Induction of macrophage migration into peritoneal cavity

Prepare Thioglycollate Medium Brewer Modified (BBL, Becton Dickinson Microbiology Systems, MD, cat nr 211716): 38.5 g in 1L. Boil for 1 min to dissolve, and autoclave.

Inject the mice with 1 ml IP, and leave them for 3-5 days.

Collection of macrophages

Reagents:

1. Syringes with 5 ml sterile PBS and 18G needles.
2. Tubes with 2ml HBSS w/o Ca^{2+} or Mg^{2+}+10% FBS+2% Penicillin / Streptomycin, on ice.
3. Tube with Ketamine/Xylazine (K/X) at 10 mg/ml K, 2mg/ml X, in PBS.

Inject the mice with 100 µl K/X intramuscular (1 mg K, 0.2 mg X).

When KO, hold them with rubber bands and inject 5ml PBS in the belly, about 0.5 cm from sternon. Collect 3 ml from there (or 4 if the liquid is too clear). Place the liquid in the tubes without making bubbles.

Centrifuge tubes 5 min 1200 rpm.

Remove SN and add 1 ml DMEM+5%FBS+2% Pen/Strep (P 200U/ml, S 200 µg/ml).

Resuspend thoroughly and transfer 20µl to 10ml counting medium.

Count and dilute to $1 \cdot 10^6$ cells/ml (If $N=6.2 \cdot 10^6$ then just need to add 5.2 ml DMEM; If N=12.3 ml, just add 11.3 ml DMEM, etc).

Place 50 µl of this dilution on columns 1 and 2 of a flat bottom (Costar) 96 well plate. The next mouse will use cols 3 and 4, next 5 and 6… ATTENTION: Cols 11 and 12 must go empty, since they are controls.

Incubate for at least 20 min at 37 °C.

Meanwhile, prepare the TLR3 agonists: Poly IC (5µg/ml, Amersham)

After the incubation period for the MΦ is over, shake the plates to lift lymphocytes and remove the supernatant. Add 100 µl of the agonist solutions. Incubate for 4 h at 37°C.

Transfer the SN to a clean plate and freeze that at -80°C.

MTT marking of Macrophages

Immediately, add 50 µl of MTT (stock 2 mg/ml in PBS) diluted 1:4 in DMEM+10%FBS+2% Pen/Strep to the macrophages. Incubate from 1 h to O/N.

L929 cells

Culture medium: DMEM+10%FBS+2%Pen/Strep

To prepare bioassay plates, rinse an 80% confluent flask with PBS. Add 3 ml Trypsine EDTA and incubate until detachment. Add 8 ml medium, pool all flasks and centrifuge 5 min 1200 rpm.

Discard supernatant and add 10-20 ml medium.

Transfer 20 µl of cells to 10 ml counting solution, and count.

TO PASS CELLS: transfer 200 µl of the cells to a large flask.

TO PREPARE BIOASSAY: Dilute to $5 \cdot 10^5$ cells/ml, and pipette 100 µl per well of a 96 well plate. Incubate 24 h.

Bioassay

Add 50 µl Cyclohexemide (CHX) (From stock at 10 mg/ml or 10,000 U/ml, dilute 1:25 in DMEM+ 10%FBS+ 2%P+S) and Incubate 20 min 37 °C.

In the meantime, prepare TNF-α standard curve: Stock aliquoted at 10 µl, corresponding to 100 ng. So, add 1 ml of medium to dilute it to 100ng/ml, from there, transfer 5 µl to 1 ml of medium (0.5 ng/ml). Prepare standard on a 96 well plate: From dilution at 0.5 ng/ml, add 30 µl to 270 µl of medium (line A). From there down, make a serial dilution 150 µl+150 µl medium until line G. REMEMBER, line H is only medium!.

From the standard prepared, add 50 µl on cols 11 and 12 (from line A to A 11 and A 12; from line B to B 11 and B 12, etc).

To the rest of the plate (cols 1-10), add 46 µl of DMEM+10%FBS+2%P+S and 4 µl of supernatant from MΦ.

Incubate the plates O/N at 37 °C.

Following day, add 70 µl of pure MTT to the wells and incubate for at least 3h.

Dry and leave at RT.

To measure, lyse cells with 100 µl lysis solution (2-propanol+10% H2O+0.5% SDS+0.04N HCl: 3,6 L isopropanol+400 ml H2O+20g SDS+4 ml HCl 4N) and incubate at RT for at least 30 min. Read at 590 nm using a reference filter of 690 nm.