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).

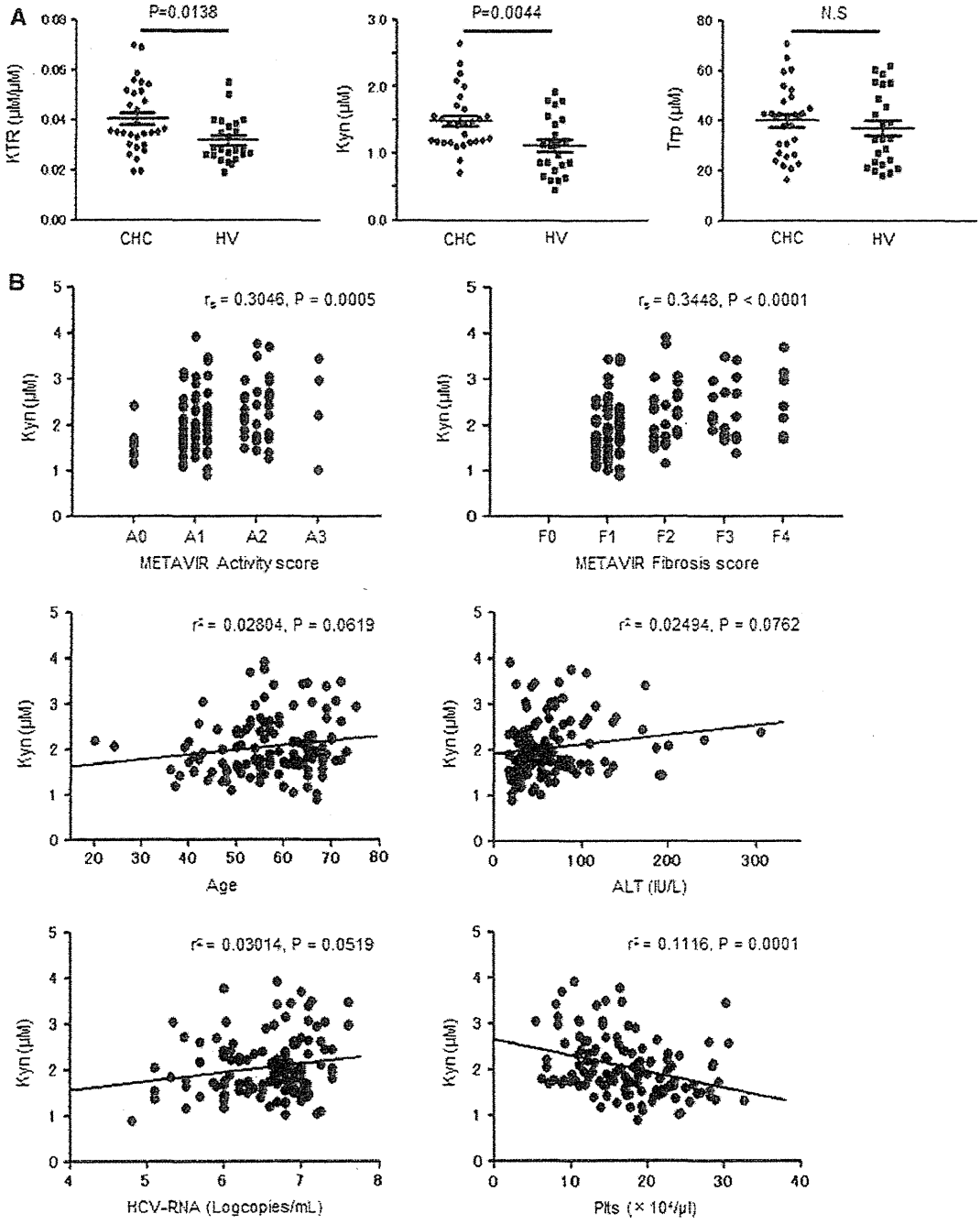


Fig. 1 Systemic indoleamine 2,3-dioxygenase (IDO) activity is enhanced in chronic hepatitis C patients. **a** Serum kynurenine (*Kyn*) and tryptophan (*Trp*) were assayed by HPLC as described in "Subjects, materials, and methods", and the kynurenine-to-tryptophan ratio (*KTR*) was calculated from their 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

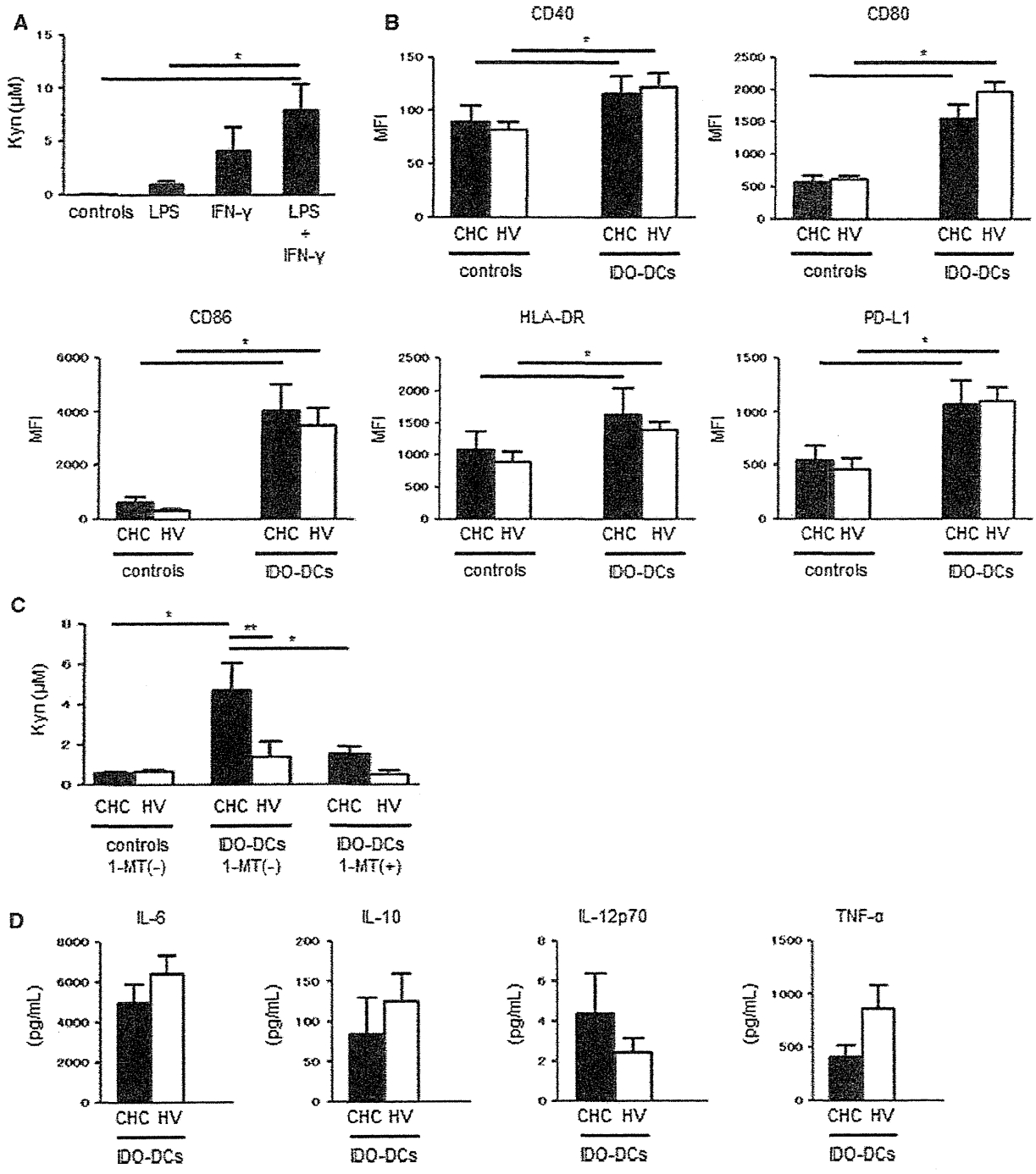
IDO is not involved in allogeneic T-cell proliferation and Th1/Th2 differentiation with DCs from chronic hepatitis C patients

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).

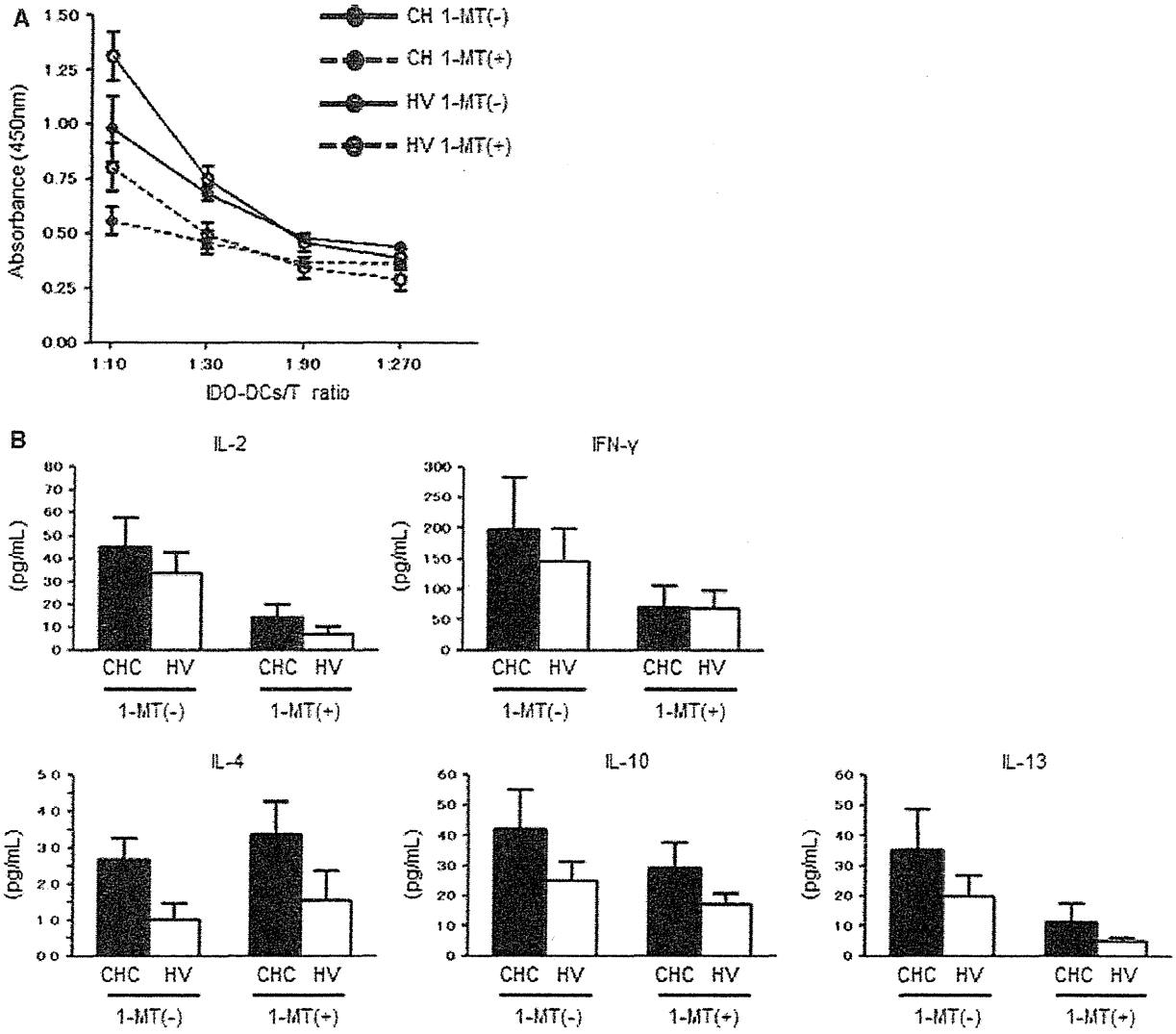


Fig. 3 IDO is not involved in lower allogeneic T-cell response and Th1/Th2 differentiation with DCs from chronic hepatitis C patients. **a** Allogeneic mixed lymphocyte reaction (MLR) with IDO-DCs was performed as described in "Subjects, materials, and methods". *Closed circles* are the 450-nm absorbance 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

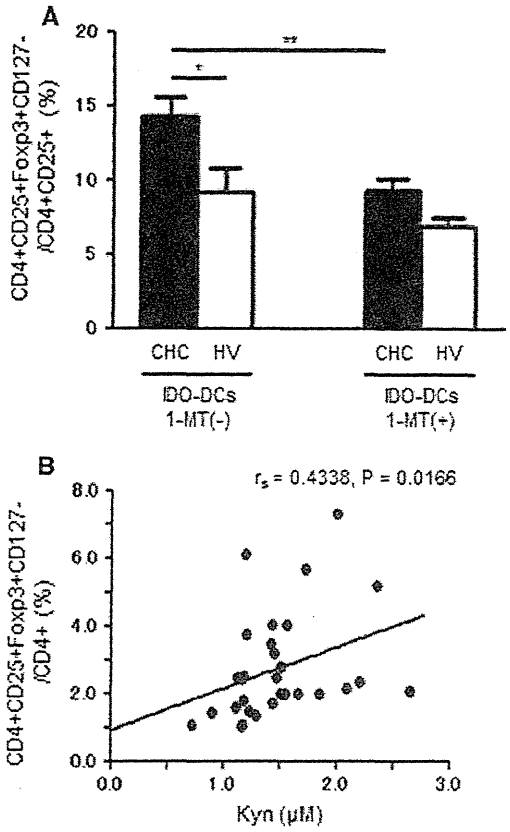


Fig. 4 IDO is involved in the induction of regulatory T cells. **a** After IDO-DCs were generated from the CHC or HV group, naive CD4+ T cells were co-cultured for 7 days with IDO-DCs in the presence or absence of 1-MT. The cultured T cells were stained with relevant antibodies (*Abs*) and analyzed with 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.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Incidence of hepatocellular carcinoma in HCV-infected patients with normal alanine aminotransferase levels categorized by Japanese treatment guidelines

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Abstract
Background This study was conducted to evaluate Japanese treatment guidelines for patients with chronic hepatitis C virus (HCV) infection and normal alanine aminotransferase (N-ALT) levels from the viewpoint of the incidence of hepatocellular carcinoma (HCC).
Methods Four groups of patients with chronic HCV infection treated with pegylated interferon (Peg-IFN) plus ribavirin, and classified according to the N-ALT guidelines, were examined for HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and platelet (PLT) $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $< 15 \times 10^4/mm^3$; group C ($n = 233$), 30 < ALT $\leq$ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), 30 < ALT $\leq$ 40 IU/L and PLT $< 15 \times 10^4/mm^3$. The mean observation period was 36.2 ± 16.5 months
Results In groups A and C, the HCC incidence was low even in patients with non-response (NR) (cumulative rates at 3 years, 0.0 and 2.9 %, respectively). In groups B and D, 14.5 and 5.3 % of NR patients had developed HCC at 3 years, but none of the patients with sustained virologic response (SVR) or relapse had developed HCC. In group B, no patients with mild fibrosis developed HCC irrespective of the antiviral effect of the treatment. Among patients with PLT $< 15 \times 10^4/mm^3$ (group B plus group D), the HCC incidence was significantly lower in patients with SVR and relapse than in NR patients ($p < 0.001$, $p = 0.021$, respectively).
Conclusion These results suggest that N-ALT patients with PLT $< 15 \times 10^4/mm^3$ could be candidates for early antiviral therapy.

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Introduction

Continuous hepatitis C virus (HCV) infection causes liver inflammation and can lead to liver fibrosis, which may progress to cirrhosis and hepatocellular carcinoma (HCC) [1–4]. Because HCV carriers with persistent normal alanine aminotransferase (PNALT) levels have minimal liver inflammation and the progression of liver fibrosis in such patients is slow, they are generally considered to be at low risk for carcinogenesis [5–7]. Moreover, patients with PNALT had not been considered as candidates for antiviral therapy in the era of interferon (IFN) monotherapy because of reports of ALT flare-up owing to antiviral therapy in some cases (47–67 %) [8–10].

However, in recent years, the antiviral efficacy of pegylated IFN (Peg-IFN) plus ribavirin combination therapy for patients with chronic HCV infection has been reported to be equivalent for patients with normal alanine aminotransferase (N-ALT) levels and those with elevated ALT levels [11–15]. In addition, for patients with PNALT, there have been fewer cases of ALT flare-up caused by Peg-IFN plus ribavirin combination therapy than with IFN monotherapy [12, 15]. Thus, patients with chronic HCV infection and N-ALT have come to be treated with Peg-IFN plus ribavirin combination therapy.

Treatment guidelines for patients with chronic HCV infection and N-ALT levels have been prepared by a Japanese group conducting "Research on Hepatitis" supported by Health and Labour Sciences Research Grants from the Japanese Government. In these guidelines, HCV carriers with N-ALT ($\leq$40 IU/L) are categorized into four groups according to their ALT levels ($\leq$30 or $\geq$31 IU/L) and platelet (PLT) counts ($\geq$15 or $<15 \times 10^4/mm^3$). Briefly, the therapeutic strategies are as follows: patients with ALT levels of more than 31 IU/L are candidates for antiviral treatment, but observation is recommended for patients with ALT levels of <30 IU/L. However, the goal of antiviral treatment is to improve the long-term prognosis, including inhibition of HCC. Therefore, the indication of antiviral therapy for patients with chronic HCV infection and N-ALT should be decided based on whether or not Peg-IFN plus ribavirin combination therapy can suppress the cumulative rate of HCC incidence and improve prognosis. It is thus very important to examine the effect of inhibition of HCC induced by antiviral therapy in patients with chronic HCV infection and N-ALT.

In the present study, we evaluated the treatment guidelines for patients with chronic HCV infection and N-ALT from the viewpoint of HCC inhibition by analyzing the differences in the cumulative rates of HCC incidence among the above four groups. The treatment guidelines also recommend that if patients with ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$ have moderate to severe liver fibrosis (F2–4), they should receive antiviral therapy. We also evaluated the effect of Peg-IFN plus ribavirin on HCC incidence according to the degree of fibrosis in this group.

Patients and methods

This retrospective study was conducted by Osaka University and institutions participating in the Osaka Liver Forum. Among patients with chronic HCV infection who had received Peg-IFN plus ribavirin combination therapy from December 2004 to December 2009, four groups of patients, classified according to the N-ALT guidelines, who had not suffered from HCC, were examined for their HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and PLT $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$; group C ($n = 233$), $30 <$ ALT ≤ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), $30 <$ ALT ≤ 40 IU/L and PLT $<15 \times 10^4/mm^3$. The Kaplan–Meier method was used to examine the cumulative rates of HCC incidence in the four groups. Excluded from this study were patients who developed HCC within 12 months from the start of Peg-IFN plus ribavirin combination therapy, patients with co-infection with hepatitis B or human immunodeficiency virus, patients with drug-induced or alcoholic liver disorders, and patients with autoimmune hepatitis. The protocol was performed after obtaining informed consent from each patient before treatment in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008. This study was approved by the Institutional Review Board and registered in the Universal Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN unique trial number, C000000197).

Treatment protocol

All patients received Peg-IFN alpha-2b (PEGINTRON; Merck & Co., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD) for the duration of the study. Peg-IFN alpha-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight $\leq$60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients. Dose modification according to the intensity of the hematological adverse effects followed, as a rule, the manufacturer's drug information. The dose of Peg-IFN alpha-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the PLT count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the PLT count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced, from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <8.5 g/dL. Both Peg-IFN alpha-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no medicine containing iron or hematopoetic growth factors, such as erythropoietin alpha, or granulocyte–macrophage colony-stimulating factor, was administered. The serum HCV RNA levels were qualitatively analyzed using the COBAS AMPLICOR HCV Test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics, Branchburg, NJ, USA), and the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml). In the patients with HCV genotype 1, as a rule, treatment duration was 48 weeks, but the patients with detectable HCV RNA (≥50 IU/mL) at week 12 and undetectable HCV RNA (<50 IU/mL) at week 24 were treated for 72 weeks. Patients with HCV genotype 2 were treated for 24 weeks.

Definition of virologic response

A sustained virologic response (SVR) was defined as undetectable HCV RNA at the end of treatment and at 24 weeks after completion of treatment. A relapse was defined as undetectable HCV RNA at the end of treatment but detectable HCV RNA at 24 weeks after completion of treatment. A non-response (NR) was defined as detectable HCV RNA at the end of treatment.

Histological evaluation

Liver biopsy was performed immediately before initiation of the Peg-IFN plus ribavirin combination therapy. Liver biopsy specimens were scored using the METAVIR system, and the grade of activity and stage of fibrosis were evaluated [16].

HCC surveillance

Ultrasonography or computed tomography (CT) was carried out before the initiation of the Peg-IFN plus ribavirin combination therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by CT or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out, with the patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

End point

The observation period was defined as the period from the start of Peg-IFN plus ribavirin combination therapy. Patients who developed HCC and patients whose treatments were switched to other types of IFN therapy were defined as censored cases at that point in time.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD. To analyze differences between baseline data among the four groups, analysis of variance or the χ^2 test was performed. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with the log-rank test. A value of $p < 0.05$ (two-tailed) was considered to indicate significance. The statistical software used for this analysis was IBM SPSS for Windows v. 19.0.0 (SPSS, Armonk, NY, USA).

Results

Baseline characteristics of patients categorized by the treatment guidelines

The baseline clinical features of the patients are shown in Table 1. There were significant differences in age; sex; body mass index (BMI); HCV genotype; past history of IFN therapy; grade and stage of liver histology; WBC, neutrophil, and PLT counts; Hb levels; and virologic response among the four groups. The mean ages of the patients in groups B and D were significantly higher than those of the patients in groups A and C. The proportion of males was lowest in group A (26 %) and highest in group C (41 %). The proportion of patients with progression of liver fibrosis (F3–4) diagnosed by the METAVIR score was 7.8 % among all patients tested and highest in group D (22.5 %). In groups B and D, peripheral blood cell counts (WBC, neutrophils, Hb, PLT) were significantly lower and the

Table 1 Baseline characteristics of the patients with chronic HCV infection and normal ALT levels

	Group A ALT ≤30 IU/L PLT count ≥15 × 10^4/mm^3	Group B ALT ≤30 IU/L PLT count <15 × 10^4/mm^3	Group C 30 < ALT ≤ 40 IU/L PLT count ≥15 × 10^4/mm^3	Group D 30 < ALT ≤ 40 IU/L PLT count <15 × 10^4/mm^3	*p* value
Number of patients	353	123	233	100	
Age (years)	55.6 ± 11.3	60.3 ± 8.4	54.6 ± 11.8	60.7 ± 8.6	<0.001
Sex: male/female	95/258	44/79	95/138	35/65	0.005
BMI (kg/m^2)	22.6 ± 3.3	22.1 ± 3.0	23.2 ± 3.4	22.3 ± 2.6	0.029
HCV genotype: 1/2	203/144	86/35	180/52	81/16	<0.001
HCV RNA (KIU/mL), mean ± SD	2333 ± 1664	2276 ± 1478	2261 ± 1599	2354 ± 1644	0.998
Past IFN therapy: naïve/experienced[a]	266/81	79/41	173/52	63/33	0.018
Histology[b]: activity: A0/A1/A2/A3	32/179/48/1	6/64/23/0	20/105/36/1	0/46/24/1	0.026
Fibrosis: F0/F1/F2/F3/F4	41/169/40/9/1	4/49/29/7/5	16/107/31/7/1	0/34/21/13/3	<0.001
White blood cell count (/mm^3)	5543 ± 1606	4405 ± 1211	5601 ± 1638	4677 ± 1337	<0.001
Neutrophil count (/mm^3)	3008 ± 1213	2332 ± 948	2999 ± 1243	2578 ± 1026	<0.001
Hemoglobin (g/dL)	13.3 ± 1.3	13.3 ± 1.4	13.9 ± 1.4	13.3 ± 1.3	<0.001
Platelet count (×10^4/mm^3)	21.1 ± 4.7	12.2 ± 2.1	21.3 ± 4.8	12.1 ± 2.2	<0.001
ALT (IU/L)	22.8 ± 5.2	23.5 ± 5.4	35.4 ± 2.9	35.8 ± 2.9	<0.001
Virologic response: SVR/relapse/NR	218/82/53	59/32/32	133/51/49	44/26/30	0.005

BMI body mass index, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *SVR* sustained virologic response, *NR* non-response, PLT platelet

[a] Virologic response to previous treatment was unknown for 22 patients

[b] Fibrosis stages are evaluated on a scale of 0–4 and activity grades are evaluated on a scale of 0–3 according to the METAVIR histological score. Fibrosis data were not available for 222 patients. Activity data were not available for 223 patients

numbers of patients with progression of liver fibrosis were significantly higher than in groups A and C. The mean duration of the observation period was 36.2 ± 16.5 months.

Antiviral efficacy of Peg-IFN plus ribavirin combination therapy

In genotype 1 patients, the rates of SVR, relapse, and NR were 50.7, 25.1, and 24.1 %, respectively, in group A; 39.5, 24.4, and 36.0 % in group B; 52.2, 23.9, and 23.9 % in group C; and 39.5, 25.1, and 35.2 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate was significantly higher in groups A and C than that in groups B and D (groups A and C: SVR 51.4 %, relapse 24.5 %, NR 24.0 %; groups B and D: SVR 39.5 %, relapse 25.1 %, NR 35.2 %, $p = 0.012$). In genotype 2 patients, the rates of SVR, relapse, and NR were 77.8, 20.1, and 2.1 %, respectively, in group A; 65.7, 31.4, and 2.9 % in group B; 75.0, 15.4, and 9.6 % in group C; and 62.5, 31.3, and 6.3 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate tended to be higher in groups A and C than that in groups B and D (groups A and C: SVR 77.0 %, relapse 18.9 %, NR 4.1 %; groups B and D: SVR 64.7 %, relapse 31.4 %, NR 8.9 %, $p = 0.152$).

Cumulative rate of HCC incidence according to the treatment effect of Peg-IFN plus ribavirin combination therapy

Eleven patients developed HCC during the observation period, and all were infected with HCV genotype 1. Figure 1 shows the cumulative rates of HCC incidence according to the treatment effect in the four groups.

In group A, no patients developed HCC during the 3 years of observation, regardless of the effect of Peg-IFN plus ribavirin combination therapy. Moreover, among those with SVR and relapse, no patients developed HCC during the 3-year observation period, while in NR patients the cumulative rate of HCC incidence at 5 years was 4.0 %. No significant difference in HCC incidence was found among the patients with SVR, relapse, and NR ($p = 0.071$) (Fig. 1a). In group C, no significant difference in HCC incidence was found among the patients with SVR, relapse, and NR (cumulative rates of HCC at 3 years, 2.2, 0.0, and 2.9 %, respectively; at 5 years, 3.7, 0.0, and 2.9 %, respectively, $p = 0.631$) (Fig. 1c). In group B, a marginally significant difference was found in HCC incidence among patients with SVR, relapse, and NR ($p = 0.054$), and patients with SVR had a significantly lower rate of HCC incidence than that of patients with NR (SVR vs. relapse, $p = 0.346$, SVR vs. NR, $p = 0.013$, relapse vs.

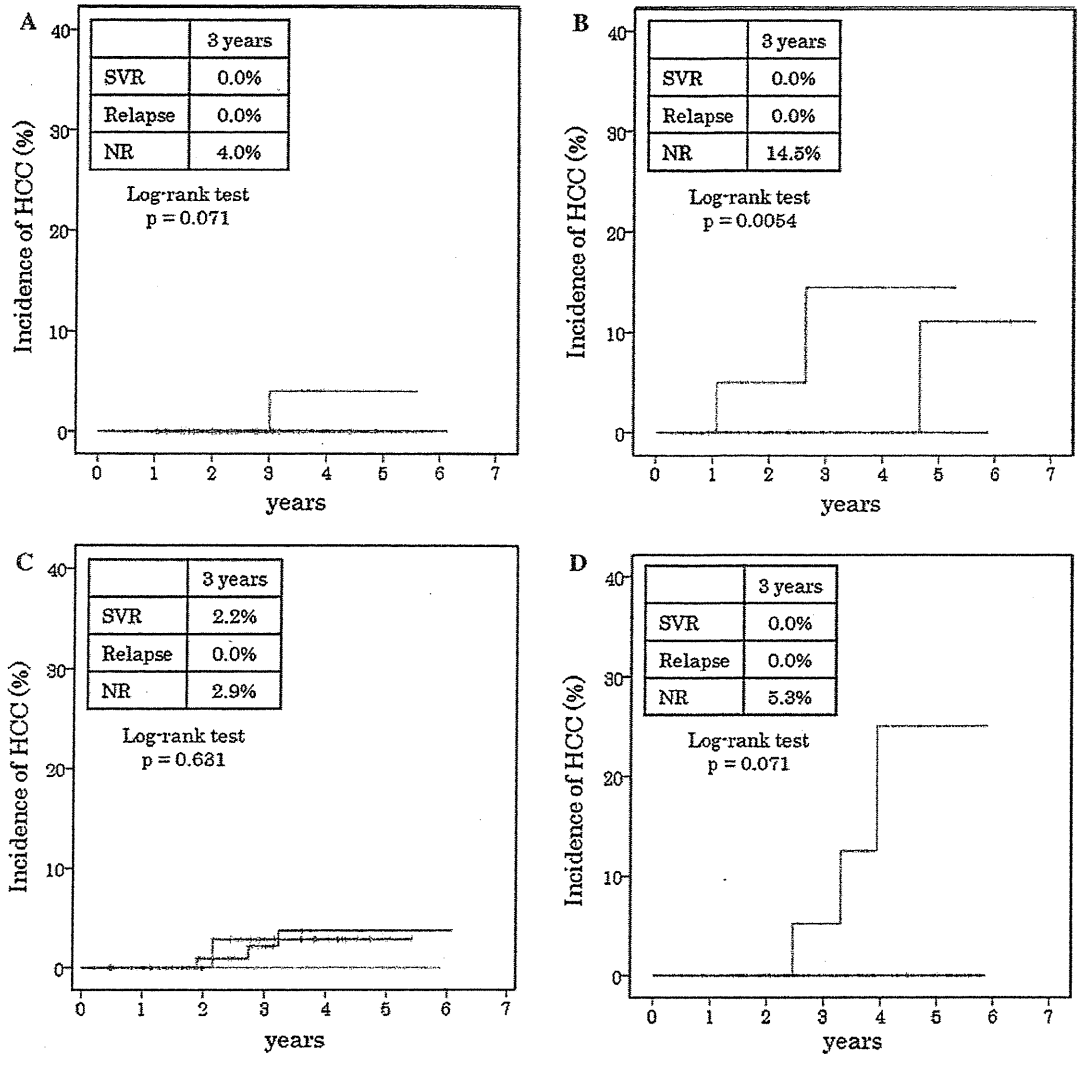

Fig. 1 Cumulative rates of hepatocellular carcinoma (*HCC*) incidence in groups A, B, C, and D, categorized according to the treatment effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy. **a** Group A (patients with alanine aminotransferase [ALT] level $\leq$30 IU/L and platelet [PLT] count $\geq 15 \times 10^4/mm^3$), **b** group B (patients with ALT $\leq$30 IU/L and PLT $< 15 \times 10^4/mm^3$), **c** group C (patients with $30 <$ ALT ≤ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$), **d** group D (patients with $30 <$ ALT ≤ 40 IU/L and PLT $< 15 \times 10^4/mm^3$). *Blue line* patients with sustained virologic response (*SVR*), *green line* patients with relapse, *red line* patients with non-response (*NR*)

NR, $p = 0.250$). Of the NR patients, 14.5 % had developed HCC at 3 years, while none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1b). In group D, there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.006$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (SVR vs. NR, $p = 0.012$, relapse vs. NR, $p = 0.047$). In the NR patients, 5.3 % had developed HCC at 3 years and 25.0 % had developed HCC at 5 years, but none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1d).

In the analysis of the differences in the cumulative rates of HCC incidence in the patients with $30 <$ ALT ≤ 40 IU/L (group C plus group D), the p value for a significant difference was 0.059 among the patients with SVR, relapse, and NR (Fig. 2). In the analysis of the differences in the cumulative rates of HCC incidence among the patients with PLT counts of less than $15 \times 10^4/mm^3$ (group B plus group D), there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p < 0.001$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (cumulative rates of HCC incidence at

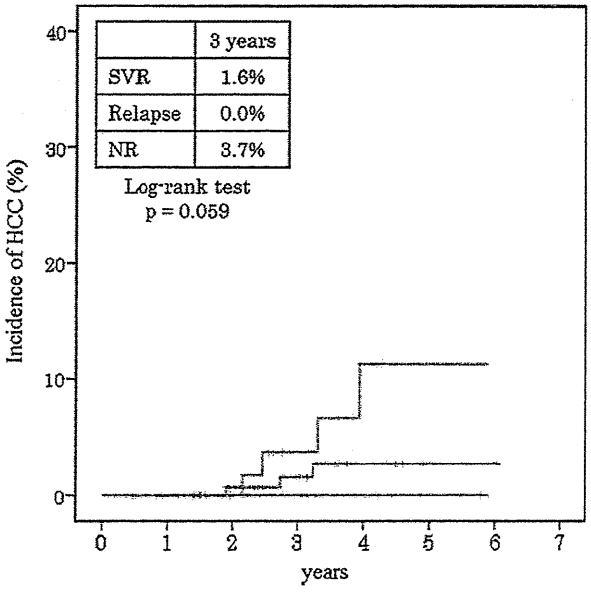

Fig. 2 Cumulative rates of HCC incidence according to ALT levels. Cumulative rates of HCC incidence in patients with ALT levels of 30 < ALT ≤ 40 IU/L (group C plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

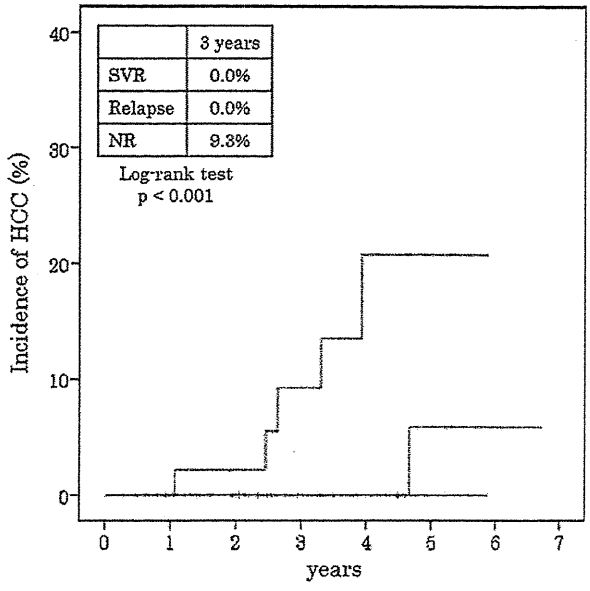

Fig. 3 Cumulative rates of HCC incidence according to PLT counts. Cumulative rates of HCC incidence in patients with PLT counts of $<15 \times 10^4/mm^3$ (group B plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

3 years, 0.0, 0.0, and 9.3 %, respectively; at 5 years, 0.0, 11.1, and 20.8 %, respectively; SVR vs. NR, $p < 0.001$, relapse vs. NR, $p = 0.021$) (Fig. 3).

Cumulative rate of HCC incidence in group B according to the stage of liver fibrosis

Based on the pattern of the Japanese treatment guidelines, we categorized the patients in group B into two groups according to the stage of liver fibrosis (F0–1 or F2–4) and compared the cumulative rates of HCC incidence. Patients with no fibrosis or mild fibrosis (F0–1) showed no HCC development regardless of the virologic response (SVR, relapse, or NR). Of note, in those with moderate to severe fibrosis (F2–4) in group B, there was no significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.174$), although SVR patients tended have a lower rate of HCC incidence than NR patients (SVR vs. relapse, $p = 0.414$, SVR vs. NR, $p = 0.071$, relapse vs. NR, $p = 0.383$). No patient in the SVR or relapse groups developed HCC, while the cumulative rate of HCC incidence at 3 years for the NR group was 25.0 % (Fig. 4).

Discussion

Patients with chronic HCV infection and N-ALT have been reported to show the possibility of ALT flare-up during the natural course of the disease (22–27 %) [17, 18] and to develop moderate to severe progression of liver fibrosis (5–30 %) [18–21]. However, very low cumulative incidences of HCC have been reported among patients with average ALT integration values less than or equal to 20 IU/L (5-year, 0.0 %, 10-year, 3.6 %) [22]. Therefore, it remains controversial whether HCV eradication by antiviral therapy can reduce the incidence of HCC in patients with chronic HCV infection and N-ALT [23–26].

The definition of N-ALT remains unclear because its cutoff value is still under consideration [22, 27, 28]. In Japan, treatment guidelines for patients with chronic HCV infection and N-ALT define N-ALT as serum ALT levels of ≤40 U/L, and the therapeutic strategy is decided after categorizing patients into four groups according to ALT levels and PLT counts. However, the indication of antiviral therapy should be based on whether or not HCC incidence can be suppressed by the antiviral therapy. Therefore, we examined the treatment guidelines from the viewpoint of inhibiting HCC in patients with chronic HCV infection and N-ALT.

In the present study, the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with chronic HCV infection and N-ALT was almost equivalent to the efficacy in those with elevated ALT levels, as previously reported [11–15]. The SVR rate was significantly higher in groups A and C than in groups B and D for patients with genotype 1, and the same tendency was found

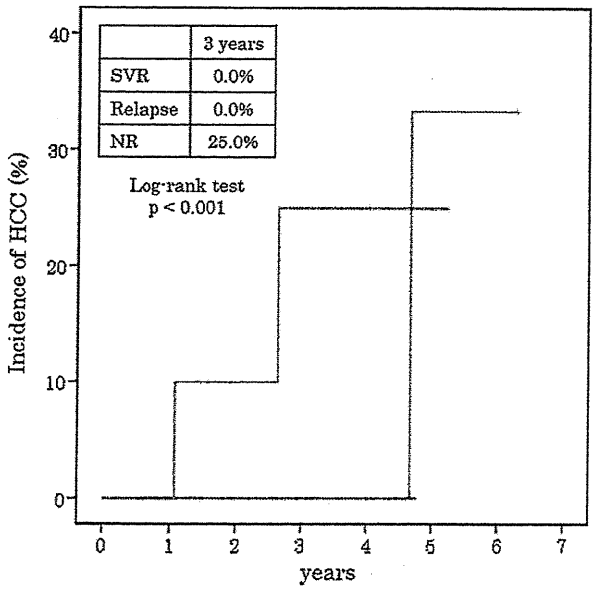

Fig. 4 Cumulative rates of HCC incidence in group B patients (ALT $\leq$ 30 IU/L and PLT $< 15 \times 10^4/mm^3$) with moderate to severe liver fibrosis (F2–4), according to the treatment effect of Peg-IFN plus ribavirin combination therapy. *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

for those with genotype 2. The reason for this was considered to be that groups B and D included many patients with moderate to severe liver fibrosis (F3–4, 17.0 %), which can lead to a lower SVR rate [23, 29, 30].

The present study revealed the cumulative rates of HCC incidence according to the treatment effect in the four groups. In group D, the cumulative rate of HCC incidence in the SVR and relapse patients was significantly lower than that for the NR patients. This result supports the recommendation by the treatment guidelines that patients in group D be managed in the same way as patients with chronic hepatitis C (CH-C) and elevated ALT levels.

In group B patients, the treatment guidelines recommend antiviral therapy for those who have moderate to severe liver fibrosis (F2–4). In our present study, patients with no fibrosis to mild fibrosis (F0–1) did not develop HCC, and in the patients with moderate to severe fibrosis (F2–4), the cumulative rate of HCC incidence tended to be lower in the SVR group than that in the NR group ($p = 0.071$). These results also indicate the appropriateness of the Japanese treatment guidelines. However, further study is needed because of the small number of cases studied here.

It appears that group A patients have time to wait for therapy with the next generation of direct antiviral agents (DAAs), such as Peg-IFN plus ribavirin plus a second-generation protease inhibitor, because none of the patients had developed HCC at 3 years. Even in group C, for which the treatment guidelines recommend antiviral therapy, there was no significant difference in the cumulative rate of HCC incidence among the SVR, relapse, and NR patients, with the incidence being below 5 % at 3 years. Accordingly, patients with PLT counts of more than $15 \times 10^4/mm^3$ (groups A or C) have time to wait until the next generation of DAAs becomes available, because patients with PLT counts of more than $15 \times 10^4/mm^3$ have a low 3-year carcinogenesis rate.

The Japanese treatment guidelines recommend antiviral therapy for patients with 30 < ALT $\leq$ 40 IU/L levels. However, in the present study, in the patients with 30 < ALT $\leq$ 40 IU/L levels, the p value for a significant difference in the cumulative rate of HCC incidence among the patients with SVR, relapse, and NR was 0.059. This result indicates that the patients with 30 < ALT $\leq$ 40 IU/L levels have the potential to be candidates for antiviral therapy, and further study is needed to clarify this. However, these patients may not be candidates for immediate antiviral therapy because the cumulative rates of HCC incidence at 3 years in the patients with SVR, relapse, and NR were low (cumulative rates of HCC at 3 years: 1.6, 0.0, and 3.7 %). On the other hand, as mentioned above, in the patients with PLT counts of $<15 \times 10^4/mm^3$, the cumulative rate of HCC incidence was significantly lower in the SVR and relapse patients than that in the NR patients (cumulative rates of HCC at 3 years: 0.0, 0.0, and 9.3 %; at 5 years: 0.0, 11.1, and 20.8 %; $p < 0.001$). This result suggests that patients with PLT counts of $<15 \times 10^4/mm^3$ may be candidates for antiviral therapy.

A limitation of this study was that the incidence of HCC was not compared between a treatment group and a non-treatment group. This study showed the suppressive effect of antiviral therapy on HCC incidence by comparing patients according to the treatment's antiviral effect. Peg-IFN plus ribavirin combination therapy has become acceptable for patients with chronic HCV infection and N-ALT levels. However, if there were no difference in HCC incidence between patients with SVR and non-SVR in the group receiving Peg-IFN plus ribavirin combination therapy, it would not be necessary for patients with chronic HCV infection and N-ALT to receive this therapy. In this study, we compared the incidence of HCC according to the treatment effect in HCV-infected patients with N-ALT levels categorized by the Japanese treatment guidelines. Indeed, although our results did not demonstrate that N-ALT patients should be treated, they indicated that it could be appropriate to treat N-ALT patients, because the incidence of HCC in these patients with SVR was suppressed compared with that in the NR patients.

In conclusion, in patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ who received Peg-IFN plus ribavirin

combination therapy, the cumulative rate of HCC incidence was significantly lower in those with SVR or relapse than in those with NR. Therefore, HCV-infected patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy for the purpose of reducing the risk of developing HCC.

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References

1. Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. Gastroenterology. 1997;112:463–72.
2. Serfaty L, Aumaitre H, Chazouilleres O, Bonnand AM, Rosmorduc O, Poupon RE, Poupon R. Determinants of outcome of compensated hepatitis C virus-related cirrhosis. Hepatology. 1998;27:1435–40.
3. Hu KQ, Tong MJ. The long-term outcomes of patients with compensated hepatitis C virus-related cirrhosis and history of parenteral exposure in the United States. Hepatology. 1999;29:1311–6.
4. National Institutes of Health Consensus Development Conference Statement. Management of hepatitis C 2002 (June 10–12, 2002). Gastroenterology. 2002;123:2082–99.
5. Nutt AK, Hassan HA, Lindsey J, Lamps LW, Raufman JP. Liver biopsy in the evaluation of patients with chronic hepatitis C who have repeatedly normal or near-normal serum alanine aminotransferase levels. Am J Med. 2000;109:62–4.
6. Marcellin P. Hepatitis C: the clinical spectrum of the disease. J Hepatol. 1999;31(Suppl 1):9–16.
7. Martinot-Peignoux M, Boyer N, Cazals-Hatem D, Pham BN, Gervais A, Le Breton V, Levy S, et al. Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. Hepatology. 2001;34:1000–5.
8. Sangiovanni A, Morales R, Spinzi G, Rumi M, Casiraghi A, Ceriani R, Colombo E, et al. Interferon alfa treatment of HCV RNA carriers with persistently normal transaminase levels: a pilot randomized controlled study. Hepatology. 1998;27:853–6.
9. Pradat P, Alberti A, Poynard T, Esteban JI, Weiland O, Marcellin P, Badalamenti S, et al. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. Hepatology. 2002;36:973–7.
10. Tassopoulos NC. Treatment of patients with chronic hepatitis C and normal ALT levels. J Hepatol. 1999;31(Suppl 1):193–6.
11. Arora S, O'Brien C, Zeuzem S, Shiffman ML, Diago M, Tran A, Pockros PJ, et al. Treatment of chronic hepatitis C patients with persistently normal alanine aminotransferase levels with the combination of peginterferon alpha-2a (40 kDa) plus ribavirin: impact on health-related quality of life. J Gastroenterol Hepatol. 2006;21:406–12.
12. Bini EJ, Mehandru S. Sustained virological response rates and health-related quality of life after interferon and ribavirin therapy in patients with chronic hepatitis C virus infection and persistently normal alanine aminotransferase levels. Aliment Pharmacol Ther. 2006;23:777–85.
13. Marcellin P, Asselah T, Boyer N. Fibrosis and disease progression in hepatitis C. Hepatology. 2002;36:S47–56.
14. Hiramatsu N, Inoue Y, Oze T, Kurashige N, Yakushijin T, Mochizuki K, Miyagi T, et al. Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case–control study. J Gastroenterol. 2011;46(11):1335–43.
15. Zeuzem S, Diago M, Gane E, Reddy KR, Pockros P, Prati D, Shiffman M, et al. Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. Gastroenterology. 2004;127:1724–32.
16. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996;24:289–93.
17. Tsuji K, Yamasaki K, Yamanishi M, Kawakami M, Shirahama S. Risk of alanine aminotransferase flare-up among asymptomatic hepatitis C virus RNA carriers: a 10-year follow-up study. J Gastroenterol Hepatol. 2001;16:536–40.
18. Puoti C, Castellacci R, Montagnese F. Hepatitis C virus carriers with persistently normal aminotransferase levels: healthy people or true patients? Dig Liver Dis. 2000;32:634–43.
19. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology. 2009;49:1335–74.
20. Puoti C, Guido M, Mangia A, Persico M, Prati D. Clinical management of HCV carriers with normal aminotransferase levels. Dig Liver Dis. 2003;35:362–9.
21. Puoti C, Magrini A, Stati T, Rigato P, Montagnese F, Rossi P, Aldegheri L, et al. Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine transaminase levels. Hepatology. 1997;26:1393–8.
22. Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, et al. Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels. J Hepatol. 2009;50:729–35.
23. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
24. Puoti C, Bellis L, Martellino F, Guarisco R, Dell' Unto O, Durola L, Galossi A. Chronic hepatitis C and 'normal' ALT levels: treat the disease not the test. J Hepatol. 2005;43:534–5.
25. Puoti C, Bellis L, Galossi A, Guarisco R, Nicodemo S, Spilabotti L, Unto OD. Antiviral treatment of HCV carriers with persistently normal ALT levels. Mini Rev Med Chem. 2008;8:150–2.
26. Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, Yasui K, et al. A follow-up study to determine

the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. J Hepatol. 2005;43:599–605.
27. Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, et al. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med. 2002;137:1–10.
28. Ahmed A, Keeffe EB. Chronic hepatitis C with normal aminotransferase levels. Gastroenterology. 2004;126:1409–15.
29. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358:958–65.
30. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.

TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlated with angiogenesis

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Keywords

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Abbreviations List

HCV, hepatitis C virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; TEMs, TIE2-expressing monocytes; ANG, Angiopoietin; PBMC, peripheral blood mononuclear cells; AFP, α-fetoprotein; LC, liver cirrhosis; CH, chronic hepatitis; ROC, receiver operating characteristic; MIF, macrophage migratory inhibitory factor; VEGF, vascular endothelial growth factor; sVEGFR-1,