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## Cytosolic Double-Stranded RNA Activates the NLRP3 inflammasome via MAVS-Induced Membrane Permeabilization and K<sup>+</sup> Efflux

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### Abstract

The Nlrp3 inflammasome plays an important role in inflammation by controlling the maturation and secretion of the cytokines IL-1 $\beta$  and IL-18 in response to multiple stimuli including pore-forming toxins, particulate matter, and ATP. Although the pathways activated by the latter stimuli lead to a decrease in intracellular K<sup>+</sup> concentration, which is required for inflammasome activation, the mechanism by which microbial RNA activates Nlrp3, remains poorly understood. Here we found that cytosolic poly(I:C), but not total RNA from healthy macrophages, macrophages undergoing pyroptosis, or mitochondrial RNA, induces caspase-1 activation and IL-1 $\beta$  release through the Nlrp3 inflammasome. Experiments with macrophages deficient in Tlr3, Myd88 or Trif, indicate that poly(I:C) induces Nlrp3 activation independently of TLR signaling. Further analyses revealed that the cytosolic sensors Rig-I and Mda5 act redundantly via the common adaptor Mav to induce Nlrp3 activation in response to poly(I:C), but not ATP or Nigericin. Mechanistically, Mav triggered membrane permeabilization and K<sup>+</sup> efflux independently of the

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inflammasome which were required for poly(I:C)-induced Nlrp3 activation. We conclude that poly(I:C) activates the inflammasome through an Mavs-dependent surveillance pathway that converges into a common  $K^+$  lowering step in the cytosol that is essential for the induction of Nlrp3 activation.

## Keywords

Nlrp3; inflammasome; IL-1, dsRNA; Mavs; innate immunity

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## Introduction

A key step in the induction of inflammatory responses during infection is the activation of pattern-recognition receptors (PRRs) expressed on innate immune cells. PRRs sense conserved molecules produced by microbes and/or endogenous changes induced during cellular injury (1, 2). Upon activation, PRRs trigger multiple signaling pathways that promote pathogen clearance and tissue repair (3). A major signaling pathway induced in response to infection is the inflammasome, a multi-protein complex that activates caspase-1 (4, 5). Once activated, caspase-1 proteolytically cleaves pro-interleukin-1 $\beta$  and pro-IL-18 into their biologically active forms (6). To date, four bona fide inflammasomes have been described of which three, the Nlrp1, Nlrp3 and Nlrc4 inflammasomes, are activated by members of the intracellular Nod-like receptor (NLR) family (5). The Nlrp3 inflammasome has received considerable attention due to its link to the pathogenesis of autoinflammatory syndromes and a variety of inflammatory diseases (7-9). Production of IL-1 $\beta$  through Nlrp3 is tightly regulated via a two-step process. The first step, referred as priming, involves the transcriptional induction of pro-IL-1 $\beta$  and Nlrp3 by Toll-like receptor (TLR) agonists or certain cytokines such as TNF- $\alpha$  or IL-1 $\beta$  (10, 11). Both reactive oxygen species (ROS) and NF- $\kappa$ B activation have been shown to regulate the priming step (10-12). The second step involves the activation of Nlrp3 to assemble an active inflammasome complex that can process pro-IL-1 $\beta$  into mature IL-1 $\beta$ . Multiple stimuli including extracellular ATP, bacterial pore-forming toxins, particulate matter and microbial RNA have been reported to activate Nlrp3 independently of the priming step (5, 13, 14).

The mechanism that activates Nlrp3 has been the subject of intense investigation. Several events have been linked to the activation of Nlrp3 including the production of ROS (15), mitochondrial damage (16), lysosomal damage (17), cell swelling (18) and cytosolic  $K^+$  efflux (19, 20). In addition, several molecules released upon mitochondrial and/or ROS-induced damage have been associated with Nlrp3 activation (21, 22). Understanding the steps required for Nlrp3 activation has been difficult because experiments often relied on chemical inhibitors or the use of activating stimuli such as ATP that trigger multiple cellular events some of which are induced in parallel or downstream of Nlrp3 activation (5, 13, 14, 20). Nonetheless,  $K^+$  efflux has been mechanistically linked to Nlrp3 activation induced by ATP, pore-forming toxin and particulate matter by multiple studies (23-25). Furthermore, recent studies showed that depletion of cytosolic  $K^+$  is sufficient to trigger the activation of Nlrp3 (24). However, the mechanism by which microbial RNA triggers activation of Nlrp3 remains unclear. Microbial RNAs activate PRRs including Tlr3 and the RIG-like receptors

(RLRs) RIG-I/Ddx58 and Mda5/Ifih1 (melanoma differentiation-associated gene 5). RIG-I and Mda5 recognize double-stranded RNA (dsRNA) generated during viral replication and polyinosinic polycytidylic acid (poly I:C), a synthetic analog of dsRNA (26, 27) and induce type I IFNs and NF- $\kappa$ B via Mavs (mitochondrial antiviral signaling protein) (28). In addition Mavs and Trif/Ticam have been linked to Nlrp3 activation in triggered by poly I:C (29), viruses (29-32) and bacteria (33), but it remains unclear whether Mavs and Trif act by priming the inflammasome (10, 11), or by regulating Nlrp3 localization (34) or activation (32). Recent studies have linked microbial RNA to Nlrp3 inflammasome activation in human macrophages through the binding of dsRNA to the DHX33 RNA helicase (35). Furthermore, stimulation of human macrophages with poly(I:C) induced the interaction of DHX33 with Nlrp3 and ASC, although it remains unclear whether such interactions are sufficient to activate the Nlrp3 inflammasome (35). Collectively, these studies suggested that dsRNA activates Nlrp3 by a mechanism that is distinct to that induced by ATP, pore-forming toxins and particulate matter. In the present studies, we have analyzed further the mechanism by which dsRNA activates the Nlrp3 inflammasome and provide evidence that cytosolic dsRNA induces membrane permeabilization via Mavs independently of inflammasome activation which in turn leads to K<sup>+</sup> efflux and Nlrp3 activation.

## Material and Methods

### Mice

*Nlrp3*<sup>-/-</sup>, *Asc*<sup>-/-</sup>, *casp1/11*<sup>-/-</sup> mice have been described (36, 37). *P2rx7*<sup>-/-</sup> mice were obtained from G. Dubyak, Case Western Reserve University (Cleveland, OH). *Tlr3*<sup>-/-</sup>, *Myd88/Ticam*<sup>-/-</sup> and *Mavs*<sup>-/-</sup>/*Ticam*<sup>-/-</sup>, *RigI/Ddx58*<sup>-/-</sup>, *Mda5/Ifih1*<sup>-/-</sup> and *RigI*<sup>-/-</sup>/*Mda5*<sup>-/-</sup> mice have been described (38-41). *Pkr*<sup>-/-</sup> mice from R. Kaufman, University of Michigan (Ann Arbor, MI). All mice were backcrossed onto the C57BL/6 background at least eight times. Wild-type (WT) C57BL/6 mice were maintained in our animal facility. All animal studies were approved by the University of Michigan Committee on Use and Care of Animals.

### Reagents

ATP was from Sigma-Aldrich. Recombinant mouse TNF- $\alpha$  was obtained from Peprotech. Ultrapure LPS from *Escherichia coli*, Pam3CSK, flagellin, high and low molecular weight polyinosinic:polycytidylic acid (hereafter referred to as poly(I:C)), CpG, double-stranded B DNA poly(dA-dT)•poly(dT-dA) (poly(dA:dT)) and nigericin were from InvivoGen. Total *Escherichia coli* RNA and mouse heart total RNA were purchased from Ambion. Lipofectamine 2000 was obtained from Invitrogen. The ROS inhibitor *N*-acetyl-L-cysteine (NAC) was purchased from Sigma. Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) and Necrostatin were from Enzo Life Sciences.

### RNA isolation

Liver mitochondria were purified as previously described (42). For isolation of total RNA from pyroptotic cells, BMDM were stimulated with Pam3CSK (10  $\mu$ g/ml) for 1 h followed by incubation with ATP (5mM) for 30 min. RNA was isolated using a DNA/RNA isolation

kit from Qiagen. To obtain RNA, total DNA/RNA was digested with RNase-Free DNase Set (Qiagen).

### Cell culture and stimulation

BMDM were isolated as previously described (43). Cells were stimulated in IMDM supplemented with 10% FCS at a density of  $1 \times 10^6$  per ml. Unless otherwise specified, BMDM were transfected with high molecular weight poly(I:C), bacterial RNA or mitochondrial RNA complexed with Lipofectamine 2000 or transfected with poly(dA:dT) complexed with Lyovec for 6-8 h at a concentration of 10  $\mu\text{g/ml}$ . Where indicated, BMDM were prestimulated with LPS (100 ng/ml) for 1 h or with TNF (100 ng/ml) for 6 h and washed before restimulation with the indicated ligands. As a positive control for caspase-1 activation, WT macrophages were incubated with 100 ng/ml LPS for 3 h and stimulated for 30 min either with 5 mM ATP or with nigericin (10  $\mu\text{g/ml}$ ). For inhibitor experiments, LPS-primed BMDM were preincubated with N-Acetyl-Cystein (NAC; 30 mM), pan-caspase inhibitor Z-VAD (40  $\mu\text{M}$ ) or necrostatin (20  $\mu\text{M}$ ) for 1 h prior to stimulation with poly(I:C) or ATP.

### Immunoblotting and cytokine measurements

For Western Blot analysis, cells were lysed by adding NP-40 to the media to a final concentration of 1 % and protease inhibitors. Cleared lysates were separated by 12% SDS-PAGE, transferred to PVDF membranes and probed with a rabbit anti-caspase-1 antibody generated in our laboratory. Concentrations of IL-1 $\beta$  (R&D Systems) and IL-18 (MBL International) were measured by ELISA in cell-free culture supernatant.

### Membrane permeabilization

Membrane permeabilization was assessed by measuring the release of cytosolic lactate dehydrogenase (LDH) into cell culture supernatants using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit from Promega according to the manufacturer's instructions.

### Determination of intracellular K<sup>+</sup> content

Intracellular K<sup>+</sup> measurements were performed by inductively-coupled plasma optical emission spectrometry (ICP-OES) with a Perkin-Elmer Optima 2000 DV spectrometer using yttrium as internal standard. The culture media were thoroughly aspirated and cells were extracted 30 min in 3% ultrapure HNO<sub>3</sub>. K<sup>+</sup> determinations were done in 48-well plates. For accurate measurement of the intracellular K<sup>+</sup>, a control was performed in every experiment to determine the extracellular amount of K<sup>+</sup> after aspiration and this value was subtracted from every measurement. In experiments using high and low extracellular K<sup>+</sup>, osmolarities were measured by freezing-point depression osmometry using a  $\mu\text{Osmette}$  (Precision systems). In experiments using high and low extracellular K<sup>+</sup>, media osmolarities were adjusted to 300 mOsm after adding the respective salts using a  $\mu\text{Osmette}$  freezing-point depression osmometer (Precision systems).

## Statistical analysis

Statistical significance between groups was determined by two-tailed Student's *t*-test. Differences were considered significant when  $p < 0.05$ .

## RESULTS

### Microbial RNA, but not mammalian RNA or RNA species generated during pyroptosis, induces the activation of the Nlrp3 inflammasome

Consistent with previous studies (36, 44, 45), we found that transfection of bone marrow-derived macrophages (BMDM) with poly(I:C), a synthetic dsRNA analog mimicking viral RNA, but not that of total RNA prepared from healthy macrophages induced the release of IL-1 $\beta$  in WT, but not in Nlrp3-deficient, macrophages (Supplemental Fig. 1A). We next tested whether RNA from macrophages stimulated with ATP to induce Nlrp3 activation could elicit inflammasome activation. We found that transfection of total RNA from macrophages stimulated with ATP to undergo Nlrp3 activation, or mitochondrial RNA did not induce IL-1 $\beta$  release in LPS-primed macrophages (Supplemental Fig. 1A). As a control, a preparation enriched in mitochondrial DNA, induced IL-1 $\beta$  release independently of NLRP3 (Supplemental Fig. 1A). Consistently, caspase-1 activation induced by bacterial RNA and poly(I:C), but not pyroptotic RNA, was abolished in LPS-primed macrophages deficient in Nlrp3 or Asc (Supplemental Fig. 1B). In contrast, stimulation with flagellin, an activator of the Nlr4 inflammasome (46, 47), or poly(dA:dT), an activator of the Aim2 inflammasome (48), induced comparable caspase-1 activation in WT and Nlrp3-deficient macrophages (Supplemental Fig. 1B). As expected, both activation of caspase-1 by cytosolic poly(dA:dT) and flagellin required the common adaptor Asc (Supplemental Fig. 1B). These results indicate that microbial RNA, but not mammalian RNA or RNA species generated during pyroptosis, induces the activation of the Nlrp3 inflammasome.

### Cytosolic poly(I:C) activates the Nlrp3 inflammasome independently of TLR signaling

Poly(I:C) activates both Tlr3-Trif and cytosolic Rig-I/Mda5 signaling pathways. We first addressed whether TLRs are required for the activation of the Nlrp3 inflammasome induced by poly(I:C). To this end, we compared the activation of caspase-1 in WT BMDM or macrophages deficient in Myd88 and Trif, the two adaptors required for TLR signaling. Priming of macrophages with extracellular poly(I:C) promoted the activation of caspase-1 by ATP in WT, but not in macrophages deficient in Myd88/Trif (Fig. 1A), which is consistent with the fact that extracellular poly(I:C) signals via Tlr3. In contrast, transfection of macrophages with poly(I:C) conjugated with lipofectamine to mediate cytosolic stimulation induced comparable activation of caspase-1 in WT and Myd88/Trif-deficient cells (Fig. 1A). Notably, cytosolic, but not extracellular, poly(I:C) induced the activation of caspase-1 even in unprimed Myd88/Trif-deficient DCs further supporting the conclusion that cytosolic poly(I:C)-induced caspase-1 activation is independent of TLR signaling (Fig. 1A). To test whether cytosolic poly(I:C) is sufficient to induce cytokine production, we assessed the production of IL-1 $\beta$  and IL-18 in unprimed BMDM, which do not express pro-IL1 $\beta$  but express low levels of pro-IL-18. In unprimed cells, cytosolic poly(I:C) induced the secretion of IL-18, but not of IL-1 $\beta$  (Supplemental Fig. 2). Consistent with these results, cytosolic poly(I:C) stimulation induced the upregulation of Nlrp3 mRNA (Supplemental Fig. 2). These

results indicate that cytosolic poly(I:C) is sufficient to induce Nlrp3 priming, activation of caspase-1 and IL-18 release, but not IL-1 $\beta$  production. In macrophages primed with LPS, transfection with poly(I:C) and lipofectamine, but not extracellular poly(I:C) alone, induced IL-1 $\beta$  release which required Myd88 consistent with the requirement of TLR signalling for LPS or extracellular poly(I:C) priming (Fig. 1B). Likewise, both ATP or cytosolic poly(I:C) stimulation induced comparable caspase-1 activation and IL-1 $\beta$  release in WT and Myd88/Trif-deficient cells primed with TNF- $\alpha$  (Fig. 1A,B). Consistent with the inability of extracellular poly(I:C) to induce caspase-1 activation, little or no secretion of IL-1 $\beta$  was detected when TNF-primed DCs were stimulated with poly(I:C) in the absence of lipofectamine (Fig. 1B). These results indicate that cytosolic poly(I:C), but not extracellular poly(I:C), induces the activation of the Nlrp3 inflammasome independently of TLR signalling.

### Microbial RNA induces Nlrp3 activation via Mavs

We next assessed the contribution of the cytosolic surveillance pathways in the activation of the Nlrp3 inflammasome induced by poly(I:C). To this end, LPS-primed WT macrophages or LPS-primed macrophages deficient in the cytosolic RNA sensors Rig-I, Mda5, or Pkr were stimulated with cytosolic poly(I:C) and the secretion of IL-1 $\beta$  was measured. We found that macrophages deficient in Rig-I, Mda5, or Pkr were not impaired in the release of IL-1 $\beta$  compared to WT cells, suggesting that the recognition of dsRNA by these receptors is dispensable for the activation of the Nlrp3 inflammasome (Fig. 2A). We next assessed the role of Mavs and Trif in inflammasome activation, as these two adaptors are used respectively by Rig-I/Mda5 and Tlr3 to initiate signal transduction pathways (49). The secretion of IL-1 $\beta$  induced by cytosolic poly(I:C) proceeded unabated in Trif-deficient macrophages (Fig. 2A). In contrast, the secretion of IL-1 $\beta$  induced by cytosolic poly(I:C) was greatly reduced in Mavs deficient macrophages and abrogated in Mavs/Trif-deficient macrophages (Fig. 2A). Consistent with the IL-1 $\beta$  results, the activation of caspase-1 induced by cytosolic poly(I:C) was greatly reduced in Mavs deficient and abolished in macrophages double deficient in Mavs and Trif (Fig. 2B). In contrast, the activation of the Nlrp3 inflammasome induced by Nigericin was comparable in WT and macrophages deficient in Mavs or Mavs/Trif (Fig. 2C). Similarly, dose response experiments showed that Mavs was dispensable for the activation of the Nlrp3 inflammasome induced by ATP or silica (Fig. 3A,B). These results indicate that Mavs plays an important role in the activation of the Nlrp3 inflammasome induced by cytosolic poly(I:C), but is dispensable for caspase-1 activation triggered by other Nlrp3 activators. To further confirm the role of Mavs in the activation of the Nlrp3 inflammasome induced by microbial RNA, we infected macrophages with *Escherichia coli* or DCs with Vesicular Stomatitis Virus (VSV), in which microbial RNA has been previously shown to induce Nlrp3 activation (29, 33, 36, 44, 50). We found that IL-1 $\beta$  secretion in macrophages infected with *E. coli* or DCs infected with VSV was severely impaired in Mavs-deficient cells compared to WT cells (Fig. 3C,D). As observed in BMDM, in BMDC the activation of caspase-1 induced by cytosolic poly(I:C) was Nlrp3 dependent (Supplemental Fig. 3). Consistent with previous results in macrophages, the secretion of IL-1 $\beta$  induced by LPS+ATP was comparable in WT and Mavs deficient DCs (Fig. 3E). Altogether, these results indicate that Mavs is important for Nlrp3 inflammasome



activation induced by microbial RNA but dispensable for the activation of Nlrp3 induced by ATP, particulate matter and bacterial pore-forming toxins.

### **Poly(I:C) induces membrane permeabilization independent of the inflammasome**

In addition to inducing the secretion of IL-1 $\beta$  and IL-18, the activation of the inflammasome triggers a form of cell death called pyroptosis, which is characterized by the caspase-1 dependent disruption of cell membrane integrity and the release of intracellular contents into the extracellular milieu(51). We next investigated whether poly(I:C) induces membrane permeabilization by measuring the release of LDH which in healthy cells is found in the cytosol. We found that cytosolic poly(I:C) and Nigericin, which was used as a positive control, compromised the cell membrane integrity as assessed by the release of LDH (Supplemental Fig. 4). As expected, membrane permeabilization induced by Nigericin was blocked in Nlrp3- or caspase-1-deficient macrophages (Supplemental Fig. 4A,B). Surprisingly, loss of cell membrane integrity induced by cytosolic poly(I:C) was unimpaired in Nlrp3 or caspase-1 deficient macrophages (Supplemental Fig. 4A,B), indicating that cytosolic poly(I:C) induced membrane permeabilization is independent of pyroptosis. To further explore the mechanism by which cytosolic poly(I:C) induces membrane permeabilization, we tested whether the release of LDH was caused by necroptosis, a form of cell death in which disruption of cell membrane integrity is dependent on RIP1 and RIP3 (52). As expected with previous studies (53), necrostatin, an inhibitor of RIP1, blocked necroptosis and membrane permeabilization induced by co-stimulation of L929 cells with TNF- $\alpha$  and ZVAD which was used as a positive control (Supplemental Fig. 4C). Necrostatin, however, had no effect on membrane damage induced by cytosolic poly(I:C) in macrophages (Supplemental Fig. 4D). Furthermore, ZVAD, a pan-caspase inhibitor that efficiently prevents apoptosis, was not effective in blocking cytosolic poly(I:C)-induced membrane permeabilization, excluding the possibility that membrane damage was caused by secondary necrosis of apoptotic cells. These results suggest that cytosolic poly(I:C)-induced membrane permeabilization is independent of caspase-1, necroptosis or apoptosis.

### **Poly(I:C) induces membrane permeabilization primarily via Mavs**

We next investigated whether membrane permeabilization was caused by the detection of poly(I:C) via sensors of dsRNA. To this end, we stimulated WT and macrophages deficient in the cytosolic RNA sensors Rig-I, Mda5, Pkr or the adaptor protein Trif and Mavs with cytosolic poly(I:C) or *Salmonella* as a control and measured membrane permeabilization by measuring the release of LDH. Cytosolic poly(I:C)-induced LDH release was reduced in macrophage deficient in Mavs and Mavs/Trif, but not in Rig-I, Mda5, Pkr or Trif (Fig.4). The role of Mavs was specific in that LDH release induced by *Salmonella* that triggers inflammasome activation via Nlr4 did not require Mavs (Fig.4). Collectively these results indicate that Mavs is important for both poly(I:C)-induced membrane permeabilization and the activation of the Nlrp3 inflammasome.

### **Poly(I:C)-induced membrane permeabilization causes potassium efflux which is required for inflammasome activation**

The  $K^+$  concentration inside cells is actively controlled by the  $Na^+/K^+$ -ATPase, a membrane transport protein that pumps  $Na^+$  outside and  $K^+$  inside the cell to achieve relatively high concentrations of  $K^+$  and low concentrations of  $Na^+$  in the cytosol. However, a compromise in cell membrane integrity can cause the efflux of  $K^+$ . Because a decrease in the intracellular concentration of  $K^+$  is important for Nlrp3 activation induced by ATP, pore-forming toxins and particulate matter(24), we investigated whether cytosolic Mavs-dependent membrane permeabilization induced by poly(I:C) could cause a net decrease of intracellular  $K^+$ . Cytosolic stimulation of macrophages with poly(I:C) induced membrane permeabilization and a decrease in the intracellular content of  $K^+$  in a dose-dependent manner (Fig.5A, B). As ROS have been previously shown to cause membrane damage(54, 55), we then tested whether poly(I:C)-induced membrane damage was prevented by inhibiting ROS with the free radical scavenger N-acetylcysteine (NAC). NAC blocked poly(I:C)-induced membrane permeabilization (Fig.5A) and prevented  $K^+$  efflux (Fig.5B). Consistent with a specific effect of NAC in blocking poly(I:C)-induced  $K^+$  efflux, the lowering of intracellular  $K^+$  induced by ATP, which occurs in response to P2RX7 receptor stimulation, was not affected by NAC (Fig.5C). To further confirm the role of ROS in Mavs-dependent activation of Nlrp3, we stimulated macrophages with cytosolic poly(I:C) or ATP in the presence of NAC. Consistent with a previous publication (12, 24), NAC did not inhibit caspase-1 activation induced by ATP in primed cells (Fig.5D), but blocked effectively cytosolic poly(I:C)-induced caspase-1 activation (Fig.5D) and IL-1 $\beta$  secretion (Fig.5E). These results confirm that in primed cells ROS are not necessary for Nlrp3 activation and suggests that in response to poly IC Mavs-dependent ROS production triggers  $K^+$  efflux by causing cell membrane damage.

### **Activation of the Nlrp3-inflammasome by poly(I:C) requires $K^+$ efflux**

We have shown that Mavs-induced membrane permeabilization triggers  $K^+$  efflux. We next investigated whether Mavs induced  $K^+$  efflux was required for Nlrp3 activation. To prevent  $K^+$  efflux caused by cytosolic poly(I:C), we stimulated macrophages in isotonic medium containing 70 mM  $K^+$  and assessed the activation of the Nlrp3 inflammasome and LDH release. As expected, transfection of high and low molecular weight poly(I:C) with lipofectamine induced  $K^+$  efflux, caspase-1 activation and IL-1 $\beta$  secretion (Fig. 6A-C). In contrast, caspase-1 and IL-1 $\beta$  secretion induced by cytosolic poly(I:C) were prevented by incubation of macrophages in high (70 mM), but not low (5 mM) extracellular  $K^+$  (Fig. 6B,C). Notably, media containing high (70 mM)  $K^+$  concentration did not prevent poly(I:C)-induced LDH release (Fig.6D), further confirming that LDH release occurs upstream of  $K^+$  efflux. Collectively, these results indicate that Mavs-induced membrane permeabilization causes  $K^+$  efflux which in turn induces Nlrp3 activation.

### **Rig-I and Mda5 act redundantly via the common adaptor Mavs to induce membrane permeabilization, $K^+$ efflux and Nlrp33 activation**

We have shown Nlrp3 activation induced by cytosolic poly(I:C) proceeds unabated in Rig-I or Mda5 deficient cells, but is greatly impaired in the Mavs deficient cells, a common



adaptor in both Rig-I and Mda5 signaling pathway. Next we explore the possibility that Rig-I and Mda5 act redundantly to induce Nlrp3 activation in response of cytosolic poly(I:C). To this end, we stimulated BMDM deficient in Mavs or deficient in both Rig-I and Mda5 and assessed membrane permeabilization, K<sup>+</sup> efflux, caspase-1 activation and IL-1 $\beta$  secretion after cytosolic poly(I:C) stimulation. We found that membrane permeabilization (Fig. 7A), K<sup>+</sup> efflux (Fig. 7B), caspase-1 activation (Fig. 7C) and IL-1 $\beta$  secretion (Fig. 7D) was impaired in BMDM deficient in both Rig-I and Mda5. Furthermore, we observed a comparable reduction of membrane permeabilization (Fig. 7A), K<sup>+</sup> efflux (Fig. 7B), caspase-1 activation (Fig. 7C) and IL-1 $\beta$  secretion (Fig. 7D) in BMDM doubly deficient in Rig-I and Mda5 and deficient in Mavs. The partial reduction in caspase-1 activation and IL-1 $\beta$  secretion in BMDM doubly deficient in Rig-I and Mda5 and deficient in Mavs is consistent with results shown in Fig. 2 that revealed the involvement of a Trif-dependent pathway in poly(I:C)-induced caspase-1 activation. In contrast, Nlrp3 activation induced by Nigericin proceeded unabated in BMDM deficient in both Rig-I and Mda5 or BMDM deficient in Mavs (Fig. 7E). Collectively, these results indicate that Rig-I and Mda5 act redundantly via the common adaptor Mavs to induce membrane permeabilization and K<sup>+</sup> efflux triggering Nlrp3 activation.

## Discussion

In this study we have identified a signaling pathway that mediates the sensing of dsRNA in the cytosol and activates the Nlrp3 inflammasome. Experiments with Tlr3 and Myd88/Trif deficient mice showed that cytosolic poly(I:C) induced Nlrp3 activation proceeds independently of TLR signalling. Furthermore, experiments using LPS-primed cells, in which pro-IL-1 $\beta$  and Nlrp3 are upregulated independently of Mavs, indicate that cytosolic poly(I:C) induces Nlrp3 activation. An important component of the dsRNA-induced Nlrp3 pathway is the adaptor Mavs which was critical for the induction of membrane permeabilization, K<sup>+</sup> efflux and caspase-1 activation. In our studies, we found that Mavs was critical for Nlrp3 activation, but RigI or Mda5 were dispensable for the induction of membrane permeabilization, caspase-1 activation and IL-1 $\beta$  release. Rig-I and Mda5 recognize dsRNA and upon activation they initiate signaling by recruiting Mavs(26). Experiment using BMDM deficient in both Rig-I and MDA5 stimulated with cytosolic poly (I:C) showed an inhibition of membrane permeabilization, K<sup>+</sup> efflux, caspase-1 activation and IL-1 $\beta$  secretion that were comparable to that observed in Mavs deficient cells, indicating that Rig-I and MDA5 act redundantly via the common adaptor Mavs to induce Nlrp3 activation. Recently, Subramanian et al. showed that Nigericin and ATP-induced Nlrp3 activation were Mavs dependent(34). In contrast, our results and recent studies (32, 56), found that Mavs was not required for Nlrp3 activation induced by Nigericin or ATP. In addition to the major Rig-I/Mda-5/Mavs pathway, we found a role for a Trif-dependent pathway in Nlrp3 activation induced by poly(I:C). Further experiments are needed to determine if the Trif pathway involves TLR3 stimulation or a different Trif-dependent pathway activated by poly(I:C). A recent model suggested that Nlrp3 is activated via a direct interaction of Nlrp3 with the DHX33 helicase induced upon recognition of dsRNA by DHX33 in human cells (35). Thus, it is possible that both Mavs-dependent K<sup>+</sup> efflux and direct interaction between DHX33 with Nlrp3 are important in triggering the activation of

Nlrp3 in response to cytosolic dsRNA. Collectively, these results indicate that recognition of dsRNA by cytosolic RLRs induces via Mavs the activation of the Nlrp3 inflammasome as well as signaling pathways leading to type I IFNs and NF- $\kappa$ B activation.

Previous studies showed that K<sup>+</sup> efflux is critical for the activation of the Nlrp3 inflammasome induced by ATP, pore-forming toxin and particulate matter (24). However, the mechanism by which dsRNA triggers activation of Nlrp3 remains unclear. We showed in the current work that cytosolic dsRNA triggers K<sup>+</sup> efflux, which was required for Nlrp3 activation. Notably, K<sup>+</sup> efflux induced by dsRNA required Mavs-mediated membrane permeabilization which was effectively blocked by NAC, but was independent of caspase-1. Intriguingly, cytosolic stimulation of DCs with poly(I:C) can induce an Mavs-dependent cell death pathway that enhances the production of type I IFNs, IL-6 and NF- $\kappa$ B activation (57). However, it is unclear whether the cytosolic pathway described by Zou *et al.* induces caspase-1 activation and IL-1 $\beta$  release. Unlike the latter Mavs-mediated pathway, the cytosolic poly(I:C)-induced pathway leading to Nlrp3 activation was unaffected by treatment with inhibitors of necroptosis (57). These results suggest that the Mavs-dependent cell death pathways enhancing type I IFNs/NF- $\kappa$ B activation and Nlrp3 activation are distinct. An interesting observation is that polyIC and bacterial dsRNA, but not RNA from pyroptotic macrophages or mitochondria, trigger Nlrp3 activation. These results suggest that RNA structures such as the 5' triphosphate group and blunt end of short dsRNAs or long-duplex RNAs that are important for Rig-I and Mda-5 recognition are critical for Nlrp3 activation (58, 59). The observation that NAC inhibits K<sup>+</sup> efflux suggests that Mavs activation induced by dsRNA mediates membrane damage through ROS which leads to K<sup>+</sup> efflux to induce Nlrp3 activation. Consistent with recent studies (12, 24), Nlrp3 activation induced by ATP was unaffected by NAC. Furthermore, extracellular media containing 70 mM K<sup>+</sup> prevented caspase-1 activation, but not dsRNA-induced membrane permeabilization, indicating that K<sup>+</sup> efflux is induced downstream of membrane damage. Collectively, these results indicate that cytosolic dsRNA sensing triggers membrane permeabilization via Mavs and ROS which in turn leads to K<sup>+</sup> efflux and Nlrp3 activation. Consistent with this model, treatment of macrophages with certain chemical agents that induce necrosis which is associated with plasma membrane damage, or high dose of lipofectamine (data not shown), can elicit caspase-1 activation and IL-1 $\beta$  release via the Nlrp3 inflammasome (60). Further studies will be necessary to better understand the mechanism by which ROS are generated in response to cytosolic dsRNA and how ROS induce membrane permeabilization.

In summary, our studies provide evidence that sensing of dsRNA in the cytosol induces a signaling pathway via the sensors RigI and Mda5 and the common adaptor Mavs that activates the Nlrp3 inflammasome by inducing membrane permeabilization and K<sup>+</sup> efflux. Thus, the cytosolic dsRNA pathway triggered by dsRNA converges into a common K<sup>+</sup> lowering step that is essential for the induction of Nlrp3 activation by a large array of stimuli including ATP, pore-forming toxins, and particulate matter.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations used in this paper

<b>BMDMs</b>	bone marrow derived macrophages
<b>LDH</b>	lactate dehydrogenase
<b>dsRNA</b>	double-stranded RNA
<b>Mavs</b>	mitochondrial antiviral signaling protein
<b>Mda5</b>	melanoma differentiation-associated gene 5
<b>NLRs</b>	nucleotide-binding oligomerization domain-like receptors
<b>Nlrp3</b>	NLR pyrin domain-containing 3
<b>poly (I:C)</b>	polyinosinic:polycytidylic acid
<b>RLRs</b>	RIG-like receptors
<b>ROS</b>	reactive oxygen species
<b>TLRs</b>	Toll-like receptors
<b>WT</b>	wild-type

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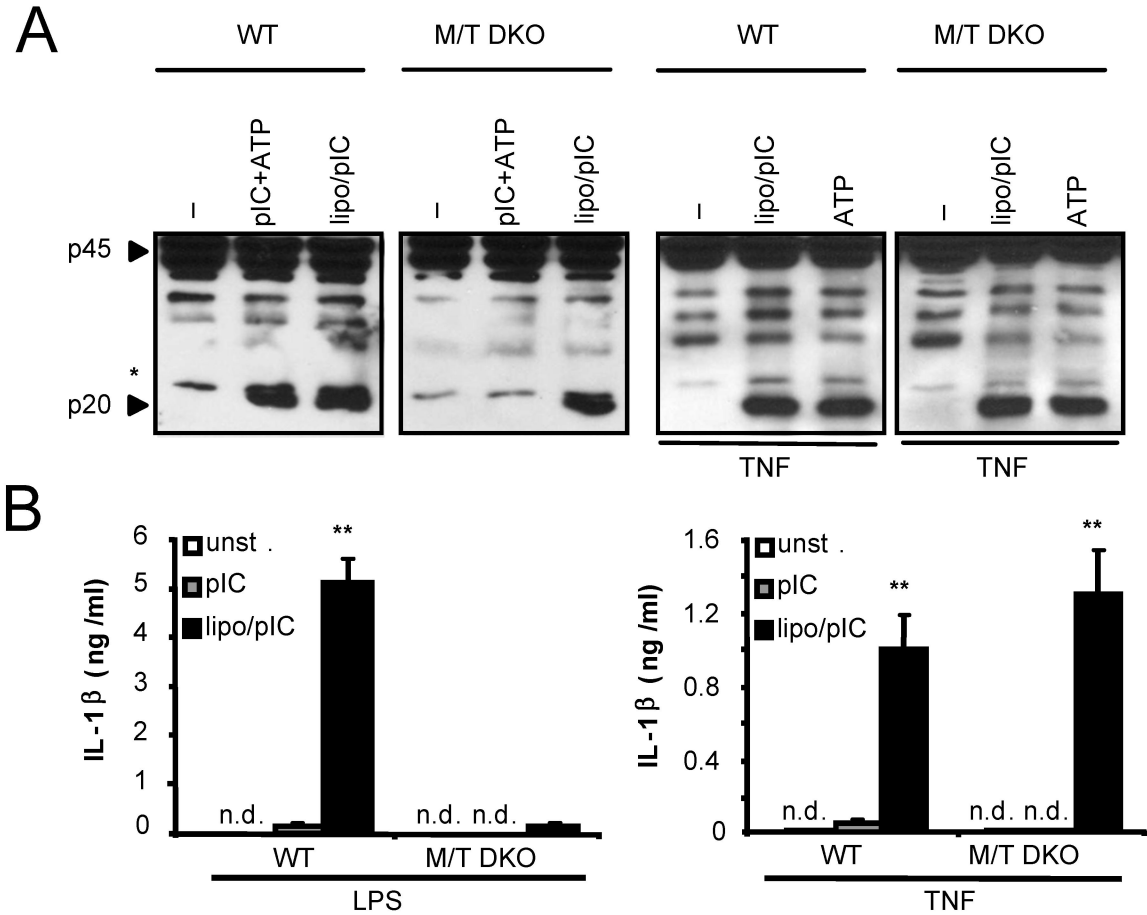
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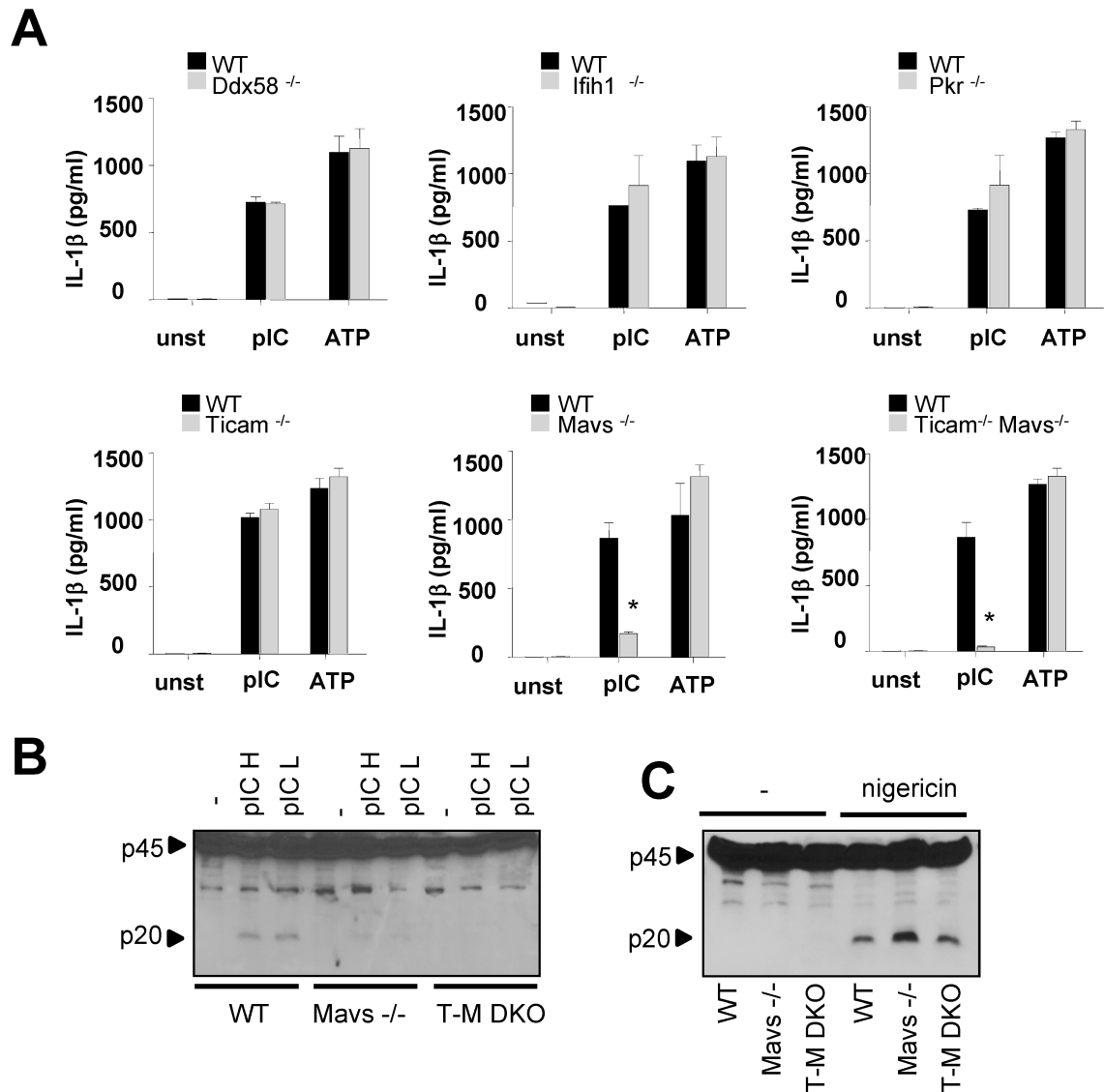
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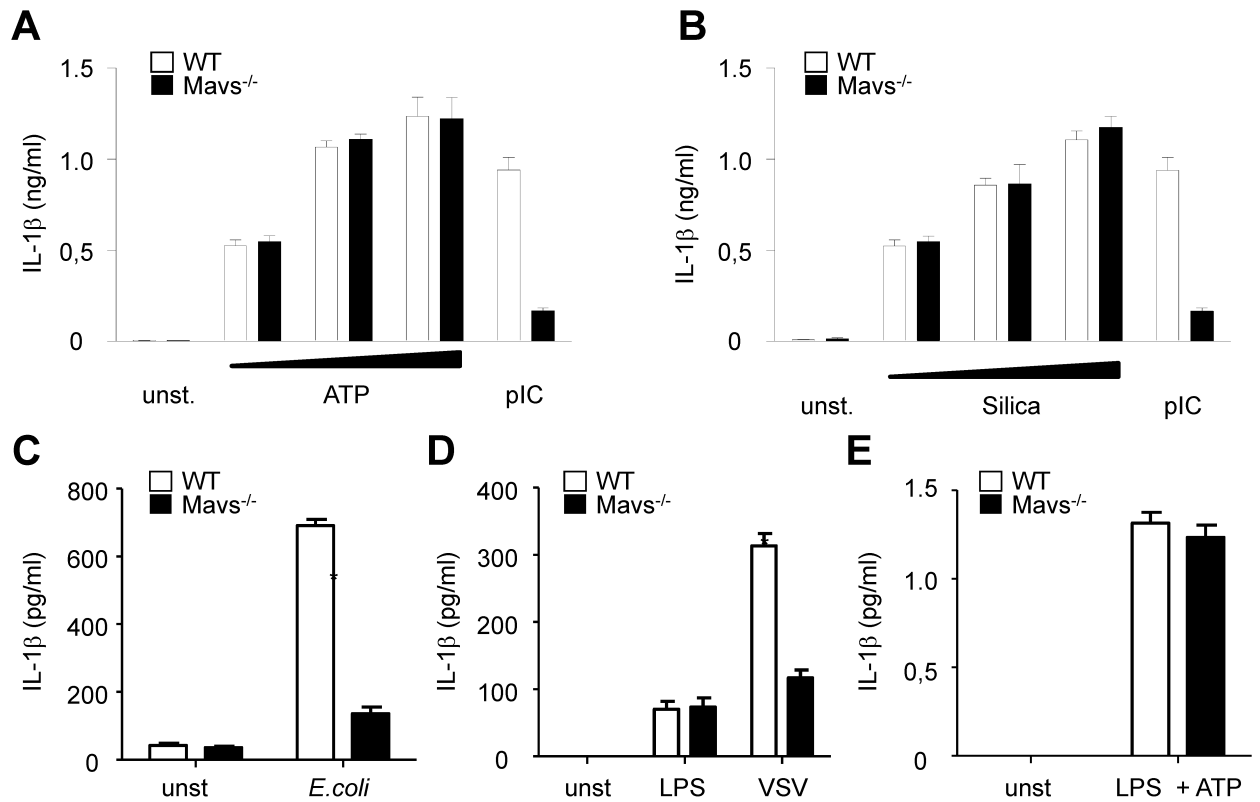


**Figure 1.**

Cytosolic poly(I:C) induces Nlrp3 activation independent of TLR signaling. (A) BMDC derived from WT or Myd88<sup>-/-</sup>/Ticam<sup>-/-</sup> DKO mice were primed with poly(I:C) (10μg/ml) or TNF (100ng/ml) for 6 hours and then stimulated with ATP (5 mM) for 30 minutes or stimulated directly with poly(I:C) conjugated with lipofectamine for 6 hours. Extracts were prepared from cells plus culture supernatants and immunoblotted with an antibody detecting active caspase-1 (p20). (B) WT or Myd88<sup>-/-</sup>/Ticam<sup>-/-</sup> DKO BMDC were primed with LPS or TNF and stimulated with poly(I:C) conjugated, or not, with lipofectamine for 6 hours. IL-1β was measured in cell-free supernatants by ELISA. Values represent the mean of triplicate wells ± SD. (\*\*\*) p<0.01. Results are representative of three independent experiments.

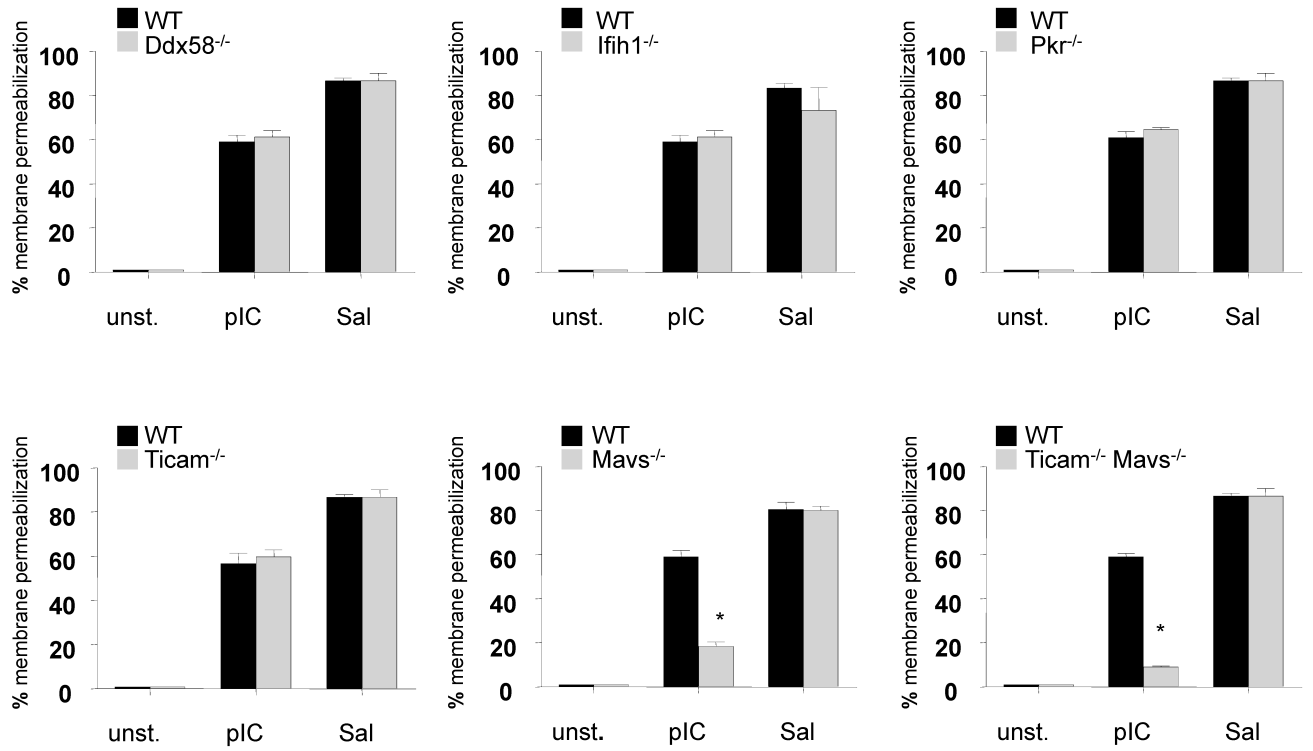


**Figure 2.** Poly(I:C) induces inflammasome activation via Mavs. (A) LPS-primed BMDM derived from WT, *Ddx58*<sup>-/-</sup>, *Ifih1*<sup>-/-</sup>, *Pkr*<sup>-/-</sup>, *Ticam*<sup>-/-</sup>, *Ips1*<sup>-/-</sup> or *Ticam*<sup>-/-</sup>/*Ips1*<sup>-/-</sup> DKO mice were stimulated with high (pIC H) or low (pIC L) molecular weight poly(I:C) conjugated with lipofectamine for 6 hours or ATP or nigericin for 30 min. (A) IL-1 $\beta$  secretion was assessed by ELISA in cell free supernatant. Values represent the mean of triplicate wells  $\pm$  SD. (\*)  $p < 0.01$ . (B-C), extracts were prepared from cells plus culture supernatants and immunoblotted with an antibody detecting active caspase-1 (p20). All results are representative of three independent experiments.



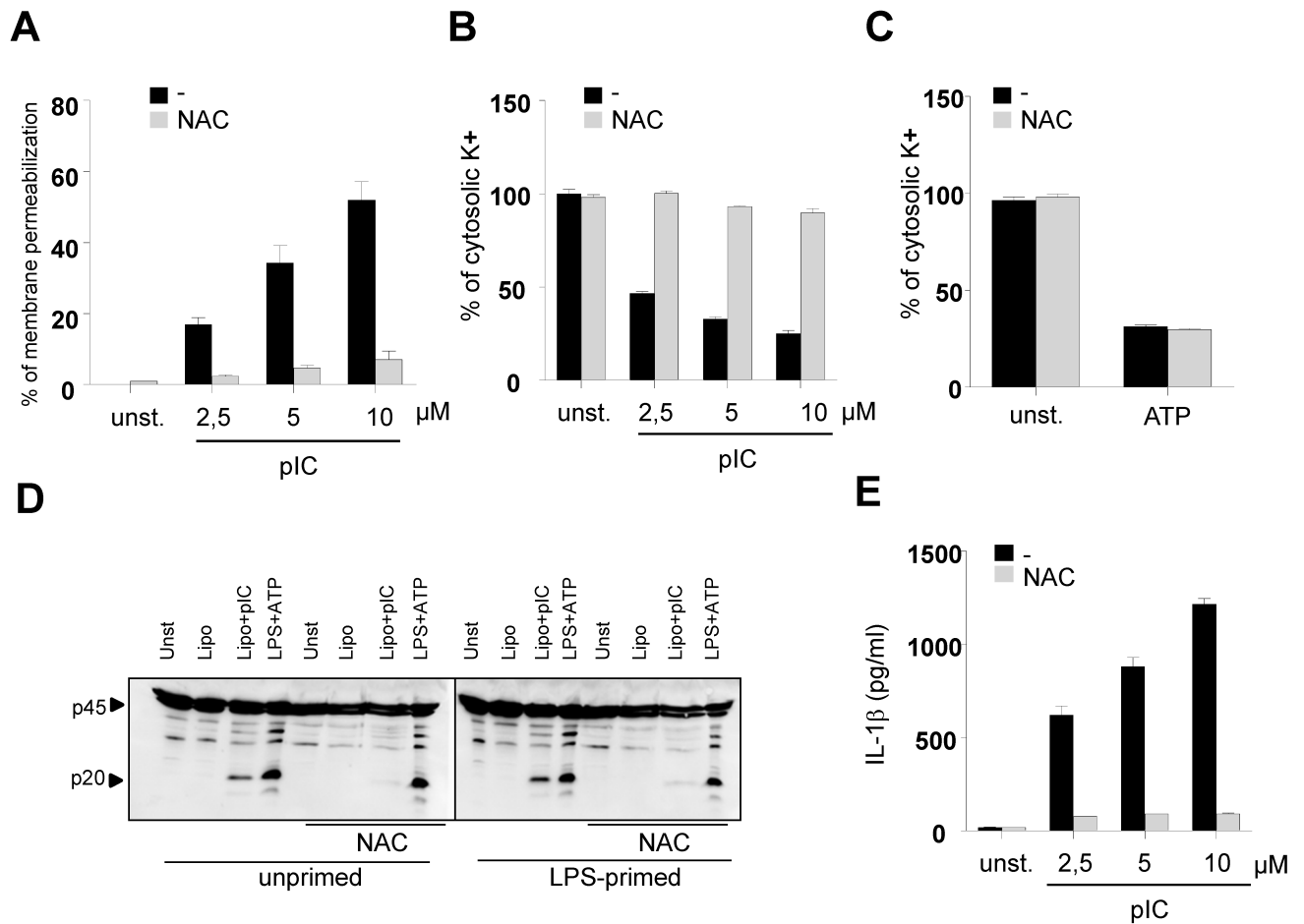
**Figure 3.**

VSV and *E. coli*, but not silica or ATP, induce inflammasome activation via Mavs. (A-C) LPS-primed BMDM or (D-E) DC derived from WT or Mavs<sup>-/-</sup> mice were stimulated with ATP (A) (1,25mM; 2,5mM; 5mM) for 30 minutes, or Silica (B) (100μg/ml; 200μg/ml, 400 μg/ml) for 6 hours, or poly(I:C) conjugated with lipofectamine for 6 hours or infected with *E. coli* or VSV for 24 hours (C-D). IL-1β secretion was assessed by ELISA in cell free supernatant. Values represent the mean of triplicate wells ± SD. (\*) p < 0.01. (A-D) Results are representative of three independent experiments.

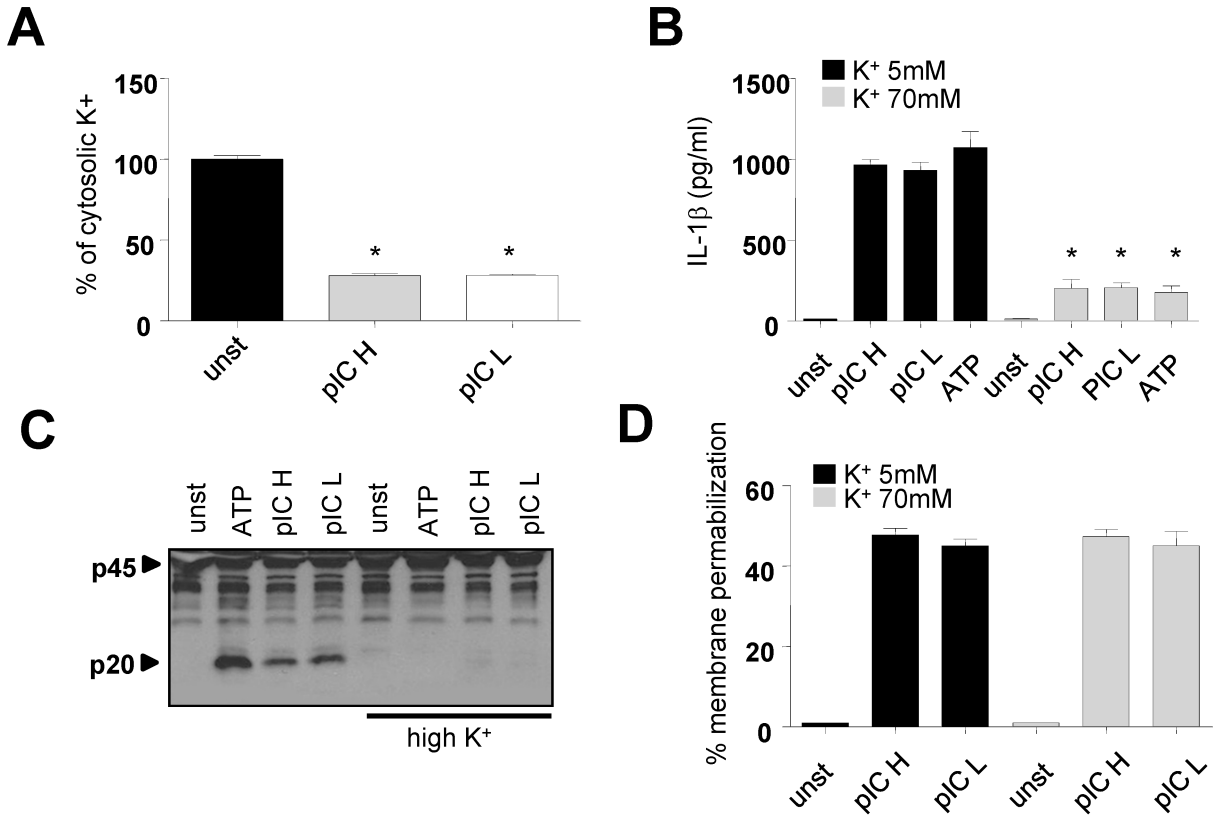


**Figure 4.**

dsRNA induces membrane permeabilization via Mavs. LPS-primed BMDM derived from WT, Ddx58<sup>-/-</sup>, Ifih1<sup>-/-</sup>, Pkr<sup>-/-</sup>, Ticam<sup>-/-</sup>, Mavs<sup>-/-</sup> or Ticam<sup>-/-</sup>/Mavs<sup>-/-</sup> DKO (T/M DKO) mice were stimulated with poly(I:C) (10 µg/ml) conjugated with lipofectamine or infected with Salmonella. Membrane permeabilization was evaluated after 6 hours by analyzing the release of cytosolic LDH in the supernatant. Values represent the mean of triplicate wells ± SD. (\*) p<0.01 Results are representative of three independent experiments.



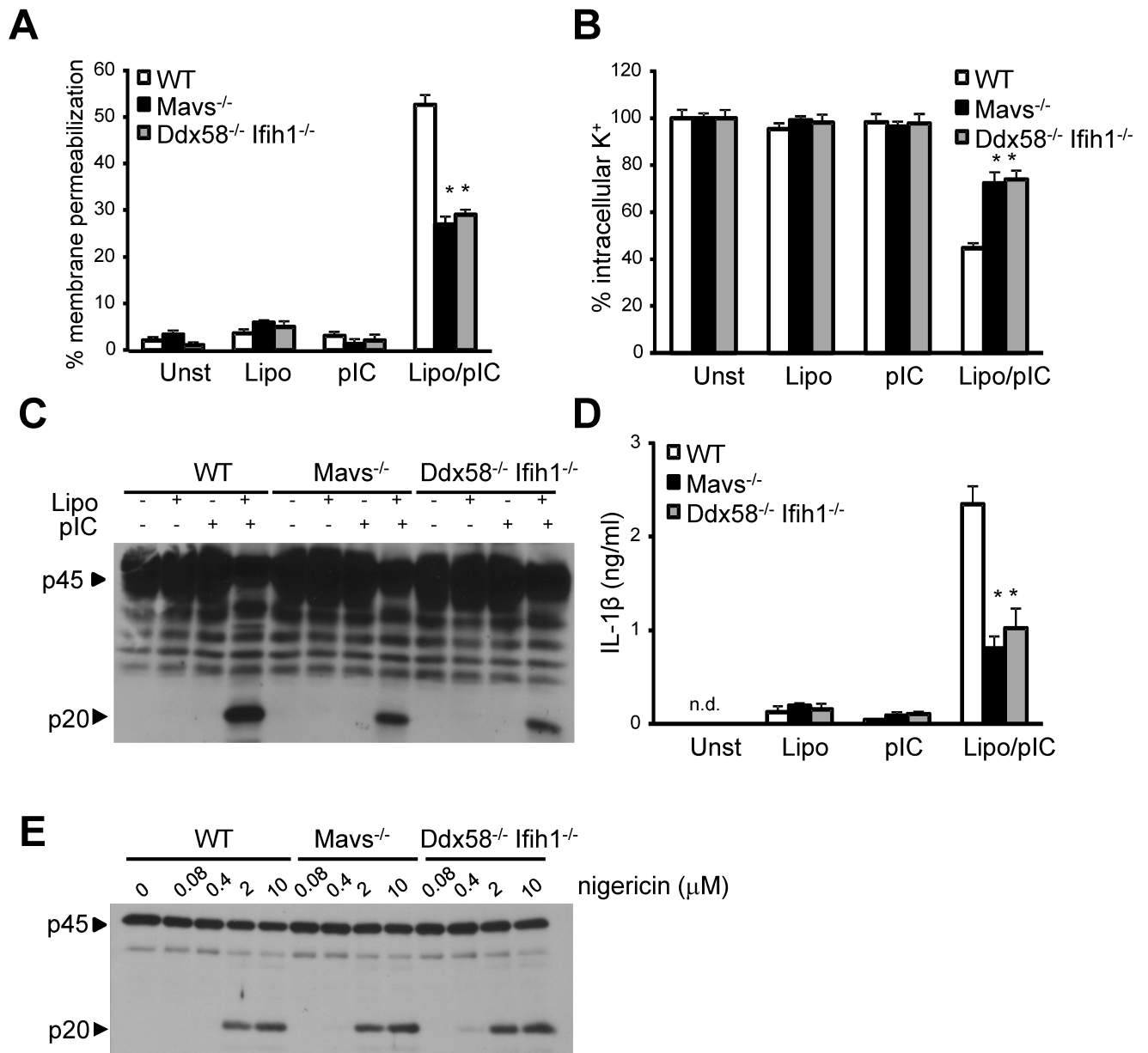
**Figure 5.** dsRNA-induced membrane permeabilization is required for K<sup>+</sup> efflux and inflammasome activation. Unprimed and LPS-primed BMDM were pretreated with NAC (30 mM) and stimulated with poly(I:C) (10 μg/ml) conjugated with lipofectamine for 6 hours (A-E) or with ATP (C-D) (5 mM) for 30 minutes. (A and E) Membrane permeabilization was evaluated by analyzing the release of cytosolic LDH in the supernatant. (B-C) intracellular K<sup>+</sup> content was evaluated by ICP/MS. (D) Caspase-1 activation was evaluated by immunoblotting with an antibody detecting active caspase-1 (p20). (E) IL-1β was evaluated by ELISA in cell free supernatant. (A-C and E) Values represent the mean of triplicate wells ± SD. (\*) p<0.01 (A-E) Results are representative of three independent experiments.



**Figure 6.**

Activation of the Nlrp3-inflammasome by poly(I:C) requires K<sup>+</sup> efflux. A, LPS- primed BMDM were stimulated with high (pIC H) or low (pIC L) molecular weight poly(I:C) conjugated with lipofectamine for 6 hours (A-D) or ATP for 30 min (B-C), in buffer containing low (5mM) or high (70mM) K<sup>+</sup> concentration (B-D). (A) Intracellular K<sup>+</sup> was evaluated by ICP/MS, (B) IL-1 $\beta$  secretion was evaluated by ELISA in cell free supernatant. (C), caspase-1 activation was evaluated by immunblotting with an antibody detecting active caspase-1 (p20) and (D) membrane permeabilization was evaluated by analyzing the release of cytosolic LDH in the supernatant. (A, B and D). Values represent the mean of triplicate wells  $\pm$  SD. (\*)  $p < 0.01$ . (A-D) Results are representative of three independent experiments.





**Figure 7.** Rig-I and Mda5 act redundantly via the common adaptor Mavs to induce NLRP3 activation. (A-D) LPS-primed BMDM derived from WT, Ddx58<sup>-/-</sup>Ifih1<sup>-/-</sup> DKO or Mavs<sup>-/-</sup> mice were stimulated poly(I:C) conjugated, or not, with lipofectamine for 6 hours (A-D) (A) membrane permeabilization was evaluated by analyzing the release of cytosolic LDH in the supernatant (B) Intracellular K<sup>+</sup> was evaluated by ICP/MS. (C) Caspase-1 activation was evaluated by immunoblotting with an antibody detecting active caspase-1 (p20). (D) IL-1 $\beta$  secretion was evaluated by ELISA in cell free supernatant. (A, B and D). Values represent the mean of triplicate wells  $\pm$  SD. (\*)  $p < 0.01$ . (A-D) Results are representative of three independent experiments.