SUPPLEMENTAL MATERIAL

Schallenberg et al., http://www.jem.org/cgi/content/full/jem.20100045/DC1

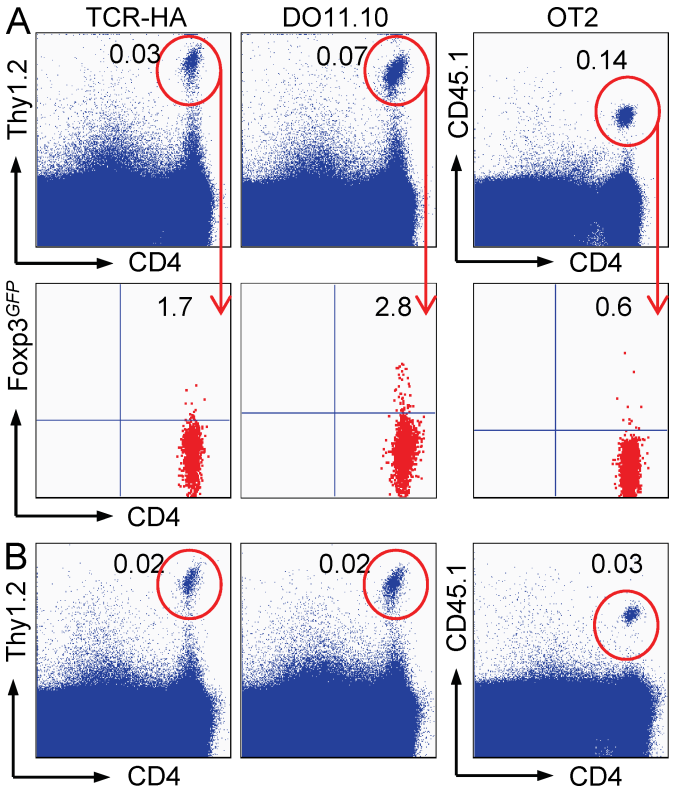

Figure S1. Phenotype of adoptively transferred CD4⁺ T cells in untreated recipients. Flow cytometry of indicated congenic marker⁺ TCR transgenic CD4⁺ T cells at day 7 (A) or day 14 (B) after adoptive transfer into recipient animals that were not treated with recombinant anti–DEC-205 antibodies fused to the respective Ag. See the legend for Fig. 1 (A and B) for details.

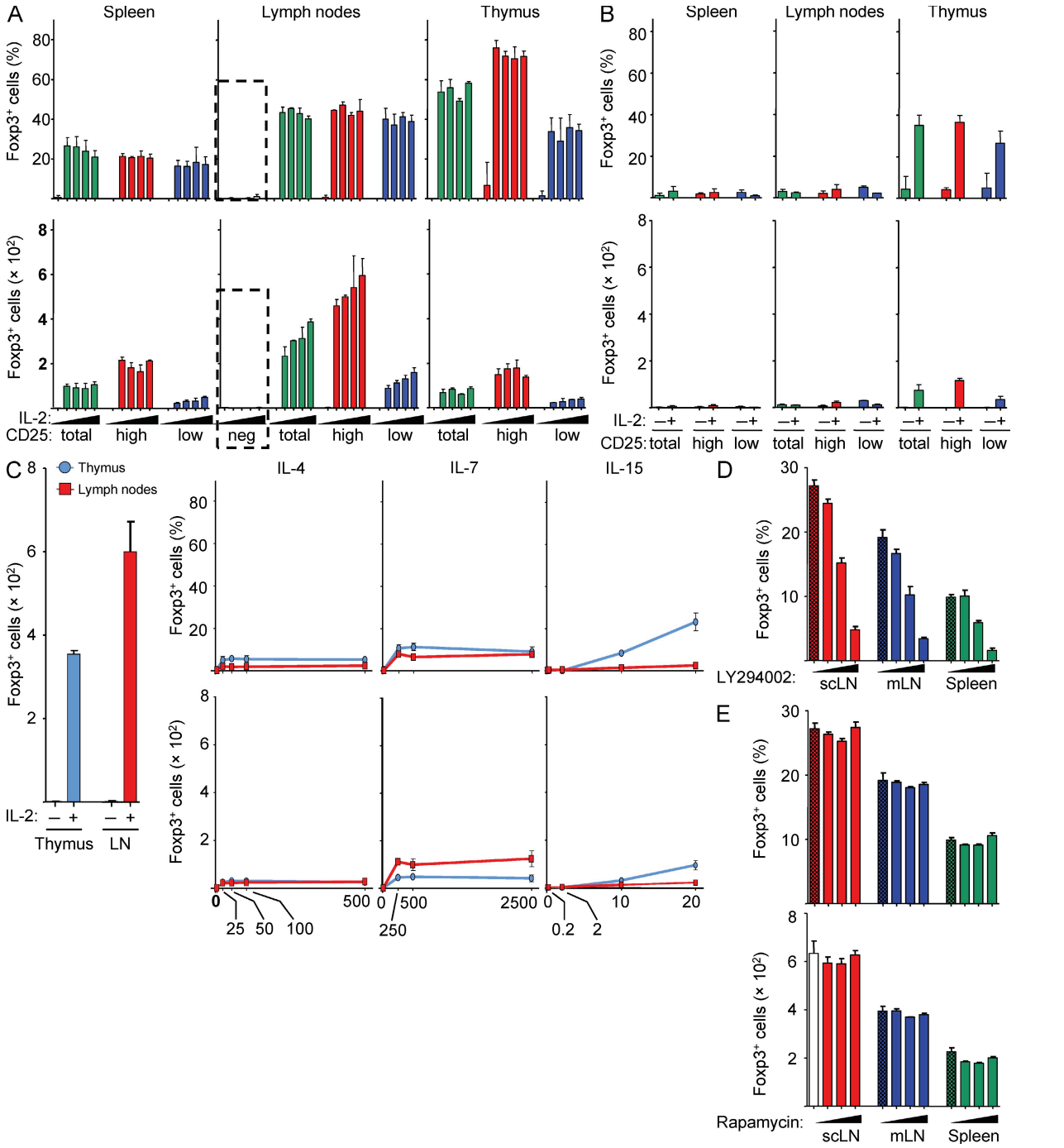

Figure S2. Effects of common gamma chain cytokines, TCR stimulation, and mTOR inhibition on precursor differentiation. (A and B) Percentages and absolute numbers of Foxp3^{GFP+} cells originating from initially CD4$^+$CD25$^+$Foxp3$^-$ cells of indicated lymphoid organs at day 3 of culture in the absence or presence of titrating amounts (10, 50, 200, or 1,000 U/ml) of IL-2 alone (A) or with added anti-CD3/CD28 T cell stimulation beads (B). CD25$^+$ total populations or cells with low or high CD25 expression levels were compared. Dotted line boxes highlight scLN-derived CD4$^+$CD25$^-$Foxp3$^-$ cells that did not appreciably up-regulate Foxp3^{GFP} expression. (C) FACS-purified CD4$^+$CD25$^+$Foxp3$^-$ cells from peripheral lymphoid organs and thymus of Foxp3^{GFP} mice were cultured with IL-2 alone (left) or in the presence of titrating amounts of IL-4, IL-7, or IL-15, as indicated. At day 3 of cultures, percentages and absolute numbers of Foxp3^{GFP} cells were determined. (D) Complementary to the experiments presented in Fig. 2 F, the percentage of initially CD4$^+$CD25$^+$Foxp3$^-$ cells from indicated lymphoid organs that up-regulated Foxp3^{GFP} expression in the presence of 1,000 U/ml IL-2 and titrating amounts of LY294002 (0, 2.5, 10, or 40 μM) at day 3 of culture is shown. (E) The impact of titrating amounts of the mTOR inhibitor rapamycin (0, 6.25, 25, or 100 nM) on in vitro up-regulation of Foxp3 expression in initially CD4$^+$CD25$^+$Foxp3$^-$ cells from indicated lymphoid organs was determined at day 3 of IL-2–containing cultures. Shown are mean values and SD of triplicate wells. Data are representative of at least two independent experiments.

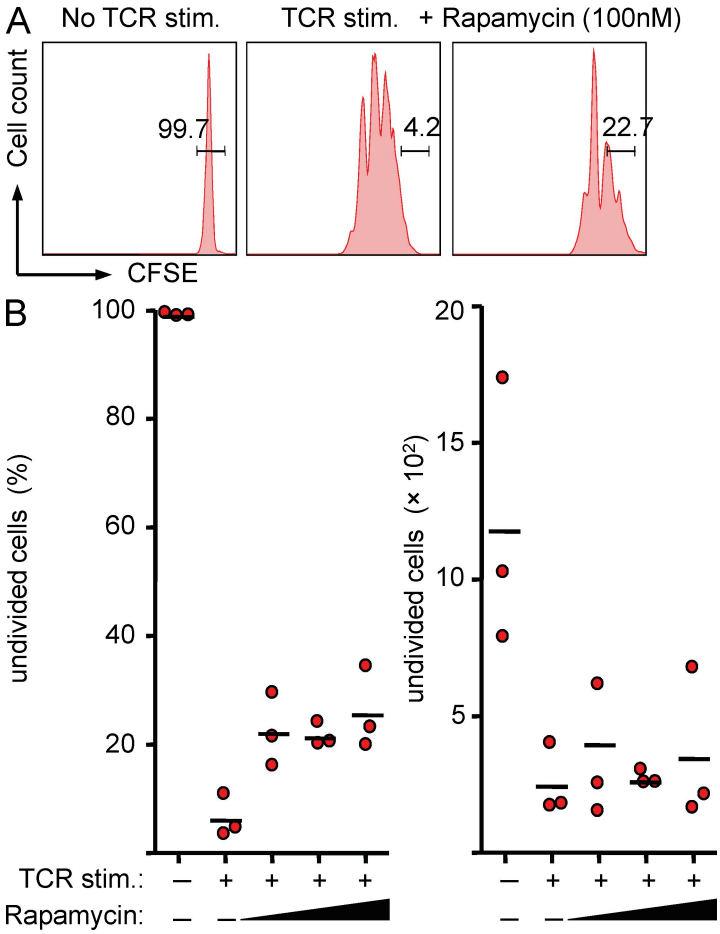

Figure S3. Activity of the mTOR inhibitor rapamycin. Flow cytometry of initially naive $CD4^+CD62L^+CD25^-$ T cells at day 3 of in vitro stimulation with anti-CD3/CD28 beads, either in the absence or the presence of titrating amounts of rapamycin (6.25, 25, or 100 nM). Before culture, cells were labeled with CFSE to track proliferation. (A) Representative flow cytometry of cell divisions. Numbers in histograms indicate the percentage of gated undivided cells. (B) Each dot represents the percentage (left) and absolute number (right) of undivided cells under indicated conditions in triplicate wells. Data are representative of at least two independent experiments. Horizontal bars indicate mean values.

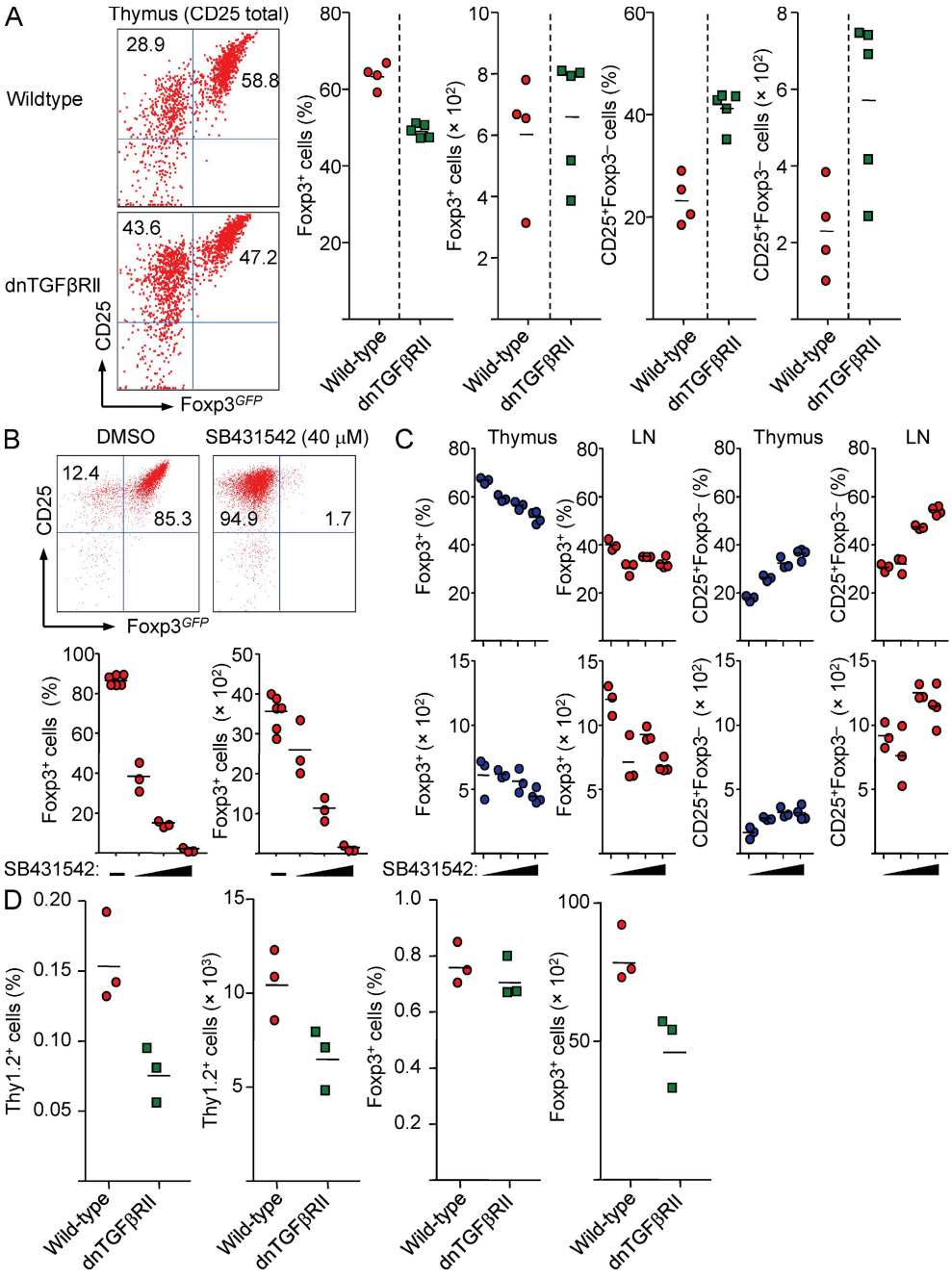

Figure S4. Impact of TGF-βR signaling on T reg precursor cell differentiation and induction of Foxp3 expression. (A) Capacity of thymic CD4+CD8−TCRβ+CD25+Foxp3− T reg precursor cell populations from dnTGF-βRII+ mice to up-regulate Foxp3 expression in IL-2–containing differentiation cultures. See the legend for Fig. 7 for details. (B) Activity of the Smad kinase inhibitor SB431542. Flow cytometry of initially naive CD4+CD62L+CD25− T cells from Foxp3^{GFP} mice at day three of in vitro stimulation with anti-CD3/CD28 beads in the presence of 5 ng/ml hTGF-β1, either in the absence or presence of titrating amounts of SB431542 (2.5, 10, and 40 μM). Top: representative flow cytometry of induced CD25 and Foxp3^{GFP} expression. Numbers in dot plots indicate the percentage of cells in the respective quadrant. Bottom: each dot represents the percentage (left) and absolute number (right) of Foxp3+ cells under indicated conditions in replicate wells. (C) Up-regulation of Foxp3 expression in initially CD4+CD25+ Foxp3− T reg precursor cells from thymus (CD8−) or LNs of Foxp3^{GFP} mice at day 3 of IL-2–containing cultures in the absence or presence of titrating amounts (2.5, 10, and 40 μM) of the Smad kinase inhibitor SB431542. See the legend for Fig. 8 (A–C) for details. (D) FACS-purified naive CD4+CD62L+CD25−Foxp3− T cells from peripheral lymphoid organs of dnTGF-βRII+ or WT Foxp3^{GFP} mice were injected into congenic recipient mice and analyzed by flow cytometry at day 7 after adoptive transfer for the ability to up-regulate Foxp3 expression. Each dot represents data from one individual mouse, and horizontal bars represent mean values. Data are representative of at least two independent experiments.

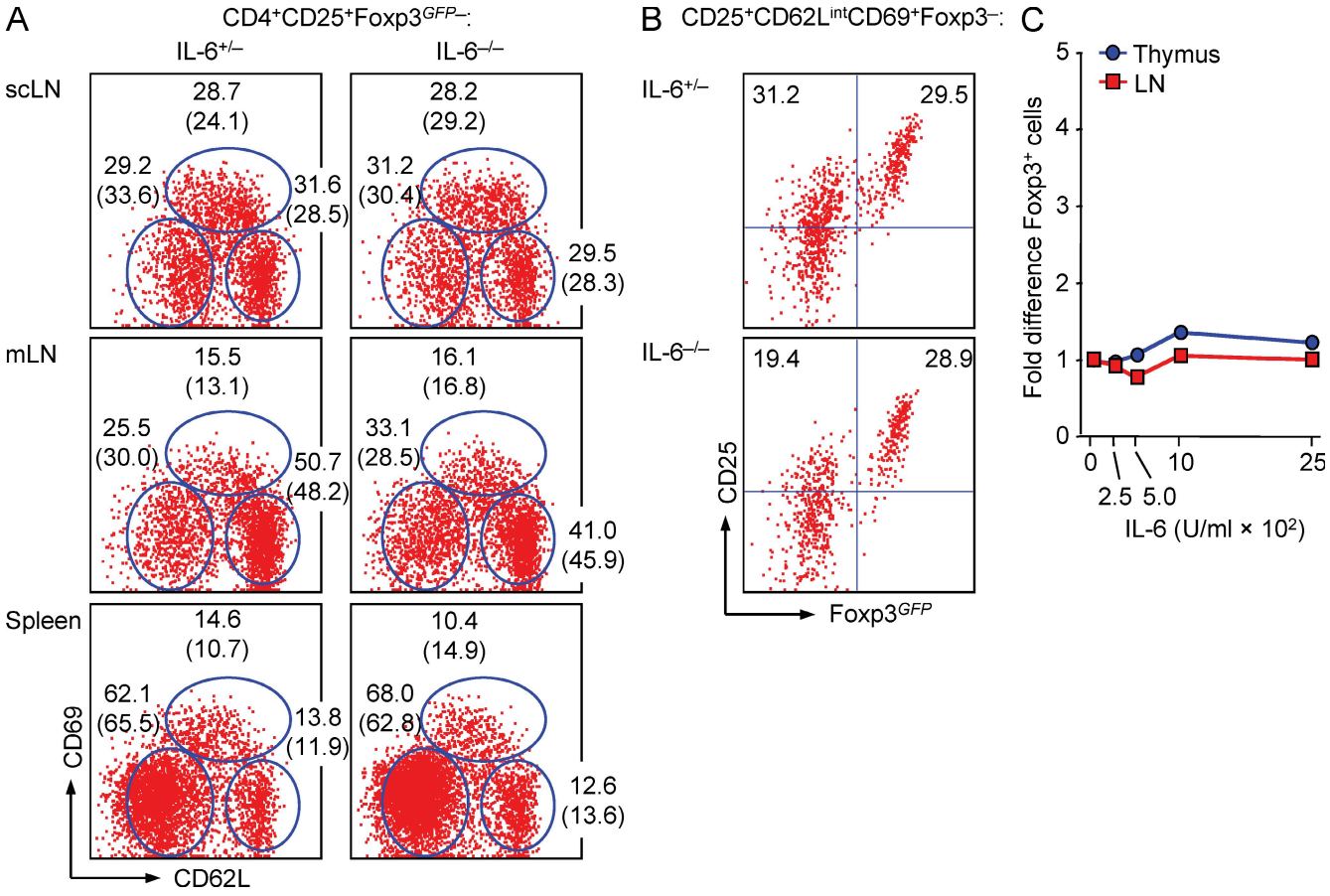

Figure S5. Characterization of extrathymic T reg cell differentiation in steady-state mice in the absence of IL-6. (A) Flow cytometry of CD62L and CD69 expression among gated CD4$^+$CD25$^+$Foxp3$^-$ cells of indicated lymphoid organs of 7-wk-old IL6$^{-/-}$ (right) and IL-6$^{+/-}$ age-matched littermate Foxp3^{GFP} mice (left). Numbers in dot plots indicate the percentage of cells in the respective gate. Numbers in brackets indicate percentages of cells in replicate mice. (B) FACS-purified CD4$^+$CD25$^+$CD62L^{int}CD69$^+$Foxp3$^-$ T cells from scLNs of Foxp3^{GFP} mice were cultured with IL-2 and analyzed at day 3 for CD25 and Foxp3^{GFP} expression. Representative flow cytometry of precursor cultures from IL-6$^{+/-}$ (top) and IL-6$^{-/-}$ (bottom) Foxp3^{GFP} mice. (C) FACS-purified CD4$^+$CD25$^+$Foxp3$^-$ cells from scLNs or CD4$^+$CD8$^-$TCRβ$^+$CD25$^+$Foxp3$^-$ cells from thymus of WT Foxp3^{GFP} mice were cultured with IL-2 in the presence of titrating amounts of IL-6, as indicated. At day 3 of cultures, the impact of IL-6 was calculated as fold difference of numbers of Foxp3$^+$ cells as follows: [absolute numbers Foxp3$^+$ cells from cultures with added IL-6] ÷ [absolute numbers Foxp3$^+$ cells from cultures without added IL-6]. Data are representative of at least two independent experiments.