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Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1β production

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Interleukin 1β (IL-1β) is a potent proinflammatory factor during viral infection. Its production is tightly controlled by transcription of *Il1b* dependent on the transcription factor NF-κB and subsequent processing of pro-IL-1β by an inflammasome. However, the sensors and mechanisms that facilitate RNA virus–induced production of IL-1β are not well defined. Here we report a dual role for the RNA helicase RIG-I in RNA virus–induced proinflammatory responses. Whereas RIG-I-mediated activation of NF-κB required the signaling adaptor MAVS and a complex of the adaptors CARD9 and Bcl-10, RIG-I also bound to the adaptor ASC to trigger caspase-1-dependent inflammasome activation by a mechanism independent of MAVS, CARD9 and the Nod-like receptor protein NLRP3. Our results identify the CARD9–Bcl-10 module as an essential component of the RIG-I-dependent proinflammatory response and establish RIG-I as a sensor able to activate the inflammasome in response to certain RNA viruses.

Viral infections are a constant threat to higher organisms, and early detection of viruses by the innate immune system is critical for host defense. Mammalian antiviral immunity is initiated by germline-encoded pattern-recognition receptors that recognize specific pathogen-associated molecular patterns such as viral nucleic acids. After recognizing viral RNA or DNA, pattern-recognition receptors activate signaling pathways that trigger the production of type I interferons and inflammatory cytokines to orchestrate immune responses for virus elimination and thereby produce the clinical symptoms of a viral infection[1]. The viral nucleic acid–recognition receptors include the transmembrane Toll-like receptors (TLRs) TLR3, TLR7, TLR8 and TLR9 (refs. 2–4), the HIN200 family member AIM2 (refs. 5–8) and the cytoplasmic RIG-I-like helicases (RLHs) RIG-I (encoded by *Ddx58*) and Mda5 (encoded by *Ifih1*)[9,10].

RLHs are responsible for the detection of viral RNA in the cytosol[1]. They are composed of an RNA-binding helicase domain, a regulatory domain and two caspase-recruitment domains (CARDs) for signal propagation to the interferon-regulatory factor (IRF) and transcription factor NF-κB signaling pathways. Despite such similarities, the RLHs RIG-I and Mda5 detect distinct RNA viruses. The viruses recognized by RIG-I include vesicular stomatitis virus (VSV) and influenza virus, whereas Mda5 controls responses to picornaviruses (encephalomyocarditis virus (EMCV) and poliovirus) and other viruses[11]. The selective ligand for RIG-I is a 5′ triphosphate on double-stranded RNA[12–15]. The natural ligand for Mda5 remains to be identified, but long stretches of polyinosinic-polycytidylic acid (poly(I:C)) can serve as an artificial agonist for this RLH[16].

To engage downstream pathways after recognizing a virus, RLHs form homotypic CARD-CARD interactions with the adaptor protein MAVS[17–19], which results in the recruitment and activation of further signaling molecules to mitochondria-associated complexes. The adaptors TRAF3, TANK and TRADD and the kinases TBK1 and IKKε are responsible for activation of the transcription factors IRF3 and IRF7 and subsequent synthesis of type I interferon[20]. RLHs additionally activate the proinflammatory NF-κB pathway for the production of cytokines such as interleukin 1β (IL-1β) and IL-6 (ref. 1), but the mechanisms that relay RLH signaling to NF-κB are not well defined.

The production of IL-1β requires, in addition to NF-κB-dependent new synthesis of pro-IL-1β, a second signal that triggers caspase-1 activation. Caspase-1 is responsible for the proteolytic processing of pro-IL-1β into mature, bioactive IL-1β. The activation of caspase-1 in response to many distinct danger signals depends on cytoplasmic multiprotein complexes called inflammasomes, which assemble

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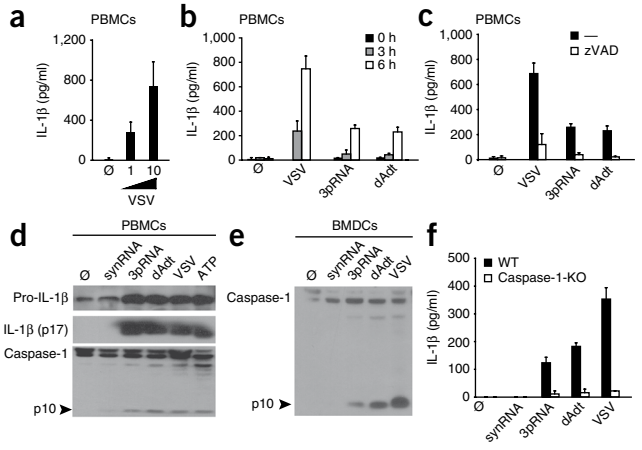

Figure 1 RIG-I signaling is required and sufficient for IL-1β production after infection with an RNA virus. (**a**,**b**) Enzyme-linked immunosorbent assay (ELISA) of IL-1β in supernatants of human PBMCs (2×10^6) left unstimulated (∅) or stimulated for 6 h with VSV at a multiplicity of infection of 1 or 10 (wedge; **a**) or for various times (key) with VSV (multiplicity of infection, 5) or 2 μg/ml of 3pRNA or poly(dA:dT) (dAdT; **b**). (**c**) ELISA of IL-1β production by PBMCs stimulated for 6 h as described in **b**, with (zVAD) or without (–) the addition of 0.05 μM z-VAD-fmk (pan-caspase inhibitor) 1 h before stimulation. (**d**) Immunoblot analysis of mature IL-1β (p17) and processed caspase-1 (p10 subunit) in supernatants of PBMCs stimulated with 2 μg/ml of synthetic dsRNA that lacks the 5′ triphosphate (synRNA), 3pRNA or poly(dA:dT), or with VSV (multiplicity of infection, 5). ATP (far right), LPS-primed PBMCs stimulated with 5 mM ATP (positive control). (**e**) Immunoblot analysis of caspase-1 processing (p10 subunit) in supernatants of BMDCs (1×10^6 cells per ml) stimulated for 6 h as described in **d**. (**f**) ELISA of IL-1β secretion by wild-type (WT) and caspase-1-deficient (Caspase-1-KO) BMDCs treated for 6 h with various stimuli (horizontal axis). Data are representative of three (**a**–**c**) or two (**f**) independent experiments (mean and s.e.m.) or are from one experiment representative of three (**d**) or at least three (**e**) experiments.

from various sensors and associated adaptor proteins in a context-dependent manner[21]. The best understood of these is the NLRP3 (also called NALP3) inflammasome, which activates caspase-1 indirectly via the inflammasome adaptor ASC (also called Pycard) in response to very diverse triggers, including crystals (such as uric acid, silica and asbestos), bacterial pore-forming toxins (such as nigericin), vaccine adjuvants, fungi, and certain DNA and RNA viruses[21–25]. However, the molecular interactions that engage the relatively non-specific NLRP3 inflammasome in response to such distinct stimuli are unclear at present. Another type of inflammasome that has been linked to viral recognition is the AIM2 inflammasome[5–8]. AIM2 is a cytoplasmic DNA receptor that directly interacts with ASC to trigger caspase-1 activation and subsequent IL-1β secretion after infection with a DNA virus. Here we demonstrate that RIG-I serves as a dual sensor that can trigger both NF-κB-dependent production of pro-IL-1β and inflammasome activation in response to certain RNA viruses. In this context, RIG-I engages the CARD9–Bcl-10 module for NF-κB activation and triggers ASC for inflammasome activation in an NLRP3-independent manner.

RESULTS

RIG-I in IL-1β production after VSV infection

To investigate the functions of RIG-I in RNA virus–induced production of IL-1β, we first infected human peripheral blood mononuclear cells (PBMCs) with VSV (**Fig. 1**). PBMCs exposed to VSV secreted mature IL-1β in a dose- and time-dependent manner (**Fig. 1a**,**b**). To selectively assess the role of RIG-I triggering in IL-1β production without considering the effects of viral RNA on other receptor systems, including TLRs, we transfected the cells with the selective RIG-I agonist 5′-triphosphate RNA (3pRNA). RIG-I ligation was sufficient to induce IL-1β production similar to that induced by transfected double-stranded DNA (poly(dA:dT)), which activates the AIM2 inflammasome[5–8] (**Fig. 1b**). Different 3pRNA species with distinct sequences resulted in similar IL-1β secretion (**Supplementary Fig. 1**), which indicated that the specific RNA sequence was not involved.

Next we pretreated PBMCs with the pan-caspase inhibitor z-VAD-fmk (**Fig. 1c**). Caspase inhibition abrogated IL-1β production after stimulation with 3pRNA and poly(dA:dT) and resulted in much less secretion of IL-1β induced by VSV (**Fig. 1c**). Then we analyzed the autocatalytic formation of the active caspase-1 subunit p10 (**Fig. 1d**). Consistent with the production of the mature (p17) form of IL-1β, caspase-1 was processed after activation of the NLRP3 inflammasome induced by ATP after priming with lipopolysaccharide (LPS) or after stimulation of cells with 3pRNA, poly(dA:dT) or VSV (**Fig. 1d**). In contrast, stimulation of cells with a synthetic double-stranded RNA (dsRNA) that lacks the 5′ triphosphate and triggers TLR7 but not RIG-I (ref. 26) did not induce IL-1β production or caspase-1 activation (**Fig. 1d**). Transfection of 3pRNA or poly(dA:dT) or infection with VSV also induced robust caspase-1 activation and IL-1β production in mouse bone marrow–derived dendritic cells (BMDCs), but transfection of the synthetic dsRNA lacking the 5′ triphosphate did not (**Fig. 1e**). As IL-1β production in response to these stimuli was defective in BMDCs from caspase-1-deficient mice (**Fig. 1f**), we conclude that caspase-1 activation is required for the 3pRNA- or VSV-induced proinflammatory responses. Together these results demonstrate that RIG-I engagement activates caspase-1 for IL-1β production.

To investigate whether RIG-I signaling is required for IL-1β production, we used DCs from RIG-I-deficient mice[16] (**Fig. 2**). Consistent with published data indicating that RIG-I ligation

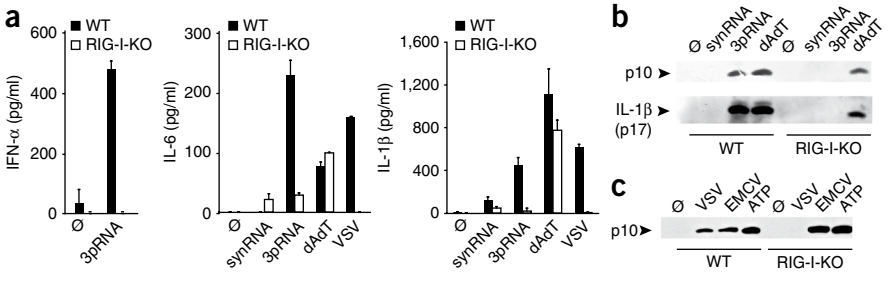

Figure 2 RIG-I controls IL-1β production and caspase-1 activation after detecting RNA viruses. (**a**) ELISA of IFN-α, IL-6 and IL-1β in supernatants of wild-type and RIG-I-deficient (RIG-I-KO) BMDCs treated for 6 h with various stimuli (horizontal axes). (**b**) Immunoblot analysis of caspase-1 processing (p10) and mature IL-1β (p17) in supernatants of the cells in **a**. (**c**) Immunoblot analysis of caspase-1 processing (p10) in wild-type and RIG-I-deficient BMDCs cells treated for 6 h with various stimuli (above lanes). ATP, LPS-primed BMDCs stimulated with 5 mM ATP (positive control). Data are representative of three independent experiments (mean and s.e.m.; **a**) or are from one experiment representative of three (**b**,**c**).

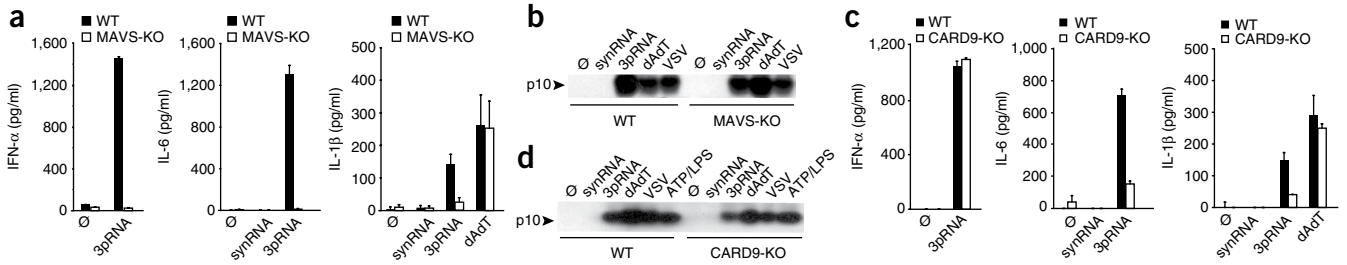

Figure 3 MAVS and CARD9 are essential for RIG-I-induced production of IL-1β but are dispensable for inflammasome activation. (**a**,**b**) ELISA of IFN-α, IL-6 and IL-1β in supernatants of wild-type and MAVS-deficient (MAVS-KO) BMDCs treated for 6 h with various stimuli (horizontal axes). (**b**) Immunoblot analysis of caspase-1 processing (p10) in supernatants of the cells in **a**. (**c**) ELISA of IFN-α, IL-6 and IL-1β in supernatants of wild-type and CARD9-deficient (CARD9-KO) BMDCs treated as described in **a**. (**d**) Immunoblot analysis of caspase-1 processing (p10) in supernatants of the cells in **c**. Data are representative of three (**a**) or at least four (**c**) independent experiments (mean and s.e.m.) or are from one experiment representative of three (**b**) or at least four (**d**) independent experiments.

activates the IRF and NF-κB transcription factors[10], RIG-I-deficient DCs were defective in IRF-controlled production of interferon-α (IFN-α) as well as in NF-κB-regulated secretion of IL-6 after stimulation with 3pRNA or VSV (**Fig. 2a**). RIG-I was required for VSV- or 3pRNA-triggered activation of caspase-1 and production of mature IL-1β p17, although poly(dA:dT) stimulation, exposure to EMCV or activation of NLRP3 with ATP and LPS induced normal activation of caspase-1 in RIG-I-deficient cells (**Fig. 2b**,**c**). Thus, RIG-I controls IRF- and NF-κB-dependent cytokine synthesis as well as inflammasome activation in response to certain RNA viruses.

RIG-I engages MAVS, CARD9 and Bcl-10

To understand the mechanisms that link RIG-I ligation to NF-κB-dependent synthesis of pro-IL-1β and to the activation of caspase-1 and inflammasomes, we investigated the role of the RIG-I effector MAVS in these pathways. Consistent with the essential role of MAVS in the activation of IRF and NF-κB[18,19], MAVS-deficient cells did not synthesize IFN-α or IL-6 after treatment with the RIG-I agonist 3pRNA (**Fig. 3a**). Moreover, MAVS-deficient cells did not secrete IL-1β after activation with RIG-I, although they produced normal amounts of IL-1β after stimulation with poly(dA:dT) (**Fig. 3a**). However, unlike RIG-I-deficient cells, MAVS-deficient BMDCs had normal activation of caspase-1 in response to stimulation with VSV or 3pRNA (**Fig. 3b**). These results indicate that although MAVS signaling is required for RIG-I-mediated production of IL-1β, MAVS engagement is not involved in RIG-I-mediated activation of caspase-1.

The E3 ubiquitin ligase TRIM25 catalyzes lysine 63–linked polyubiquitination of RIG-I to induce the recruitment of MAVS to RIG-I for the activation of effector pathways[27]. Consistent with the data reported above, TRIM25-deficient cells showed defects in IL-1β secretion after VSV infection (**Supplementary Fig. 2a**). However, caspase-1 was activated normally in VSV-infected or 3pRNA-stimulated TRIM25-deficient cells (**Supplementary Fig. 2b**).

Published work has identified the CARD coiled-coil protein CARD9 as a multifunctional adaptor that relays inputs from various pathogens to proinflammatory cascades[28]. We considered that CARD9 might also have a role in RIG-I signaling. To assess that possibility, we treated CARD9-deficient BMDCs with 3pRNA, synthetic dsRNA lacking the 5′ triphosphate, or poly(dA:dT). CARD9-deficient BMDCs showed much less of production IL-6 and IL-1β after stimulation with RIG-I, whereas the responses to poly(dA:dT) remained largely unchanged (**Fig. 3c**). Like MAVS, CARD9 was dispensable for caspase-1 activation (**Fig. 3d**). However, unlike MAVS, CARD9 was completely dispensable for IFN-α secretion (**Fig. 3c**). Thus, CARD9 selectively controls the RIG-I- and MAVS-induced proinflammatory response.

CARD9 is an upstream activator of the NF-κB pathway and of mitogen-activated protein kinases[29–31]. To define the role of CARD9 in RIG-I and MAVS signaling, we measured the activation of NF-κB in BMDCs stimulated with 3pRNA. RIG-I triggered a robust NF-κB response in wild-type BMDCs but not in cells lacking MAVS or CARD9 (**Fig. 4a**), which indicated that the two proteins act together to facilitate NF-κB activation. RIG-I engagement by 3pRNA or VSV also activated the kinases Jnk and p38, although in BMDCs this activation was independent of CARD9 (**Fig. 4b**). To determine the consequences of those findings for IL-1β production, we studied RIG-I-induced synthesis of pro-IL-1β. Consistent with the requirement for NF-κB activation in pro-IL-1β induction, both MAVS-deficient and CARD9-deficient BMDCs showed a defect in 3pRNA-induced synthesis of pro-IL-1β, although they responded normally to poly (dA:dT) (**Fig. 4c**). Accordingly, inhibition of canonical NF-κB signaling with a specific IKK kinase inhibitor, as well as CARD9 deletion, abrogated RIG-I-triggered upregulation of the secretion of pro-IL-1β mRNA and IL-1β protein after VSV infection (**Supplementary Fig. 3**). Thus, MAVS and CARD9 are essential for RIG-I-mediated activation of NF-κB and synthesis of pro-IL-1β. These findings explain why MAVS-deficient and CARD9-deficient cells fail to produce IL-1β although they regularly activate caspase-1 after RIG-I triggering.

CARD9-triggered activation of NF-κB depends on the CARD-containing adaptor Bcl-10 (refs. 28,30), which in turn can recruit the paracaspase MALT1 and use MALT1-dependent or MALT1-independent mechanisms for cell activation[32]. We next stimulated Bcl-10-deficient and MALT1-deficient cells with 3pRNA. BMDCs that lacked Bcl-10, like CARD9-deficient cells, showed severe defects in RIG-I-induced production of pro-IL-1β and secretion of IL-1β and IL-6 but had normal interferon responses (**Fig. 4d–f**) and caspase-1 activation (data not shown). The response to poly(dA:dT) remained unaffected by the Bcl-10 deletion (**Fig. 4e**,**f**). Notably, MALT1 was entirely dispensable for RIG-I-induced cytokine production (data not shown).

Together, the genetic experiments reported above demonstrated a signaling cascade downstream of RIG-I and MAVS that depended on CARD9 and Bcl-10 to control NF-κB-dependent cytokine production. To study the importance of this pathway *in vivo*, we infected wild-type and CARD9-deficient mice with VSV. Intravenous injection induced vigorous production of IFN-α, IL-6 and IL-1β in wild-type mice (**Fig. 4g**). Consistent with the *in vitro* results, the interferon responses were intact, but the concentrations of IL-6 and IL-1β were significantly lower in the serum of CARD9-deficient mice (**Fig. 4g**).

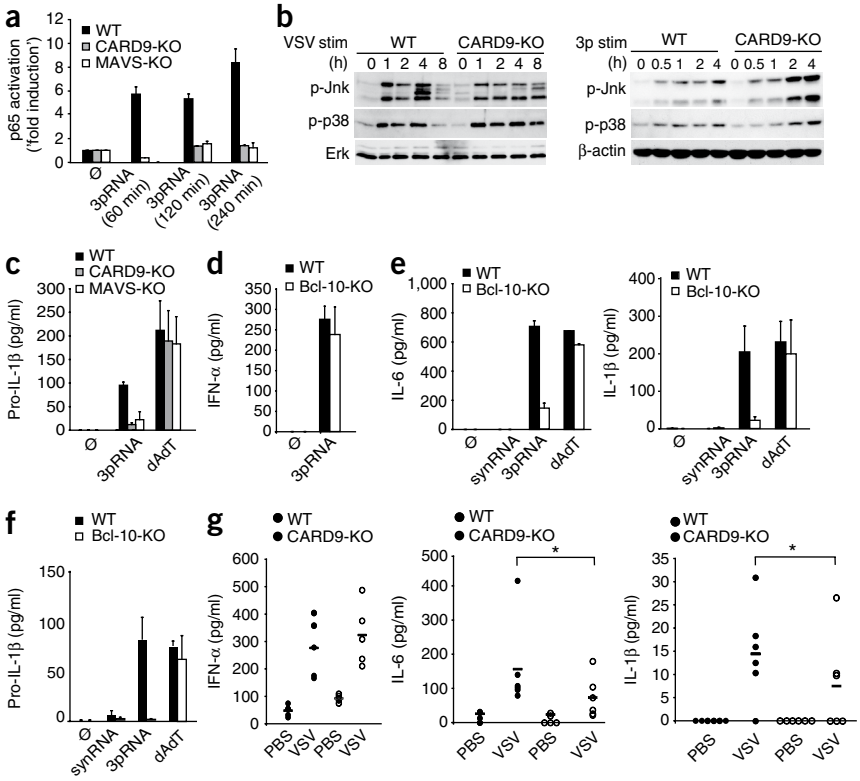

Figure 4 MAVS, CARD9 and Bcl-10 control pro-IL-1β production after RIG-I ligation. (**a**) Activation of the p65 subunit of NF-κB in nuclear protein extracts of wild-type, CARD9-deficient and MAVS-deficient BMDCs stimulated for 60, 120 or 240 min (horizontal axis) with 3pRNA, assessed by enhanced chemiluminescence and presented relative to activation in the unstimulated sample. (**b**) Immunoblot analysis of wild-type and CARD9-deficient BMDCs stimulated for various times (above lanes) with VSV (left) or 3pRNA (right), probed with antibodies specific for phosphorylated (p-) Jnk and p38. Bottom, immunoblot analysis of Erk and β-actin (loading control). (**c**) ELISA of intracellular pro-IL-1β in wild-type, CARD9-deficient and MAVS-deficient BMDCs stimulated for 6 h with 3pRNA or poly(dA:dT), assessed after cell lysis by repeated cycles of freezing and thawing. (**d**–**f**) ELISA of IFN-α (**d**), IL-6 and IL-1β (**e**), and intracellular pro-IL-1β (**f**) in supernatants of wild-type and Bcl-10-deficient (Bcl-10-KO) BMDCs treated with various stimuli (horizontal axes). (**g**) ELISA of IFN-α, IL-6 and IL-1β in serum collected from wild-type and CARD9-deficient mice 6 h after intravenous injection of 2×10^6 plaque-forming units of VSV or PBS (control). Each symbol represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.05$ (two-tailed Student's *t*-test). Data are representative of at least three (**a**,**c**) or three (**d**–**f**) independent experiments (mean and s.e.m.) or two experiments (**g**) or are from one experiment representative of at least four independent experiments (**b**).

RIG-I and ASC form an NLRP3-independent inflammasome

As the role of the MAVS–CARD9–Bcl-10 axis in RIG-I-induced production of IL-1β is restricted to the control of pro-IL-1β synthesis, it is still unclear how RIG-I activates caspase-1. Because the cytosolic bacterium *Francisella tularensis* induces a type I interferon response to indirectly activate caspase-1 (ref. 33), we considered possible indirect effects of interferon in RIG-I-mediated inflammasome activation. However, *Irf3*$^{-/-}$*Irf7*$^{-/-}$ BMDCs[34] which do not produce type I interferons, had normal production of IL-1β after stimulation with 3pRNA or DNA (**Fig. 5a**).

Many classical triggers of inflammasomes require a stimulus-induced potassium efflux for caspase-1 activation[21]. We therefore assessed RIG-I-induced production of IL-1β before and after pre-incubation with the potassium channel inhibitor glibenclamide. This treatment completely abrogated 3pRNA- or VSV-induced secretion of IL-1β, whereas IL-6 production was unaffected (**Fig. 5b**,**c**). We obtained similar results by adding excess extracellular potassium (130 mM) to the medium before RIG-I stimulation (data not shown).

Danger sensors of the NLRP family and the DNA sensor AIM2 activate caspase-1 by binding to ASC[35]. To determine whether RIG-I and ASC also form a caspase-1-activating signaling complex, we immunoprecipitated endogenous ASC from THP-1 human monocytic cells before and after infection with VSV and studied potential RIG-I interactions by immunoblot analysis. RIG-I precipitated together with ASC in uninfected and VSV-infected cells, but Mda5 did not (**Fig. 5d**), which indicated that RIG-I and ASC can form a complex.

To investigate the function of RIG-I–ASC interactions by genetic means, we studied IL-1β production in BMDCs from ASC-deficient mice. In parallel, we analyzed wild-type and NLRP3-deficient DCs. Wild-type DCs showed robust caspase-1 activation and mature IL-1β production in response to triggering of RIG-I with 3pRNA or VSV,

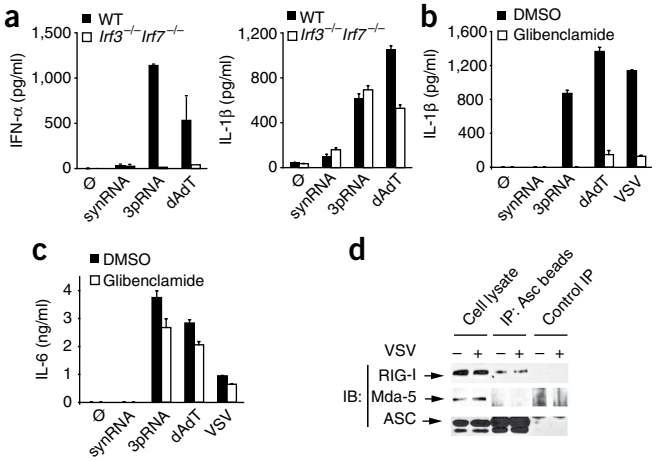

Figure 5 RIG-I engages ASC to induce inflammasome activation. (**a**) ELISA of IFN-α and IL-1β in supernatants of wild-type BMDCs and BMDCs doubly deficient in IRF3 and IRF-7 (*Irf3*$^{-/-}$*Irf7*$^{-/-}$) treated for 6 h with various stimuli (horizontal axes). (**b**,**c**) ELISA of IL-1β (**b**) and IL-6 (**c**) in supernatants of wild-type BMDCs treated for 6 h with various stimuli (horizontal axes) in the presence or absence (DMSO) of the potassium channel inhibitor glibenclamide. (**d**) Immunoassay of THP-1 cells infected with VSV (+) or left uninfected (–); immunoprecipitation of proteins from lysates with ASC-specific antibody (IP: Asc beads) or control antibody (Control IP) was followed by immunoblot analysis (IB) of immunoprecipitates (right four lanes) or total lysates (far left two lanes). Data are representative of two (**a**) or three (**b**,**c**) independent experiments (mean and s.e.m.) or are from one experiment representative of at least four independent experiments (**d**).

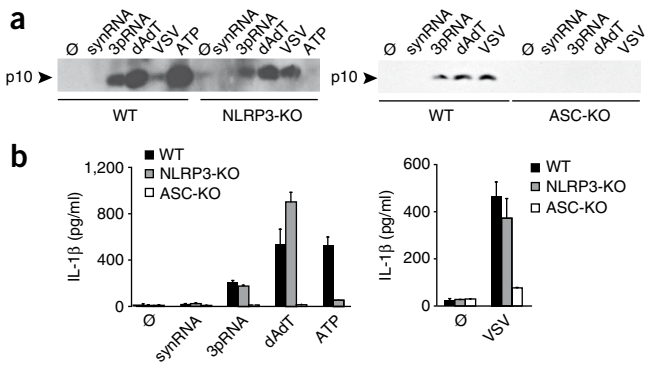

Figure 6 RIG-I-induced inflammasome activation is independent of NLRP3. (**a**) Immunoblot analysis of processed caspase-1 (p10) in supernatants of wild-type, NLRP3-deficient (NLRP3-KO) and ASC-deficient (ASC-KO) BMDCs treated with various stimuli (above lanes). ATP, cells pulsed with 5 mM ATP after 3 h of priming with ultrapure LPS (50 ng/ml). (**b**) ELISA of IL-1β in supernatants of the cells in **a**. Data are representative of at least three independent experiments (mean and s.e.m. in **b**).

after stimulation of AIM2 with poly(dA:dT) DNA, and after activation of NLRP3 with ATP after LPS priming (**Fig. 6a**). As expected, NLRP3-deficient DCs failed to activate caspase-1 (**Fig. 6a**) or to produce IL-1β (**Fig. 6b**) in response to ATP but responded normally to poly(dA:dT), as well as to triggering of RIG-I with 3pRNA or VSV. In contrast, ASC-deficient cells showed defective activation of caspase-1 and secretion of mature IL-1β after stimulation with ATP and LPS, poly(dA:dT), 3pRNA or VSV (**Fig. 6**). Thus, ASC is essential for coupling RIG-I-mediated recognition of RNA to NLRP3-independent caspase-1 activation for IL-1β production. We have proposed a model for the RIG-I-triggered proinflammatory responses and inflammasome activation (**Supplementary Fig. 4**).

Picornavirus engages Mda5, CARD9 and NLRP3

Finally, we investigated whether Mda5 engages similar mechanisms to induce IL-1β production. For this, we first infected Mda5-deficient or CARD9-deficient cells with the Mda5-engaging picornavirus EMCV[16]. Wild-type cells produced IL-1β robustly after EMCV infection (**Fig. 7a**,**b**). However, neither Mda5-deficient nor CARD9-deficient cells showed an IL-1β response (**Fig. 7b**), which indicates that CARD9 is an effector of Mda5 signaling. Further experiments demonstrated that CARD9 was required for EMCV-induced activation of NF-κB and was involved in pro-IL-1β synthesis (**Fig. 7c**,**d**). Next we assessed the role of Mda5 in inflammasome activation. In contrast to RIG-I activation in response to 3pRNA, transfection of poly(I:C) as an agonist for Mda5 (ref. 16) did not induce caspase-1 activation (**Fig. 7e**), which suggested that Mda5 activation by itself might not be sufficient for inflammasome activation. To study the requirement for Mda5 in inflammasome activation in response to the intact EMCV virus, we infected Mda5-deficient cells with EMCV (**Fig. 7f**). In parallel, we incubated the cells with the RIG-I-activating virus VSV and also infected NLRP3-deficient cells with the two viruses. Consistent with our results above (**Fig. 6a**,**b**), VSV strongly activated caspase-1 and led to potent IL-1β secretion in NLRP3-deficient cells. VSV also induced inflammasome activation in Mda5-deficient BMDCs (**Fig. 7f**). In contrast, EMCV infection required NLRP3 and Mda5 for caspase-1 activation and IL-1β production (**Fig. 7f–h**).

DISCUSSION

Here we have reported the identification of two RLH effector mechanisms that act together in the production of proinflammatory cytokines in response to recognition of RNA viruses. Although RLH-induced activation of NF-κB depended on the CARD9–Bcl-10 complex, RIG-I also activated the inflammasome by forming a signaling complex with ASC. Published work has demonstrated that RIG-I uses MAVS for activation of NF-κB and IRF[17–19]. We found that CARD9- and Bcl-10-deficient cells had profound defects in RIG-I-induced production of IL-6 and pro-IL-1β but had normal interferon responses, which provided genetic evidence that CARD9 and Bcl-10 act together downstream of MAVS to selectively control proinflammatory responses. Like RIG-I, Mda5 signals through MAVS[1]. We also observed a requirement for CARD9 in Mda5-induced production of proinflammatory cytokines. Thus, CARD9 represents a common and essential switch in RLH signaling that segregates the proinflammatory response from interferon production.

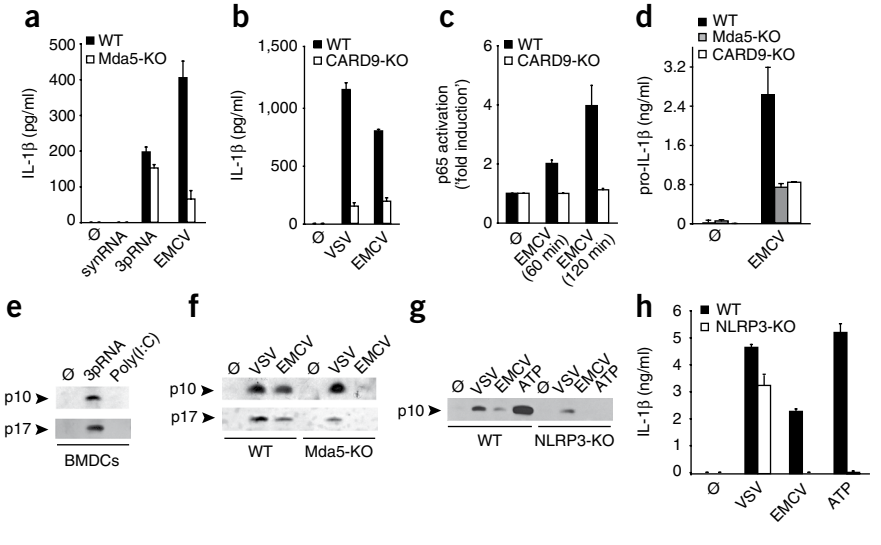

Figure 7 Mda5 requires NLRP3 for inflammasome activation. (**a**,**b**) ELISA of IL-1β in supernatants of wild-type, Mda5-deficient (Mda5-KO) and CARD9-deficient BMDCs treated for 6 h with various stimuli (horizontal axes). (**c**) Activation of the p65 subunit of NF-κB in nuclear protein extracts of wild-type and CARD9-deficient BMDCs stimulated for 60 or 120 min with EMCV, assessed by enhanced chemiluminescence and presented relative to activation in the unstimulated sample. (**d**) ELISA of intracellular pro-IL-1β in wild-type, Mda5-KO and CARD9-deficient cells, measured after cell lysis by repeated cycles of freezing and thawing. (**e**) Immunoblot analysis of caspase-1 processing (p10) and mature IL-1β (p17) in BMDCs stimulated for 6 h with 3pRNA or poly(I:C). (**f**) Immunoblot analysis of caspase-1 processing (p10) and mature IL-1β (p17) in wild-type and Mda5-deficient BMDCs infected for 6 h with VSV or EMCV. (**g**,**h**) Immunoblot analysis of caspase-1 processing (p10; **g**) and ELISA of IL-1β (**h**) in supernatants of wild-type and NLRP3-deficient BMDCs infected for 6 h with VSV or EMCV. ATP, LPS-primed BMDCs stimulated with 5 mM ATP (positive control). Data are representative of three (**a**,**b**,**d**), two (**c**) or at least three (**h**) independent experiments (mean and s.e.m.) or are from one experiment representative of QQ (**e**), three (**f**) or at least three (**g**) independent experiments.

CARD9 relays signals from various pattern-recognition receptors to proinflammatory pathways[28,36]. Together with its effector Bcl-10, CARD9 controls activation of the canonical NF-κB pathway after ligation of surface receptors containing or coupled to an immunoreceptor tyrosine-based activation motif, including dectin-1, FcγRIII, TREM-1 and Mincle[37]. This function of CARD9 is important for host defense against fungi and innate responses to certain bacteria, such as *Mycobacterium tuberculosis*[30,38]. Consistent with the function of CARD9–Bcl-10 complexes in NF-κB activation, we observed defective NF-κB signaling in CARD9-deficient and Bcl-10-deficient cells stimulated with agonists of RIG-I or Mda5. Those findings establish CARD9–Bcl-10 complexes as a missing link between RLHs and NF-κB. Previous work has shown that CARD9 can activate Jnk and p38, at least after macrophage infection with whole VSV particles[39]. However, we did not detect defects in Jnk or p38 signaling in CARD9-deficient DCs stimulated with 3pRNA. The fact that VSV triggers several innate signaling systems, including TLRs[40], and that CARD9 signaling to Jnk and p38 differs in DCs and macrophages[29] could explain why we did not observe Jnk or p38 signaling defects in our experiments, which focused specifically on the RLH-induced pathway.

Bcl-10 is also required for T cell and B cell antigen receptor signaling. In lymphocytes, Bcl-10 engages TRAF2 and TRAF6 to mediate lysine 63–linked polyubiquitinylation of IKKγ, which induces subsequent IKK activation[32]. It is therefore possible that a similar mechanism operates downstream of RLHs. Caspase-8 and FADD are also involved in antigen receptor–induced NF-κB activation[41], and a study has reported roles for caspase-8 and FADD downstream of MAVS[42]. Thus, several NF-κB activators seem to be shared by the RLH and antigen-receptor pathways.

Our work has additionally established RIG-I as a cytosolic RNA sensor for inflammasome activation. Selective RIG-I triggering was sufficient for caspase-1 activation and RIG-I was required for inflammasome activation in response to stimulation with 3pRNA or infection with VSV. In contrast to NF-κB signaling and pro-IL-1β production, this process was entirely independent of MAVS, TRIM25 and CARD9; it was also independent of NLRP3. To our knowledge, this represents the first example of MAVS-independent RIG-I signal transduction. RIG-I-induced inflammasome activation shows similarities to the activation of AIM2 inflammasomes by viral double-stranded DNA, which is also NLRP3 independent[35]. After binding double-stranded DNA, AIM2 interacts with ASC to induce ASC oligomerization and subsequent IL-1β production. Likewise, RIG-I can form a protein complex containing ASC, potentially with additional components, to induce IL-1β production in response to some RNA viruses. Thus, RIG-I and ASC can constitute a distinct inflammasome.

Influenza virus activates the NLRP3 inflammasome[22,24,25]. Influenza enters the cell via the lysosomal pathway, and lysosomal damage, which is a common NLRP3 activator, could trigger NLRP3 in this context. Consistent with that, lysosomal maturation is essential for influenza virus–induced NLRP3 activation[22]. We also observed a requirement for NLRP3 in IL-1β production after infection with EMCV. In contrast, VSV did not activate the NLRP3 inflammasome and instead relied on RIG-I and ASC. Our results and previously published data therefore indicate that inflammasome activation by RNA viruses can in principle use NLRP3-dependent or NLRP3-independent mechanisms. The precise viral ligands that trigger the NLRP3 inflammasome and the cellular sensors that detect these ligands remain to be identified. Although Mda5 was required for ECMV-induced inflammasome activation, ligation of Mda5 with poly(I:C) was not sufficient to induce inflammasome activation. In this context, the function of Mda5 might thus be restricted to priming of the NLRP3 inflammasome; that is, activation of Mda5 could potentially upregulate NLRP3, which is an essential step for activation of the NLRP3 inflammasome[43,44].

Together our findings have indicated that RLHs can trigger at least three different cellular responses: interferon production, NF-κB activation and inflammasome activation. RIG-I could in principle signal from one large signaling complex that 'fine tunes' interferon and proinflammatory responses. Alternatively, RIG-I might be a sensor in several distinct signalosomes: one may contain RIG-I together with MAVS, TRAF3 and TBK-1 for IRF activation; a second may involve MAVS, CARD and Bcl-10 for the activation of NF-κB; and a third might contain RIG-I together with ASC and potentially other factors for caspase-1 activation. Precisely how RIG-I integrates these cellular responses will be an important topic of future research, but our results offer one molecular explanation for the longstanding finding that RNA viruses are potent inducers of proinflammatory cytokines such as IL-1β and IL-6. In addition, as activation of the inflammatory responses forms a critical link to the induction of adaptive immunity, our results may have implications for the development of vaccines.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

H.P., M.B., O.G., G.H., V.H. and J.R. designed the research; H.P., M.B., O.G., K.F., S.R., N.H., M.R. and M.S. did experiments; W.B., H.K., S.A. and S.I. contributed critical reagents; H.P., M.B., O.G., S.R., S.E., C.P., V.H., G.H. and J.R. analyzed results; H.P. and M.B. prepared the figures; and H.P., M.B., O.G., G.H. and J.R. wrote the paper.

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ONLINE METHODS

Mice. Mice genetically deficient in NLRP3, ASC, RIG-I, IRF3 and IRF7, CARD9, Bcl-10, MALT1, MAVS, Mda5, TRIM25 or caspase-1 have been described[16,27,30,34,45–48]. Mice were 6–12 weeks of age at the onset of experiments and were used according to local guidelines. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Media and reagents. RPMI-1640 medium (Invitrogen) and DMEM (Invitrogen) were supplemented with 10% (vol/vol) FCS (Hyclone), 3 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Sigma-Aldrich). ATP (A6419), poly(dA:dT) sodium salt (P9764), Glyburide (glibenclamide) and Bay11-7082 (specific IKK kinase inhibitor) were from Sigma-Aldrich. OptiMEM reduced-serum medium was from Invitrogen. Both poly(I:C) and ultrapure LPS (from *Escherichia coli* strain K12; used at a concentration of 50 ng/ml) were from Invivogen. The pan-caspase inhibitor z-VAD-fmk (benzyloxycarbonyl–Val-Ala-Asp–fluoromethylketone) was from Calbiochem. Chemically synthesized RNA oligonucleotides were from MWG-Biotech. Double-stranded *in vitro*–transcribed 3pRNA was generated as described[26]. Synthetic dsRNA lacking the 5′ triphosphate (sense, 5′-GCAUGCGACCUCUGUUUGA-3′) and 3pRNA1 (sense, 5′-GCAUGCGACCUCUGUUUGAC-3′) were used in most experiments; 3pRNA2 (sense, 5′-GCAUGCGAGGACUGUUUGAC-3′) was used to exclude sequence-specific effects on inflammasome activation.

Cells. Human PBMCs were isolated from whole human blood of healthy, voluntary donors by Ficoll-Hypaque density-gradient centrifugation (Biochrom). BMDCs were generated and grown as described[23]. Experiments involving human materials were in accordance with precepts established by the Helsinki Declaration and approved by the local ethics committee.

Cell culture and stimulation. All cells were stimulated in OptiMEM reduced-serum medium (Invitrogen) at a density of 1×10^6 cells per ml. Where not indicated otherwise, cells were incubated for 6–8 h with 2 μg/ml of synthetic RNA, 3pRNA or poly(dA:dT). Cells were transfected with RNA and DNA with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). In some experiments, ultrapure LPS (50 ng/ml) and ATP (5 mM) were used as positive controls. For all conditions, cell-free supernatants were analyzed for cytokine secretion by ELISA or cells were lysed for immunoprecipitation and/or immunoblot analysis. Glibenclamide (25 μM) and z-VAD-fmk (0.05 μM) were used as described[23].

Cytokine measurement. Cell supernatants and serum were analyzed for cytokine secretion by ELISA (BD, R&D Systems or PBL Biomedical Laboratories). For analysis of intracellular pro-IL-1β, cells were lysed by repeated cycles of freezing and thawing in RPMI medium containing 10% (vol/vol) FCS, 3 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin and were analyzed by ELISA.

Immunoblot analysis. Precipitated media supernatants or cell extracts were analyzed by standard immunoblot techniques[49]. Primary antibodies were polyclonal goat antibody to mouse-IL-1β (anti–mouse IL-1β; BAF401; R&D Systems), polyclonal rabbit anti–human IL-1β (D116; Cell Signaling), monoclonal mouse anti-RIG-I (Alme-1; Alexis), polyclonal rabbit anti-Mda5 (AT113; Alexis), polyclonal rabbit anti-caspase-1 (sc-514 and sc-515; Santa Cruz), and polyclonal rabbit anti-Erk (9102), monoclonal rabbit antibody to phosphorylated p38 (12F8; 4631), polyclonal rabbit antibody to phosphorylated Jnk (9251) and polyclonal rabbit anti-β-actin (4967; all from Cell Signaling).

NF-κB activation. Nuclear extracts were prepared according to standard methods, and 1 μg nuclear protein was analyzed with a NF-κB p65 Transcription Factor Assay kit (Pierce) as described[50].

Coimmunoprecipitation. Endogenous RIG-I was immunoprecipitated from 5×10^6 THP-1 cells seeded in 10-cm dishes with or without 3 h of VSV stimulation (multiplicity of infection, 10). Cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris pH 7.0, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 1 mM Na_3VO_4, 1 mM phenylmethyl sulfonyl fluoride and a Protease Inhibitor Cocktail tablet (Roche)) and lysates were incubated overnight at 4 °C with 80 μl protein G Sepharose beads (GE Healthcare) and 2 μg antibody (polyclonal anti-ASC (AL177; Alexis Biochemicals) or polyclonal rabbit anti–Syrian hamster immunoglobulin G (307-005-003; Jackson ImmunoResearch)). Immunoprecipitates were analyzed by immunoblot.

RNA extraction and quantification. Total RNA was extracted from cells with a High Pure RNA Isolation kit as described by the manufacturer (Roche) and was analyzed by quantitative RT-PCR. RNA (1 μg) was reverse-transcribed with SuperScript II Reverse Transcriptase and oligo(dT) oligonucleotide according to the manufacturer's protocol (Invitrogen). The Universal ProbeLibrary and LightCycler 480 system (Roche) were used for quantitative PCR (primer sequences, **Supplementary Table 1**). Gene expression was calculated as a ratio of the expression of the gene of interest to that of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase) measured for the same sample.

Preparation of virus stock and plaque assay. Baby hamster kidney (BHK-21) cells were infected with VSV Indiana (Mudd-Summers strain) or EMCV (a gift from A. Krug) and cell culture supernatants were collected 20 h after infection. Virus yield in culture supernatants was determined by standard plaque assay. VSV and EMCV were used at a multiplicity of infection of 5–10.

***In vivo* viral infection.** For viral infection, mice were given intravenous injection of 2×10^6 plaque-forming units VSV per mouse in 200 μl medium or an equal amount of PBS (as a control). Serum was collected after 6 h. Cytokine concentrations were measured by ELISA.

Statistical analyses. The statistical significance of differences was determined by the paired two-tailed Student's *t*-test. Differences with a *P* value of less than 0.05 were considered statistically significant.

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