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R753Q Single Nucleotide Polymorphism Impairs Toll-like Receptor 2 Recognition of Hepatitis C Virus Core and Nonstructural 3 Proteins

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Abstract

Background—Hepatitis C virus (HCV) core and nonstructural 3 (NS3) proteins induce inflammation and immunity through a Toll-like receptor 2 (TLR2)-dependent pathway. Individuals with the R753Q single nucleotide polymorphism (SNP) in the TLR2 gene have increased risk of allograft failure after liver transplantation for chronic hepatitis C.

Methods—To test the hypothesis that R753Q SNP impairs TLR2 recognition of HCV proteins, a series of *in vitro* experiments were performed wherein stable clones of wild-type TLR2-deficient HEK293 cells, and HEK293 cells transfected with wild-type (HEK293-TLR2) or variant TLR2 genes (HEK293-TLR2-R753Q) were stimulated with HCV core and NS3 proteins. Cellular activation was assessed by nuclear factor-kappa B (NFκB)-driven luciferase activity, cytokine secretion, and gene upregulation.

Results—Compared to TLR2-deficient HEK293 cells, HEK293-TLR2 cells had marked NFκB-driven luciferase activity, had modest to marked upregulation in TLR2 signaling-associated genes, and secreted large quantities of interleukin-8 during exposure to HCV core and NS3 proteins. In contrast, HEK293-TLR2-R753Q cells did not respond to stimulation with HCV, and behaved similarly like the TLR2-deficient HEK293 cells.

Conclusion—R753Q SNP impairs TLR2-mediated immune recognition of HCV core and NS3 proteins. This biologic defect may account for the predisposition of patients to develop allograft failure after liver transplantation for chronic hepatitis C.

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Author contributions

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Keywords

single nucleotide polymorphism; hepatitis C virus; liver transplantation

INTRODUCTION

End-stage liver disease as a result of chronic infection with the hepatitis C virus (HCV) is currently the leading indication for liver transplantation in developed countries including the United States (1,2). Recurrence of HCV infection is almost universal after liver transplantation, and the clinical course of allograft HCV infection is often accelerated, with many patients developing allograft failure in the short-term (2). There have been numerous factors associated with worse outcome after liver transplantation for chronic hepatitis C (CHC), including a severely immunocompromised status either as a result of potent lymphocyte-depleting drugs or other causes of immunodeficiency (2–5).

We previously reported a clinical observation that liver transplant recipients with a Toll-like receptor 2 (TLR2) gene R753Q single nucleotide polymorphism (SNP) are at increased risk of allograft failure and mortality after liver transplantation for CHC (6). TLR2 belongs to the family of pattern recognition receptors, whose major function is to sense microbial signatures and orchestrate the innate (and adaptive) immune response to offending organisms (7–10). TLR2 specifically recognizes HCV core and nonstructural 3 proteins (NS3), through homo- or hetero-dimerization with TLR1 and TLR6 (8,9,11,12). Accordingly, TLR2 is believed to participate in orchestrating the immune response to HCV, and impairment in TLR2 function could influence the clinical course and outcome of infection (8,9,11,12).

The mechanism underlying the clinical association between TLR2 R753Q SNP and allograft failure after liver transplantation for CHC has not been investigated. We hypothesize that this association may be due to the inability of variant TLR2 protein to recognize HCV core and nonstructural 3 proteins. Indeed, this particular TLR R753Q SNP, which results in the substitution of arginine for glutamine at position 753 of expressed TLR2 protein, has been demonstrated to impair immune responses to some of its ligands, including bacterial lipopeptides (13–15). Whether the variant TLR2 also has impaired ability to recognize HCV proteins has not been studied. To address this, we performed a series of *in vitro* experiments to test the hypothesis that R753Q SNP impairs the ability of TLR2 to recognize HCV core and NS3 proteins.

MATERIALS AND METHODS

Experimental Study Design

An *in vitro* experimental model was developed utilizing stable clones of human embryonic kidney (HEK) 293 cells that do not naturally express TLR2 (herein referred to as HEK293 cells), and HEK293 cells that were experimentally manipulated to express wild-type TLR2 (HEK293-TLR2 cells) and the variant TLR2 with the R753Q SNP (HEK293-TLR2-R753Q) (16–19). These stable clones of cells were co-incubated with HCV core and nonstructural 3 proteins, and the degree of cell activation was measured using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of TLR2-associated genes, nuclear factor-kappa B (NFkB) activity, and cytokine secretion.

Cells and Reagents

Hepatitis C virus (HCV) core (5.0- μ g/ml; Sigma, St. Louis, MO), nonstructural 3 protein (5.0- μ g/ml; Sigma), peptidoglycan (PGN) derived from *Staphylococcus aureus* (1.0- μ g/ml; Sigma), lipopolysaccharide (LPS) from *Escherichia coli* (1.0- μ g/ml; Sigma), and tumor-necrosis factor (TNF)- α (10-ng/ml; R&D Systems, Minneapolis, MN) were obtained from commercial sources. HEK293 cells were initially stably transfected with a luciferase-containing NF- κ B gene construct, as we have previously reported (16–19). Our previous work demonstrated the functional absence of TLR2 in HEK293 cells (17), hence, this adherent cell line was utilized to characterize the biology of TLR2-deficient cells. HEK293 cells were maintained at 37°C and 5%CO₂ in Dulbecco's modified Eagle medium F12 (3:1) (GIBCO-BRL, Life Technologies, Gaithersburg, MD), 10% characterized FBS (Hyclone Laboratories), 5 mM HEPES (Invitrogen, Carlsbad, CA), and 1 $\times$ penicillin–streptomycin (Invitrogen).

Stable cell lines of HEK293 expressing wild-type TLR2 (HEK293-TLR2) or variant TLR2 R753Q genes (HEK293-TLR2-R753Q) were constructed by stable transfection of HEK293 cells with human pcDNA3.1-hygro (Invitrogen)-based TLR2 or TLR2-R753Q expression plasmids, respectively, and clonal selection with Hygromycin B (Invitrogen) as previously reported (16–19). HEK293-TLR2 and HEK293-TLR2-R753Q cells were maintained in a culture medium similar to that described for HEK293 cells, but with the addition of 100- μ g/ml Hygromycin B. As we have previously reported, HEK293-TLR2 cells acquired responsiveness to the prototypic ligands *S. aureus* PGN and tripalmitoyl cysteinyl lipopeptide (Pam3Cys; 10-ng/ml; Calbiochem, San Diego, CA) (16–19). All three stable cell lines remained non-responsive to purified *Escherichia coli*-derived LPS (1.0- μ g/ml; Sigma), which is consistent with the lack of TLR4 mRNA expression using RT-PCR (16–19).

Flow Cytometry Analysis of TLR2 Expression

Cell surface expression of TLR2 on various cell lines (HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q) was assessed using flow cytometry. A total of 1×10^6 cells were harvested, and incubated with fluorescein isothiocyanate (FITC)-antihuman (or anti-mouse IgG2a isotype control) TLR2 monoclonal antibody (1.0- μ g/million cells; eBioscience, San Diego, CA) for 30 minutes. After washing process, the cells were fixed with 1% paraformaldehyde, and the cells expressing TLR2 protein were counted using a FACS brand flow cytometer (Mayo Research Core Facility, Rochester, MN).

Cellular Activation

Cell densities of 1×10^5 HEK293, HEK293-TLR2, or HEK293-TLR2-R753Q cells/100 μ l/well were seeded into a 96-well flat-bottom plate (Costar 3595, Corning Incorporated, Corning, NY) and incubated overnight at 37°C and 5%CO₂. The adherent cells were stimulated with HCV core and NS3 proteins, PGN, TNF- α , or other activator controls for 4 hours (for RT-PCR analysis) or 16 hours (for luciferase assay and cytokine secretion) at 37°C and 5%CO₂.

Assessment of NF κ B Activation

HEK293 cells were initially stably transfected with a luciferase-containing NF- κ B gene construct, and the degree of NF- κ B activation was assessed using a luciferase reporter assay. After stimulation for 16 hours, HEK293, HEK293-TLR2 and HEK293-TLR2-R753Q cells were lysed with 20- μ l/well of 1 $\times$ Reporter Lysis Buffer (Promega Corporation, Madison, WI) followed by a single freeze–thaw cycle. The cell lysate was subsequently mixed with 100- μ l of Promega Luciferase Substrate (Promega) and the chemoluminescence was

measured using the Victor instrument (Perkin-Elmer Life and Analytical Sciences, Torrance, CA).

ELISA for Interleukin-8 Secretion

After stimulation for 16 hours, cell-free supernatants of HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q cells were collected and assayed for IL-8 using a single-platform cytokine-specific quantitative sandwich enzyme immunoassay technique (Quantikine for human CXCL8/IL-8; R&D Systems) as per manufacturer's instructions.

Assessment of Genes Downstream of TLR2

Cell densities of 3×10^6 HEK293, HEK293-TLR2, or HEK293-TLR2-R753Q cells/2-ml volume of media/well were seeded into a 6-well flat-bottom plate (Costar 3516, Corning Incorporated, Corning, NY) and incubated overnight at 37°C and 5% CO₂. The adherent cells were stimulated with HCV core and NS3 proteins, PGN, TNF- α , or other activator controls for 4 hours at 37°C and 5% CO₂. RNA was extracted using the RT²qPCR-Grade RNA Isolation Kit (SA Bioscience, Frederick, MD) according to manufacturer's instructions, and 1.0- μ g of RNA was converted to cDNA using RT²Profiler PCR Array System (SA Bioscience). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using GeneAmp PCR System 2400 (Perkin Elmer, Boston, MA) and genes associated with TLR2 signaling were assessed using the iCycler instrument (Biorad Laboratories, Hercules, CA).

Statistical Analysis

Unless otherwise specified, all experiments were performed in triplicate on at least two separate occasions. The results are presented as average fold-induction over unstimulated cells. The differences between groups were analyzed using a two-tailed Student's t test, with statistical significance set at $P < 0.05$.

RESULTS

R753Q SNP impairs TLR2-dependent NF- κ B activation during HCV exposure

TLR2-deficient HEK293 cells did not respond to HCV core and NS3 proteins, and to the prototypic TLR2 agonist *S. aureus* PGN (Figure 1A). When experimentally manipulated to express TLR2, the cells (HEK293-TLR2) gained responsiveness to HCV core and NS3 proteins, as indicated by a 12-fold and 11-fold higher degree of NF κ B-driven luciferase activity, respectively. Accordingly, NF κ B-driven luciferase activity in response to HCV core and NS3 proteins were significantly higher in HEK293-TLR2 compared to TLR2-deficient HEK293 cells ($p < .001$ for both comparisons). This finding concurs with previous work by others that HCV core and NS3 proteins initiate immune response through a TLR2-dependent mechanism (8, 9). In contrast, cells that were manipulated to express the variant TLR2 R753Q gene did not gain responsiveness to HCV core and NS3 proteins. In HEK293-TLR2-R753Q cells, there was only a minimal 1.6-fold and 1.5-fold induction in NF κ B-driven luciferase activity, respectively, in response to HCV core and NS3 proteins (Figure 1A). Indeed, the degree of NF κ B-driven luciferase activity during HCV antigenic exposure in HEK293-TLR2-R753Q cells was not significantly different from TLR2-deficient HEK293 cells, but was significantly lower than HEK293-TLR2 cells ($p < .001$). This finding suggests that the variant TLR2 R753Q receptor is functionally-defective. This lack of cellular activation in HEK293-TLR2-R753Q cells was not due to impaired TLR2 protein expression since robust expression of the receptor can be demonstrated by flow cytometry (data not shown). Moreover, the lack of NF κ B-driven luciferase activity in HEK293-TLR2-R753Q

cells was not due to a global unresponsive state since the cells responded to stimulation with a TLR2-independent activator, TNF- α (Figure 1A).

Impaired gene upregulation in HEK293-TLR2-R753Q cells

RT-PCR showed that HEK293-TLR2 cells exposed to HCV core protein had modest up-regulation of RNA for myeloid differentiation factor 88 (MyD88; 3-fold), interleukin-1 receptor associated kinase (7.5-fold), NF κ B (5.3-fold) and activator protein 1 (AP-1; 8.5-fold). In addition, there was modest to marked upregulation of RNA for selected cytokines in HEK293-TLR2 cells exposed to HCV core protein, including chemokine (C-C motif) ligand 2 (CCL2; 7.5-fold), chemokine (C-X-C) ligand 10 (CXCL10; 48.4-fold), interleukin-1 β (IL-1 β ; 3.6-fold), IL-8 (127-fold), and TNF- α (135-fold). In contrast, there were no significant differences in these genes between HCV core-stimulated and unstimulated HEK293-TLR2-R753Q cells.

R753Q SNP impairs TLR2-dependent IL-8 secretion during HCV exposure

Compared to TLR2-deficient HEK293 cells, HEK293-TLR2 cells secreted very high levels of IL-8 in response to HCV core (mean $\pm$ standard deviation, 33926 $\pm$ 2183 pg/ml) and NS3 proteins (31411 $\pm$ 676 pg/ml, respectively). IL-8 secretion was 32-fold higher in HCV core-stimulated, while it was 30-fold higher in HCV NS3-stimulated, compared to unstimulated HEK293-TLR2 cells (Figure 1B). In contrast, there was negligible IL-8 secretion in HCV core-stimulated HEK293 (undetectable level) and HEK293-TLR2-R753Q cells (287 $\pm$ 36 pg/ml), and in HCV NS3-stimulated HEK293 (undetectable level) and HEK293-TLR2-R753Q cells (41 $\pm$ pg/ml). The lack of cellular responses in HEK293 and HEK293-TLR2-R753Q cells were not due to global hypo-responsiveness since the cells secreted high levels of IL-8 during stimulation with TLR2-independent TNF- α .

DISCUSSION

Experimental models have implicated TLR2 as a receptor that signals the presence of HCV core and NS3 proteins (6,9). In this study, we demonstrate that the presence of R753Q SNP in the TLR2 gene paralyzes TLR2-mediated immune signaling in response to HCV core and NS3 proteins *in vitro*. This observation could serve as a putative biologic mechanism that underlies our previous clinical observation that humans who are homozygous for the TLR2 R753Q SNP have worse clinical outcome after liver transplantation for chronic hepatitis C (20).

The TLR2 gene is located in chromosome 4q32 and contains at least 89 SNPs in its promoter, intron, and exon regions. Seventeen of the SNPs modify bases in the coding sequence of exon III and could influence the function of the expressed receptor (21). Of the SNPs, R753Q has been investigated the most because of its prevalence in the Caucasian population and because of previous reports that this specific SNP is associated with impaired recognition of the prototypic ligand, peptidoglycan, and other bacterial lipoproteins (15). Because TLR2 recognizes a wide repertoire of microbial signatures from bacteria, fungi, viruses, and parasites, there has also been considerable interest in investigating the potential association between this functional impairment in TLR2 and various infections in humans (6,15,22). In this regard, previous work by others have implicated TLR2 R753Q SNP in the predisposition to develop infections due to *S. aureus* (15), *Mycobacterium tuberculosis* (23), *Borrelia burgdorferi* (24,25), *Treponema pallidum* (26), and cytomegalovirus (19,22).

We previously reported that liver transplant recipients with the TLR2 R753Q SNP were more likely to develop allograft failure and mortality after liver transplantation for chronic hepatitis C (6). In that study, all patients whose alleles both contain the R753Q SNP died or

had required re-transplantation during the median follow-up period of 32 months after liver transplantation; in the majority of cases, these outcomes were related to recurrent HCV hepatitis affecting the transplanted allograft (6). In contrast, patients who possessed at least one wild-type TLR2 alleles (i.e., including those who were heterozygous for the SNP) were less likely to develop worse outcomes (6). The present study now provides a potential biologic explanation for this clinical observation, in an *in vitro* model, by demonstrating that mutant cells with TLR2 R753Q SNP failed to recognize HCV core and NS3 proteins (9,11).

We hypothesize that the impaired innate HCV recognition by TLR2, as a result of the R753Q SNP, may have impeded the development of a robust antiviral immune mechanism that controls HCV infection (27). Indeed, our initial work demonstrated a higher, albeit non-significant, level of HCV RNA in blood samples obtained from individuals with the homozygous TLR2 R753Q SNP (6). The location of the R753Q SNP within a group of highly conserved amino acids at the C-terminal of TLR2 (i.e., cytoplasmic Toll-interleukin 1 receptor domain) suggest that R753Q SNP may not impair the ligation with HCV proteins, but instead, it may be defective in its engagement with the intracellular MyD88 adapter protein. Accordingly, this could account for the lack of MyD88 RNA upregulation in variant cells despite exposure to HCV proteins. As a consequence, genes downstream of the TLR2 molecule were not upregulated in variant cells.

Effective control of HCV and other pathogens requires the optimal functioning of proteins and molecules involved in the immune response. This *in vitro* study, which complements our prior clinical report, demonstrates one SNP that impaired the function of the TLR2 molecule and this resulted in the lack of TLR2-mediated recognition of HCV-associated molecular patterns. Having demonstrated this, we emphasize that the human immune response is very complex and involves other immune genes. Indeed, the modest prevalence of this TLR2 SNP will not account for the worse outcome in our larger HCV patient population. In this context, it is very likely that mutations in other genes that encode for other molecules involved in the immune response, such as the adapter molecules and kinases involved in TLR and other signaling pathways, may influence the pathogenesis and clinical outcomes of HCV infection.

In conclusion, this study demonstrates that R753Q SNP impairs TLR2-mediated immune signaling in response to HCV proteins *in vitro*. The collective observation from our investigations (6) and those of others (8,9,11,12) solidify the role of TLR2 in the innate immunopathogenesis of HCV infection. Moreover, this specific functional genetic variation has a potential clinical application as a prognostic marker for worse outcome in liver transplant recipients with HCV. For example, the TLR2 R753Q SNP PCR assay described in this report may be used to identify HCV-infected liver transplant candidates who are at increased risk of allograft failure and mortality so that appropriate measures are provided, including aggressive clinical surveillance and preemptive interferon-ribavirin therapy. The interaction between HCV and the innate immune system is however a highly complex phenomenon, and our observation is likely just one of the anticipated multiple genetic variations that could account for differences in how individuals respond to infectious challenges.

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Abbreviations

CHC	chronic hepatitis C
HCV	hepatitis C virus
LPS	lipopolysaccharide
NS3	nonstructural 3 protein
PGN	peptidoglycan
TLR	toll-like receptors
PRR	pathogen recognition receptors

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A

B

Figure 1.

A, Nuclear factor-kappa B driven luciferase activity in human embryonic kidney 293 (HEK293) cells with or without TLR2 genes. B, Secretion of interleukin 8 by HEK293 cells with or without TLR2 genes.