Isolating Primary $CD4^+$ T Cells from Mice & T helper subset generation

Supplies/ Buffers/ Media Needed:

Consumables/Supplies Needed:

- 24-well tissue culture plates
- Sterile 15mL conical tubes
- Sterile, frosted-end microscope slides (double-bagged and autoclaved in groups of four), 1 bag needed per biological replicate
- Sterile petri dishes, 2 per biological replicate
- Autoclaved ultrapure water
- Appropriate CD4+ T cell isolation kit (e.g. R&D Systems bulk CD4+ T cell isolation kit (cat# MAGM202); BioLegend bulk CD4+ T cell isolation kit (cat# 480033); R&D Systems Naïve CD4+ T cell isolation kit (cat# MAGM205))
- Dissection tools (curved forceps and small scissors) and platform
- Sterile 5mL, round-bottomed culture tubes

Anti-CD3 (1mg/mL) and Anti-CD28 (1mg/mL) antibodies (BD Biosciences via Fisher Scientific)

Sterile, 1X PBS

Complete Iscove's Modified Dulbecco's Medium (cIMDM)

- 500mL IMDM
- 10% FBS (50mL, add directly to 500mL purchased IMDM)
- 1% Penicillin/ Streptomycin (5mL, add directly to 500mL purchased IMDM)
- $5x10^{-5}$M BME: Add 250uL of 0.1M BME directly to 500mL purchased IMDM. (Note: To make a 0.1M BME solution dilute 69.9uL of 14.3M BME stock to 10mL IMDM; filter sterilize in hood).
- Store at 4˚C for up to 1 month.

Red Blood Cell (RBC) Lysis Buffer (0.84% NH_4Cl), filter sterilized

- 8.4g NH_4Cl
- 1000mL ultrapure water
- Filter sterilize using sterile 0.22um bottletop filter in hood, into sterile Nalgene bottle
- Store at RT

Trypan Blue

Buffers as required by $CD4^+$ T Cell Isolation Kit of Choice

Cytokines as needed to generate T helper cell subset(s) of choice (See ***T helper Subset Polarizing Conditions*** document for details.)

DAY -1: Preparing 24-well Plate(s)

In the tissue-culture hood:

1. Label a 24-well TC plate (or plates) as needed. For C57BL/6 mice, we routinely prepare 10 wells per mouse.
2. Prepare anti-CD3/anti-CD28 stimulation mastermix as follows:
   0.5mL/well sterile 1X PBS
   2.5uL/well 1ug/uL stock anti-CD3e (final concentration of 5ug/mL)
   5uL/well 1ug/uL stock anti-CD28 (final concentration of 10ug/mL)

Note: Keep antibodies on ice at all times when not in use.

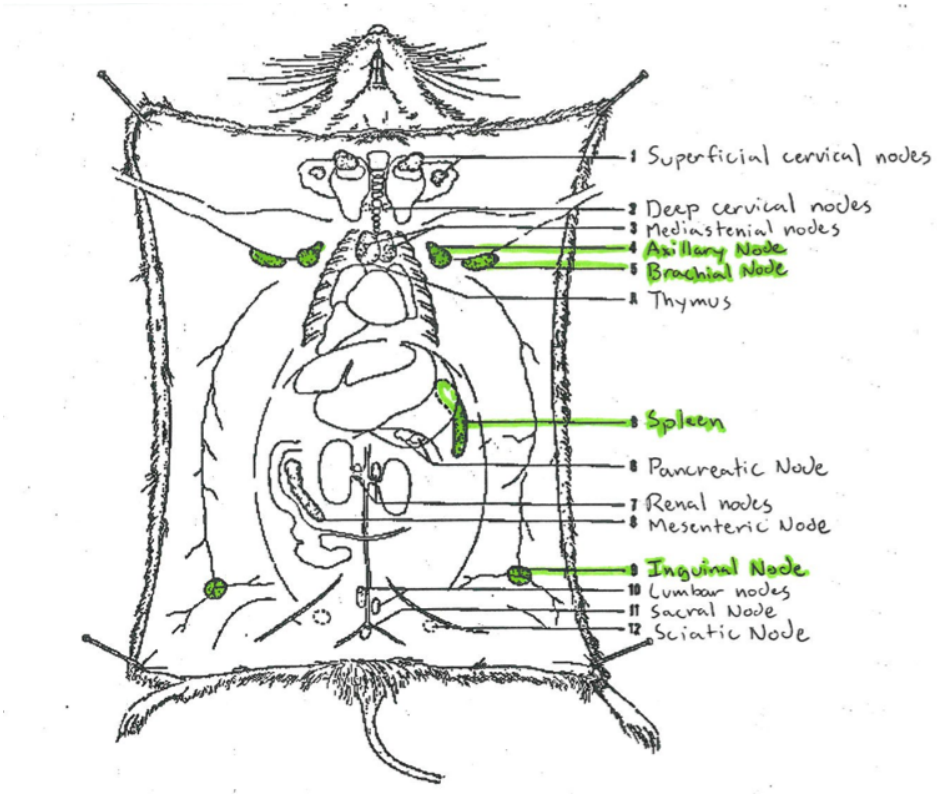
3. Mix the above mastermix by inverting gently 10-15 times and add 500uL of the antibody mixture to each well.
4. Cover plate, wrap edges with parafilm, and incubate overnight at 4˚C.

DAY 0: Spleen and Lymph Node Isolation Procedure

1. Place 5mL of cIMDM in each tube required (1 tube for lymph nodes and 1 tube for spleens *for each biological replicate*). Keep on ice.
2. Prepare 1X magnetic sorting buffer as appropriate for chosen isolation kit. Prepare 5mL of appropriate buffer for each biological replicate.
   For **R&D Systems kits**, dilute 10X MagCellect 1:10 in sterile ultrapure water;
   For **BioLegend kits**, dilute 5X MojoSort buffer 1:5 in sterile ultrapure water.

3. Sac mice and begin dissection procedure immediately. Keep as sterile as possible (spray mouse and equipment with 70% ethanol prior to dissection.) Remove lymph nodes (brachial and axillary nodes) and spleen as highlighted in green on the diagram below, and place in appropriate tube. Secondary lymphoid tissue will sink, fat will float. Keep on ice.

   Dissection Schematic:



4. Pour tube of organs and associated media into a sterile petri dish (only one tube at a time, leave the rest on ice). Save the tube. Homogenize the tissue using the frosted sides of sterile microscope slides, being careful to make the cell suspension as even as possible. Note: Be gentle—hold the slides as close to the non-frosted ends as possible to prevent over-grinding.
5. Rinse the end of one slide in the plated media by scraping with the other slide; repeat for both as needed—as soon as tissue has been even partially homogenized, washing the slides with media to remove suspended cells can

help prevent over grinding those cells. When finished, wick media from the slides by placing them end-down on an empty part of the petri dish.

6. Using a 10mL serological pipet, add 5mL of fresh cIMDM to the dish. Rinse the dish thoroughly to lift all cells from the plate (total volume will therefore be 10mL).
7. Using the same serological pipet, transfer cells and media back to the labeled tube. (Leave extraneous/connective tissue on the plate or slides during transfer.) Place cell suspension on ice.
8. Repeat with remaining tubes/organs. Note that an additional 5mL cIMDM wash (with a fresh serological pipet) for the spleen dish can be helpful for increasing yield.
9. Centrifuge cell suspensions in the large Sorvall centrifuge at 1800rpm at 4˚C for 5 minutes to pellet isolated cells (fat will float), and carefully aspirate off the media.
10. Close the tube tightly and flick or tap the bottom of the tube firmly with your finger to resuspend the pellet fully (this will break up the pellet and allow the RBC lysis buffer to reach all of the cells evenly).
11. Set a timer for 3 minutes and apply the appropriate volume of RBC lysis buffer to each tube as follows:
    a. 2mL for lymph nodes
    b. 4mL for spleen
12. Invert the tubes gently but thoroughly to ensure even exposure to the lysis buffer—there should be no visible pellet/ cells on the bottom of the tube. You can flick or tap to mix, just avoid forming bubbles as best you can.
13. Incubate suspensions at room temperature for EXACTLY 3 minutes. ((During this time it can be helpful to set up your tubes for counting the cells, see below.)) After 3 minutes, add fresh c-IMDM to each tube (to dilute the RBC lysis buffer) as follows:
    a. 4mL for lymph nodes
    b. 5mL for spleen
14. Invert gently but thoroughly to ensure that the suspension is completely mixed.
15. Leave the tubes in the hood (placed vertically) to incubate at room temperature for 5-6 minutes to allow debris (membranes, etc.) from the lysis step to settle to the bottom of the tube.
16. Pour the entirety of the (cell-containing) supernatants into a fresh 15mL conical tube (*at this time, combine the supernatants from spleen and lymph nodes from one biological replicate*). Discard the old tubes with debris at the bottom.
17. Centrifuge at 1800rpm for 5 minutes at 4˚C to pellet the harvested cells. Carefully aspirate off the media.

Continuing CD4+ Cell Isolation using Negative Selection-based Kits:

Note that while the steps below serve as a guideline, the specific protocol for your chosen kit should always be consulted/reviewed.

1. Resuspend the cell pellet thoroughly in 2mL of your prepared ice cold magnetic selection buffer.
2. Dilute 1:100 in cIMDM (2uL cell suspension + 198uL cIMDM) and pipet thoroughly to mix, then dilute again 1:2 in Trypan Blue (10uL diluted cell suspension + 10uL trypan blue). Keep original cell suspension on ice throughout this time.
3. Count the cells using a hemacytometer to determine total number of cells before the antibody step.
4. While you're counting, centrifuge at 1800rpm for 5 minutes at 4˚C to pellet the cells again (this serves as a wash step to remove residual media). Carefully aspirate off the supernatant.
5. Resuspend the cell pellet as appropriate for your chosen isolation kit:

For **R&D Systems kits**: resuspend cells at $2x10^8$ cells/mL in ice cold 1X MagCellect buffer;

For **BioLegend kits**: resuspend cells at $1x10^8$ cells/mL in 1X ice cold MojoSort buffer).

Note that the maximum volume allowable per isolation for these kits is 2.5mL. Cell harvests exceeding this amount can be split into multiple magnetic isolations, or can be isolated in the maximum volume (2.5mL).

6. Using the p1000, add the appropriate amount of Antibody Cocktail to each tube per your chosen isolation kit:

   For **R&D Systems kits**: add 200uL of Antibody Cocktail per mL of MagCellect Buffer

   For **BioLegend kits**: add 100uL of Antibody Cocktail per mL of MojoSort buffer

7. Mix gently but thoroughly by repeat pipetting with a p1000. Incubate at 4˚C in the refrigerator (**for R&D Systems kits**) or on ice (**for BioLegend kits**) for 15 minutes.
8. After 15 minutes, add the appropriate amount of Streptavidin Ferrofluid (**for R&D Systems kits**) or SAV-Nanobeads (**for BioLegend kits**) to each tube based on your isolation kit of choice:

   For R&D Systems kits: Be sure to resuspend the Streptavidin Ferrofluid by repeat pipetting with the p1000 before use. Avoid forming bubbles. Using the p1000, add 200uL of Streptavidin Ferrofluid per mL of MagCellect buffer (for MAGM202) **or** 250uL of Streptavidin Ferrofluid per mL of MagCellect buffer (for MAGM205)

   For BioLegend kits: Pulse vortex the SAV-Nanobeads 5 times (1 sec/each) on a vortex set to maximum. Add 100uL of SAV-Nanobeads/mL of Mojosort buffer.

9. Mix gently but thoroughly by repeat pipetting with a p1000. Incubate at 4˚C in the refrigerator (**for R&D Systems kits**) or on ice (**for BioLegend kits**) for 15 minutes.
10. During this second 15-minute incubation, remove your 24-well plate(s) from the refrigerator. Prepare Day 0 cytokine/blocking antibody mastermix(es) as appropriate for your subsets of interest and place on ice. (See ***T helper Subset Polarizing Conditions*** document for details.)
11. Aspirate off the PBS from each well in the 24 well plate prepared on Day -1.
12. Add 500uL sterile 1X PBS to each well. Shake the plate gently and aspirate off PBS.
13. Repeat step #10.
14. Add 500uL of appropriate cytokine mastermix to each well, and place the 24-well plate in the 37C incubator to warm.
15. *Back to the cell suspension:* At the end of the 4 degree incubation, bring the **total** final reaction volume to 3mL (in each tube) by adding the appropriate volume of ice-cold magnetic selection buffer (1X MagCellect buffer for **R&D Systems kits**, 1X MojoSort buffer for **BioLegend kits**).
16. Using a 5mL serological pipet, mix gently (no bubbles) to ensure even suspension of the sample. Transfer the cell suspension to a labeled, sterile, 5mL round-bottomed tube (discard its cap).
17. Place the reaction tube in the MagCellect Magnet, and incubate at room temperature for 6 minutes (for **R&D Systems kits**) or 5 minutes (for **BioLegend kits**). Magnetically tagged items will migrate toward the magnet (these are unwanted cells/debris, as this kit uses negative selection to isolate our cells of interest).

18. After 5-6 minutes (as appropriate), hold the tube on the magnet so that it doesn't slide and use the p1000 with sterile tips to transfer the supernatant to another sterile, round-bottomed tube. Do not touch the magnetic particles on the wall of the tube.
19. Once empty, discard the tube from the magnet (with the waste cells/magnetic particles).
20. Incubate the second round tubes containing cell suspension on the magnet for 5-6 minutes (as appropriate) to ensure that all of the magnetic nanoparticles have been removed from the cell suspension. During this incubation, place 7mL cIMDM in a labeled 15mL conical tube (1 per biological replicate).
21. After the second round of incubation on the magnet, place the cells into the prepared 15mL conical tube containing cIMDM.

Plating isolated cells:

Note that plating isolated cells from a single animal in 10 wells routinely yields plating densities of 300,000-500,000 cells per well (based on R&D Systems/BioLegend Isolation kits). Cells may be counted and resuspended appropriately to ensure even plating across subsets/experiments.

1. Centrifuge the above suspension at 1800 rpm for 5 minutes at 4˚C to pellet the collected cells, and carefully aspirate off the supernatant. (A small whitish cell pellet will be visible at the bottom of the 15mL conical tube.)
2. Resuspend cells in 0.5mL/well cIMDM by gently pipetting with a serological pipet. Ensure that cells are well-suspended without forming bubbles. (For larger volumes, it sometimes helps to resuspend the cells in 4-5mL first, and then add in the additional cIMDM.)
3. Transfer 0.5mL of cell suspension to each well—do not touch the sides of the well (or the media in each well) with the pipet tip. Do not eject all of the way when plating cells to avoid forming bubbles. Routinely repeat pipet to ensure that the cell suspension remains even throughout plating.
4. Place the plate with primary CD4+ T cells into the TC incubator, overnight.

DAY 1: Adding additional cytokines

Note: Cytokines should be added in 1mL/well cIMDM, so the final volume in each well will be 2mL after Day 1.

Prepare cytokine/blocking antibody mastermixes as appropriate for your subset(s) of interest. (See ***T helper Subset Polarizing Conditions*** document for details.)

DAY 2: Leave the cells alone.

DAY 3: Expansion of primary CD4+ Tcells

Generally, we expand as follows:
# of wells from harvest x 2 to 2.5 = number of wells after the split (see T helper cell subset conditions for split information for particular subsets). Note that these split ratios routinely yield plating densities of ~500,000 cells/well after the split.
Round to make this easier/ to fit on a given number of plates.

1. Label 24-well plates for expansion. The cells will be plated in the absence of anti-CD3/anti-CD28 stimulation.
2. Prepare cytokine/blocking antibody mastermixes as appropriate for your subset(s) of interest. (See ***T helper Subset Polarizing Conditions*** document for details.)
3. Mix by inverting 10-15 times, and avoid forming bubbles. Keep mastermixes on ice when not in use.

4. Place 1mL of mastermix into each appropriate (new) well—be sure to label where they are on the lid of the plate. Place the plates in the incubator to warm as you're working with the cells, below.
5. Harvest all cells from all wells plated on Day 1: Carefully repeat pipet in each well (~40 times, especially around the edges) to dislodge adherent cells. Check wells under the scope to ensure that cells have been thoroughly suspended if you're not sure. Avoid forming bubbles.
6. Place all cells in a 15mL or 50mL conical tube (depending on volume), and pellet by centrifugation at 1800 rpm for 5 minutes at 4C.
7. Carefully aspirate the media, and resuspend in appropriate volume (1mL of cells per well) by carefully repeat pipetting. Avoid forming bubbles.
   Note: For large volumes of 10mL or above, it is helpful to resuspend in a small volume of cIMDM FIRST, and then add more. For the 48-well example, resuspending the cell pellet thoroughly in 8mL of cIMDM, then adding 40mL of cIMDM and resuspending again ensures even suspension.
8. Without touching the pipet tip to the media in each well, or to the walls of the well, place 1mL of resuspended cells in each. (It is helpful to pipet up and down in the cell suspension periodically during plating, to ensure even distribution throughout the process.)