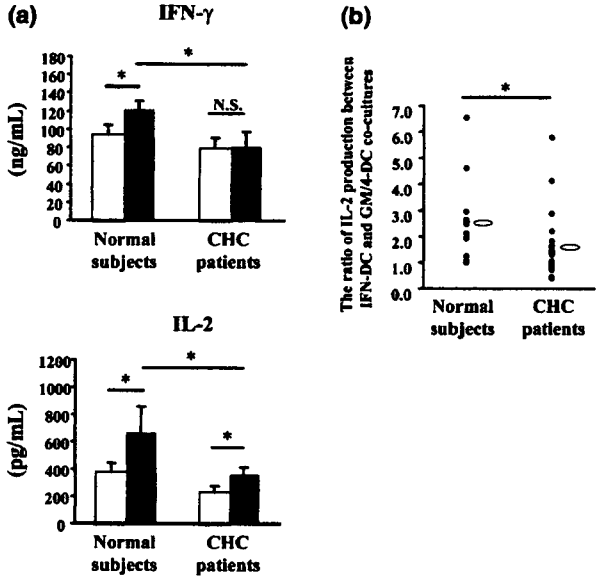

Fig. 2 Interferon-dendritic cells (IFN-DC) from hepatitis C virus-infected patients displayed the impaired capacity of inducing Th1 compared with those from normal subjects. (a) IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ cells for 6 days as described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

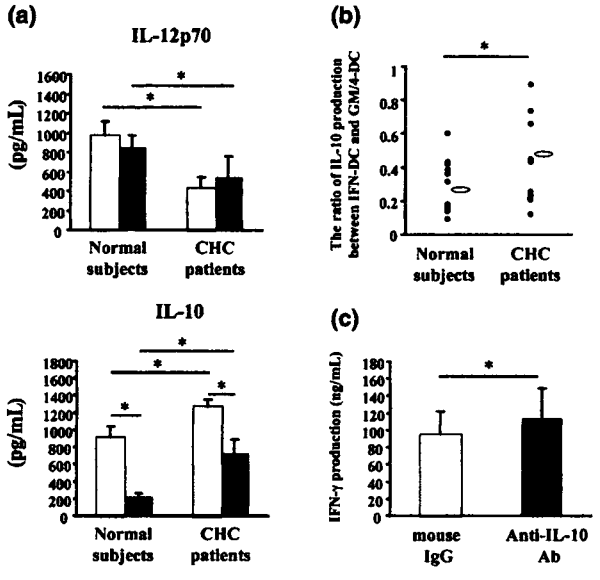

Fig. 3 The lesser IL-10 production from interferon-dendritic cells (IFN-DC) compared with GM/4-DC may be involved in Th1 induction. (a) Day 7 MoDCs were stimulated with CD40L-L-cells for 24 h. The concentrations of the IL-12p70 and IL-10 in the supernatants were determined by ELISA. Results are expressed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

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

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

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

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

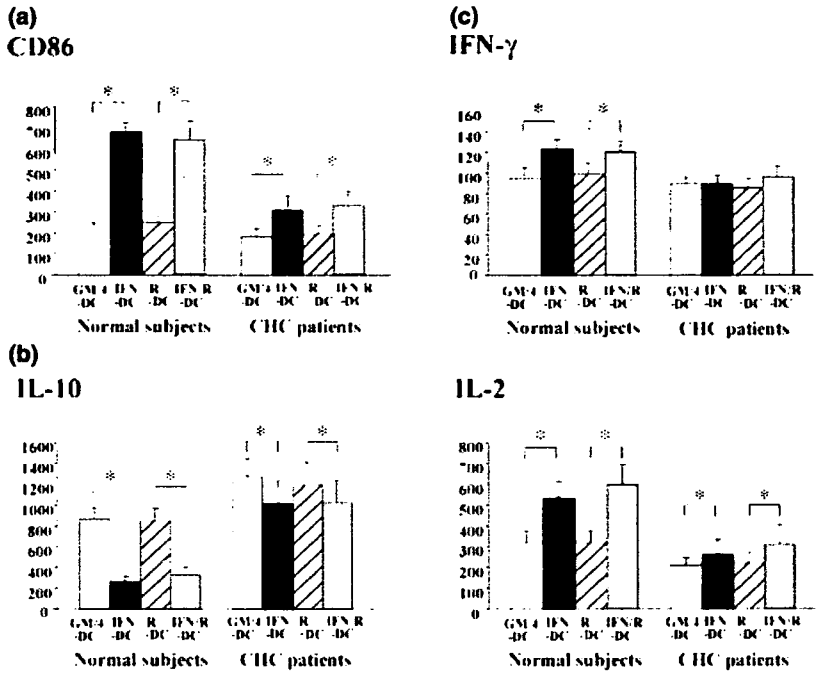

Fig. 4 Ribavirin did not significantly alter the phenotypes and functions of DC either used alone or used in combined with IFN-α. (a) GM/4-DC, IFN-DC, R-DC and IFN/R-DC were generated and analysed their CD86 expressions by fluorescence-activated cell sorter. The figures represent the mean values of MFI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

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

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

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

DISCUSSION

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

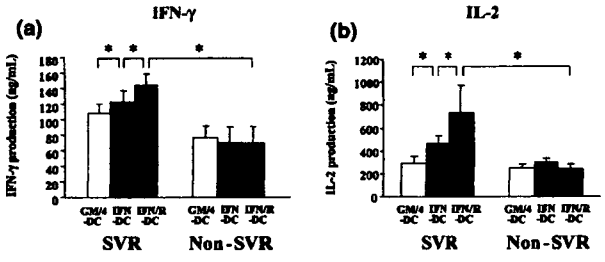

Fig. 5 IFN/R-DC from CHC patients in the SVR group induce more potent Th1 response compared with IFN-DC or GM/4-DC. IFN/R-DC, IFN-DC and GM/4-DC were generated and were cultured with allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for 6 days as described in Materials and methods. On day 4 of the co-culture,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

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

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

It is well known that DC-derived IL-12 and IL-10 may be involved in Th1 and Th2 polarization, respectively. Thus, the lesser amount of IL-12p70 from the patient' DC may be related to the lesser degree of DC-primed Th1 response in CHC patients than those in donors (Fig. 3a). What remains unknown is how the reduced IL-10 production of DC leads to the enhanced ability of DC to induce a Th1 response. IL-10 is an important key player in the pathogenesis of HCV infection, being induced by various HCV antigens [38]. Moreover, DC functions can be modulated by autocrine IL-10, which is implicated in the enhanced ability to induce Th1 response [39]. The blocking experiments using anti-IL-10 neutralizing Ab including those of our present study revealed that the inhibition of endogenous IL-10 in DC/T cell co-culture enables an increase of the Th1 response [39,40], which may be associated with the relatively enhanced activity of co-existing IL-12p70. Such a reciprocal IL-12 increase and subsequent Th1 augmentation has been observed in DC in which the IL-10 gene had been knocked down by small interference RNA [41]. However, in the present study, the IL-12 levels did not differ between the samples treated with anti-IL-10 Ab and those without it (data not shown). Thus, other DC-derived Th1-inducing cytokines, including IL-27 and IL-23 [42], may be involved in the IFN-DC-induced Th1 response, the possibility of which needs to be further evaluated.

Ribavirin has broad-spectrum activities against both DNA and RNA viruses, however, its mechanism of action for the treatment of HCV is not fully understood. Given that ribavirin has little direct activity against HCV [43–45], a number of studies have shown that ribavirin can modulate immune response by altering the Th1/Th2 bias [14,15,46]. With regard to DC, it has been previously reported that ribavirin alters cytokine production from DC [30]. However, it remains unclear whether or not ribavirin could affect Th1-driving capacity of DC. In the present study, when we analysed the patients as a whole, no additive effect was obtained with ribavirin in phenotypes and functions of DC generated with or without IFN-α. However, when the analyses had been done separately in the SVR patients and non-SVR ones, IFN/R-DC from the SVR group induced more potent Th1 response compared with IFN-DC or GM/4-DC, of which difference was not observed in the non-SVR group. In addition, the levels of IFN-γ and IL-2 released from IFN/R-DC-primed T cells were significantly higher in the SVR group than those in the non-SVR group. It is thus speculated that such better *in vitro* DC response to IFN-α and ribavirin is associated with better *in vivo* virological response in the combination therapy, as the enhancement of HCV-specific Th1 response is necessary for the clearance of HCV by IFN-α and ribavirin combination therapy. As described above, one of the mechanisms of the impairment in IFN-α-stimulated DC in HCV infection is an insufficient alteration of CD86 expression and IL-10 production. However, the addition of ribavirin to IFN-α failed to improve CD86 expression and reduce IL-10

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

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

ACKNOWLEDGEMENTS

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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

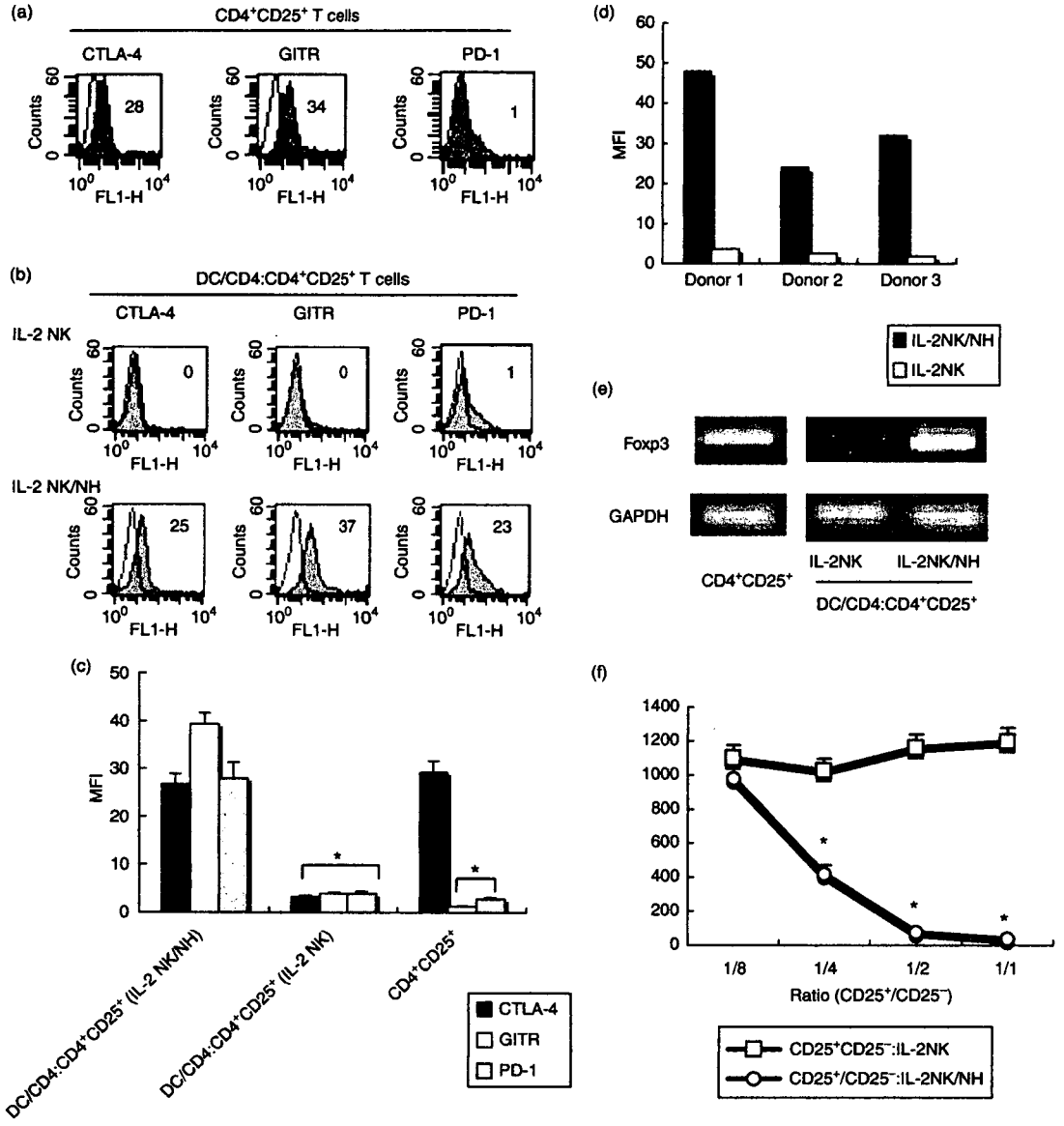

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab)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

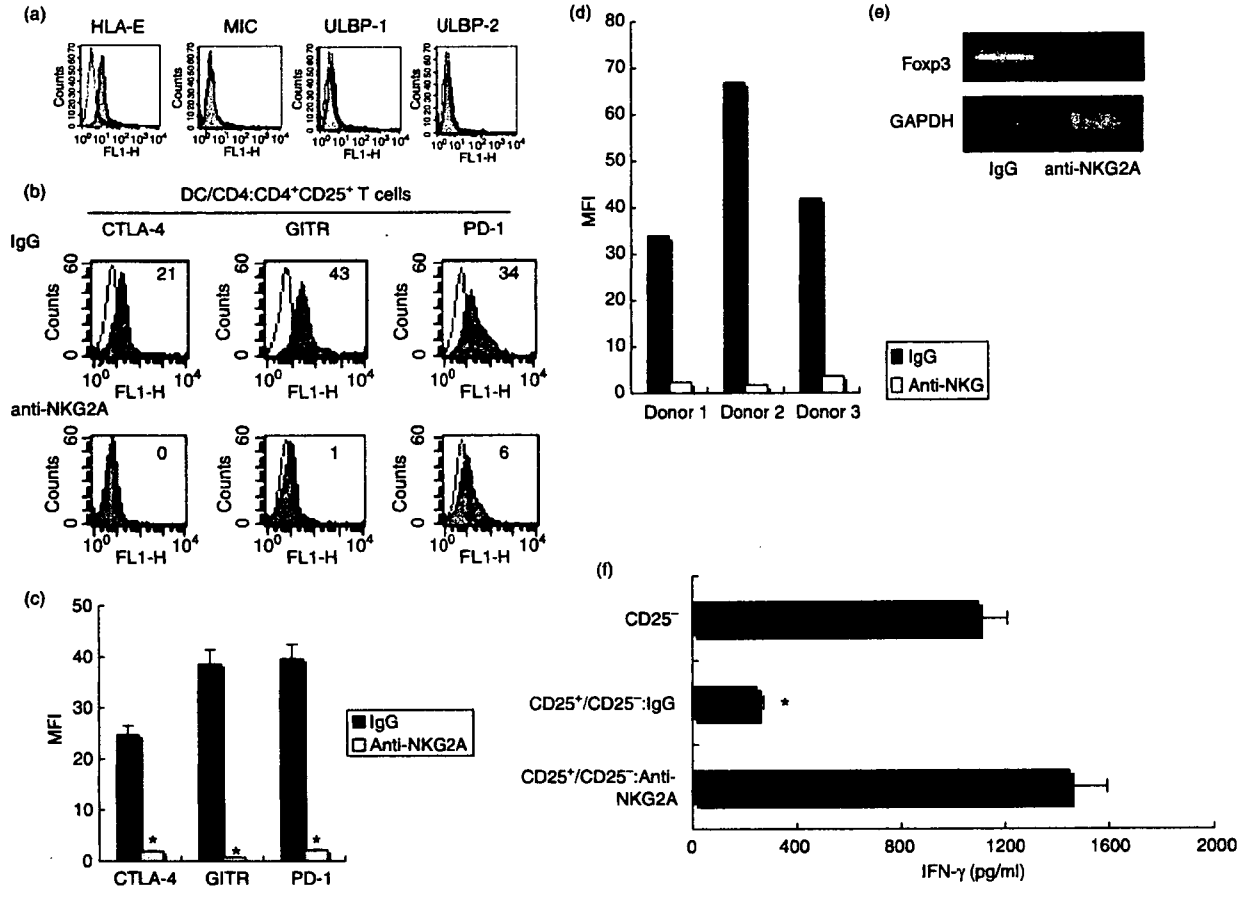

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) 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. $^*P < 0.05$ 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). $^*P < 0.05$. 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

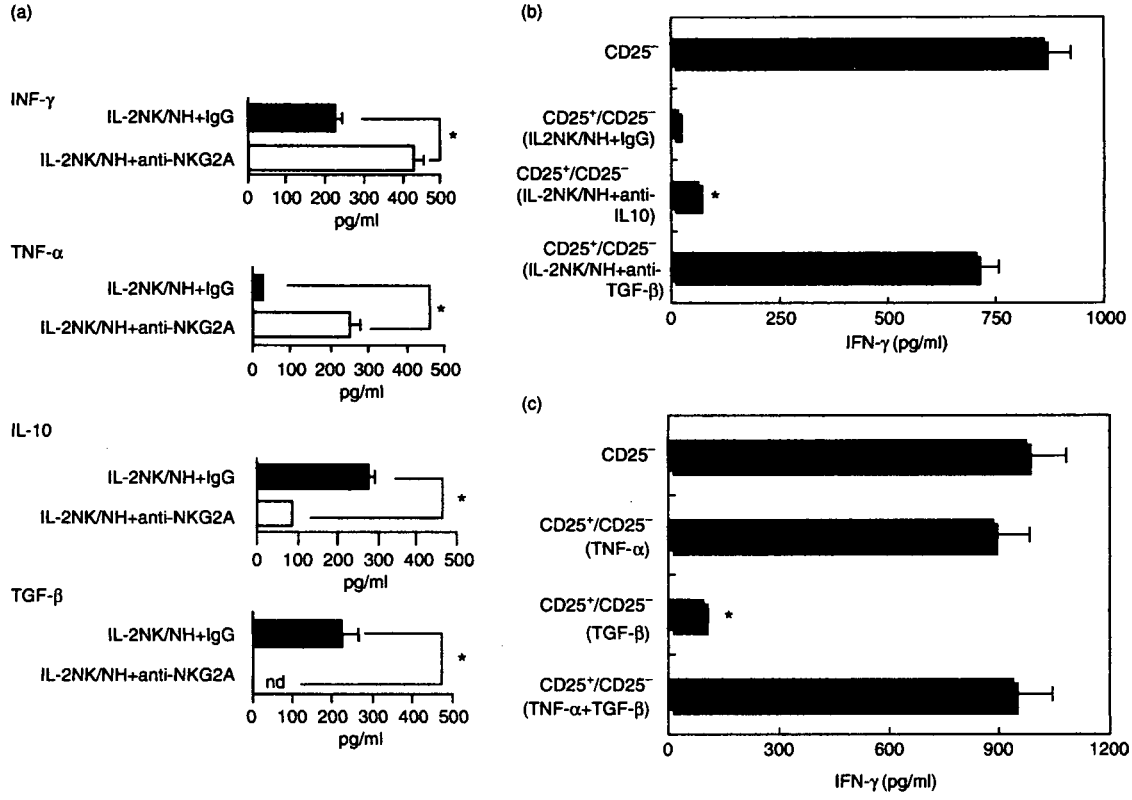

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the 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. $^*P < 0.05$. (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. $^*P < 0.05$ 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. $^*P < 0.05$ 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

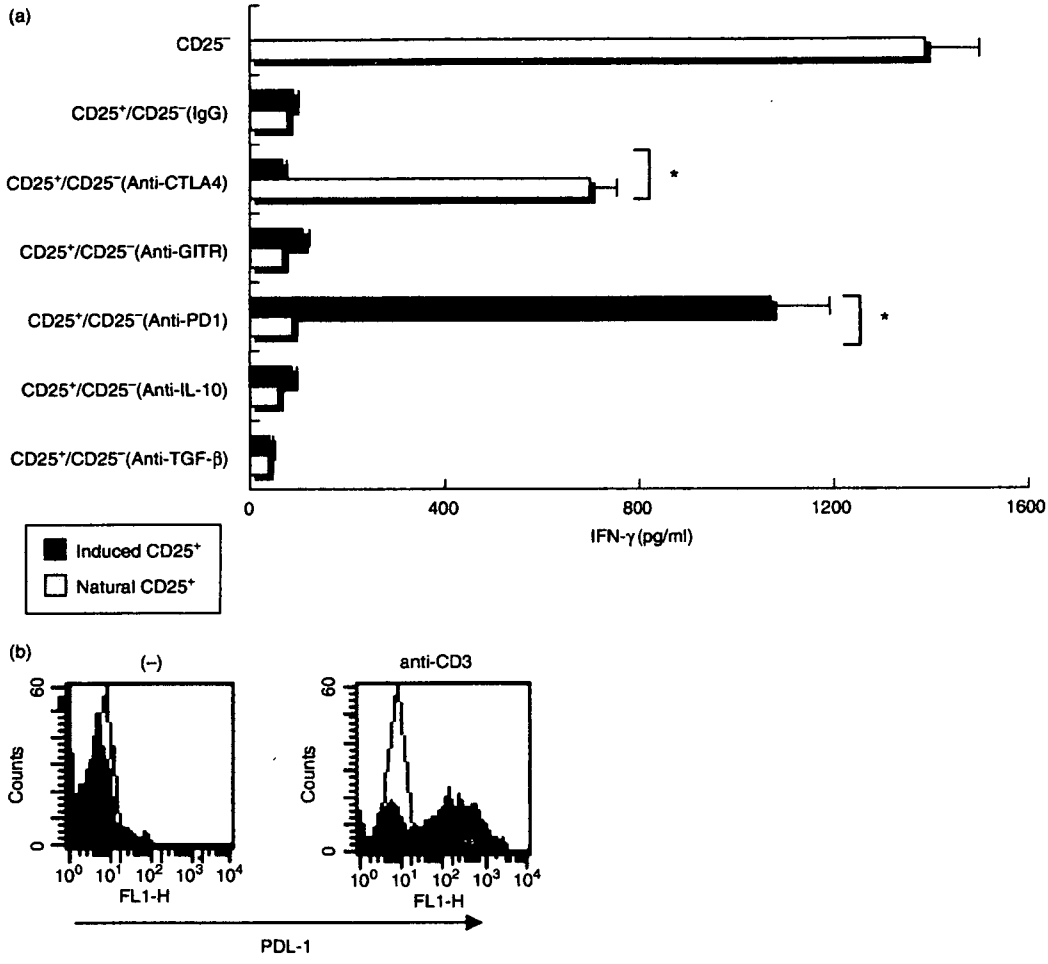

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with 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. $^*P < 0.05$ 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

signals that profoundly affect DC functions towards $CD4^+$ $CD25^+$ Treg cells. Because NK cell functions are regulated by the balance between inhibitory and activating signals, any future clarification of the role of other NK inhibitory and activating receptors in DC modulation and Treg cell activation will be of great interest.

The cross-presentation of self-antigens by major histocompatibility complex (MHC) class II pathways constitutes an important step towards generating and/or expanding peripheral Treg cells.[30] However, we initially settled our experimental design by using DCs and Treg cells from different donors, and DCs encountered $CD4^+$ T cells in an 'antigen-free' condition. Therefore, Treg cells induced by NK/NH-primed DCs are generated independently of MHC class II-mediated self-antigen recognition. These results give rise to the possibility that the cross-talk of NK cells, DCs and hepatocytes represents an alternative pathway in the generation and expansion of peripheral Treg cells. However, it should be noted that these results may not apply to all donors because of the complexity of the allogeneic system and the relatively few donors tested.

PD-1-mediated suppressive activities were characteristic for $CD4^+$ $CD25^+$ Treg cells generated by NH/IL-2 NK-primed DCs. By contrast, natural $CD4^+$ $CD25^+$ Treg cells exerted their suppressive function, at least in part, in a CTLA-4-dependent fashion. Recent reports have clarified the existence of two subtypes of Treg cells: natural and inducible $CD4^+$ $CD25^+$ Treg cells. Inducible Treg cells exert suppressive activities by using molecular mechanisms distinct from those of natural regulatory cells.[31] Our findings further identify the novel pathways by which inducible $CD4^+$ $CD25^+$ Treg cell activities triggered by NKG2A inhibitory signals are dependent on PD-1-mediated negative costimulation. A recent report identified the interaction of B7 on effector T cells with costimulatory molecules CD28/CTLA-4 on $CD4^+$ $CD25^+$ Treg cells as molecular mechanisms of their suppressor activity.[32] Thus, it is possible that reverse signalling of PDL-1 on effector cells may also be crucial for the negative costimulator-mediated suppressive action of $CD4^+$ $CD25^+$ Treg cells. In the present study, we did not address the mechanisms by which NH/IL-2 NK-primed DCs induce $CD4^+$ $CD25^+$ Treg cells with PD-1-dependent suppressive functions. Further study will be needed to clarify this issue.

We previously showed that NKG2A is expressed at higher levels from NK cells isolated from peripheral blood in patients with chronic hepatitis C virus (HCV) infection than from those in healthy donors.[20] HCV frequently persists in humans, at least in part, due to inefficient induction of NK activity as well as specific T cell responses.[33–35] The small percentage of patients who spontaneously clear the virus and recover from chronic hepatitis C mount vigorous HCV-specific $CD4^+$ and $CD8^+$ T cell responses.[36,37] Research has described an increased frequency of $CD4^+$ $CD25^+$ T cells in the blood of patients with persistent HCV infection compared with those who have spontaneously cleared HCV.[38,39] Our current findings raise the interesting possibility that increased NKG2A expression on NK cells may lead to DC-mediated induction of Treg cells, leading to the inhibition of adaptive responses to HCV and failure to eliminate this virus. Indeed, $CD4^+$ $CD25^+$ T cells induced by HCV-NK/Hep3B hepatoma cell-primed DCs expressed and suppressed effector T cell functions at greater levels than those induced by N-NK/Hep3B-primed DCs (our unpublished data). Interestingly, a recent study identified PD-1-mediated signals as a critical pathway to induce anergic $CD8^+$ T cells and impair antiviral CTL responses in chronic viral infection.[40] In this regard, the therapeutic modification of the PD-1 pathway may synergistically augment antiviral immunity by suppressing Treg activity and recovering CTL responses. It is important to establish whether the PD-1 pathway in liver lymphocytes may be operable *in vivo* and play a critical role in suppression of virus-specific immunity in HCV infection.

In conclusion, we have demonstrated that interaction of NK cells and hepatic cells via NKG2A leads to DC induction of $CD4^+$ $CD25^+$ T cells with PD-1-dependent regulatory activities. These findings also imply that NK receptor signals of NK cells may dictate DC-mediated adaptive immune responses towards tolerogenic or immunogenic status via induction of Treg cells.

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Scavenger Receptor Class B Type I Is a Key Host Factor for Hepatitis C Virus Infection Required for an Entry Step Closely Linked to CD81

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Scavenger receptor class B type I (SR-BI) has been shown to bind HCV envelope glycoprotein E2, participate in entry of HCV pseudotype particles, and modulate HCV infection. However, the functional role of SR-BI for productive HCV infection remains unclear. In this study, we investigated the role of SR-BI as an entry factor for infection of human hepatoma cells using cell culture–derived HCV (HCVcc). Anti–SR-BI antibodies directed against epitopes of the human SR-BI extracellular loop specifically inhibited HCVcc infection in a dose-dependent manner. Down-regulation of SR-BI expression by SR-BI–specific short interfering RNAs (siRNAs) markedly reduced the susceptibility of human hepatoma cells to HCVcc infection. Kinetic studies demonstrated that SR-BI acts predominately after binding of HCV at an entry step occurring at a similar time point as CD81–HCV interaction. Although the addition of high-density lipoprotein (HDL) enhanced the efficiency of HCVcc infection, anti–SR-BI antibodies and SR-BI–specific siRNA efficiently inhibited HCV infection independent of lipoprotein. *Conclusion:* Our data suggest that SR-BI (i) represents a key host factor for HCV entry, (ii) is implicated in the same HCV entry pathway as CD81, and (iii) targets an entry step closely linked to HCV–CD81 interaction. (HEPATOLOGY 2007;46: 1722-1731.)

Abbreviations: cDNA, complementary DNA; CHO, Chinese hamster ovary; HCV, hepatitis C virus; HCVcc, cell culture–derived HCV; HCVpp, HCV pseudotype particles; HDL: high-density lipoprotein; HRP, horseradish peroxidase; IgG, immunoglobulin G; LDL: low-density lipoprotein; LPDS, lipoprotein-deficient human serum; siRNA: small interfering RNA; SR-BI: scavenger receptor class B type I.

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With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health.[1] The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Attachment of the virus to the cell surface followed by viral entry is the first step in a cascade of interactions between the virus and the target cell that is required for successful entry into the cell and initiation of infection.[2] Using recombinant HCV envelope glycoproteins[3] and HCV pseudotype particles (HCVpp),[4,5] several cell surface molecules have been identified interacting with HCV during viral binding and entry. These include the tetraspanins CD81[3] and claudin-1,[6] highly sulfated heparan sulfate,[7] the low-density lipoprotein (LDL) receptor,[8] and scavenger receptor class B type I (SR-BI).[9]

SR-BI is a 509–amino acid glycoprotein with a large extracellular loop anchored to the plasma membrane at both the N- and C- termini by transmembrane domains with short extensions into the cytoplasm.[10] SR-BI is involved in bidirectional cholesterol transport at the cell membrane and can bind both native high-density lipoprotein (HDL) and LDL as well as modified lipoproteins such as oxidized LDL. SR-BI is highly expressed in liver and steroidogenic tissues[10] as well as antigen-presenting cells.[11] Furthermore, SR-BI and its splicing variant SR-BII, have been found to mediate binding and uptake of a broad range of bacteria into human epithelial cells overexpressing SR-BI and SR-BII,[12,13] suggesting that class B scavenger receptors may serve as pattern recognition receptors for bacteria.

Cross-linking studies using recombinant C-terminally truncated HCV envelope glycoprotein E2 isolated SR-BI as a cellular protein binding envelope glycoprotein E2.[9] Antibodies directed against cell surface expressed SR-BI partially inhibited cellular binding of recombinant envelope glycoproteins[14] as well as HCVpp entry.[15-17] Moreover, it has been shown that physiological SR-BI ligands, such as HDL or oxidized LDL, can modulate HCV infection either by enhancing or by inhibiting HCVpp entry, respectively.[18-20]

Recently, several laboratories succeeded in establishing a model for the efficient production of infectious HCV particles in cell culture (HCVcc),[21-23] now allowing determining of the role of cell surface molecules involved in HCV infection. Recent evidence suggests that SR-BI and CD81 may act in a cooperative manner for the initiation of HCVcc infection[24] and that overexpression of SR-BI can modulate HCVcc infection.[25] However, the functional role of SR-BI in productive HCV infection still remains elusive. In particular, it is unclear whether the impact of SR-BI for HCV entry is of key importance or optional, whether SR-BI and CD81 are involved in the same pathways of HCV entry, and which HCV entry step is targeted by SR-BI.

Therefore, in this study, we used the HCVcc system to analyze the functional role of SR-BI for productive HCV infection of human hepatoma cells. Using novel anti–SR-BI antibodies and SR-BI–specific short interfering RNAs (siRNAs), we demonstrate that SR-BI (i) represents a key host factor for HCV entry, (ii) is most likely implicated in the same HCV entry pathway as CD81, and (iii) mediates an entry step occurring postbinding and closely linked to HCV–CD81 interaction.

Materials and Methods

Cells. Human embryonic kidney cells 293T, Chinese hamster ovary cells CHO, and Huh7.5 have been described.[4,7,26,27] Primary human hepatocytes were isolated and cultured as described.[28]

Antibodies. Antibodies directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice using a pcDNA-expression vector containing the full-length human SR-BI complementary DNA (cDNA) (pcDNA SR-BI/CLA-1) (Genovac GmbH, Freiburg, Germany).[29] In brief, animals received 4 applications of 50 μg pcDNA SR-BI intradermally using a GeneGun (BioRad) at 2-week intervals. Pre-immune control serum was collected from the same animal bled before immunization. To analyze specificity of the produced anti–SR-BI polyclonal serum, CHO cells were transfected with pcDNA (control vector) or pcDNA SR-BI using liposome-mediated gene transfer (Lipofectamine; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. CHO cells were then incubated with anti–SR-BI polyclonal serum or pre-immune control serum and analyzed for cell surface SR-BI expression by flow cytometry as described.[14] R-phycoerythrin–conjugated goat anti-rat immunoglobulin G (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Because of the small amounts of pre-immune sera from individual animals used for immunization, commercially available nonimmune rat serum (PAN Biotech) was used as an additional negative control serum for experiments. Rabbit anti–SR-BI antibody (NB 400-104) was obtained from Novus Biologicals (Littleton, CO). This antibody is directed against an epitope within the SR-BI cytoplasmic C-terminal domain (CSPAAKGTVLQEAKL, corresponding to amino acids 496 through 509). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG antibodies were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), mouse anti–

β-actin antibody was from Sigma and anti-CD81 (JS-81) from BD Biosciences (Heidelberg, Germany).

RNA Interference Assay. Commercially available siRNA pools targeting SR-BI, CD81, and CD13 as well as control nontargeting siRNAs were purchased from Dharmacon (Pierce) and transfected into Huh7.5 cells using DharmaFect solution (Pierce) following the manufacturer's protocol. Silencing of SR-BI expression was assessed by western blot and flow cytometry 72 hours after transfection as described previously.[14] Seventy-two hours after transfection, cells were incubated with HCVcc, and HCV infection was assessed as described in the following sections.

Production of Viral Stocks and Infection Assays. Plasmids pJFH1, pFK-Jc1, and pFK-Luc-Jc1 have been described[21,30,31] and encode the full-length HCV Japanese fulminant hepatitis (JFH) cDNA or the chimeric HCV genome designated Jc1, which consists of J6CF and JFH1 segments. The latter construct (pFK-Luc-Jc1) represents a bicistronic reporter virus carrying a firefly-luciferase reporter gene.[30] *In vitro* HCV RNA synthesis[30] and RNA transfection was performed as described.[21,29] To study the effect of HDL on HCVcc infection, JFH1 HCVcc were also generated in lipoprotein-deficient human serum (LPDS), and HDL (30 μg/mL)[33] was added extemporaneously for infection experiments. Culture supernatants from transfected cells were cleared and concentrated as previously described using Amicon Ultra 15 (Millipore, Billerica, MA)[21] and used directly or stored at 4°C or −80°C. Viruses were titered by using the limiting dilution assay on Huh7.5 cells with a few minor modifications, and 50% tissue culture infective dose was calculated based on the method described.[22] siRNA expressing cells and naïve cells were seeded 24 hours before infection experiments in 12-well tissue culture plates at a density of 5×10^4 cells/well. Cells were preincubated in the presence or absence of anti–SR-BI serum or control serum for 1 hour at 37°C and then infected at 37°C for 3 hours with JFH1 HCVcc challenge virus titers ranging from 1×10^7 to 5×10^9 copies/mL or 4 hours with Luc-Jc1 HCVcc at low multiplicity of infection. Alternatively, Huh7.5 were inoculated with a high-titer Luc-Jc1 stock for 1 hour at 4°C in the presence or absence of anti–SR-BI serum or control serum, heparin, anti-CD81 monoclonal antibodies, or concanamycin A at concentrations indicated in the text. Subsequently, cells were washed 3 times with ice-cold phosphate-buffered saline, supplied with fresh culture fluid prewarmed to 37°C and supplemented with the respective inhibitors and shifted to 37°C. Finally, 4 hours later, cells were washed with prewarmed phosphate-buffered saline, supplied with fresh culture fluid without inhibitors, and cultured an additional 48 hours at 37°C. Depending on the experiment, cells were then washed with ice-cold phosphate-buffered saline and RNA extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). Alternatively, cells were lysed for luciferase assay as previously described.[30] HCV RNA was quantitated using VERSANT HCV-RNA 3.0 Assay (bDNA) (Bayer Corporation Diagnostic, Tarrytown, NY) or TaqMan real-time polymerase chain reaction as described.[34]

Results

Production of Antibodies Directed Against the Extracellular Loop of SR-BI Expressed on Human Hepatocytes. To assess the functional role of SR-BI for initiation of HCV infection, we first generated polyclonal anti–SR-BI sera directed against the extracellular loop of SR-BI by genetic immunization. After completion of immunization, antibodies were selected for their ability to bind to human SR-BI expressed on the cell surface of nonpermeabilized transfected CHO cells. As shown in Fig. 1, incubation of CHO cells expressing human SR-BI with rat polyclonal anti–SR-BI antibodies resulted in a specific interaction of this serum with the extracellular ectodomain of SR-BI (Fig. 1). In contrast, no interaction was present in CHO cells transfected with the pcDNA3 control vector and incubated with rat anti–SR-BI serum or in CHO cells transfected with human SR-BI cDNA and incubated with rat preimmune serum

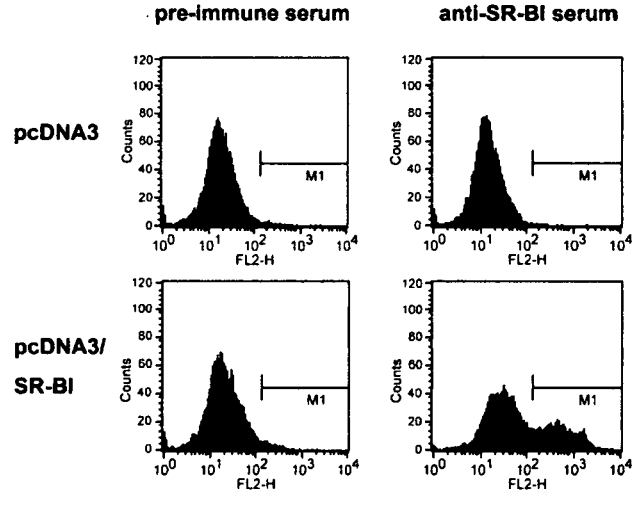

Fig. 1. Production of antibodies directed against the human SR-BI ectodomain by genetic immunization. Specific binding of rat anti-human SR-BI serum to SR-BI expressed in CHO cells. Anti-SR-BI polyclonal serum directed against the SR-BI ectodomain loop was raised by genetic immunization of Wistar rats using a plasmid harboring human SR-BI cDNA. CHO cells were transfected with pcDNA-SR-BI (pCDhSR-BI) or control vector (pcDNA). Flow cytometry of SR-BI or control transfected nonpermeabilized CHO cells incubated with rat anti-human SR-BI polyclonal serum and phycoerythrin-conjugated anti-rat IgG demonstrated specific interaction of anti-SR-BI antibodies with human SR-BI. In contrast, no interaction was present in CHO cells transfected with control vector and incubated with anti-SR-BI serum.

(Fig. 1). To study whether anti-human SR-BI recognizes SR-BI on cells susceptible to HCV infection, human hepatocytes and Huh7.5 hepatoma cells were incubated with the sera and analyzed by flow cytometry. As shown in Fig. 2, incubation of human Huh7.5 cells (Fig. 2A) and human hepatocytes (Fig. 2B) with rat polyclonal anti–SR-BI antibody demonstrated that the antibody recognized SR-BI expressed on HCV target cells, including human hepatocytes. In contrast, no interaction could be detected in the mouse cell line Hepa1.6 (Fig. 2C), confirming the species specificity of the antibody. Similar results were obtained for anti–SR-BI antibodies raised in Balb/c mice (data not shown). Taken together, these data demonstrate that anti–SR-BI sera produced by genetic immunization specifically binds to the ectodomain of human SR-BI expressed on hepatocytes.

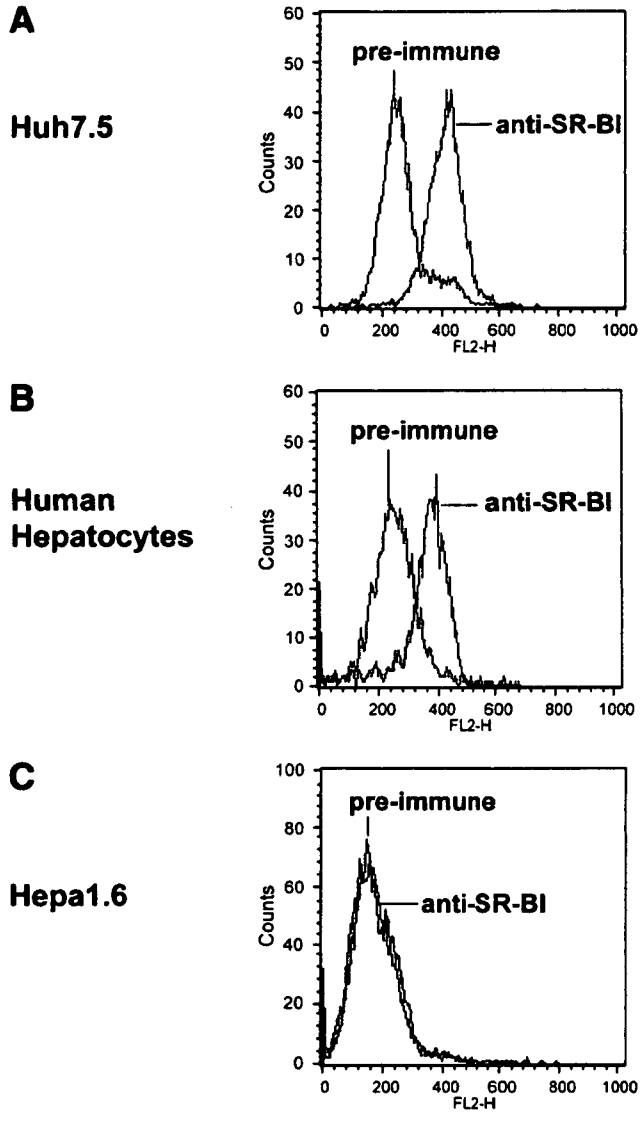

Fig. 2. Interaction of anti-SR-BI antibodies with the SR-BI ectodomain on human hepatocytes and Huh7.5 hepatoma cells. Cell surface expression of SR-BI was determined by flow cytometry using rat anti-human SR-BI serum or control pre-immune serum as described in Fig. 1. Histograms corresponding to cell surface expression of the respective cell surface molecules (open curves) are overlaid with histograms of cells incubated with the appropriate isotype control (gray shaded curves). In contrast to absent interaction on murine Hepa1.6 hepatoma cells, rat anti-human SR-BI serum specifically detected SR-BI on the cell surface of human hepatoma Huh7.5 cells and human primary hepatocytes.

Inhibition of HCV Infection of Different Isolates by Anti–SR-BI Antibodies. To assess the role of SR-BI for HCV infection, we studied JFH1 HCVcc infection of Huh7.5 cells in the presence of anti–SR-BI antibodies directed against epitopes of the SR-BI extracellular loop. Anti–SR-BI polyclonal antibodies markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a dose-dependent manner (Fig. 3A). Fig. 3A shows that anti–SR-BI serum (rat 4) inhibited JFH1 HCVcc infection by more than 70% (Fig. 3A). In contrast, the control pre-immune serum had no inhibitory effect on JFH1 HCVcc infection (Figs. 5, 6). Moreover, mouse anti–SR-BI antibodies generated by genetic immunization of Balb/c mice but not mouse control pre-immune serum were able to reduce JFH1 HCVcc infection of Huh7.5 in a similar manner (data not shown). Taken together, the data demonstrate that antibodies directed against the SR-BI ectodomain efficiently inhibit HCV infection.

To confirm that inhibition of JFH1 HCVcc infection was indeed mediated by anti–SR-BI antibodies, we purified IgG from both rat anti-SRBI (rat 4) and control serum. As shown in Fig. 3B, anti–SR-BI IgG (100 μg/mL) markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a similar manner as anti–SR-BI serum (Fig. 3B). In contrast, control IgG (100 μg/mL) purified from pre-immune serum did not inhibit JFH1 HCVcc infection (Fig. 3B). These data clearly demonstrate that the inhibitory effect of anti–SR-BI serum is mediated by anti–SR-BI antibodies and not by other substances present in the serum (such as oxidized lipoproteins potentially interfering with SR-BI function).

To study whether anti–SR-BI–mediated inhibition of HCV infection applies also to isolates other than JFH1, we performed similar experiments using chimeric J6/CF-JFH1 firefly luciferase reporter virus Luc-Jc1.[30] Figure 3C shows that, similar as for JFH1 HCVcc, both rat (rat 4) and mouse anti–SR-BI antibodies (data not shown) reduced the chimeric reporter virus infectivity in a dose-dependent manner (Fig. 3C), whereas the control pre-immune sera had no inhibitory effect (Fig. 3C and data not shown). Interestingly, we observed variations between the inhibitory effect of anti–SR-BI sera from different rats ranging from 70%-90%. Strongest inhibition was obtained with anti–SR-BI serum from rat 5 (Figs. 5 and 6), demonstrating greater than 90% inhibition of HCVcc infection, with both JFH1 isolate and Luc-Jc1 chimera.

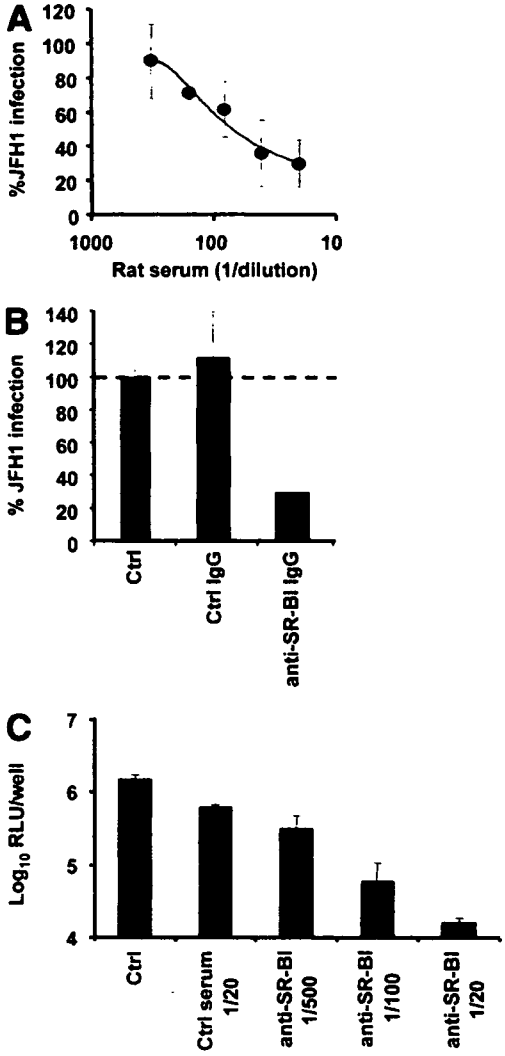

Fig. 3. Inhibition of HCV infection by anti-SR-BI antibodies. (A) Inhibition of JFH1 HCVcc infection by rat polyclonal anti-SR-BI antiserum. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI or control serum before infection with JFH1 HCVcc for 3 hours at 37°C. HCV infection was assessed by HCV RNA quantitation in lysates of infected Huh7.5 cells 72 hours post-infection. Total RNA was isolated and HCV RNA was quantified as described in Materials and Methods. Results are expressed as mean percentage HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Inhibition of JFH1 HCVcc infection by purified rat anti-SR-BI IgG. Huh7.5 cells were preincubated for 1 hour at 37°C with 100 μg/mL IgG isolated from rat anti-SR-BI or control serum before infection with JFH1 HCVcc. Results are expressed as percent HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (C) Inhibition of Luc-Jc1 HCVcc infection by anti-SR-BI. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI serum or control serum before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed by measurement of luciferase activity 48 hours after infection in lysates of infected cells. Results are expressed as mean Log_{10} RLU/well (mean ± SD; n = 4).

Silencing of SR-BI Expression Results in Markedly Reduced Susceptibility to HCV Infection. To further investigate the role of SR-BI in HCVcc infection, we silenced SR-BI expression in Huh7.5 cells using siRNAs targeting SR-BI expression. Immunoblot analysis of transfected cells shows that SR-BI–specific siRNA reproducibly down-regulates SR-BI expression, whereas β-actin expression was not affected (Fig. 4A). In contrast, a pool of negative control siRNA as well as siRNA targeting CD81 or CD13 did not significantly modulate SR-BI expression, confirming the specificity of the siRNA used (Fig. 4A). Down-regulation of SR-BI expressed on the cell surface of Huh7.5 cells by SR-BI–specific siRNA was also confirmed by flow cytometry (difference in mean fluorescence intensity (ΔMFI) of SR-BI siRNA-treated cells = 13.98 versus ΔMFI of naïve cells = 148.31). Importantly, down-regulation of SR-BI cell surface expression strongly reduced the susceptibility of human hepatoma cells to infection with HCV (Fig. 4B). As shown in Fig. 4B, siRNA targeting SR-BI or CD81 markedly inhibited JFH1 HCVcc infection of Huh7.5 cells as compared with cells without silenced cell surface molecules (Fig. 4B).

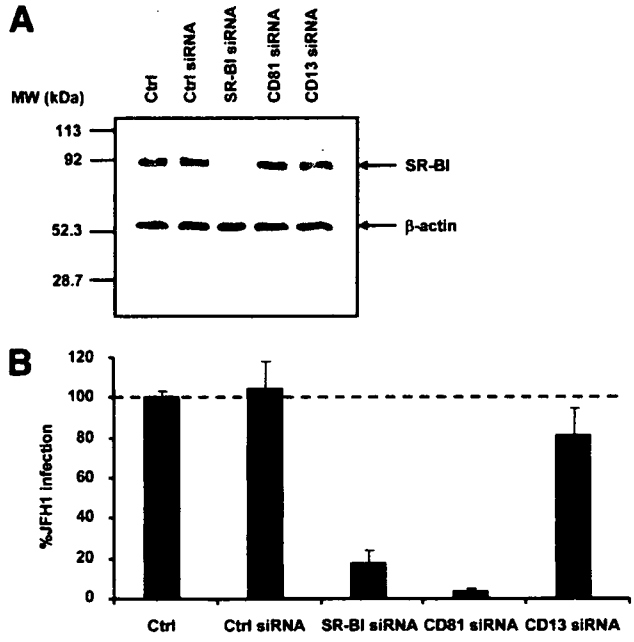

Fig. 4. Silencing of SR-BI expression results in reduced susceptibility to HCV infection. (A) Western blot analysis of siRNA mediated down-regulation of SR-BI expression in Huh7.5 cells. Lysates of control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed using rabbit anti-SR-BI antibodies (1/4000) and HRP-conjugated anti-rabbit antibodies (1/1000) or mouse anti-β-actin monoclonal antibody (1/5000) and HRP-conjugated anti-mouse antibodies (1/1000). The presence or absence of SR-BI and β-actin is indicated on the right, and molecular weight (MW) markers (kDa) are indicated on the left. (B) Susceptibility to HCVcc infection is reduced in SR-BI specific siRNA expressing Huh7.5 cells. Control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were incubated with JFH1 HCVcc. Total RNA was extracted 72 hours after infection, and HCV RNA was quantified. Data are expressed as percent HCVcc infectivity of naïve control cells (mean ± SD; n = 4).