Reliable and inexpensive expression of large, tagged, exogenous proteins in murine bone marrow-derived macrophages using a second generation lentiviral system

Matthew R. Miller, Scott D. Blystone

Department of Cell & Developmental Biology, SUNY Upstate Medical University, 750 East Adams St. Syracuse, NY 13210, USA

Corresponding author: Scott D. Blystone, PhD, Tel: 315-464-8512, Email: blystons@upstate.edu

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Abstract Over the past two decades, researchers have struggled to efficiently express foreign DNA in primary macrophages, impeding research progress. The applications of lipofection, electroporation, microinjection, and viral-mediated transfer typically result in disruptions in macrophage differentiation and function, low expression levels of exogenous proteins, limited efficiency and high cell mortality. In this report, after extensive optimization, we present a method of expressing large tagged proteins at high efficiency, consistency, and low cost using lentiviral infection. This method utilizes laboratory-propagated second generation plasmids to produce efficient virus that can be stored for later use. The expression of proteins up to 150 kDa in size is achieved in 30–70% of cells while maintaining normal macrophage differentiation and morphology as determined by fluorescence microscopy and Western blot analysis. This manuscript delineates the reagents and methods used to produce lentivirus to express exogenous DNA in murine bone marrow-derived macrophages sufficient for single cell microscopy as well as functional assays requiring large numbers of murine bone marrow-derived macrophages.

Keywords: BMDM, expression, protein, transduction, lentivirus

INTRODUCTION

Macrophages are white blood cells (leukocytes), which are essential components of the immune system. Macrophages play key roles in innate and adaptive immune responses as immediate responders to eliminate microbial infections, promote wound healing, and present antigen to lymphocytes. Macrophages are derived from circulating monocytes which, upon activation, extravasate through the vascular wall and differentiate into macrophages. After terminal differentiation macrophages can migrate through complex extracellular matrices to sites of injury or infection to elicit an immune response.

As essential components of both innate and adaptive immunity, efforts to fully understand macrophage functions involve *in vitro* studies of primary macrophage populations. Murine macrophages are generally purified for *in vitro* study utilizing two different methods. One approach entails harvesting bone marrow and differentiating multipotent stem cells into murine macrophages (BMDMs) in culture using cytokines such as murine monocyte colony-stimulating factor (MCSF) or granulocyte-macrophage colony-stimulating factor (GMCSF) [1]. Murine macrophages can also be isolated by inducing peritonitis and harvesting murine macrophages that infiltrate the peritoneal cavity, which can be directly assayed or cultured [1].

To study the structures and proteins that mediate macrophage function it is advantageous for researchers to have the ability to express constructs in these cells, including shRNA and exogenous DNA. Macrophages and macrophage cell lines are notoriously difficult to manipulate genetically. With many studies utilizing knock-out (KO) mouse generated macrophages, the ability to trace molecular mechanisms relies on reconstitution with wild type (WT), mutant, and truncated versions of the deleted protein. Most techniques utilized cause detrimental effects to macrophage differentiation and viability. One likely reason for the difficulty of expressing exogenous DNA within macrophages is that these cells undergo terminal differentiation [2]. Terminal differentiation of macrophages from precursor cells causes macrophages to adopt a quiescent state, no longer undergoing proliferation [2]. Reliable and stable expression of exogenous DNA requires gene integration into the genome; however this process is very difficult if not impossible in cells that no longer proliferate. Therefore, the most common available methods of introducing exogenous DNA in primary differentiated macrophages permit only non-integrated transient expression. Early reported studies and methods used to attempt DNA delivery and transient expression in primary murine macrophages include liposomal transfection, ionic precipitation, electroporation, and chemical transfection. However, the dawn of gene delivery viruses has yielded numerous advances in research and has begun to be utilized for macrophage research.

Multiple reports have claimed to express exogenous DNA in primary murine macrophages and murine macrophage cell lines. An early report detailed an approach to express exogenous DNA in primary murine

macrophages utilizing and optimizing various methods [3]. Rupprecht *et al.* performed calcium-phosphate, diethylaminoethyl(DEAE)-dextran, and lipofection, using an enzyme assay to determines expression profiles, and concluded that DEAE-dextran was optimal [3]. However, another study performed a few years later by Thompson *et al.* incorporated additional reagents and techniques to optimize expression in a murine macrophage cell line to measure expression using a luciferase assay. The results of this study correlated with reports of Ruprecht *et al.* concluding that electroporation yielded optimal DNA expression, while DEAE-dextran was the least efficient technique [4]. These studies were in agreement that lipofection was very ineffective, however neither study measured actual expression efficiency levels, and Thompson *et al.* reported over 75% cell death with their optimal DNA expression technique [3, 4].

Dokka *et al.* attempted to optimize lipofection techniques utilizing protamine sulfate to enhance lipid-mediated transfection of murine macrophage cell lines and additionally compared these with other transfection techniques that have been reported to be successful by other investigators [5]. Protamine sulfate is suggested to condense DNA for easier nuclear permeability as well as containing a nuclear localization sequence, which promotes trafficking of transfected DNA into the nucleus [6]. Dokka *et al.* also utilized the luciferase assay to assess transfection efficiencies and reported that lipofection combined with protamine sulfate yielded high expression levels in comparison to other transfection techniques, with little effect on cell viability [5]. Thus, there is extensive controversy found regarding these reported techniques utilized for effective transfection and expression of exogenous DNA in primary murine macrophages and murine macrophage cell lines.

Additional techniques have been described to aid in the expression of exogenous DNA in a murine macrophage cell line by blocking the silencing of foreign DNA caused by methylation after DNA has been delivered inside macrophages [7]. 5-azacytidine is a chemical that blocks the methylation of DNA by inhibiting DNA methyltransferases [7]. In one study, DEAE-dextran combined with 5-azacytidine was found to be more efficient than several liposomal reagents, concluding that DEAE-dextran was optimal for gene delivery, yet DEAE-dextran alone was reported to have poor efficiency [7]. To our knowledge, this is the only documented report utilizing 5-azacytidine to express exogenous DNA in macrophages. In our own studies 5-azacytidine treatment on murine macrophage cell lines or primary macrophages did not increase foreign DNA expression efficiencies (data not shown).

Electroporation is another method that has been reported to effectively deliver DNA into murine macrophages. This method uses electrical current to deliver charged particles, such as DNA through the plasma membrane of cells to reach the cytoplasm for expression. A variation to electroporation has been more recently developed, termed "nucleofection", that is purported to be better suited for DNA transfer into macrophages, offering more system adjustments such as voltage and cell-specific reagents for electroporation permitting additional optimization. Using nucleofection, Van De Parre *et al.* compared transfection efficiencies of DNA, dsRNA, and mRNA of green fluorescent protein (GFP) using a murine macrophage cell line. Interestingly, they showed that DNA and dsRNA caused extensive cell death through causing apoptosis and no expression of GFP was observed [8]. However, mRNA was observed to have GFP expression efficiencies up to 75% with little effect on cell viability, as evaluated by flow cytometry and Western blot analysis [8]. Another group showed success in using the nucleofection technique to transfect shRNA and DNA constructs into a murine macrophage cell line in two different studies [9, 10]. They transfected a macrophage cell line with GFP-tagged shRNA and using fluorescence microscopy to detect GFP expression generated a stably transfected macrophage cell line [9,10]. Knockdown of protein levels were demonstrated by Western blot analysis, yet efficiency levels of exogenous DNA expression were not specified [9, 10]. These studies have suggested that the more advanced electroporation system has a substantial impact on increasing exogenous DNA expression in murine macrophage cell lines. The use of nucleofection on primary cells seems unfeasible as primary murine BMDMs have a short lifespan, and are unlikely to survive a drug selection process to create a stable cell line.

The use of virus-based gene delivery into cells has gained much momentum over the last decade. Reports of increased efficacy in DNA delivery and expression in both murine macrophage cell lines and primary murine macrophages, utilizing viral systems, have begun to phase out other common techniques used for DNA transfection. The two most frequently used viral systems used for expressing exogenous DNA in murine macrophages are adenovirus and lentivirus; however, adenovirus can only be used for dividing cells, and thus is not suitable for primary bone marrow-derived macrophages. Moreover, variations in techniques and/or reagents have been utilized in efforts to increase lentiviral transduction efficiency, including polybrene and/or spinfection, also termed spinoculation [11,12]. Spinfection is thought to help sediment viral particles to increase virus contact with cell membranes, while polybrene reduces charge repulsion between viral particles and the cell membrane [11,12]. A study by Cunnick *et al.* demonstrated lentivirus transduction efficiency between 30% and 50% in murine bone marrow-derived macrophages determined using fluorescence microscopy [13]. Interestingly, this group reported that polybrene did not increase lentiviral transduction efficiencies [13]. Pan *et al.* demonstrated the use of a lentiviral system with a dual promoter, allowing for targeted and inducible expression in specific cells of macrophage lineage [14]. This study reported high expression efficiency in murine macrophage cell lines and bone marrow-derived macrophages, however multiple rounds of cell sorting was required to achieve this, which can ultimately have a detrimental impact on cell viability [14].

Prior to developing and optimizing the method presented here, we utilized a complete virus producing kit from Clontech (Cat # 631247). The expression vector ultimately utilized in our method is derived from the Clontech system. The complete Clontech kit exploited a forth generation packaging system, which allotted for a higher degree of safety, due to 4 recombination events being required for release of replicating virus. In our hands, this system did not produce infectious virus that was capable of expressing quantifiable exogenous DNA in primary macrophages. Additionally the use of four packaging vectors was probably less efficient in transferring viral DNA in to the 293T cells for viral production. These packaging vectors are purchased pre-mixed, requiring repeated purchasing instead of having the opportunity to propagate the vectors in lab, increasing lab expenses. Fortunately the expression vector containing GFP was compatible with a different packaging system that was commercially available and can be propagated in the lab. We also initially attempted to concentrate virus utilizing a concentration solution for lentivirus from Clontech (Cat # 631231), however this proved to be cytoxic to primary macrophages. We report here an efficient, consistent, inexpensive protocol of exogenous DNA expression in primary murine bone marrow-derived macrophages, using lentivirus transduction. This second generation system would require two recombination events during viral production for release of replicating virus and is considered by the CDC to be a BSL-2 level activity, suitable for most tissue culture containment hoods. While

higher generation viral systems may be suitable for many cell types, macrophages required a more simplistic viral packaging system to produce virus of sufficient infectivity.

MATERIALS

Reagents

Harvesting, culturing, and differentiating bone-marrow derived macrophages
- ✓ L929 Cell Line (Sigma-Aldrich, cat # 85011425-1VL)
- ✓ DMEM (Life Technologies, cat # 12800-017)
- ✓ FBS (Gemini Bio-Products, cat # 100-500)
- ✓ Glutamax (Life Technologies, cat # 35050-061)
- ✓ Penicillin-streptomycin, liquid (Life Technologies, cat # 15140-122)
- ✓ 50 ml Conical Tube (Corning Falcon, cat # 352098)
- ✓ 3 ml Syringe with 25G needle (Becton, Dickinson and Company, cat # 309570)
- ✓ EDTA (Sigma-Aldrich, cat # E1644)
- ✓ DPBS (Life Technologies, cat # 21600-010)
- ✓ Ammonium Chloride (Sigma-Aldrich, cat # A9434)
- ✓ Potassium Bicarbonate (Fisher Scientific, cat # P235)
- ✓ 75 cm² Vent Cap Flask (Corning, cat # 3276)
- ✓ 225 cm² Vent Cap Flask (Corning, cat # 3001)
- ✓ Sterile water (Baxter Healthcare Corporation, cat # 2F7114)
- ✓ 500 ml 0.45 µM Acetate Vacuum Filter/Storage Bottle System (Corning, cat # 430770)
- ✓ Surgical scissors (Harvard Apparatus, cat # 522474)
- ✓ Moloney Forceps (Harvard Apparatus, cat # 523407)
- ✓ Halstead-Mosquito Curved Hemostats (Harvard Apparatus, cat # 728967)
- ✓ 190 Proof Ethanol (Ultra Pure, cat # 190CSGP)

Lentivirus production
- ✓ Lenti-X 293T Cell Line (Clontech Laboratories Inc., cat # 632180)
- ✓ pLVX-AcGFP1-C1 Vector (Clontech Laboratories Inc., cat # 632155)
- ✓ psPAX2 Vector (Addgene, ID # 12260)
- ✓ pMD2.G Vector (Addgene, ID # 12259)
- ✓ 225 cm² Vent Cap Flask (Corning, cat # 3001)
- ✓ T75 Vent Cap Flask (Corning, cat # 3276)
- ✓ Cover Glasses, 12 mm Circles (Fisher Scientific, cat # 12-545-80)
- ✓ 24 well plate (Corning, cat # 3524)
- ✓ Ultracentrifuge Tubes (Beckman Coulter, cat # 344060)
- ✓ Parafilm (Fisher Scientific, cat # 13-374-10)
- ✓ JetPEI with 150 mM Sodium Chloride solution (Polyplus, cat # 101-10N)
- ✓ DMEM (Life Technologies, cat # 12800-017)
- ✓ FBS (Gemini Bio-Products, cat # 100-500)
- ✓ Glutamax (Life Technologies, cat # 35050-061)
- ✓ Penicillin-streptomycin, liquid (Life Technologies, cat # 15140-122)
- ✓ 1.7 ml Microcentrifuge tube (Corning, cat # 3622)
- ✓ 250 ml 0.45 µM Acetate Vacuum Filter/Storage Bottle System (Corning, cat #430768)
- ✓ Single beam balance (OHAUS Harvard Trip Balance)

Bone marrow-derived macrophage lentiviral infection
- ✓ 25 cm² vent cap flask (Corning, cat # 430639)
- ✓ HBSS (Sigma-Aldrich, cat # H9269)
- ✓ EDTA (Sigma-Aldrich, cat # E1644)
- ✓ Cell Scraper (Corning, cat # 3010)

Bone marrow-derived macrophage microscopy
- ✓ 24 well plate (Corning, cat # 3524)
- ✓ Cover Glasses, 12mm Circles (Fisher Scientific, cat # 12-545-80)
- ✓ HBSS (Sigma-Aldrich, cat # H9269)
- ✓ EDTA (Sigma-Aldrich, cat # E1644)
- ✓ Cell Scraper (Corning, cat # 3010)
- ✓ DAPI (Invitrogen, cat # D1306)
- ✓ 37% Formaldehyde (Fisher Scientific, cat # F-79)
- ✓ NP-40 Alternative (Calbiochem, cat # 492016)
- ✓ Goat Serum (Life Technologies, cat # 16210-064)
- ✓ Triton X-100 (Fisher Scientific, cat # BP-151)
- ✓ Polyvinyl Alcohol (Sigma-Aldrich, cat # P-8136)
- ✓ Glycerol (Sigma-Aldrich, cat # G-9012)
- ✓ DABCO (Fischer Scientific, cat # AC112471000)

BMDM Western blot analysis
- ✓ Anti-GFP mouse monoclonal (Santa Cruz Biotechnology, Inc., cat # sc-9996)
- ✓ Donkey anti-mouse HRP (Jackson ImmunoResearch, cat # 715-036-150)
- ✓ Anti-FMNL1 rabbit polyclonal (Sigma-Aldrich, cat # HPA008129)
- ✓ Donkey anti-rabbit HRP (Jackson ImmunoResearch, cat # 711-036-152)
- ✓ Anti-Transaldolase goat polyclonal (Santa Cruz Biotechnology, Inc., cat # sc-51440)
- ✓ Bovine anti-goat HRP (Jackson ImmunoResearch, cat # 805-035-180)
- ✓ Molecular Weight Marker (Amersham Systyems, cat # RPN800E)
- ✓ ECL solution (Amersham Systyems, cat # RPN2106)

Equipment

- ✓ Hemacytometer (Reichert Bright-Line)
- ✓ Tabletop Centrifuge (Labnet HERMLE Z 400 K)
- ✓ Ultracentrifuge (Beckman Coulter Optima L-90K Ultracentrifuge)
- ✓ Orbital Shaker (HOEFER SCIENTIFIC INSTRUMENT Red Rotor PR70)
- ✓ Fluorescence Microscope: (Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu ORCA-ER digital camera, Leica SP-5 Laser Scanning Confocal fluorescence microscope)

Recipes

- ✓ ACK Buffer: 150 mM Ammonium Chloride, 10 mM Potassium Bicarbonate, 10 µM EDTA in sterile water
- ✓ 50 mM EDTA in PBS Buffer: 10 mM EDTA in PBS
- ✓ 50 mM EDTA in HBSS⁻⁻ Buffer: 10 mM EDTA in HBSS⁻⁻
- ✓ L929 Cell Line Media: DMEM with 10% FBS, 2 mM Glutamax, 10 units/ml Penicillin, 10 µg/ml Streptomycin
- ✓ Lenti-X 293T Cell Line Media: DMEM with 10% FBS, 2 mM Glutamax, 10 units/ml Penicillin, 10 µg/ml Streptomycin
- ✓ Bone Marrow-Derived Macrophage Wash Media: DMEM with 2% FBS, 2 mM Glutamax, Penicillin 10 units/ml, Streptomycin

10 μg/ml
- ✓ Bone Marrow-Derived Macrophage Adhesion Exclusion Media: DMEM with 10% FBS, 2 mM Glutamax, 10 units/ml Penicillin, 10 μg/ml Streptomycin
- ✓ Bone Marrow-Derived Macrophage Differentiation Media: DMEM with 10% FBS, 15% L929 cell supernatant, 2 mM Glutamax, 10 units/ml Penicillin, 10 μg/ml Streptomycin
- ✓ 70% Ethanol Solution: 70 ml of 100% EtOH with 30 ml sterile

water
- ✓ Fixing Solution: PBS with 3.7% formaldehyde final concentration
- ✓ Permeabilization Solution: PBS with 0.002% NP-40
- ✓ Blocking Solution: PBS with 3% heat-inactivated goat serum and 1% TritonX-100
- ✓ Mounting Solution: (Gelvatol) 24 ml of distilled H_2O with 9.6 g PVA, 19.05 ml glycerol, 48 ml Tris pH 8.5, 2.5% DABCO

PROCEDURE

All reagents and materials used during viral production, harvesting, concentrating, and infection should be used in a sterile tissue culture hood equipped with ultra-violet light for decontamination. Treatment with 15% bleach was used as an additional decontamination measure.

1. Transfection of 293T-X cells for virus production.

   1.1.   The 293T-X cells are maintained in DMEM + 10% FBS + 1% penicillin-streptomycin + 10 mM glutamax. Cell passage should be performed before cells reach full confluency using trypsin. During the first wash of cell passage, an equal volume of complete media must be added to cells in trypsin to allow for proper cell pelleting.

   1.2.   To transfect plasmids for viral production, 293T-X cells are cultured in 225 cm² flasks. Cells should be at ~90% confluency for transfection. Two 225 cm² flasks of 293T-X cells should be cultured and transfected for each virus.

   1.3.   On the day of 293T-X cell transfection, media should be removed from cells, and cells should be supplemented with 20 ml of fresh complete media.

   1.4.   Transfection is performed using the transfection reagent JetPEI according to the manufacturer's protocol. The final contents of the transfection solution should be 1 ml of 150 mM Sodium Chloride JetPEI solution, 10 μg of the pLVX-vector, 20 μg of the pMD2.G vector, 30 μg of the psPAX2 vector, and 100 μl of JetPEI reagent.

   1.5.   Add the transfection solution to the cells in 225 cm² flasks in a drop-wise manner and then swirl the media in the flasks to mix the transfection solution with the media.

   1.6.   Place the flasks back in the incubator at 37°C with 5% CO_2.

   1.7.   After 6 h, replace the transfection media with 40 ml of fresh complete media and place flasks back into the incubator.

   1.8.   Harvest viral media after 72 h. Pool together viral media from identical flasks and add to a 250 ml 0.45 μM acetate filter flask to filter the viral media to remove any debris or cells in suspension.

   1.9.   Ultracentrifuge tubes are then filled with 13 ml of the filtered viral media and balanced using the single beam balance after being placed into the buckets of a SW40Ti rotor.

   1.10. Centrifuge the viral media at 100,000 × *g* for 2 h at 4°C, with a SLOW brake setting.

   1.11. After centrifugation, carefully remove buckets and transfer to hood. Remove the ultracentrifuge tubes from the rotor buckets and gently pour off supernatant. Add 500 μl of bone marrow-derived macrophage media to each tube. Wrap the top of each tube with parafilm and store at 4°C overnight.

   1.12. After overnight incubation, place tubes on orbital shaker at 100 rpm at room temperature for 1 h.

   1.13. Transfer tubes to a sterile tissue culture hood and remove parafilm. Pass media through a 5 ml serological pipet to break up remaining pellet, and pool together resuspended viral pellets from identical transfections.

2. Harvesting, culturing, and differentiation of murine bone marrow-derived macrophages for viral infection.

   2.1.   In our experiments, mice were sacrificed between the ages of 5 and 9 weeks using CO_2.

   2.2.   Spray mice down with 70% ethanol and pin mice down to dissection mat with the abdomen facing down.

   2.3.   Using forceps and surgical scissors, remove the femur and tibia and place them in a 10 cm petri dish containing wash media.

   2.4.   After removal of leg bones, in a sterile tissue culture hood further remove any remaining muscle tissue from the leg bones.

2.5. Using surgical hemostats to hold and cut the ends off of each femur and tibia using surgical scissors. Using wash media in 3 ml syringes equipped with a 25 G needle flush the bone marrow from each bone into a 50 ml conical tube.

2.6. Centrifuge the bone marrow for 5 min at 1000 rpm at room temperature.

2.7. Wash the pellet with 5 ml of 10 mM EDTA in PBS.

2.8. Centrifuge the bone marrow for 5 min at 1000 rpm at room temperature.

2.9. Resuspend the pellet with 3 ml of ACK buffer and incubate for 2 min to lyse RBCs.

2.10. After the incubation time, immediately add 10 ml of wash buffer to prevent lysis of bone marrow cells.

2.11. Centrifuge the bone marrow for 5 min at 1000 rpm at room temperature.

2.12. Resuspend the pellet with 20 ml of adhesion exclusion buffer, and transfer the cells to a 10 cm petri dish, and place dish into an incubator at 37°C with 5% CO_2 for 2 h.

2.13. Gently swirl media in the dish, and then carefully remove media with unattached cells while leaving any observable pieces of tissue.

2.14. Centrifuge the unattached bone marrow cells for 5 min at 1000 rpm at room temperature.

2.15. Resuspend pellet with 40 ml of bone marrow-derived differentiation media and plate cells evenly between two 75 cm² flasks. Incubate at 37°C with 5% CO_2 (follow this dilution of cells per number of mice used).

2.16. Allow cells to differentiate into BMDMs for 6 days. Add 5 ml of warm fresh differentiation media to each flask on day 4.

3. Viral infection of murine bone marrow-derived macrophages.

3.1. Cells must be removed from 75 cm² flasks on day 6 of BMDM differentiation. Using ice cold 10 mM EDTA in HBSS, wash the adhered cells once with 5 ml, then add 8 ml to each flask and place of 4°C for approximately 10 min.

3.2. Firmly tap the bottom of the flasks to break adhesion and also use a cell scraper to gently lift any remaining adhered cells.

3.3. Add the cells to a 50 ml conical tube and centrifuge for 5 min at 1000 rpm at room temperature.

3.4. Resuspend the pellet in 5 ml of differentiation media and centrifuge for 5 min at 1000 rpm at room temperature.

3.5. Using a hemacytometer, count the BMDMs and plate 1.0×10^6 cells per 25 cm² flasks in 4 ml of differentiation media. Macrophage viability is typically in excess of 95% by trypan blue exclusion. Incubate at 37°C with 5% CO_2.

3.6. After allowing the cells to adhere for approximately 16 h, remove differentiation media and replace with a total volume of 4 ml viral infection media. Use the entire virus concentrated from both 225 cm² flasks for one 25 cm² flask of BMDMs. Average titer used was 1.5×10^6 PFU for GFP alone and 1.0×10^5 PFU for GFP-FMNL1β and GFP-FMNL1γ. Place flasks into an incubator at 37°C with 5% CO_2.

3.7. Add 2 ml fresh macrophage differentiation media after 48 h.

3.8. After 72 h of viral infection, cells are ready for experimental analysis.

4. Performing microscopy on virally infected murine bone marrow-derived macrophages.

4.1. After 72 h cells must be removed from 25 cm² flasks. Using ice cold 10 mM EDTA in HBSS, wash the adhered cells once with 5 ml, then add 8 ml to the flasks and place in 4°C for approximately 10 min.

4.2. Firmly tap the bottom of the flasks to break adhesion and use a cell scraper to gently lift any remaining adhered cells.

4.3. Add the cells to a 50 ml conical tube and centrifuge for 5 min at 1000 rpm at room temperature.

4.4. Resuspend the pellet in 5 ml of differentiation media and centrifuge for 5 min at 1000 rpm at room temperature.

4.5. Using a hemacytometer, count the BMDMs and plate cells in 24 well plates containing glass coverslips in each well. Macrophage viability is typically in excess of 95% by trypan blue exclusion. Add a total volume of 0.5 ml of cells in differentiation media. Incubate at 37°C with 5% CO_2.

4.6. For dispersed cells, plate at 2.5×10^4 cells per well.

4.7.   For dense cells, plate 1.0×10^5 cells per well.

4.8.   After approximately 16 h, prep cells for microscopy by washing wells twice with PBS and adding 0.5 ml of ice cold fixing solution. Incubate at 4°C for 1 h.

4.9.   Wash coverslips with twice with ice cold PBS.

4.10.   Permeabilize cells by adding ice cold permeabilization solution for 10 seconds followed by three washes with ice cold PBS.

4.11.   Add blocking solution to wells at room temperature for 30 min.

4.12.   Wash wells twice with room temperature PBS.

4.13.   Add DAPI at room temperature for approximately 5 min.

4.14.   Wash wells five times with PBS.

4.15.   Mount coverslips, cell side down, on glass slides with 8 µl of mounting solution.

4.16.   Prior to imaging, store slides overnight in a slide box at 4°C to allow mounting solution to set.

ANTICIPATED RESULTS

The methods detailed here offer an optimized lentiviral system for expressing exogenous DNA in extremely hard-to-transfect cells (e.g., BMDMs) with efficiencies of ~70% with GFP alone and ~30% with large GFP-tagged proteins, as determined by visual counting of fluorescence microscopy (**Fig. 1** and **Fig. 2**) and Western blotting (**Fig. 3**). For expression efficiency shown in **Figure 2**, cells were visualized and counted at a higher magnification (60 ×) than are shown in **Figure 1**. This was necessary due to the discreet localization of FMNL1γ to macrophage podosomes. Fluorescent cells due to GFP-alone were easily visible at all magnifications (**Fig. 1** and **2**). The inset images in **Figure 2** show the difference in fluorescence distribution between GFP and GFP-FMNL1γ. GFP is broadly distributed throughout the cytoplasm while GFP-FMNL1γ is found in punctuate structures of the ventral surface, an adhesion structure termed a podosome. Fluorescence microscopy may not be sufficient to detect extremely low levels of expression in cells, thus these reported expression efficiencies may largely underestimate the actual number of expressing cells. Therefore, flow cytometry may be a more accurate method to determine actual expression levels, but was not available for this study. Furthermore, viral production may be less efficient with large, GFP-tagged proteins, so increasing the number of flasks or media harvests from the 293T-X packaging cells producing virus for concentration can increase the amount of virus. We have also periodically performed virus titers using both an ELISA-based assay to quantify levels of virus specific protein, and traditional plaque assays. Both methods appear to underestimate the total virus transduction occurring, as measured by microscopy. Two important components that contribute to the success of this protocol are concentrating the lentivirus and using it quickly after production without any freeze/thaw cycles.
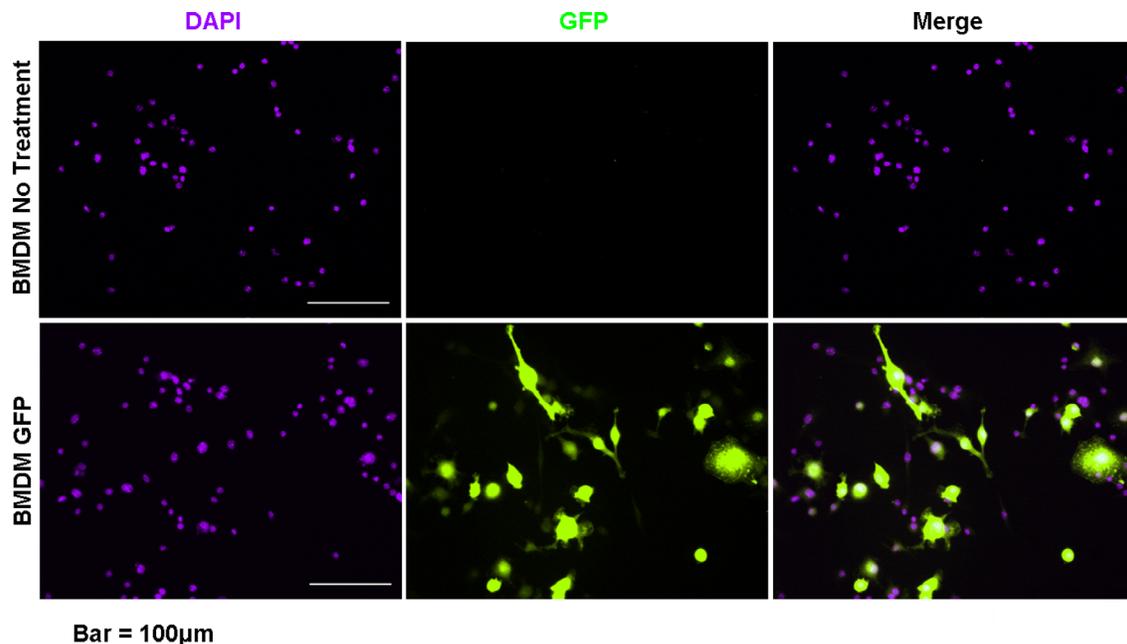

Bar = 100µm

Figure 1. Lentivirus transduction provides high expression efficiency. Murine bone marrow-derived macrophages expressing GFP were visualized utilizing fluorescence microscopy. Cells were plated at 1.0×10^5 cells per well on glass coverslips in complete media to observe expression. BMDMs were fixed, permeabilized, and stained with DAPI for nuclear visualization. Field was imaged using a 20x objective.

The GFP-tagged proteins described in these experiments are splice variants of the formin, FMNL1. Formins are actin-associated proteins that perform multiple actin cytoskeleton regulating events including actin filament nucleation, polymerization, bundling, and/or severing [15,16,17]. FMNL1 is a large protein with 1069 amino acids, making it approximately 110 kDa, and with a GFP tag (26 kDa) this protein is approximately 136 kDa. 3 different isoforms of FMNL1 have been identified, and we have shown expression of two of these using our lentiviral system (**Fig. 3**) [18,19]. PCR was used to copy Superscript-derived cDNA encoding the FMNL1 isoforms from purified macrophage RNA. Oligonucleotides specific to the 5' end of FMNL1γ (accession FJ534522) and FMNL1β (accession BC001710) incorporated a Xba1 restriction site in frame with initiating methionine. Oligonucleotides specific for the 3' end of each FMNL1 isoform were engineered to include a BamH1 restriction site. Following PCR, the product was subjected to Xba1 and BamH1 restriction and ligated into Xba1/BamH1 restricted pLVX-AcGFP1-C1 for viral production. Therefore, this expression system is well suited for expressing large proteins in primary murine bone marrow-derived macrophages with high efficiency (**Fig. 3**). Moreover, this system permits expression of full-length proteins that are fluorescently labeled, making this ideal for studies in primary murine macrophages derived from murine KO models.

Moreover, there are a few additional benefits to utilizing our developed lentiviral system for expressing exogenous DNA. For example, this system can be applied towards the expression of fluorescently labeled proteins in non-macrophage cell lines, as we have used this system to reach nearly 100% efficiency in HeLa, U2OS, and MDA cell lines (data not shown). In addition, our method incorporates transforming competent *E. coli* with viral vectors, allowing for unlimited propagation. Thus, our method is very cost-effective as it does not require the constant purchasing of viral vector packaging mixes. Also, the lentivirus produced from this procedure can be frozen and used at a later date; however, freeze/thaw cycles will likely affect some of the viral infectivity. Additionally, it may be possible to increase BMDM expression efficiency by performing a secondary transduction 48 h after the initial transduction, however this would require producing an exceptionally large volume of virus for each independent experiment.

Another very important aspect of our expression system is that it can yield high enough expression efficiencies to perform functional assays that require large numbers of cells. This would prove to be difficult if not impossible using other systems because of their lower expression efficiencies, where functional assays often require a large portion of the cell population to express the exogenous DNA for measurement and quantification. On another note, attempts to use this protocol to express foreign DNA in primary human peripheral blood monocyte-derived macrophages yielded poor expression efficiencies. However, more extensive optimizing could potentially permit higher expression levels in primary human macrophages.

Therefore, in this report, we fully detail how to produce infectious lentivirus with the capacity to express exogenous DNA in primary murine bone marrow-derived macrophages at high efficiencies, consistently, and at low cost. This easily adaptable protocol will have a profound effect on enlightening and enhancing the field of macrophage biology with more efficient and comprehensive studies working on macrophage structures and proteins.
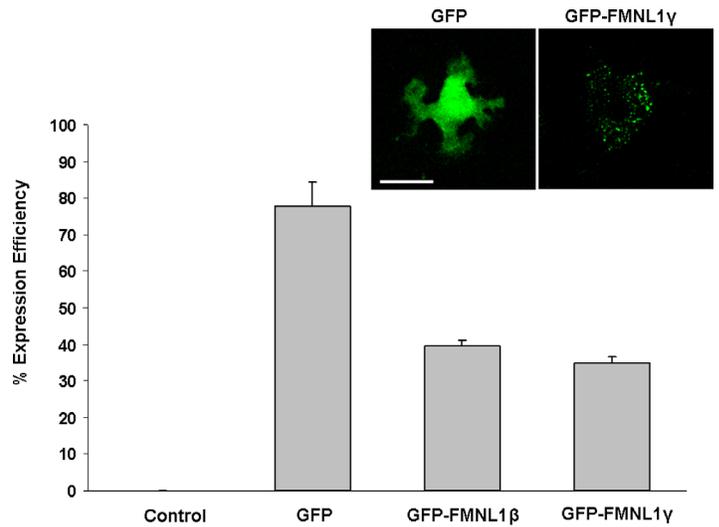

Figure 2. Lentivirus transduction provides high expression efficiency. Murine bone marrow-derived macrophages expressing nothing (Control), GFP, GFP-FMNL1β, or GFP-FMNL1γ through lentiviral transduction were quantified utilizing confocal fluorescent microscopy. The graph is representative of 4 separate experiments. The inset depicts a macrophage expressing evenly distributed GFP (left), and GFP-FMNL1γ which localizes to punctate podosomal structures (right). Scale bar is 20 μm. The inset image was taken using a 60 × objective. Total and green cells in each transduction group were counted at this magnification to derive expression efficiency since the punctuate distribution of GFP-FMNL1 is not visible at lower magnifications.
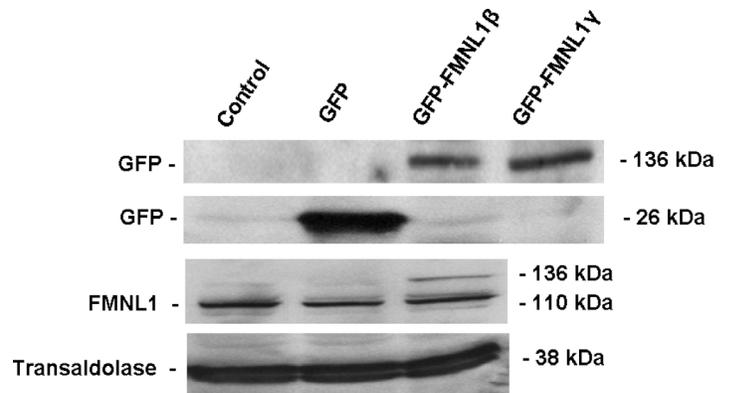

Figure 3. Lentiviral transduction of murine bone marrow-derived macrophages express exogenous protein high enough for Western blot detection. Western blot analysis of GFP and GFP tagged FMNL1 protein levels in BMDM lysates. Lysates were resolved on a 10% SDS polyacrylamide gel and then transferred to a PVDF membrane, incubated with mouse anti-GFP, followed by donkey anti-mouse HRP conjugate or rabbit anti-FMNL1 and goat anti-transaldolase, followed by corresponding HRP conjugate and exposure after treatment with ECL solution. Macrophages transduced with virus expressing GFP or GFP-FMNL1 isoforms show the select presence of each protein in full length form when lysates are blotted for the presence of GFP. Western blot analysis was also performed for FMNL1, where exogenous GFP-FMNL1β can be visualized at 136 kDa, above endogenous FMNL1 at 110 kDa. Transaldolase is shown as a loading control.

TROUBLESHOOTING

Table 1. Troubleshooting.

Step	Problem	Cause	Suggestion
1.4	Poor transfection of 293T-X cells	Poor DNA quality	EtOH precipitation of constructs
2.5	Low yield of bone marrow	Poor flushing of bones	Flush bones from both ends to ensure total harvest
2.16	Poor BMDM growth or differentiation	Low concentration of MCSF in media	Wait 10 to 14 days to collect L929 supernatant
			Do not use L929 supernatant older than 3 months
3.1, 4.2	Low yield of cells	Short time with EDTA solution	Ensure full 10 min of incubation at 4°C
		Poor scraping to lift cell	Scrape flasks thoroughly

References

1. Ezekowitz RA (1985) The role of growth factors and interferons in the induction of activated murine macrophages from bone marrow precursors. Immunol Lett 11: 135-140. PMID: 2417944

2. Rabellino EM, Ross GD, Trang HT, Williams N, Metcalf D (1978) Membrane receptors of mouse leukocytes. II. Sequential expression of membrane receptors and phagocytic capacity during leukocyte differentiation. J Exp Med 147: 434-445. PMID: 624905

3. Rupprecht AP, Coleman DL (1991) Transfection of adherent murine peritoneal macrophages with a reporter gene using DEAE-dextran. J Immunol Methods 144: 157-163. PMID: 1960412

4. Thompson CD, Frazier-Jessen MR, Rawat R, Nordan RP, Brown RT (1999) Evaluation of methods for transient transfection of a murine macrophage cell line, RAW 264.7. Biotechniques 27: 824-826. PMID: 10524325

5. Dokka S, Toledo D, Shi X, Ye J, Rojanasakul Y (2000) High-efficiency gene transfection of macrophages by lipoplexes. Int J Pharm 206: 97-104. PMID: 11058814

6. Kabanov AV, Kabanov VA (1995) DNA complexes with polycations for the delivery of genetic material into cells. Bioconjug Chem 6: 7-20. PMID: 7711106

7. Escher G, Hoang A, Georges S, Tchoua U, El-Osta A, et al. (2004) Demethylation using the epigenetic modifier, 5-azacytidine, increases the efficiency of transient transfection of macrophages. J Lipid Res 46: 356-365. doi: 10.1194/jlr.D400014-JLR200. PMID: 15520456

8. Van De Parre TJ, Martinet W, Schrijvers DM, Herman AG, De Meyer GRY (2005) mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages. Biochem Biophys Res Commun 327: 356-360. doi: 10.1016/j.bbrc.2004.12.027. PMID: 15629470

9. Vérollet C, Zhang YM, Le Cabec V, Mazzolini J, Charrière G, et al. (2010) HIV-1 Nef triggers macrophage fusion in a p61Hck- and protease-dependent manner. J Immunol 184: 7030-7039. doi: 10.4049/jimmunol.0903345. PMID: 20488787

10. Guiet R, Verollet C, Lamsoul I, Cougoule C, Poincloux R, et al. (2012) Macrophage mesenchymal migration requires podosome stabilization by filamin A. J.Biol.Chem 287: 13051-13062. doi: 10.1074/jbc.M111.307124. PMID: 22334688

11. Coelen RJ, Jose DG, May JT (1983) The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV. Arch Virol 75: 307-311. PMID: 6301409

12. O'Dohery U, Siggard WJ, Malim MH (2000) Human immundodeficiecny virus type 1 spinoculation enhances infection through virus binding. J. Virol 74: 100074-100080. PMID: 11024136

13. Cunnick J, Kaur P, Cho Y, Groffen J, Heisterkamp N (2006) Use of bone marrow-derived macrophages to model murine innate immune responses. J Immunol Methods 311: 96-105. doi: 10.1016/j.jim.2006.01.017. PMID: 16563426

14. Pan H, Mostoslavsky G, Eruslanov E, Kotton DN, Kramnik I (2008) Dual-promoter lentiviral system allows inducible expression of noxious proteins in macrophages. J.Immunol.Methods 329: 31-44. doi: 10.1016/j.jim.2007.09.009. PMID: 17967462

15. Harris ES, Li F, Higgs HN (2004) The mouse formins, FRLalpha, slows actin filament barbed end elongation, competes with capping protein, accelerates polymerization from monomers, and severs filaments. J.Biol.Chem 279: 20076-20087. doi: 10.1074/jbc.M312718200. PMID: 14990563

16. Harris ES, Rouiller I, Hanein D, Higgs HN (2006) Mechanistic differences in actin bundling activity of two mammalian formins, FRL1 and mDia2. J Biol Chem 281: 14383-14392. doi: 10.1074/jbc.M510923200. PMID: 16556604

17. Esue O, Harris ES, Higgs HN, Wirtz D (2008) The filamentous actin cross-linking/bundling activity of mammalian formins. J.Mol.Biol 384: 324-334. doi: 10.1016/j.jmb.2008.09.043. PMID: 18835565

18. Yayoshi-Yamamoto S, Taniuchi I, Watanabe T (2000) FRL, a novel formin-related protein, binds to Rac and regulates cell motility and survival of macrophages. Mol.Cell.Biol 20: 6872-6881. PMID: 10958683

19. Han Y, Eppinger E, Schuster IG, Weigand LU, Liang X, et al. (2009) Forminutes-like 1 (FMNL1) is regulated by N-terminal myristoylation and induces polarized membrane blebbing. J.Biol.Chem 284: 33409-33417. doi: 10.1074/jbc.M109.060699. PMID: 19815554