

Transcriptome profiling of peripheral blood immune cell populations in multiple sclerosis patients before and during treatment with a sphingosine-1-phosphate receptor modulator

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Summary

Aims: Fingolimod is a sphingosine-1-phosphate (S1P) receptor modulator approved for the treatment of the relapsing form of multiple sclerosis (MS). It prevents the egress of lymphocyte subpopulations from lymphoid tissues into the circulation. Here, we explored the broad effects of fingolimod on gene expression in different immune cell subsets.

Methods: Utilizing 150 high-resolution microarrays from Affymetrix, we obtained the transcriptome profiles of 5 cell populations, which were separated from the peripheral blood of MS patients prior to and following oral administration of fingolimod.

Results: After 3 months of treatment, significant transcriptome shifts were seen in CD4+ and CD8+ cells, which is mainly attributable to the selective homing of naive T cells and central memory T cells. Although the number of B cells was greatly reduced in the blood of fingolimod-treated MS patients, the analysis of differential expression in CD19+ cells identified only a small set of 42 genes, which indicated a slightly higher frequency of transitional B cells. The transcriptome signatures of CD14+ monocytes and CD56+ natural killer cells were not affected.

Conclusion: Our study corroborates changes in the composition of circulating immune cells in response to fingolimod and delineates the respective implications at the RNA level. Our data may be valuable for comparing the effects of novel S1P receptor modulating agents, which may be a therapeutic option for patients with secondary progressive MS as well.

KEYWORDS

CD19+ B cells, fingolimod therapy, peripheral blood, relapsing-remitting multiple sclerosis, transcriptome microarray analysis

1 | INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS), which is driven by auto-reactive immune cells.^{1,2} Distinct B cell and T cell subpopulations play different roles in the initiation and progression of this clinically heterogeneous disease. Activated cytotoxic T cells provoke inflammation and neurodegeneration in the CNS by targeting autoantigenic structures.³ T helper cells and memory B cells are mediating the destructive processes by presenting antigens and secreting proinflammatory cytokines to costimulate immune responses.⁴ The intrathecal formation of antibody-producing plasma cells and plasmablasts is another hallmark of the pathophysiology of MS.⁵

Fingolimod is an oral drug approved for the treatment of relapsing-remitting MS (RRMS).⁶ As a sphingosine-1-phosphate (S1P) receptor modulator, it affects the migration of lymphocytes out of secondary lymphatic tissues.^{7,8} During fingolimod therapy, certain immune cell subpopulations are sequestered in lymph nodes, because their S1P receptors are internalized and degraded. In consequence, there are less autoreactive cells circulating in the blood and hence less cells are able to migrate into the CNS. Immune cells expressing lymph node homing receptors such as CCR7 and SELL are preferentially trapped. Continued administration of fingolimod thus leads to a shift of cell populations in the peripheral blood. After 3 months of therapy, there is a significant decrease in the proportions of CD4+ T cells (from 46% to 6% of lymphocytes) and CD19+ B cells (from 11% to 6%) and a moderate decrease of CD8+ T cells (from 20% to 15%),⁹ while frequencies of CD14+ monocytes and CD56+ natural killer (NK) cells are relatively increased.^{10,11} However, although the altered trafficking of cell subsets has been well studied, the effects of fingolimod on gene expression and cellular signaling are still not fully established.

We examined the transcriptome of lymphocytes and monocytes before and during S1P receptor modulator therapy to obtain further insights on the effects on molecular signatures thought to be related to the pathophysiology of MS. To identify differentially expressed genes (DEG) in immune cells separated from the blood of treated RRMS patients, we measured all human protein-coding and noncoding transcripts at the exon level. The data allowed to analyze to which extent transcriptome changes correlate with the fingolimod-induced shift of circulating cell populations, to investigate gene regulatory programs potentially caused by downstream S1P signaling, and to define molecular markers for monitoring therapeutic responses.

2 | METHODS

Details about study design and analysis methods were published previously.^{12,13} Briefly, a large dataset comprising 150 high-resolution microarrays was obtained for specific cell populations from blood samples of MS patients. The patients were aged between 26 and 46 and diagnosed with RRMS according to the revised McDonald criteria.¹⁴ Disease duration was, on average, 8 years. All patients were

Key points

- CD4+ and CD8+ cell populations demonstrated huge shifts in the transcriptome during fingolimod therapy, reflecting the selective sequestration of smaller subpopulations in lymphoid tissues (eg, naive T cells and central memory T cells)
- CD19+ cells, although reduced in number in the blood circulation by more than 80%, showed only marginal gene expression changes, indicating that fingolimod similarly affects the trafficking of the different B cell subpopulations
- CD14+ monocytes and CD56+ natural killer cells showed fairly stable cell counts and transcriptome profiles in the course of treatment, with no evidence of significant gene regulatory effects independent of the sphingosine-1-phosphate pathway

previously treated with either glatiramer acetate or interferon-beta, and none of them received a S1P receptor modulator before. In the 12 months prior to the study, each patient experienced 1-3 relapses. For this reason, the therapy was switched to fingolimod (standard dose of 0.5 mg orally once daily), following the treatment guidelines and recommendations for routine medical care of the German Society of Neurology. As part of our research on MS, the study was approved by the local ethics committee of the University of Rostock and conducted in accordance with the ethical principles of the Declaration of Helsinki. All patients gave prior written informed consent to participate in this study.

Blood samples (~20 mL) were always taken immediately before the start of fingolimod therapy (baseline) as well as after 24 hours (before the second dose of fingolimod) and after 3 months. Five different cell subsets were magnetically labeled based on the cell surface markers CD4, CD8, CD14, CD19, and CD56 using Whole Blood MicroBeads from Miltenyi Biotec (Bergisch Gladbach, Germany). Afterward, the positively selected cell fractions were collected and then counted under a conventional microscope. Total RNA was isolated from the cells using the mirVana Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each cell population, samples of ten MS patients were prepared to analyze the gene expression in the course of therapy. In doing so, the 3 longitudinal samples for each patient and cell subset were handled at the same day by the same laboratory technician. In total, 150 high-coverage human transcriptome arrays (HTA) 2.0 from Affymetrix (Santa Clara, CA, USA) were prepared according to the manufacturer's instructions. HTA 2.0 microarrays contain over 6 million 25mer oligonucleotide probes for measuring the transcripts of all coding and noncoding genes. They are designed with 10 probes per exon fragment and 4 probes per exon-exon splice junction.¹⁵ The probes were summarized in the Expression Console 1.3.1 software (Affymetrix) to 70523 gene-level probe sets and 914585 exon-level probe sets. Quality control

and data normalization by the robust multiarray average algorithm¹⁶ including a log2 transformation were performed as described elsewhere.^{12,13} The processing of the microarray data was performed separately for each cell population. The complete raw and processed data are publicly available together with the clinico-demographic data of the patients in the Gene Expression Omnibus (GEO) database (SuperSeries GSE73174).

The Transcriptome Analysis Console (TAC) software version 1.0 (Affymetrix) was used to explore the effects of fingolimod on the transcriptome in the 5 different cell populations. For each gene-level probe set, the signal intensities of the 24-hour samples and the 3-month samples, respectively, were compared with those of the pre-treatment samples. DEG were determined by filtering probe sets with Student's two-tailed unpaired *t* test *P*-value <0.001 and fold change (FC) >1.5 or <-1.5. Probe sets not mapping to genes (*n* = 2995; hybridization controls, etc.) were discarded. The FC was calculated by TAC in linear scale, with negative FC indicating reduced gene expression. Accordingly, a statistically significant increase of more than 50% or decrease of more than 33% in the transcript levels distinguished DEG in response to the therapy. False discovery rate (FDR) correction¹⁷ was applied to adjust the *P*-values for multiple testing.

For DEG in CD19+ B cells, a gene regulatory network (GRN) model was constructed on the basis of the microarray data and information on predicted transcription factor (TF) binding sites (TFBS) in the promoter region of the genes. For this purpose, TFBS-integrating least angle regression (TILAR) was employed as described elsewhere.^{18,19} We used transcription start sites from the GeneCards database version 3.04²⁰ and evolutionarily conserved TFBS from the tfbsConsites track of the UCSC database build hg19.²¹ TFBS-integrating least angle regression (TILAR) was applied with backward stepwise selection on TF-gene interactions and no additional prior knowledge on gene-TF interactions ($\delta = 1$). All other steps of the GRN inference (eg, prevention of overfitting) were performed with the default configuration.

3 | RESULTS

Moderate differences (up to ~20%) in the numbers of cells that were separated from the individual blood samples were noted 24 hours after the first administration of fingolimod. After 3 months of therapy, however, the mean cell counts of sorted CD4-, CD8-, and CD19-expressing cells were significantly reduced by ~60%-90% compared to baseline (Table 1). The numbers of circulating CD14+ and CD56+ cells showed strong interindividual differences but, on average, remained relatively stable even after continued drug administration (Figure 1A).

The transcriptome analysis of the 5 immune cell populations revealed only a single DEG 24 hours after treatment initiation (Table 1). At this time point, solely the signal intensities of the probe set "TC10001500.hg.1" (no official gene symbol available) were significantly increased in the CD4+ cell subset. The lack of early gene regulatory effects reflects that the first dose of fingolimod does not have a lasting effect on the frequencies of blood cell populations and also indicates that S1P receptor downstream signaling does not elicit a discernible transcriptional response in these cells.

Significant alterations in the transcriptome profiles were seen after 3 months of fingolimod therapy (Table 1). The treatment effect was also well visible in a principal component analysis of the whole dataset comprising the signals of 70523 gene-level probe sets for all 150 HTA 2.0 microarrays, which were aggregated after data normalization (Figure 1B). The greatest change in gene expression was observed in the CD4+ cell subpopulation with in total 6489 DEG (890 of those even had a FC >2.0 or <-2.0). For CD8+ cells and CD19+ cells, 861 genes and 42 genes, respectively, were found to be expressed at significantly higher or lower levels relative to pretreatment levels. On the other hand, there were no significant gene expression dynamics in CD56+ cells and CD14+ cells. For the latter, only 1 probe set ("TC03002386.hg.1," no official gene symbol) fulfilled our filtering criteria (FC >1.5 or <-1.5 and *t* test *P*-value <0.001).

TABLE 1 Overview of fingolimod-induced cellular and transcriptome shifts for different blood cell populations

Cell population	24 h after the first dose		After 3 mo of therapy	
	Cell count change (%)	DEG (up/down)	Cell count change (%)	DEG (up/down)
CD4+	-21.1	1 (1/0)	-92.4	6489 (2574/3915)
CD8+	18.7	0 (0/0)	-59.5	861 (690/171)
CD14+	15.8	0 (0/0)	-5.3	1 (1/0)
CD19+	-14.2	0 (0/0)	-87.0	42 (41/1)
CD56+	15.5	0 (0/0)	-15.7	0 (0/0)

Shown are the effects of fingolimod therapy on average absolute counts of cells sorted from the peripheral blood of relapsing-remitting MS patients (see also Figure 1A). In comparison with baseline, significant changes in the number of circulating cells were only observed after 3 mo of treatment for CD4+ cells (Student's *t* test *P*-value = 0.00002), CD8+ cells (*P* = 0.027), and CD19+ cells (*P* = 0.00006). Moreover, the number of differentially expressed genes (DEG, *t* test *P*-values <0.001 and fold changes >1.5 or <-1.5) is indicated for each cell population and each time point during therapy (see also Figure 1D). There was basically no increase or decrease in expression 24 h after the first dose of fingolimod. In contrast, after 3 mo of treatment, huge transcriptome alterations were seen in CD4+ cells and CD8+ cells. For CD19+ B cells, there were 42 DEG despite a strong reduction in the respective cell count of almost 90%.

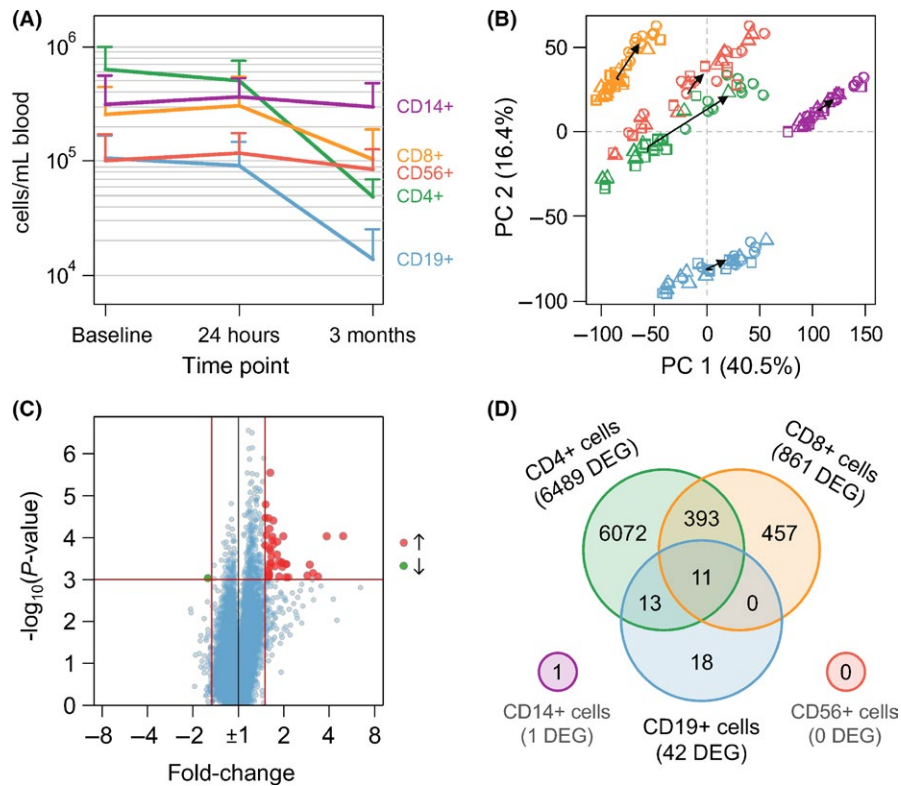


FIGURE 1 Analysis of cellular and molecular effects of fingolimod therapy in the blood of RRMS patients. Microarray-based transcriptome profiling was performed for monocytes and lymphocyte subpopulations, which are represented in the figure by different colors (CD4+ cells in green, CD8+ cells in orange, CD14+ cells in purple, CD19+ cells in blue, and CD56+ cells in red). A, Concentration of cells (mean plus standard deviation) for each of the 5 separated cell populations and each of the 3 study time points. After 3 mo of fingolimod therapy, the numbers of sorted CD4+ cells, CD8+ cells as well as CD19+ cells were significantly reduced (Student's *t* test *P*-values <0.05). B, Principal component (PC) analysis of the complete dataset comprising 150 microarrays (10 patients × 3 time points × 5 cell populations). The samples were plotted in the first two dimensions explaining most of the variation in the normalized and mean-centered gene-level probe set signal intensities (*n* = 70 523 per array). Dark arrows connect the centers of each time point (depicted by rectangles for baseline, triangles for “24 h,” and circles for “3 mo”). C, Volcano plot of gene expression changes in CD19+ cells within 3 mo after initiation of fingolimod therapy. Red and green dots display probe sets for gene transcripts expressed at significantly higher or lower levels, respectively (*n* = 42). The red vertical and horizontal lines indicate the fold change and *P*-value filtering criteria. D, Venn diagram summarizing for each cell population the sets and their intersections of differentially expressed genes (DEG) after 3 mo of fingolimod therapy relative to pretreatment levels. Eleven DEG were shared among CD4+ cells, CD8+ cells, and CD19+ cells

The huge transcriptome shifts in CD4+ cells and CD8+ cells clearly display the selective homing of T cell subpopulations to lymphoid tissues in response to sustained fingolimod therapy.^{12,13} On the contrary, the gene expression changes in CD19+ B cells were quite subtle considering the fact that their cell count was also remarkably reduced in the peripheral blood by almost 90% after 3 months (Table 1). As the purity of isolated B cells was high before and during therapy (Figure S1), this indicates that fingolimod similarly affects the trafficking of the different B cell subpopulations. Overall, 41 genes showed an up to 5-fold increase in expression, while just 1 transcript (probe set “TC15000493.hg.1”) was expressed at lower levels in the CD19+ cell subset during therapy (Figure 1C). Five of these genes remained significant at the 5% FDR level (Table 2). Predictions of TFBS revealed 5 TF to be associated with the DEG at the significance level $\alpha = 0.05$. This information was used as template for modeling the underlying GRN using TILAR.^{18,19} The resulting network linked 27 of the DEG by 15 TF-gene interactions and 42 gene-TF interactions

(Figure S2). MEF2A appeared as the most highly connected TF in the network.

A comparative analysis of the sets of DEG at the 3-month time point showed marked overlaps. Almost half of the DEG of CD8+ cells (46.9%) were also modulated in expression in CD4+ cells, and 11 genes were consistently upregulated in CD4+, CD8+, and CD19+ cells (Figure 1D). Three of these 11 shared DEG encode the proteins IQGAP2, MYBL1, and PTPN12 (Figure 2), and the other ones constitute gene fragments and less well-characterized noncoding transcripts. A subset of 18 genes was significantly differentially expressed in CD19+ B cells only.

4 | DISCUSSION

We obtained a huge dataset of 150 high-resolution HTA 2.0 microarrays to profile the transcriptome of 5 different types of circulating

TABLE 2 Genes with significant changes in expression in B cells from MS patients receiving fingolimod therapy

No	Probe set	Chromosome location	FC	P-value	Probes	Gene symbol	2 sets	3 sets
1	TC01001498.hg.1	chr1(+):171810621-172387606	2.14	0.000883	305	DNM3		
2	TC01001619.hg.1	chr1(+):192127587-192154945	4.95	0.000092	105	RGS18	✓	
3	TC02001958.hg.1	chr2(-):70476123-70476193	2.08	0.000431	30	(tRNA Gly)	✓	
4	TC02004620.hg.1	chr2(-):145143042-145143557	1.66	0.000287	30	(ZEB2)	✓	✓
5	TC02004622.hg.1	chr2(-):145251832-145254445	1.70	0.000039*	30	(ZEB2)	✓	✓
6	TC02004623.hg.1	chr2(-):145268952-145277958	1.63	0.000003*	30	(ZEB2)	✓	✓
7	TC04002285.hg.1	chr4(+):156902136-156938403	3.13	0.000705	50	–		
8	TC05002969.hg.1	chr5(-):39383148-39393457	3.37	0.000854	70	(DAB2)	✓	✓
9	TC05000368.hg.1	chr5(+):75699074-76003957	1.62	0.000409	629	IQGAP2	✓	✓
10	TC05001839.hg.1	chr5(-):138700366-138705406	1.68	0.000117	19	(SLC23A1)	✓	
11	TC06002615.hg.1	chr6(+):21597661-21598850	1.87	0.000369	15	(SOX4)	✓	
12	TC06004064.hg.1	chr6(+):27777842-27779078	3.85	0.000093	20	HIST1H3A-J		
13	TC06000759.hg.1	chr6(+):80451636-80451669	1.96	0.000503	10	RNY4	✓	
14	TC07000495.hg.1	chr7(+):77166415-77269388	1.57	0.000616	476	PTPN12	✓	✓
15	TC08001286.hg.1	chr8(-):67474410-67525529	1.60	0.000701	242	MYBL1	✓	✓
16	TC08002364.hg.1	chr8(-):67476954-67525175	1.80	0.000253	160	(MYBL1)	✓	✓
17	TC08000531.hg.1	chr8(+):86376081-86393722	1.93	0.000411	154	CA2		
18	TC08002454.hg.1	chr8(-):102193086-102195404	1.65	0.000410	30	(ZNF706)		
19	TC08002460.hg.1	chr8(-):104395306-104396082	1.81	0.000126	30	–	✓	✓
20	TC09001206.hg.1	chr9(-):74517371-74517466	1.61	0.000062	30	(Y RNA)	✓	
21	TC09001401.hg.1	chr9(-):100689073-100707138	1.99	0.000093	90	HEMGN		
22	TC09001610.hg.1	chr9(-):130594914-130595022	1.60	0.000821	30	(Y RNA)		
23	TC10001072.hg.1	chr10(-):17256238-17271983	1.63	0.000206	60	VIM-AS1	✓	
24	TC10002487.hg.1	chr10(-):17256245-17271983	1.70	0.000146	90	(VIM-AS1)	✓	✓
25	TC10001111.hg.1	chr10(-):25270908-25351208	2.07	0.000889	120	ENKUR		
26	TC10002540.hg.1	chr10(-):31109147-31110214	2.97	0.000446	27	–		
27	TC10000448.hg.1	chr10(+):73980510-73980610	1.59	0.000035*	30	(Y RNA)		
28	TC11002664.hg.1	chr11(+):48191599-48192391	1.53	0.000087	24	(PTPRJ)	✓	
29	TC11002092.hg.1	chr11(-):74971166-75062875	1.58	0.000889	197	ARRB1	✓	
30	TC12000011.hg.1	chr12(+):890299-890424	1.61	0.000185	30	(WNK1)	✓	✓
31	TC12002795.hg.1	chr12(-):24332887-24333863	1.87	0.000852	30	(SOX5)		
32	TC12000297.hg.1	chr12(+):32552463-32798984	1.51	0.000034*	454	FGD4	✓	
33	TC12000977.hg.1	chr12(+):123252646-123252747	1.75	0.000486	30	(Y RNA)		
34	TC13001561.hg.1	chr13(-):67787977-67789866	1.68	0.000096	30	(PCDH9)		
35	TC15001162.hg.1	chr15(-):31248451-31248553	1.60	0.000778	30	(MTMR10)	✓	
36	TC15000493.hg.1	chr15(+):62533117-62533147	-1.59	0.000930	7	–		
37	TC16001729.hg.1	chr16(-):223162-223620	1.95	0.000390	30	–		
38	TC16000008.hg.1	chr16(+):226679-227521	2.87	0.000807	70	HBA1-2		
39	TC16000732.hg.1	chr16(-):686736-686806	2.08	0.000431	30	(tRNA Gly)	✓	
40	TC17000978.hg.1	chr17(-):1519486-1519589	1.51	0.000154	30	(SLC43A2)	✓	
41	TC17002022.hg.1	chr17(+):5016654-5018299	1.61	0.000503	30	–		
42	TC19002594.hg.1	chr19(-):57804451-57805837	1.52	0.000016*	30	–		

In total, 42 genes were filtered as differentially expressed with *t* test *P*-values <0.001 and fold changes (FC) >1.5 or <-1.5 in CD19+ B cells when comparing the data at baseline with the data at 3 mo of treatment. The table provides the Affymetrix microarray gene-level probe set identifiers, the chromosome positions of the genes (GRCh37/hg19 genome assembly) in sorted order, FC, raw *t* test *P*-values, the number of 25mer oligonucleotide probes for each probe set as well as official gene symbols if available. Symbols in brackets (n = 21) indicate that the respective probe set interrogates only a part of a protein-coding gene or a noncoding RNA class. We marked the genes that were also expressed at significantly higher transcript levels in CD4+ cells ("2 sets," n = 24) or in both CD4+ cells and CD8+ cells ("3 sets," n = 11) as depicted in Figure 1D.

*Significant at false discovery rate of 5%.

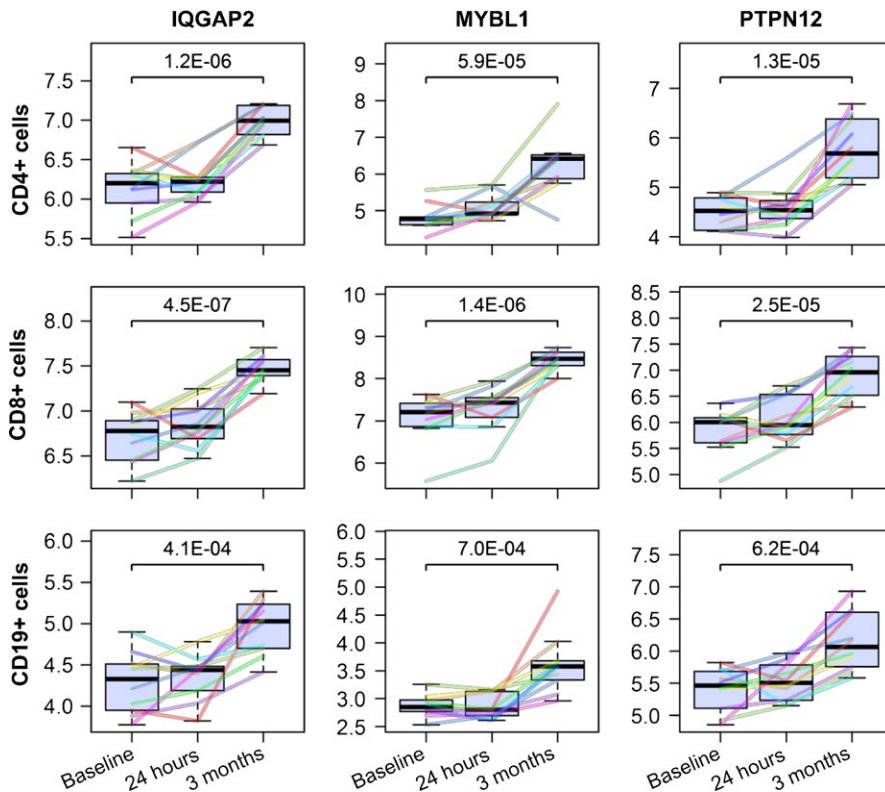


FIGURE 2 Gene expression dynamics of IQGAP2, MYBL1, and PTPN12 in the course of fingolimod therapy. Matrix of boxplots visualizing the mRNA expression of 3 selected genes (in columns) in 3 distinct immune cell populations (in rows). The cells were separated from the peripheral blood of relapsing-remitting MS patients based on cell surface markers such as CD4 and CD8 for T cells and CD19 for B cells. Pretreatment expression values as well as levels after 24 h and 3 mo of fingolimod therapy are presented in log₂ scale. Differently colored lines refer to each of the 10 individual patients per cell population. The *P*-values above the brackets were calculated by Student's *t* test. In all 3 cell populations shown, IQGAP2, MYBL1, and PTPN12 were consistently expressed at significantly higher transcript levels in response to continued administration of fingolimod in comparison with baseline (*P*-values <0.001 and fold changes >1.5)

immune cells before and during fingolimod therapy. Alterations in gene expression were not yet observed after the first dose but could be ascertained in CD4+, CD8+, and CD19+ cells (but not in CD14+ and CD56+ cells) after continued drug exposure. This goes along with a significant reduction in the number of T and B cells and, consequently, a relative increase in the frequencies of CD14+ monocytes and CD56+ NK cells in the peripheral blood of treated patients. These changes in the composition of lymphocytes, as a result of the prevention of their egress from lymphoid tissues, have already been well established in the literature.^{9-11,22} Preferential lymph node homing of naive T cells and central memory T cells, but not effector memory T cells, correlates with hundreds of DEG that we identified in the CD4+ and CD8+ cell subsets in response to fingolimod treatment. Additional gene regulatory effects independent of S1P receptor binding, however, could not be evidenced in these cells in our study. The transcriptome shift in T cells and its impact on biological processes and S1P-related pathways are presented in detail in our previous publications.^{12,13} In the following, we will focus on the results for the subset of B cells, which recently gained increasing attention in MS research.^{23,24}

Other studies have shown that the absolute numbers of naive B cells, memory B cells, and plasmablasts are significantly reduced in the peripheral blood during fingolimod treatment.²⁵⁻²⁷ As all these subpopulations are affected, only 42 DEG passed our filtering criteria when comparing the gene expression levels in separated CD19+ cells after 3 months of therapy vs baseline, although total B cell counts were decreased by 87%. Some of the DEG such as arrestin beta-1 (ARRB1), hemoglobin alpha 1/2 (HBA1-2), SRY-box 4 (SOX4), and genes of the histone H3 family (HIST1H3A-J) are known to be expressed at

higher levels in immature B cells compared to mature B cells.²⁸⁻³¹ Their elevated expression thus likely indicates a proportionally higher frequency of circulating transitional B cells in fingolimod-treated MS patients, which would also explain the emergence of TF families promoting early B cell development in the GRN (such as MEF2³²), even though classical cell surface markers such as CD24 and CD38 were not identified as DEG. Consistent with this, a relative (but not absolute) increase of newly produced immature B cells during therapy has been demonstrated by flow cytometry immunophenotyping.^{10,27,33,34} Interestingly, lower baseline percentages of transitional B cells in the peripheral blood might predict clinical response to fingolimod,³⁴ but this still has to be confirmed in larger patient cohorts.

The probe sets for the protein-coding genes IQGAP2, PTPN12, and MYBL1 were consistently filtered in the transcriptome analyses of circulating CD4+, CD8+, and CD19+ cells. This implicates that some of the 42 DEG that were identified in the CD19+ cell population after 3 months of fingolimod therapy exert various functions not only in B cells but also in other immune and non-immune cells. IQGAP2 is a broadly expressed calmodulin binding protein regulating cytoskeletal dynamics.³⁵ PTPN12, on the other hand, is a protein tyrosine phosphatase that was shown to suppress antigen-receptor signaling in B cells and T cells.³⁶ The related family member PTPRJ also plays a role in lymphocyte signaling, particularly in B cell activation and B cell development.^{36,37} Notably, the filtered probe set for PTPRJ ("TC11002664.hg.1") matches only the 3' untranslated region of the last exon, whereas the designated probe set for the full-length transcript ("TC11000412.hg.1") narrowly missed the significance threshold (*t* test *P*-value = 0.004). MYBL1 is a strong transcriptional activator, which is implicated in

Burkitt's lymphoma,³⁸ a disease associated with Epstein-Barr virus (EBV).³⁹ Three probe sets measured increased RNA levels of gene fragments from ZEB2, a transcriptional repressor that contributes to maintenance of EBV latency by inhibiting lytic reactivation.⁴⁰ Latent EBV infection of B cells is one of the strongest environmental risk factors for MS.¹ However, the possible relevance of MYBL1 and ZEB2 in MS remains to be investigated. Another differentially expressed gene in B cells during fingolimod therapy was ARRB1. An earlier study demonstrated the presence of antibodies reactive with ARRB1 in sera from patients with MS.⁴¹ Moreover, the first intron of ARRB1 harbors the precursor sequence for the microRNA hsa-miR-326, which is dysregulated in peripheral blood cells of MS patients.⁴² DNMT3 is also host gene of a microRNA, namely hsa-miR-3120. DNMT3 is preferentially expressed in the brain and as a GTP-binding protein involved in vesicular transport,⁴³ while hsa-miR-3120 regulates heat shock cognate protein 70 and vesicle uncoating.⁴⁴ Furthermore, some of the genes, for example, ARRB1, FGD4, IQGAP2, and RGS18, are known to act as regulators of GTPases.⁴⁵

Among the DEG in CD19+ cells, there were also several non-coding transcripts, which deserve further research. For instance, Ro-associated Y4 (RNY4) is affiliated with the Y RNA class, a group of small RNA acting as licensing factors for chromosomal DNA replication through interactions with chromatin and initiation proteins.⁴⁶ In addition to RNY4, we filtered 4 probe sets corresponding to paralogous Y RNA sequences in intronic regions. Moreover, 2 copies of glycine transfer RNA were found with elevated levels of expression in the B cells from patients treated with fingolimod for 3 months in comparison with baseline levels.

The strengths of our study are that we analyzed the gene expression signatures of distinct blood cell populations longitudinally in a well-characterized cohort of RRMS patients.^{12,13} Moreover, we used 150 high-resolution HTA 2.0 microarrays¹⁵ to obtain very accurate and comprehensive snapshots of the cellular transcriptomes. As limitations, our study does not give insights into RNA expression changes after the 3-month time point, and it was not designed to detect prognostic biomarkers of the long-term individual clinical response to fingolimod therapy. Subsequent studies thus may further evaluate the identified DEG in larger cohorts using focused approaches such as real-time PCR. On the other hand, a more exhaustive characterization of immune cells from treated patients is feasible as massively parallel sequencing technologies emerged that allow to measure RNA profiles at single-cell resolution.^{47,48} Other possible extensions of this study include the analysis of alternative splicing events,⁴⁹ protein levels, and other cell subpopulations. A recent study demonstrated that fingolimod also stimulates gene expression in neurons, thereby affecting axonal growth and regeneration,⁵⁰ but these effects so far have not been examined in detail at the transcriptome level.

To conclude, fingolimod selectively alters the trafficking of immune cells and, consequently, changes the gene expression profiles of CD4+, CD8+, and CD19+ cells circulating in the peripheral blood. Genes induced in B cells of MS patients receiving fingolimod therapy for 3 months comprised signaling molecules and transcriptional

regulators but not cell surface markers and cytokines. However, in comparison with T cells, the transcriptome shift in B cells was quite subtle. This reflects that the B cell subpopulations are sequestered to similar extents in lymphoid tissues. Basically, all treatments for MS modulate B cell subsets.^{51,52} Thus, further research on their roles in the pathogenesis of MS is of critical importance. Finally, our data may also be useful to compare molecular effects of fingolimod with other emerging S1P receptor modulators being examined in clinical trials of MS and other brain-related diseases.⁵³

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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