Influenza Virus-Induced Oxidized DNA Activates Inflammasomes

HIGHLIGHTS
- M2 protein triggers oxidized DNA release
- PB1-F2 protein triggers oxidized DNA release in the presence of viral RNA
- Mitochondrial localization of PB1-F2 protein is required for oxidized DNA release
- Influenza virus stimulates AIM2-dependent IL-1β secretion

Miyu Moriyama, Minami Nagai, Yuhei Maruzuru, Takumi Koshiba, Yasushi Kawaguchi, Takeshi Ichinohe
ichinohe@ims.u-tokyo.ac.jp

Moriyama et al., iScience 23, 101270
July 24, 2020 © 2020
https://doi.org/10.1016/j.isci.2020.101270
Influenza Virus-Induced Oxidized DNA Activates Inflammasomes

Miyu Moriyama, Minami Nagai, Yuhei Maruzuru, Takumi Koshiba, Yasushi Kawaguchi, and Takeshi Ichinohe

SUMMARY

Influenza virus M2 and PB1-F2 proteins have been proposed to activate the Nod-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome in macrophages by altering intracellular ionic balance or mitochondrial reactive oxygen species (ROS) production. However, the precise mechanism by which these viral proteins trigger the NLRP3 inflammasome activation remains unclear. Here we show that influenza virus stimulates oxidized DNA release from macrophages. Ion channel activity of the M2 protein or mitochondrial localization of the PB1-F2 protein was required for oxidized DNA release. The oxidized DNA enhanced influenza virus-induced IL-1β secretion, whereas inhibition of mitochondrial ROS production by antioxidant Mito-TEMPO decreased the virus-induced IL-1β secretion. In addition, we show that influenza virus stimulates IL-1β secretion from macrophages in an AIM2-dependent manner. These results provide a missing link between influenza viral proteins and the NLRP3 inflammasome activation and reveal the importance of influenza virus-induced oxidized DNA in inflammasomes activation.

INTRODUCTION

Recognition of influenza virus infection by the innate immune system plays a key role not only in limiting viral replication at early stages of infection but also in initiating the virus-specific adaptive immune responses (Iwashiki and Pillai, 2014). Toll-like receptor 7 (TLR7) recognizes influenza virus genomic RNA in endosomes (Diebold et al., 2004; Lund et al., 2004). In cytosol, retinoic acid inducible gene I (RIG-I) detects the 5’ triphosphate end of influenza genomic RNA (Hornung et al., 2006; Pichlmair et al., 2006; Rehwinkel et al., 2010; Weber et al., 2013). These two recognition pathways lead to the induction of type I interferons to restrict influenza virus replication. In contrast to TLRs and RIG-I-like helicases that recognize viral RNA, the NLRP3 senses cellular damage or distress induced by viral infection (Chen and Ichinohe, 2015). Upon activation, NLRP3 is recruited to the mitochondria (Ichinohe et al., 2013; Subramanian et al., 2013) or dispersed trans-Golgi network (Chen and Chen, 2018) to form the multi-molecular protein complex termed the NLRP3 inflammasome. The NLRP3 inflammasome activates caspase-1, which cleaves the precursor forms of proinflammatory cytokines, interleukin 1 beta (IL-1β) and IL-18, and stimulates their secretion (Evavold et al., 2018; Kayagaki et al., 2015; Shi et al., 2015). These inflammasome-dependent cytokines play a key role in the induction of adaptive immune responses and the initiation of tissue healing responses to influenza virus infection (Allen et al., 2009; Ichinohe et al., 2009, 2011; Moriyama and Ichinohe, 2019; Fang et al., 2013; Thomas et al., 2009).

Thus far, several possible mechanisms could explain how the NLRP3 detects influenza virus infection. First, influenza virus M2 protein, a proton-selective ion channel, stimulates ion flux from the trans-Golgi network and activates the NLRP3 inflammasome (Ichinohe et al., 2010). Second, phagocytosed PB1-F2 aggregates induce mitochondrial reactive oxygen species (ROS) production and stimulate the NLRP3 inflammasome-mediated IL-1β secretion from macrophages (McAuley et al., 2013; Pinar et al., 2017). Third, 2-DNA binding protein 1 (ZBP1) senses the ribonucleoprotein (RNP) complex to facilitate influenza virus-induced NLRP3 inflammasome activation (Kuriakose et al., 2016). However, the precise mechanisms of NLRP3 inflammasome activation by influenza virus M2 and PB1-F2 proteins remain unclear.

Recently, we demonstrated that the ion channel activity of influenza virus M2 protein is essential for mitochondrial DNA (mtDNA) release into the cytosol (Moriyama et al., 2019). In addition, recent studies...
highlight the importance of oxidized mtDNA in NLRP3 inflammasome activation (Shimada et al., 2012; Zhong et al., 2018). Consistent with these observations, we and others have shown that mtDNA-depleted J774A.1 or bone marrow-derived macrophages (BMMs) abrogated caspase-1 activation and IL-1β secretion (Ichinohe et al., 2013; Nakahira et al., 2011; Zhong et al., 2016). These observations prompted us to examine whether influenza virus-induced oxidized mtDNA may trigger the NLRP3 inflammasome activation. Here we examined the mechanisms by which influenza virus stimulates oxidized mtDNA release into the cytosol and role of oxidized mtDNA in influenza virus-induced NLRP3 inflammasome activation.

RESULTS
Influenza A Virus Triggers Oxidized DNA Release from Macrophages

We previously demonstrated that influenza virus stimulates cytosolic mtDNA release in mouse lung fibroblasts, human A549, and human embryonic kidney cell line 293FT (HEK293FT) cells (Moriyama et al., 2019). Consistent with these observations, we and others have shown that mtDNA-depleted p0 J774A.1 or bone marrow-derived macrophages (BMMs) abrogated caspase-1 activation and IL-1β secretion (Ichinohe et al., 2013; Nakahira et al., 2011; Zhong et al., 2016). These observations prompted us to examine whether influenza virus-induced oxidized mtDNA may trigger the NLRP3 inflammasome activation. Here we examined the mechanisms by which influenza virus stimulates oxidized mtDNA release into the cytosol and role of oxidized mtDNA in influenza virus-induced NLRP3 inflammasome activation.

Figure 1. Influenza Virus Triggers Oxidized DNA Release from Macrophages

(A) J774A.1 macrophages were subjected to digitonin fractionation as described in Methods and pellets (P) or cytosolic extracts (C) were analyzed by western blotting using the indicated antibodies.

(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 and D) or nuclear DNA (C and E) in the cytosol and supernatants were assessed by quantitative PCR.

(F) J774A.1 macrophages were infected with PR8 virus. At 24 h post infection, cells were stained with anti-dsDNA (AC-30-10) and anti-Tom20 antibodies and analyzed by confocal microscopy. Scale bars, 10 μm.

(G) J774A.1 macrophages were infected with PR8 virus. 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. These data are from three independent experiments (B–E; mean ± SEM).

*p < 0.05, **p < 0.01 and ***p < 0.001 versus mock-infected cells (one-way ANOVA and Tukey’s test). See also Figure S1.
we detected oxidized DNA in the pure cytosolic fraction of influenza virus-infected J774A.1 macrophages (Figure 1G). In addition, a recent A/Narita/1/2009 (H1N1) strain stimulated cytosolic mtDNA release and IL-1β secretion from J774A.1 macrophages (Figures S1G–S1I). Together, these data suggest that influenza virus stimulates oxidized DNA release in macrophages.

Influenza Virus M2 or PB1-F2 Proteins Trigger Oxidized DNA Release

We previously demonstrated that ion channel activity of the influenza virus M2 protein is essential for NLRP3 inflammasome activation (Ichinohe et al., 2010) or mtDNA release into the cytosol (Moriyama et al., 2019). Consistent with our previous observations (Moriyama et al., 2019), overexpression of the M2 protein was sufficient to stimulate mtDNA release into the cytosol (Figure 2A). Since influenza virus stimulates cytosolic mtDNA release in an MAVS-dependent manner (Moriyama et al., 2019), we next examined whether intracellular delivery of polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analog of double-stranded RNA, into HEK293FT cells by transfection stimulates mtDNA release into the cytosol. Interestingly, we found that, although intracellular delivery of poly(I:C) was insufficient to stimulate mtDNA release into the cytosol, the PB1-F2 protein stimulated cytosolic mtDNA release in the presence of poly(I:C) and viral RNA (Figures 2A–2C). In addition, we found that ion channel activity of the M2 protein or PB1-F2 together with poly(I:C) protein stimulated oxidized DNA release into the cytosol (Figures 2D and 2E). Furthermore, the recombinant influenza virus lacking amino acids 29–31 of the M2 protein (termed rgPR8/M2del29–31), which has lost its ion channel activity and failed to stimulate the NLRP3 inflammasome-mediated IL-1β secretion (Ichinohe et al., 2010; Moriyama et al., 2019), or the PB1-F2 protein (termed rgPR8/ΔPB1-F2) reduced its ability to stimulate mtDNA release into the cytosol (Figures 2F and 2G). These data indicate that the ion channel activity of M2 protein or PB1-F2 protein together with poly(I:C) stimulates mtDNA and oxidized DNA release into the cytosol.

We further examined the cellular mechanism by which PB1-F2 protein stimulates mtDNA and oxidized DNA release. We previously demonstrated that the PB1-F2 protein translocates into mitochondria via translocase of the outer membrane 40 (Tom40) channels (Yoshizumi et al., 2014). Consistent with our

Figure 2. Influenza Virus M2 and PB1-F2 Proteins Stimulate Oxidized DNA Release

(A–C) HEK293FT cells were transfected with the expression plasmid encoding EGFP or influenza virus proteins in the presence or absence of poly(I:C) (A, B) or influenza viral RNA (C). Cytosolic mtDNA was assessed by quantitative PCR at 24 h post transfection.

(D and E) HEK293FT cells were transfected with the expression plasmid encoding EGFP or influenza virus proteins in the presence or absence of poly(I:C). Pure cytosolic extracts were collected at 24 h post transfection. Oxidized DNA in the cytosol was detected by dot blot analysis using anti-8OH-dG antibody.

(F and G) HEK293FT cells were infected with WT (rgPR8), rgPR8/M2del29–31 (F), or rgPR8/ΔPB1-F2 virus (G). Cytosolic mtDNA was assessed by quantitative PCR at 24 h post infection. These data are from three independent experiments (A–C, F, G; mean ± SEM).

**p < 0.01 and ***p < 0.001 versus EGFP-transfected or rgPR8-infected cells (one-way ANOVA and Tukey’s test).

we detected oxidized DNA in the pure cytosolic fraction of influenza virus-infected J774A.1 macrophages (Figure 1G). In addition, a recent A/Narita/1/2009 (H1N1) strain stimulated cytosolic mtDNA release and IL-1β secretion from J774A.1 macrophages (Figures S1G–S1I). Together, these data suggest that influenza virus stimulates oxidized DNA release in macrophages.
Figure 3. Mitochondrial Localization of PB1-F2 Protein Is Required for Oxidized DNA Release

(A and B) HEK293FT cells were transfected with siRNA targeting Tom40, Tom22, or control siRNA. Two days later, cells were transfected with the expression plasmid encoding EGFP or PB1-F2 protein in the presence of poly(I:C). Cell lysates were collected at 24 h post transfection and blotted using the indicated antibodies (A). Cytosolic mtDNA was assessed by quantitative PCR at 24 h post transfection (B).

(C) HeLa cells were transfected with expression plasmids encoding PB1-F2 or its C-terminal truncated mutant. At 24 h post transfection, cells were stained with anti-PB1-F2 and anti-Tom20 antibodies to visualize the PB1-F2 protein and mitochondria, respectively, and analyzed by confocal microscopy. Scale bars, 10 μm.

(D and E) HEK293FT cells were transfected with the expression plasmid encoding EGFP, PB1-F2, or its C-terminal truncated mutant in the presence of poly(I:C). Pure cytosolic extracts were collected at 24 h post transfection. Cytosolic mtDNA was assessed by quantitative PCR at 24 h post transfection.
may be important for the NLRP3 inflammasome-mediated IL-1 for mitochondrial reactive oxygen species, inhibited IL-1 virus-infected J774A.1 and BMMs (Figures 4E, S2G, and S2H). Together, these data suggest oxidized DNA and BMMs (Figures 4Da n dS2F). Conversely, treatment with antioxidant Mito-TEMPO, a scavenger specific S2E). Furthermore, 8-oxo-dG restored IL-1b with deoxyguanosine (dG) control (Figures 4Ca n dS2E). In addition, increased levels of influenza virus-induced IL-1b secretion was significantly inhibited by Mcc950, a specific inhibitor of the NLRP3 inflammasome, or Ac-YVAD-cmk, a specific peptide inhibitor of caspase-1 (Figures 4Ca n dS2E). Furthermore, a 1,277 bp of mtDNA fragment significantly enhanced influenza virus-induced IL-1b secretion (Maruzuru et al., 2018), the recombinant herpes simplex virus 1 (HSV-1) lacking VP22 protein stimulated starting around 6 h p.i. and peaking around 9 h p.i. (Figures S2B and S2C). Consistent with our previous report (Ichinohe et al., 2010), we detected active caspase-1 in the supernatants of the virus-infected macrophages at 24 h p.i. (Figures S2B and S2C). To test whether oxidized mtDNA might enhance influenza virus-induced IL-1b secretion, we infected J774A.1 or BMMs with influenza virus in the presence of 8-oxo-dG or 8-oxo-dG+ DNA. Notably, oxidized mtDNA (8-oxo-dG+ DNA) significantly enhanced influenza virus-induced IL-1b secretion from LPS-stimulated J774A.1 and BMMs compared with control 8-oxo-dG+ DNA (Figures 4B and S2D). Similarly, 8-oxo-2'-deoxyguanosine (8-oxo-dG), an oxidized derivative of deoxyguanosine, significantly increased influenza virus-induced IL-1b secretion from LPS-stimulated J774A.1 and BMMs compared with deoxyguanosine (dG) control (Figures 4C and S2E). In addition, increased levels of influenza virus-induced IL-1b secretion by 8-oxo-dG treatment was significantly inhibited by Mcc950, a specific inhibitor of the NLRP3 inflammasome, or Ac-YVAD-cmk, a specific peptide inhibitor of caspase-1 (Figures 4C and S2E). Furthermore, 8-oxo-dG restored IL-1b secretion from rgPR8/M2del29-31 virus-infected J774A.1 and BMMs (Figures 4D and S2F). Conversely, treatment with antioxidant Mito-TEMPO, a scavenger specific for mitochondrial reactive oxygen species, inhibited IL-1b secretion or oxidized DNA release from influenza virus-infected J774A.1 and BMMs (Figures 4E, S2G, and S2H). Together, these data suggest oxidized DNA may be important for the NLRP3 inflammasome-mediated IL-1b secretion after influenza virus infection.

**DISCUSSION**

Influenza virus stimulates the NLRP3 inflammasome-mediated IL-1b secretion from macrophage through its M2 ion channel activity (Ichinohe et al., 2010) or the PB1-F2 aggregates (McAuley et al., 2013; Pinar...
et al., 2017). However, the precise mechanism by which these viral proteins trigger NLRP3 inflammasome activation remains unclear. In this study, we demonstrated that ion channel activity of the M2 protein or mitochondrial localization of the PB1-F2 through Tom40 channel was essential for oxidized DNA release. We found that these cytosolic or extracellular DNA stimulated both NLRP3 and AIM2 inflammasome-mediated IL-1β secretion from macrophages.

Mitochondria play a central role in NLRP3 inflammasome activation (Swanson et al., 2019). ROS-producing damaged mitochondria was first implicated in NLRP3 inflammasome activation (Zhou et al., 2011). Consistent with this observation, cytosolic oxidized mtDNA was found to be important for NLRP3 inflammasome activation (Shimada et al., 2012; Zhong et al., 2018). Translocation of mtDNA into the cytosol required activation of the NLRP3 or MAVS, mitochondrial ROS production, or formation of mitochondrial permeability transition (MPT) pores or BAK/BAX macropores (McArthur et al., 2018; Moriyama et al., 2019; Nakahira et al., 2011). In addition to their role in NLRP3 inflammasome activation, mitochondria also act as platforms for the NLRP3 inflammasome activation. MAVS interacts with the NLRP3 and promotes its localization to the mitochondria for full activation of the NLRP3 inflammasome (Subramanian et al., 2013). Following influenza virus infection, the NLRP3 associates with mitofusin 2 to facilitate its localization to the mitochondria (Ichinohe et al., 2013). In this study, we found that the M2 protein of influenza virus was sufficient to trigger oxidized DNA release. Since Ca²⁺ overload is sufficient to induce the MPT (Lemasters et al., 2009), we speculate that the M2-mediated perturbation of intracellular ionic concentrations may be sufficient to stimulate formation of Ca²⁺ influx-dependent MPT pores and cytosolic release of oxidized mtDNA. Interestingly, a recent study has demonstrated that disassembly of trans-Golgi network into vesicles serves as a scaffold for NLRP3 aggregation and activation (Chen and Chen, 2018). Since the M2-induced activation of inflammasomes requires its localization to the Golgi apparatus (Ichinohe et al., 2010), it is possible that the M2 protein promotes trans-Golgi network disassembly into vesicles and NLRP3 aggregation. In contrast to the M2 protein, the PB1-F2 protein required intracellular delivery of poly(I:C) or viral RNA to stimulate oxidized mtDNA release. Although the PB1-F2-mediated reduction of mitochondrial membrane potential may cause mitochondrial ROS production (Yoshizumi et al., 2014; Zhou et al., 2011), it was insufficient to...
stimulate oxidized mtDNA release. Thus, mitochondria-localized PB1-F2 protein and MAVS-dependent signals may act synergistically to stimulate oxidized mtDNA release into the cytosol. Not surprisingly, influenza virus has evolved strategies to counteract host innate antiviral immune responses. We and others previously demonstrated that the NS1 protein of influenza virus suppressed the NLPR3 inflammasome-mediated IL-1β secretion from macrophages (Chung et al., 2018; Moriyama et al., 2016; Stasakova et al., 2005). Interestingly, the RNA-binding domain of the NS1 protein was essential for the NLPR3 inflammasome-mediated IL-1β secretion (Moriyama et al., 2016). More recently, we showed that the RNA-binding domain of the NS1 protein was important for association with cytosolic mtDNA to suppress the stimulator of interferon genes (STING)-dependent interferon responses (Moriyama et al., 2019). These observations suggest that the NS1 protein of influenza virus inhibits the NLPR3 inflammasome-mediated IL-1β secretion by inhibiting the MAVS-dependent mtDNA release or sequestering cytosolic mtDNA.

Inflammasome-dependent cytokine production in the lung of influenza virus-infected mice plays an essential role in the induction of virus-specific adaptive immune responses and in the initiation of tissue repair responses following infectious damage (Allen et al., 2009; Ichinohe et al., 2009, 2011; Moriyama and Ichinohe, 2019; Pang et al., 2013). Somewhat surprisingly, induction of the influenza virus-specific adaptive immune responses was dependent of ASC and caspase-1 but not NLRP3 (Ichinohe et al., 2009). Recent reports indicated that the AIM2 plays an important role in IL-1β secretion in the lung of influenza virus-infected mice (Schattgen et al., 2016; Zhang et al., 2017). We and others detected mitochondrial or nuclear DNA in the bronchoalveolar (BAL) fluid of influenza virus-infected mice (Moriyama et al., 2019; Schattgen et al., 2016). In this study, we detected dsDNA in extracellular web-like structures of influenza virus-infected macrophages. In addition, we showed that influenza virus stimulated the AIM2 inflammasome-mediated IL-1β secretion from macrophages. These observations suggest that activation of both NLRP3 and AIM2 inflammasomes in the lung of influenza virus-infected mice may play an important role in the induction of virus-specific adaptive immune responses or influenza-associated mortality. In addition to influenza viral RNA (Kuriakose et al., 2016), mitochondrial or nuclear DNA could act as ligands for these inflammasomes in the lung tissue.

In summary, our data reveal a missing link between influenza virus M2 or PB1-F2 proteins and the NLRP3 inflammasome activation. Both M2 and PB1-F2 proteins stimulated oxidized DNA release to trigger the NLRP3 and AIM2 inflammasomes activation. Because both M2 and PB1-F2 proteins contribute virulence
of influenza A viruses (Chen et al., 2001; Conenello et al., 2007; McAuley et al., 2007, 2010, 2013), our findings suggest a possible effect of influenza virus-induced oxidized DNA in pathogenesis of influenza A virus infections. Better understanding of the role of the M2 and PB1-F2 proteins in regulating inflammatory responses in vivo and pathogenesis of influenza virus infections will aid the development of more effective interventions for the treatment of influenza virus-associated diseases.

Limitations of the Study
In the present study, we demonstrated that influenza virus stimulates oxidized DNA release from macrophages. Although we detected significant amounts of both mitochondrial and nuclear DNA in cytosol and supernatants of influenza virus-infected macrophages, the current study does not determine the relative contributions of mtDNA versus nuclear DNA in activation of the NLRP3 and AIM2 inflammasomes. In addition, we showed that ion channel activity of the M2 protein or mitochondrial localization of the PB1-F2 protein is required for oxidized DNA release. We generated recombinant influenza viruses lacking amino acids 29–31 of the M2 protein or PB1-F2 protein to examine the effect of deficiency of the M2 ion channel activity or PB1-F2 protein in influenza virus-induced IL-1β secretion. However, we found that the expression levels of the M2 and PB1-F2 proteins were different between WT and the mutant virus-infected macrophages. Thus, we cannot simply compare the ability to stimulate inflammasome-dependent IL-1β secretion from macrophages infected with rgPR8 (WT), rgPR8/M2del29-31, or rgPR8/ΔPB1-F2 virus. Since virus-encoded ion channels (viroporins) of other RNA viruses such as encephalomyocarditis virus and severe acute respiratory syndrome coronavirus activate the NLRP3 inflammasome (Chen et al., 2019; Ito et al., 2012; Nieto-Torres et al., 2015), future studies to dissect the importance of the viroporins in oxidized DNA release and activation of the NLRP3 and AIM2 inflammasomes will be important extensions of this work.

Resource Availability
Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Takeshi Ichinohe (ichinohe@ims.u-tokyo.ac.jp).

Materials Availability
All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
No custom code, software, or algorithm were used in this research.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101270.

ACKNOWLEDGMENTS
We thank Y. Kawaoka (University of Wisconsin and University of Tokyo) for providing the plasmids for reverse genetics. This work was supported by the Tokyo Biochemical Research Foundation and the SENSHIN Medical Research Foundation. M.M. is the Research Fellow of the Japan Society for the Promotion of Science.

AUTHOR CONTRIBUTIONS
M.M. and T.I. designed research; M.M., M.N., Y.M., and T.I. performed experiments and analyzed data; T.K. and Y.K. provided critical reagents and advice; T.I. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.
of viral RNA. Immunity

E.H., Pickles, R.J., and Ting, J.P. (2009). The

Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K.,

REFERENCES

Revised: May 20, 2020

Received: February 26, 2020

REVISED: May 20, 2020

McEwan, A., Maclennan-Cuadas, C.J., Pinar, A., Zeng, W., Stutz, A., Latz, E., Brown, L.E., and Mansell, A. (2013). Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. PLoS Pathog. 9, e1003392.

Morimori, M., Chen, I.Y., Kawaguchi, A., Koshiba, T., Nagata, K., Takehana, H., Hasegawa, H., and Ichinohe, T. (2016). The RNA- and TRIM25- binding domains of influenza virus NS1 protein are essential for suppression of NLRP3 inflammasome-mediated interleukin-1beta secretion. J. Virol. 90, 4105–4114.

Morimori, M., and Ichinohe, T. (2019). High ambient temperature dampens adaptive immune responses to influenza A virus infection. Proc. Natl. Acad. Sci. U S A 116, 3118–3125.

Morimori, M., Koshiba, T., and Ichinohe, T. (2019). Influenza A virus M2 protein triggers mitochondrial DNA-mediated antiviral immune responses. Nat. Commun. 10, 4624.

Nakahira, K., Haspel, J.A., Rathinam, V.A., Lee, S.J., Dolinay, T., Lam, H.C., Englert, J.A., Rabinovitch, M., Cerndas, M., Kim, H.P., et al. (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat. Immunol. 12, 222–230.

Nieto-Torres, J.L., Verdia-Bagua, C., Jimenez-Guardeno, J.M., Regla-Nava, J.A., Castano-Rodriguez, C., Fernandez-Delgado, R., Torres, J., Aguillera, V.M., and Enjuanes, L. (2015). Severe acute respiratory syndrome coronavirus E protein transports calcium ions and activates the NLRP3 inflammasome. Virology 485, 330–339.

Pang, I.K., Ichinohe, T., and Iwasaki, T. (2013). IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8(+) T cell responses to influenza A virus. Nat. Immunol. 14, 246–253.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2008). RIG-I-mediated antiviral responses to single-stranded RNA bearing S’-phosphates. Science 314, 997–1001.

Pinar, A., Dowling, J.K., Bitto, N.J., Robertson, A.A., Latz, E., Stewart, C.R., Drummond, G.R., Cooper, M.A., McEwan, J.L., Tate, M.D., et al. (2017). PB1-F2 peptide derived from avian influenza A virus H7N9 induces inflammation via activation of the NLRP3 inflammasome. J. Biol. Chem. 292, 826–836.

Rehwinkel, J., Tan, C.P., Goubau, D., Schulz, O., Pichlmair, A., Bier, K., Robb, N., Vreede, F., Barclay, W., Fodor, E., et al. (2010). RIG-I detects

McKenna, T., NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity 30, 556–565.

Chen, I.Y., and Ichinohe, T. (2015). Response of host inflammasomes to viral infection. Trends Microbiol. 23, 55–63.

Chen, I.Y., Morimori, M., Chang, M.F., and Ichinohe, T. (2019). Severe acute respiratory syndrome coronavirus 2 orin activates the NLRP3 inflammasome. Front Microbiol. 10, 50.

Chen, J., and Chen, Z.J. (2018). PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. Nature 564, 71–76.

Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Back, I., Basta, S., O’Neill, R., Schickli, J., Palese, P., et al. (2001). A novel influenza A virus mitochondrial protein that induces cell death. Nat. Med. 7, 1306–1312.

Chung, W.C., Kang, H.R., Yoon, H., Kang, S.J., Ting, J.P., and Song, M.J. (2018). Correction: influenza A virus NS1 protein inhibits the NLRP3 inflammasome. PLoS One 13, e0200624.

Conenello, G.M., Zamarin, D., Perrone, L.A., Tumpey, T., and Palese, P. (2007). A single mutation in the PB1-F2 of H5N1 (H9N2) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog. 3, 1414–1421.

Dang, E.V., McDonald, J.G., Russell, D.W., and Cyster, J.G. (2017). Oxysterol restraints of cholesterol synthesis prevent AIM2 inflammasome activation. Cell 171, 1057–1071.e11.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 302, 1529–1531.

Eavold, C.L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J.C. (2018). The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. Immunity 48, 35–44.e36.

Hornung, V., Ellegast, J., Kim, S., Brozka, K., Jung, A., Kato, H., Hoeck, A., Akira, S., Conzelmann, K.K., Schlee, M., et al. (2006). S’-Triphosphate RNA is the ligand for RIG-I. Science 314, 994–997.

Ichinohe, T., Lee, H.K., Ogura, Y., Flavell, R., and Iwasaki, A. (2009). Inflammasome recognition of influenza virus is essential for adaptive immune responses. J. Exp. Med. 206, 79–87.

Ichinohe, T., Pang, I.K., and Iwasaki, A. (2010). Influenza virus activates inflammasomes via the intracellular M2 ion channel. Nat. Immunol. 11, 404–410.

Ichinohe, T., Pang, I.K., Kuramoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. U S A 108, 5354–5359.

Ichinohe, T., Yamazaki, T., Koshiba, T., and Yang, Y. (2013). Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. Proc. Natl. Acad. Sci. U S A 110, 17963–17968.

Ito, M., Yangi, Y., and Ichinohe, T. (2012). Encapsidation of virus to influenza virus infection. Nat. Rev. Immunol. 14, 315–328.

Kagayaki, N., Stowe, I.B., Lee, B.L., O’Rourke, K., Andersson, K., Ding, S., Cuello, T., Sey, B., Roose-Girma, M., Phung, Q.T., et al. (2019). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature 526, 666–671.

Kuriakose, T., Man, S.M., Malireddi, R.K., Karki, R., Kesavardhana, S., Place, D.E., Neale, G., Vogel, P., and Kangenati, T.D. (2016). ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Sci. Immunol. 7, https://doi.org/10.1126/sciimmunol.aag2045.

Lemasters, J.J., Theruvath, T.P., Zhong, Z., and Nieminen, A.L. (2009). Mitochondrial calcium and the permeability transition in cell death. Biochem. Acta 1787, 1399–1401.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc. Natl. Acad. Sci. U S A 101, 5598–5603.

Maruizuru, Y., Ichinohe, T., Sato, R., Miyake, K., Okano, T., Suzuki, T., Koshiba, T., Koyanagi, N., Tsuda, S., Watanabe, M., et al. (2018). Herpes simplex virus 1 VP22 inhibits AIM2-dependent inflammasome activation to enable efficient viral replication. Cell Host Microbe 23, 254–265.e7.

McArthur, K., Whitehead, L.W., Heddleston, J.M., Li, L., Padman, B.S., Oorschot, V., Georgohagan, N.D., Chappaz, S., Davidson, S., San Chin, H., et al. (2018). BAK/BAX macropores facilitate mitochondrial herniation and mtDNA eflux during apoptosis. emscience 339, https://doi.org/10.1126/science.aao6047.

McAuley, J.L., Tate, M.D., McEwan, A., Pinar, A., Zeng, W., Stutz, A., Latz, E., Brown, L.E., and Mansell, A. (2013). Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. PLoS Pathog. 9, e1003392.
viral genomic RNA during negative-strand RNA virus infection. Cell 160, 397–408.

Schattgen, S.A., Gao, G., Kurt-Jones, E.A., and Fitzgerald, K.A. (2016). Cutting edge: DNA in the lung microenvironment during influenza virus infection tempers inflammation by engaging the DNA sensor AIM2. J. Immunol. 196, 29–33.

Shi, J., Zhao, Y., Wong, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., and Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526, 660–665.

Shimada, K., Crother, T.R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., Ramanujan, V.K., Wolf, A.J., Vergnes, L., Ojcius, D.M., et al. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. Immunity 36, 401–414.

Stasakova, J., Ferko, B., Kittel, C., Sereinig, S., Romanova, J., Katinger, H., and Egorov, A. (2005). Influenza A mutant viruses with altered NS1 protein function provoke caspase-1 activation in primary human macrophages, resulting in fast apoptosis and release of high levels of interleukins 1beta and 18. J. Gen. Virol. 86, 185–195.

Subramanian, N., Natarajan, K., Clatworthy, M.R., Wang, Z., and Germain, R.N. (2013). The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation. Cell 153, 348–361.

Swanson, K.V., Deng, M., and Ting, J.P. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat. Rev. Immunol. 19, 477–489.

Thomas, P.G., Dash, P., Aldridge, J.R., Jr., Ellebedy, A.H., Reynolds, C., Funk, A.J., Martin, W.J., Lamkanfi, M., Webby, R.J., Boyd, K.L., et al. (2009). The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. Immunity 30, 566–575.

Weber, M., Gawanbacht, A., Habjan, M., Rang, A., Borner, C., Schmidt, A.M., Veitinger, S., Jacob, R., Devignot, S., Kochs, G., et al. (2013). Incoming RNA virus nucleocapsids containing a 5'-triphosphorylated genome activate RIG-I and antiviral signaling. Cell Host Microbe 13, 336–346.

Zhong, Z., Umemura, A., Sanchez-Lopez, E., Liang, S., Shalapour, S., Wara, A., Dabdoub, A., Ali, S., Schnabl, B., et al. (2018). New mitochondrial DNA synthesis enables NLRP3 inflammasome activation. Nature 560, 198–203.

Zhong, Z., Yao, A., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225.
Supplemental Information

Influenza Virus-Induced Oxidized DNA Activates Inflammasomes

Miyu Moriyama, Minami Nagai, Yuhei Maruzuru, Takumi Koshiba, Yasushi Kawaguchi, and Takeshi Ichinohe
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 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<sup>−/−</sup> (B6.129P2-Aim2<sup>Gt(Csf3r<sup>Bryp</sup>)Byg</sup>/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 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′-cttagggataacagcgcaat-3′, and reverse, 5′-tagaagagcgatgtgagag-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'-ttactttataggttg-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/cm²) 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 (3519 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.
Ainai, A., Hasegawa, H., Obuchi, M., Odagiri, T., Ujike, M., Shirakura, M., Nobusawa, E., Tashiro, M., and Asanuma, H. (2015). Host Adaptation and the Alteration of Viral Properties of the First Influenza A/H1N1pdm09 Virus Isolated in Japan. PLoS One. 10(6), e0130208. DOI: 10.1371/journal.pone.0130208.

Caielli, S., Athale, S., Domic, B., Murat, E., Chandra, M., Banchereau, R., Baisch, J., Phelps, K., Clayton, S., Gong, M., et al. (2016). Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. J Exp Med. 213(5), 697-713. DOI: 10.1084/jem.20151876.

Ichinohe, T., Pang, I.K., and Iwasaki, A. (2010). Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nat Immunol. 11(5), 404-410. Published online 2010/04/13 DOI: ni.1861 [pii] 10.1038/ni.1861.

Ichinohe, T., Yamazaki, T., Koshiba, T., and Yanagi, Y. (2013). Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. Proc Natl Acad Sci U S A. 110(44), 17963-17968. Published online 2013/10/16 DOI: 1312571110 [pii] 10.1073/pnas.1312571110.

Maruzuru, Y., Ichinohe, T., Sato, R., Miyake, K., Okano, T., Suzuki, T., Koshiba, T., Koyanagi, N., Tsuda, S., Watanabe, M., et al. (2018). Herpes Simplex Virus 1 VP22 Inhibits AIM2-Dependent Inflammasome Activation to Enable Efficient Viral Replication. Cell Host Microbe. 23(2), 254-265 e257. DOI: 10.1016/j.chom.2017.12.014.

Moriyama, M., and Ichinohe, T. (2019). High ambient temperature dampens adaptive immune responses to influenza A virus infection. Proc Natl Acad Sci U S A. 116(8), 3118-3125. DOI: 10.1073/pnas.1815029116.

Moriyama, M., Koshiba, T., and Ichinohe, T. (2019). Influenza A virus M2 protein triggers mitochondrial DNA-mediated antiviral immune responses. Nat Commun. 10(1), 4624. DOI: 10.1038/s41467-019-12632-5.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., et al. (1999). Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A. 96(18), 9345-9350.

Nordmann, A., Wixler, L., Ludwig, S., and Wixler, V. (2010). Monoclonal antibodies against the PB1-F2 protein of H1N1 influenza A virus. Hybridoma (Larchmt). 29(4), 321-326. DOI: 10.1089/hyb.2010.0010.

Shimada, K., Crother, T.R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., Ramanujan, V.K., Wolf, A.J., Vergnes, L., Ocius, D.M., et al. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. Immunity. 36(3), 401-414. DOI: 10.1016/j.immuni.2012.01.009.

Tanaka, M., Kato, A., Satoh, Y., Ide, T., Sagou, K., Kinura, K., Hasegawa, H., and Kawaguchi, Y. (2012). Herpes simplex virus 1 VP22 regulates translocation of multiple viral and cellular proteins and promotes neurovirulence. J Virol. 86(9), 5264-5277. DOI: 10.1128/JVI.00913-11.

Tanifuji, K., Kurimoto, A., Sugimasa, H., Nasu, E., Takeda, Y., Iwasaki, K., Nagashima, T., Okada-Hatakayama, M., Oyama, M., Kozuka-Hata, H., et al. (2016). Long noncoding RNA UPAT promotes colon tumorigenesis by inhibiting degradation of UHRF1. Proc Natl Acad Sci U S A. 113(5), 1273-1278. DOI: 10.1073/pnas.1500992113.

West, A.P., Khoury-Hanold, W., Staron, M., Tal, M.C., Pineda, C.M., Lang, S.M., Bestwick, M., Duguay, B.A., Raimundo, N., MacDuff, D.A., et al. (2015). Mitochondrial DNA stress primes the antiviral innate immune response. Nature. 520(7548), 553-557. DOI: 10.1038/nature14156.

Yasukawa, K., Oshiumi, H., Takeda, M., Ishihara, N., Yanagi, Y., Seya, T., Kawabata, S., and Koshiba, T. (2008). Mitofusin 2 inhibits mitochondrial antiviral signaling. Sci Signal. 2(84), ra47. DOI: 10.1126/scisignal.2000287.

Yoshizumi, T., Ichinohe, T., Sasaki, O., Otera, H., Kawabata, S., Mihara, K., and Koshiba, T. (2014). Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. Nat Commun. 5, 4713. Published online 2014/08/21 DOI: ncomms5713 [pii] 10.1038/ncomms5713.