Isolating Primary T Cells from Mice: T_H1 conditions

Last updated April 2016

Supplies/ Buffers/ Media Needed:

Consumables/Supplies Needed:

- 24-well TC plates
- Sterile 15mL conical tubes
- Sterile, frosted-end microscope slides (autoclaved in groups of four and double-bagged), 1 bag per biological replicate
- Sterile petri dishes, 2 per biological replicate
- Autoclaved water
- Relevant R&D Systems MagCellect T cell isolation kit (for bulk primary CD4+ T cells: MAGM202, for Naïve primary CD4+ T cells: 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)

- 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 R&D Systems MagCellect Mouse $CD4^+$ T Cell Isolation Kit

Cytokines for T_H1 generation:
anti-IL-4 (BioLegend, catalog # 504115)
IL-12 (R&D systems, catalog # 419-ML-010)
IL-2 (Peprotech catalog # 200-02-10ug)

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

In the tissue-culture hood…

1. Label the lid of a 24-well TC plate.
2. Add 500uL/well sterile PBS to a 15mL conical tube
3. Add 5uL anti-CD3 per 1mL of PBS (final concentration of 5ug/mL; purchased stock is at 1ug/uL)
4. Add 10uL anti-CD28 per 1mL of PBS (final concentration of 10ug/mL; purchased stock is at 1ug/uL)

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

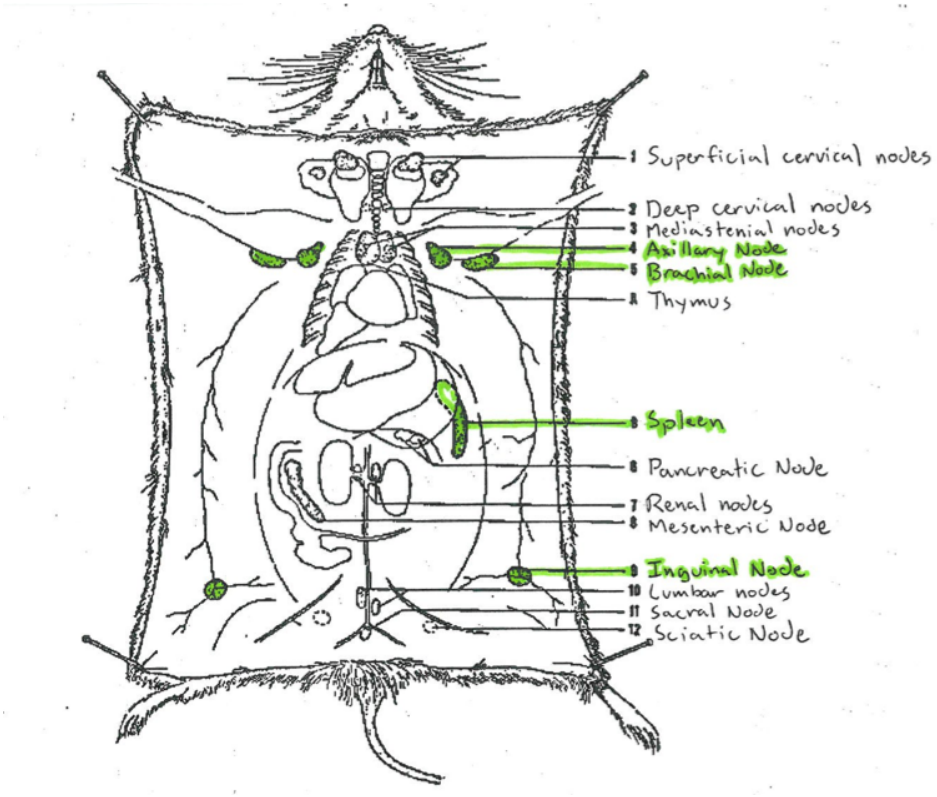
5. Mix by inverting gently 10-15 times and add 500uL of the antibody mixture to each well.
6. Cover plate, wrap edges with parafilm, and incubate overnight at 4˚C.

Our usual setup: 2 mice, 20 wells total
10mL 1X PBS
50uL anti CD3e
100uL anti CD28

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 10mL of 1X MagCellect buffer by combining 1mL of 10X MagCellect buffer (from relevant kit, stored at 4˚C) with 9mL sterile ultrapure water. Invert 5-10 times and keep on ice until needed.
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 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.
9. Centrifuge cell suspensions 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 and 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 the R&D Systems MagCellect Kit:

1. Resuspend the new cell pellet thoroughly in 2mL of your prepared ice cold 1X MagCellect 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 in ice-cold 1X MagCellect Buffer at $2x10^8$ cells/mL.
   Note: The cells should resuspend quickly and easily, however, sometimes a sticky/ flocculent debris may fail to resuspend—this is normal. The debris can be left in the tube during the following steps.
6. Using the p1000, add the appropriate amount of MagCellect Biotinylated Antibody cocktail to each tube to reach 200uL of antibody cocktail per $2x10^8$ cells, and mix gently by pipetting up and down (avoid producing bubbles).

7. Incubate at 4˚C in the refrigerator (not on ice) for 15 minutes.
8. Be sure to resuspend the Streptavidin Ferrofluid by repeat pipetting with the p1000 before use. Avoid forming bubbles. Using the p1000, add the same amount of of MagCellect Streptavidin Ferrofluid as you did Antibody Cocktail to the cell suspension, mix gently by repeat pipetting, and incubate at 4˚C in the refrigerator (not on ice) for 15 minutes.
9. During this second 15-minute incubation, remove your 24-well plate(s) from the refrigerator.

   FOR T_H1 CONDITIONS:

   Make up 0.5mL/well anti-IL-4 at 10ug/mL in cIMDM. Mix well and place on ice. (For 20 wells, add 100uL of 1mg/mL to 10mL of cIMDM.)
10. Aspirate off the PBS from each well in the 24 well plate prepared on Day -1.
11. Add 500uL sterile 1X PBS to each well. Shake the plate gently and aspirate off PBS.
12. Repeat step #10.
13. Add 500uL of the anti-IL-4 mastermix (step 9) to each well, and place the 24-well plate in the 37C incubator to warm.
14. *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 1X MagCellect Buffer.
15. 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).
16. Place the reaction tube in the MagCellect Magnet, and incubate at room temperature for 6 minutes. 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).
17. After 6 minutes, 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.
18. Once empty, discard the tube on the magnet (with the waste cells/magnetic particles).
19. Incubate the fresh tubes with cell suspension on the magnet (again) for 6 minutes to ensure that all of the magnetic nanoparticles have been removed from the cell suspension. This time, after incubation, place the cells in ~7mL cIMDM in a 15mL conical tube.

Plating isolated cells:

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.
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.

TH1 CONDITIONS

Cytokines to be added:
IL-12 (1uL of 10ng/uL stock, per well)
Anti-IL-4 (5uL of 1ug/uL stock, per well)
IL-2 (40uL of 1ng/uL working stock per well)

IL-2 working stock is made by diluting 10uL of 100ng/uL Peprotech IL-2 in 1mL of cIMDM. 100ng/uL stock is stored at -80C. Working stock should be made the day of the experiment and is stored at 4C. This working stock is only stable for 7 days at 4C.

Again, these should be prepared as mastermixes in 1mL/well cIMDM, inverted well without forming bubbles, and plated at 1mL/well.

DAY 2: Leave the cells alone.

DAY 3: Splitting primary naïve CD4+ Tcells

Generally, we split as follows:
# of wells after harvest x 2.5 = number of wells after the split.
Round to make this easier/ to fit on a given number of plates.

Under low IL-2 conditions, CD4+ T cells will divide at about half the rate of those in high IL-2 conditions. Since we want the number of cells to be equivalent for analytical purposes, we'll need to have twice as many wells prepared for low IL-2 cells as for high IL-2 cells.

Thus, for a 20-starting-well example:
20 x 2.5 = 50, but we'll round to 48 to keep them all on two plates.
16 wells will be for high IL-2 conditions
32 wells will be for low IL-2 conditions

Begin by labeling 24 well plates for the split experiment—include where Hi- and Lo-IL-2 treated cells will go.

Cytokines to be added:

For High IL-2
IL-12 (1uL of 10ng/uL stock, per well)
Anti-IL-4 (5uL of 1ug/uL stock per well)
IL-2 (see Day 1: 40uL/well of Peprotech IL-2 working stock)

For Low IL-2
IL-12 (1uL of 10ng/uL stock, per well)
Anti-IL-4 (5uL of 1ug/uL stock per well)
IL-2 (0.4uL/well of Peprotech IL-2 working stock)

1. Make up a mastermix using the information above, as appropriate for the number of wells/ conditions for your experiment.

   (For our example 16 wells for high IL-2 and 32 wells for low IL-2, using Peprotech IL-2 working stock):

   High IL-2 mastermix:
   1mL cIMDM per well x 16 wells = **16mL cIMDM**
   1uL IL-12 per well x 16 wells = **16uL IL-12**

5uL anti-IL4 per well x 16 wells = **80uL anti-IL-4**
40uL Peprotech IL-2 per well x 16 wells = **640uL Peprotech IL-2 working stock**

Low IL-2 mastermix:
1mL cIMDM per well x 32 wells = **32mL cIMDM**
1uL IL-12 per well x 32 wells = **32uL IL-12**
5uL anti-IL4 per well x 32 wells = **160uL anti-IL-4**
0.4uL Peprotech IL-2 per well x 32 wells = **12.8uL Peprotech IL-2 working stock**

2. Mix by inverting 10-15 times, and avoid forming bubbles. Keep them on ice when not in use.
3. 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.
4. 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. Avoid forming bubbles.
5. Place all cells in an appropriately-sized 15mL or 50mL conical tube (depending on volume), and pellet by centrifugation at 1800 rpm for 5 minutes at 4C.
6. Carefully aspirate the media, and resuspend in appropriate volume (we want 1mL of cells per well) by carefully repeat pipetting. Avoid forming bubbles. (In our example, between high- and low- IL-2 conditions, we have 48 wells, so we'll resuspend the cell pellet in 48mL of cIMDM).
   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.
7. 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.)