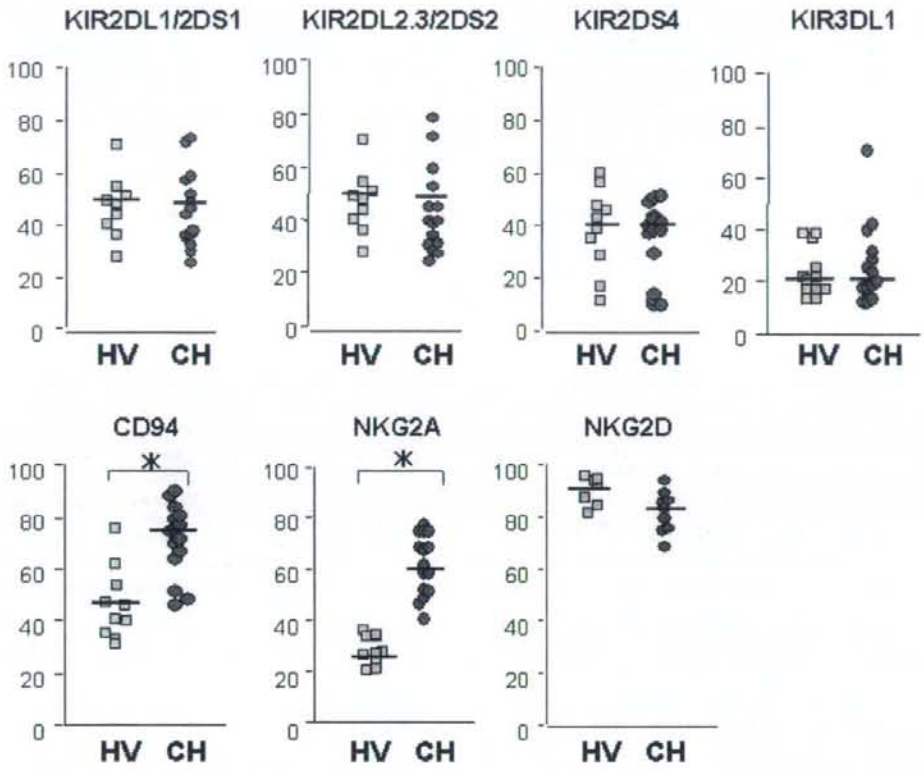

Figure 2. The expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. The percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. $*P < 0.05$ by Mann-Whitney U test.

DCs play a decisive role in shaping innate immunity by interacting with NK cells. DCs have two means to stimulate NK cells via the production of cytokines (IL-12, IL-18 or IFN-alfa) and through the expression of NK-activating ligands. In response to IFN-alfa, DCs are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D (28). Interestingly, DCs from HCV-infected patients are unresponsive to exogenous IFN-alfa to enhance MICA/B expression and fail to activate NK cells (28). It is tempting to speculate that the impairment of DCs in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN-alfa expression in the liver. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF-beta1 (27). Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts (27). Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

4.4. Natural killer T cells

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers (29). The NKT cell population is highly heterogeneous according to the differences in types and tissue distribution; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of Valfa24-JalfaQ preferentially paired with Vbeta11 in humans (29), whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DCs (29). Although endogenous ligands of iNKT cells are little known, alfa-galactosyl-ceramide (alfaGalCer) has been used as a surrogate for natural ligands. In contrast, non-invariant NKT cells are activated by CD1d-dependent manner but are not reactive to alfaGalCer. Baron *et al.* reported that, in hepatitis B virus-transgenic mice, non-invariant NKT cells are critically involved in acute liver injury (30). As for a human counterpart, Exley *et al.* observed that CD1d

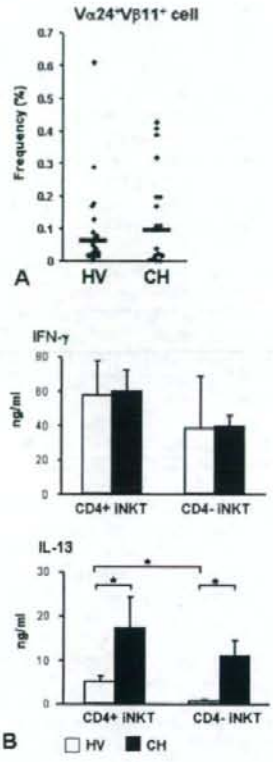

Figure 3. Frequency and cytokine production of invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. (A) The frequencies of total invariant NKT (iNKT) cells (Valfa24$^+$Vbeta11$^+$ cells) in PBMCs were determined by flow cytometry. HV, CH; See Fig 2. Horizontal bars represent the median. (B) Invariant NKT (iNKT) cells were expanded by culture with alfaGalCer-pulsed autologous monocyte-derived DCs (MoDCs) and CD4$^+$ and CD4$^-$ iNKT cells were collected by subsequent cell sorting. The activated iNKT cells were stimulated with alfaGalCer-pulsed allogeneic MoDCs for 24 h and the supernatants were collected for cytokine ELISA. The bars represent mean $\pm$ SE of 5 different subjects. HV, CH; See Figure 2. *$P < 0.05$ by Mann-Whitney U test.

restricted non-invariant NKT cells infiltrate in HCV-infected liver, where they presumably exert their promoting role in liver inflammation (31). Hepatic inflammatory cells or biliary cells up-regulate CD1d which subsequently supports NKT cell activation (32). In addition, hepatic stellate cells are capable of activating NKT cells via surface CD1d and secretion of IL-15 (33).

Although iNKT cells comprise a small portion of hemotopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings. It has been demonstrated that phenotypic as well as functional subsets exist for iNKT cells, which are CD4$^+$, CD4$^-$CD8$^-$ double negative (DN) and CD8$^+$ ones. The CD4$^+$ and DN iNKT cells produce both Th1 (IFN-gamma) and Th2 cytokines (IL-4, IL-5, IL-13). The CD4$^+$ iNKT cells secrete more Th2 cytokines than DN, while CD8$^+$ subsets predominantly secrete Th1 cytokines (34). For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells (35, 36), however, their functional roles in HCV-infected patients are largely unknown. We thus compared the frequency and the cytokine producing capacity of iNKT cells in peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by the stimulation with alfaGalCer-loaded DCs. We demonstrate that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state (Figure 3A) (37). By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls (Figure 3B) (37). Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are pro-fibrogenic to the liver (38). If this is the case, our findings suggest that iNKT cells in chronic HCV infection are pro-fibrogenic per se even in the pre-cirrhotic stage. The reason why iNKT cells in HCV infection are Th2-biased needs to be further investigated.

5. ADAPTIVE IMMUNITY IN HCV INFECTION

Many reports have been published on the importance of CD4$^+$ T cell response in the clearance and control of HCV. In chronic hepatitis C patients, HCV-specific CD4$^+$ T cells were functionally impaired and their activity was not sustained (39), which was in clear contrast with resolved cases. Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that CD4$^+$ T cell help was indispensable for the development of effective CD8$^+$ T cell response to protect from HCV persistence (40).

With regard to HCV-specific CD8$^+$ T cells observed during the chronic stages of disease, conflicting results have been reported for their roles in HCV replication and liver inflammation. Several investigators have shown that the HCV-specific CTL response is inversely correlated with viral load, suggesting its inhibitory capacity on HCV replication (41). However, others did not find a significant relationship between these parameters (42). HCV-specific CD8$^+$ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN-gamma in response to HCV antigens. Since CD8$^+$ T cells are reported to be involved in HCV-induced liver inflammation, inefficient CD8$^+$ T cells may evoke only milder hepatocyte injury, which level is not sufficient for HCV eradication (5).

Several plausible mechanisms have been proposed for T cell functional failure observed in chronic HCV infection (3): 1) HCV escape mutation, 2) primary T

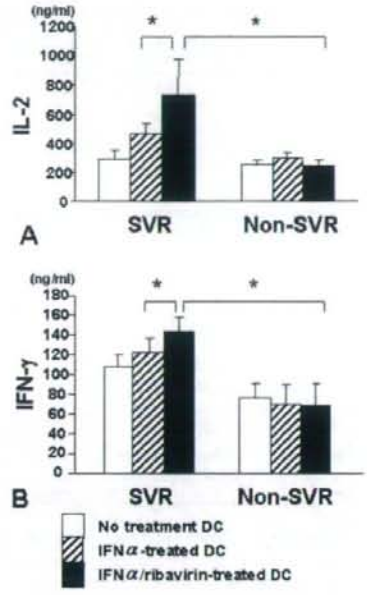

Figure 4. Improvement of Th1-inducing ability of dendritic cells by IFN-alfa and ribavirin from chronic hepatitis C patients with sustained virological response in combination therapy. Monocyte-derived dendritic cells (DC) were generated with GM-CSF and IL-4 in the presence or absence of IFN-alfa and ribavirin and were cultured with allogeneic naïve $CD4^+$ T cells for 6 days. On day 4 of the culture, half of the supernatants were collected for the assessment of IL-2. After 6 days, the cultured cells were stimulated with phorbol myrisate acetate and ionomycin for 24 hours. The results of IFN-gamma (A) and IL-2 (B) determined by ELISA were compared among them in the SVR and non-SVR group. The results are expressed as mean ± SE from five SVR and nine non-SVR patients. SVR, sustained virological responder in 24 weeks of IFN-α and ribavirin therapy. $*P < 0.05$ by Mann-Whitney U-test.

cell failure or T cell exhaustion, 3) impaired antigen presentation, 4) suppression by HCV proteins, 5) impaired T cell maturation, 6) suppression by regulatory T cells and 7) tolerogenic environment in the liver.

6. IMMUNE RESPONSE DURING ANTI-VIRAL THERAPY

Anti-viral agents, pegylated (PEG) IFN-alfa and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular caricinoma (1). In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. Earlier studies reported that HCV-specific $CD8^+$ T cell response, as examined by CTL precursor frequency, was not enhanced after IFN-alfa monotherapy (43). Furthermore, analyses of MHC class-I tetramer-positive cells in patients who underwent IFN-alfa and ribavirin therapy revealed that $CD8^+$ T cells did not increase following treatment and they were not associated with outcome (44). Combination therapy of IFN-alfa and ribavirin increases antigen-specific $CD4^+$ T cell proliferation and IFN-gamma production by $CD4^+$ T cells (45, 46). The "vigor" of the $CD4^+$ T cell response to HCV eradication is reported to be variable, something which is considered quite controversial (44).

Currently, no data is available for the involvement of innate immunity in the efficacy of IFN-alfa-based anti-HCV therapy. We thus examined whether IFN-alfa and ribavirin give a positive impact on DC capacity to induce $CD4^+$ T cell (Th1) response. By using in vitro culture system, monocyte-derived DC from chronic hepatitis C patients were impaired in the ability to drive Th1 in response to IFN-alfa. When we compared such DC capacity between patients who cleared HCV (sustained virological responders, SVR) by IFN-alfa/ribavirin therapy and those who failed to do so, impaired DC function was restored in response to IFN-alfa/ribavirin in SVR patients but not in non-SVR ones (Figure 4) (47). These results imply that DC responsiveness to anti-viral agents is restored in patients who potentially gain favorable outcomes in IFN-alfa/ribavirin therapy.

Next, we aimed to elucidate if the frequency or function of DC and innate lymphocytes is related to the outcome of pegylated IFN-alfa and ribavirin therapy. In comparison with SVR patients, non-SVR ones and transient responders (TR) showed a decline of PDC frequency from weeks 1-12 and impaired DC function at the end of treatment (Figure 5A) (48). The frequency of NK cells, as defined as $CD3^-CD56^+$ cells, in SVR patients was lower than those in TR ones (Figure 5B). In contrast, the frequency of invariant NKT cells ($Valfa24^+Vbeta11^+$ cells) did not differ between the groups in the course of the treatment (data not shown). These results show that restoration of DC function is critically involved in favorable response in pegylated IFN-alfa/ribavirin therapy. In other words, DC system could be a target of therapeutic immune modulation.

The questions remain unsolved are if impaired immune system in chronic HCV infection is restored or not by the successful HCV eradication after anti-viral therapy. Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response (49, 50), which seems to be clearly distinct from that observed in spontaneous HCV resolvers.

7. PERSPECTIVE

Protease inhibitors against HCV NS3/4A are now ready to use in clinics (51). Since they possess potent ability to suppress HCV replication, they are quite promising as an alternative approach for non-responders in PEG-IFNalfa/ribavirin therapy. In addition to that, it is anticipated that protease inhibitors are able to restore innate

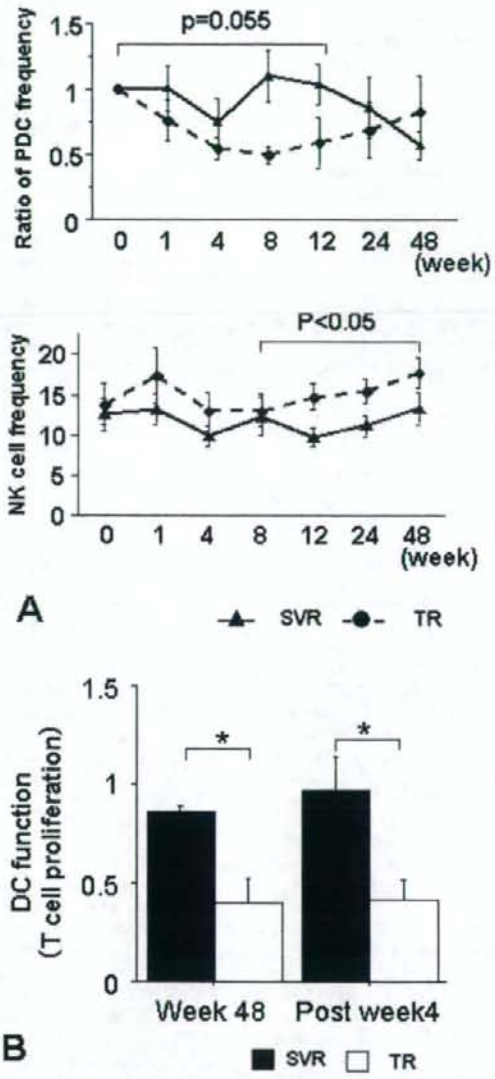

Figure 5. Early phase decline pf plasmacytoid dendritic cell frequency and sustained impairment of dendritic cell ability are related to transient response in 48-week pegylated IFN-alfa and ribavirin therapy. Frequencies and their ratios of plasmacytoid dendritic cells (PDC) and NK cells in the patients during the pegylated IFN-alfa and ribavirin therapy were determined by flow cytometric analysis. PDC were defined as Lineage-negative, HLA-DR$^+$, CD11c$^-$ and CD123^{high} cells and NK cells were as CD3-negative and CD56$^+$ cells, respectively. The results are expressed as mean $\pm$ SE. *P < 0.05 by ANOVA. At the end of treatment (Week 48) and at Week 4 after the completion of therapy, monocyte-derived DC were generated from the patients or healthy donors and their allostimulatory capacity was evaluated by mixed lymphocyte reaction (MLR). The MLR ratio between patients and controls was determined from the counts per minute of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/DC ratio of 10/1. The results are expressed as the mean $\pm$ SE of 11 SVR and 11 transient responders. SVR and TR, sustained virological responders and transient responders in 48 weeks of pegylated IFN-alfa and ribavirin therapy.*P < 0.05 by Mann-Whitney U test.

immunity by disarming NS3/4A-mediated suppression on TLR/RIG-I-dependent or -independent pathways. Therefore, extensive immunological studies on the patients treated with protease inhibitors are needed to elucidate if the therapeutic modulation of innate immunity could shape HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish HCV-specific immunotherapy are to determine the means to, direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way. Active modulation of innate immunity may be one of the strategies to gain access to the goal.

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Abbreviations: CTL, cytotoxic T lymphocytes; DC, dendritic cells; HCV, hepatitis C virus; IFN, interferon; MICA, MHC class-I related chain; MDC, myeloid dendritic cells; Mo-DC, monocyte-derived dendritic cells; NK, natural killer; PDC, plasmacytoid dendritic cells; RIG-I, retinoic acid inducible gene-I, SVR, sustained virological responders; TLR, Toll-like receptors; TCR, T cell receptor; TR, transient responders

Key Words: Dendritic cells, NK cells, NKT cells, Toll-like receptor, RIG-I, pegylated interferon-alfa, ribavirin, Review

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Impaired Cytokine Response in Myeloid Dendritic Cells in Chronic Hepatitis C Virus Infection Regardless of Enhanced Expression of Toll-Like Receptors and Retinoic Acid Inducible Gene-I

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Dendritic cells utilize various sets of Toll-like receptors (TLR) or cytosolic sensors to detect pathogens and evoke immune responses. In patients with hepatitis C virus (HCV) infection, a higher prevalence of various infectious diseases is reported; suggesting that innate immunity against pathogens is impaired. The aim of this study was to clarify whether the TLR and retinoic acid inducible gene-I (RIG-I) system in myeloid dendritic cells is preserved or not in chronic HCV infection. The expression of TLRs, RIG-I and its relatives were compared in myeloid dendritic cells between 39 patients and 52 healthy volunteers. The induction of type-I interferon (IFN) and inflammatory cytokines was examined in response to agonists for TLR2 (palmitoyl-3-cysteine-serine-lysine-4), TLR3/RIG-I (polyinosine–polycytidylic acid) or TLR4 (lipopolysaccharide). The relative expressions of TLR2, TLR4, RIG-I, and LGP2 from the patients were significantly higher than those from the volunteers, whereas TLR3 and MDA-5 expressions did not differ. In search for factors regulating TLR/RIG-I expression, it was shown that IFN-α, polyinosine–polycytidylic acid and lipopolysaccharide induced TLR3, TLR4 and RIG-I, but TNF-α, HCV core or HCV non-structural proteins did not. For the functional analyses, myeloid dendritic cells from the patients induced significantly less amounts of IFN-β, TNF-α and IL-12p70 in response to polyinosine–polycytidylic acid or lipopolysaccharide. It is noteworthy that the expression of TRIF and TRAF6, which are essential adaptor molecules transmitting TLR3 or TLR4-dependent signals, is reduced in the patients. Thus, innate cytokine responses in myeloid dendritic cells are impaired regardless of enhanced expressions of TLR2, TLR4, and RIG-I in HCV infection. ***J. Med. Virol. 80: 980–988, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; myeloid dendritic cell; innate immunity; TLR3; RIG-I

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus, which causes chronic liver disease in hosts. At primary HCV infection, approximately 80% of patients fail to eradicate HCV and eventually progress to a chronic infected state [Lauer and Walker, 2001]. It is very likely that escape mutation of the HCV genome and insufficient immune responses against HCV in hosts are involved in the persistence of infection, however, the precise mechanisms are still largely unknown. Type-I interferon (IFN) is a potent anti-viral agent that exerts its ability by suppressing viral replication or via modulating immune reactions. Gene expression analyses of HCV-infected livers obtained from chimpanzees revealed that type-I IFN and IFN-stimulated genes are highly induced even in the incubation phase [Bigger et al., 2004]. Nevertheless, HCV continues to replicate and remains at high titer levels, suggesting that HCV

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possesses some inhibitory mechanisms in IFN-inducible anti-viral responses.

As for the mechanisms of HCV persistence, the alteration or impairment of various immune cells has been reported, such as T cells, NK cells and dendritic cells [Chang et al., 2001; Wedemeyer et al., 2002; Kanto et al., 2004; Szabo and Dolganiuc, 2005]. In clear contrast with the human immunodeficiency virus, HCV does not lead to generalized immune suppression in infected hosts. Large-scale epidemiological study on US veterans revealed that the prevalence of various infectious diseases was significantly higher in HCV-positive individuals than in HCV-negative ones, including viral, bacterial, and parasite diseases [El-Serag et al., 2003]. These observations suggest that HCV infection raises the susceptibility to pathogens, not profoundly but significantly, in infected patients. However, the underlying mechanisms in the increased prevalence of infection are yet to be determined.

Toll-like receptors (TLR) are expressed in epithelial cells or antigen presenting cells and act as sensors of bacterial or viral infection. These cells utilize specific TLR for the recognition of pathogen-associated molecular patterns and eventually induce type I IFN or inflammatory cytokines. In addition to the TLR system, the existence of cytoplasmic receptors for dsRNA has been reported as virus sensors, which are retinoic acid inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) [Yoneyama et al., 2004]. Since dsRNA is a replicative intermediate of RNA virus, RIG-I and MDA-5 induce IFN-β in response to virus infection independently of TLR3. It is thus plausible that a disabled TLR/RIG-I system may be involved in the increased susceptibility to pathogens or the mechanisms of persistent virus infection [Sumpter et al., 2005]. In human hepatoma cells harboring HCV replicons, it has been shown that HCV NS3/4A protease impedes TLR3-dependent or RIG-I-dependent IFN-β induction by means of the cleavage of relevant adaptor molecules, such as TIR domain-containing adapter inducing IFN-β (TRIF) or interferon-β promoter stimulator-1 (IPS-1), respectively [Foy et al., 2005; Li et al., 2005]. However, it is not clear whether similar inhibitory machinery of HCV operates or not in immune cells, such as dendritic cells.

Dendritic cells are immune sentinels that play a central role against pathogens in inducing innate as well as adaptive immune responses. Dendritic cells consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of immune responses. Dendritic cells utilize various sets of TLR or RIG-I/MDA-5 to sense virus infection. After the recognition, dendritic cells begin to mature and gain the ability to produce type-I IFN and inflammatory cytokines. It has been reported that blood dendritic cells expresses distinct profiles of TLRs; human myeloid dendritic cells express TLR2, -3, -4, -5, -6, -7, and -8, while plasmacytoid dendritic cells express TLR7, -8 and -9 [Iwasaki and Medzhitov, 2004]. Numerical and/or functional impairment of blood dendritic cells in acute or chronic HCV infection has been reported by several investigators including us [Kanto et al., 2004; Szabo and Dolganiuc, 2005]. One of the plausible mechanisms leading to dendritic cells impairment may be direct HCV infection to blood dendritic cells or their precursors. In support for this, it was shown that myeloid dendritic cells are susceptible to HCV infection, judging from the results of an inoculation study with pseudo-HCV particles or detection of negative strand HCV-RNA [Kaimori et al., 2004]. According to another report, myeloid dendritic cells displayed impaired expression of IL-12 and TNF-α in response to poly-inosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) in patients with a large amount of cell-associated HCV [Rodrigue-Gervais et al., 2007], suggesting a possible link between direct HCV infection to myeloid dendritic cells and an impaired innate response.

Taking these reports into consideration, the current study focused on myeloid dendritic cells in order to clarify the roles of the TLR/RIG-I system in HCV infection, by comparing the expression of TLR, RIG-I, and MDA-5 and the induction of cytokines in response to specific agonists for these virus sensors. The study demonstrated that myeloid dendritic cells from HCV-infected patients induces a significantly lesser amount of cytokines in spite of enhanced expressions of TLR2, TLR4, and RIG-I. These findings imply that alteration of the TLR/RIG-I system is instrumental in impairment of innate immunity in HCV infection, where myeloid dendritic cells play a key role as immune sentinels against pathogens.

MATERIALS AND METHODS

Subjects

Thirty-nine patients (male/female: 22/17, mean age: 53.4 ± 10.3 years old, mean serum ALT levels: 93.9 ± 51.0 IU/L, HCV serotype 1/serotype 2: 39/0) with chronic hepatitis C (HCV group) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. All of them were confirmed to be positive for both serum anti-HCV antibody and HCV RNA (mean HCV RNA quantity assayed by Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics, Tokyo, Japan; [Pawlotsky et al., 2000]: $1{,}637 \pm 402$ KIU/ml) but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The presence of other liver diseases, such as alcoholic, metabolic or autoimmune hepatitis, was ruled out. Thirteen patients with chronic HBV infection determined by serum HBsAg-positive and ALT abnormality (male/female: 6/7, HBeAg+/HBeAg−: 7/6, mean age: 45.9 ± 14.4 years old, mean serum ALT levels: 95.2 ± 145 IU/L, mean HBV-DNA levels assayed by Cobas Amplicor HBV monitor Roche Diagnostics; [Noborg et al., 1999]: 6.1 ± 1.7 $\log_{10}$ copies/ml) were also enrolled as disease controls (HBV group). The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each patient. The

controls were 52 healthy volunteers or blood donors (healthy donors group) at the Osaka Red Cross Blood Center (Osaka, Japan), who were confirmed to be negative for HCV, HBV, and HIV. The background data of the blood donors were not accessible due to the confidentiality regulations of the blood center, but their serum ALT levels were confirmed to be within the normal range.

Reagents

Palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) was purchased from InvivoGen (San Diego, CA). Polyinosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Recombinant human IL-6, IL-10, and IL-12 were purchased from InvivoGen. Recombinant TNF-α was purchased from Genzyme (Framingham, MA). Recombinant HCV structural or non-structural (NS) proteins expressed by *E. coli* were purchased from Virogen (Watertown, MA). They were HCV core (amino acid positions, from 2 to 192), NS3 (from 1,450 to 1,643), and NS4 (from 1,658 to 1,863), respectively. HCV NS5B protein (from 2,421 to 2,965) was kindly provided by Japan Tobacco Corp. (Tokyo, Japan). Natural human interferon-α was purchased from Otsuka Pharmaceutical Co. (Tokyo, Japan).

Isolation of Myeloid Dendritic Cells

Peripheral blood mononuclear cells were isolated from heparinized venous blood by centrifugation on Ficoll-Hypaque cushion as described previously [Kanto et al., 2004]. Myeloid dendritic cells were magnetically isolated using a BDCA-1 Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of myeloid dendritic cells (Lineage-negative, HLA-DR^+, $CD11c^+$, and $CD123^{dim+}$ cells) was more than 95% as assessed by FACS (data not shown). Short-term culture of myeloid dendritic cells was performed in cytokine-free Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid at 37°C in 5% CO_2.

To clarify the factors influencing the expressions of TLR or RIG-I in myeloid dendritic cells, fresh myeloid dendritic cells obtained from uninfected controls were incubated for 2 hr in the presence or absence of various cytokines, agonists for TLR/RIG-I or recombinant HCV proteins. After the incubation, they were subjected to RT-PCR analyses for the comparison.

In order to compare the function of TLR/RIG-I-mediated responses in myeloid dendritic cells between the groups, myeloid dendritic cells were incubated with various agonists for 2 hr and subjected them to cytokine analysis by RT-PCR. Alternatively, myeloid dendritic cells were cultured in the presence or absence of 25 μg/ml of polyI:C for 24 hr and collected supernatants for subsequent cytokine analyses.

Flowcytometric Analysis

The phenotypes of myeloid dendritic cells were analyzed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA). For the staining, myeloid dendritic cells were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline (PBS) containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, or APC-conjugated anti-human monoclonal antibodies were used: CD11c (clone, B-ly6), HLA-DR (L243), CD80 (L307.4), CD86 (IT2.2), CD40 (5C3), and CD83 (HB15e). All were purchased from BD Biosciences.

Real-Time Quantitative PCR

Total RNA was extracted from more than 10^6 myeloid dendritic cells using RNeasy Mini kit (Qiagen, Hilden, Germany), which was subsequently reverse transcribed in 20 μl volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4, RIG-I, MDA-5, LGP2, myeloid differentiation factor 88 (MyD88), IPS-1, TRIF, TNF receptor associated factor 6 (TRAF6), TNF-α and IFN-β, ready-to-use assays (Taqman Gene Expression Assays, Applied Biosystems) were utilized, according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A calibrator sample from healthy volunteers was identified. The expressions of molecule were expressed as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, β-actin mRNA from each sample was quantified as a control of internal RNA and corrected all values with this.

Enzyme-Linked Immunosorbent Assay and Cytokine Beads Assay

The quantity of IFN-α in culture supernatants was evaluated using Human Interferon Alpha ELISA kit (PBL Biomedical Laboratories, New Brunswick, NJ) according to the manufacturer's instructions. The concentration of TNF-α, IL-6, and IL-12p70 in the supernatants was assayed by the use of BD cytokine beads assay (CBA) Flex Sets (BD Biosciences) and analyzed by FACS Calibur according to the manufacture's instructions. The detection limits of IFN-α, TNF-α, IL-6, and IL-12p70 are 10–5,000 pg/ml, respectively.

Statistical Analysis

The Mann–Whitney *U*-test was performed to evaluate differences among the groups using StatView

5.0 software (SAS Institute, Cary, NC). A P-value of <0.05 was considered to be statistically significant.

RESULTS

Expressions of TLR2, TLR4, and RIG-I Were Higher in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

With respect to the phenotypes of fresh myeloid dendritic cells, the expressions of maturation markers such as CD40, CD80, CD83, and CD86 were relatively low and were not different between the HCV group and healthy donor group (Fig. 1). The similar results were obtained from HBV group (data not shown). These results show that myeloid dendritic cells from all groups are equally immature phenotypes.

First, the expressions of TLR2, TLR3, and TLR4 in myeloid dendritic cells were examined. The relative amounts of TLR2 and TLR4 in the HCV group were higher than those in healthy donors or the HBV group (Fig. 2). In contrast, the TLR3 expression was not different among the groups (Fig. 2). In comparison between HBV and healthy donor groups, there was no difference in the expressions of these TLRs in myeloid dendritic cells (Fig. 2).

The expression of cytoplasmic receptors for dsRNA in myeloid dendritic cells was also compared. The RIG-I and LGP2 expression in the HCV or the HBV group was significantly higher than those from healthy donors, whereas MDA-5 did not differ among the groups (Fig. 2). No correlation was found among the expressions of any TLR and dsRNA receptors (data not shown).

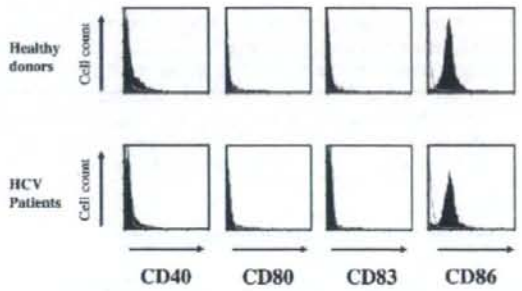

Fig. 1. Fresh myeloid dendritic cells are immature regardless of HCV infection. Myeloid dendritic cells were obtained from HCV-infective patients or healthy donors and their expressions of CD40, CD80, CD83, and CD86 were analyzed by flow cytometry. The shaded histograms are the results with specific Abs, while the open ones are those with isotype Abs. Representative results from five HCV-infected patients and five controls are shown.

IFN-α or PolyI:C Enhanced RIG-I Expression in Myeloid Dendritic Cells

To clarify the factors influencing TLR2, 3, 4, or RIG-I expression in myeloid dendritic cells, it was examined

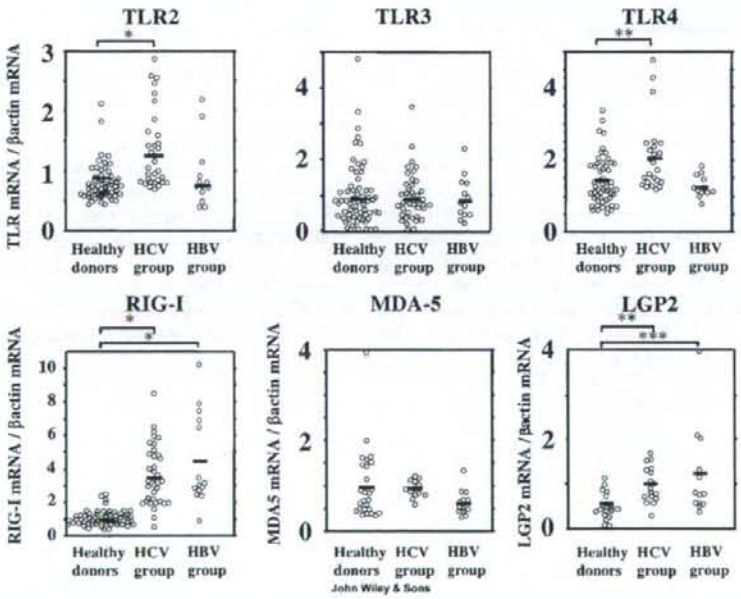

Fig. 2. Expressions of TLR2, TLR4, RIG-I, and LGP2 in patient myeloid dendritic cells from HCV-infected patients are higher than those from healthy donors, while TLR3 and MDA-5 are comparable. Expressions of TLR2, TLR3, TLR4, RIG-I, MDA-5, and LGP2 in myeloid dendritic cells were quantified by real-time RT-PCR as described in Materials and Methods Section. Horizontal bars represent the median. The statistical difference was evaluated by the Mann–Whitney U-test. $^{*}P < 0.0001$, $^{**}P < 0.0005$, $^{***}P < 0.005$.

whether they correlated with clinical parameters, such as age, serum ALT, HCV-RNA, and HBV-DNA titers. No correlation was found between any of these markers and TLR2, TLR3, TLR4, or RIG-I expressions (data not shown). Therefore, the degree of expression of these sensors is not involved in the control of virus replication or liver inflammation. Their expressions in myeloid dendritic cells cultured with and without various reagents were compared. The ratio of the quantity was determined between samples with and without treatments and their positive induction was defined as more than 2.0. The kinetics of agonist-induced TLR2, TLR3, TLR4, or RIG-I expression were preliminarily examined in myeloid dendritic cells recovered from volunteers or patients. It was found that they showed a peak at 2 hr after the stimulation, which were the same either they were HCV-infected or not (data not shown). Thus, in the following experiments, cells were obtained at this point and subsequently analyzed transcripts of target genes.

In the present study, IFN-α significantly enhanced RIG-I expression in myeloid dendritic cells (Fig. 3A). A similar effect of IFN-α was observed in TLR3 and TLR4 expression, although at much lesser degrees than those of RIG-I. In chronic hepatitis C patients, serum levels of IL-6, TNF-α, or IL-10 have been reported to be higher than those in uninfected individuals, suggesting their roles in the pathogenesis of HCV infection [Spanakis et al., 2002]. However, the addition of these cytokines or IL-12 to myeloid dendritic cell did not influence TLR or RIG-I expression (Fig. 3B). As for TLR agonists, polyI:C or LPS significantly enhanced RIG-I expression, but only slightly enhanced TLR4 (Fig. 3B). TLR2 agonist Pam_3CSK_4 did not influence the levels of TLR and RIG-I (Fig. 3B). None of the HCV proteins had a positive impact on TLR2, TLR3, TLR4, and RIG-I expressions (Fig. 3B).

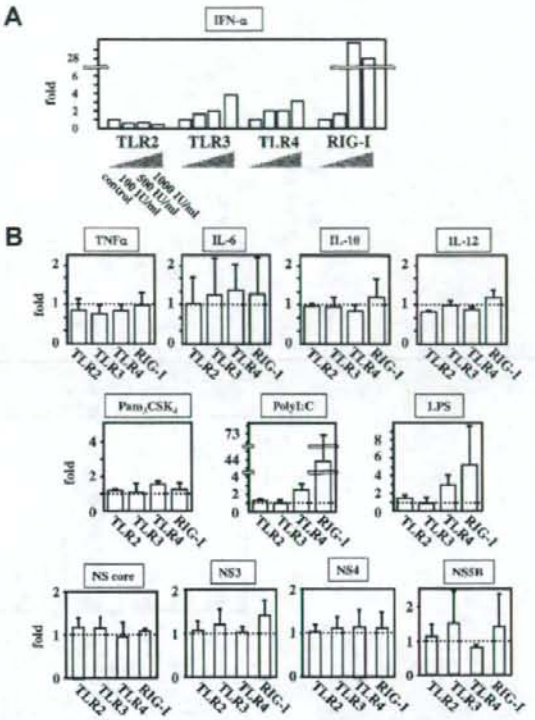

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials and Methods Section. Bars represent the mean fold increase of relevant transcripts to those of each control. Representative results from three donors are shown. **B**: Changes of TLR2, TLR3, TLR4, and RIG-I expression in myeloid dendritic cells were examined by the addition of various cytokines, TLR agonists or recombinant HCV proteins as described in Materials and Methods Section. The fold increase was determined by the ratio of each transcript of samples with reagents to those without and expressed as the mean + SEM. The concentration of reagents were 10 ng/ml of TNF-α or IL-6, 20 ng/ml of IL-10, 200 pg/ml of IL-12, 100 ng/ml of Pam3CSK4, 25 μg/ml of polyI:C, 100 ng/ml of LPS and 2.5 μg/ml each of HCV core, NS3, NS4, and NS5B. Representative results from five donors are shown.

Induction of IFN-β, TNF-α, and IL-12 p70 With TLR Agonists Is Impaired in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

First, IFN-β and TNF-α expression were examined in myeloid dendritic cells as representatives in response to specific agonists. Since the expression of these genes in myeloid dendritic cell showed a peak at 2 hr after the stimulation either they were from donors or patients (Fig. 4A), samples were collected at this point. In myeloid dendritic cells stimulated with polyI:C, IFN-β was significantly induced in the HCV, the HBV, and healthy donor groups (Fig. 4B). However, their expression from HCV or HBV-infected patients was significantly lower than that from healthy donors (Fig. 4B). Agonists for TLR3 or TLR4 significantly stimulated myeloid dendritic cells to induce TNF-α regardless of HCV or HBV infection. As the same IFN-β, TNF-α induction in myeloid dendritic cells stimulated with polyI:C or LPS was lower in the HCV or the HBV group (Fig. 4B). Therefore, in myeloid dendritic cells from hepatitis C patients, in spite of higher expression of TLR2, TLR4, and RIG-I, their levels of agonist-induced IFN-β and TNF-α were less than those in healthy donors.

To compare more precisely the cytokine response in myeloid dendritic cell between HCV-infected patients and donors, the levels of IFN-α, TNF-α, IL-6, and IL-12 p70 in supernatants were examined. Since the induction of IFN-β and TNF-α in myeloid dendritic cell was profound in the presence of polyI:C, samples were collected from myeloid dendritic cells stimulated with polyI:C. The levels of IFN-α and IL-6 were not different between the groups (Fig. 4C). In contrast, the amounts of TNF-α and IL-12 p70 from patients group were significantly lower than those from the donor group (Fig. 4C). These results suggest that some inhibitory

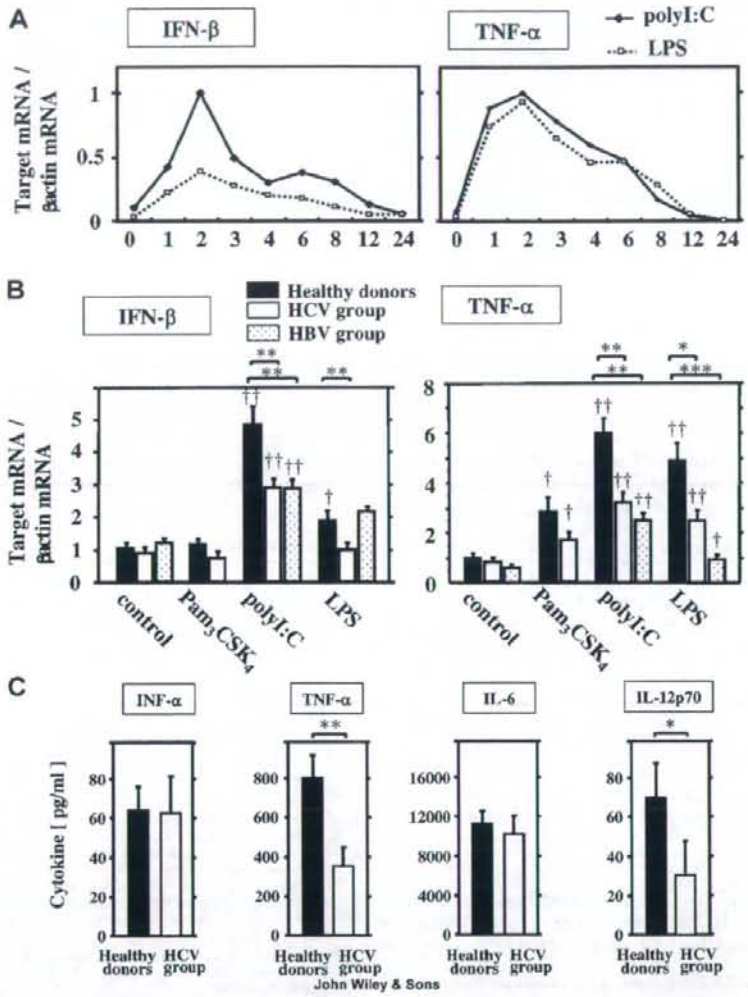

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by real-time RT-PCR as described in Materials and Methods Section. At several time points before and after the stimulation of myeloid dendritic cell with 25 μg/ml of poly I:C or 100 ng/ml of LPS, the samples were subjected to RT-PCR analyses. The results are expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. Representative results from three healthy donors are shown. **B**: Expressions of IFN-β and TNF-α in myeloid dendritic cells stimulated with various TLR agonists were quantified by real-time RT-PCR as described in Materials and Methods Section. Two hours after the stimulation of myeloid dendritic cells with Pam_3CSK_4, polyI:C or LPS, the samples were subjected to RT-PCR analyses. The results were expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. The concentrations of agonists were 100 ng/ml of Pam_3CSK_4, 25 μg/ml of polyI:C and 100 ng/ml of LPS. The bars represent mean + SEM. $^{\dagger}P < 0.05$ vs. control, $^{\dagger\dagger}P < 0.01$ versus control, $^{*}P < 0.05$ versus healthy donors, $^{**}P < 0.01$ versus healthy donors, $^{***}P < 0.001$ versus healthy donors. Representative results from 14 HCV-infected patients, 13 HBV-infected patients and 25 controls are shown. Statistical differences were evaluated by the Mann–Whitney *U*-test. **C**: Myeloid dendritic cells in both groups were stimulated with polyI:C for 24 hr. The supernatants were collected and the levels of IFN-α, TNF-α, IL-6, and IL-12p70 were examined by ELISA or cytokine beads assay as described in Materials and Methods Section. The bars represent mean + SEM. Statistical differences were evaluated by the Mann–Whitney *U*-test. Representative results from 11 HCV-infected patients and 17 controls are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

mechanisms exist downstream of TLR or RIG-I in myeloid dendritic cells from the HCV-infected patients.

Expressions of TRIF and TRAF6 Were Lower in Myeloid Dendritic Cells From the HCV-Infected Patients

In order to seek the inhibitory mechanisms of TLR or RIG-I signaling in myeloid dendritic cells, the expressions of adapter molecules, MyD88, IPS-1, TRIF, or TRAF6 were compared between the HCV and donor groups. The expressions of MyD88 and IPS-1 were higher in myeloid dendritic cells from the HCV group (Fig. 5). By contrast, the levels of TRIF and TRAF6 in myeloid dendritic cells from HCV-infected patients were significantly lower than in those from healthy counterparts (Fig. 5).

DISCUSSION

The present study demonstrated that myeloid dendritic cells from HCV-infected patients express higher levels of TLR2, TLR4, and RIG-I than those from healthy subjects. Regardless of such enhanced expression, specific agonists stimulated patient myeloid dendritic cells to induce lesser degrees of IFN-β/TNF-α/IL-12 than those from the healthy counterparts. Two conclusions were reached from the current study findings: HCV enhances expression of some TLR and RIG-I in myeloid dendritic cells, but HCV impedes TLR or RIG-I-mediated cytokine responses in them. Since dendritic cells play a role as immune sentinels, such impaired cytokine response in myeloid dendritic cell may be one of the mechanisms in enhanced susceptibility to various pathogens in HCV-infected individuals as reported elsewhere [El-Serag et al., 2003].

It has been reported that TLRs are expressed in epithelial cells and immune cells, and RIG-I is ubiquitously expressed in various cells [Yoneyama et al., 2004]. However, it remains obscure how their expressions are regulated. It is generally accepted that TLR3 and RIG-I are inducible by type-I IFN [Doyle et al., 2003; Yoneyama et al., 2004]. The current study confirmed this phenomenon also in myeloid dendritic cells, since IFN-α up-regulated TLR3, TLR4, and RIG-I expression in a dose-dependent manner. Gene expression analyses revealed that HCV infection induces type-I IFN and IFN-stimulated genes in HCV-infected liver from chimpanzees or humans [Bigger et al., 2004]. One of the triggers leading to IFN production is the presence of double-strand RNA in infected tissues, which is a replicative intermediate of HCV. The current study also showed that polyI:C is a prominent inducer of RIG-I and TLR4. Since polyI:C is a synthetic mimic of double-strand RNA, its positive impact suggests that HCV replication in myeloid dendritic cells and/or subsequent IFN production may be involved in RIG-I or TLR4 induction.

Several investigators have reported that TLR2, TLR3, or TLR4 expression is enhanced in monocytes or B cells obtained from chronic hepatitis C patients, both of which are known to be susceptible to HCV [Machida et al., 2006; Riordan et al., 2006]. Regardless of the difference in cell types, the present study offers support for the enhanced TLR2 and TLR4 expression in HCV infection described by these reports. As for the mechanisms, TNF-α or HCV NS5A has been reported to be involved in TLR2 or TLR4 up-regulation [Machida et al., 2006]. However, in this study, addition of recombinant TNF-α or the HCV proteins failed to induce any TLR or RIG-I in

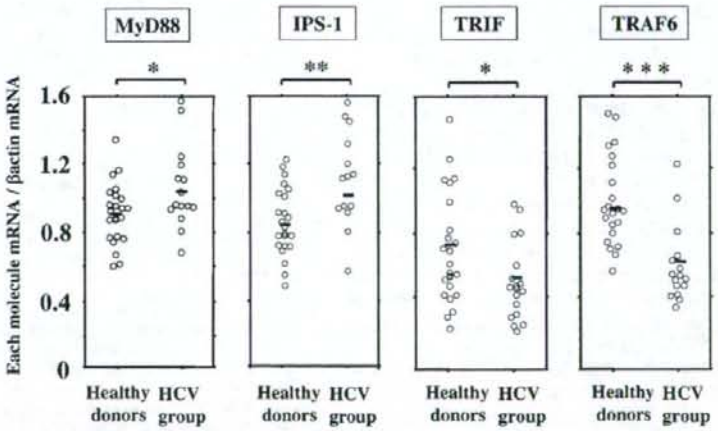

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed as the ratio of each transcript to those of β-actin. Horizontal bars represent the median. Statistical differences were evaluated by the Mann–Whitney *U*-test. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

myeloid dendritic cells. Therefore, enhanced expressions of TLR2, TLR4, and RIG-I in myeloid dendritic cells may be due to, not completely but in some part, the existence of HCV in cells or the exposure to endogenous IFN-α. To check this, it may be necessary to conduct studies with inoculation of HCV particles or transduction of the viral genome in myeloid dendritic cells.

In comparison of the results between the HCV and the HBV groups, the expressions of TLR2 and TLR4 in the HBV group were comparable with those from healthy donor group, suggesting that the induction of TLR2 and TLR4 in myeloid dendritic cells is unique in HCV infection. In contrast, the levels of RIG-I and LGP2 were comparable between the HCV and the HBV groups, both of which were higher than those from healthy donors. These results raise the possibility that, regardless of the difference of hepatitis virus, similar mechanisms may be involved in the induction of RIG-I and LGP2 in myeloid dendritic cells.In cells bearing HCV replicons, it has been reported that HCV NS3/4A inhibits TLR3 or RIG-I-mediated IFN-β induction by the cleavage of relevant adaptor molecules TRIF or IPS-1, respectively [Foy et al., 2005; Li et al., 2005]. In the present study, in myeloid dendritic cells from the HCV group, polyI:C-stimulated IFN-β, TNF-α, and IL-12 p70 induction is impaired. As for the adaptor molecules in TLR-dependent signals, TRIF and TRAF6 expression was lower in HCV-infected patients than those in healthy donors. Since it has been proven that the cleavage of TRIF hampers TLR3-mediated IFN production [Fitzgerald et al., 2003], the current study implies that lower expression of TRIF is involved in the inhibition of TLR3 or TLR4-mediated signals in myeloid dendritic cells. Of particular interest is the possibility that such reduction of TRIF and TRAF6 in myeloid dendritic cells is caused by the cleavage by NS3/4A, as shown in hepatoma cells [Foy et al., 2005; Li et al., 2005]. If this does occur, the inhibitor of NS3/4A serine protease may be able to restore TLR-dependent innate responses in myeloid dendritic cells, in addition to its potent suppressive ability of HCV replication. Machida et al. reported that enhanced expression of TLR4 in HCV-infected B cells is related to the TLR4-dependent up-regulation of IFN-β and IL-6, suggesting that TLR4-dependent signals are not impaired in B cells [Machida et al., 2006]. Further study is necessary to reveal whether HCV does actually influence innate immunity according to differences in blood cell types. In the current study, polyI:C or LPS-stimulated myeloid dendritic cells from HBV-infected patients induced lesser degree of IFN-β or TNF-α, respectively. Several investigators reported that the function of blood dendritic cells in HBV-infected patients were impaired [Tavakoli et al., 2004; van der Molen et al., 2004]. It is yet to be determined whether HBV infects to myeloid dendritic cells or not. The current study raises the possibility that distinct mechanisms are involved in the impairment of TLR or RIG-I pathway according to the difference of virus. Further study depending on expression as well as functional assay of virus recognition system in HBV infection is needed to clarify these important issues.

In contrast with RIG-I and LGP2, MDA-5 expression in myeloid dendritic cells from HCV-infected patients was comparable with that from healthy donors, suggesting that these cytosolic RNA sensors are regulated independently. Recently, it has been reported that RIG-I is expected to be involved in the detection of Flaviviridae, which HCV belong to, but MDA-5 is not [Hornung et al., 2006]. Active involvement of RIG-I in HCV infection has been reported, demonstrating that RIG-I, but not MDA-5, efficiently binds to secondary structured HCV RNA to confer induction of IFN-β [Saito et al., 2007]. In this study, although the polyI:C-stimulated cytokine response in patient myeloid dendritic cells was impeded, IPS-1 expression was higher than that in myeloid dendritic cells from the healthy donor group, suggesting a lesser possibility of IPS-1 as a cleavage target of HCV in myeloid dendritic cells. Alternatively, higher expression of LGP2 may contribute to the inhibitory machinery against RIG-I-mediated responses in myeloid dendritic cells, as reported elsewhere [Saito et al., 2007].

In summary, in myeloid dendritic cells from HCV-infected patients, innate cytokine responses were impaired regardless of the enhanced expressions of TLR2, TLR4, and RIG-I. These findings provide insights into the roles of the TLR/RIG-I system in the pathogenesis of HCV infection and their potentials as therapeutic targets for immune modulation.

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Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

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Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial emboli-zation therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

MHC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.[1] In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.[2–5] The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.[6] Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.[7] In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.[8–10] The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.[4] In addition, sera from those patients can downregulate NKG2D expression *in vitro*.[5,11] These data suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.[12,13]

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.[14,15] With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.[16] We reported previously that soluble MICA could be detected in sera of HCC patients.[17] However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

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Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

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Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH		0/5/	2/1/	4/0/
AIH/PBC/others		2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.
*$P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2-test; **$P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; ***$P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; ****$P < 0.05$ vs cirrhosis by χ^2-test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.[18] Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.[16]

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparafinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the α1 and α2 domains of MIC molecules shared by both MICA and MICB.[2] To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t-test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

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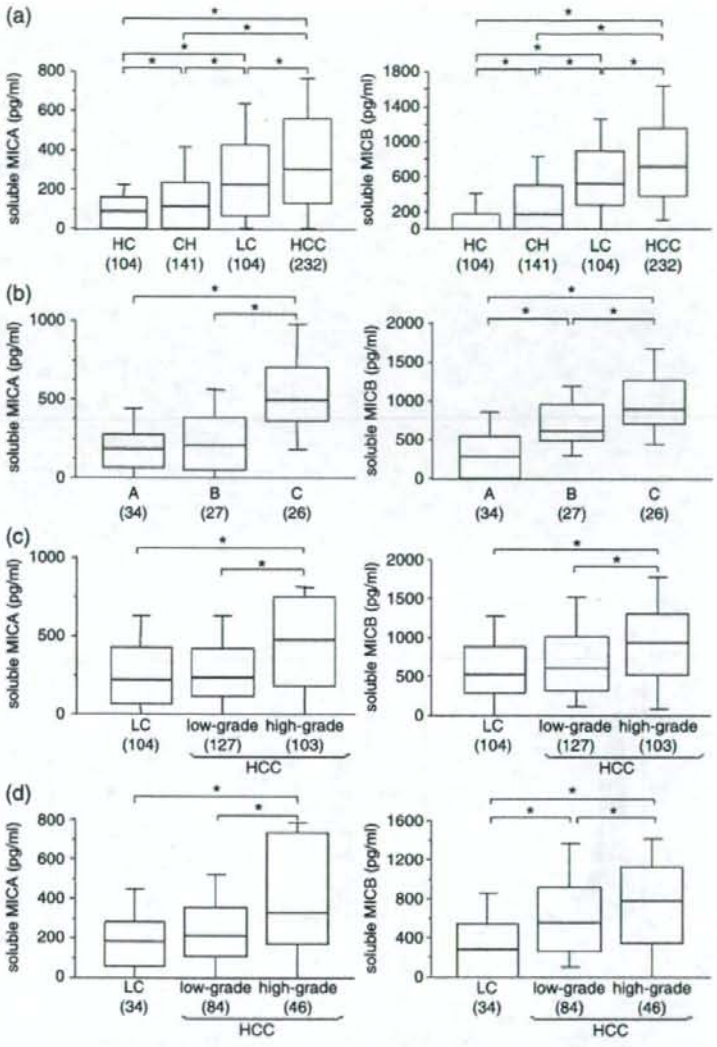

Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child–Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child–Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. $^*P < 0.05$ by Kruskal–Wallis test and post hoc Steel–Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhosis patients were stratified based on Child–Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child–Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

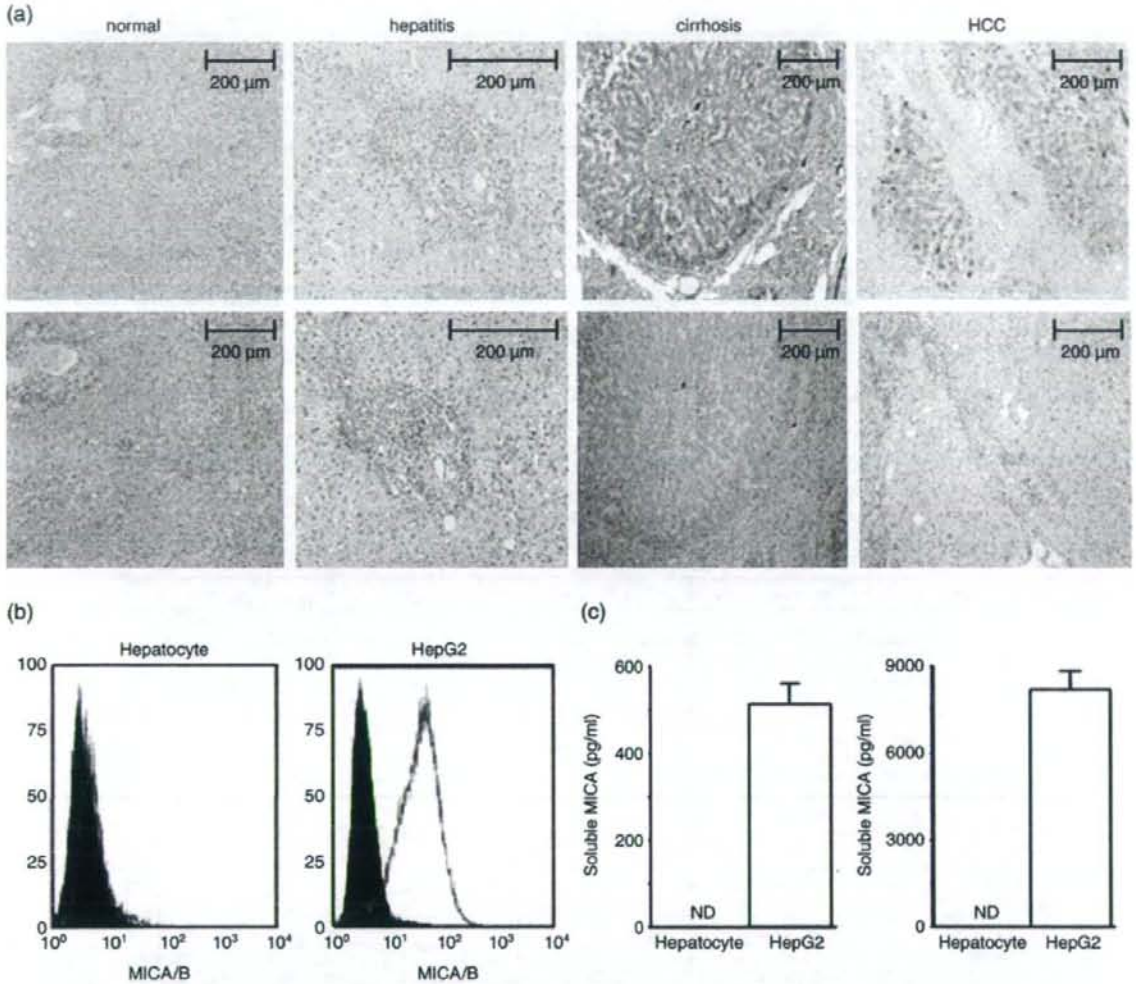

Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MICB released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.[3] Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

Downregulation of soluble MICA levels by TAE. The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

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