**Human whole blood assay to measure muramyl dipeptide (MDP)-induced cytokine response.**

**Procedure**

All procedure should be carried out in a sterile confined environment (laminar flow biological safety cabinet) possibly equipped with appropriate ventilation system to safely handle chemicals.

- Dilute the chemical probes to 10x their assay concentration in sterile PBS wo Ca++/Mg++. Recommended final volume is minimum 50 µL.
  Note: If handling many probes at the same time it is recommended to prepare 10x solutions in a sterile 96-well U or V bottom culture plate for easy delivery into the assay plate using a multichannel pipette.
- Prednisolone at the final concentration of 1 µM is used as a positive control, see M&R. Include as many vehicle controls as the DMSO final concentrations in diluted probe and prednisolone solutions. Prepare 10x solutions of both positive and vehicle controls, eg prednisolone 10 µM in PBS 1% DMSO, PBS 1% DMSO, etc.
- Dispense 10 µL of diluted probe, prednisolone and negative control 10x solutions into a 96-well flat bottom cell culture plate (assay plate) in triplicate. Dispense 10 µL of PBS in triplicate for MDP-unstimulated control, and 10 µL of PBS in triplicate for MDP-stimulation control.
- Gently invert heparin tubes containing blood 2-3 times before transferring all blood to a 50 mL sterile tube. Gently invert 2-3 times and immediately proceed to dispensing the blood.
  Note: The assay should be set up preferably within 3 hours from blood drawing.
- Pour blood into a sterile plastic reservoir and transfer 86 µL into each well of the assay plate.
- Shake at 600-700 rpm for 2 minutes.
- Incubate for 1 h at 37°C, 5% $CO_2$, >95% humidity.
- Thaw an aliquote of 1 mg/mL MDP solution (see M&R). Mix by vortexing and dilute to 10 µg/mL (25x) in PBS in a sterile tube. Mix by vortexing and inverting the tube before use.
- After 1 h incubation, transfer 4 µL of MDP 25x to each well of the assay (final MDP concentration is 0.4 ug/mL).
- OPTIONAL. Transfer 4 µL of PBS to the MDP-unstimulated control wells.
- Shake at 600-700 rpm for 2 minutes.
- Incubate for 4 h at 37°C, 5% $CO_2$, >95% humidity.
- Add 100 µL of cold PBS, either refrigerated or kept on ice, shake at 600-700 rpm for 2 minutes and spin down at 400 x g for 5 minutes.
- Transfer 100 µL of plasma supernatant to a 96-well collection plate. Cover the plate with sealing aluminum foil for storage at -20 °C.
- Discard the assay plate or cover it with sealing aluminum foil for storage at -80 °C.

Cytokine concentration in plasma supernatant was measured with a V-PLEX Human Proinflammatory Panel II (4-Plex), MesoScaleDiscovery (ref. K15053D) according to manufacturer's instruction.

- Samples were run at one dilution (1:1 in Diluent 2 buffer, provided) with no technical replicate. Sample dilution factor used for analysis was 4.
- Washing steps were performed using a solution of 0.05% Tween-20 in PBS pH=7.4 on an automated plate washer. Residual washing buffer was manually discarded by inverting the MSD plate on a piece of paper before dispensing detection antibody solution and read buffer.

**Material and reagents**

- Sterile filtered tips
- 96-well flat bottom cell culture plate (assay plate)
- 96-well U or V bottom cell culture plate (dilution plate)
- 96-well plate non-sterile (collection plate)
- Sterile (preferably endotoxin-free) tubes, eg Sarsted Biosphere
- Sterile plastic reservoirs
- PBS pH=7.4, sterile, without Ca++/Mg++
- DMSO, Hybri-Max™, sterile-filtered, BioReagent, suitable for hybridoma
  Sigma, ref. D2650-5X5ML
  Aliquote in sterile cryotubes (screw-cap) and store at -20 °C. In use aliquotes can be kept in at 4 °C for 1-2 weeks, freeze and thaw max 3 times.
- Prednisolone, Sigma, ref. P6004,
  Resuspend at 1 mM in DMSO, aliquote in sterile tubes and store at -80 °C.
- L18-MDP, Invivogen, ref. tlrl-lmdp
  Resuspend in endotoxin-free water (provided) at 1 mg/mL. Aliquote in sterile endotoxin-free tubes and store at -20 °C for max 3 months. Discard thawed aliquotes.
- 96-well plate aluminum foil sealer for cold storage
- MSD immunoassay washing buffer.
  Reconstitute PBS tablets in bi-distilled or MilliQ water. Add Tween-20 to make 0.05% solution. Shake before use.
- Plate shaker