SUPPLEMENTAL FIGURES

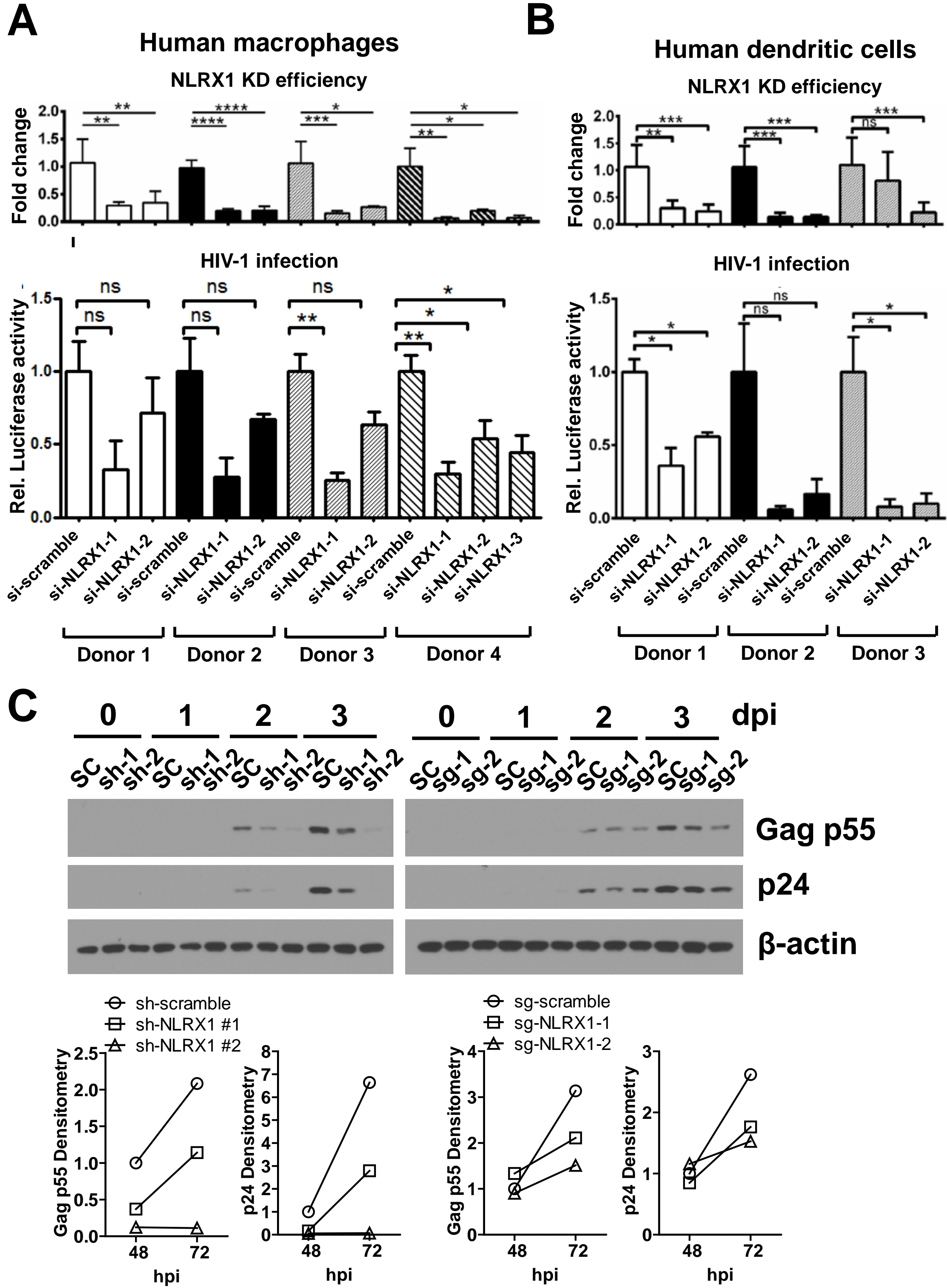


Figure S1, Related to Figure 1. NLRX1 is required for HIV-1 infection.

(A and B) Knockdown of NLRX1 decreases HIV-1 infection in human primary macrophages (A) and dendritic cells (B) from individual donors. Monocyte derived macrophage and monocyte derived dendritic cells were transfected with scramble siRNA or siRNA targeting NLRX1 followed by infection with HIV-VSV. Efficiency of gene reduction by siRNA is shown in the upper panel. HIV-1 infection as reflected by luciferase activity is shown below.

(C) THP-1 cells with scramble or shRNA or sgRNA targeting *NLRX1* were infected with a CCR5 and CXCR4 dual tropic HIV-1 strain R3A. After 24, 48, and 72 hours the expression of HIV-1 Gag p55 and p24 protein were determined by western blot. SC: scrambled control, sh-1: sh-NLRX1-1, sh-2, sh-NLRX1-2, sg-1: sg-NLRX1-1, sg-2: sg-NLRX1-2. Densitometry analysis was performed for Gag p55 and p24 levels by normalizing to β-actin.

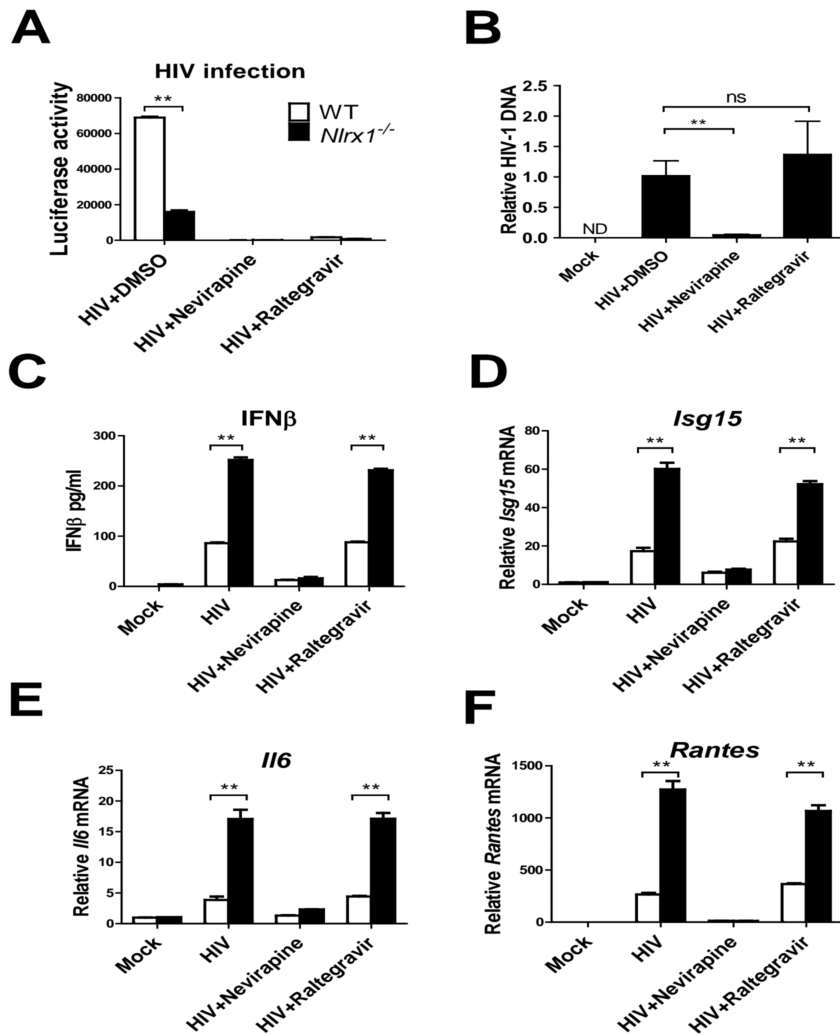


Figure S2, Related to Figure 2. NLRX1 inhibits HIV-1 reverse transcripts induced innate immune response.

(A and B) WT or *Nlrx1$^{-/-}$* MEFs were infected with HIV-VSV, MOI=1, in the absence or presence of HIV-1 reverse transcription inhibitor, nevirapine, or integration inhibitor, raltegravir. Luciferase activity (A) and HIV-1 DNA (B) was determined 24 hpi to measure viral infection. (C-F). Same as (A), except IFNβ (C), *Isg15* (D), *Il6* (E) and *Rantes* (F) were determined by ELISA or qPRC 16 hpi.

Data are presented as the mean ± standard error of the mean (SEM). **$p < 0.01$ (Student's t test). All data are representative of at least three independent experiments.

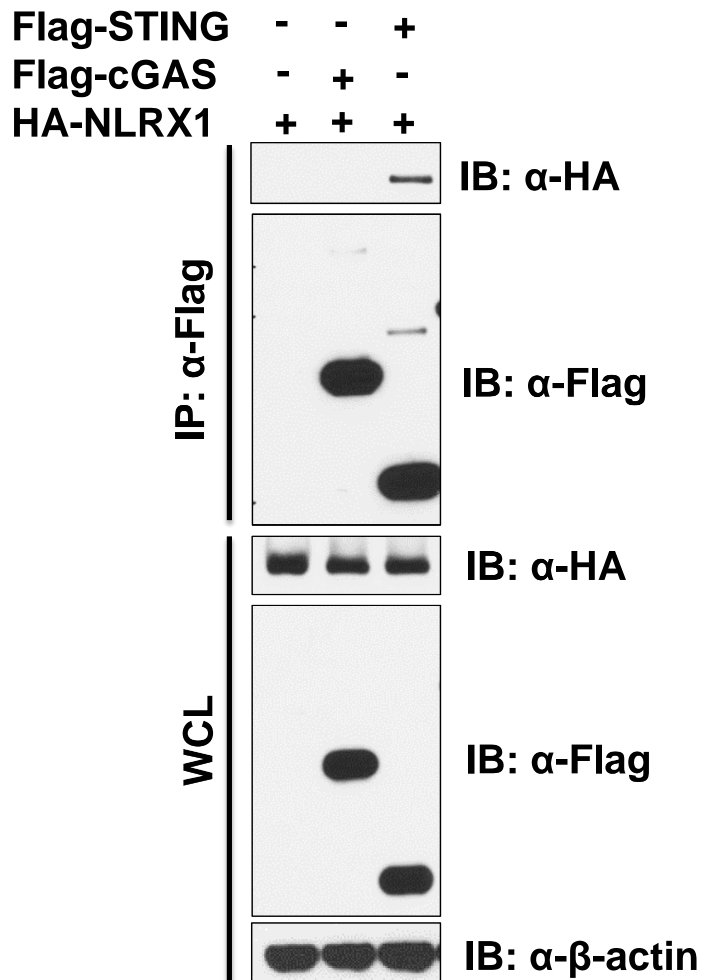


Figure S3, Related to Figure 4. NLRX1 interacts with STING but not cGAS. Association of over-expressed NLRX1 with cGAS or STING in HEK293T cells. FLAG-NLRX1 was singly expressed or co-expressed with cGAS or STING in HEK293T cells. Co-immunoprecipitaion was performed with anti-FLAG beads followed by the western blot analysis.

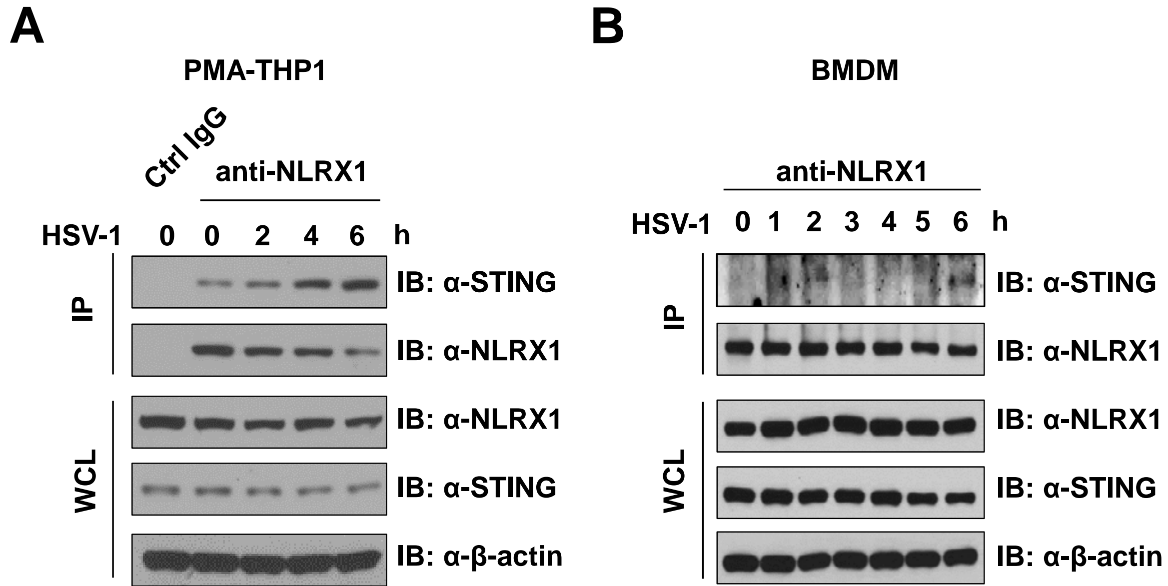
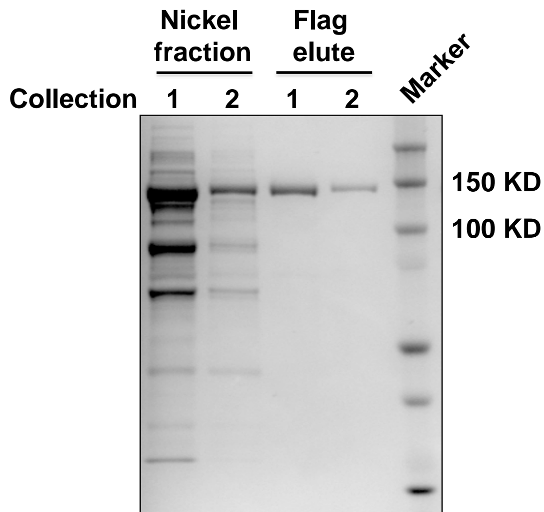


Figure S4, Related to Figure 4. HSV-1 infection enhances association of NLRX1 with STING.

(A) THP-1 were differentiated into macrophage like cells by 60 nM phorbol 12-myristate 13-acetate (PMA) for 12 hours, followed by culturing cells in regular RPMI medium for 48 hours. PMA-THP-1 were infected with HSV-1 (MOI=3). At indicated time points, cells were lyzed and co-immunoprecipitation was performed by using the normal mouse IgG or antibody against NLRX1. Immunoblots were performed with the antibodies indicated.

(B) BMDMs were infected with HSV-1 (MOI=3). At indicated time points, cells were lyzed and co-immunoprecipitation was performed by using the antibody against NLRX1. Immunoblots were performed with the antibodies indicated.



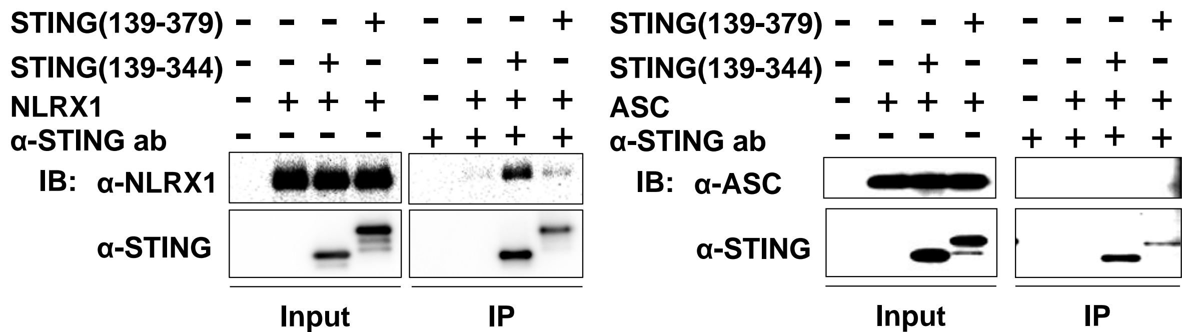


Figure S5, Related to Figure 4. Recombinant NLRX1 but not ASC interacts with STING.

(A) Recombinant NLRX1 was purified by nickel- and Flag- affinity purification.

(B) Similar to Figure 4D, *in vitro* binding assay was performed by using antibodies against STING, except this figure includes purified recombinant ASC as a negative control. Immunoblots were performed with the antibodies indicated.

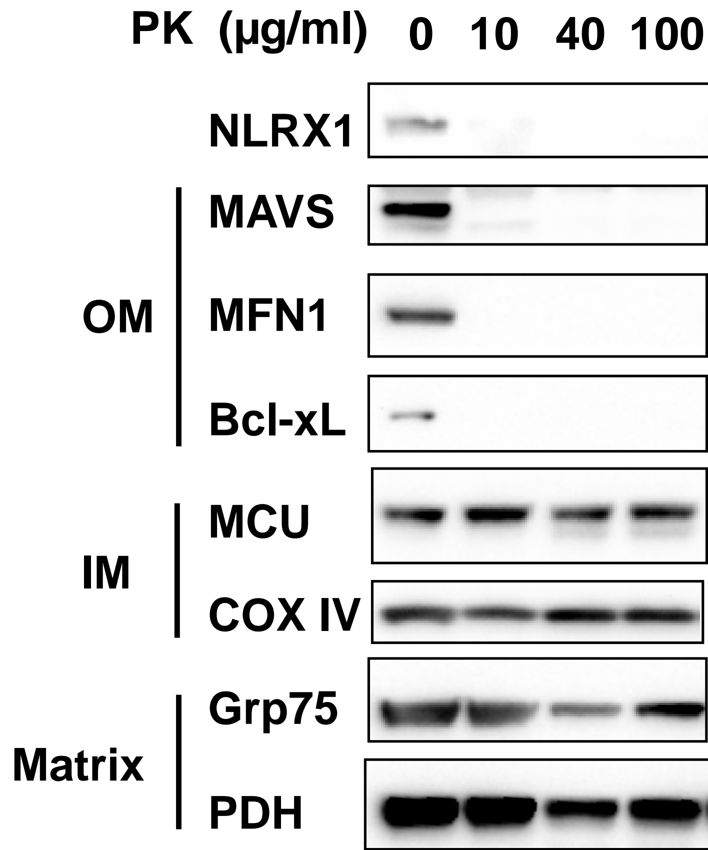


Figure S6, Related to Figure 4. Mitochondrial sublocalization of NLRX1 in HEK293T cells. Purified mitochondria from HEK293T cells were treated with increasing concentration of proteinase K. NLRX1 and other mitochondrial markers were analyzed by western blot, as indicated.

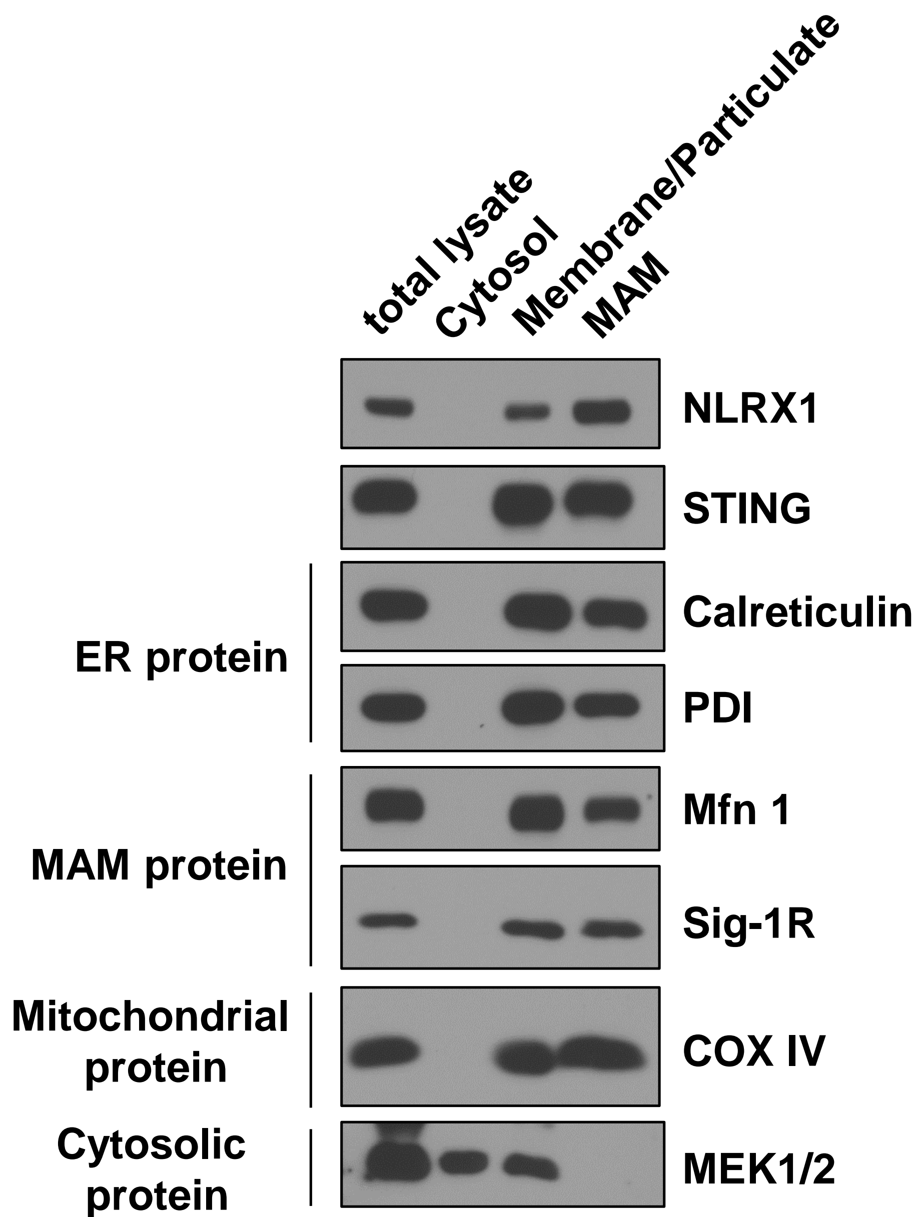


Figure S7, Related to Figure 4. NLRX1 is localized on mitochondria-associated membranes. Total, cytosolic, membrane/particulate, and MAM proteins were isolated from HFFs. 10 μg of proteins from each fractionation were resolved by SDS-PAGE and analyzed by immunoblot using indicated antibodies for ER (Calreticulin and PDI), MAM (Mfn 1 and Sig-1R), mitochondrial (COX IV), and cytosolic (MEK1/2) proteins.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture

HEK293T and THP-1 cells were purchased from ATCC. HEK293T cells, mouse peritoneal macrophages, and human foreskin fibroblast cells (HFFs) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 100μg/ml streptomycin and THP-1 cells were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin and 100μg/ml streptomycin. THP-1 cells were differentiated into macrophage like cells (PMA-THP-1) by treating with 60 nM PMA for 12 hours and culture in completed RPMI medium for 48 hours. *Nlrx1*$^{-/-}$ and the littermate control MEFs were generated from 13.5-day embryos and maintained in complete DMEM medium described above with 1 mM sodium pyruvate, 4 mM L-glutamine and non-essential amino acid. BMDMs were generated in the presence of L-929 conditional medium (50% DMEM, 20% FBS, and 30% L-929 medium). All cells were grown in a 37 °C incubator supplied with 5% CO2.

Monocyte derived macrophages (MDMs) and monocyte derived dendritic cells (MDDCs) were generated by differentiation of monocytes from human peripheral blood mononuclear cells (PBMCs). Human buffy coats were obtained from "German Red Cross Blood Donor Service Baden-Württemberg Hessen". Primary human monocytes were isolated from PBMCs by positive selection for CD14+ cells using CD14-MicroBeads (Miltenyi Biotec; purity was >94 %) according to the manufacturer's guidelines and separation from unlabeled cells was carried out in AutoMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequent differentiation into M1-like MDM or MDDC was achieved by culturing cells in T75 cell culture flasks in RPMI-1640 containing 2 mM L-Glutamine, 10 % FBS, 1% (v/v) HEPES, 1mM sodium pyruvate, which was supplemented with either 100 U/ml granulocyte-macrophage colony-stimulating

factor (GM-CSF) (PeproTech, Hamburg, Germany) or 500 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000 U/ml IL-4 (PeproTech) respectively. Medium was refreshed after 3 days and on day 5 batch lift was performed by dislodging cells from the flasks. Cells were counted and seeded at 20,000/well in 96-well format for subsequent siRNA transfection.

Reagents and antibodies

c-di-GMP and 3'3'-cGAMP were purchased from InvivoGen. Mouse IL-6 ELISA kits were from BD Biosciences. Mouse anti-IFN-β capture antibody (7F-D3) was from Abcam, anti-IFN-β detection antibody (32400-1) was from R&D system. Human IFN-β ELISA kit was from R&D system. Nevirapine and raltegravir were from the National Institutes of Health AIDS Research and Reference Reagent Program. Antibodies for HA, phospho-TBK1, TBK1, phosphor-IKKε, phospho-IRF3, IRF3, phospho-p65, p65, phospho-STAT1, STAT1, and STING were from Cell Signaling Technology, anti-HIV-1 p24 (39/5.4A) antibody was from Abcam, anti-FLAG M2 antibody was from Sigma, and anti-β-actin antibody was from Santa Cruz. IFNα/β receptor blocking antibody (MAB1155) was purchased from EMDMILLIPORE. Anti-HA and anti-FLAG antibody conjugated beads were from ThermoFisher Scientific. HSV-1 (KOS strain) from ATCC and propagated in Vero cells.

HIV-1 infection

For IFNα/β receptor blocking experiment, THP-1 cell lines were treated with 5 μg/ml of IFNα/β receptor blocking antibody 24 hours before HIV-VSV infection. Infection readout was performed 48 hr post-infection by luciferase assay. For transwell experiment, THP-1 cell lines containing scrambled shRNA or shRNA for *NLRX1* were infected with HIV-VSV at a MOI of 1 by spinoculation (1,000 × g, 25 °C) for 3 hr. Infected THP-1 sh-scramble and sh-NLRX1 cells

were cultured in the upper or lower wells of 6-well transwell plate (3450, CORNING) as indicated in the Figure 2E. THP-1 cells were infected with 50 ng of HIV-R3A by spinoculation (1,000 × g, 25 °C) for 3 hr and cultured in completed RPMI medium. HIV-R3A replication was determined 1, 2, and 3 dpi by quantifying extracellular p24 by ELISA kit from the National Institutes of Health AIDS Research and Reference Reagent Program.

NLRX1 knockdown cell lines

TRC lentivectors containing shRNA for *NLRX1* or scrambled shRNA were from Dharmacon. Scrambled (5'-CACTCACATCGCTACATGA-3') and NLRX1 targeting RNA (sgRNA) (sg-NLRX1-1: 5'-CGTTGACCCTGTGATCC-3', sg-NLRX1-2: 5'-CTGCCATTGGCCGTATC-3') were inserted into the lentivector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro containing Cas9 nuclease (Abm Inc., Canada). Lentiviruses were made by cotransfection of lentiviral vector, VSVG, and ΔNRF into 293T cells. THP-1 cells were infected by lentiviruses containing shRNA or sgRNA by spinoculation (5,500 rpm and 25 °C for 3 h) to generate the NLRX1 knockdown or knockout cell lines. Transduced cells were selected by 2 μg/ml of puromycin. To knockout STING in THP-1 cells, the construct containing sgRNA targeting *STING* (5'-CACCGGCTGGGACTGCTGTTAAACG-3') was generated based on px330 from Dr. Feng Zhang's lab at Massachusetts Institute of Technology. THP-1 cells were electroporated with plasmid expressing Cas9 and sgRNA for *STING* and single clones were selected and confirmed by sequencing and immunoblot.

HSV-1 genomic DNA copy number and viral titer measurement

Genomic DNA was extracted from cells and mouse brains using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol. HSV-1 genomic DNA copy numbers were then determined by real-time PCR using HSV-1 specific primer: 5'-

CATCACCGACCCGGAGAGGGAC-3’, 5’-GGGCCAGGCGCTTGTTGGTGTA-3’. The titer of HSV-1 was measured by plaque assay. Monolayers of Vero cells were infected with virus from brain homogenate for 1 hr and overlaid with 0.8% methylcellulose (Sigma-Aldrich) for 3 days. Plaques were then counted to determine the titer.

Plasmid and molecular cloning

FLAG-tagged and HA-tagged STING (original cloned and named as MITA) and FLAG-tagged TBK1 expression plasmids have been described (Zhong et al., 2008). C-terminal HA-tagged NLRX1 full length and domain truncation expression plasmids have been described previously (Lei et al., 2012; Moore et al., 2008). C-terminal FLAG-tagged NLRX1 was generated by traditional cloning. Briefly: two primers encoding a glycine linker and a 3x-FLAG tag (GGGGDYKDDDDKGADYKDDDDKEFDYKDDDDK) were annealed and extended to generate a dsDNA fragment (5’-ATATGGATCCTCACTTGTCGTCGTCGTCCTTGTAGTCGAATTCCTTGTCGTCGTCGTCCTTGTAGTCTGCCCCTTTATC-3’) and (5’-GCAGCTGGGAAGCTCTGGAAGCGGCGGTGGCGGTGATTATAAAGATGATGATGATAAAGGGGCAGACTACAAGG-3’), which served as the reverse primer for the PCR amplification with an NLRX1 forward primer (5’-GGGGGATATCACCATGAGGTGGGGCCACCATTTGC-3’). The 3x-Flag tagged NLRX1 amplicon was cloned into the EcoRV/BamHI site of pCIG2-IRES-PURO, a variant of pCIG2-IRES-eGFP (Petrucelli et al., 2012), generating a 3x-Flag-tagged human NLRX1 expression construct.

Real time RT-PCR and oligoes

For quantification of cellular mRNA levels of NLRX1 and pRL13A or TATBP, total cellular RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) or NucleoSpin® RNA Kit (Macherey-Nagel) according to the manufacturer's guidelines. One step q-RT-PCR was performed to determine relative expression of mRNAs from MDMs and MDDCs using QuantiTect SYBR® Green RT-PCR Kit (QIAGEN) with the respective specific primers on an ABI7900 cycler (Applied Biosystems). Relative quantification of NLRX1 and TATBP mRNA levels in HEK293T cells was achieved in a two-step q-PCR approach. Briefly, RNA samples were reverse transcribed using the QuantiTect Reverse transcription Kit (Qiagen). PCR products were detected using the Power SYBR® Green PCR Master Mix (Applied Biosystems).

The following primers were used to detect mRNA of the respective genes: hNLRX1: 5'-CAGCGACCAGATGATCGTATC-3', 5'-TGGTGGCGTATAAAGGCCCTA-3'; hpRL13A: 5'-CCTGGAGGAGAAGAGGAAAGAGA-3', 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'; hTATBP: 5'-CCACTCACAGACTCTCACAAC-3', 5'-CTGCGGTACAATCCCAGAACT-3'. Data were normalized to housekeeping gene pRL13A mRNA levels for MDMs and MDDCs and to TATBP for HEK293T cells. For gene expression analysis from THP-1, MEFs, BMDM, peritoneal macrophages, total RNA was extracted from cells or tissues using TRIzol reagent, followed by reverse transcription by MuLV reverse transcriptase (ThermoFisher Scientific) and Real-time PCR as described using SYBR green master mix. Primers used: *Ifnb*: 5'-ATGAGTGGTGGTTGCAGGC-3', 5'-TGACCTTTCAAATGCAGTAGATTCA-3'; *Ifna4*: 5'-CCTGTGTGATGCAGGAACC-3', 5'-TCACCTCCCAGGCACAGA-3'; *Il6*: 5'-ACAACCACGGCCTTCCCTACTT-3', 5'-CACGATTTCCCAGAGAACATGTG-3'; *Tnf*: 5'-CCCTCACACTCAGATCATCTTCT-3', 5'-GCTACGACGTGGGCTACAG-3'; *Rantes*: 5'-ATGAAGATCTCTGCAGCTGCC-3', 5'-

TAGGCAAAGCAGCAGGGAGTG-3’; *Isg15*: 5’-CACAGTGATGCTAGTGGTAC-3’, 5’-CTTAAGCGTGTCTACAGTCTG-3’; *Actb*: 5’-AGGGCTATGCTCTCCCTCAC-3’, 5’-CTCTCAGCTGTGGTGGTGAA-3; *IFNB1*: 5’-CATTACCTGAAGGCCAAGGA-3’, 5’-CAATTGTCCAGTCCCAGAGG-3’; *MX2*: 5’-CAGAGGCAGCGGAATCGTAA-3’, 5’-TGAAGCTCTAGCTCGGTGTTC-3’; *GAPDH*: 5’-GAAGGTGAAGGTCGGAGT-3’, 5’-GAAGATGGTGATGGGATTTC-3’; Data were normalized to housekeeping gene β-actin mRNA levels. Analysis for relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

ISD (5’-TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA-3’), HIV-1 DNA (5’-GTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTG-3’), VACV 70-mer (5'-CCATCAGAAAGAGGTTTAATATTTTTGTGAGACCATCGAAGAGAGAAAGAGATAAAACTTTTTTACGACT-3') was synthesized from Integrated DNA Technologies.

Co-immunoprecipitation

HEK293T cells were transfected with indicated expression vectors for 24-48 hr. Cells were collected after trypsinization and washed twice. Cells were resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH 7.4). Cell lysates were incubated on ice for 30 min, and cell debris was pelleted by centrifugation at 16,000 × g for 30 min at 4°C. Immunoprecipitation was performed by anti-HA or anti-FLAG agarose beads. For endogenous immunoprecipitation, MEF and THP-1 cells were resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, protease inhibitor

cocktail, phosphatase inhibitor cocktail, pH 7.4). Cell lysates were incubated on ice for 30 min, and cell debris was pelleted by centrifugation at 16,000 x g for 30 min at 4°C. Immunocprecipitation was performed by using anti-NLRX1 or anti-STING antibody plus protein G conjugated agarose beads. For endogenous immunoprecipitation upon virus infection, $2x10^7$ BMDMs or PMA-THP-1 were infected by HSV-1 at an MOI of 3, at 37 °C for 1 hr. Cells were then washed with warm PBS and cultured in complete DMEM. At indicated time points, infected cells were collected for immunoprecipitation by using anti-NLRX1 antibody plus protein G conjugated agarose beads.

Recombinant protein purification and in vitro pull-down

Recombinant baculovirus expressing NLRX1 carrying an N-terminal Halo-Tag (HALO) and C-terminal hexahistidine tag (6xHIS) was generated as previously described (Mo et al., 2012; Zhang et al., 2014). Purified NLRX1 (1 μg) was added to IP buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM β-mercaptoethanol, and 0.1% CHAPS and purified STING proteins (1 μg) (Ouyang et al., 2012). The mixture was incubated with anti-STING or anti-NLRX1 antibody while rotating at 4°C overnight. The STING protein and associated NLRX1 were captured by protein A paramagnetic beads (Miltenyi) according to the manufacturer's protocols and assayed by immunoblot for STING and NLRX1 as described.

Indirect immunofluorescence assay

HEK293T cells were transfected with FLAG-NLRX1 and HA-STING expression plasmid, applied onto slides, and fixed with 4% paraformaldehyde. The fixed cells were permeabilized with phosphate-buffered saline (PBS) plus 0.2% Triton X-100, stained with anti-HA (3724S; Cell Signaling) and anti-FLAG (F1804, Sigma) followed with AF546-conjugated anti-rabbit antibody and AF488-conjugated anti-mouse IgG1 antibody (A-11035 and A11029; Invitrogen),

and then counterstained with DAPI (1 μg/ml). Cells were analyzed with a Zeiss LSM 710 laser-scanning confocal microscope.

Subcellular fractionation and MAM isolation

Cytosolic and membrane/particulate proteins from human foreskin fibroblast cells were prepared by using FractionPREP^{TM} Cell Fractionation Kit from BioVision. Enriched heavy MAM was isolated as previously described (Zhang et al., 2011). MAM proteins were diluted IP buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH 7.4) and used for immunoprecipitation as described above.

Protease K protection assay

Briefly, mitochondria were isolated by permeabilizing the cells in digitonin buffer (0.015% digitonin , 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM HEPES, pH 7.4 with PMSF) for 3 min at 4 °C followed by centrifugation at 12,000 × g for 10 min at 4 °C. The resulting mitochondria-enriched pellet was washed once with osmotic buffer (125 mM KCl, 4 mM $MgCl_2$, 5 mM KH_2PO_4 and 10 mM HEPES, pH 7.4). The mitochondrial suspension was then aliquoted and pelleted by centrifugation at 12,000 × g for 2 min at 4 °C. Each aliquot of mitochondria were incubated for 30 minutes on ice with or without protease K in 100 μl of osmotic buffer. The reaction was terminated by the addition of 2 mM PMSF. Samples were then analyzed by immunoblotting (Itahana and Zhang, 2008; Liu et al., 2012).

Luciferase assay

HEK293T cells were seeded in 24-well plates at a density of 1.0×10^5 per well and transfected the following day by lipofectamine 2000 following the manufacturer's instruction. 10 ng of pRT-TK Renilla luciferase reporter plasmid and 100 ng of ISRE firefly luciferase reporter plasmids were transfected together with 100 ng of indicated expression plasmids. Luciferase activity was

measured 24 hr post-transfection using the Dual-Glo® Luciferase Assay System. Firefly luciferase activity was also measured from HIV-VSV infected MEFs and THP-1 cells at indicated time points according to the manufacture's instruction.

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