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Reactive oxygen species induce virus-independent MAVS oligomerization in systemic lupus erythematosus

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Abstract

The increased expression of genes induced by type I interferon (IFN) is characteristic of viral infections and systemic lupus erythematosus (SLE). We showed that mitochondrial antiviral signaling (MAVS) protein, which normally forms a complex with retinoic acid gene I (RIG-I)-like helicases during viral infection, was activated by oxidative stress independently of RIG-I helicases. We found that chemically generated oxidative stress stimulated the formation of MAVS oligomers, which led to mitochondrial hyperpolarization and decreased adenosine triphosphate production and spare respiratory capacity, responses that were not observed in similarly treated cells lacking MAVS. Peripheral blood lymphocytes of SLE patients also showed spontaneous MAVS oligomerization that correlated with the increased secretion of type I IFN and mitochondrial oxidative stress. Furthermore, inhibition of mitochondrial reactive oxygen species (ROS) by the mitochondria-targeted antioxidant MitoQ prevented MAVS oligomerization and type

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SUPPLEMENTARY MATERIALS

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Fig. S1. Measurement of mROS, mitochondrial mass, and mitochondrial phenotype.

Fig. S2. MAVS oligomers are not artificially formed during sample processing, homogenization, or lysis, and IL-6 production in the presence of ROS is independent of RIG-I helicases.

Fig. S3. MAVS oligomers are not artificially formed during the processing, homogenization, or lysis of SLE patient samples.

Fig. S4. Analysis of mitochondrial respiration in PBMC subsets.

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I IFN production. ROS-dependent MAVS oligomerization and type I IFN production were reduced in cells expressing the MAVS-C79F variant, which occurs in 30% of sub-Saharan Africans and is linked with reduced type I IFN secretion and milder disease in SLE patients. Patients expressing the MAVS-C79F variant also had reduced amounts of oligomerized MAVS in their plasma compared to healthy controls. Together, our findings suggest that oxidative stress-induced MAVS oligomerization in SLE patients may contribute to the type I IFN signature that is characteristic of this syndrome.

INTRODUCTION

Oxidative stress characterizes several infectious and autoimmune diseases, reflecting a disturbance in the normally tightly regulated balance between the production of various chemically reactive molecules, such as reactive oxygen species (ROS) and reactive nitrogen species, and antioxidants, including the glutathione and thioredoxin systems (1). During the early stages of certain RNA virus infections, retinoic acid-inducible gene I (RIG-I)-like helicases sense and bind to viral RNAs. RIG-I-RNA complexes associate with mitochondrial antiviral signaling (MAVS) protein, which is located on the cytoplasmic face of the outer mitochondrial membrane (2). The interaction between RIG-I and MAVS is facilitated by mutual N-terminal caspase recruitment domains (CARDs). RIG-I-MAVS complex formation then leads to the CARD-dependent oligomerization of MAVS and the subsequent activation of interferon (IFN) regulatory factor 3 (IRF3) and IRF7 and nuclear factor κ B (NF- κ B), which in turn induce the production of type I IFN and proinflammatory cytokines (3, 4). Findings suggest that the homotypic interaction between the CARD of MAVS and the CARD of RIG-I forms protein aggregates and filaments on the surface of the mitochondria that can further activate MAVS proteins to form functional clusters on the outer mitochondrial membrane (5). These high-molecular weight MAVS complexes amplify the formation of the cytosolic signaling complex that activates IRFs and NF- κ B (5, 6).

Virus-induced MAVS aggregates are resistant to treatment with protease and detergent but are sensitive to reducing agents, such as dithiothreitol and β -mercaptoethanol (β -Me), early in the induction process, suggesting that disulfide bond formation contributes to MAVS oligomer formation and stability (5). MAVS oligomerization may thus be a redox-sensitive process. ROS modulate various inflammatory processes (7–9), and the finding that increased cellular ROS amplifies RIG-I signaling and MAVS function at the mitochondria supports this notion (5, 10). Accordingly, the RIG-I-MAVS signaling pathway is also enhanced by nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2), a cytoplasmic source of ROS (11). Conversely, repression of mitochondrial ROS (mROS) production by cytochrome C oxidase complex subunit 5 (COX5B) inhibits MAVS aggregation and downstream signaling (12). Although work by Xu *et al.* (6) defined the structural basis of the MAVS-CARD self-interaction, how MAVS oligomerization might occur in response to oxidative stress in the absence of viral infection is unclear (6).

A study by one of our groups showed that peripheral blood lymphocytes (PBLs), particularly T cells, from patients with systemic lupus erythematosus (SLE) show an increase in ROS with corresponding mitochondrial hyperpolarization and increased mitochondrial electron

transport chain (ETC) activity at complex I (13). In turn, enhanced mROS production leads to oxidative modifications of many cellular proteins, lipids, and DNA (14, 15). Moreover, the depletion of cytosolic adenosine triphosphate (ATP) associated with mitochondrial hyperpolarization predisposes T lymphocytes to death by necrosis in SLE (16–22). The presence of increased amounts of type I IFN in the serum of SLE patients was first reported in 1982 (23), but only recently has the role of type I IFN in the pathogenesis of SLE been appreciated (24). More than half of SLE patients display excess production of type I IFN and the increased expression of a group of type I IFN–stimulated genes that are associated with active disease (25, 26). Several observations have linked SLE risk alleles to the production of, and response to, type I IFN (27). Among these is a single-nucleotide polymorphism (SNP) in the CARD of MAVS that changes a cysteine to phenylalanine at position 79 (C79F) (28). The MAVS-C79F variant occurs in 30% of the sub-Saharan healthy population, and its presence in African-American SLE patients is associated with substantially reduced amounts of type I IFN, absence of autoantibodies against RNA binding proteins, and overall milder disease (28). This suggests that Cys⁷⁹ could render MAVS susceptible to ROS-mediated modification through disulfide bridge formation among CARD domains, leading to MAVS oligomerization. Thus, those individuals bearing the MAVS-C79F variant might be somewhat protected from an overwhelming type I IFN–dependent inflammatory response to viral infections. Similarly, SLE patients with MAVS-C79F might also be protected from ROS-induced production of type I IFN.

Here, we report that MAVS oligomerization during viral infection led to decreased oxygen consumption and a higher mitochondrial membrane potential, resembling the mitochondrial phenotype observed in the T cells of SLE patients. We further showed that chemically induced oxidative stress was sufficient to stimulate MAVS oligomerization, which led to substantial production of type I IFN. Furthermore, spontaneous MAVS oligomerization was observed in the PBLs and plasma of several SLE patients but rarely in age- and sex-matched healthy controls. In addition, we showed that patients with oligomerized MAVS had a decreased capacity for oxidative phosphorylation and had increased plasma concentrations of type I IFN. MAVS oligomerization was inhibited by the mitochondria-targeted antioxidant MitoQ. Additional analysis revealed that ROS-induced oligomerization of MAVS and consequent type I IFN production were markedly reduced in cell lines expressing the MAVS-C79F variant but were not markedly affected during viral infection. Consistent with this finding, SLE patients bearing the MAVS-C79F variant showed reduced MAVS oligomerization in plasma. Together, these findings suggest a mechanism by which mitochondrial dysfunction and oxidative stress result in the type I IFN signature of SLE patients through MAVS oligomerization.

RESULTS

MAVS oligomerization decreases ATP production and spare respiratory capacity in mitochondria

ROS and redox-regulating proteins, such as COX5B and NOX2, that modify the quantities of mitochondrial and cytoplasmic ROS, respectively, inhibit the MAVS-mediated antiviral pathway by decreasing the abundance of MAVS (12). However, the role of MAVS depletion

or cleavage in cellular mitochondrial oxidative metabolism has not been investigated. Thus, we examined mitochondrial function and metabolism in wild-type (WT) or MAVS-knockout (MAVS-KO) mouse embryonic fibroblasts (MEFs) during infection with coxsackievirus B3 (CVB3) or Sindbis virus (SINV) or after transfection with an RNA containing a triphosphate incorporated at the 5' end (5'-ppp), which is a specific ligand for RIG-I. Both CVB3 and SINV are single-stranded, positive-sense RNA viruses with the ability to activate the RIG-I pathway (29). However, unlike SINV, CVB3 has the additional ability to cleave MAVS at Gln¹⁴⁸ with its 3C^{pro} protease to decrease type I IFN signaling (30). During the initial 12 hours after infection with CVB3 or SINV or transfection with 5'-ppp-RNA, we observed that both ROS and mitochondrial membrane potential (ψ_m) increased (Fig. 1, A and B), consistent with previous findings (31, 32). However, after 12 hours, in contrast to cells treated with SINV or 5'-ppp-RNA, in cells infected with CVB3, the CVB3-dependent cleavage of MAVS paralleled a marked reduction in cellular ROS and ψ_m (Fig. 1, A to C). Changes in ψ_m and ROS during viral infection or stimulation with 5'-ppp-RNA also correlated positively with MAVS oligomerization (Fig. 1D). No increase in ROS or ψ_m was observed during the viral infection of MAVS-KO cells (Fig. 1, A and B). This was also true of cells expressing a truncated form of MAVS cleaved at Gln¹⁴⁸, which lacks the CARD domain required for MAVS oligomerization (fig. S1). Using the (roGFP) as a probe of mROS (33), we determined that the increased thiol oxidation during viral infection or stimulation with 5'-ppp-RNA originated primarily in mitochondria (Fig. 1E).

We observed elongation and further swelling of mitochondria after viral infection and exposure to 5'-ppp-RNA (fig. S1), consistent with previous observations (34–36). Mitochondrial morphological alterations that occur together with changes in mitochondrial membrane polarization also lead to changes in respiration and ATP production (37). To establish whether MAVS influenced cellular metabolism during an innate immune response to viral infection, we measured total ATP production (Fig. 1F) and the ATP/adenosine diphosphate (ADP) ratio (Fig. 1G) in WT and MAVS-KO cells before and after infection with CVB3 or transfection with 5'-ppp-RNA. We observed that before infection, MAVS-KO cells had almost threefold more cellular ATP than did their WT counterparts (Fig. 1F). This high quantity of ATP in MAVS-KO cells correlated with a higher ATP/ADP ratio (Fig. 1G). After infection with CVB3, the total amount of ATP and the ATP/ADP ratio increased twofold in WT cells but did not increase further in MAVS-KO cells (Fig. 1, F and G). These findings corresponded with the cleavage pattern of MAVS during CVB3 infection (Fig. 1C), which is suggestive of a causative relationship. We did not observe the same increase in the ATP/ADP ratio in cells transfected with 5'-ppp-RNA, which can continually activate RIG-I and MAVS oligomerization. In this case, after an initial small increase in ATP abundance, the amount of ATP declined over time (Fig. 1F), suggesting that the activation of RIG-I and MAVS reduced the total quantity of ATP in these cells.

To further assess the bioenergetic profile of the cells during viral infection in the presence or absence of MAVS, we initially examined the oxygen consumption rate (OCR) as an indicator of oxidative phosphorylation with a Seahorse extracellular flux analyzer (38). To assess mitochondrial respiratory capacity, we sequentially treated cells with oligomycin (to block ATP synthesis), the ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (to uncouple mitochondria and thereby maximally stimulate the ETC), and rotenone

(to block ETC complex I) (Fig. 1H). We observed that infecting WT cells with CVB3 or treating them with 5'-ppp-RNA led to a substantially reduced basal OCR compared to that of mock-treated cells, and a very minimal response to FCCP, which suggested that these cells had impaired spare respiratory capacity (SRC) (Fig. 1, I and J). Three hours after infection, at a time when the CVB3 3C^{pro} protease had produced only a small amount of cleaved MAVS (Fig. 1C), the metabolic profile of CVB3-infected cells did not differ from that of cells transfected with 5'-ppp-RNA (Fig. 1I). However, the same metabolic profile analysis at 24 hours, when CVB3 3C^{pro} caused substantial cleavage of MAVS, showed that CVB3-infected cells, but not 5'-ppp-RNA-treated cells, had regained basal capacity of OCR and SRC (Fig. 1J). Under the same conditions, MAVS-KO cells showed higher baseline OCR and SRC than did WT cells; however, unlike in the WT cells, this state did not substantially change upon CVB3 infection or exposure to 5'-ppp-RNA (Fig. 1, K and L). The findings from three separate OCR studies comparing WT and MAVS-KO MEFs were compiled (Fig. 1, M and N). Because the prolonged culturing of MEFs affects their phenotype, we tested whether the metabolic differences were due to genotypic differences in experiments with primary splenocytes from WT and MAVS-KO mice, and we observed the same phenotype as that of the MEFs (Fig. 1, O and P). In contrast to the differences in oxygen consumption, the basal extracellular acidification rate (ECAR), a readout of lactate production by glycolysis, was very similar between WT and MAVS-KO MEFs (fig. S1C). Together, these findings suggest that MAVS oligomerization inhibits mitochondrial respiration and ROS production.

Oxidative stress induces MAVS oligomerization independently of viral infection

To further evaluate the influence of oxidative stress on MAVS oligomerization in the presence or absence of viral infection, we used two gel systems. First, we adapted a trisglycine extended (TGX) gradient polyacrylamide gel system to detect MAVS aggregates with high resolution (39). Second, to better detect the total amount of MAVS oligomers, we used trisglycine agarose gels, which enabled improved migration of large-molecular weight complexes (5). We assessed MAVS oligomerization during chemically induced oxidative stress with glucose oxidase (GOx), which generates H₂O₂ in the process of catalyzing the oxidation of glucose to D-glucono-δ-lactone, and compared it to the MAVS oligomerization that occurred in response to SINV infection (40). At concentrations of 10 to 20 μg/ml, GOx increases cytoplasmic ROS production to a quantity close to that observed during viral infection (41). We observed that GOx-mediated oxidative stress alone was sufficient to induce MAVS oligomerization, in a manner similar to that induced by SINV infection (Fig. 2A). GOx-induced MAVS oligomerization was reversible when the cell culture medium was supplemented with β-Me (Fig. 2A). This was much less apparent during SINV infection, where only a portion of the MAVS oligomers were reducible. This difference could reflect the fact that the viral infection-induced oligomerization of MAVS involves both RIG-I-CARD and MAVS-CARD dimerization and ROS-induced MAVS oligomerization, whereas GOx involves only the latter process. To address the possibility that the observed MAVS oligomerization might be an artifact of cysteine dimerization induced during cell lysis and processing for electrophoresis, we homogenized freshly prepared cells in buffer containing iodoacetamide (IAA), which covalently binds to free thiol groups of cysteines to prevent further disulfide bond formation. The presence of 100 μM IAA did not result in a decrease in

MAVS oligomers after either treatment with GOx or infection with SINV (fig. S2A). MAVS oligomerization induced by GOx also led to the formation of the MAVS signaling complex, as detected by coimmunoprecipitation of MAVS through its N-terminal His-tag. Within 3 hours after ROS induction, we observed the association of tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), TRAF family member-associated NF- κ B activator (TANK), TNF receptor type 1-associated death domain (TRADD), and receptor-interacting serine/threonine-protein kinase 1 (RIPK1) with MAVS, suggesting that both the IRF and NF- κ B branches of the MAVS signaling complex were activated (Fig. 2B).

The oligomerization of MAVS induced by GOx correlated with increased ψ_m (Fig. 2C). Consistent with the ability of β -Me to reverse GOx-induced MAVS oligomerization, supplementation of the culture medium with β -Me also prevented mitochondrial hyperpolarization in cells treated with GOx but not in cells infected with SINV (Fig. 2C). MAVS oligomerization induced by GOx led to increased secretion of IFN- β and interleukin-6 (IL-6), which was not observed in MAVS-deficient cells that were treated with GOx (Fig. 2, D and E).

To determine whether ROS derived specifically from mitochondrial respiration could also induce MAVS oligomerization, we used the compound MitoParaquat (MitoPQ), a mitochondria-targeted superoxide generator that contains a paraquat moiety tethered to a positively charged lipophilic triphenylphosphonium (TPP) cation that drives MitoPQ accumulation within negatively charged mitochondria (42). Treatment of MEFs with moderate concentrations of MitoPQ (1 to 10 nM) led to increased MAVS oligomerization (Fig. 2F), without causing substantial cell death (Fig. 2G). Treatment of cells with MitoPQ also led to a substantial increase in IFN- β and IL-6 secretion, which was not observed in MitoPQ-treated MAVS-KO cells (Fig. 2, H and I).

To further rule out a potential role for RIG-I-like receptors (RLRs) in ROS-induced MAVS oligomerization, we treated cells lacking RIG-I alone or both RIG-I and melanoma differentiation-associated gene 5 (MDA5) using GOx, MitoPQ, and specific ligands for RIG-I (5'-ppp-RNA) and MDA5 polyinosinic-polycytidylic acid [poly(I:C)]. As demonstrated by Western blotting analysis, GOx-induced MAVS oligomerization occurred in the absence of RIG-I or when both RIG-I and MDA5 were absent (Fig. 2J). MEFs lacking both RIG-I and MDA5, but not MAVS-deficient cells, had increased amounts of *Irfn* (Fig. 2K) and *Il6* mRNA (fig. S2B) after treatment with GOx and MitoPQ, which correlated with the increased secretion of these cytokines (Fig. 2L and fig. S2C). Cells deficient in RIG-I, a combination of RIG-I deficiency and MDA5 knockdown, or MAVS-deficient cells treated with RIG-I or MDA5 ligands did not show increased IFN- β (Fig. 2, K and L) or IL-6 (fig. S2, B and C) secretion. To control whether RIG-I-, MDA5-, or MAVS-deficient cells were capable of secreting proinflammatory cytokines, we treated these cells with double-stranded DNA (dsDNA), which leads to the secretion of type I IFN and IL-6 in a RIG-I-independent manner (43). We observed that dsDNA led to the secretion of equivalent amounts of IFN- β and IL-6 by RIG-I-, MDA5-, or MAVS-deficient cells (fig. S2D). These findings support the view that in the absence of viral infection, ROS alone is sufficient to induce MAVS oligomerization and downstream cytokine signaling independently of RIG-I and MDA5.

The mitochondria-targeted antioxidant MitoQ reduces MAVS oligomerization and type I IFN secretion

MitoQ {[10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl) decyl] (triphenyl)phosphonium methanesulfonate} is a mitochondria-targeted antioxidant, which contains ubiquinol as the active antioxidant component, bound to TPP (44). In mitochondria, MitoQ is continually recycled to the active ubiquinol antioxidant by complex II in the respiratory chain (44, 45). Cells pretreated with MitoQ before undergoing oxidative stress showed reduced MAVS oligomerization, which was more pronounced in cells treated with GOx than in those infected with SINV (Fig. 3A). MitoQ led to at least an 80% decrease in the amount of MAVS oligomers in cells treated with GOx, whereas in cells infected with SINV, MitoQ resulted in about a 40% decrease in the amount of MAVS oligomers (Fig. 3B). As a control, cells pretreated with decyl-TPP (dTPP), which has the same mitochondria-targeting motif as MitoQ but lacks its antioxidant properties, did not block MAVS oligomerization caused by SINV infection or ROS induction (Fig. 3A). Similar to the findings with β -Me treatment, the results from experiments with MitoQ suggest that the nature or intensity of MAVS oligomerization induced by direct oxidative stress differs from that stimulated by viral infection in which there is the additional heterodimerization caused by the interaction between the RIG-I-CARD and the MAVS-CARD. Decreased MAVS oligomerization in the presence of MitoQ correlated with decreased mitochondrial hyperpolarization (Fig. 3C) and reduced secretion of type I IFN (Fig. 3D).

MAVS oligomerizes in human PBMCs under oxidative stress and can be detected in PBMCs and plasma from SLE patients

We extended the findings from our experiments with MEFs to human peripheral blood mononuclear cells (PBMCs) from healthy donors and observed that GOx induced MAVS oligomerization that appeared to be progressive, because we identified distinct MAVS bands of ~750 and >1200 kDa (Fig. 4A). As was observed in MEFs, MAVS oligomerization did not occur in PBMCs pretreated with MitoQ or cultured in the presence of β -Me (Fig. 4A).

We previously observed that lymphocytes from SLE patients exhibit spontaneous increased ROS, hyperpolarization of mitochondria, and increased cell death (21). We thus tested whether MAVS was spontaneously oligomerized in PBMCs from SLE patients compared to PBMCs from age- and sex-matched healthy controls (Fig. 4, B to D). Spontaneous MAVS oligomerization was assessed by agarose gel separation and was identified in the PBMCs of 14 of 17 SLE patients, whereas weak oligomerization was observed in 4 of 17 healthy controls (Fig. 4, B and D). MAVS oligomers were also detected in the plasma of 12 of 17 SLE patients but not in healthy controls (Fig. 4, C and D). To exclude the possibility that the observed MAVS oligomerization spontaneously occurred during cell and plasma isolation from blood samples and processing, we added IAA, which binds covalently with the thiol group of cysteine so that the protein cannot form disulfide bonds (46). Use of IAA during sample preparation did not decrease the detection of MAVS oligomers in cells or plasma isolated from SLE patients (fig. S3). These data suggest that MAVS oligomers were not artificially formed during tissue homogenization and processing and further indicate that MAVS oligomers are stable in plasma. Plasma from SLE patients contained more type I IFN than did plasma from healthy controls (Fig. 4E), and the amount of type I IFN closely

paralleled the extent of MAVS oligomerization in the plasma (Fig. 4F). Metabolic analysis of PBMCs from SLE patients and healthy controls identified no difference in basal respiration (Fig. 4G) but showed statistically reduced maximal respiration, SRC, and rate of ATP synthesis in the PBMCs of SLE patients (Fig. 4, H to J). These findings are similar to the changes in respiratory capacity that we observed during MAVS oligomerization induced by viral infection (Fig. 1, I to N).

To further explore which subsets of cells within PBMCs contributed to MAVS oligomerization in SLE patients, we separated PBMCs into T cell, B cell, and monocyte pools, and analyzed each for the presence of MAVS aggregates. On the basis of the analysis of equivalent amounts of total cellular protein, T cells had the greatest amount of MAVS oligomers, with less in B cells, and relatively little detected in monocytes (Fig. 4K). MAVS oligomers were not observed in the same cell subsets of healthy controls. These findings are consistent with our previous findings showing that much of the increased ROS in the PBMCs of SLE patients is found in the T cell subset (20). Consistent with this observation, metabolic analysis of PBMCs isolated from healthy donors and SLE patients showed that oxidative phosphorylation in the PBMCs of SLE patients was decreased (Fig. 4L). Furthermore, analysis of subsets of these PBMCs showed that T and B cells, unlike monocytes, showed oxidative phosphorylation (fig. S4), which was consistent with previously published work (47). Although basal respiration, maximal respiration, and SRC were substantially reduced in both the B cells (Fig. 4M) and T cells (Fig. 4N) of SLE patients, the extent of the reduction of these rates in T cells was at least twofold greater. The rate of ATP synthesis was decreased only in the T cells of SLE patients and not in B cells (Fig. 4, M and N).

The MAVS-C79F variant, which is associated with milder SLE activity, shows reduced oligomerization

SLE patients bearing a MAVS-C79F SNP have milder disease and reduced production of type I IFN compared to patients with WT MAVS, and they lack autoantibodies to RNA binding proteins (28). In addition, cells expressing MAVS-C79F secrete less inflammatory mediators, such as IL-8, chemokine (C-C motif) ligand 5 (CCL5), and IFN- β , than do cells expressing WT MAVS (28). Because this SNP modifies a cysteine residue, we considered whether this might alter the ability of MAVS-C79F to form oligomers under oxidative stress. We thus initially reconstituted MAVS-KO MEFs with either WT human MAVS, the C79F variant, or a combination of the two (Fig. 5A). We observed that cells reconstituted with the MAVS-C79F variant manifested reduced amounts of oligomerized MAVS than did MAVS-KO cells reconstituted with WT MAVS. After infection with SINV, the MAVS-C79F-expressing cells showed no statistically significant decrease in high-molecular weight MAVS oligomers as compared to GOx-treated cells; however, we detected moderately sized oligomers that might reflect trimer or tetramer formation (Fig. 5, A and B). Although we have observed differences in the extent of MAVS oligomerization between SINV-infected and GOx-treated cells, the amount of type I IFN secreted was affected under both conditions (Fig. 5C). Furthermore, under oxidative stress, cells expressing the MAVS-C79F variant exhibited less mROS and ψ_m than did cells expressing WT MAVS, which suggests that the MAVS-C79F variant had little capacity to promote mitochondrial hyperpolarization (Fig. 5,

D and E). On the basis of these observations in MEFs, we examined MAVS oligomerization in six SLE patients with the MAVS-C79F variant, four SLE patients with WT MAVS, and three healthy controls, all of whom were from the original C79F SNP study (28) and whose plasma was frozen at the same time. We observed that the extent of MAVS oligomerization was statistically significantly less in the plasma of the MAVS-C79F-expressing SLE patients compared to that in the plasma of SLE patients bearing WT MAVS (Fig. 5, F and G).

MAVS oligomers from SLE patients can induce prion-like MAVS aggregates

MAVS is reported to form oligomers in a prion-like manner (5, 6). We therefore examined whether MAVS oligomers detected in the plasma of SLE patients had the ability to induce MAVS oligomerization in purified mitochondria from WT MEFs that were not exposed to oxidative stress. We first purified the MAVS oligomers from plasma by immunoprecipitation with an anti-MAVS antibody cross-linked to protein G magnetic beads (Fig. 6, A and B). Plasma samples were initially preincubated with protein G magnetic beads alone to remove nonspecifically bound proteins before being incubated with anti-MAVS-conjugated protein G beads. After the purification and elution of the MAVS aggregates, we observed that MAVS oligomers purified from the plasma of different patients had a uniform molecular weight of ~1500 kDa (Fig. 6C). Purified MAVS oligomers from the plasma of SLE patients or from the plasma of age- and sex-matched healthy controls were incubated with freshly purified mitochondria from normal MEFs for 30 min in a respiration-promoting buffer. Unbound donor MAVS oligomers were then removed by repurification of the mitochondria with anti-Tom22 magnetic beads (Fig. 6A). As a positive control, we observed that the treatment of purified mitochondria with GOx induced the oligomerization of MAVS (Fig. 6C, lane 2) similar to that of intact cells (Fig. 2A). Furthermore, we observed that MAVS oligomers isolated from the plasma of SLE patients induced MAVS oligomerization in isolated mitochondria (Fig. 6C, lane 3) and that this was decreased in extent in mitochondria pretreated with MitoQ (Fig. 6C, lane 4). Treatment of purified mitochondria with the plasma from a healthy control did not induce the formation of detectable, high-molecular weight MAVS oligomers (Fig. 6C, lane 5). As a negative control, purified MAVS oligomers from SLE patient plasma did not associate with mitochondria isolated from MAVS-KO cells (Fig. 6C, lane 6).

To further distinguish induced oligomers of endogenous MAVS from preexisting oligomers of SLE patients, we performed the same experiments with mitochondria from MAVS-KO MEFs that expressed His-tagged human WT MAVS, MAVS-C79F, or a combination of both. After treating mitochondria from WT MEFs with MAVS oligomers isolated and purified from an SLE patient, we detected high-molecular weight bands of MAVS with the anti-His antibody, again demonstrating that MAVS oligomers isolated from SLE patients induced the aggregation of endogenous MAVS (Fig. 6D). In addition, we observed reduced MAVS aggregation when mitochondria contained MAVS-C79F or a combination of both WT MAVS and the MAVS-C79F variant (Fig. 6D, lanes 3 and 4). Together, these data suggest that the MAVS oligomers in the plasma of SLE patients induce the prion-like oligomerization of endogenous MAVS.

Dendritic cells take up prion-like MAVS aggregates

The ability of dendritic cells (DCs) to take up and propagate intact prions has been described previously (48–50). Observations in transmissible spongiform encephalopathies show that internalization of fluorescently labeled prions is detected in DCs (51). However, experimental data defining the role that immune cells play in the possible uptake of exogenous prion-like aggregates of MAVS and the subsequent activation of inflammation have not been demonstrated. To examine whether human or murine DCs could internalize MAVS oligomers purified from the plasma of SLE patients, we labeled MAVS with either Alexa Fluor 546 (Alx546) or Alx488 (Fig. 7A). Before enriching and labeling MAVS oligomers from plasma, we first treated total plasma with protein G magnetic beads alone and filtered the samples through 100-kDa cutoff membranes to remove nonspecific, low-molecular weight oligomers and cytokines.

We then bound the MAVS oligomers from plasma with anti-MAVS-conjugated protein G magnetic beads and coupled them to Alx546 or Alx488 while the MAVS oligomers were still bound to the MAVS antibody tethered to the magnetic beads. This effectively removed excess dye without the need to pass the material through another size-exclusion resin. As a negative control, we also labeled plasma from healthy controls that was free of MAVS oligomers to exclude the labeling of other plasma proteins. TGX gel analysis confirmed that the labeling did not affect the stability of the MAVS oligomers from the SLE patient plasma, nor did labeling induce MAVS oligomerization in healthy control plasma (Fig. 7A). Because the extent of MAVS oligomerization was greatest in patient samples P2 to P4, we used these samples for further studies (Fig. 7A).

We then examined whether labeled MAVS aggregates could be taken up by DCs derived from human peripheral blood monocytes (hDCs) or by murine bone marrow-derived DCs (BMDCs) (52, 53). As a control, we also tested BMDCs derived from MAVS-KO mice. We initially used confocal microscopy to verify the rate of MAVS internalization and to note its intracellular location relative to mitochondria with MitoTracker. Alx546-MAVS aggregate uptake and detection in both human hDCs and BMDCs occurred within 1 hour (Fig. 7, B and C). We did not observe Alx546 cytoplasmic staining in DCs treated with Alx546-labeled plasma samples from healthy controls because freely diffusing, nonassociated MAVS yielded markedly weaker fluorescence (Fig. 7B). In contrast, exogenous MAVS oligomers required the presence of endogenous MAVS to associate with mitochondria and to induce type I IFN production from host cells. The increase in intracellular MAVS fluorescence was due to the localization and association of exogenous MAVS with mitochondria, which resulted in an increase in the local fluorescence signal. Calculation of the colocalization correlation coefficient of Alx546-MAVS and MitoTracker Green fluorescence demonstrated an association between exogenous MAVS and mitochondria; however, this was substantially reduced in MAVS-KO BMDCs (Fig. 7D). Mitochondrial colocalization of internalized MAVS aggregates also closely paralleled the amounts of type I IFN secreted by WT BMDCs and was not detected in MAVS-KO BMDCs (Fig. 7E). In addition, type I IFN production was induced by MAVS aggregates from SLE patients (P2 to P4) but not by plasma from healthy controls (C1 and C2) (Fig. 7E).

We further confirmed that internalized exogenous MAVS associated with mitochondria and endogenous MAVS by purifying mitochondria from BMDCs. We observed that labeled MAVS oligomers from the plasma of SLE patients, but not healthy control plasma, induced endogenous MAVS oligomerization (Fig. 7F, top). We confirmed the presence of exogenous SLE MAVS aggregates in the mitochondrial fraction by direct detection of labeled Alx488-MAVS in the gel. Consistent with the experiments performed with isolated mitochondria, we could not observe MAVS oligomers in the mitochondrial fraction of BMDCs isolated from MAVS-KO mice (Fig. 7F, bottom). To control whether the treatment of DCs with fluorophore alone promoted spontaneous MAVS oligomerization, we treated BMDCs with free Alx488 fluorophore, which did not lead to increased MAVS abundance or oligomerization (Fig. 7G). This demonstrated that any increased MAVS oligomerization that occurred after treatment of the BMDCs with the purified MAVS oligomers detected in samples P2 to P4 originated either from the association of exogenous MAVS aggregates with endogenous MAVS on mitochondria or from prion-like induced oligomerization. Finally, we observed that treatment of mitochondria isolated from BMDCs with purified MAVS aggregates from SLE patient plasma (P1 to P4), but not plasma from a healthy control (C1), resulted in increased mitochondrial colocalization of the MAVS signaling complex proteins TRAF2, TANK, TRADD, and RIPK1, and the formation of active IRF3 dimers (Fig. 7H), consistent with the earlier findings for type I IFN secretion by BMDCs (Fig. 7E).

DISCUSSION

Our findings support a model in which oxidative stress alone can induce the oligomerization of MAVS, resulting in the production of type I IFN and activation of the NF- κ B pathway. That spontaneous MAVS oligomerization was observed in the PBMCs of SLE patients provides a potentially important mechanistic link between the observations of increased oxidative stress and mitochondrial dysfunction in SLE patient lymphocytes and the known type I IFN signature found in many SLE patients (54). This suggests that viral- and RNA-independent activation of MAVS and type I IFN production could occur in SLE. These findings also suggest that antioxidants, which can inhibit MAVS oligomerization, might be therapeutic in SLE. Such clinical trials have been initiated (55). Finally, the inability of the MAVS-C79F variant to oligomerize under oxidative stress potentially explains why this variant is associated with milder forms of SLE (28).

Mitochondria fulfill several key functions in cellular metabolic and antiviral signaling pathways, including their central role in ATP generation. Although it has been suggested that MAVS oligomerization during viral infection or increased MAVS abundance can lead to mitochondrial dysfunction and cell death (56), a role for MAVS oligomerization in mitochondrial metabolism has not been previously addressed. We observed that MAVS was critical for the induction of ROS production during viral infection because ROS was absent in MAVS-deficient cells. This suggests a possible feed-forward mechanism in which the initial oligomerization of MAVS induced by the binding of RIG-I to MAVS through mutual CARDs stimulates ROS production, which in turn results in further MAVS oligomerization. MAVS oligomerization directly suppressed the function of mitochondrial complexes I to IV, consistent with the interaction between COX5B and MAVS (12). Our findings that cells bearing oligomerized MAVS had not only a reduced rate of ATP synthesis but also a

decreased mitochondrial SRC are consistent with this model. SRC represents the extra available mitochondrial capacity that a cell has to produce energy, and it is thought to be critical for long-term cellular survival and function (57). In agreement with this model, CVB3-mediated cleavage of MAVS or treatment of cells containing oligomerized MAVS with MitoQ restored SRC. These findings suggest that the cleavage of MAVS and the resulting restoration of high basal and SRC in mitochondria could help CVB3 to persist by preventing cell death through energetic exhaustion of the cell. Our data further support this premise by showing that cells lacking MAVS have high basal OCR and SRC and therefore could be resistant to cell death. Consistent with this hypothesis, previous studies indicate that MAVS-deficient fibroblasts are resistant to Sendai virus-induced cell death (56).

Our data show that both cellular and mROS induce the secretion of type I IFN and IL-6, which is dependent on the presence of MAVS. Furthermore, the amounts of type I IFN and IL-6 secreted by cells treated with MitoPQ were three- to fourfold greater than those secreted by cells treated with GOx, suggesting that the superoxide produced in mitochondria was potent in activating MAVS oligomerization. Another possible explanation is that GOx-induced ROS are scattered in the cell and that MAVS needs ROS to be localized in higher concentrations close to mitochondria for disulfide bridges to be formed in MAVS. Whether MAVS oligomerization is a secondary effect of mitochondrial dysfunction in SLE or an initial driving force behind mitochondrial dysfunction is not known and will be further investigated. It will be of interest to determine whether treatment of SLE patient PBMCs in culture with MitoQ, which inhibits GOx-mediated MAVS oligomerization, will restore mitochondrial vitality. That MAVS oligomerization can be inhibited by antioxidants suggests its therapeutic potential for SLE. Such antioxidant trials in SLE patients are currently under way with *N*-acetylcysteine (55).

Consistent with the data from experiments with antioxidants, which suggest a redox-mediated mechanism of MAVS oligomerization, our data showed that the MAVS-C79F variant was resistant to ROS-dependent oligomer formation. A potential explanation for how ROS induces MAVS oligomerization is through the induction of cysteine disulfide bonding, which is currently being investigated. MAVS has several cysteine residues, five of which, including Cys⁷⁹, reside within the CARD. This could explain why the MAVS-C79F variant failed to oligomerize with ROS or to induce type I IFN production. There may be other cysteines that can also form disulfide bonds, and disruption of any one could also severely disrupt the ability of MAVS to aggregate in response to ROS. Additionally, we found that although the MAVS-C79F variant did not form higher-molecular weight oligomers under oxidative stress, dimers and tetramers of MAVS-C79F may still form. Conceivably, the MAVS-C79F variant may merely alter MAVS conformation such that it cannot form oligomers. The original study describing the MAVS-C79F SNP hypothesized that this variant might alter the conformation of MAVS such that its interaction with downstream signaling molecules, such as TRAF3, might be altered (28). In addition, our data suggest that the MAVS-C79F variant, which is found in 30% of the healthy sub-Saharan population, could be a natural adaptation and a protective measure against environmentally induced oxidative stress by ultraviolet exposure, without affecting the antiviral function of MAVS.

In our model, ROS activates MAVS in a RIG-I helicase-independent manner. However, data suggest that the helicase activities of both MAVS and RIG-I play prominent roles in SLE pathogenesis, because an MDA5 gain-of-function mutant is associated with an increased type I IFN signature in several autoimmune diseases (24, 58, 59), and MAVS activation by endogenous retroviruses promotes T cell-independent B cell responses (60). It is feasible that ROS production results in the formation of an endogenous RNA ligand that stimulates the helicase activity of RIG-I, which then acts through RIG-I or MDA5 to activate MAVS. We showed that MAVS oligomerization at the early stages of exposure to oxidative stress was independent of such a scenario, because MAVS oligomerization occurred in ROS-exposed MEFs that were RIG-I-deficient and in which MDA5 was knocked down. However, it is feasible that MAVS oligomerization could, for example, lead to the release of mitochondrial RNA, which is not capped at the 5' ends, and could stimulate RIG-I helicases in a feedback loop.

Cell type distribution can be altered in the PBMCs of SLE patients (61), and comparing MAVS oligomerization in whole PBMCs from SLE patients and control donors can be biased if there are cell-specific differences. Our data are consistent with previous studies that suggest that T cells contain the greatest amounts of spontaneously oligomerized MAVS in SLE patients. Another study suggested that activation of the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)-stimulator of IFN genes (STING) pathway in neutrophils in SLE stimulates type I IFN production and NETosis, a distinct form of cell death (62). Dying cells are a likely source of plasma MAVS, given that SLE patients have an increased proportion of dying cells in their PBMCs. However, whether MAVS oligomerization occurs spontaneously in neutrophils needs to be determined. Our data suggest that MAVS is released into the plasma of SLE patients and then can be taken up by other cells, such as DCs, where they act in a prion-like manner to further amplify MAVS signaling and induce the production of proinflammatory cytokines. It will be interesting to determine how these aggregates can be eliminated by the cell and what is the stoichiometry and spatial organization of exogenous and endogenous MAVS complexes.

Together, our findings suggest that MAVS oligomerization leads to a mitochondrial metabolic phenotype that could resemble the phenotype found in the T lymphocytes of SLE patients, which manifest spontaneous mitochondrial hyperpolarization, increased ROS production, and diminished concentrations of intracellular ATP (21). The increased and sustained type I IFN signature of SLE patients may be only one determinant of overall disease activity; however, it is possible that MAVS oligomerization may contribute to the type I IFN signature in SLE. The detection of MAVS oligomers in the plasma of SLE patients suggests that this might be a useful diagnostic test for MAVS activation, which could identify subpopulations of SLE patients with type I IFN and dysfunctional mitochondria, which may be more responsive to antioxidant therapies.

MATERIALS AND METHODS

Cell culture, mice, and human subjects

MAVS-deficient and matching WT MEFs, a gift of Z. J. Chen (Howard Hughes Medical Institute, Department of Molecular Biology, University of Texas Southwestern Medical

Center) (2), were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone), supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone), 100 mM L-glutamine (Cellgro Mediatech), penicillin (10,000 U/ml), and streptomycin sulfate (10 mg/ml; Life Technologies). C57BL6 or B6;129-Mavstm1Zjc/J mice (The Jackson Laboratory) were maintained at the University of Vermont under a protocol approved by the Institutional Animal Care and Use Committee. Murine BMDCs were prepared as described by Inaba *et al.* (53). Briefly, cells derived from femur bone marrow were cultured at a density of 5×10^5 /ml in 10-cm plates in RPMI 1640 (HyClone), supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (10,000 U/ml), streptomycin sulfate (10 mg/ml), 5 μ M 2-mercaptoethanol (Sigma), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 μ g/ml; Life Technologies). Human subjects were enrolled under a protocol approved by the University of Vermont Institutional Review Board. Samples from 17 SLE patients fulfilling the American College of Rheumatology diagnostic criteria were studied (63). Of the 17 patients, 16 were female, and the age of study participants was 47 ± 3 years (Table 1). Disease activity was assessed by the SLE disease activity index (SLEDAI) scores, ranging from 0 to 10 (64). Upon informed consent, primary human PBMCs were obtained from heparinized whole-blood samples from the SLE patients. Control PBMCs were generated from age-, sex-, and ethnicity-matched healthy subjects. PBMCs were isolated by standard Ficoll gradient centrifugation as previously described (65). Cells were either used immediately to prepare lysates for Western blotting or cultured at a density of 5×10^5 /ml in a 12-well culture plate with RPMI 1640 (HyClone), supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin (10,000 U/ml)/ streptomycin sulfate (10 mg/ml). Enriched monocytes from PBMCs were used to prepare hDCs by negative magnetic bead selection (Miltenyi) and were subsequently cultivated for 6 days in RPMI 1640 culture medium containing GM-CSF (10 μ g/ml) and IL-4 (500 U/ml). All cells were maintained in a humidified chamber at 37°C and 5% CO₂.

Virus stocks

The CVB3 H3 strain was derived from an infectious complementary DNA (cDNA) clone as previously described (66). SINV strain S.A.A. R.86, a gift from R. E. Johnston (Department of Microbiology and Immunology, University of North Carolina at Chapel Hill), was derived from a genomic cDNA clone (67). Viral titers were determined by plaque assay (68). Infected MEFs were prepared by inoculating cells with CVB3 or SINV in DMEM at an MOI of 1. Cells remained in contact with the virus for 45 min to enable absorption and then were washed with phosphate-buffered saline, resuspended in fresh medium, and incubated for the times indicated in the figure legends before assays were performed.

Reagents

Poly(I:C) and 5'-ppp-dsRNA, both complexed with LyoVec, were purchased from InvivoGen. The mitochondria-targeted antioxidant MitoQ and the mitochondria-targeted superoxide generator MitoPQ were gifts of M. P. Murphy (Medical Research Council Mitochondrial Biology Unit, Wellcome Trust, Cambridge, U.K.) (44). The control reagent TPP was purchased from Sigma. Reagents were added to cells at the final concentrations indicated in the figure legends. GOx was purchased from Sigma and added to cultured cells

at a final concentration of 0.1 U/ml. IAA was from Thermo Fisher and was used at a final concentration of 100 μ M.

Flow cytometry

ROS generation was measured with oxidized, green fluorescent DHC acetoxymethylester (DHC-AM) (Life Technologies) and flow cytometry. Single-cell suspensions of MEFs were prepared, and ROS was assessed by staining the cells with 5 μ M DHC-AM in culture medium for 30 min (69). The cationic, cell-permeant dye TMRE (25 nM; Life Technologies) (70) was used to measure ψ_m . Additionally, MitoSOX Red and MitoTracker Deep Red (Life Technologies) were used to measure mROS and mitochondrial mass, respectively. Flow cytometric analysis was performed with an LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Metabolism assay

Real-time analysis of OCR and ECAR was performed with an XF24 extracellular flux analyzer (Seahorse Bioscience). Metabolic profiles were measured under basal conditions in nonbuffered DMEM (Sigma) containing 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate, in response to 1 μ M oligomycin, 0.5 μ M FCCP, and 1 μ M rotenone/ 1 μ M antimycin A (71). Total ATP was measured with the ATPlite Kit (PerkinElmer), and the ATP/ADP ratio was determined with the bioluminescence ADP/ATP Ratio Assay Kit from Sigma.

Confocal microscopy imaging

WT and MAVS-deficient MEFs were transfected by the Neon Transfection System with roGFP-Mito2 (Addgene plasmid #49437). Mito-roGFP is a form of GFP that targets the mitochondrial matrix and displays rapid changes in fluorescence in response to changes in ambient redox potential (33). After transfection, the cells were seeded on poly-L-lysine-coated coverslips (Becton Dickinson) and cultivated in DMEM overnight to enable cell recovery and expression of roGFP, before being infected with CVB3 or SINV. Mitochondrial localization and morphology were defined by staining cells with 100 nM MitoTracker Deep Red (Life Technologies) for 15 min before fixing the cells with 2% formaldehyde. Coverslips were embedded with Aqua-Mount Slide Mounting Media (Polysciences) and analyzed with a Zeiss LSM 510 Meta confocal laser scanning microscopy system (63 $\times$ numerical aperture, 1.4 objective). Images were processed with Volocity 3D Image Analysis Software (PerkinElmer).

Measurement of type I IFN and proinflammatory cytokines

ELISA was used to measure cytokine concentrations in the culture medium of MEFs and BMDCs and in the sera of SLE patients and healthy control subjects. For assays, samples were centrifuged, and IFN- β and IL-6 were measured in triplicate with murine or human ELISA kits (PBL Biosciences) according to the manufacturer's instructions. The fold changes in the abundances of type I IFN and IL-6 were calculated by directly comparing untreated cells with those infected with virus or treated with a chemical inducer of ROS.

Quantitative real-time PCR analysis

Total RNA from MEFs was isolated with the RNeasy kit (Qiagen) according to the manufacturer's instructions and was reverse-transcribed with iScript Reserve Transcription Supermix (Bio-Rad). *Ifnb* and *Ilf6* mRNA was detected with iQ SYBR Green Supermix (Bio-Rad) and prevalidated primers as described previously (72). Data were analyzed with the 2^{-C_t} method as described previously (73).

Functional reconstitution of human MAVS in MAVS-deficient MEFs

To reconstitute MAVS-deficient MEFs with His-tagged human WT MAVS or the MAVS-C79F variant, 1×10^6 MAVS-deficient MEFs were transfected with 3 μ g of pIRES plasmids encoding the appropriate constructs with the Neon Transfection System (Life Technologies). Twenty-four hours later, to select for stably transfected cells, the cells were cultured with puromycin (2.5 μ g/ml; Life Technologies) for up to 5 days.

Knockdown of MAVS and MDA5 in MEFs with siRNAs

Where indicated in the figure legends, small interfering RNA (siRNA) was used to knock down MAVS (TRC MAVS shRNA, Thermo Fisher) in MEFs or MDA5 (psiRNA-MDA5, InvivoGen) in RIG-I-KO MEFs. WT MEFs (1×10^6) were transfected with 3 μ g of the appropriate plasmid with the Neon Transfection System. Twenty-four hours after transfection, to select for cells stably expressing either MAVS- or MDA5-specific siRNA, puromycin (2.5 μ g/ml) or zeocin (2.5 μ g/ml, InvivoGen), respectively, was added to the culture medium and the cells were cultured for up to 5 days.

Anti-His-tag immunoprecipitations

MEFs were disrupted in lysis buffer, and anti-His-tag antibody (Life Technologies) was used to immunoprecipitate MAVS. Purification of precipitated MAVS was performed on a MagMAX Express Magnetic Particle Processor (Thermo Fisher), and MAVS and its coimmunoprecipitated proteins were resolved by gel electrophoresis and detected by Western blotting analysis.

Denaturing gel electrophoresis

Cultured MEFs or human PBMCs were lysed in a sample buffer containing 2% SDS, and insoluble cell fragments were removed by centrifugation. Plasma samples derived from SLE patients were collected and stored at -20°C until gel electrophoresis was performed. The BCA protein assay (Thermo Scientific) was used to determine protein concentrations in all samples, which were then resolved by SDS-polyacrylamide gel electrophoresis under denaturing conditions. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a Trans-Blot Turbo Transfer System (Bio-Rad).

Semidenaturing detergent agarose gel electrophoresis

SDD-AGE was performed according to a published protocol with minor modifications (74). Briefly, mitochondria were resuspended in sample buffer [0.5 $\times$ tris-borate EDTA (TBE), 10% glycerol, 2% SDS, and 0.0025% bromophenol blue] and loaded onto a vertical 1.5% agarose gel. After electrophoresis in running buffer (1 $\times$ TBE, 0.1% SDS) for 35 min with a

constant voltage of 75 V at 4°C, proteins were transferred to PVDF membranes with a Trans-Blot Turbo Transfer System in preparation for Western blotting analysis.

Western blotting

PVDF membranes were blocked in tris-buffered saline and 5% nonfat powdered milk. Antibodies were specific for MAVS and actin (Santa Cruz Biotechnology); RIPK1 and TRAF2 (BD Biosciences); TANK, TRADD, RIG-I, and GAPDH (Cell Signaling); MDA5 and COX4 (Abcam); Alx488 (Life Technologies); and IRF3 (Thermo Fisher). Immunoreactive proteins were visualized with horseradish peroxidase-labeled conjugates (Jackson ImmunoResearch) and developed with Clarity Western ECL Substrate (Bio-Rad). Chemiluminescence was detected and recorded with a Bio-Rad Chemidoc instrument. Densitometric measurements were performed in Image Lab image acquisition and analysis software (Bio-Rad).

Purification of MAVS oligomers, mitochondrial isolation, and functional assays

An anti-MAVS antibody was covalently coupled to protein G magnetic beads (Life Technologies) with the bifunctional amine-reactive cross-linker disuccinimidyl suberate (Thermo Scientific). To avoid sample contamination with proteins or protein complexes that non-specifically bind to protein G, plasma samples were preincubated with protein G magnetic beads without the cross-linked anti-MAVS antibody. SLE patient plasma was incubated with anti-MAVS-conjugated protein G magnetic beads for 6 hours, after which the magnetic beads were loaded onto a column in a magnetic field and extensively washed. After the beads were eluted, MAVS oligomers were released from the anti-MAVS antibody by way of increasing ionic strength buffer, lower pH, and gel filtration. Functionally intact mitochondria from cultured cells were isolated as described previously (75). This method incorporates mechanical cell rupture followed by the precipitation of mitochondria from cell lysates with magnetically labeled anti-TOM22 antibody (Miltenyi Biotec). Mitochondria were inoculated with SLE patient plasma or healthy control plasma in a respiration-promoting buffer containing 125 mM KCl, 5.0 mM KH₂PO₄ (pH 7.25), 15 mM α -ketoglutarate, and 2.0 mM ADP (76, 77). The reaction mixtures were centrifuged at 10,000g for 10 min, and the precipitates were then analyzed by SDD-AGE.

Functional assay of endogenous MAVS aggregation and activation in vitro

Before enriching the MAVS oligomers from plasma and labeling them, total plasma was treated with protein G magnetic beads alone and filtered with 100-kDa cutoff membranes to remove nonspecific, low-molecular weight oligomers and cytokines. The MAVS oligomers from plasma were then bound with anti-MAVS-conjugated protein G magnetic beads and coupled to Alx546 or Alx488 while the MAVS oligomers were still bound to the magnetic bead-tethered anti-MAVS antibody to remove any excess dye without having to pass the material through another size-exclusion resin. Purified MAVS oligomers from SLE patient plasma were fluorescently labeled with either Alx488- or Alx546-hydrazine (Life Technologies). Labeled MAVS oligomers were incubated for 1, 2, or 6 hours with BMDCs derived from either WT or MAVS-deficient mice, or with hDCs. Confocal microscopy was used to analyze Alx546 fluorescence and its location. DCs were cultured on poly-L-lysine-coated coverslips and stained with MitoTracker Green (Life Technologies) and Hoechst

33342 before being fixed with 4% formaldehyde (Ted Pella) to define mitochondria and nuclei.

Genotyping of SLE samples

Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Unique genomic primers flanking the nucleotide encoding MAVS Cys⁷⁹ were designed with Primer-Blast (FP-CCCTGGGGGCCATATTAATCC/ RP-CATCAAATCGCCTCCGAGCA) and were used to both amplify and sequence exon2.

Analysis of mitochondrial morphology by transmission electron microscopy

For ultrastructural analysis, cells were fixed for 1 hour at 65°C in 2% paraformaldehyde and 2.5% glutaraldehyde (Polysciences) in 100 mM sodium cacodylate buffer (pH 7.2). Samples were washed in cacodylate buffer and then postfixed for 1 hour in 1% osmium tetroxide (Polysciences). Samples were then extensively rinsed in distilled H₂O before undergoing en bloc staining for 1 hour with 1% aqueous uranyl acetate (Ted Pella). After several rinses in distilled H₂O, the samples were dehydrated in a graded series of ethanol and then embedded in Eponate 12 resin (Ted Pella). Sections (95 nm in thickness) were cut with an Ultracut UC7 ultramicrotome (Leica Microsystems), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1400 transmission electron microscope (JEOL USA) equipped with an XR611 high-resolution, 11-megapixel mid-mount charge-coupled device camera (Advanced Microscopy Techniques).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. MAVS oligomerization induces ROS generation and decreases cellular metabolism (A and B) WT (closed symbols) and MAVS-deficient (KO, open symbols) MEFs were infected with CVB3 or SINV at a multiplicity of infection (MOI) of 1 or were transfected with 1 μ g of 5'-ppp-RNA for the indicated times. (A) The total quantity of ROS was measured by flow cytometric analysis of cells treated with 5 μ M dihydrocalcein (DHC). (B) The mitochondrial transmembrane potential ($\Delta\psi_m$) was monitored with 25 nM tetramethylrhodamine ethylester (TMRE). Data are means $\pm$ SEM of triplicate samples from one experiment and are representative of three independent experiments. a.u., arbitrary units. (C) The kinetics of MAVS cleavage in CVB3-infected MEFs was determined by Western blotting analysis. SINV-infected MEFs and MAVS-KO MEFs served as controls. Data are representative of three independent experiments. (D) MAVS oligomerization in MEFs in response to infection with CVB3 or SINV or transfection with 5'-ppp-RNA was detected at the indicated times by nonreducing gels and Western blotting analysis. Data are representative of three independent experiments. (E) To define the cellular source of ROS, Mito-roGFP-expressing MAVS-WT and MAVS-KO MEFs were analyzed by confocal microscopy 6 hours after infection with CVB3. MitoTracker Deep Red (1 μ M) was used as a counterstain to define mitochondria. Images are representative of three independent

experiments. **(F and G)** MAVS-WT and MAVS-KO MEFs were infected with CVB3 or transfected with 5'-ppp-RNA and then cultured for the indicated times. **(F)** The total ATP content of the cells was measured with a luminescence-based assay. ATP amounts in uninfected MAVS-WT and MAVS-KO cells are shown as controls. **(G)** The ATP/ADP ratio in the same cells was measured 24 hours after treatment. Total ATP content and ATP/ADP ratios were determined in two independent experiments. **(H)** Representative scheme, adapted from Seahorse Bioscience, of OCR measurement with a Seahorse XF24 analyzer. Measurement of OCR in response to injection of the ATP synthase inhibitor oligomycin, the uncoupler FCCP, and the complex I and III inhibitors rotenone and antimycin A enabled calculation of the key metabolic parameters of basal mitochondrial respiration, maximal respiration, and SRC, as indicated. **(I to N)** OCR measurements of MAVS-WT MEFs (**I and J**, closed symbols) and MAVS-KO MEFs (**K and L**, open symbols) as a function of infection with CVB3 or transfection with 5'-ppp-RNA. Measurements were made 3 hours (**I and K**) or 24 hours (**J and L**) after treatment. Data are means $\pm$ SEM of three independent experiments. **(M and N)** Measurement of basal respiration, maximal respiration, and SRC in MAVS-WT (closed bars) and MAVS-KO (open bars) MEFs. h.p.i., hours post-inoculation. **(O and P)** OCR measurements from splenocytes of MAVS-WT (closed symbols) and MAVS-KO (open symbols) mice. Statistical analyses performed were one-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test to examine pairwise differences, whereas two-way ANOVA, or repeated-measures ANOVA followed by Tukey's multiple comparisons test, was used to examine specific comparisons, as appropriate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, and **** $P < 0.00005$.

Fig. 2. Virus-independent oxidative stress induces MAVS oligomerization and the secretion of type I IFN

(A) MAVS oligomerization in MAVS-WT and MAVS-KO MEFs after treatment with GOx (1 U/ml) or infection with SINV for 6 hours was detected by semidenaturing detergent agarose gel electrophoresis (SDD-AGE) followed by Western blotting. Data are representative of three independent experiments. (B) WT MEF cells were treated for 3 and 6 hours with GOx (1 U/ml) before being subjected to immunoprecipitation (IP) with an anti-MAVS antibody. Samples were then analyzed by Western blotting with antibodies against the indicated proteins. Data are representative of three independent experiments. (C) MAVS-WT (black bars) and MAVS-KO (white bars) MEFs were treated with GOx (1 U/ml) or were infected with SINV before ψ_m was analyzed by flow cytometry with TMRE. The ionophore FCCP (10 nM) served as a positive control in MAVS-WT and MAVS-KO cells (bars overlap). Data are representative of at least three experiments. (D and E) MAVS-WT (black bars) and MAVS-KO (white bars) MEFs were treated with GOx (1 U/ml) for 3, 6, and 12 hours before the amounts of IFN- β (D) and IL-6 (E) secreted into the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). Data are presented as fold changes in cytokine abundance rather than as absolute concentrations to enable comparability. The fold change was calculated by comparing the amounts of cytokines

secreted by treated and untreated cells. Data are means $\pm$ SEM of three independent experiments. (F) WT MEFs were left untreated or were treated with the indicated concentrations of MitoPQ (to induce mROS generation). The formation of MAVS oligomers was determined by Western blotting analysis. Data are representative of three independent experiments. (G) MAVS-WT (black bars) and MAVS-KO (white bars) MEFs were treated with the indicated concentrations of MitoPQ for 12 hours, and the extent of cell death was determined by flow cytometric analysis with live-dead stain. Data are means $\pm$ SEM of three independent experiments. (H and I) MAVS-WT (black bars) and MAVS-KO (white bars) MEFs were left untreated or were treated with the indicated concentrations of MitoPQ for 12 hours. The concentrations of type I IFN (H) and IL-6 (I) secreted by the cells were measured by ELISA. Data are means $\pm$ SEM of three experiments. (J) RIG-I-KO MEFs and RIG-I-KO MEFs in which MDA5 was knocked down (KD) were incubated in the absence or presence of GOx for 12 hours. The extent of MAVS oligomerization was determined by Western blotting analysis. Data are representative of three independent experiments. (K and L) The indicated MEFs were treated with GOx, MitoPQ, poly(I:C), or 5'-ppp-RNA for 12 hours. The relative amounts of *Irfb* mRNA were measured by quantitative polymerase chain reaction (PCR) analysis (K), whereas the amounts of IFN- β secreted by the cells were determined by ELISA. Data are means $\pm$ SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Sidak's multiple comparison test to examine pairwise differences or by repeated-measures ANOVA followed by Tukey's multiple comparisons test to examine specific comparisons. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, and **** $P < 0.00005$; ns, not significant.

Fig. 3. The mitochondria-targeted antioxidant MitoQ prevents the formation of MAVS oligomers (A) MAVS-WT MEFs were pretreated with 100 μ M MitoQ before being treated with GOx or infected with SINV. Twelve hours later, semidenaturing gel electrophoresis and Western blotting were used to detect MAVS monomers and oligomers. As a negative control, cells were treated with 100 μ M dTPP, which lacks the antioxidant quinol residue. Data are representative of three independent experiments. (B) The ratio of MAVS oligomers to monomers was determined by densitometric measurement of Western blots. Data are means $\pm$ SEM of three independent experiments. (C) WT MEFs that were pretreated in the absence or presence of MitoQ were then left untreated, treated with GOx, or infected with SINV.

Twelve hours later, ψ_m in the cells was determined by flow cytometric analysis of TMRE fluorescence. Data are means $\pm$ SEM of three independent experiments. (D) WT MEFs that were pretreated with or without MitoQ were then left untreated, treated with GOx, or infected with SINV. Twelve hours later, the amounts of IFN- β secreted by the cells were determined by ELISA. Data are means $\pm$ SEM of three independent experiments. Data in (B) and (D) were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test to examine specific comparisons. Data in (C) were analyzed by one-way ANOVA followed by Sidak's multiple comparison test to examine pairwise differences. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, and **** $P < 0.00005$.

Fig. 4. Virus-independent oxidative stress induces MAVS oligomerization in human PBMCs, whereas SLE patients manifest spontaneous MAVS oligomerization

(A) Freshly isolated human PBMCs were treated with GOx for the indicated times, and SDD-AGE was used to detect MAVS oligomerization. Cells and cell lysates were pretreated with MitoQ or β -Me, as indicated. Data are representative of three independent experiments. (B and C) Whole-cell lysates of lymphocytes (B) and plasma (C) of SLE patients ($n = 8$ patients: P1 to P8) were analyzed by SDD-AGE to detect MAVS oligomers. Sex-, age-, and ethnicity-matched subjects served as healthy controls ($n = 8$ donors: C1 to C8). (D) MAVS

oligomerization in whole-cell lysates (WCL) and plasma of SLE patients (black circles) and healthy control subjects (white circles) was quantified by densitometric measurement of Western blots. For whole-cell lysates, the ratio of MAVS monomer to oligomer was measured, whereas in plasma, MAVS oligomers were normalized to albumin abundance. Each point represents a single individual, for which MAVS oligomerization was determined on at least three independent agarose gels. **(E)** The amounts of type I IFN in plasma from control subjects (C) and in the plasma of SLE patients (P) were measured by a commercially available ELISA. Each point is an average of three independent measurements. **(F)** The plasma concentrations of type I IFN and the degree of MAVS oligomerization in SLE patients were compared. Each point represents Pearson's correlation between MAVS oligomerization and type I IFN secretion of SLE patients, as determined in (D) and (E). **(G to J)** OCR determinations of basal respiration (G), maximal respiration (H), SRC (I), and ATP synthesis (J) in PBMCs from SLE patients and healthy controls. Each parameter was determined by measuring the same patient's or healthy control's sample in at least quadruplets. **(K)** Unfractionated, whole PBMCs (W), B cells (B), T cells (T), and monocytes (M) from SLE patients and healthy controls were analyzed by SDD-AGE to detect MAVS oligomerization. Data are from three patients and three healthy control donors. **(L to N)** OCR measurements were made in whole PBMCs (L), B cells (M), and T cells (N) isolated from SLE patients (black bars) or healthy controls (white bars). Data are from three patients and three healthy control donors. The statistical analyses performed were independent *t* tests (D, E, and G to J), two-way ANOVA (L to N), and Pearson's correlation coefficient (F). **P* < 0.05, ***P* < 0.005, ****P* < 0.0005, and *****P* < 0.00005.

Fig. 5. The SLE-associated MAVS-C79F variant exhibits reduced oligomerization
(A) MAVS-WT MEFs or MAVS-KO MEFs expressing human MAVS-WT, the MAVS-C79F variant, or both were infected with SINV or treated with GOx. MAVS aggregation was then determined by 5% semidenaturing TGX gels in sample buffer in the presence or absence of 0.1% β -Me to enable detection of oligomers and intermediate lower-molecular weight MAVS oligomers after Western blotting analysis. Data are representative of at least three independent experiments. (B to E) Quantitation of MAVS oligomers (B), type I IFN production (C), ROS production (D), and mitochondrial membrane potential (E) from the

same samples as those in (A). Data are means $\pm$ SEM of three independent experiments. (F and G) Semidenaturing, 1.5% agarose gel analysis (F) and densitometry (G) of MAVS oligomers from the plasma of healthy controls, SLE patients expressing the MAVS-C79F variant, and SLE patients expressing WT MAVS. The amounts of high-molecular weight oligomers (>1000 kDa) normalized to the amount of detectable albumin in the plasma were analyzed in four independent experiments. The statistical analyses performed were two-way ANOVA (C to E), including interaction terms, followed by Tukey's multiple comparisons test to examine specific comparisons or one-way ANOVA followed by Sidak's multiple comparison test to examine pairwise differences (G). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, and **** $P < 0.00005$.

Fig. 6. MAVS oligomers from SLE patients manifest prion-like activity

(A) Experimental design for the purification of MAVS oligomers from the plasma of SLE patients and for the de novo reconstitution of SLE-MAVS oligomers with purified mitochondria from MAVS-WT MEFs, MAVS-KO MEFs, and MAVS-KO MEFs reconstituted with WT MAVS, the MAVS-C79F variant, or both. The anti-MAVS antibody was covalently linked by disuccinimidyl suberate (DSS) to protein G magnetic beads to precipitate MAVS oligomers. Similarly, mitochondria from MAVS-WT, MAVS-KO, and MAVS-C79F MEFs were isolated with Tom22 antibody-conjugated magnetic beads. (B) The size and purity of MAVS oligomers precipitated from SLE patient plasma (samples P1 to P4) were assessed by gel electrophoresis. Plasma from a healthy individual (C1) served as a control. Data are representative of three independent MAVS purification experiments. (C) Mitochondria from MAVS-WT and MAVS-KO MEFs were left untreated or treated with GOx or MitoQ and then were incubated with patient-derived MAVS oligomers. Mitochondrial lysates were then analyzed by Western blotting with anti-MAVS antibody.

Data are representative of three experiments. **(D)** Mitochondria purified from MEFs expressing WT MAVS, the MAVS-C79F variant, or both were incubated with purified MAVS from an SLE patient (P2), and samples were treated and analyzed as described in (C). Data are representative of three experiments.

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Fig. 7. SLE-derived MAVS oligomers are internalized by DCs and induce mitochondrial MAVS aggregation

(A) SLE patient plasma-derived MAVS oligomers were labeled with Alx546 or Alx488 fluorophores. Gel electrophoresis was performed to determine labeling efficiency by direct excitation of the in-gel fluorescence of control (C1 to C4) and SLE patient (P1 to P4) samples. The level of fluorescent labeling for C1 was defined as 1 in a first measurement of three independent labeling analyses. The respective number represents an average of the three measurements. The fluorescence of the remaining samples was compared to sample

C1. **(B)** Murine BMDCs or hDCs were incubated with either SLE patient–derived fluorescent MAVS oligomers (P3) or plasma from a healthy control subject (C1). Samples were then analyzed by confocal microscopy to analyze the localization of the internalized Alx546-labeled MAVS oligomers. Cells were treated with 50 nM MitoTracker Green and Hoechst 33342 (10 µg/ml) to define mitochondria and nuclei, respectively. Images are representative of two experiments. **(C)** The kinetics of MAVS internalization by the indicated cells were calculated by measuring the increase in intracellular Alx546 fluorescence intensity over time as a ratio (P3/C1) after exposure to labeled MAVS from either an SLE patient (P3) or a healthy control (C1). Data are means $\pm$ SEM of two experiments. **(D)** Correlation coefficients for mitochondria (MitoTracker Green) and Alx546-labeled MAVS oligomers were calculated to determine the colocalization of mitochondria and MAVS oligomers. For data collection, 200 individual cells were examined. **(E)** BMDCs from WT and MAVS-KO mice were incubated with MAVS isolated from the indicated SLE patients (P) and healthy controls (C) for 12 hours, and the amounts of type I IFN secreted by the cells were measured by ELISA. Data are means $\pm$ SEM of three independent experiments. **(F)** BMDCs from WT (top) and MAVS-KO (bottom) mice were exposed to fluorescently labeled MAVS isolated from SLE patient plasma. Mitochondrial fractions from the cells were analyzed by gel electrophoresis and subsequent Western blotting to detect MAVS oligomerization after treatment of the samples with β -Me. The presence of the patient-derived MAVS oligomers at the mitochondria in BMDC was confirmed by the detection of the fluorophore using an anti-Alx488 antibody or direct in-gel fluorescence (middle panel). Data are representative of three experiments. **(G)** As negative controls, BMDCs from WT mice were treated with increasing concentrations of free Alx488 dye. The mitochondrial fraction of the cells was analyzed as described in (F) to determine the amount of MAVS at mitochondria; Alx488 was detected with an anti-Alx488 antibody. Data are representative of three experiments. **(H)** The mitochondrial fraction of BMDCs from WT mice were incubated with MAVS oligomers derived from the plasma of the indicated SLE patients (P2 to P4) and healthy control (C1). Mitochondrial fractions of the cells were analyzed by Western blotting to detect the recruitment of the indicated signaling molecules downstream of MAVS activation. Western blots are representative of three independent experiments. The statistical analyses performed were two-way ANOVA (C to E) followed by Tukey's multiple comparisons test to examine specific comparisons. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.

Table 1

Genotype and activity of SLE patients.

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
C79F								nd	nd	—	—	nd	nd	—	—	—	—
Q93E								nd	nd	—	—	nd	nd	—	—	—	—
MAVS	+	+	+++	++++	+	+	+++	++	+++	++	+++	++	—	+	—	—	++
Type I IFN	+	+	++++	++++	+	+	+++	+++	++++	++++	+++	++++	+	+	—	++	++
SLEDAI	4	6	10	4	0	0	4	7	12	9	6	8	6	4	4	0	0

The extent of MAVS oligomerization and the level of type I IFN level in plasma were normalized among all 17 patients, with the highest patient value set as 1. These values were scored as +, ++, +++, and ++++ to signify that the absolute mean value after normalization was in the range of 0 to 0.25, 0.25 to 0.5, 0.5 to 0.75, and 0.75 to 1.00, respectively, nd, not determined.