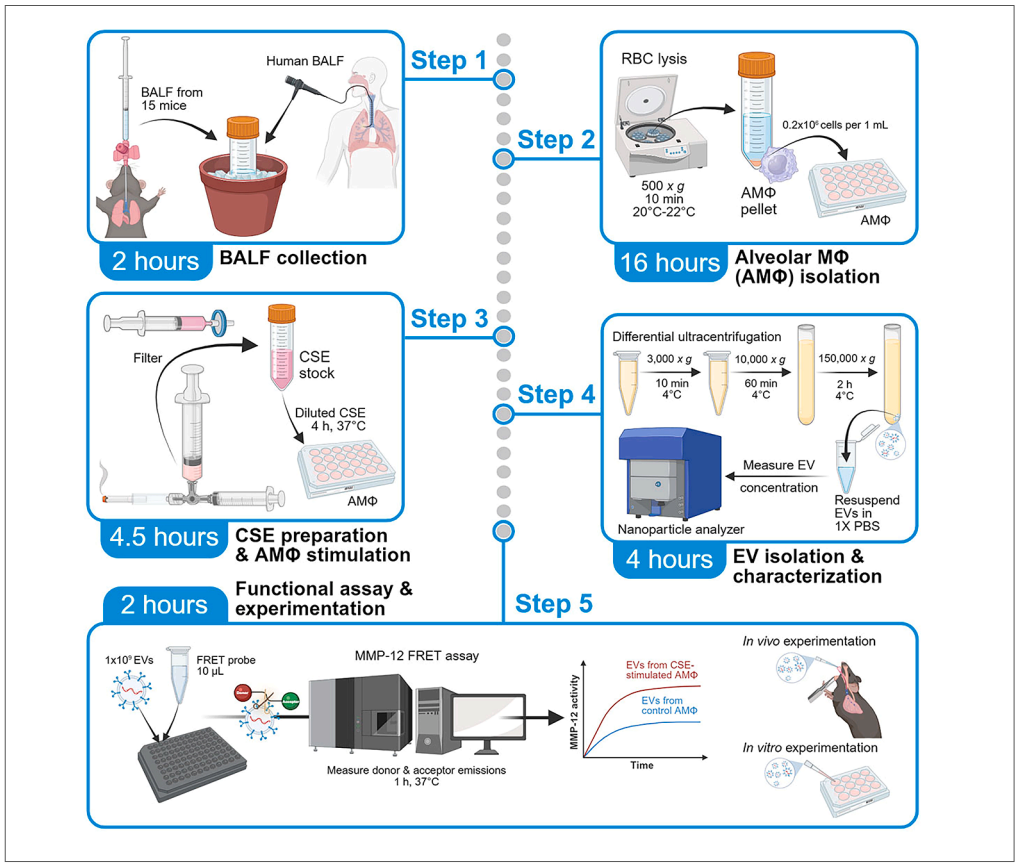
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for isolating extracellular vesicles from human or mouse airway macrophages for functional assays and *in vivo* or *in vitro* experimentation



Macrophages are key players in host defense and lung injury, and their crosstalk with other cells dictates the course of the inflammatory response and tissue remodeling. Here, we present a protocol for isolating extracellular vesicles (EVs) from human bronchoalveolar lavage fluid (BALF) and for generating EVs from primary human or murine alveolar macrophages following stimulation by cigarette smoke extract. Furthermore, we describe how to measure matrix metalloproteinase (MMP)-12 activity on macrophage-derived EVs using a Förster resonance energy transfer assay.

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

Ezgi Sari, Robert P. Richter, Delores A. Stacks, ..., Dakota C. Finley, Kristopher R. Genschmer, Camilla Margaroli

esari@uab.edu (E.S.)
cmargaroli@uabmc.edu (C.M.)

Highlights

Procedure for isolating and culturing alveolar macrophages from humans and mice

Steps to isolate EVs from bronchoalveolar lavage fluid and alveolar macrophages

Instructions for testing the MMP-12 activity on alveolar macrophage-derived EVs

Alveolar macrophage-derived EVs may be leveraged for functional experiments

Sari et al., STAR Protocols 6, 103867
June 20, 2025 © 2025 The Authors. Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2025.103867

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for isolating extracellular vesicles from human or mouse airway macrophages for functional assays and *in vivo* or *in vitro* experimentation

Ezgi Sari,[1,2,5,*] Robert P. Richter,[3] Delores A. Stacks,[2] Crystal Lewis,[2] Julian B. Smith,[4] Ningyong Xu,[4] Dakota C. Finley,[4] Kristopher R. Genschmer,[4] and Camilla Margaroli[2,6,*]

[1]University of Alabama at Birmingham, Graduate Biomedical Sciences, Birmingham, AL 35233, USA

[2]Department of Pathology, University of Alabama at Birmingham Heersink School of Medicine, Birmingham, AL 35233, USA

[3]Department of Pediatrics, University of Alabama at Birmingham Heersink School of Medicine, Birmingham, AL 35233, USA

[4]Department of Medicine, University of Alabama at Birmingham Heersink School of Medicine, Birmingham, AL 35233, USA

[5]Technical contact

[6]Lead contact

*Correspondence: esari@uab.edu (E.S.), cmargaroli@uabmc.edu (C.M.)
https://doi.org/10.1016/j.xpro.2025.103867

SUMMARY

Macrophages are key players in host defense and lung injury, and their crosstalk with other cells dictates the course of the inflammatory response and tissue remodeling. Here, we present a protocol for isolating extracellular vesicles (EVs) from human bronchoalveolar lavage fluid (BALF) and for generating EVs from primary human or murine alveolar macrophages following stimulation by cigarette smoke extract. Furthermore, we describe how to measure matrix metalloproteinase (MMP)-12 activity on macrophage-derived EVs using a Förster resonance energy transfer assay.

BEFORE YOU BEGIN

Macrophages play a critically important role in the human innate immune response, particularly within the lungs. The role of macrophage plasticity and fate in lung injury depends on their interactions with other inflammatory cells and the lung microenvironment, which collectively shape disease pathology.[1] Therefore, clarification of the granular mechanisms driving cell-to-cell crosstalk in acute and chronic lung disease is critical to advancing our understanding of the pathobiology at play and identification of novel therapeutic targets.

EVs are 50–200 nm lipid bilayer vesicles secreted from all cells that can shuttle various proteins and nucleic acids between cells.[2] EVs play a role in many cellular events, such as maintaining cellular homeostasis, immune responses, cellular communication, cell-extracellular matrix interaction, cancer formation and progression, and epithelial damage. EVs can be isolated from liquid biological specimens such as blood, urine, BALF, cerebrospinal fluid, and cell culture medium.[3–5] EVs have been identified as reliable disease biomarkers, such as in cancer or in autoimmunity, and they have also been exploited for therapeutic treatment, opening a new era of medicine.[6–8]

Isolation and characterization of quantity and quality of EVs from BALF gives an accurate representation of the lung microenvironment. Indeed, while presence of macrophage-derived and epithelium-derived EVs has been detected at homeostasis, changes in cargo of these EVs have been described in airway diseases.[9–12] On the other hand, the appearance of EVs from other inflammatory cell types, such as neutrophils and eosinophils, has been shown to be a reliable biomarker of disease

This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

progression.[9,13,14] We and others have demonstrated the important role of EVs in airway disease pathogenesis, including, but not limited to, modulation of epithelial and fibroblast activation,[12] as well as augmentation of protease activity and tissue remodeling due to increased resistance to tissue anti-proteases.[3,4]

This protocol details how to isolate primary alveolar macrophages from human and murine BALF and stimulating macrophages ex vivo using CSE for the purpose of generating macrophage-derived EVs for further experimentation (e.g., intratracheal instillation into naïve mice or treatment of human or mouse primary lung microvascular endothelial or lung epithelial cell cultures). Furthermore, we describe how to isolate EVs directly from human or mouse BALF for clinical characterization or future experimentation. Finally, we detail how to measure the enzymatic activity of MMP-12 (human macrophage elastase[15]), a well-characterized enzyme secreted by macrophages that is integrally involved in extracellular matrix remodeling in a host of inflammatory conditions,[16–19] on the surface of macrophage-derived EVs using a FRET assay to provide a functional readout of these EVs as both a biomarker of disease and confirmation of enzymatic activity prior to future experimental use.

Institutional permissions

Before proceeding, ensure that your laboratory has obtained express approval from your local Institutional Animal Care and Use Committee (IACUC) to work with mice and that each person involved in procedures involving mice has the appropriate institutional training for the care and use of mice. To work with human-derived specimens, ensure that your laboratory has obtained the appropriate approval from your local Institutional Review Board (IRB) and that all laboratory members interacting with human specimens have obtained the appropriate health and safety training according to local institutional guidelines. Data presented in this manuscript were obtained from animal studies that were performed in accordance with the Animal Welfare Act following the approval of the University of Alabama at Birmingham's IACUC (protocol 23087) and from studies involving human BALF after obtaining written, informed consent in accordance with the University of Alabama at Birmingham's IRB (protocol 161121004).

Preparation of DMEM, 10% FBS

Timing: 30 min

This step describes the preparation of DMEM, 10% v/v FBS for *in vitro* culture of primary alveolar macrophages.

1. Thaw fetal bovine serum (FBS) at 37°C.
2. In a biosafety cabinet, add 50 mL FBS to 450 mL DMEM and mix thoroughly.
3. Filter using a sterile 0.2 μm vacuum-assisted 500 mL filter bottle.
4. Store at 4°C for up to 6 months.

Note: For protection from potential contamination, aliquot 50 mL DMEM, 10% FBS into ten 50 mL sterile conical tubes and store at 4°C.

Preparation of EV-depleted 1X PBS(−/−) and DMEM (without FBS)

Timing: 1–4 h

This step describes two methods for depleting 1X PBS(−/−) or DMEM (without FBS) of residual EVs.

Note: It is essential to use EV-depleted 1X phosphate buffered saline without calcium and magnesium [PBS(−/−)] and Dulbecco's modified Eagle's medium (DMEM) during the alveolar macrophage isolation and treatment procedures in addition to the EV isolation procedure to

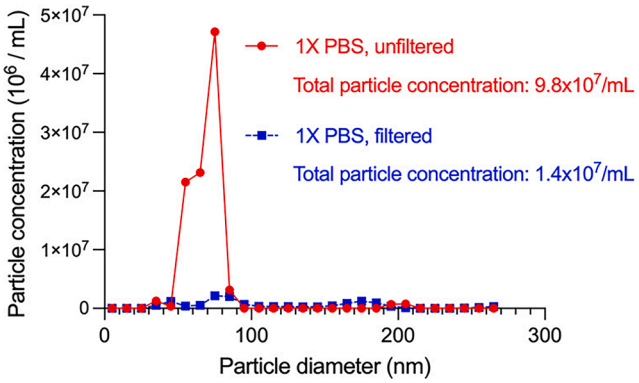


Figure 1. Effect of EV depletion on particulate concentration of buffers

ensure a pure yield of macrophage-specific EVs. Using non-EV-depleted 1X PBS(−/−) or DMEM may give a falsely elevated EV concentration reading (Figure 1).

Note: Ultracentrifugation (Step 5) is recommended when larger volumes (>20 mL) of 1X PBS or DMEM are needed, whereas the use of centrifugal filtration (Step 6) is recommended when smaller volumes (<10 mL) will be used.

⚠ CRITICAL: Following EV depletion of DMEM, DMEM must be filtered through 0.2 μm filter syringe or vacuum-assisted filter bottle before use in culture with human primary macrophages.

5. Deplete 1X PBS(−/−) or DMEM of EVs using ultracentrifugation:
   a. Centrifuge 33 mL of solution at 150,000 *x g* for 2 h using a swinging bucket ultracentrifuge with 25 x 89 mm polyallomer open-top ultracentrifuge tubes.

   Note: Ensure that the filled tubes are equivalent in mass and are inserted into the ultracentrifuge rotor in a balanced configuration.

   b. Transfer the supernatant to a sterile 50 mL conical tube, careful not to disturb the particle pellet at the bottom of the tube.
   c. Store 1X PBS at 20°C–22°C.
   d. Filter DMEM through a 0.2 μm filter syringe or vacuum-assisted filter bottle and store at 4°C.
6. Deplete 1X PBS(−/−) or DMEM of EVs using centrifugal filtration:
   a. Assemble a 4 mL 100 kDa MWCO centrifugal filter according to manufacturer specifications.
   b. Add 4 mL of the respective solution to the filter and centrifuge at 5,000 *x g* for 2 min to hydrate the filter.
   c. Discard the filtrate within the collection tube and replace the filter in the collection tube.
   d. Add 2 mL of respective solution to the filter and centrifuge at 5,000 *x g* for 5 min.
   e. Transfer the EV-depleted filtrate in the collection tube to a sterile 50 mL conical tube for future use.
   f. Store 1X PBS at 20°C–22°C.
   g. Filter DMEM through a 0.2 μm filter syringe or vacuum-assisted filter bottle and store at 4°C.

   Note: Centrifugal filters may continue to be used to deplete 1X PBS of EVs until the filter becomes clogged. We recommend single use of centrifugal filters to deplete DMEM of EVs to prevent bacterial contamination of DMEM.

Preparation of NaCl solutions for red blood cell lysis

Timing: 2 h

This step describes the preparation of NaCl solutions to lyse red blood cells (RBC) after collecting airway cells from human or mouse BALF.

7. Dissolve 0.8 g NaCl in 500 mL double-distilled water to prepare 1.6% w/v NaCl.
8. Add 62.5 mL 1.6% NaCl to 437.5 mL double-distilled water to prepare 0.2% w/v NaCl.
9. Autoclave both solutions and filter using a 0.2 μm vacuum-assisted 500 mL filter bottle.
10. Store both solutions at 4°C and use cold.

Preparation of 1X PBS(−/−), 1% v/v Tween 20 buffer for nanoparticle analysis

Timing: 10 min

11. Add 5 mL Tween 20 to 495 mL 1X PBS(−/−) and mix with a magnetic stirrer until Tween 20 dissolves.
12. For use with the Spectradyne nanoparticle analyzer bottles:
    a. Filter 480 mL PBS-T through a vacuum-assisted 0.2 μm vacuum-assisted filter bottle.
    b. Store at 20°C–22°C for up to 6 months.
13. For use during nanoparticle analysis:
    a. Filter 20 mL PBS-T using a 0.02 μm syringe filter and 20 mL Luer-Lok syringe.
    b. Store at 20°C–22°C for up to 6 months.

Note: Allow bubbles in PBS-T to resolve prior to use.

Preparation of stock solutions for the reconstitution buffer for recombinant MMP-12 and for the FRET assay buffer

Timing: 30 min

This step describes how to prepare the necessary stock solutions for the buffer to reconstitute lyophilized recombinant human MMP-12 (rhMMP-12) or recombinant mouse MMP-12 (rmMMP-12) and the buffer used in the FRET assay.

14. Dissolve 2.92 g NaCl in 50 mL molecular grade water to generate a 1 M NaCl stock solution.
15. Dissolve 1.38 g $CaCl_2$ in 50 mL molecular grade water to generate a 0.25 M $CaCl_2$ stock solution.
16. Dissolve 14.38 mg $ZnSO_4$ in 50 mL molecular grade water to generate a 1 mM $ZnSO_4$ stock solution.
17. Filter each solution using a 0.2 μm syringe filter.
18. Store at 20°C–22°C for up to 6 months.

Preparation of reconstitution buffer for recombinant MMP-12

Timing: 30 min

This step describes how to prepare a buffer that supports the enzymatic activity of both rhMMP-12 and rmMMP-12 as respective positive controls when performing the MMP-12 FRET assay using EV samples from human or mouse BALF or primary alveolar macrophages.

19. Combine 100 μL 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 1 mL 1 M NaCl, 4 μL 0.25 M $CaCl_2$, 10 μL 1 mM $ZnSO_4$, 20 μL 50% w/v polyethylene glycol (PEG), and 8.866 mL molecular grade water to generate 10 mL of a 10 mM MES, 100 mM NaCl, 100 μM $CaCl_2$, 1 μM $ZnSO_4$, 0.1% w/v PEG buffer solution.
20. Add 10 mg 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) powder to the 10 mL buffer solution prepared in Step 19 and mix thoroughly.
21. Adjust the pH of the buffer solution prepared in Step 20 to 6.0 to generate the final reconstitution buffer.
22. Filter the reconstitution buffer through a 0.2 μm syringe filter and store at 4°C for up to 6 months.

Preparation of TCNB buffer for FRET assay

Timing: 1.5 h

This step generates 10 mL of a 50 mM Tris, 10 mM $CaCl_2$, 150 nM NaCl, 0.05% Brij-35 (TCNB) buffer that is needed to prepare the FRET assay.

23. Warm 10 mL Brij-35 stock solution at 37°C for 60 min to solubilize the Brij-35.
24. Aliquot 1 mL Brij-35 stock solution into ten 1.5 mL sterile microcentrifuge tubes to facilitate warming to solubility when needed to prepare the assay buffer.
25. Combine 500 μL 1 M Tris-HCl, 400 μL 0.25 M $CaCl_2$, 1.5 mL 1 M NaCl, 17 μL Brij-35 stock solution, and 7.583 mL molecular grade water to generate 10 mL of TCNB buffer.
26. Adjust the TCNB buffer pH to 7.5.
27. Filter the TCNB buffer through a 0.2 μm syringe filter and store at 4°C for up to 6 months.

Preparation of syringes for performing mouse BALF collection

Timing: 30 min

This step describes how to prepare syringes for bronchoalveolar lavage of mice.

Note: Prepare syringes with EV-depleted 1X PBS(−/−) inside of a biosafety cabinet to limit bacterial contamination.

28. Prepare two 1 mL Luer slip tip syringes with EV-depleted 1X PBS(−/−) per mouse:
    a. Aspirate 0.5 mL EV-depleted 1X PBS(−/−) into a 1 mL syringe.
    b. Repeat Step 28a with a separate 1 mL syringe.
    c. Flick the syringes to remove all bubbles.

Note: 1X PBS(−/−), 2.5 mM EDTA, 1-2% v/v FBS may be used to perform BALF collection from mice to maintain higher alveolar macrophage viability.[20,21] However, use of FBS in BALF preparations will falsely elevate EV concentrations in the BALF. Therefore, we recommend only using 1X PBS(−/−), 2.5 mM EDTA, 2% v/v FBS for alveolar macrophage isolation and not for direct EV isolation from BALF.

Preparation of tracheostomy catheter for mouse BALF collection

Timing: 1 min

29. Carefully remove the plastic cap covering an Insyte autoguard 20-guage needle (Figure 2A).
30. Depress the white button of the needle withdrawal apparatus to safely retract the needle from the catheter (Figure 2B).

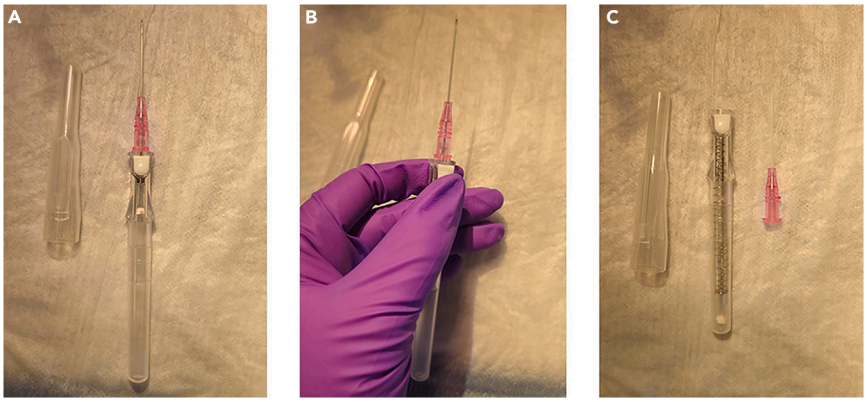


Figure 2. Preparation of the tracheostomy catheter for mouse BALF collection

(A) The plastic cap of the catheter is carefully removed.

(B) The white button of the automatic needle withdrawal apparatus is depressed to retract the needle from the catheter safely.

(C) The catheter is separated from the needle withdrawal apparatus for tracheostomy insertion.

31. Discard the retracted needle into a sharps disposal container, and retain the catheter for BALF collection (Figure 2C).

Note: The catheter length may be trimmed to facilitate easier handling during the BALF collection procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (DMEM) (alternatives available)	Corning	Cat#10-013-CV
1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)	MilliporeSigma	Cat#M1317
Polyethylene glycol (PEG), 50% (w/v) solution	Fisher Scientific	Cat#AAJ61495AE
3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) powder (alternatives available)	Fisher Scientific	Cat#BP571-1
Brij 35, 30% (w/w) aqueous wetting agent (alternatives available)	Ricca Chemical	Cat#1145-16
1 M Tris hydrochloride solution, pH 7.5 (alternatives available)	Fisher Scientific	Cat#BP1757-100
Recombinant human MMP-12 (rhMMP-12)	R&D Systems	Cat#917-MPB
Recombinant mouse MMP-12 (rmMMP-12)	R&D Systems	Cat#3467-MPB
10 mM *p*-aminophenylmercuric acetate	MedChemExpress	Cat#HY-148905
LaRee 5 powder	SiChem	Cat#SC-0043
Recombinant mouse TIMP-1 protein	R&D Systems	Cat#980-MT
Experimental models: Organisms/strains		
Mouse: C57BL/6J, 12–14 weeks, male and female	Jackson Laboratory	Cat#000664
Software and algorithms		
Analyze and Acquire software, version 3.0.0.53	Spectradyne	https://nanoparticleanalyzer.com/dlhidgen3/
Prism software, version 10.3.1	GraphPad	https://www.graphpad.com/
Cary UV workstation software	Agilent	https://www.agilent.com/en/product/molecular-spectroscopy/uv-vis-uv-vis-nir-spectroscopy/uv-vis-uv-vis-nir-software/cary-uv-workstation-software

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
SoftMax Pro 7 data acquisition and analysis software, version 7.1.2	Molecular Devices	https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software
Other		
500 mL 0.2 μm filter bottle (alternatives available)	Fisher Scientific	Cat#FB12566504
0.2 μm syringe filters (alternatives available)	Fisher Scientific	Cat#09-928-062
0.02 μm syringe filter, 25 mm diameter (alternatives available)	Fisher Scientific	Cat#09-926-13
25 × 89 mm open-top polyallomer tubes	Fisher Scientific	Cat#03-141
8 × 51 mm open-top polyallomer tubes	Seton Scientific	Cat#5009
Amicon 4 mL centrifugal filter, 100 kDa MWCO	MilliporeSigma	Cat#UFC810024
Amicon 0.5 mL centrifugal filter, 10 kDa MWCO	MilliporeSigma	Cat#UFC501096
1 mL Luer slip tip syringe	Fisher Scientific	Cat#14-955-462
10 mL Luer-Lok syringe	Fisher Scientific	Cat#B302995
20 mL Luer-Lok syringe	Fisher Scientific	Cat#22-124-967
50 mL Luer-Lok syringe	Fisher Scientific	Cat#13-689-8
Insyte Autoguard 20-guage catheter	Becton Dickinson	Cat#381434
Mouse dissecting kit	World Precision Instruments	Cat#MOUSEKIT
2 in segment of Tygon S3 E-3603 laboratory tubing, inner diameter 3/8 in	DWK Life Sciences	Cat#869791-0038
3-way stopcock	Fisher Scientific	Cat#NC9779127
Parafilm M self-sealing film	Fisher Scientific	Cat#STLPM996
6-in length forceps with serrated tip (alternatives available)	Fisher Scientific	Cat#16-100-108
Research cigarette, 3R4F	University of Kentucky	Cat#3R4F
Semi-micro spectrophotometer cell	Starna Cells	Cat#9/9-Q-10
24-well tissue culture plate	Fisher Scientific	Cat#08-772-1
200 μL extended length pipette tips	Corning	Cat#4810
Microfluidic cartridges	Spectradyne	Cat#C-400
96-well half-area, black, flat bottom polystyrene microplate	Corning	Cat#3993
Automated cell counter	DeNovix	Model#CellDropFLi
Cary 60 UV-vis spectrophotometer	Agilent	Model#G6860AA
Sorvall WX+ ultracentrifuge	Fisher Scientific	Cat#75-000-080
AH-629 swinging bucket rotor	Fisher Scientific	Cat#54-285
TFT-80.2 fixed angle rotor	Fisher Scientific	Cat#54-456
Nanoparticle analyzer	Spectradyne	Model#nCS1
SpectraMax multi-mode microplate reader	Molecular Devices	Model#iD5

MATERIALS AND EQUIPMENT

rhMMP-12 reconstitution buffer		
Reagent	Final concentration	Amount
MES (1 M)	10 mM	100 μL
NaCl (1 M)	100 mM	1 mL
$CaCl_2$ (0.25 M)	100 μM	4 μL
$ZnSO_4$ (1 mM)	1 μM	10 μL
PEG (50% w/v)	0.1% w/v	20 μL
CHAPS	0.1% w/v	10 mg
ddH_2O	N/A	8.866 mL
Total	N/A	10 mL
Adjust the buffer pH to 6.0, filter using a 0.2 μm syringe filter, and store at 4°C for up to 6 months.		

MMP-12 FRET assay TCNB buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M)	50 mM	0.5 mL
$CaCl_2$ (0.25 M)	10 mM	0.4 mL
NaCl (1 M)	150 mM	1.5 mL
Brij-35 stock (30% v/v)	0.05% v/v	17 μL
ddH_2O	N/A	7.583 mL
Total	N/A	**10 mL**

Adjust the buffer pH to 7.5, filter using a 0.2 μm syringe filter, and store at 4°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Collection of murine BALF

Timing: 1 h

This step describes a method for collecting BALF from a 12–14-week-old male or female C57BL/6J mouse. See Methods video S1 for a demonstration of this procedure.

Note: These steps may be adapted for other small animals such as rats, ferrets, or tree shrews after approval by the local IACUC and appropriate institutional training in the use of these animals.

1. Prepare the appropriate dose of anesthesia for euthanasia and euthanize the mouse according to local institutional practice (e.g., CO_2-mediated asphyxiation or cervical dislocation).

⚠ CRITICAL: Ensure euthanasia is complete before proceeding to the next steps, observing for the absence of respiratory effort or lack of movement with an aggressive toe pinch.

2. Pin the animal's front and hind paws onto a dissection tray and secure the maxillary incisors under a rubber band that is wrapped around the superior aspect of the dissection tray (Figure 3A).
3. Wash the chest and neck by spraying thoroughly with 70% ethanol. Wipe away excess ethanol with sterile gauze.
4. With scissors, cut the skin vertically from the top of the sternum to the bottom of the chin (Figure 3B).
5. Separate the submaxillary glands to expose the trachea, avoiding the veins and arteries.
6. Lift and trim the fibroelastic membrane that encloses the anterior trachea.
7. Use forceps to pass and center a 3-inch segment of 3-0 suture posterior to the trachea.
8. Create a small hole in the anterior trachea using surgical scissors.
9. Insert the tracheostomy catheter into the tracheal stoma with the catheter tip pointed toward the lungs (Figure 3C).
10. Connect a 1 mL syringe containing 0.5 mL EV-depleted 1X PBS(−/−) to the catheter and slowly inject 0.5 mL EV-depleted 1X PBS into the lungs (Figure 3D).

Note: Refer to Troubleshooting problem 1 for how to navigate the appearance of fluid into the mouse's nose and/or mouth during 1X PBS instillation into the trachea.

11. After 30 sec, slowly aspirate BALF into the 1 mL syringe.

Note: It is expected that this step may generate bubbles in the BALF and <0.5 mL of BALF may be recovered. Refer to Troubleshooting problem 2 for how to handle the inability to recover BALF.

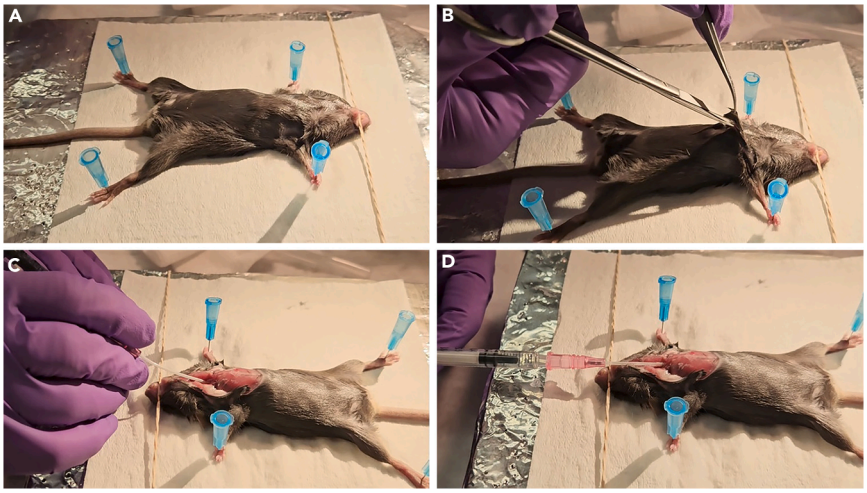


Figure 3. Preparation of a mouse for BALF collection

(A) The front and hind paws of a mouse are pinned to a dissection tray and the maxillary (upper) incisors are secured under a rubber band that is wrapped around the superior aspect of the dissection tray. The mouse's chest is then washed with 70% ethanol, and excess liquid is wiped away with sterile gauze.

(B) A 2–3 cm vertical incision is then made in the mouse's skin anterior to the trachea, and the skin is bluntly dissected from the anterior neck structures.

(C) A small stoma is created in the anterior trachea with surgical scissors, and the tracheostomy catheter is gently inserted through the stoma toward the lungs.

(D) A 1 mL syringe containing 0.5 mL sterile, EV-depleted 1X PBS(−/−) is attached to the tracheostomy catheter for bronchoalveolar lavage.

12. While keeping the tracheostomy catheter inside of the trachea, replace the 1 mL syringe containing BALF with a second, fresh 1 mL syringe containing 0.5 mL EV-depleted 1X PBS(−/−).
13. Repeat the instillation of EV-depleted 1X PBS and aspiration of BALF.
14. Transfer BALF from the two 1 mL syringes to a sterile 50 mL conical tube and place on ice.
15. Repeat Steps 1–14 using additional mice, transferring BALF from each animal into the 50 mL tube from Step 14 to pool a sufficient quantity of alveolar macrophages for experimentation.

Note: Dispose of rodent carcasses according to local institutional procedures.

Note: We recommend collecting BALF from at least 15 mice to isolate at least 0.8–1 × 10^6 alveolar macrophages for eventual EV generation.

Isolation of macrophages from human and murine BALF

Timing: 3 h

This step describes a method of isolating primary alveolar macrophages from BALF obtained from a human or mouse for cell culture.

⚠ CRITICAL: When harvesting and handling human BALF, use universal precautions according to local institutional employee health guidelines.

⚠ CRITICAL: To harvest alveolar macrophages from human BALF, a BALF specimen should be kept at 20°C–22°C and processed within 1 h of collection to preserve cell viability.

Note: Alveolar macrophages are the predominant cell type within BALF from humans and mice.[20,22] Thus, following the lysis of residual red blood cells, the overwhelming majority of

cells that isolated from this procedure are alveolar macrophages. As alveolar macrophages adhere to tissue culture plastic, use of tissue culture further selects alveolar macrophages from lymphocytes, the next most prevalent cell type within BALF from humans and mice.[20,22]

Note: BALF from humans may be tenacious with high viscosity, which may limit alveolar macrophage pelleting and eventual isolation. When this is encountered, BALF may be diluted 1:4-1:10 v/v in sterile, EV-depleted 1X PBS(−/−) or diluted 1:2-1:4 v/v in sterile, EV-depleted 1X PBS(−/−), 2.5 mM EDTA. Addition of EDTA chelates cations and may reduce protein-protein interactions. Further adjustment of BALF to pH 7.5 using $NaHCO_3$ or NaOH may also further reduce BALF viscosity.

16. Centrifuge BALF at 500 *x g* for 10 min at 4°C.
17. Carefully remove and discard the supernatant, careful not to disturb the cell pellet.
18. Lyse RBCs within the cell pellet:
    a. Resuspend the cell pellet in 0.5 mL 0.2% NaCl.
    b. Incubate at 20°C–22°C for 30 sec.
    c. Add 0.5 mL 1.6% NaCl and mix well.
    d. Centrifuge the cell suspension at 500 *x g* for 10 min at 4°C.
    e. Remove and discard the supernatant.

Note: If RBCs are still present, as evidenced by an RBC pellet after centrifugation, repeat Step 18. Refer to Troubleshooting problem 3 for the persistence of an RBC pellet after repeating Step 18.

19. Resuspend the cell pellet in 0.5 mL DMEM, 10% FBS.
20. Determine the cell concentration using standard hemocytometry.
21. Dilute the cell suspension to 0.2 × 10^6 cells per mL with DMEM, 10% FBS.
22. Seed 0.2 × 10^6 cells per well in a 24-well tissue culture plate until the cell suspension is exhausted.
23. Incubate overnight at 37°C in a humidified chamber with 5% CO_2.

Generation of CSE stock

Timing: 1.5 h

This step details the assembly of the apparatus used to generate CSE and the procedure for preparing CSE stock from a single cigarette. See Methods video S2 for a demonstration of this procedure.

⚠ CRITICAL: Due to the volatile substances within CSE, CSE should be prepared on the day that alveolar macrophages will be stimulated with CSE and used within 30 min of preparation.

24. Autoclave the 2 in piece of Tygon vacuum tubing that will be used as the cigarette holder in a single-use autoclave bag.

Note: The Tygon vacuum tubing is reusable and should be autoclaved between uses.

25. Allow 5 mL EV-depleted DMEM to warm to 20°C–22°C.
26. In a biosafety cabinet, aspirate 5 mL EV-depleted warm DMEM into a 50 mL syringe.
27. With a 3-way stopcock oriented such that the end ports are pointed left-to-right, the middle port is pointed upward, and the valve faced toward the proceduralist, screw a sterile 10 mL Luer-Lok syringe onto the right port of 3-way stopcock valve (Figure 4A).
28. Slide one end of the Tygon tubing over the left port of the 3-way stopcock (Figure 4B).

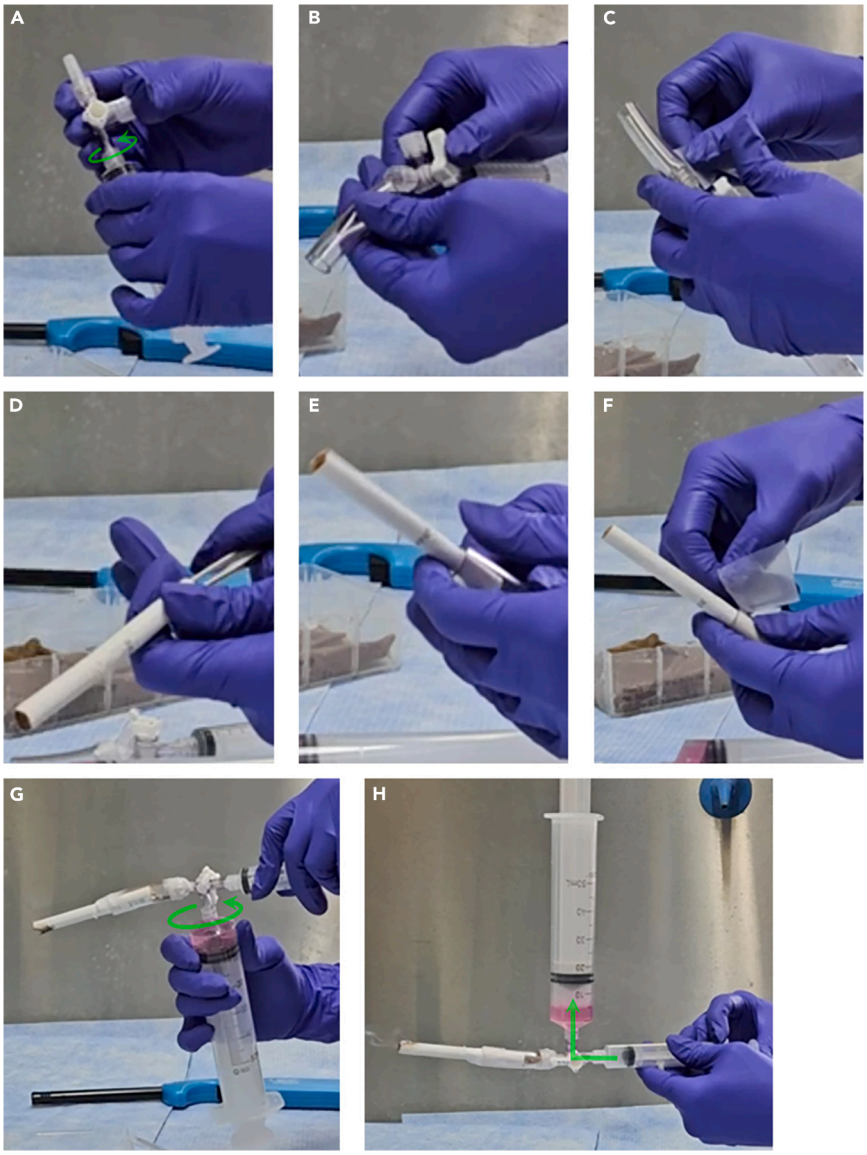


Figure 4. Preparation of CSE

(A) A 10 mL Luer-Lok syringe is attached to the right port of a 3-way stopcock.
(B) Tygon tubing is inserted over the left port of the 3-way stopcock.
(C) The connection between the Tygon tubing and the 3-way stopcock is sealed with a Parafilm wrap.
(D) The filter is removed from a cigarette with forceps.
(E) The cigarette is inserted into the open end of the Tygon tubing with the filter side facing the 3-way stopcock.
(F) The connection between the cigarette and Tygon tubing is sealed with Parafilm wrap.
(G) After the cigarette has been lit and cigarette smoke has been aspirated into the 10 mL syringe twice, a 50 mL syringe filled with 5 mL of DMEM, 10% FBS is attached to the open port of the 3-way stopcock.
(H) After cigarette smoke is aspirated into the 10 mL syringe, the stopcock is closed to the cigarette and opened to both the 10 mL and 50 mL syringes. The cigarette smoke is then bubbled through the cell culture medium by depressing the plunger of the 10 mL.

29. Use Parafilm to seal the end of the Tygon tubing overlaying the left port of the 3-way stopcock (Figure 4C).
30. Use forceps to remove the filter from a single cigarette (Figure 4D).

31. Insert the end of the cigarette from which the filter was removed into the open end of the Tygon tubing (Figure 4E).
32. Use Parafilm to seal the connection between the cigarette and the Tygon tubing (Figure 4F).
33. Make a homemade ashtray by lining an empty 200 mL pipette tip box with a wet paper towel to safely collect the cigarette ashes and the cigarette butt when finished smoking.

Note: A similar ashtray may be prepared by forming a 6 in x 6 in tray from aluminum foil.

⚠ CRITICAL: Perform Steps 34–41 in a chemical fume hood.

34. Ignite the free end of the cigarette with a lighter.
35. Turn the stopcock valve so that the stopcock is open from left-to-right and closed to the middle port.
36. Pull the plunger of the 10 mL syringe to aspirate cigarette smoke into the syringe.

Note: Refer to Troubleshooting problem 4 for difficulty with aspirating cigarette smoke into the 10 mL syringe.

37. Remove the 10 mL syringe and depress the plunger to release smoke into the hood.
38. Re-attach the 10 mL syringe to the right port of the stopcock.
39. Repeat Steps 36–38.
40. Screw the 50 mL syringe containing 5 mL culture medium onto the middle port (Figure 4G).
41. Push cigarette smoke through the culture medium:
    a. Aspirate smoke from the cigarette into the 10 mL syringe.
    b. Turn the stopcock valve closed to the left port and open to the middle and right ports.
    c. Depress the plunger of the 10 mL syringe to push cigarette smoke through the 5 mL DMEM in the 50 mL syringe (Figure 4H).
    d. Turn the stopcock valve closed to the middle port and open to the left and right ports.
    e. Repeat Steps 41a-d until the cigarette is completely burned through.

⚠ CRITICAL: If the 50 mL syringe is nearly full of smoke and the 5 mL of media before the cigarette is completely burned through, turn the stopcock valve closed to the middle port, rotate the 50 mL syringe 180° downwards, unscrew the 50 mL syringe from the stopcock, and depress the plunger while keeping the Luer opening pointed upward until only media remains in the syringe. Then, re-attach the 50 mL syringe to the middle port of the stopcock to resume Step 41a.

42. Deplete CSE of potential endotoxin contamination by passing the CSE through a 10 kDa MWCO 15 mL centrifugal filter according to manufacturer specifications.
43. In a biosafety cabinet, filter the CSE through a 0.2 μm syringe filter.
44. Keep the filtered CSE stock at 20°C–22°C until use.

Standardized dilution of CSE

Timing: 10 min

This step describes how to dilute the CSE stock in EV-depleted DMEM for treatment of alveolar macrophages.

Note: This step typically generates a 2% v/v CSE preparation for cell culture. However, we choose to normalize the diluted CSE to the optical density of the stock CSE at 320 nm wavelength to ensure CSE standardization.[23,24]

45. In a biosafety cabinet, add 50 μL CSE stock to 950 μL EV-depleted DMEM to generate 1 mL 5% v/v CSE solution.
46. Using 1 mL of EV-depleted DMEM as a spectrophotometric blank, measure the optical density (OD) of the 5% CSE solution at 320 nm wavelength using a spectrophotometer.
47. Calculate the dilution factor (DF) of the CSE stock using the formula: DF = 166.67 x OD_{320}.
48. Calculate the volume of CSE stock needed per 0.5 mL of culture medium using the formula: (volume of CSE stock) = [(0.5 mL) x (number of wells on a tissue culture plate for CSE treatment)] / DF.
    a. For example, if the OD_{320} of a 5% v/v stock CSE solution is 0.3 (DF = 166.67 x 0.3 = 50), 40 μL of CSE stock would be needed to treat alveolar macrophages in four wells of a tissue culture plate with 0.5 mL of diluted CSE per well [(0.5 mL) x (4 wells) / 50 = 0.04 mL or 40 μL].
49. In a biosafety cabinet, add the volume of CSE stock calculated in Step 48 to the total volume of EV-depleted DMEM needed for all wells designated for CSE treatment on the tissue culture plate minus the CSE stock volume.
    a. For example, 40 μL of CSE stock would be added to 1.96 mL of EV-depleted DMEM to prepare a sufficient volume of diluted CSE to add 0.5 mL dilute CSE to 4 wells.

Alveolar macrophage stimulation by CSE

Timing: 4.5 h

This step describes a method for stimulating primary human or murine alveolar macrophages with CSE in cell culture.

50. Aspirate the culture medium from alveolar macrophages.
51. Wash the alveolar macrophages with 0.5 mL warm EV-depleted 1X PBS(−/−) twice.

⚠ CRITICAL: Avoid disrupting adherent macrophages on the cell culture plate while aspirating the medium and 1X PBS.

Note: Prepare the diluted CSE before the last wash of 1X PBS(−/−). Do not let the wells become dry.

52. To the respective wells designated for control or CSE treatment, add 0.5 mL EV-depleted DMEM (control) or 0.5 mL CSE and incubate at 37°C in a humidified chamber with 5% CO_2 for 4 h.
53. Harvest cell culture medium from each well into individually labeled, sterile 1.5 mL microcentrifuge tubes and place on ice.

EV isolation from BALF or primary cell culture medium

Timing: 4 h

This step describes a method for isolating EVs from either BALF or culture medium from primary alveolar macrophages using differential ultracentrifugation.

Note: Among the various methods available for EV isolation (e.g., differential ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography, membrane affinity), we recommend using differential ultracentrifugation when isolating EVs from BALF or primary cell culture supernatant for functional assays and experimentation to avoid EV exposure to proprietary buffers or nonaqueous solutions that may impact the activity of EV-associated enzymes such as MMP-12 in an unpredictable manner.

⚠ CRITICAL: Store ultracentrifuge rotors at 4°C prior to use.

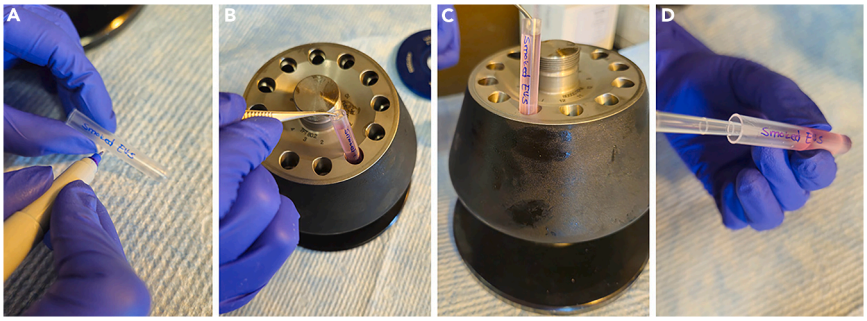


Figure 5. Preparing samples for ultracentrifugation

(A) Labeling of the side of the ultracentrifuge tube with the sample number or experimental condition.
(B) Insertion of the ultracentrifuge tube containing supernatant.
(C) Depiction of the ultracentrifuge tube positioned so that the labeled side faces the outside of the rotor.
(D) Supernatant removal using a 200 μL extended length pipette tip while avoiding touching the EV pellet that accumulated on the labeled side of the tube.

⚠ CRITICAL: BALF should be stored at 4°C until ready for EV isolation.

54. Centrifuge BALF or cell culture medium at 3000 *x g* for 10 min at 4°C to pellet cell debris.
55. Transfer the supernatant into fresh, individually labeled, sterile 1.5 mL microcentrifuge tubes.
56. Centrifuge supernatant at 10,000 *x g* for 60 min at 4°C to pellet larger, denser particles such as large EVs (>200 nm).[25]
57. During Step 56, label a sufficient number of ultracentrifuge tubes with the sample number or experimental condition (Figure 5A).

Note: For cell culture supernatant volumes <10 mL, we recommend using 8 x 51 mm open-top polyallomer ultracentrifuge tubes for a fixed angle rotor. When using 8 x 51 mm ultracentrifuge tubes, we recommend adding 1.7 mL supernatant to each tube and ensuring filled tubes are equivalent in mass by adding EV-depleted 1X PBS(−/−) as needed. This volume should prevent the ultracentrifuge tubes from collapsing.

Note: For human BALF supernatant or cell culture supernatant volumes >10 mL, we recommend using 25 x 89 mm open-top polyallomer tubes for a swinging bucket rotor. When using 25 x 89 mm ultracentrifuge tubes, we recommend bringing the supernatant volume to 33 mL with EV-depleted 1X PBS(−/−) and transferring the diluted supernatant to the ultracentrifuge tube.

58. Transfer the supernatant to labeled ultracentrifuge tubes.

⚠ CRITICAL: Ensure filled tubes are equivalent in mass by adding EV-depleted 1X PBS(−/−) as needed.

59. Insert ultracentrifuge tubes into the rotor in a balanced configuration with the label facing outwards (Figures 5B and 5C).

Note: For odd numbers of ultracentrifuge tubes that will not permit a balanced configuration in the rotor, a spare, appropriately sized ultracentrifuge tube may be filled with 1X PBS(−/−) to equivalent mass to achieve a balanced rotor configuration.

60. Centrifuge supernatant at 150,000 *x g* for 2 h at 4°C.

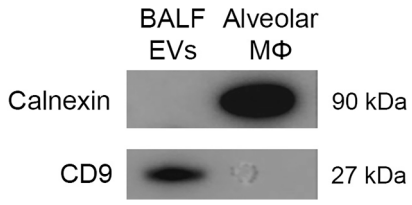


Figure 6. Confirmation of a canonical EV marker in BALF-derived EVs using western blot

Protein from 8 × 10^{10} EVs isolated from the BALF of 16-week-old male C57BL/6J mice after 30 d of daily cigarette smoke exposure using differential ultracentrifugation were resolved with SDS-PAGE and transferred to a nitrocellulose membrane. As a control, EV protein was resolved alongside protein from mouse primary alveolar macrophage cell lysate (2 μg per lane). Protein from BALF-derived EVs and cell lysate were probed for the canonical EV marker CD9 (Thermo Fisher Scientific, Cat#10626D; RRID: AB_2532982) (1:500 v/v in blocking buffer) and calnexin (Proteintech, Cat#10427-2-AP; RRID: AB_2069033) (1:1,000 v/v in blocking buffer), a protein localized to the membrane of cellular endoplasmic reticula. See Figure S1 for complete membranes.

Note: EVs will pellet on the labeled side of the tube.

61. Aspirate and discard the supernatant without disturbing the EV pellet (Figure 5D).

Note: We recommend using 200 μL extended length pipette tips to remove supernatant from and resuspend EVs in the 8 x 51 mm ultracentrifuge tubes.

Note: Steps 62–65 clarify EVs of protein aggregates that may pellet with the EVs following the first 150,000 *x g* centrifugation.

62. Resuspend the EV pellet in 1.7 mL EV-depleted 1X PBS(−/−) within the ultracentrifuge tube.
63. Re-insert the ultracentrifuge tube into the rotor in a balanced configuration.
64. Centrifuge EVs again at 150,000 *x g* for 2 h at 4°C.
65. Aspirate and discard the supernatant without disturbing the EV pellet.
66. Resuspend the EV pellet with 50 μL EV-depleted 1X PBS(−/−) by pipetting up and down 6 times and vortexing 6 times twice.
67. Keep the EV suspension on ice or at 4°C until ready for characterization or experimentation.

Note: If the user wishes to bank EVs for later use or analysis, EVs may be stored for up to 1 year at −80°C.

EV characterization and concentration determination

Timing: 1 h

This step describes a method for characterizing the size distribution and concentration of EVs using a Spectradyne nCS1 nanoparticle tracking analyzer.

Note: This procedure enumerates the EV concentration within each sample necessary to prepare standardized EV concentrations used in functional assays. In addition, nanoparticle tracking analysis provides a size distribution of the EVs within each sample, helping clarify whether the EV sample size distribution is consistent with "small EVs" (<200 nm diameter).[25] To confirm that the particles are "small EVs," we refer the reader to our prior methods publication[26] for a protocol to assess the protein yield of the EV sample and confirm the presence of canonical EV markers[25] (e.g., TSG101, ALIX, syntenin-1, CD9, CD81, CD63) using western blot (see Figure 6 as an example).

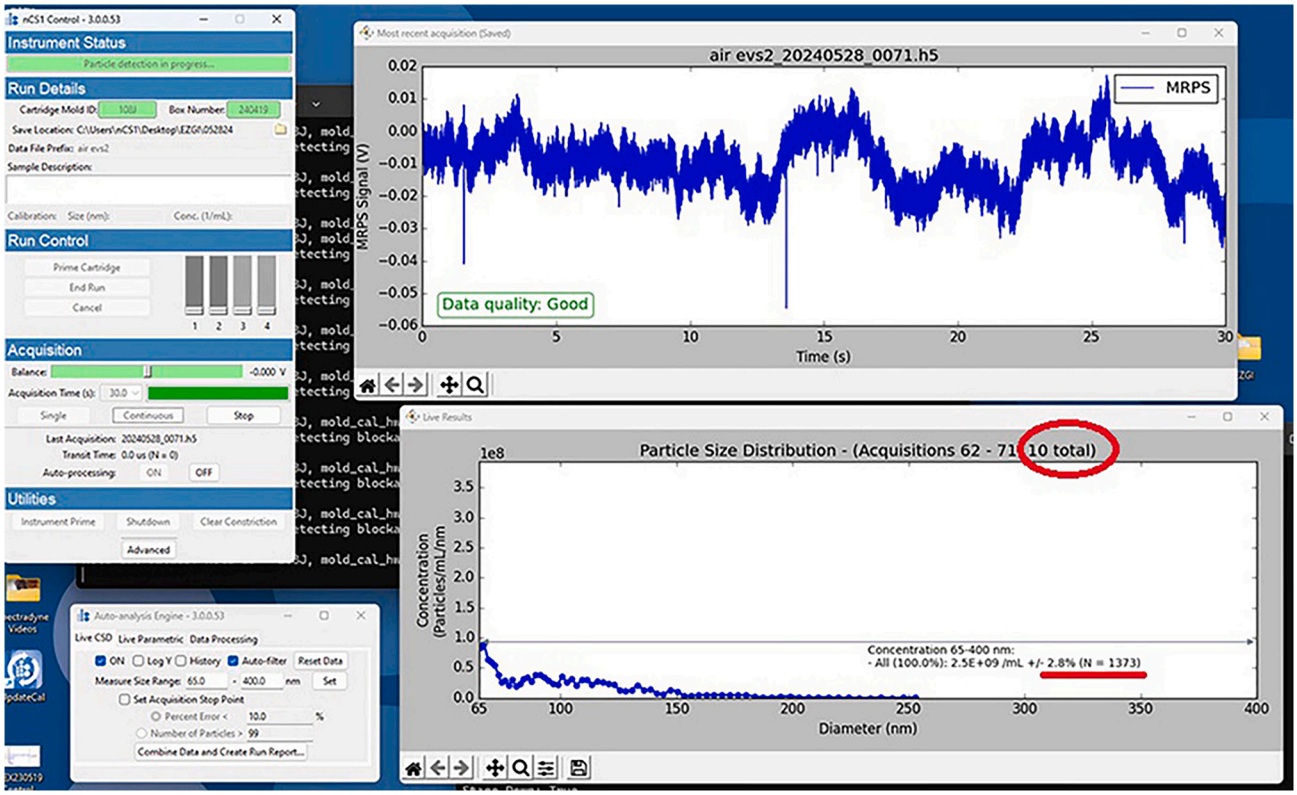


Figure 7. Screenshot of the Spectradyne software demonstrating the EV concentration readout with a minimum of 10 total reads and accurate reading percentage of ±2%

Note: Alternative nanoparticle tracking analyzers may be used in place of a Spectradyne instrument, such as the ZetaView Evolution (Particle Metrix GmbH, Inning am Ammersee, Germany) or the NanoSight Pro (Malvern Panalytical, Worcestershire, UK).

68. Turn on the machine and exchange the two diethyl pyrocarbonate (DEPC)-treated water bottles for bottles filled with EV-depleted PBS-T (filtered with 0.2 μm filter).
69. Run the reusable cleaning cartridge to start the system.
70. Measure the particle concentration and size distribution of the PBS-T buffer filtered through a 0.02 μm filter:
    a. Add 5 μL PBS-T (filtered with 0.02 μm filter) to a new single-use, disposable cartridge without introducing bubbles.
    b. Tap the cartridge on the bench gently to rid the sample of small bubbles.
    c. Insert the cartridge into the machine.
    d. Enter the run details with the information on the cartridge box, naming the sample as 'blank.'
    e. Obtain readings until the accurate reading percentage normalizes to, at most, 5% with a minimum of 10 readings (Figure 7).
    f. Stop the instrument reading and save the data.
    g. Eject and dispose of the used cartridge.

Note: Refer to Troubleshooting problem 5 for handling residual bubbles within the cartridge that interfere with particle analysis and Troubleshooting problem 6 for navigating an unexpectedly high concentration of particles (>1 × 10^8/mL) within the PBS-T buffer that was filtered with a 0.02 μm filter.

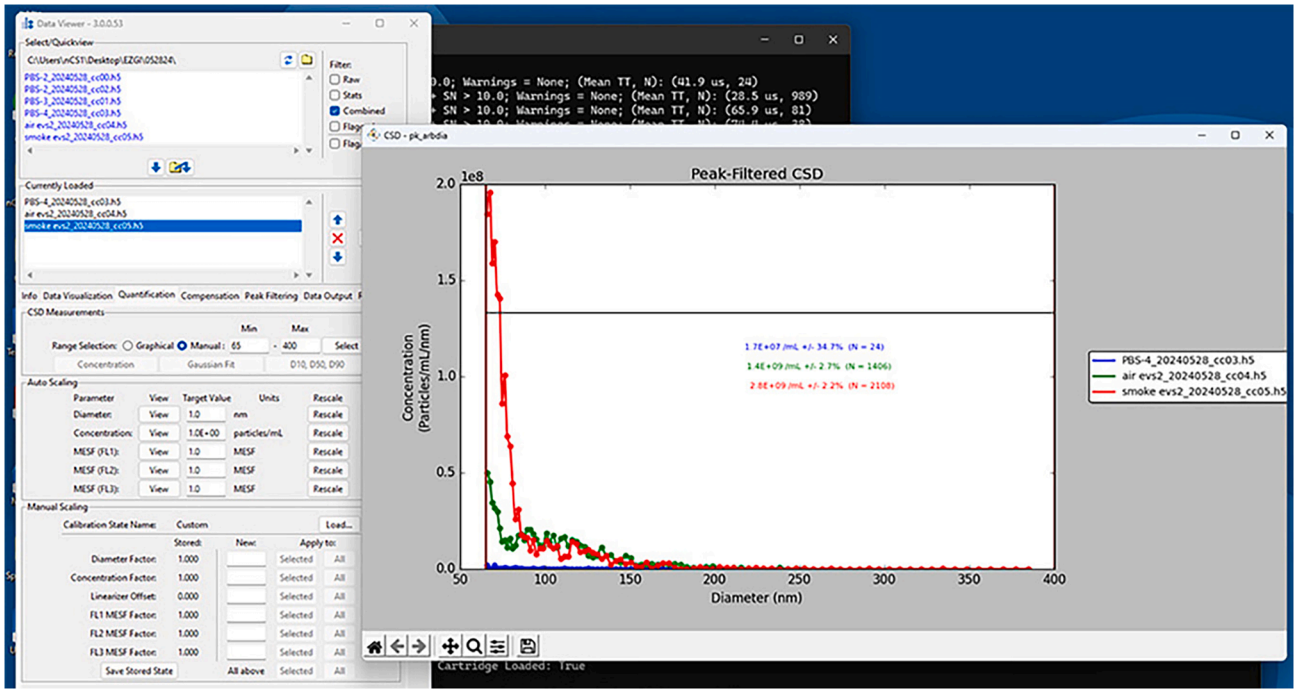


Figure 8. Screenshot of the Spectradyne software demonstrating the size distribution readouts of EVs isolated from the culture medium of primary alveolar macrophages stimulated with CSE (red line), EVs isolated from the culture medium of unstimulated primary alveolar macrophages (green line), and PBS-T buffer filtered through a 0.02 μm filter that serves as a 'blank' to adjust EV concentrations for background particulates (blue line)

71. Measure the particle concentration and size distribution of the first EV sample:
    a. Dilute a sample of the EV solution 1:100 v/v by adding 2 μL of EV sample to 198 μL PBS-T (filtered with 0.02 μm filter) in a sterile 1.5 mL microcentrifuge tube and mixing well.
    b. Add 5 μL of dilute EV preparation from Step 71a into a new cartridge.
    c. Tap the cartridge on the bench gently to rid the sample of small bubbles.
    d. Insert the cartridge into the machine.
    e. Enter in the run details with the information on the cartridge box, naming the sample with an appropriate label.
    f. Obtain readings until the accurate reading percentage normalizes to, at most, 5% with a minimum of 10 readings.
    g. Stop the instrument reading and save the data.
    h. Eject and dispose of the used cartridge.
72. Repeat Step 71 until the concentrations/size distributions of all samples have been measured.
73. Determine the EV concentration within each sample:
    a. Subtract the total particle concentration of the 'blank' specimen (0.02 μm filtered PBS-T) in Step 70 from the total particle concentrations of the diluted samples obtained in Steps 71 and 72 to calculate the adjusted EV concentrations of each sample.
    b. Multiply the adjusted EV concentrations of each sample in Step 73a by 100 (i.e., the dilution factor in Step 71a).

Note: Refer to Troubleshooting problem 7 for handling EV concentrations that are not higher than the 'blank' sample (i.e., background reading) and Troubleshooting problem 8 for how to handle size distributions that demonstrate peaks of particle sizes >200 nm.

Note: Only the total EV concentration of each sample is required to perform functional analyses of and experimentation with EVs. However, particle size distribution data (Figure 8)

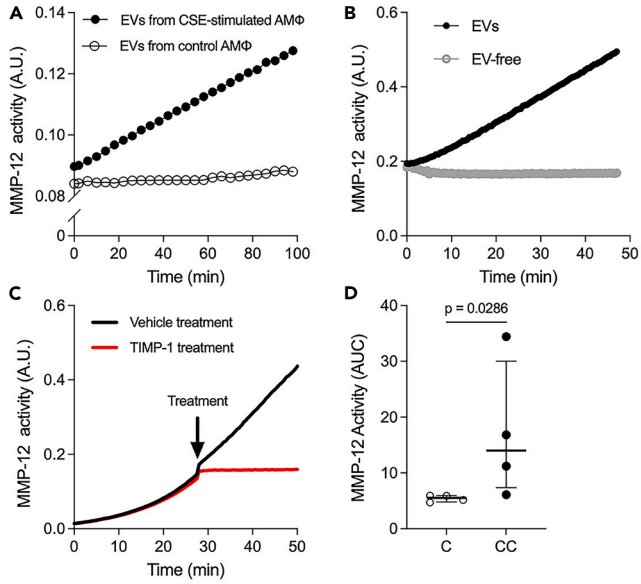


Figure 9. Results of MMP-12 activity on various populations of EVs using FRET assay

(A) Alveolar macrophages isolated from mouse BALF were stimulated with CSE or culture medium alone (control), and EVs were isolated from the cell culture supernatant using differential ultracentrifugation. Isolated EVs were tested for MMP-12 activity, which is reported as arbitrary units (A.U.) of the FRET donor:acceptor emission ratio.

(B) EV and EV-free fractions of the cell culture supernatant from CSE-stimulated alveolar macrophages in (A) were tested for MMP-12 activity, demonstrating the specificity of the FRET assay for MMP-12 on EVs.

(C) EVs directly isolated from the BALF of mice exposed to cigarette smoke for 3 months were treated with or without TIMP-1 (an MMP-12 inhibitor) during the FRET assay to further demonstrate the specificity of the assay for MMP-12 on EVs.

(D) EVs directly isolated from the BALF of current smokers without COPD (C) and current smokers with COPD (CC) using differential ultracentrifugation were tested for MMP-12 activity. Data as median (interquartile range). Comparison was made using the Mann-Whitney *U* test.

may be exported from the Spectradyne software as a Microsoft Excel file for a more detailed characterization of the EV sample and confirmation of sample's expected size distribution (<200 nm diameter for small EVs[25]).

MMP-12 activity measurement with FRET

Timing: 2.5–26 h

This step describes how to measure the enzymatic activity of MMP-12 on the surface of alveolar macrophage-derived EVs using the highly sensitive probe LaRee-5 in a FRET-based assay.[27]

Note: Though we detail a method for characterizing EVs from BALF or cultured primary airway macrophages using an enzyme activity assay for MMP-12, EV preparations may also be characterized using western blot,[28] RT-qPCR,[29] mass spectrometry,[30] or transmission electron microscopy.[3] More selective isolation of unique EV populations may be performed using magnetic bead pulldown as we have described previously.[3,26] When selecting for macrophage-specific EVs using the magnetic bead pulldown technique, we recommend conjugating streptavidin-labeled magnetic beads to biotinylated antibody against human or mouse macrophage markers (e.g., CD11b/integrin α M, CD14, CD68/SR-D1, CD163, or CD206). Moreover, EVs may be used for further experimentation as we have shown previously.[3,4]

Table 1. Serial dilutions to generate recombinant MMP-12 standards

Recombinant MMP-12 standard	TCNB volume	Recombinant MMP-12 volume
250 ng/mL	195 µL	5 µL of 10 µg/mL substock
125 ng/mL	100 µL	100 µL of 250 ng/mL standard
62.5 ng/mL	100 µL	100 µL of 125 ng/mL standard
31.25 ng/mL	100 µL	100 µL of 62.5 ng/mL standard
15.6 ng/mL	100 µL	100 µL of 31.25 ng/mL standard
7.8 ng/mL	100 µL	100 µL of 15.6 ng/mL standard
3.9 ng/mL	100 µL	100 µL of 7.8 ng/mL standard
1.95 ng/mL	100 µL	100 µL of 3.9 ng/mL standard
0.98 ng/mL	100 µL	100 µL of 1.95 ng/mL standard
0 ng/mL	100 µL	N/A

Note: We recommend using at least 1×10^9 EVs to measure MMP-12 activity as the use of fewer EVs may be insufficient to obtain robust readings.

Note: In Steps 74–77, we detail how to prepare rhMMP-12 or rmMMP-12 standards should the user wish to quantify the level of MMP-12 activity of EVs relative to a respective recombinant enzyme control (i.e., rhMMP-12 for EVs derived from human samples/cells and rmMMP-12 for EVs derived from mouse samples/cells). Alternatively, the user may also begin the procedure at Step 78 when performing relative comparisons between EVs from two or more separate experimental conditions (Figures 9A and 9B).

74. Allow p-aminophenylmercuric acetate (APMA) and TCNB to come to 20°C–22°C.
75. Reconstitute 20 µg lyophilized, recombinant MMP-12 in 80 µL reconstitution buffer to generate a stock concentration of 0.25 mg/mL.
76. Reconstitute 1 mg LaRee-5 in 68 µL dimethyl sulfoxide (DMSO) to generate a 10 mM LaRee-5 stock.

⚠ CRITICAL: Reconstitute LaRee-5 in dim light as LaRee-5 is susceptible to degradation by light.

Note: We recommend aliquoting the reconstituted recombinant MMP-12 stock and LaRee-5 stock into separate 10 µL aliquots and storing at −20°C for up to 6 months until ready to perform the FRET assay. Thaw an aliquot of recombinant MMP-12 and LaRee-5 stock to 20°C–22°C prior to use or use freshly prepared substrate.

77. Prepare recombinant MMP-12 standards for the FRET assay:
    a. Add 2 µL 0.25 mg/mL recombinant MMP-12 stock solution to 8 µL TCNB buffer to generate 10 µL of 50 µg/mL recombinant MMP-12 substock.
    b. Combine 2 µL 50 µg/mL recombinant MMP-12 substock prepared in Step 77a with 1 µL 10 mM APMA and 7 µL TCNB buffer to generate 10 µL of 10 µg/mL recombinant MMP-12 substock.
    c. Incubate at 37°C for 24 h in a closed microcentrifuge to activate the recombinant MMP-12.
    d. Generate recombinant MMP-12 standards using serial dilutions of the 10 µg/mL recombinant MMP-12 substock with TCNB buffer according to Table 1.

⚠ CRITICAL: Fresh LaRee-5 substock must be prepared immediately prior to use.

⚠ CRITICAL: Protect LaRee-5 substock from light.

78. Dilute LaRee-5 stock 1:100 v/v with TCNB buffer by mixing 2 µL 10 mM LaRee-5 stock with 198 µL TCNB buffer to prepare 200 µL of 100 µM LaRee-5 substock.

79. Prepare a 10 μM LaRee-5 substock to add to each standard (if used) and sample by adding 1 μL of 100 μM LaRee-5 substock (Step 78) to 9 μL TCNB buffer per sample and standard.
    a. For example, if there are 10 standards run in duplicate with 5 EV samples (total of 25 standards and samples), add 25 μL 100 μM LaRee-5 substock to 225 μL TCNB buffer and mix well.
80. Incubate the 10 μM LaRee-5 substock at 37°C for 10 min in the dark.
81. Dilute 1×10^9 EVs per sample in 40 μL TCNB buffer.

Note: Refer to Troubleshooting problem 9 for how to handle EV concentrations that are too dilute to permit the addition of 1×10^9 EVs in <40 μL and Troubleshooting problem 10 for how to handle having an insufficient quantity of EVs for analysis.

82. Transfer 40 μL of each standard (if used) and the diluted EVs into separate wells in the half-surface area black plates.

Note: When using recombinant MMP-12 standards, we recommend performing the standards in duplicate.

83. Bring the EVs in the assay plate to 37°C by incubating the plates in the plate reader at 37°C for 10 min.

Note: If an inhibitor is used for the analysis, it can be added to the system either before or during the assay. If adding an inhibitor to the EV sample before the assay is started, add the EVs to a well on the microplate, inject the inhibitor into the same well, and adjust the final well volume to 40 μL with TCNB buffer; then incubate the plate at 37°C for 30 min. If performing the inhibition during the assay, add the inhibitor diluted in TCNB buffer with a final volume of 5–10 μL at any time course. Adding the inhibitor can be done either by manual pipetting or by using an autoinjector (preferred method, as available). If the blockade of MMP-12 is during the reaction, the same volume of TCNB buffer should be added to the control (vehicle) wells.

84. After 10 min of incubation, begin taking measurements ever 2 min for 1 h.
85. After the first reading, pause the plate reader and eject the plate.
86. Add 10 μL of 10 μM LaRee-5 substock from Step 80 to each standard (if used) and sample.
87. Re-insert the plate into the plate reader and resume measurements.

Note: Adjust the plate reader to shake the plate 2–3 times before each reading.

88. After 1 h, stop the plate reader and export the results as a Microsoft Excel spreadsheet.
89. Divide the 400 nm readings by the 490 nm readings to generate donor:acceptor ratios that are measured in arbitrary units, or A.U.
90. To visualize the enzyme kinetics of MMP-12, plot donor:acceptor ratios on the y-axis and respective time intervals on the x-axis (Figure 9A).

Note: EV MMP-12 activity may be semi-quantified from the donor:acceptor ratio-versus-time curve generated in Step 90 by calculating the area under the curve (AUC) of this curve.

Note: Refer to Troubleshooting problem 11 for how to handle FRET signal saturation.

91. Quantify EV MMP-12 activity using the recombinant MMP-12 standards (if used):
    a. For duplicates, calculate the average donor:acceptor ratio of each recombinant MMP-12 standard.
    b. Perform a 4-parameter logistic regression analysis of the average donor:acceptor ratios relative to the respective concentration of the recombinant MMP-12 standards.

c. Interpolate the donor:acceptor ratios of the EV MMP-12 activity using the 4-parameter logistic regression analysis in Step 91b.

EXPECTED OUTCOMES

MMP-12 is mainly expressed by alveolar macrophages and plays a key role in smoking-induced chronic obstructive pulmonary disease (COPD) by cleaving extracellular matrix components and increasing monocyte and neutrophil recruitment to the lung.[15,31,32] Thus MMP-12 is central to disease pathology in COPD. We have previously shown that *in vivo* exposure to cigarette smoke increases the amount of MMP-12 on macrophage-derived EVs isolated from mouse BALF.[4] Here, we present a method for isolating EVs from human and mouse BALF as well as EVs derived from primary airway macrophages *in vitro*. Moreover, we detail how to measure the functional MMP-12 activity on these EVs using a highly sensitive FRET assay. Showcasing these techniques, we demonstrate for the user that MMP-12 activity is higher in alveolar macrophage-derived EVs from CSE-treated versus untreated EVs (Figure 9A) and that MMP-12 activity is present solely in the EV fraction of the cell culture medium (Figure 9B). Importantly, the FRET assay described in this protocol is specific for MMP-12 as demonstrated by the abrogation of MMP-12 activity by TIMP-1, a natural MMP-12 inhibitor, on the surface of EVs derived from the BALF of mice who were exposed daily to cigarette smoke for 3 months (Figure 9C). Finally, we offer the measurement of MMP-12 on BALF-derived EVs as a potentially helpful functional readout of pulmonary disease in humans by demonstrating that EVs from the BALF of current smokers with COPD have increased MMP-12 activity compared to BALF-derived EVs from smokers without COPD (Figure 9D).

LIMITATIONS

Our laboratory uses differential ultracentrifugation to isolate EVs from BALF and primary alveolar macrophage culture supernatant to limit EV loss and to control the buffers to which the EVs are exposed. However, despite our use of a clarification centrifugation to separate EVs from protein aggregates, differential ultracentrifugation has the inherent limitations of potentially pelleting protein aggregates with EVs and causing EV aggregation/clumping. EVs may be isolated using alternative methods such as membrane affinity isolation or size exclusion chromatography but at the risk of exposing EVs to proprietary buffers. To overcome this limitation, a final buffer exchange may be considered using centrifugal filtration with a 10 kDa centrifugal filter. Moreover, in this protocol, we detail how to assess the functional characteristics of EV samples from BALF or primary airway macrophages for the purpose of biological comparisons or future experimentation. For further description on how to characterize EV samples in a more robust manner using magnetic bead pull-down for flow cytometry or western blot, we refer the reader to our prior protocol publication.[26]

TROUBLESHOOTING

Problem 1

Fluid is observed to be coming from mouse's mouth or nose during 1X PBS instillation to the tracheal catheter for BALF collection (see Step 10).

Potential solution

The catheter is either obstructed by the tracheal wall or is not sufficiently advanced into the trachea. The catheter should be advanced into the trachea and twisted clockwise a quarter turn to move the catheter toward the lung and overcome tracheal wall obstruction. Also consider repeating Step 10 with a fresh 1 mL syringe filled with 0.5 mL EV-depleted 1X PBS(−/−) if there was substantial loss of fluid through the mouth and/or nares.

Problem 2

Unable to withdraw any 1X PBS back into the syringe from the lungs after 1X PBS instillation into the trachea (see Steps 11 and 13).

Potential solution

If no fluid is recovered with the first attempt, the catheter may either have shifted or be obstructed by the tracheal wall. Make further attempts at aspirating BALF while twisting the tracheal catheter in a clockwise motion and pulling back on the catheter by 3–5 mm.

Problem 3

RBCs are still present after repeating the RBC lysis procedure (see Step 18).

Potential solution

Repeat the RBC lysis procedure a third time. Do not incubate cells with 0.2% NaCl longer than 30 sec. Longer incubation might affect the viability and yield of the alveolar macrophages.

Problem 4

Cigarette smoke will not flow into the 10 mL syringe (see Steps 36 and 41a).

Potential solution

If smoke from a lit cigarette does not flow into the 10 mL syringe after pulling back on the plunger with the stopcock valve open to the left and right ports, there is likely an air leak in the apparatus or the filter was not removed from the cigarette. Extinguish the cigarette for safety, and perform the following.

- Remove the Parafilm wrapping from the Tygon tubing and stopcock.
- Obtain a fresh cigarette and ensure the filter has been fully removed.
- Ensure the 10 mL syringe is screwed in completely to the stopcock.
- Tightly re-wrap the Tygon tubing and the cigarette as well as the Tygon tubing and the 3-way stopcock with fresh Parafilm.

Problem 5

There are bubbles in the cartridges for the Spectradyne nCS1 nanoparticle tracking analyzer (see Steps 70–72).

Potential solution

If bubbles are visually apparent in the bottom of the cartridge or the system gives an error in Step 70e, perform the following.

- Remove the cartridge.
- Carefully take the solution out from the cartridge with a pipette.
- Add fresh solution, avoiding double pipetting.
- Tap the cartridge slowly and firmly on the bench to remove any bubbles.

Problem 6

The concentration of the PBS-T buffer used as a 'blank' is $>1 \times 10^8$/mL (see Step 70).

Potential solution

If the PBS-T buffer used as the 'blank' has a high background ($>1 \times 10^8$ particles per mL), filter the PBS-T through a 0.02 μm filter or 100 kDa MWCO centrifugal filter a second time to remove any additional particles.

Problem 7

The EV concentration of the sample(s) is not higher than background (i.e., the concentration of the 'blank') (see Step 73).

Potential solution

If the EV concentration is significantly higher different than the 'blank,' it is likely that the EV pellet was lost during supernatant removal following ultracentrifugation or inadequate EV pellet resuspension. To avoid losing the EV pellet with the supernatant removal following centrifugation, we recommend that supernatant be removed promptly following ultracentrifugation to prevent loosening of the EV pellet with gradual EV resuspension as the ultracentrifuge tube sits undisturbed. Moreover, we recommend removing supernatant using a 200 μL pipette tip with a 20–200 μL pipette with the pipette tip pointed toward the side of the ultracentrifuge tube opposite of the tube label, which should keep the pipette tip away from the EV pellet. To effectively resuspend the EV pellet, point the 200 μL pipette tip toward the side of the tube with the EV pellet, making sure not to skip the pipetting and vortexing steps as they are very important to the final EV yield.

Problem 8

Peaks are observed in the EV sample size distribution >200 nm (see Step 73).

Potential solution

Peaks in the size distribution of a sample beyond 200 nm suggests that a pure sample of EVs were not isolated. Particulate contamination can occur for different reasons, such as a disturbance in the large EV/particle pellet after the 10,000 *x g* centrifugation while collecting the supernatant for ultracentrifugation or from protein aggregates in your sample. To deplete the sample of large EVs/particles, repeat Step 56. To deplete the sample of protein aggregates, repeat Step 64 or add a sucrose gradient.

Problem 9

The EV concentration is too dilute (see Step 81).

Potential solution

Concentrate the EV sample using a 10–100 kDa MWCO centrifugal filter according to manufacturer specifications. The concentration of the pooled EV sample will need to be measured to confirm that a sufficient number of EVs are available for the FRET assay.

Problem 10

Insufficient number of EVs to perform the FRET assay (see Step 81).

Potential solution

EV samples from the same experimental condition may be pooled and concentrated using a 10–100 kDa MWCO centrifugal filter according to manufacturer specifications. The concentration of the pooled EV sample will need to be measured to confirm that a sufficient number of EVs are available for the FRET assay.

Problem 11

MMP-12 activity is saturated (see Step 90).

Potential solution

Repeat the MMP-12 activity assay using fewer EVs and/or changing the plate reader PMT to 'medium/low.'

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Camilla Margaroli (cmargaroli@uabmc.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Ezgi Sari (esari@uab.edu).

Materials availability

Please contact the lead contact for requests as above.

Data and code availability

This study did not generate any datasets or code.

ACKNOWLEDGMENTS

The authors would like to acknowledge startup funding from the University of Alabama at Birmingham (to C.M.) and funding from the NIH (K08 GM144788 to R.P.R. and R01 HL162705 to K.R.G.). The graphical abstract/figures were created using BioRender; GraphPad Prism, version 10.3.1; and Adobe Photoshop.

AUTHOR CONTRIBUTIONS

Conceptualization, K.R.G. and C.M.; methodology, E.S., R.P.R., D.A.S., C.L., J.B.S., N.X., D.C.F., K.R.G., and C.M.; writing, E.S. and R.P.R.; review and editing, E.S., R.P.R., K.R.G., and C.M.; funding, C.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103867.

REFERENCES

1. Sari, E., He, C., and Margaroli, C. (2022). Plasticity towards Rigidity: A Macrophage Conundrum in Pulmonary Fibrosis. Int. J. Mol. Sci. *23*, 11443. https://doi.org/10.3390/ijms231911443.

2. Kalluri, R., and LeBleu, V.S. (2020). The biology, function, and biomedical applications of exosomes. Science *367*, eaau6977. https://doi.org/10.1126/science.aau6977.

3. Genschmer, K.R., Russell, D.W., Lal, C., Szul, T., Bratcher, P.E., Noerager, B.D., Abdul Roda, M., Xu, X., Rezonzew, G., Viera, L., et al. (2019). Activated PMN Exosomes: Pathogenic Entities Causing Matrix Destruction and Disease in the Lung. Cell *176*, 113–126.e15. https://doi.org/10.1016/j.cell.2018.12.002.

4. Madison, M.C., Margaroli, C., Genschmer, K.R., Russell, D.W., Wells, J.M., Sari, E., Soto-Vazquez, Y.M., Guo, Y.Y., Mincham, K.T., Snelgrove, R.J., et al. (2023). Protease-armed, Pathogenic Extracellular Vesicles Link Smoking and Chronic Obstructive Pulmonary Disease. Am. J. Respir. Crit. Care Med. *208*, 1115–1125. https://doi.org/10.1164/rccm.202303-0471OC.

5. Margaroli, C., Madison, M.C., Viera, L., Russell, D.W., Gaggar, A., Genschmer, K.R., and Blalock, J.E. (2022). An in vivo model for extracellular vesicle-induced emphysema. JCI Insight *7*, e153560. https://doi.org/10.1172/jci.insight.153560.

6. Wu, F., Gao, J., Kang, J., Wang, X., Niu, Q., Liu, J., and Zhang, L. (2022). Knowledge Mapping of Exosomes in Autoimmune Diseases: A Bibliometric Analysis (2002-2021). Front. Immunol. *13*, 939433. https://doi.org/10.3389/fimmu.2022.939433.

7. Shin, H., Choi, B.H., Shim, O., Kim, J., Park, Y., Cho, S.K., Kim, H.K., and Choi, Y. (2023). Single test-based diagnosis of multiple cancer types using Exosome-SERS-AI for early stage cancers. Nat. Commun. *14*, 1644. https://doi.org/10.1038/s41467-023-37403-1.

8. Zhang, W., Wan, Z., Qu, D., Sun, W., Zhang, L., Liang, Y., Pan, L., Jiang, H., Ye, Z., Wei, M., et al. (2024). Profibrogenic macrophage-targeted delivery of mitochondrial protector via exosome formula for alleviating pulmonary fibrosis. Bioact. Mater. *32*, 488–501. https://doi.org/10.1016/j.bioactmat.2023.09.019.

9. St-Laurent, J., Turmel, V., Boulet, L.P., and Bissonnette, E. (2009). Alveolar macrophage subpopulations in bronchoalveolar lavage and induced sputum of asthmatic and control subjects. J. Asthma *46*, 1–8. https://doi.org/10.1080/02770900802444211.

10. Letsiou, E., and Bauer, N. (2018). Endothelial Extracellular Vesicles in Pulmonary Function and Disease. Curr. Top. Membr. *82*, 197–256. https://doi.org/10.1016/bs.ctm.2018.09.002.

11. Mathiesen, A., Hamilton, T., Carter, N., Brown, M., McPheat, W., and Dobrian, A. (2021). Endothelial Extracellular Vesicles: From Keepers of Health to Messengers of Disease. Int. J. Mol. Sci. *22*, 4640. https://doi.org/10.3390/ijms22094640.

12. Srinivasan, A., and Sundar, I.K. (2021). Recent updates on the role of extracellular vesicles in the pathogenesis of allergic asthma. Extracell. Vesicles Circ. Nucl. Acids *2*, 127–147. https://doi.org/10.20517/evcna.2021.03.

13. Ambrozej, D., Stelmaszczyk-Emmel, A., Czystowska-Kuzmicz, M., and Feleszko, W. (2022). "Liquid biopsy" - extracellular vesicles as potential novel players towards precision medicine in asthma. Front. Immunol. *13*, 1025348. https://doi.org/10.3389/fimmu.2022.1025348.

14. Ren, J., Chen, W., Zhong, Z., Wang, N., Chen, X., Yang, H., Li, J., Tang, P., Fan, Y., Lin, F., et al. (2023). Bronchoalveolar Lavage Fluid from Chronic Obstructive Pulmonary Disease Patients Increases Neutrophil Chemotaxis Measured by a Microfluidic Platform. Micromachines *14*, 1740. https://doi.org/10.3390/mi14091740.

15. Shapiro, S.D., Kobayashi, D.K., and Ley, T.J. (1993). Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. J. Biol. Chem. *268*, 23824–23829.

16. Gronski, T.J., Jr., Martin, R.L., Kobayashi, D.K., Walsh, B.C., Holman, M.C., Huber, M., Van Wart, H.E., and Shapiro, S.D. (1997). Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. J. Biol. Chem. *272*, 12189–12194. https://doi.org/10.1074/jbc.272.18.12189.

17. Matsumoto, S., Kobayashi, T., Katoh, M., Saito, S., Ikeda, Y., Kobori, M., Masuho, Y., and Watanabe, T. (1998). Expression and localization of matrix metalloproteinase-12 in the aorta of cholesterol-fed rabbits: relationship to lesion development. Am. J. Pathol. *153*, 109–119. https://doi.org/10.1016/s0002-9440(10)65551-4.

18. Suomela, S., Kariniemi, A.L., Snellman, E., and Saarialho-Kere, U. (2001). Metalloelastase (MMP-12) and 92-kDa gelatinase (MMP-9) as well as their inhibitors, TIMP-1 and -3, are

expressed in psoriatic lesions. Exp. Dermatol. *10*, 175–183. https://doi.org/10.1034/j.1600-0625.2001.010003175.x.

19. Belvisi, M.G., and Bottomley, K.M. (2003). The role of matrix metalloproteinases (MMPs) in the pathophysiology of chronic obstructive pulmonary disease (COPD): a therapeutic role for inhibitors of MMPs? Inflamm. Res. *52*, 95–100. https://doi.org/10.1007/s000110300020.

20. Busch, C.J., Favret, J., Geirsdottir, L., Molawi, K., and Sieweke, M.H. (2019). Isolation and Long-term Cultivation of Mouse Alveolar Macrophages. Bio Protoc *9*, e3302. https://doi.org/10.21769/BioProtoc.3302.

21. Devkota, S.P., Onah, C., Joshi, P.R., Adhikari, S., and Baral, P. (2024). Optimized method for higher yield of alveolar macrophage isolation for ex vivo studies. Heliyon *10*, e37221. https://doi.org/10.1016/j.heliyon.2024.e37221.

22. Heron, M., Grutters, J.C., ten Dam-Molenkamp, K.M., Hijdra, D., van Heugten-Roeling, A., Claessen, A.M.E., Ruven, H.J.T., van den Bosch, J.M.M., and van Velzen-Blad, H. (2012). Bronchoalveolar lavage cell pattern from healthy human lung. Clin. Exp. Immunol. *167*, 523–531. https://doi.org/10.1111/j.1365-2249.2011.04529.x.

23. Kono, Y., Colley, T., To, M., Papaioannou, A.I., Mercado, N., Baker, J.R., To, Y., Abe, S., Haruki, K., Ito, K., and Barnes, P.J. (2021). Cigarette smoke-induced impairment of autophagy in macrophages increases galectin-8 and inflammation. Sci. Rep. *11*, 335. https://doi.org/10.1038/s41598-020-79848-0.

24. Xu, S.W., Zhang, Y.J., Liu, W.M., Zhang, X.F., Wang, Y., Xiang, S.Y., Su, J.C., and Liu, Z.B. (2023). Cigarette smoke extract-induced inflammatory response via inhibition of the TFEB-mediated autophagy in NR8383 cells. Exp. Lung Res. *49*, 39–48. https://doi.org/10.1080/01902148.2022.2164674.

25. Welsh, J.A., Goberdhan, D.C.I., O'Driscoll, L., Buzas, E.I., Blenkiron, C., Bussolati, B., Cai, H., Di Vizio, D., Driedonks, T.A.P., Erdbrügger, U., et al. (2024). Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. J. Extracell. Vesicles *13*, e12404. https://doi.org/10.1002/jev2.12404.

26. Richter, R., Sari, E., Stacks, D., Lewis, C., Smith, J., Xu, N., Langster, T., Finley, D., Margaroli, C., and Genschmer, K.R. (2025). Protocol for the isolation of human neutrophil-derived extracellular vesicles for characterization and functional experimentation. STAR Protoc. *6*, 103739. https://doi.org/10.1016/j.xpro.2025.103739.

27. Cobos-Correa, A., Trojanek, J.B., Diemer, S., Mall, M.A., and Schultz, C. (2009). Membrane-bound FRET probe visualizes MMP12 activity in pulmonary inflammation. Nat. Chem. Biol. *5*, 628–630. https://doi.org/10.1038/nchembio.196.

28. Dlugolecka, M., Szymanski, J., Zareba, L., Homoncik, Z., Domagala-Kulawik, J., Polubiec-Kownacka, M., and Czystowska-Kuzmicz, M. (2021). Characterization of Extracellular Vesicles from Bronchoalveolar Lavage Fluid and Plasma of Patients with Lung Lesions Using Fluorescence Nanoparticle Tracking Analysis. Cells *10*. Cells *10*, 3473. https://doi.org/10.3390/cells10123473.

29. Batochir, C., Kim, I.A., Jo, E.J., Kim, E.B., Kim, H.J., Hur, J.Y., Kim, D.W., Park, H.K., and Lee, K.Y. (2024). Discrimination of Lung Cancer and Benign Lung Diseases Using BALF Exosome DNA Methylation Profile. Cancers (Basel) *16*, 2765. https://doi.org/10.3390/cancers16152765.

30. Shaba, E., Landi, C., Carleo, A., Vantaggiato, L., Paccagnini, E., Gentile, M., Bianchi, L., Lupetti, P., Bargagli, E., Prasse, A., and Bini, L. (2021). Proteome Characterization of BALF Extracellular Vesicles in Idiopathic Pulmonary Fibrosis: Unveiling Undercover Molecular Pathways. Int. J. Mol. Sci. *22*, 5696. https://doi.org/10.3390/ijms22115696.

31. Demedts, I.K., Morel-Montero, A., Lebecque, S., Pacheco, Y., Cataldo, D., Joos, G.F., Pauwels, R.A., and Brusselle, G.G. (2006). Elevated MMP-12 protein levels in induced sputum from patients with COPD. Thorax *61*, 196–201. https://doi.org/10.1136/thx.2005.042432.

32. Van Lint, P., and Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J. Leukoc. Biol. *82*, 1375–1381. https://doi.org/10.1189/jlb.0607338.