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TITLE:
Lentiviral vector preparation for efficient gene and microRNA modulation of peritoneal cavity tissue-resident macrophages *in vivo* in mice.

AUTHORS AND AFFILIATIONS:
Gurney M[1], Davies LC[1,2], Jones RE[1], Bart VM[1], Jenkins RH[1], Brennan P[1], Taylor PR[1,3], Czubala MA[1]

[1]Systems Immunity Research Institute, Division of Infection and Immunity, Cardiff University, UK [2]Biomedical Sciences Unit, Faculty of Medicine, Health and Life Science, Swansea University, UK
[3]UK Dementia Research Institute, Cardiff, UK

Corresponding Author:
Czubala MA (CzubalaMA@cardiff.ac.uk)

Co-Authors:
Gurney M (GurneyM1@cardiff.ac.uk)
Davies LC (l.c.davies@swansea.ac.uk)
Jones RE (jonesre27@cardiff.ac.uk)
Bart VM (valentina.bart.mail@gmail.com)
Jenkins RH (JenkinsRH2@cardiff.ac.uk)
Brennan P (BrennanP@cardiff.ac.uk)
Taylor PR (TaylorPR@cardiff.ac.uk)

SUMMARY:
We demonstrate a step-by-step protocol for the investigation of gene function in peritoneal tissue-resident macrophages *in vivo*, using lentiviral vectors.

ABSTRACT:

Peritoneal tissue-resident macrophages have broad functions in the maintenance of homeostasis and are involved in pathologies within local and neighboring tissues. Their functions are dictated by microenvironmental cues; thus, it is essential to investigate their behavior in an *in vivo* physiological niche. Currently, specific peritoneal macrophage-targeting methodologies employ whole mouse transgenic models. Here, a protocol for effective *in vivo* modulation of mRNA and small RNA species (e.g., microRNA) expression in peritoneal macrophages using lentivirus particles is described. Lentivirus preparations were made in HEK293T cells and purified on a single sucrose layer. *In vivo* validation of lentivirus effectivity following intraperitoneal injection revealed predominant infection of macrophages, restricted to local tissue. Targeting of peritoneal macrophages was successful during homeostasis and thioglycolate-induced peritonitis. The limitations of the protocol, including low level inflammation induced by intraperitoneal delivery of lentivirus, and time restrictions for potential experiments, are discussed. In summary, we present a quick and accessible protocol for the rapid assessment of gene function in peritoneal macrophages *in vivo*.

INTRODUCTION:

Tissue-resident macrophages (Mϕ) are a heterogeneous population of phagocytic immune cells that sense and respond to invading pathogens [1,2]. In addition, they play an essential role in tissue development, remodeling, and maintenance of the homeostasis[1,3]. Many tissue Mϕ derive from yolk sac progenitors during embryogenesis and persist in the tissue throughout the life[4,5]. The phenotype and functions of these cells are dictated by collaborative and hierarchical interactions of specific transcription factors and the local microenvironment[6-9]. Growing understanding of this dependency increases the need for effective *in vivo* methods for gene manipulation of Mϕ within their physiologically relevant niche.

Lentiviral vectors are a frequently employed tool for the manipulation of nucleic acids in specific cell populations *in vivo*[10-12], particularly due to their ability to infect both dividing and non-dividing cells and to stably integrate into host genome[13,14]. Over the last two decades, lentivirus delivery technology has been optimized, and alternative envelopes and synthetic promoters have been investigated to increase lineage specific targeting[8,15]. Owing to its broad cell tropism vesicular stomatitis virus envelop glycoprotein (VSV-G)[16,17] has become the "gold-standard" envelope used in lentivirus technology.

In this protocol[18], VSV-G pseudotyped lentiviral particles are employed to demonstrate targeted and effective delivery of short hairpin RNA (shRNA) and microRNA (miR) to mouse peritoneal Mϕ (pMϕ) *in vivo,* at steady state[19]. Transgene expression was driven by the spleen focus forming virus (SFFV) promoter. Productive infection of cells was defined by expression of lentivirus-derived enhanced green fluorescent protein (GFP). Utilization of this approach allowed easy readout for *in vivo* lentivirus experiments to define the optimal dose and the experimental timeframe. Finally, *in vivo* lentiviral challenge of mice during thioglycolate-induced inflammation, revealed the natural propensity for selective pMϕ infection.

PROTOCOL:

All animal work was conducted in accordance with Institutional and UK Home Office guidelines.

NOTE: All *in vivo* studies with lentivirus should be performed according to local and national guidelines on the ethical use of animals in research, as well as adhering to all regulations associated with the use of category II infectious materials. Animal welfare should also be monitored in accordance with local regulations. In this step of the protocol extreme care needs to be taken when working with lentiviral particles and sharps.

1. Preparation of HEK293T cells for transfection

NOTE: Perform these steps under a sterile tissue culture biological safety cabinet.

1.1 Prepare complete Dulbecco's Modified Eagle Medium (cDMEM) for HEK293T cells by combining the 450 mL DMEM with 10 % v/v fetal calf serum (50 mL), and final concentration of 100 U/mL penicillin/streptomycin.

1.2 Defrost HEK293T cells at least one week prior to planned transfection. Maintain healthy growing conditions and passage every 2 - 3 days using trypsin + EDTA to detach the cells from the flask.

1.3 One day prior to transfection, carefully aspirate the growth medium from the cells and gently wash cells with sterile DPBS. Add 1 – 5 mL of trypsin to the flask and incubate at 37 °C in a 5 % CO_2 incubator for 2-5 minutes. Add 5 – 10 mL of cDMEM and spin the cells at 350 x g for 5 min. Remove the liquid and resuspend the cell pellet in 10 mL of cDMEM. Count the cells and seed 10 - 11 x 10^6 viable HEK293T cells per T175 flask in 20 mL of cDMEM.

1.4 Incubate at 37 °C in a 5 % CO_2 incubator overnight to allow the HEK293T cells to reach 70 - 80 % confluency.

1.5 The following day, confirm the confluency of cells under a light microscope.

1.6 Remove the media from the flask with a 25 mL serological stripette without disturbing the cell monolayer.

1.7 Gently, add 10 mL DPBS and rock the plate to wash the cells, taking care not to disturb the cell monolayer.

1.8 Remove DPBS with a 25 mL serological stripette.

1.9 Add 15 mL of new cDMEM on the wall per T175 flask not to disturb the cell monolayer, and return the plate to the incubator at 37 °C.

2. Transfection of HEK293T cells using non-liposomal lipid transfection reagent

2.1 In a 15 mL centrifuge tube, prepare the lentiviral components: 2 µg lentiviral plasmid, 1.5 µg pΔ8.91 (pCMV-Δ8.91) and 1 µg pMD2.G (pCMV-MD2.G) with Buffer EC to a final volume of 600 µL.

2.2 Add 36 µL of Enhancer solution. Mix the components using a 1 mL pipette by repeatedly sucking up a part of the liquid and putting it back drop by drop directly on the remaining solution.

2.3 Leave for 5 min at room temperature.

2.4 Add 120 µL of transfection reagent and mix as above approximately 20 times.

2.5 Incubate for 10 min at room temperature.

2.6 Add 5.2 mL of cDMEM and mix as above with a 5 mL serological stripette.

2.7 Using a disposable transfer pipette, add the transfection mix dropwise directly onto the HEK293T cells monolayer (avoiding the flask walls), spreading the mix at different spots in the flask.

2.8 Distribute the plasmid by gently rocking the flask from side to side. Avoid getting any liquid in the flask lid.

2.9 Incubate the flask at 37 °C in a 5 % CO_2 incubator for 48 h. Effective transfection of HEK293T cells is confirmed by the appearance of fluorescent marker, here GFP, within 24 hours of transfection. This can be monitored on a cell imaging fluorescent microscope. For constructs without the fluorescent marker, HEK293T cells must be tested for the presence of used marker gene or by expression change in the gene/protein of interest by appropriate techniques, e.g., qPCR. Alternatively, successful transfection can be indirectly verified during testing of the lentivirus collection on Jurkat cells at the later stages of the protocol by the above techniques.
CAUTION: Transfected HEK293T cells are considered infectious and appropriate safety precautions should be implemented when handling these cells. Local rules should be followed, which could typically include: working under a category II biology safety cabinet; the use of double gloves; and appropriate decontamination solution for disinfection, for example increased concentration of waste bleach (2,000 ppm) or equivalent solution according to institutional biosafety guidelines. All solutions and plastics coming in contact with lentivirus preparation should be disinfected accordingly to institutional biosafety procedures.

3. Collection of lentiviral particles

3.1 Prepare decontamination solution, a high concentration (2,000 ppm) bleach (sodium hypochlorite) solution or equivalent agent according to institutional biosafety guidelines in a bucket and place it in the biology safety cabinet.

3.2 Prepare the biology safety cabinet by removing any excess items, such as empty tube racks etc.

3.3 Pre-label a 50 mL centrifuge tube per T175 HEK293T cells flask and remove the lid from the centrifuge tube. Place the tubes in a stable rack.

3.4 Retrieve the flask from the incubator and confirm GFP expression using a fluorescent microscope with a 488 nm laser (**Figure 1A**). The intensity of the signal depends on the transfection efficiency of the procedure and used constructs.

3.5 Collect the media from the cells into the pre-labelled 50 mL centrifuge tube. Tilt the T175 HEK293T flask with transfected cells so the medium collects in the bottom corner of the flask. Using a 25 mL serological stripette, collect the medium without disturbing cell monolayer. Some floating cells might be visible at this point. Transfer the medium to clean 50 mL centrifuge tube and close the lid.

3.5.1 Using 25 mL serological stripette, add 25 mL of fresh, warm cDMEM to the flask. Make sure to direct the medium flow on the walls of the flask not to disturb the cell monolayer. Return the flask with transfected HEK293T cells to the 37 °C in a 5 % CO_2 incubator. A Second collection of lentivirus and further purification can be performed after additional 24 h following the procedure outlined in the steps above.

3.6 When lentivirus collection is completed after 24 or 48 hours, add decontamination solution to the cells, making sure it covers the cells monolayer. Keep the flask horizontal for the next 24 hours, then discard it following appropriate category II regulations.

4. Purification of lentivirus

4.1 Filter collected medium from step 3.5. Remove the plunger from the 50 mL syringe and insert a low protein binding polyether sulfone or polyvinylidene fluoride 0.45 µm, sterile filter into the syringe end. . Using 25 mL serological stripette add collected medium from step 3.5.1 to syringe and gently return the plunger. Push the plug slowly to pass the medium through the filter into fresh 50 mL centrifuge tube. If the flow reduces significantly, position syringe with the filter pointing upwards and pull out the plunger enough to clear the medium from the filter. Carefully, replace the filter on the syringe with a new one and dispose used filter in the decontamination solution. Continue medium filtration. When finished, decontaminate the filter and the syringe by submerging the filter and filling the syringe with the decontamination solution.

4.2 Pre-cool the ultracentrifuge by setting the temperature to 4 °C and ensure the lid is shut to allow the temperature to acclimatize.

4.3 Add 3 mL of 20 % sucrose solution into the bottom of the conical ultracentrifuge tube.

4.4 Using a 25 mL serological stripette, overlay filtered medium on top of the sucrose layer, being careful not to disturb the layers. Use the lowest speed setting on the pipette boy. Angle the conical ultracentrifuge tube to about 45 degrees and slowly add filtered lentivirus containing medium from step 4.1 to the tube wall. Medium and sucrose should not mix and clear separation of the layers should be visible (**Figure 1B**). As the volume of the medium layer increases, slowly tilt the ultracentrifuge tube back to an upright position.

4.5 If the total volume, including the sucrose and the medium, in the ultracentrifuge tube is less than 29 mL, top up with fresh cDMEM to avoid the tube collapsing during the spin. For multiple T175 mL collections, mix filtered medium preparations and use multiple ultracentrifuge tubes. Top up the final ultracentrifuge tube with medium as required.

4.6 Make sure the ultracentrifuge buckets are clean, there is no residue medium on the bottom of the bucket or on the caps. If required, wiped out with ~70 % alcohol. Put in appropriate tube adapters in the bottom of each bucket (**Figure 1C**) and gently lower the ultracentrifuge tubes into the buckets.

4.7 Close the buckets securely and hang them on the allocated spaces on the spin-out rotor.

4.8 Ensure that the buckets are balanced with the same volumes of sucrose and medium. A spin-out rotor must never be run without buckets, although opposing buckets may be left empty[20] (**Figure 1D**).

4.9 Carefully, insert the rotor into the ultracentrifuge and turn on the vacuum.

4.10 Set the ultracentrifuge acceleration and deceleration rate to the lowest setting, and run the lentivirus preps at 85,000 x *g* for 90 min at 4 °C.

4.11 Wait for the ultracentrifuge to reach the required speed (about 3-5 min) before walking away to ensure the rotor is inserted properly and the spin will not terminate.

4.12 Whilst the ultracentrifuge is running, clean up the workspace accordingly to category II safety requirements and prepare the space for the next steps.

4.13 When the spin finishes, disable the vacuum, and carefully remove the rotor not to disturb the samples.

4.14 Investigate the ultracentrifuge for any spills and confirm no leaks from the buckets. In case of spillage, double glove and decontaminate the centrifuge and external surface of buckets with antiviral products.

4.15 Remove the buckets from the ultracentrifuge and carefully transport the buckets to the category II biology safety cabinet.

NOTE: Lentivirus particles collect at the bottom of the ultracentrifuge tube. The pellet is not visible to the naked eye.

4.16 Pour the sucrose and medium with one smooth motion directly into the waste container with decontamination solution.

4.17 Keeping the ultracentrifuge tube inverted, transfer it to the double layer of tissue and leave it to dry for 10 min. Meantime, wipe the inside of the buckets with water-soaked tissue and air-dry upside down.

4.18 Carefully dry any remaining liquid from the rim of the ultracentrifuge tube with tissue, before turning it upright. Disinfect the tissue and the surface underneath.

4.19 Resuspend the virus pellet in 1 mL of serum free media, or solution required for further experimentation, by gently pipetting the solution up and down. Leave it for 15 min at room temperature inside the category II biology safety cabinet.

4.20 Gently mix the lentivirus preparations using 1 mL pipette before aliquoting into 1.5 mL screw top tubes (e.g., cryotubes). Prepare the aliquots for the *in vivo* work (multiplicity of 100 µL) and for lentivirus titer in Jurkat T cells (1 x 20 µL) (see step 5).

4.21 Avoid freeze-thaw cycles, lentiviral preparations can be stored at -80 °C for up to 6 months without negative effect on infectivity.

5. Titration of lentivirus production in Jurkat T cells

5.1 Prepare complete Roswell Park Memorial Institute 1640 medium (cRPMI-1640) for Jurkat T cells by supplementing 500 mL RPMI-1640 with 10 % (v/v) fetal calf serum, and final concentration of 100 U/mL penicillin/streptomycin.

5.2 To ensure a healthy culture of Jurkat T cells, maintain the cells in culture for up to 1 week before infection.

5.3 On the day of the lentivirus titration, plate out viable Jurkat T cells in a 24-well plate at 2 x 10^5 cells/well in 200 µL per well of cRPMI-1640.

5.4 Use freshly collected lentivirus or thaw the lentivirus vial containing 20 µL on ice and mix gently by pipetting up and down.

5.5 Using serial dilution in cRPMI-1640, infect Jurkat T cells with 0.25, 0.5, 1, 2.5, 5, and 10 µL of lentivirus stock. Gently rock the plate to assure equal distribution of the lentivirus.

5.6 Non-infected Jurkat T cells serve as a negative control.

5.7 Incubate the plate at 37 °C. After 4 h, top up each well with cRPMI to a total volume of 400 µL. Return the plate to the incubator at 37 °C for 3 days.

5.8 After 48 h post infection, confirm GFP expression under a fluorescent imaging system.

5.9 3 days after infection, collect each well of the 24-well plate into a separate 1.5 mL collection tubes and centrifuge the cells at 350 x *g* at 4 °C for 5 min.

5.10 Discard the supernatant . Resuspend the pellet with 300 µL of 2 % paraformaldehyde (PFA) prepared to 2% (w/v) in DPBS and leave it for 15 min on ice in the dark.

5.11 Centrifuge the cells at 350 x *g* at 4 °C for 5 min and resuspend the pellet in flow cytometry (FACS) buffer.

5.12 Analyze the GFP expression frequency and mean fluorescent intensity of the lentivirus infected and its control cells using flow cytometry (**Figure 2**).

6. *In vivo* lentivirus infection of tissue-resident peritoneal macrophages

6.1 In a category II biological safety cabinet, load insulin needles with a total volume 200 µL serum free media medium containing required amount of lentivirus (use single-use needles, 30 G for animal welfare and to avoid fluid loss in the needle).

6.2 Place the needle sheath back on the needle using the one-hand scoop technique. Keep the needle on ice and inject within 30 minutes.

6.3 In the animal facility, set up a category II biological safety cabinet prior to injections (**Figure 1E**): lay down a sheet of clean tissue, loosen the sheath on the insulin needle containing

lentivirus, prepare a petri dish containing small pieces of tissue and chlorhexidine gluconate-based disinfectant, a 50 mL tube containing decontamination solution (2,000 ppm bleach solution) and a sharp safe container.

6.4 Restrain the mouse by grasping the skin at the nape of the neck[21]. Properly restrained mice should be immobile, and this is required for safety.

6.5 With the abdomen facing up, point the animal's head slightly down. Intraperitoneally inject the lentivirus into the lower right quadrant of the abdominal cavity, avoiding injection into any peritoneal cavity organs.

6.6 Before releasing the mouse, fill the syringe with decontamination solution and safely dispose of it in the sharp safe box.

6.7 Wipe the injection site on the mouse abdomen with tissue soaked in disinfectant and return the animal to the cage.

6.8 Lentivirus-injected mice should be housed in category II scantainer or individually ventilated cages (IVC) (isolated cages with high efficiency air filtration) for a minimum of 72 h after injection. An appropriate information card should be placed on the front of the cage.

6.9 Mice can be moved to new category I holding cages after 72 h post infection, depending on local safety approvals.

6.10 Monitor mice daily for a total of 3 days from injection.

7. Collection of peritoneal cells from lentivirus infected mice

CAUTION: For collections within 72 h post lentivirus injection, follow institutional category II biological safety rules. Bedding and holding cage where infected animals were kept in the first 72 h post lentivirus injection must be decontaminated accordingly to institutional category II biological safety rules.

7.1 Euthanize mice in accordance with institutional regulations, for example by inhalation of increasing concentration of CO_2, followed by confirmation of death by cervical dislocation.

7.2 Clean the mouse abdomen with 70 % isopropanol and carefully cut the skin to expose the peritoneal cavity membrane.

7.3 Lavage the peritoneal cavity with 6 mL of ice-cold FACS buffer using a 10 mL syringe and 23 G needle. Avoid puncturing any organs.

7.4 Gently massage the peritoneum to dislodge cells to FACS buffer.

7.5 Collect the peritoneal fluid using the same syringe and needle.

7.6 Remove the needle and transfer the cells to a 15 mL centrifuge tube.

7.7 Keep the peritoneal lavages on ice.

7.8 Collect other required organs and store them as appropriate to the study.

7.9 Dispose of animal carcasses according to local animal and safety guidelines.

8. Peritoneal cells staining and analysis

8.1 Centrifuge the peritoneal lavage at 350 x *g* at 4 °C for 5 min, discard the supernatant in decontamination solution and resuspend the cells in 1 mL of FACS buffer.

8.2 Count the collected cells and plate 4 x 10^5 cells per well in a V-bottom 96-well plate.

8.3 Perform viability staining using fixable reagent (e.g., Fixable Near-IR Dead Cell Stain Kit used here) accordingly to manufacturer instructions.

8.4 If cells were infected within the last 72 h, fix the cells in 2 % PFA for 15 min on ice. Add an equal volume of cold DPBS and centrifuge again at 350 x *g* at 4 °C for 5 min.

8.5 Prepare the blocking buffer: mix 4 µg/mL 2.4G2 antibody in 10 % (v/v) rat serum in FACS buffer for surface staining or in permeabilization buffer for intracellular staining.

8.6 Resuspend each well containing cell pellet in 50 µL blocking buffer and incubate at 4 °C for 15 min.

8.7 Prepare antibody mix in either 50 µL of FACS buffer (for surface staining) or permeabilization buffer (for intracellular staining) per each sample. Final staining volume for each sample including 50 µL blocking buffer will total 100 µL and antibody concentrations should be calculated accordingly.

8.8 Add 50 µL of antibody mix to samples and 50 µL of isotype controls and control buffer to control samples. Incubate the samples for 30 min on ice in dark.

8.9 Include unstained cells and isotype controls as required.

8.10 Wash each well with 100-200 µL ice-cold DPBS and centrifuge the plate at 350 x *g* at 4 °C for 5 min. Repeat this step to wash away any unbound antibody.

8.11 Analyze the samples on a flow cytometer.

9. Extraction of cells from organs

9.1 Prepare 1 mL of digestion mix per organ: mix Hank's balanced salt solution (HBSS), 2 mg/mL collagenase type IV and 0.03 mg/mL DNase I (include 1.5 mg/mL of hyaluronidase for lung digestion).

9.2 Transfer collected organ to 1 mL digestion mix and mince it with scissors.

9.3 Filter the cells through a 40 µm strainer and centrifuge at 350 x *g* at 4 °C for 5 min.

9.4 If isolating cells from lung, spleen, or liver, lyse red blood cells using ACK lysis buffer. Filter the cells through a 40 µm strainer and centrifuge at 350 x *g* at 4 °C for 5 min.

9.5 Stain and analyze the cells as described in step 8.

REPRESENTATIVE RESULTS:

When followed fully and correctly, this protocol yields a total of 1.5 mL of high-quality lentivirus stock per single preparation, sufficient for twelve *in vivo* injections at the optimal volume determined in this study[18]. Success of the transfection can be evaluated early in the protocol. Healthy and confluent HEK293T cells should display, if present in the plasmids, an easily detectable marker signal (e.g., GFP used in this study) after 48 h post plasmid transfection (**Figure 1A**). Low intensity of the signal, excessive cell detachment and low confluency at early steps of the protocol, could indicate cell death and will result in low yield of the lentivirus preparation. Some of the cell detachment prior to optional collection II (**step 3.5.1**.) is visible and expected.

Three plasmids are used in this protocol for generation of lentivirus particles: pCMV-ΔR8.91 packaging plasmid encoding structural HIV-1 protein (Gag), accessory proteins Tat and Rev, and reverse transcriptase polymerase (Pol)[22]; pMD2.G encoding VSV-G envelop under CMV promoter[13]; and pHR'SIN-cPPT-SEW plasmid (encoding enhanced GFP marker)[19] modified accordingly for the expression of shRNA or microRNA.

Lentivirus preparations are titrated in the Jurkat T cell line due to their high infectivity[23]. Successful lentivirus preparation will achieve infection rate over 95 % with dose as small as 5 µL (**Figure 2A**). Mean fluorescent intensity of the infected cells continues to increase with the higher doses (**Figure 2B, C**). If required, viral titers can be measured using real-time PCR of integrated viral components e.g., the SFFV promoter, which correlated linearly with percentage GFP-expressing cells and logarithmically with GFP MFI (**Figure 2D**). Depending on the construct, particularly those with a large insert[24], some lentivirus preparations can display reduced infectivity in Jurkat T cells as demonstrated for Cre-GFP construct used in this study (**Figure 2E,F**). In such case, multiple preparations of lentivirus particles could be combined and resuspended in 1 mL. We recommend validating immune responses to those preparations *in vivo* prior to experimentation. Naturally, lentivirus preparations with alternative entry receptors to VSV-G used here, might display different infection efficiency in the Jurkat T cell line, depending on the receptor expression on the cells. Cell lines used for the titration should be selected so they express the receptor used by the lentivirus particles to enter the cells and would preferentially lack or have very low expression of the restriction factors[25].

Successful production of lentiviral particles is further evidenced by infection of pMϕ (defined as $CD11b^+$ $F4/80^+$ and $Tim4^+$ population)[18] (**Figure 3A**). We determined that intraperitoneal

injection of 100 µL lentivirus preparation (in total volume of 200 µL of serum free media) yields the highest percentage and intensity of the GFP signal in these cells (**Figure 3B, C**). Injections of higher doses (150 µL and 200 µL of lentivirus preparation) had no beneficial impact on GFP expression in the pMϕ. Time course experiments at 4 h, 3, 7 and 14 days post intraperitoneal (i.p) injection with 100 µL lentivirus revealed a significant percentage of GFP-expressing resident pMϕ at days 3 and 7, followed by the disappearance of the infected population at day 14 (**Figure 3D, E**). Interestingly, GFP-expressing pMϕ mostly disappears by day 14 post infection, due in part to immune recognition of the GFP marker. Indeed, lentivirus experiments with GFP marker in T-Reg selective, Foxp3-DTR-eGFP mice prevent rejection of infected resident pMϕ until at least day 21 (**Figure 3F**). For lentivirus preparations with diminished effectiveness in Jurkat cells, higher amount would be required to achieve the expected infection rate *in vivo*. However, as demonstrated with Cre-GFP lentivirus preparation, depending on the construct design, even a dose of 300 µL at 7 days post i.p injection might yield a poor outcome (**Figure 3G**). We have previously demonstrated a successful use of this protocol for overexpression and knockdown of genes *in vivo* in mouse pMϕ, including lentiviral shRNA-mediated *Map3k8* and *Gata6* knockdown, and *Gata6* overexpression[19]. Here we show that this protocol can be also successfully employed for overexpression of murine microRNA 146b (mmu-miR-146b) and for knockdown of intercellular adhesion molecule 1 (ICAM1, CD54) using lentiviral derived shRNA in resident pMϕ (**Figure 3H, I**).

Infection of primary cells, such as macrophages, requires higher lentivirus input, presumably due to presence of the restriction factors in these cells. Restriction factors are natural protective mechanisms of cells, which interfere with viruses' life-cycle steps, such as reverse transcription or integration, leading to inhibition of gene expression from the constructs.

Further *in vivo* validation of the protocol demonstrated no effect of the i.p lentivirus delivery on peritoneal immune cell viability (**Figure 4A**) and indicated distinct infectivity of resident pMϕ subpopulations (defined by the expression of CD73 and Tim4 markers) (**Figure 4B, C**). Importantly, productive lentivirus infection was limited to resident Mϕ at the site of injection as evidenced by lack of significant GFP expression in mesenteric lymph node (mLN), lung, liver, or spleen Mϕ after 7 days post challenge (**Figure 4D**). Considering that in many cases genetic targeting of Mϕ is required to be performed under inflammatory conditions, we investigated the effectiveness of this protocol in mice challenged intraperitoneally with 0.1 mL of 4 % thioglycolate. Thioglycolate injection triggers an influx of inflammatory monocyte-derived Mϕ and monocytic cells that can be divided into 5 distinct populations (**Figure 4E**). Flow cytometry analysis revealed refractory phenotype of monocytic-like cells ($Ly6C^{hi}$ populations 1 and 2) to lentivirus infection, in line with previous findings in human cells[26]. In contrast, susceptibility of resident Mϕ and monocytes (groups 3 - 5) corresponded to their phenotypic convergence on the recognized phenotype of resident pMϕ, which remained most easily infected (**Figure 4F**).

Detailed flow cytometric analysis detected GFP expression predominantly in resident peritoneal Mϕ and major histocompatibility complex (MHC) class II^+ resident pMϕ ($MHCII^+$ $F4/80^+$ $Tim4^+$) (up to 60 % at day 3 after injection) (**Figure 5A**). Little to no GFP signal was detected in other peritoneal cell populations, including bone marrow derived peritoneal Mϕs/DCs ($MHCII^+$, $CD11b^+$, $CD11c^+$), B cells ($CD19^+$), T cells ($CD3^+$), mast cells ($CD11b^-$, $Fc\varepsilon R1^+$),

eosinophils (Siglec-F$^+$), NK cells (CD19$^-$, NK1.1$^+$) and neutrophils (Ly6G$^+$) (**Figure 5A, B**). GFP expression longevity (**Figure 5C**) in all populations followed this of resident peritoneal Mϕ (**Figure 1D**). A transient increase in neutrophils frequency was recorded after 4 h post i.p injection of 100 µL (in a total volume of 200 µL serum free media) lentivirus (**Figure 5D**) indicating early mild inflammation present in challenged animals. However, neither interferon alpha (IFN-α) nor beta (IFN-β), normally released in response to viral infection, were detected in the peritoneal cavity effluent when tested at 4 h, 3, 7, and 14 days post injection (data not shown). Finally, resident pMϕ experienced a major drop in the frequency between days 7 and 14 post infection (**Figure 5E**), suggesting the best experimental window between days 3 and 7 post injection.

FIGURE AND TABLE LEGENDS:

Figure 1: Lentivirus production in HEK293T cells. (**A**) Representative immunofluorescence pictures of HEK293T cells 48h after successful transfection (fluorescent microscope (488 nm excitation peak, 510 nm emission peak), x20 magnification, scale bar = 400 µm). (**B**) Photograph of ultracentrifuge conical tube containing a layer of 20 % sucrose (bottom, clear) and a layer of medium collected from transfected HEK293T cells (top, red). (**C**) Photograph of correct insertion of the conical ultracentrifuge in the bucket, including adaptor. (**D**) Photographs showing correct and incorrect balancing of the ultracentrifuge rotor. (**E**) Photograph of the optimal setup of the Cat II cabinet and materials for *in vivo* injections for a right-handed individual. This figure has been modified from Ipseiz N *et al*., 2020[18].

Figure 2: Lentivirus titration in Jurkat T cells. (**A**) Representative flow cytometric analysis of Jurkat T cells 72 h after infection with increasing doses of lentivirus containing a GFP plasmid showing percentage of GFP$^+$ cells (GFP$^+$), (**B**) mean fluorescence intensity (MFI) of GFP$^+$ cells and (**C**) a representative histogram of GFP expression. (**D**) Scatter plot showing MFI (left Y) and the percentage of cells infected with an GFP-expressing lentivirus (right Y) versus the copy number of virus detected per pg of DNA (x axis). (**E**) A representative histogram of Jurkat T cell infection with suboptimal Cre-GFP lentivirus (Cre-GFP LV) preparation and control GFP lentivirus (GFP LV) and (**F**) summary data showing percentage of Cre-GFP lentivirus infected Jurkat T cells (GFP$^+$). This figure has been modified from Ipseiz N *et al*., 2020[18].

Figure 3: Infection efficiency of the resident pMΦ. (**A**) Gating strategy of resident pMϕ (CD11b$^+$, Tim4$^+$, F4/80$^+$) and Tim4$^-$, F4/80$^+$ cells. Cells were gated on singlets, followed by CD11b$^+$. (**B**) Representative dot plots, and (**C**) a summary of infection frequency (% GFP+ cells) and intensity (MFI) of GFP$^+$ cells isolated 3 days after *in vivo* infection with different amount of GFP lentivirus preparation. (**D**) Representative dot plot, and (**E**) a summary of infection frequency (% GFP+ cells) and intensity (MFI) of GFP+ cells isolated at different timepoints after *in vivo* infection with 100 µL lentivirus in a total volume of 200 µL serum free media medium. (**F**) Representative dot plots at days 7, 14 and 21 after intra-peritoneal delivery of GFP-expressing lentivirus in Foxp3-DTR-eGFP mice showing the number of GFP-expressing pMϕ, and inflammatory macrophages and dendritic cells (InfMØs/DCs (F480^{low})). (n= 1-2 per group). (**G**) Representative dot plot of suboptimal *in vivo* infection of *Gata6*-KO^{mye} [19] resident pMϕ with 300 µL of Cre-GFP lentivirus 7 days post i.p injection. (**H**) A representative dot plot of successful downregulation of ICAM1 on pMϕ in female 129S6 mice, 7 days after i.p injection of lentivirus containing targeting shRNA. Control shRNA is shown. Overlay shows isotype control. (**I**) RT-

qPCR quantification of mmu-miR-146b-5p expression of resident peritoneal Mϕ challenged *in vivo* with 100 µL lentivirus encoding murine microRNA-146b (miR-146b) or control (C). Resident pMϕ (white circles) and $Tim4^-$, $F4/80^+$ cells (grey circles). Data expressed as mean ± SEM, n≥3 mice. This figure has been modified from Ipseiz N *et al*., 2020[18].

Figure 4: Infection efficiency of the resident pMϕ subpopulations. (**A**) Percentage of total single cells and resident Mϕ viable 7 days after 100 µL serum free media (-) or GFP lentivirus (+) i.p injection. (**B**) Gating strategy showing four major populations of pMϕ found *in vivo*: $CD73^+Tim4^+$, $CD73^-Tim4^-$, $CD73^+Tim4^-$, $CD73^-Tim4^+$, and (**C**) corresponding infection frequency (% GFP+ cells) and intensity (MFI of GFP+ cells) of these populations. (**D**) Percentage of GFP+ cells in multiple organs 7 days after 100 µL lentivirus i.p injection. mLN, mesenteric lymph node. (**E**) Mice were injected i.p with 0.1 mL of 4% thioglycolate for 5 days followed by i.p injection with lentivirus. Gating strategy of pMϕ and monocytes post 3 days after lentivirus i.p injection. (**F**) Infection frequency, MFI, and cell number analysis of GFP+ monocytes ($Ly6C^+$) and Mϕ ($Ly6C^-$). Data expressed as mean ± SEM, n≥3 mice. This figure has been modified from Ipseiz N *et al*., 2020[18].

Figure 5: Impact of lentivirus injection on peritoneal inflammation. Mice were injected *in vivo* i.p with GFP expressing lentivirus. (**A**) Infection frequency of cell populations in the peritoneal cavity following various amount of lentivirus injections (50, 100, 150 or 200 µL). (**B**) Intensity of GFP expression in productively infected cell populations at 7 days post i.p injection. (**C**) Infection frequency of cell populations in the peritoneal cavity at various time points after i.p injection. (**D**) and (**E**) Percentage of cells at various timepoints after i.p injection. All lentivirus injections were performed with the same total volume of 200 µL completed by serum free media medium. Unless specified otherwise, 100 µL lentivirus dose was used. Control mice ("C") received 200 µL of neat serum free media medium. Data expressed as mean ± SEM, n≥3 mice. This figure has been modified from Ipseiz N *et al*., 2020[18].

Table 1: List of Antibodies

DISCUSSION:

Tissue-resident macrophages perform a range of homeostatic and inflammatory tissue-specific functions[1,2] dictated by their physiological environment[6-9]. In this protocol, an effective method[18] for manipulation of peritoneal resident macrophages *in vivo* using lentivirus particles was introduced to investigate macrophage function in their biological microenvironment.

It is essential for the success of the protocol to use healthy HEK293T cells. It is the best practice to defrost the cells at least a week prior to the start of this protocol to ensure cells recovery and good numbers. Cells should be seeded in appropriate volume of medium one day before planned transfection and should reach about 80 % confluency on the day of transfection. Under- or over-confluent cell preparations will result in reduced lentivirus yield. We recommend transfecting HEK293T cells with the transfection reagent accordingly to manufacturer's instructions for the best results. Essential steps of the transfection include appropriate mixing of the plasmids and reagents and drop-wise addition of the mix directly

to HEK293T cell monolayer. Calcium phosphate transfection of HEK293T cells[27,28] could be employed in this protocol. However, the user should be aware that the effectiveness of this method can vary, and it can result in diminished transfection efficiency.

Safety precautions should be considered in the protocol from the day of HEK293T cells transfection. These include working in category II safety cabinet, wearing double gloves when handling contaminated material, and appropriate bleaching of the contaminated material (with decontamination solution, for example 2,000 ppm bleach solution) for minimum 4 h. Users should refer to their institutional regulations regarding work with category II pathogens and waste.

In this protocol, the lentivirus preparation is first purified using a 0.45 µm filter to remove HEK293T cells debris. Use of smaller filters (0.22 µm) and cellulose ester membranes should be avoided as it will result in the loss of lentivirus particles. It is recommended to use low protein binding polyether sulfone or polyvinylidene fluoride filters[29]. The second purification step is performed on the single 20 % sucrose layer in an ultracentrifuge to remove remaining impurities, which is particularly important for the consequent *in vivo* administration of the lentivirus preparation. In the institutions where an ultracentrifuge is not available, others[30] have described the sucrose-based lentivirus purification using a standard laboratory centrifuge. This could be implemented in this protocol as an alternative. Lentivirus preparation is titrated in Jurkat T cells on the expression of the marker signal (e.g., GFP used in this protocol). For constructs without markers, physical lentivirus particles could be evaluated by quantification of HIV-1 p24gag protein by ELISA kit[31], flow cytometry analysis of HIV-1 core antigen[18], or measurement of the changes to the targeted gene (preferentially using the methods allowing measurement of changes in individual cells, e.g., flow cytometry or microscopy). If titration of the control and lentivirus differ significantly in Jurkat T cells, the volumes used for the *in vivo* studies can be adjusted to reach the most comparable infection between lentivirus preparations.

The major limitation of the protocol is the observed disappearance of GFP^+ resident peritoneal Mϕ within 14 days of i.p lentivirus injection. For the long term studies, mouse lines with stable genetic alteration of genes in a specific cell type or tissues should be considered[32]. For example, as peritoneal macrophages do not express Foxp3 (a T-Reg protein) we utilized Foxp3-DTR-eGFP mice[33] and confirmed that expression of GFP is maintained in peritoneal Mϕ at 21 days post infection (**Figure 3F**). However, it is important to note that the lentiviruses contain other foreign components and this extension in persistence of infected cells in the Foxp3-DTR-GFP mice may not be permanent. An additional weakness of this method is a low level of inflammation that can be observed in the peritoneal cavity following lentivirus injection, as witnessed by influx of neutrophils at 4 h post injection (**Figure 5D**), which may mean repeated infections would accelerate the inflammation-associated loss of GFP-expressing resident peritoneal Mϕ. Although, we have not detected type I interferons (IFNs) in the peritoneal cavity, VSV-G pseudotyped lentivirus particles were previously demonstrated to induce some of the IFN-stimulated genes in human Mϕ in the absence of detectable IFNs[34]. This should be considered when using this protocol for experiments investigating antiviral immune responses. The occurrence of acute and sustained inflammation after i.p injection of lentivirus preparation might indicate contamination of the preparation.

Areas of troubleshooting include: 1) for low lentivirus titer, ensure the health of HEK293T cells and effective transfection (e.g., marker expression, if present, in HEK293T cells). If infection rate remains low, consider the size of the construct. Constructs with larger inserts or more complex secondary structures can affect the final lentivirus titer and infectivity[24]. Therefore, each construct should be tested independently and in parallel to its respective control vector; 2) If high amount of the lentivirus is required, it is recommended to prepare a multiple T175 flasks of HEK293T cells and to scale up the production accordingly. To avoid variation of the lentivirus preparations, it is the best practice to merge the final collections prior to aliquoting and storage. For such larger production, plasmids mix (**step 2**) should be prepared in 50 mL tubes to ensure effective mixing of the components.

Despite its broad tropism, VSV-G pseudotyped lentiviral particles predominantly target tissue macrophages as demonstrated previously for alveolar[35], and here[18] for pMΦ when administered by the respective routes. Alterations to the envelope on lentivirus particles can, in some cases, result in diminished transduction of macrophages *in vivo*[36] and is unnecessary for this protocol. Compared to other viral approaches for *in vivo* gene manipulation in macrophages (reviewed in[37]), use of lentiviral vectors offers stable integration of the transgene in tissue macrophages[35], efficient transgene expression, and the largest vector size limit (approximately 8 Kb).

Peritoneal Mϕ play an important role in the prevention, onset, progression and resolution of various diseases, including abdominal cancers, pancreatitis and peritonitis[38]. This protocol describes an effective tool for gene modification in murine pMϕ, allowing investigation of the biological processes behind these pathologies within a physiologically relevant microenvironment. While lentiviral vectors themselves are becoming a tool of interests for clinical interventions[39], due to the origin from immunodeficiency virus and a stable genome integration, the safety concerns impede their therapeutic implementation. Further understanding of the macrophage responses to lentiviral gene modulation could advance application of this highly effective tool in a clinical setting.

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The authors have nothing to disclose.

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