Siponimod enriches regulatory T and B lymphocytes in secondary progressive multiple sclerosis

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Abstract

**Background:** Siponimod (BAF312) is a selective sphingosine 1-phosphate receptor 1 and 5 (S1PR1, S1PR5) modulator recently approved for active secondary progressive multiple sclerosis (SPMS). The immunomodulatory effects of siponimod in SPMS have not been previously described.

**Methods:** We conducted a multi-centered randomized, double-blind, placebo-controlled AMS04 mechanistic study with 36 SPMS participants enrolled in the EXPAND trial. Gene expression profiles were analyzed using RNA derived from whole blood with Affymetrix Human Gene ST 2.1 microarray technology. We performed flow cytometry based assays to analyze the immune cell composition and microarray gene expression analysis on peripheral blood from siponimod-treated participants with SPMS relative to baseline and placebo during the first year randomization phase.

**Results:** Microarray analysis showed that immune-associated genes involved in T and B cell activation and receptor signaling were largely decreased by siponimod, which is consistent with the reduction of CD4+ T cells, CD8+ T cells, and B cells. Analysis done by flow cytometry showed that within the remaining lymphocyte subsets, there was a reduction in the frequencies of CD4 and CD8 naïve T cells and central memory cells, while T effector memory cells, anti-inflammatory Th2, and T regulatory (Treg) cells were enriched. Transitional Bregs (CD24hiCD38hi) and B1 cell subsets (CD43+CD27+) were enriched, shifting the balance in favor of regulatory B cells over memory B cells. The pro-regulatory shift driven by siponimod treatment included a higher proliferative potential of Tregs compared with non-Tregs, and upregulated expression of PD-1 on Tregs. Additionally, a positive correlation was found between regulatory T cells and regulatory B cells in siponimod treated participants.

**Conclusion:** The shift toward an anti-inflammatory and suppressive homeostatic immune system may contribute to the clinical efficacy of siponimod in SPMS.

Clinical Trials Registration: NCT02330965
Introduction:

Secondary progressive multiple sclerosis (SPMS) follows an initial course of relapsing-remitting MS (RRMS) and is characterized by chronic disability progression not associated with relapses (1). MS involves both inflammation and neurodegeneration, and although the underlying mechanisms are not well understood, progressive MS is thought to be driven primarily by neurodegenerative processes (2). The Phase 3 EXPAND clinical trial demonstrated that siponimod (BAF312; trade name Mayzent®) is clinically effective for SPMS based on the primary outcome of reduction of 3-month confirmed disability progression (CDP) [defined by a 0.5 or 1 point increase in expanded disability status scale (EDSS) relative to baseline] (3). 288 (26%) of 1096 patients receiving siponimod and 173 (32%) of 545 patients receiving placebo had 3-month CDP (hazard ratio 0.79, 95% CI 0.65-0.95; relative risk reduction 21%; p=0.013). In addition, its key secondary objective was met with a 26% reduction 6-month CDP as well as a significant reduction in the annualized relapse rate and MRI activities (3). The positive EXPAND data are encouraging for a disease with such a high unmet need, as no other drug trial in SPMS has shown positive results.

Siponimod is a second generation sphingosine-1-phosphate (S1P) receptor modulator selective for S1PR1 and S1PR5 (4). The first generation S1P receptor modulator, fingolimod, is approved for RRMS, but failed to show efficacy in the Phase 3 INFORMS trial for primary progressive MS (PPMS) (5). Most of the other immunomodulatory therapies approved for RRMS have also failed to show clinically meaningful efficacy for SPMS (6), thus the relevance of immune modulation to disease progression in SPMS has been unclear.

The success of siponimod in slowing progression in active SPMS (3) provides the first opportunity to determine the relevance of immune system modulation to disease progression in SPMS. In order to identify the most relevant changes, it is critical to understand the immune changes induced by siponimod treatment and how they compare to previous therapies which
failed to slow progression. It is anticipated that, similar to fingolimod, siponimod reduces inflammation by trapping S1P-sensitive subsets of lymphocytes in lymph nodes through functional antagonism of S1PR1 (7). However, compared to fingolimod, siponimod has greater receptor (S1PR1 and S1PR5) specificity, a shorter half-life, and does not require in vivo phosphorylation for biological activity (4, 8). These differences may be critical for its efficacy in SPMS, therefore it is necessary to understand the specific immune changes associated with siponimod treatment. Here, we report the longitudinal prospective changes of the peripheral immune cell profile of a cohort of participants in the AMS04 sub-study of the EXPAND trial following treatment with placebo or siponimod for up to 12 months.
Results:

Participant demographics: The AMS04 multi-centered mechanistic study consisted of a total of 36 participants, with 13 participants randomly assigned to placebo and 23 participants assigned to siponimod (Figure 1). Baseline samples were collected from all study participants, and post-treatment samples were not available for 4 participants in the siponimod treated and 1 in placebo group due to patient drop out. The baseline demographic and disease-associated characteristics of participants in the placebo and siponimod-treated groups were comparable (see Table 1). Our cohort is in line with the US cohort which is slightly different from the global cohort, i.e. our participants tended to be older with a higher proportion of females, fewer participants had relapses prior to enrollment, and the vast majority of participants were pre-treated with other disease modifying therapies and had a high degree of disability with EDSS of 6.0 and 6.5.

Gene expression analysis: We examined how siponimod affected the mRNA expression profile of peripheral blood from SPMS participants using microarray analysis, and detected differential expression in 1531 out of a total of 13399 genes with measured expression as shown in a volcano plot (Figure 2A). Our data showed clear separation between baseline untreated (green dots) and 12 months siponimod treated groups (blue dots) on the 3D PCA plots; and similar clustering was seen with the 12 months placebo treated group (grey dots) compared to baseline untreated (green dots) (Figure 2B). The significantly representative pathways are shown in Figure 2C. The majority of differentially expressed target genes are associated with T cell and B cell function, and showed decreased expression following 12 months of siponimod treatment, indicating a preferential effect on lymphocytes. The affected genes, which include, CD28, CCR7, ICOS, CARD11, IGHG1, and CD40LG, encompass a variety of essential lymphocyte functions, including co-stimulation, T/B cell interaction, antibody production, antigen receptor-mediated signaling pathways, and cytokine-cytokine receptor interaction (Figure 3).
Effects of siponimod on WBCs, PMBC and lymphocyte counts: Since fingolimod treatment induces lymphopenia, and gene expression analysis suggested that siponimod similarly depletes lymphocytes, we examined the absolute counts of peripheral blood cells at baseline, 6 months, and 9-12 months post-treatment. Siponimod treatment significantly decreased the absolute numbers of circulating white blood cells (WBC), peripheral blood mononuclear cells (PBMCs), and lymphocytes (Figure 4A-C). On average, the white blood cell (WBC) count decreased 37%, to the low end of the normal range, at 4050 ± 1553 cells/µl at 9-12 months of siponimod treatment. A high frequency of siponimod treated participants developed leukopenia (10/17 at 6 months, and 11/17 at 9-12 months). Siponimod treatment also led to a 55% (6 months) and 58% (9-12 months) reduction in PBMCs, and a more than 71% (6 months) and 69% (9-12 months) reduction in absolute lymphocyte count (ALC). 24% (6 months) and 35% (9-12 months) of siponimod-treated individuals in this cohort developed Grade 2 lymphopenia (ALC: 500-800/µl), while 67% (6 months) and 53% (12 months) of siponimod treated participants developed Grade 3 lymphopenia (ALC: 200-500/µl).

Siponimod reduces circulating T and B lymphocytes: Immunophenotyping analysis was conducted to determine which peripheral immune cell subsets are most affected by siponimod. Consistent with our peripheral blood expression analysis, the most drastic reductions took place within the CD4+ T cell and CD19+ B cell populations in the siponimod-treated group compared with those in placebo-treated group. CD4+ T cells were decreased by 97% to 25±15 cells/µl at 6 months (P≤0.0001) and 96% to 33 cells/µl at 9-12 months (P≤0.0001) with siponimod treatment (Figure 5A). The numbers of CD8+ T cells were also decreased to a lesser degree by 67% of their baseline levels at 6 months (P=0.0003) and 59% at 9-12 months (P=0.0015) (Figure 5B) leading to an overall 3 fold reduction of the CD4/CD8 T cell ratio (P < 0.0001 at 6 months and P=0.0008 at 9-12 months) (Figure 5C). CD19+ B cells were reduced by 93% of their baseline levels to 9 cells/µl at 6 months (P≤0.0001) and by 89% to 12 cells/µl at 9-12 months (P<0.0001).
(Figure 5D). Notably, the average absolute numbers of CD4\(^+\) T cells and B cells, as well as CD8 T cells at 6 months were lower than those at 9-12 months, suggesting ongoing homeostatic proliferation (Supplemental Figure 3) which is induced by lymphopenia. Siponimod treatment did not significantly change the absolute number of NK, NKT, or monocyte cell populations compared with placebo-treated group (Figure 5E-G).

Siponimod depletes circulating naïve T cells while enriching memory T cells: Within the CD3\(^+\) T cell population, siponimod treatment led to a shift toward fewer naïve and more differentiated memory cells (Figure 6-7). In the CD4\(^+\) T cell population (Figure 6), siponimod significantly reduced the frequency of naïve T cells (T naïve, Figure 6A) by 46\% at 6 months (P=0.0048) and 41\% at 9-12 months (P=0.0058) compared to the placebo group. Within the siponimod-treated group, naïve T cells were reduced by approximately 50\% at 6 months and 9-12 months (both P<0.0001, Figure 6E). No significant frequency changes were found for central memory T cells (Tcm) between the siponimod-treated and placebo groups (Figure 6B), or before and after siponimod treatment (Figure 6F). The relative increase of memory cells in the siponimod-treated group was driven primarily by the effector memory (Tem) subset, which were 2.4 fold higher at 6 months (P=0.016) and 2.1 fold higher at 9-12 months (P=0.0005) compared with the placebo-treated group (Figure 6C). Within the siponimod-treated group, the frequency of Tem was increased about 2.9 fold (P=0.0006) and 2.5 fold (P=0.0011) at 6 months and 9-12 months, respectively, compared to pre-treatment levels (Figure 6G). There was an overall relative increase of CD45RO\(^+\) effector memory T cell (Temra) frequency within the siponimod-treated group compared with the placebo group (Figure 6D P=0.0.0058 9-12 months, Figure 6H).

Within the CD4\(^-\) T cells, which is primarily CD8\(^+\) T cells, T naive and Tcm were significantly reduced with siponimod treatment relative to baseline and the placebo group (Figure 7A-B, E-F). There was an over 75\% reduction in CD4\(^-\) T naive at both 6 months (P<0.0001) and 9-12 months (P<0.0012) and Tcm at 6 months (P=0.0051); while Tem (Figure 7C and 7G) was moderately increased. Percentage of Temra within CD4\(^-\) T cells were increased significantly in
Siponimod-treated patients compared with those of placebo-treated patients (Figure 7D P<0.0001 for both 6 months and 9-12 months. There was a 3-fold increase in CD4⁺ Temra from 19% to 55% at 6 months (P<0.0001) and to 58% at 9-12 months (P=0.0002, Figure 7H) compared with pre-siponimod levels.

**Siponimod increases the relative frequency of Th2 and regulatory T cells:** Within the CD4⁺ effector T cell population, there were no detectable changes in the relative frequencies of pathogenic Th1, Th1-Th17 and Th17 subsets between siponimod-treated and placebo-treated groups (Supplemental Figure 4). However, there were significant increases in the relative frequencies of Th2 (<0.0001 at both 6 months and 9-12 months, Figure 8A) and T regulatory cells (Tregs) (6 months P=0.0002, 9-12 months P=0.0148; Figure 8C) compared with the placebo-treated group. Longitudinally, Th2 frequencies (Figure 8E) increased 4 fold at 6 months (P=0.0007) and 3.6 fold at 9-12 months (P=0.0031), while Tregs (Figure 8G) increased 2.4 fold at 6 months (P=0.0012) and 3 fold at 9-12 months (P=0.0026) after siponimod treatment. Correspondingly, the ratios of Th2:Th17 (Figure 8B and 8F) and Treg:Th17 cells (Figure 8D and 8H) were also significantly increased, which may act to temper pathogenic immune responses in MS patients.

**Siponimod boosts the relative frequency of regulatory B cells:** Within the CD19⁺ B cells, naïve B cell frequencies were significantly reduced (P=0.0409, Figure 9A), while no significant differences were detected in the relative frequencies of CD27⁺ memory B cell populations, including both unswitched and switched memory B cell subsets (Figure 9B-D). In contrast, the percentages of regulatory transitional Bregs (CD24<sup>hi</sup>CD38<sup>hi</sup>, P=0.0056 at 9-12 months; Figure 9E and 9G) and to a lesser extent, B1 cells (CD43⁺CD27⁺), P=0.0229 at 9-12 months; Figure 9F) were also increased, thereby effectively shifting the balance in favor of these putative IL-10 producing subsets (Figure 9G) over effector switched memory B cells (Figure 9H).

**Effect of siponimod on Treg and Breg are correlated:** Both transitional B cells and B1 cells are regulatory in nature, and siponimod treatment increased their frequency collectively (Figure
10A). The shift toward more Tregs and Bregs is consistent with a shift toward an overall more pro-regulatory immune environment following siponimod treatment. Unlike placebo-treated participants (Figure 10B), the increase in the Breg and Treg populations derived from siponimod-treated participants exhibits a positive correlation (Figure 10C, R=0.5203, P=0.0019). The increase in Bregs may play a role in driving the proliferation of Tregs, as IL-10 producing Breg may modulate T cells by enhancing FoxP3 and PD-1 expression (9) or vice versa. Following siponimod treatment, the FoxP3+CD4+ T cells proliferated more robustly (P=0.0001 at 6 months and <0.0001 at 9-12 months, Figure 11A-B) compared to conventional Fox P3- CD4+ T cells (Figure 11A, Supplemental Figure 3), based on Ki67 expression (Figure 11B). Furthermore, the frequency of CD4+ T cells expressing the immune checkpoint inhibitor--costimulatory PD-1 receptor was increased in siponimod treated participants as compared with those treated with placebo (Figure 11C), which may be indicative of enhanced self-tolerance of these cells. PD-1 ligation-induced suppressive activity can inhibit TCR-induced activation of the Akt-mTOR pathway, which supports the downregulation of the mTOR pathway in peripheral blood cells in response to siponimod treatment (Supplemental Figure 5). S1P1 has previously been shown to inhibit Treg suppressive function via this pathway (10, 11).
Discussion

As the first disease modifying therapy approved for active SPMS siponimod is expected to affect pathways and processes that drive the inflammation-associated etiology of SPMS. Our study characterized the peripheral immune related changes induced by siponimod treatment and revealed a dominant effect on regulatory immune cell frequency and function.

Our microarray gene expression study is the first report in human demonstrating the profile of differentially expressed genes in response to siponimod treatment in SPMS. We found co-signaling molecules e.g. CARD11, PRKCA, RPS6, RPTOR, CD28, CTLA4 and ICOS were decreased by siponimod in SPMS. Using a similar whole blood microarray method, several other groups have reported gene expression changes in response to various DMTs, including the related S1P receptor modulator, fingolimod, however, these were done in the context of RRMS. A study assessing 78 RRMS participants treated with glatiramer acetate, Interferon-β, or fingolimod, identified 8 common differentially expressed genes, in which 7 out of the 8 genes (ITGA2B, ITGB3, CD177, IGJ, IL5RA, MMP8, P2RY12) were downregulated, and one, S100β, was upregulated (12). In contrast, our study showed that S100Z (not S100β), ITGA2B, and P2RY12 were increased in expression with siponimod. The discrepancy may be related to the clinical population, as we examined SPMS participants. As expected, genes related to the mechanism of action of a S1P receptor modulator, such as S1PR1 and CCR7, were downregulated, as reported with fingolimod (13, 14). Furthermore, we found that siponimod exerted an anti-inflammatory effect through the downregulation of AKT3, CD19, CD40, CD40L, IL23A, CXCR5, IL2RA, IL7R, IL23A, IL21R, IL11RA, IL6ST, CR2 and IRF4 genes, which is similar to what has been observed in RRMS patients treated with fingolimod (15).

Consistent with the microarray, significant reductions in the CD19+ B cell and CD4+ T cell populations were detected via immunophenotyping analysis. The overall profile of the major
immune subsets in siponimod treated participants was largely similar to what has previously been reported for fingolimod (16), and thus is consistent with a primary mechanism of S1P1 mediated peripheral lymphocyte trapping. In line with fingolimod (17), siponimod produced a shift toward relatively fewer naïve and more CD4+ Tem and CD4- Temra cells within the T cell subsets, although siponimod led to a greater reduction in CD4+ Tcm. Within CD19+ B cells, siponimod treatment led to a rise in circulating transitional Bregs (CD24hiCD38hi). This is also consistent with fingolimod (18-20) and the broader mechanism of S1PR1 mediated lymphocyte trapping because transitional Bregs do not home to lymph nodes, and are thus not subject to trapping. However, we did not detect the overall shift toward fewer memory and more naïve B cells found with fingolimod (19, 21). While transitional Bregs (CD24hiCD38hi) were studied with fingolimod treatment, our finding on enrichment of B1 regulatory cells (CD27+CD43+CD19+) with siponimod in SPMS is novel. The shift toward a relative increase of total regulatory B cells (transitional and B1) may contribute to a shift toward an overall more pro-regulatory immune environment, as the increased total Breg frequency correlated significantly with the increased Treg frequency post-siponimod treatment (Figure 10). Most notably, we found that siponimod shifts the balance of the CD4+ effector population by increasing the percentage of anti-inflammatory Th2 and Treg subsets, while leaving the pathogenic Th1 and Th17 populations relatively unchanged (Figure 8, Supplemental Figure 4). The pro-regulatory profiles in the blood could be a mirror image of the profile of cells sequestered in the lymph nodes, as our results suggest that pathogenic T effectors are likely to be sequestered in the lymph nodes. While some studies have found that fingolimod treatment reduces the percentage of Th1 and Th17 subsets (22), there is considerable patient-to-patient variation, which can range from a significant decrease to a slight increase depending on the particular patient cohort and duration of treatment (23, 24). In our SPMS cohort, we did not detect a significant change in the percentage of these pathogenic
effector subsets with siponimod treatment. This shift in balance toward Tregs likely stems, in part, from the role of S1PR1 signaling in Treg and T effector cell differentiation, mediated through Akt-mTOR (11, 25). In mice, S1P plays important roles in T cell differentiation and survival (26-28). mTOR has been implicated as the downstream effector of S1PR1 signaling mediating this effect based on the ability of the mTOR inhibitor rapamycin to phenocopy the effect of FTY720 in directly controlling the differentiation of Th1 cells to Tregs in an immunological hepatic injury mouse model (29). S1PR1 also inhibits Treg differentiation and function through activation of Akt-mTOR (10). Our microarray analysis revealed that the Akt-mTOR pathway was downregulated in the peripheral blood by siponimod in SPMS participants (Supplemental Figure 5), and likely contributes to the enhancement of Treg function.

In addition to increased Treg proliferation, we also found increased expression of PD-1 after siponimod treatment (Fig 11), which is indicative of increased suppressive capacity (30). Tregs are critical for the development and maintenance of tolerance to self-antigens, and PD-1 activation inhibits the expansion of self-reactive T cells (30). Polymorphisms that decrease PD-1 function are linked to disease severity and progression (31, 32). Interestingly, anti-PD-1 and anti-CTL4A therapies for melanoma are linked to autoimmune demyelinating disorders (33-36) along with enhanced responses of myelin specific CD4+ T cells (36). Our data are consistent with these observations and suggest that increases in both regulatory lymphocyte quantity and function contribute to the siponimod treatment effect.

Although shifting the peripheral immune profile toward a more regulatory state is a common feature of many of the disease modifying therapies that are efficacious for RRMS, these therapies have historically failed to translate to progressive MS (37). Preclinical studies indicate a role of Tregs in promoting remyelination (38), suggesting that the yet to be determined effects of siponimod on the immune profile within the CNS may account for its efficacy in SPMS. Additionally, while fingolimod and siponimod both primarily affect the peripheral immune cell composition via S1P1 mediated lymphocyte trapping, their effects on cell activation stem from
different affinity and engagement of S1P receptors (39). These differences in cell activation, particularly with the lymphoid tissues and/or the CNS, likely contribute to siponimod’s efficacy. Immunoprofiling of the lymphoid tissue and CNS will ultimately be needed to fully understand how siponimod slows disease progression in SPMS.

Furthermore, CNS neurons and glia express S1P receptors, and S1P modulators are neuroprotective in various preclinical models (40, 41), and siponimod is CNS penetrant (42). While clear evidence for neuroprotective activity within MS patients has not yet been established, it was proposed that an S1P receptor mediated reduction of glial activation may synergize with the peripheral immune modulation to prevent disease-associated inflammatory activity in the CNS (43). Notwithstanding these potential additional dimensions of the effects of siponimod, our study shows that siponimod reduces the inflammatory profile in the peripheral blood through enhancement of regulatory cell populations. These effects of siponimod are expected to partially drive its efficacy in SPMS.
Methods:

Study design and patients. Subjects participating in the “EXPAND” clinical trial, “A multicenter, randomized, double-blind, parallel-group, placebo-controlled variable treatment duration study exploring the efficacy and safety of siponimod (BAF312) in patients with secondary progressive multiple sclerosis followed by extended treatment with open-label BAF312” (protocol number CBAF312A2304 sponsored by Novartis Pharmaceuticals; clinicaltrials.gov identifier NCT01665144) were eligible to participate in the Autoimmunity Center of Excellence Multiple Sclerosis Study 04 (AMS04), which is “Mechanistic Studies of Phase III Trial with BAF312 in Secondary Progressive Multiple Sclerosis” sponsored by Division of Allergy, Immunology, and Transplantation (DAIT), National Institute of Allergy and Infectious Diseases (NIAID) (clinicaltrials.gov identifier NCT02330965). Participants were recruited from multiple study sites in the United States in a sequential manner as they consented. There were two phases in the EXPAND study. The randomized treatment phase (RTP) was the double-blind treatment phase in which participants were assigned at random to siponimod or placebo at 2:1 ratio. Novartis ended the RTP of EXPAND on October 07, 2015 when planned events of progression was met. Following this, participants entered open-label phase (OLP) (Figure 1) and offered treatment with siponimod.

Inclusion and Exclusion criteria: AMS04 study recruited subjects age 18 to 60 who had a clinical diagnosis of SPMS and were enrolled in the EXPAND trial. Details could be found in https://clinicaltrials.gov/NCT02330965. Twelve study centers in the United States participated in recruiting subjects in the AMS04 study. The University of Michigan Autoimmunity Center of Excellence was the central site for conducting AMS04 including sample collection, processing, data collection and analysis. Site contracting was facilitated through University of California, San Francisco.
Enrollment in the parent study (EXPAND) ended in May 2015; at that time, 40 subjects were screened in AMS04, and 36 were randomized (Figure 1). The first subject enrolled for AMS04 study was May 21, 2014 and the last subject completed AMS04 was July 12, 2017.

**PAXgene Blood RNA Isolation.** Whole blood of subjects was collected into PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany) to perform RNA isolation. The blood was initially mixed into the PAXgene tube buffer to lyse the cells, and stored at -80°C prior to RNA isolation and batch analysis. To isolate blood RNA, the PAXgene tubes with cell lysate were thawed and centrifuged to pellet nucleic acids. The pellet was washed and resuspended, and the RNA was purified according to manufacturer’s suggested protocol. RNA concentration was determined by nanodrop.

**Microarray Gene expression analysis.** mRNA levels derived from total blood RNA isolated from the PAXgene Blood RNA tubes was analyzed using microarray technology (Affymetrix Human Gene ST 2.1 Array) at the University of Michigan Core facility. Each RNA sample was analyzed using a 2100 bioanalyzer (Agilent, USA) to assess RNA quality. Expression values were calculated for the pre and post-siponimod treatment using the Robust Multi-array Average (RMA) technique, implemented through the “oligo” package of bioconductor in R version 3.3.0. Probe sets not annotated as a 'main' probe sets by Affymetrix were removed as well as probe sets with a variance less than 0.1 across all sample types. The "limma" package was used to fit weighted linear models to the expression values. iPathwayGuide (Advaita Corporation) software was used to perform pathway and GO analysis. A volcano plot with the log2 fold change data on the X axis and -log10 (adjusted p value) data on the Y-axis was created to show all the tested probe sets on the array. The heatmaps were generated using the ggplots package in R from genes with at least a 1.5-fold change (without using the array quality weights) that were part of these GO terms as defined by the bioconductor GO.db version 3.4.0 package. For genes with more than 1 probe set, the probe set with the largest inter quartile range was used to represent that gene. The GEO accession number for our microarray data is GSE141381.
**Immunophenotyping analysis.** 60 ml of heparinized blood was collected from each patient in BD Vacutainer™ Sodium Heparin® green top tubes. Samples were shipped to the University of Michigan central laboratory for analysis within 24hrs. Tubes were centrifuged at 400g for 10 minutes, plasma was collected aliquoted and stored at -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque density gradient centrifugation method. PBMCs were stained with antibodies to mark cell subsets and analyzed by flow cytometry using the BD FACSCanto II system and FlowJo, as previously described (44, 45). Details of the antibodies used in this study are described in Supplemental Table 1. Markers used to define major immune cell subsets are described in Table 2. Examples of flow cytometry gating strategies are shown in Supplemental Figures 1A (Th1, Th2, Th1-Th17, Th17), 1B (Treg) and Supplemental Figure 2 (B cell subsets).

**Statistical analysis.** The immune cell subset analyses included the first year RTP randomized participants for which there was flow cytometric data from blood draws taken for at least two of the time points. Due to shipping delays two samples could not be processed until over 2 days from the time of the blood draw, and these samples were excluded from this analysis. Participant samples that deviated from our trial design protocol were also excluded. Also excluded from the analysis are major outliers calculated using the interquartile range method (see figure legends for details). For cross-sectional analysis between siponimod-treated and placebo-treated, data were first analyzed for their normality using the D’Agostino & Pearson test. If normality was confirmed, unpaired t-tests were performed. If the two sets of data had unequal variance, then the unpaired t-tests were corrected with Welch’s method. If data were not normally distributed, Mann-Whitney U tests were performed. For within treatment group longitudinal comparison between different time points, one-way ANOVA followed by Tukey’s multiple comparison tests were performed if normality of the data were confirmed, otherwise data were analyzed using Friedman tests with Dunn’s correction. Significant P values (<0.05) derived from these statistical analyses are shown in the appropriate figures. All the statistical
analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc. La Jolla, CA, USA).

**Study Approval:** The AMS04 study was conducted according to the Declaration of Helsinki in accordance with good clinical practice guidelines, and approved by independent institutional review boards at each participating clinical center. Informed consent was obtained from participants prior to participation in the study.
Author Contributions:
QW, QW, SL conducted experiments, QW, QW, EM, SL, DF, Y-MD contributed to study design and analyzed data, CD, CF, BK served as clinical coordinators, QW, QW, EM, SL, EM, YM-D wrote manuscript.

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Figure 1. AMS04 patient allocation and disposition. RTP: randomized treatment phase. OLP: open-label phase. Blue boxed part is the RTP which we presented in this manuscript.
Figure 2. Microarray analysis for pre- and post-siponimod treatment. In this experiment, 1531 differentially expressed genes were identified out of a total of 13399 genes with measured expression as shown in volcano plot (A). A threshold of 0.05 for statistical significance (p-value) and a log fold change of expression with absolute value of at least 0.58 was chosen. Blue dots and arrow down indicate genes with decreased expression. Red dots and arrow up indicate genes with increased expression. (B) 3D PCA analysis: With PC1-47%, PC2-29%, PC3-24%, separation of SPMS baseline group (green dots) with group of siponimod-treated (blue dots) and placebo-treated (grey dots) were shown indicating different gene expression profiles for SPMS patients before and after siponimod treatment. (C) The significantly representative pathways are shown generated by iPathwayGuide with over-representation on the x-axis (pORA) and the total pathway accumulation on the y-axis (pAcc).
Figure 3. Heatmap of cellular signaling pathway changes post-siponimod treatment.

Heatmaps with significant changes of genes comparing siponimod-treated (T) with SPMS baseline (BL) and placebo treated (PL) in plasma membrane (A) and antigen-receptor (B) signaling pathways ordered by expression level from bottom (low) to top (high). These two pathways were chosen because they were classified in iPathwayGuide. BL and PL were largely unchanged while T showed a general reduction in expression (blue) (COLOR KEY: blue indicates decreased gene expression and red indicates increased gene expression). The rows are different genes. Blue labeled genes: co-signaling molecules e.g. CTLA4, ICOS, and CXCR5; Red labeled genes: immunoglobulin and interleukin related; Green labeled genes: cell surface markers; and purple labeled genes: other signaling molecules. The columns reflect: BL (baseline; N=21); PL (placebo; N=7); T (siponimod treatment; N=12).
Figure 4. Changes in blood cell counts from baseline to 12 months in study participants assigned to siponimod or placebo. (A) Absolute white blood cell (WBC) counts. Placebo N: 0 month=13, 6 months=12, 9-12 months=12; Siponimod N: 0 month=23, 6 months=20, 9-12 months=18. (B) Absolute peripheral blood mononuclear cell (PBMC) counts calculated by subtracting granulocytes from WBC counts. Placebo N: 0 month=13, 6 months=12, 9-12 months=12; Siponimod N: 0 month=23, 6 months=20, 9-12 months=17. (C) Absolute Lymphocyte counts. Placebo N: 0 month=13, 6 months=12, 9-12 months=12; Siponimod N: 0 month=23, 6 months=20, 9-12 months=17. Points represent individual participants. Lines indicates mean for placebo (black) and siponimod (red). P value above the data dots represent statistically significant difference between placebo and siponimod at the same time point. Statistical tests used are: Mann-Whitney U test for (A), (B) 9-12 months, and (C) 6 months and 9-12 months; unpaired t test for (B) 0 month and 6 months.
Figure 5. Siponimod treatment primarily decreases absolute levels of CD4+ T cells and CD19+ B cells in blood. Absolute cell counts per milliliter blood were calculated using clinical complete blood cell counts and the percentages as determined by flow cytometry following staining with the markers: TCRαβ, CD4, CD14, CD8, CD19, CD56, Lin, and HLA-DR. (A) CD4+ T cells (TCRαβ+ CD4+). (B) CD8+ T cells (TCRαβ+ CD8+). Data of one patient at all time points were excluded because they are statistically outliers. (C) CD4: CD8 ratio. Data of one patient at all time points were excluded because they are statistically outliers. (D) B cells (CD19+). (E) NK cells (TCRαβ-CD56-). (F) NKT cells (TCRαβ+CD56+). (G) Monocytes (CD14+). Points represent individual participants, lines show means for placebo (black) and siponimod (red). Numbers above the data dots represent statistically significant differences between placebo (black) and siponimod (red). Statistical tests used are: Mann-Whitney U test for (A-B), (C-D) 0 month and 9-12 months, (E) 9-12 months, (F) and (G) 0 month and 9-12 months; unpaired t test for (E) 0 month and 6 months, (G) 6 months; unpaired t-test with Welch’s correction for (C-D) 6 months. (A-B, D-G) Placebo N: 0 month=9, 6 months=9, 9-12 months=12; (C) Placebo N: 0 month=8, 6 months=8, 9-12 months=11; (C, E-G) Siponimod N: OM=16, 6 months=16, 9-12 months=16; (B) Siponimod N: 0 month=15, 6 months=15, 9-12 months=16; Siponimod N: 0 month=16, 6 months=16, 9-12 months=17; (A & D).
Figure 6. Changes in CD4+ naïve and memory T cell populations from baseline to 12 months following treatment with placebo or siponimod. Cross-sectional representation (A-D) and longitudinal representation (E-H) of frequencies of subsets as a fraction of total CD3+CD4+ T cells. (A, E) Tnaïve (CD45RO-CCR7+). (B, F) Tcm (CD45RO+CCR7+). (C, G) Tem (CD45RO+CCR7-). (D, H) Temra (CD45RO-CCR7-). A-D: Points represent individual participants, lines show means for placebo (black) and siponimod (red). A-D: Cross-sectional representation; Numbers above the data dots represent statistically significant differences between placebo (black) and siponimod (red) at the same time point. Statistical tests used are unpaired t-test for (A-B); Mann-Whitney U test for (C) 0 month and (D); unpaired t-test with Welch’s correction for (C) 6 months and 9-12 months. Placebo N: 0 month=10, 6 months=9, 9-12 months=12; Siponimod N: 0 month=17, 6 months=16, 9-12 months=14. E-H: Longitudinal representation. Data are mean ± SEM. P values represent statistically significant difference within group comparisons for placebo (black) and siponimod (red) using Tukey’s or Dunn’s multiple comparison. Placebo N=9; (E-G) Siponimod N: 0 month=14, 6 months=15, 9-12 months=14; (H) Siponimod N=13.
Figure 7. Changes in CD4⁺ naïve and memory T cell populations from baseline to 12 months following treatment with placebo or siponimod. Cross-sectional representation (A-D) and longitudinal representation (E-H) of frequencies of subsets as a fraction of total CD3⁺CD4⁺ T cells. (A, E) T naïve (CD45RO⁻CCR7⁺). (B, F) T cm (CD45RO⁺CCR7⁺). (C, G) Tem (CD45RO⁺CCR7⁻). (D, H) Temra (CD45RO⁻CCR7⁻). A-D: Cross-sectional representation; points represent individual participants, lines show means for placebo (black) and siponimod (red). Numbers above the data dots represent statistically significant differences between placebo (black) and siponimod (red) at the same time point. Statistical tests used are unpaired t-test for (A) 0 month and 6 months, (C) 6 month and 9-12 months; Mann-Whitney U test for (A) 9-12 months, (B), (D) 0 month; unpaired t test with Welch’s correction for (C) 0 month, (D) 6 months and 9-12 months. E-H: Longitudinal representation; data are shown as mean ± SEM. P values represent statistically significant differences within group comparisons for placebo (black) and siponimod (red) using Tukey’s or Dunn’s multiple comparison. (A-D) Placebo N: 0 month=10, 6 months=9, 9-12 months=12; Siponimod N: 0 month=17, 6 months=16, 9-12 months=18. (E-H) Placebo N=9; (E-F) Siponimod N=13; (G-H) Siponimod N: 0 month=14, 6 months=15, 9-12 months=14.
Figure 8. Siponimod shifts the balance of effector T cells toward more Th2 and Treg cells in blood. Frequencies of effector T cell subsets shown as a fraction of total CD3+CD4+ T cells. 

(A, E) Th2 (CD4+CXCR3−CCR6−CD161−CRTH2+) (B, F) Th2: Th17 (CD4+CXCR3−CCR6+CD161+) ratio. (C, G) Treg (CD4+CD25+FoxP3+). (D, H) Treg:Th17 ratio. A-D: Points represent individual participants, lines show means for placebo (black) and siponimod (red). Numbers above the data dots represent P values where the difference between placebo (black) and siponimod (red) is statistically significant at the same timepoints. Statistical tests used are unpaired t test for (A-C) 0 month, (D) 6 months; Mann-Whitney U test for (A) 6 months, (B) 6 months and 9-12 months, (C) 9-12 months; unpaired t-test with Welch’s correction for (A) 9-12 months, (C) 6 month, (D) 0 month and 9-12 months. E-H: Longitudinal representation; data are shown as mean ± SEM. P values represent statistically significant differences within group comparisons for placebo (black) and siponimod (red) using Tukey’s or Dunn’s multiple comparison. (A-D) Placebo N: 0 month=10, 6 months=9, 9-12 months=12; (A-B) Siponimod N: 0 month=17, 6 months=16, 9-12 months=18; (C-D) Siponimod N: 0 month=17, 6 months=14, 9-12 months=18; (E-H) Placebo N: 0 month=9, 6 months=9, 9-12 months=9; Siponimod N: 0 month=14, 6 months=15, 9-12 months=14.
Figure 9. Siponimod treatment increases percentage of regulatory B cell populations in blood. Cross-sectional representation (A-F) and longitudinal representation (G-H) of frequencies of B cell subsets as fraction of total CD19+ B cells. (A) Naïve B cells (CD19+IgD+CD27-). (B) CD27+ B cells (CD19+CD27+). (C) Unswitched memory B cells (CD19+IgD+CD27+). (D) Switched memory B cells (IgD-CD27+). Regulatory B cell populations: (E, G) Transitional Bregs (CD19+CD24++CD38++). (F) B1 cells (CD19+CD43+CD27+). (H) Transitional: Switched memory B cell ratio. (A-F): Cross-sectional representation; points represent individual participants, lines show means for placebo (black) and siponimod (red). Numbers above the data dots represent P values where the difference between placebo and siponimod is statistically significant at the same time points. Statistical tests used are unpaired t test for (B), (D) 0 month, 9-12 months; (E) 6 months; Mann-Whitney U test for (A), (C), (D) 6 months, (E) 0 month and 6 months, (F); unpaired t-test with Welch’s correction for (E) 9-12 months. Placebo N: 0 month=10, 6 months=8, 9-12 months=12; Siponimod N: 0 month=17, 6 months=15, 9-12 months=17. (G-H): Longitudinal representation; data are shown as mean ± SEM. P values represent statistically significant differences within group comparisons for placebo (black) and siponimod (red) using Tukey’s or Dunn’s multiple comparison. (G-H) Placebo N: 0 month=10, 6 months=8, 9-12 months=12; Siponimod N: 0 month=15, 6 months=14, 9-12 months=17.
Figure 10 Increased frequency of combined Breg (sum of transitional B cells and B1 cells) are closely correlated with increased frequency of Treg (CD25+FoxP+ CD4+ T cells) in siponimod-treated, but not placebo-treated patients. (A) Transitional Breg plus B1 cells cross-sectional representation; points represent individual participants, lines show means for placebo (black) and siponimod (red). Numbers above the data dots represent statistically significant P value difference between placebo and siponimod at the same time points. Statistical tests used are Mann-Whitney U test for (A) 0 month and 6 months; unpaired t-test with Welch’s correction for (A) 9-12 months. Placebo N: 0 month=10, 6 months=8, 9-12 months=12; Siponimod N: 0 month=15, 6 months=14, 9-12 months=17. (B) Spearman Correlation analysis of combined Breg frequencies of B cells with Treg frequencies of CD4+ T cells was done using data derived from patients before and after 6 months and 9-12 months placebo treatment. Treg and Breg pairs N=27 (0 month=9, 6 months=9, 9-12 months=9). (C) Spearman Correlation analysis of combined regulatory B cells (Transitional Breg+ B1) frequencies of B cells with Treg frequencies of CD4+ T cells was done using data derived patients before (black dot) and after 6 months (red open dot) and 9-12 months (red square) siponimod treatment. Spearman correlation coefficient R and p value are shown. Treg vs Breg pairs N=33 (0 month=12, 6 months=11, 9-12 months=10). The line in the graph is the best fit linear regression line.
Figure 11. Treg that are increased in frequency after siponimod treatment exhibit increased proliferation. (A) Representative FACS profile of CD4 T cells from patient before and after treatment of siponimod (12 months) and placebo (10 months, end of study) (B) Cross-section comparison of Ki67+% of FoxP3+ CD4 T cells at baseline, 6 months and 9-12 months after treatment with placebo (0 month: N=8; 6 months: N=8; 9-12 months: N=12) or siponimod (0 month: N=13; 6 months: N=13; 9-12 months: N=16). (C) Increased PD-1+ frequency of CD4 T cells in 9-12 months siponimod-treated patients (N=10) compared with 9-12 months placebo-treated patients (N=7). Numbers above the data dots represent P values where the difference between placebo and siponimod is statistically significant at the same time points. Statistical tests used are unpaired t test for (A) 0 month and 9-12 months; Mann-Whitney U test for (A) 9-12 months, (C).
Table 1. Demographics of AMS04 study.

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### Table 2. Cell Surface Lineage Markers Used

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