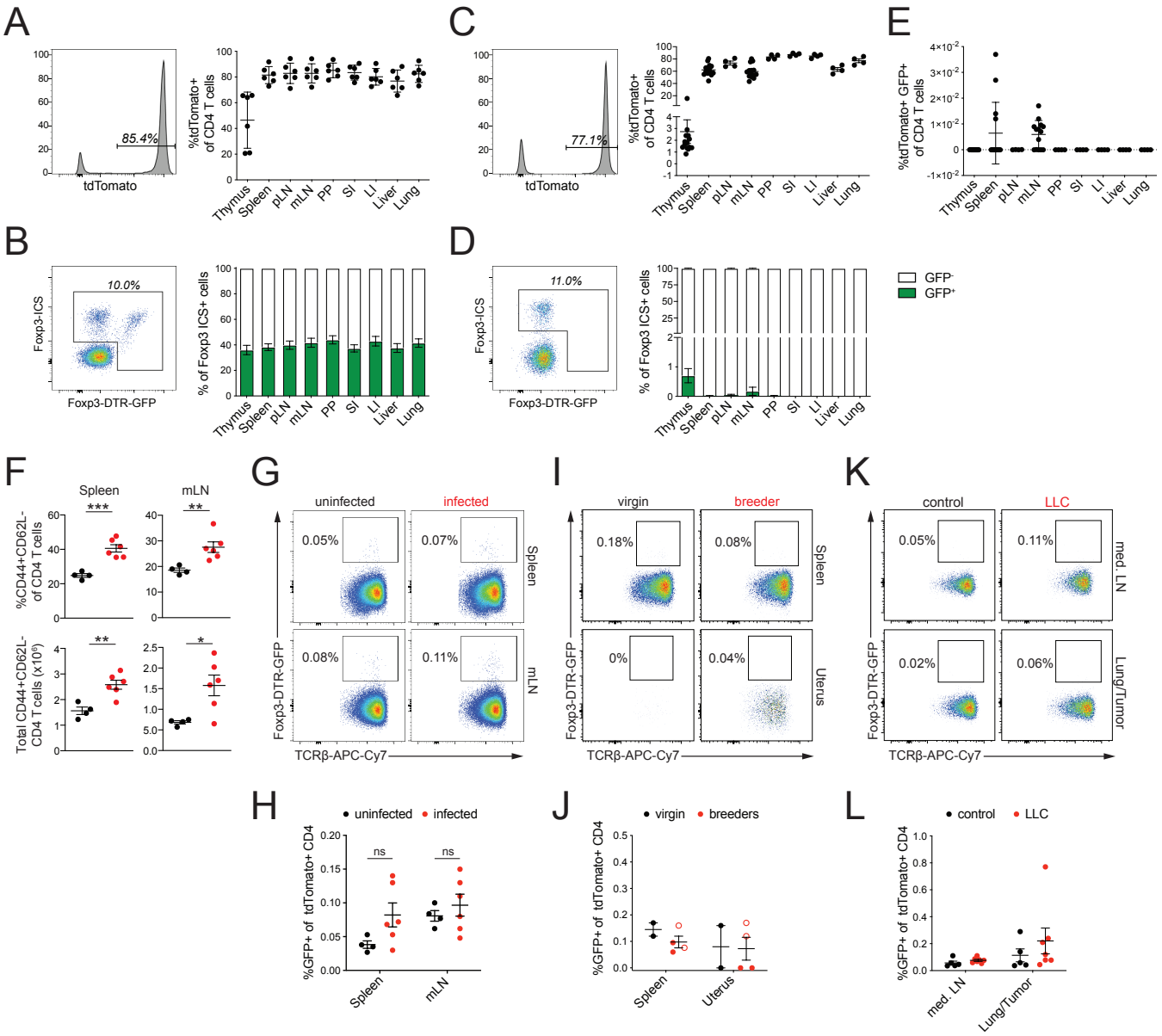

Figure S1: Genetic labeling of extrathymic CD4 T cells, related to Figure 1. A-B) Female *Foxp3*$^{DTR\text{-}GFP/wt}$*CD4*$^{CreER/wt}$*R26*$^{lsl\text{-}tdTomato/wt}$ mice were analyzed by flow cytometry one week after receiving tamoxifen by oral gavage. **C-E)** Female *Foxp3*$^{DTR\text{-}GFP/wt}$*CD4*$^{CreER/wt}$*R26*$^{lsl\text{-}tdTomato/wt}$ mice were analyzed by flow cytometry three weeks after receiving tamoxifen by oral gavage and weekly administration of DT via intraperitoneal injection. Pooled data from two independent experiments (n=4-14 mice per group). pLN: peripheral lymph nodes; mLN: mesenteric lymph nodes; PP: Peyer's patches; SI: small intestine lamina propria; LI: large intestine lamina propria. **F-H)** Following labeling, mice were infected with 200 *H. polygyrus* L3 larvae by oral gavage. spleens and mLN were analyzed by flow cytometry 3 weeks post-infection. **F)** Frequency and total number of activated CD4 T cells. **G-H)** Frequency of GFP^{+} cells among tdTomato^{+} CD4 T cells. Pooled data from two independent experiments (n=4 to 6 mice per group). P-values from multiple t-tests

(*:p<0.05, **:p<0.01, ***:p<0.001). **I-J)** Following labeling, female *Foxp3*$^{DTR\text{-}GFP/wt}$*CD4*$^{CreER/wt}$*R26*$^{lsl\text{-}tdTomato/wt}$ mice were moved to breeding cages containing SPRET/EiJ males (breeders) or housed without a male (virgins) for 25 days. Open red circles represent mice that became visibly pregnant during the experiment. Frequency of GFP^{+} cells among tdTomato^{+} CD4 T cells. **K-L)** Following labeling, mice were intravenously injected with 150.000 Lewis Lung Carcinoma cells (LLC), or left uninjected (control) and analyzed 17-20 days later (two independent experiments).

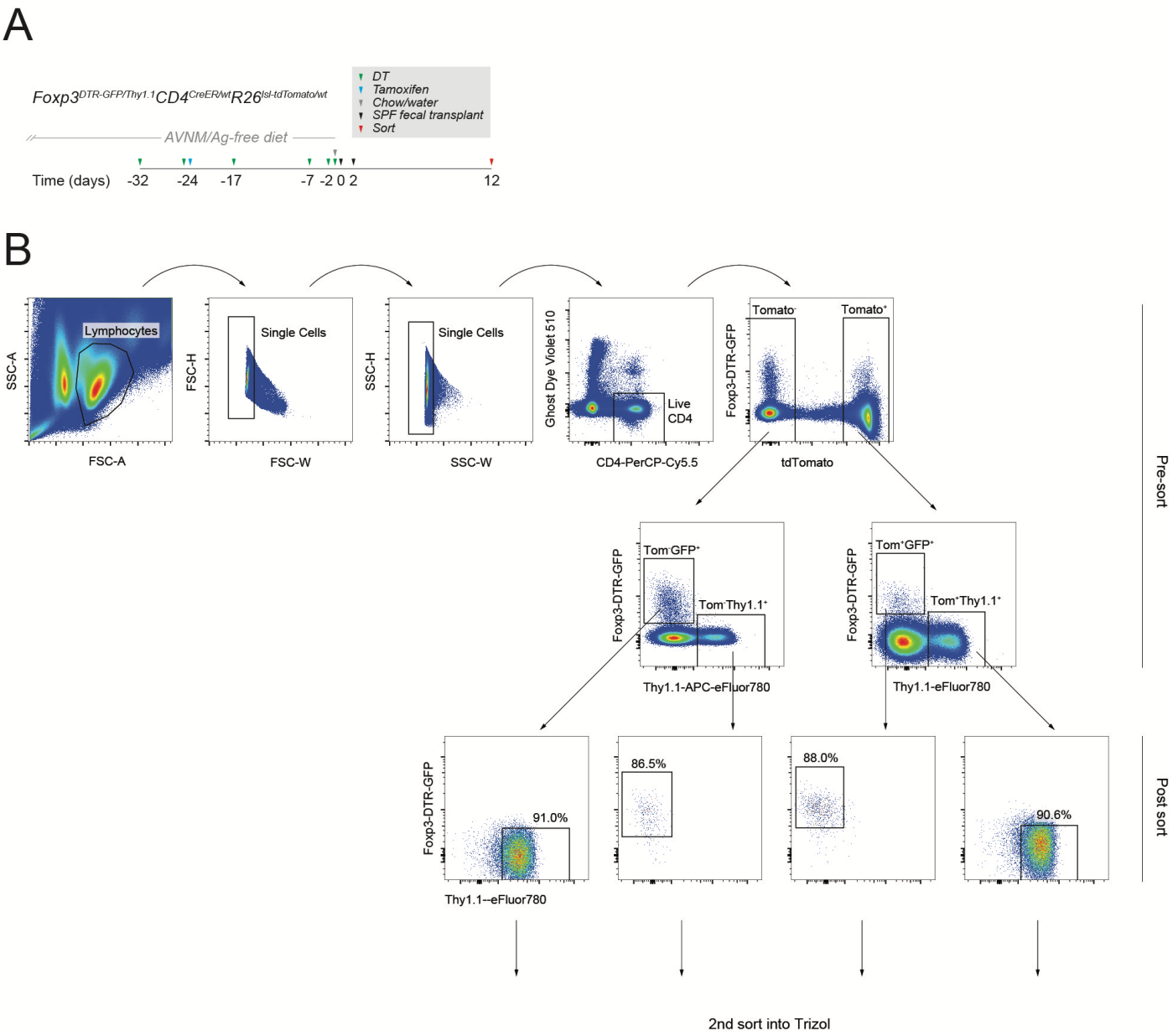

Figure S2: Isolation of fate-mapped Treg cell subsets from the mLN, related to Figure 2. A) Labeling scheme used for the isolation of fate-mapped pTreg cells for RNA-seq analysis in Figure 2A-G. **B)** Sorting strategy for isolation of mLN Treg cell populations used as input for RNA-seq analysis in Figure 2A-G. Indicated populations were sorted once into cell culture media (~86-91% purity) followed by a second sort into Trizol.

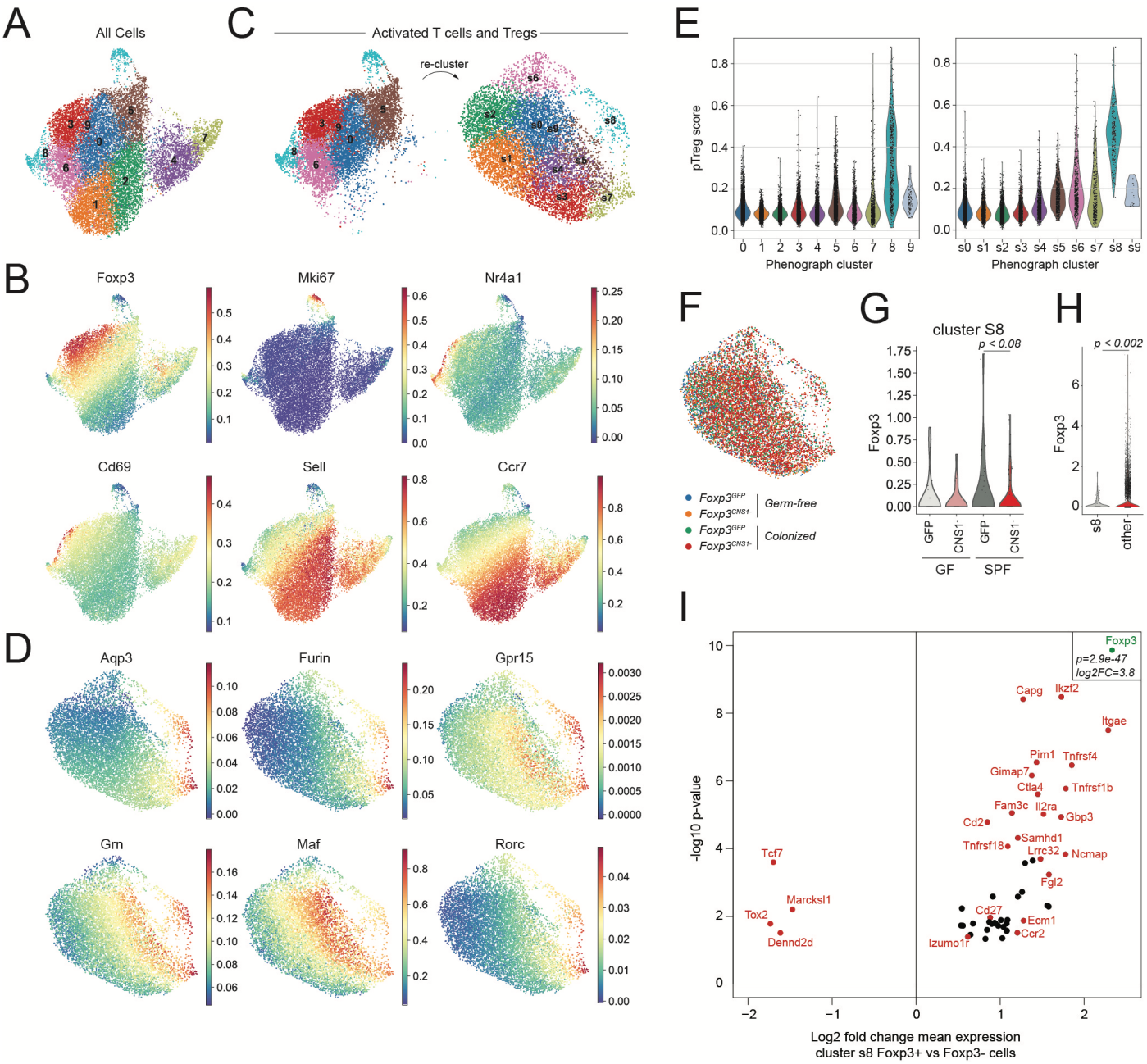

Figure S3: Characterization of mLN CD4 T cells in germ-free and colonized *Foxp3*^{GFP} and *Foxp3*$^{GFP\Delta CNS}$, related to Figure 2. Male germ-free *Foxp3*^{GFP} *and* *Foxp3*$^{GFP\Delta CNS}$ mice were colonized with SPF fecal microbiota or kept germ-free. CD4 T cells from the mLN were analyzed by scRNA-seq on day 9 post-colonization. **A)** UMAP of cells from all 4 experimental groups. **B)** Imputed expression levels of Foxp3 and selected T cell activation and proliferation markers. **C)** Re-clustering of select populations. **D)** Imputed expression levels of selected genes. **E)** pTreg signature score across the clusters. A Mann-Whitney U test was used to compare scores between cluster 8 and each of the other clusters (p<1e-17 for all comparisons) or cluster s8 and each of the other subclusters (p<1e-11 for all comparisons). **F)** Distribution of cells from 4 different experimental groups

across the UMAP. **G)** Foxp3 expression in cluster s8 across 4 experimental groups. P-value from Mann-Whitney U test. **H)** Foxp3 expression in cells from cluster s8 versus cells from other clusters. P-value from Mann-Whitney U test. **I)** Differentially expressed genes in $Foxp3^+$ and $Foxp3^-$ cells from cluster s8.

A

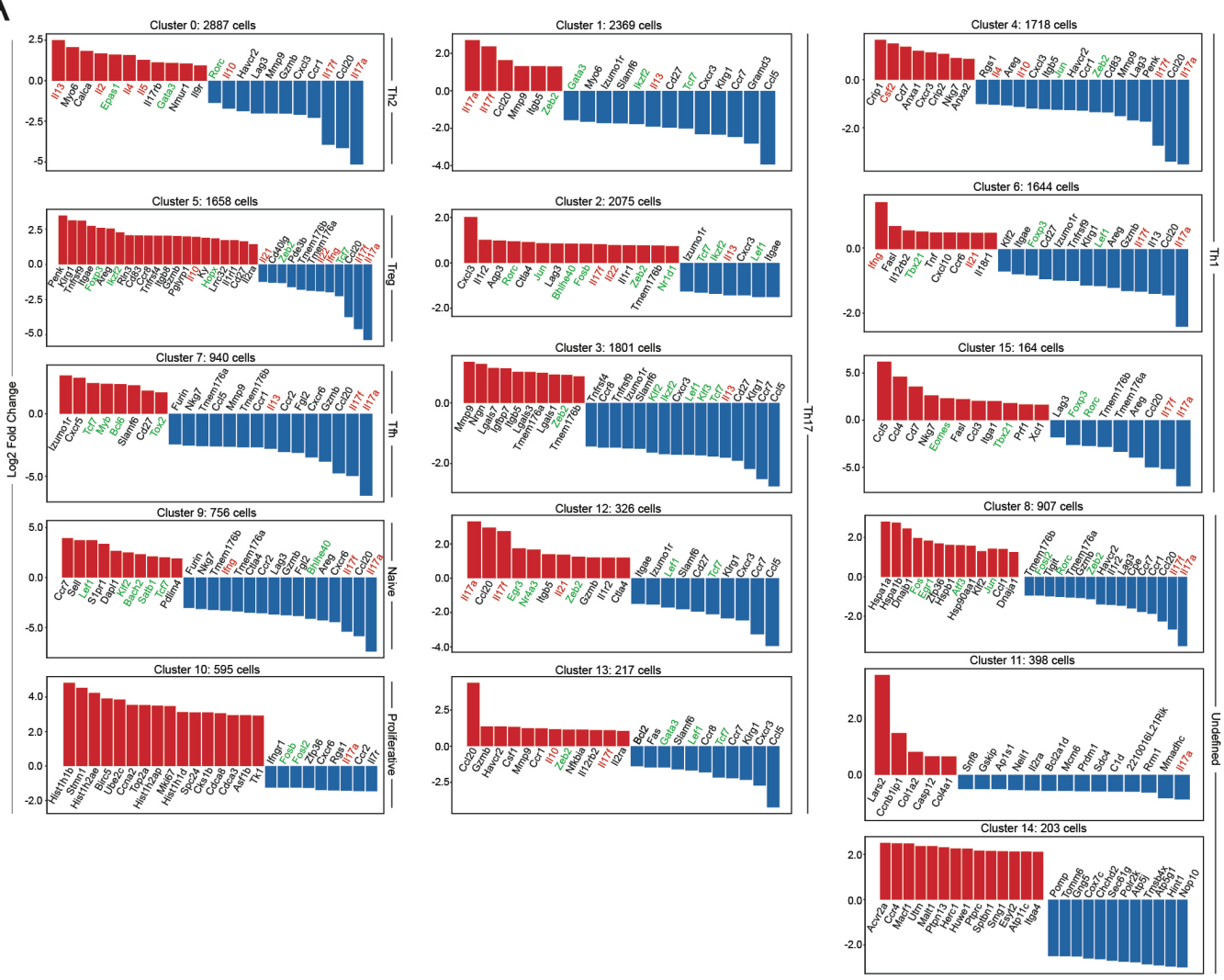

Figure S4: Differential gene expression in colonic CD4 T cell clusters, related to Figure 4.

A) Strongly differentially expressed genes for cells belonging to 16 PhenoGraph clusters of colonic CD4 T cells. Differential expression analysis was run for cells in each cluster vs. all other cells outside the cluster, by calculating log2 fold change (log2FC) of average expression, then performing Mann-Whitney U test (only for genes with absolute log2FC > 0.5) and then dividing the resulting p-values by the number of tested genes to correct for multiple hypothesis testing. Selected genes from the top 30 most strongly differentially over- or under-expressed genes (sorted by log2FC) in each cluster (adjusted p-value < 0.01) are shown. Selected cytokines and transcription factors are shown in red and green, respectively.

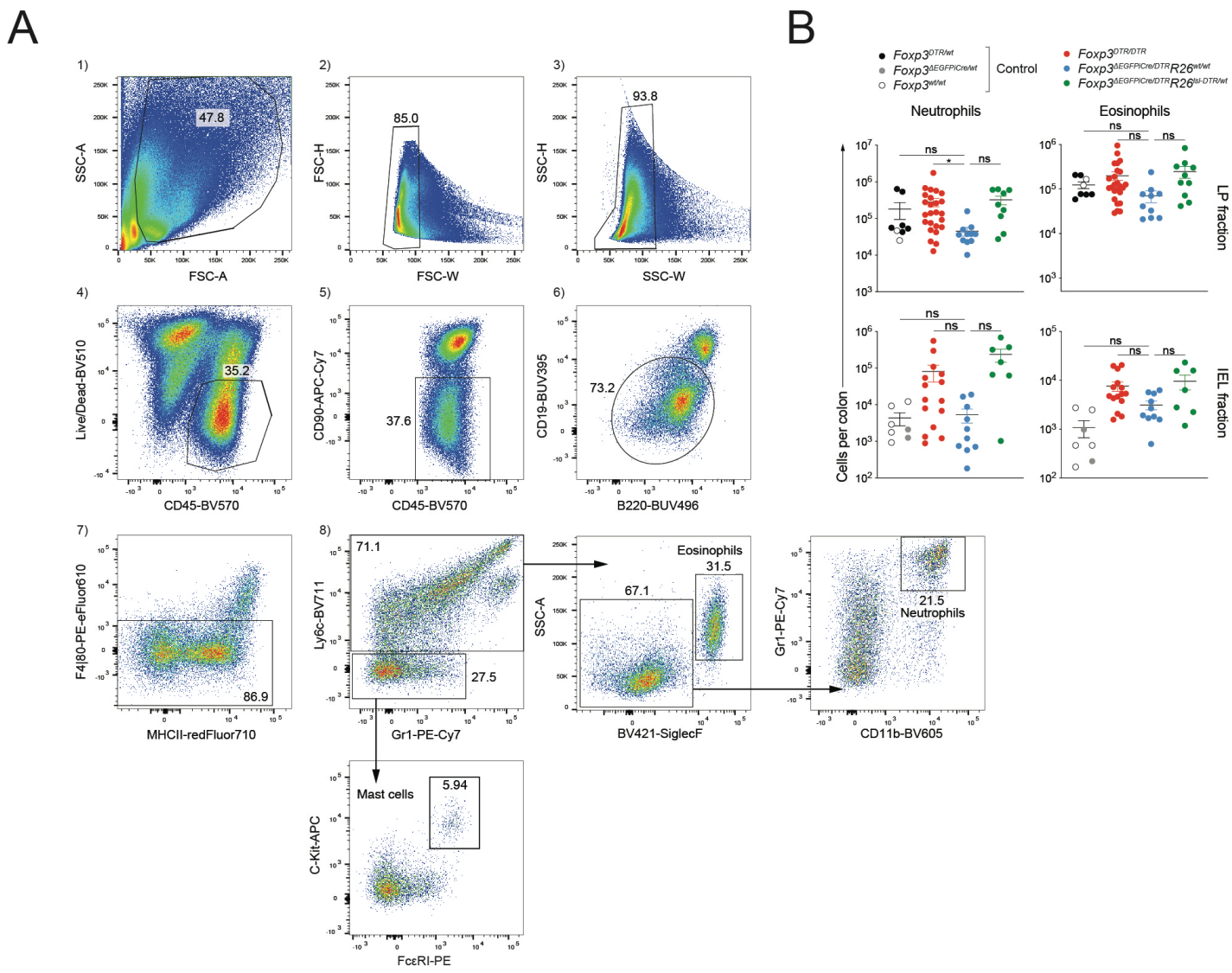

Figure S5: Effect of Treg cell depletion and Foxp3 insufficiency on immune cells in the colon, related to Figure 5. A) Gating strategy for identification of mast cells, neutrophils and eosinophils in the colon. **B)** Flow cytometry analysis of flow cytometry analysis of neutrophils and eosinophils in the colonic lamina propria (LP) and intraepithelial (IEL) fractions of female *Foxp3*$^{DTR/DTR}$, *Foxp3*$^{CreKO/DTR}$*R26*$^{wt/wt}$, *Foxp3*$^{CreKO/DTR}$*R26*$^{lsl\text{-}DTR/wt}$ and control animals 2 weeks after DT treatment. Pooled data from 4 (LP) or 3 (IEL) independent experiments with n=8 to 25 mice per group (LP) or n=7 to 15 mice per group (IEL). P-values from Brown-Forsythe and Welch ANOVA (*:p<0.05).