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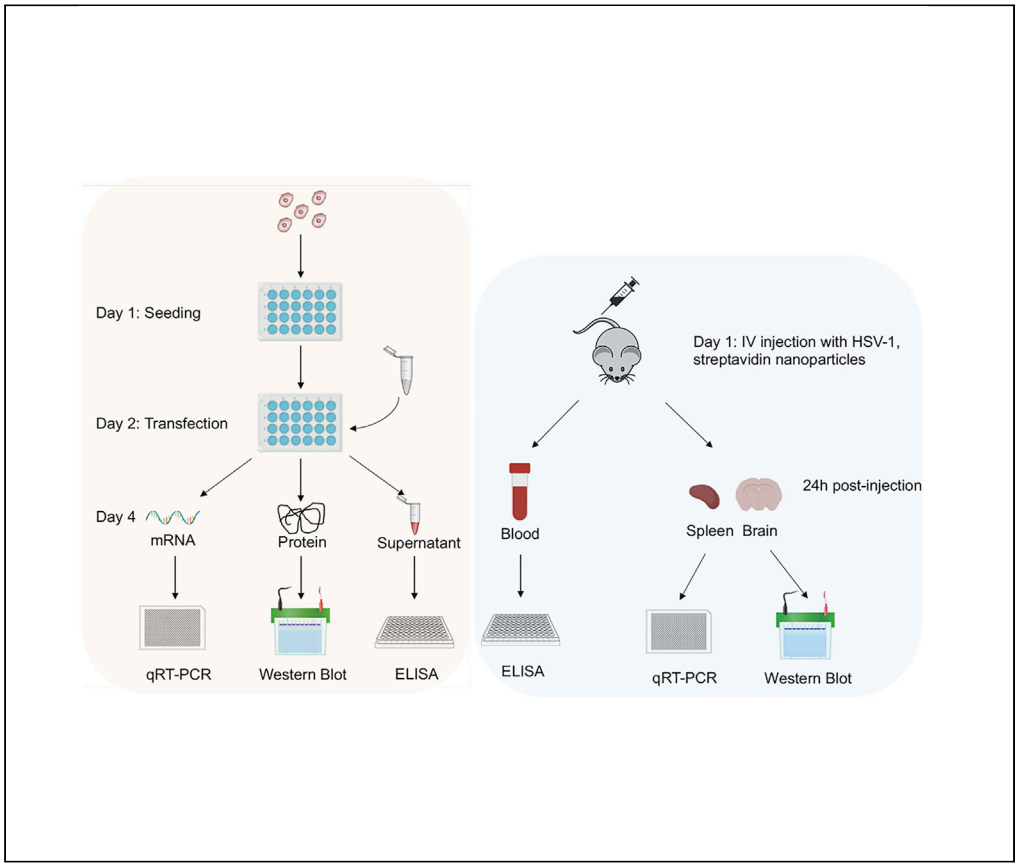
Protocol for Monitoring DNA-Triggered cGAS/STING Signaling in Mammalian Cells and Mice



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HIGHLIGHTS

A protocol to monitor cGAS/STING signaling at protein and mRNA levels

Western blotting analyses to monitor key phosphorylation events

RT-PCR and ELISA analyses to determine IFNβ levels

Mouse model of HSV-1 infection to measure cGAS/STING *in vivo*

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Protocol for Monitoring DNA-Triggered cGAS/STING Signaling in Mammalian Cells and Mice

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SUMMARY

Innate immunity is the first layer of defense against infection in mammals and is tightly regulated. We monitored cGAS/STING signaling upon ISD90 or 2′,3′-cGAMP stimulation in EA.hy926 cells by western blotting, RT-PCR, and ELISA analyses to reveal signaling activation and IFNβ production. In addition, we also include an HSV-1 infected mouse model to further reveal procedures in analyzing cGAS/STING signaling in mice. This protocol could be applied to studies focusing on cell culture or mouse models to investigate cGAS/STING signaling.
For complete details on the use and execution of this protocol, please refer to Zhang et al. (2020).

BEFORE YOU BEGIN

EA.hy926 Cell Culture

Timing: 2 days

1. We have generated EA.hy926 cells (ATCC) stably expressing pLenti-blasticidin-GFP or pLenti-blasticidin-streptavidin as described (available upon request from (Zhang et al., 2020)). Prepare at least 1 × 10 cm dish of 50%–70% confluent EA.hy926 cells (ATCC) stably expressing pLenti-blasticidin-GFP or pLenti-blasticidin-streptavidin (DMEM medium containing 10% FBS, pen/strep and 100 μg/mL blasticidin, temperature: 37°C, 5% CO_2). GFP-expressing cells serve as a negative control to investigate effects of streptavidin expression in cells upon DNA challenge.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
ISD90 (Nucleotide stimulants) 5′-TACAGATCTACTAGTGATCTA TGACTGATCTGTACATGATCTACA TACAGATCTACTAGTGATCTATGAC TGATCTGTACATGATCTACA-3′	Eurofins Genomics	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
2′,3′-cGAMP (Nucleotide stimulants)	InvivoGen	cat# tlrl-nacga23-02
Primer: mouse IFNβ-F 5′-GCTCCAAGAAAGGACGAACA-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Primer: mouse IFNβ-R 5′-CATCTCTTGGATGGCAAAGG-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Primer: human β-actin-F 5′-CCTGGCACCCAGCACAAT-3′	Home designed and synthesized by IDT	N/A
Primer: human β-actin-R 5′-GCCGATCCACACGGAGTA-3′	Home designed and synthesized by IDT	N/A
Primer: human IFNβ-F 5′-TCTCCTCCAAATTGCTCTCC-3′	Home designed and synthesized by IDT	N/A
Primer: human IFNβ-R 5′-CTCCCATTCAATTGCCACAG-3′	Home designed and synthesized by IDT	N/A
Primer: mouse β-actin-F 5′-TACCACAGGCATTGTGATGG-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Primer: mouse β-actin-R: 5′-TCTCAGCTGTGGTGGTGAAG-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Primer: HSV-1 ICP27-F 5′-GCGTCCTTCGTGTTTGTCATT-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Primer: HSV-1 ICP27-R 5′-GCATCTTCTCTCCGACCCCG-3′	Home Designed and synthesized by Eurofins Genomics	N/A
Antibodies		
Anti-cGAS antibody	Cell Signaling Technology	cat#15102, lot#3
Anti-STING antibody	Cell Signaling Technology	cat#13647, lot#1
Anti-phospho-Ser172-TBK1 antibody	Cell Signaling Technology	cat#5483, lot1#1
Anti-phospho-Ser396-IRF3 antibody	Cell Signaling Technology	cat#29047, lot#1
Anti-TBK1 antibody	Cell Signaling Technology	cat# 51872
Anti-IRF3 antibody	Cell Signaling Technology	cat# 5478
Monoclonal anti-Tubulin antibody	Sigma	cat#T-5168, lot#115M4828V
Peroxidase-conjugated anti-mouse secondary antibody	Sigma	cat#A-4416, lot#SLBW4917
Peroxidase-conjugated anti-rabbit secondary antibody	Sigma	cat#A-4914, lot#SLBV6850
Chemicals, Peptides, and Recombinant Proteins		
Blasticidin	Fisher BioReagents	cat# 3513-03-9, lot# 171370
DMEM	Gibco	cat# 11995065, lot#2186870
OPTI-MEM	Gibco	cat# 31985070, lot#2185843
FBS	Gibco	cat# 26140, lot# 2139222
BSA	Goldbio	cat# A-420-500
SDS	Sigma-Aldrich	cat# 436143
Glycerol	Sigma-Aldrich	cat# G7893
DTT	Sigma-Aldrich	cat# D9779
BPB (Bromophenol Blue)	Sigma-Aldrich	cat# B0126
Tris base	Sigma-Aldrich	cat# 11814273001
Glycine	Sigma-Aldrich	cat# G7126
DEPC water	vwr	cat# 10220-384

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pen/strep (penicillin-streptomycin solution)	Corning	cat# 30-002-Cl
Experimental Models: Cell Lines		
EA.hy926	From Tissue Culture Facility at UNC Chapel Hill	N/A
Experimental Models: Organisms/Strains		
Mice: C57BL/6	Jackson Laboratory	Stock# 000664
Mice: *cGAS*$^{-/-}$	Jackson Laboratory	Stock# 026554
Bacterial and Virus Strains		
HSV-1	From Dr. Steve Bachenheimer Lab at UNC Chapel Hill	KOS strain
Software and Algorithms		
Origin7 (Microcal)	OriginLab Corporation	https://microcal-origin.joydownload.com/
SPSS Statistics	IBM Corporation	SPSS 11.5 Statistical Software
Graph Pad	GraphPad Prism 7	N/A
Critical Commercial Assays		
RNeasy Mini Kit	QIAGEN	cat# 74106
iScript™ Reverse Transcription Supermix for RT-qPCR	Bio-Rad	cat# 170-8891
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems by Thermo Fisher Scientific	cat# A25742, lot# 00718807
Human IFNβ ELISA kit	R&D systems	cat# DY814-05, lot#P181016
DNeasy Blood & Tissue Kits	QIAGEN	cat# 69506
Protease Inhibitor Cocktail	Bimake	cat# B14012, lot# 411013
Phosphatase inhibitor cocktail A and B	Bimake	cat# B15001-A /B15001-B, lot# 510028
Protein Bradford Assay reagent	Bio-Rad	cat# 5000006
lipofectamine 3000	Invitrogen by Thermo Fisher Scientific	cat# L3000150
Streptavidin nanoparticles	Sigma-Aldrich	cat# 53134-1ML
Other		
PVDF membrane	Bio-Rad	cat# 1620177
ECL-Millipore	Millipore	cat# WBKLS0500
ECL-Femto	Thermo Scientific	cat# 34096
ECL-Pierce	Thermo Scientific	cat# 32106
NanoDrop One^{C}	Thermo Scientific	cat# ND-ONEC-W
Eppendorf Centrifuge	Eppendorf	cat# 022620444
Power supply	Bio-Rad	cat# 1645050
SDS-PAGE gel tank	Bio-Rad	cat# 1658004
SDS-PAGE transfer device	Bio-Rad	cat# 1703930
QUANT imager	Kindle Bioscience	cat# D1001
ViiA^{TM}6 Real-Time PCR system	Thermo Scientific	cat# 4453545
Cytation 5 Cell-imaging multimode reader	BioTek	N/A
Labnet shaker ProBlot 35 delux rocking platform	Labnet	cat# S2035-D

MATERIALS AND EQUIPMENT

3× SDS sample buffer (stocks can be kept at −20°C)

Reagents	Final Concentration	Amount (for a 60 mL Stock)
10% SDS	6.7%	40 mL
glycerol	33.3%	20 mL
DTT	300 mM	3 g
BPB		1–5 mg
ddH_2O		to 60 mL

5× SDS-PAGE running buffer (stocks can be kept at 25°C)

Reagents	Final Concentration	Amount (for a 1 L Stock)
Tris-HCl	125 mM	15 g
Glycine	1 M	72 g
SDS	0.5%	5 g
ddH_2O		to 1 L

10× SDS-PAGE transfer buffer (stocks can be kept at 25°C)

Reagents	Final Concentration	Amount (for a 4 L Stock)
Tris-HCl	250 mM	107.3 g
Glycine	2 M	512.6 g
SDS	1%	3.56 g
ddH_2O		to 4 L

10× TBST buffer (stocks can be kept at 25°C)

Reagents	Final Concentration	Amount (for a 4 L Stock)
Tris-HCl, pH8.0	100 mM	200 mL of 2 M stock
NaCl	120 mM	351 g
Tween 20	1%	40 mL
ddH_2O		to 4 L

EBC buffer (stocks can be kept at 25°C and should be supplemented with protease inhibitors and phosphatase inhibitors before usage; EBC buffer with protease inhibitors and phosphatase inhibitors should be stored at −20°C)

Reagents	Final Concentration	Amount (for a 1 L Stock)
Tris-HCl pH 7.5	50 mM	50 mL of 1 M stock
NaCl	120 mM	24 mL of 5 M stock
NP-40	0.5%	5 mL
ddH_2O		to 1 L

Triton X-100 buffer (stocks can be kept at 25°C and should be supplemented with protease inhibitors and phosphatase inhibitors before usage; Triton X-100 buffer with protease inhibitors and phosphatase inhibitors should be stored at −20°C)

Reagents	Final Concentration	Amount (for a 1 L Stock)
Tris-HCl pH 7.5	50 mM	50 mL of 1 M stock
NaCl	150 mM	30 mL of 5 M stock
Triton X-100	1%	10 mL
ddH_2O		to 1 L

STEP-BY-STEP METHOD DETAILS

Cell Preparation for Transfection

Timing: 5 min

1. Split sub-confluent EA.hy926 cells into 24-well plates (~ 5 × 10^4 cells/well). Cells are left untreated for 8–10 h and cells reach ~60%–70% confluence upon transfection.

   Note: Duplicates/triplicates are needed for western blot analyses and triplicates are needed for mRNA and ELISA assays.

   ⚠ CRITICAL: Ensure cell confluence is ~60%–70% to achieve optimal transfection efficiency.

ISD90 or 2′,3′-cGAMP Transfection

Timing: ~20 min

2. Transfection:
   a. Preparation of DNA complex for a single well in 24-well plates: mix desired amounts (starting from 2.5 μg for general purpose) of ISD90 or 2′,3′-cGAMP with P3000 in a 1:2 ratio in 65 μL OPTI-MEM medium (low serum and antibiotics free) using a sterile 1.5 mL Eppendorf tube and vortex at medium speed. For example, for a transfection of 2.5 μg ISD90, 5 μL P3000 is added with ISD90 to 65 μL OPTI-MEM medium.
   b. Preparation of lipid complex: mix lipofectamine 3000 (in a 1:3 ratio for ISD90/2′,3′-cGAMP to lipofectamine 3000) with 65 μL OPTI-MEM medium and vortex at medium speed. For example, for a transfection of 2.5 μg ISD90, 5 μL lipofectamine 3000 is added to 65 μL OPTI-MEM medium.
   c. Mix DNA and lipid complex and vortex at medium speed.
   d. Leave at room temperature (~25°C) for 15 min.
   e. Carefully and slowly drop the mixture to cells. 5–6 h post transfection, cells are washed with sterile PBS and cultured in DMEM medium with 10% FBS and antibiotics (pen/strep) until cell collection.

   ⚠ CRITICAL: Optimal DNA/P3000/lipofectamine 3000 ratios are critical for transfections with high efficiency.

   ⚠ CRITICAL: Too much lipofectamine used in a transfection reaction may cause cell toxicity.

   ⚠ CRITICAL: Dripping transfection mixture into dishes should be slowly and evenly performed with minimal disturbance to the cell monolayer.

Cell Collection to Examine cGAS/STING Signaling by Western Blotting

Timing: ~2 days

3. **Cell collection:** At desired time periods post transfection, wash cells with 1× PBS and lyse cells by EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40) or Triton X-100 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors and phosphatase inhibitors (diluted to 1× following manufacturer's instructions: https://www.bimake.com/product/protease-inhibitor-cocktail-mini-tablet.html and https://www.bimake.com/product/phosphatase-inhibitor-cocktail.html). Incubate cells at 4°C for 10 min with gentle rotation. Spin down cell debris in an Eppendorf 5424R centrifuge at 4°C for 10 min at maximum speed

(~21,300 × *g*). Transfer supernatants to a new tube and save as whole cell lysates (WCL) with no visible cell debris.

⚠ CRITICAL: Inhibitors for proteases and phosphatases are important to preserve protein phosphorylation signals.

4. **Protein concentration determination:** Add 1 μL of WCL into 1 mL of 1:5 diluted Bradford protein assay reagent and determine protein concentrations by Nanodrop One[C]. Establish a standard curve using 1, 3, 6 and 9 μg/μL BSA.

⚠ CRITICAL: Careful and thorough mixture of WCL with diluted Bradford assay reagent is important to obtain reliable protein concentration measurements.

5. **SDS-PAGE:** Add 1/3 volume of 3× SDS sample buffer into WCL and boil samples at 95°C for 10 min. Load same amounts of WCLs (usually ~50 μg) on 10% SDS-PAGE gels in SDS-PAGE running buffer and run at 128 V constant voltage for 70 min, or until the dye front reaches the bottom of the gel.

⚠ CRITICAL: Samples should be spun down briefly after boiling to collect any evaporates on lids or tube walls, and should be vortexed before sample loading.

6. **Transfer of proteins onto PVDF membrane:** Assemble transfer sandwiches by positioning the SDS-PAGE gel next to the PVDF membrane per manufacture instructions in the transfer buffer. Potential air bubbles should be removed by careful rolling with rollers purchased from Bio-Rad. Insert the transfer sandwiches into the western blot transfer device in SDS-PAGE transfer buffer and cooling pad at 128 V for 2 h.

⚠ CRITICAL: Rolling out air bubbles in the assembled transfer sandwiches is important to ensure a successful transfer.

7. **Western blotting:** Upon completion of transfer, block PVDF membranes with 5% non-fat milk in TBST buffer for 30 min on a Labnet shaker at room temperature (~25°C).
   a. Dilute desired primary antibodies in 5% non-fat milk in a 1:1,000 dilution and incubate with PVDF membranes at 4°C for 8–10 h with gentle shaking.
   b. On the next day, wash PVDF membranes thoroughly with 1× TBST buffer with shaking on a Labnet shaker at room temperature (~25°C) for 10 min and repeat this four times.
   c. Dilute HRP-conjugated secondary antibodies into 5% non-fat milk in TBST buffer in a 1:3,300 dilution and incubate with PVDF membrane for 1 h at room temperature (~25°C) with gentle shaking.
   d. Wash PVDF membranes thoroughly with 1× TBST buffer for four times with 10 min/time with shaking on a shaker at room temperature (~25°C).
   e. Afterwards, incubate PVDF membranes with ECL reagents for 5 min at room temperature (~25°C) before imaging by QUANT imager.

Note: The signal intensity for ECL-Pierce is the weakest and the signal intensity for ECL-Femto is the strongest. The choices of these ECL reagents can be determined by the expression level of the protein target or if this is unknown, start with the ECL-Pierce to avoid overexposure.

⚠ CRITICAL: Primary antibody dilution affects WB signal intensity. This should be determined by manufacturer instructions or amounts of samples loaded.

⚠ CRITICAL: These ECL reagents sustain reliable and stable signals for ~20 min so ECL detection should be done within 20 min.

RT-PCR Analyses to Examine IFNβ mRNA Changes

Timing: ~4–5 h

8. Extract total RNA using RNeasy mini kit (QIAGEN) according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-mini-kit/#orderinginformation). Dissolve/Elute extracted RNA in DNase-free and RNase-free DEPC water.
9. Determine RNA concentrations by a spectrophotometer (Nanodrop One^{C}, Thermo Scientific).
10. **cDNA synthesis from mRNA:** Use iScript^{TM} Reverse Transcription Supermix for RT-qPCR to reversely transcribe mRNA into cDNA following manufacturer's instructions (https://www.bio-rad.com/en-us/product/iscript-reverse-transcription-supermix-for-rt-qpcr?ID=M87EVMKG4). A total of 1 μg RNA is used as the template in this step.
11. **RT-PCR and data analyses:** Mix iTaq^{TM} universal SYBR Green Supermix (https://www.bio-rad.com/en-us/product/itaq-universal-sybr-green-supermix?ID=M87FTF8UU) with cDNA template (with a final amount of 500 ng), forward and reverse primers (500 nM of each primer) together in a 20 μL reaction volume and perform the PCR reaction on the ViiA^{TM}6 Real-Time PCR system with the settings as below:

PCR Cycling Conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing, extension, and read fluorescence	60°C	1 min	
Melting curve validation	95°C	15 s	1
	60°C	1 min	
	95°C	15 s	
Hold	4°C	forever	

IFNβ mRNA levels are calculated by using the comparative Ct (Livak $2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). The expression of IFNβ mRNAs is normalized to the expression of β-actin.

⚠ CRITICAL: Although much less cells are needed for RT-PCR analysis, increasing number of cells used for RNA extraction increases quality and quantity of RNA.

ELISA for Extracellular IFNβ Protein Measurement

Timing: 24 h

12. Preparation of plates:
    a. Reconstitute Capture Antibody with 0.5 mL PBS to the working concentration, and dilute Capture Antibody reconstitution solution (480 μg/mL) with PBS without carrier protein to working solution concentration (4.00 μg/mL). Coat high binding 96-well plate with Capture Antibody working solution (100 μL/well), cover the plate and incubate at 4°C for 8–10 h.
    b. Aspirate wells and wash wells three times with Wash Buffer (0.05% Tween-20 in PBS, pH 7.2–7.4, 400 μL/well). After washing, invert and tap the plate on clean paper towels to remove remaining Wash Buffer.
    c. Block the plate with blocking solution (1% BSA in PBS, pH 7.2–7.4, 300 μL/well) for 1 hour at room temperature (~25°C).
    d. Repeat step 12b.

⚠ CRITICAL: Avoid freeze/thaw of reconstitute Capture Antibody

13. Establishment of the standard curve using recombinant IFN-β proteins provided in the ELISA kit:
    a. Reconstitute standard with 0.5 mL Reagent Diluent (1% BSA in PBS, pH 7.2–7.4), and dilute standard reconstitution solution (55 ng/mL) with Reagent Diluent to working solution concentration (500 pg/mL). Perform a seven-point standard curve by using 2-fold serial dilutions from 500 pg/mL.
14. IFNβ protein measurements:
    a. Collect culture media from samples and centrifuge in a table top centrifuge at top speed (~21,300 × *g*) briefly to eliminate cell debris or dead cells.
    b. Add 100 μL standard (two replicates each concentration) and centrifuge-cleared supernatant (1:1 mixed with Reagent Diluent) to designated wells. Seal the plate and incubate for 2 h at room temperature (~25°C).
    c. Repeat step 12b in the preparation of plates.
    d. Reconstitute Detection Antibody with 1 mL Reagent Diluent, and dilute Detection Antibody reconstitution solution (15 μg/mL) with Reagent Diluent to working solution concentration (250 ng/mL). Add Detection Antibody working solution to each well (100 μL/well), seal the plate and incubate the plate for 2 h at room temperature (~25°C).
    e. Repeat step 12b in the preparation of plates.
    f. Dilute Streptavidin-HRP with Reagent Diluent to a working solution concentration (a 40-fold dilution), and add 100 μL Streptavidin-HRP working solution to each well. Seal the plate and incubate the plate in the dark for 30 min at room temperature (~25°C).
    g. Repeat step 12b in the preparation of plates.
    h. Add 100 μL substrate solution to each well, seal the plate and incubate the plate in the dark for 20 min at room temperature (~25°C).
    i. Add 50 μL of Stop Solution to each well.
    j. Measure absorbance with a microplate reader (BioTek Cytation 5) at 450 nm after adding Stop Solution.

⚠ CRITICAL: Sample dilution may increase variances among measurements.

15. Date Analyses

Generate the standard curve (y axis: protein concentration, x axis: absorbance) with a 4-parameter algorithm using Excel. Subtract background from the readings by using the blank samples and use the standard curve to calculate concentrations of samples.

HSV-1 Infection and Streptavidin Nanoparticles Injection into Mice

Timing: 24 h

16. Six-week-old C57BL/6 WT and *cGAS*$^{-/-}$ female mice were used for this study.
17. Inject 100 μL of purified HSV-1 viruses (in PBS) with indicated pfu as described in (Zhang et al., 2020) into indicated mice through tail vein injection.
18. Inject 100 μL of streptavidin nanoparticles into indicated mice through tail vein injection.
19. Twenty-four hours post infection, euthanize mice using carbon dioxide for at least 4 min followed by cervical dislocation according to the approved IACUC protocol.

⚠ CRITICAL: Tail vein injection requires experience and training.

Collection of Mouse Tissues for Analyses

Timing: 15–20 min per mouse

20. Collect blood by cardiac puncture as described (Parasuraman et al., 2010) right after mice are euthanized. Proper aseptic technique is recommended during the process. Store collected blood in clean sterile Eppendorf tubes.

⚠ CRITICAL: Blood collection should be performed immediately after euthanization.

21. To obtain the serum, centrifuge the tube with the collected blood for at least 15 min at 1,000 × *g*. Pipette the serum or plasma into a clean Eppendorf tube and attach the label. Avoid transferring red blood cells into the final tube.

Note: The serum can be stored at −80°C (should be used as soon as possible) before it is subjected to ELISA to detect interferon and cytokines.

22. Harvest spleen and brain tissues after euthanasia. Split the harvested tissues into three sections.

Note: Tissue sections can be stored at −80°C (should be used as soon as possible) before being subjected to further analysis.

23. The first aliquot is subjected to RNA extraction using QIAGEN RNeasy Mini Kits following manufacturer's instructions (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-mini-kit/#orderinginformation) and qRT-PCR as described above to detect cellular gene expression at the RNA levels.
24. The second aliquot is subjected to DNA extraction using QIAGEN DNeasy Blood & Tissue Kits following manufacturer's instructions (https://www.qiagen.com/us/shop/pcr/dneasy-blood-and-tissue-kit/) and qPCR as described above to detect HSV-1 genome copy numbers.
25. The last aliquot is subjected to western blot analysis by extracting proteins using a plastic pestle to grind tissues in a 1.5 mL Eppendorf tube in lysis buffer (EBC buffer) used above and follow the exact steps as described in step 3 to detect cellular protein or HSV-1 viral protein expression levels.

EXPECTED OUTCOMES

Activation of the cGAS/STING signaling pathway can be validated at cell signaling levels by western blotting for increased IRF3-pS396 and TBK1-pS172 signals and correlates with super-shifts of STING signals. As a result, IRF3 phosphorylation promotes IFNβ transcription monitored by RT-PCR and subsequently increases secreted levels of IFNβ in cell culture media that is monitored by ELISA (Zhang et al., 2020) (Figure 1).

LIMITATIONS

- Western blotting, RT-PCR, and ELISA only measure average changes for these desired signals in a population of cells. Given that each cell responds differentially to stimuli, this method cannot monitor real-time signaling changes for single cells.
- This method relies on WCL that cannot distinguish signals from cytoplasm and nuclei. Nuclear and cytoplasmic extraction kits can be used to distinguish signals from cytoplasm and/or nuclei.
- This method does not include a direct measurement of cellular 2′,3′-cGAMP, the direct product of cGAS, as an evidence for cGAS activation.

TROUBLESHOOTING

Problem 1

Low transfection efficiency (step 2).

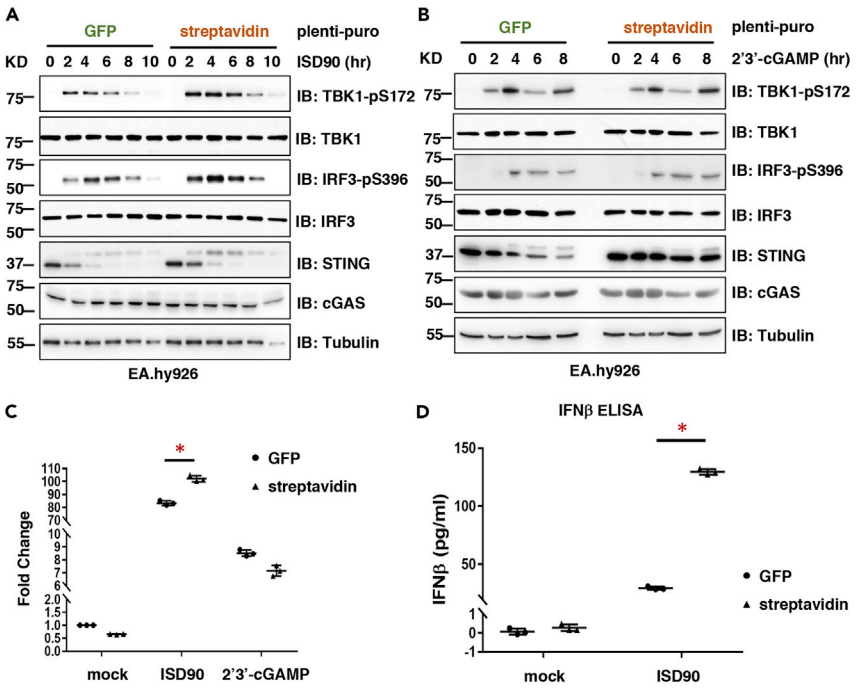

Figure 1. Streptavidin Expression in EA.hy926 Cells Facilitates ISD90-Induced cGAS Activation

(A and B) IB analyses of WCL derived from EA.hy926 cells transfected with ISD90 (A) or cGAMP (B). (C) RT-PCR analysis of IFNβ mRNA levels in EA.hy926 cells stably expressing either GFP or streptavidin treated with ISD90 or 2′,3′-cGAMP for 6 h. *p < 0.05 from Student's t tests.

(D) ELISA assays using cell culture media from EA.hy926 cells stably expressing either GFP or streptavidin treated with ISD90 for 16 h. *p < 0.05 from Student's t tests. These data are from the original Figures 2A–2D in Zhang et al. (2020).

Potential Solution

Increase lipofectamine amount (be cautious that extra amounts of lipofectamine cause cell toxicity), change the ratio of DNA:P3000 (for example use a 1:3 ratio instead of 1:2), use optimized transfection conditions for a given cell line (for example, for a 24-well plate transfection, treat each well with 150 μL of transfection mixture composed of 2 μg DNA with 4 μL P3000 in 65 μL OPTI-MEM, and 6.5 μL lipofectamine 3000 in 65 μL OPTI-MEM), remove the transfection mix by replacing with fresh medium 6–7 h post transfection, start the transfection with lower cell confluency (no more than 75% confluency as the higher the cell confluency, the lower the transfection efficiency) or try other transfection reagents such as PEI.

Problem 2

Cell death upon transfection (step 2).

Potential Solution

Reduce amounts of ISD90 or 2′,3′-cGAMP in transfection, or reduce amounts of lipofectamine used in each reaction. Remove the transfection mix by replacing with fresh medium 6–7 h post transfection.

Problem 3

Low cGAS signaling activation (step 7).

Potential Solution

Increase amounts of ISD90, extend treatment periods, try harvest samples at different time points, or check mycoplasma contamination.

Problem 4
Large variations in RT-PCR data (steps 8–11).

Potential Solution
Optimize transfection protocols to increase transfection efficiency and reproducibility, increase reaction volumes to reduce pipette errors, try other internal controls, increase number of replicates, or control cell conditions.

Problem 5
Large variations in ELISA data (steps 12–15).

Potential Solution
Optimize transfection protocols to increase transfection efficiency and reproducibility, optimize supernatant recovery methods (for example, centrifuge supernatants at high speed before freezing to remove cell debris and/or dead cells), use non-diluted supernatant instead of diluted supernatant to mix with Reagent Diluent, reduce pipette errors, increase number of repeats.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pengda Liu (pengda_liu@med.unc.edu)

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
This study did not generate and/or analyze any datasets.

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AUTHOR CONTRIBUTIONS
Conceptualization, Y.W., B.D. and P.L.; Writing – Original Draft, Y.W. and P.L.; Writing – Review & Editing, Y.W., Z.M., B.D., and P.L.; Funding Acquisition, B.D. and P.L.; Supervision, B.D. and P.L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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