Induction of Mouse Foxp3$^+$ iTregs *in vitro*

A protocol developed following original reports of TGFβ induction of Foxp3 in naive mouse CD4+ T cells [1,2], adapted here to also use the *Heligmosomoides polygyrus* mimic of TGFβ, TGM [3-5].

Reagents and Equipment

1. Foxp3-GFP reporter mice (see *Note 1*)
2. T cell medium:
   500 ml RPMI 1640 (Gibco Cat.No. 31870-025)
   50 ml FBS (to 10%) Heat Inactivated (Gibco Cat.No.10500064)
   5 ml Penicillin/Streptomycin, 100x (Gibco Cat.No.15140-122)
   5 ml L-Glutamine 100x (200mM) (Gibco Cat.No.25030-024)
   5 ml MEM Non-Essential Amino Acids (NEAA) 100x (Gibco Cat.No. 11140-035)
3. Red Blood Cell lysis buffer (Sigma R7757)
4. Antibodies for FACS sorting:
   Anti-CD4-PerCP/Cy5.5 (Biolegend, clone GK1.5, diluted 1:25) (*Note 2*)
   Anti-CD25-APC (eBioscience, clone PC61.5, 1:200),
   Anti-CD44-PE/cy7 (Biolegend, clone IM7, 1:200)
6. Anti-CD3: 0.5 or 1 mg/ml, clone 145-2C11 (Invitrogen)
   Make up working stock of eg 2.5 µg/ml, according to starting concentration.
7. Murine IL-2: 250,000 U/ml in sterile 0.1% BSA/PBS (Miltenyi Cat.No. 130-098-221))
   Make up 1600 U/ml working stock, 32 µL 250,000 U/ml IL-2 in 5 ml T cell media

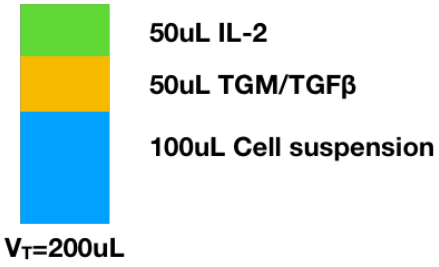
8a.. TGF-β: typically 100 µg/ml (UCB)
   Make up 80 ng/ml working stock, eg 4 µl 100 µg/ml TGFβ in 5 ml T cell media

8b. TGM stock, varying concentrations (In house)

9. Flat-bottom 96-well plate (Corning Costar 3596 – polystyrene TC treated)

Protocol:

1. Coat 96-well plate with 50 µl/well of 10 µg/ml anti-CD3 in sterile PBS, parafilm and put at 4°C overnight (*Note 3*). This step can also be carried out at 37°C for 2 hr.
2. Wash plate 3x with 100 µl/well of sterile PBS
3. Make single cell suspension of splenocytes from Foxp3-GFP reporter mice by passing through a 70 µm cell strainer and resuspending in T cell medium. Centrifuge at 400 *g* for 5 min and resuspend the pellet in 2 ml of RBC lysis buffer at RT° for 2 min, then add 8 ml of T cell medium, centrifuge again (400 *g*, 5 min) and take up pellet in T cell medium.
4. Sort CD4$^+$CD25^{low}Foxp3$^-$ T cells by flow (see below).
5. Count cells; expect 1-2x10^7 cells per spleen.
5. Wash cells 3 x in T cell media - 400 *g* for 5 min at 4°C.
6. Resuspend cells at a concentration of 2x10^6 cells/ml in T cell media
7. To each well add:
   100 µl of cell suspension (2x10^5 cells/well) (*Note 4*).
   50ul of 1600 U/ml IL-2 working stock (final concentration 400 U/ml).
   50 µl of 80 ng/ml TGFβ or TGM working stock (final concentration 20 ng/ml),
   or 50 µl of T cell media to IL-2 only wells (*Note 5*)



8. Incubate 37°C in 5% CO_2 for 72-96 hours and stain for flow cytometry

Notes:

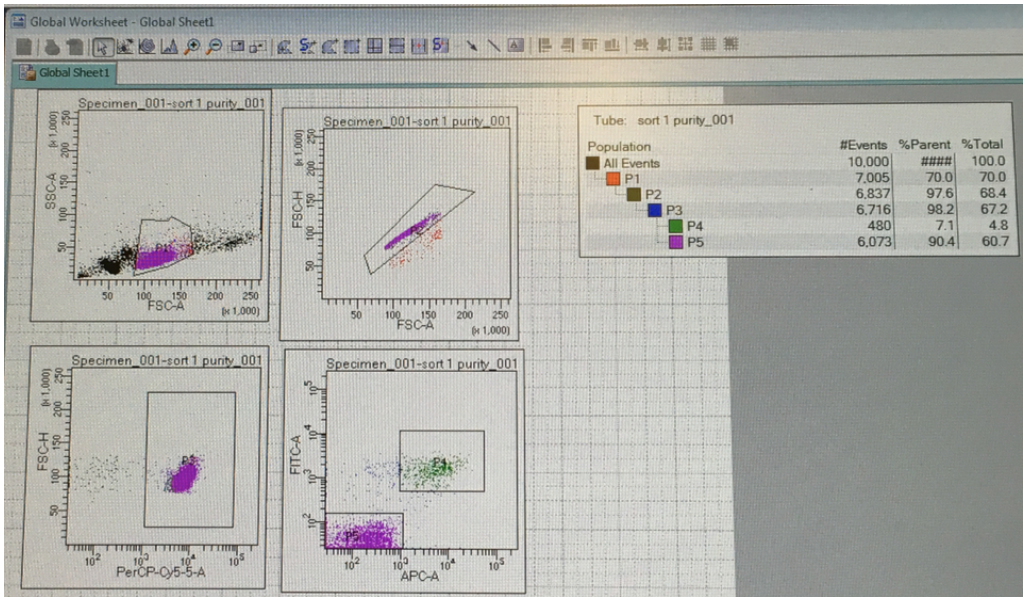
1. This protocol will work equally well with non-transgenic T cells, in which case sorting for CD4+ cells by AutoMACS can be used, but the baseline of Foxp3 expression will be considerably higher. For MACS purification, use the Naive $CD4^+CD25^-$ isolation kit (Miltenyi, Cat.No. 130-104-453) for a lower $Foxp3^+$ starting population.
2. Anti-CD4 used at higher concentration than normal (1:400).
3. Results are better with plate-bound anti-CD3, for overnight coating of plates, concentrations down to 2.5 µg/ml are effective. Some protocols use anti-CD3 and anti-CD28 coated beads (Miltenyi, 130-093-627).
4. If other materials (eg inhibitors, antibodies) are to be added, reduce volume of cell suspension to 50 µL of $4x10^6$ cells/ml and add other materials in 50 µl of T cell media.
5. IL-2 alone controls are also required to set up Foxp3 negative gating.

4. Monoclonal antibody 1D11 can be added to block mammalian TGFβ. Dilute to 40 µg/ml in media, and add 50 µl/well (final concentration 10 µg/ml))
   Include control mouse IgG1 (MOPC31C, BD 557273) at same dilution. Preincubate TGFβ with the mAb or control antibody for 1hr at 37°C.

5. Addition of Retinoic Acid can drive higher Treg induction [6].
   Retinoic Acid stock, 100 µM (Sigma, R2625, 3.0 g/100 ml))
   Make up 4 nM working stock, first dilute 1/10 in T cell media to get 10 µM; add 2 µl diluted RA to 5 ml T cell media for 4 nM working stock (4x conc). Add 50 µl/well.
6. Cells from C57BL/6 and BALB/c mice differ, and C57BL/6 cells can be seeded at a higher concentration i.e. $3\text{-}4x10^5$ cells/well.
7. To scale up (e.g. to produce iTregs for in vivo transfer), coat a 24 well plate (Corning Costar CLS3527) with 500 µl of 10 µg/ml anti-CD3, add cells at $1x10^6$ per well or higher ($1.5\text{-}2x10^6$) for C57BL/6 background mice.

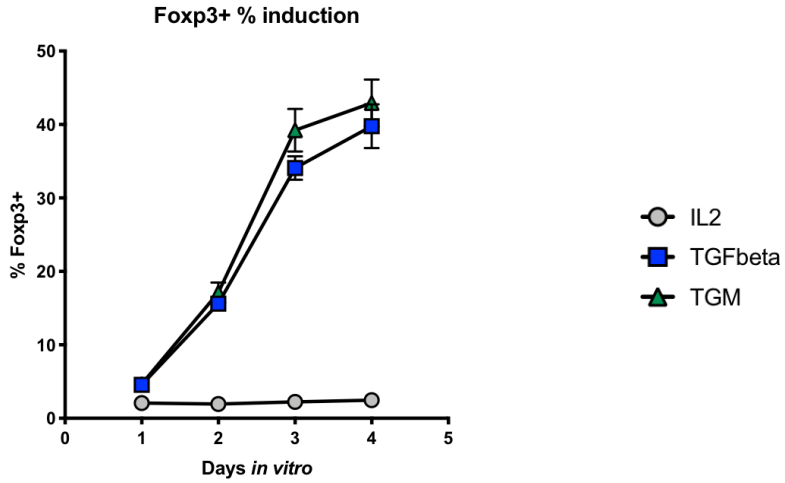
FACS Sorting:

Typical sort for $CD4^+$(PerCP)$Foxp3^-$(FITC)$CD25^-$APC) T cells using the Aria with a 70 µm nozzle, cells at $30x10^6$/ml in Automacs buffer containing 20 U/ml DnaseII, having passed through 40 µm cell strainer prior to running on the instrument. Sort into 15 ml falcons preloaded with 700 µl FBS (vortex tubes to wet the sides with protein).



Sorting for $Foxp3^+$ iTregs at the end of an experiment, use the 85 µm nozzle because the cells are activated and sort into tubes preloaded with 0.5 ml of media with 0.5 ml FBS, to provide high protein environment for cells that are sorted in PBS.

Example iTreg induction:



References:

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