SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents, antibodies and flow cytometry. Fluorochrome-conjugated antibodies were purchased from BD-Biosciences, eBiosciences, and Biolegends. For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) and ionomycin (1.0 μg/ml) for 4-6 hours in the presence of Brefeldin A and Monensin. Intracellular staining for Foxp3, RORγt, CTLA-4 and Ki-67 was performed by Foxp3 staining kit provided by eBiosciences. Samples were analyzed by FACS Calibur or LSR II Fortessa or FACS Verse (BD Biosciences) and data were analyzed with FlowJo software.

Isolation of ICOS^{hi}CCR6^{+} Treg cells and tetramer staining. Foxp3$^{YFP\text{-}Cre}$ or Foxp3^{GFP} reporter mice were immunized with 150 μg $MOG_{35\text{-}55}$ peptide (RN Synthesis, KY) emulsified in incomplete Freund's adjuvant (IFA) supplemented with *Mycobacterium tuberculosis* extract (H37Ra, Difco). Seven days after immunization, total CD4^{+} T cells were isolated from draining lymph nodes by positive selection by AutoMACS (Miltenyi Biotec). CD4^{+} T cells were further sorted into YFP^{+}(or GFP^{+})ICOS^{hi}CCR6^{+}CD4^{+} cells, YFP^{+}(or GFP^{+})ICOS^{lo}CCR6^{-}CD4^{+} cells, YFP^{+}(or GFP^{+})ICOS^{hi}CCR6^{-}CD4^{+} cells or YFP^{-}(or GFP^{-})ICOS^{hi}CCR6^{+}CD4^{+} cells by FACS ARIA II or FACS Fusion (BD Biosciences). For MOG-specific CD4^{+} T cell detection, draining lymph node cells were incubated with $MOG_{38\text{-}49}$-I-A^{b} or $hCLIP_{103\text{-}117}$-I-A^{b} tetramer (NIH Tetramer Core Facility) at room temperature for 1 hour.

RNA preparation and RT-PCR. Total RNA was extracted with TRIZOL reagent (Invitrogen). cDNA was prepared with Superscript III reverse transcriptase (Invitrogen) and gene expression was analyzed by iCycler Optical System (Roche) and SYBR green kit (Bio-Rad). The primer sequences were previously described (Yang et al., 2008).

RNA-sequencing. Total RNA was extracted from FACS-sorted cells with TRIZOL reagent. Raw read counts were obtained by SOLiD v4 sequencing system and then mapped to mouse genome. After a normalization of read counts, fold changes were calculated using genes only with normalized read count greater than or equal to 10 at least in one sample. For fold change/fold change comparison between LN and colonic RORγt^{+} Treg cells, transcriptome profiling data of colonic RORγt^{+} Treg were adopted (Sefik et al., 2015). Any gene that has a fold change value bigger than or equal to +2.0 and smaller than or equal to -2.0 was considered differentially expressed.

In vitro induction of RORγt^{+}CCR6^{+} Treg cells. FACS-sorted CD4^{+}CCR6^{-}GFP^{+}(or CD25^{hi}) Treg cells (5.0 x 10^4 cells/well) isolated from splenocytes of Foxp3^{GFP} or Stat3$^{f/f}$CD4^{Cre} mice were stimulated with plate-bound anti-CD3 Ab and plate-bound anti-CD28 Ab in the presence of titrated dose of recombinant mouse IL-6 (Peprotech) or recombinant mouse IL-10 (Peprotech). Four days after culture, RORγt and CCR6 expressions in gated Foxp3^{+} cells were analyzed by flow cytometry.

Retroviral transduction. CD4^{+}CCR6^{-}CD25^{hi} Treg cells isolated from RORγ$^{f/f}$ or RORγ$^{f/f}$CD4^{Cre} mice were stimulated with plate-bound anti-CD3 Ab and plate-bound anti-CD28 Ab in the presence of recombinant human IL-2. Twenty-four hours later, culture supernatant containing GFP-expressing empty retroviral vector or RORγt-GFP-expressing retroviral vector produced by 293T cells was added and the cells were infected with the vectors by spin infection as previously described (Ichiyama et al., 2015). Cells were further stimulated for three days after spin infection and then CCR6 expression in GFP^{+} cells was analyzed by flow cytometry.

In vitro Treg suppression assay. FACS-sorted, CD4^{+}ICOS^{hi}CCR6^{+}GFP^{-} Th17 cells or CD4^{+}ICOS^{hi}GFP^{-} effector T cells isolated from MOG/CFA immunized CD45.2^{+}Foxp3^{GFP} mice were stimulated with soluble anti-CD3 Ab (1.0 μg/ml) plus irradiated CD45.1^{+} T cell-depleted splenocytes (TdS) or splenic dendritic cells. Serially diluted numbers of ICOS^{hi}CCR6^{+}CD4^{+}GFP^{+} cells, ICOS^{lo}CCR6^{-}CD4^{+}GFP^{+} cells and ICOS^{hi}CCR6^{-}CD4^{+}GFP^{+} cells isolated from CD45.1^{+}CD45.2^{+} Foxp3^{GFP} mice were added to the culture. In some experiments, effector T cells were labeled with 2.0 μM carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher Scientific). Three days later, IL-17A, IL-17F, IL-22 and IFN-γ production in the culture supernatant was measured by ELISA. For Ag-specific Th17 cell suppression, MOG_{35-55} peptide (50 μg/ml) instead of anti-CD3 Ab was added to the culture. To test the involvement of IL-10 signaling, IL-10R Ab (1B1.3A, BioXCell) or ratIgG (HRPN, BioXCell) was added to the culture. Cytokine production in the culture supernatant was analyzed at day 3 after culture by ELISA.

SUPPLEMENTAL REFERENCES

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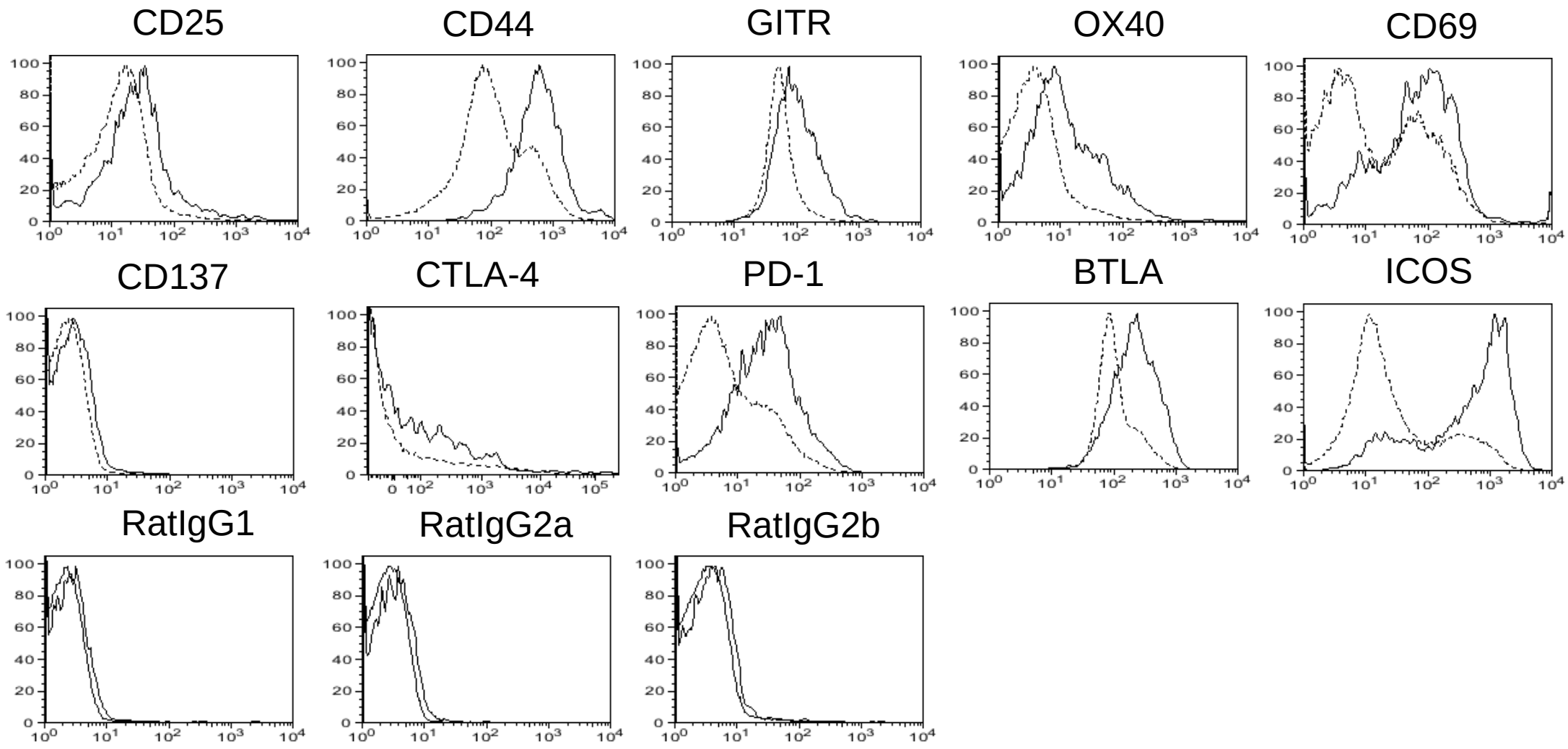


Figure S1. Cell surface marker expression in RORγt^{+}CCR6^{+} non-Treg cells. Related to Figure 2.
Indicated cell surface markers expression in gated CD4^{+}Foxp3^{-}RORγt^{+}CCR6^{+} cells (Solid) and CD4^{+}Foxp3^{-} RORγt^{-}CCR6^{-} (Dashed) were analyzed as described in Figure 2A. Data are representatives of two independent experiments.

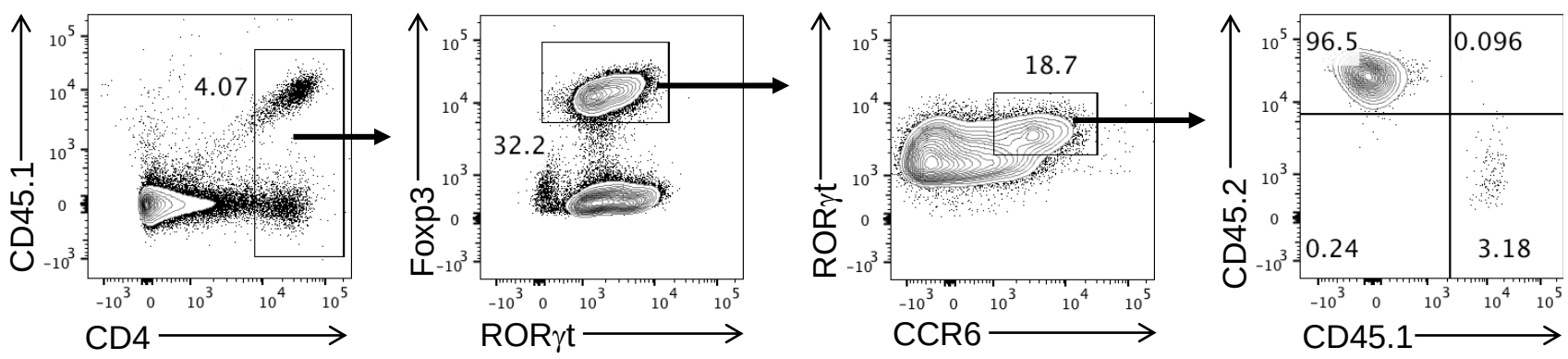


Figure S2. Thymic origin of RORγt^{+}CCR6^{+} Treg cells. Related to Figure 3. Thymic CD4^{+}GFP^{+} Treg cells isolated from Foxp3^{GFP} reporter mice (CD45.2^{+}) were mixed with CD4^{+}CD25^{-} CD44^{lo}CD62L^{+} naïve T cells isolated from B6SJL mice (CD45.1^{+}) in the 1:9 ratio and then were adoptively transferred to TCRβ$^{-/-}$ recipient mice. One day after the adoptive transfer, recipient mice were immunized with MOG/CFA. Seven days after immunization, CD45.2 and CD45.1 expression in gated CD4^{+}Foxp3^{+}RORγt^{+}CCR6^{+} cells in draining inguinal LNs were analyzed. Data are representatives of two independent experiments.

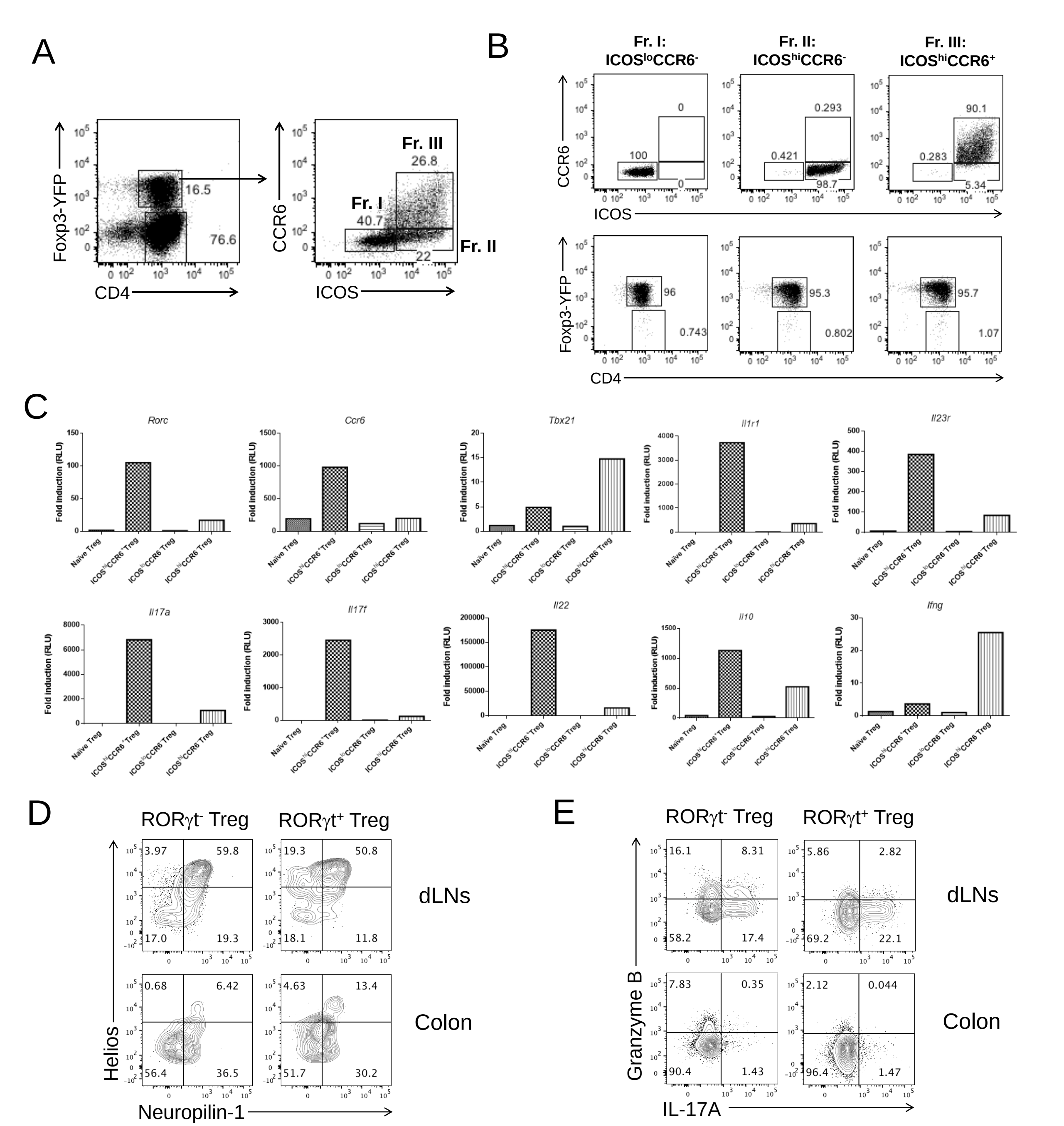
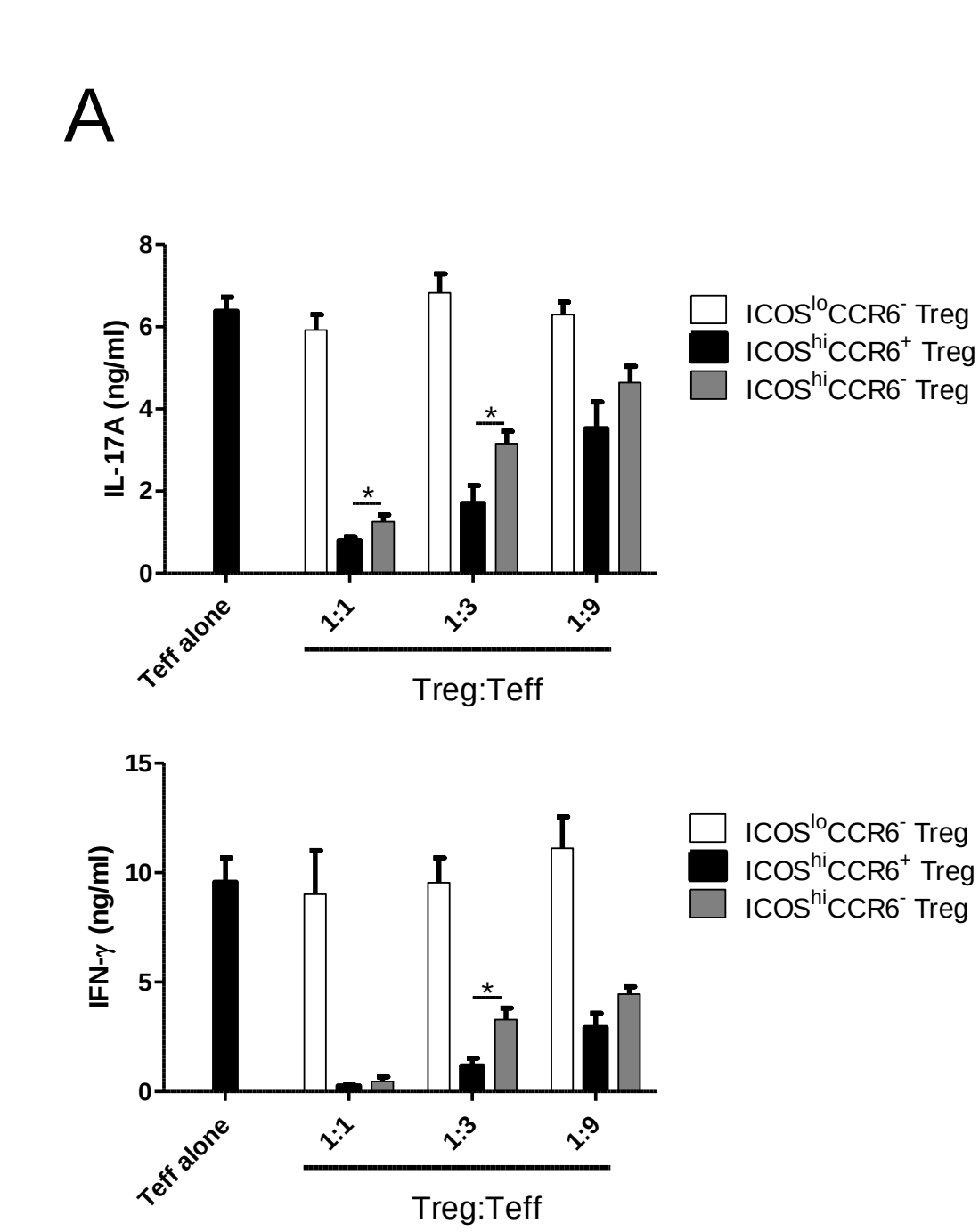
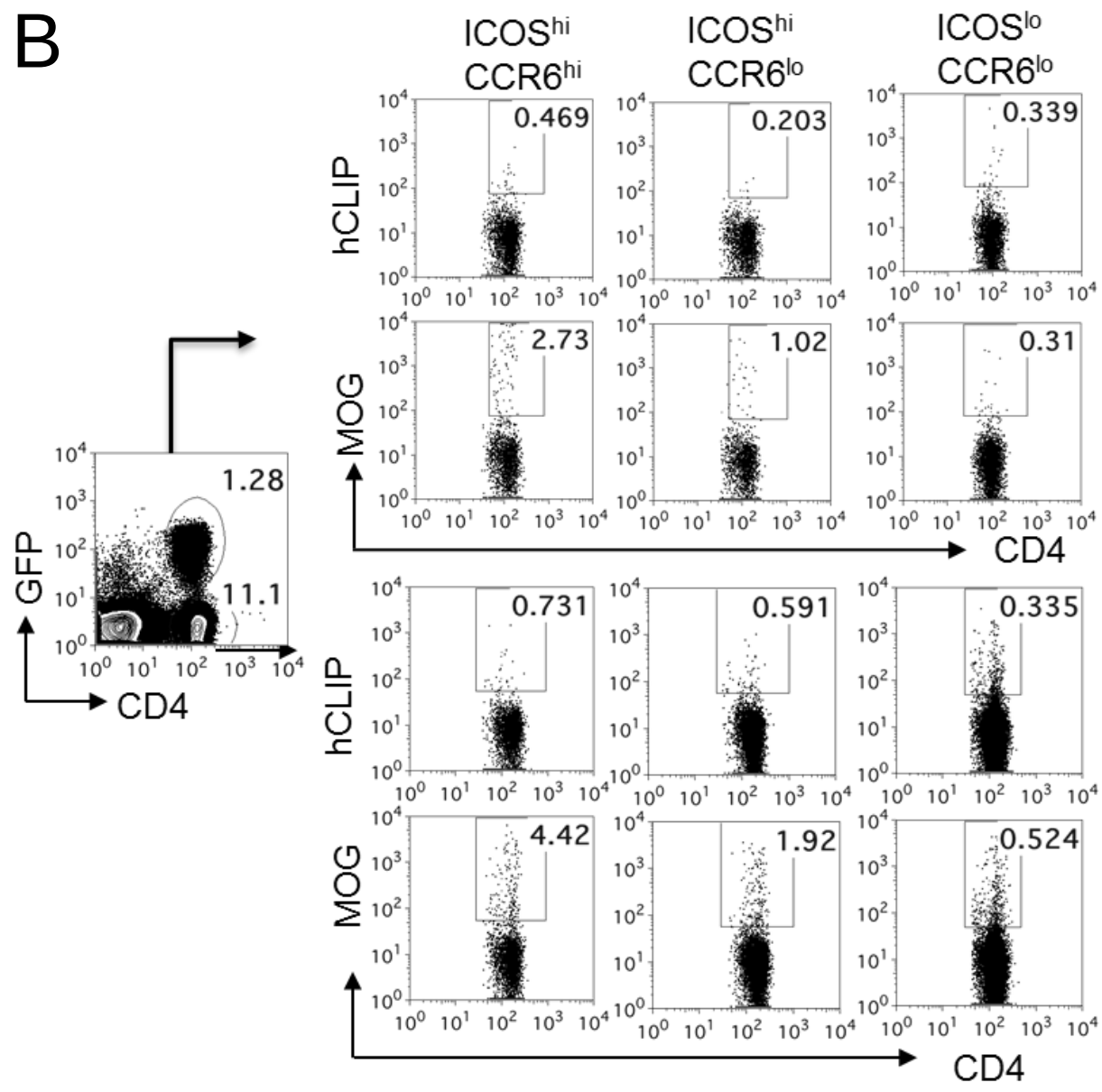
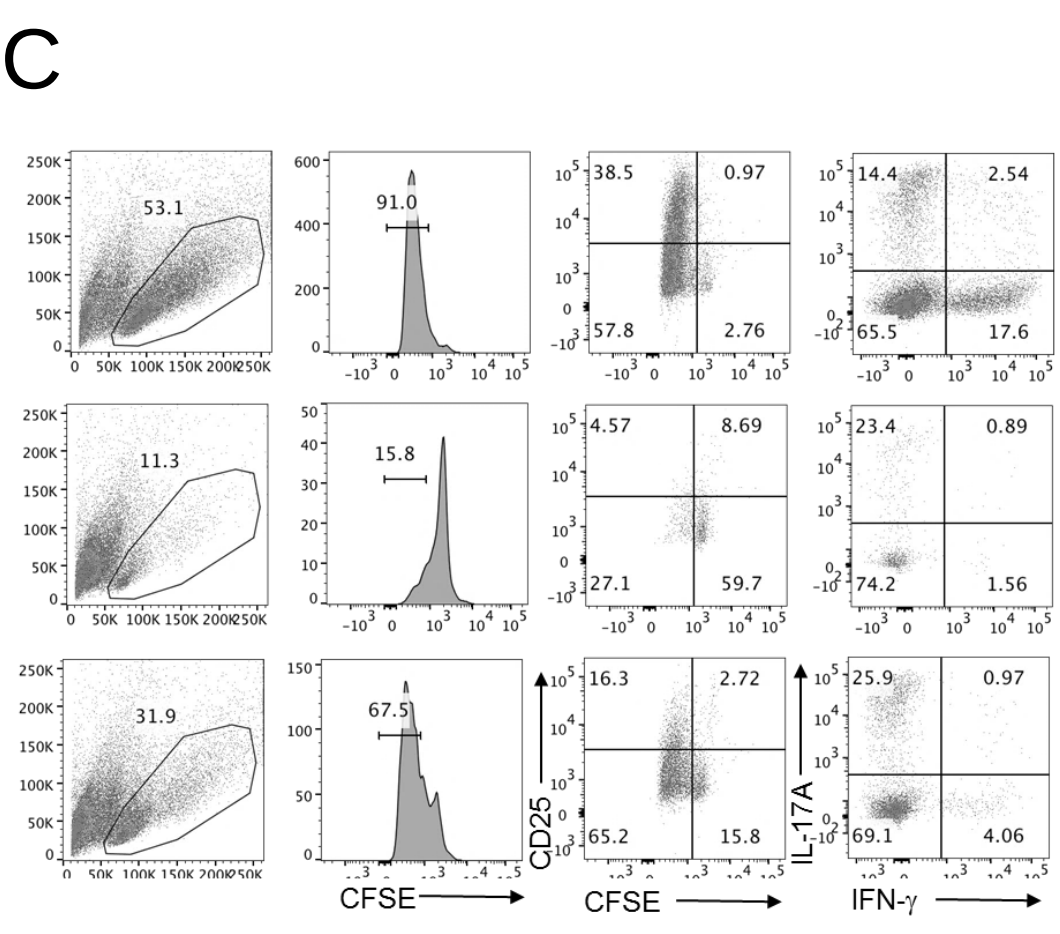


Figure S3. Characterization of RORγt$^+$ Treg cells with different anatomic locations. Related to Figure 4. (A) Seven days after MOG/CFA s.c immunization, dLNs were isolated from Foxp3$^{YFP\text{-}Cre}$ reporter mice. MACS-sorted CD4$^+$ T cells were further purified into three independent Treg cell populations based on YFP, ICOS and CCR6 expression. (B) Purity of the FACS-sorted populations. (C) Cells were isolated as described in A and *Supplemental Experimental Procedures*. Relative expression of indicated genes in different cell populations was analyzed by real time RT-PCR. Data are representatives of two independent experiments. (D) Intracellular expression of Helios and cell surface expression of Neuropilin-1 in RORγt$^+$ or RORγt$^-$ Treg cells in draining LN (dLN) or colon of MOG/CFA-immunized mice were analyzed by flow cytometry. (E) IL-17A and Granzyme B expression was analyzed as in D after PMA/Ionomycin restimulation.







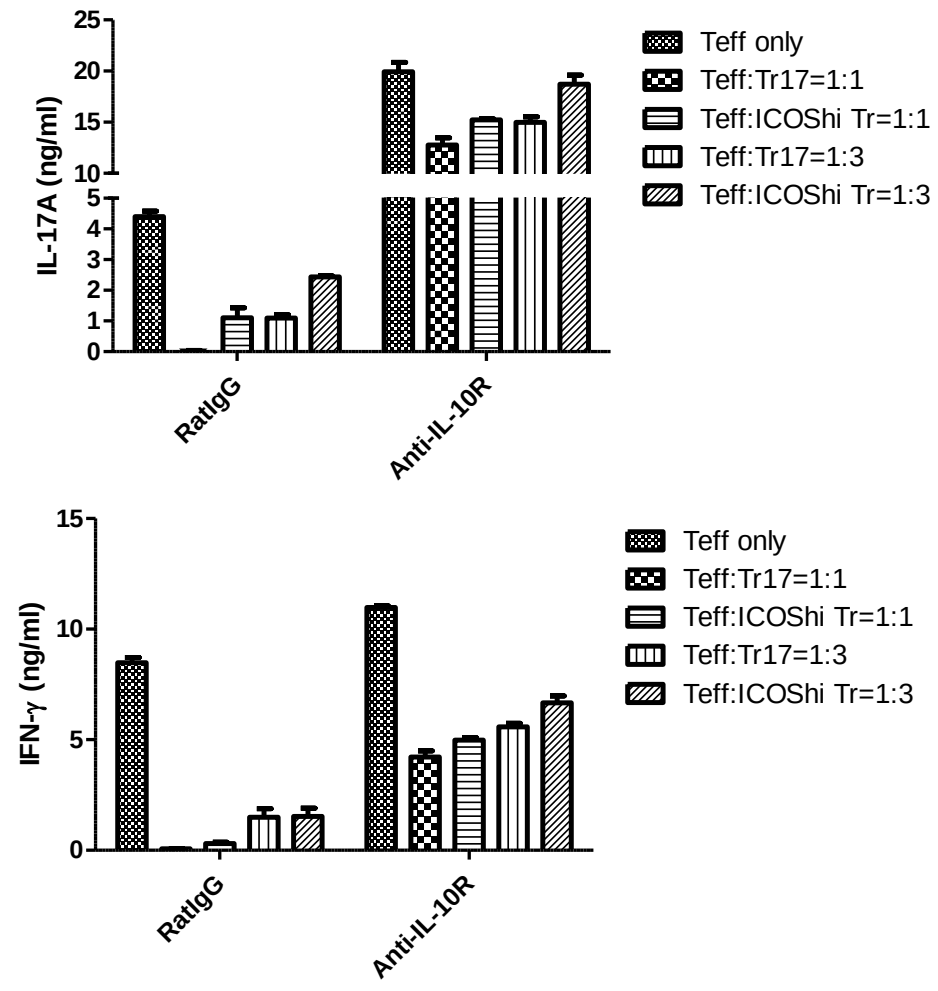
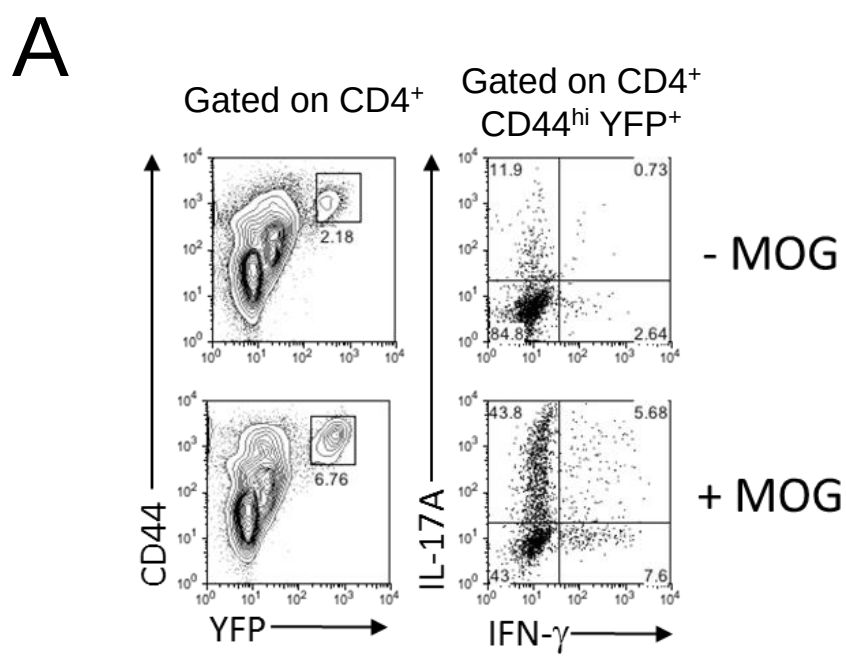


Figure S4. Involvement of IL-10/IL-10R signaling in the suppression of Th17 and Th1 cells by ICOS^{hi}CCR6^{+}RORγt^{+} Treg cells. Related to Figure 6. (A) ICOS^{hi}GFP^{-}CD4^{+} T effector cells (Teff) were stimulated with irradiated splenocytes in the presence of MOG peptide. Titrated doses of indicated Treg cells were added to the culture. Cytokine levels in the culture supernatant were analyzed by ELISA three days after culture. (B) Representative flow cytometry plot for tetramer staining in Figure 6B. (C) Suppressive activity of ICOS^{hi}CCR6^{+} Treg (Tr17) cells against ICOS^{hi}GFP^{-}CD4^{+} T effector cells (Teff) was analyzed as described Figure 6C except that MOG peptide was replaced with soluble anti-CD3 Ab. (D) ICOS^{hi}GFP^{-}CD4^{+} T effector cells (Teff) were stimulated as in (A). Indicated numbers of Tr17 cells or ICOS^{hi}CCR6^{-} Treg (ICOS^{hi} Tr) cells were added to the culture. Anti-IL-10R or control ratIgG was added. Cytokine levels in the culture supernatant were analyzed by ELISA three days after culture. Data are representatives of at least two independent experiments. *, $p<0.05$. **, $p<0.005$.



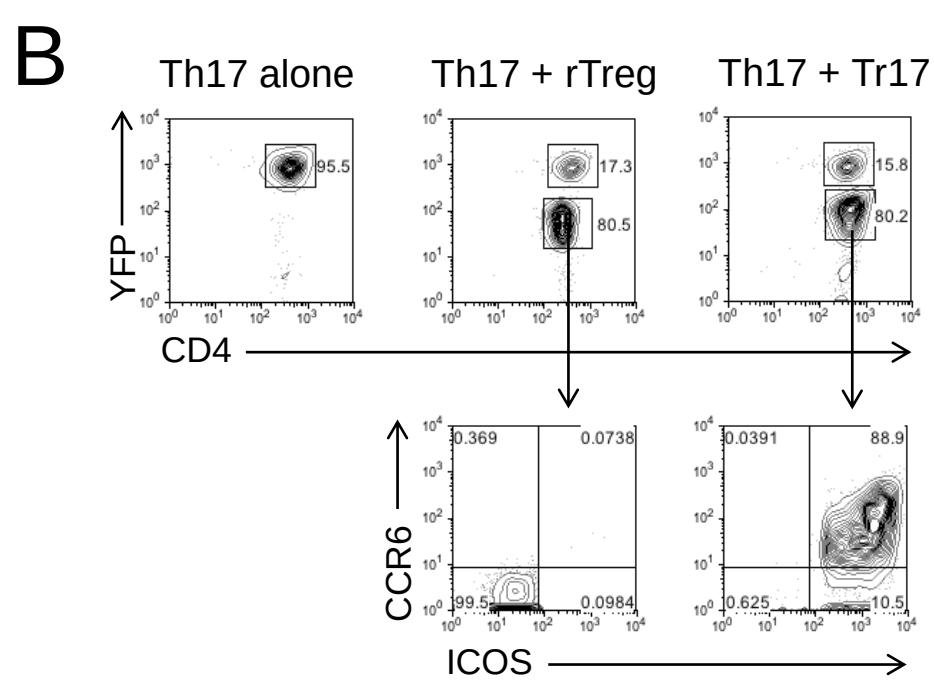


Figure S5. Purity of the FACS-sorted Th17 alone, Th17+Tr17 and Th17+rTreg cells. Related to Figure 7. (A) Draining lymph node cells isolated from MOG/CFA-immunized IL-17F^{Cre}xRosa26^{YFP} fate mapping mice were restimulated with MOG peptide for 48hrs. Brefeldin A and monensin were added for last 6hrs of culture. IL-17A and IFN-γ production by gated CD4^{+}CD44^{hi}YFP^{hi} cells was analyzed by flow cytometry. (B) Purity of FACS-sorted Th17 alone, Th17+Tr17 or Th17+rTreg cells described in Figure 7 was analyzed by flow cytometry before adoptive transfer into RAG1$^{-/-}$ mice.