Supporting Information

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SI Materials and Methods

DNA Constructs for tRORγt-TMD and tTbet-TMD. The RORγt-DBD (RORγt-TMD) and RORγt-LBD that encode amino acids 1–99 and 293–495, respectively, of the wild-type RORγt (1–495) were amplified from the RORγt plasmid (from D. R. Littman) by PCR. The tTbet-TMD that encode amino acids 120–336, of the wild-type Tbet were amplified from the Tbet plasmid (from L. H. Glimcher) by PCR. The conditions of amplification were 94 °C for 5 min; 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 32 cycles; and then 72 °C for 10 min. Purified PCR fragments were cloned into the NheI-HindIII–digested pRSET-B vector. The fidelity of the reading frame was verified by sequencing.

Expression and Purification of tRORγt-TMD and tTbet-TMD. The cloned DNA was transformed into *Escherichia coli* strain BL21 star (DE3) pLysS competent cells (Novagen) for expression. Inoculation of the transformed cells was in LB media. Cells were grown at 37 °C to an OD value of 0.45 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 5 h. The harvested cells were sonicated at 4 °C in lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). Lysates were clarified by centrifugation (Effendorf centrifuge, model 5415R, rotor F45-24-11) and loaded onto a Ni^{2+}-nitrilotriacetic acid sepharose affinity column (Qiagen) under soluble conditions. Bound proteins were washed with wash buffer (30 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and eluted with elution buffer (3 M imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). The recombinant proteins contained in each fraction were desalted on PD-10 Sephadex G-25 (Amersham Pharmacia). Then, endotoxin was eliminated by using SP Sepharose Fast Flow (GE Healthcare). Bound proteins were washed with wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 7.2) and eluted with elution buffer (2 M NaCl, 50 mM NaH_2PO_4, pH 8.0). Finally, each protein fraction was desalted on PD-10, separated into aliquots, and flash-frozen at −83 °C. The purity of the proteins was determined by limulus amebocyte lysate (LAL; Lonza) and Picogreen assays (Invitrogen). The endotoxin level was 27.0 endotoxin units (Eu)/mg, and the bacterial DNA level was 8.6 ng/mg of protein in four independent preparations.

Cell Cultures and Experimental Treatments. Primary $CD4^+$ T cells were maintained in RPMI 1640 media containing 10% heat-inactivated FBS (GIBCO), 100 μg/mL penicillin/streptomycin, and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 in air at 37 °C. For the transduction of fusion proteins, primary $CD4^+$ T cells were harvested and resuspended in serum-free medium. Approximately 2×10^6 cells were grown to confluence in a 12-well plate and then treated with the recombinant proteins. The human cervical cancer cell line, HeLa cells, were maintained in DMEM supplemented with 10% heat-inactivated FBS (GIBCO), 100 μg/mL penicillin/streptomycin, 1 mM sodium pyruvate, 1× NEAA Mixture, and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 in air at 37 °C. For the transduction of fusion proteins, $0.5–1 \times 10^6$ HeLa cells were grown to confluence on a four-well LabTek II chamberslide (Nunc) for 12–16 h.

Immunoblot Analysis. Transduced cells were lysed in lysis buffer (10 mM Tris·HCl pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 400 μM EDTA pH 8.0, 10 mM NaF, 10 mM Na_3VO_4, 10 mM PMSF, 5 μg/mL leupeptin, and 5 μg aprotinin). An equal volume of 5× SDS gel loading buffer was added to samples, which were then boiled for 10 min and loaded onto a 10–15% SDS-polyacrylamide gel. Proteins were electrophoresed and blotted onto a nitrocellulose membrane. The membrane was blocked with 4% bovine albumin in Tris-buffered saline containing 1% Tween 20 (TBST). The membrane was then probed with mouse anti-FLAG and anti-PARP (nuclear marker) antibody diluted 1:1,000 in blocking solution. After washing in TBST solution, membranes were incubated with secondary antibodies (anti-mouse and anti-rabbit IgG; (Sigma). Bound antibodies were then visualized by using WEST-ZOL plus.

Luciferase Reporter Assay. HEK293 cells were transfected with a Lipofectamine reagent (Invitrogen). Both 1 μg of luciferase reporter construct (*Il17*-, *apolipoprotein A5*-, *Ifn*-γ-promoter-linked) and 1 μg of pEGFP-N1 plasmid along with wild-type RORγt, RORα1 (from S. H. Baek) or Tbet were mixed with Plus Reagent (Invitrogen) and diluted into Opti-MEM (Gibco) without serum. At the same time, Lipofectamine reagent was diluted into Opti-MEM. After 15 min, the premixtures were combined and incubated for 15 min at room temperature, and then HEK293 cells (1×10^5 cells per well) were incubated with the DNA plus Lipofectamine reagent mixture. After 3 h, 100 nM–2 μM tRORγt-TMD, tRORγt-LBD, RORγt-TMD, or 1–4 μM tTbet-TMD proteins were added and incubated 24 h. The luciferase activity was performed according to the manufacturer's instructions, and the value was normalized by Renilla activity measured with a luminometer (Promega). Each transfection was done more than three times.

Immunocytochemistry. HeLa cells were grown in four-well LabTek (Nunc) plates and incubated with 500 nM tRORγt-TMD proteins for 1 h at 37 °C under an atmosphere of 95% air and 5% CO_2. Cells were washed twice with PBS, then fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked by blocking solution (1% BSA, 0.1% gelatin, and 0.001% sodium azide). Anti-FLAG antibody at a 1:100 dilution (Qiagen) was used to detect the tRORγt-TMD proteins. FITC-conjugated anti-mouse IgG (1:100 dilution; Qiagen) was then incubated with the cells for 2 h at room temperature. Samples were stained with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) solution to visualize the nuclei. Finally, cells were washed with distilled water and analyzed by confocal microscopy (Nikon).

Cell Sorting. Mouse naïve $CD4^+$ T cells were isolated from spleens from C57BL/6 mice on a magnetic-activated cell sorter (MACS) column by using CD4, CD25, and CD62L microbeads (Miltenyi Biotec). To isolate $CD4^+$ $CD25^-$ $CD62L^+$ T cells, we performed three-step purification with CD4-negative beads, CD25-positive beads, and CD62L-positive beads. To isolate T_{reg}, we purified with CD4-negative and CD25-positive beads. To isolate $CD4^+$ $CCR6^+$ T cells, CD4-negative beads for MACS and anti-$CCR6^+$ antibody for FACS sorter were used.

PBMCs Stimulation and Cytokine Analysis. PBMCs were isolated from healthy adult volunteer donor's blood by using BD vacutainer CPT cell preparation tube with sodium citrate. Lymphocyte layer was collected and washed with PBS. PBMCs were activated with plate-bound anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) mAb in the presence of 100 pM, 100 nM, or 1 μM RORγt-TMD(h) or tRORγt-TMD(h) for 72 h (RORγt-TMD(h), tRORγt-TMD(h); human form of RORγt). Cells were reactivated with PMA/ionomycin for 4 h and then stained with anti-CD4 and intracellular-stained anti–IL-17A mAb followed by FACS analysis.

Analysis of T-Cell Activation and Differentiation. Primary mouse splenocytes were treated with tRORγt-TMD or Tbet-TMD for 1 h, then washed with PBS three times and incubated with 0.5 μg of anti-CD28 mAb (Pharmingen) for 20 min at 4 °C. The anti-CD28 mAb-bound cells were further activated on the plate coated with 1 μg of anti-CD3 mAb (Pharmingen) for 72 h. Each cell was stained with anti–CD69-PE or anti–CD25-FITC antibodies and analyzed by FACScaliber (BD Biosciences). Naïve $CD4^+$ T cells were maintained in RPMI 1640 medium supplemented with 10% FBS and stimulated with 1 μg of plate-bound anti-CD3 and 0.5 μg of soluble anti-CD28 under conditions formulated to obtain the following cell types: T_H17 (TGF-β1 [3 ng/mL] and IL-6 [30 ng/mL] with anti–IL-4/IFN-γ antibody), T_H1 (IL-12 [10 ng/mL] and anti–IL-4 antibody), T_H2 (IL-4 [40 ng/mL] and anti–IFN-γ antibody), and T_{reg} (TGF-β1 [10 ng/mL] and IL-2 [50 U/mL] with anti–IL-12/IFN-γ antibody). The supernatant in each well was analyzed for the level of secreted IL-2, IL-17A/F, IFN-γ, IL-13, and IL-10 by ELISA.

Measurement of Cytokines. After 72 h of incubation, the culture supernatants from the stimulated $CD4^+$ T cells or the sera from cells transduced with RORγt-TMD, tRORγt-LBD, tRORγt-TMD, or tRORγt-TMD (RR-AG) were analyzed by ELISA for murine IL-2, IL-4, IL-13, IL-17A/F, and IFN-γ using paired antibodies in accordance with the manufacturer's instructions (eBioscience).

Animals. Female C57BL/6 mice were purchased from Orient Bio and housed in individually ventilated micro isolation cages in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center (YLARC). All animals were maintained on a 12-h:12-h light/dark cycle with access to food and water ad libitum. All behavioral procedures were conducted during the light phase of the cycle. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of YLARC and performed in accordance with the YLARC-IACUC guidelines for the ethical use of animals (YLARC2010-0035).

EAE Induction and Scoring. Female C57BL/6 mice (6- to 8-wk-old) were purchased from the Orient Bio and maintained at the YLARC. Myelin oligodendrocyte glycoprotein (MOG) peptide (MEVGWYRSPFSRVVHLYRNGK), corresponding to the fragment of mouse MOG from amino acid residues 35–55, was synthesized by Anygen. Mice were immunized with two s.c. injections of a total of 300 μg of the $MOG_{35–55}$ peptide emulsified in 0.2 mL of complete Freund's adjuvant (Difco Laboratories) containing with 400 μg of heat-killed *Mycobacterium tuberculosis* (Difco Laboratories) on day 0 and day 7. A total of 500 ng of pertussis toxin (List Biological Laboratory) was administered i.p. on day 0 and day 2. Mice were scored daily starting at day 7. Scoring was as follows: 0, normal; 1, weakness of tail; 2, definite tail paralysis and hind limb weakness; 3, partial paralysis of hind limb; 4, complete paralysis of hind limb; 5, complete paralysis of hind limbs with incontinence and partial or complete paralysis of forelimbs; 6, dead.

Isolation of Mononuclear Cells from Spinal Cords. Before spinal cord dissection, mice were perfused with PBS to remove blood from internal organs. The spinal columns were dissected, cut open, and intact spinal cords were separated carefully from the vertebrae. The spinal cords were cut into several small pieces and placed in 2 mL of digestion solution containing 10 mg/mL collagenase D (Roche) in PBS. Digestion was performed for 45 min at 37 °C with short vortexing every 15 min. At the end of the digestion, the solution was passed through a 40-μm cell strainer. The cells were washed once in PBS, placed in 6 mL of 38% percoll solution, and pelleted for 20 min at 1,500 × *g*. Pellets were resuspended in T-cell medium and used for subsequent experiments.

Histologic Studies. Brains and spinal cords from tRORγt-TMD–treated and MOG-immunized mice were obtained and fixed by perfusion with 4% phosphate-buffered paraformaldehyde. These samples were embedded in paraffin, and paraffin sections were stained with H&E, Periodic acid-Schiff (PAS), and Luxol fast blue for visualization of inflammatory cells and demyelination. Deparaffinized sections were immersed in Luxol Fast Blue (LFB; ID Labs, 0.1% wt/vol in 95% ethanol with 0.05% acetic acid) and incubated 2 h at 60 °C. Slides were rinsed in 95% ethanol and rehydrated in water. Sections were differentiated first in 0.05% $LiCO_3$ and then in 70% ethanol until the contrast between gray and white matter was maximal. Sections were stained with Cresyl Echt Violet and then rinsed in water. Slides were dehydrated in 100% ethanol, cleared in xylene/S3-Histo, and mounted with Vectamount. In brief, to quantify H&E or PAS-positive cells, the images were acquired from the series of adjacent sections in the white matter of spinal cord stained for immunohistochemistry and observed by an Olympus microscope connected to a digital camera.

CIA Induction and Scoring. All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea. Forty male DBA/1 mice at 8 wk of age were evenly divided into four groups (group 1, (-) controls; group 2, (+) untreated; group 3, 35 mg/kg MTX-treated; group 4, 2.5 mg/kg tTbet-TMD-treated, group 5, 2.5 mg/kg tTbet-TMD (R164A)-treated). All mice except controls were given an intradermal injection of 100 mg of bovine type II collagen emulsified in complete Freund's adjuvant (Arthrogen-CIA) (1:1, wt/vol) to the base of the tail. Two weeks later, the mice were given a booster intradermal injection of 100 mg of bovine type II collagen in incomplete Freund's adjuvant (DIFCO) (1:1, vol/vol). The (-) control mice were treated with Freund's adjuvant without bovine type II collagen. Mice were observed twice a week for 60 d after primary immunization. Arthritis severity was evaluated by visual inspection. All four legs of the mice were evaluated and scored from 0 to 4 according to the following scale: 0, no evidence of erythema and swelling; 1, erythema and mild swelling from the ankle and/or redness of the paw or one digit; 2, from the ankle to the tarsals or two joints involved; 3, moderate swelling from the ankle to metatarsal joints or more than two joints involved; and 4, severe swelling from the ankle, foot, and digits or ankylosis of the limb, severe arthritis of the entire paw and all digits. Each paw was graded, and the grades were summed to yield the arthritis score for each animal (maximum scores = 16). Paw thickness was measured with a Vernier caliper. Arthritis scoring and paw thickness measurement were performed by two independent observers.

T-Cell Transfer Colitis Model. To induce colitis, $CD4^+$ $CD45RB^{high}$ cells were FACS-purified from the spleen and lymph nodes of C57BL/6 wild-type mice, using a FACSAria II (BD Biosciences). A total of 4×10^5 $CD4^+$ $CD45RB^{high}$ cells were injected i.v. in 200 μL of PBS into C57BL/6 RAG-$1^{-/-}$ recipient mice (>7 wk). Mice were monitored for 5 wk for clinical signs and body weight and then killed. Colons were flushed with 10% formalin to remove feces and fixed in PBS containing 4% formaldehyde. Parraffin-embedded sections (5 μm) were stained with H&E. Colitis development was evaluated by histology by two independent experts in a blinded fashion.

Statistical Analysis. The results are expressed as a mean ± SEM ($n = 3$ or more). Statistical analysis of group differences was examined by using an unpaired Student *t* test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered to be significant.

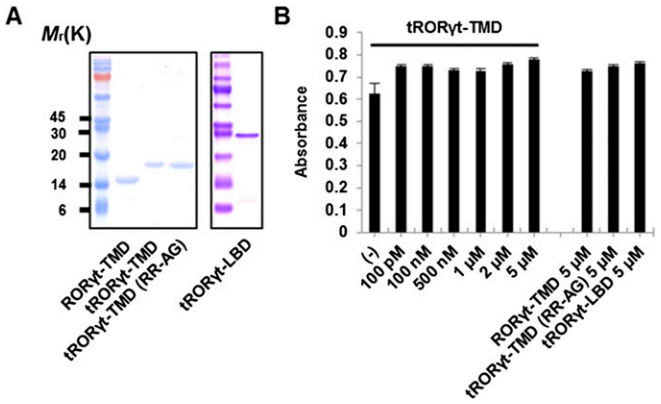

Fig. S1. Generation of tRORγt-TMD. (*A*) Purified proteins of RORγt-TMD, tRORγt-TMD, tRORγt-TMD (RR-AG), and tRORγt-LBD were visualized in 10% SDS/PAGE gel. (*B*) In vitro cytotoxicity of purified proteins on mouse primary naïve $CD4^+CD25^-CD62L^{high}$ T cells. RORγt-TMD, tRORγt-TMD, tRORγt-TMD (RR-AG), or tRORγt-LBD (100 pM–5 μM) was transduced into the cells for 1 h, and then viability was assessed by CCK-8. Data are representative of at least three independent experiments.

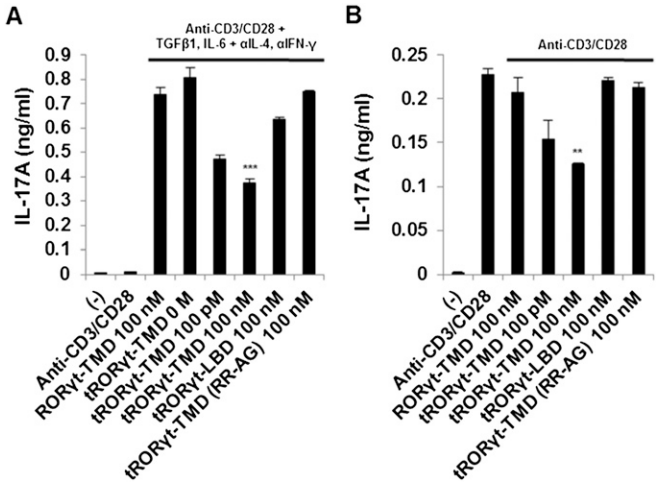

Fig. S2. Inhibition of IL-17A secretion from the differentiated T_H17 cells by tRORγt-TMD. (*A*) The mouse primary naïve $CD4^+CD25^-CD62L^{high}$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 72 h under T_H17-polarizing condition and then incubated with 100 pM or 100 nM of RORγt-TMD, tRORγt-TMD, tRORγt-LBD, or tRORγt-TMD (RR-AG) for 1 h. The cells were washed two times and incubated with T_H17-polarizing condition again for 48 h. (*B*) $CD4^+$ $CCR6^+$ T cells were isolated from spleen and lymph nodes of C57BL/6 mice. $CD4^+$ $CCR6^+$ T cells were incubated with 100 pM or 100 nM RORγt-TMD, tRORγt-TMD, tRORγt-LBD or tRORγt-TMD (RR-AG) for 1 h, and then stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 72 h. The level of IL-17A in the culture media was analyzed by ELISA. Data are representative of at least three independent experiments. Error bars denote SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

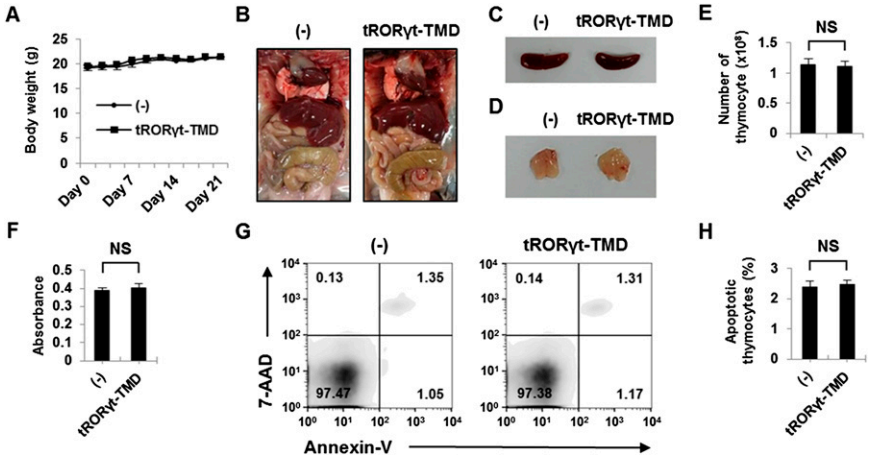

Fig. S3. In vivo toxicity of tRORγt-TMD in mice. C57BL/6 mice were injected with PBS (-) or tRORγt-TMD (2 mg/kg) every other day from day 1 to day 21. All experiments were performed 21 d after the first injection. (*A*) Weight was monitored every other day for 3 wk. (*B*) Morphology of thymus, heart, lung, liver, or small/large intestine was examined. (*C* and *D*) The size of spleen (*C*) or thymus (*D*) was examined. (*E*) Total number of thymocytes was analyzed. (*F*) Cytotoxicity of tRORγt-TMD on thymocytes. tRORγt-TMD (1 μM) was retransduced into the cells for 1 h, and then viability was assessed by CCK-8. (*G*) Effect of tRORγt-TMD on survival of thymocytes. Thymocytes were stained with Annexin-V and 7-AAD and analyzed by FACS. (*H*) Percentage of apoptotic thymocytes was analyzed. Data are representative of five mice per group. NS, not significant.

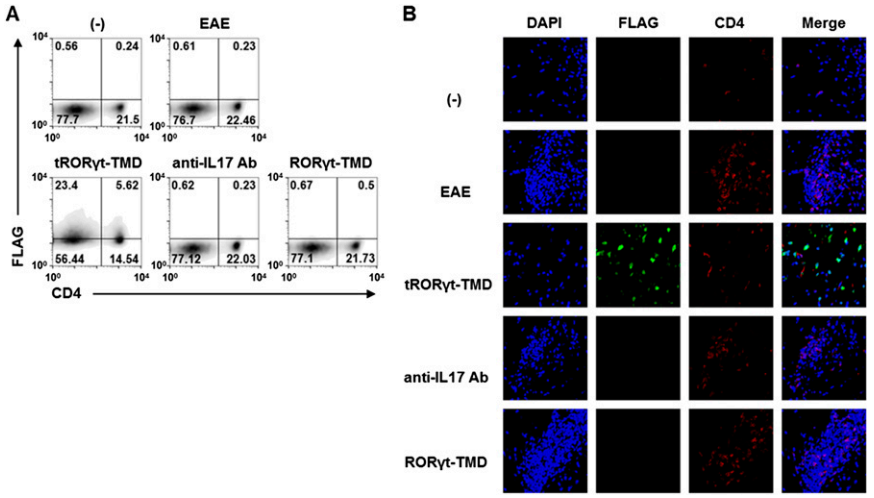

Fig. S4. In vivo transduction efficiency of tRORγt-TMD in splenic $CD4^+$ T cells and spinal cord-infiltrating $CD4^+$ T cells. EAE mice were injected with PBS (EAE), RORγt-TMD (control), tRORγt-TMD, or anti-IL17 mAb every other day from day 1 after EAE induction. (*A*) Splenocytes were removed at day 5 and staining with anti-CD4 antibody and anti-FLAG antibody. The samples were analyzed by flow cytometry. The FLAG tag was fused to the C terminus of RORγt-TMD and tRORγt-TMD. (*B*) Spinal cords were removed at day 21. The presence of tRORγt-TMD in spinal cords was examined by staining with anti-CD4 and anti-FLAG antibody. The samples were then analyzed by confocal microscopy. The FLAG tag was fused to the C terminus of RORγt-TMD and tRORγt-TMD. The cells were stained with DAPI to visualize the nuclei.

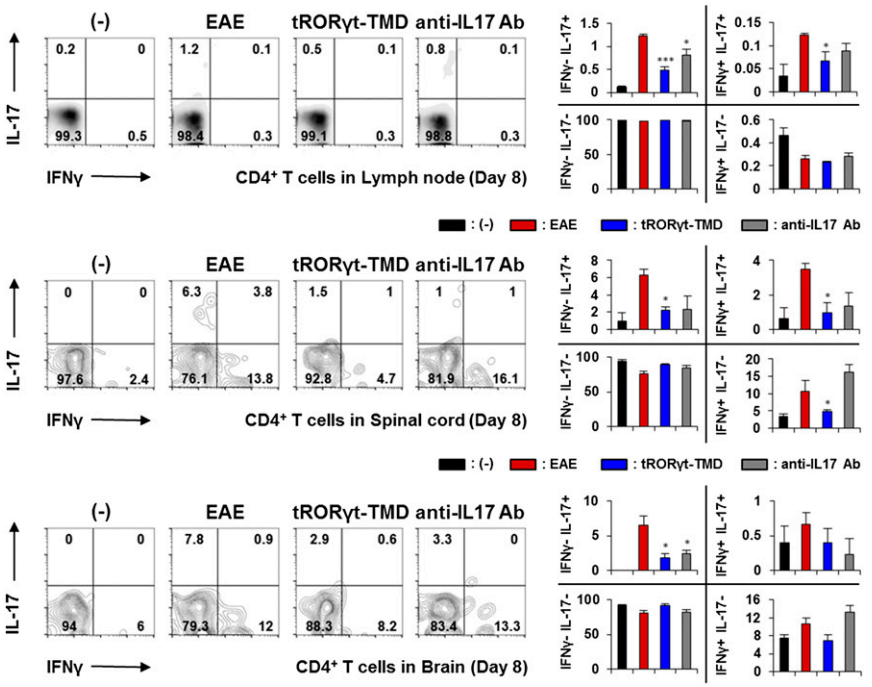

Fig. S5. Preventive potential of tRORγt-TMD in the amelioration of EAE through inhibition of T_H17 differentiation. Lymphocytes and mononuclear cells from spinal cord and brain were removed at day 8, reactivated with PMA/ionomycin for 4 h, and then stained for anti-CD4 and intracellular-stained with anti–IL-17A/IFN-γ mAb followed by FACS analysis. Percentages of $CD4^+$ $IL\text{-}17^+$/$IFN\text{-}\gamma^+$ T cells were measured. Data are representative of at least three to five mice per group. Error bars denote SEM. $^*P < 0.05$, $^{***}P < 0.001$.

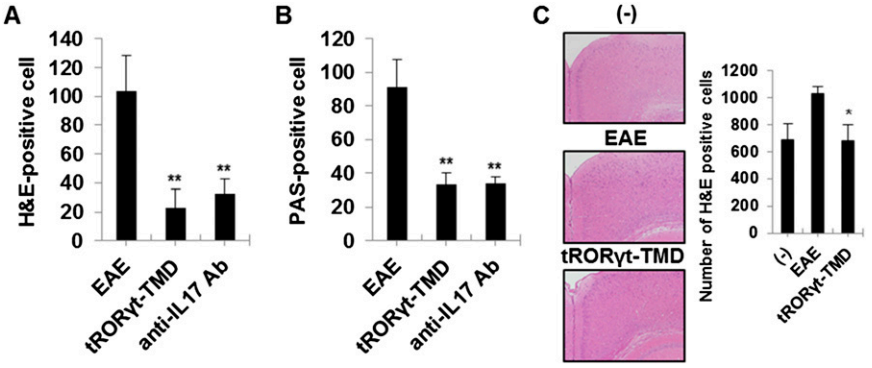

Fig. S6. Inhibition of inflammatory cell infiltration into the CNS by tRORγt-TMD during the amelioration of EAE. (*A* and *B*) Spinal cord sections obtained from each mouse at day 21 after EAE induction were quantified and compared among the groups by counting the number of H&E-positive (*A*) and PAS-positive (*B*) cells in the field. (*C*) Brains were removed at day 21, and their sections were stained with H&E to visualize the infiltration of inflammatory cells. Three to five random fields at 40× fields were independently counted by two researchers. Error bars denote SEM. $^*P < 0.05$, $^{**}P < 0.01$.

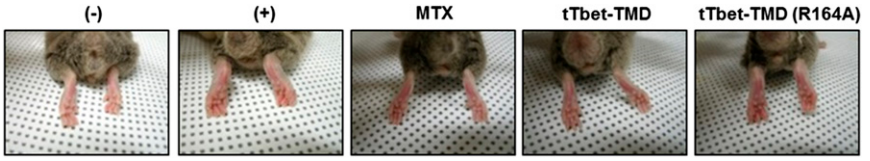

Fig. S7. Preventive potential of tTbet-TMD in the alleviation of CIA. Microscopic analysis of arthritis was assessed by paw thickness.

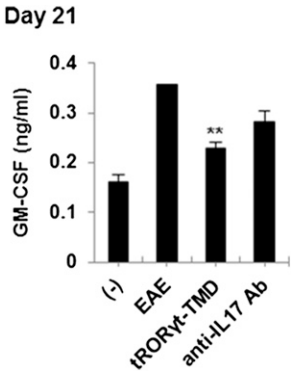

Fig. S8. Inhibition of GM-CSF secretion by tRORγt-TMD in EAE. EAE mice were injected with PBS (EAE), RORγt-TMD (control), tRORγt-TMD, or anti–IL-17 mAb every other day from day 1 after EAE induction. Splenocytes were removed at day 21 and stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 72 h. The level of GM-CSF in the culture media was analyzed by ELISA. Error bars denote SEM. $^{**}P < 0.01$.

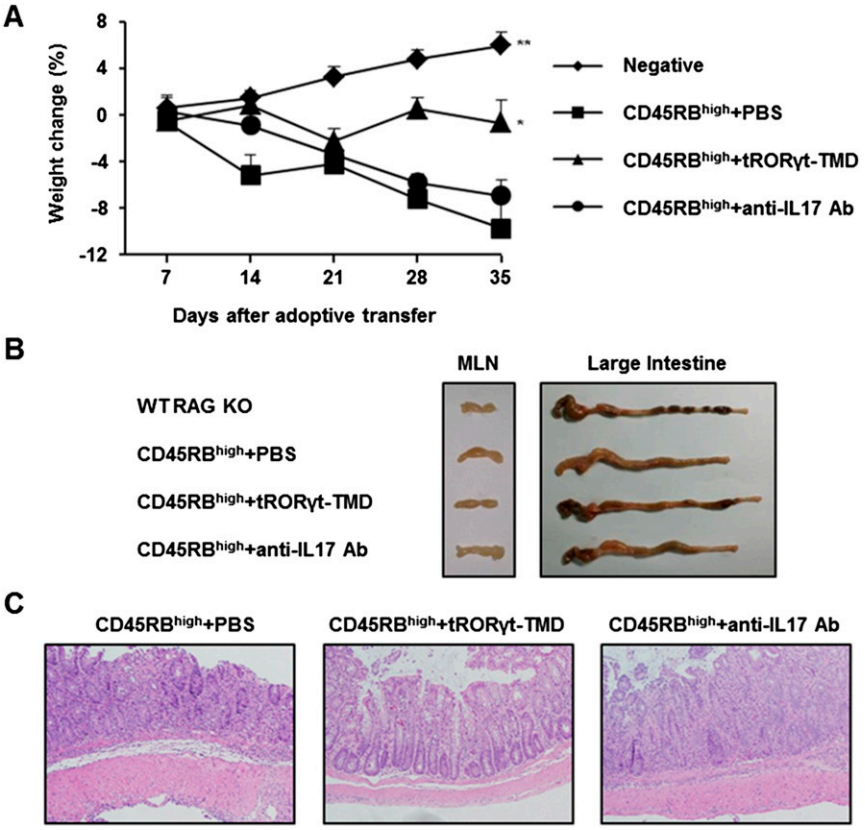

Fig. S9. Prevention of colitis by tRORγt-TMD. tRORγt-TMD injection inhibits colitis induced by $CD4^+$ $CD45RB^{high}$ T cells. A total of 5×10^5 wt $CD4^+$ $CD45RB^{high}$ T cells were transferred into RAG-2 KO mice, and tRORγt-TMD or anti–IL-17 mAb was injected with 2 mg/kg every other day until the day of killing. (*A*) Weight was monitored every week for 5 wk after the cell transfer. (*B*) The size of mesenteric lymph node (MLN) and the shape of large intestine were examined. (*C*) Infiltration of inflammatory cells into intestine is reduced by tRORγt-TMD. Colon tissue of each mouse shown was stained by H&E and is shown at 10× magnification. Four mice from each group were used for the analysis.

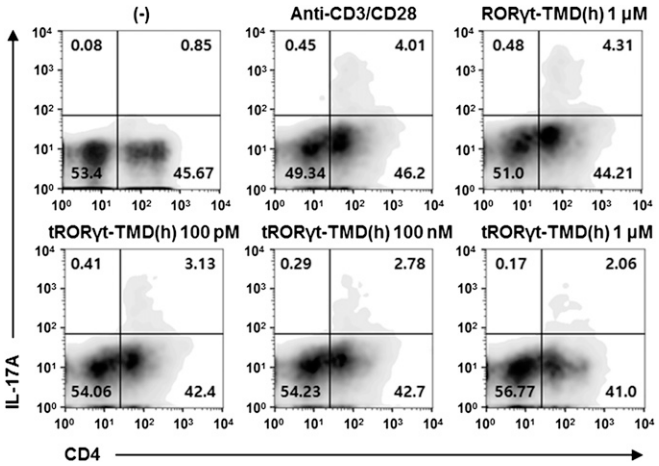

Fig. S10. Specific inhibition of IL-17A secretion from TcR-stimulated human PBMC by tRORγt-TMD(h) (human form of RORγt). Human PBMCs were activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of 100 pM, 100 nM, or 1 μM RORγt-TMD(h) or tRORγt-TMD(h) for 72 h. Cells were reactivated with PMA/ionomycin for 4 h, and then stained with anti-CD4 and intracellular-stained with anti–IL-17A cytokine mAb by FACS analysis. Data are representative of at least three independent experiments.