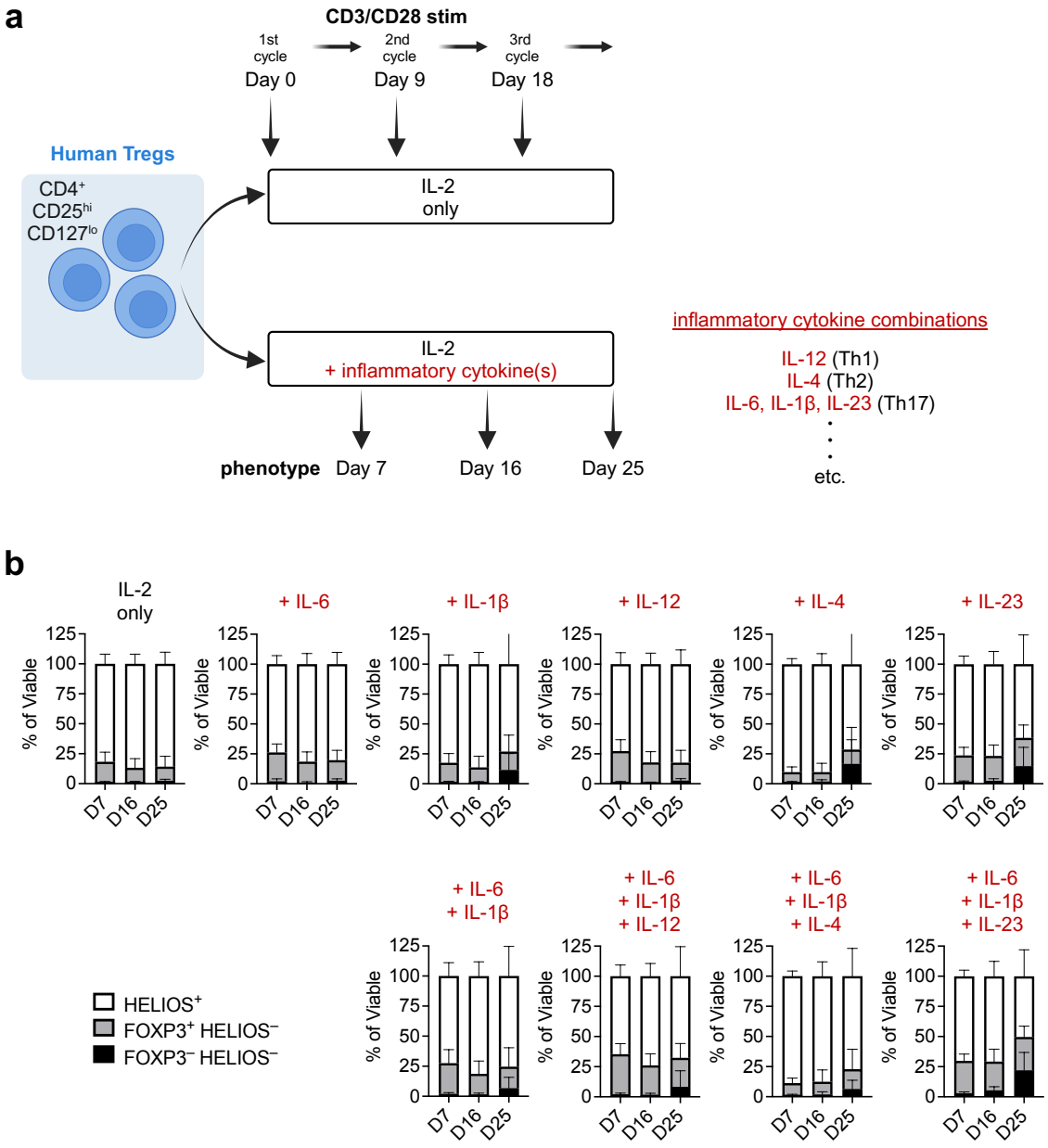
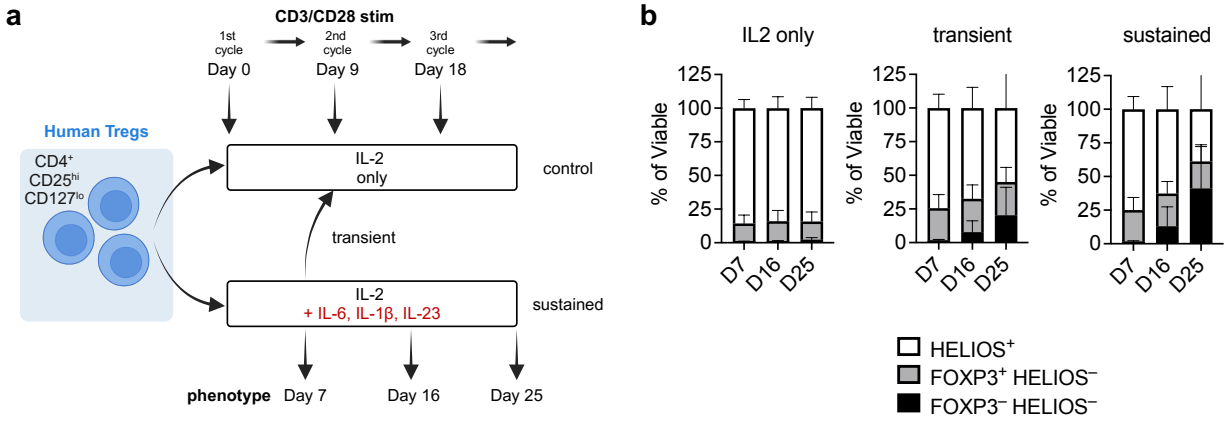
Extended Data Figures

Extended Data Figure 1



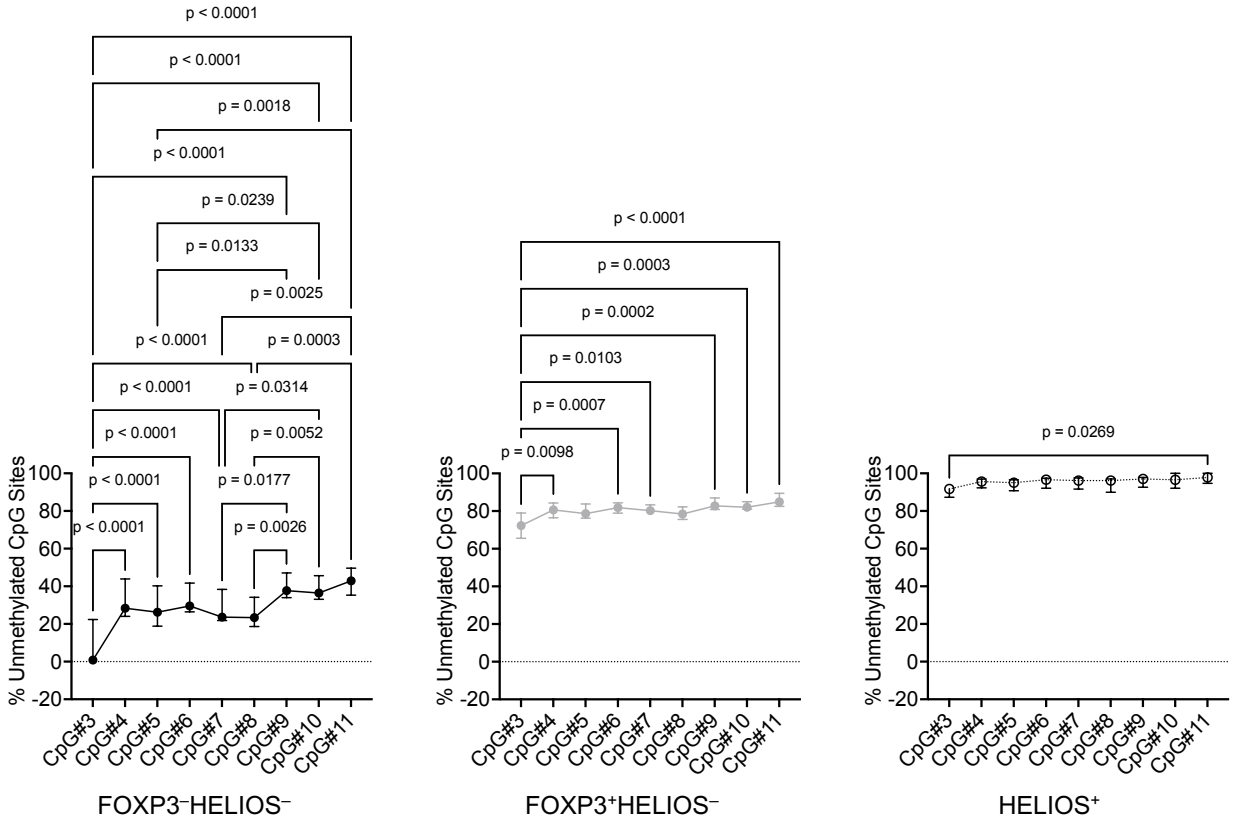
Extended Data Figure 1. Primary human Treg instability can be induced by different inflammatory cytokines. (a) Human Tregs were expanded either with IL-2 alone or IL-2 plus the indicated cytokine(s). **(b)** Average distribution of cells (n = 4-5 normal human donors) within the gated HELIOS^{+}, FOXP3^{+}HELIOS^{-}, and FOXP3^{-}HELIOS^{-} sub-populations at days 7, 16, and 25 of culture is summarized. Results are shown as mean ± standard deviations.

Extended Data Figure 2



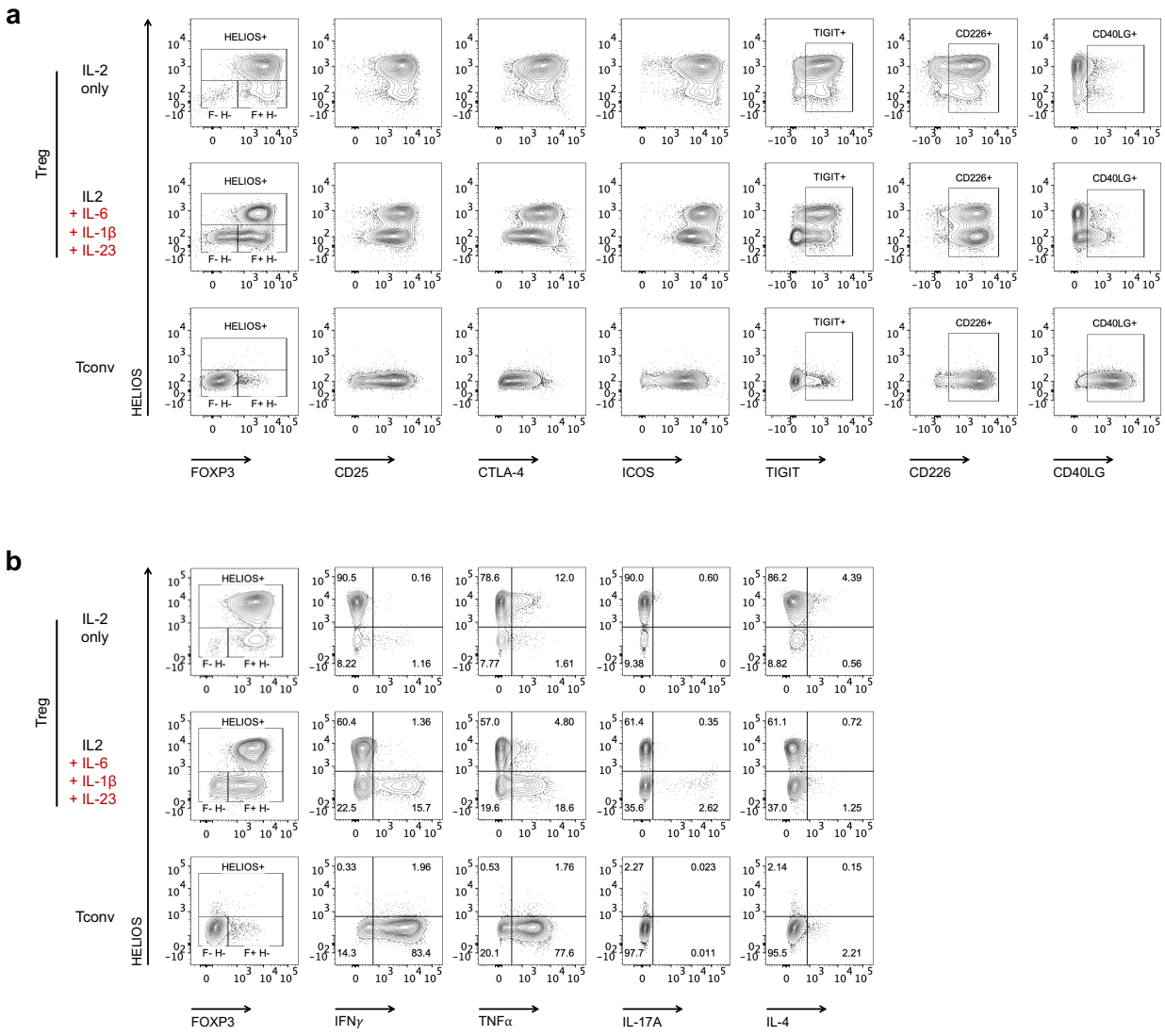
Extended Data Figure 2. Prolonged inflammatory cytokine exposure induces human Treg destabilization to a greater degree. (a) Human Tregs were expanded in IL-2 only or challenged with the added presence of IL-6, IL-1β, and IL-23. The inflammatory cytokine cocktail was supplied either transiently for 7 days (transient) or for the duration of the 25-day culture (sustained). For the transiently challenged samples, cells were washed and subsequently expanded in IL-2 only conditions for the remainder of the culture period. **(b)** Average distribution of cells (n = 8 healthy human donors) amongst HELIOS^{+}, FOXP3^{+}HELIOS^{-}, and FOXP3^{-}HELIOS^{-} populations, as determined via flow cytometry at days 7, 16, and 25 of culture is summarized. Results are shown as mean ± standard deviations.

Extended Data Figure 3



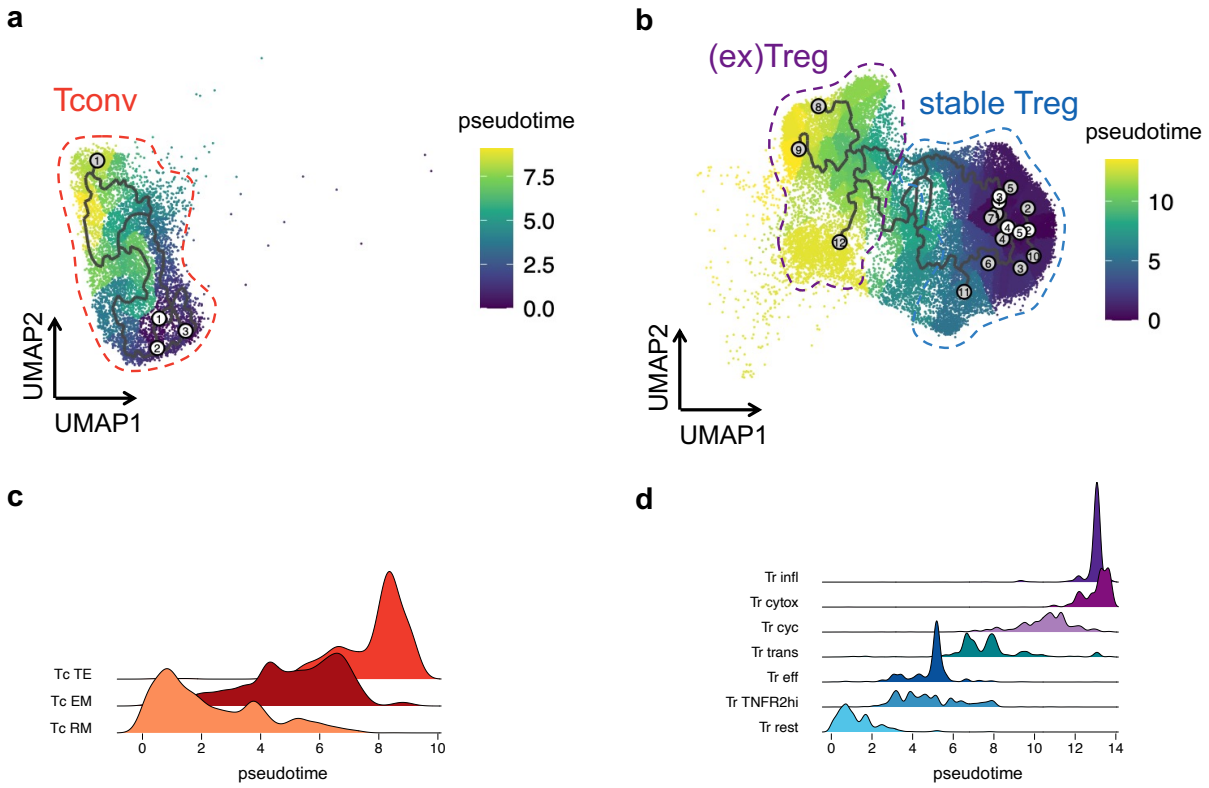
Extended Data Figure 3. DNA re-methylation of CpG sites in FOXP3⁻HELIOS⁻ cells occurs at different rates across the TSDR (corresponding to Fig. 1d). Percentage of unmethylated CpGs sites at each assayed CpG within the *FOXP3* CNS2 (TSDR) from each of the FACS-purified FOXP3⁻HELIOS⁻ (left), FOXP3⁺HELIOS⁻ (middle), or HELIOS⁺ (right) sub-populations, as determined by pyrosequencing of bisulfite-converted genomic DNA. Results are shown as mean ± standard deviations. Statistical significance was calculated via two-way ANOVA with post Tukey test comparing the sorted sub-populations ($p < 0.0001$ for each comparison between sub-populations, not shown) and the individual CpG sites.

Extended Data Figure 4



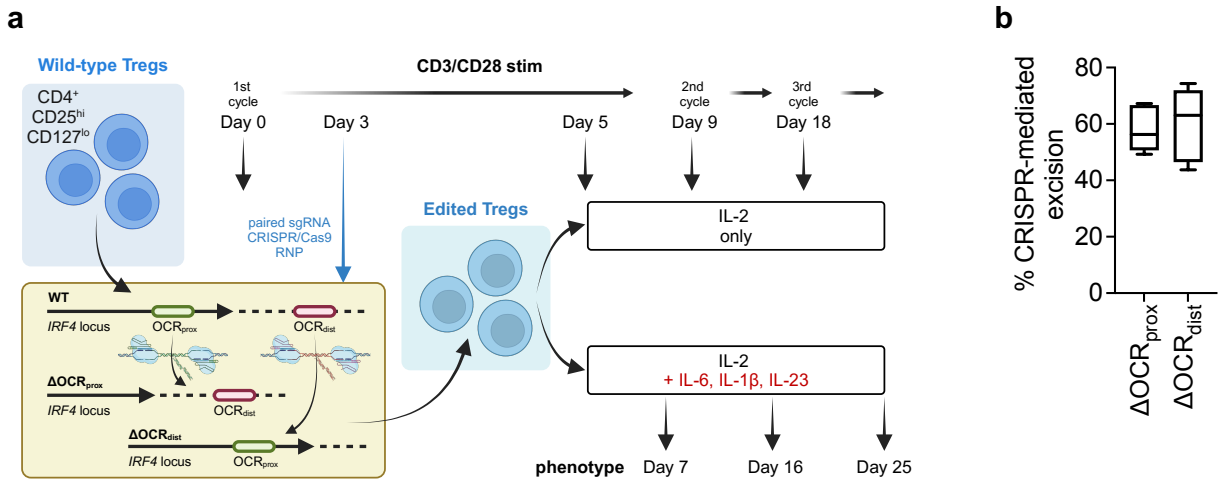
Extended Data Figure 4. Inflammatory cytokine exposure induces dysregulated expression of Treg phenotypic markers and pro-inflammatory cytokines (corresponding to Figure 2). **(a)** Tregs were stimulated with anti-CD3/CD28 through repeated 9-day cycles either in IL-2 only or in the added presence of IL-6, IL-1β, and IL-23. Representative expression profiles and gating for common phenotypic markers, including CD25, CTLA-4, ICOS, TIGIT, CD226, and CD40LG. **(b)** After 25 days of *in vitro* culture, cells were stimulated for 5h with PMA and ionomycin prior to intracellular flow staining for pro-inflammatory cytokine expression. Representative intracellular flow staining profiles for the expression of FOXP3, HELIOS, and pro-inflammatory cytokine, including IFNγ, TNFα, IL-17A, and IL-4.

Extended Data Figure 5



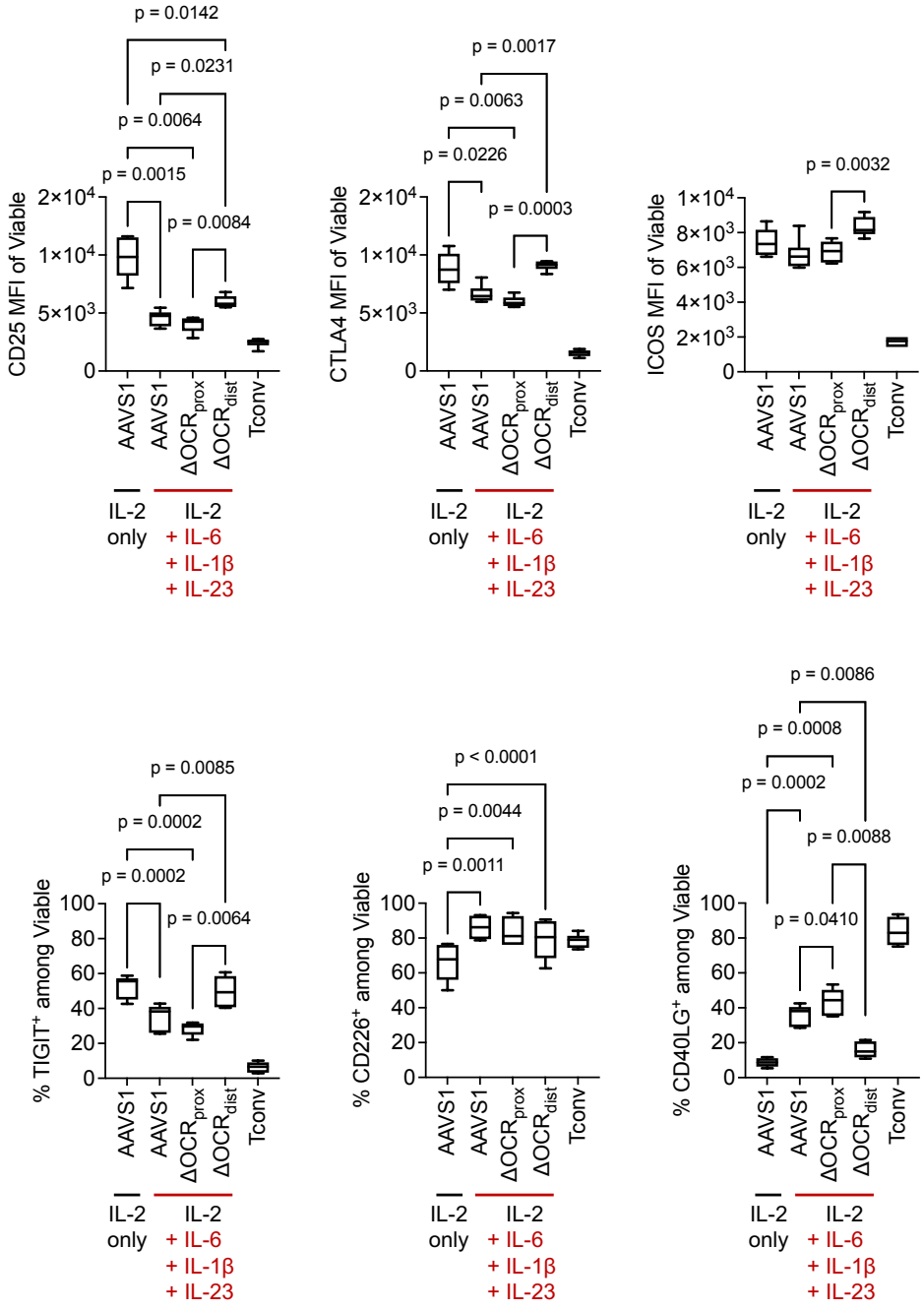
Extended Data Figure 5. Pseudotime trajectory inference indicates a transition from stable Tregs to (ex)Tregs (corresponding to Figure 4). (a, b) Pseudotime trajectories inferred via Monocle3 reveal an ordering of cell states for Tconvs (a) and Tregs (b). **(c, d)** Ridge plots for the distribution of each cluster from Tconv samples (a) and Treg samples (b) along the inferred pseudotime.

Extended Data Figure 6



Extended Data Figure 6. CRISPR/Cas9-mediated excision of OCR_{prox} and OCR_{dist} in primary human Tregs (corresponding to Figure 5e, 5f). (a) Paired CRISPR sgRNAs were designed to flank each target OCR. After initial activation in IL-2 only conditions, Tregs were treated with CRISPR/Cas9 RNPs to target AAVS1 (as a mock editing control) or to excise either OCR_{prox} or OCR_{dist}. The edited cells were then split into cultures with IL-2 alone or IL-2 plus IL-6, IL-1β, and IL-23, and re-stimulated with anti-CD3/CD28 on days 9 and 18. **(b)** Each CRISPR-targeted OCR was PCR amplified from genomic DNA with primers flanking the sgRNA cut sites. The deletion efficiency for each OCR was determined as the fraction of PCR amplicons with lengths corresponding to the loss of the target region between the cut sites specified by the sgRNA pair.

Extended Data Figure 7



Extended Data Figure 7. ΔOCR_{dist} Tregs retain a suppressive Treg phenotype following even after prolonged inflammatory cytokine exposure (corresponding to Figure 6). Primary human Tregs were edited with CRIPSR/Cas9 RNPs and subsequently challenged with repeated anti-CD3/CD28 stimulation in the presence or absence of IL-6, IL-1β, and IL-23 to induce destabilization. Average geometric mean of fluorescence intensity (CD25, CTLA-4, ICOS) or percent positive (TIGIT, CD226, CD40LG) of common Treg phenotypic markers at day 25. Data are from n = 5-6 normal human donors. Statistical significance was calculated via one-way ANOVA with post Tukey test.