Supplemental figures and legends
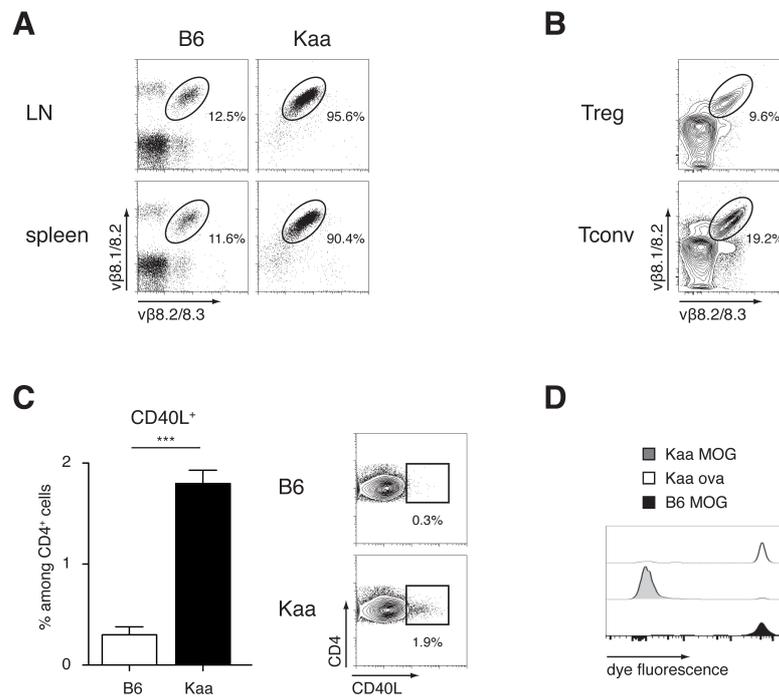
Figure S1, related to Figure 1 | Kaa: A transgenic mouse with increased frequencies of MOG-reactive protective Treg and pathogenic Tconv cells.
A. Representative FACS plots of vβ8.2$^+$ cells among CD3$^+$ cells in LN and spleen of Kaa and C57BL/6 mice. **B.** Representative FACS plots of vβ8.2$^+$ cells among CD4$^+$Foxp3$^+$ Treg cells and CD4$^+$Foxp3$^-$ Tconv cells in the CNS of C57BL/6 mice on day 40 after EAE induction. **C.** Frequency of CD40L$^+$ cells among CD4$^+$ cells in spleen from Kaa (n=5) and C57BL/6 (n=3) mice after *in vitro* stimulation with MOG(35-55) for 5 h. FACS plots are gated on CD4$^+$ cells. Representative results of more than three experiments. **D.** Proliferation dye-labelled Treg cells from Kaa or C57BL/6 mice were injected into CD45.1$^+$ C57BL/6 mice and immunized with either MOG(35-55) or OVA(323-339) peptide in CFA. The histogram shows a representative staining of dye dilution of CD45.2$^+$CD4$^+$Foxp3$^+$ cells in the draining LN six days after transfer.
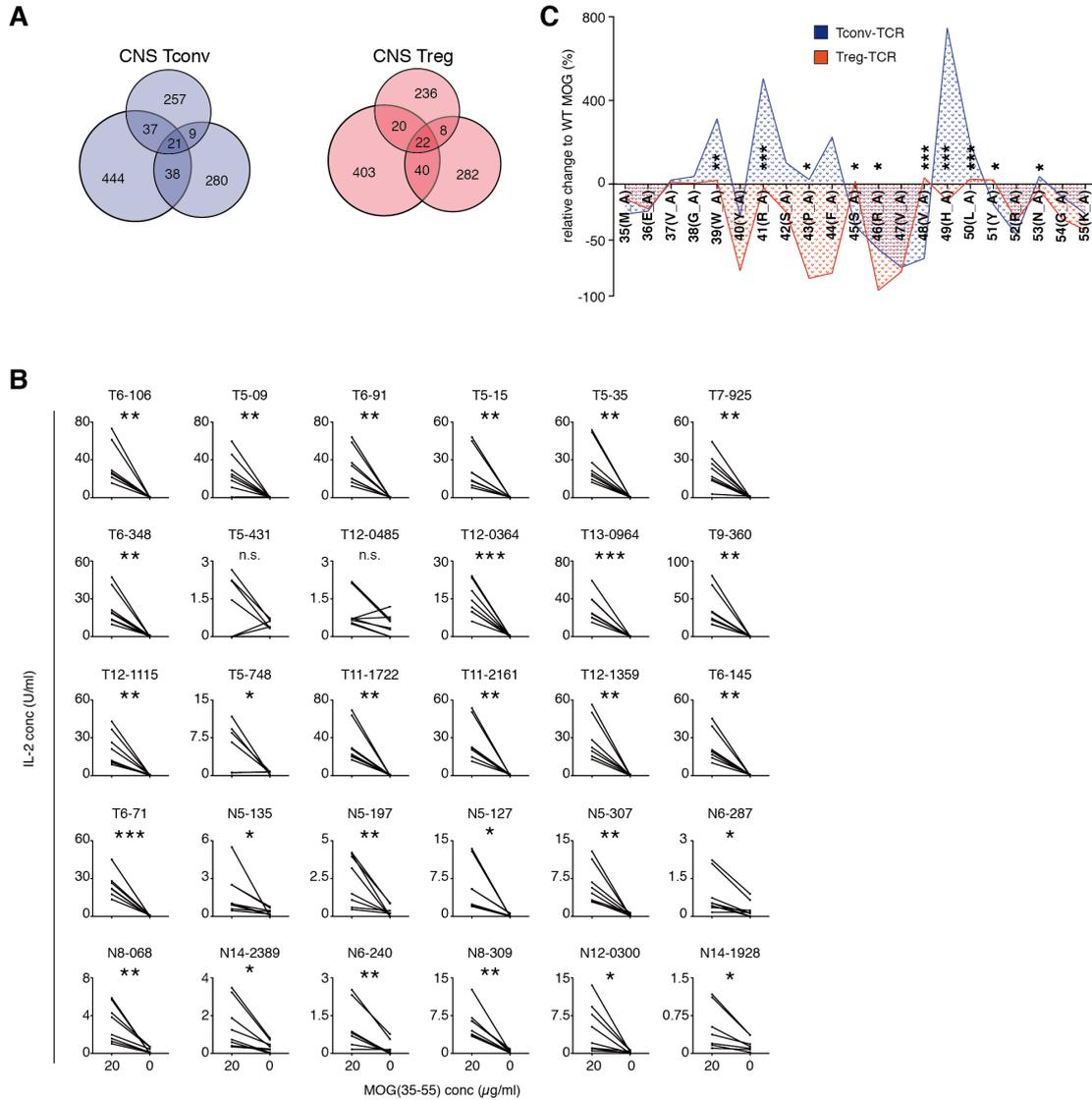
Figure S2, related to Figure 2 │ Antigen recognition by TCR from MOG-reactive Treg and Tconv cells.
A. Venn diagram showing the numbers of unique TCRα sequences cloned from Tconv and Treg cells from the CNS of Kaa.*Foxp3*.eGFP.*Tcra*$^{+/-}$*Tcrb*$^{+/-}$ mice with EAE in each of the three independent experiments. The overlaps indicate sequences found in more than one experiment. **B.** T.54ζ17 cell lines expressing 30 distinct TCR (19 from Treg cells, 11 from Tconv cells) were stimulated with splenic APCs loaded with 20 µg/ml MOG(35-55) (left) or no peptide (right) for 48 h, and IL-2 concentrations in supernatants were determined by ELISA. Data are pooled from four experiments each performed with two independent T cell lines for each TCR. **C.** A set of randomly selected T cell lines expressing Treg (n=7) or Tconv (n=6) cell TCR were stimulated for 48 h with the 21 variants of alanine-substituted MOG peptide, and the WT MOG(35-55) peptide at 2 µg/ml. Data show the change of IL-2 secretion upon stimulation with each altered peptide compared to the original peptide for Treg and Tconv cell TCRs. Asterisks indicate substitutions leading to significantly altered responses for the Treg vs. Tconv cell TCRs (unpaired t-test). One representative out of two experiments.
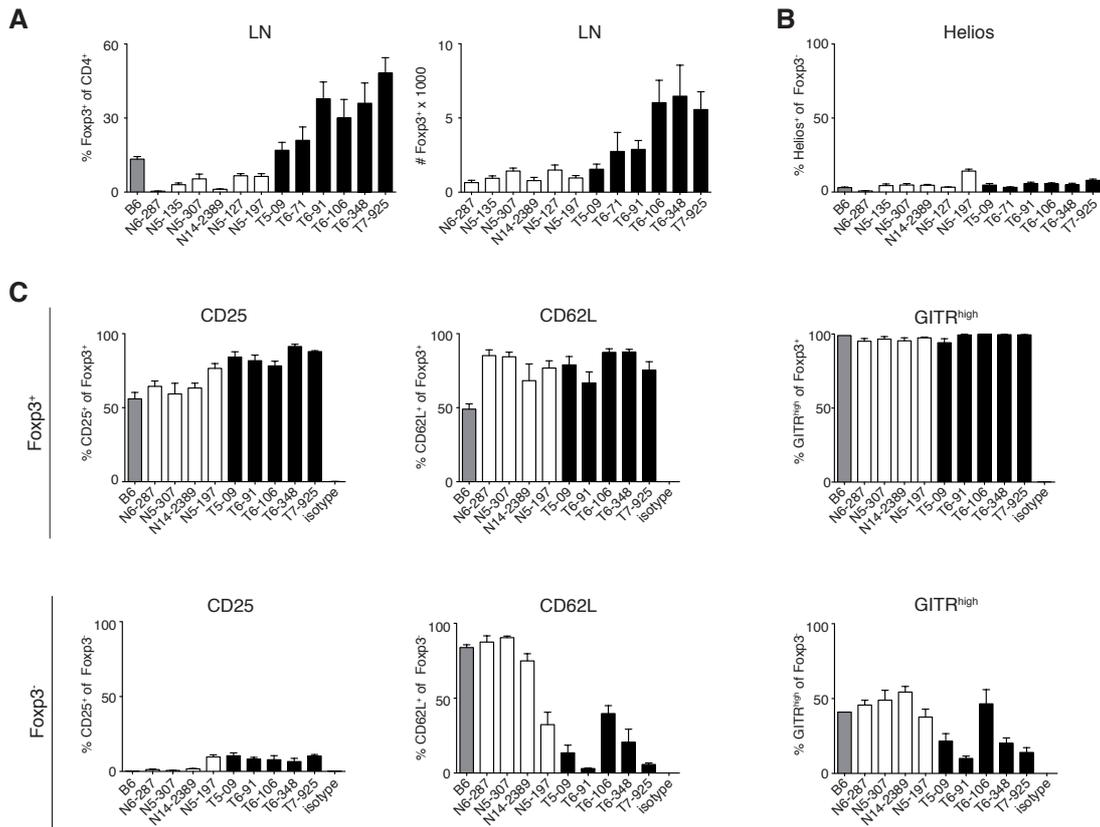
Figure S3, related to Figure 3 │ Instructive role of the TCR for MOG-reactive Treg cell development.

This supplemental figure provides complementary information on the retrogenic mice described in Figure 3. **A.** Nine weeks after reconstitution LN were analysed for percentage of Foxp3$^+$ cells among donor-derived CD45.2$^+$CD4$^+$ T cells (left) and absolute number (right) of donor-derived CD45.2$^+$CD4$^+$Foxp3$^+$ cells. **B.** Expression of Helios by splenic donor-derived CD45.2$^+$Foxp3$^-$ Tconv cells was determined by flow cytometry. **C.** Donor-derived splenic CD45.2$^+$CD4$^+$Foxp3$^+$ Treg cells (top) and CD45.2$^+$CD4$^+$Foxp3$^-$ Tconv cells (bottom) were analysed by flow cytometry for expression of CD25, CD62L and GITR. Black bars: Treg cell TCR. White bars: Tconv cell TCR. Data show mean + sem and are pooled from two experiments (n=9/10 per TCR).
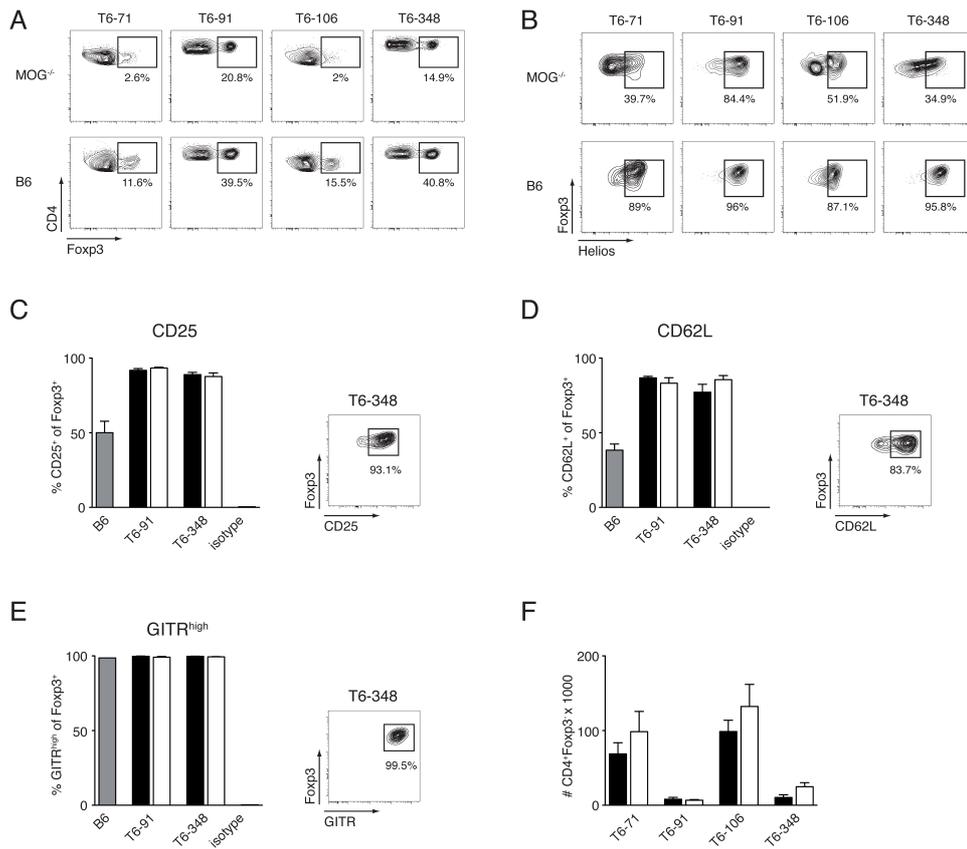
Figure S4, related to Figure 4 | Endogenous MOG controls the selection of MOG-reactive Treg cells.

This supplemental figure provides complementary information on the retrogenic mice described in Figure 4. **A.** Representative FACS plots showing expression of Foxp3 by donor-derived CD45.1$^+$CD4$^+$ T cells in spleen in reconstituted MOG$^{-/-}$ (upper row) or C57BL/6 mice (lower row). Numbers indicate the percentage of Foxp3$^+$ cells. **B.** Representative FACS plots showing expression of Helios and Foxp3 in CD45.1$^+$CD4$^+$Foxp3$^+$ Treg cells in spleen of reconstituted MOG$^{-/-}$ (upper row) or C57BL/6 mice (lower row). Numbers indicate the percentage of Helios$^+$ cells. **C.-E.** Expression of CD25, CD62L and GITR on splenic CD45.1$^+$CD4$^+$Foxp3$^+$ Treg cells (left) and representative FACS plots of retrogenic C57BL/6 mice reconstituted with HSC expressing the TCR T6-348 (right). **F.** Absolute number of donor-derived CD4$^+$Foxp3$^-$ Tconv cells in the spleen of retrogenic mice.
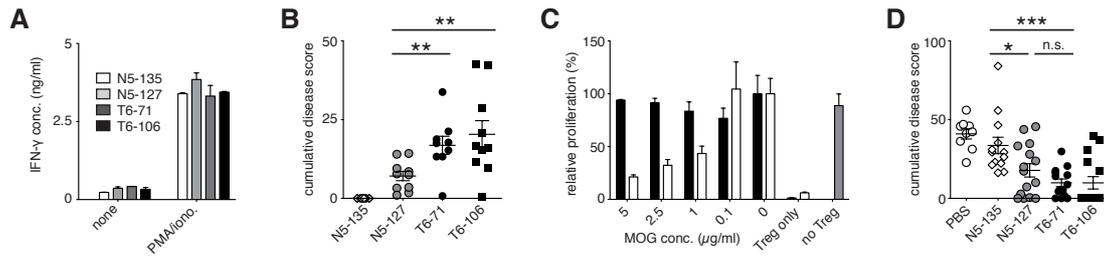
Figure S5, related to Figure 5 │ TCR functional avidity determines the function of MOG-reactive Treg and Tconv cells.
A. CD4$^+$CD25$^-$ Tconv cells were transduced with retroviral vectors encoding for one of four different MOG-reactive TCR (N5-135, N5-127, T6-71, T6-106), and subsequently stimulated or not with PMA and ionomycin for 24 h. IFN-γ concentration in supernatant was measured by ELISA. Values show means of duplicates + sem. One representative of two experiments. **B.** Data show the cumulative disease scores for the groups of mice shown in Figure 5D, and are pooled from two experiments. **C.** CD4$^+$ T cells from OT-II TCR-transgenic mice were activated with OVA(323-339) peptide (2 µg/ml) for three days in the presence of Tregs transduced to express the T6-106 TCR and different amounts of MOG(35-55). Proliferation was measured by ^{3}H-thymidine incorporation, and standardized relatively to cultures not containing Treg cells (100%). Black bars correspond to conditions with untransduced polyclonal Treg cells. White bars correspond to conditions with transduced Treg cells expressing T6-106 TCR. Bars show means of duplicates + sem. One representative of two experiments. **D.** Data show the cumulative disease scores for the groups of mice shown in Figure 5F, and are pooled from three experiments.
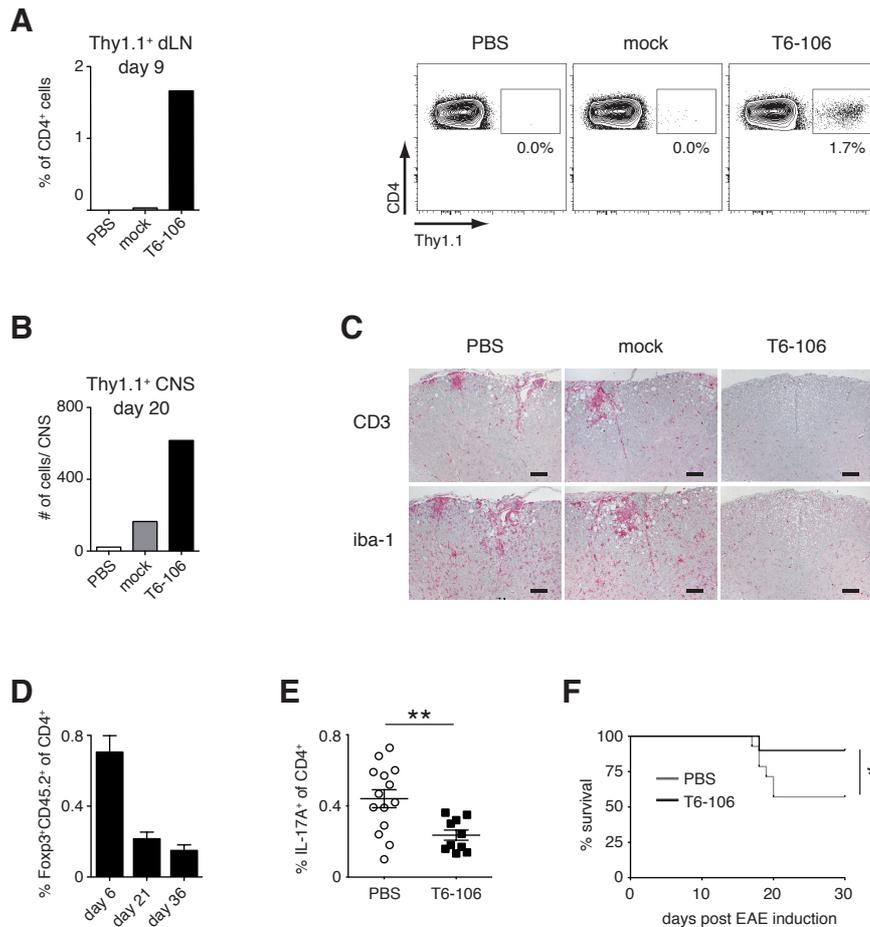
Figure S6, related to Figure 6 │ Protective effect of engineered MOG-reactive Treg cells in EAE.

This supplemental figure provides complementary information for the experiments described in Figure 6. **A.** Data show percentages of transduced Thy1.1^{+} cells among total CD4^{+} T cells in draining LN (popliteal and inguinal), and representative stainings for expression of CD4 and Thy1.1 among CD4^{+} T cells, for the mice analysed in Figure 6B. **B.** Data show absolute numbers of transduced Thy1.1^{+} T cells in CNS (brain and spinal cord) of mice shown in Figure 6B analysed on day 20 after EAE induction. One representative of two experiments. **C.** Immunohistochemistry of spinal cord sections from Treg-treated and control mice collected on day 20 after EAE induction, as in B. **D.** Naïve CD45.1[+] C57BL/6 mice received 2x10^{6} T6-106 TCR-modified CD45.2^{+} Treg cells (n=4) or PBS (n=5), and persistence of the cells in blood was followed over time. Shown is the percentage of CD45.2^{+}Foxp3^{+} cells among total CD4^{+} cells. One representative of two experiments. **E.** Mice from the experiment shown in Figure 6D were bled on day 10 after immunization, and blood samples were re-stimulated with MOG(35-55) for 5 h to quantify the percentage of IL-17A-secreting cells among CD4^{+} cells by flow cytometry. Data pooled from two experiments. **F.** Survival curves of mice from Figure 6D. Data pooled from two experiments.

Sequence ID	V	CDR3	J
T5-15	TRAV4D-3*03	CAAELTGNTGKLIF	TRAJ37*01
T5-35	TRAV4D-3*03	CAASNTNKVVF	TRAJ34*02
T5-431	TRAV4D-3*03	CAAEHRNYQLIW	TRAJ33*01
T5-748	TRAV4D-3*03	CAAEPARSNAKLTF	TRAJ42*01
T5-9	TRAV4D-3*03	CAAEVAGNTGKLIF	TRAJ37*01
T6-106	TRAV7D-2*02	CAASGANTGKLTF	TRAJ27*01
T6-145	TRAV4D-3*03	CAAGNSAGNKLTF	TRAJ17*01
T6-348	TRAV4D-3*03	CAAGNSNNRIFF	TRAJ31*01
T6-71	TRAV7D-2*02	CAASGANTGKLTF	TRAJ52*01
T6-91	TRAV4D-3*03	CAAEITGNTGKLIF	TRAJ37*01
T7-925	TRAV4D-3*03	CAADGGSNAKLTF	TRAJ42*01
T9-360	TRAV4D-3*03	CAANYAQGLTF	TRAJ26*01
T11-1722	TRAV4D-3*03	CAADYANKMIF	TRAJ47*01
T11-2161	TRAV7D-2*02	CAASKANTGKLTF	TRAJ27*01
T12-0364	TRAV4D-3*03	CAAPGNTGKLIF	TRAJ37*01
T12-0485	TRAV6N-7*01	CALGSYQGGRALIF	TRAJ15*01
T12-1115	TRAV4D-3*03	CAAEYNQGKLIF	TRAJ23*01
T12-1359	TRAV4D-3*03	CAAEAGNNRIFF	TRAJ31*01
T13-0964	TRAV4D-3*03	CAASNYQLIW	TRAJ33*01
N5-127	TRAV4D-3*03	CAARNTNTGKLTF	TRAJ27*01
N5-135	TRAV13N-1*01	CAMGGGSNAKLTF	TRAJ42*01
N5-197	TRAV4D-3*03	CAATASSSFSKLVF	TRAJ50*01
N5-307	TRAV4N-3*01	CAAGNYNVLYF	TRAJ21*01
N6-240	TRAV4N-4*01	CAAGNSAGNKLTF	TRAJ17*01
N6-287	TRAV13-2*02	CAPTNSAGNKLTF	TRAJ17*01
N8-068	TRAV4N-3*01	CAARNTNTGKLTF	TRAJ27*01
N8-309	TRAV4D-3*03	CAAGNYNVLYF	TRAJ21*01
N12-0300	TRAV13D-1*02	CAFFNSAGNKLTF	TRAJ17*01
N14-1928	TRAV7D-2*02	CAASPGNTGKLIF	TRAJ37*01
N14-2389	TRAV7D-2*02	CAGGTGNTGKLIF	TRAJ37*01

Table S1, related to Figure 2 | List of TCRα chains tested for MOG reactivity. This table provides the Vα-CDR3-Jα usage for the TCRα chains from Treg and Tconv cells that were re-expressed in T.54ζ17 cells (Figure 2).

Supplemental experimental procedures

Mice

C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Rag$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}), Rag$^{-/-}$CD45.1 (B6.SJL(129S6)-Ptprc^a/BoyCrTac-Rag2^{tm1Fwa}), B6.CD45.1 (B6.SJL-Ptprc^a/BoyAiTac) were bred under specific pathogen-free conditions at the Bundesinstitut für Risikobewertung (Berlin, Germany). MOG$^{-/-}$ (MOGi-cre) (Hovelmeyer et al., 2005) mice were a kind gift of A. Waisman (Universitätsmedizin Mainz, Germany). Kaa mice express the public TCRβ chain (TRBV13-2/TRBD2, TRBJ2-1, CDR3: CASGETGGNYAEQFF (Fazilleau et al., 2006). This TCRβ was amplified from the P32.2 MOG-reactive hybridoma (Sweenie et al., 2007), and cloned into the huCD2 minigene vector (Zhumabekov et al., 1995), which was used to generate the transgenic Kaa mouse. In brief, DNA was injected at a concentration of 2 ng/μl into the pronucleus of donor zygotes from superovulated 5-6 weeks old C57BL/6J female mice. Subsequently, 2-cell stage embryos were transferred into the oviducts of pseudopregnant CD1 recipients. Kaa mice were crossed to Foxp3.IRES.eGFP (Wang et al., 2008) and Tcra$^{-/-}$Tcrb$^{-/-}$ mice (Mombaerts et al., 1992) to obtain Kaa$^+$Foxp3.IRES.eGFP$^+$Tcra$^{+/-}$Tcrb$^{+/-}$ mice.

Peptides used for active EAE induction and *in vitro* assays

MOG(35-55) peptide (MEVGWYRSPFSRVVHLYRNGK) and the alanine-substituted 49(H_A) peptide variant used for immunization experiments were produced at the Institut für Medizinische Immunologie, Charité (Berlin, Germany). OVA(323-339) peptide (ISQAVHAAHAEINEAGR) was used as an unspecific control. Peptides used for *in vitro* stimulation assays were obtained from JPT Peptide Technologies or Biosyntan (Germany).

Antibodies and Flow cytometry

After blocking of Fc receptors (2.4G2), surface stainings were done with mAbs against CD3 (145-2C11), CD4 (GK1.5/RM4-5), CD8α (53-6.7), CD19 (6D5), CD45.1 (A20), CD45.2 (104), Thy1.1 (OX-7) Vβ8.1/8.2 (KJ16-133.18), Vβ8.2/8.3 (CT8E), CD49d (R1-2), CD25 (PC61), GITR (DTA-1) and CD62L (MEL-14). For intracellular stainings, cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience, San Jose, CA, USA) or Foxp3 staining buffer set (eBioscience, San Diego, CA, USA) and stained with mAbs against IFN-γ (XMG1.2), TNF-α (MP6-XT22), IL-17A (TC11-18H10.1), GM-CSF (MP1-22E9), CD40L (MR1), CTLA-4 (UC10-4B9), Foxp3 (FJK-16s), and Helios (22F6). Antibodies were from Biolegend (San Diego, CA, USA), eBioscience, BD, Miltenyi Biotec (Bergisch Gladbach, Germany) or produced in our facility. For staining with MOG-I-A^b or unspecific tetramer (from NIH facility) 10^6 cells were incubated for 3 h at 37 °C with 5 μl tetramer in 500 μl RPMI medium. Data were acquired on FACS Calibur, LSRII or CantoII (BD) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). Flow cytometry was performed according to standard procedure (Shen et al., 2014).

Treg cell adoptive transfers

For experiments described in Fig. 1F and 1G, Treg cells were isolated from pooled spleen and LN from naïve C57BL/6 or Kaa mice using a Dynabeads® Untouched^{TM} Mouse CD4 Isolation kit (Life technologies). The untouched fraction was then used to isolate CD25$^+$ cells using biotinylated anti-CD25 (BD Pharmingen) and biotin-magnetic beads (Miltenyi Biotech). For some experiments, the isolated Treg cells were labelled with 10 μM eFluor450 proliferation dye (eBioscience). C57BL/6 mice received 2-3x10^6 Treg cells intravenously and were immunized 12-14 h later with MOG(35-55) to induce EAE(Fig. 1F), or received 2x10^5 labelled Treg cells intravenously and were immunized immediately (Fig. 1G).

For experiments described in Fig. 5-7, LN and spleen were collected from naïve C57BL/6 mice, subjected to a mouse CD4$^+$ T cell pre-enrichment EasySep kit (STEMCELL technologies), and CD25$^+$ cells were subsequently obtained using a mouse CD25 positive selection EasySep kit (STEMCELL technologies). The obtained cells were then activated on anti-CD3/anti-CD28-coated plates in presence of IL-2 (750 U/ml), and transduced on day 2 and 3 with MP71 retroviral supernatants. C57BL/6 mice received 2x10^5 Foxp3$^+$Thy1.1$^+$ transduced Treg cells for prophylactic treatments (Fig. 5F and G, 6A and B, and 7), and 2x10^6 Foxp3$^+$Thy1.1$^+$ transduced Treg cells for therapeutic treatment (Fig. 6C) and for the experiment described in Fig. 6D.

Proliferation assay of CD25-depleted Kaa splenocytes
Complete or CD25–depleted naïve Kaa splenocytes were stimulated in 96 well-plates ($8x10^5$ cells/well) with MOG(35-55) in indicated amounts, and 48 h later 1 Ci ^{3}H-thymidine was added per well. Proliferation was quantified after an additional 18 h incubation with a Top-count NXT liquid scintillation counter (Perkin Elmer). CD25$^+$ cells were depleted via magnetic cell sorting using biotinylated anti-CD25 and anti-biotin magnetic beads (Miltenyi Biotec).

Preparation of mononuclear cells from CNS
Brain and spinal cord were removed from perfused mice and digested with collagenase (Worthington Biochemical) and DNase (Sigma) followed by mechanical disaggregation. Mononuclear cells were isolated on a 30% Percoll gradient (GE Healthcare) by centrifugation for 20 min at 2000 rpm.

Immunohistochemistry
Sections of 1-2 µm of formalin-fixed, paraffin-embedded spinal cord tissue were deparaffinized, and subjected to heat-induced epitope retrieval. T cells and activated microglia were stained with polyclonal rabbit antibodies against either CD3 (Dako) or iba-1 followed by detection with biotinylated donkey anti-rabbit antibody (Dianova) and the LSAB$^+$, Dako REAL™ Detection System using Fast Red as chromogen (Dako). Scale bar size is 100 µm.

Detection of cytokine production by ELISA and intracellular cytokine staining
TCR-expressing T.54ζ17 cells were seeded in 96-well flat-bottom plates (10^4 cells/ well) together with either MOG(35-55) or one of the alanine-substituted MOG peptides (2 µg/ml), except if otherwise indicated, and $3x10^5$ irradiated splenic APCs. Supernatants were harvested after 48 h, and IL-2 concentration in supernatant was measured by ELISA, as previously described (Calderon-Gomez et al., 2011).

For detection of CD40L, IFN-γ, IL-17A, and GM-CSF expression by blood CD4$^+$ T cells, blood was collected and immediately subjected to red blood cell lysis. Cells were subsequently stimulated in 96-well flat bottom plates at $6x10^5$ cells / well with 20 µg/ml MOG(35-55) for 6 h in the presence of GolgiStop (BD Biosciences). Cells were then stained as previously described (Calderon-Gomez et al., 2011).

For detection of IFN-γ, IL-17A, TNF-α, and GM-CSF expression by Kaa CD4$^+$ T cells (Fig. 1A and B), activated cells were re-stimulated on day 3 of culture with PMA and ionomycin (Sigma) for 5 h in the presence of Golgi stop (BD Pharmingen). Cells were then stained as previously described (Calderon-Gomez et al., 2011).

IFN-γ was quantified in cell supernatant after 24 h of cultures using a BD OptEIA ELISA kit.

Sequences of miRNA for silencing of CD49d, GITR and control miRNA
The *Cd49d*-specific antisense sequence was TGA ACA GTC AGC ATA ACC TCA, which was inserted into two different miRNA environments. The *Tnfrsf18*-specific antisense sequences AAT CCA AAC TGA GAA CAG TTG and AAC AGT TGG TCC AAA GTC TGC were inserted into miR-155 and the artificial miRNA, respectively. Control miRNAs were generated by inserting the antisense sequence AAA TTA TTA GCG CTA TCG CGC into the same two environments.

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