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TITLE:

Expression of Fluorescent Fusion Proteins in Murine Bone Marrow-Derived Dendritic Cells and Macrophages

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KEYWORDS:

Dendritic cells, macrophages, myeloid cells, differentiation, murine bone marrow cells, cytokines, M-CSF, GM-CSF, viral infection, GFP tagged protein

SUMMARY:

In this article, we provide a detailed protocol for the expression of fluorescent fusion proteins in murine bone marrow derived dendritic cells and macrophages. The method is based on the transduction of bone marrow progenitors with retroviral constructs followed by differentiation into macrophages and dendritic cells *in vitro*.

ABSTRACT:

Dendritic cells and macrophages are crucial cells that form the first line of defense against pathogens. They also play important roles in the initiation of an adaptive immune response. Experimental work with these cells is rather challenging. Their abundance in organs and tissues is relatively low. As a result, they cannot be isolated in large numbers. They are also difficult to transfect with cDNA constructs. In the murine model, these problems can be partially overcome by *in vitro* differentiation from bone marrow progenitors in the presence of M-CSF for macrophages or GM-CSF for dendritic cells. In this way, it is possible to obtain large amounts of these cells from very few animals. Moreover, bone marrow progenitors can be transduced with retroviral vectors carrying cDNA constructs during early stages of cultivation prior to their differentiation into bone marrow derived dendritic cells and macrophages. Thus, retroviral

transduction followed by differentiation *in vitro* can be used to express various cDNA constructs in these cells. The ability to express ectopic proteins substantially extends the range of experiments that can be performed on these cells, including live cell imaging of fluorescent proteins, tandem purifications for interactome analyses, structure-function analyses, monitoring of cellular functions with biosensors and many others. In this article, we describe a detailed protocol for retroviral transduction of murine bone marrow derived dendritic cells and macrophages with vectors coding for fluorescently-tagged proteins. On the example of two adaptor proteins, OPAL1 and PSTPIP2, we demonstrate its practical application in flow cytometry and microscopy. We also discuss the advantages and limitations of this approach.

INTRODUCTION:

Myeloid cells represent an indispensable part of our defense mechanisms against pathogens. They are able to rapidly eliminate microbes, as well as dying cells. In addition, they are also involved in regulating tissue development and repair and in maintaining homeostasis[1-3]. All myeloid cells differentiate from common myeloid progenitors in the bone marrow. Their differentiation into many functionally and morphologically distinct subsets is to a large extent controlled by cytokines and their various combinations[4]. The most intensively studied myeloid cell subsets include neutrophilic granulocytes, macrophages and dendritic cells. Defects in any of these populations lead to potentially life-threatening consequences and cause severe dysfunctions of the immune system in humans and mice[1-3,5,6].

Unlike neutrophilic granulocytes, dendritic cells and macrophages are tissue resident cells and their abundance in immune organs is relatively low. As a result, the isolation and purification of primary dendritic cells and macrophages for experiments requiring a large number of these cells is expensive and often impossible. To solve this problem, protocols have been developed to obtain large amounts of homogenous macrophages or dendritic cells *in vitro*. These approaches are based on the differentiation of murine bone marrow cells in the presence of cytokines: macrophage colony-stimulating factor (M-CSF) for macrophages and granulocyte-macrophage colony-stimulating factor (GM-CSF) or Flt3 ligand for dendritic cells[7-12]. Cells generated by this method are commonly described in the literature as bone marrow derived macrophages (BMDMs) and bone marrow derived dendritic cells (BMDCs). They have more physiological properties in common with primary macrophages or dendritic cells than with corresponding cell lines. Another major advantage is the possibility of obtaining these cells form genetically modified mice[13]. Comparative studies between wild-type cells and cells derived from genetically modified mice are often critical for uncovering novel functions of genes or proteins of interest.

Analysis of subcellular localization of proteins in living cells requires the coupling of a fluorescent label to the protein of interest *in vivo*. This is most commonly achieved by expressing genetically encoded fusion construct composed of an analyzed protein coupled (often via a short linker) to a fluorescent protein (*e.g.*, green fluorescent protein (GFP))[14-16]. The expression of fluorescently tagged proteins in dendritic cells or macrophages is challenging. These cells are generally difficult to transfect by standard transfection procedures and the efficiencies tend to be very low. Moreover, the transfection is transient, it generates cellular stress and achieved intensity of fluorescence might not be sufficient for microscopy[17]. In order to obtain a reasonable fraction of

these cells with a sufficient level of transgene expression, the infection of bone marrow progenitor cells with retroviral vectors and their subsequent differentiation into BMDMs or BMDCs has become a very efficient approach. It has allowed for the analysis of the proteins of myeloid origin in their native cellular environment, both in a steady state or during processes that are critical for immune response such as phagocytosis, immunological synapse formation or migration. Here, we describe a protocol that allows stable expression of fluorescently tagged proteins of interest in murine bone marrow derived macrophages and dendritic cells.

PROTOCOL:

All methods described here have been approved by the Expert Committee on the Welfare of Experimental Animals of the Institute of Molecular Genetics and by the Academy of Sciences of the Czech Republic.

1. Reagent Preparation

1.1. Prepare the ammonium-chloride-potassium (ACK) buffer. Add 4.145 g of NH_4Cl and 0.5 g of $KHCO_3$ to 500 mL of ddH_2O, then add 100 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA) and filter-sterilize.

1.2. Prepare polyethylenimine (PEI) solution. Add 0.1 g of PEI to 90 mL of ddH_2O. While stirring, add 1 M HCl dropwise until the pH is lower than 2.0. Stir for up to 3 h until PEI is dissolved and then adjust pH to 7.2 with 1 M NaOH. Adjust the volume to 100 mL with ddH_2O and filter-sterilize. Make 1-2 mL aliquots and store at -20°C.

Note: After thawing, PEI can be stored at 4°C for up to 2 weeks but should not be re-frozen.

1.3. Prepare cell culture supernatants containing M-CSF or GM-CSF. These supernatants can be made in advance and stored in -80°C. To make these supernatants, grow the cytokine-producing cells (J558 cells for GM-CSF [18] or CMG 14-12 cells for M-CSF [19]) in a 10 cm Petri dish in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) to confluence. Then transfer all cells to 200 mL of media in T150 tissue culture flask and culture for additional 4 days at 5% CO_2/37 °C. Collect the supernatant and filter over 0.2 µm sterilization filter. Make aliquots and store these at -80 °C.

1.4. Prepare 100 mL of cell culture medium: DMEM supplemented with 10% heat inactivated FBS and cell culture supernatants from cells secreting GM-CSF (for BMDC differentiation) or M-CSF (for BMDM differentiation).

Note: The amount of cytokines in these supernatants can vary and the working concentration has to be determined empirically. Typically, 2-3% supernatant from cell lines producing GM-CSF (the recommended starting concentration is 2%) or 5-10% supernatant from CMG 14-12 cells producing M-CSF (the recommended starting concentration is 10%) is used. Alternatively, purified commercially available M-CSF at 10 ng/mL and GM-CSF at 20 ng/mL can be used with results virtually identical to cytokine-containing supernatants, *i.e.*, without any effect on the rate

of differentiation, infection efficiency and subcellular localization of EGFP-tagged constructs. Antibiotics, including penicillin G (100 IU/mL), streptomycin (100 µg/mL), and gentamicin (40 µg/mL), can be used for cell culture at any step of the protocol unless otherwise stated.

2. Production of Retrovirus

CAUTION: Although retroviral vectors are relatively safe when compared to other types of viral vectors, they still pose a potential safety hazard. Therefore, it is crucial to work with the utmost care and appropriate protective equipment, and to adhere to all safety regulations and legal requirements for working with viral particles.

2.1. Plate a single cell suspension of Platinum-Eco (Plat-E) packaging cells in a 10 cm Petri dish and cultivate in 15 mL of DMEM containing 10% FBS until 50-60 % confluent (~24 h). Cells should grow in a monolayer and should not form clumps in culture.

2.2. Pipette 20 µg of retroviral construct (*e.g.*, the construct expressing the fluorescently tagged protein of interest in the pMSCV vector) and 10 µg of pCL-Eco packaging vector[20] into 1 mL of DMEM (without serum and antibiotics) and gently mix.

2.3. To a second tube, add 75 µL of PEI in 1 mL of DMEM (without serum and antibiotics). Incubate 5 min at room temperature (RT) and then mix the contents of both tubes together and incubate for additional 10 min at RT.

Note: Addition of pCL-Eco is optional. It is coding for the ecotropic viral receptor and may increase the virus titer.

2.4. Carefully replace the medium on Plat-E cells with 8 mL of fresh DMEM supplemented with 2% FBS. Pre-warm the medium to 37 °C before use. Do not use antibiotics during transfection, since antibiotics may reduce the transfection efficiency.

2.5. Carefully add (in drops) the mixture prepared in Step 2.3 on the Plat-E cells, and incubate for 4 h at 37 °C.

2.6. After the incubation, exchange the medium on Plat-E cells for 10 mL of pre-warmed DMEM containing 10% FBS, and cultivate the cells for 24 h at 37 °C. During this incubation, Plat-E cells will produce virus into the media.

2.7. After 24 h, collect the medium containing retroviral particles from Platinum Eco cells using a 10 mL serological pipette and transfer it to a 15 mL centrifuge tube (= “supernatant 1” containing ecotropic retroviral particles).

2.8. To avoid contamination by Plat-E cells in viral supernatants, spin the collected virus at 1250 × g for 5 min at 4 °C. For the best results, the virus should be used immediately for infection.

Note: Aliquots of virus can also be stored at -80 °C for later use. However, it will result in certain reduction in transduction efficiency. Avoid repetitive freezing/thawing of the virus, since it leads to virus degradation.

2.9. Add 10 mL of pre-warmed DMEM with 10% FBS to Plat-E cells and cultivate for another 24 h at 37 °C.

2.10. Repeat steps 2.7. and 2.8. to obtain “supernatant 2”.

3. Murine Bone Marrow Cell Isolation

3.1. Sacrifice the mouse using cervical dislocation or other approved method. Spray the mouse with 70% ethanol.

3.2. Using tweezers and scissors, remove the skin as well as part of muscles from hind legs. Carefully dislodge the acetabulum from the hip joint without breaking the femur. Cut the paw in the ankle joint. Spray the bones (femur connected to tibia) with 70% ethanol and remove the rest of the muscles using a paper towel.

3.3. Place the bones in a 5 cm Petri dish containing sterile phosphate buffered saline (PBS) with 2% FBS (PBS-FBS) and keep on ice until processed.

3.4. For securing cultivation sterility, perform all the following steps in a tissue culture hood.

3.5. Separate the femur from the tibia without breaking the bone ends (bend in the knee joint and carefully cut with scissors).

3.6. Process the bones one by one. Cut off a very small part of the epiphyses (approximately 1 - 2 mm) with scissors while holding the bone in tweezers.

3.7. Use a 30G needle and a 2 or 5 mL syringe filled with PBS-FBS to flush the bone marrow cells from both ends of the bone into a 15 mL centrifuge tube. Move the needle inside the bone during the flushing in order to remove all the cells. If the needle gets clogged, change it.

Note: Bones should turn from red to white during flushing. This indicates that the majority of the cells were removed from the bone. Use approximately 2-3 mL of PBS-FBS per bone.

3.8. Centrifuge the cells at 500 × g for 5 min at 4 °C.

3.9. Discard the supernatant and lyse the red blood cells by resuspending the pellet in 2.5 mL of ACK buffer for 2-3 min at room temperature. During the lysis, filter the bone marrow cells through a 100 µm cell strainer into a fresh 15 mL centrifug tube. Restore the tonicity by adding

12 mL of PBS-FBS.

Note: Do not exceed 5 min of hypotonic lysis with ACK buffer to avoid cell death.

3.10. Centrifuge immediately at 500 × g for 5 min at 4 °C.

4. Bone Marrow Cell Differentiation into Bone Marrow Derived Macrophages

4.1. Resuspend the pellet of bone marrow cells in DMEM supplemented with 10 % FBS and antibiotics (see the note after Step 1.4. for antibiotic concentration) and count the cells. For differentiation into BM derived macrophages, plate 5-10 × 10^6 of bone marrow cells in a 10 cm non-tissue culture treated (bacterial) Petri dish with 10 mL of pre-prepared DMEM media with serum and M-CSF from Step 1.4.

Note: The yield of the bone marrow cells is approximately 4 × 10^7 per 6-8 week-old C57BL/6J mouse.

4.2. Incubate the cells in cell culture incubator for 3 days at 5% CO_2, 37 °C.

Note**:** During the first 2 days, cells do not look very vital, as a large number of apoptotic cells is present (cells unable to differentiate into myeloid cells and terminally differentiated cells).

4.3. After 3 days, the bone marrow cell culture begins to look vital and clusters of dividing cells are formed. First adherent cells can already be observed. At this point, supplement the cells with fresh cytokine media.

4.4. Add 10 mL of pre-warmed DMEM media with serum and M-CSF (from Step 1.3) into each 10 cm Petri dish and return it in the cell culture incubator. There is no need to remove the old media during this step.

Note: Bone marrow macrophages are fully differentiated after 5-7 days in culture. The best time for harvesting is at day 6-8, where majority of cells are adherent and the Petri dish is completely covered.

4.5. At day 5, take the Petri dish into the cell culture hood and incline the dish until the media is almost reaching the edge of the dish. Carefully take out 15 mL of the media from the surface near the edge, and the cells tend to stay in the middle of the dish. Add the same volume of pre-warmed media with M-CSF and place the dish back into the incubator.

Note: If media is aspirated slowly and carefully, almost no cells are lost. However, it is also possible to centrifuge the aspirated media and add the cells back to the culture, to ensure that no non-adherent (*i.e.*, incompletely differentiated) cells are lost.

4.6. For experiments, only adherent cells (macrophages) are used. To harvest cells, on day 6

or 7, remove all media and floating cells. Wash the dish once with pre-warmed PBS without serum.

4.7. Add 5 mL of 0.02% EDTA in PBS, and incubate for 3-5 min at 37 °C in tissue culture incubator.

4.8. Using a 5 mL pipette, remove the cells from the dish by a stream of PBS-EDTA and place them in a 50 mL centrifuge tube with 25 mL of PBS. If needed, pool more dishes together.

4.9. Centrifuge immediately at 500 × g for 5 min at 4 °C.

4.10. Resuspend the macrophage pellet in DMEM media and count the cells. Verify the expression of macrophage surface differentiation markers (CD11b and F4/80) by flow cytometry.

4.11. For experiments requiring the cells to be in suspension, *e.g.*, flow cytometry experiments, qPCR or western blot analysis, use the macrophages directly. For experiments with adherent macrophages, plate the cells in the tissue culture plate according to the experimental setup.

4.12. The cells are already fully differentiated. Keep them in the media suitable for the intended experiment or in the original growth and differentiation media with M-CSF.

Note: For working with adherent macrophages, transfer them into a new plate at least 6 h before use (ideally overnight) to allow for the full adhesion to the new surface. The small fraction of floating cells can be removed before experiment.

5. Bone Marrow Cell Differentiation into Bone Marrow Derived Dendritic Cells

5.1. Follow the protocol for BMDMs with adjustments specific for BMDCs described below in steps 5.2. – 5.4.

5.2. Resuspend the obtained pellet of bone marrow cells in DMEM supplemented with 10% FBS and antibiotics and count the cells (by following Steps 4.1. - 4.2. of macrophage protocol). For differentiation into dendritic cells, plate 1 - 1.5 × 10^7 bone marrow cells in a 10 cm non-tissue culture treated (bacterial) Petri dish in 10 mL of pre-prepared DMEM media with serum and GM-CSF (from Step 1.4.).

5.3. Follow the same cultivation steps as in BMDM protocol (steps 4.3. - 4.5. of macrophage protocol). Use DMEM media with serum and GM-CSF instead of M-CSF. Since for BMDCs the cultivation time is longer (typically 10-12 days), add 1-2 additional feedings in 3 day intervals (by removing the supernatant and adding a new cultivation media as described in Step 4.5.).

5.4. This part of the protocol is virtually the same as the corresponding part of the macrophage protocol (Steps 4.6. – 4.12. of macrophage protocol). For experiments use only adherent cells. On day 10-12, collect the cells using EDTA, count and plate them on a new surface. Verify the

expression of surface differentiation markers of dendritic cells (CD11c+, CD11b+, F4/80-) by flow cytometry.

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6. Production of BMDMs and BMDCs Expressing EGFP-tagged Protein of Interest

6.1. Resuspend the pellet of bone marrow cells obtained in Step 3.10. in DMEM supplemented with 10 % FBS and antibiotics and count the cells. For the infection, use 2-5 × 10^6 of BM cells per well of a 6-well tissue culture treated plate.

6.2. Plate the cells in 1 mL of the prepared DMEM media per well, supplemented either with M-CSF for differentiation into BMDMs or with GM-CSF for differentiation in BMDCs. Keep the cells for 4-6 h in a tissue culture incubator with 5% CO_2 at 37 °C.

6.3. Add 2 mL of freshly collected virus ("supernatant 1") supplemented with polybrene (12 µg/mL, final concentration 8 µg/mL after addition to the cells).

Note: Frozen aliquot of the virus-containing supernatant can also be used, but efficacy will be lower.

6.4. Centrifuge the plate at 1250 × g for 90 min at 30 °C (with slow acceleration and deceleration). Then, incubate for 4 h with 5% CO_2 at 37 °C.

6.5. Optional: Replace 2 mL of the culture media with fresh medium containing respective cytokine (M-CSF or GM-CSF) and culture with 5% CO_2 at 37 °C. On the second day, remove 2 mL of culture media and repeat the whole infection procedure (Step 6.3-6.4) with 2 mL of freshly collected virus ("supernatant 2").

Note: This step may increase infection efficacy. Improvement after the second infection is dependent on the cell type and target protein and in our experience can vary from 30% increase in efficiency to no improvement at all.

6.6. Collect the non-adherent cells, transfer to a 15 mL centrifuge tube, and spin at 500 × g for 5 min (4 °C). Discard the supernatant.

6.7. Resuspend the cell pellet in 10 mL of culture media with M-CSF or GM-CSF, place the cells into a 10 cm non-tissue culture treated Petri dish and culture at 37 °C, 5% CO_2. Optimal number of cells for a 10 cm dish is 5-10 × 10^6 for BM-derived macrophages and 10-15 × 10^6 for BM-derived dendritic cell.

Note: Smaller dishes or plates can be used, but cell numbers must be adjusted accordingly.

6.8. Follow the macrophage and dendritic cell cultivation and differentiation protocol described in step 3 and 4.

REPRESENTATIVE RESULTS:

Signaling adaptor proteins are usually small proteins without any enzymatic activity. They possess various interaction domains or motifs, which mediate binding to other proteins involved in signal transduction, including tyrosine kinases, phosphatases, ubiquitin ligases and others[21]. For the demonstration of the functionality of this protocol myeloid cell adaptors PSTPIP2 and OPAL1 were selected. PSTPIP2 is a well characterized protein involved in the regulation of inflammatory response[22]. It is a cytoplasmic protein which can also be recruited to cellular membranes via its F-bar domain. Second protein is a transmembrane adaptor OPAL1, expected to be associated with cellular membranes. Its physiological function is still unknown. However, in acute lymphoblastic leukemia, expression of OPAL1 is associated with better prognosis[23].

cDNA constructs coding for PSTPIP2 or OPAL1 fused via a short linker (GSGGGS or Myc-tag, respectively) to EGFP at the C-terminus were cloned into the pMSCV retroviral vector using standard methods of cDNA cloning. This construct was then transfected into Plat-E cells together with the packaging vector pCL-Eco. The resulting supernatants containing retroviruses were used for the transduction of bone marrow cells, followed by the differentiation into BMDMs and BMDCs. The efficacy of Plat-E transfection was evaluated by flow cytometry after the collection of the second virus-containing supernatant. Mean transfection efficiency was 62% for PSTPIP2-EGFP and 53% for OPAL1 and the results were highly reproducible (**Figure 1A,** B). OPAL1 construct seemed to be more toxic for Plat-E cells (assessed by the appearance of floating/dying cells in culture), resulting in a reduction in the percentages of transfected cells.

Differentiation status of the bone marrow derived macrophages and dendritic cells (transduced with PSTPIP2-EGFP and OPAL1-EGFP retroviral constructs) was assessed by flow cytometry. Mature macrophage population is defined by CD11b and F4/80 expression, while dendritic cells express the CD11c lineage marker. More than 90% of cells in both types of culture were positive for their respective markers (**Figure 2A, B**). Finally, we determined the expression level of PSTPIP2-EGFP and OPAL1-EGFP constructs in BMDMs and BMDCs by a simple flow cytometry measurement of EGFP fluorescence. The mean percentage of EGFP-positive macrophages was 71% for PSTPIP2-EGFP and 62% for OPAL1-EGFP (**Figure 3A**). In case of dendritic cells, the efficiency was lower, 32% for PSTPIP2 and 9% for OPAL1 (**Figure 3B**). The results of multiple experiments demonstrate the reproducibility of this method (**Figure 3C**).

We typically do not determine the virus concentration in the supernatants that we use in infections. We prefer to use the virus supernatants fresh, immediately after collection, while the virus titer determination requires three additional days. As a result, the information on virus titer can only be obtained *ex post.* However, it can still be useful when addressing technical issues and problems. To assess the virus concentration in supernatants from Plat E cells transfected with PSTPIP2-EGFP and OPAL1-EGFP constructs, we incubated NIH-3T3 cells with serially diluted virus-containing supernatants collected from these transfected Plat-E cells and determined virus titer exactly as described by Zjablovskaja *et al* in previously published JoVE article[24]. In three independent experiments, the virus titer ranged from 1.1×10^6 to 4.4×10^6 TU/mL. We did not observe any substantial differences between PSTPIP1-EGFP and OPAL1-EGFP constructs and between supernatants from day 1 and day 2. When these supernatants were used for bone

marrow cell infections according to the protocol we are describing in this article, the multiplicity of infection (MOI) ranged from 1.1 to 4.4. Interestingly, within this range, we did not observe any correlation between MOI and infection efficiency.

In **Figure 4**, PSTPIP2-EGFP and OPAL1-EGFP expressed in BMDMs and BMDCs were visualized by confocal microscopy. Fully differentiated macrophages and dendritic cells have a characteristic shape. The change in morphology from small rounded progenitor cells to the large cells of irregular shapes confirms successful differentiation. In macrophages, PSTPIP2 was cytoplasmic with partial localization at the plasma membrane. OPAL1 appeared to be also partially targeted to the plasma membrane. The rest was likely associated with intracellular membranes, such as the endoplasmic reticulum and Golgi complex. However, to confirm this localization, specific organelle markers would have to be used. In dendritic cells, the membrane localization was less apparent.

FIGURE LEGENDS:

Figure 1. Efficiency of Plat-E cell transfection. For transfection of Plat-E cells, two constructs encoding adaptor proteins PSTPIP2 and OPAL1 fused with EGFP (PSTPIP2-EGFP and OPAL1-EGFP) were cloned into the pMSCV vector. Standard PEI transfection was performed. The efficacy of transfection was evaluated by flow cytometry of the Plat-E cells after the collection of the second viral supernatant. (**A**). Representative flow cytometry plot. (**B**). Graph showing results of four independent experiments.

Figure 2. Assessment of the differentiation status of BMDMs (A) and BMDCs (B). Surface expression of specific macrophage and dendritic cell lineage markers was measured by flow cytometry at day 8 of cultivation. Dead cells were gated out based on their side and forward scatter properties and staining with Hoechst 33258. The results are representative of at least 3 independent experiments.

Figure 3. Assessment of the expression of PSTPIP2-EGFP and OPAL1-EGFP. EGFP fluorescence in BMDMs (**A**) and BMDCs (**B**) retrovirally transduced with PSTPIP2-EGFP and OPAL1-EGFP constructs was measured by flow cytometry at day 8 of cultivation. (**C**)**.** Graph showing the results of multiple independent experiments. BMDMs and BMDCs were gated as in **Figure 2**.

Figure 4. Representative images of macrophages and dendritic cells expressing PSTPIP2 or OPAL1. BMDMs (**A**) and BMDCs (**B**) expressing PSTPIP2 and OPAL1 were visualized by live imaging confocal microscopy. EGFP fluorescence in green is shown on the left side of each panel, bright field image on the right. Bar = 10 µm. The results are representative of at least three independent experiments.

DISCUSSION:

The expression of protein of interest in target cells is a key step in many types of biological studies. Differentiated macrophages and dendritic cells are difficult to transfect by standard transfection and retroviral transduction techniques. Bypassing the transfection of these differentiated cells with retroviral transduction of bone marrow progenitors, followed by

differentiation when they already carry the desired construct, is a critical step allowing the expression of ectopic cDNAs in these cell types. An example of successful use of this method can be found in our recent publication[25]. Here, we provide a cost-effective protocol for achieving stable expression of the construct of choice in bone marrow-derived dendritic cells and macrophages using this approach. The procedure we present is relatively inexpensive and simple, yet delivering very good results. Reagents used in this protocol allow for its routine use even under a relatively restrictive budget. The protocol for Plat-E transfection employs PEI as a transfection reagent. Compared to other chemical transfection agents, PEI is of a very low cost, while its efficiency is similar to the majority of other widely used compounds. However, PEI can be replaced with many different commercially available transfection reagents in this step without any loss of efficiency. As a guiding principle, transfection protocols known to work with commonly used HEK293 cells typically perform well with Plat-E cells, too. Another cost-effective measure is the utilization of cytokine-containing supernatants instead of purified recombinant cytokines. The use of these supernatants requires some optimization. However, when the standard protocol for their preparation is established and followed, the variability between individual batches of these supernatants becomes very low, usually requiring no changes in working concentrations between individual lots. The efficiencies of BMDM and BMDC differentiation with these supernatants are, in our experience, identical to purified cytokines.

In addition to retroviral transduction, other well-established methods of mammalian cell transfection exist, including chemical transfection (typically using cationic lipids or cationic polymers forming complexes with DNA), electroporation and the use of other types of viral vectors, mainly adenoviral and lentiviral systems[26-30]. Although very high titers and efficiencies can be achieved with adenovirus-based gene delivery, the preparation of corresponding plasmids and viral particles are more difficult and time-consuming than in the case of retroviral systems[29]. In addition, adenovirus elicits inflammatory response in dendritic cells and macrophages[29,31,32] and for optimum performance in murine hematopoietic cells mouse strain carrying transgenic adenovirus receptor is required[33]. On the other hand, the generation of lentiviral particles carrying the gene of interest is a relatively simple process, virtually identical to the one utilized for retroviruses. In contrast to the ecotropic retroviral vectors used in this protocol, lentiviruses are capable of infecting non-proliferating cells of multiple species, including humans[34]. This may be an important advantage under specific experimental conditions. However, this feature also greatly compromises the safety of these vectors. In our opinion, for gene delivery to BMDMs and BMDCs, retroviruses provide the best balance of efficiency, safety and ease of use. Retroviral DNA constructs can be easily prepared using simple standard molecular cloning techniques. Virus is produced by packaging the cell lines directly to the culture supernatant and further virus purification is usually not necessary. Ecotropic retroviruses also do not readily infect human cells, which makes their use relatively safe. However, there also are some general disadvantages associated with the use of retroviral vectors. The major limiting factor is that these viruses infect only proliferating cells[35]. This feature does not significantly affect the protocol described here, but it limits the range of applications where retroviral vectors can be used. The size of the gene of interest that can be cloned into these vectors is also limited and the viral particle titer decreases with increasing insert size. With pMSCV vectors, we usually start seeing effects of insert size at around 3 kbp. With further increases in insert size, the infection efficiency gradually

declines.

The chemical transfection and electroporation are easier to use, safer and less time-consuming than any of the virus-based procedures[26-28,30]. However, in BMDMs and BMDCs, they can stimulate responses to foreign nucleic acids[31] and they generate more cellular stress. In addition, some chemical transfection reagents increase cell auto-fluorescence that may interfere with flow cytometry or microscopy analyzes. Due to the transient nature of expression, they can only be used with mature differentiated BMDMs or BMDCs. In contrast, the sequences introduced with retroviral vectors are permanently integrated into the genome of the target cells and allow for a stable long-lasting expression compatible with the time scale of the differentiation protocols[34,35]. However, this feature also increases the risk of insertional mutagenesis. Due to the relatively random nature of the vector integration, its effects on large populations of cells are limited. However, they may be visible at the level of individual cells. Additional problems may arise when a construct expressed from the retroviral vector affects dendritic cell or macrophage differentiation, resulting in failure to generate differentiated BMDMs or BMDCs from the infected progenitors. To some extent, this may be overcome by adjusting the infection conditions to achieve low expression levels (*e.g.*, by reducing the virus titer) or through the use of an inducible expression system. The use of EGFP fused to the protein of interest or as a reporter also allows for sorting of cells with expression level corresponding to experiment goals and limitations. Finally, we should also mention problems common to all transfection/transduction procedures. These include mainly overexpression artefacts, such as protein misfolding, mislocalization and toxicity[36]. Protein toxicity could be the reason why OPAL1 was relatively difficult to express. Its example clearly illustrates that the nature of the expressed protein can substantially affect the effectiveness of this method. However, despite this, we were able to obtain sufficient quantities of OPAL1-EGFP expressing cells for microscopy analysis with this method, demonstrating its usefulness even when dealing with difficult targets. In addition, it would be possible to increase the percentages of transduced cells by FACS sorting if required by a particular application. The low infection efficiency can also be partially overcome by increasing viral particle concentration using various methods or ready-to-use kits. In our hands, ultrafiltration of the viral supernatant on centrifugal filters with a molecular weight cut off of 100 kDa has proven to provide the best balance between efficiency and required effort.

Bone marrow derived macrophages and dendritic cells are widely used tools in phagocyte immunology. They are more physiologically relevant than available cell lines. They can be generated in relatively high numbers and, at the same time, lack the genetic heterogeneity and instability characteristic of cell lines. Another advantage is that they can be generated from genetically modified mice to study the effects of genetic modification on a relatively abundant and homogenous cell population. This is particularly useful in biochemical studies, where relatively large amounts of cells are typically required. The ability to transduce these cells with cDNA constructs opens up additional possibilities of research based on the reconstitution or complementation of genetic defects in these cells and structure-function analysis.

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DISCLOSURES:

The authors have nothing to disclose.

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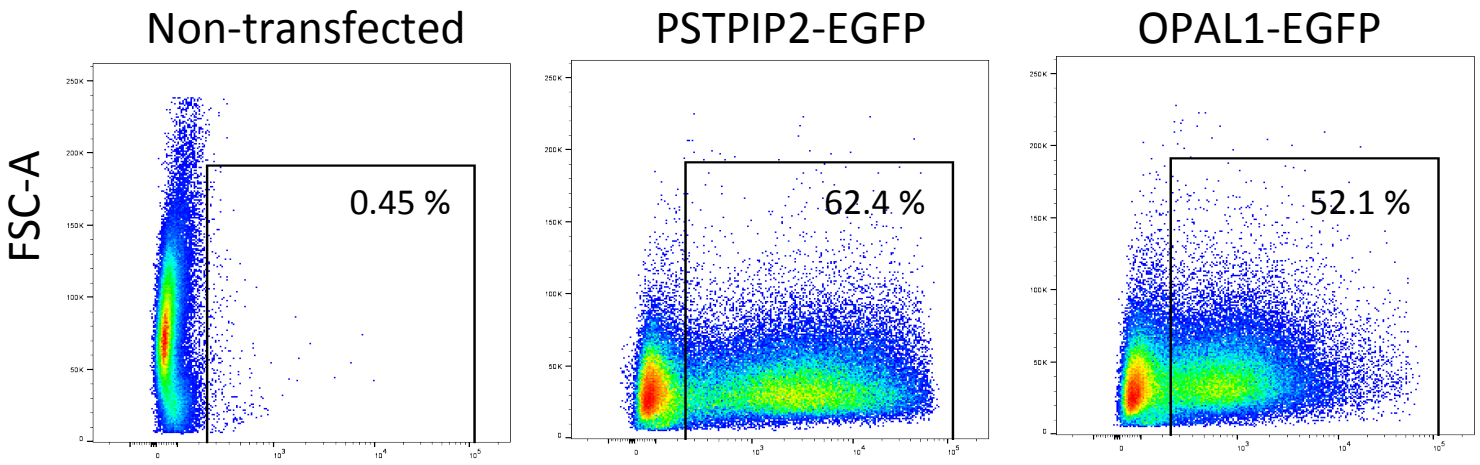

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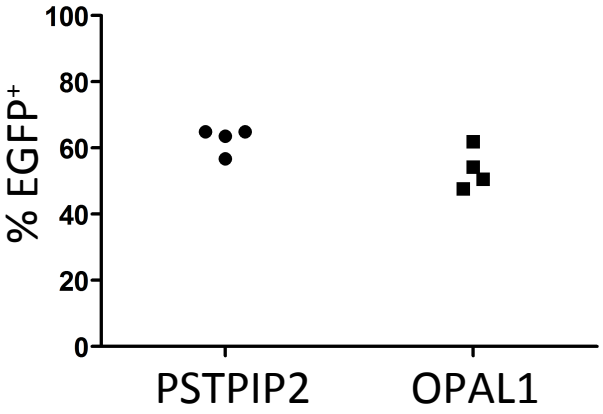
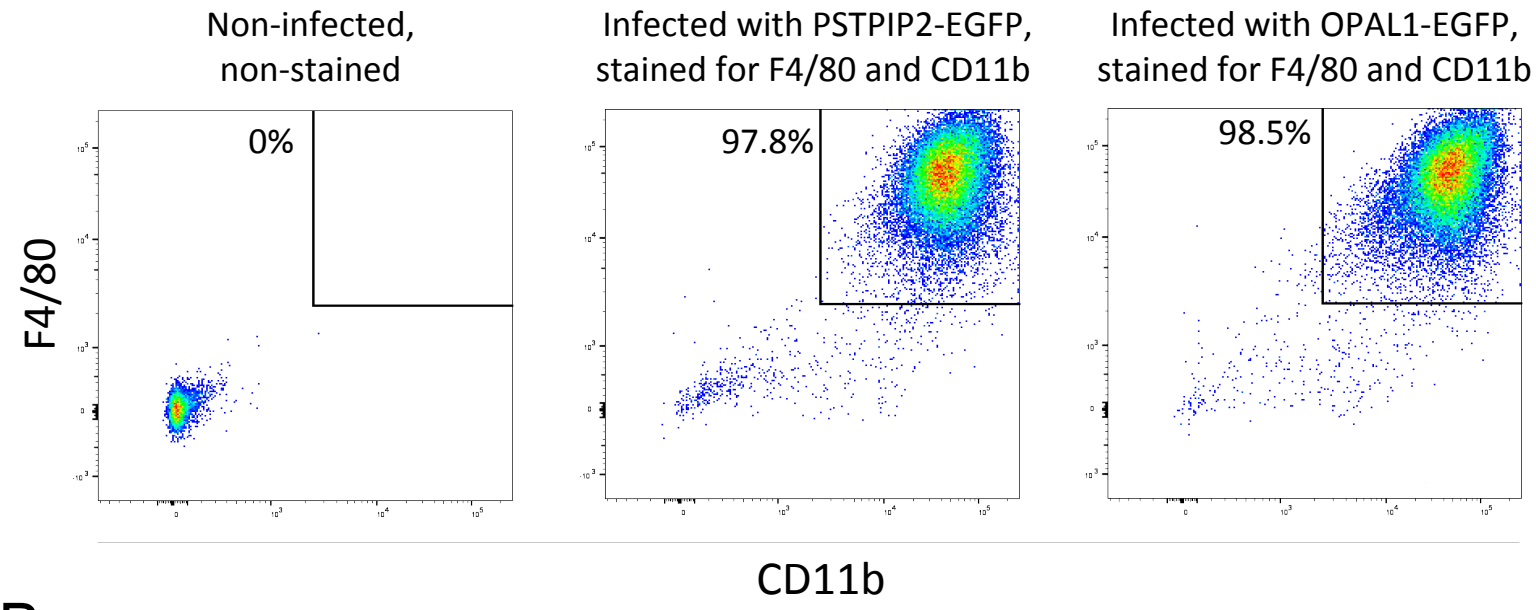


A

Bone marrow derived macrophages



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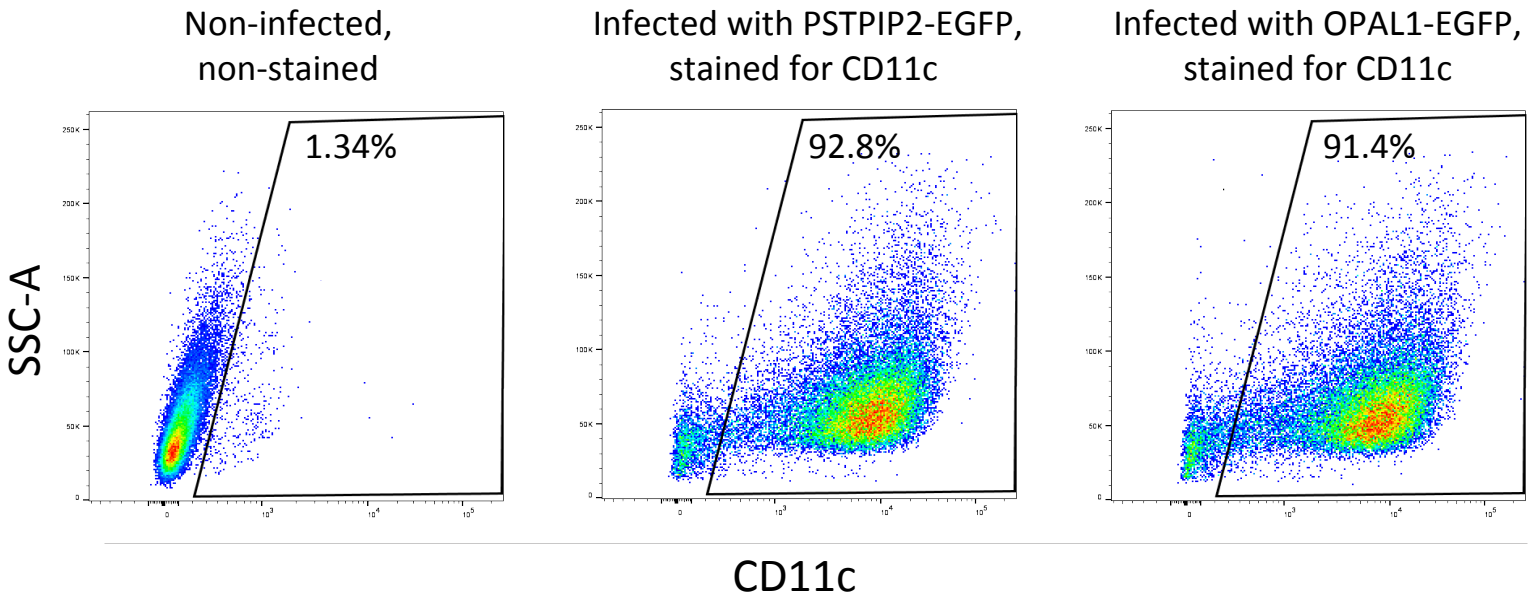
Bone marrow derived dendritic cells



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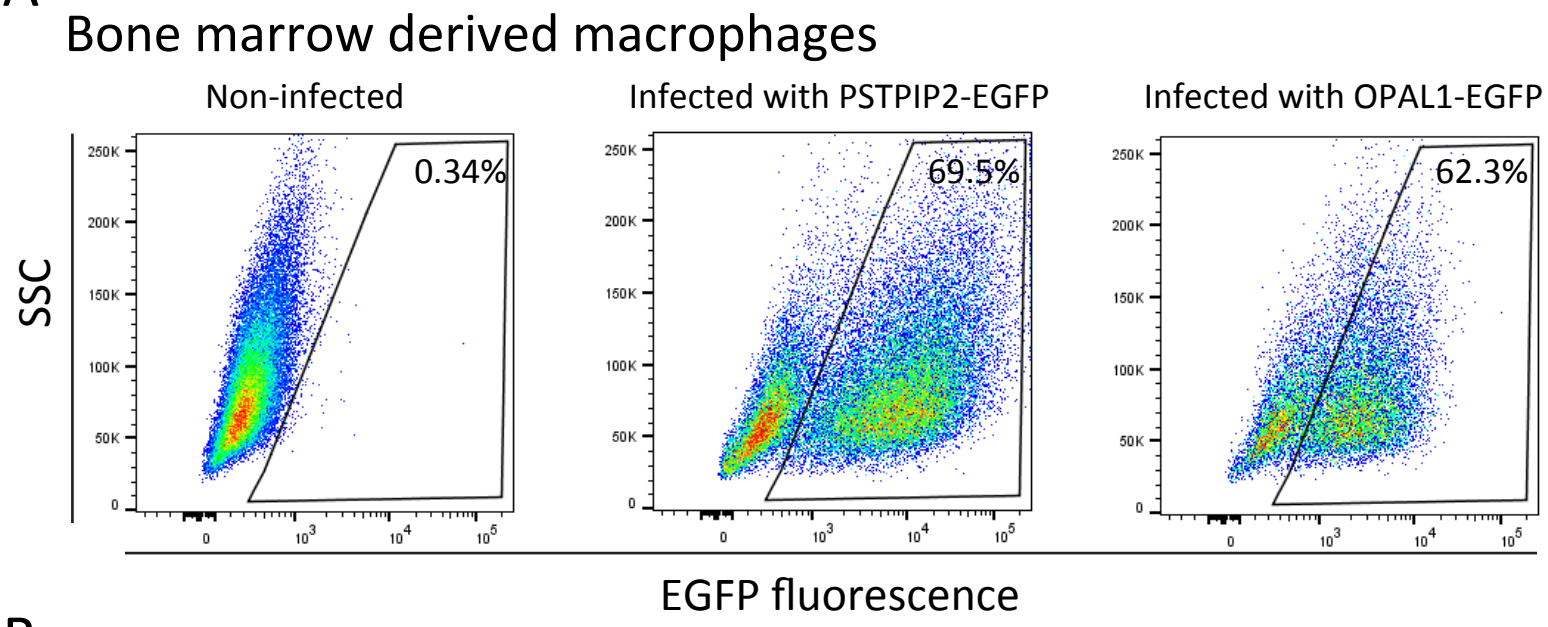

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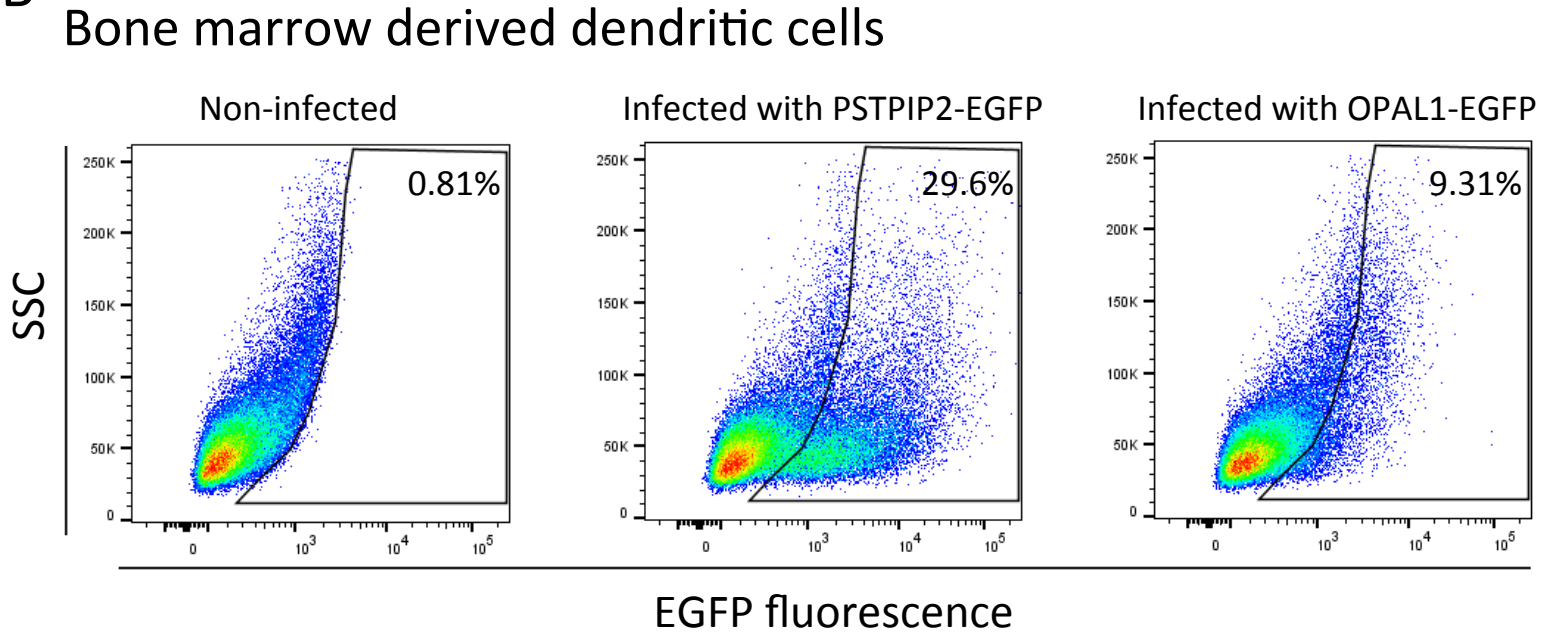

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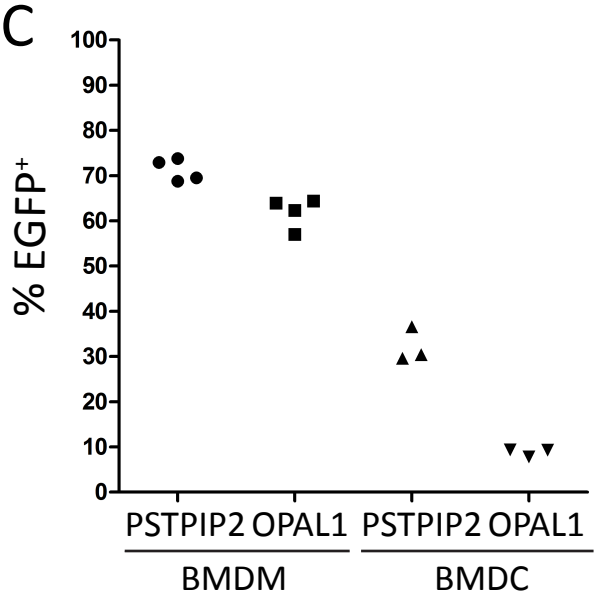
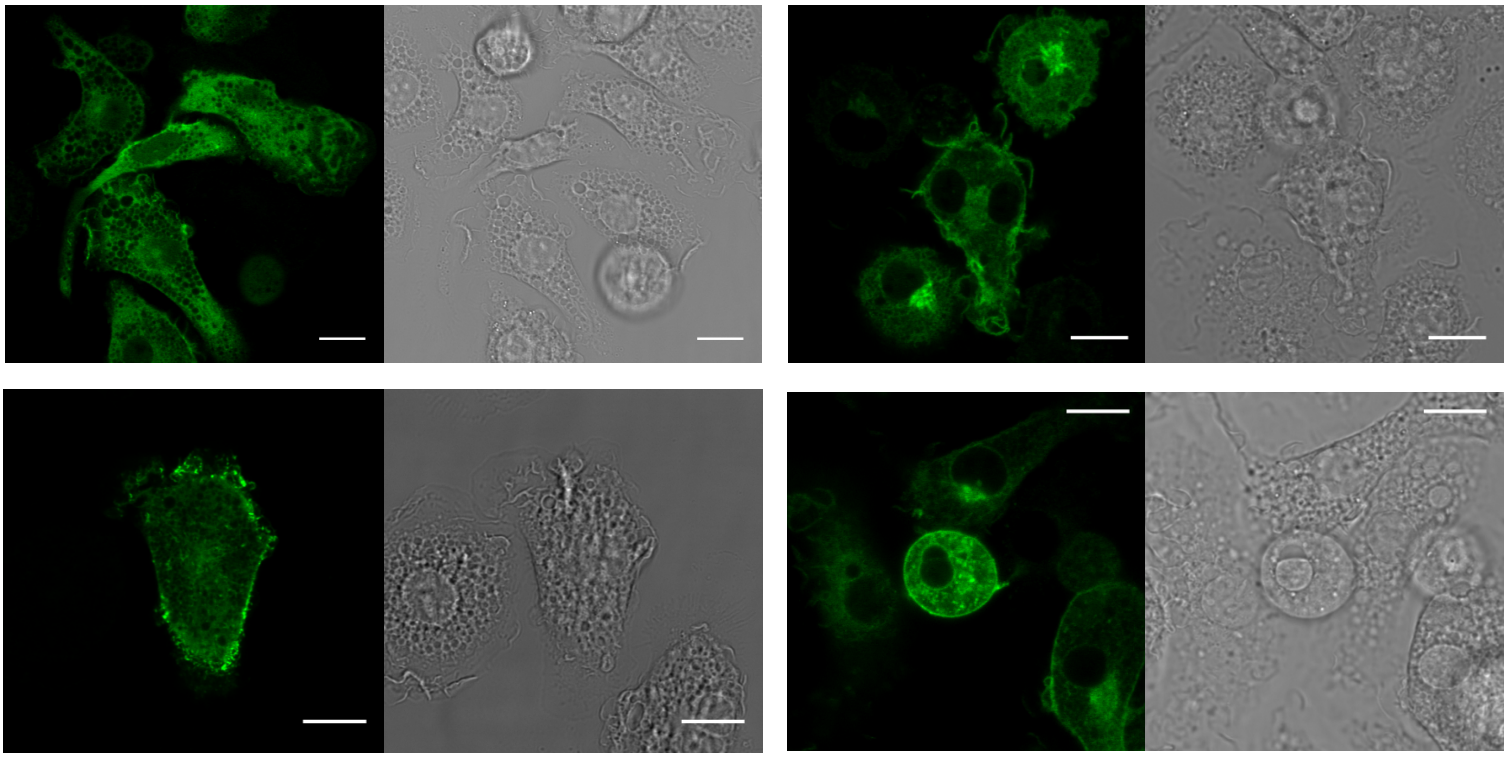


A

Bone marrow derived macrophages

PSTPIP2-EGFP

OPAL1-EGFP



B

Bone marrow derived dendritic cells

PSTPIP2-EGFP

OPAL1-EGFP

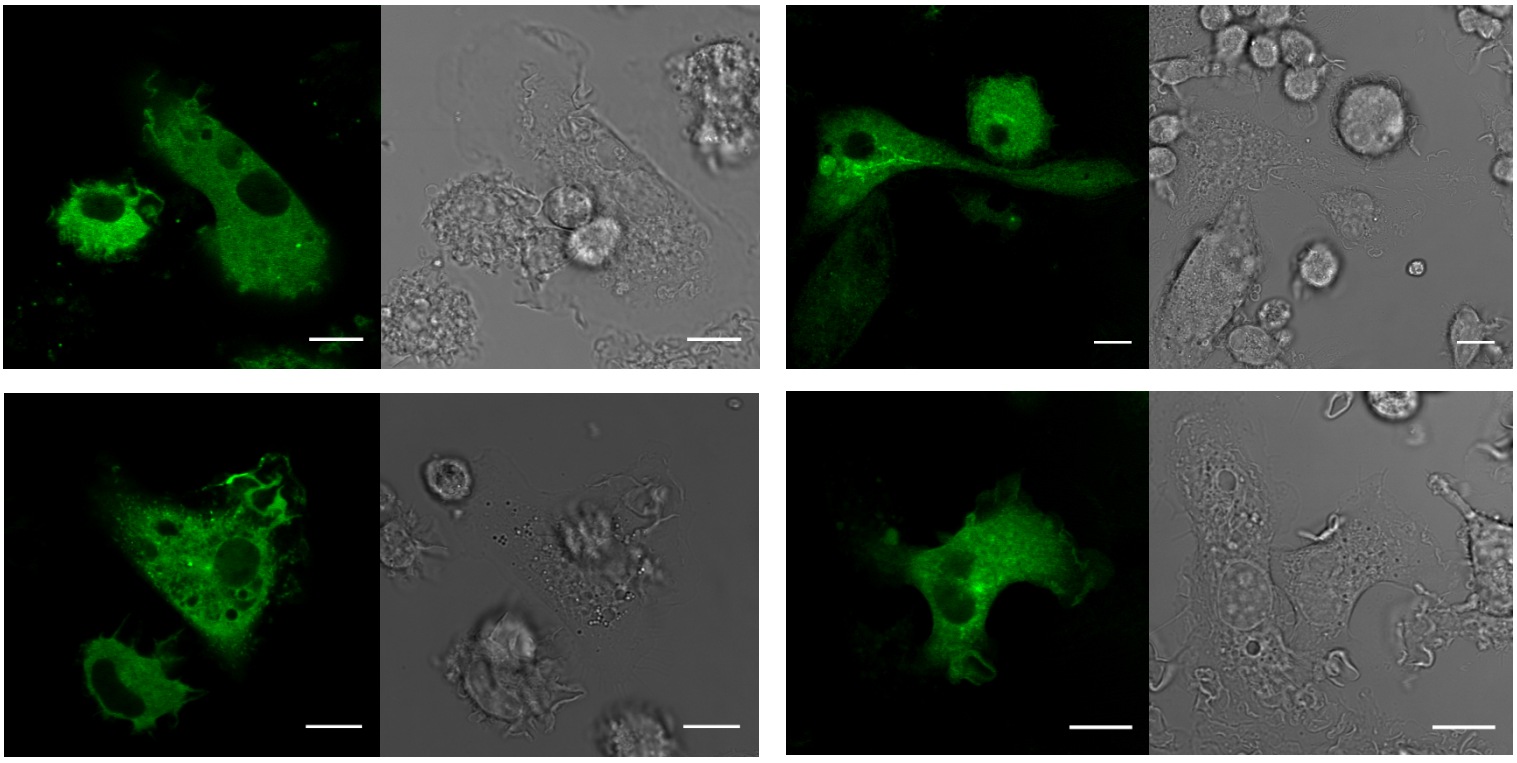


Table of Materials

Click here to download Table of Materials Table of Materials and Equipment.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DMEM	Thermo Fisher Scientific, Waltham, MA, USA	15028	
Fetal bovine serum (FBS)	Thermo Fisher Scientific, Waltham, MA, USA	10270	For media suplementation
$KHCO_3$	Lachema, Brno, Czech Republic	N/A	
NH_4Cl	Sigma-Aldrich (Merck, Kenilworth, NJ, USA)	A9434	
Penicillin	BB Pharma AS, Prague, Czech Republic	N/A	PENICILIN G 1,0 DRASELNÁ SOĽ BIOTIKA
Streptomycin	Sigma-Aldrich (Merck, Kenilworth, NJ, USA)	S9137	Streptomycin sulfate salt powder
Gentamicin	Dr. Kulich Pharma, Hradec Králové, Czech Republic	N/A	
Polyethylenimine, linear, MW 25,000	Polyscience, Warrington, PA, USA	23966	
Polybrene	Sigma-Aldrich (Merck, Kenilworth, NJ, USA)	H9268	
EDTA	Sigma-Aldrich (Merck, Kenilworth, NJ, USA)	E5134	
PBS	Prepared in-house by media facility of IMG ASCR, Prague, Czech Republic	N/A	
APC anti-mouse/human CD11b Antibody, clone M1/70	BioLegend (San Diego, CA, USA)	101212	flow cytometry analysis
PE anti-mouse F4/80 Antibody, clone BM8	BioLegend (San Diego, CA, USA)	123110	flow cytometry analysis
APC anti-mouse CD11c Antibody, clone N418	BioLegend (San Diego, CA, USA)	117310	flow cytometry analysis

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To: **Bing Wu, Ph.D.**
Review Editor,
JoVE

June 12th, 2018

Dear Dr. Wu

Thank you very much for your letter of May 31st, 2018 and for the editorial comments. We have addressed all the issues raised in these comments. Details can be found in the point by point response bellow. We have also made a few additional edits to increase the clarity of the text. The changes are all highlighted as tracked changes in the manuscript text. We now hope that the manuscript will be acceptable for publication.

Looking forward to hearing from you

Yours sincerely,

Tomáš Brdička, Ph.D.

Response to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread again and several mistakes corrected.

2. Please define all abbreviations before its first use, e.g., EDTA, etc.

All the abbreviations are now defined before the first use.

3. Please specify euthanasia method.

Cervical dislocation – we have added the information in step 3.1.

4. Step 1.2: What's the concentration of HCl, NaOH.

We have added the concentrations to step 1.2.

5. 3.3: What's the size of the Petri dish?

It is 5 cm, we have adjusted the text accordingly.

6. 3.6: What's the size of the small part of the epiphyses?

1 – 2 mm, we have adjusted the text accordingly.

7. 4.1: Please specify the antibiotics and the concentration.

The antibiotic concentration was specified in the note after step 1.4. We have now added the reference to this note into step 4.1.

8. 4.12: Please ensure that all text is written in imperative tense.

We have adjusted the text accordingly.

9. 4.13: Please ensure that all text is written in imperative tense.

We moved parts of step 4.13. to previous steps where its addition was more appropriate. We have changed the tens to its imperative form.

10. 5.1: Please ensure that all text is written in imperative tense.

We have re-written the whole step 5 to conform to this requirement.

11. 5.4: Please ensure that all text is written in imperative tense.

We have re-written the whole step 5 to conform to this requirement.

12. 5.5: Please ensure that all text is written in imperative tense.

We have re-written the whole step 5 to conform to this requirement.

13. 6.8: Chapter 3 and 4? Do you mean step 3 and 4?

Yes we do, we have adjusted the text accordingly.