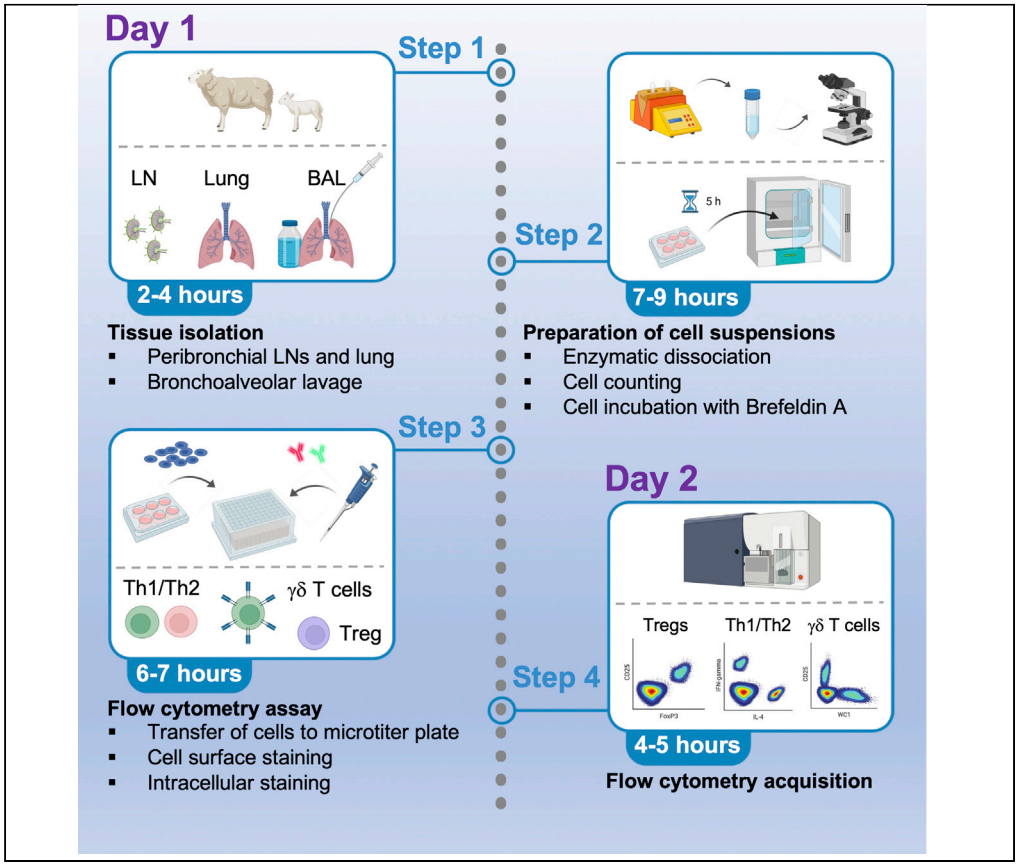
STAR Protocols

CellPress
OPEN ACCESS

Protocol

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



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.

Thomas Démoulins, Melanie Brügger, Beatrice Zumkehr, Blandina I. Oliveira Esteves, Nicolas Ruggli, Marco P. Alves

thomas.demoulins@vetsuisse.unibe.ch (T.D.)
marco.alves@vetsuisse.unibe.ch (M.P.A.)

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

Démoulins et al., STAR Protocols 3, 101688 December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101688

STAR Protocols

CellPress
OPEN ACCESS

Protocol

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

Thomas Démoulins,[1,2,3,4,*] Melanie Brügger,[1,3] Beatrice Zumkehr,[1,3] Blandina I. Oliveira Esteves,[1,3] Nicolas Ruggli,[1,3] and Marco P. Alves[1,3,5,*]

[1]Institute of Virology and Immunology, Bern, Switzerland

[2]Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland

[3]Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

[4]Technical contact

[5]Lead contact

*Correspondence: thomas.demoulins@vetsuisse.unibe.ch (T.D.), marco.alves@vetsuisse.unibe.ch (M.P.A.)
https://doi.org/10.1016/j.xpro.2022.101688

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 Démoulins 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, Veterinärdienst 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.

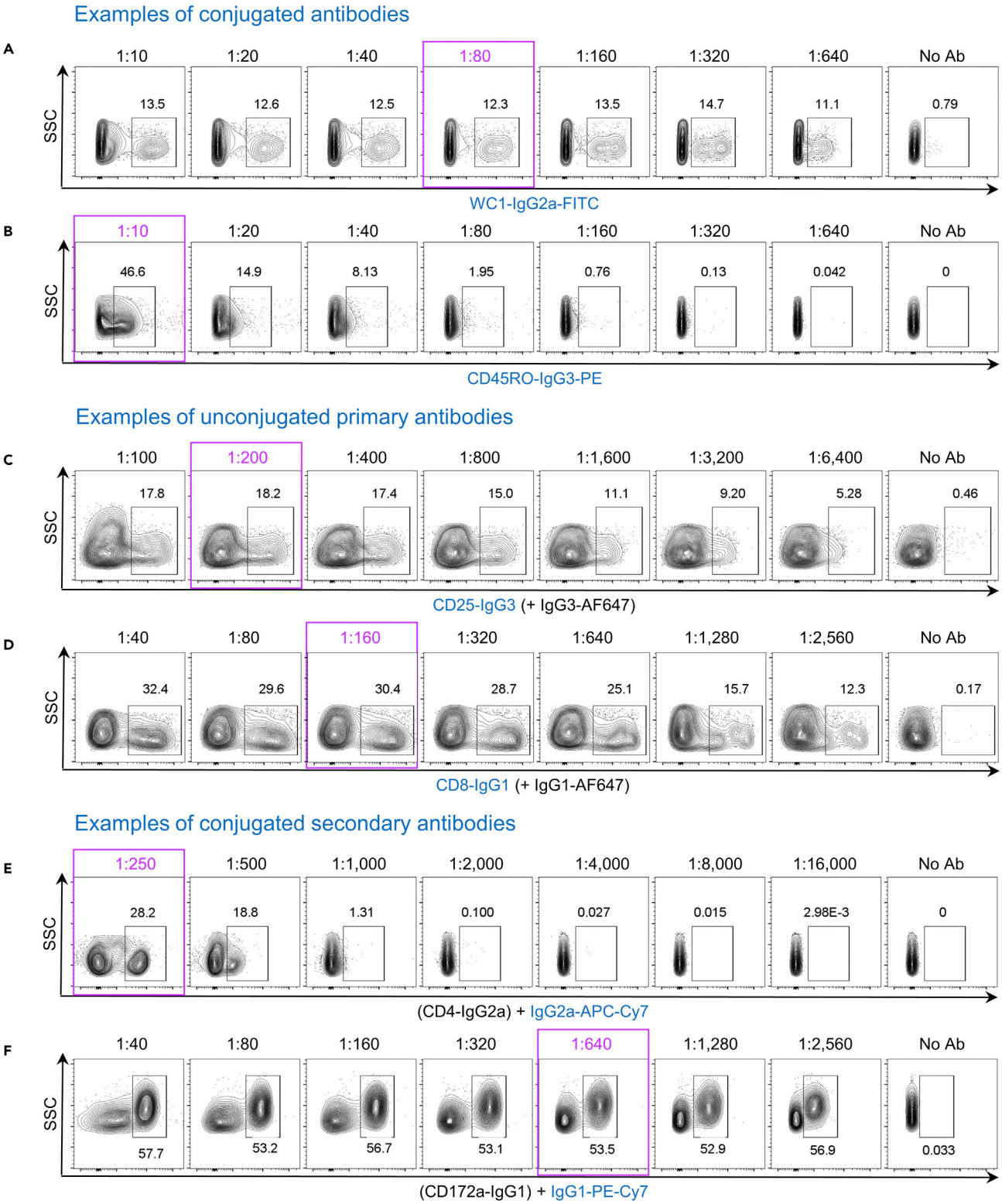


Figure 1. Antibody titration and validation

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 μg/mL.

Figure 1. *Continued*
(B) CD45RO-IgG3-PE, the manufacturer does not provide the concentration corresponding to the recommended dilution 1:10.
(C) CD25-IgG3: recommended dilution 1:100 corresponds to 10 μg/mL.
(D) CD8-IgG1: recommended dilution 1:40 corresponds to 25 μg/mL.
(E) IgG2a-APC-Cy7: recommended dilution 1:250 corresponds to 1 μg/mL.
(F) IgG1-PE-Cy7: recommended dilution 1:40 corresponds to 5 μg/mL. Highlighted are the working dilutions that were defined for further use in the study.

⚠ 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 7×10^6 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.

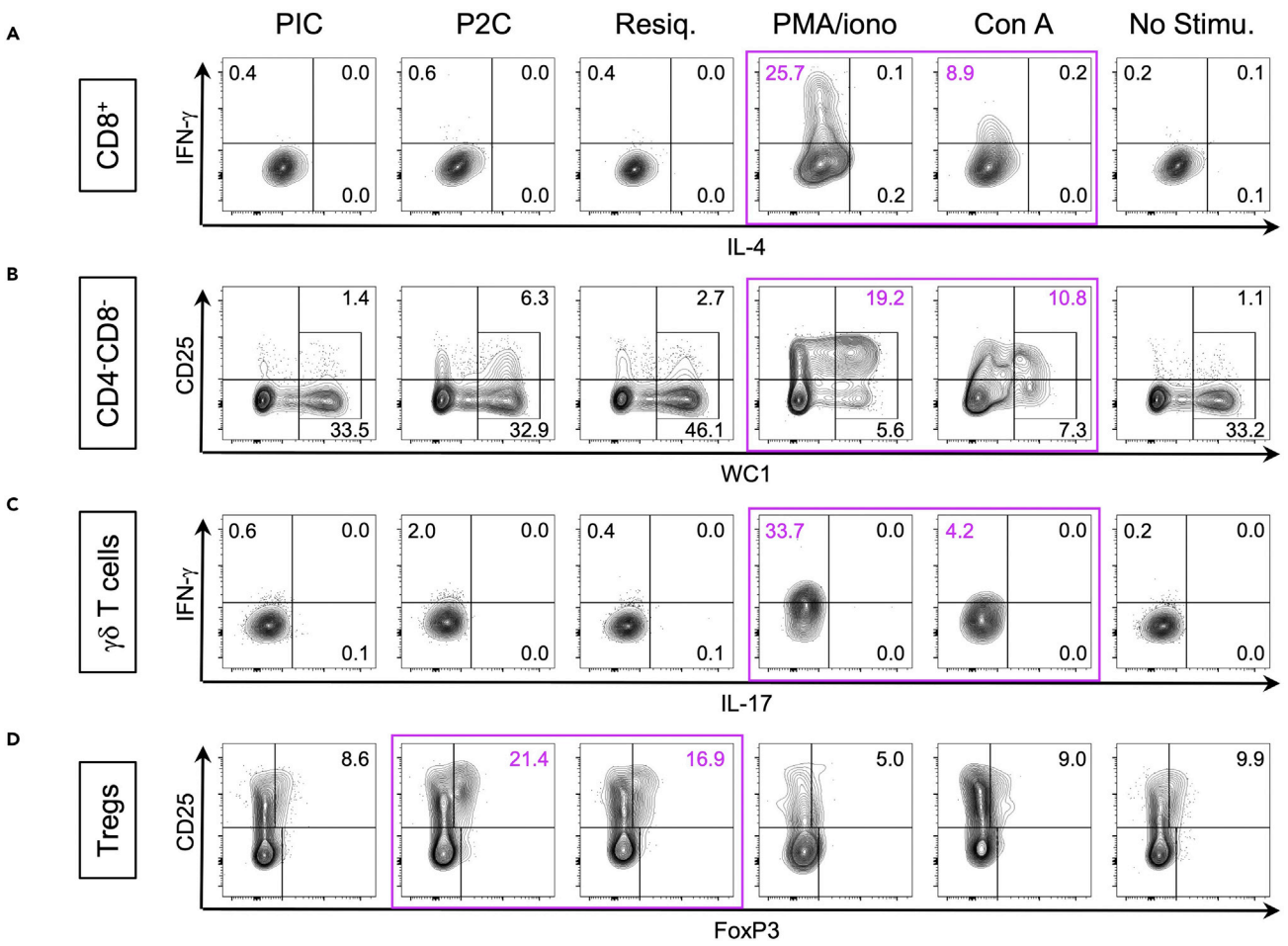


Figure 2. *Ex vivo* stimulation of peripheral blood mononuclear cells by toll-like receptor ligands or mitogens

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.

3. Leave for 24 h at 37°C, 5% CO_2 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% CO_2 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.

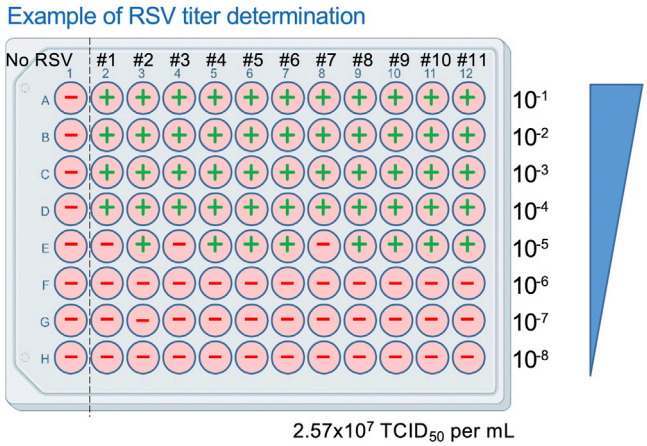


Figure 3. RSV titers are determined by a PFU assay

Here is shown the titration plate layout for one RSV stock preparation. Illustration created with BioRender.com.

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% CO_2.
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% CO_2.
19. Add 100 μL of growth medium (refer to materials and equipment for detailed recipe) and incubate for 48 h at 37°C, 5% CO_2.
20. Gently wash the cells once with 100 μL Dulbecco's PBS per well.
21. 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).
22. Wash the cells twice with 100 μL Dulbecco's PBS per well.
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. Add 50 μL per well of 3,3′-diaminobenzidine (DAB) substrate; leave for 30–120 min at room temperature (20°C).
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. Add 100 μL Dulbecco's PBS per well to prevent the cells drying.
30. 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.
31. Calculate RSV titers as follows: RSV [PFU/mL] = (number of plaques × 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 10^7 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 (10×)	Gibco	Cat# 14200067
Dulbecco's PBS, calcium, magnesium (1×)	Gibco	Cat# 14040-174
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat# 15140-122
DMEM (1×) + GlutaMax	Gibco	Cat# 32430-027
Fetal bovine serum, qualified	Gibco	Cat# 10270
UltraPure™ 0.5 M EDTA, pH 8.0 (500 ×)	Thermo Fisher Scientific	Cat# 15575020
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	Cat# 25300054
Brefeldin A Solution (1000×)	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[a]	Sigma-Aldrich	Cat# 34860

(*Continued on next page*)

Continued

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™	BD Biosciences	Cat# 554714
Ficoll® Paque Plus	Cytiva / Merck Millipore	Cat# 17-1440-03
ChromPure Mouse IgG, whole molecule	Lubio Kingfisher	Cat# 015-000-003
Midazolam	Roche	Cat# Dormicum®
Butorphanol	Dr. E. Graeub AG	Cat# Morphasol®
Experimental models: Cell lines		
HEp-2 cells	ATCC	Cat# CCL-23
Antibodies		
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-FOXP3-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, a647 (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# 406612
Anti-mouse IgG1, PE-Cy7 (Clone RMG1-1; rat IgG; 1:640 dilution)	BioLegend	Cat# 406614
Anti-mouse IgG1, APC-Cy7 (Clone RMG1-1; rat IgG; 1:40 dilution)	BioLegend	Cat# 406620
Anti-mouse IgG1, BV421 (Clone RMG1-1; rat IgG; 1:80 dilution)	BioLegend	Cat# 406616
Anti-Mouse IgG3, PerCP-Cy5.5 (Goat IgG; 1:200 dilution)	SouthernBiotech	Cat# 1100-13

(*Continued on next page*)

Continued

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 cm^2 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 BioWizard Golden GL-130	Kojair	Cat# 027407
Flow cytometry system FACSCanto II	BD Biosciences	N/A
CO_2 incubator ICO	Memmert	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

[a]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.

[b]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 (1×) + 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 (1×) + 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 gentleMACsTM 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.

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).

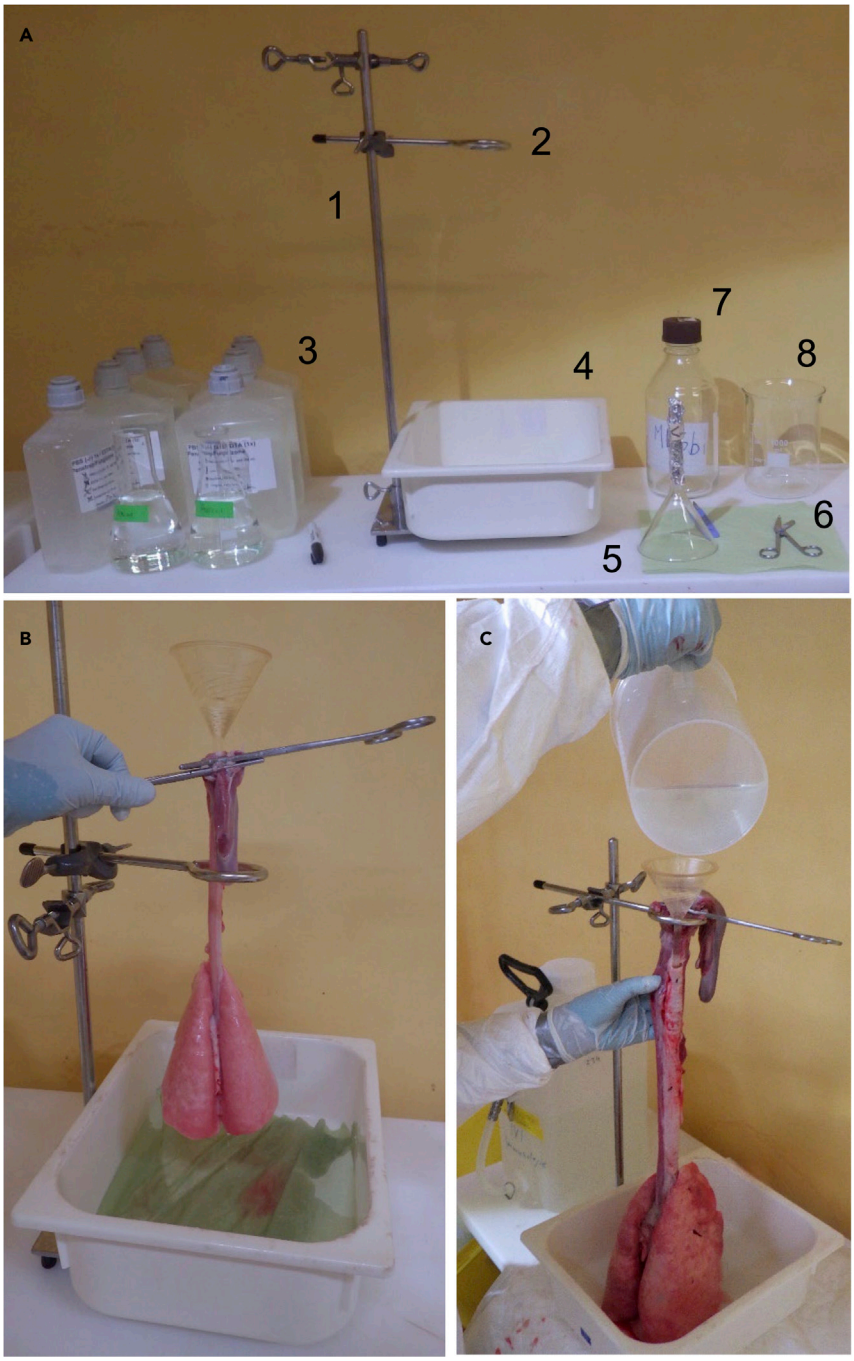


Figure 4. Bronchoalveolar lavage procedures

(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 H_2O (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.

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 CD4-IgG2a CD25-IgG3	IgG1-PE-Cy7 IgG2a-APCy-Cy7 IgG3- PE-Cy5	+	WC1-IgG1-FITC	+	IL17A-IgG1-PE IFN-γ-IgG1-AF647	-
Th2/Tc2	+	CD8-IgG1 CD4-IgG2a CD25-IgG3	IgG1-PE-Cy7 IgG2a-APC-Cy7 IgG3- PE-Cy5	+	CD45RO-IgG3-PE	+	IL-4-IgG2a-FITC IFN-γ-IgG1-AF647	-
Tregs	+	CD4-IgG2a CD25-IgG3	IgG2a-APC-Cy7 IgG3- PE-Cy5	+	CD45RO-IgG3-PE	+	TGF-β-IgG1 IL10-IgG2b	IgG1-PE-Cy7 IgG2b-AF647 FOXP3-IgG2a-AF488
Compensations[a]								
Unstained	-	-	-	-	-	+	-	-
AF488	-	CD8-IgG1	IgG1-AF488	-	-	+	-	-
PE	-	CD8-IgG1	IgG1-PE	-	-	+	-	-
PE-Cy5	-	CD8-IgG1	IgG1-PE-Cy5	-	-	+	-	-
PE-C	-	CD8-IgG1	IgG1-PE-Cy7	-	-	+	-	-
AF647	-	CD8-IgG1	IgG1-AF647	-	-	+	-	-
APC-Cy7	-	CD8-IgG1	IgG1-APC-Cy7	-	-	+	-	-
BV421	-	CD8-IgG1	IgG1-421	-	-	+	-	-
Live/Dead Aqua	+ (Cells 5 min at 65°C)	-	-	-	-	+	-	-

[a]This sample compensation matrix applies to all series of tubes.

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 × 10^6 cells/mL.
    b. Incubate 5 mL of cell suspension (10 × 10^6 cells) per well in 6-well plates for 5 h.
35. Then, transfer 1 mL (2 × 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.

Note: The following steps can be performed outside of a biosafety cabinet.

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.

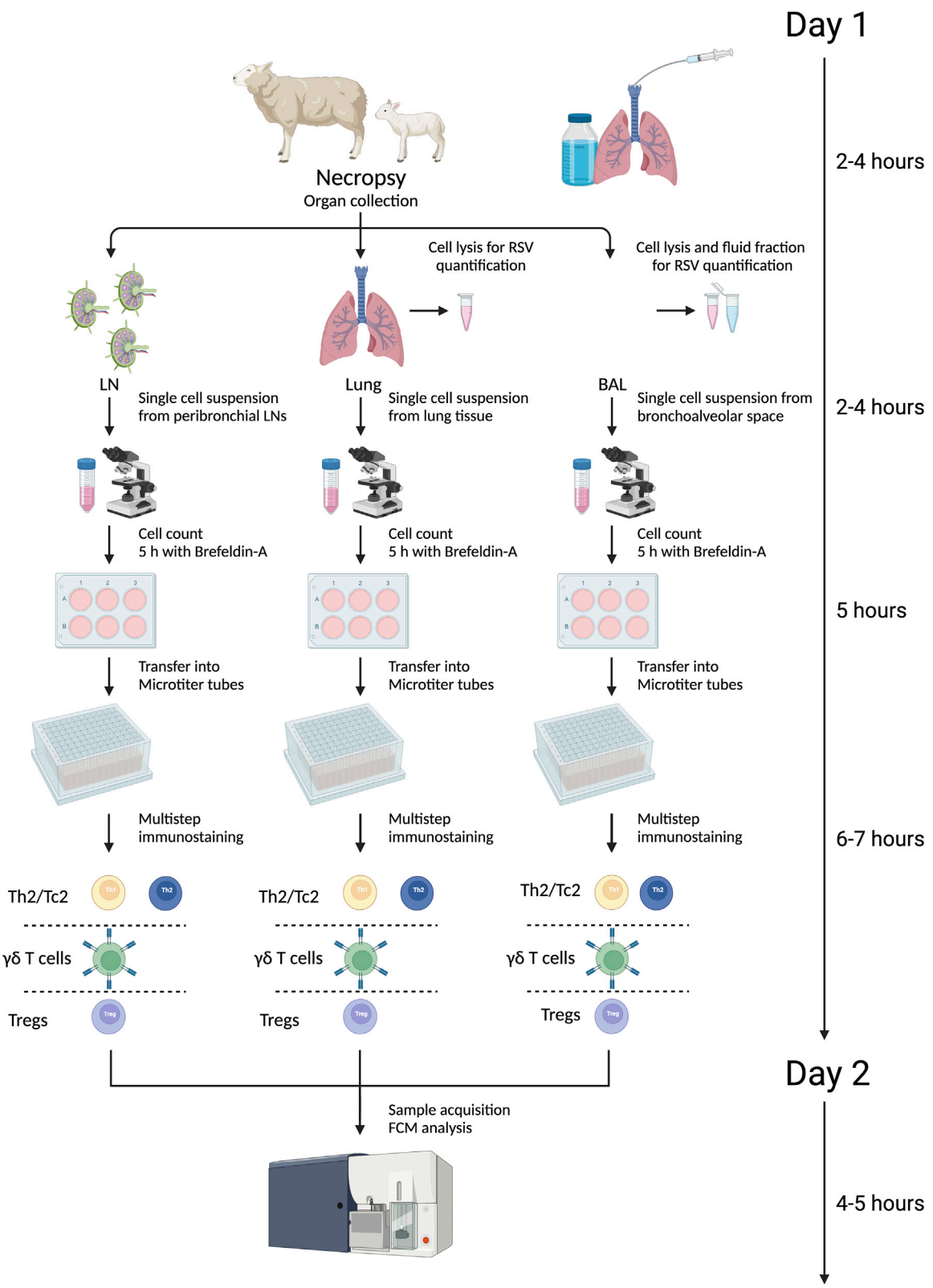


Figure 5. Flow chart depicting the experimental timeline from euthanasia to sample acquisition by FCM

LN = lymph nodes; BAL = Bronchoalveolar lavage. Illustration created with BioRender.com.

Note: 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.

Note: 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).

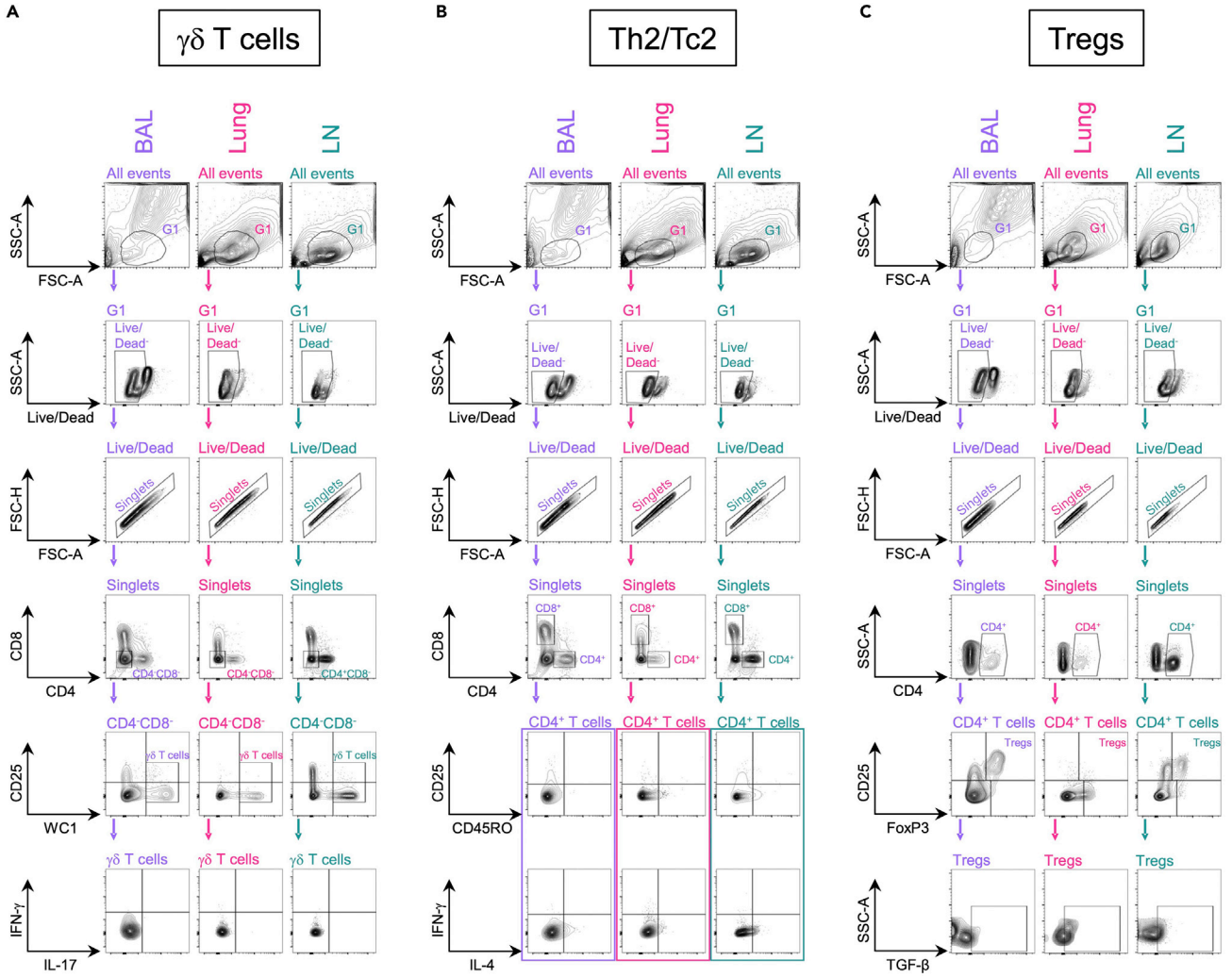


Figure 6. FCM gating strategy for components of the pulmonary T cell compartment

(A–C) Example of gating strategy for multiparameter FCM analysis to identify ovine γδ T cells (A), $CD4^+$ and $CD8^+$ T cells (B), and Tregs (C) among cell suspension isolated from BAL, lung tissue and LN cell suspensions. The neonatal lambs were infected with RSV A2 and euthanized at day 3 post-infection. (A) Phenotype of ovine γδ T cells. Gating strategy following multiparameter FCM staining uses Abs against CD4, CD8, CD25, WC1, IFN-γ and IL-17. Cells showing low forward scatter and side scatter profiles were gated. Then, the dead cell population (positive for Live/Dead) was excluded, followed by a FSC-H/FSC-A contour plot (to exclude doublets from the analysis). Then, we defined among this population the cells negative for both CD4 and CD8 markers. Among these cells, γδ T cells were defined as the $WC1^+$ cells. Maturation was evaluated based on CD25 expression, whereas activation was evaluated on the basis of IFN-γ and/or IL-17 production. (B) Phenotype of ovine T cells. Gating strategy following multicolor FCM staining uses Abs against CD4, CD8, CD25, CD45RO, IFN-γ and IL-4. Cells showing low forward scatter and side scatter profiles were gated. Then, the dead cell population (positive for Live/Dead) was excluded, followed by a FSC-H/FSC-A contour plot (to exclude doublets from the analysis). Then, we defined among this population either the cells positive for CD4 and negative for CD8 markers ($CD4^+$ T cells), either cells positive for CD8 and negative for CD4 markers ($CD8^+$ T cells). Herein, example is given for the $CD4^+$ T cell subset, where maturation was evaluated based on CD25 expression, whereas activation was evaluated based on IL-4 or IFN-γ production. (C) Phenotype of ovine Tregs. Gating strategy following multicolor FCM staining uses Abs against CD4, CD25, FoxP3 and TGF-β. Cells showing low forward scatter and side scatter profiles were gated. Then, the dead cell population (positive for Live/Dead) was excluded, followed by a FSC-H/FSC-A contour plot (to exclude doublets from the analysis). Next, we defined among this population the cells positive for CD4 marker ($CD4^+$ T cells). Among this cell subset, Tregs were defined as the $FoxP3^+CD25^+$ cells. Activation was evaluated based on TGF-β production.

Potential solution

- 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.

ACKNOWLEDGMENTS

T.D. was supported in part by the EU FP7 Project UniVax (HEALTH-F3-2013-60173). This project was funded by the Swiss National Science Foundation (grant no. 310030_172895 to M.P.A.).

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.

REFERENCES

Blockus, S., Sake, S.M., Wetzke, M., Grethe, C., Graalmann, T., Pils, M., Le Goffic, R., Galloux, M., Prochnow, H., Rox, K., et al. (2020). Labyrinthopeptins as virolytic inhibitors of respiratory syncytial virus cell entry. Antiviral Res. *177*, 104774. https://doi.org/10.1016/j.antiviral.2020.104774.

Démoulins, T., Brügger, M., Zumkehr, B., Oliveira Esteves, B.I., Mehinagic, K., Fahmi, A., Borcard, L., Taddeo, A., Jandrasits, D., Posthaus, H., et al. (2021). The specific features of the developing T cell compartment of the neonatal lung are a determinant of respiratory syncytial virus immunopathogenesis. PLoS Pathog. *17*, e1009529. https://doi.org/10.1371/journal.ppat.1009529.

Radi, Z.A., Meyerholz, D.K., and Ackermann, M.R. (2010). Pulmonary cyclooxygenase-1 (COX-1) and COX-2 cellular expression and distribution after respiratory syncytial virus and parainfluenza virus infection. Viral Immunol. *23*, 43–48. https://doi.org/10.1089/vim.2009.0042.