* 1. **Isolation of fresh PBMCs – HV86**

1. Collect 75ml blood in 9ml EDTA tubes. Meanwhile prepare Leucocep tubes by adding 15ml leucocep to the tubes and centrifuging 1 min 1000g.
2. Decant blood into 4 leucocep tubes (max 30ml per tube) and centrifuge at 1000 rcf 15 min full acc brake at 1, at RT.
3. Resuspend pellet in 3ml rbc lysis buffer and incubate on ice for 3 mins.
4. Quench by adding 35ml RPMI 5% FCS then spin down 300g 7 mins 4C
5. Resuspend in 10ml RPMI, do 1in5 dilution for counting whilst spin down
6. Resuspend cells in required volume for plating

Cell count: **40x10^6** – plate 40x10^6/40ml (200,000 cells per 200ul well = 19.2x10^6 per plate)

8 conditions, 3 lanes each condition (4.8x10^6)

*UT, LPS HD, LPS LDHD, hydrocortisone, hydrocortisone + HD LPS, IL10, IL10 + HD LPS, IL6 + hydrocortisone + HD LPS*

* 1. **Plating PBMCs**

Plate layout (2 plates)

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
| unstim | unstim | unstim | LPS HD | LPS HD | LPS HD | LPS LDHD | LPS LDHD | LPS LDHD | Hydrocortisone | Hydrocortisone | Hydrocortisone |
|  |  |  |  |  |  |  |  |  |  |  |  |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |
| Hydro + LPS | Hydro + LPS | Hydro + LPS | IL10 | IL10 | IL10 | IL10 + LPS | IL10 + LPS | IL10 + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS | IL6 + hydro + LPS |

1. Add stimulus to cells in falcon (5ul to 5ml):
   1. LPS HD: 100 ng/ml
      1. Stock = 5 mg/ml

Do 1in 50 dilution (1ul +49ul) to give 100ug/ml working stock. Add at 1000X to cells i.e. 5ul

* 1. LPS LD: 10ng/ml
     1. Dilute LPS HD above 1in10 then use at 1000X i.e. 5ul
  2. IL-6: 100ng/ml
     1. Use 100ug/ml stock at 1000X for final 100ng/ml i.e. 5ul
  3. IL10
     1. Use 200ug/ml stock at 2000X for final 100ng/ml i.e. 2.5ul
  4. Hydrocortisone 100nM
     1. Dilute 10mM stock 1in100 then use at 1000X for final 100nM i.e. 5ul

1. Plate 200,000 cells per 200ul well with stimulus (1 million/ml) in a 96 well U-bottomed plate.
2. Culture for 72 hours @ 37C 5% CO2.

48h later: LDHD LPS

1. Slowly remove media, not touching cells. Transfer media to falcon and spin down in case cells were removed
2. Add 200ul fresh media containing HD LPS
   * 1. 1in50 dilution of stock then use at 1000X (3 lanes = 5ml so 5ul)
3. Resuspend potential pellet and add back to wells if necessary
4. Culture for a final 24h
   1. **Cell harvest and FACS staining**
5. Harvest cells after 72h culture of each condition using multichannel and reservoirs into a single Falcon. Ensure that adherent monocytes at the bottom of the 96 well plates are harvested by pipetting up and down to dislodge cells and use PBS/media to wash wells to ensure collect all cells
6. Resuspend in 1-2ml PBS + 0.2% BSA, count and transfer into eppendorfs – remember to do single staining too
7. Spin down eppendorfs 400g 5 mins and aspirate supernatant
8. Flick to resuspend pellet then add required volume of Fc block (5ul ish) and incubate for 10 mins RT
9. Add PBS then spin down
10. Resuspend in 1ml viability dye mix (diluted 1000X in PBS) and incubate RT for 30 mins
11. Split into further eppendorfs for the surface staining then spin down.
12. Meanwhile prepare antibody mixes (all abs, HLADR FMO, IL1R2 FMO)
13. Flick to resuspend pellets then add antibody mix and incubate RT for 30 mins
14. Add 1ml PBS ontop then spin down
15. Resuspend pellet in 500ul Lyse&fix (diluted 1in10 with water) and incubate RT 10 mins
16. Add 1.5ml PBS+0.2% BSA on top and spin down
17. Resuspend in 400ul PBS+0.2% BSA and proceed to acquisition on X20 or place in fridge until ready

|  |  |
| --- | --- |
| Staining conditions | Cell numbers |
| Full stain | 500k |
| FMO1 (HLA-DR) | 300k |
| FMO2 (IL1R2) | 300k |
| unstained | 300k |
| single stain BV421/450 | 300k |
| single stain FITC | 300K |
| single stain APC | Compensation beads |
| single stain AF 700 | Compensation beads |
| single stain PE-Cy7 | Compensation beads |
| single stain PE | Compensation beads |

* 1. **FACS acquisition**

Turn on computer and log on

Turn on X20

Load …to start up lasers

Open DIVA, log on AliceJKnightGroup password: KNIGHT

New experiment – Put PBS tube in, set Run, Low

Set up parameters for FSC,SSC, (-– remember to tick ‘W’ box for these) and all colours using

Setup compensation controls

Load unstained sample, acquire and set parameters for each colour (for negative signals) and FSC vs SSC then record

Load each single staining and adjust parameters for positive signals and record

Calculate compensation

Create new specimen, new sample and proceed with recording events

When finished, proceed with Rinse, Clean, Water cycle (Run on high for2- 3 mins)

Then change X20 to standby, low. Then log off Diva, close down laser set up, shut down computer, turn off X20