**Supplementary information**

# IL-23p19 and CD5 antigen-like form a possible novel heterodimeric cytokine and contribute to experimental autoimmune encephalomyelitis development

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## Supplementary Methods

**Human naive CD4$^+$ T cell preparation.** Fresh human peripheral blood was collected from healthy volunteers and mononuclear cells were immediately purified by using Lympholyte-H (Cedarlane) density gradient centrifugation. Naive CD4$^+$ T cells were further purified from peripheral blood mononuclear cells by negative selection using the naive CD4$^+$ T cell isolation kit and AutoMACS Pro (Miltenyi Biotec). The purity was analyzed by flow cytometry and routinely more than 95%. This study was approved by the institutional review board of Tokyo Medical University (no. 3339). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

**Th differentiation assay.** Human naive CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 using Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) under Th1-polarizing conditions with human IL-12 (kindly provided from Dr. Gately, 10 ng/ml) and anti-human IL-4 (MP4-25D2, BioLegend, 10 μg/ml), Th2-polarizing conditions with human IL-4 (BioLegend, 20 ng/ml) and anti-human IFN-γ (AF-285-NA, R&D, 5 μg/ml), and Th17-polarizing conditions with human IL-1β (Peprotech, 10 ng/ml), human IL-6 (BioLegend, 20 ng/ml), human IL-23 (eBioscience, 10 ng/ml), anti-human IFN-γ, anti-human IL-4, and Th conditions without any addition of cytokine and antibody.

**Flow cytometry.** For intracellular human cytokine staining, single-cell suspensions were restimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 5 μg/ml brefeldin A. Cells were stained with FICT-conjugated anti-human CD4 (OKT4, BioLegend), fixed with Fixation Buffer (eBioscience) for 15 min, and permeabilized with Permeabilization Buffer (eBioscience) for 30 min. These cells were then stained intracellularly with PE-conjugated anti-human p19 (727753, R&D), APC-conjugated anti-human CD5L (CD5L/2951, Novusbio), Pacific blue-conjugated anti-human GM-

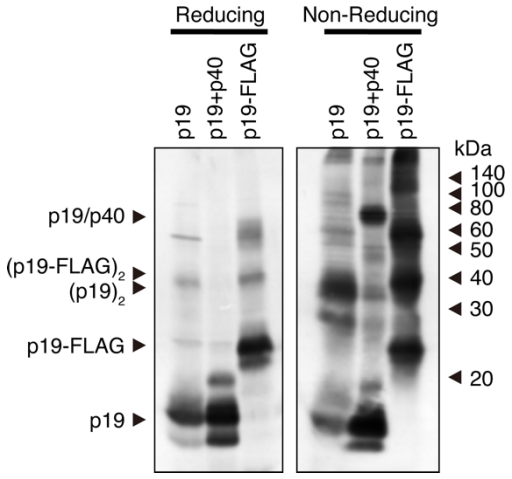
CSF (BVD2-21C11, BioLegend), and PE-Cy7-conjugated anti-human IL-17A (eBio64DE17, eBioscience).

**Supplementary Table 1.** Comparison of EAE susceptibility between control $p19^{flox/flox}$ mice and $CD4^+$ T-cell-specific conditional p19-deficient (CD4-Cre/$p19^{flox/flox}$) mice.

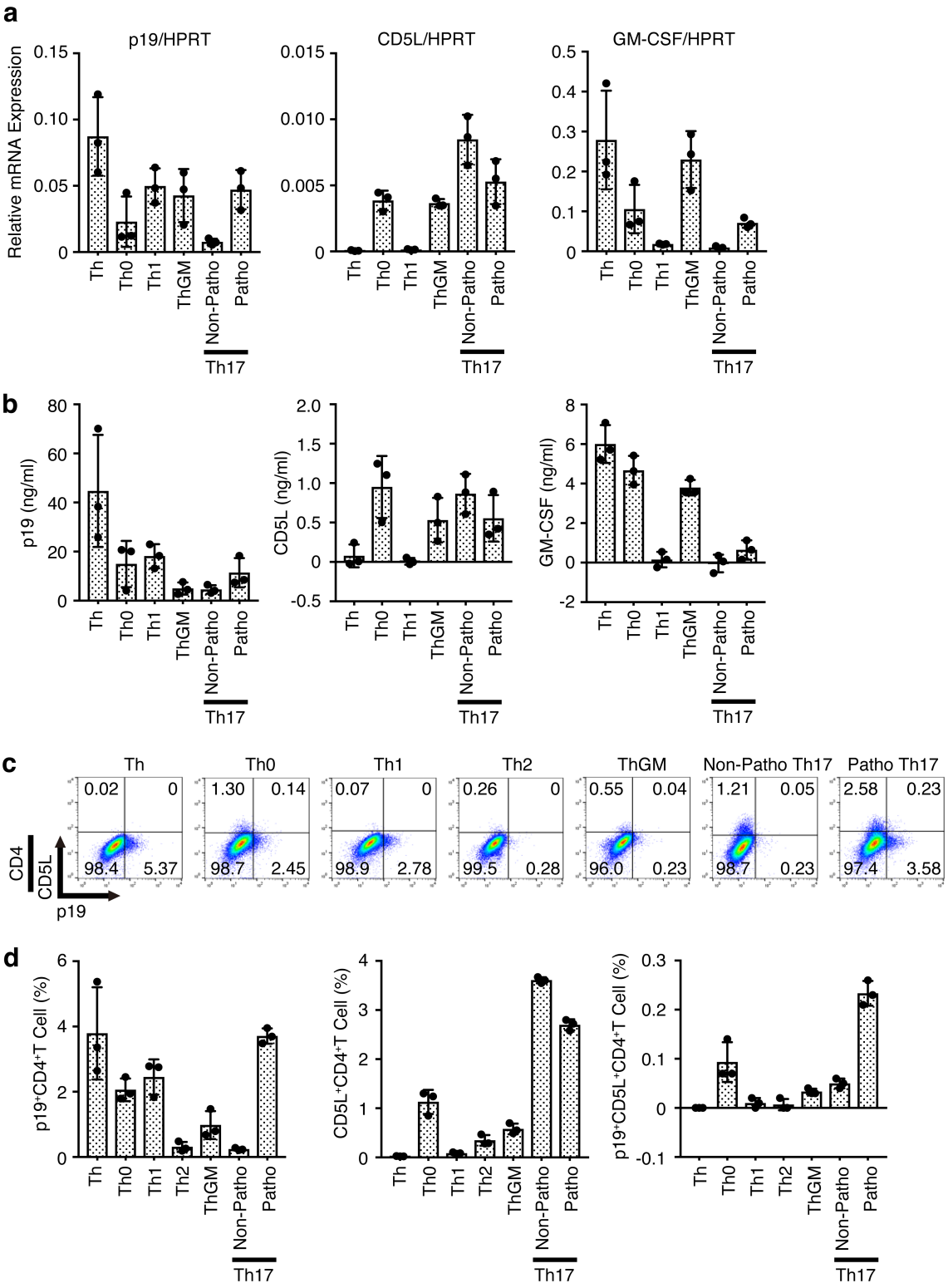
| Mice | Incidence Rate | Day of Onset | Maximum Score |
|---|---|---|---|
| $p19^{flox/flox}$ | 7 / 7 | 15.3 ± 3.5 | 3.0 ± 0.9 |
| CD4-Cre/$p19^{flox/flox}$ | 6 / 7 | 18.6 ± 4.6 | 1.3 ± 0.7 |
| *P* value | | $P = 0.259$ | $P < 0.05$ |

**Supplementary Table 2.** Comparison of EAE susceptibility between control WT mice and CD5L-deficient mice.

| Mice | Incidence Rate | Day of Onset | Maximum Score |
|---|---|---|---|
| WT | 6 / 6 | 9.8 ± 2.3 | 3.2 ± 0.4 |
| CD5L-deficient | 5 / 6 | 14.6 ± 4.3 | 1.0 ± 0.6 |
| *P* value | | $P = 0.061$ | $P < 0.001$ |

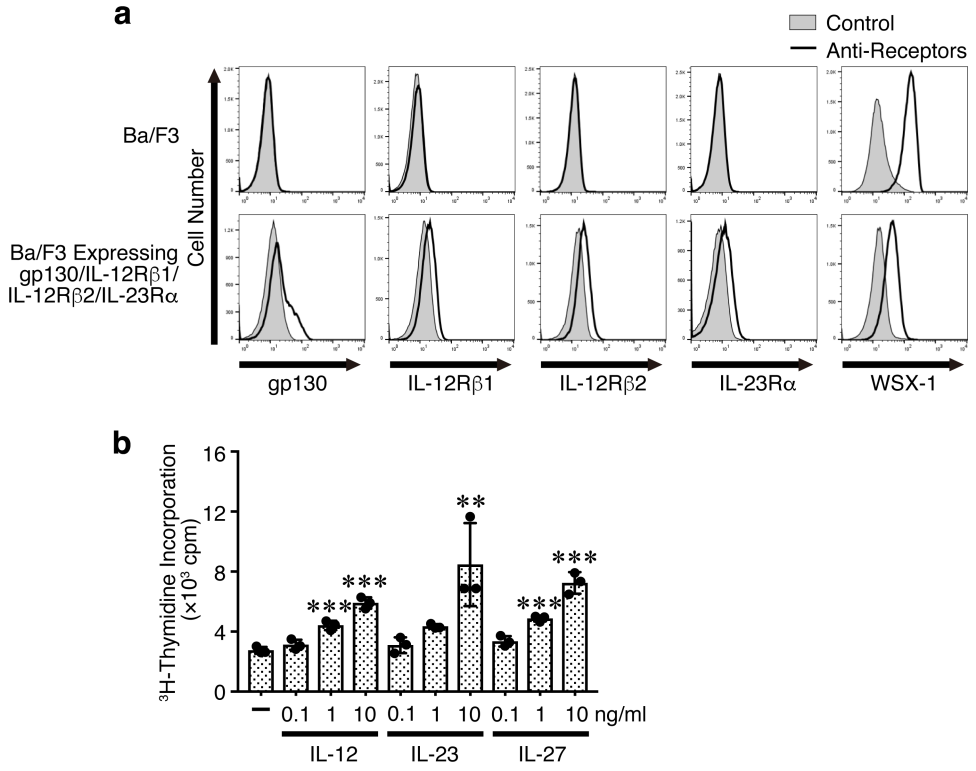



**Supplementary Figure 1.** Possible formation of a p19 homodimer in the culture supernatants of cells overexpressed with the p19 expression vector. HEK293T cells were transfected with expression vectors of p3×FLAG-CMV-14-p19 without a FLAG tag or p3×FLAG-CMV-14-p19 alone and cotransfected with expression vectors of p3×FLAG-CMV-14-p19 without a FLAG tag or p3×FLAG-CMV-14-p40 without a FLAG tag. After 3 days, the culture supernatants were subjected to western blotting using anti-p19 under reducing and nonreducing conditions. Data are representative of two independent experiments.

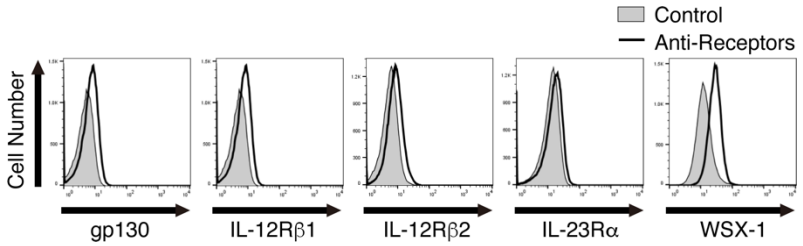



**Supplementary Figure 2.** Correlation of p19, CD5L, p19/CD5L, and GM-CSF under various Th-polarizing conditions. **a**, **b** Naive CD4$^+$ T cells from WT mice were stimulated with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 2 days under various Th-polarizing conditions; Th, Th0, Th1, ThGM, non-pathogenic Th17, and pathogenic

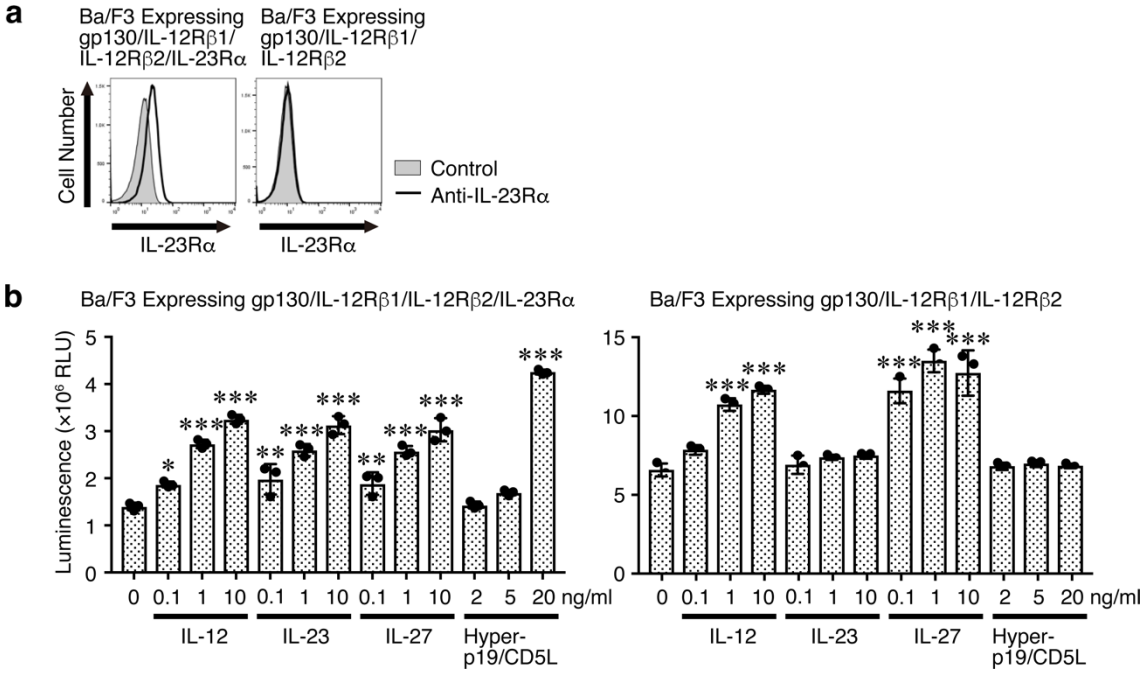
Th17. Total RNA was extracted and subjected to quantitative RT-PCR analysis (**a**). After 3 days later, culture supernatants were collected and analyzed for protein expression of p19 and CD5L by ELISA (**b**). Note that a recombinant single-chain IL-23 (10 ng/ml), which can be detected by p19-specific ELISA, was included in the culture medium under pathogenic Th17-polarizing conditions. **c**, **d** Naive $CD4^+$ T cells were also stimulated with plate-coated anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) under various Th-polarizing conditions for 2 days, then intracellularly stained for p19 and CD5L after restimulation with PMA and ionomycin. Representative dot plots were shown (**c**). Data are shown as the mean ± SD (n = 3) and are representative of at least three independent experiments.



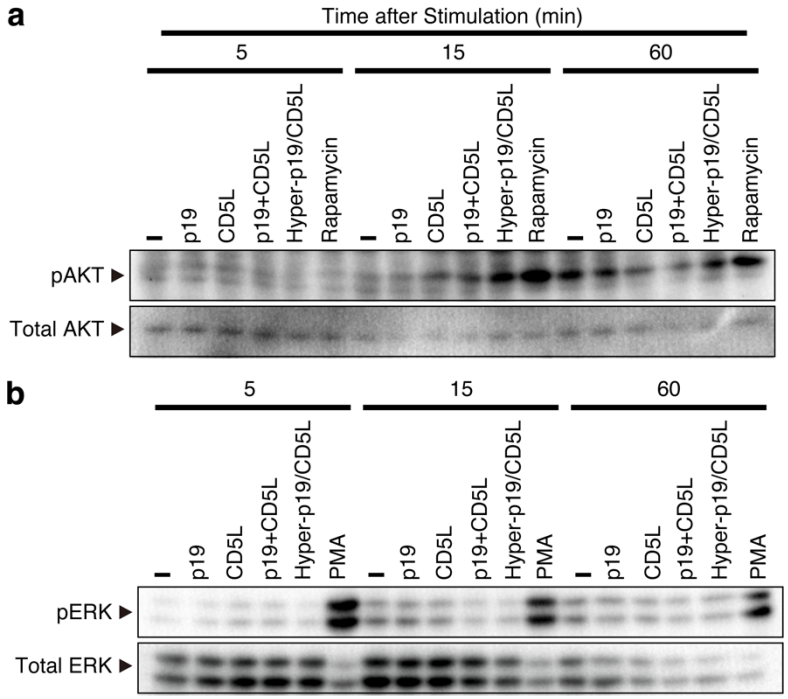

**Supplementary Figure 3.** Characterization of Ba/F3 cells expressing gp130/IL-12Rβ1/IL-12Rβ2/IL-23Rα. Cell surface expressions of gp130, IL-12Rβ1, IL-12Rβ2, IL-23Rα and WSX-1 on parental Ba/F3 and Ba/F3 cells transfected with expression vectors for gp130, IL-12Rβ1, IL-12Rβ2 and IL-23Rα were analyzed via FACS (**a**). Proliferative activity of these cells in response to IL-12, IL-23 and IL-27 was determined (**b**). Data are shown as the mean ± SD in triplicate and are representative of three independent experiments. *P* values were determined using one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$.



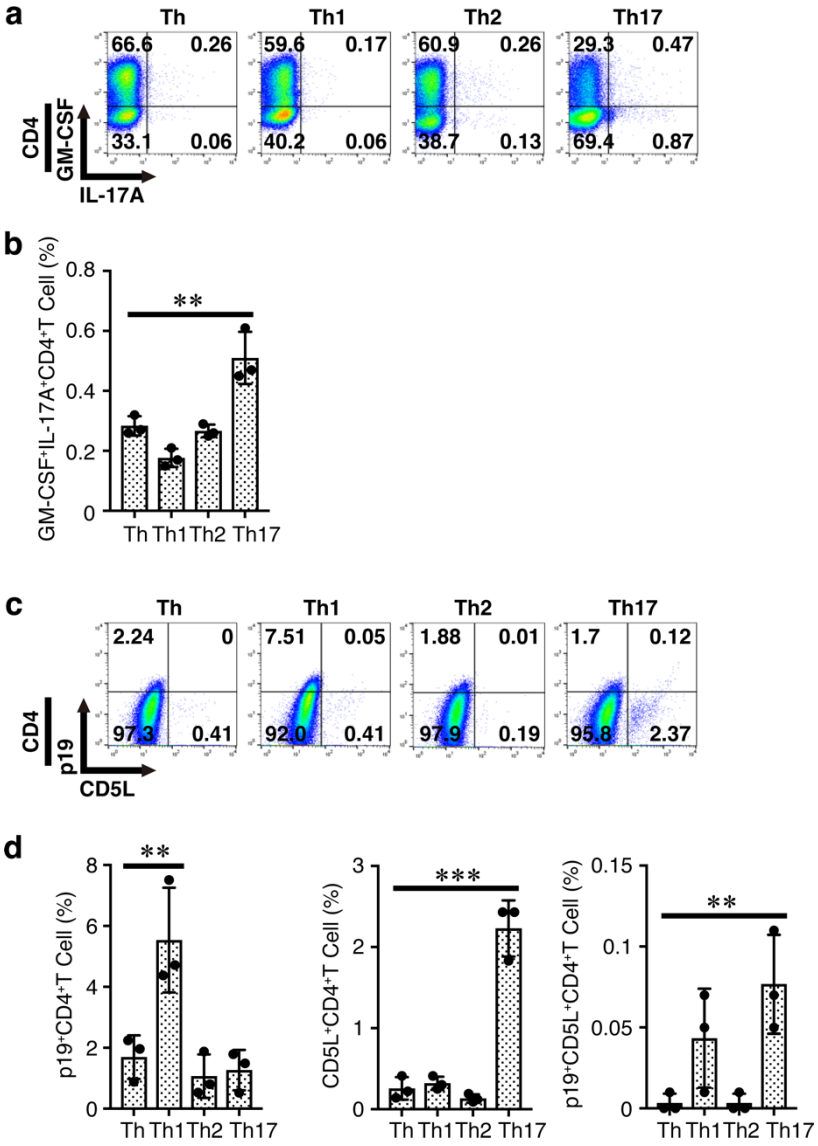

**Supplementary Figure 4.** Cell surface expression of activated $CD4^+$ T cells. Naive $CD4^+$ T cells from WT mice were stimulated with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) under Th conditions for 3 days, and their cell surface expressions of gp130, IL-12Rβ1, IL-12Rβ2, IL-23Rα and WSX-1 were analyzed via FACS. Data are representative of two independent experiments.



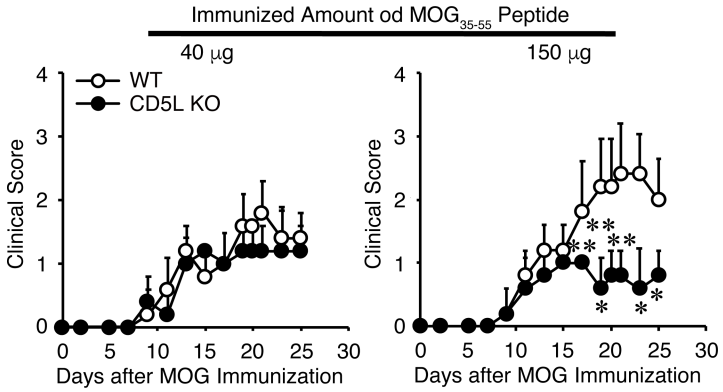

**Supplementary Figure 5.** IL-23Rα is possibly one of the receptor subunits for p19/CD5L.Ba/F3 cells ($1 \times 10^4$ cells/200 μl) expressing gp130/IL-12Rβ1/IL-12Rβ2/IL-23Rα and Ba/F3 cells ($3 \times 10^3$ cells/200 μl) expressing gp130/IL-12Rβ1/IL-12Rβ2 were analyzed for their cell surface expression of IL-23Rα (**a**) and their proliferative activities in response to IL-12, IL-23, IL-27, and hyper-p19/CD5L were determined after 3 days using CellTiter-Glo 2.0 Viability Assay kit (Promega) (**b**). Data are shown as the mean ± SD in triplicate and are representative of three independent experiments. *P* values were determined using one-way ANOVA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.



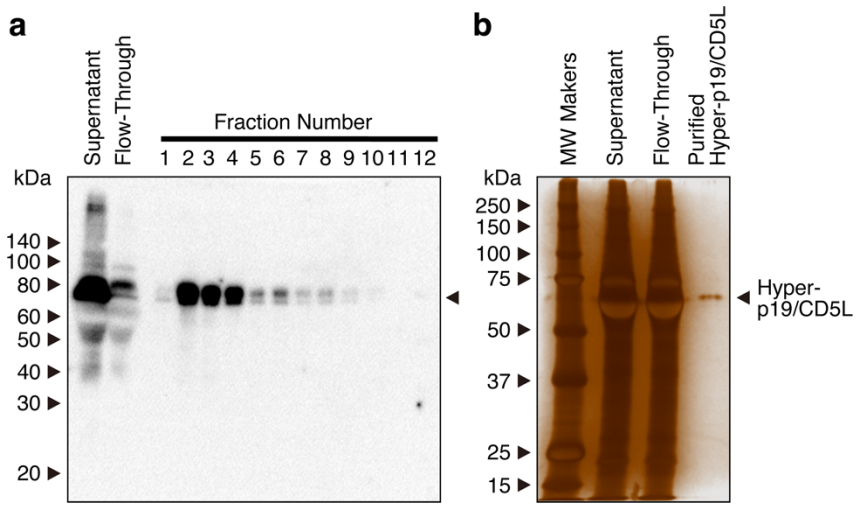

**Supplementary Figure 6.** Hyper-p19/CD5L induced phosphorylation of AKT but not ERK. Ba/F3 cells expressing gp130/IL-12Rβ1/IL-12Rβ2/IL-23Rα were unstimulated (−) or stimulated with purified recombinant p19, CD5L, a mixture of p19 and CD5L, hyper-p19/CD5L (all 20 ng/ml), and rapamycin (Sigma-Aldrich, positive control for AKT phosphorylation, 5 μM) (**a**) or PMA (positive control for ERK phosphorylation, 5 μM) (**b**) for 5, 15 and 60 min, then subjected to western blotting with anti-pAKT or anti-pERK and subsequently anti-total AKT or anti-total ERK. Data are representative of two independent experiments.



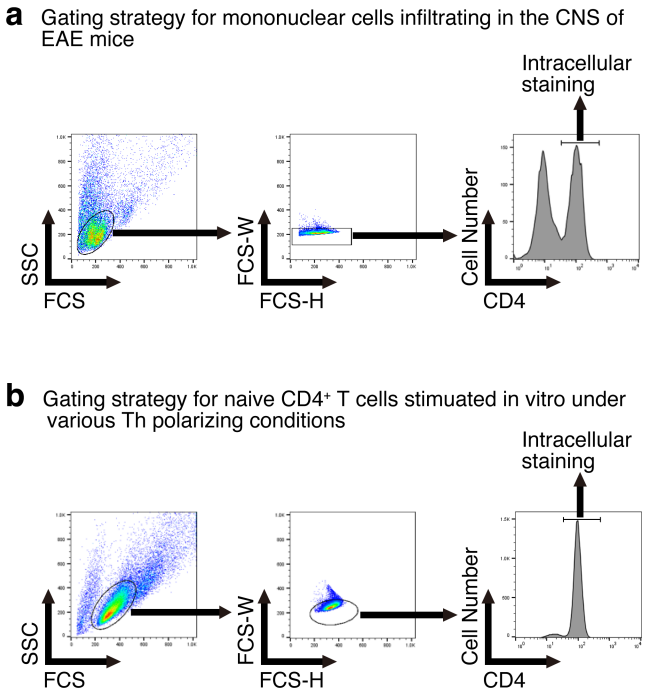

**Supplementary Figure 7.** Higher frequency of $p19^{+}CD5L^{+}CD4^{+}$ T cells in human $CD4^{+}$ T cells activated under pathogenic Th17-polarizing conditions and expressing higher frequency of $GM\text{-}CSF^{+}IL\text{-}17A^{+}CD4^{+}$ T cells. Human peripheral blood-derived naive $CD4^{+}$ T cells were stimulated under Th1-, Th2-, and Th17-polarizing conditions together with Th conditions for 6 days with IL-2, and then intracellularly stained for GM-CSF, IL-17A (**a**, **b**), p19, and CD5L (**c**, **d**) after restimulation with PMA and ionomycin. Data are shown as the mean ± SD in triplicate and are representative of two independent experiments. *P* values were determined using one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$.




**Supplementary Figure 8.** Effect of dose of $MOG_{35-55}$ peptide used for immunization on the susceptibility of WT and CD5L-deficient mice to develop EAE. WT mice or CD5L-deficient mice were immunized with the $MOG_{35-55}$ peptide 40 μg or 150 μg, and their clinical scores were monitored over time. Data are shown as the mean ± SD (n = 5) and representative of two independent experiments. *P* values were determined using unpaired two-tailed Student's *t*-tests. $^*P < 0.05$.




**Supplementary Figure 9.** Characterization of purification procedure by western blotting with anti-CD5L and silver staining of recombinant hyper-p19/CD5L protein. HEK293T cells were transiently transfected with the p3×FLAG-CMV-14-hyper-p19/CD5L expression vector. After 3 days, the culture supernatant was harvested, and hyper-p19/CD5L was purified by affinity chromatography with an anti-p19 affinity column. Each fraction eluted from the affinity column, the culture supernatant before applying the column, and the flow-through were subjected to western blotting analysis with anti-CD5L (Santa Cruz) (**a**). The purity was determined by silver staining following SDS-PAGE and estimated to be >95% via densitometrical analysis using Image Lab software (**b**).




**Supplementary Figure 10.** Gating strategy with flow cytometry. Gating strategy for mononuclear cells infiltrating in the CNS of EAE mice (**a**) and for naive $CD4^+$ T cells stimulated in vitro under various Th-polarizing conditions (**b**).