BASIC AND TRANSLATIONAL—LIVER

Differential, Type I Interferon-Mediated Autophagic Trafficking of Hepatitis C Virus Proteins in Mouse Liver

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BACKGROUND & AIMS: The hepatitis C virus (HCV) serine protease NS3/4A can cleave mitochondria-associated antiviral signaling protein (MAVS) and block retinoic acid–inducible gene I–mediated interferon (IFN) responses. Although this mechanism is thought to have an important role in HCV-mediated innate immunosuppression, its significance in viral persistence is not clear. **METHODS:** We generated transgenic mice that express the HCV NS3/4A proteins specifically in the liver and challenged the animals with a recombinant vesicular stomatitis virus, a synthetic HCV genome, IFN alfa, or IFN beta. We evaluated the effects of HCV serine protease on the innate immune responses and their interactions. **RESULTS:** Expression of HCV NS3/4A resulted in cleavage of intrahepatic MAVS; challenge of transgenic mice with vesicular stomatitis virus or a synthetic HCV genome induced strong, type I IFN-mediated responses that were not significantly lower than those of control mice. Different challenge agents induced production of different ratios of IFN alfa and beta, resulting in different autophagic responses and vesicular trafficking patterns of endoplasmic reticulum- and mitochondria-associated viral proteins. IFN beta promoted degradation of the viral proteins by the autolysosome. Variant isoforms of MAVS were associated with distinct, type I IFN-mediated autophagic responses; these responses have a role in trafficking of viral components to endosomal compartments that contain Toll-like receptor–3. **CONCLUSIONS: IFN beta mediates a distinct autophagic mechanism of antiviral host defense. MAVS has an important role in type I IFN-induced autophagic trafficking of viral proteins.**

Keywords: Autophagy; TLR3; Liver Disease; RIG-I.

Hepatitis C virus (HCV) has infected an estimated 130 million people worldwide.[1] Exposure to HCV typically leads to chronic liver disease and is the most common cause of cirrhosis, chronic liver failure, and hepatocellular carcinoma. The immediate host response to acute HCV infection is triggered through cellular pattern recognition receptors, such as the intracytoplasmic retinoic acid–inducible gene I (RIG-I) and the Toll-like receptor 3 (TLR3). These molecules signal through their respective adaptors, the mitochondria-associated antiviral signaling protein (MAVS) (also called IPS-1, Cardif, and VISA) and the Toll/interleukin-1 receptor domain containing adaptor-inducing interferon (IFN) beta (TRIF) to activate synthesis of IFN beta, IFN alfa, and proinflammatory cytokines. The secreted IFN beta and alfa are recognized by a shared IFN alfa/beta receptor. Their initial induction leads to activation of an autocrine/paracrine-positive feedback pathway, which results in the amplification of the response and induction of an antiviral state in the infected as well as neighboring bystander cells.[2,3]

The mechanism of HCV persistence is complex and has often been linked to its ability to thwart the host immune response.[4] Molecular studies have revealed several levels of host immune modulation by HCV.[5] In one such mechanism, the HCV NS3/4A serine protease has been shown to interfere with the innate immune signaling pathways through the specific cleavage of the adaptor proteins MAVS and TRIF.[4] A signaling blockade imposed by this mechanism has been suggested to limit the level and diversity of IFN-stimulated gene (ISG) expression, leading to significant attenuation of host immunity.[5–8] Although the role of NS3/4A-mediated MAVS cleavage in HCV-mediated innate immune suppression has been largely substantiated in vitro and in vivo,[9,10] its significance in the clinical course of the disease remains unclear. TLR3 is up-regulated during the ISG response in HCV-infected cells, and cleavage of TRIF has been demonstrated in vitro.[4] However, the issue of how virus-derived double-stranded RNA encounters TLR3, which is present on the cell surface or in intracellular endosomes, remains unresolved. In this context, the cellular autophagy machinery has been suggested to play a role in delivering viral genetic

Abbreviations used in this paper: AdLacZ, recombinant adenovirus LacZ; ER, endoplasmic reticulum; HCV, hepatitis C virus; IFN, interferon; IRF-3, IFN regulatory factor-3; ISG, IFN-stimulated gene; LAMP1, lysosome-associated membrane protein 1; LC3B, microtubule-associated protein I light chain 3B; MAVS, mitochondria-associated antiviral signaling protein; mRNA, messenger RNA; qRT-PCS, quantitative reverse transcription polymerase chain reaction; RFP, red fluorescence protein; RIG-I, retinoic acid-inducible gene I; TLR3, Toll-like receptor 3; TRIF, Toll/interleukin-1 receptor domain containing adaptor inducing IFN beta; VSV, vesicular stomatitis virus.

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material to endosomal TLRs.[11] Autophagy is a cellular degradation pathway by which cytoplasmic constituents, including organelles, are directed to the lysosome. Although autophagic pathways can regulate viral replication by influencing the antiviral responses, many viruses have evolved to evade, subvert, or exploit this innate response.[12] HCV requires the autophagy machinery to initiate its replication.[13] It induces an incomplete autophagic process at the surface of the endoplasmic reticulum (ER) and uses it as a membranous scaffold for RNA replication.[14] Alternatively, the Atg5-Atg12 conjugate, a key regulator of autophagy, negatively regulates the type I IFN signaling by direct association with RIG-I and MAVS.[15] Yet, it is not known if these 2 virus-related autophagic responses traverse.

HCV is seen to persist in infected patients, despite potent IFN induction and ISG expression.[9,10] Currently, the standard therapy with pegylated IFN alfa and ribavirin is far from optimal; the sustained virological response is <50% among patients with genotype 1 HCV infection.[4] However, high pretreatment ISG levels in hepatitis C patients are more frequently associated with nonresponsiveness to the IFN alfa–based therapy,[16,17] which is rather perplexing, as an active IFN system is expected to potentiate the IFN alfa treatment. To date, an understanding of the correlation between MAVS cleavage and innate immune response, as well as IFN treatment outcomes, has been largely impeded by the absence of robust small animal models suitable to address these issues in vivo.

To specifically delineate the role of the HCV NS3/4A-mediated innate immunocompromise in viral infection, we generated a novel transgenic mouse with hepatocyte-specific expression of NS3/4A proteins, and demonstrated that cleavage of MAVS by the HCV NS3/4A protease by itself does not impose an overwhelming blockade of the type I IFN response in the liver. Our results further show, for the first time, a differential effect of IFN alfa and beta on HCV proteins in vivo due to the disparate up-regulation of autophagic proteolysis and suggest an essential role of MAVS in the delivery of viral RNA to the endosomal compartments.

Materials and Methods

Mice

Inducible NS3/4A transgenic mice were generated on the C57BL/6 × C3H background as detailed in Supplementary Materials and Methods and bred with Alb-Cre mice[18] to generate mice with liver-specific, constitutive expression of HCV NS3/4A proteins. Double-transgenic (NS3/4A × Alb-Cre) mice were euthanized around 6 to 8 weeks of age, and liver, spleen, and lung tissues were analyzed for constitutive expression of the NS3/4A transgene by Western blot and immunohistochemistry. To determine the time-course induction of transgene expression in the NS3/4A single transgenic mice, a pCMVCre plasmid[19] was hydrodynamically injected through the tail vein and livers were harvested for Western blot at 2-day intervals up to day 12. Mice were euthanized by CO_2 asphyxiation. All experiments described were performed on 6- to 12-week-old animals, and control and transgenic groups were age- and sex-matched. All animal care and experimentation were performed according to the National Institute of Health Guidelines and with the approval of the Institutional Animal Care and Use Committee.

Challenge Studies

Double transgenic and littermate control mice were challenged with various inducers of type I IFN response, including a recombinant vesicular stomatitis virus, vesicular stomatitis virus tagged with red fluorescence protein (VSV-RFP) (Indiana strain) (10^7 pfu), HCV genotype 1b genome complexed with a lipid-based in vivo transfection reagent (100 μg; Altogen, Las Vegas, NV), poly(I:C) (100 μg; InvivoGen, San Diego, CA); poly(I:C)/LyoVec (50 μg; InvivoGen); a replication-deficient adenovirus (AdLacZ, 2×10^9 pfu; ViraQuest, North Liberty, IA); IFN alfa (1000 IU; HyCult, Plymouth Meeting, PA); and IFN beta (10,000 IU; PBL InterferonSource, Piscataway, NJ). All challenge agents, except for the HCV genome, were delivered intravenously through the tail vein. The HCV genome was diluted in 5% glucose and hydrodynamically delivered. Mice were euthanized at 5 or 8 hours postchallenge, and the blood and livers were collected for analysis.

Statistical Analysis

For statistical analyses, the 2-tailed Student *t* test was used. Analysis of variance was performed. *P* values <.05 were considered significant. *P* values >.05 were considered not significant.

Additional Methods

Procedures described in Supplementary Materials and Methods include those for VSV passage and titration; in vitro transcription of HCV genome; quantitative reverse transcription polymerase chain reaction (qRT-PCR) for relative quantitation of IFN beta and ISG56 messenger RNA (mRNA) levels and transgene transcription analysis; qRT-PCR for AdLacZ copy numbers; immunostaining and fluorescent microscopy; Western blot; enzyme-linked immunosorbent assay; immunoprecipitation; and isolation of cellular vesicles.

Results

Characterization of Transgenic Mice

To delineate the effect of HCV NS3/4A on the innate immune responses, we generated Cre/loxP-inducible, NS3/4A transgenic mice by using the pLIVE-NS3/4A liver-specific construct (Figure 1*A*). To render the mice capable of expressing NS3/4A constitutively, we bred the transgenic founders with an ALB-Cre partner that has liver-specific expression of Cre-recombinase.[18] Double transgenic mice (NS3/4A × Alb-Cre) from 6 founder lines were analyzed for transgene expression. Of the 6 lines screened, only one line showed detectable transgene expression (Figure 1*B*−*D*). Both the NS3/4A fusion protein and the cleaved NS3 (the major species) were detected in Western blots (Figure 1*B*). Cleavage of the NS3/4A junction indicated that the NS3 serine protease was functionally active. NS3 expression was further confirmed by immunoprecipitation in the double transgenic livers (Figure 1*C*). Immunohistochemical analyses demonstrated the pe-