

Supplementary Information for

Nitro-fatty acids are formed in response to virus infection and are potent inhibitors of STING palmitoylation and signaling

Anne Louise Hansen, Gregory J. Buchan, Michael Rühl, Kojiro Mukai, Sonia R. Salvatore, Emari Ogawa, Sidsel D. Andersen, Marie B. Iversen, Anne L. Thielke, Camilla Gunderstofte, Mona Motwani, Charlotte T. Møller, Andreas S. Jakobsen, Katherine A. Fitzgerald, Jessica Roos, Rongtuan Lin, Thorsten J. Maier, Raphaela Goldbach-Mansky, Cathrine A. Miner, Wei Qian, Jonathan J. Miner, Rachel E. Rigby, Jan Rehwinkel, Martin R. Jakobsen, Hiroyuki Arai, Tomohiko Taguchi, Francisco J. Schopfer, David OLAGNIE, Christian K. Holm.

Christian Kanstrup Holm
Email: holm@biomed.au.dk

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SI Materials and Methods

Animals. Pathogen-free C57BL/6 (WT) mice were bred at animal vendor, Janvier Labs, France and *nos2*^{-/-} or *inos*^{-/-} (B6.129S2–*Nos2tm1MrI* N12) mice were bred at the animal facility, Department of Biomedicine, Aarhus University. All experiments were carried out at Aarhus University. Prior to infection experiments, mice were kept at the animal facility for at least 5 days. All female mice used for experiments were age-matched and 8 weeks old at time of infection. Animals received proper care in agreement with animal protocols approved by Animal Welfare Bodies at Health, Aarhus University and conducted with ethical permission from the Animal Experiments Inspectorate, Danish Veterinary and Food Administration to perform vaginal HSV-2 infection.

Cells lines and Cell Culture. Murine macrophage cell line, RAW264.7, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 100 U ml⁻¹ Penicillin, 100 ug ml⁻¹ Streptomycin and 292 µg ml⁻¹ L-glutamine (Gibco) hereafter termed complete DMEM. RAW264.7 cells deficient in iNOS (*Nos2*^{-/-}) or Nrf2 (*Nrf2*^{-/-}) were generated using CRISPR/Cas9 system, more specifically by employing the pLentiCRISPR/Cas9 V2 vector (Addgene). The guide RNAs (gRNA) used had the sequence GAAGTCTCGAACTCCAATCT (*nos2*, exon 9) or the sequence GACTTGGAGTTGCCACCGCC (*nrf2*, exon 1), respectively. For controls, the pLentiCRISPR/Cas9 V2 vector was used without a gRNA sequence. Lenti-virions were produced by transfecting HEK293T cells with pLentiCRISPR/Cas9 V2 vector, pMD.2G (Addgene) pRSV.Rev (Addgene) and pMDlg/p-RRE (Addgene). Knock-out of the targeted gene-products were validated by immunoblotting or other functional assays. Murine bone marrow-derived macrophages (BMM) were prepared from either freshly harvested or frozen bone marrow. The cells were differentiated over 6 days using 20 – 40 % L929 supernatant (M-CSF producing murine fibroblast cell line) in RPMI 1640 (Lonza) supplemented with 10 % heat-inactivated FCS (Sigma-Aldrich), 100 U ml⁻¹ Penicillin, 100 ug ml⁻¹ Streptomycin and 292 µg ml⁻¹ L-glutamine (Pen-Strep-Glut, Gibco) hereafter termed complete RPMI. Murine bone marrow-derived dendritic cells (BMDC) were obtained by collecting non-adherent cells after 7 days culture of bone marrow cells in the presence of GM-CSF (R&D systems in complete RPMI. The human acute monocytic leukemia cell line, THP-1, was cultured in complete RPMI. To differentiate THP-1 cells into adherent macrophages, cells were stimulated with 150-200 nM Phorbol 12-myristate 13-acetate, (PMA, Sigma-Aldrich) in complete RPMI for 24 hours/over-night and then rested for an additional day in complete media without PMA before stimulation. Human Embryonic Kidney 293T, HEK293T, cells were cultured in complete DMEM. Mouse embryonal fibroblasts (MEFs) from STING KO mice genetically modified to express EGFP-STING (Mukai et al., 2016). Fibroblasts prepared from patients suffering from STING-associated vasculopathy with onset in infancy (SAVI) all have a mutation in the coding region, c.461A>G, of STING (*TMEM173*) leading to an Asn to Ser substitution in protein, p.N154S. The SAVI fibroblasts were cultured in complete DMEM, using a 50 % media change at passing and seeded 48 hours prior to stimulation. The murine fibroblast cell line, L929, was cultured in complete DMEM with 5-10 % supplementation of FCS. The L929 cells were used to measure murine IFN- α/β bioactivity and the supernatant of confluent L929 was harvested and used for BMM differentiation. HEK-Blue IFN- α/β (InvivoGen) cells were cultured in complete DMEM further supplemented with 30 µg ml⁻¹ Blasticidin, 100 µg ml⁻¹ Normocin and 100 µg ml⁻¹ Zeocin (InvivoGen). Blasticidin and Zeocin were left out of the test-medium during IFN- α/β assay.

Viruses and Reagents. HSV-2 virus used was laboratory strain 333, propagated in Vero cells using standard conditions. Nitrated oleic acids, 9-NO₂-OA and 10-NO₂-OA were obtained from Cayman Chemicals together with control lipids oleic acid (OA), linoleic acid (LA) and

conjugated linoleic acid (cLA). HSV-2 virus used was laboratory strain 333, propagated in Vero cells using standard conditions. NO₂-cLA, isotopically labeled NO₂-FA and biotinylated 10-NO₂-OA were synthesized as previously described. The concentration of NO₂-cLA was determined in methanol using $\lambda_{312} = 11.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 312nm in methanol(1). Biotinylated 10-NO₂OA and bionylated OA was kindly provided by Francisco Schopfer (University of Pittsburgh). LPS was purchased from Invivogen (tlrl-b5lps), murine IFN γ was purchased from R&D systems (485-MI). HEK293T were transfected with various plasmids (see below) using either PEI (2 μ l/ml, Sigma-Aldrich) using Lipofectamine2000 (4 μ l/mL, Invitrogen). PPAR γ inhibitors were purchased from Cayman Chemicals (GW9662 and T0070907). Sendai virus was acquired from Charles River. Plasmid expression wild type hSTING Flag-tagged was kindly provided by professor Rongtuan Lin (McGill University, Canada). Plasmid expression wild type hSTING was kindly provided by professor Cambier(2). Plasmids with mutations in human STING related to STING-associated vasculopathy with onset in infancy (SAVI); V147L SAVI, N154S SAVI and V155M SAVI was kindly provided by Kate Fitzgerald (University of Massachusetts Medical School, USA).

Vaginal HSV-2 Infection. Mice were injected sub-cutaneous with 2 mg Depo-Provera (Pfizer) 5 days before infection. For infection, mice were anesthetized with Isoflurane (Sigma Aldrich) and inoculated intra-vaginally with 20 μ l HSV-2 (strain 333, 6.7×10^4 pfu) suspended in sterile PBS (Sigma-Aldrich). Hereafter, the mice were positioned on their backs and maintained under light anesthesia for 10 min. Inoculation intra-vaginally with either 1 mM cLA or LA in PBS were completed with lightly anesthetized mice positioned under their back on a heating pillow for 30 min. Vaginal lavages were collected after infection (p.i.) under anesthesia by gently washing the intra-vaginally lumen with 2 x 40 μ l sterile PBS. The lavages were diluted to a final volume of 250 μ l. The lavages sent for mass spectrometry for investigation of NO₂cLA production were snap-frozen immediately after harvest. The lavages and harvest vaginal tissue used for flow cytometric investigation of iNOS production in cellular subsets were stained immediately.

Analytical Determination of NO₂-FAs Levels. Plasma (200 μ l), lavage (500 μ l) and tissue homogenates (approx. 25 mg/500 μ L) were spiked with 10 pmoles of internal standard (10-¹⁵NO₂-OA-d4) followed by mercury (II) chloride (10-20 mM) and incubated at 37 °C for 30 min with shaking to release NO₂-cLA from small peptide and protein adducted cysteines. After incubation, plasma samples were extracted with 1 mL isopropanol/hexane/formic acid (10:15:1), 0.5 mL water, and 1 mL hexanes. Then mixed and spun at 2800 x g for 10 min at 4°C. Top organic layer was dried under stream of nitrogen. Saturated sulfanilamide (7.5 mg/mL) was added to plasma prior to extraction and during mercury chloride addition to avoid any artifactual nitration of residual nitrite during the acidic extraction. Urine was extracted using solid phase extraction (C18), and tissue homogenate using 3 ml of chloroform/methanol (2:1). Dried extracts were solvated in methanol for LC-MS/MS analysis. Samples were quantified by isotopic dilution LC-MS/MS using a 6500+ Qtrap (Sciex) or a API 5000 (Applied Biosystems) using the following settings: curtain gas 40, CAD medium or 4, source temp 650°C or 600°C, GS1 60 or 55, GS2 45 or 60, declustering potential -80, entrance potential -10 or -5, CE -42 or -35, CXP -12 or -3 for 6500+Qtrap and 5000 respectively. NO₂-cLA was resolved using a reverse phase Luna C18 analytical column (2x100 mm, 5 μ m, Phenomenex) using a flow rate of 0.65 mL/min or a Gemini C18 column (2x20 mm) with a flow rate of 0.7 mL/min. Gradient consisted of solvent A (water/0.1% acetic acid) and solvent B (acetonitrile/0.1% acetic acid). For samples resolved using the Luna column, a 15 min method was used. Samples were injected at 20% B and B increased to 100% over 10 min, maintained at 100% for 2 min, and then re-equilibrated for 3 min at initial conditions. A shorter method (5 min) was developed using the Gemini column consistent of the same solvents and using a flow rate of 0.7 mL/min. The short gradient ramped B from 35 to 100% from 0.1 to 3 min, maintained 100 % for a minute to re-equilibrate at 35% for an additional minute. Data were collected and analyzed with Analyst Software. Quantification was done using

a standard curve using NO₂-cLA concentrations ranging from 1 to 500 nM in the presence of 20 nM of isotopically labeled 10-¹⁵NO₂-OA-d4 or ¹⁵NO₂-cLA. Peak integration was manually confirmed. The following transitions were used: 324/46 (NO₂-cLA), 331/47 (10-NO₂-OA-d4), and 325/47 (¹⁵NO₂-cLA). All solvents were LC-MS grade.

Detection of Nitro-Alkylation by Mass Spectrometry. STING samples were bound to NHS-modified magnetic beads (NHS Mag Sepharose magnetic beads, GE healthcare, Great Britain) according to the manufacturer's protocol. After washing the beads twice with 50 mM Ammonia bicarbonate (Sigma Aldrich, Taufkirchen, Germany), STING loaded beads were pipetted onto the 384-well MALDI-MS stainless steel target plate (Thermo Fisher Scientific, Bremen, Germany), mixed with proteomics grade trypsin (Promega, Mannheim, Germany) and then subsequently digested for 1h at 37°C as described previously(3). After digestion beads were removed and the sample was mixed with 3 mg/mL α -cyanohydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) solution (70% acetonitrile, 0.1% trifluoroacetic acid) and cocrystallized with the analytes. Crystals were washed with 5% FA and then recrystallized with 80%/0.1% acetonitrile/trifluoroacetic acid. MALDI MS measurements were conducted using a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) in Fourier transformation mode, optimized laser energy, positive polarity, 30000 resolution, automatic gain control value of 5×10^5 and a mass-to-charge range of 800-4000. For data analysis, RAW files were converted to mzML files by MSConvert (Version 5.5.0) mzML were loaded into mMass³ (Version to perform signal processing like peak picking, deisotoping and generating a mass-intensity list. Database search was performed using the following parameters: peptide mass tolerance 10 ppm, fixed modifications none, variable modifications nitrooleic acid, nitrosyloleic acid (NO-OA), amino oleic acid (NH₂-OA) on cysteine residues due to reduction and laser desorption and methionine oxidation, enzyme for digestion trypsin with maximum 1 missed cleavage, protein sequence Stimulator of interferon genes protein (Uniprot, updated: 04/2017).

Mass Spectrometric Measurement of Synthetic Peptides for Mass Shift Determination.

Synthetic peptide AAAACAAAAR (JPT Peptide Technologies) was diluted to 20 μ M and mixed with 20 μ M NO₂-OA in a total volume of 2 μ L directly on the MALDI sample plate. Incubation at 37° C for 1 h was conducted as previously described in Rühl et al., 2017, α -cyano-cinnamic acid was added to stop the reaction and MALDI-mass spectrometry was conducted as described in above. Spectra were investigated for characteristic mass shifts.

Metabolic Labelling with [³H]-Palmitate. Sting^{-/-} MEFs reconstituted with EGFP-tagged mouse STING (WT) were pretreated with 10-NO₂-OA in DMEM containing 0.1% essentially fatty acid-free BSA (BSA-DMEM) for 4 h at 37 °C, and starved with BSA-DMEM for 1 h. Cells were then metabolically labelled with 0.1 mCi ml⁻¹ [³H] palmitate at 37 °C. One hour after the labelling, DMXAA (final 25 mg ml⁻¹) was added to the cell medium. After incubation for appropriate times, cells were washed with ice-cold PBS, scraped in immunoprecipitation buffer composed of 50 mM HEPES-NaOH (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% SDS, 1% Triton X-100, protease inhibitors (Complete EDTA- free Protease inhibitor cocktail (Roche)) and phosphatase inhibitors (8 mM NaF, 12 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1.2 mM Na₂MoO₄, 5 mM cantharidin and 2 mM imidazole). The lysates were then sonicated on ice and diluted to 0.1% SDS. After centrifugation at 15,000 r.p.m. for 10 min at 4 °C, the resultant supernatants were incubated for overnight at 4 °C with anti-GFP (3E6), and then incubated for 3 h with protein G plus agarose (ThermoFisher scientific). The beads were washed four times with immunoprecipitation wash buffer (50 mM HEPES-NaOH (pH 7.2), 150 mM NaCl, 0.1% Triton X-100) and eluted with 2xLaemmli sample Buffer. The immunoprecipitated proteins were separated with SDS-PAGE and transferred to PVDF membrane, then autoradiographed with BAS-IP TR2040 and Typhoon9000 (GE Healthcare).

Immunoblotting. Cells were lysed in 100 μ L of ice-cold Pierce RIPA lysis buffer (ThermoFisher Scientific) supplemented with 10 mM NaF, 1x complete protease cocktail inhibitor (Roche) and 5 IU mL⁻¹ benzonaze (Sigma), respectively. Protein concentration was determined using a BCA protein assay kit (ThermoFisher Scientific). Whole-cell lysates were either left non-reduced and non-denatured (for semi-native condition in the detection of (STING)₂, and (IRF)₂) or denatured for 3 min at 95°C in presence of 1x XT Sample Buffer (BioRad) and 1x XT reducing agent (BioRad). 5-10 mg of samples were separated by SDS-PAGE on 4-20% Criterion TGX precast gradient gels (BioRad). Each gel was run initially for 15 min at 70V and 45 min at 120V. Transfer onto PVDF membranes (BioRad) was done using a Trans-Blot Turbo Transfer system for 7 min. Membranes were blocked for 1h with 5 % skim-milk (Sigma Aldrich) at room temperature in PBS supplemented with 0.05 % Tween-20 (PBST). Membranes were fractionated in smaller pieces and probed overnight at 4 °C with any of the following specific primary antibodies in PBST: anti-TBK1/NAK (3013, Cell Signaling 1:1000), anti-phospho-TBK1/NAK (5483, Cell Signaling 1:1000), anti-IRF3 (11904, Cell Signaling 1:1000), anti-phospho-IRF3 (4947, Cell Signaling 1:500), anti-STING (13647, Cell Signaling 1:1000), anti-Flag /Sigma, F3165, 1:2000) and anti-Vinculin (18799, Cell Signaling 1:1000) used as loading control. For MEF cells, rabbit polyclonal anti-STING(4) (1:100) or rabbit anti-STING antibody (19851-1-AP, Proteintech, 1:1000) and mouse anti- α -tubulin (DM1A Sigma, 1:1000). After three washes in PBST, secondary antibodies, peroxidase-conjugated F(ab)₂ donkey anti-mouse IgG (H+L) (715-036-150, 1:10000) or peroxidase-conjugated F(ab)₂ donkey anti-rabbit IgG (H+L) (711-036-152, 1:10000) and for MEF cells, Donkey anti-mouse IgG (H+L) conjugated with DyLight405 (715-475-150, 1:2000), colloial gold (CG) particle-conjugated donkey anti-rabbit antibody (12 nm, 711-205-152, 1:20), (Jackson ImmunoResearch) were added to the membrane in PBST 1% milk for 1h at room temperature. All membranes were washed three times and exposed using either the SuperSignal West Pico PLUS chemiluminescent substrate or the SuperSignal West Femto maximum sensitivity substrate (Both ThermoScientific) and an ImageQuant LAS4000 mini Imager (GE Healthcare).

Flow cytometry. Single-cell suspension of mouse vaginal tissue were achieved by both mechanical and enzymatic processing using 1 mg/ml Collagenase/Dispase (Roche) and 100 μ g/ml DNaseI (Roche) Cells were stained with LiveDead Violet (Invitrogen) in PBS (Sigma-Aldrich) for 10 min on ice and in the dark to evaluate viability. Subsequently, cells were incubated with primary antibodies for surface-markers in staining buffer consisting of PBS containing 2 % BSA (Company) for 30 min on ice. Cells were fixed using 2 % formaldehyde (Sigma) for 15 min at RT and before permeabilizing cells for intracellular staining using 0.05 % Saponin solution in staining buffer. Staining for intracellular markers were conducted in 0.05 % Saponin solution in staining buffer for 30 min on ice. All staining procedures were completed in the dark. The following anti-mouse antibodies were used for flow analysis: CD45-V500 (clone 30-F11, BD Horizon), NOS2-APC (clone CXNFT, eBioscience), IgG2a-APC kappa-isotype (eBioscience). Samples were analyzed using BD LSRFortessa Cell Analyzer with BD FACSDiva Software (Becton Dickinson) and data was processed using FlowJo software version 10.0.8 (Tree Star Inc.).

Nitrite measurements. Griess Reagent Kit for Nitrite Determination (Molecular Probes) was used to measure levels of nitrite production to the recommendations the manufacture. Samples for nitrite measurements were prepared in DMEM without phenol-red (Lonza). Nitrite-containing samples were assessed by measuring optical density at 540 nm on a microplate reader (ELx808, BioTEK). The detection limit of the assay is 1 μ M nitrite.

Cytokine measurements. Human IL-1 β and MIP-1 β , together with mouse and human IL-6 was detected by DuoSet ELISA development System for R&D according to manufacturer's instructions.

RNA isolation and quantitative RT-PCR. RNA was extracted using High Pure RNA Isolation Kit (Roche) according to the recommendations of the manufacturer. RNA quantity was controlled using NanoDrop spectrometry (ThermoFisher Scientific). Gene expression was determined by quantitative real-time PCR. mRNA encoding of *hIFN- β* (ID Hs01077958_s1), *hIFIT1* (ID Hs03027069_s1), *hISG15* (ID Hs01921425_s1) and *h β -Actin* (ID Hs01060665_g1) was quantified TaqMan RNA-to-Ct 1-step Kit (Applied Biosystems) following recommended procedures by the manufacturer. All primer/probe mixes were obtained from TaqMan Gene Expression Assays.

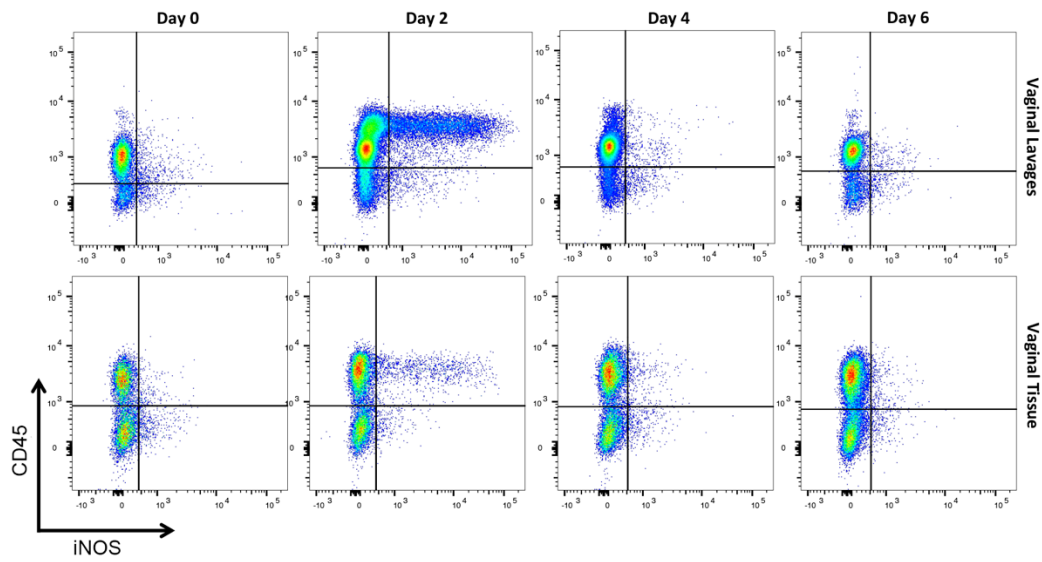


Fig. S1. Cellular expression of iNOS during vaginal HSV-2 infection. Representative dot plots of CD45 and iNOS expression in C57BL/6 (WT) mice infected intra-vaginally with HSV-2 (6.7×10^4 pfu/mouse). Vaginal lavages together with vaginal tissue were harvested from uninfected mice (day 0) or at day 2, 4 and 6 p.i. Debris, doublets and dead cells have been excluded and analyzed for iNOS induction by flow cytometry.

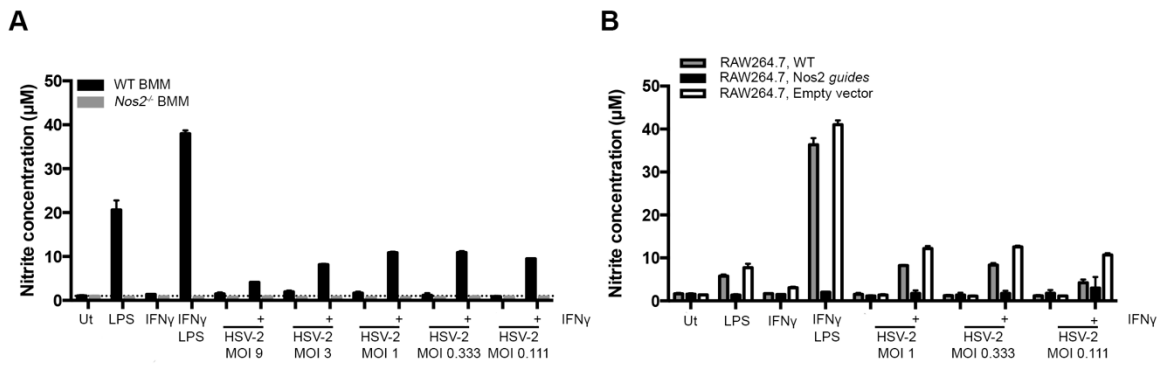


Fig. S2. Nitrite release in response to HSV-2 infection. (A) BMM from WT or *Nos2*^{-/-} mice and (B) RAW264.7 WT, RAW264.7 control cells (Empty vector) or RAW264.7 cells with CRISPR/Cas9-mediated deletion of *Nos2* expression were stimulated with combinations of IFN γ (10 ng/mL), LPS (1 μ g/mL) and HSV-2 (various MOIs). After 24 h of stimulation, supernatants were analyzed for content of nitrite using Griess Reagent. Data are presented as mean \pm SEM.

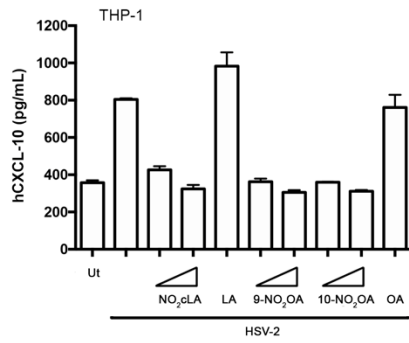


Fig. S3. Nitro-fatty acids suppress release of CXCL10 after HSV-2 infection. THP-1 cells were infected with HSV-2 (MOI 1) 2 h prior to treatment with indicated NO₂-FAs (5-10 μM) or OA/LA (10 μM). After 20 h incubation in total, supernatants were analyzed for CXCL-10. Data are presented as mean ± SEM.

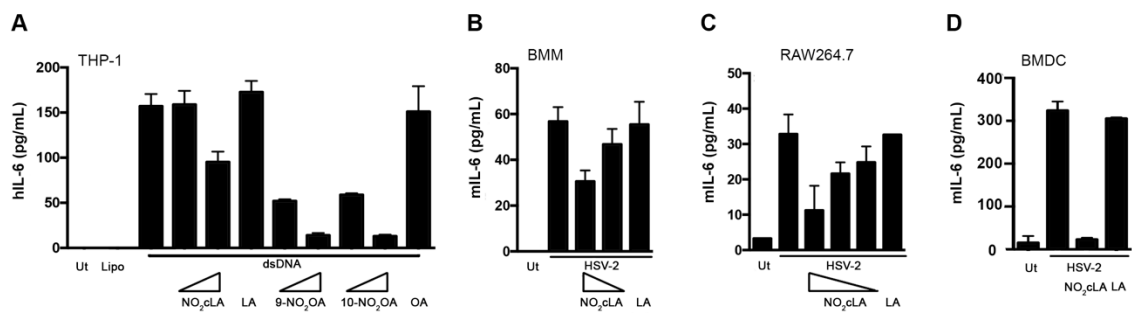


Fig. S4. Nitro-fatty acids suppress release of IL-6. (A) THP-1 cells, (B) BMM (WT mouse), (C) RAW264.7 WT and (D) BMDC (WT mouse) were treated with indicated NO₂-FAs (5-10 μ M) or OA/LA (10 μ M) 15 min prior to stimulation with either dsDNA (4 μ g/mL) or infection with HSV-2 (MOI 1). After 20 h, supernatants were analyzed for IL-6 by ELISA. Data are presented as mean \pm SEM.

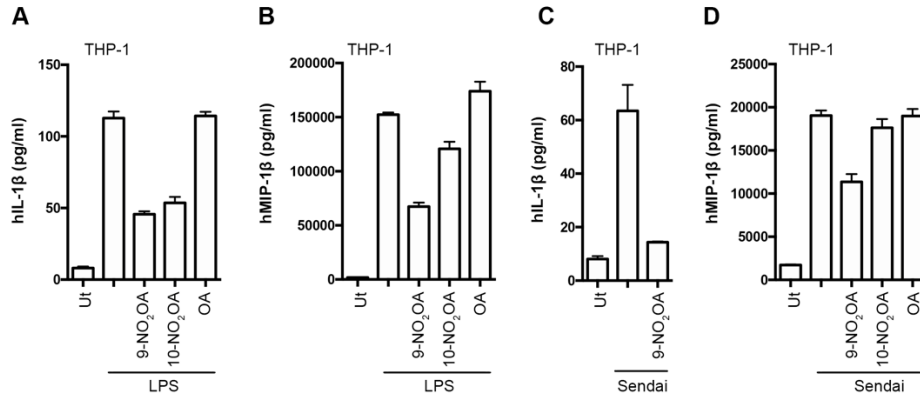


Fig. S5. Nitro-fatty acids suppress release of IL-1 β and MIP-1 β after LPS stimulation and Sendai virus infection. THP-1 cells stimulated with (A, B) LPS (0.5 μ g/mL) or (C, D) infected with Sendai virus. After 8 h, supernatants were analyzed for (A, C) IL-1 β or (B, D) MIP-1 β by ELISA. Data are presented as mean \pm SEM.

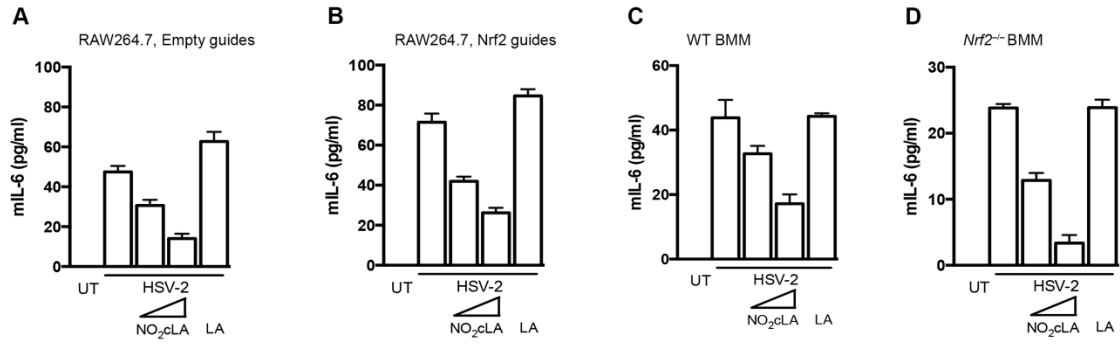


Fig. S6. Nitro-fatty acids inhibit cytokine release independently of Nrf2. (A) RAW264.7 control cells (Empty vector) or (B) RAW264.7 cells with CRISPR/Cas9-mediated deletion of *Nrf2* expression and (C) BMM from WT or (D) *Nrf2*^{-/-} mice were treated with NO₂cLA (5-10 μM) or LA (10 μM) prior to infection with HSV-2 (MOI 1). After 20 h of stimulation, supernatants were analyzed for IL-6 by ELISA. Data are presented as mean ± SEM.

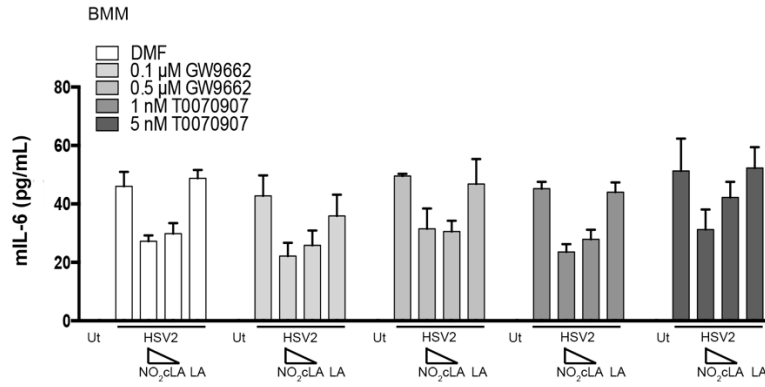


Fig. S7. Nitro-fatty acids inhibit cytokine release independently of PPAR γ . BMM from WT mice were treated with the PPAR γ inhibitors, GW9662 (0.1 or 0.5 μ M) or T0070907 (1 nM or 5 nM) for 1 h prior to treatment with NO₂cLA (5-10 μ M) or LA (10 μ M) preceding infection with HSV-2 (MOI 1). After 20 h of stimulation, supernatants were analyzed for IL-6 by ELISA. Data are presented as mean \pm SEM.

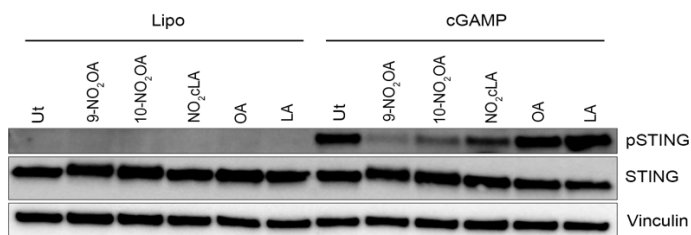


Fig. S8. Nitro-fatty acids suppress phosphorylation of STING. THP-1 cells were treated with NO₂-FAs (10 μ M) or OA/LA (10 μ M) 15 min prior to stimulation with cGAMP (4 μ g/mL) using Lipofectamine2000 (Lipo). After 3 h, lysates were separated by SDS-PAGE for detection of pSTING and STING by western blotting using specific antibodies. Vinculin was used as loading control.

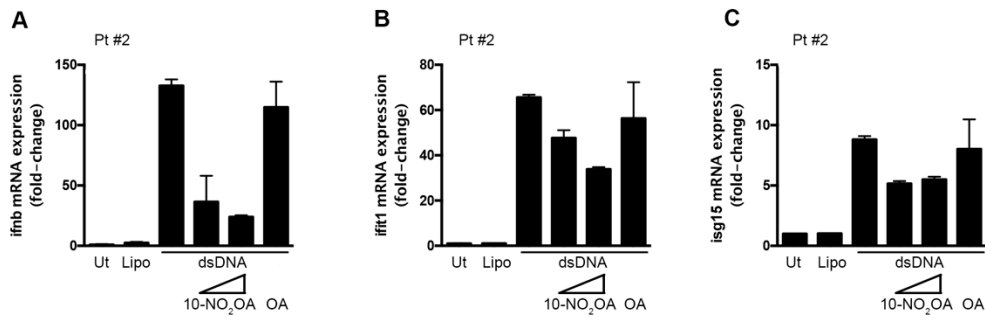


Fig. S9. Nitro-fatty acids inhibit expression of IFN β and ISG in SAVI fibroblasts. Immortalized fibroblasts derived from one SAVI patient (Pt #2) were treated with 10-NO₂OA (5-10 μ M) or OA (10 μ M) 15 min prior to stimulation with dsDNA (4 μ g/mL). After 6 h, RNA was isolated and mRNA expression of (A) IFN β and the two ISGs, (B) IFIT1 and (C) ISG15 were assessed by qPCR. Data are displayed as mean \pm SEM.

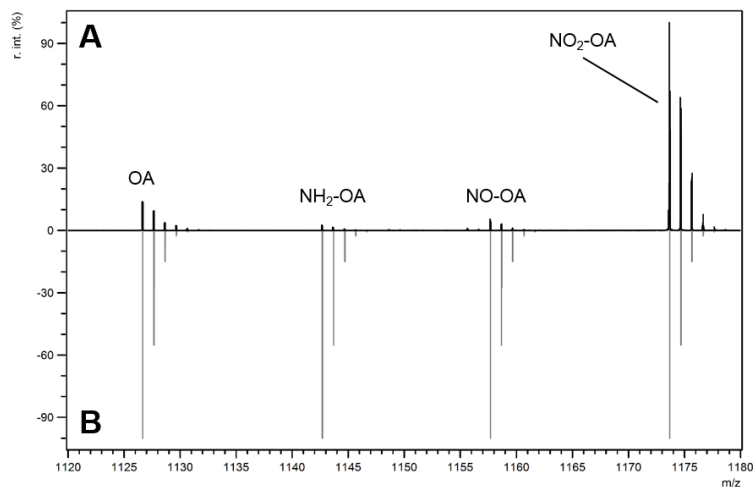


Fig. S10. Nitro-fatty shows mass shifts of reduced nitro-moiety in MALDI-MS spectra.

Synthetic peptide AAAACAAAAR was incubated for 1 h at 37 °C directly on the MALDI sample plate. Mass spectrum shows the main ion of the NO₂-OA modified with a mass shift of 327 Da as previously described. The neighboring mass shifts of 310 Da, 293 Da and 278 Da could be identified as Nitrosyl-OA (NO-OA), Amino-OA (NH₂-OA) and the nitric acid loss and conversion to oleic acid (OA). **(A)** Upper panel in black shows the measured spectrum and **(B)** lower panel in grey shows the theoretical spectrum calculated with mMass. None of these reductive modifications occurred in spectra obtained from histidine-containing peptides. The r.int. (%) denotes relative intensity in %.

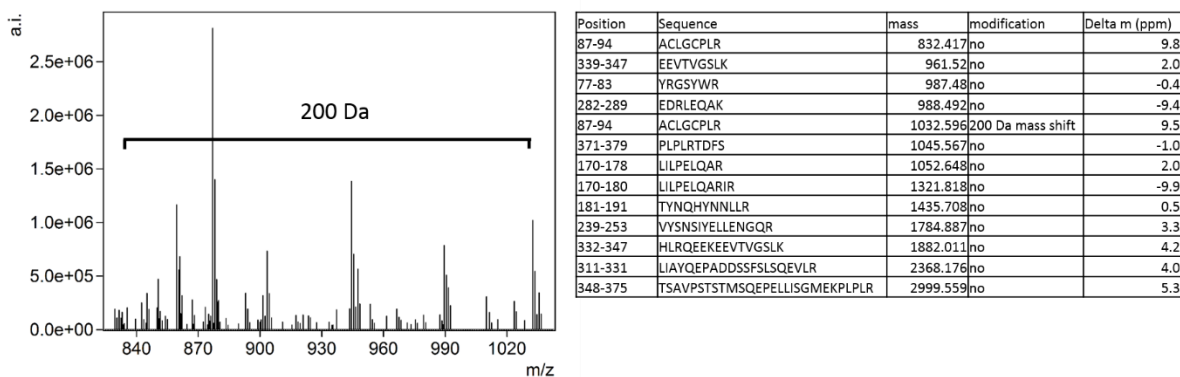


Fig. S11. Investigation of NO₂-OA modified peptides using previously reported mass shifts by Gil et al., 2013.

The spectrum obtained from NO₂-OA treated samples were reinvestigated to confirm our experiment with mass shifts, Gil et al described in 2013. In the spectrum, we found the Peptide ACLGCPLR modified, which confirms our findings. The spectrum (*left*) displays the 200 Da mass shift of the peptide, The table (*right*) gives an overview of the matched peptides using this variable modification. The peptide with the 200 Da mass shift did not occur in the untreated sample. The a.i. denotes absolute intensity.

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