PROPAGATION & CULTURING OF RAW264.7 CELLS

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BACKGROUND

These cells are macrophage-like cell line derived from Balb/c mice. They maintain many of the properties of macrophages including NO production, phagocytosis (beads, other), extreme sensitivity to TLR agonists and motility. They are susceptible to genetic drift so freezer stocks must be made from early passage number cells. They are genetically fairly heterogeneous, which can be a benefit as clones that have reduced binding to certain ligands can be isolated and propagated. They express SRA but not MARCO. They may be transfected, although mortality is high and expression is not guaranteed.

NOTES

Freezer Stocks: These cells are fairly resistant to freeze/thaw.

Propagation: These cells should never be completely confluent (60-75% max). In general macrophages do not like to be too dense. These cells are known to suffer from genetic drift – they are genetically heterogeneous and there will be subclones within the mix that differ in receptor expression, TLR responsiveness, etc. To avoid genetic drift use low passage numbers (i.e. less than 20) and ensure that all cells (or almost all) are lifted during subculture. Lifting these cells can be tricky (they are macrophages after all), trypsin EDTA is a possibility, lidocaine + EDTA works sometimes and scraping is possible.

Culturing stably expressing cell lines: Stable expression is hard to achieve in this cell line and cells must be continuously tested for expression. When taking stably expressing cells (usually because transfected with linearized construct in pcDNA3.1), start culture at 0.2mg/ml G418, after 2 days increase to 0.6mg/ml).

EQUIPMENT

- Equipment:
  - Level 2 Biosafety Cabinet (BSC)
  - 37°C, 5% CO_2 Incubator
  - Centrifuge
- Materials
  - DMEM + 10% FBS (+L-glutamine, Pen/Strep is optional). **Note:** RPMI + 10% FCS (+L-glutamine if necessary, Pen/Strep is optional) can also be used.
  - PBS (heated to 37°C)
    - Trypsin-EDTA/Trypsin (optional)
  - FischerSci cell lifter (or any suitable alternatives)

Protocol

All activities with cells should be conducted under a clean, sterile biosafety cabinet (level 2). Do not expose cells to cold media or violent movement as this can cause dislodging or cell death. Cells should not be handled outside of incubator for long periods of time.

To Propagate cells:

These cells are most suitable to culture in a 75 cm^2 flask. Total volume in flasks should be 10 mL. The growth media should be refreshed every 2-3 days but no more than 4 days without causing significant cell death. The average doubling time of RAW cells is 15 hours.

1. Aspirate all media by tilting flask to allow media to collect in a corner of the flask
2. Gently eject fresh media into the same corner

To Split cells:

Using a light microscope and if cell confluency is estimated to be around 60-70%, the cells need to be split. Warm trypsin, media and PBS for at least 20 minutes in a 36^oC waterbath prior to this procedure. Remember to thoroughly spray all containers coming in and out of BSC with 70% ethanol for sterility.

1. Aspirate all growth media by tilting flask to allow media to collect in a corner
2. Wash once with PBS (half of culture volume) by gently aspirating PBS into a corner, gently shaking the PBS around the flask and aspirate
3. Eject half of culture volume of trypsin directly onto cells (the cells will need to be lifted at this point) and incubate for 5 minutes at 37^oC
    a. RAW cells are quite adherent to tissue culture flask; if the trypsin does not immediately lift the cells, do not proceed to wait longer as this risks the viability of cells
4. Eject (half of culture volume+0.5mL) warm media directly onto cells
5. Gently use the cell lifter and scrape cells off the flask. These cells do not lyse easily with a cell lifter. Collect the cell suspension from flask
6. Spin for 8 minutes at 1500 rpm. These parameters can be changed – the goal is to completely pellet the cells without causing damage to cells. Be sure to properly balance the centrifuge (use a scale!) to avoid damage to the rotor
7. Resuspend in fresh media. It is important to triturate repeatedly to allow for a single-cell suspension. The volume of resuspension should be the same volume that was originally aspirated from all flasks (e.g. 10ml/flask collected – 4 flasks worth of cells should be resuspended in 40ml media)
8. Seed cells into a new flask with dilution and place back into incubator
    a. 1:3, 1:4 or 1:5 dilutions are all acceptable. The greater the dilution, the longer cells will reach confluence. Do not heavily dilute as cells seeded with extremely low confluence exhibit impaired growth and proliferation
9. Monitor for growth and refresh media when necessary

To Freeze cells:

Considering the nature of these cells, it is suggested that freezer stocks be made as soon as a new vial of low-passage numbered cells are cultured.

1. Grow to a density of 1 x 10^6 cells in 20 mL of media (see Cell Counting protocol). This can be done in a larger 175 cm^2 flask. Collect the cells in a similar manner as the 'Split cells' section above.

2. Centrifuge at 1000rpm x 5 min (or however long to pellet the cells).

3. Resuspend in 3.6mL of FCS/FBS and add 400uL of DMSO in a drop-wise manner. This step needs to be done quickly as DMSO is cytotoxic but necessary in preparing for freezing conditions

    a. DMSO mitigates the formation of sharp water crystals which can puncture cells at negative temperature conditions

4. Pipette 1mL into a cryovial

5. Quickly place on ice and store at -80˚C overnight. Transfer to liquid nitrogen or keep in -80˚C for up to 6 months.

To Defrost cells:

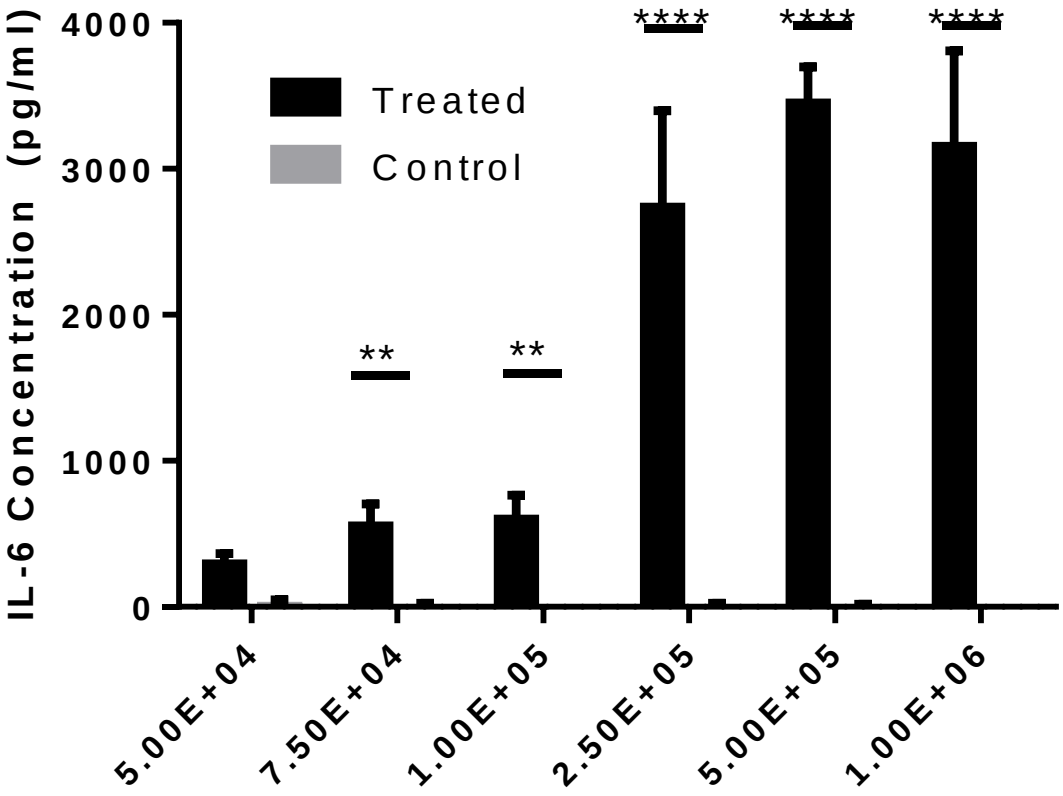
Cells should be warmed as quickly as possible. This can be done by quickly submerging in a clean waterbath or by hands.

1. Warm media (see recipe above) to 37˚C.
2. Pipette 30 mL of media into a 50 ml conical tube and keep warm
3. Pour the contents into the warmed media and centrifuge at 1000 rpm x 5 min.
4. Resuspend in 10 mL media and seed into a 75 cm^2 flask overnight. Check cell density the next day and treat accordingly

Supplementary

RAW cells can be plated at various densities. The following are concentrations of RAW cells in 96-well plates and their associated IL6 release as measured by ELISA. Cells were stimulated with 1000 ng/ml Pam_3Csk_4 to elicit IL6 secretion.

Cell Concentration (cells/ml) in 120 ul	Cell Density (cells/well)	Cell Density (cells/well) @ time of harvest (assuming doubling time is ~15hour; harvest cells 48hr post plating)
$5x10^4$	6 000	55 138
$7.5x10^4$	9 000	82 706
$1x10^5$	12 000	110 275
$2.5x10^5$	30 000	275 688
$5x10^5$	60 000	551 375
$1x10^6$	120 000	1 102 750



**p<0.001, ****p<0.0001