Please cite this article as: Rommereim, L. *et al.* (2021). Lentivirus-mediated Conditional Gene Expression. *Bio-protocol* 11(21): e4205. DOI: 10.21769/BioProtoc.4205.

bio-protocol www.bio-protocol.org/e4205 Bio-protocol 11(21): e4205. DOI:10.21769/BioProtoc.4205

Lentivirus-mediated Conditional Gene Expression

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[Abstract] The ability to identify the role of a particular gene within a system is dependent on control of the expression of that gene. In this protocol, we describe a method for stable, conditional expression of Nod-Like receptors (NLRs) in THP-1 cells using a lentiviral expression system. This system combines all the necessary components for tetracycline-inducible gene expression in a single lentivector with constitutive co-expression of a selection marker, which is an efficient means for controlling gene expression using a single viral infection of cells. This is done in a third generation lentiviral expression platform that improves the safety of lentiviruses and allows for greater gene expression than previous lentiviral platforms. The lentiviral expression plasmid is first engineered to contain the gene of interest driven by a TRE (tetracycline response element) promoter in a simple gateway cloning step and is then co-transfected into HEK293T cells, along with packaging and envelope plasmids to generate the virus. The virus is used to infect a cell type of interest at a low MOI so that the majority of the transduced cells contain a single viral integration. Infected cells are grown under selection, and viral integration is validated by qPCR. Gene expression in stably transduced cells is induced with doxycycline and validated by qPCR, immunoblot, and flow cytometry. This flexible lentiviral expression platform may be used for stable and robust induction of a gene of interest in a range of cells for multiple applications.

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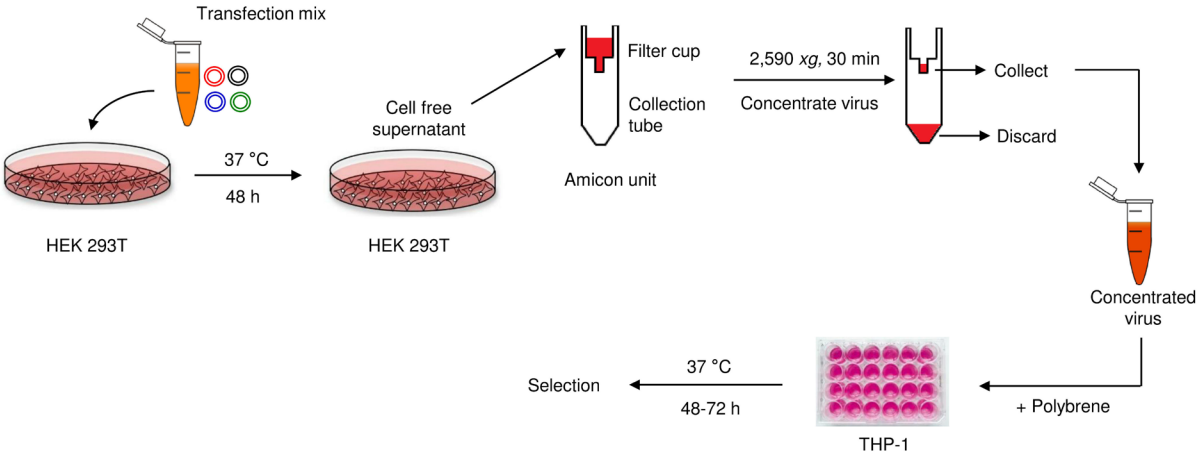
Graphic abstract:



Schematic overview of lentiviral transduction of THP-1 cells.

Keywords: NLR, NOD1, Lentivirus, Stable expression, TRE, Tetracycline

[Background] A crucial technique for studying functional responses in molecular biology is the ability to regulate the expression of a gene. Constitutive gene expression systems have allowed researchers to explore gene responses only if prolonged overexpression is non-toxic. Such systems can also lead to the development of compensatory mechanisms within a cell that can mask functional phenotypes (El-Brolosy *et al.*, 2019). Inducible expression systems are a favored alternative to constitutive expression, giving researchers the ability to switch genes on and off or titrate the level of gene expression, with fewer side effects and greater efficiency. One advantage of such conditional expression is the ability to regulate gene expression in a quantitative, temporally-regulated, and reversible manner that more accurately reveals the direct result of a given genetic change. The tetracycline-controlled operator system containing the TRE promoter provides reversible gene expression with a large induction range at low concentrations of a drug that is non-toxic to mammalian cells. The TRE promoter contains a modified Tet response element consisting of seven repeats of a 36-nt sequence that contains the 19-bp Tet operator sequence (TCCCTATCAGTGATAGAGA). This Tet response element is positioned upstream of a minimal CMV promoter sequence, which lacks the enhancer present in the native CMV promoter. Consequently, the TRE promoter is silent in the absence of binding of the tetracycline responsive rtTA3 regulatory protein. Advances in the system have optimized the reverse Tet transactivator (rtTA) component for improved drug sensitivity and activity and, more importantly, to show no activity in the absence of tetracycline or doxycycline (DOX, a tetracycline analog), thus reducing the leakage of the system (Zhou *et al.*, 2006; Yamada *et al.*, 2018). Nevertheless, it should be noted that tetracycline-derived contaminants are often present in cell culture sera; this poses a challenge for Tet-based systems, but this issue can be avoided by using tetracycline-free serum.

Lentiviruses provide an efficient vehicle for facilitating the establishment of inducible gene expression within a cell, particularly because they are able to infect many different dividing and non-dividing cell

types (Naldini *et al.*, 1996; Reiser *et al.*, 1996), making lentiviral systems an essential tool for introducing exogenous genes into difficult to transduce targets such as primary cells. Although lentiviral platforms do pose the risk of endogenous gene disruption, the system described here packages all components into a single vector platform so that only a single viral infection per cell is necessary for tet-inducible gene expression (Figure 1A). This eliminates the need for multiple viral infections and limits the possible number of random genome integrations, thereby limiting the chance of off-target effects on cell physiology (Connolly *et al.*, 2002). The constitutively expressed Neo/Venus transduction marker contained in the system allows for easy enrichment of the transduced cell population and can also be easily replaced with another selection marker (*e.g.*, any antibiotic or cell surface marker) to optimize the system for a range of cell types and applications. This not only makes the system highly tractable, but the development of third generation lentiviral vectors, such as pSLIK (Shin *et al.*, 2006), which separate the minimal genetic elements of HIV into three plasmids (pMDL containing gag and pol, pRSV containing the rev protein, and pVSV containing the envelope protein), increases the safety of lentiviruses and decreases the need for highly specialized training (Barde *et al.*, 2010). While this means that the lentivector system described here requires the transfection of four separate plasmids to generate functional lentiviral particles, this design substantially increases its safety for common laboratory use over second generation platforms that expressed the Gag, Pol, Rev, and Tat genes from a single packaging plasmid. Importantly, third generation lentiviral platforms are self-inactivating in nature due to a deletion in the long terminal repeat (LTR) region that results in the loss of the pro-viral enhancer sequence upon integration. This further improves biosafety because replication-competent viruses are not generated within the target cells (Li *et al.*, 2005). Finally, our lentiviral system utilizes a hybrid 5'LTR fused to a CMV promoter that increases gene expression. This is in contrast to second generation systems, which relied on a weak viral 5' LTR and required the presence of Tat to activate gene expression. Overall, the third generation lentiviral platform described in this protocol ensures robust gene expression from a single infection of cells and increases the laboratory safety of lentiviruses.

Combining inducible expression with such a flexible lentiviral vector platform allows for tetracycline-regulated gene expression from a minimal viral infection of a wide range of cell targets (Shin *et al.*, 2006). We utilized the Tet-on configuration of such a system in our recent paper (Rommereim *et al.*, 2020) to express Nod-like receptors in THP-1 cells and provide the detailed protocol here.

Materials and Reagents

A. Consumables and reagents
   1. 10× PBS (Gibco, catalog number: 70011044), store at 4°C
   2. 293T culture dishes; 100 × 15 mm TC-treated Petri dishes (Corning Falcon, catalog number: 353003)
   3. Amicon Ultra-15 Centrifugal Filter Units (Millipore, catalog number: UFC903024)
   4. DMEM (Gibco, catalog number: 11995-065), store at 4°C
   5. FBS (Gibco, catalog number: A31605), store at -20°C

6. G418, Geneticin (Invivogen, catalog number: ant-gn-1), store at 4°C
7. Glutamine (Gibco, catalog number: 25030149), store at 4°C
8. HEPES (Gibco, catalog number: 15630-080), store at 4°C
9. Lipofectamine 2000 (Invitrogen, catalog number: 52887), store at 4°C
10. Opti-MEM (Gibco, catalog number: 51985-034), store at 4°C
11. Penicillin G (Sigma, catalog number: P3032), store at 4°C
12. Polybrene (Sigma, catalog number: H9268)
13. Poly-L-Lysine Hydrobromide (Sigma, catalog number: P1274), store at 4°C
14. RPMI (Gibco, catalog number: 11875-101), store at 4°C
15. Sodium Pyruvate (Gibco, catalog number: 11360-070), store at 4°C
16. Valmark Ultra-Dish Petri dishes 100 mm × 15 mm (Midwest Scientific, catalog number: 900)

B. Plasmids

1. Entry Vector pEN_TmiRc3 (Addgene Plasmid #25748)
   Entry vector for cloning a gene of interest, such that its expression is driven by a TRE promoter.
2. Lentiviral expression vector pSLIK_Neo (Addgene Plasmid #25735)
   3rd generation lentiviral expression vector that allows for inducible Tet-based gene expression and contains a constitutive neomycin resistance cassette.
3. pMDL (Addgene Plasmid #12251)
   3rd generation lentiviral packaging plasmid that contains gag and pol.
4. pRSV (Addgene Plasmid #12253)
   3rd generation lentiviral packaging plasmid that contains rev.
5. pVSV (Addgene Plasmid #138479)
   Lentiviral packaging plasmid that contains the VSV envelope protein.

C. Cell lines and media

1. HEK293T cells (ATCC, catalog number: CRL-3216)
2. THP-1 cells (ATCC, catalog number: TIB-202)
3. HEK293T Growth Media (see Recipes)
4. THP-1 Growth Media (see Recipes)

D. Primers

See Table 1

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Table 1. List of primers and probes used for evaluating the expression of endogenous and 3X-FLAG tagged NOD1 and NLRP2 by qPCR

Gene Name/Product	5'-3' Sequence	Type
FLAG-NOD1 Exon 3	ATCGATTACAAGGATGACGATGAC	Forward Primer
	GGGTGAGACTCTGATGGGATTATT	Reverse Primer
NOD1 Exon 2	GATGGCAAGAGGTGGAGATTG	Forward Primer
	TTCCCATAAAAACAGCAACTTGTCT	Reverse Primer
FLAG-NLRP2 Exon 1	ATCGATTACAAGGATGACGATGAC	Forward Primer
	CCAGGAGAGCCTGCAGGTT	Reverse Primer
NLRP2 Exon 14 3'-UTR	CTCCATGAAGTCATCGATTTTCC	Forward Primer
	ACATCTAGCCCAGCAATGAACTC	Reverse Primer

Equipment

1. Centrifuge (Eppendorf, model: 5810R, catalog number: 022625004)
2. DNA Spectrophotometer (Nanodrop, model: ND-1000, catalog number: THERMO-ND1000)
3. Flow Cytometer (Becton Dickinson, model: BD FACSCalibur™)
4. Gel Imaging System (Bio-Rad, model: ChemiDoc XRS+, catalog number: 1708265)
5. Protein gel tank (Invitrogen, model: Mini gel tank, catalog number: A25977)
6. Real-time PCR system (Applied Biosystems, model: Quant Studio 6 Flex, catalog number: 4485691)
7. Thermal Cycler (Bio-Rad, model: T100, catalog number:1861096)
8. Transfer apparatus (Bio-Rad, model: Trans-blot SD Semi-Dry Transfer Cell, catalog number: 1703940)

Software

1. GraphPad Prism Version 9

Procedure

A. Prepare plates with Poly-L-Lysine

Note: Poly-L-Lysine enhances cell adhesion, decreasing the probability of HEK293T cells detaching from the plate during transfection.

1. Dilute 20 mg/ml Poly-L-lysine (PLL) to 20 µg/ml PLL in 1× PBS by combining 50 µl of PLL (20 mg/ml) with 5 ml of 10× PBS and 45 ml of ddH_2O.
2. Add 4 ml of diluted PLL (20 µg/ml) to a 10 cm 293T culture dish.
3. Incubate for 1 h at 37°C.
4. Aspirate PLL solution and wash three times with 5 ml of 1× PBS. To store dishes, rinse three times with sterile ddH_2O and air dry plates in a sterile tissue culture hood at room temperature.

B. Passage HEK293T cells

1. Aspirate media from a T75 flask of HEK293T cells at 90% confluency. One T75 flask should contain enough cells to plate three 10 cm dishes at 4.5 × 10^6 cells per dish.
2. Wash flask with 5 ml of 2 mM EDTA PBS.
3. Add 5 ml of warm Trypsin-EDTA and gently rock the flask to detach cells.
4. Collect cells in a 15 ml conical tube and add 5 ml of complete media to inhibit trypsin.
5. Spin at 300 × *g* for 5 min.
6. Aspirate media and flick the pellet to loosen cells, resuspending in 10 ml of media and count cells.
7. Plate 4.5 × 10^6 cells per PLL-coated 10 cm dish; cells should quickly adhere to the dish.

C. Transfect Cells

1. Change media on cells with 10 ml of regular growth media per plate 1 h prior to transfection.
2. Warm Opti-MEM (OM) at 37°C.
3. Dilute DNA constructs in OM to a total of 1.5 ml at the amounts listed in Table 2.

Table 2. Amount of DNA constructs required for lentiviral production

Plasmid	DNA (µg)
pMDL	7.5
pRSV	7.5
pVSV	5
Expression Plasmid (Figure 1A)	10

4. Dilute 60 µl of Lipofectamine 2000 (LF2000) with 1.44 ml OM. Let stand at room temperature for 5 min.
5. Add the 1.5 ml solution of LF2000/OM to the diluted DNA and let stand for 20 min at room temperature.
6. Carefully add 3 ml of DNA/LF2000/OM to the 10 ml media in the plate, one drop at a time.
7. Incubate overnight (12-18 h) at 37°C.
8. Aspirate media and replace with 10 ml of warm culture media per plate.
9. Incubate for an additional 36 h.

D. Virus Collection

1. Transfer media from 10 cm 293T culture plate to a 15 ml conical tube.
2. Centrifuge supernatant for 5 min at 500 × *g* at 4°C.
3. Filter supernatant through a 0.45 µm non-pyrogenic filter.
4. Store supernatant at 4°C while the Amicon unit is prepared.
5. Sterilize the Amicon unit by adding 15 ml of 70% ethanol to the filter cup. Let it sit for 10 min.

6. Centrifuge the Amicon unit for 15 min at 2,590 × *g*.
7. Discard ethanol from the collection tube.
8. Add 15 ml of sterile water to the filter cup. Let it sit for 5 min.
9. Centrifuge the Amicon unit for 15 min at 2,590 × *g*. Discard water from the collection tube.
10. Add the viral supernatant to the Amicon unit.
11. Centrifuge for 30 min at 2,590 × *g* at 4°C. The supernatant should completely pass through the filter, with none remaining in the filter cup portion.
    Alternative: If Amicon unit is unavailable, aliquot the supernatant from Step D4 into a 50 ml conical tube and set up an overnight centrifugation. Spin with labels facing out to know where to look for the pellet. Spin with slow acceleration (4) and normal braking (9) at 8,000 × *g* for 12 to 18 h at 4°C.
12. Add 1 ml of RPMI to the filter cup. Collect the viscous concentrated virus and store at 4°C.
    Alternative: If concentrating the virus by centrifugation, aspirate the supernatant. Resuspend pellet in 1 ml of RPMI and store at 4°C.

E. Lentivirus titration in 293T cells

Notes:

a. To achieve optimal lentiviral infection of target cells, it is important to use the correct ratio of cells to infectious viral particles. This ratio is also known as the multiplicity of infection (MOI). The number of viable infectious viral particles in the concentrated lentivirus stock is determined from a viral titration experiment. Viral infection can also be scored by flow cytometry to detect the presence of a fluorescent protein co-expressed in the lentiviral construct or staining for a gene encoded by the lentivector. While we describe the use of the pSLIK-Neo viral backbone, which co-expresses a Neomycin resistance gene, a similar pSLIK parental plasmid, which co-expresses the Venus yellow fluorescent protein (Addgene# 25734), is available.

b. The infectious viral titer is determined in the unconcentrated culture supernatant collected from the packaging cells, the concentrated viral stock, and in the concentrated viral stock following a freeze-thaw cycle. Comparison of the titer of these different preparations aids in the identification of sources of viral loss, which can be an important factor to control.

1. Plate 293T cells harvested from log phase cultures in 12-well tissue culture plates at a concentration of 1.44 × 10^5 cells/well and incubate overnight. Visually confirm that the 293T cells are healthy, evenly distributed, and at 40-50% confluence at the time of infection.
2. Aspirate medium from 293T cells and add 0.9 ml of pre-warmed 293T growth medium containing 8 µg/ml polybrene. Return plates to incubator.
3. Dilute viral solutions in 293T growth medium containing 8 µg/ml polybrene. A final volume of 100 µl is needed for each dilution, and each sample will be diluted another 10-fold when added to the cells. A set of serial dilutions (usually three) over the range 10-10^3 is tested for unconcentrated virus, and a range of 10^2-10^5 is tested for concentrated virus.

4. Add 100 µl of each virus dilution to wells of 293T cells (in 0.9 ml of media). To serve as matched uninfected controls, add 100 µl of 293T growth media/polybrene that does not contain virus to several wells. Incubate cells for 24 h.
5. After 24 h, replace the media with fresh 293T growth medium and return plates to the incubator.
6. Harvest cells for flow cytometric analysis 48-72 h after infection. Pool the media supernatant, washes, and dislodged cells from each sample well into FACS tubes to ensure that all cells are included.
7. Pellet cells by centrifugation (300 × *g*, 5 min, 4°C).
8. Determine the percentage of Venus-positive cells by flow cytometric analysis by comparison of infected samples with uninfected 293T cells. Identify samples in which the infection rate is 3-10% and calculate the number of cells infected on the day of infection. This is approximately equivalent to the number of infectious virus particles added per well, assuming a 1:1 infection ratio. The number of active viruses detected is used with the volume and dilution of virus stock to calculate the virus concentration in the stock solution. This provides the lentiviral titer with respect to 293T cell infection.

   Lentiviral titer = number of infected cells × volume of virus stock × dilution of virus stock

   Notes:

   a. *The titer for the user's target cells of interest can be calculated by side-by-side infection of such target cells, with 293T cells using identical cell numbers and viral quantity. Many cell types may infect less efficiently than 293T cells, but we have found the relative infection ratio between cells to be quite consistent. Thus, if the user's target cells typically show 50% of the 293T infection level, this ratio can be assumed for future viral preparations.*
   b. *Similarly, we have found side-by-side comparison of the pSLIK-Venus and pSLIK-Neo parental plasmids to yield very similar viral titers. Thus, titration of a pSLIK-Venus control alongside pSLIK-Neo based viruses should yield comparable titers.*

   Our average viral titer from this protocol is 1.175×10^7 viral particles from 1 plate of 293T cells. Although each lab should carry out several viral titrations to determine the level and consistency of their titers (Kutner *et al*., 2009), we use this average value for THP-1 cell infections and only run titrations if we find our infection rates drop unexpectedly.

F. THP-1 Cell Transduction

1. Calculate viral particles per milliliter by dividing 1.175×10^7 by the total volume of concentrated virus recovered per plate.
2. Calculate the volume of virus needed to infect cells at 10 MOI by dividing 5.0×10^6 by the concentration of viral particles per ml. We typically infect 0.5×10^6 THP-1 cells with an approximate MOI of 10 (293T transduction units).

   Volume of virus (ml) = (number of cells per well × MOI)/viral particles per ml, *i.e*.

   $(0.5 \times 10^6 \times 10)$/viral particles per ml.

   This equates to an MOI of <1 for THP-1 cells, usually around 30% transduction efficiency [where

transduction efficiency (%) = number of transduced cells/total number of cells]. Transduction efficiency can be measured by FACS to count the number of THP-1 cells that are positive for expression of the lentivirus-encoded transgene or a constitutively expressed Venus transduction marker as a fraction of the total number of cells. We aim for this relatively low infectivity to ensure that most cells do not have multiple viral integrations.

3. Plate 0.5 × 10^6 cells per well into two wells of a 24-well dish.
4. Infect cells 0.5-3 h after seeding.
5. Infect one well by diluting virus up to 500 µl with serum-free growth medium supplemented with 4 µg/ml polybrene and 100 U/ml penicillin G.
6. Aspirate media and replace it with diluted virus.
7. After 4 h, add 1.5 ml regular growth media with penicillin.
8. The next day, split cells and add 1 × 10^6 cells into Valmark dishes.
9. One to two days after splitting, select for cells by growing in complete media supplemented with G418 (1 mg/ml).

G. Gene Expression Validation in THP-1 Cells

1. Plate cells in a 6-well plate and incubate at 37°C for 6 h.
2. Add DOX to the cells (1 µg/ml) and incubate for 6-18 h.
3. Harvest cells and isolate RNA or protein.
4. Validate viral integration by qPCR (Figure 1B) and DOX-induced overexpression by qPCR, immunoblot, or flow cytometry (Figure 1C-1E).

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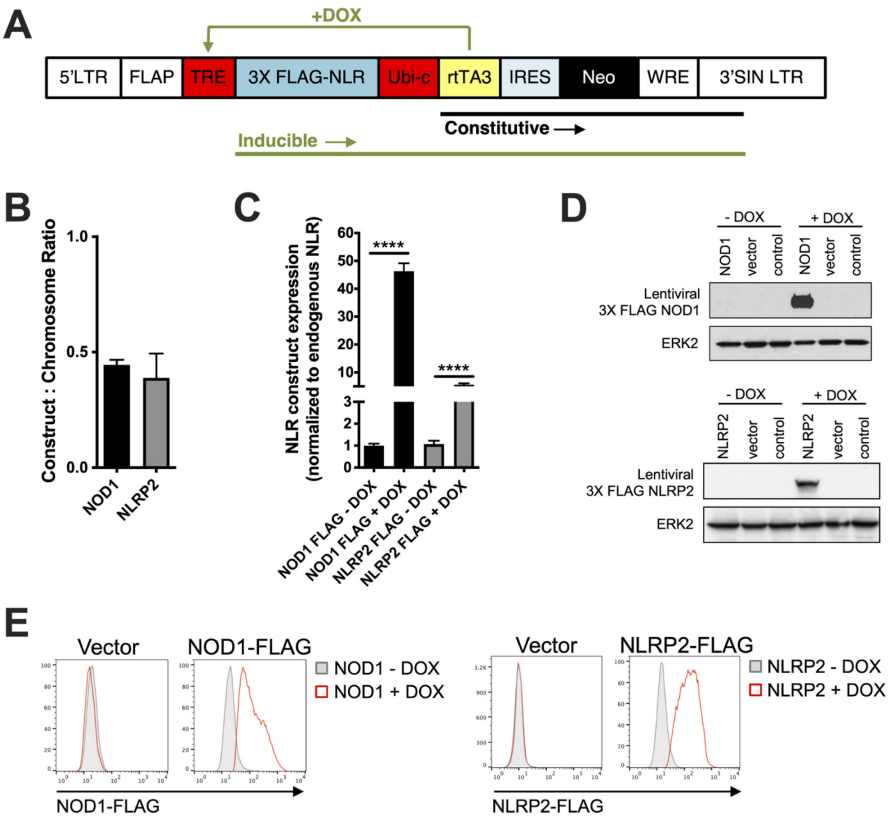

Figure 1. Lentivirus-mediated conditional expression of NOD1 and NLRP2. (**A**) Lentiviral system for DOX-inducible expression of NLRs. Expression of 3X-FLAG-tagged *NLR* coding sequence was transcribed under the control of an inducible tetracycline promoter, called a tetracycline response element (TRE). To engineer the lentiviral expression vectors pSLIK_Neo_NOD1 or pSLIK_Neo_NLRP2, N-terminal 3X-FLAG-tagged NOD1 or NLRP2 were first cloned between the Spe1 and Mfe1 restriction sites of the entry vector pEN_TmiRc3, so that their gene expression was driven by a TRE promoter. Entry vectors were then recombined with the lentiviral expression vector, pSLIK_Neo, via the Gateway LR cloning reaction. This recombination reaction shuttles the TRE-driven NOD1 and NLRP2 gene cassettes into the lentiviral backbone, resulting in the generation of pSLIK_Neo_NOD1 and pSLIK_Neo_NLRP2 lentiviral expression vectors. Lentiviral plasmids were co-transfected with pMDL, pRSV, and pVSV into HEK293T cells to generate lentiviruses used to infect THP-1 cells. (**B**) qPCR showing construct:chromosome ratio of the conditionally expressed NLRs. THP-1 cells were infected with lentivirus at low infectivity. qPCR probes specific for the endogenous or the FLAG-tagged *NLR* (Table 1) were used to determine the ratio of the NLR transgene (construct) to the endogenous gene (chromosome), as an estimate of the number of integrations per genome. A construct:chromosome ratio of 0.5 indicates one integration per cell, and a ratio of 1 indicates two integrations per cell. (**C**) qPCR showing overexpression of *NLRs* upon DOX addition. THP-1 cells harboring DOX-inducible *NOD1* or *NLRP2* were left untreated or were treated with DOX for 6 h. RNA was harvested, and NLR expression was determined by qPCR. NLR expression in the absence of DOX was set at 1. DOX-induced NLR overexpression was measured relative

to that in the absence of DOX. (**D** and **E**) Immunoblot (D) and flow cytometry (E) showing protein expression of FLAG-tagged NLRs in the indicated THP-1 lines with or without addition of DOX for 6 h. 'Vector' refers to cells transduced with empty vector and 'control' refers to THP-1 cells with no lentiviral transduction. Data are presented as means ± SEM. Statistical analyses were performed using a Student's unpaired *t*-test (two-tailed) in GraphPad Prism. *P* values <0.05 were considered significant. ****$P < 0.0001$.

Recipes

1. HEK293T Growth Media
   DMEM supplemented with 10% FBS, 10 mM HEPES, and 2 mM glutamine.
2. THP-1 Growth Media
   RPMI supplemented with 10% FBS, 10 mM HEPES, and 2 mM glutamine

Acknowledgments

This work was funded by the ISB, U.S. NIH grant R21-AI138258 to N.S. and the Intramural Research Program of NIAID, NIH (R.N.G and I.D.C.F). A.S.A. was supported by an American Association of Immunologists (AAI) Careers in Immunology Fellowship. We thank S. Porcella and the Genomics Unit at Rocky Mountain Laboratories for primer design and qPCR support. This protocol was adapted with minor modifications from Shin *et al.* (2006) and Rommereim *et al.* (2020).

Competing interests

Leah Rommereim is an employee and stockholder of SEngine Precision Medicine. None of the other authors have any competing financial interests.

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