FLOW CYTOMETRY: INTRACELLULAR STAINING (ICS) FOR CYTOKINES

I. Materials Required

1. 1x phosphate saline buffer (PBS), Corning, 21-031-CM
2. Bovine serum albumin (BSA), Gemini, 700-101P
3. Sodium azide, Fosher Scientific, S227-500
4. FACS buffer (1xPBS, 10g/L BSA, 1g/L sodium azide)
5. RPMI, Corning, 10-040-CM
6. Fetal calf serum (FCS), Gemini, 900-108
7. Penicillin/streptomycin, 10,000U/ml penicillin, 10,000µg/ml streptomycin, Lonza, 17-602E
8. "R10" medium (RPMI, 10% FCS, 1% penicillin/streptomycin)
9. DNAseI, Roche, 04716728001
10. BD Cytofix/Cytoperm buffer, BD Biosciences, 554722
11. BD Perm/wash buffer, BD Biosciences, 554723
12. Live/Dead fixable Aqua Dead Cell Stain kit, Invitrogen, L34966, diluted 1:60 in 1x PBS (prepare fresh each day from DMSO stock)
13. Paraformaldehyde (PFA), EMS, 15712-S, diluted to 1% in PBS
14. Fluorophore-labelled antibodies of choice

II. Procedure

A. Thawing

1. Thaw samples and rest overnight at a concentration of $2x10^6$/ml in R10 medium + 1µl/ml DNAseI in the incubator at 37°C, 5% CO2.
2. On the next day, re-suspend cells and transfer to 15ml or 50ml conical tube.
3. Count the cells and spin at 500g for 5 minutes at room temperature (RT)
4. Re-suspend cells in R10 medium at $1\text{-}5x10^6$/ml and transfer to 5ml FACS tubes

B. Stimulation

1. Stimulate cells according to the T Cell Stimulation SOP
2. After incubation proceed to viability/ECS and ICS steps

C. Viability and extracellular staining (ECS)

1. spin tubes at 500g for 5 minutes at RT
2. discard supernatant and blot tube over paper towel
3. loosen cell pellet by tapping the tube and add 5µl of 1:60 Aqua viability dye directly to cell pellet
4. incubate for 10 minutes at RT in the dark

5. prepare antibody cocktail master mix with fluorophore-labelled antibodies for extracellular markers and adjust volume to 50µl per test with FACS buffer
6. add 50µl of ECS antibody cocktail to cells and incubate for 20 minutes at RT in the dark (prepare fixation buffer in the meantime)
7. wash cells with 3ml FACS buffer
8. spin tubes at 500g for 5 minutes at RT
9. discard supernatant and blot tubes over paper towel

D. Fixation and permeabilization

1. Add 500µl BD Cytofix/Cytoperm buffer to cell pellet and re- suspend with pipet.
2. Incubate for 18 minutes at RT in the dark
3. Prepare perm/wash buffer in the meantime by mixing 1 part of 10x BD perm/wash buffer with 9 parts distilled water (calculate 4.1ml per sample)
4. Wash cells with 2ml 1x BD perm/wash buffer
5. Spin tubes at 800g for 5 minutes at RT
6. Discard supernatant and blot tube over paper towel

E. Intracellular staining (ICS)

1. prepare antibody cocktail master mix with fluorophore-labelled antibodies for intracellular markers and adjust volume to 50µl per test with 1x BD perm/wash buffer
2. Add 50µl of ICS antibody cocktail to cell pellet
3. Incubate for 1 hour at RT in the dark
4. Wash cells with 2ml 1x BD perm/wash buffer
5. Spin tubes at 800g for 5 minutes at RT
6. Discard supernatant and fix cells with 250µl of 1% PFA.
7. Run samples on flow cytometer.