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RESEARCH ARTICLE

Fingolimod treatment promotes regulatory phenotype and function of B cells

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Abstract

Objective: To evaluate the influence of Fingolimod treatment on B-cell subset composition and function in multiple sclerosis patients and its potential clinical relevance. **Methods**: Subset composition and cytokine production of B cells derived from peripheral blood mononuclear cells from multiple sclerosis patients under Fingolimod treatment, untreated multiple sclerosis patients and healthy controls were analyzed by flow cytometry and ELISA. Migration of lymphocyte subsets across primary human brain microvascular endothelial cells was assessed in an in vitro transmigration assay. Cell numbers and composition of B-cell subsets in cerebrospinal fluid and peripheral blood were determined by flow cytometry. Regulatory B-cell frequencies were correlated with parameters of disease stability. **Results**: Within the peripheral B-cell compartment of Fingolimod-treated patients, the proportion of regulatory B cells ($CD38^+CD27^-CD24^+CD5^+$) was significantly increased as compared to treatment-naïve multiple sclerosis patients and to healthy controls, and significantly more regulatory B cells produced Interleukin-10. Fingolimod treatment enhanced the capacity of regulatory B cells to transmigrate across brain endothelial cells in an in vitro model of the blood-brain-barrier. In line with these findings, the cerebrospinal fluid/blood ratio of total B cells and regulatory B cells was strongly increased by Fingolimod treatment, and patients exhibited increased regulatory B-cell frequencies in the cerebrospinal fluid. Finally, elevated regulatory B-cell percentages in the periphery significantly correlated with clinical and paraclinical disease stability. **Interpretation**: These data suggest a novel and as yet unrecognized role of Fingolimod in correction of the imbalance between regulatory and effector B-cell functions in multiple sclerosis both by direct effects and indirect partitioning effects on B-cell subpopulations.

Introduction

In Multiple Sclerosis (MS), an immune-mediated disorder of the CNS, the antibody-independent pathogenic role of B cells has recently been increasingly acknowledged.[1,2] Immunopathogenic relevance beyond their capacity to produce auto-antibodies comprises antigen presentation as well as dysregulated cytokine production, both resulting in enhanced $CD4^+$ T-cell activation.[3] Recently, protective functions of regulatory B cells have been characterized, and impaired functions of this subpopulation have been implicated in several autoimmune diseases.[4,5]

Fingolimod (FTY720) is an approved treatment for relapsing-remitting MS. It acts as a functional antagonist of the sphingosine-1-phosphate (S1P) receptor, rendering lymphocytes insensitive to S1P-mediated signals, necessary for lymphocyte egress from secondary lymphoid structures.[6,7] As a consequence naïve and central memory T cells are trapped within the lymphatic tissue. Although a significant drop in peripheral blood (PB) cell counts has been described for B cells, as well, functional consequences

of Fingolimod treatment on B-cell subsets have not been elucidated.[8]

The aim of this study was to characterize the influence of Fingolimod treatment on B-cell subsets with a focus on regulatory B-cell frequencies and function in the PB and CSF of MS patients. To address this, we evaluated regulatory B-cell frequencies, cytokine responses, and migratory activity and compared these data with those from untreated MS patients and healthy controls (HCs).

Subjects/Materials and Methods

Details on standard protocol approvals, registration and patient consents, antibodies, cells and reagents, biomaterials as well as protocols for cytokine secretion assay, HBMEC culture and transmigration assay, flow cytometry and statistics applied in this study are given in Data S1 and Table S1.

Patients and HCs

Blood samples of Fingolimod-treated MS patients were collected before and at different time points after treatment initiation. Patients were under regular clinical observation and neurological examination including Expanded Disability Status Scale (EDSS) was performed every 3 months by an experienced neurologist. All patients received cMRI assessments in yearly intervals. After ≥18 months of treatment, patients were divided into either "active" (at least one relapse or new/enlarging T2 lesion or Gadolinium enhancing lesion in cMRI during Fingolimod treatment) or "stable" (complete absence of criteria defining "active" patients).

As controls, age- and sex-matched untreated patients with MS, healthy donors (no previous history of neurologic or immune-mediated diseases) and CSF-Ctrl. patients were included in the analysis. Individuals designated as "Ctrl." underwent lumbar puncture with suspected presence of a neurological disorder but turned out to be healthy. The inclusion criteria for the CSF-Controls and the assessment of further controls are specified in the Data S1. Table 1 gives an overview of patients and controls included in this study.

Assessment of the migrational propensity of whole lymphocytes, B cells and B-cell subsets in vivo

To compare and visualize the propensity of B cells of the three groups to migrate across the blood-brain-barrier (BBB), column graphs of CSF/blood ratios were developed:

The average *whole B-cell* counts of the Ctrl.-group in blood and CSF were set to one, and in relation to those, the values of the individual cell counts in the according compartment were recalculated. By dividing the CSF-values by the according blood values individual blood/CSF ratios were obtained. The columns in the according graphs reflect the averages of the individual ratios per group; the latter are displayed as points in front of the columns.

The above-described procedure was carried through for whole-lymphocyte counts, as well. Only they were normalized to the average *lymphocyte* counts of the Ctrl.-group.

Results

Fingolimod treatment increases the proportion of IL-10-producing regulatory B cells in the PB of MS patients

In our cohort we investigated the influence of Fingolimod treatment on absolute B-cell counts and percentages in the PB of MS patients. In accordance with the literature, both were significantly decreased in the PB mononuclear cell (PBMC) compartment of Fingolimod-treated MS patients (Fig. S1).

Further analysis of B-cell subsets (gating strategy see Fig. 1A) revealed that the percentage of naïve B cells (HC 61.7%; MS 64.4%; Fingolimod-treated patients [FTY] 55.5%) and of memory B cells (HC 30.8%; MS 30.8%; FTY 20.6%) within the B-cell compartment was slightly reduced in FTY compared to the untreated MS (MS) and the HC group (Fig. 1B and C). In contrast, the percentage of regulatory B cells, as defined by the expression pattern $CD38^{+}CD27^{-}CD24^{+}CD5^{+}$,[9,10] was significantly increased in response to Fingolimod treatment (HC 5.2%; MS 3.7%; FTY 19.8%) (Fig. 1B and C).

Table 1. Participant data.

	Untreated patients with MS	Fingolimod-treated patients with MS	Healthy controls	CSF controls
Samples	74	48	70	9
Sex F/M	51/23	34/14	45/25	7/2
Mean age ± SD (range)	39 ± 11 (23–63)	38 ± 10 (23–59)	39 ± 12 (22–68)	41 ± 16 (22–69)

MS, multiple sclerosis; SD, standard deviation.

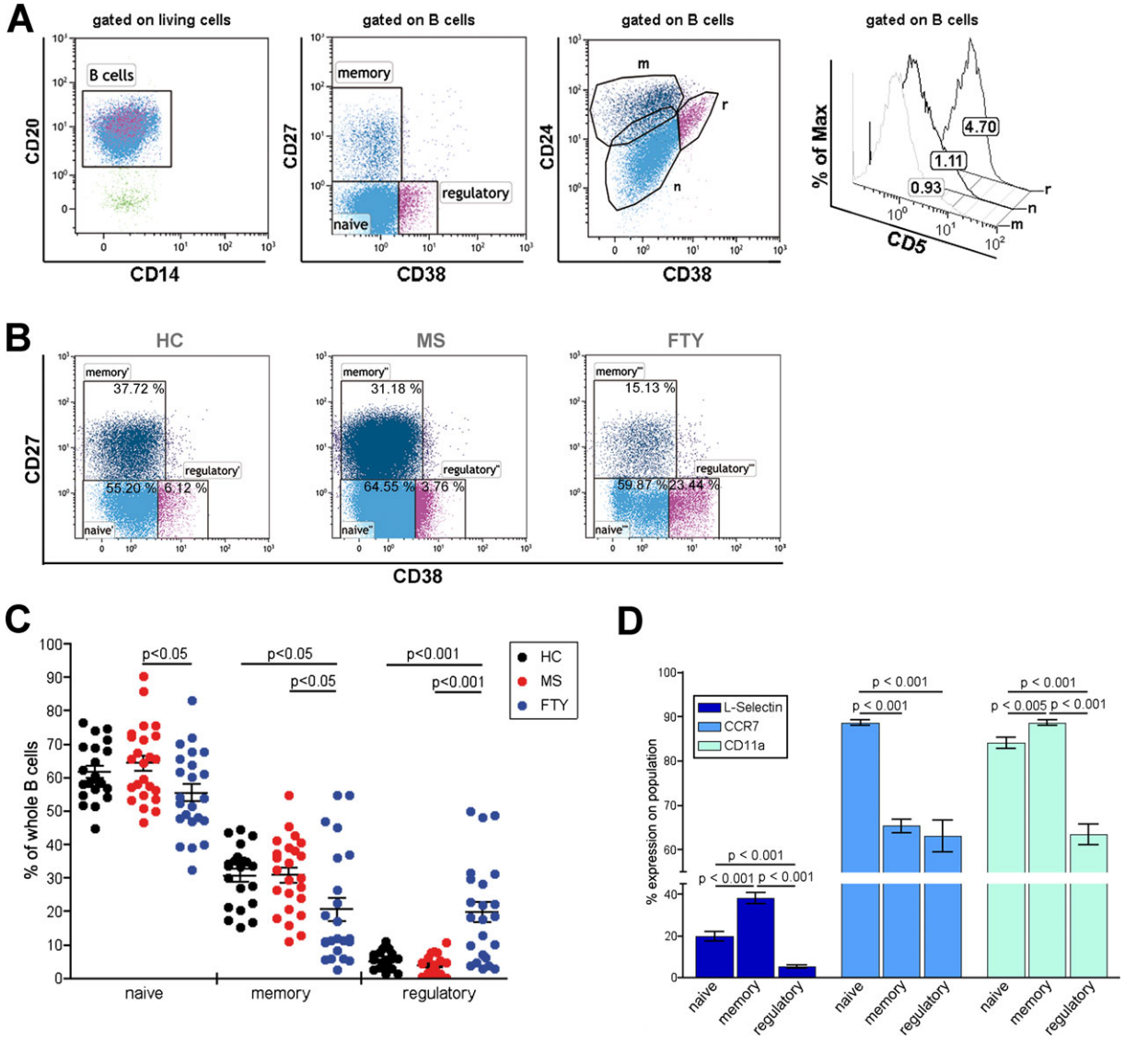


Figure 1. Effect of Fingolimod on B-cell subpopulation composition in the peripheral blood. (A–C) Frequencies of B-cell subpopulations in peripheral blood of MS, FTY and HC, determined by flow cytometry. (A) B-cell subset characterization. Naïve B cells (n): $CD38^{-}$ $CD27^{-}$ $CD24^{-/dim}$; memory B cells (m): $CD38^{-}$ $CD27^{+}$ $CD24^{+}$, regulatory B cells (r): $CD38^{+}$ $CD27^{-}$ $CD24^{+}$ $CD5^{+}$. Numbers in histogram plot represent MFI. (B) Representative examples of B-cell subsets of the three groups (HC, MS, FTY) are shown. (C) Percentages of B-cell subpopulations within the three groups. (D) Expression levels of L-Selectin (CD62L), CCR7 (CD197), and LFA-1 (CD11a) on B-cell subpopulations of HC determined via flow cytometry. (C and D) Horizontal lines indicate mean ± SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with multiple sclerosis; HC, healthy controls; MFI, mean fluorescence intensity; MS, untreated patients with MS; SEM, standard error of the mean.

To evaluate whether this shift in B-cell subpopulation composition is caused by differential sequestration due to differing lymph node-homing properties, we analyzed the expression of CCR7, L-Selectin, and LFA-1 on naïve, memory, and regulatory B cells from HC, MS, and FTY.

Regarding the general homing propensities of B-cell subsets in HC we saw that naïve B cells displayed a markedly higher expression of CCR7 than memory and regulatory B cells (naive: 88.7%; memory: 65.4%; regulatory: 63.2%). Further, the expression of L-Selectin was highest on memory B cells (38.4%) and lowest on regulatory B cells (5.3%), and CD11a expression was high in naïve and memory B cells (naïve: 84.2%; memory: 88.7%) while considerably lower on regulatory B cells (63.5%) (Fig. 1D).

To assess how Fingolimod might alter the cytokine production of B cells, freshly isolated B cells were stimulated either with CD40L or with CpG-B, and production of IL-10, the most relevant anti-inflammatory B-cell cytokine,[10] was analyzed by flow cytometry. Without stimula-

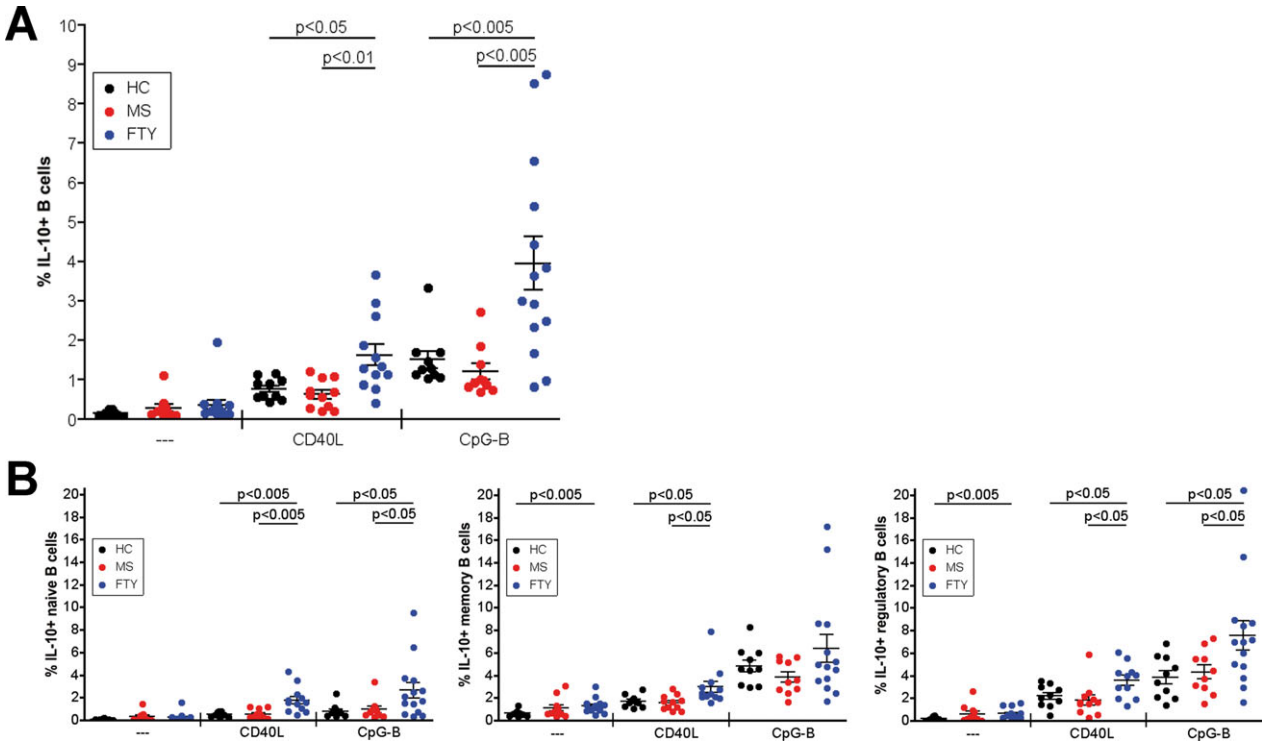


Figure 2. Fingolimod-treatment promotes B-cell IL-10 production. Isolated CD19$^+$ B cells from HC, MS and FTY remained unstimulated (—) or were stimulated with either CD40L or CpG-B. IL-10 production was determined by flow cytometry. (A) Frequencies of IL-10-producing whole B cells. (B) Frequencies of IL-10-producing naïve B cells (left), memory B cells (middle) and regulatory B cells (right). Horizontal lines indicate mean ± SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with multiple sclerosis; HC, healthy controls; MS, untreated patients with MS; SEM, standard error of the mean.

tion, the number of IL-10-producing B cells was not altered by Fingolimod (Fig. 2A). Upon stimulation, however, significantly more B cells from FTY than from the other groups produced IL-10 (Fig. 2A). Furthermore, we found that within the naïve and regulatory population from Fingolimod-treated MS patients a significantly elevated proportion of B cells produced IL-10, when compared to the MS and HC groups (Fig. 2B). Interestingly, production of TNFα and lymphotoxin remained unaffected. Consequently, the ratio of normalized values of the production of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines TNFα and lymphotoxin per patient was shifted considerably toward the anti-inflammatory side (Fig. S2).

Fingolimod treatment enhances regulatory B-cell migration across brain endothelium

Considering the effects of Fingolimod treatment on lymphocyte migration in the periphery, we asked whether it also influences lymphocyte transmigration across the BBB. We therefore investigated the migratory properties of PBMCs in an in vitro model of the BBB (Fig. 3A). Neither under noninflamed nor under inflamed conditions did Fingolimod treatment affect the migration of T cells (Fig. 3B). In contrast, B cells from Fingolimod-treated MS patients displayed a significantly increased migration across brain endothelial cells when compared to the untreated MS as well as the HC group (Fig. 3B and C). This difference was more pronounced during transmigration across a noninflamed endothelial cell layer (FTY 18.4%, MS 8.1%, HC: 3.8%) but could be observed under inflamed conditions, as well.

We next assessed the transmigratory properties of B-cell subsets. While there was no difference in the transmigratory capacity of naïve and memory B cells between the groups, we observed a profoundly increased transmigration of regulatory B cells derived from FTY compared to HC and MS (Fig. 3D; regulatory B cells: HC 3.9%, MS 5.3%, FTY 12.3%). Also under inflammatory conditions, there was an

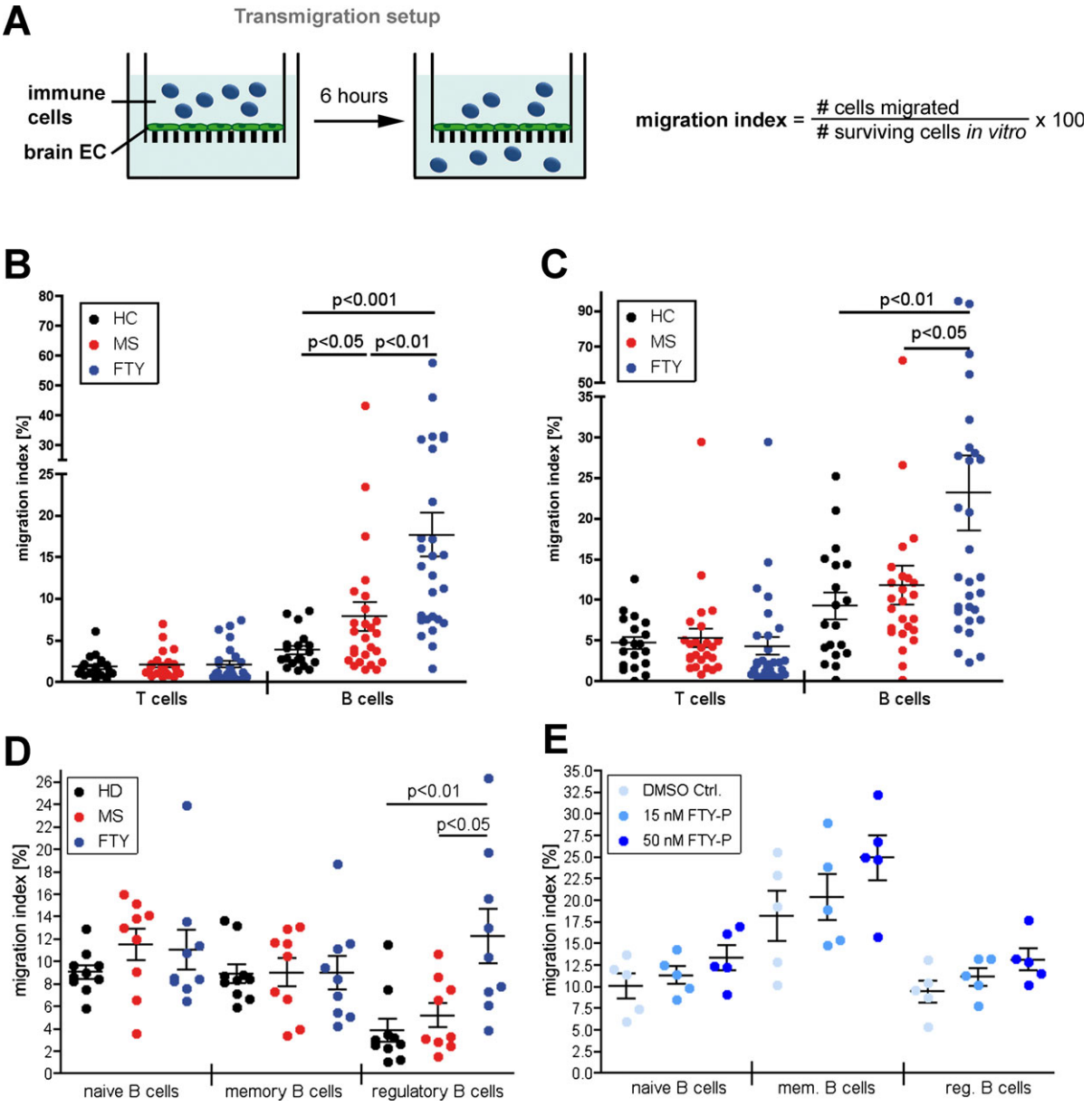


Figure 3. Fingolimod treatment improves transendothelial migration of B cells. Transendothelial migration of B cells and T cells of HC, MS, and FTY across a primary human brain microvascular endothelial cell monolayer and subsequent evaluation of B-cell subset migration via flow cytometry. (A) Experimental setup. (B) Transendothelial migration of peripheral blood cells under homeostatic conditions (no endothelial cell inflammation). (C) Transendothelial migration of peripheral blood cells under inflammatory conditions (i.e., endothelial cell preincubation with TNFα and Interferon γ). (D) Transendothelial migration of isolated B cells under homeostatic conditions. (E) Transendothelial migration of isolated B cells with and without pretreatment with Fingolimod phosphate under homeostatic conditions. Horizontal lines indicate mean ± SEM. *P* values display significant differences. Brain EC, brain endothelial cells; FTY, Fingolimod-treated patients with MS; HC, healthy controls; MS, untreated patients with MS; SEM, standard error of the mean.

enhanced migration of regulatory but not memory B cells in the FTY group compared to the HC group (Fig. S3).

To assess whether the observed migratory phenotype is a direct effect of Fingolimod on B-cell function, we performed transmigration assays with B cells from HD with and without pretreatment with Fingolimod phosphate (FTY-P). Our measurements revealed a consistent albeit nonsignificant trend toward better migration with increasing FTY-P concentration for all three B-cell subpopulations (Fig. 3E).

Fingolimod treatment reduces naïve and memory B-cell counts in the CSF to control levels while retaining regulatory B-cell numbers

Based on the results of our in vitro transmigration assays, we investigated the influence of Fingolimod treatment on B-cell subset composition in the CSF. Here, we observed an increased proportion of regulatory B cells in the FTY group as compared to controls and untreated MS patients, thus mirroring the proportions found in the PB (Fig. 4A).

Next, we evaluated absolute cell numbers of different lymphocyte populations in the CSF as compared to the periphery. Whereas total B-cell numbers in the CSF of MS patients were significantly increased as compared to control individuals (Fig. 4B and D), Fingolimod patients exhibited normalized B cell counts, that were – in contrast to the periphery – equal to those of the controls (Fig. 4B).

Further evaluation of CSF B-cell subsets revealed that despite the peripheral reduction to less than 3% compared to controls, absolute numbers of CSF-derived naïve and memory B-cell counts from Fingolimod-treated MS patients were not decreased. Regulatory B-cell counts from Fingolimod-treated MS patients, reduced to ~20% in the PB, were even increased in the CSF compared to those of controls (Fig. 4E). When compared to untreated MS patients, FTY displayed normalized naïve and memory B-cell counts in the CSF, whereas regulatory B-cell counts were retained (Fig. 4E).

To indirectly conclude on the migratory capacity of B-cell subsets across the BBB in vivo, we calculated the CSF/blood ratio of absolute B-cell counts for each individual. Interestingly, the average ratio of CSF-derived to blood-derived B cells was markedly elevated in FTY when compared to controls and untreated MS patients, which corroborates our in vitro data indicating an enhanced transmigratory activity of B cells under Fingolimod treatment (Fig. 4C). In contrast to that, the transmigration of whole lymphocytes/monocytes remained unaffected by Fingolimod treatment (Fig. 4C).

Analysis of B-cell subset ratios revealed that Fingolimod treatment resulted in an enhanced migration of all B-cell subsets as reflected by the increased CSF/blood ratios of naïve B cells, memory B cells as well as regulatory B cells (Fig. 4F). These data suggest that also in vivo, Fingolimod treatment results in enhanced migration of all B-cell subsets, however, not of lymphocytes in general (see also Fig S4A and B).

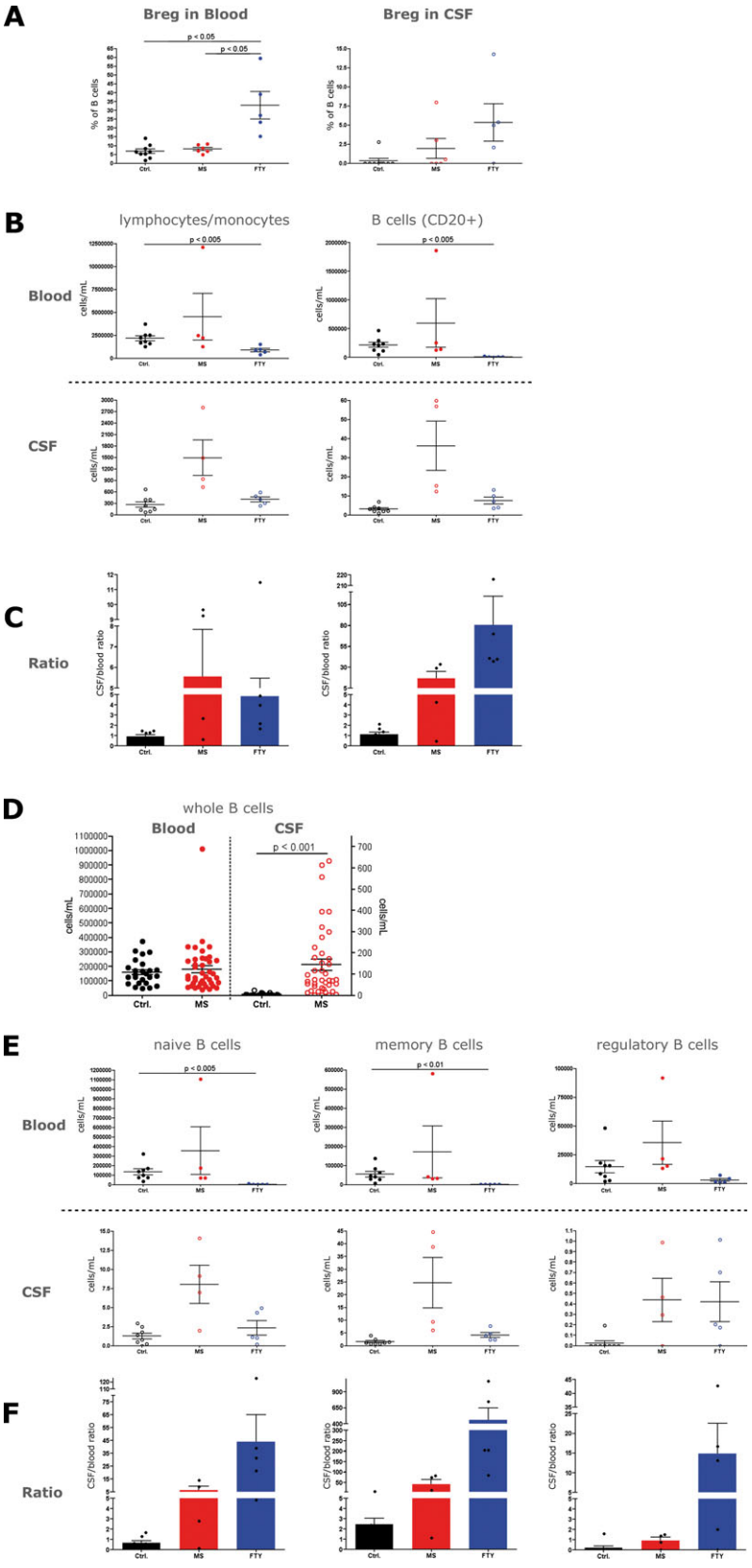
Elevated proportions of regulatory B cells in the PB of FTY correlate with disease stability

Analysis of the proportions of regulatory B cells in the PB of Fingolimod-treated MS patients over time revealed that they rose slowly from initially normal levels, reaching a plateau ~1 year after treatment initiation (Fig. 5A). Based on this observation we wondered whether there was a correlation between the individual percentages and the treatment response. Therefore, patients of our cohort having received Fingolimod treatment for at least 18 months were divided into a "stable" and an "active" group as defined by clinical and radiologic criteria (specified in Subjects/Material and Methods). Here, we observed that after ≥12 months of treatment the group of "stable" patients exhibited significantly higher percentages of regulatory B cells in the PB than the group of "active" patients, while before treatment initiation, there was no difference in this respect between both groups (Fig. 5B).

Discussion

In accordance with the literature, we found a significant reduction in total B cells in the PB of Fingolimod-treated MS patients.[8,11] When further analyzing B-cell subpopulations, we observed a highly significant increase in the proportion of regulatory B cells, while naïve and memory B-cell percentages were slightly decreased, as also observed by Nakamura et al. This reduction was less pronounced than that described for T-cell subsets of FTY,[11–13] which has been shown to originate from the predominance of CCR7-mediated lymphocyte immigration into peripheral lymphoid organs combined with the impairment of S1P1-

Figure 4. Fingolimod treatment preserves CSF B-cell subset counts in spite of reduction in peripheral blood. B-cell subset composition and absolute numbers in peripheral blood and CSF of MS, FTY, and controls (Ctrl.) as determined via flow cytometry. (A) Regulatory B-cell proportions. (B) Absolute counts of all $CD45^+$ lymphocytes/monocytes (left) and B cells only (right). (C) Ratio of lymphocytes/monocytes (left) and B cells (right) in CSF and peripheral blood (CSF/blood ratio), calculated from normalized cell counts as described in the methods section. (D) Absolute $CD19^+$ B-cell counts. Values of stable MS patients and patients in relapse were pooled, because there was no difference in B cell counts between both groups. (E) Absolute counts of B-cell subsets, that is, naïve B cells (left), memory B cells (middle), regulatory B cells (right). (F) CSF/blood ratio of B-cell subsets. (A, B, D, and E) Horizontal lines indicate mean ± SEM. (C and F) Columns represent mean + SEM of depicted individual values. *P* values display significant differences. FTY, Fingolimod-treated patients with MS; Ctrl., controls; MS, untreated patients with MS; SEM, standard error of the mean.



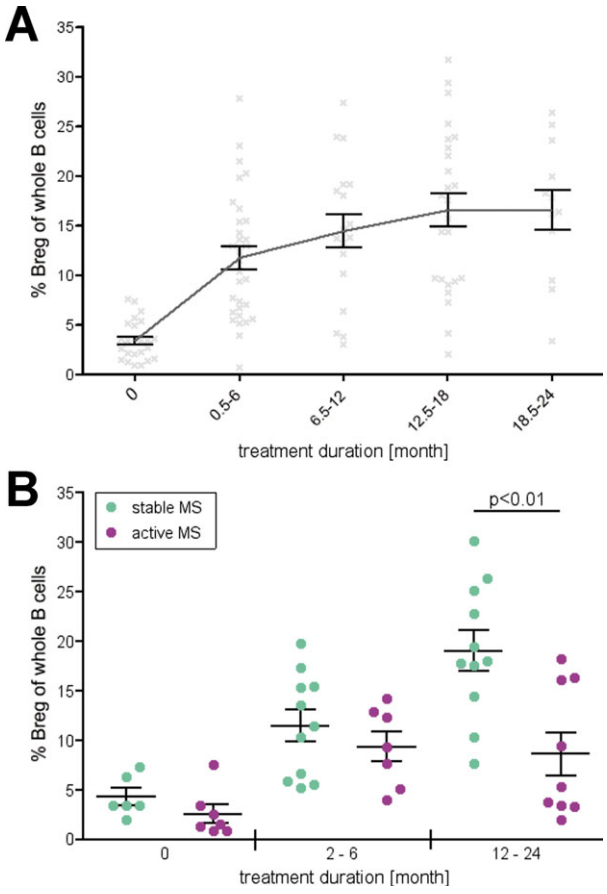


Figure 5. Regulatory B-cell proportions under Fingolimod treatment correlate with disease stability. The percentage of regulatory B cells within the B-cell compartment of Fingolimod-treated patients was determined by flow cytometry, before and at different time points after treatment initiation. (A) Development of regulatory B-cell proportions of all available patients over time. (*n* [0 months] = 24; *n* [0.5–6 months] = 31; *n* [6.5–12 months] = 18; *n* [12.5–18 months] = 25; *n* [18.5–24 months] = 12). (B) Correlation of regulatory B-cell percentages at three different time points before/after initiation of Fingolimod treatment with disease activity determined over a period of at least 18 months after initiation of Fingolimod treatment. Patients were divided into two groups, either "stable", that is, absence of clinical relapses AND no new/enlarging T2 lesions in cMRI AND no detection of Gadolinium enhancing lesions in cMRI, or "active", that is, presence of at least one relapse AND/OR new/enlarging T2 lesions AND/OR detection of Gadolinium enhancing lesions in cMRI. *P* values display significant differences. Depicted are mean ± SEM. Breg, regulatory B cells; MS, multiple sclerosis; SEM, standard error of the mean.

mediated lymphocyte egress.[6,14] We could show that naïve and memory B cells display markedly higher expressions of the "lymph node homing triad" molecules L-Selectin, CCR7 and LFA-1[15] than regulatory B cells, indicating a lower LN-homing propensity for regulatory B cells and thus a lower susceptibility to Fingolimod-mediated sequestration within the LN. Further, the expression of L-Selectin and CCR7 was decreased on B-cell subpopulations of the FTY group as compared to the other two groups (Fig. S5). We therefore conclude that the observed shift in B-cell subset composition in Fingolimod-treated MS patients is at least partly due to subset-specific LN-homing propensities resulting from differential expression of L-Selectin and CCR7.

Besides the observed increase in regulatory B-cell proportions as a result of Fingolimod treatment, these cells were found to produce significantly more IL-10, the anti-inflammatory signature cytokine of regulatory B cells.[9,16,17] This increase not only restored impaired IL-10 production in MS patients, but even exceeded production by B cells from HC. In accordance with our finding, it has been shown that B cells derived from MS patients exhibit an impaired IL-10 production compared to B cells from HC,[5,18] suggesting that insufficient IL-10 production by B cells might contribute to dysregulated immune functions in MS.

The increase in the proportion and function of regulatory B cells in Fingolimod-treated MS patients is particularly intriguing with regard to their role for control of autoimmunity: In mice, regulatory B cells are essential for control of autoimmune responses in models of arthritis,[19] MS[16,20] and type 1 diabetes.[21] In humans, their role is less obvious. Only recently, their capacity to control T-cell responses has been demonstrated – for instance the ability of regulatory B cells to suppress T_H1 and T_H17 differentiation while favoring the generation of T_{reg}. This function was impaired in patients with rheumatoid arthritis.[22] Regulatory B cells from SLE-patients also exhibit functional deficits.[9] Additionally, abnormalities in peripheral and CSF B-cell homeostasis have been described in active MS patients.[23]

In line with this studies provide evidence that B-cell ablation in active MS patients by the anti-CD20 antibody rituximab and subsequent B-cell reconstitution results in enhanced IL-10 and reduced lymphotoxin production by B cells compared to pretreatment levels.[5] This is probably due to a preponderance of naïve and regulatory B cells after repopulation.[24]

It should be noticed that we observed the highest increase in proportion of IL-10-producing cells within the regulatory B-cell population, thereby providing evidence that the increased IL-10 production by total B cells is not simply a secondary effect caused by changes in peripheral B-cell distribution patterns, but instead, that Fingolimod has a direct impact on regulatory B-cell functions. Indeed, the connection of the S1P pathway with immunologically relevant cellular functions beyond immune cell trafficking has been demonstrated, before, as for example it is

involved in the intrinsic modulation of $CD4^+$ T-cell differentiation toward encephalitogenic T_H17 cells by influencing intracellular Jak-STAT3 signaling.[25] Moreover, Fingolimod enhances the suppressive function of T_{reg} in vitro and in vivo.[26] In line with these findings, we conclude that Fingolimod exerts subset-specific functional effects on B cells beyond its influence on B-cell trafficking.

Given the relevance of the S1P1 pathway in modulation of B- and T-lymphocyte trafficking,[27] we investigated whether Fingolimod treatment influences lymphocyte transmigration across the BBB as a key step in MS disease pathogenesis. In contrast to T cells, B cells derived from FTY displayed a significantly enhanced migratory activity, which could be attributed to a selective increase of the migration of regulatory B cells in subsequent experiments. We only observed a slight but consistent enhancing effect of in vitro FTY-P on B-cell transmigration; however, the effect might be larger in vivo due to continuous exposure to FTY-P as compared to our in vitro assay. We therefore propose that the effects of Fingolimod treatment in vivo on B cell migratory capacity might result from both, indirect partitioning effects on B cell subpopulations as well as direct effects on B cell migration.

The in vitro data are supported by our in vivo data, as Fingolimod treatment resulted in an increase in the CSF/blood B-cell ratio, whereas the ratio of total lymphocytes remained unaffected, at least in the small cohort of patients whose CSF we were able to study. In other words, although Fingolimod strongly reduces absolute numbers of B-cell subpopulations in the periphery, in the CSF FTY exhibit naïve and memory B-cell counts comparable to those of control individuals and even increased numbers of regulatory B cells. These findings support our perception that Fingolimod treatment enhances migration of B cells across the BBB. We are fully aware that we cannot formally prove that the increased CSF/blood ratio of B cells in FTY results from enhanced migration rather than prolonged B-cell survival or increased B-cell proliferation in the CSF. However, at least in vitro, we did not observe any influence of Fingolimod treatment on B-cell survival (not shown), nor has any influence of Fingolimod on immune cell proliferation been described, as yet. Interestingly, Fingolimod treatment results in a correction of increased frequencies of naïve and memory B cells in the CSF of MS patients, whereas absolute numbers of regulatory B cells are preserved; indicating that Fingolimod treatment might correct the local imbalance between inflammatory and protective B-cell subpopulations. It should be mentioned, that Fingolimod treatment also markedly reduces absolute numbers of plasmablasts and plasma cells in the periphery and CSF, hinting toward more complex effects of Fingolimod in the correction of impaired B-cell functions, as yet another beneficial effect of Fingolimod treatment (Fig. S4C). Our data extend previous findings from Kowarik et al., who described preserved whole B-cell proportions in contrast to $CD4^+$ T cell percentages in the CSF of FTY.[8] Further studies are warranted to better elucidate the presence and function of regulatory B cells within the CNS.

Finally, our data suggest that an increase of regulatory B-cell proportions in the PB of FTY might indicate a good treatment response; as at least in our small cohort, those patients fulfilling the criteria of stable disease exhibited significantly higher percentages of regulatory B cells than those with active disease. Although this finding needs verification in an independent cohort, it is tempting to speculate that (1) the increased regulatory B-cell percentages indicate a novel and so far unrecognized mechanism of Fingolimod-mediated correction of perturbed immune functions in MS, and (2) in the future, assessment of regulatory B-cell proportions in the PB might help to evaluate the treatment response of MS patients under treatment with Fingolimod.

In summary, our data provide first evidence of a novel and as yet unrecognized role of Fingolimod in correction of impaired B-cell functions and the potential relevance of regulatory B cells as indicators of good treatment response to Fingolimod in MS patients.

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Author's Contributions

B. G. designed and performed research, collected and analyzed data, and wrote the manuscript; S. H., C. G., and M. H. helped to conceptualize the study, analyzed and interpreted the data; A. P.-F., B. W., B. C. K., and T. D. organized patient recruitment and logistics, and provided clinical information; L. K. designed the project, was responsible for the concept, designed research, generated

funding, organized patient recruitment, provided clinical information and wrote the manuscript; H. W. helped to conceptualize the project, generated funding, and critically edited the manuscript. All authors wrote the manuscript.

Conflict of Interest

B.G., S.H., M.H., A.P-F. have nothing to disclose. C.C.G. has received honoraria for lecturing and travel expenses for attending meetings from Bayer Health Care, Genzyme and Novartis Pharma and is funded from the German research foundation (DFG, single grant GR3946/2-1). B.T.W. has received funding for travel and speaker honoraria from Biogen Idec/Elan Corporation, Merck Serono, Teva Pharmaceutical Industries Ltd., Novartis; has served on advisory boards for Biogen Idec/Elan corporation and Novartis; and has received research support from Biogen Idec/Elan corporation, Biotest, Merck Serono, Novartis, from the German Ministry of Education and Research and from the Dietmar Hopp Stiftung. B.K. has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Bayer Health Care,Biogen Idec, Genzyme/Sanofi Aventis, Grifols, Merck Serono, Mitsubishi Europe, Novartis, Roche, Talecris, and TEVA. T.D. has received funding for travel expenses from Genzyme and Novartis Pharma GmbH, Germany. H.W. received compensation for serving on Scientific Advisory Boards/Steering Committees for Bayer Healthcare, Biogen Idec, Genzyme, Merck Serono, Novartis and Sanofi Aventis. He has received speaker honoraria and travel support from Bayer Vital GmbH, Bayer Schering AG, Biogen Idec, CSL Behring, Fresenius Medical Care, Genzyme, Glaxo Smith Kline, GW Pharmaceuticals, Lundbeck, Merck Serono, Omniamed, Novartis and Sanofi Aventis. He has received compensation as a consultant from Biogen Idec, Merck Serono, Novartis and Sanofi Aventis. Prof. Wiendl received research support from Bayer Vital, Biogen Idec, Genzyme Merck Serono, Novartis, Sanofi Aventis Germany, Sanofi US. L.K. received compensation for serving on Scientific Advisory Boards for Genzyme. She received speaker honoraria and travel support from Novartis, Merck Serono and CSL Behring. She receives research support from Novartis and the DFG.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fingolimod treatment reduces the amount of circulating B cells. Frequencies of B and T cells in peripheral blood of HC, MS, and FTY, determined via flow cytometry. (A) Proportions of B cells and T cells of whole PBMC. (B) Absolute B-cell counts. Horizontal lines indicate mean ± SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with MS; HC, healthy controls; MS, untreated patients with MS, SEM, standard error of the mean.

Figure S2. Fingolimod treatment shifts the cytokine secretion profile toward anti-inflammation. (A) Correlation of pro- and anti-inflammatory cytokine production of B cells from HC, MS, and FTY. Isolated B cells were stimulated with CpG-B. IL-10 production was determined via flow cytometry; TNFα and LT secretion were measured by ELISA. Values have been normalized based on a reference-sample set to one. The displayed ratios represent the average of the individual TNFα/IL-10 or LT/IL-10 quotients ±SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with multiple sclerosis; HC, healthy controls; LT, lymphotoxin; MS, untreated patients with multiple sclerosis; SEM, standard error of the mean.

Figure S3. Migration of B cells across inflamed endothelium. (A) Transendothelial migration of B cells of HC and FTY across a primary human brain microvascular endothelial cell monolayer, inflammatory stimulated with IFNγ and TNFα and subsequent evaluation of B-cell subset migration via flow cytometry. Horizontal lines indicate mean ± SEM. FTY, Fingolimod-treated patients with MS; HC, healthy controls; SEM, standard error of the mean.

Figure S4. Influence of Fingolimod treatment on the in vivo migrational propensity of whole lymphocytes, B cells, and B-cell subsets and on PB/PC frequencies in blood and CSF. Absolute plasmablast and plasma cell (PB/PC) counts in peripheral blood and CSF of MS, FTY and controls (Ctrl.) as determined via flow cytometry. (A and B) Before-after plots with normalized ordinates, for comparison and visualization of the propensity of lymphocytes, B cells and B-cell subsets of the three groups to migrate across the BBB. The cell count per mL blood of each individual is plotted on the left ordinate, the cell count per mL CSF is plotted on the right ordinate. Solid lines connect blood/CSF-pairs of variates of individuals; dotted lines horizontally connect the *y*-value for the average B-cell count per mL blood of the control group (Ctrl.) with the *y*-value for the average B-cell count per mL CSF of the Ctrl. Thereby the scaling of the two ordinates is normalized to the to the average migrational propensity of *whole B cells* of the Ctrl. (represented by that horizontal line). The migrational propensities of the B-cell subpopulations of each individual can thus be directly compared to that of the Ctrl. B cells – a positive gradient of the connecting line indicates comparatively increased migration, whereas a negative gradient represents reduced migration – and to that of any other individual and/or B-cell subpopulation (with a higher gradient indicating better migration and a lower gradient poorer migration).

Displayed CSF/Blood ratios are the same as depicted in Figure 3. (A) Migrational propensity of whole lymphocytes (left) and B cells (right). (B) Migrational propensity of naïve (left), memory (middle), and regulatory (right) B cells. (C) Absolute counts of PB/PC in blood (left) and CSF (right). Solid horizontal lines indicate mean ± SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with multiple sclerosis; HC, healthy controls; MS, untreated patients with multiple sclerosis; PB/PC, plasmablasts and plasma cells; SEM, standard error of the mean.

Figure S5. Effect of Fingolimod treatment on the expression of LN-homing markers on B-cell subpopulations. (A) Comparison of expression levels of L-Selectin (left) and CCR7 (right) on B-cell subpopulations between HC, MS, FTY, determined via flow cytometry. Horizontal lines indicate mean ± SEM. *P* values display significant differences. FTY, Fingolimod-treated patients with multiple sclerosis; HC, healthy controls; MS, untreated patients with MS; SEM, standard error of the mean.

Data S1. Subjects/Material and Methods. In this section details on standard protocol approvals, registration and patient consents, antibodies, cells and reagents, biomaterials as well as protocols for cytokine secretion assay, HBMEC culture and transmigration assay, flow cytometry, and statistics applied in this study are given.

Table S1. List of monoclonal, fluorochrome-coupled antibodies used for flow cytometry. *BD Biosciences, Heidelberg, Germany; **Beckman Coulter, Krefeld, Germany; ***BioLegend GmbH, Fell, Germany.

