Dysregulated proinflammatory circulating follicular helper T cells and suppressive follicular regulatory T cells in patients with multiple sclerosis

Razaul Haque[1], Yeseul Kim[1], Hyunmin Jang[1], So Yeon Kim[1], Ho Lee[1], and Ho Jin Kim[1]

[1]National Cancer Center

June 22, 2020

Abstract

Follicular T helper (Tfh) and regulatory (Tfreg) cells are distinct subsets of CD4+T lymphocytes, regulating humoral immune responses in the germinal center. Dysregulated Tfh and Tfreg cells are believed to associate with autoimmunity. Here, we evaluated the frequencies of circulating CXCR5+PD-1+ Tfh (cTfh) and CXCR5+PD-1+FoxP3+CD25+ Tfreg (cTfreg) cells, and their corresponding cytokines from the peripheral blood mononuclear cells of 20 patients with relapsing-remitting multiple sclerosis (MS) and 12 age- and sex-matched healthy controls (HC). Subsets of cTfh cells by Th1 and Th17 related surface markers (CXCR3 and CCR6) were also evaluated. We found the frequency of cTfh cells was significantly higher in MS patients compared to HC (p=0.002). Conversely, cTfreg cells were downregulated in MS patients (p<0.0001). IL-21 producing cTfh cells were significantly increased in MS patients (p=0.003), while IL-10 secreting cTfreg cells were decreased in MS compared to HC (p=0.02). Among cTfh cells, cTfh17.1 cells were major subtypes that were significantly increased in MS compared to HC (p=0.002) and the frequency of IL-21 secreting cells were highest. These results suggest that an imbalanced distribution of cTfh and cTfreg exist in MS, which contributes to reciprocally altered IL-21 and IL-10 production.

Dysregulated proinflammatory circulating follicular helper T cells and suppressive follicular regulatory T cells in patients with multiple sclerosis

Razaul Haque[1, 2, 3], Yeseul Kim[2, 3], Hyunmin Jang[2], So Yeon Kim[2, 3], Ho Lee[1] and Ho Jin Kim*[1, 2, 3]

Authors' information

[1] Department of Cancer Biomedical Science, Graduate School of Cancer Science and Policy, National Cancer Center, Goyang, South Korea.

[2] Department of Neurology, National Cancer Center, Goyang, South Korea·

[3] Division of Clinical Research, National Cancer Center, Goyang, South Korea.

Correspondence: Ho Jin Kim

Head of Department of Neurology Head of Division of Clinical Research Research Institute & Hospital of National Cancer Center Professor of Department of Cancer Biomedical Science National Cancer Center Graduate School of Cancer Science & Policy 323 Ilsan-ro, Ilsandong-gu, Goyang, 10408, Korea E-mail: *hojinkim@ncc.re.kr*Tell: +82-31-920-2438

Abbreviations

MS - Multiple sclerosis

CNS - Central nervous system

HC - Healthy control

Tfh - Follicular T helper cells

cTfh - Circulating T follicular helper

Tfreg - Follicular regulatory T cells

CD - Cluster of differentiation

CXCR5 - Chemokine receptor type 5

CCR - Chemokine receptor

ICOS - Inducible T cell co-stimulator

PD-1 - Program death 1

Bcl-6 - B cell lymphoma 6

FoxP3 - Forkhead box P3

GC - Germinal center

SLE - Systemic lupus erythematous

RA - Rheumatoid arthritis

MG- Myasthenia gravis

PBMC Peripheral blood mononuclear cell

ICS Intracellular cytokine staining

Summary

Follicular T helper (Tfh) and regulatory (Tfreg) cells are distinct subsets of $CD4^+$T lymphocytes, regulating humoral immune responses in the germinal center. Dysregulated Tfh and Tfreg cells are believed to associate with autoimmunity. Here, we evaluated the frequencies of circulating $CXCR5^+PD\text{-}1^+$ Tfh (cTfh) and $CXCR5^+PD\text{-}1^+FoxP3^+CD25^+$Tfreg (cTfreg) cells, and their corresponding cytokines from the peripheral blood mononuclear cells of 20 patients with relapsing-remitting multiple sclerosis (MS) and 12 age- and sex-matched healthy controls (HC). Subsets of cTfh cells by Th1 and Th17 related surface markers (CXCR3 and CCR6) were also evaluated. We found the frequency of cTfh cells was significantly higher in MS patients compared to HC ($p=0.002$). Conversely, cTfreg cells were downregulated in MS patients ($p<0.0001$). IL-21 producing cTfh cells were significantly increased in MS patients ($p=0.003$), while IL-10 secreting cTfreg cells were decreased in MS compared to HC ($p=0.02$). Among cTfh cells, cTfh17.1 cells were major subtypes that were significantly increased in MS compared to HC ($p=0.002$) and the frequency of IL-21 secreting cells were highest. These results suggest that an imbalanced distribution of cTfh and cTfreg exist in MS, which contributes to reciprocally altered IL-21 and IL-10 production.

Keywords: Multiple sclerosis, Follicular T helper cells, Follicular regulatory T cells.

Background

Multiple sclerosis (MS) is a chronic demyelinating disease of central nervous system (CNS) characterized by destruction of myelin sheath and nerve axons [1]. $CD4^+$ T cells are considered a key player in MS pathogenesis. Different subsets of $CD4^+$ T cells orchestrate an inflammatory wave to initiate and progress MS [2, 3]. Additionally, B cells are known to play an important role in MS through antibody production or influencing autoreactive T cell generation [4-6]. T follicular helper (Tfh) cells, a newly described $CD4^+$ T cell subset that resides in the germinal center (GC), are specialized for B cell help and antibody mediated immune responses [7]. Tfh cells possess unique phenotypic attributes and express chemokine receptor type 5 (CXCR5), inducible T cell co-stimulator (ICOS), and program death-1 (PD-1) at their surfaces, all controlled

by a B cell lymphoma 6 transcription factor (Bcl-6) [7-11]. IL-21 is the signature cytokine produced by Tfh cells, which is required for Tfh maintenance, B cell differentiation and antibody production [11-13]. Conversely, follicular regulatory T (Tfreg) cells have been recently identified as a novel subtype of regulatory T cells that share some common phenotypes with Tfh, along with conventional Treg markers, such as forkhead box P3 (FoxP3) and CD25 [14, 15]. Similar to the canonical Treg, Tfreg cells also secrete IL-10 [16] and their basic role is to regulate the functional activity of Tfh cells in the GC [17, 18].

Since their discovery, Tfh and Tfreg cells have become crucial parts of the antigen specific immune responses and self-tolerance, which led to explore their relevance in human health and disease [17, 19]. The characterization of bona fide Tfh and Tfreg in humans has been poorly studied because of sampling difficulties. Therefore, studies have focused on their counterparts in peripheral blood, termed as circulating Tfh (cTfh) cells [20]. These cTfh cells are identified in the memory T cell chamber, sharing common phenotypes with GC-Tfh except Bcl-6 [21]. Like resident Tfh, cTfh cells are also capable of providing help to B cells [22]. Recently, cTfh cells have been reported to exert more potent cytokine mediated immune responses than GC-Tfh [23]. Moreover, armed with memory-like properties, cTfh cells may provide more rapid cellular immune response and undergo homeostatic proliferation, where they show phenotypic heterogeneity, accessing a broader range of immune compartments and expressing a variety of surface markers and secreting an array of cytokines [24]. Morita et al. initially described the existence of Th1 (CXCR3) and Th17 (CCR6) related surface markers on cTfh cells. cTfh cells were divided into cTfh1 ($CXCR3^+CCR6^-$), cTfh2 ($CXCR3^-CCR6^-$), and cTfh17 ($CXCR3^-CCR6^+$), with each involved in producing different sets of cytokines, namely IFN-γ, IL-4, and IL-17 respectively, as well as secreting IL-21 [25]. These diverse phenotypic markers and cytokines collectively reshape cTfh cells to provide more robust help to B cells and induce high affinity maturation of plasma cells and antibody production [20, 25].

Emerging evidence suggests that Tfh cells should be tightly controlled to maintain immune tolerance and avoid autoimmune responses. Uncontrolled activation of Tfh cells and deactivation of Tfreg cells lead to a breakdown of self-tolerance. Imbalance between Tfh and Tfreg cells has been found in number of autoimmune diseases, including systemic lupus erythematous (SLE), rheumatoid arthritis (RA), and myasthenia gravis (MG) [26-29]. Moreover, subsequent polarization of Tfh subsets has reportedly been involved in several autoimmune diseases, such as SLE, RA and IgG_4-related disease which are associated with autoantibody production [30-32]. The relevance of Tfh and Tfreg cells has recently been recognized in MS patients, suggesting their potential roles in MS pathogenesis [33, 34]. To address this issue we investigated the frequency of cTfh ($CD4^+CXCR5^+PD\text{-}1^+$) cells and their subsets, as well as cTfreg ($CD4^+CXCR5^+PD\text{-}1^+FoxP3^+CD25^+$) cells in MS patients compared to healthy controls (HC). We also investigated the frequency of respective cytokines secreted by these cellular subtypes. We found that patients with MS display alterations to their cTfh and cTfreg cells, both in terms of their frequencies and cytokine secretions.

Methods

Study participants

This study enrolled 20 relapsing-remitting MS (RRMS) patients from the Department of Neurology, National Cancer Center, South Korea. The diagnosis of MS was made according to 2017 McDonald criteria [35]. Twelve age- and sex-matched healthy controls (HC) were also recruited. No patients had co-existing central nervous system (CNS) diseases or other autoimmune diseases. Among 20 MS patients, 10 patients were treated with β-interferon and the rest received glatiramer acetate. None of the patients had received high-dose steroids in the 2 months preceding blood sampling. Demographic as well as clinical characteristics of participants are summarized in Table 1. This study was approved by the Institutional Review Board and written informed consent was obtained from all participants. Statement on informed consent from the legally authorized representatives were also obtained as patients with RRMS are involve in this study. All the methods were performed according to the guidelines and regulations of Declaration of Helsinki.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by density gradient centrifuga-

tion over Ficoll-Paque^{TM} PLUS (GE Healthcare, Sweden) according to the manufacturer's protocols.

Surface staining and intracellular staining

A total of 1.5x10^6 and 2x10^6 fresh PBMCs were used for Tfh and Tfreg analysis, respectively. PBMCs were stained with a fixable viability dye-700-alexa fluorophore at 4^{0}C for 15 minutes in the dark (for dead cell discrimination) prior to surface and intracellular staining. PBMCs were subsequently stained with surface markers at 4^{0}C for 30 minutes in the dark with the following monoclonal antibodies: anti-CD3-PE-cy7, anti-CD4-Buv496, anti-CD8-FITC, anti-CXCR5-Percp-cy5.5, anti-PD-1-BV786, anti-CXCR3-PE, anti-CCR6-BV711, and anti-CD25-APC-cy-7. All monoclonal antibodies were purchased from BD Bioscience. For intracellular cytokine staining (ICS), PBMCs were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (500 ng/mL) (Sigma-Aldrich), and golgistop (monensin; 0.6 µL/mL) (BD Bioscience). Following stimulation, PBMCs were fixed and permeabilized with cytofix/cytoperm cell permeabilization buffer (BD Bioscience). ICS was then performed using monoclonal antibodies against IFN-γ-APC, IL-17-BV650, IL21-BV421, IL-10-PE-CF594 (BD Bioscience) and IL-4-BV605 (Bio-legend) following incubation under the same conditions described above for surface staining. Simultaneously, FoxP3 staining was performed for 30 minutes in dark at 4^{0}C using anti-FoxP3-PE antibody according to the manufacturer's instructions (eBioscience).

Flow cytometry

After surface and intracellular staining, PBMCs were acquired for analysis using an LSRFortessa^{TM} flow cytometer (BD Bioscience), followed by analysis using FlowJo software (Tree Star). At least 500,000 live events were acquired for the lymphocyte and monocyte populations. Fine singlets were constructed from PBMCs by removing doublets and cell clumps (Fig.1a).

Statistical analysis

The data were statistically analyzed using Graphpad Prism version 5 (Graphpad software Inc, San Diego, CA, USA). All mean values are expressed as mean±SEM. Non-parametric *Mann-Whitney U* test was performed to compare between healthy controls and MS patients descriptively. A probability value of less than 0.05 ($p<0.05$) was considered statistically significant.

Results

Increased frequency of cTfh cells in MS patients

Circulating Tfh cells were analyzed by gating CXCR5$^+$PD-1$^+$ cells from CD3$^+$CD4$^+$ T cells. As shown in Fig. 1b*(right panel)* , d, the average frequency of cTfh cells in MS patients was significantly higher than that observed in HC (7.88±0.42% vs. 5.73±0.48%, p=0.002). The percentage of CD4$^+$ T cells in the PBMC of HC vs. MS patients was not significantly different (Fig. 1b*(left panel)* , c).

Tfh cells have been categorized into three different subsets based on Th1 (CXCR3) and Th17 (CCR6) related surface markers [25]. Previous work has shown that CXCR3$^+$ Th1 and CCR6$^+$ Th17 cells play essential roles in MS [36]. Here, we explored the expression of CXCR3 and CCR6 markers in Tfh cells to classify and evaluate the frequency of different subsets in MS patients. Our data demonstrate that cTfh17 (CXCR3$^-$CCR6$^+$ Tfh) cells were significantly increased in MS patients compared to HC (50.45±1.36% vs. 45.18±1.44%, p=0.02) (Fig. 2a,d). Recently, CXCR3$^+$CCR6$^+$ T cells have been defined as T helper 1 like Th 17 (Th17.1) cells [37, 38]. We also observed CXCR3$^+$CCR6$^+$ cells among cTfh cells and recognized them as cTfh17.1 cells. Interestingly, the frequency of cTfh17.1 (CXCR3$^+$CCR6$^+$Tfh) cells was significantly higher in MS patients than that in HC (9.66±1.32% vs. 4.58±1.14%, p=0.005) (Fig. 2a,e). In contrast, the frequencies of cTfh1 (CXCR3$^+$CCR6$^-$Tfh) and cTfh2 (CXCR3$^-$CCR6$^-$ Tfh) did not show significant difference between HC and MS (Fig. 2a-c).

IL-21 secreting cTfh cells were higher in MS patients

Aberrant secretion of inflammatory cytokines plays an important role in autoimmune disease. We evaluated

some signature cytokines, such as IFN-γ, IL-4, IL-17 and IL-21. Our data indicate that the frequency of IL-21 secreting PBMCs were significantly higher in MS patients than those in HC (2.62±0.34% vs. 0.93±0.33%, p=0.0004) (Fig. 3a,c), whereas no significant difference was observed in IFN-γ, IL-4 and IL-17 production between MS patients and HC (Fig. S1). The frequency of IL-21 secreting $CD3^+$ T cells was higher in MS patients compared to HC (1.95±0.32% vs. 0.81±0.49%, p=0.0009) (Fig. 3c), whereas no significant differences in the frequencies of IL-21 producing B cells and monocytes were observed between MS patients and HC (data not shown). Comparative analysis of IL-21 secretion by $CD4^+$ T cells and $CD8^+$ T cells showed that significantly higher percentage of $CD4^+$ T cells secreted IL-21 in MS patients compared to HC (2.32±0.23% vs. 0.9±0.41%, p=0.002), while IL-21 producing $CD8^+$ T cells showed no significant difference between HC and MS patients (Fig. 3b, c).

Next, we analyzed the secretion of cytokines by cTfh cells and compared their frequencies between MS patients and HC. The frequency of IL-21 secreting cTfh cells was remarkably higher in MS patients compared to HC (8.83±1.29% vs. 3.53±1.25%, p=0.003) (Fig. 3d, e), whereas no significant difference was observed in IFN-γ, IL-4 and IL-17 secreting cTfh cells between MS patients and HC (Fig. S2). Comparative analysis of IL-21 production by follicular vs. non-follicular Th cells revealed that, higher percentage of cTfh cells secreted IL-21 compared to their counterpart non-follicular Th cells in both HC (3.53±1.25% vs. 1.66±0.64%, p=0.06) and MS (8.83±1.29% vs. 1.35±0.25%, p<0.0001). Interestingly, IL-21 secreting cTfh cells was significantly higher in MS compared to HC (Fig. 3d,e). Collectively, these findings suggest that cTfh cells may engage with elevated secretion of IL-21 in MS patients over other immune cells.

cTfh17.1 cells were the major subtype for IL-21 production in MS patients

As shown in Fig. 3d and e, the level of IL-21 secreting cTfh cells was significantly higher in MS patients. We then examined the secretion of IL-21 by different subsets of cTfh cells and compared between HC and MS patients. Although all the four subtypes of cTfh cells produce IL-21, cTfh17.1 cells were associated with the greatest production of IL-21 in both HC and MS (Fig. 4a,b). Interestingly, IL-21 secreting cTfh17.1 cells was significantly higher in MS patients (52.52±4.86% vs. 24.81±5.34%, p=0.002) than that in HC (Fig. 4a,b). However, other subsets of IL-21 secreting cTfh cells did not differ significantly between HC and MS. We also analyzed and compared IL-17 secreting cTfh17 cells in HC and MS patients, followed by a comparative analysis of IL-17 production by cTfh17 vs. non-follicular Th17 cells. We found no noticeable difference in IL-17 secreting cTfh17 cells between HC and MS (Fig. 4c and S3a). Moreover, IL-17 secreting cTfh17 versus non-follicular Th17 cells did not show any differences between HC and MS (Fig. 4d and S3a,b,e,f). However, a significant increase of $CXCR3^+CCR6^+$ cTfh17.1 cells in MS patients led us to investigate them further based on the cytokine types, especially IFN-γ and IL-17. Along with IL-21, cTfh17.1 cells also produced IFN-γ and IL-17 with different frequencies (Fig. S3c,d and Fig. 4e,f), although no significant difference was observed between HC and MS regarding IFN-γ and IL-17 secreting cTfh17.1 cells. Importantly, IL-21 was the major cytokine secreted by the cTfh17.1 subtype over others suggesting that cTh17.1 cells are the major secretor of IL-21 in MS patients.

Reduced frequency of cTfreg cells in MS patients.

Next, we evaluated the frequency of cTfreg cells, which was determined by selecting $FoxP3^+CD25^+$ cells from cTfh cells (Fig. 1). The frequency of cTfreg cells was significantly reduced in MS patients compared to HC (1.38±0.14% vs. 3.0±0.35%, p<0.0001) (Fig. 5a$_{(right\ panel)}$,b), whereas the percentage of non-follicular cTfreg cells did not differ between HC and MS (Fig. 5c and S4a). However, the frequency of cTreg ($CD4^+FoxP3^+CD25^+$) cells among $CD4^+$ T cells was significantly lower in MS patients compared to HC (1.87±0.29% vs. 3.11±0.35%, p=0.003) (Fig. S4b$_{(left\ panel)}$,c). We also examined the expression of $CXCR5^+PD\text{-}1^+$ markers in cTreg cells. Interestingly, the percentage of $CXCR5^+PD\text{-}1^+$ cells among cTreg cells was also significantly reduced in MS patients compared to that observed in HC (13.49±1.48% vs. 25.54±1.8%, p<0.0001) (Fig. S4b$_{(right\ panel)}$, d). Taken together, these data suggest that cTfreg cells were down regulated in MS patients.

IL-10 producing cTfreg cells were decreased in MS patients

Being the most common anti-inflammatory cytokine, we analyzed and compared the secretion of IL-10 between HC and MS patients. In PBMCs, IL-10 secreting cells were significantly lower in MS patients compared to HC (0.77±0.14% vs. 2.15±0.38%, p=0.004) (Fig. 6a,d). Next, we investigated the production of IL-10 by follicular and non-follicular cTreg cells, followed by comparative observation between HC and MS patients. The results showed that compared to non-follicular cTreg cells, higher percentage of cTfreg cells secreted IL-10 in both HC (19.19±2.71% vs. 4.41±0.87%, p=0.0001) and MS patients (11.85±1.24% vs. 2.39±0.23%, p<0.0001), respectively (Fig. 6b,c,e). Similar to the trend of decreased frequency of cTfreg cells in MS, IL-10 producing cTfreg cells were significantly reduced in MS patients compared to HC (11.85±1.24% vs. 19.19±2.71%, p=0.02) (Fig. 6b,c,e). The percentage of IL-10 producing non-follicular cTreg cells were also reduced in MS patients compared to HC (Fig. 6c,e).

Discussion

Tfh and Tfreg cells are the latest members of $CD4^+$ T cells that constitute a compact immune regulatory system and disturbance of this system can cause autoimmune disease [26, 29-31]. Despite ongoing research, little is known about the role of Tfh and Tfreg cells in MS pathogenesis.

In the present study, we showed an increased frequency of circulating $CXCR5^+PD\text{-}1^+$ Tfh cells in MS patients compared to HC. Along with higher frequency of cTfh cells, IL-21 secretion was also elevated significantly in MS patients. Subsequent analysis of IL-21 production by follicular and non-follicular Th cells revealed that cTfh cells were engaged with highest production of IL-21, where the frequency was remarkably increased in MS patients. The role of Tfh cells in autoimmune disease was initially described in murine model [39]. However, recent reports with human data in RA, SLE, MG, and autoimmune thyroid disease have revealed that frequency of Tfh cells and their cytokine IL-21 were elevated where they associated with disease activity as well as severity through the amplification of autoreactive B cells and pathogenic autoantibody production [28-30, 40]. Our data are consistent with those results and more importantly for MS, we convincingly connect cTfh cells to high levels of IL-21 production, suggesting that these cells may play a vital role in the immunopathogenesis of MS. Our results also supported by previous study in MS, where the frequency of memory Tfh and plasma as well as CSF IL-21 levels were higher in MS patients [33, 41, 42]. However, we have to admit that we lacked treatment-naïve MS patients who provide us clearer picture about cTfh and IL-21 in the disease context. Interestingly, despite our patients were on first-line injectable disease modifying therapy, the frequency of cTfh cells and the production of IL-21 remained elevated, while other subsets of Th cells showed no significant difference between HC and MS. These results suggest first-line injectable therapies may have minimal effects on cTfh and IL-21regarding their frequency and secretion in MS. Recently, Nicolas et al. reported that elevated cTfh cells can be reverted in patients with neuromyelitis optica after treatment with rituximab [43], suggesting to explore new therapeutic solution for MS.

Proportional bias among different subtypes of Tfh cells have been reported to be associated with several autoimmune diseases, such as SLE, MG, RA and IgG_4-related disease [29-32]. cTfh17 cells have been reported to be enriched in primary progressive MS compared to HC and RRMS [41]. The exact role of cTfh17 cells in MS is not clear. Morita et al. have reported that cTfh17 cells proficiently help naïve and memory B cells to produce Igs (IgG, IgA, and IgM), a process that needs to be elucidated in MS [25]. Cunill et al. have recently described an increased percentage of cTfh17.1 cells in untreated RRMS compared to HC, but no significant difference was observed in the cTfh17 frequency [45]. In our study, both cTfh17 and cTfh17.1 cells were highly elevated in MS patients compared to HC. However, increased frequency of cTfh17.1 cells, but not of cTfh17 cells were positively associated with significantly higher production of IL-21 in MS patients. Additionally, among the four subtypes, only cTfh17.1 cells were involved in producing substantial amount of various cytokines including IFN-γ, IL-17 and IL-21, where the level of IL-21 secretion was greatest in MS patients. These observations collectively suggest cTfh17.1 cells may be one of the key players in disease pathogenesis. However, an extensive study with transcriptional profiling and evaluation of a broader range of cytokines may provide more insights on the role of cTfh17.1 cells in MS pathogenesis.

IL-17 producing Th17 cells were reported to be increased in untreated MS patients compared to HC [43, 45, 46]. In this study, there was no remarkable difference in the frequency of non-follicular Th17 cells and

IL-17 secretion between HC and MS patients. This discrepancy might be attributed to enrolled patients who were continuously undergoing disease-modifying treatments. Indeed, recent reports have shown that IL-17 producing $CD4^+$ T cells were reduced in MS patients after treatment with β-interferon or dimethyl fumarate [47, 48].

Alternatively, it is known that the pathogenesis of MS is linked to the failure of regulatory activities by Treg cells [15, 49, 50]. In this study, one of our main focuses was to investigate whether the proportion of $CXCR5^+PD\text{-}1^+FoxP3^+CD25^+$cTfreg cells is altered in MS patients. The data revealed that the frequency of cTfreg cells was significantly lower in MS patients compared to HC. Additionally, reduced $CXCR5^+PD\text{-}1^+$ phenotypes among cTreg cells in MS patients compared to HC implies the unique behavior of cTfreg cells in MS. Unlike GC-Tfreg, cTfreg does not express Bcl-6 and ICOS although they share the same functional property to control excessive humoral-mediated immunity [21]. Recently, an in vitro co-culture assay with responder T cells and resident Tfreg and cTfreg cells has shown that both cTfreg and resident Tfreg cells provide equal suppressive activity over the responder cells [34]. The frequency of cTfreg cells was found to be reduced in different autoimmune diseases [26, 29, 34]. These findings are consistent with our result and therefore suggest that impaired cTfreg cells may participate in uncontrolled immunoregulation in MS.

The mechanism underlying the suppressive activity of Tfreg cells over other effector T cells is still elusive. It is not clear whether Tfreg cells directly inhibit Tfh cells by blocking their transcriptional activation or they use the IL-10 signaling pathway to set up their inhibitory milieu. Our data has shown that the frequency of IL-10 producing cTfreg cells was significantly higher than their non-follicular cTreg counterpart in both HC and MS patients, suggesting that cTfreg cells were the primary producer of IL-10 among different subtypes of cTreg cells. Interestingly, we observed a decreased frequency of cTfreg cells associated with a parallel reduction of IL-10 in MS patients compared to HC. Taken together, these data demonstrate that the lower frequency of cTfreg is likely linked to reduced IL-10 secretion in MS patients. The exact role of IL-10 in MS remains elusive. Recently, depleted IL-10 secretion was reported to increase the severity of MS [51, 52]. One possible hypothesis might be that, IL-10 exerts beneficial effect in MS by regulating other cytokines, resulting in less tissue damage and minor lesion [53]. Failure of cytokine regulation by defective IL-10 has been reported in progressive MS [54]. Our findings together with previous reports suggest that lower level of IL-10 secreted by cTfreg cells may be involved in MS pathogenesis and might serve as a novel therapeutic target to treat MS.

Conclusions

While our study has some limitations, including a small sample size, absence of treatment naïve patients, and the lack of confirmative studies using animal model, we found an impressive interaction between the increased frequency of cTfh cells, particularly cTfh17.1 cells and the enhanced production of IL-21 in MS patients. Contrarily, cTfreg cells and their signature cytokine IL-10 were remarkably impaired in MS patients. Taken together, our study demonstrates an alteration of cTfh and cTfreg cells, which is reflected in the dysregulated cytokine production in MS. An additional study with a larger cohort is warranted to validate our observations regarding the role of Tfh cells and their subsets as well as Tfreg cells in MS. Functional analysis through *in vitro* co-culture of purified cTfh, B and cTfreg cells may provide a better understanding of MS and other autoimmune diseases that could potentially guide us to new therapeutic interventions.

Ethics approval and consent to participate

The Institutional Review Board of NCC approved the present study, and written informed consent was obtained from all participants.

Consent for publication

Not Applicable

Availability of data and material

All data generated or analyzed during this study are included this published article

Competing interest

Haque R, Kim YS, Jang H, Kim SY, Lee H and Kim HJ report no conflict of interest. Dr. Kim received a grant from the National Research Foundation of Korea; received consultancy/speaker fees from Alexion, Celltrion, Eisai, HanAll BioPharma, Merck Serono, Novartis, Sanofi Genzyme, Teva-Handok, and Viela Bio; serves on a steering committee for MedImmune/Viela Bio; is a co-editor for the Multiple Sclerosis Journal and an associated editor for the Journal of Clinical Neurology.

Funding

This study was supported by the National Research Foundation of Korea (NRF-20143A9B6069336 & NRF-2018R1A5A2023127) and International Cooperation & Education Program (NCCRI.NCCI 52210-52211, 2019) of National Cancer Center, South Korea.

Authors' Contribution

Haque R and Kim HJ had full access to all of the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analysis. Haque R and Kim HJ contributed to the study concept and design, and drafting of the manuscript. Haque R performed the experiment and data analysis. Haque R and Kim YS carried out data acquisition. Jang H helped to collect blood from participants. Haque R and Kim SY carried out the statistical analysis. Haque R and Lee H provided technical instruction and drew the figures. All authors were involved in critical revision of the manuscript for intellectual content. Kim HJ supervised the study. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

References

1. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. N Engl J Med 2018; 378(2):169-180.

2. Chitnis T. The role of CD4 T cells in the pathogenesis of multiple sclerosis. Int Rev Neurobiol 2007; 79:43-72.

3. Kaskow BJ, Baecher-Allan C. Effector T cells in multiple sclerosis. Cold Spring Harbor perspectives in medicine 2018; 8(4):a029025.

4. Franciotta D, Salvetti M, Lolli F *et al* . B cells and multiple sclerosis. The Lancet Neurology 2008; 7(9):852-858.

5. Li R, Patterson KR, Bar-Or A. Reassessing B cell contributions in multiple sclerosis. Nature Immunology 2018; 19(7):696-707.

6. Pappalardo JL, O'Connor KC. B cells drive auto-T cells to the brain. Sci Immunol 2018; 3(28):eaav4512.

7. Crotty S. Follicular Helper CD4 T Cells (TFH). Annu Rev Immunol 2011; 29(1):621-663.

8. Yu D, Rao S, Tsai LM *et al* . The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity 2009; 31(3):457-468.

9. Bossaller L, Burger J, Draeger R *et al* . ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol 2006; 177(7):4927-4932.

10. Shi J, Hou S, Fang Q *et al* . PD-1 Controls Follicular T Helper Cell Positioning and Function. Immunity 2018; 49(2):264-274.e264.

11. Chtanova T, Tangye SG, Newton R *et al* . T Follicular Helper Cells Express a Distinctive Transcriptional Profile, Reflecting Their Role as Non-Th1/Th2 Effector Cells That Provide Help for B Cells. J Immunol 2004; 173(1):68.

12. Nurieva RI, Chung Y, Hwang D *et al* . Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 2008; 29(1):138-149.

13. Bryant VL, Ma CS, Avery DT *et al* . Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol 2007; 179(12):8180-8190.

14. Chung Y, Tanaka S, Chu F *et al* . Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat Med 2011; 17(8):983-988.

15. Venken K, Hellings N, Thewissen M *et al* . Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology 2008; 123(1):79-89.

16. Laidlaw BJ, Lu Y, Amezquita RA *et al* . Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. Sci Immunol 2017; 2(16):eaan4767.

17. Wollenberg I, Agua-Doce A, Hernandez A *et al* . Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol 2011; 187(9):4553-4560.

18. Linterman MA, Pierson W, Lee SK *et al* . Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 2011; 17(8):975-982.

19. Linterman MA, Vinuesa CG. T follicular helper cells during immunity and tolerance. Prog Mol Biol Transl Sci 2010; 92:207-248.

20. Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. Trends Immunol 2014; 35(9):436-442.

21. Sage PT, Alvarez D, Godec J *et al* . Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest 2014; 124(12):5191-5204.

22. Simpson N, Gatenby PA, Wilson A *et al* . Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum 2010; 62(1):234-244.

23. Asrir A, Aloulou M, Gador M *et al* . Interconnected subsets of memory follicular helper T cells have different effector functions. Nat commun 2017; 8:847.

24. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. Front. immunol 2015; 6:16.

25. Morita R, Schmitt N, Bentebibel SE *et al* . Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011; 34(1):108-121.

26. Xu B, Wang S, Zhou M *et al* . The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. Clin Immunol 2017; 183:46-53.

27. Liu C, Wang D, Lu S *et al* . Increased Circulating Follicular Treg Cells Are Associated With Lower Levels of Autoantibodies in Patients With Rheumatoid Arthritis in Stable Remission. Arthritis Rheumatol 2018; 70(5):711-721.

28. Liu R, Wu Q, Su D *et al* . A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. Arthritis Res Ther 2012; 14(6):R255.

29. Zhang CJ, Gong Y, Zhu W *et al* . Augmentation of Circulating Follicular Helper T Cells and Their Impact on Autoreactive B Cells in Myasthenia Gravis. J Immunol 2016; 197(7):2610-2617.

30. Le Coz C, Joublin A, Pasquali JL *et al* . Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. PLoS One 2013; 8(9):e75319.

31. Arroyo-Villa I, Bautista-Caro M-B, Balsa A *et al* . Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis 2014; 16(6):500.

32. Akiyama M, Suzuki K, Yamaoka K *et al* . Number of Circulating Follicular Helper 2 T Cells Correlates With IgG4 and Interleukin-4 Levels and Plasmablast Numbers in IgG4-Related Disease. Arthritis Rheumatol 2015; 67(9):2476-2481.

33. Fan X, Jin T, Zhao S *et al* . Circulating CCR7+ICOS+ Memory T Follicular Helper Cells in Patients with Multiple Sclerosis. PLoS One 2015; 10(7):e0134523.

34. Dhaeze T, Peelen E, Hombrouck A *et al* . Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. J Immunol 2015; 195(3):832.

35. Thompson AJ, Banwell BL, Barkhof F *et al* . Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol 2018; 17(2):162-173.

36. Arellano G, Acuña E, Reyes LI *et al* . Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. Front Immunol 2017; 8:753.

37. van Langelaar J, van der Vuurst de Vries RM, Janssen M *et al* . T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. Brain 2018; 141(5):1334-1349.

38. Ramstein J, Broos CE, Simpson LJ *et al* . IFN-gamma-Producing T-Helper 17.1 Cells Are Increased in Sarcoidosis and Are More Prevalent than T-Helper Type 1 Cells. American journal of respiratory and critical care medicine 2016; 193(11):1281-1291.

39. Linterman MA, Rigby RJ, Wong RK *et al* . Follicular helper T cells are required for systemic autoimmunity. J Exp Med 2009; 206(3):561-576.

40. Zhu C, Ma J, Liu Y *et al* . Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. J Clin Endocrinol Metab 2012; 97(3):943-950.

41. Christensen JR, Börnsen L, Ratzer R *et al* . Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17-and activated B-cells and correlates with progression. PloS One 2013; 8(3):e57820.

42. Tzartos JS, Craner MJ, Friese MA *et al* . IL-21 and IL-21 Receptor Expression in Lymphocytes and Neurons in Multiple Sclerosis Brain. Am J Pathol 2011; 178:794–802.

43. Nicolas P Ruiz A, Cobo-Calvo A *et al* . The Balance in T Follicular Helper Cell Subsets Is Altered in Neuromyelitis Optica Spectrum Disorder Patients and Restored by Rituximab. Front Immunol 2019; 10:2686 .

44. Cunill V, Massot M, Clemente A *et al* . Relapsing-Remitting Multiple Sclerosis Is Characterized by a T Follicular Cell Pro-Inflammatory Shift, Reverted by Dimethyl Fumarate Treatment. Front immunol 2018; 9:1097.

45. Babaloo Z, Aliparasti MR, Babaiea F *et al* . The role of Th17 cells in patients with relapsing-remitting multiple sclerosis: Interleukin-17A and interleukin-17F serum levels. Immunol Lett 2015; 164(2):76-80.

46. Matusevicius D, Kivisäkk P, He B *et al* . Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Mult Scler 1999; 5(2):101-104.

47. Zhang X, Markovic-Plese S. Interferon beta inhibits the Th17 cell-mediated autoimmune response in patients with relapsing–remitting multiple sclerosis. Clin Neurol Neurosurg 2010; 112(7):641-645.

48. Wu Q, Wang Q, Mao GA-O *et al* . Dimethyl Fumarate Selectively Reduces Memory T Cells and Shifts the Balance between Th1/Th17 and Th2 in Multiple Sclerosis Patients. J Immunol 2017; 198(8):3069-3080.

49. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004; 199(7):971-979.

50. Mexhitaj I, Nyirenda MH, Li R *et al* . Abnormal effector and regulatory T cell subsets in paediatric-onset multiple sclerosis. Brain 2019; 142(3):617-632.51.

51. van Boxel-Dezaire AH, Hoff SC, van Oosten BW *et al* . Decreased interleukin-10 and increased interleukin-12p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. Ann Neurol 1999; 45:695–703.

52. Waubant E, Gee L, Bacchetti P *et al* . Relationship between serum levels of IL-10, MRI activity and interferon beta-1a therapy in patients with relapsing remitting MS. J Neuroimmunol 2001; 112:139–145.

53. Petereit H, Pukrop R, Fazekas F *et al* . Low interleukin-10 production is associated with higher disability and MRI lesion load in secondary progressive multiple sclerosis. J Neurol Sci 2003; 206:209– 214.

54. Balashov KE, Comabella M, Ohashi T *et al* . Defective regulation of IFN- g and IL-12 by endogenous IL-10 in progressive MS. Neurology 2000; 55: 192– 8.

Figure Legends

Fig. 1. Frequency of circulating Tfh cells was significantly higher in MS patients. Fresh PBMCs from HC and MS patients were surface stained with anti-CD3, anti-CD4, anti-CXCR5 and anti-PD-1 antibodies. a) Construction of fine singlet's; representative example of by flow cytometry gating of total live PBMC by exclusion of debris, doublets and dead cells. b) gating strategy: $CD3^+CD4^+$ T cells*(left panel)* were defined by gating them on PBMCs after constructing fine singlets, $CXCR5^+PD\text{-}1^+$*(right panel)* cells defined as cTfh cells which were selected by gating from $CD3^+CD4^+$ T cells *(left panel),* c) percentage of $CD4^+$ T cells in MS and HC were calculated and compared, d) percentage of cTfh cells in MS patients was calculated and compared with that of HC. **p =0.002. Each data point represents each individual.

Fig. 2. cTfh17 and cTfh17.1 cells were the major subtypes of cTfh cells that increased in MS patients. Fresh PBMCs from HC and MS patients were surface stained with anti-CD3, anti-CD4, anti-CXCR5 anti-PD-1, anti-CXCR3, anti-CCR6 antibodies a) gating strategy; CXCR3 and CCR6 were gated from $CXCR5^+PD\text{-}1^+$cells to delineate cTfh subtypes, where $CXCR3^+CCR6^-$ defined as cTfh1, $CXCR3^-CCR6^-$ as cTfh2, $CXCR3^-CCR6^+$ as cTfh17 and $CXCR3^+CCR6^+$ as cTfh17.1 cell, b-e) percentages of different subtypes of cTfh cells were calculated in MS patients and compared with those in HC, *p=0.02 and **p=0.005 respectively.

Fig. 3. Frequency of IL-21 was significantly increased in MS patients. Fresh PBMCs from 12 HC and 20 MS patients were surface stained with monoclonal antibodies with anti-CD3, anti-CD4, anti-CD8, anti-CXCR5 and anti-PD-1 antibodies. Intracellular cytokine staining was performed using anti-IL-21 antibody after stimulating 5 hours with PMA, ionomycin and golgistop, a-b) gating strategy; IL-21 positive population was selected from PBMCs, $CD4^+$ T cells and $CD8^+$ T cells, c) percentage of IL-21 secretion in PBMCs, $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells from MS patients was calculated and compared with those in HC, ***p=0.0004 and 0.0009 respectively and **p=0.002, d) gating strategy: IL-21 positive cells were selected from $CXCR5^+PD\text{-}1^+$ Tfh cells and $CD4^+CXCR5^-$ non-follicular cTh cells, e) average frequencies of IL-21 producing cTfh and non-follicular cTh cells were calculated and compared between HC and MS. **p=0.003 indicates the difference of IL-21 secreting cTfh cells between MS patients and HC. ****p<0.0001 represents the statistical analysis of IL-21 producing cTfh vs. non-follicular cTh cells in MS patients. Mean±SEM are shown.

Fig. 4. Increased frequency of IL-21 producing cTh17.1 cells were observed in MS patients. Fresh PBMCs from 12 HC and 20 MS patients were surface stained with aforementioned antibodies described in Fig. 2. ICS was additionally performed using anti-IFN-γ, anti-IL-17, anti-IL-4 and anti-IL-21 antibodies after stimulating 5 hours with PMA, ionomycin and golgistop a) gating strategy; IL-21 population was positively selected from different subsets of cTfh cells, b) frequencies of IL-21 production by different subtypes of cTfh

cells were calculated in MS and compared with those of HC ***p=0.002, c) proportion of IL-17 producing cTfh17 cells in MS and HC were calculated and compared, d) percentages of IL-17 production by follicular and non-follicular cTh17 cells were calculated and compared in MS patients, e) percentage of IL-17 producing cTfh17.1 cells in MS patients was calculated and compared with that of HC, f) frequency of IFN-γ producing cTfh17.1 cells was calculated in MS patients and compared with that in HC. Mean±SEM are shown. Each data point represents each individual.

Fig. 5. Frequency of cTfreg cells was significantly reduced in MS patients. Fresh PBMCs from both HC and MS were surface stained with anti-CD3, anti-CD4, anti-CXCR5, anti-PD-1 and anti-CD25 antibodies. FoxP3 staining with anti-FoxP3 antibody was performed according to manufacturer's protocol (eBioscience), a) gating strategy: cTfreg$_{(right\ panel)}$ cells were selected by gating FoxP3^{+}CD25^{+} cells on CXCR5^{+}PD-1^{+}cTfh$_{(left\ panel)}$ cells, b) percentage of cTfreg cells was calculated in MS patients and compared with that of HC ****p<0.0001, c) percentage of non-follicular cTreg cells was calculated in MS patients and compared with that in HC. Mean±SEM are shown. Each data point indicates each individual.

Fig. 6. Frequency of IL-10 producing cTfreg cells was reduced in MS patients. Fresh PBMCs from 12 HC and 20 MS patients were surface stained with monoclonal antibodies as described in Fig 5. ICS was additionally performed using anti-IL-10 antibody after stimulating PBMCs with PMA, ionomycin and golgi stop for 5 hours, a-c) gating strategy: IL-10 positive cells were gated on PBMCs $_{(top\ panel)}$, cTfreg cells $_{(mid\ panel)}$ and non-follicular cTreg cells $_{(bottom\ panel)}$respectively, d) percentage of IL-10 secretion in the PBMC of MS patients was calculated and compared with those of HC **p=0.004, e) average frequencies of IL-10 producing cTfreg cells and non-follicular cTreg cells were calculated in MS patients and compared with those in HC, *p=0.02 represents the comparative analysis of IL-10 secretion by cTfreg cells between HC and MS, whereas *p=0.05 indicates the statistical difference of IL-10 production by non-follicular cTreg cells in HC vs. MS, and finally ***p= 0.0001 and ****p<0.0001 demonstrate the statistical difference of IL-10 secretion by follicular vs. non-follicular cTreg cells in HC and MS patients respectively. Mean±SEM are shown. Each data point indicates each individual.

Table 1. Demographic and clinical representation of participants.

Characteristics	Healthy control	MS patient
Sample size (n)	12	20
Age (years, mean±SD)	35±7	34.3±9.9
Women: men (n:n)	8:4	12:8
Disease onset age (years, mean±SD)		29.29±7.81
Disease duration (years, mean±SD)		5.49±4.33
EDSS (years, median±SD)		1.5±1.35
Treatment duration (years, mean±SD)		4.1±3.97

Hosted file

`List of figures.ppt` available at https://authorea.com/users/334995/articles/460931-dysregulated-proinflammatory-circulating-follicular-helper-t-cells-and-suppressive-follicular-regulatory-t-cells-in-patients-with-multiple-sclerosis