Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7

Yi-Liang Zhang¹, Ying-Jun Guo¹, Bin Li², Shu-Han Sun¹,*

¹Department of Medical Genetics, Second Military Medical University, 800 Xiang’Yin Road, 200433 Shanghai, China
²Department of Secondary Hepatic Surgery, Eastern Hepatic Biliary Hospital, Secondary Military Medicine University, 800 Xiang’Yin, Shanghai, China

Background/Aims: Innate immune responses to HCV infection are triggered through host recognition of pathogen-associated molecular patterns. Interferons are critical for the protection against HCV infection. However, the pathways linking virus recognition to IFN induction remain poorly understood.

Methods: Immune cells and Huh-7 cells were infected with HCV cell culture (HCVcc) or transfected with HCV-derived immunostimulatory RNA oligonucleotides (ORNs), and immune activation was assessed.

Results: We found that HCVcc suppressed immune responses because the HCVcc protein impaired the PBMC and pDC responses. However, HCVcc genomic RNA had an immunostimulatory effect. HCV encodes G/U-rich ssRNA TLR7 ligands that significantly activate innate immunity, and induced IFN-α production. Moreover, HCV-derived ORNs also activated IRF7 and NF-κB in Huh-7 cells. In particular, the HCV 3'-UTR strongly induced cytokine production. Different lengths of polyuridine tract in the 3'-UTR of different HCV strains induced IFN-α production. These data demonstrate that the HCV-specific G/U fragment is a motif sequence, and is recognized by TLR7 as a PAMP. The requirement for TLR7 to recognize HCV RNA was confirmed using specific inhibitors, RNAi and by TLR7 overexpression.

Conclusion: These results provide an insight into the development of immune adjuvant for vaccines and for the production of new antiviral drugs.

Keywords: Hepatitis C virus; Innate immunity; RNA; Toll-like receptor 7

1. Introduction

More than 170 million people are infected with HCV and 55–85% of patients become chronically infected [1]. However, HCV is immunogenic and triggers immune responses in most healthy adults. Intrinsic components of the virus presumably activate the innate immune system, which recognizes the presence of invading patho-
virus-specific molecular structures, long dsRNA and CpG motif, are detected via TLR3 and TLR9, respectively. Unsurprisingly, some viruses have also evolved strategies to sequester dsRNA by a variety of mechanisms to avoid activation of these antiviral pathways. For example, most human pathogenic viruses are single-stranded RNA virus that can avoid detection by TLR3.

Recent studies have demonstrated that TLR7/8 can recognize some single-stranded RNA viruses and activate innate responses. Sandra et al. reported that influenza virus stimulates effector cells of the immune system to secrete proinflammatory cytokines and IFN-α via TLR7 [8]. Some synthetic single-stranded RNA molecules of the influenza virus also induce TLR7-dependent production of inflammatory cytokines. The results from other studies also suggest that high concentration of ssRNA or their degradation products of some ssRNA viruses (Human Immunodeficiency Virus type 1, Respiratory Syncytial Virus, Foot-and-Mouth disease virus) are detected via TLR7/8 [8–13].

As a positive ssRNA virus, how does HCV ssRNA activate the immune response in HCV infection? In HCV-infected patients, signal transduction of TLR2 and TLR4 is profoundly impaired, even though the expression of TLR2 and TLR4 is higher compared with that in uninfected patients [14]. Therefore, other innate immune responses must be activated in most healthy adults. pDCs possess a dsRNA-independent pathway for recognizing HCV and produce high levels of type I-IFN after virus infection [15]. TLR expression pathway for recognizing HCV and produce high levels of type I-IFN after virus infection [15]. TLR expression in human pDCs is limited to TLR7 and TLR9 [16]. As a consequence, TLR7-mediated pDC recognition of HCV ssRNA may be an important antiviral pathway.

In this study, we found that HCV RNA induces innate immune responses and that TLR7 is required for the recognition of HCV ssRNA. These results present direct evidence for the induction of innate immunity of HCV RNA via TLR7.

2. Materials and methods

2.1. Cell isolation and culture

PBMCs were obtained by density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). pDCs were isolated from healthy donor PBMCs using a BDCA-4 isolation kit (Miltenyi Biotec, Germany) and were cultured in RPMI 1640 medium (Invitrogen, CA, USA) containing 10% FBS (Gibco, CA, USA) and 1% Pen-Strep (Invitrogen). Purity was confirmed by staining with CD303-PE (Miltenyi Biotec) and was typically >90%. THP-1 cells were maintained in RPMI 1640 supplemented with 10% FBS. The human hepatoma cell lines Huh-7, Huh-7.5 (ATCC, USA) and Human Embryonic Kidney (HEK)-293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS.

2.2. Preparation of HCVcc

The preparation of HCVcc was performed as described elsewhere [17]. Briefly, the plasmid pJFH-1 was digested with XbaI. Two micrograms of the linearized DNA served as a template for in vitro transcription (MEGAscript; Ambion, Austin, TX) and the transcripts were purified by an RNeasy kit (Qiagen, Valencia, CA). RNA was quantified by absorbance at 260 nm. Then, in vitro transcribed JFH-1 RNA was transfected into Huh7.5 cells using Lipofectamine 2000 (Invitrogen). Viral stocks were obtained by harvesting cell culture supernatants at 1 week post-transfection. Secondary viral stocks were obtained by additional amplifications of native Huh7.5 cells and harvesting cell culture supernatants. The infectious titer of viral stocks was estimated to be between 10^5 and 10^6 infectious units per ml, based on immunofluorescent detection of infected foci after infection of Huh7.5 cells with serial dilutions of the viral stocks.

2.3. Preparation of HCV RNA/HCV-derived ORN, stimulation of cells and cytokine detection

HCV RNA was extracted from the Huh-7 culture supernatant using the AxyPrep viral RNA miniprep kit (Axygen, USA), concentrated and quantified using spectrophotography. G/U-rich regions containing more than 60% G/U within the ssRNA sequence of HCV were identified and synthesized (Generpharma, Shanghai, China). The regions named according to the first RNA nucleotide position relative to the coding domain (Table 1). Different lengths of poly(U) were produced using the T7 RiboMAX™ Express system (Promega, USA). PBMCs, pDCs, THP-1 cells and Huh-7 cells were incubated with HCV RNA or HCV–ORN complexed with DOTAP (Sigma, MS, USA) at the indicated concentration for 18 h. The following reagents were also used to stimulate cells in vitro: R-848 (InvivoGen, USA) at 10 μM, imiquimod (InvivoGen) at 10 μg and ODN2216 (Invitrogen) at 0.1 μM. The supernatants were then collected and the levels of IFN-α and TNF-α were measured by ELISA using a PBL human IFN-α ELISA kit (PBL Biomedical, NJ) and a human TNF-α ELISA kit (Jingmei, China), respectively. Chloroquine (Sigma) was added, as indicated below, at a final concentration of 100 μM to inhibit lysosomal maturation. The TLR7-specific inhibitor IRS661 (5'-TGCTTTGCAAGCTTGGCAAGCA-3') and the TLR9-specific inhibitor IRS869 (5'-TCTGGAGGGTTGTG-3') were added at a concentration of 5 μM.

2.4. Expression vectors for human TLR7

Human TLR7 was cloned in pcDNA3.1-V5-his-TOPO with a six-his tag at the N terminus and sequenced to confirm the correct open reading frame.

<table>
<thead>
<tr>
<th>Region in HCV and ORN name</th>
<th>Sequence</th>
<th>Mutation (G/U to A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORN311</td>
<td>GCCCCGGGAGGUCUGCUGAGA</td>
<td>ACCCCAAAAAACACAAAAA</td>
</tr>
<tr>
<td>ORN1000</td>
<td>CCCCCUGUCUGAACCUGUGUC</td>
<td>CCAAAAAAAAACACAAAAA</td>
</tr>
<tr>
<td>ORN3131</td>
<td>UGCUGCUGUAGCGGCGGGGG</td>
<td>AAAAAAAAAAAAAAAAAAAAAA</td>
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<td>ORN4871</td>
<td>GUGAAAAGACCCUGUCAUGAU</td>
<td>AAAAAAAACCAACCAAAAA</td>
</tr>
<tr>
<td>ORN8961</td>
<td>CUUUUGAAAUUGUACGGGGGCCA</td>
<td>CAAAAAAACCAACCAAAA</td>
</tr>
<tr>
<td>ORNhevtail</td>
<td>UUUUUUUUUUUUUUUUUUUUU</td>
<td>AAAAAAAAAAAAAAAAAAAAAA</td>
</tr>
</tbody>
</table>
2.5. NF-κB and IRF7 electrophoretic mobility-shift assays (EMSA) and NF-κB reporter assay

EMSA [18] and reporter assays for NF-κB [19] were performed as described elsewhere. Briefly, HEK-293 cells were cultured in DMEM supplemented with 10% FBS, plated in 48-well plates and transfected the next day with Lipofectamine 2000 (Invitrogen). The following plasmids were then added: human TLR7 expression vectors or empty vector (0.1 μg), 0.1 μg NF-κB-Luc, and 0.01 μg pRL-TK for normalization. Luciferase activity was detected by a Dual-Luciferase Reporter Assay System (Promega). Luciferase assay of the activation of IRF7 in Huh7 was performed after Huh7 cells were transfected with 50 ng of plasmids expressing Gal4-IRF7, 50 ng of Gal4-Luc reporter plasmid and 50 ng of pRL-TK.

2.6. RNA interference

The previously published siTLR7 sequences are as follows: TLR7-sense 5'-GCCUUGAGGCCCAACAAUCdTdT-3', TLR7-antisense 5'-AUGUUGUUUGCCCUAAGGCdTdT-3' and were synthesized by Genepharm. SiGAPDH was purchased from Genepharm. Huh7 was performed after Huh7 cells were transfected overnight with 30 mM of siRNAs using Lipofectamine 2000.

2.7. Statistical analysis

Experiments were repeated at least three times. P-values were determined using Student’s t tests (SAS Institute, Cary, NC). P-values less than 0.05 were considered statistically significant.

3. Results

3.1. HCV RNA triggers innate immune responses

The recognition of some ssRNA virus by pDCs cells through TLR7 results in the activation of costimulatory molecules and cytokine production [10]. Recent studies have also demonstrated that SM360320, a ligand for TLR7, induces anti-HCV immunity and has a potential role in controlling HCV infection [20].

To assess the innate immune activity of HCV, PBMCs that were obtained from healthy uninfected blood donors were exposed to HCVcc. As shown in Fig. 1A and C, HCVcc did not induce IFN-α or TNF-α. Although HCVcc did not induce immune responses, exposure of PBMCs to protease-treated HCVcc resulted in IFN-α and TNF-α production (Fig. 1B and C). As a control, exposure of PBMCs to native Huh7.5 cells, with or without protease treatment, did not elicit cytokine production. These data suggest that HCV RNA may possess stimulatory activity. Levels of the anti-inflammatory mediator, IL-10, were measured because the immune responses were suppressed by HCVcc infection. HCVcc did not induce IL-10 production in PBMCs (Fig. 1D). Therefore, the immune responses were inhibited in HCVcc-infected cells, probably because HCV protein blunts the ability of HCV RNA to activate immunity (Fig. 5D).

The recognition of PAMP by TLR7 involves single-stranded RNA. Therefore, we first assayed the ability of HCV genomic RNA to trigger the innate immunity. The intact HCV genome enhanced IFN-α and TNF-α production by PBMCs in the presence of the cationic transfection agent DOTAP (Fig. 2A and B). We then assessed the dose–response characteristics. At 0.01 μg/ml, the HCV genome did not induce cytokine production. However from 0.05 to 0.5 μg/ml, the HCV genome induced continually increasing production of IFN-α and TNF-α (Fig. 2C and D). The pDCs were enriched and stimulated with the HCV genome, and IFN-α and TNF-α production were similar to that in PBMCs (data not shown). These results suggest that HCV genomic RNA can induce the activation of immune cells and result in cytokine production.

3.2. HCV-derived ORNs induce cytokine production

In the endosome, viral genome may be degraded to RNA oligonucleotides (ORNs), and some immunostimulatory ORNs can then activate immune responses. Previous studies have shown that pDCs can sense a uridine-rich region within the HIV-1 genome, a sequence termed RNA40 [9]. Therefore, we hypothesized that some guanosine (G) and uridine (U)-rich ssRNA ORNs encoded by the HCV genome are responsible for HCV induced immune activation. We screened the HCV RNA sequence and synthesized some G/U-rich 20-nucleotide ssRNA ORNs (Table 1).

Two ORNs in the HCV genome, ORN1000 and ORNhcvtail, significantly enhanced the production of IFN-α and TNF-α by human PBMCs (Fig. 3A and B). Activated THP-1 cells are macrophage-like cells, and exposure of THP-1 cells to ORN1000 and ORNhcvtail also resulted in TNF-α production (Fig. 3C). The activation of pDCs also exhibit ORN immunostimulation. Fig. 3D indicates that ORN1000 and ORNhcvtail enhanced IFN-α production by pDCs. Taken together, these results indicate that the HCV-derived ORNs can trigger innate immunity.

3.3. Immunostimulatory ability of the HCV RNA-specific poly(U) tail

HCV genomic RNA possesses a specific structural element, a conserved poly(U) tail in the 3'-untranslated region (UTR). Previous studies have demonstrated that the poly(U) region is a critical determinant of HCV replication [21]. In our study, the immunostimulatory ORNhcvtail is the 20-U oligonucleotide from the poly(U) region in the 3'-UTR.

To demonstrate the innate immune activity of different lengths of the poly(U) region or the 3'-UTR, we used 10, 20, 30, 50, 60 and 100-U oligonucleotide to stimulate immune cells and we measured cytokine production. Fig. 4A and B shows that from 10 to 100-U, all poly(U) ORNs induced the production of IFN-α and TNF-α by
PBMCs. Furthermore, pDCs up-regulated costimulatory molecules, including CD80 and CD86, in response to these poly(U) ORNs (data not shown), and resulted in enhanced IFN-α expression (Fig. 4C). Each HCV strain contains a 20–200 nucleotide poly(U/UC) region. Therefore, the HCV poly(U) tail in the 3'-UTR may encode viral immunostimulatory motifs that induce innate immune responses.

3.4. HCV-derived ORNs activate NF-κB and IRF7 in Huh-7 cells

TLR7 mRNA was expressed in Huh-7 cells, and an anti-TLR7 antibody specifically recognized human TLR7 expressed in Huh-7 cells (Fig. 5A), whereas TLR8 expression was not detected (data not shown). NF-κB is a transcriptional regulator of genes involved in immune and inflammatory responses. NF-κB activation was detected in Huh-7 cells incubated with the ORNs–DOTAP complex using EMSA and a NF-κB-luciferase reporter assay. When the nuclear extract from Huh-7 cells was cultured with the ORN1000/ORNhcvtail–DOTAP complex and incubated with a biotin-labeled NF-κB DNA probe, the band corresponding to the DNA–NF-κB complex was very intense (Fig. 5B), whereas the density of the NF-κB band was very faint in the other groups of cells cultured with ORN311, ORN3131, ORN4871, ORN8961 and the DOTAP control did not activate NF-κB (Fig. 5C). However, HCV genomic RNA, ORN1000

Fig. 1. HCV RNA triggers innate immune responses. (A) Human PBMCs (70,000 cells) were stimulated with HCVcc (MOI = 0.5) or 0.1 μM ODN2216, and IFN-α was measured. (B) PBMCs were cultured with 0.1 μM ODN2216, HCVcc pretreated with RNase or HCVcc pretreated with protease complexed to DOTAP. (C) Human PBMCs were stimulated with HCVcc (MOI = 0.5), HCVcc pretreated with RNase, HCVcc pretreated with protease complexed to DOTAP or 0.1 μM ODN2216, and TNF-α was measured. (D) PBMCs were cultured with HCVcc (MOI = 0.5) or 0.1 μM ODN2216, and IL-10 was measured. Medium, native Huh-7.5 cell culture supernatants. Values are means ± standard deviation of three independent experiments. *P < 0.05.
and ORNhcvtail did not activate NF-κB signaling in HCVcc-infected Huh7 cells (Fig. 5D). These results confirmed that HCVcc can inhibit TLR7 responses induced by ssRNA. Meanwhile, treatment of Huh7 cells with HCV ORNs also induced IRF7 activation (Fig. 5E). Taken together, these data demonstrate that HCV-derived ORNs can activate IRF7 and NF-κB signaling in Huh-7 cells.

3.5. TLR7 is required for immune activation

The immune activation may be mediated by TLR7 because pDCs were activated by ORNs, and only TLR7 and TLR9 are expressed in endosome within pDCs [16]. Agents that interfere with endosomal acidification, such as chloroquine, can block signaling by these intracellular TLRs. To determine whether the immune activation induced by the ORNs was mediated by TLR7, we pretreated PBMCs and pDCs with chloroquine before incubation with ORN1000 and ORNhcvtail. Chloroquine inhibited the production of IFN-α by PBMCs and pDCs that were stimulated by ORNs (Fig. 6A and B). To further assess the function of TLR7, PBMCs and pDCs that were stimulated by ORNs (Fig. 6C and D) reveal that, for ORNs, the addition of IRS661 reduced the production of IFN-α by PBMCs and pDCs, whereas the addition of IRS869 did not significantly inhibit IFN-α production.

Fig. 2. Cytokine production by human PBMCs in response to HCV genomic RNA. (A and B) Freshly isolated PBMCs (70,000 cells) were cultured with HCV genomic RNA, and IFN-α and TNF-α were determined by ELISA after incubation for 18 h. (C and D) Freshly isolated PBMCs (70,000 cells) were plated in a 96-well plate. Increasing doses of HCV genomic RNA (from 0.01 to 0.5 μg/ml) were used to stimulate PBMCs, and IFN-α and TNF-α were determined by ELISA after incubation for 18 h. Values are means ± standard deviation of three independent experiments.
Fig. 3. Cytokine production by human PBMCs, pDCs or THP-1 cells in response to HCV-ORNs. (A and B) Freshly isolated PBMCs (70,000 cells) were cultured with 2 μg/ml of HCV-derived ORNs, and IFN-α and TNF-α were determined by ELISA after incubation for 18 h. (C) THP-1 cells (20,000 cells) were differentiated for 18 h in conditioned medium with 20 ng/ml PMA per well in a 96-well plate and then cultured with 2 μg/ml of HCV-derived ORNs, and TNF-α was determined by ELISA after incubation for 18 h. (D) pDCs (20,000 cells) were cultured with 2 μg/ml of HCV-derived ORNs and IFN-α was determined by ELISA after incubation for 18 h. Values are means ± standard deviation of three independent experiments.

Fig. 4. Effects of different lengths of the poly(U) region on cytokine production. (A and B) Freshly isolated PBMCs (70,000 cells) were cultured with 2 μg/ml of different lengths of poly(U) sequences, and IFN-α and TNF-α were determined by ELISA after incubation for 18 h. (C) pDCs (20,000 cells) were cultured with 2 μg/ml of different lengths of poly(U) sequences, and IFN-α was determined by ELISA after incubation for 18 h. Values are means ± standard deviation of three independent experiments.
production. To investigate the possible role of TLR8 in activating PBMCs and THP-1 cells (shown in Fig. 3), these cells were co-incubated with ORNs in the presence of imiquimod (TLR8 inhibitor, TLR7 agonist). Fig. 6E and F shows that imiquimod suppressed TNF-α production, and suggest that TLR8 can mediate the activation of PBMCs and THP-1 cells in addition to TLR7. In fact, TLR7 and 8 usually play similar roles in mediating immune responses as members of the same superfamily.

RNA interference was used to knock-down TLR7 gene expression before stimulation. siTLR7 significantly knocked down the expression of TLR7 in Huh-7 cells (Fig. 7A) and siRNA silencing of TLR7 in the presence of imiquimod (TLR8 inhibitor, TLR7 agonist). Fig. 6E and F shows that imiquimod suppressed TNF-α production, and suggest that TLR8 can mediate the activation of PBMCs and THP-1 cells in addition to TLR7. In fact, TLR7 and 8 usually play similar roles in mediating immune responses as members of the same superfamily.

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4. Discussion

Despite extensive research efforts, characterization of the interactions between HCV and the host immune responses has been difficult owing to the lack of efficient cell culture and small animal models. The recent establishment of the HCV culture system [17,23,24] has allowed the generation of the complete HCV particle. In this study, we generated HCVcc and studied its effects on freshly isolated PBMCs and pDCs obtained from healthy blood donors. The complete HCV particles did not induce cytokine production because the HCV protein impaired PBMC and pDC responses, whereas HCV RNA possesses immunostimulatory activity. Although HCVcc did not induce immune responses in vitro, HCV infection resulted in persistent immune activation in vivo. The underlying mechanisms, however, are not fully understood. Previous studies provided indirect evidence for the interaction between TLR7 and natural HCV infection. Once-daily treatment for 7 days with intravenous isatoribine, an agonist of TLR7, reduced plasma virus concentrations in patients chronically infected with HCV [25]. Lee et al. also showed that TLR7 can mediate HCV immunity, not only by interferon induction but also through an interferon-independent mechanism [20]. Schott et al. further analyzed the prevalence of single nucleotide polymorphisms (SNPs) of TLR7 in patients chronically infected with HCV and the effect of these SNPs on liver fibrosis. They showed that specific TLR7 SNPs have a significant effect
on fibrosis progression in patients with chronic HCV infection [26].

In this study, we provided direct evidence that HCV RNA components can greatly enhance immune activation via TLR7. We demonstrated that HCV RNA possesses immunostimulatory effects, and HCV encodes G/U-rich ssRNA TLR7 ligands that induce immune activation of PBMCs and pDCs, and THP-1 and Huh7 cells. In the processes of immune activation, cell attachment of HCV generally leads to endocytosis of the bound virions. Some HCV particles are then degraded by endosomal proteases, exposing the viral genome and the immunostimulatory RNA fragments, thus allowing TLR7 signaling, which is known to occur in endosomes [8,27]. Therefore, pDCs and other cells in the innate immune system exploit the necessity of HCV to enter acidified compartments in order to detect the presence of the virus [8]. In immune individuals, the lipoproteins or immunoglobulins may be directly targeted to endosomal compartments via low-density lipoprotein receptors (LDLr) or Fc receptors, leading to immune activation [28].

Of note, ORN1000 encoded in HCV E1 and ORNhcvtail encoded in HCV 3’-UTR greatly enhanced immune activation. The 3’-UTR is composed of three sequence elements that are believed to be involved in RNA replication: a nonconserved variable region, a 20–200 nucleotide poly(U/UC) region and a conserved 98 nucleotide sequence. You and Rice showed that the poly(U) region does not function simply as an unstructured spacer to position the kissing-loop elements [21]. Our study reveals a new role of the poly(U) region and the 3’-UTR in triggering innate immune signaling. As a PAMP motif and a potential substrate of TLR7 signaling, the poly(U) region of the HCV genome can induce strong immune responses. The poly(U) motif is an essential determinant of the HCV replication ability. Therefore, because the virus must maintain this motif for its viability, the host takes advantage of this requirement and targets the poly(U) region as a discriminator for the PAMP motif through interaction with TLR7.

In general, TLR3 can recognize the viral dsRNA structure and initiate the innate immune response. For example, antiviral immunity against HBV mediated by

Fig. 6. TLR7 participates in the activation of immune responses. (A and B) Chloroquine blocked the immune stimulatory effects of HCV-derived ORNs in human PBMCs and pDCs. (C and D) IRS661 blocked the immune activation of HCV-derived ORNs, whereas IRS869 did not affect immune activation in human PBMCs or pDCs. Values are means ± standard deviation of three independent experiments. (E and F) PBMCs and THP-1 cells were stimulated with ORN1000 or ORNhcvtail in the presence of imiquimod, and TNF-α was determined. Values are means ± standard deviation of three independent experiments. ‘’P < 0.01, ’’P < 0.05.
TLR3 plays a very important role in antiviral host defense [29]. However, HCV suppresses this response. Thus another effective pathway must be available to recognize the HCV-specific genome structure. Because PRRs recognize RNA, TLR7 provides an important role in detecting the ssRNA structure. Heil et al. described that the primary structural feature recognized by TLR7 is G/U-rich ssRNA [9]. HIV-derived ssRNA40 (GCCCGUCUGUUGUGUGACUC) contains more than 60% G/U. Angela et al. showed that uridine is crucial for immunostimulation and identified several immunostimulatory ORNs in which more than 50% of the nucleotides were uridine [30]. In addition, several studies have shown that the ssRNA motif responsible for activating the innate immune response via TLR7 is sequence-specific [31,32]. Adam et al. identified that 5′-UGUGU-3′ is one such motif and showed that the sequence recognition mechanism is stringent enough that minimal base substitutions can have profound effects on the immunostimulatory capacity of ssRNAs.

In our study, the immunostimulatory ORN1000 (CCGCUCUGUGUACCUUGUGUC) contains two UGUGU motifs. These data, along with the previous study, indicate that the specific base composition or sequence-dependent motif provides the foundation for TLR7 recognition. However, we found that ORNhcvtail and 10, 30, 50, 60 and 100-U, which comprised poly(U) regions of different lengths, enhanced cytokine production, which was mediated by TLR7. Therefore, it is possible that the poly(U) region and these sequence-specific motifs possess similar or identical conformations that are only recognized by TLR7. The structural basis of TLR7 signaling with ssRNA must be investigated through crystal structure analysis of a complex between TLR7 and ssRNA.

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