**Handling of blood samples of adult PNOIT study (U19, AADCRC project 2)**

- These are whole blood samples of ~450 ml.

- Set aside 2 ml of blood for basophil test.

- Use 200 ul of blood for DNA isolation.

- Put the blood in 50 ml tubes and spin at 800g, 15 min. Harvest all the plasma, put in five 5 ml polypropylene tubes (4 ml/tube) and the rest in 50 ml tubes.

- Add equal volume of PBS, resuspend, do Ficoll separation, and isolate PBMC, per the usual protocol. Resuspend PBMC in 1 ml PBS and count.

**Distribution of the PBMC sample:**

1. 65x106 PBMC for cell culture.

2. 200x106 PBMC for positive isolation of CD4+ cells and CD19+ cells. Give negative fraction to David Alvarez.

- Give 30x106 CD4+ cells to David, cryopreserve the rest, in two vials.

- Cryopreserve all CD19+ cells, in two vials.

3. Cryopreserve rest of the PBMC (~200x106).

- Flow analysis by Sanofi (10x106 in one vial).

- Flow analysis by Patrick Brennan (10x106 in one vial).

- Rest in aliquots of 30x106 (~6 vials).

**Culture of PBMC:**

1. Culture cells in 24-wells plate, 3 wells per variable, 5x106 cells/well, in 1 ml AIM-V medium per well. Variables are Unstim, Golden PE, anti-CD3/CD28, and Ara h 1/Ara h 2, so 4x3=12 wells total. Make suspension of 65x106 PBMC in 13 ml medium, then add 1 ml per well.

2. Add 16 ul Golden PE (100 ug/ml), 6 ul anti-CD3/CD28 beads (1:20 bead-to-cell ratio), or

30 ul Ara h 1/136 ul Ara h 2 (both 50 ug/ml) in the appropriate wells, and mix with P1000 pipette.

3. Incubate for ~18h total.

4. Add 20 ul CD154-PE antibody to all wells for the last ~3h of culture, and mix with P1000 pipette.

5. Harvest cells, resuspend in 150 ul staining buffer, and stain with the following panel for sorting. Use the indicated amounts per tube (~15x106 cells). Remember to prepare compensation controls, and FMO control for CD69.

Live/dead-violet 3ul (do not add live/dead-violet to cells cultured with Ara h 1/Ara h 2).

CD3-AF700 8ul

CD4-APCCy7 8ul

CD45RA-FITC 30ul

CD154-PE 30ul

CD69-AF647 8ul

Gate on forward/side scatter, then live CD3+ cells, then CD3+CD4+ cells, then CD4+CD45RA- cells.

- For the Unstim, Golden PE, and anti-CD3/CD28 cultures, sort CD154+CD69+/- cells, CD154-CD69+ cells, and CD154-CD69- cells (3 tubes total). Spin down the cells, lyse in buffer RLT + b-ME and store in -80C freezer.

- For the Ara h 1/Ara h 2 culture, sort CD154+ and CD69+ cells (in one tube), and CD154-CD69- cells (2 tubes total). Then spin down and cryopreserve in FBS + 10% DMSO.