TITLE: Ovine FAANG Project Harvest of Rambouillet Sheep Tissues from Benz2616

Collection: April 26, 2016 at Utah Veterinary Diagnostic Laboratory

Protocol for isolating specific leukocyte populations from fluids:

A) Peripheral blood mononuclear cell (PBMC) isolation

Collect whole blood into a container with an anticoagulant (EDTA).

Centrifuge whole blood in sterile 50 mL conical tubes at ~700 x g for 10 minutes at room temperature to form a "buffy coat" layer of leukocytes.

Remove buffy coat and an equal volume of plasma to a new tube.

Carefully layer the buffy coat/plasma over ~20 mL of a Ficoll gradient and centrifuge ~400 x g for 20 minutes at 4° C with no brake.

Remove lymphocyte layer to a new tube and add at least 2x volume of 1 x PBS, centrifuge ~400 x g for 7 minutes, and then decant supernatant.

Remove red blood cells: Resuspend cells in 1 mL 1 x PBS with 5 mM EDTA and then add 19 mL H_2O mixing by inversion for 20 seconds. Add 2 mL of 10x PBS to stop lysis. Centrifuge ~400 x g for 7 minutes.

Decant supernatant. Resuspend cell pellet with 1 x PBS then centrifuge @~400 x g for 7 minutes.

Resuspend cell pellet in 1-5 mL 1 x PBS, depending on the size of the pellet. Count the number of cells.

B) Magnetic bead isolation of specific leukocyte populations

Perform Miltenyi Biotec (Germany) protocol. Briefly:

Keep all reagents and cells on ice when not processing them.

Pellet cells by centrifuging at 300 x g for 10 minutes at 4° C.

Decant supernatant and resuspend cells in an appropriate volume of buffer for the cell count:

10^7 cells = 0.085 mL

10^8 cells = 0.850 mL

Add your choice of primary antibody ensuring that it is the appropriate isotype for the microbead antibody to the following concentration:

10^7 cells = 15 µM

10^8 cells = 15 µM

Incubate on ice or at 4 ° C for 30 minutes. Gently mix at 10 minutes and 20 minutes.

Wash by adding approximately 2 mL of buffer per 10^7 cells and centrifuge at 300 x g for 10 minutes. Decant supernatant.

Resuspend cells in 80 µL buffer per 10^7 cells. Add 20 µL of Anti-Mouse MicroBeads per 10^7 cells. Mix and incubate for 15 minutes at ~4 ° C. Mix at ~7 minutes.

Wash as above. Resuspend cells in 500 µL buffer for up to 10^8 cells.

Select appropriate sized column and place in MACS Separator.

Rinse column with appropriate amount of buffer: MS- 500 µL LS- 3 mL

If processing more than 1 animal/primary antibody at a time add 1 mL to additional columns to allow for appropriate time to process all samples. Add resuspended cells to column when there is a small amount of buffer in the reservoir of the column. Make sure no bubbles enter the column.

Once cells have nearly all passed through the column add appropriate amount of buffer 3 consecutive times to wash unlabeled cells out of the column.

Remove column from MACS Separator to new tubes. Add appropriate amount of buffer, MS: 1 mL LS: 5 mL, to the column and apply plunger to rinse selected cells into the tube.

Count cells again.

Snap freeze in liquid nitrogen or process for your specific experiment.

Cells were snap frozen dispersed in ~0.5 to 0.75 mL of PBS.

Antibodies that were used for this sample collection were purchased from Bio Rad (Hercules, CA, USA):

CD4 MCA2213F
CD8 MCA2216PE
CD14 MCA920GA
WC1 MCA2222F

Approximate total number of live cells collected per tube:

PBMC	2.68x10^7 cells
CD4+	4.14x10^7 cells
CD8+	2.45x10^7 cells
CD14+	1.03x10^7 cells
WC1+	1.06x10^7 cells

C) Bronchoalveolar lavage for alveolar macrophages

Approximately 2.5 liters of sterile PBS were poured into excised lungs 1 liter at a time. With PBS in the lungs, tissue was gently massaged for about 30 seconds to aid in alveolar macrophage harvest.

Lavage fluid was then decanted into sterile liter bottles for processing.

Under antiseptic conditions in a laminar flow hood, lavage fluid was passed through 100 μM cell strainers to remove surfactant and mucus.

Strained lavage fluid was centrifuged at 400 x g for 10 minutes at room temperature in 50 mL conical tubes. Supernatant was removed and cell pellets were combined.

Red blood cells were lysed by resuspending cells in 1 mL 1 x PBS with 5 mM EDTA and then adding 19 mL H_2O mixing by inversion for 20 seconds. Add 2 mL of 10 x PBS to stop lysis. Then centrifuge for 5 minutes at 400 x g.

Cell pellet was resuspended in PBS and total number of cells counted with a hemocytometer.

Cells were snap frozen dispersed in approximately 0.5 to 0.75 mL of PBS.

Total number of live cells collected per tube:

Alveolar macrophages	7.2x10^8 cells