cells and that ADAM9 knockdown by siRNA resulted in the decrease in the production of soluble MICA from IL-1β-treated HepG2 cells. Our results suggested at least that the increase in ADAM9 might resulted in the increase in the shedding of soluble MICA in the IL-1β-treated HCC cells.

Recent studies have identified various metalloproteinases responsible for MICA/B cleavage in various cancers [31]. We previously found that ADAM9 plays critical roles in the shedding of MICA in human HCC. ADAM9 was directly associated with decreasing the expression of membrane-bound MICA and increasing the production of soluble MICA in human HCC [19]. Thus, it would be interesting to examine the activity of ADAM9 in IL-1β-treated HCC cells to understand how IL-1β regulates the production of soluble MICA from HCC cells. We demonstrated that IL-1β treatment could increase the mRNA and protein expression of ADAM9 in HCC cells and that ADAM9 knockdown in HCC cells resulted in decreasing of the soluble MICA production. These results suggested that ADAM9 played an important role in the increase in soluble MICA production from IL-1β-treated HCC cells. Both ADAMs and ADAMs with thrombospondin motifs (ADAMTS) are proteinases closely related to matrix metalloproteinases (MMPs). Structure of ADAMs and ADAMTS is highly conserved and involves metalloproteinase and disintegrin domains endowing them with features of both proteinases and adhesion molecules [32]. Several ADAMTSs including ADAMTS1 and ADAMTS9 were activated by IL-1β via NFATc1 transcription factor in chondrosarcoma [33, 34]. Although IL-1β may regulate such transcription factors in HCC cells, the detail mechanism of the activation of ADAM9 by IL-1β remains unclear. The concentration of IL-1β in our in vitro study was high compared with the serum IL-1β concentration level. However, the local IL-1β concentration in the liver tissues still remains unknown and may differ from the serum IL-1β concentration. Our in vitro study at least demonstrated that IL-1β could enhance the production of soluble MICA via up-regulating the expressions of ADAM9 in HCC cells, which might support the possible role of IL-1β in the survival of HCC cells.

Cai et al. [35] demonstrated that the numbers of CD56+ NK cells reduced in HCC tissues compared with healthy donors and CD56+ NK cells in HCC patients displayed impairments in cytotoxicity and IFN-γ production. This suggests that immunological microenvironment in liver tissues of CH patients might be favorable for the survival of HCC cells. We demonstrated that serum IL-1β levels correlated with soluble MICA in CH patients, which is consistent with our in vitro data. This suggests that the chronic elevation of IL-1β in CH patients might impair the function of NK cells by accelerating the production of soluble MICA. We also demonstrated that IL-1β treatment resulted in the inhibition of the cytolytic activity of NK cells against HCC cells. Intrahepatic activated macrophages and plasma cells could produce IL-1β inducing the inflammatory process in chronic liver disease [36]. If we could control the production of IL-1β with new reagents, it might be possible to develop a new therapeutic strategy against HCC. IL-1β receptor antagonist (IL-1RA) has been reported to apply clinically to the treatment of rheumatoid arthritis [37]. We believe the future clinical application of IL-1RA in HCC treatment as a new agent.

In spite of recent progress in the understanding of HCC, there remains to be unknown mechanism of the escape of HCC cells from innate immunity. We have shown here that ADAM9 was directly associated with increasing the production of soluble MICA in IL-1β-treated human HCC. These findings might indicate that IL-1β contributes to the survival of HCC cells by inhibiting innate immunity.

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References

1. Dinarello CA (1996) Biologic basis for interleukin-1 in disease. Blood 87:2095–2147
2. Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW (1996) Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology 24:759–765
3. Powell EE, Edwards-Smith CJ, Hay JL, Clouston AD, Crawford DH, Shorthouse C, Purdie DM, Jonsson JR (2000) Host genetic factors influence disease progression in chronic hepatitis C. Hepatology 31:828–833
4. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, Huber C (1992) Serum levels of cytokines in chronic liver diseases. Gastroenterology 103:264–274
5. Bidwell J, Keen L, Gallagher G et al (1999) Cytokine gene polymorphism in human disease: on-line databases. Genes Immun 1:3–19
6. El-Omar EM, Carrington M, Chow WH et al (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 404:398–402
7. Howell WM, Calder PC, Grimble RF (2002) Gene polymorphism, inflammatory disease and cancer. Proc Nutr Soc 61:447–456
8. Wang Y, Kato N, Hoshida Y et al (2003) Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis C virus infection. Hepatology 37:65–71
9. Tanaka Y, Furuta T, Suzuki S, Orito E, Yeo AE, Hirashima N, Sugauchi F, Ueda R, Mizokami M (2003) Impact of interleukin-1β genetic polymorphism on the development of hepatitis C virus-related hepatocellular carcinoma in Japan. J Infec Dis 187:1822–1825
10. Hirankarn N, Kimkong I, Kummee P, Tangkijvanich P, Poovorawan Y (2006) Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection. World J Gastroenterol 12:776–779

11. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC (2008) Overexpression of interleukin-1β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell 14:408–419
12. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T (1999) Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$T cells of MICA and MICB. Proc Natl Acad Sci USA 96:6879–6884
13. Jinushi M, Takehara T, Tatsumi T et al (2003) Expression of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acids. Int J Cancer 104:354–361
14. Ogasawara K, Lanier LL (2005) NKG2D in NK and T cell-mediated immunity. J Clin Immunol 25:534–540
15. Caudert JD, Held W (2006) The role of the NKG2D receptor for tumor immunity. Semin Cancer Biol 16:333–343
16. Groh V, Wu J, Yee C, Spies T (2002) Tumor-derived soluble MIC ligands impair expression of NKG2D and T cell activation. Nature 419:734–738
17. Salih HR, Rammensee HG, Steinle A (2002) Downregulation of MICA on human tumors by proteolytic shedding. J Immunol 169: 4098–4102
18. Kohga K, Takehara T, Tatsumi T et al (2008) Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver disease and changes during transcatheter arterial embolization for hepatocellular carcinoma. Cancer Sci 99:1643–1649
19. Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A, Hayashi N (2010) Sorafenib inhibits the shedding of MICA on hepatocellular carcinoma cell by downregulating ADAM9. Hepatology 51:1264–1273
20. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR (2006) Soluble MICA in malignant disease. Int J Cancer 118: 684–687
21. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in human liver. Immunol Rev 174:5–20
22. Mehal WZ, Azzaroli F, Crispe IN (2001) Immunology of the healthy liver: old questions and new insights. Gatsroenterology 120:250–260
23. Guerra N, Tan YX, Joncker NT et al (2008) NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 28:571–580
24. Kohga K, Takehara T, Tatsumi T, Miyagi T, Ishida H, Ohkawa K, Kanto T, Hiramatsu N, Hayashi N (2009) Anti-cancer chemotherapy inhibits MICA ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma. Cancer Res 69:8050–8057
25. Oyanagi Y, Takahashi T, Matsui S, Takahashi S, Boku S, Takahashi K, Furukawa K, Arai F, Asakura H (1999) Enhanced expression of interleukin-6 in chronic hepatitis C. Liver 19:464–472
26. Lapinski TW (2001) The levels of IL-1β, IL-4 and IL-6 in the serum and the liver tissue of chronic HCV-infected patients. Arch Immunol Ther Exp 49:311–316
27. Bortolami M, Kotsafti A, Cardin R, Farinati F (2008) Fas/FasL system, IL-1β expression and apoptosis in chronic HBV and HCV liver disease. J Viral Hepat 15:515–522
28. Migita K, Abiru S, Maeda Y et al (2005) Serum levels of interleukin-6 and its soluble receptors in patients with hepatitis C virus infection. Human Immunol 67:27–32
29. Nakagawa H, Maeda S, Yoshida H et al (2009) Serum IL-6 levels and the risk for hepatocarcinogenesis in chronic hepatitis C patients: an analysis based on gender difference. Int J Cancer 125:2264–2269
30. Wong VW, Yu J, Cheng AS et al (2009) High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. Int J Cancer 124:2766–2770
31. Waldhauer I, Goehlsdorf D, Gieseke F et al (2008) Tumor-associated MICA is shed by ADAM proteases. Cancer Res 68: 6368–6376
32. Seals DF, Courtneidge SA (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17:7–30
33. Yaykasli KO, Oohashi T, Hirohata S, Hatipoglu OF, Inagawa K, Demircan K, Ninomiya Y (2009) ADAMTS9 activation by interleukin 1 beta via NFATc1 in OUMS-27 chondrosarcoma cells and in human chondrocytes. Mol Cell Biochem 323:69–79
34. Kalinski T, Krueger S, Sel S, Wemer K, Ropke M, Roessner A (2007) ADAMTS1 is regulated by interleukin-1beta, not by hypoxia, in chondrosarcoma. Hum Pathol 38:86–94
35. Cai L, Zhang Z, Zhou L et al (2008) Functional impairment in circulating and intrahepatic NK cell and relative mechanism in hepatocellular carcinoma patients. Clin Immunol 129:428–437
36. Hassan G, Moreno S, Massimi M, Di Biagio P, Stefanini S (1997) Interleukin-1-producing plasma cells in close contact with hepatocytes in patients with chronic active hepatitis. J Hepatol 27:6–17
37. Gabby C, Lamacchia C, Palmer G (2010) IL-1 pathway in inflammation and human disease. Nat Rev Rheumatol 6:232–241

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin

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Abstract
Background The timing to the first undetectable hepatitis C virus (HCV) RNA level is strongly associated with sustained virologic response in pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) with genotype 1. This study was conducted to clarify the impact of drug exposure to Peg-IFN on the timing of HCV RNA negativity in Peg-IFN plus ribavirin combination therapy for CH-C patients with genotype 1.

Methods A total of 1409 patients treated with Peg-IFN alfa-2b plus ribavirin were enrolled and classified into four categories according to the Peg-IFN dosage. Furthermore, 100 patients were extracted from each Peg-IFN dosage category to adjust for characteristic factors, using the propensity score method.

Results Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$). The HCV RNA negative rate at week 4 decreased from 12% with a Peg-IFN dose of >1.5 μg/kg/week to 1–3% with a dose of <1.5 μg/kg/week ($p \leq 0.001$), and at week 12 the rate had decreased from 44% with a dose of

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≥1.2 μg/kg/week to 18% with a dose of <1.2 μg/kg/week ($p = 0.001$). Treatment failure (patients without a 1-log decrease of HCV RNA at week 4 or a 2-log decrease of HCV RNA at week 12, or positive at week 24) was found in 54–66% of patients given <1.2 μg/kg/week ($p \leq 0.001$), and these patients accounted for 64% of the non-responders.

Conclusions The timing of HCV RNA negativity depends significantly on the Peg-IFN dose. Reducing the Peg-IFN dose can induce a later virologic response or non-response in HCV genotype 1 patients treated with Peg-IFN plus ribavirin.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · Drug adherence · HCV RNA negativity · Propensity score matched study

Introduction

The timing to the first undetectable hepatitis C virus (HCV) RNA level during pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) genotype 1 is strongly associated with a sustained virologic response (SVR), defined as undetectable HCV RNA at 24 weeks after the finishing of the treatment. The SVR rate was 87–100% in patients with undetectable HCV RNA at week 4, 73–81% at week 12, and 14–44% between weeks 12 and 24 in patients receiving the standard 48-week treatment [1–8]. These results suggest that HCV RNA negativity should be achieved as soon as possible during treatment in order to attain a higher SVR rate.

Previous studies have revealed that many factors affect the complete virologic response (c-EVR) and SVR, such as age, gender, degree of liver fibrosis, HCV genotype, HCV viral load, and the amount of drug exposure [1–3, 9–16]. Of these factors, only the amount of drug exposure can be controlled in order to try to improve the antiviral effect, as the other factors are fixed for individual patients. Also, recently, the single-nucleotide polymorphisms (SNPs) of the *IL28B* gene have been revealed to be associated with the antiviral effects of pegylated interferon-alpha and ribavirin therapy [17–19].

Peg-IFN has been reported to be dose-dependently correlated with c-EVR [13]. Patients' characteristic factors can be related to drug adherence, as aged patients, female patients, and patients with progression of liver fibrosis have a tendency to show low drug adherence. This suggests that patients with low drug adherence could be those who are difficult to treat. Therefore, patients with similar characteristic factors should be compared in order to precisely assess the actual impact of drug exposure on the timing to the first undetectable HCV RNA level.

Only a few randomized controlled trials (RCTs) have examined the relationship between drug dose reduction and antiviral effect with Peg-IFN plus ribavirin combination therapy [1–3, 20–23], and the findings are controversial. Manns et al. [1] reported that the SVR rate was significantly lower in patients given 0.5 μg/kg/week of Peg-IFN than in those given 1.5 μg/kg/week of Peg-IFN (34 vs. 42%, $p < 0.05$). McHutchison et al. [3] reported that the SVR rate did not differ between groups given 1.0 and 1.5 μg/kg/week of Peg-IFN (38 vs. 40%, $p = 0.20$). No detailed study of the relationship between dose reduction and delay of HCV RNA negativity or the relationship between dose reduction and an increase of non-responders to the treatment has been reported, and the real impact of drug exposure on the anti-viral effect remains unclear.

In this present work, we conducted a matched study in which characteristic factors other than drug exposure were adjusted using propensity scores. We investigated the impact of drug exposure to Peg-IFN on the timing to the first undetectable HCV RNA level.

Patients and methods

Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1409 Japanese patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin were enrolled in this study between December 2004 and July 2008.

Patients eligible for this study were those who were infected with HCV genotype 1 and had a viral load of $\geq 10^5$ IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study, which was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight

(body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients.

Dose reduction

As a rule, dose modification, which was performed according to the intensity of the adverse hematologic effects, was done by following the manufacturer's drug information. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the platelet (Plt) count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the Plt count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600 mg, or from 800 to 600 mg, or from 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The HCV RNA level was evaluated every 4 weeks during treatment. A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 24. Patients with <a 1-log decrease in the HCV RNA level at week 4 or <a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 were considered to have experienced treatment failure (non-response, NR) and had to stop treatment. If patients discontinued the treatment due to adverse events, without HCV RNA negativity being attained, they were also regarded as having had treatment failure.

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin actually taken were evaluated by reviewing the medical records and calculating the amount taken from the start until the timing of the first undetectable HCV RNA level for the patients achieving HCV RNA negativity, and calculating the amount taken throughout the treatment for the patients not attaining HCV RNA negativity. For patients who discontinued the treatment, if their HCV RNA had become negative before discontinuation, the drug amount data were calculated from the start of treatment until the timing of the first undetectable HCV RNA level, and if HCV RNA had not become negative before discontinuation, the data throughout the treatment before discontinuation were used. The amounts of both drugs were divided individually on the basis of body weight at baseline as the average: Peg-IFN alfa-2b was expressed as μg/kg/week and ribavirin as mg/kg/day.

Evaluation of impact of drug exposure on HCV RNA negativity

We evaluated the relationship between the exposure to both drugs and HCV RNA negativity at week 24 by univariate and multivariate analyses for the patients who completed 24 weeks of treatment, using the mean administration doses of both drugs during the first 24 weeks and the characteristic factors other than drug exposure at baseline.

The patients were divided into four categories according to the Peg-IFN dose: up to 0.9 μg/kg/week of Peg-IFN; from 0.9 to less than 1.2 μg/kg/week; from 1.2 to less than 1.5 μg/kg/week; and from 1.5 μg/kg/week. The propensity score matching method was used to adjust the patients' characteristic factors among these categories. This score was calculated for each patient by logistic regression analysis, with four patient characteristic factors as independent variables; age, gender, Plt values, and history of IFN treatment. We then performed 1:1 nearest neighbor matching within a caliper of 0.15 standard deviation of the propensity score: one patient in each group with 0.9–1.2 μg/kg/week, 1.2–1.5 μg/kg/week, and ≥1.5 μg/kg/week to one patient with <0.9 μg/kg/week, and extracted 100 patients from each category.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD or median values. Factors associated with HCV RNA negativity at week 24 were assessed by univariate analysis using the Mann–Whitney U-test or the χ^2 test, and by multivariate analysis using logistic regression analysis. To analyze the difference between baseline data among the four Peg-IFN groups, analysis of variance (ANOVA) or the χ^2 test was performed. The significance of trends in values for the timing to the first undetectable HCV RNA level was determined with the Mantel–Haenszel χ^2 test. A two-tailed p value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Table 1 Baseline characteristics of patients before matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	≥1.5 μg/kg/week of Peg-IFN	*p* value
Number	1409	153	159	670	427	
Age (years)	56.3 ± 10.4	58.0 ± 9.9	57.3 ± 10.2	55.9 ± 10.6	56.3 ± 10.4	0.069
Sex: male/female	722/687	70/83	69/90	376/294	207/220	0.004
History of IFN treatment: naïve/experienced	862/547	98/55	96/63	408/262	260/167	0.894
White blood cells (/mm^3)	5060 ± 1532	4325 ± 1419	4566 ± 1394	5246 ± 1562	5215 ± 1456	<0.001
Neutrophils (/mm^3)	2578 ± 1073	2129 ± 1049	2258 ± 949	2699 ± 1080	2667 ± 1052	<0.001
Red blood cells (×10^4/mm^3)	440 ± 46	424 ± 42	429 ± 45	445 ± 46	441 ± 45	<0.001
Hemoglobin (g/dl)	14.0 ± 1.4	13.6 ± 1.2	13.7 ± 1.5	14.1 ± 1.4	14.1 ± 1.4	<0.001
Platelets (×10^4/mm^3)	16.3 ± 5.6	11.9 ± 3.9	13.2 ± 4.8	17.4 ± 5.7	17.4 ± 5.2	<0.001
ALT (IU/l)	78 ± 61	93 ± 64	87 ± 68	75 ± 58	73 ± 60	0.001
Serum HCV RNA (KIU/ml)[a]	1450	1300	1900	1700	1900	0.176
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	810/186 (17%)	78/34 (30%)	70/30 (30%)	392/77 (16%)	270/45 (14%)	<0.001
Activity, 0–1/2–3	527/468	43/67	38/62	259/210	187/129	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 413 patients

[c] Percent of patients with 3–4

Results

Clinical characteristics of all patients according to Peg-IFN dosage before matching

A total of 1409 patients were enrolled in this study, and the baseline characteristics of the patients are shown in Table 1. Based on the Peg-IFN dosage, these patients were classified into four categories. With the decrease of Peg-IFN dosage, the ratio of female-to-male patients increased, the peripheral blood cell count decreased, and the number of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) increased significantly ($p < 0.001$). Patients with a lower Peg-IFN dosage tended to be older ($p = 0.07$).

Next, we analyzed the factors associated with HCV RNA negativity at week 24 for the 1226 patients who completed 24 weeks of treatment, using the baseline characteristic variables, excluding liver histology, shown in Table 1 and the mean doses of both drugs during the first 24 weeks. The HCV RNA negative rate at week 24 was 68% (829/1226). The results of univariate analysis are shown in Table 2. The factors evaluated by multivariate analysis were those for which the *p* value was <0.10 by univariate analysis for HCV RNA negativity at week 24: age, gender, history of IFN treatment, WBC, neutrophils, red blood cells (RBC), Hb, Plt, alanine aminotransferase, and the mean doses of Peg-IFN and ribavirin during the first 24 weeks. By the multivariate analysis, in addition to the RBC value ($p = 0.02$), Plt value ($p < 0.001$), and history of IFN treatment ($p = 0.04$), the factor of Peg-IFN exposure was an independent factor for HCV RNA negativity at week 24 ($p < 0.001$) (Table 3). The mean dose of ribavirin did not show a significant correlation with HCV RNA negativity at week 24 ($p = 0.07$).

Table 2 Univariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Negative	Positive	*p* value
Number	829	397	
Age (years)	55.1 ± 10.5	57.6 ± 10.1	<0.001
Sex: male/female	437/392	189/208	0.094
History of IFN treatment: naïve/experienced	523/306	229/168	0.069
White blood cells (/mm^3)	5175 ± 1498	4566 ± 1394	<0.001
Neutrophils (/mm^3)	2665 ± 1087	2429 ± 1059	<0.001
Red blood cells (×10^4/mm^3)	445 ± 44	434 ± 47	<0.001
Hemoglobin (g/dl)	14.1 ± 1.4	13.9 ± 1.4	0.004
Platelets (×10^4/mm^3)	17.2 ± 5.6	14.8 ± 5.3	<0.001
ALT (IU/l)	73 ± 55	83 ± 63	0.001
Serum HCV RNA (KIU/ml)	1750	1800	0.673
Mean Peg-IFN dose (μg/kg/week)	1.39 ± 0.24	1.25 ± 0.32	<0.001
Mean ribavirin dose (mg/kg/day)	10.6 ± 1.8	9.7 ± 2.2	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

Clinical characteristics of patients extracted from each Peg-IFN dosage category after matching

Patients in the four Peg-IFN categories were matched by the propensity score method and 100 patients were extracted from each category. The c-statics for the propensity score model between the patients with <0.9 μg/kg/week of Peg-IFN and those given different levels of Peg-IFN were 0.62 for 0.9–1.2 μg/kg/week of Peg-IFN, 0.82 for 1.2–1.5 μg/kg/week of Peg-IFN, and 0.82 for ≥1.5 μg/kg/week of Peg-IFN.

Table 3 Multivariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Category	Odds ratio	95% CI	*p* value
Age	1 year	–	–	NS
Sex	Male/female	–	–	NS
History of IFN treatment	Naïve/experienced	0.756	0.581–0.984	0.037
White blood cells	$1 \times 10^3/mm^3$	–	–	NS
Neutrophils	$1 \times 10^3/mm^3$	–	–	NS
Red blood cells	$1 \times 10^4/mm^3$	1.004	1.001–1.007	0.02
Hemoglobin	1 g/dl	–	–	NS
Platelets	$1 \times 10^4/mm^3$	1.054	1.026–1.083	<0.001
ALT	1 IU/l	–	–	NS
Mean Peg-IFN dose	0.1 μg/kg/week	1.096	1.045–1.149	<0.001
Mean ribavirin dose	1 mg/kg/day	1.060	0.994–1.130	0.074

ALT alanine aminotransferase, *CI* confidence interval, *IFN* interferon, *NS* not significant, *Peg-IFN* pegylated interferon

The baseline characteristics of the patients extracted according to the Peg-IFN dosage category are shown in Table 4. There was no significant difference among the four Peg-IFN categories in any of the factors, indicating that the extracted cohort of 400 patients was well matched according to propensity score methods.

Timing to the first undetectable HCV RNA level according to Peg-IFN dosage

We evaluated the relationship between the virologic response during the treatment and the drug exposure to Peg-IFN using our matched cohort of 400 patients (Fig. 1). Of the 400 patients, 23 had discontinued treatment due to adverse events by week 24 (<0.9 μg/kg/week, $n = 5$; 0.9–1.2 μg/kg/week, $n = 4$; 1.2–1.5 μg/kg/week, $n = 5$; ≥1.5 μg/kg/week, $n = 9$). The proportion of patients with treatment failure increased according to the decrease in the dose of Peg-IFN: 66% among patients with <0.9 μg/kg/week of Peg-IFN, 54% among those with 0.9–1.2 μg/kg/week of Peg-IFN, 35% among those with 1.2–1.5 μg/kg/week of Peg-IFN, and 32% among those with ≥1.5 μg/kg/week of Peg-IFN ($p < 0.001$). Additionally, the timing to the first undetectable HCV RNA level tended to shift to an earlier time

Table 4 Baseline characteristics of patients after matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	>1.5 μg/kg/week of Peg-IFN	*p* value
Number	400	100	100	100	100	
Age (years)	56.9 ± 9.6	57.4 ± 10.0	56.6 ± 9.9	56.8 ± 9.5	56.7 ± 9.1	0.941
Sex: male/female	190/210	47/53	47/53	46/54	50/50	0.948
History of IFN treatment: naïve/experienced	286/114	70/30	71/29	73/27	72/28	0.970
White blood cells ($/mm^3$)	4557 ± 1344	4331 ± 1310	4532 ± 1372	4642 ± 1399	4725 ± 1280	0.186
Neutrophils ($/mm^3$)	2261 ± 955	2070 ± 855	2200 ± 883	2416 ± 1068	2357 ± 972	0.054
Red blood cells ($\times 10^4/mm^3$)	429 ± 43	423 ± 40	427 ± 42	432 ± 44	434 ± 46	0.300
Hemoglobin (g/dl)	13.8 ± 1.5	13.7 ± 1.3	13.7 ± 1.6	13.9 ± 1.4	14.0 ± 1.5	0.300
Platelets ($\times 10^4/mm^3$)	12.1 ± 3.7	11.8 ± 3.9	12.0 ± 3.8	12.1 ± 3.5	12.4 ± 3.6	0.625
ALT (IU/l)	89 ± 67	98 ± 65	92 ± 70	86 ± 61	80 ± 69	0.254
Serum HCV RNA (KIU/ml)[a]	1700	1400	1800	1700	1750	0.742
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	186/89 (32%)	47/22 (32%)	46/22 (32%)	48/21 (30%)	45/24 (35%)	0.958
Activity, 0–1/2–3	115/159	25/42	26/42	32/37	32/38	0.585

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 125 patients

[c] Percent of patients with 3–4

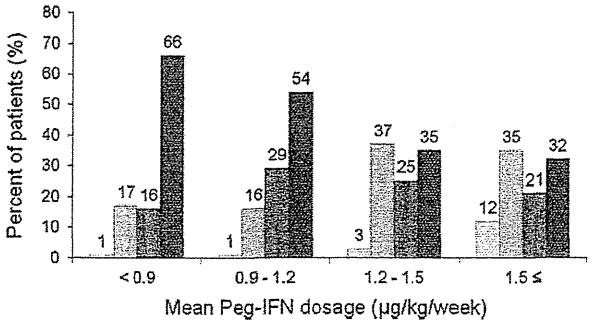
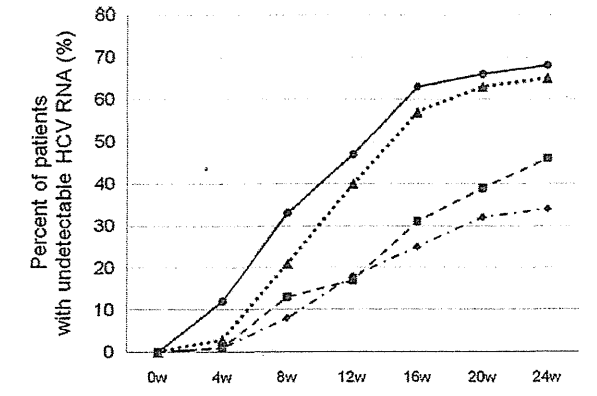


Fig. 1 Timing to the first undetectable hepatitis C virus (HCV) RNA level according to pegylated interferon (*Peg-IFN*) dosage. *Light gray bars* patients with undetectable HCV RNA at week 4. *Medium gray bars* patients with undetectable HCV RNA during 5 to 12 weeks. *Dark gray bars* patients with undetectable HCV RNA during 13–24 weeks. *Black bars* patients with treatment failure (patients with less than a 1-log decrease in HCV RNA level at week 4 or less than a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 and those with treatment discontinuance without HCV RNA negativity). Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$)



Peg-IFN (μg/kg/week), %						
< 0.9	1	8	18	25	43	34
0.9 ≤ < 1.2	1	13	17	31	39	46
1.2 ≤ < 1.5	3	21	40	57	63	65
1.5 ≤	12	33	47	63	66	68

Fig. 2 Longitudinal negative HCV RNA rates from the start to 24 weeks of the treatment. *Filled circles* Peg-IFN ≥1.5 μg/kg/week, *filled triangles* Peg-IFN 1.2–1.5 μg/kg/week, *filled squares* Peg-IFN 0.9–1.2 μg/kg/week, *filled diamonds* Peg-IFN <0.9 μg/kg/week. The HCV RNA negative rate at week 4 was significantly higher among the patients with Peg-IFN ≥1.5 μg/kg/week than among those with Peg-IFN <1.5 μg/kg/week ($p \leq 0.001$). The HCV RNA negative rates at weeks 12 and 24 were significantly higher among the patients with Peg-IFN ≥1.2 μg/kg/week than among those with Peg-IFN <1.2 μg/kg/week ($p = 0.001$, $p = 0.002$, respectively). *w* week

during the treatment according to the increase in the Peg-IFN dose ($p \leq 0.001$).

Figure 2 shows the longitudinal data of the HCV RNA negative rate. The data for patients with treatment failure were included until the end of each patient's treatment. The percentage of patients with undetectable HCV RNA at week 4 decreased from 12 to 1–3% if they were given <1.5 μg/kg/week of Peg-IFN ($p \leq 0.001$). As for the HCV RNA negative rates at week 12 and week 24, there was no significant difference between patients with 1.2–1.5 μg/kg/week and those with >1.5 μg/kg/week of Peg-IFN. The two groups with <1.2 μg/kg/week of Peg-IFN showed significantly lower HCV RNA negative rates than the other two groups given ≥1.2 μg/kg/week of Peg-IFN (week 12, 18 vs. 44%, $p = 0.0001$, week 24, 40 vs. 67%, $p = 0.0002$). The patients with <0.9 μg/kg/week tended to show a decreased HCV RNA negative rate at week 24 compared to the patients given 0.9–1.2 μg/kg/week (34 vs. 46%, $p = 0.08$).

Figure 3 shows the proportion of patients, according to Peg-IFN exposure, among those with undetectable HCV RNA at week 4 ($n = 17$) and at week 12 ($n = 122$), as well as the proportion of patients with detectable HCV RNA at week 24 ($n = 213$). The patients given ≥1.5 μg/kg/week of Peg-IFN accounted for 70% of the patients with undetectable HCV RNA at week 4, and those given ≥1.2 μg/kg/week of Peg-IFN accounted for 71% of the patients with undetectable HCV RNA at week 12. On the other hand, the patients given <1.2 μg/kg/week of Peg-IFN accounted for 64% of the patients with detectable HCV RNA at week 24.

Discussion

The association between drug exposure and HCV RNA negativity has been reported [9–13]. However, most studies have shown only the fixed-point relationship at week 12, and it remains unclear whether dose modification accelerates or delays the timing to the first undetectable HCV RNA level. The present study is the first to clarify this. Induction regimens in which a high dose (360 μg/week) of Peg-IFN alfa-2a was administered for the first 12 weeks failed to improve SVR rates compared to treatment with a standard dose of Peg-IFN in the CHARIOT [22] and PROGRESS [23] RCTs. In contrast, the adherence study of McHutchison et al. revealed that patients who received ≥80% of the planned dose of Peg-IFN and ribavirin for ≥80% of the full 48 weeks of treatment had a significantly higher SVR rate (51%) than those who received <80% of the planned dose of one or both drugs for ≥80% of the full 48 weeks of treatment (34%) ($p = 0.011$) [10]. These apparently paradoxical results for the relationship between drug dosage and antiviral effect imply that the dose-dependent increase of the antiviral effect was observed up to a certain dose and the antiviral effect then reached a plateau above the regular dose. This paradoxical effect could explain the impact of Peg-IFN reduction from a regular dose on the timing to the first undetectable HCV

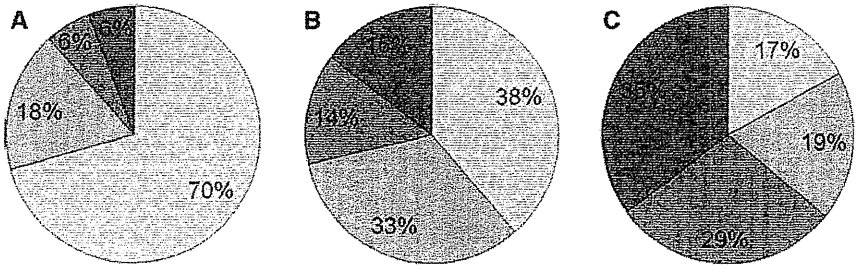


Fig. 3 Proportions of patients according to Peg-IFN exposure among patients with undetectable HCV RNA at weeks 4 and 12 and those with detectable HCV RNA at week 24. **a** Week 4 ($n = 17$), **b** week 12 ($n = 122$), **c** week 24 ($n = 213$). *Light gray segments* Peg-IFN <0.9 μg/kg/week. *Medium gray segments* Peg-IFN 0.9–1.2 μg/kg/week. *Dark gray segments* Peg-IFN 1.2–1.5 μg/kg/week. *Black segments* Peg-IFN ≥1.5 μg/kg/week

RNA level in the present study differing from that of the induction therapy with a high Peg-IFN dose in the above two studies.

In the present study, characteristic matched patients were extracted from a large retrospective cohort to examine the impact of Peg-IFN dosage on viral dynamics. The reason for using a matched cohort was that performing an RCT according to Peg-IFN doses poses an ethical problem, because a low dose of Peg-IFN is known to show little efficacy. The reason for our focusing on Peg-IFN dosage was based on the finding that ribavirin was indeed a significant factor for HCV RNA negativity at week 24 on univariate analysis, but not on multivariate analysis, and Peg-IFN was significantly correlated with HCV RNA negativity at week 24 in an independent manner in this study cohort. Our previous report that Peg-IFN, but not ribavirin, was correlated with c-EVR supports this [13].

To calculate the propensity score, we chose four covariates as candidates for adjustment: age, gender, Plt values, and history of IFN treatment, because there was a need to match universal features such as age, gender, and factors associated with HCV RNA negativity at week 24, such as Plt values and the history of IFN treatment. As shown in Table 4, the baseline characteristic factors in the different Peg-IFN patient categories were well matched after propensity score adjustment. That is, c-statics, the hallmark of application to logistic regression analysis, was regarded as adequate for random assignment. Only the c-statics for the patients given <0.9 μg/kg/week of Peg-IFN and the patients given 0.9–1.2 μg/kg/week of Peg-IFN showed a low value (0.62), because the number of patients in the Peg-IFN category of 0.9–1.2 μg/kg/week ($n = 153$) was not very large. However, the patient characteristic factors in two categories after extraction were well matched and were considered to be adequate for further analysis. In this study, the populations extracted after matching were composed of patients with relatively advanced liver fibrosis compared to the original population; the mean Plt value was lower and the proportion of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) was higher in the extracted population than in the original one (mean Plt value $12.1 \times 10^4/mm^3$ vs. $16.3 \times 10^4/mm^3$, proportion of patients with progression of liver fibrosis, 32 vs. 19%, respectively). The patients with <0.9 μg/kg/week of Peg-IFN, which was the smallest population among the four Peg-IFN categories and included more patients with advanced liver fibrosis, were used as the control for the propensity score matching.

Recently, the usefulness of extended therapy has been revealed for patients with LVR, defined as HCV RNA negativity between week 12 and week 24 (or week 36). In addition, we have reported that, even with extended treatment of 72 weeks, the timing of HCV RNA disappearance showed a strong correlation with relapse after treatment [24]. Accordingly, at present, it is necessary to verify how reducing drug doses affects the delay of the timing to the first undetectable HCV RNA level or treatment failure, and in the present study we demonstrated the appropriate dose of Peg-IFN required to attain HCV RNA negativity by 24 weeks. As shown in Fig. 1, Peg-IFN dose-dependently affected the timing to the first undetectable HCV RNA level during the treatment. These results indicate that dose reduction of Peg-IFN can cause a shift from c-EVR to LVR and a shift from LVR to HCV RNA-positivity at week 24. The proportion of patients with treatment failure among those given <0.9 μg/kg/week of Peg-IFN (66%) was decreased by half among the patients given ≥1.2 μg/kg/week of Peg-IFN (32–35%). Considering that the effectiveness of extended treatment for patients with LVR is obvious, if patients without a c-EVR were to attain HCV RNA negativity by 24 weeks, those patients would have the potential to attain an SVR with extended treatment. However, if patients do not attain HCV RNA negativity, those patients must discontinue the treatment. Therefore, causing patients to shift from HCV RNA negativity by week 24 to being HCV RNA-positive at week 24 would be missing the chance to obtain SVR even with extended treatment. As shown in Fig. 2, the longitudinal negative

rate of HCV RNA was dose-dependently affected by Peg-IFN at all points during the treatment. Therefore, a marked dose reduction of Peg-IFN should not be done at the start of treatment even for patients with lower Plt values (which are indicative of advanced fibrosis), because dose reduction of Peg-IFN before HCV RNA negativity is attained can lead to an increased possibility of treatment failure.

Next, as shown in Fig. 3, 70% of the patients with undetectable HCV RNA at week 4 were given ≥1.5 μg/kg/week of Peg-IFN, 71% of those with undetectable HCV RNA at week 12 were given ≥1.2 μg/kg/week, and 64% of those with detectable HCV RNA at week 24 were given ≤1.2 μg/kg/week. Therefore, in HCV genotype 1 patients treated with Peg-IFN plus ribavirin, the treatment goal for c-EVR or non-NR should be to maintain a Peg-IFN dose of ≥1.2 μg/kg/week, and that for RVR should be to maintain a Peg-IFN dose of ≥1.5 μg/kg/week. Using granulocyte–macrophage colony-stimulating factor for patients who develop a severe decrease of blood cells and are forced to decrease Peg-IFN can be beneficial, as long as HCV RNA is positive.

A limitation of the present study is that the actual SVR rate could not be compared among the four Peg-IFN categories because some patients with LVR were treated for 72 weeks and some were treated for 48 weeks; actual SVR rates were 20% in patients with Peg-IFN <0.9 μg/kg/week, 18% in those with 0.9–1.2 μg/kg/week, 36% in those with 1.2–1.5 μg/kg/week, and 48% in those ≥1.5 μg/kg/week. On the assumption that the SVR rate for patients with RVR is 90%, the SVR rate for those with c-EVR without RVR is 75% for 48-week treatment, and the SVR rate for those with LVR is 60% for 72-week treatment, the SVR rate of response-guided therapy was calculated to be 23% for patients given <0.9 μg/kg/week of Peg-IFN, 30% for those given 0.9–1.2 μg/kg/week of Peg-IFN, 45% for those given 1.2–1.5 μg/kg/week of Peg-IFN, and 50% for those given ≥1.5 μg/kg/week of Peg-IFN in the matched cohort in the present study. Thus, dose reduction of Peg-IFN can reduce the SVR rate even if response-guided therapy is done. Another limitation of this study is that the *IL28B* SNP, which is known to be a host factor affecting the antiviral effect, could not be examined in all cases, because the characteristic matched patients were extracted from a large retrospective cohort. However, we had the result of the *IL28B* SNP (rs8099917) for 290 patients; 214 patients had TT and 76 had TG or GG. The proportions of patients with the *IL28B* SNP TT were similar among the four Peg-IFN categories (≤0.9 μg/kg/week, 76%, 31/41; 0.9–1.2 μg/kg/week, 71%, 27/38; 1.2–1.5 μg/kg/week, 67%, 99/147; ≥1.5 μg/kg/week, 77%, 57/74, $p = 0.853$). Therefore, it would appear that there was no bias for any cases. Among the patients with the *IL28B* SNP TT, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/58), 33% (19/58), and 69% (40/58) among the patients with <1.2 μg/kg/week of Peg-IFN and 4% (7/156), 62% (97/156), and 82% (128/156) among those with ≥1.2 μg/kg/week. There were significant differences between these two Peg-IFN groups in the HCV RNA negative rates at weeks 12 and 24 ($p = 0.002$, $p = 0.04$, respectively). Similarly, among the patients with *IL28B* SNP TG or GG, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/21), 0% (0/21), and 10% (2/21) among the patients with <1.2 μg/kg/week of Peg-IFN and 2% (1/55), 9% (5/55), and 27% (15/55) among those with ≥1.2 μg/kg/week. The HCV RNA negative rates at weeks 12 and 24 tended to be higher in the patients with ≥1.2 μg/kg/week of Peg-IFN ($p = 0.06$, $p = 0.13$, respectively). From the above-mentioned results, it appears that the dose-dependent effect of Peg-IFN on the timing of HCV RNA negativity could be considered regardless of the *IL28B* SNP.

In conclusion, this matched study has demonstrated that, in patients with CH-C with genotype 1 receiving Peg-IFN plus ribavirin combination therapy, Peg-IFN dose-dependently affects the timing to the first undetectable HCV RNA level and the failure to attain HCV RNA negativity. Dose reduction of Peg-IFN to <1.2 μg/kg/week before HCV RNA negativity is attained delays HCV RNA clearance dose-dependently and increases the rate of treatment failure. Maintaining the Peg-IFN dose at ≥1.2 μg/kg/week, and preferably at ≥1.5 μg/kg/week, can accelerate the timing to the first undetectable HCV RNA level for CH-C genotype 1 patients treated with Peg-IFN plus ribavirin.

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References

1. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001; 358:958–65.

2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347: 975–82.
3. McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med. 2009;361: 580–93.
4. Jensen DM, Morgan TR, Marcellin P, Pockros PJ, Reddy KR, Hadziyannis SJ, et al. Early identification of HCV genotype 1 patients responding to 24 weeks peginterferon alpha-2a (40 kd)/ ribavirin therapy. Hepatology. 2006;43:954–60.
5. Mangia A, Minerva N, Bacca D, Cozzolongo R, Ricci GL, Carretta V, et al. Individualized treatment duration for hepatitis C genotype 1 patients: a randomized controlled trial. Hepatology. 2008;47:43–50.
6. Yu ML, Dai CY, Huang JF, Chiu CF, Yang YH, Hou NJ, et al. Rapid virological response and treatment duration for chronic hepatitis C genotype 1 patients: a randomized trial. Hepatology. 2008;47:1884–93.
7. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
8. Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. Hepatology. 2007;46:1688–94.
9. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
10. McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology. 2002;123:1061–9.
11. Shiffman ML, Ghany MG, Morgan TR, Wright EC, Everson GT, Lindsay KL, et al. Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. Gastroenterology. 2007;132:103–12.
12. Reddy KR, Shiffman ML, Morgan TR, Zeuzem S, Hadziyannis S, Hamzeh FM, et al. Impact of ribavirin dose reductions in hepatitis C virus genotype 1 patients completing peginterferon alfa-2a/ ribavirin treatment. Clin Gastroenterol Hepatol. 2007;5:124–9.
13. Oze T, Hiramatsu N, Yakushijin T, Kurokawa M, Igura T, Mochizuki K, et al. Pegylated interferon alpha-2b (Peg-IFN alpha-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN alpha-2b plus ribavirin. J Viral Hepat. 2009;16: 578–85.
14. Hiramatsu N, Oze T, Yakushijin T, Inoue Y, Igura T, Mochizuki K, et al. Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin. J Viral Hepat. 2009;16:586–94.
15. Oze T, Hiramatsu N, Yakushijin T, Mochizuki K, Oshita M, Hagiwara H, et al. Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy. J Hepatol. 2011;54:604–11.
16. Inoue Y, Hiramatsu N, Oze T, Yakushijin T, Mochizuki K, Hagiwara H, et al. Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses. J Viral Hepat. 2010;17: 336–44.
17. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
18. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1100–4.
19. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105–9.
20. Lindsay KL, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, et al. A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology. 2001;34:395–403.
21. Fried MW, Jensen DM, Rodriguez-Torres M, Nyberg LM, Di Bisceglie AM, Morgan TR, et al. Improved outcomes in patients with hepatitis C with difficult-to-treat characteristics: randomized study of higher doses of peginterferon alpha-2a and ribavirin. Hepatology. 2008;48:1033–43.
22. Roberts SK, Weltman MD, Crawford DH, McCaughan GW, Sievert W, Cheng WS, et al. Impact of high-dose peginterferon alfa-2A on virological response rates in patients with hepatitis C genotype 1: a randomized controlled trial. Hepatology. 2009;50:1045–55.
23. Reddy KR, Shiffman ML, Rodriguez-Torres M, Cheinquer H, Abdurakhmanov D, Bakulin I, et al. Induction pegylated interferon alfa-2a and high dose ribavirin do not increase SVR in heavy patients with HCV genotype 1 and high viral loads. Gastroenterology. 2010;139:1972–83.
24. Oze T, Hiramatsu N, Yakushijin T, Mochizuki K, Imanaka K, Yamada A, et al. The efficacy of extended treatment with pegylated interferon plus ribavirin in patients with HCV genotype 1 and slow virologic response in Japan. J Gastroenterol. 2011;46: 944–52.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Dynamics of regulatory T cells and plasmacytoid dendritic cells as immune markers for virological response in pegylated interferon-α and ribavirin therapy for chronic hepatitis C patients

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Abstract
Background For the treatment of chronic hepatitis C, a combination of pegylated interferon-α (PEG-IFNα) and ribavirin has been widely used as a standard of care. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. Our aim was to elucidate whether or not the frequency or function of blood cells is related to the outcome of the therapy.
Methods Sixty-seven chronic hepatitis C patients with high viral load of HCV genotype 1 infection who underwent 48 weeks of PEG-IFNα2b and ribavirin therapy were examined. During the treatment, frequencies of myeloid or plasmacytoid dendritic cells, Th1, Th2 cells, NK cells, and regulatory T cells were phenotypically determined.
Results Among the patients enrolled, 29 showed a sustained virological response (SVR), 18 a transient response (TR) and 17 no response (NR). The clinical and immunological markers were compared between the SVR and non-SVR patients, including TR and NR. Based on clinical, histological, immunological parameters, and cumulative dosage of PEG-IFNα2b and ribavirin, multivariate analyses revealed that higher platelet counts and higher regulatory T cell frequency at week 12 are indicative of SVR. Even in patients who attained complete early virological response at week 12, multivariate analyses disclosed that higher platelet counts and higher plasmacytoid dendritic cell frequency are indicative of SVR.
Conclusions In PEG-IFNα and ribavirin combination therapy for chronic hepatitis C patients, the increments of regulatory T cells and plasmacytoid dendritic cell frequency are independently related to favorable virological response to the therapy.

Keywords Early virological response · Plasmacytoid dendritic cells · Regulatory T cells

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Introduction

Hepatitis C virus (HCV) is one of the major causative agents of chronic liver diseases and hepatocellular

carcinoma (HCC) in the world [1, 2]. In order to prevent the development of HCV-induced liver diseases, eradication of HCV from infected patients may be required. For the treatment of chronic hepatitis C, a combination of pegylated interferon-α (PEG-IFNα) and ribavirin treatment has been used as a standard of care (SOC) [3, 4]. However, in patients with HCV genotype 1 and high viral load, approximately 50% of them are able to clear the virus by 48 weeks of SOC [5, 6]. In addition to HCV genotype and quantity, several demographic factors have been reported as therapeutic determinants in PEG-IFNα and ribavirin therapy, such as age, gender, ethnicity, and liver fibrosis [5, 6]. In addition, it is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., early virological response (EVR), correlate well with the clinical outcomes of the treated patients [5, 7]. It has been reported that the patients who fail to clear HCV at week 24 are not likely to attain SVR after 48 weeks of the therapy, suggesting that non-EVR can serve as a negative predictor of SVR [8]. Even in patients who attained EVR, 30% of them eventually relapse during the 48 weeks of therapy. Prolongation of the duration of PEG-IFNα and ribavirin therapy from 48 to 72 weeks is recommended to improve the SVR rate by decreasing relapsers [9]. Thus, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, because it can offer them a better chance of attaining SVR.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells (DC), NK cells, and T cells [10, 11]. Some investigators have reported that the dynamics of immune cells throughout the therapy are involved in the efficacy of PEG-IFNα and ribavirin. In chronic HCV infection, the enhancement of HCV-specific Th1 response or DC function has been reported to be involved in therapeutic HCV eradication [12, 13]. We have previously demonstrated that plasmacytoid dendritic cell (PDC) frequency and DC function are involved in HCV eradication in patients who underwent 48 weeks of PEG-IFNα and ribavirin therapy [14]. These reports have supported the possibility that the enhancement of certain immune responses is a prerequisite for therapeutic HCV clearance. However, one of the limitations of these studies is that the conclusions were drawn from relatively small numbers of patients and evaluated by univariate analysis. Therefore, multivariate analyses are arguably required in order to validate the significance or independence of immune cell markers in the therapeutic efficacy.

In this study, we have extended our investigation to elucidate whether or not the dynamics of immune cells are involved in therapeutic outcomes. Consequently, the independent significance of regulatory T cell or plasmacytoid DC frequency is revisited in the efficacy of PEG-IFNα and ribavirin therapy for chronic hepatitis C patients.

Materials and methods

Subjects

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Kosei-nenkin Hospital, Higashi Osaka Municipal Hospital, and Osaka National Hospital, 67 patients who received PEG-IFNα2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethics committee of the Osaka University Graduate School of Medicine and all the hospitals listed above (approval no. 08156). Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody (Ab) and HCV RNA, but were negative for hepatitis B virus and human immunodeficiency virus. All of them were infected with HCV genotype 1b with serum HCV RNA quantity of more than 100 kilo international units (KIU)/ml, as determined by methods described elsewhere [15]. All patients had shown persistent or fluctuating serum alanine aminotransferase (ALT) abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune, alcoholic, and metabolic disorders was excluded by laboratory and imaging analyses. A combination of biochemical markers and ultrasonography (US) or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver in all patients. Histological analyses of liver disease were performed with liver tissue obtained by US-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the METAVIR scoring system [16].

Treatment

All patients were treated with PEG-IFNα2b subcutaneously at a dose of 75 μg/week (body weight >40 and ≤60 kg), 105 μg/week (body weight >60 and ≤80 kg), or 135 μg/week (body weight >80 and ≤100 kg) and oral ribavirin at a dose of 600 mg/day (body weight >40 and ≤60 kg), 800 mg/day (body weight >60 and ≤80 kg), or 1000 mg/day (body weight >80 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.

Dose reduction of PEG-IFNα and ribavirin

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematological adverse effects. The dose of PEG-IFNα2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to less than 1500/mm^3, the

neutrophil count to less than 750/mm^3, or the platelet (Plt) count to less than 8×10^4/mm^3, and was discontinued if the WBC count declined to less than 1000/mm^3, the neutrophil count to less than 500/mm^3, or the Plat count to less than 5×10^4/mm^3. Ribavirin was also reduced from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg if the hemoglobin (Hb) level decreased to less than 10 g/dl, and was discontinued if the Hb level decreased to less than 8.5 g/dl. Both PEG-IFNα2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During the therapy, ferric medicine or hematopoietic growth factors, such as erythropoietin alpha or granulocyte–macrophage colony-stimulating factor were not administered.

Quantification of HCV RNA and assessment of virological response

Serum HCV RNA titers were quantified using the COBAS AMPLICOR HCV MONITOR Test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed by the COBAS AMPLICOR HCV Test, version 2.0 (detection threshold 50 IU/ml).

Virological response during and after the therapy was determined according to the American Association for the Study of Liver Diseases (AASLD) practice guideline [17]. The complete early virological responders (c-EVR) 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 (SVR) was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response (TR) 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 (NR) meant that there was persistently positive serum HCV RNA throughout the therapy period. The non-SVR group comprised TR and NR patients.

Assessment of drug exposure

The amounts of PEG-IFNα2b and ribavirin actually taken by patients during the first 12 weeks of the treatment were evaluated by reviewing the medical records as reported previously [18, 19]. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline. The dose of PEG-IFNα2b and ribavirin was expressed as micrograms per kilogram per week and milligrams per kilogram per day, respectively.

Analysis of DC subsets, helper T cells, NK cells, and regulatory T cells

For the numerical analyses of blood DC, helper T cells, NK cells, and regulatory T cells (Tregs), venous blood was drawn from patients before treatment and at weeks 8, 12, 24, and 48 during the therapy. Blood samples taken from patients in relevant hospitals were transferred to Osaka University within 6 h and were processed on the same day. 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 Abs for phenotypic markers. All immunological assays were performed in Osaka University.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA): anti-Lineage marker [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). The antibodies for CD25 (clone B1.49.9) and CD4 (clone 1 3B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA). Anti-CXCR3 (clone 49801) monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously [14]. The acquisitions and analyses of data were performed with FACS Calibur (BD Biosciences) and CellQuest software.

Blood DCs were defined as Lin$^-$ and HLA-DR$^+$ cells. Myeloid DCs (MDC) are Lin$^-$, HLA-DR$^+$, CD11c$^+$, and CD123^{low} cells, and plasmacytoid DCs (PDC) are Lin$^-$, HLA-DR$^+$, CD11c$^-$, and CD123^{high} cells. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are CD4$^+$, CD45RO$^+$, and CXCR3$^+$, and Th2 cells are CD4$^+$, CD45RO$^+$, and CCR4$^+$. NK cells were defined as CD3$^-$ and CD56$^+$ cells. Regulatory T cells (Tregs) were defined as CD4$^+$, CD25^{high+} cells as reported previously [20]. The percentages of DC subsets and NK cells in PBMCs or Th1, Th2 cells and Tregs in CD4$^+$ T cells were determined by FACS. In order to examine the dynamics of immune cells after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy [14].

Allogeneic mixed leukocyte reaction with DC

In some patients, we examined whether the allostimulatory ability of DCs was related to the clinical outcomes. Before, at the end of treatment, and at week 4 after completion of

the treatment, monocyte-derived DCs were generated from PBMC obtained from the patients according to methods reported previously [21]. As controls, monocyte-derived DCs were simultaneously generated from healthy donors. As responder cells in mixed lymphocyte reactions (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 DC was performed as reported previously [21]. In order to compare the ability of DC among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of [^{3}H]thymidine incorporated into CD4 T cells at the T cell/DC ratio of 10:1.

Statistical analyses

To analyze the relationship between clinical and immunological data at the baseline and virological response, univariate analysis using the Mann–Whitney *U* test or chi-squared test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. Differences of continuous variables between groups were compared by two-way analysis of variance (ANOVA). A two-railed *P* value less than 0.05 was considered significant. These statistical analyses were performed with SPSS version 15.0 (SPSS Inc. Chicago, IL, USA).

Results

Outcome of the PEG-IFNα2b and ribavirin therapy

In 67 patients who had been treated for 48 weeks, 29 (43%) achieved SVR, 18 (27%) were TR, 17 (25%) were NR, and 3 (4%) were unknown (Fig. 1). The clinical backgrounds of these patients are summarized in Table 1. Among these cohorts, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR group) and 8 TR (EVR-TR group). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR and 3 were unknown. Details of the therapeutic response in the current study are shown in Fig. 1.

Higher platelet counts and Treg increase are involved in SVR in patients who underwent PEG-IFNα2b and ribavirin therapy

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, we first compared these parameters between SVR and non-SVR groups. Representative dot plots of the immune cell populations are shown in Fig. 2. The identification and enumeration of immune cells were determined by FACS. The pretreatment percentages of DC in SVR were higher than those in the non-SVR group. However, those of PDC, NK cells, Th1, Th2, Treg, and DC function as judged by MLR were not different between them (Fig. 3).

As for the changes of DC subsets during the therapy, in the SVR group, the frequencies of PDC increased after the

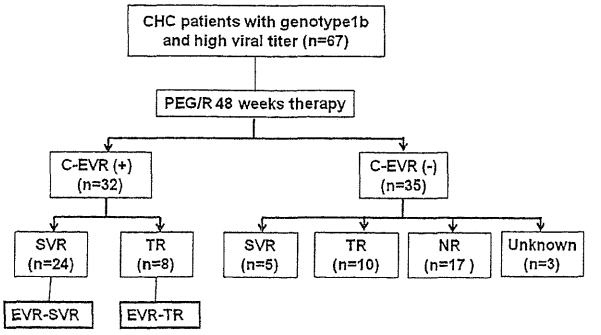


Fig. 1 Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. In 67 patients who had been treated for 48 weeks, 29 achieved SVR, 18 were TR, 17 were NR, and 3 were unknown. The complete early virological responders (c-EVR) were defined as those who show a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at week 12 of the therapy. According to this criterion, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR) and 8 TR (EVR-TR). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR, and 3 were unknown. *SVR* sustained virological responder, *TR* transient responder, *NR* non-responder

Table 1 Demographics and clinical backgrounds of the subjects

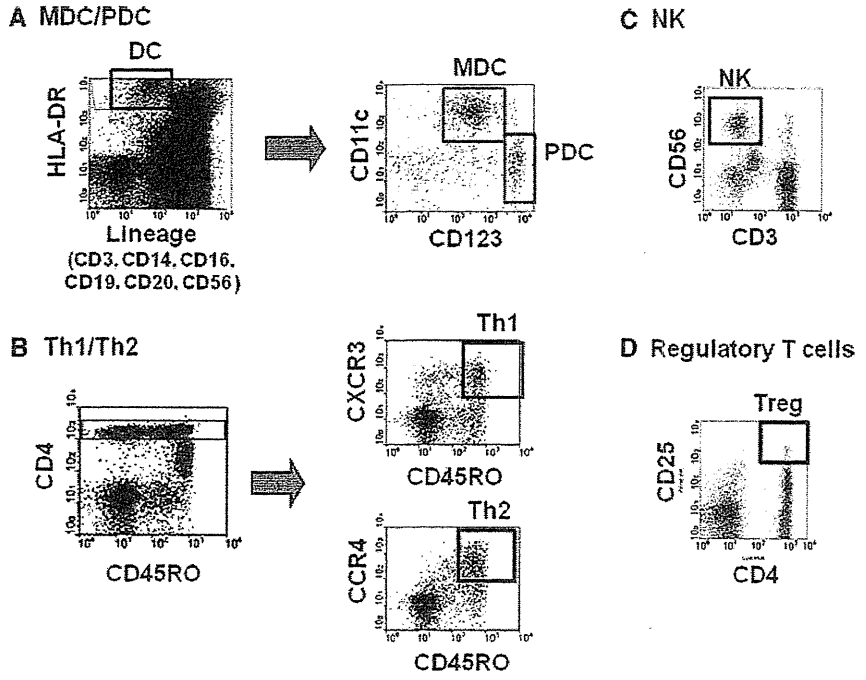
Factors	Value	Range
Number	67	
Age (years)	51.0 ± 10.3	(24–67)
Gender (M/F)	44/23	
HCV RNA (KIU)[a]	2415	
Activity: A0/1/2/3[b]	0/35/30/1	
Fibrosis: F0/1/2/3/4[b]	2/27/27/9/1	
WBC (/ml)	5229 ± 1299	(2960–9400)
Neutro (/ml)	2663 ± 826	(1077–4516)
Hb (g/dl)	14.6 ± 1.2	(12.0–18.0)
Platelets ($\times 10^4$/mm^3)	16.6 ± 4.6	(5.0–31.0)
ALT (IU/l)	83.1 ± 53.9	(14–269)
T. chol (mg/dl)	172 ± 29	(118–238)
Cr (mg/ml)	0.8 ± 0.2	(0.4–1.3)

All results are expressed as mean ± SD and range

T. chol total serum cholesterol, *Cr* creatinine

[a] Amplicore HCV monitor

[b] Ishak's histological scores



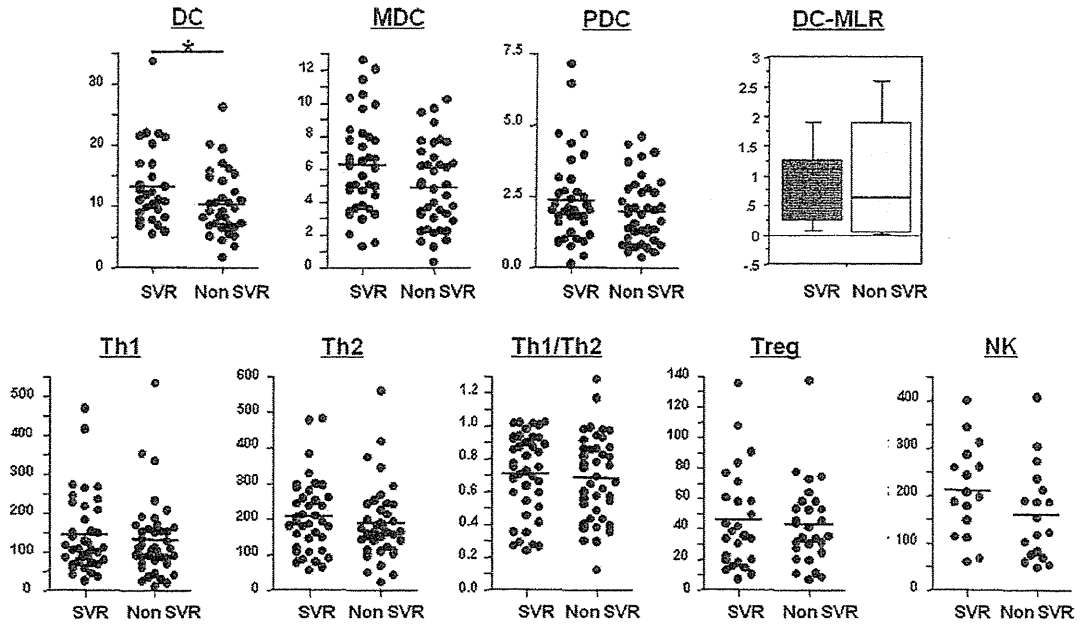


Fig. 2 Phenotypic identification of blood cells by flow cytometry. Representative analyses of myeloid and plasmacytoid dendritic cells (MDC and PDC), type 1 and type 2 helper T cells (Th1 and Th2), natural killer (NK) cells, and regulatory T cells are shown. The combination of surface molecules for the identification of cells is described in "Materials and methods"

Fig. 3 Comparison of pretreatment frequency of blood cells and allostimulatory capacity of monocyte-derived dendritic cells between SVR and non-SVR patients who had been treated with 48-week PEG-IFNα2b and ribavirin therapy. The frequencies of MDC, PDC, Th1 and Th2 cells, Th1/Th2 ratio, NK cells, regulatory T cells, and allogeneic MLR were compared between SVR and non-SVR patients. 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/DC ratio of 10:1. *$P < 0.05$ by Mann–Whitney U test

beginning of therapy and showed a peak at week 12 of therapy (T12W), which subsided to the end-of-treatment (EOT). Such a PDC increase at the early phase was not observed in the non-SVR group (Fig. 4a). In contrast, the MDC frequency remained at a similar level throughout the therapy, regardless of viral response (data not shown). Alternatively, in the SVR group, the percentages of Treg (CD4$^+$CD25^{high+} cells) increased through the therapy,

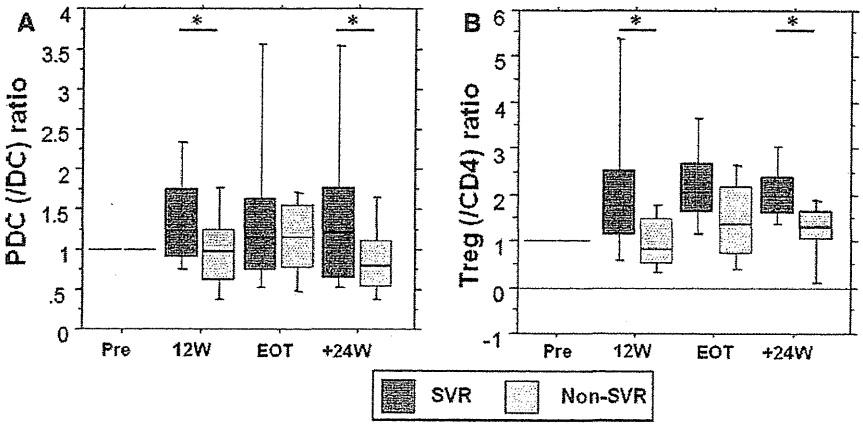


Fig. 4 Changes in frequencies of plasmacytoid dendritic cells and regulatory T cells during and after 48-week PEG-IFNα2b and ribavirin therapy in SVR and non-SVR patients. The ratios of frequencies of PDC (**a**) and Tregs (**b**) at each time point to the pretreatment values were compared between SVR and non-SVR patients. *Boxes* represent lower and upper quartiles, *solid line* within each *box* the median value, *whiskers* the minimum and maximum values. $*P < 0.05$ by Mann–Whitney *U* test. *EOT* end-of-treatment (at 48 weeks of the therapy), *+24W* 24 weeks after the completion of therapy

with cell levels being higher than those in the non-SVR group (Fig. 4b). The other cells, including Th1, Th2, and NK cells, did not differ between the groups (data not shown). Univariate and multivariate analyses were performed to assess the significance of various factors, including demographic, biochemical, virological, immunological parameters, and drug adherence. The allostimulatory capacity of DC after the completion of therapy, whose significance was demonstrated in the previous paper [21], was not included in this study because the numbers of patients examined for it were limited. In univariate analyses, platelet counts, histological activity and fibrosis, dose of PEG-IFNα2b, and attainment of c-EVR were found to be significant in SVR (Table 2). As for immunological markers, pretreatment DC frequency, PDC frequency, their ratio at T12W, and Treg frequency ratio at T12W are significant (Table 3). Based on these parameters, multivariate analysis revealed that platelet counts and Treg frequency at T12W were independent factors involved in SVR (Table 4). These results show that higher platelet counts and Treg increment may be related to SVR in 48 weeks of PEG-IFNα and ribavirin treatment.

Table 2 Univariate analyses of clinical factors involved in SVR

Factors	SVR	Non-SVR
N	29	38
Age (years)	48.0 ± 11.8	53.3 ± 8.6
Gender (M/F)	20/9	24/14
WBC (/mm^3)	5361 ± 1314	5127 ± 1295
Neutro (/mm^3)	2969 ± 861	2461 ± 753
Hb (g/dl)	14.6 ± 1.2	14.5 ± 1.2
Platelets (×10^4/mm^3)	18.2 ± 4.4*	15.2 ± 4.4
ALT (IU/l)	72 ± 54	92 ± 53
HCV RNA (KIU/ml)	2103	2654
Activity: 0–1/2–3/n.d.	29/0/0$^\#$	27/10/1
Fibrosis: 0–2/3–4/n.d.	20/9/0*	15/22/1
PEG-IFN dose (μg/kg/day)	1.43 ± 0.14$^\#$	1.31 ± 0.22
Ribavirin dose (mg/kg/day)	10.6 ± 1.5	9.9 ± 1.4
c-EVR: +/−	24/5$^\#$	8/27

Mann–Whitney *U* test, chi-square test

n.d. not determined

$*P < 0.05$, $^\#P < 0.01$

Higher platelet counts and PDC increase are independent factors involved in SVR after attainment of c-EVR

Next, we examined the above-mentioned immunological parameters in patients who attained c-EVR, as they were considered to be comparable with respect to the virological response to the therapy. Among 32 patients in the c-EVR group, 24 developed to SVR (EVR-SVR) and the remaining 8 to TR (EVR-TR) (Fig. 1). Univariate analysis disclosed that lower age is a characteristic of the EVR-SVR patients compared with those in the EVR-TR group (Table 5). As for immunological markers, pretreatment DC frequency, PDC frequency, and PDC ratio at T12W were higher in EVR-SVR patients than those in EVR-TR (Table 6). The pretreatment percentages of MDC, PDC, Th1, Th2, NK cells, and Tregs and those at any all points during the therapy did not differ between EVR-SVR and EVR-TR patients (data not shown). Multivariate analyses revealed that higher platelet counts and PDC increase at T12W were independent factors involved in EVR-SVR (Table 7). These results indicate that the dynamics of PDC

Table 3 Univariate analyses of immunological factors involved in SVR

Factors	SVR	Non-SVR	*P* value
N	29	38	
DC pre (/μl)	13.3 ± 6.5	10.3 ± 5.4	0.038
PDC-12W (/DC)	0.23 ± 0.09	0.18 ± 0.07	0.017
PDC-12W (/DC) ratio	1.42 ± 0.72	1.04 ± 0.63	0.028
Treg-12W (/CD4) ratio	2.49 ± 2.62	1.03 ± 0.64	0.016

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre DC number before therapy, *PDC-12W (/DC)* PDC frequency in DC at T12W, *PDC-12W (/DC) ratio* the ratio of PDC frequency in DC at T12W to the pretreatment value, *Treg-12W (/CD4) ratio* the ratio of regulatory T cell frequency in CD4 at T12W to the pretreatment value

Table 4 Multivariate analyses of clinical and Immunological factors involved in SVR

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.531	0.322–0.875	0.013
Treg-12W (/CD4) ratio	<1.2/>1.2	0.026	0.001–0.750	0.033

Logistic regression analysis, stepwise method

Table 5 Univariate analyses of clinical factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	EVR-SVR	EVR-TR
N	24	8
Age (years)	46.9 ± 12.3*	57.6 ± 6.5
Gender (M/F)	17/7	6/2
WBC (/mm^3)	5442 ± 1382	5211 ± 805
Neutro (/mm^3)	2975 ± 890	2587 ± 759
Hb (g/dl)	14.7 ± 1.1	15.1 ± 1.2
Platelets (×10^4/mm^3)	18.7 ± 4.5	15.0 ± 3.8
ALT (IU/l)	69 ± 56	91 ± 61
HCV RNA (KIU/ml)	1723	1296
Activity: 0–1/2–3/n.d.	24/0/0	6/2/0
Fibrosis: 0–2/3–4/n.d.	16/8/0	5/3/0
PEG-IFN dose (μg/kg/day)	1.43 ± 0.15	1.39 ± 0.23
Ribavirin dose (mg/kg/day)	10.8 ± 1.5	10.1 ± 2.1

Mann–Whitney *U* test, chi-square test

n.d. not determined, *EVR-SVR* SVR patients who attained complete EVR at T12W, *EVR-TR* TR patients who attained complete EVR at T12W

*$P < 0.05$

frequency during therapy serve as an independent immunological predictor for SVR in patients who attained c-EVR with PEG-IFNα and ribavirin therapy.

Table 6 Univariate analyses of immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	EVR-SVR	EVR-TR	*P* value
N		24	8	
DC pre (/μl)		13.5 ± 6.8	8.9 ± 4.5	0.030
PDC-12W (/DC) ratio	<0.8/>0.8	3/21	4/4	0.047

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre, *PDC-12 (/DC) ratio*: see Table 3

Table 7 Multivariate analyses of clinical and immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.627	0.402–0.978	0.040
PDC-12W (/DC)	<0.18/≥0.18	0.028	0.001–0.787	0.036
PDC-12W (/DC) ratio	<0.8/≥0.8	0.032	0.002–0.673	0.027

Logistic regression analysis, stepwise method

PDC-12W (/DC), PDC-12W(/DC) ratio: see Table 3

Discussion

In this study, we demonstrated that the increase of Treg frequency during therapy is involved in SVR, and that of PDC is in SVR patients who attained c-EVR in 48 weeks of PEG-IFNα and ribavirin therapy. Of particular importance is that such significance is independent of viral dynamics (c-EVR), host factors (fibrosis, gender), and drug adherence.

Regulatory T cells (Treg) are immune suppressors that are supposed to alleviate HCV-induced liver inflammation. In chronic HCV infection, the increment of Tregs has been reported by several investigators, including us, although the underlying mechanisms were unspecified [20, 22]. The increase of Treg in SVR patients observed herein seems to be inconsistent with the previous reports regarding Treg as a tolerance inducer in chronic hepatitis C patients. Several controversial reports have been published with regard to the involvement of Tregs in the efficacy of PEG-IFNα and ribavirin therapy for chronic hepatitis C. Soldevila et al. [23] showed that the pretreatment frequency of Treg is higher in patients with non-response (NR) than those in the non-NR groups. Akiyama et al. [24] reported that Tregs in PBMC increased in SVR patients at earlier time points, while Tregs in liver-infiltrating lymphocytes decreased. By contrast, another group disclosed that frequency, phenotype, and function of Tregs are comparable regardless of the outcomes of PEG-IFNα and ribavirin therapy [25].

The current observation raises the possibility that the reduction of HCV load and/or liver inflammation correlates with the increment of Treg frequency, or vice versa. Recently, it was reported that liver inflammation caused by HCV induces PD-L1 on hepatocytes, which then suppress Treg proliferation in liver [26]. If such a scenario is operative as well in PEG-IFNα and ribavirin therapy, alleviation of liver inflammation may reduce PD-L1 expression on hepatocytes, thereby stimulating Treg proliferation. However, most of the TR patients, who were categorized as being in the non-SVR group, displayed normalized serum ALT levels and negative HCV RNA during treatment, of which conditions are equivalent with the SVR patients. Thus, it is still uncertain whether or not such mechanisms are applicable to the present results.

The other possibility is that phenotypically determined Tregs in this study partly consist of activated T cells. It is well known that $CD127^-$ and $FOXP3^+$ are reliable markers of Tregs [27]. In order to examine whether or not the increment of Treg frequency in this study is a contamination of activated T cells, we determined Tregs as $CD4^+CD25^{high}FOXP3^+CD127^-$ cells instead of $CD4^+CD25^{high}$ cells in some patients. In the comparison of the ratio of $CD4^+CD25^{high}FOXP3^+CD127^-$ cell frequency between the SVR and non-SVR groups at T12W, similar results were obtained with those of $CD4^+CD25^{high}$ cells (SVR vs. non-SVR, 10 patients in each group, 2.50 ± 1.20 vs. 1.54 ± 0.53, $P < 0.05$ by Mann–Whitney U test). These results suggest that the analytical results of $CD4^+CD25^{high}$ T cells reflect those of $FOXP3^+$ Tregs. Further investigation is needed to show that such Tregs are functionally suppressive and to see if the change of frequency parallels with suppressor capacity or not.

According to the AASLD practice guidelines for the treatment of chronic hepatitis C, a combination of PEG/R for 48 weeks is recommended for patients who attained c-EVR at week 12 of therapy [17]. However, in some cohorts with large numbers of patients, approximately 30% of them eventually relapse after cessation of the therapy [5]. The factors involved in post-therapeutic relapse have not been fully explored. We and others have reported that liver fibrosis, female gender, late virological response, and dosage of ribavirin (drug adherence) are critically involved in relapse [19, 28, 29]. It is well known that platelet counts in patients with chronic liver disease are well correlated with the degree of fibrosis. In the present study, multivariate analyses revealed that platelet counts but not fibrosis stage are involved in SVR. The reasons for such discrepant contributions to SVR are not clear; however, it demonstrates that the degree of fibrosis is involved in the therapeutic response in this cohort. In addition, the current study showed that the changes of PDC frequency are also somewhat involved in virological relapse in patients that once attained c-EVR.

Plasmacytoid DCs (PDC) play crucial roles in antivirus immune responses by producing IFN-β and -α [30]. In the previous study by us [14], the increment of PDC is observed in patients with SVR, of which change is more significant in those with c-EVR. No concrete explanation is available for the mechanisms of PDC increase in SVR patients. One of the possibilities is that the PDC increase is a consequence of better response to exogenous IFN-α in patients who have a higher chance of attaining SVR. IFN-α is reported to act as a regulatory factor on $CD11c^-$ DCs to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [31]. Such a possibility is supported by the findings that higher induction of IFN-stimulated genes (ISGs) in hepatocytes after PEG-IFNα and ribavirin therapy, but not higher ISG levels before therapy, is critically involved in successful outcome [32]. Thus, patients who respond well to IFN-α, as demonstrated by better PDC survival during the treatment, are likely to have better chances to eradicate HCV.

Another possible reason for the PDC increase in the periphery of SVR patients is that PDC alter their localization during the treatment. Mengshol et al. [33] reported that PDC and myeloid DC (MDC) are accumulated in inflamed liver through the interactions of chemokines and their receptors. Of particular interest is that the expression of such chemokine receptors on DCs decreased in SVR patients, but not in non-SVR ones [33]. Therefore, it is plausible that PDC may migrate from the liver to periphery/lymphoid tissue after being unleashed from chemokines in the liver. In support for this, it is reported that IFN-α alters the profiles of chemokine receptors on DC, resulting in changes of the DC migrating ability [34].

Recently, numerous other factors were reported to be involved in therapeutic response in chronic hepatitis C patients, such as mutations of HCV genome (core region) [35] or host genetic variation (single nucleotide polymorphisms near the IL28B gene) [36]. In the current study, we were unable to analyze such factors because of the limited numbers of patients. A prospective study is warranted to analyze the involvement of such factors in relation to immune cell markers, in the outcomes of SOC, or the treatment with direct-acting antiviral agents.

In summary, we demonstrated that the increase of Treg frequency is an independent factor involved in SVR in 48 weeks of SOC for chronic hepatitis C patients. In addition, the increase of PDC gains similar significance in SVR patients who attained c-EVR. The assessment of the dynamics of such cells during therapy could offer some clues to identify potential relapsers and give them a better chance of attaining SVR by rescheduling the therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Nordenstedt H, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. Dig Liver Dis. 2010;42(Suppl 3):S206–14.
2. Kanwal F, Hoang T, Kramer JR, Asch SM, Goetz MB, Zeringue A, et al. Increasing prevalence of HCC and cirrhosis in patients with chronic hepatitis C virus infection. Gastroenterology. 2010:140: 1182–8.e1.
3. Poynard T, Colombo M, Bruix J, Schiff E, Terg R, Flamm S, et al. Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology. 2009;136:1618–28.e2.
4. Jacobson IM. Treatment options for patients with chronic hepatitis C not responding to initial antiviral therapy. Clin Gastroenterol Hepatol. 2009;7:921–30.
5. Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. J Gastroenterol. 2006;41:17–27.
6. Poynard T. Treatment of hepatitis C virus: the first decade. Semin Liver Dis. 2004;24(Suppl 2):19–24.
7. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
8. Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales FL Jr, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin. J Hepatol. 2005;43:425–33.
9. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
10. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest. 2009;119:1745–54.
11. Kanto T, Hayashi N. Immunopathogenesis of hepatitis C virus infection: multifaceted strategies subverting innate and adaptive immunity. Intern Med. 2006;45:183–91.
12. Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. Gastroenterology. 2002;123:1070–83.
13. Pachiadakis I, Chokshi S, Cooksley H, Farmakiotis D, Sarrazin C, Zeuzem S, et al. Early viraemia clearance during antiviral therapy of chronic hepatitis C improves dendritic cell functions. Clin Immunol. 2009;131:415–25.
14. Itose I, Kanto T, Inoue M, Miyazaki M, Miyatake H, Sakakibara M, et al. Involvement of dendritic cell frequency and function in virological relapse in pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C patients. J Med Virol. 2007;79:511–21.
15. Pawlotsky JM, Bouvier-Alias M, Hezode C, Darthuy F, Remire J, Dhumeaux D. Standardization of hepatitis C virus RNA quantification. Hepatology. 2000;32:654–9.
16. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19:1513–20.
17. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology. 2009;49:1335–74.
18. Oze T, Hiramatsu N, Yakushijin T, Kurokawa M, Igura T, Mochizuki K, et al. Pegylated interferon alpha-2b (Peg-IFN alpha-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN alpha-2b plus ribavirin. J Viral Hepat. 2009;16:578–85.
19. Hiramatsu N, Oze T, Yakushijin T, Inoue Y, Igura T, Mochizuki K, et al. Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin. J Viral Hepat. 2009;16:586–94.
20. Itose I, Kanto T, Kakita N, Takebe S, Inoue M, Higashitani K, et al. Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis. J Viral Hepat. 2009;16:844–52.
21. Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol. 1999;162:5584–91.
22. Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. Hepatology. 2003;38:1437–48.
23. Soldevila B, Alonso N, Martinez-Arconada MJ, Morillas RM, Planas R, Sanmarti AM, et al. A prospective study of T- and B-lymphocyte subpopulations, CD81 expression levels on B cells and regulatory CD4(+) CD25(+) CD127(low/−) FoxP3(+) T cells in patients with chronic HCV infection during pegylated interferon-alpha2a plus ribavirin treatment. J Viral Hepat. 2011;18:384–92.
24. Akiyama M, Ichikawa T, Miyaaki H, Motoyoshi Y, Takeshita S, Ozawa E, et al. Relationship between regulatory T cells and the combination of pegylated interferon and ribavirin for the treatment of chronic hepatitis type C. Intervirology. 2010;53:154–60.
25. Burton JR Jr, Klarquist J, Im K, Smyk-Pearson S, Golden-Mason L, Castelblanco N, et al. Prospective analysis of effector and regulatory CD4+ T cells in chronic HCV patients undergoing combination antiviral therapy. J Hepatol. 2008;49:329–38.
26. Franceschini D, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G, et al. PD-L1 negatively regulates CD4+CD25+ Foxp3+Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. J Clin Invest. 2009;119:551–64.
27. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203: 1701–11.
28. Oze T, Hiramatsu N, Yakushijin T, Mochizuki K, Oshita M, Hagiwara H, et al. Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy. J Hepatol. 2011;54:604–11.
29. McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology. 2002;123:1061–9.
30. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol. 2008;8:594–606.
31. Ito T, Amakawa R, Inaba M, Ikehara S, Inaba K, Fukuhara S. Differential regulation of human blood dendritic cell subsets by IFNs. J Immunol. 2001;166:2961–9.
32. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A. 2008;105:7034–9.