Protocol

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Here, we present a protocol to analyze the T cell profiles of the neonatal ovine lung during respiratory syncytial virus (RSV) infection. The protocol delivers standardized multiparameter flow cytometry (FCM) analysis of CD4+, CD8+, regulatory, and γδ T cells isolated from lung, lymph nodes, and bronchoalveolar lavages (BALs). We detail the preparation of RSV and transtracheal inoculation of newborn lambs. We then describe tissue isolation and preparation of cell suspensions, followed by FCM acquisition to identify different T cell subsets.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Highlights
- Procedure to generate a translational neonatal model of RSV disease
- Approach to assess T cell responses from lung, lymph nodes, and BAL of newborn lambs
- Steps to isolate tissue and prepare cell suspensions
- Standardized multiparameter FCM assay to identify different T cell subsets

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Protocol

Multiparameter flow cytometry assay to analyze the pulmonary T cell profiles in the ovine model of respiratory syncytial virus infection

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SUMMARY

Here, we present a protocol to analyze the T cell profiles of the neonatal ovine lung during respiratory syncytial virus (RSV) infection. The protocol delivers standardized multiparameter flow cytometry (FCM) analysis of CD4+, CD8+, regulatory, and γδ T cells isolated from lung, lymph nodes, and bronchoalveolar lavages (BALs). We detail the preparation of RSV and transtracheal inoculation of newborn lambs. We then describe tissue isolation and preparation of cell suspensions, followed by FCM acquisition to identify different T cell subsets. For complete details on the use and execution of this protocol, please refer to Démoúlins et al. (2021).

BEFORE YOU BEGIN

We provide a procedure to characterize the T cell responses of the neonatal lung in a translational model of respiratory syncytial virus (RSV) infection. Since RSV is a natural pathogen of ruminants, we use lambs as the animal model of which the lung development is comparable to human, and infection recapitulates the key features of pediatric disease.

Ethical and safety aspects

The experiments in sheep were performed in compliance with the Swiss animal protection law (TSchG SR 455; TSchV SR 455.1; TVV SR 455.163) under the authorization BE125/17. The experiments were reviewed by the cantonal committee on animal experiments of the canton of Bern, Switzerland, and approved by the cantonal veterinary authority (Amt für Landwirtschaft und Natur LANAT, Veterinárdienst VeD, Bern, Switzerland). Work under biosafety level 2 conditions is required to carry out this protocol. Also, when in contact with infected animals, we recommend appropriate personal protective equipment such as disposable coveralls with hood and FFP3 face masks.

Determination of the antibody working concentrations

© Timing: 5–6 h for the titration of a set of antibodies

1. Perform all staining reactions in 1.2 mL Microtiter tubes, with a reaction volume of 50 μL.
Figure 1. Antibody titration and validation

All antibodies were titrated separately; here are a few examples to illustrate. We started with the manufacturer recommended dilution and made at least 6-point 1:2 dilution series plus unstained control.

(A) WC1-IgG2a-FITC: recommended dilution 1:10 corresponds to 1 mg/mL.

Examples of conjugated primary antibodies

| Dilution | WC1-IgG2a-FITC | CD45RO-IgG3-PE |
|----------|----------------|----------------|
| 1:10     | 13.5           | 46.6           |
| 1:20     | 12.6           | 14.9           |
| 1:40     | 12.5           | 8.13           |
| 1:80     | 12.3           | 1.95           |
| 1:160    | 13.5           | 0.76           |
| 1:320    | 14.7           | 0.13           |
| 1:640    | 11.1           | 0.042          |
| No Ab    | 0.79           | 0              |

Examples of unconjugated primary antibodies

| Dilution | CD25-IgG3 (+ IgG3-AF647) | CD8-IgG1 (+ IgG1-AF647) |
|----------|--------------------------|------------------------|
| 1:100    | 17.8                     | 32.4                   |
| 1:200    | 18.2                     | 29.6                   |
| 1:400    | 17.4                     | 30.4                   |
| 1:800    | 15.0                     | 28.7                   |
| 1:1600   | 11.1                     | 25.1                   |
| 1:3200   | 9.20                     | 15.7                   |
| 1:6400   | 5.28                     | 12.3                   |
| No Ab    | 0.46                     | 0.17                   |

Examples of conjugated secondary antibodies

| Dilution | (CD4-IgG2a) + IgG2a-APC-Cy7 | (CD172a-IgG1) + IgG1-PE-Cy7 |
|----------|-----------------------------|-----------------------------|
| 1:250    | 28.2                        | 57.7                        |
| 1:500    | 18.8                        | 53.2                        |
| 1:1000   | 1.31                        | 56.7                        |
| 1:2000   | 0.100                       | 53.1                        |
| 1:4000   | 0.027                       | 53.5                        |
| 1:8000   | 0.015                       | 52.9                        |
| 1:16000  | 2.98E-3                     | 56.9                        |
| No Ab    | 0                           | 0.033                       |
CRITICAL: An important consideration when building multicolor panels is titration of antibodies to define their working concentration. A high antibody concentration creates background that reduces the resolution of the results. Moreover, titration of antibodies can save resources by using smaller concentrations than the one stated in the manufacturer’s instructions.

a. Perform antibody titrations by starting with the commercial concentration (saturated in most cases) and then performed 1:2 dilution series, as shown in Figure 1.

b. Define the working concentration as the concentration when the separation between the positive and negative population is still clearly visible, without losing mean fluorescence intensity (MFI).

Note: The representative examples in Figure 1 shows the optimal concentrations that generate specific staining with a negligible background. For instance, we found that the working concentration for WC1-FITC was 1:80 instead of 1:10, as stated by the manufacturer; however, for CD86-PE, the working concentration was identical to the one stated by the manufacturer (1:10).

Note: Before moving to pulmonary tissue, we recommend using peripheral blood samples to confirm the efficacy of the flow cytometry (FCM) assay. Shortly, to measure T cell maturation (CD25, CD45RO and/or FoxP3 expression) and/or activation (cytokine production), we first stimulated ovine peripheral blood mononuclear cells (PBMCs) for 16 h with a panel of selected toll-like receptor (TLR) ligands (poly I:C (PIC, 10 μg/mL), Pam2Cys-SK4 (P2C, 10 μg/mL) or Resiquimod (Resiq., 10 μg/mL)) or lymphocyte mitogens (PMA/ionomycin (PMA, 100 ng/mL; Iono, 1 μg/mL) or Concanavalin A (Con A, 20 μg/mL)). Figure 2 depicts the typical immunomodulatory effect on the T cell subsets of interest.

Virus propagation and concentration

Timing: 48–72 h depending on HEp-2 cell confluence and cytopathic effect

Note: To modulate respiratory syncytial virus (RSV) disease severity in neonatal lambs, we used two different strains, namely RSV-ON1-H1 and RSV A2 (Démoulins et al., 2021). RSV-ON1-H1 was the major genotype circulating in central Europe during the RSV seasons of 2012–2017, whereas RSV A2 is a well characterized prototypical strain of RSV. RSV-ON1-H1 is a primary clinical isolated from nasopharyngeal aspirates of a child below the age of five years with confirmed RSV infection (Blockus et al., 2020). The following procedure for the propagation, concentration, and titrations of RSV stocks can be performed similarly for both strains.

2. At day 0, seed out $7 \times 10^6$ HEp-2 cells per T-150 cm$^2$ flask in culture media (refer to materials and equipment for detailed recipe).

Note: Adjust the number of flasks based on the desired amount of virus. Typically, around 2–5 $\times 10^6$ TCID$_{50}$ per mL is obtained per T-150 cm$^2$ flask.
3. Leave for 24 h at 37°C, 5% CO₂ to reach a confluence of 60%–80%.

4. At day 1, wash the HEp-2 cells once with 10 mL of infection medium (refer to materials and equipment for detailed recipe).

5. To recover frozen virus stored at −150°C, thaw in 1–2 min at 37°C. Add the virus to the cells, at a multiplicity of infection (MOI) of 0.02 plaque-forming unit (PFU) per cell in a total volume of 8 mL of infection medium.

**Note:** Calculations are based on the number of cells seeded at day 0.

**Note:** We recommend a range of 5–15 passage numbers.

6. Adsorb for 2 h at 37°C and 5% CO₂ on an orbital shaker.
7. Add 9.1 mL of DMEM and 900 μL FBS per flask (18 mL final volume).
8. Incubate for 36–44 h until the cytopathic effect (CPE) reaches approximately 60%. This comprises large syncytia formation, cell rounding and extensive cellular monolayer disruption.
9. Freeze the cultures overnight at −70°C, to disrupt the cells and release the infectious virions.

**Figure 2.** *Ex vivo* stimulation of peripheral blood mononuclear cells by toll-like receptor ligands or mitogens

PBMCs from individual animals were stimulated for 16 h with various stimulants.

(A–D) Next, our multiparameter FCM assay was applied to analyze the specific responses of CD8⁺ T cells (A), CD4⁺CD8⁻ cells (B), γδ T cells (C) and Tregs (D). Highlighted are the contour plots were clear activation (intracellular cytokine detection) or maturation (modulation of cell surface marker expression) was detected.
10. Thaw the cell culture flasks quickly at 37°C.

11. Scrape the HEp-2 cell monolayer using a sterile cell scraper and spin down the total content of the flask for 10 min at 1,000 g, 4°C to collect the supernatant and get rid of the cell debris.

12. Layer the clarified supernatants (typically 180 mL, from the processing of 10 flasks) over 20% sucrose (w/v) solution (typically 35 mL) prepared in sterile Dulbecco’s PBS (no calcium, no magnesium) (refer to materials and equipment for detailed recipe) in a 230 mL Polycarbonate bottle with screw-on cap, and centrifuged for 3.5 h at 71,000 g, at 4°C.

13. Discard smoothly the supernatant by tube inversion. 

Note: Be careful while inverting the tubes because the pellet is not always visible.

14. Resuspend the pellet in virus resuspension buffer (refer to materials and equipment for detailed recipe).

△ CRITICAL: To ensure an optimal resuspension of the virus, pipet gently up and down at least 50 times.

15. Aliquot the resultant virus stock and store at −150°C until further use.

△ CRITICAL: To prevent a loss in the virus biological titer, we recommend storage up to 6 months at −150°C.

Virus titer determination

⊙ Timing: 48–96 h

Note: The Dulbecco’s PBS used in this section contains no calcium, no magnesium.

16. At day 0, transfer 2–3 × 10^4 HEp-2 cells per well in growth media (refer to materials and equipment for detailed recipe) at a rate of 100 μL per well, into a 96 well flat-bottomed plate; leave for 24 h at 37°C, 5% CO2.

17. At day 1, titrate the virus stock with a ten-fold serial starting from 10^{-1} (upper row A) to 10^{-8} (lower row H), use infection medium for dilutions (refer to materials and equipment for detailed recipe) and do 11 replicates for each dilution. Dilutions are done directly in the 96-well plate with a multi-channel micropipette. Only column 1 is free of virus (negative control, Figure 3).
18. Wash the cells once with Dulbecco’s PBS and add 100 μL of the above virus stock dilutions per well, incubate for 2 h at 37°C, 5% CO2.
19. Gently wash the cells once with 100 μL Dulbecco’s PBS per well.
20. Fix the cells for 20 min at room temperature (20°C) with 200 μL fixation solution per well (refer to materials and equipment for detailed recipe).
21. Wash the cells twice with 100 μL Dulbecco’s PBS per well.
22. Incubate for 1 h at room temperature (20°C).
23. Add 100 μL per well of biotinylated anti-RSV antibody at a working dilution of 1:400 (200 μg/mL), in Dulbecco’s PBS, BSA 1%; incubate for 1 h at room temperature (20°C).
24. Wash the cells twice with 150 μL Dulbecco’s PBS, BSA 1% per well.
25. Add 100 μL per well of ExtrAvidin peroxidase (working dilution of 1:500, in Dulbecco’s PBS); incubate for 30 min at RT.
26. Wash the cells twice with 150 μL Dulbecco’s PBS, BSA 1% per well.
27. Wash the cells twice with 100 μL Dulbecco’s PBS per well.
28. When the plaques appear and have acquired the desired coloration, stop the reaction by washing the cells with 100 μL Dulbecco’s PBS per well.
29. Count plaques in the whole well, then choose the virus dilutions that give 50–100 plaques per well and count all replicates of that dilution.
30. Calculate RSV titers as follows: RSV [PFU/mL] = (number of plaques \times dilution factor)/(0.1 mL).

**Transtracheal RSV inoculation**

**Timing:** 2–3 h depending on the number of animals

*Note:* Postpartum, the acclimatization of the newborn lambs to the respective stables lasted 3–4 days prior RSV infection. In accordance with the principles of the 3Rs, suckling lambs were held together with their mothers and fed *ad libitum*. For further details on the procedures associated with gestation and group allocation, please refer to (Démoulins et al., 2021). Screening for the initial presence of RSV-specific neutralizing antibodies was done for all ewes. Shortly, the collected serums were serially diluted and co-incubated with RSV (100 PFU) prior being applied to HEp-2 cells for 48 h. The results show that only one ewe presented an RSV-neutralization. For further details, please see (Démoulins et al., 2021).

*Note:* The Dulbecco’s PBS used in this section contains no calcium, no magnesium.

32. To perform analgesia on 5–10 days old lambs, inject intramuscularly a mixture of midazolam (0.2 mg/kg) and butorphanol (0.2 mg/kg). Typically, the animals are under sleep 5–10 min post-injection.
33. Shave the injection site with an electric trimmer and disinfected with 70% ethanol.
34. Puncture the trachea between the proximal tracheal rings with a 20G needle (0.9 × 40 mm) and inject in 5–10 s a volume of 2 mL of Dulbecco’s PBS or 10⁷ PFU of RSV per animal in 2 mL Dulbecco’s PBS.
35. Monitor the rectal body temperature and clinical status daily.

*Note:* The clinical status of the lambs is assessed by a veterinarian, whenever possible always the same person to ensure unbiased clinical assessment. The rectal body temperature is measured with a digital thermometer, respiratory and heart rates with a stethoscope. For further details on infection procedure, clinical parameters, study termination, euthanasia, and necropsy, please see (Démoulins et al., 2021).
Enzymatic dissociation of peribronchial lymph nodes and lungs

**Timing:** 10–15 min

△ CRITICAL: The enzyme dissociation mixes must always be reconstituted the day of experiment.

36. For one lymph node (LN) (typically 4–5 g) sample to be dissociated, weigh:
   a. Collagenase D (1:10, final concentration = 0.36 mg/mL): 5.4 mg of Collagenase D powder;
   b. DNase I (1:10, final concentration = 0.1 mg/mL): 1.5 mg of DNase I powder.

   **Note:** The two enzymes must be reconstituted in 15 mL Dulbecco’s PBS containing calcium and magnesium.

37. For one piece of lung (typically 10–20 g) to be dissociated, weigh:
   a. Collagenase I (final concentration = 1.25 mg/mL): 12.5 mg of Collagenase I powder;
   b. Collagenase II (final concentration = 2.5 mg/mL): 25 mg of Collagenase II powder;
   c. DNase I (final concentration = 0.16 mg/mL): 1.6 mg of DNase I powder.

   **Note:** The three enzymes must be reconstituted in 10 mL Dulbecco’s PBS containing calcium and magnesium.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Biological samples  |        |            |
| Peribronchial lymph nodes, Bronchoalveolar lavages, lung tissue | Institute of Virology and Immunology (University of Bern, Switzerland) | N/A |

| Experimental models: Organisms/strains |        |            |
|----------------------------------------|--------|------------|
| Sheep (Skudde, 2–5 days old, males and females) | Institute of Virology and Immunology (University of Bern, Switzerland) | N/A |

| Bacterial and virus strains |        |            |
|-----------------------------|--------|------------|
| RSV A2 | ATCC | VR-1540P™ |
| RSV-ON1-H1 (Blockus et al., 2020) | Hannover Medical School, Germany | N/A |

| Chemicals, peptides, and recombinant proteins |        |            |
|-----------------------------------------------|--------|------------|
| Sterile water | Bichsel | Cat# 7689602 |
| Dulbecco’s PBS, no calcium, no magnesium (10x) | Gibco | Cat# 14200067 |
| Dulbecco’s PBS, calcium, magnesium (1x) | Gibco | Cat# 14040-174 |
| Penicillin-Streptomycin (10,000 U/mL) | Gibco | Cat# 15140-122 |
| DMEM (1X) + GlutaMax | Gibco | Cat# 32430-027 |
| Fetal bovine serum, qualified | Gibco | Cat# 10270 |
| UltraPure™ 0.5 M EDTA, pH 8.0 (500 x) | Thermo Fisher Scientific | Cat# 15575020 |
| Trypsin-EDTA (0.05%), phenol red | Thermo Fisher Scientific | Cat# 25300054 |
| Brefeldin A Solution (1000X) | Thermo Fisher Scientific | Cat# 00-4506-51 |
| eBioscience™ Foxp3 / Transcription Factor Staining Buffer | Thermo Fisher Scientific | Cat# 00-5523-00 |
| Albumin from bovine serum (BSA) | Sigma-Aldrich | Cat# A7030 |
| ExtrAvidin Peroxidase | Sigma-Aldrich | Cat# E2886 |
| 3,3’-diaminobenzidine (DAB) tablets | Sigma-Aldrich | Cat# D4293 |
| Methanol* | Sigma-Aldrich | Cat# 34860 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Hydrogen peroxide solution, 30% b | Sigma-Aldrich | Cat# H1009 |
| Collagenase D | Sigma-Aldrich | Cat# 11088866001 |
| Sucrose | Sigma-Aldrich | Cat# S0389 |
| Deoxyribonuclease (DNAse) I | Worthington Biochemical | Cat# LS002139 |
| Collagenase, Type 1 | Worthington Biochemical | Cat# LS004196 |
| Collagenase, Type 2 | Worthington Biochemical | Cat# LS004176 |
| CellWASH | BD Biosciences | Cat# 349524 |
| BD Cytofix/Cytoperm<sup>n</sup> | BD Biosciences | Cat# 554714 |
| Ficoll<sup>n</sup>/Paque Plus<sup>c</sup> | Cytiva / Merck Millipore | Cat# 17-1440-03 |
| ChromPure Mouse IgG, whole molecule | Lubio Kingfisher | Cat# 015-000-003 |
| Midazolam | Roche | Cat# Dormicum<sup>k</sup> |
| Butorphanol | Dr. E. Graeub AG | Cat# Morphasol<sup>k</sup> |
| Experimental models. Cell lines | | |
| HEp-2 cells | ATCC | Cat# CCL-23 |

### Antibodies

| Antibodies | SOURCE | IDENTIFIER |
|------------|--------|------------|
| Anti-CD45RO-PE (Clone IL-A116A; mouse IgG3; 1:10 dilution) | Bio-Rad | Cat# MCA2434PE |
| Anti-CD4 (Clone 44.38; mouse IgG2a; 1:400 dilution) | Bio-Rad | Cat# MCA2213GA |
| Anti-CD8 (Clone CC58; mouse IgG1; 1:160 dilution) | Bio-Rad | Cat# MCA1654G |
| Anti-IL-4-FITC (Clone CC303; mouse IgG2a; 1:10 dilution) | Bio-Rad | Cat# MCA1820F |
| Anti-IFN-γ-AF647 (Clone CC302; mouse IgG1; 1:160 dilution) | Bio-Rad | Cat# MCA1783A647 |
| Anti-TGF-β (Clone TB21; mouse IgG1; 1:100 dilution) | Bio-Rad | Cat# MCA797 |
| Anti-IL-10 (Clone CC318; mouse IgG2b; 1:40 dilution) | Bio-Rad | Cat# MCA2110 |
| Anti-WC1-FITC (Clone CC15; mouse IgG2a; 1:80 dilution) | Bio-Rad | Cat# MCA838F |
| hRSV-biotin (Goat IgG; 1:400 dilution) | Bio-Rad | Cat# 7950-0104 |
| Anti-CD25 (Clone LCTB2A; mouse IgG3; 1:200 dilution) | Kingfisher | Cat# WS0597B-100 |
| Anti-CD172a (Clone DH59B; mouse IgG1; 1:400 dilution) | Kingfisher | Cat# WS0567B-100 |
| Anti-FOX3-FITC (Clone FJK-16s; rat IgG2a; 1:50 dilution) | Thermo Fisher Scientific | Cat# 11-5773-82 |
| Anti-IL-17A-PE (Clone Ebio64DEC17; mouse IgG1; 1:20 dilution) | Thermo Fisher Scientific | Cat# 12-7179-42 |
| Anti-mouse IgG1, FITC (Goat IgG; 1:1,000 dilution) | Thermo Fisher Scientific | Cat# A-21121 |
| Anti-mouse IgG1, PE (Goat IgG; 1:200 dilution) | Thermo Fisher Scientific | Cat# P-21129 |
| Anti-mouse IgG1, a467 (Goat IgG; 1:1,000 dilution) | Thermo Fisher Scientific | Cat# A-21240 |
| Anti-mouse IgG2b, a647 (Goat IgG; 1:1,000 dilution) | Thermo Fisher Scientific | Cat# A-21242 |
| Anti-mouse IgG1, PerCP-Cy5.5(Clone RMG1-1; rat IgG; 1:80 dilution) | BioLegend | Cat# 406662 |
| Anti-mouse IgG1, PE-Cy7 (Clone RMG1-1; rat IgG; 1:640 dilution) | BioLegend | Cat# 4066614 |
| Anti-mouse IgG1, APC-Cy7 (Clone RMG1-1; rat IgG; 1:40 dilution) | BioLegend | Cat# 4066620 |
| Anti-mouse IgG1, BV421 (Clone RMG1-1; rat IgG; 1:80 dilution) | BioLegend | Cat# 4066616 |
| Anti-Mouse IgG3, PerCP-Cy5.5 (Goat IgG; 1:200 dilution) | SouthernBiotech | Cat# 1100-13 |

(Continued on next page)
**MATERIALS AND EQUIPMENT**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-mouse IgG2b, PE-Cy7 (Goat IgG; 1:250 dilution) | SouthernBiotech | Cat# 1090-17 |
| Anti-mouse IgG2a, APC-Cy7 (Goat IgG; 1:250 dilution) | SouthernBiotech | Cat# 1080-19 |
| **Other** | | |
| T-150 cm² flask | TPP | Cat# 90552 |
| Cell culture plates, 6-well | TPP | Cat# Z707767 |
| Cell culture plates, 96-well | TPP | Cat# 92096 |
| Serological pipettes 5 mL | Sarstedt | Cat# 86.1253.001 |
| Serological pipettes 10 mL | Sarstedt | Cat# 86.1254.001 |
| Serological pipettes 25 mL | Sarstedt | Cat# 86.1685.001 |
| Filter tip, 10 µL | Sarstedt | Cat# 70.1130.210 |
| Filter tip, 20 µL | Sarstedt | Cat# 70.760.213 |
| Filter tip, 200 µL | Sarstedt | Cat# 70.3031.255 |
| Filter tip, 1000 µL | Sarstedt | Cat# 70.3060.255 |
| 1.2 mL microtiter tubes | Thermo Fisher Scientific | Cat# 3492 |
| Trypan blue strain (0.4%) | Gibco | Cat# 15250-061 |
| gentleMACS C tube | Miltenyi | Cat# 130-093-237 |
| gentleMACS Octo Dissociator | Miltenyi | Cat# 130-095-937 |
| Class II Biosafety cabinet | Kojair | Cat# 027407 |
| BioWizard Golden GL-130 | | |
| Flow cytometry system FACSCanto II | BD Biosciences | N/A |
| CO₂ incubator | ICM | N/A |
| 37°C water bath | Hecht | Cat# 8911275 |
| Metal Stand | N/A | N/A |
| Funnel holder | N / A | N/A |
| Surgical clamp | Hammacher | N/A |
| Sterile glass bottle | Simax | N/A |
| Sterile beaker | DWK Life Sciences | N/A |
| **Software and algorithms** | | |
| FlowJo V10 | FlowJo LLC | https://www.flowjo.com |
| BD FACSDiva™ Software | BD Biosciences | https://www.bdbiosciences.com/en-ch/products/software/instrument-software/bd-facsdiva-software |

* Methanol is highly inflammable and toxic if swallowed, in contact with skin, or if inhaled. Avoid direct exposure and wear safety gear covering the face, eyes, and skin. If working where methanol vapors are present, proper ventilation is imperative for safety.

* Hydrogen peroxide is harmful if swallowed, irritates the respiratory system and, if inhaled, may cause inflammation and pulmonary edema. Avoid direct exposure and wear safety gear covering the face, eyes, and skin.

**MATERIALS AND EQUIPMENT**

**Culture media for HEp-2 cells**

| Reagent | Final concentration | Amount (for 100 mL) |
|---------|---------------------|---------------------|
| DMEM (1X) + GlutaMax | N/A | 90 mL |
| Fetal bovine serum, qualified | 10% | 10 mL |

Keep sterile and store at 4°C for up to 1 month.

**Infection media for HEp-2 cells**

| Reagent | Final concentration | Amount (for 100 mL) |
|---------|---------------------|---------------------|
| DMEM (1X) + GlutaMax | N/A | 100 mL |

Keep sterile and store at 4°C for up to 1 month.
**Fixation solution for HEp-2 cells**

| Reagent                              | Final concentration | Amount (for 1 plate) |
|--------------------------------------|---------------------|----------------------|
| Methanol                             | 98%                 | 18.7 mL              |
| Hydrogen peroxide solution           | 2%                  | 1.3 mL               |

Prepare freshly.

**RSV resuspension buffer**

| Reagent                              | Final concentration | Amount (for 10 mL) |
|--------------------------------------|---------------------|--------------------|
| Phosphate buffer solution (no calcium, no magnesium) | N/A                 | 9 mL               |
| Fetal bovine serum, qualified        | 10%                 | 1 mL               |

Keep sterile and store at 4°C for up to 1 month.

**0.2 μm filtered 20% sucrose (w/v) solution**

| Reagent                              | Final concentration | Amount (per well) |
|--------------------------------------|---------------------|-------------------|
| Sucrose                              | 20%                 | 100 g             |
| Phosphate buffer solution (no calcium, no magnesium) | N/A                 | 500 mL            |

Keep sterile and store at 20°C for up to 3 months.

**Enzyme dissociation mix for lymph nodes**

| Reagent                              | Final concentration | Amount (for 1 sample) |
|--------------------------------------|---------------------|-----------------------|
| Dulbecco’s PBS, calcium, magnesium   | N/A                 | 15 mL                 |
| Collagenase D                        | 0.36 mg/mL          | 5.4 mg                |
| Deoxyribonuclease I                  | 0.1 mg/mL           | 1.5 mg                |

Prepare freshly.

**Dulbecco’s PBS solution (no calcium, no magnesium) containing EDTA and antibiotics**

| Reagent                              | Final concentration | Amount (for 1 liter) |
|--------------------------------------|---------------------|----------------------|
| Sterile water                        | N/A                 | 1,000 mL             |
| Dulbecco’s PBS, no calcium, no magnesium (10×) | N/A                 | 110 mL               |
| UltraPure™ 0.5 M EDTA, pH 8.0 (500×)  | 1 mM                | 2.2 mL               |
| Penicillin-Streptomycin (10,000 U/mL) | 100 units/mL        | 11 mL                |

Keep sterile and store at 4°C for up to 1 month.

**Enzyme dissociation mix for lung tissue**

| Reagent                              | Final concentration | Amount (for 1 piece) |
|--------------------------------------|---------------------|----------------------|
| Dulbecco’s PBS, calcium, magnesium   | N/A                 | 10 mL                |
| Collagenase I                        | 1.25 mg/mL          | 12.5 mg              |
| Collagenase II                       | 2.5 mg/mL           | 25 mg                |
| Deoxyribonuclease I                  | 0.15 mg/mL          | 1.5 mg               |

Prepare freshly.

**Culture media for lymph nodes / bronchoalveolar lavage / lung tissue isolated cells**

| Reagent                              | Final concentration | Amount (per well) |
|--------------------------------------|---------------------|-------------------|
| DMEM (1×) + GlutaMax                 | N/A                 | 4.5 mL             |
| Fetal bovine serum, qualified        | 10%                 | 500 μL             |
| Brefeldin A Solution (1000×)         | 10 μg/mL            | 15 μL              |

Prepare freshly.
STEP-BY-STEP METHOD DETAILS
Preparation of cell suspensions of ovine peribronchial lymph nodes

© Timing: 2–3 h

This step describes how to prepare a single-cell suspension of LNs using an automated tissue dissociator. This step should be performed inside a biosafety cabinet.

1. Transfer the LNs to 2 mL of enzyme dissociation mix (refer to materials and equipment for detailed recipe).
   a. Remove the fat tissue.
   b. Cut the whole LN with surgical scissors into pieces.
   c. Transfer into a gentleMACS C tube.
2. Place the tubes in the gentleMACS Octo Dissociator, proceed two times with the spleen program: [59 s, rpm: 1260].
3. Add 13 mL of enzyme dissociation mix. Place the tubes in the gentleMACS Octo Dissociator, proceed to the 37C_m_LDK_1 program: [31 min:13 s, temp: ON, rpm: 2844].

Note: Alternatively, the cell suspension can be obtained by mincing the tissue with surgical scissors prior enzymatic dissociation. However, this approach is significantly less efficient and more harmful for the cells compared to the use of an automated tissue dissociator.

Note: The Dulbecco’s PBS supplemented with 1 mM EDTA in the subsequent steps contains no calcium, no magnesium.

4. Pass the suspension through a 100 μm cell strainer, placed in a 50 mL conical bottom tube, with light pressure using the rubber end of a syringe plunger.

Note: Leave to stand for 30 s and remove the small clumps which settle to the bottom of the tube.

5. Rinse cell strainer with some Dulbecco’s PBS. Fill the tube up to 50 mL.
6. Centrifuge the cell suspension 10 min at 350 g, 4°C. Resuspend the pellet in 1–2 mL cold (4°C) Dulbecco’s PBS. Then, once the cells are resuspended homogeneously, make up to 20 mL per pellet with cold (4°C) Dulbecco’s PBS.
7. Pass through a 70 μm cell strainer, placed in another 50 mL conical bottom tube.
8. Pass through a 40 μm cell strainer, placed in another 50 mL conical bottom tube.
9. Rinse cell strainer with some Dulbecco’s PBS. Fill the tube up to 50 mL.
10. Centrifuge the cell suspension 10 min at 250 g, 4°C. Resuspend the pellet in 5 mL cold (4°C) DMEM supplemented with 10% FBS.
11. Count using a Neubauer chamber or an automated cell counter.

Preparation of cell suspensions of ovine lung tissue

© Timing: 3–4 h

This step describes how to prepare a single-cell suspension of immune cells from lung tissue using an automated tissue dissociator. This step should be performed inside a biosafety cabinet.

Note: One specimen per lung region (cranial, middle, and caudal) were pooled and processed (typically, one piece of lung tissue weighs 4–6 g).
12. Rinse tissue in a Petri dish containing cold Dulbecco’s PBS to remove erythrocytes.
13. Replace the Dulbecco’s PBS with 2 mL of enzyme mix (refer to materials and equipment for detailed recipe). Cut the lung tissue with scissors into pieces and transfer into a gentleMACS tube.
14. Place the tubes in the gentleMACS Octo Dissociator, proceed two times with the Spleen program: [59 s, rpm: 1260].
15. Add 8 mL of enzyme mix. Place the tubes in the gentleMACS Octo Dissociator, proceed with the 37C_m_LDK_1 program: [31 min:13 s, temp: ON, rpm: 2844].

**Note:** The Dulbecco’s PBS supplemented with 1 mM EDTA in the subsequent steps contains no calcium, no magnesium.

16. Pass through a sieve and collect into a beaker.
17. Pass the preparation through a 100 μm cell strainer, placed in a 50 mL conical bottom tube, with light pressure using the rubber end of a syringe plunger.

**Note:** Leave to stand for 30 s and remove the small clumps which settle to the bottom.

18. Rinse cell strainer with some Dulbecco’s PBS. Fill the tube up to 50 mL.
19. Centrifuge the cell suspension 10 min at 350 g, 4°C. Resuspend the pellet in 1–2 mL cold (4°C) Dulbecco’s PBS and make up to 20 mL per pellet with cold (4°C) Dulbecco’s PBS.
20. Pass through a 70 μm cell strainer, placed in another 50 mL conical bottom tube. Rinse cell strainer with some Dulbecco’s PBS. Fill the tube up to 50 mL.
21. Pass through a 40 μm cell strainer and transfer to another 50 mL tube.
22. Centrifuge the cell suspension 250 g/10 min/4°C. Resuspend the cells in 13 mL of DMEM supplemented with 10% FBS. Put 10 mL of Ficoll-Paque (density 1.077 g/mL) into fresh 50 mL conical bottom tubes. Tilt the tubes and overlay the 13 mL of single-cell suspension from ovine lungs carefully onto the Ficoll-Paque.
23. Centrifuge the Ficoll density gradient 25 min at 800 × g, at room temperature (20°C).
24. After centrifugation, collect the desired ring of PBMCs into 50 mL falcon tubes and fill up to 50 mL with cold Dulbecco’s PBS.
25. Centrifuge 10 min at 250 g, at 4°C. Resuspend the pellet in 20 mL cold (4°C) DMEM containing 10% FBS.
26. Count using a Neubauer chamber or an automated cell counter.

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**Preparation of cell suspensions from bronchoalveolar space**

**Timing:** 2–3 h

This step describes how to prepare a single-cell suspension from immune cells isolated from the bronchoalveolar space through bronchoalveolar lavages (BALs) of sheep lung as illustrated in Figure 4. This step is performed just after euthanasia of the animal. The staff present is equipped with appropriate personal protective equipment, including a Tyvek coverall and hood (both 3 M), and a Jupiter air respirator equipped with an HEPA filter system (3 M). Once the BALs are isolated, this step should be performed inside a biosafety cabinet.

△ **CRITICAL:** The lungs are isolated with the trachea, which is clamped before cutting, to prevent blood from entering the lungs. Then, Dulbecco’s PBS solution (no calcium, no magnesium) containing EDTA and antibiotics is poured through the trachea using a sterile funnel. Depending on the BAL volume, single-cell suspensions are distributed in the appropriate number of 50 mL conical bottom tubes (total volumes collected typically: 150–200 mL for neonatal lambs; 2–3 L for ewes).

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Figure 4. Bronchoalveolar lavage procedures
(A) Material required for the isolation of BAL: metal stand (1), funnel holder (2), Dulbecco’s PBS solution (no calcium, no magnesium) containing EDTA and antibiotics (3), plastic tray (4), funnel (5), surgical clamp (6), Sterile glass bottle (7) and one sterile beaker (8). The volume of Dulbecco’s PBS solution is for the respective number of lungs processed at this specific necropsy day.
(B) The trachea is clamped with a surgical clamp and the lungs are hanged using the funnel holder.
(C) Dulbecco’s PBS at room temperature is poured through the trachea using a sterile funnel. Once the lungs are full of PBS, gently massage the lungs for 20–30 s and pour the BAL inside a sterile beaker. Transfer the BAL from the beaker into the sterile glass bottle for safe transportation to the laboratory equipped with biological safety cabinet.
27. Pour the required volume of Dulbecco’s PBS in the lungs through a sterile funnel. Gently massage the lungs for 20–30 s. Pour the lungs content inside a sterile beaker Figure 4.

△ CRITICAL: Use Dulbecco’s PBS at room temperature rather than cold Dulbecco’s PBS to avoid any undesired lung tissue constriction. The Dulbecco’s PBS supplemented with 1 mM EDTA in the subsequent steps contains no calcium, no magnesium.

Note: At this step, to avoid damage to the lung tissue, invert the clamped lung a few times rather than rigorous kneading (repeat lavage with 100 mL as many times as required).

28. Distribute the cells in suspension in 50 mL conical bottom tubes. Centrifuge 10 min at 350 g, at 4°C. Resuspend the pellet in 1–2 mL cold (4°C) Dulbecco’s PBS.

29. Combine 4 tubes into a new 50 mL conical tube (to get rid of any fat, contaminant, or mucus). Centrifuge 10 min at 350 g, at 4°C.

Note: For adult animals, larger volumes of Dulbecco’s PBS are required. Repeat step 3 as many times as needed.

30. Resuspend the cell pellets, pass through a 100 μm cell strainer and centrifuge at 350 g for 10 min, 4°C.

31. Resuspend The pellets are, pass through a 70 μm cell strainer and centrifuge at 350 g for 10 min, 4°C.

Note: If needed, erythrocytes can be lysed by resuspending the pellet with 1–2 mL of H2O (up and down during a few seconds). Then wash immediately with 48 mL of cold (4°C) Dulbecco’s PBS and centrifuge 10 min at 350 g, 4°C. Alternatively, an ammonium chloride lysis of erythrocytes can be performed.

32. Resuspend the pellet in 15 mL cold (4°C) DMEM containing 10% FBS.

33. Count using a Neubauer chamber or an automated cell counter.

Flow cytometry acquisition

⊙ Timing: 6–7 h for staining

⊙ Timing: 4–5 h for acquisition

This step describes how the different T cell subsets were identified by FCM using an eight-step, seven-color assay (Table 1). This procedure should be performed inside a biosafety cabinet until step “Fixation and Permeabilization”. The experimental timeline of a typical necropsy day is summarized in Figure 5.

Note: As this protocol implies the handling of a large number of samples, we recommend having enough people in the team. The necropsy and organ collection requires at least 5–6 people. Also, a minimum of 4 people are required for organ dissociation, preparation of cell suspensions and cell counting. Finally, the multiparameter FCM procedure can be performed by a single researcher. Notably, upon storage of the stained cells at 4°C, the FCM acquisition can be performed the following day.

△ CRITICAL: We strongly recommend performing the assay on freshly isolated cells. Indeed, cryopreservation induces significant cell death, renders the cells quiescent rather than cytokine producing cells, as well as modulates the expression levels of cell surface canonical markers.
34. Once all the samples have been processed to single cell suspension and are stored on ice:
   a. Count the cells and resuspend all samples in DMEM, 10% FBS supplemented with Brefeldin A (10 μg/mL), at a density of $2 \times 10^6$ cells/mL.
   b. Incubate 5 mL of cell suspension ($10^3 \times 10^6$ cells) per well in 6-well plates for 5 h.
35. Then, transfer 1 mL ($2 \times 10^6$ cells) per staining condition into microtiter tubes.
36. Centrifuge 7–8 min at 350 g, 4°C.
37. Incubate the cell pellets with 100 μL LIVE/Dead Aqua, 20 min on ice (step “Live/Dead Aqua”, Table 1). Wash with 800 μL cold Dulbecco’s PBS without calcium and magnesium, centrifuge 7–8 min at 350 g, 4°C.
38. Incubate the cell pellets with 50 μL unconjugated primary antibodies, 15 min on ice (step “Surface 1”, Table 1). Wash with 800 μL Cell Wash, centrifuge 7–8 min at 350 g, 4°C.
39. Incubate the cell pellets with 50 μL conjugated secondary antibodies, 15 min on ice (step “Surface 2”, Table 1). Wash with 800 μL Cell Wash, centrifuge 7–8 min at 350 g, 4°C.
40. Perform an Ig blocking using 25 μL ChromPure mouse IgG, 5 min on ice (step “IgG block”).
41. Proceed directly by adding 25 μL conjugated antibodies, let the cells incubate 15 min on ice (step “Surface 3”, Table 1). Wash with 800 μL Cell Wash, centrifuge 7–8 min at 350 g, 4°C.
42. Incubate the cell pellets with 100 μL Fixation/Permeabilization solution, 25 min on ice (step “Fix & Perm”, Table 1). Wash with 800 μL cold BD Perm/Wash™ Buffer, centrifuge 7–8 min at 350 g, 4°C.
43. Incubate the cell pellets with 50 μL unconjugated primary antibodies, 15 min on ice (step “Intracellular 1”, Table 1). Wash with 800 μL cold BD Perm/Wash™ Buffer, centrifuge 7–8 min at 350 g, 4°C.
44. Incubate the cell pellets with 50 μL conjugated secondary antibodies, 15 min on ice (step “Intracellular 2”, Table 1). Wash with 800 μL cold BD Perm/Wash™ Buffer, centrifuge 7–8 min at 350 g, 4°C.
45. Resuspend the pellet in 70 μL cold BD Perm/Wash Buffer. At this step, you can either proceed to the sample acquisition or either store the tube holder overnight at 4°C.

*This sample compensation matrix applies to all series of tubes.

### Table 1. Staining procedure using an eight-step, seven-color assay

| Subset | Live/Dead Aqua | Surface 1 | Surface 2 | IgG block | Surface 3 | Fix & Perm | Intracellular 1 | Intracellular 2 |
|--------|----------------|-----------|-----------|-----------|-----------|------------|----------------|----------------|
| γδ T   | +              | CD8-IgG1  | IgG1-PE-Cy7 | +         | WC1-IgG1-FITC | +           | IL17A-IgG1-PE | -              |
| Th2/Tc2| +              | CD4-IgG2a | IgG2a-APC-Cy7 | +         | CD45RO-IgG3-PE | +           | IL-4-IgG2a-FITC | -              |
| Tregs  | +              | CD25-IgG3 | IgG3-PE-Cy5 | +         | CD45RO-IgG3-PE | +           | TGF-β-IgG1     | IgG1-PE-Cy7 |

Compensations:

- Unstained
- AF488
- PE
- PE-Cy5
- PE-C
- AF647
- APC-Cy7
- BV421
- Live/Dead Aqua

Compensation for Th2/Tc2:

- BD Perm/Wash

**Note:** The following steps can be performed outside of a biosafety cabinet.
Figure 5. Flow chart depicting the experimental timeline from euthanasia to sample acquisition by FCM

LN = lymph nodes; BAL = Bronchoalveolar lavage. Illustration created with BioRender.com.
When the intracellular staining involves directly labeled antibodies (such as IL-4-IgG2a-FITC and IFN-γ-IgG1-AF647 for “Th2/Tc2” identification), then skip step 10 to proceed directly to sample resuspension.

After cell resuspension, the content of 1.2 mL Microtiter tubes must be transferred into 5 mL FCM tube before acquisition on a FACS Canto II (BD Bioscience) or any other 6 colours flow cytometer using the BD FACSDIVA software (or any other proprietary software) and further analyzed with FlowJo (TreeStar).

Note: BD FACSDiva software, used to acquire the samples, can export data files in FCS 3.0 default formats. FCS 3.0 files can be analyzed by other software applications, including FlowJo™.

EXPECTED OUTCOMES

The goal of the present protocol is to provide a solid basis to analyze the pulmonary T cell profiles following RSV infection in lamb and adult sheep. We also describe the preparation of highly concentrated RSV to be inoculated in sheep for a large-scale animal experimentation. The main strength of this experimental approach is the ease sampling and the full accessibility to immune cell subsets in the distal lung throughout the course of RSV disease by performing necropsy at different time post-infection, while sampling in infants is mainly restricted to the upper airways, the latter representing only an approximate surrogate of the distal lung where RSV disease occurs. The subsequent cell suspensions collected from BALs, lung tissues and draining LN can be stained with our multiparameter FCM assay for a precise characterization of a large fraction (WC1+) of γδ T cells, CD4+ and CD8+ T cells, and Tregs (Figure 6). The acquired knowledge is applied in distinct research fields such as neonatal immunity and rationalization of new vaccine designs against RSV (still challenging after five decades). Also, this experimental approach is of a high potential to provide new insight in other human respiratory virus infections, such as parainfluenza virus (Radi et al., 2010).

LIMITATIONS

The number of antibodies specific for or that cross-react with ovine cells is limited, with most of them available only in unconjugated form. Thus, although the newborn lamb is a classical model of human lung development and a translational model of RSV infection, one cannot envisage the same precise delineation of immune cell subsets that the one currently performed in mouse and human. Also, to run an animal trial in large animals requires specialized facilities and an experienced staff including at least one veterinarian.

TROUBLESHOOTING

Problem 1
Insufficient number of people involved to process the organs, insufficient laboratory infrastructure to deal with the high number of tubes (steps 1–30).

Potential solution

- Make sure that a clear role is allocated to all the staff involved (either responsible for all samples of a given animal, or responsible for a given type of samples such as BAL, etc.).
- Make sure that the required facility is fully available (biosafety cabinets, centrifugations, bench, etc.).
- Facilitate the organization by preparing as much as possible the weeks before necropsy dates (tubes with enzyme powder, box containing all the material for necropsy, clear labeling of collecting tubes with stickers, tubes containing Ficoll-Paque or Dulbecco’s PBS, etc.).

Problem 2
Contamination of processed samples (step 31).
Whenever possible, carry out the whole procedure inside a biosafety cabinet. All items placed inside the biosafety cabinet must be sprayed with 70% ethanol.
All the workbench surfaces must be sprayed with 70% ethanol. Instruments such as scissors and forceps can be soaked in 70% ethanol for 20 min prior use and stored in 70% ethanol between uses. It is recommended to air-dry the instruments before use. Filter sterilize the solutions and work aseptically. We recommend adding antibiotics to the Dulbecco’s PBS used to collect the samples in the necropsy room.

Problem 3
Due to the high number of tubes, there is a high risk to mix up samples as well as introducing a mistake in a specific mix (steps 31–42).

Potential solution
- Prepare all the antibody mixes in advance with appropriated buffers, during the 5 h incubation time with Brefeldin A.
- Tick in the experimental template each time an antibody and/or buffer is added to a mix.
- Leave at least one empty row on the tube holder between series and always put back at the same place on the tube holder after vortexing, centrifugation, etc.
- Number the tubes and be sure to rigorously keep the same order along the whole staining procedure.
- Discard the antibody mix tubes just after use, put back Dulbecco’s PBS or Cell Wash in the fridge just after use, to minimize the risk of an error.

Problem 4
No clear in vivo immune activation is observed, which is possible since in vitro restimulation is not preformed (step 42).

Potential solution
- Make sure that the flow cytometer is properly calibrated.
- Design the animal groups for the necropsy dates to have at least one control uninfected animal; in the unlikely case that the flow cytometer shows some instability (voltage, fluidic) between acquisition dates, this ensures the possibility to relate the results observed in RSV-infected animals to values measured in the same conditions in control animals.
- Redo compensations control for all acquisition dates, to ascertain that all the fluorochromes are correctly detected by the machine. Ideally, to keep consistent with the measurement done at previous time-point we recommend using the same parameters (PMT voltages for all channels) for all acquisition series.
- After saving the compensation matrix, have a brief look with a few samples to ascertain that all cell phenotypic markers are satisfactory detected in the expected gated region.

Problem 5
The acquisition step involves more than 100 tubes and lasts up to six hours. Thus, it is very likely that at one point a sample fails to display any events, despite cell pellet was easily visible at all staining steps (step 42).

Potential solution
- Verify that the FCM tube is not cracked, in this case transfer the cell solution into a new FCM tube.
- Verify that cells are not clumped. If it is the case, then proceed to a vigorous treatment with vortex to achieve resuspension and filter them through a 100 µm cell strainer.
- Proceed to a cleaning procedure by placing a tube with 3 mL of 10% bleach and acquire at high flow rate for 5 min. Afterward, acquire a tube with 3 mL of deionized water at high flow rate for 5 min. However, this is specific for each instrument: some can invert the flow and others can detect
partial blockages or bubbles. If a partial blockage is suspected (e.g., FSC/SSC or fluorescence MF shift), SSC or fluorescence signals over time can be monitored to verify interruptions in the signal.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Marco P. Alves (marco.alves@vetsuisse.unibe.ch).

**Materials availability**
The study did not generate new unique reagents.

**Data and code availability**
No data or code was generated or analyzed in this protocol.

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**AUTHOR CONTRIBUTIONS**
Conception & design, T.D., N.R., and M.P.A. Data acquisition, T.D., M.B., B.Z., and B.I.O.E. Data analysis & interpretation, T.D. and M.P.A. Drafting the manuscript, T.D. and M.P.A. Final approval of the manuscript, all authors.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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