**Screening protocol for detection of IL-10 release from activated murine BMDCs**

**Step 1 - Harvesting bone marrow to differentiate into BMDCs**

1. Collect femurs and tibias from 2 x male C57BL/6 mice (Jackson labs strain number 000664) in 10 mL PBS into 10 cm petri dish

2. Fill another 10 cm dish with 10 mL DMEM + 10% FBS (D-10)

3. Trim off tips of bones

4. Take up 10 mL D-10 with 20.5 gauge needle

5. Switch needle to tip to 27.5 gauge tip

6. Use fine tip to flush bone marrow from bones into 2nd petri dish

7. Disperse cells in media using 20.5 gauge and filter bone fragments using 70 um filter

8. Wash petri dish with additional 5 mL D-10 media and pass through 70 um filter

9. Pellet cells 300 x g for 4 min

10. RBC lysis: Resuspend in 3 mL of 0.2% NaCl for 30 sec, then dilute with 7 mL 1.2% NaCl (note: can do 2 mice worth of bones per RBC lysis reaction)

11. Pellet cells 300 x g for 4 min

12. Count cells

13. Plate cells at 4-5x10^6 cells in 10 mL D-10 supplemented with 20-50 ng/mL rGM-CSF (Peprotech Cat# 315-03) per 10 cm petri dish (not TC treated)

14. Add additional 5 mL D-10 media supplemented with 20-50 ng/mL rGM-CSF on Day 4

15. Differentiated BMDCs can be used on day 7 or 8 after start of cultures

16. Differentiated BMDCs can be analyzed for >90% CD11c (allophycocyanin (APC) antiCD11c clone HL3; BD Biosciences) positivity by flow cytometry before use in experiments.

**Step 2 - Harvesting BMDCs and plating in 384-well plates**

1. Harvest differentiated BMDCs by scraping, typically pool 4-5 plates per 50 mL conical vial

3. Pellet harvested BMDCs 300 x g for 4 min

4. Resuspend BMDCs in 8 mL D-10 media without rGM-CSF

5. Pool all resuspended BMDCs

6. Count BMDCs

7. Resuspend BMDCs at 0.5 x 10^6 cells/mL D-10 media.

8. Disperse BMDCs into 384 well tissue culture treated plates (Fisher cat# 3712) in 40 uL/well using a Multidrop Combi Liquid Dispensor (Note: Can also pipet manually).

 -Use large cassette

 -Use standard speed settings

 -Need 16 mL media per 384w plate + 12 mL dead volume

9. Spin down plates at 150 x g for 3 min

10. Return plates to 37C incubator for minimum of 2 hr, but typically overnight, to allow BMDCs to adhere to plates

**Step 3 - Compound treatment and microbial stimulation**

1. Remove 384w from 37 C incubator

2. Compounds (100 nL per well) were pin-transferred from concentrated DMSO stocks (typically 10 mM) using CyBi-Well Vario (CyBio) into duplicate plates for a final concentration of 25 uM for test compounds

3. For each treatment plate, 32 out of 384 wells were pinned with DMSO as negative control (final %DMSO = 0.25%) and 32 out of 384 wells were pinned with PGE2 (Sigma Cat #P0409; 5-μM final concentration).

4. 48 hr after compound treatment, BMDCs were stimulated with zymosan (Sigma cat# Z4250;

4 μg/mL final concentration) dispersed in D-10 medium (20 μL per well) using a Multidrop Combi reagent dispenser.

5. After 18 h, 5 μL of resulting supernatant was transferred from the culture plate to shallow well 384-well AlphaLISA plates (Perkin-Elmer Cat # 6008350) using a CyBi-Well Vario.

6. Note: A larger volume of cell culture media can be transferred to an intermediary 384w standard volume plate

**Step 4 - AlphaLISA-based detection of secreted IL-10**

1. Dilute 50-100 mL of 1X AlphaLISA buffer by diluting 10X stock with MilliQ dH2O

2. Addition of 1st set of reagents for AlphaLISA reaction

-Add 5 uL of Acceptor beads + mAb-btn solutions per well using Combi with small

volume cassette

-Need 2 mL (384 \* 0.005 mL) per assay plate, plus 2.5 mL of dead volume

-Calculate appropriate volume of 1st set of reagents and add both Acceptor beads and

mAb-btn each at 1/200 dilutions.

3. After additions are complete, spin plates at 300 x g rpm for 5 min

4. Incubate for 1 hr at room temp protected from light

5. Addition of 2nd reagent for AlphaLISA reactions. IMPORTANT: Alpha donor beads are light sensitive so turn off overhead lights, close blinds and cover plates after additions

 -Add 6.5 uL of donor bead solution per well using Combi with small volume cassette

 -Need 2.5 mL (384 \* 0.0065 mL) per assay plate, plus 2.5 mL of dead volume

 -Calculate appropriate volume of 2nd reagent and add donor bead at 1/62.5 dilution

 -COVER PLATES AFTER ADDITION

6. After additions are complete, spin plates at 300 x g for 5 min

7. Incubate for a total of approximately 30 min at room temp protected from light

8. Levels of AphaLISA signal intensity read on an EnVision multimode plate reader

9. Compound activity was expressed as a percent of the differences between the mean abundance of IL-10 in PGE2 versus DMSO wells on a per-plate basis.

**Step 5 - Cell viability assays**

1. Aspirate remaining media from 384w plates of treated BMDCs

2. Add 50 mL of solution (50% (vol/vol) CellTiter-Glo (Promega Cat# G7570) in PBS) using a Multidrop Combi reagent dispenser.

3. Plates were incubated with moderate shaking for 20 min at room temperature

4. Luminescence was read using an Envision multimode plate reader

5. Signal intensity calculated relative to the mean of DMSO control wells on a per-plate basis.