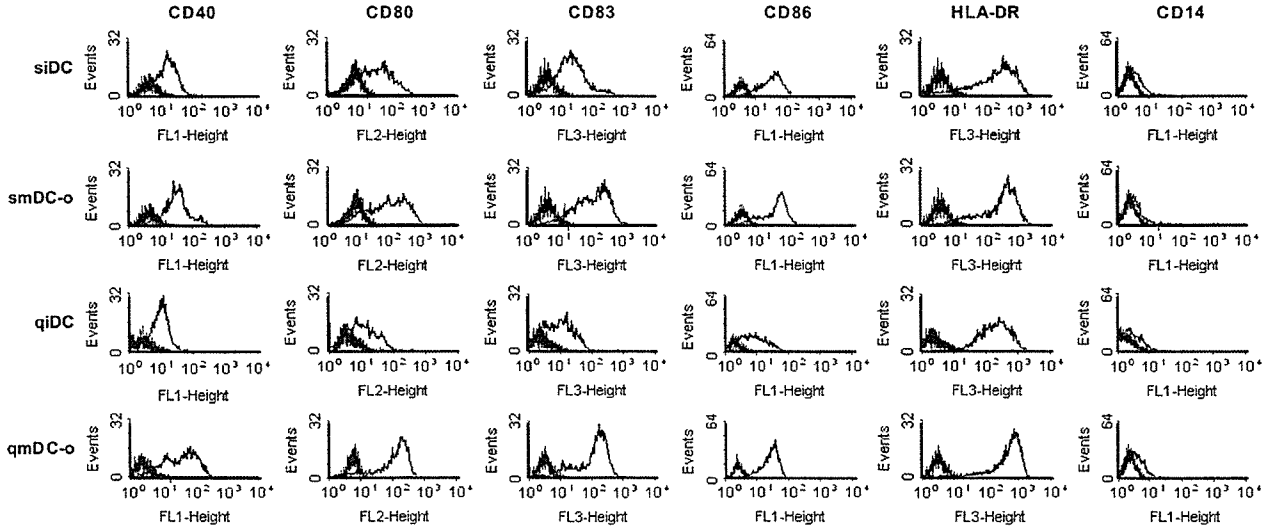


FIGURE 2. OK432 induces maturation on qiDCs and siDCs. Cells differentiated under distinct culture conditions were analyzed by flow cytometry for the expression of CD40, CD80, CD83, CD86, HLA-DR, and CD14 (thin lines with a shaded portion). Thick lines with an unshaded portion show the results of cells without staining. Various DCs were generated, as described in Fig. 1.

system (Fig. 3). CCR7 expression and the migrating ability of DCs were slightly enhanced with the addition of OK432 alone to qmDC-o and smDC-o compared with those without. Recently, Scandella et al[20] reported that PGE_2 is an important factor for enhancing the migratory capacity of MoDCs toward CCL21. Therefore, to induce more potent migrating ability of DCs, we added PGE_2 combined with OK432 to qiDCs or siDCs. We found that the combination of OK432 and PGE_2 upregulated CCR7 expression on DCs more than OK432 alone (smDC-op and qmDC-op). In parallel with CCR7 expression, DCs cultured with the combination of OK432 and PGE_2 showed significantly higher migratory potential toward CCL21 than those cultured with OK432 alone on qiDCs and smDCs. Interestingly, the qmDC-op generated in only 3 days showed more significant migration than the smDC-op generated in 7 days.

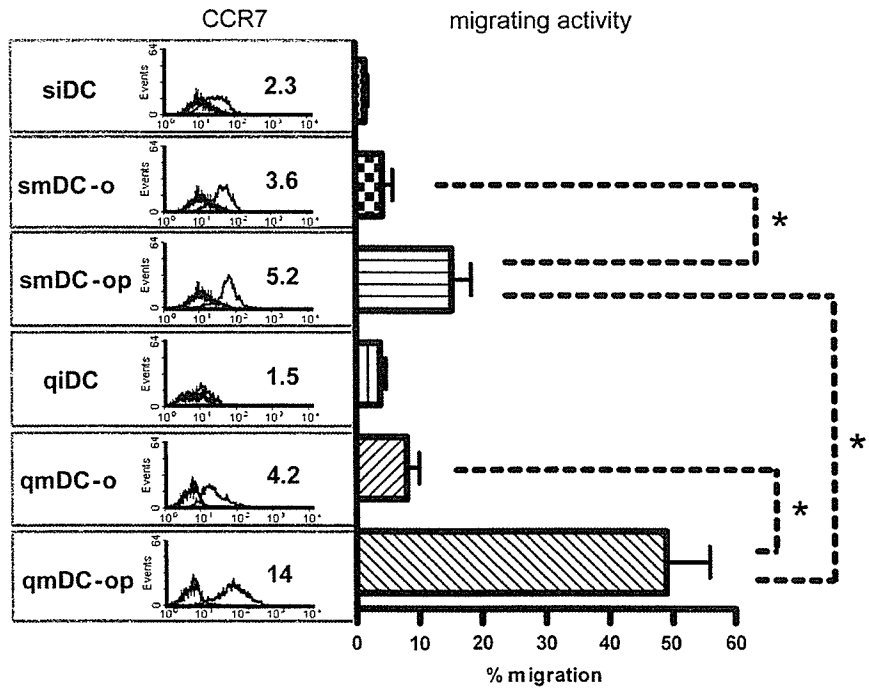


FIGURE 3. PGE_2 combined with OK432 enhances CCR7 expression and migration to CCL21 of qiDCs and siDCs. Various DCs were generated, as described in Fig. 1. They were analyzed by flow cytometry for the expression of CCR7. Thin lines with a shaded portion show CCR7 expression of various DCs. Thick lines with an unshaded portion show the results of various DCs without staining. The numbers indicate the ratios between the mean fluorescence intensity of each sample with and without staining. In addition, the migratory potential of various DCs toward CCL21 was analyzed, as described in the Materials and Methods section. The results are expressed as the mean ± SEM of 5 experiments ($*P < 0.05$).

Combination of OK432, Interferon-α, and Low-Dose Prostaglandin E2 Induces Potent T Helper 1–Inducing Dendritic Cells

Although the combination of OK432 and PGE_2 is beneficial for the induction of mature DCs with potent migration ability, some investigators have reported that PGE_2 reduces the T_H1 priming ability of MoDCs.[21] To examine such an effect of PGE_2 on DCs matured with OK432, we quantified IFNγ production from $CD4^+$ T cells primed with various DCs. DCs stimulated with OK432 alone (smDC-o and qmDC-o) significantly enhanced IFNγ secretion from $CD4^+$ T cells compared with immature DCs (siDCs and qiDCs), respectively (Fig. 4A). In addition, qmDC-o had a similar level of T_H1-inducing ability to that of smDC-o. The addition of PGE_2 tended to reduce the ability of these DCs to prime T cells to secrete IFNγ (smDC-op and qmDC-op), however (see Fig. 4A). Because qmDC-op demonstrated better migratory ability than smDC-op as mentioned in the previous section, we modified the qmDC-op generation protocol accordingly to alleviate T_H1 suppression by PGE_2. First, we titrated down the concentration of PGE_2. Next, we added T_H1-inducing reagent, IFNα, to the culture. In our preliminary experiment, IFNα enhanced the T_H1-stimulating ability of DCs in a dose-dependent manner over a range of 100 to 500 IU/mL and reached a plateau at 500 IU/mL (data not shown). Thus, we used 500 IU/mL IFNα in the following experiments to obtain a potent T_H1 response. We generated qmDCs with OK432 and different concentrations of PGE_2 (0–1000 ng/mL) in the presence or absence of IFNα. In the absence of IFNα, PGE_2 reduced the ability of qmDCs with OK432 to prime T_H1 in a dose-dependent manner (see Fig. 4B). The addition of IFNα enhanced the ability of priming T cells to produce IFNγ even in the presence of PGE_2, however, most significantly with a lower concentration of PGE_2 (50–100 ng/mL; see Fig. 4B).

As mentioned previously, the combination of 0.1 KE/mL OK432, 50 ng/mL PGE_2, and 500 IU/mL IFNα produced qmDCs with potent abilities of migration and T_H1 induction. We designated this combination of reagents as "OPA" (OK432, low-dose PGE_2, and IFNα) and used it as a maturation stimulus for quickly induced DCs in the following experiments. The qmDCs with OPA (OPA-DCs) expressed CD40, CD80, CD83, and CD86 comparable with qmDC-o and had potent migrating ability comparable with qmDC-op (data not shown).

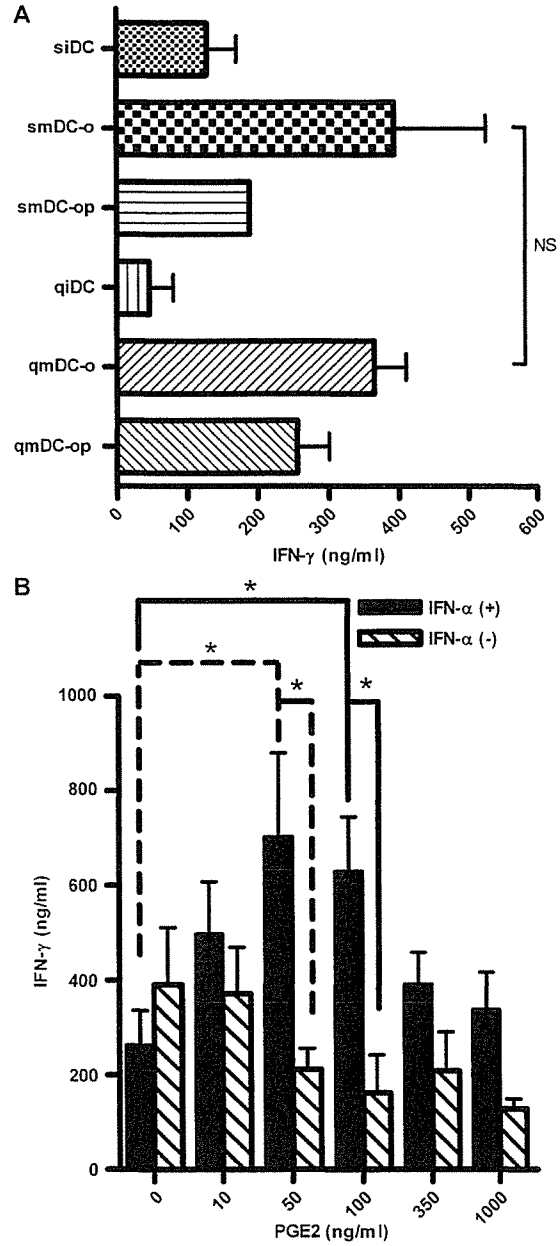


FIGURE 4. Addition of IFNα and reduction of PGE_2 alleviate the T_H1 suppressive effect of PGE_2 on qmDCs. Allogeneic naive $CD4^+$ T cells were cultured with various DCs for 7 days. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ in each supernatant was measured by ELISA. A, Various DCs were generated, as shown in Fig. 1, with 0.1 KE/mL OK432 or 350 ng/mL PGE_2. The results are expressed as the mean ± SEM of 6 experiments. B, qmDCs with 0.1 KE/mL OK432 and a different concentration of PGE_2 were generated in the presence (shaded bars) or absence (striped bars) of 500 IU/mL IFNα. The results are expressed as the mean ± SEM of 6 experiments (*$P < 0.05$).

Quick Induction or the Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Stimuli Enhanced Yield of Monocyte-Derived Dendritic Cells

The yield of DCs is important for the potential clinical application of DC vaccine, because the number of DCs administered is one of the critical determinants for successful immunization. Thus, we compared the yield of various DCs (Fig. 5). With or without maturation stimuli, the quick induction method gave rise to the DCs with better yield compared with the conventional method. Furthermore, OPA-DCs gained

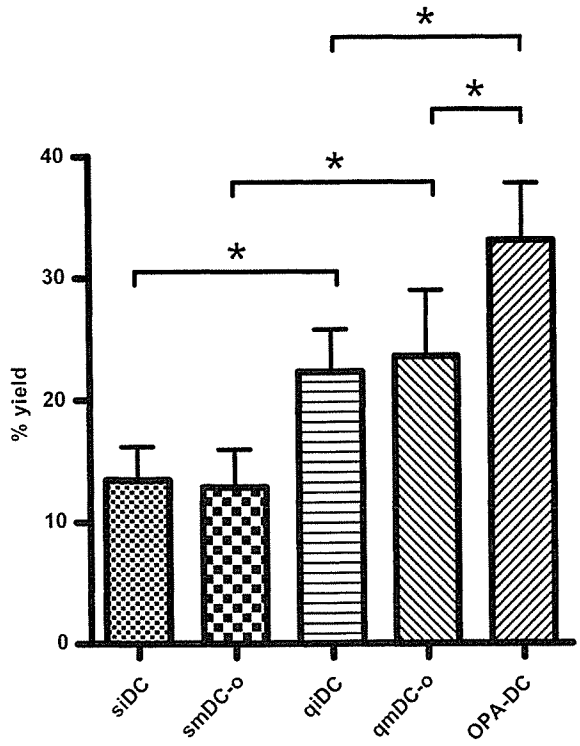


FIGURE 5. The quick induction and maturation with OPA enhanced the yield of MoDCs. Various DCs were generated with serum-free media, as described in Fig. 1. The yields of DCs were determined, as described in the Material and Methods section. The results are expressed as the mean ± SEM of 7 experiments (*$P < 0.05$).

higher yield than the other quickly induced DCs (qiDC and qmDC-o).

Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Is Comparable to the Monocyte-Conditioned Medium Mimic in Ability to Promote Migration and T Helper 1 Induction of Quickly Induced Mature Dendritic Cells

The combination of TNFα, IL-1β, IL-6, and PGE_2 is well known as an MCM-mimic cytokine cocktail, which many investigators have reported is effective for maturation of DCs.[2] Thus, we compared OPA (0.1 KE/mL OK432, 50 ng/mL PGE_2, and 500 IU/mL IFNα) with the MCM-mimic (10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 350 ng/mL PGE_2) with respect to their ability to improve migration and T_H1 induction of qiDCs. Interestingly, qmDCs with OPA (OPA-DCs) had a more potent migration ability than qmDCs with MCM-mimic (MCMm-DCs) (Fig. 6A). In addition, OPA-DCs primed $CD4^+$ T cells to produce T_H1 cytokine (IFNγ) more significantly than MCMm-DCs (see Fig. 6B), whereas their ability to stimulate $CD4^+$ T cells to produce IL-10 did not differ.

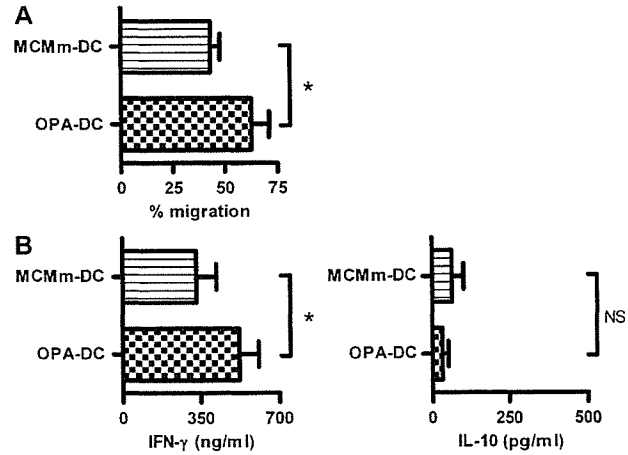


FIGURE 6. OPA has more potent ability than the MCM-mimic in stimulating DCs for migration and T_H1 induction. qiDCs were matured with OPA or the MCM-mimic for the last 24 hours of generation, as shown in Fig. 1. A, Migratory potential of these DCs toward CCL21 was analyzed, as described in the Materials and Methods section. B, Allogeneic naive $CD4^+$ T cells were cultured with each DC for 7 days. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amounts of IFNγ and IL-10 in each supernatant were measured by ELISA. The results are expressed as the mean ± SEM of 10 experiments (*$P < 0.05$).

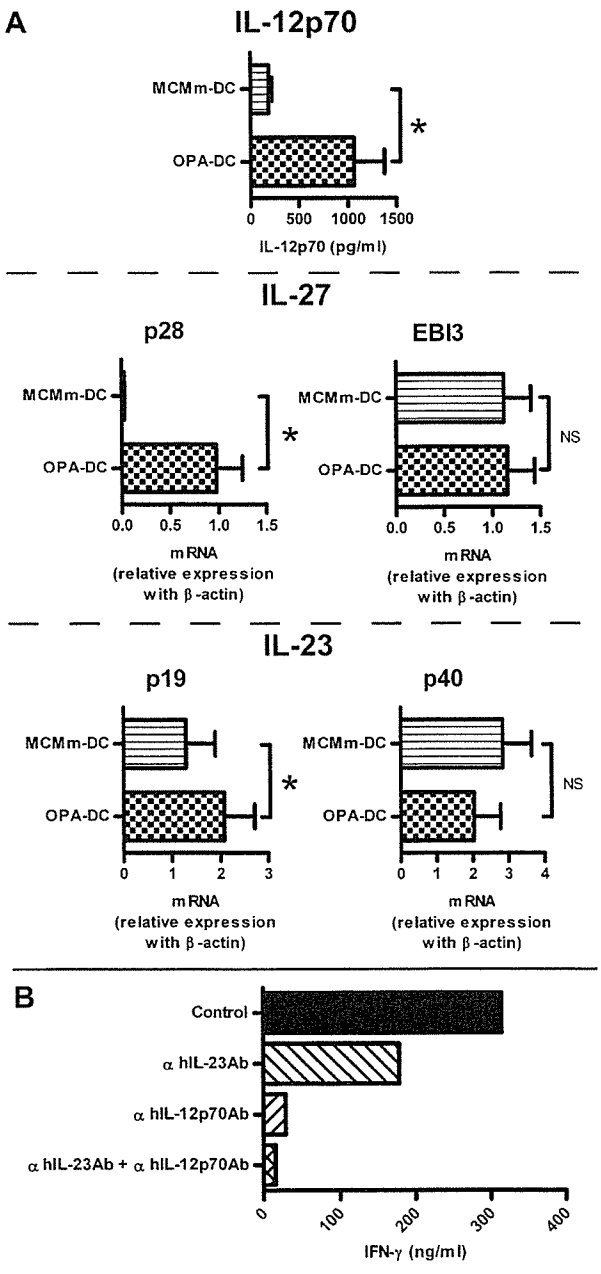
Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Produce More Interleukin-12–Related Cytokines Than That With the Monocyte-Cultured Medium-Mimic

The cytokine-producing profile of DCs is one of the most important factors determining the helper T-cell responses. DC-derived IL-12 family members (IL12p70, IL-23, or IL-27) play a major role in orchestrating T_H1 responses.[22] OPA promoted DCs to secrete more IL-12p70 and to express more transcripts of p28 (a subunit of IL-27) and p19 (a subunit of IL-23) than the MCM-mimic (Fig. 7A). Neutralization of IL-12p70 in the coculture of DCs and $CD4^+$ T cells significantly reduced the T_H1-inducing ability of OPA-DCs (see Fig. 7B). The addition of anti-IL-23 antibody to the culture reduced DC-primed T_H1 induction, but much less than the additional of IL-12p70. Although the roles of IL-27 in the T-cell response are yet to be determined, these results indicate that IL-12p70 and IL-23 are primarily involved in OPA-DC–induced T_H1 polarization.

Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Show Potent Natural Killer and Cytotoxic T-Lymphocyte–Inducing Ability

Many investigators have reported that NK cells and CTLs are major effector cells in the antitumor immune response. According to the results described previously, we compared OPA-DCs with MCMm-DCs for induction of NK cells and CTLs. We found that OPA-DCs activated NK cells

more significantly than MCMm-DCs (Fig. 8A). In addition, OPA-DCs primed CTLs to gain more potent lytic activity against CEA-peptide–pulsed target cells than MCMm-DCs (see Fig. 8B). In the presence of anti-human HLA-ABC antibody or anti-human CD8 antibody, the cells primed by OPA-DCs reduced their lytic activity to 25% to 30% of that without the antibody treatments, showing that they are HLA class I–restricted and CD8-positive cells. In addition, OPA-DC–primed cells showed limited lytic activity against unpulsed cells or K562 (data not shown). Thus, OPA-DCs could induce conventional CEA-peptide–specific CTLs with potent lytic capacities.



Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Obtained From Cancer Patients Have Comparable Activity to Those From Healthy Donors in Migration and T Helper 1 Stimulation

In advanced cancer patients, some functional disorders of DCs have been reported. To verify the feasibility of OPA-DCs as a therapeutic tool, we compared the abilities of OPA-DCs generated from cancer patients with those from healthy subjects. The profiles of the patients with untreated and advanced colonic or gastric cancer are shown in Figure 9A. The OPA enhanced CD40, CD80, CD83, CD86, and CCR7 expression on qiDCs of cancer patients as well as healthy donors (data not shown). In addition, OPA-DCs generated from cancer patients possessed comparable abilities to those from healthy volunteers in terms of migration and T_H1 induction (see Fig. 9B).

DISCUSSION

In clinical trials of DC vaccine against cancers, most have been carried out with MoDCs loaded with tumor lysate or a combination of peptides.[23] Unfortunately, the outcomes of these trials were not always satisfactory. Such limited responses of DC vaccine may be partly attributable to the immaturity of the DCs used. Because it has been demonstrated that mature DCs can induce more effective CTLs against cancers than immature DCs, several maturation stimuli for DCs have been examined to generate potent DCs in clinical settings. The protocols of DC generation still need to be improved to establish functionally mature DCs capable of enhancing in vivo anticancer immune responses, however.

Conventionally, MoDCs are generated in 5 to 7 days with subsequent maturation in 1 to 2 days, thus requiring a total of 7 to 9 days. Several drawbacks have been raised for this method with respect to clinical application. One is that the

FIGURE 7. qmDCs with OPA express more T_H1-inducing IL-12 family cytokines than those with the MCM-mimic. A, OPA-DCs and MCMm-DCs were stimulated by IFNγ and CD40L/L cells for 24 hours. The amount of IL-12p70 in each supernatant was measured by ELISA. The amounts of mRNA of IL-23 and IL-27 subunits in OPA-DCs and MCMm-DCs were measured by means of real-time quantitative RT-PCR. p19 and p40 are subunits of IL-23. p28 and EBI3 are subunits of IL-27. The mRNA expression of each subunit was standardized by β-actin mRNA expression as an internal standard. The results are expressed as the mean ± SEM of 6 experiments ($^*P < 0.05$). B, Allogeneic naive $CD4^+$ T cells were cultured with OPA-DCs for 7 days in the presence or absence of 25 ng/mL anti-human IL-12p70 antibody (αhIL-12Ab) and 20 ng/mL anti-human IL-23 antibody (αhIL-23Ab). As a control, isotype mouse IgG was used as a substitute for antibodies. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ in each supernatant was measured by ELISA. The representative results of 6 experiments are shown.

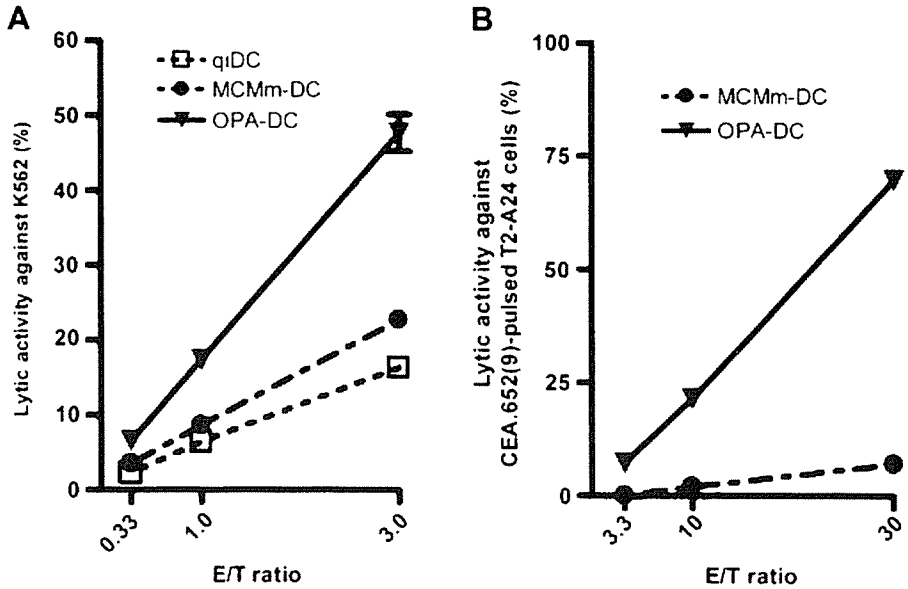


FIGURE 8. qmDCs with OPA significantly activate autologous NK cells and CEA-peptide–specific CTLs. A, Various qiDCs (qiDCs, MCMm-DCs, and OPA-DCs) were generated, as described in Fig. 1. After being stimulated, NK cells were cultured with K562 cells labeled with $Na_2{}^{51}CrO_4$ for 4 hours at 37°C. Supernatants were then harvested, and radioactivity was counted. The representative results of 5 experiments are shown. B, $CD14^-$, $CD56^-$, and $CD19^-$ cells were cultured with CEA 652(9)-loaded various DCs (MCMm-DCs and OPA-DCs) for 28 days with repetitive stimulation of IL-2 and IL-7, as described in the Materials and Methods section. They were then cocultured with CEA 652(9)-pulsed T2-A24 cells for 4 hours at 37°C. The supernatants were harvested, and radioactivity was counted. The representative results of 8 experiments are shown.

average yield of MoDCs cultured with media containing FCS or human serum by this protocol was 15% to 30%,[24,25] which limits the administration of sufficient numbers of DCs. Wong et al[26] reported that the yield of day 5 MoDCs was better than that of day 7, suggesting that a shorter culture period gives rise to larger numbers of DCs. In addition, quicker generation of DCs is beneficial for the maintenance of cell quality, because the apoptosis rate of DCs is reported to increase from 10% to 25% over 1 week of DC culture.[27] Thus, MoDCs should be generated in short-time culture if the cells are phenotypically and functionally comparable with mature DCs. Recently, Dauer et al[15] reported that mature DCs are inducible in only 2 days using a cytokine cocktail of TNFα, IL-1β, IL-6, and PGE_2, which is well known as an MCM-mimic.[2] In support of this report, we demonstrated in this study that OK432 can be used as a substitute for the MCM-mimic to generate mature DCs in 3 days. The average yield of DCs generated by this short-term protocol was more than 30%, even with serum-free media.

The migratory capability of DCs toward DLs is one of the essential factors dictating the successful induction of anti-tumor immune responses.[11,23] In this process, the interactions of chemokines expressed in secondary lymphoid tissue (CCL21) and its receptor (CCR7) on DCs play a major role. Recently, PGE_2 combined with TNFα or IL-1β was found to promote CCR7 expression of mature MoDCs.[20] In the present study, we have shown that a combination of PGE_2 and OK432 significantly enhanced CCR7 expression and migration of DCs to CCL21. Interestingly, qmDC-op demonstrated better CCR7 expression and migrating ability than smDC-op. Although the molecular mechanisms for these processes remain to be determined, one of the reasons may be that qmDC-op maintained better biologic capacity than smDC-op, because the viability of qmDCs was better as a result of the short culture period (data not shown).

Regardless of the positive impact of PGE_2 on DC migration, many investigators have reported that PGE_2 inhibits the T_H1-stimulating activity of MoDCs.[21,28] In the present study, PGE_2 also suppressed the T_H1-stimulating activity of day 7 and day 3 OK432-primed DCs in a dose-dependent manner. To overcome such an inhibitory effect of PGE_2, we reduced the concentration of PGE_2 and added IFNα to generate day 3 mature DCs. Consequently, the addition of IFNα and low dose of PGE_2 to OK432 at the early phase of DC differentiation significantly enhanced the T_H1-inducing activity of MoDCs. Recently, several investigators have reported that IL-12 family members (IL-27, IL-12p70, and IL-23) play major roles in the differentiation of T_H1 cells.[22] Although the MCM-mimic has been widely used as a DC maturation stimulus, it is not always sufficient to endow DCs with the ability to produce biologically active IL-12.[6] In this study, OPA-DCs significantly secreted more IL-12p70 and induced more transcripts of p28 and p19 (subunits of IL-27 and IL-23, respectively) than MCMm-DCs. In addition, neutralization of IL-23 and IL-12p70 with antibodies reduced the T_H1-inducing ability of OPA-DCs. Therefore, enhanced IL-12p70 and IL-23 expression may play important roles in the potent T_H1 induction with OPA-DCs. Further examination is

A

Patient No.	Age	Gender	Primary lesion	Metastasis	CEA* (ng/ml)
1	67	male	colon	(-)	37.0
2	66	male	stomach	(+) p.c.*	413.0
3	61	male	stomach	(+) p.c.*	2.3
4	64	female	colon	(-)	6.4
5	68	female	stomach	(-)	1.1
6	68	female	colon	(-)	12.7
7	62	male	colon	(-)	2.7
mean	65.1				67.9

* p.c. = peritonitis carcinomatosa

*The normal range of CEA is less than 5 ng/ml.

B

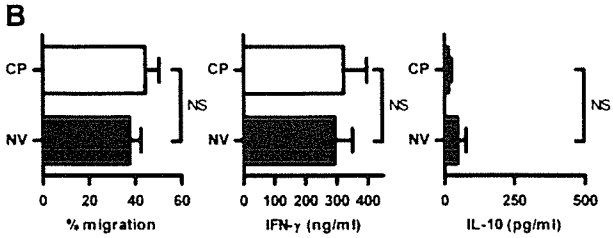


FIGURE 9. OPA can quickly generate mature DCs with potent abilities of migration and T_H1 induction even from monocytes of cancer patients. *A,* Clinical backgrounds of the advanced cancer patients are presented. None of the patients was infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). *B,* OPA-DCs were generated from cancer patients (CP) and normal volunteers (NV). The normal volunteers were blood donors who were negative for human immunodeficiency virus (HIV), HBV, and HCV. The migratory potential of OPA-DCs derived from CP or NV was analyzed, as described in the Materials and Methods section. Conversely, $CD4^+$ T cells primed with OPA-DCs for 7 days were stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ and IL-10 in each supernatant was measured by ELISA. The results are expressed as the mean ± SEM of 7 experiments.

needed to determine whether or not IL-27 is involved in the OPA-DC–induced T_H1 response.

It is still unknown why an optimum concentration of PGE_2 (50–100 ng/mL) exists in the combination of IFNα and OK432 to enhance the T_H1-inducing ability of qmDCs. In our unpublished data, IFNα significantly enhanced IL-12p70 and IL-27 expression in qmDCs but showed limited enhancement of IL-23 transcripts. Conversely, PGE_2 significantly increased IL-23 transcripts but reduced IL-12p70 and IL-27 expression in a dose-dependent manner (data not shown). Therefore, in the presence of OK432 and IFNα, it is conceivable that low-dose PGE_2 is required for qmDCs to maintain sufficient expression of IL-23 without decreasing IL-12p70 production for T_H1 polarization.

We found that OPA enhanced the ability of qmDCs to stimulate NK cells as well as to induce CEA-peptide–specific CTLs more than the MCM-mimic. A plausible reason for this is the profound ability of OPA-DCs to secrete IL-12p70 and induce T_H1, because it has been demonstrated that IL-12p70 and T_H1-derived IFNγ contribute to the activation of NK cells and CTLs, respectively.

Some investigators have reported that DCs obtained from cancer patients have functional disorders,[29] including impaired maturation in response to inflammatory stimuli. Such DC dysfunction may be related, at least in part, to unsatisfactory outcomes of DC vaccine trials against advanced cancers. We demonstrated in the present study that the OPA cocktail could induce phenotypically and functionally mature MoDCs even from advanced cancer patients, suggesting that OPA is a feasible immune adjuvant for a DC vaccine.

In summary, we successfully generated novel mature DCs with OPA. This type of DC possesses the characteristics of quick inducibility, potent migrating ability, and potent stimulating activity for T_H1, CTL, and NK cells, which are desirable for DC vaccines against cancers.

ACKNOWLEDGMENTS

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Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection

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Abstract

Background: Dendritic cells (DCs) utilize Toll-like receptors (TLRs) to sense virus and initiate immune responses. We aimed at elucidating the roles of TLRs on DCs in hepatitis C virus (HCV) infection.
Methods: Monocyte-derived DCs were obtained from 32 healthy volunteers (HV) and 30 chronically HCV-infected patients (CH). TLR2, TLR3 and TLR4 expressions on immature DCs were quantified by real-time quantitative RT-PCR. We stimulated DCs with specific TLR ligands and examined DC maturation, cytokine production and ability to stimulate allogeneic $CD4^+$ T cells.
Results: TLR2 expression on immature DCs was lower in the CH group, whereas those of TLR3 or TLR4 were not different between the groups. Each TLR ligand induced DC maturation and stimulated them to release comparable levels of IL-12p70, IL-6, IL-10, TNF-α and IFN-β between the groups. TLR2 and TLR4 ligands enhanced DC ability to stimulate T cell proliferation, with the degree due to the TLR2 ligand being lower in the CH group.
Conclusions: In HCV infection, the TLR2 expression on DCs is reduced and TLR2-stimulated DCs show lesser ability to proliferate T cells than healthy counterparts, suggesting that the TLR2 system is involved in HCV-induced immunopathogenesis.

Keywords: Dendritic cell; Hepatitis C virus; Toll-like receptor; Mixed lymphocyte reaction

1. Introduction

Hepatitis C virus (HCV), a single-stranded RNA virus that belongs to the flaviviridae family, is a major causative agent of chronic liver disease [20]. Approximately 80% of newly HCV-infected patients are not able to eradicate HCV, and subsequently develop to a chronically infected state. One of the most important factors involved in HCV persistence is inadequate cell-mediated immune response in hosts. Several studies have demonstrated that a vigorous and sustained $CD4^+$ T cell response is correlated with spontaneous viral clearance in acute HCV infection [10,24]. In chronic HCV infection, the $CD8^+$ T cell response to HCV proteins as well as that of $CD4^+$ T cell was less vigorous and narrowly focused on some restricted epitopes [30,36]. Although the precise mechanism of such impaired T cell responses in HCV infection is yet to be determined, impaired function of dendritic cells (DCs) may be involved, since DCs are the most potent antigen presenting cells (APCs) that regulate immune responses [12]. We previously reported that monocyte-derived DCs (MDDCs)

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from HCV-positive patients are impaired in allogeneic $CD4^+$ T cell stimulation compared with those from healthy volunteers [19]. One of the causes of such DC dysfunction may be the failure of DC to mature in response to some inflammatory stimuli, which may be due to direct HCV infection to DCs [3,25] or the influences of HCV proteins [7] as reported elsewhere.

Toll-like receptors (TLRs) recognize molecular patterns specific to microbial pathogens [1]. DCs show subset-specific expressions of TLRs on their cell surface or endosomal membrane [16,18]. Cumulative reports have shown that DC activation via TLR signaling is prerequisite for the subsequent induction of vigorous T cell responses. Some viruses have been shown to interact with TLRs or their downstream molecules and interfere with the signaling cascade [9,13]. These reports suggest that the alteration of TLR-mediated signals is one of the mechanisms of virus-induced immune modulation. We thus hypothesize that HCV infection influences the profiles of TLR expressions as well as their functions in DCs resulting in aberrant DC-mediated immune response. To address this issue, we compared the expressions of TLRs on MDDCs between patients with chronic HCV infection and healthy subjects. To study their functions, we stimulated MDDCs with TLR-specific ligands and then examined DC phenotypes, cytokine release and DC-induced T cell responses.

2. Materials and methods

2.1. Subjects

Thirty-two healthy volunteers (HV) and 30 patients with chronic hepatitis C (CH) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. Informed consent was obtained from each patient or donor included in the study, and the study protocol conformed to The Code of Ethics of the World Medical Association for experiments involving humans. The healthy volunteers include buffy coats provided from the Osaka Red Cross Blood Center (Osaka, Japan) and they were confirmed to be negative for hepatitis C virus, hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The characteristics of patients are shown in Table 1. All patients were confirmed to be positive for both serum anti-HCV antibody and HCV RNA but were negative for other viral infections, including HBV and HIV.

Table 1
The characteristics of HCV-infected patients enrolled in the present study

Gender (male/female)	21/9
Age (years)	57.1 ± 1.9
Serotype (1/2/N.D.)	23/0/7
HCV-RNA (Meq/mL)	8.1 ± 1.7
Serum ALT (IU/L)	62.7 ± 9.3

Data were expressed as mean ± standard error. N.D.: not determined.

2.2. Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were purchased from PeproTech (Rocky Hill, NJ). Palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) was purchased from InvivoGen (San Diego, CA). Polyinosine-polycytidylic acid (poly(I:C)) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO).

2.3. Generation of MDDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density gradient centrifugation using Ficoll–Hypaque. $CD14^+$ monocytes were purified from PBMCs using the MACS system (Miltenyi Biotec, Gladbach, Germany) with anti-CD14 antibody-conjugated microbeads. MDDCs were generated from $CD14^+$ cells (purity was >96%) by the culture in DC media (Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY)) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid) containing 25 ng/mL GM-CSF and 10 ng/mL IL-4 for 7 days at 37 °C in 5% CO_2. On day 4, half of the culture supernatants were replaced with fresh media containing equal concentrations of cytokines. On day 7, MDDCs were used to analyze the expression of TLRs without any stimulus. The remaining cells were stimulated with selective TLR ligands, Pam3CSK4 for TLR2, poly(I:C) for TLR3 and LPS for TLR4, respectively. After 24 h of incubation, the supernatants were harvested for the measurement of cytokines by enzyme-linked immunosorbent assay (ELISA). The cells were then subjected to immunophenotyping and RNA extraction.

2.4. Flow cytometric analysis

The phenotypes of MDDCs were analyzed by FACS Calibur (BD Biosciences, San Jose, CA). For the staining, MDDCs were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, PerCP-, or PC5-conjugated anti-human monoclonal antibodies were used: CD11c (KB90, Dako Cytomation, Glostrup, Denmark), HLA-DR (L243, BD Biosciences), CD80 (L307.4, BD Biosciences), CD86 (IT2.2, BD Biosciences) and CD83 (HB15a, Beckman Coulter, Flullerton, CA). The expression of each molecule was expressed by mean fluorescence intensity (MFI), which was determined using CellQuest software (BD Biosciences).

2.5. Real-time quantitative PCR

Total RNA from MDDCs was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the

manufacturer's instructions. Total RNA (0.3–1 μg) was reverse transcribed in a 20 μL volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4 and IFN-β, we used ready-to-use assays (Assay-on-Demands Gene Expression Products, Applied Biosystems), according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. We identified a calibrator sample from healthy volunteers and the other samples expressed relative values to the calibrator. To normalize the amount of total RNA added to each reaction mixture, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values using this.

2.6. ELISA

The cytokines released from MDDCs stimulated with TLR ligands were measured by means of ELISA with matched antibody pairs for human IL-12p70, IL-6, IL-10, TNF-α (Endogen, Woburn, MA), according to the manufacturer's instructions (The ranges of the detection were 4–1000 pg/mL for IL-12p70, IL-10 and TNF-α and 31–1000 pg/mL for IL-6).

2.7. Allogeneic mixed lymphocyte reaction (MLR)

Responder $CD4^+$ T cells were purified from PBMCs of one healthy volunteer using the MACS system with anti-CD4 monoclonal antibody-conjugated microbeads (Miltenyi Biotec). After 7 days of culture, the graded numbers of MDDCs stimulated with each TLR ligand for 6 h were co-cultured with 1×10^5 $CD4^+$ T cells for 5 days. In the final 16–20 h of co-culture, 1 μCi/well of [^{3}H]-thymidine (ICN Biomedicals, Costa Mesa, CA) was pulsed. The uptake of [^{3}H]-thymidine to T cells was measured using a β-counter (Wallac, Gaithersburg, MD).

2.8. Statistical analysis

The values were compared by Mann–Whitney *U*-test and Wilcoxon signed rank test, using StatView 5.0 software (SAS Institute, Cary, NC). A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. The expression of TLR2 was lower in CH

In this study, we obtained highly pure (>98%) immature MDDCs ($CD14^-$, $CD11c^+$ and $HLA\text{-}DR^+$ cells) after 7 days of culture from both HV and CH groups. It is known that immature MDDCs express transcripts spanning from TLR1 to TLR8 [16,18]. We examined TLR2, TLR3 and TLR4 as representatives of TLR to clarify their roles in MDDCs in HCV infection. We evaluated the expression of TLRs using real-time quantitative RT-PCR. The relative amounts of TLR2 in MDDCs from CH were lower than those from HV. In contrast, the relative amounts of TLR3 and TLR4 revealed no significant differences between the groups (Fig. 1). We investigated the correlation between each TLR expression and clinical parameters among HCV-infected patients. Both HCV viral load and serum ALT level were not correlated with each TLR expression (Data not shown).

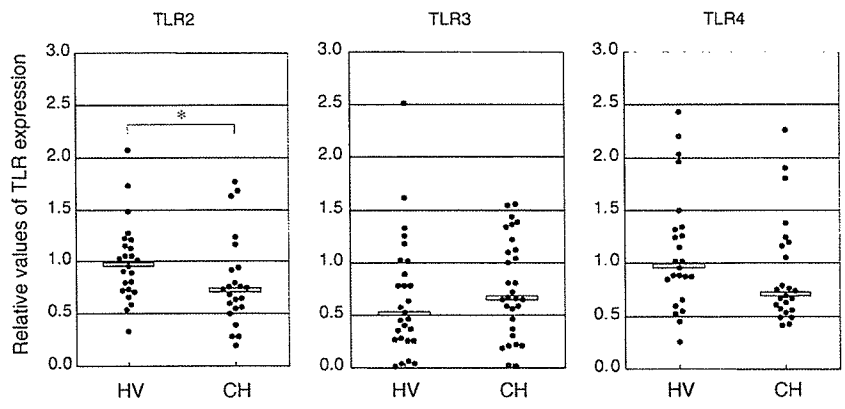

Fig. 1. TLR2 expression in MDDCs from HCV-infected patients is lower than those from healthy donors. The expressions of TLR2, TLR3 and TLR4 mRNA in day 7 MDDC were quantified by real-time quantitative RT-PCR as described in Section 2. The values were determined as the ratio of the results between the samples and a calibrator sample from healthy volunteers after they had been corrected for the amount of β-actin mRNA from each sample as an internal control. The horizontal bars indicate the median. $^*p<0.05$ by Mann–Whitney *U*-test. HV: healthy volunteers; CH: chronic hepatitis C patients.

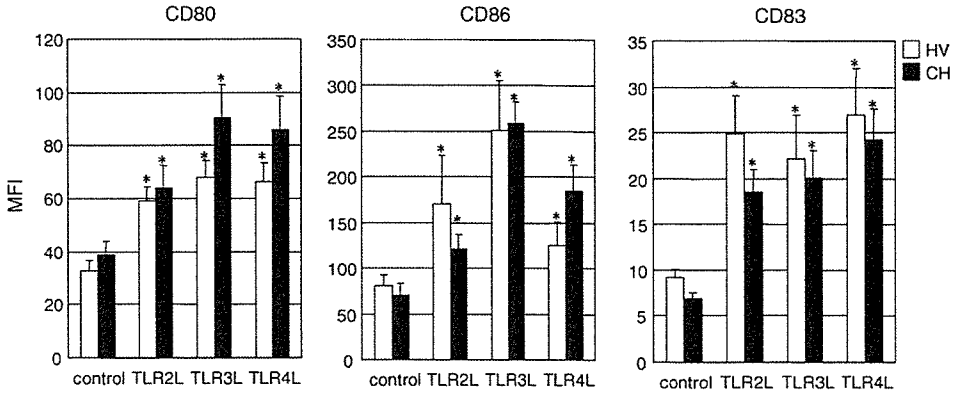

Fig. 2. TLR ligands induce comparable levels of MDDC maturation in healthy donors and HCV-infected patients. The expression of CD80, CD86 and CD83 on MDDC were analyzed by flow cytometry before and after the stimulation with TLR ligands. The values were expressed as mean fluorescence intensity (MFI). The MFI of each marker is represented as the mean + standard error (S.E.) of six healthy donors (open bars) and six patients (closed bars). $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

3.2. Immature MDDC from CH matured on stimulation with TLR ligands

To examine the capacity of TLR ligands to induce MDDC maturation, we performed flow cytometric analysis of cell surface markers (CD80, CD86 and CD83). The cells from HV and CH displayed the characteristic of immature MDDC in the absence of any stimulus (Fig. 2). Pam3CSK4, poly(I:C) and LPS significantly up-regulated these markers on MDDCs regardless of HCV infection. The expressions of all maturation markers did not statistically differ between the groups.

3.3. The ability of cytokine production in response to TLR ligands was preserved in MDDC from CH

Mature MDDCs release inflammatory cytokines in response to TLR ligands. We thus examined the production of IL-12p70, IL-6, IL-10 and TNF-α or the expression of IFN-β in TLR-stimulated MDDCs. Each TLR ligand induced the production of IL-10, IL-6 and TNF-α from MDDCs, with the degree induced by LPS being the most significantly (Fig. 3). As for IL-12p70, poly(I:C) strongly induced the production, whereas Pam3CSK4 and LPS did not. In the preliminary experiments, we examined the kinetics of IFN-β expression in TLR-stimulated MDDC. In both HV and CH groups, the IFN-β expression in MDDC showed a peak at 3 h after the stimulation with TLR ligands (data not shown). Thus, we compared its expression at this point in the following experiments. Only poly(I:C) was able to stimulate MDDC to express IFN-β (Fig. 4). Although there was a trend that TLR3L-stimulated MDDC from the patients released more IL-12p70, TNF-α than those from healthy donors, the data for the cytokine production and IFN-β expression were not statistically different between the groups.

3.4. TLR2 specific ligand strongly enhanced the ability of MDDC to stimulate T cell proliferation in MDDC

The ability of DC to stimulate $CD4^+$ T cell proliferation is one of the most important characteristics of DCs as professional APCs. We thus examined allogeneic MLR by MDDC stimulated with various TLR ligands (Fig. 5). In both HV and CH groups, Pam3CSK4 and LPS enhanced the MDDC ability to stimulate allogeneic $CD4^+$ T cell proliferation compared to those without stimulation. In the CH group, the degree of $CD4^+$ T cell proliferation with Pam3CSK4-stimulated MDDCs was significantly lower than those from the HV group. In contrast, poly(I:C) did not enhance MDDC capacity to stimulate $CD4^+$ T cells either in the CH or HV group.

4. Discussion

TLRs recognize various pathogens and are able to initiate the chain of immune responses by linking innate and adaptive immune systems. Several lines of evidence have demonstrated that TLR-mediated signaling which leads to the induction of type-I interferon plays an important role in the eradication of intracellular pathogens, including viruses [15,17,21]. DCs express distinct TLRs according to the differences in their ontogeny and maturation stage [16,18]. In general, TLR ligands induce DC maturation and enhance their ability to produce inflammatory cytokines [4,29,32] and to stimulate T cell responses [4,27], thus contributing to viral eradication. However, it has not been clarified whether TLR expression on DC as well as their function is preserved or not in disease conditions, such as HCV infection.

In the present study, we demonstrated that TLR2 expression on immature MDDC from HCV-infected patients is lower than those from healthy subjects. As for the correlation

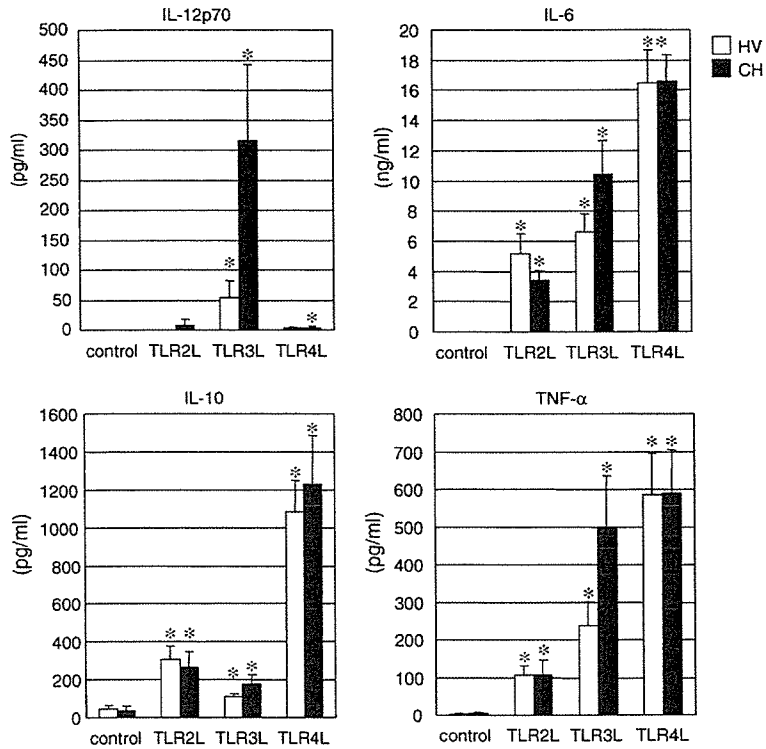

Fig. 3. TLR ligands stimulate MDDC to secrete comparable amounts of inflammatory cytokines between samples from healthy donors and HCV-infected patients. The concentrations of IL-12p70, IL-6, IL-10 and TNF-α in media from DC cultured with TLR ligands for 24 h were measured by ELISA. The data from 16 healthy donors (open bars) and 18 patients (closed bars) are shown as the mean + S.E. $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

with TLR expression and DC functions, TLR2-stimulated DC from HCV-infected patients showed lesser ability to proliferate allogeneic $CD4^{+}$ T cells than those from the healthy counterparts. In contrast, TLR3 and TLR4 expressions on MDDC from the patients and their functions in DC maturation are comparable with those from healthy donors.

In spite of recent research progress on the innate immune system, it has been largely unknown how TLR expression is regulated. In several disease conditions, increased TLR2 expression has been reported, which may be due to inflammatory mediators derived from inflamed tissue where virus replication or autoimmunity occurs. It is considered that IFN-γ, IL-12 or IL-18 are responsible for TLR2 up-regulation on epithelial cells [5] or PBMCs [28]. With regard to TLR2 down-regulation, there is one report on all-trans retinoic acid [23]. Why TLR2 expression is decreased on MDDC from HCV-infected patients is currently unknown. It is conceivable that direct HCV infection to DC or the influence of HCV-derived proteins is involved in TLR2 down-regulation, since these are mechanisms reported to lead to DC disability in HCV infection [3,7,25].

TLR2 ligand induced phenotypic as well as functional DC maturation as judged by up-regulation of co-stimulators and enhancement of MLR. MLR is generally accepted as one of the reliable values for assessing comprehensive DC function [11,33]. However, the precise factors dictating the magnitude of MLR are still under debate. Plausible MLR determinants are the expressions of CD86 on DC [37] or DC-derived cytokines including IL-12 [14,26]. In the present study, the expression of CD86 and CD83 on MDDCs stimulated with Pam3CSK4 tended to be lower in CH group than those in HV group, though it failed to reach statistical significance. As for DC-derived cytokines, we could not measure bioactive IL-12p70 produced by MDDCs stimulated with Pam3CSK4, of which level is much less than IL-12 p40 as reported elsewhere [34]. In addition, we did not observe any difference in IL-10 levels, which is reported to subvert T cell proliferation [6]. Therefore, it is still unclear about the precise mechanisms of the lower level of MLR induced by TLR2-stimulated MDDC in patients compared to those in healthy subjects. Lesser degree of CD86 expression on MDDC in HCV-infected patients may be involved. Further examination is still needed to disclose the involvement of other factors in MLR impairment with HCV-DC.

In this study, no significant enhancement in MLR was obtained with TLR3 ligand-stimulated MDDC regardless of HCV infection, which is in clear contrast with previous reports [22,35]. Such contradictory results may be due to the difference in duration of the poly(I:C) treatment. We matured

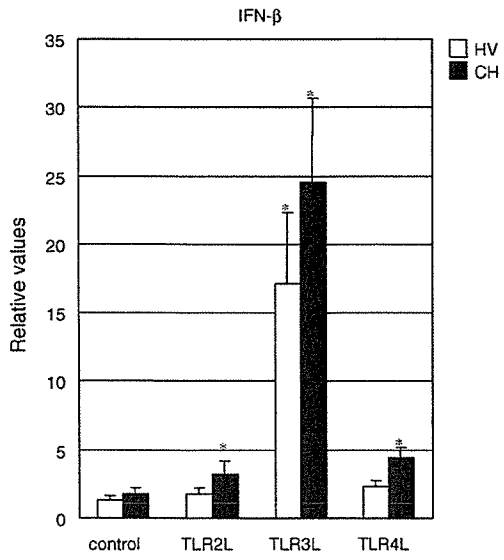

Fig. 4. TLR ligands induce comparable levels of IFN-β mRNA expression in MDDC from healthy donors and HCV-infected patients. The expression of IFN-β in MDDC was analyzed by real-time quantitative RT-PCR 3 h after stimulation with TLR ligands as described in Section 2. The values of IFN-β expression were determined as the same in TLR expression analyses. The results of 11 healthy donors (open bars) and 11 patients (closed bars) are shown as the mean + S.E. $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

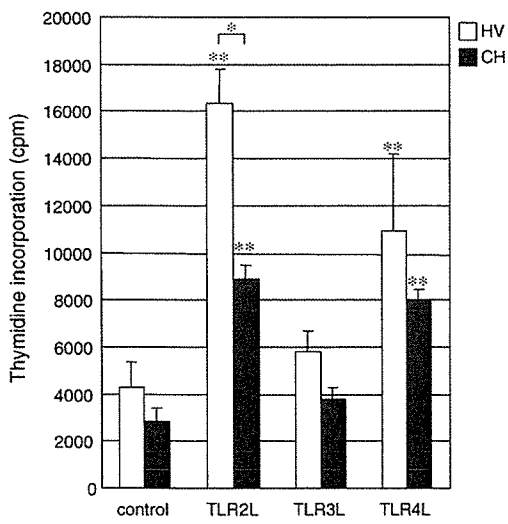

Fig. 5. MDDCs from HCV-infected patients fail to gain comparable ability with those from healthy counterparts to stimulate allogeneic $CD4^+$ T cell proliferation in response to TLR2 ligand. After MDDC had been stimulated with TLR ligands for 6 h, allogeneic $CD4^+$ T cells were co-cultured with DC for 5 days (DCs:T cells = 1:20). The $CD4^+$ T cell proliferation was determined by their uptake of [^{3}H]-thymidine as described in Section 2. The amounts of thymidine incorporation to T cells stimulated with DC from six healthy donors (open bars) and six patients (closed bars) are expressed as the mean + S.E. $^{*}p < 0.05$ by Mann–Whitney U test, $^{**}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

MDDCs by 6 h stimulation of poly (I:C), which was shorter than the condition performed in other studies.

Evoking of the T cell response against allo-antigens has been shown to parallel a successful T cell response against specific antigens [2]. Thus, impaired MLR with TLR2-stimulated DC from HCV-infected patients may be related to impaired ability of HCV-DC to mount a vigorous T cell response, as reported by several groups including us [3,19,31]. Recently, Dolganiuc et al. [8] reported that HCV core and NS3 proteins were directly associated with TLR2 on monocytes and subsequently stimulated them to release TNF-α, suggesting some role of TLR2-mediated signals in the pathogenesis of HCV infection. To elucidate this, further work is needed to examine the association between TLR2 and other HCV-related proteins, and whether the expression and function of TLR2 are changed when HCV is eradicated by IFN-α-based therapy.

In conclusion, in HCV infection, TLR2 expression on DC is reduced and DC maturation via TLR2 fails to induce a level of $CD4^+$ T cell proliferation comparable to that observed with DC those from healthy subjects, suggesting that the TLR2 system is involved in HCV-induced immunopathogenesis.

Acknowledgements

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Enhanced ability of peripheral invariant natural killer T cells to produce IL-13 in chronic hepatitis C virus infection

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***Background/Aims*: Human invariant natural killer T (iNKT) cells express a TCR Vα24-JαQ paired with Vβ11 and are activated by a surrogate ligand, α-galactosylceramide (αGalCer). The iNKT cells are involved in the regulation of antiviral immune responses; however, little is known about their roles in hepatitis C virus (HCV) infection.**

***Methods*: We compared the frequency of peripheral iNKT cells and their cytokine producing capacity reactive to αGalCer between chronically HCV-infected patients and healthy subjects. Cytokine production of freshly isolated iNKT cells were analyzed by ELISPOT. Activated iNKT cells were obtained by culture with αGalCer-loaded dendritic cells (DCs) and re-stimulated with them for the measurement of cytokine production.**

***Results*: The frequencies of iNKT cells were not different between HCV-infected patients and healthy subjects. The number of fresh IFN-γ-producing iNKT cells reactive to αGalCer was not different between the patients and controls, whereas fresh iNKT cells produced negligible amounts of Th2 cytokines regardless of HCV infection. In response to αGalCer, expanded iNKT cells from the patients secreted IFN-γ comparable in amount to controls, whereas they released significantly more IL-13 than cells from controls.**

***Conclusions*: Activated iNKT cells from HCV-infected patients gain more ability to secrete IL-13 than those from healthy subjects.**

Keywords: **Hepatitis C virus; Natural killer T cell; Th1; Th2; α-Galactosylceramide**

1. Introduction

Hepatitis C virus (HCV) frequently gives rise to chronic liver disease, which varies from asymptomatic HCV carriers to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Cumulative reports have demonstrated that innate as well as adaptive immune responses are involved in the pathogenesis of HCV-induced liver injury and the development of liver disease [2,3].

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers [4]. The NKT cell population is highly heterogeneous; invariant NKT (iNKT) cells express an invariant TCR, composed of Vα24-JαQ preferentially paired with Vβ11 in humans [4,5], whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on the non-polymorphic MHC class I-like molecule CD1d [6,7], which is expressed by antigen presenting cells, such as dendritic cells (DCs). Although endogenous ligands of iNKT cells are little known, α-galactosylceramide (αGalCer) has

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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 (interferon (IFN)-γ) and Th2 cytokines (interleukin (IL)-4, IL-5, IL-13). The CD4+ iNKT cells secrete more Th2 cytokines than DN, while CD8+ subsets predominantly secrete Th1 cytokines [8].

Although iNKT cells comprise a small portion of hemotopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings, such as autoimmune disease, viral infection or cancer [9–13]. For chronic HCV infection, there have been some controversial reports about the frequency of peripheral iNKT cells [14–16], however, their functional roles in HCV-infected patients are largely unknown. We compared the frequency and the cytokine producing capacity of iNKT cells in fresh peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by stimulation with αGalCer-loaded DCs. Of note, in response to αGalCer-pulsed DCs, the activated iNKT cells obtained from chronic hepatitis C patients secreted a significantly larger amount of IL-13 and tend to produce more IL-4 and IL-5 than those from healthy subjects, indicating that peripheral iNKT cells may be involved in the pathogenesis of chronic hepatitis C.

2. Materials and methods

2.1. Subjects

After informed consent had been obtained, 19 patients who were positive for both anti-HCV Ab and serum HCVRNA were enrolled in this study (chronic hepatitis [CH] group). All patients were negative for hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and had no apparent history of other types of liver diseases. The HCV serotype of all patients was type 1. None of them had been treated with anti-viral agents, such as IFN-α or ribavirin. As controls, 18 age-matched healthy subjects (HS group) who were negative for HCV, HBV and HIV were examined. The clinical backgrounds of the patients and healthy subjects are shown in Table 1.

Table 1
Clinical backgrounds of healthy subjects and chronic hepatitis C patients

	Healthy subjects	Chronic hepatitis C patients
N (M/F)	18 (13/5)	19 (11/8)
Age[a]	38 ± 9	48 ± 14
Serum ALT (IU/L)[b]	ND	58 (23–238)
HCVRNA (Mequiv./ml)[b]	ND	2.5 (0.5–15.0)

[a] Values are expressed as mean ± SD.

[b] Median with range in parentheses. ALT, alanine aminotransferase; Mequiv./ml, million genome equivalents per milliliter; ND, not determined.

2.2. Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from Peprotech (London, UK). Recombinant human IL-2 was from Genzyme (Minneapolis, MN). αGalCer was provided by Kirin Brewery (Gumma, Japan).

2.3. Generation of monocyte-derived DCs

To stimulate iNKT cells, monocyte-derived DCs (Mo-DCs) were generated from CD14+ cells. CD14+ cells were separated from peripheral blood mononuclear cells (PBMCs) with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). They were cultured at 5×10^5/well in the DC culture media (DCM) (IMDM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM non-essential amino acid and 2 mM L-glutamine) containing 100 ng/ml GM-CSF and 20 ng/ml IL-4 for 7 days. For maturation of the DCs, they were given 50 μg/ml *Staphylococcus aureus Cowan I* (SAC) on day 6 of the culture. αGalCer (100 ng/ml) was pulsed to the DCs on the same day.

2.4. Frequency and phenotype analyses of iNKT cells in peripheral blood

PBMCs were isolated from the venous blood of HCV-positive patients or healthy subjects by Ficoll–Hypaque density-gradient centrifugation. For staining, PBMCs were incubated with fluorescence-labeled Abs for 30 min at 4 °C in PBS supplemented with 1% BSA and 0.1% NaN_3. The stained cells were analyzed using FACS Calibur and Cell Quest software (Becton Dickinson, San Jose, CA). For the measurement of iNKT cell subsets, the cells were stained with a combination of anti-Vα24, anti-Vβ11 and anti-CD4 mAbs (Immunotech, Marseilles, France). The frequencies of total (Vα24+, Vβ11+), CD4-positive (Vα24+, Vβ11+, CD4+) and CD4-negative (Vα24+, Vβ11+, CD4–) iNKT cells were determined. To examine iNKT cell phenotypes, they were further stained with mAbs against CCR7, CXCR3 (R&D Systems, Minneapolis, MN), CCR4, CD62L (BD Pharmingen, San Jose, CA) and analyzed by FACS Calibur.

2.5. Cytokine analysis of peripheral iNKT cells in response to αGalCer

For enumeration of the peripheral IFN-γ-producing cells in response to αGalCer, we used enzyme-linked immunospot (ELISPOT) assay. MultiScreen® ELISPOT plates (Millipore, Bedford, MA) were coated with 10 μg/ml mouse anti-human IFN-γ mAb (1-D1K, Mabtech, Sweden). Monocyte-depleted PBMCs were cultured at 5×10^5/well with 5×10^4/well autologous Mo-DCs pulsed with or without αGalCer for 24 h on Ab-coated plates. PBMCs pulsed with PHA (1 μg/ml) were used as positive controls. After 24 h, the culture supernatants were collected for ELISA. Subsequently, the plates were washed and then incubated with biotin-labeled anti-human IFN-γ mAb (7-B6-1, Mabtech). After addition of streptavidin-HRP to the plates, spots were developed using 3-amino-9-ethylcarbazole (Sigma–Aldrich, St Louis, MO). Spots corresponding to IFN-γ-secreting cells were identified by microscopy and counted by two independent observers. The number of αGalCer-reactive IFN-γ-producing cells was determined by subtracting the number of spot-forming cells with αGalCer-unpulsed Mo-DCs from those with αGalCer-pulsed ones. To confirm that cytokines were released from iNKT cells, we also examined Vα24-depleted and/or Vβ11-depleted cells stimulated with αGalCer-pulsed Mo-DCs. Vα24-positive or Vβ11-positive cells was depleted with mouse anti-human Vα24 or Vβ11 mAbs (Immunotech) and subsequently anti-mouse IgG microbeads (Miltenyi Biotec). The Vα24-positive

or Vβ11-positive cells remaining in the treated samples were less than 5% as assessed by FACS (data not shown).

2.6. Expansion of iNKT cells from peripheral blood and analyses of cytokine production from them

To investigate the ability of activated iNKT cell subsets to proliferate and produce cytokines in response to αGalCer, we expanded them according to a method described previously [17] with some modifications. Monocyte-depleted PBMCs were cultured at 3×10^6/well with 3×10^5/well αGalCer-loaded autologous mature Mo-DCs for 2 weeks in DCM containing 5 ng/ml IL-2. For stimulation of the cells, 2.5 ng/ml IL-2 was added to the culture every 3 days. Subsequently, Vα24+ cells were magnetically separated and cultured in DCM for additional 3 weeks, which were fed with 2.5 ng/ml IL-2 every 3 days. Finally, the cells were stained with fluorescence-labeled mAbs against Vα24, Vβ11 and CD4. At this point, the rates of increase of total, CD4-positive and CD4-negative iNKT cells were calculated from the absolute numbers of relevant cells before and after the culture. Vα24+Vβ11+CD4+ cells and Vα24+Vβ11+CD4− cells were sorted by FACS Vantage (Becton Dickinson). The sorted cells whose purity was more than 85%, were used for cytokine producing analysis. Sorted iNKT cells were cultured at 1×10^5/well with 1×10^4/well αGalCer-pulsed or unpulsed allogenic mature Mo-DCs for 24 h. Mo-DCs as stimulators were obtained from the same donor. The supernatants of the culture were collected for cytokine ELISA.

2.7. ELISA

IFN-γ, IL-4, IL-5 and IL-13 concentrations of the supernatants were measured by ELISA. The paired Abs and standards were purchased from Endogen (Woburn, MA). The ranges of the assay were 0–1000 pg/ml for IFN-γ and IL-13, and 0–500 pg/ml for IL-4 and IL-5, respectively.

2.8. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test (StatView, SAS Institute, Cary, NC). A *P*-value of less than 0.05 was taken as statistically significant.

3. Results

3.1. Frequencies of peripheral iNKT cell subsets in chronic hepatitis C patients are comparable to those in healthy subjects

Human CD1d-restricted iNKT cells express a conserved canonical TCR α-chain (Vα24-JαQ) paired with TCR β-chain (Vβ11). Thus, we examined the frequencies of peripheral Vα24+Vβ11+ cells as iNKT cells in the CH and HS groups. Although the frequencies of these cells showed a wide range of distribution in both groups (HS = 0.01–0.61%, CH = 0.01–0.43%), no difference was observed in total iNKT cells and their CD4+ and CD4− subsets between the CH and the HS group (Fig. 1). In both CH and HS groups, there was no correlation between any of the iNKT frequencies and age, serum HCVRNA titers or alanine aminotransferese (ALT) levels (data not shown).

3.2. Peripheral iNKT cell subsets in chronic hepatitis C patients express higher levels of CXCR3 than in healthy subjects

Next, we examined the expressions of some chemokine receptors and homing receptor on peripheral iNKT cell subsets. The expression of CXCR3 on both subsets in the CH group was higher than that in the HS group (Fig. 2), whereas those of the others (CCR4, CCR7 or CD62L) were not different between the groups (Fig. 2).

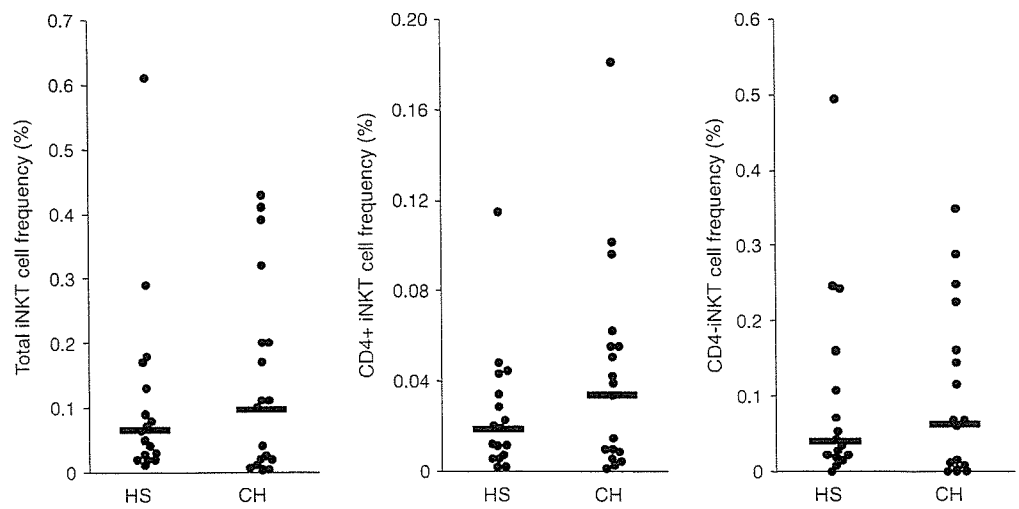

Fig. 1. Frequency of peripheral iNKT cell subsets in healthy subjects and chronic hepatitis C patients. The frequencies of total, CD4-positive and CD4-negative iNKT cells in PBMCs were determined by flow cytometry as described in Section 2. HS, healthy subjects; CH, chronic hepatitis C patients. Horizontal bars represent the median.

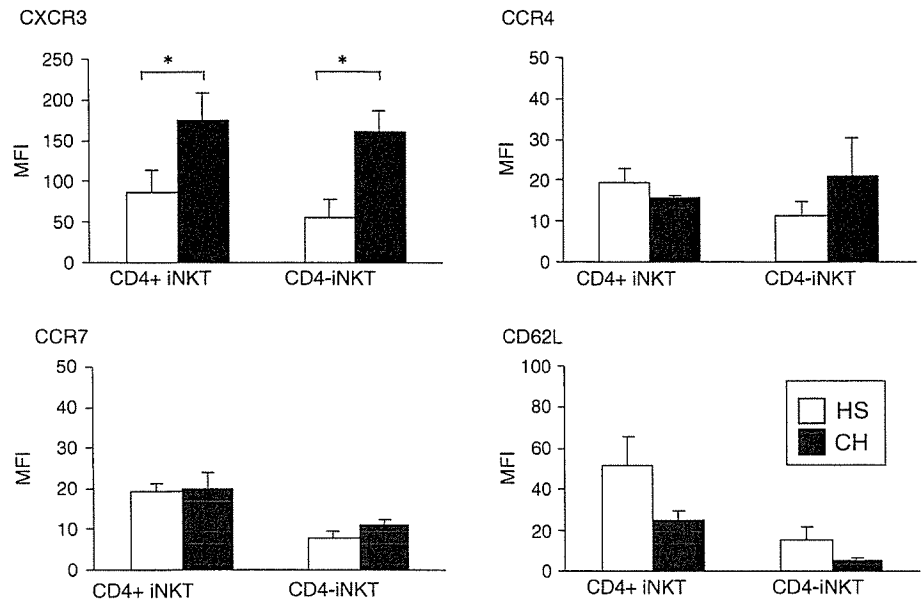

Fig. 2. Expressions of chemokine receptors and homing receptor on peripheral iNKT cell subsets in healthy subjects and chronic hepatitis C patients. The *y*-axis indicates the mean fluorescence intensity (MFI) of the expression of each receptor as determined by flow cytometry. The bars represent mean + SE of six different subjects. The white column represents the healthy subject group and the black one represents the chronic hepatitis C group. HS, CH, as in Fig. 1. *$P < 0.05$ by Mann–Whitney *U*-test.

3.3. The IFN-γ-producing capacity of peripheral iNKT cells in response to αGalCer in chronic hepatitis C patients is comparable with those in healthy subjects

With respect to the αGalCer-responsive IFN-γ-producing cells in PBMCs, the ELISPOT assay in this study detected as few as 10 spots/5×10^5 cells. When samples depleted of Vα24+ or Vβ11+ cells were used, the numbers of spots were significantly reduced, indicating that Vα24+ or Vβ11+ cells mainly released IFN-γ in response to αGalCer (data not shown). The numbers of IFN-γ-producing cells reactive to αGalCer in the peripheral blood were not different between the CH and the HS groups (Fig. 3). No correlation was observed between IFN-γ-producing cell number and serum HCVRNA titers or ALT levels. The levels of IL-4 and IL-13 in the supernatants were below the thresholds of ELISA (data not shown).

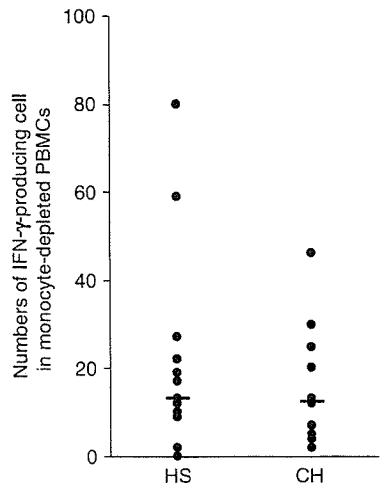

Fig. 3. Numbers of IFN-γ-producing cells in fresh PBMC stimulated with αGalCer-pulsed Mo-DCs. After monocyte-depleted PBMCs were co-cultured with αGalCer-pulsed autologous Mo-DCs for 24 h on IFN-γ mAbs-coating plates, the spots were developed and were counted as IFN-γ-producing cells as described in Section 2 (ELISPOT). The numbers of IFN-γ-producing cells in monocyte-depleted PBMCs from the HS and CH groups were counted as described above. Horizontal bars represent median. HS, CH, as in Fig. 1.

3.4. Peripheral iNKT cells from chronic hepatitis C patients proliferate in response to αGalCer at a level comparable to those from healthy subjects

Next, we compared the ability of peripheral iNKT cells to proliferate in response to αGalCer-loaded DCs between the CH and the HS groups. In all subjects, the absolute number of iNKT cells was significantly

Table 2
Increase of iNKT cell numbers expanded with αGalCer-loaded Mo-DCs after 5 weeks of culture

	Healthy subjects (-fold)	Chronic hepatitis C patients (-fold)
Total iNKT cells	148 (21–2143)	249 (87–2220)
CD4+ iNKT cells	182 (3–630)	254 (45–2209)
CD4− iNKT cells	124 (1–5856)	319 (113–2277)

Data express median (range).

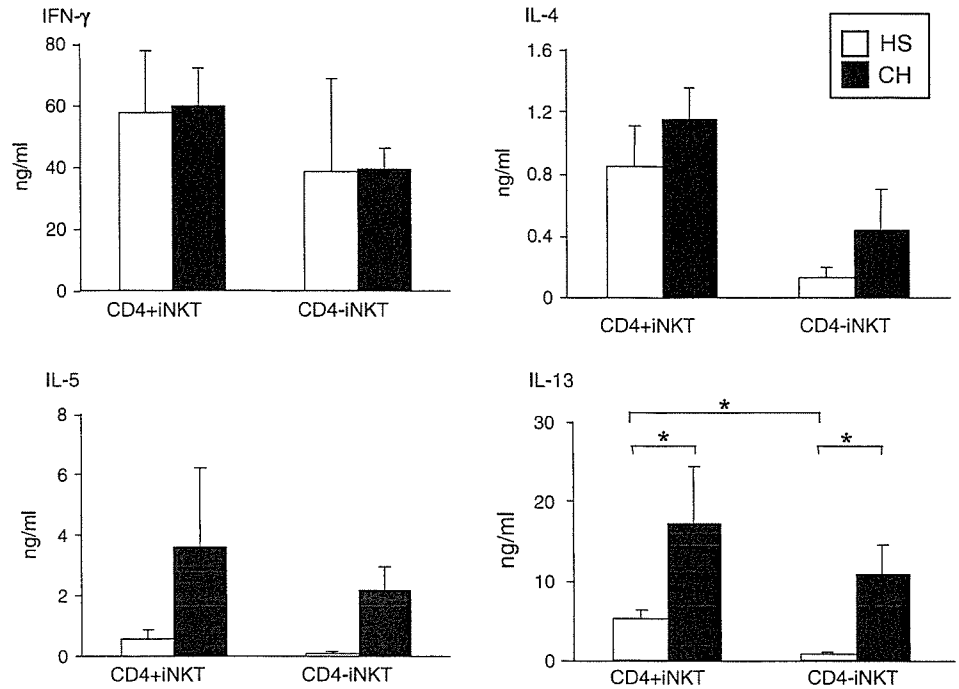

Fig. 4. Cytokine production from expanded iNKT cells stimulated with αGalCer-loaded Mo-DCs. Invariant NKT cells were expanded by culture with αGalCer-pulsed autologous Mo-DCs and subsequent cell sorting as described in Section 2. The activated iNKT cells were stimulated with αGalCer-pulsed allogeneic Mo-DCs for 24 h and the supernatants were collected for cytokine ELISA. The white column represents the healthy subject group and the black one represents the chronic hepatitis C group. The bars represent mean + SE of five different subjects. *$P < 0.05$ by Mann–Whitney U-test. HS, CH, as in Fig. 1.

increased after 5 weeks of culture. Although there was considerable variation among individuals in the proportion of iNKT cell increase (21–2220-fold), the total number of increased iNKT cells as well as their CD4 + or CD4− subsets in the CH group was comparable with those in the HS group (Table 2).

3.5. Expanded iNKT cells obtained from chronic hepatitis C patients produce more IL-13 in response to αGalCer than those from healthy subjects

In some reports, fresh isolated iNKT cells produce Th1 cytokines but not Th2 cytokines, while αGalCer-activated iNKT cells are able to produce both Th1 and Th2 cytokines [17,18]. Therefore, we compared the cytokine producing capacity of expanded iNKT cells. In contrast with the results of peripheral iNKT cells prior to in vitro expansion, the expanded iNKT cells produced considerable amounts of IL-4, IL-5 and IL-13 as well as IFN-γ in response to αGalCer (Fig. 4). The level of IFN-γ production from expanded iNKT cells did not differ between the CH and the HS groups, either from CD4 + or CD4− subsets (Fig. 4). With regard to Th2 cytokines, CD4 + iNKT cells in the HS produced significantly more IL-13 and tended to produce more IL-4 and IL-5 than the CD4− iNKT cells. In contrast, in the CH group, similar predominance of CD4 + iNKT cells over CD4− cells in Th2 cytokine production was observed, but did not reach a significant level. Of particular interest is the finding that a significantly larger amount of IL-13 was released from CD4 + as well as CD4− iNKT cells in the CH group as compared to those in the HS group (Fig. 4). A similar tendency was observed as the enhanced production of IL-4 and IL-5 from iNKT cells in the CH group. Thus, the activated iNKT cells obtained from chronic hepatitis C patients release more Th2-type cytokines, most significantly IL-13, than those from healthy subjects.

4. Discussion

Invariant NKT cells play distinctive roles in the regulation of immune responses in various diseases. In HIV infection, iNKT cells decrease in parallel with an increase in the viral load, which is due to direct HIV infection to these cells [11,13]. As for the frequency of peripheral iNKT cells in HCV infection, some conflicting results have been published. It has been reported that the number of iNKT cells in HCV-infected patients was in the same range as that in healthy subjects [15,16]. In contrast, other report showed that their number was

less in HCV viremic patients than non-viremic patients or healthy individuals [14]. In the present study, the frequencies of iNKT cells and their subsets in HCV-infected patients were comparable with those in healthy subjects. Several investigators reported that there are certain factors influencing iNKT cell numbers, such as gender and age in the study population [19,20]. Although no significant correlation was found between these background factors and iNKT cell frequency in our study, demographic as well as ethnic differences in the subjects might be involved in the discordant observations among these studies.

Limited numbers of peripheral iNKT cells have hampered the progress of research on the function of these cells. We used ELISPOT assay to analyze IFN-γ production from freshly isolated peripheral iNKT cells. The IFN-γ producing capacity of fresh peripheral iNKT cells from HCV-infected patients was comparable with those from healthy subjects. However, fresh iNKT cells produced negligible amounts of Th2 cytokines regardless of the presence or absence of HCV infection. Several investigators have reported that fresh iNKT cells are capable of predominantly producing Th1 cytokines compared to Th2 cytokines in response to αGalCer [17,18]. However, iNKT cells cultured with αGalCer are reported to gain the capacity to produce both Th1 and Th2 cytokines [17,18,21], implying that activated iNKT cells are able to secrete Th2 cytokines.

To analyze the capacity of cytokine production of activated iNKT cells, we thus expanded fresh iNKT cells with αGalCer-loaded DCs. However, the possibility remains that the experimental conditions may influence on iNKT cell functions, resulting in the functional difference between in vitro cultured iNKT cells and fresh iNKT cells in vivo. In the present study, the proliferative capacity of iNKT cells from HCV-infected patients was comparable with those from healthy donors, implying that expanded cells reflect the cell functions in vivo either in patients or donors. In clear contrast with fresh iNKT cells, the expanded iNKT cells from HCV-infected patients were able to produce a comparable amount of IFN-γ but more Th2 cytokines, most significantly IL-13, than those from healthy subjects. The mechanisms that induce Th2 bias of iNKT cells in HCV infection are yet to be demonstrated. Since iNKT cells were activated with αGalCer-pulsed autologous DCs, the functional alteration of DCs in HCV infection [22,23] may be responsible for the Th2 bias of activated iNKT cells.

With regard to the involvement of iNKT cells in the pathogenesis of chronic hepatitis C, several possibilities can be considered. First, Th2-biased iNKT cells may impede HCV clearance by suppressing the Th1 response. In this study, IL-13 production from expanded iNKT cells was not correlated with serum HCVRNA titres. However, IFN-γ from CD4+ iNKT cells tended to be inversely correlated with serum HCVRNA levels ($P = 0.07$, data not shown), while IL-4 from CD4− iNKT cells was positively correlated with HCVRNA quantity ($P = 0.07$, data not shown), suggesting an active role of iNKT cells in the control of HCV replication. Second, iNKT cells are involved in the progression of fibrosis in HCV-infected liver. Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are profibrogenic to the liver [21,24,25]. If this is the case, the present study suggests that iNKT cells in chronic HCV infection are profibrogenic per se even in the pre-cirrhotic stage. Third, it is conceivable that the secretion of Th2 cytokines from iNKT cells is one of the compensatory mechanisms of liver inflammation. In patients with multiple sclerosis, a chronic inflammatory disease of the central nervous system, who are in remission, the iNKT cells produce a larger amount of IL-4 than those from patients in relapse [10], suggesting that iNKT cells may alleviate Th1-mediated inflammation by releasing Th2 cytokines. Analyses of liver-infiltrating lymphocytes in HCV-infected liver have disclosed that Th1-type CD4 T cells are compartmentalized [26], suggesting that the Th1 response is related to liver injury. Our study showed that CXCR3 expression on peripheral iNKT cells was higher in the CH group than those in the HS, whereas CCR7 or CD62L expressions were low in both groups. These results suggest that peripheral iNKT cells in HCV infection are prone to be of the tissue-infiltrating type, not the lymphoid-homing type. It has been reported that the expressions of IP-10 or MIG, which are ligands of CXCR3, are increased in HCV-infected liver, in correlation with the degree of inflammation [27–29]. Additionally, enhanced expression of CD1d is detected on the inflammatory infiltrates in HCV-infected liver [21,29,30]. Therefore, our results indicate the following hypothetical pathway of iNKT cell recruitment. Invariant NKT cells expressing CXCR3 tend to be mobilized in an inflamed liver, where they are activated via CD1d-expressing cells, and subsequently secrete large amount of Th2 cytokines.

Of particular importance is the large population of iNKT cells in liver-infiltrating lymphocytes compared to peripheral blood even in the steady state [31,32]. Thus, further examinations of liver-infiltrating iNKT cells are arguably necessary to understand the precise roles of iNKT cells in HCV-infected liver.

In summary, we demonstrated that the number and functions of peripheral iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state, but activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls. The altered functions of these cells in chronic hepatitis C may be involved in the pathogenesis of HCV-induced liver disease.