SOP: **Isolation of primary mouse naïve Tn and regulatory Tr cells**
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Summary

For isolation of highly pure $CD4^+/CD25^-$ naive Tn and $CD4^+/CD25^+$ regulatory Tr cells from pooled mouse splenocytes and lymphocytes, two Miltenyi magnetic bead column purification kits are utilized (CliniMACS affinity-based technology, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Regulatory Tr cells are isolated from the positive fraction of the final step using the first kit (regulatory T cell isolation kit, see "Materials List"), and the negative fraction, $CD4^+/CD25^-$ cells, undergo an additional round of positive selection for CD4 to increase the purity of this population.

The negative selection step in the regulatory T cell isolation kit negatively selects for everything except for $CD4^+$ T cells, which flow through, by retaining in the column all the cells (red blood cells, B cells, granulocytes, macrophages, and CD8 T cells).

Negative selection utilizes a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against:
CD8a (Ly-2; isotype: rat IgG2a),
CD11b (Mac-1; isotype: rat IgG2b),
CD45R (B220; isotype: rat IgG2a),
CD49b (DX5; isotype: rat IgM)
Ter-119 (isotype: rat IgG2b)

Following elution of the CD4 fraction, these cells are labeled and positively selected for CD25 (a specific marker of regulatory Tr cells). The CD25 negative fraction is then labeled for positive selection on CD4 to improve the purity of the $CD4^+/CD25^-$ population.

Materials List

1. Miltenyi $CD4^+CD25^+$ regulatory T cell isolation kit, mouse (Miltenyi, Cat# 130-091-041)
2. Miltenyi CD4 (L3T4) microbeads (Miltenyi, Cat# 130-049-201)
3. See Miltenyi product information sheets (below website) for necessary buffers and reagents
4. C57BL/6 mice from Jackson labs
5. RPMI 1640, 1X, with 2mM L-glutamine (Cellgro, Cat# 10-040-CM)
6. Characterized Fetal Bovine Serum (HyClone, Cat# SH30071)
7. 100 µm cell strainers (BD Biosciences, Cat# 352360)
8. 6 cm tissue culture dishes
9. 50mL Corning conical centrifuge tubes
10. 1mL syringes
11. Eppendorf Refrigerated Centrifuge 5810R
12. 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 1640 supplemented with 10% FBS 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.

2. Follow procedure for $CD4^+CD25^+$ regulatory T cell isolation kit.

3. Set aside $CD4^+CD25^+$ regulatory Tr cell fraction for freezing (or immediately freeze regulatory Tr cells during a break in step 3) as per freezing protocol (SOP: Cryopreservation of mouse naïve Tn, regulatory Tr, and B cells 03/04/2011 R.S. Hansen, T.K. Canfield (UW)).

4. Utilizing the CD25 negative fraction from step 1 (naïve Tn cells), use the Miltenyi CD4 microbeads to further enrich $CD4^+$ cells, followed by freezing as per freezing protocol (SOP: Cryopreservation of mouse naïve Tn, regulatory Tr, and B cells 03/04/2011 R.S. Hansen, T.K. Canfield (UW)).

E. It is recommended that purity of the isolation be tested by staining a small aliquot of purified cells for CD4 and CD25 and analyzing by FACS. Purity should be >95% of cells in lymphocyte gate (non-debris). Also, intracellular staining can be performed for the lineage specifying transcription factor for the Treg cell lineage, Foxp3, in order to determine the frequency of bona fide $Foxp3^+$ Tr cells among the $CD4^+$/ $CD25^+$ cells. Per cent of Foxp3 cells should be >85%. We have observed that % Foxp3 positive cells is typically higher in younger (4-8 week old mice) compared to older mice.

Resources (datasheets for the above Miltenyi kits):

http://www.miltenyibiotec.com/download/datasheets_en/47/MiltenyiBiotec_DataSheet_CD4-(L3T4)-MicroBeads,-mouse_130-049-201.pdf
http://www.miltenyibiotec.com/download/datasheets_en/287/DS130_091_301.pdf