BRIEF REPORT

The R753Q Polymorphism Abrogates Toll-Like Receptor 2 Signaling in Response to Human Cytomegalovirus

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Toll-like receptor 2 (TLR2) serves as a pattern recognition receptor that signals the presence of cytomegalovirus. Herein, we report that R753Q polymorphism paralyzes TLR2-mediated immune signaling in cells exposed to cytomegalovirus glycoprotein B. This immunologic impairment could serve as a biologic mechanism underlying the association between the TLR2 R753Q polymorphism and cytomegalovirus disease in humans.

Toll-like receptors (TLRs) are germline-encoded innate immune sensors of microbial pathogens [1]. Activation of TLR signaling results in induction of proinflammatory cytokines and antimicrobial peptides [1]. Experimental observations suggest that TLR2 is involved in the innate immune response to cytomegalovirus (CMV) [2, 3]. Stimulation of TLR2 by CMV in vitro, specifically by its envelope glycoproteins B (gB) and H [2], resulted in nuclear factor-κB (NF-κB) activation and cytokine secretion [2, 3]. In vivo, mice deficient for TLR2 had higher hepatic and splenic levels of CMV [4]. Moreover, NK cell–mediated control of CMV in mice was dependent on functional TLR2 [4]. To provide clinical relevance to these findings, we reported that transplant recipients with the R753Q single nucleotide polymorphism (SNP) in TLR2 had a significantly higher degree of CMV replication and were more likely to develop CMV disease [5]. However, the underlying mechanism of why R753Q SNP was associated with CMV disease has not been investigated. We hypothesized that the association was attributable to impaired TLR2-mediated immune recognition of CMV-associated molecular patterns.

Materials and methods. To address the hypothesis, we developed an experimental model with use of stable clones of human embryonic kidney (HEK) 293 cells (a TLR2-deficient cell line [6, 7]) transfected with luciferase-containing an NF-kB gene construct. Stable cells expressing TLR2 (HEK293-TLR2) or TLR2-R753Q (HEK293-TLR2-R753Q) were constructed by transfecting HEK293 with human pcDNA3.1-hygro (Invitrogen)–based TLR2 or TLR2-R753Q expression plasmids, respectively, and clonal selection with Hygromycin B (Invitrogen) [6, 7]. Full genetic sequencing of the TLR2 coding region confirmed the change from CGG to CAG at nucleotide position 2257 in HEK293-TLR2-R753Q cells (translating to an amino acid substitution from arginine to glutamine at position 753 [R753Q]) (GenBank accession NM-003264); no other genetic polymorphisms were observed. All cells were maintained at 37°C and at 5% CO_2 in a culture medium as described elsewhere [6, 7]. HEK293-TLR2 cells acquired responsiveness to *Staphylococcus aureus* peptidoglycan (Sigma) [6, 7]. All cells were unresponsive to *Escherichia coli*–derived purified lipopolysaccharide (Sigma) [6, 7].

For flow cytometry analysis of TLR2 expression, a total of 1×10^6 cells were incubated for 30 min with fluorescein isothiocyanate anti-human (or anti-mouse immunoglobulin G2a isotype control) TLR2 monoclonal antibody (1.0 μg/million cells; eBioscience); this antibody specifically binds to wild-type and mutant TLR2 protein expressed on the cell surface. After washing, cells were fixed with 1% paraformaldehyde, and TLR2-expressing cells were counted using a FACS Brand flow cytometer (BD).

For Western blot analysis, a total of 1×10^6 cells were lysed in 950 μL of Cell Extraction Buffer (Biosource) and 50 μL of Protease Inhibitor Cocktail (Sigma). Protein extracts (10 μg) were separated on NuPage 4%–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane with use of the iBlot Dry Blotting System (Invitrogen). Protein expression was analyzed using WesternBreeze Chemiluminescence Western Blot Immunodetection Kit (Invitrogen).

NF-κB activation and interleukin (IL)-8 secretion were analyzed. Cell densities of 1×10^5 HEK293, HEK293-TLR2, or HEK293-TLR2-R753Q cells/100 μL/well were seeded into a 96-well plate (Costar 3595; Corning Incorporated) and incubated overnight at 37°C with 5% CO_2. Adherent cells were stimulated

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with CMVgB (5.0 μg/mL; DevaTal), peptidoglycan (1.0 μg/mL), lipopolysaccharide (1.0 μg/mL), or tumor necrosis factor (TNF)–α (10 ng/mL; R&D Systems) for 16 h at 37°C with 5% CO_2.

To assess NF-κB activation, HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q cells were lysed with 20-μL/well of 1× Reporter Lysis Buffer (Promega Corporation), followed by a single freeze–thaw cycle. The cell lysate was mixed with 100 μL of Promega Luciferase Substrate, and chemoluminescence was measured using the Victor instrument (Perkin-Elmer Life and Analytical Sciences). To quantify IL-8 secretion, cell-free supernatants of HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q cells were assayed using a single-platform IL-8–specific quantitative sandwich enzyme immunoassay (Quantikine for Human CXCL8/IL-8; R&D Systems).

Genes downstream of TLR2 were also assessed. Cell densities of 3×10^6 HEK293, HEK293-TLR2, or HEK293-TLR2-R753Q cell/2 mL/well were seeded into a 6-well plate (Costar 3516) and incubated overnight at 37°C with 5% CO_2. Adherent cells were stimulated with CMVgB for 4 h at 37°C with 5% CO_2. RNA was extracted using RT^{2}qPCR-Grade RNA Isolation Kit (SuperArray Bioscience Corporation), and 1.0 μg of RNA was converted to complementary DNA with use of RT^{2}Profiler PCR Array System. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using the GeneAmp PCR System 2400 (Perkin Elmer), and genes associated with TLR2 signaling (such as myeloid differentiation factor 88 [MyD88], IL-1 receptor associated kinases [IRAK], and TNF receptor-associated factor 6 [TRAF6]) were assessed using the iCycler instrument (Biorad Laboratories) [8].

All experiments were performed in triplicate on at least 2 different occasions. Results are presented as mean (± standard deviation) fold induction in stimulated versus unstimulated cells. Differences between groups were analyzed using a 2-tailed Student's *t* test, with statistical significance set at $P < .05$.

Results. The R753Q SNP impairs NF-κB activation in response to CMVgB. CMVgB induced a 12-fold higher degree of NF-κB–driven luciferase activity in HEK293-TLR2 cells (Figure 1*A*). In contrast, no significant NF-κB–driven luciferase activity was observed in TLR2-deficient HEK293 cells (0.7 fold) and HEK293-TLR2-R753Q (1.2 fold) cells during exposure to CMVgB. Accordingly, the degree of NF-κB–driven luciferase activity in response to CMVgB was significantly higher in HEK293-TLR2 cells, compared with TLR2-deficient HEK293 cells ($P < .001$) or the mutant HEK293-TLR2-R753Q cells ($P < .001$). A similar pattern of cellular response was observed in peptidoglycan-stimulated cells (Figure 1*A*). The lack of cellular activation in HEK293-TLR2-R753Q cells was not attributable to impaired TLR2 protein expression, as determined by Western Blot analysis and flow cytometry (Figure 1*B*). Moreover, the lack of NF-κB–driven luciferase activity in HEK293-TLR2-R753Q cells was not attributable to a global unresponsive state, because cells responded to stimulation with a TLR2-independent activator, TNF-α (Figure 1*A*).

The R753Q SNP impairs the regulation of cytokines during CMVgB stimulation. RT-PCR demonstrated no significant up-regulation in genes encoding for IL-1β, IL-6, IL-8, and TNF-α in HEK293-TLR2-R753Q cells stimulated for 4 h with CMVgB (Figure 1*C*). In contrast, HEK293-TLR2 cells had modest up-regulation of genes encoding IL-1β (4.5 fold) and IL-6 (2 fold) and marked up-regulation in genes encoding IL-8 (85 fold) and TNF-α (90 fold). Furthermore, enzyme-linked immunosorbent assay demonstrated that HEK293-TLR2 cells secreted high levels of IL-8 (mean ± standard deviation, 30,801 ± 2017 pg/mL). IL-8 secretion was 30-fold higher in CMVgB-stimulated cells, compared with unstimulated HEK293-TLR2 cells (Figure 1*D*). In contrast, there was negligible IL-8 secretion in CMVgB-stimulated HEK293 and HEK293-TLR2-R753Q cells. However, in response to TLR2-independent stimulation with TNF-α, HEK293-TLR2-R753Q demonstrated a 23-fold higher level of IL-8 secretion, compared with unstimulated cells.

Discussion. This experimental study provides in vitro evidence that the R753Q SNP paralyzes TLR2-mediated immune signaling in response to CMV. This observation could serve as a putative biological mechanism underlying the clinical association between TLR2 R753Q SNP and a higher degree of CMV replication and higher incidence of CMV disease in humans.

The human TLR2 gene, which is located in chromosome 4q32, contains at least 89 SNPs, including 17 that modify bases in the coding sequence of exon III [9]. Nine SNPs are nonsynonymous, and 2 (R677W and R753Q) were characterized as impairing TLR2 function [10–12]. Because TLR2 recognizes a wide repertoire of pathogen-associated molecular patterns from Gram-positive bacteria (peptidoglycan), mycobacteria (lipoarabinomannan), parasites (glycophosphatidylinositol), fungi (zymosan), and viruses (envelope proteins), there is considerable interest in investigating the potential association between functional impairment in this receptor and various human infections [9, 13]. In this regard, the TLR2 R753Q SNP has been suggested to influence the risk of infection due to *S. aureus* [10], *Mycobacterium tuberculosis* [11], *Borrelia burgdorferi* [14], and *Treponema pallidum* [15].

Previously, we reported that patients with the TLR2 R753Q SNP were more likely to develop CMV disease after liver transplantation [5]. Our present study provides the biological explanation for this clinical observation by demonstrating that variant cells with the TLR2 R753Q SNP failed to recognize CMVgB [2]. This impaired innate viral recognition may impede development of a more robust antiviral immune mechanism, thereby translating to a higher incidence of clinical disease. It is unclear whether the R753Q SNP results in an impaired li-

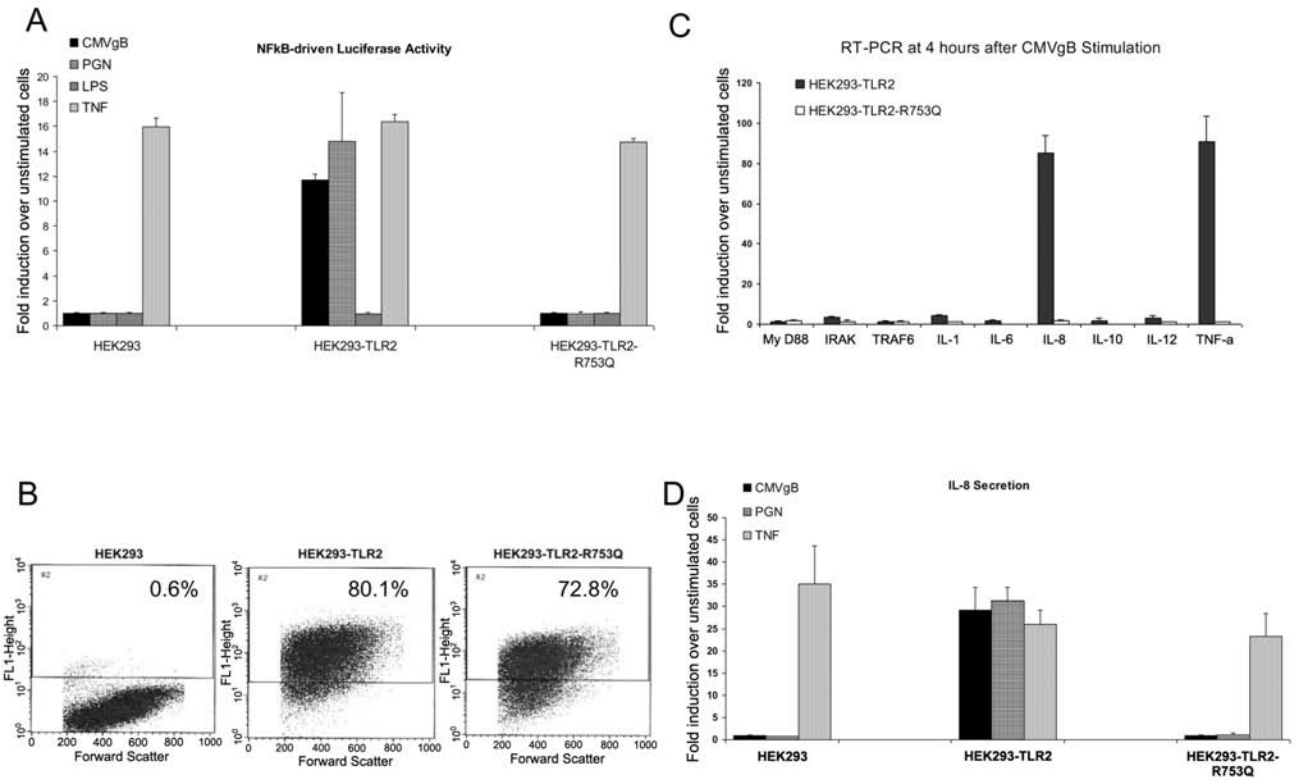

Figure 1. *A,* Nuclear factor-κB (NFκB) activation, as measured by luciferase assay, in human embryonic kidney (HEK)293, HEK293-TLR2, and HEK293–Toll-like receptor (TLR)2–R753Q cells activated with cytomegalovirus envelope glycoprotein B (CMVgB), *Staphylococcus aureus*–derived peptidoglycan (PGN), *Escherichia coli*–derived lipopolysaccharide (LPS), and tumor necrosis factor (TNF)-α. Cells that express normal TLR2 (HEK293-TLR2) exhibited significant NFκB activity during exposure to the TLR2 agonists CMVgB and PGN, whereas cells that do not express TLR2 (HEK293) or that express mutant TLR2 (HEK293-TLR2-R753Q) did not respond to PGN and CMVgB. All cells did not respond to the negative control LPS, thereby indicating the lack of TLR4 expression in all the cell lines; whereas all cells responded to activation with positive TLR2-independent control stimulus TNF-α. *B,* Flow cytometry analysis demonstrates the absence of TLR2 protein expression in HEK293 cells and the comparably robust cell surface expression of TLR2 protein in HEK293-TLR2 and mutant HEK293-TLR2-R753Q cells. *C,* Reverse-transcription polymerase chain reaction analysis of genes downstream of TLR2 suggests a modest to marked cytokine gene up-regulation during 4-h stimulation with CMVgB in HEK293-TLR2 cells, compared with HEK293-TLR2-R753Q cells. *D,* Enzyme-linked immunosorbent assay for interleukin (IL)-8 in HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q cells activated with CMVgB, PGN, and TNF-α. Cells that express normal TLR2 (HEK293-TLR2) secreted high levels of IL-8 during exposure to the TLR2 agonists CMVgB and PGN, whereas cells that do not express TLR2 (HEK293) and cells that express mutant TLR2 (HEK293-TLR2-R753Q) did not secrete IL-8 during stimulation with PGN and CMVgB. Data are presented as mean (± standard deviation) fold-induction in stimulated versus unstimulated HEK293, HEK293-TLR2, and HEK293-TLR2-R753Q cells.

gand-receptor attachment or impaired engagement of downstream adapter molecules. The location of the R753Q SNP within a group of highly conserved amino acids at the C-terminal end of TLR2 (ie, the cytoplasmic Toll-IL-1 receptor domain) suggests that the R753Q SNP may impede the engagement of the MyD88 adapter protein; however, this remains to be demonstrated. Currently, what is known is that, in vitro, the R753Q SNP in TLR2 is associated with impaired NF-κB activation during exposure to CMV and bacterial lipoproteins [12]. These findings expand on our previous observations [5] and those of others [3] that TLR2 is an important component in the immunopathogenesis of CMV in humans. We emphasize, however, that the clinical relevance of TLR2 has been suggested only in immunocompromised transplant recipients, and its importance in the pathogenesis of CMV in healthy individuals has yet to be demonstrated.

In conclusion, this study demonstrates that the R753Q SNP impairs TLR2-mediated immune signaling in response to CMV. This specific functional genetic variation, which is reported in 3%–12% of humans, has a potential clinical application as a prognostic marker for a heightened risk of CMV disease. As subunit CMV vaccines are being developed clinically, with gB as a major component, it is anticipated that immune responses to vaccination may be impaired in individuals with the TLR2 R753Q SNP. These potential clinical implications should encourage the conduct of more studies to confirm our observations and to assess other functional TLR SNPs and their relevance to human infections.

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