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

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

## MATERIALS AND METHODS

### *Subjects*

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

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

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

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

### *Reagents*

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

### *Generation of MoDC*

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

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

### *Phenotypic analysis of MoDC*

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

### *Analysis of cytokine production from MoDC*

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

### *Analysis of T-cell polarization by MoDC*

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

### *Enzyme-linked immunosorbent assay*

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

### *Statistical analysis*

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

## RESULTS

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

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

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

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

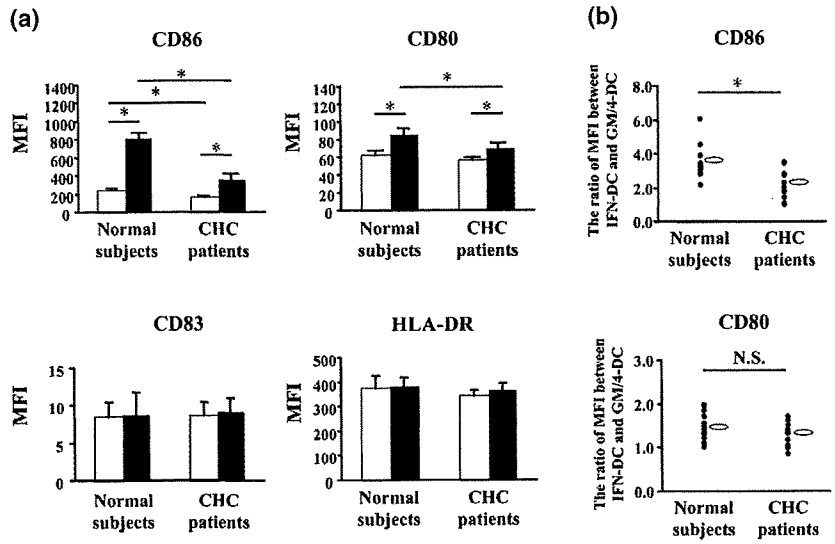


Fig. 1 Interferon (IFN)-α enhanced CD86 and CD80 expression on monocyte-derived DC, in which the degrees of CD86 was higher in healthy subjects than those in chronic hepatitis C (CHC) patients. (a) Monocyte-derived DC were generated from monocytes by 7-day culture with granulocyte–macrophage colony-stimulating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

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

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

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

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

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

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

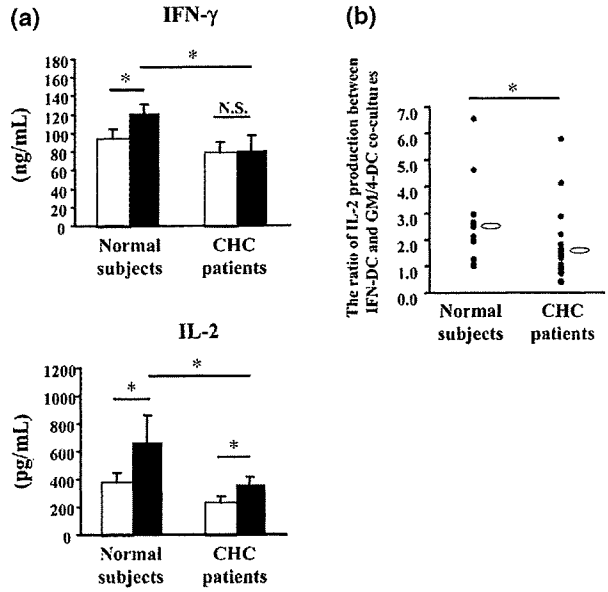


Fig. 2 Interferon-dendritic cells (IFN-DC) from hepatitis C virus-infected patients displayed the impaired capacity of inducing Th1 compared with those from normal subjects. (a) IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ cells for 6 days as described in Materials and methods. On day 4 of the co-cultures, half of the supernatants were collected for assessment of IL-2 release from the cells. After 6 days, the cultured cells were stimulated with phorbol myristate acetate and ionomycin for 24 h. IFN-γ and IL-2 concentrations in the supernatants were determined by ELISA. The results were expressed as mean ± SEM from 15 healthy donors and 20 chronic hepatitis C (CHC) patients. Open bars, GM/4-DC; close bars, IFN-DC. (b) The ratios of IL-2 production between IFN-DC co-culture and GM/4-DC co-culture, from 15 healthy donors and 20 CHC patients are shown. The horizontal bars indicate median. *$P < 0.05$ by Mann–Whitney *U*-test.

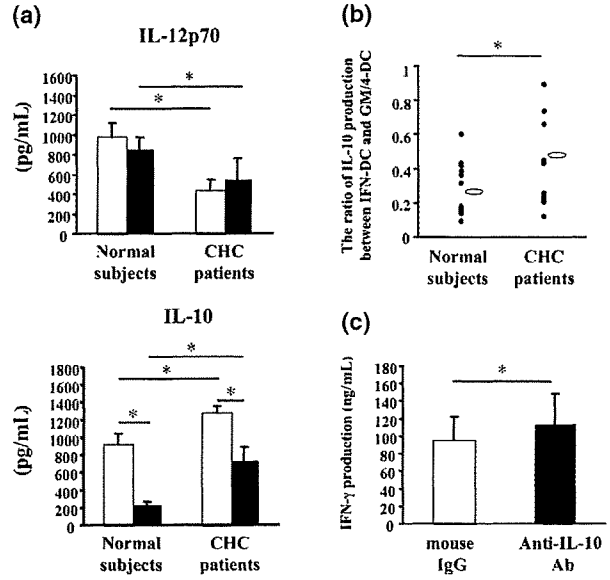


Fig. 3 The lesser IL-10 production from interferon-dendritic cells (IFN-DC) compared with GM/4-DC may be involved in Th1 induction. (a) Day 7 MoDCs were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-12p70 and IL-10 in the supernatants were determined by ELISA. Results are expressed as mean ± SEM of 12 healthy donors and 15 chronic hepatitis C (CHC) patients. Open bars, GM/4-DC; close bars, IFN-DC. (b) Day 7 MoDC were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-10 in the supernatants were determined by ELISA. The ratios of IL-10 between IFN-DCs and GM/4-DCs from 12 healthy donors and 15 CHC patients are shown. The horizontal bars indicate median. (c) Neutralizing anti-IL-10 Ab or isotype mouse IgG was added to the co-culture of day 7 GM/4-DC from healthy subjects and naïve CD4 T cells as described in Materials and methods. After 6 days of co-culture, CD4 T cells were stimulated with phorbol myristate acetate and ionomycin and the concentrations of IFN-γ in the supernatants were analysed by ELISA. The results are the mean ± SEM of five experiments. *$P < 0.05$ by Mann–Whitney *U*-test.

anti-IL-10 Ab to the MoDC/CD4 T cell co-culture and then measured IFN-γ levels from CD4 T cells. The addition of anti-IL-10 Ab increased CD4-derived IFN-γ production, suggesting an inhibitory role of DC-derived IL-10 in DC-primed Th1 response (Fig. 3c).

### *Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or in combined with IFN-α*

To investigate whether ribavirin alone or its combination with IFN-α gives significant impact on DC, we compared phenotypes and functions among GM/4-DC, IFN-DC, R-DC and IFN/R-DC in all patients and donors. In comparison with GM/4-DC, the expressions of CD86, CD80, HLA-DR and CD83 on R-DC did not differ either in normal donors or CHC patients (Fig. 4a and data not shown). The CD86 expression on IFN-DC were significantly higher than those on GM/4-DC but were comparable with those on IFN/R-DC regardless of HCV infection (Fig. 4a).

Similar trends were observed in the functions of DC generated in the same culture conditions. Compared with GM/4-DC, R-DC did not differ in the ability to stimulate CD4 T cells to release IFN-γ and IL-2 or in the production of IL-10 and IL-12p70 in both groups (Fig. 4b,c and data not shown). There was no difference in the priming ability of T cells between IFN-DC and IFN/R-DC either in donors or the patients (Fig. 4c). IFN-DC produced lesser amount of IL-10 than GM/4-DC, the levels of which were not different from IFN/R-DC either in volunteers or in the patient group

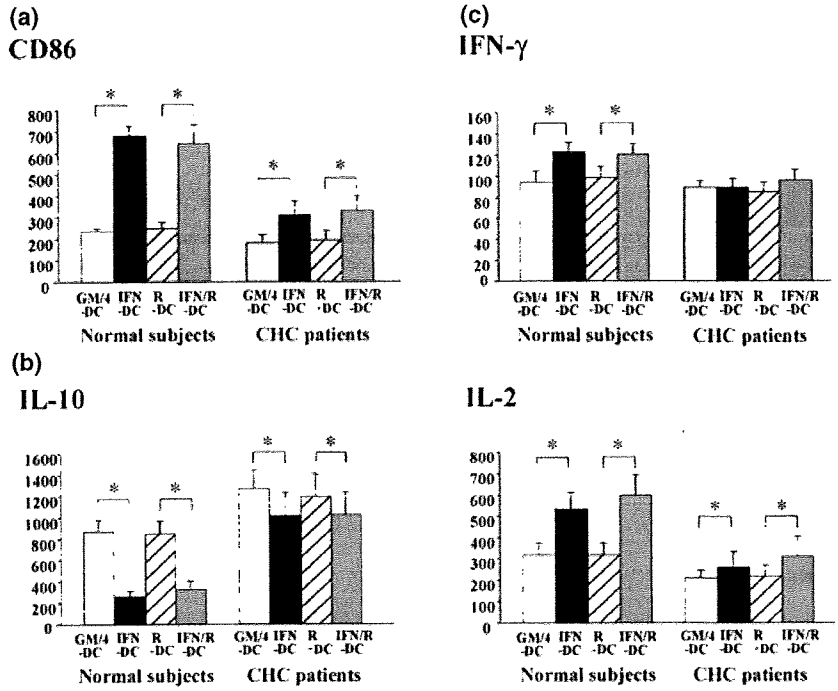


**Fig. 4** Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or used in combined with IFN-α. (a) GM/4-DC, IFN-DC, R-DC and IFN/R-DC were generated and analysed their CD86 expressions by fluorescence-activated cell sorter. The figures represent the mean values of MFI ± SEM, from six healthy donors and six CHC patients. (b) Day 7 GM/4-DC, IFN-DC, R-DC and IFN/R-DC were stimulated with CD40L-L-cells for 24 h. The concentrations of IL-10 in the supernatants were determined by ELISA. Results are expressed as mean ± SEM of six healthy donors and six CHC patients. (c) Day 7 GM/4-DC, IFN-DC, R-DC and IFN/R-DC were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture, half of the supernatants were collected for assessment of IL-2 release from the cells. After 6 days, the cultured cells were stimulated with phorbol myristate acetate and ionomycin for 24 h. IFN-γ and IL-2 concentrations in the supernatants were determined by ELISA. The results were expressed as mean ± SEM from six healthy donors and six CHC patients. Open bars, GM/4-DC; close bars, IFN-DC; striped bars, R-DC; gray bars, IFN/R-DC. *$P < 0.05$ by Mann–Whitney *U*-test.

(Fig. 4b). Therefore, in the analysis of all patients as subjects, ribavirin did not give positive impact on phenotypic DC maturation and DC function *in vitro* either which was used alone or in combined with IFN-α.

### *IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC*

Subsequently, 14 of 20 patients were treated with a combination of IFN-α2b and ribavirin for 24 weeks. Five of 14 patients achieved SVR (the SVR group) while four patients were transient responders and five patients were nonresponders (the non-SVR group). In order to verify the relationship between the *in vitro* responsiveness of DC to these anti-viral reagents and therapeutic outcomes in chronic HCV infection, we retrospectively compared the IFN-γ and IL-2 production from DC-primed CD4 T cells *in vitro* between the patients who attained SVR and those who did not. As R-DC were not different from GM/4-DC in phenotypes and functions, we compared Th1-inducing ability among GM/4-DC, IFN-DC and IFN/R-DC. In the SVR group, IFN-γ and IL-2 secretion from IFN/R-DC-primed CD4 T cells was increased in comparison with IFN-DC-primed T cells. Such enhancement was not observed in the non-SVR group (Fig. 5). Additionally, IFN-γ or IL-2 release from IFN/R-DC-primed CD4 T cells was significantly higher in the SVR group compared with the non-SVR group (Fig. 5). These results disclosed that the patients who successfully eradicated HCV by IFN-α and ribavirin combination therapy had tended to show better *in vitro* DC ability to induce Th1 in response to these agents, suggesting an involvement of DC in therapeutic efficacy.

## DISCUSSION

Interferon-α has been shown to act as a differentiation or maturation factor of DC [28,29]. Cumulative reports have demonstrated that the addition of IFN-α at the later phase of DC development promotes phenotypic and functional DC maturation, as evidenced by the enhancement of CD80, CD86, and HLA-DR expressions [28,30–32] and enhanced ability to release IL-12 [30].

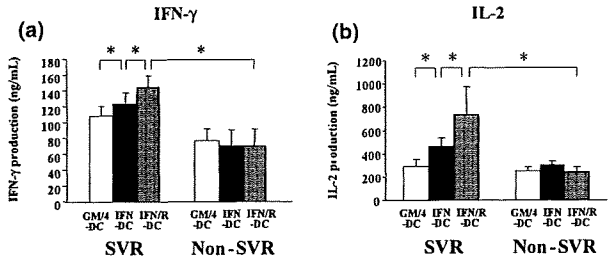


**Fig. 5** IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC. IFN/R-DC. IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture, half of the supernatants were collected for assessment of IL-2 release from the cells. After 6 days, the cultured cells were stimulated with phorbol myristate acetate and ionomycin for 24 h. IFN-γ and IL-2 concentrations in the supernatants were determined by ELISA. The levels of IFN-γ (a) and IL-2 (b) were compared among them in the SVR and the non-SVR group. The results were expressed as mean ± SEM from five SVR and nine non-SVR patients. Open bars, GM/4-DC: close bars, IFN-DC: gray bars, IFN/R-DC; SVR, sustained virological response. $^*P < 0.05$ by Mann–Whitney *U*-test.

In this study. we first intended to elucidate the role of IFN-α in the DC differentiation and its subsequent impact on the ability of DC to stimulate T cells. We added IFN-α from the beginning of DC generation from monocytes in the presence of both GM-CSF and IL-4. Here, we demonstrate that IFN-α is a unique DC differentiation factor in the setting of MoDC generation driven by GM-CSF and IL-4, as it gave rise to MoDC capable of preferentially priming Th1 cells. Of particular interest is the finding that IFN-DC from HCV-infected patients are less able to induce a Th1 response than the healthy counterparts, as evidenced by the analysis of IFN-γ and IL-2 production (Fig. 2a–c). Our results suggest that the IFN-α-induced alterations of DC involving in priming Th1 response are (1) an upregulation of CD86. and (2) a decrease in IL-10 production. However, in CHC patients, such IFN-α-driven alterations in MoDC occur to a lesser degree, thus resulting in impaired DC-primed Th1 response.

As for possible mechanisms of such hyporesponsiveness of patients' DC to IFN-α. the expression of IFN-α receptor on monocyte and DC may be lower in HCV infection. However, this is unlikely as FACS analyses revealed no significant difference in the expression of IFN-α receptor 1 on monocytes or MoDC between the patients and healthy donors (data not shown). Thus. as reported in hepatocytes. signal transduction in DC after binding of IFN-α to its receptor might be hampered by HCV-associated proteins. although the precise pathways linking IFN-α with CD86 or IL-10 remain unclear [33–35]. One of the mechanisms of DC impairment in the ability to prime Th1 in response to IFN-α may be direct HCV infection to monocytes or DC. as reported elsewhere [36,37].

It is well known that DC-derived IL-12 and IL-10 may be involved in Th1 and Th2 polarization. respectively. Thus. the lesser amount of IL-12p70 from the patient' DC may be related to the lesser degree of DC-primed Th1 response in CHC patients than those in donors (Fig. 3a). What remains unknown is how the reduced IL-10 production of DC leads to the enhanced ability of DC to induce a Th1 response. IL-10 is an important key player in the pathogenesis of HCV infection, being induced by various HCV antigens [38]. Moreover, DC functions can be modulated by autocrine IL-10, which is implicated in the enhanced ability to induce Th1 response [39]. The blocking experiments using anti-IL-10 neutralizing Ab including those of our present study revealed that the inhibition of endogenous IL-10 in DC/T cell co-culture enables an increase of the Th1 response [39.40], which may be associated with the relatively enhanced activity of co-existing IL-12p70. Such a reciprocal IL-12 increase and subsequent Th1 augmentation has been observed in DC in which the IL-10 gene had been knocked down by small interference RNA [41]. However. in the present study. the IL-12 levels did not differ between the samples treated with anti-IL-10 Ab and those without it (data not shown). Thus. other DC-derived Th1-inducing cytokines. including IL-27 and IL-23 [42], may be involved in the IFN-DC-induced Th1 response, the possibility of which needs to be further evaluated.

Ribavirin has broad-spectrum activities against both DNA and RNA viruses. however. its mechanism of action for the treatment of HCV is not fully understood. Given that ribavirin has little direct activity against HCV [43–45], a number of studies have shown that ribavirin can modulate immune response by altering the Th1/Th2 bias [14.15.46]. With regard to DC. it has been previously reported that ribavirin alters cytokine production from DC [30]. However, it remains unclear whether or not ribavirin could affect Th1-driving capacity of DC. In the present study, when we analysed the patients as a whole, no additive effect was obtained with ribavirin in phenotypes and functions of DC generated with or without IFN-α. However, when the analyses had been done separately in the SVR patients and non-SVR ones. IFN/R-DC from the SVR group induced more potent Th1 response compared with IFN-DC or GM/4-DC. of which difference was not observed in the non-SVR group. In addition. the levels of IFN-γ and IL-2 released from IFN/R-DC-primed T cells were significantly higher in the SVR group than those in the non-SVR group. It is thus speculated that such better *in vitro* DC response to IFN-α and ribavirin is associated with better *in vivo* virological response in the combination therapy. as the enhancement of HCV-specific Th1 response is necessary for the clearance of HCV by IFN-α and ribavirin combination therapy. As described above. one of the mechanisms of the impairment in IFN-α-stimulated DC in HCV infection is an insufficient alteration of CD86 expression and IL-10 production. However, the addition of ribavirin to IFN-α failed to improve CD86 expression and reduce IL-10

production from patient' DC in the current study, suggesting that other factors may be involved in the mechanisms of ribavirin. In the present study, IL-2 produced in IFN/R-DC and T-cell co-culture was higher than those in IFN-DC culture in the SVR group. Although IL-2 is not a primary Th1-driving factor, it supports Th1 differentiation by promoting T-cell response or survival. Thus, it is plausible that a combination of IFN-α and ribavirin may increase DC-primed IL-2 secretion from CD4 T cells, resulting in enhanced IFN-γ production by T cells.

In summary, in chronic HCV infection, IFN-DC is less able to prime CD4 T cells to produce IFN-γ and IL-2 compared with those in healthy subjects. We also showed the possibility that ribavirin may restore the impaired responsiveness of DC to IFN-α *in vitro* in some HCV-infected patients. Further prospective analyses in large number of patients are warranted to elucidate if a combination of IFN-α and ribavirin directly improves DC function to stimulate Th1 response, thus contributing to HCV eradication from the treated patients.

## ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

# Injection of IL-12 gene-transduced dendritic cells into mouse liver tumor lesions activates both innate and acquired immunity

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*Dendritic cell (DC)-based vaccines have been applied clinically in the setting of advanced-stage cancer. To date, the clinical efficacy of these vaccines has been limited, possibly owing to the impairment of transferred DC function in cancer-bearing patients. In this study, we examined the therapeutic efficacy of interleukin-12 (IL-12) gene-transfected DCs isolated from tumor-bearing hosts against liver tumor. The endogenous DCs isolated from subcutaneous (s.c.) CMS4 tumor-bearing mice (CMS4DC) exhibited decreased expression levels of antigen-presenting molecules and low-allostimulatory capacity. CMS4DC produced less IL-12p70 than DCs isolated from normal mice. Adenoviral transfection of IL-12 gene into CMS4DC (AdIL12DC) restored the expression of antigen-presenting molecules and allostimulatory capacity. Intratumoral (i.t.) delivery of AdIL12DC resulted in complete rejection of intrahepatic CMS4 tumors and activation of innate and acquired immune cells. Antibody depletion studies revealed that both CD4+ and CD8+ T cells as well as natural killer cells play critical roles in mediating liver tumor rejection. I.t. treatment of AdIL12DC resulted in long-term protection against s.c. rechallenge with CMS4 tumor cells. These results revealed that IL-12 gene transfer is capable of improving the impaired functions of DC isolated from tumor-bearing hosts, and support the preclinical therapeutic efficacy of intrahepatic injection of AdIL12DC.*

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**Keywords:** *dendritic cell; interleukin-12; immunotherapy; liver tumor*

## Introduction

Dendritic cells (DCs) effectively elicit immune responses to self and foreign antigens.[1] As these specialized antigen-presenting cells (APCs) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper cells, DC-based vaccines are attractive strategies for the treatment of cancer. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole-cell lysates,[2] peptides,[3] proteins,[4] RNA,[5] or DNA,[6] have proven effective in eliciting protective and therapeutic tumor antigen-specific immunity in murine models. The results of several DC-based tumor vaccine trials have also recently been reported for late-stage cancer patients with B-cell lymphoma, melanoma, prostate cancer and renal cell carcinoma.[7–10] Although tumor-specific T cells were promoted by vaccination in most patients, objective clinical responses have thus far only been observed in a minority of treated individuals. In several cancers, the functional activity of DCs isolated from advanced cancer patients has been reported to be significantly impaired, and this might be associated with the limited clinical efficacy of DC-based cancer vaccines applied to these individuals.[11,12] Hence, these modest current clinical successes observed for DC-based cancer vaccines might be expected to improve if study designs were modified in order to optimize DC promotion of Th1-type immunity in cancer-bearing hosts.

Interleukin-12 (IL-12) exhibits a number of immunologically important activities, including the ability to enhance NK cell and CTL functionality,[13,14] to polarize CD4+ T-cell responses by preferentially supporting the Th1/Tc1-type, and to suppress Th2-type immunity.[15] Previous reports demonstrated potent antitumor effects associated with IL-12 gene therapy using IL-12 gene-modified tumor cells[16] and DCs[17] in murine tumor models. In cancer patients, DCs are often found in an immature and hypostimulatory state.[18] Grohmann *et al.*[19] demonstrated that IL-12 directly activates DC by signals mediated by IL-12 receptors expressed on DCs. Thus, IL-12 transfection into DCs isolated from cancer patients might be expected to lead to recovery of their impaired function via a positive feedback loop.

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The liver contains a large compartment of innate immune cells (NK cells, NKT cells, as well as DCs). DCs contribute to innate immune responses by activating NK cells in both cell-to-cell contact-dependent and -independent manners.[20–22] Thus, DCs secreting abundant IL-12 would be expected to activate both T cells and NK cells in the liver that could prove therapeutic. Primary and metastatic liver tumors are the most common malignancies that resist conventional chemotherapy and radiotherapy, and present with a poor prognosis. In this setting, the development of novel and more effective immunotherapy has been necessitated especially for advanced liver cancer. Toward this end, we present two novel findings in the present report: (1) IL-12 transfection of DCs restores the impaired phenotype/function of endogenous DCs isolated from tumor-bearing animals and (2) intratumoral (i.t.) injection of IL-12 gene-transduced DCs activates both innate and acquired immunity that is, therapeutic in the setting of a liver tumor model.

## Results

### *Phenotype, allostimulatory capacity and cytokine production of endogenous DCs isolated from tumor-bearing mice*

We initially validated the phenotype and allostimulatory capacity of, and IL-12p70 production by DCs isolated from subcutaneous (s.c.) CMS4 tumor-bearing mice (CMS4DC). Flow cytometric examination revealed that CMS4DC displayed significantly decreased levels of expression of the major histocompatibility complex (MHC) class II antigens and the CD80 costimulatory molecules when compared to DCs isolated from normal mice (N-DC) ($P<0.05$, Figure 1a). The expression of CD86 molecule on CMS4DC tended to decrease although it was not significant ($P=0.07$). The decreased expressions of these molecules on DCs were also observed in Colon26 mouse colon cancer tumor-bearing mice (data not shown). Mixed leukocyte reaction (MLR) experiments revealed that CMS4DC exhibited significantly lower allogeneic T-cell proliferation than N-DC ($P<0.05$, Figure 1b). To evaluate IL-12 production from DCs, CMS4 DC and N-DC were treated with or without LPS. Two days later, the supernatants of the DC cultures were harvested and analyzed using mouse IL-12p70-specific enzyme-linked immuno-sorbent assay (ELISA). Notably, IL-12p70 production from LPS-treated CMS4DC was significantly lower than that from LPS-treated N-DC ($P<0.05$, Figure 2a).

### *Phenotypical and functional normalization of CMS4DC after* ex vivo *infection with AdIL12*

CMS4DC were infected with recombinant adenoviruses and then analyzed 48 h later for their phenotype and IL-12p70 production capacity (Figure 2b). As expected, CMS4DC infected with adenoviruses encoding mouse IL-12 (AdIL12DC) secreted significantly higher levels of IL-12p70 than CMS4DC infected with adenoviral mock vector (Adψ5DC) or noninfected N-DC ($P<0.05$). AdIL12DC also displayed significantly elevated levels of the MHC class II antigens when compared to Adψ5DC, approximating the levels observed for N-DC

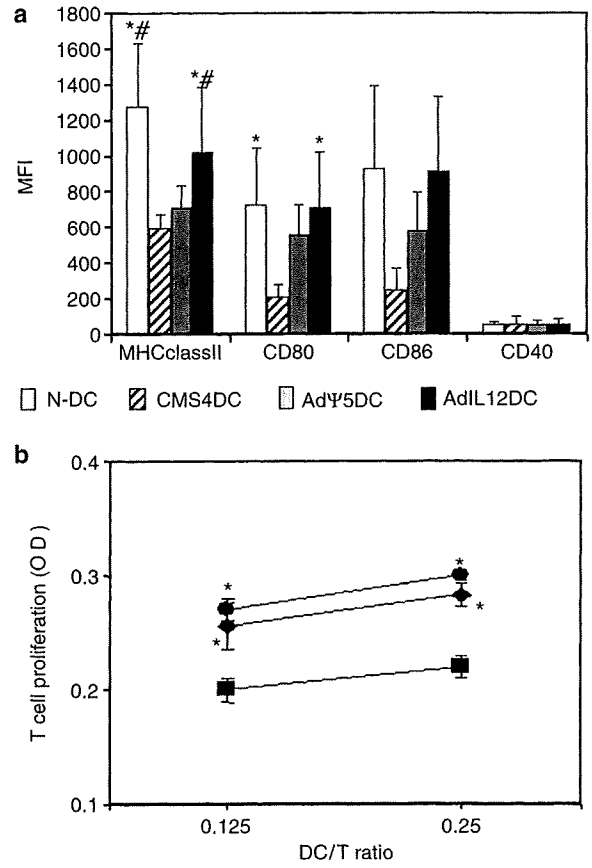


**Figure 1** Phenotype and allostimulatory capacity of DCs generated from CMS4 tumor-bearing mice or adenovirus-infected DCs generated from CMS4 tumor-bearing mice. Bone marrow-derived DCs were generated from mice bearing established s.c. 28 day CMS4 tumor (CMS4DC) or normal mice (N-DC). CMS4DC were transfected with AdIL12 or Adψ5. (**a**) The phenotype of DCs was evaluated by flow cytometry. The expression levels of MHC and costimulatory molecules are reported in arbitrary MFI (mean±s.d. of triplicate samples). *$P<0.05$ vs CMS4DC. #$P<0.05$ vs Adψ5DC. (**b**) Allostimulatory function of DCs was evaluated by MLR. The results are expressed as the mean OD±s.d. of three independent experiments. (●) N-DC (■) CMS4DC (◆) AdIL12DC. *$P<0.05$ vs CMS4DC.

($P<0.05$, Figure 1a). The expressions of CD80 and CD86 costimulatory molecules on AdIL12DC also tended to increase to the levels of N-DC. MLR experiments revealed that AdIL12DC possessed significantly more stimulatory capacity than CMS4DC for the proliferation of allogeneic T cells ($P<0.05$, Figure 1b). These results suggest that AdIL12 gene transfer into CMS4DC restores their impaired antigen presenting function of CMS4DC.

### *I.t. delivery of AdIL12DC was therapeutic in the CMS4 sarcoma liver tumor model*

We next examined the therapeutic potential of i.t. injection of AdIL12DC in CMS4 sarcoma liver tumor-bearing animals. BALB/c mice were injected intrahepatically with $5\times10^5$ CMS4 cells. At the same time, these animals were also treated with an i.t. injection of $1\times10^6$ AdIL12DC or Adψ5DC. Two weeks after tumor injection, the livers of treated mice were removed and weighed to examine intrahepatic tumor growth. As shown in Figure

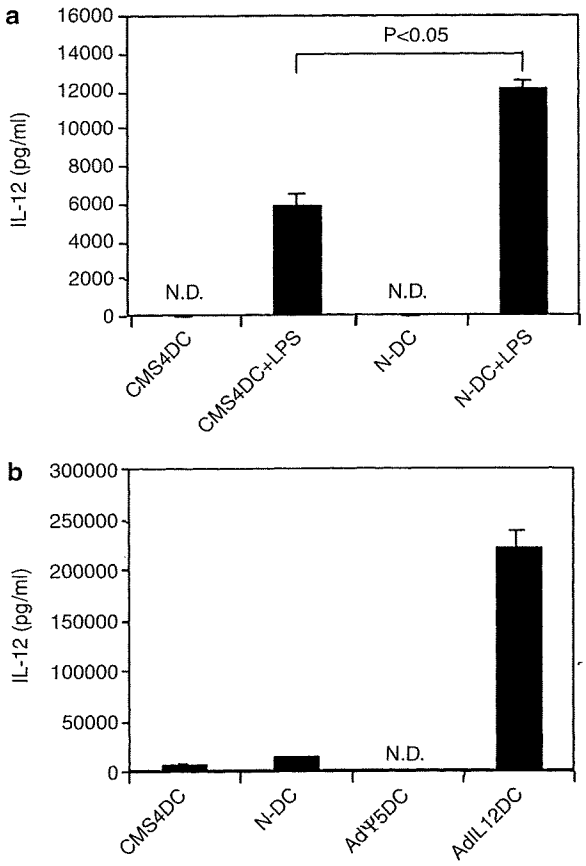


**Figure 2** IL-12 production from DCs generated from CMS4 tumor-bearing mice (**a**) and from adenovirus-infected DCs generated from CMS4 tumor-bearing mice (**b**). (**a**) Bone marrow-derived DCs were generated from mice bearing established s.c. 28 day CMS4 tumor (CMS4DC) or normal mice (N-DC). On day 7, DCs were stimulated with (CMS4DC+LPS, N-DC+LPS) or without LPS (CMS4DC, N-DC). On day 9, the supernatants of DC cultures were harvested and subjected to mouse IL-12p70 ELISA. (**b**) CMS4DC were adenovirally infected with AdIL12 (AdIL12DC) or Ad$\psi$5 (Ad$\psi$5DC). At 48 h after adenoviral transfection, the supernatant of DCs cultures were harvested and subjected to mouse IL-12p70 ELISA. Cytokine levels are reported in pg/ml (mean$\pm$s.d. of triplicate samples). Similar results were obtained in two independent experiments. N.D., not detected.

3a and b, although the liver weight of the Ad$\psi$5DC treatment group was significantly lighter than that of the phosphate-buffered saline (PBS) treatment group ($P<0.05$), large CMS4 liver tumors were formed in both PBS-treated and Ad$\psi$5DC-treated mice. The liver weight of the AdIL12DC treatment group was significantly lighter than that of Ad$\psi$5DC or PBS treatment group ($P<0.05$), and no CMS4 tumor formation was observed in livers of AdIL12DC-treated mice, suggesting the strong therapeutic efficacy of i.t.-delivered AdIL12DC.

We evaluated the degree of systemic (i.e. serum) interferon gamma (IFN-$\gamma$) produced in the treated animal as a result of IL-12-based gene therapy, that might reflect the Th1-type immune responses in treated animals. As shown in Figure 3c, the serum IFN-$\gamma$ level detected in AdIL12DC-treated mice was significantly higher than that observed in either Ad$\psi$5DC- or PBS-treated mice ($P<0.05$).

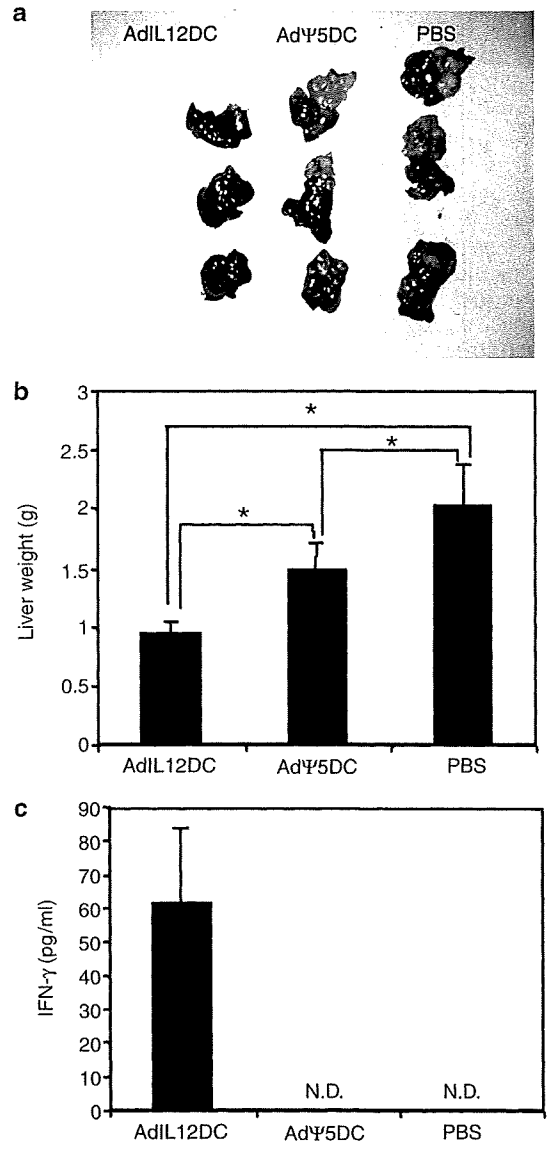


**Figure 3** Therapeutic effect of i.t.delivered AdIL12DC in CMS4 liver tumor model. BALB/c mice were injected intrahepatically with $5\times10^5$ CMS4 cells. At the same time, BALB/c mice were treated with i.t. injection of $1\times10^6$ adnovirus-infected CMS4DCs or PBS (each treatment group $N=6$). Two weeks after the 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**) The mice sera were harvested 2 weeks after intrahepatic tumor injection and AdIL12DC or Ad$\psi$5DC treatment and subjected to mouse IFN-$\gamma$ ELISA. Cytokine levels are reported in pg/ml (mean$\pm$s.d. of triplicate samples). Similar results were obtained in two independent experiments. N.D., not detected.

### *AdIL12DC-based treatment efficiently activated hepatic NK cells and generated CTL against CMS4 tumor*

To examine whether hepatic NK cells were activated by i.t. injection of AdIL12DC, we investigated the cytotoxic activity of hepatic mononuclear cells (MNCs) against YAC-1 cells (Figure 4a) and CMS4 cells (Figure 4b) after i.t. injection of adenovirally transduced DCs. MNCs from Ad$\psi$5DC-treated mice yielded higher levels of cytotoxi-

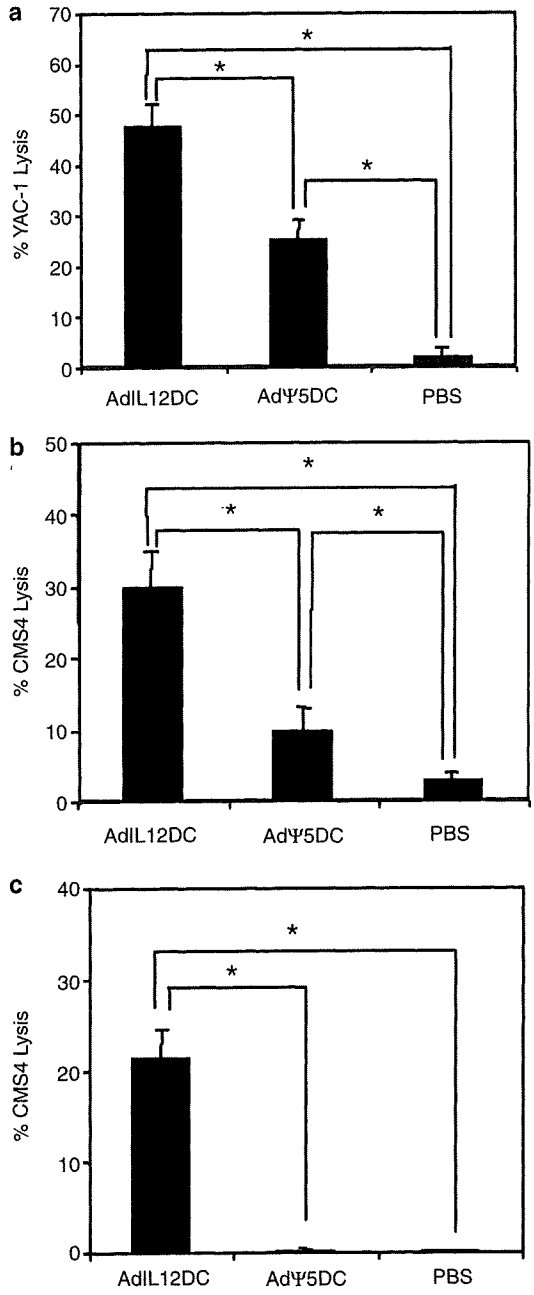


Figure 4 I.t. injection of AdIL12DC activates hepatic NK cells and generated CMS4-specific CTL. (a and b) BALB/c mice were treated with an i.t. injection of $1 \times 10^6$ adenovirally transduced DCs against CMS4 liver tumor. After 48 h, hepatic MNC were isolated from the liver to evaluate the cytotoxicity against YAC-1 cells (a) or CMS4 (b) at 1:1 effector/target cells ratio. (c) 14 days after AdIL12DC treatment, splenocytes were isolated from treated mice and co-cultured with MMC-treated CMS4 cells for 5 days. Lymphocytes were harvested and subjected to 4 h $^{51}Cr$ release assay against CMS4 cells at 60:1 effector/target ratio. Similar results were obtained in two independent experiments (*$P<0.05$).

city than those from PBS-treated mice against both YAC-1 cells and CMS4 cells. MNCs from AdIL12DC-treated mice showed the highest level of cytotoxicity when compared with those from Adψ5DC- or PBS-treated mice ($P<0.05$).

We also examined whether the treatment of AdIL12DC against CMS4 liver tumor generated anti-CMS4 CTL. Splenocytes were obtained from treated mice 14 days after i.t. injection of AdIL12DC, and were cocultured with mitomycin C (MMC)-treated CMS4 cells for 5 days. As shown in Figure 4c, splenocytes from AdIL12DC-treated mice displayed significantly stronger cytolytic activity against CMS4 cells than those from either Adψ5DC- or PBS-treated mice ($P<0.05$). The cytolytic activity of splenocytes from AdIL12DC-treated mice was completely reduced by $CD8^+$ T-cell depletion, but not by $CD4^+$ T-cell depletion, demonstrating that $CD8^+$ T cells were the main effector cells of the cytolytic activity of splenocytes from AdIL12DC-treated mice (data not shown).

These results suggested that i.t. injection of AdIL12DC in the liver could activate hepatic NK cells and generate anti-CMS4 CTL most efficiently.

### *Depletion of $CD4^+$, $CD8^+$ T cells or NK cells abolished the antitumor efficacy of AdIL12DC-based treatment*

To examine that the therapeutic benefit of the AdIL12DC-based regimen in CMS4 liver tumor model was T cell and/or NK cell dependent, we performed T-cell subset depletion and NK-cell depletion studies. Depletion of either $CD4^+$ or $CD8^+$ T cells significantly inhibited the antitumor effects associated with i.t. injections of AdIL12DC ($P<0.05$, Figure 5a). NK cell depletions also significantly inhibited the antitumor efficacy of this therapy ($P<0.05$, Figure 5b). These results suggested that both $CD8^+$ and $CD4^+$ T cells as well as NK cells each play critical roles in the benefits associated with AdIL12DC-based therapy.

### *AdIL12DC was superior to the combination of CMS4DC and AdIL12 injection in the liver tumor treatment*

To examine whether i.t. injection of AdIL12DC was superior to the i.t. combined injection of CMS4DC and AdIL12 vector against CMS4 sarcoma liver tumor, we performed additional control experiments. Mice bearing CMS4 liver tumor were treated with AdIL12DC or the combination of CMS4DC and AdIL12. The amount of each Ad injected and the numbers of DCs were equivalent in both treatment groups. As shown in Figure 5c, although the liver weight of the combination of CMS4DC and AdIL12 treatment group was significantly lighter than that of the PBS treatment group ($P<0.05$), the liver weight of the AdIL12DC treatment group was significantly lighter than that of the combination of CMS4DC and AdIL12 treatment group ($P<0.05$). These results suggest that i.t. delivered AdIL12DC was superior to the combination of i.t. delivered CMS4DC and AdIL12 in the therapeutic efficacy.

### *Prolonged, systemic CMS4 tumor-specific immunity resulted from i.t. injection of AdIL12DC*

We next tried to determine whether the treatment of a CMS4 sarcoma lesion in the liver would impact the progression of a consequent s.c. injection of CMS4 tumor cells. BALB/c mice were intrahepatically injected with CMS4 tumors and treated with i.t. injection of AdIL12DC. After 42 days, $1 \times 10^6$ CMS4 cells or Colon26 cells were injected subcutaneously into the right flank of

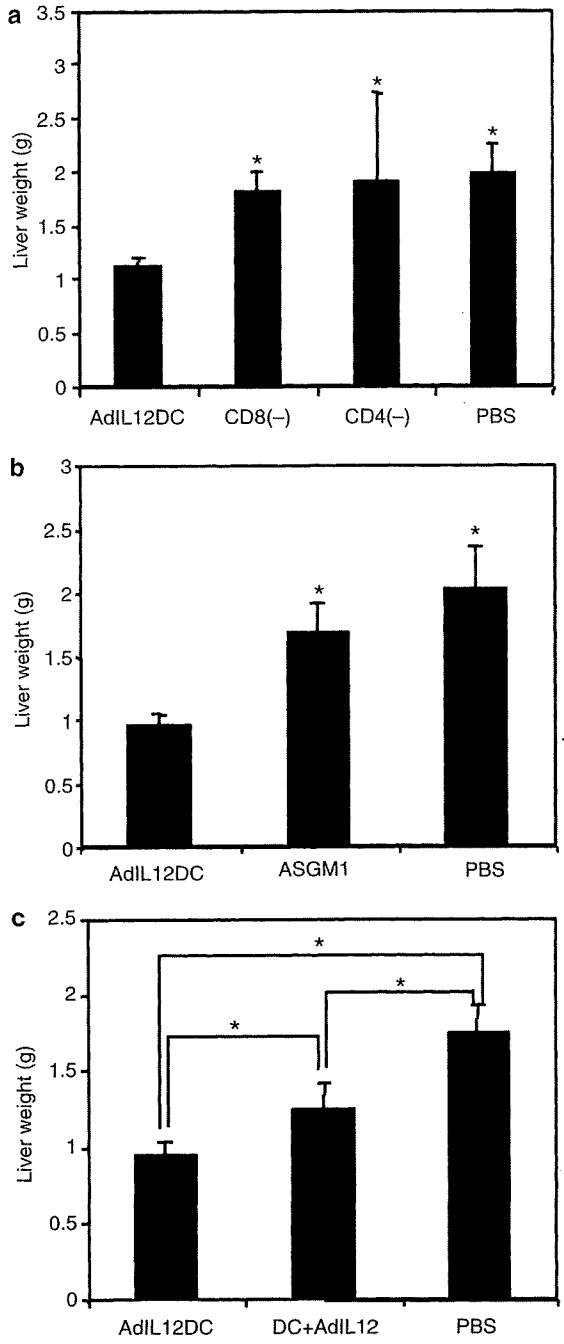


**Figure 5** The antitumor efficacy of i.t. AdIL12DC delivery is dependent on $CD4^+$ and $CD8^+$ T cells as well as NK cells, and i.t. injection of AdIL12DC is superior to coinjection of CMS4DC and AdIL12. (**a** and **b**) To prove that the therapeutic benefit of AdIL12DC-based regimen in the CMS4 liver tumor model is T cell- and NK cell-dependent, $CD4^+$ (CD4(−)) and $CD8^+$ (CD8(−)) T cell subset or NK cell (ASGM1) depletion were performed, and the specific T cell- or NK cell-depleted mice were intrahepatically injected with CMS4 cells and treated with AdIL12DC. Ab-mediated *in situ* depletion of $CD4^+$, $CD8^+$ T cells or NK cells markedly reduces the therapeutic efficacy of AdIL12DC therapy (each treatment group $N=6$). $^*P<0.05$ vs AdIL12DC. (**c**) BALB/c mice bearing CMS4 liver tumor were treated with an i.t. injection of $1\times10^6$ AdIL12DC or the combination of $1\times10^6$ CMS4DC and the same amount of AdIL12 vector as we generated AdIL12DC (each treatment group $N=6$). Two weeks after the tumor injection, the livers of treated mice were removed. $^*P<0.05$.

these previously treated animals. As shown in Figure 6a, the s.c. CMS4 sarcoma tumors in mice receiving the AdIL12DC regimen were significantly smaller on day 14 through the chosen end point of these experiments on day 28 after tumor inoculation when compared with tumors in non-treated mice ($P<0.05$). In contrast, the growth of s.c. Colon26 tumor in mice that rejected CMS4 liver tumor by i.t. Injection of AdIL12DC was not inhibited when compared with Colon26 tumors in non-treated mice (Figure 6b). These results suggest that i.t. AdIL12DC treatment against CMS4 liver tumors resulted in generating durable and CMS4-specific systemic acquired immunity against CMS4 sarcoma tumors.

## Discussion

We have demonstrated that endogenous DCs in mice bearing large sarcoma tumor burdens exhibited decreased expression of molecules related to antigen

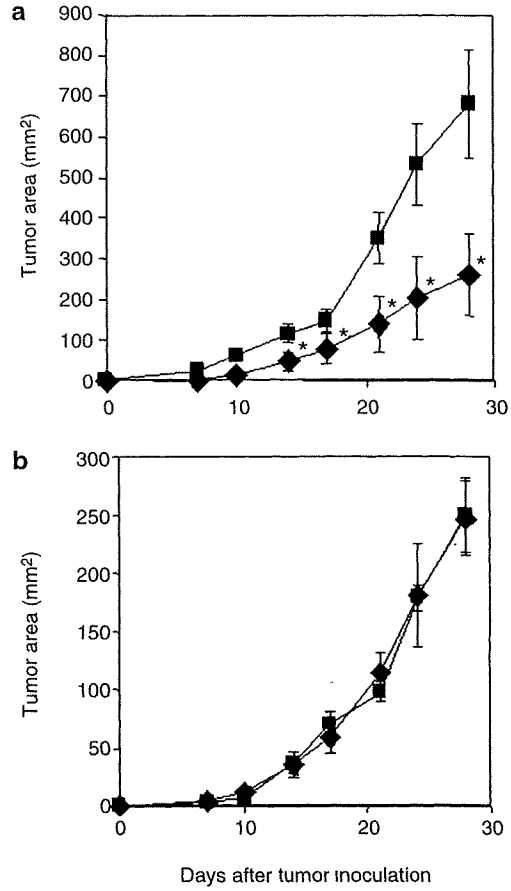


**Figure 6** AdIL12DC-based intrahepatic therapy results in the development of systemic antitumor immunity that protects distal tumor rechallenge. On day 0, BALB/c mice were injected intrahepatically with CMS4 tumors and then treated with an i.t. injection of AdIL12DC. Forty-two days after treatment, mice were challenged s.c. with $1\times10^6$ CMS4 cells (**a**) or Colon26 cells (**b**) in the right flank (each treatment group $N=5$). (◆) AdIL12DC treated mice, (■) non-treated mice. Each data point represents the mean tumor size ± s.d. CMS4 tumors, but not Colon26 tumors, in AdIL12DC-treated mice were significantly smaller than those in non-treated mice. $^*P<0.05$.

presentation and that these APC produced less IL-12p70 than DCs isolated from healthy donor mice, which may lead to inferior type-1 immunity in the tumor microenvironment. Although the functional impairment of DCs might be correlated with the tumor-derived immunosuppressive factors, such as TGF$\beta$, IL-10 and vascular endothelial growth factor,[18] details of the dominant mechanisms underlying impaired DC function are still a topic of active investigation. Most of the current clinical trials using DCs were conducted for patients with advanced cancer. Several human studies showed that the deficient antigen-presenting capacity of the DCs found in patients with advanced cancer induces a cellular-immune deficit.[11,12] These studies suggested that DCs from advanced cancer patients might not have sufficient capacity for inducing tumor antigen-specific immune responses. This is consistent with our tumor experiments that DCs from tumor-bearing mice did not elicit sufficient antitumor effect in CMS4 liver tumor. Clearly, there is a great need to enhance the impaired capacity of DCs from advanced-stage cancer patients in order to provide an optimized DC-based vaccine.

Currently, although many reports of DC-based vaccine in mouse tumor models have been published, few have evaluated the antitumor effect of DCs prepared from tumor-bearing mice. Our present data demonstrated that AdIL12DC generated from cancer-bearing animals exhibit normalized functionality; that is, restored expressions of molecules associated with antigen-presenting capacity and restore MLR function. It has been reported that activation of IL-12 receptor on DCs by IL-12 promotes nuclear localization of NF-$\kappa$B, which regulates the expression of these (MHC and costimulatory) molecules and that IL-12 also regulates DC immunostimulatory function.[19] Thus, IL-12 production as a consequence of AdIL12 infection may serve as a positive feedback stimulus correcting tumor-induced dysfunction in DCs.

Although both Ad$\psi$5 infection and AdIL12 infection enhanced MHC class I expression on CMS4 sarcoma cells and Colon26, colon cancer cells, only AdIL12, but not Ad$\psi$5, enhanced MHC class I expression on DCs (Tatsumi T, unpublished data). These data were consistent with Nielsch's report that high levels of MHC class I expression in adenovirus-infected tumor cells were due to a combinatorial stimulation of two *cis*-regulatory sequences of the MHC class I promoters: NF-$\kappa$B binding site and the interferon stimulated response element.[23] In the current study, for tumor experiments, both AdIL12DC and Ad$\psi$5DC were washed twice before use. After these procedures, both AdIL12DC and Ad$\psi$5DC were injected intrahepatically with tumor cells. Thus, in our tumor model, the influence of adenovirus transfection on tumor cells seems to be weak.

Our results also demonstrated that CMS4 sarcoma tumors in the liver can be treated effectively by i.t. injection with AdIL12DC. Significant systemic toxicities have been previously reported for combined recombinant IL-12+recombinant IL-18 protein-based therapy in murine tumor models,[24] with similar complications anticipated in human clinical trials.[25] Furumoto *et al.*[26] demonstrated that DCs offer the best cytokine delivery system because the DC delivery system minimizes systemic toxicity of cytokines, such as IL-12, and is simultaneously capable of promoting the most efficient immune responses affecting the tumor microenvironment. Clearly, local production of IL-12 in the tumor microenvironment would be expected to induce effective antitumor immunity and prevent systemic toxicity. In this study, although i.t. co injection of DCs and AdIL12 resulted in significant inhibition of CMS4 liver tumor, i.t. injection of AdIL12DC is significantly superior to that of combination of DCs and AdIL12 in the antitumor effect in the liver. Taken together, we believe that i.t. delivery of AdIL12DC may optimally promote antitumor effects in the mouse liver tumor microenvironment.

We have shown that serum IFN-$\gamma$ concentrations in AdIL12DC-treated mice were significantly elevated vs those in control mice. Zitvogel *et al.*[27] reported that the antitumor effects of DC-based vaccination were dependent on the production of Th1-associated cytokines such as IFN-$\gamma$, tumor necrosis factor alpha and IL-12. Therefore, enhanced IFN-$\gamma$ production resulting from i.t. injection of AdIL12DC may also play an important role in the increased antitumor activity *in vivo*.

A normal liver contains lymphocytes that are usually enriched with NK cells and NKT cells; that is, 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK cells and 5% NKT cells.[28,29] Recently, DCs have been implicated in the activation of NK cells in both mice and human.[20,21,30] During NK cell activation by DCs, both soluble factors and cell-to-cell contact seem to be important. Of the soluble factors involved in this process, IL-12 exhibits a number of important biological activities, including the ability to enhance NK cell and CTL activities. Based on these results, intrahepatic injection of AdIL12DC would be anticipated to induce strong activation of both NK cells and CTL. Our present report shows that both $CD8^+$ and $CD4^+$ T cells, as well as, NK cells play critical roles in the therapeutic benefits associated with a comparable therapy of intrahepatic tumors. We also found that early activation of liver NK cells by i.t. injection of AdIL12DC were observed in the cytolytic activity against both YAC-1 (NK sensitive) cells and CMS4 cells, and that CMS4 tumor-specific CTL were generated after rejection of CMS4 liver tumor by AdIL12DC treatment. These findings support the ability of AdIL12DC to activate the liver-associated NK cells, which may partially contribute to eradication of tumor cells and to generate CMS4 tumor-specific CTL. Additional experiments using a subcutaneous (s.c.) rechallenge with tumor suggested that i.t. AdIL12DC treatment of liver sarcoma tumors not only blocks treated liver tumor progression, but protects against consequent 'recurrence' of that same tumor at a distal site via the induction of systemic immunity mediated by $CD4^+$ and $CD8^+$ T cells.

In spite of recent progress and early successes reported for DC-based cancer immunotherapies, there is significant room for improvement in these regimens, especially in the setting of advanced primary and metastatic liver cancer. Thus, new therapeutic approaches of DC-based immunotherapy to advanced liver tumor need to be developed. Recently percutaneous liver tumor ablation methods, radiofrequency ablation therapy and ethanol injection therapy, were well established in hepatocellular carcinoma treatment, and these methods encourage us to apply i.t. injection-immunotherapy to liver tumor treatment. We have shown here that adenoviral transfection of IL-12 cDNA into DCs could

restore the impaired function of tumor-bearing animal-derived DCs and that i.t. injection of IL-12-transfected DCs in a hepatic tumor model resulted in the coordinate activation of both innate and acquired immunity in the liver, providing superior antitumor efficacy. These results suggested that the use of i.t.-delivered AdIL12DC developed from the patients own cells as cancer therapy may represent a particularly promising approach to suppress liver tumor growth and to promote the regression of disseminated lesions.

## Materials and methods

### *Mice*

Six-to-eight-week-old female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan), and maintained in micro-isolator cages. Animals were handled under aseptic conditions. 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 is a chemically induced BALB/c sarcoma and has been described previously.[17] 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 in complete medium (CM, Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10 mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, NY, USA) in a humidified incubator at 5% $CO_2$ tension and 37°C.

### *Generation of DCs* in vitro *from the bone marrow of tumor-bearing mice*

BALB/c mice were injected subcutaneously with $1 \times 10^6$ CMS4 cells into the right flank on day 0. On day 28, the tumor size reached approximately 200 $mm^2$ Bone marrow-derived DCs from CMS4 tumor-bearing mice (CMS4DC) and normal mice (N-DC) were generated as previously described with minor modifications.[17] Briefly, BALB/c bone marrow cells were cultured in CM supplemented with 1000 U/ml of granulocyte-macrophage colony-stimulating factor and mIL-4 (PeproTech EC, London, UK) for 7 days. $CD11c^+$ DCs were then isolated from whole bone marrow culture by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

### *Flow cytometry*

For phenotypic analysis of DCs, PE- or fluorescein isothiocyanate-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80 and CD86 (all from BD-Pharmingen, San Diego, CA, USA), MHC class II antigens (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 arbitrary mean fluorescence intensity (MFI) units.

### *MLR*

MLR were performed to evaluate allostimulatory function of DCs as previously described with minor modification.[31] DCs were seeded in triplicate ($10–2.5 \times 10^3$ cells/well) for use as stimulator cells. Allogeneic (C57/BL6) splenic responder T cells ($2 \times 10^4$) were added to DCs (at indicated T cell: DC ratios) and cultured for 48 h. To evaluate the proliferation of allogeneic T cells, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium, salt) assay (Nacalai Tesque, Kyoto, Japan) was performed according to the manufacturer's protocol. The 450 nm absorbance was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), with the results expressed as the mean optical density (O.D.) $\pm$ s.d. of three independent experiments.

### *Mouse IL-12 production from adenovirally transduced CMS4DCs*

Five million (day 7 cultured) CMS4DCs were infected with recombinant adenoviruses encoding mouse IL-12 (AdIL12DC; MOI = 50) and mock vector (Ad$\psi$5DC, MOI = 50), as previously reported.[17] After 48 h, adenovirus-infected CMS4DCs were harvested and analyzed for phenotype and function. Culture supernatants were also collected for measurement of mouse IL-12 production using a species-specific IL-12p70 ELISA kit (BD-Pharmingen), with a lower level detection limit of 62.5 pg/ml. For tumor experiments, both AdIL12DC and Ad$\psi$5DC were washed twice with PBS before use.

### *Animal experiments*

BALB/c mice were intrahepatically injected with $5 \times 10^5$ CMS4 cells on day 0. At the same time, BALB/c mice were treated with or without an i.t. injection of $1 \times 10^6$ adenovirus-infected CMS4DCs in a total volume of 100 $\mu$l of PBS. Two weeks after the tumor injection, the livers of the treated mice were removed and weighed to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.t. treatment of AdIL12DC, BALB/c mice were injected intrahepatically with $5 \times 10^5$ CMS4 cells on day 0 and treated with AdIL12DC. On day 42 after intrahepatic tumor injection, $1 \times 10^6$ CMS4 cells or Colon26 cells were injected into the right flank. Tumor size was assessed every 3 or 4 days and recorded in $mm^2$ by determining the product of the largest perpendicular diameters measured with Vernier calipers. Data are reported as the average tumor area $\pm$ s.d.

### *IFN-$\gamma$ ELISA*

Mice serum was harvested 2 weeks after intrahepatic tumor injection and AdIL12DC treatment, and subjected to mouse IFN-$\gamma$ ELISA (BD-Pharmingen), with lower detection limit of 31.3 pg/ml.

### *Cytotoxic assay*

BALB/c mice were treated with an i.t. injection of $1 \times 10^6$ AdIL12DC against CMS4 liver tumor. After 48 h, hepatic MNCs were prepared as described previously.[32] To evaluate the activation of hepatic NK cells, WST-8 was performed as described previously with minor modifications.[17] After 24 h coculture of hepatic MNC and NK-susceptible YAC-1 target cells or CMS4 cells at a 1.1 ratio in 96-well plates, 10 $\mu$l WST-8 was added into each well

and the cells were incubated for another 1 h. The 450 nm absorbance was measured using a microplate reader as described above. NK cell cytotoxicity was calculated as described previously.[17] To evaluate the generation of CTL against CMS4 cells by treatment of AdIL12DC, $^{51}Cr$ release assay was performed. Splenocytes were harvested from treated mice 14 days after i.t. injection of AdIL12DC against CMS4 liver tumor, and were cocultured with MMC-treated CMS4 cells for 5 days as described previously.[31] Lymphocytes were harvested after 5 days *in vitro* stimulation and subjected to 4 h $^{51}Cr$ release assay against CMS4 target.[31] Assays were performed in triplicate, with spontaneous release of all assays never exceeding 25% of the maximum release.

### *T-cell and NK-cell depletion experiments*

On days −6, −1, 5 and 10 after tumor inoculation, mice were injected intraperitoneally with anti-CD4 (GK1.5 hybridoma, American-Type Culture Collection, ATCC, Rockville, MD, USA) or anti-CD8 (53–6.72 hybridoma, ATCC) as previously described.[17] For NK-cell depletion, mice were injected with anti-asialo GM-1 (WAKO, Osaka, Japan) on days −1, 5 and 10 after tumor inoculation. The efficiency of specific subset depletions was validated by flow cytometry analysis of splenocytes using PE-conjugated anti-CD4 and anti-CD8 mAbs or PE-conjugated anti-DX5 mAbs (all Pharmingen). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

### *Statistical analysis*

Statistical significance of differences between the groups was determined by applying a Student's *t*-test or two-sample *t*-test with Welch correction after each group had been tested with equal variance and Fisher's exact probability test. The statistical significance of the differences in more than two groups was determined by applying one-way analysis of variance. Statistical significance was defined as $P<0.05$.

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

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

## INTRODUCTION

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

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

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

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

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

## MATERIALS AND METHODS

### Patients

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

### Study Design

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

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

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

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

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

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

### Allogeneic Mixed Leukocyte Reaction With Dendritic Cells

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

### Statistical Analyses

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

## RESULTS

### Outcome of the PEG-IFNα and Ribavirin Therapy

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

TABLE I. Baseline Clinical Characteristics of the Patients

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

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