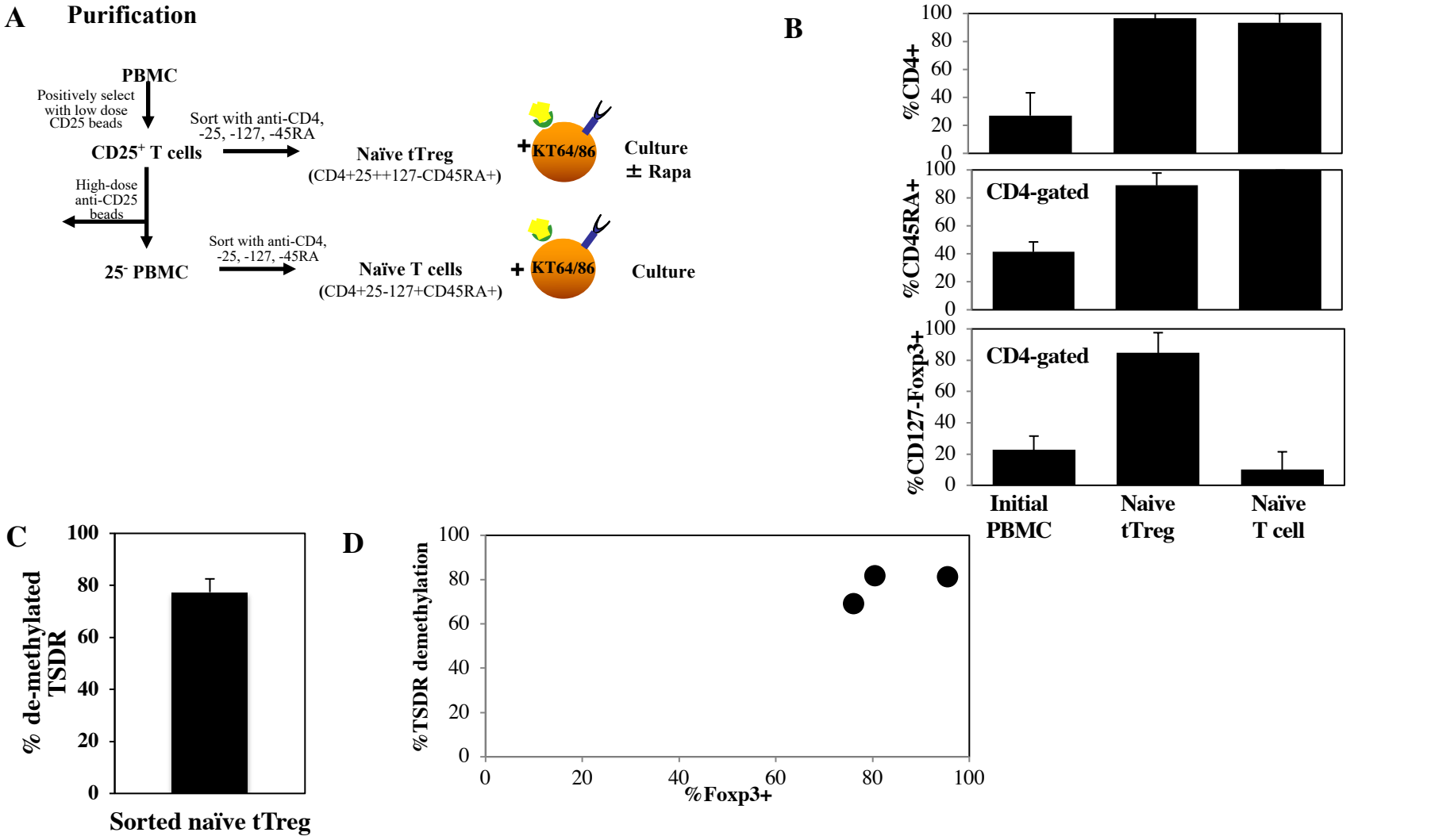


Figure S1: Purification schema and characterization of naïve human CD4+ T cells and tTreg. Peripheral blood mononuclear cells were isolated from human apheresis products and were purified by flow sorting. (A) Schema outlining the purification of naïve tTreg and naïve CD4 Teff used in this study. (B) Summary of flow phenotyping for purity. Purified naïve conventional CD4 T cells were 93 ± 7% CD4+ and >99% CD45RA+, while naive tTreg were 97 ± 3% CD4+, 89 ± 9% CD45RA+, and 85 ± 13% CD127-Foxp3+. (C) Genomic DNA was isolated from sorted naïve tTreg and Foxp3 TSDR demethylation status was assessed with bisulfite sequencing. Data represent the average of all 11 methylation sites in the TSDR. (D) Comparison of % Foxp3+ by flow with degree of TSDR demethylation for the 3 purifications used in this analysis.

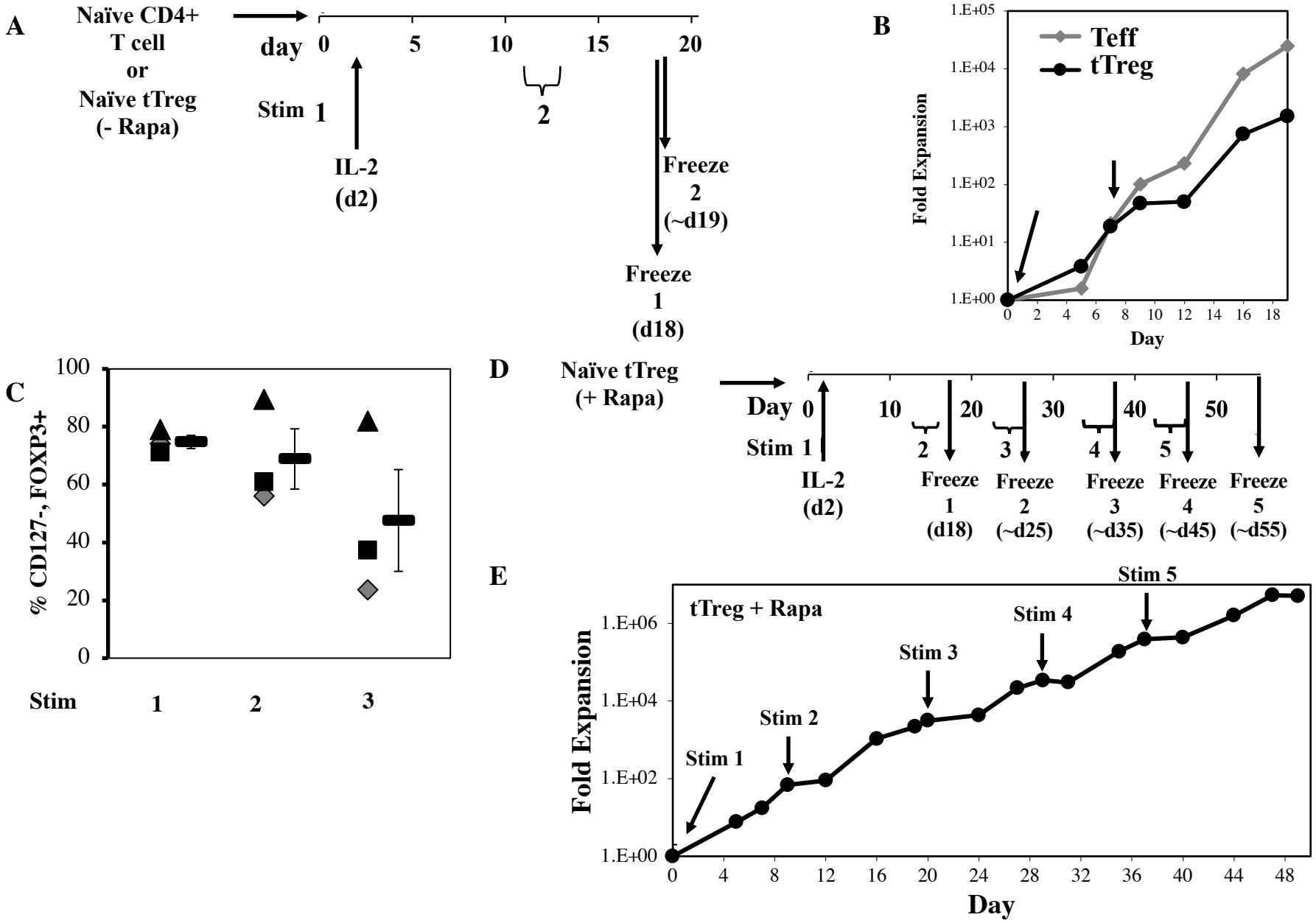


Figure S2: Characterization of naïve human tTreg expanded in vitro in the absence or presence of rapamycin. (A) Schema outlining the timeline for stimulation and assay of expanded naïve tTreg and naïve CD4 Teff. (B) Representative example of expansion for CD4 T cells (Teff) and tTreg-No Rapa. (C) Schema outlining the timeline for stimulation and assay of expanded naïve tTreg expanded in the presence of rapamycin. (D) Representative example of expansion for tTreg stimulated 1 to 5 times in the presence of rapamycin.

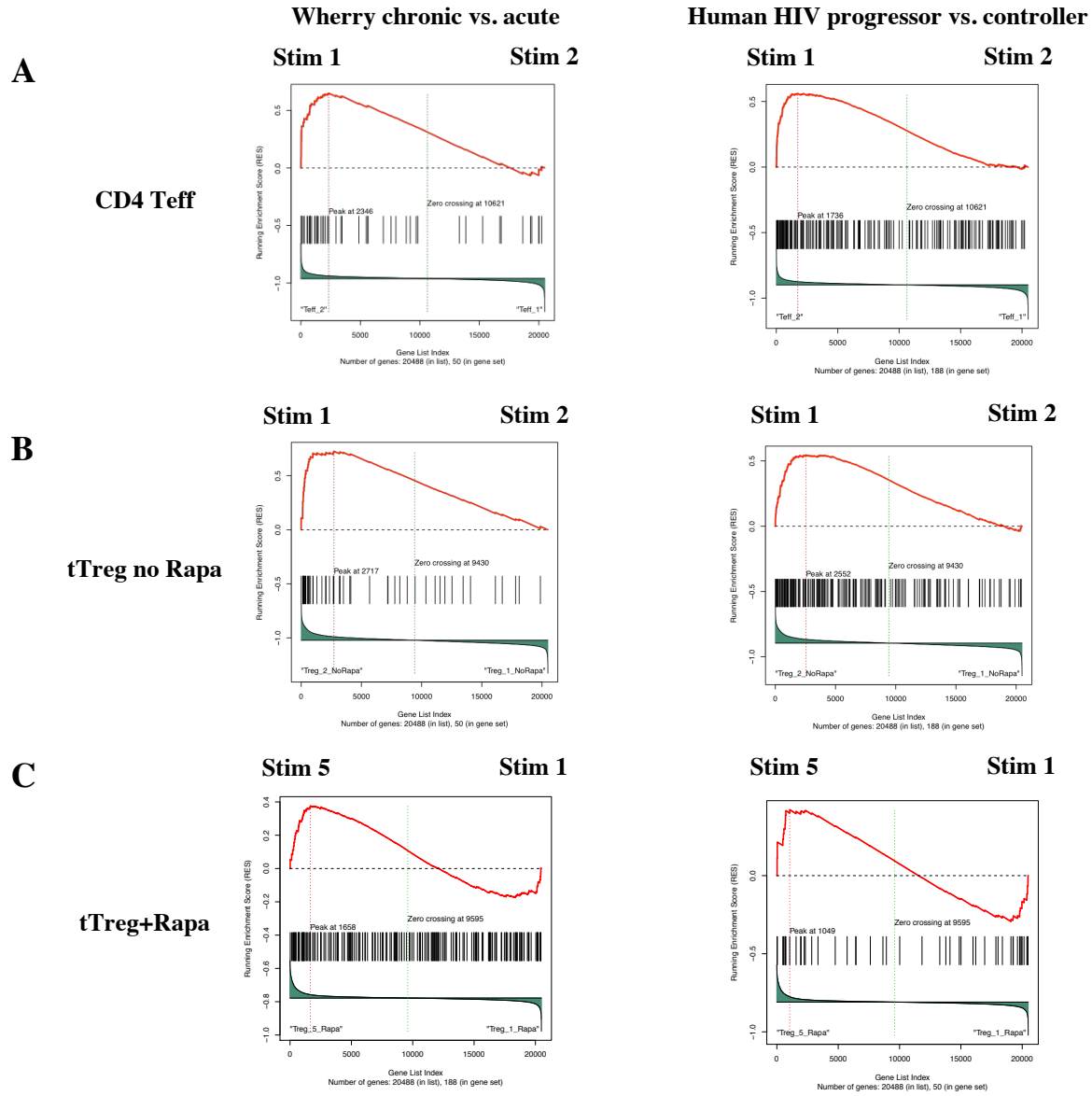


Figure S3: Naïve CD4+ T cells and tTreg cultured in the absence of rapamycin stimulated twice develop an exhaustion signature , in contrast to naïve tTreg-Rapa stimulated 5 times. GSEA of transcripts previously identified as being expressed in exhausted murine CD4 and CD8 T cells (Wherry chronic vs. acute), and transcripts previously identified as being expressed in human CD8 T cells from HIV patients that progress to, or control, AIDS. Comparisons are made with CD4 Teff expanded with 1 vs. 2 stimulations (A), tTreg expanded in the absence of rapamycin with 1 vs. 2 stimulations (B), and tTreg expanded in the presence of rapamycin with 1 vs. 5 stimulations (C).

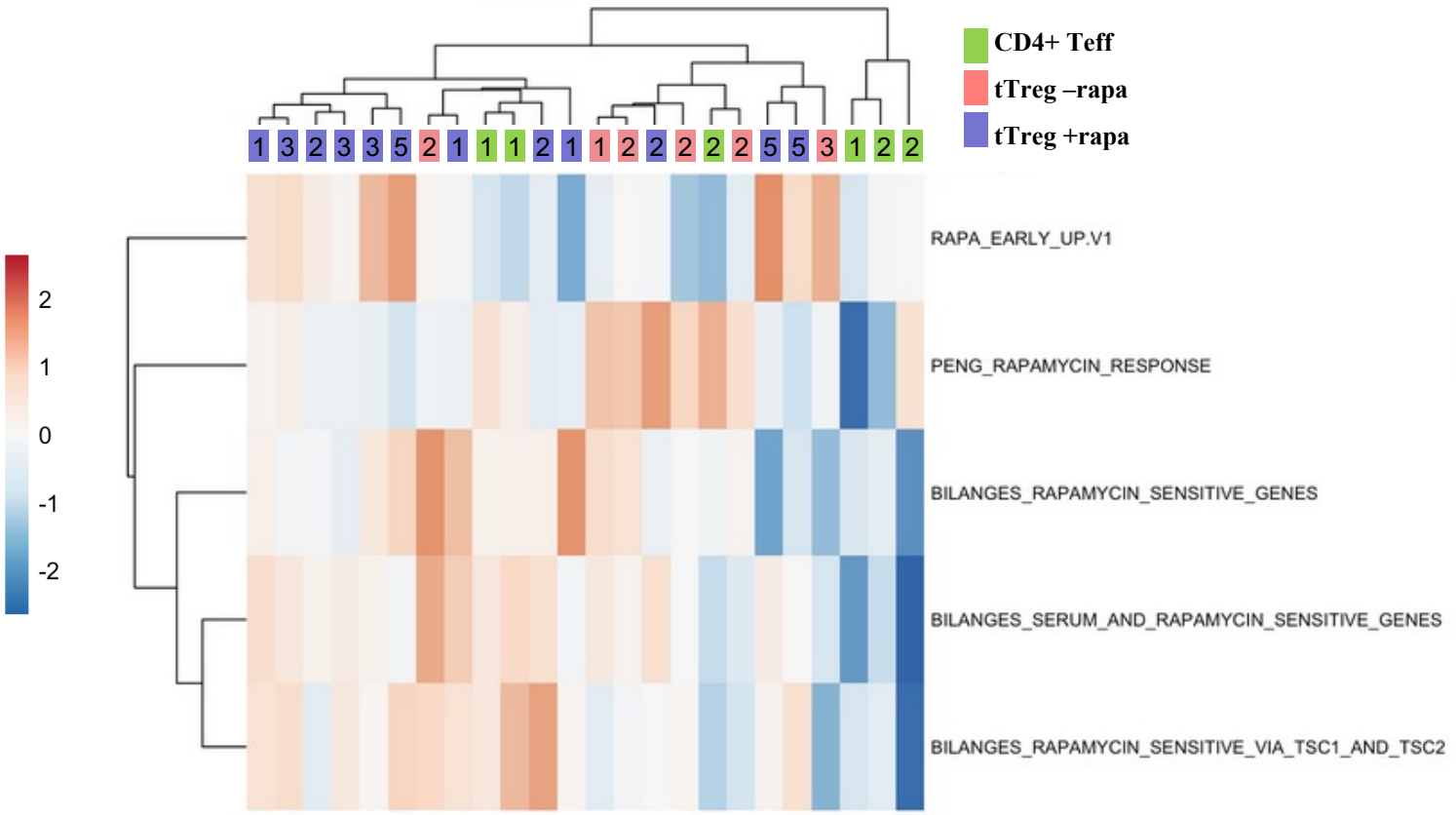


Figure S4: tTreg-Rapa expanded 3 or 5 rounds of stimulation do not preferentially align with previously defined rapamycin-related gene expression. Naïve PB CD4+ T cells and tTregs were sort-purified (CD4+25-127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using up to 5 rounds of stimulation. (A) Heatmap of rapamycin-related gene sets enriched between CD4 Teff, tTreg-No Rapa and tTreg+Rapa stimulated the indicated number of times using ssGSEA.

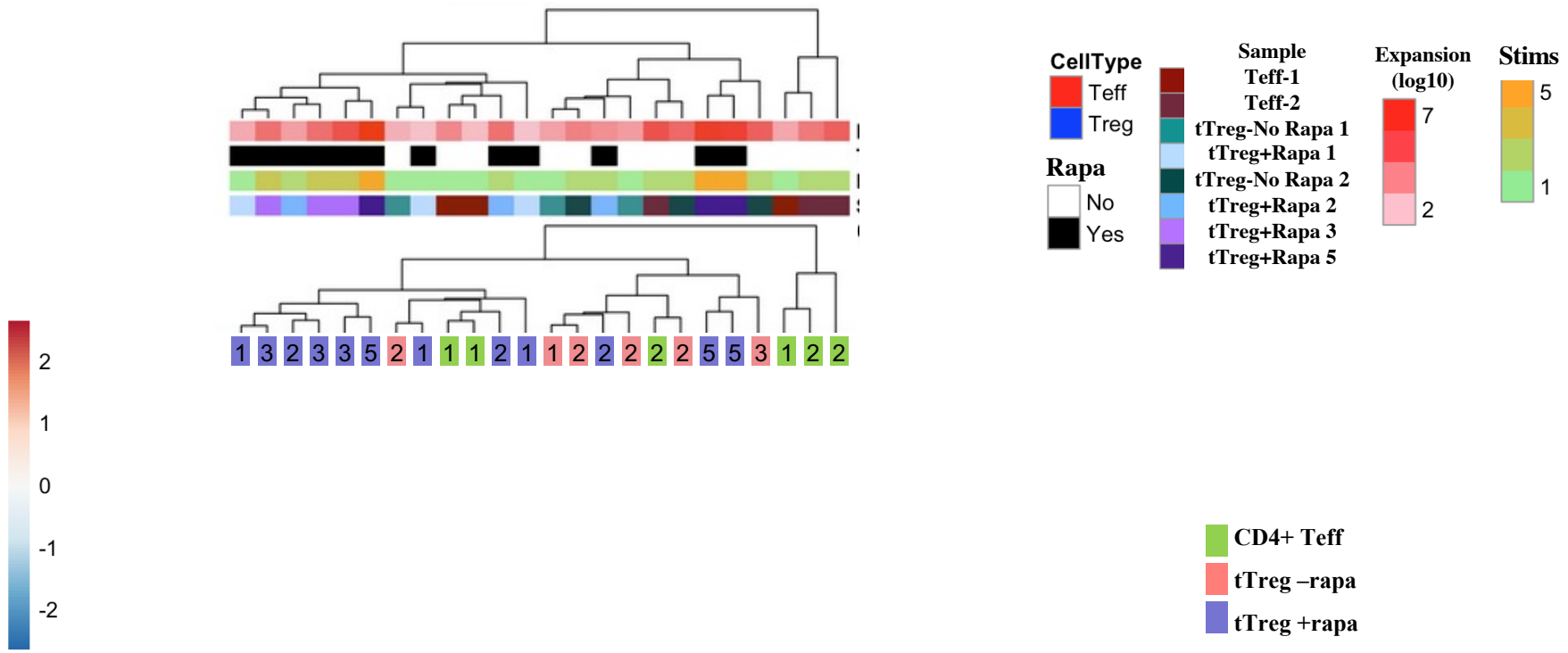
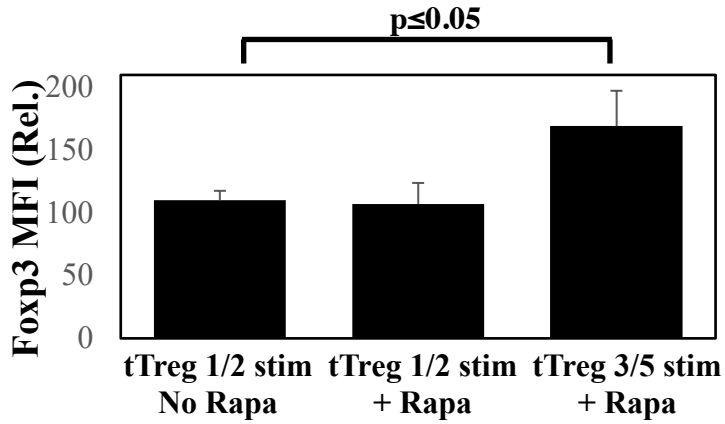


Figure S4: tTreg-Rapa expanded 3 or 5 rounds of stimulation do not preferentially align with previously defined rapamycin-related gene expression. Naïve PB CD4+ T cells and tTregs were sort-purified (CD4+25-127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using up to 5 rounds of stimulation. (A) Heatmap of rapamycin-related gene sets enriched between CD4 Teff, tTreg-No Rapa and tTreg+Rapa stimulated the indicated number of times using ssGSEA.

A



B

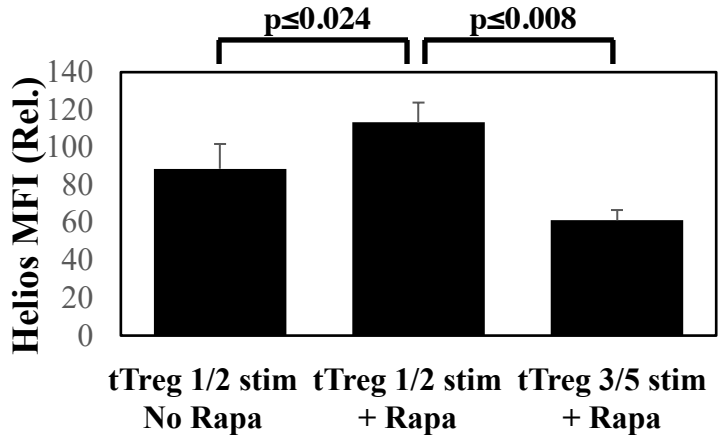


Figure S5: tTreg-Rapa stimulated 3 or 5 times have higher Foxp3 and lower Helios expression than tTreg-No Rapa stimulated 1 or 2 times. To assess (A) Foxp3 and (B) Helios protein expression changes following stimulation, cultured cells from tTreg-No Rapa (1 and 2 stimulations), tTreg+Rapa (1 and 2 stimulations)and tTreg+Rapa (3 and 5 stimulations) were stained with antibodies to the respective transcription factors and analyzed by flow cytometry. Results shown are paired data from 3 donors.