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1 **TITLE:**
2 Multicellular Human Alveolar Model Composed of Epithelial Cells and Primary Immune Cells
3 for Hazard Assessment
4
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20 **KEYWORDS:**
21 human lung cocultures, 3D human cocultures, air-liquid interface, isolation of human
22 peripheral blood monocytes, primary macrophages and dendritic cells, cryopreserved
23 primary immune cells
24

25 **SUMMARY:**
26 Presented here is a protocol for primary human blood monocyte isolation as well as their
27 differentiation into macrophages and dendritic cells and assembly with epithelial cells into a
28 multicellular human lung model. Biological responses of cocultures composed of immune
29 cells differentiated from either freshly isolated or thawed monocytes, upon exposure to
30 proinflammatory stimuli, are compared.
31

32 **ABSTRACT:**
33 A human alveolar cell coculture model is described here for simulation of the alveolar
34 epithelial tissue barrier composed of alveolar epithelial type II cells and two types of immune
35 cells (i.e., human monocyte-derived macrophages [MDMs] and dendritic cells [MDDCs]). A
36 protocol for assembling the multicellular model is provided. Alveolar epithelial cells (A549
37 cell line) are grown and differentiated under submerged conditions on permeable inserts in
38 two-chamber wells, then combined with differentiated MDMs and MDDCs. Finally, the cells
39 are exposed to an air-liquid interface for several days. As human primary immune cells need
40 to be isolated from human buffy coats, immune cells differentiated from either fresh or
41 thawed monocytes are compared in order to tailor the method based on experimental
42 needs. The three-dimensional models, composed of alveolar cells with either freshly isolated
43 or thawed monocyte-derived immune cells, show a statistically significant increase in
44 cytokine (interleukins 6 and 8) release upon exposure to proinflammatory stimuli
45 (lipopolysaccharide and tumor necrosis factor α) compared to untreated cells. On the other
46 hand, there is no statistically significant difference between the cytokine release observed in
47 the cocultures. This shows that the presented model is responsive to inflammatory stimuli in

48   the presence of MDMs and MDDCs differentiated from fresh or thawed peripheral blood
49   monocytes (PBMs). Thus, it is a powerful tool for investigations of acute biological response
50   to different substances, including aerosolized drugs or nanomaterials.
51
52   **INTRODUCTION:**
53
54   In vitro lung cell cultures offer cost-effective, robust, and well-controlled platforms to assess
55   the hazards of aerosols[1]. As a model cell system for human alveolar pneumocytes, the
56   epithelial A549 cell line isolated from a pulmonary adenocarcinoma is often used[2]. These
57   cells represent squamous type II epithelial cells from the alveolar region[3] and are a widely
58   used lung cell line for hazard and toxicity assessment[1,4-10]. The A549 cell line possesses
59   relevant features of alveolar epithelial type II cells, such as the presence of characteristic
60   lamellar bodies containing densely packed phospholipids[3].
61
62   It has been shown that when the cells are cultured at an air-liquid interface (ALI), a
63   surfactant is released on the apical side of air-exposed epithelial cells, reducing surface
64   tension[11-13]. This feature is particularly important in nanomaterial respiratory hazard and
65   toxicity investigations. Once inhaled nanomaterials/toxicants are deposited in the alveolar
66   region, they first interact with pulmonary surfactant and are displaced by wetting forces into
67   the aqueous hypophase, where the interaction with pulmonary cells takes place[14,15]. Even
68   though A549 cells form a monolayer (which can overgrow into multilayers at later
69   timepoints when cultivated at ALI) and produce surfactant, a drawback is their insufficient
70   tight junction formation, resulting in low transepithelial electrical resistance values[16-18].
71
72   In the lungs, there is a variety of immune cell populations, including phagocytic and
73   professional antigen-presenting cells (i.e., macrophages and dendritic cells) that directly
74   communicate through cell-cell contact or intercellular signaling to control and maintain
75   homeostasis. Macrophages and dendritic cells are critical innate immune effectors and
76   initiators of the adaptive immune response[19]. Dendritic cells residing within or beneath the
77   epithelium can form protrusions across the epithelium to the lumen to catch antigens.
78   Alveolar macrophages are located on the apical surface of the epithelium and act as sentinel
79   cells, representing the first cellular defense against foreign material as well as bacterial, viral
80   and fungal infections. Their phenotypic plasticity allows rapidly induction of
81   proinflammatory reactions in response to such stimuli as well as shifting into triggering anti-
82   inflammatory (i.e., inhibitory) reactions[20].
83
84   To simulate the human alveolar epithelial tissue barrier, we established a triple coculture
85   model with A549 cells supplemented with human blood monocyte-derived macrophages
86   (MDMs) and dendritic cells (MDDCs) on the apical and basal sides, respectively[17]. Cultivation
87   of this model at ALI has been previously reported[16], even up to 72 h post-exposure[21]. Acute
88   immune responses to carbon nanotubes exposures was significantly enhanced in cell culture
89   exposed to the ALI as compared to submerged conditions[22]. The coculture model, cultured
90   and exposed to different materials at ALI, has been previously used to investigate
91   cytotoxicity, oxidative stress and inflammatory responses upon exposures to zinc oxide,[23]
92   graphene-related materials[24], gold nanoparticles[25,26], carbon nanotubes[21], and volcanic ash
93   and diesel exhaust particles[27].
94

95 Furthermore, the important role of macrophages and dendritic cells as immune effector cells
96 in an in vitro human lung model has been confirmed. In particular, an increased
97 proinflammatory response in the model was observed only in the presence of immune cells
98 in comparison to monoculture systems[7]. Potential drawbacks of using primary monocyte-
99 derived immune cells are the limited accessibility of PBMs as well as donor-to-donor
100 variation. As a solution to these potential drawbacks, presented here is a protocol
101 introducing cryopreservation of freshly isolated PBMs[28] for the cell culture model assembly.
102 The aim of this study is to demonstrate the 3D human alveolar epithelial tissue model
103 assembly, including the isolation of PBMs from human buffy coats. The responsiveness to
104 proinflammatory stimuli is compared to the model composed of MDMs and MDDCs
105 differentiated from fresh PBMs or differentiated from frozen/thawed PBMs.
106
107 Working with untested human blood samples involves specific care to prevent the potential
108 transmission of infectious diseases, such as HIV (human immunodeficiency virus), hepatitis
109 B, and hepatitis C. Therefore, the use of personal protective measures such as gloves, gowns,
110 masks, and eye protection are crucial and must be in accordance with good laboratory
111 practice principles. These protections reduce the risk of exposing the skin or mucous
112 membranes to potentially infectious fluids. Additionally, for those involved in handling buffy
113 coats and PBMs, vaccination against the hepatitis B virus is mandatory, and blood titer levels
114 of antibodies anti-hepatitis B must be above 100 IU/L (country-specific legislative
115 requirements need to be addressed). In addition, all work must be performed in biosafety
116 level 2 laboratories (country-specific legislative requirements need to be addressed).
117 Standard health and safety precautions associated with working in a laboratory environment
118 and handling mammalian cell culture, including handling of waste, should be adopted when
119 conducting the entire protocol.
120
121 **PROTOCOL:**
122
123 The work involving primary monocytes isolated from human blood was approved by the
124 committee of the Federal Office for Public Health Switzerland (reference number: 611-1,
125 Meldung A110635/2) for the Adolphe Merkle Institute.
126
127 **1. Isolation of peripheral blood monocytes (PBMs) from human buffy coats**
128
129 NOTE: The following section describes the isolation of immune cells from one 50 mL bag of a
130 buffy coat, purchased from the Swiss Transfusion Centre in Bern, Switzerland.
131
132 **1.1. Preparation of reagents**
133
134 1.1.1. Prepare 100 mL of magnetic separation buffer per buffy coat: 0.5% (w/v) bovine
135 serum albumin (BSA; in phosphate-buffered saline [PBS]) with 2 mM
136 ethylenediaminetetraacetic acid (EDTA; adjust to pH = 7.2, sterile filter with 0.22 μm pore
137 size). Keep at 4 °C throughout the procedure.
138
139 1.1.2. Prepare the cell culture medium (CCM): RPMI 1640 with 10% fetal bovine serum (FBS),
140 1% L-glutamine (here, 2 mM L-glutamine), and 1% penicillin-streptomycin (here, 100
141 units/mL penicillin and 100 μg/mL streptomycin).

142

143 NOTE: The required amount of each reagent depends on the number of cells to be seeded in
144 the following steps.

145

146 **1.2. Isolation of PBMs**

147

148 NOTE: All glass and plasticware needs to be sterilized before use. For safety reasons, the use
149 of plasticware is recommended when handling human blood samples to reduce the risk of
150 injury with glassware.

151

152 1.2.1. Use scissors to cut open the hose end of the bag containing the buffy coat.

153

154 1.2.2. Distribute the buffy coat by pouring the bag's contents through the bag's duct directly
155 into two conical centrifuge 50 mL tubes (~25 mL each).

156

157 1.2.3. Gently pour or pipette PBS into the tubes to reach 50 mL volumes. Mix the contents
158 by turning the tube gently upside down 3x.

159

160 1.2.4. Divide the buffy coat-PBS mixture into four new 50 mL conical centrifuge tubes by
161 pipetting 25 mL of the mixture in each fresh tube.

162

163 1.2.5. Slowly lay 13 mL of density gradient medium underneath the buffy coat-PBS mixture
164 using a 10 mL serological pipette. Detach the filled pipette from the pipette holder and
165 immediately plug the upper opening of the pipette with a thumb to prevent any additional
166 leaking of the density gradient medium into the buffy coat-PBS mixture.

167

168 NOTE: Holding the upper opening with the thumb, place the filled pipette at the bottom of
169 the conical centrifuge tube so that the density gradient medium slowly flows beneath the
170 buffy coat-PBS mixture, leaving approximately 1 mL of the density gradient medium inside
171 the pipette.

172

173 1.2.6. Repeat steps 1.2.4–1.2.6 with the other three tubes containing the buffy coat-PBS
174 mixture.

175

176 1.2.7. Centrifuge all four tubes containing the mixtures for 20 min at 1,000 $x g$ and 25 °C at a
177 slow braking mode (here, maximum brake = 4). Use holders for centrifugation.

178

179 1.2.8. Open the lid of each tube, remove the upper layer containing plasma and platelets
180 using a serological pipette, and dispose in a biohazard liquid waste container.

181

182 1.2.9. Use a serological pipette for collecting the peripheral blood mononuclear cell layer,
183 which appears as a whitish turbid small fraction (~2–3 mm in thickness) between the plasma
184 and the density gradient medium layers. The pellet contains red blood cells at the bottom.
185 Avoid transferring erythrocytes that form the bottom-most layer. Repeat this for all four
186 tubes.

187

188 NOTE: Peripheral blood mononuclear cells consist of PBMs and lymphocytes. PBMs will be
189 separated from lymphocytes later during magnetic CD14+ separation.

190

191 1.2.10. Pool peripheral blood mononuclear cells from two tubes into one 50 mL tube.

192

193 1.2.11. Fill up the two tubes with PBS to 50 mL and cover with a lid.

194

195 1.2.12. Discard the leftover erythrocytes and plasma from the original four tubes in a
196 biohazard liquid waste container.

197

198 1.2.13. Centrifuge the two tubes for 8 min at 500 x g and 18–20 °C at a regular centrifuge
199 speed.

200

201 1.2.14. After centrifugation, remove the supernatant with a serological pipette and discard it
202 in a biohazard liquid waste container.

203

204 1.2.15. Resuspend the cells with 5 mL of PBS using a serological pipette.

205

206 1.2.16. Pool the cell suspensions into one 50 mL conical centrifuge tube and fill to mL with
207 PBS.

208

209 1.2.17. Use 5 µL of the cell suspension to count the cells with a cell counter using the trypan
210 blue (45 µL) exclusion method.

211

212 1.2.18. Pipette 10 µL of the trypan blue-PBMs solution into a cell counter chamber and
213 count the number of cells per the standard counting protocol. Use Equation 1 to calculate
214 the total cell number, C_T.

215

216 $$c_T = \frac{\text{counted cells}}{\text{mL}} * 50 \qquad (1)$$

217

218 1.2.19. After counting the cells, centrifuge the 50 mL tubes as done in step 1.2.13.

219

220 **1.3. CD14 positive selection**

221

222 1.3.1. Gently open the lid of each tube, then remove and discard the supernatant using a
223 serological pipette without disturbing the pellet.

224

225 1.3.2. Add the calculated amount (Equation 2) of magnetic separation buffer (here, 80 µL of
226 buffer per 1 x 10^7 total cells) and resuspend the cell pellet by pipetting the solution up and
227 down.

228

229 $$buffer\ volume = \frac{c_T * 80}{10^7} = µL \qquad (2)$$

230

231 1.3.3. Calculate using Equation 3 (here, 10 µL per 1 x 10^7 total cells) the corresponding
232 volume of CD14+ magnetic beads and pipette the appropriate volume.

233

$$beads\ volume = \frac{c_T*10}{10^7} = \mu L \qquad (3)$$

1.3.5. Upon incubation, fill the tube up to 50 mL with magnetic separation buffer.

1.3.6. Centrifuge as done in step 1.2.13.

1.3.7. Aspirate and discard the supernatant using a serological pipette without disturbing the cell pellet.

1.3.8. Pipette the corresponding amount of magnetic separation buffer (Equation 4; here, 500 μL of buffer per 1 x 10^8 cells) and gently mix by pipetting up and down 3x.

$$buffer\ volume = \frac{c_T*50}{10^7} = \mu L \qquad (4)$$

1.3.9. Disinfect the magnetic separation station by spraying and wiping it with a sterilizing agent. Place in the laminar flow hood along with the column for magnetic separation.

1.3.10. Place the magnetic separation column in the magnetic field and place an empty 50 mL conical centrifuge tube directly under the column for collecting the wash and unlabeled cells (i.e., waste).

1.3.11. Rinse the magnetic separation column by pipetting 3 mL of magnetic separation buffer into the column. Do not let the column dry out throughout the procedure.

1.3.12. Prepare a 15 mL conical centrifuge tube and pipette 1 mL of magnetic separation buffer.

1.3.13. Apply the cell suspension (prepared in step 1.3.8) to the magnetic separation column. In the 50 mL conical centrifuge tube under the filter, collect unlabeled cells that passed through.

NOTE: Do not exceed 2 x 10^9 cells per column to avoid blocking of the column.

1.3.14. As soon as the column reservoir is empty (i.e., when the cells have passed through the column), apply 3 mL of magnetic separation buffer using a serological pipette and let it pass through the column. Repeat this 3x.

1.3.15. Remove the magnetic separation column from the magnetic separator by gently pulling with hands, then place it into a 15 mL tube containing pre-pipetted 1 mL of magnetic separation buffer (prepared in step 1.3.12).

1.3.16. Add 5 mL of magnetic separation buffer to the column and flush out the magnetically labeled cells by firmly pushing the plunger into the column.

1.4. Reagent preparation for MDM and MDDC differentiation

1.4.1. Count the cells with a cell counter using the trypan blue exclusion method as done in steps 1.2.17 and 1.2.18.

1.4.2. Calculate the required volumes of CCM or FBS for further steps as follows: either the volume of CCM corresponding to a cell density of 1 x 10^6 cells/mL (step 1.4.4.1), or the volume of FBS corresponding to a cell density of 6 x 10^6 cells per 0.9 mL of FBS (step 1.4.4.2).

Total cell number (c$_T$)

$$c_T = \frac{counted\ cells}{mL} * 6$$

CCM volume (step 1.5.1)

$$CCM\ volume = \frac{c_T}{10^6} = \mu L$$

FBS volume (step 1.6.3)

$$FBS\ volume = \frac{c_T}{6*10^6} * 0.9 = \mu L$$

1.4.3. Close the lid, place the tube in the centrifuge, and centrifuge as done in step 1.2.13. Remove and discard the supernatant without disturbing the cell pellet. Proceed to step 1.5 for cell seeding or step 1.6 for cell freezing.

1.5. PBM seeding and differentiation into MDMs and MDDCs

1.5.1. Resuspend the cell pellet in the calculated volume of cell CCM as calculated in step 1.4.2 (here, a final concentration of 1 x 10^6 cells/mL) by pipetting up and down 3x.

1.5.2. Pipette the amount of cells intended to differentiate into MDMs and MDDCs in separate conical centrifuge tubes using a serological pipette.

1.5.3. Pipette differentiating factors to the CCM with PBMs and mix well by pipetting up and down. Differentiating factors are applied as follows:

1.5.3.1. For MDDCs: final concentration of 10 ng/mL interleukin-4 (IL-4) and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).

1.5.3.2. For MDMs: final concentration of 10 ng/mL macrophage colony-stimulating factor (M-CSF).

1.5.4. Pipette the cell suspensions in CCM with the added differentiating factors into 6 well plates by distributing 3 mL of the suspension per well (corresponds to 3 x 10^6 cells/mL).

1.5.5. Place the 6 well plates in a cell culture incubator (37 °C, 5% CO$_2$) and let them differentiate for 6 days without refreshing the CCM.

NOTE: Differentiation ranges from 5–8 days depending on the local availability of buffy coats and experimental setup, provided that the differentiation efficiency is determined using the appropriate techniques (refer to the discussion section).

1.6. PBM freezing

1.6.1. Resuspend the cell pellet in cryoprotective medium (here, FBS and dimethyl sulfoxide [DMSO; cytotoxic]) at a ratio of 9:1 (v/v) by pipetting a volume of prewarmed FBS. This corresponds to a final cell concentration of 6×10^6 cells/mL, considering a further addition of 10% DMSO (v/v).

1.6.2. Mark the desired number of cryovials in the laminar flow hood (i.e., record the date, isolation code, and number of cells).

1.6.3. Pipette 0.9 mL of cell suspension in pure FBS (here, 6×10^6 cells in 0.9 mL of FBS) to each cryovial. Subsequently, slowly pipette 0.1 mL of DMSO and mix the suspension well by turning the cryovials up and down 3x.

1.6.4. Transfer the cryovials to a cell-freezing container and immediately set it to -80 °C for 24 h.

1.6.5. After 24 h, remove the cryovials from the -80 °C freezer and container and place them into the liquid nitrogen tank suitable for cell storage.

1.7. PBM thawing and differentiation into MDMs and MDDCs

1.7.1. Warm all the required reagents to 37 °C in a water bath (~20–30 min).

1.7.2. Prepare the appropriate number of 6 well plates corresponding to the number of thawed cells (here, one plate per 1.8×10^7 cells). Pipette 2 mL of CCM to each well under aseptic conditions. Place plates in the incubator (5 % CO_2, 37 °C) for 15 min to allow the pH to equilibrate.

1.7.3. Take the required amount of cryovials with frozen cells from a liquid nitrogen tank and gently swirl them in a 37 °C water bath (1–2 min) to ensure uniform thawing of the cell suspension.

1.7.4. Remove the cryovial from the water bath and decontaminate with a sterilizing agent, ensuring that the agent does not interact with the lid and O-ring.

NOTE: Hereon, all steps must be completed under aseptic conditions.

1.7.5. Prepare the appropriate number of 15 mL conical centrifuge tubes corresponding to the number of cryovials to be thawed (here, 6×10^6 cells/tube). Pipette 9 mL of prewarmed CCM into each tube.

1.7.6. Pipette slowly (drop-by-drop) the contents of the cryovial into a tube containing CCM. Close the lid, repeat for each tube, and centrifuge at 200 x g for 5 min at a regular centrifuge speed.

1.7.7. Discard the supernatant without disturbing the pellet.

374

375 1.7.8. Resuspend the pellet from each tube (which contains cells from one cryovial) in 2 mL
376 of prewarmed CCM by pipetting up and down using a serological pipette (cell density
377 corresponding to 3 x 10^6 cells/mL).

378

379 1.7.9. From each tube, pipette the resuspended cells into two wells of a 6 well plate
380 containing 2 mL of previously prepared CCM (1 mL per well) to reach a cell density of 3 x 10^6
381 cells/well (corresponding to a final concentration of 1 x 10^6 cells/mL). Repeat this for all
382 other tubes.

383

384 1.7.10. Proceed with differentiation as described in section 1.5.3.

385

386 1.7.11. Place the 6 well plates in a cell culture incubator (37 °C, 5% CO_2) and let them
387 differentiate for 6 days without refreshing the CCM.

388

389 **2. Triple cell coculture model of human alveolar epithelial tissue**

390

391 NOTE: This section provides instructions on volumes and cell numbers corresponding to 12
392 well plate inserts. **Figure 1** summarizes a proposed timeline for model assembly.

393

394 **2.1. Epithelial cell (A549 cell line) seeding**

395

396 2.1.1. Culture the epithelial cells according to the recommendations provided by the supplier
397 (ATTC). Briefly, subculture the cells in CCM at 80% cell confluency (here, 2x–3x per week).

398

399 NOTE: Subculture A549 for at least four passages before the coculture model composition,
400 using A549 cells in a passage range of 5–25.

401

402 2.1.2. Pipette 1.5 mL of prewarmed CCM into 12 well plates (the number of wells
403 corresponds to the desired number of models).

404

405 2.1.3. Place individual 12 well cell culture inserts into wells of a 12 well plate using sterilized
406 tweezers.

407

408 2.1.4. Detach the cells from a flask according to the subcultivation protocol (i.e., using a
409 detaching agent, remove the agent by centrifugation as done in step 1.2.13). Resuspend in
410 the appropriate volume of CCM corresponding to the final cell concentration of A549 (here,
411 50 x 10^4 cells/mL in 0.5 mL per insert and seeding density of 2.78 x 10^5 cells/cm^2).

412

413 2.1.5. Pipette 0.5 mL of the cell suspension (i.e., 5.0 x 10^5 cells/mL) into the apical side of the
414 insert using a 1 mL pipette.

415

416 2.1.6. Cover the plates with lids and place them in a cell culture incubator (37 °C, 5 % CO_2)
417 for 4 days.

418

419 NOTE: Regularly check the confluency of A549 cells under a phase-contrast microscope.

420

2.2. MDDC seeding

2.2.1. Aspirate CCM with unattached cells in the MDDCs containing 6 well plates.

2.2.2. Add 1 mL of fresh prewarmed CCM to each well.

2.2.3. Use a cell scraper, detach (scrape) adherent MDDCs from each well, gently wash the wells with the existing 1 mL of CCM 3x, and combine them into one conical centrifuge tube.

2.2.4. Count the cells with a cell counter using the trypan blue exclusion method using 10 μL of cell suspension and 10 μL of trypan blue solution.

2.2.5. Centrifuge the cell suspension as done in step 1.2.13.

2.2.6. Calculate the CCM volume required for resuspension (Equation 5):

Cell density (CD)   =   _____cells/mL
Cell suspension volume (Vs) =   _____mL

$$c_T = \frac{CD}{mL} * Vs \qquad (5)$$

Required MDDC density = 4.2×10^5 cells/mL; each insert requires 6.3×10^4 cells, which corresponds to a seeded cell density of 7×10^4 cell/cm^2.

CCM volume for cell resuspension (Vm; Equation 6):

$$CCM\ volume = \frac{CD}{42 \times 10^4} * Vs = mL \qquad (6)$$

2.2.6. Gently aspirate and discard the CCM from the upper chamber of 12 well plates with growing A549 on the inserts.

2.2.7. Place the inserts with A549 cells in an upside-down position in a sterile Petri dish using sterilized tweezers. Prepare a conical centrifuge tube (50 mL) with PBS and pre-moisten a cell scraper.

2.2.8. Scrape off A549 cells from the basal surface of the insert (i.e., the upper part at the upside-down position), which should grow through the pores of the inserts.

NOTE: Rinse the scraper with PBS (prepared in a tube) between scraping individual samples and keep it wet throughout the procedure.

2.2.9. Upon centrifugation (step 2.2.5), aspirate and discard the supernatant, then redisperse the MDDC pellet in the calculated amount of CCM (step 2.2.6) and pipette up and down 3x.

2.2.10. Pipette 150 μL of the cell suspension on top of each insert so that the entire basal surface of the insert is equally covered with the liquid and not containing bubbles.

468

469 2.2.11. Cover the dish with a lid and place in a cell culture incubator for 70 min. Aspirate the
470 CCM from the cell culture plates (where the inserts have initially been placed), discard it in a
471 biohazard liquid waste, and pipette 1.5 mL of fresh CCM into each well. Cover the plate with
472 a lid and place in the cell incubator (37 °C, 5% CO_2).

473

474 NOTE: Do not exceed the time period mentioned above to avoid drying out the cells.

475

476 2.2.12. After the incubation, carefully hold each insert with sterilized tweezers and place
477 them to the plates containing CCM in an ordinary position. Cover the plate with a lid and
478 return it to the cell incubator (37°C, 5% CO_2).

479

480 **2.3. Macrophage (MDM) seeding**

481

482 2.3.1. Take 6 well plates containing predifferentiated MDMs. Place them from the cell
483 incubator and place them to a laminar flow hood.

484

485 2.3.2. Aspirate and discard CCM with unattached MDMs grown in 6 well plates, and pipette
486 1 mL of fresh prewarmed CCM in each well.

487

488 2.3.3. Using a cell scraper, gently remove adherent MDMs from individual wells (as done in
489 step 2.2.3 for the MDDCs).

490

491 2.3.4. Pipette 10 µL of trypan blue into a well or tube and add 10 µL of the MDM suspension
492 to achieve the final dilution of 1:1 (v/v). Count the number of MDMs using the appropriate
493 counting protocol.

494

495 2.3.5. Centrifuge the cell suspension as done in step 1.2.13.

496

497 2.3.6. Calculate the required volume (Equation 7):

498

499 Cell density (CD)   = _____ cells/mL
500 Cell suspension volume (Vs) = _____ mL
501 Required MDM density   = 2.5 x 10^4 cells/mL in CCM (here, each insert requires 1.25 x
502 10^4 cells, which corresponds to a seeded cell density of 2.5 x 10^4 cell/cm^2).

503

504 $$CCM \ volume = \frac{CD}{2.5 \times 10^4} * Vs = \ mL \qquad (7)$$

505

506 2.3.7. Upon centrifugation, aspirate and discard the supernatant, redisperse the MDM pellet
507 in the calculated amount of CCM (step 2.3.6), and pipette up and down 3x.

508

509 2.3.8. Carefully pipette 0.5 mL of the MDM suspension (prepared in step 2.3.7) on the wall
510 of the cell culture inserts with A549 and MDDCs (not directly on epithelial cells) using a 1 mL
511 pipette. Cover plates with lids and place in a cell culture incubator (37 °C, 5 % CO_2) for 24 h.

512

513 **2.4. Transfer of coculture model to air-liquid interface (ALI)**

514

515 2.4.1. Upon the end of the 24 h (±2 h) incubation period of the assembled model in a cell
516 culture incubator, aspirate and discard CCM from both apical and basal parts of cell culture
517 inserts and from the wells.

519 2.4.2. Using sterilized tweezers, lift individual inserts from the wells and pipette 0.6 mL of
520 fresh prewarmed CCM to each well using a 1 mL pipette. Do not add CCM to the apical side
521 of the insert.

3. Exposure to selected positive controls (known stimuli for inducing proinflammatory response)

NOTE: Exposure of the coculture models to a known proinflammatory stimulus endotoxin lipopolysaccharide (LPS)[7] and proinflammatory cytokine tumor necrosis factor α (TNF-α)[7] is used to illustrate responsiveness of the model. Furthermore, exposure to a detergent (Triton X-100) is used to confirm the sensitivity of a lactate dehydrogenase (LDH) assay.

3.1. Prepare the positive control solutions: LPS stock (1 mg/mL in distilled water), TNF- α stock (100 µg/mL in distilled water) and Triton-X 100 (2% [v/v] in PBS).

3.2. Upon 24 h incubation of the coculture model at ALI conditions, aspirate and discard the supernatant from the basal compartment. Using sterilized tweezers, lift individual inserts from the wells and pipette 0.6 mL of fresh prewarmed CCM into each well.

3.3. Prepare individual positive controls working solutions by diluting the stocks in CCM in conical centrifuge tubes as follows: 1 µg/mL LPS, 1 µg/mL TNF- α, and 0.2% Triton-X 100. The volumes correspond to the number of tested inserts (here, 100 µL/insert). Mix the solutions well by pipetting up and down 3x.

3.4. Apply 100 µL of each positive control solution by slowly pipetting onto the wall of the cell culture insert. Cover the well plate with a lid and place in the cell culture incubator (37 °C, 5% CO_2) for 24 h. Upon incubation, aspirate and discard the liquid at the apical side of the insert by holding individual inserts using tweezers.

3.5. Collect CCM in the basal compartments and store at 1) 4 °C for further LDH analysis, denoting cell membrane rupture-mediated cytotoxicity, and/or 2) store at -80 °C for further analysis of protein release via enzyme-linked immunosorbent assay (ELISA). Run the assays according to the kit supplier recommendations.

3.6. Upon the removal of CCM, wash the inserts with PBS 3x and fix the cells on cell culture inserts in 4% paraformaldehyde (in PBS, 15 min at room temperature [RT]) by ensuring both insert sides are well-covered with PFA solution. Subsequently wash 3x with PBS to remove PFA. Store the samples submerged in PBS at 4 °C for further immunostaining (an example of this method has been described previously[17]).

REPRESENTATIVE RESULTS:

Human lung coculture models, composed of alveolar epithelial cells and immune cells, were assembled either from fresh or frozen MDDCs and MDMs progenitors (here, human

563 peripheral blood-derived monocytes). As presented in **Figure 1**, A549 cells were seeded 3
564 days after the first section involving monocyte isolation/thawing. After 6 days of
565 differentiation, the differentiated MDMs appeared round-shaped, whereas MDDCs formed a
566 more elongated shape with observable protrusions. They also appeared as agglomerates,
567 especially when differentiated from fresh monocytes (**Figure 2, Figure 3**). Epithelial cells
568 formed a dense cell layer of cells after 3 days of growth on membrane inserts (**Figure 4**),
569 when the cocultures were assembled. After 24 h of assembling and an additional 24 h of
570 being subjected to ALI conditions, the cocultures were prepared for exposures.

572 Responsiveness of the 3D cell culture models was investigated upon exposure to known
573 proinflammatory stimuli using a pseudo-ALI approach, as described previously[29]. The
574 proinflammatory stimuli, LPS and TNF-α, were added in low volumes (100 µL) onto the apical
575 surface of the air-exposed cell model. In parallel, the absence of membrane rupture as a
576 measure of cytotoxicity was assessed via LDH assay. A significant increase in LDH release in
577 CCM of the basal compartment was observed upon exposure to the positive control for
578 membrane rupture, a detergent Triton-X 100 (**Figure 5**). These results proved responsiveness
579 of the model to a cytotoxic substance, whereas no increase in LDH release was observed
580 upon apical stimulation with TNF-α or LPS.

582 A possible reason for the different measured values of LDH in the samples assembled with
583 either fresh or previously frozen PBMs can be attributed to the sample storage. Samples
584 from fresh PBMs were stored for a longer time at -80 °C; therefore, the activity of LDH
585 enzyme may drop. Notably, LDH is stable for only up to 4 days in CCM; thus, it is
586 recommended to perform the assay the latest 2 days after collecting the supernatants.
587 Alternatively, it is possible to freeze down the supernatants directly after collection.
588 However, it is important to consider that freezing can decrease the enzymatic activity of
589 LDH.

591 Secretion of proinflammatory mediators (here, TNF-α and interleukins 6 [IL-6] and 8 [IL-8])
592 into the basal CCM was quantified via ELISA. Statistically significant ($p < 0.05$, one-way
593 ANOVA) increases in the release of IL-6 and IL-8 were observed in both LPS- and TNF-α-
594 treated samples compared to respective untreated cells, as well as in the cell culture models
595 assembled from either PBMs source (**Figure 6**). Although concentrations (pg/mL) of all the
596 tested cytokines in the basal CCM were higher in the cocultures composed of fresh PBMs,
597 differences among the two cocultures and monocultures were not statistically significant (p
598 > 0.05) (**Figure 6**). To confirm the added value of coculture models with respect to a 2D
599 epithelial cell culture, A549 monocultures were also exposed to LPS or TNF-α. As expected,
600 the release of all investigated mediators from A549 monocultures was lower compared to
601 both coculture models; although, the difference between them was not statistically
602 significant ($p > 0.05$, one-way ANOVA).

604 Cellular morphology of the 3D human alveolar epithelial tissue barrier was assessed via
605 confocal laser scanning microscopy (LSM). To visualize the composition of each model,
606 macrophages within the coculture models (MDMs) were stained with mature macrophage
607 marker 25F9 (also known as cluster of differentiation CD163). MDDCs were stained with
608 CD83, which is an important marker for activated dendritic cells[30]. Regarding cellular
609 morphology, no difference was observed between the coculture models using MDMs and

610 MDDCs from fresh PBMs compared to those using thawed PBMs. In LPS- and TNF-α-exposed
611 cocultures, both composed of fresh and frozen immune cells, a disrupted epithelial layer in
612 LSM images was observed, which was not the case in untreated cells (**Figure 7**, **Figure 8**).
613
614 **FIGURE LEGENDS:**
615
616 **Figure 1: Schematic timeline of the protocol.** Presentation of the 3D coculture model
617 preparation, assembly, and application (exposure to a tested substance). ALI = air-liquid
618 interface, MDDCs = monocyte-derived dendritic cells, MDMs = monocyte-derived
619 macrophages, PBMs = peripheral blood monocytes.
620
621 **Figure 2: MDMs and MDDCs differentiated from fresh PBMs.** Phase-contrast microscopy
622 image of differentiated (**A**) MDMs and (**B**) MDDCs from fresh PBMs (6 days after cell
623 isolation). MDMs are round-shaped, while MDDCs are often observed as agglomerates.
624
625 **Figure 3: MDMs and MDDCs differentiated from frozen PBMs.** Phase-contrast microscopy
626 image of differentiated (**A**) MDMs and (**B**) MDDCs from thawed PBMs (6 days after thawing).
627 MDMs are round-shaped, but some elongated cells can be observed. MDDCs also appear
628 round-shaped with protrusions.
629
630 **Figure 4: Epithelial cells growth on membrane inserts.** Phase-contrast microscopy image of
631 confluent A549 growing on a membrane insert 4 days after seeding, forming a dense layer of
632 cells.
633
634 **Figure 5: Cytotoxicity results investigated via membrane rupture-based (LDH) assay.** The
635 data is presented as a fold increase over untreated cells (mean ± SD, n = 3, asterisk denotes
636 statistically significant increase compared to untreated cells, **p < 0.01, ****p < 0.0001). In
637 green models MDMs and MDDCs from fresh PBMs are represented, and in purple models
638 those assembled from thawed PBMs are represented.
639
640 **Figure 6: Proinflammatory reactions in the cocultures and monocultures.** The
641 proinflammatory mediators (TNF-α, IL-6, and IL-8) release in the cocultures upon 24 h
642 challenge with LPS or TNF-α. The data is presented as relative to untreated cells (mean ± SD,
643 n = 3, **p < 0.01, ***p < 0.001, ****p < 0.0001). In green models MDMs and MDDCs from
644 fresh PBMs are represented, and in purple models those assembled from thawed PBMs are
645 represented. Grey represents A549 monocultures.
646
647 **Figure 7: Morphology of cocultures composed of fresh immune cells.** LSM images of apical
648 and basal sides of the coculture model with xz projections of apical sides of the model using
649 MDMs and MDDCs from fresh PBMs. Cyan represents nuclei (DAPI), magenta represents
650 cytoskeleton (rhodamine-phalloidin), white represents MDMs (25F9, also known as CD163),
651 and green represents MDDCs (CD 83). The white arrow denotes MDM, whereas the green
652 arrow denotes MDDC.
653
654 **Figure 8: Morphology of cocultures composed of frozen immune cells.** LSM images of the
655 apical side of the coculture model with corresponding xz projections, and basal side of the
656 model using MDMs and MDDCs from thawed PBMs. Cyan represents nuclei (DAPI), magenta

657 represents cytoskeleton (rhodamine-phalloidin), white represents MDMs (25F9, also known
658 as CD163), and green represents MDDCs (CD 83). The white arrow denotes MDM, whereas
659 the green arrow denotes MDDC.
660
661 **DISCUSSION:**
662
663 Emerging production of novel materials, including chemicals and drugs, gradually increases
664 the need for predictive in vitro models. To comply with the three principles of replacement,
665 reduction, and refinement of animal testing[32], in vitro cell models have become powerful
666 tools for elucidating mechanisms of a drug's or material's action[8-11]. Presented here is a
667 detailed protocol of assembling the multicellular model using immune cells that are either
668 freshly isolated or thawed from previously frozen monocytes. Also described is the
669 cultivation of the model at ALI. Finally, the protocol illustrates an example of exposure to
670 proinflammatory stimuli and compares the response of the two models containing either
671 fresh or frozen monocytes.
672
673 Various studies have been performed to confirm and justify the added value of the
674 enhanced complexity of the models grown and exposed under ALI conditions compared to
675 conventional submerged exposure[7,22,31]. Observation of the higher proinflammatory
676 response in cocultures compared to monocultures of epithelial cells confirm a previous
677 study. The study used the presented coculture model (stimulated with LPS) and showed a
678 higher response at gene expression levels of *TNF* and *IL1B* compared to the A549
679 monoculture equivalent model[7]. On the other hand, both models showed higher variations
680 within measured proinflammatory mediator release values compared to A549
681 monocultures. This can be explained by the use of immune cells from different donors (buffy
682 coats) within biological replicates (i.e., one repetition, one donor), as previously shown[7]. If
683 desired, the variations among the replicates can be overcome by 1) using thawed PBMs from
684 the same donor or 2) pooling PBMs from different donors prior to freezing the cells, then
685 subsequent usage of the same pool in each repetition. Including more biological replicates is
686 also recommended.
687
688 The cell-freezing technique can be considered as a critical step; however, it is a common
689 laboratory procedure for preserving cells for phenotypic and functional analysis. Various
690 studies have demonstrated that the quality of frozen PBMs is vital to their survival, and an
691 appropriate freezing technique is key to the success of subsequent assays with the same
692 cells[28,32]. Modification of the protocol can be performed by freezing PBMs, which provides
693 flexibility in the experimental setup, as the availability of buffy coats is usually limited.
694 Another advantage of using frozen PBMs (in several vials) over freshly isolated ones is that
695 they can be used in subsequent experiments even after 1 year. This decreases the potential
696 issue of donor-to-donor variability if this is a desired or required parameter in an
697 experimental.
698
699 Outcomes of an interlaboratory comparison performed after up to 13 months show that
700 PBMs, when properly stored in a liquid nitrogen tank, can be used over a long period
701 without any effect on cell viability or cell recovery[33]. Longer storage times (over 1 year) may
702 be possible upon careful validation of cell viability and cell responsiveness before performing
703 an experiment. Also, the temperature in the liquid nitrogen tank must remain stable at all

704   times. The main factor affecting the viability of cryopreserved PBMs was found to be DMSO
705   concentration, with an optimal concentration of 10%–20 % (v/v)[28]. To minimize potentially
706   harmful effects of freezing, different sources of proteins, FBS or BSA (with a broad range of
707   concentration from 40% up to 100 %[34]) are often added to the freezing medium as natural
708   protective components that can increase cell survival.
709
710   Due to the high cytotoxic potential of DMSO, it is recommended first to disperse PBMs in
711   FBS, then add DMSO to PBMs already dispersed in FBS. Notably, although higher FBS
712   concentrations (>40%) did not show any improvement in cell viability. However, at the same
713   time, they did not cause harm to the cells[28]. Nevertheless, freezing monocytes is a possible
714   approach to overcoming issues of limited buffy coat availability. However, if the use of
715   MDDCs and MDMs from fresh PBMs is desired, the immune cells can be differentiated and
716   used 5–8 days after isolation[7,16,17,35-37]. If experimental planning allows, at least 6 days of
717   differentiation in both MDDCs and MDMs is recommended. However, consistency among
718   different repetitions in the same experiment, along with routine inspections of their specific
719   surface marker expressions, are crucial. The responsiveness to a proinflammatory stimulus,
720   such as LPS, after the differentiation time should also be regularly checked.
721
722   Many investigations using the A549 cell line have been performed at ALI, either as a
723   monoculture or combined with other cell types (macrophages, dendritic cells, or fibroblasts)
724   into 3D coculture model[22,24,29,38]. Using this 3D coculture model, the cytotoxicity, oxidative
725   stress, or proinflammatory effectd of (nano-)materials have been investigated for up to
726   72 h[1,17,21,24,29]. The model's resemblance to in vivo tissue has previously been investigated
727   based on confocal laser scanning imaging of the model[16]. When assembling the model, it is
728   important to consider both cell proliferation (which can affect A549 in the model presented
729   here) as well as performance of the primary (not proliferating) immune cells (here, MDDCs
730   and MDMs). It is also important to consider that not all the CD14 positive monocytes
731   differentiate into MDDCs and MDMs, and that the cells can be present both attached and
732   suspended forms. Based on the nature of the coculture assembly (here, both cell types need
733   to attach to the existing epithelial layer), it is recommended to use only the adherent sub-
734   populations of both immune cell types. Additionally, routine analyses of monocytes, MDDC
735   and MDM monoculture responsiveness to LPS, and expression of specific surface markers
736   (CD14, CD163, CD86, CD93, or CD206, data not shown) have suggested that 6 and 7 days of
737   differentiation are the optimum timepoints.
738
739   Although a realistic number of alveolar epithelial cells in human lungs corresponds to
740   ~160,000 cells/cm^2, the number of A549 cells counted in the model is ~1,000,000 cells/cm^2
741   after 9 days cultured on the insert[16,18]. Thus, this in vitro model's limitations need to be
742   considered. First, the density of epithelial cells was established based on their ability to form
743   a confluent layer on the growing membrane. It is also important to mention that the A549
744   represents an epithelial type II cell with a cuboidal form, contrary to epithelial type I cells,
745   which are flat and outspread. On the other hand, the required number of immune cells was
746   established based on the literature and presented in this protocol as cell number/surface
747   area[39-41]. The cell density of MDDCs in the range of 400 cells/mm^2 (4 cells/cm^2)[16] is
748   comparable to the steady-state cell density of 500–750 cells/mm^2 (5- 7 cells/cm^2) reported
749   from in vivo studies[39]. The density of MDMs in this model is within the same range of in vivo
750   situation in the human alveolar region[40].

751

752 Mature macrophage staining (25F9) was observed both in the apical side (where MDMs are
753 present) as well as basal side (i.e., at the site of dendritic cells). Translocation of immune
754 cells through the membrane inserts pores is possible and has also been observed using this
755 model[16], which may explain the observed differences in staining intensities. However,
756 another possible explanation is that the mature macrophage marker can also be expressed
757 on dendritic cells, but the expression is highly donor-specific[42]. Also, the intensity of the
758 25F9/CD163 expression is much higher in MDMs (**Figure 7, Figure 8**). Both proinflammatory
759 stimuli (LPS and TNF-α) affected the integrity of the pulmonary epithelial barrier in both
760 cocultures (**Figure 7, Figure 8**). This was expected based on previous publications[43,44]
761 showing that proinflammatory cytokines and bacterial products disrupt the integrity of
762 epithelial barriers.
763

764 The 3D multicellular model of the human alveolar epithelium, established and characterized
765 previously[17], has served as a powerful and useful tool for assessing biological responses (i.e.,
766 acute proinflammatory reactions, oxidative stress response, particle distribution, and cellular
767 communication) in vitro[21,24,25,45]. The results confirm the responsivness of coculture models
768 to proinflammatory stimuli (here, LPS and TNF-α). The response was slightly increased when
769 using immune cells from fresh PBMs; however, there was no statistically significant
770 difference between cocultures using fresh vs. thawed PBMs. Furthermore, the
771 proinflammatory reactions of both coculture models were higher than those of epithelial cell
772 monocultures cultivated under the same (ALI) conditions. In summary, the protocol
773 describes the assembly of a 3D human alveolar epithelial tissue coculture model using either
774 fresh or thawed PBMs for differentiation into MDMs and MDDCs. It is shown that both
775 models are highly responsive to proinflammatory stimuli; therefore, they can serve as
776 powerful tools for potential hazard and toxicity assessments.
777

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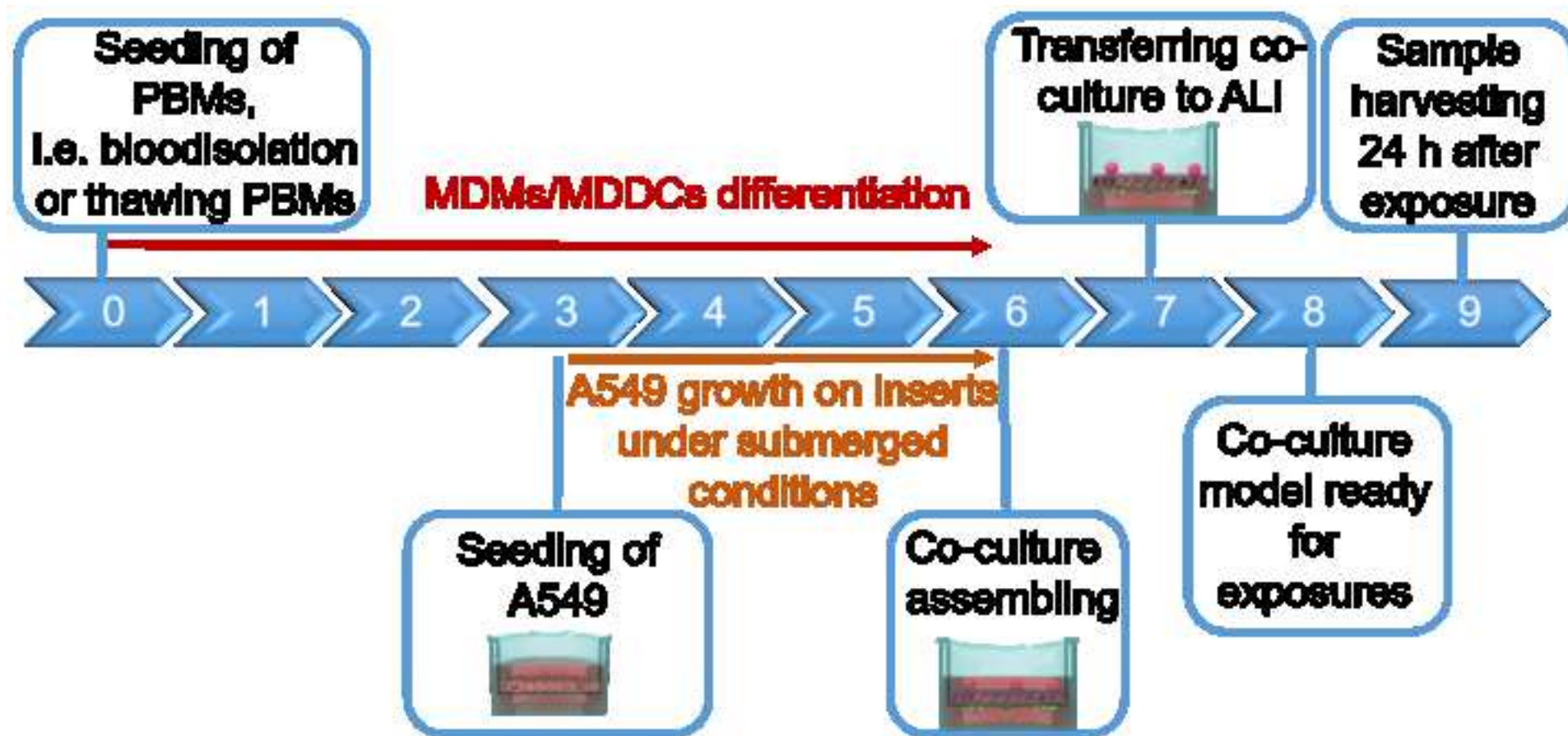837   **19**, (2006).
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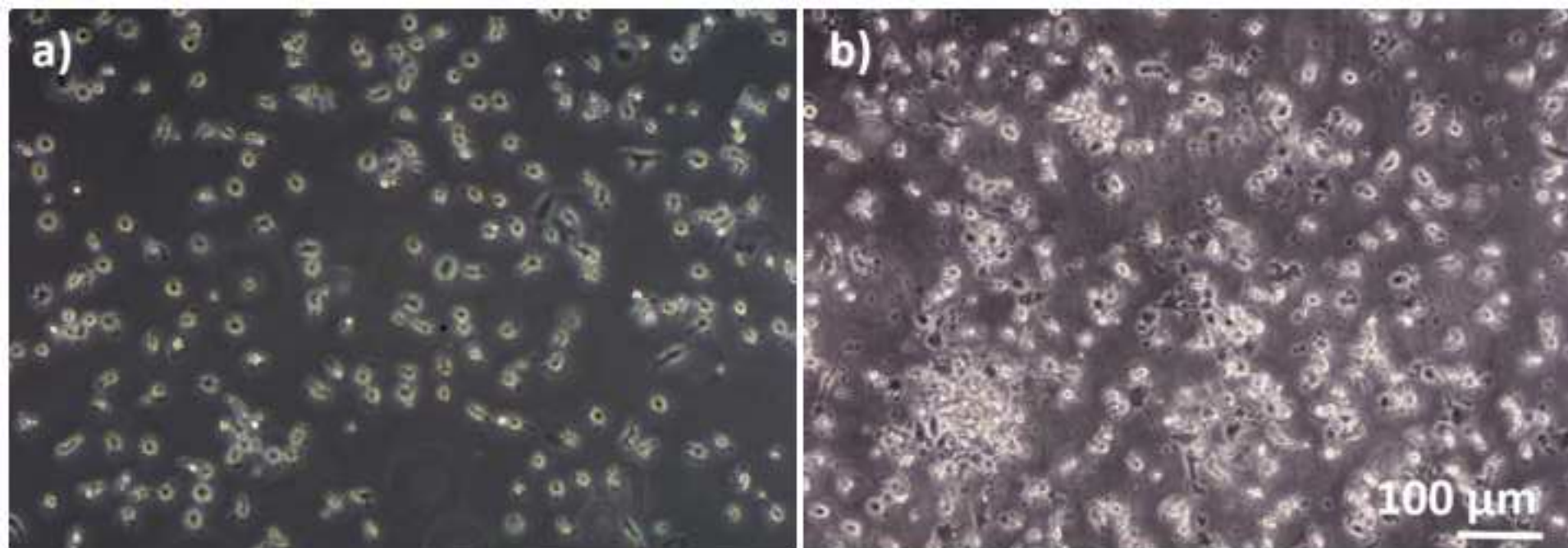840   20   Kopf, M., Schneider, C., Nobs, S. P. The development and function of lung-resident
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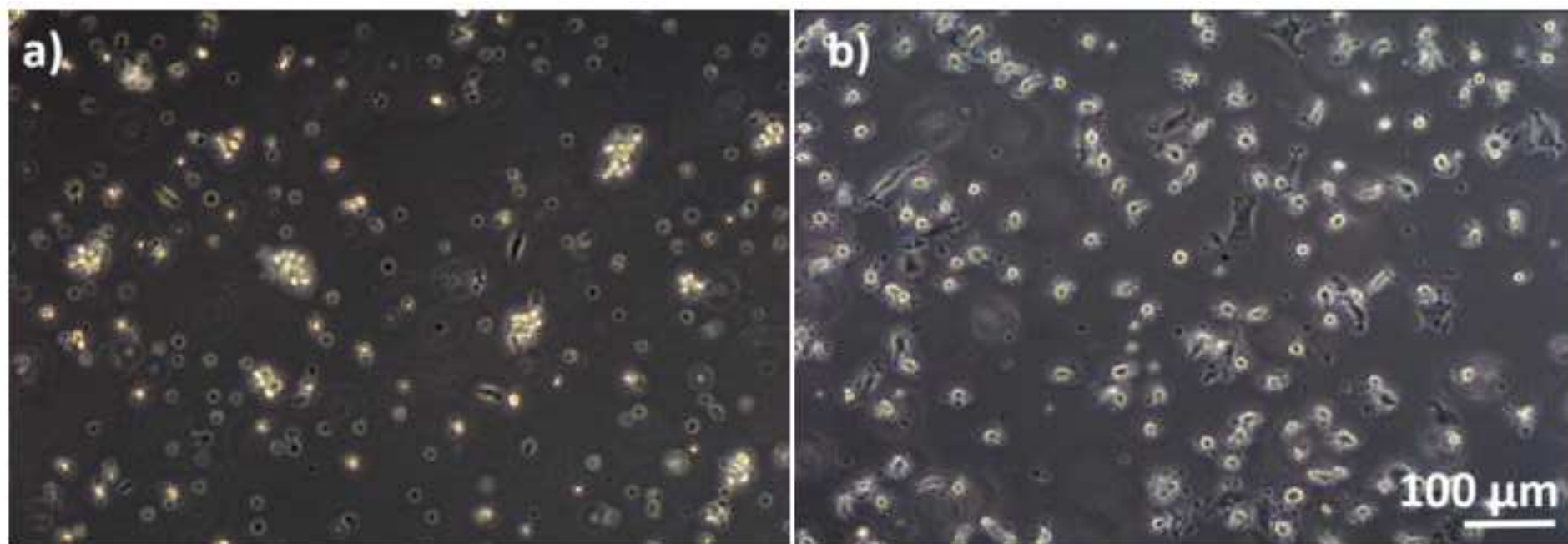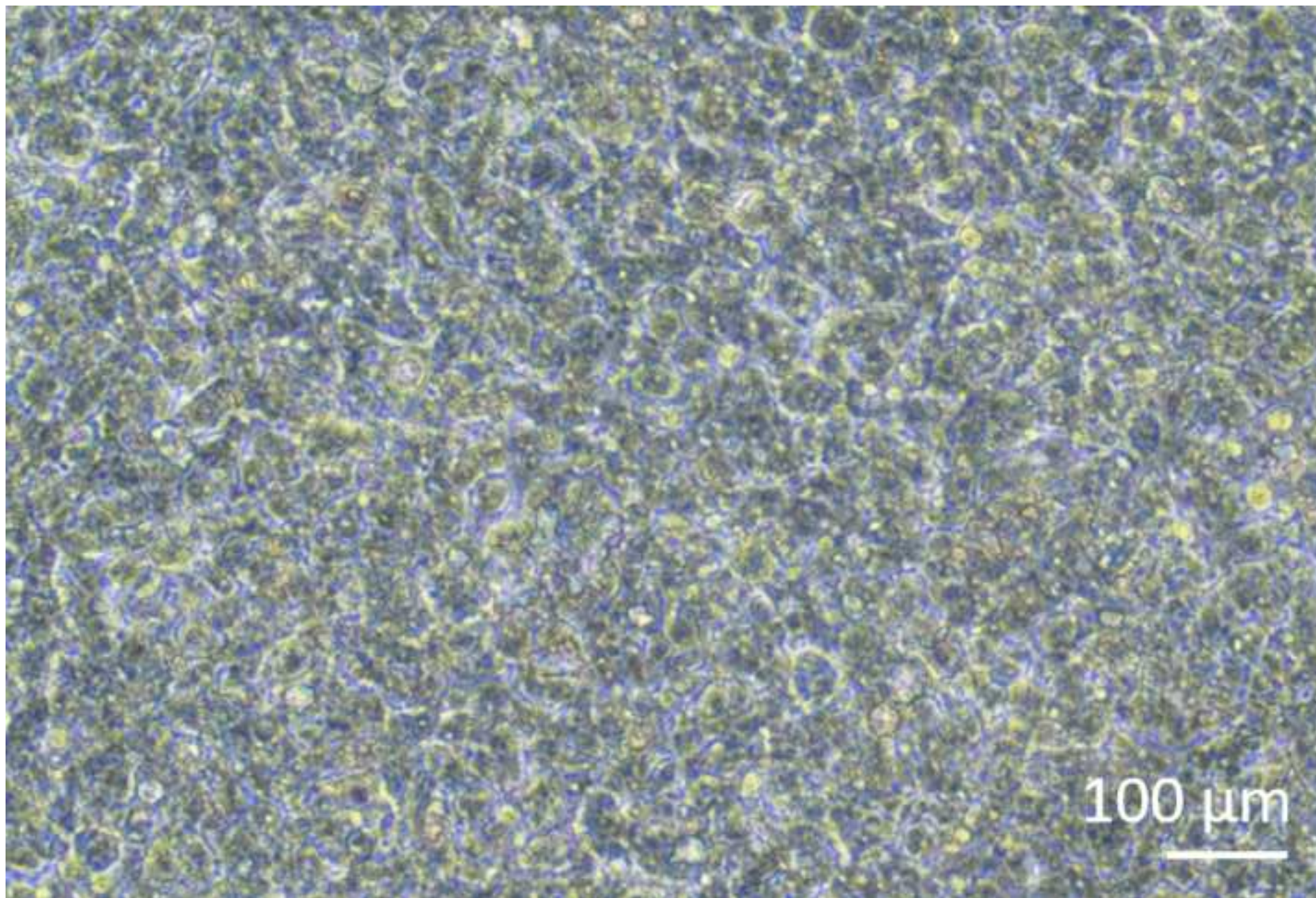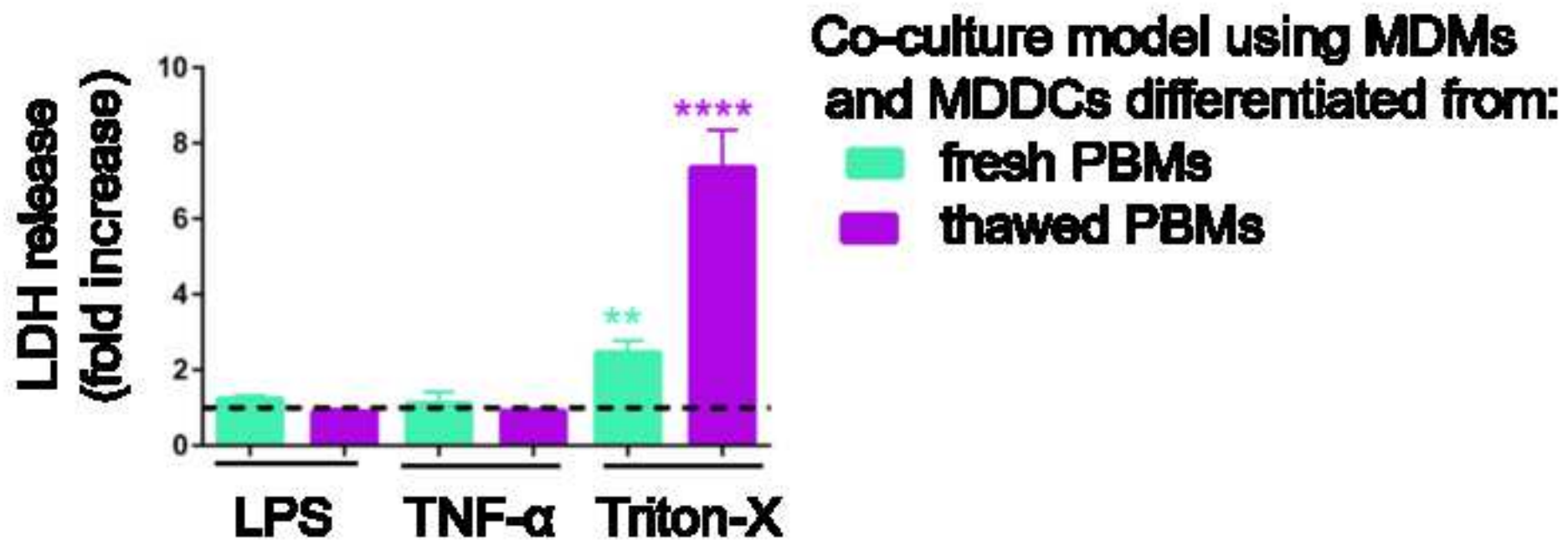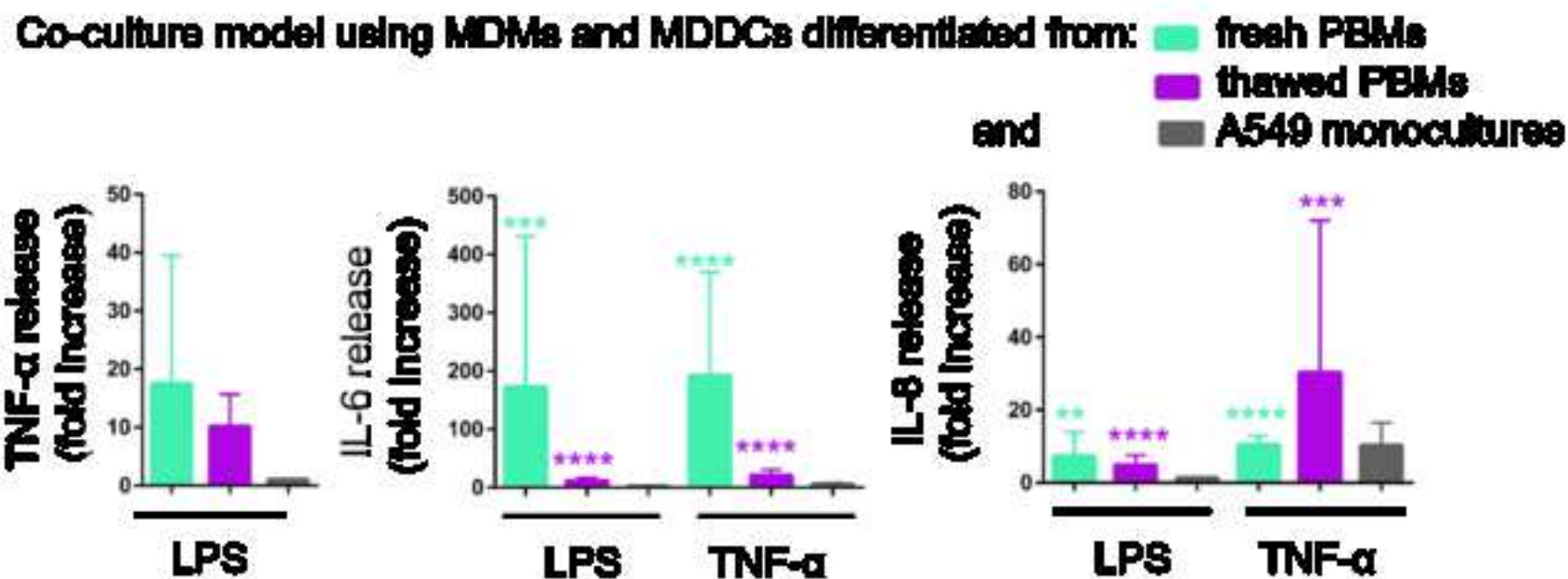912   *Toxicology.* **14** (49) (2017).
913

Figure 2

Figure 3

Figure 4

Figure 5

Click here to access/download;Figure;Figure 5.tif ±

Co-culture model using MDMs and MDDCs differentiated from:
- fresh PBMs
- thawed PBMs

Figure 6   Click here to access/download;Figure;Figure 6.tif ⬇

Co-culture model using MDMs and MDDCs differentiated from: fresh PBMs / thawed PBMs / and A549 monocultures

Figure 7

Untreated cells

LPS treated cells

TNF-α

Apical side

Basal side

30 µm

Figure 8   Click here to access/download;Figure;Figure 8.tif ⬇

Laboratory equipment
Benchmark microplate reader
Cell culture Incubator
Cell freezing container (for example Mr. Frosty)
Centrifuge
Confocal laser scanning microscope
Heamatocytometer, or automatic cell counter
Laminar bio-safety hood class II
MultiStand Macs (Macs Cell Separator)
pH meter
Phase contrast inverted light microscope
Pipette boy, pipettors (different volumes)
Scissors
Vacuum pump
Water bath

Disposable small equipment/glassware
15 mL and 50 mL conical centrifuge tubes
6- and 12-well cell culture plates, flat bottom, low evaporation lid, sterile
Cell culture inserts, transparent PET membrane, 12-well, 3 µm pore size
Cell scrapper
Cryovials
Glass autoclaved Petri Dishes
LS Columns
Sterile filtration cup for vacuum filtration, 0.2 µm pore size
Sterile Lab Bottle compatible with Filtration cup (min. 100 mL)
Sterile pipettes

Chemicals
Bovine serum albumine (BSA)
CD14+ MicroBeads human - magnetic beads
Deattachnig agent Trypsin-EDTA, 0.05%, phenol red
Density gradient medium Lymphoprep
Dimethyl Sulfoxide (DMSO)
Ethylenediaminetetraacetic acid (EDTA)
Fetal bovine serum (heat inactivated)

Human granulocyte-macrophage colony-stimulating factor (GM-CSF), premium grade

Human Interleukin 4 (IL-4), premium grade
Human macrophage colony-stimulating factor (M-CSF), premium grade
L-glutamine
Lipopolysaccharid (LPS) from Escherichia coli
Paraformaldehyde (PFA)
Penicilin-Streptomycin
Phosphate Buffer Saline (PBS)
Roswell Park Memorial Institute-1640 Medium (RPMI)
Triton X-100
Trypan blue solution (0.4%)

Tumor necrosis factor alpha (TNF-α)

Assays used for cytotoxicity, (pro-)inflammatory response
Cytotoxicity Detection Kit (LDH)
Human IL-6 DuoSet ELISA
Human IL-8/CXCL8 DuoSet ELISA

Immunostaining
4',6-diamidino-2-phenylindole (DAPI), concentration 2 µg/mL

Goat anti-mouse IgG (H+L) Alexa Fluor 647 conjugated, concentration 20 µg/mL

Goat anti-rabbit IgG antibody (H+L) Dylight 488 conjugated, concentration 10 µg/mL

Mature Macrophage Marker Monoclonal Antibody, concentration 50 µg/mL
Phalloidin rhodamine, concentration 0.264 µM
Recombinant Anti-CD83 antibody, 1:50 dillution

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Miltenyi, Germany
Gibco, Switzerland
Alere Technologies AS, Norway
Sigma Aldrich, Switzerland
Sigma-Aldrich, Switzerland
Gibco, Switzerland

Miltenyi, Germany

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Gibco, Switzerland
Sigma-Aldrich, Switzerland
Sigma-Aldrich, Switzerland
Gibco, Switzerland
Gibco, Switzerland
Gibco, Switzerland
Sigma-Aldrich, Switzerland
Sigma Aldrich, Switzerland

Immunotools

Roche, Switzerland
R&D, Biotechne, Switzerland
R&D, Biotechne, Switzerland

Sigma-Aldrich, Switzerland

Abcam, UK

Agrisera, Sweden

eBioScience, Thermo Fischer, Switzerland
Molecular Probes, Life Technologies, Switzerland
Abcam, UK

Type or catalogue number

Zeiss LSM 710 meta

130-042-303

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353046 and 353043
353181
353085

130-042-401
10040-446

A7030-100g
130-097-052
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E6758-100g
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130-095-373
130-096-485
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DY208

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R415
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Fribourg, 27.2.2020

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Dear Dr. Bajaj,

Please find attached author's letter to the reviewers' comments and the revised manuscript entitled "Multicellular human alveolar model composed of epithelial cells and primary immune cells for hazard assessment" by Hana Barosova, Barbara Drasler, Alke Petri-Fink, and myself.

We have carefully addressed all the Editorial comments in the revised version. We hope that you will find these changes adequate and further consider our article for publication in the highly regarded journal JoVE. Please do not hesitate to contact us if you have any queries or concerns regarding this rebuttal letter or changes to the manuscript.

We look forward to hearing from you at your earliest convenience.

On behalf of all authors, our kindest and sincerest regards,

B. Rothen

Prof. Barbara Rothen-Rutishauser

Co-Chair BioNanomaterials

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