iScience, Volume *23*

Supplemental Information

Influenza Virus-Induced Oxidized DNA Activates Inflammasomes

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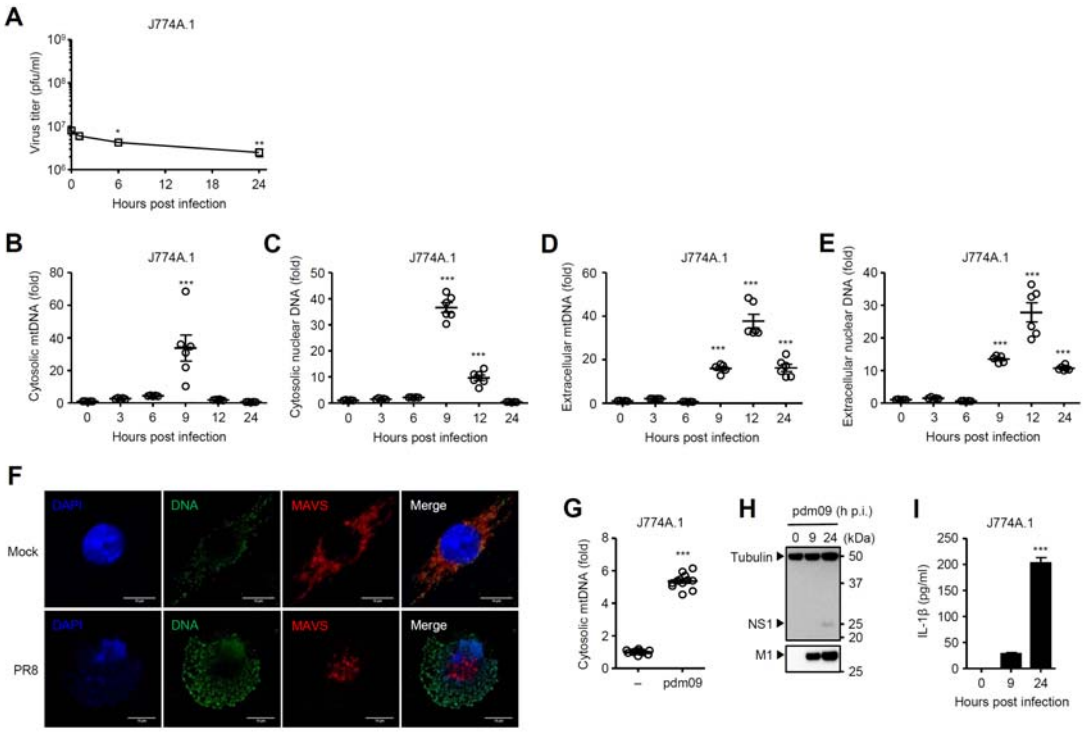

Figure S1. Influenza virus triggers DNA release from macrophages. Related to Figure 1.

(A) J774A.1 macrophages were infected with PR8 virus. Supernatants were collected at indicated time points and viral titers were determined by standard plaque assay.
(B-E) J774A.1 macrophages were infected with PR8 virus. Pure cytosolic extracts and supernatants were collected at indicated time points. Relative levels of mtDNA (B, D) or nuclear DNA (C, E) in the cytosol and supernatants were assessed by quantitative PCR.
(F) BMMs were infected with PR8 virus. At 24 h post infection, cells were stained with anti-dsDNA (AC-30-10) and anti-MAVS antibodies and analyzed by confocal microscopy. Scale bars, 10 μm.
(G-I) J774A.1 macrophages were infected with A/Narita/1/2009 (H1N1) virus (pdm09) at MOI of 3. Pure cytosolic extracts were collected at 9 h p.i.. Relative levels of mtDNA in the cytosol were assessed by quantitative PCR (G). Cell lysates were collected at indicated time points and blotted using the indicated antibodies (H). Cell-free supernatants were collected at indicated time points and analyzed for IL-1β by ELISA (I).
These data are from two independent experiments (A-E, G, I; mean ± s.e.m.). **P < 0.01 and ***P < 0.001 vs. mock-infected cells (one-way ANOVA and Tukey's test).

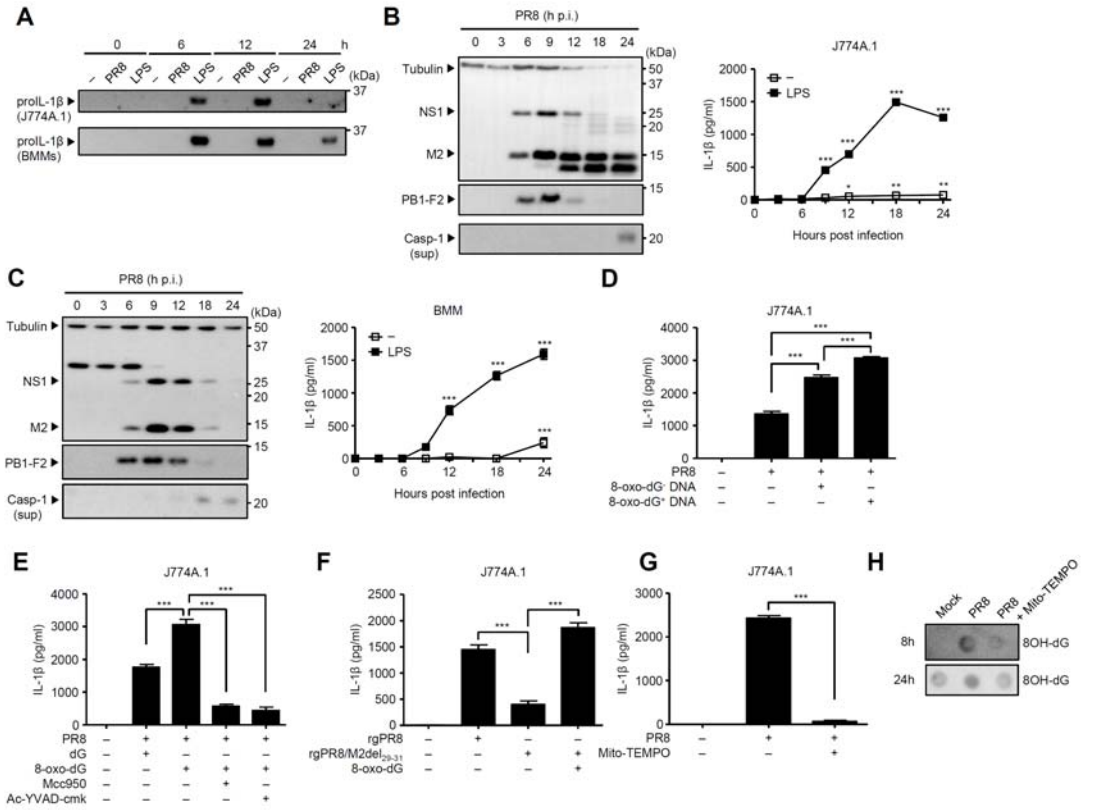

Figure S2. Role of oxidized DNA in influenza virus-induced IL-1β secretion. Related to Figure 4.

(A) J774A.1 (upper panel) and BMMs (lower panel) were stimulated with PR8 or LPS. Cell lysates were collected at indicated time points and analyzed by immunoblotting with rabbit polyclonal antibody to mouse IL-1β (AB1413; Millipore).
(B) LPS-stimulated J774A.1 macrophages were infected with PR8 virus. Cell lysates were collected at indicated time points and analyzed by immunoblotting with indicated antibodies (left panel). Cell-free supernatants were collected and analyzed for IL-1β by ELISA (right panel).
(C) LPS-stimulated BMMs were infected with PR8 virus. Cell lysates were collected at indicated time points and analyzed by immunoblotting with indicated antibodies (left panel). Cell-free supernatants were collected and analyzed for IL-1β by ELISA (right panel).
(D) LPS-stimulated J774A.1 macrophages were infected with PR8 for 24 h in the presence or absence of oxidized or control DNA. Cell-free supernatants were collected and analyzed for IL-1β by ELISA.
(E, F) LPS-stimulated J774A.1 macrophages were infected with PR8 (E), rgPR8, or rgPR8/M2del29–31 virus (F) for 24 h in the presence or absence of dG (246 μM), 8-oxo-dG (246 μM), Mcc950 (20 μM), or Ac-YVAD-cmk (20 μM). Cell-free supernatants were collected and analyzed for IL-1β by ELISA.
(G, H) LPS-stimulated J774A.1 macrophages were infected with PR8 virus for 24 h in the presence or absence of Mito-TEMPO (500 μM). Cell-free supernatants were collected and analyzed for IL-1β by ELISA (G). Pure cytosolic extracts were collected at indicated time points. Oxidized DNA in the cytosol was detected by dot blot analysis using anti-8OH-dG antibody (H).
These data are from two independent experiments (B-G; mean ± s.e.m.). ***P < 0.001; (one-way ANOVA and Tukey's test).

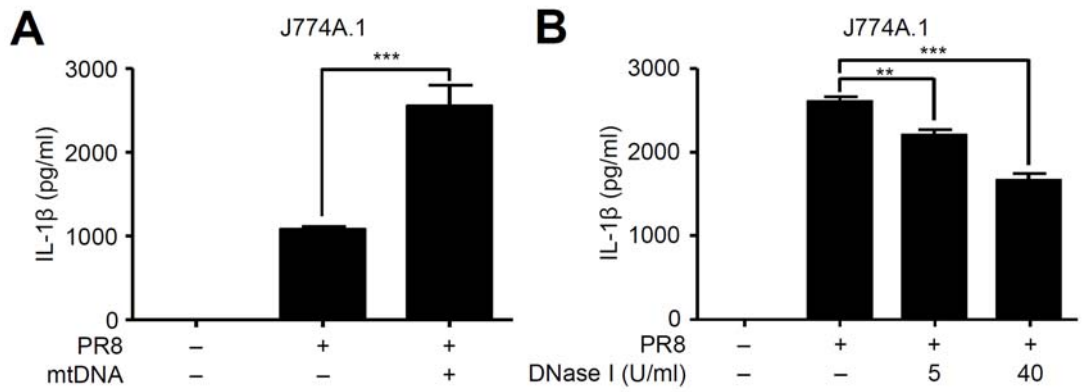

Figure S3. Role of DNA in influenza virus-induced IL-1β secretion. Related to Figure 5.

(A, B) LPS-stimulated J774A.1 macrophages were infected with PR8 virus for 24 h in the presence or absence of mtDNA fragment prepared as described in Materials and Methods (A) or DNase I (B). Cell-free supernatants were collected and analyzed for IL-1β by ELISA.
These data are from three independent experiments (mean ± s.e.m.). ***P < 0.001; (one-way ANOVA and Tukey's test).

Transparent Methods

Ethics statement

All animal experiments were performed in strict accordance with the recommendations in Guidelines for Proper Conduct of Animal Experiments of Science Council of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Medical Science, the University of Tokyo (Approval number PA17-68).

Mice

Age- and sex-matched C57BL/6J obtained from Japan SLC, Inc. were used as WT controls. *AIM2*$^{-/-}$ (B6.129P2-Aim2$^{Gt(CSG445)Byg}$/J) mice were purchased from Jackson Laboratory (013144).

Cells and viruses

BMMs were prepared as described previously (Ichinohe et al., 2010; Ichinohe et al., 2013). Briefly, bone marrows from the tibia and femur were obtained by flushing with Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque). Bone marrow cells were cultured with DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine and 30% L929 cell (a gift from Akiko Iwasaki) supernatant containing the macrophage colony-stimulating factor at 37°C for 5 days. HEK293FT cells (Invitrogen, Cat#R70007), HeLa cells (a gift from Katsuyoshi Mihara) and J774A.1 macrophages (a gift from Hitomi Mimuro) were maintained in DMEM supplemented with 10% FBS.

WT A/Puerto Rico/8/34 (A/PR8) and A/Narita/1/09 (H1N1pdm09) influenza virus (a gift from Hideki Hasegawa and Hideki Asanuma, respectively) was grown in allantoic cavities of 10-d-old fertile chicken egg for 2d at 35˚C (Ainai et al., 2015; Moriyama and Ichinohe, 2019). Recombinant influenza viruses including WT A/PR8 influenza virus (termed rgPR8) used in this study were generated by using plasmid-based reverse genetics, as described previously (Moriyama et al., 2019; Neumann et al., 1999). These influenza viruses were propagated in MDCK cells (a gift from Hideki Hasegawa) for 2 days at 37˚C. The recombinant HSV-1 lacking VP22 protein (YK451) was described previously (Maruzuru et al., 2018; Tanaka et al., 2012). Viruses were stored at -80˚C, and the viral titer was quantified in a standard plaque assay using MDCK cells for influenza virus and Vero cells for HSV-1ΔVP22.

Virus infection

BMMs or J774A.1 macrophages were infected with influenza virus or HSV-1ΔVP22 at a multiplicity of infection of 3-10 for 1 h at 37˚C, and cultured for an additional 23 h with complete DMEM in the presence of LPS (1μg/ml). Unless otherwise stated, all experiments were performed in LPS-stimulated J774A.1 or BMMs.

ELISA

Cell-free supernatants were collected at 24 h postinfection. The supernatants were analyzed for the presence of IL-1β using an enzyme-linked immunosorbent assay (ELISA) utilizing paired antibodies (eBiosciences) (Ichinohe et al., 2010; Ichinohe et al., 2013).

Isolation of cytosolic or extracellular DNA

Digitonin extracts from HEK293FT cells or J774A.1 macrophages were generated as described previously (Moriyama et al., 2019; West et al., 2015). Cytosolic or extracellular DNA was isolated from these pure cytosolic fractions or supernatants of influenza virus-infected J774A.1 macrophages using QIAquick Nucleotide Removal kit (QIAGEN).

Quantitative PCR

TB Green Premix Ex Taq II (TaKaRa) and a LightCycler instrument (Roche Diagnostics) were used for quantitative PCR with the following primers: human mtDNA forward, 5′ -cctagggataacagcgcaat-3′ , and reverse, 5′ -tagaagagcgatggtgagag-3′ ; mouse mtDNA forward, 5′ -gccccagatatagcattccc-3′ , and reverse, 5′ -gttcatcctgttcctgctcc-3′ ; mouse nuclear DNA (*Tert*) forward, 5′ -ctagctcatgtgtcaagaccctctt-3′ , and reverse, 5′ -gccagcacgtttctctcgtt-3′ (Moriyama et al., 2019; West et al., 2015). The relative mtDNA or nuclear DNA levels in mock-infected or EGFP-transfected cells was set to 1.

Preparation of oxidized mtDNA fragment

Oxidized mtDNA fragment was prepared as described previously (Caielli et al., 2016; Shimada et al., 2012) with slight modifications. Briefly, total mtDNA was isolated from whole-cell extracts of J774A.1 macrophages using QIAamp DNA Mini Kit (QIAGEN). The mtCOX1 gene fragment (1,277 bp) was amplified with unmodified dNTPs and 8-oxo-dGTP (Jena Bioscience GmbH, Germany) using Taq DNA polymerase,

isolated mtDNA, and following primers: mouse mtDNA forward, 5′ - gccccagatatagcattccc-3′ , and reverse, 5′ -tttacttttacataggttgg-3′ .

Western blot analysis

Cells were washed with PBS and lysed in 200 µl of 1× TNT buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol) containing protease inhibitors (Sigma). Lysates were centrifuged at 20,630 ×g for 10 min at 4˚C. Each supernatant was mixed with sodium dodecyl sulfate (SDS) loading buffer [50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol] and boiled for 5 min. These samples were fractionated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were incubated with mouse anti-tubulin (DM1A, Cat#sc-32293; 1:2000), rabbit anti-calnexin (Cat#4731; 1:2000), rabbit anti-Tom20 (FL-145, Cat#sc-11415; 1:1000), mouse anti-Tom22 (1C9-2, Cat#sc-58308; 1:1000), mouse anti-Tom40 (D-2, Cat#sc-365467; 1:1000), rabbit anti-influenza virus M1 (Cat#GTX127356; 1:3000), mouse anti-influenza virus NS1 (NS1-23-1, Cat#sc-130568; 1:1000), mouse anti-influenza virus M2 (14C2, Cat#ab5416; 1:1000), rabbit anti-influenza PB1-F2 (Yoshizumi et al., 2014), anti-GFP (GF200, Cat#04363-66; 1:10,000), or mouse anti-Flag (M2, Cat#F1804; 1:1000) antibody, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories) or anti-rabbit IgG (Invitrogen). The PVDF membranes were then treated with Chemi-Lumi One Super (Nacalai Tesque) to elicit chemiluminescent signals, which were detected and visualized using an LAS-4000 Mini apparatus (GE Healthcare).

Dot blot analysis

Dot blot analysis was performed as described previously (Taniue et al., 2016) with slight modifications. Briefly, cytosolic DNA was spotted onto a Hybond-N^+ membrane (GE Healthcare). The membrane was UV cross-linked (70,000 µJ/cm^2) and then blocked with 10% (wt/vol) skimmed milk, 1% BSA (wt/vol) in TBS-Tween overnight at 4˚C. The membranes were incubated with mouse anti-8OHdG (E-8, Cat#sc-393871; 1:200) or mouse anti-dsDNA (35I9 DNA, Cat#ab27156; 1:1000), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories; 1:10,000). The membranes were then treated with Chemi-Lumi One Super (Nacalai Tesque) to elicit chemiluminescent signals, which were detected and visualized using an LAS-4000 Mini apparatus (GE Healthcare).

Confocal microscopy

HeLa cells or J774A.1 macrophages were seeded on 35-mm glass bottom dishes (IWAKI, Shizuoka, Japan) and infected with PR8 virus or transfected with the expression plasmid encoding PB1-F2 or its C-terminal truncated mutant. At 24 h after infection or transfection, cells were fixed and permeabilized with PBS containing 4% formaldehyde and 1% Triton X-100. Cells were then washed with PBS and incubated with mouse anti-dsDNA (AC-30-10, Cat#CBL186; Chemicon International, Inc.; 1:500), rabbit anti-Tom20 (Cat#NBP1-81556; 1:200), rabbit anti-MAVS (1:200) (Yasukawa et al., 2009), or mouse anti-influenza virus PB1-F2 (a gift from V. Wixler, the Münster University Hospital Medical School) (Nordmann et al., 2010), followed by incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (H+L) (Cat#A21202; Life Technologies; 1:5000), Alexa Fluor 488-conjugated goat anti-mouse IgM (Cat#ab150121; Abcam; 1:5000), Alexa Fluor 568-conjugated goat anti-mouse IgG (Cat#A11031; Life Technologies; 1:5000), or Alexa Fluor 568-conjugated goat anti-rabbit IgG (H+L) antibodies (Cat#A11036; Life Technologies; 1:5000). Stained cells were observed under a confocal microscope (LSM5; Zeiss).

Statistical analysis

Statistical significance was tested using nonparametric one-way analysis of variance (ANOVA), using PRISM software (version 5; GraphPad software). P values of <0.05 were considered statistically significant.

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