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LETTERS

Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses

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The innate immune system senses viral infection by recognizing a variety of viral components (including double-stranded (ds)RNA) and triggers antiviral responses[1,2]. The cytoplasmic helicase proteins RIG-I (retinoic-acid-inducible protein I, also known as Ddx58) and MDA5 (melanoma-differentiation-associated gene 5, also known as Ifih1 or Helicard) have been implicated in viral dsRNA recognition[3–7]. *In vitro* studies suggest that both RIG-I and MDA5 detect RNA viruses and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue[3]. Although a critical role for RIG-I in the recognition of several RNA viruses has been clarified[8], the functional role of MDA5 and the relationship between these dsRNA detectors *in vivo* are yet to be determined. Here we use mice deficient in MDA5 (*MDA5*$^{-/-}$) to show that MDA5 and RIG-I recognize different types of dsRNAs: MDA5 recognizes poly(I:C), and RIG-I detects *in vitro* transcribed dsRNAs. RNA viruses are also differentially recognized by RIG-I and MDA5. We find that RIG-I is essential for the production of interferons in response to RNA viruses including paramyxoviruses, influenza virus and Japanese encephalitis virus, whereas MDA5 is critical for picornavirus detection. Furthermore, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice are highly susceptible to infection with these respective RNA viruses compared to control mice. Together, our data show that RIG-I and MDA5 distinguish different RNA viruses and are critical for host antiviral responses.

Host pattern recognition receptors, such as Toll-like receptors (TLRs) and helicase family members, have an essential role in the recognition of molecular patterns specific for different viruses, including DNA, single-stranded (ss)RNA, dsRNA and glycoproteins[1,9,10]. dsRNA can be generated during viral infection as a replication intermediate for RNA viruses. TLR3, which localizes in the endosomal membrane, has been shown to recognize viral dsRNA as well as the synthetic dsRNA analogue poly(I:C) (refs 11, 12). The cytoplasmic proteins RIG-I and MDA5 have also been identified as dsRNA detectors[3–5,7,13]. RIG-I and MDA5 contain two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. RIG-I recruits a CARD-containing adaptor, IPS-1 (also known as MAVS, VISA or Cardif)[14–17]. IPS-1 relays the signal to the kinases TBK1 and IKK-i, which phosphorylate interferon-regulatory factor-3 (IRF-3) and IRF-7, transcription factors essential for the expression of type-I interferons[18–22]. In contrast, TLR3 activates TBK1 and IKK-i through the TIR-domain-containing adaptor TRIF (also known as Ticam1)[12].

In vitro studies have shown that both RIG-I and MDA5 can bind to poly(I:C) and respond to poly(I:C) and RNA viruses[6]. We have generated *RIG-I*$^{-/-}$ mice, and show that RIG-I is essential eliciting the immune responses against several RNA viruses, including Newcastle disease virus (NDV), Sendai virus (SeV) and vesicular stomatitis virus (VSV), in various cells except for plasmacytoid dendritic cells (pDCs)[8]. Hepatitis C virus and Japanese encephalitis virus are also reported to be recognized by RIG-I *in vitro*[23,24].

The *in vivo* functional relationship between RIG-I and MDA5 remains to be determined. To investigate a functional role for MDA5 *in vivo*, we generated *MDA5*$^{-/-}$ mice and investigated viral recognition (Supplementary Fig. 1). In contrast to *RIG-I*$^{-/-}$ mice, which are mostly embryonic lethal, *MDA5*$^{-/-}$ mice are born in a mendelian ratio, grow healthily and do not show gross developmental abnormalities until 24 weeks of age. Flow cytometric analysis of leukocytes from the spleen and lymph nodes (staining for CD3, B220 and CD11c) revealed that the composition of lymphocytes and dendritic cells is similar in wild-type and *MDA5*$^{-/-}$ mice (data not shown).

TLR3, RIG-I and MDA5 have been implicated in the recognition of poly(I:C) and the subsequent induction of antiviral responses. However, their exact contribution to *in vivo* responses against dsRNA has yet to be clarified. We therefore examined the *in vivo* responses to poly(I:C) in mice lacking RIG-I, MDA5 or TRIF, or both MDA5 and TRIF. Administration of poly(I:C) led to rapid induction of the cytokines interferon-α (IFN-α), IFN-β, interleukin-6 (IL-6) and IL-12 in sera of both wild-type and *RIG-I*$^{-/-}$ mice (Fig. 1a and Supplementary Fig. 2a). In contrast, *MDA5*$^{-/-}$ mice failed to produce IFN-α and IFN-β in response to poly(I:C), and production of IL-6 and IL-12p40 was also significantly impaired (Fig. 1b). Although *Trif*$^{-/-}$ mice produced normal amounts of IFN-α, they also showed severely impaired production of IL-12p40 and partial impairment in IL-6 production. *MDA5*$^{-/-}$; *Trif*$^{-/-}$ double-knockout mice failed to induce IFN-α, IL-6 and IL-12p40 in response to poly(I:C). These results indicate that MDA5 is essential for poly(I:C)-induced IFN-α production and TLR3 signalling is critical for IL-12 production, whereas both MDA5 and TLR3 regulate IL-6 production.

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When bone-marrow-derived dendritic cells generated by granulocyte–macrophage colony-stimulating factor (GMCSF) were incubated in the presence of poly(I:C), production of IFN-α and IFN-β was severely impaired in *MDA5*$^{-/-}$, but not in *RIG-I*$^{-/-}$ or *Trif*$^{-/-}$, GMCSF-DCs (Fig. 1c and Supplementary Fig. 2b). Even when poly(I:C) was transfected into GMCSF-DCs using lipofectamine, poly(I:C) induced IFN-β production in an MDA5-dependent, but not a RIG-I- or TRIF-dependent, manner (Fig. 1d). IFN-β production in response to poly(I:C) was also impaired in *MDA5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Fig. 1e), indicating that poly(I:C) is primarily recognized by MDA5, not RIG-I and TLR3, in these cells.

dsRNAs transcribed *in vitro* (Supplementary Fig. 2c) also stimulated MEFs to produce IFN-β. Unlike for poly(I:C), wild-type and *MDA5*$^{-/-}$ MEFs produced comparable amounts of IFN-β (Fig. 1e) in response to *in vitro* transcribed dsRNAs. In contrast, *RIG-I*$^{-/-}$ MEFs did not produce detectable amounts of IFN-β, indicating that RIG-I is essential for the detection of *in vitro* transcribed dsRNAs. As RIG-I, but not MDA5, is responsible for IFN-β production in response to dsRNAs of various lengths, these helicases probably distinguish nucleotide structure or sequence, but not length. Together, these results indicate that MDA5 and RIG-I are involved in the detection of poly(I:C) and *in vitro* transcribed dsRNAs, respectively.

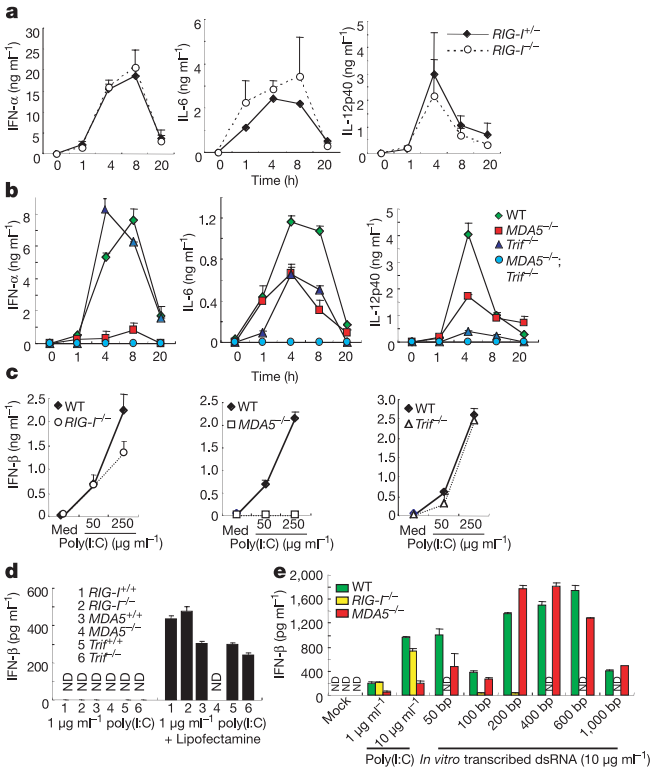

Figure 1 | Roles of MDA5, RIG-I and TRIF in the recognition of synthesized dsRNAs and dsRNA analogues. a, **b**, *RIG-I*$^{-/-}$ and littermate *RIG-I*$^{+/-}$ mice (**a**) or wild-type (WT), *MDA5*$^{-/-}$, *Trif*$^{-/-}$ or *MDA5*$^{-/-}$; *Trif*$^{-/-}$ double-knockout mice (**b**) were injected intravenously with 200 μg poly(I:C) for the indicated periods, and IFN-α, IL-6 and IL-12p40 production was measured in serum by ELISA. Data show mean ± s.d. **c**, GMCSF-DCs from *RIG-I*$^{-/-}$, *MDA5*$^{-/-}$, *TRIF*$^{-/-}$ and littermate control mice were incubated in the presence of 50 or 250 μg ml^{-1} poly(I:C) for 24 h. IFN-β production in the cell culture supernatants was measured by ELISA. Med, medium only. **d**, GMCSF-DCs were treated with 1 μg ml^{-1} poly(I:C) complexed with or without lipofectamine 2000 for 24 h, and IFN-β production was measured. **e**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were treated with poly(I:C) or *in vitro* transcribed dsRNAs of indicated lengths complexed with lipofectamine 2000 for 12 h, and IFN-β production was measured. Error bars indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

This finding led us to hypothesize that RIG-I and MDA5 are involved in the detection of different RNA viruses. We have previously shown that a set of negative-sense RNA viruses are recognized by RIG-I[8]. We first examined IFN-β and IFN-α production in *MDA5*$^{-/-}$ MEFs in response to a set of negative-sense ssRNA viruses, including NDV, SeV, VSV and influenza virus. As infection with most of the wild-type viruses (except NDV) failed to induce type-I interferons in MEFs, owing to suppression of interferon responses by viral proteins (data not shown), we also used mutant viruses lacking viral interferon-inhibitory proteins. As shown in Fig. 2a and Supplementary Fig. 4b, wild-type MEFs produce IFN-β and IFN-α in response to these mutant viruses. Production of type-I interferons was severely impaired in *RIG-I*$^{-/-}$ MEFs compared to wild-type cells, but MDA5 was dispensable for the production of type-I interferons. Japanese encephalitis virus (JEV), a positive-sense ssRNA virus belonging to the flavivirus family, also required RIG-I, but not MDA5, for IFN-β production (Fig. 2b).

We then examined the interferon responses of MEFs to encephalomyocarditis virus (EMCV), a positive-sense ssRNA virus belonging to the picornavirus family. EMCV-induced IFN-β production was abrogated in *MDA5*$^{-/-}$ MEFs (Fig. 2c). In contrast, wild-type and *RIG-I*$^{-/-}$ MEFs produced comparable amounts of IFN-β, indicating that EMCV is specifically recognized by MDA5. The induction of genes encoding IFN-β, IP-10 and IL-6 in response to EMCV was abrogated in *MDA5*$^{-/-}$ macrophages (Supplementary Fig. 3d). The synthesis of cellular proteins in *MDA5*$^{-/-}$ MEFs was progressively inhibited during EMCV infection, to an extent and with kinetics similar to wild-type MEFs (Supplementary Fig. 5), indicating that the EMCV infection was established in wild-type and *MDA5*$^{-/-}$ MEFs in a similar manner. Moreover, other viruses belonging to the picornavirus family (Theiler's and Mengo viruses) also induced IFN-α through MDA5 (Supplementary Fig. 4d). Furthermore, the production of IFN-β in response to SeV and EMCV was impaired in *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ GMCSF-DCs, respectively (Fig. 2d, e), indicating that conventional dendritic cells (cDCs) also use these helicases for the differential recognition of viruses. EMCV-induced production of IL-6 was also abrogated in *MDA5*$^{-/-}$, but not *RIG-I*$^{-/-}$, cDCs (Supplementary Fig. 4c). Therefore, MDA5 is critical for the regulation of pro-inflammatory cytokines as well as type-I interferons in response to EMCV.

We next examined whether viral RNAs derived from VSV and EMCV recapitulate the production of interferons through MDA5 and RIG-I. When transfected into GMCSF-DCs by lipofection, RNAs prepared from VSV or EMCV induced production of IFN-α in a RIG-I- or MDA5-dependent manner, respectively (Fig. 2f). We also performed reconstitution experiments by transfecting RIG-I or MDA5 expression vectors into *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ MEFs, in which IFN-β induction was completely abrogated in response to infection with EMCV or SeV Cm (SeV with a mutated C protein) (Fig. 2g). The ectopic expression of human RIG-I, but not MDA5, activated the *Ifnb* promoter in response to SeV Cm. Reciprocally, cells expressing human MDA5, but not RIG-I, activated the *Ifnb* promoter in response to EMCV in a dose-dependent manner (Fig. 2h). These results indicate that human RIG-I and MDA5 recognize different RNA viruses by recognizing viral RNAs.

Previous studies have shown that pDCs use mainly the TLR system instead of RIG-I in the recognition of several RNA viruses[8]. MyD88 is an adaptor protein essential for TLR signalling (except through TLR3). We purified B220^{+} pDCs from Flt3L-generated bone-marrow-derived dendritic cells (Flt3L-DCs) and infected them with EMCV. pDCs from *Myd88*$^{-/-}$, but not *MDA5*$^{-/-}$, mice showed a profound defect in IFN-α production (Supplementary Fig. 6). Reciprocally, MDA5, but not MyD88, is required for the production of IFN-α in B220^{-} cDCs purified from Flt3L-DCs (Supplementary Fig. 6). These results indicate that both MDA5 and RIG-I are

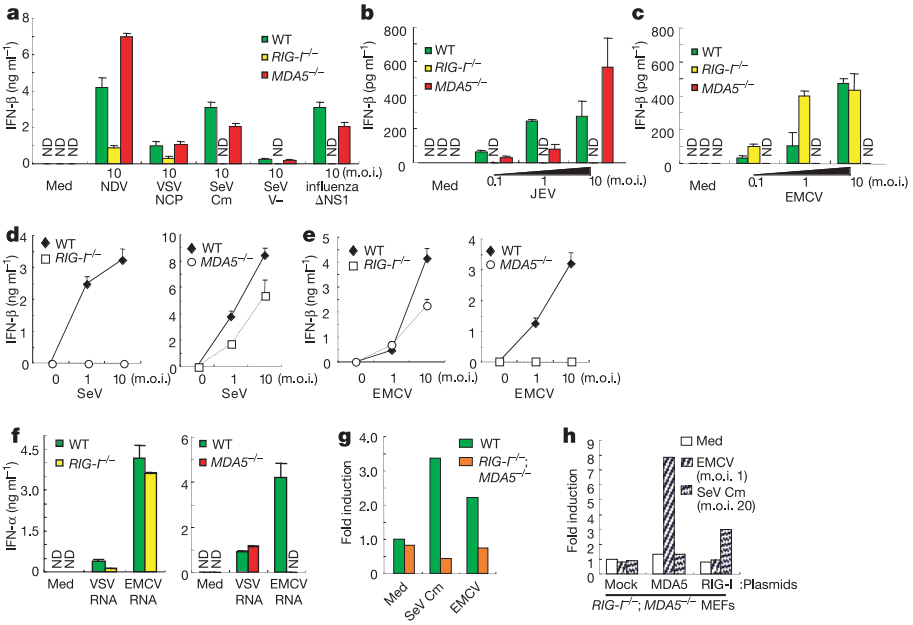

Figure 2 | Differential viral recognition by RIG-I and MDA5. **a**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV lacking a variant of M protein (NCP), SeV with a mutated C protein (Cm), SeV lacking V protein (V^{-}), and influenza virus lacking the NS1 protein (ΔNS1) for 24 h. IFN-β production in the culture supernatants was measured by ELISA. **b**, **c**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were exposed to the positive-sense ssRNA viruses JEV (**b**) and EMCV (**c**), and IFN-β production was measured. **d**, **e**, GMCSF-DCs from *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice and their littermate wild-type mice were infected with an increasing m.o.i. of SeV V^{-} (**d**) or EMCV (**e**) for 24 h, and IFN-β production was measured. **f**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ GMCSF-DCs were treated with RNAs directly prepared from VSV and EMCV (complexed with lipofectamine 2000) for 24 h, and IFN-α production was measured. **g**, Wild-type and *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ MEFs were transiently transfected with a reporter construct containing the *Ifnb* promoter and exposed to SeV Cm or EMCV for 24 h. Cell lysates were then prepared and subjected to a luciferase assay. **h**, RIG-I$^{-/-}$; MDA5$^{-/-}$ MEFs were transiently transfected with the *Ifnb* promoter construct together with expression plasmids encoding human RIG-I or MDA5. The cells were then infected with EMCV or SeV Cm for 24 h and were subjected to a luciferase assay. Error bars in **a**–**g** indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

dispensable for the viral induction of IFN-α in pDCs.

We next examined the *in vivo* roles of MDA5 and RIG-I in host defence against viral infection. Although most *RIG-I*$^{-/-}$ mice are embryonic lethal[8], we could efficiently obtain live adult mice by intercrossing the *RIG-I*$^{+/-}$ mice obtained after *RIG-I*$^{+/-}$ × ICR crosses (Supplementary Table 1). When the mice were infected with JEV, serum IFN-α levels were markedly decreased in *RIG-I*$^{-/-}$ mice compared to littermate *RIG-I*$^{+/-}$ mice. In contrast, *MDA5*$^{-/-}$ mice did not show a defect in JEV-induced systemic IFN-α production (Fig. 3a). IFN-α production was partially impaired in *Myd88*$^{-/-}$ mice compared to wild-type mice, but the extent of this impairment was far less than in *RIG-I*$^{-/-}$ mice (Fig. 3a). These data suggest that the TLR system is not critical for the induction of serum IFN-α *in vivo* in response to JEV. Consistent with this finding, *RIG-I*$^{-/-}$ mice, but not *MDA5*$^{-/-}$ or *Myd88*$^{-/-}$ mice, were more susceptible to JEV infection than control mice (Fig. 3b). Furthermore, *RIG-I*$^{-/-}$ mice, but not *MDA5*$^{-/-}$ mice, succumbed to VSV infection, consistent with abrogated interferon responses (Supplementary Fig. 7). Thus, RIG-I-mediated recognition of a specific set of viruses has a critical role in antiviral host defence *in vivo*.

We next challenged the mice with EMCV as a model virus that is recognized by MDA5. Induction of IFN-β, IFN-α, RANTES and IL-6 was severely impaired in the sera of *MDA5*$^{-/-}$ mice (Fig. 4a and Supplementary Fig. 8). *MDA5*$^{-/-}$ mice and mice null for the IFN-α/β receptor (*Ifnar1*$^{-/-}$) were highly susceptible to EMCV infection (viral titre of 1×10^2 plaque-forming units (p.f.u.)) compared to littermate controls ($P < 0.01$) (Fig. 4b). In contrast, deficiency of neither RIG-I nor TLR3 affected the survival of mice infected with EMCV. Consistent with a previous report[22], *Myd88*$^{-/-}$ mice were modestly susceptible to EMCV infection compared to wild-type mice, implying that pDC-mediated responses are not critical for eliminating EMCV (Fig. 4b).

It is known that EMCV preferentially infects cardiomyocytes and causes myocarditis. Consistent with increased susceptibility to EMCV, viral titre in the heart was much higher in *MDA5*$^{-/-}$ mice compared to control mice (Fig. 4c). Histological analysis of hearts two days after EMCV infection revealed that focal necrosis of

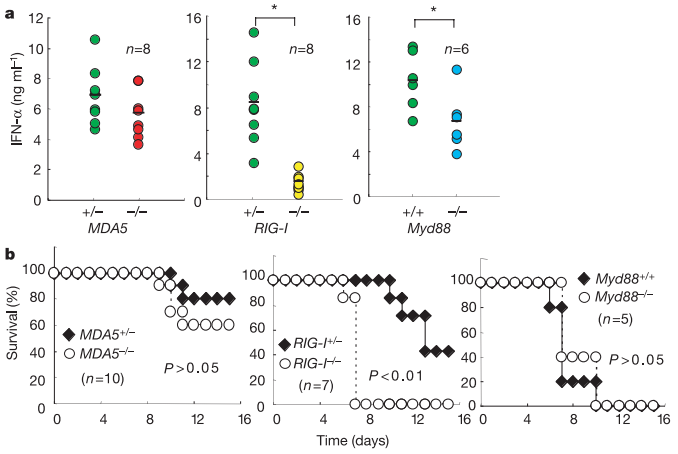

Figure 3 | Susceptibility of *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice to JEV infection. **a**, *RIG-I*$^{+/-}$, *RIG-I*$^{-/-}$, *MDA5*$^{+/-}$ and *MDA5*$^{-/-}$ mice ($n = 8$), and *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice ($n = 6$), were injected intravenously with 2×10^7 p.f.u. JEV. Sera were collected 24 h after injection, and IFN-α production levels measured by ELISA. Circles represent individual mice, bars indicate mean values. Asterisk, $P < 0.05$ versus controls (*t*-test). **b**, The survival of 6-week-old mice (genotypes as indicated) infected intravenously with 2×10^7 p.f.u. JEV. Mice were monitored for 15 days ($P < 0.01$ between *RIG-I*$^{-/-}$ mice and their littermate controls, generalized Wilcoxon test).

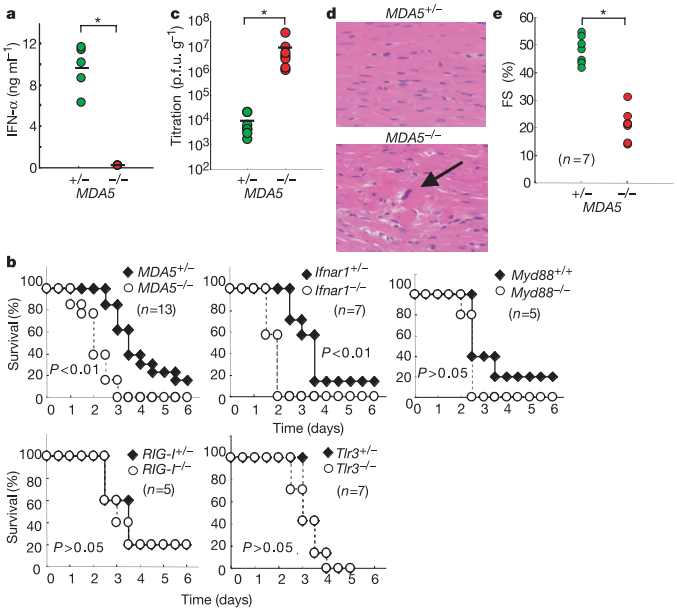

Figure 4 | Role of MDA5 in host defence against EMCV infection. **a**, *MDA5*$^{+/-}$ and *MDA5*$^{-/-}$ mice ($n = 5$) were inoculated intravenously with 1×10^7 p.f.u. EMCV. Sera were prepared 4 h after injection and IFN-α production levels determined by ELISA. **b**, The survival of 6-week-old mice (genotypes as indicated) infected with 1×10^2 p.f.u. EMCV intraperitoneally was monitored every 12 h for six days ($P < 0.01$ between *MDA5*$^{-/-}$ or *Ifnar1*$^{-/-}$ mice and their littermate controls, generalized Wilcoxon test). **c**, *MDA5*$^{+/-}$ and *MDA5*$^{-/-}$ mice were infected intraperitoneally with 1×10^2 p.f.u. EMCV. After 48 h, mice were killed and virus titres in hearts were determined by plaque assay. **d**, Heart sections of *MDA5*$^{+/-}$ and *MDA5*$^{-/-}$ mice, two days after infection, were assessed for histological changes using haematoxylin and eosin staining. Arrow indicates the focal necrosis of cardiomyocytes. **e**, Cardiac function of mice 48 h after EMCV infection was assessed by echocardiography (see Supplementary Fig. 8b). The fractional shortening (FS) after infection determined by transthoracic M-mode echocardiographic tracings is shown. Asterisk, $P < 0.05$ versus *MDA5*$^{+/-}$ mice (*t*-test).

cardiomyocytes had developed in *MDA5*$^{-/-}$ mice, but wild-type hearts showed no histological abnormalities at this time point (Fig. 4d). Notably, no infiltration of immune cells was observed in either wild-type or *MDA5*$^{-/-}$ heart sections at this time point. However, when cardiac performance was analysed by echocardiography two days after infection (Fig. 4e), cardiac contractility was severely depressed in *MDA5*$^{-/-}$ mice (fractional shortening 48.2 ± 4.9% in *MDA5*$^{+/-}$ mice, 21.2 ± 5.8% in *MDA5*$^{-/-}$ mice), indicating that *MDA5*$^{-/-}$ mice developed severe heart failure due to virus-induced cardiomyopathy. Thus, MDA5-mediated recognition of EMCV is a prerequisite for triggering antiviral responses as well as for prevention of myocardial dysfunction.

Together, our results demonstrate that RIG-I and MDA5 have essential roles in the recognition of different groups of RNA viruses, as well as in the subsequent production of type-I interferons and pro-inflammatory cytokines. We have found that poly(I:C) and *in vitro* transcribed dsRNA are recognized by MDA5 and RIG-I, respectively; this is in contrast to results from previous *in vitro* studies. RIG-I probably recognizes dsRNA generated over the course of RNA virus replication, as all *in vitro* transcribed dsRNAs tested except for poly(I:C) induced type-I interferons through RIG-I. In contrast, the endogenous ligand of MDA5 remains enigmatic. Moreover, how RIG-I and MDA5 differentially recognize natural dsRNAs is undetermined. Given that the helicase domains of RIG-I and MDA5 bind to dsRNA, analyses of the crystal structures of these domains should to help achieve a better understanding of the molecular mechanisms underlying this differential recognition. Furthermore, it is still possible that unknown dsRNA-binding proteins also function as direct receptors for viral RNAs.

Finally, the picornavirus family contains several viruses that are pathogenic for humans, including poliovirus, rhinovirus and the virus causing foot-and-mouth-disease. Our studies suggest that human MDA5 and RIG-I also recognize RNA viruses. Thus, identification of therapeutic agents that modify RIG-I or MDA5 may lead to antiviral strategies against selected viruses.

METHODS

Mice, cells and reagents. The generation of *MDA5*$^{-/-}$ mice is described in the Supplementary Information. *Myd88*$^{-/-}$, *Tlr3*$^{-/-}$ and *Trif*$^{-/-}$ mice have been described previously[12]. *Ifnar1*$^{-/-}$ mice have also been described previously[25]. *RIG-I*$^{+/-}$ mice in a 129Sv × C57BL/6 background were crossed with ICR mice, and the resulting *RIG-I*$^{+/-}$ mice were further intercrossed. Interbreeding of these *RIG-I*$^{+/-}$ mice produced healthy and fertile *RIG-I*$^{-/-}$ offspring, although their number was less than half that of *RIG-I*$^{+/+}$ progeny (Supplementary Table 1). *RIG-I*$^{-/-}$ and *RIG-I*$^{+/-}$ littermate mice were used for *in vivo* experiments. *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ mice in a 129Sv × C57BL/6 background were lethal at embryonic day 12.5. Additional details regarding cells, reagents and the preparation of *in vitro* transcribed dsRNA are provided in the Supplementary Information.

Viruses. NDV (ref. 3), VSV, VSV lacking a variant of M protein (NCP) (ref. 8), influenza virus lacking the NS1 protein (ΔNS1) (ref. 26), JEV (ref. 27) and EMCV (ref. 3) have been described previously. SeV and SeV lacking the V protein (V^-) or with mutated C proteins (Cm) were provided by A. Kato[28].

Luciferase assay. Wild-type or *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ MEFs were transiently transfected with a reporter construct containing the *Ifnb* promoter together with an empty vector (mock), or *RIG-I* or *MDA5* expression vectors. As an internal control, a *Renilla* luciferase construct was transfected. Transfected cells were untreated or infected with EMCV or SeV Cm (m.o.i. 20) for 24 h. The cells were lysed and subjected to a luciferase assay using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Analysis of mice after EMCV infection. Methods for plaque assays, histological analysis and echocardiography are described in the Supplementary Information.

Measurement of cytokine production. Cell culture supernatants were collected and analysed for IFN-β, IFN-α, IL-6 or IL-12p40 production using enzyme-linked immunosorbent assays (ELISAs). ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories, and those for IL-6, IL-12p40 and RANTES were obtained from R&D Systems.

Statistical analysis. Kaplan–Meier plots were constructed and a generalized Wilcoxon test was used to test for differences in survival between control and mutant mice after viral infection. Statistical significance of any differences in cytokine concentration and ECMV titres was determined using Student's *t*-tests.

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1. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124,** 783–801 (2006).
2. Katze, M. G., He, Y. & Gale, M. Jr. Viruses and interferon: A fight for supremacy. *Nature Rev. Immunol.* **2,** 675–687 (2002).
3. Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature Immunol.* **5,** 730–737 (2004).
4. Kang, D. C. *et al. mda-5*: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc. Natl Acad. Sci. USA* **99,** 637–642 (2002).
5. Andrejeva, J. *et al.* The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the *IFN-β* promoter. *Proc. Natl Acad. Sci. USA* **101,** 17264–17269 (2004).
6. Yoneyama, M. *et al.* Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **175,** 2851–2858 (2005).
7. Rothenfusser, S. *et al.* The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J. Immunol.* **175,** 5260–5268 (2005).
8. Kato, H. *et al.* Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23,** 19–28 (2005).
9. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature Immunol.* **5,** 987–995 (2004).
10. Beutler, B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* **430,** 257–263 (2004).
11. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413,** 732–738 (2001).

12. Yamamoto, M. *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301,** 640–643 (2003).
13. Kovacsovics, M. *et al.* Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. *Curr. Biol.* **12,** 838–843 (2002).
14. Kawai, T. *et al.* IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature Immunol.* **6,** 981–988 (2005).
15. Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* **122,** 669–682 (2005).
16. Xu, L. G. *et al.* VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol. Cell* **19,** 727–740 (2005).
17. Meylan, E. *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437,** 1167–1172 (2005).
18. Fitzgerald, K. A. *et al.* IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nature Immunol.* **4,** 491–496 (2003).
19. Sharma, S. *et al.* Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300,** 1148–1151 (2003).
20. Hemmi, H. *et al.* The roles of two IκB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J. Exp. Med.* **199,** 1641–1650 (2004).
21. Sato, M. *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13,** 539–548 (2000).
22. Honda, K. *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434,** 772–777 (2005).
23. Chang, T. H., Liao, C. L. & Lin, Y. L. Flavivirus induces interferon-beta gene expression through a pathway involving RIG-I-dependent IRF-3 and PI3K-dependent NF-κB activation. *Microbes Infect.* **8,** 157–171 (2006).
24. Melchjorsen, J. *et al.* Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J. Virol.* **79,** 12944–12951 (2005).
25. Hoshino, K., Kaisho, T., Iwabe, T., Takeuchi, O. & Akira, S. Differential involvement of IFN-β in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol.* **14,** 1225–1231 (2002).
26. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303,** 1529–1531 (2004).
27. Mori, Y. *et al.* Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. *J. Virol.* **79,** 3448–3458 (2005).
28. Kato, A. *et al.* Characterization of the amino acid residues of sendai virus C protein that are critically involved in its interferon antagonism and RNA synthesis down-regulation. *J. Virol.* **78,** 7443–7454 (2004).

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