SOP: **Isolation of resting and activated primary mouse regulatory T cells and CD4 effector cells *ex vivo***
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Summary

Below (in **Supplementary Figure 1**) is a schematic of the Foxp3 DTR knock-in allele. Due to restricted Foxp3 expression to regulatory T cells, only regulatory Tr cells express DTR. Therefore, treatment of mice with Diphtheria toxin (DT) results in specific depletion of regulatory Tr cells resulting in multiorgan autoimmunity and death within 3 weeks if DT is continuously delivered. For activated regulatory Tr cell isolation, we administer DT twice (D0=day 0 and D1=day 1) and then 10-12 days later harvest the regulatory Tr cells that "bounce back" (these will be regulatory Tr cells that escaped initial depletion and have expanded to fill the now empty Tr niche, or regulatory Tr cells that come out of the thymus during this time period). These regulatory Tr cells are, upon harvest D10-12, participating in suppression of a severe inflammatory process. In this way we can isolate regulatory Tr cells *ex vivo* under physiologic conditions without resorting to *in vitro* activation. For FACS sorting, regulatory Tr cell populations from these mice we stain for CD4 and CD25 and then gate on $CD4^+CD25^+Foxp3GFP^+$ cells (based on the reported expressed at the endogenous Foxp3 locus). Control regulatory Tr cells are also sorted from untreated mice for "resting" regulatory Tr cell controls. For isolation of "resting" naïve $CD4^+$ T cells, $CD4^+Foxp3^-$ cells are sorted from unmanipulated Foxp3-IRES-DTR-GFP mice. For isolation of "activated" effector $CD4^+$ T cells, $CD4^+Foxp3^-$ cells are sorted from DT-treated Foxp3-IRES-DTR-GFP mice (note: activation status is evident from increase in the frequency of $CD25^+Foxp3^-$ cells).

Materials List

1. RPMI 1640, 1X, with 2mM L-glutamine (Cellgro, Cat# 10-040-CM)
2. Characterized Fetal Bovine Serum (HyClone, Cat# SH30071)
3. 100 µm cell strainers (BD Biosciences, Cat# 352360)
4. 6 cm tissue culture dishes
5. 50mL Corning conical centrifuge tubes
6. 1mL syringes
7. Diphtheria toxin from *Corynebacterium diphtheriae* was purchased from Sigma-Aldrich (Cat# D0564) and was reconstituted according to the manufacturer's protocol.
8. See **Materials and Methods** in "Resources" website below for additional information on reagents.
9. Foxp3-IRES-DTR-GFP mice from Alexander Rudensky lab, Sloan-Kettering Institute (see below in "Procedure" section).
10. Eppendorf Refrigerated Centrifuge 5810R
11. Microscope

Medium (for primary cell suspension maintenance, on ice, before MACS separation)

RPMI 1640, 1X
10% FBS

Procedure

1. All peripheral lymph nodes and spleens are collected from C57BL/6 mice from Jackson labs and pooled in a 6 cm dish containing RPMI 1640 supplemented with 10% FBS. Tissues and cell suspensions should be kept on ice at all times. Following removal of all media from the dish (retain this media as it contains some cells), the pooled lymph nodes and spleens are chopped into a fine pulp. The pulp is then

scraped into a 100 µm cell strainer placed on a 50mL conical tube. Alternative rinsing cell strainer with RPMI supplemented with 10% FB and mashing tissue through cell strainer with the back of a 1ml syringe plunger, until only white residue remains above strainer. Spin cells down and then resuspend in buffer as instructed in magnetic isolation protocol (CliniMACS affinity-based technology, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

2. Frozen diphtheria toxin stocks were frozen and thawed once and 50 µg/kg of diphtheria toxin was injected intraperitoneally on day 0 and day 1.

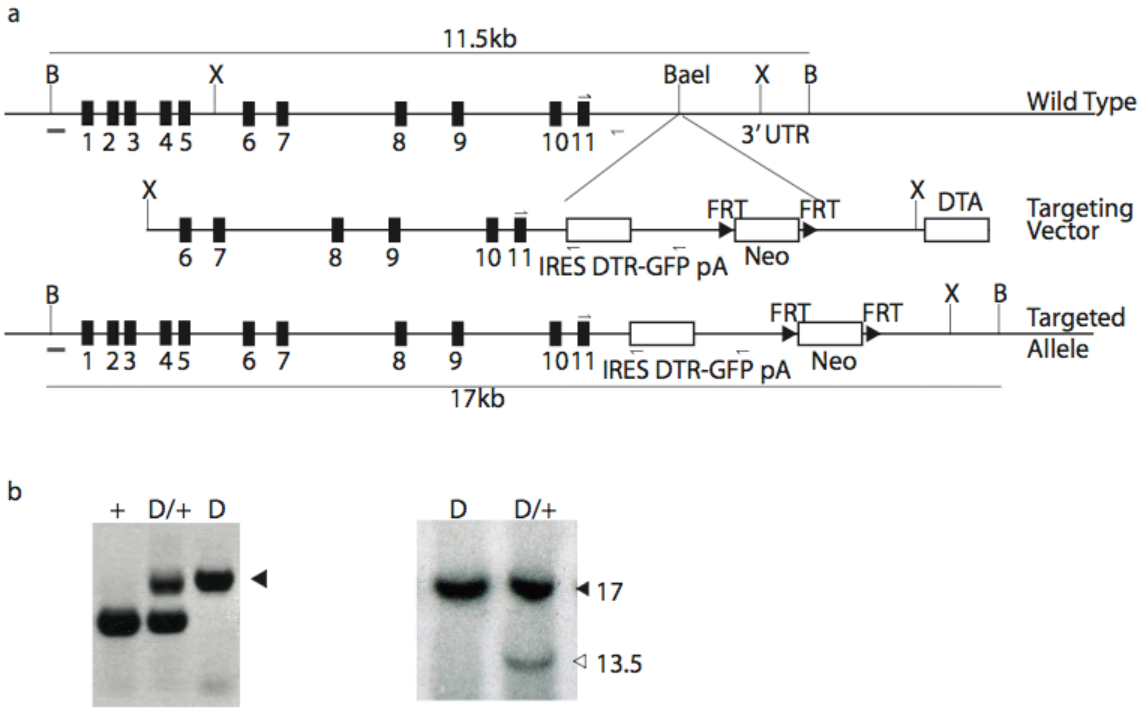
3. Single cell suspensions are stained with CD4 and CD25 antibodies.

4. The following populations were sorted from pooled single cell suspension to high purity (>98%):
   $CD4^+Foxp3GFP^+$ cells
   $CD4^+Foxp3GFP$- cells

Resources (manuscript and protocol for DT-mediated depletion of regulatory Tr cells in Foxp3-IRES-DTR-GFP mice):

http://www.nature.com/ni/journal/v8/n2/full/ni1428.html

SOP: Isolation of mouse resting and activated regulatory T cells and effector CD4 T cells using the DTR depletion system



Supplementary Figure 1 Generation of the *Foxp3*^{DTR} knockin allele. (a) Schematic diagram of the gene targeting strategy to insert DTR-GFP cDNA into the 3' UTR of the Foxp3 locus, showing the maps for the Foxp3 locus, targeting vector, and targeted allele. Positions of PCR genotyping primers are depicted as arrows and the location of the Southern probe as a gray line. (b) PCR (left) and Southern blot (right) analysis of ES cells containing the targeted allele (filled arrowhead). The 13.5kb fragment (open arrowhead) in the Southern blot represents the wild-type Foxp3 allele, and is present as a result of feeder cell contamination. Two independently derived ES cell clones were utilized to generate chimeric mice.