**Protocol User-Defined ID\* T cell response to Influenza vaccination in aged populations - Vac2012**

**USER\_DEF\_STUDY\_ID T cell response to Influenza vaccination in aged populations - Vac2012**

**Part 1**

**Human PBMC Purification with Ficoll from Whole Blood**

Materials:

DPBS without Mg and Ca at RT and 4C

Ficoll-Paque Plus at RT

ACK lysis buffer

RPMI with 10% FBS, Pen/Strep and L-glutamine at 4C

Protocol:

Place 20ml room temperature Ficoll-Paque Plus into 50ml conical

In another 50ml conical place approximately 2 vacutainers (max 30 mL) human blood

Bring total volume of human blood to 30ml with PBS if necessary

Using a 25ml serological pipette, layer the blood/PBS mixture over the Ficoll-Paque Plus

Spin at 2000 rpm, 20 min at RT with no brake

Remove buffy coat and place in new 50ml conical

combine multiple tubes tube for larger bleeds with PBS

you can add PBS to bring each tube to equal volume

Spin at 2000 rpm, 15 min, aspirate

Add 5ml ACK lysis buffer to each tube

can use up to 10ml for high RBC contamination

make sure to dislodge pellet efficiently

can repeat this step if there is inefficient RBC lysis

Incubate at RT for 5 min

Add 20ml RPMI

Spin at 2000 rpm, 15 min

Aspirate

Resuspend in 2 to 5ml RPMI (depending on pellet size) and count cells using trypan blue

make sure to dislodge pellet efficiently

Rest cells for 16 to 24 hours at 37C at concentration of 5x10^6 per ml

**Part 2**

**In Vitro Stimulation**

**Materials**

SEF/TSST from Toxin technologies Cat# TT 606

Influenza Peptide Library

**Influenza Peptide Stimulation**

Peptide working stocks should be 2mg/mL (2000x)

Final peptide concentration should be 1ug/mL,

and no less than 0.1ug/mL for pooled peptides

Large peptide pools should be made to maximize peptide concentration

e.g. the HA peptide pool has 137 peptides each peptide should be dissolved at the highest possible concentration (20 – 50 mg/mL) since it will be diluted 137 fold (.145 - .365 mg/mL) when making the pool.

**SEF Stimulation**

SEF/TSST from Toxin technologies Cat# TT 606

1mg dissolved in DMSO @ 1mg/mL and aliquotted (labeled 10,000x)

Use from 1ug/ml to 1ng/mL normal working stock 100ug/mL (1,000x)

1 to 0.1ug/mL for 4 hour stimulation

0.01ug/mL to 1.0ng/mL for long term culture/proliferation

**Part 3**

**Surface staining**

Make an antibody mix:

Dilute fluorochomes into FACS buffer (HBSS + 1% FBS + 0.02% Sodium Azide).

Based on 50 μL total staining volume per well, which is enough for up to 10 million cells

Protect from light and keep at 4C until needed.

Keep concentrated fluorochromes undiluted if not using within 6-8 hours.

Remember to make 10% extra to account for pipet error and adherence to the walls of the tube.

Add cells to a 96 round bottom well plate (you want to have around 106 cell/well/100 μl). Spin at 2000 rpm for 5 minutes.

After spin, flick the supernatant into the sink in one fluid movement, which will leave the cells behind.

Add 50μL of the staining mix to each sample. Mix by pipetting up and down using a multichannel.

Protect from light and incubate at RT for 20 min.

Add 150μl FACS buffer to each well and spin at 2000 rpm for 5 minutes. Flick off supernatant.

Continue to intracellular staining (or fix cells if not doing intracellular stains).

**Intracellular staining using BD CytoFix/CytoPerm kit (BD 554714):**

Prepare intracellular staining fluorochrome mix

Dilute fluorochromes in BD PermWash and protect from light.

Add 50 uL of BD Cytofix/Cytoperm to each well to permeabilize cells.

Incubate at RT in the dark for 20 min.

Add 150 uL of BD PermWash. Centrifuge 2000 rpm x5 min. Flick off supernatant.

Add 50 uL of intracellular staining mix. Incubate at RT in the dark for 60 min.

Add 150 uL of PermWash. Centrifuge 2000 rpm x5 min. Flick off supernatant.

If streptavidin counter stain, then step 7. Otherwise go to fixing step.

Add 50 uL of Streptavidin counter stain mix at RT in the dark for 10 min. Other wells get 50 uL PermWash.

Add 150 uL of PermWash. Centrifuge 2000 rpm x5 min. Discard supernatant.

Stain with secondary antibody or go to fixing step.

**Intracellular staining using eBiosciences FoxP3 permeabilization kit (eBio 00-5521-00):**

Prepare intracellular staining fluorochrome mix

Dilute fluorochromes in eBiosciences Permeabilization Buffer (00-8333) and protect from light

Dilute Foxp3 Perm concentrate with the Foxp3 diluent in a 1:3 ratio (i.e. 25 uL+ 75 uL)

Add 50 uL of the diluted Foxp3 Perm to each well

Incubate at RT in the dark for 20 min.

Add 150 uL of Perm Buffer. Centrifuge 2000 rpm x5 min. Flick off supernatant.

Add 50 uL of the diluted fluorochrome mix.

Incubate at RT in the dark for 60 min.

Add 150 uL of Perm Buffer. Centrifuge 2000 rpm x5 min. Flick off supernatant.

Stain with secondary antibody or go to fixing step.

**Fixing with para-Formaldehyde (1% PFA)**

Add 150 μL of 1%PFA to each well. Transfer this to FACS tubes.

Add 150 uL of 1%PFA to each well again and transfer this to FACS tubes (final vol 300 uL).

Store in the dark at 4C.

Acquire sample on flow cytometer within 2-5 days. (The sooner the better)