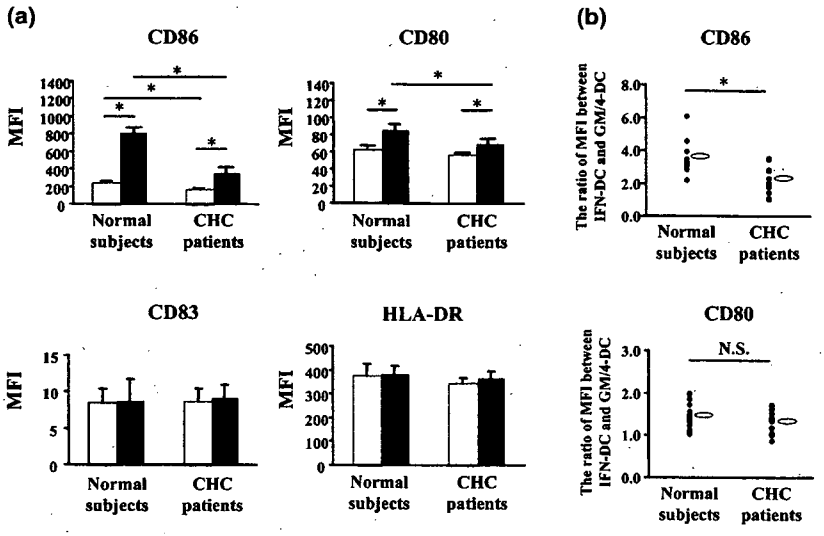

Fig. 1 Interferon (IFN)-α enhanced CD86 and CD80 expression on monocyte-derived DC, in which the degrees of CD86 was higher in healthy subjects than those in chronic hepatitis C (CHC) patients. (a) Monocyte-derived DC were generated from monocytes by 7-day culture with granulocyte–macrophage colony-stimulating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

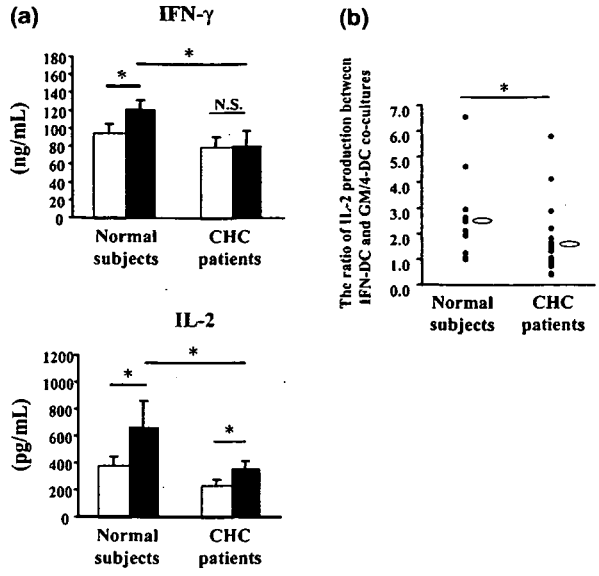

Fig. 2 Interferon-dendritic cells (IFN-DC) from hepatitis C virus-infected patients displayed the impaired capacity of inducing Th1 compared with those from normal subjects. (a) IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ cells for 6 days as described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

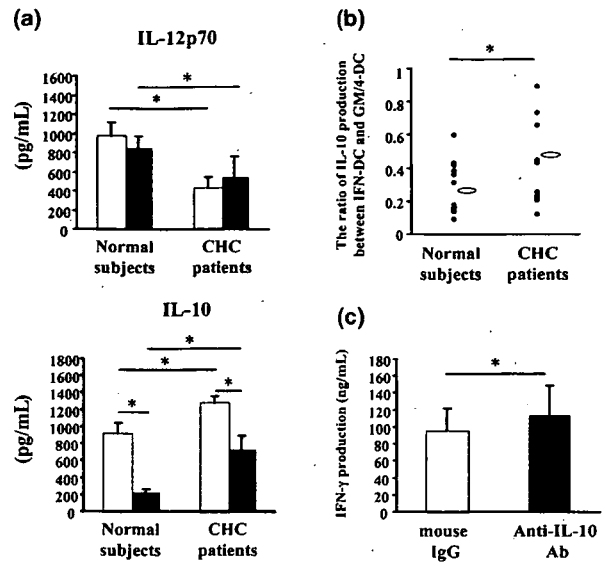

Fig. 3 The lesser IL-10 production from interferon-dendritic cells (IFN-DC) compared with GM/4-DC may be involved in Th1 induction. (a) Day 7 MoDCs were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-12p70 and IL-10 in the supernatants were determined by ELISA. Results are expressed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

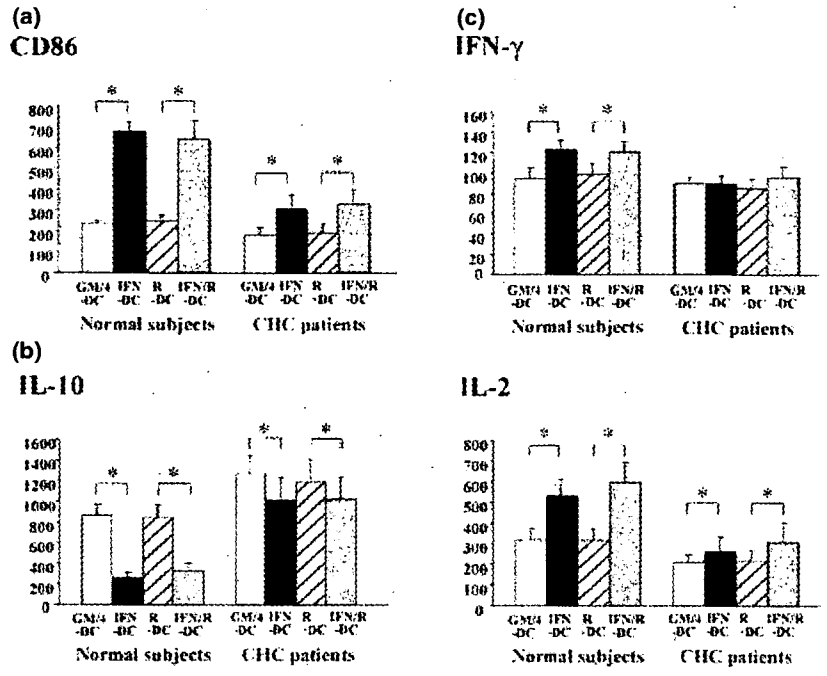

Fig. 4 Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or used in combined with IFN-α. (a) GM/4-DC, IFN-DC, R-DC and IFN/R-DC were generated and analysed their CD86 expressions by fluorescence-activated cell sorter. The figures represent the mean values of MFI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

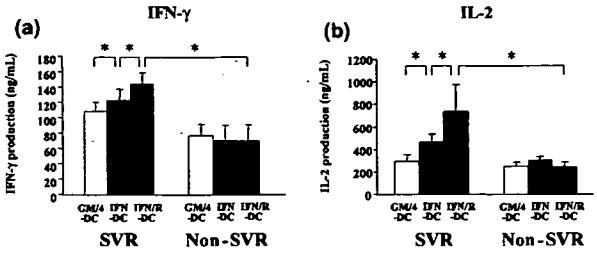

Fig. 5 IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC. IFN/R-DC, IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture,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

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Sequential activation of innate and adaptive immune response is crucial for virus elimination. We thus sought to clarify the role of innate immune system in the pathogenesis of hepatitis C virus (HCV) infection. Dendritic cells (DC) sense virus infection via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I), resulting in the secretion of type-I interferons (IFN) and inflammatory cytokines. Blood DC consist of two subsets; myeloid DC (MDC) and plasmacytoid DC (PDC). In MDC from HCV-infected patients, regardless of higher expression of TLR2, TLR4 and RIG-I compared to the controls, the levels of TLR/RIG-I-mediated IFN-β or TNF-α induction are lower than those in uninfected donors. These results suggest that the signal transduction in the downstream of TLR/RIG-I in MDC is profoundly impaired in HCV infection. In response to IFN-α, DC are able to express MHC class-I related chain A/B (MICA/B) and activate natural killer (NK) cells following ligation of NKG2D. Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells. Alternatively, NK cells from HCV-infected patients downregulate DC functions in the presence of human leukocyte antigen E-expressing hepatocytes by secreting interleukin (IL)-10 and transforming growth factor-β1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts. Invariant NKT cells activated by CD1d-positive DC secrete both T-helper (Th)1 and Th2 cytokines, serving as immune regulators. The frequency of NKT cells in chronic HCV infection does not differ from those in healthy donors. Activated NKT cells produce higher levels of IL-13 but comparable levels of IFN-γ with those from healthy subjects, showing that NKT cells are biased to Th2-type in chronic HCV infection. In conclusion, cross-talks among DC, NK cells and NKT cells are critical in shaping subsequent adaptive immune response against HCV.

Key words: α-galactosyl-ceramide, IL-β, MICA/B, NKG2A, TLR

INTRODUCTION

HEPATITIS C VIRUS (HCV) is one of major causes of chronic liver disease worldwide. HCV is hepatotrophic, but not directly cytopathic and elicits progressive liver injuries resulting in end-stage liver disease unless effectively eradicated.[1] Epidemiological studies have revealed that more than 80% of acutely HCV-infected patients fail to eradicate the virus and they subsequently develop chronic hepatitis.[1] It has been proposed that the ability of infected hosts to mount vigorous and sustained cellular immune reactions to HCV is necessary for control in primary infection.[2] Once HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection. First, the virus alters its antigenic epitopes recognized by T cells and neutralizing antibodies to escape immune surveillance. Second, HCV also subverts immune functions in an antigen-specific manner, from innate to adaptive immunity.[3]

Cumulative reports have shown that innate immune system dictates the direction and magnitude of subsequent adaptive immune response. It is generally accepted that HCV-specific $CD8^+$ T cells are responsible for HCV elimination by inducing hepatocyte apoptosis.[2] Innate immune cells, including natural killer (NK) cells and

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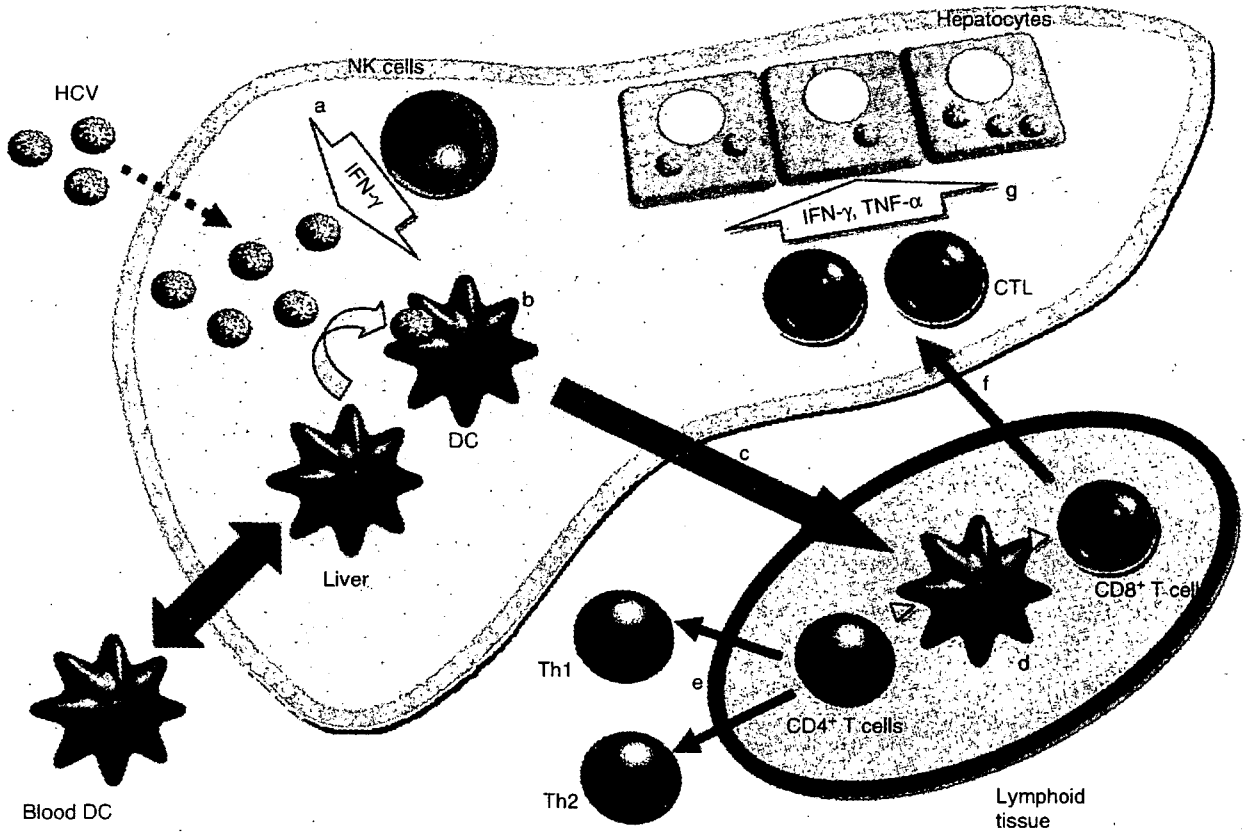

Figure 1 Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HCV, hepatitis C virus; NK, natural killer cell; Th, helper T cell. (1–7), see text.

NKT cells, may contribute to HCV eradication after primary infection; however, their roles in a chronically infected state remain elusive. Because dendritic cells (DC) orchestrate anti-HCV immune response by linking innate and adaptive arms of the immune system,[4] functional impairment of DC leads to failure of NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells. Infiltration of disabled $CD8^+$ T cells to the infected liver may result in weak liver inflammation that is not sufficient for HCV eradication.[5]

In this paper, we discuss the current understandings of the roles of innate immunity in the pathogenesis of HCV infection, especially focused on interferons (IFN), DC, NK cells and NKT cells.

KEY PLAYERS IN IMMUNE RESPONSES TO VIRAL HEPATITIS

AFTER HCV INFECTS the liver, viral replication continues and viral particles are continuously released into the circulation. The first lines of defense are provided by NK and NKT cells, of which populations are relatively increased in the liver compared to the periphery. These cells are activated in the liver, where expression of IFN-α and IFN-inducible genes are extremely high during the early phase of hepatitis virus infection.[6] Activated NK and NKT cells secrete IFN-γ, which inhibits replication of HCV through a non-cytolytic mechanism (Fig. 1a).[7]

Dendritic cells or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Fig. 1b).[4] Because DC express distinct sets of toll-like receptors (TLR),[8] it is likely that some viral components stimulate DC through cytosolic ligation of TLR. DC develop a mature phenotype and migrate to lymphoid tissues (Fig. 1c), where they stimulate effectors, including T cells and B cells (Fig. 1d). Following the encounter of DC with other cells, DC secrete various cytokines (interleukin [IL]-12, tumor necrosis factor [TNF]-α, IFN-α and IL-10) instructing or regulating the functions of the adja-

cent cells.[4] In addition to these cytokines, DC express various costimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported; that is, myeloid DC (MDC) and plasmacytoid DC (PDC).[9] MDC predominantly produce IL-12 or TNF-α following proinflammatory stimuli, while PDC release a considerable amount of IFN-α upon virus infection depending on the immune stimulus; both cytokines in actuality can be made by both cells. Helper T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTL) generation (T-helper [Th]1 with secretion of IL-2, IFN-γ and TNF-α) or B-cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Fig. 1e). DC ontogeny and DC-derived cytokines are crucially associated with the polarization of helper T-cell subsets.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific $CD4^+$ T cells in HCV eradication has been well described during both acute or chronic infection.[10] However, there is little evidence that $CD4^+$ T cells mediate direct liver cell injury in HCV infection. Thus, it is likely that $CD4^+$ T cells play a critical role in facilitating other antiviral immune mechanisms, such as enhancing $CD8^+$ effector function. The antigen-primed CTL recruit to the liver (Fig. 1f) and constitute the critical element in the eradication of virus-infected cells (Fig. 1g).

INNATE IMMUNITY IN HCV INFECTION

Toll-like receptors and retinoic acid inducible gene-I as sensors for virus infection

GENE EXPRESSION ANALYSES in HCV-infected liver revealed that HCV triggers expression of type I IFN and IFN-induced genes during primary infection regardless of the outcomes.[6] However, the HCV viral load does not decrease in the early phase, suggesting that HCV impedes the execution of antiviral machineries. Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing antiviral proteins, such as interferon regulatory factor (IRF)-3,[11] nuclear factor (NF)-κB and double-stranded RNA-dependent protein kinases (PKR).[12] Mammalian TLR sense some pathogen-associated molecular patterns embedded in virus components and then induce inflammatory cytokines or type-I IFN, resulting in the augmentation of antivirus immune reactions.[8] Retinoic acid inducible gene-I (RIG-I) is a cytosolic molecule that senses double-stranded RNA (dsRNA) of virus replicative intermediate, which subsequently activates IRF-3 and NF-κB pathways.[13] By using the HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4 A proteins influence the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN-β as well as subsequent IFN-stimulated genes.[14,15] However, it is yet to be proven whether the results obtained from HCV replicon are applicable or not for HCV-infected individuals.

To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and functions in MDC and PDC between patients and donors. In MDC from HCV-infected patients, TLR2, TLR4 and RIG-I expression were significantly higher than those in healthy counterparts. Of particular interest, regardless of the higher expressions, specific agonists for these sensors stimulated patients' MDC to induce lesser amounts of IFN-β and TNF-α compared to donor MDC (Miyazaki *et al.*, 2007, unpublished data). These results show that the signal transduction via these receptors is strongly impeded in HCV infection. Inconsistent with the findings of MDC, we previously reported that TLR2 expression on monocyte-derived DC (MoDC) in chronic hepatitis C is lower than those in healthy donors.[16] Because MoDC is an *in vitro*-generated DC mimic, the opposite results of TLR2 in HCV infection might be explained by impaired ability of MoDC to mature in response to cytokines, as reported elsewhere.[17] Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

Blood DC subsets

Impaired antigen presentation by DC might be involved in the failure of the maintenance of sustained HCV-specific T-cell response. MoDC generated from hepatitis C patients have an impaired ability to stimulate allogeneic $CD4^+$ T cells.[18,19] Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability.[18] In addition to *in vitro*-generated DC, the alterations in number and function of circulating blood DC have been reported in HCV infection.[20]

Direct HCV infection of DC might be one of the plausible mechanisms of DC dysfunction in chronic hepatitis C. The HCV genome has been reported to be isolated

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

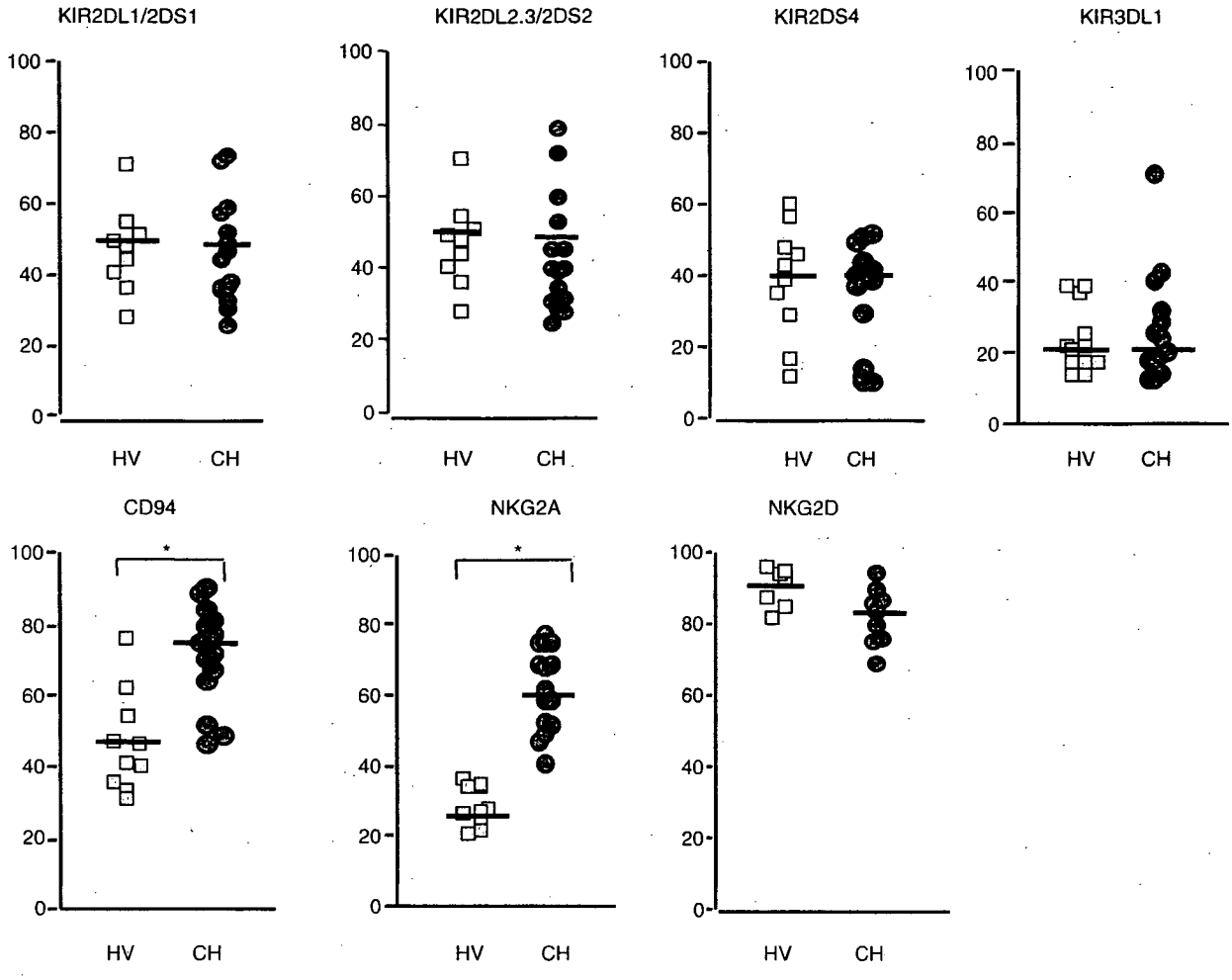

Figure 2 Expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. Percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. *$P < 0.05$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

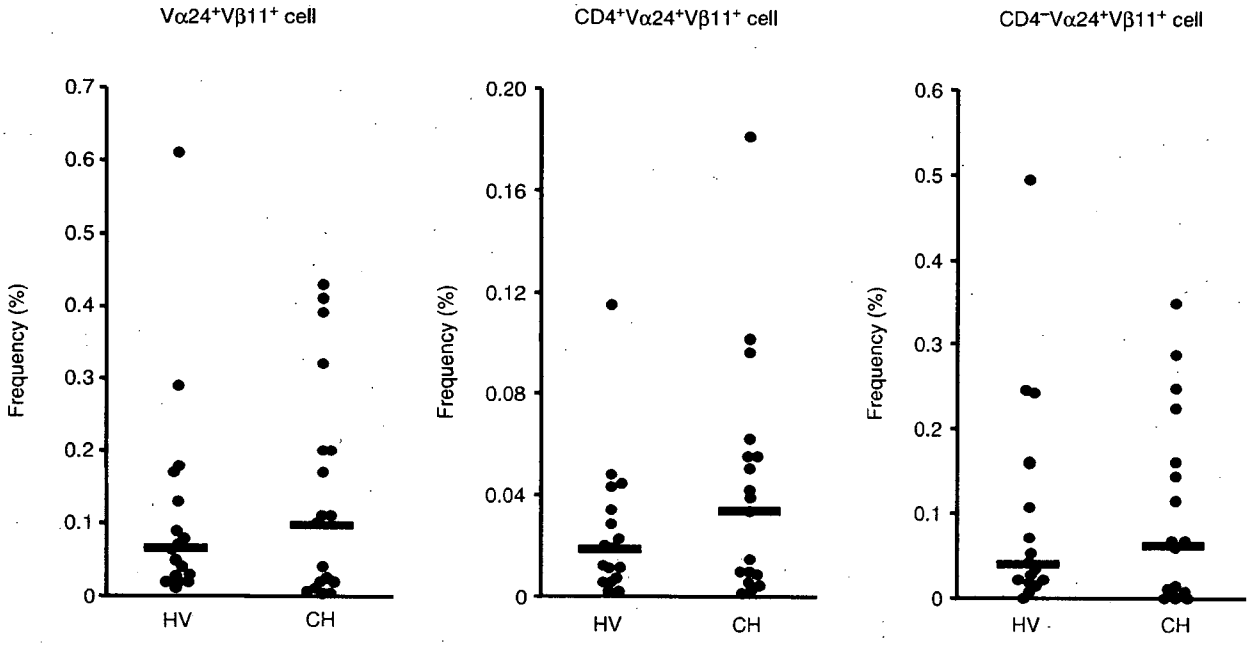

Figure 3 Frequency of peripheral invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. The frequencies of total invariant NKT (iNKT) cells ($V\alpha24^+V\beta11^+$ cells), $CD4^+$ and $CD4^-$ iNKT cells in PBMC were determined by flow cytometry. HV, healthy volunteers; CH,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

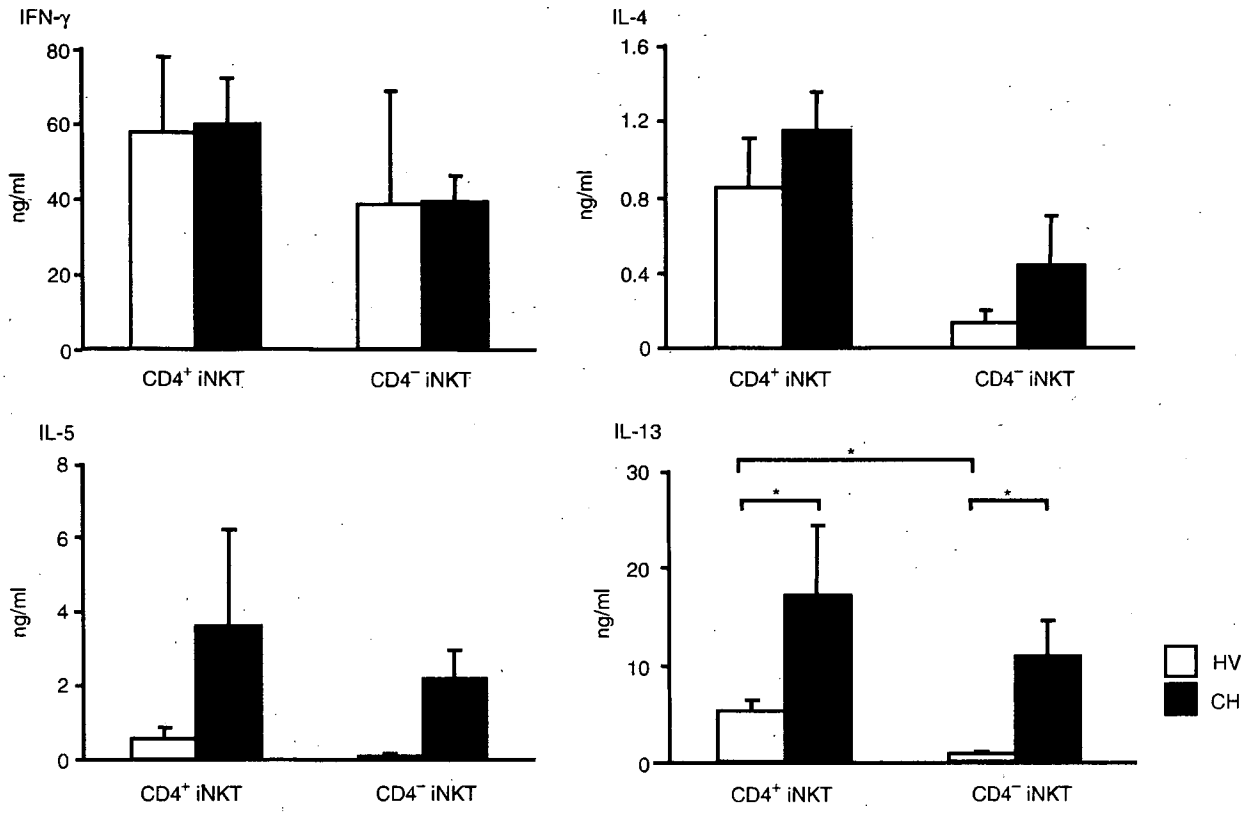

Figure 4 Cytokine production from expanded invariant NKT cells stimulated with α-galactosyl-ceramide (αGalCer)-loaded monocyte-derived DC. Invariant NKT (iNKT) cells were expanded by culture with αGalCer-pulsed autologous monocyte-derived DC (MoDC) and subsequent cell sorting. Activated iNKT cells were 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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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 $< 0{\cdot}05$.

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

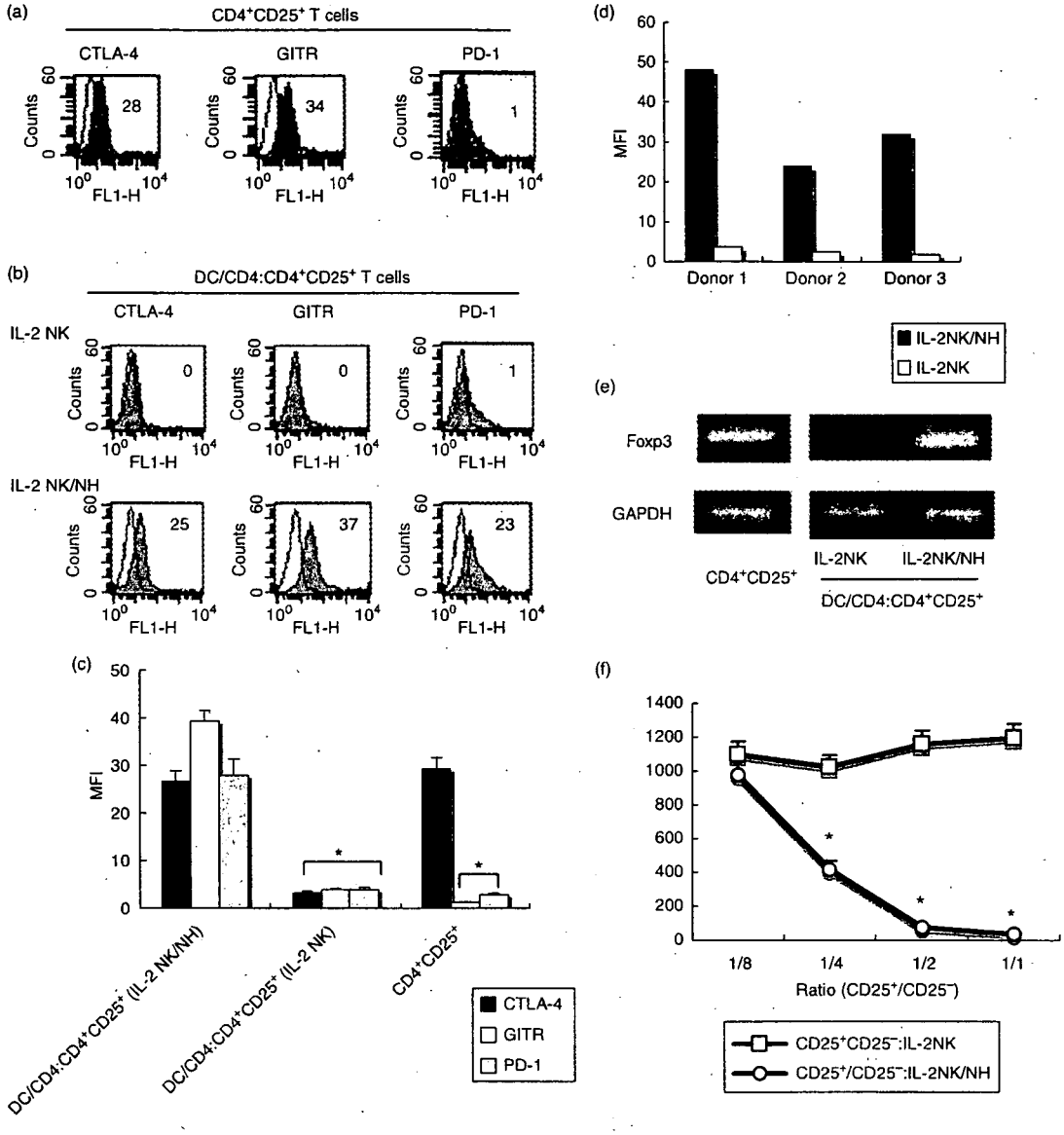

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) 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. $^*P < 0.05$ 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. $^*P < 0.05$.

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.

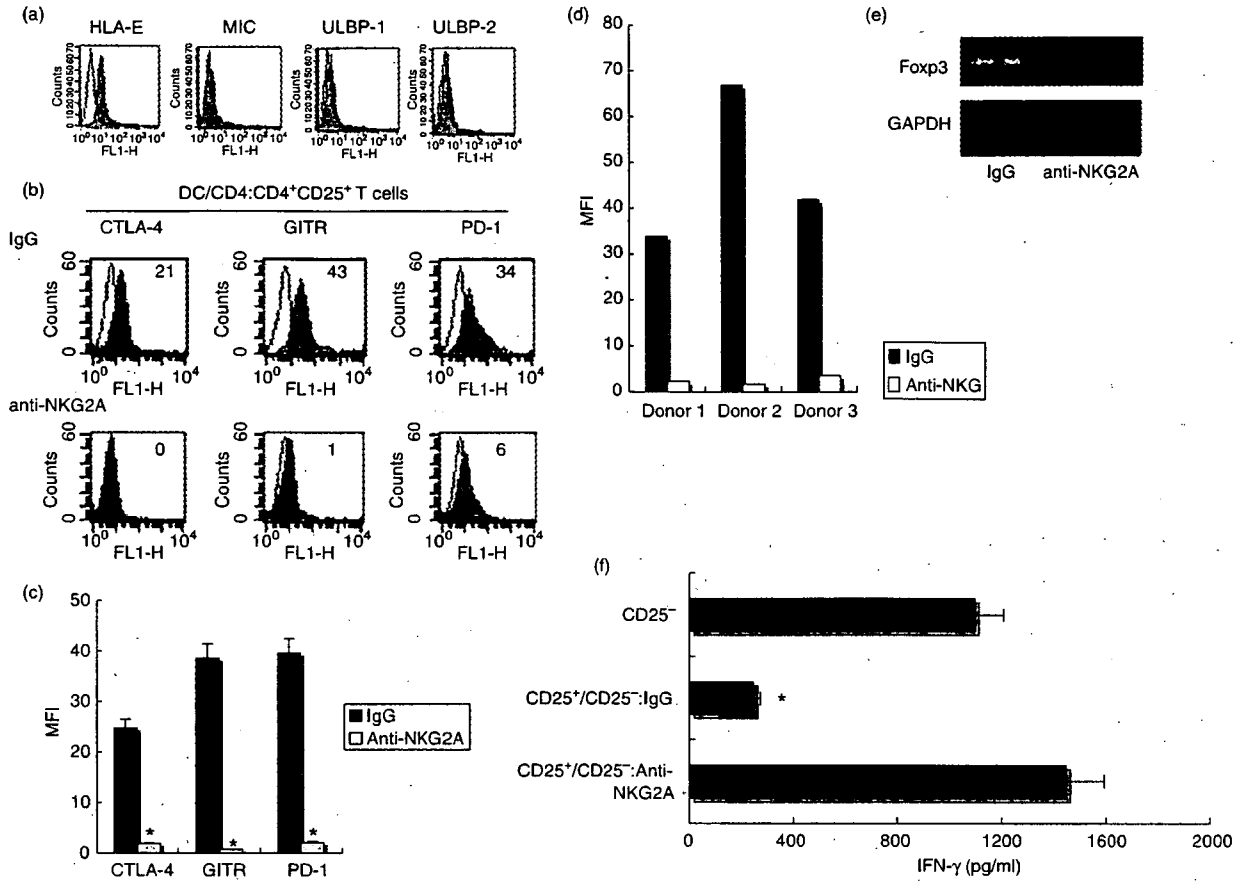

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) 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