Defective sphingosine-1-phosphate receptor 1 ($S1P_1$) phosphorylation exacerbates T_H17-mediated autoimmune neuroinflammation

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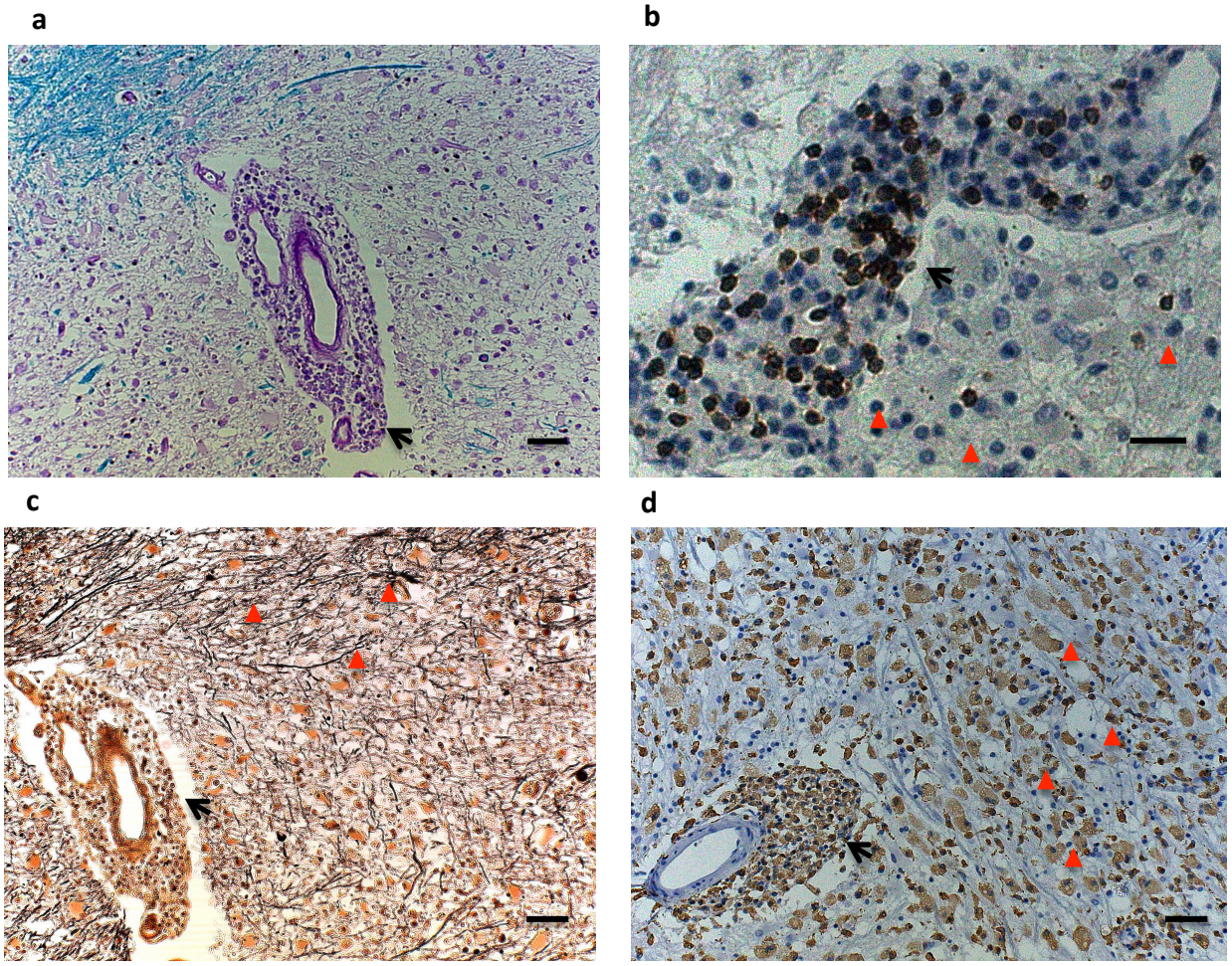
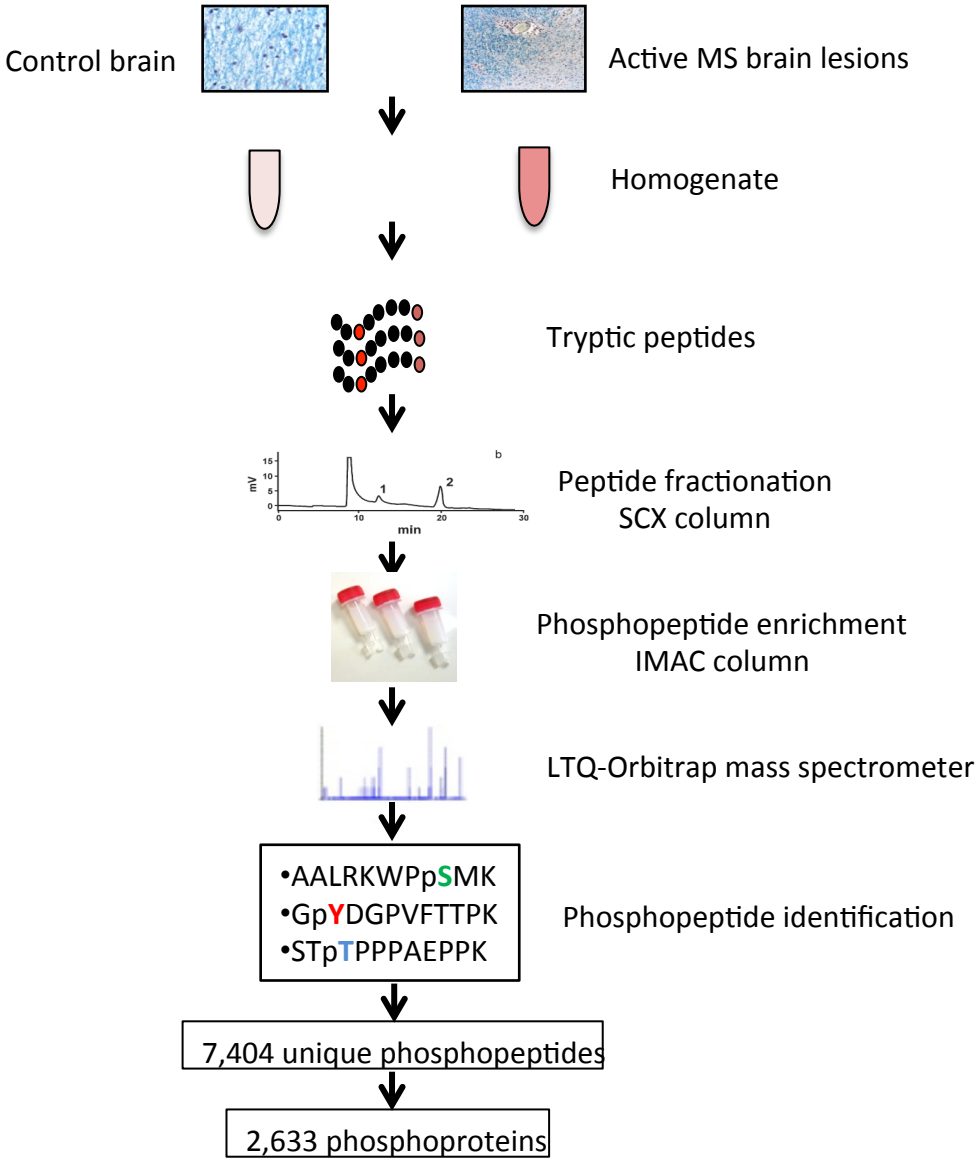
Supplementary Figure 1



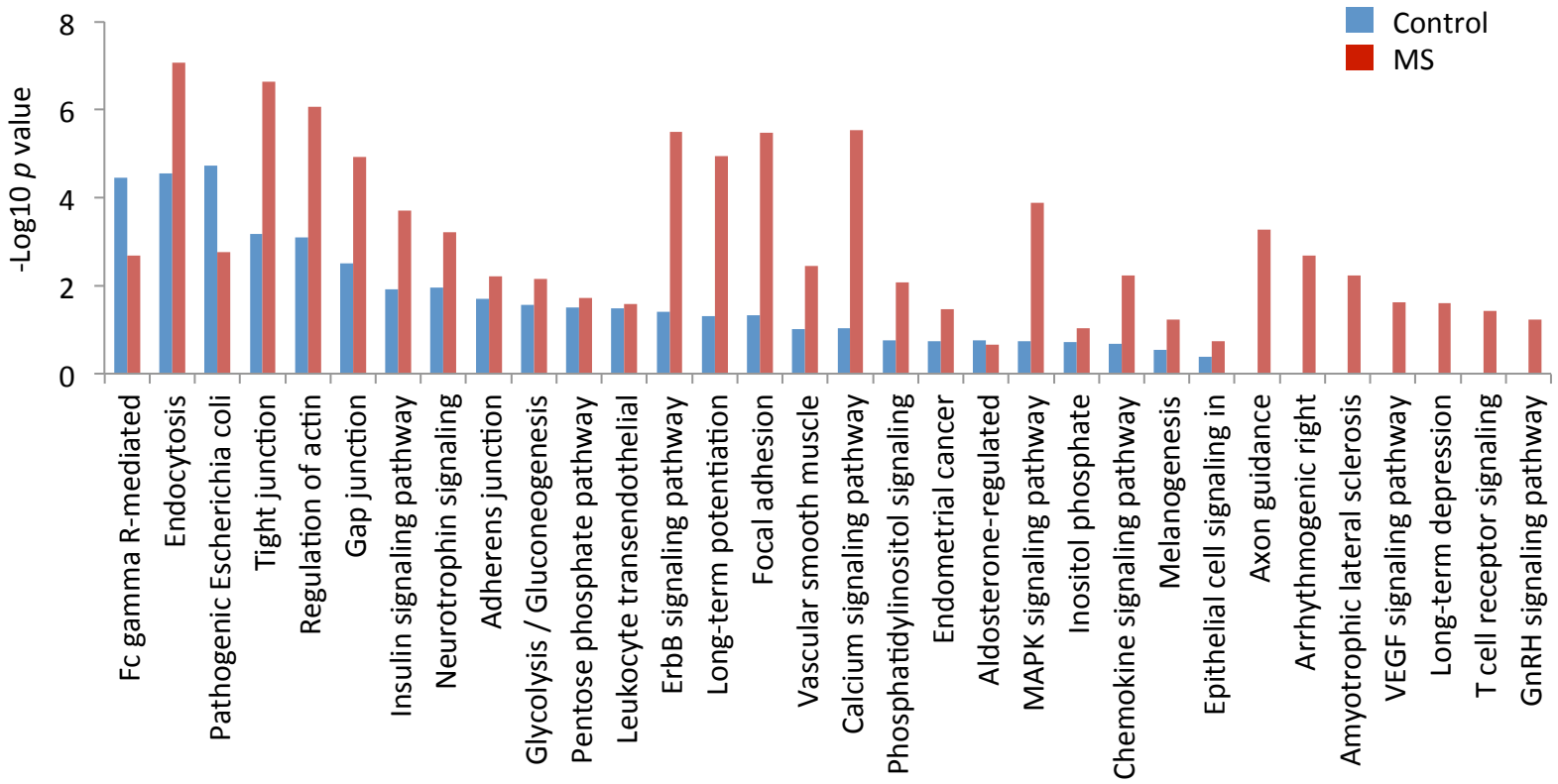
Histological characterization of MS brain lesions included in phosphoproteomic analysis. (**a-d**) Histopathological characteristics of active multiple sclerosis (MS) brain lesions. **a**) Luxol Fast Blue-Periodic acid Schiff (PAS) stain depicting myelin loss (myelin; blue). Demyelinated areas around the perivascular cuff (arrow) appeared as pale pink. **b)** Immunohistochemistry depicting $CD3^+$ cells (dark brown) in the perivascular cuff (black arrow) and brain parenchyma (red arrowhead). **c**) Bielschowsky preparation depicting axonal (dark brown staining, red arrowheads) loss especially around the perivascular cuff (black arrow). **d**) $CD68^+$macrophages and microglia in the perivascular cuff (black arrow) and brain parenchyma (red arrowhead). Scale bars = 100μm. (*n*=4 slides per tissue sample, total of 3 MS brain samples were analyzed).

Supplementary Figure 2



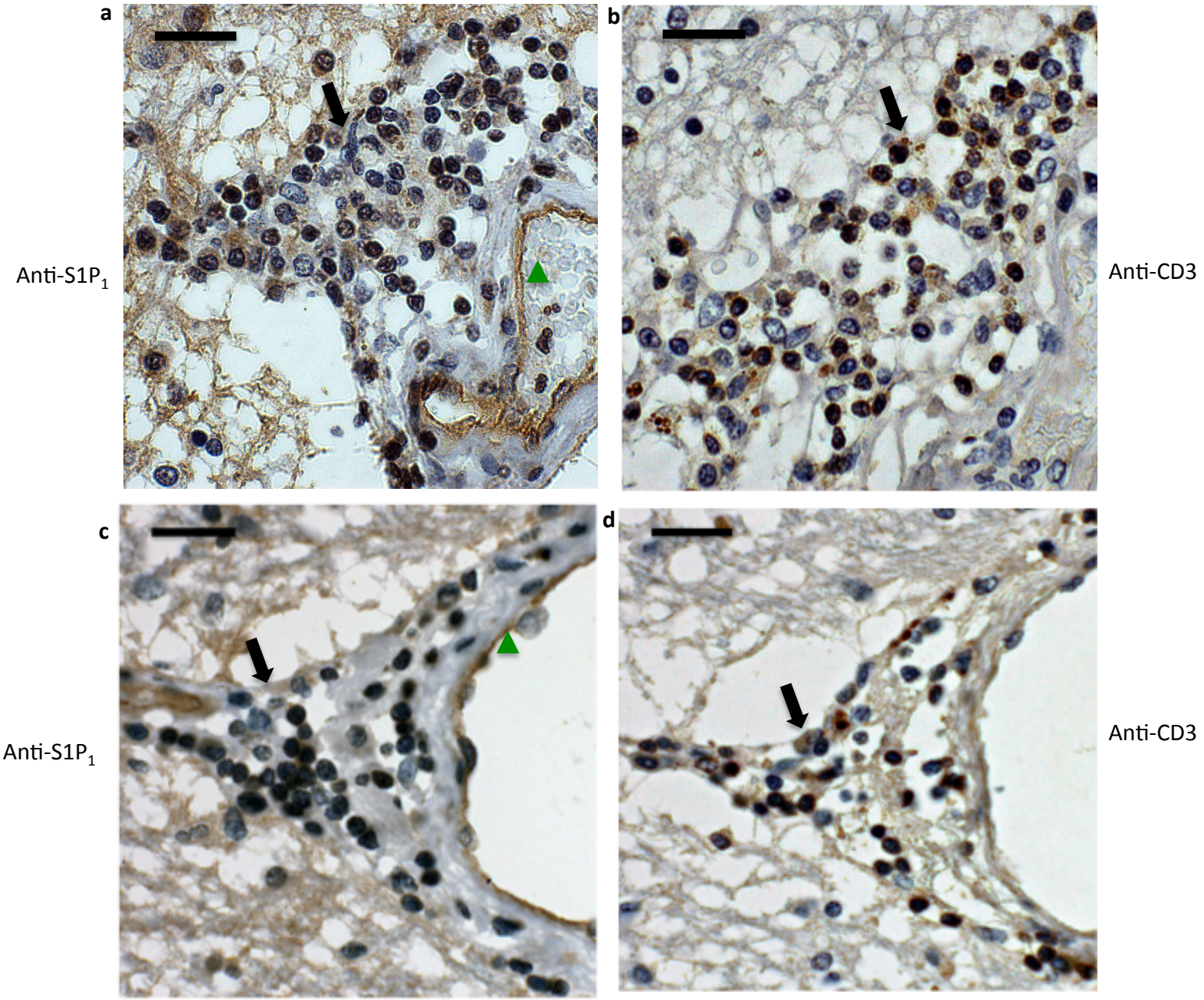
Schematic outline of phosphoproteomic study of multiple sclerosis (MS) brain lesion. Fresh-frozen MS and healthy control brain samples were characterized by histopathological analysis. Protein extracts from MS and control brain samples were digested with trypsin, phophopeptides were enriched, peptide pools were analyzed by mass spectrometry and phosphorylation sites were assigned. SCX; strong cation exchange, IMAC; immobilized metal affinity chromatography. Samples from 3 MS and 2 control subjects were analyzed. Data was pooled from two independent mass spectrometric analysis (each) of MS and control samples.

Supplementary Figure 3



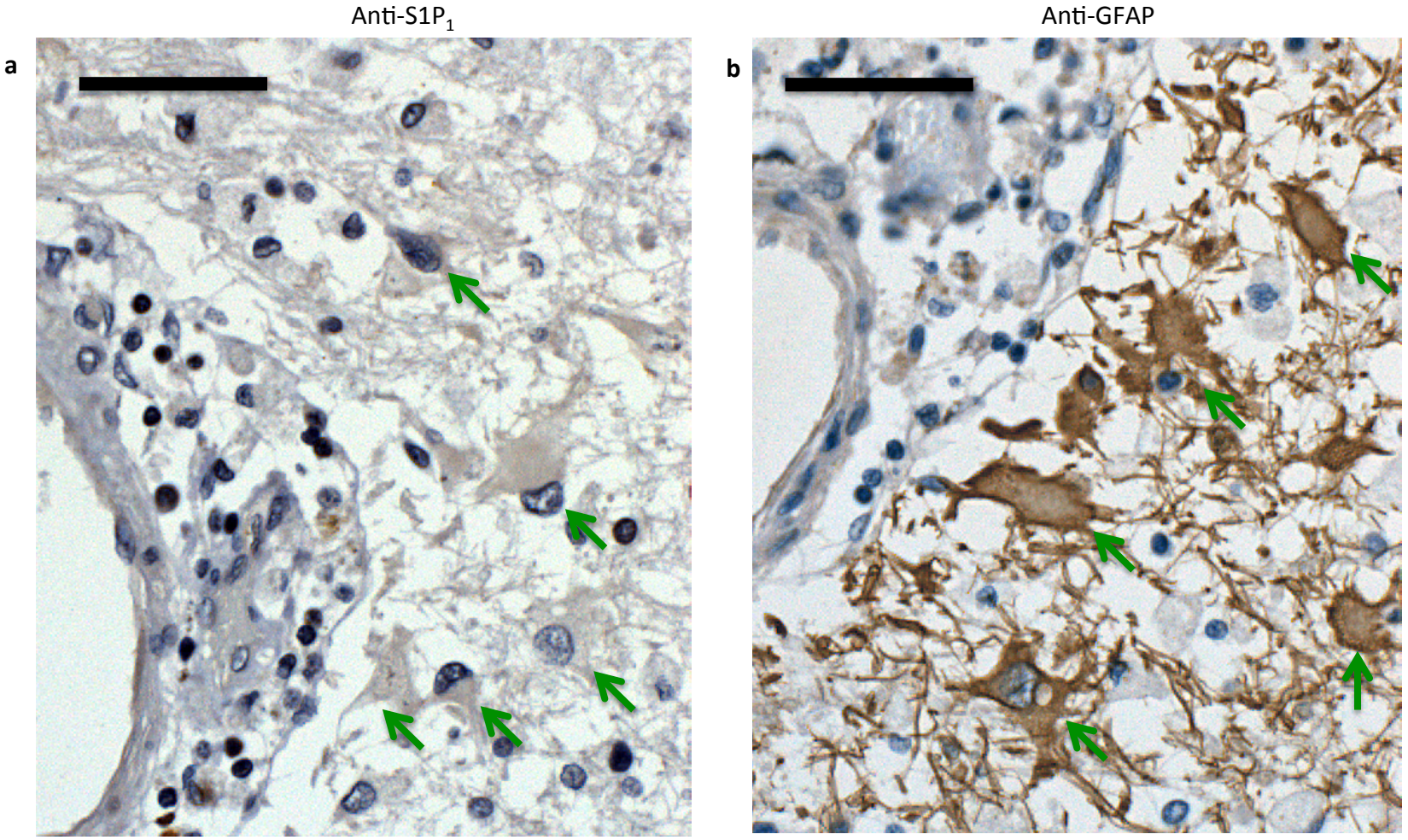
Multiple signaling pathways were differentially regulated in MS brain lesions. Quantification of the MS brain lesion phosphoproteome was performed based on the spectral counts of individual phosphorylation sites from the phosphopeptides originating from MS and control brain samples. Upregulation; MS> control, downregulation; control > MS. Data from this analysis was uploaded into the Database for Annotation, Visualization, Integrated Discovery (DAVID), respectively. Proteins from MS and control samples were then subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation enrichment analysis utilizing the total identified phosphoproteins as background and significance was determined by the –Log10 of the Benjamini *p* values.

Supplementary Figure 4



S1P$_1$ was expressed in T lymphocytes. **a, c**) Immunohistochemistry of formalin-fixed, paraffin-embedded sections from active MS brain lesions using an antibody against S1P$_1$. Immune cells within the perivascular cuff (black arrow) and endothelial cells (green arrow) were labeled for S1P$_1$. **b, d**) The same cells in a serial section were also labeled with anti-CD3. Scale bars = 50 μm. Three MS samples were analyzed.

Supplementary Figure 5



S1P$_1$ was also expressed in astrocytes. Immunohistochemistry of formalin-fixed, paraffin-embedded sections from an active MS brain lesion utilizing antibodies against S1PR$_1$ (**a**) and glial fibrillary acidic protein (GFAP) (**b**). Astrocytes were labeled with both anti- S1P$_1$ (**a**) and anti-GFAP (**b**) (green arrow heads). Scale bar = 50 μm. Three MS brain samples were analyzed.

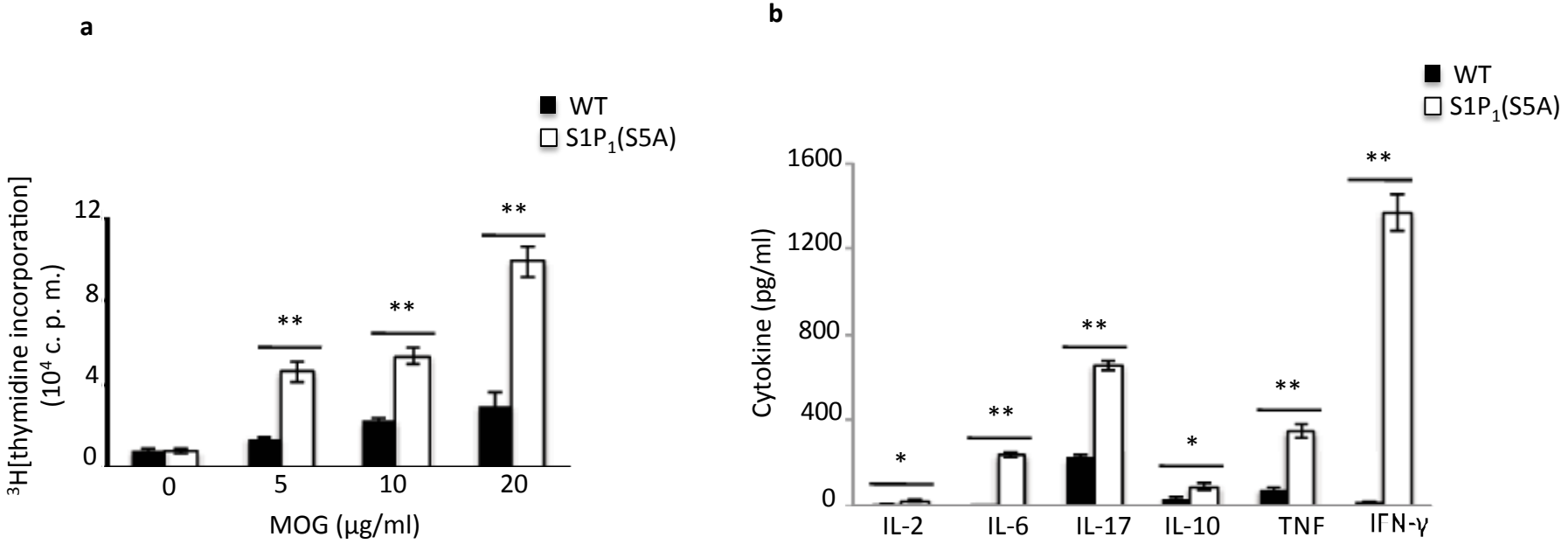
	$S1P_1$(S5A)	WT (C57BL/6J)	$p<0.05$*
Incidence	100% (9/9)	90% (9/10)	n.s.
Time after immunization (d)	10.7 ± 0.36	13 ± 0.62	0.007*
Maximum score	4	3.3 ± 0.4	n.s.
Score at peak EAE	3.67 ± 0.33	3 ± 0.44	n.s.
C.D.I.	78.3 ± 6.63	50.5 ± 6.24	0.006*

Supplementary Table 2. $S1P_1$(S5A) mice developed more severe EAE. EAE clinical parameters of $S1P_1$(S5A) and wild-type (WT) C57BL/6J mice following immunization with MOG_{35-55} peptide. *$p<0.05$, Mann-Whitney *U*-test. Experiment repeated three times, n=9-10/arms. d= days, C.D.I.=Cumulative disease index.

	$S1P_1$(S5A) recipients	WT (C57BL/6J) recipients	$p<0.05$*
Incidence	100% (5/5)	100% (5/5)	n.s.
Time after transfer (d)	8 ± 0.55	10.2 ± 0.58	0.02*
Maximum Score	4	3	0.01*
Score at Peak EAE	3.8 ± 0.2	3	0.047*
C.D.I.	54.8 ± 1.65	32.2 ± 2.46	6.15×10^{-5}*

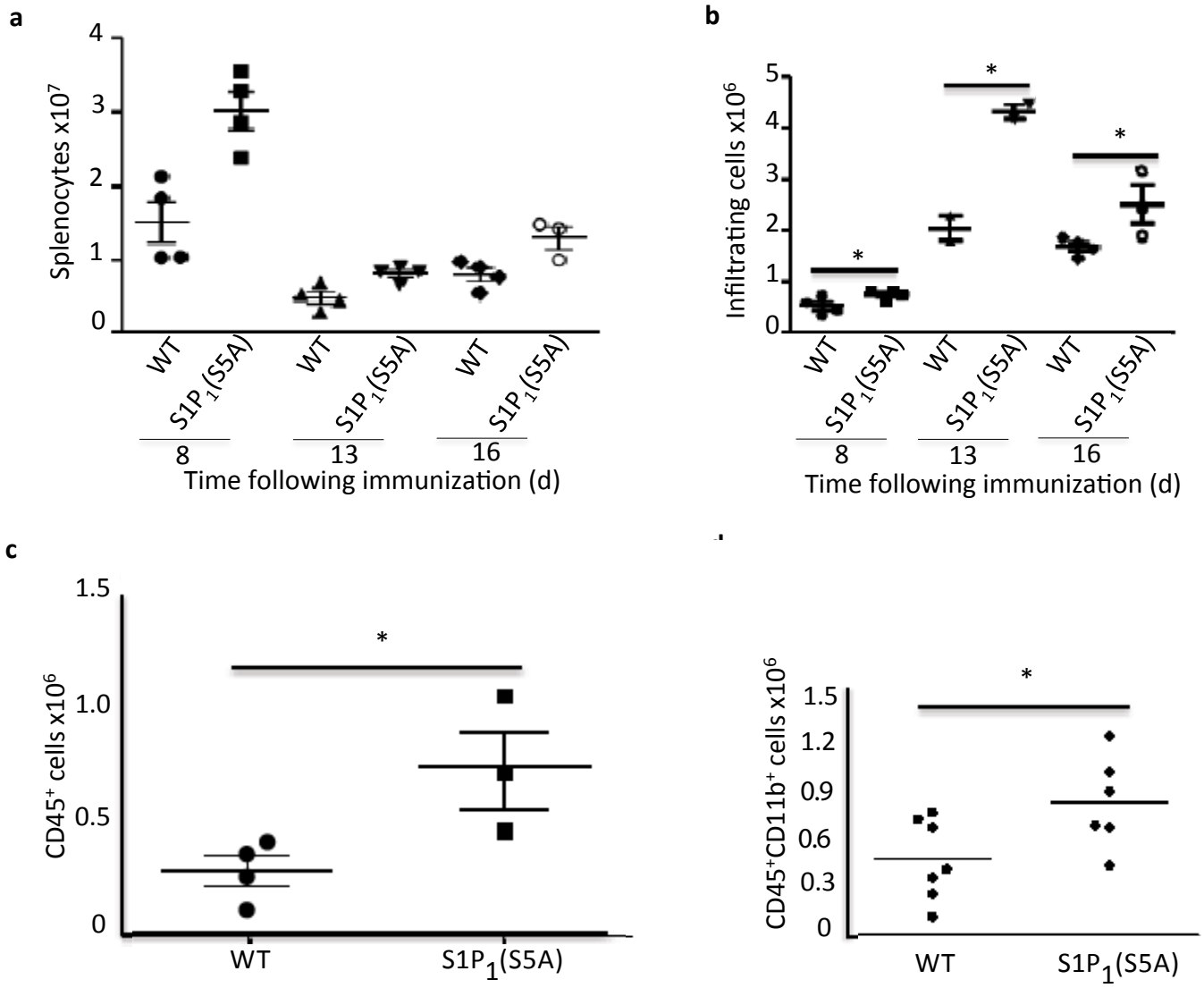
Supplementary Table 3. $S1P_1$(S5A) recipient *Rag1*$^{-/-}$ mice developed more severe EAE. Clinical parameters of adoptive transfer from MOG_{35-55} primed $S1P_1$(S5A) and WT (C57BL/6J) donor (females, 8-9 weeks old) enccphalitogenic cells into *Rag1*$^{-/-}$ (females, 5-6 weeks old) recipients. *$p<0.05$, Mann-Whitney *U*-test. Experiment repeated twice, n=9-10/arms (donors) and 5/arm (recipients). d= days, C.D.I.=Cumulative disease index.

Supplementary Figure 6



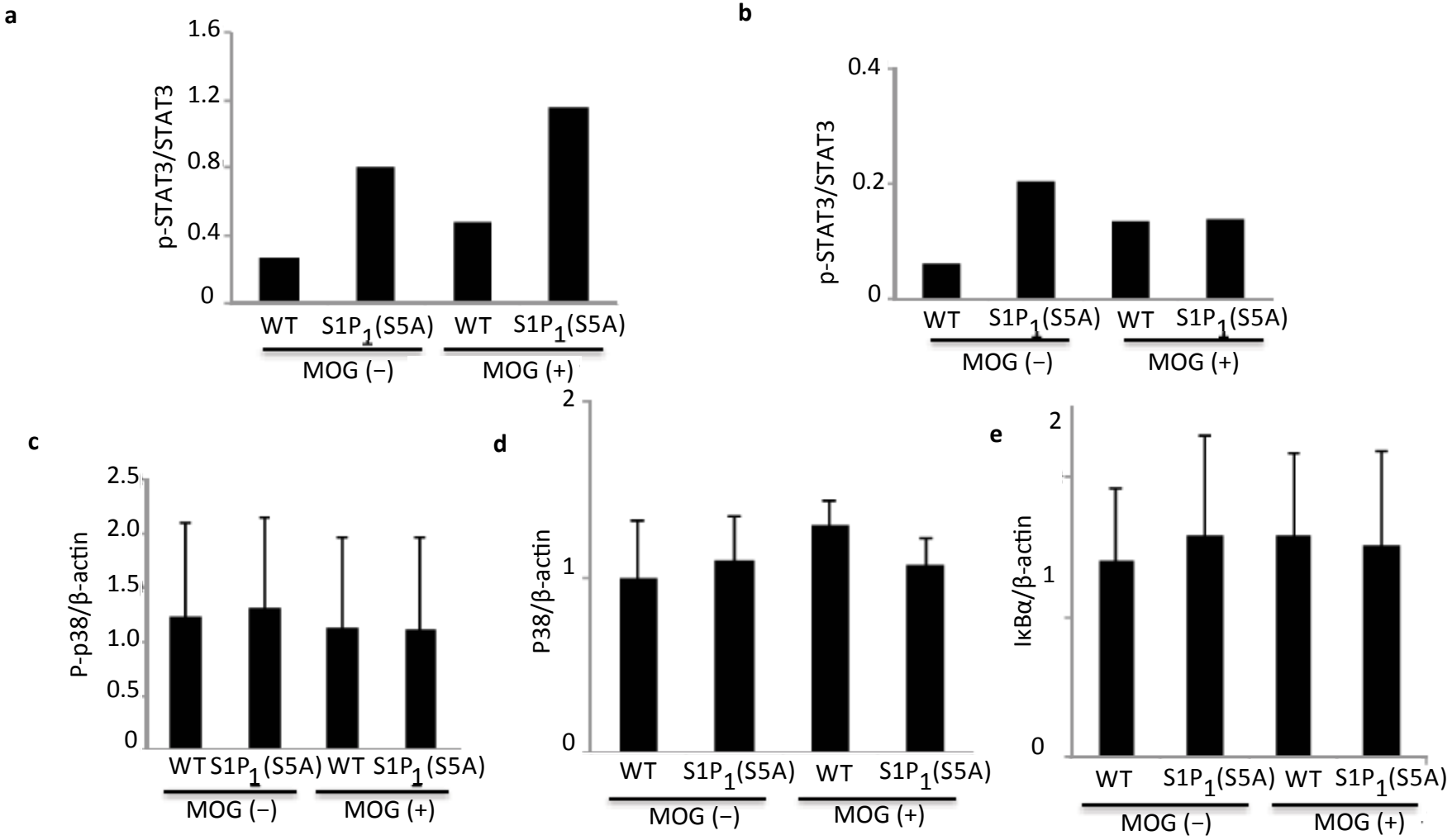
Lymph node cells of S1P$_1$(S5A) EAE mice demonstrated a severe inflammatory response. Immune cell proliferation (measured by ^{3}H[thymidine] incorporation) (**a**) and cytokine expression (measured by ELISA) (**b**) of *ex vivo* recall assay from MOG$_{35-55}$-immunized S1P$_1$(S5A) and wild-type (WT) EAE lymph node cells (day 8 post-immunization). MOG; myelin oligodendrocyte glycoprotein, c.p.m.; counts per minute. *p<0.05, **p<0.01, Student's t-test. n=3-5/arm. These experiments were repeated 3 times.

Supplementary Figure 7



Splenocyte and brain infiltrating immune cell counts in WT and S1P$_1$(S5A) mice during EAE disease course. Splenocytes (**a**) and brain infiltrating immune cells (**b-d**) were harvested from WT (C57BL6/J) and S1P$_1$(S5A) EAE mice on days 8, 13 and 16 following immunization with MOG$_{35-55}$ peptide. Cell counts were quantified by hemocytometer **(a, b)** and flow cytometry (**c, d**). Dots indicate cell numbers from each mouse. Brain infiltrating immune cells were labeled with antibodies against CD45 (**c**) and CD45 and CD11b (**d**) for cells of myeloid lineage, and analyzed by flow cytometry. Each experiment was performed three times with n=3-5 mice / arm. *p<0.05 by Student's t-test.

Supplementary Figure 8



High expression of p-STAT3 in S1P$_1$(S5A) EAE mice. Splenocytes from MOG$_{35-55}$-immunized WT(C57BL6/J) and S1P$_1$(S5A) EAE mice (Day 8 post-immunization) were activated with MOG$_{35-55}$ peptide *in vitro*, protein extracts were analyzed by immunoblot analysis utilizing antibodies against p-STAT3 and STAT3 (**a** and **b**), p-p38MAPK (**c**), p38MAPK (**d**) and IκBα (**e**). Chemiluminescence bands corresponding to the respective proteins were quantified by densitometry and normalized with β-actin. (**a**, **b**) demonstrate two independent experiments. (**c-e**) cumulative data from three independent experiments. Error bars = mean ± S. D. STAT3; signal transducer and activator of transcription, MAPK; mitogen activated kinase, IκBα; NFκB inhibitor α.