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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.

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 [3–5]. 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 [4–7]. 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 [8–10]. 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].

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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 [25–27]. 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 [28–30]. 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 [5,10,32–35]. 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% CO_2.

2.2. Infection in MEFs

MEFs (1×10^6) were infected by NoV with the same amount of viral genome copies (5×10^6). MEFs were harvested at 12, 24, 48, 72-h 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 ± 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 h 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 h after infection (Fig. 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