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The mitochondrial anti-viral protein MAVS associates with NLRP3 and regulates its inflammasome activity[1]

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Abstract

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 (mtROS) have been proposed to play a critical role in NLRP3 activation. Here, we provide evidence that the mitochondrial anti-viral 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, while 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 mtROS.

Introduction

The NLRP3 inflammasome is a multiprotein complex that assembles rapidly in response to a wide range of danger signals, including pathogens, microbial toxins, crystalline materials and endogenous danger signals (1). The assembled NLRP3 inflammasome recruits the adaptor protein ASC to activate caspase-1, which processes pro-IL-1β and pro-IL-18 into their active IL-1β and IL-18 cytokines, respectively. Although, the NLRP3 inflammasome plays an important role in the innate immune response to pathogenic infection, its inappropriate activation can lead to a number of human inflammatory diseases (2), including gouty arthritis, silicosis, neurodegeneration and many metabolic disorders such as atherosclerosis, type 2 diabetes, and obesity (3, 4). Inappropriate activation of the NLRP3

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inflammasome due to mutations in NLRP3 can also lead to a number of autoinflammatory diseases or periodic fever syndromes (5).

Assembly of the NLRP3 inflammasome requires a priming signal derived from pattern recognition or cytokine receptors, followed by an activation signal derived from extracellular ATP, pore-forming toxins, or crystalline materials (6, 7). However, the detailed molecular mechanism of how these two signals activate the NLRP3 inflammasome and how they are regulated are not yet clear (8). Recent studies suggested that mitochondrial reactive oxygen species (mtROS), which is generated in response to a wide range of stimuli, might be critical for NLRP3 activation (7, 9). It has been suggested that ROS might induce conformational changes in redox sensing proteins that then associate with NLRP3 resulting in its activation (10). More recently a NLRP3 deubiquitination mechanism has also been proposed to explain the activation of NLRP3 by diverse stimuli (7, 11,12).

The mitochondrial antiviral signaling protein MAVS (also known as VISA, IPS-1 or cardif) mediates type I interferon (IFN) and NF-κB signaling in response to viral infection (13-16). During infection with RNA viruses, cytoplasmic RIG-I or MDA-5 senses viral 5’ ppp-RNA or double-stranded RNA, respectively, and associates with MAVS through CARD-CARD homotypic interaction on the outer mitochondrial membrane (17). The mechanism of activation of MAVS by the upstream adaptor RIG-I involves the formation of very large prion-like functional aggregates of MAVS (18). These MAVS aggregates then activate the cytosolic kinases TBK1 and IKK, which in turn activate IRF3 and NF-κB, respectively, leading to the induction of type I IFN and other antiviral molecules (17).

Considering recent evidence that a fraction of NLRP3 is recruited to the mitochondria upon activation (9), and that mitochondria-derived molecules such as mtROS and mitochondrial DNA might be involved in the activation of NLRP3 (9, 19, 20), we investigated whether MAVS aggregation on the outer mitochondrial membrane might also regulate NLRP3 activity. Here, we demonstrate that activated MAVS associates with NLRP3 and induces its oligomerization, leading to the activation of caspase-1. Knocking down MAVS expression lead 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 antibodies

Rotenone, nigericin, LPS, poly dA:dT, Poly I:C and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma. Anti-NLRP3, Anti-human-caspase-1 (p20), anti-AIM2 and anti-pyrin antibodies were generated in the lab as described previously (7, 21-23). All other antibodies detecting Myc (Abcam), Flag (Sigma), T7 (Bethyl Laboratories), mouse caspase-1 (Adipogen), phospho-IRF3 (Cell Signaling), IRF-3 (Santa Cruz), human MAVS (Alexis), mouse MAVS (Cell Signaling), human IL-1β (Cell Signaling), mouse IL-1β (R&D), IκB (Cell Signaling), VDAC1 (Abcam) and ASC (Santa Cruz) were obtained from commercial sources.

Cell culture

293T cells stably expressing ASC and procaspase-1 (293T-C1A cells) and THP-1 cells stably expressing ASC-GFP were generated as described previously (22, 24). 293T cells and 293T-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 RPMI1640 supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium

pyruvate, 0.05 mM 2-mercaptoethanol and 100 U/ml penicillin and streptomycin. Transfections of plasmids were performed in 6 well or 12 well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Wild type and NLRP3-deficient immortalized mouse bone marrow-derived macrophages (BMDM) were described previously (23). NLRP3- or NLRP3-GFP-expressing mouse BMDMs were generated by retrovirus infection of NLRP3-deficient BMDM 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 MAVS-ΔTM (pcDNA3-MAVS-ΔTM-myc) were generated by PCR using appropriate PCR primers and human MAVS cDNA, and cloned in the BglII-Xho I sites of Myc-pcDNA3. The human NLRP3 constructs, pcDNA3-Flag-NLRP3 and NLRP3-ΔLRR, were generated by PCR using human NLRP3-cDNA as a 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 phorbol-12-myristate-13-acetate (PMA, 0.4 μM) for 3 h, and the next day cells were stimulated with LPS or Sendai virus 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 Hepes (pH 7.5) buffer containing 0.5% 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, and then immunoblotted with the appropriate antibodies. To determine inflammasome activity in macrophages, culture supernatants were precipitated and immunoblotted for caspase-1 as described previously (23).

Coimmunoprecipitation

Cells were lysed in 10 mM Hepes buffer (pH 7.4) containing 0.2% NP-40, 100 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, 1 mM DTT and protease inhibitors, and centrifuged to remove cell debris. The resulting lysates were pre-cleared with protein G-sepharose (GE) for 30 min and immunoprecipitated with the indicated antibodies. The protein-antibody complexes were then precipitated with protein G-sepharose bead, and the bead-bound proteins were fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies.

Confocal microscopy

Cells grown on cover slips in 12 well plate were fixed with 4% formaldehyde and then permeabilized with 0.2% Triton X-100. After blocking with 4% bovine serum albumin (Sigma), cells were incubated with primary antibody against MAVS or NLRP3, followed by incubation with fluorescein-conjugated anti-rabbit IgG or Cy3-conjugated anti-mouse IgG antibodies (Jackson). Cover slips were then mounted using the ProLong Gold reagent (Invitrogen) containing the nuclear stain DAPI and examined using a confocal microscope (Olympus, FV1000). 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 Hepes (pH 7.8), 10 mM KCl, 2 mM MgCl2, 0.1 mM EGTA, 1 mM DTT, 0.1% NP-40 and protease inhibitors. The lysates were centrifuged for 10 min at 700 g to remove nuclei. The supernatants were collected and then centrifuged for 10 min at 12,500 g. The resultant supernatants were used for cytosolic fraction analysis and the remaining pellets were washed with buffer A and then resuspended in 10 mM Hepes buffer (pH 7.4) containing 0.5% NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA and protease inhibitors. The resuspended pellets were re-centrifuged for 10 min at 12,500 g, and the resulting supernatants were used as mitochondrial-protein enriched fraction for immunoblot analysis and for immunoprecipitation.

Knockdown of MAVS expression

To knockdown MAVS expression in THP-1 macrophages, cells were transfected with siRNA oligonucleotides (Bioneer, 50 nM) targeting human MAVS or nontargeting scrambled siRNA control using Lipofectamine 2000. To knockdown MAVS expression in mouse macrophages, lentiviral particles containing shRNA targeting mouse MAVS or nontargeting shRNA control (Sigma) were used to infect immortalized mouse BMDMs. Cells stably expressing shRNA were cloned by puromycin selection and used for experiments.

Determination of ASC oligomerization

Chemical crosslinking was performed using discuccinimidyl suberate (DSS, Pierce) as described previously (24). Precipitated pellets and soluble lysates were simultaneously immunoblotted using anti-ASC antibody. To quantify *in situ* ASC oligomerization, ASC specks were counted after different treatments in THP-1-ASC-GFP cells in several random fields containing more than 250 cells each using a fluorescence microscope (Olympus IX71/DP71). 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 I IFN production

For reporter gene assay, cells were transfected with an IFN-β promoter-luciferase reporter plasmid (100 ng), pβ-gal (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 to unstimulated control with MAVS, poly I:C or Sendai virus. To measure the mRNA levels of IFN-β, quantitiative real-time PCR assay was performed. Briefly, total cellular RNA was isolated using the PureLink RNA kit (Invitrogen) and reverse transcribed using the SuperScript cDNA synthesis kit (Invitrogen). Template DNA was then amplified by quantitative real-time PCR using iTaq universal SYBR green supermix (BioRad). Primers were as follows: 5’-TTC CTG CTG TGC TTC TTC AC-3’ and 5’-CTT TCC 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 GTG AA-3’ for mouse β-actin.

Statistics

All values were expressed as the mean and s.d. of individual samples. Data were analyzed with Student’s *t*-test. *p* values of 0.05 or less were considered significant.

Results

MAVS associates with NLRP3

Mitochondria-derived ROS (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 to 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 does not occur in the cytosol but occurs on the outer mitochondrial membrane. These findings were also corroborated by confocal immunofluorescence imaging, which showed filamentous/punctate staining of full-length MAVS that co-localized with NLRP3 staining, and diffused cytosolic MAVS-ΔTM staining that did not co-localize 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 interaction region is located in the PYD-NACHT domains.

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 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, co-expression 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 and C), co-expression 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, 4th lane), but did not promote caspase-1 processing in the absence of NLRP3 (Fig. 2C, 2nd lane). These results suggest that formation of the MAVS signaling complexes in response to Sendai virus infection can lead to NLRP3-dependent caspase-1 activation.

To further validate the above 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 above 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 knock-down (Fig. 3C, and 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, and 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 above 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 un-stimulated 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 above in mouse WT and NLRP3-KO bone marrow-derived macrophages (BMDM). 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 WT BMDM with Sendai virus alone, or stimulation with LPS alone (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, 4th to 6th 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 to 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 speck-like 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 induced the NLRP3 speck-like structures in these cells (Fig. 4C). These results indicate that Sendai virus infection provides a NLRP3 priming signal (signal 1) similar to that provided by TLR signaling.

To provide additional evidence for a role for MAVS in the activation of the NLRP3 inflammasome by Sendai virus infection in mouse BMDM, 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 knockout (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 co-transfected 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 both in the cytosol and 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 above. 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 suggest that ROS plays an important role in the activation of the NLRP3 inflammasome (7, 9,10). Our recent results showed that ROS scavenging by anti-oxidants such as NAC or mito-TEMPO inhibits priming and activation of NLRP3, whereas induction of mitochondrial ROS (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 co-transfection with MAVS plus NLRP3 expression constructs. Interestingly, NAC almost completely inhibited NLRP3 oligomerization, whereas rotenone greatly enhanced its oligomerization (Fig. 5F and 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 above results demonstrate that the interaction of MAVS with NLRP3 clearly impacts the function of NLRP3. 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 more than 80% inhibition of MAVS-induced activation of the IFN-β promoter (Fig. 6A). This inhibition was specific for NLRP3, as 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 and C). 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 and E). Collectively, these results indicate that the interaction between NLRP3 and MAVS negatively regulates MAVS-induced type-1 interferon 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 which cleave uncharacterized cellular proteins, which in turn directly or indirectly activate NLRP3 (26). The mitochondrial damage model on the other hand 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 which 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 mitochondrial ROS production and NLRP3 inflammasome activation (9).

The work presented here provides further evidence for the involvement of mitochondria in the regulation of NLRP3 inflammasome. The mitochondrial anti-viral protein MAVS is critically involved in the activation of type I interferons 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 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 mtROS production as stimulation of mtROS production with rotenone increases MAVS-induced NLRP3 oligomerization, whereas scavenging ROS with NAC inhibits it. Since 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 non-transcriptional ROS-dependent mechanism without upregulation of NLRP3 (7). The results presented here 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 a NLRP3 priming signal (signal 1). This signal could prime NLRP3 by upregulating NLRP3 expression through induction of NF-κB, and/or by a posttranslational 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 interferon 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 non-functional MAVS oligomers or inhibition of MAVS oligomer formation by NLRP3. This mechanism might be important to inhibit type I interferon production in order 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* reported that residues 2-7 at the N-terminus of human NLRP3 controls its mitochondrial targeting and interaction with MAVS (30). 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 which 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 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, since 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 to the results of Subramanian *et al* (30), our data do not support a role for MAVS in the activation of the NLRP3 inflammasomes by non-viral stimuli such as LPS plus nigericin (Supplemental Fig. 2), or LPS plus ATP (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-1 interferon pathway) is triggered when activated MAVS engages IKK and TBK1, to insure 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 apoptosome activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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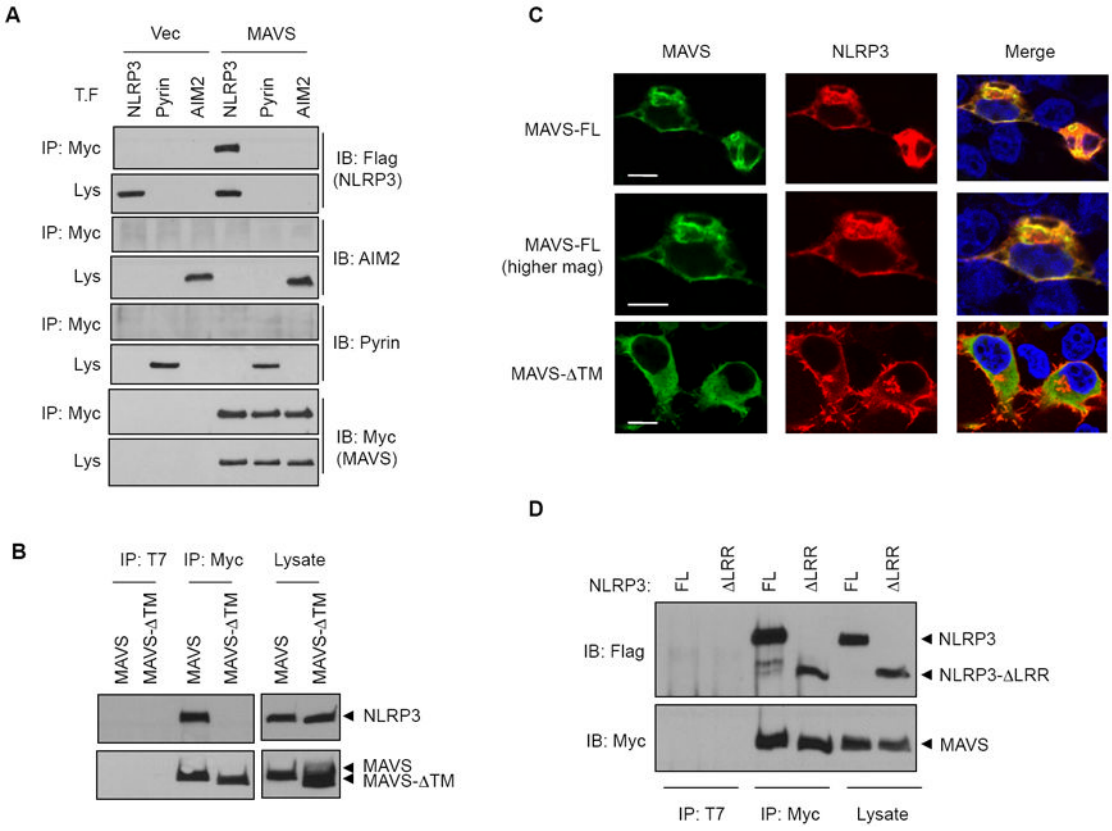

FIGURE 1.

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 antibody, and the immunoprecipitates were then immunoblotted with anti-Flag (NLRP3), anti-AIM2, anti-pyrin or anti-Myc (MAVS) antibody, 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 antibody. The immunoprecipitates were immunoblotted with anti-Flag (upper) or anti-Myc (lower) antibody. (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”. Bar: 10 μm. (D) 293T cells were transfected with Myc-MAVS and Flag-NLRP3 full-length or Flag-NLRP3-ΔLRR constructs (0.3 μg). Coimmunoprecipitation was performed as described in (B).

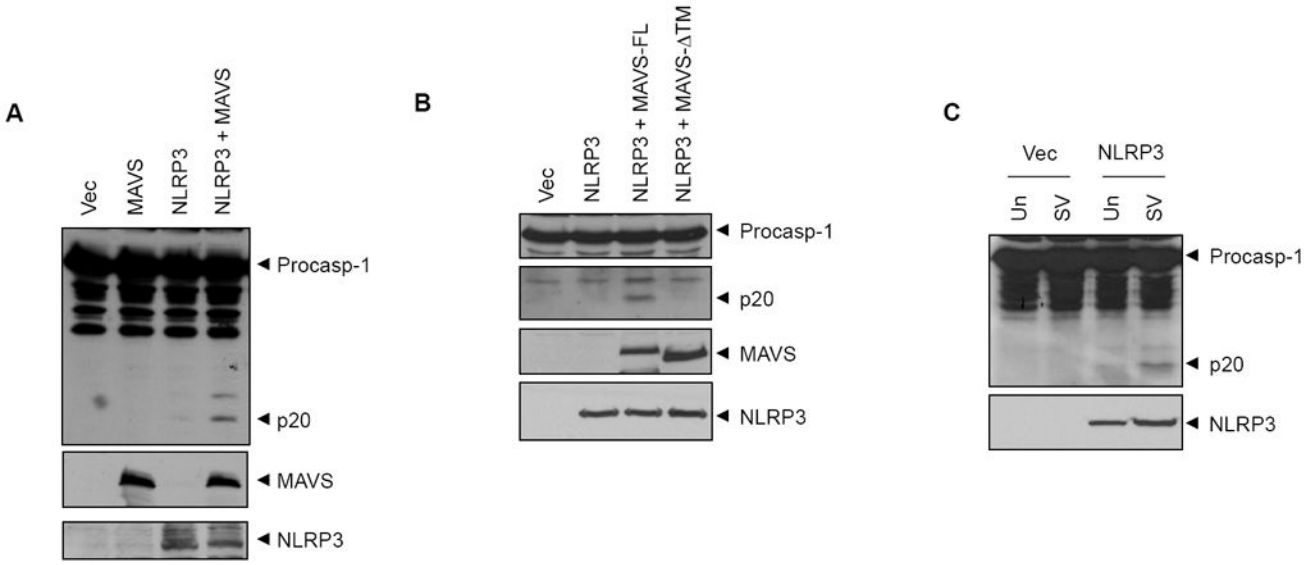

FIGURE 2.

Activation of NLRP3 inflammasome by MAVS. (A, B) Immunoblots of caspase-1 in cell lysates of 293T-C1A cells transfected with empty vector (Vec) MAVS, NLRP3, NLRP3 plus MAVS full length, or NLRP3 plus MAVS-ΔTM constructs as indicated. (C) Immunoblots of caspase-1 in cell lysates of 293T-C1A cells transfected with empty vector (Vec) or NLRP3 followed by no treatment (Un) or infection with Sendai virus (SV, 9 HA/ml) for 8 h as indicated. Cell lysates were also immunoblotted with anti-Myc (MAVS) or anti-Flag (NLRP3) antibody (Lower panels, A and B) or anti-Flag (NLRP3) antibody (lower panel, C) as indicated.

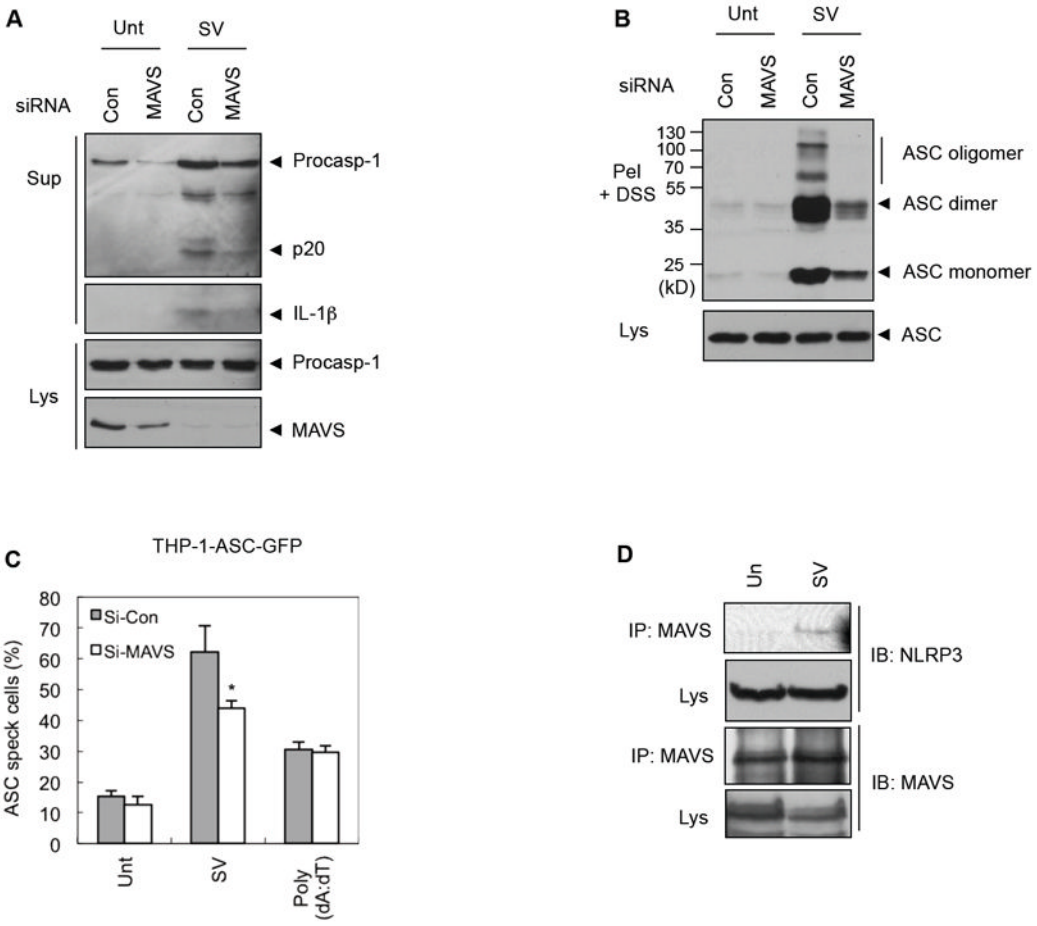

FIGURE 3.

Sendai virus-mediated activation of NLRP3 inflammasome in THP-1 cells. (A) Immunoblots of caspase-1 and IL-1β in culture supernatants (Sup) of PMA-differentiated THP-1 cells transfected with scrambled siRNA (Con) or human MAVS-specific (MAVS) siRNA oligonucleotides (50 nM) for 48 h, followed by infection with Sendai virus (10 HA/ml, 6 h). Cell lysates (Lys) were immunoblotted with anti-caspase-1 or anti-MAVS antibody as indicated. (B) Immunoblot of ASC in DSS-crosslinked NP-40 insoluble pellets of PMA-differentiated THP-1 cells transfected with scrambled siRNA (Con) or human MAVS-specific (MAVS) siRNA oligonucleotides followed by infection with Sendai virus (10 HA/ml, 6 h) as indicated. The lower panel shows an immunoblot of ASC in the soluble lysates from the same cells. (C) PMA-differentiated THP-1-ASC-GFP cells were transfected with siRNA as in (A), followed by treatment with Sendai virus (10 HA/ml, 6 h) or transfection with poly dA:dT (2 μg, 6 h). ASC pyroptosome-containing cells (ASC specks) were counted using fluorescence microscopy and represented as percentages of total cells counted. Asterisk indicates significant difference compared to Si-Con cells (*, $p<0.05$, n = 5). (D) PMA-differentiated THP-1 cells were left untreated or infected with Sendai virus (10 HA/ml) for 4 h. Cell lysates were fractionated and the mitochondria-enriched fractions were immunoprecipitated with anti-MAVS antibody. Immunoprecipitates and cytosolic lysates were immunoblotted with anti-NLRP3 or anti-MAVS antibody as indicated.

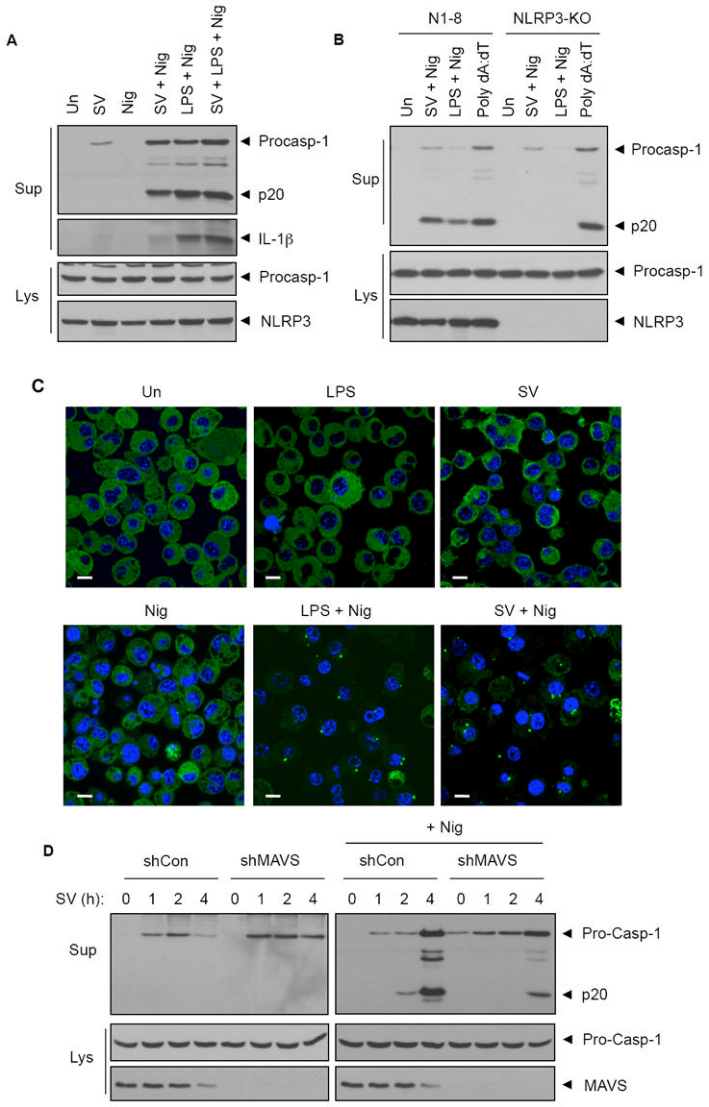

FIGURE 4.

Sendai virus-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 Sendai virus (SV, 10 HA/ml, 6 h), nigericin (Nig, 5 μM, 45 min), Sendai virus (6 h) followed with nigericin (45 min) (SV+Nig), LPS (0.25 μg/ml, 4 h) followed with nigericin (LPS+Nig), or Sendai virus (6 h) followed with LPS (4 h) and then nigericin (45 min) (LPS+SV+Nig) as indicated. Cell lysates (Lys) were immunoblotted with anti-caspase-1 or anti-NLRP3 antibody 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 Sendai virus (SV, 10 HA/ml, 6 h) plus nigericin (Nig, 5 μM, 45 min), LPS (0.25 μg/ml, 4 h) plus nigericin (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, Sendai virus (SV), nigericin (Nig), LPS plus nigericin (LPS+Nig), or Sendai virus plus nigericin (SV+Nig) as described in A and B above. The green and blue signals represent NLRP3 and nuclear fluorescence, respectively. Bar: 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 Sendai virus alone (10 HA/ml, 0-4 h, *left panels*) or Sendai virus (10 HA/ml, 0-4 h) followed by nigericin (45 min, *right panels*). Cell lysates were immunoblotted as indicated.

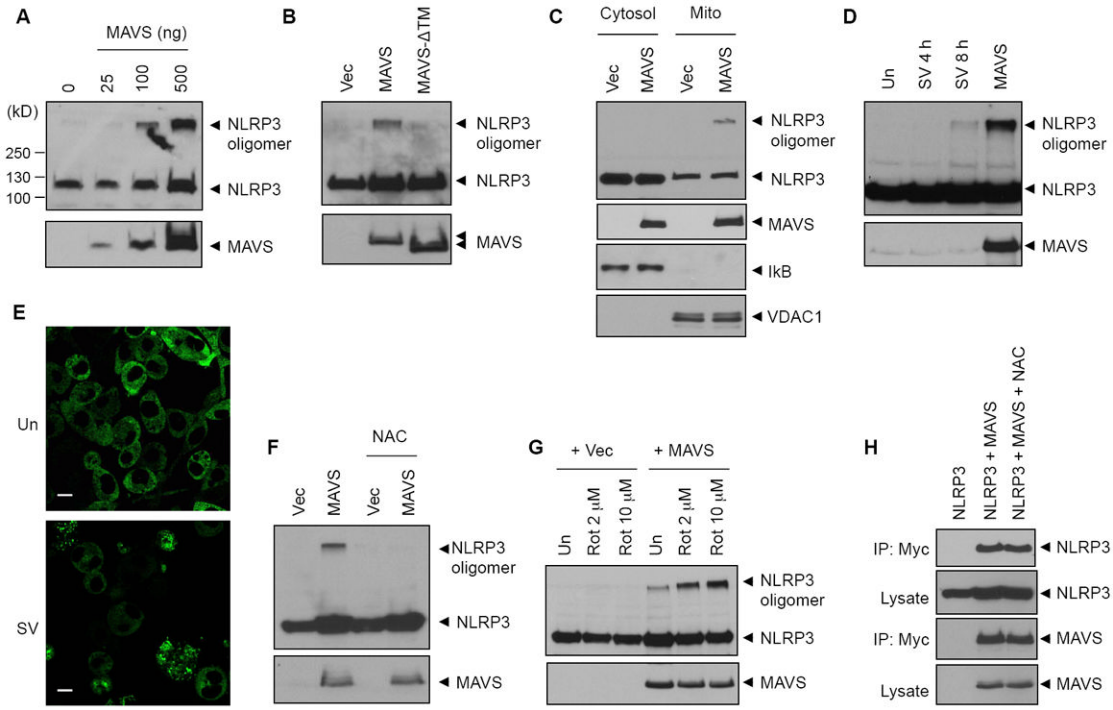

FIGURE 5.

NLRP3 oligomerization by MAVS. (A, 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) antibodies. (C) 293T cells were transfected with a 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) antibodies. (D) 293T cells were transfected with NLRP3 (0.3 μg) followed by infection with Sendai virus (SV, 10 HA/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 Sendai virus (SV, 10 HA/ml, 8 h) as indicated. Bar: 10 μm. (F-G) 293T cells were transfected with a 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 above. (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 antibody, and the immunoprecipitates were then immunoblotted with anti-Flag (NLRP3) or anti-Myc (MAVS) antibody, respectively.

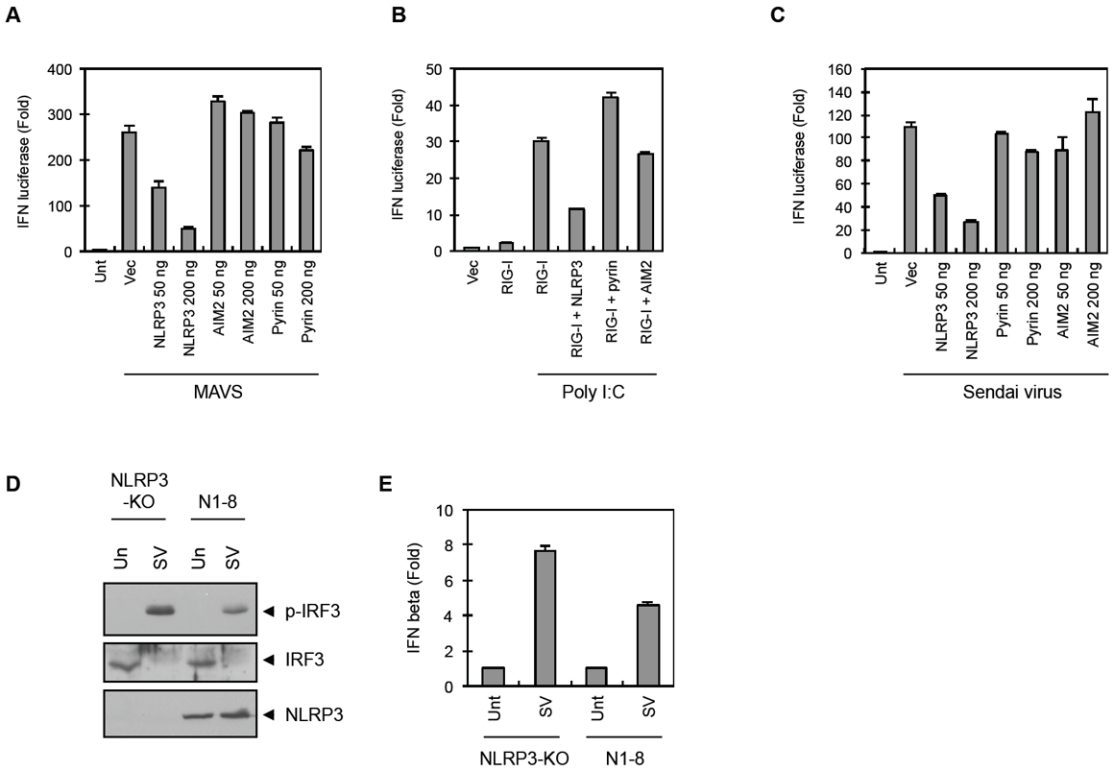

FIGURE 6.

Inhibition of MAVS-induced Type I IFN signaling by NLRP3. (A) 293T cells were transfected with IFN-β promoter-luciferase reporter plasmid (100 ng), pβ-gal (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 the "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. 24 h after transfection cells were transfected with poly I:C (10 μg, 20 h, B) or infected with Sendai virus (SV, 10 HA, 8 h, C) as indicated. Reporter luciferase activity was then determined as in A. (D, E) NLRP3-deficient BMDMs or NLRP3-restored (N1-8) BMDMs were left untreated or infected with Sendai virus (SV, 5 HA/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". p-IRF3, phospho-IRF3.