

## Fraction of IL-10<sup>+</sup> and IL-17<sup>+</sup> CD8 T cells is increased in MS patients in remission and during a relapse, but is not influenced by immune modulators

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### ABSTRACT

In the present study, circulating proportions of CD8<sup>+</sup> T (Tc) cell subsets, including IL-17 (Tc17) and IL-10 (Tc10) producing cells, were assessed in relapsing–remitting MS (RRMS) patients and a possible effect of beta interferon (IFN-β), glatiramer acetate (GA), and vitamin D (VitD) on these cell subsets was investigated. We show that both Tc17 and Tc10 cell fractions are elevated in the circulation of RRMS patients in remission compared to healthy subjects and that these Tc subsets remain unaffected by current immune modulating regimens.

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### 1. Introduction

Multiple sclerosis (MS) is considered to be a T cell mediated autoimmune disease. Although research focused mainly on CD4<sup>+</sup> T helper (Th) cells, CD8<sup>+</sup> cytotoxic T (Tc) cells may be importantly involved in MS pathogenesis as well. It was demonstrated that adoptively transferred myelin specific Tc cells could induce experimental autoimmune encephalomyelitis (EAE) (Huseby et al., 2001; Sun et al., 2001), the animal model for MS. Certain MHC class I alleles were identified as MS disease risk alleles (reviewed by Martin, 2008) and MHC class I expression was higher on astrocytes and oligodendrocytes in MS lesions compared to normal brain tissue (Hoftberger et al., 2004). Tc cells could be detected in MS brain lesions and they even outnumbered Th cells. These Tc cells were found in the vicinity of damaged oligodendrocytes and myelin containing antigen presenting cells (Neumann et al., 2002; Serafini et al., 2006). In addition, the intraliesional Tc cells, as well as Tc cells in the cerebrospinal fluid (CSF), are clonally expanded, suggesting antigen specific reactivity (Babbe et al., 2000; Jacobsen et al., 2002).

Together, these data suggest active involvement of Tc cells in MS pathogenesis.

Like Th cells, Tc cells can be divided into different subsets based on their cytokine profile. In MS patients, research focused mainly on peripheral IFN-γ<sup>+</sup> and IL-4<sup>+</sup> Tc cells (Tc1 and Tc2, respectively). Studies regarding the Tc1 cell percentages show contradictory results, whereas studies regarding the Tc2 cell fractions show no difference between MS patients and healthy controls (Becher et al., 1999; Inoges et al., 1999; Furlan et al., 2000; Killestein et al., 2001; Ochi et al., 2001; Sepulcre et al., 2005). Recently, an IL-17 producing cell subset within the Tc cell compartment (Tc17) was identified. Interestingly, Wang et al. showed that Tc17 cells are increased in the peripheral blood of relapsing–remitting MS patients (RRMS) (Wang et al., 2011) and more than 70% of the Tc cells in MS brain tissue are IL-17 positive (Tzartos et al., 2008). Next to Tc17 cells, IL-10 producing Tc (Tc10) cells have been described as cells important in tissue protection during a viral infection (reviewed by Zhang and Bevan, 2011). These cells might also be importantly involved in controlling inflammation in MS patients.

In this study, we investigated the Tc cell compartment, including Tc17 and Tc10 cells, in healthy volunteers and RRMS patients in remission and during a relapse. We measured fractions of Tc cell subsets and investigated the effects of first line immune modulating treatments for RRMS, i.e. beta interferon (IFN-β) and glatiramer acetate (GA). Moreover, we examined the effect of high dose vitamin

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D supplementation, as add-on therapy to IFN- $\beta$ , on Tc10 and Tc17 cell fractions.

## 2. Methods

### 2.1. Patients and blood sampling

In total, 73 RRMS patients were included in this study. Sixty-four RRMS patients were enrolled from the Academic MS Center at Orbis Medical Center (Sittard, The Netherlands), and 9 RRMS patients were enrolled from the MS Center at Amphia Hospital (Breda, The Netherlands). RRMS was defined by the revised McDonald criteria (Polman et al., 2011). Only RRMS patients with a disease duration < 6 years were included and RRMS patients in remission had to be relapse free for at least 6 weeks. Written informed consent was obtained from each study subject and the study was approved by the regional ethical committee 'Atrium-Orbis-Zuyd', 'Local Advisory Board on Scientific Research' of Orbis Medical Center and 'the medical ethical committee of Amphia' of Amphia Hospital.

Tc cell fractions in HC and MS patients in remission or during a relapse and the effects of IFN- $\beta$  and GA were studied in a cross-sectional cohort study in which 59 RRMS patients and 30 HC were enrolled. Gender and age of the MS patients matched that of the HC. Characteristics of these populations are given in Table 1. Forty-seven MS patients were in disease remission and were either using no immune modulatory drugs ( $n = 16$ ), or were treated with IFN- $\beta$  1a/1b ( $n = 18$ ) or GA ( $n = 13$ ). Twelve RRMS patients were included while having a relapse. None of these patients were on immune modulating therapy. A relapse was defined as occurrence of new symptoms which lasted at least 24 h and required treatment with intravenous methylprednisolone. Blood samples were retrieved before methylprednisolone infusion.

The association of vitamin D with different Tc cell subsets was studied in the above described cross-sectional cohort and the influence of vitamin D supplementation on the composition of the Tc cell compartment was studied in 14 RRMS patients who received a daily dose of 20,000 IU vitamin D<sub>3</sub> for 12 weeks. All 14 supplemented patients were in remission and were treated with IFN- $\beta$  1a/1b. Blood samples for T cell phenotyping were retrieved at baseline and at 12 weeks. Detailed population characteristics are described by Smolders et al. (2010b).

### 2.2. Serum 25(OH)D assessment

Serum samples were stored directly after sampling until analysis at -20 °C. A radioimmunoassay kit (Immunodiagnostic Systems, Boldon, UK) was used to determine serum 25-hydroxyvitamin D (25(OH)D) levels (representing the vitamin D status).

### 2.3. Intracellular flow cytometry

Ficoll density gradient centrifugation (Histopaque; Sigma Aldrich, Zwijndrecht, The Netherlands) was used to isolate peripheral blood mononuclear cells (PBMC). To assess the intracellular CD8<sup>+</sup> T cell cytokine profile, PBMC were stimulated with 1  $\mu$ g/mL calcium ionomycin (Sigma Aldrich) and 50 ng/mL PMA (Sigma Aldrich) for 5 h. Monensin (BD Biosciences, Breda, The Netherlands) was added simultaneously to block cytokine secretion. After stimulation, cells were stained extracellularly with anti-CD3-horizon V450 and anti-CD8-APC-H7 (both BD Biosciences). Next, the cells were fixed and permeabilized using fixation/permeabilization buffer (BD Biosciences). Cells were then stained intracellularly with anti-IFN- $\gamma$ -FITC (BD Biosciences), anti-IL-4-PE, anti-IL-10-APC and anti-IL-17A-PerCP-Cy5.5 (all Biolegend, Uithoorn, The Netherlands). Samples were acquired on a FACS Canto II flow cytometer (BD Biosciences) and data analysis was performed using the FACS Diva software (BD Biosciences). On average 10,000 events within the CD8<sup>+</sup> T cell gate were acquired. A representative example of our gating strategy and specificity of the staining is illustrated in Fig. 1.

### 2.4. Statistics

Statistical analysis was performed with Statistical Package for Social Sciences version 17.0 software (SPSS Inc., Chicago, IL). Continuous data are given as median with corresponding interquartile range (IQR). Differences in continuous variables between groups were tested using the Mann-Whitney *U* test. Associations between the vitamin D status and the different cytokine producing CD8<sup>+</sup> T cells were assessed with the Spearman correlation. Paired variables were analyzed with the Wilcoxon Signed Ranks test. A *p*-value < 0.05 was considered statistically significant.

## 3. Results

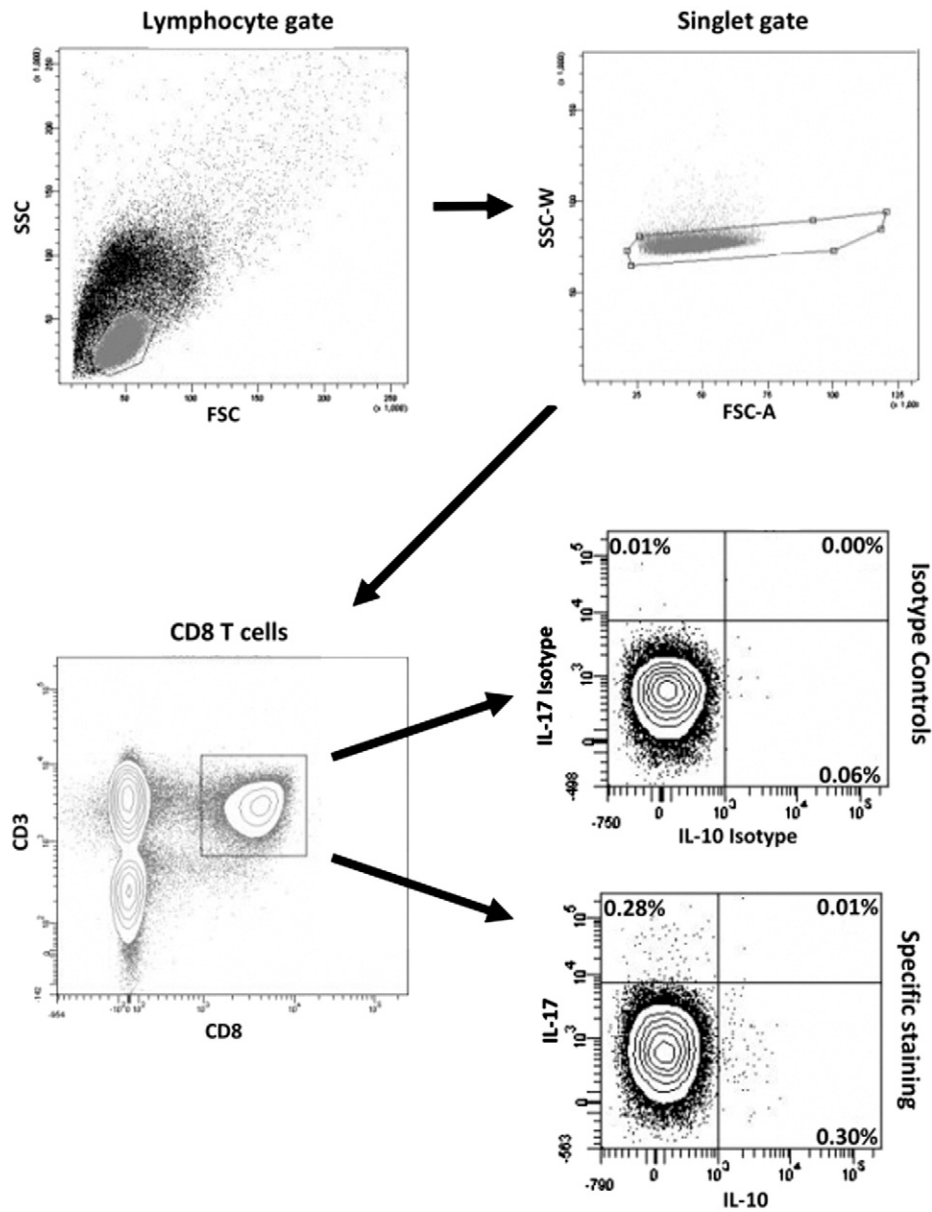
### 3.1. The fraction cytokine producing CD8<sup>+</sup> T cell subsets in RRMS patients

First, we assessed the percentages of IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-10<sup>+</sup> and IL-17<sup>+</sup> cells within the CD8<sup>+</sup> T cell population (Tc1, Tc2, Tc10 and Tc17, respectively) in HC, in untreated RRMS patients in remission, and in untreated RRMS patients during a relapse. The fraction Tc1 cells (Fig. 2A) was similar between HC (24.2% [16.9–35.0]) and untreated RRMS patients in remission (34.2% [14.8–42.2]) and between untreated RRMS patients in remission and during a relapse (23.8% [17.6–34.8]). In addition, Tc2 cell percentages (Fig. 2B) did not differ between untreated RRMS patients in remission (1.6% [0.8–2.9]) and HC (1.2% [0.8–1.6]), or untreated RRMS patients during a relapse (1.1% [0.7–1.3]). Interestingly, the median Tc10 cell percentage (Fig. 2C) was higher in untreated RRMS patients in remission (0.5% [0.2–0.5]) compared to HC (0.2% [0.2–0.3], *p* = 0.011), while there was no

**Table 1**  
Population characteristic cross-sectional study cohort.

Treatment	Number (%) / median [IQR]				
	HC	MS remission No	IFN- $\beta$	GA	MS relapse No
Number of subjects	30	16	18	13	12
Gender:					
Male	8 (26.7)	2 (12.5)	4 (22.2)	1 (7.7)	3 (25.0)
Female	22 (73.3)	14 (87.5)	14 (77.8)	12 (92.3)	9 (75.0)
Age (y)	41.0 [30.8–47.3]	38.4 [29.0–46.8]	43.0 [32.5–51.0]	39.5 [33.7–46.2]	32.0 [28.3–38.0]
Disease duration (y)	–	2.6 [0.7–4.1]	3.6 [1.9–4.4]	1.2 [0.1–2.1]	2.7 [0.4–3.9]
Relapse rate (relapse/y)	–	1.0 [0.0–1.0]	0.0 [0.0–0.3]	0.0 [0.0–1.0]	1.0 [0.0–1.0]
Time since last relapse (y)	–	0.8 [0.2–1.0]	2.5 [0.6–4.4]	0.9 [0.4–1.2]	1.0 [0.4–2.2]
EDSS score	–	1.5 [1.0–2.5]	1.5 [1.0–3.3]	1.5 [1.0–1.5]	2.0 [1.3–2.5]

HC = healthy controls; MS = multiple sclerosis; EDSS = expanded disability status scale; IQR = interquartile range; IFN- $\beta$  = beta interferon; GA = glatiramer acetate; No = no treatment.



**Fig. 1.** Representative gating strategy for Tc10 and Tc17 cells. A representative gating strategy is depicted starting at the lymphocyte gate. Within this gate singlets were selected. From the singlet gate, the fraction CD8 CD3 positive cells were determined. Within the CD8 T cell gate we investigated the IL-10 and IL-17 positive cells. The specific staining for IL-10 and IL-17 as well as their isotype controls are shown.

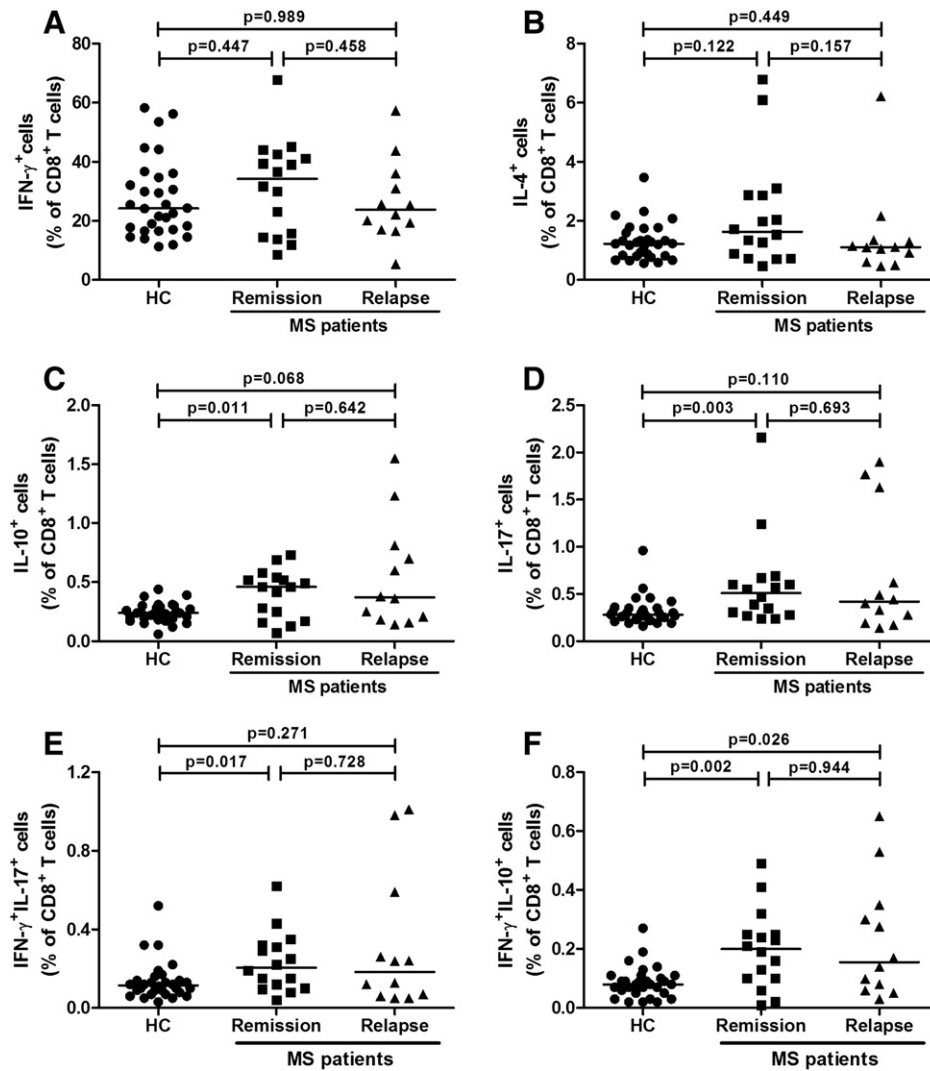
difference between untreated RRMS patients in remission and during a relapse (0.4% [0.2–0.8]). Also, the percentage Tc17 cells (Fig. 2D) was higher in RRMS patients in remission (0.5% [0.3–0.7]) compared to HC (0.3% [0.2–0.4],  $p = 0.003$ ). Again, there was no difference between untreated RRMS patients in remission and during a relapse (0.4% [0.2–1.4]).

Next to the total Tc10 and Tc17 cell subsets, double producing Tc cell subsets, e.g. IL-10<sup>+</sup>IL-17<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> Tc cells, might be of interest. IL-10<sup>+</sup>IL-17<sup>+</sup> Tc cell percentages are hardly detectable (Fig. 1) and overall numbers were too low for reliable analysis (data not shown). The IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> Tc cell percentages are increased in untreated RRMS patients in remission (0.21% [0.11–0.32] and 0.20% [0.10–0.25], respectively) compared to HC (0.12% [0.08–0.15];  $p = 0.017$  and 0.08% [0.05–0.11];  $p = 0.002$ , respectively), while these percentages were similar compared to untreated RRMS patient during a relapse (0.18% [0.06–0.51];  $p = 0.728$  and 0.16% [0.07–0.34];  $p = 0.944$ , respectively; Fig. 2E–F). Since the percentage IFN- $\gamma$ <sup>+</sup> cells within both Tc10 and Tc17 cell

subsets was on average 40%, the changes observed in the IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> Tc cell percentages might just parallel with the changes in the fraction of Tc10 and Tc17 cells, respectively.

### 3.2. Treatment effects on the fraction cytokine producing CD8<sup>+</sup> T cell subsets

Next, we assessed if RRMS patients in remission on IFN- $\beta$  or GA treatment differed in the relative presence of cytokine producing CD8<sup>+</sup> T cell subsets as compared to untreated RRMS patients in remission. Compared to untreated RRMS patients (34.2% [14.8–42.2]), Tc1 cell percentages (Fig. 3A) are lower in patients on IFN- $\beta$  (12.4% [8.9–23.1],  $p = 0.011$ ), but not in patients on GA treatment (30.1% [14.0–42.1]). Tc2, Tc10 and Tc17 cell percentages did not differ between the treatment groups and the untreated RRMS patients (Fig. 3B–D). In addition, IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> Tc cell frequencies were similar between the different treatment cohorts (data not shown).



**Fig. 2.** The percentage cytokine producing CD8<sup>+</sup> T cell subsets in MS patients. The percentage of IFN- $\gamma$ <sup>+</sup> (A), IL-4<sup>+</sup> (B), IL-10<sup>+</sup> (C), IL-17<sup>+</sup> (D), IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> (E) and IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> (F) cells within CD8<sup>+</sup> T cells from healthy controls (HC, n = 30), MS patients in remission (n = 16) and MS patients during a relapse (n = 12). Black horizontal lines indicate the median. Differences between the groups were assessed using the Mann–Whitney U test. A p-value <0.05 was considered statistically significant.

### 3.3. The effect of vitamin D on the fraction cytokine producing CD8<sup>+</sup> T cell subsets

Next to the first line immune modulatory therapies, vitamin D has been proposed to function as a natural immune modulator in RRMS. Hence, we investigated the possible association between the vitamin D status (serum 25(OH)D level) and the fractions of the different cytokine producing Tc cell subsets in our cross-sectional cohort. In HC and total RRMS patients in remission, serum 25(OH)D levels were neither associated with Tc1 and Tc2 cell percentages ( $r < 0.350$  and  $p > 0.100$  for all correlations) nor with Tc10 and Tc17 cell percentages ( $r < 0.350$  and  $p > 0.100$  for all correlations, Fig. 4A–D). Also, the double producing Tc cell percentages were not associated with serum 25(OH)D levels neither in HC nor in total RRMS patients in remission ( $r < 0.350$  and  $p > 0.100$  for all correlations, data not shown). Next, we investigated if the vitamin D status was associated with the Tc cell subsets within the different treatment groups. Serum 25(OH)D levels were not associated with Tc1, Tc2, Tc10, and Tc17 cells, or double producers in RRMS patients in remission without and with IFN- $\beta$  treatment ( $r < 0.350$  and  $p > 0.100$  for all correlations). In RRMS patients in remission with GA treatment, serum 25(OH)D levels were positively correlated with the fraction

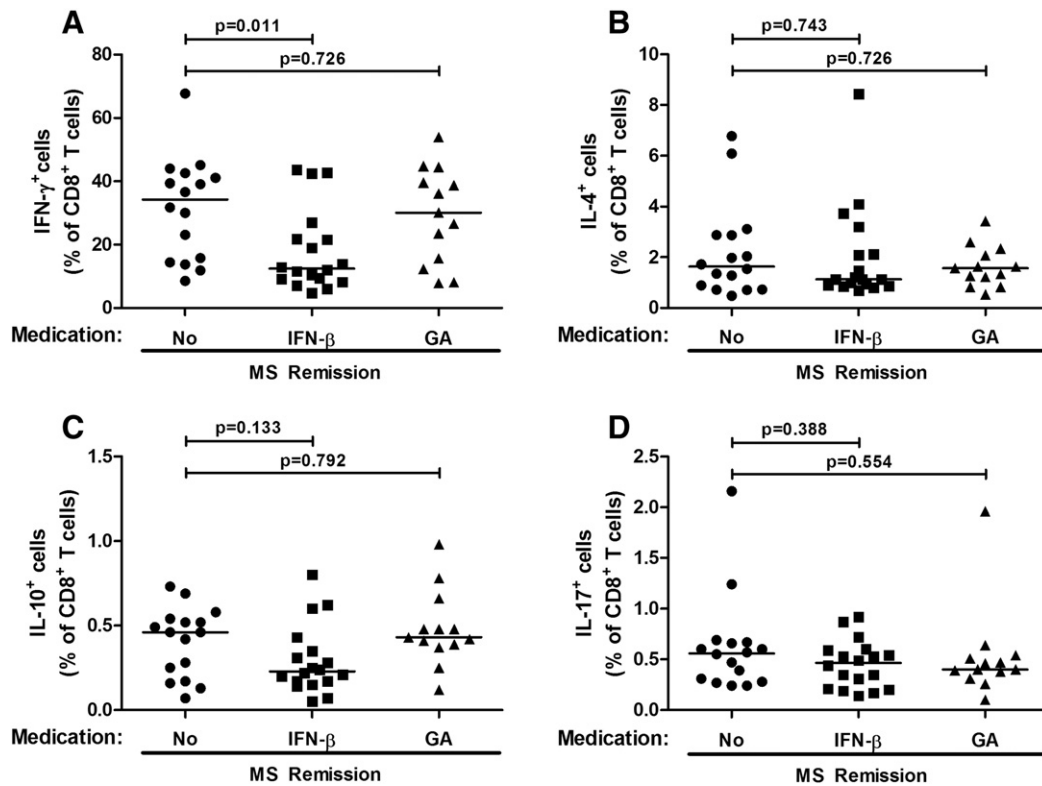
Tc10 cells ( $r = 0.597$ ,  $p = 0.031$ ), but not with the other Tc cell fractions ( $r < 0.350$  and  $p > 0.100$  for all correlations).

Next, a possible add-on effect of vitamin D<sub>3</sub> on the fraction of cytokine expressing Tc cell subsets was assessed in 14 RRMS patients in remission treated with IFN- $\beta$ . These patients received daily 20,000 IU vitamin D<sub>3</sub> for 12 weeks and the different Tc cell subsets were determined at baseline and at 12 weeks. Percentages of Tc1, Tc2, Tc10 and Tc17 cells were similar at baseline (17.5% [11.2–24.9], 0.8% [0.5–1.6], 0.2% [0.1–0.3] and 0.2% [0.1–0.3], respectively) and after 12 weeks of vitamin D<sub>3</sub> supplementation (18.1% [10.8–27.2], 0.9% [0.5–1.5], 0.2% [0.1–0.3] and 0.2% [0.2–0.3], respectively; Fig. 4E–F). The double producing Tc cell frequencies at baseline did not differ from these Tc cell frequencies 12 weeks after vitamin D<sub>3</sub> supplementation (data not shown).

## 4. Discussion

In the present study, we show first that Tc10 and Tc17 Tc cell percentages are higher in untreated RRMS patients in remission compared to HC. Second, percentages of the different Tc cell subsets were similar between untreated RRMS patients in remission and during a relapse. Third, IFN- $\beta$ -treated RRMS patients in remission





**Fig. 3.** Treatment effects on the fraction cytokine producing  $CD8^+$  T cell subsets. The percentage of  $IFN-\gamma^+$  (A),  $IL-4^+$  (B),  $IL-10^+$  (C) and  $IL-17^+$  (D) cells within  $CD8^+$  T cells from MS patients in remission without ( $n = 16$ ) or with beta interferon ( $IFN-\beta$ ;  $n = 18$ ) or glatiramer acetate therapy (GA;  $n = 13$ ). Black horizontal lines indicate the median. Differences between the groups were assessed using the Mann–Whitney  $U$  test. A  $p$ -value  $<0.05$  was considered statistically significant.

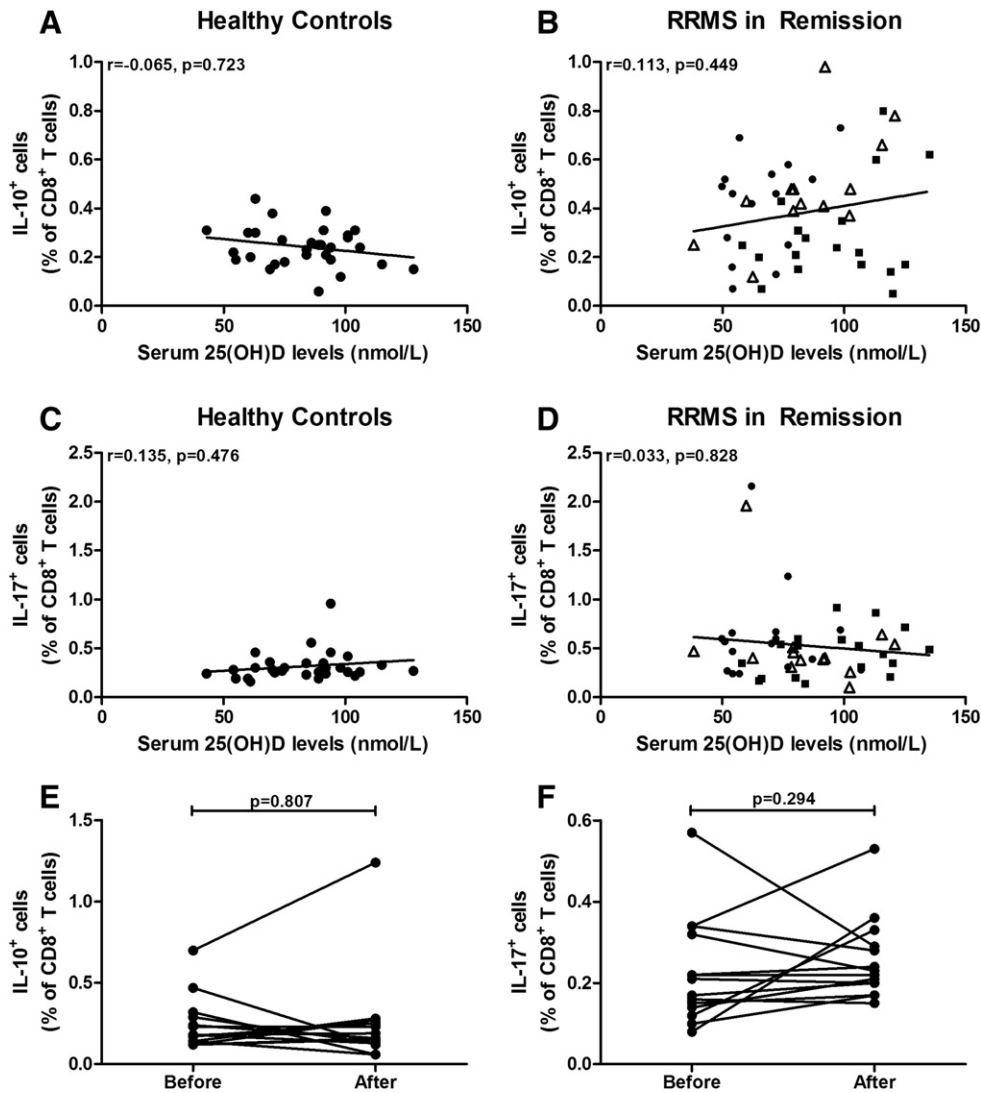
showed lower Tc1 cell percentages, but no different Tc2, Tc10 or Tc17 cell percentages, compared to untreated RRMS patients in remission. GA treatment did not affect any of the Tc cell subsets. Fourth, vitamin D supplementation, as add-on to  $IFN-\beta$  therapy, did not influence fractions of the different Tc cell subsets.

Tc cells are T cells with cytotoxic properties which are especially important in controlling viral infections (reviewed by Zhang and Bevan, 2011). Epstein–Barr virus (EBV), a risk factor for MS, might be a pivotal link between Tc cells and MS (Ascherio and Munger, 2007). An *in vitro* study showed that Tc cells are able to induce apoptosis in oligodendrocytes (Jurewicz et al., 1998). This could eventually result in a decreased myelination of the axons and this might add to MS pathogenesis. Next to the classical cytotoxic function, Tc cells might exert helper functions through cytokine secretion, which could play an important role in MS as well. In addition, cytotoxicity and cytokine production can be produced in the same cell. Tc1 cells seem to produce next to  $IFN-\gamma$  also perforin, while Tc17 cells seem to be unable to produce this effector molecule. In MS, little research has focused on cytokine producing Tc cell subsets and these studies preferentially addressed Tc1 and Tc2 cells. Therefore, the main focus of the present study was on Tc10 and Tc17 cell subsets, since these subsets may have regulatory and pathogenic potential, respectively.

Our results show that untreated RRMS patients in remission had an expanded Tc17 cell population compared to healthy controls, while the percentage of this cell subset did not differ between untreated RRMS patients in remission and during a relapse. Wang et al. showed an elevated Tc17 cell fraction in MS patients during a relapse compared to healthy controls, but did not include MS patients in remission (Wang et al., 2011). In addition, a recent study showed an increased Tc17 cell fraction in the CSF of clinically isolated syndrome and early MS patients, but not in the periphery (Huber

et al., 2013). The discrepancy between this and our study could be explained by the differences in patient cohorts. Huber et al. included patients during their first clinical symptoms, while our patients were in remission and would have, on average, a longer disease duration. Moreover, their control cohort consisted of patients with non-infectious headache, while we included HC. Tc17 cells can, like their T helper counterpart (Th17 cells), be expected to play a pathogenic role in MS. The observation that  $>70\%$  of Tc cells in MS lesions express  $IL-17$  supports this. Moreover, recently Tc17 cells have been shown to be important in the migration and accumulation of Th cells into the central nervous system (CNS) and therefore seem essential in EAE induction (Huber et al., 2013). To further investigate the role of these cells in the pathogenesis, more functional studies should be performed. It would be interesting to investigate the Tc17 cell responses to a probably more representative antigen for MS, like myelin oligodendrocyte glycoprotein or myelin basic protein.

Next to Tc17 cells, we observed an expanded Tc10 cell population in untreated RRMS patients in remission. In contrast, Killestein et al. observed a reduction in Tc10 cell percentages in RRMS patients (Killestein et al., 2001). The discrepancy between the two studies might be explained by the used patient materials (freshly isolated PBMC in our study and thawed PBMC in Killestein's study).  $IL-10^+$  T cell percentages might be more susceptible to differences in activation conditions, since we recently showed that monensin reduced  $IL-10^+$  T cell percentages while it enhanced the other cytokine producing T cell fractions (Muris et al., 2012). In line with this, we speculate that Tc10 cell percentages can be affected by a freezing–thawing cycle. Indeed, cytokine secretion or the fraction of cytokine positive cells might differ between fresh and thawed PBMC (Mallone et al., 2011). The results of the present study suggest that the immune system tries to counterbalance the increase in pro-inflammatory cells by



**Fig. 4.** The effect of vitamin D on the fraction cytokine producing CD8<sup>+</sup> T cell subsets. Correlations of serum 25(OH)D levels and either the percentage of IL-10<sup>+</sup> (A, B) and IL-17<sup>+</sup> (C, D) cells within CD8<sup>+</sup> T cells from healthy controls ( $n = 30$ ; A, C) and all RRMS patients in remission ( $n = 47$ ; B, D). In B and D, black dots are RRMS patients in remission without treatment ( $n = 16$ ), black squares are RRMS patients in remission with beta-interferon (IFN- $\beta$ ,  $n = 18$ ) treatment and open triangles are RRMS patients with glatiramer acetate (GA,  $n = 13$ ) treatment. The black lines are the best fitted lines through the data points of HC (A, C) and all MS patients in remission (B, D). The percentage of IL-10<sup>+</sup> (E) and IL-17<sup>+</sup> (F) cells within CD8<sup>+</sup> T cells from MS patients in remission ( $n = 14$ ) at baseline (before) and after 12 weeks (after) of 20,000 IU vitamin D<sub>3</sub> per day. An association was tested with the Spearman correlation. The correlation coefficient and p-values are given. Differences between before and after were tested with a Wilcoxon Signed Ranks test. A p-value <0.05 was considered statistically significant.

expanding the Tc10 fraction. Tc10 cells have been described to be regulatory cells which are important in protection from tissue damage during infections (reviewed by Zhang and Bevan, 2011). We speculate that this function also exists during inflammation in autoimmune diseases and that the expanded Tc10 cell population could protect the brain tissue from damage in MS. This might be insufficient during disease exacerbation.

Th cells can express multiple cytokines simultaneously, even cytokines which have been assigned to different Th cell subpopulations. These double producing cells might exert distinct functions (Boniface et al., 2010; Zielinski et al., 2012). Also, Th17 cells have been described to be pathogenic cells in EAE, while Th cells producing both IL-17 and IL-10 seem to be non-pathogenic (McGeachy et al., 2007). This might also be true for Tc cells. Therefore, we assessed 3 different double producing Tc cell subsets, i.e. IL-10<sup>+</sup>IL-17<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> Tc cells. Our results showed that the frequency of the first is too low for reliable analysis, whereas the latter two Tc subsets were elevated in untreated RRMS patients in remission. The low cell

frequencies of these double producing Tc cells implicate, however, that the findings of these subsets in our cohort should be approached with caution. Future studies investigating double producing Tc cells should be more accurately designed for this purpose and if possible elucidate the function of these cells.

GA and IFN- $\beta$  are first line immune modulatory drugs, which have been shown to reduce the relapse rate (PRISMS, 1998; Comi et al., 2001). Studies indicate that the induction of anti-inflammatory and regulatory immune cells and the inhibition of pro-inflammatory cells might contribute to the beneficial effects of these therapies (reviewed by Goodin, 2005; Lalive et al., 2011). In the present study, we only observed a reduction in the Tc1 cell fraction in the IFN- $\beta$ -treated RRMS population. This is in agreement with previous studies (Becher et al., 1999; Franciotta et al., 2003; Mei et al., 2006). The Tc1 signature cytokine, IFN- $\gamma$ , is involved in various immune stimulating processes (Pouly et al., 2000). These observations suggest a pathogenic role for this Tc cell subset and the observed reduction in this subset might contribute to the clinical effect of IFN- $\beta$ . The clinical

effect of GA does not seem to be due to modulation of Tc cell subset numbers. However, a local effect of IFN- $\beta$  or GA cannot be excluded. It is possible that IFN- $\beta$  and GA do not affect circulating Tc cell percentages, but instead influence their function, e.g. cytokine secretion, prevent pathogenic Tc cells from entering the CNS or act locally on Tc cells, or act on other pathogenic T cells.

Next to first line medication, vitamin D is being advocated as a possible add-on therapy because of its immune modulatory properties (Smolders et al., 2010a, 2011). In autoimmune diseases, vitamin D skews the immune system towards a more anti-inflammatory profile (reviewed by Peelen et al., 2011). Studies investigating the effects of vitamin D on Tc cells are limited. Veldman et al. showed that Tc cells express higher levels of the vitamin D receptor compared to Th cells (Veldman et al., 2000). However, vitamin D has been shown to prevent EAE independently of Tc cells (Meehan and DeLuca, 2002). Nonetheless, vitamin D might have a beneficial effect on Tc cells in MS patients. Interestingly, Lysandropoulos et al. showed *in vitro* that vitamin D was able to reduce IFN- $\gamma$  and TNF- $\alpha$  secretion when Tc cells were stimulated with either an EBV-peptide or anti-CD3 and anti-CD28 antibodies (Lysandropoulos et al., 2011). Our data do not show an association of the different Tc cell subsets and the vitamin D status in HC and RRMS patients in remission. Recently, Stewart et al. showed that MS patients with IFN- $\beta$  treatment with a higher vitamin D status had a reduced relapse rate (Stewart et al., 2012). This suggests a synergistic effect of vitamin D and IFN- $\beta$  treatment, which might also be true for effects on the immune system. Therefore, we investigated the associations in the different treatment groups. However, we did not observe an association between the vitamin D status and the different Tc cell subsets in RRMS patients in remission with IFN- $\beta$  treatment nor in the patients without treatment. Also, high dose vitamin D supplementation in RRMS patients with IFN- $\beta$  treatment did not affect these Tc cell frequencies. In RRMS patients in remission with GA, we only observed a positive association between the vitamin D status and Tc10 cell percentages. This might indicate that vitamin D and GA have a synergistic effect on Tc10 cells.

This paper provides new insights in the frequency of circulating Tc10 and Tc17 cells in RRMS patients in remission and during a relapse. Furthermore, as far as we know, this is the first paper that assessed the effect of first line treatment and vitamin D on Tc10 and Tc17 cells in RRMS patients directly *ex vivo*. The present study has some short comings. First, except for the vitamin D supplementation, our study had a cross-sectional design, which gives no information on causality. Second, we only assessed cells in the periphery and not in CSF or brain tissue. Third, we investigated Tc cell percentages, which do not give information on the amount of cytokines secreted or other functional properties of these cells.

In conclusion, untreated RRMS patients in remission have an augmented Tc10 and Tc17 cell fraction in the circulation, while during a relapse the Tc cell subset percentages are similar to the percentages of the patients in remission. Whether Tc10 and Tc17 cells are actively involved in the pathogenesis of RRMS remains to be determined. If so, new therapeutic modalities will be required since our data show that current first line medication does not affect the relative presence of these Tc cell subsets.

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## Conflict of interest statement

The authors declare no conflict of interest.

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