

Hepatitis C Virus Core Protein Induces Homotolerance and Cross-Tolerance to Toll-Like Receptor Ligands by Activation of Toll-Like Receptor 2

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Background. Hepatitis C virus (HCV) activates host innate immune responses mediated by retinoic acid inducing gene-1 (RIG-I) and Toll-like receptors (TLRs). Although the nonstructural protein 3/4A (NS3/4A) of HCV disrupts interferon responses by inhibiting RIG-I signaling, the effects of TLR activation by HCV-associated proteins on host innate immune responses are poorly understood.

Methods. Proinflammatory cytokine responses to various TLR ligands in human antigen-presenting cells (APCs) were examined either with or without prestimulation by HCV core protein.

Results. TLR2 activation by the HCV core protein leads to a decrease in interleukin 6 (IL-6) production by human APCs after subsequent stimulation with TLR2 (homotolerance) ligands and TLR4 (cross-tolerance) ligands. This hyporesponsiveness induced by preexposure to the HCV core protein was partially mediated by the negative regulation of nuclear factor- κ B activation by the induction of IRAK-M. TLR ligand-induced IL-6 production was significantly reduced in peripheral blood monocytes isolated from HCV-infected patients, compared with those of healthy control subjects. Alloantigen presentation by monocytes isolated from HCV-infected patients results in impaired production of interleukin 17 by naive CD4⁺ T cells in the presence of TLR ligands.

Conclusions. Chronic stimulation of APCs with HCV core protein is associated with hyporesponsiveness in TLR-mediated innate immunity.

Hepatitis C virus (HCV) is a successful pathogen that establishes persistent infection and causes chronic liver disease in the host [1, 2]. The mechanisms by which HCV avoids elimination by the host immune system are poorly understood. One proposed mechanism accounting for the high rate of persistent infection is that HCV infection inhibits the production of type I interferons that constitute the antiviral host defense [3]. HCV RNA is recognized by innate virus-sensing molecules, such as retinoic acid inducing gene-1 and Toll-

like receptor 3 (TLR3), which then induce rapid interferon responses [4–6]. The HCV nonstructural protein 3/4A protease is reported to blunt the innate antiviral interferon responses mediated by these virus-sensing molecules [5, 7]. However, defective interferon responses are not sufficient to explain the development of the abnormal immunological environments permissive to persistent HCV infection. This notion is supported by the clinical outcome showing that only ~50% of patients with HCV infection are successfully treated with pegylated type I interferon and ribavirin [8].

Several bacterial infections such as sepsis and cellulitis are more common in HCV-infected patients than in those without HCV infection [9–11]. Because TLR-mediated proinflammatory cytokine responses are necessary for host defenses against bacteria [12], it is likely that chronic HCV infection generates an immune environment in which TLR-mediated proinflammatory cytokine production is impaired after exposure to bacterial antigens. With respect to TLR activation by HCV-associated antigens, the core protein activates TLR2 on antigen-presenting cells (APCs) to induce cytokine re-

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sponses through nuclear translocation of nuclear factor- κ B (NF- κ B) subunits [13, 14]. However, the type of immune response that is finally generated by TLR activation in HCV-infected patients remains largely unknown. If the HCV core protein stimulates TLR2, then this TLR pathway will be constantly activated in peripheral blood APCs in HCV-infected patients. Preactivation of a single TLR pathway results in reduced cytokine responses after restimulation with TLR ligands [15]. Therefore, this study investigated whether the activation of TLR2 by the HCV core protein induces tolerogenic cytokine responses after subsequent stimulation with TLR ligands. We found that core protein-mediated activation of TLR2 in human APCs reduces interleukin 6 (IL-6) production by these cells after restimulation with TLR4 ligands (cross-tolerance), or TLR2 ligands (homotolerance). More importantly, IL-6 production mediated by these TLR ligands is significantly reduced in the peripheral blood monocytes of chronic HCV patients compared with healthy control subjects, resulting in impaired allogeneic interleukin 17 (IL-17) production.

METHODS

Stimulation of cell lines with core protein. Human embryonic kidney 293 (HEK293) cells (ATCC) and isolated clones of HEK293 cells stably expressing the human TLR2 gene (HEK293-TLR2; InvivoGen) or TLR4 and MD2 genes (HEK293-TLR4-MD2; InvivoGen) were stimulated with core protein (2 or 20 μ g/mL; Biodesign International), peptidoglycan (10 μ g/mL; Fluka), Pam₃CSK4 (10 μ g/mL; InvivoGen) or lipopolysaccharide (1 μ g/mL; Sigma-Aldrich). The purity of the core protein was >95%. The human monocytic cell line MonoMac 6 (MM6; 1×10^6 cells/mL) [16] was stimulated with core protein (5 μ g/mL), peptidoglycan (10 μ g/mL), Pam₃CSK4 (10 μ g/mL), or lipopolysaccharide (1 μ g/mL) in the presence of a neutralizing anti-TLR2 monoclonal antibody (T2.5; 2 or 20 μ g/mL; eBioscience) or mouse IgG1 control antibody (eBioscience). Cells were cultured for 24 h, and supernatants were analyzed for production of IL-6 and interleukin 8 (IL-8).

Prestimulation of cells with core protein. Human monocyte-derived dendritic cells (DCs) from healthy control subjects were generated as described elsewhere [17]. DCs or MM6 cells (1×10^6 cells/mL) were incubated with core protein (10 μ g/mL) or medium alone for 24 h. The cells were then washed 3 times and restimulated with microbial antigens as described above. Culture supernatants were collected 24 h after restimulation and analyzed for cytokine production.

Flow cytometry. MM6 cells (1×10^6 cells/mL) were incubated with core protein (10 μ g/mL) or culture medium alone for 24 h. Cell surface expression of TLR2 and TLR4 was analyzed using a PE-conjugated anti-human TLR2 monoclonal antibody (TL2.1; eBioscience), an anti-TLR4 monoclonal antibody (HTA125; eBioscience) or a PE-conjugated mouse IgG2a

control antibody (eBioscience). Apoptotic cell death was assessed using an Annexin V assay as described elsewhere [18]. Cell-surface expression of CD80 and CD86 was analyzed by using a PE-conjugated anti-human CD80 or CD86 monoclonal antibody (eBioscience) as described elsewhere [19].

Enzyme-linked immunosorbent assay. The concentrations of cytokines and chemokines were determined by enzyme-linked immunosorbent assay kits for human IL-6, IL-8, interleukin 10 (IL-10), interleukin 12p40 (IL-12p40), interferon γ (IFN- γ) (BD Bioscience), and IL-17 (eBioscience) as described elsewhere [20].

NF- κ B activation assay. Nuclear extracts were prepared from MM6 cells (1×10^6 cells/mL) preincubated with either core protein (10 μ g/mL) or medium for 24 h and then stimulated with core protein (5 μ g/mL) or lipopolysaccharide (1 μ g/mL) for 1 h. The binding of the nuclear extract (30 μ g/well) to NF- κ B consensus oligonucleotides was measured using a Mercury Transfactor kit (BD Bioscience) as described elsewhere [21].

Immunoblot analysis. Immunoblot analysis was performed as described elsewhere [21]. The blotted membranes were incubated with anti-MyD88 (Active Motif), anti-interferon regulatory factor 3 (IRF3; Santa Cruz Biotechnology), anti-interferon regulatory factor 5 (IRF5; Abcam), anti-IRAK-M (Cell Signalling), or anti-actin (Santa Cruz Biotechnology) antibodies.

Assays with small interfering RNA specific to IRAK-M. MM6 cells (5×10^5 cells/mL) were transfected with either IRAK-M small interfering RNA (siRNA) (Santa Cruz Biotechnology) or control siRNA (25 nmol/L) using the TransIT-TKO transfection reagent (Mirus), followed by stimulation with core protein (10 μ g/mL) for 24 h and restimulation with core protein (5 μ g/mL) and lipopolysaccharide (1 μ g/mL).

Studies using peripheral blood cells from patients. Ethical permission for this study was granted by the review board of Kinki University. Healthy control subjects ($n = 10$) and treatment-naïve patients with chronic HCV infection ($n = 10$) were enrolled in this study after informed consent was obtained. Peripheral blood monocytes (1×10^6 cells/mL) isolated from each patient were stimulated with HCV-associated proteins and TLR ligands as described. Monocytes were purified from peripheral blood mononuclear cells (PBMCs) using a monocyte isolation kit (Miltenyi Biotec). Culture supernatants were collected after 24 h and analyzed for cytokine production. In some experiments, monocytes (1×10^6 cells/mL) isolated from HCV patients or healthy control subjects were cocultured for 7 days with naïve CD4⁺ T cells (1×10^6 cells/mL) isolated from PBMCs of healthy control subjects. Culture supernatants were then analyzed for cytokine production. Naïve CD4⁺ T cells were purified using a naïve CD4⁺ T cell isolation kit (Miltenyi Biotec).

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Figure 1. Hepatitis C virus (HCV) core protein is a specific activator of Toll-like receptor 2 (TLR2).

Statistical analysis. A Student *t* test was used to evaluate statistical significance. Statistical analysis was performed using the StatView software, version 4.5 (Abacus Concepts). A value of $P < .05$ was regarded as statistically significant.

RESULTS

Activation of TLR2 by core protein. Our initial studies determined whether core protein functions as a specific activator of TLR2. Core protein induced IL-8 production only in HEK293-TLR2 cells, which suggests that this protein activates TLR2 (Figure 1A). We next used a different approach to confirm this finding in the human monocytic cell line, MM6. Production of IL-6 and IL-8 induced by core protein was reduced by the addition of an anti-TLR2 monoclonal antibody as in the case of stimulation with a conventional TLR2 ligand, peptidoglycan (Figure 1B). To further confirm the activation of TLR2 by core protein, we used splenocytes lacking MyD88, a downstream effector molecule of the TLR2 pathway [12]. MyD88-deficient splenocytes failed to produce IL-6 after stimulation with core protein (Figure 1C). These data suggest that the core protein is a specific activator of the TLR2-MyD88 signaling pathway.

Induction of cross-tolerance by core protein. Although the ligation of TLRs on APCs induces proinflammatory responses, preexposure to TLR2 or TLR4 ligands has been shown to desensitize APCs to subsequent stimulation by TLRs [22–25]. In the case of the TLR4 signaling pathway, preexposure of APCs to lipopolysaccharide reduces responsiveness not only to lipopolysaccharide (homotolerance) but also to TLR2 ligands (cross-tolerance) [22–25]. It remains controversial whether preactivation of TLR2 leads to cross-tolerance in the TLR4 signaling pathway [22–25]. Identification of core protein as a specific TLR2 activator prompted us to address whether preexposure of APCs to core protein leads to tolerogenic responses not only to TLR2 ligands, but also to TLR4 ligands. For this purpose, MM6 cells were incubated with core protein, followed by stimulation with core protein, peptidoglycan, Pam₃CSK4, or lipopolysaccharide, to measure production of proinflammatory cytokines. Production of IL-6 and IL-8 induced by core protein was markedly reduced in cells prestimulated with core protein compared with cells that were not prestimulated (Figure 2A). Interestingly, the production of IL-6 and IL-8 induced by a TLR4 ligand (lipopolysaccharide), as well as TLR2 ligands (peptidoglycan or Pam₃CSK4), was markedly reduced in MM6 cells

preincubated with core protein. Preexposure of cells to core protein did not alter the production of IL-12p40 after restimulation with TLR ligands (data not shown). Thus, exposure of MM6 cells to core protein induces homotolerance and cross-tolerance after subsequent stimulation with TLR ligands. We next examined whether this was the case with primary human APCs. Human monocyte-derived DCs from healthy control subjects were activated with core protein and then restimulated with TLR ligands. The production of IL-6 and IL-8 was markedly reduced in DCs after restimulation with TLR2 and TLR4 ligands (Figure 2B). Moreover, induction of cross-tolerance by exposure to core protein was impaired by blockade of the TLR2 pathway, because IL-6 production was significantly increased in MM6 cells treated with an anti-TLR2 monoclonal antibody (Figure 2C). Taken together, these data strongly suggest that preactivation of TLR2 on human APCs by core protein induces both homotolerance and cross-tolerance after subsequent stimulation with TLR ligands.

Expression of TLRs and activation markers in core protein-tolerant cells. We investigated whether preactivation by core protein alters the expression of costimulatory molecules and TLRs. No significant alteration in the expression of CD80, CD86, TLR2, or TLR4 was seen in MM6 cells by preactivation with core protein (Figure 3A and 3C). In addition, the expression of CD80 and CD86 was not changed by treatment with core protein after restimulation with core protein or lipopolysaccharide (Figure 3A). It is unlikely that the inhibition of TLR responses by core protein prestimulation is due to the induction of apoptotic cell death because stimulation of MM6 cells with core protein did not alter the percentage of Annexin V⁺ apoptotic cells (Figure 3B).

Molecular mechanisms of cross-tolerance by core protein. NF- κ B is a crucial transcription factor for the regulation of IL-6 and IL-8 gene expression. Tolerogenic responses seen after preexposure to Pam₃CSK4 or lipopolysaccharide have been shown to be mediated by a reduction in NF- κ B activation [22, 23]. Figure 4A shows that after restimulation with either core protein or lipopolysaccharide, the binding of the NF- κ B subunits, p65 and p50, from nuclear extracts isolated from MM6 cells preincubated with core protein to consensus sequences is markedly reduced compared with that of cells that had not been preincubated with core protein. In contrast, there was no significant difference in the binding of c-Rel in the nuclear extracts either with, or without, core protein prestimulation. Furthermore, no difference was seen in the binding activity of any of the NF- κ B subunits when cells were restimulated with phorbol myristate acetate, which activates NF- κ B independent of TLR signaling. Neither the core protein-induced nor lipopolysaccharide-induced binding activity of c-Fos and c-Jun in the nuclear extracts was affected by preincubation with core protein (data not shown). Moreover, p65 nuclear translocation

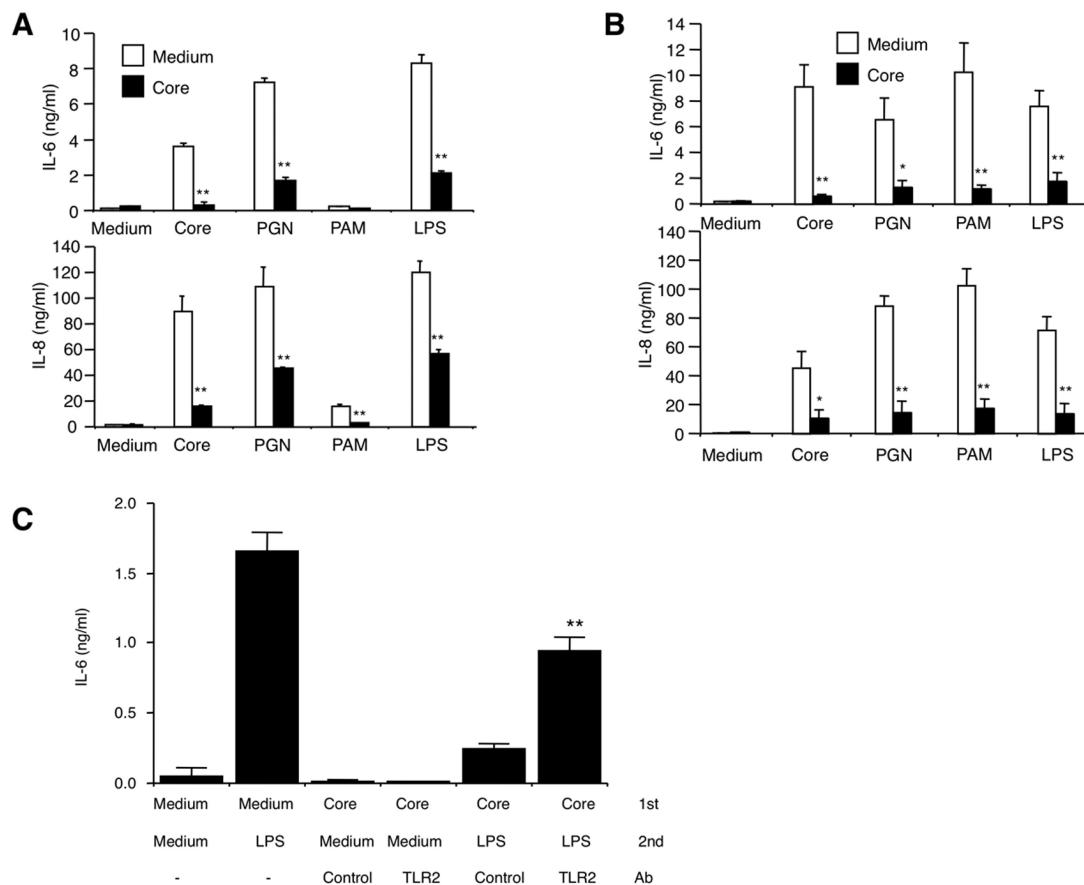


Figure 2. Induction of cross-tolerance to Toll-like receptor (TLR) ligands by hepatitis C virus (HCV) core protein. MM6 cells (1×10^6 cells/mL) (panels A and C) or human monocyte-derived dendritic cells (DCs) (1×10^6 cells/mL) (panel B) were incubated with HCV core protein ($10 \mu\text{g/mL}$) or culture medium alone for 24 h, washed 3 times, and then stimulated with HCV core protein ($5 \mu\text{g/mL}$), peptidoglycan (PGN) ($10 \mu\text{g/mL}$), Pam₃CSK4 (PAM) ($10 \mu\text{g/mL}$), or lipopolysaccharide (LPS) ($1 \mu\text{g/mL}$). In some experiments, MM6 cells were treated with anti-TLR2 monoclonal antibody (mAb) ($50 \mu\text{g/mL}$) or control antibody (Ab) ($50 \mu\text{g/mL}$) for 12 h before stimulation with core protein. Culture supernatants were collected 24 h later, and production of interleukin 6 (IL-6) and interleukin 8 (IL-8) was measured. A, C, Results represent 1 of 3 independent experiments and are shown as mean \pm standard deviation. B, Results are shown as pooled DCs isolated from 4 healthy control subjects and are expressed as mean \pm standard error. * $P < .05$, ** $P < .01$ compared with cells preincubated with medium alone (panels A and B). *** $P < .01$, compared with cells treated with control Ab (panel C).

was restored in MM6 cells stimulated with core protein followed by lipopolysaccharide in the presence of an anti-TLR2 monoclonal antibody (Figure 4B). These data suggest that core protein prestimulation reduces subsequent activation of NF- κ B by TLR ligands.

We performed immunoblot analyses to determine the expression levels of the signaling molecules, or negative regulators, involved in TLR signaling pathways [15] in cells incubated either with, or without, core protein. As shown in Figure 5A, there was no difference in the expression of TLR signaling molecules, such as MyD88, IRF3, or IRF5, in cells with or without core protein stimulation. In contrast, core protein clearly enhanced the expression of the negative regulator IRAK-M. A significant reduction in IRAK-M expression was seen in cells treated with an anti-TLR2 monoclonal antibody. We could not detect the expression of other negative regulators such as IRF4 or SOCS-

1 by immunoblotting (data not shown). Because IRAK-M is responsible for the induction of endotoxin tolerance [26], we looked at whether IRAK-M expression induced by core protein is involved in the induction of homotolerance and cross-tolerance. We investigated whether gene silencing of IRAK-M expression by siRNA [17] abrogates core protein-mediated inhibitory effects. Figure 5B shows that transfection of MM6 cells with IRAK-M siRNA substantially reduced the expression of IRAK-

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Figure 3. Cell-surface expression of costimulatory molecules and Toll-like receptors (TLRs) in cells stimulated with hepatitis C virus (HCV) core protein.

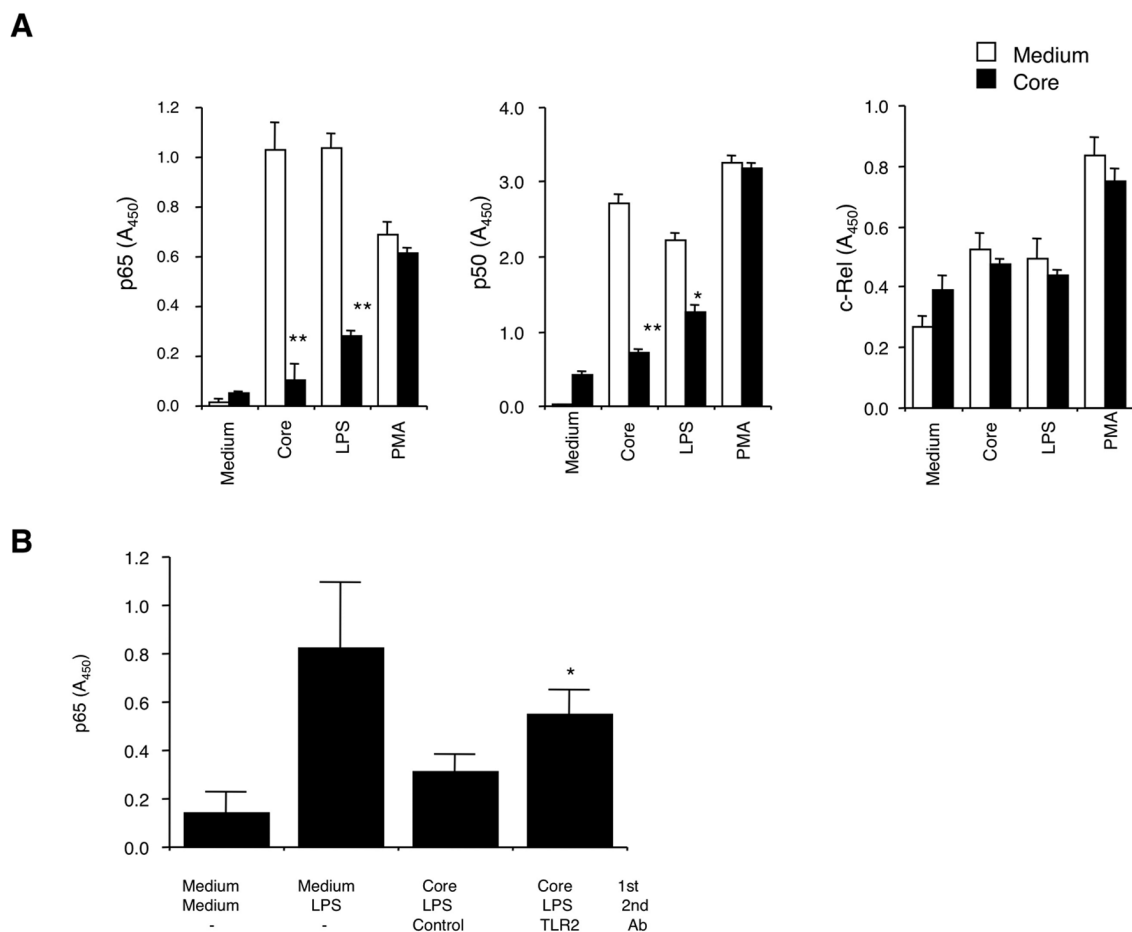


Figure 4. Activation of NF- κ B in cells preincubated with hepatitis C virus (HCV) core protein and stimulated with core protein or lipopolysaccharide (LPS). *A*, MM6 cells (1×10^6 cells/mL) were incubated with HCV core protein (10 μ g/mL) or culture medium alone for 24 h and then stimulated with HCV core protein (5 μ g/mL), LPS (1 μ g/mL), or phorbol myristate acetate (PMA) (50 ng/mL) for 1 h. Nuclear extracts were isolated, and the expression of NF- κ B subunits was determined. *B*, Nuclear extracts were isolated from MM6 cells treated with an anti-TLR2 monoclonal antibody (mAb), or control antibody (Ab), followed by stimulation with HCV core protein and lipopolysaccharide. The expression of p65 was determined. Results shown are representative of 2 experiments (panel *B*) or 3 experiments (panel *A*) and are expressed as mean \pm standard deviation. * $P < .05$, ** $P < .01$ compared with cells preincubated with medium alone (panel *A*). * $P < .05$, compared with cells treated with control Ab (panel *B*).

M at the protein level in core protein-stimulated MM6 cells. Transfection of IRAK-M siRNA led to a significant increase in IL-6 production in core protein-prestimulated MM6 cells after subsequent stimulation with either core protein or lipopolysaccharide, and these effects were associated with restored nuclear translocation of p65 (Figure 5C and 5D). These studies clearly show that induction of IRAK-M expression is involved in the inhibitory effects mediated by core protein prestimulation.

Production of IL-6 by monocytes from HCV-infected patients. Given the fact that circulating peripheral blood APCs in patients with HCV infection are exposed to core protein in the blood, it is interesting to examine whether core protein modulates the responsiveness of APCs by the mechanisms outlined above. To address this, we stimulated peripheral blood monocytes isolated from patients with HCV infection

with core protein and TLR ligands and measured the production of IL-6 and IL-8. The production of IL-6 and IL-8 by monocytes isolated from HCV-infected patients was significantly reduced compared with that of healthy control subjects when cells were stimulated with core protein, peptidoglycan, Pam₃CSK4, or lipopolysaccharide (Figure 6). In contrast, no difference was seen in IL-12p40 production between the 2 populations. Thus, the continuous activation of TLR2 by core protein results in reduced cytokine responses to TLR ligands in monocytes from HCV-infected patients.

Impaired production of IL-17 by CD4⁺ T cells cocultured with monocytes from HCV-infected patients in the presence of TLR ligands. Because APC-derived IL-6 is essential for Th17 differentiation [27], it is possible that chronic activation by core protein induces development of APCs with limited

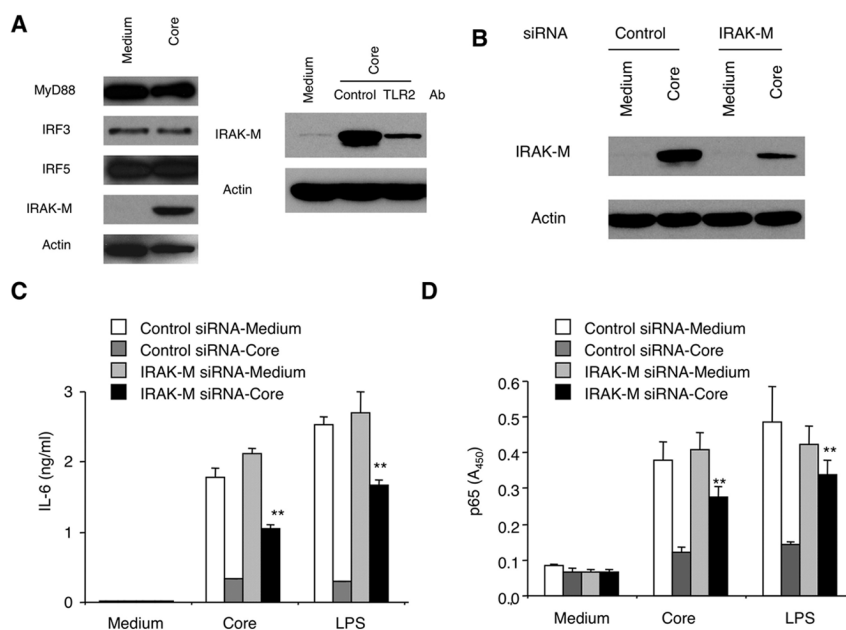


Figure 5. Core protein stimulation is associated with IRAK-M expression. *A*, Whole extracts were prepared from MM6 cells (1×10^6 cells/mL) stimulated with hepatitis C virus (HCV) core protein ($10 \mu\text{g/mL}$) or culture medium alone for 24 h. Whole extracts were immunoblotted with antibodies against the indicated proteins. The expression of IRAK-M after treatment with anti-TLR2 monoclonal antibody (mAb) ($50 \mu\text{g/mL}$) or control antibody (Ab) ($50 \mu\text{g/mL}$) is shown in the right panel. *B*, IRAK-M expression in MM6 cells transfected with IRAK-M small interfering RNA (siRNA). MM6 cells (5×10^5 cells/mL) were transfected with IRAK-M siRNA or control siRNA (25 nmol/L), followed by stimulation with HCV core protein ($10 \mu\text{g/mL}$) for 48 h. Whole extracts were subjected to immunoblot analysis. *C*, MM6 cells (5×10^5 cells/mL) transfected with IRAK-M siRNA or control siRNA (25 nmol/L) were incubated with HCV core protein ($10 \mu\text{g/mL}$) for 24 h and then stimulated with HCV core protein ($5 \mu\text{g/mL}$) or lipopolysaccharide (LPS) ($1 \mu\text{g/mL}$) for another 24 h. Culture supernatants were analyzed for interleukin 6 (IL-6) production. *D*, Nuclear extracts were isolated from MM6 cells transfected with siRNAs after stimulation with HCV core protein and LPS for 1 h. The expression of p65 was determined. Results shown are representative of 2 experiments (panel *D*) or 3 experiments (panels *A*, *B*, and *C*) and are expressed as mean \pm standard deviation. ****** $P < .01$, compared with cells preincubated with core protein and treated with control siRNA.

ability to drive Th17 differentiation in response to TLR ligands. To address this issue, we examined allospecific adaptive immune responses in naive CD4^+ T cells cocultured with monocytes isolated from HCV-infected patients or healthy control subjects in the presence of TLR ligands. Figure 7 shows that IL-17 production is markedly enhanced during antigen presentation by monocytes isolated from healthy control subjects in the presence of core protein, Pam₃CSK4, and lipopolysaccharide. Thus, the stimulation of TLR signaling in monocytes leads to increased production of IL-17 by CD4^+ T cells. In contrast, this enhancement of IL-17 production was absent in CD4^+ T cells stimulated by monocytes isolated from HCV-infected patients. Therefore, alloantigen presentation by monocytes from HCV-infected patients decreases IL-17 production by T cells in the presence of TLR ligands. Interestingly, IFN- γ production was similarly enhanced by alloantigen presentation by monocytes from both healthy control subjects and HCV-infected patients in the presence of TLR ligands. Furthermore, this reduction of IL-17 production was not due to counterregulation of immunosuppressive cytokines, because the production of IL-10 or TGF- β was not increased by coculture with

monocytes isolated from HCV-infected patients (Figure 7) (data not shown). Therefore, these data suggest that impaired production of IL-6 by monocytes isolated from HCV-infected patients is associated with a defective IL-17 response by CD4^+ T cells in the presence of TLR ligands.

DISCUSSION

This study demonstrates that activation of TLR2 by core protein induces not only homotolerance to subsequent TLR2 stimulation but also cross-tolerance to TLR4 stimulation. Consistent with this is the finding that monocytes isolated from HCV-infected patients show defective production of IL-6 after stimulation with TLR ligands, presumably due to chronic exposure to core protein. Impaired production of IL-6 by monocytes from HCV-infected patients is associated with reduced production of IL-17 by allogeneic T cells in the presence of TLR ligands. These results are supported by those of Villacres et al [28], who report a reduced IL-6 response to TLR ligands by PBMCs isolated from patients with HCV infection. They found that IL-6 production by PBMCs from patients with HCV in-

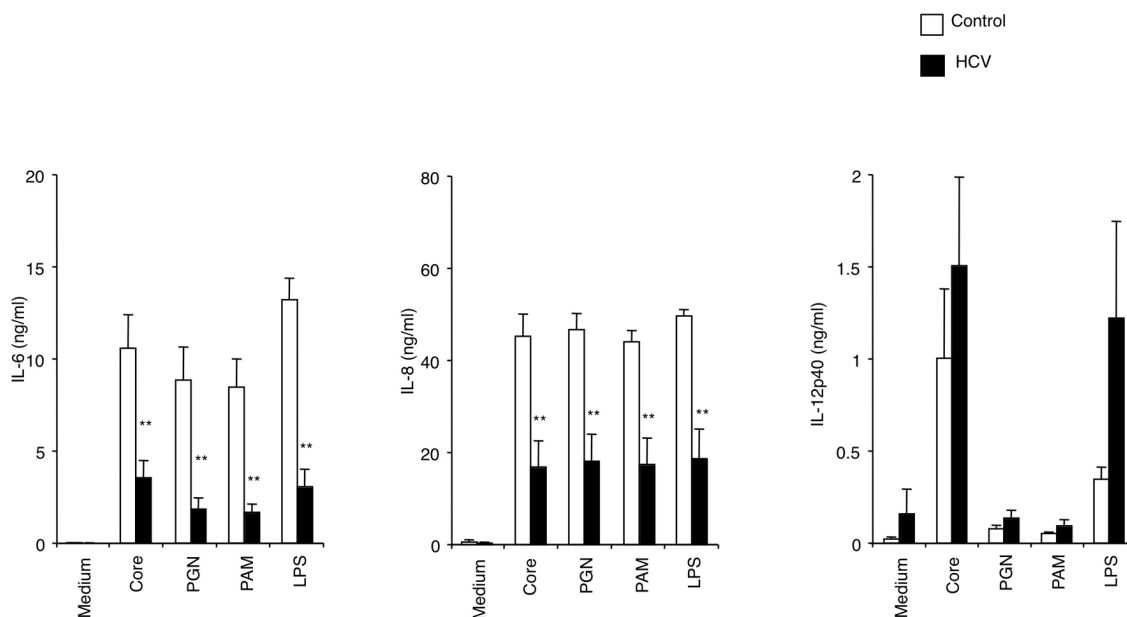


Figure 6. Production of interleukin 6 (IL-6) and interleukin 8 (IL-8) by monocytes isolated from patients infected with hepatitis C virus (HCV). Monocytes were isolated from 10 patients with HCV infection or 10 healthy control subjects. Monocytes (1×10^6 /mL) were stimulated with HCV core protein ($5 \mu\text{g}/\text{mL}$), peptidoglycan (PGN) ($10 \mu\text{g}/\text{mL}$), Pam₃CSK4 (PAM) ($10 \mu\text{g}/\text{mL}$), or lipopolysaccharide (LPS) ($1 \mu\text{g}/\text{mL}$) for 24 h. Culture supernatants were analyzed for production of IL-6, IL-8, and interleukin 12p40 (IL-12p40). Results are expressed as mean \pm standard deviation. ** $P < .01$, compared with monocytes from healthy control subjects.

fection was significantly decreased after stimulation with TLR4 ligands [28]. In addition, another report shows that DCs isolated from HCV-infected patients exhibit an impaired production of TNF- α in response to TLR4 ligands [29]. These results, taken together with our data, show impaired cytokine responses to TLR2 and TLR4 ligands in APCs isolated from HCV-infected patients.

We clearly show that antigen presentation by APCs isolated from HCV-infected patients affects T helper (Th) cell differentiation in the presence of TLR ligands. Chronic exposure to core protein results in the development of APCs with a limited ability to drive Th17 differentiation in the presence of TLR ligands. IL-17 (but not IFN- γ) production by allogeneic naive CD4⁺ T cells was markedly reduced when T cells were cocultured with monocytes from HCV-infected patients and with TLR ligands. This selective impairment of the adaptive IL-17 response can be explained by profiles of cytokine production by these monocytes. IL-6 production induced by core protein and TLR ligands was significantly reduced in monocytes from HCV-infected patients compared with those from healthy control subjects, whereas IL-12p40 production was comparable in monocytes from both populations. Consistent with the results of the patient study (Figure 6), preincubation of APCs with core protein results in reduced production of IL-6 (but not IL-12p40) after restimulation with TLR ligands (Figure 2). Because IL-6 and IL-12 play an essential role for Th17 and Th1 differ-

entiation, respectively [18, 27, 30], the defective IL-17 response seen in allogeneic CD4⁺ T cells may be due to impaired IL-6 production by APCs from HCV-infected patients. Thus, chronic exposure to core protein appears to impair the adaptive IL-17 response (through the development of APCs with a limited ability to produce IL-6 after stimulation with TLR ligands) without affecting adaptive IFN- γ or TGF- β responses. However, it should be noted that we cannot exclude the involvement of TGF- β in reduced IL-17 production by CD4⁺ T cells in the presence of TLR ligands and monocytes from HCV-infected patients. Rowan et al [31] show the indispensable role played by virus-induced TGF- β in the suppression of HCV-specific Th17 cells. This discrepancy regarding the role played by TGF- β may be explained by the differences in target antigens, responses to virus-specific antigens [31] or to allogeneic antigens, or by the difference in types of TGF- β tested—bioactive form [31], cell-surface, [31] or total.

Activation of NF- κ B is impaired in APCs prestimulated with core protein after subsequent restimulation with TLR ligands. Nuclear translocation of p65 and p50 is reduced in cells prestimulated with core protein, whereas the translocation of c-Rel is not. Impaired nuclear translocation of p65 and p50 is responsible for a marked decrease in production of IL-6, because transcription of IL-6 is mediated by activation of the p65-p50 heterodimer [32]. In contrast, APCs that were prestimulated with core protein produced comparable levels of IL-12p40,

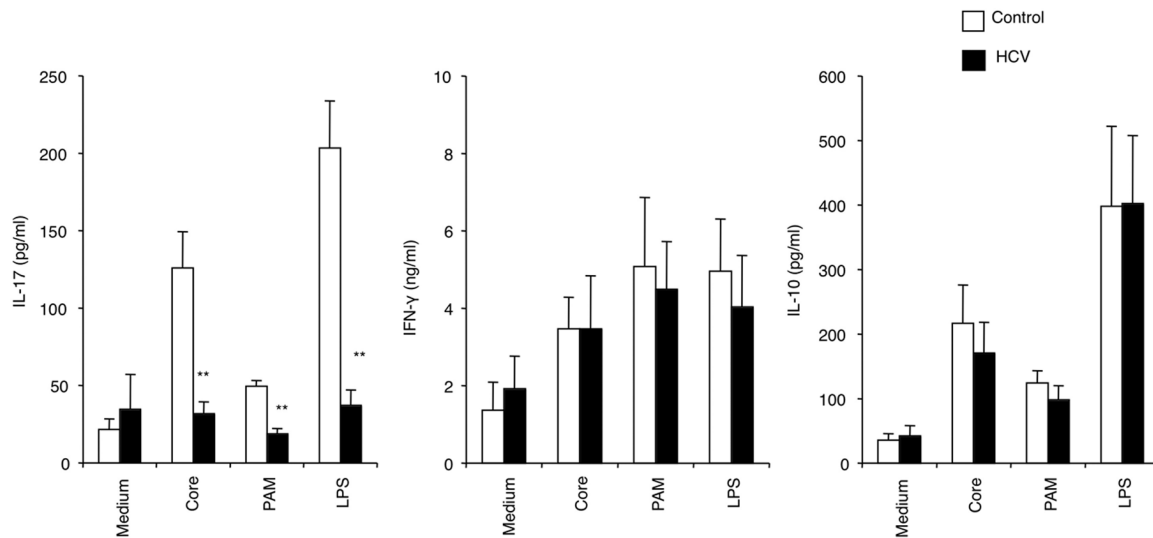


Figure 7. Production of interleukin 17 (IL-17) by allogeneic naive CD4⁺ T cells in the presence of Toll-like receptor (TLR) ligands and monocytes from patients infected with hepatitis C virus (HCV). Naive CD4⁺ T cells were isolated from the peripheral blood of healthy control subjects. Naive CD4⁺ T cells (1×10^6 cells/mL) were cocultured with peripheral blood monocytes (1×10^6 cells/mL) from 8 HCV-infected patients and 8 healthy control subjects in the presence of HCV core protein (5 μ g/mL), Pam₃CSK4 (PAM) (10 μ g/mL), or lipopolysaccharide (LPS) (1 μ g/mL) for 7 days. Culture supernatants were analyzed for production of interleukin 17 (IL-17), interferon γ (IFN- γ), and interleukin 10 (IL-10). Results are expressed as means \pm standard deviation. ** $P < .01$, compared with culture with monocytes from healthy control subjects.

the transcription of which depends on activation of the c-Rel subunit [21]. Therefore, preexposure of APCs to core protein results in reduced production of IL-6 because of the impaired nuclear translocation of p65 and p50 subunits. Impaired activation of NF- κ B by prestimulation with core protein is associated with up-regulation of IRAK-M. Our results show that core protein-mediated activation of TLR2 leads to IRAK-M expression and that knockdown of IRAK-M expression by specific siRNA restores production of IL-6 by APCs prestimulated with core protein. Because IRAK-M is one of the most important negative regulators in TLR signaling [15], these data suggest that IRAK-M expression, induced by core protein-mediated TLR2 activation, modulates the cytokine responses mediated by multiple TLR ligands by the inhibition of NF- κ B activation. However, it should be noted that transfection of IRAK-M siRNA did not completely restore the production of IL-6 by APCs prestimulated with core protein. Thus, other mechanisms of negative regulation of TLR signaling may also operate in the induction and maintenance of homotolerance and cross-tolerance by HCV core protein.

The impaired production of proinflammatory cytokines mediated by TLR2 and TLR4 might be involved in persistent infection by HCV. In fact, the activation of TLR2 and TLR4 plays a protective role in the case of respiratory syncytial virus and cytomegalovirus infection [33]. However, the reduction in IL-6 production by monocytes isolated from HCV-infected patients did not correlate with the HCV load in the serum (data

not shown). This finding may be explained by the fact that the doses of core protein used in this study are much higher than those in the serum of HCV-infected patients. Indeed, serum levels of IL-6 are comparable between HCV-infected patients and healthy control subjects [34, 35]. Similarly, Shiina et al [36] report that infectious cell culture-produced HCV did not inhibit TLR4-mediated IL-6 production by DCs, which suggests that the dose of core protein in this system is not enough to cause cross-tolerance.

Given the fact that TLR2 and TLR4 play critical roles in host defense against microbial infection [12], and that Th17 cells are involved in host defense against bacterial infection [37], our results suggest that TLR2 activation by core protein may contribute to an increased susceptibility to microbial infection in individuals with chronic HCV infection. However, most patients with HCV infection are asymptomatic, although bacterial infections are more common among HCV-infected patients than among those without HCV infection [9–11]. Thus, impaired proinflammatory responses through TLRs might be compensated by other mechanisms in patients with HCV infection. In this regard, Foster et al [38] report that proinflammatory cytokine responses and antimicrobial effectors are differently regulated by TLR-induced chromatin modifications. Therefore, it is possible that antimicrobial effectors rather than proinflammatory cytokines play an important role in host defense against bacterial infection in HCV-infected patients.

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