Role of mitochondrial anti-viral signaling protein and dynamin-related protein 1 for the NLRP3 inflammasome activation

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Role of mitochondrial anti-viral signaling protein and dynamin-related protein 1 for the NLRP3 inflammasome activation

Directed by Professor Je-Wook Yu

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박사과정을 무사히 마칠 수 있게 도와주신 실험실 후배 분들께도 큰 감사를 드립니다. 졸업을 나보다 빨리 한 후배 인화야, 되지도 않는 개그를 해도 반응해줘서 고마워. 술 마실 때 빼고 늘 조용한 은주야, 어서 좋은 인연 만나길 바란다. 옆자리에서 노인 모시는 정민아, 재미있는 걸 찾았을 때는 늦지 않게 형을 부르렴. 우리 귀여운 막내 승환아…4 년동안 힘내라. 모든 것을 다 알고 있는 선아야, 어떻게 나만큼 알고 있는 건지 참 궁금하다. 그리고 지금은 실험실을 떠나신 김은희 박사님과 우리 현정 누나, 지희 누나, 영은 선생님까지도 소중했던 인연들에 머리 숙여 감사 드립니다.

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마지막으로 지금의 제가 있을 수 있게 곁에서 너무 많이 헌신해주고, 의지 박약한 저를 학문의 길로 올 수 있게 이끌어준 수정이에게는 특별히 고맙다는 말을 전하고 싶습니다.

박사과정은 이로써 마침표를 찍지만, 더 넓은 세상을 향해 나아가기 위해 쉼표를 찍었다는 느낌으로 앞으로 주어지는 시간들에도 끝까지 최선을 다하는 사람이 되도록 노력하겠습니다. 저와 함께해준 모든 인연들께 좋은 일만 가득하기를 바라며 그분들께 감사의 마음을 담아 이 논문을 바칩니다.

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박상준 배상

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ABSTRACT

Role of mitochondrial anti-viral signaling protein and dynamin-related protein 1 for the NLRP3 inflammasome activation

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NLRP3 inflammasome is an intracellular protein complex composed of NLRP3, adaptor protein ASC and procaspase-1, and activates caspase-1 in response to various stimuli such as pathogens, microbial toxin, crystalline materials, and endogenous danger signals. Many studies have tried to figure out how NLRP3 inflammasome is activated, but molecular mechanisms are still unclear. Recent studies suggest that mitochondria play an important role in NLRP3 inflammasome activation by releasing of mitochondrial product from damaged mitochondria such as mitochondrial ROS (mtROS) or mitochondrial DNA (mtDNA). However, despite diverse studies, how mitochondria regulate NLRP3 inflammasome remains to be explored. Therefore, I attempt to demonstrate the mitochondrial regulation of NLRP3 inflammasome activation by focusing mitochondrial protein MAVS and abnormal mitochondrial dynamics induced by decrease of mitochondrial fission-associated protein Drp1 protein expression.

In PART1, I demonstrated that mitochondrial anti-viral signaling protein MAVS interacts with NLRP3, and mitochondrial MAVS promoted NLRP3 inflammasome activation by facilitating ROS-dependent NLRP3 oligomerization on

mitochondrial outer membrane in response to Sendai virus infection. Supporting this, knockdown of MAVS expression in macrophages showed a significant decrease in NLRP3-mediated caspase-1 activation in response to Sendai virus infection. Moreover, both mouse macrophages and human THP-1 cells exhibited MAVS-mediated NLRP3 inflammasome assembly and activation in response to Sendai-virus infection. Thus, mitochondrial MAVS facilitates ROS-dependent NLRP3 oligomerization, and promotes NLRP3 inflammasome activation in response to Sendai virus infection.

In PART 2, I demonstrated that abnormal mitochondrial dynamics caused by knockdown of Drp1 protein expression was associated with NLRP3 inflammasome activation. Drp1 knockdown macrophages exhibited elongated mitochondria and increase NLRP3 inflammasome activation in response to LPS followed by ATP. Furthermore, Drp1 knockdown-mediated mitochondrial elongation enhanced the formation of NLRP3 inflammasome assembly. On the other hand, mitochondrial fragmentation induced by mitochondrial fission inducer CCCP treatment show highly attenuated NLRP3 inflammasome assembly and activation. At molecular levels, Drp1 knockdown macrophages show increase of the release of mitochondrial product such as mtROS or cytosolic mtDNA. However, caspase-1-selective inhibitor YVAD treatment prevented the release of mitochondrial products. Thus, this phenomenon might be the consequences of caspase-1 activation. Interestingly, Drp1 knockdown macrophages show constitutively phosphorylated extracellular signal-regulated kinase (ERK) without any stimulation, suggesting that ERK has a critical role in NLRP3 inflammasome assembly by localizing NLRP3 into mitochondria. Supporting this, ERK phosphorylation inhibitor U0126 treatment induced markedly impaired NLRP3 inflammasome assembly and activation. Therefore, these data show that Drp1 knockdown-mediated abnormal mitochondrial elongation enhances NLRP3 inflammasome activation in response to classical NLRP3 activating stimuli such as LPS plus ATP in an ERK signaling dependent manner.

Collectively, I demonstrated MAVS- and Drp1-dependent mitochondrial regulation to the NLRP3 inflammasome activation.

Key words: inflammasome, NLRP3, mitochondria, mitochondrial dynamics, MAVS, Drp1

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Ⅰ. INTRODUCTION

Innate immunity plays an important role in host protection against pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP) by inducing inflammatory responses.[1] During infection, innate immune cells activate interferon regulatory factor 3 (IRF3) to produce type Ⅰ interferons (IFNs), and nuclear factor kappa-light chain-enhancer of activated B cells (NFκB) for proinflammatory cytokines.[2-4] IL-18 and IL-1β are known as major inflammatory cytokines in inflammatory immune response and these cytokines are regulated by specific protease called 'caspase-1', and caspase-1 is also regulated by a specific protein complex called 'inflammasome'.[1,5-7]

Inflammasome is an intracellular protein complex composed of sensor proteins such as NOD-like receptor family, pyrin domain-containing 3 (NLRP3), NLR family, CARD-containing 4 (NLRC4), or absent in melanoma 2 (AIM2), adapter protein named apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and effector protein procaspase-1.[8,9] So far, depending on their sensor proteins, four inflammasomes including NLRP1, NLRC4, AIM2, and NLRP3 have been described to activate caspase-1 which processes pro-IL-1β and pro-

IL-18 into their mature forms in response to various stimuli involving pathogens, microbial toxin, crystalline materials, and endogenous danger signals.[9-14]

Currently, NLRP3 inflammasome is the most well-characterized of the four inflammasomes. It has been known that two types of signals are essential for activation of NLRP3 inflammasome.[8,15] The priming signal is induction of NLRP3 or assembly of NLRP3 inflammasome, such as ASC oligomerization and interaction with NLRP3 by activating NFκB signaling derived from pattern recognition receptors such as Toll like receptors (TLRs),[16] followed by an activation signal derived from extracellular ATP, pore-forming toxins, various type of bacteria, or crystalline materials, leading to conformational change of NLRP3 to the activating form.[17-19] However, the molecular mechanism of NLRP3 inflammasome activation still remains elusive.

During pathogenic infection, activation of NLRP3 inflammasome provides advantage to host innate immune responses.[6] However, deregulated NLRP3 inflammasome activation could lead to chronic inflammatory diseases or metabolic disorders such as type 2 diabetes or obesity.[20-23] Furthermore, the excessive activation of NLRP3 inflammasome is involved in neurodegenerative diseases such as Alzheimer's disease.[24-27] Therefore, determination of NLRP3 inflammasome activation mechanism is important for establishing therapeutic strategy in inflammatory diseases.

Until now, few models have suggested to explain NLRP3 inflammasome activation. The lysosomal disruption model proposes that large crystalline particles or protein aggregates such as alum, MSU, and various types of cholesterols induce disruption of phagosomes or lysosomes, in turn, triggers the release of lysosomal enzymes that cleave nonspecific cellular proteins, and leads to NLRP3 inflammasome activation directly or indirectly.[28] Potassium efflux has been known as an essential step of NLRP3 inflammasome activation induced by many stimuli. Nigericin is a molecule which is originally derived from fungus, and it is considered as a strong

potassium efflux inducer.[29] This molecule triggers a number of micro pores on cellular membrane, leading to intracellular potassium efflux into extracellular space, which in turn induces NLRP3 inflammasome activation. Recently, mitochondrial damage model has raised interest in NLRP3 inflammasome activation.[30] Many studies have suggested that mitochondria play an important role in NLRP3 inflammasome activation by release of mitochondrial products such as mitochondrial reactive oxygen species (mROS) or mitochondrial DNA (mtDNA) into the cytosol during a number of mitochondrial stress conditions.[30-35] In the case of mtROS, it activates NLRP3 directly or indirectly through potential NLRP3-interacting proteins such as TXNIP.[21] On the other hand, release of oxidized mtDNA leads to the activation of the NLRP3 inflammasome through direct interactions between oxidized mtDNA and NLRP3.[35] Furthermore, inhibition of mitochondrial voltage-dependent anion channel attenuates mtROS production and NLRP3 inflammasome activation.[31] These studies support the role of mitochondria in NLRP3 inflammasome activation. However, despite extensive investigations, the molecular mechanisms of mitochondrial regulation to NLRP3 inflammasome activation need to be explored. Therefore, I try to demonstrate mitochondrial regulation of NLRP3 inflammasome signaling by targeting mitochondrial anti-viral signaling protein (MAVS) and Dynamin-related protein 1 (Drp1)-deficient abnormal mitochondrial elongation.

Mitochondrial anti-viral signaling protein MAVS is one of the mitochondrial outer membrane proteins,[36] which is also known as IFN-β promoter stimulator-1 (IPS-1),[37] virus-induced signaling adaptor (VISA),[38] and recruitment domain adaptor inducing IFN-β (Cardif).[39] MAVS consists of 540 amino acids and has been reported to contain N-terminal caspase activation and recruitment domain (CARD) to interact with other proteins, C-terminal mitochondrial-targeting transmembrane domain (TM) for localization to the mitochondrial outer membrane, and internal proline-rich domain for downstream signaling.[36-39]

Previous studies about MAVS reported that MAVS has an important role in innate immune response such as type Ⅰ IFN production and NFκB activation in response to viral infection.[40,41] During RNA viral infection, RLR like RIG-I or MDA-5 senses viral 5'ppp-RNA or dsRNA, and binds to MAVS through CARD-CARD homotypic interaction on the mitochondrial outer membrane. Interaction between MAVS and RLR induces the formation of large prion-like functional aggregation of MAVS,[42] and MAVS aggregation activates the cytosolic kinase TBK1 and IKK, in turn, this event induces the activation of IRF3 and NFκB.[37] As a result, the activation of IRF3 and NFκB produce type Ⅰ IFN and other antiviral molecules.[42,43] Hence, MAVS has been considered as a critical regulator of antiviral innate immune signaling. Despite the reporting of these studies about the role of MAVS in innate immune signaling, little is known about the association between MAVS and inflammasome. Furthermore, recent studies have suggested that NLRP3 is recruited to the mitochondria upon activation.[42] Thus, mitochondrial outer membrane protein MAVS might regulate NLRP3 inflammasome activation.

This study also investigated the association between abnormal mitochondrial elongation and NLRP3 inflammasome activation. Mitochondria are mobile organelles that exist in dynamic networks.[44] They continuously join by the process of fusion and divide by the process of fission. These continuous two processes called 'mitochondrial dynamics'. How mitochondrial dynamics are regulated is still not completely explained, but key proteins and models of mitochondrial dynamics were uncovered considerably. In the case of fusion, mitochondrial outer membrane protein MFN 1/2 induces outer membrane fusion by homotypic or heterotypic interaction between MFN 1 and 2 on targeted mitochondria,[44-46] in turn, inner membranes of mitochondria are fused by Opa1 protein interaction.[47-50] In contrast, mitochondrial fission protein Drp1 and Fis1 are involved in division of mitochondria. Drp1 is originally localized in the cytosol, but the phosphorylation of Drp1 by various stimuli induces translocation into mitochondria by Fis1 dependent manner. [51-54] Continuously,

phosphorylated Drp1 forms ring-shaped oligomer on target site of mitochondria, which leads to mitochondrial fission by their GTPase activity dependent hydrolysis.[54-57] Since mitochondria maintain their DNA contents, shape, and function through the mitochondrial dynamics,[44-46,58] the balance of mitochondrial dynamics is critical for mitochondrial homeostasis.

So far, the association between mitochondrial dynamics and innate immune signaling was fairly well-studied. According to previous studies, viral infection induces mitochondrial elongation, leading to inhibition of intrinsic pathway of apoptosis by preventing the release of cytochrome C from mitochondrial matrix into the cytosol,[58,59] appropriate mitochondrial movement, and removal by autophagy.[34,60] Furthermore, hyperfused mitochondria were reported to increase NFκB activation and type Ⅰ IFN production in response to viral infection.[61-63] On the other hand, various stresses such as UV stimulation induce mitochondrial fission, leading to inhibition of intra-mitochondrial mixing of mitochondrial components such as proteins, lipids, and DNA.[44,46] Moreover, previous studies reported that fragmented mitochondria show decrease in NFκB activation and type Ⅰ IFN production in response to viral infection.[62]

Abnormal mitochondrial dynamics caused by mutation or deficiency in mitochondrial dynamics associated proteins such as Drp1, MFN 1/2, and Opa1 potentially contributes to a number of neurodegenerative diseases such as Parkinson's or Alzheimer's disease.[64-66] Especially, Drp1, which is a key protein of mitochondrial fission process, is associated with various developmental disorders, and has strong correlation to neonatal death.[27,50,67] A recent study reported that a female infant with mutation on Drp1 exhibited abnormal brain development and died suddenly at the age of 37 days. The skin fibroblasts from this patient showed abnormal elongated mitochondria and peroxisome compared to healthy control.[68] In addition, few studies have reported that Alzheimer's disease patient exhibits largely decreased Drp1 expression.[65,68]

Unlike the association between abnormal mitochondrial dynamics and other immune responses, the connection between abnormal mitochondrial elongation and NLRP3 inflammasome activation has not been explored in depth. According to recent studies, MFN2 could form NLRP3-MFN2-MAVS complex during RNA virus infection, and this complex is required for activation of NLRP3 inflammasome.[41,61,62] Another study suggests that RNA virus infection induces Drp1-mediated mitochondrial fragmentation and subsequent production of mitochondria-derived products.[31,40,69] It was also reported that the reduction of Drp1 protein expression by shRNA interference in 293T cells exhibits largely increased NFκB activation.[61] Considering these studies, abnormal mitochondrial elongation might be involved in NLRP3 inflammasome activation.

Collectively, I tried to demonstrate the differential contribution of mitochondria to the NLRP3 inflammasome signaling by focusing mitochondrial protein MAVS and Drp1-deficient abnormal mitochondrial elongation.

Ⅱ. MATERIALS AND METHODS

1. Reagents and antibodies

Reagents: LPS, ATP, Rotenone, nigericin, cycloheximide (CHX), CCCP, mdivi-1, U0126, Poly(dA:dT), Poly(I:C), N-acetyl-L-cysteine (NAC) and MAVS-targeting (shMAVS), Drp1-targeting (shDrp1) or non-targeting (shScr) shRNA lentiviral plasmids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alum was purchased from InvivoGen (SanDiego, CA, USA). Ac-YVAD-chloromethylketone (Ac-YVAD-cmk) and z-VAD-fluoromethylketone (zVAD-fmk) were obtained from Bachem (Bachem SA, Vionnaz, Switzerland). Mouse IL-1β enzyme-linked immunoassay (ELISA) kits were obtained from R&D System (Minneapolis, MN, USA). MitoSOX, MitoTracker Green, MitoTracker Deep Red, and mouse recombinant TNF-α were purchased from Invitrogen (Gran Island, NY, USA).

Antibodies: Anti-human caspase-1 (p10), ASC, p-ERK, β–actin, and IRF antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-mouse caspase-1 (p20) and NLRP3 antibodies were obtained from Adipogen (San Diego, CA, USA). Anti-Drp1 and JNK1/2 were obtained from BD Biosciences (San Jose, CA, USA). Anti-VDAC1 and Myc were obtained from Abcam (Massachusetts, MA, USA). Anti-human IL-1β, caspase-3, ERK, IκB, p-IRF3, human caspase-1, and mouse MAVS were purchased from Cell Signaling Technology (Beverly, MA, USA). All other antibodies detecting phospho-JNK1/2 (Invitrogen), Flag (Sigma), T7 (Bethyl Laboratories, Alabama, AL, USA), human MAVS (Alexis, Gran Island, NY, USA), and mouse IL-1β (R&D systems) were obtained from commercial sources.

2. Cell culture

Mouse bone marrow-derived macrophages (BMDMs) were prepared as the following: bone marrow cells were forced from the femurs and tibias of C57BL/6

mice (7 wk) by PBS. The cells were then cultured in petri-dish with L929-conditioned Dulbecco's Modified Eagles Medium (DMEM, Hyclone, Logan, UT, USA) including 10% FBS and 100 U/mL penicillin and streptomycin (Gibco, Gran Island, NY, USA). Three days after, culture medium was replaced with fresh medium to remove non-adherent cells. Next day, cells were detached by trypsinization and plated 1 x 10^6 cells per well in appropriate plates. Protocols for the animal experiments were approved by the Institutional Ethical Committee, Yonsei University College of Medicine. All experiments involving BMDM preparation were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

Human THP-1 and THP-1–ASC–GFP cells were grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Hyclone) including 10% FBS (Hyclone), 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-ME, and 100 U/mL penicillin and streptomycin (Gibco). 293T and 293T-C1A cells were maintained in DMEM/F12 medium supplemented with 10% FBS and 100 U/ml of penicillin/streptomycin.

Wild-type immortalized and NLRP3-GFP-expressing mouse BMDMs were kindly provided by Dr. E.S. Alnemri (Thomas Jefferson University, Philadelphia, PA, USA).

3. Production of knockdown cells

To produce Drp1 or MAVS protein knockdowned mouse BMDMs, I transfected 293T cells with pLP1, pLP2, pLP VSVG (kindly gifted from Dr. HP, Kim, Yonsei University, Seoul, Korea), and targeting mouse Drp1, or mouse MAVS or non-targeting short hairpin RNA (shRNA) control (Sigma) to produce lentivirus particles (Table.1). Then, lentiviral particles containing supernatant were collected after 48 – 72 hr incubation. Lentiviral particles containing shRNA were infected to immortalized mouse BMDMs with 8 μg/ml polybrene (Sigma), and incubated for 48 hr. Cells stably expressing shRNA were cloned by puromycin selection and used for experiments.

To knockdown MAVS protein expression in THP-1-derived macrophages, cells were transfected with small interfering RNA (siRNA) oligonucleotides (Bioneer, Daejeon, Korea) targeting human MAVS or non-targeting scrambled siRNA control using Lipofectamine 2000 (Invitrogen) or lentiviral particles containing non-targeting, Drp1-targeting, or Opa1-targeting shRNA were used to infect THP-1 cells.

4. Immunoblot analysis

Cells were lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were subjected to SDS-PAGE and transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA), and immunoblotted with the appropriate Abs. Supernatants were collected, and proteins were precipitated by methanol-chloroform extraction then were subjected to immunoblot with appropriate Abs.

5. Co-immunoprecipitation (Co-IP)

To determine protein-protein interaction, cells were lysed in 10 mM HEPES buffer (pH 7.4) containing 0.2% NP-40, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, and protease inhibitors, and centrifuged at 10 min for 12,500 x rpm to remove cell debris. The resulting lysates were pre-cleared to remove nonspecific binding proteins with protein G-Sepharose (GE Healthcare Life Science, Piscataway, NJ, USA) for 1 hr, and immunprecipitated overnight with primary Ab at 4°C. The protein-Ab complexes were then precipitated for 2 hr with protein G-Sepharose bead, and the bead-bound proteins were fractionated by SDS-PAGE and immunoblotted with the appropriate Abs.

6. Subcellular fractionation

To fractionate mitochondrial protein-enriched fraction and cytosolic protein-enriched fraction, cell were lysed in buffer A solution containing 250 mM sucrose, 10

mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.1% NP-40 and protease inhibitors. The lysates were centrifuged for 10 min at 700 x g to discard nuclear fraction. Supernatants were centrifuged for 10 min at 12,500 x g to obtain cytosolic protein-enriched fraction, and the remaining pellets were washed with buffer A solution and resuspended with 10 mM HEPES (pH 7.5) buffer containing 0.2% NP-40, 100 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, and protease inhibitors. Finally, resuspended pellets were centrifuged for 10 min at 12,500 x g to acquire mitochondrial protein-enriched fraction. These two fractions were used for immunoblot analysis and for determination of NLRP3 distribution.

7. Determination of co-localization

To determine the interaction between MAVS and NLRP3, cells grown on coverslips in a 12-well plate were fixed with 4% formaldehyde for 30 min and then permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 4% BSA (Sigma) for 30 min, cells were incubated with anti-MAVS Ab and anti-NLRP3 Ab for 2 hr, followed by incubation with Cy3-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG Ab (Jackson, West Grove, PA, USA) for 1 hr. Coverslips were then mounted using the ProLong Gold reagent (Invitrogen) containing the nuclear stain DAPI and examined using a confocal microscope (FV1000; Olympus, Tokyo, Japan, LSM700; Carl Zeiss, Jena, Germany).

8. Activation of inflammasomes

To activate NLRP3 inflammasome, cells were primed with LPS (0.25 μg/ml, 3 hr), followed with ATP (1-2.5 mM, 30-40 min) or nigericin (2.5 μM, 40 min). In the case of THP-1, cells were treated with PMA (0.4 mM, 3 hr) for differentiation, and after at least 12 hr (ON incubation), cells were treated with LPS (0.5 μg/ml, 6 hr) or Alum (250 μg/ml, 6 hr) for NLRP3 inflammasome activation. For activation of NLRC4 inflammasome, *S. typhimurium* (kindly gifted from Dr. SS, Yoon, Yonsei

University, Seoul, Korea) was grown overnight at 37 °C with aeration, and then diluted (1:20) and grown for additional 2 hr. Cells were infected with *S. typhimurium* at the indicated MOI (multiplicity of infection) for 30 min, washed and incubated with gentamicin (100 μg/ml)-containing medium to remove extracellular bacteria. Finally, cells were harvested after 150 min incubation. Poly(dA:dT) transfection for 6 hr by using Lipofectamine 2000 (Invitrogen) was performed for AIM2 inflammasome activation.

9. Assessment of inflammasome assembly

To determine ASC oligomerization, cells were lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Cell lysates were centrifuged at 12,000 rpm for 10 min at 4℃. Then, soluble lysates and insoluble pellets were separated. Pellets were resuspended with PBS containing 0.75 mM disuccinimidyl suberate (Pierce, Gran Island, NY, USA), then incubated for 30 min at RT at dark condition, and centrifuged at 12,000 rpm for 10 min. Pellets and soluble lysates were simultaneously immunoblotted using anti-ASC Ab. For assessment of the interaction between ASC and NLRP3, co-immunoprecipitation analysis was performed as described above. For the NLRP3 subcellular distribution assay, cells were fractionated by subcellular fractionation analysis, then immunoblotted with appropriate Abs.

10. Determination of type Ⅰ IFN production

To determine type Ⅰ IFN production, cells were transfected with an IFN-β promoter luciferase reporter plasmid (100 ng), p–β-galactosidase (100 ng), and transfected with indicated plasmids (Promega, Madison, WI) for 24 hr. Then, IFN-β promoter activity levels were measured using a Luciferase assay kit (Promega). β-Galactosidase assay kit (Promega) was used for normalizing transfection efficiencies.

IFN luciferase activity was represented as the relative fold compared with unstimulated with MAVS, Poly(I:C), or Sendai virus infection.

11. Quantification of NLRP3 mRNA transcription

To assess NLRP3 mRNA transcription, total cellular RNA was extracted using the TRIzol reagent (Invitrogen) and reverse transcribed using PrimeScript™ RT Master Mix (Takara Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Template DNA was amplified by quantitative real-time PCR using SYBR *Premix Ex Taq*™ II (Takara) or iTaq universal SYBR green supermix (BioRad, Hercules, CA, USA). Primers (Genotech, Deajeon, Korea) were as the following: 5′ -ATG CTG CTT CGA CAT CTC CT-3′ and 5′ -AAC CAA TGC GAG ATC CTG AC-3′ (*Nlrp3*); 5′ -AAC TTT GGC ATT GTG GAA GG-3′ and 5′ -ACA CAT TGG GGG TAG GAA CA-3' (*GAPDH*).

12. Determination of cell death

To induce apoptosis or necroptosis, cells were treated with TNF-α (30 ng/ml) and cycloheximide (CHX, 0.4 μg/ml) with or without zVAD-fmk (20 μM) for 20 hr. To induce pyroptosis, cells were treated with LPS (0.25μg/ml, 3 hr) for priming, and followed by nigericin (5 μM, 45 min). Cell death was determined by the extracellular release of lactate dehydrogenase (LDH) using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega). LDH release was calculated as [extracellular LDH/(intracellular LDH + extracellular LDH) × 100].

13. Determination of mitochondrial damage and mitochondria-derived products

To determinate mitochondrial damage, I used MitoTracker Deep Red (membrane potential dependent mitochondrial dye, Invitrogen), MitoTracker Green (membrane potential independent mitochondrial dye, Invitrogen) double staining. Cells were treated with indicated stimulation, and washed with PBS, then trypsinized.

Detached cells were incubated with PBS containing mitoTracker Deep Red 25 nM and mitoTracker Green 50 nM for 10 min at 37℃. Emitted fluorescence was measured by FACSverse analyzer (BD) and analyzed with FlowJo analytical software (TreeStar, Ashland, OR, USA)

To measure mitochondrial ROS, I used MitoSOX (mitochondrial superoxide indicator, Invitrogen) staining according to the manufacture's protocol. Cells were stimulated and detached as described above. Detached cells were incubated with HBSS solution containing MitoSOX 2.5 μM for 10 min at 37℃. Emitted fluorescence was detected by FACSverse analyzer.

To assess cytosolic mtDNA, cells were homogenized with a 21 G syringe in buffer A containing 250 mM sucrose, 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors. The lysates were centrifuged for 10 min at 700 x g to remove nuclear fraction. The supernatant were normalized before centrifuged for 30 min at 10,000 x g for the production of a supernatant corresponding to the cytosolic fraction. DNA was isolated from 200 μl of the cytosolic fraction with a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The copy number of DNA encoding cytochrome C oxidase 1 was measured by quantitative real-time PCR using SYBR *Premix Ex Taq*™ II (Takara) with the same volume of the DNA solution. The following primers were used: forward, 5′-GCC CCA GAT ATA GCA TTC CC-3′, and reverse, 5′-GTT CAT CCT GTT CCT GCT CC-3′. (*COX1*, cytochrome C oxidase I)

14. Observation of mitochondrial morphology

To observe mitochondrial morphology, immunofluorescence analysis was performed with anti-TOM20 Ab (Santa Cruz), and examined using a confocal microscope (LSM700; Carl Zeiss). For transmission electron microscopy (TEM), cells were fixed with 2% glutaraldehyde paraformaldehyde in 0.1 M phosphate buffer

pH 7.4 for 2 hr, then washed with PBS. Cells were post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 2 hr and dehydrated in ascending gradual series (50~100%) of ethanol. Specimens were embedded using a Poly/Bed 812 kit (Polysciences, Warrington, PA, USA). Seventy-nanometer-thin sections were stained with uranyl acetate and lead citrate. Stained sections were then observed using a transmission electron microscope (JEM-1011; JEOL, Tokyo, Japan).

15. Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) of individual sample or independent experiments. The data were analyzed with Student's *t*-test, and p values ≤ 0.05 were considered significant.

Table 1. shRNA sequences to knockdown target genes

		Sequences
MAVS	Forward	5’-CCGGAGTGCACTCTTGGCTGTATTCCTCGAGGAATACAGCCAAGAGTGCACTTTTTTG-3’
	Reverse	5'-AATTCAAAAAAGTGCACTCTTGGCTGTATTCCTCGAGGAATACAGCCAAGAGTGCACT-3'
Drp1 (#2)	Forward	5’-CCGGCGGTGGTGCTAGGATTTGTTACTCGAGTAACAAATCCTAGCACCACCGTTTTTG-3’
	Reverse	5’-AATTCAAAAACGGTGGTGCTAGGATTTGTTACTCGAGTAACAAATCCTAGCACCACCG-3’
Drp1 (#3)	Forward	5’-CCGGCGGTGGTGCTAGGATTTGTTACTCGAGTAACAAATCCTAGCACCACCGTTTTTG-3’
	Reverse	5’-AATTCAAAAACGGTGGTGCTAGGATTTGTTACTCGAGTAACAAATCCTAGCACCACCG-3’
Drp1 (#4)	Forward	5’-CCGGGGCAATTGAGCTAGCGTATATCTCGAGATATACGCTAGCTCAATTGCCTTTTTG-3’
	Reverse	5’-AATTCAAAAAGGCAATTGAGCTAGCGTATATCTCGAGATATACGCTAGCTCAATTGCC-3’

Ⅲ. RESULTS

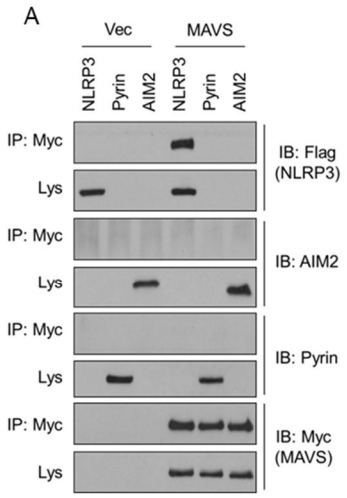
PART Ⅰ: Mitochondrial anti-viral signaling protein is required for the NLRP3 inflammasome activation in response to Sendai virus infection

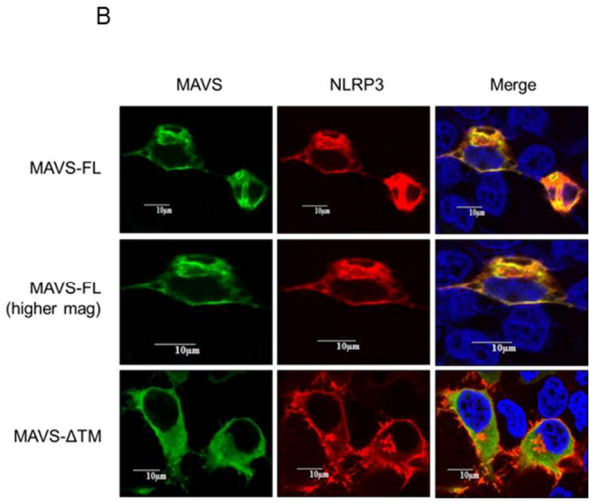
1. Molecular association of NLRP3 with MAVS

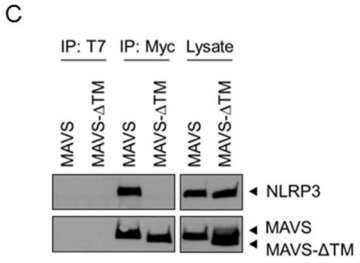
Recent studies have reported that NLRP3 is recruited to the mitochondria during activation, and mitochondria-derived molecule such as mtROS or mtDNA which are released from damaged mitochondria in response to various stimulations are involved in NLRP3 inflammasome activation, indicating that NLRP3 might be associated with damaged mitochondrial outer membrane protein.[31,35] To explore this possibility, I examined the interaction between NLRP3 and the mitochondrial outer membrane protein MAVS, which has been known to play an important role in innate immune responses in response to viral infection.[36] I performed co-immunoprecipitation in transfected 293T cells, and I observed the interaction between MAVS and NLRP3, while AIM2 or Pyrin inflammasomes did not interact with MAVS protein (Fig. 1A). Because the location of MAVS on mitochondrial outer membrane is critical for its signaling,[36] I wondered whether MAVS localization is required for the interaction between MAVS and NLRP3. To investigate this, I examined the interaction between NLRP3 and MAVS-ΔTM, which lacks the C-terminal mitochondrial-targeting transmembrane domain. Confocal immunofluorescence image shows that MAVS localization is critical for co-localization with NLRP3 (Fig. 1B). And co-immunoprecipitation data shows that the full-length MAVS interacts with NLRP3 in transfected 293T cells, while MAVS-ΔTM did not (Fig. 1C), suggesting that the localization of MAVS on mitochondrial outer membrane is essential for the interaction between MAVS and NLRP3.

Since previous studies reported that Sendai virus infection to 293T cells and mouse embryonic fibroblasts induce the formation of large MAVS signaling complexes composed of functional prion-like MAVS aggregates,[42] I investigated

whether Sendai virus infection could induce the interaction between MAVS and NLRP3. Interestingly, I observed the interaction between endogenous MAVS and NLRP3 in response to Sendai virus in THP-1 cells (Fig. 4D). Together, these results indicate that mitochondrial MAVS interacts with NLRP3 in response to Sendai virus infection.







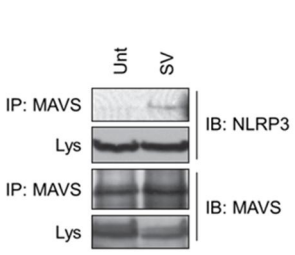


Figure 1. MAVS interacts to NLRP3 on the mitochondria. (A) 293T cells were transfected with empty vector (Vec) or Myc-MAVS expression plasmid together with Flag-NLRP3, T7-pyrin, or T7-AIM2 expression plasmid (0.3 μg) as indicated. Immunoprecipitation was performed with cell lysates using anti-Myc Ab, and the immunoprecipitates were immunoblotted with anti-Flag (NLRP3), anti-AIM2, anti-pyrin, or anti-Myc (MAVS) Abs. **(B)** 293T cells were transfected with NLRP3 and MAVS full-length (MAVS-FL) or truncated (MAVS-ΔTM) expressing plasmid (0.1 μg). Immunofluorescence was performed with anti-MAVS (Green) or NLRP3 (Red). Cells were observed using confocal microscopy. Scale bar, 10 μm. **(C)** 293T cells were transfected with Flag-NLRP3 and Myc-MAVS or Myc-MAVS-ΔTM plasmids (0.3 μg) as indicated. Cell lysates were immunoprecipitated with anti-T7 (negative control) or anti-Myc Ab, and the immunoprecipitates were immunoblotted with anti-Flag or Myc Abs. (**D**) PMA-differentiated THP-1 cells were treated with Sendai virus (10 HA/ml, 4 hr). Co-immunoprecipitation was performed with the cell lysates using anti-MAVS Ab, and the immunoprecipitates and cell lysates (Lys) were immunoblotted with anti-NLRP3 or MAVS Ab as indicated.

2. Activation of NLRP3 inflammasome by Sendai virus infection

Since I observed the interaction between MAVS and NLRP3 in response to Sendai virus infection, I investigated whether MAVS is involved in NLRP3 inflammasome activation. To investigate this, I infected Sendai virus to NLRP3 transfected caspase-1 and ASC expressing 293T (293T-C1A) cells and performed immunoblot analysis. Sendai virus infection potentiated the activation of caspase-1, but without the NLRP3 transfection, 293T-C1A cells could not activate caspase-1. These results indicate that the aggregation of MAVS by Sendai virus infection leads to NLRP3-mediated caspase-1 activation (Fig. 2A). Supporting this, I treated wild-type bone marrow-derived macrophages (BMDM) with Sendai virus or LPS, followed with nigericin stimulation, which is known as a NLRP3 inflammasome activating stimulus. As a result, Sendai virus plus nigericin or LPS plus nigericin treatments showed activation of caspase-1, but Sendai virus infection or nigericin treatment alone did not activate caspase-1 (Fig. 2B).

Notably, NLRP3 KO macrophages did not exhibit Sendai virus-mediated caspase-1 activation, but NLRP3 restored BMDM could activate caspase-1 (Fig. 2C), suggesting that Sendai virus-mediated caspase-1 activation occurs in NLRP3 dependent manner. Confocal immunofluorescence image also support these results. NLRP3 KO cells stably reconstituted with NLRP3-GFP protein showed more aggregation of NLRP3 in response to Sendai virus plus nigericin or LPS plus nigericin stimulation than Sendai virus or nigericin treatment alone (Fig. 2D). These data indicate that Sendai virus infection contributes to NLRP3 priming signal in NLRP3 inflammasome activation.

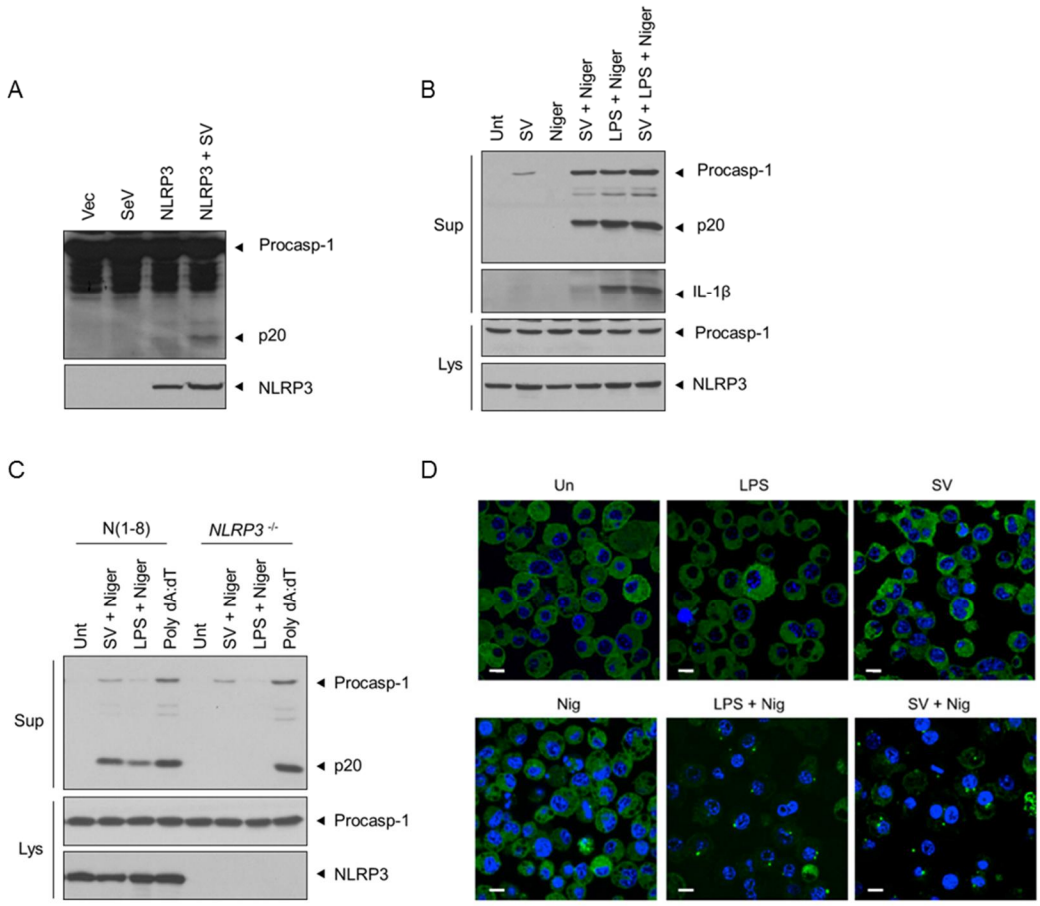


Figure 2. Sendai virus causes NLRP3-depnedent caspase-1 activation. (**A**) 293T-C1A cells were transfected with empty vector (Vec) or NLRP3 expression plasmid followed with no treatment (Un) or Sendai virus infection (SV; 9 hemagglutination activity, HA/ml) for 8 hr. Cell lysates were immunoblotted with anti-caspase-1 or Flag (NLRP3) Ab as indicated. (**B**) Wild-type mouse BMDMs were treated with Sendai virus (SV; 10 HA/ml, 6 hr), nigericin (Nig; 5 μM, 45 min), SV(6 hr) followed with nigericin (45 min; SV+Nig), LPS (0.25 μg/ml, 4 hr) followed with nigericin (LPS+Nig), or SV (6 hr) followed with LPS (4 hr) and nigericin (45min; LPS+SV+Nig) as indicated. Cell culture supernatants (Sup) or Cell lysates (Lys) were immunoblotted with indicated Abs. (**C**) NLRP3-deficient (NLRP3 KO) or NLRP3-restored (N1-8) BMDMs were treated with SV (10 HA/ml, 6 hr) plus nigericin (5 μM,45 min), LPS (0.25 μg/ml, 4 hr) plus nigericin (LPS+Nig), or transfected with Poly(dA:dT) (2 μg, 6 hr) as indicated. Cell culture supernatants (Sup) or Cell lysates (Lys) were immunoblotted with indicated Abs. (**D**) NLRP3-GFP–expressing BMDMs were treated with LPS, SV, Nig, LPS+Nig, or SV+Nig as described in (A) and (B). NLRP3 aggregates were observed by confocal microscope. NLRP3 (Green), nuclear (Blue). Scale bars, 10 μm.

3. Role of MAVS in the NLRP3 inflammasome activation and assembly by Sendai virus

Next, in order to assess whether MAVS requires Sendai virus-mediated caspase-1 activation, I produced MAVS knockdown macrophages by using short-hairpin RNA interference (Fig. 3A). Knockdown of MAVS in macrophages (shMAVS) showed markedly decrease of Sendai virus-mediated caspase-1 activation level but had no effects on NLRP3 or AIM2 inflammasome activation induced by their activating stimulation, such as LPS plus nigericin stimulation or Poly(dA:dT) transfection (Fig. 3B,C). Moreover, MAVS knockout BMDM exhibits complete dependency to MAVS for Sendai virus-mediated caspase-1 activation, while LPS followed nigericin stimulation did not exhibit difference in both cells (Fig. 3D). To provide an additional evidence for the role of MAVS in Sendai virus-mediated caspase-1 activation, I examined whether PMA-differentiated THP-1 macrophages could also induce Sendai virus-mediated caspase-1 activation in MAVS dependent manner. I observed that Sendai virus infection alone is sufficient to induce capsase-1 activation in THP-1-derived macrophages. On the other hand, shMAVS exhibited highly attenuated caspase-1 activation in response to Sendai-virus infection (Fig. 3E). These results indicate that MAVS has an essential role in Sendai virus-mediated caspase-1 activation and dispensable for classical activation of NLRP3 inflammasome induced by LPS plus nigericin stimulation.

The assembly of NLRP3 inflammasome components is required for NLRP3 inflammasome activation.[10] Especially, ASC oligomerization has critical role in caspase-1 activation in response to NLRP3 activating stimuli.[70] Supporting the earlier reports, PMA-differentiated THP-1 macrophages infected with Sendai virus infection showed robust ASC oligomerization, but cells with reduced level of MAVS expression exhibited significantly decreased level of ASC oligomerization (Fig. 4A). Furthermore, over 60% of THP-1-ASC-GFP cells showed ASC specks during Sendai virus infection, while knockdown of MAVS significantly prevented from forming

ASC specks in response to Sendai virus infection (Fig. 4B). Together, these results indicate that MAVS promote assembly and activation of NLRP3 inflammasome in response to Sendai virus infection.

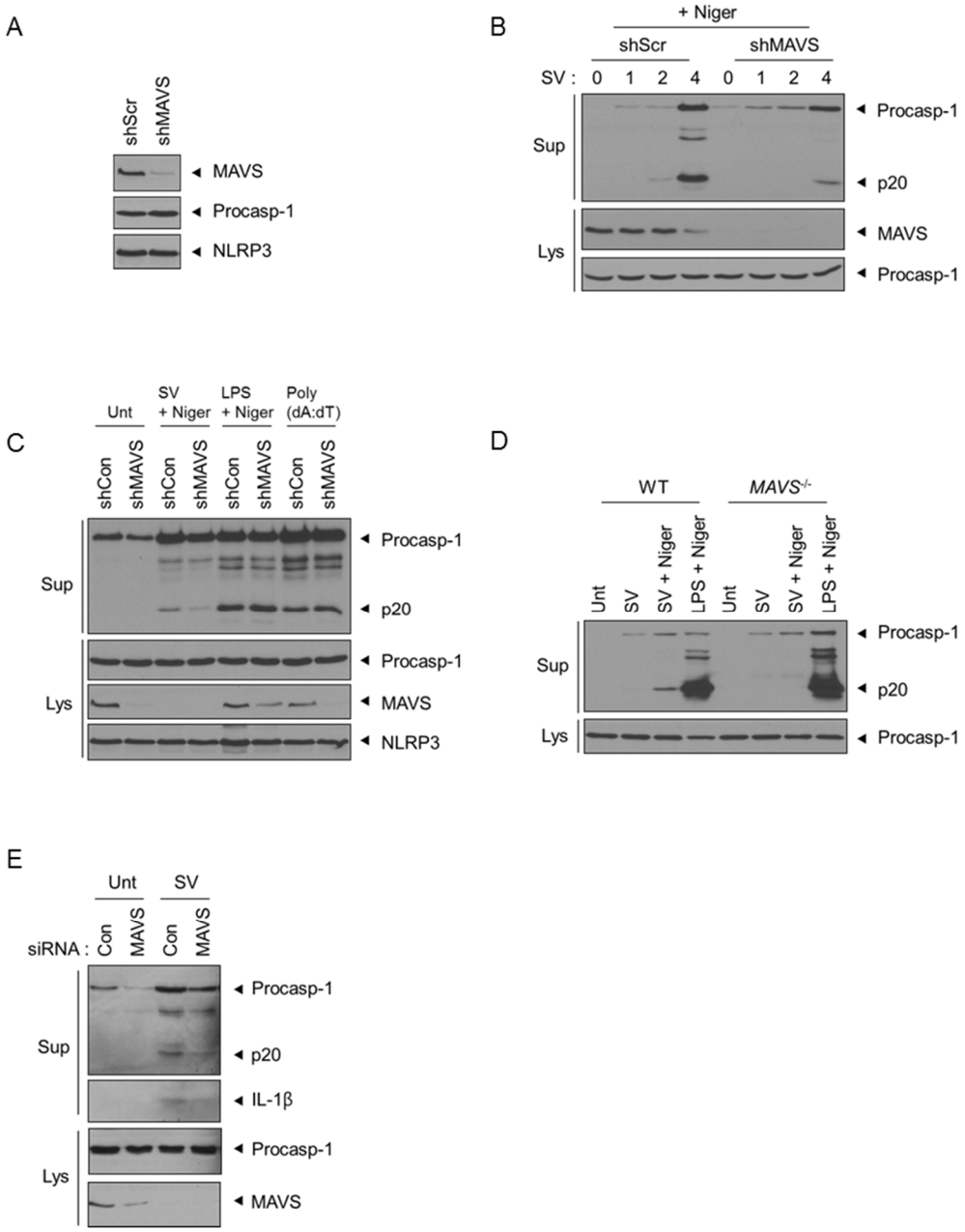


Figure 3. Deficiency of MAVS abrogates the Sendai virus-induced caspase-1 activation. (**A**) Stable knockdown of MAVS protein expression macrophages were produced by lentiviral particles containing short hairpin RNA (shRNA) targeting mouse MAVS (shMAVS) or non-targeting control (shScr). Cell lysates were immunoblotted with indicated Abs. (B and C) shScr or shMAVS BMDMs were treated with (**B**) Sendai virus (10 HA/ml, 0–4 hr) followed with nigericin (5 μM, 45 min) or (**C**) treatment with SV plus nigericin (SV+Nig), or LPS plus nigericin (LPS+Nig), or transfection with Poly(dA:dT) (2 μg, 6 hr). Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs. (**D**) Wild-type or MAVS KO BMDMs were treated with SV, Sendai virus plus nigericin (SV+Nig), or LPS plus nigericin (LPS+Nig). Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs. (**E**) PMA-differentiated THP-1 cells were transfected with scrambled (Con) or human MAVS-specific (MAVS) siRNA oligonucleotides (50 nM) for 48 hr, followed with Sendai virus infection(10 HA/ml, 6 hr). Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs.

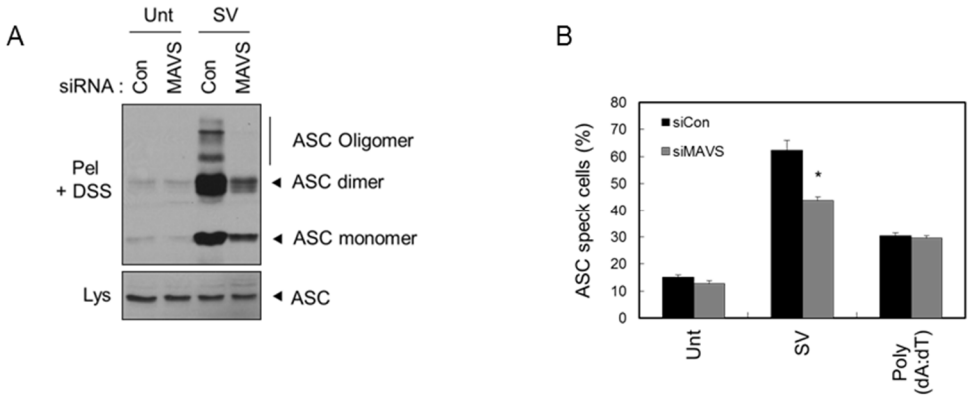


Figure 4. Knockdown of MAVS attenuates the Sendai virus-induced oligomerization of ASC. (**A**) PMA-differentiated THP-1 cells were transfected with scrambled (Con) or human MAVS-specific (MAVS) siRNA oligonucleotides (50 nM) for 48 hr, followed with Sendai virus infection(10 HA/ml, 6 hr). cell lysates were performed disuccinimidyl suberate (DSS) cross-linking analysis. DSS added insoluble pellet (Pel + DSS) or soluble lysates from the same cells (Lys) were immunoblotted with indicated Abs. (**B**) PMA-differentiated THP-1–ASC–GFP cells were transfected with siMAVS, followed with Sendai virus infection (10 HA/ml, 6 hr) or transfected with Poly(dA:dT) (2 μg, 6 hr). ASC speck-containing cells (ASC specks) were counted using fluorescence microscopy and represented as percentages of total cells counted. Asterisk indicates significant difference compared with siCon cells (*$p < 0.05$, $n = 5$).

4. Role of MAVS in the oligomerization of NLRP3

Previous studies reported that Sendai virus infection induces the formation of large SDS-resistant MAVS oligomers[42] and I found that Sendai virus infection also induces the interaction between MAVS and NLRP3. Therefore, I hypothesized that the activation of MAVS might influence the priming step of NLRP3 inflammasome activation by inducing formation of SDS-resistant NLRP3 oligomers on the mitochondria. To test this possibility, I transfected 293T cells with NLRP3 and MAVS together, and assessed whether NLRP3 could form SDS-resistant oligomers by immunoblot analysis. Surprisingly, I could detect SDS-resistant NLRP3 oligomers in MAVS dependent manner (Fig. 5A). Furthermore, co-transfected 293T cells with NLRP3 and MAVS-ΔTM did not form NLRP3 oligomers, indicating that the localization of MAVS on mitochondria is essential for MAVS dependent NLRP3 oligomerization (Fig. 5B). Considering a recent study which reported that a fraction of NLRP3 is recruited to the mitochondria during activation,[42] I thought that NLRP3 oligomerization might occur on the mitochondrial outer membrane. To examine this, I transfected NLRP3 to 293T cells with or without MAVS, and fractionated these cell lysates into cytosolic and mitochondria-enriched fraction. Although MAVS expression levels were almost the same between cytosolic and mitochondria-enriched fraction, NLRP3 protein expression was mainly observed in the cytosolic fraction. However, by using immunobolt analysis, I could detect MAVS-mediated NLRP3 oligomers solely in the mitochondria-enriched fraction. These indications suggest that the MAVS-mediated NLRP3 oligomerization occurs on the mitochondrial outer membrane (Fig. 5C). To support these data, I tested whether Sendai virus infection also induces endogenous NLRP3 oligomerization. I infected NLRP3 expressing 293T cells with Sendai virus and assessed NLRP3 oligomers by immunoblot analysis. Same as the earlier results, Sendai virus infection also induced endogenous NLRP3 oligomerization (Fig. 5D). Together, these results suggest that Sendai virus infection induces NLRP3 oligomerization on the mitochondrial outer membrane in a MAVS dependent manner.

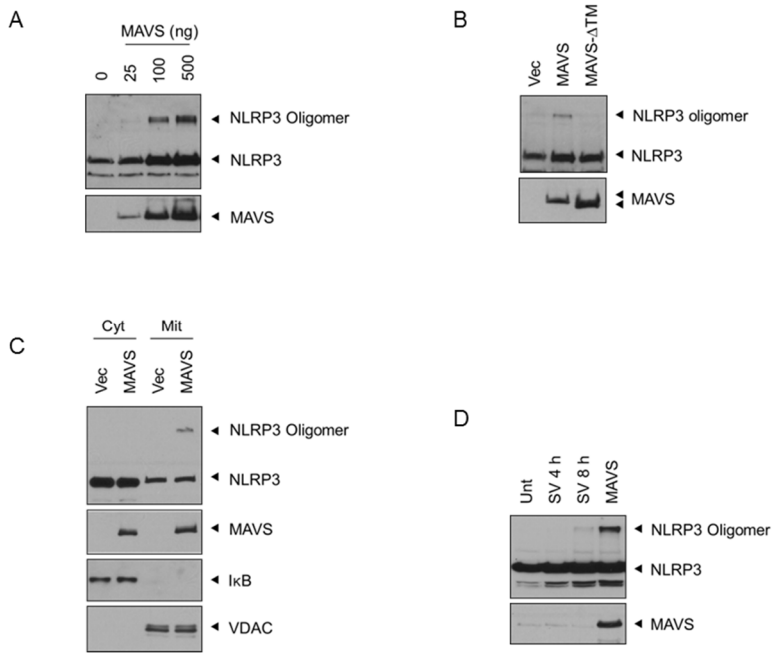


Figure 5. MAVS causes the NLRP3 oligomerzation on the mitochondria. (**A**) 293T cells were transfected with NLRP3 (0.3 μg) and increasing amount of MAVS expression plasmid as indicated. Cell lysates were immunoblotted with anti-Flag (NLRP3) or Myc (MAVS) Abs. (**B**) 293T cells were transfected with NLRP3 expression plasmid together with an empty vector (Vec) or MAVS full-length or truncated (-ΔTM) construct. Cell lysates were immunoblotted with indicated Abs. (**C**) Cell lysates were fractionated into cytosolic or mitochondrial protein-enriched fraction, and immunoblotted with anti-Flag (NLRP3), Myc (MAVS), IκB (cytosol), or VDAC1 (mitochondria) Abs. (**D**) 293T cells were transfected with NLRP3 (0.3 μg) followed with Sendai virus infection (10 HA/ml) for the indicated times, or transfected with NLRP3 together with MAVS (0.3 μg). Cell lysates were immunoblotted with anti-NLRP3 or MAVS Abs.

Since mtROS has been implicated in the activation of the NLRP3 inflammasome,[31] I examined whether ROS also influences MAVS-mediated NLRP3 oligomerization. To test the effect of ROS on MAVS-mediated NLRP3 oligomerization, I transfected NLRP3 with or without MAVS to 293T cells, and induced mtROS by rotenone treatment, which is known as a mitochondrial complex Ⅰ inhibitor.[71] Notably, rotenone treatment could induce MAVS-mediated NLRP3 oligomerization (Fig. 6A). To confirm the role of ROS in MAVS-mediated NLRP3 oligomerization, I treated ROS scavenger N-acetyl cysteine (NAC) to NLRP3 expressing 293T cells transfected with MAVS or empty vectors. As I expected, removal of ROS by NAC treatment completely inhibited MAVS-mediated NLRP3 oligomerization (Fig. 6B). However, NAC treatment did not affect the interaction between MAVS and NLRP3 (Fig. 6C). Together, these data suggest that ROS has a critical role in MAVS-mediated NLRP3 oligomerization.

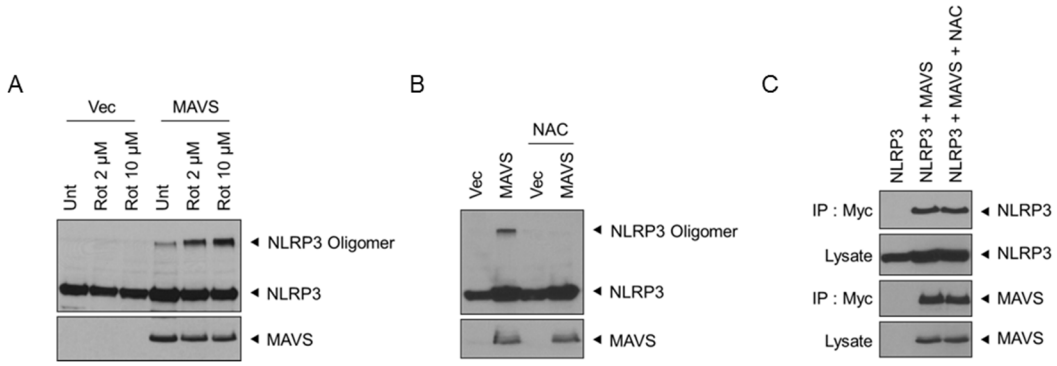


Figure 6. Mitochondrial ROS is involved in the MAVS-induced oligomerization of NLRP3. (**A**-**C**) 293T cells were transfected with an NLRP3 expression plasmid (0.3 μg) together with an empty vector or a MAVS construct (0.3 μg), and treated with (**A**) rotenone for 6 hr or (**B** and **C**) NAC (15 mM, 18 hr) as indicated. Cell lysates were immunoblotted with anti-Flag (NLRP3) or Myc (MAVS) Abs. (**C**) Cell lysates were immunoprecipitated using anti-Myc Ab, and the immunoprecipitates were then immunoblotted with anti-Flag (NLRP3) or anti-Myc (MAVS) Ab.

5. Inhibition of MAVS-dependent type Ⅰ interferon production by NLRP3

The preceding results show that MAVS interacts with NLRP3, and this interaction implies that MAVS and NLRP3 might influence their signaling reciprocally. In earlier data, I have demonstrated that MAVS promotes NLRP3 inflammasome activation in response to Sendai virus infection. Next, I wondered whether NLRP3 could regulate MAVS signaling. To examine the role of NLRP3 in MAVS signaling, I used luciferase activity assay for measuring MAVS-mediated type Ⅰ IFN production. Surprisingly, overexpression of NLRP3 in 293T cells induced significant inhibition of MAVS-mediated activation of the IFN-β promoter. Moreover, this inhibition was specific for NLRP3. Overexpression of AIM2 or pyrin failed to inhibit MAVS-mediated IFN-β promoter activation (Fig. 7A). Furthermore, overexpression of NLRP3 also inhibited Poly(I:C) or Sendai virus-mediated activation of IFN-β promoter (Fig. 7B,C). To confirm these data, I assessed IRF3 phosphorylation in response to Sendai virus infection in NLRP3 KO or NLRP3 restored (N1-8) BMDM. The phosphorylation of IRF3 in response to Sendai virus infection was increased in NLRP3 KO BMDM compared with N1-8 cells (Fig. 7D). Therefore, NLRP3 could negatively regulate MAVS-mediated type Ⅰ IFN production in response to Sendai virus infection.

Collectively, I demonstrated the role of MAVS in NLRP3 inflammasome activation in response to Sendai virus infection by facilitating NLRP3 oligomerization on the mitochondria. In addition, I also demonstrated that NLRP3 could play as a negative regulator of MAVS signaling during Sendai virus infection.

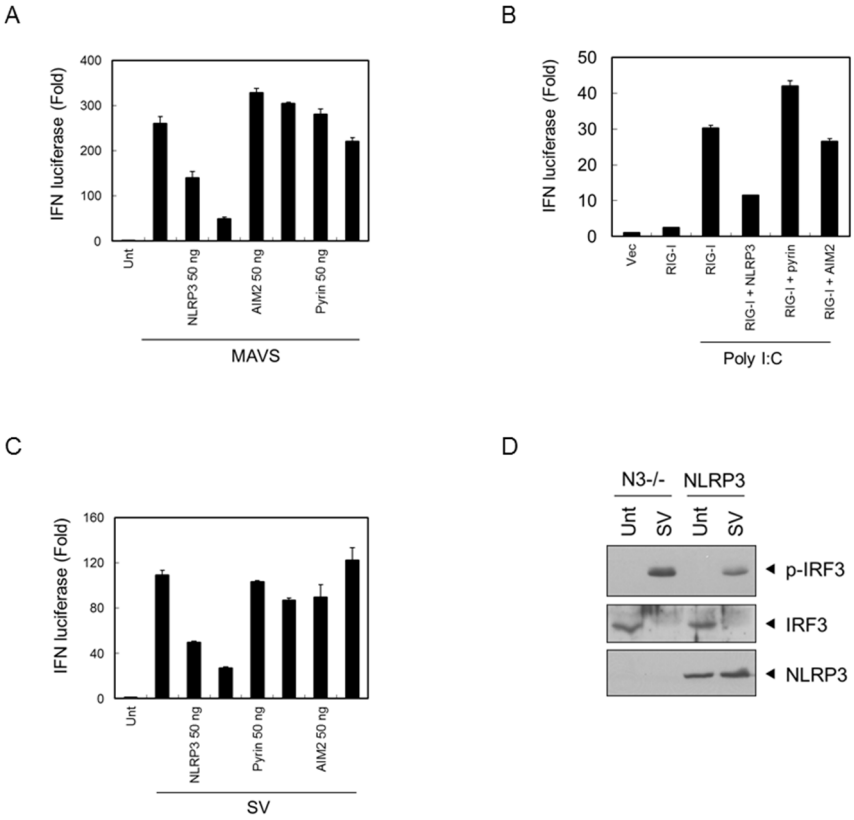


Figure 7. NLRP3 inhibits MAVS-mediated type I IFN production. (**A**) 293T cells were transfected with IFN-β promoter-luciferase reporter plasmid (100 ng), p–β-galactosidase (100 ng), and MAVS expression plasmid (200 ng) together with empty vector (Vec), NLRP3, AIM2, or pyrin as indicated. Luciferase assay was performed with cell lysates. (**B** and **C**) 293T cells were transfected as in (A) together with RIG-I expression plasmid with or without NLRP3, Pyrin, or AIM2 expression plasmid. Twenty-four hr after transfection, (**B**) cells were transfected with Poly(I:C) (10 μg, 20 h) or (**C**) infected with Sendai virus (10 HA/ml, 8 hr) as indicated. Reporter luciferase activity was then performed as indicated (**D**). NLRP3-deficient (NLRP3-KO) or NLRP3-restored BMDMs (NLRP3) were infected with Sendai virus (5 HA/ml, 8 hr), and cell lysates were immunoblotted with indicated Abs.

PART Ⅱ. Mitochondrial elongation caused by Drp1 knockdown augments the activation of NLRP3 inflammasome

1. Induction of mitochondrial elongation by Drp1 knockdown in macrophages

To examine whether abnormal mitochondrial dynamics affects inflammasome, I established Drp1 knockdown macrophages (shDrp1) by shRNA interference. Since Drp1 has been known to play an important role in mitochondrial fission,[58] I induced abnormal mitochondrial elongation by reducing the expression of Drp1 protein in macrophages. Immunoblot analysis show that the expression of Drp1 protein level was significantly reduced in shDrp1 macrophages, while the level in non-targeting (shScr) macrophages was not changed compared to wild-type macrophages (Fig. 8A).

It has been well known that preventing mitochondrial fission leads to elongation of mitochondria.[44] As similar to these reports, I observed shDrp1 macrophages exhibit elongated and hyperfused mitochondria compared to shScr cells by using microscopy (Fig. 8B,C). Together, knockdown of Drp1 leads to abnormal mitochondrial elongation in macrophages.

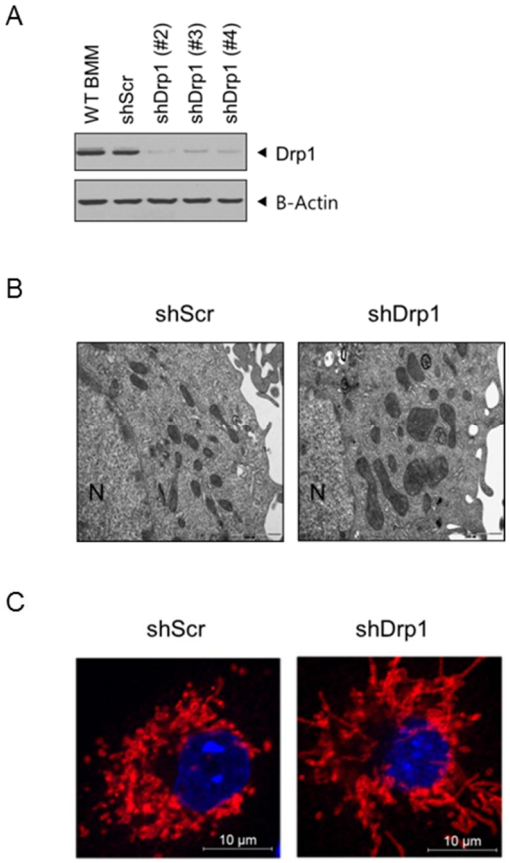


Figure 8. Knockdown of Drp1 induces mitochondrial elongation. (**A**) Stable knockdown of Drp1 protein expression macrophages were produced by lentiviral particles containing short hairpin RNA (shRNA) targeting mouse Drp1 (shDrp1) or non-targeting control (shScr). Cell lysates were immunoblotted with indicated Abs. (**B** and **C**) shScr or shDrp1 BMDMs mitochondria were observed by (**B**) transmission electron microscopy (TEM) (Scale bars, 2 μm. magnification, 20,000×) or (**C**) immunofluorescence confocal microscopy (TOM20: red, nuclear: blue, Scale bars, 10 μm).

2. Potentiation of pyroptotic cell death by Drp1 knockdown

Previous studies reported that mitochondria have an important role in regulating programmed cell death.[58,72] In addition, recent studies reported that elongated mitochondria prevent intrinsic pathway of apoptosis by inhibiting the release of cytochrome C into the cytosol in response to TNF-α and cycloheximide (TC) stimulation.[58] I examined the effect of mitochondrial elongation in various type of cell death such as apoptosis, necroptosis, or pyroptosis. To compare shDrp1 and shScr macrophages in cell death, I treated TC or zVAD plus TNF-α and cycloheximide (TCZ) in order to induce apoptotic and necroptotic cell death. shDrp1 macrophages exhibited significantly decreased LDH release and caspase-3 processing, as similar to the previous reports. However, shDrp1 did not affect necroptotic cell death (Fig. 9A,B). Notably, shDrp1 macrophages exhibit largely increased pyroptosis induced by LPS followed ATP or nigericin stimulation (Fig. 9C). These data indicate that knockdown of Drp1 increases pyroptotic cell death in response to NLRP3 inflammasome activating stimulation.

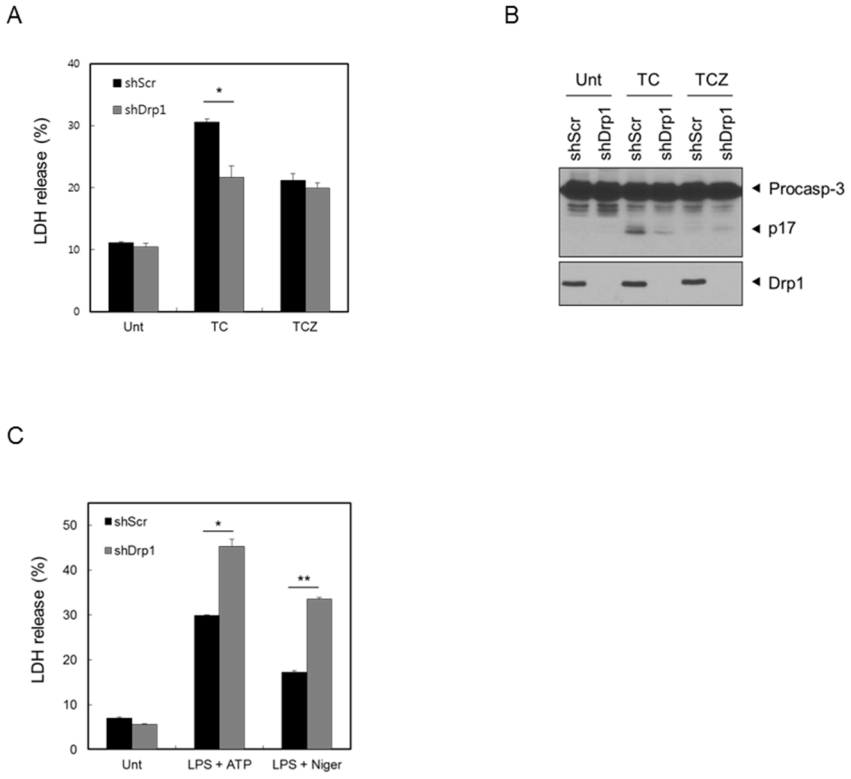


Figure 9. Knockdown of Drp1 increases the susceptibility to pyroptosis-inducing stimulation. (**A** and **B**) shScr or shDrp1 BMDMs were treated with TNF-α and CHX in the absence (TC) or presence of zVAD-fmk (TCZ), or (**C**) primed by LPS (0.25 μg/ml, 3 hr), followed with ATP (2.5 mM, 45 min) or nigericin (5 μM, 45 min). LDH release assay was performed with cell lysates and immunoblotted with anti-caspase-3 or Drp1 Abs. Asterisks indicate significant differences (n = 6, *p< 0.0005, A; n = 6, *p < 0.0005, **p < 0.0001).

3. Potentiation of NLRP3 inflammasome signaling by mitochondrial elongation

Since pyroptosis was deeply associated with activation of caspase-1,[16] above results imply that abnormal mitochondrial elongation might be involved in NLRP3 inflammasome activation. To test this possibility, I examined whether mitochondrial elongation affects the activation of caspase-1. I primed shDrp1 macrophages with LPS, and treated with increasing amount of ATP for the activation of caspase-1. As a result, shDrp1 macrophages showed significant increase in caspase-1 processing and IL-1β secretion in response to LPS plus ATP stimulation (Fig. 10A). To confirm this, I measured IL-1β secretion in response to LPS followed by ATP stimulation in both cells. Similar to the earlier data, shDrp1 macrophages exhibited significantly increased IL-1β secretion in response to LPS plus ATP stimulation compared to shScr cells (Fig. 10B). To test whether mitochondrial elongation promotes NLRP3 inflammasome activation, I treated Drp1-selective chemical inhibitor called mitochondrial division inhibitor-1 (mdivi-1) to wild-type BMDMs.[73] I could detect mdivi-1 treatment increased the activation of caspase-1 in response to LPS plus ATP stimulation in wild-type macrophages (Fig. 10C).

Next, I investigated whether abnormal mitochondrial elongation affects other inflammasome activation. To examine this, I infected *Salmonella* with shDrp1 macrophages to induce NLRC4 inflammasome activation.[11] And I found that *Salmonella* infection was not considerably affected by Drp1 knockdown. This result indicates that increased caspase-1 activation in shDrp1 macrophages is specifically associated with NLRP3 inflammasome (Fig. 10D). Surprisingly, ATP stimulation only in shDrp1 macrophages could activate caspase-1 without LPS priming signal, while shScr macrophages could not (Fig. 10A,C). This implies abnormal mitochondrial elongation might induce priming signal of NLRP3.

Not only macrophages, but also human monocyte THP-1 exhibited increased caspase-1 activation caused by Drp1 knockdown. I produced non-targeting (shScr), Drp1-targeting (shDrp1), or Opa1-targeting (shOpa1) knocked down cells by shRNA

interference. These cells were treated by PMA to differentiate into macrophages, and were examined for NLRP3-mediated caspase-1 activation in response to LPS or Alum stimulation. Similar to shDrp1 macrophages, THP-1-differentiated shDrp1 macrophages exhibited largely increased caspase-1 activation and IL-1β secretion in response to LPS or Alum stimulation. However, shOpa1 macrophages exhibited significant decrease in caspase-1 activation and IL-1 β secretion much less than wild-type cells (Fig. 10E).

As I mentioned earlier, the assembly of NLRP3 inflammasome is essential for NLRP3 inflammasome activation. Thus, I examined Drp1 knockdown effect on the assembly of NLRP3 inflammasome in both cells. As expected, shDrp1 macrophage showed robust enhancement of the interaction between ASC and NLRP3 in response to LPS or LPS plus ATP stimulation. ASC oligomerization also markedly increased compared to shScr cells (Fig. 11A,B). Together, knockdown of Drp1 induced increase of caspase-1 activation in response to NLRP3 activating stimulation.

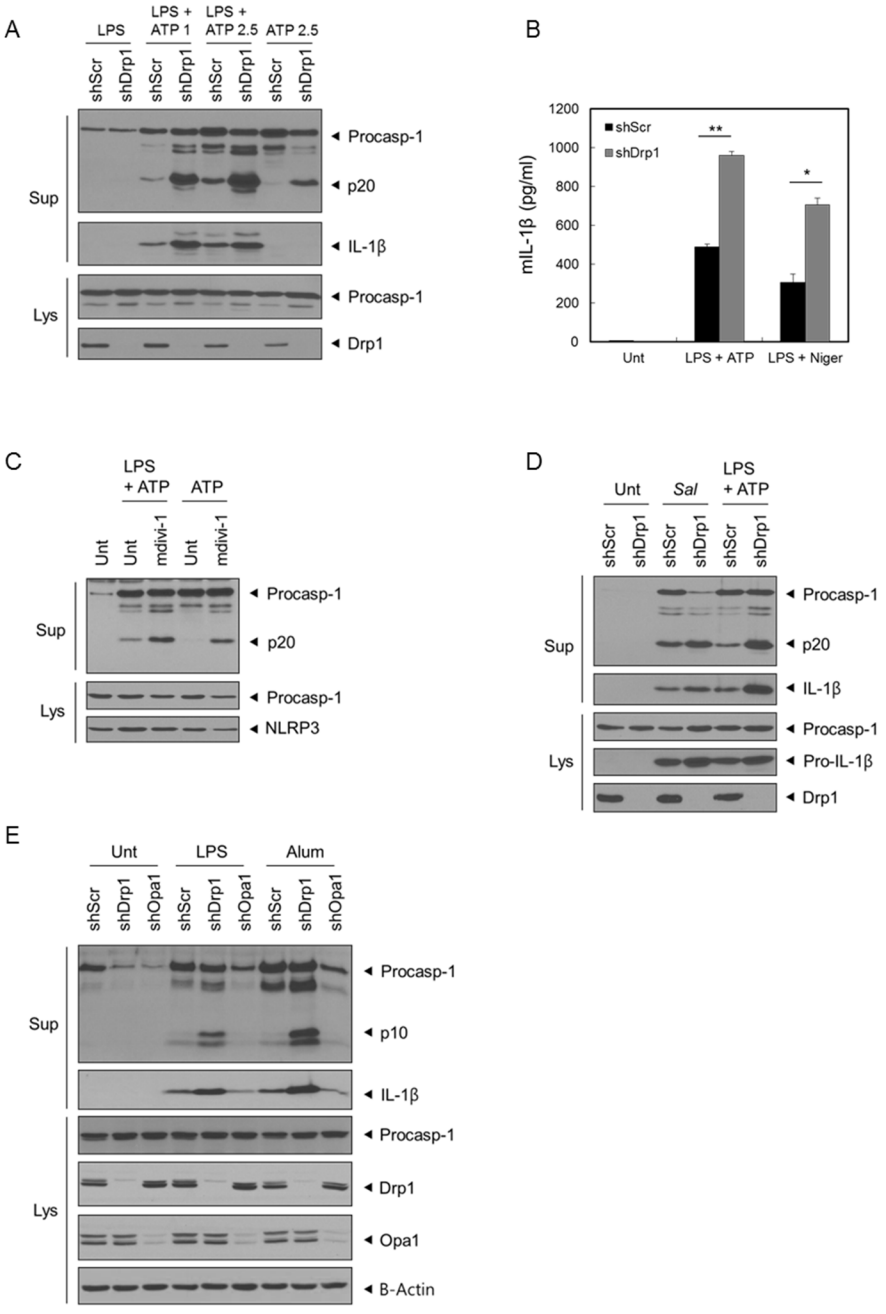


Figure 10. Mitochondrial elongation enhances the NLRP3-dependent caspase-1 activation by NLRP3 agonist. (**A**) shScr or shDrp1 BMDMs were primed by LPS, followed with ATP (1 or 2.5 mM, 45 min) or ATP alone. Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs. (**B**) ShScr or shDrp1 BMDMs were primed by LPS, followed with ATP, or nigericin (5 μM, 45 min). Culture supernatants were assayed for IL-1β secretion by ELISA. Asterisks indicate significant differences (n = 3, *p < 0.05, **p < 0.005). (**C**) Wild-type BMDMs were treated with nothing (Unt), ATP alone, or LPS (0.25 μg/ml, 3 hr) in the presence or absence of mdivi-1 (25 μM, 30 min) pretreatment, followed with ATP (2.5 mM, 45 min). (**D**) shScr or shDrp1 BMDMs were treated with *S. typhimurium* (*Sal*, MOI 20) as described in Materials and Methods or LPS (0.25 μg/ml, 3 hr) followed by ATP (2.5 mM, 40 min). (**E**) PMA-differentiated shScr, shDrp1 or shOpa1 THP-1 cells were treated with LPS (0.5 μg/ml) or Alum (250 μg) for 6 hr. All cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs.

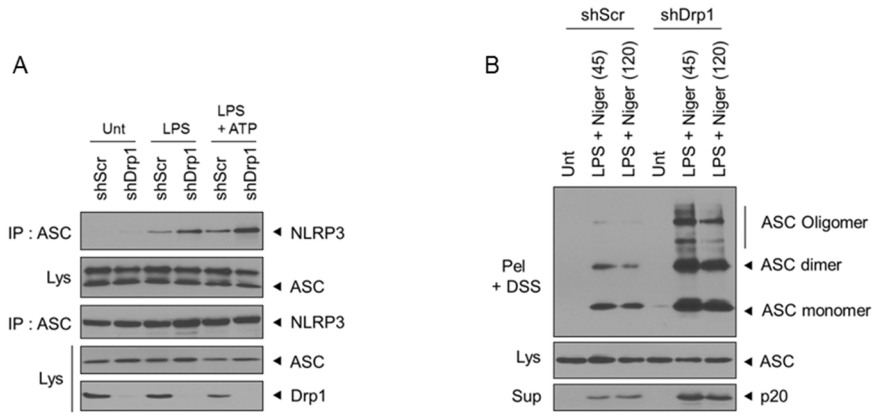


Figure 11. Knockdown of Drp1 potentiates the NLRP3 inflammasome assembly by NLRP3 agonists. (**A**) shScr or shDrp1 BMDMs were treated with LPS (0.25 μg/ml, 3 hr), followed with ATP (2.5 mM, 30 min) as indicated. Soluble cell lysates were immunoprecipitated with anti-ASC antibody, and the immunoprecipitates or lysates were immunoblotted with indicated Abs. (**B**) shScr or shDrp1 BMDMs were treated with LPS (0.25 μg/ml, 3 hr), followed with nigericin (5 μM, 45 min or 120 min). Disuccinimidyl suberate (DSS) cross-linking analysis was performed with cell lysates. DSS added insoluble pellet (Pel + DSS) or soluble lysates from the same cells (Lys) and culture supernatants (Sup) were immunoblotted with anti-ASC or caspase-1 Abs.

4. Impairment of NLRP3 inflammasome signaling by CCCP-induced mitochondrial fission

For the activation of NLRP3 inflammasome, NFκB-mediated NLRP3 protein induction is important.[12] Since shDrp1 macrophages exhibit increase of caspase-1 activation, I assessed the expression of *nlrp3* gene in response to LPS stimulation. However, the expression of *nlrp3* mRNA levels showed no difference in both cells, indicating that the increase of caspase-1 activation in shDrp1 cells was not caused by induction of NLRP3 mRNA levels (Fig. 12A).

To confirm abnormal mitochondrial elongation induces the increase of caspase-1 activation in shDrp1 cells, I treated macrophages with carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), which is known as a mitochondrial fission inducer.[74] As a result, CCCP treatment induced highly attenuated NLRP3 inflammasome activation in response to LPS plus ATP stimulation (Fig. 12B). Consistently, CCCP treatment also inhibit the interaction between ASC and NLRP3, and ASC oligomerization was also significantly reduced in wild-type BMDMs after CCCP treatment (Fig. 12C,D). Therefore, contrast to mitochondrial elongation, CCCP-induced mitochondrial fragmentation attenuates the activation of NLRP3 inflammasome, and I confirmed again that abnormal mitochondrial elongation induce increase of caspase-1 activation in response to NLRP3 activating stimulation.

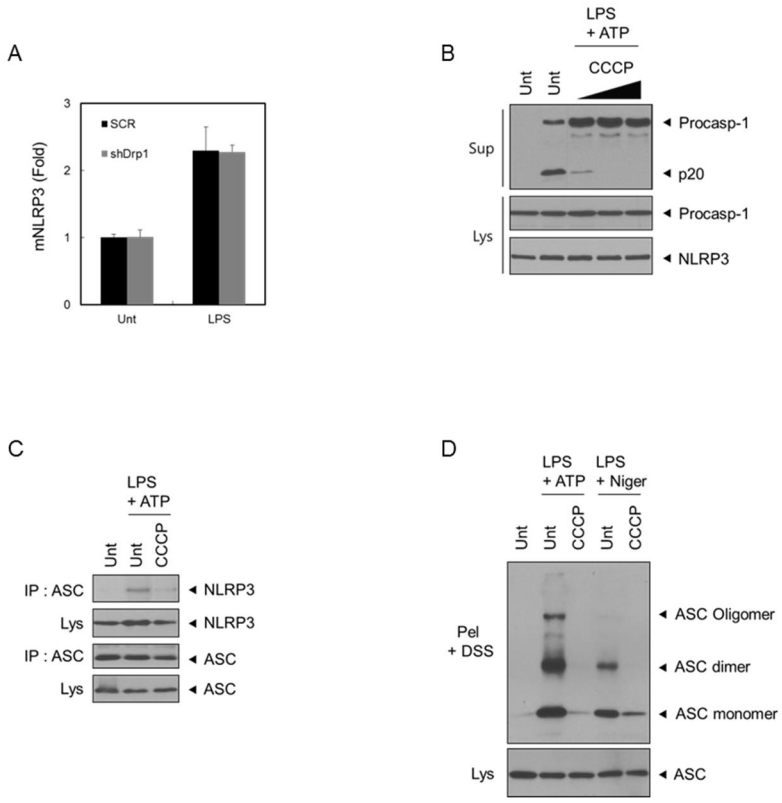


Figure 12. CCCP inhibits the assembly and activation of NLRP3 inflammasome. (**A**) shScr or shDrp1 BMDMs were treated with LPS (0.5 μg/ml, 3 hr), and mRNA production was determined by quantitative real-time PCR (qPCR) as described in Materials and Methods (n = 3). (**B** and **C**) Wild-type BMDMs were treated with (**B**) LPS (0.25 μg/ml, 3 hr) in the presence of CCCP (1–40 μM), followed with ATP (2.5 mM, 40 min) or (**C**) treated with LPS in the presence or absence of CCCP (5 μM) for 3 hr, followed by ATP or nigericin (5 μM, 40 min). Soluble cell lysates were immunoprecipitated as described before. Cell lysates (Lys) were immunoblotted with indicated Abs. (**D**) Wild-type BMDMs were treated with LPS, followed with ATP or nigericin in the presence or absence of CCCP. ASC oligomerization was examined by disuccinimidyl suberate (DSS) cross-linking analysis.

5. Role of mitochondrial damage in the augmented activation of NLRP3 inflammasome seen in Drp1-knockdown macrophages

Currently, a lot of studies have suggested that mitochondria-derived products such as mtDNA or mtROS influence NLRP3 inflammasome activation.[30] I examined the generation of mitochondria-derived products in shDrp1 macrophages compared to shScr cells. To determine mitochondrial ROS generation, I used mitochondrial ROS-sensitive MitoSOX staining. LPS, followed by ATP stimulation induces the production of mtROS in shScr cells, but shDrp1 macrophages exhibited significantly increased mtROS production compared to shScr cells (Fig. 13A). Furthermore, the release of mtDNA into the cytosol during LPS plus ATP stimulation also significantly increased in shDrp1 macrophages (Fig. 13B). Next, I measured damaged mitochondria by using mitochondria sensitive MitoTracker Green (mitochondria specific dye) and MitoTracker Deep Red (mitochondrial membrane potential dependent dye) double staining. As a result, I found that shDrp1 macrophages have more severely damaged mitochondria than shScr cells in response to LPS plus ATP stimulation (Fig. 13C,D).

Although mitochondrial damages were increased in shDrp1 macrophages treated with LPS followed ATP, it could not fully explain how shDrp1 macrophages exhibit enhanced caspase-1 activation than shScr cells. Moreover, mitochondrial-derived products such as mtROS or cytosolic mtDNA were reduced by selective caspase-1 inhibitor YVAD treatment (Fig. 14A-C). In addition, NLRP3 knockout BMDMs exhibit completely inhibited mitochondrial damages in response to LPS followed ATP stimulation (Fig. 14D,E). Therefore, mitochondrial damage was not a cause of NLRP3-mediated caspase-1 activation, but were consequences of augmented caspase-1 activation.

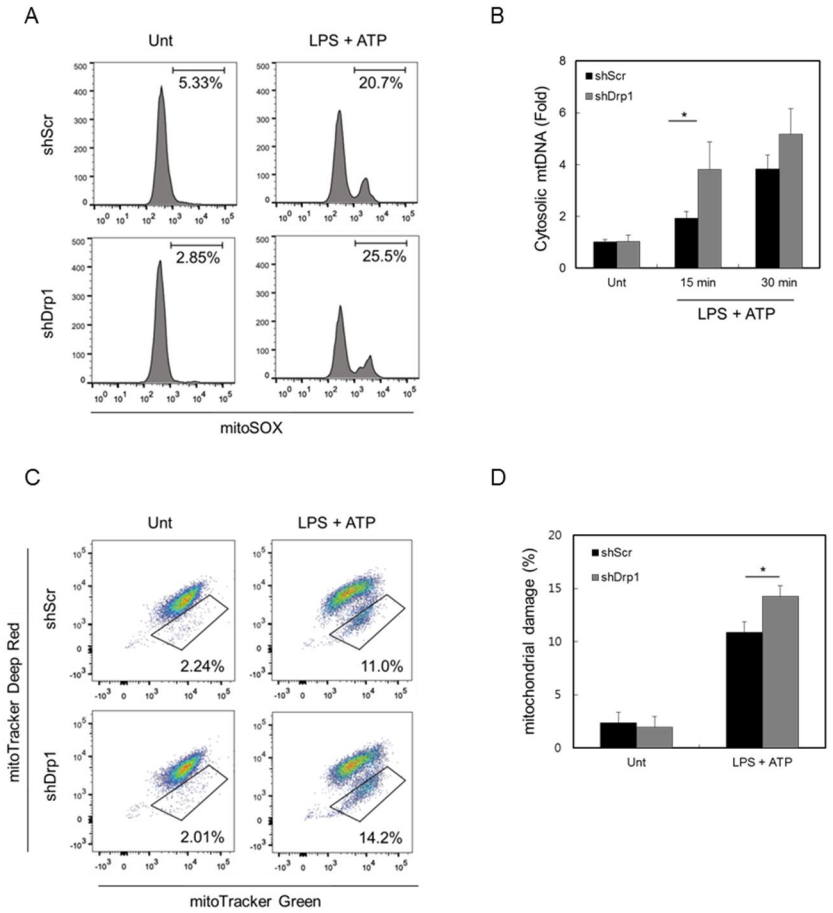


Figure 13. Drp1-knockdown cells show the increased indications of mitochondrial damage by NLRP3 agonists. (**A**-**D**) shScr or shDrp1 BMDMs were (**A**,**C**, and **D**) primed with LPS (0.25 μg/ml, 3 hr) followed by ATP (2.5 mM, 30 min, or 15-30 min, **B**) treatment.(**A**) Cells were stained with MitoSox (2.5 μM, 10 min) and emitted fluorescence was measured by FACSverse analyzer. (**B**) Cytosolic mtDNA were measured by quantitative real-time PCR (qPCR) as described in Materials and Methods (**C**) Cells were costained with MitoTracker Green (50 nM, 10 min) and MitoTracker Deep Red (25 nM, 10 min), and then analyzed by flow cytometer. (**D**) Representative histograms of three-independent experiments were presented as in (**C**). Asterisk indicates a significant difference ($n = 3$, $*p < 0.05$).

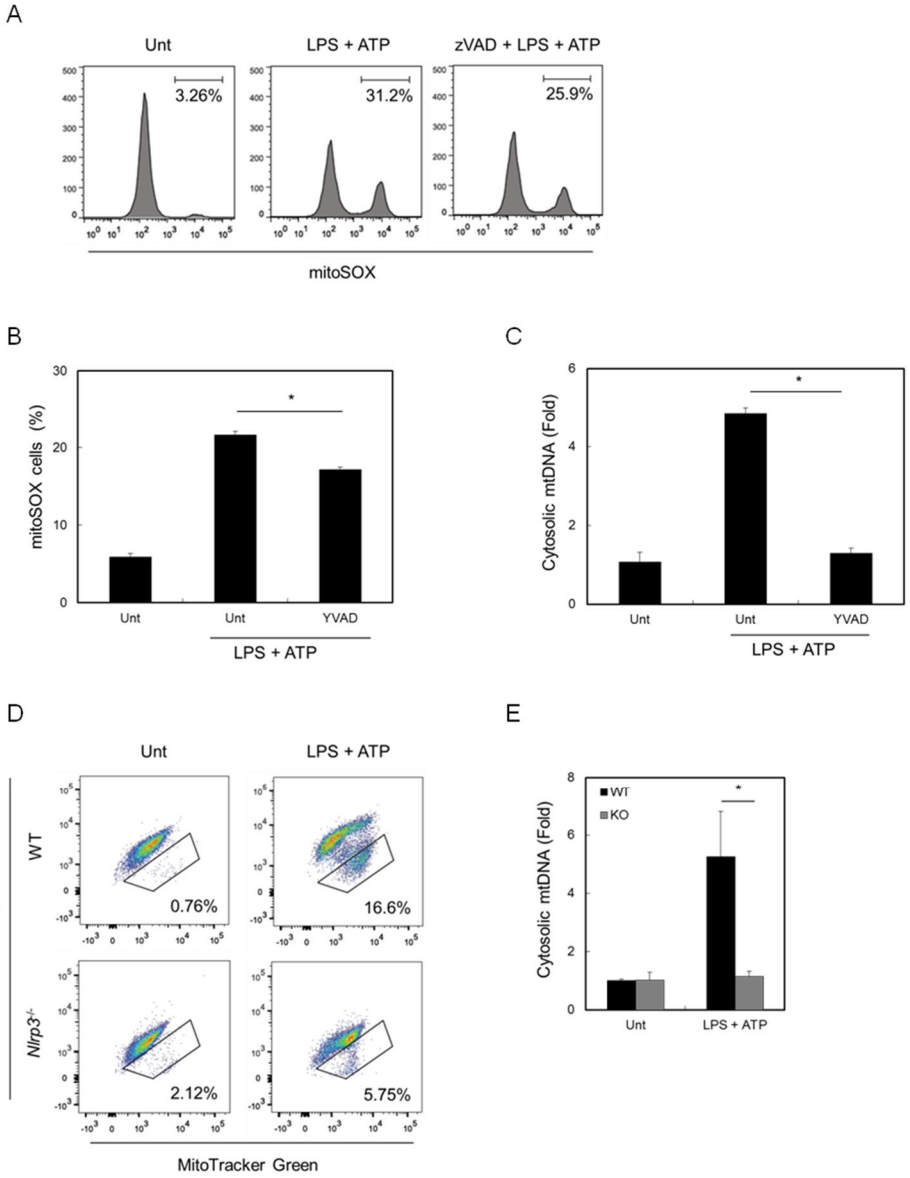


Figure 14. Mitochondrial damage is, at least partially, caused by the caspase-1 activation. (**A**-**C**) Wild-type BMDMs were treated with (**A**) LPS (0.25 μ g/ml, 3 hr), followed with ATP (2.5 mM, 30 min) in the presence or absence of zVAD-fmk or (**B** and **C**) YVAD-cmk (20 μM, 30 min pretreatment before LPS) as indicated. (**A** and **B**) Cells were stained with MitoSOX (2.5 μM, 10 min) and analyzed by FACSverse analyzer. Asterisk indicates a significant difference ($n = 4$, $^*p < 0.01$). (**C**) Cytosolic mtDNA was then assayed as described. Asterisk indicates a significant difference when compared to the absence of zVAD treatment samples ($n = 3$, $^*p < 0.0001$). (**D** and **E**) Wild-type or NLRP3 KO BMDMs were treated with LPS plus ATP as in (**A**). (**D**) Mitochondrial damages were assayed by costaining with MitoTracker Green and MitoTracker Deep Red, and then analyzed by flow cytometry. (**E**) Cytosolic mtDNA were measured by quantitative real-time PCR analysis as described before. Asterisk indicates a significant difference ($n = 3$, $^*p < 0.05$).

6. Role of ERK signaling in the augmented activation of NLRP3 inflammasome seen in Drp1-knockdown macrophages

Recently, it was reported that extracellular signal-regulated kinase (ERK) signaling was involved in the LPS-mediated NLRP3 priming signal.[75] My earlier data show that ATP stimulation only in shDrp1 macrophages could activate caspase-1 without priming signal (Fig. 10A,C), indicating that ERK signaling might be implicated in this phenomenon. To investigate this, I examined the implication of ERK signaling in NLRP3 inflammasome. Interestingly, although LPS stimulation induced ERK 1/2 phosphorylation in shScr macrophages, shDrp1 macrophages displayed a constitutive basal phosphorylation of ERK. However, c-Jun N-terminal kinase (JNK) phosphorylation levels showed no difference between shScr and shDrp1 macrophages (Fig. 15A). Indeed, inhibition of the ERK signaling by U0126, a selective inhibitor of MEK1 and MEK2,[76] induced highly attenuated caspase-1 activation and IL-1β secretion in response to LPS plus ATP stimulation in both cells (Fig. 15B,C). In addition, U0126 treatment also inhibited the interaction between ASC and NLRP3 in wild-type macrophages (Fig. 16A). These results support that ERK signaling is involved in NLRP3 inflammasome activation.

Previous studies suggested that mitochondria could serve as a platform to assemble NLRP3 inflammasome components.[30] So, I examined the subcellular distribution of NLRP3 inflammasome component. LPS stimulation induced NLRP3 translocation from cytosol to mitochondria in shScr macrophages, but U0126 treatment inhibited mitochondrial distribution of NLRP3. Interestingly, in shDrp1 macrophages, the basal localization of NLRP3 in mitochondria was increased without any stimulation, and inhibited by U0126 treatment. Consistent with this, localization of ASC were also higher in shDrp1 macrophages (Fig. 16B), indicating that elongated mitochondria might provide a larger platform than normal mitochondria to assemble the NLRP3 inflammasome. Therefore, abnormal mitochondrial elongation induces the

activation of ERK signaling by unknown mechanism, and facilitates the assembly of NLRP3 inflammasome.

Collectively, I demonstrated that abnormal mitochondrial elongation caused by Drp1 deficiency induces the activation of ERK signaling, which in turn the phosphorylation of ERK leads to recruitment of NLRP3 onto mitochondria. These events increase the susceptibility to NLRP3 inflammasome activation.

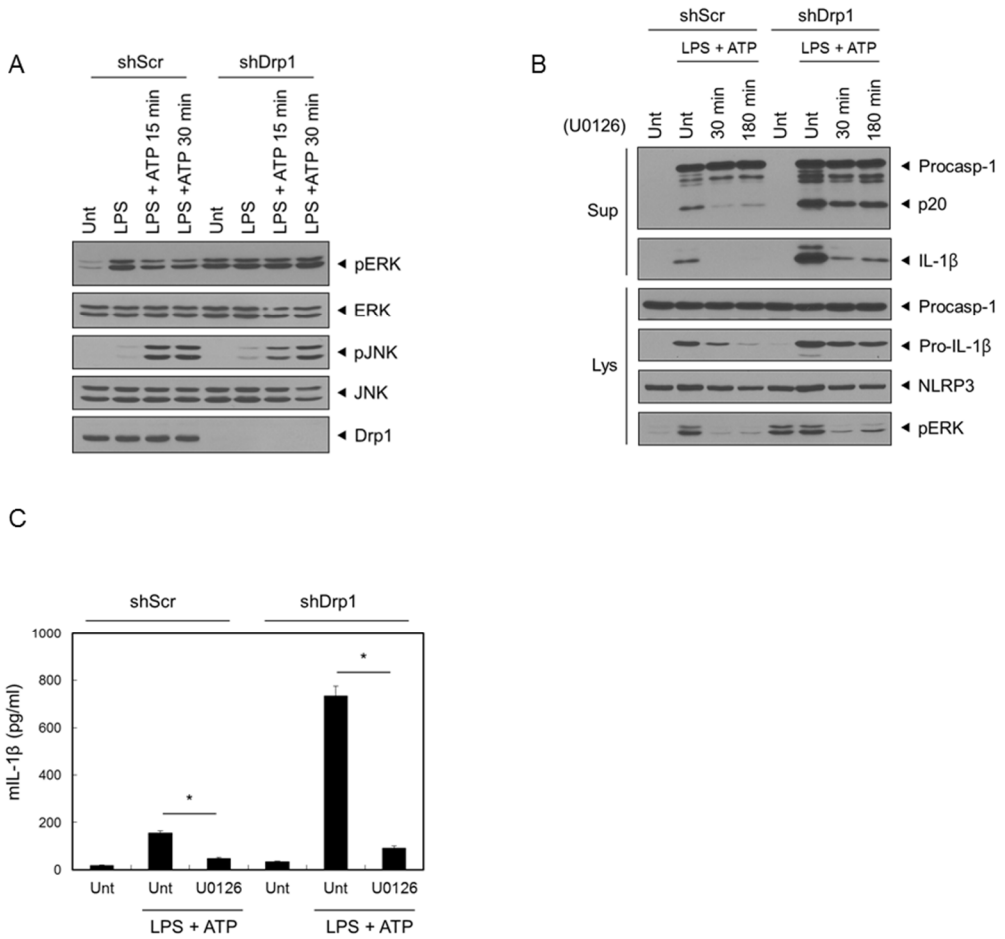


Figure 15. ERK phosphorylation is involved in the activation of NLRP3 inflammasome. (**A**) shScr or shDrp1 BMDMs were primed by LPS (0.25 μg/ml, 3 hr), followed with ATP (2.5 mM, 15 min or 30 min) as indicated. (**B-C**) shScr or shDrp1 BMDMs were treated with LPS, followed by ATP in the presence or absence of U0126 (10 μM, B, 30 or 180 min, or C, 30 min pretreated). (**B**) Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with indicated Abs. (**C**) Culture supernatants were assayed for IL-1β secretion by ELISA. Asterisks indicate significant difference ($n = 3$, $*p < 0.005$).

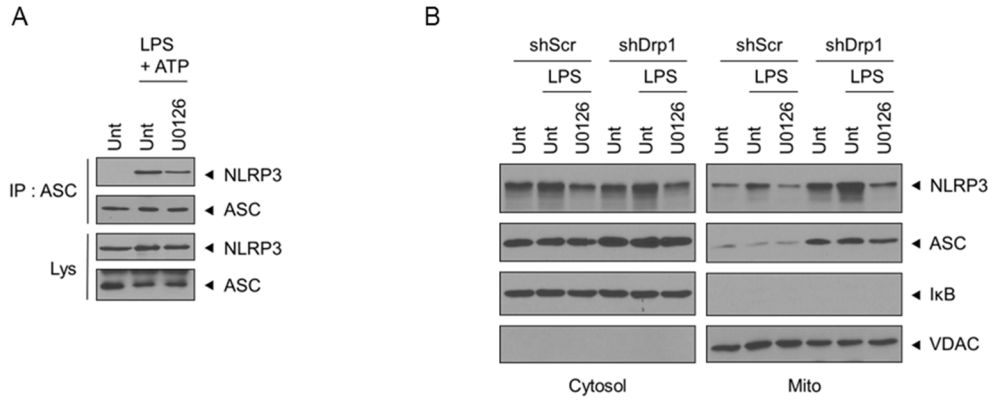


Figure 16. U0126 inhibits the assembly of NLRP3 inflammasome. (**A**) Wild-type BMDMs were primed with LPS in the presence of U0126 pretreatment (10 μM, 30 min), followed by ATP treatment. Soluble lysates were immunoprecipitated with anti-ASC Ab, and the immunoprecipitates or lysates were immunoblotted with indicated Abs. (**B**) ShScr or shDrp1 BMDMs were primed with LPS in the presence or absence of U0126 pretreatment (10 μM, 2 hr). fractionated into mitochondria (Mito) or cytosolic protein-enriched fractions (Cytosol) as described Materials and Methods. Samples were immunoblotted with indicated Abs.

IV. DISCUSSION

In this study, I tried to demonstrate mitochondrial contribution to the NLRP3 inflammasome signaling by targeting mitochondrial protein MAVS and abnormal mitochondrial elongation caused by Drp1 knockdown. Since previous studies reported that the translocation of cytosolic NLRP3 onto mitochondria during activation, and another study reported that abnormal mitochondrial dynamics due to the mutation or deficiency of mitochondrial dynamics-associated protein was associated with innate immune signaling such as NFκB activation or type Ⅰ IFN production,[69] I hypothesized that mitochondrial outer membrane protein might associate with NLRP3, and abnormal mitochondrial elongation also might associate with NLRP3 inflammasome activation.

In PART 1, I demonstrated that mitochondrial outer membrane protein MAVS promotes NLRP3 inflammasome signaling in response to Sendai virus infection. The mitochondrial antiviral protein MAVS, which localized on mitochondrial outer membrane, is important in innate immune signaling such as NFκB activation or type Ⅰ IFN production in response to viral infection.[36] During viral infection, RNA virus is sensed by cytosolic sensor protein RIG-I, which then interacts with MAVS through homotypic CARD-CARD interactions leading to the oligomerization of MAVS into large SDS-resistant aggregates on the outer membrane of the mitochondria.[42] In PART 1, I showed that MAVS interacts with NLRP3 and regulates NLRP3 inflammasome activation in response to Sendai virus infection followed by nigericin stimulation. MAVS knockdown macrophages exhibit highly impaired Sendai virus-mediated caspase-1 activation. In contrast to mitochondrial MAVS, cytosolic MAVS (-ΔTM) could not interact with NLRP3 and could not promote Sendai virus-mediated caspase-1 activation. Thus, the localization of MAVS on outer membrane of mitochondria is essential for Sendai virus-mediated caspase-1 activation. Not only macrophages, but also THP-1 cells showed that MAVS promotes

NLRP3 inflammasome activation in response to Sendai virus infection. In THP-1-derived macrophages, Sendai virus infection induced the assembly of NLRP3 inflammasome component, but the assembly of NLRP3 inflammasome in MAVS knockdown THP-1 cells was significantly reduced. In addition, by using MAVS knockout BMDM, I found that MAVS is required for Sendai virus-mediated caspase-1 activation. However, MAVS is dispensable for NLRP3 inflammasome activation in response to their classical activator such as LPS plus nigericin stimulation. Interestingly, Sendai virus-mediated NLRP3 inflammasome activation shows the low levels of IL-1β secretion than LPS plus nigericin stimulation, indicating that Sendai virus-mediated NLRP3 inflammasome activation is different compared to classical activation of NLRP3 inflammasome.

Regarding the mechanisms of Sendai virus-mediated NLRP3 inflammasome activation, I proved that full-length of MAVS, not cytosolic MAVS, facilitates the oligomerization of NLRP3 on the outer membrane of mitochondria by overexpressing NLRP3 in the presence of MAVS or Sendai virus infection. NLRP3 oligomer requires mtROS production because the stimulation of mtROS production with rotenone, a mitochondria complex Ⅰ inhibitor, treatment increases MAVS-mediated NLRP3 oligomerization, whereas ROS scavenger NAC treatment completely prevents MAVS-mediated NLRP3 oligomerization. Since NLRP3 oligomerization is essential step for NLRP3 inflammasome activation,[15] it is likely that these MAVS-mediated NLRP3 oligomers form molecular platforms on mitochondria to recruit and oligomerize ASC and procaspase-1, leading to caspase-1 activation. Therefore, mtROS play a critical role in Sendai virus-mediated NLRP3 inflammasome activation.

As I mentioned above, the activation of NLRP3 inflammasome requires a priming signal from pattern recognition receptor such as TLR followed by an activation signal derived from various stimuli.[8] The results in PART 1 show that Sendai virus infection with macrophages in the absence of activation signal does not induce caspase-1 activation, however, Sendai-virus infection followed by nigericin

treatment lead to robust NLRP3 inflammasome activation. This indicates that Sendai-virus infection provides a NLRP3 priming signal MAVS dependently.

Since I presented in PART 1 showing the interaction between MAVS and NLRP3, I thought that not only MAVS affects NLRP3, but also NLRP3 might influence MAVS signaling. MAVS was originally known as the critical regulator of innate immune signaling by interaction and activation of downstream signaling proteins such as TBK1 and IKK complex in response to viral infection.[38] Surprisingly, I could detect NLRP3 protein negatively regulating MAVS-mediated type Ⅰ IFN production in response to Poly(I:C) stimulation or Sendai virus infection. Furthermore, NLRP3 is the only protein that could inhibit MAVS-mediated type Ⅰ IFN production among the other sensor proteins of other inflammasomes. Supporting this, NLRP3 knockout macrophages exhibited largely increased phosphorylation of IRF-3 in response to Sendai virus infection compared to NLRP3 restored cells. It might be that NLRP3 competes with other signaling protein for type Ⅰ IFN production by direct interaction with MAVS in response to Sendai virus infection. Although NLRP3 inflammasome activation could influence the MAVS-mediated type Ⅰ IFN production, a hypothesis which requires further investigation, NLRP3 has potential role in a negative regulator of MAVS-mediated type Ⅰ IFN production.

While this work was in progress, Subramanian *et al.* reported that MAVS promotes NLRP3 inflammasome activation in response to non-viral stimuli such as LPS followed by nigericin treatment, which is known as classical activating condition of NLRP3.[77] However, in my experiment, using MAVS knockout macrophages did not show notable difference in caspase-1 activation in response to LPS plus nigericin treatment compared to wild-type macrophages. Therefore, I thought the role of MAVS in NLRP3 inflammasome activation is limited to a specific condition such as viral infection.

In PART 2, I demonstrated that abnormal mitochondrial elongation could increase susceptibility to NLRP3 inflammasome activation. As I mentioned above, mitochondrial dynamics play an important role in homeostasis of mitochondria such as maintaining their size, number, signaling, and content of DNA.[44] Furthermore, mitochondrial dynamics are associated with many innate immune responses. Previous studies reported that mitochondrial elongation caused by viral infection, leads to increase of NFκB activation and type Ⅰ IFN production.[61] On the other hand, mitochondrial fragmentation caused by a number of stress such as UV like apoptotic stimulation to the mitochondria, leads to decrease of NFκB activation and type Ⅰ IFN production.[44] Mitochondrial dynamics are strongly associated with intrinsic pathway of apoptosis or autophagy pathway. However, despite the extensive studies, association between mitochondrial dynamics and inflammasome was still unclear.

Previous studies showed that the deficiency or genetic mutations in the protein related to mitochondrial dynamics are associated with severe defects in brain development or embryonic lethality.[44] In addition, a recent study reported that a female infant with mutation on Drp1 exhibited abnormal brain development and died suddenly at the age of 37 days. In this patient, the skin fibroblast showed markedly elongated mitochondria and peroxisome compared to healthy control.[68] Another study reported that Drp1 protein expression levels were significantly decreased in Alzheimer's disease patients.[65] Considering that inflammasome-mediated neuroinflammation significantly contributes to the neurodegenerative diseases such as Alzheimer's disease, Drp1-mediated abnormal mitochondrial elongation might be associated with NLRP3 inflammasome activation.

In this study, I established Drp1 knockdown macrophages by using shRNA interference to examine the role of abnormal mitochondrial elongation in NLRP3 inflammasome activation. shDrp1 cells exhibit elongated and hyperfused mitochondria in morphology, and significant increase in pyroptotic cell death compared to shScr macrophages. I also detected the increase of caspase-1 activation

in shDrp1 macrophages. These results imply that mitochondrial elongation induces the increase of caspase-1 activation in response to LPS followed by ATP stimulation. Supporting this, Drp1 selective inhibitor mdivi-1 treatment[73] with wild-type macrophages exhibited highly increased caspase-1 activation. By using mitochondrial dynamics-associated protein knockdown THP-1 cells, I found out the role of abnormal mitochondrial dynamics in NLRP3 inflammasome activation. Drp1 knockdown THP-1-derived macrophages exhibited markedly increased caspase-1 activation in response to LPS or Alum stimulation. However, shOpa1 THP-1 showed attenuated caspase-1 activation, even less than wild-type cells. shDrp1 macrophages also increased the assembly of NLRP3 inflammasome component in response to LPS stimulation. In addition, mitochondrial fission inducer CCCP treatment abolished NLRP3-dependent caspase-1 activation and NLRP3 inflammasome assembly. Although previous study has already shown CCCP stimulation could inhibit NLRP3 inflammasome, this study revealed that CCCP-mediated mitochondrial fission appears to attenuate NLRP3 inflammasome assembly and activation in a transcription independently. These data strongly supports that the abnormal mitochondrial elongation induces enhanced NLRP3 inflammasome activation.

A recent study suggested that mitochondria-derived products such as mtROS and mtDNA influence the activation of NLRP3 inflammasome.[31] In my experiment, shDrp1 macrophages exhibited significant increase of mitochondrial products during LPS plus ATP stimulation. However, these results could not fully explain the increase of NLRP3 inflammasome activation in shDrp1 macrophages. Moreover, mitochondrial damages reflect caspase-1 activation because selective caspase-1 inhibitor, YVAD treatment prevents the generation of mitochondrial products in response to LPS followed ATP stimulation. Furthermore, NLRP3 knockout macrophages showed complete inhibition of mitochondrial damages. Therefore, enhanced mitochondrial damages in shDrp1 macrophages might be the consequences of caspase-1 activation. In PART 2, I also found that the ERK signaling was critical for priming step of NLRP3 inflammasome activation. I detected LPS stimulation

could induce phosphorylation of ERK in shScr macrophages. Surprisingly, Drp1 knockdown macrophages exhibited phosphorylation of ERK without any stimulation, and my earlier data showed that shDrp1 macrophages could activate caspase-1 by ATP stimulation alone. These data imply that shDrp1 cells might be already primed without LPS stimulation. Moreover, enhanced ERK signaling in shDrp1 macrophages promotes the recruitment of NLRP3 components on mitochondria. ERK phosphorylation inhibitor U0126, which prevent the activation of ERK upstream signal proteins MEK 1/2, could highly reduce the assembly and activation of NLRP3 inflammasome in both cells. These results might explain the activation of caspase-1 in shDrp1 cells in response to ATP alone. Therefore, increase of caspase-1 activation in shDrp1 macrophages was caused by enhanced ERK signaling. Although how abnormal mitochondrial elongation induces the phosphorylation of ERK requires further investigation, it is sure that ERK activation contributes to increased susceptibility to NLRP3 inflammasome in Drp1 knockdown macrophages.

Collectively, this work provides new insights in mitochondrial regulation to the NLRP3 inflammasome activation. Although MAVS was known as a critical regulator of type Ⅰ IFN production pathway in response to viral infection, I demonstrated that MAVS could engage NLRP3 to form an inflammasome complex by facilitating NLRP3 oligomerization in response to Sendai virus infection. I also demonstrated that abnormal mitochondrial elongation caused by the reduction of Drp1 expression increases the susceptibility to the NLRP3 inflammasome activation in an ERK signaling dependently. This work could not completely explain how mitochondria regulate NLRP3 inflammasome activation, but it demonstrated that MAVS- and Drp1-dependent mitochondrial regulation to NLRP3 inflammasome activation.

Ⅴ. CONCLUSION

These studies demonstrated that MAVS- and Drp1-dependent contribution of mitochondria to the regulation of NLRP3 inflammasome activation. Mitochondrial anti-viral protein MAVS induce ROS-dependent NLRP3 oligomerization on mitochondria in response to Sendai virus infection, and promotes NLRP3 inflammasome activation. Abnormal mitochondrial elongation caused by Drp1 knockdown increases susceptibility to the assembly and activation of NLRP3 inflammasome in response to NLRP3 activating stimuli such as LPS plus ATP in an ERK signaling dependent manner.

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ABSTRACT (IN KOREAN)

NLRP3 인플라마좀 활성화에 대한 mitochondrial anti-viral signaling protein 과 dynamin-related protein 1 의 조절기전

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박상준

NLRP3 인플라마좀 (inflammasome)은 NLRP3 와 ASC 그리고 procaspase-1 으로 구성되어 있는 세포 내 단백질 복합체로 병원균, 미생물로부터 유래되는 독성물질, 결정화 물질이나 내부적 위험신호 같은 자극에 대하여 비활성화 상태인 caspase-1 을 활성화 시킬 수 있다고 알려져 있다. 지금까지 많은 연구들이 NLRP3 인플라마좀이 어떻게 활성화 되는지에 대하여 밝히려 했지만 아직까지도 그 기전은 불명확하다. 최근의 연구들은 손상된 미토콘드리아로부터 나오는 mtROS 나 mtDNA 같은 물질들의 세포질 유출이 NLRP3 인플라마좀 활성에 중요한 역할을 할 것이라고 제시하고 있지만, 미토콘드리아가 어떻게 NLRP3 인플라마좀 활성을 조절하는가에 대한 기전은 아직도 더 많은 연구가 필요하다. 따라서, 본 연구에서는 미토콘드리아 단백질인 MAVS 와 Drp1 단백질 발현 저해로 인한 미토콘드리아의 비정상적인 증대가 어떻게 NLRP3 인플라마좀 활성을 조절할 수 있는지에 대하여 연구를 수행하였다.

PART 1 에서는, 미토콘드리아 외막 단백질인 MAVS 가 Sendai virus 감염 시, NLRP3 와 결합하여 ROS 의존적인 NLRP3 의 oligomerization 을 유도함으로써 NLRP3 인플라마좀을 활성화 시킬 수 있다는 것을

보여주었다. 또한 MAVS 가 미토콘드리아에 위치하지 못하면 이런 활성을 나타내지 못하는 결과를 통해서 미토콘드리아에서의 MAVS 와 NLRP3 의 결합이 Sendai virus 감염에 의한 NLRP3 인플라마좀 활성에 중요하다는 것을 확인하였다. 이런 결과들을 뒷받침 하기 위해 MAVS 의 발현을 억제시킨 macrophages 를 제작, 실험을 통해서 Sendai virus 감염에 의한 caspase-1 의 활성이 MAVS 의존적임을 보여주었다. 이를 통해 MAVS 가 Sendai virus 감염에 대해서 ROS 의존적인 NLRP3 의 oligomerization 을 유도하고, NLRP3 인플라마좀을 활성화 시킬 수 있다는 것을 증명하였다.

PART 2 에서는, 미토콘드리아의 비정상적인 증대가 같은 자극에 의한 NLRP3 인플라마좀의 활성을 증가시킬 수 있다는 것을 보여주었다. Drp1 단백질 발현을 억제시킨 macrophages 는 비정상적으로 증대한 미토콘드리아를 나타내었고, 또한 ATP 나 nigericin 처리에 의한 NLRP3 인플라마좀의 활성이 증가하는 것을 확인하였다. 더욱이, 미토콘드리아의 증대는 NLRP3 인플라마좀의 형성을 증가시키는 반면, CCCP 처리를 통해 미토콘드리아의 분열을 유도하였을 때는 NLRP3 인플라마좀의 형성과 활성이 크게 감소하는 현상을 보였다. Drp1 의 발현을 억제한 macrophages 는 LPS 와 ATP 처리에 의해 mtROS 또는 세포질 mtDNA 의 생성이 증가하지만, caspase-1 활성 저해제인 YVAD 를 처리하거나 NLRP3 KO macrophages 를 이용한 실험에서 미토콘드리아 손상이 감소하는 현상을 통해 손상된 미토콘드리아로부터 유래되는 물질들은 caspase-1 의 활성에 의한 결과인 것을 보여주었다. 또한 본 연구에서는 ERK 의 인산화가 NLRP3 를 미토콘드리아로 집결시키는 과정에 중요한 역할을 한다는 것을 확인하였다. 이를 뒷받침하는 증거로 ERK 의 인산화를 저해하는 물질인 U0126 을 처리하였을 때 NLRP3 인플라마좀의 형성과 활성이 크게 저해되는 것을 보여주었다. 이런 결과들을 바탕으로 Drp1 단백질의 발현

억제로 인한 비정상적인 미토콘드리아의 증대는 LPS 와 ATP 처리에 의한 NLRP3 인플라마좀의 활성을 증가시키고, 이때 ERK 신호의 활성화가 중요한 역할을 한다는 것을 밝혀내었다.

종합적으로, 본 연구는 미토콘드리아 단백질인 MAVS 가 Sendai virus 감염 시 NLRP3 인플라마좀의 활성을 증가시킨다는 것과 Drp1 단백질 발현 억제로 인한 미토콘드리아의 증대가 LPS 와 ATP 처리에 의한 NLRP3 인플라마좀 활성을 증가시킨다는 내용을 밝혀내었고, 이를 통해 미토콘드리아가 어떻게 NLRP3 인플라마좀 활성 조절에 중요한 역할을 수행하는가에 대한 기전을 밝혀내었다.

핵심되는 말: 인플라마좀, NLRP3, 미토콘드리아, 미토콘드리아 역동성, MAVS, Drp1

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