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Hepatitis C Virus Core Protein Inhibits Interleukin 12 and Nitric Oxide Production from Activated Macrophages

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A characteristic feature of hepatitis C virus (HCV) infection is a high frequency of persistence and the progression to chronic liver diseases. Recent data suggest that prevalent T helper (Th) 2 immunity as well as weak HCV-specific T-cell response is associated with viral persistence. Here, we showed that the production of interleukin 12 (IL-12) and nitric oxide (NO) that is critical for the induction of Th1 and innate immunity, but not that of tumor necrosis factor α (TNF-α), was significantly suppressed in both HCV core-expressing macrophage cell lines and mouse peritoneal macrophages treated with recombinant core protein. In addition, IL-12 p40 promoter activity was repressed by the presence of HCV core in macrophages stimulated with lipopolysaccharide (LPS) following IFN-γ treatment, indicating that IL-12 production may be downregulated at the transcriptional level. We also found that proliferation of T cells and IFN-γ production in mixed lymphocyte reactions (MLR) with core-expressing cells were inhibited. Taken together, our results suggest that HCV core protein could play roles in suppressing the induction of Th1 immunity through inhibition of IL-12 and NO production.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus belonging to the flaviviridae family and chronically infects about 170 million person worldwide. HCV infection induces a high frequency of persistence (up to 85% of acute infection) and the progression to chronic liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (Cohen, 1999). The failure of a host immune response to eliminate HCV has been mainly explained by rapid generation of viral variants (Farci *et al.,* 2000) and weak immunogenicity resulting from the low level of viral replication (Christie *et al.,* 1999) and the replication of the virus in the immune-privileged sites of the body. Alternatively, HCV clearance by host can be inhibited by HCV gene products such as envelope protein 2 (E2) and nonstructural (NS) 5 proteins, which interact with double-stranded RNA-activated protein kinase (PKR) to inhibit antiviral activity of interferon α (IFN-α) (Song *et al.,* 1999; Taylor *et al.,* 1999).

These explanations, however, are insufficient to account for recent evidence that weak T cell responses and a prevalent Th2 pattern of cytokine production are associated with persistence of HCV and chronic evolution of diseases (Diepolder *et al.,* 1995; Missale *et al.,* 1996; Tsai *et al.,* 1997). In general, the differentiation of naive $CD4^+$ T helper (Th0) cells into Th1 or Th2 cells is often decisive for the outcome of a lot of diseases, and the polarization of Th subtypes depends on the costimulatory attributes of the antigen-presenting cells (APCs) as well as the nature and abundance of cytokines provided by them at the initiation of immune response. It was reported that the level of IFN-γ was extremely low in the mixed lymphocyte reaction (MLR) with the dendritic cells (DCs), one of APCs, from HCV patients (Kanto *et al.,* 1999) and that patients with chronic hepatitis C have a lower frequency of HCV core-reactive IFN-γ-producing T cells compared with that of anti-HCV positive healthy subjects (Lechmann *et al.,* 1996; Woitas *et al.,* 1996). In addition, lymphoid DCs expressing HCV structural genes were demonstrated to have the impaired allostimulatory capacity (Hiasa *et al.,* 1998). These data suggest that HCV gene products could play a role in the dysfunction of APCs, leading to defective cellular immunity including HCV-specific Th1 and cytotoxic T lymphocyte (CTL) responses. Therefore, it is necessary to investigate which HCV gene products are involved in the negative regulation of APCs' function.

In this study, we focused on the effect of HCV core protein on the function of macrophages because there are increased mortality, depressed production of IFN-γ and IL-2, and suppression of vaccinia-specific CTL response in mice inoculated with recombinant vaccinia/HCV core virus (Large *et al.,* 1999). Moreover, HCV core protein was known to be a highly conserved protein among HCV gene products and has many functions such as transcriptional regulation of several genes and modulation of cell growth and apoptosis, in addition to its role in viral morphogenesis (Ray *et al.,* 1995, 1997, 1998;

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Srinivas *et al.,* 1996; Chang *et al.,* 1998; Zhu *et al.,* 1998). Here, we found that production of IL-12 and NO, but not of TNF-α, was dramatically suppressed in core-expressing macrophages and DCs as well as in macrophages treated with recombinant core protein produced in *E. coli.* In addition, the stimulatory capacity of macrophages in MLR was demonstrated to be impaired by the presence of core protein.

RESULTS

The production of IL-12 and NO, but not TNF-α, was significantly reduced in HCV core-expressing macrophages compared with their control cells

To examine whether HCV core protein has any effects on the function of APCs, we established *in vitro* tissue culture systems in which HCV core was expressed in macrophage cell lines. Murine macrophage cell lines, RAW 264.7 and J774 cells, and human promonocytic cell line, THP-1 cells, were stably transfected with pCI-Neo/core 1–191 encoding the full length of HCV core protein. Through immunoblotting analysis with rabbit anti-HCV core antibody, RAW 264.7/Core, J774/Core, and THP-1/Core were isolated (data not shown). Cells transfected with pCI-Neo vector, RAW 264.7/Neo, J774/Neo, and THP-1/Neo were also established to use as negative controls. The established macrophage cells were stimulated with LPS (5 μg/ml, 30 h) following pretreatment of recombinant IFN-γ (1000 U/ml, 8 h), and then the amount of IL-12 in the supernatants of the activated macrophages was measured through ELISA. As shown in Fig. 1A, core-expressing macrophages RAW 264.7/Core and J774/Core showed marked reduction of IL-12 secretion in response to IFN-γ and LPS treatment, whereas RAW 264.7/Neo and J774/Neo yielded 450 and 100 pg/ml of IL-12 production, respectively.

To exclude the possibility that the responsiveness of core-expressing macrophage cells to LPS could be modified in the process of establishing stable cell lines, RAW 264.7/Neo and RAW 264.7/Core cells were also stimulated with other stimulants such as zymosan and p1826. Zymosan is a ghost yeast cell that is a kind of particle to be phagocytosed by macrophages, and p1826, CpG motif-containing oligodeoxynucleotide, is known to have an immunostimulatory capacity (Yi *et al.,* 1998). In these experiments, we also observed the dramatic suppression of IL-12 production when core-expressing macrophage cells were stimulated with either zymosan or p1826. These results suggest that the inhibitory effect of HCV core protein on IL-12 production from activated macrophages is stimulant-independent. In contrast, TNF-α production from RAW 264.7/Core or J774/Core was almost equal to that from their control cells (Fig. 1A), reinforcing that HCV core protein caused the impairment of IL-12 production from activated macrophage cells without the general unresponsiveness to various stimulants. These results are partially consistent with a recent clinical observation that DCs from HCV-infected patients showed a low allogeneic capacity with no sign of a generalized immune suppression (Kanto *et al.,* 1999).

NO also has been known to play a major role in the effector phase of the Th1 response as well as to be required for IL-12 signaling in innate immunity (Holscher *et al.,* 1998; Diefenbach *et al.,* 1999; Bogdan *et al.,* 2000). So, we measured the level of NO accumulated during the activation of macrophages (Fig. 1A). Treatment of RAW 264.7/Neo and J774/Neo cells with three different stimulants led to the accumulation of 20 to 40 μM NO within 24 h. However, the concentration of NO released from RAW 264.7/Core and J774/Core was as low as 5 μM, indicating that HCV core protein downregulates NO release as well as IL-12 production. The suppression of both IL-12 and NO secretion by HCV core protein was also observed in human promonocytic cells. IL-12 and NO production from THP-1/Core cells were significantly reduced compared with that from THP-1/Neo, a control cell (Fig. 1B).

To further confirm our observations, we isolated splenic DCs of transgenic mice carrying HCV core gene under the control of a transcriptional regulatory region from hepatitis B virus. HCV core protein was detectable in the splenic monocytes of transgenic mice as well as in the liver and kidney by Western blot analysis (data not shown). Consistent with our previous results, IL-12 secretion from splenic DCs of transgenic mice treated with IFN-γ and LPS was significantly suppressed compared with that from splenic DCs of nontransgenic littermate mice of the same age (Fig. 1C). However, the level of TNF-α production was similar in both mice, indicating that HCV core protein can exert its inhibitory effect on the function of splenic DCs in terms of IL-12 production.

The suppression by HCV core protein was dose-dependent and occurred at the transcriptional level

To determine whether the suppression of IL-12 and NO production is related with the expression level of core protein, we isolated several clones of RAW 264.7/Core cells with different expression levels (Fig. 2A). After activation with either LPS or zymosan in the presence or absence of IFN-γ pretreatment, IL-12 and NO released into supernatants were measured. We found that IL-12 and NO productions were further suppressed at higher concentrations of core protein (Fig. 2B). In contrast, the complete repression of NO production from RAW 264.7/Core clones after zymosan treatment was achieved with the lowest concentration of core protein. Since IL-12 and NO inductions were markedly suppressed in different clones of macrophage cells and operative in RAW 264.7/Core (L), which produces core protein about 20-fold lower than that of RAW/Core (H) (Fig. 2B), it is unlikely that the results observed here are an artifact caused by

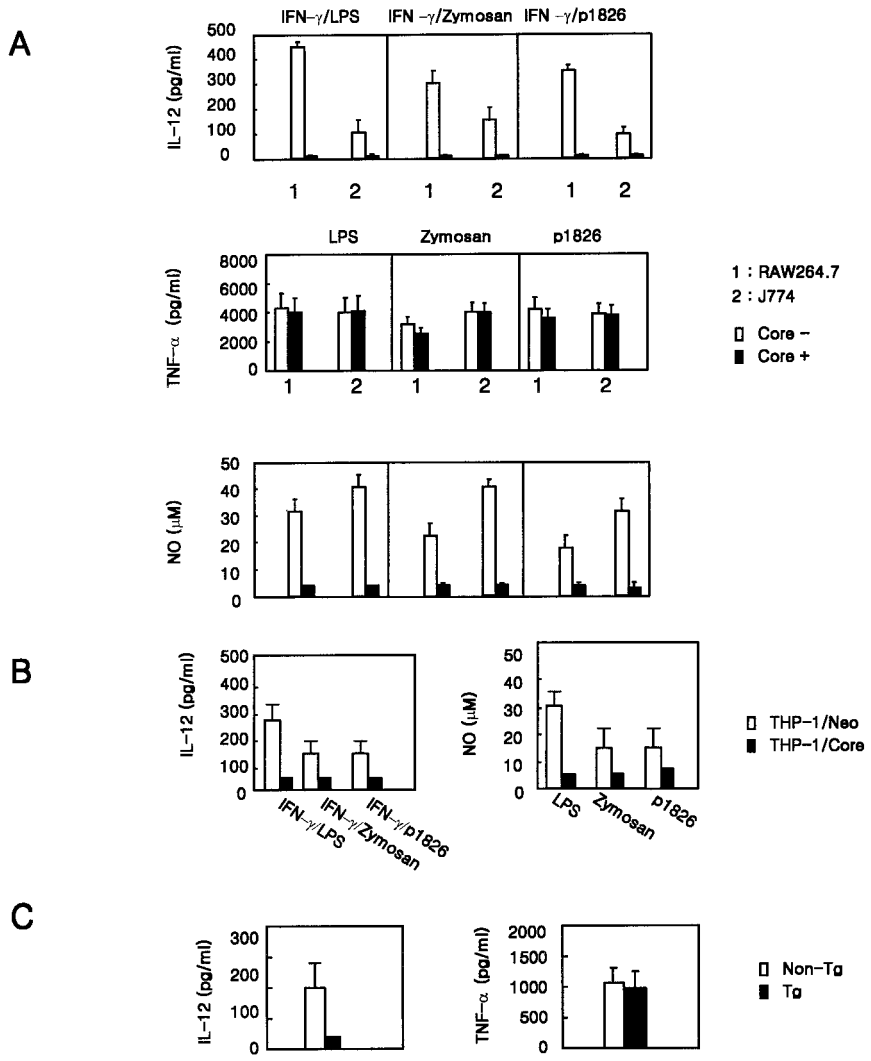

FIG. 1. Inhibition of IL-12 and NO production, but not TNF-α, in the various macrophages expressing HCV core gene. (A) For the induction of IL-12, core-expressing macrophages [RAW 264.7/Core (H) and J774/Core] and their control cells (RAW 264.7/Neo and J774/Neo) were stimulated by LPS (5 μg/ml), zymosan (100 μg/ml), or p1826 (10 μg/ml) for 30 h, following the IFN-γ pretreatment (1000 U/ml, 8 h). For the induction of TNF-α, LPS, zymosan, or p1826 were treated at the same condition as described above without IFN-γ pretreatment. IL-12 and TNF-α production were measured by ELISA using the cultured supernatants from activated macrophage cells. NO production was determined using Griess reagent after LPS stimulation (24 h), except that J774 cells were stimulated with IFN-γ and LPS. (B) After activation under the same conditions, production of IL-12 and NO from THP-1/Neo and THP-1/Core cells was measured. (C) DCs derived from the splenic monocytes of transgenic mice (8-week-old) expressing HCV core protein were activated with IFN-γ and LPS. Production of IL-12 and TNF-α from these cells was measured through ELISA. The splenic DCs of nontransgenic littermates of the same age were used as controls. The values represent the averages of assay from three transgenic and nontransgenic mice, respectively.

the positional effect of the integrated DNA and/or the overexpression of core in the cell line *in vitro*.

Having established the specificity of the HCV core effect upon the production of IL-12, we further confirmed the downregulation of IL-12 production by HCV core protein at the transcription level. IL-12 p40-luciferase construct (Fig. 3A) was transiently cotransfected into RAW 264.7/Neo and RAW 264.7/Core cells together with β-galactosidase expressing vector as an internal control. As shown in Fig. 3B, when the transfected cells were stimulated with IFN-γ (1000 U/ml, 8 h) and LPS (5 μg/ml, 8 h), IL-12 p40 promoter-driven luciferase activity was markedly decreased in RAW 264.7/Core compared with that in RAW 264.7/Neo. This observation implicates that IL-12 production from HCV core-expressing RAW 264.7 cells may be transcriptionally downregulated.

NO production from mouse peritoneal macrophages, but not TNF-α, was suppressed by the exogenous addition of purified core protein

Evidence of the existence of extrahepatic sites supporting HCV replication continues to accumulate (Wang *et al.,* 1992; Lerat *et al.,* 1996; Laskus *et al.,*

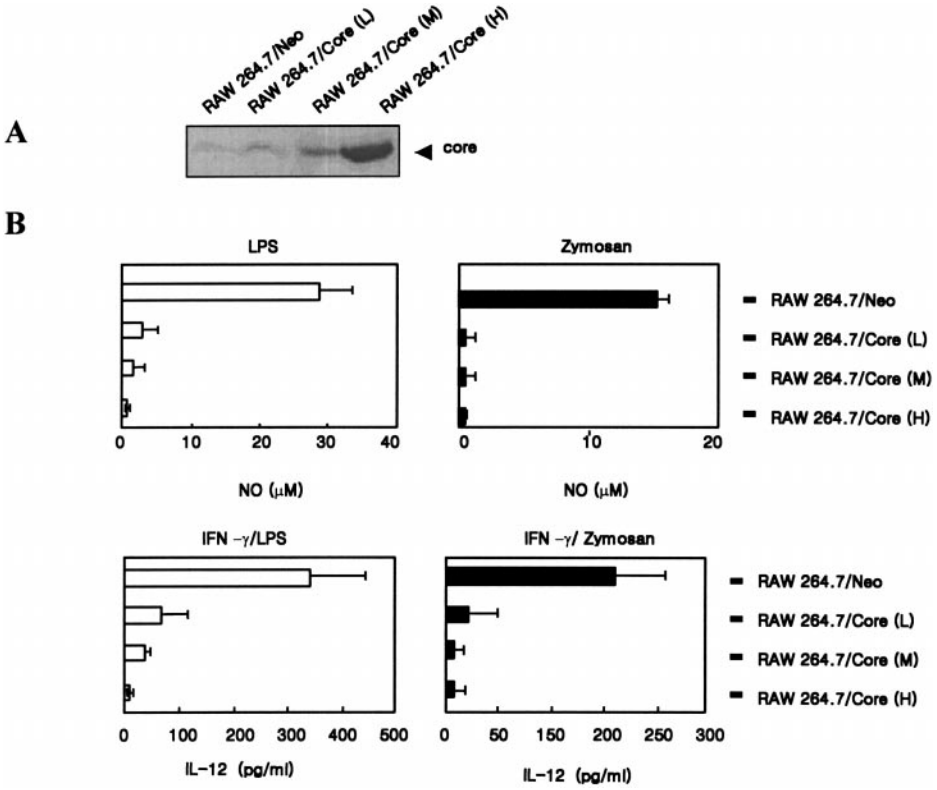

FIG. 2. Dose-dependent suppression of IL-12 and NO productions in HCV-core-expressing cells. (A) Core expression levels from three different RAW 264.7/Core clones were compared by immunoblotting with anti-HCV core antisera. Among the isolated clones, RAW 264.7/Core (H) represents a clone expressing the highest level of core protein. RAW 264.7/Core (M) and RAW 264.7/Core (L) represent clones with medium and the lowest expression level of HCV core protein compared with that of RAW 264.7/Core (H), respectively. (B) Production of NO and IL-12 was compared among several RAW 264.7/Core clones, represented as low (L), medium (M), and high (H), according to their relative expression levels of HCV core protein. Assays were performed as described in Fig. 1.

2000). Detection of HCV genomic sequences, both the positive- and the negative-strand RNA, from monocyte/macrophage that is one of the pheripheral blood mononuclear cell (PBMC)-associated cell subsets, was previously reported (Bouffard *et al.,* 1992; Gabrielli *et al.,* 1994; Lerat *et al.,* 1998). So, it is possible that HCV core protein expressed within APCs during replication exerts its inhibitory effect on function of APCs. Alternatively, APCs such as macrophages and DCs may take up viral particles and/or core proteins released from damaged hepatocytes. To investigate whether HCV core protein by itself could be taken by APCs, presumably through endocytosis, and then dysregulate the function of APCs, we added recombinant core protein to the mouse peritoneal macrophages, and then measured the level of NO. Recombinant core protein that was overexpressed in *E. coli* and purified with more than 95% homogeneity was used for this study (data not shown). Interestingly, NO release, but not TNF-α production from LPS-stimulated peritoneal macrophages was dramatically inhibited by preincubation with 20 μg/ml of exogenous core protein for 1 h. However, heat-inactivated core protein or control buffer did not suppress NO production (Fig. 4), sup-

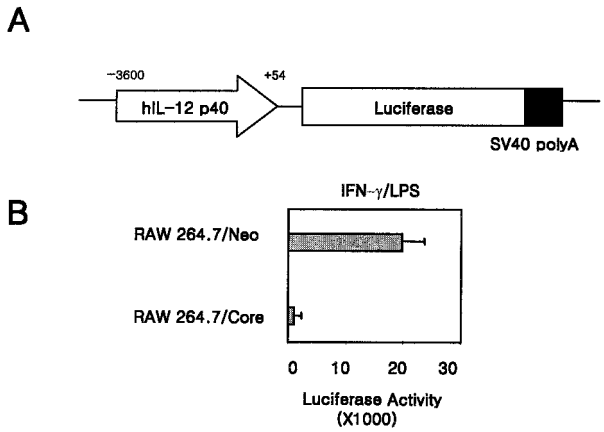

FIG. 3. The repression of IL-12 p40 promoter activity in HCV core-expressing RAW 264.7 cells. (A) The IL-12 p40 promoter-luciferase plasmid, pGL2-p40/Luc, which contains 3.6 kb of upstream region of IL-12 p40 transcription start site, was constructed. (B) The pGL2-p40/Luc (20 μg) and pRC/CMV β-Gal (5 μg), β-galactosidase expression vector as an internal control, were transiently cotransfected into the various macrophage cell lines (5×10^6) by electroporation. After 24 h, cells were stimulated with IFN-γ (1000 U/ml, 8 h) and LPS (5 μg/ml, 8 h), and cell extracts were prepared with 100 μl of lysis buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10% glycerol). The values shown are based on the activity of pGL2-p40/Luc (luciferase) relative to that of pRc/CMV-β-gal (β-galactosidase) and are averages of five independent experiments.

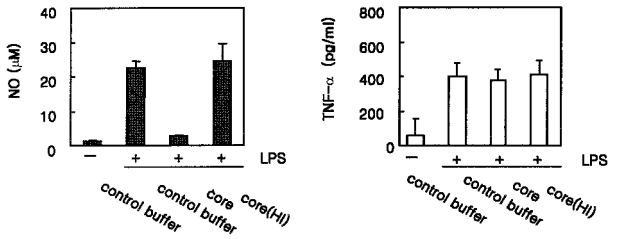

FIG. 4. Inhibition of NO production from mouse peritoneal macrophages, but not TNF-α, by exogenous HCV core protein. Mouse peritoneal macrophages were prepared from 8- to 10-week-old BALB/c mice. The primary macrophages (0.5 to 1 $\times$ 10^6) were stimulated either with or without LPS (5 μg/ml) for 20 h after preincubation with control buffer (PBS with 0.1% Triton X-100, 1 mM EDTA, and 1 mM DTT), core protein (2 μg/ml), or heat-inactivated (HI) core protein (2 μg/ml) for 1 h. TNF-α production and nitrate accumulation in culture medium were measured.

porting that exogenous core protein also exerts its inhibitory effect on the function of peritoneal macrophages.

THP-1/Core cells showed impaired allostimulatory capacity and reduced IFN-γ production in MLR

To examine the effect of core protein on the allostimulatory capacity of macrophages in MLR, both THP-1/Core and THP-1/Neo cells as a negative control were treated with 5 μg/ml of LPS for 24 h after pretreatment of 1000 U/ml IFN-γ for 8 h, and then used as stimulators to activate purified $CD3^+$ primary human T cells. After 5 days of coculture, the degree of T-cell proliferation and cytokine production were measured. Thymidine incorporation and the production of IFN-γ were markedly lower in MLR with THP-1/Core than in that with THP-1/Neo at two different ratios of primary human T cells to DNA-transfected THP-1 cells (Figs. 5A and 5B), indicating that HCV core protein is associated with defective function of macrophages. Since the reduced allostimulatory potential of THP-1/Core could be the result of the enhanced apoptosis of primary T cells during MLR, the cells at Day

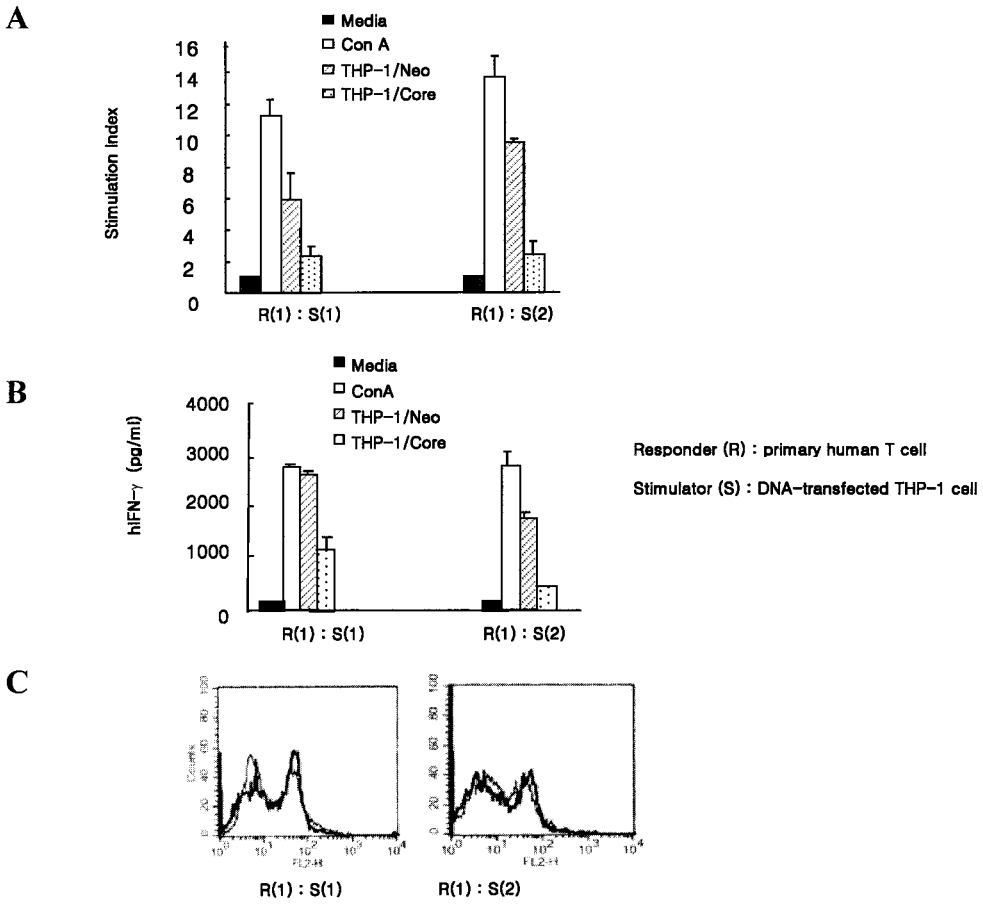

FIG. 5. Impaired allostimulatory capacity and reduced IFN-γ production of macrophages expressing HCV core. (A) The amount of [^{3}H]thymidine incorporated into primary human T cells (1 to 2 $\times$ 10^5 cells) cocultured with THP-1/Core or THP-1/Neo cells (2 $\times$ 10^5 cells) in MLRs was measured. The stimulation index (SI) was defined as the ratio of cpm between the samples with stimulated macrophages and those with unstimulated macrophages. Con A (5 μg/ml)-stimulated T cells were used as positive controls. The results from three independent assays using T cells from three subjects were shown to be similar. The data shown here represent the averages in triplicate cultures with T cells from one of the subjects. (B) The production of IFN-γ from MLR was measured through ELISA using the supernatants harvested on Day 5. (C) The cells recovered on Day 5 in the MLR culture were stained with FITC-conjugated Annexin V (10 μg/10^6 cells). Annexin V-bound cells were assessed using FACS analysis. Thick and thin lines indicate the cells with THP-1/Core and with THP-1/Neo from MLR, respectively.

5 of MLR were stained with FITC–Annexin V. Flow cytometric analysis showed a similar profile in the Annexin V-positive macrophages and T cells after cocultures with either THP-1/Neo or THP-1/Core (Fig. 5C), suggesting that downmodulation of T cell proliferation and IFN-γ production by core protein did not result from the susceptibility or inducibility of apoptosis. Furthermore, to rule out the possibility that HCV core protein exerts its suppressive effects on IL-12 and NO production via the secretion of antiinflammatory cytokine such as IL-10, we measured IL-10 production from DNA-transfected macrophages in response to various stimulations, but could not detect any significant change of IL-10 production in the same conditions tested (data not shown). Thus, these results suggest that HCV core protein could downregulate the allostimulatory capacity of macrophages, which is independent on the proapoptosis or upregulation of antiinflammatory cytokine.

DISCUSSION

The present study demonstrated that HCV core protein, which is either expressed within macrophage cells or exogenously added to them, is capable of suppressing production of IL-12 and NO, but not of TNF-α, from APCs treated with IFN-γ and LPS. In addition, HCV core protein was shown to exert an inhibitory effect on the stimulatory capacity of macrophages in MLR. These results are partially consistent with the following observations that the allostimulatory capacity of peripheral DCs recovered from chronic hepatitis C patients was impaired (Kanto *et al.,* 1999) and that lymphoid DCs expressing structural genes of HCV have a lower stimulatory capacity (Hiasa *et al.,* 1998). It has been known that IL-12 promotes cell-mediated immunity by facilitating Th1 responses, including IFN-γ production from both T and NK cells, enhances the lytic activity of NK cells, and augments specific CTL responses (Trinchieri, 1994). Some viruses such as human immunodeficiency virus (HIV) and measles virus (MV) were reported to inhibit the secretion of IL-12 from PBMC and/or APC, which appear to be one of the strategies to evade host immune response, even though exact molecular mechanisms are still unclear (Clerici *et al.,* 1993; Karp *et al.,* 1996).

Other viruses encode viral gene products that are able to cause cytokine imbalance. For example, Epstein–Barr virus encodes BCRF-1, an IL-10 homolog which suppresses cytokine production from the Th1 subset like IL-10 (Moore *et al.,* 1990). This strategy can give the virus a distinct advantage, helping it to evade host immune defenses by inhibiting the generation of effective antiviral immunity. Since HCV core protein was known to be a structural capsid protein, it raises an interesting question about the biological role of core protein. It was recently reported that HCV core protein functions as a Raf-1 kinase-activating protein through its interaction with 14-3-3 protein (Aoki *et al.,* 2000). Raf-1 activation by core protein resulted in the extracellular signal-related kinase (ERK) activation, which may lead to the suppression of IL-12 production. In addition, it was reported that p38 mitogen-activated protein (MAP) kinase is required for the induction of IL-12 p40 mRNA, and that macrophages and DCs from Mkk3(−/−) mice showed defective production of IL-12 (Feng *et al.,* 1999; Lu *et al.,* 1999). Therefore, it is likely that the direct or indirect interference of Raf-1 and/or p38 MAPK by HCV core protein may be possible mechanisms involved in the suppression of the IL-12 production. As a preliminary result, we demonstrated that phosphorylation of p38 MAPK in macrophages stimulated with LPS was significantly reduced by both endogenous and exogenous core proteins, although that of ERK was not affected by HCV core protein (our unpublished results). These results are similar to the effect of p15E, a retroviral envelope protein, in terms of inhibition of signal transduction by viral structural protein, leading to negative regulation of the host immune responses. The p15E protein was known to block signal transduction by protein kinase C during T cell activation (Dezzutti *et al.,* 1990; Ruegg *et al.,* 1990).

It is of interest to note that recombinant core protein that was exogenously added to APCs can downregulate NO production, but not that of TNF-α. A few recombinant proteins such as HIV-1 Tat and human formy virus Bel-1 are known to have biological activity when these proteins were treated to cells (Lee *et al.,* 1994; Chen *et al.,* 1997; Gibellini *et al.,* 1998; Kumar *et al.,* 1998). However, it is still an open question how exogenous recombinant protein enters cells to exert its biological activities. Whatever the mechanisms are, our results provide a new insight that HCV is capable of suppressing Th1 immunity through downregulation of IL-12 production presumably by core protein released from either broken hepatocytes or extrahepatic reservoirs such as PBMCs and viral particles, in spite of lack of endogenous synthesis in APCs.

In addition to core protein, HCV has been known to encode E2 and NS5A proteins that inactivate PKR (Song *et al.,* 1999; Taylor *et al.,* 1999), which may overcome the antiviral effect of the interferon, a major nonspecific host defense against viruses. Recent observation that the production of IL-12 and IFN-γ in patients with viremic chronic hepatitis C is weaker than that in control subjects suggests that defective production of IL-12 and IFN-γ may contribute to the persistence of HCV infection (Sarih *et al.,* 2000). Therefore, together with the previous reports about the function of HCV E2 and NS5A protein, our results may provide possible explanations about the establishment and maintenance of high persistent HCV infection in humans.

MATERIALS AND METHODS

Cell lines and plasmid

The murine macrophagic cell lines RAW 264.7 and J774, and the human promonocytic cell line THP-1 were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) and RPMI 1640 (Gibco BRL) supplemented with 10% fetal calf serum (HyClone, Logan, UT), respectively. Plasmid pCI-Neo/core 1–191 encoding the full length of HCV core protein was constructed by inserting the PCR-amplified core region of the HCV-K isolate (genotype 1b) into the eukaryotic expression vector pCI-Neo (Promega, Madison, WI).

Establishment of stable cell lines expressing HCV core protein

For stable expression of the HCV core protein, pCI-Neo/core 1–191 was transfected into RAW 264.7, J774, and THP-1 cells by the electroporation method as previously described (Ma *et al.,* 1997), after which transfected cells were isolated as each of single clones. Briefly, cells were collected and washed once with serum-free media, and resuspended in the complete media containing 10% serum at a concentration of 2×10^6 cells/250 μl. Cell suspension (250 μl) and plasmid DNA (20 μg) were placed in 0.45-cm electroporation cuvettes (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) and electroporation was carried out at 960 μF and 250 V. Transfected cells were selected with 400 μg/ml (for RAW 264.7 and J774 cells) or 700 μg/ml (for THP-1 cells) of G418 sulfate (Gibco BRL). After 2 weeks of selection, several clones were isolated and whole cell lysates of each clone were immunoblotted with rabbit anti-HCV core antibody. Stable cell clones transfected with empty vector, pCI-Neo, were also established to serve as controls. All of the permanent cell clones were maintained in the presence of G418 sulfate (200 μg/ml) throughout the experiment.

Reagents

Human and murine IFN-γ were purchased from R&D Systems (Minneapolis, MN), and LPS (*Escherichia coli* 0127:B8) was purchased from Sigma Chemical Co. (St. Louis, MO).

Induction of cytokines and ELISAs

To induce IL-12 production from macrophages, the established and primary macrophage cells were pretreated with 1000 U/ml of IFN-γ for 8 h before LPS addition (5 μg/ml). After 30 h of LPS stimulation, the supernatants were harvested and the amount of IL-12 or TNF-α secreted from activated macrophages was quantified with ELISA kits (R&D Systems). Results are expressed as pg/ml, based on recombinant cytokine standards.

Analysis of NO accumulation

NO accumulation by activated macrophage cells was determined as previously reported (Ding *et al.,* 1988). Briefly, 100 μl of supernatant was combined with an equal volume of Griess reagent, and the samples were incubated at room temperature before measuring the absorbance at 550 nm. Using the standard curve, the absorbance of the sample was converted to the μmol of NO.

Measurement of IL-12 p40 promoter activity

A 3.6-kb genomic DNA fragment of the human IL-12 p40 promoter (−3600 to +54) was isolated from λ-phage genomic clone (Lambda Fix Library; Stratagene, La Jolla, CA), and then cloned into a plasmid encoding luciferase gene, pGL2-control (Promega), at *Bam*HI and *Xho*I sites by replacing with its original SV40 early promoter to obtain IL-12 p40 promoter-luciferase construct. This promoter-reporter plasmid was designated as pGL2-IL-12 p40/Luc. To measure promoter activity, RAW 264.7/Neo and RAW 264.7/Core were transiently cotransfected with 20 μg of the IL-12 p40-luciferase construct and 5 μg of a plasmid containing β-galactosidase under the control of CMV promoter (electroporation at 960 μF and 250 V). The transfected cells were incubated for 16 h at 37°C in 5% CO_2 atmosphere and treated with 1000 U/ml of recombinant IFN-γ for 8 h before the addition of LPS (5 μg/ml). After 8-h stimulation with LPS, cells were harvested and lysed with 100 μl of lysis buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10% glycerol). Lysates were used for both luciferase and β-galactosidase assay. Lysates were mixed with equal volumes of assay buffer (50 μl) containing 1 mM luciferin (Promega), and luciferase activity was measured as light units using a luminometer (Luminol). β-Galactosidase expression was assayed using an assay buffer [1 mg/ml of CPRG (BMB), 1 mM $MgCl_2$, 100 mM DTT, and 20 mM Tris–HCl, pH 7.2]. Luciferase expression was corrected based on the simultaneous expression of β-galactosidase.

Preparation of splenic DCs from transgenic mice carrying HCV core gene

The transgene construct, pRc/CMV-HBVCore, which encodes the core gene under the control of a transcriptional regulatory region from hepatitis B virus, was introduced into FVB mouse embryos. Mice were maintained in a specific pathogen-free state. The transgenic mice lineage was screened by PCR and Southern blot analyses. The expression of core protein from various tissues was tested by immunoblotting with anticore antibody. Nontransgenic littermate mice that showed no expression of core protein were used as negative controls.

Splenic DCs of both transgenic and nontransgenic mice were prepared as previously described (Ridge *et al.,* 1996; Singh *et al.,* 1998). Briefly, splenocytes adhered to a tissue culture plates for 2 h, after which nonadherent cells were removed. Adherent cells were cultured overnight in 5 ng/ml of recombinant mouse GM-CSF (R&D Systems) to differentiate into DCs. After 12 to 16 h incubation, the weakly adherent cells were harvested and purified again with 55% Percoll density-gradient centrifugation. The resulting low-density cells from interface were used as splenic DCs-enriched populations.

Mixed lymphocyte reaction

To evaluate the allostimulatory capacity of HCV core-expressing THP-1 cells, MLR was performed. PBMC of healthy donors were obtained by Ficoll–Hypaque density-gradient centrifugation. These cells were incubated with mouse anti-human CD3 antibody (10 μg/10^6 cells) and magnetic bead-tagged goat anti-mouse IgG antibody to collect primary human T cells. After IFN-γ (1000 U/ml, 8 h) treatment and LPS (5 μg/ml, 24 h) stimulation, THP-1/neo or THP-1/core 1–191 was inactivated with mitomycin C (50 μg/ml, 40 min). The inactivated cells (2×10^6 cells/well) were placed on 96-well flat-bottom culture plates and mixed with primary $CD3^+$ T cells (1 to 2×10^6 cells/well) and cocultured for 5 days. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h before being harvested, and then the amounts of [^{3}H]thymidine incorporated into cells were measured with a beta counter. The supernatants from MLR were harvested at Day 5 and assayed to measure the production of IFN-γ and IL-10 through ELISA.

Purification of HCV core protein produced in *E. coli*

A plasmid expressing HCV core gene (type 1a, amino acids 1–191), pET-3a/core191, was introduced into *E. coli* BL21(DE3). The expression of HCV core gene in cultured cells was induced by the addition of 0.5 mM IPTG. The harvested cells were resuspended in column buffer (20 mM sodium phosphate, pH 7.0, 1 mM DTT, 1 mM EDTA) and disrupted by sonication. After centrifugation of the cell lysates, the precipitated insoluble proteins were dissolved in column buffer containing 8 M urea and loaded onto a column packed with a cation exchanger resin, SP–Sepharose Fast Flow (Pharmacia, Piscataway, NJ). At the end of sample loading, the column was washed and selectively eluted with column buffer containing 0.1 to 1 M NaCl in stepwise manner. To renature the core protein, urea was gradually removed through dialysis. The purity of the obtained protein was over 95%, based on the Coomassie blue staining. The circular dichroism spectral analysis of the purified core protein showed that estimated compositions of the secondary structure coincided with the predicted pattern, based on their amino acid sequences (manuscript in preparation). In addition, the *in vitro* binding assay (Hsieh *et al.,* 1998) demonstrated that the purified GST-hnRNP K efficiently interacted with the core protein, but not with the heat-inactivated core protein (boiled for 10 min).

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REFERENCES

Aoki, H., Hayashi, J., Moriyama, M., Arakawa, Y., and Hino, O. (2000). HCV core protein interacts with 14-3-3 protein and activates the kinase Raf-1. *J. Virol.* **74,** 1736–1741.

Bogdan, C., Rollinghoff, M., and Diefenbach, A. (2000). The role of nitric oxide in innate immunity. *Immunol. Rev.* **173,** 17–26.

Bouffard, P., Hayashi, P. H., Acevedo, R., Levy, N., and Zeldis, J. B. (1992). Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. *J. Infect. Dis.* **166,** 1276–1280.

Chang, J., Yang, S.-H., Cho, Y. G., Hwang, S. B., Hahn, Y. S., and Sung, Y. C. (1998). Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. *J. Virol.* **72,** 3060–3065.

Chen, P., Mayne, M., Power, C., and Nath, A. (1997). The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. *J. Biol. Chem.* **272,** 22385–22388.

Christie, J. M., Chapel, H., Chapman, R. W., and Rosenberg, W. M. (1999). Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. *Hepatology* **30,** 1037–1044.

Clerici, M., Lucey, D. R., Berzofsky, J. A., Pinto, L. A., Wynn, T. A., Blatt, S. P., Dolan, M. J., Hendrix, C. W., Wolf, S. F., and Shearer, G. M. (1993). Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science* **262,** 1721–1724.

Cohen, J. (1999). The scientific challenge of hepatitis C. *Science* **285,** 26–30.

Dezzutti, C. S., Lafrado, L. J., Lewis, M. G., and Olsen, R. G. (1990). Inhibition of phorbol ester-induced neutrophil chemiluminescence by FeLV. *Arch. Virol.* **111,** 75–85.

Diefenbach, A., Schindler, H., Röllinghoff, M., Yokoyama, W. M., and Bogdan, C. (1999). Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science* **284,** 951–955.

Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Wierenga, E. A., Santantonio, T., Jung, M. C., Eichenlaub, D., and Pape, G. R. (1995). Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* **346,** 1006–1007.

Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141,** 2407–2412.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J. C., Strazzera, A., Chien, D. Y., Munoz, S. J., Balestrieri, A., Purcell, R. H., and Alter, H. J. (2000). The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* **288,** 339–344.

Feng, G. J., Goodridge, H. S., Harnett, M. M., Wei, X. Q., Nikolaev, A. V., Higson, A. P., and Liew, F. Y. (1999). Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* **163,** 6403–6412.

Gabrielli, A., Manzin, A., Candela, M., Caniglia, M. L., Paolucci, S., Danieli, M. G., and Clementi, M. (1994). Active hepatitis C virus infection in bone marrow and peripheral blood mononuclear cells from patients with mixed cryoglobulinaemia. *Clin. Exp. Immunol.* **97,** 87–93.

Gibellini, D., Bassini, A., Pierpaoli, S., Bertolaso, L., Milani, D., Capitani, S., La Placa, M., and Zauli, G. (1998). Extracellular HIV-1 Tat protein induces the rapid Ser133 phosphorylation and activation of CREB transcription factor in both Jurkat lymphoblastoid T cells and primary peripheral blood mononuclear cells. *J. Immunol.* **160,** 3891–3898.

Hiasa, Y., Horiike, N., Akbar, S. M., Saito, I., Miyamura, T., Matsuura, Y., and Onji, M. (1998). Low stimulatory capacity of lymphoid dendritic cells expressing hepatitic C virus genes. *Biochem. Biophys. Res. Commun.* **249,** 90–95.

Holscher, C., Kohler, G., Muller, U., Mossmann, H., Schaub, G. A., and Brombacher, F. (1998). Defective nitric oxide effector functions lead to extreme susceptibility of Trypanosoma cruzi-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. *Infect. Immun.* **66,** 1208–1215.

Hsieh, T. Y., Matsumoto, M., Chou, H. C., Schneider, R., Hwang, S. B., Lee, A. S., and Lai, M. M. (1998). Hepatitis C virus core protein interacts with heterogeneous nuclear ribonucleoprotein K. *J. Biol. Chem.* **273,** 17651–17659.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., and Hori, M. (1999). Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* **162,** 5584–5591.

Karp, C. L., Wysocka, M., Wahl, L. M., Ahearn, J. M., Cuomo, P. J., Sherry, B., Trinchieri, G., and Griffin, D. E. (1996). Mechanism of suppression of cell-mediated immunity by measles virus. *Science* **273,** 228–231.

Kumar, A., Manna, S. K., Dhawan, S., and Aggarwal, B. B. (1998). HIV-Tat protein activates c-Jun N-terminal kinase and activator protein-1. *J. Immunol.* **161,** 776–781.

Large, M. K., Kittlesen, D. J., and Hahn, Y. S. (1999). Suppression of host immune response by the core protein of hepatitis C virus: Possible implications for hepatitis C virus persistence. *J. Immunol.* **162,** 931–938.

Laskus, T., Radkowski, M., Wang, L. F., Nowicki, M., and Rakela, J. (2000). Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: Confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. *J. Virol.* **74,** 1014–1017.

Lechmann, M., Langhans, B., Bär, S., Woitas, R., Leifeld, L., Ihelfeldt, H. G., Jung, G., Effenberger, W., Sauerbruch, T., and Spengler, U. (1996). Deficient secretion of Th 1 cytokines in patients with chronic hepatitis C. *Hepatology* **24,** 266A.

Lee, C. W., Chang, J., Lee, K. J., and Sung, Y. C. (1994). The Bel1 protein of human foamy virus contains one positive and two negative control regions which regulate a distinct activation domain of 30 amino acids. *J. Virol.* **68,** 2708–2719.

Lerat, H., Berby, F., Trabaud, M. A., Vidalin, O., Major, M., Trepo, C., and Inchauspe, G. (1996). Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J. Clin. Invest.* **97,** 845–851.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M. A., Trepo, C., and Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: Influence of viral load, viral genotype, and cell phenotype. *Blood* **91,** 3841–3849.

Lu, H. T., Yang, D. D., Wysk, M., Gatti, E., Mellman, I., Davis, R. J., and Flavell, R. A. (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J.* **18,** 1845–1857.

Ma, X., Neurath, M., Gri, G., and Trinchieri, G. (1997). Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J. Biol. Chem.* **272,** 10389–10395.

Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M. G., Houghton, M., Fiaccadori, F., and Ferrari, C. (1996). Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the antiviral cell-mediated immune response. *J. Infect. Dis.* **98,** 706–714.

Moore, K. W., Vieira, P., Fiorentino, D. F., Trounstine, M. L., Khan, T. A., and Mosmann, T. R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein–Barr virus gene BCRFI. *Science* **248,** 1230–1234.

Ray, R. B., Lagging, L. M., Meyer, K., Steele, R., and Ray, R. (1995). Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. *Virus Res.* **37,** 209–220.

Ray, R. B., Meyer, K., Steele, R., Shrivastava, A., Aggarwal, B. B., and Ray, R. (1998). Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. *J. Biol. Chem.* **273,** 2256–2259.

Ray, R. B., Steele, R., Meyer, K., and Ray, R. (1997). Transcriptional repression of p53 promoter by hepatitis C virus core protein. *J. Biol. Chem.* **272,** 10983–10986.

Ridge, J. P., Fuchs, E. J., and Matzinger, P. (1996). Neonatal tolerance revisited: Turning on newborn T cells with dendritic cells. *Science* **271,** 1723–1726.

Ruegg, C. L., Clements, J. E., and Strand, M. (1990). Inhibition of lymphoproliferation and protein kinase C by synthetic peptides with sequence identity to the transmembrane and Q proteins of visna virus. *J. Virol.* **64,** 2175–2180.

Sarih, M., Bouchrit, N., and Benslimane, A. (2000). Different cytokine profiles of peripheral blood mononuclear cells from patients with persistent and self-limited hepatitis C virus infection. *Immunol. Lett.* **74,** 117–120.

Singh, N., Bhatia, S., Abraham, R., Basu, S. K., George, A., Bal, V., and Rath, S. (1998). Modulation of T cell cytokine profiles and peptide-MHC complex availability in vivo by delivery to scavenger receptors via antigen maleylation. *J. Immunol.* **160,** 4869–4880.

Song, J., Fujii, M., Wang, F., Itoh, M., and Hotta, H. (1999). The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. *J. Gen. Virol.* **80,** 879–886.

Srinivas, R. V., Ray, R. B., Meyer, K., and Ray, R. (1996). Hepatitis C virus core protein inhibits human immunodeficiency virus type 1 replication. *Virus Res.* **45,** 87–92.

Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N., and Lai, M. M. (1999). Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* **285,** 107–110.

Trinchieri, G. (1994). Interleukin 12: A cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* **84,** 4008–4027.

Tsai, S. L., Liaw, Y. F., Chen, M. H., Huang, C. Y., and Kuo, G. C. (1997). Detection of type 2-like T-helper cells in hepatitis C virus infection: Implications for hepatitis C virus chronicity. *Hepatology* **25,** 449–458.

Wang, J. T., Sheu, J. C., Lin, J. T., Wang, T. H., and Chen, D. S. (1992). Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *J. Infect. Dis.* **166,** 1167–1169.

Woitas, R. P., Lchmann, M., Langhans, B., Bär, S., Ihlenfeldt, H. G., Jung, G., Sauerbruch, T., and Spengler, U. (1996). Cytokine response to hepatitis C virus core peptides in patients with chronic hepatitis C infection and healthy anti-HCV seropositives. *Hepatology* **24,** 266A.

Yi, A. K., and Krieg, A. M. (1998). Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161,** 4493–4497.

Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. M. (1998). Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J. Virol.* **72,** 3691–3697.