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The Mitochondrial Antiviral Protein MAVS Associates with NLRP3 and Regulates Its Inflammasome Activity

Sangjun Park,* Christine Juliana,† Sujeong Hong,* Pinaki Datta,† Inhwa Hwang,* Teresa Fernandes-Alnemri,† Je-Wook Yu,*† and Emad S. Alnemri†

NLRP3 assembles an inflammasome complex that activates caspase-1 upon sensing various danger signals derived from pathogenic infection, tissue damage, and environmental toxins. How NLRP3 senses these various stimuli is still poorly understood, but mitochondria and mitochondrial reactive oxygen species have been proposed to play a critical role in NLRP3 activation. In this article, we provide evidence that the mitochondrial antiviral signaling protein MAVS associates with NLRP3 and facilitates its oligomerization leading to caspase-1 activation. In reconstituted 293T cells, full-length MAVS promoted NLRP3-dependent caspase-1 activation, whereas a C-terminal transmembrane domain–truncated mutant of MAVS (MAVS-ΔTM) did not. MAVS, but not MAVS-ΔTM, interacted with NLRP3 and triggered the oligomerization of NLRP3, suggesting that mitochondrial localization of MAVS and intact MAVS signaling are essential for activating the NLRP3 inflammasome. Supporting this, activation of MAVS signaling by Sendai virus infection promoted NLRP3-dependent caspase-1 activation, whereas knocking down MAVS expression clearly attenuated the activation of NLRP3 inflammasome by Sendai virus in THP-1 and mouse macrophages. Taken together, our results suggest that MAVS facilitates the recruitment of NLRP3 to the mitochondria and may enhance its oligomerization and activation by bringing it in close proximity to mitochondrial reactive oxygen species. The Journal of Immunology, 2013, 191: 4358–4366.

*Department of Microbiology, Brain Korea 21 Project for Medical Science, Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, Seoul 120-752, Korea; and †Department of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, BLSB904, Philadelphia, PA 19107

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Address correspondence and reprint requests to Dr. Emad S. Alnemri or Dr. Je-Wook Yu, Department of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, BLSB904, Philadelphia, PA 19107 (E.S.A.) or Department of Microbiology, Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, 250 Seongsanno, Seodaemun-gu, Seoul 120-752, Korea (J.-W.Y.). E-mail addresses: emad.alnemri@jefferson.edu (E.S.A.) or jewooky@yuhs.ac (J.-W.Y.)

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CARD, caspase activation and recruitment domain; KO, knockout; LRR, leucine-rich repeat; MAVS-ΔTM, C-terminal transmembrane domain–truncated mutant of MAVS; mtROS, mitochondrial reactive oxygen species; NAC, N-acetyl-l-cysteine; NP-40, Nonidet P-40; ROS, reactive oxygen species; shRNA, short hairpin RNA; sRNA, small interfering RNA.

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Knocking down MAVS expression leads to decreased activation of NLRP3 in response to Sendai virus infection. Our results suggest that MAVS facilitates the recruitment of NLRP3 to the mitochondria, and may enhance its activation by allowing efficient sensing of ROS from damaged mitochondria.

Materials and Methods

Reagents and Abs

Roterone, nigericin, LPS, poly dA:dT, poly(I:C), and N-acetyl-t-cysteine (NAC) were purchased from Sigma. Anti-NLRP3, anti-human caspase-1 (p20), anti-AIM2, and anti-pyrin Abs were generated in the laboratory as described previously (7, 21–23). All other Abs detecting Myc (Abcam), Flag (Sigma), T7 (Bethyl Laboratories), mouse caspase-1 (Adipogen), p-IRF3 (Cell Signaling), Flag-IRF3 (Santa Cruz), human MAVS (Alexis), mouse MAVS (Cell Signaling), human IL-1β (Cell Signaling), mouse IL-1β (R&D Systems), Ibα (Cell Signaling), VDAC1 (Abcam), and ASC (Santa Cruz) were obtained from commercial sources.

Cell culture

293T cells stably expressing ASC and procaspase-1 (293T-ASC, cells) and THP-1 cells stably expressing ASC-GFP were generated as described previously (22, 24). 293T cells and 293T-ASC-C1A cells were maintained in DMEM/F12 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. THP-1 and THP-1–ASC-GFP cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPESS, 1 mM sodium pyruvate, 0.05 mM 2-ME, and 100 U/ml penicillin and streptomycin. Transfections of plasmids were performed in 6- or 12-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Wild-type and NLRP3-deficient immortalized mouse bone marrow–derived macrophages (BMDMs) were described previously (23). NLRP3- or NLRP3-GFP–expressing mouse BMDMs were generated by retrovirus infection of NLRP3-deficient BMDMs as described previously (7, 25). Mouse BMDMs were maintained in L929-conditioned DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

Expression constructs

The cDNAs for human full-length MAVS (pcDNA3-MAVS-myc) and C-terminal transmembrane domain–truncated mutant of MAVS (MAVS-ΔTM) (pcDNA3-MAVS-ΔTM-myc) were generated by PCR using appropriate PCR primers and human MAVS cDNA, and cloned in the BglII-XhoI sites of Myc-pcDNA3. The human NLRP3 constructs, pcDNA3-Flag-NLRP3 and NLRP3-Aucine-rich repeat (LRR), were generated by PCR using a human cDNA template. All other constructs including human pyrin (pcDNA3-pyrin-T7) and AIM2 (pcDNA3-AIM2-T7) were generated as described previously (22, 23).

Cell treatment

Sendai virus was cultured using eggs containing chick embryos and titrated by hemagglutination assay. For determination of inflammasome activity, THP-1 cells were differentiated with PMA (0.4 μg/ml) for 4 h before stimulation with nigericin for 6 h. In some experiments, mouse BMDMs were infected with Sendai virus for 6 h or primed with LPS (0.25 μg/ml) for 4 h before stimulation with nigericin for 45 min.

Immunoblot analysis

Cells were lysed in 20 mM HEPESS buffer containing 0.5% Nonidet P-40 (NP-40), 50 mM KCl, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, and protease inhibitors. Soluble lysates were subjected to SDS-PAGE and transferred onto PVDF membranes, then immunoblotted with the appropriate Abs. Culture supernatants were precipitated and immunoblotted for caspase-1 as described previously (23) to determine inflammasome activity in macrophages.

Confocal microscopy

Cells grown on coverslips in a 12-well plate were fixed with 4% formaldehyde and then permeabilized with 0.2% Triton X-100. After blocking with 4% BSA (Sigma), cells were incubated with primary Ab against MAVS or NLRP3, followed by incubation with fluorescein-conjugated anti-rabbit IgG or Cy3-conjugated anti-mouse IgG Abs (Jackson). Coverslips were then mounted using the ProLong Gold reagent (Invitrogen) containing the nuclear stain DAPI and examined using a confocal microscope (FV1000; Olympus). Confocal microscopy of ASC-GFP–expressing THP-1 cells or NLRP3-GFP–expressing BMDMs was performed as described previously (23, 24).

Subcellular fractionation

For subcellular fractionation experiments, cells were lysed in buffer A containing 250 mM sucrose, 10 mM HEPESS (pH 7.8), 10 mM KCl, 2 mM Lipofectamine 2000. To knock down MAVS expression in mouse macrophages, we used lentiviral particles containing short hairpin RNA (shRNA) targeting mouse MAVS or nontargeting shRNA control (Sigma) to infect immortalized mouse BMDMs. Cells stably expressing shRNA were cloned by puromycin selection and used for experiments.

Determination of ASC oligomerization

Chemical cross-linking was performed using disuccinimidyl suberate (Pierce) as described previously (24). Precipitated pellets and soluble lysates were simultaneously immunoblotted using anti-ASC Ab. ASC specks were counted after different treatments in THP-1–ASC–GFP cells in several random fields containing >250 cells each using a fluorescence microscope (Olympus IX71/DP71), to quantify in situ ASC oligomerization. Relative percentage of ASC speck-containing cells was calculated by dividing the number of ASC speck-containing cells by the total number of cells in each field. Five independently treated wells were analyzed per experiment.

Type 1 IFN production

For reporter gene assay, cells were transfected with an IFN-β promoter-luciferase reporter plasmid (100 ng), β-galactosidase (100 ng), and the appropriate plasmids as indicated. After transfection for 24 h, cells were lysed and the luciferase activity was determined 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 control with MAVS, poly (I:C), or Sendai virus. To measure the mRNA levels of IFN-β, we performed quantitative real-time PCR assay. In brief, total cellular RNA was isolated using the PureLink RNA kit (Invitrogen) and reverse transcribed into cDNA, and the resultant cDNA was subjected to quantitative real-time PCR using iTag universal SYBR green supermix (BioRad). Primers were as follows: 5'-CTT TCC TTC TTC AC-3' and 5'-CTT TTC ATT CAG CTG CTC CA-3' for mouse IFN-β, and 5'-AGC CAT GTA CGT AGC CAT CC-3' and 5'-CTC TCA GCT GTG GTG AA-3' for mouse β-actin.

Statistics

All values were expressed as the mean and SD of individual samples. Data were analyzed with Student t test. The p values ≤ 0.05 were considered significant.

Results

MAVS associates with NLRP3

mtROS and mitochondrial DNA have been implicated in the activation of the NLRP3 inflammasome (9, 19), suggesting that NLRP3 might sense these products and/or ROS-damaged mitochondrial.
outer membrane proteins. To explore the latter possibility, we analyzed the interaction of NLRP3 with the outer mitochondrial membrane-associated protein MAVS, because MAVS plays a crucial role in the innate immune responses to viruses and might thus play a direct role in NLRP3 inflammasome activation. Immunoprecipitation experiments in transfected 293T cells showed that MAVS could indeed interact specifically with the NLRP3 inflammasome, but not with the AIM2 or pyrin inflammasomes (Fig. 1A).

To investigate whether the interaction between NLRP3 and MAVS requires the localization of MAVS to the mitochondrial outer membrane, we examined the interaction of NLRP3 with MAVS-ΔTM, which lacks the C-terminal mitochondrial-targeting transmembrane domain. In contrast with the mitochondrially localized full-length MAVS, the cytosolic MAVS-ΔTM failed to associate with NLRP3, indicating that mitochondrial localization of MAVS is essential for the interaction with NLRP3 (Fig. 1B), and that the interactions between NLRP3 and MAVS do not occur in the cytosol but occur on the outer mitochondrial membrane. These findings were also corroborated by confocal immunofluorescence imaging, which showed filamentous/punctate staining of full-length MAVS that colocalized with NLRP3 staining, and diffused cytosolic MAVS-ΔTM staining that did not colocalize with the NLRP3 staining in any specific structures (Fig. 1C).

Further examination of the interactions between NLRP3 and MAVS revealed that truncated NLRP3-ΔLRR, which lacks the C-terminal LRR domain, could still interact with MAVS, indicating that the LRR domain is dispensable for this interaction (Fig. 1D), and that the interactions between NLRP3 and MAVS do not occur in the cytosol but occur on the outer mitochondrial membrane. These findings were also corroborated by confocal immunofluorescence imaging, which showed filamentous/punctate staining of full-length MAVS that colocalized with NLRP3 staining, and diffused cytosolic MAVS-ΔTM staining that did not colocalize with the NLRP3 staining in any specific structures (Fig. 1C).

**MAVS regulates NLRP3 inflammasome activity**

Previous studies showed that a fraction of NLRP3 associates with the mitochondria and mitochondria-associated ER membranes upon activation (9), suggesting that this redistribution of NLRP3 might be important for its activation. To examine whether the observed interactions between MAVS and NLRP3 on the mitochondrial membrane (Fig. 1) might facilitate NLRP3 inflammasome activation, we transiently transfected 293T cells that stably express procaspase-1 and ASC (293T-C1A cells) (22) with a low amount of NLRP3 plasmid together with or without MAVS plasmid (Fig. 2A). Expression of NLRP3 alone in these cells caused negligible processing of procaspase-1 into the active caspase-1 p20 subunit (Fig. 2A, lane 3). However, coexpression of MAVS together with NLRP3 resulted in marked increase in processing of procaspase-1 (Fig. 2A, lane 4), whereas expression of MAVS alone had no effect on procaspase-1 (Fig. 2A, lane 2). Consistent with the observed lack of interaction between NLRP3 and MAVS-ΔTM (Fig. 1B, 1C), coexpression of MAVS-ΔTM with NLRP3 failed to induce processing of procaspase-1 (Fig. 2B). Together, these results indicate that the interaction between MAVS and NLRP3 facilitates the activation of the NLRP3 inflammasome.

**Sendai virus promotes MAVS- and NLRP3-dependent caspase-1 activation**

Infection of 293T cells and mouse embryonic fibroblasts with Sendai virus has been shown to induce the formation of large MAVS signaling complexes composed of functional prion-like MAVS aggregates (18). To examine whether formation of these MAVS aggregates in response to Sendai virus infection could lead to activation of the NLRP3 inflammasome, we infected NLRP3-transfected cells with Sendai virus. Similar to ectopic overexpression of MAVS, Sendai virus infection potentiated NLRP3-mediated caspase-1 activation (Fig. 2C, fourth lane), but did not promote caspase-1 processing in the absence of NLRP3 (Fig. 2C, second lane). These results suggest that formation of the MAVS signaling

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**FIGURE 1.** Association of MAVS with NLRP3. (A) 293T cells were transfected with empty vector (Vec) or Myc-MAVS expression plasmid together with Flag-NLRP3, T7-pyrin, or T7-AIM2 (0.3 μg) expression plasmid as indicated. Cell lysates were immunoprecipitated using anti-Myc Ab, and the immunoprecipitates were then immunoblotted with anti-Flag (NLRP3), anti-AIM2, anti-pyrin, or anti-Myc (MAVS) Ab, respectively. (B) 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. The immunoprecipitates were immunoblotted with anti-Flag (upper) or anti-Myc (lower) Ab. (C) 293T cells were transfected with NLRP3- and MAVS full-length (FL) or ΔTM-expressing constructs (0.1 μg). MAVS or NLRP3 was visualized by immunofluorescence confocal microscopy as described in **Materials and Methods.** Scale bar, 10 μm. (D) 293T cells were transfected with Myc-MAVS and Flag-NLRP3 FL or Flag-NLRP3-ΔLRR constructs (0.3 μg). Immunoprecipitation was performed as described in (B).
complexes in response to Sendai virus infection can lead to NLRP3-dependent caspase-1 activation.

To further validate the earlier findings, we examined whether infection of PMA-differentiated THP-1 macrophages with Sendai virus could lead to MAVS-dependent caspase-1 activation. As shown in Fig. 3A, infection of THP-1 cells with Sendai virus caused caspase-1 activation as evidenced by formation of the active caspase-1 p20 subunit and IL-1β p17 fragment. Knockdown of MAVS expression in these cells remarkably reduced the formation of active caspase-1 p20 subunit and IL-1β p17 in response to Sendai virus infection (Fig. 3A). These results suggest that MAVS signaling is important for caspase-1 activation in response to Sendai virus infection in macrophages.

ASC oligomerization is a critical step for caspase-1 activation and caspase-1–dependent pyroptosis in response to NLRP3 stimuli (22). Consistent with the earlier caspase-1 activation results, Sendai virus infection of THP-1 cells induced strong oligomerization of ASC, which was largely reduced when MAVS expression was knocked down by siRNA (Fig. 3B), indicating that MAVS is required for ASC oligomerization by Sendai virus. Similarly, formation of the oligomeric ASC speck–like pyroptosomes in THP-1–ASC–GFP cells upon infection with Sendai virus was significantly reduced after MAVS knockdown (Fig. 3C, Supplemental Fig. 1). In contrast, ASC pyroptosome formation in response to poly(dA:dT), which activates the AIM2 inflammasome, was not affected by knockdown of MAVS (Fig. 3C, Supplemental Fig. 1). These results suggest that MAVS is important for the assembly of the NLRP3 inflammasome in response to Sendai virus infection, but dispensable for the assembly of the AIM2 inflammasome in response to poly(dA:dT).

In agreement with the interaction studies in transfected 293T cells (Fig. 1A), and the earlier activation studies in THP-1 macrophages, we found that endogenous MAVS associates with endogenous NLRP3 in THP-1 cells upon stimulation of these cells...
with Sendai virus, but not in unstimulated cells (Fig. 3D). These results indicate that activation of MAVS by Sendai virus infection promotes its association with NLRP3.

To provide additional evidence that caspase-1 activation by Sendai virus infection in macrophages is dependent on NLRP3, we performed similar experiments as described earlier in mouse wild-type and NLRP3-knockout (KO) BMDMs. Unlike THP-1 macrophages, mouse BMDMs require, in addition to the primary NLRP3 stimulus (also known as signal 1), a second stimulus (signal 2) from pore-forming toxins (e.g., nigericin) or purinergic receptors to induce efficient NLRP3-dependent caspase-1 activation (6, 7). Consistent with this mechanism, infection of wild-type BMDMs with Sendai virus alone, or stimulation with LPS alone (data not shown), did not show detectable caspase-1 activation (Fig. 4A). However, when these cells were infected with Sendai virus or treated with LPS followed with stimulation with nigericin, they showed robust caspase-1 activation and IL-1β secretion (Fig. 4A, fourth to sixth lanes). The relatively weaker secretion of IL-1β is attributed to the lower induction of pro–IL-1β in response to Sendai virus infection compared with LPS. Sendai virus–mediated caspase-1 activation was not observed in NLRP3-KO macrophages, but was restored when these macrophages were stably reconstituted with NLRP3 (N1-8 macrophages) (7) (Fig. 4B), indicating that Sendai virus–mediated caspase-1 activation is dependent on the NLRP3 inflammasome. Consistent with these results, Sendai virus plus nigericin stimulation of NLRP3-KO cells stably reconstituted with NLRP3-GFP protein induced aggregation of NLRP3 into one or two specklike structures per cell, which was also observed after LPS plus nigericin stimulation (Fig. 4C). In contrast, LPS, nigericin, or Sendai virus treatments alone did not induce the NLRP3 speck–like structures in these cells (Fig. 4C). These results indicate that Sendai virus infection provides an NLRP3 priming signal (signal 1) similar to that provided by TLR signaling.

**FIGURE 4.** Sendai virus (SV)–mediated activation of NLRP3 inflammasome in mouse macrophages. (A) Immunoblots of caspase-1 and IL-1β in culture supernatants (Sup) of wild-type mouse BMDMs treated with SV (10 hemagglutination activity/ml, 6 h), nigericin (Nig; 5 μM, 45 min), SV (6 h) followed with Nig (45 min; SV+Nig), LPS (0.25 μg/ml, 4 h) followed with Nig (LPS+Nig), or SV (6 h) followed with LPS (4 h) and then Nig (45 min; LPS+SV+Nig) as indicated. Cell lysates (Lys) were immunoblotted with anti–caspase-1 or anti-NLRP3 Ab as indicated. (B) Immunoblot of caspase-1 in culture supernatants (Sup) and cell lysates (Lys) of NLRP3-deficient (NLRP3-KO) or NLRP3-restored (N1-8) BMDMs treated with SV (10 hemagglutination activity/ml, 6 h) plus Nig (5 μM, 45 min), LPS (0.25 μg/ml, 4 h) plus Nig (LPS+Nig), or transfected with poly(dA:dT) (2 μg, 6 h) as indicated. (C) Confocal images of stable NLRP3-GFP–expressing BMDMs left untreated (Un) or treated with LPS, SV, Nig, LPS+Nig, or SV+Nig as described in (A) and (B). Green and blue signals represent NLRP3 and nuclear fluorescence, respectively. Scale bars, 10 μm. (D) Immunoblot of caspase-1 in culture supernatants (Sup) and cell lysates (Lys) of stable mouse BMDMs cell lines expressing scrambled shRNA- (shCon) or mouse MAVS-specific shRNA (shMAVS) after treatment with SV alone (10 hemagglutination activity/ml, 0–4 h, left panels) or SV (10 hemagglutination activity/ml, 0–4 h) followed by Nig (45 min, right panels). Cell lysates were immunoblotted as indicated.
To provide additional evidence for a role for MAVS in the activation of the NLRP3 inflammasome by Sendai virus infection in mouse BMDMs, we reduced the expression of MAVS in these cells by shRNA-mediated knockdown. Knockdown of MAVS largely diminished caspase-1 activation induced by Sendai virus plus nigericin (Fig. 4D), but not by LPS plus nigericin or the AIM2 inflammasome stimulus poly(dA:dT) (Supplemental Fig. 2A). Similar experiments in MAVS-KO macrophages showed that MAVS is required for caspase-1 activation by Sendai virus plus nigericin, but not by LPS plus nigericin (Supplemental Fig. 2B). Collectively, these results indicate that Sendai virus infection requires MAVS to prime the NLRP3 inflammasome.

**MAVS facilitates NLRP3 oligomerization in mitochondria**

The reported ability of MAVS to form large SDS-resistant oligomers upon activation by Sendai virus infection or enforced expression (18), and its ability to interact with NLRP3 (Fig. 1) suggest that MAVS might prime the NLRP3 inflammasome by inducing formation of SDS-resistant NLRP3 oligomers on the mitochondrial membrane. To examine this possibility, we cotransfected 293T cells with NLRP3 plasmid together with increasing amounts of MAVS plasmid and then analyzed NLRP3 expression by immunoblot analysis. Notably, enforced expression of MAVS resulted in the formation of large SDS-resistant NLRP3 oligomers (Fig. 5A). These oligomers were not detected when cells were transfected with NLRP3 and MAVS-ΔTM, indicating that formation of these NLRP3 oligomers requires close interaction between NLRP3 and MAVS on the outer mitochondrial membrane (Fig. 5B). To validate these results, we fractionated 293T cell lysates into cytosolic and mitochondria-enriched fractions after transfection with NLRP3 and MAVS, and then analyzed the expression of NLRP3 in these fractions. Although the majority of NLRP3 was detected in the cytoplasmic fraction, a small amount of NLRP3 was also found in the mitochondrial fraction (Fig. 5C). However, MAVS was equally detected in the cytosol and in the mitochondrial fractions. Notably, enforced expression of MAVS caused the formation of NLRP3 oligomers in the mitochondrial, but not the cytosolic fraction (Fig. 5C), indicating that MAVS-mediated oligomerization of NLRP3 occurs on the outer mitochondrial membrane.

To examine whether infection of cells with Sendai virus can also lead to formation of the NLRP3 oligomers, we infected NLRP3-expressing 293T cells with Sendai virus and analyzed the oligomerization status of NLRP3 as described earlier. As shown in Fig. 5D, Sendai virus infection induced formation of some NLRP3 oligomers, albeit to a lesser extent than seen with MAVS overexpression. These NLRP3 oligomers were also detected by confocal microscopy in some NLRP3-KO macrophages stably reconstituted with NLRP3-GFP protein (Fig. 5E). Knocking down MAVS by siRNA transfection reduced the formation of the NLRP3 oligomers (Supplemental Fig. 2C), indicating that MAVS is required for NLRP3 oligomerization.

Accumulating evidence suggests that ROS play an important role in the activation of the NLRP3 inflammasome (7, 9, 10). Our recent results showed that ROS scavenging by antioxidants such as NAC or mito-TEMPO inhibits priming and activation of NLRP3, whereas induction of mtROS by rotenone can lead to its potent priming and activation (7). To examine whether ROS regulates MAVS-induced NLRP3 oligomerization, we treated 293T cells with NAC or rotenone after cotransfection with MAVS plus NLRP3 expression constructs. Interestingly, NAC almost completely inhibited

**FIGURE 5.** NLRP3 oligomerization by MAVS. (A and B) 293T cells were transfected with a construct for NLRP3 (0.3 μg) and increasing amount of MAVS construct (A), or NLRP3 and MAVS or MAVS-ΔTM constructs (0.3 μg) (B) as indicated. Cell lysates were immunoblotted with anti-Flag (NLRP3) or anti-Myc (MAVS) Abs. (C) 293T cells were transfected with an NLRP3 construct together with an empty vector (Vec) or MAVS construct. Cell lysates were fractionated into cytosolic or mitochondrial fraction, and then immunoblotted with anti-Flag (NLRP3), anti-Myc (MAVS), anti-IκB (cytosol), or anti-VDAC1 (mitochondria) Abs. (D) 293T cells were transfected with NLRP3 (0.3 μg) followed by infection with Sendai virus (SV; 10 hemagglutination activity/ml) for the indicated times, or transfected with NLRP3 together with MAVS (0.3 μg) as indicated. Cell lysates were immunoblotted for NLRP3 and MAVS. (E) Confocal images of NLRP-GFP–expressing BMDMs left untreated (Un) or infected with SV (10 hemagglutination activity/ml) for the indicated times, or transfected with NLRP3 together with MAVS (0.3 μg) as indicated. Scale bar, 10 μm. (F and G) 293T cells were transfected with an NLRP3 construct (0.3 μg) together with an empty vector or MAVS construct (0.3 μg), in the presence or absence of NAC (15 mM, 18 h) (F) or followed by treatment with rotenone for 6 h as indicated. Cell lysates were immunoblotted for NLRP3 or MAVS as described earlier. (H) 293T cells were transfected with a Flag-NLRP3 (0.3 μg) plasmid alone, or together with a Myc-MAVS (0.3 μg) plasmid in the presence or absence of NAC (15 mM, 18 h) as indicated. Cell lysates were immunoprecipitated using anti-Myc Ab, and the immunoprecipitates were then immunoblotted with anti-Flag (NLRP3) or anti-Myc (MAVS) Ab, respectively.
NLRP3 oligomerization, whereas rotenone greatly enhanced its oligomerization (Fig. 5F, 5G). Notably, NAC did not affect MAVS interaction with NLRP3 (Fig. 5H). Together, these observations indicate that ROS is critical for MAVS-induced NLRP3 oligomerization.

NLRP3 negatively regulates MAVS signaling

The earlier results demonstrate that the interaction of MAVS with NLRP3 clearly impacts the function of MAVS. To examine whether this interaction could also impact the function of MAVS itself, we tested whether NLRP3 could inhibit MAVS-induced activation of an IFN-β promoter. Indeed, overexpression of NLRP3 in 293T cells resulted in >80% inhibition of MAVS-induced activation of the IFN-β promoter (Fig. 6A). This inhibition was specific for NLRP3, because overexpression of AIM2 or pyrin proteins failed to inhibit MAVS-induced activation of the IFN-β promoter (Fig. 6A). Similarly, overexpression of NLRP3, but not AIM2 or pyrin, also inhibited activation of the IFN-β promoter by poly(I:C)/RIG-I and Sendai virus infection (Fig. 6B, 6C). Supporting these results, infection with Sendai virus induced more phosphorylation of IRF3 and IFN-β synthesis in NLRP3-KO macrophages compared with the NLRP3-reconstituted N1-8 macrophages, which express physiological level of NLRP3 (7) (Fig. 6D, 6E). Collectively, these results indicate that the interaction between NLRP3 and MAVS negatively regulates MAVS-induced type-1 IFN production.

Discussion

Activation of caspase-1 by the NLRP3 inflammasome is a critical step in the innate immune response against a wide variety of stimuli including pathogens, microbial products, crystalline materials, and endogenous danger signals (1). How NLRP3 senses these diverse stimuli is still unknown. Two models have been proposed to explain NLRP3 inflammasome activation. The lysosomal disruption model suggests that the rupture of phagosomes triggered by phagocytosis of large crystalline particles or protein aggregates releases lysosomal enzymes that cleave uncharacterized cellular proteins, which, in turn, directly or indirectly activate NLRP3 (26). The mitochondrial damage model, in contrast, suggests that mitochondria damage, which can be triggered by a large number of stimuli, causes the releases of ROS, which stimulates the activation of NLRP3 directly or indirectly through potential NLRP3-interacting proteins such as TXNIP (10). Yet another version of the mitochondrial damage model suggests that the release of oxidized DNA from damaged mitochondria leads to the activation of the NLRP3 inflammasome through direct interactions between oxidized mtDNA and NLRP3 (19). Support for a role for mitochondria in NLRP3 activation was based on studies that showed that a fraction of NLRP3 translocates from the cytosol to the mitochondria upon activation with NLRP3 stimuli (9). In addition, inhibition of mitochondrial voltage-dependent anion channel suppressed mtROS production and NLRP3 inflammasome activation (9).

The work presented in this article provides further evidence for the involvement of mitochondria in the regulation of NLRP3 inflammasome. The mitochondrial antiviral protein MAVS is critically involved in the activation of type I IFNs by RNA viruses (13–16). This pathway is activated when viral RNA is recognized by RIG-I, which then binds to MAVS through homotypic CARD–CARD interactions leading to the oligomerization of MAVS into large SDS-resistant aggregates on the outer mitochondrial membrane (18). Our work shows that knockdown of MAVS or MAVS deficiency in macrophages causes a marked reduction in NLRP3 inflammasome activation in response to Sendai virus infection. This indicates that mitochondria use MAVS to regulate NLRP3 activation in response to infection with RNA viruses.

The mechanism by which MAVS regulates NLRP3 inflammasome activation appears to involve association of MAVS with

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![Image](https://example.com/image3.png)

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**FIGURE 6.** Inhibition of MAVS-induced type I IFN signaling by NLRP3. (A) 293T cells were transfected with IFN-β promoter-luciferase reporter plasmid (100 ng), β-galactosidase (100 ng), and the indicated expression constructs for MAVS (200 ng) together with empty vector (Vec) NLRP3, AIM2, or pyrin. Reporter luciferase activity was then determined as in Materials and Methods. (B) 293T cells were transfected as in (A) together with an expression construct for RIG-I with or without NLRP3, pyrin, or AIM2 constructs (B) or with constructs for NLRP3, pyrin, or AIM2 (C) as indicated. Twenty-four hours after transfection, cells were transfected with poly(I:C) (10 µg, 20 h, B) or infected with Sendai virus (SV; 10 hemagglutination activity, 8 h, C) as indicated. Reporter luciferase activity was then determined as in (A). (D and E) NLRP3-deficient BMDMs or NLRP3-restored (N1-8) BMDMs were left untreated or infected with SV (5 hemagglutination activity/ml) for 8 h. Cell lysates were immunoblotted for the indicated proteins (D), and mRNA levels of IFN-β were quantitated by real-time PCR as described in Materials and Methods.
NLRP3 on the outer mitochondrial membrane. This association triggers NLRP3 oligomerization as enforced expression of MAVS, or infection of cells with Sendai virus leads to the formation of SDS-resistant NLRP3 oligomers. The formation of these NLRP3 oligomers requires mROS production because stimulation of mROS production with rotenone increases MAVS-induced NLRP3 oligomerization, whereas scavenging ROS with NAC inhibits it. Because oligomerization of NLRP3 is a critical step in its activation (27), it is likely that these MAVS-induced NLRP3 oligomers form molecular platforms to recruit and oligomerize ASC and procaspase-1, leading to caspase-1 activation.

In mouse macrophages, the assembly of the NLRP3 inflammasome requires a priming signal (signal 1) from pattern recognition or cytokine receptors followed by a second signal (signal 2) derived from extracellular ATP, pore-forming toxins, or crystalline materials (6, 7). Although it is thought that signal 1 primes NLRP3 by inducing its expression (6), our recent studies indicate that signal 1 can prime NLRP3 by a nontranscriptional RO-S-dependent mechanism without upregulation of NLRP3 (7). The results presented in this article show that stimulation of mouse macrophages with Sendai virus alone in the absence of signal 2 (e.g., nigericin) does not cause detectable caspase-1 activation, whereas stimulation with Sendai virus followed by signal 2 leads to robust caspase-1 activation. This indicates that MAVS provides an NLRP3 priming signal (signal 1). This signal could prime NLRP3 by upregulating NLRP3 expression through induction of NF-κB and/or by a post-translational mechanism through induction of NLRP3 oligomerization. Our confocal imaging studies of mouse macrophages expressing a GFP-tagged mouse NLRP3 revealed that NLRP3 is entirely cytosolic in untreated cells. However, upon stimulation of these macrophages with Sendai virus and nigericin or LPS and nigericin, NLRP3 aggregates into one or two NLRP3 foci or clusters per cell resembling the ASC pyroptosome or speckles (24). These results suggest that the role of signal 2 is to induce clustering of the primed oligomeric NLRP3 to allow the recruitment of ASC and procaspase-1 to this cluster. Whether these NLRP3 clusters are formed by coalescing of the primed NLRP3 around mitochondrial MAVS aggregates or other mitochondrial aggregates requires further investigation.

Interestingly, the interaction of NLRP3 with MAVS appears to negatively impact its type I IFN signaling. Our results are reminiscent of previous findings, which showed that the mitochondrial NLR protein NLRX-1 can also suppress MAVS signaling (28). One possible explanation for the inhibition of MAVS signaling by NLRP3 or NLRX-1 is the formation of nonfunctional MAVS oligomers or inhibition of MAVS oligomer formation by NLRP3. This mechanism might be important to inhibit type I IFN production to prevent their reported inhibitory effect on NLRP3 inflammasome activation (29).

How NLRP3 is targeted to the mitochondria is not fully clear at present. However, while this work was in progress, Subramanian et al. (30) reported that residues 2–7 at the N terminus of human NLRP3 control its mitochondrial targeting and interaction with MAVS. Although our results are in general agreement with their findings, we wish to point out that residues 2–3 at the N terminus of human NLRP3 that contain a critical basic lysine residue are missing in mouse NLRP3. A PSORT analysis (31) of the mouse NLRP3 N terminus similar to that performed by Subramanian et al. (30) assigned the highest certainty score to the cytoplasm. This assignment is in agreement with their finding that deletion of residues 2–3 at the N terminus of human NLRP3 (Δ2-3) reduced its mitochondrial localization and interaction with MAVS. Nevertheless, because our data and theirs are consistent with a role for MAVS in regulating NLRP3 activation in mouse cells, it is possible that additional mechanisms might be involved in targeting mouse NLRP3 on activation to the mitochondria and controlling its interaction with MAVS. Contrary with the results of Subramanian et al. (30), our data do not support a role for MAVS in the activation of the NLRP3 inflammasomes by nonviral stimuli such as LPS plus nigericin (Supplemental Fig. 2) or LPS plus ATP (data not shown). Our data suggest that MAVS regulates NLRP3 activation primarily in response to stimuli that directly engage MAVS such as infection with Sendai virus.

Collectively, this work provides new insights into the regulation of NLRP3 inflammasome activation by the mitochondria and strengthens the significance of mitochondria in the control of inflammation. It identifies MAVS as a critical regulator of the NLRP3–ASC–caspase-1 inflammasome. The strategic localization of MAVS to the outer mitochondrial membrane provides it with a molecular platform to sense, coordinate, and assemble large complexes to respond to many danger signals especially those arising from viral infection. Our results combined with previously published results suggest that MAVS coordinates two signaling pathways simultaneously in response to viral infection. The first pathway (type-I IFN pathway) is triggered when activated MAVS engages IKK and TBK1, to ensure the syntheses of antiviral proteins. This pathway also leads to the syntheses of pro–IL-1β, which is processed by caspase-1 to generate the potent proinflammatory cytokine IL-1β. The second pathway is triggered when activated MAVS engages NLRP3 to form an inflammasome complex that recruits and activates procaspase-1. It is thus intriguing that MAVS not only stimulates the transcriptional induction of pro–IL-1β and other proinflammatory cytokines, but also is directly involved in the assembly of the NLRP3 inflammasome that processes pro–IL-1β into its active cytokine. The regulation of NLRP3 inflammasome activation by mitochondrial MAVS indicates that mitochondrial proteins play a central role in inflammasome activation similar to their role in apoptosis activation.

Disclosures
The authors have no financial conflicts of interest.

References


