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Running Head:

"MCMV-induced type I IFN response"

Measurement of mouse cytomegalovirus induced interferon-β with immortalized luciferase reporter cells

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Summary

The production of cytokines is a crucial element of the host response to viral and bacterial infections. To follow these events *in vivo,* transgenic mice have become a valuable tool to study cytokine production through induction of reporter genes. We describe here the generation and immortalization of cells derived from transgenic reporter mice for the development of a high-throughput assay system for virus or bacteria induced cytokine induction. As an example we describe mouse cytomegalovirus (MCMV) infection of immortalized fibroblasts derived from mice expressing the firefly luciferase reporter downstream of the IFN-β promoter. Common methods to determine IFN-β production, including ELISA, quantitative real-time PCR (qPCR), and transient reporter assays using plasmid based reporter constructs, have disadvantages and limitations. Transient transfections influence type I IFN responses in most cell types, and IFN-β ELISA as well as qPCR are both laborious and expensive. The method presented here is highly sensitive as well as cost effective, and allows monitoring of a robust and dose-dependent induction of IFN-β upon virus infection in cell lysates as well as living cells.

Key words: type I interferon (IFN), IFN-β, mouse cytomegalovirus (MCMV), luciferase reporter assay, ear fibroblast, luminometer, immortalization, lentiviral transduction

1. Introduction

Reporter gene systems enable quantitative determination of the induction and expression of genes of interest. Technologies to detect these reporters have rapidly improved and assays to monitor luciferase activity or fluorescent protein expression are now standard methods in many laboratories. In accordance, recombinant mice equipped with reporter functions have become available in recent years which allow monitoring of gene induction *in vivo* or in *ex vivo* assays. Isolation and immortalization of cells obtained from such mice provide an easily expandable pool of reporter cells that can be used in *in vitro* systems to address a multitude of scientific questions.

We previously generated reporter mice to study analysis of IFN-β promoter induction upon infection *in vivo* ***(1)***. A myc-tagged firefly luciferase was integrated into the mouse genome by targeted mutagenesis to replace the coding sequence of the *ifnβ* gene, leaving the upstream region intact. This strategy ensures that reporter activity mimics the induction of IFN-β in an optimal fashion. The *ifnβ* wild type allele that is still present in cells of heterozygous reporter mice provides functional IFN-β and therefore a physiological response to viral infection.

We describe here the generation of conditionally immortalized cells derived from mice heterozygous for the luciferase transgene, and subsequent *in vitro* luciferase reporter assays. Primary ear fibroblasts were conditionally immortalized by lentiviral-mediated expression of the SV40 large T antigen (TAg) ***(2,3)*** and used for downstream analysis of IFN-β induction upon MCMV infection. We compare luciferase activity measured in cell lysates to luciferase activity measured in living cells. Both methods are robust, reproducible, highly sensitive and cost effective, and therefore versatile alternatives to methods such as ELISA and qPCR.

2. Materials

2.1 Cell culture general

Tissue culture-treated dishes (10 cm dishes, 6-well plates, 96-well plates, T25 flasks), 15 ml conical tubes, scalpels, cover slips (square 20x20m), cryovials, 70% ethanol, isopropanol, Dulbecco's Modified Eagle Medium (DMEM) with 4500 mg/ml glucose, cell freezing container, tissue culture centrifuge.

2.2 Preparation of lentiviruses in HEK 293T cells

1. HEK (human embryonic kidney) 293T cells (ATCC CRL-11268).
2. DMEM 2+: DMEM, 10% fetal bovine serum (FBS), 2 mM glutamine.
3. Phosphate buffered saline (PBS).
4. Trypsin/EDTA solution (trypsin).
5. Lentiviral helper plasmids e.g. ViraPower Lentiviral Expression System containing pLP1 (gag/pol), pLP2 (rev), pLP/VSVg (VSVg) (Invitrogen by LifeTechnologies K4975-00).
6. Lentiviral expression plasmid (pLV): uni-TAg or bi-TAg ***(3)*** (*see* **Note 1**).
7. For 293T transfection: 2.5 M $CaCl_2$ solution and HEBS solution: 280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with 1 N NaOH.
8. Luer Lock plastic syringe (10 ml), 0.45 µm syringe filter.

2.3 Lentiviral transduction, selection, and expansion of IFN-β luciferase reporter ear fibroblasts

1. DMEM 5+: DMEM, 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U penicillin/100 µg/ml streptomycin.
2. G418 (100 mg/ml stock) dissolved in PBS, store at -20°C.
3. Doxycycline hyclate (2 mg/ml stock) dissolved in 100% ethanol, store at -20°C.
4. Polybrene (Hexadimethrine bromide, 4 mg/ml stock) dissolved in H_2O, store at -20°C.

2.4 Maintenance and freezing of cells

1. DMEM 5+ Dox: DMEM 5+, 2 µg/ml doxycycline hyclate (for both uni-TAg and bi-TAg), and 0.4 mg/ml G418 (only for bi-TAg).
2. PBS, trypsin.
3. Freezing solution: final 90% FBS, 10% dimethyl sulfoxide (DMSO).

2.5 Infection with Murine cytomegalovirus (MCMV)

Purified MCMV (e.g. strain Smith, ATCC VR-1399), titered (*see* **Note 2**).

2.6 Luciferase assay of cell lysates

1. White well flat bottom tissue culture treated polystyrene 96-well plates (*see* **Note 3**).
2. Luciferase assay system (e.g. Promega E4530) containing cell lysis buffer, e.g. Reporter Lysis 5x Buffer, and reconstituted luciferase assay reagent.
3. 96-well plate luminometer (e.g. Promega GloMax 96 Microplate Luminometer with Dual Injectors, E6521).

2.7 Luciferase assay of living cells

1. White well flat, transparent bottom tissue culture treated polystyrene 96-well plates (*see* **Note 3**).

2. 200x D-luciferin Stock Solution: Dissolve 1 g D-luciferin potassium salt in 33.3 ml of PBS and sterile filter (0.2 µm). Aliquots can be stored at -20°C.
3. Sensitive light detecting device compatible with 96-well plates (e.g. IVIS®, PerkinElmer).

3. Methods

Murine cells can be efficiently immortalized by ectopic expression of the viral oncogene SV40 large T antigen (TAg). TAg inhibits the activity of the tumor suppressors p53 and retinoblastoma protein ***(4)***. Since constant activation of TAg leads to cellular alterations, we present a conditional immortalization method which is based on transcriptional regulation of TAg mediated by the tet-system ***(2)***. TAg expression is activated by the addition of doxycycline, which leads to cell proliferation. Here, we use continuously proliferating immortalized cells that are grown in the presence of doxycycline. Withdrawal of doxycycline from the cell culture medium (~ one week) leads to complete arrest of cell proliferation which might be advantageous in some assay systems. Cells can be kept in culture without doxycycline for at least two weeks.

3.1 Isolation of primary IFN-β luciferase reporter ear fibroblasts

1. Sacrifice mouse (e.g. IFN-β luciferase reporter mouse, **Fig. 1**).
2. Cut off ear, rinse in 70% ethanol, and transfer to one 10 cm dish with 5 ml PBS (*see* **Note 4**).
3. Cut ear in 40-50 small pieces with a scalpel.
4. Transfer 10 pieces into each well of a 6-well plate and cover with a cover slip to immobilize the ear pieces.
5. Add 2 ml of DMEM 5+ per well of a 6-well plate and allow outgrowth of the cells (*see* **Note 5**).
6. Renew medium every four to five days until >5 colonies (with more than 100 cells) have formed, and then carefully remove the coverslip with forceps.
7. Wash colonies twice with 2 ml PBS, detach with 0.5 ml trypsin for 3-5 min at 37°C, add 2 ml DMEM 5+, and transfer to a new well of a 6-well plate.
8. When cells have reached confluency, transfer to a T25 flask as described in **Subheading 3.1., item 7** (with a final volume of 5 ml).
9. For long term storage the primary cells can be frozen. One T25 flask gives one cryovial. Detach cells from a confluent T25 flask (wash with PBS, add 1 ml of trypsin for 3-5 min at 37°C, add 5 ml DMEM 5+), transfer to 15 ml conical tube and centrifuge at 200xg for 5 min. Remove medium and resuspend pellet in 0.5 ml FBS. Slowly and dropwise add 0.5 ml FBS with 20% DMSO to have a freezing solution with a final concentration of 90% FBS and 10% DMSO. Mix cell suspension gently after addition of each drop. Transfer to cryovials and put vials in cell freezing

container. Put container in -70°C freezer overnight and transfer vials to liquid nitrogen storage the next day.

3.2 Production of SV40 large T antigen expressing lentivirus in 293T cells

The TAg expression cassette is incorporated into a third generation lentiviral vector system which has an improved safety profile due to deletion of most of the viral sequences. Lentiviral transduction allows high immortalization efficiency, as well as transduction and immortalization of different cell types (*see* **Note 6**).

1. Plate HEK293T at a density of 40 000 cells/cm^2 (day 0) in DMEM 2+.
2. On day 1 transfect 293T cells with the three helper plasmids (pLP1, pLP2, pLP/VSVg) and either UniTAg or biTAg (pLV) by calcium phosphate transfection. The following amounts and/or volumes are given for lentiviral production in a 10 cm dish (adjust the amounts accordingly if using other cell culture plates): combine the plasmids (pLV 8 µg, pLP1 5.6 µg, pLP2 1.8 µg, pLP/VSVg 3 µg) with 15 µl 2.5 M $CaCl_2$ and use H_2O to adjust to a final volume of 150 µl. Add the DNA solution dropwise to 150 µl HEBS under vigorous vortexing and incubate at room temperature for 10 min before addition to the cells.
3. On day 2 aspirate medium and add 6 ml of DMEM 2+ (0.1 ml/cm^2).
4. Allow lentivirus production to proceed until day 3.
5. On day 3 collect supernatant containing lentiviral particles. Add fresh medium to cells.
6. Filter harvested supernatant through 0.45 µm pore size filter and store in aliquots at -70°C until use.
7. On day 4 collect supernatant containing lentiviral particles. Filter and store as described in **Subheading 3.2., item 6**.

3.3 Transduction of primary ear fibroblasts with SV40 large T antigen expressing lentivirus

1. For establishment of an immortalized cell line, primary cells can be used directly after isolation (see 3.1) or can be thawed (*see* **Subheading 3.5**).
2. For lentiviral transduction plate $1x10^5$ of primary ear fibroblasts per well of a 6-well plate (day 0) in DMEM 5+ (2 ml/well) (*see* **Note 6**).
3. On day 1 remove medium and add 1 ml undiluted supernatant containing lentiviral particles (*see* **Subheading 3.2**) expressing either uni-TAg or bi-TAg in the presence of 8 µg/ml polybrene and incubate overnight.
4. On day 2 aspirate lentiviral-containing medium and add 2 ml DMEM 5+ Dox.

5. On day 4 or after the cells reach confluence, passage transduced cells with a ratio of 1:3 (wash cells twice with 2 ml PBS, trypsinize with 0.5 ml trypsin for 3-5 min at 37°C, resuspend cells in DMEM 5+ Dox).
6. Plate cells in desired cell culture plate in DMEM 5+ Dox and either select with 0.4 mg/ml G418 (bi-TAg) or by growth advantage (uni-TAg) (*see* **Note 7**).

3.4 Cell maintenance

1. For routine maintenance cells are cultivated with DMEM 5+ Dox (*see* **Note 8**).
2. Passage cells when confluent as follows: wash cells once with 0.2 ml/cm^2 PBS and detach with 0.05 ml/cm^2 trypsin. Gently tilt the plate to distribute trypsin equally and incubate at 37°C, 7.5% CO_2 for 3-5 minutes. Add 0.2 ml/cm^2 DMEM 5+ Dox and detach cells with vigorous pipetting or a cell scraper. Pipette up and down to minimize clumps.
3. In the first four weeks after infection the cells should be passaged with a ratio of 1:3. At later time points the ratio can be adjusted to 1:5 or 1:10.
4. Cells can be frozen as described in **Subheading 3.1**.

3.5 Thawing ear fibroblasts

1. Remove vial of frozen cells from liquid nitrogen and transfer to a 37°C water bath.
2. After thawing, transfer the solution into a 15 ml conical tube and add 9 ml pre-warmed medium dropwise (DMEM 2+ for primary fibroblasts and DMEM 5+ Dox for immortalized fibroblasts).
3. Mix gently and centrifuge at 200xg for 5 minutes at room temperature.
4. Aspirate supernatant, resuspend the pellet in 10 ml of pre-warmed DMEM 2+ or DMEM 5+ Dox, and transfer to a 10 cm tissue culture dish.
5. Check cells the next day and change medium if necessary.
6. When cells are confluent split 1:3 or 1:5. Cells will grow more quickly after complete recovery and can be split 1:10.

3.6 Infection of immortalized ear fibroblasts with MCMV

The luciferase assay is designed for a 96-well plate format to allow high-throughput analysis. The experimental workflow is illustrated in **Fig. 2**.

1. One day prior to infection, seed 6.25x10^3 cells/well in 75 µl of DMEM 5+ Dox in a 96-well tissue-culture treated plate. For subsequent detection of luciferase in living cells, use white well transparent bottom 96-well plates (*see* **Note 3**). Cells should be ~80% confluent the next day for infection.
2. Dilute MCMV in DMEM 5+ Dox to infect with the desired multiplicity of infection (MOI) (*see* **Note 9**). Prepare enough virus suspension to be able to add 75 µl per well.
3. Add 75 µl virus suspension directly to the 75 µl medium on the cells.
4. Spin-infect for 1 hour at 800xg at 4°C (*see* **Note 10**).

5. Carefully remove virus-containing supernatant without touching the cell layer. Add 150 µl of pre-warmed DMEM 5+ Dox.
6. Proceed directly to **Subheading 3.7** or **3.8** (for a 0 h time point measurement) or incubate at 37°C and 7.5% CO_2 for the desired time, before proceeding to **Subheading 3.7** or **3.8** (*see* **Note 11**).

3.7 Luciferase assay with cell lysates

1. To make 1x Reporter Lysis Buffer (RLB) add 4 volumes of deionized water to 1 volume of 5x RLB. Equilibrate to RT before use. 50 µl/well of 1x buffer are needed.
2. Carefully remove supernatant and wash cells once with PBS. Then add 50 µl of 1x RLB to each well and incubate at RT for 10 minutes (*see* **Note 12**). Perform a freeze-thaw cycle to ensure complete lysis (*see* **Note 13**).
3. Thaw Luciferase Assay Reagent and equilibrate to room temperature (you will need 100 µl of reagent/sample) (*see* **Note 14**).
4. Transfer 20 µl of each cell lysate to individual wells of a white 96-well plate. Analyze light production according to the manufacturer's instructions. For the Promega Glomax 96 with Dual Injectors, we have utilized automatic injection of 100 µl reconstituted luciferase assay reagent followed by a 2 second measurement delay and a 10 second measurement read (**Fig. 3**, left panel). The values given are relative light units (RLU).

3.8 Luciferase assay in living cells

This assay variation does not require cell lysis and therefore allows further downstream analysis such as immunofluorescence or secondary stimulations. In contrast to cell lysates, living cells provide necessary cofactors for the luciferase reaction, allowing the use of a cost effective substrate solution rather than a complex and expensive assay reagent as required for **Subheading 3.7**.

1. Using cell culture medium, prepare a 4x luciferin working dilution (600 µg/ml) from the 200x (30 mg/ml) D-luciferin stock (*see* **Subheading 2.7**). Equilibrate to 37°C.
2. Add 50 µl of the 4x working dilution to each well containing cells (with 150 µl of cell culture medium) to yield a final concentration of 150 µg/ml luciferin.
3. Incubate cells for 5 min at 37°C.
4. Read the light emission of the plate using high sensitivity settings on the reader (*see* **Note 15**). For the IVIS®, we used the following settings: 4 min integration, binning 16, f/stop 1 (**Fig. 3**, right panel). Using the unit average radiance (p/sec/cm2/sr) allows comparison of the values to readings that are acquired with other settings.

9

Notes

1. Plasmids uni-TAg and bi-TAg are available upon request from Tobias May. The uni-TAg plasmid harbors a unidirectional Tet-dependent promoter that drives the expression of TAg and a reverse transactivator (tet-on). The bi-TAg plasmid is based on the bidirectional Tet-dependent promoter driving two mRNAs, one encoding for TAg and the other bicistronic mRNA for the reverse transactivator and a fusion gene comprised of eGFP and Neomycin phosphotransferase. The plasmids can be propagated using standard bacterial strains/techniques.
2. MCMV was purified as described in ***(5)*** with slight modifications: a) virus was harvested from cell culture supernatant and not from intact cells and b) virus was purified through a 10% Nycodenz cushion instead of a sucrose cushion. Work with MCMV requires biosafety level 1.
3. For detection of luminescent signals white plates are advantageous due to their reflective properties. In addition, scattering of light emitted from one well to neighboring wells is reduced. The use of white well plates with transparent bottoms allows microscopic monitoring of cell growth and viability throughout the experiment. A white piece of paper beneath the plate during measurement increases the signal by reflecting the emitted light to the detector, almost as good as a white bottom.
4. Virus and cell preparation, infections, and stimulations are performed under sterile conditions in a laminar flow hood (*see* **Note 6**). Cells are incubated at 37°C in a humidified atmosphere with 7.5% CO_2.
5. To avoid or reduce the risk of bacterial contamination we recommend adding gentamycin to the primary cells for the first two weeks in culture (a final concentration of 500 µg/ml is obtained from a 1:200 dilution of a 10 mg/ml stock solution).
6. For the production and use of recombinant lentiviruses (especially for lentiviruses encoding immortalization genes) biosafety level 2 is required. Lentiviral safety guidelines for the applicable country must be followed.
7. Selection of bi-TAg transduced fibroblasts with G418 usually requires 7-10 days. Selection of uni-TAg transduced fibroblasts by growth advantage requires several subcultivation steps and typically 3-4 weeks to lose untransduced primary cells. Alternatively, cells can be selected by FACS sorting of GFP-positive cells (bi-TAg).
8. The established immortalized fibroblast cell line is cultivated with doxycycline (2 µg/ml) to activate the immortalizing SV40 large T antigen. This activation induces proliferation of the cell

line. Without doxycycline the SV40 large T antigen becomes inactivated which leads to growth arrest. The cells stay alive in this state for at least fourteen days.

9. The day after seeding cells have approximately doubled and cell numbers are about $1.25x10^4$ cells/well. To confirm the cell number at the time of infection, trypsinize one well and count cells. To make a virus suspension of an MOI of 0.1 from a given virus stock with a titer of 10^7 PFU/ml, dilute 0.125 µl of the virus stock in 75 µl medium (MOI x cell number divided by PFU/ml). Prepare enough virus suspension to be able to add 75 µl per well. If less volume is added to cells, they will dry out during the following centrifugation step.
10. Viral attachment to cells is highly enhanced by centrifugation. Centrifugation is performed at 4°C to avoid virus entry into the cells. Entry will be synchronized upon warming.
11. Prepare separate plates for each time point. This allows freezing of the entire plate to complete cell lysis.
12. Although only 20 µl are needed for analysis, 50 µl of lysis buffer are added to allow an additional luciferase assay or further downstream analysis such as immunoblotting.
13. Reporter lysis buffer requires a single freeze-thaw cycle to fully lyse cells. Cells in 1x RLB are frozen once at -20°C and thawed either at RT or 37°C to ensure complete cell lysis.
14. Leftover luciferase assay reagent (LAR) can be frozen at -80°C and mixed with frshly prepared LAR for subsequent measurements. Repeated freeze-thaw cycles will eventually reduce efficacy.
15. White plates show phosphorescence below 560 nm. To avoid interference of this signal with the luciferase signal, a 620 nm emission filter can be selected on the IVIS® system. Sensitivity can be enhanced by putting a reflective plate (e.g. white paper) underneath the 96-well plate while reading in the IVIS®.

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Figure legends

Fig. 1: Scheme of the targeted gene locus of the IFN-β reporter mice. After homologous recombination with the targeting construct, the *ifnβ* locus contains a myc-tagged luciferase gene allowing visualization of virus-induced IFN-β expression ***(1)***. To maintain IFN-β production, one wildtype (wt)

allele is preserved in the mice/cells. In the reporter allele the 5' untranslated region of the IFN-β message is interrupted by a loxP site, which remains from the targeting strategy. Except for this alteration, the sequence preceding the reporter coding sequence is identical to the sequence of the wt locus to avoid interference with regulatory elements driving IFN-β production. The luciferase sequence is derived from pGL3 (Promega), a vector commonly used in reporter gene assays. To facilitate the detection of IFN-β induction in other assays, an oligonucleotide coding for a myc tag was inserted in front of the luciferase coding sequence. The myc-luciferase sequence is followed by an SV40 poly(A) site originating from pGL3. The sequence displayed in the lower panel of the figure gives the reporter cassette in the *ifnβ* locus starting from the TATA-box, including the loxP site and a part of the coding sequence for the N-terminally myc-tagged luciferase. The genetic background of the embryonic stem cells as well as the mice used to obtain the reporter cells is C57BL/6.

Fig. 2: Luciferase assay experimental setup. $6.25x10^3$ cells/well of immortalised ear fibroblasts derived from IFN-β reporter mice are plated the day before infection in 75 µl of DMEM 5+ Dox in a 96-well plate. Cells are incubated overnight at 37°C and then infected with MCMV at different MOI or left uninfected. For the luciferase assay of cell lysates, cells are lysed in reporter lysis buffer at different time points post infection and luciferase activity is analysed in a standard luminometer. To assess luciferase activity in living cells luciferase substrate is directly added to cells without prior cell lysis, and luciferase activity is measured in the IVIS® system.

Fig. 3: Kinetics of IFN-β induction in immortalised ear fibroblasts upon MCMV infection. Immortalised ear fibroblasts derived from IFN-β luciferase reporter mice were infected with MCMV at different MOI as indicated in the figures or left uninfected and incubated for 0, 4, 8, and 24 hours. Left panel: Luciferase activity in cell lysates. After incubation, cells were lysed in reporter lysis buffer and luciferase activity was determined in a standard luminometer using a commercial luciferase assay system. The graph shows one representative experiment with samples analyzed in triplicate. Right panel: Luciferase activity of living cells. In a parallel experiment, instead of lysing the cells, luciferin was added directly to living cells and light emission was assessed in an IVIS® system. Both readout

systems are very well suited to follow MCMV-induced IFN-β expression and show high sensitivity and reproducibility.

13

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