Method Sheet 13

Challenging macrophages with *Phytotitre* extracts and LPS for anti-inflammatory molecule discovery

Overview

This method sheet explains how to challenge an adherent macrophage cell-line, such as RAW, J774 or THP-1, with bacterial endotoxin (lipopolysaccharide, LPS) for the purpose of screening for anti-inflammatory molecules in plant extracts. LPS is a structural component of the outer membrane of Gram-negative bacteria. It is recognised as foreign by the mammalian innate immune system via its interaction with Toll-like receptor (TLR)-4 on the surface of macrophages and several other cell-types. The triggering of TLR4 signalling by LPS results in the secretion of numerous pro-inflammatory cytokines from macrophages. Measuring these cytokines by ELISA can be costly for a multi-plate drug-discovery screening programme. Usefully, however, the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) can be measured using the inexpensive L929-cell bioassay. This method explains how to stimulate secretion of TNF-α by adherent macrophages, prior to measurement of this cytokine using such a bioassay.

Reagents

- 96-well plates containing adherent J774, RAW or THP-1 cells
- RPMI with 10% FCS and antibiotics
- Defrosted *Phytotitre* or *Puretitre* library
- *E. coli* LPS at 1 mg/ml in H_2O (keep in -20°C freezer until use)
- Polymyxin-B at 1 mg/ml in H_2O (keep in -20°C 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 1 - 10 µl
- 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°C
- -80°C freezer for storage of supernatants

Challenging cells with *Phytotitre* extracts

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) Fully defrost a *Phytotitre* or *Puretitre* library plate in a 37°C incubator (follow Method Sheet 01).

3) Carefully remove the cap mat from the library plate and place with the round domes facing upwards (do not discard the cap mat).
4) Column 1 of the library plate is left empty to allow space for negative controls - you can make use of this by pipetting 100 µl of sterile DMSO into each well of this column in the library plate to use as a vehicle only control.
5) Place both library and culture plates side by side and ensure both are in the correct orientation (i.e. check that well A1 is in the top left position).
6) Set a small volume multichannel pipette to dispense 2 µl (this is a common starting point, but can vary depending on your own experiment, see notes below).
7) Attach tips to the pipette and ensure all are firmly seated.
8) Work from left to right, starting at column 1 and moving across, one pipetting cycle at a time, to column 11.
9) It can be difficult to keep track of which column is being pipetted from and to when challenging plates for screening, so you can cover the wells that have already been completed with a sterile 96-well plate lid to help keep your place as you move along the plate from column to column.
10) Push the plunger down to the first stop (not all the way to the second stop).
11) Place the tips below the surface of the liquid in each well of the respective column of the library plate (containing stock extracts).
12) Slowly allow the plunger to return to the top position.
13) Look carefully across all the tips to ensure the level of liquid in each tip is the same, if not, dispense the suspension back into the correct wells of the library plate and try again.
14) Likewise, if there are any large air bubbles in any of the tips, dispense and try again.
15) Move the pipette to the plate containing the mammalian cell culture and dispense all the liquid into the respective wells, being careful to pipette into the liquid and not onto the side of the well.
16) When dispensing, push the plunger past the first stop all the way to the second stop.
17) Discard the tips after pipetting into each column (this is essential to prevent cross-contamination of the library contents).
18) Place fresh tips on the pipette before moving onto the next column.
19) Move the plate lid marker one column to the right to make it easier to see which column is next to work from.
20) Continue until every well of the plate between columns 1 and 11 have been challenged.
21) Move the culture plate backwards and forwards, then side to side, gently several times to mix the compound into the suspension.
22) Avoid jarring movements that may splash liquid between wells and cause cross-contamination.
23) Proceed directly to LPS challenge.

Challenging cells with LPS

1) For each 96-well plate, supplement 12 ml of RPMI / 10% FCS with 2.4 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 to supplement every well in columns 1 to 11 with 100 µl of the working LPS solution.
4) Use a single-channel pipette to supplement every well in column 12 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 37°C 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 -80°C freezer for storage before assay (note, a -20°C freezer is not cold enough to properly store the supernatants for subsequent cytokine measurement).
17) Alternatively, if no space is available in a -80°C freezer, it is possible to proceed directly to challenging L929 cells for TNF-α bioassay without having to store the supernatants first.

Plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	01	09	17	25	33	41	49	57	65	73	RPMI
B	DMSO	02	10	18	26	34	42	50	58	66	74	RPMI
C	DMSO	03	11	19	27	35	43	51	59	67	75	RPMI
D	DMSO	04	12	20	28	36	44	52	60	68	76	RPMI
E	DMSO	05	13	21	29	37	45	53	61	69	77	RPMI
F	DMSO	06	14	22	30	38	46	54	62	70	78	RPMI
G	DMSO	07	15	23	31	39	47	55	63	71	79	RPMI
H	DMSO	08	16	24	32	40	48	56	64	72	80	RPMI

1 µl DMSO + LPS

1 µl each plant extract or stock compound, + LPS

No DMSO No LPS

Notes

- The protocol assumes that the user is trained in the basic principles and techniques of mammalian cell culture - please seek such training if you have not received it already before attempting to follow the method.
- When dispensing extracts, ensure the liquid goes directly into the larger culture volume, and does not touch and then adhere to the plastic side of the well.
- If using the *Phytotitre* library, the stock plant extracts are at 10 mg/ml, so transfering 2 µl of stock extract into 198 µl of culture represents a 1:100 dilution, which means the final concentration of plant extract in each culture will be 100 µg/ml.
- Accordingly, the final concentration of DMSO (the solvent, also called vehicle, used to dissolve the plant extracts) will be 1% (wt:vol) when following this protocol.
- If using the *Puretitre* collection instead, the stock compounds are at 10 mM, which means the final concentration of each compound in each culture will be 100 µM.
- The final concentration of LPS tested in this experiment is 100 ng/ml.
- If there are too many hits per screen, further dilutions of extracts are possible using intermediate plates containing complete medium (e.g. 1 µl extract into 24 µl DMEM per well in plate 1, then 5 µl from plate 1 onto 95 µl of each cell culture in plate 2, for a 1:500 dilution from stock).