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RIG-I-dependent antiviral immunity is effective against an RNA virus encoding a potent suppressor of RNAi

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Abstract

Nodamura virus (NoV) lethally infects suckling mice and contains a segmented positive-strand RNA genome that encodes a potent suppressor of RNA interference (RNAi). Recent studies have demonstrated immune detection and subsequent processing of NoV dsRNA replicative intermediates by the mouse RNAi machinery. However, diverse RNA viruses, including Encephalomyocarditis virus that also triggers Dicer-dependent biogenesis of viral siRNAs in mouse cells, are targeted in mammals by RIG-I-like receptors that initiate an IFN-dependent antiviral response. Using mouse embryonic fibroblasts (MEFs) for NoV infection, here we show that MEFs derived from mice knockout for RIG-I, but not those knockout for MDA5, LGP2, TLR3 or TLR7, exhibited an enhanced susceptibility to NoV. Further studies indicate that NoV infection induced an IFN-dependent antiviral response mediated by RIG-I. Our findings suggest that RIG-I directs a typical IFN-dependent antiviral response against an RNA virus capable of suppressing the RNAi response.

Keywords

NoV; RIG-I; Type I Interferons; RNAi

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1. Introduction

Host innate immune system provides protection against virus attack by recognizing pathogen-associated molecular patterns (PAMPs) and generates both inflammatory and antiviral responses through pattern recognition receptors (PRRs)[1,2]. PRRs are classified into several families. The family of RIG-I-like receptors (RLRs) contains RIG-I, MDA5, LGP2. RIG-I, retinoic acid inducible gene 1 protein is required for the innate immune sensing of many RNA viruses including Influenza A, B virus, Paramyxoviruses, Vesicular Stomatitis virus (VSV), Respiratory syncytial virus (RSV), Japanese encephalitis virus [3,4,5]. Melanoma differentiation associated gene-5 (MDA5) preferentially recognizes Picornaviruses including Encephalomyocarditis virus (EMCV) [5]. Several viruses, such as West Nile virus, Sendai virus (SeV), Dengue virus, are detected by both RIG-I and MDA5 [4,6,7]. By contrast, the role of Laboratory of Genetics and Physiology 2 (LGP2) in virus sensing is yet to be clearly defined; some studies suggest that LGP2 is required for the virus-induced production of type-1 interferons (IFNs) whereas others indicate a negative regulatory role [8,9,10]. During the RLR signaling, mitochondrial anti-viral signaling protein (MAVS) [11], also known as IPS-1 [12], VISA [13], Cardif [14], functions downstream of RIG-I and MDA5 as an essential adapter protein to mediate IRF3 and IRF7 activation, leading to IFN production and subsequent transcriptional induction of IFN-stimulated genes (ISGs). The family of Toll-like receptors (TLRs) consists of more than 10 members. TLR3 is known to participate in the ligand recognition of viruses such as RSV [15], West Nile virus [15], IAV [16]. TLR7 is essential for the recognition of IAV [17], HIV [18], Dengue virus [19], SeV [20], whereas TLR8 shares phylogenetic and functional similarity to TLR7 and recognizes HIV [17,21].

Recent studies have provided evidence for an antiviral function of RNA interference (RNAi) in mammals [22,23]. Antiviral RNAi, characterized extensively in plants and invertebrates, begins with the processing of virus-specific dsRNA by the Dicer nuclease into small interfering RNAs (siRNAs), which are subsequently assembled into RNA-induced silencing complex (RISC) to guide specific virus clearance by an Argonaute protein [24]. Production of abundant viral siRNAs was detected in both mouse embryonic stem cells (mESCs) and suckling mice infected by a mutant Nodamura virus (NoV) defective in the expression of its B2 protein [22,23], a known viral suppressor of RNAi (VSR) that acts by inhibiting Dicer processing of long dsRNA into siRNAs [25,26,27]. Although wildtype NoV is lethal to suckling mice, the VSR-deficient NoV mutant fails to establish infection in suckling mice and mESCs, but replicates to high levels in mESCs knockout of the four mouse Argonaute genes [22,23]. Dicer-dependent production of the viral siRNAs was also readily detectable in mESCs infected with EMCV [23], indicating dual recognition of EMCV dsRNA by both MDA5 and Dicer.

In this work, we investigated if NoV infection triggers innate immune recognition by RLRs and TLRs known to restrict RNA virus infection in mammals. NoV contains a positive sense single-stranded RNA genome and is the type species of the genus *Alphanodavirus* in the *Nodaviridae*. Unlike other nodaviruses that are pathogens of insects and fishes, NoV can lethally infect both insects and mammals [28,29,30]. The genome of NoV is divided into RNA1 and RNA2 that encode RNA-dependent RNA polymerase (RdRP) and the viral

capsid precursor protein, respectively [31]. The VSR protein B2 is translated from RNA3, which is a subgenomic RNA of RNA1. Here, we first developed a model for NoV infection in cultured mouse embryonic fibroblasts (MEFs). Use of MEFs derived from wildtype and mutant mouse strains knockout for individual RLRs and TLRs allowed us to examine the role of these innate immune receptors in the mouse response to NoV infection. Our findings indicate a key role for RIG-I in the induction of an IFN-dependent response against NoV infection.

2. Materials and methods

2.1 Cells and viruses

Stocks of Nodamura virus (NoV) were produced by intraperitoneal injection of BALB/c suckling mice as previously reported [22]. We followed the guidelines described under the federal Animal Welfare Regulations Act with the protocol approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. Mouse embryonic fibroblasts (MEFs) cell lines were generated from the wild-type (WT) mice with a C57BL/6 background and RIG-I^{-/-}, MDA5^{-/-}, LGP2^{-/-}, MAVS^{-/-}, TLR3^{-/-}, TLR7^{-/-} knockout mice as previously described [5,10,32,33,34,35]. C57BL/6, MDA5^{-/-}, MAVS^{-/-}, TLR3^{-/-}, and TLR7^{-/-} mice were purchased from Jackson Laboratory whereas RIG-I^{-/-} and LGP2^{-/-} were kindly provided by Drs. Adolfo García-Sastre, Shizuo Akira and Michael Gale, Jr. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine solution, 37°C, 5% CO₂.

2.2 Infection in MEFs

MEFs (1×10⁶) were infected by NoV with the same amount of viral genome copies (5×10⁶). MEFs were harvested at 12, 24, 48, 72-hour post infection (hpi). For IFN pre-treatment, mouse IFN-β at final concentration of 1 IU (International Units)/ml (PBL assay science) was added to MEFs 8 h prior to infection. Total RNA from MEFs was extracted using TRIzol reagent (Invitrogen). First strand cDNA synthesis was performed using iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's instruction. The copy numbers of the viral genome RNA1 of NoV were analyzed by real-time PCR as previously described [22].

2.3 Real-time RT-PCR assay

Quantitative real-time PCR was used to determine the gene expression changes in MEFs. One μg of extracted total RNA was reverse-transcribed with iScript Reverse Transcription Supermix (Bio-Rad), and 1/10 of the cDNA products was mixed with iQ SYBR green Supermix (Bio-Rad). Mouse β-actin was used as an endogenous control. Primer sequences were as follows. Mouse β-actin forward primer 5'-ATT GGC AAC GAG CGG TTC C-3' and reverse primer 5'-AGC ACT GTG TTG GCA TAG AGG-3'. Mouse RIG-I forward primer 5'- GAG AGT CAC GGG ACC CAC T -3' and reverse primer 5'- CGG TCT TAG CAT CTC CAA CG -3'. Mouse MDA5 forward primer 5'- TGA TGC ACT ATT CCA AGA ACT AAC A -3' and reverse primer 5'- TCT GTG AGA CGA GTT AGC CAA G -3'. Mouse LGP2 forward primer 5'- CAG CCT AGT CTG CTG CTA TTC -3' and reverse primer 5'- CCA GAG CAG GTA AGA TCA CTT -3'. Mouse MAVS forward primer 5'-

CTG GCT GAT CAA GTG ACT CG -3' and reverse primer 5'- AAT GCA GAG GGT CCA GAA AC -3'. Mouse Dicer forward primer 5'- TGA ACC TTT TGA CAC CTC GG C-3' and reverse primer 5'- TGA TGC TGG GAT TGG ATG TAT AG -3'. Mouse Argonaute 2 forward primer 5'- ATT CAG TTC TAC AAG TCC ACC C -3' and reverse primer 5'- CTG ATA GTC CTT CTC CAG CTT G -3'. Mouse TRBP2 forward primer 5'- GGA GGG AAT GAG TGA AGA GG -3' and reverse primer 5'- GGC GTC TTT CCT ATT CTG GTC -3'. Mouse PACT forward primer 5'- CCG AAC ACA GAC TAC ATC CAG -3' and reverse primer 5'- CTC TGC GAG ACA CTG ATA CTG -3'. Changes in gene expression were expressed as a ratio of the level observed in mock-infected MEFs by Real-time PCR performed as previously described [22].

2.4 Statistical analysis

Data were expressed as mean \pm S.E.M. from at least three independent experiments. Statistical analysis was done using student's test where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

3. Results

3.1 RIG-I^{-/-} MEFs are more susceptible to NoV infection than wildtype MEFs

NoV has a limited cell and tissue tropism and was reported infectious in cultured BHK-21 and CHO cells that support high levels of replication [36]. Since fibroblasts from skeletal muscles of suckling mice were permissive to NoV [37], we isolated mouse embryonic fibroblasts (MEFs) from C57BL/6 mice for NoV infection. MEFs seeded in 6-well plate were infected with 5×10^6 genome copies of NoV as previously described [22]. Following infection with NoV, MEFs were collected without supernatants and total RNA was extracted at 12, 24, 48, 72 hours post infection (hpi) for real-time RT-PCR analysis of NoV genomic RNA accumulation. We detected an approximately 10-fold increase of NoV accumulation in the wildtype MEFs 48 hour after infection (Figure 1). This indicated that MEFs were susceptible to NoV although MEFs appeared more resistant to NoV than BHK-21 cells.

We next generated MEFs from RIG-I^{-/-}, MDA5^{-/-}, LGP2^{-/-}, MAVS^{-/-}, TLR3^{-/-}, and TLR7^{-/-} mice and determined whether any of these MEFs was more susceptible to NoV infection. We found that NoV RNA1 levels increased approximately 46 and 89 folds in RIG-I^{-/-} MEFs at 48 and 72 hpi, respectively (Fig. 1). In contrast, no obvious differences in the accumulation of NoV were observed at 48 hpi in MEFs from WT, MDA5^{-/-}, LGP2^{-/-}, MAVS^{-/-}, TLR3^{-/-}, and TLR7^{-/-} mice (Fig. 1). However, NoV accumulated to approximately 24-fold higher levels in MAVS^{-/-} MEFs at 72 hpi when NoV accumulation remained low in MEFs from WT, MDA5^{-/-}, LGP2^{-/-}, TLR3^{-/-}, and TLR7^{-/-} mice (Fig. 1). These findings indicate that MEFs from RIG-I^{-/-} and MAVS^{-/-} mice were more susceptible to NoV infection than other MEFs. Therefore, NoV infection in MEFs may be detected by RIG-I, triggering a MAVS-dependent antiviral response.

3.2 RIG-I is necessary for the expression of IFN- β and ISGs induced by NoV

To investigate the role of RIG-I in the innate immune response to NoV, we next compared the expression of key innate immune genes in WT and RIG-I^{-/-} MEFs following NoV

infection. It is known that nonprofessional immune cells such as fibroblasts produce IFN- β in response to viral infection [7]. Therefore, we focused on IFN- β and four IFN-stimulated genes (ISGs), including RIG-I, MDA5, LGP2 and STAT1. As a control, we also analyzed the expression of MAVS, which is not induced by IFN. Our time course analyses by real-time RT-PCR at 12, 24, 48 and 72 hpi showed that RIG-I, MDA5, LGP2, and STAT1 as well as IFN β expressed to significantly higher levels in WT MEFs after NoV infection (Fig. 2A, 2B, 2C, 2E, 2F). However, expression of MDA5, LGP2, STAT1 and IFN β remained at the background levels in RIG-I^{-/-} MEFs as found for RIG-I during NoV infection (Fig. 2A, 2B, 2C, 2E, 2F). In contrast, no significant difference in MAVS expression was detected between WT and RIG-I^{-/-} MEFs in response to NoV infection (Fig. 2D). These findings together suggest that NoV infection induces the expression of IFN β and ISGs in a manner dependent on RIG-I.

3.3 IFN β pre-treatment rescues NoV resistance in RIG-I^{-/-} MEFs

The above results suggest that RIG-I is necessary for NoV-triggered expression of IFN β and the downstream signaling to the induction of ISGs and that NoV replicated to higher levels in MEFs derived from RIG-I^{-/-} mice than in WT MEFs. Therefore, we investigated whether IFN β pre-treatment could enhance the resistance of RIG-I^{-/-} MEFs to NoV infection. We found that NoV replication was markedly inhibited in RIG-I^{-/-} MEFs pre-treated with IFN β at the final concentration of 1 IU (International Units)/ml (Fig. 3). The inhibitory effect of IFN β on NoV infection was clearly detectable at all of the four time points examined after inoculation (Fig. 3). This result suggests that, in the absence of RIG-I, a potent antiviral state could be rescued in MEFs by IFN β pre-treatment. Taken together, our findings indicate that NoV infection of MEFs induces an IFN-dependent antiviral response mediated by RIG-I.

3.4 IFN β pre-treatment does not alter expression of key RNAi pathway genes

We next determined if IFN β pre-treatment altered the expression of RNAi pathway genes in NoV-infected RIG-I^{-/-} MEFs. To this end, we examined the expression level of four RNAi pathway genes by real-time RT-PCR in RIG-I^{-/-} MEFs at four time points after NoV inoculation with and without IFN β pre-treatment. Dicer and Argonaute 2 as well as the two dsRNA-binding proteins found in RISC, PACT and TRBP, were selected. The results showed that NoV infection does not alter the expression of these RNAi pathway genes in RIG-I^{-/-} MEFs either with or without IFN β pre-treatment (Fig. 4A–D). This suggests that the enhanced resistance of RIG-I^{-/-} MEFs pre-treated with IFN β is not associated with an altered expression of key RNAi pathway genes.

4. Discussion

In this manuscript we report the first investigation on the role of innate immune receptors RLRs and TLRs in the control of an RNA virus that encodes a VSR characterized in mouse infection. We showed previously that lethal infection of suckling mice with NoV is associated with the suppression of the biogenesis of viral siRNAs by the viral B2 protein [22]. Production of abundant viral siRNAs is readily detectable in mESCs infected with the B2-deficient mutant NoV, but not with wildtype NoV, indicating B2 suppression of viral siRNA biogenesis in the cultured mouse cells [23]. This study aimed to determine whether

mouse RLRs and TLRs restrict the infection of NoV, an RNA virus that potently suppresses the processing of the viral dsRNA into siRNAs during infection. We found that NoV accumulation reached a peak at 48 hpi in WT MEFs and declined at 72 hpi, suggesting restriction of NoV infection in MEFs by an antiviral response. However, NoV replicated to much higher levels in RIG-I^{-/-} MEFs than WT MEFs and the accumulation of NoV in RIG-I^{-/-} MEFs continued to increase from 48 hpi to 72 hpi. In contrast, genetic knockout of other RLRs and the two TLRs had no apparent effect on the infection of MEFs by NoV. These observations suggest that RIG-I acts to suppress the infection of NoV, similarly to those reported previously on the infection of other RNA viruses [3,4,5,7,38]. Our quantitative RT-PCR analysis showed that NoV infection induced the expression of IFN β and four ISGs in WT MEFs, but not in RIG-I^{-/-} MEFs. We found that unlike WT MEFs, the resistance to NoV infection was not maintained beyond 48 hpi in MEFs from MAVS^{-/-} mice, which are deficient in the production of type 1 IFNs triggered by RIG-I in response to virus infection [39,40]. Notably, IFN β pre-treatment significantly enhanced the resistance of RIG-I^{-/-} MEFs to NoV infection. These findings together suggest that NoV is targeted by an IFN-dependent antiviral immunity initiated by RIG-I.

Our results indicate that the IFN-dependent antiviral immunity mediated by RIG-I remains effective to target an RNA virus capable of strong suppression of antiviral RNAi during infection. One interpretation of this finding is that distinct mechanisms restrict NoV infection in IFN-dependent immunity and antiviral RNAi, but only the latter is targeted for suppression by the B2 protein. Future studies are necessary to investigate the possible functional interactions of these two antiviral responses. For example, recent studies have shown that two RLR proteins are essential for antiviral RNAi in *Caenorhabditis elegans* by controlling the biogenesis of viral siRNAs [41,42,43]. Interestingly, suppression of antiviral RNAi in plants by VSRs triggers a distinct counter-counter defense known as effector-triggered immunity [44,45,46]. Therefore, IFN-dependent response may have evolved in mammals to inhibit the infection of those viruses capable of escaping restriction by antiviral RNAi.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- RIG-I^{-/-} MEFs are more susceptible to NoV infection than wildtype MEFs.
- RIG-I is necessary for the expression of IFN-β and ISGs induced by NoV.
- IFNβ pre-treatment rescues NoV resistance in RIG-I^{-/-} MEFs.
- IFNβ pre-treatment does not alter expression of key RNAi pathway genes.

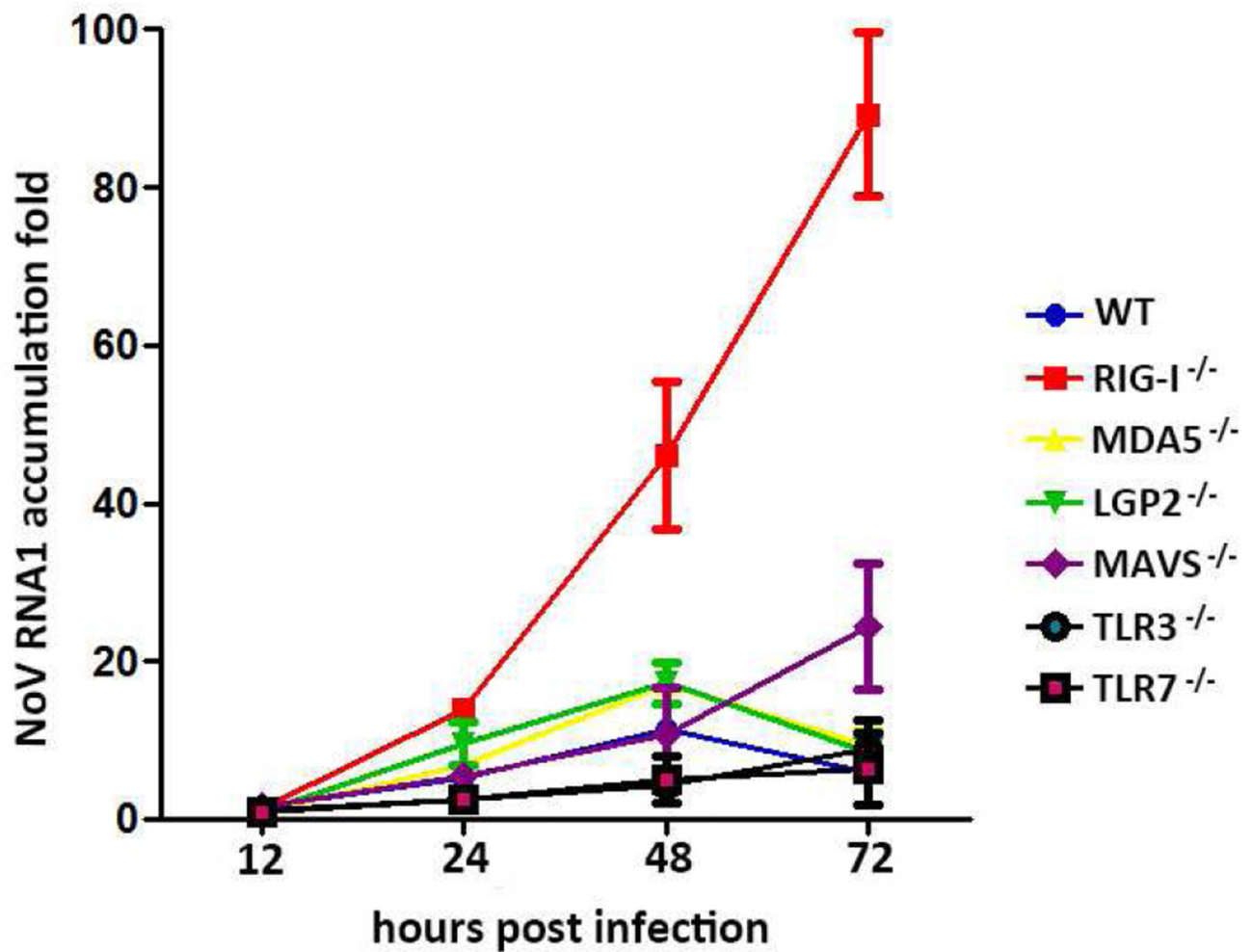


Figure 1. RIG-I deficiency leads to increased NoV replication in MEFs
Mouse embryo fibroblasts from wild type (WT), RIG-I^{-/-}, MDA5^{-/-}, LGP2^{-/-}, MAVS^{-/-}, TLR3^{-/-} and TLR7^{-/-} were inoculated with NoV at amount of viral genome copies (5×10^6). RNA1 levels of NoV were determined at 12, 24, 48 and 72 hpi for each sample by real-time RT-PCR.

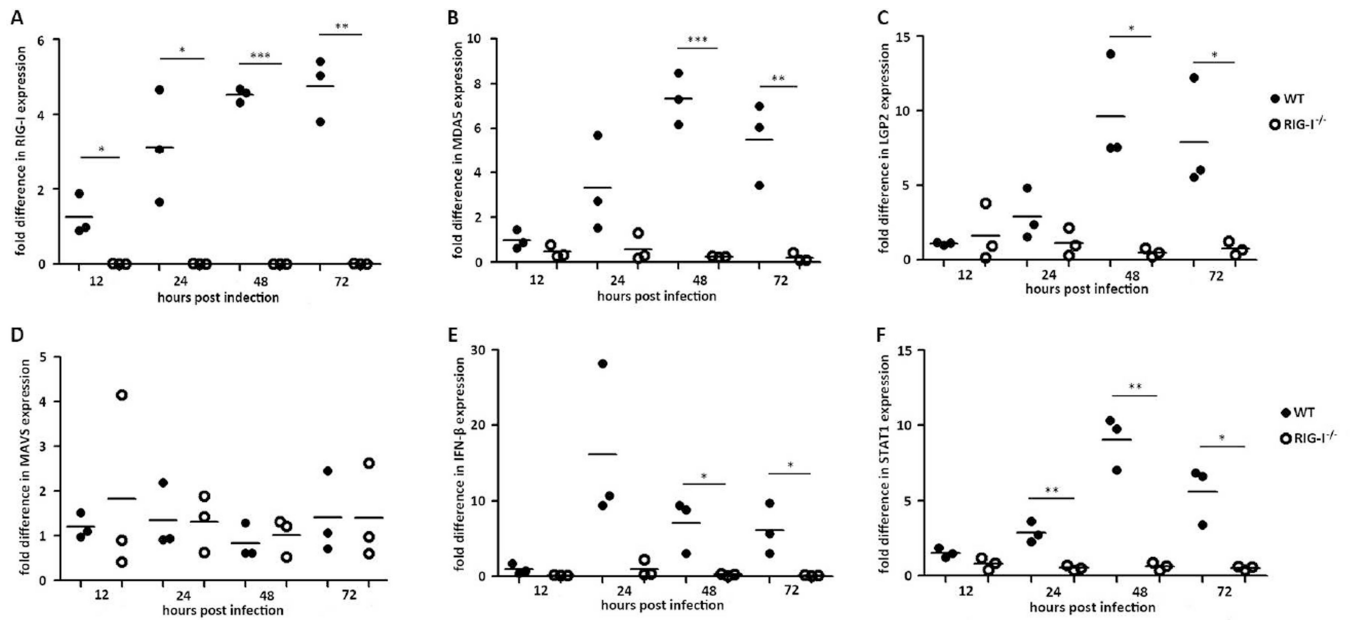


Figure 2. Expression profiles of innate immune-related genes in RIG-I^{-/-} MEFs

WT and RIG-I^{-/-} MEFs were infected with NoV for 12, 24, 48 and 72hpi. Cells of each sample were harvested and total RNA extracted. RIG-I (A), MDA5 (B), LGP2 (C), MAVS (D), IFN-β (E) and STAT1 (F) mRNA levels were determined by real-time RT-PCR and normalized by mRNA levels expressed in mock-inoculated MEFs.

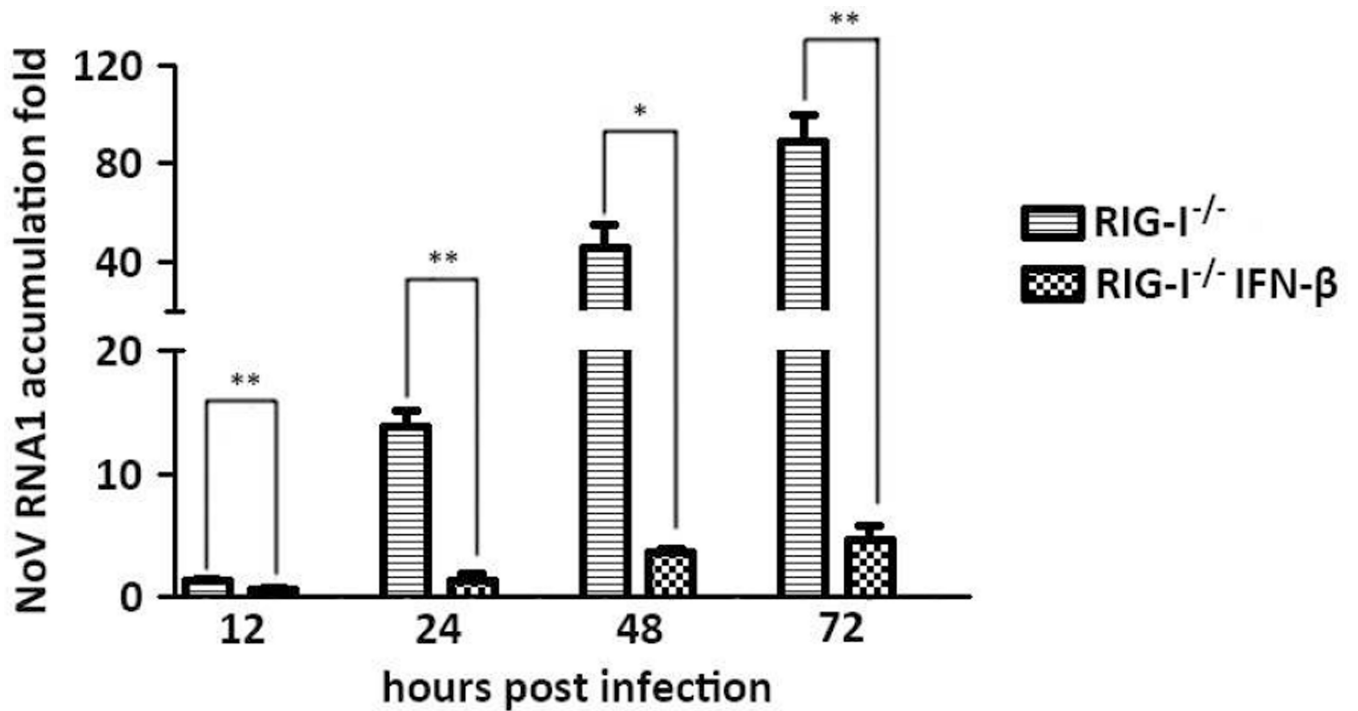


Figure 3. Suppression of NoV replication in RIG-I^{-/-} MEFs by IFN pre-treatment
 IFN-β (1unit/mL) was added in IFN pre-treated fibroblasts 8 hours prior to NoV infection. RNA1 levels of NoV were determined at 12, 24, 48 and 72 hpi for each sample by real-time RT-PCR. Data are shown as mean±S.E.M of triplicate samples from three independent experiments.

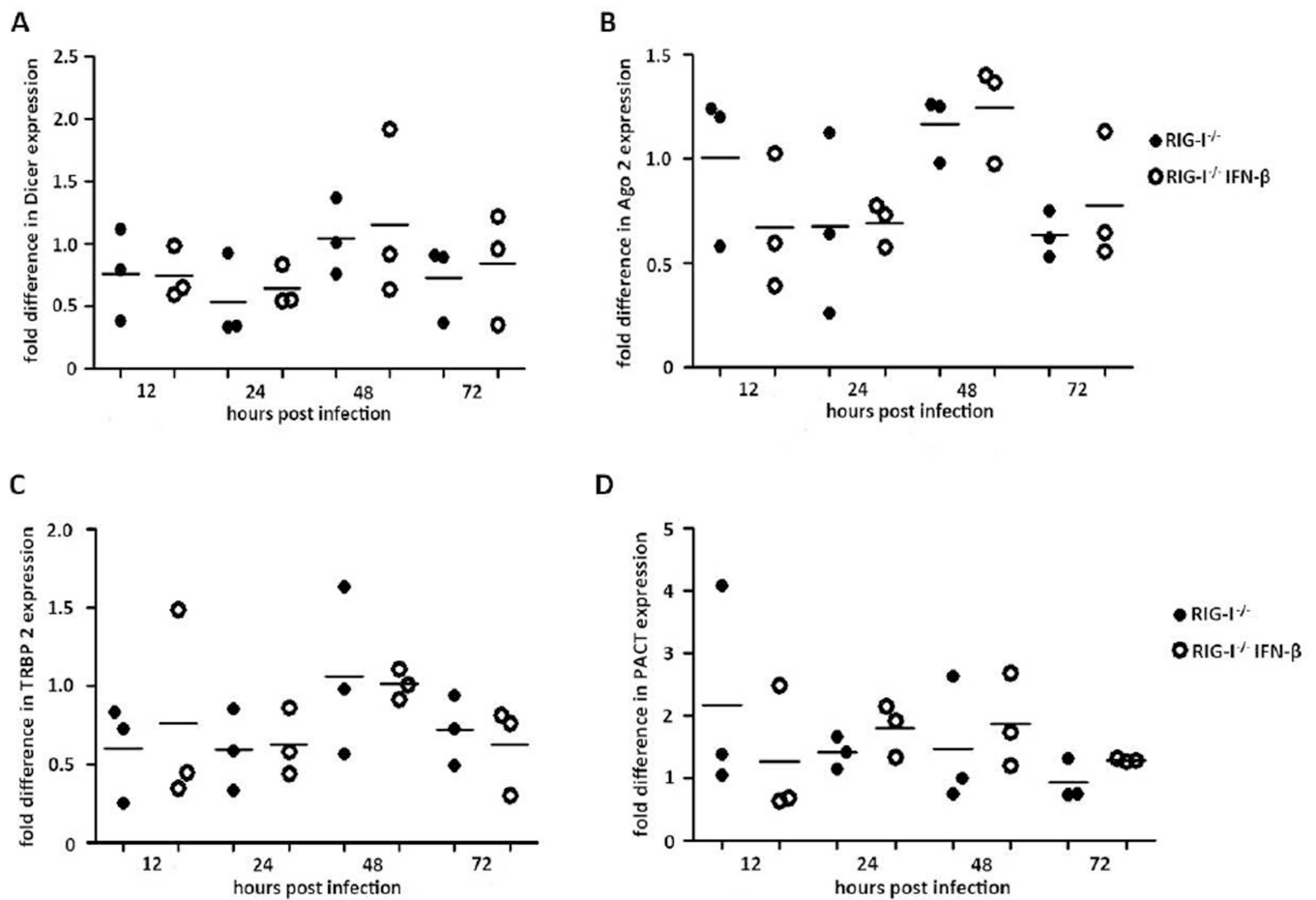


Figure 4. RNAi related gene expression profiles in IFN pre-treated RIG-I^{-/-} fibroblasts
Fibroblasts with and without IFN treatment were infected with NoV for 12, 24, 48 and 72hpi. Cells of each sample were harvested and total RNA was extracted. Dicer (A), Ago 2 (B), TRBP2 (C) and PACT (D) mRNA levels were determined by real-time RT-PCR and normalized by mRNA levels expressed in mock fibroblasts.