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Cytosolic Double-Stranded RNA Activates the NLRP3 Inflammasome via MAVS-Induced Membrane Permeabilization and K$^+$ Efflux

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The nucleotide-binding oligomerization domain–like receptor pyrin domain–containing 3 (Nlrp3) inflammasome plays an important role in inflammation by controlling the maturation and secretion of the cytokines IL-1β 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$^+$ concentration, which is required for inflammasome activation, the mechanism by which microbial RNA activates Nlrp3, remains poorly understood. In this study, 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-1β 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 melanoma differentiation–associated gene 5 act redundantly via the common adaptor mitochondrial antiviral signaling (Mavs) to induce Nlrp3 activation in response to poly(I:C), but not ATP or nigericin. Mechanistically, Mavs triggered membrane permeabilization and K$^+$ efflux independently of the 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. *The Journal of Immunology*, 2014, 193: 4214–4222.

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 multiprotein complex that activates caspase-1 (4, 5). Once activated, caspase-1 proteolytically cleaves pro–IL-1β and pro–IL-18 into their biologically active forms (6). To date, four bonafide 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 because of its link to the pathogenesis of autoinflammatory syndromes and a variety of inflammatory diseases (7–9). Production of IL-1β through Nlrp3 is tightly regulated via a two-step process. The first step, referred as priming, involves the transcriptional induction of pro–IL-1β and Nlrp3 by TLR agonists or certain cytokines such as TNF-α or IL-1β (10, 11). Both reactive oxygen species (ROS) and NF-κB 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-1β into mature IL-1β. 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

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; DC, dendritic cell; ICP, inductively coupled plasma; LDH, lactate dehydrogenase; Mavs, mitochondrial antiviral signaling; Mda5, melanoma differentiation–associated gene 5; NAC, *N*-acetyl-L-cysteine; NLR, nucleotide-binding oligomerization domain–like receptor; Nlrp3, NLR pyrin domain–containing 3; poly(dA:dT), poly(dA-dT)•poly (dT-dA); poly (I:C), polyinosinic:polycytidylic acid; PRR, pattern-recognition receptor; ROS, reactive oxygen species; VSV, vesicular stomatitis virus; WT, wild-type.

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 Rig-I/Ddx58 and Mda5/Ifih1 (melanoma differentiation–associated gene 5). Rig-I and Mda5 recognize 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-κB via mitochondrial antiviral signaling (Mavs) (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^+ efflux and Nlrp3 activation.

Materials and Methods

Mice

$Nlrp3^{-/-}$, $Asc^{-/-}$, $casp1/11^{-/-}$ mice have been described previously (36, 37). $P2rx7^{-/-}$ mice were obtained from G. Dubyak (Case Western Reserve University, Cleveland, OH). $Tlr3^{-/-}$, $Myd88/Ticam^{-/-}$ and $Mavs^{-/-}/Ticam^{-/-}$, $RigI/Ddx58^{-/-}$, $Mda5/Ifih1^{-/-}$, and $RigI^{-/-}Mda5^{-/-}$ mice have been described previously (38–41). $Pkr^{-/-}$ mice were obtained 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 purchased from Sigma-Aldrich. Recombinant mouse TNF-α was obtained from Peprotech. Ultrapure LPS from *Escherichia coli*, Pam3CSK, flagellin, high and low m.w. poly(I:C), CpG, double-stranded B DNA poly (dA-dT)•poly(dT-dA) [poly(dA:dT)], and nigericin were obtained 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-Aldrich. Carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone and Necrostatin were from Enzo Life Sciences.

RNA isolation

Liver mitochondria were purified as described previously (42). For isolation of total RNA from pyroptotic cells, bone marrow–derived macrophage (BMDM) were stimulated with Pam3CSK (10 μg/ml) for 1 h, followed by incubation with ATP (5 mM) 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 described previously (43). Cells were stimulated in IMDM supplemented with 10% FCS at a density of 1×10^6/ml. Unless otherwise specified, BMDM were transfected with high m.w. 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 μg/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 μg/ml). For inhibitor experiments, LPS-primed BMDM were preincubated with NAC (30 mM), pan-caspase inhibitor Z-VAD (40 μM), or necrostatin (20 μM) 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 Nonidet P-40 to the media to a final concentration of 1% and protease inhibitors. Cleared lysates were separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with a rabbit anti–caspase-1 Ab generated in our laboratory. Concentrations of IL-1β (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 The CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit from Promega according to the manufacturer's instructions.

Determination of intracellular K^+ content

Intracellular K^+ measurements were performed by inductively coupled plasma (ICP) optical emission spectrometry with a Perkin-Elmer Optima 2000 DV spectrometer using yttrium as an internal standard. The culture media were thoroughly aspirated, and cells were extracted 30 min in 3% ultrapure HNO_3. K^+ determinations were done in 48-well plates. For accurate measurement of the intracellular K^+, a control was performed in every experiment to determine the extracellular amount of K^+ after aspiration, and this value was subtracted from every measurement. In experiments using high and low extracellular K^+, osmolarities were measured by freezing-point depression osmometry using a μOsmette (Precission systems). In experiments using high and low extracellular K^+, media osmolarities were adjusted to 300 mOsm after adding the respective salts using a μOsmette freezing-point depression osmometer (Precission systems).

Statistical analysis

Statistical significance between groups was determined by two-tailed Student 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 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-1β 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-1β release in LPS-primed macrophages (Supplemental Fig. 1A). As a control, a preparation enriched in mitochondrial DNA induced IL-1β 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 Nlrc4 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 sig-
naling 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 re-
quired for TLR signaling. Priming of macrophages with extra-
cellular 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 dendritic cells (DC), fur-
ther 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-1β and IL-18 in
unprimed BMDM, which do not express pro–IL-1β 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-1β (Supplemental
Fig. 2). Consistent with these results, cytosolic poly(I:C) stimu-
lation induced the upregulation of Nlrp3 mRNA (Supplemental
Fig. 2). These results indicate that cytosolic poly(I:C) is suffi-
cient to induce Nlrp3 priming, activation of caspase-1 and IL-18
release, but not IL-1β production. In macrophages primed with
LPS, transfection with poly(I:C) and Lipofectamine, but not ex-
tracellular poly(I:C) alone, induced IL-1β release, which required
Myd88 consistent with the requirement of TLR signaling 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-1β release in WT- and Myd88/Trif-deficient
cells primed with TNF-α (Fig. 1A, 1B). Consistent with the in-
ability of extracellular poly(I:C) to induce caspase-1 activation,
little or no secretion of IL-1β was detected when TNF-primed
DCs were stimulated with poly(I:C) in the absence of Lipofect-
amine (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 signaling.

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-1β was measured. We found that macrophages
deficient in Rig-I, Mda5, or Pkr were not impaired in the release of
IL-1β compared with 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 because these two adaptors
are used, respectively, by Rig-I/Mda5 and Tlr3 to initiate signal
transduction pathways (49). The secretion of IL-1β induced by
cytosolic poly(I:C) proceeded unabated in Trif-deficient macro-
phages (Fig. 2A). In contrast, the secretion of IL-1β induced by
cytosolic poly(I:C) was greatly reduced in Mavs-deficient macro-
phages and abrogated in Mavs/Trif-deficient macrophages (Fig. 2A).
Consistent with the IL-1β 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, 3B).
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 acti-
vation of the Nlrp3 inflammasome induced by microbial RNA, we
infected macrophages with *Escherichia coli* or DC with vesicular
stomatitis virus (VSV) in which microbial RNA has been previ-
ously shown to induce Nlrp3 activation (29, 33, 36, 44, 50). We
found that IL-1β secretion in macrophages infected with *E. coli*
or DC infected with VSV was severely impaired in Mavs-deficient
cells compared with WT cells (Fig. 3C, 3D). As observed in
BMDM, in BMDC, the activation of caspase-1 induced by cyto-
solic poly (I:C) was Nlrp3 dependent (Supplemental Fig. 3). Con-
sistent with previous results in macrophages, the secretion of IL-1β
induced by LPS+ATP was comparable in WT- and Mavs-deficient
DCs (Fig. 3E). Altogether, these results indicate that Mavs is im-
portant 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-1β and IL-18, the ac-
tivation of the inflammasome triggers a form of cell death called
pyroptosis, which is characterized by the caspase-1–dependent
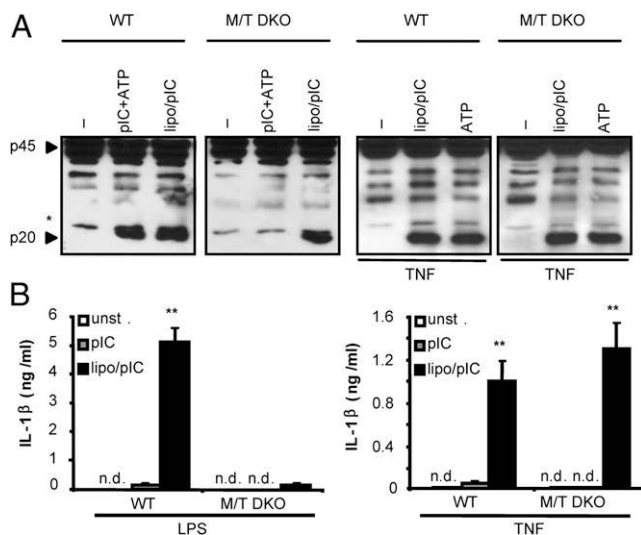disruption of cell membrane integrity and the release of intracel-

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

FIGURE 2. Poly(I:C) induces inflammasome activation via Mavs. (**A**) LPS-primed BMDM derived from WT, Ddx58$^{-/-}$, Ifih1$^{-/-}$, Pkr$^{-/-}$, Ticam$^{-/-}$, Ips1$^{-/-}$, or Ticam$^{-/-}$/Ips1$^{-/-}$ DKO mice were stimulated with high (pIC H) or low (pIC L) m.w. poly(I:C) conjugated with Lipofectamine for 6 h or ATP or nigericin for 30 min. (A) IL-1β secretion was assessed by ELISA in cell-free supernatant. Values represent the mean of triplicate wells ± SD. *$p < 0.01$. (**B** and **C**) Extracts were prepared from cells plus culture supernatants and immunoblotted with an Ab detecting active caspase-1 (p20). All results are representative of three independent experiments.
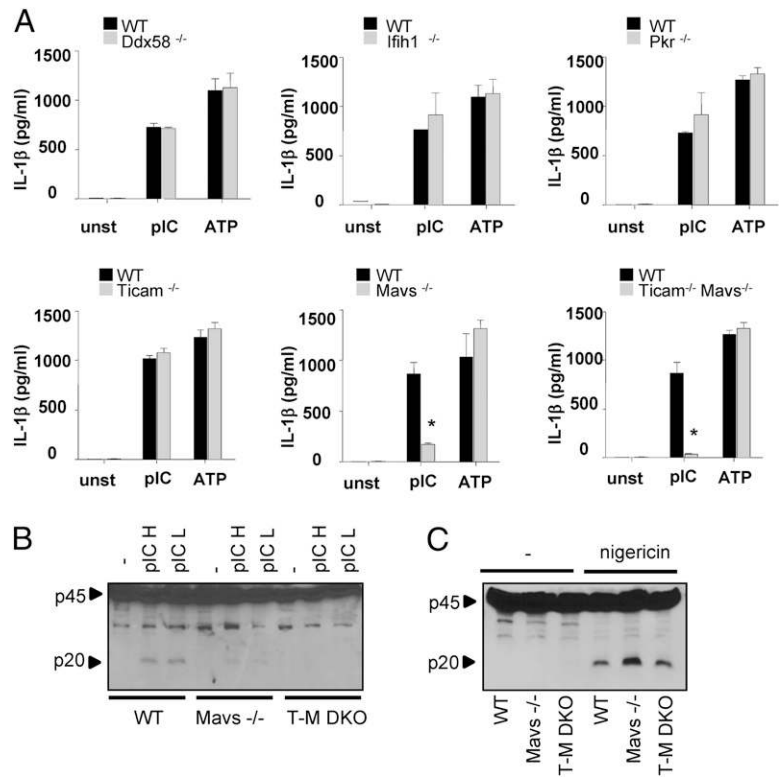
lular contents into the extracellular milieu (51). We next investigated whether poly(I:C) induces membrane permeabilization by measuring the release of LDH, which in healthy cells is found in the cytosol. We found that cytosolic poly(I:C) and nigericin, which was used as a positive control, compromised 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, 4B). Surprisingly, loss of cell membrane integrity induced by cytosolic poly(I:C) was unimpaired in Nlrp3 or caspase-1–deficient macrophages (Supplemental Fig. 4A, 4B), 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 costimulation of L929 cells with TNF-α 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.
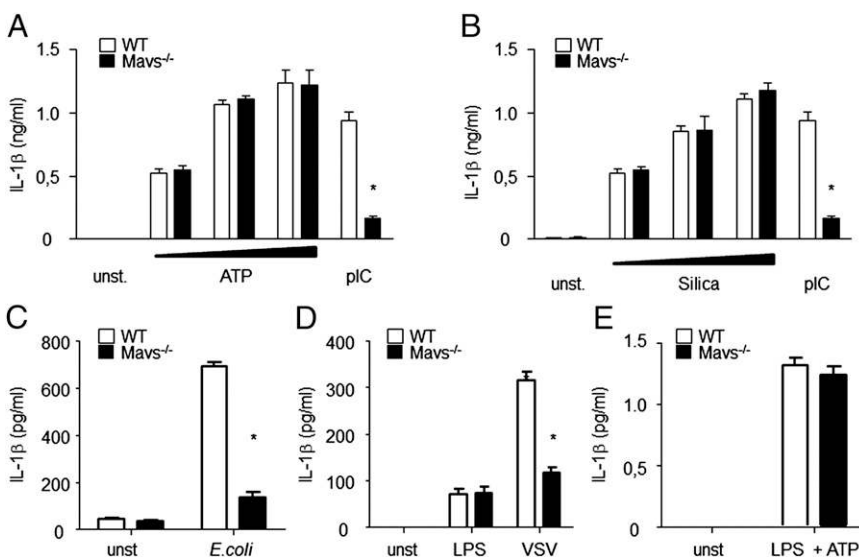

FIGURE 3. VSV and *E. coli*, but not silica or ATP, induce inflammasome activation via Mavs. LPS-primed BMDM (**A–C**) or DC (**D** and **E**) derived from WT or Mavs$^{-/-}$ mice were stimulated with ATP (A) (1.25, 2.5, and 5 mM) for 30 min or silica (B) (100, 200, and 400 μg/ml) for 6 h or poly(I:C) conjugated with Lipofectamine for 6 h or infected with *E. coli* or VSV for 24 h (C and D). IL-1β secretion was assessed by ELISA in cell-free supernatant. Values represent the mean of triplicate wells ± SD. (A–D) Results are representative of three independent experiments. *$p < 0.01$.

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 Nlrc4 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, 5B). 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 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-1β secretion (Fig. 5E). These results confirm that in primed cells ROS are not necessary for Nlrp3 activation and suggests that in response to poly (I:C) 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 m.w. poly(I:C) with Lipofectamine-induced K^+ efflux, caspase-1 activation, and IL-1β secretion (Fig. 6A–C). In contrast, caspase-1 and IL-1β 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, 6C). 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 that 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 ot 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^+ efflux, caspase-1 activation, and IL-1β secretion after cytosolic poly(I:C) stimulation. We found that membrane permeabilization (Fig. 7A), K^+ efflux (Fig. 7B), caspase-1 activation (Fig. 7C), and IL-1β 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^+ efflux (Fig. 7B), caspase-1 activation (Fig. 7C), and IL-1β secretion (Fig. 7D) in BMDM doubly deficient in Rig-I and Mda5 and deficient in Mavs. The partial reduction in

FIGURE 4. dsRNA induces membrane permeabilization via Mavs. LPS-primed BMDM derived from WT, Ddx58$^{-/-}$, Ifih1$^{-/-}$, Pkr$^{-/-}$, Ticam$^{-/-}$, Mavs$^{-/-}$, or Ticam$^{-/-}$/Mavs$^{-/-}$ double-knockout (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 h by analyzing the release of cytosolic LDH in the supernatant. Values represent the mean of triplicate wells ± SD. Results are representative of three independent experiments. *$p < 0.01$.
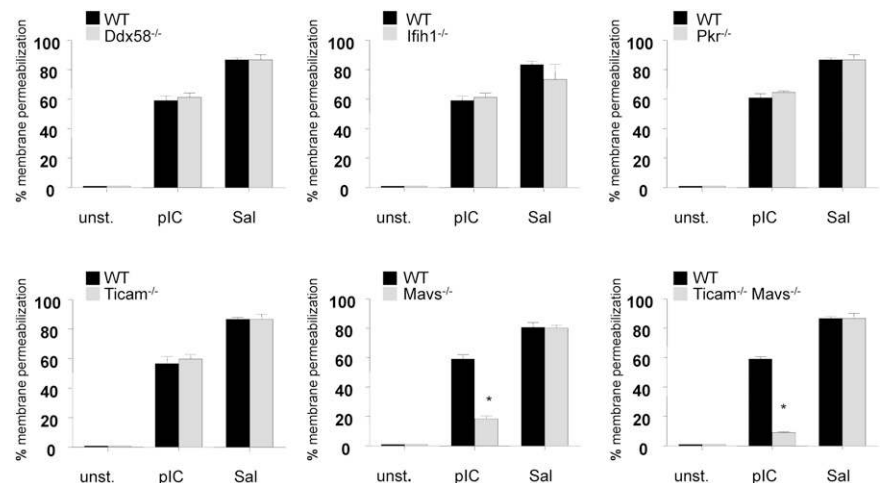
FIGURE 5. dsRNA-induced membrane permeabilization is required for K$^+$ ellux and inflammasome activation. Unprimed and LPS-primed BMDM were pretreated with NAC (30 mM) and stimulated with poly(I:C) (10 μg/ml) conjugated with lipofectamine for 6 h (**A**, **B**, **D**, and **E**) or with ATP (**C** and D) (5 mM) for 30 min. (A and E) Membrane permeabilization was evaluated by analyzing the release of cytosolic LDH in the supernatant. (B and C) Intracellular K$^+$ content was evaluated by ICP-mass spectroscopy. (D) Caspase-1 activation was evaluated by immunblotting with an Ab 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. Results are representative of three independent experiments. *$p < 0.01$ (A–E).
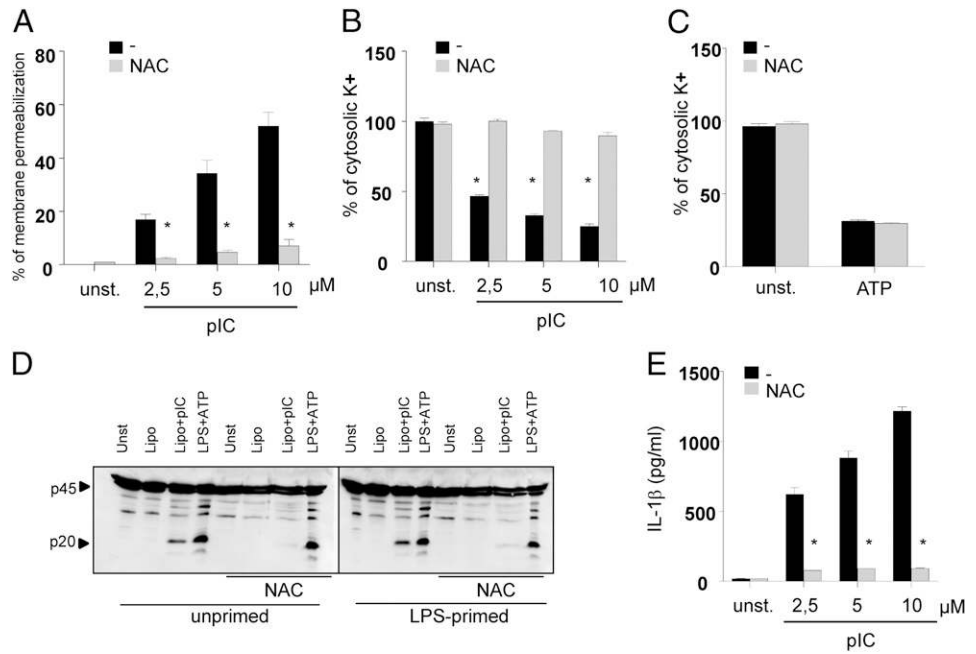
caspase-1 activation and IL-1β secretion in BMDM doubly deficient in Rig-I and Mda5 and deficient in Mavs is consistent with results shown in Fig. 2 that revealed the involvement of a Trif-dependent pathway in poly(I:C)-induced caspase-1 activation. In contrast, Nlrp3 activation induced by nigericin 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$^+$ 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 signaling. Furthermore, experiments using LPS-primed cells, in which pro–IL-1β 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 that was critical for the induction of membrane permeabilization, K$^+$ 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-1β release. Rig-I and Mda5 recognize dsRNA, and upon activation, they initiate signaling by recruiting Mavs (26). An experiment using BMDM deficient in both Rig-I and MDA5 stimulated with cytosolic poly (I:C) showed an inhibition of membrane permeabilization, K$^+$ efflux, caspase-1 activation, and IL-1β 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. (34) showed that nigericin and ATP-induced Nlrp3 activation were Mavs dependent. In contrast, our results and recent studies (32, 56) found that Mavs was not required
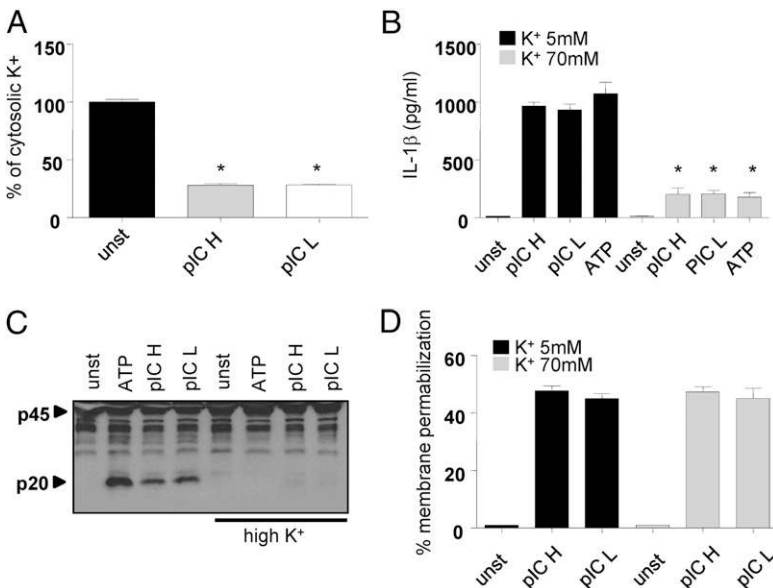
FIGURE 6. Activation of the Nlrp3 inflammasome by poly(I:C) requires K$^+$ efflux. (**A**) LPS-primed BMDM were stimulated with high (pIC H) or low (pIC L) m.w. poly(I:C) conjugated with Lipofectamine for 6 h (A–D) or ATP for 30 min (B and C) in buffer containing low (5 mM) or high (70 mM) K$^+$ concentration (B–D). Intracellular K$^+$ was evaluated by ICP-mass spectroscopy (A), and IL-1β secretion was evaluated by ELISA in cell-free supernatant (B). Caspase-1 activation was evaluated by immunoblotting with an Ab detecting active caspase-1 (p20) (C), and membrane permabiliziation was evaluated by analyzing the release of cytosolic LDH in the supernatant (D). (A, B, and D) Values represent the mean of triplicate wells ± SD. (A–D) Results are representative of three independent experiments. *$p < 0.01$.

FIGURE 7. Rig-I and Mda5 act redundantly via the common adaptor Mavs to induce NLRP3 activation. (**A–E**) LPS-primed BMDM derived from WT, $Ddx58^{-/-}Ifih1^{-/-}$ DKO, or $Mavs^{-/-}$ mice were stimulated poly(I:C) conjugated, or not, with Lipofectamine for 6 h or Nigericin for 1 h. (A) Membrane permeabilization was evaluated by analyzing the release of cytosolic LDH in the supernatant. (B) Intracellular K^+ was evaluated by ICP/MS. (C and E) Caspase-1 activation was evaluated by immunoblotting with an Ab detecting active caspase-1 (p20). (D) IL-1β secretion was evaluated by ELISA in cell-free supernatant. (A, B, and D) Values represent the mean of triplicate wells ± SD. Results are representative of three independent experiments (A–D). $*p < 0.01$.
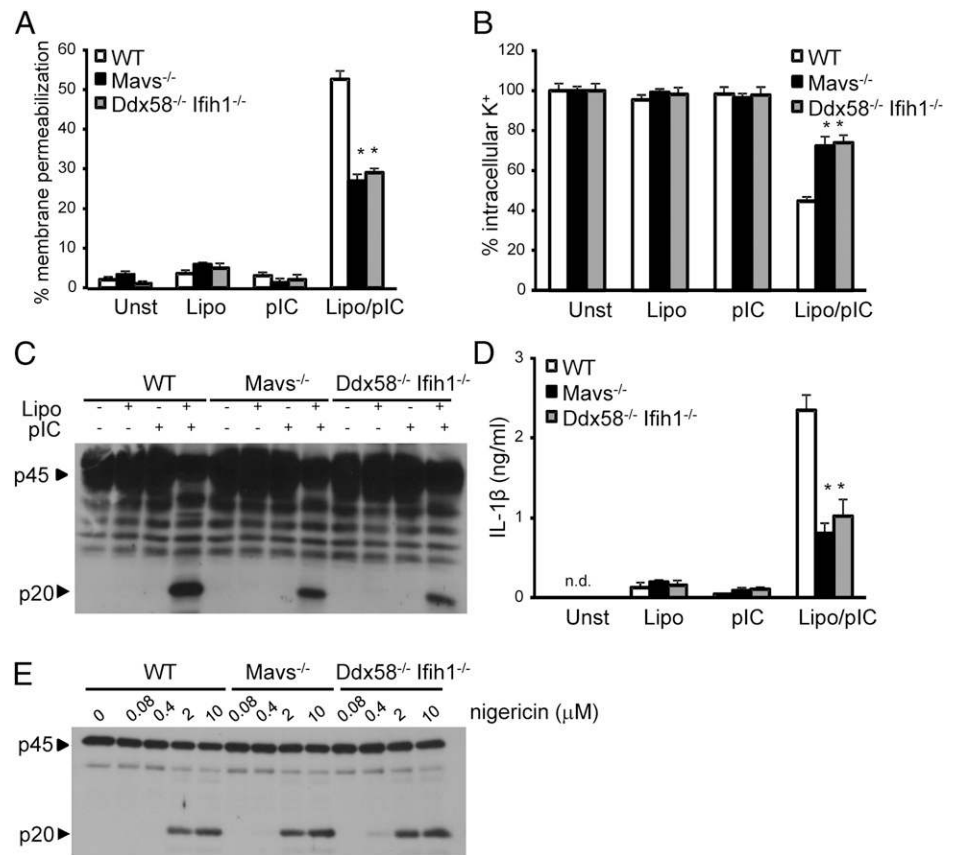
for Nlp3 activation induced by nigericin or ATP. In addition to the major Rig-I/Mda-5/Mavs pathway, we found a role for a Trif-dependent pathway in Nlrp3 activation induced by poly(I:C). Further experiments are needed to determine whether the Trif pathway involves TLR3 stimulation or a different Trif-dependent 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^+ 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 RIG–like receptors induces via Mavs the activation of the Nlrp3 inflammasome as well as signaling pathways leading to type I IFNs and NF-κB activation.

Previous studies showed that K^+ 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^+ efflux, which was required for Nlrp3 activation. Notably, K^+ 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-κB activation (57). However, it is unclear whether the cytosolic pathway described by Zou et al. (57) induces caspase-1 activation and IL-1β 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-κB activation and

Nlrp3 activation are distinct. An interesting observation is that poly (I:C) 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^+ efflux suggests that Mavs activation induced by dsRNA mediates membrane damage through ROS, which leads to K^+ 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^+ prevented caspase-1 activation but not dsRNA-induced membrane permeabilization, indicating that K^+ 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^+ 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 a high dose of Lipofectamine (data not shown), can elicit caspase-1 activation and IL-1β 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 Rig-I and Mda5 and the common adaptor Mavs that activates the Nlrp3 inflammasome by inducting membrane permeabilization and K^+ efflux. Thus, the cytosolic dsRNA pathway triggered by dsRNA converges into a common K^+ 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.

Acknowledgments

Disclosures

References

1. Chen, G. Y., and G. Nuñez. 2010. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10: 826–837.
2. Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11: 373–384.
3. Inohara, N., and G. Nuñez. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* 3: 371–382.
4. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* 27: 229–265.
5. Franchi, L., R. Muñoz-Planillo, and G. Núñez. 2012. Sensing and reacting to microbes through the inflammasomes. *Nat. Immunol.* 13: 325–332.
6. Franchi, L., T. Eigenbrod, R. Muñoz-Planillo, and G. Nuñez. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 10: 241–247.
7. Masters, S. L., A. Simon, I. Aksentijevich, and D. L. Kastner. 2009. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annu. Rev. Immunol.* 27: 621–668.
8. Ozkurede, V. U., and L. Franchi. 2012. Immunology in clinic review series; focus on autoinflammatory diseases: role of inflammasomes in autoinflammatory syndromes. *Clin. Exp. Immunol.* 167: 382–390.
9. Hao, L. Y., X. Liu, and L. Franchi. 2013. Inflammasomes in inflammatory bowel disease pathogenesis. *Curr. Opin. Gastroenterol.* 29: 363–369.
10. Bauernfeind, F. G., G. Horvath, A. Stutz, E. S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B. G. Monks, K. A. Fitzgerald, et al. 2009. Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* 183: 787–791.
11. Franchi, L., T. Eigenbrod, and G. Nuñez. 2009. Cutting edge: TNF-α mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J. Immunol.* 183: 792-796.
12. Bauernfeind, F., E. Bartok, A. Rieger, L. Franchi, G. Núñez, and V. Hornung. 2011. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *J. Immunol.* 187: 613–617.
13. Wen, H., E. A. Miao, and J. P. Ting. 2013. Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity* 39: 432–441.
14. Latz, E., T. S. Xiao, and A. Stutz. 2013. Activation and regulation of the inflammasomes. *Nat. Rev. Immunol.* 13: 397–411.
15. Dostert, C., V. Pétrilli, R. Van Bruggen, C. Steele, B. T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320: 674–677.
16. Zhou, R., A. S. Yazdi, P. Menu, and J. Tschopp. 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469: 221–225.
17. Hornung, V., F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* 9: 847–856.
18. Compan, V., A. Baroja-Mazo, G. López-Castejón, A. I. Gomez, C. M. Martínez, D. Angosto, M. T. Montero, A. S. Herranz, E. Bazán, D. Reimers, et al. 2012. Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity* 37: 487–500.
19. Kahlenberg, J. M., K. C. Lundberg, S. B. Kertesy, Y. Qu, and G. R. Dubyak. 2005. Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-κB-driven protein synthesis. *J. Immunol.* 175: 7611–7622.
20. Perregaux, D., and C. A. Gabel. 1994. Interleukin-1β maturation and release in response to ATP and nigericin: evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J. Biol. Chem.* 269: 15195–15203.
21. Shimada, K., T. R. Crother, J. Karlin, J. Dagvadorj, N. Chiba, S. Chen, V. K. Ramanujan, A. J. Wolf, L. Vergnes, D. M. Ojcius, et al. 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36: 401–414.
22. Iyer, S. S., Q. He, J. R. Janczy, E. I. Elliott, Z. Zhong, A. K. Olivier, J. J. Sadler, V. Knepper-Adrian, R. Han, L. Qiao, et al. 2013. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39: 311–323.
23. Franchi, L., T. D. Kanneganti, G. R. Dubyak, and G. Núñez. 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J. Biol. Chem.* 282: 18810–18818.
24. Muñoz-Planillo, R., P. Kuffa, G. Martínez-Colón, B. L. Smith, T. M. Rajendiran, and G. Núñez. 2013. K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38: 1142–1153.
25. Pétrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* 14: 1583–1589.
26. Loo, Y. M., and M. Gale, Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34: 680–692.
27. Yoneyama, M., and T. Fujita. 2009. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol. Rev.* 227: 54–65.
28. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6: 981–988.
29. Rajan, J. V., D. Rodriguez, E. A. Miao, and A. Aderem. 2011. The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection. *J. Virol.* 85: 4167–4172.
30. Delaloye, J., T. Roger, Q. G. Steiner-Tardivel, D. Le Roy, M. Knaup Reymond, S. Akira, V. Petrilli, C. E. Gomez, B. Perdiguero, J. Tschopp, et al. 2009. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. *PLoS Pathog.* 5: e1000480.
31. Pothlichet, J., I. Meunier, B. K. Davis, J. P. Ting, E. Skamene, V. von Messling, and S. M. Vidal. 2013. Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells. *PLoS Pathog.* 9: e1003256.
32. Park, S., C. Juliana, S. Hong, P. Datta, I. Hwang, T. Fernandes-Alnemri, J. W. Yu, and E. S. Alnemri. 2013. The mitochondrial antiviral protein MAVS associates with NLRP3 and regulates its inflammasome activity. *J. Immunol.* 191: 4358–4366.
33. Rathinam, V. A., S. K. Vanaja, L. Waggoner, A. Sokolovska, C. Becker, L. M. Stuart, J. M. Leong, and K. A. Fitzgerald. 2012. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 150: 606–619.
34. Subramanian, N., K. Natarajan, M. R. Clatworthy, Z. Wang, and R. N. Germain. 2013. The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation. *Cell* 153: 348–361.
35. Mitoma, H., S. Hanabuchi, T. Kim, M. Bao, Z. Zhang, N. Sugimoto, and Y. J. Liu. 2013. The DHX33 RNA helicase senses cytosolic RNA and activates the NLRP3 inflammasome. *Immunity* 39: 123–135.
36. Kanneganti, T. D., N. Özören, M. Body-Malapel, A. Amer, J. H. Park, L. Franchi, J. Whitfield, W. Barchet, M. Colonna, P. Vandenabeele, et al. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440: 233–236.
37. Özören, N., J. Masumoto, L. Franchi, T. D. Kanneganti, M. Body-Malapel, I. Ertürk, R. Jagirdar, L. Zhu, N. Inohara, J. Bertin, et al. 2006. Distinct roles of TLR2 and the adaptor ASC in IL-1β/IL-18 secretion in response to *Listeria monocytogenes. J. Immunol.* 176: 4337–4342.
38. Kumar, H., S. Koyama, K. J. Ishii, T. Kawai, and S. Akira. 2008. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. *J. Immunol.* 180: 683–687.
39. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19–28.
40. Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc. Natl. Acad. Sci. USA* 103: 8459–8464.
41. Errett, J. S., M. S. Suthar, A. McMillan, M. S. Diamond, and M. Gale, Jr. 2013. The essential, nonredundant roles of RIG-I and MDA5 in detecting and controlling West Nile virus infection. *J. Virol.* 87: 11416–11425.
42. Frezza, C., S. Cipolat, and L. Scorrano. 2007. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat. Protoc.* 2: 287–295.
43. Franchi, L., N. Kamada, Y. Nakamura, A. Burberry, P. Kuffa, S. Suzuki, M. H. Shaw, Y. G. Kim, and G. Núñez. 2012. NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat. Immunol.* 13: 449–456.
44. Kanneganti, T. D., M. Body-Malapel, A. Amer, J. H. Park, J. Whitfield, L. Franchi, Z. F. Taraporewala, D. Miller, J. T. Patton, N. Inohara, and G. Núñez. 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* 281: 36560–36568.
45. Allen, I. C., M. A. Scull, C. B. Moore, E. K. Holl, E. McElvania-TeKippe, D. J. Taxman, E. H. Guthrie, R. J. Pickles, and J. P. Ting. 2009. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30: 556–565.
46. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozören, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, et al. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in *Salmonella*-infected macrophages. *Nat. Immunol.* 7: 576–582.
47. Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller, and A. Aderem. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1β via Ipaf. *Nat. Immunol.* 7: 569–575.
48. Schattgen, S. A., and K. A. Fitzgerald. 2011. The PYHIN protein family as mediators of host defenses. *Immunol. Rev.* 243: 109–118.
49. Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637–650.
50. Sander, L. E., M. J. Davis, M. V. Boekschoten, D. Amsen, C. C. Dascher, B. Ryffel, J. A. Swanson, M. Müller, and J. M. Blander. 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* 474: 385–389.
51. Fink, S. L., and B. T. Cookson. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect. Immun.* 73: 1907–1916.

52. Kaczmarek, A., P. Vandenabeele, and D. V. Krysko. 2013. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 38: 209–223.

53. Vandenabeele, P., W. Declercq, and T. Vanden Berghe. 2008. Necrotic cell death and 'necrostatins': now we can control cellular explosion. *Trends Biochem. Sci.* 33: 352–355.

54. Demidchik, V., S. N. Shabala, K. B. Coutts, M. A. Tester, and J. M. Davies. 2003. Free oxygen radicals regulate plasma membrane Ca^{2+}- and K^+-permeable channels in plant root cells. *J. Cell Sci.* 116: 81–88.

55. van der Zee, J., T. M. Dubbelman, and J. van Steveninck. 1985. Peroxide-induced membrane damage in human erythrocytes. *Biochim. Biophys. Acta* 818: 38–44.

56. Allam, R., K. E. Lawlor, E. C. Yu, A. L. Mildenhall, D. M. Moujalled, R. S. Lewis, F. Ke, K. D. Mason, M. J. White, K. J. Stacey, et al. Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Rep.* In press.

57. Zou, J., T. Kawai, T. Tsuchida, T. Kozaki, H. Tanaka, K. S. Shin, H. Kumar, and S. Akira. 2013. Poly IC triggers a cathepsin D- and IPS-1-dependent pathway to enhance cytokine production and mediate dendritic cell necroptosis. *Immunity* 38: 717–728.

58. Schlee, M., A. Roth, V. Hornung, C. A. Hagmann, V. Wimmenauer, W. Barchet, C. Coch, M. Janke, A. Mihailovic, G. Wardle, et al. 2009. Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31: 25–34.

59. Kato, H., K. Takahasi, and T. Fujita. 2011. RIG-I-like receptors: cytoplasmic sensors for non-self RNA. *Immunol. Rev.* 243: 91–98.

60. Li, H., A. Ambade, and F. Re. 2009. Cutting edge: necrosis activates the NLRP3 inflammasome. *J. Immunol.* 183: 1528–1532.