Protocol 601: Mouse CD62L-Assay to determine granulocyte sensitivity to TLR-agonists

Material needed:

- 100µl heparin blood
- Stimulus plates (see below for preparation)
- Pyrogen-free pipette tips
- APC-anti-mouse CD62 (Biolegend) and FITC-anti-mouse Gr-1 (Biolegend)
- FACS RBC Lysis Solution (BD)
- FACS Buffer

Mice:

1. Anaesthesise mice with Isoflurane
2. Take a minimum of 100µl Vena cava blood with heparin flushed syringes into heparin tubes (NOTE: Assay does not work with retro-orbital blood as contact with skin (and thus bacteria) around the eye activates cells)
3. Immediately put on ice for transport

Assay:

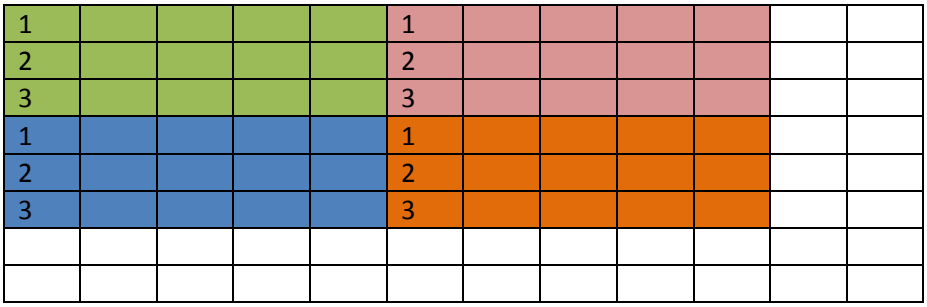
1. 20µl whole blood added per well of thawed stimulus plate (avoid contact of pipette tip with agonist!)
2. Incubate plate at 37°C for 45mins
3. Recover plate and wash all wells with 150µl of FACS buffer, centrifuge (3min, 2500 rpm), flick out supernatant
4. Re-suspend cell pellets in 25µl FACS buffer containing APC-anti-mCD62L (1:100) and FITC-anti-Gr1 (1:100)
5. Incubate at RT for 5-10mins
6. Add 200µl per well of freshly diluted 1x BD FACSLysis solution.
7. Acquire on FACSArray

Preparation of plates (use pyrogen-free, U-bottom, 96-well plates):

Add 20µl of serially-diluted agonist in RPMI to plates. Plates can be frozen and kept for several weeks at -80°C prior to use.

Plate Design for 4 mice:
(this is what I used, but virtually any TLR-agonist can be tested):

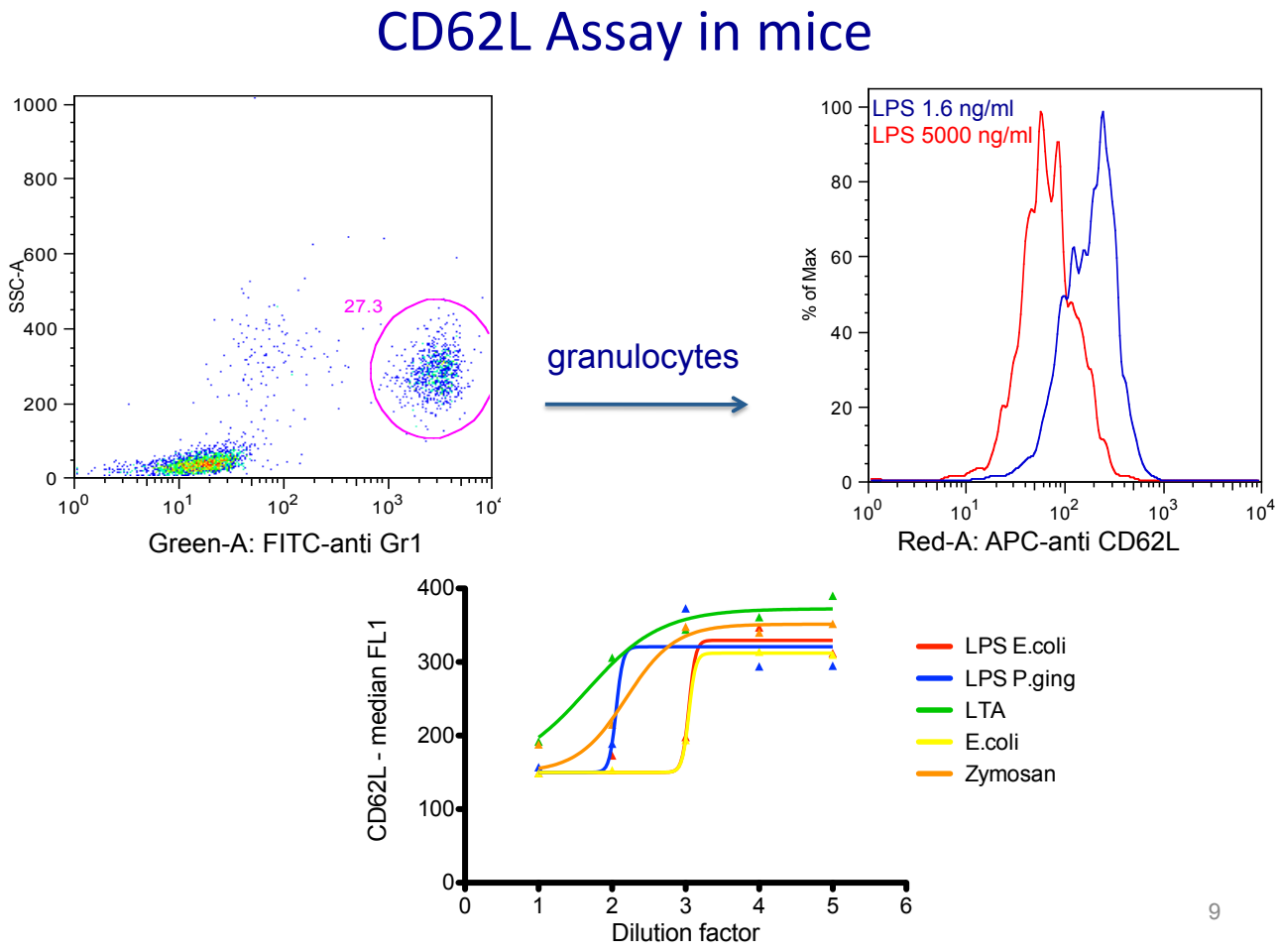
1) *E. coli* LPS: 1000, 200, 40, 8, 1.6 ng/ml
2) Live *E. coli* JM83: 10^8, 2x10^7, 4x10^6, 8x10^5, 1.6x10^4 bact/ml
3) Zymosan: 100, 20, 4, 0.8, 0.16 µg/ml



Important Notes:

- Shedding of CD62L is a very sensitive measurement of myeloid cell activation. The fresher the sample, the better the data quality.
- Washing off of antibody is omitted as it avoids the need for further washes and re-suspensions of the blood which minimizes the loss of cells and allows smaller blood volumes to be worked with. It was also found to have no effect on quality of staining or background with these antibodies.
- Working absolutely sterile is key to good results: use pyrogen free material and reagents, work in a laminar flow hood!
- Other TLR agonists that worked in my hands are lipoteichoic acid, live Staphylococcus xylosus and LPS *P. gingivalis*. When ordering TLR-agonists make sure they are ultrapure, endotoxin-free (Invivogen has good ones).

Analysis:



LC50 values can then be calculated according to the equation

$$LC50 = \frac{[agonist]max}{dilution\ factor^{(LogEC50 - 1)}}$$

where *agonist.max* is the top-concentration of agonist added and *dilution factor* the dilution factor of serially-diluted agonists (i.e. 5)