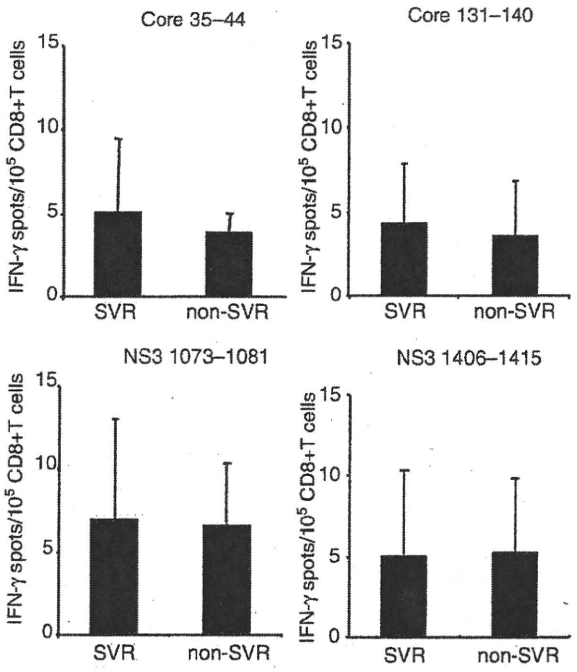


**Figure 3** Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We analyzed the association between the HCV-specific CD8+ T cell responses and the achieving of SVR. SVR: patients who were observed SVR, non-SVR: patients who were not observed SVR.

SVR.[1,12] We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides ($Core_{35-44}$, $Core_{131-140}$, $NS3_{1406-1415}$) increased significantly 4 weeks after the starting treatment and those against $NS3_{1073-1081}$ peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results suggested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

CD8+ CTL activities in pre-treated HCV patients have been reported to be very low.[7,18,19] Consistent with the previous observations, the frequencies of HCV specific CD8+ T cell in pre-treated patients were also low in our study. The frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the HCV viral load and the serum ALT levels of patients before treatment. Several reports demonstrated that the baseline presence of HCV-specific CTLs prior to treatment was associated with viral clearance.[7,18] However, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the achievement of SVR in our study. In previous other reports, whole PBMC isolated from treated patients were used to evaluate the antiviral activity of HCV-specific CD8+ T cells. In our study, enriched CD8+ T cells obtained by magnetic sorting methods were used to enhance the sensitivity for the detection of HCV-specific CD8+ T cells. Both ELISPOT and staining with tetramers/pentamers could be applied for immunological monitoring for peptide-specific CTLs.[20] ELISPOT can detect activated functional CTLs, and tetramers/pentamers staining can detect peptide-specific CTLs.[20] In our study, we assessed the HCV-specific CD8+ T cell responses by IFN-γ ELISPOT, which is the most well-established methods and has already applied for immunological monitoring in cancer patients.[11] Recently perforin- or granzyme B-ELISPOT assays have also been reported. However, due to limitations in cell numbers of PBMC isolated from HCV patients, we were unable to apply another system of immunological monitoring and test other functional molecules. If we can apply these ELISPOT assays, we could directly evaluate the cytotoxic activity of HCV-specific CTLs.

In our study, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were similar between SVR and non-SVR patients. In contrast, significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. Caetano et al. evaluated the HCV-specific CD8+ T cells by HLA class I pentamers specific for the one HCV-Core epitope and one NS3 epitope which were same as we used.[16] They demonstrated that the increase of the frequencies of HCV-specific CTLs at 1 month after starting treatment was mainly due to terminally differentiated cells as well as, to a lesser extent, central memory cells in SVR patients and, in contrast, the increase of HCV-specific

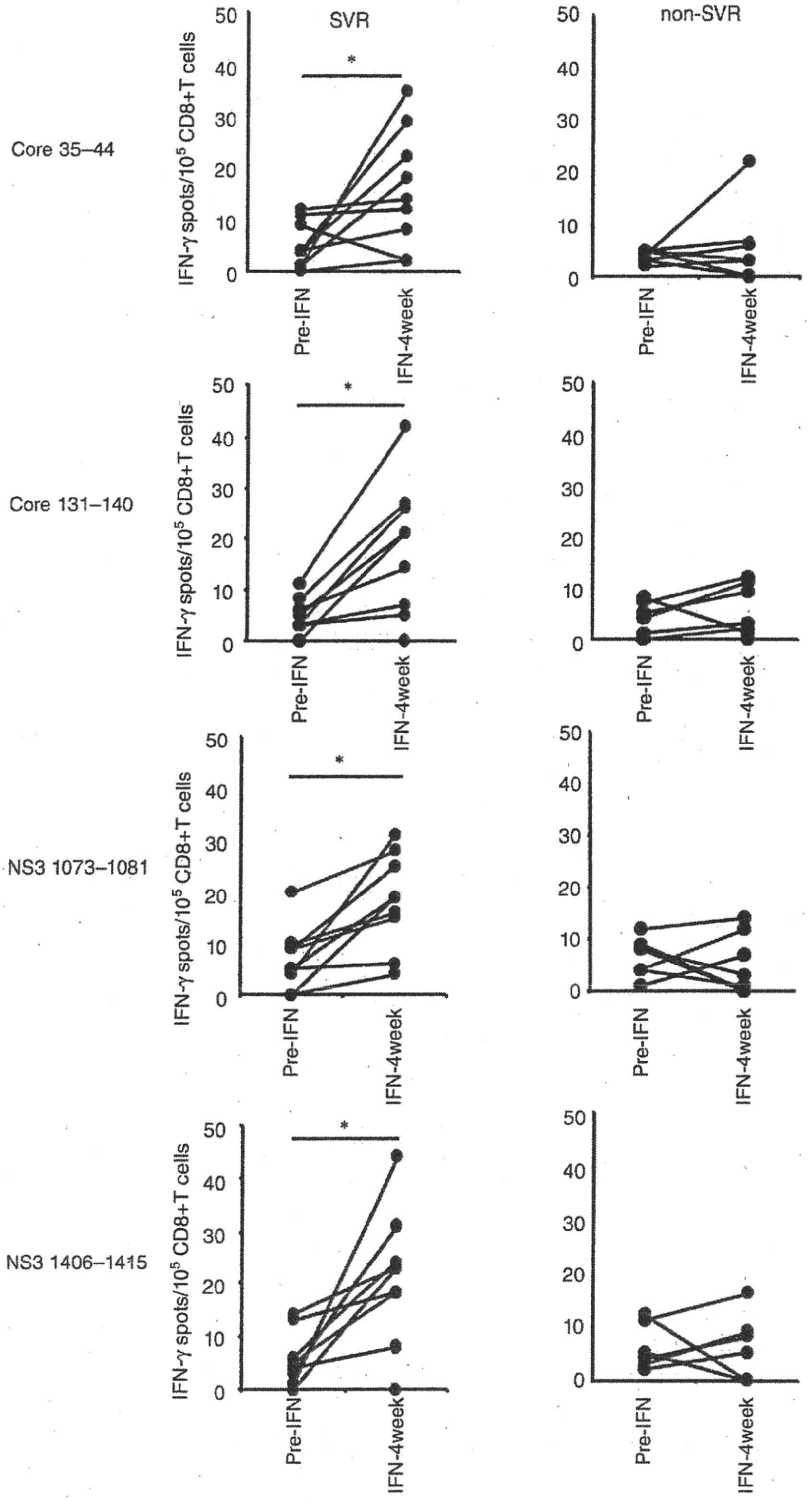


**Figure 4** Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week patients. HCV-specific CD8+ T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. We analyzed the association of HCV-specific CD8+ T cell responses in treated CH-C patients with the achieving SVR. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core or NS3 protein-derived peptides. The treated patients were divided into two groups; SVR group and non-SVR group. $^*P < 0.05$.

pre-terminally differentiated CD8+ T cells was also observed in non-SVR patients.[16] These results suggested that CTLs maturation efficiently occurred in SVR patients. HCV or HCV-gene products have been reported to inhibit the maturation pathway of CTLs.[5,21] Thus the decrease of viral load during this combination therapy may induce CTL maturation.

We demonstrated that the achievement of SVR in this combination therapy was associated with the early elevation of HCV-specific CD8+ T cell responses, but not with the pre-treated levels of HCV-specific CD8+ T cell responses. These results suggested, at least, that the enhancement of HCV-specific CD8+ T cell responses might play critical roles in the second slope of viral clearance by this combination therapy. The increasing frequencies of HCV-specific CD8+ T cells have also been reported to be associated with SVR during the combination therapy by evaluating with pentamers of HCV-specific peptides.[16] Ribavirin has immunomodulatory effect with a switch from Th2 to Th1 cytokine profile.[22] The combined use of pegIFNα and ribavirin might have more immunomodulatory effect to generate HCV specific CTLs. However, even now, this should be elucidated to develop better treatment of chronic hepatitis C.

Although CTL responses to HCV are multi-specific,[13,23] we and others tested only small part of the known CTL epitopes of HCV, which do not comprise all potential HLA A2-restricted CTL epitopes of HCV. HCV may have mutated and escaped from the CTL responses to the corresponding epitopes in the chronically infected patients. The epitopes used in our study have been applied to the detection of HCV-specific CTLs in several other previous studies,[5,15,16] which support the usefulness of the selected epitopes. Our results demonstrated that the increases of the frequencies of CD8+ T cells against four synthesized peptides were associated with the antiviral activity of this combination therapy. Thus the selected epitopes used in our experiments were probably stable, at least, during the 4 weeks after starting treatment.

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.[2] To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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## REFERENCES

1 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; 40: 8–13.
2 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41: 17–27.
3 Price DA, Klenerman P, Booth BL, Phillip RE, Sewell AL. Cytotoxic T lymphocytes, chemokines and antiviral immunity. *Annu Rev Immunol* 1999; 14: 207–32.
4 Gruener NH, Lechner F, Jung MC *et al.* Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; 75: 5550–8.
5 Wedemeyer H, He XS, Nascimbeni M *et al.* Impaired effector function hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; 169: 3447–58.
6 Takaki A, Wiese M, Maertens G *et al.* Cellular immune responses persist, humoral responses decrease two decade after recovery from a single source outbreak of hepatitis C. *Nat Med* 2000; 6: 578–82.
7 Pilli M, Zerbini A, Penna A *et al.* HCV-specific T-cell response in relation to viral kinetics and treatment outcome (DITTO-HCV project). *Gastroenterology* 2007; 133: 1132–43.
8 Ishii S, Koziel MJ. Immune responses during acute and chronic infection with hepatitis C virus. *Clin Immunol* 2008; 128: 133–47.
9 Altman JD, Moss PAH, Goulder PJR *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996; 274: 94–6.
10 Lalvani A, Brookes R, Hambleton S, Britton WJ, Hill AV, McMicheal AJ. Rapid effector function in CD8+ memory T cells. *J Exp Med* 1997; 186: 859–65.
11 Tatsumi T, Herrem CJ, Olson WC *et al.* Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor

tyrosine kinase EphA2 in patients with renal cell carcinoma. *Cancer Res* 2003; **63**: 4481–9.
12 Oze T, Hiramatsu N, Yakushijin T *et al.* Pegylated interferon alpha-2b (Peg-IFN α-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN α-2b plus ribavirin. *J Viral Hepat* 2009; **16**: 578–85.
13 Rehermann B, Chang KM, McHutchinson JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocytes response in patients with chronic hepatitis C virus infection. *J Clin Invest* 1996; **98**: 1432–40.
14 Nelson DR, Marousis CG, Ohno T, Davis GL, Lau JY. Intrahepatic hepatitis C virus-specific cytotoxic T lymphocytes activity and response to interferon alfa therapy in chronic hepatitis C. *Hepatology* 1998; **28**: 225–30.
15 Lohr HF, Schmitz D, Arenz M, Weyer S, Gerken G, Meyer zum Buschenfelde KH. The viral clearance in interferon-treated chronic hepatitis C is associated with increased cytotoxic T cell frequencies. *J Hepatol* 1999; **31**: 407–15.
16 Caetano J, Martinho A, Paiva A, Pais B, Valente C, Luxo C. Differences in hepatitis C virus (HCV)-specific CD8 T-cell phenotype during pegylated alpha interferon and ribavirin treatment are related to response to antiviral therapy in patients chronically infected with HCV. *J Virol* 2008; **82**: 7567–77.
17 Fried MW, Schiffman ML, Reddy KR *et al.* Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. *N Eng J Med* 2002; **347**: 975–82.
18 Freeman AJ, Marinos G, Ffrench RA, Lloyd AR. Intrahepatic and peripheral blood virus-specific cytotoxic T lymphocyte activity is associated with a response to combination IFN-α and ribavirin treatment among patients with chronic hepatitis C virus infection. *J Viral Hepat* 2005; **12**: 125–9.
19 Barnes E, Harcourt G, Brown D *et al.* The dynamics of T-lymhocyte responses during combination therapy for chronic hepatitis C virus infection. *Hepatology* 2002; **36**: 743–54.
20 Sato N, Hirohashi Y, Tsukahara T *et al.* Molecular pathological approaches to human tumor immunology. *Pathol Int* 2009; **59**: 205–17.
21 Lechner W, Wong DK, Dubar PR *et al.* Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499–512.
22 Tam RC, Pai B, Bard J *et al.* Ribavirin polarizes human T cell responses toward a type 1 cytokine profile. *J Hepatol* 1999; **30**: 376–82.
23 He XS, Rehermann B, Lopez-Labradoe XL *et al.* Quantitative analysis of chronic hepatitis C virus-specific CD8+ T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci USA* 1999; **96**: 5692–7.

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***Original Article***

# α-Galactosylceramide activates antitumor immunity against liver tumor

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*Aim:* α-Galactosylceramide (α-GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. We investigated the detailed process of activating liver dendritic cells (DC) and immune cells after α-GalCer treatment in the mouse liver tumor model.

*Methods:* BALB/c mice bearing CMS4 liver tumor (p53 peptide-expressing tumor) were treated by α-GalCer. We evaluated the activation of liver DC and immune cells after α-GalCer treatment. Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay was performed to detect p53 peptide-specific cytotoxic T lymphocytes (CTL). To assess the impact of systemic acquired immunity by α-GalCer treatment, 28 days after liver tumor treatment, CMS4 cells or Colon26 cells were re-challenged s.c.

*Results:* The liver weights of α-GalCer-treated mice were significantly lighter than those of vehicle-treated mice. Depletion experiments revealed that natural killer (NK) cells were essential for the antitumor effect of α-GalCer. α-GalCer treatment significantly increased the population of DC and NK cells in the liver. The expressions of co-stimulatory molecules on liver DC significantly increased with the peak at 1 day after α-GalCer administration. IFN-γ ELISPOT assay demonstrated that p53 peptide-specific CTL was generated efficiently in α-GalCer-treated mice. $^{51}$Cr-release assay revealed that $CD8^+$, not $CD4^+$, CTL against CMS4 cells were generated in α-GalCer-treated mice. The mice that had been protected from CMS4 liver tumor by α-GalCer injection became resistant against s.c. CMS4 re-challenge, but not against Colon26 re-challenge.

*Conclusion:* These results demonstrated the therapeutic potential of α-GalCer against liver cancer through activating liver DC and immune cells in the liver.

**Key words:** α-galactosylceramide, cytotoxic T lymphocytes, dendritic cells, liver, natural killer cells.

## INTRODUCTION

THE GLYCOLIPID ANTIGEN α-galactosylceramide (α-GalCer) induced the activation of natural killer (NK) T cells in a CD1d-dependent manner.[1,2] Recently, α-GalCer has been attracting attention as a novel antitumor therapy. In *in vivo* animal studies, systemic administration of α-GalCer showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma and lymphoma) in hepatic and lung metastasis models.[3,4] Based on the promising results of preclinical studies demonstrating the antitumor potential of α-GalCer, several phase 1 clinical studies on cancer immunotherapy by the i.v. administration of α-GalCer has been carried out, but clinical responses of α-GalCer has been limited.[5] No clinical trial against liver cancer has been reported to date. In view of future α-GalCer treatment of liver cancer, the precise mechanism of activation of innate and acquired immunity in the liver by α-GalCer should be examined. However, these are still not fully understood.

The liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells).[6,7] Dendritic cells (DC) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) cells.[8,9] Recent research of DC biology revealed that DC also contribute

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to innate immune responses by activating NK cells[10–14] and NKT cells[1,15,16] through interleukin (IL)-12 secretion and direct cellular interaction. Thus, DC can be expected to play critical roles in activating abundant T cells, NK cells and NKT cells in the liver after α-GalCer administration. We previously reported that administration of α-GalCer stimulated hepatic NKT cells and led to activation of hepatic NK cells,[4] and that α-GalCer activated liver DC have higher ability to generate acquired immunity.[17] However, the detailed process of activating liver DC and immune cells after α-GalCer treatment should be elucidated.

In this study, we evaluated the liver DC activation and antitumor effect mediated by both innate and acquired immunity against mouse liver tumor after administration of α-GalCer. Administration of α-GalCer induced early activation of liver DC with upregulation of antigen presenting-related molecules and resulted in complete rejection of local liver tumor by NK cells. Followed by early rejection of liver tumor, tumor antigen-specific CTL were generated and complete rejection in s.c. re-challenge of tumor cells was observed. Sequential activation of liver DC, innate and acquired immune cells in the liver may be an attractive strategy for treatment of local and distant tumor of liver cancer.

## METHODS

### Mice

SIX-TO-EIGHT-WEEK-OLD female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). All mice were maintained in micro-isolator cages. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

### Cell lines and culture

CMS4 sarcomas (H-$2^d$) express mutated p53 and present the wild-type $p53_{232-240}$ epitope recognized by H-$2K^d$-restricted CTL.[18] Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained as previously described.[19,20]

### α-GalCer

α-Galactosylceramide was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*[1]

### Animal experiments

BALB/c mice were injected in the liver with $5 \times 10^5$ CMS4 cells on day 0. On day 1, BALB/c mice were injected i.p. with α-GalCer (2 μg/100 μL) or 100 μL of vehicle. Two weeks after the tumor injection, the livers of treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.p. injection of α-GalCer, mice were injected intrahepatically with $5 \times 10^5$ CMS4 cells on day 0 and i.p. treated with α-GalCer on day 1. On day 28 after α-GalCer treatment, $1 \times 10^6$ CMS4 cells or Colon26 cells were injected in the right flank of treated mice. To confirm the involvement of $CD8^+$ T cells in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice. On day 1 and day 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. as previously described.[19] Tumor size was assessed every 3 or 4 days and recorded in $mm^2$ by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± standard deviation (SD).

### NK cell depletion experiments

For NK cell depletion, mice were injected with anti-asialo GM-1 (ASGM1) antibody (Ab) (Wako, Osaka, Japan) on day 1, 5, 10, 15 and 20 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using phycoerythrin (PE)-conjugated anti-DX5 monoclonal antibody (BD-Pharmingen, San Diego, CA, USA). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

### Preparation of hepatic mononuclear cells and liver DC

Hepatic mononuclear cells (MNC) were prepared as previously described.[4] $CD11c^+$ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

### Flow cytometry

For phenotypic analysis of liver DC, NK cells, NKT cells and $CD4^+$ and $CD8^+$ T cells, PE- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80, CD86, T-cell receptor [TCR]-β, CD49b [DX5], CD4, CD8, CD69 [all from BD-Pharmingen], major his-

tocompatibility complex [MHC] class II [Miltenyi Biotec]), and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results of flow cytometric analysis are reported in positive cell rates (%) determined by using isotype controls. DC were identified as $CD11c^+$/MHC class $II^+$ cells. NK cells were identified as $DX5^+$/TCR-$\beta^-$ cells, NKT cells as $DX5^+$/TCR-$\beta^+$ cells, as previously described.[20]

## IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays for $p53_{232-240}$ peptide-reactive CD8+ T-cell responses

Bone marrow derived DC (BMDC) were generated from normal mice as previously described[21] and were used as peptide-presenting cells. On day 14 after treatment of α-GalCer or vehicle, $CD8^+$ T cells were isolated from the spleen cells of treated mice by using magnetic beads (Miltenyi Biotec). We used a mouse IFN-γ ELISPOT kit (R&D systems, Minneapolis, MN, USA) to detect the $p53_{232-240}$ peptide-specific $CD8^+$ T-cell responses. To evaluate the $p53_{232-240}$ peptide, strongly expressing on CMS4 cells,[18] specific CTL induction, isolated $CD8^+$ T cells ($1 \times 10^5$ cells/well) were co-cultured with syngeneic BMDC ($2 \times 10^4$ cells/well) pulsed with $p53_{232-240}$ peptide in an ELISPOT culture plate. BMDC cells without $p53_{232-240}$ peptide served as the negative control, and this value was subtracted from all experimental determinations to establish $p53_{232-240}$ peptide-specific spot numbers. The data are represented as mean IFN-γ spots ± SD per 100 000 $CD8^+$ T cells analyzed.

## Cytolytic assays

Splenocytes from α-GalCer or vehicle-treated mice were harvested 14 days after tumor inoculation. After 5 days of *in vitro* stimulation with mitomycin-C (Kyowa Hakko, Tokyo, Japan)-treated CMS4 cells, lymphocytes were analyzed for their ability to kill CMS4 cells in 4-h $^{51}Cr$-release assays (effector cells/target cells ratio, 60:1), as previously described.[21] $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively.

## Statistical analyses

All experiments with three or more groups in which treatment was applied with a completely random design were first analyzed by a one-way factorial ANOVA. If the resulting *P*-value was less than 0.05, specific pairwise

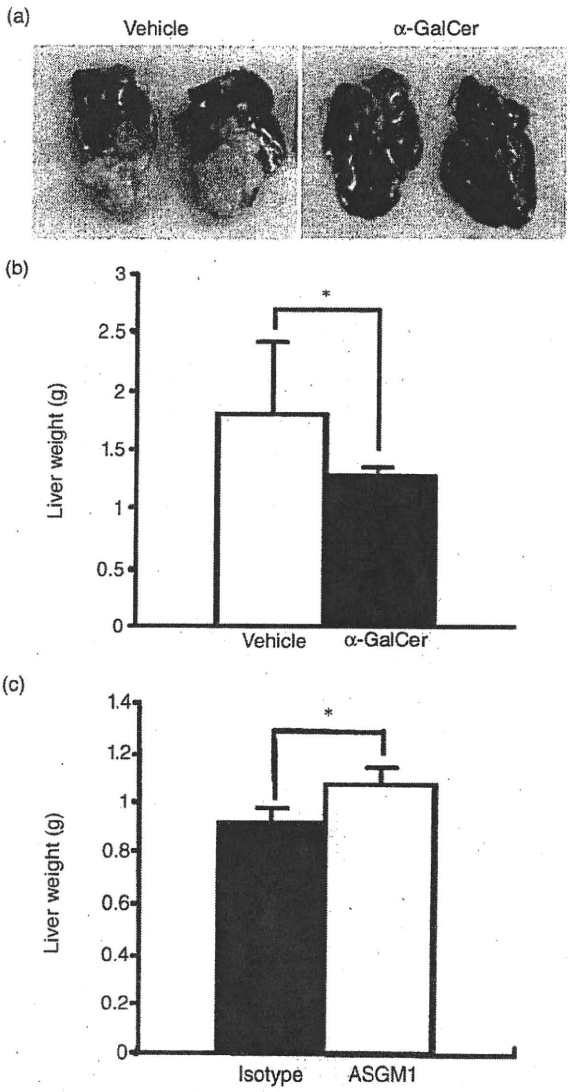



**Figure 1** Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intro-hepatically with $5 \times 10^5$ CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, $n = 7$). Two weeks after the CMS4 tumor injection, the livers of treated mice were removed. (a) Representative liver macroscopic view of each group. (b) Comparison of liver weight of each group. $^*P < 0.05$. (c) To prove that the therapeutic benefit of α-GalCer treatment in the CMS4 liver tumor model is natural killer (NK)-cell dependent, *in vivo* depletion of NK cells was performed (as described in Methods, ASGM1). In control mice, isotype antibody (Ab) was injected i.p. (isotype). Both mice were treated by α-GalCer. Ab-mediated *in situ* depletion of NK cells markedly reduces the therapeutic efficacy of α-GalCer treatment (all treatment groups $n = 5$). $^*P < 0.05$.

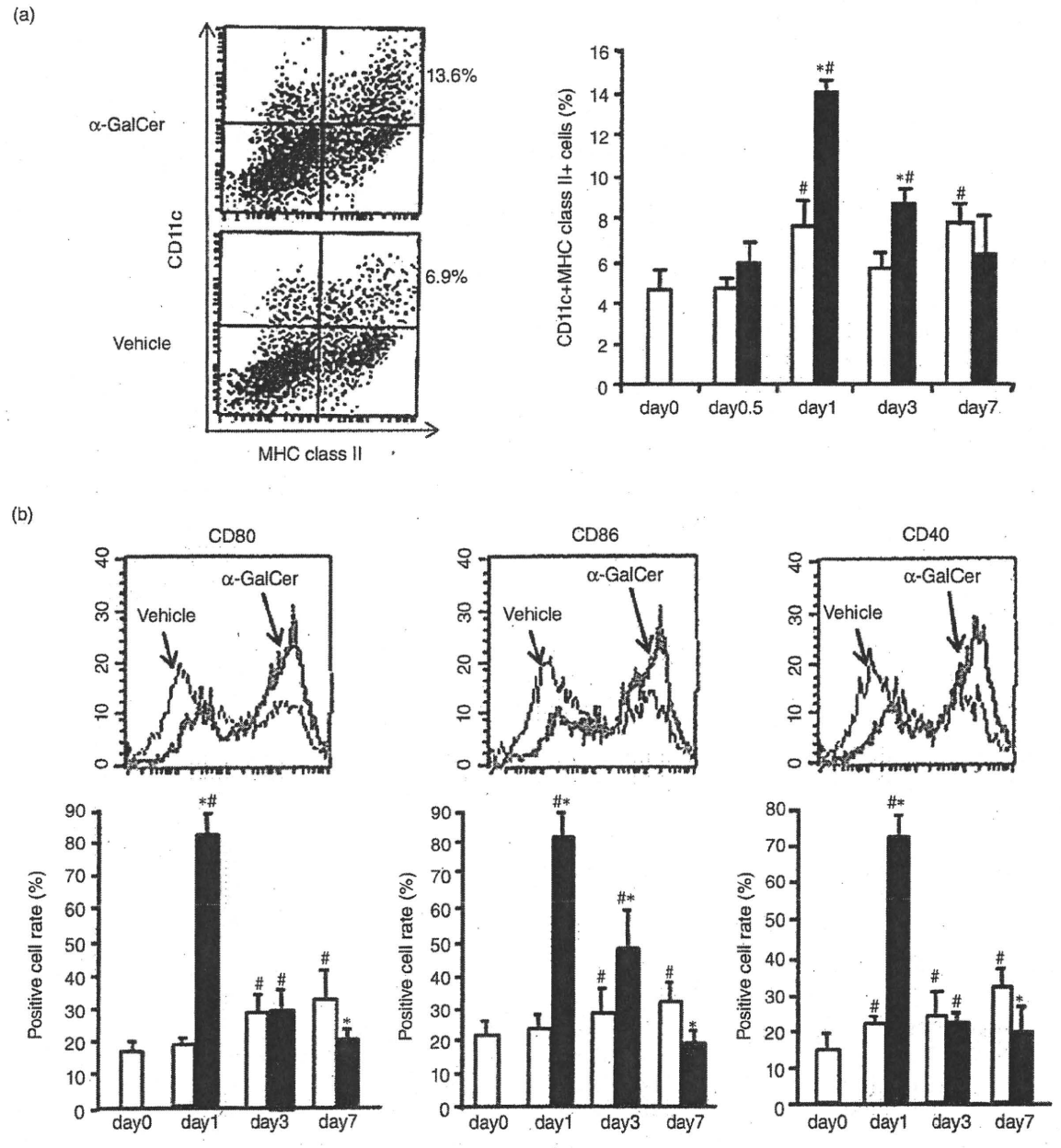



**Figure 2** α-Galactosylceramide (α-GalCer) treatment increased dendritic cell (DC) population in the liver mononuclear cells (MNC) and activated DC functions. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 0.5, 1, 3 and 7. DC ($CD11c^+$ major histocompatibility complex [MHC] class $II^+$ cells) population was evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. Representative dot plots of liver DC ($CD11c^+$ MHC class $II^+$ cells) at day 1 after α-GalCer or vehicle administration are shown in the left panels. The calculated percentages of liver DC are shown in the right. (b) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 and DC were isolated from liver MNC by a magnetic cell sorting system. For phenotypic analysis, liver DC were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86), and the expressions of these molecules were analyzed by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $^*P < 0.05$ vs vehicle-treated mice, $\#P < 0.05$ vs non-treated mice. Representative histograms of the expressions of CD80, CD86 and CD40 on liver DC at day 1 after α-GalCer or vehicle administration are shown in the upper panels.

contrasts were tested with a Student's *t*-test with Welch's correction for unequal variance as needed.

## RESULTS

### α-GalCer administration inhibited CMS4 liver tumor mediated by NK cells

WE INITIALLY EXAMINED whether α-GalCer administration could induce antitumor effect against CMS4 liver tumor. As shown in Figure 1(a), no tumors were observed in the livers of α-GalCer-treated mice whereas large tumors were observed in the livers of vehicle-treated mice. The liver weight of the α-GalCer treatment group was significantly lighter than that of the vehicle treatment group (Fig. 1b). Depletion of NK cells significantly inhibited the antitumor efficacy of α-GalCer treatment (Fig. 1c), whereas depletion of neither $CD4^+$ nor $CD8^+$ T cells was inhibited (data not shown). These results suggested that administration of α-GalCer was therapeutic against CMS4 liver tumor and NK cells were the main effector cells in this antitumor immunity.

### Administration of α-GalCer increased DC population in the liver MNC and activated DC functions

We investigated the population changes of DC in the liver MNC after α-GalCer or vehicle treatment. On day 1 after α-GalCer administration, liver DC proportion in α-GalCer-treated mice was higher than that in vehicle-treated mice (Fig. 2a). Liver DC proportion increased with the peak at 1 day after α-GalCer administration and the liver DC proportion at 7 days decreased to the same level with that from non-treated mice (Fig. 2a). In contrast, liver DC proportion in vehicle-treated mice exhibited weaker change than those in α-GalCer-treated mice (Fig. 2a). The liver DC number also exhibited increase at the peak of 1 day after α-GalCer administration whereas that from vehicle-treated mice exhibited no change (data not shown). We examined the CD40, CD80 and CD86 expressions of liver DC after administration of α-GalCer, which is an indicator of DC activation. On day 1 after α-GalCer administration, CD40, CD80 and CD86 on liver DC from α-GalCer-treated mice expressed more strongly than those from vehicle-treated mice (Fig. 2b). The expressions of all these molecules on liver DC increased with the peak at 1 day after α-GalCer administration and the expression levels of these molecules at 7 days decreased to the same levels on liver DC from non-treated mice (Fig. 2b). In contrast, the expressions of these molecules on liver DC exhibited weaker change in vehicle-treated mice.

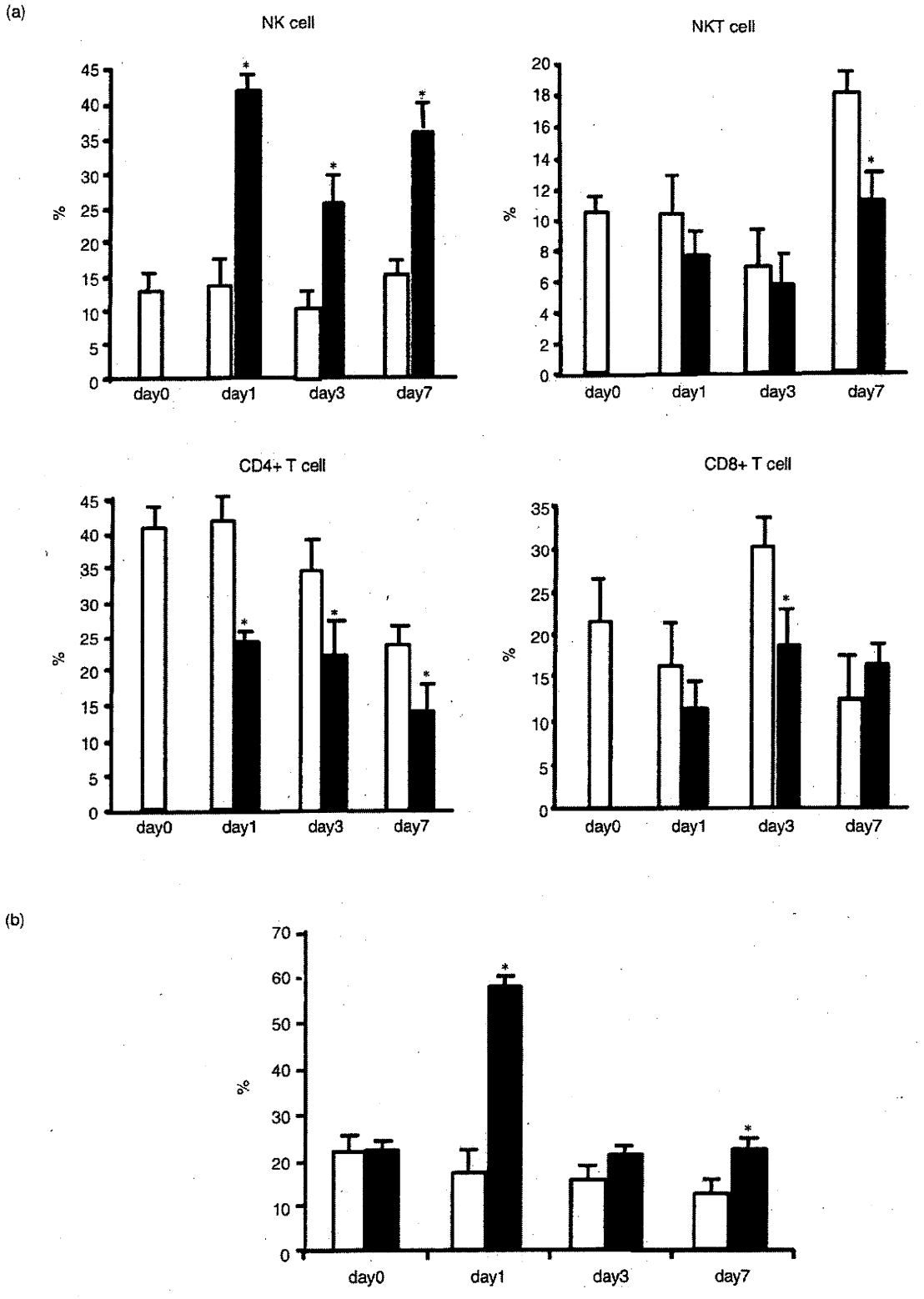
### Activated NK cells composed the major subpopulation of hepatic MNC that increased after α-GalCer treatment

We examined the population change of MNC from the livers after α-GalCer or vehicle administration. It is notable that NK cells strikingly increased in proportion after α-GalCer administration, but not after vehicle administration (Fig. 3a). In contrast, the NKT cells decreased at 1 and 3 days after α-GalCer administration and recovered at day 7 after α-GalCer administration. Both $CD4^+$ and $CD8^+$ T cells decreased in proportion after α-GalCer administration, but not after vehicle administration. We also examined the CD69 expressions of NK cells, which is an indicator of lymphocyte activation. The CD69 expressions on liver NK cells increased with the peak at 1 day and gradually decreased at 7 days after α-GalCer administration (Fig. 3b). In contrast, those did not change after vehicle administration. These results demonstrated that the activated NK cells were the major subpopulation of MNC that increased in the liver after α-GalCer administration.

### $p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor

We evaluated whether $p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor. $CD8^+$ T cells were isolated from the spleen cells of treated mice and then co-cultured with syngeneic DC pulsed with $p53_{232-240}$ peptide strongly expressed on CMS4 cells. As shown in Figure 4(a), the number of

Figure 3 Activated natural killer (NK) cells composed the major subpopulation of hepatic mononuclear cells (MNC) that increased after α-galactosylceramide (α-GalCer) treatment. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 1, 3 and 7. NK cells, NKT cells, $CD4^+$ T cells and $CD8^+$ T cells in liver MNC were evaluated by flow cytometry. (b) The expressions of CD69 on liver NK cells were also evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $^*P < 0.05$ vs vehicle-treated mice.



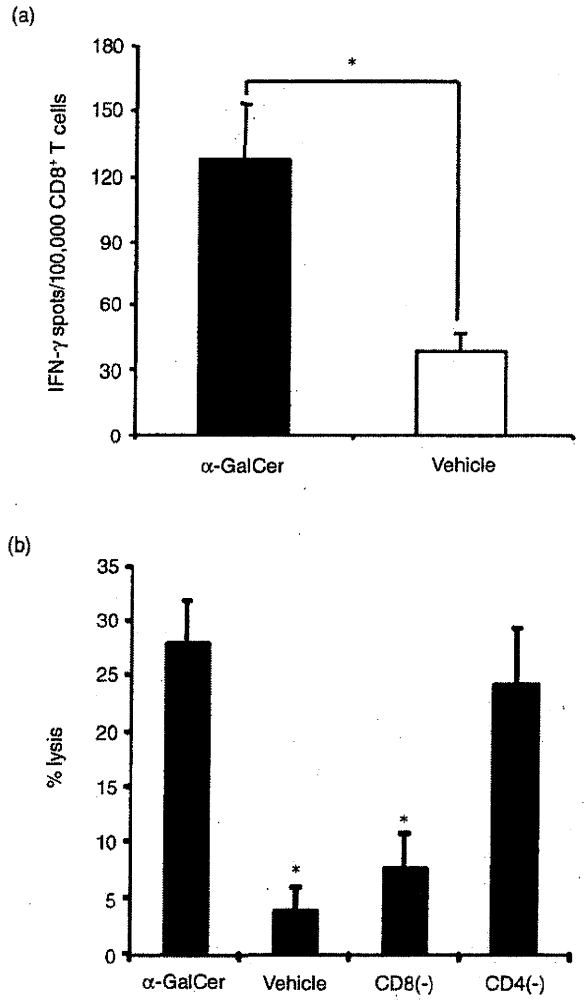



Figure 4 Evaluation of $p53_{232-240}$ peptide-specific $CD8^+$ cytotoxic T lymphocytes (CTL) in α-galactosylceramide (α-GalCer)-treated mice. (a) $CD8^+$ T cells were isolated from the spleen cells of treated mice 14 days after α-GalCer or vehicle treatment. The frequency of $p53_{232-240}$ peptide-specific $CD8^+$ CTL was evaluated by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay. The results are shown in spots/100 000 $CD8^+$ T cells; mean ± standard deviation of triplicate samples. $^*P < 0.05$. (b) Splenocytes from α-GalCer- or vehicle-treated mice were harvested 14 days after tumor inoculation and were analyzed for their ability to kill CMS4 cells in 4 h $^{51}Cr$-release assays (effector cells/target cells ratio, 60:1). $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively. $CD8^-$, $CD8^+$ T-cell-depleted splenocytes. $CD4^-$, $CD4^+$ T-cell-depleted splenocytes. $^*P < 0.05$ vs the cytolytic activity of splenocytes from α-GalCer-treated mice.

IFN-γ spots (per 100 000 $CD8^+$ T cells) observed for T-cell responses against $p53_{232-240}$ peptide in α-GalCer-treated mice were significantly higher than that in vehicle-treated mice. These results suggested that strong $p53_{232-240}$ peptide-specific CTL were generated by α-GalCer treatment of liver tumor. Splenocytes from α-GalCer-treated mice displayed strong cytolytic activity against CMS4 cells, while those from vehicle-treated mice did not (Fig. 4b). $CD8^+$ T-cell-depleted splenocytes from α-GalCer-treated mice displayed significant weak cytolytic activity against CMS4 cells, but $CD4^+$ T-cell-depleted splenocytes did not. These results demonstrated that $CD8^+$ T cells (i.e. CTL) played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice.

## Systemic therapeutic antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

Because strong $p53_{232-240}$ peptide-specific CTL were generated in α-GalCer-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in the liver would impact the progression of subcutaneous untreated CMS4 tumors. BALB/c mice were intrahepatically injected with CMS4 tumors and treated by administration of α-GalCer. Twenty-eight days later, $1 \times 10^6$ CMS4 cells or Colon26 cells were injected s.c. in the right flank. As shown in Figure 5(a), the non-treated CMS4 tumors in mice receiving α-GalCer treatment were completely rejected in all mice. The growth of non-treated Colon26 tumor in α-GalCer-treated mice was not inhibited (Fig. 5b). These results suggested that systemic CMS4-specific antitumor immunity could be induced by α-GalCer treatment. To confirm the involvement of CTL in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells (s.c. injection of $1 \times 10^6$ CMS4 cells) in α-GalCer-treated mice bearing CMS4 liver tumor. On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. As shown in Figure 5(c), antitumor effect against re-challenged CMS4 subcutaneous tumor was diminished in $CD8^+$ T-cell-depleted mice. These results supported that $CD8^+$ T cells (i.e. CTL) play essential roles in the antitumor effect against re-challenge of CMS4 cells in α-GalCer-treated mice.

## DISCUSSION

WE PREVIOUSLY DEMONSTRATED that administration of α-GalCer activated both NKT cells and NK cells in the liver, and that liver NK cells were the

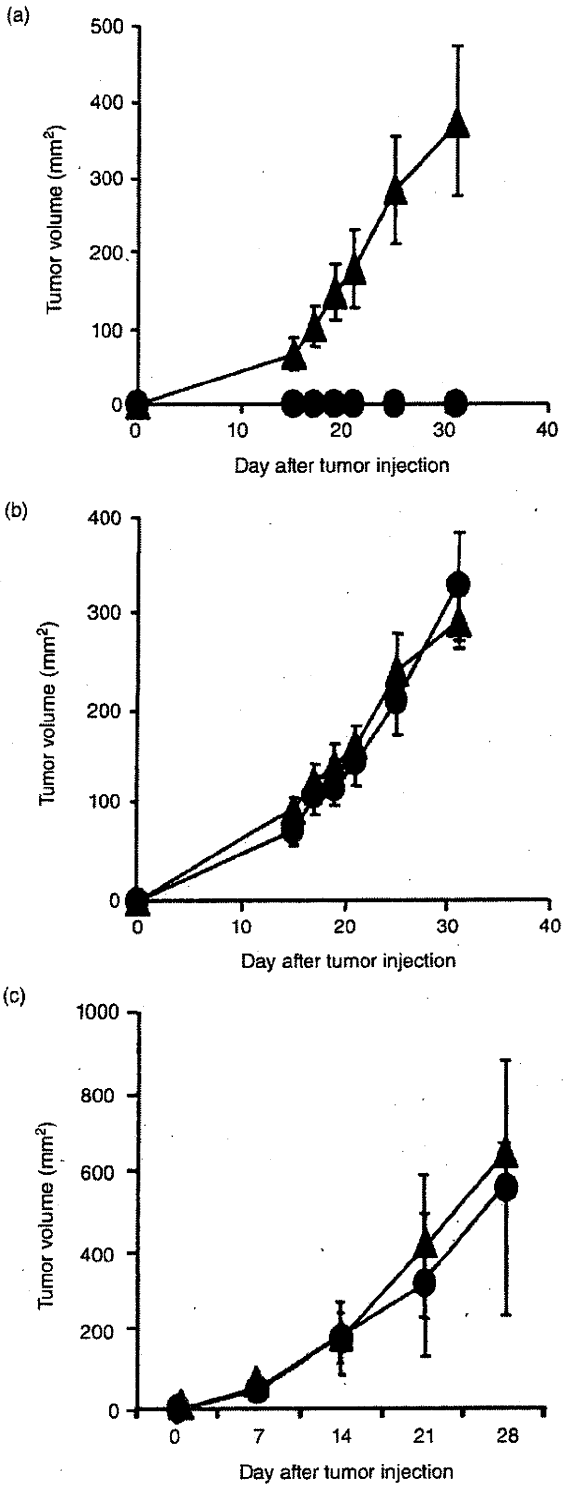



**Figure 5** α-Galactosylceramide (α-GalCer) therapy results in the development of systemic antitumor immunity that protects distal tumor re-challenge. BALB/c mice were injected intrahepatically with CMS4 tumors. Twenty-four hours later, the mice were treated with α-GalCer. Twenty-eight days after treatment, α-GalCer-treated mice were re-challenged s.c. with $1 \times 10^6$ CMS4 cells (a) or Colon26 cells (b) in the right flank (all treatment groups, $n = 8$). To confirm the involvement of cytotoxic T lymphocytes (CTL) in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice bearing CMS4 liver tumor (c). On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody was injected i.p. Tumor size was assessed every 3 or 4 days after s.c. injection of tumor cells (on day 0). As control mice, naïve mice were injected s.c. with $1 \times 10^6$ CMS4 cells (a, $n = 8$; c, $n = 6$) or Colon26 cells (b) ($n = 8$) on day 0. (●) α-GalCer-treated mice, (▲) control mice. Each data point represents the mean tumor size ± standard deviations.

main effector cells to kill disseminated hepatoma cells injected from spleen in α-GalCer treatment.[4] In this study, we evaluated α-GalCer treatment in local injected liver tumor, and the α-GalCer treatment resulted in complete rejection of local liver tumor, which had a similar antitumor effect as α-GalCer in a previous metastatic liver tumor model. These findings suggested the ability of α-GalCer treatment to activate the liver NK cells efficiently, which may mainly contribute to eradication of local liver tumor cells. A normal liver contains lymphocytes that are usually enriched with NK and NKT cells; namely, 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells.[6,7] Thus, activation of innate immune cells, NK cells and NKT cells must be important to develop more effective immunotherapy against liver cancer. We believe that α-GalCer treatment must be a good candidate for human liver cancer treatment.

Recently, activated DC have been implicated in the activation of NKT and NK cells in both mice and humans,[1,5,8–12,22] suggesting that DC play crucial roles in the activation of abundant immune cells in the liver. To establish more efficient α-GalCer treatment in liver cancer, the precise mechanism of liver DC activation is needed. Our results demonstrated that the proportion of liver DC in liver MNC increased immediately and reached the peak 1 day after α-GalCer treatment. The infiltration of tumors by mature DC has been reported to correlate with a better prognosis in cancer patients.[23,24] Thus, the increase of liver DC by α-GalCer might contribute to generation of antitumor effect against liver cancer. The expressions of co-stimulatory

molecules on liver DC also increased early after administration of α-GalCer. IL-12 production from DC is key Th1-cytokine to enhance NK and CTL functionality,[25,26] IL-12 production from liver DC after α-GalCer treatment was significantly higher than that after vehicle treatment.[17] These results suggested that α-GalCer treatment resulted in rapid activation of liver DC, which might play important roles in activating liver NK cells and might contribute to the subsequent establishment of acquired immunity against liver cancer. Pillarisetty *et al.* identified new DC subsets, NK–DC, which presented in the liver of mice,[27] which may affect the interpretation of the activation of liver NK cells by α-GalCer. However, we previously demonstrated that α-GalCer had no direct effect on liver NK cells in mice.[4] These results supported the idea that α-GalCer activated liver DC, which activated the liver NK cells secondary.

Interferon-γ ELISPOT assay revealed that the frequency of $CD8^+$ T cells isolated from α-GalCer-treated mice in liver tumors in response to $p53_{232-240}$ peptide were much higher than that from vehicle-treated mice. Mayordomo *et al.* reported that immunization of $p53_{232-240}$ peptide-pulsed DC induced peptide-specific CTL in immunized mice that showed cytolytic activity against CMS4, p53 overexpressing cells.[18] In this study, $^{51}Cr$-release assay demonstrated that $CD8^+$ T cell, not $CD4^+$ T cells, played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice, which is consistent with the IFN-γ ELISPOT results. The detection of $p53_{232-240}$ peptide-specific CTL means the generation of CMS4 tumor-specific CTL after eradication of liver tumor by α-GalCer treatment. The activation of NKT cells was associated with an expansion of antigen-specific CTL, as might be expected if the DC that matured *in vivo* in response to NKT cells were capturing antigens.[28–31] Our results suggested that the activation of hepatic DC might be associated with the efficiency of generation of tumor antigen-specific CTL.

Additional experiments using an s.c. re-challenge with tumor demonstrated that α-GalCer treatment of liver tumors not only blocked treated CMS4 liver tumor progression but completely protected against consequent "recurrence" of that same tumor at a distant site. In contrast, Colon26 re-challenge tumor was not inhibited in treated mice, suggesting that CMS4-specific immunity was generated after liver tumor treatment. These results were consistent with the activation of acquired immunity evaluated by IFN-γ ELISPOT assay with increase of the frequency of $p53_{232-240}$ peptide-specific CTL. Taken together, we believe that α-GalCer treatment of liver tumors resulted in rejection of both local liver tumor and distant metastatic tumor.

In summary, we have shown that α-GalCer treatment activated both innate and acquired immune cells in the liver. These findings suggested that the use of α-GalCer might represent a particularly promising approach to suppress tumor growth and to promote regression of metastatic lesions in liver cancer patients.

## ACKNOWLEDGMENTS

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## REFERENCES

1 Kawano T, Cui J, Koezuka Y *et al.* CD1d-restricted and TCR-mediated activation of Vα14NKT cells by glycosylceramides. *Science* 1997; **278**: 1626–9.
2 Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ- producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 2002; **3**: 867–74.
3 Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M *et al.* α-Galactosylceramide-activated Vα 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci USA* 2000; **97**: 8461–6.
4 Miyagi T, Takehara T, Tatsumi T *et al.* CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. *Int J Cancer* 2003; **106**: 81–9.
5 Giaccone G, Punt CJ, Ando Y *et al.* A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002; **8**: 3702–9.
6 Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in human liver. *Immunol Rev* 2000; **174**: 5–20.
7 Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gatsroenterology* 2001; **120**: 250–60.
8 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**: 271–96.
9 Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 1997; **90**: 3245–87.
10 Fernandez NC, Lozier A, Flament C *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* 1999; **5**: 405–11.

11 Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* 2002; **195**: 327–33.
12 Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting NK cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002; **195**: 343–51.
13 Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* 2002; **195**: 335–41.
14 Miller G, Lahrs S, Dematteo RP. Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity. *FASEB J* 2003; **17**: 728–30.
15 Kawano T, Cui J, Koezuka Y *et al.* Natural killer-like non-specific tumor cell lysis mediated by specific ligand-activated Vα14NKT cells. *Proc Natl Acad Sci USA* 1998; **95**: 5690–3.
16 Kitamura H, Iwakabe K, Yahata T *et al.* The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopoteniating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 1999; **189**: 1121–8.
17 Sasakawa A, Tatsumi T, Takehara T *et al.* Activated liver dendritic cells generates strong acquired immunity in α-galctosylceramide treatment. *J Hepatol* 2009; **50**: 1155–62.
18 Mayordomo JI, Loftus DJ, Sakamoto H *et al.* Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. *J Exp Med* 1996; **183**: 1357–65.
19 Tatsumi T, Huang J, Gooding WE *et al.* Intratumoral delivery of dendritic cells engineered to secrete both interleukin(IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res* 2003; **63**: 6378–86.
20 Uemura A, Takehara T, Miyagi T *et al.* Natural killer cell is a major producer of IFN-γ that is critical for the IL-12-induced anti-tumor effect in mice. *Cancer Immunol Immunother* 2010; **59**: 453–63.
21 Yamaguchi S, Tatsumi T, Takehara T *et al.* Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide pulsed dendritic cell vaccines. *Cancer* 2007; **110**: 1469–77.
22 Ferlazzo G, Munz C. NK cell compartments and their activation by dendritic cells. *J Immunol* 2004; **172**: 1333–9.
23 Takagi S, Miyagawa S, Ichikawa E *et al.* Dendritic cells, T-cell infiltration, and Grp94 expression in cholangiocellular carcinoma. *Hum Pathol* 2004; **35**: 881–6.
24 Iwamoto M, Shinohara H, Miyamoto A *et al.* Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int J Cancer* 2003; **104**: 92–7.
25 Kobayashi M, Fitz L, Ryan M *et al.* Identification and purification of natural killer cell stimulatory factors (NKSF), a cytokine with multiple biological effects on human lymphocytes. *J Exp Med* 1989; **170**: 827–45.
26 Gately MK, Wolitzky AG, Quinn PM, Chizzonite R. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell Immunol* 1992; **143**: 127–42.
27 Pillarisetty VG, Katz SC, Bleier JI, Shah AB, DeMatteo RP. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. *J Immunol* 2005; **174**: 2612–18.
28 Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 2003; **198**: 267–79.
29 Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 2004; **199**: 1607–18.
30 Hermans IF, Silk JD, Gileadi U, Salio M *et al.* NKT cells enhance CD4+ and CD8+ T cells responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 2003; **171**: 5140–7.
31 Nishimura T, Kitamura H, Iwakabe K *et al.* The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* 2000; **12**: 987–94.

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# Amino Acid Substitution in the Core Protein has no Impact on Relapse in Hepatitis C Genotype 1 Patients Treated With Peginterferon and Ribavirin

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Previous reports demonstrated that amino acid (aa) substitutions in the hepatitis C virus (HCV) core protein are predictors of non-virological responses to pegylated interferon (Peg-IFN) and ribavirin combination therapy. The aim of this study was to investigate the impact of core aa substitutions on viral kinetics during the treatment and relapse after the treatment. The 187 patients with HCV genotype 1 enrolled in this study were categorized into four groups according to core aa substitution patterns: double-wild group (n = 92), Arg70/Leu91; 70-mutant group (n = 42), Gln70/Leu91; 91-mutant group (n = 31), Arg70/Met91; and double-mutant group (n = 22), Gln70/Met91. The relationship between the core aa substitutions and the virological response was examined. Multivariate logistic regression analyses showed that substitution at aa 70 was significantly associated with a poor virological response during the first 12 weeks (decline of < 1 log from baseline at week 4, < 2 log at week 12), and substitution at aa 91 was significantly associated with detectable HCV RNA at week 24. With respect to relapse, only the ribavirin exposure (odds ratio (OR), 0.77; 95% confidence interval (CI), 0.60–0.98) and HCV RNA disappearance between weeks 13 and 24 (OR, 23.69; 95% CI, 5.44–103.08) were associated independently with relapse, with no correlation being found with the core aa substitutions and relapse. In conclusion, the results showed that core aa substitutions can be strong predictive factors at pretreatment of the non-response, but not for relapse, for virological responders with HCV RNA disappearance during treatment. *J.*



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**KEY WORDS:** amino acid substitution; core protein; hepatitis C virus; peginterferon and ribavirin combination therapy; relapse

## INTRODUCTION

The current standard of care for chronic hepatitis C patients is combination therapy using pegylated interferon (Peg-IFN) and ribavirin [Anonymous, 2002; Strader et al., 2004; Dienstag and McHutchison, 2006]. However, the treatment outcome in response to this combination therapy among patients infected with hepatitis C virus (HCV) genotype 1 is still unsatisfactory and the chance of sustained virological response ranges from 42% to 52% [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004]. Therefore, tailoring treatment regimens for individual patients has become an important issue.

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Outcome of therapy is influenced by various factors. Some host factors, such as age, sex, body weight, insulin resistance, and liver fibrosis have been reported as pretreatment factors affecting virological response to this combination therapy [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Romero-Gomez et al., 2005]. Recently, several genome-wide association studies identified single nucleotide polymorphisms (SNPs) near the interleukin (IL)-28B gene, which encodes interferon (IFN) lambda-3, as associated with response to Peg-IFN plus ribavirin treatment among patients infected with HCV of European [Suppiah et al., 2009], African [Ge et al., 2009], and Asian ancestry [Tanaka et al., 2009]. These studies suggest that host genetic variants may be associated strongly with response to IFN-alpha-based therapy. However, the ethical problem to perform host genetic search for all patients remains, and the sustained virological response rate is only 48–69% in patients having favorable IL-28B genotype to this combination therapy [Thompson et al., 2010].

Response-guided therapy is a dynamic approach to management of chronic hepatitis C patients based on the virological response at weeks 4 and 12 of treatment. At present, it is regarded as an excellent strategy for optimizing the treatment duration for individual patients. Earlier HCV RNA disappearance has been shown to lead to a higher sustained virological response rate [Ferenci et al., 2005; Berg et al., 2006; McHutchison et al., 2009], while patients without an early virological response, defined as showing an at least 2 log decrease from the baseline of HCV RNA levels at week 12 is recommended for discontinuing the treatment under the current guidelines [Anonymous, 2002; Strader et al., 2004; Dienstag and McHutchison, 2006].

In addition to viral kinetics during treatment, other viral factors have also been reported to be associated with this combination therapy outcome [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Shirakawa et al., 2008]. Previous studies indicated that amino acid (aa) 70 and/or 91 substitutions in the HCV core protein were independent pretreatment predictors of null or weak response to this combination therapy in genotype 1 patients [Akuta et al., 2007b,c]. The HCV core protein has been reported to inhibit signal transducer and activator of transcription (STAT)-1 phosphorylation, and disrupt the normal IFN-stimulated transcriptional response to viral infection [Lin et al., 2006]. It is supposed that the HCV core region might be associated with resistance to IFN therapy involving the Janus activated kinase (Jak)-STAT signaling cascade [Blindenbacher et al., 2003; Bode et al., 2003; Melen et al., 2004; de Lucas et al., 2005]. Recently, Okanoue et al. [2009] have demonstrated that wild type of core aa 70 and 91 are important for positive prediction of the virological response. However, the impact of core aa substitutions on the extent of HCV RNA decline during the treatment or virological relapse after completion of treatment has not yet been investigated in detail. Approximately 30% of genotype 1 patients who become HCV RNA negative at the end of the treatment will experience relapse [Hadziyannis et al., 2004]. Being able to distinguish between end-of-treatment responders with a high probability of relapse and those with a low probability of relapse will be useful in reducing relapse rates and improving treatment outcome.

The aim of this study was to evaluate the impact of aa substitutions in the HCV core protein on viral kinetics and virological relapse in patients with HCV genotype 1 treated by Peg-IFN alpha-2b and ribavirin combination therapy.

## PATIENTS AND METHODS

### Patient Selection and Study Design

Patients considered to be eligible for this study were those who were infected with HCV genotype 1, had a viral load more than $10^5$ IU/ml, had started Peg-IFN alpha-2b (Schering-Plough K.K. Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to June 2008 at Osaka University Hospital and three other medical institutions taking part in the Osaka Liver Forum, and had been examined with respect to the aa sequences at positions 70 and 91 in the HCV core protein with pretreatment serum samples. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus co-infection; decompensated liver disease; severe cardiac, renal, hematological, or chronic pulmonary disease; poorly controlled psychiatric disease; poorly controlled diabetes; and immunologically mediated disease. As a result of screening at the institutions concerned, 187 patients with HCV genotype 1 were enrolled in this study. Liver biopsy had been performed within 12 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [Bedossa and Poynard, 1996].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by Institutional Review Boards at the respective sites.

Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; and 91 kg or more, 150 μg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; and 81 kg or more, 1,000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the severity of the adverse hematologic effects.

### Detection of Amino Acid Substitutions in Core Region

The nucleotide sequence encoding aa 1–191 (the core protein of HCV) was analyzed by direct sequencing as described by Akuta et al. [2005, 2007b]. In brief, HCV

RNA was extracted from the serum samples and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows: the first PCR was performed using cc11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers. The second PCR was performed using cc9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. All samples were denatured initially at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. The conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR Purification Kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer, Tokyo, Japan). The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession No. D90208) [Kato et al., 1990]. Wild types virus encoded arginine (Arg) and leucine (Leu) at aa 70 and 91, respectively, and the aa substitutions were glutamine (Gln) or histidine (His) at aa 70 and methionine (Met) at aa 91. If the intensities of the band were similar, the case was regarded as competitive. Two patterns of mutant and competitive were labeled as mutant. In this study, patients were categorized into four groups according to aa substitution patterns: double-wild group, Arg70/Leu91; 70-mutant group, Gln or His70/Leu91; 91-mutant group, Arg70/Met91; and double-mutant group, Gln or His70/Met91.

### Virological Tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5,000 IU/ml and a dynamic range from 5,000 to 5,000,000 IU/ml.

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/ml.

### Efficacy Assessments

Patients who achieved negative HCV RNA at week 12 were defined as having a complete early virological response. Patients who became HCV RNA negative between weeks 13 and 24 were defined as having a late virological response. According to the established guidelines, the treatment was considered to have failed if the patients showed an insufficient virological response at week 12 (a detectable HCV RNA and a decrease of <2 log from the baseline level) or at week 24 (a detectable HCV RNA), and therapy was discontinued. The end-of-treatment response was defined as undetectable HCV RNA at week 48. Patients with end-of-treatment response and undetectable HCV RNA 24 weeks after completion of therapy were defined as having sustained virological response. Relapse was defined as a case in which HCV RNA had been undetectable at the end-of-treatment, but detectable during the 24-week follow-up after the treatment.

### Drug Exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

### Data Collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

### Statistical Analysis

Continuous variables are reported as the mean with standard deviation (SD) or median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test (between two groups) or Kruskal–Wallis test (among more than three groups) was used to analyze continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. For all tests, two-sided *P* values were calculated, and the results were considered statistically significant if $P < 0.05$. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict a virological response. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL).

## RESULTS

### Baseline Characteristics of Study Groups

The total study population was predominately male (55.6%), with a mean age of 56.2 years. The baseline characteristics of all patients and the four study groups according to core aa substitution patterns are shown in Table I. Mean age of patients in the double-mutant group was higher than the other three groups ($P = 0.003$). More patients in the double-wild group had

TABLE I. Baseline Demographic and Viral Characteristics of Patients

| Characteristic | Total (n = 187) | Double-wild (n = 92) | 70-Mutant (n = 42) | 91-Mutant (n = 31) | Double-mutant (n = 22) | P value[a] |
|---|---|---|---|---|---|---|
| Age (years) | 56.2 ± 9.3 | 55.7 ± 9.2 | 57.0 ± 9.8 | 52.4 ± 9.9 | 61.8 ± 4.7 | 0.003 |
| Sex (male/female) | 104/83 | 51/41 | 26/16 | 18/13 | 9/13 | 0.444 |
| Body weight (kg) | 60.9 ± 11.6 | 60.9 ± 11.7 | 62.2 ± 11.7 | 62.5 ± 13.2 | 56.0 ± 7.5 | 0.193 |
| Body mass index (kg/m$^2$) | 22.8 ± 3.1 | 22.8 ± 3.0 | 22.8 ± 3.1 | 23.1 ± 3.6 | 22.1 ± 2.4 | 0.627 |
| Past IFN therapy (naïve/experienced) | 118/69 | 45/47 | 34/8 | 20/11 | 19/3 | <0.001 |
| HCV RNA (×10$^3$ IU/ml)[b] | 1,700 | 2,100 | 1,400 | 1,500 | 1,230 | 0.122 |
| Fibrosis (0–2/3–4)[c] | 105/29 | 56/11 | 22/6 | 14/7 | 13/5 | 0.366 |
| Activity (0–1/2–3)[d] | 83/50 | 42/24 | 18/10 | 11/10 | 12/6 | 0.771 |
| White blood cell (×10$^6$/l) | 4,980 ± 1,520 | 4,990 ± 1,420 | 5,180 ± 1,760 | 4,890 ± 1,430 | 4,660 ± 1,560 | 0.795 |
| Red blood cell (×10$^{12}$/l) | 4.34 ± 0.46 | 4.33 ± 0.46 | 4.41 ± 0.52 | 4.39 ± 0.42 | 4.18 ± 0.32 | 0.145 |
| Hemoglobin (g/dl) | 13.9 ± 1.4 | 13.9 ± 1.4 | 14.0 ± 1.7 | 14.2 ± 1.4 | 13.5 ± 1.1 | 0.253 |
| Platelet (×10$^9$/l) | 161 ± 54 | 167 ± 49 | 165 ± 65 | 154 ± 60 | 138 ± 30 | 0.067 |
| ALT (IU/l) | 74 ± 61 | 73 ± 67 | 79 ± 56 | 81 ± 64 | 57 ± 37 | 0.263 |
| γ-GTP (IU/l) | 62 ± 74 | 47 ± 54 | 81 ± 89 | 70 ± 93 | 78 ± 78 | 0.032 |

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase.
[a]P value for comparison among double-wild, 70-mutant, 91-mutant, and double-mutant.
[b]Values expressed as median.
[c]Data for 53 patients are missing.
[d]Data for 54 patients are missing.

been treated previously for HCV infection ($P < 0.001$). Patients in the double-wild group had significantly lower gamma-glutamyl transpeptidase (γ-GTP) levels ($P = 0.032$).

### Progress of Patients

The progress of patients in this study is shown in Figure 1. Of the 187 patients, 183 completed 4 weeks of treatment. Among them, 133 were assessed based on HCV RNA dynamics between baseline and week 4. Those completing 12 weeks of treatment totaled 181, of which 154 were assessed for HCV RNA dynamics between baseline and week 12. Those completing 24 weeks of treatment totaled 153, and all were assessed for HCV RNA quantitatively or qualitatively at week 24. Those completing 48 weeks of treatment totaled 114. These 114 patients and the 55 patients who had discontinued treatment because of treatment failure entered a follow-up period. Among these 169 patients, 164 completed 24 weeks follow-up and the sustained virological response (SVR) rate

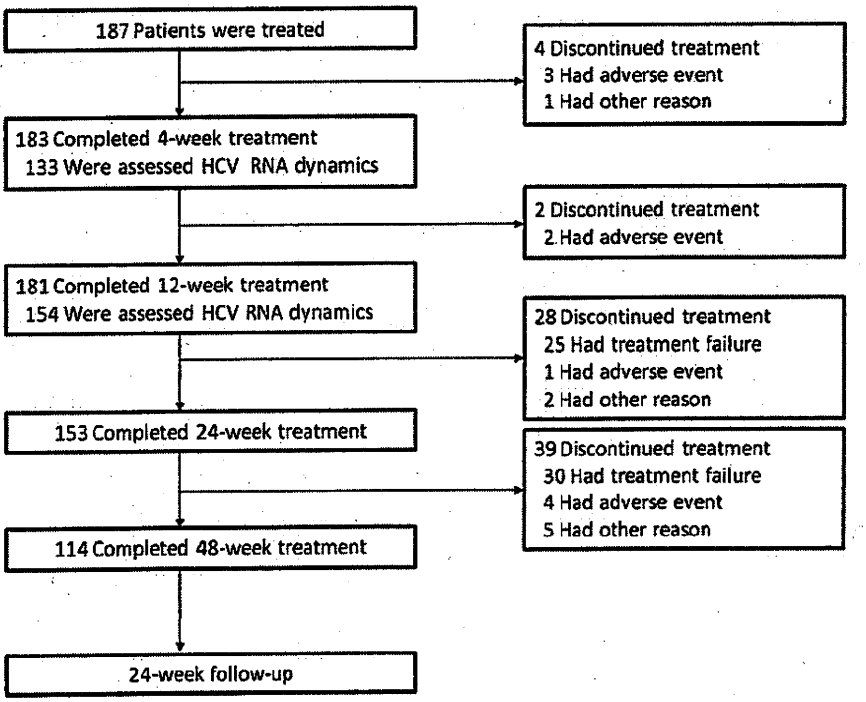


Fig. 1. Treatment and follow-up of the study patients. Treatment was discontinued for patients with <2 log decrease from the baseline HCV RNA level at week 12 or detectable HCV RNA at week 24.

TABLE II. Multivariate Analysis for Factors Associated With <1 log Decrease in HCV RNA Level at Week 4, <2 log Decrease at Week 12, Detectable HCV RNA at Week 24, and Relapse After Treatment

| Factor | Category | Odds Ratio | 95% CI | *P* value |
|---|---|---|---|---|
| HCV RNA <1 log decrease at week 4 | | | | |
| White blood cells ($\times 10^6$/l) | <5,000/5,000≤ | — | — | NS |
| γ-GTP (IU/l) | <40/40≤ | — | — | NS |
| Peg-IFN dose (μg/kg/week) | By 0.1 μg/kg/week | 0.80 | 0.67–0.97 | 0.020 |
| Core aa 70 | Wild/mutant | 1/2.80 | 1.16–6.75 | 0.022 |
| HCV RNA <2 log decrease at week 12 | | | | |
| γ-GTP (IU/l) | <40/40≤ | — | — | NS |
| Peg-IFN dose (μg/kg/week) | By 0.1 μg/kg/week | — | — | NS |
| Core aa 70 | Wild/mutant | 1/2.72 | 1.09–6.78 | 0.032 |
| Detectable HCV RNA at week 24 | | | | |
| Platelet ($\times 10^9$/l) | <150/150≤ | — | — | NS |
| γ-GTP (IU/l) | <40/40≤ | 1/2.46 | 1.02–5.95 | 0.045 |
| Core aa 91 | Wild/mutant | 1/4.11 | 1.73–9.78 | 0.001 |
| Relapse after treatment | | | | |
| Ribavirin dose (mg/kg/day) | By 1 mg/kg/day | 0.77 | 0.60–0.98 | 0.036 |
| Virological response | Complete early virological response/late virological response | 1/23.69 | 5.44–103.08 | <0.001 |

CI, confidence interval; NS, not significant difference; γ-GTP, gamma-glutamyl transpeptidase; Peg-IFN, pegylated interferon; aa, amino acid.

was 48.2% (79/164), based on per-protocol set. Among the 106 patients who had an end-of-treatment response and completed follow-up, 27 showed relapse during the follow-up period; the relapse rate was 25.5% (27/106).

## IMPACT OF CORE-RELAPSE AFTER TREATMENT (TABLE II)

Impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of the core aa substitutions on <1 log viral decrease at week 4, which is a predictor of non-sustained virological response; fewer than 5% of patients without 1 log decrease at week 4 had an sustained virological response [McHutchison et al., 2009] was examined. Among the 133 patients who completed 4 weeks of treatment, 31 failed to show a ≥1 log decrease of HCV RNA level at week 4. Univariate analysis for factors associated with <1 log decrease of HCV RNA level at week 4 was performed on the following variables: age, sex, body weight, BMI, history of past IFN therapy, baseline HCV RNA level, histological fibrosis and activity, white blood cell count, red blood cell count, hemoglobin level, platelet count, alanine aminotransferase (ALT) level, γ-GTP level, dose exposure of Peg-IFN and ribavirin, and aa substitutions in the HCV core protein. The results indicated that pretreatment white blood cell count, γ-GTP level, the mean dose of Peg-IFN during the first 4 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <1 log decrease of HCV RNA level at week 4. Analysis of these factors by multivariate logistic regression analysis showed that substitution of aa 70 (odds ratio (OR) 2.80, 95% confidence interval (CI) 1.16–6.75, $P = 0.022$) as well as the mean dose of Peg-IFN (OR 0.80, 95% CI 0.67–0.97, $P = 0.020$) was independently associated with viral decline (<1 log) at week 4.

Next, the impact of the core aa substitutions on <2 log viral decrease rate at week 12, which is presently considered to be the most reliable predictor of non-sustained virological response [Fried et al., 2002; Davis et al., 2003] was examined. Among the 154 patients who completed 12 weeks of treatment, 25 failed to show a ≥2 log decrease of HCV RNA level at week 12. Univariate analysis was performed on the same factors in the preceding examination. As a result, pretreatment γ-GTP level, the mean dose of Peg-IFN during the first 12 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <2 log decrease of the HCV RNA level. These factors were then analyzed by multivariate logistic regression analysis; only substitution of aa 70 (OR 2.72, 95% CI 1.09–6.78, $P = 0.032$) was found to be independently associated with an insufficient virological response (<2 log HCV RNA decrease from baseline level) at week 12.

The impact of the core aa substitutions on detectable HCV RNA at week 24, which is another non-sustained virological response predictor [Davis et al., 2003] was also examined. Among 153 patients who completed 24 weeks of treatment, 30 still had detectable HCV RNA at week 24. Univariate analysis revealed that pretreatment platelet count, γ-GTP level, and single-spot substitution in the HCV RNA core position at aa 91 contributed to the HCV RNA remaining positive. Multivariate logistic regression analysis, using these factors, indicated that substitution of aa 91 (OR 4.11, 95% CI 1.73–9.78, $P = 0.001$) as well as γ-GTP level (>40 IU/l) (OR 2.46, 95% CI 1.02–5.95, $P = 0.045$) was

independently associated with detectable HCV RNA at week 24.

Next, the factors associated with virological relapse after the treatment was examined. Univariate analysis was performed on the virological response (complete early virologival response or late virological response) in addition to the factors in the preceding examination, revealing the mean dose of ribavirin during the full treatment period and a late virological response, but not aa substitutions (single-spot substitution in the HCV RNA core position at aa 70, $P = 0.467$; aa 91, $P = 0.776$).

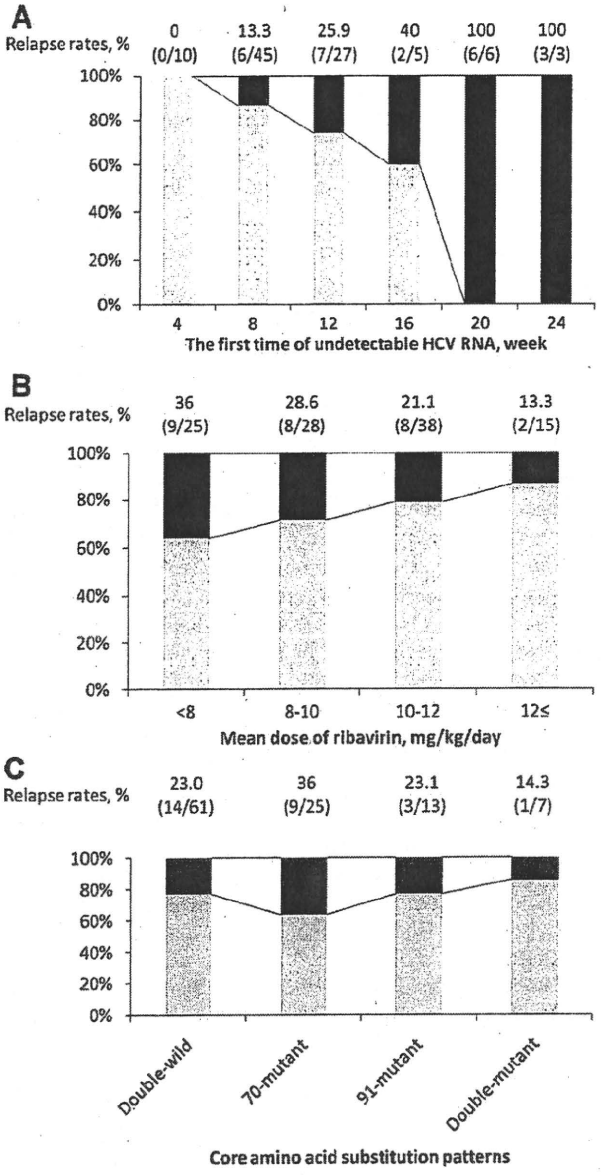


Fig. 2. Relapse rates according to the timing of HCV RNA disappearance (A), mean ribavirin dose (B), and core amino acid substitution patterns (C) in patients who had end-of-treatment response and completed 24-week follow-up. Relapse rates are shown as percentages and the number of patients with relapse in relation to the total number of patients examined is shown at the top of each column. Gray bar, sustained virological response; black bar, relapse.

These factors were analyzed by multivariate logistic regression analysis. This analysis revealed that the mean ribavirin dose (OR 0.77, 95% CI 0.60–0.98, $P = 0.036$) and a late virological response (OR 23.69, 95% CI 5.44–103.08, $P < 0.001$) were independently associated with relapse.

### Relapse Rates According to the Timing of HCV RNA Disappearance, Ribavirin Dose, and Core aa Substitution Patterns

The relapse rates were indicated according to the time to the first non-detection of HCV RNA, mean ribavirin dose and core aa substitution patterns (Fig. 2). The relapse rate was 0% (0/10) in patients with undetectable HCV RNA during 1–4 weeks, and increased 13.3% (6/45) during 5–8 weeks, 25.9% (7/27) during 9–12 weeks, 40% (2/5) during 13–16 weeks, 100% (6/6) during 17–20 weeks, and 100% (3/3) during 21–24 weeks (Fig. 2A). Similarly, the relapse rates increased as the mean ribavirin dose decreased; 13.3% (2/15) in patients receiving ≥12 mg/kg/day of ribavirin, 21.1% (8/38) at 10–12 mg/kg/day, 28.6% (8/28) at 8–12 mg/kg/day, and 36% (9/25) at <8 mg/kg/day (Fig. 2B). On the other hand, the relapse rates were similar among the four core aa substitution patterns; 23.0% (14/61) in patients in the double-wild group, 36% (9/25) in 70-mutant group, 23.1% (3/13) in 91-mutant group, and 14.3% (1/7) in double-mutant group (Fig. 2C). In the subgroup of patients receiving <10 mg/kg/day of ribavirin, no significant difference of the relapse rates was observed between double-wild group and 70-mutant and/or 91-mutant group (31.3% (10/32) in double-wild group vs. 33.3% (7/21) in 70-mutant and/or 91-mutant group), and also in the patients receiving ≥10 mg/kg/day of ribavirin (13.8% (4/29) in double-wild group vs. 25% (6/24) in 70-mutant and/or 91-mutant group) (Fig. 3). Among patients with complete early virological response, the relapse rates were also similar between double-wild group and 70-mutant and/or 91-mutant group (13.7% (7/51) in double-wild vs. 18.4% (7/38) in 70-mutant and/or 91-mutant group). The impact of core aa substitutions on relapse rates in patients with late virological response could not be assessed because of the small number of patients.

## DISCUSSION

Kobayashi et al. [2010] investigated the clinical and virological factors influencing these core aa substitutions in patients infected with HCV genotype 1 who had not received antiviral therapy, and found that HCV variants with wild type of core aa 70 and 91 significantly decreased with age, while those with the mutant type of core aa 70 and/or 91 significantly increased with age. Furthermore, they demonstrated that the proportion of patients with the mutant type of core aa 70 HCV variant significantly increased with an elevated γ-GTP level and a decrease in platelet counts. In this study, the significant differences of baseline demographics between patient groups according to core aa substitution pat-