

Table S1. RNA Sequencing Raw CPMs. Spreadsheet containing raw counts per million (CPMs) from RNA Sequencing of eTregs. FL= FOXP3+Hel-FL, d3B= FOXP3+Hel-Δ3B.

Table S2. Gene Fold Changes. Spreadsheet of top 2000 genes changed when comparing RNA Sequencing of eTregs described in Chapter 2. FL= FOXP3+Hel-FL, d3B= FOXP3+Hel-Δ3B.

Figure S1: Helios overexpression downregulates expression of cDNA on the same vector.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors. T cells were activated with anti-CD3 and anti-CD28 antibody stimulation, cultured in IL-2 containing media and transduced with retroviral particles containing one of the following vectors: SFG-FOXP3- Δ CD19, SFG-Hel-FL-FOXP3- Δ CD19 or SFG-Hel- Δ 3B-FOXP3- Δ CD19. The cDNA on these cells were expressed on a single SFG retroviral vector separated by 2A linkers. Two days post-transduction, transduced cells were purified with antibody-coated magnetic bead particle separation specific for the transduction marker Δ CD19 and cultured for 7 days. **A)**

Representative dot plots of CD19 and FOXP3 expression for FOXP3, FOXP3-Hel-FL and FOXP3-Hel- Δ 3B eTregs. **B)** Graphs summarizing the geometric mean fluorescence intensity (GMFI) of FOXP3 and CD19 of the indicated eTreg population normalized to empty vector control cells. n=2.

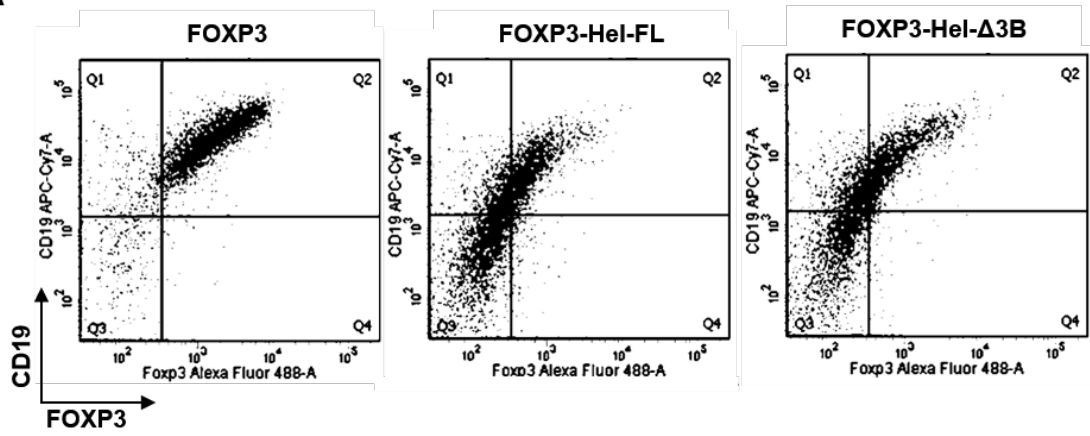
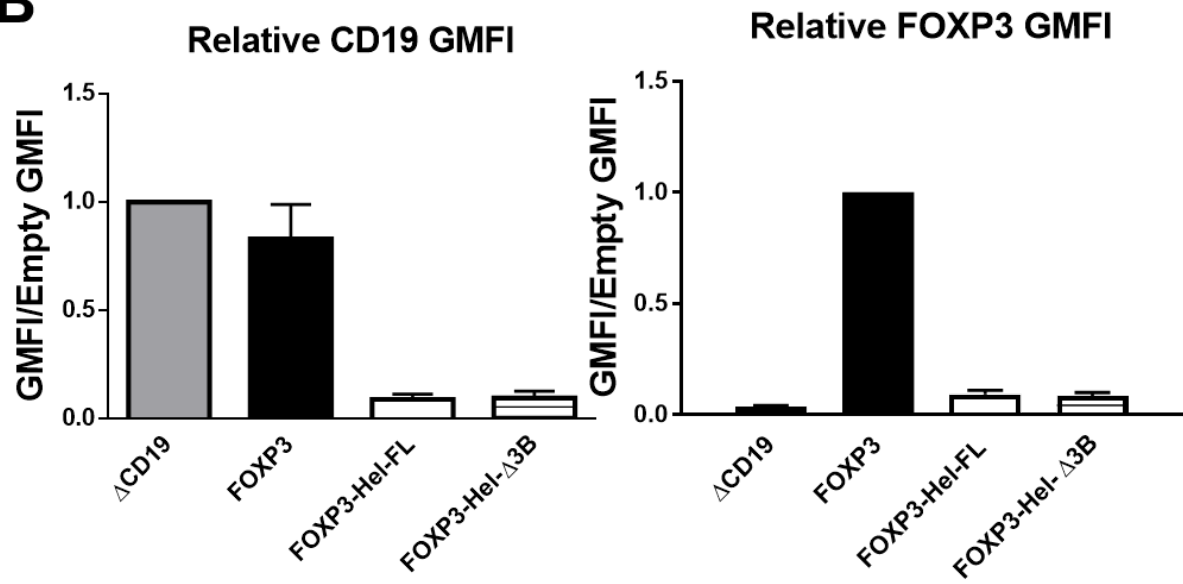
A**B**

Figure S2: Transduction marker expression pre and post-magnetic bead purification. (A-
B) Expression of transduction Δ CD34 and Δ CD19 before CD34 and CD19 magnetic bead purification (Pre) and after CD19 bead purification (Post). Marker expression was assessed via flow cytometry and plotted as percent of total eTregs positive for the indicated marker. **A)** Summary of Δ CD34 and Δ CD19 expression with % positive of non-transduced control subtracted out. n=3-7 and 5 different donors. Some experiments were performed with two separated cell transductions with the same donor cells and some groups did not have all eTreg cell types. * p<0.05 compared to empty vector control based on one-tailed Mann-Whitney test. **B)** Representative histograms of Δ CD34 and Δ CD19 expression. **C)** Representative dot plots of after Δ CD34 vs Helios and Δ CD19 vs FOXP3 following CD19 bead purification for each eTreg cell line.

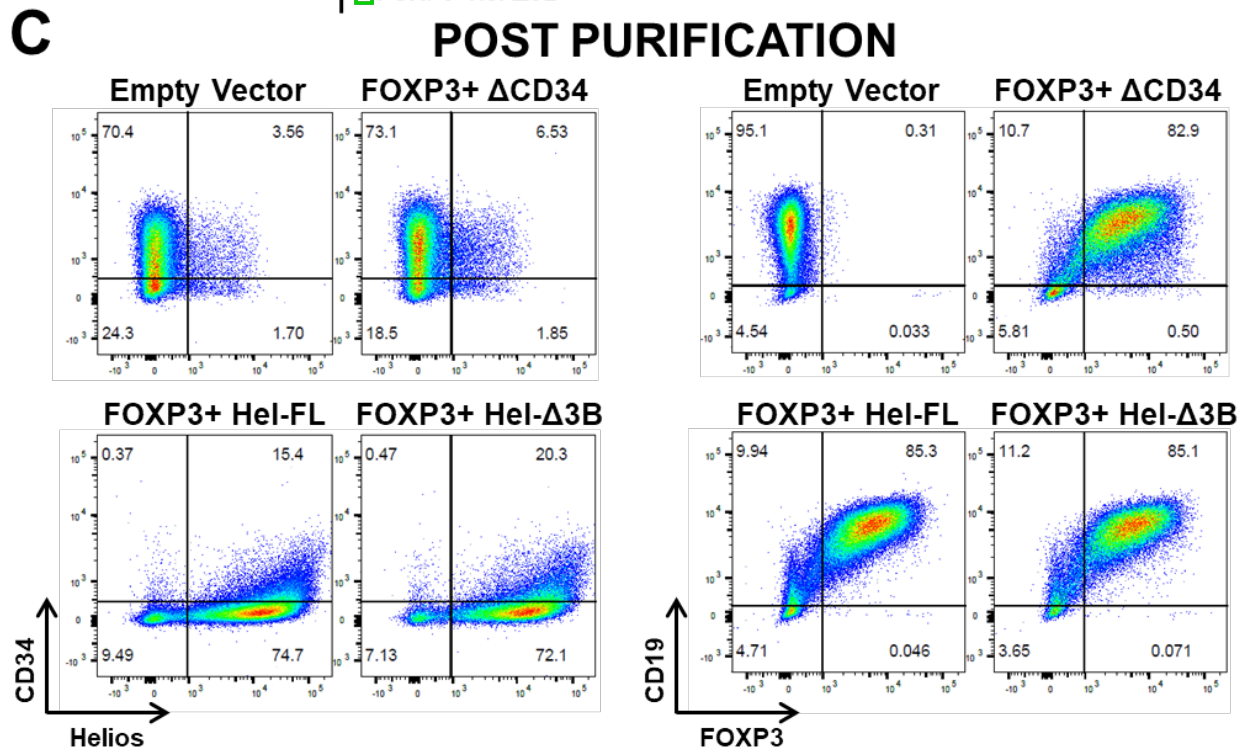
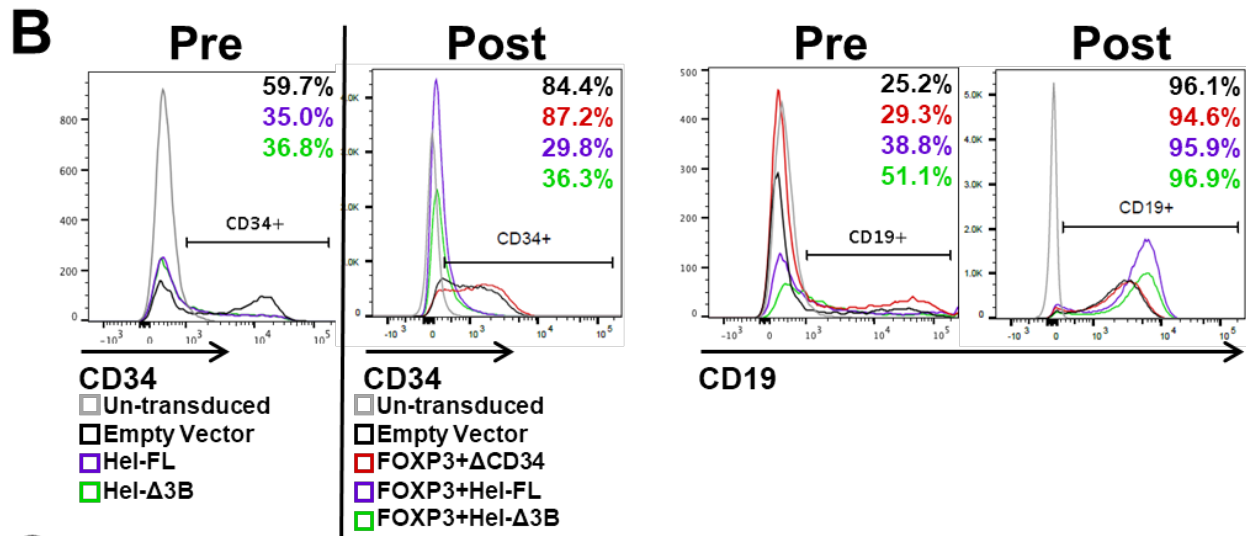
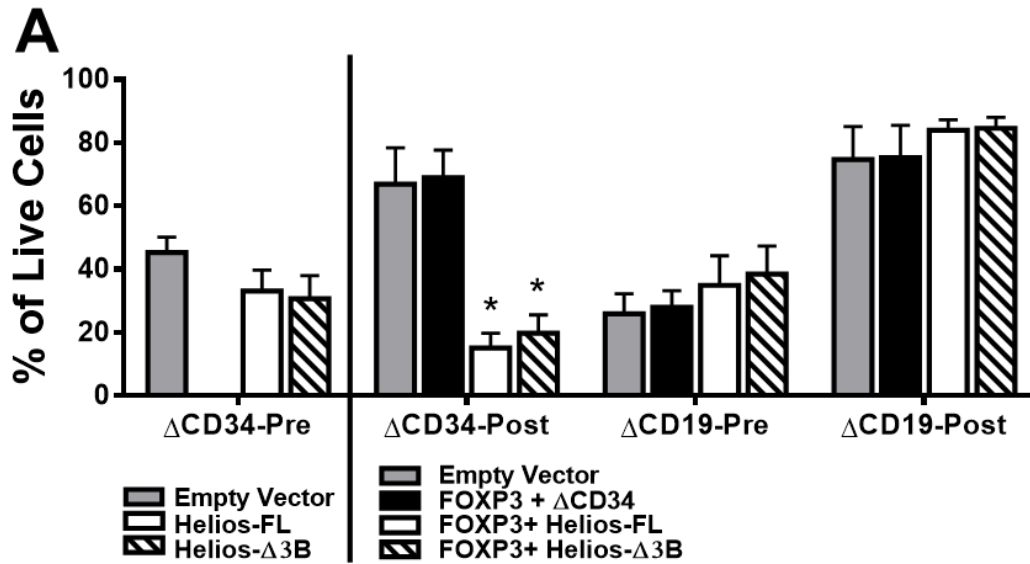


Figure S3: CD4:CD8 ratios were comparable across different vectors in xenoGVHD experiments. Prior to injection into mice for xenoGVHD experiments, empty vector control cells (n=6), FOXP3 eTreg (n=6), FOXP3+HEL-FL eTregs (n=7) and FOXP3+ Hel- Δ 3B eTregs (n=7) were assessed for surface expression of CD4 and CD8 expression via flow cytometry. T cells from 4 different donors were used. Comparison of all groups was performed using one-tailed Mann-Whitney test $p \leq 0.05$.

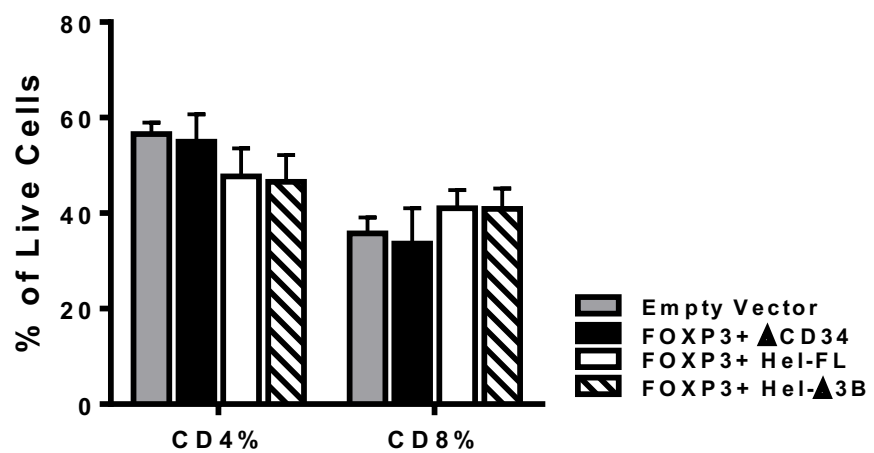


Figure S4: FOXP3+Hel-FL and FOXP3+Hel-Δ3B differentially mediate CD4+ and CD8+ eTreg suppression of CD4+ T cell proliferation. Labeled autologous target Tconv cells were co-cultured at a 1:1 ratio with each eTreg cell strain or empty vector control cells with no stimulation or stimulation with anti-CD3 and anti-CD28 coated beads. After 96 hours, target cell proliferation was assayed via flow cytometry. **A)** Percent suppression of CD4+ T cells for each eTreg cell strain. Cells were plated as follows: 5×10^4 target Tconvs alone, 5×10^4 target Tconvs + 5×10^4 empty vector control cells, 5×10^4 target Tconvs + 5×10^4 FOXP3 eTreg, 5×10^4 target Tconvs + 5×10^4 FOXP3+Hel-FL eTregs, or 5×10^4 target Tconvs + 5×10^4 FOXP3+ Hel-Δ3B eTregs. eTregs were either total T cells (n=5 for each condition), CD4+ only (n=7) or CD8+ only (n=6). T cells from 4 different donors were used. Negative percent suppression was plotted as 0% suppression. $p \leq 0.05$ in each comparison based on a one-tailed Wilcoxon test. **B)** Representative dot plots of CD4+ responder cell proliferation 96 hours after co-culture with eTregs or empty vector control. ND= not detectable. NS=not statistically significant.

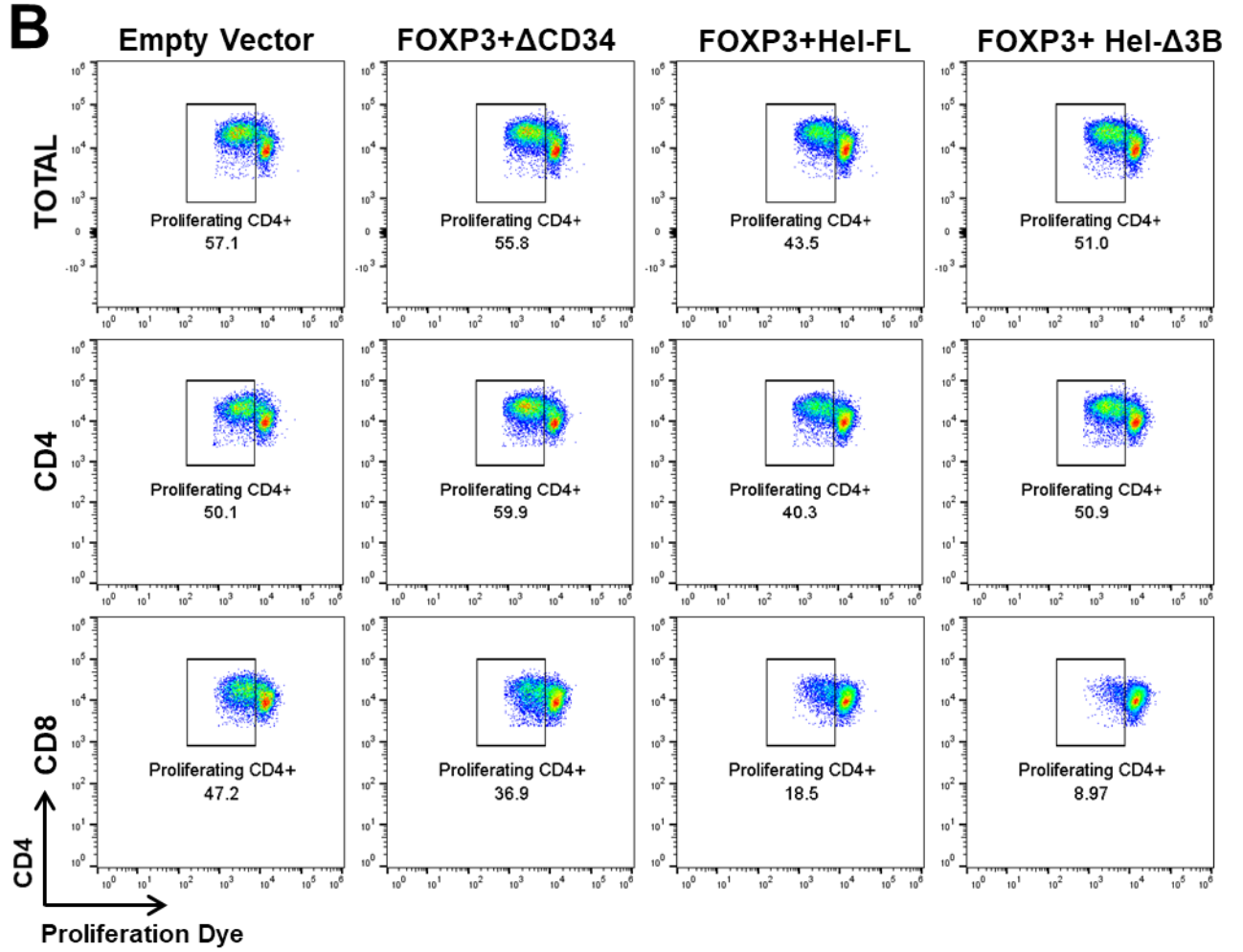
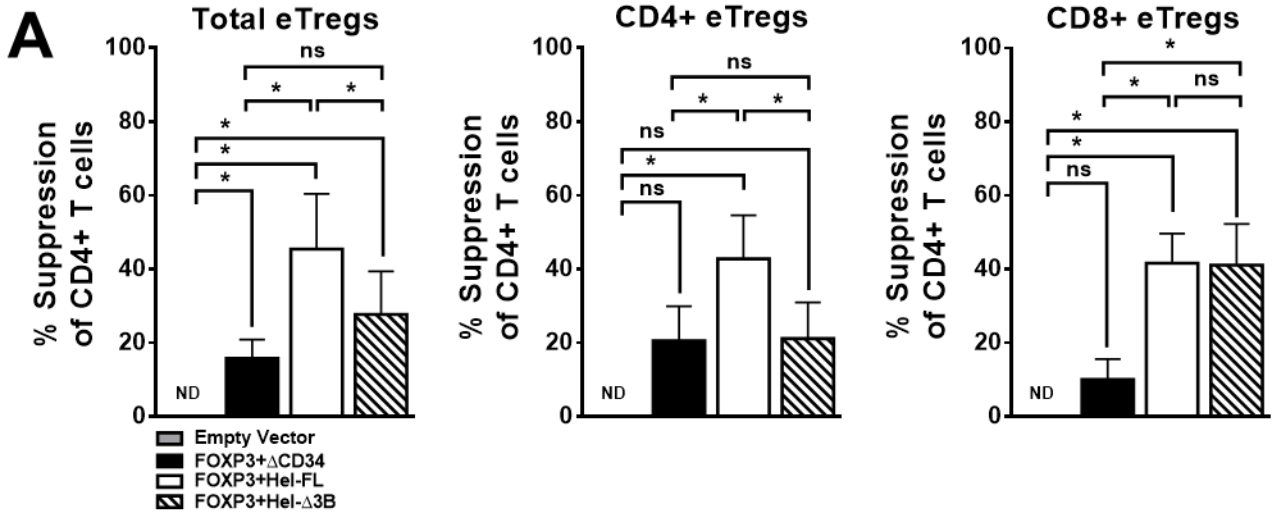


Figure S5: FOXP3+Hel-FL and FOXP3+Hel-Δ3B differentially mediate CD4+ and CD8+ eTreg suppression of CD8+ T cell proliferation. Labeled autologous target Tconv cells were co-cultured at a 1:1 ratio with each eTreg cell strain or empty vector control cells with no stimulation or stimulation with anti-CD3 and anti-CD28 coated beads. After 96 hours, target cell proliferation was assayed via flow cytometry. **A)** Percent suppression of CD8+ T cells for each eTreg cell strain. Cells were plated as follows: 5×10^4 target Tconvs alone, 5×10^4 target Tconvs + 5×10^4 empty vector control cells, 5×10^4 target Tconvs + 5×10^4 FOXP3 eTreg, 5×10^4 target Tconvs + 5×10^4 FOXP3+Hel-FL eTregs, or 5×10^4 target Tconvs + 5×10^4 FOXP3+ Hel-Δ3B eTregs. eTregs were either total T cells (n=5 for each condition), CD4+ only (n=7) or CD8+ only (n=6). T cells from 4 different donors were used. Negative percent suppression was plotted as 0% suppression. $p \leq 0.05$ in each comparison based on a one-tailed Wilcoxon test. **B)** Representative dot plots of CD8+ responder cell proliferation 96 hours after co-culture with eTregs or empty vector control. ND= not detectable. NS=not statistically significant.

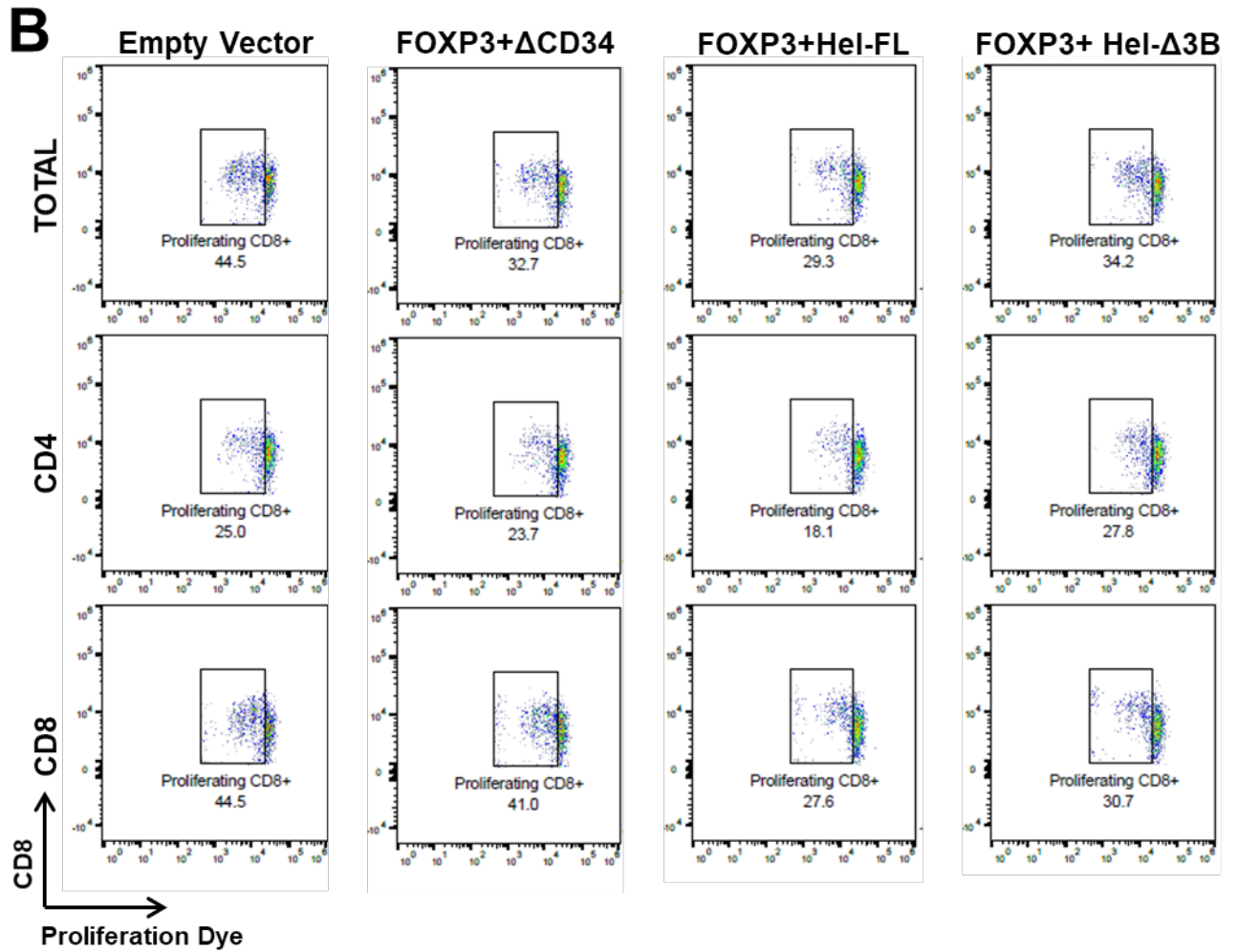
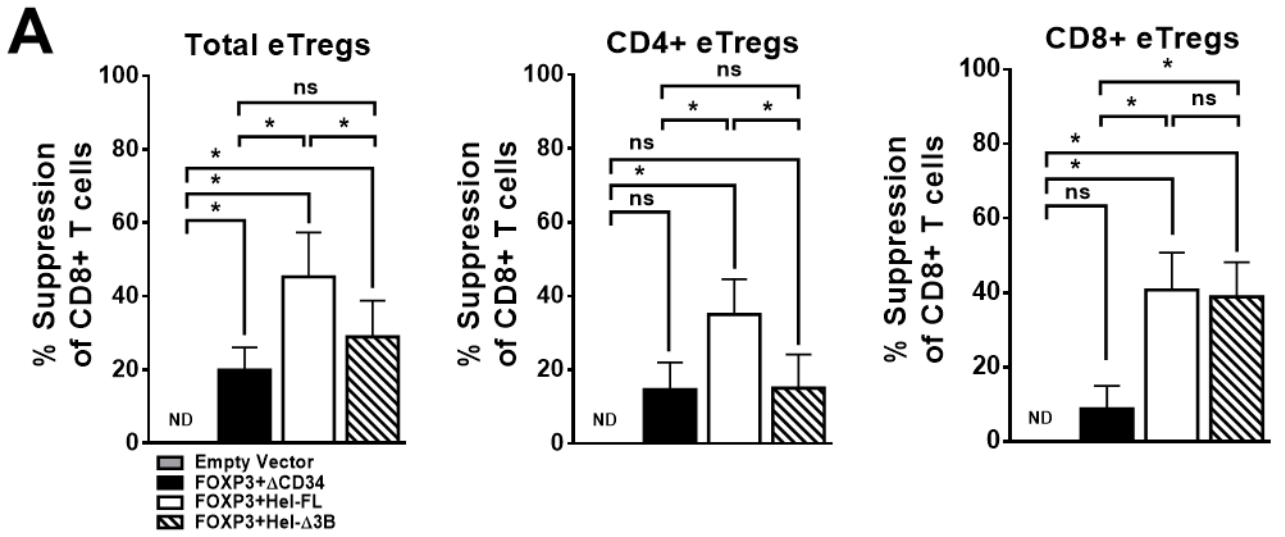


Figure S6: Groups within individual suppression assays reflect the cumulative trend.

Labeled autologous target Tconv cells were co-cultured at a 1:1 ratio with each eTreg cell strain or empty vector control cells with no stimulation or stimulation with anti-CD3 and anti-CD28 coated beads. After 96 hours, target cell proliferation was assayed via flow cytometry. The following graphs depict eTreg percent suppression of **A)** total target T cells, **B)** CD4+ T cells or **C)** CD8+ T cells. Each line in the graphs represents an individual experiment within the cumulative suppression assay data presented in Figures 3, S4 and S5. eTregs were either total T cells (n=5 for each condition), CD4+ only (n=7) or CD8+ only (n=6). T cells from 4 different donors were used. Negative percent suppression was plotted as 0% suppression.

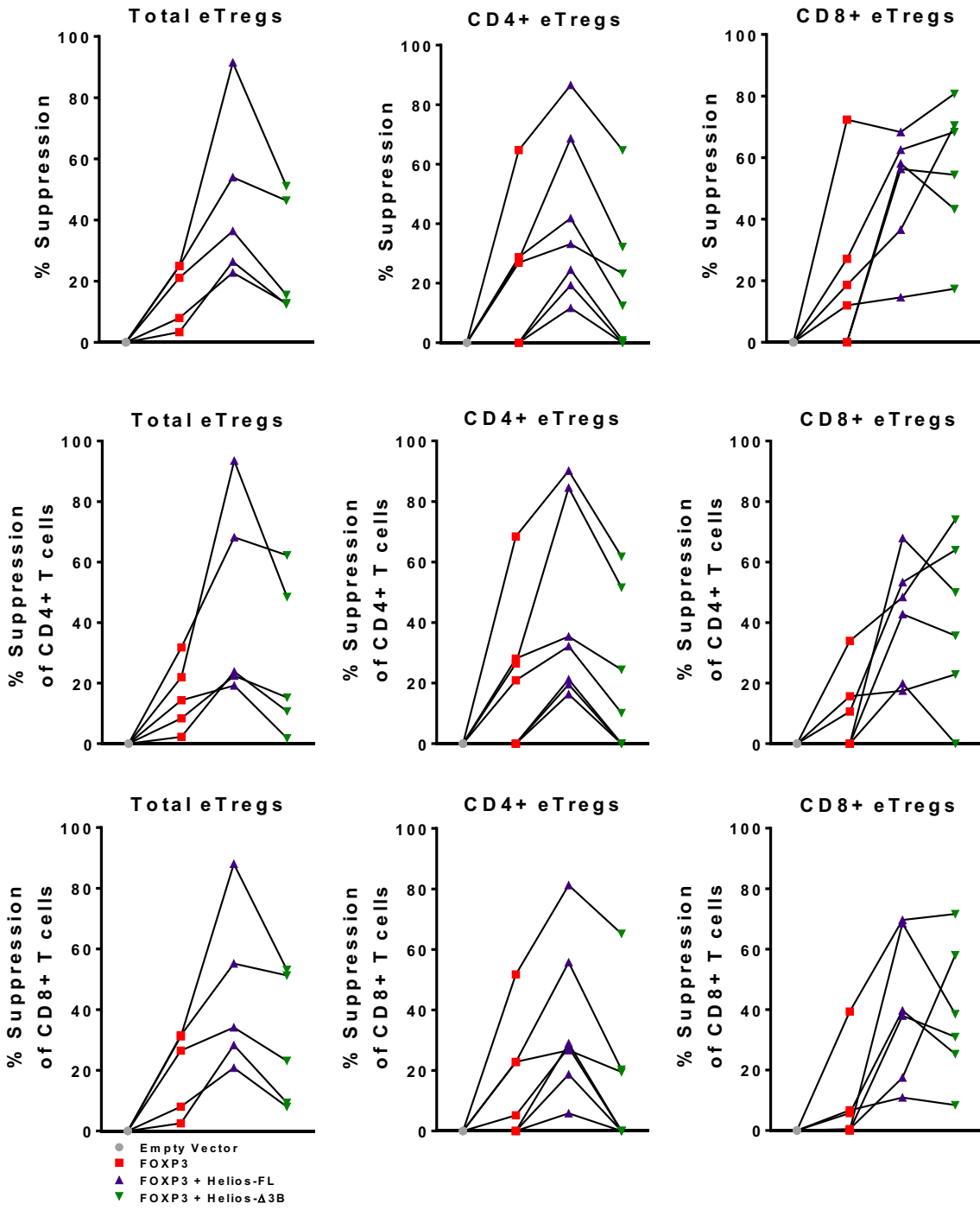


Figure S7: Overexpressing Hel-FL and Hel-Δ3B does not affect T cell proliferation. A)

Average cell counts of eTregs growing in IL-2 supplemented media for 2 days starting on Day 3 post-transduction. n=5 for each group from 4 different donors. * $p \leq 0.05$ in each comparison based on a one-tailed Wilcoxon test for each time point. **B)** Graph of individual replicates for each group depicted in Figure S7A. Average fold change reported above each graph.

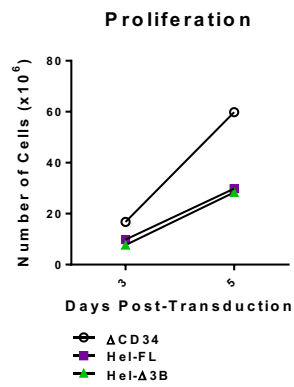
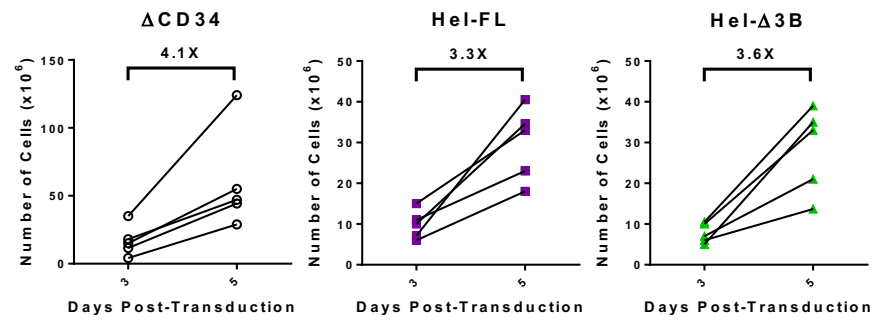
A**B**

Figure S8: FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs express regulatory T cell markers. **A)** Expression of Treg markers by CD4⁺ and CD8⁺ eTregs. Marker expression was assessed via flow cytometry and plotted as percent of CD4⁺ or CD8⁺ eTregs positive for the indicated marker. n=3-7 and 5 different donors. Some experiments were performed with two separated cell transductions with the same donor cells and some groups did not have all eTreg cell types. * p<0.05 compared to empty vector control based on one-tailed Mann-Whitney test.

B) Representative histograms of Treg marker expression by CD4⁺ and CD8⁺ eTregs.

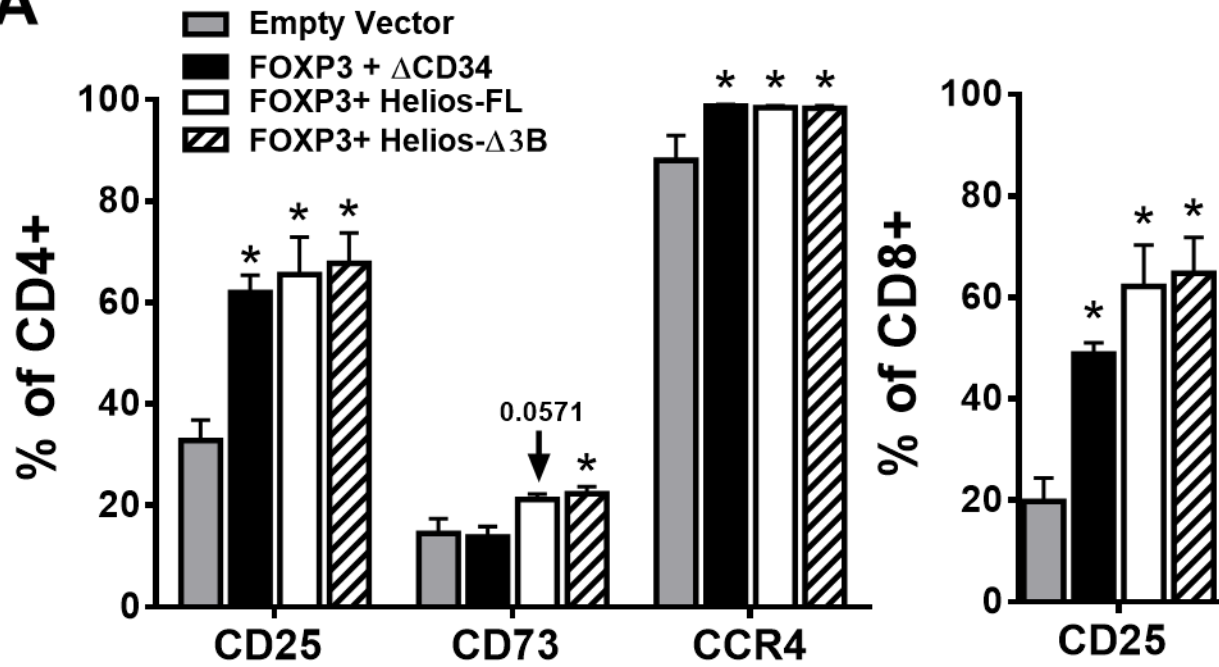
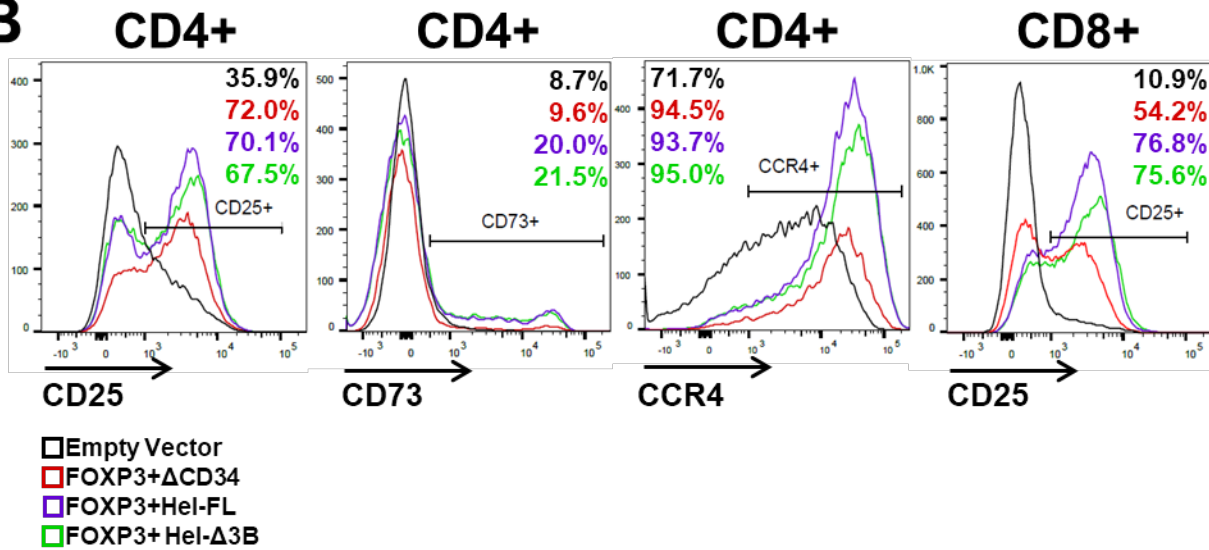
A**B**

Figure S9: CD4+ FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs have reduced cytokine production. A) Cytokine production by CD4+ eTregs. eTregs were stimulated for 6 hours with anti-CD3 and anti-CD28 plate bound antibody and Brefeldin A and Golgi Stop. Cells were assessed for cytokine production via intracellular cytokine staining and flow cytometry. Values normalized to empty vector control and n=4-9 with 4-6 different donors. Some experiments were performed with two separated cell transductions with the same donor cells and some groups did not have all eTreg cell types. *p<0.05 compared to empty vector control based on one-tailed Mann-Whitney test. B) Representative dot plots of CD4+ eTreg intracellular cytokine production.

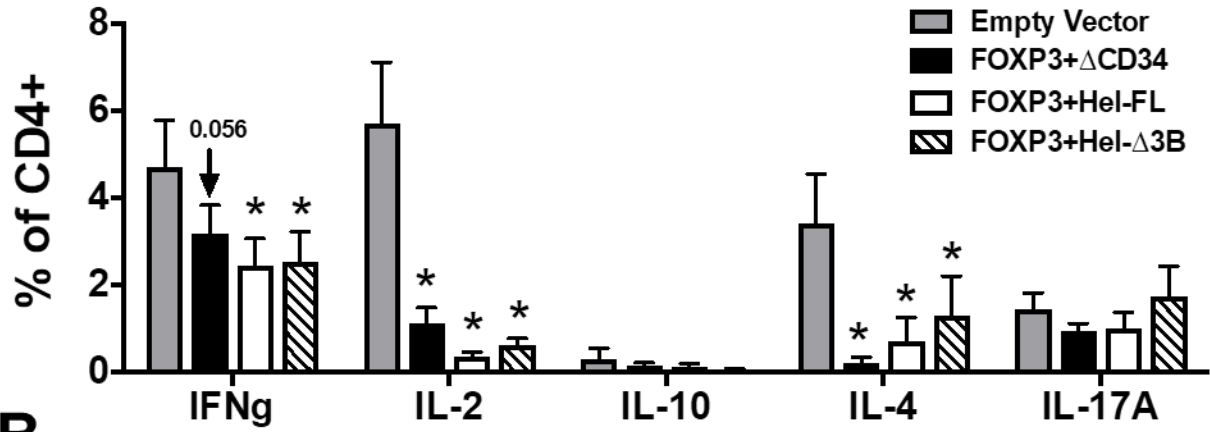
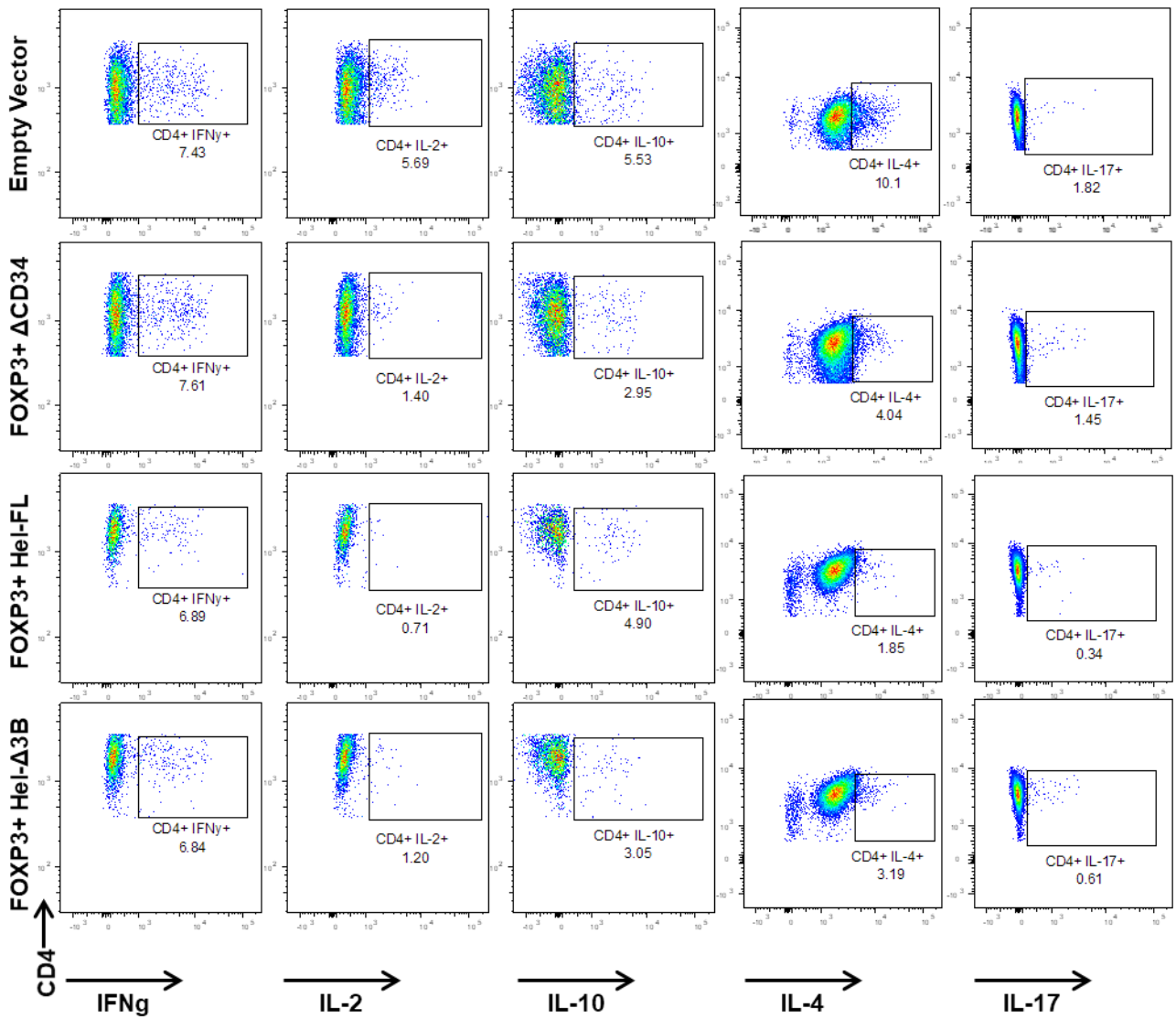
A**CD4⁺ eTregs****B**

Figure S10: CD8+ FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs have reduced cytokine production. **A)** Cytokine production by CD8+ eTregs. eTregs were stimulated for 6 hours with anti-CD3 and anti-CD28 plate bound antibody and Brefeldin A and Golgi Stop. Cells were assessed for cytokine production via intracellular cytokine staining and flow cytometry. Values normalized to empty vector control and n=4-9 with 4-6 different donors. Some experiments were performed with two separated cell transductions with the same donor cells and some groups did not have all eTreg cell types. *p<0.05 compared to empty vector control based on one-tailed Mann-Whitney test. **B)** Representative dot plots of CD8+ eTreg intracellular cytokine production.

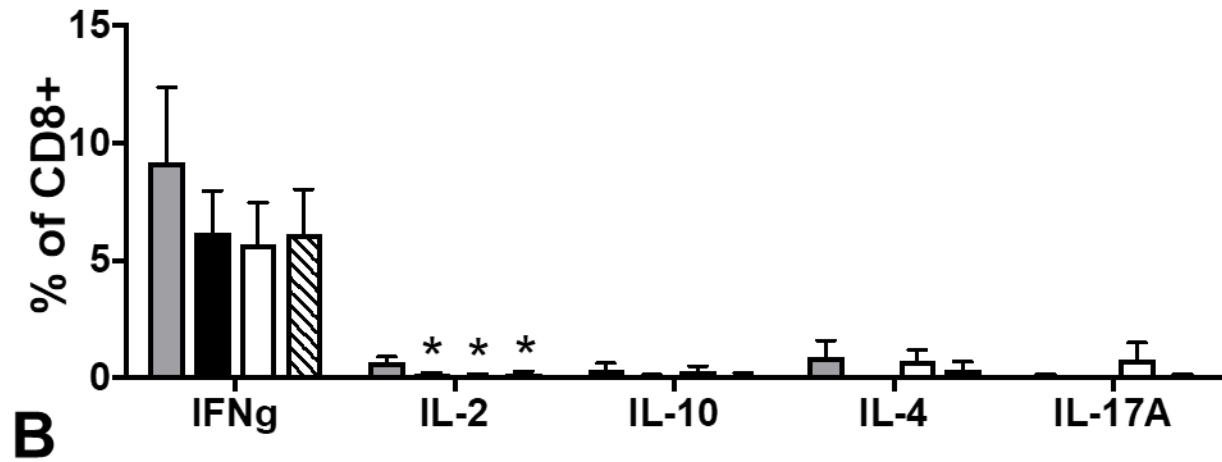
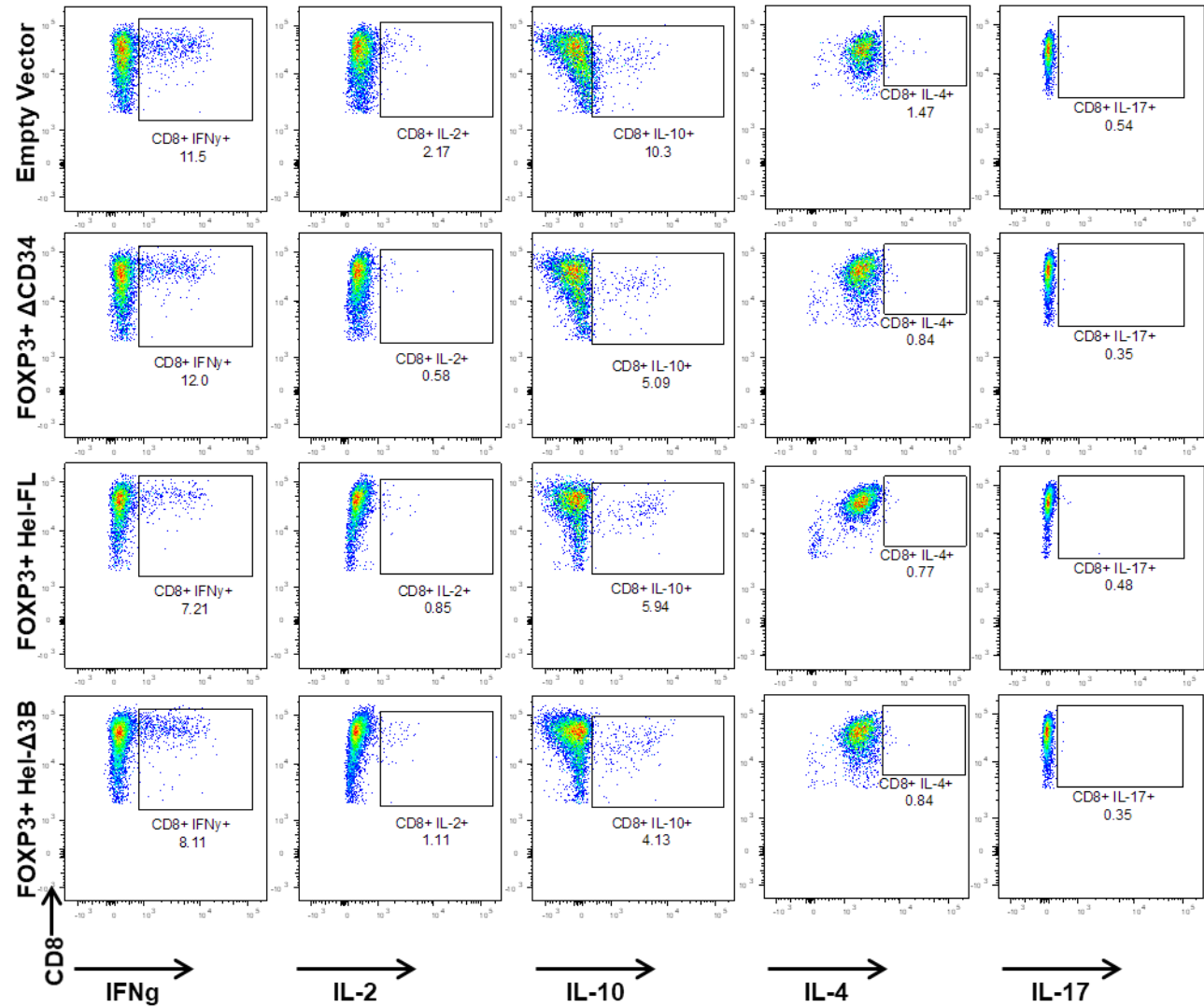
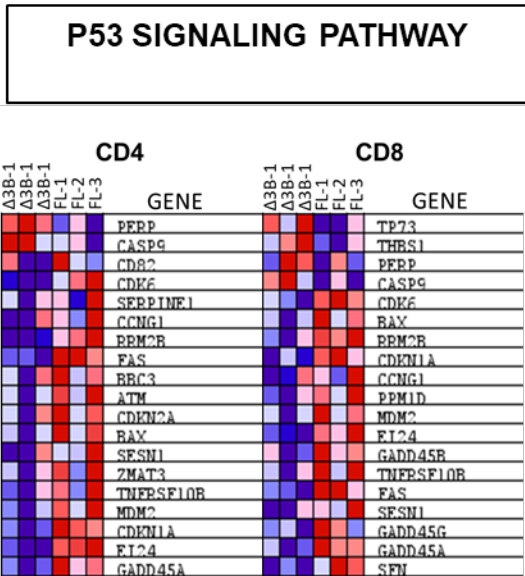
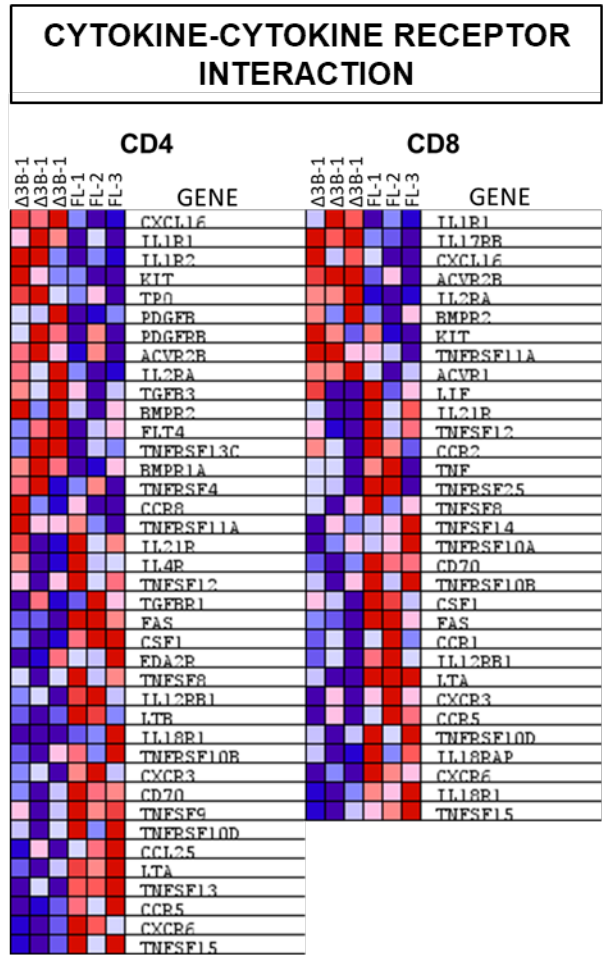
A**CD8+ eTregs****B**

Figure S11: Different genes are altered in pathways enriched in FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD4+ eTregs compared to the corresponding CD8+ eTregs. Heatmaps of genes altered in common pathways enriched in CD4+ and CD8+ eTregs in each of the three donors for the FOXP3+Hel-FL (FL1-FL3) vs FOXP3+Hel-Δ3B (Δ3B1-Δ3B1) comparison. These pathways are **A)** p53 signaling, **B)** cytokine-cytokine receptor interaction and **C)** cell adhesion molecules CAMs.

A



B



C

