Method Sheet 41

Dose response assays for macrophage TNF-α production

Overview

This method sheet explains how to challenge an adherent macrophage cell-line with bacterial endotoxin (lipopolysaccharide, LPS) in the presence of serial doubling dilutions of natural extracts with suspected anti-inflammatory activity, for measurement of their capacity to inhibit macrophage TNF-α production. Performing this experiment will shed light on which of the hit extracts is the most potent in terms of their potential to inhibit secretion of TNF-α by the macrophage cell-line you have chosen to work with.

Reagents

- 96-well plates containing adherent J774, RAW or THP-1 cells
- RPMI with 10% FCS and antibiotics
- One or more hit natural extracts at 10 mg/ml in DMSO
- *E. coli* LPS at 1 mg/ml in H_2O (keep in -20^oC freezer until use)

Equipment

- Sterile, clear plastic 96-well microplate(s) with lids
- Sterile pipette tips (compatible with each pipette)
- Sterile reservoir(s) to dispense challenge compound (LPS)
- Multichannel pipette (8- or 12-channel) capable of dispensing 100 - 200 µl
- Tissue culture suite with a Class 2 biosafety cabinet and an incubator capable of maintaining a 5% CO_2 atmosphere at 37^oC
- -80^oC freezer for storage of supernatants

Method

1) Prepare one or more 96-well plates containing adherent J774, RAW or THP-1 cells 1-3 days before challenge (follow Method Sheet 12).
2) Defrost the hit *Phytotitre* extract vial(s) and a control vial of sterile DMSO alone.
3) Label 8 sterile 1.5 ml microtubes with the following DMSO concentrations: D0, D2, D4, D8, D16, D32, D64 and D128.
4) Label 8 sterile 1.5 ml microtubes with the following extract concentrations: E0, E2, E4, E8, E16, E32, E64 and E128.
5) Add 40 µl of RPMI into tubes 0 through to 64 in both rows (i.e. for both the DMSO and extract tubes).
6) Add 59.5 µl of RPMI into the D128 and E128 tubes.
7) Add 20.5 µl of DMSO into the D128 tube, and 20.5 µl of hit extract into the E128 tube, then mix both tubes well by gently pipetting up and down.

8) This gives a concentration of 2.56 mg/ml extract in these stock tubes, but that concentration will fall by a factor of ten to 256 µg/ml when adding 10 µl of the suspension to 90 µl of each cell culture, and then later it will be halved to 128 µg/ml as the final concentration when we add 100 µl of LPS to the cells later.

9) Now perform serial doubling dilutions in each row by transferring 40 µl of the suspension in the 128 tube to the 64 tube, mixing gently by pipetting several times, then doing the same to the next tube until reaching the D2 or E2 tube, but do not put any extract in the D0 or E0 tubes, these must remain RPMI only.

10) Using a multichannel pipette, gently remove 10 µl of supernatant from the middle wells of the plate, according to the map shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		No treatment (negative control)	DMSO 0	DMSO 2	DMSO 4	DMSO 8	DMSO 16	DMSO 32	DMSO 64	DMSO 128		
C			DMSO 0	DMSO 2	DMSO 4	DMSO 8	DMSO 16	DMSO 32	DMSO 64	DMSO 128		
D			DMSO 0	DMSO 2	DMSO 4	DMSO 8	DMSO 16	DMSO 32	DMSO 64	DMSO 128		
E			Extract 0	Extract 2	Extract 4	Extract 8	Extract 16	Extract 32	Extract 64	Extract 128		
F			Extract 0	Extract 2	Extract 4	Extract 8	Extract 16	Extract 32	Extract 64	Extract 128		
G			Extract 0	Extract 2	Extract 4	Extract 8	Extract 16	Extract 32	Extract 64	Extract 128		
H												

11) Now, using a single channel pipette, add 10 µl of each DMSO or Extract dilution into the respective well as shown in the plate map above.

12) Add 10 µl of fresh RPMI only to each well in column 2 of the plate (this will be the no treatment, negative control).

Challenging the cells with LPS

1) For each 96-well plate, supplement 6 ml of RPMI / 10% FCS with 1.2 µl stock LPS (at 1 mg/ml, this should be kept frozen until use).

2) Pour the LPS solution into a suitable sterile plastic reservoir.

3) Use a multi-channel pipette with 8 tips attached to supplement every well in columns 3 to 10, as shown in the plate map above, with 100 µl of the working LPS solution.

4) Use a single-channel pipette to supplement every well in column 2 with 100 µl of RPMI / 10% FCS only, without LPS (these wells will be negative controls for TNF-α production).

5) Return the plate to the 37ºC incubator for 3 hours.

6) Repeat for as many plates as necessary.

7) After 3 hours incubation, remove the plate from the incubator into a sterile biosafety cabinet.

8) Place an empty, sterile 96-well plate next to the plate containing cells, and align both plates so that they are in the same orientation (i.e. with well A1 at the top left).

9) Use a multichannel pipette to transfer 150 µl of supernatant from every well of the plate containing challenged cells into the corresponding wells in the empty plate.

10) Be careful not to push the tips so far down that they touch the cell layer on the bottom of the well, this helps to avoid accidental aspiration of cells into the recipient plate.
11) Change tips in between each column transferred from one plate to the other.
12) Once supernatant has been transferred from all wells in the culture plate to the collecting plate, discard the plate containing remaining cells and media.
13) Use a marker to label the lid of the collection plate carefully with the date, experimental conditions, and the extract plate number (i.e. 1-5).
14) Place the lid on the collection plate and use the marker to draw vertical lines on the side of the plate, connecting both the lid and the base - draw one line for plate 1, five lines for plate 5, etc.
15) These lines will help you to keep track of which plate base maps to which experimental condition, since it is easy to accidentally swap lids if you are screening multiple plates on the same day.
16) Transfer the collection plate(s) containing supernatants to a -80ºC freezer for storage before assay (note, a -20ºC freezer is not cold enough to properly store the supernatants for subsequent cytokine measurement).
17) Alternatively, if no space is available in a -80ºC freezer, it is possible to proceed directly to challenging L929 cells for TNF-α bioassay without having to store the supernatants first.

Measurement of TNF-α production by crystal violet assay

1) Follow the directions in Method Sheet 14 to measure TNF-α production from each of the cultures in the plate by L929 cell crystal violet assay.
2) Plot the levels of TNF-α production as two lines on a scatter plot, one showing the dose response to DMSO and the other to the chosen Extract.

Notes

- This method shows how to test the relative inhibitory activity of one hit extract, if you are examining more than one hit extract, you will have to prepare and challenge more than one 96-well plate (one plate per extract).
- The final concentration of LPS tested in this experiment is 100 ng/ml.