Imperial College London

INFECTION, IMMUNITY IMMUNOPHENOTYPING

Aim

To measure cellular inflammation by total and differential cell counts in the BAL fluid of knockout mice, compared to wildtype (control) mice 10 days after infection with X-31 influenza.

Equipment

Sodium pentobarbital (Sigma)
Surgical tools
Cannula (Venisystems, Abbocath-T 20G, Cat # 4535-40)
Thread
1 ml syringes (3 per group)
Ice
Eppendorf tubes
Haemocytometer (FastRead 102 disposable counting slides, Immune Systems)
Benchtop centrifuge
Cell-Prep Cytospin centrifuge (Centurion, model 4050), including cassettes and filter paper
Microscope slides (HAD, Cat # 7107)
Coverslips (VMR, Cat # 631-0137)
Mounting media (Leica, Surgipath Sub-X, Cat # 3801740)
Microscope for total cell count (Zeiss)
Microscope for differential cell count (Leica, DFC300FX)

Reagents

Autoclaved 1×PBS
White cell counting fluid
Methanol
A+B PBS Buffer for Wright Giemsa Staining
Wright-Giemsa Stain (Sigma Aldrich, Cat # WG16-500ML)

Media recipes

A+B PBS Buffer: 50 ml A + 50 ml B and make up to 1 L with dH_2O. A = 9.47 g/L NaH_2PO_4; B = 13.609 g/L KH_2PO_4

White cell counting fluid: 2 ml acetic acid, 1 ml 1% crystal violet in methanol, 47 ml PBS

Preparation

- Mice need to be sacrificed by an overdose of an anaesthetic (injection). The mice cannot be killed by dislocation of the neck or exposure to CO_2 gas.
- Complete killing by cardiac puncture. Can be performed blind or open chest cavity and be careful not to puncture the lungs.
- Carefully make an incision in the neck through the skin and muscles to expose the trachea (Fig.1). Isolate the trachea and insert a thread between the trachea and the oesophagus (located behind the trachea). Make a small incision in the trachea and insert the cannula. Tie the cannula with the thread.

BAL

1) Lavage lungs with 3 × 0.4 ml PBS (autoclaved PBS). Use a cannula and 1 ml syringe.
2) Place the lavage fluid (should be about 1ml) in a pre-labelled 1.5 ml eppendorf tube, and put the tube on ice.
3) Spin tubes at 450 × *g* for 5 min in a benchtop centrifuge.
4) Discard the supernatant and resuspend cells in 0.5 ml PBS, and count on haemocytometer with white cell counting fluid (1/10 dilution).
5) Count cells and record. Count three quadrants/sample and calculate average.

   Total cell count = average number of cells $\times 10^5$/ml

Cytospins

1) Vortex cells as they may have settled to the bottom.
2) Pipette 100 µl of resuspended cells for each slide (approximately 5×10^4 cells per slide) into the hole of the cassette and make up to 250 µl by adding 150 µl of PBS. Note that the cassettes for this particular cytospin require a total volume of 250-500 µl to be loaded.
3) Spin at 32 × *g* (500 rpm) for 4 min.
4) Remove and air dry.
   Check slides to see the density of cells. Dilute cells if necessary (or centrifuge cells again and resuspend in <500 µl if there are not enough cells) and cytospin again on a new slide.
5) When dry, fix in methanol for 5 min.
   Let the slides dry and store in a storage box (slides can be stained at a later time).

Staining with Wright Giemsa

1) Rehydrate in PBS (A+B PBS buffer) for 30 min.
2) Whilst slides still in the rack stain dunk in box with
   a. Wright-Giemsa stain for 7 min,
   b. PBS (A+B PBS buffer) for 7 min
   c. Wash under gently running tap
3) Leave to dry and then coverslip.

Analysis

Examine each slide using a light microscope (Leica, DFC300FX) and count 300-400 cells in 4–8 random fields (×40 magnification). Identify macrophages, monocytes, lymphocytes, eosinophils and neutrophils by their distinct nuclear morphologies. Record the number of cells and calculate the percentage and absolute number of each leukocyte subtype for each BAL sample.

Percentage of each subtype = (no. of subtype/total cells counted)*100

Absolute number of each subtype = total number of cells × (no. of cell type/total cells counted)

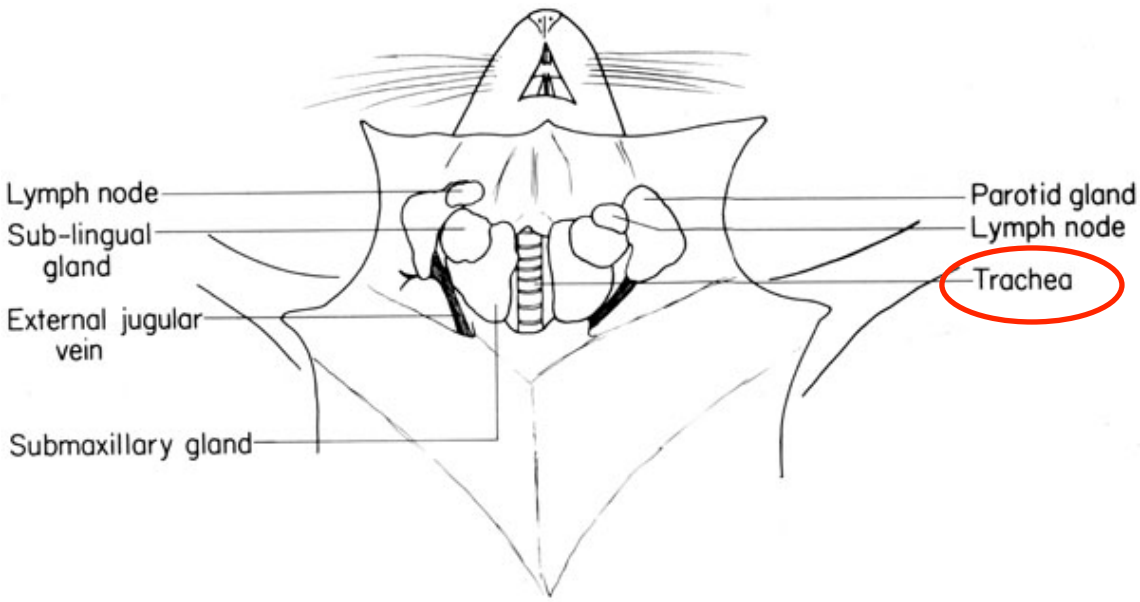

Figure 1. Mouse neck dissected to display trachea