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MDA-5 Recognition of a Murine Norovirus

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Abstract

Noroviruses are important human pathogens responsible for most cases of viral epidemic gastroenteritis worldwide. Murine norovirus-1 (MNV-1) is one of several murine noroviruses isolated from research mouse facilities and has been used as a model of human norovirus infection. MNV-1 infection has been shown to require components of innate and adaptive immunity for clearance; however, the initial host protein that recognizes MNV-1 infection is unknown. Because noroviruses are RNA viruses, we investigated whether MDA5 and TLR3, cellular sensors that recognize dsRNA, are important for the host response to MNV-1. We demonstrate that MDA5−/− and TLR3−/− mice have a defect in cytokine response to MNV-1. In addition, MNV-1 replicates to higher levels in MDA5−/− DCs as well as in MDA5−/− mice in vivo. Interestingly, TLR3−/− DCs do not have a defect in vitro, but TLR3−/− mice have a slight increase in viral titers. This is the first demonstration of an innate immune sensor for norovirus and shows that MDA5 is required for the control of MNV-1 infection. Knowledge of the host response to MNV-1 may provide keys for prevention and treatment of the human disease.

Introduction

Norwalk virus and other human noroviruses are common human pathogens responsible for most of the nonbacterial epidemic gastroenteritis in both developed and developing countries [1,2,3,4,5]. In humans, norovirus infection can result in vomiting, diarrhea, fever, malaise, and abdominal pain within 24 hours after infection. These symptoms usually clear within 48 hours, but the virus can persist asymptptomatically for 3–6 weeks post-infection [6,7]. Until recently the inability to culture human noroviruses has prevented investigation into its pathogenicity. The discovery and subsequent routine culture of murine norovirus-1 (MNV-1) has led to advances in understanding of both the norovirus lifecycle as well as the host response to norovirus infection [8,9].

Noroviruses are in the Caliciviridae family and are nonenveloped viruses containing a single-stranded positive-sense RNA genome. Norovirus genomes are covalently linked at the 5′ end to a viral nonstructural protein VPg [10]. Norovirus genomes encode three open reading frames (ORFs) [11,12,13,14]. ORF1 encodes a polypeptide that is cleaved into at least six nonstructural proteins by the viral 3C-like protease [15,16,17,18]. ORF2 encodes the major capsid protein, viral protein 1 [11,19], while ORF3 encodes the small basic protein, viral protein 2 [20,21]. An additional ORF, ORF4 was recently discovered in the MNV genome although the function of this ORF has yet to be characterized [14].

The rapid clearance of MNV-1 infection in immunocompetent mice indicates an important role for the innate immune system, since clearance precedes the timeframe normally associated with the initiation of adaptive immunity [22]. Previous work has revealed that MNV-1 infection of mice lacking either the type I and type II interferon (IFNα/β/γ) receptors or the STAT-1 molecule results in lethality [9,22]. Several proteins are known to initiate the IFN response to viruses [23], including Toll-like receptors (TLR) [24], Rig-I-like helicases (RLH) [25,26], PKR [27], and RNase L [28]. However, the initial sensor responsible for recognition of noroviruses and subsequent activation of cytokine response has not yet been determined.

TLRs are located on the plasma membrane and in endosomal compartments. Among the TLRs, TLR 7 and 8 recognize ssRNA [29,30,31], TLR9 recognizes DNA [32,33], while TLR3 signals in response to dsRNA [34]. The RLHs are sensors located within the cytoplasm [26], which include Rig-I and MDA-5 [23,35,36] and signal through IPS-1/MAVS/Cardiffl/VISA [37,38,39,40]. Rig-I has recently been shown to preferentially recognize 5′-phosphorylated RNA [41,42], while MDA5 responds to dsRNA [43]. Recently it has been shown that the lack of Rig-I does not confer susceptibility to human norovirus in vitro [44]. Because MDA5 [45,46,47,48], and TLR3 [49,50] have been shown to play a role in host response to other RNA viruses we investigated if these sensors might be involved in norovirus recognition in vitro and in vivo using the MNV-1 model system. In this study we demonstrate that indeed MDA5 is the predominant sensor of MNV-1 and initiates the innate immune response against the virus, and that TLR3 may also play a role in the response to MNV-1 in certain tissues.

Results

MDA-5 is required for cytokine response to MNV-1 by Bone Marrow-Derived DC

Previous studies have shown a requirement for the type I IFN response for control of MNV-1 infection in vitro [8]. Since both
MDA-5 and TLR3 have been shown to be involved in type I IFN and cytokine signaling in response to infection with other RNA viruses, we were interested to see if they may play a role in MNV-1 infection. MNV-1 infection has a limited cell tropism—infecting only DC and macrophage lineages in vitro [8,51]. In order to test whether the MDA5 or TLR3 sensors were important, BMDCs from Wild Type as well as TLR3−/− and MDA5−/− mice were cultured for 7 days and then inoculated with various MOI of MNV-1. After 24 hours supernatants from the in vitro infections were harvested and tested for cytokine secretion from the BMDCs.

Interestingly, although WT and TLR3 DCs produced similar levels of IFNα and inflammatory cytokines in response to MNV stimulation, MDA5 deficient DCs produced significantly less IFNα, IL-6, MCP-1, TNFα (Figure 1) and IFNβ (data not shown). In this cell type MDA5 appears to be the primary sensor responsible for type I IFN production in response to MNV-1, however, we cannot rule out that other sensors may play a role in other cell types.

MDA5 limits MNV-1 replication in vivo

MNV-1 infection naturally occurs after fecal-oral transmission [8]. In order to test whether MDA5 and TLR3 play a role in MNV-1 detection in vivo we infected WT, MDA5−/−, or TLR3−/− mice with MNV-1.CW3 perorally. Organs were then harvested in titers. This may reflect a cell type-specific role for viral sensors.

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deficient mice were then stimulated with the harvested RNA, as well as RNA treated with RNase A which degrades ssRNA, and proteinase K (PK), which degrades proteins—in this case the VPg cap. As expected, RNase treatment degraded the viral RNA, while PK treatment did not degrade the RNA as seen in Figure 4a. Consistent with the results of in vitro MNV-1 infections shown in Figure 1, both WT and TLR3−/− BMDCs produced type 1 IFN in response to purified viral RNA, while MDA5−/− BMDCs had a significant decrease in IFN response (Figure 4b,c). However, the addition of PK or RNase to the RNA abrogated the cytokine response in WT and TLR3−/− BMDCs. This data demonstrates that VPg is required for MDA5 recognition of MNV-1, and suggests that MDA5 either directly recognizes RNA linked to VPg or, since VPg is required for norovirus replication [44], that MDA5 recognizes dsRNA generated during viral replication. Because MDA5 has been previously shown to recognize uncapped poly I:C [46,47], it is most likely that the result of PK treatment reflects the requirement for viral replication and the subsequent generation of dsRNA that is recognized by MDA5. Consistent with this hypothesis, WT BMDCs inoculated with UV-inactivated MNV-1 did not produce IFNβ (data not shown).

**Discussion**

We have provided the first description of an initial sensor of norovirus infection. MDA5 recognizes MNV-1 and stimulates antigen presenting cells to produce type I interferon as well as IL-6, MCP-1, and TNFα that function to recruit other immune cells as well as activate antiviral pathways in host cells. Deficiency of this sensor results in lack of cytokine production as well as increased MNV-1 replication in deficient cells and mice.

It is interesting to note that although MDA5 deficient cells have a severe defect in IFNα production, MDA5−/− mice contain and clear MNV-1 infection. This is in contrast the severe systemic infection and survival phenotype as the IFNαβR or STAT1 deficient mice, which lack type I and type II IFN signaling pathways. STAT-1−/− and IFNαβR−/− mice have a 4 log increase in viral titers in vivo and a 2 log increase in viral titers in vitro as seen in previously published data [22]. In our study MDA5−/− mice have a 1-log increase in viral titers in vivo and in vitro, while TLR3−/− mice have a 0.5 log increase, but only in one organ in vivo. This indicates to us that although MDA5 may be the dominant sensor in BMDCs, it is likely that in other cell types additional sensors can detect MNV-1, such as Rig-I, PKR, TLR7, and perhaps other unknown sensors. Further investigation is needed to determine if mice and cells that are deficient in multiple nucleic acid sensors lack all ability to respond to MNV-1 and whether they therefore have a more severe phenotype. Data from our lab and others [44] from in vitro experiments suggest that lack of TLR3 and Rig-I seem to have little effect on MNV-1 recognition individually, however, we cannot rule out that their involvement is masked by MDA5.

Although the putative recognition structure for Rig-I has previously been determined [41,42], the RNA structure recog-
nized by MDA5 in viral infection remains unclear. We demonstrated that MDA5 recognition of MNV RNA is abrogated by treatment with PK, which degrades VPg, preventing viral replication. This data suggests that VPg is essential for MDA5 recognition of MNV-1. Although we cannot rule out the possibility that MDA5 recognizes the VPg-RNA structure itself, this is less likely because MDA5 is known to respond to poly I:C which has no protein cap. It is more likely that since VPg is essential for viral replication of the ssRNA norovirus genome, loss of VPg prevents MDA5 recognition of dsRNA produced during viral replication. Learning more about which viruses are recognized by MDA5 may provide hints as to what this protein recognizes. This information could then be used to design adjuvants to manipulate the immune response for both vaccine design as well as in treatment of viral infection.

Materials and Methods

Cell lines
RAW264.7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (HyClone), 100 U penicillin/ml, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine.

Viruses
All experiments were performed with MNV-1.CW3 [14]. Virus stocks were generated using RAW 264.7 cells that were inoculated with a multiplicity of infection (MOI) of 0.05 in VP-SFM media (Gibco) and harvested approximately 40 hours after inoculation. Infected cell lysates were frozen at -80°C and thawed three times. Cell lysates were clarified by low-speed centrifugation for 20 min at 3,000 rpm. To generate a concentrated virus stock, clarified cell lysates were concentrated by centrifugation at 4°C for 3 h at 27,000 rpm (90,000 g) in a SW32 rotor.

Bone marrow-derived DC
Bone marrow was flushed from the femurs of mice and cultured as described previously [52]. Briefly, cells were cultured in RPMI (Gibco) with 10% fetal calf serum (HyClone), Glutamax, Na Pyruvate, Non-Essential AAs, and Kanamycin for 7–8 days at 37 degrees.
Mice
MDA5−/− mice were described previously [47]. For the infection studies mice backcrossed onto a pure 129/SVJ background were used. Control WT mice were age and sex matched and were obtained from littermate controls and from Jackson Lab for 129/SVJ and C57BL/6. TLR3−/− mice were kindly provided by Richard Flavell [34]. All mice were bred and housed in a pathogen free facility and regularly tested for MNV-1 antibodies.

In vitro stimulations
BMDCs were counted and plated at 200,000 cells/well in a 96 well plate. MNV-1 was added at various MOI to the cultures, or alternatively 500 ng RNA was complexed with lipofectamine 2000 (invitrogen) and added according to manufactures instructions. After 20–24 hours supernatants were harvested and stored at −20 degrees until cytokine analysis. IFNα and IFNβ levels from the supernatants were measured by ELISA (PBL Biomedical Laboratories, New Brunswick, NJ), while IL-6, MCP-1, and TNFα levels were determined by cytokine bead array (BD Biosciences).

In vivo infections
WT, TLR3−/−, or MDA5−/− mice were infected perorally with 3×10⁷ PFU MNV1.CW3 [14] or mock-infected with media only. Three days post-infection the following organs were harvested and stored at −80 degrees until assayed: spleen, liver, mesenteric lymph node, lung, proximal intestine, distal intestine, stool, and serum.

MNV-1 plaque assay
Tissue samples were homogenized in 1 ml complete DMEM by bead beating with 1.0-mm zirconia/silica beads (BioSpec Products, Inc.). Tissue homogenates were diluted 1:10 in complete DMEM and tested for viral titers by using a plaque assay that has been previously described [8]. Briefly, 2×10⁶ RAW264.7 cells were seeded into each well of six-well plates, and infected the next day with 10-fold dilutions of tissue homogenate in duplicate. After a 1-hr infection, the inoculum was removed and wells were overlaid with 1.5% SeaPlaque agarose (Cambridge Biosciences) in complete minimal essential medium and incubated at 37°C. After 48 hrs, a second overlay was added containing 1.5% SeaKem agarose (Cambridge Biosciences) and 0.01% neutral red in complete minimal essential medium. After 8 hrs, plaques were then visualized.

RNA preparation
Total viral RNA was harvested from concentrated virus stock using Trizol reagent (Invitrogen) according to manufacturer’s instructions. Purified RNA was incubated with either 10 units RNase A (Sigma) in NEB buffer 3 (New England Biolabs) or with 200 μg/ml protease K (Sigma) in 0.1 M NaCl, 10 mM Tris

Figure 3. MDA5 deficiency leads to increased MNV titers in vitro. Bone marrow-derived dendritic cells from wild type (WT), MDA5−/−, or TLR3−/− mice were inoculated with MNV at an MOI of 5 (A) or 0.05 (B) or pre-treated with 20 U IFNαx and then inoculated with an MOI of 0.05 (C). Viral titers were done at 6 hour time-points for each sample and statistical significance was determined using student’s t test. There was no significant difference between WT and TLR3−/− titers, statistical significance is marked between WT and MDA5−/− titers where * = p<0.05. Data shown is the average of four independent experiments (A and B) or three independent experiments (C). In (D) supernatants from WT BMDC infected at MOI 0.05 were harvested at various time-points and tested for IFNβ by ELISA. Data shown is the average of three independent experiments.
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(pH 8), 1 mM EDTA, 0.5% sodium dodecyl sulfate or left untreated in NEB buffer 3 for 30 minutes at 37°C then stopped with 0.1 mM EDTA. To test for RNA degradation, samples were run on a 1% agarose gel and visualized using a UV light box.

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Author Contributions

Conceived and designed the experiments: SAM MC. Performed the experiments: SAM. Analyzed the data: SAM LBT HWV. Contributed reagents/materials/analysis tools: LBT LG SG HWV. Wrote the paper: SAM.

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