Table 3 Univariate analyses of immunological factors involved in SVR

Factors	SVR	Non-SVR	*P* value
N	29	38	
DC pre (/μl)	13.3 ± 6.5	10.3 ± 5.4	0.038
PDC-12W (/DC)	0.23 ± 0.09	0.18 ± 0.07	0.017
PDC-12W (/DC) ratio	1.42 ± 0.72	1.04 ± 0.63	0.028
Treg-12W (/CD4) ratio	2.49 ± 2.62	1.03 ± 0.64	0.016

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre DC number before therapy, *PDC-12W (/DC)* PDC frequency in DC at T12W, *PDC-12W (/DC) ratio* the ratio of PDC frequency in DC at T12W to the pretreatment value, *Treg-12W (/CD4) ratio* the ratio of regulatory T cell frequency in CD4 at T12W to the pretreatment value

Table 4 Multivariate analyses of clinical and Immunological factors involved in SVR

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.531	0.322–0.875	0.013
Treg-12W (/CD4) ratio	<1.2/>1.2	0.026	0.001–0.750	0.033

Logistic regression analysis, stepwise method

Table 5 Univariate analyses of clinical factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	EVR-SVR	EVR-TR
N	24	8
Age (years)	46.9 ± 12.3*	57.6 ± 6.5
Gender (M/F)	17/7	6/2
WBC (/mm^3)	5442 ± 1382	5211 ± 805
Neutro (/mm^3)	2975 ± 890	2587 ± 759
Hb (g/dl)	14.7 ± 1.1	15.1 ± 1.2
Platelets (×10^4/mm^3)	18.7 ± 4.5	15.0 ± 3.8
ALT (IU/l)	69 ± 56	91 ± 61
HCV RNA (KIU/ml)	1723	1296
Activity: 0–1/2–3/n.d.	24/0/0	6/2/0
Fibrosis: 0–2/3–4/n.d.	16/8/0	5/3/0
PEG-IFN dose (μg/kg/day)	1.43 ± 0.15	1.39 ± 0.23
Ribavirin dose (mg/kg/day)	10.8 ± 1.5	10.1 ± 2.1

Mann–Whitney *U* test, chi-square test

n.d. not determined, *EVR-SVR* SVR patients who attained complete EVR at T12W, *EVR-TR* TR patients who attained complete EVR at T12W

$^*P < 0.05$

frequency during therapy serve as an independent immunological predictor for SVR in patients who attained c-EVR with PEG-IFNα and ribavirin therapy.

Table 6 Univariate analyses of immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	EVR-SVR	EVR-TR	*P* value
N		24	8	
DC pre (/μl)		13.5 ± 6.8	8.9 ± 4.5	0.030
PDC-12W (/DC) ratio	<0.8/>0.8	3/21	4/4	0.047

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre, PDC-12 (/DC) ratio: see Table 3

Table 7 Multivariate analyses of clinical and immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.627	0.402–0.978	0.040
PDC-12W (/DC)	<0.18/≥0.18	0.028	0.001–0.787	0.036
PDC-12W (/DC) ratio	<0.8/≥0.8	0.032	0.002–0.673	0.027

Logistic regression analysis, stepwise method

PDC-12W (/DC), PDC-12W(/DC) ratio: see Table 3

Discussion

In this study, we demonstrated that the increase of Treg frequency during therapy is involved in SVR, and that of PDC is in SVR patients who attained c-EVR in 48 weeks of PEG-IFNα and ribavirin therapy. Of particular importance is that such significance is independent of viral dynamics (c-EVR), host factors (fibrosis, gender), and drug adherence.

Regulatory T cells (Treg) are immune suppressors that are supposed to alleviate HCV-induced liver inflammation. In chronic HCV infection, the increment of Tregs has been reported by several investigators, including us, although the underlying mechanisms were unspecified [20, 22]. The increase of Treg in SVR patients observed herein seems to be inconsistent with the previous reports regarding Treg as a tolerance inducer in chronic hepatitis C patients. Several controversial reports have been published with regard to the involvement of Tregs in the efficacy of PEG-IFNα and ribavirin therapy for chronic hepatitis C. Soldevila et al. [23] showed that the pretreatment frequency of Treg is higher in patients with non-response (NR) than those in the non-NR groups. Akiyama et al. [24] reported that Tregs in PBMC increased in SVR patients at earlier time points, while Tregs in liver-infiltrating lymphocytes decreased. By contrast, another group disclosed that frequency, phenotype, and function of Tregs are comparable regardless of the outcomes of PEG-IFNα and ribavirin therapy [25].

The current observation raises the possibility that the reduction of HCV load and/or liver inflammation correlates with the increment of Treg frequency, or vice versa. Recently, it was reported that liver inflammation caused by HCV induces PD-L1 on hepatocytes, which then suppress Treg proliferation in liver [26]. If such a scenario is operative as well in PEG-IFNα and ribavirin therapy, alleviation of liver inflammation may reduce PD-L1 expression on hepatocytes, thereby stimulating Treg proliferation. However, most of the TR patients, who were categorized as being in the non-SVR group, displayed normalized serum ALT levels and negative HCV RNA during treatment, of which conditions are equivalent with the SVR patients. Thus, it is still uncertain whether or not such mechanisms are applicable to the present results.

The other possibility is that phenotypically determined Tregs in this study partly consist of activated T cells. It is well known that $CD127^-$ and $FOXP3^+$ are reliable markers of Tregs [27]. In order to examine whether or not the increment of Treg frequency in this study is a contamination of activated T cells, we determined Tregs as $CD4^+CD25^{high}FOXP3^+CD127^-$ cells instead of $CD4^+CD25^{high}$ cells in some patients. In the comparison of the ratio of $CD4^+CD25^{high}FOXP3^+CD127^-$ cell frequency between the SVR and non-SVR groups at T12W, similar results were obtained with those of $CD4^+CD25^{high}$ cells (SVR vs. non-SVR, 10 patients in each group, 2.50 ± 1.20 vs. 1.54 ± 0.53, $P < 0.05$ by Mann–Whitney U test). These results suggest that the analytical results of $CD4^+CD25^{high}$ T cells reflect those of $FOXP3^+$ Tregs. Further investigation is needed to show that such Tregs are functionally suppressive and to see if the change of frequency parallels with suppressor capacity or not.

According to the AASLD practice guidelines for the treatment of chronic hepatitis C, a combination of PEG/R for 48 weeks is recommended for patients who attained c-EVR at week 12 of therapy [17]. However, in some cohorts with large numbers of patients, approximately 30% of them eventually relapse after cessation of the therapy [5]. The factors involved in post-therapeutic relapse have not been fully explored. We and others have reported that liver fibrosis, female gender, late virological response, and dosage of ribavirin (drug adherence) are critically involved in relapse [19, 28, 29]. It is well known that platelet counts in patients with chronic liver disease are well correlated with the degree of fibrosis. In the present study, multivariate analyses revealed that platelet counts but not fibrosis stage are involved in SVR. The reasons for such discrepant contributions to SVR are not clear; however, it demonstrates that the degree of fibrosis is involved in the therapeutic response in this cohort. In addition, the current study showed that the changes of PDC frequency are also somewhat involved in virological relapse in patients that once attained c-EVR.

Plasmacytoid DCs (PDC) play crucial roles in antivirus immune responses by producing IFN-β and -α [30]. In the previous study by us [14], the increment of PDC is observed in patients with SVR, of which change is more significant in those with c-EVR. No concrete explanation is available for the mechanisms of PDC increase in SVR patients. One of the possibilities is that the PDC increase is a consequence of better response to exogenous IFN-α in patients who have a higher chance of attaining SVR. IFN-α is reported to act as a regulatory factor on $CD11c^-$ DCs to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [31]. Such a possibility is supported by the findings that higher induction of IFN-stimulated genes (ISGs) in hepatocytes after PEG-IFNα and ribavirin therapy, but not higher ISG levels before therapy, is critically involved in successful outcome [32]. Thus, patients who respond well to IFN-α, as demonstrated by better PDC survival during the treatment, are likely to have better chances to eradicate HCV.

Another possible reason for the PDC increase in the periphery of SVR patients is that PDC alter their localization during the treatment. Mengshol et al. [33] reported that PDC and myeloid DC (MDC) are accumulated in inflamed liver through the interactions of chemokines and their receptors. Of particular interest is that the expression of such chemokine receptors on DCs decreased in SVR patients, but not in non-SVR ones [33]. Therefore, it is plausible that PDC may migrate from the liver to periphery/lymphoid tissue after being unleashed from chemokines in the liver. In support for this, it is reported that IFN-α alters the profiles of chemokine receptors on DC, resulting in changes of the DC migrating ability [34].

Recently, numerous other factors were reported to be involved in therapeutic response in chronic hepatitis C patients, such as mutations of HCV genome (core region) [35] or host genetic variation (single nucleotide polymorphisms near the IL28B gene) [36]. In the current study, we were unable to analyze such factors because of the limited numbers of patients. A prospective study is warranted to analyze the involvement of such factors in relation to immune cell markers, in the outcomes of SOC, or the treatment with direct-acting antiviral agents.

In summary, we demonstrated that the increase of Treg frequency is an independent factor involved in SVR in 48 weeks of SOC for chronic hepatitis C patients. In addition, the increase of PDC gains similar significance in SVR patients who attained c-EVR. The assessment of the dynamics of such cells during therapy could offer some clues to identify potential relapsers and give them a better chance of attaining SVR by rescheduling the therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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Comparative analyses of regulatory T cell subsets in patients with hepatocellular carcinoma: A crucial role of $CD25^{-}FOXP3^{-}$ T cells

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Regulatory T cells (Tregs) play pivotal role in cancer-induced immunoediting. Increment of $CD25^{high+}FOXP3^{+}$ natural Tregs has been reported in patients with hepatocellular carcinoma (HCC); however, the involvement of other type of Tregs remain elusive. We aimed to clarify whether $FOXP3^{-}$ Tregs are increased and functionally suppressive or not in patients with HCC. We enrolled 184 hepatitis C-infected patients with chronic liver diseases or HCC, 57 healthy subjects and 27 HCC patients with other etiology. Distinct Treg subsets were phenotypically identified by the expression of CD4, CD25, CD127 and forkhead/winged helix transcription factor (FOXP3). Their gene profiles, frequency and suppressor functions against T cell proliferation were compared among the subjects. To examine the molecules involving in Treg differentiation, we cultured naive $CD4^{+}$ T cells in the presence of HCC cells and dendritic cells. We determined two types of $CD4^{+}CD127^{-}$ T cells with comparable regulatory ability; one is $CD25^{high+}$ cells expressing FOXP3 ($CD25^{high+}FOXP3^{+}$ Tregs) and the other is $CD25^{-}$ cells without $FOXP3^{-}$ expression ($CD25^{-}FOXP3^{-}$ cells). The peripheral or intrahepatic frequency of $CD25^{-}FOXP3^{-}$ Tregs in HCC patients is higher than those in other groups, of which significance is more than $CD25^{high+}FOXP3^{+}$ cells. Of importance, $CD25^{-}FOXP3^{-}$ Tregs, but not $CD25^{high+}FOXP3^{+}$ cells, dynamically change in patients accompanied by the ablation or the recurrence of HCC. $CD25^{-}FOXP3^{-}$ T cells with $CD127^{-}IL\text{-}10^{+}$ phenotype are inducible *in vitro* from naive $CD4^{+}$ T cells, in which programmed cell death 1 ligand 1, immunoglobulin-like transcript 4 and human leukocyte antigen G are involved.. In conclusion, $CD25^{-}FOXP3^{-}$ Tregs with suppressive capacity are increased in patients with HCC, suggesting their distinct roles from $CD25^{+}FOXP3^{+}$ Tregs.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths in the world.[1] One of the most prevalent risk factors for HCC is hepatotropic viruses, such as hepatitis B (HBV) or C (HCV) virus.[2,3] In the process of HCC development, the involvement of tumor-induced immune suppression; *i.e.*, immunoediting, has been implicated. Regulatory T cells (Tregs) are unique subset of T cells, playing essential roles in the maintenance of immune homeostasis or in the protection of hosts from virulent infections and cancers.[4] Generally, the existence of two types of Tregs has been reported. One is naturally occurring $CD4^{+}CD25^{high+}$ Tregs, which are derived from the thymus and suppress auto-reactive T cells. The other is inducible or adaptive Tregs, including interleukin (IL)-10-secreting type-1 regulatory T cells (Tr1) and transforming growth factor (TGF)-β-producing Th3. These are inducible in the

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Key words: HCC, regulatory T cells, FOXP3, CD25, CD127

Abbreviations: CTLA-4: cytotoxic T-lymphocyte antigen 4; DC: dendritic cell; FOXP3: forkhead/winged helix transcription factor; GITR: glucocorticoid-induced TNF receptor family-regulated gene; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; IL-T4: immunoglobulin-like transcript 4; LAG-3: lymphocyte-activation gene 3; PBMC: peripheral blood mononuclear cell; PD-1: programmed cell death 1; PD-L1: programmed cell death 1 ligand 1; RFA: radiofrequency ablation; RT-PCR: reverse transcription polymerase chain reaction; Tr1: type-1 regulatory T cells; Tregs: regulatory T cells

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Table 1. Clinical backgrounds of the patients enrolled in the study[1]

	HV	CH (C)	LC (C)	HCC (C)	HCC (B)	HCC (NBNC)
N	57	66	39	79	12	15
Gender (M/F)	35/22	44/22	23/16	44/35	8/4	9/6
Age (years)	56 ± 11	56 ± 18	61 ± 9	66 ± 11	56 ± 9	62 ± 13
ALT (IU/l)	ND	70 ± 15	44 ± 13	56 ± 17	65 ± 7	45 ± 11
Platelets (10^4/μl)	ND	15 ± 4	11 ± 4	12 ± 4	13 ± 4	12 ± 4
Total bilirubin (mg/ml)	ND	0.9 ± 0.4	1.6 ± 0.4[2]	0.9 ± 0.3	0.6 ± 0.1	0.7 ± 0.3
Alb (g/dl)	ND	3.7 ± 0.5	3.3 ± 0.4	3.1 ± 0.6[2]	3.5 ± 0.2	3.6 ± 0.3
AFP (ng/ml)[3]	ND	2-115 (15)	2-347 (16)	4-33357 (43)	7-12 (10)	10-16520 (23)
TNM stage[4] (I + II/III + IV)	–	–	–	55/24	9/3	9/6

[1]All values except for AFP are expressed as mean ± standard deviation. [2]$p < 0.05$ *vs.* CH (C) group. [3]Values are expressed as range (median).
[4]Seventh edition of International Union Against Cancer TNM staging system of HCC.
Abbreviations: HV, healthy volunteers; CH (C), LC (C), HCC (C), HCV-positive chronic hepatitis, liver cirrhosis and hepatocellular carcinoma; HCC (B), HBV-positive hepatocellular carcinoma; HCC (NBNC), non-B, non-C hepatocellular carcinoma; ALT, alanine aminotransferase; Alb, albumin; AFP, alfa-fetoprotein; ND, not determined.

periphery and are endowed with the ability to suppress antigen-specific T cells.[5] Several reports have shown that natural Tregs are increased in peripheral blood and/or tumor in patients with various types of cancer.[6] In HBV-infected HCC patients, an increase in natural Tregs and their suppressor functions against antigen-specific CTLs has been reported.[7] A correlation has been observed between natural Treg frequency and recurrence-free or overall survival of HCC patients.[8] However, it is yet to be determined if a distinct Treg subset is involved or not in the development of HCC.

The forkhead/winged helix transcription factor, FOXP3, is acknowledged as a major and specific marker of Tregs, the cellular expression of which is correlated with suppressive activities.[9] However, in the differentiation from naive T cells to effector/memory T cells, FOXP3 is transiently expressed but not sustained, suggesting that some proportion of FOXP3$^+$ T cells are not regulatory but activated ones.[10] These observations suggest that using FOXP3 as a marker of functionally regulatory cells would be limited and not suitable for adaptive Tregs. In recent studies, the expression of IL-7 receptor alpha chain (CD127) was found to be downregulated in Tregs and CD127 expression to be inversely correlated with FOXP3 expression.[11,12] Moreover, CD127-negative T cells are endowed with suppressive ability irrespective of their CD25 expression.[13] Alternatively, several studies have shown that CD127 is downregulated on FOXP3$^-$ Tr1 cells.[14,15] Due to the lack of specific or appropriate markers for identification of adaptive Tregs, it is yet to be confirmed that FOXP3$^-$ T cells are adaptive Tregs. Furthermore, little is known about the precise roles of FOXP3$^-$ regulatory cells in the development of HCC.

In this study, we focused on FOXP3$^-$ Tregs and tried to elucidate whether or not such cells are associated with the presence of HCC. To assess the feasibility of FOXP3$^-$ cells as a therapeutic target for immunological control of HCC, we tried to clarify the molecular mechanisms of its induction.

Material and Methods

Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, we enrolled 184 patients who were further categorized into three groups according to the stages of liver disease: chronic hepatitis (CH), liver cirrhosis (LC) and HCC groups. The clinical stage of HCC was determined according to the TNM classification system of the International Union against Cancer (seventh edition). The study protocol was approved by the ethical committee at the Osaka University Graduate School of Medicine. At enrollment, written informed consent was obtained from all patients and volunteers. Some of HCC patients in this study received radiofrequency ablation (RFA) therapy. Indication for RFA therapy was based on therapeutic guidelines for HCC promoted by the Japan Society of Hepatology.[16] After the RFA session, the efficacy of tumor ablation or HCC recurrence thereafter was evaluated by computed tomography or magnetic resonance imaging scanning. In some of the HCC patients who underwent surgical resection, cancerous and adjacent noncancerous tissues were obtained at operation for further Treg analyses. As controls, 57 healthy subjects (HS) without history of liver diseases, 27 HCC patients with HBV infection (HBV–HCC group), those without HBV and HCV (non-B–, non-C [NBNC]–HCC group). The clinical backgrounds of the subjects are shown in Table 1.

Frequency analyses of peripheral and liver-infiltrating Tregs

Peripheral blood mononuclear cells (PBMCs) were stained with a combination of various fluorescence-labeled anti-human mouse or rat monoclonal antibodies (mAbs) as reported previously (17). The mAbs for CD4, CD25, CD127, FOXP3 and IL-10 were purchased from Becton Dickinson Biosciences (San Jose, CA). Fresh liver specimens were

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washed twice with phosphate-buffered saline and were diced into 0.5 mm pieces. After these pieces were passed through a nylon mesh, liver-infiltrating lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation. These cells were stained with fluorescence-labeled Abs as performed for PBMC. For the analyses of FOXP3 and IL-10, we performed intracellular staining using a human FOXP3 staining kit (BD Biosciences) according to the manufacturer's instructions. The stained cells from PBMC or liver were analyzed by FACS Canto (BD Biosciences) and Cell Quest software.

Functional analysis of regulatory T cell subsets

To obtain live Tregs for functional analyses, we collected four populations of $CD4^+$ T cells according to the patterns of CD25 and CD127 expressions by FACS Aria (BD Biosciences). We cocultured various numbers of sorted cells with 1×10^5 allogenic naive $CD4^+CD25^-$ T cells in the presence of agonistic anti-CD3 and anti-CD28 Abs (BD Biosciences Pharmingen) on 96-well flat-bottom plates (Corning, Corning, NY) for 5 days. The proliferation of cells was assessed by incorporation of [3H]-thymidine. To clarify the suppression mechanism by Tregs, the cells were cultured with or without separation by transwell inserts (pore size 0.4 μm, Corning). Alternatively in some experiments, the cells were cultured in the presence or absence of neutralizing 10 ng/ml anti-IL-10 or anti-TGF-β Abs (R&D Systems, Mckinley, MN) or isotype IgG.

To examine regulatory cells possess suppressive function on recall antigen-specific $CD4^+$ T cell responses, we cocultured 1×10^4 each of sorted cells from some HCC patients with 1×10^5 autologous $CD4^+$ T cells in the presence or absence of 20 μg/ml of tetanus toxoid (Sigma) for 5 days, stimulated with 10 IU/ml of recombinant human IL-2 (BD Pharmingen). The proliferation of cells was assessed using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl) -5 -(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reagent in the Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instructions.

Real-time RT-PCR

To analyze gene profiles of Tregs, we collected $CD4^+CD25^{high+}CD127^-$ and $CD4^+CD25^-CD127^-$ T cells using FACS Aria. Extraction of total RNA and subsequent real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as reported previously with some modifications.[17] Assays-on-demand primers and probes (Applied Biosystems, Foster City, CA) were used to quantify FOXP3, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor family-regulated gene (GITR), lymphocyte-activation gene 3 (LAG3), IL-21, programmed cell death 1 (PD-1) and c-masculoaponeurotic fibrosarcoma (c-Maf) expression. The expressions of molecules were given as the relative values to the calibrator samples. To standardize the amount of total RNA, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values with this.

Induction of $CD4^+CD25^-CD127^-FOXP3^-$ T cells from PBMC

To clarify the molecular mechanisms of Treg induction, we cultured 1×10^6 naive $CD4^+CD25^-$T cells with 1×10^5 autologous monocyte-derived dendritic cells (DCs) and mitomycin C (Sigma-Aldrich, St. Louis, MO)-treated 1×10^5 HCC cell lines, Huh7 or HepG2 (AmericanType Culture Collection, Manassas, VA) on 24-well flat-bottom plates for 5 days. Monocyte-derived DCs were generated from $CD14^+$ cells as reported previously.[18] On days 2 and 4 of the coculture, recombinant human IL-2 (10 IU/ml), IL-10 (20 IU/ml) and IL-15 (20 IU/ml; BD Pharmingen) were added to the cells. On day 6, they were stimulated with phorbol 12-myristate 13 acetate (PMA; 1 ng/ml) and ionomycin (1 μmol/l) in the presence of anti-CD3 mAb (1 μg/ml) and breferdin A (1 μg/ml) (BD Pharmingen). In some experiments, we separated relevant cells by transwell inserts (pore size 0.4 μm) or added 10 μg/ml neutralizing Abs against TGF-β (R&D), HLA-DR (BD), PD-1 (R&D), programmed cell death 1 ligand 1 (PD-L1; e-Bioscience) or immunoglobulin-like transcript 4 (IL-T4) (e-Bioscience) during the culture. Subsequently, the cells were stained with Abs for CD4, CD25, CD127, FOXP3 and IL-10 and then were subjected to FACS analysis.

Knockdown of PD-L1 and HLA-G genes in HCC cell lines by siRNA

To confirm the molecules involving Treg induction, we knocked down PD-L1 and HLA-G genes in Huh7 cells by means of RNA interference. We used the small interfering RNA (siRNA) cocktail targeting human CD274 (PD-L1) or human leukocyte antigen G (HLA-G), provided by COSMO BIO (Tokyo, Japan). Transfection of siRNA to Huh7 or HepG2 cells was performed using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. To assess the efficiency of transfection, we compared the mRNA expression of target genes before and after the procedure by real time RT-PCR.

Statistical analyses

The Jonckheere-Terpstra test was used for the analysis of dose-dependent tendency. The Mann-Whitney nonparametric *U* test was used to compare differences in unpaired samples and Kruskal-Wallis nonparametric tests were used to compare differences among multiple groups, respectively. Friedman test with Bonferroni multiple comparison tests was used to compare differences in paired samples. All tests were two-tailed, and a $p < 0.05$ was considered statistically significant.

Results

$CD4^+$ T cells with distinct patterns of CD127 and FOXP3 expression were identified

According to the expression of CD25 and CD127 in $CD4^+$ T cells, we separated them into four groups: $CD25^{high+}CD127^-$, $CD25^-CD127^-$, $CD25^{high+}CD127^+$ and $CD25^-CD127^+$ cells, respectively (Fig. 1*a*). Most of the $CD4^+CD25^{high+}CD127^-$

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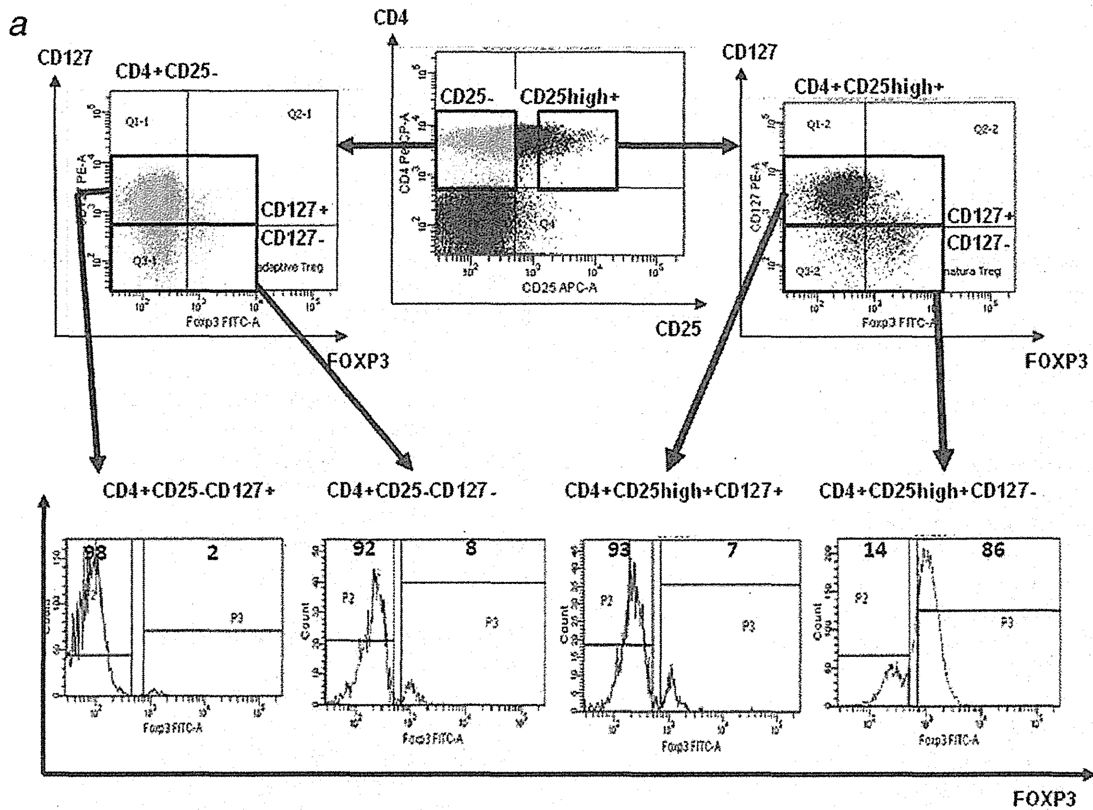

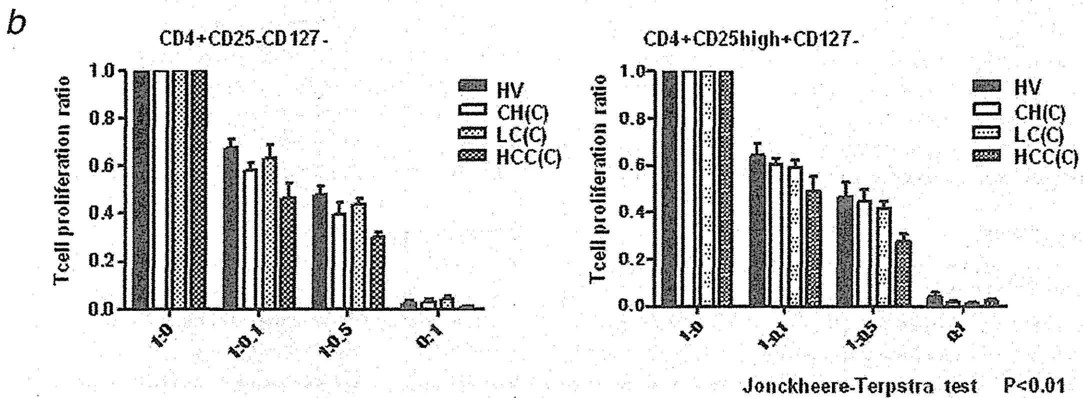

Figure 1. $CD4^+CD25^-CD127^-$ and $CD4^+CD25^{high+}CD127^-$ T cells are Tregs. (*a*) $CD4^+$ T cells are separated into four subpopulations: $CD4^+CD25^{high+}CD127^+$, $CD4^+CD25^{high+}CD127^-$, $CD4^+CD25^-CD127^+$ and $CD4^+CD25^-CD127^-$ cells, respectively. These cells were examined for FOXP3 expression. The numbers in the histograms depict the percentages of gated cells. Representative plots from three patients and donors are shown. (*b*) Sorted $CD4^+CD25^-CD127^-$ T cells and $CD4^+CD25^{high+}CD127^-$ T cells obtained from patients and healthy donors were added at various ratios to allogenic $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 Abs. After 5 days of culture, $CD4^+$ T cell proliferation was evaluated by incorporation of ^{3}H-thymidine. The bars indicate the ratio of counts per minutes (cpm) in various responders to regulatory cells ratio to those at 1:0. The results are shown as mean + SEM of ten patients or donors in each group. The dose dependency was analyzed by Jonckheere Terpstra test and comparison among the disease statuses was analyzed by Wilcoxon rank sum test with Bonferroni multiple comparison test. HV, healthy volunteers; CH(C), LC(C), HCC (C), HCV-infected chronic hepatitis, liver cirrhosis or hepatocellular carcinoma, respectively.

cells express FOXP3 (>80%). In contrast, the populations of $CD4^+CD25^-CD127^-$, $CD4^+CD25^{high+}CD127^+$ and $CD4^+CD25^-CD127^+$ lack FOXP3 expression (<10%). These results show that, except for $CD4^+CD25^{high+}CD127^-$ cells, the remaining $CD127^-$ cells lack FOXP3 and CD25 expression ($CD4^+CD25^-CD127^-FOXP3^-$).

$CD4^+CD25^{high+}CD127^-$ cells and $CD4^+CD25^-CD127^-$ T cells are suppressors against allogeneic T cells with distinct mechanisms

To examine which cell populations exert a suppressive capacity, we added each phenotype of cells separated from the subjects to allogeneic $CD4^+$ T cells. The sorted $CD4^+CD127^+$ T cells had no regulatory activities regardless of CD25 expression (data not shown). In contrast, $CD127^-$ cells, either $CD25^-$ or $CD25^{high+}$, significantly inhibited allogenic $CD4^+$ T cell proliferation in a dose-dependent manner, at comparable levels (Fig. 1*b*). Of note is that their suppressive capacity did not differ at the single cell level between patients and donors, regardless of the stage of liver disease (Fig. 1*b*). In addition, $CD127^-$ cells are anergic irrespective of CD25 expression (Fig. 1*b*). The suppressive ability of $CD4^+CD25^{high+}CD127^-$ cells was significantly abrogated by transwells and anti-TGF-β Ab, suggesting that they work in cell–cell contact-dependent and TGF-β-dependent manners (Supporting Information Fig. 1). By contrast, suppression by $CD4^+CD25^-CD127^-$ cells was alleviated by anti-IL-10 Ab but not by transwells, showing that they are contact-independent but IL-10-dependent (Supporting Information Fig. 1). These results show that $CD4^+CD25^-CD127^-$ cells possess a suppressive capacity with distinct machinery from $CD4^+CD25^{high+}CD127^-$ cells. In the setting of tetanus toxoid-reactive $CD4^+$ T cell response, each type of cells tended to be comparably suppressive (Supporting Information Fig. 2).

$CD4^+CD25^{high+}CD127^-$ and $CD4^+CD25^-CD127^-$ T cells display distinct gene profiles

$CD4^+CD25^{high+}CD127^-$ and $CD4^+CD25^-CD127^-$ T cells were sorted by FACS Aria and were subjected to real-time RT-PCR analyses. The expressions of FOXP3, CTLA-4 and GITR in $CD4^+CD25^{high+}CD127^-$ cells were higher than those in $CD4^+CD25^-CD127^-$ T cells, while those of LAG-3, IL-21, PD-1 and c-Maf in $CD4^+CD25^-CD127^-$ T cells were higher than those in $CD4^+CD25^{high+}CD127^-$ cells, respectively (Fig. 2). Thus, these two types of regulatory cells have distinct molecular profiles. As we described in the previous sections, $CD4^+CD25^-CD127^-$ cells with regulatory capacity lack FOXP3 expression (Figs. 1 and 2). Thus, we tentatively defined such cells as $CD25^-FOXP3^-$ Tregs in the following parts.

$CD25^-FOXP3^-$ Tregs are increased in HCC patients and their increments are associated with cancer progression

We compared the frequency of Treg subsets among healthy donors and HCV-infected patients. In HCC patients, $CD25^-FOXP3^-$ Tregs or $CD4^+$ $CD127^-$ $CD25^{high+}$ $FOXP3^+$ cells ($CD25^{high+}FOXP3^+$ Tregs) frequency in the periphery was significantly higher than those in other groups (Fig. 3*a*). The frequency of each type of Tregs is not correlated with HCV quantity (Supporting Information Fig. 3). These results show that the increase in $CD25^-FOXP3^-$ or $CD25^{high+}FOXP3^+$ Tregs is correlated with the development of liver cancer, but not with HCVRNA titers. Such increment of peripheral Tregs is also observed in HBV-HCC or NBNC-HCC patients (Fig. 3*a*).

Next, we compared the frequency of Tregs between PBMC and liver-infiltrating lymphocytes in HCC patients. Both $CD25^-FOXP3^-$ and $CD25^{high+}FOXP3^+$ Tregs are detected in liver-infiltrating lymphocytes, and $CD25^-FOXP3^-$ Tregs are higher in tumor-infiltrating lymphocytes than those in nontumor-infiltrating and circulating lymphocytes (Fig. 3*b*). These results demonstrate that $CD25^-FOXP3^-$ Tregs increase both in the liver and in the periphery in parallel with the development of cancer.

We serially examined the frequency of $CD25^-FOXP3^-$ Tregs and $CD25^{high+}FOXP3^+$ Tregs before and after RFA therapy. The $CD25^-FOXP3^-$ Tregs frequency dramatically decreased after successful HCC ablation and further subsided in patients without intrahepatic recurrence (Fig. 4*a*). In clear contrast, in patients with subsequent HCC recurrence, $CD25^-FOXP3^-$ Tregs increased before apparent radiological identification of HCC (Fig. 4*a*). Such dynamic frequency changes in parallel with HCC recurrence were not apparent in $CD25^{high+}FOXP3^+$ Tregs (Fig. 4*b*). Therefore, $CD25^-FOXP3^-$ Treg frequency is more closely correlated than $CD25^{high+}FOXP3^+$ Tregs with the presence or absence of HCC.

PD-L1, IL-T4 and HLA-G are involved in the induction of $CD4^+CD25^-CD127^-FOXP3^-$ $IL\text{-}10^+$ T cells

After the culture of naive $CD4^+$ T cells, DC and Huh7 or HepG2, we found that $CD4^+CD25^-CD127^-FOXP3^-$ cells produce IL-10 (Fig. 5*a*), whereas $CD4^+CD25^+CD127^-FOXP3^+$ cells do not (Supporting Information Fig. 4). Since $CD4^+CD25^-CD127^-$ cells use IL-10 as one of suppressor mechanisms (Supporting Information Fig. 1), such $IL\text{-}10^+$ $CD4^+CD25^-CD127^-FOXP3^-$ T cells are functionally competent $CD25^-FOXP3^-$ Tregs (Fig. 5*a*). In culture, the frequency of $IL10^+$ $CD25^-FOXP3^-$ T cells decrease in the presence of anti-TGF-β, anti-PD-1, anti-PD-L1 or anti-ILT4 Abs, with the difference being the most significant with anti-PD-L1 or anti-ILT4 Abs (Fig. 5*b*). Next, in the absence of DC or the separation of T cells from HCC cell lines significantly reduced $IL10^+$ $CD25^-FOXP3^-$ T cell induction, whereas separation of T cells from DC did not change it (Fig. 5*c*). These results indicate that the contact between T cells and HCC cell lines is indispensable for $IL\text{-}10^+$ $CD25^-FOXP3^-$ T cell induction, but the contacts between T cells and DC or between DC and HCC cell lines are not, respectively. Similarly, the addition of anti-PDL1 or anti-IL-T4 Abs to this culture resulted in suppression of $IL\text{-}10^+$ $CD25^-FOXP3^-$ T cell induction, regardless of the presence of transwells (Fig. 5*c*).

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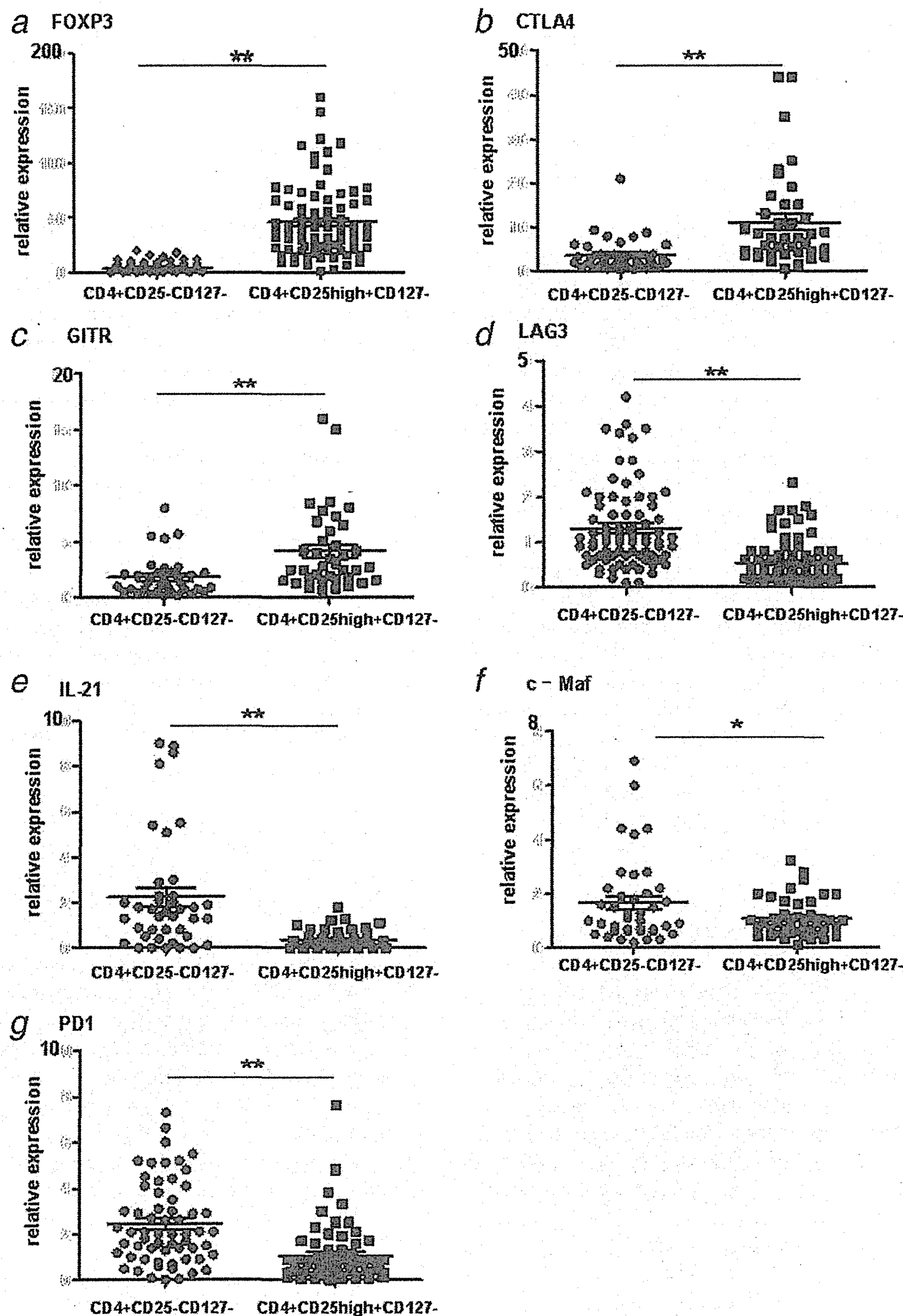

Figure 2. CD4$^+$CD25$^-$CD127$^-$ T cells and CD4$^+$CD25^{high+}CD127$^-$ T cells display distinct gene profiles. Sorted CD4$^+$CD25$^-$CD127$^-$ cells and CD4$^+$CD25^{high+}CD127$^-$ cells from PBMC of HCC patients were subjected to real-time RT-PCR for the analyses of FOXP3 (*a*), CTLA4 (*b*), GITR (*c*), LAG3 (*d*), IL-21 (*e*), c-Maf (*f*) and PD1 (*g*). The results are shown in relative expression of relevant genes to those of β-actin. *: $p < 0.05$; **: $p < 0.01$ by Mann-Whitney *U* test with Welch's correction.

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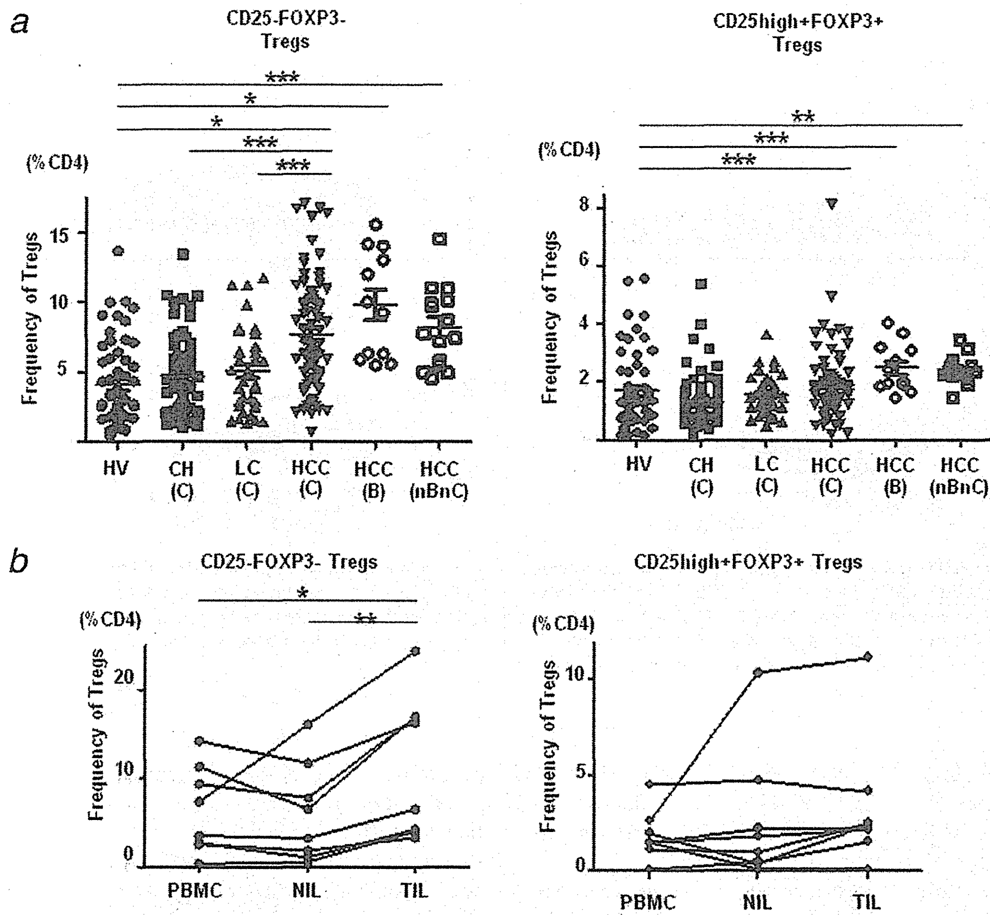

Figure 3. $CD25^-FOXP3^-$ Tregs and $CD25^{high+}FOXP3^+$ Tregs increase in HCC patients both in the periphery and in the liver. (*a*) The frequencies of $CD25^-FOXP3^-$ Tregs ($CD4^+CD25^-CD127^-$ $FOXP3^-$) and $CD25^{high+}FOXP3^+$ Tregs ($CD4^+CD25^{high+}CD127^-FOXP3^+$) in $CD4^+$ T cells were compared among the groups. HV, healthy volunteers; CH(C), LC(C), HCC (C), HCV-infected chronic hepatitis, liver cirrhosis or hepatocellular carcinoma, respectively; HCC (B), HBV-positive; HCC (nBnC), HCV-negative and HBV-negative HCC patients. The horizontal bars indicate mean ± standard deviation. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ by Kruskal–Wallis test with Dunn's multiple comparison test. (*b*) $CD25^-FOXP3^-$ Tregs and $CD25^{high+}FOXP3^+$ Tregs are present in tumor-infiltrating lymphocytes of HCC patients. Lymphocytes from HCC, nontumor liver tissue and PBMC were collected from identical nine HCC patients, and the frequency of $CD25^-FOXP3^-$ Tregs and $CD25^{high+}FOXP3^+$ Tregs in them was compared. PBMC, peripheral blood mononuclear cells; NIL, nontumor tissue infiltrating lymphocytes; TIL, tumor-infiltrating lymphocytes. *: $p < 0.05$; **: $p < 0.01$, by Friedman test with Bonferroni multiple comparison test.

IL-T4 is expressed on DC and transmits inhibitory signals after ligation with HLA-G. To confirm that the HLA-G and PD-L1 expressed in HCC are responsible for $IL10^+$ $CD25^-FOXP3^-$ T cell induction, we knocked down HLA-G and/or PD-L1 in Huh7 cells by siRNA and subjected them to the abovementioned *in vitro* cultures (Fig. 5*d*). As a result, $IL10^+$ $CD25^-FOXP3^-$ T cell frequency is significantly decreased in the presence of siRNA-treated HCC, but not with mock-transfected HCC (Fig. 5*e*). These results demonstrate that DC and HCC cells are actively involved in $IL\text{-}10^+$ $CD25^-FOXP3^-$ T cell induction, in which PD-L1, IL-T4 and HLA-G are indispensible.

Discussion

In this study, we focused on $CD25^-FOXP3^-$ Tregs in HCC patients, which are distinct from $CD25^{high+}FOXP3^+$ natural Tregs in cellular phenotypes, genetic profiles and functional aspects. We demonstrated that; (*i*) $CD4^+CD127^-CD25^-$ cells (as defined as $CD25^-FOXP3^-$ cells in this study) are endowed with suppressive capacity comparably with

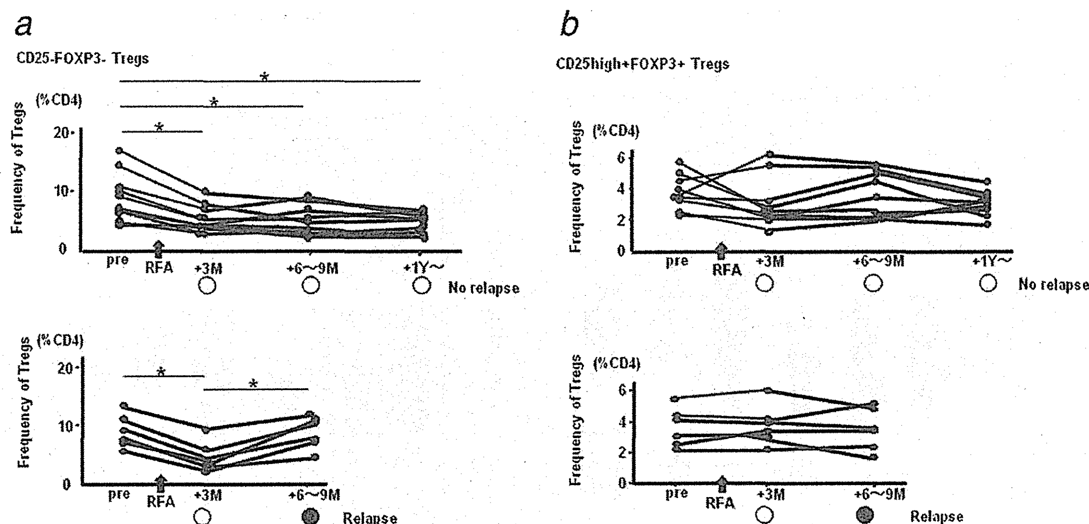

Figure 4. $CD25^-FOXP3^-$ Tregs increase in parallel with post therapeutic HCC recurrence. In HCC patients who underwent RFA therapy, frequencies of $CD25^-FOXP3^-$ Tregs (*a*) and $CD25^{high+}FOXP3^+$ Tregs (*b*) in $CD4^+$ T cells are examined serially before RFA sessions and after confirmation of complete ablation of relevant HCC lesions. Open circles (○)depict the time points without HCC recurrence under CT/MRI examinations and closed circles (●)are those with detectable HCC recurrence, respectively. Arrows indicate the time points of RFA sessions. *: $p < 0.05$ by Friedman test with Bonferroni multiple comparison test.

$CD4^+CD127^-CD25^{high+}$ cells ($CD25^{high+}FOXP3^+$ cells) and (*ii*) the frequency of $CD25^-FOXP3^-$ Tregs changes more dynamically than those of $CD25^{high+}FOXP3^+$ Tregs in correlation with post-therapeutic HCC recurrence.

Extensive studies have been carried out on the role of natural Tregs in cancer patients, of which are conventionally defined as $CD25^{high+}FOXP3^+$ T cells. Pharmaceutical deprivation of $CD25^+$ T cells *in vivo* were tried to improve immune reactivity against cancers; however, most of the study results were unsatisfactory.[19,20] Such experiences raise the possibility that the involvement of $CD25^-$ Tregs in the pathogenesis of certain cancers. In support for this, the existence of $CD25^-FOXP3^-$ Tregs has been reported in mice and human, in relation to viral infection or cancers.[21–23] The comparative roles of $CD25^{high+}FOXP3^+$ natural Tregs and $CD25^-$ Tregs in human diseases are still largely unknown. It is reported that CD127 expression is inversely correlated with a FOXP3 and CD127 negative population broadly encompassing regulatory cells.[11,12] Several investigators reported that CD127 expression on T cells is aberrantly regulated with regard to their functional relevance.[24,25] Taking these findings into consideration, we aimed to identify distinct type of Tregs in $CD4^+CD127^-$ population. Consequently, we found a functional regulatory subset in $CD4^+CD25^-CD127^-$ T cells, which differ from $CD4^+CD25^{high+}CD127^-$ Tregs in molecular profiles and inhibitory mechanisms. The profile of $CD4^+CD25^-CD127^-$ T cells is quite unique; they express more LAG-3, IL-21, c-Maf and PD-1 but less FOXP3, CTLA-4 and GITR than $CD4^+CD25^{high+}CD127^-$ Tregs do. In support of our results, Pot *et al.* reported that IL-27 induces IL-21 and c-Maf, which are critically involved in the differentiation of IL-10-producing Tr1.[26] As for functional aspects, we showed that $CD4^+CD25^-CD127^-$ cells use IL-10 as suppressive machineries, not completely but in part. Based on these characteristics, it is likely that $CD4^+CD25^-CD127^-FOXP3^-$ cells, as defined as $CD25^-FOXP3^-$ Tregs in this study, are presumed to be aforementioned Tr1 cells. Such phenotype of T cells are compatible with Tr1-like cells in human, as reported by Haringer *et al.*[14] To confirm that, several additional examinations, such as antigen-specific suppressive capacity, need to be carried out. Using tetanus toxoid as a representative of general recall antigens in this study, $CD4^+CD25^-CD127^-$ cells and $CD4^+CD25high^+CD127^-$ cells tended to be suppressive on autologous $CD4^+$ T cell proliferation (Supporting Information Fig. 2). Further analysis needs to be performed on this issue, using other sets of recall antigens.

To therapeutically control Tregs *in vivo*, extensive studies have been carried out to disclose the mechanisms of the induction or attraction of $FOXP3^+$ Tregs.[27,28] Likewise, it is tempting to consider that $CD25^-FOXP3^-$ Tregs depletion would have a favorable impact on the clinical features of the patients. Thus, identifying the molecules involved in $CD25^-FOXP3^-$, Treg induction should be carried out for the future development of Treg-oriented therapeutic approach. For this purpose, we successfully expanded $CD4^+CD25^-CD127^-FOXP3^-IL10^+$ cells from naive $CD4^+CD25^-$T cells. Such cultured cells contained approximately 10% of $IL\text{-}10^+$ cells, which subsequently

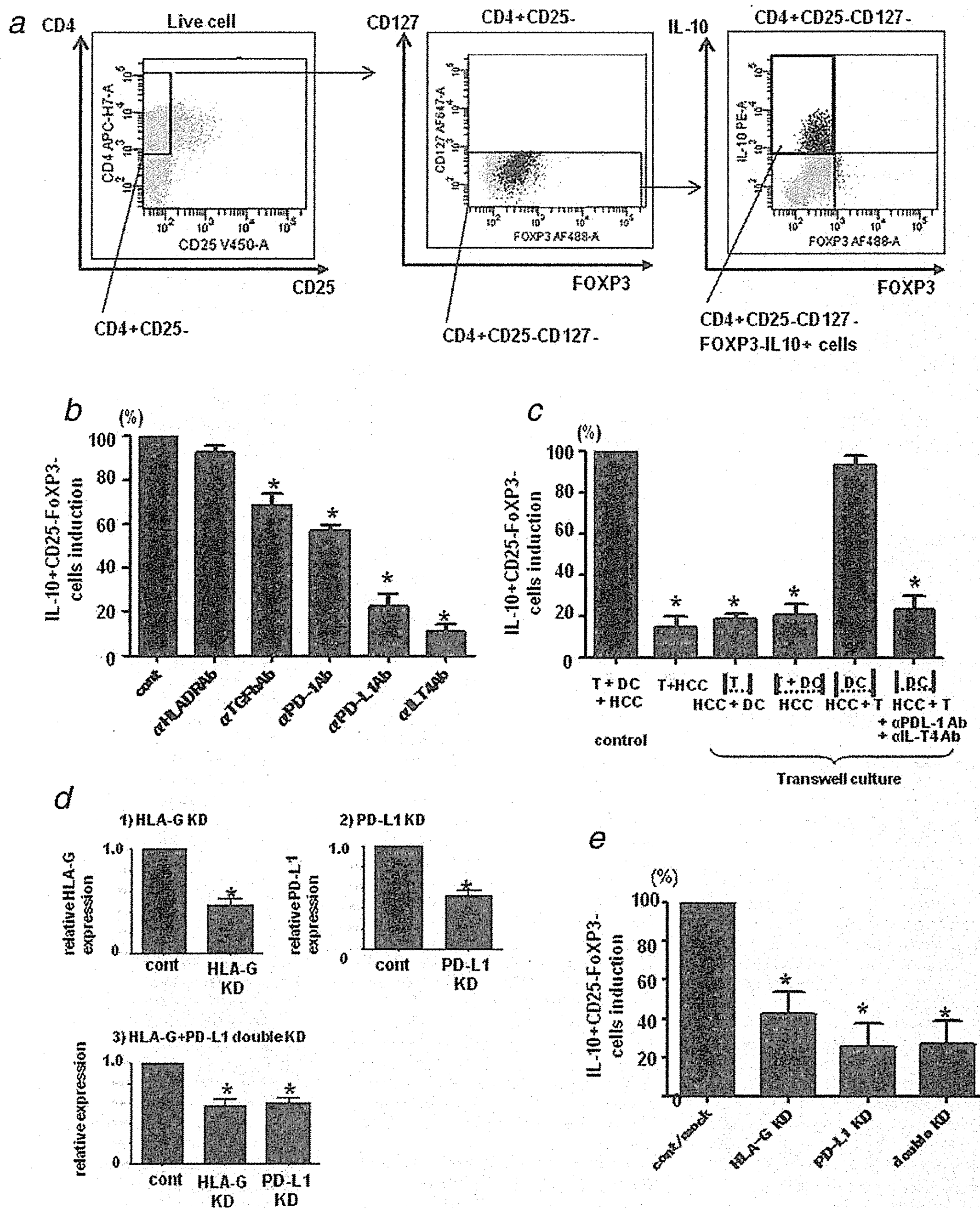

Figure 5. HLA-G and PD-L1 in HCC and IL-T4 in DC are involved in the induction of IL-10$^+$CD25$^-$FOXP3$^-$ Tregs. After culture of CD4$^+$CD45RA$^+$ naive T cells with autologous monocyte-derived dendritic cells and Huh-7 or HepG2, CD4$^+$ CD127$^-$ CD25$^-$FOXP3$^-$IL-10$^+$ T cells (IL-10$^+$CD25$^-$FOXP3$^-$ Tregs) were generated. (*a*) Representative dot plots from results of seven healthy volunteers are shown. In the abovementioned coculture system, various neutralizing/masking Abs (*b*) or transwell inserts (*c*) were added and the results were compared with the frequencies of IL-10$^+$CD25$^-$FOXP3$^-$ Tregs with or without treatments. In addition, we transfected siRNA against HLA-G and/or PD-L1 to Huh-7 and cocultured them with naive CD4$^+$ T cells and DC as the same as above. The efficiency of gene silencing was evaluated by the comparison of transcripts of HLA-G or PD-L1 with or without siRNA transfection (*d*). The frequency of IL-10$^+$CD25$^-$FOXP3$^-$ Tregs after the culture was compared with mock-transfected ones (*e*). In Figures 5-B, 5-C and 5-E, the bars indicate the ratio of IL-10$^+$CD25$^-$FOXP3$^-$ Tregs frequency (mean + standard deviation) between those with treatment and without from three series of experiments. *: $p < 0.05$ by Wilcoxon rank sum test.

tended to inhibit proliferation of allogeneic $CD4^+$ T cells (data not shown). Using this culture, we demonstrated that DCs are indispensible for IL-10^+ $CD25^-FOXP3^-$ Tregs induction *in vitro* by way of PD-1/PD-L1 and IL-T4/HLA-G pathways. Several reports showed that such molecular interactions are involved in the generation of regulatory cells in cancer patients.[29,30] In patients with HCC, a positive correlation is observed between the expression of PD-L1 or HLA-G in cancer tissue and the poorer prognosis of the patients,[31,32] suggesting that such molecules are involved in cancer development. As for HLA-G in this study, direct cellular contact between DC and HCC is not necessary in IL-10^+ $CD25^-FOXP3^-$ Tregs induction, suggesting that soluble HLA-G released from HCC may play an active role. In our hands, soluble HLA-G was measurable in culture supernatants of HCC cell lines and in serum samples from HCC patients (data not shown). Further investigation is arguably needed to elucidate whether soluble HLA-G is functional or not in HCC patients.

In summary, we demonstrate that $CD25^-FOXP3^-$ Tregs are increased in HCC patients, which change dynamically in response to HCC occurrence and post-therapeutic recurrence. Cross-talks among HCC cells, DC and $CD4^+$ T cells are required for IL-10^+ $CD25^-FOXP3^-$ Tregs induction, in which PD-L1, HLA-G and IL-T4 are critically involved. Although further investigation is needed to prove that deprivation or inactivation of $CD25^-FOXP3^-$ Tregs improves immune responses *in vivo*, such molecules could serve as targets of Treg-oriented therapeutic intervention for HCC.

Acknowledgements

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Fibroblast growth factor-2 enhances NK sensitivity of hepatocellular carcinoma cells

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The roles of fibroblast growth factor-2 (FGF-2) in the hepatocellular carcinoma (HCC) development are still controversial. In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. The FGF-2 expressions were detected in the liver tissues of patients, but not in normal liver. The serum FGF-2 levels of the patients with CH, liver cirrhosis (LC) or HCC were significantly higher than those of healthy volunteers. The serum FGF-2 levels of patients decreased with the progression of chronic liver disease. HCC occurrence of LC patients with high levels of serum FGF-2 was significantly lower than that with low levels of serum FGF-2. Proinflammatory cytokines, such as IL-1β and IL-6, induced FGF-2 expressions in HCC cells and normal hepatocytes. FGF-2 stimulation resulted in increasing the expression of the membrane-bound major histocompatibility complex class I-related chain A (MICA), an NK activating molecule, and decreasing that of human leukocyte antigen (HLA) class I, an NK inhibitory molecule, on HCC cells. This did not occur with normal hepatocytes. Adding anti-FGF receptor-2 neutralizing antibody resulted in inhibiting the change of MICA and HLA class I expressions on FGF-2 stimulated HCC cells. FGF-2 stimulation on HCC cells resulted in increasing NK sensitivity against HCC cells. These findings indicate that FGF-2 produced by HCC cells or normal hepatocytes of chronic liver disease may play critical roles in eliminating HCC cells by innate immunity.

Fibroblast growth factor (FGF)-2 is one of a family of FGFs that includes 22 structurally related members.[1] FGF-2 has been shown to exert a potent angiogenic effect by interacting with tyrosine kinase receptors, FGFR1, FGFR2 and FGFR3, in various cancers including hepatocellular carcinoma (HCC).[2–4] Aside from its angiogenic effect, FGF-2 has also been shown to act as a mitogen for HCC cell proliferation *via* an autocrine mechanism.[5] Uematsu *et al.* reported that the serum FGF-2 of chronic liver disease patients without HCC tended to be higher than that of those with HCC.[6] Decrease of serum FGF-2 could be observed prior to the emergence of HCC, and this suggests that FGF-2 may play a critical role in the surveillance of HCC. However, the immunological significance of elevating the FGF-2 levels in chronic liver disease patients remains unclear.

HCC is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and nonalcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis (LC), in particular, being considered a premalignant condition.[7,8] The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells),[9,10] but the activation process of these immune cells in HCC development remains unclear. A recent study has demonstrated that the innate immune system may play a critical role in tumor surveillance *via* an NKG2D signal.[11] Knowing the details of how to activate the abundant NK cells in the liver could lead to the establishment of attractive new strategies for HCC treatment.

In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. Of importance are the findings that serum FGF-2 levels in patients with CH and LC without HCC were significantly higher than that in those with HCC and that FGF-2 enhanced the NK sensitivity of HCC cells. The present study

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Table 1. Clinical backgrounds

	Normal	Hepatitis	Cirrhosis	HCC
Number	24	80	84	112
				Stage I/II 51
				Stage III/IV 61
Sex (M/F)	12/12	45/35	44/40	67/45
Age	64 ± 15	56 ± 13	62 ± 13	66 ± 11
Etiology		HCV	HCV	HCV

Abbreviations: Stage: TNM stage; M: male; F: female; HCV: hepatitis C virus.

sheds light on previously unrecognized immunological effects of FGF-2 on HCC cells and thus suggests a role of FGF-2 in HCC development in patients with CH type C.

Material and Methods

Liver tissues and immunohistochemistry

Human HCC tissues ($n = 6$) and normal liver tissues ($n = 2$) were obtained at surgical resection. CH tissues ($n = 4$) and LC tissues ($n = 4$) were obtained as liver biopsy samples. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. Liver sections were subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA). The primary antibody (Ab) was antihuman FGF-2 Ab (Abcam, Cambridge, MA). To confirm the specificity of the staining, the primary antibody was incubated with recombinant human FGF-2 protein (R&D Systems, Minneapolis, MN) for 3 hr and then applied onto liver sections in parallel with staining of the primary antibody as the absorption test.

HCC cell lines

HepG2 and PLC/PRF/5, human hepatoma cell lines, were purchased from American Type Culture Collection (Rockville, MD) and were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C.

ELISA

The sera from CH patients ($n = 80$), LC patients ($n = 84$), HCC patients ($n = 112$, StageI/II $n = 51$ and StageIII/IV $n = 61$) and age-matched healthy volunteers (HVs) ($n = 24$) were subjected to analysis of the FGF-2 level. Clinical backgrounds of patients were summarized in Table 1. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. The level of FGF-2 and soluble major histocompatibility complex class I-related chain A (MICA) were determined using Quantikine Human FGF basic (R&D Systems) and DuoSet MICA eELISA kit (R&D Systems), respectively.

HCC cells and normal hepatocyts cultures

Both HepG2 and PLC/PRF/5 cells or normal hepatocytes (ScienCell Research Laboratories, Carlsbad, CA) were cultured for 72 hr in the presence or absence of human interleukin-1β (IL-1β) (50 ng/ml, Peprotech, Rocky Hill, NJ), human IL-6 (300 ng/ml, Peprotech), human transforming growth factor-β1 (TGF-β1) (50 ng/ml, R&D Systems) and human tumor necrosis factor-α (TNF-α) (100 ng/ml, Peprotech), and the treated cells were harvested and evaluated for expression of FGF-2. In some experiments, HepG2 and PLC/PRF/5 cells were cultured in the presence or absence of recombinant human FGF-2 protein (250 ng/ml, R&D Systems) with or without antihuman FGFR2 neutralizing Ab (10 μg/ml, R&D Systems) for 48 hr, and the hepatoma cells were harvested and evaluated for the immunological regulation of the NK cells.

Flow cytometry

For the detection of membrane-bound MICA, cells were incubated with anti-MICA specific Ab (2C10, Santa Cruz Biotechnology, Santa Cruz, CA) and stained with Goat F(ab′)2 fragment anti-Mouse IgG(H+L)-PE (Beckman Coulter, Fullerton, CA) as a secondary reagent and then subjected to flow cytometric analysis. For the detection of human leukocyte antigen (HLA) class I, cells were incubated with PE-conjugated antihuman HLA-A,B,C Ab (w6/32, BD Biosciences, San Jose, CA). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Western blotting

The total cellular protein was electrophoretically separated using sodium dodecyl sulfate-12% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked in Tris-buffered saline-Tween20 containing 5% skim milk for 1 hr and then probed with rabbit polyclonal Ab to FGF-2 (Abcam) at room temperature overnight. Horseradish peroxidase-conjugated anti-rabbit IgG and SuperSignal West Pico System (Pierce, Rockford, IL) were used for the detection of blots.

Real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assay (Applied Biosystems) was used for the quantification of FGF-2 (ID: Hs00960934_ml), MICA (Hs00792195_ml) and β-actin (Hs:99999903_ml) mRNAs according to the manufacturer's instructions. β-actin mRNA from each sample was quantified as endogenous control of internal RNA.

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NK cell analysis

NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyi Biotech, Auburn, CA).[12] The cytolytic ability of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr ^{51}Cr-release assay with or without antihuman MICA/B Ab (BD Biosciences) as previously described.[12] The expressions of NKG2D and NKG2A on NK cells were analyzed by flow cytometry with PE-conjugated antihuman NKG2D Ab (BD Biosciences) and PE-conjugated IgG antihuman NKG2A Ab (R&D Systems).

Statistics

For human sample data, values were expressed as the median and interquartile range using box plots and the 10th and 90th percentiles as horizontal bars. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel-Dwass procedure. Differences between retreatment and post-treatment values were tested by the paired *t*-test. FGF-2 mRNA values were expressed as the mean and SD, and the statistical significance of differences between the groups was determined by applying Student's *t* test after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $p < 0.05$.

Results

FGF-2 is expressed in the liver and serum of patients with chronic liver diseases

We first examined the FGF-2 expressions in the livers of normal volunteers and the patients with chronic liver diseases. Immunohistochemical analysis revealed that FGF-2 was not expressed in normal liver tissues. In contrast, the expressions of FGF-2 were detected in chronic liver tissues (Fig. 1*a*). We evaluated the serum FGF-2 levels by specific ELISA. All of the chronic liver disease patients were hepatitis C virus (HCV)-RNA positive. As shown in Figure 1*b*, the serum FGF-2 levels in CH and LC patients were significantly higher than those of HV, but those in HCC patients were not. Those in CH patients were also significantly higher than those in LC or HCC patients. Those in LC patients tended to be higher than those in HCC patients, although this was not significant. The serum FGF-2 levels in HCC patients were low and significant difference between StageI/II patients and III/IV patients was not observed (data not shown). We compared the serum FGF-2 levels before and after the

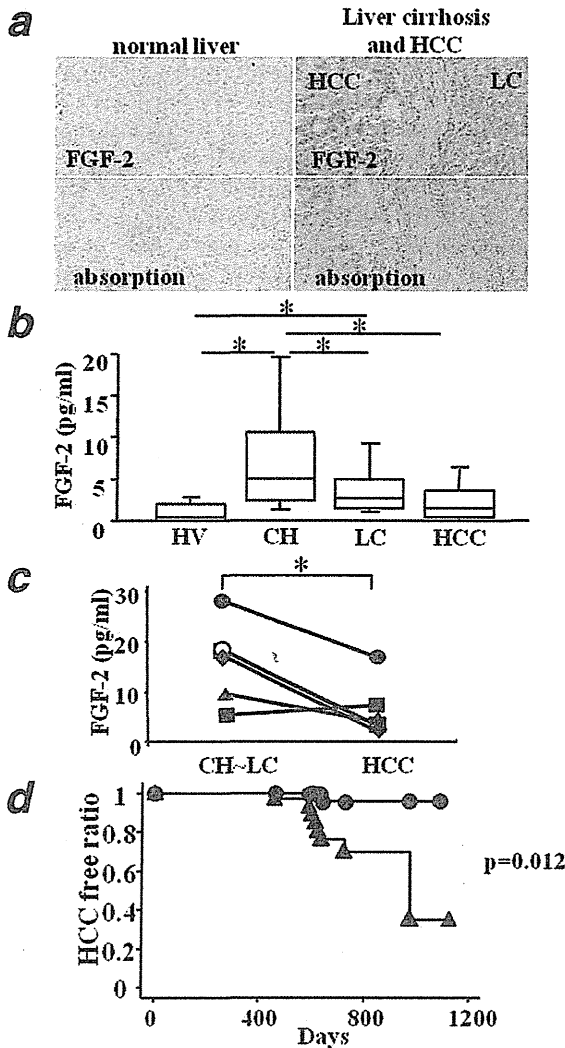

Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (*a*) Immunohistochemical analysis of FGF-2 in normal liver tissues ($N = 2$), chronic hepatitis tissues ($N = 4$), liver cirrhosis (LC) tissues ($N = 4$) and hepatocellular carcinoma (HCC) tissues ($N = 6$). Liver sections were stained with the FGF-2 Ab (*upper panels*). The primary Ab was incubated with recombinant FGF-2 protein and then applied to liver sections in parallel as the absorption test (*lower panels*). Representative pictures are shown. (*b*) Serum FGF-2 levels in chronic hepatitis patients (CH, $N = 80$), liver cirrhosis patients (LC, $N = 84$) and HCC patients ($N = 112$) were evaluated by specific ELISA. All patients were HCV-RNA positive. Comparison of serum FGF-2 levels of each group. * $p < 0.05$. (*c*) Serum FGF-2 levels were compared between before and after HCC development in six chronic liver disease patients. The mean follow-up period was nine years. * $p < 0.05$. (*d*) The correlation of the FGF-2 level and HCC incidence was evaluated. 84 LC patients were divided into two groups according to serum FGF-2 levels; high (serum FGF-2 concentration > 1.8 pg/ml; 40 patients, ●) and low (≦1.8 pg/ml; 44 patients, ▲). We followed these LC patients for three years and compared the rate of HCC-free survival in these groups.

development of HCC in six chronic liver disease patients. The mean follow-up period was nine years. The serum FGF-2 levels of the patients before the occurrence of HCC were significantly higher than those of the same patients after the occurrence of HCC (Fig. 1*c*). These results demonstrated that the serum FGF-2 levels were highest in CH patients and significantly decreased as the liver disease progressed.

FGF-2 levels were associated with the incidence of HCC in chronic liver disease patients

The earlier results suggested that increased FGF-2 levels might prevent HCC tumor development. We investigated the correlation of the serum FGF-2 level and HCC incidence. The 84 LC patients were divided into two groups according to serum FGF-2 levels, high (serum FGF-2 concentration $>$ 1.8 pg/ml; 40 patients) and low ($\leq$1.8 pg/ml; 44 patients), because the median of FGF-2 levels in these patients was 1.8 pg/ml. We followed these LC patients for three years and compared the rates of HCC-free survival. As shown in Figure 1*d*, the HCC free ratio of the high FGF-2 patients was significantly higher than that of the low FGF-2 patients. These results suggested that FGF-2 production from chronically diseased liver tissues might be associated with the occurrence of HCC.

Inflammatory cytokines increased FGF-2 expression in HCC cells and normal hepatocytes

Previous reports demonstrated that FGF-2 expressions were detected in both tumor cells and normal hepatocytes in addition to sinusoidal endothelial cells in HCC tissues.[5] Some inflammatory cytokines, such as IL-1β, IL-6, TGF-β and TNF-α, are known to increase in CH patients.[13–15] To examine the effect of such inflammatory cytokines on FGF-2 expression in liver cells, we cultured HepG2 and PLC/PRF/5 HCC cells for 72 hr in the presence or absence of these cytokines. As shown in Figure 2*a*, IL-1β and IL-6 increased FGF-2 protein levels in both HepG2 and PLC/PRF/5 cells. FGF-2 mRNA levels in HepG2 and PLC/PRF/5 cells treated with IL-1β and IL-6 were significantly higher than those in nontreated control HCC cells (Fig. 2*b*). We also examined FGF-2 levels in the supernatants of the HCC cells cocultured with inflammatory cytokines. FGF-2 levels of IL-1β- or IL-6-treated HepG2 cells or PLC/PRF/5 cells tended to increase compared with those of nontreated HCC cells (data not shown). FGF-2 mRNA levels in normal hepatocytes treated with IL-1β, but not IL-6, were also significantly higher than those in nontreated control cells (Fig. 2*c*). These results suggested that both IL-1β and IL-6 were capable of inducing FGF-2 expression in HCC cells and normal hepatocytes. We also examined whether TGF-β1 and TNF-α could induce FGF-2 expressions on HCC cells. We found that FGF-2 expression levels in treated HCC cells did not change in Western blotting or real-time RT-PCR analysis (data not shown).

FGF-2 induced the expression of membrane-bound MICA and suppressed the expression of HLA class I on HCC cells, but FGF-2 did not change the expressions of NKG2D and NKG2A on NK cell

The above findings suggested that decreasing FGF-2 might affect the HCC development in the patients with chronic liver disease. To investigate whether or not FGF-2 protein directly activates NK cells, we examined whether FGF-2 affected the expression of NKG2D (activating receptor) or NKG2A (inhibitory receptor) on NK cells. We cultured CD56+ NK cells obtained from HVs with FGF-2 for 24 hr and then subjected them to flow cytometric analysis. The expressions of both NKG2D and NKG2A on NK cells did not change by adding FGF-2 protein (Fig. 3*a*), suggesting that FGF-2 did not have a direct effect on NK cells. We next examined the immunological modification of human HCC cells by adding human FGF-2 protein. We evaluated the expressions of membrane-bound MICA (NK activating molecule) and HLA class I (NK inhibitory molecule) in HepG2 and PLC/PRF/5 cells by flow cytometry. The expressions of MICA on FGF-2-treated cells were higher than those on nontreated cells in both HepG2 and PLC/PRF/5 cells (Fig. 3*b*). In contrast, those of HLA class I on FGF-2-treated cells were lower than those on nontreated cells in both types of HCC cells (Fig. 3*b*). FGF-2-treatment could modify the expressions of MICA and HLA class I on HCC cells in a dose-dependent manner (data not shown). The mRNA level of MICA in FGF-2-treated HepG2 cells was also significantly higher than that in nontreated HepG2 cells. The mRNA level of MICA in FGF-2-treated PLC/PRF/5 tended to be higher than that in nontreated cells, although the difference was not statistically significant (Fig. 3*b*). We examined the expressions of MICA and HLA class I on FGF-2-treated normal hepatocytes. The expressions of both molecules did not change in FGF-2-treated normal hepatocytes (Fig. 3*c*). We also evaluated FGF-2-dependent MICA regulation on a gastric cancer cell line (KATOIII), colon cancer cell lines (HCT116, HT29) and a cervical cancer cell line (Hela). The MICA expression was induced in FGF-2-treated HCT116 cells and weakly in FGF-2-treated Hela cells, but not in the other two cell lines (data not shown). These results suggested that FGF-2 could modify the MICA expressions in several types of cancers.

The signal via FGF-2/FGF-receptor2 is essential for the induction of MICA and HLA class I expressions on HCC cells

We examined the FGF receptors (FGFR1, FGFR2, and FGFR3) on both types of HCC cells by flow cytometry. The expressions of FGFR2 were high for both cell types. While FGF-2 has cross-reactivity with FGFR1 and FGFR3, the expressions of FGFR1 and FGFR3 were very low on both types of HCC cells (Fig. 4*a*). To examine whether the interaction between FGF-2 and FGFR2 could induce the expressions of MICA and HLA class I on both types of HCC cells, we evaluated the expressions of both molecules on FGF-2-treated

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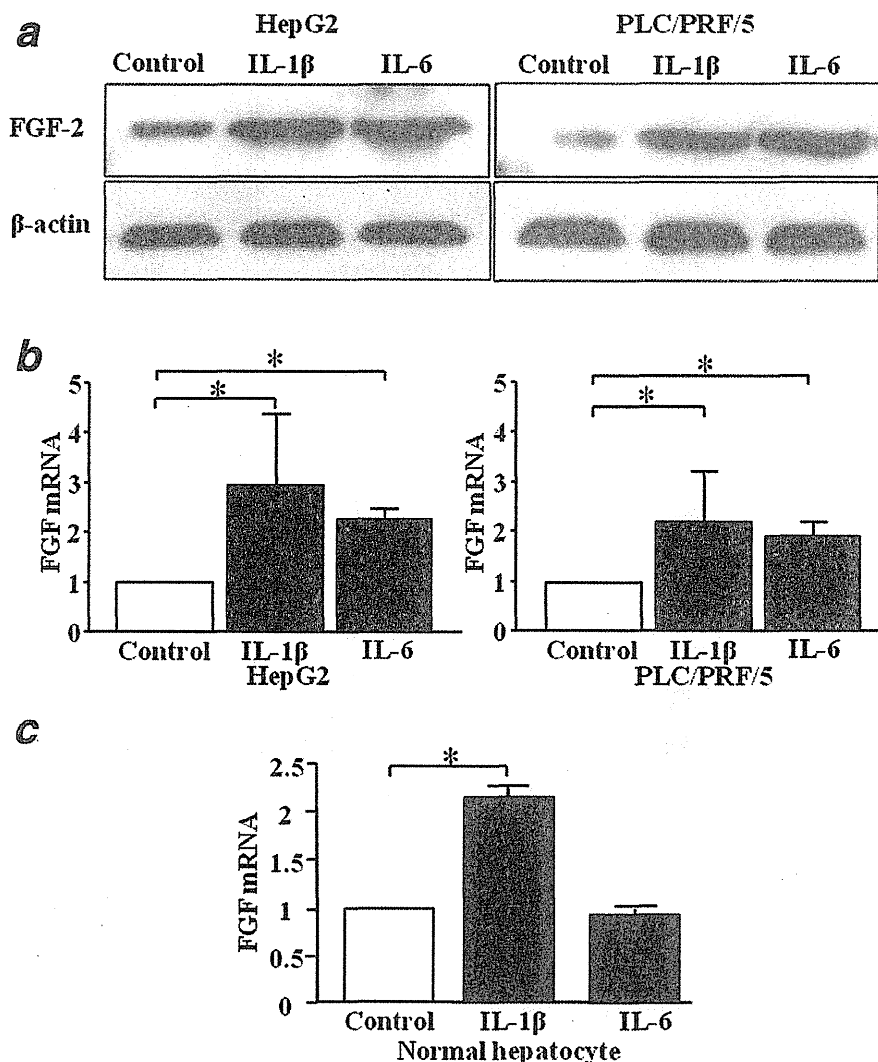

Figure 2. IL-1β and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1β and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (*a,b*) or normal hepatocytes (*c*) were cultured for 72 hr in the presence or absence of IL-1β (50 ng/ml) and IL-6 (300 ng/ml). FGF-2 expression in these cells was evaluated by Western blotting analysis (*a*) and real-time RT-PCR analysis (*b,c*). (*a*) The proteins were subjected to Western blot assay using each specific Ab. *Upper panel* is FGF-2 and *lower panel* is β-actin. (*b,c*) Total RNA was extracted and reverse transcribed. Relative copy numbers of FGF-2 were determined by real-time PCR analysis and normalized with β-actin expression. Results are expressed as mean ±SD. Similar results were obtained in two independent experiments. * $p < 0.05$.

HCC cells with anti-FGFR2 neutralizing Ab. The anti-FGFR2 Ab blocks the ability of FGF-2 to modulate MICA and HLA class I on both HepG2 and PLC/PRF/5 cells (Fig. 4*b*).

FGF-2 enhanced susceptibility to NK cells of HCC cells and the correlation of serum FGF-2 and soluble MICA levels in patients with chronic liver disease

The earlier results suggested that FGF-2 might enhance the susceptibility to NK cells of HCC cells. We next examined whether FGF-2 could modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against FGF-2-treated HepG2 and FGF-2-treated PLC/PRF/5 cells were higher than those against nontreated HCC cells (Fig. 5*a*). The cytolytic activity against FGF-2-treated HCC cells decreased to the control levels on addition of anti-MICA/B blocking antibody (Fig. 5*a*) but not on addition of isotype IgG antibody (Fig. 5*b*). These results demonstrated that adding FGF-2 enhanced the NK sensitivity of HCC cells *via*