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

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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 multicentered, 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 in CD4+ T cells, CD8+ T cells, and B cells. Flow cytometric analysis showed that within the remaining lymphocyte subsets there was a reduction in the frequencies of CD4+ and CD8+ naive T cells and central memory cells, while T effector memory cells, antiinflammatory Th2, and T regulatory cells (Tregs) were enriched. Transitional regulatory B cells (CD24hiCD38hi) and B1 cell subsets (CD43+CD27+) were enriched, shifting the balance in favor of regulatory B cells over memory B cells. The proregulatory 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 Tregs and regulatory B cells in siponimod-treated participants.

CONCLUSION. The shift toward an antiinflammatory and suppressive homeostatic immune system may contribute to the clinical efficacy of siponimod in SPMS.

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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 III EXPAND clinical trial demonstrated that siponimod (BAF312; trade name Mayzent) is clinically effective for SPMS based on the primary outcome of a reduction in 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). Two hundred eighty-eight (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 in 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 receptor (S1PR) modulator selective for S1PR1 and S1PR5 (4). The first-generation S1PR modulator, fingolimod, is approved for RRMS, but failed to show efficacy in the phase III 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 that failed to slow progression. It is anticipated that, similarly to fingolimod, siponimod reduces inflammation by trapping S1P-sensitive subsets of lymphocytes in lymph nodes through functional antagonism of S1PR1 (7). However, compared with 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 in the peripheral immune cell profile of a cohort of participants in the AMS04 substudy of the EXPAND trial following treatment with placebo or siponimod for up to 12 months.

Results

Participant demographics. The AMS04 multicentered 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 posttreatment samples were not available for 4 participants in the siponimod-treated and 1 in the placebo group due to patient dropout. 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 before enrollment, and the vast majority of participants were pretreated 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 13,399 genes with measured expression, as shown in a volcano plot (Figure 2A). Our data showed clear separation between baseline untreated (green dots) and 12-month siponimod-treated groups (blue dots) on 3D principal component analysis (PCA) plots, and similar clustering was seen with the 12 months placebo-treated group (gray dots) compared with 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 costimulation, T and B cell interaction, antibody production, antigen receptor–mediated signaling pathways, and cytokine–cytokine receptor interaction (Figure 3).

**Effects of siponimod on WBC, PBMC, and lymphocyte counts.** Because 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 after treatment. Siponimod treatment significantly decreased the absolute numbers of circulating white blood cells (WBCs), peripheral blood mononuclear cells (PBMCs), and lymphocytes (Figure 4, A–C). On average, the 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 of 17 at 6 months, and 11 of 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). Twenty-four percent (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 the 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, although 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 in 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 ($P \leq 0.0001$) 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 that is induced by lymphopenia (Supplemental Figure 3; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.134251DS1). Siponimod treatment did not significantly change the absolute number of NK, NKT, or monocyte cell populations compared with the placebo-treated group (Figure 5, E–G).

**Siponimod depletes circulating naive T cells while enriching memory T cells.** Within the CD3+ T cell population, siponimod treatment led to a shift toward fewer naive and more differentiated memory cells (Figures 6 and 7). In the CD4+ T cell population (Figure 6), siponimod significantly reduced the frequency of naive T cells (Figure 6A) by 46% at 6 months ($P = 0.0048$) and 41% at 9–12 months ($P = 0.0058$) compared with the place-
bo group. Within the siponimod-treated group, naive 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 in 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 with pretreatment levels (Figure 6G). There was an overall relative increase in CD45RO– Tem (Temra) frequency within the siponimod-treated group compared with the placebo group (Figure 6D, $P = 0.00058$ for 9–12 months; and Figure 6H). Within the CD4– T cells, which are primarily CD8+ T cells, naive T and Tcm were significantly reduced with siponimod treatment relative to baseline and the placebo group (Figure 7, A, B, E, and F). There was a greater than 75% reduction in CD4– naive T cells 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 7, C and G) were moderately increased. Percentages 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 presiponimod 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 ($P < 0.0001$ 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 3.7-fold at 9–12 months ($P = 0.0026$) after siponimod treatment. Correspondingly, the Th2/Th17 (Figure 8, B and F) and Treg/Th17 cell ratios (Figure 8, D and H) 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, naive 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 9, B–D). In contrast, the percentages of transitional regulatory B cells (Bregs, CD24hiCD38hi) ($P = 0.0056$ at 9–12 months; Figure 9, E and G) 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 Tregs and Bregs 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 proregulatory 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 Bregs 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 \( P < 0.0001 \) at 9–12 months; Figure 11, A and B) compared with conventional FoxP3–CD4+ T cells (Figure 11A and Supplemental Figure 3), based on Ki67 expression (Figure 11B). Furthermore, the frequency of CD4+ T cells expressing the immune checkpoint inhibitor 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 to our knowledge in humans demonstrating the profile of differentially expressed genes in response to siponimod treatment in SPMS. We found that cosignaling 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 disease-modifying treatments, including the related S1PR 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, and P2RY12) were downregulated, and 1, 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 an S1PR modulator, such as S1PR1 and CCR7, were downregulated, as reported with fingolimod (13, 14).

Furthermore, we found that siponimod exerted an antiinflammatory 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 analysis, 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 naive 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 (CD24+CD38hi). 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 naive B cells found with fingolimod (19, 21). Although transitional Bregs (CD24+CD38hi) were studied with fingolimod treatment, our finding on enrichment of B1 regulatory cells (CD27+CD43+CD19+) with siponimod in SPMS is potentially novel.

The shift toward a relative increase in total Bregs (transitional and B1) may contribute to a shift toward an overall more proregulatory immune environment, as the increased total Breg frequency correlated significantly with the increased Treg frequency after 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 and Supplemental Figure 4). The proregulatory 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.
Although 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 into 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 (Figure 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-CTLA4 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.

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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 (6). Preclinical studies indicate a role for Tregs in promoting remyelination (37), 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 S1PR1-mediated lymphocyte trapping, their effects on cell activation stem from different affinity and engagement of S1PRs (38). These differences in cell activation, particularly with the lymphoid tissues and/or the CNS, likely

Figure 7. Changes in CD4+ naive 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 and E) Naive T (CD45RO–CCR7+). (B and F) Tcm (CD45RO–CCR7+). (C and G) Tem (CD45RO–CCR7–). (D and H) Temra (CD45RO–CCR7–). (A–D) Symbols represent individual participants, lines show means for placebo (black) and siponimod (red); numbers above the data symbols represent statistically significant differences between placebo (black) and siponimod (red) at the same time point. Unpaired t test was used for A, 0 and 6 months; C, 6 and 9–12 months. Mann-Whitney U test was used for A, 0 and 6 months; B, and D, 0 months. Unpaired t test with Welch’s correction was used for C, 0 months; D, 6 and 9–12 months. (E–H) 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-comparisons test. (A–D) Placebo n: 0 months = 10, 6 months = 9, 9–12 months = 12. Siponimod n: 0 months = 17, 6 months = 16, 9–12 months = 18. (E–H) Placebo n = 9. (E and F) Siponimod n = 13. (G and H) Siponimod n: 0 months = 14, 6 months = 15, 9–12 months = 14.
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 S1PRs, S1P modulators are neuroprotective in various preclinical models (39, 40), and siponimod is CNS penetrant (41). Although clear evidence for neuroprotective activity within MS patients has not yet been established, it was proposed that an S1PR-mediated reduction in glial activation may synergize with the peripheral immune modulation to prevent disease-associated inflammatory activity in the CNS (42). 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 the 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 2 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 a 2:1 ratio. Novartis ended the RTP of EXPAND on October 07, 2015 when planned events of progression were met. Following this, participants entered open-label phase (OLP) (Figure 1) and were offered treatment with siponimod.
Inclusion and exclusion criteria. The AMS04 study recruited subjects age 18 to 60 who had a clinical diagnosis of SPMS and were enrolled in the EXPAND trial. Details can 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 the 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 in the AMS04 study was May 21, 2014 and the last subject completed AMS04 on July 12, 2017.

PAXgene blood RNA isolation

Whole blood of subjects was collected into PAXgene Blood RNA Tubes (Qiagen) to perform RNA isolation. The blood was initially mixed into the PAXgene tube buffer to lyse the cells, and stored at −80°C before 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) to assess RNA quality. Expression values were calculated for before and after 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 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.

Figure 9. Siponimod treatment increases percentage of regulatory B cell populations in blood. Cross-sectional representation (A–F) and longitudinal representation (G and H) of frequencies of B cell subsets as fraction of total CD19+ B cells. (A) Naive 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 and G) Transitional Bregs (CD19+CD24+CD38−). (F) B1 cells (CD19+CD43−CD27+). (H) Transitional/switched memory B cell ratio. (A–F) Symbols represent individual participants, lines show means for placebo (black) and siponimod (red), numbers above the data symbols represent P values where the difference between placebo and siponimod is statistically significant at the same time points. Unpaired t test was used for B; D, 0 and 9–12 months; and E, 6 months. Mann-Whitney U test was used for A; C, D, 6 months; E, 0 and 6 months; and F. Unpaired t test with Welch’s correction was used for A, 9–12 months. Placebo n: 0 months = 10, 6 months = 8, 9–12 months = 12. Siponimod n: 0 months = 15, 6 months = 14, 9–12 months = 17. (G and H) Placebo n: 0 months = 10, 6 months = 8, 9–12 months = 12. Siponimod n: 0 months = 17, 6 months = 15, 9–12 months = 17. 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-comparisons test. (G and H) Placebo n: 0 months = 10, 6 months = 8, 9–12 months = 12. Siponimod n: 0 months = 15, 6 months = 14, 9–12 months = 17.
iPathwayGuide (Advaita Corporation) software was used to perform pathway and Gene Ontology (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 interquartile range was used to represent that gene. The NCBI Gene Expression Omnibus (GEO) accession number for our microarray data is GSE141381.

Immunophenotyping analysis

Heparinized blood (60 mL) 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 24 hours. Tubes were centrifuged at 400 g for 10 minutes, and plasma was collected, aliquoted, and stored at –80°C. PBMCs were isolated using the Ficoll-Hypaque density gradient centrifugation method. PBMCs

Figure 10. Increased frequency of combined Bregs (sum of transitional B cells and B1 cells) are closely correlated with increased frequency of Tregs (CD25+FoxP+CD4+ T cells) in siponimod-treated, but not placebo-treated patients. (A) Transitional Breg plus B1 cells cross-sectional representation. Symbols represent individual participants, lines show means for placebo (black) and siponimod (red); numbers above the data symbols represent statistically significant P values for differences between placebo and siponimod at the same time points. Mann-Whitney U test was used for A, 0 and 6 months. Unpaired t test with Welch's correction was used for A, 9–12 months. Placebo n: 0 months = 10, 6 months = 8, 9–12 months = 12. Siponimod n: 0 months = 15, 6 months = 14, 9–12 months = 17. (B) Spearman’s 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 of placebo treatment. Treg and Breg pairs n = 27 (0 months = 9, 6 months = 9, 9–12 months = 9). (C) Spearman’s correlation analysis of combined regulatory B cell (transitional Breg + B1) frequencies of B cells with Treg frequencies of CD4+ T cells was done using data derived patients before (black symbols) and after 6 months (red open symbols) and 9–12 months (red squares) of siponimod treatment. Spearman's correlation coefficient r and P value are shown. Treg versus Breg pairs n = 33 (0 months = 12, 6 months = 11, 9–12 months = 10). The line in the graph is the best-fit linear regression line.

Figure 11. Tregs 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 with siponimod (12 months) or placebo (10 months, end of study). (B) Cross-sectional comparison of Ki67+ percentage of FoxP3+CD4+ T cells at baseline, 6 months, and 9–12 months after treatment with placebo (0 months, n = 8; 6 months, n = 8; 9–12 months, n = 12) or siponimod (0 months, n = 13; 6 months, n = 13; 9–12 months, n = 16). (C) Increased PD-1+ frequency of CD4+ T cells in 9–12 month siponimod-treated patients (n = 10) compared with 9–12 month placebo-treated patients (n = 7). Numbers above the data symbols represent P values where the difference between placebo and siponimod is statistically significant at the same time points. Unpaired t test was used for A, 0 and 9–12 months. Mann-Whitney U test was used for A, 9–12 months; and C.
were stained with antibodies to mark cell subsets and analyzed by flow cytometry using the BD FACSCanto II system and FlowJo, as previously described (43, 44). 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 Figure 1A (Th1, Th2, Th1-Th17, and Th17), Supplemental Figure 1B (Treg), and Supplemental Figure 2 (B cell subsets).

**Statistics**

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 2 of the time points. Due to shipping delays, 2 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 2 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, 1-way ANOVA followed by Tukey’s multiple-comparisons test was performed if normality of the data was confirmed; otherwise, data were analyzed using Friedman’s test 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.

**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 before participation in the study.
Author contributions
Q. Wu, Q. Wang, and SKL conducted experiments. Q. Wu, Q. Wang, EAM, SKL, DAF, and YMD contributed to study design and analyzed data. CAD, CF, and BK served as clinical coordinators. Q. Wu, Q. Wang, EAM, SKL, and YMD wrote manuscript.

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