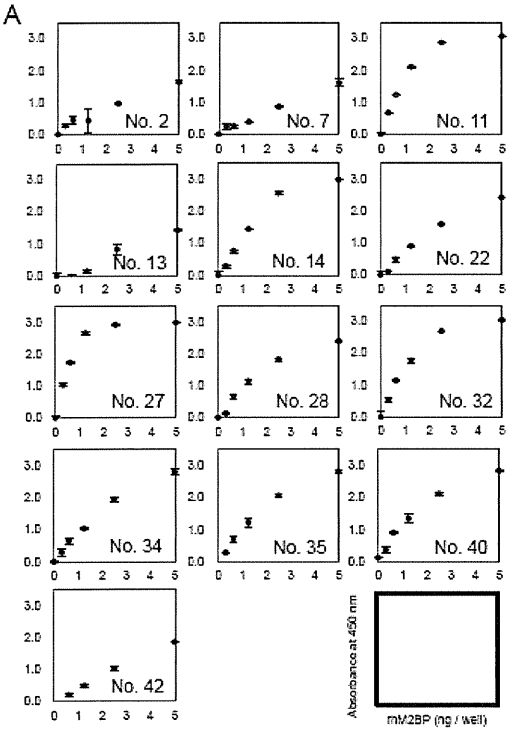
Figure 2. Sequential screening of the most reliable anti-hM2BP mAb using a lectin microarray with twofold serial dilution in a WFA–antibody sandwich ELISA (A), accelerated stability test (B) and spiking experiment (C). In the spiking experiment, appropriate amounts of rhM2BP were dissolved in the reaction buffer in the presence (open squares) or absence (closed squares) of serum samples where endogenous hM2BP had been depleted by immunoprecipitation.

A



B

Clone No.	IgG subclass	Stability (%)			
		Serum A		Serum B	
		Day 7	Day 14	Day 7	Day 14
2	IgG1	83	75	84	77
7	IgG1	83	73	85	76
11	IgG2a	94	82	100	87
13	IgG1	92	81	89	84
14	IgG1	87	73	90	77
22	IgG1	95	86	92	82
27	IgG2a	86	72	82	74
28	IgG1	93	80	95	85
32	IgG1	85	77	84	73
34	IgG1	79	75	79	72
35	IgG1	77	73	80	70
40	IgG1	93	77	98	83
42	IgG1	81	72	82	74

C

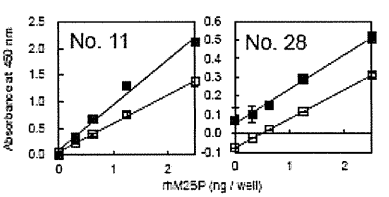
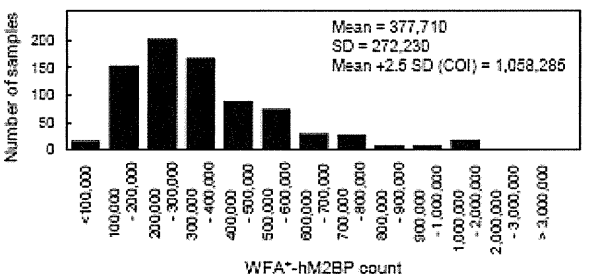


Figure 3. Cutoff index (COI) values for standardization of WFA^+-hM2BP counts showing the distribution of WFA^+-hM2BP counts of 800 samples from healthy volunteers.



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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection

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Abstract

Background Altered functions of dendritic cells (DCs) and/or increases of regulatory T cells (Tregs) are involved in the pathogenesis of chronic hepatitis C virus (HCV) infection. A tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), is reported to be an inducer of immune tolerance. Our aim was to clarify whether or not IDO is activated in chronic hepatitis C patients and its role in immune responses.

Methods This study enrolled 176 patients with chronic HCV infection and 37 healthy volunteers. Serum kynurenine concentration was evaluated by high-performance liquid chromatography, and its correlation with clinical parameters was examined. Monocyte-derived DCs were prepared from the subjects and subsequently stimulated with a combination of lipopolysaccharide and interferon-gamma to induce functional IDO (defined as IDO-DCs). The phenotypes, kynurenine or cytokine production, and T-cell responses with IDO-DCs were compared between the patients and healthy volunteers.

Results The serum kynurenine level in the patients was significantly higher than that in the healthy volunteers, and the level of serum kynurenine was positively correlated with the histological activity or fibrosis score. IDO activity in IDO-DCs from the patients was significantly higher than that in IDO-DCs from the volunteers. Furthermore, IDO-DCs from the patients induced more Tregs in vitro compared with those from the volunteers, and the frequency of induced Tregs by IDO-DCs was decreased with an IDO-specific inhibitor.

Conclusions Systemic IDO activity is enhanced in chronic hepatitis C patients in correlation with the degree of liver inflammation and fibrosis. In response to inflammatory stimuli, DCs from the patients tend to induce Tregs, with some of this action being dependent on IDO.

Keywords Hepatitis C virus · Dendritic cell · Regulatory T cell · Indoleamine 2,3-dioxygenase

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is estimated that 170 million people

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are chronically infected with HCV and are at risk of developing liver cirrhosis and/or hepatocellular carcinoma [1]. Approximately 70 % of those exposed to HCV progress to a chronically infected state [2]. The mechanisms of HCV leading to persistent infection have been ascribed to escape mutations of the HCV genome and insufficient immune responses to HCV in hosts, but the precise mechanisms are still largely unknown.

Dendritic cells (DCs) are key regulators of the immune system and are capable of promoting or suppressing T-cell responses depending on their environment [3, 4]. One of the crucial machineries of HCV-induced immune dysfunction is impaired abilities of DCs. Several research groups, including ours [5, 6] have demonstrated that DCs from chronically HCV-infected patients have lower ability to stimulate T cells and to drive T-helper 1 (Th1) polarization than those from healthy controls [7, 8]. Regulatory T cells (Tregs) are specialized suppressor cells that maintain immune tolerance against auto-reactive T cells or against pathogens [9]. In patients with chronic HCV infection, the frequency of Tregs in peripheral blood mononuclear cells (PBMCs) is higher than that in healthy individuals, suggesting the active roles of Tregs in immune alteration or alleviation of inflammation [10, 11]. However, the mechanisms of DC dysfunction or Treg expansion in chronic HCV infection have not been completely elucidated.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the initial and rate-limiting steps in the catabolism of the essential amino acid tryptophan (Trp), resulting in the generation of kynurenine (Kyn). IDO is widely expressed in human tissues [12] and cell subsets [13] and is induced during inflammation by interferon-gamma (IFN-γ) and/or other inflammatory cytokines [14–16]. Recent studies have demonstrated a crucial role of IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and cancers [17–21]. IDO expressed by DCs promotes immune tolerance by inhibiting T-cell activation and proliferation or by inducing Tregs through Trp starvation and/or the accumulation of Trp catabolites, such as Kyn, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid [22–25]. With respect to chronic HCV infection, a small-sized study showed that IDO expression was up-regulated in the liver and was associated with increased serum IDO activity [26]. However, the functions of IDO in immune cells in HCV infection still remain obscure.

In this study, we aimed to clarify whether or not IDO in DCs has a role in chronic HCV infection. We found that systemic IDO activity was enhanced in chronic hepatitis C patients. By comprehensively comparing the function of IDO-expressing DCs between the patients and healthy volunteers, we showed that IDO in DCs may be related to the induction of Tregs.

Subjects, materials, and methods

Subjects

This study enrolled 176 patients chronically infected with HCV serotype 1 (CHC group) who had been followed at Osaka University Hospital (Suita, Japan), National Hospital Organization Osaka National Hospital (Osaka, Japan), or Ikeda Municipal Hospital (Ikeda, Japan). All of them were confirmed to be positive for both serum anti-HCV antibody and HCV-RNA but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus. The presence of other liver diseases, such as alcoholic, metabolic, or autoimmune hepatitis was ruled out, and the presence of liver cirrhosis and hepatocellular carcinoma was excluded by the use of laboratory and imaging analyses. As controls, we examined 37 healthy volunteers (HV group), working as medical staff at Osaka University Hospital, who were negative for HCV and HBV markers. As disease controls, 13 patients with chronic HBV infection followed at National Hospital Organization Osaka National Hospital were also enrolled. They were positive for hepatitis B surface (HBs) antigen and had abnormal levels of alanine aminotransferase (ALT). The characteristics of the group were: male/female 10/3, hepatitis B envelope (HBe) antigen-positive/HBe antigen-negative 6/7, mean age 43.9 $\pm$ 15.0 years, mean serum ALT level 218.7 $\pm$ 282.5 IU/L, and mean HBV-DNA level [assayed by the COBAS AmpliPrep^{TM}/COBAS TaqMan^{TM} HBV test (Roche, Branchburg, NJ, USA)] 6.1 $\pm$ 2.3 Log copies/mL. At enrollment, written informed consent was obtained from each subject. The study protocol was approved by the ethics committee of each institution.

In this study, because of the limitations of sampling from multiple centers, the conditions for blood collection and preservation differed among the facilities. Thus, for the precise comparison of IDO activity between the patients and healthy volunteers, firstly, we examined the samples collected and preserved under the same conditions at Osaka University Hospital (Cohort I, Table 1). Secondly, because liver biopsy was not carried out in Cohort I patients, we used another cohort (Cohort II, Table 1) for our analysis of the correlation between IDO activity and clinical parameters. Cohort II consisted of the remaining 127 patients, whose samples were collected at National Hospital Organization Osaka National Hospital or Ikeda Municipal Hospital. Histological examination was performed according to the METAVIR scoring system. The clinical backgrounds of the patients in Cohorts I and II, except for HCV-RNA quantity, were not different.

Table 1 Clinical backgrounds of subjects

	HV (Cohort I)	CHC (Cohort I)	CHC (Cohort II)
N	37	49	127
Male/female	20/17	24/25	58/69
Age (years)[a]	44.3 ± 14.6[b]	57.8 ± 12.6	56.5 ± 10.9
ALT (IU/L)[a]	ND	55.8 ± 39.9	64.6 ± 47.9
Plts ($\times 10^4/\mu L$)[a]	ND	16.8 ± 6.4	17.3 ± 6.1
HCV-RNA[c] (Log copies/mL)[a]	ND	6.1 ± 1.0	6.6 ± 0.6[b]
METAVIR activity (A0/1/2/3)	ND	ND	10/78/35/4
METAVIR fibrosis (F0/1/2/3/4)	ND	ND	0/70/29/21/7

CHC chronic hepatitis C patients, *HV* healthy volunteers, *ALT* alanine aminotransferase, *Plts* platelets, *ND* not determined

[a] Values are expressed as means ± SD

[b] Statistical significance was analyzed by the Mann–Whitney *U*-test ($P < 0.05$), compared with CHC group (Cohort I)

[c] Serum HCV-RNA titer was quantitated using the COBAS AmpliPrep™/COBAS TaqMan™ HCV test (Roche)

Reagents and antibodies

Recombinant human interleukin-4 (IL-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) from *Escherichia coli*, L-tryptophan, L-kynurenine, and 1-methyl-L-tryptophan (1-MT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein monoclonal antibodies (mAbs) against human CD4 (clone, SK3), CD11c (B-ly6), CD25 (M-A251), CD40 (5C3), CD80 (L307.4), CD86 (IT2.2), CD127 (HIL-7R-M21), CD274/PD-L1 (MIH1), HLA-DR (L243), Foxp3 (259D/C7), and isotype control Abs were purchased from BD Biosciences (San Jose, CA, USA).

Generation of CD14+ monocyte-derived dendritic cells

Monocyte-derived DCs (MoDCs) were generated from CD14+ cells as reported previously [27]. In brief, CD14+ cells were cultured for 7 days at 37 °C and 5 % CO_2 in DC culture medium [Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of Hepes buffer, and 10 mM of nonessential amino acids] in the presence of 20 ng/mL of IL-4 and 50 ng/mL of GM-CSF. On day 5 of the culture, cells were stimulated with 50 ng/mL of LPS and/or 50 ng/mL of IFN-γ to induce functional IDO, and cultured for 48 h. On day 7, cells were harvested and subjected to phenotypic and functional analysis. At the same time, the supernatant of the culture was also collected and subjected to cytokine assays. As controls, unstimulated MoDCs were also prepared.

Flow cytometric analysis

For the analysis of cell surface markers, cells were stained as reported previously [27]. In this study, Tregs were defined as CD4+CD25+CD127-Foxp3+ cells, the frequency of which in PBMCs was analyzed as reported previously [11]. Flow cytometric analyses were performed with the use of a FACSCantoII flow cytometer (BD Biosciences). Analyses of data were done with FACSDiva 6.1 software (BD Biosciences).

Analysis of IDO activity by high-performance liquid chromatography (HPLC)

For the measurement of Kyn and Trp, the HPLC analysis was performed according to the procedure developed by Takikawa et al. [28]. As an index of IDO activity in vivo, the serum kynurenine-to-tryptophan ratio (KTR) was determined by HPLC [26, 29], after deproteinization by the addition of one-tenth volume 2.4 M perchloric acid and centrifugation at $20000 \times g$ for 10 min. To assay the functional IDO in MoDCs in vitro, the cells were harvested on day 7 of the culture, washed, and resuspended in Hanks' balanced salt solution (HBSS; Gibco Laboratories) containing 100 μM L-Trp. The cells were incubated for an additional 24 h, and Kyn in the culture supernatants was determined by HPLC. IDO activity in vitro was expressed as the concentration of Kyn (μM) in the supernatant, converted from 100 μM L-Trp by IDO.

T-cell stimulation and cytokine analyses

Naive CD4+ T cells were isolated from the allogeneic healthy volunteer using a Naive CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. After 7 days of the culture, the

graded numbers of IDO-DCs (MoDCs stimulated with LPS and IFN-γ for 48 h) were co-cultured with 1×10^5 naive CD4+ T cells in DC culture medium for 4 days. An IDO-specific inhibitor, 1-MT, was used to confirm the specificity of the IDO activity in the T-cell responses. On day 0 of the co-culture, 1-MT was added to IDO-DCs and T-cell cultures at a final concentration of 1 mM. On day 4, half of the supernatants were collected to assess the Th1/Th2 polarization, which was done by measuring the various cytokines. Next, WST-8 reagent in the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added to the cultures, followed by incubation for 4 h. The T-cell proliferation index was measured at the absorbance 450 nm of reduced WST-8 using the plate reader. Assays were performed in triplicate wells.

Cytokine bead assay

To analyze the cytokine secretion of IDO-DCs and of naive CD4+ T cells primed with IDO-DCs, the concentrations of IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFN-γ, or tumor necrosis factor-alpha (TNF-α) in the supernatants were assayed using the Cytometric Bead Array System (BD Biosciences) according to the manufacturer's instructions.

Treg induction

To assess the potential effects of IDO on Treg induction from naive CD4+ T cells, the cells were primed with allogeneic IDO-DCs at a 10:1 ratio in HBSS containing 100 μM L-Trp. After 7 days, the primed T cells were harvested and assessed for their surface phenotype and intracellular Foxp3 expression. Phenotyping of the cells after the co-culture was performed using anti-CD4-PerCP, anti-CD25-APC, and anti-CD127-PE. To exclude dead lymphocytes after the co-culture, Near-IR LIVE/DEAD Fixable Dead Cell Stain (Invitrogen, Carlsbad, CA, USA) was used, according to the manufacturer's instructions. Next, the cells were fixed, permeabilized, and stained with anti-Foxp3-Alexa Fluor 488, using the Human FoxP3 Buffer Set (BD Biosciences) according to the manufacturer's instructions. The frequency of CD4+CD25+ CD127-Foxp3+ Tregs generated from each priming culture condition was determined by flow cytometry. As described above, 1 mM of 1-MT was added on day 0 to test for IDO-dependent effects.

Statistical analysis

The values were analyzed by nonparametric tests–the Mann–Whitney *U*-test, the Wilcoxon signed rank test, or Spearman's rank correlation test–or by linear regression analysis, using GraphPad Prism software, version 5.04 (Graph Pad Software, San Diego, CA, USA). A P value of <0.05 was considered to be statistically significant.

Results

Systemic IDO activity is enhanced in chronic hepatitis C patients

To examine whether or not IDO activity is up-regulated in chronically HCV-infected patients, we compared the serum Kyn and Trp levels between the groups in Cohort I. The serum KTR was significantly higher in the CHC group than that in the HV group (Fig. 1a). Furthermore, we found that the concentration of Kyn in the CHC group was significantly higher than that in the HV group, whereas the levels of Trp were comparable in the two groups (Fig. 1a). These results show that the KTR level in serum, as a surrogate for systemic IDO activity, was higher in chronic hepatitis C patients than in uninfected controls. Furthermore, as the KTR and Kyn levels were correlated (data not shown), the serum Kyn level can be regarded as a surrogate marker for systemic IDO activity.

Next, in order to examine whether or not the enhanced systemic IDO activity was specific for chronically HCV-infected patients, we compared serum Kyn concentrations among chronic hepatitis B patients, chronic hepatitis C patients (Cohort II), and healthy subjects. The serum Kyn concentration in chronic hepatitis B patients was significantly higher than those in the healthy subjects and the patients with chronic hepatitis C (chronic hepatitis B patients: 2.42 ± 0.11 μM, healthy subjects, 1.12 ± 0.09 μM, chronic hepatitis C patients in Cohort II: 2.04 ± 0.06 μM), suggesting that systemic IDO activity is enhanced in chronic HBV infection as well.

Systemic IDO activity correlates with activity grade and fibrosis stage in the liver

Next, to investigate the underlying mechanisms of enhanced IDO activity in chronically HCV-infected patients, we assessed whether or not serum Kyn levels in Cohort II were correlated with various clinical parameters and the METAVIR scores. A significant positive correlation was observed between serum Kyn levels and the histological activity or fibrosis scores (Fig. 1b). However, there was no correlation between the Kyn level and age, ALT level, or HCV-RNA quantity (Fig. 1b). These results show that the more advanced the inflammation and fibrosis of the liver, the higher the serum Kyn, and vice versa. The inverse correlation between serum Kyn and platelet counts was consistent with the correlation between Kyn and the fibrosis score (Fig. 1b).

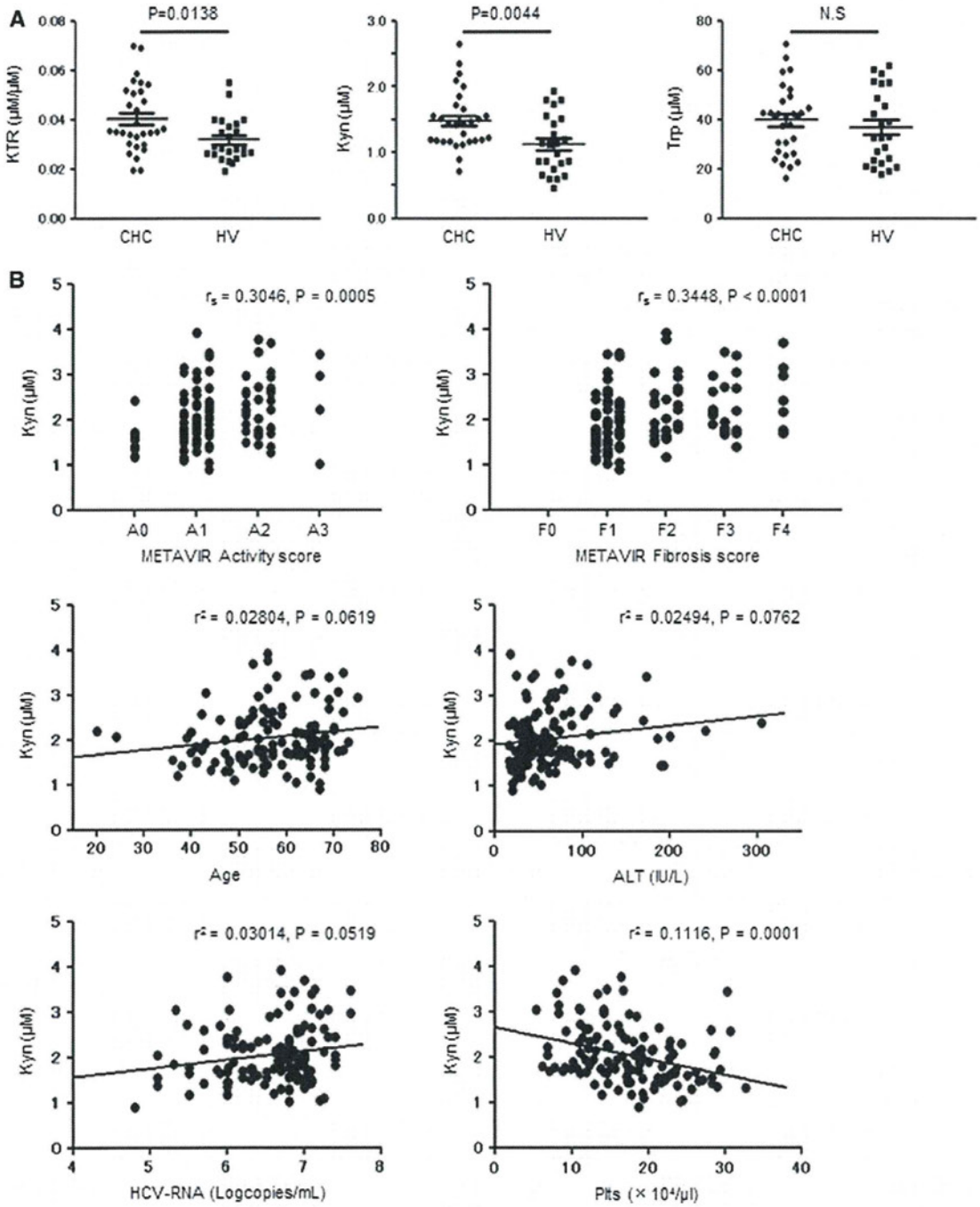


Fig. 1 Systemic indoleamine 2,3-dioxygenase (IDO) activity is enhanced in chronic hepatitis C patients. **a** Serum kynurenine (*Kyn*) and tryptophan (*Trp*) were assayed by HPLC as described in "Subjects, materials, and methods", and the kynurenine-to-tryptophan ratio (*KTR*) was calculated from their concentrations. Scatter plots of 30 chronic hepatitis C patients (*CHC*) and 24 healthy volunteers (*HV*) are shown. *Horizontal bars* depict mean ± SEM. Statistical analyses were performed using the nonparametric Mann–Whitney *U*-test. **b** Correlation analyses were performed between the serum Kyn concentration and histological scores in the liver, and clinical parameters (age, alanine aminotransferase [*ALT*], hepatitis C virus [*HCV*]-RNA titers, and platelet counts [*Plts*]) in 127 chronic hepatitis C patients. Spearman's correlation or simple linear regression analyses were performed. r_s Spearman's correlation coefficient, r^2 linear regression coefficient. *N.S* not significant

Lipopolysaccharide and IFN-γ induce functional IDO in DCs

DCs have been reported to be the most prominent IDO inducer in blood cells in response to inflammatory stimuli [13]. We first assayed the IDO activity (i.e., production of Kyn) of unstimulated MoDCs from chronic hepatitis C patients and found that they did not induce functional IDO (Fig. 2a). In order to simulate the inflammatory condition of DCs in vivo, we examined whether or not IDO was inducible in MoDCs with different combinations of cytokines for various incubation times. In this context, we examined the IDO activity of MoDCs stimulated with LPS alone, IFN-γ alone, or LPS plus IFN-γ for 48 h. The Kyn concentration in media from MoDCs stimulated with LPS alone did not differ from that in unstimulated MoDCs, whereas Kyn concentrations in media from MoDCs stimulated with IFN-γ alone or LPS plus IFN-γ were elevated (Fig. 2a). These results show that the combination of LPS and IFN-γ for 48 h significantly induces functional IDO in MoDCs. Therefore, in the following experiments, we used a combination of LPS and IFN-γ to induce functional IDO.

DCs from chronic hepatitis C patients induce more IDO in response to LPS and IFN-γ than those from healthy volunteers

First, we compared the phenotype of IDO-DCs and unstimulated MoDCs from each group. The expressions of CD40, CD80, CD86, HLA-DR, and CD274/PD-L1 on IDO-DCs were significantly up-regulated compared with those on unstimulated MoDCs, and their expression levels were not different between the CHC and HV groups (Fig. 2b).

Next, we examined the concentration of Kyn in the culture supernatants. In the CHC group, Kyn levels from MoDC culture were significantly enhanced by the stimulation with LPS and IFN-γ (Fig. 2c). Moreover, the Kyn levels in the IDO-DC culture from the CHC group were significantly higher than those in the HV group, whereas those in unstimulated MoDCs did not differ between the groups (Fig. 2c). This increase of Kyn was blocked by the addition of 1-MT, showing that the production of Kyn is specifically dependent on IDO activity (Fig. 2c). These results show that IDO activity is enhanced more in DCs from chronic hepatitis C patients than in DCs from healthy subjects.

Finally, we compared the ability of IDO-DCs to produce various cytokines. The levels of IL-6, IL-10, IL-12p70, and TNF-α from IDO-DCs were not different between the hepatitis C patients and healthy controls (Fig. 2d).

Fig. 2 Enhanced induction of IDO in dendritic cells (DCs) from chronic hepatitis C patients in response to a combination of lipopolysaccharide (*LPS*) and interferon-γ (*IFN-γ*). **a** The levels of Kyn in the culture supernatants of monocyte-derived DCs (MoDCs) in the presence of LPS (50 ng/mL) and/or IFN-γ (50 ng/mL) were determined by HPLC, as described in "Subjects, materials, and methods". The results are expressed as the mean ± SEM from 4 chronic hepatitis C patients. $*P < 0.05$ by nonparametric Wilcoxon signed rank test. *Controls*, unstimulated MoDCs. **b** Phenotype analysis of IDO-DCs was performed as described in "Subjects, materials, and methods". The values are expressed as mean fluorescence intensity (*MFI*). The MFI of each marker is represented as the mean ± SEM from 9 patients and 7 healthy volunteers. $*P < 0.05$ by nonparametric Wilcoxon signed rank test. *IDO-DCs*, MoDCs stimulated with LPS and IFN-γ for 48 h. **c** The levels of Kyn in the culture supernatants were assayed by HPLC as described in "Subjects, materials, and methods". The samples were obtained from MoDCs in the presence (IDO-DCs) or absence (controls) of a combination of LPS and IFN-γ. In parallel, the same experiments were performed in the presence or the absence of 1 mM of 1-methyl-L-tryptophan (*1-MT*). The results are expressed as the mean ± SEM from 12 chronic hepatitis C patients and 10 healthy controls. $*P < 0.05$ by Wilcoxon signed rank test, $**P < 0.05$ by Mann–Whitney *U*-test. **d** The levels of cytokines in the culture supernatants from IDO-DCs were assayed with the Cytometric Bead Array System, as described in "Subjects, materials, and methods". *Bars* depict the mean concentration of each cytokine ± SEM from 10 healthy volunteers and 10 chronic hepatitis C patients. *IL* interleukin, *TNF-α* tumor necrosis factor-α

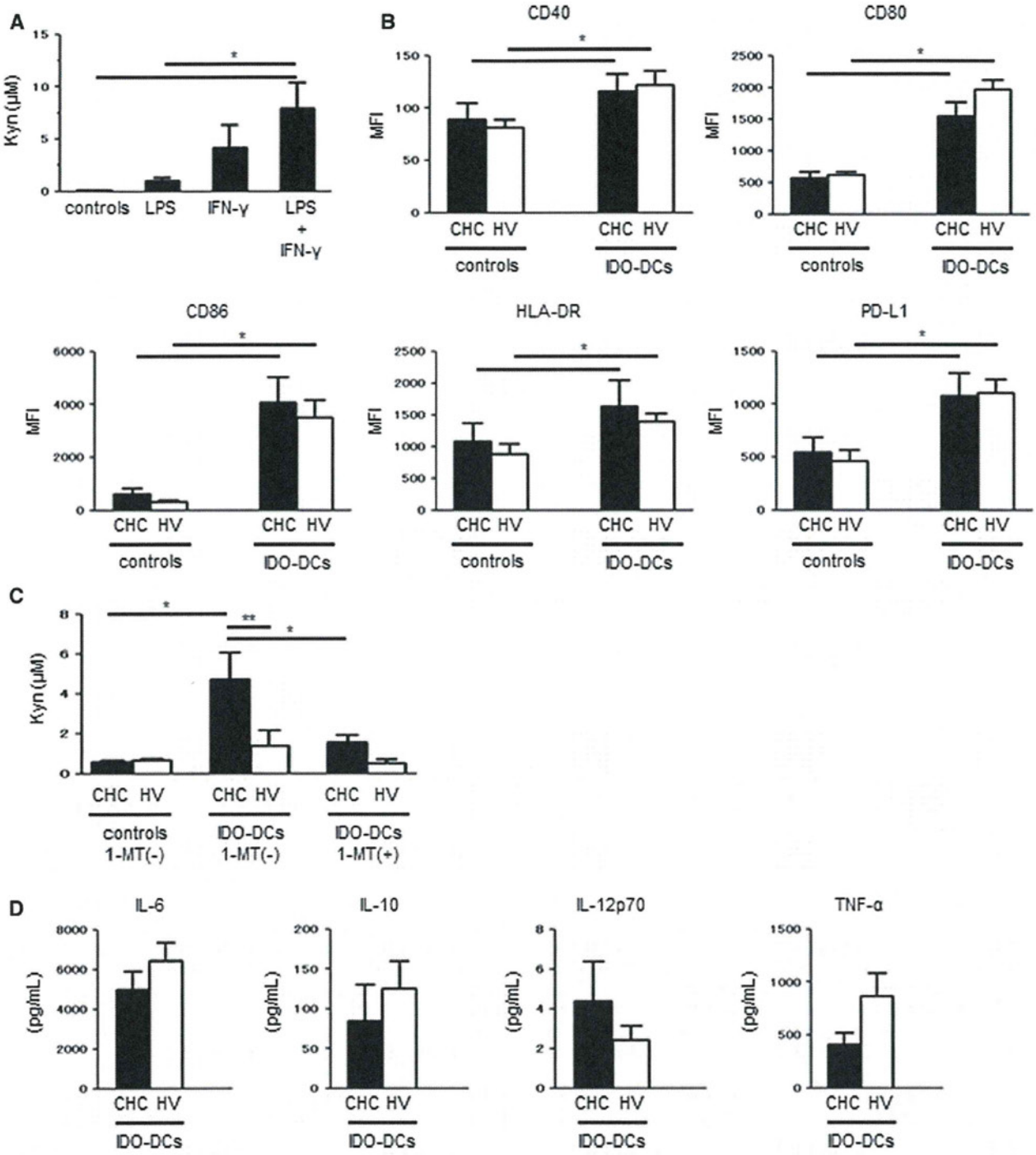
IDO is not involved in allogeneic T-cell proliferation and Th1/Th2 differentiation with DCs from chronic hepatitis C patients

With regard to the allogeneic CD4+ T-cell response, IDO-DCs from the CHC group tended to have a lower stimulatory capacity than those from the HV group (Fig. 3a). To examine whether this phenomenon was dependent on IDO activity, we compared T-cell proliferation with IDO-DCs in the presence and absence of 1-MT. The CD4+ T-cell responses with IDO-DCs were not restored by the addition of 1-MT, regardless of HCV infection (Fig. 3a).

In order to examine whether functional IDO in DCs is involved in Th1/Th2 differentiation, we quantified cytokines in the supernatants obtained from the co-culture of IDO-DCs and CD4+ T cells. In samples from chronic hepatitis C patients, the levels of Th1 cytokines (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-10, IL-13) tended to be higher than the levels in samples from healthy volunteers, though the difference was not significant. The levels of all cytokines, except for IL-4, tended to decrease with the addition of 1-MT (Fig. 3b). Thus, IDO in DCs is not actively involved in Th1/Th2 differentiation.

IDO is involved in the induction of regulatory T cells

We examined whether or not IDO in DCs was involved in the generation of Tregs. With IDO-DCs from the CHC



group, the frequency of Tregs after the co-culture was significantly higher than that with IDO-DCs from the HV group (Fig. 4a). Such Treg frequency from the culture of the CHC group was significantly reduced in the presence of 1-MT (Fig. 4a). These results show that functional IDO in DCs is partially involved in the generation of Tregs in vitro.

A significant correlation exists between peripheral Treg frequency and serum IDO activity

Finally, we examined whether or not the frequency of Tregs in PBMCs and serum Kyn levels were correlated in our subjects. In the chronic hepatitis C patients, a positive correlation was observed between these parameters (Fig. 4b).

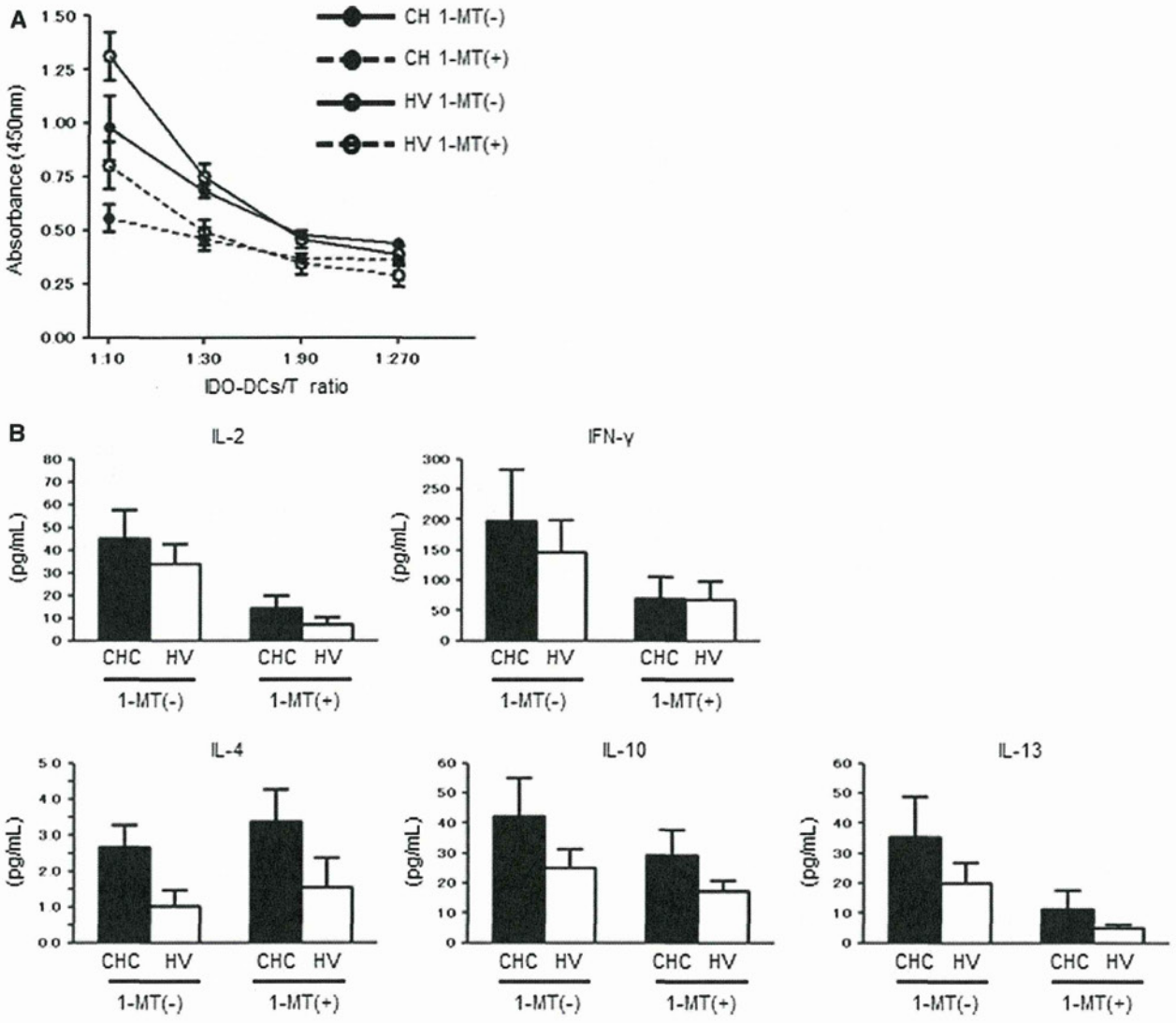


Fig. 3 IDO is not involved in lower allogeneic T-cell response and Th1/Th2 differentiation with DCs from chronic hepatitis C patients. **a** Allogeneic mixed lymphocyte reaction (MLR) with IDO-DCs was performed as described in "Subjects, materials, and methods". *Closed circles* are the 450-nm absorbance obtained with IDO-DCs from the CHC group, and *open circles* are that obtained with IDO-DCs from the HV group. *Dotted lines* are the 450-nm absorbance obtained with IDO-DCs from both groups with the addition of 1-MT. *Vertical bars* indicate the mean ± SEM from 5 chronic hepatitis C patients and 5 healthy volunteers. **b** The levels of cytokines in the supernatants of co-culture of IDO-DCs and naive CD4+ T cells in the presence or absence of 1-MT were assayed with the Cytometric Bead Array System. Results are expressed as the mean ± SEM from 5 patients and 5 healthy controls. *IDO-DCs*; see Fig. 2 legend

However, no significant correlation was observed between peripheral Treg frequency and clinical parameters (i.e., age, ALT, HCV-RNA titers, or platelet counts) (data not shown). These results suggest that an increase in serum Kyn, or enhanced IDO activity, is involved in the increased frequency of Tregs in the PBMCs of HCV-infected patients.

Discussion

In comparison with healthy subjects, we have shown that in chronic hepatitis C patients: (1) systemic IDO activity is enhanced; (2) DCs from these patients exhibit enhanced IDO activity in response to LPS and IFN-γ; (3) IDO-DCs from these patients are more capable than IDO-DCs from healthy volunteers of inducing Tregs in vitro; and (4) the frequency of Tregs in PBMCs is positively correlated with the serum Kyn concentration. Based on these data, it seems that enhanced IDO activity in chronic HCV infection may be one of the mechanisms of Treg induction.

Mammals have two enzymes that catabolize the first and rate-limiting step in the degradation of Trp, resulting in the production of downstream metabolites collectively known as Kyn. The first enzyme is tryptophan 2,3-dioxygenase

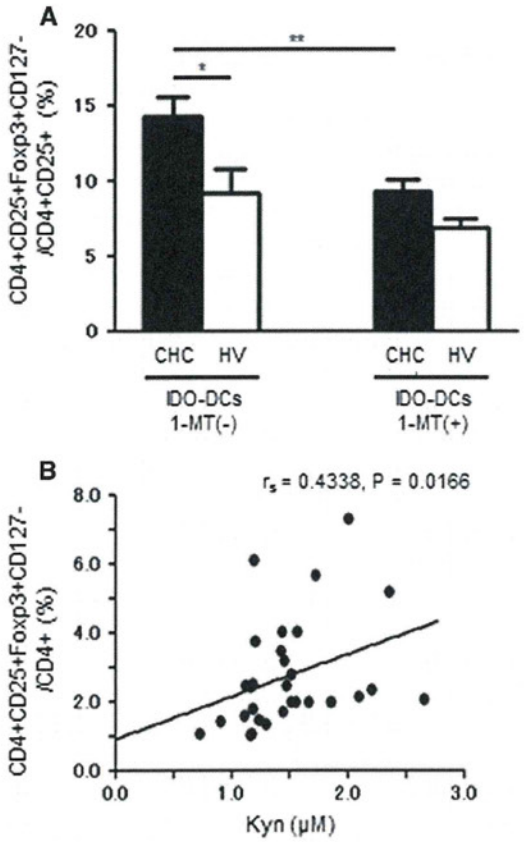


Fig. 4 IDO is involved in the induction of regulatory T cells. **a** After IDO-DCs were generated from the CHC or HV group, naive CD4+ T cells were co-cultured for 7 days with IDO-DCs in the presence or absence of 1-MT. The cultured T cells were stained with relevant antibodies (*Abs*) and analyzed with a FACSCantoII flow cytometer. The percentage of regulatory T cells was determined by the positive ratio of CD4+CD25+CD127-Foxp3+ cells to CD4+CD25+ T cells, as described in "Subjects, materials, and methods". Results are expressed as the mean ± SEM from 9 chronic hepatitis C patients and 5 healthy controls. $*P < 0.05$ by Mann–Whitney *U*-test, $**P < 0.05$ by the Wilcoxon signed rank test. *IDO-DCs*; see Fig. 2 legend. **b** The correlation between the serum Kyn level and the frequency of regulatory T cells was analyzed in 30 chronic hepatitis C patients. The frequency of regulatory T cells was expressed as the percentage of CD4+CD25+CD127-Foxp3+ T cells in CD4+ T cells assessed by FACS. r_s Spearman's correlation coefficient

(TDO), which is expressed primarily in the liver and catabolizes excess dietary Trp to maintain its serum concentration. The second one is IDO, which is expressed in a wider range of tissues, but by a limited range of cell types. In general, TDO is constitutively expressed and is not regulated by inflammatory mediators, while IDO expression is inducible by antigen-presenting cells and is subject to complex regulation by various immunological signals. For the analysis of IDO activity, several modalities have been used, including HPLC and colorimetric and mass spectrometric assays [29, 30]. In the present study, to measure Trp and Kyn, we utilized HPLC owing to its reproducibility, as well as its high-throughput feature. By measuring large numbers of samples, we demonstrated that systemic IDO activity (as expressed by serum KTR) in chronic hepatitis C patients was enhanced compared with that in healthy controls. In addition, we found that increases in KTR were dependent on increased serum Kyn, but not on Trp. Thus, we used Kyn levels as a surrogate for IDO activity.

It is yet to be clarified which type of cell is the source of Kyn in chronic hepatitis C patients. Two possibilities exist for its origin; one is the liver and the other is DCs. We observed positive correlations between serum Kyn levels and the degree of liver inflammation or fibrosis in the present study, suggesting that IDO in the liver may play some role in Kyn production. In support of this possibility, up-regulation of IDO in the liver and increased serum KTR have been reported in patients with chronic HCV infection [26]. It is well known that the inflamed liver is infiltrated by numerous activated immune cells, such as T cells, natural killer (NK) cells, macrophages, and DCs. Thus, it is likely that activated T cells or NK cells release IFN-γ or other cytokines and subsequently induce IDO in hepatocytes or co-existing DCs.

Several investigators have reported that some of the critical stimuli for inducing IDO are inflammatory cytokines or Toll-like receptor (TLR) agonists [14–16, 30–34]. Among them, IFN-γ is reported to play a prominent role in inducing IDO in cancer cells, and the origin of the IFN-γ is presumed to be infiltrated lymphocytes [31]. Furthermore, LPS is regarded as a potent stimulant that induces and sustains IDO in DCs. Therefore, we hypothesized that DCs exposed to some inflammation or fibrosis-related factors express IDO, thereby regulating the immune response in chronic hepatitis C patients. In this study, we used MoDCs for functional assays of IDO in DCs. In order to simulate the inflammatory condition in vivo, we stimulated MoDCs with various combinations of factors, as described above. We found that a combination of IFN-γ and LPS strongly enhanced IDO activity in MoDCs, with this activity being more significantly enhanced in the MoDCs from chronically HCV-infected patients than in those from the healthy controls (Fig. 2a, c). However, the other cytokines failed to enhance IDO activity in MoDCs. Moreover, we confirmed that IDO activity was also enhanced in myeloid dendritic cells (MDCs), stimulated with a combination of IFN-γ and LPS, from the healthy volunteers (Supplementary Figure 1). Because blood MDCs and plasmacytoid DCs (PDCs) are scarce in PBMCs, we used MoDCs as representative cells for the functional analysis of IDO. Thus, in this study, we used a combination of LPS and IFN-γ for MoDCs to induce functional IDO and termed these cells 'IDO-DCs'.

It is intriguing that MoDCs from chronic hepatitis C patients expressed more functional IDO in response to

IFN-γ and LPS than the MoDCs from the healthy controls. The simplest reason for this finding would be that such a difference occurs owing to a difference in receptor expressions on DCs. However, this is unlikely, because our previous work showed that TLR4 transcripts in immature MoDCs did not differ between patients with chronic hepatitis C and healthy controls [27]. In addition, in the present study, flow cytometric analysis revealed that the expression of CD119 (IFN-γ receptor α chain) on MoDCs did not differ between the two groups (data not shown). The next possible explanation of the finding that MoDCs from chronic hepatitis C patients expressed more functional IDO in response to IFN-γ and LPS than those from the healthy controls is that there was an influence of other cytokines produced from the stimulated MoDCs in an autocrine fashion. It has been reported that a balance between Th1 and Th2 cytokines has some impact on IDO expression [31]. Finally, the signaling pathways downstream of IFN-γ and LPS may differ between the groups. Jung et al. [32] reported that LPS-induced IDO expression was mediated by IFN-γ-independent mechanisms, including phosphatidylinositol-3-kinase (PI3K) and Jun-N-terminal kinase (JNK) pathways, in murine bone marrow-derived DCs, while IFN-γ-induced IDO expression was regulated by the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathways. As shown in the present study (Fig. 2a), the levels of IDO activity in MoDCs were additively enhanced with LPS and IFN-γ, suggesting the presence of some cross-talk between these signals. Further investigation focusing on the signaling pathway of functional IDO induction is needed to clarify this issue.

Numerous reports have shown that IDO is involved in immune tolerance. As for the mechanisms underlying its involvement, the starvation of Trp could inhibit T-cell proliferation by way of the general control nonrepressed 2 (GCN2) kinase and eukaryotic initiation factor 2α (eIF2α) pathway [35] or the mammalian target of rapamycin (mTOR) and PI3K pathway [36]. Accumulation of Kyn and its metabolites could exert an immune-modulating effect. In the present study, serum Kyn levels were higher in HCV-infected patients than in the healthy controls, whereas Trp levels were comparable in the two groups, suggesting that an increase of Kyn derivatives contributes to immune modulation.

In chronic HCV infection, the mechanisms of IDO-mediated immune tolerance remain unclear. In the present study, we have shown that IDO-DCs are involved in the generation of Tregs in vitro, and the specificity of this involvement was confirmed by the effect of 1-MT. In order to exclude the possibility that 1-MT is cytotoxic to DCs and naive CD4+ T cells, we performed a dye exclusion test or WST-8 assay. Even at the highest concentration of 1-MT, the percentages of viable DCs and the proliferation of T cells were not decreased compared with the findings at the lower concentrations, suggesting that 1-MT was not cytotoxic to cells (Supplementary Figure 2A,B). A possible link between enhanced IDO activity and an increase in Treg frequency was observed in the chronic hepatitis C patients in this study. Thus, it is possible that IDO activity may be partially involved in Treg induction.

Several research groups, including ours, have reported that the frequency and the suppressor function of Tregs are higher in chronic hepatitis C patients than in controls [10, 11]. However, the mechanisms of Treg induction or activation are still largely unknown. Various molecules in DCs, including IL-10, transforming growth factor-beta (TGF-β), programmed cell death 1 ligand 1 (PD-L1), and IDO, are key differentiation molecules for Tregs in various clinical settings. Although the level of TGF-β from DCs was not evaluated in the present study, the levels of IL-10 production and PD-L1 expression did not differ between the HCV-infected patients and the healthy controls (Fig. 2b, d). In this study, the addition of 1-MT did not completely suppress Treg induction by IDO-DCs in vitro. Thus, it is suggested that other factors, such as IL-10, TGF-β, and PD-L1, are also involved in Treg induction. Cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is capable of inducing functional IDO in DCs, has been reported as one of the key molecules for Treg induction [37]. In the present study, the induction of Tregs with IDO-DCs was not altered in the presence of masking anti-CTLA-4 antibody (data not shown), suggesting that CTLA-4 is not involved in this setting.

In conclusion, we have demonstrated that systemic IDO activity is enhanced in chronic hepatitis C patients, and this activity is influenced by histological activity and fibrosis. DCs express functional IDO in response to inflammatory stimuli and, presumably, induce Tregs. Targeting IDO with its specific inhibitor 1-MT could serve as a potential modality to improve the immune response to HCV.

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

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med. 2001;345:41–52.
2. Seeff LB. Natural history of chronic hepatitis C. Hepatology. 2002;36:S35–46.

3. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245–52.
4. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003;21:685–711.
5. Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol. 1999;162:5584–91.
6. Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, et al. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis. 2004;190: 1919–26.
7. Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology. 2001;120:512–24.
8. Murakami H, Akbar SM, Matsui H, Horiike N, Onji M. Decreased interferon-alpha production and impaired T helper 1 polarization by dendritic cells from patients with chronic hepatitis C. Clin Exp Immunol. 2004;137:559–65.
9. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004;22:531–62.
10. Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology. 2004;40: 1062–71.
11. Itose I, Kanto T, Kakita N, Takebe S, Inoue M, Higashitani K, et al. Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis. J Viral Hepat. 2009;16:844–52.
12. Yamazaki F, Kuroiwa T, Takikawa O, Kido R. Human indolylamine 2,3-dioxygenase. Its tissue distribution, and characterization of the placental enzyme. Biochem J. 1985;230:635–8.
13. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol. 2004;4: 762–74.
14. Taylor MW, Feng GS. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. FASEB J. 1991;5:2516–22.
15. Puccetti P. On watching the watchers: IDO and type I/II IFN. Eur J Immunol. 2007;37:876–9.
16. Braun D, Longman RS, Albert ML. A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. Blood. 2005;106:2375–81.
17. Boasso A, Herbeuval JP, Hardy AW, Anderson SA, Dolan MJ, Fuchs D, et al. HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells. Blood. 2007;109:3351–9.
18. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281:1191–3.
19. Hainz U, Jurgens B, Heitger A. The role of indoleamine 2,3-dioxygenase in transplantation. Transpl Int. 2007;20:118–27.
20. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science. 2005;310:850–5.
21. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003;9:1269–74.
22. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science. 2002;297: 1867–70.
23. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med. 2002;196: 447–57.
24. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol. 2008;181:5396–404.
25. Jurgens B, Hainz U, Fuchs D, Felzmann T, Heitger A. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. Blood. 2009;114:3235–43.
26. Larrea E, Riezu-Boj JI, Gil-Guerrero L, Casares N, Aldabe R, Sarobe P, et al. Upregulation of indoleamine 2,3-dioxygenase in hepatitis C virus infection. J Virol. 2007;81:3662–6.
27. Yakushijin T, Kanto T, Inoue M, Oze T, Miyazaki M, Itose I, et al. Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection. Hepatol Res. 2006;34:156–62.
28. Takikawa O, Yoshida R, Kido R, Hayaishi O. Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. J Biol Chem. 1986;261:3648–53.
29. Suzuki Y, Suda T, Furuhashi K, Suzuki M, Fujie M, Hahimoto D, et al. Increased serum kynurenine/tryptophan ratio correlates with disease progression in lung cancer. Lung Cancer. 2010;67:361–5.
30. Suh HS, Zhao ML, Rivieccio M, Choi S, Connolly E, Zhao Y, et al. Astrocyte indoleamine 2,3-dioxygenase is induced by the TLR3 ligand poly(I:C): mechanism of induction and role in antiviral response. J Virol. 2007;81:9838–50.
31. Godin-Ethier J, Pelletier S, Hanafi LA, Gannon PO, Forget MA, Routy JP, et al. Human activated T lymphocytes modulate IDO expression in tumors through Th1/Th2 balance. J Immunol. 2009;183:7752–60.
32. Jung ID, Lee CM, Jeong YI, Lee JS, Park WS, Han J, et al. Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells. FEBS Lett. 2007;581:1449–56.
33. Fujigaki H, Saito K, Fujigaki S, Takemura M, Sudo K, Ishiguro H, et al. The signal transducer and activator of transcription 1alpha and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB pathways, and synergistic effect of several proinflammatory cytokines. J Biochem. 2006;139:655–62.
34. Fujigaki S, Saito K, Sekikawa K, Tone S, Takikawa O, Fujii H, et al. Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism. Eur J Immunol. 2001;31:2313–8.
35. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity. 2005;22:633–42.
36. Cobbold SP, Adams E, Farquhar CA, Nolan KF, Howie D, Lui KO, et al. Infectious tolerance via the consumption of essential amino acids and mTOR signaling. Proc Natl Acad Sci USA. 2009;106:12055–60.
37. Li R, Perez N, Karumuthil-Melethil S, Prabhakar BS, Holterman MJ, Vasu C. Enhanced engagement of CTLA-4 induces antigen-specific CD4+CD25+Foxp3+ and CD4+CD25− TGF-beta 1+ adaptive regulatory T cells. J Immunol. 2007;179:5191–203.

Research Article

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Comparison of resection and ablation for hepatocellular carcinoma: A cohort study based on a Japanese nationwide survey

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Background & Aims: The treatment of choice for early or moderately advanced hepatocellular carcinoma (HCC) with good liver function remains controversial. We evaluated the therapeutic impacts of surgical resection (SR), percutaneous ethanol injection (PEI), and radiofrequency ablation (RFA) on long-term outcomes in patients with HCC.

Methods: A database constructed on the basis of a Japanese nationwide survey of 28,510 patients with HCC treated by SR, PEI, or RFA between 2000 and 2005 was used to identify 12,968 patients who had no more than 3 tumors (⩽3 cm) and liver damage of class A or B. The patients were divided into SR (n = 5361), RFA (n = 5548), and PEI groups (n = 2059). Overall survival and time to recurrence were compared among them.

Results: Median follow-up was 2.16 years. Overall survival at 3 and 5 years was respectively 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA, and 78.9%/56.3% in the PEI. Time to recurrence at 3 and 5 years was 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively. On multivariate analysis, the hazard ratio for death was significantly lower in the SR group than in the RFA (SR *vs.* RFA:0.84, 95% confidence interval, 0.74–0.95; $p = 0.006$) and PEI groups (SR *vs.* PEI:0.75, 0.64–0.86; $p = 0.0001$). The hazard ratios for recurrence were also lower in the SR group than in the RFA (SR *vs.* RFA:0.74, 0.68–0.79; $p = 0.0001$) and PEI groups (SR *vs.* PEI:0.59, 0.54–0.65; $p = 0.0001$).

Conclusions: Our findings suggest that surgical resection results in longer overall survival and shorter time to recurrence than either RFA or PEI in patients with HCC.

Keywords: Hepatectomy; Surgical resection; Radiofrequency ablation; Percutaneous ethanol injection.

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Abbreviations: HCC, hepatocellular carcinoma; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transcatheter hepatic arterial chemoembolization.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, worldwide [1]. Outcomes remain disappointing, despite recent progress in the techniques of diagnosis and therapy. Japanese [2], European [3] and American [4] clinical practice guidelines strongly recommend surgical resection (SR) and percutaneous ablation, including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI), for the management of early or moderately advanced HCC (i.e., up to 3 tumors 3 cm or less in diameter) in patients with adequately maintained liver function. Although comparative studies of these treatments have been conducted previously [5–7], the most suitable treatment strategy still remains controversial.

By nationwide surveys initiated in 1965, the Liver Cancer Study Group of Japan has prospectively collected data on patients with HCC in Japan. The Group conducted two retrospective analyses to define the treatment with the best outcomes [8,9]. However, each of the analyses was flawed, and had several problems: data on RFA were not included in the first report [8], and the follow-up period was short in the second one [9]. Although the second analysis demonstrated that surgical resection was superior to RFA and PEI for preventing recurrence [9], no apparent difference in the overall survival could be discerned between surgery and percutaneous ablation therapies (RFA and PEI). Thus, the treatment of choice for less advanced HCC still remains under debate.

Before starting this study, the results of 2 randomized controlled trials (RCT) were available [10,11]. As we pointed out in a previous report [12], however, the study designs of these 2

trials were critically flawed by factors such as insufficient sample size, excessively optimistic hypotheses, and high conversion ratios. Because of these problems, the results of the two RCTs do not allow firm conclusions to be drawn concerning the important clinical question: is surgery or percutaneous ablation the treatment of choice for early or moderately advanced HCC? To answer this question, we conducted this cohort study based on the latest data available from a Japanese nationwide survey.

Patients and methods

Patients and settings

The Liver Cancer Study Group of Japan has performed nationwide surveys of patients with primary liver cancer since 1965. Patients are registered and followed up, as reported previously [9]. Although this study protocol was not submitted to the Institutional Review Board of each institution participating in the nationwide survey, the collection and registration of data of patients with HCC were performed with the approval of each institution. Because RFA has been available for clinical use since 1999 in Japan, we set the study period from 2000 to 2005, to exclude preliminary experiences with RFA. During this period, a total of 28,510 patients with HCC were registered and received surgical resection, RFA or PEI as the primary treatment with curative intent for HCC. We identified 12,968 patients who met the following criteria: (1) liver function classified as liver damage A or B defined by the Liver cancer Study Group of Japan [13]; (2) number of tumors 3 or less; (3) maximum tumor diameter ⩽3 cm. The 12,968 patients were divided into 3 groups according to the treatment received: SR group (n = 5361, 41.3%), RFA group (n = 5548, 42.8%), and PEI group (n = 2059, 15.9%). The diagnostic criteria and details of follow-up were described previously [8]. Because it has been unusual for biopsies to be performed in cases treated by percutaneous ablation in Japan, histological findings such as microscopic vascular invasion, tumor grading, and microscopic intrahepatic metastasis were not evaluated in this study. Relevant clinical data were collected and analyzed.

Statistical analyses

The baseline characteristics of the three groups (Table 1) were compared by analysis of variance for continuous variables and by Chi-square or Mantel-trend tests for categorical variables. Consistent with our preliminary report [9], the SR group had a higher proportion of younger patients and male patients than the RFA and PEI groups. Hepatitis C virus infection was less prevalent in the SR group than in the RFA and PEI groups. Based on the liver damage class, serum albumin and total bilirubin levels, platelet counts, and the indocyanine green retention rate at 15 min, liver function was better in the SR group than in the RFA and PEI groups, consistent with our previous report [9]. As for tumor-related factors, the number of tumors was smaller, and the maximum tumor diameter was larger in the SR group than in the RFA or PEI group. The SR group had the lowest proportion of patients with abnormally elevated alpha-fetoprotein levels (⩾15 ng/ml) and the highest proportion of patients with abnormally elevated des-γ-carboxy prothrombin levels (⩾40 AU/ml).

Overall survival and time to recurrence curves were plotted using the Kaplan–Meier method and compared with the use of the log-rank test. Recurrence was diagnosed on the basis of imaging studies, clinical data, and/or histopathological studies at each institution [9].

The therapeutic impacts of surgical resection, RFA and PEI were estimated using a Cox proportional hazards model including the following 10 covariates: age, gender, liver damage class, hepatitis C virus antibody, hepatitis B surface antigen, platelet count, number of tumors, tumor size, and serum alpha-fetoprotein and des-γ-carboxy prothrombin levels. The results of multivariate analysis were expressed as hazard ratios with 95% confidence intervals. p values of <0.05 were considered to indicate statistical significance.

For the subgroup analyses, the study populations were classified into 8 subgroups according to the tumor size (< or ⩾2 cm), tumor number (single or multiple), and liver damage class (A or B). Macroscopic vascular invasion was excluded from the subgroup analyses because its presence is a contraindication to percutaneous ablation therapies. The therapeutic impacts of the three treatments were evaluated in each of these subgroups, and hazard ratios with 95% confidence intervals and p values were calculated according to the above three factors (tumor size, number of tumors, and liver damage class).

Results

The median follow-up after treatment was 2.16 years, and the 5th and 95th percentiles were 0.14 and 5.19 years, respectively. The overall survival rates at 3/5 years were 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA group, and 78.9%/56.3% in the PEI group (Fig. 1). The median survival times were 8.4, 5.9, and 5.6 years in the three groups, respectively. The time to recurrence rates at 3/5 years in the 3 groups were 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively (Fig. 2).

According to the results of the multivariate analysis, the hazard ratio for death in the SR group was 0.84 (0.74–0.95, $p = 0.006$) relative to that in the RFA group, and 0.75 (0.64–0.86, $p = 0.0001$) relative to that in the PEI group (Table 2A). The hazard ratios for recurrence in the SR group were 0.74 (0.68–0.79, $p = 0.0001$) and 0.59 (0.54–0.65, $p = 0.0001$) relative to those in the RFA and PEI groups, respectively (Table 2B). These results indicated that the overall survival and time to recurrence rates were both significantly better in the SR group than in the RFA and PEI groups.

The overall survival rates following surgical resection, RFA and PEI in the 4 subgroups with a single tumor are shown in Fig. 3A–D. The results of the subgroup analyses (summarized in Fig. 4A) showed that the overall survival was significantly longer in the SR group than in the RFA group in 2 subgroups of patients, namely, those who had a single tumor smaller than 2 cm in diameter with liver damage class A, and those who had a single tumor 2 cm or larger in diameter with liver damage class B.

As shown in Fig. 4B, the time to recurrence was shorter in the SR group than that in the RFA group in the 4 following subgroups: patients with a single tumor with liver damage class A (regardless of the tumor size), those with multiple tumors 2 cm or larger in diameter with liver damage class A, and those with a single tumor 2 cm or larger in diameter with liver damage class B.

Discussion

Our study showed that surgical resection was associated with significantly lower risk of both death and recurrence as compared to RFA and PEI in patients with early or moderately advanced HCC. Our previous preliminary report [9] suggested that surgery reduces the risk of recurrence, but failed to demonstrate any difference in the overall survival between surgery and percutaneous ablation therapies in patients with early or moderately advanced HCC. The present study reconfirms that surgery is associated with a reduced recurrence rate and newly shows that surgery yields a longer overall survival than percutaneous ablation therapies.

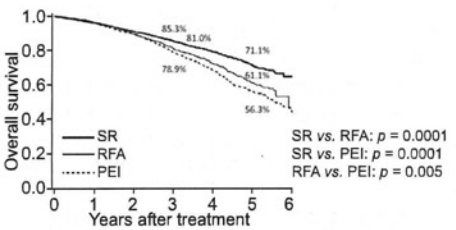
Differences in the results between the present study and previous investigations are most likely related to the sample size and length of follow-up. The total number of subjects increased markedly from 7185 in our previous study to 12,968 in this study, and the median follow-up period increased from 10.4 months to 2.16 years (25.9 months). These factors are considered not only to have enhanced the reliability of our findings, but also to have strengthened our conclusions. We believe that our results, which are, of course, subject to the inherent drawbacks of the study design, are meaningful, given the current lack of credible data derived from well-designed RCTs.

The large sample size and prolonged follow-up period also allowed us to perform several subgroup analyses, which were not feasible in our previous study [9]. We classified the patients

Table 1. Baseline characteristics.

Variables	SR n = 5361	RFA n = 5548	PEI n = 2059	*p* value
Age, median (5, 95 percentile), yr	66 (48, 77)	69 (52, 80)	69 (52, 80)	<0.0001[a]
Sex				<0.0001[b]
Male, No. (%)	3967 (74.0)	3569 (64.3)	1303 (63.3)	
Female, No. (%)	1394 (26.0)	1979 (35.7)	756 (36.7)	
Hepatitis virus infection				<0.0001[b]
HBs Ag(+)/HCV-Ab(-), No. (%)	908 (16.9)	462 (8.3)	141 (6.8)	
HBs Ag(-)/HCV-Ab(+), No. (%)	3393 (63.3)	4263 (76.8)	1632 (79.3)	
HBs Ag(+)/HCV-Ab(+), No. (%)	106 (2.0)	87 (1.6)	32 (1.6)	
HBs Ag(-)/HCV-Ab(-), No. (%)	760 (14.2)	512 (9.2)	160 (7.8)	
Unknown	194 (3.6)	224 (4.0)	94 (4.6)	
Liver damage				<0.0001[b]
A, No. (%)	4000 (74.6)	3349 (60.4)	1204 (58.5)	
B, No. (%)	1361 (25.4)	2199 (39.6)	855 (41.5)	
Serum albumin, median (5, 95 percentile), g/dl	3.9 (3.1, 4.6)	3.7 (2.9, 4.4)	3.7 (2.8, 4.4)	<0.0001[a]
Serum total bilirubin, median (5, 95 percentile), mg/dl	0.8 (0.4, 1.5)	0.9 (0.4, 1.9)	0.9 (0.4, 2.2)	<0.0001[a]
Platelet count, median (5, 95 percentile), x 10^4/μl	12.6 (5.8, 24.0)	9.9 (4.5, 20.4)	9.5 (4.4, 19.6)	<0.0001[a]
ICG R15, median (5, 95 percentile), %	15 (5, 35)	22 (7, 51)	24 (8, 51)	<0.0001[a]
Tumor number				<0.0001[c]
Single, No. (%)	4458 (83.2)	4068 (73.3)	1449 (70.4)	
Two, No. (%)	706 (13.2)	1096 (19.8)	443 (21.5)	
Three, No. (%)	197 (3.7)	384 (6.9)	167 (8.1)	
Tumor size, median (5, 95 percentile), mm	23 (12, 30)	20 (10, 30)	17 (10, 30)	<0.0001[a]
Alpha-fetoprotein				<0.0001[b]
≥15 ng/ml, No. (%)	2726 (50.9)	3028 (54.6)	1125 (54.6)	
<15 ng/ml, No. (%)	2457 (45.8)	2301 (41.5)	828 (40.2)	
Unknown, No. (%)	178 (3.3)	219 (3.9)	106 (5.2)	
Des-γ-carboxy prothrombin				<0.0001[b]
≥40 AU/ml, No. (%)	2182 (40.7)	1593 (28.7)	541 (26.3)	
<40 AU/ml, No. (%)	2651 (49.5)	3322 (59.9)	1169 (56.8)	
Unknown, No. (%)	528 (9.9)	633 (11.4)	349 (17.0)	

HBsAg, hepatitis B virus antigen; HCV-Ab, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min.
[a]ANOVA.
[b]Chi-square.
[c]Mante-trend test.

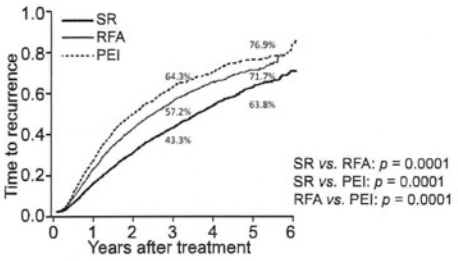


Patients at risk

SR	5361	3833	2570	1680	894	400	29
RFA	5548	3780	2328	1264	569	160	5
PEI	2059	1595	1112	718	444	247	58

Fig. 1. Overall survival curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

into 8 subgroups according to 3 factors (liver damage class, tumor size, and number of tumors), which have repeatedly been shown to be clinically relevant prognostic factors. The results of the subgroup analyses indicated that surgical resection would effectively prevent recurrence in patients with relatively advanced HCC (2–3 cm in diameter) among the study populations, irrespective of liver damage class or number of tumors. This finding suggests that surgery might be superior to percutaneous ablation therapies in patients with a more advanced tumor stage. As for the subgroups with a single tumor, surgical resection yielded better overall survival and time to recurrence rates than RFA or PEI. Especially in the subgroup with a single tumor smaller than 2 cm in diameter, both the overall and time to recurrence rates were statistically significantly better after surgery than after RFA, whereas no such statistically significant differences in these two parameters between the two treatment groups were detected in a few subgroups with a single tumor, maybe due to the insufficient sample size of the subgroups. Thus, surgical resection would be considered as the treatment modality of first choice for a single HCC, as recommended by the Japanese clinical practice guideline [2]. Overall, there was a trend toward superior



Patients at risk							
SR	5361	3265	1844	1039	451	189	15
RFA	5548	2954	1396	591	225	62	4
PEI	2059	1154	583	304	172	90	15

Fig. 2. Time to recurrence curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

overall and time to recurrence rates after surgery than after RFA and PEI.

The reason why the long-term outcomes of the SR group were better than those of the PEI and RFA groups cannot be definitely clarified from the results of this study, however, in theory, surgical resection has the advantage of offering better local control of HCC over PEI and RFA, both of which have some potential risks of local recurrence associated with insufficient ablation. In addition, anatomic resection to remove minute tumor satellites [14] might have decreased the recurrence rate in the SR group, although this remains a speculation.

Recently, the latest trial from China [15], which had an adequate sample size (total 230 patients), reported that surgical resection yielded significantly better long-term outcomes than RFA. Although the study design was better than that of the two previously reported RCTs [10,11], it appeared to have limitations with respect to the results, such as drop in the overall survival in the RFA group as compared with that in the surgery group during the early period after treatment. The early deaths in the RFA group could have been treatment-related rather than cancer-related. Thus, no conclusion can be drawn from the three currently available RCTs.

One of the limitations of our study is the diversity of demographic factors in the study population, which would have been caused by the selection process of treatment modalities. As sim-

Table 2. Hazard ratios for death and recurrence adjusted by multivariate analysis.

A For death

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.84	0.74, 0.95	0.006
	SR *vs.* PEI	0.75	0.64, 0.86	0.0001
	RFA *vs.* PEI	0.88	0.77, 1.01	0.08
Age	<65 *vs.* ≥65	0.71	0.63, 0.79	0.0001
Sex	Female *vs.* male	0.87	0.78, 0.98	0.03
HBsAg	Positive *vs.* negative	0.91	0.74, 1.11	0.34
HCV Ab	Positive *vs.* negative	0.93	0.79, 1.10	0.40
Liver damage	A *vs.* B	0.62	0.56, 0.69	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4$/μl	0.76	0.68, 0.85	0.0001
Tumor size	<2 *vs.* ≥2 cm	0.82	0.73, 0.92	0.0007
Tumor number	Single *vs.* multiple	0.72	0.64, 0.80	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.66	0.59, 0.74	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.59	0.53, 0.66	0.0001

B For recurrence

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.74	0.68, 0.79	0.0001
	SR *vs.* PEI	0.59	0.54, 0.65	0.0001
	RFA *vs.* PEI	0.81	0.74, 0.88	0.0001
Age	<65 *vs.* ≥65	0.83	0.78, 0.89	0.0001
Sex	Female *vs.* male	0.88	0.82, 0.95	0.0001
HBsAg	Positive *vs.* negative	1.04	0.92, 1.17	0.53
HCV Ab	Positive *vs.* negative	1.15	1.04, 1.27	0.007
Liver damage	A *vs.* B	0.87	0.81, 0.93	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4$/μl	0.92	0.86, 0.98	0.02
Tumor size	<2 *vs.* ≥2 cm	0.84	0.79, 0.90	0.0001
Tumor number	Single *vs.* multiple	0.69	0.64, 0.74	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.71	0.67, 0.76	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.72	0.67, 0.77	0.0001

HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; Ab, antibody; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; CI, confidence interval.

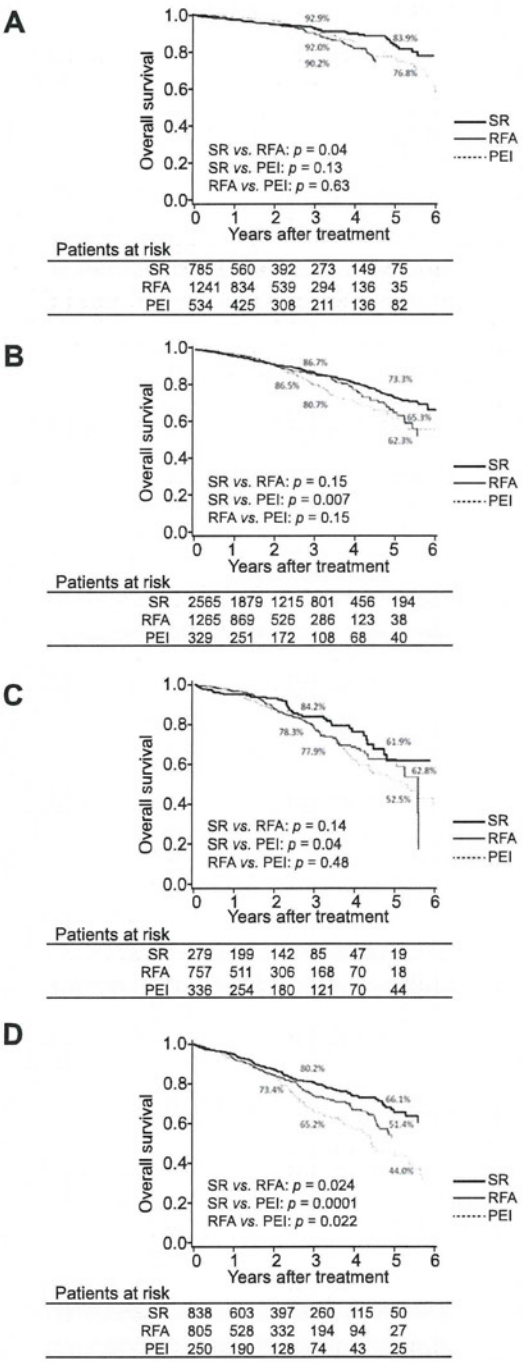


Fig. 3. Overall survival rates after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI) in the subgroup of cases with single tumor and liver damage class A and B. (A) Liver damage class A, a single tumor (<2 cm); (B) liver damage class A, a single tumor (2–3 cm); (C) liver damage class B, a single tumor (<2 cm); (D) liver damage class B, a single tumor (2–3 cm).

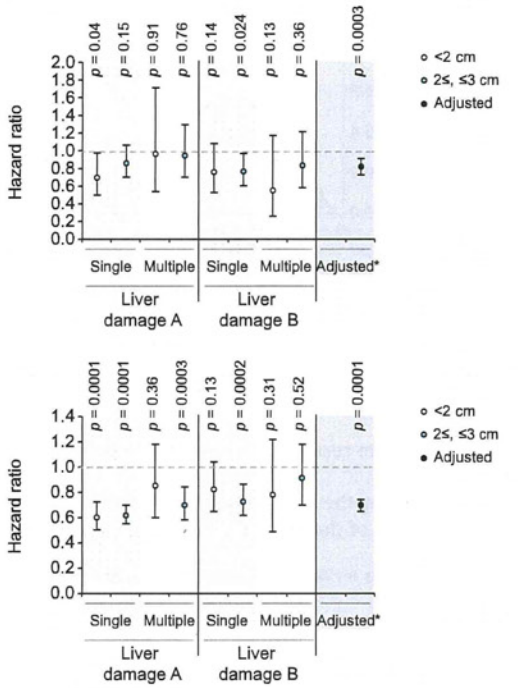


Fig. 4. Hazard ratios for death and recurrence with 95% confidence intervals and *p* values after surgical resection relative to those after radiofrequency ablation in the 8 subgroups. *The adjusted values for death and recurrence were calculated according to the three factors (tumor size, number of tumors, and liver damage class), as done in each subgroup. (A) Hazard ratios for death; (B) hazard ratios for recurrence.

ilar to the previous retrospective studies [5–9], the patients amenable to surgery had had younger age, less prevalence of hepatitis C virus infection, better liver function, less association with portal hypertension, fewer number of tumors and lower alpha-fetoprotein level, whereas their tumor size was larger and their des-γ-carboxy prothrombin level was higher. To minimize potential effects of confounding factors, we studied patients who had similar tumor-related and liver function-related factors and performed multivariate analysis using 10 clinically important factors, similar to our previous study [9]. Although it is impossible to completely eliminate potential negative impacts of demographic diversity, we believe that our results are clinically meaningful, because of the large sample size of our study. In Japan, a nationwide RCT in patients with HCC is now ongoing, and the results are expected to lead to more definitive conclusions [16].

Another potential limitation of our study is the lack of data on liver function during the follow-up, which precluded assessment of the relationship between the liver function status and the choice of treatment at recurrence. In HCC, the influence of the first treatment is considered to be smaller than that in other primary malignant diseases, because the liver function remarkably affects the recurrence rate. Further investigations, particularly prospective clinical trials, are needed to address these issues.

In conclusion, this large cohort study based on data obtained by a nationwide survey in Japan, suggests that surgical resection may offer some advantage over RFA and PEI in terms of both overall survival and time to recurrence in patients with less advanced HCC. Although our results are considered as being more reliable than those of previous studies comparing the treatment

outcomes in HCC, our conclusions need to be confirmed by future RCTs.

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Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

[1] El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011;365:1118–1127.
[2] Makuuchi M, Kokudo N. Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan. World J Gastroenterol 2006;12:828–829.
[3] Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL Conference. European association for the study of the liver. J Hepatol 2001;35:421–430.
[4] Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–1236.
[5] Montorsi M, Santambrogio R, Bianchi P, Donadon M, Moroni E, Spinelli A, et al. Survival and recurrences after hepatic resection or radiofrequency for hepatocellular carcinoma in cirrhotic patients: a multivariate analysis. J Gastrointest Surg 2005;9:62–67.
[6] Hong SN, Lee SY, Choi MS, Lee JH, Koh KC, Paik SW, et al. Comparing the outcomes of radiofrequency ablation and surgery in patients with a single small hepatocellular carcinoma and well-preserved hepatic function. J Clin Gastroenterol 2005;39:247–252.
[7] Guglielmi A, Ruzzenente A, Valdegamberi A, Pachera S, Campagnaro T, D'Onofrio M, et al. Radiofrequency ablation versus surgical resection for the treatment of hepatocellular carcinoma in cirrhosis. J Gastrointest Surg 2008;12:192–198.
[8] Arii S, Yamaoka Y, Futagawa S, Inoue K, Kobayashi K, Kojiro M, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. Hepatology 2000;32:1224–1229.
[9] Hasegawa K, Makuuchi M, Takayama T, Kokudo N, Arii S, Okazaki M, et al. Surgical resection vs. percutaneous ablation for hepatocellular carcinoma: a preliminary report of the Japanese nationwide survey. J Hepatol 2008;49:589–594.
[10] Huang GT, Lee PH, Tsang YM, Lai MY, Yang PM, Hu RH, et al. Percutaneous ethanol injection versus surgical resection for the treatment of small hepatocellular carcinoma: a prospective study. Ann Surg 2005;242:36–42.
[11] Chen MS, Li JQ, Zheng Y, Guo RP, Liang HH, Zhang YQ, et al. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for hepatocellular carcinoma. Ann Surg 2006;243:321–328.
[12] Hasegawa K, Kokudo N, Makuuchi M. Surgery or ablation for hepatocellular carcinoma. Ann Surg 2008;247:557–558.
[13] Ikai I, Arii S, Kojiro M, Ichida T, Makuuchi M, Matsuyama Y, et al. Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. Cancer 2004;101:796–802.
[14] Makuuchi M, Hasegawa H, Yamazaki S. Ultrasonically guided subsegmentectomy. Surg Gynecol Obstet 1986;161:346–350.
[15] Huang J, Yan L, Cheng Z, Du L, Wang J, Xu Y, et al. A randomized trial comparing radiofrequency ablation and surgical resection for HCC conforming to the Milan criteria. Ann Surg 2010;252:903–912.
[16] Hasegawa K, Kokudo N, Shiina S, Tateishi R, Makuuchi M. Surgery vs. radiofrequency ablation for small hepatocellular carcinoma: start of a randomized controlled trial (SURF trial). Hepatol Res 2010;40:851–852.

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FGF3/FGF4 Amplification and Multiple Lung Metastases in Responders to Sorafenib in Hepatocellular Carcinoma

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The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7%-3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinico-pathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of 13 responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time polymerase chain reaction–based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ($P = 0.006$). Fluorescence *in situ* hybridization analysis confirmed *FGF3* amplification. In addition, the clinico-pathological features showed that multiple lung metastases (5/13, $P = 0.006$) and a poorly differentiated histological type (5/13, $P = 0.13$) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3/FGF4*-amplified and three *FGFR2*-amplified cancer cell lines exhibited hypersensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion:* *FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies. (HEPATOLOGY 2012;00:000-000)

Abbreviations: 5FU, 5-fluorouracil; CGH, comparative genomic hybridization; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ *hybridization; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist-II; RPMI-1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse-transcription PCR.*

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