from MoDC or blood DC.[18] However, these results need to be interpreted carefully, because contamination with free virus in blood cannot be ruled out when amplifying polymerase chain reaction techniques are used. To exclude this possibility, HCV pseudovirus has been developed to investigate the cell tropisms of HCV as well as to determine putative HCV entry receptors to cells. By using this, MDC, but not PDC, displayed susceptibility to HCV pseudovirus possessing chimeric HCV E1/E2 proteins.[21]

Several criticisms have been raised recently about DC dysfunction in the setting of chronic HCV infection,[22] failing to demonstrate any DC defects which may have to do with differences in the populations studied. Cohort studies on chimpanzees following HCV infection showed that functional impairment of DC was observed in some cases but was not a prerequisite of persistent infection.[23] Further study needs to be done to clarify whether DC are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or if it is simply a consequence of active HCV infection.

Natural killer cells

Natural killer cells express various functional receptors; the one group that transduces inhibitory signals (killer inhibitory receptors [KIR], CD94, NKG2A) and the other performs activating signals (NKG2D). The function of NK cells is dynamically regulated *in vivo* by the balance between expressions of counteracting receptors and their association with relevant ligands.[24] First, we compared the expressions of NK cell receptors between HCV-infected patients and healthy donors. As for inhibitory receptors, KIR expressions are not different between the groups; however, CD94 and NKG2A expressions are higher in patients than controls.[25] In contrast, activating receptor NKG2D expression is comparable between the groups (Fig. 2). It is yet to be determined how the expression of the NK cell receptor is regulated. In our experience, HCV pseudovirus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (Kaimori A *et al.*, 2004, unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

Dendritic cells play a decisive role in shaping innate immunity by interacting with NK cells. DC have two means to stimulate NK cells via the production of cytokines (IL-12, IL-18 or IFN-α) and through the expression of NK-activating ligands. In response to IFN-α, DC are able to express major histocompatibility complex (MHC) class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D.[26] Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells.[26] It is tempting to speculate that the impairment of DC in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN-α expression in the liver. Alternatively, NK cells from HCV-infected patients downregulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and transforming growth factor-β1.[25] Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts.[25] Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

Natural killer T cells

Natural killer T cells are a unique lymphocyte subset coexpressing T-cell receptors (TCR) and NK cell markers.[27] The NKT cell population is highly heterogeneous; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of Vα24-JαQ preferentially paired with Vβ11 in humans,[27] whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DC.[27] Although endogenous ligands of iNKT cells are little known, α-galactosyl-ceramide (αGalCer) has been used as a surrogate for natural ligands. It has been demonstrated that phenotypic as well as functional subsets exist for iNKT cells, which are $CD4^+$, $CD4^-CD8^-$ double negative (DN) and $CD8^+$ ones. The $CD4^+$ and DN iNKT cells produce both Th1 (IFN-γ) and Th2 cytokines (IL-4, IL-5, IL-13). The $CD4^+$ iNKT cells secrete more Th2 cytokines than DN, while $CD8^+$ subsets predominantly secrete Th1 cytokines.[28]

Although iNKT cells comprise a small portion of hematopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings. For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells,[29,30] however, their functional roles in HCV-infected patients are largely unknown. We thus compared the frequency and the cytokine producing capacity of iNKT cells in peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by the stimulation with

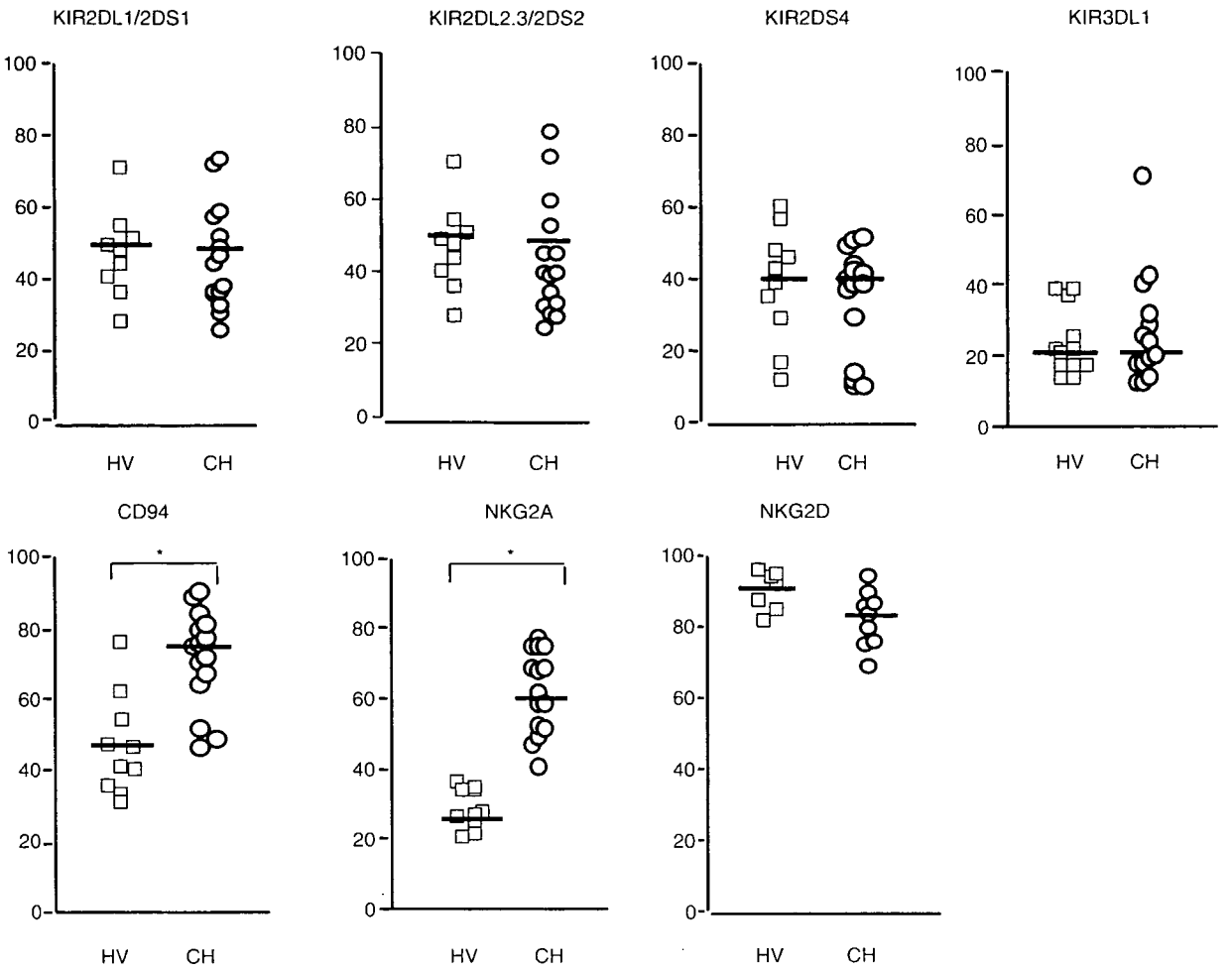

Figure 2 Expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. Percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. $^{*}P 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

αGalCer-loaded DC. We demonstrated that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state (Fig. 3).[31] By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls (Fig. 4).[31] Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by hepatitis B virus or HCV, implying that these cells are pro-fibrogenic to the liver.[32] If this is the case, our findings suggest that iNKT cells in chronic HCV infection are pro-fibrogenic per se even in the pre-cirrhotic stage. The reason why iNKT cells in HCV infection are Th2-biased needs to be further investigated.

ADAPTIVE IMMUNITY IN HCV INFECTION

MANY REPORTS HAVE been published on the importance of $CD4^+$ T-cell response in the clearance and control of HCV. In chronic hepatitis C patients, HCV-specific $CD4^+$ T cells were functionally impaired and their activity was not sustained,[33] which was in clear contrast with resolved cases. Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that $CD4^+$ T-cell help was indispensable for the development of effective $CD8^+$ T cell response to protect from HCV persistence.[34]

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

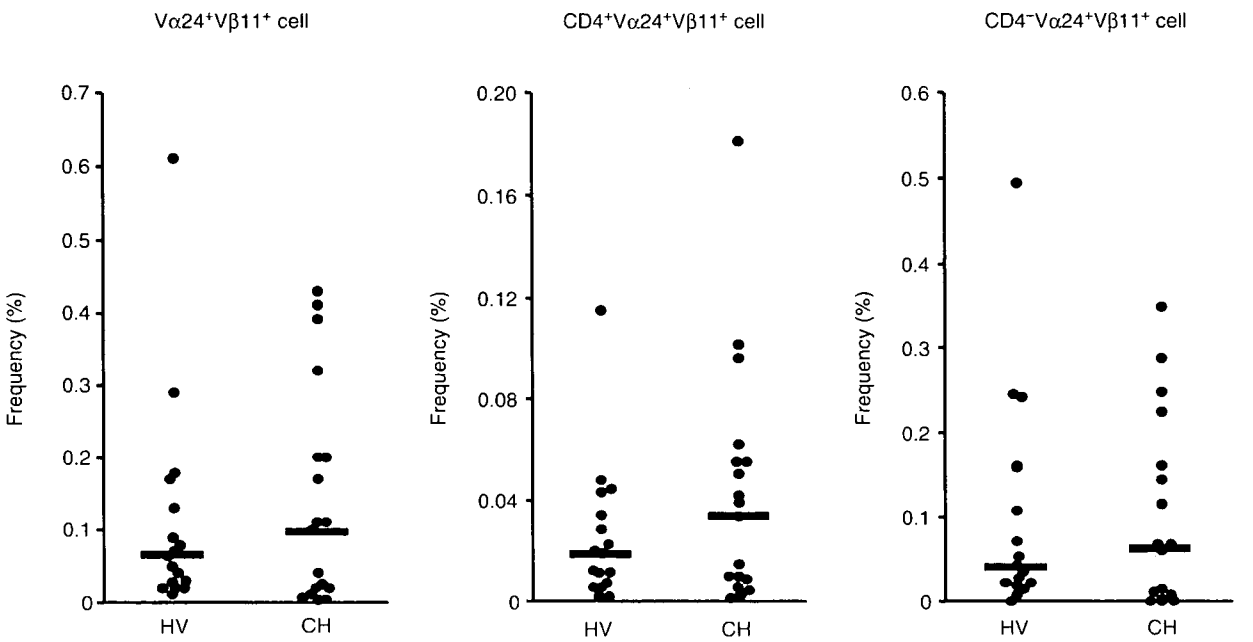

Figure 3 Frequency of peripheral invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. The frequencies of total invariant NKT (iNKT) cells ($V\alpha24^+V\beta11^+$ cells), $CD4^+$ and $CD4^-$ iNKT cells in PBMC were determined by flow cytometry. HV, healthy volunteers; CH,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

have been reported for their roles in HCV replication and liver inflammation. Several investigators have shown that the HCV-specific CTL response is inversely correlated with viral load, suggesting its inhibitory capacity on HCV replication.[35] However, others did not find a significant relationship between these parameters.[36] HCV-specific $CD8^+$ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN-γ in response to HCV antigens. Because $CD8^+$ T cells are reported to be involved in HCV-induced liver inflammation, inefficient $CD8^+$ T cells may evoke only milder hepatocyte injury, which level is not sufficient for HCV eradication.[5]

Several plausible mechanisms have been proposed for T-cell functional failure observed in chronic HCV infection:[3] (i) HCV escape mutation; (ii) primary T-cell failure or T-cell exhaustion; (iii) impaired antigen presentation; (iv) suppression by HCV proteins; (v) impaired T-cell maturation; (vi) suppression by regulatory T cells; and (vii) tolerogenic environment in the liver.

PERSPECTIVES

ANTIVIRAL AGENTS, PEGYLATED (PEG)-IFN-α and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular carcinoma.[1] In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. The questions remain unsolved whether an impaired immune system in chronic HCV infection is restored or not by successful HCV eradication after antiviral therapy. Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response,[37,38] which seems to be clearly distinct from that observed in spontaneous HCV resolvers. Protease inhibitors against HCV NS3/4A are now ready to use in clinics. Because they possess potent ability to suppress HCV replication, they are quite promising as an alternative approach for non-responders in PEG-IFN-α/ribavirin therapy. In addition to that, it is anticipated that protease inhibitors are able to restore innate immunity by disarming NS3/4A-mediated suppression on TLR/RIG-I-dependent or -independent pathways. Therefore, extensive immunological studies on the patients treated with protease inhibitors are needed to elucidate if the therapeutic modulation of innate immunity could shape HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish HCV-specific immunotherapy are to determine the

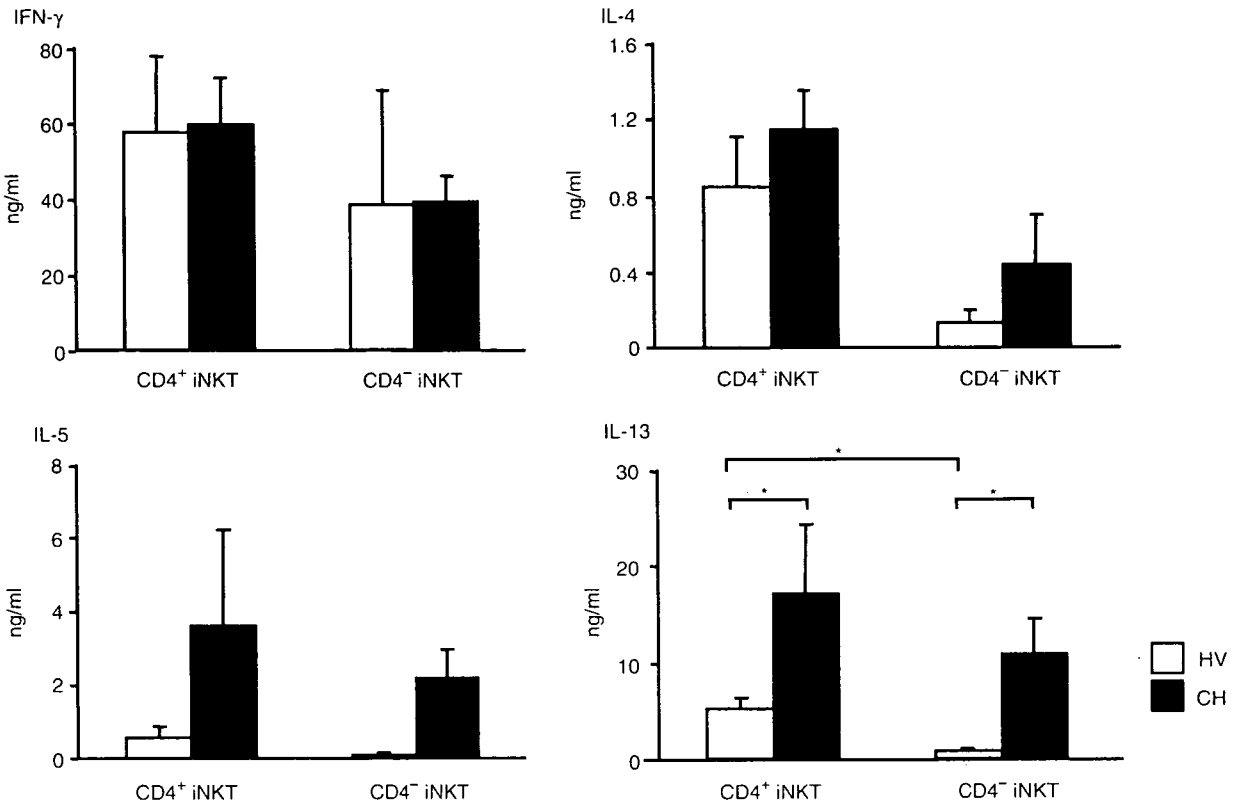

Figure 4 Cytokine production from expanded invariant NKT cells stimulated with α-galactosyl-ceramide (αGalCer)-loaded monocyte-derived DC. Invariant NKT (iNKT) cells were expanded by culture with αGalCer-pulsed autologous monocyte-derived DC (MoDC) and subsequent cell sorting. Activated iNKT cells were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

means to direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way. Active modulation of innate immunity may be one of the strategies to gain access to the goal.

CONFLICT OF INTEREST

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

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

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Subjects

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

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

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

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

Reagents

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

Generation of MoDC

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

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

Phenotypic analysis of MoDC

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

Analysis of cytokine production from MoDC

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

Analysis of T-cell polarization by MoDC

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

Enzyme-linked immunosorbent assay

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

Statistical analysis

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

RESULTS

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

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

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

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

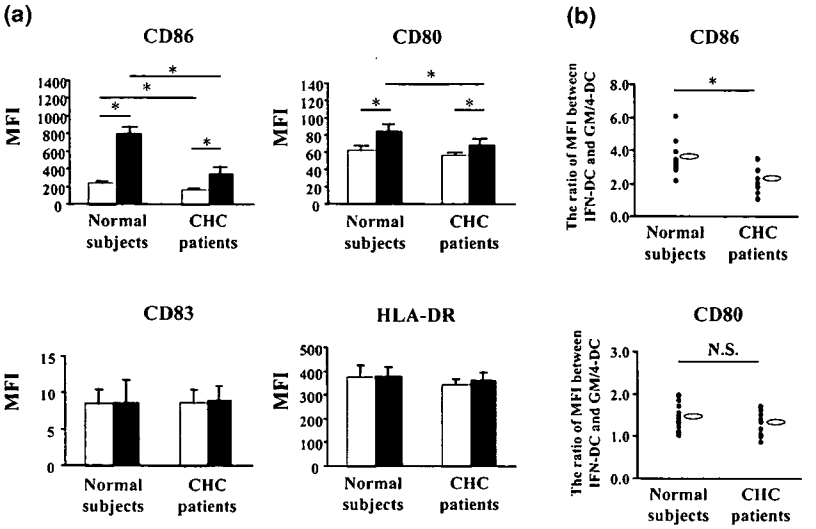

Fig. 1 Interferon (IFN)-α enhanced CD86 and CD80 expression on monocyte-derived DC, in which the degrees of CD86 was higher in healthy subjects than those in chronic hepatitis C (CHC) patients. (a) Monocyte-derived DC were generated from monocytes by 7-day culture with granulocyte–macrophage colony-stimulating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

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

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

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

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

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

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

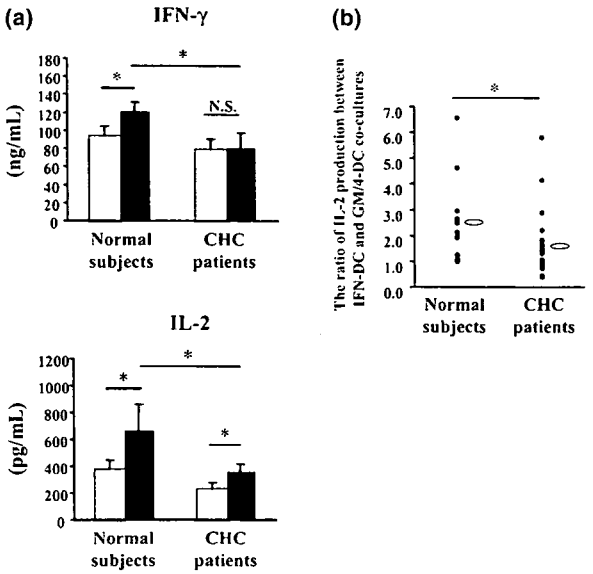

Fig. 2 Interferon-dendritic cells (IFN-DC) from hepatitis C virus-infected patients displayed the impaired capacity of inducing Th1 compared with those from normal subjects. (a) IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ cells for 6 days as described 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.

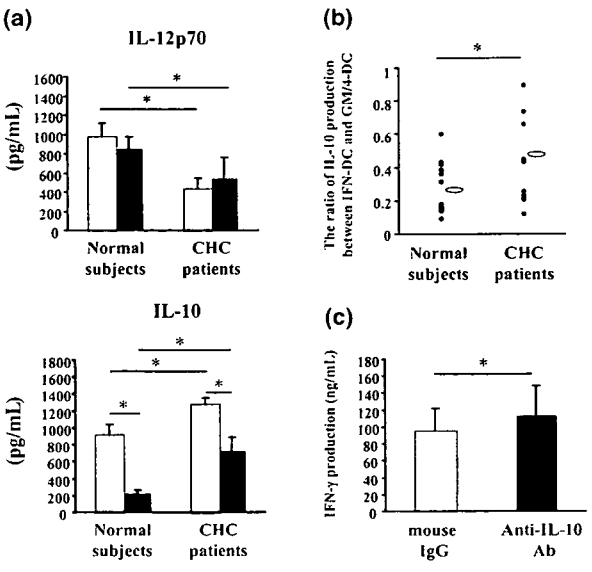

Fig. 3 The lesser IL-10 production from interferon-dendritic cells (IFN-DC) compared with GM/4-DC may be involved in Th1 induction. (a) Day 7 MoDCs were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-12p70 and IL-10 in the supernatants were determined by ELISA. Results are expressed 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

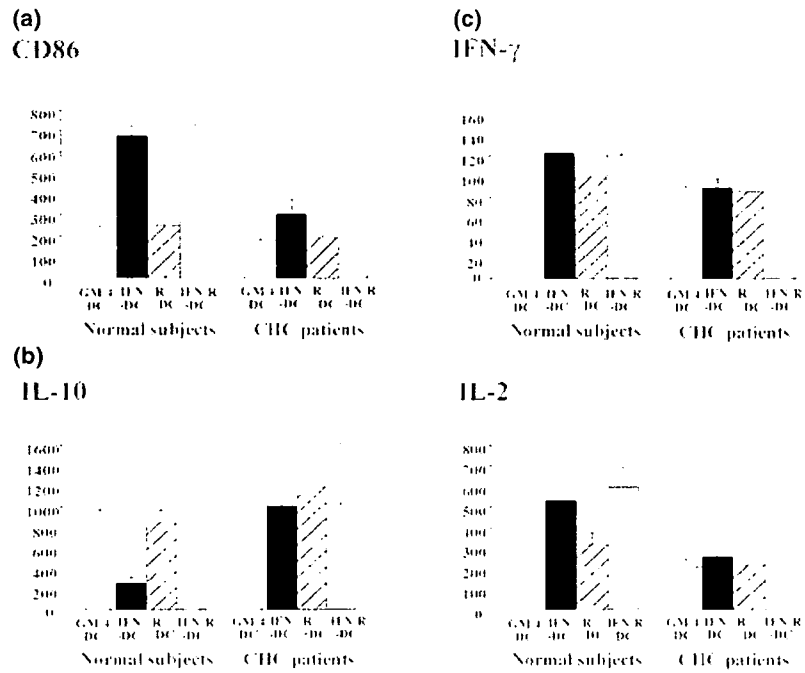

Fig. 4 Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or used in combined with IFN-α. (a) GM/4-DC, IFN-DC, R-DC and IFN/R-DC were generated and analysed their CD86 expressions by fluorescence-activated cell sorter. The figures represent the mean values of MFI ± 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].

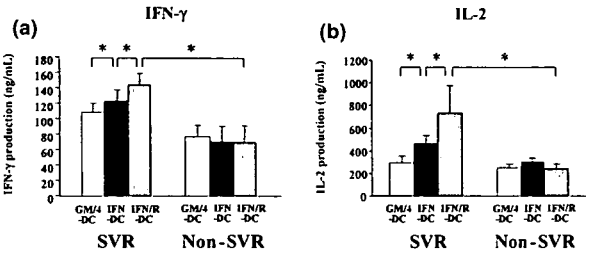

Fig. 5 IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC. IFN/R-DC, IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture, 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.

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Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNα gene to mice

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Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. To evaluate the therapeutic efficacy and mechanisms of IFNα on hepatic metastasis of tumor cells, we hydrodynamically injected naked plasmid DNA encoding IFNα1 (pCMV-IFNa1) into Balb/cA mice having 2 days hepatic metastasis of CT-26 cells. Single injection of pCMV-IFNa1 efficiently enhanced the natural killer (NK) activity of hepatic mononuclear cells, induced production of IFNγ in serum and led to complete rejection of tumors in the liver. Mice protected from hepatic metastasis by IFNα therapy displayed a tumor-specific cytotoxic T cell response and were resistant to subcutaneous challenge of CT-26 cells. NK cells were critically required for IFNα-mediated rejection of hepatic metastasis, because their depletion by injecting anti-asialo GM1 antibody completely abolished the antimetastatic effect. To find whether NK cells are directly activated by IFNα and are sufficient for the antimetastatic effect, the responses to IFNα were examined in SCID mice lacking T cells, B cells and NKT cells. IFNα completely rejected hepatic metastasis in SCID mice and efficiently activated SCID mononuclear cells, as evidenced by activation of STAT1 and a variety of genes, such as MHC class I, granzyme B, tumor necrosis factor-related apoptosis-inducing ligand and IFNγ, and also enhanced Yac1 lytic ability. Study of IFNγ knockout mice revealed that IFNγ was not necessary for IFNα-mediated NK cell activation and metastasis protection. In conclusion, IFNα efficiently activates both innate and adaptive immune responses, but NK cells are critically required and sufficient for IFNα-mediated initial rejection of hepatic metastasis of microdisseminated tumors.

Key words: DNA; innate; adaptive; immunity NK

The liver is the most common site of metastatic malignancy and the status of this organ is an important determinant of survival in patients with advanced disease. The risk of hepatic metastasis remains high in many patients after potentially curative surgery at primary sites.[1] This suggests that the spread of tumor cells can occur in the liver even when they cannot be detected by current diagnostic modalities. To suppress the incidence of liver metastasis, whole liver therapy against microdisseminated tumors should be considered.[2] Since the liver contains an abundance of immune cells, the cytokine-meditated activation of these cells may be a promising approach toward this end.[3,4]

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. IFNα as well as IFNγ are primarily induced in response to viral infection of cells and ligate a cognate receptor for the Type 1 IFN expressed on target cells.[5] On the other hand, Type 2 IFN, IFNγ, is produced predominantly by T lymphocytes, natural killer (NK) cells and NKT cells and uses a distinct receptor. IFNα-mediated antiviral activity includes induction of 2′-5′ oligoadenylate synthetases, double-stranded RNA-activated protein kinase (PKR) and Mx proteins. IFNα can exert direct effects on tumor cells by inhibiting proliferation, inducing apoptosis and inhibiting the release of proangiogenic factors such as vascular endothelial growth factor.[6] IFNα-mediated immunomodulation includes dendritic cell maturation, NK cell activation, MHC Class I induction and cytokine production.[7] Most, if not all, of these actions are mediated by the Jak-STAT signaling pathway downstream of the Type 1 IFN receptor.[8–10] Type 1 IFN receptor upon ligand ligation phosphorylates Jack1 and then phosphorylates STAT1, which activates a variety of IFN-regulated genes. IFNα and IFNβ have been shown to elicit antitumor effects in various murine models of cancer.[11–14] IFNβ was also shown to be effective for retarding metastatic tumor growth in murine liver, but the underlying mechanisms have not been elucidated.[15]

In the present study, we investigated the efficacy of hydrodynamics-based expression of IFNα in the liver against a murine model of hepatic metastasis of CT-26 colon cancer cells and the mechanisms of an IFNα-mediated therapeutic effect of hepatic metastasis. Mice treated with IFNα completely rejected hepatic metastasis and became resistant to rechallenge by CT-26 cells. Although IFNα induced a variety of host responses including increased NK activity, increased IFNγ production and tumor-specific T cell responses, the initial rejection of hepatic metastasis was solely dependent on NK cells. Our study has shed light on NK cell activation as an important mechanism by which IFNα ablates microdisseminated tumors in the liver.

Material and methods

Mice

Specific pathogen-free female Balb/cA mice, SCID mice and their wild-type control mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/cA background was kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo).[16] All mice were used at the age of 5 to 8 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intrasplenic injection of tumor cells was used to establish microdisseminated liver tumors in mice.[17] CT-26 colon cancer cells originating from Balb/cA mice were maintained in DMEM supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 150 μl of PBS and injected into the spleen. For subcutaneous tumor models, CT-26 cells (5×10^5) were injected into the back of the mice under light anesthesia.

NK cell depletion

For depletion of NK cells *in vivo*, anti-asialo GM1 antibody (Wako, Osaka, Japan) was intraperitoneally administered.[17] We

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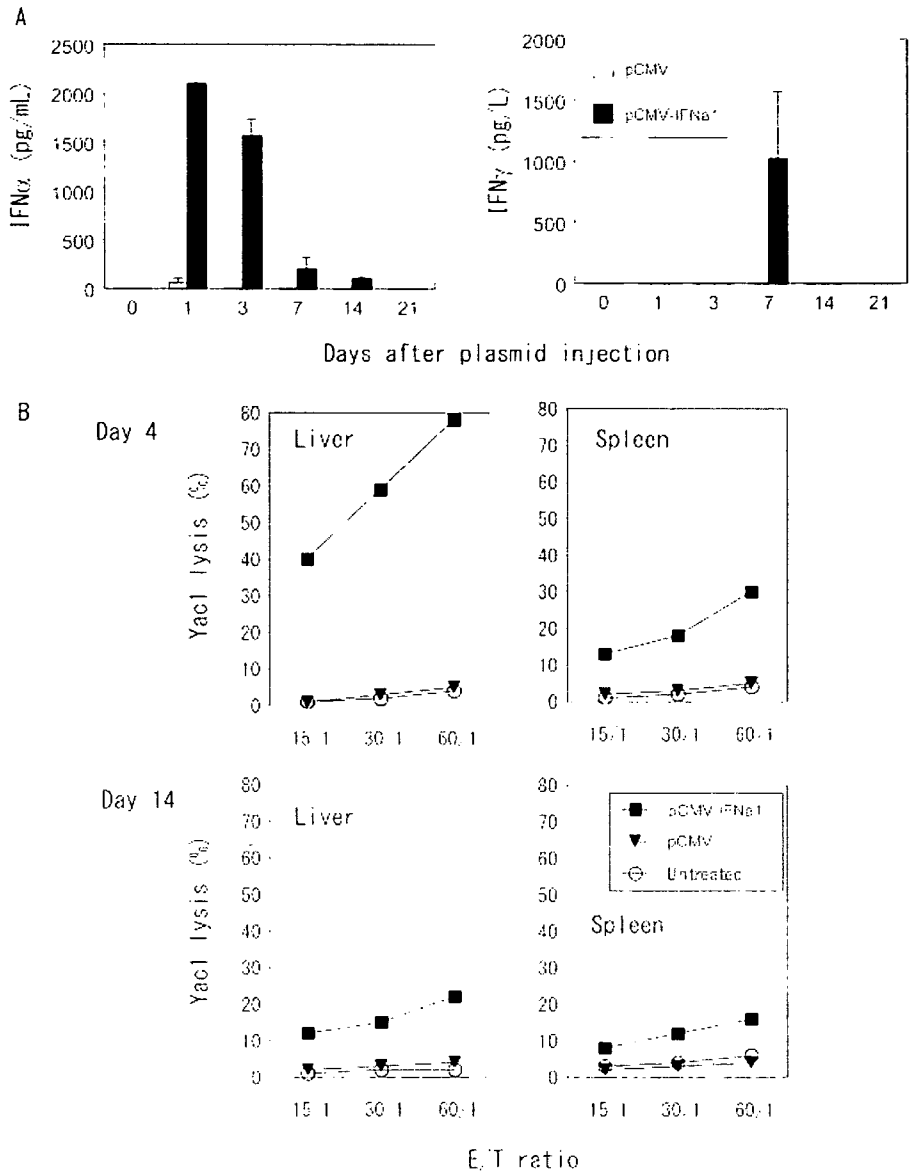

FIGURE 1 – Effects of hydrodynamic injection of IFNα-expressing plasmid. (*a*) Serum IFNα and IFNγ concentration. Balb/cA mice were hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) and bled at indicated time points to measure the levels of serum IFNα and IFNγ. The results are indicated as mean and SD (n = 3/group). Shown are representative data for 2 independent experiments. (*b*) Yac1 lytic ability. Hepatic or splenic mononuclear cells were isolated from naive Balb/cA mice (open circles) and those injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Yac1 lytic ability was measured by a standard chromium-release assay at indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown.

determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. Injection of this dose of anti-asialo GM1 antibody depleted more than 95% of DX-5 positive, TCRβ-negative cells (NK cells) in the liver. NKT cells were less affected than NK cells, because 40% of Cd1d-tetramer positive cells, which are invariant NKT cells, still remained in the liver after the treatment. Anti-asialo GM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Injection of naked plasmid DNA

A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr. Daniel J. J. Carr (University of Oklahoma, Health Science Center).[18] Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described.[19] In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 sec.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IFNα and IFNγ were measured using commercially available ELISA kits (Biomedical Laboratories for murine IFNα; Endogen for murine IFNγ).

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described.[20] The NK activity of mononuclear cells was assessed with standard 4-hr ^{51}Cr-releasing assay using Yac1 cells as targets. To examine CT-26-specific responses, splenocytes were stimulated with CT-26 cells for 5 days in the presence of 30 U/ml

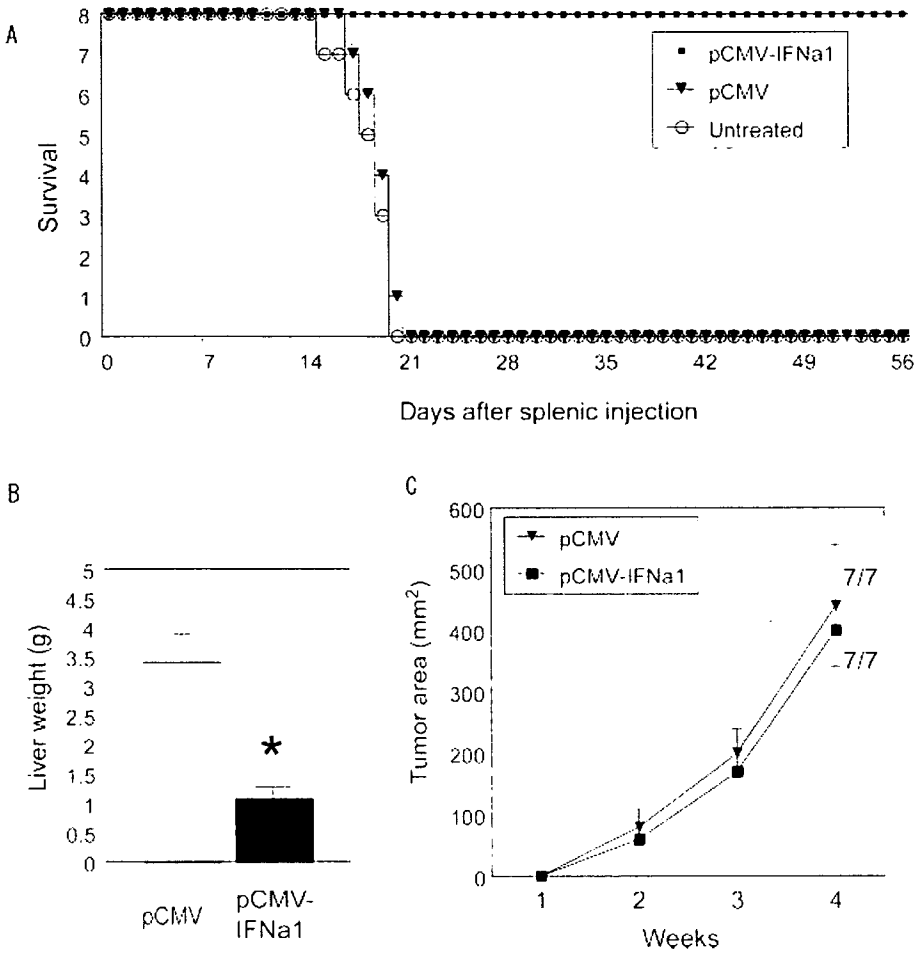

FIGURE 2 – Anti-tumor effects of IFNα therapy. (*a*) Survival. Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomly assigned to 3 groups and received hydrodynamic injection of either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) or untreated (open circles). The number of survivors in each group was monitored. (*b*) Anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. At 14 days after the splenic injection, the mice were sacrificed to examine liver tumor development by measuring liver weight. All experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*c*) Anti-tumor effects on subcutaneous tumors. Balb/cA mice were subcutaneously injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) 2 days later. Tumor growth was examined every week. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 4 weeks is shown in the figure.

of murine IL-2 and subjected to analysis for lytic activity against CT-26 cells or BNL A.7 murine hepatoma cells by 4-hr ^{51}Cr-releasing assay. In some experiments, mononuclear cells were separated into CD90-positive cells (T cells) and CD90-negative cells (non-T cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blotting

Mouse recombinant IFNα was generously provided by Fujisaki Institute, Hayashibara Biochemical Laboratories (Okayama, Japan). Mononuclear cells were treated with or without IFNα. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was stained with anti-STAT1 antibody (Upstate Biotechnology, Lake Placid, NY) or antiphospho-sepcific STAT1 (Y701) antibody (Upstate Biotechnology) and visualized by chemiluminescence. The specificities of STAT1 and phosphorylated STAT1 signals were confirmed by siRNA experiment using BNL A.7 cells in the presence or absence of IFNα treatment (data not shown). Anti-STAT antibody recognizes STAT1α, whereas antiphospho-STAT1 antibody recognizes phosphorylated form of both STAT1α and STAT1β.

Microarray analysis

Total RNA was isolated from cultured SCID splenocytes in the presence or absence of IFNα by ISOGEN. RNA was analyzed using the GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA). Analysis of difference expression was performed by GeneChip Operating Software Ver. 1.1. Genes were considered to be significantly upregulated according to the following criteria: (*i*) the mean fold increase was more than 4-fold; (*ii*) the expression of a gene was significant in NK cells after IFNα treatment; (*iii*) a significant increase was registered based on the algorithm of the software.

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. *Post hoc* tests were done using the Bonferroni's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Single intravenous injection of IFNa1 gene enhances NK activity and completely rejects hepatic metastasis of CT-26 cells

Hydrodynamics-based gene delivery establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes.[21,22] Serial measurement of serum IFNα demonstrated that pCMV-IFNa1 injection led to substantial IFNα production on Day 1. The levels of serum IFNα then declined but were still detectable at Day 14 (Fig. 1*a*). To examine biological effects of the produced IFNα, we evaluated the NK activity of mononuclear cells from the liver and spleen. pCMV-IFNa1 injection, but not

control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells and, to a lesser extent, splenic mononuclear cells at 4 days. The levels of Yac1 lytic activity declined but were still higher at 14 days after the injection (Fig. 1*b*). We also measured IFNγ production in serum, since IFNα is known to activate IFNγ production.[23,24] pCMV-IFNa1 injection, but not pCMV injection, increased serum IFNγ at 7 days (Fig. 1*a*). Since serum IFNγ increased relatively at a later time point, it may be an indirect effect rather than a direct effect of IFNα. These data indicated that hydrodynamic injection of pCMV-IFNa1 efficiently produced biologically active IFNα for a while in mice.

To evaluate the therapeutic effects of IFNα, Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomized into 3 groups and intravenously injected with either pCMV-IFNa1 or pCMV or were not treated. All pCMV-injected mice or untreated mice died within 3 weeks (Fig. 2*a*). They exhibited massive liver tumor in the liver. In contrast, all mice receiving pCMV-IFNa1 survived more than 2 months. To evaluate tumor metastasis, we sacrificed another cohort of mice at 2 weeks after tumor inoculation. There were no macroscopic or microscopic liver tumors in the pCMV-IFNa1-injected mice. In contrast, livers from pCMV-injected mice had massive tumors and were significantly heavier than those from pCMV-injected mice (Fig. 2*b*).

These results clearly indicated the striking therapeutic effects of IFNα on hepatic metastasis of CT-26 cells. To examine this therapeutic effect at a site other than the liver, Balb/cA mice were subcutaneously injected on the back with CT-26 cells and hydrodynamically injected 2 days later with pCMV-IFNa1 or pCMV. No difference in tumor growth was noted between pCMV-IFNa1-injected mice and pCMV-injected mice (Fig. 2*c*).

Mice protected from hepatic metastasis by IFNα gene therapy were resistant to subcutaneous challenge of CT-26 cells and exhibited a tumor-specific T cell response

We next investigated the possibility of IFNα-mediated rejection of hepatic metastasis being followed by induction of an adaptive immune response to the original tumor. To this end, we subcutaneously injected CT-26 cells into the mice that had been protected from CT-26 hepatic metastasis by IFNα therapy. The mice were rechallenged with CT-26 cells 1 month after the initial splenic injection. The controls were naïve Balb/cA mice as well as those receiving pCMV-IFNa1 but not CT-26 splenic inoculation. The incidence of tumor formation was lower in mice that had rejected hepatic metastasis by IFNα therapy than in the control mice. Even if they developed subcutaneous tumors, tumor size was significantly smaller than in the control mice (Fig. 3*a*).

To examine the tumor-specific response, splenocytes were isolated 3 weeks after tumor inoculation and restimulated *in vitro* with CT-26 cells. Splenocytes isolated from CT-26 bearing mice treated with IFNα showed significant levels of killing ability against CT-26 cells, but not against BNL A.7 cells (Fig. 3*b*). When mice were intrasplenically injected with UV-irradiated CT-26 cells, the splenocytes did not show significant killing activity regardless of the subsequent IFNα therapy (Fig. 3*c*). Separation of effector cells into T cells and non-T cells based on CD90 expression revealed that this killing ability was mediated by T cells, but not by non-T cells (data not shown). Thus, a tumor-specific cytotoxic T cell response was established in mice that had rejected hepatic metastasis of CT-26 cells by IFNα therapy.

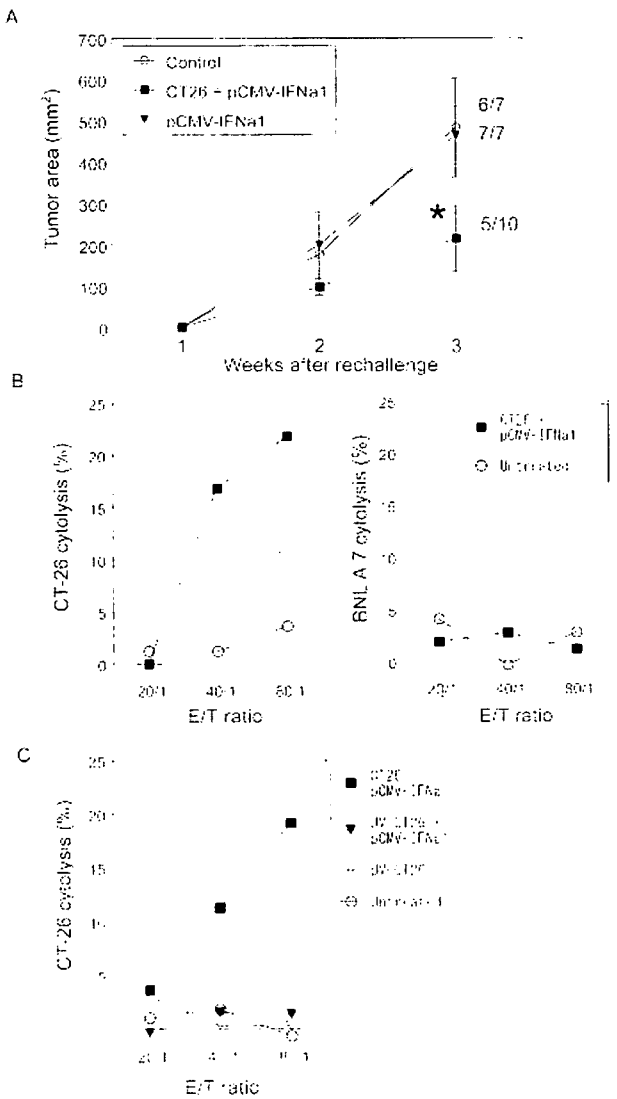

FIGURE 3 – Systemic immunity and tumor-specific T cell response. (*a*) Anti-tumor effects on rechallenged tumors. Balb/cA mice that had rejected hepatic metastasis of CT-26 cell by IFNα (closed squares), those treated with IFNα alone (closed triangles), and naïve mice (open circles) were challenged with subcutaneous injection of CT-26 cells 1 month after the previous treatment. Subcutaneous tumor growth was examined every week by measuring tumor area. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 3 weeks is shown in the figure. *, $p < 0.05$ *vs.* control or pCMV-IFNa1 injection only group. (*b*) *In vitro* tumor-specific killing ability. Balb/cA mice were intrasplenically injected with CT-26 cells and then treated with pCMV-IFNa1 2 days later. Splenocytes were isolated from CT-26 plus pCMV-IFNa1-injected mice at 3 weeks (closed squares) or naïve mice (open circles), restimulated with CT-26 cells for 5 days and then subjected to analysis for the lytic ability against CT-26 cells (left) or BNL A.7 cells (right). Shown are representative data for 3 independent experiments. (*c*) Requirement of CT-26 cells and IFNα on induction of tumor-specific killing ability. Balb/cA mice were intrasplenically injected with live CT-26 cells (squares) or UV-irradiated CT-26 cells (triangles) and then treated with (closed symbols) or without (open symbols) pCMV-IFNa1 2 days later. Splenocytes were isolated from mice at 3 weeks, restimulated with CT-26 cells for 5 days and then subjected to the analysis for the lytic ability against CT-26 cells. Mice injected with live CT-26 cells without following injection of pCMV-IFNa1 did not survive for 3 weeks naïve mice were included as controls (open circles). Shown are representative data for 3 independent experiments.

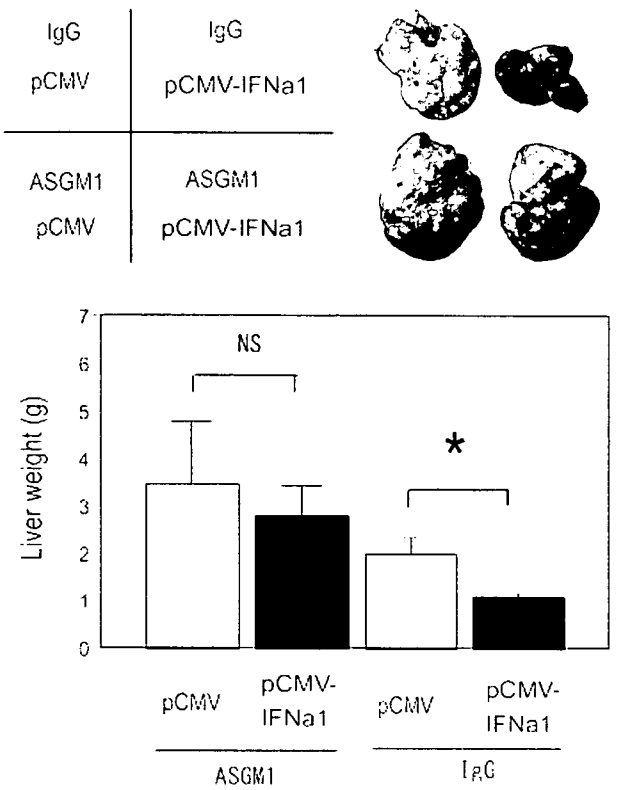

FIGURE 4 – Requirement of NK cells on IFNα-mediated anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells, intraperitoneally injected with either anti-ASGM1 or control IgG at 1 day, and hydrodynamically injected with either pCMV-IFNa1 (closed bars, n = 8/group) or pCMV (open bars, n = 7/group). Mice were sacrificed at 14 days to examine tumor growth in the liver. Top, a representative picture of the liver in each group. Bottom, comparison of liver weight among treatment groups. Experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$. NS, not significant.

NK cells are required for IFNa-mediated initial rejection of hepatic metastasis

To examine whether the observed increase in NK activity of hepatic mononuclear cells is involved in the complete rejection of hepatic metastasis, we induced depletion of NK cells by injecting anti-asialo GM1 antibody. pCMV-IFNa1 injection completely abrogated hepatic tumor formation in control immunoglobulin-injected mice. In sharp contrast, pCMV-IFNa1 injection did not offer antimetastatic effects in anti-aialo GM1 antibody-injected mice, suggesting the critical contribution of NK cells to the antimetastatic effects of IFNα (Fig. 4). We examined the possibility that hepatic mononuclear cells can serve as direct effectors cells for CT-26 eradication. Although CT-26 cells were more resistant to hepatic mononuclear cells than Yac1 cells, pCMV-IFNa1 injection clearly enhanced the killing ability of hepatic mononuclear cells against CT-26 cells (data not shown). This result indicated that CT-26 is potentially susceptible to hepatic mononuclear cells upon IFNα therapy.

IFNα directly activates NK cells

IFNα is known to be able to activate a variety of immune cells. To examine whether NK cells can be directly activated by IFNα, we analyzed SCID mice that lack T cells, B cell and NKT cells due to spontaneous DNA-dependent protein kinase point mutation.[25] SCID or wild-type splenocytes were cultured with IFNα and examined for STAT1 phosphorylation, which peaked at 30 min and declined at 6 hr after IFNα stimulation in both mice (Fig. 5*a*). However, the signals of STAT1 phosphorylation were weaker in SCID splenocytes than in wild-type cells. Of interest is the finding that STAT1 expression was reduced in SCID cells compared to wild-type cells. Similar data were also obtained from experiments on Rag2 KO mice, another model of deficiency for T cells, B cells and NKT cells. To examine the reasons for SCID or Rag2 KO cells expressing low levels of STAT1, we separated wild-type splenocytes into T cells and non-T cells based on CD90 expression. The levels of STAT1 expression were weaker in non-T cells than in T cells (Fig. 5*b*). Taken together, the difference in the levels of STAT1 expression among lymphocyte subsets could explain the reduced phosphorylation signals after IFNα treatment in SCID or Rag2 KO cells.

To examine the gene profiles activated by IFNα in NK cells, we used Affymetrix DNA array analysis on SCID hepatic mononuclear cells. Six hours treatment of IFNα (1,000 U/ml) upregulated 243 of 45,101 genes in SCID cells by more than 4-fold. They included well known IFNα-regulated genes such as H2, 2′-5′ oligoadenylate synthetases, Mx1, IRF and suppressor of cytokine signaling (SOCS). Among the effector molecules for cytotoxicity, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B were activated. Various cytokines such as IL-15 and IFNγ were also upregulated. These data revealed that NK cells upon IFNα stimulation produced well-characterized IFN-inducible genes and others that are relatively specific to killer cells or immune cells.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in SCID mice

We examined the *in vivo* effects of IFNα in SCID mice. In agreement with SCID cell activation *in vitro*, pCMV-IFNa1 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in SCID mice (Fig. 5*c*). To examine whether NK cells are sufficient for IFNα-mediated rejection of hepatic metastasis, we injected pCMV-IFNa1 or pCMV into mice that had been intrasplenically injected with CT-26 cells 2 days earlier. pCMV-IFNa1 completely suppressed tumor formation in the liver (Fig. 5*d*). As described in the *Material and methods* section, anti-asialo GM1 injection reduces the number of NKT cells. However, this SCID experiment clearly showed that NKT cells are not required for NK cell activation by IFNα and its antimetastatic effects.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in GKO mice

IFNγ has been established as an endogenous inhibitor of tumor development and progression.[26] Exogenous administration of IFNγ suppresses tumor formation in a variety of models.[15,27] To examine the possibility of IFNγ being involved in antimetastatic effects on IFNα, we injected pCMV-IFNa1 or pCMV plasmid into GKO mice exposed to 2 days of metastasis of CT-26 cells. IFNα treatment led to complete rejection of CT-26 cells in GKO mice (Fig. 6*a*). pCMV-IFNa1 injection, but not pCMV injection, augmented the Yac1 lytic ability of mononuclear cells (Fig. 6*b*).

Discussion

Here we report that a single injection of pCMV-IFNa1 could lead to complete rejection of preexisting hepatic metastasis of colon cancer cells. This partly agrees with a previous report by Kobayashi et al.,[15] who hydrodynamically injected IFNβ- or IFNγ-expressing plasmid into CT-26 bearing mice and reported the antimetastatic effects of IFNβ or IFNγ. In contrast to our study, all mice died within 45 days due to metastasized tumor growth even if plasmid injection was began one day after tumor inoculation and repeated every other day. The complete protection against hepatic metastasis observed in the present study allowed

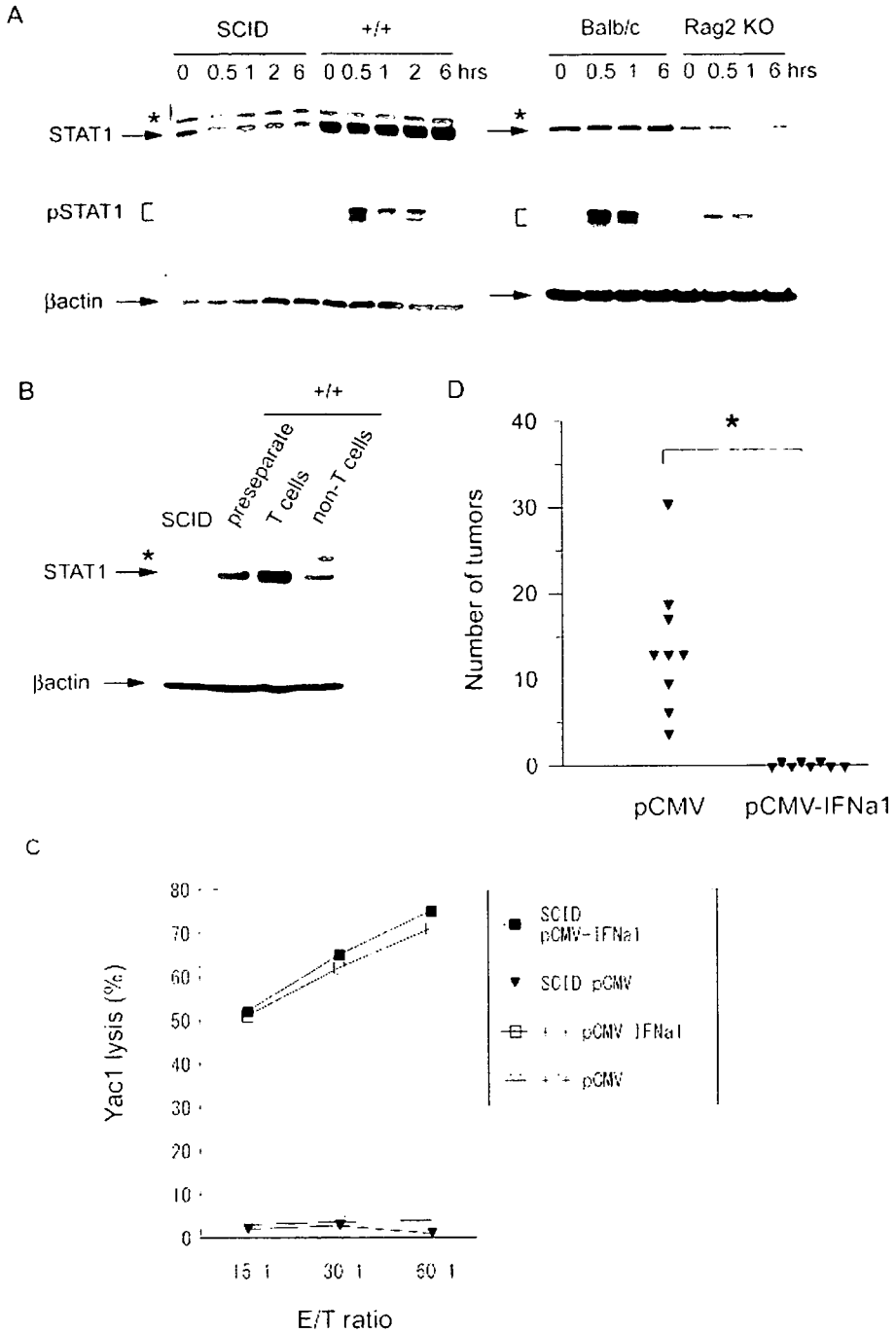

FIGURE 5 – IFNα-mediated NK cell activation and anti-metastatic effects in SCID mice. (*a*) STAT1 and phospho-STAT1 expression of splenocytes from SCID or Rag2 KO mice. Splenocytes were treated with 1,000 U/ml of IFNα and lysed at indicated time points (0 to 6 hr). Expression of STAT1 and phospho-STAT1 (pSTAT1) was analyzed by Western blot. +/+ and Balb/c indicate wild-type controls for SCID and Rag2 KO mice, respectively. *, non-specific band (see the *Material and methods* section). (*b*) STAT1 expression in T cells and non-T cells. Splenocytes from wild-type mice (+/+) were separated into T cells and non-T cells based on expression of CD90. Expression of STAT1 was analyzed by Western blot. SCID and preseparated wild-type splenocytes were included as controls. *, non-specific band (see the Material and methods section). (*c*) Yac1 lytic ability. SCID mice (closed symbols) or wild-type mice (open symbols) were hydrodynamically injected with either pCMV-IFNa1 (squares) or pCMV (triangles). Four days later, splenocytes isolated form the mice were examined for the lytic ability for Yac1 cells. Experiments were done at least 3 times and representative data are shown. (*d*) Anti-metastaic effects. SCID mice or wild-type mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 or pCMV 2 days later. After 14 days, mice were sacrificed to examine tumor development in the liver. The numbers of hepatic tumors were compared among the groups. Experiments were performed 3 times and representative data are shown. *, $p < 0.05$.

us to investigate the adaptive response after antimetastatic effects. Mice that had rejected CT-26 cells by IFNα showed a tumor-specific T cell response and suppressed tumor growth of rechallenged skin tumor. Therefore, pCMV-IFNa1 injection not only caused initial rejection of metastasized tumors but also induced durable and systemic adaptive immunity. Interestingly, splenic injection of UV-irradiated CT-26 cells, even if followed by pCMV-IFNa1 injection, did not elicit significant tumor-specific T cell responses. Therefore, the efficient induction of adaptive T cell responses requires IFNα-mediated rejection of live tumor cells and cannot be recapitulated by simple injection of dead tumor cells and IFNα.

NK cells are present in a high percentage in the liver.[28] In the present study, we focused on NK cells which were rapidly activated by IFNα to examine the cellular mechanisms of protection against hepatic metastasis. Critical requirement of NK cells was demonstrated by anti-asialo GM1 antibody-injected mice which did not show protection against CT-26 metastasis. In contrast, T cells, B cells or NKT cells were dispensable for IFNα-mediated antimetastatic effects since IFNα therapy did show antimetastatic