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Methods of Assessing STING Activation and Trafficking

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Abstract

The signaling adapter protein STING is crucial for the host immune response to cytosolic DNA and cyclic dinucleotides. Under basal conditions, STING resides on the endoplasmic reticulum (ER), but upon activation, it traffics through secretory pathway to cytoplasmic vesicles, where STING activates downstream immune signaling. Classical STING activation and trafficking are triggered by binding of cyclic dinucleotide ligands. STING signaling can also be activated by gain-of-function mutations that lead to constitutive trafficking of STING. These gain-of-function mutations are associated with several human diseases such as STING-associated vasculopathy with onset in infancy (SAVI), systemic lupus erythematosus (SLE), or familial chilblain lupus (FCL). This dynamic activation pathway presents a challenge to study. We describe methods here for measuring ligand-dependent and ligand-independent activation of STING signaling in HEK293T cells. We also describe a retroviral-based reconstitution assay to study STING protein trafficking and activation in immune competent cells such as mouse embryonic fibroblasts (MEF), which avoids the use of plasmid DNA. These methods will expedite research regarding STING trafficking and signaling dynamics in the settings of infection and autoimmune diseases.

Keywords

Sting; Interferon response; Innate immunity; Cytosolic DNA sensing

1 Introduction

Extracellular and intracellular innate immune receptors alert the host of infection or self-damage. STING is an intracellular protein indispensable for the pathway involved in detecting cytosolic DNA of viral, bacterial, or self-origin [1]. Under basal conditions, STING resides on the endoplasmic reticulum (ER) as a transmembrane protein. Upon the introduction of DNA into the cellular cytosol, the intracellular DNA-sensor, cGAS, binds to the DNA ligand, and converts ATP and GMP to produce the STING ligand cyclic di-GMP-AMP (cGAMP). cGAMP diffuses throughout the cell and binds to the cytosolic carboxy-terminal-domain of STING. After cGAMP binding, STING translocates from the ER to the ER-Golgi intermediate compartment (ERGIC) and the Golgi, then to cytoplasmic vesicles, during which time it recruits TBK1 and IRF3 and activates downstream type I IFN signaling [2, 3]. The cGAS-STING pathway is critical for sensing a variety of microbial pathogens, including DNA viruses such as herpes simplex virus 1 (HSV-1), bacterial pathogens such as

Listeria monocytogenes, *Shigella flexneri*, and *Mycobacterium tuberculosis*, and retroviruses such as human immunodeficiency virus 1 (HIV-1) [3–9].

STING may also be activated independently of upstream activator cGAS or cyclic dinucleotide binding, through gain-of-function mutations in the gene encoding for STING, *TMEM173*. Several STING mutations have been reported in STING-associated vasculopathy with onset in infancy (SAVI), patients with SLE-like syndromes or familial chilblain lupus [10–12]. These STING mutations constitutively activate STING trafficking and type I IFN signaling and are associated with high childhood morbidity and mortality.

Here, we describe a HEK293T cell-based assay to measure ligand-dependent and ligand-independent activation of STING signaling. We also describe a retroviral-based reconstitution assay to study STING-GFP trafficking and activation in immune competent cells to avoid the use of plasmid DNA for the expression of STING.

2 Materials

2.1 Cells

1. HEK293T cells.
2. *Sting*^{−/−} mouse embryonic fibroblasts (MEF).
3. Lenti-X 293T cells (Clontech).

2.2 Plasmids

1. pGL3-IFN β -firefly.Luc (IFN β -Luc), reporter plasmid, IFN β promoter-driven firefly luciferase.
2. pRL-CMV-renilla.Luc (CMV-Luc), reporter plasmid, CMV promoter-driven renilla luciferase.
3. pMCSV-hSTING, mammalian expression plasmid for human STING.
4. pMRX-ibsr, retroviral expression plasmid.
5. pCMV-VSV-G (VSVG), retroviral packaging plasmid.
6. pCMV-Gag-Pol (Gagpol), retroviral packaging plasmid.

2.3 Media and Reagents

1. Opti-MEM reduced serum media.
2. Cell culture medium: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum.
3. Dual luciferase assay system.
4. Microplate reader with dual injection system.
5. Lipofectamine 2000.
6. 2'3'-cGAMP.

7. Blasticidine S hydrochloride.
8. Lenti-X Concentrator.
9. Sterile syringe filter, 0.45 μ m, PVDF.
10. Hexadimethrine bromide (Polybrene).

3 Methods

3.1 Ligand-Dependent STING Activation

3.1.1 Day 1 (STING Plasmid Transfection)

1. Plan to have at least the following conditions in each experiment, with at least two duplicates. Each experimental transfection will require its own 1.5 mL tube when setting up the transfection:
 - 293T cells alone.
 - 293T cells + IFN β -Luc + CMV-Luc plasmids.
 - 293T cells + IFN β -Luc + CMV-Luc + STING plasmid.
 - 293T cells + IFN β -Luc + CMV-Luc + STING plasmid + cGAMP.
2. Utilize Table 1 to calculate amounts of each reagent required for 1 well in a 24-well plate. Scale up or down as necessary.
3. Create a single master mix consisting of Opti-MEM media and the reporter plasmids, IFN β -Luc and CMV-Luc. Count the total number of wells which will be transfected and calculate the volumes of Opti-MEM and reporter plasmids to add into a single tube. Take into account pipetting errors. Mix homogenously and distribute appropriate amounts to separate 1.5 mL tubes to be used for distinct transfection conditions.
4. To each prepared tube containing reporter plasmids, add the appropriate amount of STING plasmid, to account for the number of wells to be transfected.
5. Count the total number of wells to be transfected, and in a separate tube, add a scaled amount of Opti-MEM. Scale the amount of lipofectamine-2000 to add by this same factor. Take into account for pipetting errors. Mix well.
6. Incubate the tubes with plasmids and the tube with lipofectamine separately for 5 min at room temperature.
7. After 5 min of incubation, add in a 1:1 ratio the lipofectamine and Opti-MEM mixture to each tube with reporter plasmids. Mix and incubate for 20 min at room temperature.
8. Prepare 293T cells for reverse plasmid transfection during plasmid and lipofectamine incubation. Trypsinize 293T cells, wash with PBS, and then plate 200,000, 293T cells per well in a 24-well plate. Bring the final volume to 0.4 mL in DMEM + 10% FBS. Incubate cells at a 37 °C incubator (see **Note 1**).

9. After 20 min plasmid and lipofectamine incubation, carefully pipette 100 μ L of the incubated transfection mixture to each well containing 293T cells. Incubate for 18–24 h at 37 °C.

3.1.2 Day 2 (cGAMP Transfection)

1. The following day, ensure cells are 75–90% confluent prior to beginning transfection of cGAMP. Change media to 0.4 mL fresh media (*see Note* ²).
2. In separate tubes incubate a scaled amount of Opti-MEM with cGAMP, and Opti-MEM with lipofectamine. Calculate the amount of cGAMP to utilize given the final concentration to be 0.5–2 μ g/mL (1–4 μ g per well of the 24-well plate).
3. Perform separate 5 min incubation, then combine in a 1:1 ratio the lipofectamine and Opti-MEM mixture to the tube with cGAMP and Opti-MEM. Incubate for 20 min, then add 100 μ L of the reaction per well of the 24-well plate. Incubate for 18–24 h at 37 °C.

3.1.3 Day 3 (Measuring Luciferase Activity)

1. The following day, thaw the Dual Luciferase Kit's reagents prior to removing cells from the incubator. Follow the manufacturer's protocol to reconstitute the kit components.
2. Aspirate media from cells, and add 100 μ L of 1 $\times$ lysis buffer (diluted 1:5 with nuclease-free H₂O) to each well of the plate.
3. Place the plate on a shaker at room temperature for 15 min.
4. Transfer cell lysates to 1.5 mL tubes and spin at top speed at room temperature for 1 min.
5. Transfer the supernatant to PCR tubes (*see Note* ³).
6. Pipette 20 μ L with a multi-channel pipette to an opaque 96-well plate. Pipette in technical duplicates.
7. Follow the Dual-Luciferase Kit protocol and plate reader instructions to measure firefly and renilla luciferase activities.
8. Normalize IFN β -Luc to CMV-Luc values to calculate specific activation of IFN β promoter (*see Note* ⁴).

¹If the 20 minimum incubation time is insufficient for preparing the cells, 293T cells can be prepared before assembling the plasmid DNA/lipofectamine complex. We found that reverse transfection yields higher efficiency than conventional transfection.

²Be very gentle when changing the media because 293T cells can easily detach after overnight transfection.

³Clear cell lysate from this step can be stored at –20 °C for luciferase activity measurement at a later time.

⁴Make sure the reading for both luciferases are within range of the instrument. If the readings are too high or too low, adjust sensitivity of the instrument accordingly or adjust amount of each luciferase plasmid.

3.2 Ligand-Independent STING Activation

1. Plan to have at least the following conditions in each experiment, with at least two duplicates. Each experimental transfection will require its own 1.5 mL tube when setting up the transfection (*see Note* ⁵):

293T cells alone.

293T cells + IFN β -Luc + CMV-Luc plasmids.

293T cells + IFN β -Luc + CMV-Luc + STING plasmid (wild type or mutants).

2. Utilize Table 2 to calculate amounts of each reagent required for 1 well in a 24-well plate. Scale up or down as necessary.

3. **Steps 3–9** are the same as above in Subheading 3.1.

4. Follow the steps above in Subheading 3.1.3.

3.3 Establish Stable STING-GFP Expressing Cells for Microscopy Studies of STING Trafficking

3.3.1 Generate Retrovirus Expressing Wild-Type or Mutant STING-GFP

1. Day 1: Plate Lenti-X 293T cells onto a 10 cm dish so that they will be 60–75% confluent the next day (*see Note* ⁶).
2. Day 2: Obtain pCMV-VSV-G, pCMV-Gag-Pol, and pMRX-STING-GFP (wild-type or mutant) plasmids and calculate the volume needed to transfect 4 μ g of VSVG +6 μ g of Gagpol +6 μ g of pMRX-STING plasmids into one 10 cm dish of Lenti-X 293T cells.
3. Add the calculated amount into 0.5 mL Opti-MEM media in a 1.5 mL tube. In a separate tube, add 0.5 mL Opti-MEM media and 15 μ L of lipofectamine-2000.
4. Incubate both tubes separately for 5 min, then combine into one tube and incubate for 20 min.
5. Pipette the entire 1 mL of mixture carefully onto 293T Lenti-X cells so as to not disrupt the monolayer. Incubate for 18–24 h at 37 °C.
6. Day 3: In the morning, discard the cell media and add 8 mL fresh media per 10 cm plate.
7. Day 4: Collect media in the morning by pipetting it into a 15 mL centrifuge tube and spin at 1500 $\times g$ for 5 min to pellet any suspending cells. Transfer supernatant media containing retroviruses into a 50 mL centrifuge tube and store in 4 °C. Add 8 mL fresh media to cell culture. Repeat these steps in the evening, and once again the next day in the morning (*see Note* ⁷).

⁵This protocol for ligand-independent STING activation essentially removes cGAMP transfection from previous protocol ligand-dependent STING activation.

⁶Other HEK293T cells can also be used for producing pseudo-typed retrovirus, although the efficiency may not be as good as Lenti-X 293T cells.

8. Day 5: After three collections of virus, use a 30 mL syringe and a 0.45 syringe filter to filter the ~24 mL media containing retrovirus.
9. (*Optional*) To this filtered media, add 1/3 the volume of Lenti-X concentrator and incubate in 4 °C for 12–24 h.
10. (*Optional*) At 4 °C, spin the media with viral concentrator for 45 min at 1500 × *g*. After spin a white pellet will be visible. Resuspend in approximately 1/10 of original media or less depending on desired viral concentration.
11. Aliquot virus and store in –80 °C.

3.3.2 Establish *Sting*^{–/–}MEFs Stably Expressing Wild-Type or Mutant STING-GFP

1. Infect *Sting*^{–/–} MEFs with wild-type or mutant STING-GFP virus supplemented with 1 µg/mL of polybrene. Incubate for 24 h, then remove media and add new media containing the 15 µg/mL of BSH. Maintain cells for 2–3 days to ensure complete killing of control untransduced cells (*see Note* ⁸).
2. (*Optional*) Using FACS, sort for GFP-positive cells with a narrow window of GFP fluorescence intensity. Expand sorted cells and freeze aliquots. This will help to achieve uniform STING-GFP expression for microscopy.
3. Cell line expressing STING-GFP is now available for microscopy, flow cytometry, and other biochemical studies (*see Note* ⁹).

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⁷We recommend three collections to maximize production of retrovirus from each plate of cells. The first collection usually contains the highest titer virus. The viral titer usually declines substantially 3 days after plasmid transfection.

⁸We recommend testing the dose of BSH required for complete kill of parent cell line of interest. 15 µg/mL BSH is usually sufficient to kill untransduced MEFs in 2 days.

⁹For microscopy studies, wild-type STING-GFP will localize to the ER at basal state, and translocates to vesicles after DNA or cGAMP transfection within a few hours. Gain-of-function STING constitutively localize to the ERGIC and Golgi at basal state. An example can be seen at [3].

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Table 1

Reagents required for ligand-dependent STING plasmid transfection

Reagent	Amount needed/well
293T cells	200,000 cells
DMEM + 10%FBS media	400 μ L
Opti-MEM media for plasmid	50 μ L
Opti-MEM media for lipofection	50 μ L
Lipofectamine 2000	1 μ L
IFN β -Luc plasmid	100 ng
CMV-Luc plasmid	20 ng
MCSV-hSTING plasmid	50 ng
cGAMP	0.5–2 μ g/mL

Table 2

Reagents required for ligand-independent STING plasmid transfection

Reagent	Amount needed/well
293T cells	200,000 cells
DMEM + 10%FBS media	400 μ L
Opti-MEM media for plasmid	50 μ L
Opti-MEM media for lipofection	50 μ L
Lipofectamine 2000	1 μ L
IFN β -Luc plasmid	100 ng
CMV-Luc plasmid	20 ng
MCSV-hSTING plasmid (wild type or mutants)	50–200 ng