Supplemental Materials for

Effects of sphingosine-1-phosphate receptor 1 phosphorylation in response to FTY720 during neuroinflammation

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Supplemental Figure 11. Control IgG had no significant effects on the improvement of EAE clinical score.

Supplemental Table 1. EAE clinical parameters of C57BL/6J (WT) mice following immunization with MOG_{35-55} peptide.

	WT (C57BL6/J) Vehicle	WT (C57BL6/J) FTY720	*p* Value
Incidence	100% (10/10)	50% (5/10)	0.010*
Onset (days after immunization)	14 ± 2.0	17 ± 3.1	0.034*
Maximum score	3.5 ± 0.34	1.7 ± 0.44	0.0004*
Score at Peak EAE	2.7 ± 0.67	0.7 ±1.06	0.0013*
C.D.I.	37.3 ± 5.34	7.4±9.9	0.0002*

*p<0.05, Mann-Whitney *U*-test. Experiment repeated three times, n=10/arm. C.D.I.=Cumulative disease index.

Supplemental Table 2. EAE clinical parameters of f S1PR1(S5A) mice following immunization with MOG_{35-55} peptide.

	S1PR1(S5A) Vehicle	S1PR1(S5A) FTY720	*p* Value
Incidence	100%(12/12)	83% (10/12)	n.s.
Onset (days after immunization)	13 ±1.9	14 ± 2.7	n.s.
Maximum score	3.9 ± 0.29	2.6 ± 1.44	0.011*
Score at Peak EAE	3.5 ± 0.80	2.25 ± 1.36	0.018*
C.D.I.	55.08±12.73	30.17±20.23	0.0039*

*p<0.05, Mann-Whitney *U*-test. Experiment repeated three times, n=12/arm. C.D.I.=Cumulative disease index.

Supplemental Table 3. EAE clinical parameters of C57BL/6J (WT) mice following adoptive transfer of IL-23-polarized (T_H17) WT splenocytes.

	WT T_H17	WT T_H17	*p* value
	Vehicle	FTY720	
Incidence	8/10(80%)	60% (6/10)	n.s.
Onset (days post transfer)	9.6 ± 1.69	19.83 ± 8.86	0.013*
Maximum score	2.2 ± 1.23	1.6 ± 1.51	n.s.
Score at Peak EAE	2.0 ± 1.33	1.3±1.42	n.s.
C.D.I.	39.7±26.39	16.8±24.28	0.05*

*$p<0.05$, Mann-Whitney *U*-test. Experiment repeated three times, n=10/arm. C.D.I.=Cumulative disease index.

Supplemental Table 4. EAE clinical parameters of S1PR1(S5A) mice following adoptive transfer of IL-23-polarized (T_H17) S1PR1(S5A) splenocytes.

	S1PR1(S5A)T_H17	S1PR1(S5A)T_H17	*p* value
	Vehicle	FTY720	
Incidence	73% (11/15)	87% (13/15)	n.s.
Onset (days post transfer)	8.9 ± 0.83	10.5 ± 1.98	0.02*
Maximum score	3.1 ± 2.05	3.0± 1.41	n.s.
Score at Peak EAE	2.7 ±1.83	2.6 ±1.35	n.s.
C.D.I.	60.5 ± 47.94	56.7 ± 31.78	n.s.

*$p<0.05$, Mann-Whitney *U*-test. Experiment repeated three times, n=15/arm. C.D.I.=Cumulative disease index.

Supplemental Table 5. EAE clinical parameters of C57BL/6J (WT) mice following adoptive transfer of IL-12-polarized (T_H1) WT splenocytes.

	WT T_H1	WT T_H1	*p* value
	Vehicle	FTY720	
Incidence	60% (6/10)	50%(5/10)	n.s.
Onset (days post transfer)	10 ± 1.27	13.6 ± 9.87	n.s.
Maximum score	1.3 ± 1.16	1.2 ± 1.40	n.s.
Score at Peak EAE	1.2 ±1.14	0.8±1.32	n.s.
C.D.I.	17.7± 15.99	12.1± 15.31	n.s.

*$p<0.05$, Mann-Whitney *U*-test. Experiment repeated three times, n=10/arm. C.D.I.=Cumulative disease index.

Supplemental Table 6. EAE clinical parameters of S1PR1(S5A) mice following adoptive transfer of IL-12-polarized (T_H1) S1PR1(S5A) splenocytes.

	S1PR1(S5A)T_H1	S1PR1(S5A)T_H1	*p* value
	Vehicle	FTY720	
Incidence	53% (8/15)	67% (10/15)	n.s.
Onset (days post transfer)	10.4 ± 1.69	12.44 ± 3.88	n.s.
Maximum score	1.5 ± 1.5	1.5 ± 1.54	n.s.
Score at Peak EAE	1.3 ±1.45	1.2 ±1.15	n.s.
C.D.I.	17.5 ± 23.98	21.13 ± 20.37	n.s.

*$p<0.05$, Mann-Whitney *U*-test. Experiment repeated three times, n=15/arm. C.D.I.=Cumulative disease index.

Supplemental Table 7. EAE clinical parameters of S1PR1(S5A) mice following immunization with MOG_{35-55} peptide and FTY720 treatment.

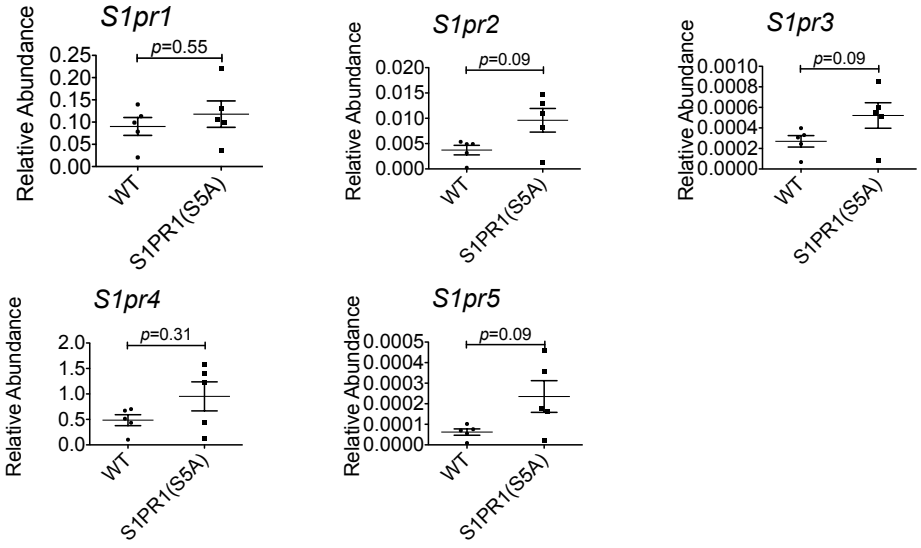
	S1PR1(S5A) FTY720 treatment		*p* value
	Control IgG	Anti-CCR6	
Incidence	100% (7/7)	80% (6/7)	n.s.
Onset (days after immunization)	15 ± 1.4	17 ± 1.5	0.036*
Maximum score	2.9 ± 0.69	2.1 ± 1.35	n.s.
Score at Peak EAE	2.9 ± 0.69	2.1 ± 1.35	n.s.
C.D.I.	22 ± 4.0	14 ± 7.8	0.016*

Control IgG and Anti-CCR6 mAb (100μg per mice) were administrated twice as indicated. *$p<0.05$, Mann-Whitney *U*-test. n=7/arm. C.D.I.=Cumulative disease index.

Supplemental Table 8. Primers for SYBRGreen quantitative PCR (synthesized in Stanford PAN Facility)

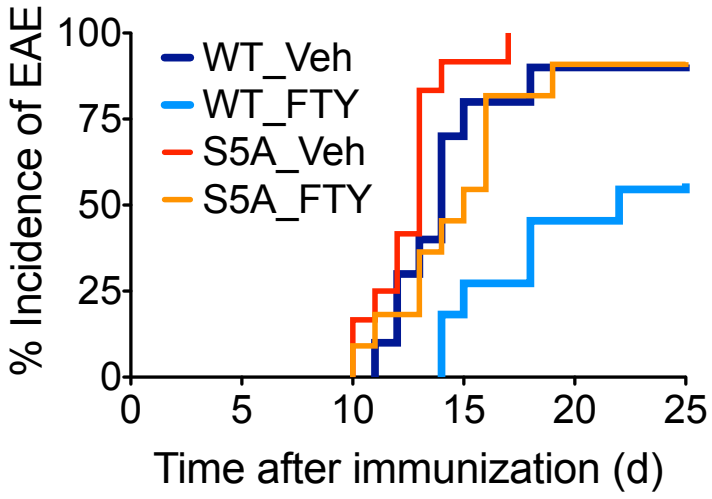
Transcript	Forward primer	Reverse primer
Il17a	TACCTCAACCGTTCCACGTC	TTTCCCTCCGCATTGACACA
S1pr1	GTGTAGACCCAGAGTCCTGCG	AGCTTTTCCTTGGCTGGAGAG
S1pr2	CCAAGGAGACGCTGGACATG	TGCCGTAGAGCTTGACCTTG
S1pr3	GCAACTTGGCTCTCTGCGAC	GACGATGGTCACCAGAATGG
S1pr4	GTGTATGGCTGCATCGGTCTGTG	GGATTAATGGCTGAGTTGAACACG
S1pr5	GTGGCGCTCGCCGCGTCGGTG	GAAGGTGTAGATGATGGGATTCAG

Supplemental Figure 1



Relative RNA expression of S1P receptors in splenic CD4$^+$ T cells from naïve WT (C57BL/6J) and S1PR1(S5A) mice. Total RNA was isolated from splenic CD4$^+$ T cells and analyzed by SYBRGreen quantitative PCR. Data was normalized with RNA expression of β-actin (n=4 per group, Mean± SEM, *p<0.05, 2-tailed Student's *t*-test).

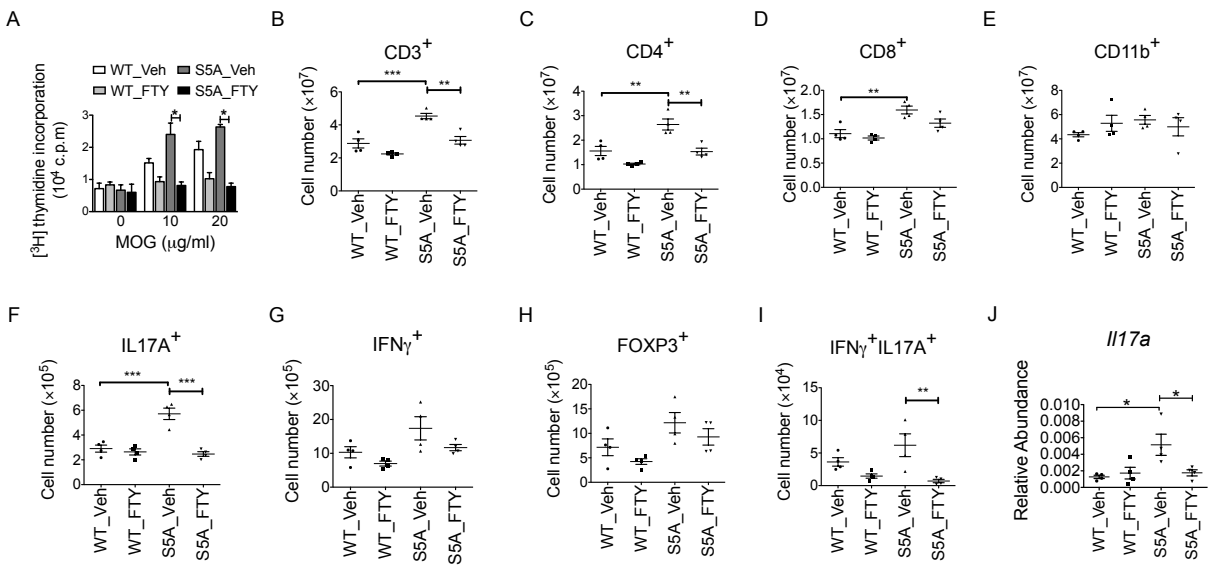
Supplemental Figure 2



S1PR1(S5A) mice showed higher incidence and early onset of EAE compared to C57BL/6J (WT) mice following FTY720 treatment.

MOG_{35-55}-immunized C57BL/6J (WT) and mice carrying phosphorylation defective S1PR1 (S1PR1(S5A)) (females, 8-12 weeks) were treated with either vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily intraperitoneal injections. Mice were followed clinically throughout EAE disease course. n= 10-12 mice/arm.

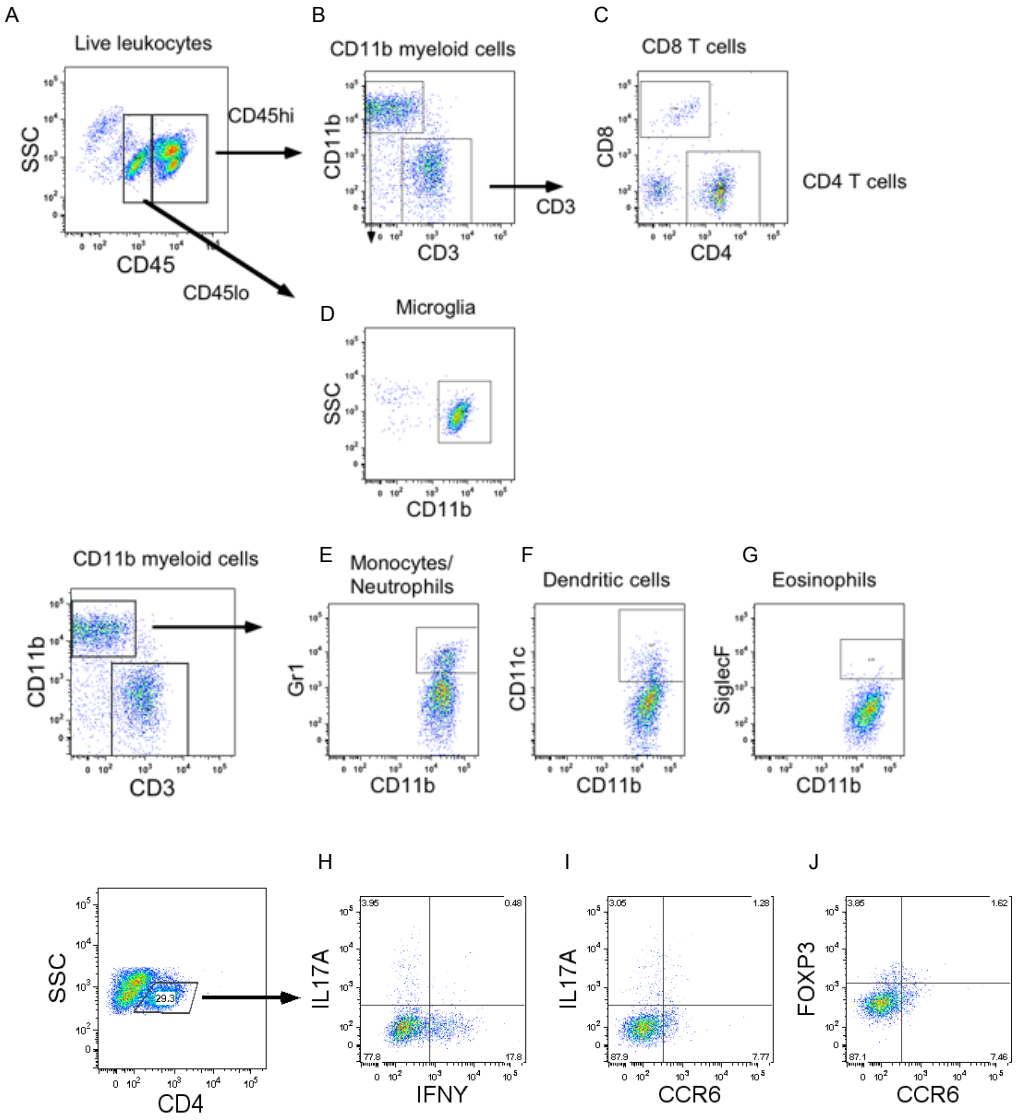
Supplemental Figure 3



FTY720 treatment decreased lymphocyte counts in the spleens of both WT and S1PR1(S5A) EAE mice.

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) at the day of immunization by daily i.p injection. Splenocytes were isolated at D8 post-immunization. (**A**) Immune cells proliferation in response to ex vivo MOG_{35-55} reactivation was measured by [^{3}H] thymidine incorporation (c.p.m., counts per minute). The numbers of $CD3^+$ **(B)**, $CD4^+$ **(C)**, $CD8^+$ **(D)**, and $CD11b^+$ **(E)** cells were quantified by flow cytometry. The cell number was calculated by multiplying the total viable cell number of splenocytes by the percentage of gated cells. Cells were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 4 h and intracellular staining was performed to measure IL-17A (**F, I**), IFN-γ (**G, I**), and FOXP3 (**H**) expression among $CD4^+$ T cells. $CD4^+$ T cells were isolated from splenocytes of MOG_{35-55}-immunized EAE mice at D8. The expression of IL-17A RNA transcripts **(J)** in the $CD4^+$ T cells was analyzed by SyBrGreen quantitative PCR and normalized to β-actin. (n=4 per group, Mean± SEM, *p<0.05, ANOVA analysis with Turkey's multiple comparison test).

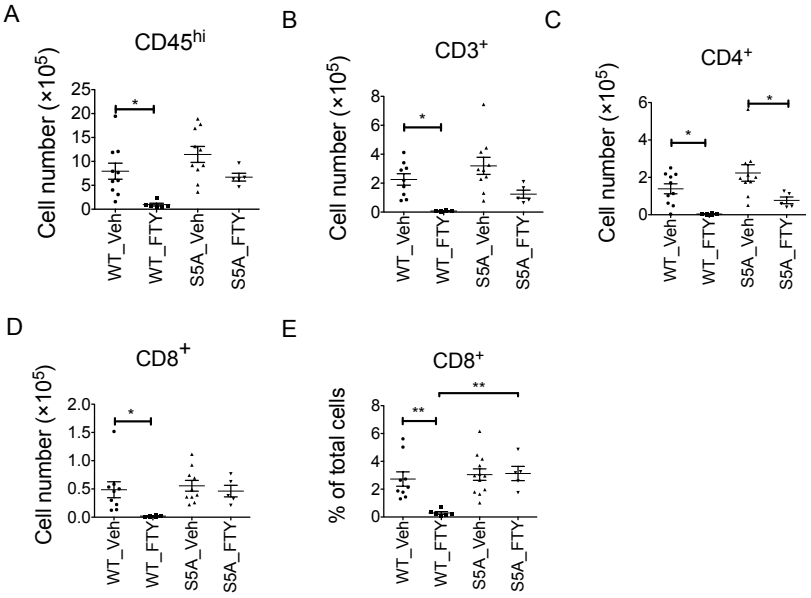
Supplemental Figure 4



Gating strategy of CNS immune cells by multi-dimensional flow cytometry

(**A**) $CD45^{low}$ and $CD45^{hi}$ cells were gated from live leukocytes. $CD45^{hi}$ cells were gated to CNS-infiltrating myeloid cells ($CD45^{hi}CD11b^{+}$) and $CD3^{+}$ cells (**B**). $CD3^{+}$ cells were then gated to $CD4^{+}$ and $CD8^{+}$ T cells (**C**). $CD45^{low}$ cells were gated to microglia cells ($CD45l^{o}CD11b^{+}$) (**D**). Infiltrating myeloid cells ($CD45^{hi}CD11b^{+}$) were further gated to neutrophils/monocytes ($CD45^{hi}CD11b^{+}Gr1^{+}$) (**E**), dendritic cells ($CD45^{hi}CD11b^{+}CD11c^{+}$) (**F**), eosinophils ($CD45^{hi}CD11b^{+}SiglecF^{+}$) (**G**). The effector functions of $CD4^{+}$ T cells were gated by CCR6, IL-17A, IFN-γ, and FOXP3 markers (**H-J**).

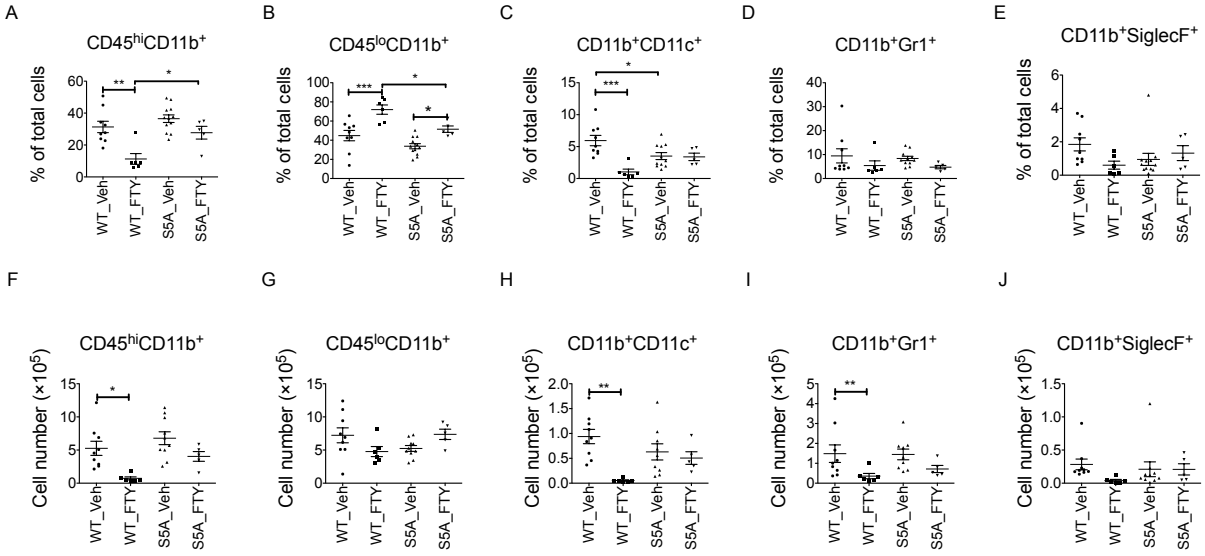
Supplemental Figure 5



The profile and cell counts of CNS-infiltrating lymphocytes in WT and S1PR1(S5A) EAE mice

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections. Immune cells from the brains and spinal cords were collected when EAE clinical scores reached 2-3. Numbers of $CD45^{hi}$ **(A)**, $CD3^+$ **(B)**, $CD4^+$ **(C)**, $CD8^+$ **(D)** cells and percentage of $CD8^+$ **(E)** cells were quantified by flow cytometrty. The cell number was calculated by multiplying the total viable cell number of splenocytes by the percentage of gated cells. Data represent three independent experiments ($n \geq 5$, Mean± SEM, $*p<0.05$, ANOVA analysis with Turkey's multiple comparison test).

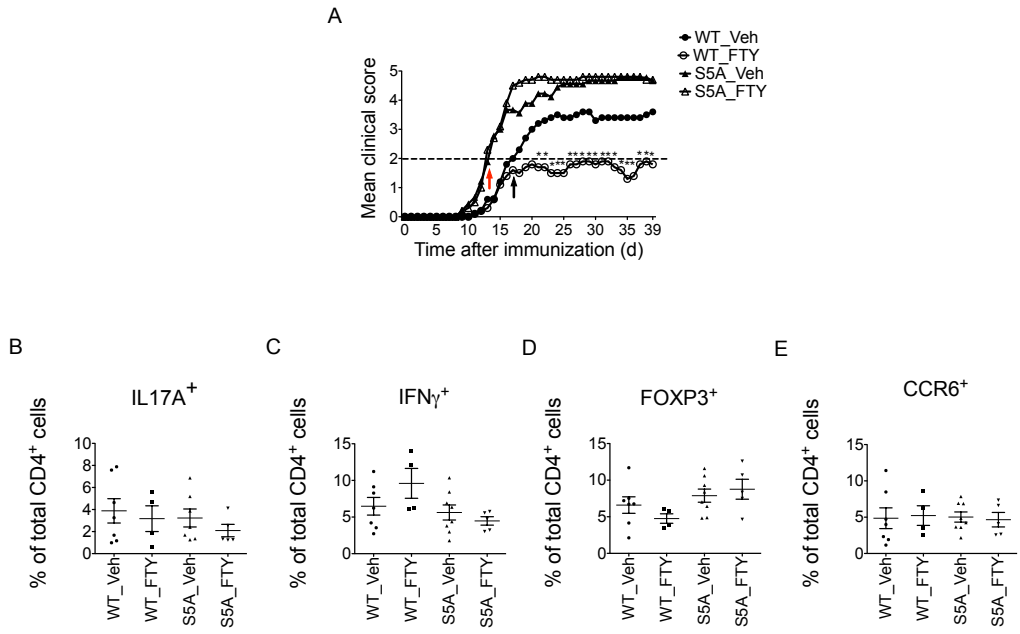
Supplemental Figure 6



The profile and number of CNS-infiltrating myeloid cells in C57BL/6J (WT) and S1PR1(S5A) EAE mice

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections. Immune cells from the brains and spinal cords were collected when EAE clinical scores reached 2-3. The percentage and number of CNS-infiltrating myeloid cells ($CD45^{hi}CD11b^{+}$) (**A, F**), microglia cells ($CD45^{lo}CD11b^{+}$) (**B, G**), dendritic cells ($CD45^{hi}CD11b^{+}CD11c^{+}$) (**C, H**), monocytes/neutrophils ($CD45^{hi}CD11b^{+}Gr1^{+}$) (**D, I**), and eosinophils ($CD45^{hi}CD11b^{+}SiglecF^{+}$) (**E, J**) were quantified by flow cytometry. The cell number was calculated by multiplying the total viable cell number of cells by percentage of gated cells. Data represent three independent experiments ($n \geq 5$, Mean± SEM, $*p<0.05$, ANOVA analysis with Turkey's multiple comparison test).

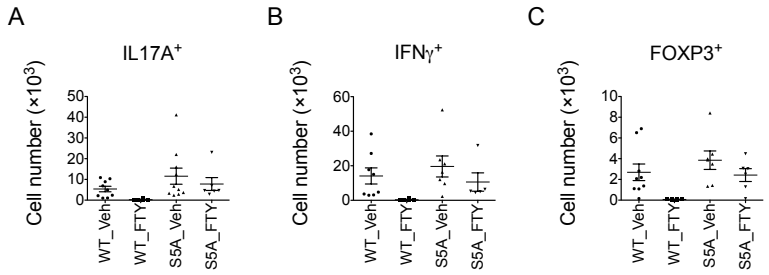
Supplemental Figure 7



S1PR1(S5A) mice with established EAE were refractory to FTY720 treatment.

(**A**) Mean clinical score of MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) mice (females, 8-12 weeks) treated with either vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections when mice first displayed an EAE clinical score of 2. Arrows (➔WT and ➔ S1PR1(S5A)) indicate the time of initiation of therapy. *p<0.05, Mann-Whitney *U*-test. This experiment was performed 2 times with n= 9-10 mice/ arm. (**B**-**E**) MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) at the day of immunization by daily i.p. injections. Immune cells from the spleen were collected when EAE clinical scores reached 2-3. Cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and intracellular staining was performed to measure the expression of IL-17A (**B**), IFN- γ (**C**), FOXP3 (**D**) and CCR6 (**E**) among $CD4^+$ T cells. This experiment was performed three times with n≥4 mice/ arm, Mean± SEM, *p<0.05, ANOVA analysis with Turkey's multiple comparison test.

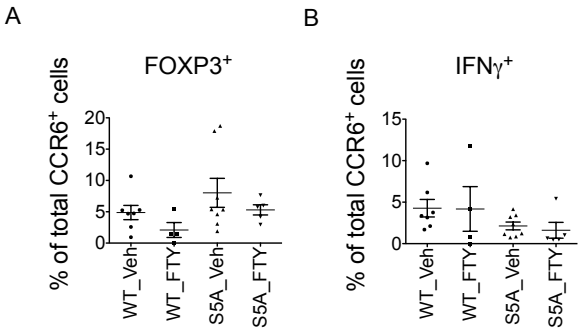
Supplemental Figure 8



The functions and cell counts of CNS-infiltrating T_H cells in WT and S1PR1(S5A) EAE mice

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections. Immune cells from the brains and spinal cords were collected when EAE clinical scores reached 2-3. Cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and intracellular staining was performed to measure the expression of IL-17A (**A**), IFN- γ (**B**), and FOXP3 (**C**) among $CD4^+$ T cells. The cell number was calculated by multiplying the total viable cell number of cells by percentage of gated cells. Data represent three independent experiments (n≥6, Mean± SEM, **p*<0.05, ANOVA analysis with Turkey's multiple comparison test).

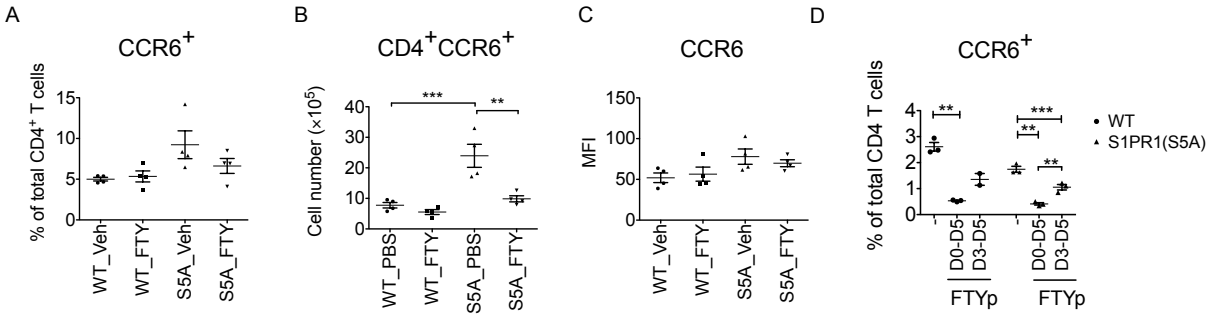
Supplemental Figure 9



The percentage of FOXP3-positive and IFNγ-positive cells among CCR6-positive CNS-infiltrating CD4 T lymphocytes

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections at the day of immunization. Immune cells from the brains and spinal cords were collected when mice display an EAE score of 2 to 3. CNS immune cells were re-stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and then cells were labeled with antibodies against CD4, CCR6, IFN-γ, and FOXP3 antibodies. Percentage of $FOXP3^+$ (**A**) and IFN-γ^+ (**B**) cells in $CD4^+CCR6^+$ population were quantified by flow cytometry (n≥4, Mean± SEM, *p<0.05, ANOVA analysis with Turkey's multiple comparison test).

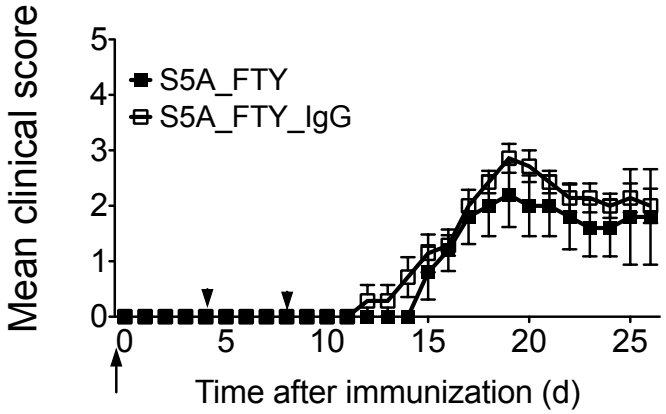
Supplemental Figure 10



FTY720 suppressed CCR6$^+$ cell generation both in vivo and in vitro.

MOG_{35-55}-immunized C57BL/6J (WT) and S1PR1(S5A) EAE mice were treated with vehicle (1% cyclodextrin in PBS) or FTY720 (0.5mg/kg) by daily i.p. injections up to D8 post-immunization. Splenocytes were harvested and labeled with anti-CD4 and CCR6 antibodies. The percentage (**A**), total number (**B**), and MFI (**C**) of CCR6$^+$ cells among CD4$^+$ T cells from the spleen were quantified by flow cytometry. The cell number was calculated by multiplying the total viable cell number of cells by percentage of gated cells. Data represent two independent experiments (n=4, Mean± SEM, *p<0.05, ANOVA analysis with Turkey's multiple comparison test) CD4$^+$ T cells were isolated from spleens of naïve C57BL/6J (WT) and S1PR1 (S5A) mice and activated in vitro with anti-CD3 (5μg/ml) and anti-CD28 (2μg/ml) for 5 days in RPMI1640 media supplemented with IL-6 (20ng/ml), IL-23 (20ng/ml), TGF-β (1ng/ml) in the presence of FTYp (1μM). Cells were stained with anti-CCR6 antibody and percentage of CCR6$^+$ cells (**D**) among CD4$^+$ T cells was analyzed by flow cytometry. This experiment was performed three times (n=3, Mean± SEM, *p<0.05, ANOVA analysis with Turkey's multiple comparison test).

Supplemental Figure 11



Control IgG had no significant effects on the improvement of EAE clinical score.

Mean clinical score ± SEM of MOG_{35-55}-immunized S1PR1(S5A) mice (females, 8-12 weeks) treated with FTY720 (0.5mg/kg) by daily i.p. injections. Arrow indicates time of initiation of FTY720 therapy. Additionally, mice were also i.p. injected with IgG control (100µg per mouse) at D4 and D8. Arrowheads indicates time of antibody injection ($n \geq 5$ per arm $*p<0.05$, Mann-Whitney *U*-test).