Stain-and-sort screen for GITR expression in primary mouse Regulatory T-cells

ATAC-seq peaks were selected for guide RNA design using GuideScan v2.0. The resulting gRNAs were filtered to keep those with specificity score ≥ 0.2, to remove repeats of GGGGG, TTTTT, and ranked by chromatin-accessibility score derived from the sum of overlapping ATAC-seq fragments. gRNA were selected for to restrict overlap by more than either 5bp or 10bp. 5% of the total library were GC-matched, nontargeting gRNAs and added as negative controls.

The gRNA library was cloned into a Murine Stem Cell Virus (MSCV) retroviral mU6 promoter-driven expression system using NEBuilder HiFi DNA Assembly (NEB, cat# E2621L). This retrovirus contains a Thy1.1 reporter gene under control of a separate PGK promoter (Addgene #209613).

gRNA containing retrovirus libraries were produced using Platinum-E (Plat-E) Retroviral Packaging Cell Line (Cell Bio Labs cat# RV-101) following transient transfection with lipofectamine 3000. Retroviral supernatant was 50x concentrated with Retro-X and stored in liquid nitrogen.

Naive CD4+ T cells were harvested from spleen and lymph nodes of dCas9-KRAB[1] crossed to Foxp3-eGFP CD4-CRE C576BL/6 mice using magnetic selection (Thermo Cat# 8804-6821-74). 4 mice were used as independent biological replicates.

Naïve T cells were seeded at 0.5e6 cells/mL and cultured in complete RPMI (10% FBS, 1% Penicillin, 1% Streptamycin, 1% Gentamicin, 1% L-glutamine, 1% HEPES, 1% sodium pyruvate, 55nM 2-mercaptoethanol) and activated with Th0 conditions (250ng/mL αCD3, 1µg/mL αCD28, 2µg/mL αIL-4, 2µg/mL αIFNg) for 24 hours. Cells were transduced with gRNA library containing virus and 6.66ng/µL polybrene in RPMI at a low multiplicity of infection (MOI=0.3) using spinfection 900 x g for 2 hours at 30C. Cells were then cultured in Treg polarizing conditions (Th0 conditions + 10ng/mL IL-2, 10ng/mL hTgfβ) for 96 hours. Live cells were stained for viability-e780 (Thermo Cat# 65-0865-14), Gitr-PE (BD Bioscience Cat# 558140), CD4-e450 (Thermo, Cat# 48-0042-80), Thy1.1-APC (Stem Cell Technologies, Cat# 60024AZ) for 30 minutes on ice and sorted using a Sony SH800Z with a 70µm chip. At least 40,000 cells were sorted from the top and bottom 15% of Gitr signal (Gating: Lymphocytes / Live / Singlets / $CD4^+$ / $THY1.1^+$ / $FOXP3\text{-}eGFP^+$ / $GITR^{hi/lo}$). gDNA was recovered using Zymo Quick-DNA Miniprep Plus Kit (Zymo Cat# D4068) and gRNA were recovered via PCR. Libraries were sequenced on an Illumina Miseq using 20bp single end reads.

References:

1. Gemberling, M.P. et al. Transgenic mice for in vivo epigenome editing with CRISPR-based systems. *Nat Methods* **18**, 965-974 (2021).