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Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis

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Follicular regulatory T cells (TFR) have been extensively characterized in mice and participate in germinal center responses by regulating the maturation of B cells and production of (auto)antibodies. We report that circulating TFR are phenotypically distinct from tonsil-derived TFR in humans. They have a lower expression of follicular markers, and display a memory phenotype and lack of high expression of B cell lymphoma 6 and ICOS. However, the suppressive function, expression of regulatory markers, and FOXP3 methylation status of blood TFR is comparable with tonsil-derived TFR. Moreover, we show that circulating TFR frequencies increase after influenza vaccination and correlate with anti-flu Ab responses, indicating a fully functional population. Multiple sclerosis (MS) was used as a model for autoimmune disease to investigate alterations in circulating TFR. MS patients had a significantly lower frequency of circulating TFR compared with healthy control subjects. Furthermore, the circulating TFR compartment of MS patients displayed an increased proportion of Th17-like TFR. Finally, TFR of MS patients had a strongly reduced suppressive function compared with healthy control subjects. We conclude that circulating TFR are a circulating memory population derived from lymphoid resident TFR, making them a valid alternative to investigate alterations in germinal center responses in the context of autoimmune diseases, and TFR impairment is prominent in MS. The Journal of Immunology, 2015, 195: 000–000.

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Abbreviations used in this article: AID, autoimmune disease; BCL-6, B cell lymphoma 6 (BCL-6), signaling lymphocytic activation molecule–associated protein (SAP), and B cells (4). Phenotypic analysis of TFR overlapped with the surface profile of TFR cells (CD4+CXCR5+PD-1+ICOS3), and both cell types also express BCL-6. In contrast with TFFH, TFR do not express IL-20, IL-4, or CD40L. Besides sharing surface markers with TFFH cells, TFR cells also express regulatory markers such as Foxp3, CD25, CTLA-4, glucocorticoid-induced TNFR-related protein, and IL-10 (4–6). The transcription factor NFAT2 has been identified as crucial for TFR development in mice, because it is essential for the induction of CXCR5 in these cells (7). CXCR5 directs the migration of TFFH into the GC where they suppress the magnitude of the GC response by regulating the number of TFFH B cells, and Abs. Taken together, TFFH and TFR are a distinct population of Tregs located primarily in the GC, showing overlap with both Tregs and TFFH.

Because regulation of (auto)antibody production primarily takes place in the GC, it is probable that an aberrant GC response can contribute to autoimmunity. In the absence of TFFH, overwhelming outgrowth of nonspecific B cells leads to lower amounts of Ag-specific B cells (8). Furthermore, a lack of CXCR5+ Tregs in mice results in increased GC activity, including affinity maturation and differentiation toward plasma cells. In addition, PD-1 was found to be crucial for the homeostasis of TFFH, because PD-1–deficient mice showed increased numbers of TFFH in lymph nodes. Also, the PD-1 pathway seems to impair TFFH function, considering PD-1 deficiency results in an increased TFFH suppressive capacity (9). NFAT2-deficient TFFH fail to control lupus-like disease manifestations in mice, supporting the proposition that TFFH play a role in maintaining self-tolerance (7).

In this study, we sought to identify the involvement of TFR in human disease by using multiple sclerosis (MS) as a model for an autoimmune disease (AID) with Treg disturbances (10) and elevated autoantibody levels (11, 12). Peripheral blood (PB) is the
most accessible source to analyze immune responses in healthy control subjects (HCs) and patients with AID. Therefore, we first determined whether blood TFR are a good representation to investigate TFR responses ongoing in secondary lymph nodes. To do so, a pairwise comparison was made between TFR derived from blood and tonsils of HCs. Next, circulating TFR were monitored after influenza vaccination to define GC-induced changes in this subset. Finally, circulating TFR alterations were investigated in MS patients and compared with HCs to evaluate their involvement in AID.

Materials and Methods

Human samples

Ethics approvals were obtained from each institute’s human ethics committee. Tonsils and blood were obtained from adult patients without AIDs who were undergoing routine tonsillectomies at Hospital East-Limburg (Genk, Belgium; for detailed information, see Supplemental Table I). Adult healthy volunteers were recruited for the vaccination of inactivated influenza vaccine Influvac S 2013/2014 (ABBOTT BIOLOGICALS B.V., Brussels, Belgium; for detailed information, see Supplemental Table I). MS patients were recruited from the Rehabilitation and MS-Center (Oeperelt, Belgium). Detailed clinical characteristics are listed in Supplemental Table I.

PBMC purification and flow-cytometric analysis

PB was collected in heparin-coated tubes (VenoSafe plastic tubes; Terumo Europe N.V., Leuven, Belgium). Tonsils were cut into small pieces and single cells were obtained using a cell strainer (EASYstrainer 70 μm; Greiner Bio-One BVBA/SPRL, Wemmel, Belgium). After collection of the plasma, density centrifugation was used to isolate the PBMCs (Lymphocyte; Cederlane Laboratories, SanBio B.V., Uden, the Netherlands). In line with recent publications, specific flow-cytometric markers were used to identify circulating Tregs and TFR in human blood (13, 14). For flow-cytometric analysis of the T cell subsets, the following Abs were used: CD4 allophycocyanin, CD4 PE-CF594, CD4 FITC, CD45RO PE-CF594, CXCR5 Alexa Fluor 488, CXCRI3 PE-CF594, CCR6-PerCP-Cy5.5, BCL6 PE-CF594, Foxp3 PE-CF594 (all from BD Biosciences, Erembodegem, Belgium), CD25 PerCP-Cy5.5, CD127 PE, PD-1 PE-Cy7, CD45RA allophycocyanin-H7, CD31 allophycocyanin, CCRI7 PE, CD62L allopheocyanin-Cy7, SAP-PE, Helios allophycocyanin (all from eBioscience, San Diego, CA); and CD25 allophycocyanin-Cy7 and CD27 allophycocyanin (from Biologend, ImTec Diagnostics N.V., Antwerp, Belgium). B cell analysis was performed with CD19 PerCP-Cy5.5, IgD allophycocyanin-Cy7, and CD27 PE-Cy7 (all from BD Biosciences). Appropriate isotype controls were used to establish the proper gating strategies (all from BD Biosciences). All flow-cytometric analyzes were performed on a FACSAriaII flow cytometer and analyzed with FACSDiva software (BD Biosciences). For Foxp3 intranuclear staining, the eBioscience kit was used; for other intracellular stains, the BD Cytofix/Cytoperm kit (BD) was used according to the manufacturers’ guidelines.

Purification of CD4 T cell subsets

CD4+ T cells were purified using CD4 negative selection (STEMCELL Technologies Sarl, Grenoble, France). CD25 positive selection (STEMCELL Technologies Sarl) was used to obtain a CD25-enriched population and a CD25+ population. CD4+CD25+CD127+ responder T cells (Tresp) and CD4+CD25+CD127- CXCR5-PD-1+ TFR were sorted from the CD25+ population. CD4+CD25+CD127+ Tresp and CD4+CD25+CD127-CXCR5-PD-1+ TFR were sorted from the CD25+ population using the following Abs: CD4 FITC (BD), CD25 PerCP-Cy5.5 (eBioscience), CD127 PE (eBioscience), CXCR5 Alexa Fluor 647 (BD), and PD-1 PE-Cy7 (eBioscience) using a FACSAria II (BD). Purity of the isolated cells was confirmed. Flow-cytometric analysis was performed using FACSDiva software (BD Biosciences) and Flowjo V10.

Suppression assays

A 96-well round-bottom plate (Nunc, Roskilde, Denmark) was coated for 2 h at 37°C with 0.01 μg/ml anti-CD3 (HT3; BD) and washed with PBS. CD4+CD25+CD127+ Tresp (labeled with 4 μM CFSE; Invitrogen) were cultured at 1 × 10^6 cells/well with 1 × 10^5 irradiated autologous PBMCs (feeder cells) in the presence or absence of the same number of Tregs or TFR in duplicate. The isolated Treg population thus also includes the CXCR5-PD-1+ TFR population. Cell cultures were also stimulated with soluble anti-CD28 (BD) for 4 d. The following controls were used: 1) a nonlabeled stimulated control to serve as reference for setting the Treg gate, 2) a labeled nonstimulated control to serve as reference for setting the nonproliferated gate, and 3) a labeled stimulated control with double amount of responder cells to exclude possible nutrient deprivation effects. Cocultures were analyzed on a FACSAriaII on day 4. The suppressive capacity (percentage) of Tregs toward Tresp in coculture was calculated relative to the maximal proliferation of the Tresp alone: [(0 – (% proliferation Tresp alone)/% proliferation Tresp + Treg)]*100.

Foxp3 methylation assay

CD4+CD25+ T cells (purified as described earlier) were sorted to obtain CD4+CD25+CD127+ Tregs and CD4+CD25+CD127+CXCRI5-PD-1+ TFR, and CD4+CD25- T cells were sorted to obtain CD4+CD25+CD127-CXCR5-PD-1+ TFR cells using FACSAria II (BD). Purity of the obtained cells was confirmed. Purified cells were pelleted and frozen at −80°C. Next, the proportions of cells with a demethylated FOXP3 intron 1 allele were quantified by quantitative PCR on bisulfite-treated genomic DNA, as described previously (15).

Hemagglutination assay

Plasma samples from HCs were obtained before and 21 d after vaccination, for Ab titration against the 2013–2014 influenza H1N1 pdm vaccine strain A/California/7/2009 (A/California/7/2009 NYMC X-179A, Cat. No. FR-1184; Influenza Reagent Resources, Centers for Disease Control and Prevention) and the 2013–2014 influenza H1N2 vaccine strain A/Texas/50/2012 (A/Texas/50/2012 X-223, Cat. No. FR-1185; Influenza Reagent Resources, Centers for Disease Control and Prevention), using a hemagglutination inhibition (HAI) assay. In brief, 1:2 serial dilutions of inactivated human plasma samples were preincubated with a standardized amount of virus before the addition of 1% RBC (glutaraldehyde-stabilized freshly prepared guinea pig RBCs, Cat. No. 88R-P001; Bio-connect). After incubation, HAI titers are recorded. Controls samples were included in all analyses. Samples were tested in duplicate, and assays were independently repeated. The titer analyzed was the geometric mean of these test results. Patients who showed nonspecific agglutination of RBCs were excluded.

Statistics

Statistical analyses were done using SAS 9.3, SAS Jump, and GraphPad Prism 6. Graphics were made using GraphPad Prism 6. Data sets were checked for normality, and for effect of age and sex. Analyses of the vaccination study was done using a mixed model (multiple measurements SAS 9.3). Analysis of multiple groups was done using ANOVA, nonparametric testing (Kruskal–Wallis), or linear correlations using SAS Jump. A Mann–Whitney U test was used for nonparametric unpaired data. Wilcoxon matched-pair test was used for nonparametric paired data. Tests were considered significant when p < 0.05 (two-sided tests).

Study approval

All human blood samples and tonsils were obtained with ethical approval of each institute’s human ethics committee, the Medical Ethical Commission of University Hospital Leuven and Hospital East-Limburg, respectively. Written, informed consent was obtained from all study subjects.

Results

Human circulating TFR comprise a phenotypically distinct population

The presence and origin of genuine follicular subsets in the circulation remains controversial (2, 16). This led us to question whether circulating TFR are phenotypically bona-fide TFR or rather represent a distinct population.

First, circulating TFR were phenotypically characterized in detail comparing blood and tonsils of HC (for detailed information, see Supplemental Table I). Distinct subpopulations of Treg (CD4+CD25+CD127+) and conventional T cells (CD4+CD25+CD127-) were found to express the follicular markers CXCR5 and PD-1 in blood and in tonsils (Fig. 1A). Based on that, CD4+CD25+CD127−CXCR5+PD-1− and CD4+CD25+CD127−CXCR5+PD-1+ were defined as (circulating) counterparts of, respectively, TFR and TFr. In tonsils, TFr and TFr comprise a much larger population compared with the blood (Fig. 1A, 1B).
FIGURE 1. Circulating TFR comprise a distinct population compared with tonsil-derived TFR. (A) Gating strategy to identify circulating TFR (CD4+CD25+CD127+CXCR5+PD-1+) and TFH (CD4+CD25−CD127+CXCR5+PD-1+) within the Tregs and conventional T cells (Tconv) gate, respectively, in tonsils and blood. (B) Percentage of TFR and TFH in blood and in tonsils. (C) Percentage of follicular markers (ICOS, SAP, and BCL-6) on both TFR and TFH from blood and tonsils. The MFI of BCL-6 is shown. (D) Expression levels of follicular markers CXCR5, PD-1, BCL-6, and (Figure legend continues)
We next analyzed whether circulating follicular cells are phenotypically similar to those derived from tonsils using both follicular (ICOS, SAP, and BCL-6) and regulatory (Foxp3, Helios, and CD31) markers (Fig. 1C–E). Circulating TFR and TFH did not express ICOS, whereas tonsil-derived cell subsets did. SAP, essential for T–B cell interaction, was significantly less expressed on circulating compared with tonsil-derived TFH, whereas no significant differences were found for TFR. A significantly higher proportion of TFR from tonsil express BCL-6 compared with circulating TFR, whereas TFH did not significantly differ in percentage of BCL-6+ cells. In contrast, the mean fluorescent intensity (MFI) of BCL-6 is significantly decreased on both blood TFR and TFH compared with their tonsil-derived counterparts. In addition, the expression levels of essential follicular markers (CXCR5, PD-1, BCL-6, and ICOS) are shown in Fig. 1D, again highlighting the phenotypical difference in follicular expression on blood TFH and TFR. The regulatory markers Foxp3 and Helios were equally expressed by TFR in tonsils and blood. No expression of regulatory markers was seen in TFH from any source. CD31 is a key molecule for the regulation of T cell homeostasis, effector function, and trafficking (17–19). We found an increased expression of CD31 on the surface of circulating follicular cells, whereas the tonsil-derived counterparts did not express this marker. Taken together, these data show that human circulating TFR are not bona fide TFR because they lack high expression of CXCR5, PD-1, BCL-6, and ICOS, all essential follicular marker proteins.

Lastly, we determined the differentiation stage and effector phenotype of human circulating TFR. As a negative and positive control, we compared these cells with naïve T cells and memory T cells, respectively, based on their CD45RO expression. Tonsil-derived TFR and TFH cells have a CD45RO+ and CD45RO2 cell population unlike the circulating follicular cell subsets, which are all CD45RO+ indicating a memory phenotype (Fig. 2A). To further characterize this memory phenotype, we used CCR7 and CD62L to distinguish effector memory T cells (TEM) (Fig. 2B) from central memory T cells (TCM) (Fig. 2C). Although all tonsil-derived follicular T cells have a TEM phenotype (CCR72 and CD62L2), only half of the circulating TFR have a TEM and a minority is TCM. We next examined the effector phenotype based on the expression of chemokine receptors CXCR3 and CCR6. On TFH (CD4+CXCR5+), CXCR3 expression is reported to represent a Th1 phenotype, whereas CCR6 indicates a Th17 phenotype (20). Combining both markers gives a more elaborate view on the effector phenotype (Th2; CXCR32 CCR62, Th17; CXCR3+CCR6+, and Th1; CXCR3+CCR62). We found that circulating TFR have a significantly higher percentage of CXCR3+CCR6+ cells (Th1-like phenotype) compared with tonsil-derived TFR (p = 0.03; Fig. 2D). A trend toward an increase in the percentage of CXCR3+CCR6+ cells (Th17-like phenotype) was found (Fig. 2E), whereas the percentage of CXCR3+CCR62 cells (Th2-like phenotype) was significantly decreased (p = 0.0079; Fig. 2F). An overview of the gating strategy of the flow-cytometric markers can be found in Supplemental Fig. 1.

Together, these data suggest that human circulating TFR are phenotypically distinct from their counterparts in the secondary lymphoid organs (tonsils were used as a model) because they

ICOS on TFR and TFH in blood (dark gray) and tonsils (light gray). (E) Percentage of regulatory markers (Foxp3, Helios, and CD31) on both TFR and TFH from blood and tonsils. Data are shown as median for n = 5 HCs. *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon matched-pair test.
express lower levels of follicular markers, are a memory population, and have a proinflammatory effector phenotype.

Influenza vaccination boosts the number of circulating TFR

To investigate whether follicular cell activity is measurable in the blood, we next assessed the effect of vaccination on circulating follicular and B cell subsets. PB from HCs (n = 24) were collected before (D0), and after influenza vaccination (Influvac S 2013/2014) at different time points: after 1 d (D1), after 1 wk (D7), and after 3 wk (D21). Circulating TFR and TFH were identified as CD4+CD25+CD127−CXCR5+PD-1+ and CD4+CD25−CD127−CXCR5−PD-1−, respectively, gated as shown in Fig. 1A. (A and C) The percentage of both circulating TFR and TFH (phenotyped as shown in Fig. 1) after vaccination was assessed. (B and D) The percentage of CD45RO+ subpopulation in the TFR and TFH before and after vaccination. (E-H) The MFI of both CXCR5 and PD-1 in the circulating follicular cells. (I) The percentage of plasmablast (CD19+CD27+CD138+) at different time points. (J) The ratio of non–class-switched B cells (NCS B cells, CD19+IgD−CD27+) on class-switched B cells (CS B cells, CD19+IgD+CD27+) at different time points. (K–N) The percentage of both circulating TFR and plasmablast at D7 was correlated with the plasma titers for H1N1 strain and H3N2 strain after D21. Data are mean ± SEM. Correlation analyses were done using a linear regression model. Analyses were done using a mixed model (multiple measurements SAS 9.3) with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
the percentage of circulating TFR at D7 was positively correlated with postvaccination (D21) geometric mean titers (GMTs; \( p = 0.05 \) and \( p = 0.03 \); Fig. 3K, 3L). Moreover, the percentage of plasmablasts significantly correlated with postvaccination H1N1pdm GMTs titers \( (p = 0.02; \text{Fig. 3M}) \), but not with postvaccination H3N2 GMTs (Fig. 3N).

In summary, we show that seasonal influenza vaccination in HCs leads to a significant increase in circulating (memory) TFR and TFH, which significantly correlate with titers of anti-vaccine Abs. We therefore conclude that circulating TFR are a relevant source to measure TFR activity and regulation of Ab responses in response to GC reactions.

**Human circulating and tonsil-derived TFR are equally suppressive in vitro**

To assess the functionality of circulating TFR, we made a pairwise comparison of the suppressive capacity of sorted blood- and tonsil-derived TFR and total Tregs of HCs \( (n = 7, \text{Supplemental Table I}) \) using an in vitro coculture suppression assay. In brief, CFSE-labeled Tresp were cocultured in a 1:1 ratio with TFR, total Tregs, or no Tregs, and this in the absence or presence of CD3/CD28 stimulation for 4 d \( (23–25) \). Fig. 4A shows a representative example with the various conditions that were incorporated in the assay. Blood-derived TFR and Tregs were equally suppressive as their tonsil-derived counterparts, indicative of an equal functional activity (Fig. 4B). In addition, the methylation status of the FOXP3 gene was assessed as previously described \( (15) \). No significant difference in demethylated FOXP3 was found between TFR from blood compared with tonsils, indicating equal differentiation into Treg lineage (Fig. 4C). Sorted total Tregs and TFH were used as a positive and negative control, respectively. Together, these results show that circulating TFR are equally suppressive and have the same level of demethylated FOXP3 as TFR of secondary lymphoid organs, governing them as a good alternative for functional analyses in the context of (auto)immunity.

**Circulating TFR frequencies are decreased in patients with MS**

Various groups, including ours, have reported an impairment in the Treg compartment of MS patients \( (10, 26–28) \). Moreover, elevated levels of autoantibodies have been reported in MS \( (29, 30) \). In light of these findings, we investigated whether the TFR com-
The compartment known to regulate humoral immunity is disturbed in MS. We show a decreased frequency of circulating TFR in MS patients ($n = 172; p < 0.0001, n = 172$ for MS patients and $n = 107$ for HCs) compared with HCs ($n = 107$ for more information, see Supplemental Table I). (C) Correlation of clinical scores (Expanded Disability Status Scale) and percentage TFR. (D) The percentage of TFR in different MS disease types (one-way ANOVA). (E) Correlation between the percentage of circulating TFR and the disease duration in patients with MS. (F) Within the circulating TFR, the percentage of effector cells was investigated using CXCR3 and CCR6: Th2-like; CXCR3$^+$CCR6$^-$, Th17-like; CXCR3$^+$CCR6$^+$, and Th1-like; CXCR3$^+$CCR6$^+$ in MS patients and HCs ($n = 14$ for MS patients and $n = 16$ for HC, $p = 0.0033$, Mann–Whitney test). Data are mean $\pm$ SEM. Correlations were made using a standard linear regression model. **$p < 0.01$, ****$p < 0.0001$.

FIGURE 5. Frequency of circulating TFR in MS patients and HCs. (A) The percentage of circulating TFR in HCs and MS patients compared with HCs ($p < 0.0001, n = 172$ for MS patients and $n = 107$ for HCs). (B) The TFH/TFR ratio MS patients and HCs ($p < 0.0001, n = 172$ for MS patients and $n = 107$ for HC). (C) Correlation of clinical scores (Expanded Disability Status Scale) and percentage TFR. (D) The percentage of TFR in different MS disease types (one-way ANOVA). (E) Correlation between the percentage of circulating TFR and the disease duration in patients with MS. (F) Within the circulating TFR, the percentage of effector cells was investigated using CXCR3 and CCR6: Th2-like; CXCR3$^+$CCR6$^-$, Th17-like; CXCR3$^+$CCR6$^+$, and Th1-like; CXCR3$^+$CCR6$^+$ in MS patients and HCs ($n = 14$ for MS patients and $n = 16$ for HC, $p = 0.0033$, Mann–Whitney test). Data are mean $\pm$ SEM. Correlations were made using a standard linear regression model. **$p < 0.01$, ****$p < 0.0001$.

Circulating TFR are functionally impaired in patients with MS

The functionality of MS-derived circulating TFR was tested using the in vitro coculture suppression assay described earlier (Fig. 6A). MS-derived TFR displayed a strongly impaired suppression compared with HCs (HC: $n = 15$, MS: $n = 12, p \leq 0.0001$; for more information, see Supplemental Table I; Fig. 6B). We further confirmed that conventional Tregs from these MS patients were also significantly impaired ($p < 0.0001$; Fig. 6B).

When correlating the suppressive function of both Tregs and TFR from the same donor, a significant positive association was found for both MS patients and HCs (Fig. 6C). For one MS patient (relapsing-remitting MS, no treatment), the suppressive capacity of Tregs and TFR (at a 1:1 ratio) at both time points was in the same range (data not shown), confirming the reproducibility and stability of the assay, as well as the constant nature of Treg/TFR function.

In conclusion, we found that both Tregs and TFR isolated from MS patients had a reduced capacity to suppress the proliferation of Tresp showing a functional impairment of circulating TFR in MS patients.
Discussion

In this study, we provide an insight into the phenotype and function of circulating TFR in humans. Although circulating TFR represent a population that is phenotypically distinct from their tonsil-derived counterparts, they are an eligible source to measure GC responses ongoing in secondary lymphoid organs. We showed a decreased frequency and impaired functionality of circulating TFR in MS patients, indicating their involvement in breakdown of self-tolerance in human AIDs. These conclusions led us to two main questions: What is the origin and fate of these cells? And what explains the impairment of the circulating TFR population in MS?

Circulating TFR are not considered bona-fide TFR because they lack high expression of essential follicular markers, such as BCL-6 and ICOS. These results are consistent with findings in circulating TFH and likely indicate that follicular markers upregulate only after homing to the GC (16). This notion is further supported by the decreased expression of CXCR5 and PD-1 on circulating TFR after influenza vaccination, which could reflect homing of CXCR5hiPD-1hi TFR to the GC. In mice, TFR were shown to originate from thymic-derived Tregs (4). In line with these findings, we showed that human blood TFR express Helios and demethylated FOXP3 to a similar extent as tonsil-derived TFR and are fully functional. Furthermore, we characterized these cells as central memory with a higher expression of both CCR7 and CD62L compared with tonsil-derived TFR, allowing recirculation to the lymph nodes. He et al. (16) showed that circulating TFH also have a higher expression of these markers. In addition, we found a significant increase in the percentage of memory TFR after seasonal influenza vaccination. It is therefore possible that they originate from GC TFR, migrate to the circulation after a GC response, and become a central effector memory population that is long-lived and has the capacity to recirculate. Another theory proposed by He et al. (16) suggested that circulating TFH cells are a population of cells that leave the GC response in its early phase, before developing into mature TFH cells, governing a “precursor memory” population. We showed that although the expression of Foxp3 and Helios was the same in TFR, blood-derived follicular cells are CD31+ whereas the tonsil-derived follicular cells are negative. It has been reported that CD31 expression regulates T cell activation and can thus prevent hyperactivation (17, 19). In addition, recent evidence showed that the loss of CD31 after activation leads to a stable interaction of T cells with B cells (18). This could indicate that circulating TFR are indeed a central memory population that is quiescent by nature and will lose CD31 expression upon activation. In line with this, a recent mouse study (31) reported that circulating TFR are a long-lived memory population that homes to GC after reactivation. A human study showed that follicular T cell populations do not need an ongoing GC response for their maintenance because treatment with rituximab, known to eliminate GC B cells, had no effect on the follicular T cell compartment (32). Together all these data indicate that circulating TFR are a distinct effector memory population that persists for a long time and is able to recirculate to the lymph nodes when needed.

MS, an AID of the CNS, was used as a model to investigate the role of circulating TFR in autoimmunity. A functional impairment of Tregs in MS was shown by various groups (26–28, 33, 34). We
confirmed an impairment of Tregs in MS patients and found that T_{FR} from the same patients are also defective in their capacity to suppress Tresp. Also, a decreased percentage of blood T_{FR} in MS patients was found compared with HCs. Based on the chemokine markers suggested by Morita et al. (20), we show that the amount of Th17-like T_{FR} is increased in MS patients. A more proinflammatory phenotype could explain the decreased suppressive function of circulating T_{FR}. Furthermore, this impairment could originate from a defect in CTLA-4 signaling because CTLA-4 is essential in T_{FR} function (35). An alternative explanation for the reduced frequency and suppressive activity in the circulating T_{FR} compartment could be that the most potent T_{FR} homed to the lymph nodes to suppress the ongoing GC response. Sage et al. (31) showed that circulating T_{FR} in mice require dendritic cells for their development and cytokine production. Circulating dendritic cells in MS were shown to have a decreased regulatory character and could in this way contribute to an impairment of functional circulating T_{FR} (36).

In conclusion, we believe that follicular populations in the blood form a source of responsive memory cells that quickly react when encountering an Ag again. Impairment of circulating T_{FR} and T_{FR} could contribute to the pathogenesis of various AIDs, including MS, highlighting their importance in conserving normal immunity.

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Disclosures

The authors have no financial conflicts of interest.

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