Protocol – Flow cytometry analysis of IL-10 and TGF-β

- Plate BMDMs in 12-well plates in L-cell conditioned medium at a density of 0.4 M cells/well.
- The next day, incubate the cells with PKH67-labeled ACs at a 5:1 AC:macrophage ratio.
- After 45 min, rinse the macrophages with PBS and incubate them overnight in DMEM with 10% FBS containing 2 µL/mL Protein Transport Inhibitor Cocktail (eBioscience; Invitrogen, cat. 00-4975) to prevent exosomal release of secreted proteins.
- Put the plate of macrophages on ice for 10-15 minutes, and then release the cells from the plate by using a cell scraper. Resuspend them by pipetting the cells in medium up and down and collect in Eppendorf tubes.
- Separate a little bit of the cells that were not incubated with labeled ACs from each tube for an unstained control and compensation controls (one for each antibody)
- Spin the cells down at 800 g for 5 min.
- Aspirate and resuspend in 4% paraformaldehyde. Incubate for 15 min on ice.
- Spin the cells down at 800 g for 5 min.
- Aspirate and rinse the cells with 1 mL FACS BSA stain buffer.
- Spin the cells down at 800 g for 5 min.
- Resuspend the cells in 100 µL FACS BSA stain buffer (BD Biosciences, cat. 554657) containing 1 µL diluted Fc receptor blocker (1:100, TruStain FcX antibody, BioLegend, cat. 101319), and incubate for 30 min on ice.
- Spin the cells down at 800 g for 5 min.
- Aspirate and resuspend in BD Perm/Wash buffer (10x diluted 1:10 with ddH_2O) to permeabilize the cells. Incubate for 15 min on ice.
- Proceed with intracellular staining of IL-10 and TGF-β using fluorescent antibodies; PE anti-mouse LAP (TGF-β1; Biolegend cat. 141403) and PB anti-mouse IL-10 (Biolegend cat. 505019), resuspended in <u>BD Perm/Wash buffer</u> at a 1:50 dilution (2 µL antibody in 100 µL stain buffer per sample). Incubate for 45-60 min on ice.
- After incubation, spin cells down at 800 g for 5 min at 4 °C
- Wash the cells with 1 mL of FACS BSA stain buffer and spin down again
- Remove supernatant, resuspend in 200 µL of FACS BSA stain buffer and transfer the samples to special flow tubes
- Analyze the results on the 17th floor Cantos II machine, set up for the appropriate wavelengths (PE=red, PB=blue, PKH67=green). Collect at least 50,000 cells per sample with flow.