SUPPLEMENTAL FIGURES

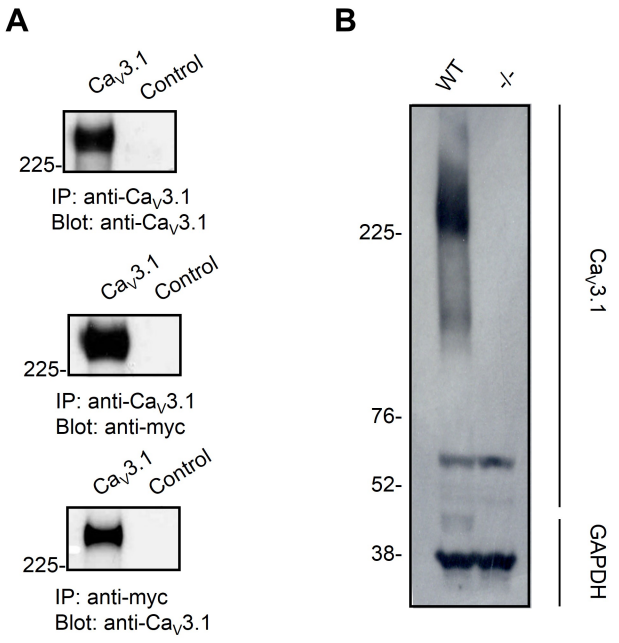

Figure S1, related to Figure 1. Characterization of $Ca_V3.1$ expression using the anti-$Ca_V3.1$ antibody

(A) Full-length $Ca_V3.1$ ($Ca_V3.1$) or empty vector control (control) myc-tagged cDNAs were transiently transfected into HEK-293 cells. Overexpressed proteins were detected in whole cell lysates via immunoprecipitation (IP) and immunoblotting (blot) with anti-$Ca_V3.1$ or anti-myc.

(B) Total protein tissue lysates from WT and *Cacna1g*$^{-/-}$ cerebellum were analyzed by immunoblotting with anti-$Ca_V3.1$ antibody or anti-GAPDH antibodies to verify equal protein loading.

Data are from one experiment representative of three independent experiments with similar results (A-B).

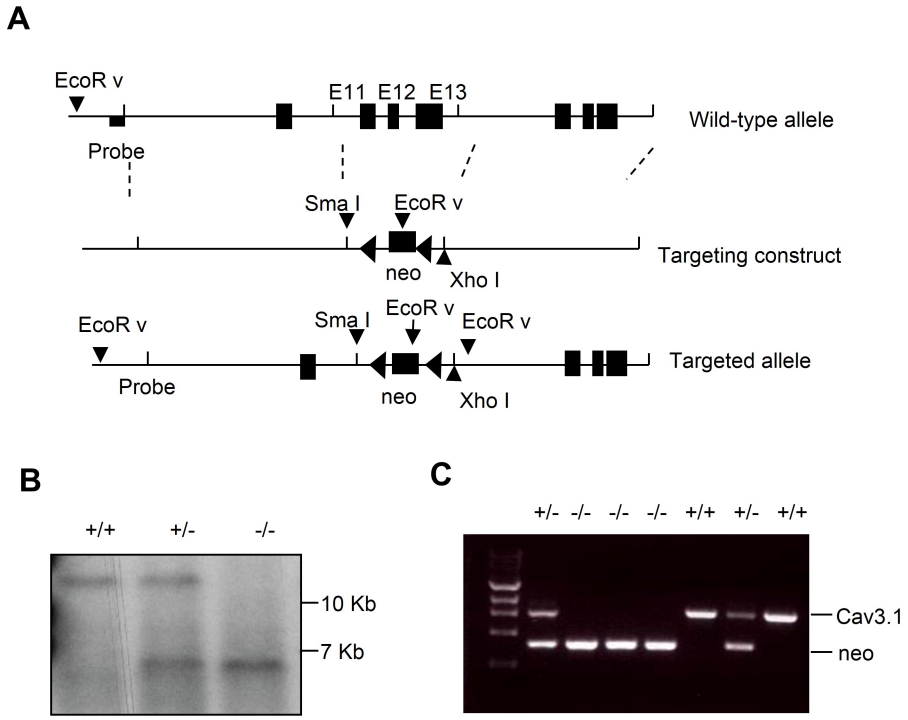

Figure S2, related to Figure 1. Generation of *Cacna1g*$^{-/-}$ mice
(A) A targeting construct was designed to genetically delete the pore region of *Cacna1g* using a neomycin cassette to replace exons 11 to 13.
(B-C) Targeted disruption of the *Cacna1g* gene was verified by Southern blotting (B) and by PCR (C) of genomic DNA isolated from candidate mice.
Data are from one experiment representative of three independent experiments with similar results (B-C).

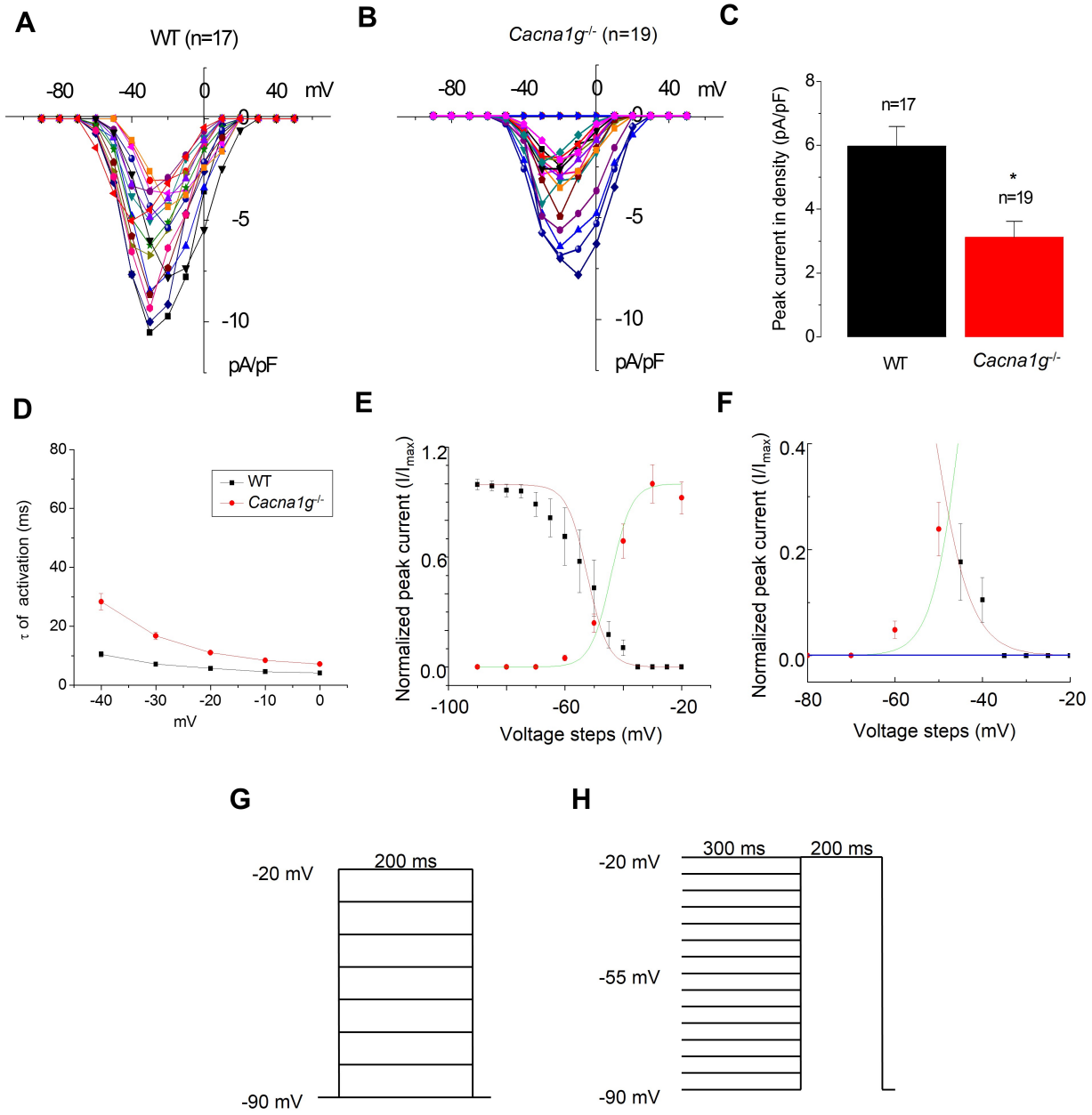

Figure S3, related to Figure 2. The signature current for Ca_V3 channels is present in WT CD4$^+$ T cells and reduced in *Cacna1g*$^{-/-}$ CD4$^+$ T cells

(A-B) Current-voltage (I-V) relationships of currents recorded from 17 WT cells (A) and 19 *Cacna1g*$^{-/-}$ cells (B).

(C) Peak currents (means ± SEM) recorded from 17 WT cells, and 19 *Cacna1g*$^{-/-}$ cells (*$p < 0.05$).

(D) Time constant (τ) of voltage-dependent activation of currents in CD4$^+$ T cells elicited by voltage steps from a holding potential of -90 mV. Data represent means ± SEM from 7 WT and 8 *Cacna1g*$^{-/-}$ cells.

(E-H) T-type channel window currents in WT CD4$^+$ T cells. Steady-state activation and inactivation curves (E) were determined from peak T-type currents and normalized to maximal currents. Maximal T-type currents were calculated from a voltage step to -20mV from a resting potential of -90mV. The values of normalized peak current are means ± SEM. Expanded figure (F) from plots in (E) is showing a range from -65 mV to -25 mV for T-type channel window currents. The protocols of voltage steps for activation and inactivation curves are shown in G and H, respectively.

Data are from one experiment representative of three independent experiments with similar results (A-H).

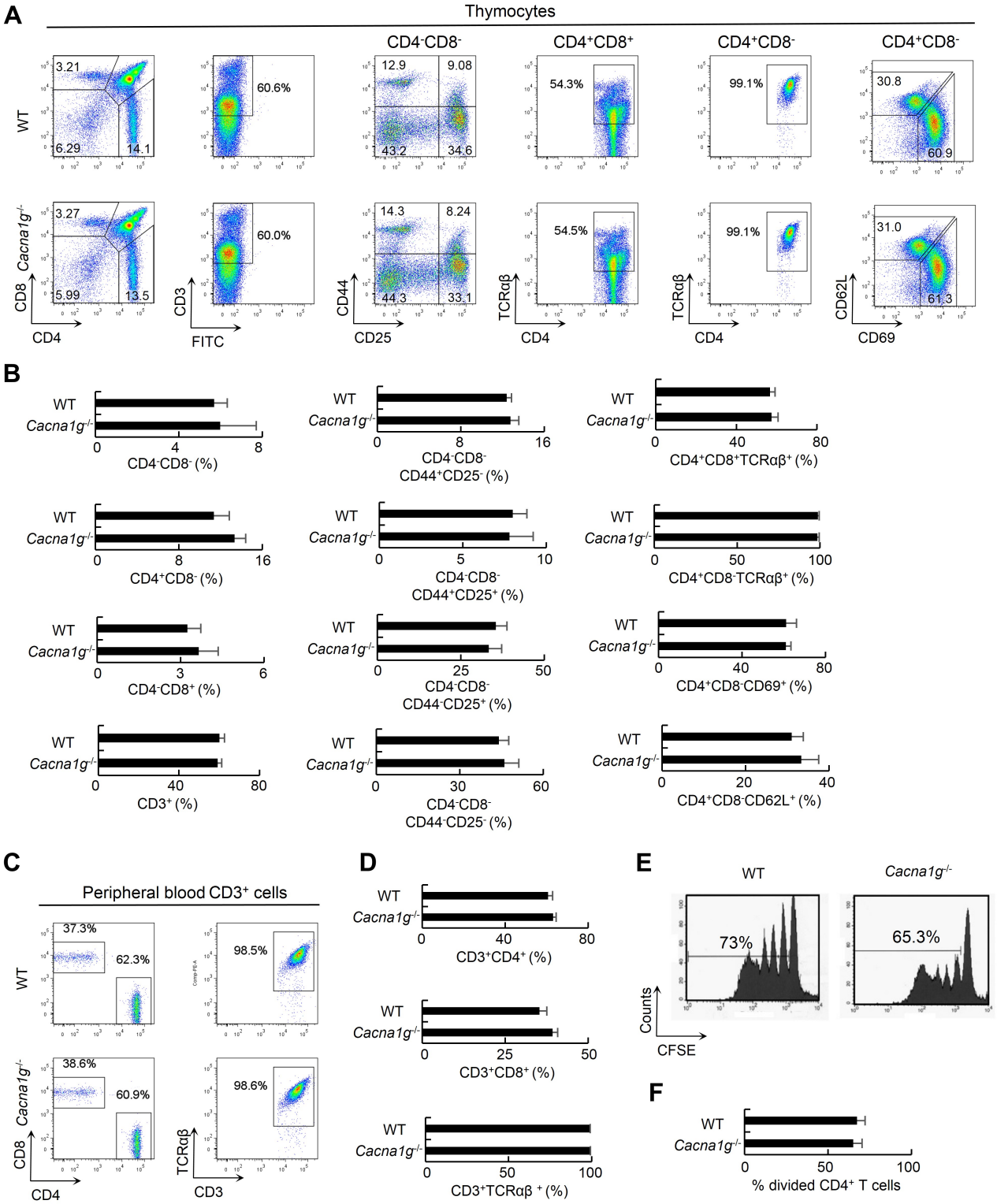

Figure S4, related to Figure 4. T cell development and proliferation

(A-B) Thymocytes were isolated from WT and *Cacna1g*$^{-/-}$ mice and analyzed by flow cytometry. Representative flow cytometry data (A) and analysis (B) of surface marker expression profile on WT or *Cacna1g*$^{-/-}$ thymocytes are shown.

(C-D) Representative flow cytometry data (C) and analysis (D) of surface marker expression profile on WT or *Cacna1g*$^{-/-}$ peripheral blood T cells are shown.

(E-F) Representative flow cytometry data (E) and analysis (F) of proliferation of splenic CD4^{+} T cells from WT and *Cacna1g*$^{-/-}$ mice in response to anti-mouse CD3 and CD28 Dynabeads are shown.

Data are from one experiment representative of three independent experiments with similar results (A-F; mean and s.e.m. in B,D,F).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Anti-$Ca_V3.1$ antibody generation

We designed a unique peptide located in the C terminus of $Ca_V3.1$, CPGVEGPDSPDSPKPGA. A rabbit polyclonal antibody with reactivity to this sequence was generated and purified by Invitrogen.

T cell preparation

Mouse thymus, spleen and lymph nodes were dissected from WT, *Cacna1g* $^{-/-}$ and *Cacna1g*$^{flox/-}$*Lck-cre* mice. Cells in suspension were recovered. Untouched $CD4^+$ T cells or untouched $CD4^+CD25^-D62L^{high}CD44^{low}$ naïve T cells were isolated using mouse $CD4^+$ T cell isolation kit and naïve $CD4^+$ T cell isolation kit, respectively (Miltenyi Biotec). Untouched $CD4^+$ T cells from thymus, spleen and lymph nodes of WT mice were used for comparing RNA level $Ca_V3.1$expression in $CD4^+$ T cell from these lymphatic tissues. Untouched $CD4^+$ T cells from spleen were used for electrophysiology studies and untouched naïve $CD4^+$ T cells from spleen were used for *in vitro* Th polarization studies. The typical purities of recovered $CD4^+$ T cells or naïve $CD4^+$ T cells were in the range of 95% to 98%.

Quantitative real-time RT-PCR

Total RNA was extracted from $CD4^+$ or naïve $CD4^+$ T cells of different lymphoid tissues, or cells from cerebellum or cerebrum, and then was reverse-transcribed. Multiplex quantitative real-time PCR was performed in ABI Prism 7700 Sequence Detector or 7500 Fast Real Time PCR System (Applied Biosystems). 18S internal control was used to normalize the quantities of target genes. Taqman primer and probe sequence for mouse $Ca_V3.1$ are 5'-

TGAGGCCAAGAGTTCCTTTGA -3' (sense), 5'- GAAGCCGACTTGCCATTACAG -3' (anti-sense), and 6FAM TCGGAGCTCTGCCTCTGAACACCA TAMRA (probe). Taqman primers and probes Mm00445382_ml for mouse $Ca_V3.2$ and Mm01299033_m1 for mouse $Ca_V3.3$ were purchased from Life Technologies.

Immunoblotting and immunoprecipitation

Cells were lysed in RIPA buffer containing protease inhibitors. The lysates were separated by 3-8% SDS-PAGE and transferred to PVDF membranes, then blotted with the primary antibodies and visualized by ECL detection (GE Healthcare). Anti-mouse pStat3 antibody was purchased from Cell Signaling, anti-mouse RORγt antibody was from Millpore and anti-mouse NFATc1, NFATc2 and T-bet were from Santa Cruz Biotechnology. For immunoprecipitation (IP), polyclonal anti-$Ca_V3.1$ antibody or irrelevant rabbit IgG was bound to Dynabeads (Invitrogen) and then incubated with RIPA cell lysates. IPs were resolved on SDS-PAGE gels and proteins were detected by immunoblotting with antibodies as indicated.

$Ca_V3.1$ overexpression

The full-length cDNA encoding $Ca_V3.1$ was subcloned into the pcDNA4TO-myc-his expression vector and transiently transfected into HEK 293 cells. Control clones were generated in parallel with empty vector. $Ca_V3.1$ over-expression was verified by immunoblotting.

Cell surface protein isolation

Cell surface proteins were isolated and purified with The Cell Surface Protein Isolation Kit (Thermo Scientific Pierce) according to the manufacturer's suggestions. $CD4^+$ cells were labeled with EZ-Link™Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent, for 30 minutes at 4 ℃. Cells were subsequently lysed with a mild detergent containing proteinase inhibitors and the biotinylated proteins were then isolated with NeutrAvidin™Agarose in a spin column. The bound proteins are released by incubating with SDS-PAGE sample buffer containing 50mM DTT.

CRAC channel recording

For CRAC channel recording, pipette solution containing 145 mM Cs-methanesulfonate, 20 mM Cs-BAPTA, 8 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.2 with CsOH) and bath solution containing 135 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 20 mM $CaCl_2$, and 10 mM glucose (pH was adjusted to 7.4 with NaOH) were used. Cells were maintained at a 0 mV holding potential during experiments and subjected to reverse voltage ramps from +100 to −140 mV lasting 250 ms every 2 s.

Fluorometric Imaging Plate Reader Assay (FLIPR)

$CD4^+$ T cells were stained with fluo-4 (4 μM) at 37 ℃ and 5% CO_2 for 1 hour in cell culture medium, then washed with Calcium-free HBSS. The plate was then placed in the Fluorometric Imaging Plate Reader (FLIPR) instrument (Molecular Devices, Sunnyvale, CA, USA) where baseline fluorescence was recorded, then 1.8 mM calcium with thapsigargin (TG) were added. Calcium entry was calculated relative to the baseline-subtracted signal derived from wells in Calcium-free HBSS (F/F0).

Flow cytometric calcium flux assay

Splenocytes were suspended in HBSS buffer containing 1.25 mM calcium, 1 mM magnesium, 0.5% BSA were loaded with 2 mM Indo‐1 mixed with Pluronic F-127 at 37℃ for 45 minutes. The cells were washed and resuspended in HBSS buffer containing 1.25 mM Ca, 1 mM Mg and 0.5% BSA, then labeled with anti-CD4-FITC on ice for 30 min, and then washed and loaded with biotinylated anti-mouse CD3 antibody (10 μg/ml). $CD4^+$ T cells were gated and analyzed on a Becton Dickinson LSRII with FACSDiva software using the violet and blue channels. After measuring the baseline for 2 min, streptavidin (20 μg/ml) was added.

Antibodies and flow cytometry

Antibodies for cell surface antigens and Th1, Th2, Th17 and Treg related intracellular cytokines as well as isotype-matched controls were purchased from eBioscience and BD Biosciences. Anti-CD3, CD4, CD8, CD25, CD44, CD62L, CD69, Foxp3, IL-17A, IL-17F, and IL-21 antibodies were purchased from eBiosciences. Anti-TCRαβ, IFN-γ, TNF-α, IL-4 and GM-CSF antibodies were purchased from BD Biosciences. All these antibodies were fluorescence conjugated monoclonal anti-mouse antibodies. Cell surface marker staining was performed prior to intracellular cytokine staining. Cells were washed twice in ice-cold PBS with 2% fetal calf serum, then incubated with each antibody at 4 °C for 30 min in the dark and washed twice. All samples were analyzed on a five laser LSRII (Becton Dickinson) and using the FlowJo software (Tree Star Inc., San Carlos, CA, USA).

T cell proliferation assay

CellTrace™ CFSE Cell Proliferation Kit from Invitrogen was used for T cell proliferation assay. $CD4^+$ cells were isolated form mouse spleen and stained with 5 μM CFSE for 20 minutes at room temperature. Then cells were washed and stimulated with Dynabeads mouse T-Activator CD3/CD28 (Life Techologies) in 1:1 of cell and beads ratio in a 96-well plate. Cells were incubated for 5 days and then harvested and analyzed by flow cytometry.

Standard EAE score

0: Normal. No signs of neurological disease. 1: Flaccid paralysis of the tail; partial or no tail muscle tone. Mouse is unable to curl tail around finger or pencil. No significant gait abnormalities. 2: Hind limb paresis, weak or wobbling gait, impaired righting reflex. 3: Bilateral hind limb paresis. Mouse drags its hind limbs over flat surface. Mouse exhibits incontinence. 4: Hind and forelimb paralysis. Mouse barely moves around. 5: Complete hind and complete front leg paralysis, no movement.

Isolation of mononuclear cells from the central nervous system of EAE mice

Mice were deeply anesthetized. After cardiac perfusion with PBS to remove cells from blood vessels, the brain and spinal cord were removed and put in a 100 mm cell strainer placed in a 10 cm petri dish containing 10 ml of ice-cold PBS. Then each piece of organ was pressed through the cell strainer using the back of a sterile 1 ml syringe plunger. The single cell suspension was collected into a 50 ml tube and centrifuged. The cell pellet was resuspended in 20 ml of 30% Percoll in PBS and overlaid onto 10 ml of 70% Percoll in PBS in a 50ml tube. Then the cells were centrifuged at 390 g for 20 min at room temperature. The

mononuclear cells were collected from the interface of 30% and 70% Percoll. The cells were stimulated with 50ng/ml PMA and 500ng/ml of Ionomycin in the presence of 1ug/ml brefildin A for 5 hours before intracellular cytokine staining.

Naïve $CD4^+$ T cell *in vitro* polarization

To obtain subset phenotypes differentiated towards the Th1, Th17 and Treg, naïve $CD4^+$ T cells were activated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) in the presence of polarizing cytokines and blocking antibodies. For Th1, 5 ng/ml IL-12 and 10 μg/ml anti-IL-4 were used. For Treg, 10 ng/ml IL-2, 5 ng/ml TGF-β, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4 were used. For Th17, 1 ng/ml TGF-β, 30 ng/ml IL-6, 10 ng/ml IL-23 (added on day3), 10 μg/ml anti-IFN-γ and 10 μg/ml anti-IL-4 were used. After 3 days differentiation, the cells were expanded by adding fresh medium with relevant cytokines and blocking antibodies and then transferred to a bigger plate without plate-bound anti-CD3 and cultured for 2 more days. To obtain subsets differentiated towards the Th2 phenotype, naïve $CD4^+$ cells were cultured with plate-bound anti-CD3 (25 μg/ml) and soluble anti-CD28 (2 μg/ml) in the presence of 10 ng/ml IL-2, 50 ng/ml IL-4, 10 μg/ml anti–IFN-γ and 10 μg/ml anti–IL12 for 2 days and then washed and cultured with only 10 ng/ml IL-2, 50 ng/ml IL-4 cytokines for 3 more days. After 5 days differentiation, the cells were either collected for protein extraction or restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (1 μg/ml) for 5 hours for intracellular cytokine detection. All antibodies were purchased from BD Pharmingen. Recombinant cytokine of mouse IL-23 was from R&D Systems and all the others were from Peprotech.

Intracellular cytokine staining

For intracellular staining of GM-CSF and Th signature cytokines, the *in vitro* differentiated or PMA and Ionomycin-activated CNS-infiltrating cells were fixed and permeabilized with BD Cytofix/Cytoperm fixation/permeabilization solution (BD Biosciences) that contains mild detergents along with a formaldehyde-based fixative for 20 minutes at 4 ℃ in the dark. Then the cells were washed twice with BD Perm/Wash™ buffer that contains a cell permeabilizing agent, saponin. Cells were resuspended with BD Perm/Wash buffer and stained with the fluorochrome-conjugated anti-cytokine antibodies at 4 ℃ for 30 minutes in the dark. The positive cells were analyzed by flow cytometry. For Foxp3 staining, Foxp3/Transcription Factor Staining Buffer Set (from eBioscience) for the detection of nuclear antigens such as transcription factors was used according to the manufacturer's suggestions.

Fura-2 single-cell calcium imaging

Naive $CD4^+$ T Cells or those following *in vitro* Th17 polarization for12 hours were loaded at 1×10^6 cells/ml with 1 μM Fura-2/AM (Molecular Probes) in culture medium for 30 min at 37 ℃, then were washed with Hanks Balanced Salt Solution (HBSS, 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 11 mM D-glucose, 10 mM Hepes, pH 7.4). Cells were suspended with HBSS and settled into poly-d-lysine–coated glass bottom No. 1.5 MatTek tissue culture dishes (MatTek Corp. Ashland, MA) for 15 minutes at 37 ℃ in a dark cell culture incubator. The dish was then washed with calcium free HBSS and images were collected with a 40x S-Fluor NA 1.3 oil objective on a Nikon Ti inverted microscope with an

environmental chamber to keep the samples at 37 ℃. Fura-2 was excited in 10 second intervals using a Lambda DG-4 unit (Sutter) with 340 and 380 nm excitation filters. Emission was captured through at 510/40 nm bandpass filter (Semrock) by a Clara Interline transfer CCD camera (Andor Technology). Image acquisition and hardware was controlled by Andor iQ computer software. Fura-2 emission was first recorded in calcium free HBSS for 3 minutes and then in HBSS with 2 mM calcium for 5 minutes. Fluorescence ratio images were calculated subsequently using ImageJ analysis software. For each sample, 50–100 individual cells were analyzed. Values of $[Ca^{2+}]_i$ were calibrated according to the formula $[Ca^{2+}]_i = Kd [(R - R_{min})/(R_{max} - R)]$ (Sf2/Sb2) (Grynkiewicz et al., 1985). The Kd for the Ca^{2+}-Fura-2 complex was assumed to be 225 at 37 ℃. The Rmax or Rmin was measured under Ca^{2+}-saturating condition (2 mM Ca^{2+} and 10 μM Ionomycin) or Ca^{2+}-free condition (0 mM Ca^{2+}, 2 mM EGTA, and 10 μM Ionomycin), respectively. Sf2 was the fluorescence at 380 nm excitation under Ca^{2+}-free condition, and Sb2 was the fluorescence at 380 nm excitation under Ca^{2+}-saturating condition.

Nuclear and cytoplasmic protein extraction

Cytoplasmic and nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Life technologies) according to the manufacturer's suggestions. Naïve $CD4^+$ T cells or those stimulated by anti-CD3 and anti-CD28 antibodies for 12 hours were washed with PBS and pelleted. The Cytoplasmic Extraction Reagents which contained proteinase inhibitor were added to the cells to disrupt cell membrane to release of cytoplasmic contents. After recovering the intact nuclei from the cytoplasmic extract by centrifugation, the proteins were extracted out of the nuclei with Nuclear Extraction Reagent.

Acute deletion of *Cacna1g*

Naïve $CD4^+$ cells isolated from spleens of *Cacna1g*$^{floxed/floxed}$ mice were transfected with GFP-CRE recombinase Lentiviral Particles (GeneCopoeia) or empty control lentiviral particles with GFP alone and 8ug/ml polybrene. $CD4^+GFP^+$ cells were sorted by flow cytometry 72 hours after transfection. Quantitative PCR for $Ca_V3.1$ were performed with total RNA isolated from sorted cells to confirm $Ca_V3.1$ knock-down ratio. For studying cytokine profile in the *Cacna1g* acutely deleted cells, sorted $CD4^+GFP^+$ cells were *in vitro* differentiated to Th1 or th17 cells for 5 days.

Supplemental References

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. Journal of Biological Chemistry *260*, 3440-3450.