Supporting Information

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SI Materials and Methods

Generation of *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ Mice. Because *Nrp1* is essential for nervous system development, systemically deleting *Nrp1* is embryonically lethal (1). To subvert this constraint, we used a *Cre*-loxP conditional knockout system (1). To produce these conditional knockouts, we bred mice with loxP sites flanking exon 2 of the *Nrp1* gene to mice containing the gene for Cre recombinase under the control of the *CD4* promoter. The excision of exon 2 of *Nrp1* results in a frameshift mutation, abrogating *Nrp1* protein expression in any *Cre* expressing tissue (1). *Nrp1* should be absent from all T cells of the resulting *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice, because all thymocytes must pass through a CD4 expressing phase during development. *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice exhibit no intrinsic defect in fertility or health, and the lack of *Nrp1* expression on CD4^{+} T cells was confirmed by real-time PCR.

Retroviral Overexpression. Retroviral infections were carried out by using the Phoenix Retroviral Expression System (Orbigen) according to the manufacturer's instructions. Briefly, *Nrp1* cDNA was inserted into the pBMN-GFP multicloning site and transfected into Phoenix viral producer cells. To infect primary T cells, viral producer cells successfully transduced with either the pBMN-GFP-*Nrp1* or pBMN-GFP-empty vector were centrifuged with Ac1-11–specific CD4^{+}CD25^{-} T cells from MBP-TCR-Tg mice previously activated in vitro with Ac1-11. By sorting for GFP, 10^6 *Nrp1* retrovirally infected cells or GFP-empty vector containing cells were isolated and transferred into B10.PL-TCR$\alpha^{-/-}$ mice lacking endogenous T cells. In addition, 10^6 Ac1-11 activated, untransduced CD4^{+} cells were cotransferred into both groups of the same B10.PL-*TCR*$\alpha^{-/-}$ mice, as well as an additional group of recipient mice given no transduced cells to act as a negative control for the retroviral infection.

Epicutaeneous Immunization (ECi). The backs of mice were shaved with an electric razor 1 d before applying 1 mg of $MOG_{35–55}$ in PBS to gauze on an occlusive patch (Duo-DERM; Convatec) and affixing to the shaved area. PBS ECi mice were used as controls. The patch was left in place for 1 wk, and this process was repeated for a second week. At the end of the second week, the second patch was removed and the mice were immunized for EAE.

CD4^{+} T-Cell Isolation and T_H-Cell Polarization Harvested lymphocytes from spleens and lymph nodes of wild-type and *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice were incubated with a CD4 selection mixture (antibodies to: CD8 (TIB-105); IA^{b,d,v,p,q,r} (212.A1); FcR (2.4-G2); B220 (TIB-164); NK1.1 (HB191); all generated in our laboratory) and then incubated with magnetic beads [BioMag goat anti-mouse IgG, goat anti-mouse IgM, and goat anti-rat IgG (Qiagen)]. Unlabeled CD4^{+} cells were removed and cultured in Bruff's media under T_H skewing conditions. For differentiation into T_H-17 cells (without additional IL-23), IL-2 (25 U/mL, generated in our laboratory), IL-6 (20 ng/mL; eBioscience), TGF-β (1 ng/mL; R&D Systems), anti–IL-4 (10 μg/mL Clone 11B11; eBioscience), anti–IL-12 (10 μg/mL, Clone JES6-1A12; BD Biosciences), and anti–IFN-γ (10 μg/mL, Clone XMG 1.2; eBioscience) were added to the cell cultures. For assays requiring the presence of IL-23 during differentiation into T_H-17 cells (as noted in the text), naïve CD4^{+} T cells were cultured with IL-2 (25 U/mL), IL-6 (20 ng/mL), IL-23 (20 ng/mL; eBioscience), TGF-β (3 ng/mL), anti–IL-4 (10 μg/mL), anti–IL-12 (10 μg/mL), and anti–IFN-γ (10 μg/mL). In both conditions, cells were stimulated with immobilized mouse anti-CD3 (1 μg/mL, Clone 145–2C11; BD Biosciences) and soluble anti-mouse CD28 (1 μg/mL, Clone 37.51; BD Biosciences). At day 3 after stimulation, cells were expanded for an additional 4 d in fresh media containing 25 U/mL mouse IL-2. At day 7, cells were washed and restimulated with anti-mouse CD3/CD28 (1 μg/mL each) plus mouse IL-2 (25 U/mL) for 48 h. Cell culture supernatant was collected for ELISA, and differentiated T cells were collected for either proliferation or intracellular cytokine staining.

Suppressor Cell Isolation and in Vitro Suppression Assay CD4^{+} responder cells were primed in vivo by immunization of WT mice with a 1:1 CFA:MOG (3 mg/mL in PBS) emulsion (50 μg in both flanks of the mouse) on day 0 and day 5 and then isolated (as described above) on day 7. CD4^{+} responder cells from 2D2-Tg mice were also used in certain assays (2). For suppressor cells, CD4^{+} cells were first isolated (as described above) from naïve WT or *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ mice. Then, CD25^{+} cells were isolated by using a CD4^{+}CD25^{+} Regulatory T Cell Isolation Kit (Miltenyi) according to the manufacturer's instructions. Following the removal of the WT CD25^{+} cell population, the remaining WT CD25^{-} cells were incubated with a rabbit anti-mouse Nrp1 antibody (AbCam) for 20 min on ice, washed, then incubated with goat anti-rabbit IgG microbeads (Miltenyi) and selected for according to the manufacturer's instructions. The primed CD4^{+} responder cells (10^5) (primed in vivo with MOG or isolated from 2D2-Tg mice) were cultured with suppressor cells (WT CD4^{+}CD25^{-}Nrp1^{+}, WT CD4^{+}CD25^{+}, or *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ CD4^{+}CD25^{+}) and irradiated APCs (1:5 T-cell:APC) in the presence of 10 μg/mL MOG at ratios of 1:1, 1:2, 1:4, and 1:8 responder to suppressor cells. To assess the role of IL-10 and TGF-β in suppression by CD4^{+}CD25^{-}Nrp1^{+} cells, cultures were treated with either 10 μg/mL anti–IL-10 (eBioscience), 10 μg/mL sIL-10 receptor (R&D Systems), 10 μg/mL anti–TGF-β (eBioscience), or 10 μg/mL of the appropriate isotype control. Proliferation was measured by ^{3}H-thymidine incorporation.

Intracellular Staining. Intracellular staining was performed by first stimulating T cells for 4 h with PMA/ionomycin in the presence of brefeldin A. After staining of cell surface receptors, cells were fixed in 2% paraformaldehyde for 10 min at room temperature, washed in 0.05% saponin, stained with intracellular antibodies in a 0.5% saponin solution on ice for 30 min, and washed again in 0.05% saponin.

ELISA. ELISA experiments were performed by using OptEIA mouse IL-10 kit (BD Biosciences) following the manufacturer's protocol. ELISA plates were read at 450 nm with a Bio-TEK ELx800 (Bio-TEK). IL-17 ELISA data were generated by using a DuoSet ELISA Development System mouse IL-17 kit (R&D Systems).

RT-PCR. Total RNA was extracted from CD4^{+} cells by TRIzol fractionation according to the manufacturer's instructions. For *Nrp1*, cDNA was synthesized by using total RNA and random primers (hexamers) with Moloney murine leukemia virus reverse transcriptase (Life Technologies) as recommended by the manufacturer. Forward and reverse primers for *Nrp1* are described in ref. 1. The primer binding sites are identical between mice and humans. PCR products were then subjected to electrophoresis and visualized by SYBR Green staining, which was then analyzed by PhosphorImager and quantified by ImageQuant. Relative copy numbers of mRNA abundance were normalized against

GAPDH. For *RORγT*, cDNA was synthesized by using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. Real-time PCR was performed by using SYBR Green technology (SYBR FAST Master Mix KAPAbiosystems) on a CFX96 Real Time System Thermal Cycler (Bio-Rad). The expression level of *RORγT* (forward-TGT CCT GGG CTA CCC TAC TG, reverse-GTG CAG GAG TAG GCC ACA TT) was normalized to the internal control gene GAPDH (forward-CCC CAA TGT GTC CGT CGT G, reverse-GCC TGC TTC ACC ACC TTC T). The cycling conditions were as follows: Enzyme activation 95 °C for 3 min followed by 40 cycles of denaturation 95 °C for 3 s, annealing 60 °C for 30 s, elongation 72 °C for 5 s.

1. Gu C, et al. (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 5:45–57.

2. Bettelli E, et al. (2003) Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197: 1073–1081.

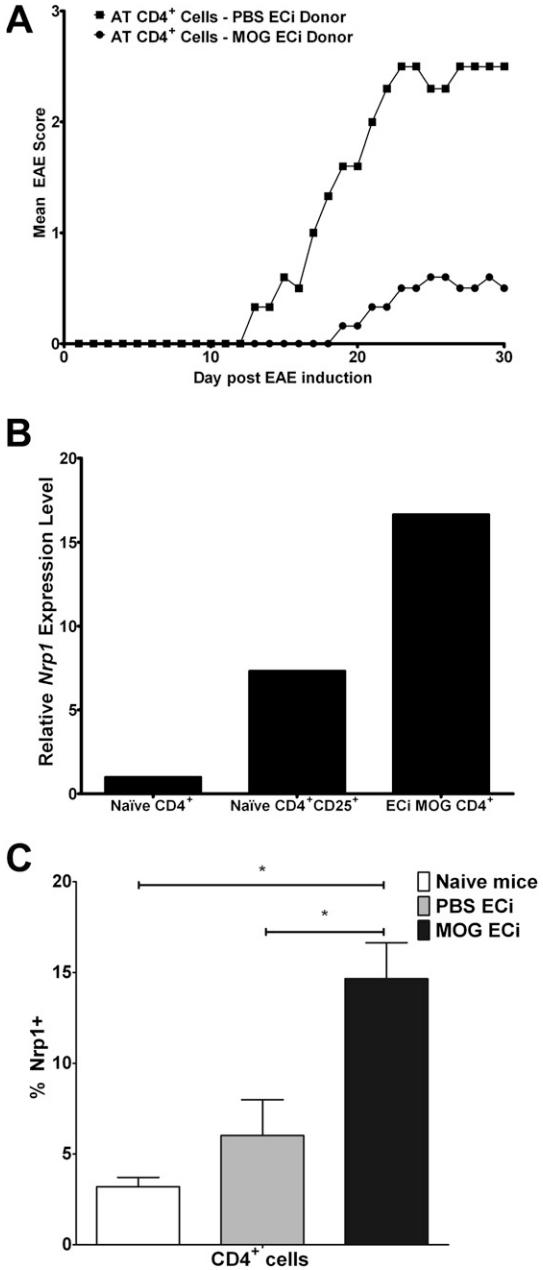

Fig. S1. *Nrp1* expression is protective against EAE. (*A*) C57BL/6 mice were ECi with MOG peptide or PBS for two consecutive weeks before EAE induction. On day 30 after EAE induction, $CD4^+CD25^-$ T cells were sorted from these mice and adoptively transferred (i.v.) into naive C57BL/6 recipients at 2×10^6 cells per mouse ($n = 3$), concomitant with EAE induction. EAE progression was followed for 30 d. Results from one experiment are displayed as mean (± SEM) EAE score. (*B*) WT mice ($n = 50$) were ECi with MOG. On day 7, RNA was extracted from purified $CD4^+$ T cells of ECi mice, as well as from total naïve $CD4^+$ T cells and $CD4^+CD25^+$ T cells. cDNA transcripts were analyzed for *Nrp1* expression by RT-PCR and normalized to GAPDH. Data from one experiment is shown as fold induction relative to *Nrp1* expression levels in total naïve $CD4^+$ cells. (*C*) Wild-type mice ($n = 3$) were ECi for 3 wk with either 1 mg of MOG or PBS. Lymphocytes were isolated from naïve (white bar), PBS ECi (gray bar), or MOG ECi (black bar) mice and analyzed by flow cytometry for CD4 and *Nrp1* expression. Results are displayed as mean (±SEM) % of $CD4^+$ cells, which are $Nrp1^+$. *P* values are calculated by using Student's *t* test (*$P < 0.05$). Data are representative of four experiments.

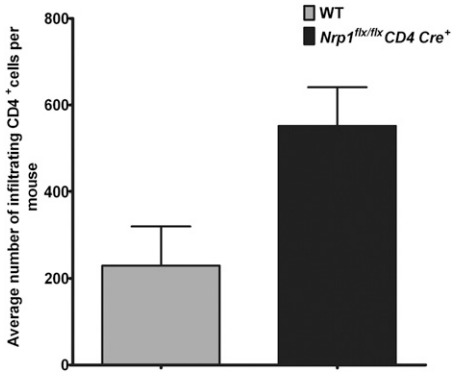

Fig. S2. *Nrp1flx/flx* mice exhibit more immune cell infiltrates in the central nervous system than WT mice. Brains of mice were isolated on day 30 after EAE induction, cryopreserved, sectioned, and stained with a monoclonal antibody to CD4. Sections from four WT or *Nrp1flx/flx* mice were visualized microscopically, and $CD4^+$ T cells (stained red) were counted 15 fields per slide encompassing areas of the cerebellum and the hippocampus. Anatomically similar fields per brain per mouse were analyzed at 10× magnification (n = 4 mice). Results are presented as mean (± SEM) number of $CD4^+$ infiltrating cells per mouse.

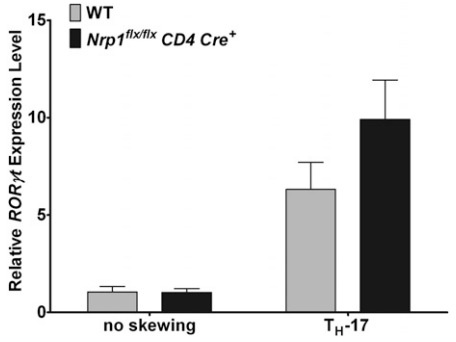

Fig. S3. *RORγT* expression in T_H-17–skewed cultures. $CD4^+$ T cells from WT (gray bars) and *Nrp1*$^{flx/flx}$*CD4 Cre*$^+$ (black bars) mice (n = 5 mice each) were purified and cultured under neutral or T_H-17 polarizing conditions. Both groups were stimulated with anti-CD3 and anti-CD28 and harvested on day 9 for RNA extraction, cDNA synthesis, and real-time PCR by using primers specific for *RORγT* in combination with SYBR Green. Results are depicted as expression normalized to GAPDH and presented as mean (± SEM) fold change relative to unskewed cells. Data are representative of two separate experiments.

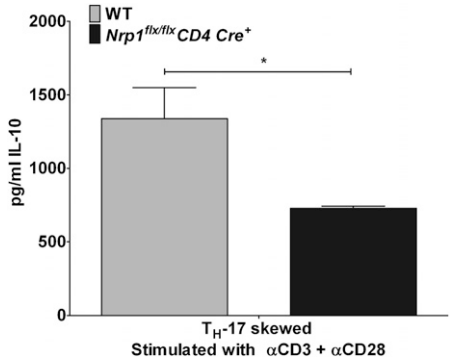

Fig. S4. *Nrp1*-deficient $CD4^+$ T cells display a decreased production of IL-10 compared with WT. $CD4^+$ T cells from naïve wild-type (gray bar) and *Nrp1*$^{flx/flx}$*CD4 Cre*$^+$ (black bar) mice (n = 5 each) were harvested, stimulated with anti-CD3/anti-CD28, and skewed toward T_H-17. Cultures were refreshed with cytokines on day 3 and restimulated on day 7. Restimulated cells were harvested on day 9 for ELISA analysis. Results are displayed as mean (±SEM) IL-10 pg/mL produced by *Nrp1*$^{flx/flx}$*CD4 Cre*$^+$ $CD4^+$ cells (black bars) compared with WT (gray bars) under T_H-17 skewing conditions. Data are representative of five experiments. P values are calculated by using the Student's t test (*$P < 0.05$).

Table S1. Mice that received *Nrp1*$^{+}$ transduced CD4^{+} T cells are protected from EAE

CD4 T cells transferred	Incidence, %	Mean day of onset	Maximum mean EAE score
Activated CD4^{+} T cells	100 (3/3)	10	5.0 ± 0.0
GFP^{+} vector only	100 (3/3)	8	5.0 ± 0.0
Nrp1^{+}GFP^{+} cells	100 (3/3)	10	1.3 ± 0.2

See Fig. 1*B*. CD4^{+}CD25^{-} T cells were isolated from MBP-TCR-Tg mice and then stimulated with 5 μg of Ac1-11 and then transduced with a retroviral *GFP* construct containing *Nrp1* (n = 3) or an empty vector (n = 3). Retrovirally transduced cells (10^6) were adoptively transferred into B10.PI *TCR*$\alpha^{-/-}$ recipient mice concomitant with 10^6 (untransduced) Ac1-11–activated CD4^{+}CD25^{-} cells. Untransduced cells served as a control (n = 3). Results from one experiment are expressed as mean EAE score (±SEM).

Table S2. EAE incidence in *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice compared with WT

Mouse strain	Day of earliest onset of EAE	Mean day of onset of EAE	Maximum EAE score	Maximum mean EAE scores	No. of mice with EAE
WT	10	12.75 ± 0.95	3.5	2.1	4/4
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$	8	9.25 ± 0.48	5	4.4	4/4

See Fig. 1*C*. EAE was induced in *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ (n = 7) and WT mice (n = 4). Representative (1 of 4) results are expressed as mean EAE score (±SEM).

Table S3. CD4^{+} T cells from *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ and WT mice transferred into *TCR*-$\alpha^{-/-}$ recipients and immunized for EAE

CD4 T cells transferred (n)	Earliest day of EAE onset	Maximum EAE score	Mean maximum EAE scores	Incidence
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ (10×10^6)	9	3	2.6	4/4
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ (5×10^6)	9	3	1.9	4/4
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ (10^6)	12	3	1.7	3/3
WT (10^6)	12	1.5	0.4	2/4
WT (10×10^6)	14	1.5	1.4	4/4

See Fig. 1*D*. CD4^{+} cells from *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ (n = 20) and WT mice (n = 5) primed with MOG were isolated and transferred into C57BL/6-*TCR*$\alpha^{-/-}$ recipient mice (n = 4 mice each) followed by immunization with MOG to induce EAE. Results from one experiment are displayed as mean (±SEM) EAE score.

Table S4. Anti–T$_H$-17 treatment of wild-type and *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice concomitant with EAE induction

Mice with or without anti T$_H$-17 antibody treatment	Avg. time of recovery*	Day of EAE onset†	No. of mice with EAE	Maximum mean EAE score	Maximum EAE score
Wild type plus treatment	23	18	4/5	1	2.0
Wild type no treatment	23	12	4/5	1.5	2.5
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ plus treatment	24	15	5/5	1.2	1.5
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ no treatment	30	11	5/5	2	3.0

*First sign of steady decline in clinical signs within a group.

†First clinical signs of EAE.

See Fig. 2*F*. EAE was induced in WT and *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice followed by treatment with anti–IL-6, anti–IL-23, and anti–TGF-β antibodies i.v. on days 1, 3, 5, and 7.

Table S5. In vivo suppression by CD4^{+}Nrp1^{+} T cells

Cells transferred	Day of EAE onset*	No. of mice with EAE	Maximum mean EAE score	Maximum EAE score
WT CD4^{+}CD25^{+}Nrp1^{+}	14	3/5	0.2	1
WT CD4^{+}CD25^{-}Nrp1^{+}	15	4/6	0.4	1.5
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ CD4^{+}CD25^{-}	11	3/3	3.3	5
Nrp1$^{flx/flx}$*CD4 Cre*$^{+}$ CD4^{+}CD25^{+}	12	5/5	2.4	3.5

*Day of onset for first clinical signs of EAE.

See Fig. 3*B*. CD4^{+}CD25^{+}Nrp1^{+} or CD4^{+}CD25^{-} Nrp1^{+} T cells (1×10^6) from WT mice or 1×10^6 CD4^{+}CD25^{+} or CD4^{+}CD25^{-} cells from naïve *Nrp1*$^{flx/flx}$*CD4 Cre*$^{+}$ mice were cotransferred with in vitro skewed T$_H$-17 responder/effector cells into C57BL/6-TCR-$\alpha^{-/-}$ recipients. EAE was induced concomitant with cell transfer. Results from one experiment are displayed.