In vitro culture of Human Tonsil/Spleen Organoids from cell suspension

I – Principle

This SOP provides a guide for the generation of in vitro organoids using human tonsil/spleen cell suspensions. Here, tonsils or spleen organoids will be used as in vitro functional systems that mimic key features of the germinal centres in vivo. Here, we will stimulate organoids with various antigens (for example influenza vaccine, BCG vaccine, lysates of *Plasmodium*-parasitised red blood cells (pRBC in schizont stage) and lysates of uninfected red blood cells (uRBC)) and determine adaptive immune response to each of the stimuli. Immune responses characterized include the phenotypic profiling of B and T cells by identifying surface markers and antigens expressed and the functional profiling of B and T cells by measuring cytokine and antibody production.

Note: this protocol will be under optimisation and therefore optional steps are listed within the protocol

II – Safety Overview

- It is the responsibility of all personnel handling human blood samples to ensure they are vaccinated against Hepatitis B and are trained to treat all biological samples as potentially infectious.
- The Safety and Ethics Committee must approve all work before commencement.
- Standard personal protective equipment (PPE) must be worn when performing laboratory work unless otherwise stated. Standard PPE includes.
    - Long-sleeved lab gown, enclosed footwear, safety glasses and gloves.
- Please be aware of the risks involved in working with human samples.
    - Care must be taken when handling blood and purified cell populations obtained from clinical samples.
- Class II Biosafety Cabinet must be used when handling unfixed blood.
    - Cabinet must be sealed and exposed to UV light for 20 min prior to use.
    - Cabinet surface must then be wiped down with 80% (v/v) ethanol.

Any items for use in the cabinet (including pipettes) must be decontaminated with 80% (v/v) ethanol.

III – Equipment, Reagents and Consumables

Equipment

- Finnpipette™ F2: P2, P20, P200, and P1000
- Finnpipette™ F2: Multichannel pipette 30-300uL
- Eppendorf Centrifuge 5910
- Esky – for transport of samples between facilities
- Class 2 Biosafety Cabinet
- CellDrop DeNovix (Automatic Cell counter)
- CO2 incubator (at 37C)

- Fridge 4C
- Freezer -20C
- Freezer -80C
- Liquid Nitrogen tank
- Inverted Microscope
- Neubauer Haemocytometer Cell Counting Chamber
- Water Bath (at 37C)

Reagents/Chemicals

- 100 x NEAA (Non-essential Amino Acids) (Thermo Fisher – Cat.no # 11140050)
- Acridine Orange / Propidium Iodide Assay Protocol (DeNovix - Cat.no # CD-AO-PI1.5)
- Bovine Serum Albumin (BSA) (Sigma – Cat no# A7906)
- Benzonase Nuclease (Sigma Aldrich - Cat.no # E1014-25KU)
- Foetal bovine serum (FBS) Heat-inactivated (Heat inactivated 56°C, 45 min)(Thermo Fisher Cat.no # 10099141)
- ITSG (Thermo Fisher – Gibco # 41400045)
- Matrigel Matrix for Organoid Culture, Phenol Red-free, LDEV-free, (Corning – Cat no# 356255)
- PBS- 1X Filtered Phosphate Buffer Saline
- Normocin (Jomar Life Sciences - Cat.no # ant-nr-2)
- Penicillin-streptomycin solution (Thermo Fisher – Cat.no # 15140122)
- Recombinant-human B Cell Activating Factor – (RH BAFF) (Carrier Free) (Biolegend Cat.no # 559606)
- RPMI 1640 Medium with Glutamax (Thermo Fisher – Cat.no # 61870036)
- Sodium Azide (powder) – Thermo Fisher
- Sodium Pyruvate (Thermo Fisher – Cat.no # 11360070)
- Trypsin-EDTA (Thermo Fisher – Cat.no # 15400054)
- Y-27632 (Merck – Cat.no # 688000)
- BCG vaccine: Engerix B Adult pre-filled syringe 20cmg 1ml
- Flu vaccine: FluQuadri vaccine injection
- RPMI- DMEM, high glucose, GlutaMAX™ Supplement, HEPES (Thermo Fisher – Cat no# 10564011)
- RPMI- Advanced DMEM (Thermo Fisher – Cat no# 12491015)
- ImmunoCult™-ACF Human B Cell Expansion Supplement (Stem Cell Technologies Cat.no# 10974)
- B-27™ Supplement (50X), serum-free (Thermo Fisher – Cat. no# 17504044)
- Y-27632 (Dihydrochloride) - ROCK inhibitor (Stem Cell Technologies Cat.no# 72304)

Consumables

- Tips (various manufacturers) 10, 20, 200 and 1000 µL for use with Finnpipette™ F2 pipettes (listed above)
- 1.5 ml polypropylene tubes (Various manufacturers)
- 10 ml polypropylene tubes (Various manufacturers)
- 50 ml polypropylene tubes (Various manufacturers)
- 1ml disposable transfer pipette (Various manufacturers)
- Cryovials (Corning)
- FACS tubes (Various manufacturers)
- 96-well Clear Flat Bottom Ultra-Low Attachment Microplate (Corning Cat no# 3474)

IV – SOPS

Preparation of Organoid Culture Media (OCM):

This media is for growing organoids.

Steps
1. Thaw Normocin and Pen-Strep overnight at 4°C. 2. Thaw heat-inactivated FBS at 37°C 3. Remove 65 mL media from RPMI bottle, and store at 4°C 4. To the remaining 450 mL RPMI, add. • 50 mL FBS 100% • 1 mL of Normocin (50 mg/mL) 100 ug/mL • 2.5 mL Pen-Strep (10,000 U/mL) 50 U/mL ug/mL • 5 mL 100X NEAA 1X • 5 mL 100X ITS-G 1X • 5 mL of 100 mM Sodium Pyruvate 1mM 5. Protect media from light, label, date, and store at 4°C. 6. Use within 1 month. <u>Other reagents that will be utilized during the optimization process:</u> - Y-27632 (Dihydrochloride) - ROCK inhibitor (amount to be determined) - B-27™ Supplement (50X), serum free (amount to be determined) - RPMI DMEM , high glucose, GlutaMAX™ Supplement, HEPES - RPMI- Advanced DMEM
Notes
This media is for growing organoids only.

- Because GlutaMAX is expensive
- Processing media contains L-glutamine rather than GlutaMAX. You *can* use the "Processing Media" to grow organoids instead – but need to do daily media changes.
- With GlutaMAX – media change is every 3-4 days.
- Normocin is stable at room temperature for 2 weeks.
- Stability of Pen-Strep a 4°C is unknown. Stability at -5°C is 12 months.

Preparation of Organoid Culture Media (OCM) with BAFF:

To prepare BAFF Media for organoid culture. BAFF improves total B cell survival and induces B cell maturation, proliferation, survival, and immunoglobulin production.

Steps
1. Aliquot required volume of media into 50 Ml (Make little excess – BAFF is expensive) ▪ Usually 1 mL media per well (12 well plate) 2. Check the concentration of BAFF on vial ▪ It is batch dependant. 3. Quickly spin vial before opening 4. Add BAFF to Organoid Culture Media to achieve 0.5 ug/mL BAFF or 1 ug/mL BAFF (1).
Notes
○ Always make fresh. ○ Storage ○ At -70°C until labelled expiry ○ At -20°C for 6 months ○ At 4°C for 2 weeks ○ Avoid repeat Freeze/Thaw

1. Tissue Collection and Processing and Cell Isolation and Suspension:

Process of isolating cells from tonsils, followed by storage in liquid nitrogen.

Steps
Please refer to the SOP: **BoyleLab_SOP_Tonsils_Processing** File is in: Lab Archives > Boyle Lab > SOPs and Risk Assessments

2. Thaw and count cells:

Steps
1. Collect vials from the Liquid Nitrogen Tank. 2. Warm Organoid Culture Media (OCM) in water bath 3. Half thaw cells in 37°C water bath a. Expect 30-70% recovery from most samples post-thaw; keep on dry ice until ready to thaw. 4. Transfer to 10 mL tubes 5. Add 7 mL OCM. 6. Rinse cryovial with 1 mL OCM, then transfer to 10 mL tube. 7. Centrifuge at 1,500 rpm x 10 min at RT (acceleration speed 6 and deceleration speed 6) 8. Aspirate supernatant 9. Resuspend in 1-2 mLs OCM. 10. Make a 1/100 dilution and count this. a. Add 10 uL cell suspension to 990 uL OCM. b. Add 10 uL of diluted cells to 10 uL AO/PI c. Count this on Cell Drop 11. Resuspend cells to 6 × 10^7 cells per ml for larger cultures or 2 × 10^7 cells per ml for smaller cultures (1).

3. Start, maintenance and passaging of the organoid culture:

Start culture
1. Cells were plated, 100µl per well, into permeable (0.4-µm pore size) membranes (24-well size PTFE or polycarbonate membranes in standard 12-well plates or 96-well polycarbonate membrane plates with single-well receiver trays; Corning or Millipore) (1) Or Add 200 uL cell suspension containing **7.5x10^6 cells/mL** cells into volume in ultra-low attachment plates (2) (1.5x10^6 cells/well – 12 well plate). Or isolated cells were embedded in Matrigel in a 48-well plate (**Seeding density (cells /well) = 0.03 x10^6)** and incubated at 37 ◦C for 10 min to polymerize the matrices (3). 2. Incubate plate in a humidified incubator at 37°C with 5% CO2 (2)
Maintenance
1. Change the medium every 2-7 days by exchanging 30% of the volume with fresh organoid media (2).

- Most optimal: try change media at 3 days

2. Observe the organoids under a microscope regularly to monitor growth and morphology:
   - see morphology of culture days 1-7

Passaging

3. Once the organoids reach an appropriate size (usually after 7-10 days), they can be passaged. Gently disrupt the organoids using enzymatic treatment (0.25% trypsin- EDTA) and transfer them to a new Matrigel-coated plate (3).
4. For the first 2 days at every passage, 10 µM Y-27632 is added to the culture medium.
   - Y-27632 Enhances survival & cloning efficiency of ESC without affecting their pluripotency.

4. Stimulations:

Steps

1. Thaw Schizont Lysate or PfRBCs and matched uRBC lysate at RT
   - Please refer to the SOP: BoyleLab_SOP_in vitro culturing of P. falciparum parasite
2. Prepare vaccine (Influenza and BCG) dilutions and add to each well accordingly.
   - Transfer vaccine inner chamber of required trans wells.
3. Prepare pRBC and uRBC lysate dilution and add to each well accordingly.
   - pRBC: $12x10^6$ cells/mL- $6x10^6$ cells/mL - $3x10^6$ cells/mL- $1.5x10^6$ cells/mL
   - uRBC: $12x10^6$ cells/mL- $6x10^6$ cells/mL - $3x10^6$ cells/mL- $1.5x10^6$ cells/mL
   - Transfer 95 uL of diluted lysate to inner chamber of required transwells

5. Passaging Organoids:

Steps

1. When the organoids reach an appropriate size (usually 7-14 days), carefully collect the Matrigel droplets containing organoids.
2. Break the Matrigel into smaller pieces and wash the organoids with PBS to remove residual Matrigel.
3. Process organoids as in steps 2 and 3 to generate new organoid cultures.

6. Flow cytometry surface staining:

Steps
4. Harvest organoid cells. 5. Wash cells with FACS buffer (PBS+0.1% BSA, 0.05% sodium azide, and 2 mM EDTA) (1) 6. Perform antibody staining. • Please refer to the SOP: BoyleLab_SOP_Ex_vivo_cell_staining • File is in: Lab Archives > Boyle Lab > SOPs and Risk Assessments

References

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(2) Kastenschmidt JM, Sureshchandra S, Jain A, Hernandez-Davies JE, de Assis R, Wagoner ZW, Sorn AM, Mitul MT, Benchorin AI, Levendosky E, Ahuja G, Zhong Q, Trask D, Boeckmann J, Nakajima R, Jasinskas A, Saligrama N, Davies DH, Wagar LE. **Influenza vaccine format mediates distinct cellular and antibody responses in human immune organoids**. Immunity. 2023 Aug 8;56(8):1910-1926.e7. doi: 10.1016/j.immuni.2023.06.019. Epub 2023 Jul 20. PMID: 37478854; PMCID: PMC10433940.

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