body weight of all CsA-treated mice was reduced by more than 20% during this period (Fig. 4C). The concentration of human serum albumin in the mice treated with CsA did not change significantly (data not shown). This toxicity was not observed with DEBIO-025 and Peg-IFN.

Quantification of Hepatic HCV RNA and Core Protein Levels and Immunohistochemistry at the End of Treatment in Chimeric Mice Infected with Genotype 1a. At the end of treatment, hepatic HCV RNA was quantified by real-time reverse transcription PCR, and core protein levels were quantified by enzyme-linked immunosorbent assay (Fig. 6A,B). DEBIO-025 monotherapy (1a-3 mouse) reduced HCV RNA by 3-fold compared with the nontreated mouse (1a-4 mouse). Peg-IFN reduced both HCV RNA and core protein levels by approximately 10-fold (1a-2 mouse). Combined treatment with DEBIO-025 and Peg-IFN resulted in an approximately 100-fold reduction in HCV RNA and HCV core protein levels (1a-1 mouse). Moreover, immunohistochemistry was performed. In 1a-4 mouse, HCV core protein was detected in human hepatocytes. In 1a-1 mouse, HCV core protein was not detected by immunohistochemistry; however, reduced HCV core protein was quantified by enzyme-linked immunosorbent assay, which is more sensitive than immunohistochemistry (Fig. 6C, D).

Discussion

Development of new anti-HCV drugs has been significantly impeded by the lack of a suitable cell culture model for the propagation of HCV in laboratories. This obstacle has been partially overcome by the development of the replicon system, which can be used for evaluating the *in vitro* anti-HCV effect of compounds. However, because adaptive mutation into the replicon genome and host permissiveness enable particularly efficient replication in cultured hepatoma cell lines,[25] evaluation of HCV drugs using replicon systems alone is considered insufficient. The only animal species readily infected with HCV has been the chimpanzee, which is labor-intensive and expen-

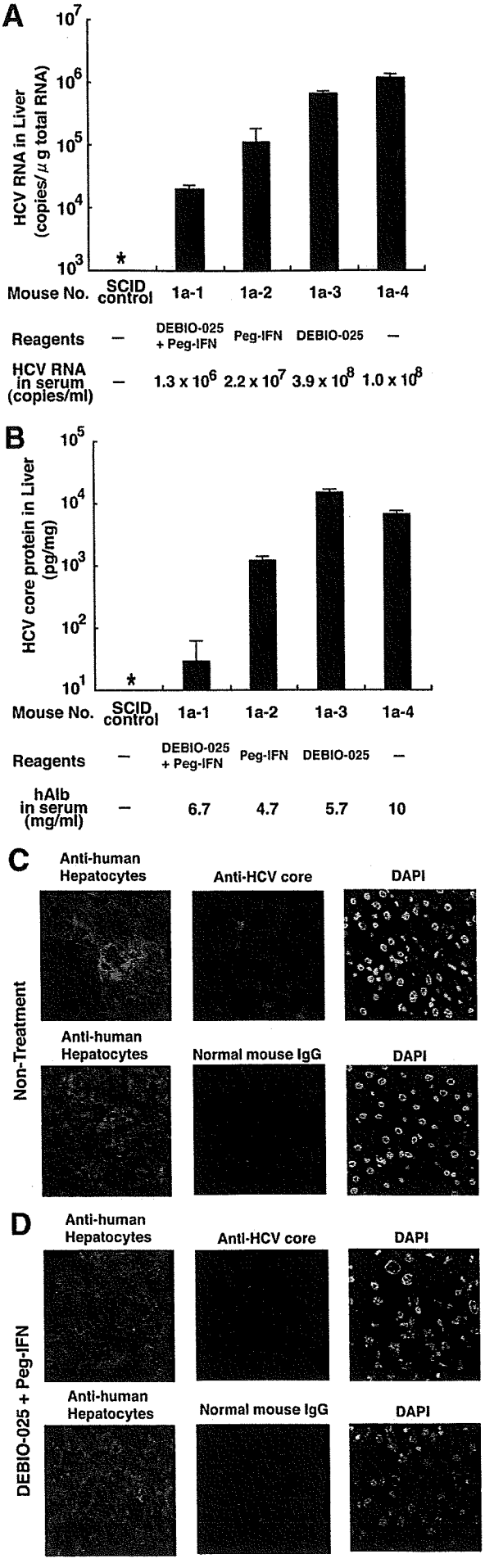

Fig. 6. Analysis of liver tissue from chimeric mice infected with HCV genotype 1a. (A) HCV RNA, and (B) HCV core protein, measured in triplicate in the livers of mice undergoing different treatment protocols. Severe combined immunodeficient (SCID) control: noninfected SCID mouse; 1a-1, mouse treated with DEBIO-025 combined with Peg-IFN; 1a-2, mouse treated with Peg-IFN; 1a-3, mouse treated with DEBIO-025; 1a-4, nontreated mouse infected with HCV. (C,D) Immunofluorescent labeling of human hepatocytes and HCV core protein, and fluorescent staining of nuclei. HCV core protein was labeled in human hepatocytes of nontreated chimeric mouse (C), but was not apparent in chimeric mouse treated with DEBIO-025 combined with Peg-IFN (D).

sive to use, and is associated with ethical problems. The chimeric mouse with human hepatocytes has recently been developed as a practical small animal model that can be infected with HCV.[20] This model is promising for the evaluation of new anti-HCV drugs because the mice are easy to handle, grow rapidly, and are well characterized genetically and immunologically. In this study, we used chimeric mice to bridge the gap between the replicon system and naive HCV replication in human liver, and to examine the anti-HCV effect of DEBIO-025, a novel cyclophilin inhibitor and non-immunosuppressive cyclosporin.

We found that HCV from our patient sera were able to infect the chimeric mice and persistently replicate over several weeks. HCG9 (1a) and HCR6 (1b) reached 10^8 to 10^9 copies/ml and 10^6 to 10^7 copies/ml, respectively, resulting in HCV RNA levels in serum that were higher than those previously reported.[20] This was probably because of a high substitution rate of human hepatocytes in the chimeric mice. When Mercer et al.[20] initially developed chimeric mice infected with HCV, they reported that human albumin concentrations in sera of the mice reached 2 mg/ml and that the substitution rate of liver from mouse to human was approximately 50%. In our study, the human albumin concentration in the chimeric mice reached 6.5 mg/ml, which would be consistent with a higher substitution rate of 80% to 90%.[21] In addition, our findings also indicate that the plateau point of HCV RNA in serum depends on the type of inoculum, because the HCV RNA levels were different for HCG9 and HCR6. Taken together, the results suggest that our chimeric mice propagated large amounts of HCV in their livers.

Although DEBIO-025 strongly inhibited replication of the HCV replicon, it did not affect the replication of naive HCV *in vivo* when given as monotherapy. These results probably indicate differences between the replication of naive HCV *in vivo* and the replicon system. The sensitivity of HCV strains to CsA and non-immunosuppressive cyclosporins was variable, depending on their cyclophilin requirement for their replication.[26] Cyclophilin polymorphism and its role in HCV replication will be the focus of future study.

The HCV RNA levels are known to decline biphasically in most patients treated with IFN.[27] During the first phase, there is a rapid drop in viremia that reflects the direct inhibition of HCV replication. During the second phase, there is a slower decline in serum HCV RNA levels, which appears to reflect the elimination of infected cells by host immune responses. In chimeric mice, the second-phase decline is not obvious, because they lack T cells and B cells (being SCID). Thus, it appears that DEBIO-025 accelerates the decline in HCV RNA levels induced by Peg-IFN during the first phase. There is no evidence that DEBIO-025 enhances the interferon pathway. Also, recent *in vitro* findings show that cyclosporins do not modify the IFN-α signal transduction pathway as assessed by 2′, 5′-oligoadenylate synthetase (2′, 5′-OAS) levels.[28] It therefore seems likely that the apparent synergistic effect of DEBIO-025 seen in our *in vivo* model is not solely related to the antiviral effect mediated by IFN. The DEBIO-025 inhibition of cyclophilin may produce a proper anti-HCV effect by interacting with the RNA-dependent RNA polymerase.[11]

CsA was originally used as an immunosuppressive agent, and we previously demonstrated in clinical trials that CsA has an anti-HCV effect.[9] However, CsA is not devoid of adverse effects, such as hypertension, neurotoxicity, and nephrotoxicity, limiting its therapeutic usefulness against HCV.[29] The immunosuppressive action of CsA occurs by inhibition of calcineurin. Our findings showing that DEBIO-025 exhibits a 7,000-fold lower immunosuppressive activity than CsA suggest that it has less affinity to calcineurin and may lead to fewer adverse effects in patients.

In conclusion, our results indicate that naïve HCV replication *in vivo* is inhibited by the combined administration of the cyclophilin inhibitor DEBIO-025 and Peg-IFN. These findings support further evaluation of DEBIO-025 as a promising drug for the treatment of chronic hepatitis C.

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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).

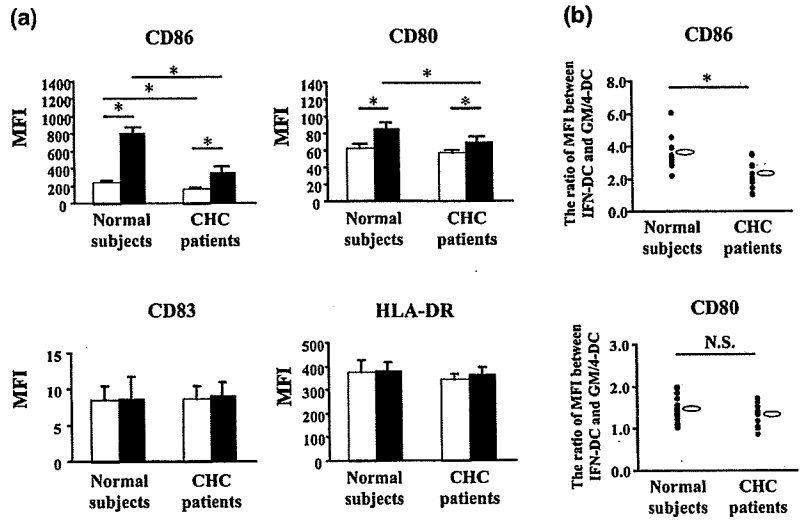

Fig. 1 Interferon (IFN)-α enhanced CD86 and CD80 expression on monocyte-derived DC, in which the degrees of CD86 was higher in healthy subjects than those in chronic hepatitis C (CHC) patients. (a) Monocyte-derived DC were generated from monocytes by 7-day culture with granulocyte–macrophage colony-stimulating 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

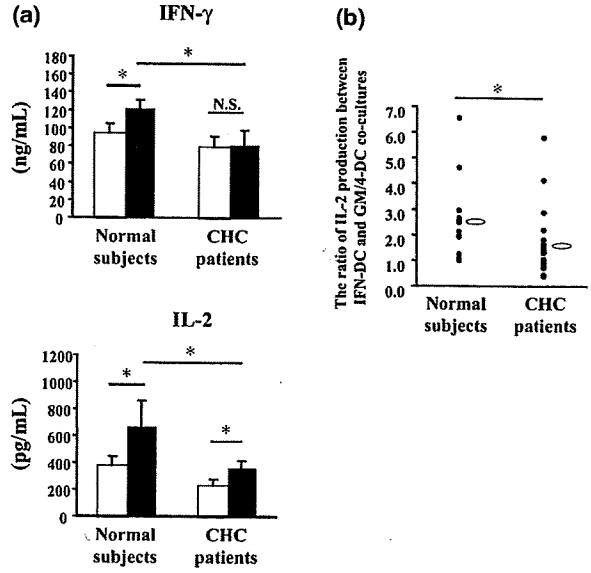

Fig. 2 Interferon-dendritic cells (IFN-DC) from hepatitis C virus-infected patients displayed the impaired capacity of inducing Th1 compared with those from normal subjects. (a) IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ cells for 6 days as described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

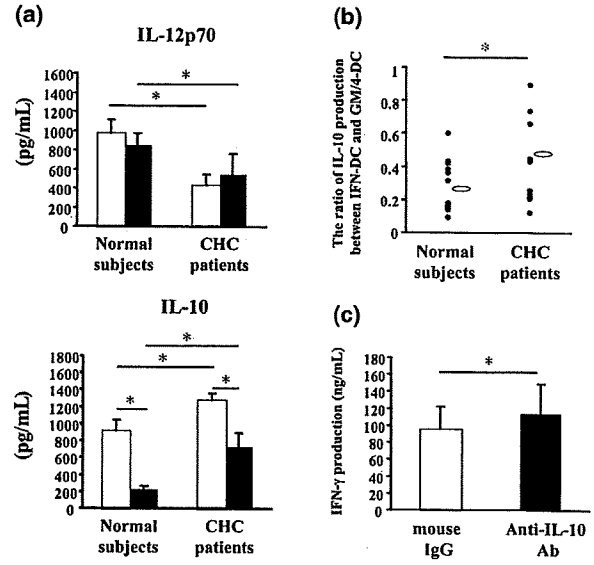

Fig. 3 The lesser IL-10 production from interferon-dendritic cells (IFN-DC) compared with GM/4-DC may be involved in Th1 induction. (a) Day 7 MoDCs were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-12p70 and IL-10 in the supernatants were determined by ELISA. Results are expressed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

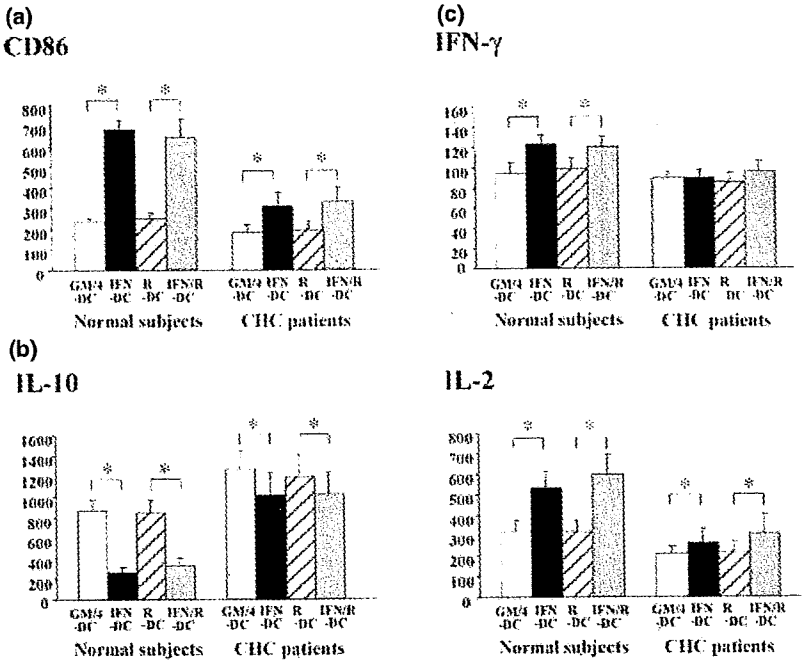

Fig. 4 Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or used in combined with IFN-α. (a) GM/4-DC, IFN-DC, R-DC and IFN/R-DC were generated and analysed their CD86 expressions by fluorescence-activated cell sorter. The figures represent the mean values of MFI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

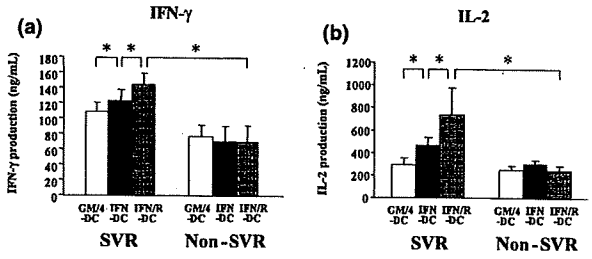

Fig. 5 IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC. IFN/R-DC, IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture,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

Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4$^+$ CD25$^+$ T cells with PD-1-dependent regulatory activities

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Summary

Natural killer (NK) cells have the ability to control dendritic cell (DC)-mediated T cell responses. However, the precise mechanisms by which NK receptor-mediated regulation of NK cells determines the magnitude and direction of DC-mediated T cell responses remain unclear. In the present study, we applied an *in vitro* co-culture system to examine the impact of NK cells cultured with hepatic cells on DC induction of regulatory T cells. We found that interaction of NK cells and non-transformed hepatocytes (which express HLA-E) via the NKG2A inhibitory receptor resulted in priming of DCs to induce CD4$^+$ CD25$^+$ T cells with regulatory properties. NKG2A triggering led to characteristic changes of the cytokine milieu of co-cultured cells; an increase in the transforming growth factor (TGF)-β involved in the generation of this specific type of DC, and a decrease in the tumour necrosis factor-α capable of antagonizing the effect of TGF-β. The regulatory cells induced by NK cell-primed DCs exert their suppressive actions through a negative costimulator programmed death-1 (PD-1) mediated pathway, which differs from freshly isolated CD4$^+$ CD25$^+$ T cells. These findings provide new insight into the role of NK receptor signals in the DC-mediated induction of regulatory T cells.

Keywords: NK receptor; regulatory T cell; HLA-E; liver; HCV

Introduction

CD4$^+$ CD25$^+$ regulatory T (Treg) cells have been identified as the main suppressors of immune responses.[1–5] Although the mechanisms by which CD4$^+$ CD25$^+$ Treg cells exert their suppressive actions have not been fully elucidated, negative costimulatory signals via cytotoxic T lymphocyte antigen-4 (CTLA-4) or inducible costimulator (ICOS)-mediated signals, have been suggested to play a key role in the activation of CD4$^+$ CD25$^+$ Treg cells.[6,7] Programmed death-1 (PD-1), another molecule identified as a negative costimulatory receptor, has also serves as a negative regulator for effector immune responses.[8] Recent reports have demonstrated that PD-1 is expressed in CD4$^+$ CD25$^+$ Treg cells, suggesting its potential roles in the regulation of T cell tolerance.[9] However, the precise roles of PD-1 in CD4$^+$ CD25$^+$ Treg cell functions remain elusive.

The mechanisms by which CD4$^+$ CD25$^+$ Treg cells are generated have been extensively investigated. Dendritic cells (DCs), the sentinels between innate and adaptive immunity, have recently emerged as candidate cells involved in the differentiation and/or activation of CD4$^+$ CD25$^+$ Treg cells.[10] Various kinds of factors have been identified as involved in DC induction of CD4$^+$ CD25$^+$ Treg cells. Mouse immature DC promotes the differentiation of CD4$^+$ CD25$^+$ Treg cells through the DEC 205-mediated targeting of self-antigen in the steady state.[10,11] The immune regulatory cytokines interleukin (IL)-10/transforming growth factor (TGF)-β have also been reported to play important roles in DC generation and activation of CD4$^+$ CD25$^+$ Treg cells.[12–14]

Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; GITR, glucocorticoid-induced TNF receptor; HCV, hepatitis C virus; HLA, human leucocyte antigen; NH, human non-transformed hepatocyte; NK, natural killer; PD-1, programmed death-1; PDL-1, programmed death ligand 1; PBMC, peripheral blood mononuclear cell; Treg, regulatory T.

Several lines of evidence have revealed that natural killer (NK) cell-mediated innate immunity regulates DC functions to determine the direction and magnitude of adaptive T cell immunity.[15–18] It has also been established that NK cell function is regulated by positive and negative signals through their receptor and ligand interactions.[19] We previously reported that, upon exposure to non-transformed hepatocytes (NHs), IL-2-primed NK cells negatively regulated DC functions, which appeared to be dependent on NKG2A inhibitory signals during co-culture of NK cells and NHs. Immunosuppressive cytokines such as IL-10 and TGF-β, but not direct NK–DC contact, were responsible for this action.[20] However, it remains unclear whether these NK/hepatocyte co-cultures can also influence the induction as well as activation of $CD4^+$ $CD25^+$ Treg cells.

In the present study, we investigated whether DCs stimulated with the co-culture supernatant of IL-2-prestimulated NK cells and NHs can modulate Treg cell functions. We found that TGF-β produced from NK cell/hepatocyte co-culture via NKG2A activation is responsible for modulating DCs to induce and maintain regulatory phenotypes and functions of $CD4^+$ $CD25^+$ Treg cells. Furthermore, the generated $CD4^+$ $CD25^+$ Treg cells suppressed T cell activation via interaction between PD-1 and programmed death ligand 1 (PDL-1). These findings represent new evidence that NK receptor-mediated modulation of NK cells may dictate DC-induced adaptive immunity toward an immunogenic or tolerogenic status via induction of Treg cells.

Materials and methods

Antibodies

Anti-NKG2A monoclonal antibody (mAb) (Z199), PC5-labelled CD25 mAb or isotype-matched control IgG1 and IgG2a mAb were purchased from Beckmann-Coulter (Fullerton, CA). Anti-IL-10, anti-TGF-β, anti-CTLA-4, anti-GITR (glucocorticoid-induced TNF receptor) and anti-PD-1 polyclonal Abs were purchased from R & D Systems (Minneapolis, MN) and phycoerythrin (PE)-labelled mAb CTLA-4 from BD Biosciences (San Jose, CA). Anti-HLA-E mAb 3D12 was kindly provided by Dr E. Geraghty (Fred Hutchinson Cancer Research Institute, Seattle, WA) and used as reported previously.[21] Anti-MIC mAb 6D4, anti-ULBP1 mAb 3F1 and anti-ULBP2 mAb DH1 were kindly provided by Drs T. Spies and V. Groh (Fred Hutchinson Cancer Research Institute) and used as reported previously.[22]

Human hepatic cells

Human non-transformed hepatocytes (NHs) derived from mixed heterogeneous donors were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and cultured in CS-C complete medium according to the manufacturer's instructions.

Isolation of peripheral blood lymphocyte populations

Resting NK cells ($CD56^+$ $CD3^+$), naive $CD4^+$ T cells ($CD45RA^+$ RO^+) or $CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) with a positive cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ $CD25^+$ T cells were further separated from naive $CD4^+$ T cells using anti-CD25 microbeads (Miltenyi Biotech). Their purity was >90% by flow cytometry. Informed consent was obtained from all blood donors.

Generation of monocyte-derived DC

Monocytes were isolated by plastic adherence from PBMCs and cultured in RPMI-1640 supplemented with granulocyte–macrophage colony stimulating factor (GM-CSF) (PeproTech, London, UK) and IL-4 (PeproTech). At day 6, they were stimulated with or without the co-culture supernatant of NK cells and hepatic cells. At day 7, non-adherent cells were harvested and used as described below.

Stimulation of DCs by co-culture supernatants of NK cells and hepatic cells

Freshly isolated NK cells were cultured with or without IL-2 for 24 hr. IL-2-prestimulated or non-stimulated NK cells were seeded in 24-well plates and then co-cultured for 24 hr with NHs (1×10^5 cells/well), respectively. Monocyte-derived DCs were cultured for 24 hr with 1 ml of the co-culture supernatant of IL-2-prestimulated NK cells and NHs (NH/IL-2 NK-primed DC). In some experiments, anti-NKG2A mAb (Z199) or isotype-matched control Ab was added during the co-cultures of NK cells and hepatic cells. Z199 mAb was previously confirmed to block the NKG2A-mediated signal.[23] In some experiments, the supernatant of NK/hepatic cell co-cultures was also treated with anti-IL-10 or anti-TGF-β neutralizing Ab and used for DC stimulation for 24 hr. In some experiments, tumour necrosis factor (TNF)-α, TGF-β or both were used for DC stimulation for 24 hr.

Isolation of $CD4^+$ $CD25^+$ T cells

DCs (1×10^5) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr; $CD4^+$ $CD25^+$ fractions were isolated from DC and $CD4^+$ co-culture and subjected to further analysis. $CD4^+$ $CD25^+$ fractions were also isolated

from PBMCs and cultured with 1 μg/ml plate-bound anti-CD3 mAb (UCHT1; Beckmann-Coulter) for 24 hr to efficiently induce their suppressive properties as described previously.[3] These cells are referred to as natural $CD4^+$ $CD25^+$ T cells.

Flow cytometry

The expression of NK inhibitory ligands (human leucocyte antigen, HLA, class I, HLA-E) was examined on NHs by using w6/32 or 3D12, respectively. MIC, ULBP1 or ULBP2 expression on hepatocytes was also evaluated by mAb 6D4, 3F1 or DH1, respectively. For $CD4^+$ $CD25^+$ T cell staining, the cells were costained with PC5-labelled CD25 mAb with PE-labelled mAb of CTLA-4, GITR or PD-1 polyclonal Ab. The cells were analysed by flow cytometry using a fluorescence-activated cell sorter (FACScan) system, and data analysis was performed using CELLQUEST software.

Measurements of cytokine production in culture supernatant

The culture supernatants of interferon (IFN)-γ, TNF-α, IL-10 and TGF-β were examined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (IFN-γ, TNF-α and IL-10, Endogen, Tokyo, Japan; TGF-β, R & D Systems).

Analysis of Foxp3 mRNA expression

Polymerase chain reaction (PCR) analysis was performed to determine Foxp3 mRNA expression of $CD4^+$ T cells using a commercial PCR panel according to the manufacturer's instructions (Gibco BRL, Rockville, MD). The following primers were used: 5′-CCCACTTACAGGCACT CCTC-3′ (forward) and 5′-CTTCTCCTTCTCCAGCAC CA-3′ (reverse).[24] Amplification was carried out for 35 cycles of 20 seconds at 95°, 20 seconds at 58 ° and 30 seconds at 72°. As a control for the integrity of mRNA, primers specific for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as follows: 5′-GCCACCCAGAAGACTGTGGATGGC-3′ (forward) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (reverse). The PCR products were analysed by ethidium bromide-stained 1·5% agarose gel electrophoresis.

Analysis of $CD4^+$ $CD25^+$ T cell suppressor functions

DCs (5×10^4/well) were cultured with allogeneic $CD4^+$ T cells (5×10^5/well) for 48 hr, after which $CD4^+$ $CD25^+$ T cells were isolated from the co-cultured cells. $CD4^+$ $CD25^-$ T cells were freshly isolated from the same donors and activated with 1 μg/ml plate-bound anti-CD3 mAb in the presence or absence of autologous $CD4^+$ $CD25^+$ T cells for 48 hr. The ability of $CD4^+$ $CD25^+$ T cells to suppress proliferation and IFN-γ production of activated $CD4^+$ $CD25^-$ T cells was determined by [^{3}H]thymidine incorporation and ELISA assay, respectively. To further examine the mechanisms of $CD4^+$ $CD25^+$ T cell suppressive actions, neutralizing Ab of IL-10 or TGF-β, anti-CTLA-4, anti-GITR or anti-PD-1 was added at the beginning of $CD4^+$ $CD25^+$ T cell and $CD4^+$ $CD25^-$ T cell co-cultures.

Statistical analysis

Comparisons between groups were analysed by *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. Differences were considered significant when the *P*-value was < 0.05.

Results

IL-2-primed NK cells upon exposure to NH-modulated DCs on the induction of regulatory $CD4^+$ $CD25^+$ T cells

Natural $CD4^+$ $CD25^+$ T cells from human peripheral blood lymphocytes (PBLs) expressed CTLA-4 and GITR, both of which have been identified as regulatory markers,[6,25] but did not express PD-1 (Fig. 1a). To examine whether DCs can modulate the expression of these regulatory markers on $CD4^+$ $CD25^+$ T cells, we stimulated monocyte-derived DCs for 24 hr, either by the culture supernatant of IL-2-stimulated NK cells (IL-2 NK) or by the co-culture supernatant of NH/IL-2 NK. After washing, the resulting DCs were cultured for 48 hr with $CD4^+$ T cells isolated from allogeneic donors. $CD4^+$ $CD25^+$ T cells were isolated from the DC and $CD4^+$ T cell co-culture and subjected to analysis for regulatory markers. The expression levels of CTLA-4 and GITR decreased on $CD4^+$ $CD25^+$ T cells after stimulation of IL-2 NK-primed DCs (Fig. 1b). By contrast, $CD4^+$ $CD25^+$ T cells stimulated with NH/IL-2 NK-primed DCs remained positive for CTLA-4 and GITR on their surface. Of note is the finding that PD-1 was induced on these cells, showing their phenotypic properties to differ from natural $CD4^+$ $CD25^+$ T cells (Fig. 1b, c). The induction of PD-1 on $CD4^+$ $CD25^+$ T cells was further confirmed when IL-2NK/NH-primed DCs from different donors were used as stimulators (Fig. 1d). The supernatant of NH without NK cells had little effect on phenotypic changes of $CD4^+$ $CD25^+$ T cells by DCs (data not shown).

The forkhead transcription factor Foxp3 has been recently identified as a master gene for defining Treg cells.[26] We therefore performed reverse transcription-PCR (RT-PCR) analysis of $CD4^+$ T cells to evaluate the mRNA expression of Foxp3. Foxp3 expression was detected in natural $CD4^+$ $CD25^+$ T cells. When $CD4^+$ T cells were

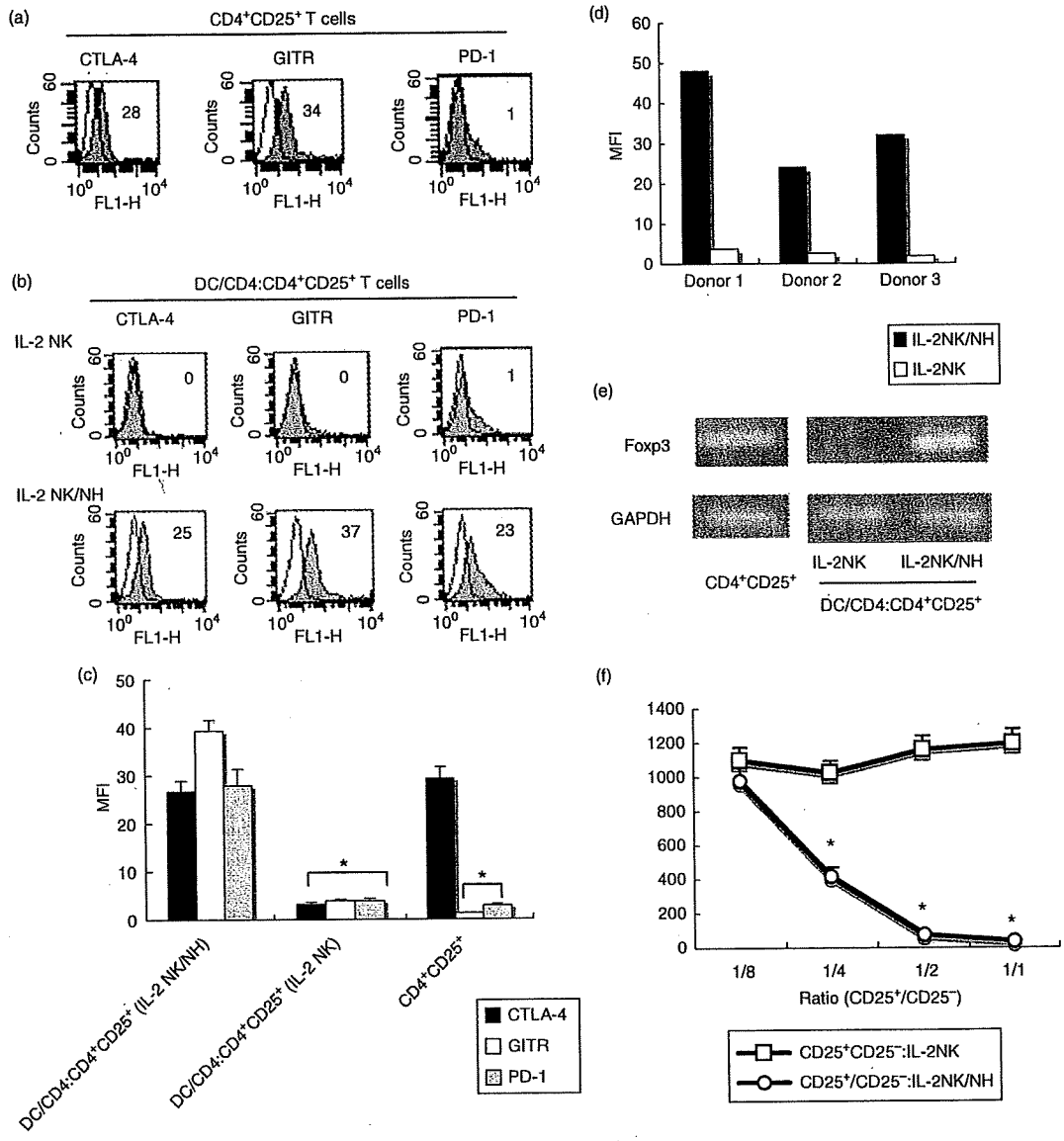

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) for 24 hr, and then subjected to flow cytometry to examine their expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms represent the staining of control Ab. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cells. (b) NK cells were preactivated with 50 ng/ml interleukin (IL)-2, and co-cultured in the absence (IL-2 NK) or presence (IL-2 NK/NH) of NHs at a ratio of 1 : 1 for 24 hr. DCs (1×10^5) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T co-culture and subjected to flow cytometry for expression of CTLA-4, GITR or PD-1 (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the MFI of each type of stained cell. (c) All experiments in (a) and (b) were performed three times and the composite results with statistical analysis are shown as the MFI of the staining cells. $^*P < 0{\cdot}05$ vs. responses of IL-2 NK/NH group. The experiment was performed with a different set of donors and similar results were obtained. (d) PD-1 expression on $CD4^+$ $CD25^+$ T cells stimulated with allogeneic DCs from three different donors, shown as the MFI. (e) $CD4^+$ $CD25^+$ T cells were prepared as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR). (f) $CD4^+$ $CD25^+$ fractions were isolated from DC/$CD4^+$ T cell co-cultures. Different numbers of these $CD4^+$ $CD25^+$ T cells were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells (1×10^5/well) in the presence of plate-bound anti-CD3 Ab ($CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$). The anti-CD3 Ab-activated $CD4^+$ $CD25^-$ T cells alone were used as a positive control ($CD4^+$ $CD25^-$). IFN-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$.

stimulated with IL-2 NK-primed DCs for 24 hr, Foxp3 was not expressed on $CD4^+$ $CD25^+$ T cells. By contrast, they dominantly transcribed Foxp3 at levels comparable with those of natural $CD4^+$ $CD25^+$ T cells when stimulated with NH/IL-2 NK-primed DCs (Fig. 1e). Taken together, $CD4^+$ $CD25^+$ T cells, when stimulated by NH/IL-2 NK-primed DCs, maintained regulatory phenotypes such as CTLA-4, GITR and Foxp3, and properties distinct from those of natural $CD4^+$ $CD25^+$ Treg cells in terms of PD-1 expression.

$CD4^+$ $CD25^+$ T cells on stimulation of NH/IL-2 NK-primed DC suppressed effector cell functions

We next analysed the functions of $CD4^+$ $CD25^+$ T cells stimulated by NH/IL-2 NK-primed DC. $CD4^+$ $CD25^+$ T cells were co-cultured for 72 hr with $CD4^+$ $CD25^-$ T cells freshly isolated from the same donors. During the co-cultures, $CD4^+$ $CD25^-$ T cells were stimulated with plate-bound anti-CD3 Ab. The $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs dose-dependently suppressed the proliferation of co-cultured cells, whereas those induced by IL-2 NK-primed DC did not (data not shown). $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs also dose-dependently inhibited IFN-γ production of the co-cultured cells, by contrast with those induced by IL-2 NK-primed DCs (Fig. 1f). The suppressive activities of these $CD4^+$ $CD25^+$ Treg cells were similar to those of natural $CD4^+$ $CD25^+$ Treg cells (data not shown). These results demonstrate that $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs exert suppressive actions to effector cell functions, consistent with their expression of regulatory markers. Taken together, these results indicated that NK cell modulation of DCs leads to the $CD4^+$ $CD25^+$ Treg cell-mediated suppression of effector cell responses when NK cells encounter hepatocytes.

NKG2A signal of NK cells is responsible for the modulation of DCs to activate $CD4^+$ $CD25^+$ Treg cells

We examined the expression of various ligands for NK cell receptors on NHs. NHs expressed HLA-E, the ligand of NKG2A, but did not express NKG2D receptor ligands, MIC and ULBP1-2 (Fig. 2a). Given our previous findings that NHs negatively regulated IL-2 NK-mediated modulation of DC functions through the interaction of the NKG2A inhibitory receptor and its ligand HLA-E,[20] we evaluated the role of these receptor signals in the induction of $CD4^+$ $CD25^+$ Treg cells by DCs. When anti-NKG2A Ab was added during the co-culture of NH and IL-2 NK and DCs were stimulated with the resultant supernatant, the expression of CTLA-4, GITR and PD-1 was diminished on $CD4^+$ $CD25^+$ T cells (Fig. 2b, c). NKG2A blockade also suppressed PD-1 expression on $CD4^+$ $CD25^+$ T cells stimulated with IL-2NK/NH-primed DCs from three different donors (Fig. 2d). The anti-NKG2A neutralizing Ab treatment also abrogated Foxp3 expression in $CD4^+$ $CD25^+$ Treg cells (Fig. 2e). Moreover, the blockade of NKG2A signals during NH and IL-2 NK co-cultures resulted in inhibition of the DC ability to induce $CD4^+$ $CD25^+$ T cells with regulatory functions; these $CD4^+$ $CD25^+$ T cells did not suppress proliferation or IFN-γ production (Fig. 2f and data not shown) of $CD4^+$ $CD25^-$ T cells. Altogether, the activation of NKG2A inhibitory signals during NK cell and hepatocyte interaction was required for the DC induction of $CD4^+$ $CD25^+$ T cells with regulatory phenotypes and functions.

Change of cytokine milieu, triggered by NKG2A signals, plays a critical role in DC-mediated induction of $CD4^+$ $CD25^+$ Treg cells

TNF-α has been well known as a critical factor for NK cell-mediated maturation of DCs.[27] By contrast, IL-10 and TGF-β are known to act as suppressive factors of effector immune responses, and their roles in modulating DCs for Treg cell induction has recently been validated.[12–14] These findings led us to evaluate the change in cytokine production patterns in NH and IL-2 NK co-cultures in the presence or absence of anti-NKG2A Ab. ELISA data showed that the production of IFN-γ and TNF-α from NH and IL-2 NK co-cultures were substantially increased in the presence of anti-NKG2A Ab. By contrast, the addition of NKG2A masking Ab during the co-culture resulted in the marked reduction of IL-10 and TGF-β from co-cultured cells (Fig. 3a).

We next examined whether these changes of cytokine profiles were responsible for the DC induction of the $CD4^+$ $CD25^+$ Treg cells. For this purpose, the NH and IL-2 NK co-culture supernatant was treated with neutralizing Ab of IL-10 or TGF-β before DC stimulation, and suppressive activity was evaluated by analysing $CD4^+$ $CD25^+$ T cells obtained from $CD4^+$ and DC mixtures. The neutralization of IL-10 did not reverse the suppressive actions of $CD4^+$ $CD25^+$ Treg cells, but the blockade of TGF-β led to reversal of $CD4^+$ $CD25^+$ Treg cell activities (Fig. 3b).

We directly examined the effect of TGF-β on the modulation of DC ability to induce $CD4^+$ $CD25^+$ Treg cells. TGF-β endowed DCs with the ability to induce $CD4^+$ $CD25^+$ Treg cells. TNF-α inhibited TGF-β-mediated DC induction of $CD4^+$ $CD25^+$ Treg cells (Fig. 3c). By contrast, IFN-γ had little effect on the modulation of DC by TGF-β (data not shown). Taken together, these results strongly suggest that increased TGF-β and decreased TNF-α production, the change of cytokine profiles mediated by the NKG2A signals, are involved in DC-mediated $CD4^+$ $CD25^+$ Treg cell induction.

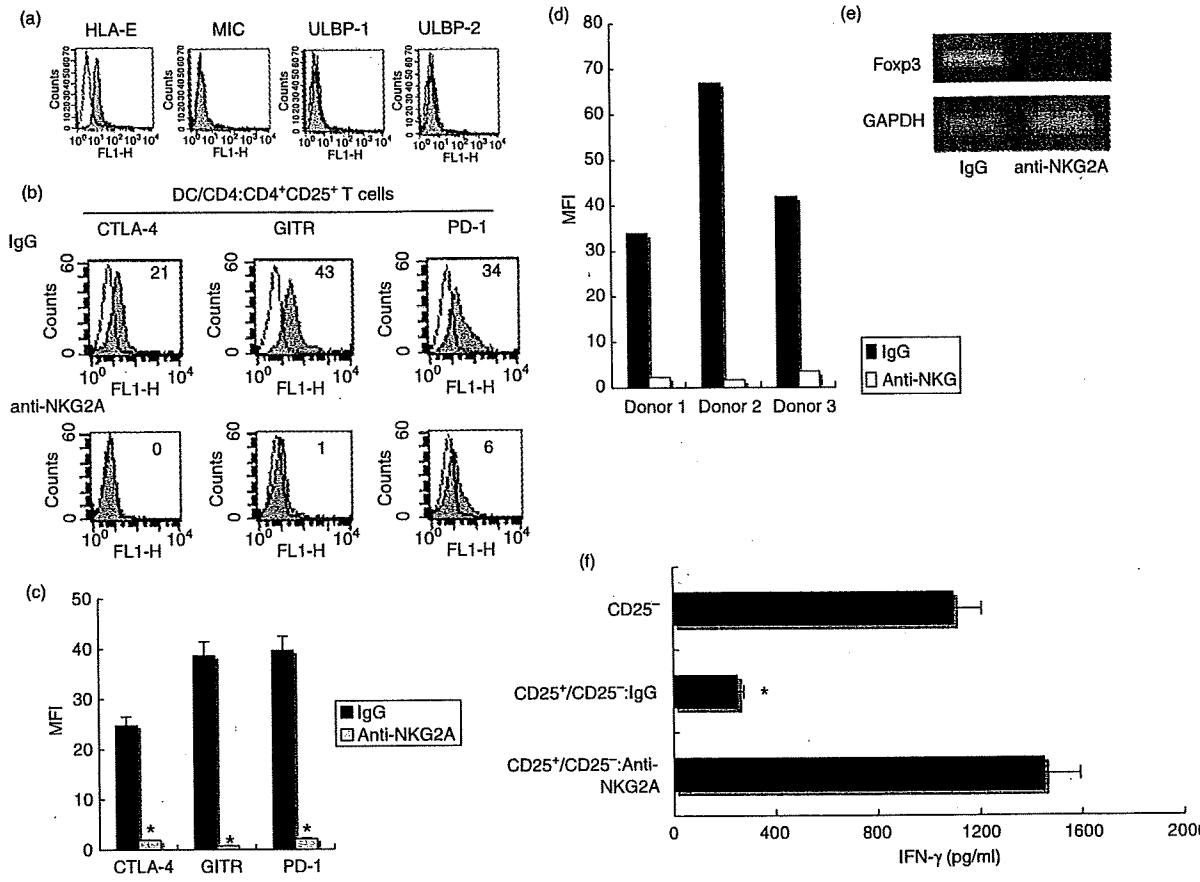

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) were assessed by flow cytometry (closed histograms). Open histograms show isotype control staining. (b, c) Interleukin (IL)-2-preactivated NK cells were co-cultured with NHs in the presence of 30 μg/ml of anti-NKG2A neutralizing antibody (Ab) (anti-NKG2A) or control IgG. DCs (1×10^5) were then stimulated with the supernatant obtained from the co-cultured medium for 24 hr. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ cells isolated from the co-culture were subjected to FCM for their surface expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cell. All experiments were performed three times. Representative data (b) and composite results with statistical analysis (c) are shown as the MFI of the staining cells. $^*P < 0{\cdot}05$ vs. responses of IgG group. The experiment was performed in different set of donors and similar results were obtained. (d) The inhibitory effect of anti-NKG2A Ab on PD-1 expression of $CD4^+$ $CD25^+$ T cells stimulated with allogeneic DCs from three different donors. Data are shown as MFI. (e) $CD4^+$ $CD25^+$ T cells were stimulated and purified as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR. (f) $CD4^+$ $CD25^+$ T cells (1×10^5/well) isolated from DC and $CD4^+$ T cell co-cultures were cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab ($CD25^+/CD25^-$). The anti-CD3 Ab-activated $CD4^+$ $CD25^-$ T cells alone were used as a positive control ($CD25^-$). Interferon (IFN)-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay (ELISA). $^*P < 0{\cdot}05$. All experiments were performed three times; representative results are shown.

Suppressive actions of $CD4^+$ $CD25^+$ Treg cells, induced by NH/IL-2 NK-primed DCs, depends on PD-1-mediated negative costimulatory signals

The suppressive activities of $CD4^+$ $CD25^+$ Treg cells reportedly depend on various kinds of mediators, such as CTLA-4, IL-10 and/or TGF-β, but the exact mechanisms of the actions have not been fully elucidated.[1,6,12–14] PD-1, recently identified as a negative costimulatory receptor of the B-7 family, is expressed in $CD4^+$ $CD25^+$ Treg cells, indicating that PD-1-mediated negative signals may be involved in the regulatory functions of $CD4^+$ $CD25^+$ Treg cells.[9] Thus, we evaluated the involvement of these molecules in the suppressive activities of $CD4^+$ $CD25^+$ Treg cells. For this purpose, the blocking Ab of CTLA-4, GITR, PD-1, TGF-β or IL-10 was added

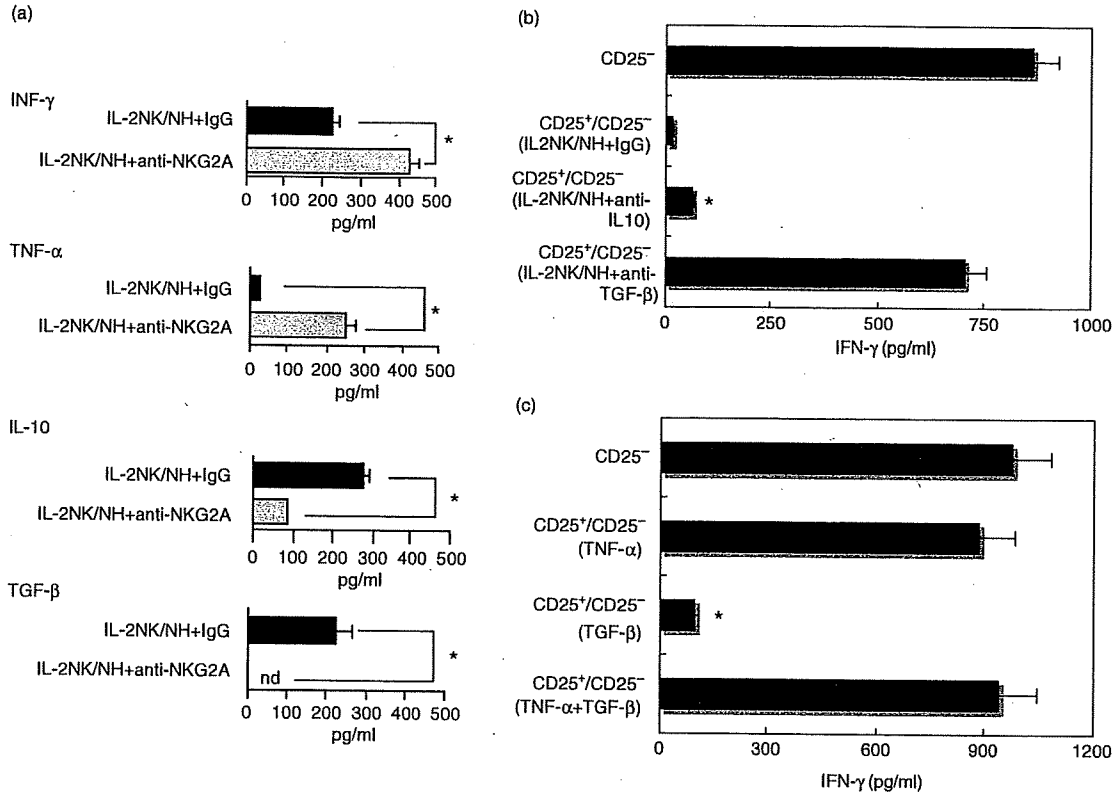

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the presence of masking antibodies (Abs) of NKG2A (IL-2 NK/NH + anti-NKG2A) or isotype control IgG (IL-2 NK/NH + IgG) for 24 hr. *$P < 0{\cdot}05$. (b) IL-2 activated NK cells were co-cultured with NHs (IL-2 NK/NH). DCs (1×10^5) were stimulated with the culture supernatant in the presence of anti-IL-10, anti-transforming growth factor (TGF)-β neutralizing Ab or control IgG for 24 hr. DCs were washed thoroughly and co-cultured with allogeneic $CD4^+$ T cells for 48 hr. Next, the isolated $CD4^+$ $CD25^+$ T cells (1×10^5/well) were co-cultured with autologous $CD4^+$ $CD25^-$ T cells in the presence of plate-bound anti-CD3 Ab at a ratio of 1 : 1. Interferon (IFN)-γ production from the culture supernatant was examined by enzyme-linked immunosorbent assay. *$P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (c) DCs (1×10^5) were stimulated with 50 ng/ml TNF-α, 100 ng/ml TGF-β or both for 24 hr. After thorough washing, they were co-cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ T cells (1×10^5/well) were isolated from the DC and $CD4^+$ co-cultures and cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab. IFN-γ production was examined as described above. *$P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells.

during co-cultures of $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cells in the presence of anti-CD3 Ab. In case of natural $CD4^+$ $CD25^+$ T cells, their suppressive action was partially reversed on addition of anti-CTLA-4 Ab. By contrast, they preserved their suppressive capacity even in the presence of the blocking Ab of GITR, PD-1, TGF-β or IL-10 (Fig. 4a). When $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were used instead of natural $CD4^+$ $CD25^+$ T cells, their suppressive activity was markedly reduced on addition of the blocking Ab of PD-1 but not CTLA-4, IL-10, TGF-β or GITR (Fig. 4a). The regulatory functions of these Treg cells were required for direct cell-to-cell contact because separation of $CD4^+$ $CD25^+$ Treg cells and $CD4^+$ $CD25^-$ T cells in transwell chambers virtually abolished their suppressive effects (data not shown). We also confirmed the presence of PDL-1 expression on $CD4^+$ $CD25^-$ T cells when they were activated with anti-CD3 Ab (Fig. 4b), suggesting that effector cells themselves induce suppressive activities of $CD4^+$ $CD25^+$ Treg cells. Taken together, these results further reinforced the hypothesis that $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were different from natural $CD4^+$ $CD25^+$ Treg cells in their PD-1-dependent suppressive functions.

Discussion

Recent studies have revealed that activated NK cells positively regulate DC activation and maturation either through direct contact via NK cell receptors (NKp30, NKG2D, etc.) or in co-ordination with various kinds of cytokines (IFN-γ, TNF-α, etc.).[15–18] However, the issue of

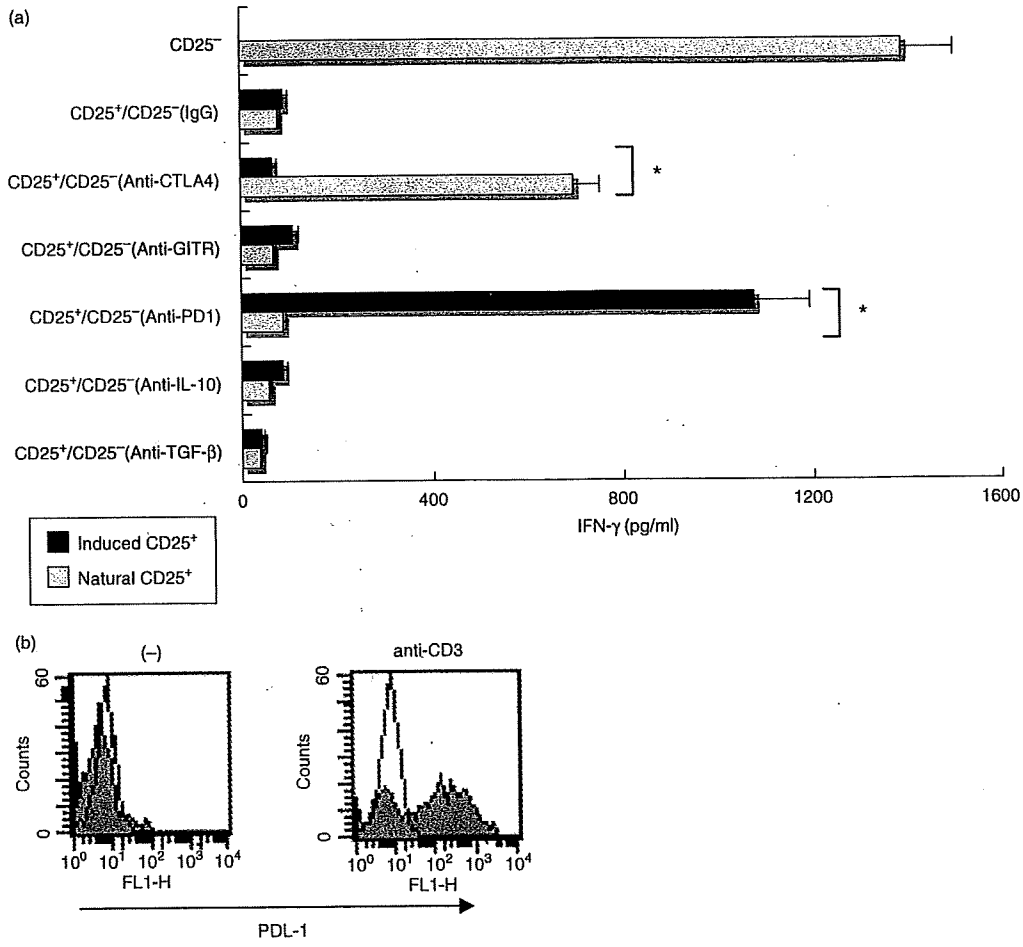

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with the IL-2 NK/NH supernatant for 24 hr, and then cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T cell mixtures. Freshly isolated $CD4^+$ $CD25^+$ T cells (natural $CD25^+$) or $CD4^+$ $CD25^+$ T cells induced by NK/NH-primed DCs (induced $CD25^+$) were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 upon stimulation of plate-bound anti-CD3 antibody (Ab). Anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) Ab, anti-GITR (glucocorticoid-induced TNF receptor) Ab, anti-PD-1 Ab, anti-IL-10 Ab, anti-TGF-β Ab or isotype control IgG (20 μg/ml for each) were incubated during $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cell co-cultures. Interferon (IFN)-γ was measured for each supernatant obtained after 72 hr of co-culture by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (b) Freshly isolated $CD4^+$ $CD25^-$ T cells were incubated with (anti-CD3) or without (–) plate-bound anti-CD3 Ab for 24 hr. PDL-1 expression was assessed by flow cytometry (closed histograms). Open histograms show isotype control staining.

whether NK cells are involved in DC-mediated Treg cell induction has not been resolved. In the present study, we report that the expression of regulatory markers and functions was markedly decreased on $CD4^+$ $CD25^+$ T cells upon exposure to IL-2 NK-primed DCs. By contrast, the interaction of activated NK cells and NH through the NKG2A inhibitory receptor led to DC induction of $CD4^+$ $CD25^+$ T cells with regulatory properties. Furthermore, NKG2A-mediated increase in TGF-β as well as decrease in TNF-α in an NH and NK cell mixture contributed to DC induction of $CD4^+$ $CD25^+$ Treg cells. This is consistent with previous reports showing that TGF-β plays a role in generating the specific DC that activates $CD4^+$ $CD25^+$ Treg cells.[10,11] The findings that TNF-α suppressed TGF-β-mediated priming of DCs to induce Treg cells also extended the previously identified role of TNF-α as a positive regulator of DC activation. In line with our findings, previous reports showed that impairment of $CD4^+$ $CD25^+$ Treg cell activities restored their suppressive functions after blocking TNF-α signals in non-obese diabetic (NOD) mice or in patients with Crohn's disease.[28,29] To our knowledge, the present study is the first description of modulation of NK cells and human hepatocytes through NKG2A-mediated inhibitory

