Article Title:
A novel *in vitro* mouse model to study *Mycobacterium tuberculosis* dissemination across brain vessels: a combination of mouse granuloma and blood-brain barrier model

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Running title
Novel mouse in vitro BBB and granuloma model to study Mycobacterium dissemination to the brain

Significance statement (Social media promotion)
Tuberculosis (TB) of the central nervous system (CNS) is the most dangerous form of *Mycobacterium tuberculosis* (Mtb) infection. Despite its public health importance, due to the lack of appropriate models, current understanding of the dissemination of Mtb into the brain is limited. We present an *in vitro* mouse model of the blood brain barrier (BBB) that can be applied to study Mtb dissemination across the BBB.

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Keywords
Primary mouse brain endothelial cell astroglia isolation, blood-brain barrier, dendritic cell, peripheral blood mononuclear cell, *Mycobacterium tuberculosis*, transmigration, central nervous system tuberculosis

Abstract

In vitro culture models of the blood-brain barrier (BBB) provide a useful platform to test the mechanisms of cellular infiltration and pathogen dissemination into the central nervous system (CNS). We present a mouse *in vitro* model of the BBB to test *Mycobacterium tuberculosis* (Mtb) dissemination across brain endothelial cells. One-third of the global population is infected with Mtb and in 1-2 % of these cases bacteria invade the CNS through a largely unknown process. The "Trojan horse" theory supports the role of a cellular carrier that engulfs and carries the bacteria to the brain without being recognized. We present for the first time a protocol of an *in vitro* BBB-granuloma model that supports the Trojan horse mechanism of Mtb dissemination into the CNS. Handling of bacterial cultures, *in vivo* and *in vitro* infections, isolation of primary astroglia and endothelial cells and the assembly of the *in vitro* BBB model is presented. These techniques can be used to analyze the interaction of adaptive and innate immune system cells with brain endothelial cells, cellular transmigration, BBB morphological and functional changes and methods of bacterial dissemination.

Basic protocol 1. Isolation of primary brain astrocytes and endothelial cells

Basic protocol 2. Isolation of mouse bone marrow-derived dendritic cells (DC)

Basic protocol 3. Isolation of mouse peripheral blood mononuclear cells (PBMCs)

Basic protocol 4. Handling of *Mycobacterium tuberculosis* (Mtb) culture, *in vivo* and *in vitro* infections

Basic protocol 5. Protocol for the *in vitro* granuloma and BBB model assembly

Support protocol 1. Assembly of the blood brain barrier (BBB) co-culture model

Support protocol 2. Flow cytometry analysis to validate the purity of the DC culture

Support protocol 3a. Purification of CD4+ T-cells from primary mouse PBMCs

Support protocol 3b. Flow cytometry analysis to validate the purity of the isolated CD4+ T-cells

Support protocol 3c. Spleen cell isolation

Support protocol 4. Liver granuloma supernate isolation and determination of organ load colony forming unit (CFU)

INTRODUCTION

Brain endothelial cells form the functional basis of the brain capillaries which separate the brain parenchyma from the circulation, and form the neurovascular unit together with pericytes, astroglia, microglia and neurons (Engelhardt et al., 2017; Mastorakos & McGavern, 2019). *In vitro* modeling of the blood-brain barrier (BBB) provides a method to study many aspects of brain function: cell-cell interactions between cell types of the BBB, drug permeability and metabolism, brain vessel related diseases and microbial pathogenesis involving cellular transmigration across the brain endothelial cell monolayer (Helms et al., 2016; DeStefano et al., 2018). *Mycobacterium tuberculosis* (Mtb) primarily infects the lung resulting in pulmonary granuloma formation, however disseminating bacteria can spread to the central nervous system (CNS) and form lesions in the parenchyma causing serious neurological symptoms and high mortality (Jain et al., 2018). Previous studies from our laboratory have shown that dendritic cells (DCs) *in vivo* are capable of escaping the primary granuloma and contribute to Mtb dissemination (Harding et al., 2015). The mechanism how Mtb enters the CNS is not yet clear. The presented model allows to test different mechanisms, including the "Trojan Horse" theory which favors Mtb dissemination into the CNS via cellular transport.

In order to model cellular and bacterial transmigration across the brain endothelial monolayer, we developed an integrated *in vitro* BBB and granuloma model combining multiple primary cells derived from mice. The current protocol details the isolation process of mouse primary brain endothelial cells and primary astroglia and the assembly of the *in vitro* BBB co-culture model using these two cell types on Transwell culture inserts. To study Mtb dissemination with a cellular carrier, primary bone marrow-derived DC isolation and infection with mycobacteria is described. The assembly of the *in vitro* granuloma system also involves the addition of primary peripheral blood mononuclear cells and primary spleen cells besides the infected DCs to the luminal (apical) compartment of the Transwell inserts. Here in the Transwell model the apical compartment represents the vessel lumen, while the bottom (basal) compartment corresponds to the brain environment. Using this method several key factors of bacterial dissemination can be identified, including immune cell and BBB interactions, BBB disruption and inflammatory characteristics, cellular transmigration, bacterial dissemination and background mechanisms.

STRATEGIC PLANNING

To establish this *in vitro* model consisting of at least four different primary cell cultures, careful consideration of biological variables such as sexes and ages are important (newborn mice for astroglia and 2-6 month-old mice for endothelial cell, dendritic cells and PBMCs/spleen cells; Figure 1). In the current article, we do not detail animal housing and breeding at the appropriate biosafety levels; local protocols must be established and animal facilities consulted. The handling of Mtb has to take place in a special Biosafety Level 3 facility, for which local facilities and protocols need to be established. Alternatively, pilot experiments can be performed with the attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Pasteur strain, for which a BSL2 facility and special decontamination is needed (see Basic protocol 4.). We provide the schematic (Figure 1) for the general timeline of the isolation of cell types, mycobacterial infections and model assembly to help the understanding of protocols and planning.

Experiments need to be executed in a sterile microbiological hood with laminar airflow. All reagents and equipment need to be sterile or sterilized before use. During all isolations, except for glial cells, donor mice need to be sex-matched for all cell types to avoid incompatibility. We did not observe differences when using female or male animals, but in general cell yield from males of the same age is higher.

[*Insert Figure 1 near here]

Basic protocol 1. Isolation of primary brain astrocytes and endothelial cells

The isolation of primary mouse brain endothelial cells (ECs) and astroglia cultures was previously published in detail (Lénárt et al., 2015; Barna et al., 2020). The isolation procedure and the assembly of the BBB model require precision and expertise in handling primary cultures. Isolation of glial cells is done by mechanical dissociation of brain tissue with a long thin needle and an additional filtration step. The final glia culture contains maximum 20 % microglia cells besides astroglia. The purity of cultures should be confirmed by immunohistochemistry or flow cytometry. Isolation of ECs is performed by a two-step enzymatic digestion and a 3-step gradient centrifugation. The high purity of the EC culture is ensured by selective elimination of other contaminating cell types using puromycin (Perrière et al., 2005).

Materials:

- Mice used for cell isolations:
  - EC: For one isolation, 13, 2-6 months old C57BL/6 wild type mice, male or female (The Jackson Laboratory, cat. no. 000664), yield is max. 1.8 $\times 10^6$ cells
  - Astroglia: For one isolation, 2 newborn, maximum 3-day-old C57BL/6 wild type mice, yield is 2×T25 flask confluent cell layer, about 3 $\times 10^6$ cells / flask
- Isoflurane anesthetic
- 70 % ethanol prepared from 100 % absolute ethanol (EtOH)
- Styrofoam pad, sterile needles for the dissection
- Sterile dissecting instruments: 2 curved forceps with fine tip, 1 big straight forceps, 1 small curved blunt forceps, 2 small scissors, 1 big scissors, 2 scalpels with blades
- glass beakers, glass petri dish
- fine filter paper or WHATMAN Chromatography paper 3MM (CHR 3030 909)
- 10 mL sterile syringe (Braun)
- long needle (21G 4 ¾; Braun, 4665643)
- 6-well tissue culture plates (Corning)
- T25 tissue culture flask (Corning)
- 50-mL conical centrifuge tubes (e.g. BD Falcon)
- Cell strainer (40 µm, Corning, Falcon, cat.no. 352340)
- Phosphate buffered saline solution (PBS)
- Collagenase 2 (Sigma, stock concentration 10 mg/mL, final concentration: 1 mg/mL)
- Collagenase/dispase (Roche, stock concentration 10 mg/mL, final concentration: 1 mg/mL)
- DNase I (Sigma, stock concentration: 2000 U/mL)
- Puromycin (Sigma, stock concentration: 4 mg/mL; final concentration 4 µg/mL)
- 20 % bovine serum albumin (BSA)-DMEM/F12 solution
- Refrigerated, swing-out rotor centrifuge (Eppendorf 5810R)

Primary astroglia culture medium components:

- DMEM with 1 g/L glucose containing stabile L-glutamine (GlutaMAX or equivalent, Life Technologies, Gibco)
- FBS (10 %; Corning Cellgro, 35-010-CV)
- HEPES (1 M solution from Corning or Sigma powder, used concentration: 10 mM),
- Gentamicin (Sigma, 50 mg/mL, final concentration: 50 µg/mL)

Primary brain endothelial cell (EC) culture medium components:

- DMEM/F12 containing stable L-glutamine (GlutaMAX or equivalent, Life Technologies, Gibco)
- Fetal bovine serum (FBS) (10 %; Corning Cellgro, 35-010-CV for use in the US); Plasma Derived Bovine Serum (PDS) (10 %; First Link UK Ltd., 60-00-850 for use inside Europe)
- HEPES (1 M solution from Corning, final concentration: 10 mM),
- Gentamicin (Sigma, 50 mg/mL, final concentration: 50 µg/mL)
- hbFGF (human basic fibroblast growth factor, Sigma, final concentration: 1 ng/mL)
- Heparin (Sigma, final concentration: 100µg/ml; 20U)
- 1 % ITS (Insulin-transferrin-sodium selenite supplement, Sigma or Thermo Fisher Scientific, 100x)
- *During the first 3 days after isolation this medium is supplemented by another 5 % of PDS or FBS and 4 µg/mL puromycin.*

Protocol for primary mouse astroglia isolation

1. Sacrifice mice according to local animal handling regulations. Rinse the head of the animals twice in 70% ethanol, then cut the heads with scissors and put them into a big Petri dish on ice.

   Keep the tissue and the isolate on ice during the steps of the protocol when appropriate. This ensures better viability and higher yield at the end of the process.

2. Put the dissection equipment into two beakers filled with 70 % EtOH. Place one small scissors and curved forceps into one beaker and two fine curved forceps and one small scissors into the other beaker. Prepare two small glass Petri dishes on ice: one of them should contain sterile PBS, the other one 2 mL DMEM/F12.
3. Brains need to be removed from the skull in a sterile way. For this pin down the nose with a needle to the Styrofoam pad. Cut up the skin with a pair of small scissors in the middle of the head and fix the skin edges to the dissecting pad with needles firmly. Use another pair of scissors for cutting the bone with a sagittal incision. Keep the scissors in ethanol when not in use. Open the skull with the curved forceps, remove the olfactory bulb and the cerebellum. Take the forebrain and put it into a dish containing PBS on ice. Collect all brains to this same dish.

4. Cut the brains in half and remove the meninges by rolling brain fragments on wet sterile chromatography paper and place the brain tissue in 1 mL of DMEM.
5. When meninges are removed from all brain tissue pieces, place them with a 1 mL pipette into a 50 mL tube containing 10 mL of DMEM with 10 % FBS. Dissociate the tissue mechanically using a 10mL syringe with a 21G 4 ¾ long needle. Suck up the entire 10mL into the syringe then push it back three times.
6. When the mechanical dissociation is done, place the needle and syringe to an empty tube and allow the medium with the tissue pieces to sit for 1 min. Remove the top 7 mL to another tube. Add another 7 mL of media with serum and repeat the dissociation step twice more.

   During the 1 min waiting time following the mechanical dissociation bigger tissue clumps settle in the tube. After moving the dissociated tissue with the upper part of the medium to another tube, the settled clumps at the bottom of the tube will be disrupted with the needle in the further rounds of dissociation.
7. At the end all volumes are pooled adding up to a total of 24 mL medium containing the dissociated tissue.
8. Pipette the contents of the tube through a 40 µm nylon mesh into a new 50mL tube to filter out remaining bigger tissue pieces. Wash the mesh with another 5 mL of medium.
9. Centrifuge the cells at 700g, 5 min, at 4 °C.
10. Resuspend the cells in 1 mL of primary astroglia culture medium. Prepare as many T25 flasks as many mice were sacrificed: 1 mouse/T25 flask. Seed cells to the flask(s) in 5mL of medium each.
11. On Day 2 gently wash glial cells twice with PBS and refresh medium. By this time small islands of cells are attached (see Figure 2). On Day 3 wash cells firmly 4-5 times with PBS and add fresh medium. Repeat this every 2-3 days until confluency, which is reached within 6-10 days (Figure 2).
12. Utilize these cells in the construction of the BBB model described in Support protocol 1.

[*Insert Figure 2 near here]

Protocol for primary mouse brain EC isolation

13. Autoclave pieces of filter/chromatography paper for the removal of the meninges step. Thiese will be placed into a sterile big Petri dish later. Alternatively, the chromatography paper can be sterilized in aluminum foil and the foil can be rolled back to expose one side of the paper when needed.
14. Autoclave or sterile filter bi-distilled water in a glass bottle.
15. Sterilize the equipment for dissection with heat (160˚C, for 3 h, metal and glassware). Sterilize extra aluminum foil to be able to wrap the Styrofoam dissecting pad.
16. Prepare 100 mL of 70 % ethanol (EtOH, v/v %) with sterile distilled water.
17. Prepare the stock solutions that should be kept at -20 ˚C: enzymes, collagen type IV, heparin, hbFGF, 20 % BSA-DMEM/F12 solution (for this see *Reagents and Solutions* section).
18. Coat cell culture dishes before isolation with collagen type IV (steps 18-23). No coating needed for astroglia cells. For mouse brain EC, prepare one 6-well plate (Corning) for 13 mice. (surface of 6 wells is ~60 cm^2/13 mice, ≈ 4.5 cm^2/mouse).
18. One 6-well plate should be coated with collagen type IV (stock: 1 mg/mL; final cc. 100 µg/mL in sterile distilled water).
19. Prepare only the necessary amount; 1 mL of coating solution is enough for one 6-well plate.
20. Put the solution into a dish, cover the whole surface and pipet up & down minimum 5 times (avoid bubbles!). When ready, transfer the solution to the next well, a thin fluid layer of the matrix will cover the bottom of the dishes/plates. Discard the leftover coating solution in the end.
21. Put the dishes/plates for drying either into the CO_2 incubator until the isolation of the microvessel fragments is finished or dry it on the bench beforehand (airflow or under UV light for 30 min). The surface should be dry when the cells are put on it.
22. Always prepare coating and coating solutions freshly. Do not store the coated dishes/plates or the diluted coating mixture.
23. Start the isolation with deeply anesthetizing mice with isoflurane according to local animal handling regulations and protocols. Remove brains using the same technique as during the astroglia isolation, collect them to PBS on ice.
24. Place the sterile chromatography paper into a large sterile dish. Take off the meninges by spreading one hemisphere on the paper by gently rolling. Also remove the white

matter and choroid plexus. Put the tissue pieces in the other dish on ice that contains 2 mL DMEM/F12.

Here only one brain should be removed from the PBS at a time to avoid drying out the tissue. Separate the two hemispheres and lay down the brain with the gray matter facing the filter paper. Handle the tissue very gently. Small pieces of meninges can be removed also by pinching them off of the surface of the brain.

25. When ready with all brains, cut the tissue in the 2 mL DMEM/F12 into very small pieces with scalpels. Transfer the tissue to a 50 mL tube.

    The tissue is cut small enough when it can be transferred using a 1 mL regular pipette tip. Additional volume of medium can be added to help the transfer of the tissue pieces.

26. Start the first enzymatic digestion step by adding a total of 13.5 mL DMEM/F12 to the minced tissue along with 1.5 mL Collagenase 2 solution (final conc. 1 mg/mL) and 400 µl DNase I. Dissociate the tissue pieces in the solution by pipetting 50 times up and down with a 10 mL serological pipette. Incubate at 37 °C for 55 mins.

    When pipetting take care not to leave big tissue pieces in the solution. During the enzymatic digestion step put the tube on a horizontal shaker or a rocker to move around the liquid completely in the tube to be able to ensure good mixing for optimal enzyme activity. DNase I is important to avoid clumping of the tissue during digestion. At the end of the incubation the homogenate will look more "creamy", like "milk coffee".

27. When the digestion is ready, add 10 mL DMEM/F12 to the mixture and centrifuge at 1000 g for 8 min.

    All centrifugation steps need to be performed at 4 °C, in a swing-out rotor centrifuge.

28. Aspirate the supernatant with a pipette and add 12 mL 20 % BSA-DMEM/F12 solution to the cell pellet. Mix very thoroughly with the 10 mL serological pipette, 25 times up/down. Centrifuge this mixture at 1000 g for 20 min.

    When the centrifugation is finished, the reddish cell pellet containing the microvessels should be the at the bottom of the tube. Above it the gradient solution and on top the myelin rich layer.

29. Gently rotate the tube to detach the myelin layer from the wall of the tube. Aspirate the myelin rich layer (containing neurons, glia) at the top and the BSA solution and put it into another tube. Collect the microvessels in a clean separate tube by suspending them in 1 mL DMEM/F12 and washing out the tube with another 1 mL of DMEM/F12. Place this tube on ice during the next centrifugation steps. Close the tube containing the myelin and the BSA solution tightly and shake it vigorously 20 times, centrifuge it as before, and collect the microvessels at the bottom again. Repeat this step a total of 3 times. After the 3rd repetition the myelin rich layer and the BSA solution can be discarded.
30. Start the second enzymatic digestion with the isolated microvessels. When the 3 fractions from the three gradient centrifugations are pooled in one tube, it will contain 6 mL of medium. For the second digestion add an additional 3 mL of DMEM/F12 (total DMEM/F12 volume: 9 mL) along with 1 mL Collagenase/Dispase solution (final conc. 1 mg/mL) and 400 µl DNase I. Mix the solution with a 10 mL serological pipette. Incubate at 37 °C for 35 mins.

    During the enzymatic digestion step put the tube on a horizontal shaker or rocker to agitate the liquid completely to ensure the mixture of the enzyme. At the end of the incubation the solution will be hazy from the cells and look like a raspberry drink.
31. When the second digestion step is ready, add 10 mL DMEM/F12 to the mixture and centrifuge at 700 g for 6 min.
32. Repeat this washing step using 15 mL DMEM/F12, 700 g, 8 min and the cells are ready to plate.
33. Take up the final pellet in a total of 3 mL primary EC medium containing an extra 5 % PDS or FBS and 4 µg/mL puromycin and seed the cells to the pre-coated 6-well plate. Prepare 1.5 mL medium into the wells and add 500-500 µl of cell suspension in each well.

    Puromycin eliminates all cell types not expressing the P-glycoprotein efflux pump and ensures that only brain endothelial cells grow in the culture. On rare occasions, if at the dissection step the choroid plexus was not removed properly, epithelial cells from the choroid plexus can contaminate the culture. The presence of some of the myelin rich

fragments is normal during the isolation and does not disturb the growth nor the purity of the isolate.

34. On Day 2 after isolation wash the cells 3 times with PBS and give cells fresh medium containing puromycin. The next day (Day 3 after isolation) wash the cultures 4-5 times with PBS and give cells primary EC medium not containing puromycin. Figure 3 shows phase contrast pictures taken at different days of the culture process demonstrating the growth of the primary cells.
    i. *If cells grow slowly the days of culture can be prolonged one extra day either in the with puromycin or without puromycin phase. Cultures have to reach confluence in 5 days maximum. Never use puromycin treatment in the co-cultures, since it will kill all P-glycoprotein non-expressing cells, including astrocytes.*
35. When cells reach 95 % confluence normally on Day 4 (Figure 3), they are ready to be used to assemble the BBB model.
36. Utilize these cells in the construction of the BBB co-culture model described in support protocol 1.

[*Insert Figure 3 near here]

Support protocol 1. Assembly of the BBB co-culture model

In this support protocol, the establishment of the co-culture BBB model is described consisting of the barrier forming primary mouse brain ECs and primary mouse astroglia cultures. Glial cells are cultured on the bottom of 24-well plates for at least 2 weeks to ensure proper secretion of BBB inducing factors. ECs are passaged to the top of Transwell inserts and are monitored for barrier integrity properties by transendothelial electrical resistance (TEER) measurement. Hydrocortisone is added to the culture to strengthen interendothelial junctions and to enhance barrier integrity.

Materials:

- Primary ECs: cultures are ready 4 days after isolation (See Basic protocol 1).
- Primary astroglia: cultures are ready at passage #1, 2-weeks after passage; ~3 weeks after isolation

- EVOM-2 Volt/Ohm, transendothelial electrical resistance (TEER) Meter with ENDOHM-6G cell culture cup chamber-type electrode (World Precision Instruments)
- Transwell inserts 3 µm pore size, polycarbonate membrane, 0.33 cm^2 surface, with 24-well plates (Corning, 3415)
- Trypsin-EDTA solution (0.05% with EDTA)
- Hydrocortisone (powder, Sigma, H4001, 1 mg/mL stock in ethanol)
- Collagen type IV. (Sigma, 1 mg/mL stock)
- Fibronectin solution (Sigma, 1 mg/mL stock)
- Sterile distilled water
- Phosphate buffered saline solution (PBS)
- Neubauer or Bürker chamber for cell counting
- Primary brain EC culture medium
- Primary astroglia culture medium

1. For the timing of astroglia isolation, see Figure 1. Glial cells reach confluency after one week on average in culture in a T25 flask. Passage glial cells to the bottom of collagen type IV coated wells in 24-well, plates. To coat the plate, prepare 100 µg/mL collagen (10x dilution) in sterile distilled water and suspend coating solution up and down 4-5 times/well. Remove excess coating solution from the wells and dry the plates under laminar flow or UV light for 20 mins.

   The amount of 24-well plates needed is decided by the user, but from one T25 flask a maximum of four 24-well plates can be prepared. Passaging glial cells in a higher ratio increases growth time. The use of UV light during coating helps to cross-bind collagen fibers in the coating solution to each other and to the surface of the multiwell plate.

2. Keep glial cells in the 24-well plate for up to two months. Cells are kept in DMEM containing a lower amount of glucose (1 g/L) than the medium for ECs.

   Passage glial cells at least 2 weeks before the experiment. This step gives enough time for glial cells to grow and secrete appropriate factors to induce BBB properties when co-cultured with ECs.

3. Isolate primary brain ECs and culture them according to the protocol. Cells reach confluency in about 4-5 days. Prepare Transwell inserts for ECs by coating them with

8:1:1 ratio of sterile distilled water : collagen type IV : fibronectin (100 µg/mL concentration each). Dry inserts under laminar flow or UV light for 20 mins.

4. Place Transwell inserts into the 24-well plate. Change the astroglia medium in the bottom compartment to 900 µL of primary brain EC medium. Passage ECs to the top of Transwell inserts by washing the monolayers once with PBS, then adding Trypsin-EDTA and incubating cells gently for 60-90 seconds on 37 °C. Observe cell detachment by phase contrast microscopy. Gently collect trypsinized cells, pellet ECs by centrifugation (5 min, 400 g, 4 °C) and suspend them up in full medium to count in a Neubauer/Buerker chamber. Place 25,000 cells / Transwell insert in 200 µL of primary brain EC medium to the top compartment (Figure 4).

   As a rule of thumb, counting should be performed so that about 100,000 cells are suspended in 100 µl of culture medium. At this point cells tend to aggregate into small clusters containing no more than 10-15 cells. This is normal and should not be suspended to single-cell stage due to the damaging effect of mechanical dissociation.

5. Change medium from both compartments after 48 hours. Supplement the full medium with 550 nM (0.2 µg/mL) hydrocortisone.

   Addition of hydrocortisone to confluent monolayers on inserts inhibits dedifferentiation and increases junctional tightness and BBB properties.

   [*Insert Figure 4 near here]

6. Measure TEER after 72 hours. Fill the chamber electrode set with 70 % Ethanol-distilled water mix for 20 min, remove ethanol, wash the chamber once with medium and place 1.5 mL culture medium into it. Put inserts one-by-one into the chamber, measure and register TEER.

7. After 96 hours of co-culture (48 h hydrocortisone) the system is ready to use to test transmigration across the brain EC monolayers. Optimally by this time TEER should reach on average at least 100 $\Omega \times cm^2$.

   TEER is calculated from its raw value minus the background, multiplied by the surface of the culture insert (for 24-well format it is 0.33 cm^2).

Basic protocol 2. Isolation of primary mouse bone marrow derived dendritic cells

Primary mouse bone marrow derived dendritic cells (DCs) are most frequently isolated from the marrow of the femur, tibia or both. From several methods available in the literature here

we describe the details of primary DC isolation from mouse femur (Karman et al., 2004; Clarkson et al., 2015) with a culture medium containing granulocyte-macrophage stimulating factor (GM-CSF). With our isolation method up to 70 % of CD11c+ yield is possible. The following protocol describes the isolation procedure along with the explanation of how to clean the femur and gain the bone marrow. We also focus on the composition of the special primary DC medium (cRPMI medium) and the culture method using different cell culture treated surfaces to elevate purity. In the support protocol a short flow cytometry summary is given how CD11c+ cells are identified in the culture. Using this isolation protocol immature, DCs are gained, which are able to take up antigens effectively and thus are suitable to model bacterial infection.

Materials:

- Donor mice: one adult, 2-6 months old C57BL/6 wild type or CD11c-eYFP (B6.Cg-Tg(Itgax-Venus)1Mnz/J), male or female (The Jackson Laboratory, cat. no. 000664 & 008829 respectively)
- Isoflurane anesthetic
- 70 % ethanol prepared from 100 % absolute ethanol (EtOH)
- RPMI 1640 cell culture medium (Corning Cellgro, cat.no. 10-040-CV)
- GM-CSF (granulocyte-macrophage colony stimulating factor, Peprotech, 315-03)
- FBS (Corning Cellgro, 35-010-CV)
- ACK (Ammonium-Chloride-Potassium) lysing buffer (Thermo Fisher Scientific)
- Blue absorbent pad for surgical surfaces
- Styrofoam pad
- Sterile gauze
- Sterile dissecting instruments: forceps, scissors, scalpels, blades, lancets
- 3mL sterile syringes (Braun)
- 23 G needle (BD or any sterile brand, 1.25”/32mm)
- 6-well tissue culture plates
- 60 mm cell culture dishes
- 100 mm bacteriological petri dishes (Corning, 351029) and tissue culture treated dishes (Corning)
- 50-mL conical centrifuge tubes (e.g. BD Falcon)
- Cell strainers (40 µm, Corning, Falcon, cat.no. 352340)

- Refrigerated centrifuge (Eppendorf 5810R)

cRPMI dendritic cell culture medium components:

- 10% FBS (50mL)
- 1% HEPES (Corning, final concentration: 10 mM)
- 1% Penicillin-Streptomycin (Corning, 30-001-CL)
- 1% GlutaMAX (Life Technologies, cat.no. 35050061)
- 1% Na-Pyruvate (Corning, 25-000-CL)
- 1% non-essential amino acids (Corning, 25-025-CL),
- 1% essential amino acids (Corning, 25-030-CL)
- 0.5 % 2-Merkaptoethanol (Sigma, M6250-10ML, prepared as a stock using 13.5 mL RPMI medium + 100 µl 2/ME stock, prepare this solution freshly every month)

Isolation of primary mouse bone marrow derived dendritic cells

1. Mix RPMI 1640 cell culture medium with 10 % FBS.
2. Fill a beaker with 70 % EtOH to keep the sterile dissecting instruments except the scalpels. Prepare the surgical surface with the absorbent pad.
3. Prepare a Styrofoam box, fill with ice and place four 60 mm cell culture dishes on top. Fill up two dishes with 4 mL RPMI, one with 2 mL RPMI and one with 70 %EtOH / bone.

   For two femurs 8 dishes are needed, 6 with RPMI, 2 with EtOH.
4. Euthanize mice according to your institutions protocol, check anesthesia effectiveness with tail and toe pinch.
5. Remove the femur by cutting in the middle of the joint with a lancet between the femur and tibia at the knee precisely.

   By removing the muscles around the hip joint and moving the femur in a circular way very gently the bone will be released from the joint.
6. Put the femurs into the first Petri dish containing RPMI medium. Soak gauze in 70 % EtOH and try to remove as much leftover muscle from the bone as possible. Then place the bone in 70 % EtOH containing Petri dish for exactly 2 min, then transfer the bone to another dish containing RPMI medium.

   Keeping to the 2 min time in the EtOH is key, since in this way the outside of the bone gets clean enough but EtOH still does not harm the tissue itself.

7. Cut off the two ends of the bone (epiphysis) careful not to break the bone. When successful, only the round cross-section of the bone should be visible (diaphysis).
8. Fill the 3 mL syringe with RPMI medium, attach the needle and push the needle inside the bone cavity containing the reddish bone marrow. With a firm push, flush out the marrow into a dish containing 2 mL RPMI medium. Small pieces of red tissue will be visible in the dish. This step can be repeated 2-3 times if needed.
9. When the bone marrow from both femurs is collected, dissociate the tissue pieces in the medium by suspending them up and down using the syringe with needle. Pool the marrow into one 50 mL centrifuge tube and spin it down for 5 min at 300 g, 4 °C.
10. Discard the supernatant, and suspend the cells in 1 mL ACK lysing buffer for exactly 1 min to lyse red blood cells. Add 20mL 10 % FBS-RPMI mix to stop this reaction.

    This method helps to eliminate contaminating red blood cells from the pellet.
11. Centrifuge again (5 min, 300 g, 4 °C), and resuspend the pellet in culture medium (cRPMI).
12. Count cells with the cell counter. Plate 7-10 × 10^6 cells per 100 mm low-attachment ("bacteriological") Petri dish in 10mL cRPMI medium.

Cell culture and handling of dendritic cells

13. During the first 6 days in culture in the low attachment plate, supplement the cRPMI medium with 1000 x dilution from the GM-CSF stock (stock: 20 µg/mL, used concentration: 20 ng/mL).
14. Medium needs to be changed partially on the 3rd day (Figure 5A): carefully remove half of the medium, spin it down, collect the pellet in 5 mL of fresh cRPMI containing the 20 ng/mL GM-CSF and place the cells back to the dish.
15. On the 6th day, passage cells by gently swirling the dish to detach the semi-adherent cells then remove the media so as to take only the semi and non-adherent cells and put into a 50 mL centrifuge tube. Centrifuge them at 300 g, 5 min, 4 °C. Take up the pellet in cRPMI containing 10 ng/mL GM-CSF and plate the cells in 1:1 ratio to a new 100 mm Petri dish with cell culture treated surface.

    This step helps to purify the culture by eliminating attached cells, which are mostly macrophage precursors. For experiments only the floating cell population is used.
16. Cells are ready to be used on day 8.

[*Insert Figure 5 near here]

Support protocol 2. Flow cytometry analysis to validate the purity of the DC culture

Utilize this support protocol after completing steps 15 and 16 from basic protocol 2. Validation of the purity of the DC culture is crucial, especially at the beginning of the experimental optimization. Usually when cells are ready to be used (day 8), part of the population is reserved for flow cytometry test. Later during the experiments this step does not need to be performed every time, cellular purity and the presence of the CD11c reporter eYFP protein can be observed using fluorescent or confocal microscopy.

Materials:

- 3 % paraformaldehyde solution (diluted with PBS from 32 % solution, Electron Microscopy Sciences)
- PBS buffer
- 3 % bovine serum albumin (BSA)-PBS solution
- 15-mL conical centrifuge tubes (e.g. BD Falcon)

1. Put the desired number of cells in an Eppendorf or 15 mL centrifuge tube and spin them down for 5 min, at 300 g, 4 °C.
2. Take up the pellet in 3 % paraformaldehyde solution and fix the cells for 15 min at room temperature. Then spin down cells again for 5 min, at 300 g, 4 °C.
3. Suspend up the pellet in 1 mL 3 % bovine serum albumin (BSA)-PBS solution
4. For the analysis prepare the unstained cell population and another eYFP tube for proper compensation.
5. Run cells with appropriate flow cytometry protocol and analyze data with FlowJo. (Figure 5B)

    During the analysis of the flow cytometry data cells need to be plotted forward scatter vs. side scatter to see the distribution of the cell population (Figure 5B, left plot). After gating for lymphocytes, additional gating for doublets and viability stain can be performed (not shown here). Purity of the DC culture was evaluated by gating for the CD11c-eYFP positive cells (Figure 5B, right plot).

Basic protocol 3. Isolation of primary mouse peripheral blood mononuclear cells (PBMCs)

In vitro work with isolated PBMCs is crucial during immunological research in the study of the interaction of phagocytic and antigen presenting cell types and adaptive immune cell interactions (Riedhammer et al., 2016). Here we detail a well-known Ficoll Paque PLUS based gradient separation protocol which yields the separation of T-cells, B-cells and monocytes from whole blood obtained from mice. The difficulty in the protocol is the low blood-amount that is compensated for by the use of high volume of base buffer. The yield of PBMCs is also decreased by the two extra centrifugation steps which aim to remove the excessive number of platelets in the culture. We also detail the isolation of CD4+ T-cells from the isolated lymphocytes using the standard positive selection Miltenyi isolation kit. For the Mtb/BCG experiments, P25 T-cell receptor transgenic mice are used which contain CD4+ cells expressing a transgenic T-cell antigen receptor recognizing Mtb's $Ag85b_{240-254}$ epitope restricted to the MHC class II allele H-2-IAb.

Materials

- P25 TCR-Tg transgenic mice (P25Ktk/JC57BL/6-Tg(TcraTcrb)Ktk/J, The Jackson Laboratory, cat. no. 011005)
- Isoflurane anesthetic
- 10 mL PBS with 20 U/mL heparin (Sigma Aldrich)
- Ficoll Paque PLUS solution (GE Healthcare)
- 10 mL syringe with 20 G, 1.5" needle
- 2 x 50 mL PBS with 2% FBS (Corning Cellgro, 35-010-CV)
- Cell culture medium (used during experimental setup for Basic Protocol 4 and 5.)
- Blue absorbent pad for surgical surfaces
- Styrofoam pad
- Sterile dissecting instruments: forceps, scissors, needles
- Refrigerated centrifuge (Eppendorf 5810R)
- Household bleach (diluted to 10 % with distilled water)
- 50 mL conical centrifuge tubes (e.g. BD Falcon)
- Neubauer or Bürker chamber for cell counting

Protocol:

1. Deeply anesthetize mice according to approved protocol, check anesthesia effectiveness with tail and toe pinch.
2. Perform blood collection using approved protocols into the 10 mL PBS-heparin solution, keep at room temperature and shake it gently so the collected blood mixes in the solution.

   Heparin in the buffer stops blood coagulation. The larger buffer volume helps to dilute blood if being collected from multiple animals and promotes the separation on the gradient.
3. Estimate buffer volume containing the blood. For 3 mice the volume will be around 12 mL when collected into the 10mL PBS-heparin. Blood : Ficoll ratio should be approx. 2 : 1, therefore for 12 mL blood, 6 mL Ficoll solution is used.
4. Pipet the Ficoll solution to the bottom of the 50 mL centrifuge tube then layer the blood on top with a serological pipette very slowly and carefully so the gradient does not mix with the buffer containing the blood.
5. Centrifuge for 30 min, 740 g, 20 °C, acceleration 5, deceleration 0, without break. At the end the gradient should be visible with red pellet on the bottom and a hazy layer containing the cells of interest in the middle (Figure 6).

   When using any kind of gradient centrifugation, it is crucial to switch off the brake of the centrifuge. If brake is applied, gradient might swirl up when stopping and the cell separation can be disturbed.
6. Carefully remove and discard the top compartment containing the plasma and platelets with a serological pipette. Be careful not to disturb the lymphocyte layer.
7. PBMCs are collected directly from the gradient with the 10 mL syringe and needle to 10 mL 2% FBS-PBS solution. When finished, fill this tube up to 25 mL. Discard the rest of the gradient using 10 % bleach.

   It is important to pull the plunger of the syringe very slowly when collecting the cells with the needle. If sucked up too fast, cells can be damaged when entering and exiting the needle resulting in cell number decrease.

[*Insert Figure 6 near here]

8. Spin down this 25 mL buffer with cells to remove platelet contamination: 10 min, 120 g, 20 °C, acceleration 5, deceleration 0 (brake off). Remove the top 20 mL of the

supernate with serological pipette. Resuspend the remaining 5 mL with serological pipette and fill it up again to 20 mL with 2 % FBS-PBS for another round of centrifugation at 10 min, 120 g, 20°C, acceleration 5, deceleration 0 (brake off).

PBMC isolation protocols often neglect the importance of the variability in final platelet content. Besides the upper layer of the gradient, the buffy coat also contains millions of platelets which need to be decreased by these two extra centrifugation steps.

9. After the second low speed centrifugation, remove the top 20 mL of the supernate again with serological pipette, then again 5 mL remains in the tube. Mix up the pellet and fill up the tube with 2% FBS-PBS to 15 mL. Collect the cells with centrifugation: 600 g, 5 min, 4 °C, acceleration 9, deceleration 9, brake can be used.
10. The collected cells should be taken up in the appropriate medium used for experiments. Cells are counted and used according to the protocol of the experiment.

- *On average 1 million cells / animal is the yield. Blood derived from male mice yield up to 1.5 million cells / animal, females up to 0.8 million cells / animal. Pooling blood from more animals at the beginning elevates the yield / animal.*
- *Between the two extra steps for platelet removal the sample can be centrifuged (600 g, 5 min, 4 °C, acceleration 9, deceleration 9, brake on) and the cell number can be checked. If the cell number is low, the second low speed centrifugation can be skipped.*
- *Collect and save all discarded supernates during the procedure until the end in case there is cell loss at any of the steps. At the end these tubes can be discarded if the yield is normal.*
- *The Ficoll Paque PLUS solution should be kept at room temperature. The PBMC layer at the gradient after the first centrifugation is much more clear if the solution is not cold.*
- *5-10% erythrocyte contamination among the PBMCs is normal. Also the presence of platelets at the end is still normal, although their number should be minimalized by the washing steps. More tips and tricks for appropriate erythrocyte and platelet contamination assessment and cell counting can be found at https://www.nexcelom.com/applications/cellometer/blood-based-samples/peripheral-blood-mononuclear-cells-pbmc/.*

Support protocol 3a. Purification of CD4+ T-cells from primary mouse PBMCs

Utilize this support protocol after completing step 9 from basic protocol 3. The following description is based on the Miltenyi Biotec company's mouse CD4+ T-cell isolation kit (catalog number: 130-104-454). This separation allows the purification of up to 10^9 cells by the depletion of other cell types targeted by a biotin-conjugated antibody cocktail. These cells are then magnetically labeled with anti-biotin microbeads and separated on a magnetic field separator, while the unlabeled desired cell population flows through the column. This kit is appropriate for lower cell numbers such as PBMCs from mouse blood. If higher yield is needed, CD4+ cells can be also separated from the spleen cell suspension of P25 transgenic animals (for isolation see support protocol).

Materials

- Mouse CD4+ T-cell isolation kit (Miltenyi Biotec, catalog number: 130-104-454), containing antibody cocktail against many cell types but CD4+ T-cells; anti-biotin microbeads for magnetic labeling
- MACS LS column (Miltenyi Biotec)
- MidiMACS Separator with MultiStand (Miltenyi Biotec)
- Cell separation buffer: sterile PBS with 0.5 % BSA, 2 mM EDTA
- Eppendorfs
- 15 mL conical centrifuge tubes (BD Falcon)
- Thin needle (20-21G)

Cell labeling steps:

1. Isolate PBMCs according to the first part of the protocol and count them. From this step on keep cells on ice or at 4 °C during the procedure.
2. Take up the needed amount of cells in an Eppendorf tube (max 10^7 / isolation) in cell separation buffer and pellet them by centrifugation at 700 g for 5 min (4°C if possible).

   Spinning down in an Eppendorf tube is the easiest to reach exact volumes for the next step.
3. Take up the pellet in exactly 40 µL cell separation buffer and add the 10 µL biotin-Ab cocktail to this suspension. Mix it well, but do not vortex the sample and incubate it for 5 min at 4 °C.

4. After this incubation add 30 µL of cell separation buffer to the current 50 µL volume then add 20 µL of anti-biotin microbeads. Mix this well, but do not vortex and incubate for 10 min at 4 °C.
5. Next, add at least 400 µL cell separation buffer to the mixture, but no more than 900 µL. Total volume should not exceed 1 mL. Cells are then ready for the column separation step.
6. Place the LS-column in the MidiMACS magnetic field separator. When inserted check the orientation of the column: wings need to face the front. Attach a 20 G thick needle to the end of the column to slow down the dripping.
7. Prepare the column by rinsing with 3mL cell separation buffer. Put a 15 mL centrifuge tube under the column to be able to collect washing buffer. Do not let the column to dry out.
8. After the washing buffer has stopped dripping, replace the 15 mL centrifuge tube under the column with a new one. Apply the labeled cell suspension to the column (500-1000 µl), collect the liquid which goes through the column which will contain the enriched CD4+ cells.
9. Wash the column two times with 3 mL cell separation buffer into the same 15 mL tube. In the end CD4+ cells will be in a total of 7 mL buffer. Spin down cells at 700 g, 5 min, 4 °C. Count the cells and use them for the experiment.
10. Additional step:

    It is recommended, that the quality of the cells derived from the column is checked occasionally. Also positively selected cells can be collected by removing the LS column from the magnetic separator, adding 5mL buffer to it and flushing out the non-CD4+ cells with the plunger into another 15 mL centrifuge tube. Spin down cells at 700 g, 5 min, 4 °C and take them up in the appropriate buffer for analysis (see Support protocol 3a).

Support protocol 3b. Flow cytometry analysis to validate the purity of the isolated CD4+ T-cells

Utilize this support protocol after completing step 9 from support protocol 3a. Validating of the purity of the CD4+ T-cell isolate is important at the beginning of the experiment to be able to determine the quality of our isolate. This step is not crucial to be performed every time.

Materials:

- 3 % paraformaldehyde solution (diluted with PBS from 32 % solution, Electron Microscopy Sciences)
- 3 % BSA-PBS solution
- Ghost viability dye UV450 (Tonbo Biosciences)
- Anti-CD4+ antibody (PE labeled, BD Pharmingen, 553048)
- PBS buffer
- 96-well U-bottom plates
- 15-mL conical centrifuge tubes (e.g. BD Falcon)
- Refrigerated centrifuge (Eppendorf 5810R) with multiwell plate-rotor
- UltraComp eBeads Compensation Beads (Thermo Fisher)
- ArC™ Amine Reactive Compensation Bead Kit (Thermo Fisher)

1. After the cell isolation, take up the cells in 3 % BSA-PBS buffer and put the desired number of cells into wells of the 96-well U-bottom plate (at least 10^5 cells / well). One well will stay unstained, one well receives only viability dye, one only CD4+ stain and one will be stained with all markers. Therefore, a total of 4 wells are needed at least. Spin down the plate for 5 min, at 400 g, 4 °C.
2. Aspirate the buffer and take up the cells in PBS or appropriate staining buffer. Cells which do not receive the Ghost viability dye receive only PBS. Ghost dye is used according to the manufacturer's protocol and added to the wells in 50 µl volume. Incubate at 4 °C for 10 min.
3. Add the CD4+ PE conjugated antibody (100 x) in 3 % BSA-PBS solution to the wells (50 µl/well). Unstained and viability stain only cells receive 3 % BSA-PBS buffer. Incubate at 4 °C for 30 min.
4. At the end of the 30 min add 100 µl 3 % BSA-PBS to the wells and spin down the plate for 5 min, at 400 g, 4 °C.
5. Aspirate the supernate and fix cells by suspending them in 100 µl of 3 % paraformaldehyde-PBS solution for 15 min at room temperature. Then spin down cells again for 5 min, at 700 g, 4 °C.

6. Aspirate the supernate and suspend the pellet in 200 µl 3 % BSA-PBS buffer, filter the cells into FACS running tubes through 40 µm nylon mesh to create a single cell suspension. Add another 300 µl of buffer into each tube.
7. For the analysis prepare the unstained beads, Ghost beads and CD4+ PE antibody labeled-beads for proper compensation according to the manufacturer's protocol. Analyze with flow cytometry (Figure 7).

   When analyzing the flow cytometry data cells need to be plotted forward scatter vs. side scatter to see the distribution of the PBMCs (Figure 7, left plot). The very small population in the bottom left corner can be platelets or other debris. To gate them out, we select for the PBMCs by gating to the more distinct cell population, then we gate for single cells (Figure 7, middle plot). Purity of the CD4+ isolated cells is identified by plotting living cells vs. CD4+ cells, where we showed 85% CD4+ staining positivity (Figure 7, right plot).

[*Insert figure 8]

Support protocol 3c. Spleen cell isolation

Generally, from 1 million PBMCs about 2×10^5 CD4+ T-cells are gained. If this cell amount for certain experiments is not enough, CD4+ T-cells can also be isolated from the spleen. In this case the cellular yield is much higher – about 10^9 cells / spleen from which about 20 % are CD4+ T-cells. In this support protocol we present a quick method to isolate primary spleen cells.

Materials:

- 50 mL conical centrifuge tubes
- 40 µm cell strainer (Millipore)
- Blue absorbent pad for surgical surfaces
- Styrofoam pad
- Sterile dissecting instruments: forceps, scissors, needles
- Refrigerated centrifuge (Eppendorf 5810R)
- 60 mm cell culture dish (Corning)
- Base RPMI medium (Corning)
- ACK (Ammonium-Chloride-Potassium) lysing buffer (Thermo Fisher Scientific)

- FBS (Corning Cellgro, 35-010-CV)

1. Deeply anesthetize mice with approved protocol, check anesthesia effectiveness with tail and toe pinch.
2. Remove the spleen and place it in RPMI medium in a 60 mm dish on ice. Cut it into 10-12 pieces. Put the 40 µm cell strainer to the mouth of a 50 mL centrifuge tube and transfer the small spleen pieces to the strainer.
3. Remove the plunger of a 3 mL syringe and with the rubber-end grind the tissue through the mesh. Always wash the mesh, so cells can drip down to the bottom of the tube.
4. Collect the cells with centrifugation with 300 g, 4 °C, 7 min. Discard the supernatant.
5. Add 1 mL ACK buffer to lyse red blood cells to the pellet, shake it gently for 1 min, then dilute with 20 mL of 10 % FBS-RPMI.
6. Centrifuge with 300 g, 4 °C, 7 min. Remove supernatant to another tube, resuspend the pellet in 3-4 mL of medium, and wait until the big clumps settle.
7. Transfer the top of the cell suspension without the clumps to another 50 mL tube, dilute and count cells and use for CD4+ isolation and the experiment.

Basic protocol 4. Handling of bacteria: culture, *in vivo* and *in vitro* infections

The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Pasteur strain was provided to our lab by Dr. Glen Fennelly (Albert Einstein College, Bronx, NY). Mtb H37Rv was purchased from ATCC (strain ATCC 27294/H37Rv). Both BCG and Mtb strains were electroporated with E2-Crimson (pTEC19) plasmids which were generated by Dr. Lalita Ramakrishnan (Addgene plasmid #30178, Cambridge, MA, USA). It is important to mention that Mtb must be handled in specific biosafety level 3 laboratories equipped with an appropriate microbiological biosafety cabinet and personal protective equipment. Protocols need to be established for each laboratory and according to institutional and NIH guidelines. Only plastic consumables, sealable containers are being used while the sterilization of surfaces with Vesphene Ilse and bleach are crucial. BCG cultures are kept under biosafety level 2 conditions. These bacteria also need to be handled under the biosafety cabinet and all equipment and liquids getting in contact with the bacteria need to be decontaminated with 10 % household bleach. Working with *mycobacteria* is not difficult, but one needs to be cognizant about their growth time in shaking cultures (4-5 days starting from optical density

(OD)=0.2) or on agar plates (first colonies appear after 2.5-3 weeks). Here we detail the general *mycobacterial* culture procedures for *in vivo* and *in vitro* infections; however, the in-depth guidelines for BSL3 and BSL2 work is not detailed.

Materials

- Special contained environment for BSL2 and BSL3 ~~animal~~ infections and sample handling
- Strains of bacteria:
    - *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) electroporated with E2-Crimson (pTEC19) plasmid
    - Mycobacterium tuberculosis (Mtb H37Rv) electroporated with E2-Crimson (pTEC19) plasmid
- Donor mice: adult, 2-6 months old C57BL/6 wild type, male or female (The Jackson Laboratory, cat. no. 000664)
- Incubator shaker for sterile flasks (New Brunswick Scientific C24 incubator)
- Bacterial culture medium (see Reagents and Solutions)
- Spectrophotometer to measure cell density based on OD (WPA Biowave CO8000 Cell density meter; 80-3000-45)
- Disposable cuvettes for OD measurement (BioExpress)
- 10 % household bleach
- Insulin syringe and needle (1 mL syringe with 25G ×5/8”)
- Ultrasonic Homogenizer (Biologics, Inc. Model 300 V/T)
- Low-attachment 48-well flat bottom plate (Corning)
- Eppendorf tubes (1.5 ml)
- Refrigerated centrifuge (Eppendorf 5810R)
- 2 % FBS-RPMI medium without antibiotics
- Bacterial culture medium (see Reagents and Solutions)
- Prepared 7H10 agar plates (see Reagents and Solutions)
- Medium needed for the assembly of the model (“Running medium without antibiotics”, see Reagents and Solutions)

1. Thaw one vial of bacteria from the −80 °C stock. Bacterial strains are frozen in simple bacterial culture medium at OD=1. Special freezing medium or cryoprotection is not needed if bacteria are frozen at this density.

   In our case bacteria were transfected by a plasmid containing E2-Crimson protein. To keep up and select out this phenotype, hygromycin B (100 μg/mL) is added to the base culture broth, which eliminates all bacteria not carrying the resistance gene.

2. Grow BCG and Mtb strains at 37 °C in a shaking incubator. Check OD every day and establish a growth protocol to be able to schedule experiments. When the culture reaches OD=1, in vivo or in vitro infections can be performed as detailed below.

   In our hand a frozen stock of OD=1 placed into 9 mL of bacterial growth medium reach OD=1 in 4-5 days. Scaling down the final volume results in reaching the desired OD faster. Do not let cultures overgrow since that enhances bacterial aggregation and results in false OD measurements. Besides this physical quality, after leaving log growth phase a change in metabolic activity, virulence can affect experimental results.

***In vivo* infections**

The current protocol details the intraperitoneal infection of mice with BCG for liver granuloma supernate isolation (see support protocol 4).

3. Take 1 mL out of an OD=1 culture. 3.14×10^7 bacteria should be present. Spin this down at 10,000g, 5 min and discard the supernate.
4. Take up bacteria in 630 μl of PBS, which is enough for the infection of 3 animals (10^7 bacteria/animal).

   Infection needs to be performed in a sterile microbiological hood under BSL2 conditions. Bacteria have to be contained all the time within the syringe. Syringe and needle should be disposed to a sharps container after proper disinfection with 10 % household bleach.

5. Sonicate bacteria at medium force for 30 seconds to break up clumps in the bacterial culture. Inject mice intraperitoneally (i.p.) with the bacteria (200 μl of PBS with bacteria).

6. Mice have to be kept for 3 weeks in a separate infection room to develop liver granulomas. Animals can be sacrificed after this time to be able to isolate liver granuloma supernate.

***In vitro* infections**

The current protocol details the infection process of isolated DCs in vitro.

3. BCG and Mtb strains are grown in a shaking incubator at 37 °C. Infection can be performed at OD=1.

   If there is a small deviation from the OD number above 1, sample can be slightly diluted and adjusted.

4. Culture primary bone marrow derived DCs until day 8. For details see Basic protocol 2. Collect DCs from the cell culture dish and spin them down at 300 g, 5 min, 4 °C. Plate the appropriate number of DCs for the experiment, and add extra wells for uninfected and infected cells for flow cytometry compensation and infection controls.
5. Add 500,000 DCs / well in 500 µl 2 % FBS-RPMI without antibiotics and add BCG or Mtb to this in PBS in a 1:1 or 1:0.5 ratio, respectively.

   For the preparation of bacteria follow "In vivo infection" protocol's 1-3. Points. Take 1 mL of OD=1 bacterial suspension with 3.14 ×10^7 bacteria, spin it down and take it up in 1mL of PBS. Sonicate and add appropriate number of bacteria to each well.

6. After 4 hours remove cells from the wells by suspending the medium and pool appropriate number of wells needed for the experiment. Spin them down at 300 g for 5 min, 4 °C and take up cells in experimental medium (see Basic protocol 5.), in a ratio of 100,000 DCs in 100 µl medium and use for experiments.

Support protocol 4. Liver granuloma supernate isolation and determination of organ load colony forming unit (CFU)

Utilize this protocol after step 6 of *in vivo* infections of basic protocol 4. The aim of the *in vivo* infection is to induce BCG infection in healthy mice leading to the formation of granulomas in the liver. By isolating and culturing these liver granulomas we can obtain the cellular supernate, which contains important factors for our *in vitro* granuloma model. By

plating part of the liver tissue we can validate the success of our infection by counting the CFU on agar plates.

Materials

- PBS buffer
- Sterile dissection equipment as detailed above
- Tray, containers for disinfectant, proper cleaning equipment
- Motor driven tissue grinder for bigger volumes and for smaller volumes
- Round bottom plastic tube for organ collection
- Collagenase type II (C6885-100MG)
- 60 mm Petri dish for organ storage
- RPMI cell culture medium (Corning)
- FBS (Corning)
- ACK (Ammonium-Chloride-Potassium) lysing buffer (Thermo Fisher Scientific)
- cRPMI cell culture medium mix (for recipe see Basic Protocol 2.)
- Neubauer or Buerker chamber for cell counting
- 96-well U-well multiwell plate
- 15 mL and 50 mL centrifuge tubes
- Eppendorf tubes (1.5 ml)
- 0.22 µm low-bind syringe filter

1. Wait three weeks after the i.p. bacterial injection. Euthanize mice with approved protocol, perfuse with 20 mL PBS.

   During manual perfusion take care to slowly introduce the buffer to the body. Organs start to lose color during this perfusion. Always prepare containment, cleanable tray and equipment for proper disinfection with 10 % bleach. Infection of the animal is instantly visible by the enlargement of the spleen compared to uninfected mice.

2. Harvest the liver and measure the weight of the organ. Cut a 400 mg piece of liver tissue and place it in a small plastic tube with 2 mL PBS on ice for colony forming unit (CFU) bacterial load evaluation.

   Parallel with this to confirm infection, a piece of organ can be fixed in 3 % paraformaldehyde-PBS and transferred to 30 % sucrose-PBS solution for

dehydration. Tissue can be frozen with the cryoembedding medium OCT and sectioned with a cryotome and stained using immunohistochemistry. Procedure is not detailed here.

3. Homogenize the tissue in a cleaned, sterile, sealable tissue blender in 25 mL RPMI medium and grind the tissue into small pieces. Repeat this step with another 20 mL of medium. Pour these to a 50 mL centrifuge tube and wait 2 minutes until the aggregated cell clusters (liver granulomas) settle and are separated from hepatic cells based on size.
4. Discard the top 30 mL of the supernate and refill the tube with 30 mL fresh medium. Centrifuge this for 5 min, 450 g, 4 °C. Discard the supernate and add 2.4 mL 2.5 mg/mL collagenase type II to the mixture. Shake this for 40 min at 37 °C.
5. Deactivate digestion with the addition of 25 mL of 2 % FBS-RPMI. Dissociate cells mechanically by moving up and down the cell suspension with a 10 ml syringe 25 times. After this, wait 1 min for the bigger clumps to settle and transfer the supernate to a new 50 mL centrifuge tube. Centrifuge this for 5 min, 450 g, 4 °C.
6. Discard the supernate carefully. Add 2 mL ACK lysis buffer to remove erythrocytes from the suspension, and wait 1 min while gently tapping the tube. Add 25 mL of 2 % FBS-RPMI to stop the reaction and centrifuge again for 5 min, 450 g, 4 °C.
7. Discard the supernate, take up the pellet in 2 % FBS-PBS. Prepare a new 50 mL tube, and put the 40 µm cell strainer to its mouth and pipette the cell suspension through this to remove remaining bigger cell clumps.
8. Centrifuge again for 5 min, 450 g, 4 °C and take up the pellet in 3 mL cRPMI medium (for recipe see Basic Protocol 2.). Count cells and plate them to a 96-well U-bottom plate with 5×10^5 cells/well in 200 µl of 10 % FBS and RPMI.

   Here 50 µg/mL antibiotics are kept in the cell culture medium to eliminate potential extracellular bacteria. BCG is obligate intracellular pathogen and survives inside the cells.
9. After 2 days in culture spin down the 96-well plate for 5 min, 300 g, 4 °C to pellet cells. Collect supernatant into a 15 mL centrifuge tube, filter it with a 0.22 µm low-bind syringe filter, aliquot it to Eppendorf tubes (1 mL/vial) and store them at -80 °C until use.

   If possible always collect liver tissue samples for CFU organ load determination. Only those supernatants can be used later during the

experiments, where appropriate infection rate of the animals was confirmed both with immunohistochemistry showing liver granulomas and proven appropriate infection level with liver CFU organ load (LogCFU/organ load > 5 as optimal infection load; Harding JS, personal communication).

10. To determine CFU organ load weigh then homogenize a piece of liver in 2 ml of PBS with a motor driven tissue homogenizer with a small head. Dilute and plate the homogenate in serial dilutions onto agar plates (for recipe see reagents & solutions) and incubate them at 37°C for 3 weeks. Count the colonies, calculate back the number according to the dilution and calculate the proportion of CFU/g tissue.

Basic protocol 5. Assembly of the model from the four different primary cells

All cellular components of the *in vitro* granuloma model are primary isolated, therefore isolation times and assembly have to be precisely scheduled and performed for each experiment. This means planning ahead for cultures which need a longer growth time (see strategic planning), but also for the day of the model assembly (Figure 8).

The *in vitro* co-culture BBB model is established from primary brain endothelial cells and astroglia, and is ready to be used after four-five days of co-culture (see Basic Protocol 1.). By this time, bone marrow-derived DCs are differentiated in culture for 8 days (maximum 9, see Basic Protocol 2.) and passaged to be infected with BCG or Mtb on the day of the model assembly for 4 h (see Basic Protocol 4.). During the infection time PBMCs are freshly isolated and used for the model setup and/or for CD4+ cell isolation simultaneously (see Basic Protocol 3.). In each experiment performed using the Transwell insert setup, 10^5 uninfected or infected DCs are cultured on the top of brain endothelial cells with or without the addition of 10^5 P25 PBMCs. Alternatively, wild type or other knock-out PBMCs can be isolated, combined with 10^4 P25 CD4+ cells, and added to mimic the same conditions. All groups receive 20 % liver granuloma supernate (see Support protocol 4.) to enhance *in vitro* granuloma formation. Cells are kept in culture for 2 days while several functional tests and morphological studies can be performed such as flow cytometry for the evaluation of transmigration, immunofluorescence to detect cell-cell interactions and barrier damage. Cell culture supernates can be collected and measured with ELISA for secreted factors and cells can be used for Western blot and other morphological studies.

Materials

All cells and materials need to be prepared and ready to be used which have been detailed in Basic protocols 1-4. Here we list the specific materials for this particular basic protocol:

- Running medium w/o antibiotics (see Reagents & Solutions)
- Running medium w/ antibiotics (see Reagents & Solutions)
- 3% paraformaldehyde solution (diluted with PBS) from 32 % solution, Electron Microscopy Sciences)
- 24-well plates (Corning), Eppendorf
- EVOM-2 Volt/Ohm (TEER) Meter with ENDOHM-6G cell culture cup chamber-type electrode (World Precision Instruments)

Note: By the beginning of Basic protocol 5, the following sections of Basic Protocol 1-4 need to be ready: 1.) Assembled and matured *in vitro* BBB model on its day 4 of assembly. 2.) Primary bone marrow-derived DCs on their cultivation day 8. 3.) Liver granuloma supernates isolated from BCG infected mice, 3 weeks post-infection, supernates are stored at -80°C. 4.) Bacterial culture grown to OD=1 for infections.

Note: In this section an example of a full 24-well plate with the complete *in vitro* granuloma & *in vitro* BBB model is presented containing infected DCs + P25 PBMCs with their controls (Figure 7). The model is assembled using a 24-well plate containing the ready-to-go *in vitro* BBB model on 3 μm Transwell inserts (see Basic Protocol 1.). This protocol includes the groups "uninfected DCs", "infected DCs", "uninfected DCs + P25 PBMCs" as controls for the "infected DCs + P25 PBMCs" group. The control groups can be modified according to the experimental plan. Besides these variations, DCs and PBMCs isolated from knock-out animals can be included.

1. First, measure TEER of the *in vitro* BBB model (96 h timepoint). Change the medium of co-culture model to the running medium containing antibiotics. By this time TEER should reach on average at least 100 $\Omega \times cm^2$.

   Until the 96 h timepoint cells receive 48 h hydrocortisone treatment. Before the experiment this treatment is removed and a preconditioning without hydrocortisone with lower, 10 μg/mL, gentamicin containing medium is performed during the DC infection and PBMC isolation interval.

   Generally during the establishment of the model and throughout the

experiment brain endothelial cells are kept in co-culture with astrocytes. In this case no extra chemokine is added to bottom compartment of the system since glial cells naturally secrete chemokines important for the assay (Banks et al., 2018). If the researcher wants to study the cellular transmigration in a controlled environment, glial cells can be removed and 200 ng/mL CCL-2 can be added to the bottom compartment as chemoattractant.

2. Infect cells according to Basic Protocol 4. In total prepare enough cells for 12 $\times 10^5$ DCs for both uninfected and infected groups and prepare two extra wells with 5 $\times 10^5$ DCs for flow cytometry and infection validation.
3. During the 4 h infection perform the PBMC isolation and if needed the CD4+ separation. At the end of this step, take up PBMCs at a concentration of 10^5/60 µl of running medium without antibiotics.

   During the PBMC isolation's first gradient centrifugation, fix one well of DCs from the infected group in 3 % PFA-PBS (~2.5-3 h infection) to check infection progress using confocal microscopy.
4. Follow the last instructions of "Basic Protocol 4. *In vitro* infections", take out the cells from the wells by suspending the medium and pool the four wells needed for the experiment (4 well $\times 5 \times 10^5$ infected with MOI 1:1 (BCG) or MOI 1:0.5 (Mtb)). Centrifuge at 300 g for 5 min, 4 °C and take them up in running medium without antibiotics, at a concentration of 10^5 DCs per 100 µL medium.
5. Assemble the system. Seed DCs to the top compartment at 10^5 cells/100µL per insert, PBMCs at 10^5 cells/60µL per insert, add 40µL (20 %) granuloma supernate which will add up to 200µL media per insert total volume (900 µL to the bottom compartment). Where no PBMCs are added their volume is replaced by running medium without antibiotics (for outline see Figure 9).

   Two conditions of running media are used: with and without antibiotics. Bottom compartment receives medium with antibiotics, top receives medium alone. The reason behind this step is that the granuloma supernate's medium contains 50 µg/mL gentamycin (see "Liver granuloma isolation" protocol), which is diluted 5 times when added to the system. To keep up the same conditions in the top and bottom compartments, lower amount of antibiotics is also added to the bottom. Also this serves to eliminate potential extracellular bacteria.

6. Incubate the system for 24 and 48 hours. After these time-points TEER can be measured again and supernates can be collected for analysis. To collect cells for flow cytometry or protein assays, they should be centrifuged at 300 g, 5 min, 4°C in Eppendorf tubes and fixed with 3% PFA-PBS for at least 1 h or lysed. Supernates can be collected for further analysis. For flow cytometry they can be centrifuged once more after fixation and taken up in 3 % BSA-PBS for measurement. For immunohistochemistry cells are fixed in 3% PFA-PBS for at least for 1 h or overnight and used for junctional or intracellular marker labeling.

[*Inset figure 9 near here]

Reagents and Solutions

For detailed reagent list see Supplementary Tables 1., 2. and 3.

20% BSA-DMEM/F12 (w/v %, BSA: Sigma, A7906)

- Dissolve 20 g BSA in 100 mL DMEM/F12: put the powder into a sterile glass bottle with wide mouth, add the medium gently and leave this solution overnight at 4 °C. On the next day dissolve leftover clumps by stirring the solution with sterilized magnetic stirrer for a few hours.
- Sterilize with low-protein binding syringe filter (0.22 µm) with 12 mL/50 mL tube, store at -20 °C.

Collagenase-dispase (C/D) solution (Sigma/Roche, COLLDISP-RO/11097113001, 500 mg; 10 mg/mL stock)

- Dissolve 500 mg enzyme in 50 mL DMEM/F12, dissolves quickly.
- Sterilize with low-protein binding syringe filter (0.22 µm).
- Aliquot the solution with 1 mL / microtube, store at -20 °C.

Collagenase 2 (CLS2) solution (Sigma, C6885, 100 mg; 10 mg/mL stock)

- Dissolve 500 mg enzyme in 50 mL DMEM/F12 + 50 mg BSA by flipping up and down the tube after the addition of the medium.
- Sterilize with low-protein binding syringe filter (0.22 µm).
- Aliquot the solution with 1 mL and 0.5 mL / microtube, store at -20 °C.

DNase I solution (Sigma, D4513-1VL, 2000 U/mL stock)

- For each bottle specific amount and enzyme activity needs to be checked using the Certificate of Analysis.
- Dissolve the given amount of enzyme according to enzyme activity. For example, if there is 11 mg enzyme in one bottle with a 4500 U/mg enzyme activity, to reach 2000 U/mL we need to add 24.75 mL ice-cold PBS to the powder.
- Sterilize with low-protein binding syringe filter (0.22 µm).
- Aliquot the solution with 800 µL / microtube, store at -20 °C.

Human basic fibroblast growth factor (hbFGF) (Sigma, F0291, 25 µg; 10 µg/mL stock)

- add 2500 µL DMEM/F12 to 25 µg bFGF vial
- No need to further sterile filter this vial. Aliquot the solution with 100 µL / microtube, store
  at -20 °C.

Bacterial culture medium for shaking cultures

- Middlebrook 7H9 media (BD Biosciences, Difco)
- 10 % oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD Biosciences)
- 0.05 % Tween 80 (Sigma Aldrich) in the presence of hygromycin B (100 µg/mL; InvivoGen)
- 0.2 %glycerol (Fisher Bioreagents)
- Hygromycin B (50 µg/mL, InvivoGen)
- Sterile filtered

Bacterial culture medium for agar plates

- Middlebrook 7H10 agar medium (BD Biosciences, Difco)
- 10 % OADC and 0.5 % glycerol (Fisher Scientific)
- 0.2 %glycerol (Fisher Bioreagents)
- Hygromycin B (50 µg/mL, InvivoGen)

Collagen type IV. (Sigma, C3355, 5 mg; 1 mg/mL stock)

- Stock to prepare: 1mg/ml (used concentration: 100µg/ml)

- Dissolve in 0.25% acetic acid-sterile distilled water, turn it upside down several times. Put it to 4 °C overnight.
- Next day aliquot it into Eppendorf tubes and store at -20 °C. Do not freeze and thaw. Can be stored at 4 °C up to one week

Heparin (Sigma, H3149, 100kU (512 mg); used concentration: 100µg/ml; 20U)

- Add to the bottle 10mL sterile DW, then sterile filter it with 0.22 µm syringe filter.
- Aliquot it as 500 µl / Eppendorf, store at -20°C
- After thawing store at 4 °C

Running medium for Basic protocol 5. *for 50 mL total*

- 5 mL FBS
- 500 µL ITS
- 500 µL HEPES
- 100 µL heparin
- Fill up to 50 mL with DMEM/F12, sterile filter
    - Split 34 mL of this and add 6.8 µL gentamicin stock (used: 10 µg/mL): "Running medium w/ antibiotics"
    - Keep the remaining 16 mL solution as antibiotic-free: "Running medium w/o antibiotics"

COMMENTARY

General discussion

Several *in vitro* models of BBB exist with many advantages and drawbacks which are used in various research methodologies from pharmaceutics to pathology (Helms et al., 2016). The primary mouse *in vitro* BBB model is well established and widely used by our laboratory (Sandor et al., 2014; Lenart et al., 2015). Tuberculosis meningitis is a well known, but understudied disease since its description in 1933 (Rich et al., 1933), with an under-defined mechanism regarding the entry of bacteria to the brain. Most protocols studying this phenomenon use free-floating bacteria (Jain et al., 2006), study the effect of supernates (Brilha et al., 2018) or phagocytic cells *in vivo* (van Leeuven et al., 2018), but no models have combined these aspects by integrating a primary brain EC based co-culture model with

primary DCs as phagocytic and antigen presenting cell type with primary PBMCs in combination with *Mycobacterium* infection. Previous data from our group showed that after *in vivo* BCG infection, primary granulomas are formed from where immune cells carrying live bacteria exit. These cells were identified as CD11c+ DCs (Harding et al., 2015), which arrest with CD4+ T-cells in the body at different distances from the primary focus forming new, secondary granulomas and promoting dissemination. Concurrently, the migratory capacity of infected cells isolated from liver granulomas were studied and revealed that phagocytic cells carrying mycobacteria are hindered in motion (Harding et al., 2015). The present model allows to test the idea of a cellular carrier facilitating dissemination of mycobacteria across the brain endothelial monolayer.

The current method is also based on the idea of previous *in vitro* granuloma models established by the co-culture of blood-derived mononuclear cells and monocytes (Birkness et al., 2007). In the present model, microscopy and flow cytometry analysis were facilitated by the use of specialized transgenic animals expressing CD11c-eYFP protein. The utilization of BCG and Mtb strains transfected with plasmids providing fluorescently detectable bacterial strains highly enhances the visualization and the tracking of infection. The P25 transgenic mouse strain enables us to utilize CD4+ T-cells specific to the Mtb peptide 25 (aa 240-254) of Antigen 85B bound to MHC class II. I-Ab. The combination of DCs and mononuclear cells in the system enables us to study cell-cell interactions near the *in vitro* granuloma formation site, which is very important due to the inherent role these cells play during the course of tuberculosis infection (McShane et al., 2002; Norris&Ernst, 2018).

Critical parameters and Troubleshooting

See Table 1. for troubleshooting guides to Basic Protocols 1-4.

Understanding results

When the model is assembled on Day 9 of the strategic planning (Figure 1.) four to five primary mouse cell types are combined into one model to understand cellular transmigration, cell-cell interactions and BBB morphological changes during *mycobacterial* infection. These factors probably will affect the brain endothelial barrier integrity, morphology, gene expression, signaling pathways and more. We find that this model which is assembled using phagocytic and antigen presenting DCs and surrounding PBMCs may provide a general platform to study dissemination of pathogens across vessels on a systemic level.

Time Considerations

For general timing and strategic planning consult Figures 1 & 8. These provide a detailed experimental schedule weeks and days before and after the start of the isolation procedures and show timing required for the model assembly.

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Internet resources

https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-isolation-of-mononuclear-cells.pdf

https://www.nexcelom.com/applications/cellometer/blood-based-samples/peripheral-blood-mononuclear-cells-pbmc/.

Figure legends

Figure 1. Timeline for the establishment of the *in vitro* mouse granuloma and blood-brain barrier model. For the isolation of liver granuloma supernates animals have to be infected with mycobacteria 3-4 weeks before experiments. Primary astroglia cultures need to be isolated 3 weeks before the isolation time of endothelial cells and dendritic cells to give time for growth, passage and then stabilization of glial cultures in the experimental setup. Isolation of the last 3 cell types, assembly of the model and incubation times take up to 11 days to perform.

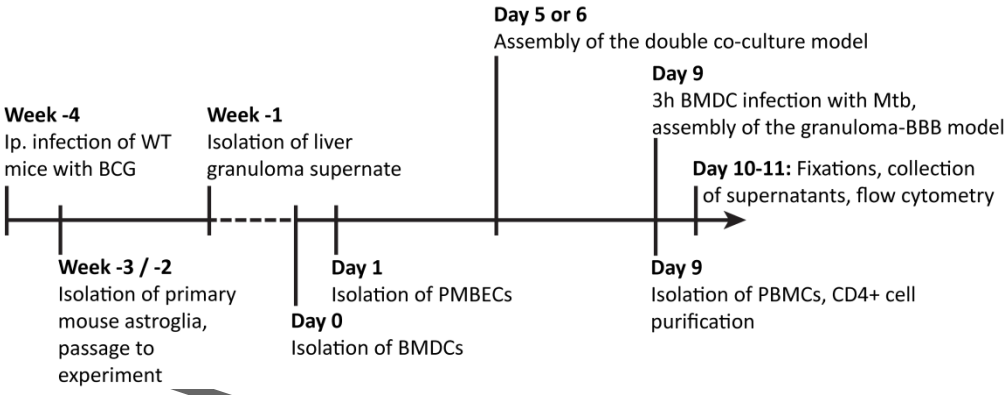

Figure 2. Example phase contrast pictures showing the growth timeline of primary mouse astroglia cultures on day 2, 4 and 7 after isolation. Pictures were taken on each day after performing appropriate washing steps. Bar: 250 µm.

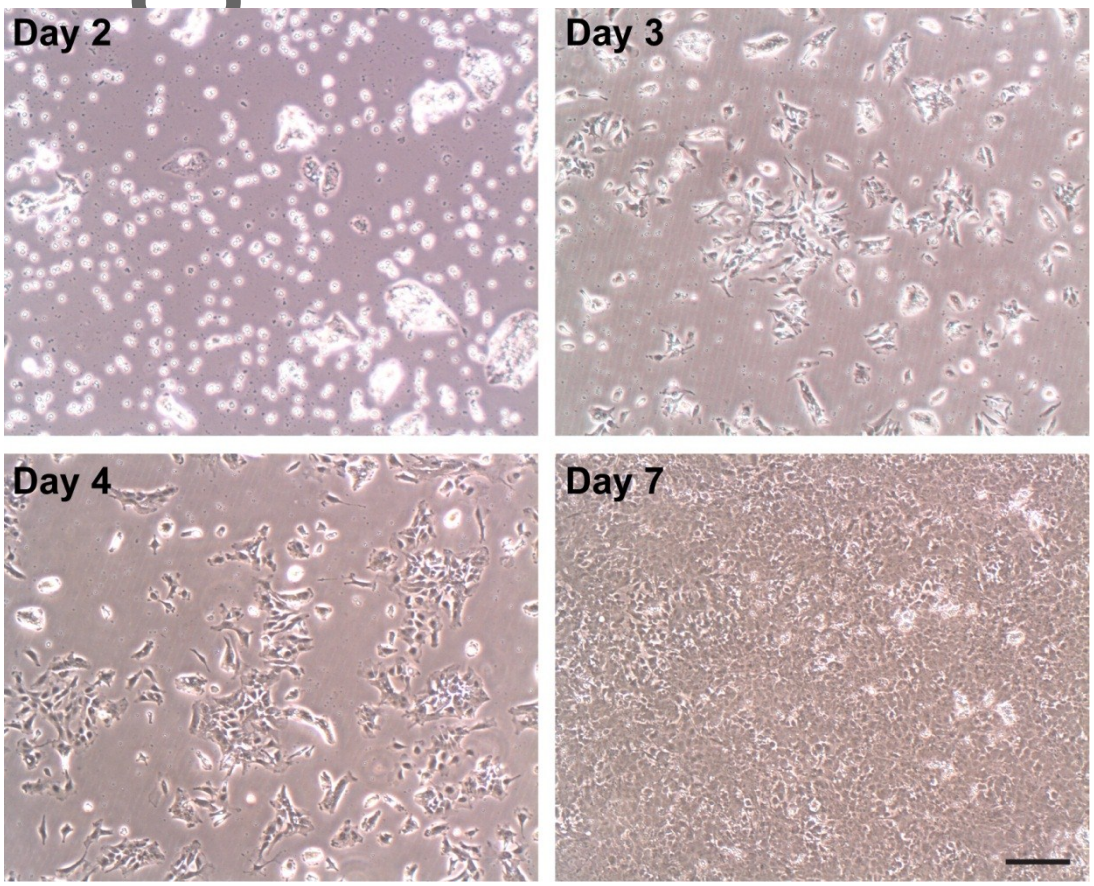

Figure 3. Example phase contrast pictures showing the morphology of primary isolated brain microvascular endothelial cells (EC) right after isolation on (Day 0) to full confluency (Day 4). Freshly isolated microvessels should be seen in the culture as floating shiny cell strings (pointed out by arrowheads). On Day 1 no medium change occurs yet, but the outlines of attached cellular islands should be visible below the floating dead "contaminating" cell types. Between Days 2-4 phase contrast pictures show cells after appropriate washing. Cellular islands are growing together and forming a confluent layer, which has a typical whirlpool-like growth pattern. Bar: 250 µm.

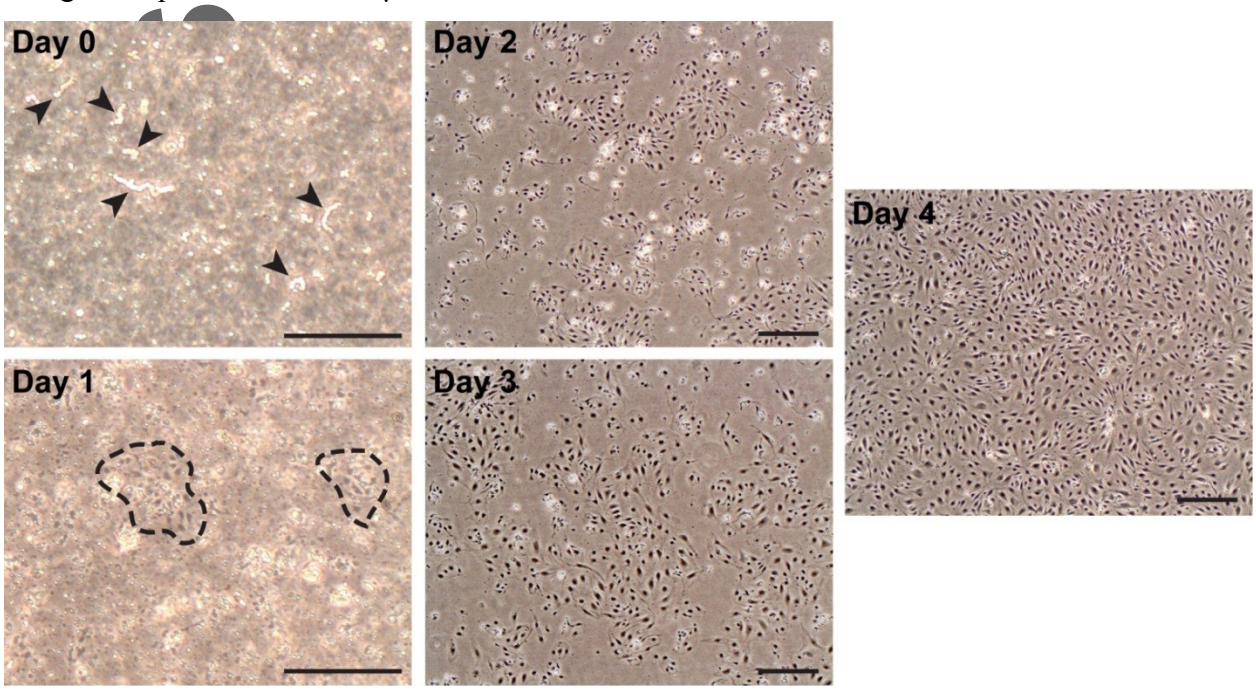

Figure 4. Schematic showing the assembled co-culture model of the BBB and resistance measurement. (A) Cartoon shows the position of brain endothelial cells (EC) and astroglia established with the Transwell cell culture insert method. Immunohistochemistry was performed on ECs to validate purity and proper cellular marker expression, which stained positive for tight junction marker molecule Claudin-5 (Cl-5). (B) Example transendothelial electrical resistance (TEER) values from one experiment during the growth and tightening phase of brain EC monolayer. Zero hour represents culture assembly; 96 hours means 4 days in culture. n=22.

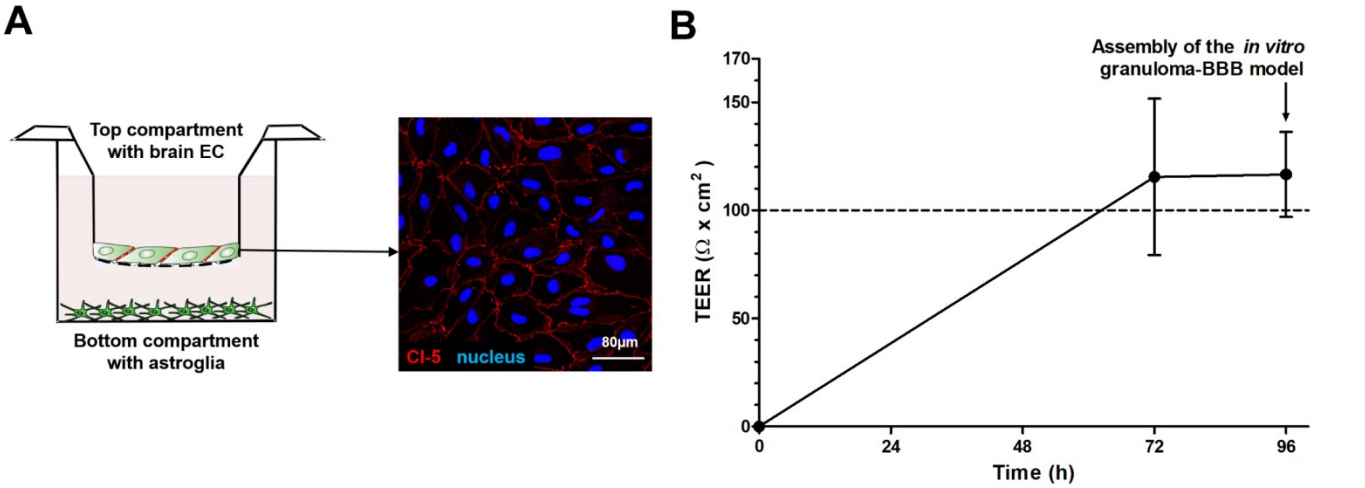

Figure 5. Sample cell culture light microscopy picture and flow cytometry data. A) CD11c eYFP+ dendritic cells forming small clusters in culture on day 3. B) eYFP expressing dendritic cell culture purity validation with flow cytometry on culture day 8. Analysis was performed using the FlowJo software to gate live cells (Lymphocytes) and CD11c eYFP+ cells.

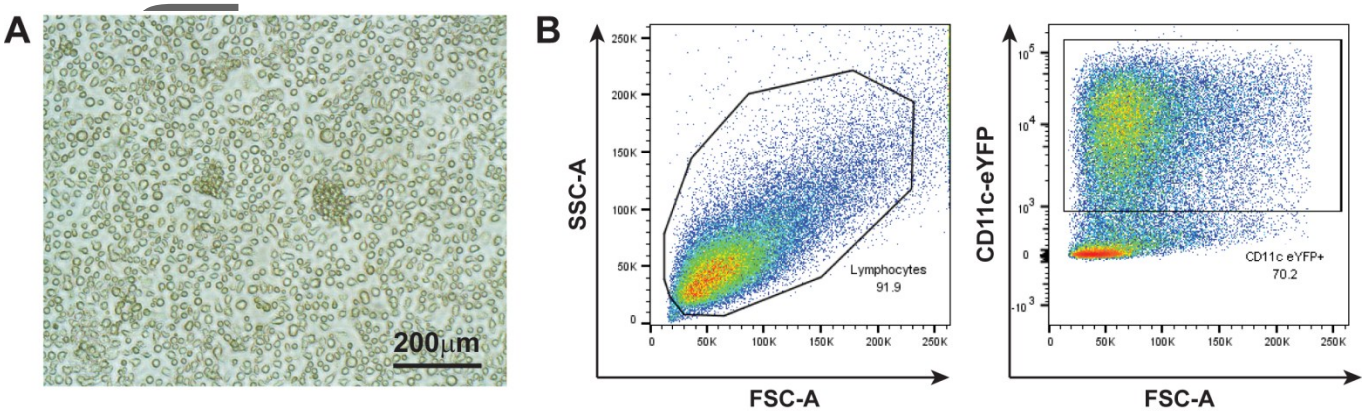

Figure 6. Lymphocyte isolation using Ficoll Paque PLUS density gradient centrifugation. At the bottom of the gradient red blood cells with granulocytes are present. Above that the gradient solution is visible, while the hazy PBMC rich layer is visible above this containing cells of our interest. Above the lymphocyte layer the blood plasma is present containing the majority of the platelets.

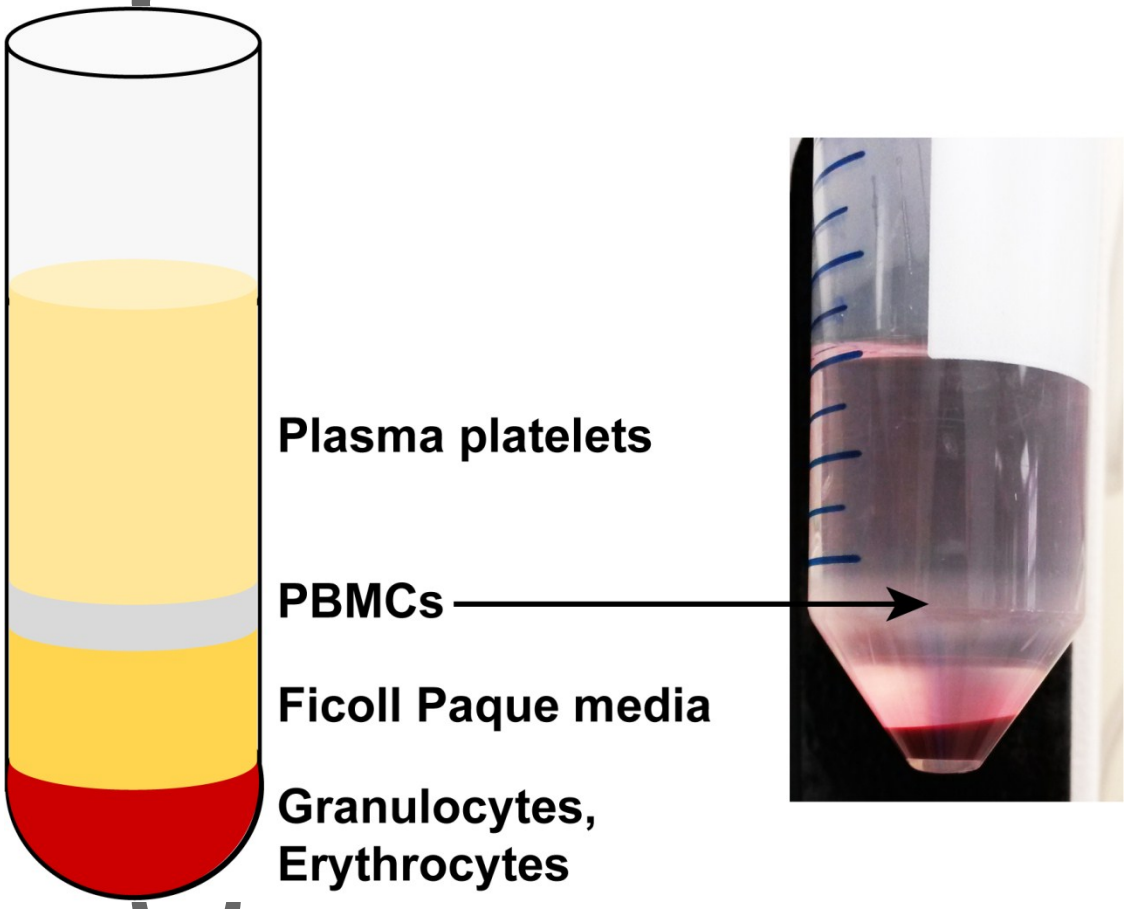

Figure 7. Sample flow cytometry data after Miltenyi mouse CD4+ T-cell isolation kit method. Cells were first gated for size, then for single cells. For this sample experiment purified PBMCs were labeled with viability dye and CD4+ antibody. It is clear that no cell-death occurs during the method and a high level of CD4+ purity can be achieved. Analysis was performed using the FlowJo software to gate single cells, live cells and CD4+ T-cells.

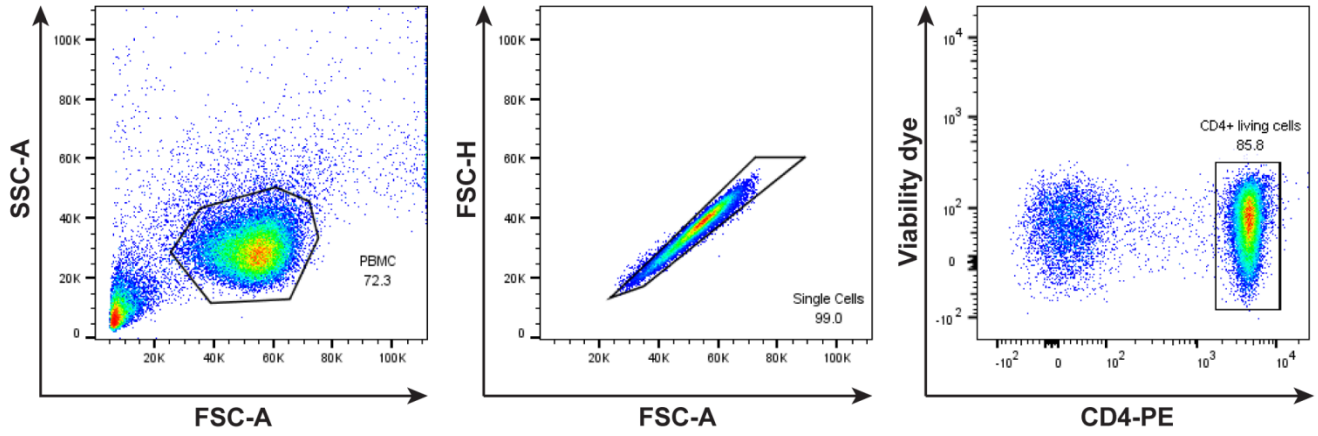

Figure 8. Timeline for the establishment of the *in vitro* mouse granuloma and blood-brain barrier model within Day 9 of the "Strategic planning" outline (Figure 1.). The day starts with the transendothelial electrical resistance (TEER) measurement on the BBB model, then medium is changed for less antibiotics and no hydrocortisone containing medium. Then DCs are passaged and infected. After this step the PBMC isolation is upcoming, with or without extra CD4+ cell isolation. After 4 h of DC infection cells are collected, and the whole model is assembled. These culture model could be used up to two days.

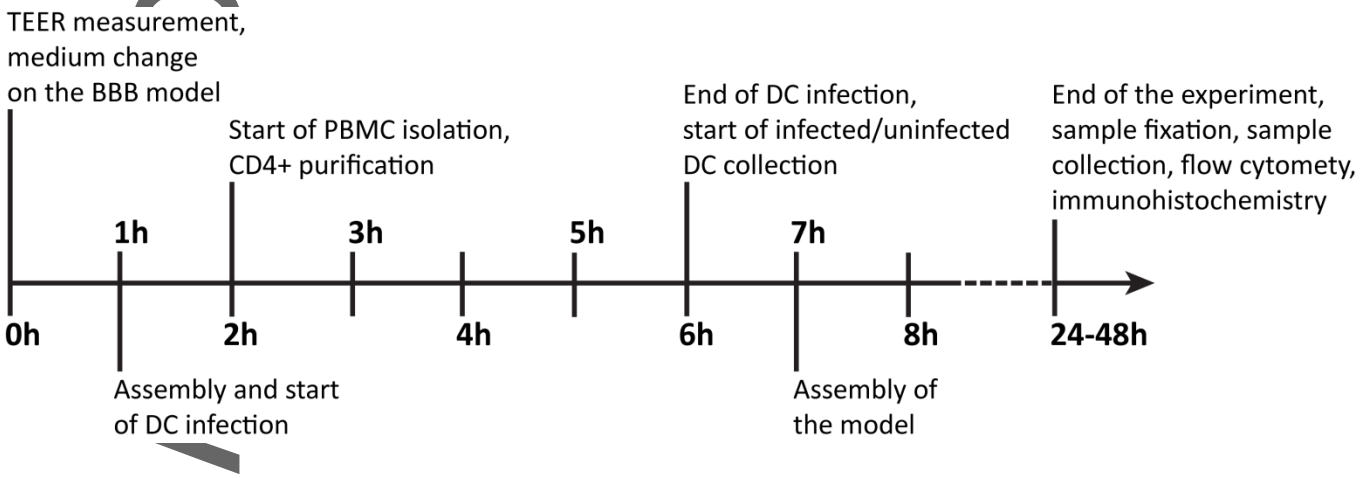

Figure 9. Example for an *in vitro* granuloma & *in vitro* BBB combination experiment to model cell-cell interactions and cellular transmigration across the brain endothelial monolayer.

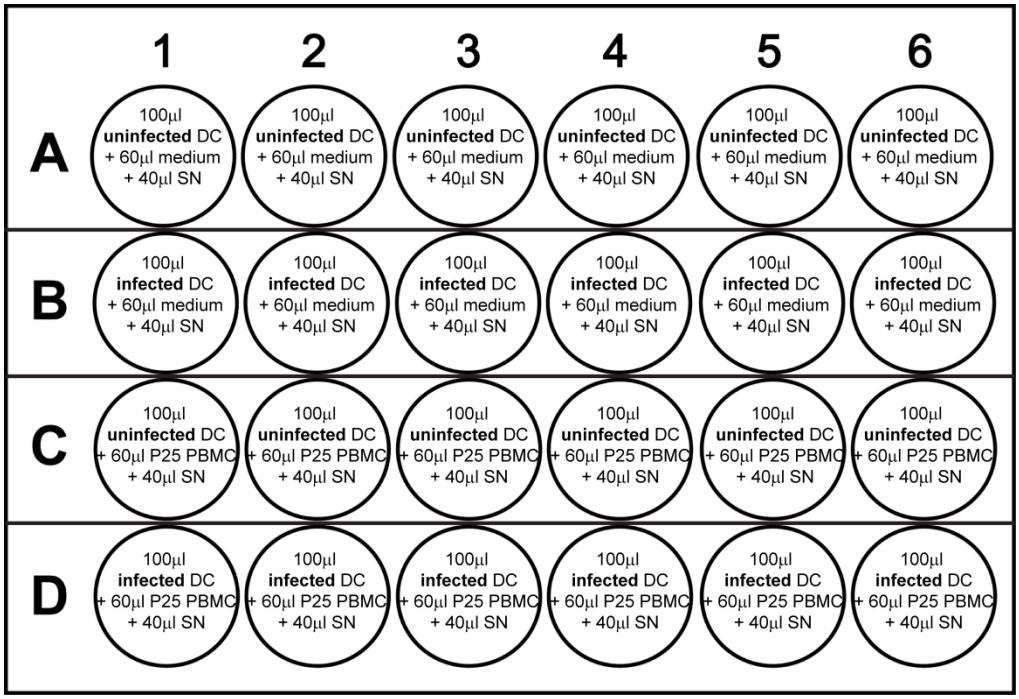

Table 1. Troubleshooting for basic protocols.

Problem	Possible cause	Solution
General: contamination in cell cultures	Pathogens: fast reproducing bacteria or fungi are introduced in the system	Use proper aseptic technique, combination of antibiotics and antimycotics. Except for the end of the experiment when using mycobacteria, which do not tolerate high concentrations of these agents. Perform regular maintenance of equipment, use proper disinfectant like 70 % ethanol. Discard contaminated samples.
Basic protocol 1.	Primary isolated endothelial cells are not growing properly	When handling primary brain endothelial cells an efficient quick, but gentle treatment is needed. Several factors need to be considered: 1. Proper digestion of the cells, 2. Proper gradient centrifugation, 3. Quality of the coating, 4. Quality of the plastic the cells are seeded to, 5. Concentration of each reagent used, especially antibiotics, sera and growth factors. Contamination of the culture needs to be checked very thoroughly. When isolating primary cells cultures can get very easily infected which can lead to a complete stop of cell growth.
	Glial cells are not attaching and growing	During the isolation the most critical step is the speed of the mechanical dissociation. If it is too fast, cells do not survive, but if it is too slow, many tissue clumps stay in the medium and the yield will be low. As a rule of thumb by the third round of mechanical dissociation not many tissue clumps should be visible by eye.
	Endothelial barrier is not established, cells are not visible	Cells spending too much time in trypsin, exposed to too harsh pipetting or which are kept too long at room temperature tend to be damaged leading to impaired barrier formation.
Basic protocol 2.	The femur bone breaks during isolation	During the dissection steps muscles need to be removed and joints need to be freed from its pocket very carefully.

		Later, when the bone spends too much time in the disinfectant ethanol, the tissue dries out and is prone to break.
Basic protocol 3.	No buffy coat, PBMCs do not separate	When using gradient centrifugation, brake at the centrifuge needs to be switched off. Too quick stop leads to the stirring of the gradient. Check expiry date of all components, here fresh gradient solution is key.
Basic protocol 4.	Bacteria clump too much	Mycobacteria tend to form big aggregates, clumps, which can be eliminated by longer sonication. If sonication does no help, bacterial clumps can be broken up by mechanical dissociation through a thin needle.

Reagent list

Animal/bacterial strains; Reagents and Consumables list.

Table S1.

Animal strains	**Company**	**Catalog number**
C57BL6 wild type mice	The Jackson Laboratory	000664
CD11c-eYFP (B6.Cg-Tg(Itgax-Venus)1Mnz/J)	The Jackson Laboratory	008829
P25Ktk/JC57BL/6-Tg(TcraTcrb)Ktk/J	The Jackson Laboratory	011005
Bacterial strains	**Company**	**Catalog number**
Mtb H37Rv	ATCC	27294/H37Rv
TMC 1011 [BCG Pasteur]	ATCC	35734

Table S2.

Reagent name	**Producer**	**Catalog number**
2-Mercaptoethanol	Sigma Aldrich	M6250-10ML
32 % paraformaldehyde solution	Electron Microscopy Sciences	15714-S
Accucheck counting beads	Thermo Fisher Scientific	PCB100
ACK lysing buffer	Thermo Fisher Scientific	A1049201
Anti-CD4+ antibody	BD Pharmingen	553048

ArC™ Amine Reactive Compensation Bead Kit	Thermo Fisher Scientific	A-10346
Bovine serum albumin powder	Sigma Aldrich	A7906
Collagen type IV	Sigma Aldrich	C3355
Collagenase type II	Sigma Aldrich	C6885
Collagenase-Dispase	Sigma Aldrich/ Roche	COLLDISP-RO/ 11097113001
Deoxyribonuclease I (DNase I)	Sigma Aldrich	D4513-1VL
Difco™ Middlebrook 7H10 Agar	BD Biosciences	262710
Difco™ Middlebrook 7H9 Broth	BD Biosciences	271310
DMEM (low glucose) GlutaMAX	Thermo Fisher Scientific	10567-014
DMEM/F12 GlutaMAX	Thermo Fisher Scientific	10565-018
EDTA solution	Thermo Fisher Scientific	AM9260G
Essential amino acid solution	Corning	25-030-CL
Fetal bovine serum (FBS)	Corning	35-010-CV
Fibronectin	Sigma Aldrich	F1141
Ficoll Paque PLUS solution	GE Healthcare	17144002
Gentamicin	Sigma Aldrich	G1397
GHOST viability dye	Tonbo Biosciences	13-0868-T500
GlutaMAX solution	Thermo Fisher Scientific	35050061
Glycerol	Fisher Bioreagents	BP229-1
granulocyte-macrophage stimulating factor (GM-CSF)	Peprotech	315-03
Heparin	Sigma Aldrich	H3149
HEPES solution	Corning	25-060-CI
Household bleach	-	-
Human basic fibroblast growth factor (hbFGF)	Sigma Aldrich	F0291
Hydrocortisone	Sigma Aldrich	H4001
Hygromycin	Invivogen	ant-hm-1
Insulin-transferrin-sodium selenite (powder)	Sigma Aldrich	I1884-1VL
Insulin-transferrin-sodium selenite (solution)	Thermo Fisher Scientific	41400045
Isoflurane	Santa Cruz Biotech	SC-363629RX
Middlebrook OADC enrichment	BD Biosciences	212351
Mouse CD4+ T-cell isolation kit	Miltenyi Biotec	130-104-454
Na-Pyruvate	Corning	25-000-CL
Non-essential amino acid solution	Corning	25-025-CL
Penicillin-Streptomycin solution 100x	Corning	30-001-CL
Phosphate buffered saline solution (PBS)	Corning	21-040-CV
Plasma Derived Bovine Serum (PDS)	First Link UK Ltd.	60-00-850
Puromycin	Sigma Aldrich	P7255
RPMI 1640	Corning	10-040-CV
Trypsin-EDTA solution (0.05 % with EDTA)	Corning	25-052-CI

Tween 80	Sigma Aldrich	P1754
UltraComp eBeads Compensation Beads	Thermo Fisher Scientific	01-2222-42
Vesphene Ilse	Steris Life Sciences	646108

Table S3.

Consumable name	**Producer**	**Catalog number**
0.22 µm low-bind syringe filter	Millipore	SLGP033RS
0.22 µm syringe filter	Corning	431218
0.45 µm syringe filter	Corning	431220
40 µm cell strainer	Corning	352340
100 mm bacteriological dishes, non-treated	Corning	351029
100 mm tissue culture dishes, TC treated	Corning	430167
60 mm cell culture dishes	Corning	430166
6-well tissue culture plates, TC treated	Corning	3516
24-well tissue culture plates, TC treated	Corning	3524
48-well tissue culture plates, non-treated	Corning	351178
96-well U-bottom plates	Corning	353077
T25 cell culture flask, TC treated	Corning	430168
15 mL conical centrifuge tubes	Corning	14-959-70C
50 mL conical centrifuge tubes	Corning	352070
1.5 mL microcentrifuge flex tubes	Eppendorf	022364111
Blue absorbent pad for surgical surfaces (Fisherbrand Absorbent underpads)	Thermo Fisher Scientific	14-206-62
Disposable cuvettes for OD measurement	BioExpress	E-5022-3
EVOM-2 Volt/Ohm Meter	World Precision Instrument	ENDOHM-6G
Glass beakers: 50 mL, 100 mL, 250 mL	Fisher Scientific	
Glass bottles for medium: 100 mL, 250 mL, 500 mL	Fisher Scientific	
MidiMACS Separator with MultiStand	Miltenyi Biotec	130-042-301
Miltenyi MACS LS column	Miltenyi Biotec	130-042-401
Motor driven tissue grinder		
Neubauer or Buerker chamber		
OD spectrophotometer (CO8000 Cell density meter)	WPA Biowave	80-3000-45
Refrigerated centrifuge	Eppendorf	5810R
Sterile dissecting instruments: forceps, scissors, scalpels, blades, lancets	World Precision Instruments / Kent Scientific	
Sterile gauze		
Sterile needles	BD Biosciences	
Special sterile needle, 21G 4 ¾	Braun	4665643
Sterile syringes	Braun	
Transwell inserts 3 µm pore size, with 24-well plates	Corning	3415
Ultrasonic Homogenizer (Model 300 V/T)	Biologics	