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Supplemental information

An IL-2 mutein increases regulatory T cell suppression of dendritic cells via IL-10 and CTLA-4 to promote T cell anergy

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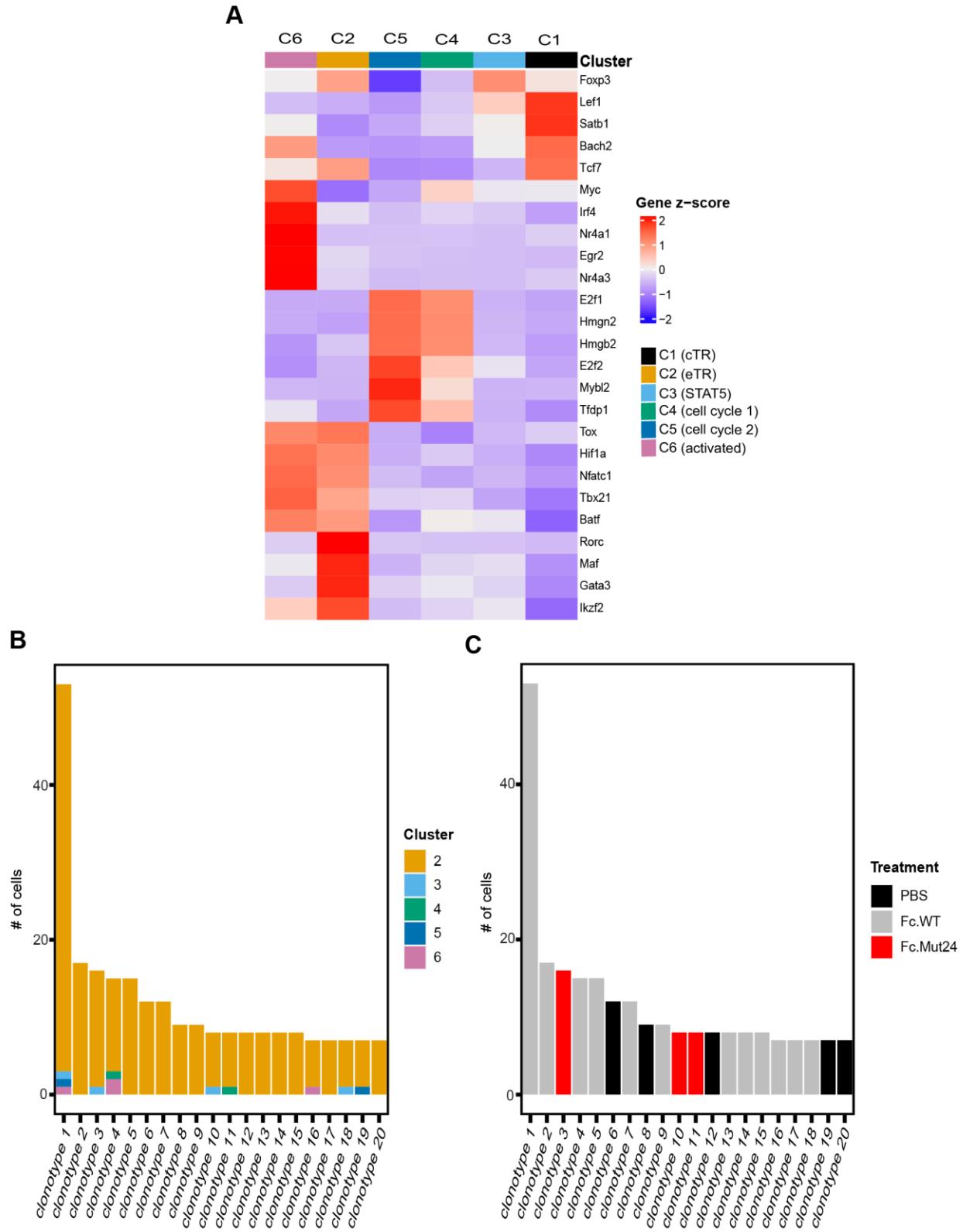

Figure S1. Fc.Mut24 does not impact Foxp3⁺ T_R cell clonal expansion, related to Figure 1. Splenocytes from C57BL/6 Foxp3-mRFP treated with PBS, Fc.WT, or Fc.Mut24 were harvested 3 days later and sorted to obtain CD4⁺ Foxp3⁺ regulatory T (T_R) cells for single-cell RNA-seq (scRNA-seq). **A.** Heat map showing the mean expression (z-score) of representative transcription factor genes (rows) for each cluster (columns). **B&C.** Bar graphs showing the number of cells identified for each of the top twenty T_R clonotypes colored by cluster **(B)** or treatment group **(C).**

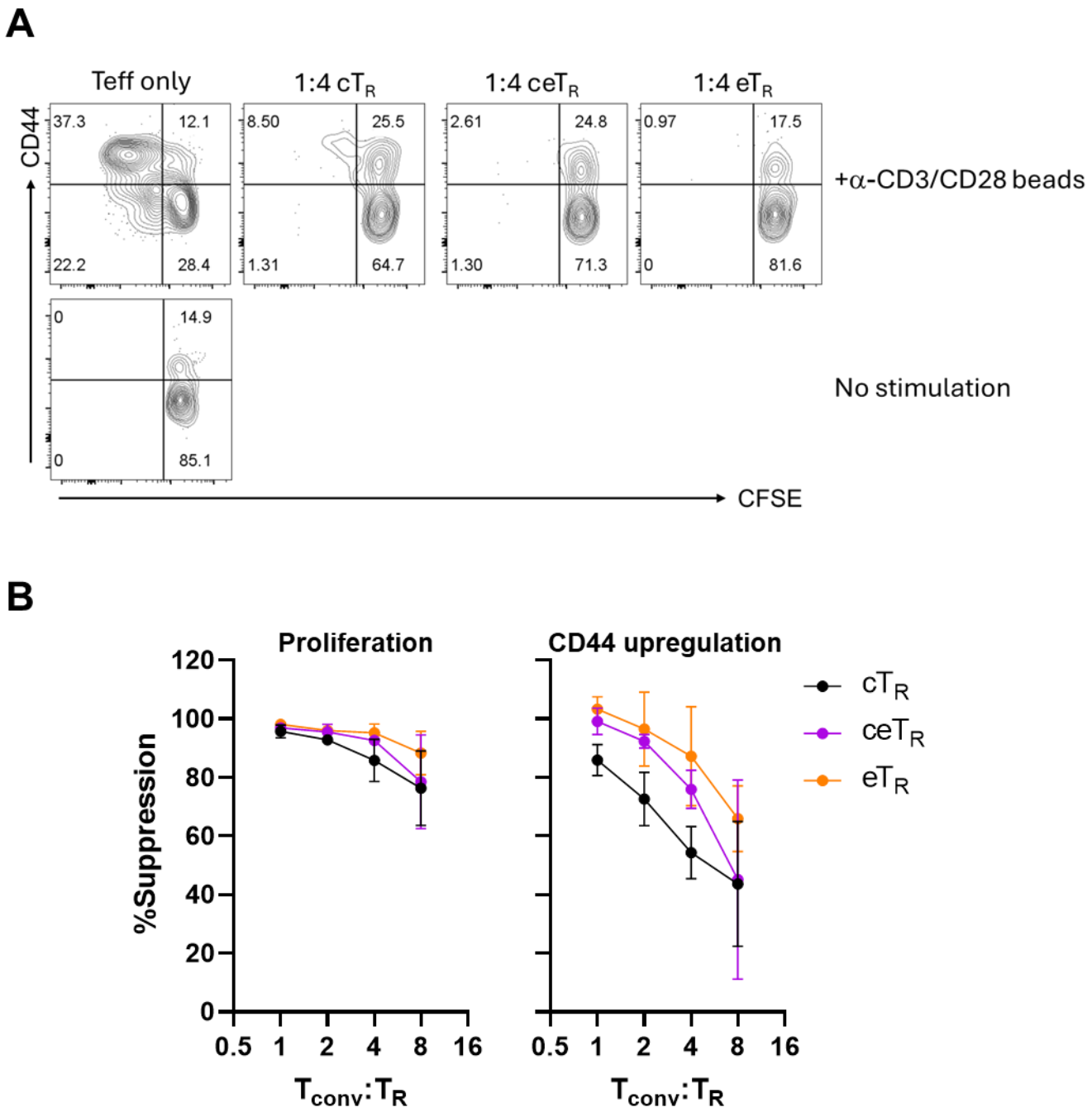

Figure S2. Fc.Mut24-expanded cT_R, ceT_R and eT_R are highly suppressive, related to Figure 2. cT_R, ceT_R or eT_R were sorted from the spleens of B6.Foxp3-mRPF mice treated with Fc.Mut24 3 days previously (gated as in Figure 2), and their ability to suppress activation-induced proliferation and upregulation of CD44 by CFSE-labeled and sorted Foxp3-mRFP-negative T_{conv} was assessed. **A.** Representative flow cytometric analysis of cell proliferation (based on CFSE-dilution) and CD44 expression by gated Teff cells activated alone or mixed in a 1:4 T_R:T_{conv} ratio with the indicated population of sorted T_R. **B.** Analysis of %Suppression of T_{conv} proliferation and CD44 upregulation by each of the sorted T_R populations as indicated. No statistical significance was observed in the ability of any of the T_R populations to suppress either proliferation or CD44 upregulation by one-way ANOVA with Tukey's multiple comparison test.

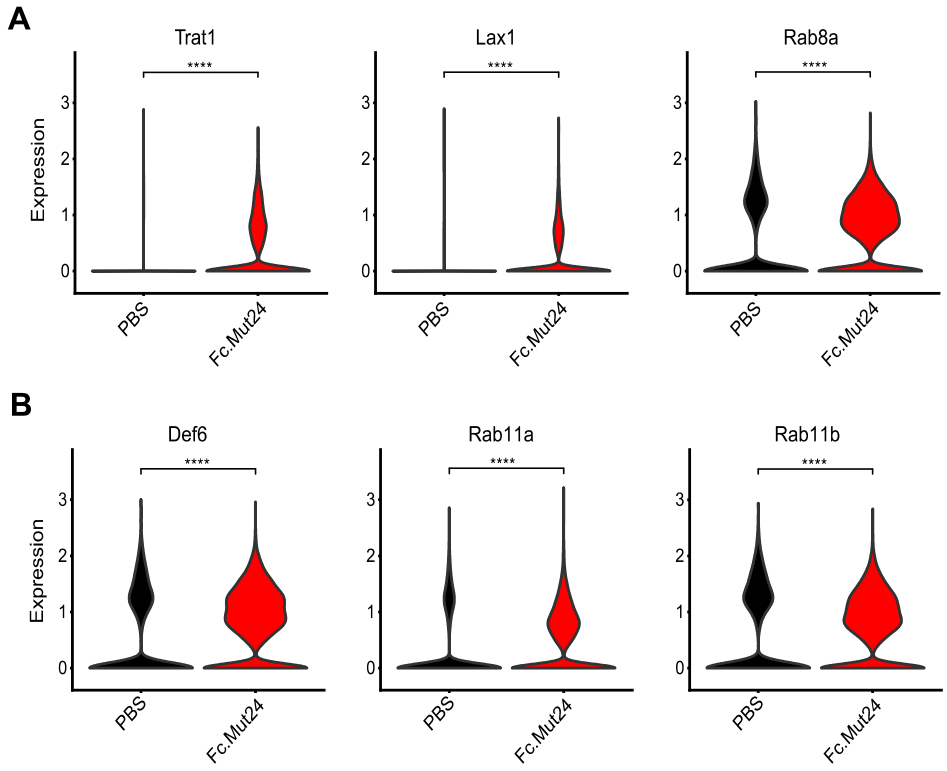

Figure S3. Fc.Mut24 increases the expression of genes involved in CTLA-4 trafficking and recycling in Foxp3⁺ T_R cells, related to Figure 3. Splenocytes from C57BL/6 Foxp3-mRFP treated with PBS, Fc.WT, or Fc.Mut24 were harvested 3 days later and sorted to obtain CD4⁺ Foxp3⁺ regulatory T (T_R) cells for single-cell RNA-seq (scRNA-seq). Violin plots showing normalized expression for the indicated genes involved in CTLA-4 trafficking to the cell surface **(A)** or endosomal recycling of CTLA-4 **(B)**.

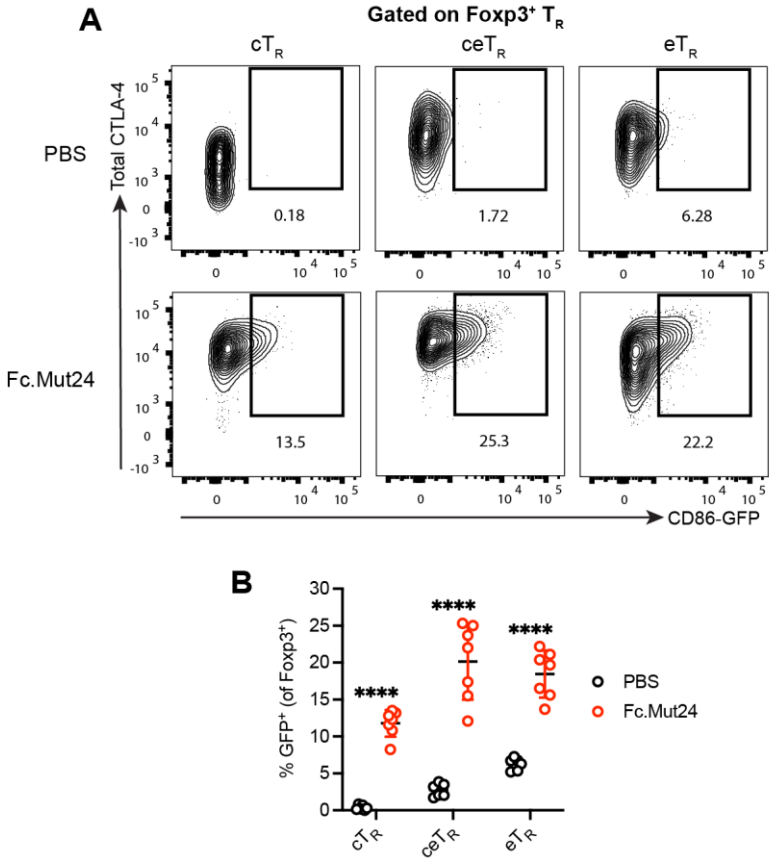

Figure S4. Fc.Mut24 increases CTLA-4-dependent transendocytosis by different Foxp3$^+$ T$_R$ cell populations, related to Figure 4. **A.** Capture of CD86-GFP by CD44^{lo} CD62L^{hi} central T$_R$ (cT$_R$), CD44^{hi} CD62L^{hi} central effector T$_R$ (ceT$_R$), and CD44^{hi} CD62L^{lo} effector T$_R$ (eT$_R$) cells between different treatment groups. See Figure 4A for the experimental design schematic. Representative flow cytometry plots are shown. **B.** Graph shows mean ± SD with individual data points (n = 7); ****$P \leq 0.0001$, multiple unpaired *t*-tests. Data are representative of 2-3 independent experiments.

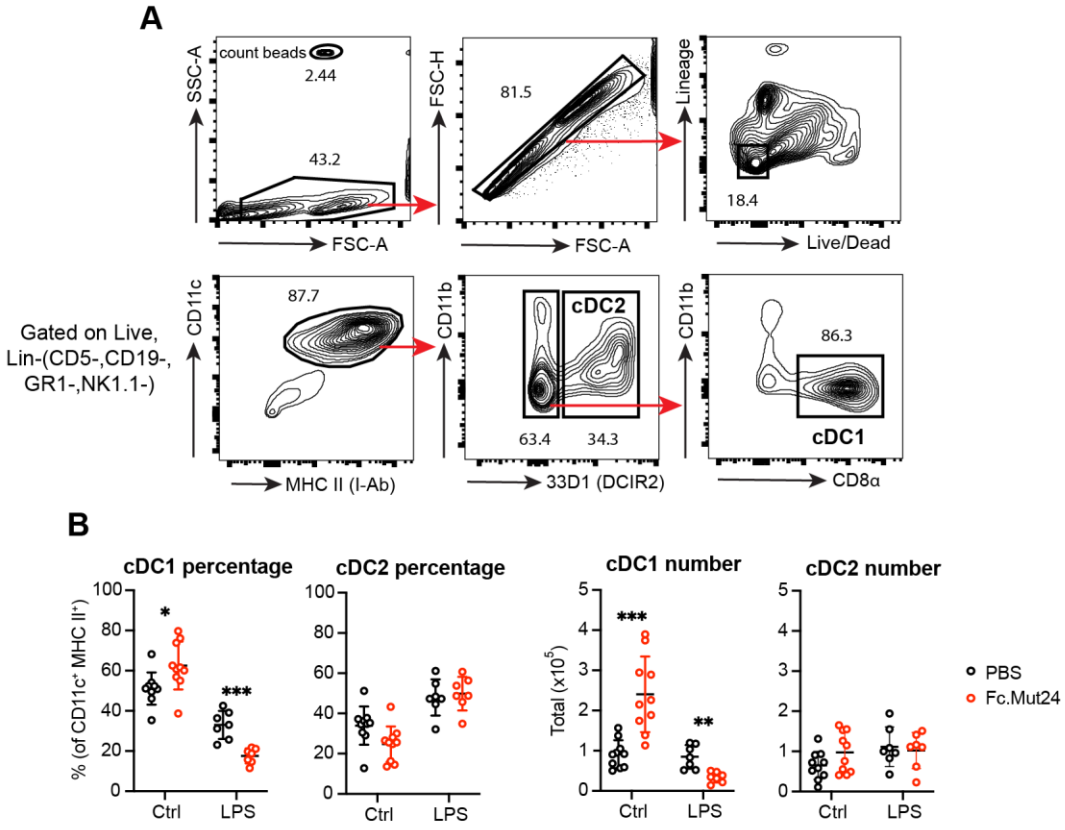

Figure S5. Fc.Mut24 expands cDC1s in C57BL/6 mice, related to Figure 5. A. Representative gating strategy for CD11c$^+$MHC II$^+$33D1$^-$ CD8α^+ cDC1 and CD11c$^+$MHC II$^+$33D1$^+$ cDC2. **B.** Percentage and number of cDC1 and cDC2 between different treatment groups. See Figure 5A for the experimental design schematic**.** Graphs show mean ± SD with individual data points (n = 7 to 10); **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, multiple unpaired *t*-tests. Ctrl, Control. Data are representative of 2-3 independent experiments.

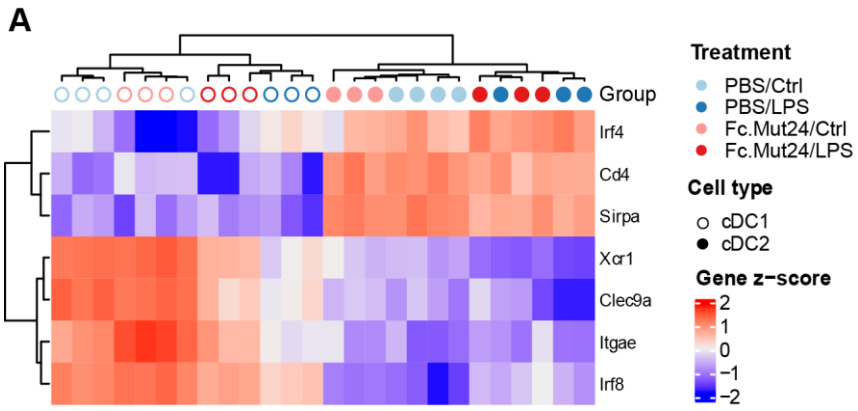

Figure S6. Expression of known identity markers in dendritic cells after Fc.Mut24 treatment, related to Figure 5. Unstimulated (Ctrl) or LPS-stimulated splenocytes from C57BL/6 mice treated with PBS or Fc.Mut24 were harvested 4 days post-treatment and sorted to obtain cDC1 or cDC2 populations for bulk RNA-seq. See Figure 5A for the experimental design schematic**. A.** Heatmap showing the mean expression (z-score) of select genes differentially expressed between cDC1 and cDC2 for all treatment groups.

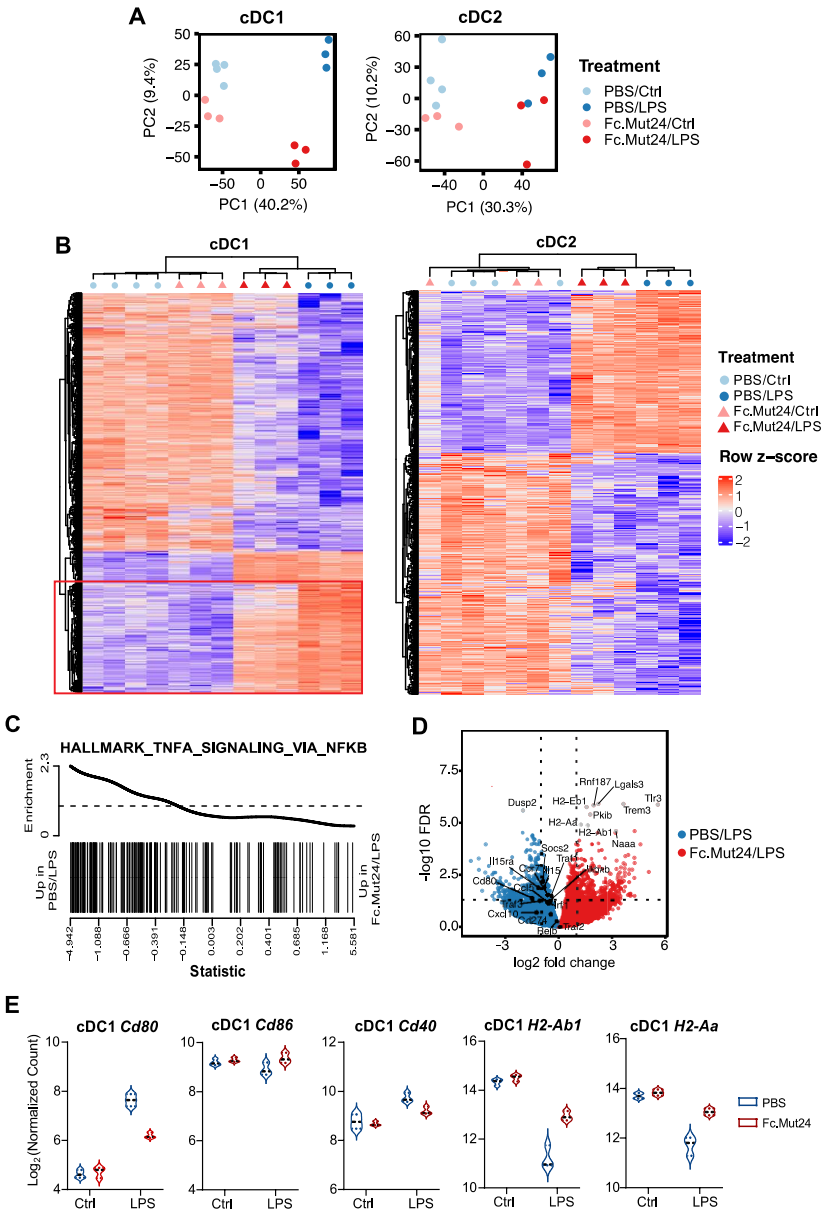

Figure S7. Impact of Fc.Mut24 treatment of the LPS-mediated transcriptional response in dendritic cells, related to Figure 5. Unstimulated (Ctrl) or LPS-stimulated splenocytes from C57BL/6 mice treated with PBS or Fc.Mut24 were harvested 4 days post-treatment and sorted to obtain cDC1 or cDC2 populations for bulk RNA-seq. See Figure 5A for the experimental design schematic**. A.** Principal component analysis of global transcriptional signatures from indicated cell types and treatment groups. **B.** Heatmap showing the mean expression (z-score) of all genes differentially expressed between LPS and Ctrl samples in cDC1 and cDC2 (FDR < 0.01, Log_2 fold-change > 1). In cDC1s, a subset of genes (red rectangle) have diminished LPS-mediated upregulation in the presence of Fc.Mut24. **C.** Gene set enrichment analysis of cDC1s showing that the Hallmark TNFA Signaling via NFKB pathway is enriched in PBS/LPS relative to Fc.Mut24/LPS samples (FDR = 0.005). **D.** Volcano plot of cDC1s comparing Fc.Mut24 treatment versus PBS with the top ten differentially expressed genes by FDR (gray) and select NFκB-regulated genes (black) highlighted. **E.** Violin plots of cDC1s showing Log_2 normalized counts for select genes: *Cd80*, *Cd86*, *CD40*, *H2-Ab1* (I-A^b beta chain), and *H2-Aa* (I-A^b alpha chain).

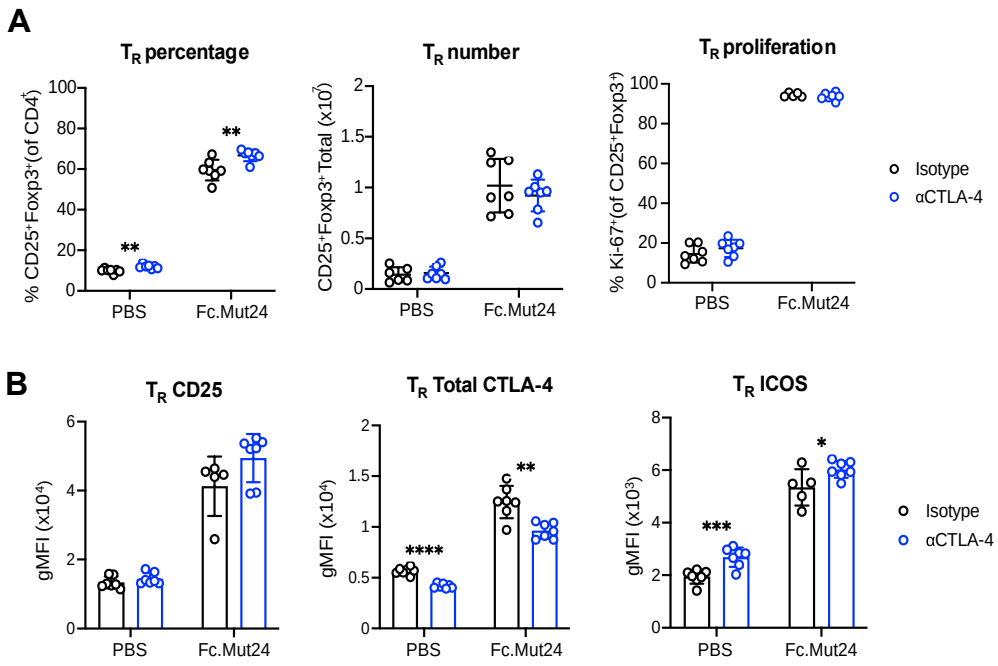

Figure S8. The anti-CTLA-4 antibody 4F10 does not cause Foxp3⁺ T_R cell depletion during Fc.Mut24 treatment, related to Figure 5. Splenocytes from C57BL/6 mice treated with PBS or Fc.Mut24 followed by isotype or anti-CTLA-4 antibody and LPS stimulation were analyzed by flow cytometry. Gates were set on live, CD4⁺ cells. **A&B.** Graphs show mean ± SD with individual data points (n = 7); **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001, multiple unpaired *t*-tests. Data are representative of 2-3 independent experiments. **B.** Gates were set on CD25⁺ Foxp3⁺ T_R for analysis of the expression of indicated T_R activation markers.

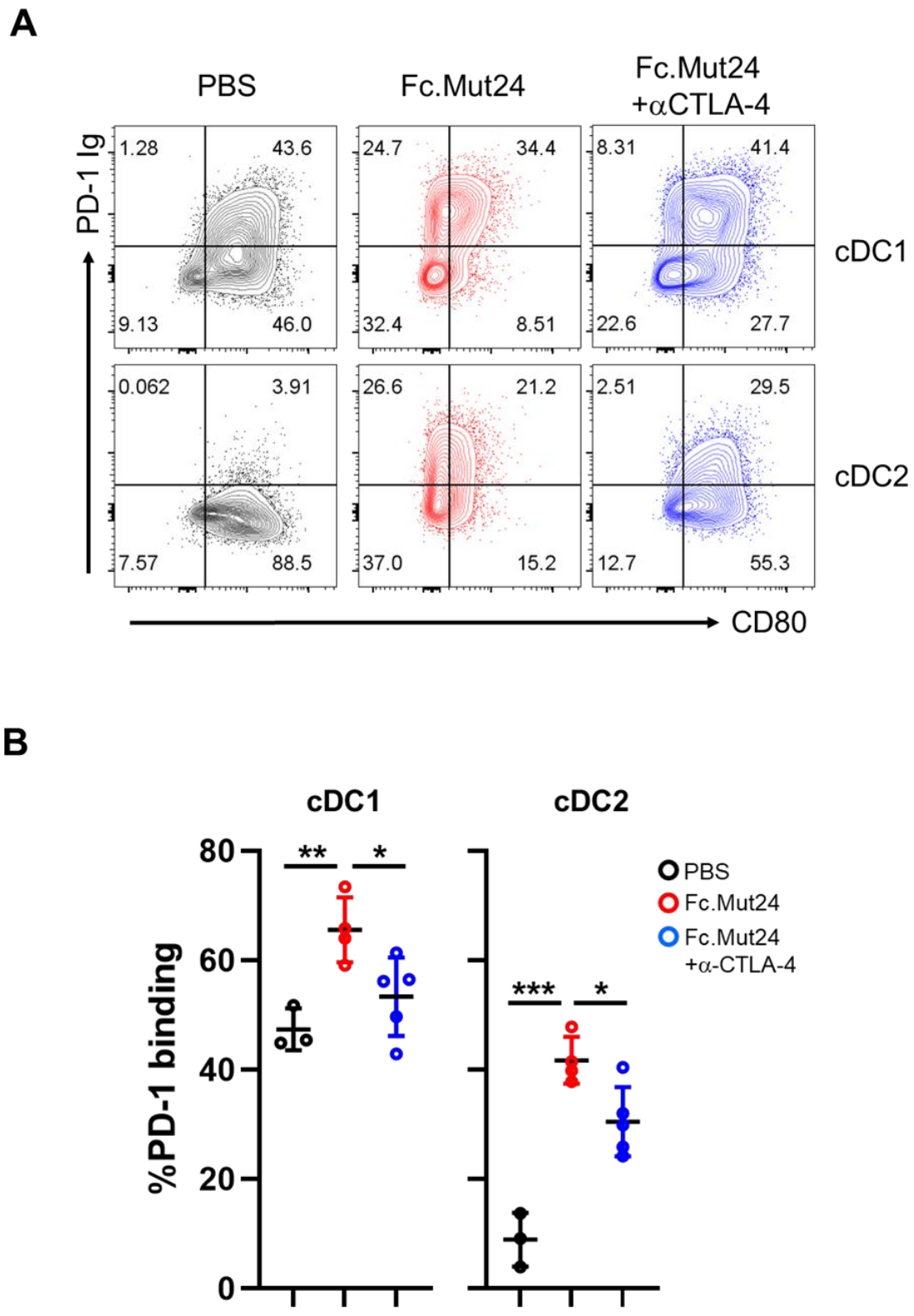

Figure S9. Fc.Mut24 treatment enhances PD-1 binding to splenic DCs, related to Figure 5. Splenic DCs (gated as in Fig. S5) from C57BL/6 mice treated with PBS, Fc.Mut24, or Fc.Mut24 + anti-CTLA-4 antibody followed by LPS were analyzed by flow cytometry. **A.** Representative flow cytometry plots showing CD80 expression and PD-1 binding by gated cDC1 and cDC2. **B.** Graphs show mean ± SD with individual data points in each group as indicated (n = 3-5 mice/group); **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, by one-way ANOVA with Tukey's multiple comparison test. Data are representative of 2 independent experiments.

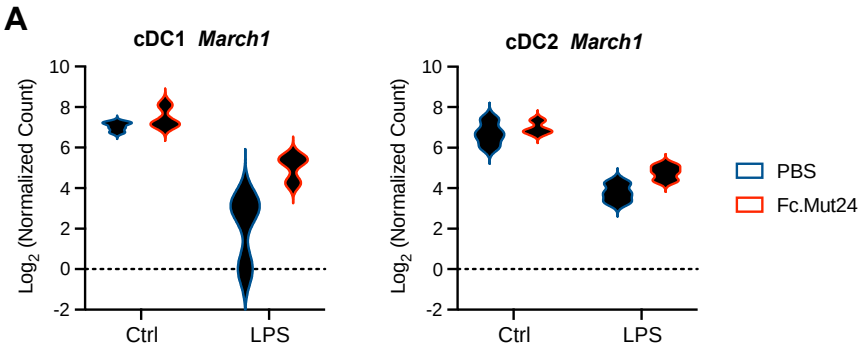

Figure S10. Fc.Mut24 increases March1 gene expression in cDC1s from C57BL/6 mice, related to Figure 6. Unstimulated (Ctrl) or LPS-stimulated splenocytes from C57BL/6 mice treated with PBS or Fc.Mut24 were harvested 4 days post-treatment and sorted to obtain cDC1 or cDC2 populations for bulk RNA-seq. See Figure 5A for the experimental design schematic **A.** Violin plots showing Log_2 normalized counts for *March1* gene expression between treatment groups.

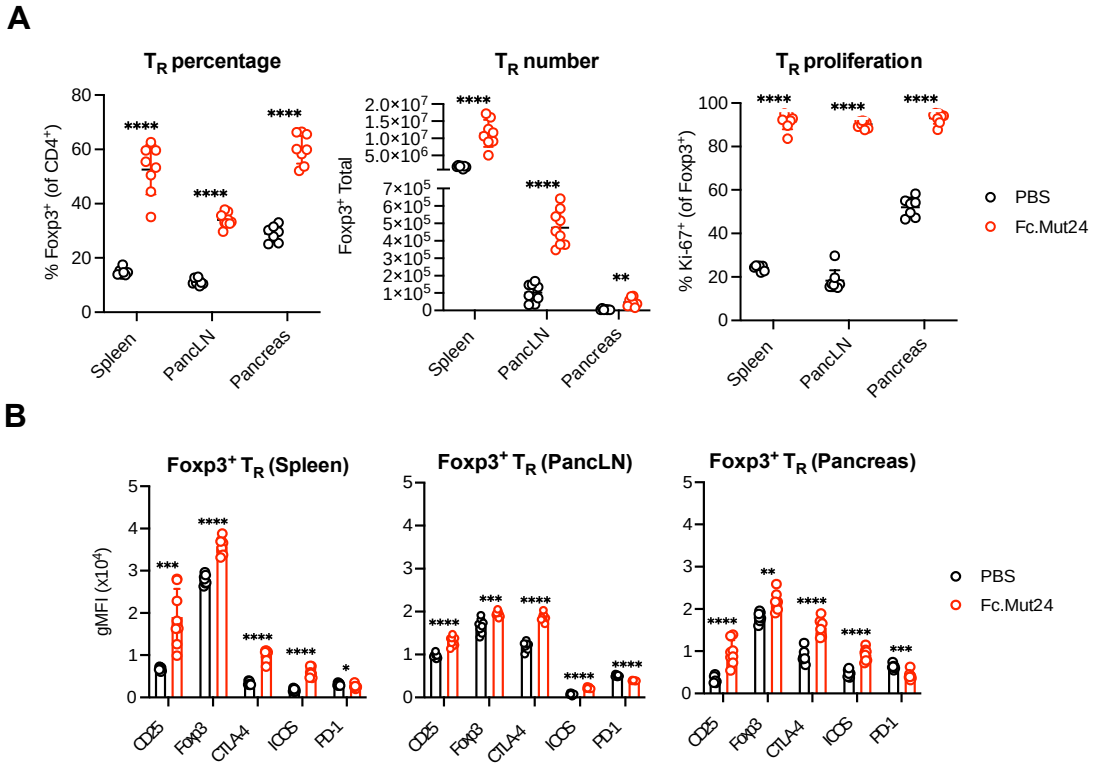

Figure S11. Fc.Mut24 promotes Foxp3$^+$ T_R cell activation in NOD mice, related to Figure 7. The spleen, pancreatic lymph node (PancLN), and pancreas of 8-10-week-old female non-obese diabetic (NOD) mice treated with PBS or Fc.Mut24 were harvested 4 days later and analyzed by flow cytometry. Gates were set on live, CD45$^+$, CD4$^+$ cells. Percentage, number, and proliferation (Ki-67$^+$) of Foxp3$^+$ T_R cells **(A)** and expression of indicated T_R activation markers **(B)**. **A&B.** All graphs show mean ± SD with individual data points (n = 6 to 9); **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001, multiple unpaired *t*-tests. Data are representative of 2-3 independent experiments.

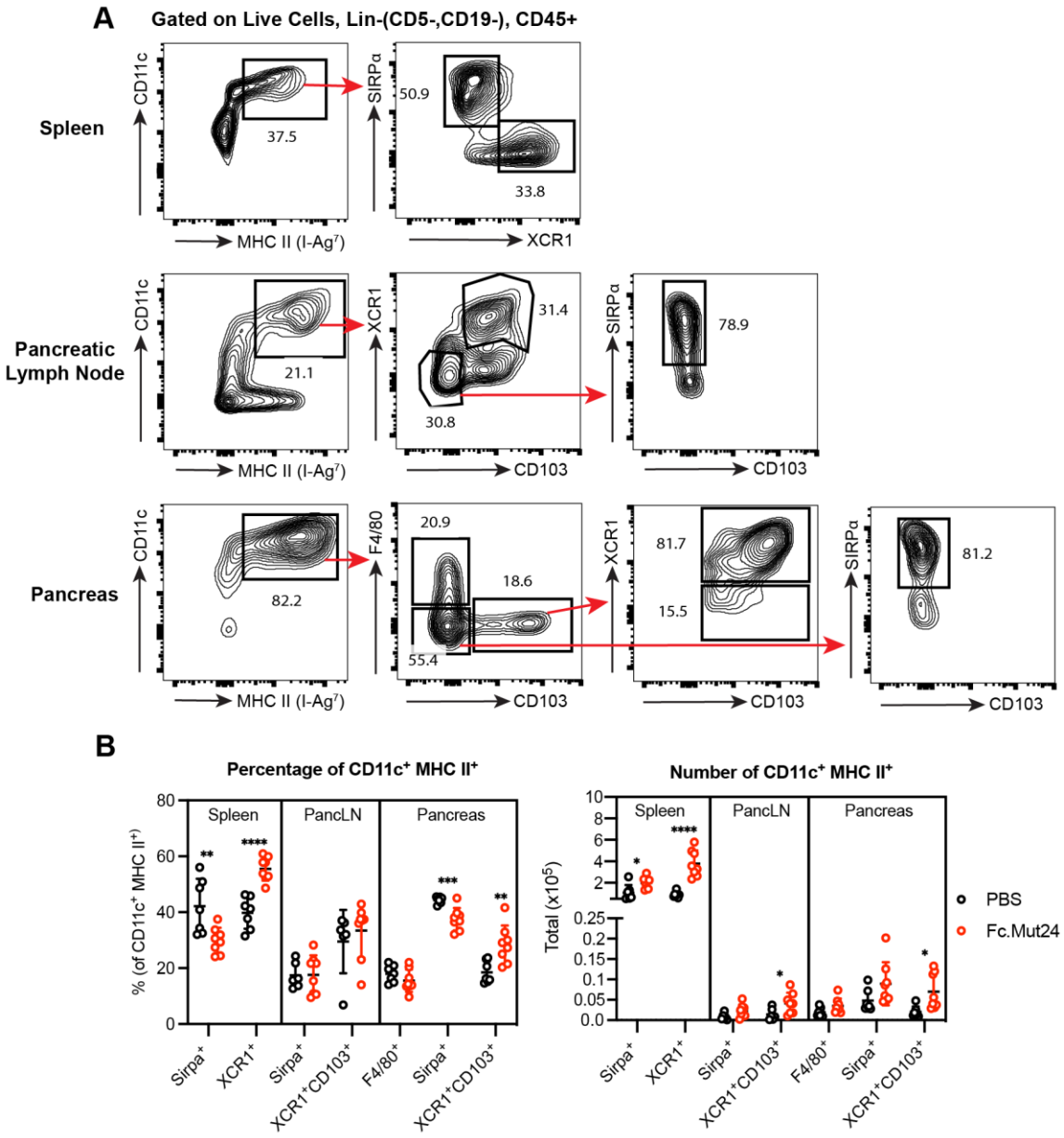

Figure S12. Fc.Mut24 expands cDC1s in NOD mice, related to Figure 7. The spleen, pancreas, and pancreatic lymph node (PancLN) of 8-10-week-old female non-obese diabetic (NOD) mice treated with PBS or Fc.Mut24 were harvested 4 days later and analyzed by flow cytometry. **A.** Representative gating strategy for the CD11c$^+$ MHC II$^+$ antigen-presenting cell subsets in different tissues. **B.** Percentage and number of different subsets of CD11c$^+$ MHC II$^+$ antigen-presenting cells. Graphs show mean ± SD with individual data points (n = 6 to 8); *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, multiple unpaired *t*-tests. Data are representative of 2-3 independent experiments.

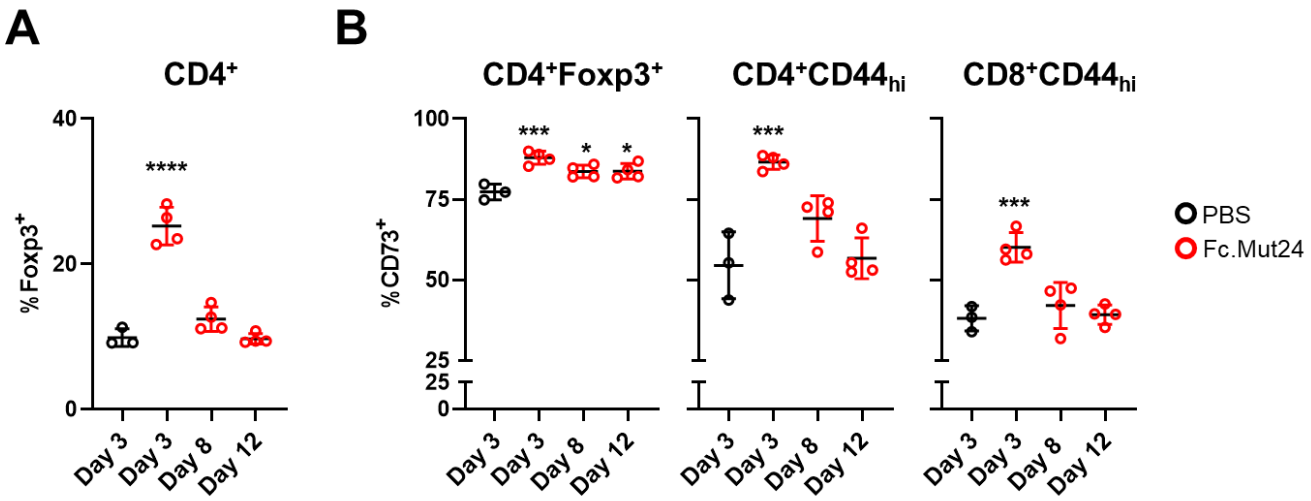

Figure S13. CD73 expression is transiently upregulated by CD4⁺ and CD8⁺ effector T cells after Fc.Mut24 treatment of NOD mice, related to Figure 7. T cells from the pancLN of NOD mice treated with either PBS (black symbols) or Fc.Mut24 (red symbols) were analyzed by flow cytometry. **A.** Frequency of Foxp3⁺ T_R among total CD4⁺ T cells at the indicated times after treatment. **B.** Frequency of CD73⁺ cells among CD4⁺Foxp3⁺, CD4⁺Foxp3⁻CD44^{hi}, and CD8⁺CD44^{hi} populations at the indicated times after treatment. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001, one-way ANOVA with Tukey's multiple comparison test. Data are from 1 experiment.