from MoDC or blood DC.[18] However, these results need to be interpreted carefully, because contamination with free virus in blood cannot be ruled out when amplifying polymerase chain reaction 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.[21]

Several criticisms have been raised recently about DC dysfunction in the setting of chronic HCV infection,[22] 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 DC was observed in some cases but was not a prerequisite of persistent infection.[23] Further study needs to be done to clarify whether DC are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or if it is simply a consequence of active HCV infection.

Natural killer cells

Natural killer cells express various functional receptors; the one group that transduces inhibitory signals (killer inhibitory receptors [KIR], CD94, NKG2A) and the other performs 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.[24] First, we compared the expressions of NK cell receptors 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.[25] In contrast, activating receptor NKG2D expression is comparable between the groups (Fig. 2). It is yet to be determined how the expression of the NK cell receptor is regulated. In our experience, HCV pseudovirus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (Kaimori A *et al.*, 2004, unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

Dendritic cells play a decisive role in shaping innate immunity by interacting with NK cells. DC have two means to stimulate NK cells via the production of cytokines (IL-12, IL-18 or IFN-α) and through the expression of NK-activating ligands. In response to IFN-α, DC are able to express major histocompatibility complex (MHC) class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D.[26] Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells.[26] It is tempting to speculate that the impairment of DC 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-α expression in the liver. Alternatively, NK cells from HCV-infected patients downregulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and transforming growth factor-β1.[25] 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.[25] Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

Natural killer T cells

Natural killer T cells are a unique lymphocyte subset coexpressing T-cell receptors (TCR) and NK cell markers.[27] The NKT cell population is highly heterogeneous; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of Vα24-JαQ preferentially paired with Vβ11 in humans,[27] whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DC.[27] Although endogenous ligands of iNKT cells are little known, α-galactosyl-ceramide (αGalCer) has been used as a surrogate for natural ligands. 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-γ) 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.[28]

Although iNKT cells comprise a small portion of hematopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings. For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells,[29,30] 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

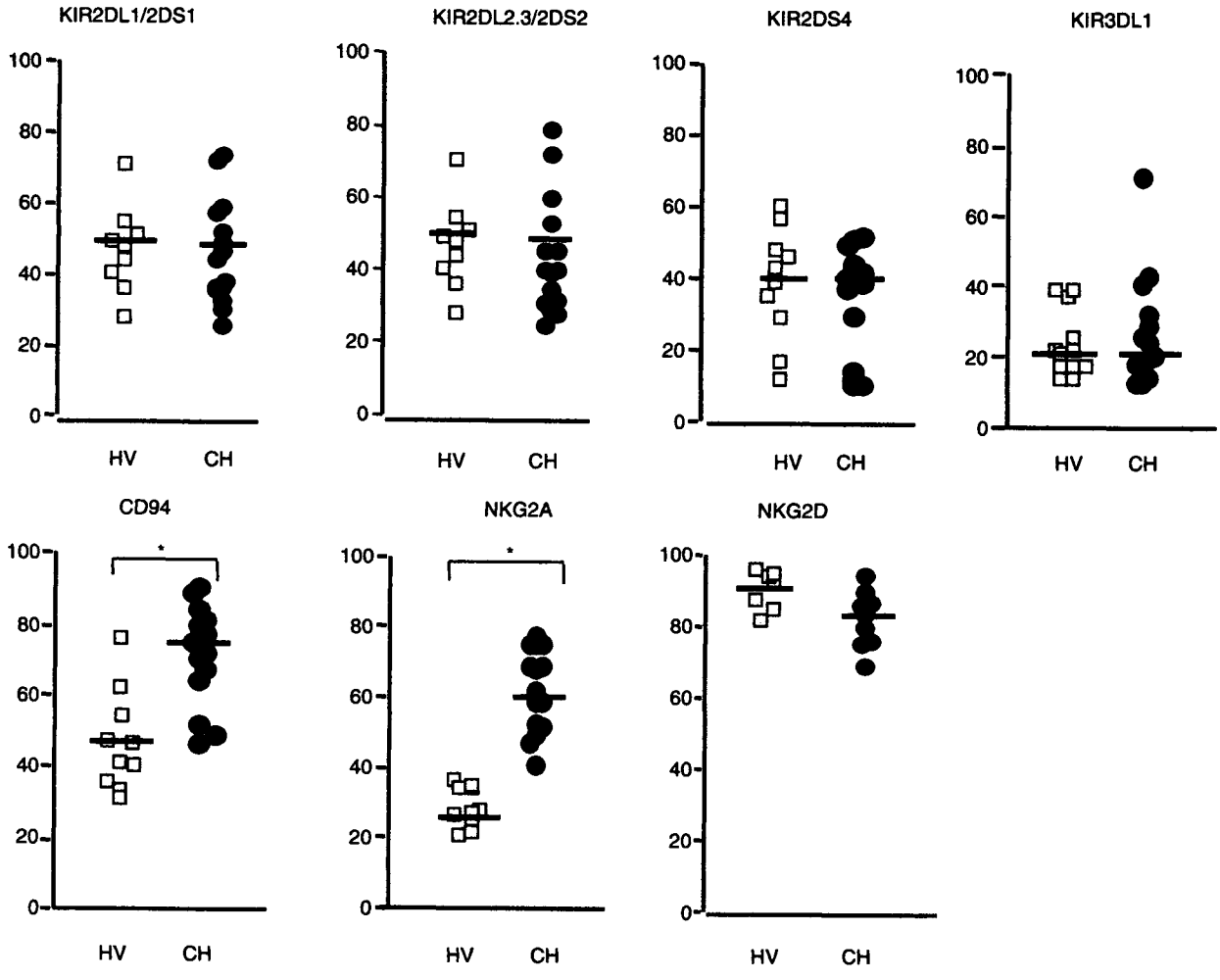

Figure 2 Expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. Percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. $^*P < 0.05$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

αGalCer-loaded DC. We demonstrated that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state (Fig. 3).[31] By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls (Fig. 4).[31] Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by hepatitis B virus or HCV, implying that these cells are pro-fibrogenic to the liver.[32] 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.

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,[33] 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.[34]

With regard to HCV-specific $CD8^+$ T cells observed during the chronic stages of disease, conflicting results

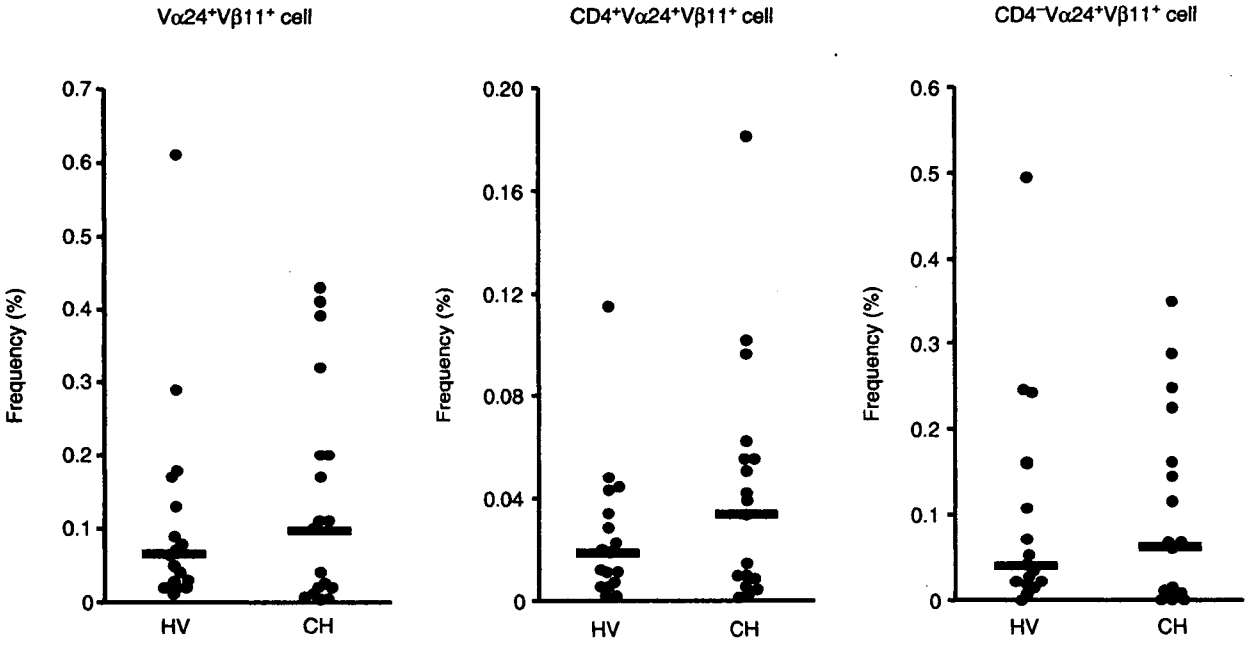

Figure 3 Frequency of peripheral invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. The frequencies of total invariant NKT (iNKT) cells ($V\alpha24^+V\beta11^+$ cells), $CD4^+$ and $CD4^-$ iNKT cells in PBMC were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median.

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.[35] However, others did not find a significant relationship between these parameters.[36] HCV-specific $CD8^+$ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN-γ in response to HCV antigens. Because $CD8^+$ 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] (i) HCV escape mutation; (ii) primary T-cell failure or T-cell exhaustion; (iii) impaired antigen presentation; (iv) suppression by HCV proteins; (v) impaired T-cell maturation; (vi) suppression by regulatory T cells; and (vii) tolerogenic environment in the liver.

PERSPECTIVES

ANTIVIRAL AGENTS, PEGYLATED (PEG)-IFN-α and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular carcinoma.[1] In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. The questions remain unsolved whether an impaired immune system in chronic HCV infection is restored or not by successful HCV eradication after antiviral therapy. Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response,[37,38] which seems to be clearly distinct from that observed in spontaneous HCV resolvers. Protease inhibitors against HCV NS3/4A are now ready to use in clinics. Because they possess potent ability to suppress HCV replication, they are quite promising as an alternative approach for non-responders in PEG-IFN-α/ribavirin therapy. In addition to that, it is anticipated that protease inhibitors are able to restore innate 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

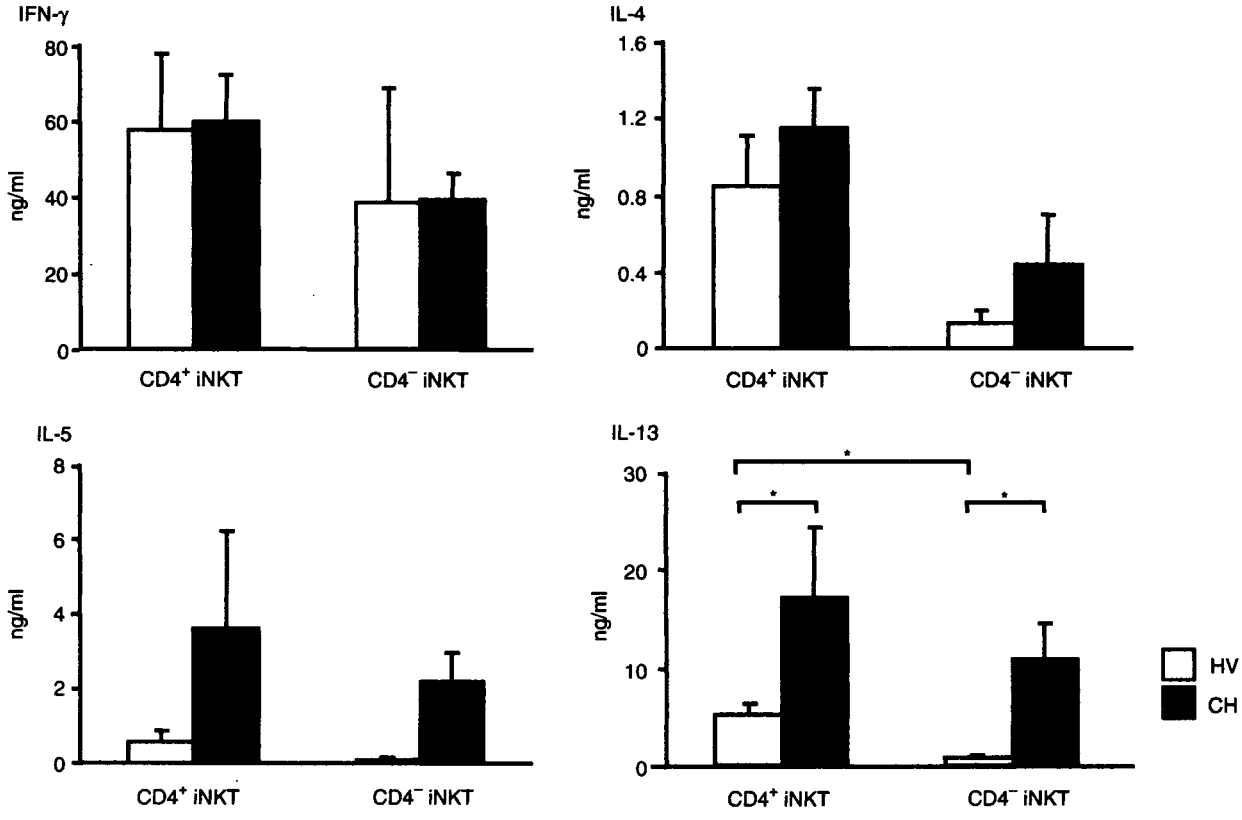

Figure 4 Cytokine production from expanded invariant NKT cells stimulated with α-galactosyl-ceramide (αGalCer)-loaded monocyte-derived DC. Invariant NKT (iNKT) cells were expanded by culture with αGalCer-pulsed autologous monocyte-derived DC (MoDC) and subsequent cell sorting. Activated iNKT cells were stimulated with αGalCer-pulsed allogeneic MoDC for 24 h and the supernatants were collected for cytokine enzyme-linked immunosorbent assay. HV, healthy volunteers; CH, chronic hepatitis C patients. Bars represent mean ± SE of five different subjects. $^*P < 0.05$ by Mann–Whitney *U*-test.

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.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by T Kanto and N Hayashi.

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Involvement of Dendritic Cell Frequency and Function in Virological Relapse in Pegylated Interferon-α and Ribavirin Therapy for Chronic Hepatitis C Patients

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A combination of pegylated interferon α (PEG-IFNα) and ribavirin has been used widely. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. The aim of the study was to elucidate whether the frequency or function of immunocompetent blood cells is related to the outcome of the therapy. Twenty-five chronic hepatitis C patients with high viral load of HCV genotype 1 who underwent 48 weeks of PEG-IFNα2b and ribavirin therapy were examined. During the treatment, frequencies of dendritic cell subsets, helper T cell subsets, and NK cells were phenotypically determined. In some patients, the ability of dendritic cells to stimulate allogeneic $CD4^+$T cells was examined at the end and after the therapy. Among the 25 patients, 11 showed a sustained virological response, 11 a transient response, and 3 no response. In comparison with sustained virological responders, non-sustained virological responders showed impaired dendritic cell function at the end and after the treatment. The transient responders showed a decline of plasmacytoid dendritic cell frequency from Weeks 1–12 and impaired dendritic cell function as well. Even in patients who attained negative serum HCV RNA at Week 12, the transient responders showed a significant decrease of plasmacytoid dendritic cell frequency and impaired dendritic cell function. In conclusion, in PEG-IFNα and ribavirin combination therapy for chronic hepatitis C patients, the early-phase plasmacytoid dendritic cell frequency and/or end-of-treatment dendritic cell function are related to the virological outcome of the therapy.
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KEY WORDS: chronic hepatitis C; PEG interferon; ribavirin; dendritic cell

INTRODUCTION

Hepatitis C virus (HCV) infection causes various types of liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [Seeff, 2002]. The most effective way to prevent the progression of disease is to eradicate HCV from the infected hosts [Alter et al., 1989]. At present, combination therapy with pegylated interferon alpha (PEG-IFNα) and ribavirin is considered as the standard treatment for chronic HCV infection. The rate of the sustained virological response achieved by the combination therapy has been up to 50% in patients with HCV genotype 1 and a high HCV RNA titer; however, half of the patients do not attain sustained virological response [Manns et al., 2001; Fried et al., 2002]. In addition to HCV genotype and HCV quantity, several factors have been reported as

Abbreviations: HCV, hepatitis C virus; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; NK, natural killer; MLR, mixed leukocyte reaction

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therapeutic determinants in PEG-IFNα and ribavirin combination therapy, such as liver fibrosis, age, gender, and ethnicity [Manesis et al., 1997; Poynard et al., 1998; Jacobson et al., 2005]. It is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., HCV dynamics, correlates well with the clinical outcomes of the treated patients [Davis et al., 2003; Hayashi and Takehara, 2006]. In PEG-IFNα and ribavirin therapy, an early virological response is defined as a reduction in serum HCV RNA quantity by at least 2 $\log_{10}$ units or to an undetectable level by a sensitive qualitative PCR after the first 12 weeks of the treatment or negative serum HCV RNA at Week 24 of the therapy [Davis et al., 2003]. It has been reported that the patients who fail to attain early virological response at Week 12 or 24 are not likely to gain sustained virological response after 48 weeks of the combination therapy, suggesting that early virological response can serve as a negative predictor of sustained virological response [Ferenci, 2004; Ferenci et al., 2005]. Prolongation of the duration of PEG-IFNα and ribavirin combination therapy from 48–72 weeks is likely to improve sustained virological response rate by decreasing relapsers [Berg et al., 2006]. Therefore, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, since it can offer them a better chance of attaining sustained virological response. However, no reliable marker is currently available for predicting virological relapse in PEG-IFNα and ribavirin therapy.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells, NK cells, and T cells [Kanto et al., 1999; Auffermann-Gretzinger et al., 2001; Rosen et al., 2002; Nattermann et al., 2006]. It is reported that sustained viral responders maintained vigorous and multispecific HCV-specific $CD4^+$ Th1 responses, suggesting that the restoration of $CD4^+$ T cell responses may be related to successful HCV eradication [Kamal et al., 2002]. However, it is not known whether the frequency or the function of other immune cells during the combination therapy has any relationship to the therapy outcome.

In the present study, in order to determine immunological markers correlated with the efficacy of the treatment, the frequency of peripheral blood cell subsets and their dynamics were studied during and after the combination therapy. The function of dendritic cells from the patients was examined to clarify whether it was correlated with the therapeutic efficacy. This study supports the view that the reactivity of the immune system to the combination therapy is involved critically in the outcome of the treatment.

MATERIALS AND METHODS

Patients

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Koseinenkin Hospital, and Osaka National Hospital, 32 patients who received PEG-IFNα2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethical committee of the Osaka University Graduate School of Medicine. Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody and HCV RNA, but were negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. All the patients were infected with HCV genotype 1b with a serum HCV RNA quantity of more than 100 kilocopies/ml, as determined by methods described elsewhere [Pawlotsky et al., 2000]. All patients had shown persistent or fluctuating serum alanine aminotransferase abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, alcohol abuse, and metabolic disorders was excluded by laboratory and imaging analyses. With all patients, a combination of biochemical markers and ultrasonography or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver. Histological analyses of liver disease were performed with liver tissue obtained by ultrasonography-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the classification proposed by Desmet [Desmet et al., 1994].

Study Design

All patients were treated with PEG-IFNα2b subcutaneously at a dose of 75 μg/week (body weight > 40 kg and ≤ 60 kg) or 105 μg/week (body weight > 60 kg and ≤ 80 kg) or 135 μg/week (body weight > 80 kg and ≤ 100 kg) and oral ribavirin at a dose of 600 mg/day (body weight > 40 kg and ≤ 60 kg) or 800 mg/day (body weight > 60 kg and ≤ 80 kg) or 1000 mg/day (body weight > 80 kg and ≤ 100 kg). Ribavirin was administered divided into two doses per day. All patients were treated for 48 weeks and followed for 24 weeks after the cessation of therapy. The early responders were defined as those who showed a reduction in serum HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. Virological response was estimated at 24 weeks after cessation of the treatment. Sustained virological response was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response was defined as the reappearance of serum HCV RNA within 6 months after cessation of therapy in patients who had achieved negative serum HCV RNA at the end of the treatment. No response meant that there was persistently positive serum HCV RNA throughout the therapy period. Non-sustained virological response group is comprised of transient responders and no responders.

Analysis of Dendritic Cell Subsets, Helper T Cells, and NK Cells

For the numerical analyses of blood dendritic cells, helper T cells, and NK cells, venous blood was drawn

from patients before treatment and at Weeks 1, 4, 8, 12, 24, and 48 during the therapy. Peripheral blood mononuclear cells (PBMCs) were collected by density-gradient centrifugation on a Ficoll–Hypaque cushion. After viable PBMCs had been counted, the cells were stained with combinations of various antibodies for phenotypic markers.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA): anti-lineagemarker (Lin; CD3 (clone SK7), CD14 (clone MφP9), CD16 (clone 3G8), CD19 (clone SJ25C1), CD20 (clone L27), and CD56 (clone NCAM16.2)), anti-CD4 (clone RPA-T4), anti-CD11c (clone B-ly6), anti-CD123 (clone 7G3), anti-CD3 (clone UCHT1), anti-CD45RO (clone UCHL1), anti-CD56 (clone B159), anti-HLA-DR (clone L243), anti-CCR4 (clone 1G1). Anti-CXCR3 (clone 49801) monoclonal antibody was purchased from R&D Systems (Minneapolis, MN). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously. The acquisitions and analyses of data were performed with FACSCalibur (BD Biosciences) and CellQuest software.

Blood dendritic cells were defined as Lin^- and $HLA\text{-}DR^+$ cells. Myeloid dendritic cells are Lin^-, $HLA\text{-}DR^+$, $CD11c^+$, $CD123^{low}$ cells, and plasmacytoid dendritic cells are Lin^-, $HLA\text{-}DR^+$, $CD11c^-$, and $CD123^{high}$ cells, respectively. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are $CD4^+$, $CD45RO^+$, $CXCR3^+$, and Th2 cells are $CD4^+$, $CD45RO^+$, and $CCR4^+$, respectively. NK cells were defined as $CD3^-$, $CD56^+$ cells. The percentages of dendritic cell subsets and NK cells in PBMCs or Th1 and Th2 cells in $CD4^+$ T cells were determined by FACS. In order to examine the dynamics of dendritic cell subsets after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy.

Allogeneic Mixed Leukocyte Reaction With Dendritic Cells

In some patients, we examined whether the allostimulatory ability of dendritic cells was related to the clinical outcomes. At the end of treatment and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from PBMC obtained from the patients according to methods reported previously [Romani et al., 1994]. As controls, monocyte-derived dendritic cells were generated simultaneously from healthy donors. As responder cells in mixed leukocyte reaction (MLR), naive $CD4^+$ T cells were isolated from PBMC of irrelevant healthy donors by using a naive $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). Allogeneic MLR with monocyte-derived dendritic cells was performed as reported previously [Kanto et al., 1999]. In order to compare the ability of monocyte-derived dendritic cells among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of ^3H-thymidine incorporated into $CD4^+$ T cells at the T cell/dendritic cell ratio of 10/1.

Statistical Analyses

For statistical analysis, the non-parametric Mann–Whitney *U*-test was used between the groups. To analyze paired data, we used Wilcoxon's signed rank test. Differences of continuous variables between groups were compared by two-way ANOVA. *P*-values of less than 0.05 were considered to be statistically significant. These statistical analyses were performed with StatView software (Cary, NC).

RESULTS

Outcome of the PEG-IFNα and Ribavirin Therapy

Among the 32 patients who received PEG-IFNα2b and ribavirin combination therapy, 25 completed the therapy while 7 patients dropped out due to various adverse effects. Among the 25 patients who completed the therapy, 11 (44%) achieved sustained virological response, 11 (44%) showed transient response, and 3 (12%) showed no response (Table I). There was no difference in the baseline clinical parameters among these groups (Table I). With regard to HCV RNA at Week 12 in patients who completed the therapy, 11 were negative for HCV RNA (early responders), while the remaining 14 were not. Among 11 patients with early response, 7 were sustained virological responders and 4 were transient responders. Among 14 patients who were positive for serum HCV RNA at Week 12, 4 patients achieved sustained virological response, 7 showed transient response, and 3 showed no response. Details of the therapeutic response in the current study are shown in Figure 1.

TABLE I. Baseline Clinical Characteristics of the Patients

	All patients	SVR	TR	NR
Age[a]	50.0 ± 10.9	46.7 ± 12.4	54.1 ± 8.9	46.7 ± 9.3
Sex (M/F)	20/5	9/2	8/3	3/0
ALT (IU/l)[a]	99.3 ± 47.8	97.5 ± 50.9	103 ± 51.3	94.0 ± 34.6
HCV RNA (kilo copies/ml)[a]	3146 ± 2675	3685 ± 3023	2743 ± 2338	2647 ± 3163
Activity (minimal/mild/moderate)	7/7/11	5/3/3	1/4/6	1/0/2
Fibrosis (mild/moderate/severe)	11/12/2	6/5/0	3/7/1	2/0/1

ALT, alanine aminotransferase.
Histological activity and fibrosis were assessed according to the classification proposed by Desmet.
[a]Mean ± SD.

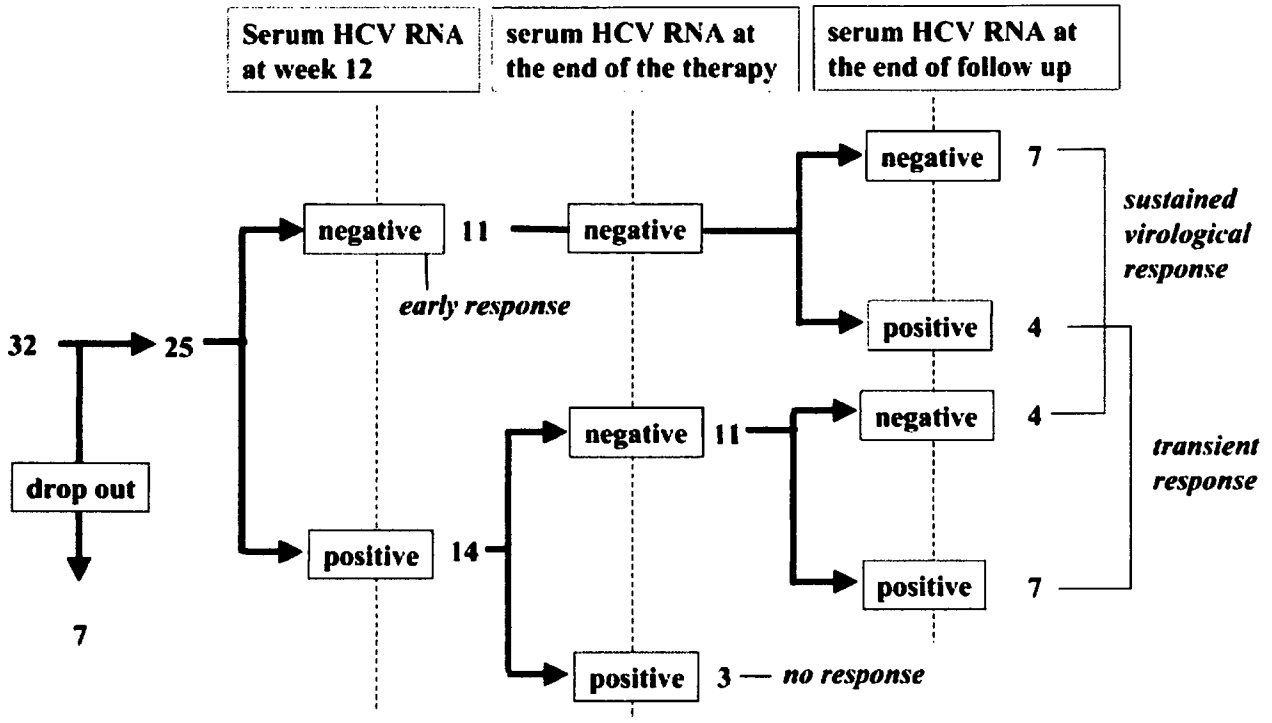

Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 11 were transient responders, and 3 were non-responders. The early responders were defined as those who showed a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. According to this criterion, 11 patients were early responders and were further categorized into 7 sustained virological response (sustained virological responders with early response) and 4 transient response (transient responders with early response). Of the other 14 patients who were not early responders, 4 were sustained virological responders, 7 were transient responders, and 3 were non-responders.

Non-Sustained Virological Responders Had a Lower MLR Ratio Than Sustained Virological Responders

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, these parameters were compared between sustained virological responders and non-sustained virological responders, including transient responders and no responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and non-sustained virological responders (Fig. 2A). As for the changes of dendritic cell subsets during the therapy, frequencies of both plasmacytoid dendritic cells and myeloid dendritic cells at each time point did not differ between sustained virological responders and non-sustained virological responders (Fig. 2B,C). The percentages of NK cells in non-sustained virological responders tended to be higher than those in sustained virological responders from Weeks 4–48, which did not reach statistical significance ($P = 0.0533$ ANOVA) (Fig. 2F). The frequencies of Th1 and Th2 did not differ between these two groups (Fig. 2G,H). As for dendritic cell function, dendritic cells from the non-sustained virological responders showed a lower MLR ratio than those from the sustained virological responders at the end ($P < 0.01$) and at 4 weeks after the completion of therapy ($P < 0.005$) (Fig. 3). These results show that lesser ability of dendritic cells at the end of treatment may be related to non-sustained virological response.

Transient Responders Had a Lower MLR Ratio in Dendritic Cell Function Than Sustained Virological Responders in the Course of Combination Therapy

In order to elucidate if the above-mentioned immunological markers are related to virological relapse, a

Fig. 2. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (A), during the combination therapy (B, C, F–H) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (D, E) were determined as described in Materials and Methods, which were compared between sustained virological responders and non-sustained virological responders. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict non-sustained virological responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. PBMC, peripheral blood mononuclear cells; NK, natural killer.

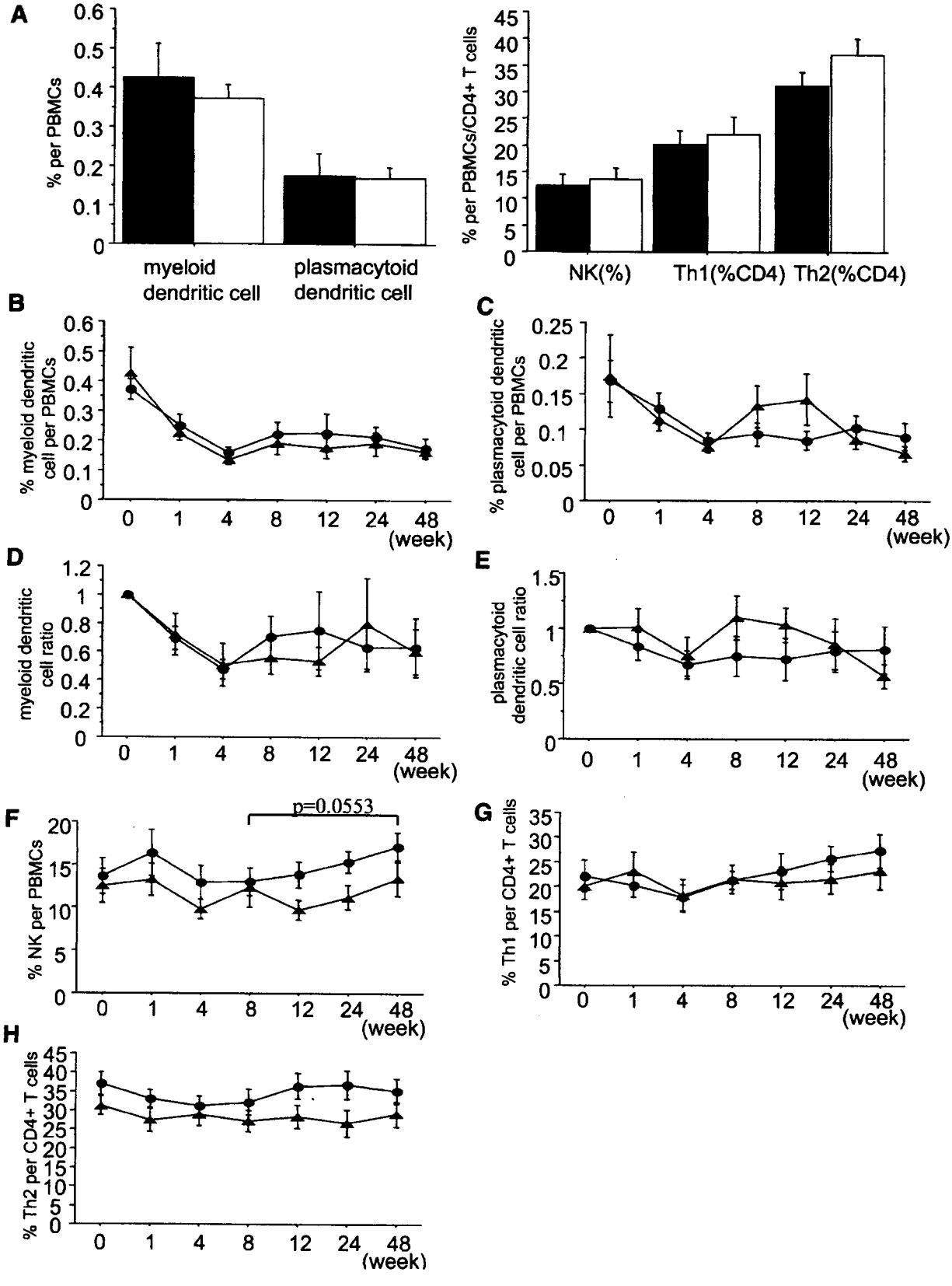
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
p=0.0553
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
0 1 4 8 12 24 48
(week)

Fig. 2.

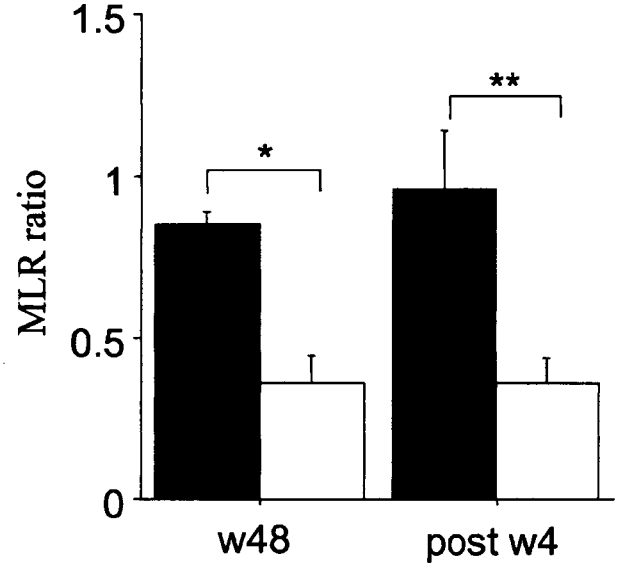

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/dendritic cell ratio of 10/1. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. Black bars indicate sustained virological responders and white bars indicate non-sustained virological responders. *$P < 0.01$, **$P < 0.005$.

comparison was undertaken between sustained virological responders and transient responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and transient responders (Fig. 4A).

The percentages of myeloid dendritic cells and plasmacytoid dendritic cells were not different between the sustained virological responders and transient responders at each time point (Fig. 4B,C). The transient responders tended to show a lower plasmacytoid dendritic cell ratio than sustained virological responders from Weeks 1–12 ($P = 0.0553$, ANOVA) (Fig. 4E), suggesting that plasmacytoid dendritic cell is likely to decrease in the early phase in transient responders whereas those in sustained virological responders tend to be maintained. By contrast, no difference was observed in the myeloid dendritic cell ratio between the groups (Fig. 4D). The percentages of NK cells in transient responders were significantly higher than those in sustained virological responders from Weeks 8–48 ($P < 0.05$) (Fig. 4F). The frequencies of Th1 or Th2 at each point during therapy did not differ between the sustained virological responders and transient responders (Fig. 4G,H).

With regard to the dendritic cell function, the transient responders showed a lower MLR ratio than the sustained virological responders from Weeks 4–48 after the end of the therapy ($P < 0.05$) (Fig. 5). These results suggest that sustained impairment of dendritic cell function at the end and after the treatment may be related to the virological relapse after cessation of the therapy.

Early-Phase Decline of Plasmacytoid Dendritic Cell Frequency and Sustained Impairment of Dendritic Cell Ability Are Related to Transient Response in the Combination Therapy Even in Patients Who Lost Serum HCV RNA at Week 12 of the Treatment

In order to estimate more precisely the involvement of immunological markers in the outcomes of the combination therapy, we examined the above-mentioned parameters in patients who attained negative serum HCV RNA at Week 12 (early response group), as they were considered to be comparable with respect to the virological response to the therapy. Among 11 patients who were clear of serum HCV at Week 12, 7 were categorized into sustained virological response (sustained virological responders with early response) and the remaining 4 into transient response (transient responders with early response) (Fig. 1). Among patients with early response, the pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, Th1, Th2, and NK cells (Fig. 6A) and those at any points during the therapy did not differ between sustained virological responders and transient responders (Fig. 6B,C,F–H). The plasmacytoid dendritic cell ratios in transient responders were lower than those in sustained virological responders from Weeks 1–12 ($P < 0.05$, ANOVA) (Fig. 6E), whereas the myeloid dendritic cell ratio did not differ between the groups (Fig. 6D).

As for MLR, dendritic cells from the transient responders showed a lower MLR ratio than those from the sustained virological responders at the end and at 4 weeks after the completion of therapy (Fig. 7) ($P < 0.001$).

DISCUSSION

In the PEG-IFNα and ribavirin therapy for chronic hepatitis C, viral and host factors are critically involved in the efficacy of treatment. As for viral factors, HCV

Fig. 4. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B, C, F–H**), and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D, E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. PBMC, NK are shown in Figure 2. *$P < 0.05$ (sustained virological responders vs. transient responders).

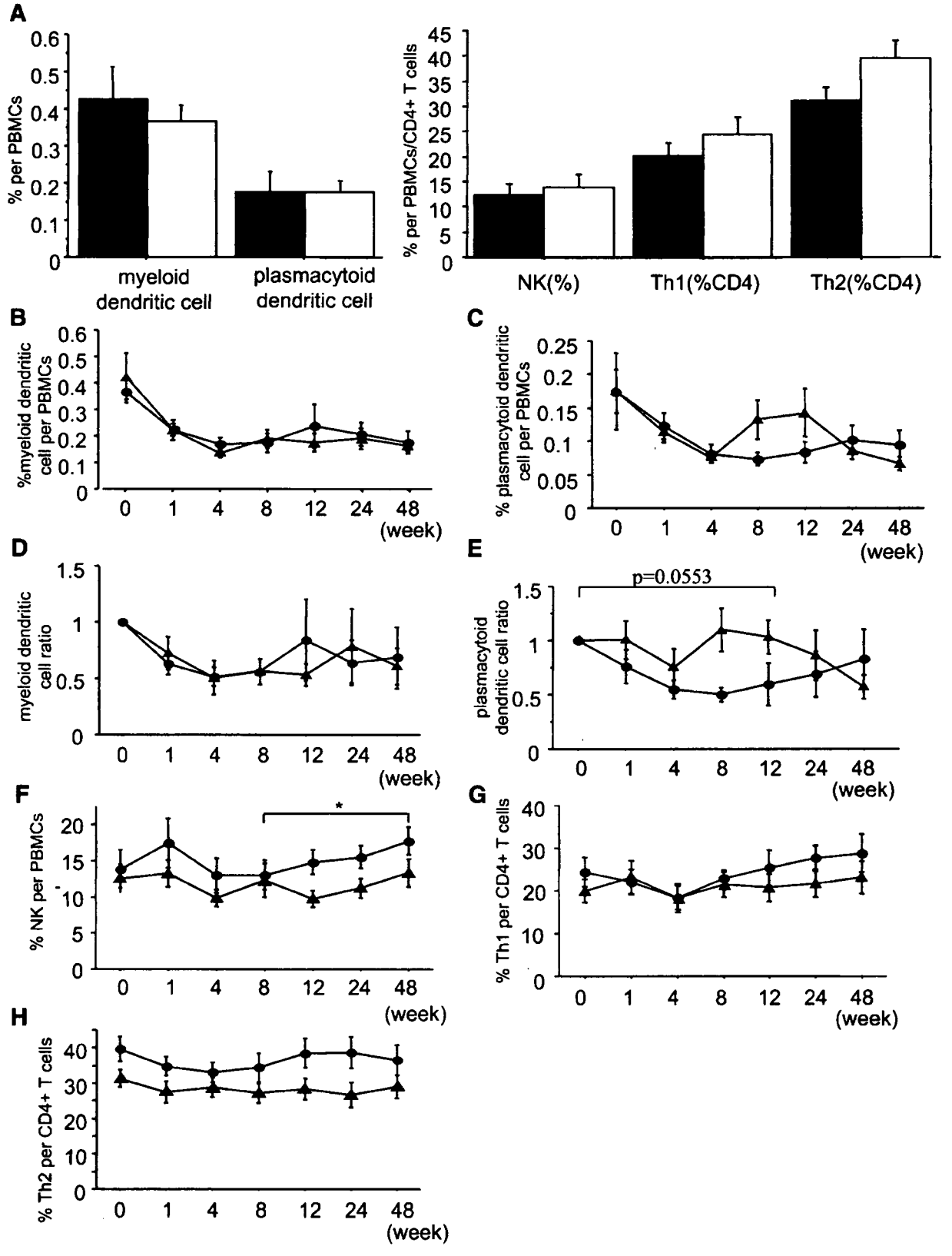
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
%myeloid dendritic cell per PBMCs
(week)
C
% plasmacytoid dendritic cell per PBMCs
(week)
D
myeloid dendritic cell ratio
(week)
E
p=0.0553
plasmacytoid dendritic cell ratio
(week)
F
*
% NK per PBMCs
(week)
G
% Th1 per CD4+ T cells
(week)
H
% Th2 per CD4+ T cells
(week)

Fig. 4.

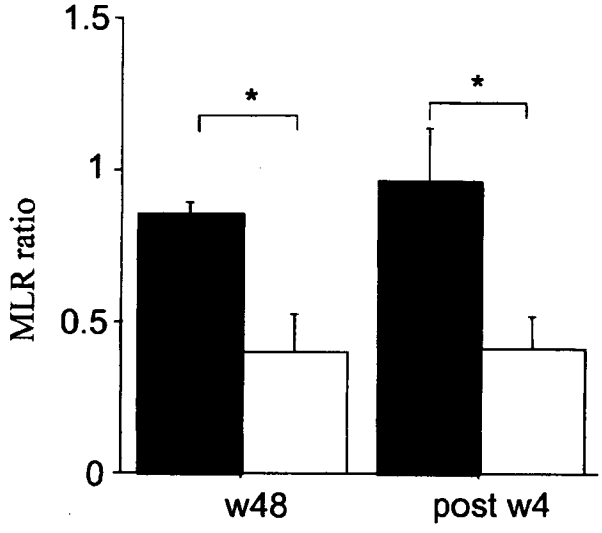

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. Black bars indicate sustained virological responders and white bars indicate transient responders. *$P < 0.05$.

genotypes and baseline HCV RNA titers are major determinants dictating therapeutic outcomes. In addition, failure of rapid decline in serum HCV RNA from the beginning of the treatment, i.e., non-early virological response, has been used as a negative predictor for sustained virological response. Alternatively, the enhancement of immunity has been implicated to play a key role in the successful responses in PEG-IFNα and ribavirin therapy. However, it is yet to be determined which parameters are practically feasible for the assessment of treatment-induced immune responses correlating with therapeutic efficacy.

In the present study, it was determined whether the frequencies of dendritic cells, NK cells, Th1 and Th2 cells, as well as dendritic cell function in patients are related to the outcome of the PEG-IFNα and ribavirin therapy. By comparing these markers in the course of the treatment between sustained virological responders and non-sustained virological responders, it was demonstrated that non-sustained virological responders showed impaired dendritic cell function in MLR than sustained virological responders. When the analyses were extended to comparison between sustained virological responders and transient responders, transient responders exhibited (1) lower plasmacytoid dendritic cell ratio, (2) higher NK cell frequency, and (3) impaired dendritic cell function than sustained virological responders. Of particular interest were the findings of a lower plasmacytoid dendritic cell ratio as well as lower MLR even in transient responders with early response compared to sustained virological responders with early response. Since patients with early response are defined as those who showed negative serum HCV RNA at Week 12, they are considered to be similar in virological response to the combination therapy. Thus, such parameters could serve as immunological markers for virological relapse, presumably being independent of the early virological response.

In general, homeostasis of blood cell number is regulated by their life span and their recruitment from the bone marrow to circulating blood. A reduction of blood cell numbers is frequently observed in patients who are treated with PEG-IFNα and ribavirin combination therapy, which may be due to bone marrow suppression, enhancement of cellular apoptosis, or alteration of localization. However, the dynamics of dendritic cell subsets or NK cells under combination therapy is yet to be clarified. Some investigators have reported that the frequency or the absolute number of blood dendritic cell is dynamically changed by various stresses, such as infection [Hotchkiss et al., 2002] or surgery [Ho et al., 2001]. The present study showed that reduction of plasmacytoid dendritic cells after the introduction of combination therapy is much greater in the transient responders than in the sustained virological responders. IFNα is reported to act as a regulatory factor on $CD11c^-$ dendritic cells to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [Ito et al., 2001]. Thus, patients who respond well to IFNα, as demonstrated by better plasmacytoid dendritic cell survival during the treatment, are likely to have better chances to eradicate HCV. Limited information is available about the factors influencing the number of NK cells. In chronic HCV infection, it has been reported that the progression of liver disease is associated with a decrease of peripheral as well as liver-residing NK cells [Kawarabayashi et al., 2000]. It is plausible that the lower frequency of peripheral NK cells in the sustained virological responders compared to the transient responders, as shown in this study, may be related to the accumulation of NK cells in the liver, where they presumably produce IFNγ to suppress HCV replication. Further study is needed to disclose the reasons for the dynamics of these cells being related to the virological response in the combination therapy.

Fig. 6. Pretreatment frequency of blood cells and changes during 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F–H**) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean±SEM of seven sustained virological responders with early response and four transient responders with early response. PBMC, NK are shown in Figure 2. *$P < 0.05$ (sustained virological responders vs. transient responders).

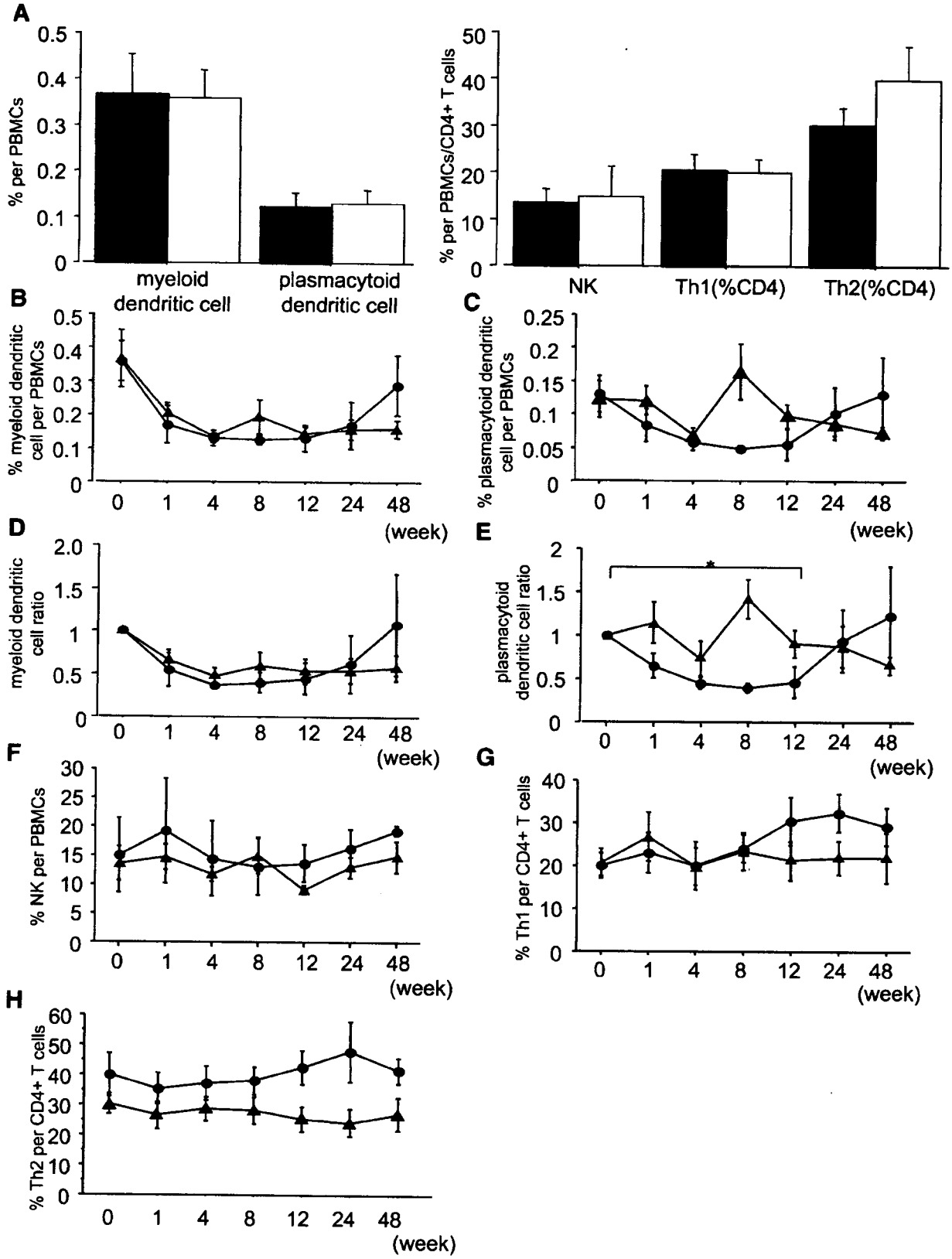
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
(week)

Fig. 6.

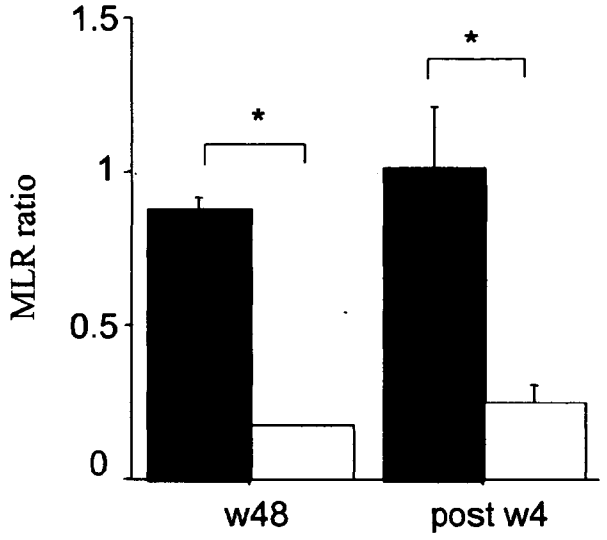

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of seven sustained virological responders with early response and four transient responders with early response. Black bars indicate sustained virological responders and white bars indicate transient responders, respectively. *$P < 0.05$.

In the present study, non-sustained virological responders or transient responders showed a lesser capacity for dendritic cell function than sustained virological responders at the end and after cessation of the therapy. Even in the patients who lost serum HCV RNA at Week 12, the dendritic cell function was lower in transient responders than sustained virological responders. One of the mechanisms of impaired dendritic cell function in non-sustained virological responders or transient responders may be residual HCV both in serum and in cells. It is reported that the relapse rate was higher in the patients who were positive for HCV RNA by sensitive transcription-mediated amplification (TMA) at the end of combination therapy than those who were negative for it, even when they were negative for HCV RNA by conventional PCR [Gerotto et al., 2006]. Other investigators have shown that residual HCV is detectable by means of sensitive PCR in blood cells from patients who cleared HCV from the serum by IFNα and ribavirin combination therapy [Pham et al., 2004], supporting the possibility that blood cells are reservoirs of HCV replication. Taking these findings into consideration, it is conceivable that a small quantity of HCV might exist in the blood cells in some transient responders. Since direct HCV infection of monocytes or blood dendritic cells is considered to be one of the mechanisms of the functional impairment of dendritic cell [Navas et al., 2002; Goutagny et al., 2003; Ducoulombier et al., 2004], persistent HCV may delay the restoration of dendritic cell function in non-sustained virological responders or transient responders compared to sustained virological responders.

In summary, it was shown that the frequencies of plasmacytoid dendritic cells or NK cells and dendritic cell function might be related to the outcomes of the combination therapy. Since the present study was performed with a relatively small number of patients, a greater number of patients should be examined in order to validate the feasibility of using these as immunological markers of relapse. The prediction of virological non-response or relapse during therapy can help improve the clinical outcomes of treated patients, as prolongation of combination therapy offers potential relapsers a better chance of sustained virological response by suppressing HCV reappearance.

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Impaired ability of interferon-alpha-primed dendritic cells to stimulate Th1-type CD4 T-cell response in chronic hepatitis C virus infection

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SUMMARY. In interferon-alpha (IFN-α)/ribavirin combination therapy for chronic hepatitis C (CHC), an enhanced T helper 1 (Th1) response is essential for the eradication of hepatitis C virus (HCV). We aimed to elucidate the role of IFN-α or IFN-α/ribavirin in dendritic cell (DC) ability to induce Th1 response in HCV infection. We generated monocyte-derived DC from 20 CHC patients and 15 normal subjects driven by granulocyte–macrophage colony-stimulating factor and interleukin 4 (IL-4) without IFN-α (GM/4-DC), with IFN-α (IFN-DC), with ribavirin (R-DC) or with IFN-α/ribavirin (IFN/R-DC) and compared their phenotypes and functions between the groups. We also compared them in 14 CHC patients between who subsequently attained sustained virological response (SVR) and who did not (non-SVR) by 24 weeks of IFN-α/ribavirin therapy. Compared with GM/4-DC, IFN-DC displayed higher CD86 expression, but lesser ability to secrete IL-10 and were more potent to prime $CD4^+$ T cells to secrete IFN-γ and IL-2. Such differences were more significant in healthy subjects than in CHC patients. No additive effect of ribavirin was observed in DC phenotypes and functions *in vitro* either which was used alone or in combined with IFN-α. However, in the SVR patients, an ability of IFN/R-DC to prime T cells to secrete IFN-γ and IL-2 was higher than those of IFN-DC and those of IFN/R-DC in the non-SVR group, respectively. In conclusion, DC from CHC patients are impaired in the ability to drive Th1 in response to IFN-α. Such DC impairment is restored *in vitro* by the addition of ribavirin in not all but some patients who cleared HCV by the combination therapy.

Keywords: chronic hepatitis C, dendritic cells, hepatitis C virus, interferon-alpha, ribavirin, Th1.

INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection is evident with 170–200 million being affected worldwide [1,2]. Approximately 30% of those exposed to HCV are able to eradicate it after the initial exposure, while the remaining 70% cannot, subsequently developing to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [3]. In the early phase of acute HCV infection, HCV continues to replicate in the liver, where interferon-alpha (IFN-α) and IFN-inducible genes are significantly induced, suggesting that HCV hampers the execution of IFN-α-mediated anti-virus or immune response [4,5]. In order to eradicate HCV from chronically infected patients, IFN-α has been used. However, IFN-α monotherapy successfully eradicates HCV in only 10–20% of treated patients [6], the efficacy being lower in patients infected with HCV genotype 1 than those with other genotypes [7]. Pegylated IFN-α in combination with ribavirin has been widely used as the first-line anti-HCV therapy, as the rate of HCV clearance has been improved to be 46–56% of the treated patients [8]. These clinical results show that IFN-α alone is not sufficient to initiate anti-HCV activity in some chronically infected patients.

Both IFN-α and ribavirin have an immunomodulatory effect on immune cells in addition to their direct antiviral effects; however, the mechanisms of action of these drugs during the therapy are poorly understood. IFN-α directly or indirectly stimulates T helper 1 (Th1) cell development and

Abbreviations: CHC, chronic hepatitis C; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; GM-CSF, granulocyte–macrophage colony-stimulating factor; HCV, hepatitis C virus; IFN-α, interferon-alpha; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MoDC, monocyte-derived DC; PBMC, peripheral blood mononuclear cells; SVR, sustained virological response; Th1, T helper 1

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appears to suppress Th2 cell development [9–13]. Ribavirin has been shown to enhance antiviral type 1 and suppress type 2 cytokine expression in human T cells [14] and may significantly promote the Th1 immune response *in vivo* [15]. Several investigators have reported that the enhancement of a HCV-specific Th1 response is necessary for HCV eradication by IFN-α and ribavirin combination therapy [16–19]. As dendritic cells (DC) are the most potent antigen-presenting cells (APC) that regulate Th1 or Th2 differentiation *in vivo* [20,21], it is possible that IFN-α or a combination of IFN-α and ribavirin may cause DC to modulate Th1 differentiation. In chronic HCV infection, we as well as others have demonstrated that monocyte-derived DC (MoDC) have impaired allostimulatory capacity [22–24]. However, it is still uncertain whether or not IFN-α or a combination of IFN-α and ribavirin affects DC development and alters DC function in chronic HCV infection.

In the present study, we hypothesize that IFN-α influences on DC differentiation and subsequently enhances the DC capacity to induce the Th1 response. To clarify whether or not DC in HCV infection similarly respond to IFN-α or a combination of IFN-α and ribavirin, we generated MoDC in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 without IFN-α (GM/4-DC), with IFN-α (IFN-DC), with ribavirin (R-DC), or with IFN-α and ribavirin (IFN/R-DC) and compared their phenotypes and functions between HCV-infected patients and normal subjects. We demonstrate here that MoDC generated in the presence of IFN-α gain the ability to induce a Th1 response. However, with chronic HCV infection, MoDC fail to respond sufficiently to IFN-α, resulting in a lesser ability to induce a Th1 response than those from healthy counterparts. We show that IFN-α and ribavirin in combination enhance the ability of DC to induce a Th1 response *in vitro* in some HCV-infected patients, which may be associated with a subsequent sustained virological response (SVR) by the combination therapy.

MATERIALS AND METHODS

Subjects

Twenty patients who were both positive for anti-HCV Ab and serum HCV RNA were enrolled in the present study. All of them were infected with HCV serotype 1 and had shown elevated or fluctuated serum alanine aminotransferase levels for more than 6 months at the enrollment. They were negative for HBV and HIV, and displayed no sign of other liver diseases. None of the patients had previously been treated with IFN-α-based therapy. The controls were 15 age-matched normal subjects who were negative for anti-HCV Ab, HBsAg, and anti-HIV Ab. The clinical backgrounds of these subjects are shown in Table 1. Informed consent was obtained from each patients included in the study. Fourteen of 20 patients were subsequently treated with 6 MU of IFN-α2b (Schering-Plough, Kenilworth, NJ, USA) three times a week with 600–1000 mg of ribavirin (Schering-Plough) for 24 weeks. Virological response to IFN-α and ribavirin combination therapy was assessed 24 weeks after the completion of the therapy. The 'SVR group' was defined as the patients who showed negative serum HCV RNA at the end of therapy and continued to be negative for 24 weeks thereafter. Transient responders were defined as those who showed negative serum HCV RNA at the end of therapy but displayed HCV RNA reappearance within 24 weeks after the therapy cessation. Non-responders showed positive serum HCV RNA throughout the treatment. The 'non-SVR group' consisted of transient responders and nonresponders in this study.

Table 1 The clinical backgrounds of normal subjects and chronic hepatitis C patients*

	Normal subjects (n = 15)	CHC patients (n = 20)
Men/women	12/3	15/5
Age (years)	41 ± 9	47 ± 12
ALT level (IU/L)	ND	77 ± 47
Serum HCV-RNA (Meq/mL)	ND	6.0 ± 1.5

ALT, alanine aminotransferase; ND, not determined. *Values are expressed as the mean ± SD.

Reagents

Recombinant human GM-CSF and interleukin 4 (IL)-4 were purchased from Peprotech (Rocky Hill, NJ, USA). Human IFN-α was provided by Otsuka Pharmaceuticals (Tokyo, Japan). Ribavirin was obtained from Sigma-Aldrich (St Louis, MO, USA). Neutralizing mouse anti-human IL-10 Ab (clone #23738) and isotype mouse IgG were obtained from R&D Systems (Minneapolis, MN, USA).

Generation of MoDC

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood or buffy coats using Ficoll–Hypaque density gradient centrifugation. Monocytes were immunomagnetically separated from PBMC by using anti-CD14 monoclonal antibody (mAb)-coated microbeads (Miltenyi Biotec, Bergish-Gladbach, Germany). To generate MoDC, monocytes were cultured for 7 days at 37 °C with 5% CO_2 in iscove's modified dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal calf serum, 50 IU/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 10 mM Hepes buffer, 10 mM nonessential amino acid in the presence of 50 ng/mL GM-CSF and 10 ng/mL IL-4. To examine the influence of IFN-α

with or without ribavirin on the development of MoDC, we added 100 U/mL IFN-α or 3 μg/mL ribavirin or a combination of these to the cells from the beginning of the culture as 100 U/mL of IFN-α and 3 μg/mL of ribavirin are close to the peak serum concentration of these drugs in the patients who were administrated intramuscularly at 5 MU of IFN-α and 400 mg/day of ribavirin, respectively [25,26]. On day 4 of the culture, half of the medium was replaced with fresh medium containing equal concentrations of GM-CSF, IL-4, IFN-α or ribavirin. The cells were harvested on day 7 and subjected to phenotypic and functional analysis. In order to examine the relationship between *in vitro* DC function and the therapeutic response to a combination of IFN-α and ribavirin therapy, we generated MoDC as described above from PBMC obtained before the treatment and compared DC function between the patients who attained SVR and those who did not.

Phenotypic analysis of MoDC

The cells were incubated in phosphate-buffered saline containing 2% bovine serum albumin and 0.1% sodium azide with FITC-, PE-, or PerCP-conjugated mouse monoclonal anti-human Ab against CD86 (clone #IT2.2), CD80 (clone #L307.4) (BD PharMingen, San Diego, CA, USA), human leukocyte antigen-DR(HLA-DR) (clone #L243) (BD Biosiences, San Jose, CA, USA), or CD83 (clone #HB15a) (Immunotech, Marseille, France) or isotype Abs for 20 min at 4 °C. The expressions of these markers on MoDC were analysed by fluorescence-activated cell sorter (FACS) calibur (Becton Dickinson Immunocytometry Systems, San Diego, CA, USA) using CellQuest software (Becton Dickinson Immunocytometry Systems).

Analysis of cytokine production from MoDC

On day 7 of culture, 10^4/well of MoDC were stimulated with 5×10^4/well of human CD40L-transfected mouse L-cells (CD40L-L-cells) for 24 h at 37 °C, 5% CO_2. The supernatants were stored at −80 °C until being subjected to ELISA.

Analysis of T-cell polarization by MoDC

To examine the capacity of DC to polarize CD4 T cells, day 7 MoDC were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days (DC/T cell ratio = 1/10). Naïve $CD4^+$ T cells were separated from PBMC of healthy donors by immunomagnetic separation using a human naïve $CD4^+$ T-cell enrichment cocktail and anti-CD45RO mAb (Stemcell Technologies Inc., Seattle, WA, USA) according to the manufacturer's instructions. More than 98% of the collected cells were $CD4^+$ and $CD45RO^-$ as assessed by FACS (data not shown). In some series of experiments, 50 μg/mL of anti-human IL-10 Ab or mouse IgG was added to the cells from the beginning of the co-culture. On day 4 of the culture, half of the supernatants were collected to assess the IL-2 release from the cells. On day 6 of co-culture, the cells were harvested and stimulated with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich). For ELISA, the supernatants were collected 24 h after the stimulation of cells.

Enzyme-linked immunosorbent assay

The concentrations of IL-10, IL-12p70, IL-2, and IFN-γ in the supernatants were determined by ELISA using matched pairs of relevant mAbs (Endogen, Woburn, MA, USA) according to the manufacturer's instructions. The detection thresholds of IL-10, IL-12p70, IL-2, and IFN-γ are 10, 10, 10 and 16 pg/mL, respectively.

Statistical analysis

Statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). The unpaired two-tailed Mann–Whitney *U*-test was used to compare differences in the level of cytokine and surface marker expression.

RESULTS

IFN-α significantly enhanced CD86 expression on MoDC from chronic hepatitis C patients and normal subjects

First, in order to examine the role of IFN-α in GM-CSF and IL-4-driven DC development, we compared the phenotypes and functions between GM/4-DC and IFN-DC. After 7 days of culture with GM-CSF, IL-4, with or without IFN-α, the cells were negative for CD14 (data not shown), but were strongly positive for CD86 and HLA-DR, and moderately positive for CD80, whereas their expression of CD83 was barely detectable (Fig. 1a).

In this study, we added IFN-α to the cells for DC generation from the beginning of the culture. In the preliminary experiments for the assessment of IFN-α dose–response relationship, we examined the expressions of CD86 and CD80 as representatives on DC cultured with different concentrations of IFN-α and fixed concentrations of GM-CSF and IL-4. The expressions of these molecules on DC were upregulated even as low as 100 U/mL of IFN-α, the degree of which did not differ even at higher concentrations up to 1000 U/mL (data not shown).

The comparison of the expressions of these markers showed that CD86 expression on the cells generated in the presence of GM-CSF and IL-4 from HCV-infected patients was lower than those from normal donors (Fig. 1a). IFN-α upregulated the levels of CD86 on MoDC regardless of HCV infection (Fig. 1a). The CD86 upregulation was more significant in normal donors as demonstrated by comparison of the ratios of mean fluorescence intensity (MFI) between IFN-DC and GM/4-DC (Fig. 1a,b).

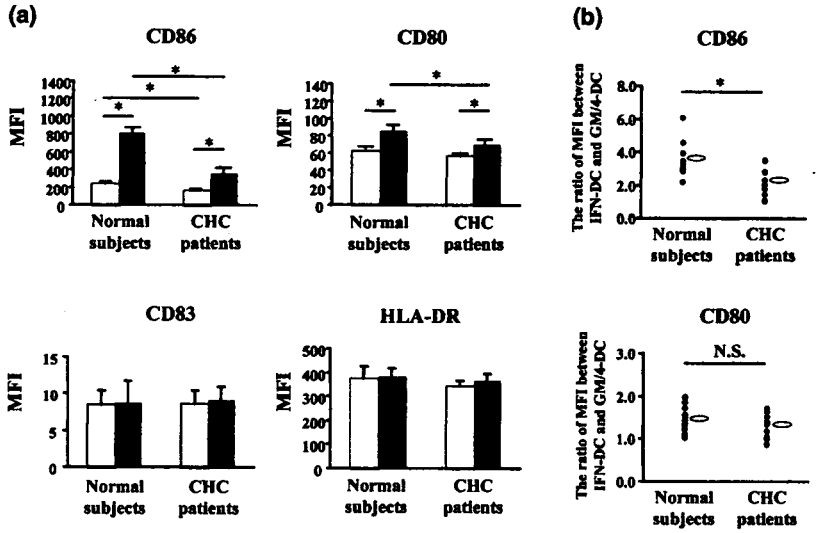

Fig. 1 Interferon (IFN)-α enhanced CD86 and CD80 expression on monocyte-derived DC, in which the degrees of CD86 was higher in healthy subjects than those in chronic hepatitis C (CHC) patients. (a) Monocyte-derived DC were generated from monocytes by 7-day culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 (GM/4-DC) or with GM-CSF, IL-4 and IFN-α (IFN-DC). On day 7, the mean fluorescence intensity (MFI) of CD86, CD80, CD83, and HLA-DR was determined by fluorescence-activated cell sorter analysis. The figures represent the mean values of MFI ± SEM, from 12 healthy donors and 15 CHC patients. Open bars, GM/4-DCs; close bars, IFN-DC. (b) The ratios of MFI of CD86 and CD80 between IFN-DC and GM/4-DC, from 12 healthy donors and 15 CHC patients are shown. The horizontal bars indicate median. *$P < 0.05$ by Mann–Whitney *U*-test. N.S., not significant.

As for CD80, IFN-α enhanced CD80 expression on MoDC from either patients or healthy donors; however, the ratios of MFI of CD80 between IFN-DC and GM/4-DC were not different between them (Fig. 1a,b). In contrast, there was no significant difference in CD83 and HLA-DR expression either in the presence or in the absence of IFN-α regardless of HCV infection (Fig. 1a). These results show that IFN-DC are mature but not full-matured, as evidenced by their enhanced CD86 but limited CD83 expression, respectively [27]. Thus, IFN-DC from HCV-infected patients showed a lesser degree of phenotypic maturation than those from healthy donors as judged by CD86 expression.

MoDC from chronic hepatitis C patients displayed impaired capacity to induce Th1 cells in response to IFN-α

To investigate whether IFN-α affects the capacity of MoDC to induce a Th1 response, we examined the IFN-γ and IL-2 production from CD4 T cells primed by IFN-DC. With MoDC from normal subjects, IFN-DC stimulated allogeneic naïve CD4 T cells to produce more IFN-γ than GM/4-DC (Fig. 2a). In contrast, with MoDC from chronic hepatitis C (CHC) patients, IFN-DC failed to enhance IFN-γ secretion from DC-primed CD4 T cells compared with GM/4-DC (Fig. 2a). The levels of IL-2 in the IFN-DC co-culture were significantly elevated compared with those of GM/4-DC in both patients and donors (Fig. 2a). However, the IL-2 levels from IFN-DC culture in the patients were significantly lower than those in healthy donors (Fig. 2a). Furthermore, the ratios of IL-2 levels between IFN-DC and GM/4-DC co-culture were significantly lower in CHC patients than those of normal subjects (Fig. 2b). These results show that MoDC from CHC patients are less able to induce Th1 cells in response to IFN-α than the healthy counterparts.

IFN-DC showed lesser ability to produce IL-10, more significantly in those from normal donors

To analyse the mechanisms by which IFN-DC from HCV-infected patients displayed an impaired ability to induce a Th1 response, we examined MoDC-derived cytokines stimulated with CD40L-L-cells. In both GM/4-DC and IFN-DC, the levels of IL-12p70 production from MoDC of the patients were significantly lower than those from normal DC (Fig. 3a). However, no enhancement of IL-12p70 release was observed from IFN-DC compared with GM/4-DC regardless of HCV infection (Fig. 3a).

In contrast, with GM/4-DC or IFN-DC, the levels of IL-10 in the patients were higher than those in normal subjects (Fig. 3a). IFN-DC showed lesser ability to release IL-10 than GM/4-DC regardless of HCV infection, with the degree being more significant in healthy donors (Fig. 3a,b). To examine whether the reduced IL-10 production from MoDC is involved in Th1 augmentation, we added neutralizing