enhance the E2 protein-binding activity of C-s3-33 by the multiplication of C-s3-33. Initially, we made pMAL-c2X-based expression vectors encoding two, three, and four repeats of C-s3-33 as MBP-fused proteins, and then expressed them in *E. coli*. We successfully purified two repeats $(C\text{-}s3\text{-}33)_2$ and three repeats $(C\text{-}s3\text{-}33)_3$ of C-s3-33 as soluble forms of the MBP-fused protein; the purification of the four repeats of C-s3-33 failed due to problems with solubility. Using the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, we performed Far-Western blot analysis to compare their E2 protein-binding activities. The result revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$ (Fig. 1). Although the E2 protein-binding activity of C-s3-33 was weaker than that of human LF, the binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$

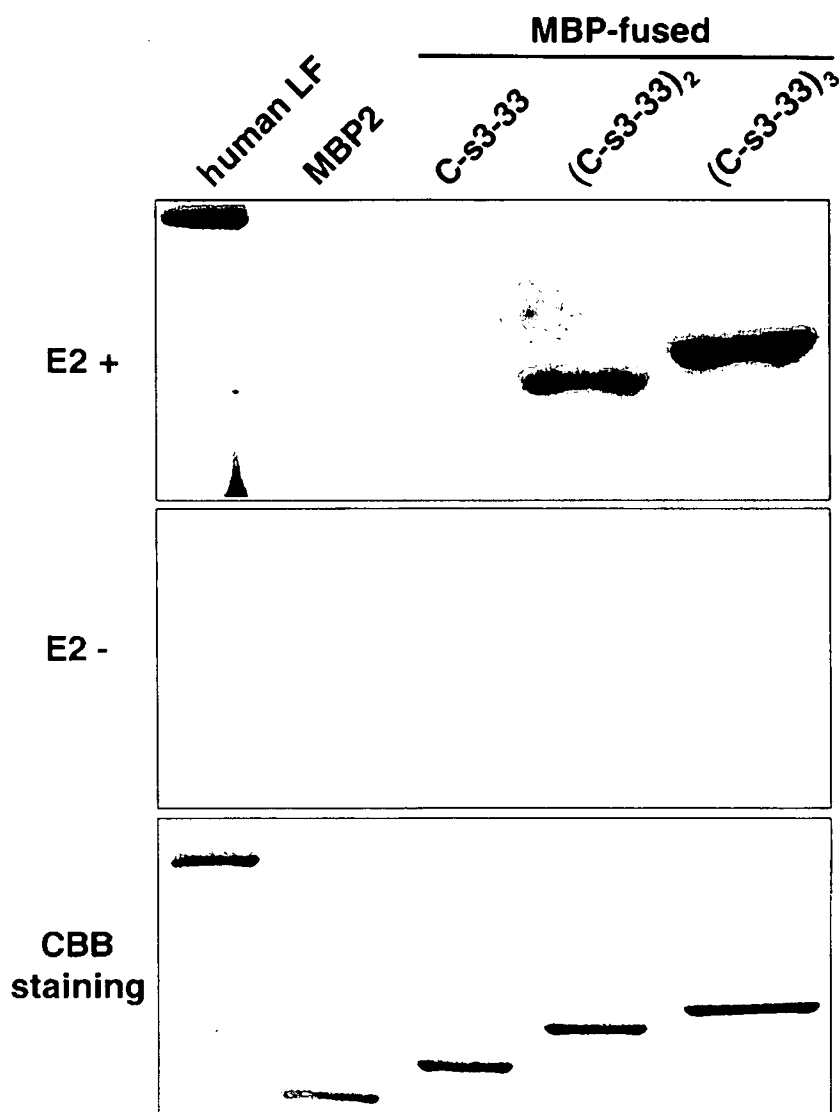

Fig. 1. Comparison of the E2 protein-binding activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$. MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ (0.5 μg each) were resolved by 10% SDS-PAGE. Human LF and MBP2 (0.5 μg each) were also used for the assay as control materials. Far-Western blot analysis using the E2 protein expressed in Chinese hamster ovary cells (14) as a probe was performed as described under "Materials and Methods." Rat monoclonal antibody MO-12 (13) against the E2 protein was used for the detection of the E2 protein bound to MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ as well as human LF. Far-Western blot analysis in the absence of the E2 protein was also performed. The bottom panel shows the results for human LF, MBP2, and MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ detected by staining with Coomassie Brilliant Blue.

became comparable with that of human LF (Fig. 1). To exclude the possibility of cross-reactions between C-s3-33 and the anti-E2 antibody, we performed a Far-Western blot analysis in the absence of the E2 protein. No significant bands were obtained in this control experiment (Fig. 1). The Far-Western blot analysis using normal rat serum instead of anti-E2 antibody also detected no significant bands (data not shown). These results suggest that the specific E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ increase with the degree of multiplication of C-s3-33.

$(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ Efficiently Prevented HCV Infection in PH5CH8 Cells

Since we obtained the expected results that the E2-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33, we next compared their anti-HCV activities in our HCV infection system using PH5CH8 cells (10, 25). The obtained result (Fig. 2) revealed that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ (IC_{50}=10 μM in both) became stronger than that of the C-s3-33 (IC_{50}=23 μM), although their activities were somewhat weaker than that of human LF (IC_{50}=5 μM). These results support the previous suggestion that the E2 protein-binding activity of C-s3-33 contributes to the inhibition of HCV infection (inoculum HCV-O) in human hepatocyte cells (25). However, in our HCV infection system, we failed to clearly show a difference in inhibiting activities between $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, because each standard deviation became somewhat large value due to the low level of cell culture-based HCV infection (20, 25, 31). In order to improve this point, we developed an infection system with VSVΔG*(HCV), a VSV pseudotype bearing the native E1 and E2 proteins derived from HCV-O (30), and this VSV pseudotype was used for further analysis as described below.

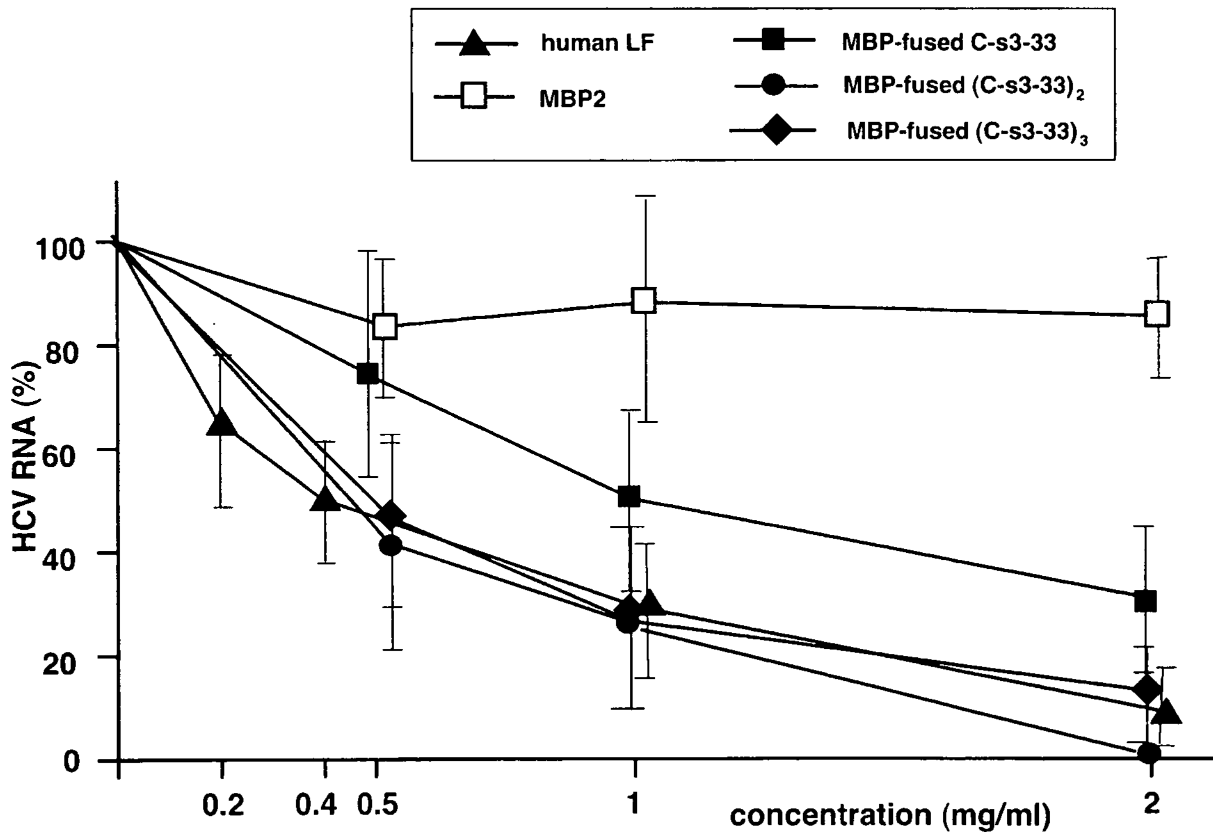

Fig. 2. Anti-HCV activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in an HCV infection system using PH5CH8 cells. PH5CH8 cells and the inoculum HCV-O were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the percent of HCV RNA determined by real-time LightCycler PCR (26). Approximately 2,000 copies of HCV RNA per μg of cellular RNA were reproducibly obtained using this HCV infection system (10, 26). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF and MBP2 were also used for the assay as control materials. The data are means ± SD of triplicates from three independent experiments.

Antiviral Effects of (C-s3-33)$_2$ and (C-s3-33)$_3$ against VSVΔG(HCV) Infection in PH5CH8 Cells*

Since PH5CH8 cells showed good susceptibility to our developed VSV pseudotype, VSVΔG*(HCV) (30), we examined the antiviral effects of (C-s3-33)$_2$ and (C-s3-33)$_3$ against VSVΔG*(HCV) infection in PH5CH8 cells, and compared them with those of the C-s3-33 and human LF. In this experiment, the antiviral effects of human TF and a C-s3-33-relevant fragment of human TF were also examined. The results (Fig. 3) clearly showed that human LF (IC_{50}=0.6 μM) strongly inhibited VSVΔG*(HCV) infection, but that human TF and the C-s3-33-relevant fragment of human TF did not, nor did MBP2, suggesting that inhibition against VSVΔG*(HCV) infection also occurred in an LF-specific manner as observed previously in the HCV infection system (25, 31). These results support previous findings (23, 30) using the VSV pseudotype infection system. Furthermore, we obtained clear results that C-s3-33 showed inhibiting activity against VSVΔG*(HCV) infection, and that its inhibiting activity was increased with multiplication of C-s3-33. The IC_{50} doses of C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ were 17 μM, 5.0 μM, and 3.0 μM, respectively. This result indicates that antiviral activity of C-s3-33 is improved by the duplication and triplication of C-s3-33, although the antiviral activity of (C-s3-33)$_3$ is still weaker than that of human LF. We confirmed that these LF fragments did not inhibit VSVΔG*G (control virus without E1 and E2 proteins) infection in PH5CH8 cells (data not shown). In summary, our results suggest that direct interaction of the C-s3-33 fragment with the E2 protein in VSVΔG*(HCV) prevents the virus infection in PH5CH8 cells.

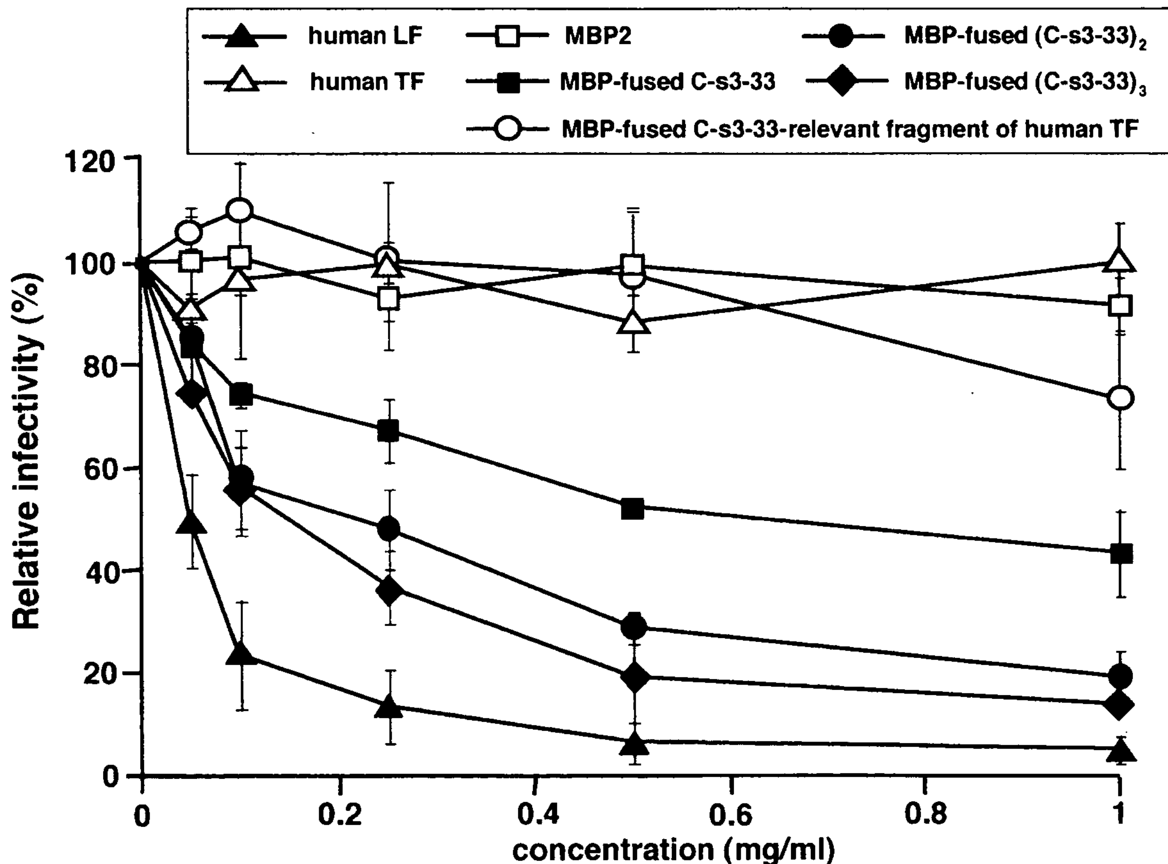

Fig. 3. Antiviral activity of the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ in the infection system of pseudotype virus using PH5CH8 cells. PH5CH8 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$, human LF, human TF, MBP2, and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay as controls. The data are means ± SD of three independent experiments.

Antiviral Effects of (C-s3-33)₂ and (C-s3-33)₃ against VSVΔG(HCV) Infection in HepG2 Cells*

We have shown the inhibiting activities of LF fragments against HCV infection or VSV pseudotype infection in PH5CH8 cells; however, it is not clear whether or not the LF fragments used in this study show inhibiting activities against virus infection in cells other than PH5CH8 cells. To clarify this point, HepG2 cells were used for the analysis, because HepG2 cells showed the highest susceptibility to VSVΔG*(HCV) among 25 cell lines examined (30). As a consequence, we obtained similar results (Fig. 4) with those obtained in the infection system using PH5CH8 cells. The IC_{50} doses of C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ were >12 μM, 7.6 μM, and 3.9 μM, respectively, indicating that, again, the inhibiting activity was increased with multiplication of C-s3-33, although antiviral activity of $(C\text{-}s3\text{-}33)_3$ was still weaker than that of human LF (IC_{50}= 1.2 μM). In conclusion, our results indicated that tandem repeats of C-s3-33 enhanced the inhibiting activity in cell culture-based HCV infection.

Discussion

In our previous (30) and present studies, we showed that pretreatment of VSV pseudotypes with bovine and human LFs reduced the infectivity of VSVΔG*(HCV) and VSVΔG*(E2) bearing only the E2 protein in a dose-dependent manner, whereas pretreatment with TF did not. In contrast, LFs partially inhibited the infectivity of VSVΔG*(E1) bearing only the E1 protein (30). These results suggested that the interaction of LF and the E2 protein is the main contributing factor to the prevention of HCV infection. This idea has been strongly supported by the results obtained in this study. We demonstrated that tandem repeats of C-s3-33, an anti-HCV peptide derived from human LF, enhanced the E2 protein-binding activity and the inhibiting activity

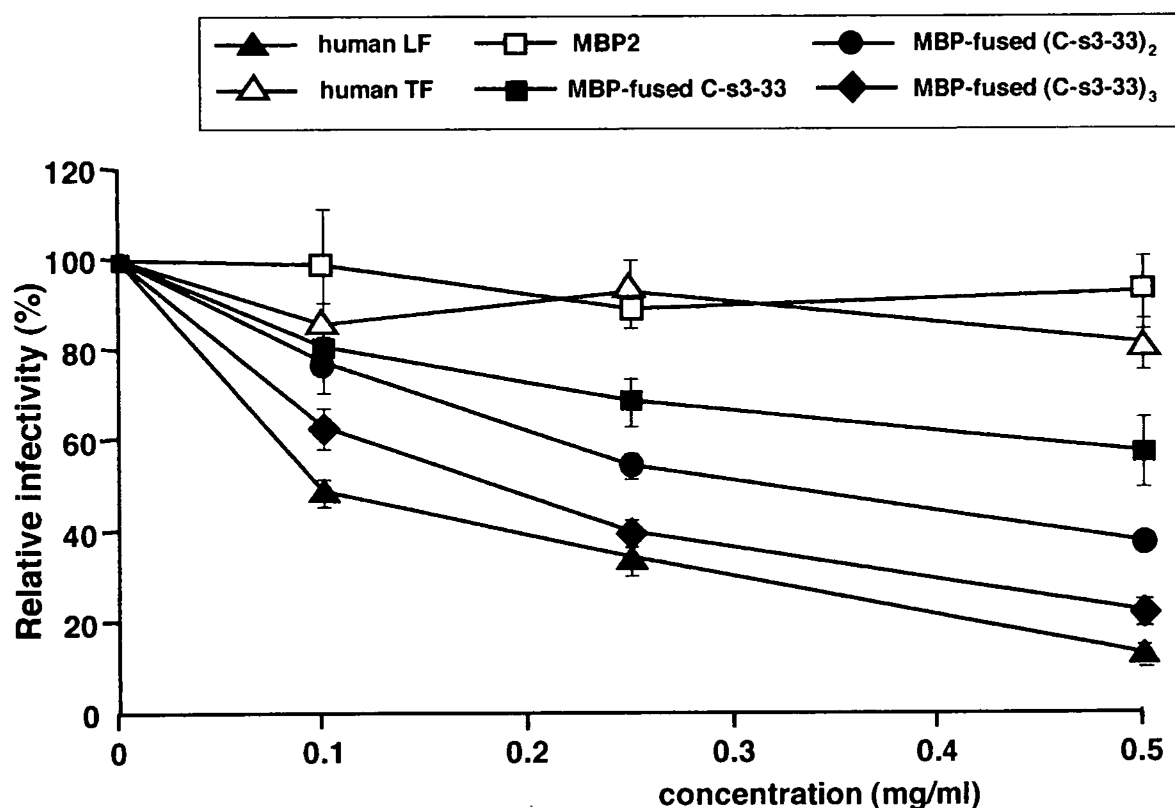

Fig. 4. Antiviral activity of the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in the infection system of the pseudotype virus using HepG2 cells. HepG2 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described in "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF, human TF, and MBP2 were used for the assay as controls. The data are means ± SD of three independent experiments.

against infection by HCV or the VSV pseudotype, VSVΔG*(HCV), in human hepatic cell lines. These results strongly suggest that the direct interaction between C-s3-33 and the E2 protein plays a central role in the inhibition of HCV infection by LF.

Since C-s3-33 or repeated forms of C-s3-33 could prevent HCV and VSVΔG*(HCV) infection, C-s3-33 must bind to a region other than the region (aa 441–500 of E2 protein) required for heteromeric complex formation between E1 and E2 proteins. Our preliminary results suggested that the C-s3-33 bound to aa 411–500 and aa 600–661 of the E2 protein, indicating that the target sites of C-s3-33 may be plural. This result suggests a rather complex interaction between C-s3-33 and the E2 protein. To clarify this point, further comprehensive analysis will be needed.

Although tandem repeats of C-s3-33 enhanced the anti-HCV activity compared with that of the C-s3-33, the fact that their antiviral activities were still several-fold weaker than that of original human LF remains a subject to be resolved. As one approach to increase anti-HCV activity, tandem repeats of C-s3-33-relevant fragment of bovine LF may be useful, because we previously observed that the anti-HCV activity of bovine LF (IC_{50}=1.5 μM) was stronger than that of human LF (IC_{50}=5.0 μM) (26), and that the E2 protein-binding activity of the C-s3 (93 aa)-relevant fragment of bovine LF was stronger than that of C-s3 (25). Since 10 aa out of 33 aa differ between C-s3-33 and its relevant fragment of bovine LF, some aa substitutions between both fragments may help to further increase the anti-HCV activity of LF-derived peptides. Alternatively, some spacer between the C-s3-33 repeats may be needed. Therefore, further trials will be needed to achieve the maximum anti-HCV activity of C-s3-33.

In conclusion, the results of the present study demonstrated that tandem repeats of human LF-derived 33 aa prevented HCV infection more strongly than the 33 aa, and suggest that this repeated form will be useful as a novel anti-HCV reagent.

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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. ***J. Med. Virol. 79:511–521, 2007.***

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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.

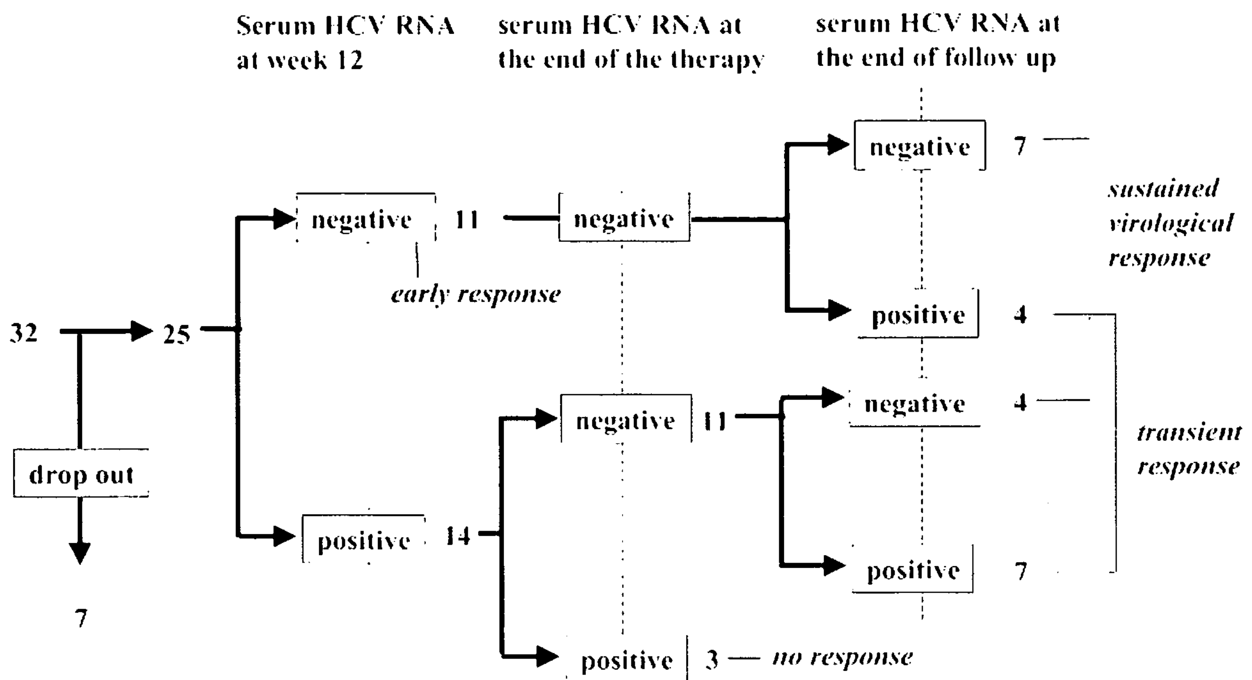

Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 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.

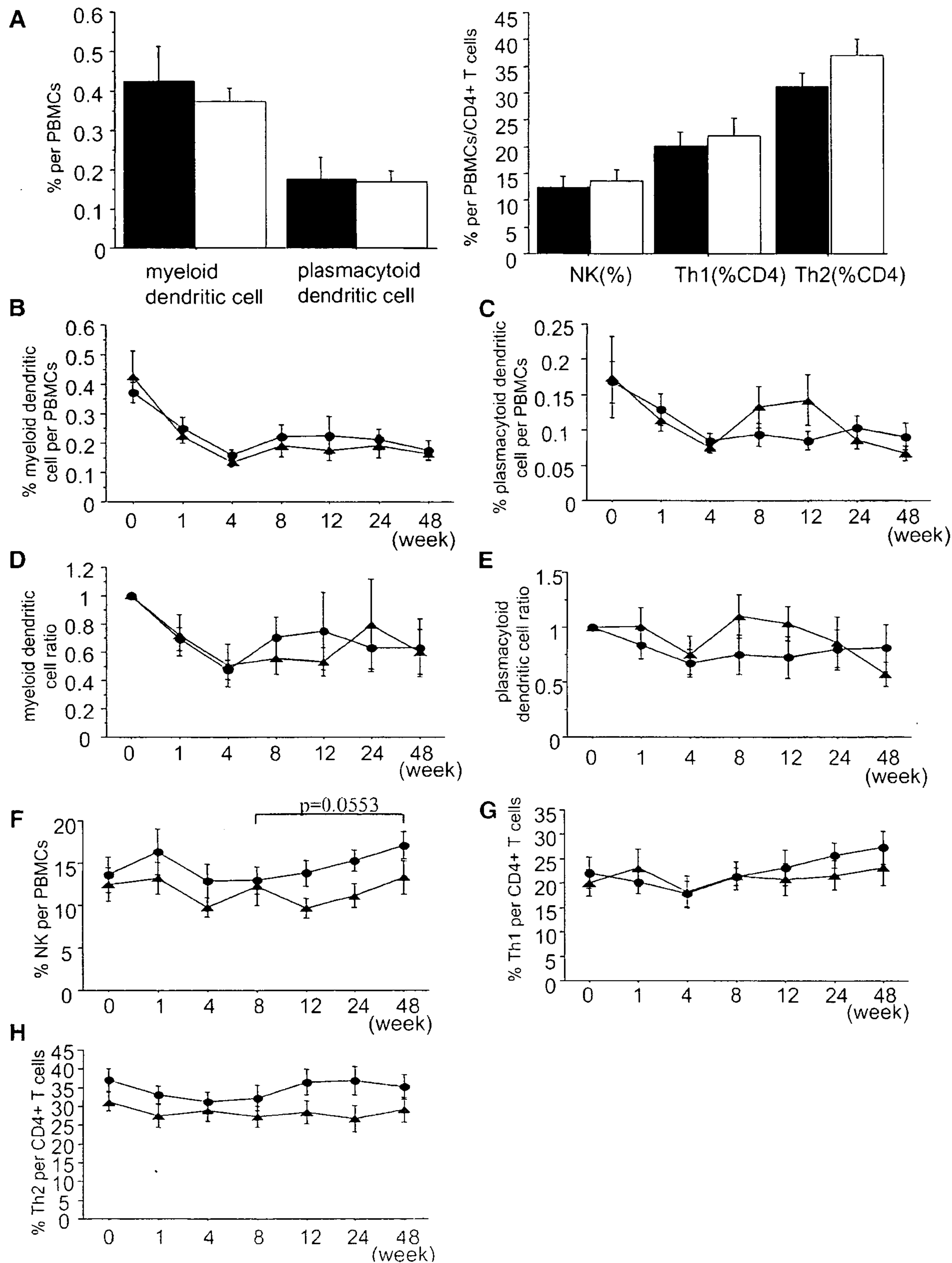

Fig 2.

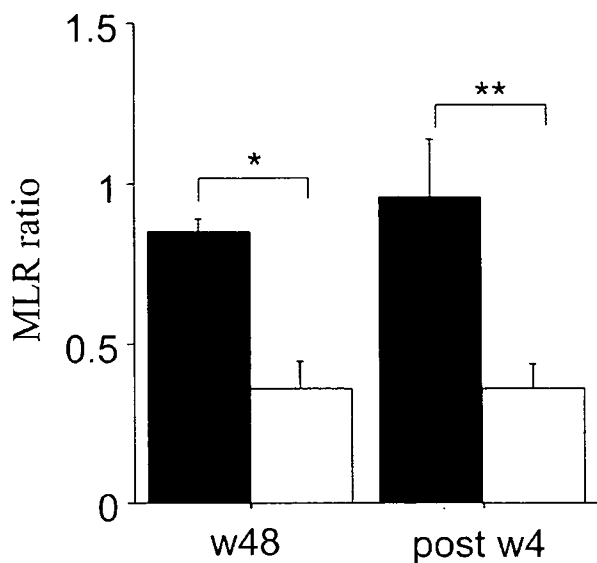

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic 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).

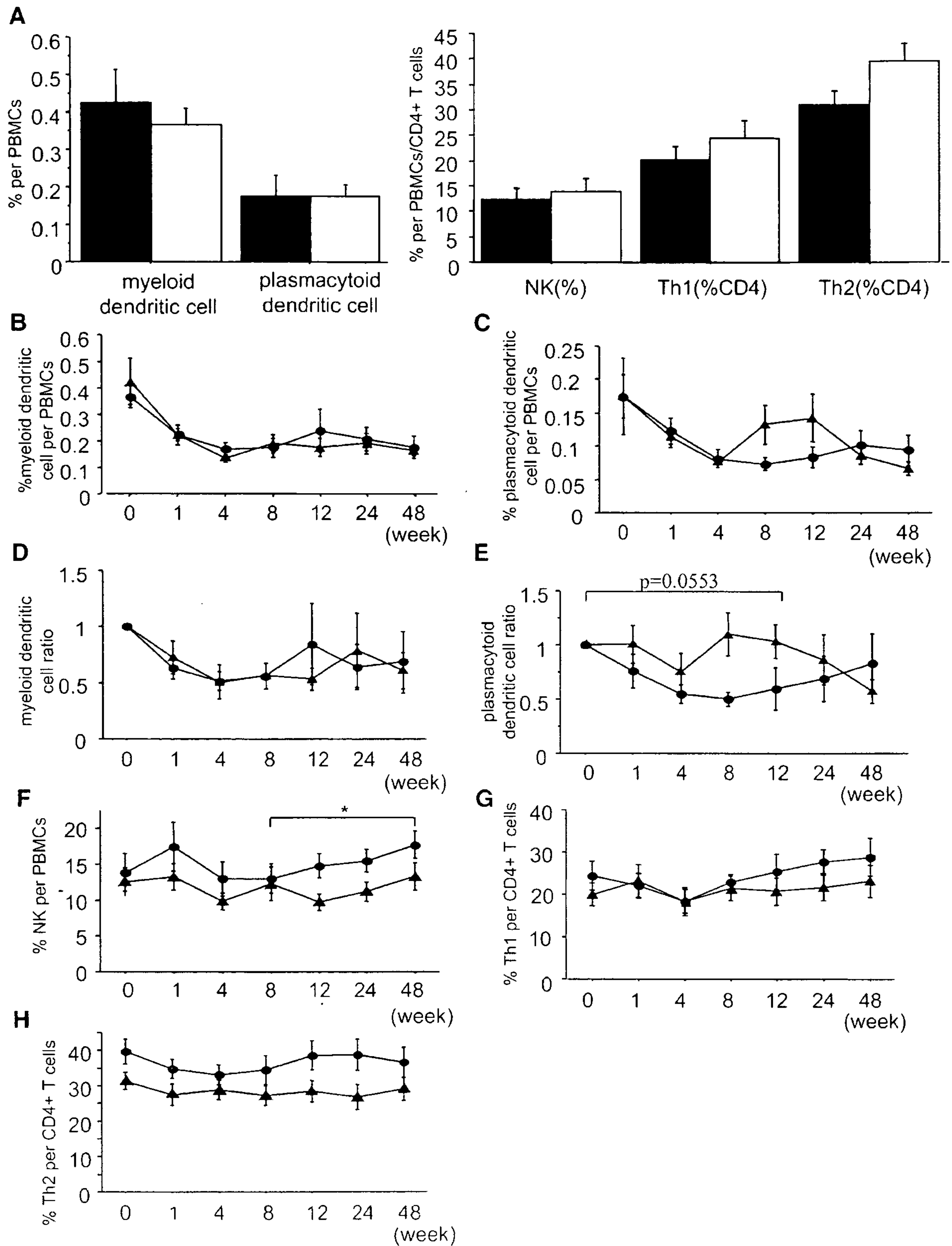

Fig. 4.

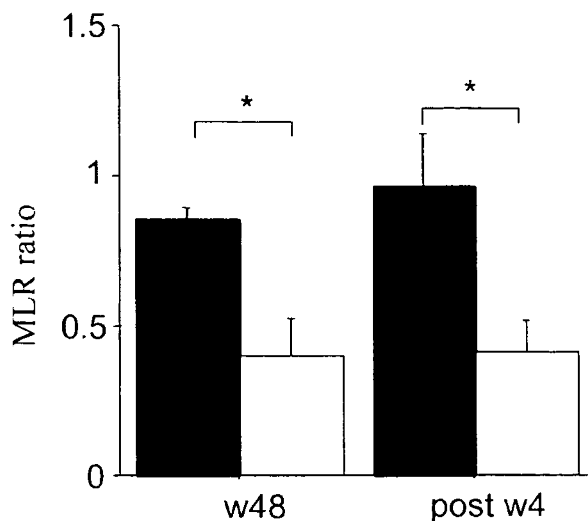

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were 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).

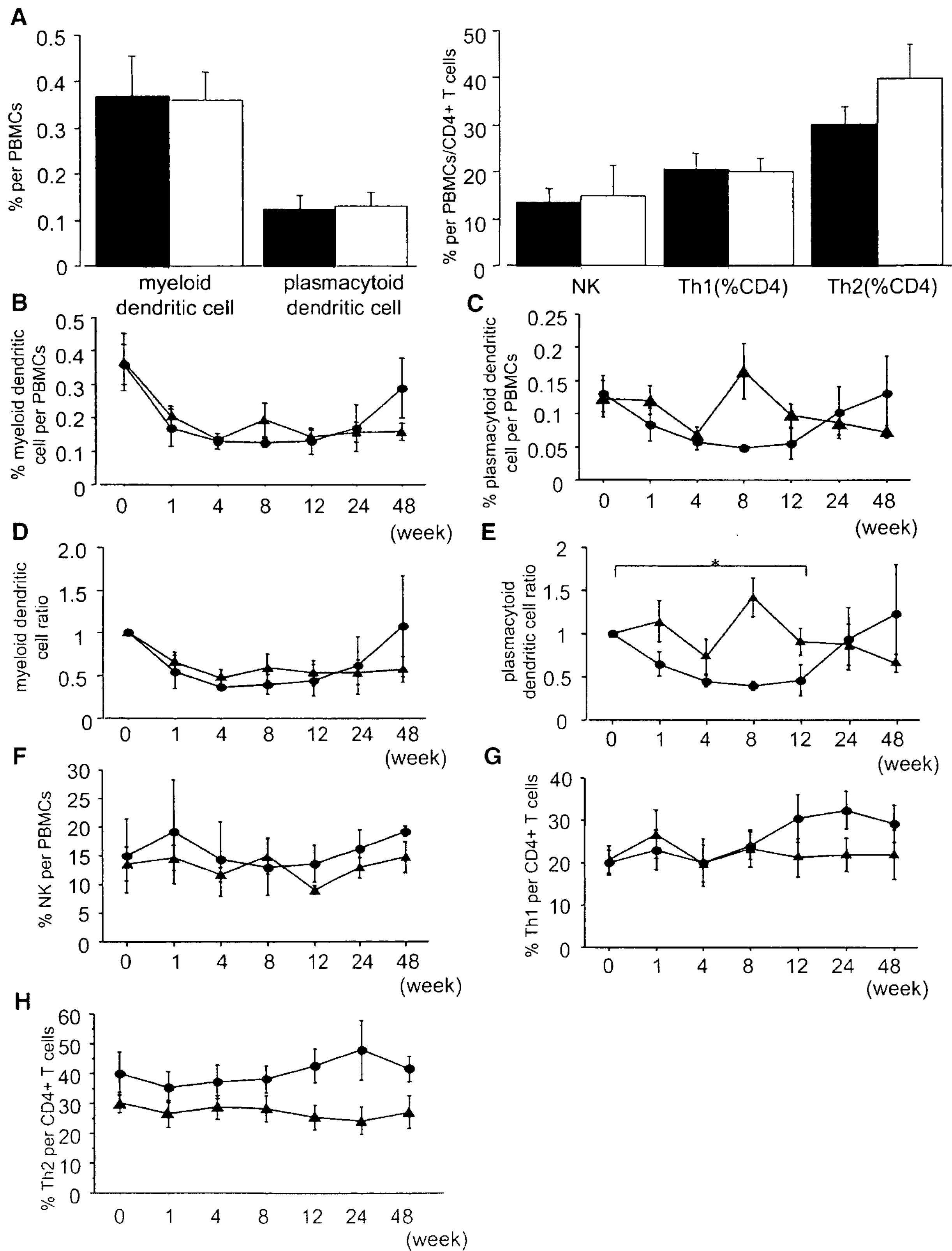

Fig. 6.

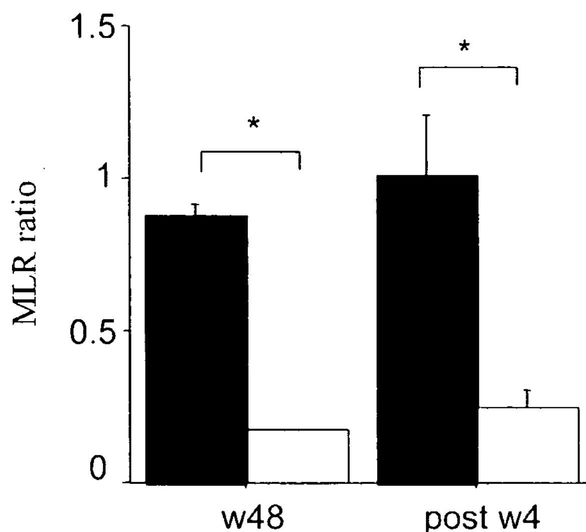

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic 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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Innate immunity in hepatitis C virus infection: Interplay among dendritic cells, natural killer cells and natural killer T cells

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Sequential activation of innate and adaptive immune response is crucial for virus elimination. We thus sought to clarify the role of innate immune system in the pathogenesis of hepatitis C virus (HCV) infection. Dendritic cells (DC) sense virus infection via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I), resulting in the secretion of type-I interferons (IFN) and inflammatory cytokines. Blood DC consist of two subsets; myeloid DC (MDC) and plasmacytoid DC (PDC). In MDC from HCV-infected patients, regardless of higher expression of TLR2, TLR4 and RIG-I compared to the controls, the levels of TLR/RIG-I-mediated IFN-β or TNF-α induction are lower than those in uninfected donors. These results suggest that the signal transduction in the downstream of TLR/RIG-I in MDC is profoundly impaired in HCV infection. In response to IFN-α, DC are able to express MHC class-I related chain A/B (MICA/B) and activate natural killer (NK) cells following ligation of NKG2D. Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells. Alternatively, NK cells from HCV-infected patients downregulate DC functions in the presence of human leukocyte antigen E-expressing hepatocytes by secreting interleukin (IL)-10 and transforming growth factor-β1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts. Invariant NKT cells activated by CD1d-positive DC secrete both T-helper (Th)1 and Th2 cytokines, serving as immune regulators. The frequency of NKT cells in chronic HCV infection does not differ from those in healthy donors. Activated NKT cells produce higher levels of IL-13 but comparable levels of IFN-γ with those from healthy subjects, showing that NKT cells are biased to Th2-type in chronic HCV infection. In conclusion, cross-talks among DC, NK cells and NKT cells are critical in shaping subsequent adaptive immune response against HCV.

Key words: α-galactosyl-ceramide, IL-β, MICA/B, NKG2A, TLR

INTRODUCTION

HEPATITIS C VIRUS (HCV) is one of major causes of chronic liver disease worldwide. HCV is hepatotrophic, but not directly cytopathic and elicits 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 for HCV elimination by inducing hepatocyte apoptosis.[4] Innate immune cells, including natural killer (NK) cells and

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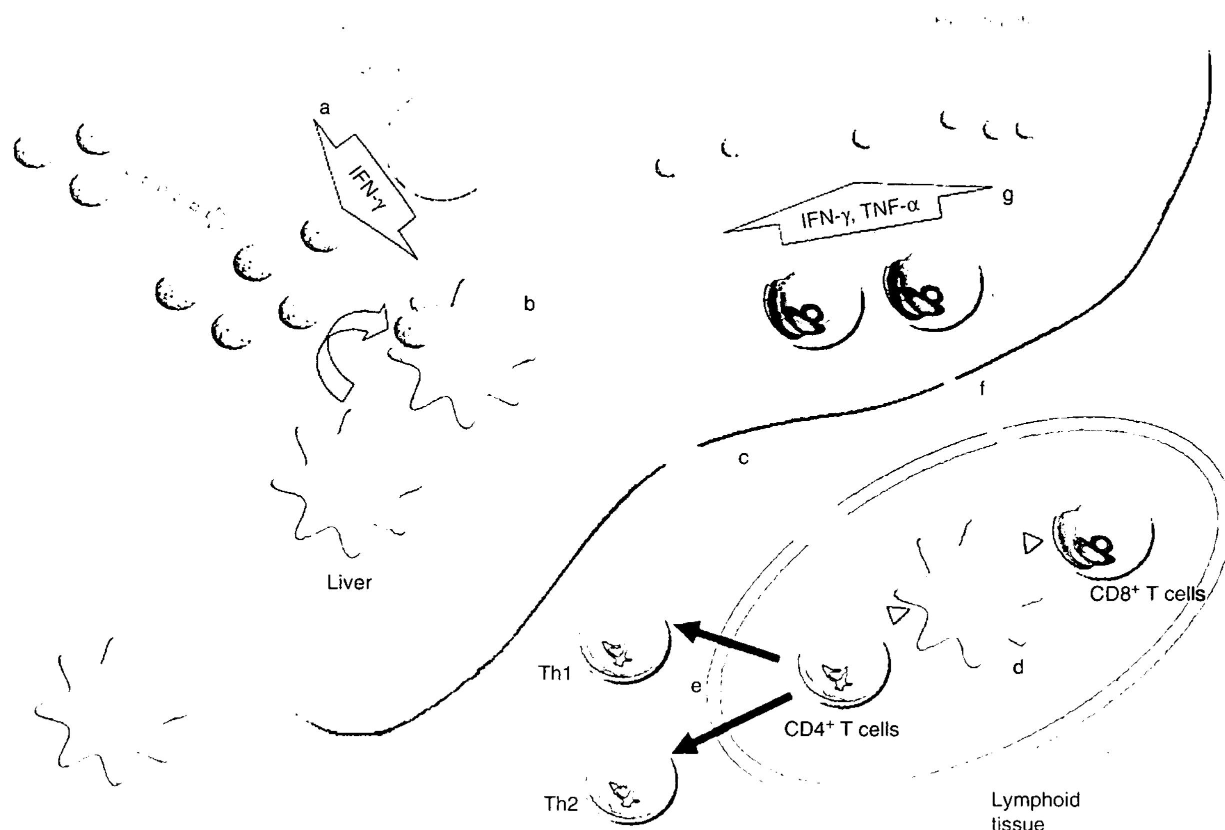

Figure 1 Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HCV, hepatitis C virus; NK, natural killer cell; Th, helper T cell. (1–7), see text.

NKT cells, may contribute to HCV eradication after primary infection; however, their roles in a chronically infected state remain elusive. Because dendritic cells (DC) orchestrate anti-HCV immune response by linking innate and adaptive arms of the 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, especially focused on interferons (IFN), DC, NK cells and NKT cells.

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-α and IFN-inducible genes are extremely high during the early phase of hepatitis virus infection.[6] Activated NK and NKT cells secrete IFN-γ, which inhibits replication of HCV through a non-cytolytic mechanism (Fig. 1a).[7]

Dendritic cells or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Fig. 1b).[4] Because DC express distinct sets of toll-like receptors (TLR),[8] it is likely that some viral components stimulate DC through cytosolic ligation of TLR. DC develop a mature phenotype and migrate to lymphoid tissues (Fig. 1c), where they stimulate effectors, including T cells and B cells (Fig. 1d). Following the encounter of DC with other cells, DC secrete various cytokines (interleukin [IL]-12, tumor necrosis factor [TNF]-α, IFN-α and IL-10) instructing or regulating the functions of the adja-

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cent cells.[4] In addition to these cytokines, DC express various costimulatory 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; that is, myeloid DC (MDC) and plasmacytoid DC (PDC).[9] MDC predominantly produce IL-12 or TNF-α following proinflammatory stimuli, while PDC release a considerable amount of IFN-α 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 (CTL) generation (T-helper [Th]1 with secretion of IL-2, IFN-γ and TNF-α) or B-cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Fig. 1e). 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 CTL recruit to the liver (Fig. 1f) and constitute the critical element in the eradication of virus-infected cells (Fig. 1g).

INNATE IMMUNITY IN HCV INFECTION

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 antiviral machineries. Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing antiviral proteins, such as interferon regulatory factor (IRF)-3,[11] nuclear factor (NF)-κB and double-stranded RNA-dependent protein kinases (PKR).[12] Mammalian TLR sense some pathogen-associated molecular patterns embedded in virus components and then induce inflammatory cytokines or type-I IFN, resulting in the augmentation of antivirus immune reactions.[8] Retinoic acid inducible gene-I (RIG-I) is a cytosolic molecule that senses double-stranded RNA (dsRNA) of virus replicative intermediate, which subsequently activates IRF-3 and NF-κB pathways.[13] By using the HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4 A proteins influence the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN-β as well as subsequent IFN-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.

To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and functions in MDC and PDC between 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 amounts of IFN-β and TNF-α compared to donor MDC (Miyazaki *et al.*, 2007, 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 DC (MoDC) in chronic hepatitis C is lower than those in healthy donors.[16] Because 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.[17] Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

Blood DC subsets

Impaired antigen presentation by DC might be involved in the failure of the maintenance of sustained HCV-specific T-cell response. MoDC generated from hepatitis C patients have an impaired ability to stimulate allogeneic CD4⁺ T cells.[18,19] Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability.[18] In addition to *in vitro*-generated DC, the alterations in number and function of circulating blood DC have been reported in HCV infection.[20]

Direct HCV infection of DC might be one of the plausible mechanisms of DC dysfunction in chronic hepatitis C. The HCV genome has been reported to be isolated

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