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MDA5 plays a critical role in interferon response during hepatitis C virus infection

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Background & Aims: Hepatitis C virus (HCV) is a human pathogen that can evade host immunity to cause persistent infection, leading to liver cirrhosis and hepatocellular carcinoma. The transfected 3′UTR of HCV genomic RNA can be recognized by host protein RIG-I to activate interferon production in hepatocytes. However, it is difficult to demonstrate the RIG-I mediated sensing of HCV genomic RNA in the context of HCV infection because HCV-encoded NS3-4A protease can inactivate MAVS, a critical adaptor protein in interferon signaling. Our aim was to identify the viral sensor that triggers interferon response in hepatocytes during HCV infection.

Methods: We generated a hepatic cell line that stably expressed mutant MAVS resistant to the NS3-4A cleavage. This cell line allowed us to investigate the interferon signaling pathway in the context of HCV infection. By using the knockdown and knockout technology together with biochemical approaches, we were able to identify the actual viral sensor in hepatocytes during HCV infection.

Results: We showed that HCV infection induced robust interferon response in the cells expressing MAVS resistant to the NS3-4A cleavage. Unexpectedly, the interaction between HCV′s 3′UTR and RIG-I seemed to play a minor role in this activation, while another helicase MDA5 played a more important role in sensing HCV infection to trigger interferon response.

Conclusions: Our data demonstrate that MDA5 recognizes HCV to initiate host innate immune response during HCV infection. This study provides insight into how host senses HCV to initiate innate immunity during HCV infection.

Keywords: HCV; MDA5; Interferon; Innate immunity.

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Abbreviations: HCV, hepatitis C virus; RIG-I, retinoic acid inducible gene I; MDA5, melanoma differentiation-associated gene 5; IFN, interferon; UTR, untranslated region; MAVS, mitochondrial antiviral signaling protein; PAMP, pathogen-associated molecular patterns; HCVcc, HCV cell culture; MOI, multiplicity of infection.

Introduction

The reaction of host to invading microbes is an innate immune response initiated by host pattern recognition receptors (PRRs) that recognize components specific to microorganisms called pathogen-associated molecular patterns (PAMPs) [1]. There are three major classes of PRRs: Toll-like receptors (TLRs), RIG-I–like helicases (RLHs) and NOD-like receptors (NLRs). RLHs comprise of RIG-I, MDA5, and LGP2, which all contain DExD/H helicase domain. RIG-I and MDA5 both contain two CARD domains at the N-terminal [2]. The binding of viral RNA to the C termini of RIG-I and MDA5 presumably induces a conformational change that exposes the N-terminal CARD domains, which interact with the CARD domain of the mitochondrial adaptor protein MAVS (also known as IPS1, VISA, or CARDIF) [3,4]. MAVS then activates the cytosolic kinases IKK and TBK1 [5], which activate the transcription factors NF-κB and IRF3, respectively. NF-κB and IRF3 translocate into the nucleus collectively eliciting innate antiviral immune responses, including production of type I and III interferons [6,7]. RIG-I and MDA5 sense different types of ligands and distinct subsets of RNA viruses. Cytoplasmic 5′-triphosphorylated ssRNA and short poly(I:C) are recognized by RIG-I [8,9]. Long cytoplasmic dsRNAs, such as long poly(I:C), are recognized by MDA5 [8]. This distinct ligand preference has been shown to confer specific recognition of individual viruses: RIG-I is required for interferon production in response to several paramyxoviruses, influenza virus, and Japanese encephalitis virus [10], whereas MDA5 is crucial for the host defense against picornaviruses [11]. RIG-I and MDA5 are individually dispensable for signaling in response to reovirus or dengue virus (DEN) infection. RIG-I and MDA5 cooperate to trigger an innate immune response to West Nile virus (WNV) [11].

Hepatitis C virus (HCV) infects approximately 170 million people worldwide, and 80% of the infected individuals develop persistent infection. HCV contains a 9.6-kb positive-sense RNA

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genome encoding a 3000-amino acids polyprotein, which is cleaved into at least ten structural and nonstructural (NS) proteins by cellular and viral proteases. The 3′ untranslated region (3′UTR) of HCV genomic RNA, essential for viral genome replication, contains a 100-nt polyU/UC tract flanked by a variable region at the 5′ end and a conserved region at the 3′ end. Previous studies showed that *in vitro* transcribed HCV 3′UTR RNA can be recognized by RIG-I to trigger innate immune response after it is delivered into HuH7 hepatic cells by transfection [12,13]. Biochemical analyses revealed the direct physical interaction between RIG-I and HCV 3′UTR RNA, and the polyU/UC tract plays an essential role [14]. However, the physiological relevance of these results remains to be addressed as the RIG-I's recognition of HCV RNA to initiate the interferon response was difficult to demonstrate in the context of HCV infection, in spite of availability of an infectious cell culture model (HCVcc) [15–17]. HCVcc infection in HuH7 cells fails to trigger the production of detectable amounts of type I interferon [3,18], because HCV-encoded NS3-4A serine protease can cleave MAVS at cysteine of position 508, right before its mitochondrial targeting domain. This cleavage releases MAVS from the mitochondria and thus blocks the downstream interferon activation [3,6,19]. Therefore, it remains elusive whether HCV does possess the ability to trigger the interferon response during its replication life cycle, and whether this activation is mediated by RIG-I.

To address these questions, we generated a HuH7 hepatoma cell line that expresses a MAVS mutant form resistant to the NS3-4A cleavage. HCV infection induces robust interferon production in this cell line. However, we found that this interferon activation is mainly dependent upon MDA5. Our work provides important insight into how host cells sense HCV to initiate the interferon signaling during HCV infection.

Materials and methods

Preparation of HCV 3′UTR RNA, RNA isolation, RNA transfection, coimmunoprecipitation, Western blot, and HCVcc stock preparation

The protocols were as previously described [6,20].

Knockdown assays

The RIG-I or MDA5 siRNAs, the mix of 3–5 siRNAs targeting different sites were purchased from Santa Cruz. Control siRNA was a nontargeting pool (D-001810-10-20) from Dharmacon (Lafayette, CO, USA). siRNAs were transfected into cells following the manufacture's instruction.

Generation of RIG-I or MDA5 knockout HuH7-MAVSR cells by lenti-CRISPR-Cas9

HEK293T cells were seeded into 6-well plates one day prior to transfection at a density of 7×10^5 cells per well. The cells were co-transfected with 1.3 μg VSV-G expressing plasmid, 2.5 μg pCMV-dR8.91 plasmid expressing gag, pol and rev genes, and 2.5 μg lenti-Cas9-sgRNAs [21] targeting EGFP, RIG-I or MDA5, using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested at 48 h post transfection, filtered and used to infect HuH7-MAVSR cells. After 48 h post-infection, 0.75 μg/ml puromycin was added into medium for long time culture. The knockout of RIG-I and MDA5 was verified by genomic DNA sequencing and Western blot.

Luciferase reporter assay

HuH7-MASVR cells were seeded into 48-well plates overnight prior to infection with JFH1 at the indicated MOI. At 24 h post infection, the cells were transfected with 20 ng/well of the IFN-β-Luc construct [22] and 20 ng/well of the CMV promoter driven-Renilla luciferase vector (pRL-CMV; Promega) used for normalizing transfection efficiency. One day after the plasmid transfection, the cells were transfected with 400 ng/well HCV 3′UTR or L-poly(I:C) and harvested at 18 h post-transfection. Cell lysates were assayed for luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

Primary human hepatocytes culture, knockdown and infection

Primary human hepatocytes (purchased from Research Institute for Liver Diseases, Shanghai, China) were plated in complete DMEM at a density of 1×10^5 cells/cm^2 on 48-well plates coated with type 1 collagen. The medium was changed to serum-free differentiation (SFD) medium [23] at 12 h after plating. To generate lentiviruses expressing shRNA targeting RIG-I (5′-CCA GAA TTA TCC CAA CCG ATA-3′) or MDA5 (5′-CCA ACA AAG AAG CAG TGT ATA-3′), empty pLKO.1 plasmid or plasmids bearing the RIG-I or MDA5 shRNA were transfected into HEK293T cells respectively, together with packaging plasmid pCMV-dR8.91 and envelope plasmid pMD2.G. The produced lentiviruses were collected at day 2 post-transfection, passed through a 0.45 μm filter, and then inoculated to primary human hepatocytes. Two days later the lentivirus-transduced cells were infected with HCVcc at an MOI of 2. The cells were collected at 48 h post infection for RT-qPCR analysis of interferon activation.

Results

Generation of a HuH7 cell line expressing MAVS mutant resistant to HCV NS3-4A protease cleavage

HCV NS3-4A protease can cleave the MAVS protein at Cys508 to subvert the innate immune response [3,19]. As the result, HCV infection does not trigger detectable amounts of interferon production in the recently developed HCV *in vitro* cell culture model [15,16,22]. To identify the host sensor that recognizes HCV infection, we used a lentiviral vector to generate HuH7 cell lines that stably express the Flag-tagged wild-type MAVS or mutant MAVS in which the cysteine at position 508 was changed to arginine (C508R). The C508R mutation has been shown previously to block the NS3-4A proteolytic cleavage while maintaining the function of MAVS in the interferon signaling [3]. The exogenously introduced wild-type or C508R mutant MAVS were mildly expressed in the stable cell lines, designated HuH7-MAVS and HuH7-MAVSR respectively, and did not significantly increase the basal levels of *IFN-β* mRNA (Supplementary Fig. 1).

Next, we compared the abilities of these cell lines to produce IFN-β following stimulation with HCV 3′UTR RNA or long poly(I:C) (L-poly(I:C)), which are recognized by RIG-I or MDA5, respectively [8,12,13]. As shown in Fig. 1A, the IFN-β levels in the two cell lines stimulated by HCV 3′UTR RNA or L-poly(I:C) were comparable, suggesting that the C508R mutation in MAVS did not alter RIG-I- or MDA5-mediated interferon signaling.

Next we tested how these two cell lines responded to the stimuli in the presence of the HCV NS3-4A. The NS3-4A expression plasmids were transduced into HuH7-MAVS and HuH7-MAVSR cells prior to the stimulation with HCV 3′UTR RNA and L-poly(I:C). Immunoblotting assays were performed to analyze the NS3-4A expression and the MAVS cleavage, and *IFN-β* mRNA was determined by RT-qPCR assay. As illustrated in Fig. 1A and B, NS3-4A cleaved MAVS and dramatically inhibited 3′UTR RNA- and L-poly(I:C)-induced interferon production in HuH7-MAVS cells, but failed to do so in HuH7-MAVSR cells. The slight decrease of IFN-β levels in the NS3-4A transfected HuH7-MAVSR cells could result from the cleavage of endogenous MAVS by NS3-4A or other previously reported NS3-4A blocking mechanism independent from the MAVS cleavage [24,25]. These results show