F**) Anti-MADCAM1 monoclonal antibody or isotype-matched antibodies (control) were administrated to mice ($n = 15$ per group). EAE was induced (Day 0) and clinical scores were assessed (mean and SEM). The data are a combination of three experiments. (**E** and **F**) $^{****}P < 0.001$, $^{**}P < 0.01$, $^*P < 0.05$ by two-way ANOVA with Bonferroni's post-test.

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 ROR$\gamma$t and produced elevated levels of IL-17 and IFN$\gamma$ in SPMS. Huber *et al.* (2014) reported the frequencies of IL-17 and IFN$\gamma$-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 ROR$\gamma$t 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 ROR$\gamma$t$^+$ 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). Upregulation of ROR$\gamma$t 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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# Competing interests

The authors declare no competing financial interests.

# Supplementary material

Supplementary material is available at *Brain* online.

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