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Hepatitis C virus NS3/4A protease blocks IL-28 production

Qiang Ding[1], Bing Huang[2], Jie Lu[1], Yong-Jun Liu[2,3] and Jin Zhong[1]

[1] Unit of Viral Hepatitis, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
[2] Unit of Dendritic Cell Biology and Viral Sensor, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
[3] Baylor Institute for Immunology Research, Dallas, TX, USA

Type I interferons (IFNs), including IFN-α, -β, and -ω, play a critical role in innate immune responses against viral infection. IFN-λ, including IL-29, IL-28A, and IL-28B, recently identified as a new subfamily of IFN named type III IFN, has also been demonstrated to suppress virus replication in vitro and in vivo. However, the molecular mechanisms that regulate the induction of type III IFNs during viral infection remain elusive. Here, we demonstrate that IL-28 (IFN-λ 2/3) IFN production, similar to type I IFN, represents a primary and direct host response to HCV genomic RNA transfection. IL-28 (IFN-λ2/3) induction by HCV genomic RNA was dependent upon the activation of NF-κB and IRF3. We identified a minimal IL-28 promoter region consisting of putative NF-κB and IRF3-binding sites. Furthermore, we showed that HCV infection can inhibit HCV genomic RNA-induced IL-28 expression, and that the viral NS3/4A protease activity was responsible for this inhibitory effect. Our results present important evidence for the control of type III IFN response by HCV, and shed more light on the molecular mechanisms underlying the persistence of HCV infection.

Keywords: Hepatitis C virus · IL-28 · Interferon lambda (IFN-λ) · NS3/4A protease

Introduction

Viral infection can trigger innate immune responses, and interferon (IFN) production plays critical roles in the antiviral program and immune response regulation. Currently, there are three kinds of IFNs: type I (IFN-α and IFN-β), type II (IFN-γ), and type III (IFN-λ). IFN-λ was a recently identified IFN subclass that includes IFN-λ1, IFN-λ2, and IFN-λ3, encoded by IL-29, IL-28A, and IL-28B, respectively [1, 2]. Viruses can be sensed in infected cells through the interaction of pathogen-associated molecular patterns (PAMPs) with host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). After engaging with ligands, these PRRs activate IKKε/TANK-binding kinase 1 (TBK-1), the IKKα/β/γ complex as well as MAPK, leading to the activation of IFN regulatory factor 3 (IRF3), IRF7, NF-κB, and AP-1. The activated transcription factors translocate to the nucleus and activate the transcription of type I IFN. In an autocrine as well as paracrine manner, the IFN-α/β activates expression of IFN-stimulated genes (ISGs) through the JAK-STAT signaling pathway to establish an antiviral state in the host cells [3, 4]. IFN-λs are believed to activate the JAK-STAT signaling pathway that promotes the expression of a common set of antiviral proteins [5]. However, in contrast to type I IFN that signals through a universally expressed receptor, IFN-λ uses a distinct receptor complex (IL-28R) for signaling,

Correspondence: Dr. Jin Zhong
e-mail: jzhong@sibs.ac.cn

which consists of a unique IFN-λR1 subunit and IL-10R2 subunit [1, 6]. The IFN-λR complex is expressed on a limited range of cell types, such as epithelial cells of respiratory and gastrointestinal tracts [7]. Differences in their receptor distribution suggest that the type I and III IFNs may not be functionally redundant.

Viruses also evolve multiple mechanisms to evade the host IFN responses. The influenza A virus NS1 protein or the poxvirus E3L protein can sequester viral double-stranded RNA (dsRNA) and prevent activation of intracellular dsRNA sensors, such as RIG-I and MDA5, suppressing type I IFN induction pathway [8, 9]. In addition, the type I IFN-activated JAK-STAT signaling pathway may also be targeted by viruses. For example, Japanese encephalitis virus counteracts the effect of IFN-α/β by blocking Tyk2 activation [10]. Hepatitis C virus (HCV) NS3/4A, the viral protease responsible for cleavage of the viral polyprotein [11, 12], can also cleave the key adaptor protein MAVS [13, 14] — also named VISA, Cardif, or IPS-1[14–17] — that transmits signals from upstream sensor molecules RIG-I or MDA5 to downstream TBK-1/IKKε and IKKα/β/γ complex; HCV NS3/4A also cleaves TRIF, another key adaptor protein responsible for transmitting signals from TLR3 [18], therefore blocking both RIG-I- and TLR3-mediated activation of type I IFNs. HCV NS2 protein was also found to inhibit IFN-α, IFN-β, IL-29, and chemokine gene promoter activity [19].

Type III IFNs have been reported to have antiviral function through the JAK-STAT signal pathway, which is similar to IFN-α/β. Recent work demonstrated that genetic variations of the IL-28 locus in the human genome are strongly associated with spontaneous clearance of HCV [20] and with the therapeutic outcomes of chronic HCV patients treated by pegylated type I IFN plus ribavirin [21–23]. This association suggests that type III IFN may contribute to type I IFN-mediated HCV control.

Here, we demonstrate that IL-28 production, similar to type I IFN, represents a primary and direct host response to viral infection. HCV genomic RNA was able to induce the IL-28 expression in a RIG-I-dependent manner, which can be suppressed by HCV NS3/4A protease. Our results present important evidence for the control of type III IFN response by HCV, and shed more light on the molecular mechanisms underlying the persistence of HCV infection.

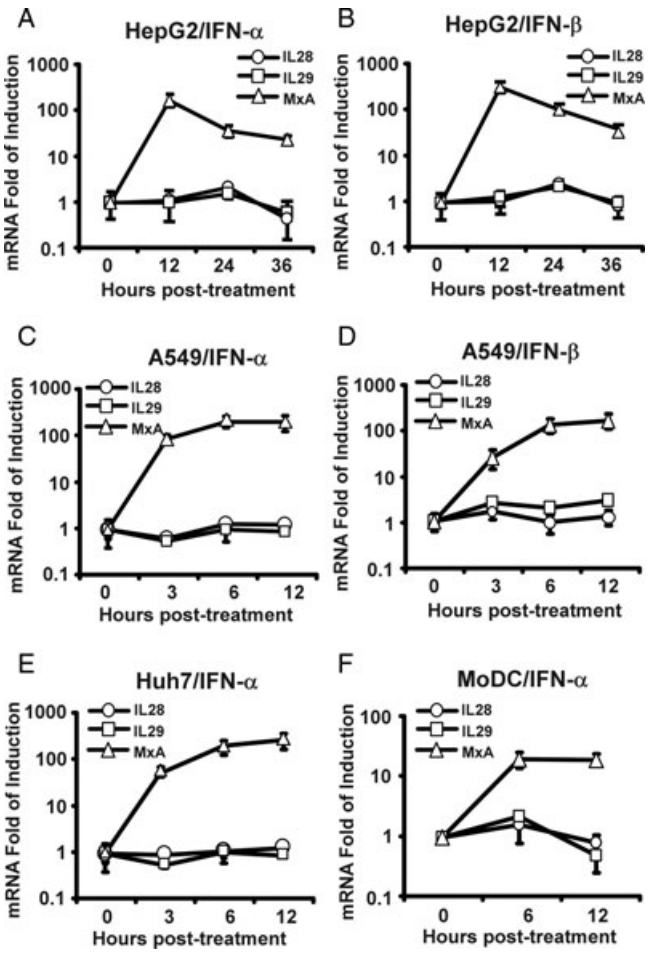

Figure 1. IL-28 and IL-29 are not induced by type I IFN. The indicated cell types were treated with 200 IU/mL of IFN-α or IFN-β, and collected at the indicated time points for determining the mRNA levels of IL-28 (circles), IL-29 (squares), and MxA (triangles) by RT-qPCR. All data were normalized against cellular GAPDH mRNA levels, and expressed as the fold of induction as compared with mock-treated cells. Data are shown as the mean ± SD of six samples pooled from three independent experiments. (A) HepG2 cells were treated with IFN-α; (B) HepG2 cells were treated with IFN-β; (C) A549 cells were treated with IFN-α; (D) A549 cells were treated with IFN-β; (E) Huh7 cells were treated with IFN-α; (F) Human monocyte-derived dendritic cells (MoDCs) were treated with IFN-α.

Results

IL-28 and IL-29 are not classical type I IFN-stimulated genes

Previous studies showed that IL-28 and IL-29 expression can be upregulated upon virus infection or poly(I:C) stimulation that also induces type I IFN production [1, 2]. Another study showed that IL-28 and IL-29 can be induced by IFN-α treatment in hepatoma-derived cell line HepG2, suggesting type III IFNs may act against virus infection as the type I IFN-stimulated gene [24]. Recent genome-wide association studies on chronic HCV patients receiving the standard IFN-α-based therapy revealed a remarkable association of SNPs (single-nucleotide polymorphism) near and within the IL-28B gene with the outcome of the therapy [21–23] also suggesting that type I IFN may act against HCV by inducing type III IFN.

To investigate whether type III IFNs (IL-28 and IL-29) are type I IFN induced, HepG2 and a lung epithelial cell line (A549) were treated with IFN-α or IFN-β for 3, 6, and 12 h. RT-qPCR assay was performed to determine the IL-28, IL-29, and MxA mRNA levels. As shown in Fig. 1A–D, IL-28 and IL-29 mRNA levels barely changed upon either the IFN-α or IFN-β treatment; whereas MxA, one of well characterized type I IFN ISGs, was dramatically upregulated (> 100-fold). Our data (not shown) also indicated that the IL-28 and IL-29 mRNA levels did not increase at later time points (24 and 36 h) in HepG2 cells. Furthermore, we tested whether IFN-α can induce IL-28 and IL-29 in another hepatic cell line

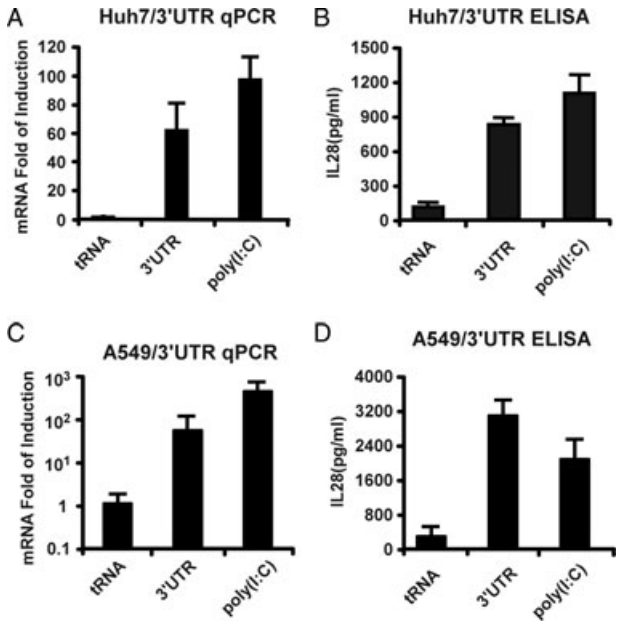

Figure 2. HCV 3′UTR RNA transfection induces type III IFN expression. (A-B) Huh7 cells were transfected with HCV 3′UTR RNA. Sixteen hours posttransfection, (A) the cells were collected for determining the IL-28 mRNA levels by RT-qPCR, and (B) the culture supernatants were collected for determining the IL-28 protein levels by ELISA. (C-D) A549 cells were transfected with HCV 3′UTR RNA. (C) The cells were collected for determining the IL-28 mRNA levels by RT-qPCR, (D) the culture supernatants were collected for determining the IL-28 protein levels by ELISA. Data are shown as the mean + SD of six samples pooled from three independent experiments.

(Huh7) and human monocyte-derived dendritic cells (MoDCs). As shown in Fig. 1E and F, IL-28 and IL-29 were not upregulated in response to the IFN-α treatment. These data clearly indicated that, in contrast to the previous report [24], IL-28 and IL-29 were not classical type I IFN stimulated genes under the conditions tested.

IL-28 can be upregulated upon HCV 3′UTR stimulation

Previous studies showed that IL-28 and IL-29 were upregulated upon viral infection or poly(I:C) stimulation in PBMCs [1], and HCV 3′UTR was the PAMP motif recognized by RIG-I to trigger IFN-β transcription [25]. To examine whether HCV 3′UTR can induce IL-28, we synthesized 3′UTR RNA of JFH-1, a genotype 2a HCV strain [26–28], and transfected it into Huh7 or A549 cells. Yeast tRNA and poly(I:C) were also transfected in parallel as the controls. The IL-28 mRNA and protein levels were determined by RT-qPCR and ELISA respectively. Figure 2A–D shows that HCV 3′UTR RNA and poly(I:C) transfection can activate IL-28 in both cells. IFN-β and MxA mRNAs were also strongly induced by the treatment, and IL-28 and IL-29 mRNA induction displayed similar kinetics with IFN-β (data not shown).

IL-28A and IL-28B cDNA sequences share over 90% homology; therefore, they could not be distinguished in the RT-qPCR or ELISA assays. To answer this question, we subcloned the RT-qPCR products of IL-28 mRNA induced by HCV 3′UTR. The sequencing analysis of 20 and 16 individual clones revealed that the ratio of IL-28A/IL-28B mRNA in A549 and Huh7 cells upon the induction was about 1.5:1 and 1.7:1 respectively, indicating that both IL-28A and IL-28B were induced.

Taken together, our data indicated that IL-28 may be directly induced by HCV 3′UTR suggesting that the type III IFN production likely constituted a primary rather than a secondary antiviral response to HCV infection.

RIG-I is essential for HCV 3′UTR RNA-induced IL-28 production

Previous studies showed that HCV 3′UTR could be sensed by RIG-I to activate type I IFN response[25]. To examine the role of RIG-I in the type III IFN response to HCV 3′UTR, we transfected HCV 3′UTR RNA into Huh7 cells and RIG-I deficient Huh7.5.1 cells [29], and the IL-28 protein levels were determined by ELISA. As presented in Fig. 3A, both poly(I:C) and HCV 3′UTR RNA significantly induced IL-28 in Huh7 cells, but not in Huh7.5.1 cells, suggesting that RIG-I was the essential PRR that recognizes HCV 3′UTR RNA to trigger the type III IFN response. To further confirm this, we transfected Huh7.5.1 cells with plasmids expressing the wild-type or a dominant-negative mutant RIG-I (RIG-IC) in which the CARD domain was deleted [30]. At 24 h posttransfection, the cells were transfected again with the HCV 3′UTR RNA. Sixteen hours later, culture supernatants were collected for IL-28 ELISA analysis, and the cells were harvested for RT-PCR to quantify the transfected 3′UTR RNA and for western blotting assay to determine the RIG-I expression levels. As presented in Fig. 3B, the wild-type but not the dominant-negative mutant RIG-I can restore the IL-28 induction in Huh7.5.1 cells upon the HCV 3′UTR RNA stimulation. In consistent with this result, the overexpression of the dominant-negative mutant RIG-I can significantly reduce IL-28 induction in Huh7 cells upon the stimulation (data not shown).

IL-28 and type I IFNs share a similar activation pathway upon viral infection

NF-κB and IRF3 are essential transcription factors for IFN-β transcription in response to virus infection [31]. Therefore next we tested whether the induction of IL-28 was also mediated by these two key transcription factors.

To examine whether the NF-κB pathway was activated upon HCV 3′UTR RNA stimulation in A459 cells, we transfected A549 cells with 3′UTR RNA and cells lysates were subjected to western blotting assay to detect IκBα. As shown in Fig. 4A, IκBα was degraded at 4 h posttransfection and then restored to normal levels at 8 h postinfection, an indicator of NF-κB activation [32], suggesting that HCV 3′UTR RNA could activate the NF-κB pathway. Next, we examined whether the NF-κB activation led to IL-28 induction. We transfected increasing doses of plasmids expressing IKK-β, which is responsible for phosphorylation and degradation of IκBα, or p65, the transactivation subunit of

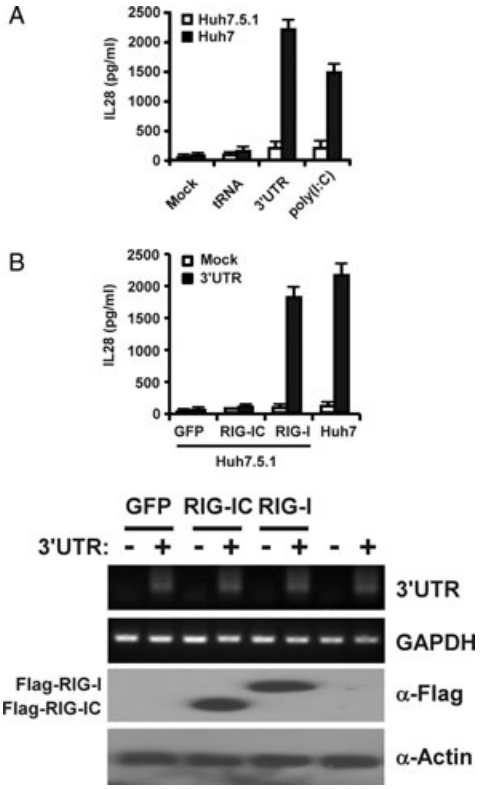

Figure 3. RIG-I is essential for HCV 3′UTR RNA-induced IL-28 production. (A) Huh7 and RIG-I deficient Huh7.5.1 cells were transfected with 2 μg in vitro transcribed HCV 3′UTR RNA, with an equal dose of yeast tRNA and poly(I:C) included as the controls. Sixteen hours later, the culture supernatants were collected for the IL-28 ELISA assay. (B) The plasmids expressing FLAG-tagged wild-type or a dominant-negative mutant RIG-I (RIG-IC), or GFP were transfected into Huh7.5.1 cells. Twenty-four hours posttransfection, the cells were transfected with the HCV 3′UTR RNA for an additional 16 hours, with Huh7 cells included as the control. After stimulation, culture supernatants were collected for IL-28 ELISA analysis (upper panel), and the cells were harvested for RT-PCR to quantify the transfected 3′UTR RNA and for western blotting assay to determine the Flag-RIG-I and Flag-RIG-IC expression levels (lower panels). The ELISA data are presented as absolute quantities. Data are shown as the mean + SD of six samples pooled from three independent experiments.

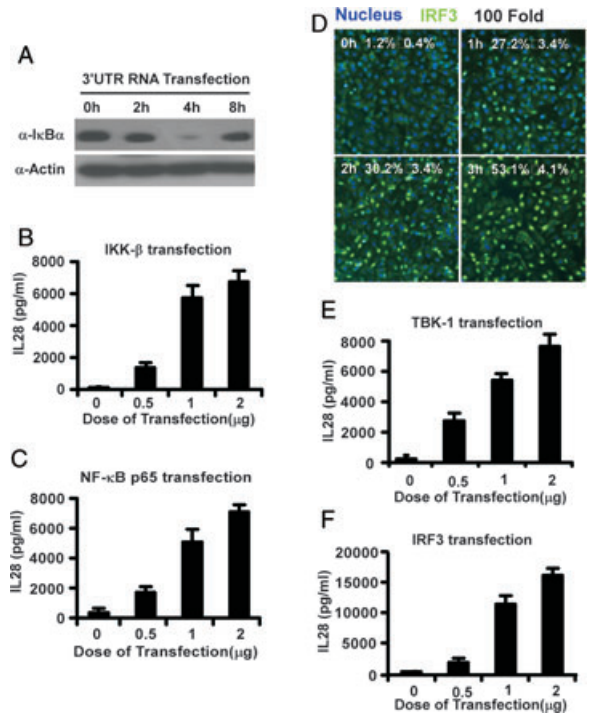

Figure 4. NF-κB and IRF3 mediate HCV 3′UTR RNA-induced IL-28 activation. (A) Western blot analysis of IκBα in A549 cells at the indicated time points after 3′UTR RNA transfection. Cells lysates were separated by 12% SDS-PAGE and probed with an IκBα antibody. β-actin was also examined as the protein loading control. (B, C) Increasing amounts of plasmids expressing (B) IKK-β or (C) p65 subunit of NF-κB was transfected into A549 cells, respectively. Culture supernatants were collected 24 h posttransfection for IL-28 analysis by ELISA. (D) Immunofluorescence analysis of the IRF3 subcellular localization in A549 cells upon 3′UTR RNA transfection. 3′UTR RNA transfected A549 cells were fixed at the indicated time points and then were probed with an antibody against IRF3, followed by a secondary Alexa FluorR 488 conjugated antibody (green). Cell nuclei were visualized by staining with Hoechst dye (blue). Two hundred cells from three randomly selected images were counted to quantify the nuclear localization of IRF-3, and the numbers were shown in white on the top of the representative images. Data are shown as means ± SD of three randomly selected images and are representative of three independent experiments. The magnification is 100×. (E and F) Increasing amounts of plasmids expressing (E) TBK-1 or (F) IRF3 were transfected into A549 cells. Culture supernatants were collected 24 h posttransfection for IL-28 analysis by ELISA. The ELISA data are presented as absolute quantities. Data are shown as the mean ± SD of six samples pooled from three independent experiments.

NF-κB into A549 cells, and measured the IL-28 protein levels at 24 h posttransfection. As shown in Fig. 4B and C, overexpression of IKK-β or p65 significantly increased the IL-28 expression in a dose-dependent manner. These data suggested that the NF-κB activation-mediated HCV 3′UTR RNA induced type III IFN production.

Next, we examined whether IRF3 activation was also involved in HCV 3′UTR induced IL-28 production. First, we analyzed the subcellular localization of IRF3 upon HCV 3′UTR RNA transfection. As shown in Fig. 4D, the frequency of cells with IRF3 in nucleus reached over 50% at 3 h posttransfection from less than 2% at the baseline, indicative of IRF3 activation[31]. Then we examined whether the activation of IRF3 led to IL-28 induction. We transfected A549 cells with increasing doses of plasmids expressing IRF3 or TBK-1 that is responsible for phosphorylation of IRF3, and measured the IL-28 protein levels at 24 h posttransfection. As shown in Fig. 4E and F, overexpression of TBK-1 or IRF3 significantly increased the IL-28 expression in a dose-dependent manner, suggesting that the IRF3 activation-mediated HCV 3′UTR RNA induced type III IFN production.

Altogether, our results indicated that IL-28 and type I IFNs shared a very similar induction pathway that is dependent upon NK-κB and IRF3.

Mapping of a minimal region of IL-28 promoter for transcription activation

To map a minimal region in the IL-28 promoter responsible for IL-28 transcription activation, we analyzed the DNA

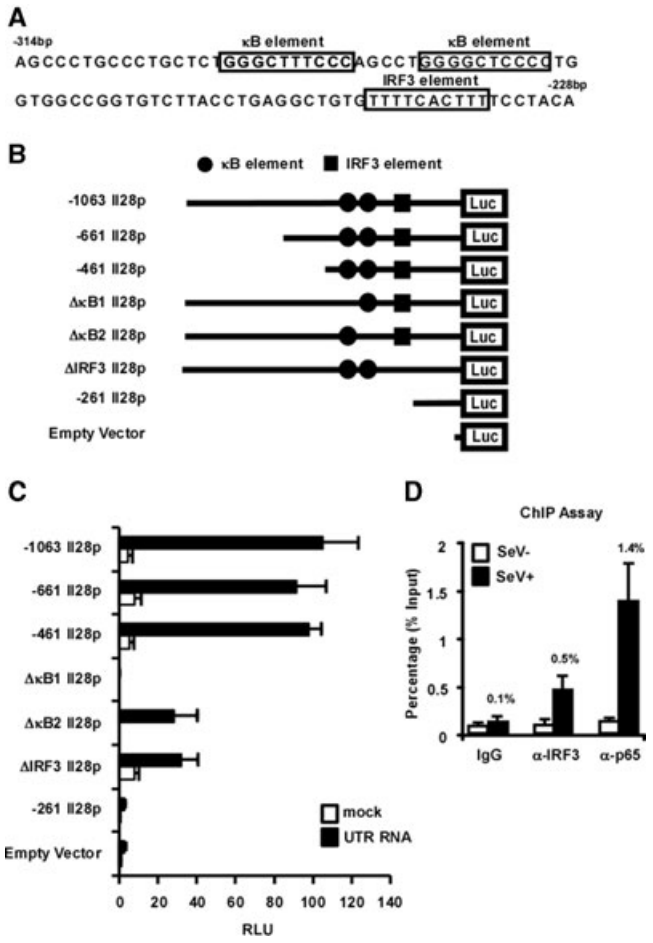

Figure 5. NF-κB p65 and IRF3 can directly bind to the putative elements located within the IL-28B promoter. (A) Sequence of the minimal IL-28B promoter. One putative IRF3-binding site, at −243 nt and two putative NF-κB-binding sites, at −298 nt and −283 nt, are indicated with open boxes. The position is numbered according to the start site of IL-28B translation. (B) Schematic diagram of a series of IL-28B promoter luciferase reporter constructs. Putative NF-κB and IRF3-binding sites are marked. (C) A549 cells were cotransfected with various IL-28B promoter luciferase reporter plasmids and the internal control plasmid pRL-TK. Twenty-four hours later, cells were transfected with or without HCV 3′UTR RNA (2 ug/mL) for 12 h. The cell lysates were subjected to a dual luciferase assay, and the results were expressed as fold induction of luciferase activity relative to the empty vector without HCV 3′UTR RNA transfection. Data are shown as the mean + SD of six samples pooled from three independent experiments. (D) A549 cells were infected with SeV for 4 h, cross-linked, and sonicated to generate chromatin fragments. The sheared chromatin was immunoprecipitated with monoclonal antibodies against NF-κB p65, IRF3, or a mouse IgG isotype control followed by qPCR analysis using the primer set flanking the IL-28 promoter. The data are expressed as the percentage of immunoprecipitated chromatin DNA versus the total input. Data are shown as the mean + SD of six samples pooled from three independent experiments.

sequences 1200-bp upstream of IL-28 start codon by TFsearch program (http://www.cbrc.jp) and TESS Tool (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Two putative κB and one putative IRF3-binding sites were identified (Fig. 5A). To confirm this bioinformatical result, we constructed a series of truncated IL-28 promoters (Fig. 5B) upstream of a promoterless luciferase ORF in the pGL3-enhancer vector [33]. The engineered pGL3-IL28P plasmids were transfected into A549 cells prior to the HCV 3′UTR RNA transfection. The luciferase activities were measured to evaluate the promoter activity of the inserted IL-28 promoter sequences. As shown in Figure 5C, deletion of the κB and IRF3-binding sites (-261IL28p) completely abolished the luciferase activity induced by the HCV 3′UTR RNA transfection. The deletion of any of these κB and IRF3-binding sites also significantly impaired the HCV 3′UTR RNA stimulation, with the first κB-binding site as the most critical one. All the data suggested that the key elements responsible for IL-28 transcription activation were located in a region between 461-bp and 261-bp upstream of IL-28 ORF. Furthermore, we performed the chromatin immunoprecipitation (ChIP) assay to confirm this result. The Sendai virus (SeV) infected A549 cells were harvested at 4 h postinfection, and the cell lysates were immunoprecipitated with antibodies for IRF3 or p65, followed by qPCR using the primer sets specifically for the IL-28 promoter sequence to quantify the amount of the IRF3 or NF-κB-bound DNA. As shown in Figure 5D, the amount of IL-28 promoter precipitated by α-NF-κB p65 or α-IRF3 increased for 15- and 5-fold respectively in the SeV infected cells as compared with the isotype lgG control, whereas no difference was observed in the mock infected cells. These results revealed direct and specific binding of NF-κB and IRF3 to cis-regulatory sequences in the IL-28 promoter region.

HCV NS3/4A protease suppresses viral genomic RNA-induced IL-28 activation

Next, we tested whether IL-28 could be induced during HCV infection. Huh7 cells were inoculated with cell culture-grown HCV (HCVcc; strain (JFH-1) at an MOI of 0.2. IL-28 mRNA levels were determined by RT-qPCR at 3, 6, 12, 24, 48, and 72 h postinfection. IL-28 mRNA levels were not induced in the HCV infected Huh7 cells (data not shown), indicating that HCV may have evolved a mechanism to block the induction of IL-28. Therefore, we examined the impact of HCV on its own 3′UTR RNA-induced IL-28 expression. Huh7 cells were first infected with HCVcc. When greater than 80% cells had been infected at day 5 postinfection (data not shown), the cells were then transfected with HCV 3′UTR RNA for 16 h. The culture supernatants were collected for the IL-28 ELISA, and cells were collected for western blot to probe HCV NS3 and MAVS (also named as VISA, IPS-1, or Cardif) [14–17], a known host target of NS3 protease [13, 14]. As illustrated in Fig. 6A, HCV 3′UTR RNA induced the IL-28 expression in the uninfected Huh7 cells, whereas this induction was significantly reduced in the HCV infected cells in which MAVS was cleaved. These results suggested that HCV infection could block the IL-28 activation induced by its own genomic RNA.

To confirm whether the inhibitory effect of HCV infection on the IL-28 induction was indeed exerted by NS3/4A protease, we transfected Huh7 cells with plasmids expressing wild-type NS3/4A or a protease defective H57A mutant [34, 35]. At 48 h posttransfection, Huh7 cells were transfected again with HCV 3′UTR RNA

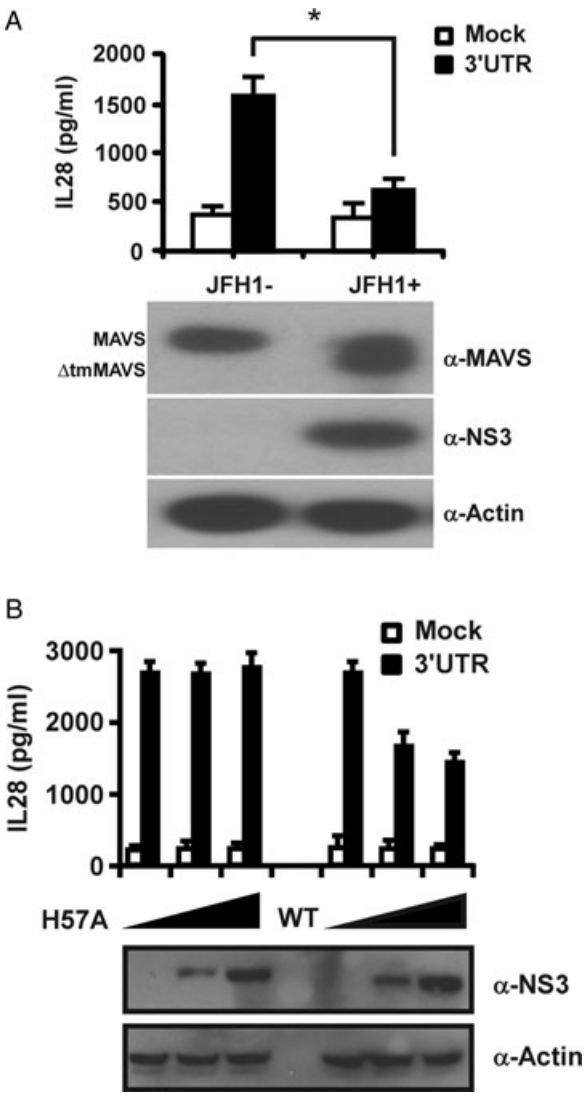

Figure 6. HCV NS3/4A protease blocks HCV 3′UTR RNA-induced IL-28 production. (A) The HCVcc (JFH-1) infected Huh7 cells were transfected with HCV 3′UTR RNA. Sixteen hours later, the cells were lysed for western blotting analysis to probe MAVS and HCV NS3 expression. The culture supernatants were collected for IL-28 analysis by ELISA. Data are shown as means + SE of duplicate wells and are representative of three independent experiments.* $p < 0.05$ (two-tailed unpaired t-test). (B) Huh7 cells were transfected with the indicated dose of plasmids expressing wild-type or protease defective H57A mutant NS3/4A. Forty-eight hours later, the cells were transfected again with HCV 3′UTR RNA for an additional 16 h. Cell lysates were subjected for western blotting assay to detect NS3 expression, and the culture supernatants were harvested for the IL-28 ELISA. Data are shown as the mean + SD of six samples pooled from three independent experiments.

for 16 h. The culture supernatants and cell lysates were collected to measure the IL-28 and NS3 protein levels respectively. As illustrated in Fig. 6B, the wild-type NS3/4A protease could reduce the HCV 3′UTR RNA induced IL-28 expression in a dose-dependent manner, while the protease mutant NS3/4A could not.

Collectively, our data suggested that HCV 3′UTR RNA acted as the PAMP motif recognized by RIG-I to induce IL-28 production, and HCV NS3/4A protease was able to block this activation by targeting the key adaptor molecules MAVS.

Discussion

Host cells respond to virus infection by producing type I and type III IFNs. It remains controversial whether type III IFN production represents a primary or a secondary host response to virus infection. One study showed that the IFN-α treatment induced the IL-28 and IL-29 expression in HepG2 cells [24], and recent genome-wide association studies revealed a remarkable correlation between IL-28B SNPs and the outcome of IFN-α-based therapy of chronic hepatitis C patients [21–23], favoring the concept that type III IFN represents a secondary type I IFN-induced antiviral response, at least in hepatocytes. Our study, however, demonstrates that type III IFN cannot be induced by IFN-α or IFN-β stimulation in hepatic cell lines (HepG2 and Huh7), lung epithelial cell line (A549), and monocyte-derived dendritic cells. Instead, our data demonstrate that IL-28 production represents a direct antiviral response, which was consistent with the results from several other groups. A cluster of functional IFN regulatory factor-binding sites and a NF-κB-binding site were found within the promoter region of IL-29 (IFN-λ1) [36]. Stoltz et al. found that Hantaan Virus could induce IFN-λ1 expression in African green monkey epithelial Vero E6 cells that cannot produce type I IFNs, clearly indicating that HTNV can induce IFN-λ1 independent of IFN-α/β production [37]. Our study provided the molecular mechanisms by which virus infection directly induces IL-28 (IFN-λ2/3) transcription. We found that HCV PAMP induces IL-28 through both NK-κB and IRF3, the key transcription factors also required for transcription of IFN-β. A minimal promoter region of IL-28 consisting of two NK-κB and one IRF3-binding sites was identified. It is worth noting that the NF-κB elements identified in our study were different from the element identified by Österlund et al. [38]. All of these data indicate that type III IFN, similar to that of type I IFN, constitutes the primary host antiviral response.

Although type I and type III IFNs share similar antiviral activities and molecular regulation of their production, several lines of evidence suggest that type I and type III IFNs have some important differences. While the type I IFN receptors are expressed in almost all types of cells, the type III IFN receptors are primarily expressed on epithelial cells, including hepatocytes, but not on hemopoietic cells and neural cells, suggesting more restricted roles of type III IFNs [39]. Type I and III IFNs may also exert their antiviral effects at different stages of host responses or against different types of viruses [7, 40, 41]. Recent studies revealed some unique roles of type III IFNs in regulating adaptive immunity. IL-29 and IL-28B were found to promote Th1 and CTL responses, while inhibiting regulatory T-cell responses [42]. A recent study found that IL-28A could promote DC IL-12p70 production and Th1 responses, while suppress Th2 and Th17 responses and ameliorated allergic airway disease [43]. These data suggested type III IFNs may have a distinct role in regulating adaptive immunity than the type I IFNs.

Our work found that HCV 3′UTR can act as the PAMP motif to trigger type III IFN expression. As a counter measurement, HCV blocks this antiviral response by using its own NS3/4A protease to target the key adaptor molecules that mediate the activation of

NK-κB and IRF3. In order to maintain a persistent viral infection, HCV has developed multiple mechanisms to evade both innate and adaptive immune responses. Previous studies had revealed that HCV can block type I IFN production by cleaving two key adaptor molecules, TRIF [18] and MAVS [13, 14], which play important roles in TLR3 and RIG-I mediated IFN-β production respectively. Here our results provide important evidence that HCV can employ a similar mechanism to block IL-28 production, which has also been observed by Lamarre and colleagues in freshly isolated human normal primary hepatocytes through microarray analysis [44]. In addition to suppression of innate immune response, the blockade of type III IFN production may potentially disrupt its modulation on Th1/Th2/Th17 responses to skew Th1/Th2/Th17 responses in favor of Th2 and Th17 [42, 43]. The lack of a Th1 response may contribute to the inadequate CTL response that plays an essential role in eliminating HCV infection [45]. Therefore, our findings should shed more light on the mechanisms underlying the persistence of HCV infection. A deeper understanding of these processes may eventually lead to development of efficient immune therapy for chronic hepatitis C patients.

Materials and methods

Cell culture

A549, HepG2, Huh7, and Huh7.5.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Human MoDCs were maintained in HyQ®RPMI 1640 medium (HyClone, Logan, UT, USA) with the same supplements.

Plasmid construction

To construct the IL-28 promoter reporter plasmid, a 1063 bp sequence upstream of the translation start codon was amplified from cDNA of A549 cells using primers containing KpnI and HindIII sites. To generate the IL-28 promoter truncations, a series of DNA fragments were PCR amplified by using a common reverse primer and a forward primer. The PCR product was then cloned into pGL3-enhancer vector (Promega, Madison, WI, USA). To make the pUC-3′UTR construct, JFH-1 3′UTR (9443–9678nt) was PCR amplified. The PCR products were cloned into pUC19 behind a T7 promoter. All constructs were verified by DNA sequencing analysis. The primer information is available upon request.

In vitro transcription, RT-qPCR, and indirect immunofluorescence

These assays were performed as previously described [26, 46]. The primer for RT-qPCR information is available upon request.

HCVcc genesis and HCV 3′UTR RNA generation

The methods of producing HCVcc virus has been previously described [26]. The HCV infectious clone (JFH1) was derived from a Japanese patient with fulminant hepatitis [47]. Wakita and colleagues [48] cloned this HCV cDNA behind a T7 promoter to create the plasmid pJFH-1. To generate the full-length genomic JFH1 or 3′UTR RNA, the pJFH-1 or pUC-3′UTR plasmids were linearized at the 3′ end of the HCV cDNA by XbaI digestion, and used as a template for in vitro transcription (Ambion, Austin, TX, USA). In vitro transcribed JFH1 RNA was delivered to cells by electroporation [26]. Cells were passaged every 3–5 days, and the produced viruses (HCVcc) were collected and titrated [26].

Western blot analysis

Cells were lysed and cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The primary antibodies included mouse anti-IκBα (Cell Signaling, Danvers, MA, USA), mouse anti-HCV NS3 (Abcam, Cambridge, MA, USA), mouse anti-MAVS (Santa Cruz, CA, USA), mouse anti-Flag (Sigma, St. Louis, MO, USA) and mouse anti-β-actin (Santa Cruz). The secondary antibodies were HRP-conjugated anti-mouse lgG (CHEMICO, Orchard Hills, Australia). The Amersham® ECL Western Blotting Detection System (GE healthcare, Uppsala, Sweden) was used to detect chemiluminescent signals.

ELISA

Cell culture supernatants were collected and analyzed using Human IL-28 DuoSet® ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Promoter assay

Ten thousand A549 cells were seeded into 48-well plates overnight prior to transfection with 50 ng/well of pGL3-IL28 constructs and 50 ng/well of CMV promoter driven *Renilla* luciferase vector (pRL-CMV; Promega) for normalization of transfection efficiency. One day later, the cells were transfected with HCV 3′UTR RNA (2 ug/mL) and harvested twelve later. Cell lysates were assayed for luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

ChIP assay

A549 cells infected with SeV were harvested at 8 h postinfection, cross-linked, and sonicated to generate chromatin fragments according to a previously described protocol [49]. In particular, the cells were crosslinked with 1% formaldehyde, then lysed, and

sonicated to shear DNA. Twenty percent of the sheared chromatins were kept as "input control". The rest were incubated with antibodies against NF-κB p65 (Upstate Biotech, Waltham, MA, USA), IRF3 (Santa Cruz), or normal mouse IgG isotype control followed by additional incubation with protein A agarose. The bead-bound protein-DNA complexes were eluted and crosslinks were reversed. Precipitated DNA was subjected to qPCR using the IL-28 promoter primers: 5′-AGAAGGGCAGTCCCAGCTGATGT-3′ and 5′-ACCAGAGCAGGTGGAATCCTCCT-3′. Enrichment values were normalized with corresponding input control.

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Abbreviations: **HCVcc**: cell-culture grown HCV · **ISG**: IFN-stimulated gene · **SNP**: single-nucleotide polymorphism.

Full correspondence: Dr. Jin Zhong, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai, 200025, China.
Fax: +86-21-6385-9365
e-mail: jzhong@sibs.ac.cn

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