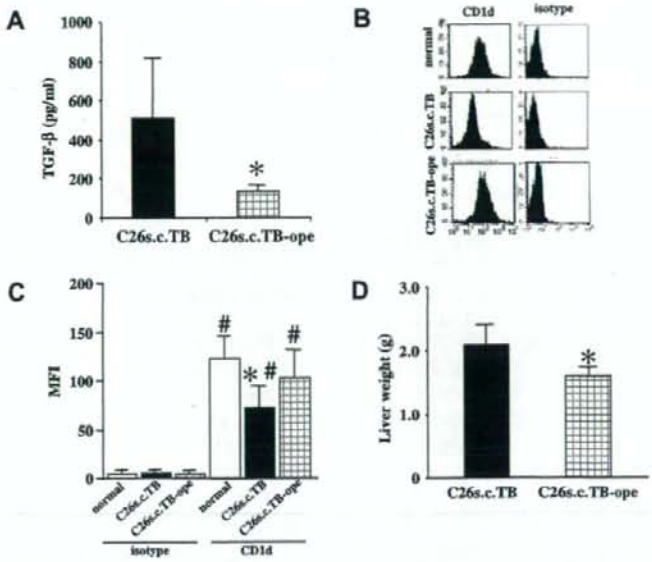

Fig. 6. Evaluation of serum TGF-β and CD1d expression on liver DCs and the antitumor effect of α-GalCer against metastatic liver tumor in surgical treated C26s.c.TB-mice. At 42 days, Colon26 subcutaneous tumors in C26s.c.TB-mice were surgically excised. Fourteen days later, liver DCs from surgically treated mice were prepared for comparison with liver DCs isolated from 42-day C26s.c.TB-mice. (A) Mice sera from C26s.c.TB-mice (C26s.c.TB) or surgically treated C26s.c.TB-mice (C26s.c.TB-ope) were harvested and were subjected to mouse TGF-β ELISA. Cytokine levels are reported in pg/ml (mean ± SD of triplicate samples). $^{*}p < 0.05$. (B, C) The expressions of CD1d on liver DCs from C26s.c.TB-mice (C26s.c.TB) or surgically treated C26s.c.TB-mice (C26s.c.TB-ope) were evaluated by flow cytometry. The representative flow cytometry data of CD1d expressions on liver DC were shown in Fig. 6B. The expression levels of CD1d molecules are reported as arbitrary MFI (mean ± SD of triplicate samples, Fig. 6C). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD1d expression in normal mice. (D) C26s.c.TB-ope mice or C26s.c.TB-mice were injected into spleen with 5×10^5 Colon26 cells, and 24 h later α-GalCer was administered intraperitoneally ($N = 4$ in each group). Ten days after treatment, the livers were removed from treated mice and the liver weights of the groups were compared. $^{*}p < 0.05$. α-GalCer treated C26s.c.TB-ope mice vs α-GalCer treated C26s.c.TB-mice.

patients with advanced cancer, and encouraged us to investigate the detailed mechanism of the markedly reduced antitumor effect of α-GalCer in TB-mice to establish better α-GalCer treatment for cancer patients.

DCs have been implicated in the activation of NKT and NK cells in both mice and humans [1,6,12–17]. α-GalCer presented by CD1d molecules expressed on DCs activates NKT cells via recognition between CD1d molecules and Vα14–Jα281 invariant antigen receptor in mice [18]. Thus the expression of CD1d molecules on DCs is believed to be important for activation of NKT cells. Our study demonstrated that CD1d expressions on bone marrow-derived DCs were similar between normal and C26s.c.TB-mice, suggesting that the ability of differentiating DCs from precursor cells in bone marrow were same in both normal and C26s.c.TB-mice. In contrast, the CD1d expressions of liver DCs and spleen DCs in C26s.c.TB-mice were lower than those in normal mice. This is not unique to C26s.c.TB-mice, because decreased expression of CD1d molecules on liver DCs (not bone marrow-derived DCs) was also observed in CMS4 mouse sarcoma or BNL mouse hepatoma TB-mice (Tatsumi, unpublished data). These results suggested that some systemic immunosuppressive factors might modify the CD1d expression on DCs in TB-mice. Osman et al. demonstrated that α-GalCer administration resulted in activation of liver NKT cells with significant early disappearance of liver NKT cells in normal mice [19]. They also demonstrated that these phenomenon were not observed in CD1d(−/−) mice, suggesting that CD1d expressions play essential roles of liver NKT activation [19]. In our study, the early decreases of liver NKT cells were not observed after α-GalCer treatment in C26s.c.TB-mice. Based on these observations, the decreased expression of CD1d molecules on DCs might be associated with the impaired activation of liver innate immunity, thus resulting in an impaired antitumor effect of α-GalCer.

A normal mice liver contains lymphocytes that are usually enriched with NK and NKT cells; i.e., 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells

[20,21]. Efficient activation of abundant NKT cells and NK cells in the liver might be important in an antitumor effect against liver tumor. We and others have previously reported that sequential activation of both NKT cells and NK cells could be observed in the liver after α-GalCer administration. Although most NKT cells had disappeared from the liver within 12 h of α-GalCer administration [4,19], the antitumor effect against disseminated liver tumor depends on NK cells in the α-GalCer treatment, evidenced by that depletion of NK cells abolished the anti-metastatic tumor effect [4]. In the present study, we found the impairment of both the cytolytic activity of NK cells and an increase of the NK cell proportion in whole liver MNC in α-GalCer-treated C26s.c.TB-mice. These findings also offer the evidence that insufficient activation of liver NK cells might be associated with a poor antitumor effect of α-GalCer in TB-mice. The expressions of antigen-presenting related molecules, CD80 and CD86, on liver DCs in C26s.c.TB-mice were also lower than those in normal mice. Taken together, the presence of a tumor mass might modify the innate immune response in the liver and the maturation of liver DCs in TB-mice.

Several previous reports have demonstrated that TGF-β and IL-10 inhibit CD1d expression on DCs [10,11]. We hypothesize that the decreased expressions of CD1d might be associated with these immunosuppressive cytokines derived from the tumor mass. Our study demonstrated that Colon26 cells produce a large amount of TGF-β, but not IL-10, and that serum TGF-β level in C26s.c.TB-mice was significantly higher than that in normal mice, while the serum IL-10 level was not. Our results suggested that tumor-derived TGF-β might decrease CD1d expressions on liver DCs in C26s.c.TB-mice. Biswas et al. demonstrated that administration of anti-TGF-β neutralizing antibody inhibited metastatic cancer [22], suggesting that if the tumor-derived TGF-β had decreased in TB-mice, the liver immunological environment might be improved to develop antitumor immunity. Based on these results, we next examined serum TGF-β levels and the CD1d expression on liver DCs after surgical subcutaneous mass resection. Fourteen days after surgical resection, serum TGF-β in treated C26s.c.TB-mice had significantly decreased and the expressions of CD1d on liver DCs from treated C26s.c.TB-mice had significantly increased and recovered to the level of normal mice, suggesting that Colon26 tumor tissue derived TGF-β might modify the CD1d expression on liver DCs. More importantly, we demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was significantly improved in C26s.c.TB-ope mice. We believe that if complete resection of primary tumor could be achieved, the liver immune microenvironment might be expected to recover dramatically and cancer immunotherapy using α-GalCer might lead to better outcomes.

de Lalla et al. reported that the human invariant NKT cells are significantly enriched in chronically inflamed livers as compared with noninflamed ones although human liver harbors significantly less invariant NKT cells than the mouse one [23], suggesting that human invariant NKT cells might also play important roles in developing the chronic liver disease. Although the frequency of invariant Vα24 NKT cells is very low in humans, Vα24 NKT cells can be expanded by the stimulation of α-GalCer in cancer patients [7]. These suggested that the effector function of invariant NKT cells in human liver might be important for the establishing of new cancer treatments of α-GalCer.

The liver is the most common site of metastasis of gastrointestinal cancers (i.e., colorectal cancer, gastric cancer and pancreatic cancer). Thus, new therapeutic approaches of cancer immunotherapy for advanced liver tumor need to be developed. Our report is the first report demonstrating that the presence of a tumor mass might inhibit the activation of liver innate immune cells by α-GalCer due to decreased expression of CD1d on liver DCs. These findings indicate that α-GalCer treatment may represent a promising approach to preventing liver metastasis if the primary tumor can be completely controlled.

Acknowledgements

The authors thank Kirin Pharma Co. Ltd. (Gunma, Japan) for providing the α-galactosylceramide. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan and Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Agency.

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[Frontiers in Bioscience 13, 6183-6192, May 1, 2008]

Virus associated innate immunity in liver

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TABLE OF CONTENTS

1. ABSTRACT

Dendritic cells (DCs) sense virus via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I) and evoke a cascade of immune reactions. In myeloid DC (MDC) from hepatitis C virus (HCV)-infected patients, the levels of TLR/RIG-I-mediated IFN-beta or TNF-alfa induction are lower than those in uninfected donors, suggesting that their signal transduction in MDC is impaired. Dendritic cells in HCV infection are unresponsive to interferon (IFN)-alfa, thus failing to enhance MHC class-I related chain A/B and subsequent NK cell activation. Alternatively, NK cells from the patients down-regulate DC in the presence of HLA-E-expressing hepatocytes by secreting IL-10 and TGF-beta1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of NKG2A/CD94. Activated NKT cells from the patients produce higher levels of IL-13 but comparable IFN-gamma with those from controls, showing their bias to Th2-type. In pegylated IFN-alfa/ribavirin therapy for chronic hepatitis C, improved DC function is related with successful HCV eradication. In conclusion, cross-talks among DCs and innate lymphocytes are critical in shaping immune response against HCV, either spontaneously or therapeutically.

2. INTRODUCTION

Hepatitis C virus (HCV) is one of major causes of chronic liver disease worldwide. HCV is hepatotrophic, but not directly cytopathic and elicit progressive liver injuries resulting in end-stage liver disease unless effectively eradicated (1). Epidemiological studies have revealed that more than 80% of acutely HCV-infected patients fail to eradicate the virus and they subsequently develop chronic hepatitis (1). It has been proposed that the ability of infected hosts to mount vigorous and sustained cellular immune reactions to HCV is necessary for control in primary infection (2). Once HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection. First, the virus alters its antigenic epitopes recognized by T cells and neutralizing antibodies to escape immune surveillance. Second, HCV also subverts immune functions in an antigen-specific manner, from innate to adaptive immunity (3).

Cumulative reports have shown that innate immune system dictates the direction and magnitude of subsequent adaptive immune response. It is generally accepted that HCV-specific $CD8^+$ T cells are responsible

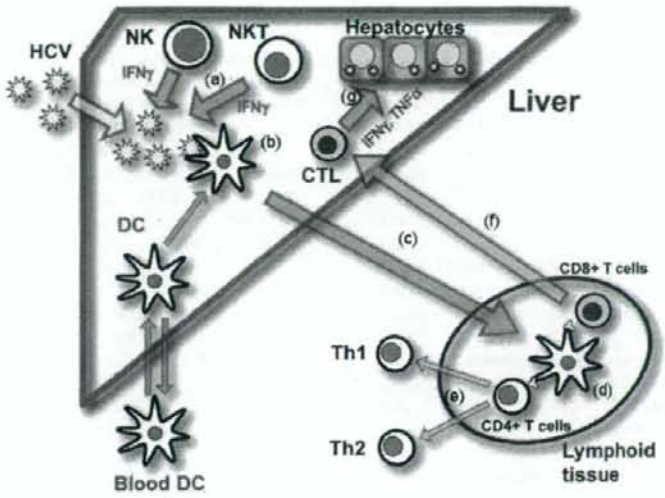

Figure 1. Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HCV, hepatitis C virus; NK, natural killer cell; Th, helper T cell. (a)- (g), see text.

for HCV elimination by inducing hepatocyte apoptosis (2). Innate immune cells, including NK cells and NKT cells, may contribute to HCV eradication after primary infection; however, their roles in chronically-infected state remain elusive. Since dendritic cells (DCs) orchestrate anti-HCV immune response by linking innate and adaptive arms of immune system (4), functional impairment of DC leads to failure of NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells. Infiltration of disabled $CD8^+$ T cells to the infected liver may result in weak liver inflammation that is not sufficient for HCV eradication (5).

In this paper, we discuss the current understandings of the roles of innate immunity in the pathogenesis of HCV infection as well as efficacy of anti-HCV therapy, especially focused on interferons (IFN), DCs, NK cells and NKT cells.

3. KEY PLAYERS IN IMMUNE RESPONSES TO VIRAL HEPATITIS

After HCV infects the liver, viral replication continues and viral particles are continuously released into the circulation. The first lines of defense are provided by NK and NKT cells, of which populations are relatively increased in the liver compared to the periphery. These cells are activated in the liver, where expression of IFN-alfa and IFN-inducible genes are extremely high during the early phase of hepatitis virus infection (6). Activated NK and NKT cells secrete IFN-gamma, which inhibits replication of HCV through a non-cytolytic mechanism (Figure 1-a) (7).

Dendritic cells (DCs) or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Figure 1-b) (4). Since DCs express distinct sets of toll-like receptors (TLRs) (8), it is likely that some viral components stimulate DCs through cytosolic ligation of TLRs. DCs develop a mature phenotype and migrate to lymphoid tissues (Figure 1-c), where they stimulate effectors, including T cells and B cells (Figure 1-d). Following the encounter of DCs with other cells, DCs secrete various cytokines (IL-12, TNF-alfa, IFN-alfa and IL-10) instructing or regulating the functions of the adjacent cells (4). In addition to these cytokines, DCs express various co-stimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported; i.e., myeloid DC (MDC) and plasmacytoid DC (PDC) (9). MDC predominantly produce IL-12 or TNF-alfa following pro-inflammatory stimuli, while PDC release a considerable amount of IFN-alfa upon virus infection depending on the immune stimulus; both cytokines in actuality can be made by both cells. Helper T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTLs) generation (Th1 with secretion of IL-2, IFN-gamma and TNF-alfa) or B cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Figure 1-e). DC ontogeny and DC-derived cytokines are crucially associated with the polarization of helper T cell subsets.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific $CD4^+$ T cells in HCV eradication has been well described during both acute or chronic infection (10). However, there is little evidence that $CD4^+$ T cells mediate direct liver cell injury in HCV infection. Thus, it is likely that $CD4^+$ T cells play a critical role in facilitating other antiviral immune

mechanisms, such as enhancing $CD8^+$ effector function. The antigen-primed CTLs recruit to the liver (Figure 1-f) and constitute the critical element in the eradication of virus-infected cells (Figure 1-g).

4. INNATE IMMUNITY IN HCV INFECTION

4.1. Toll-like receptors and retinoic acid inducible gene-I as sensors for virus infection

Gene expression analyses in HCV-infected liver revealed that HCV triggers expression of type I IFN and IFN-induced genes during primary infection regardless of the outcomes (6). However, the HCV viral load does not decrease in the early phase, suggesting that HCV impedes the execution of anti-viral machineries. Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing anti-viral proteins, such as interferon regulatory factor (IRF)-3 (11), NF-kappa B and RNA-dependent protein kinases (PKR) (12). Mammalian toll-like receptors (TLRs) sense some pathogen-associated molecular patterns embedded in virus components and then induce inflammatory cytokines or type-I IFNs, resulting in the augmentation of anti-virus immune reactions (8). Retinoic acid inducible gene-I (RIG-I) is a cytosolic molecule that senses dsRNA of virus replicative intermediate, which subsequently activates IRF-3 and NF-kappa B pathways (13). By using HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4A proteins influences on the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN-beta as well as subsequent interferon-stimulated genes (14, 15). However, it is yet to be proven whether the results obtained from HCV replicon are applicable or not for HCV-infected individuals.

Large-scale cohort study on US veterans revealed that the prevalence of various infectious diseases, including virus, bacteria and parasites, in HCV-infected individuals is significantly higher than those in uninfected controls (16). These observations suggest that first-line defense against pathogens, of which system is initiated by TLR/RIG-I stimulation, is functionally impaired in HCV infection. To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and the functions in MDC and PDC between the patients and donors. In MDC from HCV-infected patients, TLR2, TLR4 and RIG-I expression were significantly higher than those in healthy counterparts. Of particular interest, regardless of the higher expressions, specific agonists for these sensors stimulated patients MDC to induce lesser amount of IFN-beta and TNF-alfa compared to donor MDC (unpublished data). These results show that the signal transduction via these receptors is strongly impeded in HCV infection. Inconsistent with the findings of MDC, we previously reported that TLR2 expression on monocyte-derived DCs (MoDCs) in chronic hepatitis C is lower than those in healthy donors (17). Since MoDC is an in vitro-generated DC mimic, the opposite results of TLR2 in HCV infection might be explained by impaired ability of MoDC to mature in response to cytokines, as reported elsewhere (18). Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

4.2. Blood DC subsets

Impaired antigen presentation by DC might be involved in the failure of the maintenance of sustained HCV-specific T cell response. Monocyte-derived DCs (MoDCs) generated from hepatitis C patients have an impaired ability to stimulate allogeneic $CD4^+$ T cells (19, 20). Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability (19). In addition to in vitro-generated DCs, the alterations in number and function of circulating blood DC have been reported in HCV infection (21, 22).

Direct HCV infection of DCs might be one of the plausible mechanisms of DC dysfunction in chronic hepatitis C. The HCV genome has been reported to be isolated from MoDCs or blood DCs (19). However, these results need to be interpreted carefully, since contamination with free virus in blood cannot be ruled out when amplifying PCR techniques are used. To exclude this possibility, HCV pseudovirus has been developed to investigate the cell tropisms of HCV as well as to determine putative HCV entry receptors to cells. By using this, MDC, but not PDC, displayed susceptibility to HCV pseudovirus possessing chimeric HCV E1/E2 proteins (23).

Several criticisms have been raised recently about DC dysfunction in the setting of chronic HCV infection (24), failing to demonstrate any DC defects which may have to do with differences in the populations studied. Cohort studies on chimpanzees following HCV infection showed that functional impairment of DCs was observed in some cases but was not a prerequisite of persistent infection (25). Further study needs to be done to clarify whether DCs are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or it is simply a consequence of active HCV infection.

4.3. Natural killer cells

Natural killer cells express various functional receptors; the one group that transduces inhibitory signals (Killer Inhibitory Receptors/KIRs, CD94, NKG2A) and the other does activating signals (NKG2D). The function of NK cells is dynamically regulated in vivo by the balance between expressions of counteracting receptors and their association with relevant ligands (26). First, we compared the expressions of NK cell receptor between HCV-infected patients and healthy donors. As for inhibitory receptors, KIR expressions are not different between the groups; however, CD94 and NKG2A expressions are higher in patients than controls (27). In contrast, activating receptor NKG2D expression is comparable between the groups (Figure 2). It is yet to be determined how the expression of NK cell receptor is regulated. In our hands, HCV pseudovirus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

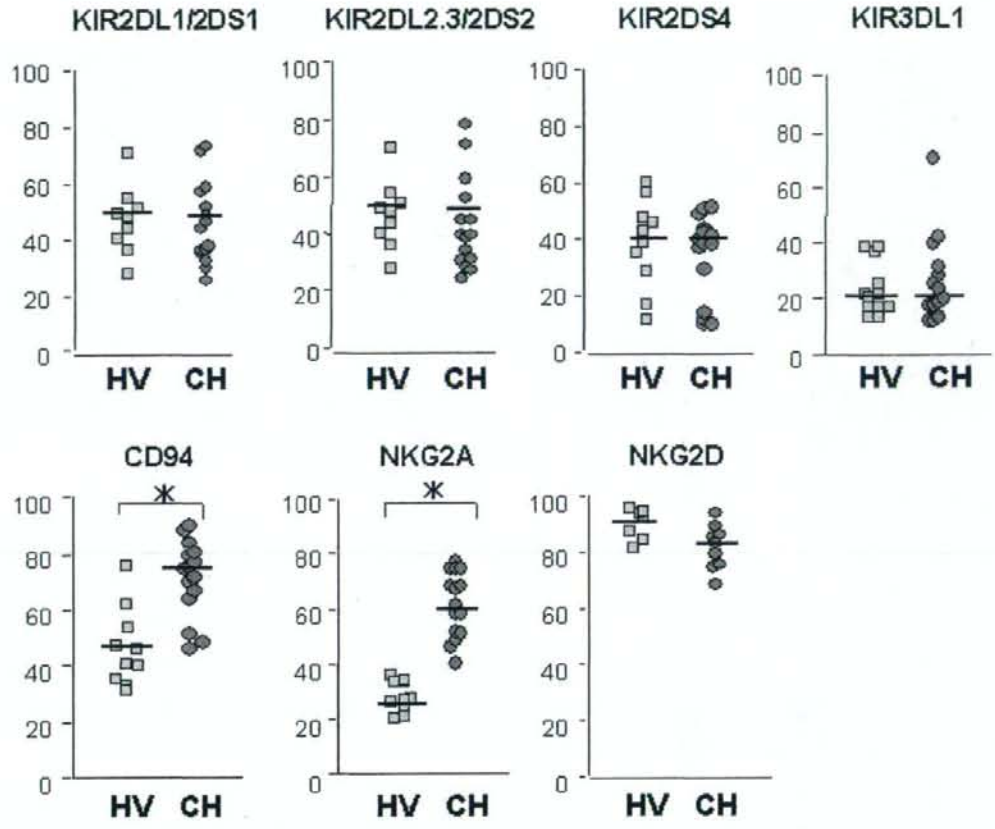

Figure 2. The expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. The percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median.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

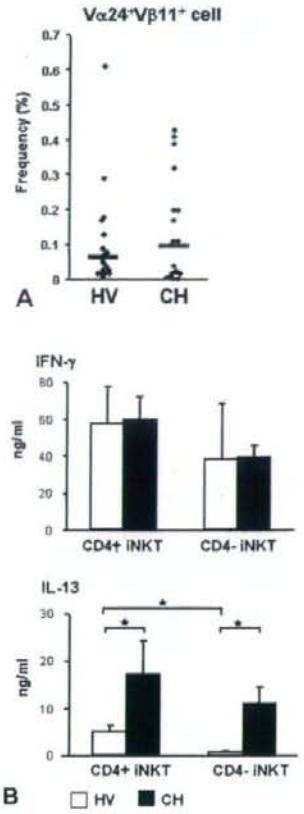

Figure 3. Frequency and cytokine production of invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. (A) The frequencies of total invariant NKT (iNKT) cells (Valfa24$^+$Vbeta11$^+$ cells) in PBMCs were determined by flow cytometry. HV, CH; See Fig 2. Horizontal bars represent 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

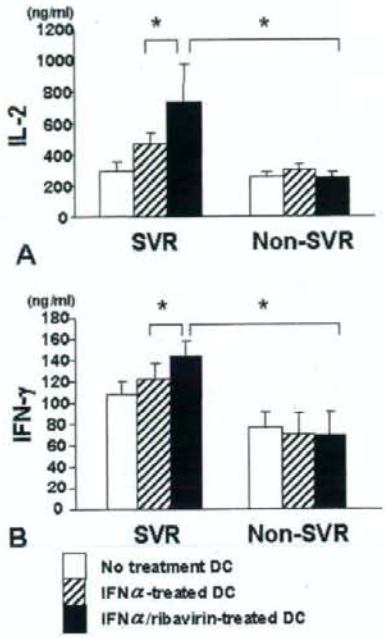

Figure 4. Improvement of Th1-inducing ability of dendritic cells by IFN-alfa and ribavirin from chronic hepatitis C patients with sustained virological response in combination therapy. Monocyte-derived dendritic cells (DC) were generated with GM-CSF and IL-4 in the presence or absence of IFN-alfa and 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 $\pm$ 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

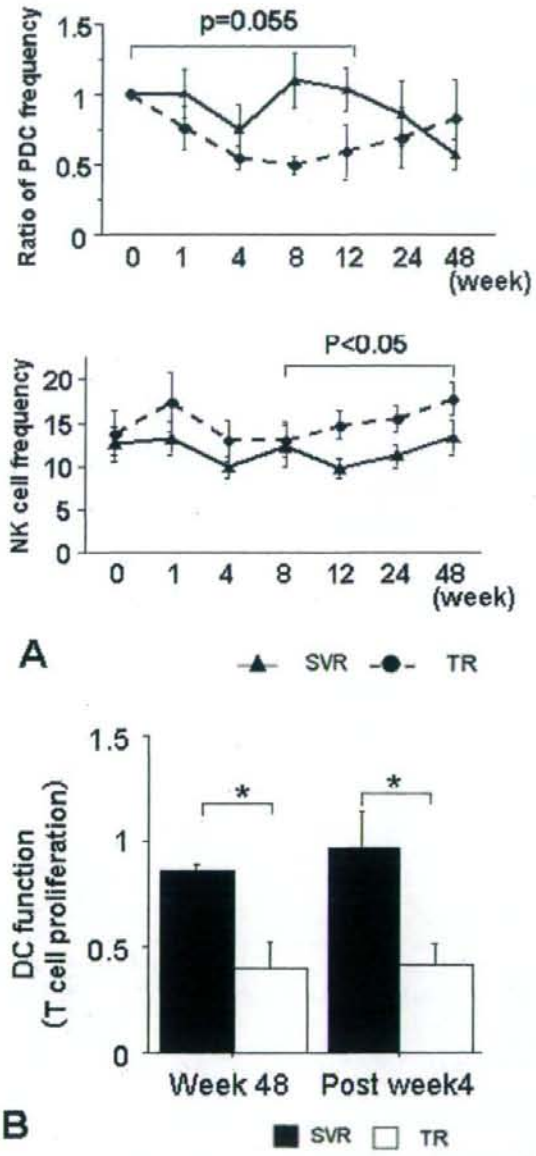

Figure 5. Early phase decline pf plasmacytoid dendritic cell frequency and sustained impairment of dendritic cell ability are related to transient response in 48-week pegylated IFN-alfa and ribavirin therapy. Frequencies and their ratios of plasmacytoid dendritic cells (PDC) and NK cells in the patients 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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Journal of Medical Virology 80:980–988 (2008)

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

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: Ministry of Health, Labor and Welfare of Japan.

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Accepted 5 February 2008
DOI 10.1002/jmv.21174
Published online in Wiley InterScience (www.interscience.wiley.com)

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 ± 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).

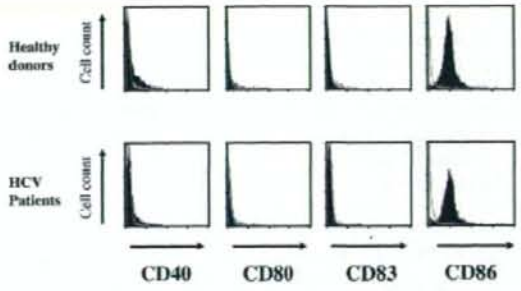

Fig. 1. Fresh myeloid dendritic cells are immature regardless of HCV infection. Myeloid dendritic cells were obtained from HCV-infective patients or healthy donors and their expressions of CD40, CD80, CD83, and CD86 were analyzed by flow cytometry. The shaded histograms are the results with specific 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

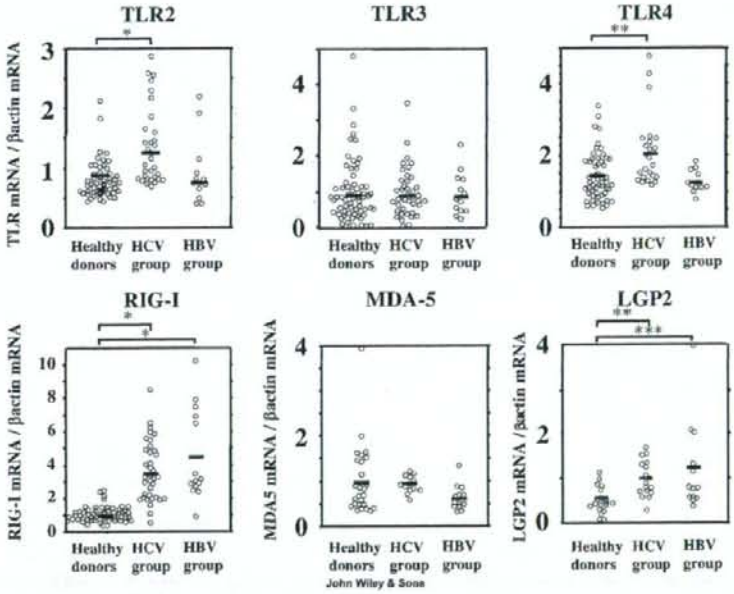

Fig. 2. Expressions of TLR2, TLR4, RIG-I, and LGP2 in patient myeloid dendritic cells from HCV-infected patients are higher than those from healthy donors, while TLR3 and MDA-5 are comparable. Expressions of TLR2, TLR3, TLR4, RIG-I, MDA-5, and LGP2 in myeloid dendritic cells were quantified by real-time 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).

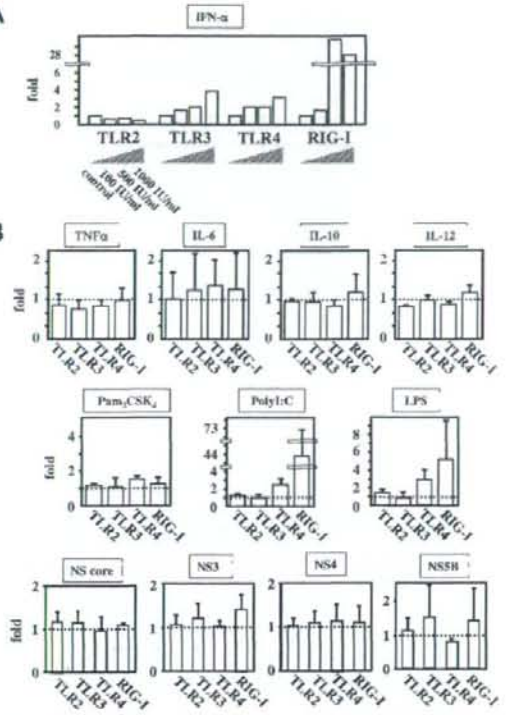

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials 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

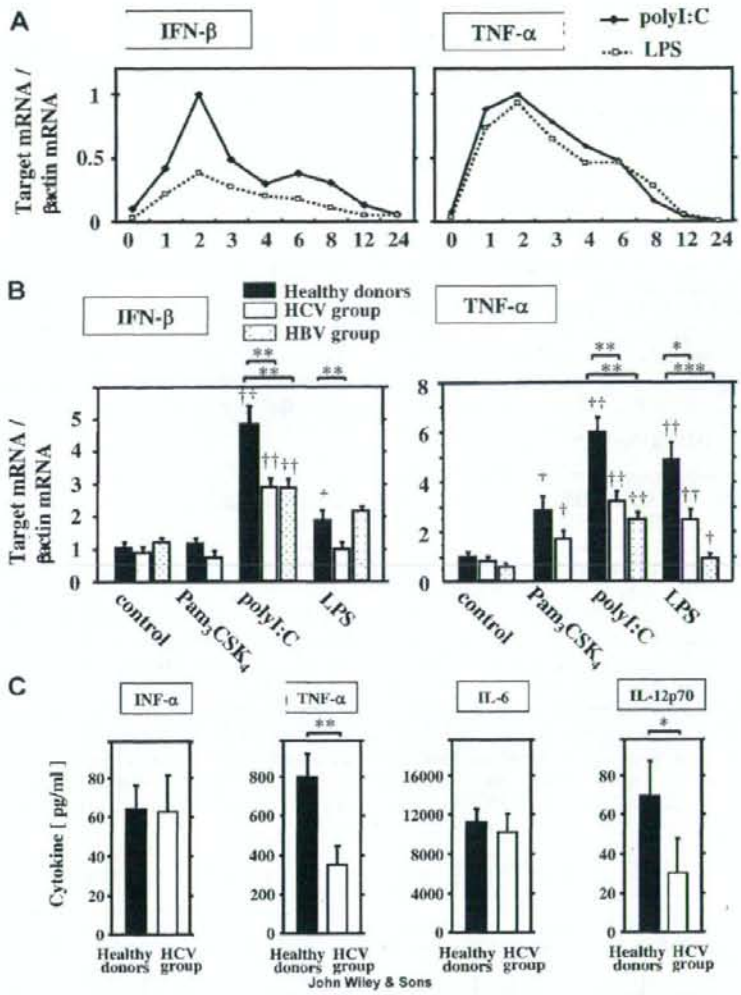

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by 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

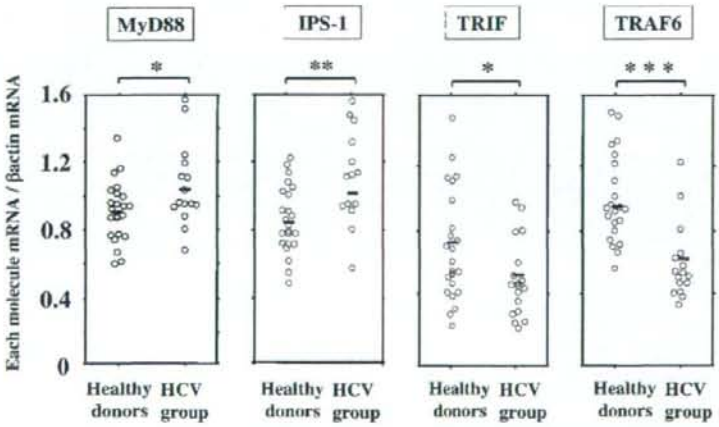

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed 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. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.