レベルB 表1… 核酸系抗HBV剤

薬剤	Wild-type HBV	YMDD Mutation	HIV治療での使用
ラミブジン(3TC)	有効	無効	あり
アデホビル(ADV)	有効	有効	なし
エンテカビル(ETV)	有効 (0.5mg)	有効(1mg) だが単剤では 耐性化しやすい#	なし
エムトリシタビン(FTC)	有効	無効	あり
テノホビル(TDF)	有効	有効	あり
Telbivudine	有効	無効	なし

＊：HBVのYMDD変異を有する場合、エンテカビルに切り替えても耐性化しやすいため、ラミブジン・アデホビル併用療法もしくはエンテカビル・アデホビル併用療法をするべきです。

文献8改変

2 HIV/HBV重複感染例のマネジメント・治療 レベルB

1. HIV感染者のHBV慢性感染 レベルA

HBV感染の側からみると、HIVを重複感染していると、高HBV血症になりやすく、肝硬変・肝癌への進展率が高くなります[4, 5]。またHIV/HBV重複感染例における肝疾患関連死は、HIV単独感染例の約8倍、HBV単独感染例の約19倍も高いと報告されています[6]。したがって、HIV/HBV重複感染者に対して、両ウイルスに対し有効な2剤を含むARTを開始することが米国保健福祉省(Department of Health and Human Services：DHHS)の抗HIVガイドラインで推奨されています[7]。HIVとHBVともに複製過程で逆転写酵素を必要とし、両ウイルスに対し有効な逆転写酵素阻害剤が存在するからです(表1)。ただ、単剤で治療を行うと耐性化を起こすことがあるため、必ずHIV/HBV重複感染者に対しては単剤での治療は行わないように注意すべきです。

2. HIV/HBV重複感染例に対するB型慢性肝炎の治療適応 レベルA

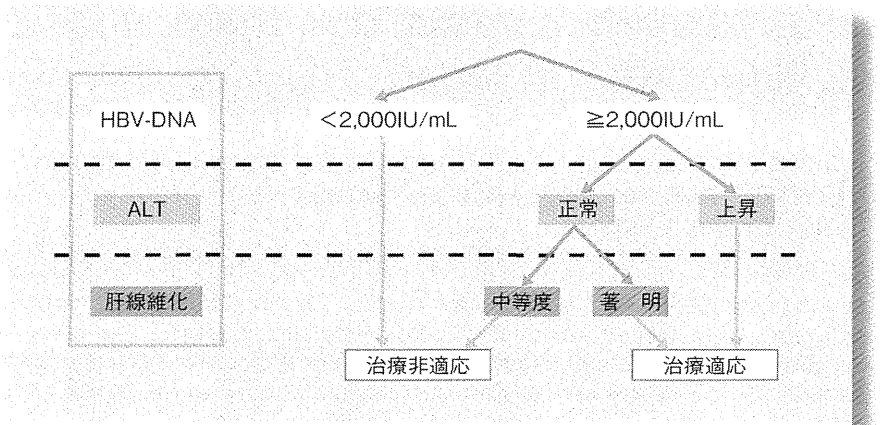
日本ではHIV非感染者に対するB型肝炎治療の治療指針(他項参照 ▶p.138～)がありますが、HIVが重複感染したB型慢性肝炎に対する明確な治療導入基準は示されていません。したがってHIV/HBV重複感染例には海外の治療適応基準が参考になるでしょう(図1)。その中でHBV単独感染に比べ早期に治療を開始することが推奨されています[8]。

HIVとHBVの両方に治療適応がある場合は、2剤以上の抗HBV活性を有する薬剤を含むARTレジメを採用します。現状では、テノホビル・エムトリシタビンの配合剤、もしくはテノホビルとラミブジンをバックボーンにしたARTを行います。HIV感染症にだけ治療適応があり、B型肝炎には治療の必要がないケースでも同様のARTを行います。

一方B型肝炎に対しては核酸アナログの治療適応がなく、「HIV感染症に治療適応がない、もしくは治療を望まない」ケースではPEG-IFNα-2aの単独治療が考慮されます。HIV/HBV重複感染例に多いゲノタイプAはゲノタイプCに比べるとIFN治療効果が良いからです。もちろん、IFN治療に際しては、肝線維化進展度、血小板数、年齢などの要素を考慮し、総合的に適応を決める必要があります。

テノホビルを含んだレジメでARTを行い、腎

レベルB 図1… HIV感染患者における治療指針



＊：HBV-DNA 1 IU/mLは約5.8 copies/mLに相当します。したがって、換算すると2,000 IU/mLは4.06 log copies/mL（11,600 copies/mL）となります。

文献2改変

障害のためテノホビルを他の薬剤に切り替える必要が出た場合は、ARTに加えエンテカビルを併用することが一般的です。

3. おわりに レベルA

今後、HIV/HBV重複感染例は増加するものと思われます。現状ではHIV診療に習熟した施設でのフォローアップ・加療が適切ですが、近い将来多くの肝臓専門医が対応できるようになりたいものです。

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Special Report

A multicenter survey of re-treatment with pegylated interferon plus ribavirin combination therapy for patients with chronic hepatitis C in Japan

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Aim: This study aimed to clarify the factors associated the efficacy of re-treatment with pegylated interferon (PEG IFN) plus ribavirin combination therapy for patients with chronic hepatitis C who had failed to respond to previous treatment.

Methods: One hundred and forty-three patients who had previously shown relapse ($n = 79$), non-response ($n = 34$) or intolerance ($n = 30$) to PEG IFN plus ribavirin were re-treated with PEG IFN plus ribavirin.

Results: Twenty-five patients with intolerance to previous treatment completed re-treatment and the sustained virological response (SVR) rates were 55% and 80% for hepatitis C virus (HCV) genotype 1 and 2, respectively. On re-treatment of the 113 patients who completed the previous treatment, the SVR rates were 48% and 63% for genotype 1 and 2, respectively. Relapse after previous treatment and a low baseline HCV RNA level on re-treatment were associated with SVR in genotype 1 ($P < 0.001$). Patients with the interleukin-28B major genotype responded significantly better and earlier to re-treatment, but the difference in the SVR rate did not reach a significant level between the major and minor genotypes ($P = 0.09$). Extended treatment of 72 weeks raised the SVR rate among the patients who attained complete early virological response but not rapid virological response with re-treatment (72 weeks, 73%, 16/22, vs 48 weeks, 38%, 5/13, $P < 0.05$).

Conclusion: Relapse after previous treatment and a low baseline HCV RNA level have predictive values for a favorable response of PEG IFN plus ribavirin re-treatment for HCV genotype 1 patients. Re-treatment for 72 weeks may lead to clinical improvement for genotype 1 patients with complete early virological response and without rapid virological response on re-treatment.

Key words: chronic hepatitis C, pegylated interferon and ribavirin combination therapy, re-treatment

INTRODUCTION

PEGYLATED INTERFERON (PEG IFN) plus ribavirin combination therapy can show antiviral efficacy for patients with chronic hepatitis C (CH-C). However, a sustained virological response (SVR), which is defined as undetectable serum hepatitis C virus (HCV) RNA at 24 weeks after the treatment, remains at 50% for patients with HCV genotype 1 and 80% for those with HCV genotype 2 treated with PEG IFN plus ribavirin.[1–6] The number of patients who fail to achieve a SVR increases over time, requiring urgent action to eradicate HCV in them.

Recently, addition of the first-wave protease inhibitor telaprevir to PEG IFN plus ribavirin combination therapy, which has been reported to improve antiviral efficacy, has become commercially available, but this

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triple therapy increases side-effects, especially severe anemia and skin rash.[7–11] Second-wave protease inhibitors, such as TMC435, which not only improve antiviral efficacy but also decrease side-effects, have been developed and are undergoing clinical trials.[12] Also, IFN-free regimens, such as protease inhibitor and polymerase inhibitor combination therapy, have been developed.[13,14] In Japan, HCV carriers are increasing in an aging population, and large numbers of patients are ineligible for triple therapy with telaprevir due to potential anemia. That is why re-treatment with PEG IFN plus ribavirin is a possible choice for patients who failed to achieve SVR to previous antiviral therapy or patients ineligible for triple therapy with telaprevir who must wait until next-generation antiviral therapies, such as triple therapy with second-wave protease inhibitors or IFN-free regimens, become commercially available.

As for re-treatment with PEG IFN plus ribavirin, some studies have been reported but the subjects and treatment protocols were varied.[15–20] According to past reports, the previous treatment response is associated with the efficacy of the re-treatment[17,20] and the SVR rates in re-treatment ranged 4–23%.[16–18] Recently, host factors, such as single nucleotide polymorphisms (SNP) located near the interleukin (IL)-28B gene, and virus factors, such as the amino acid substitutions in the HCV core region, were revealed to have a strong impact on SVR in PEG IFN plus ribavirin combination therapy for naïve CH-C patients.[21–26] Moreover, response-guided therapy which extends treatment duration until 72 weeks for patients with a slow virological response can raise the SVR rate for naïve CH-C patients.[27–29] However, the value of IL-28B SNP has been uncertain in re-treatment and the most appropriate treatment duration in re-treatment is still unclear. Although it remains obscure which factors are associated with SVR in re-treatment with standard PEG IFN plus ribavirin therapy as pointed out above, some patients do respond to re-treatment and it is very important to be able to identify them. Such findings will be valuable for optimizing the antiviral treatment for CH-C patients by making it possible to decide which patients should be considered for re-treatment with PEG IFN plus ribavirin therapy and which should wait for next-generation antiviral treatment.

In the present study, we tried to determine which patients could benefit from re-treatment and to identify the factors associated with SVR in re-treatment, including the host genome SNP and treatment duration.

METHODS

Patients

THIS RETROSPECTIVE, MULTICENTER study was conducted by the Study Group of Antiviral Therapy for Difficult-to-Treat Chronic Hepatitis C supported by the Ministry of Health, Labor and Welfare, Japan. This study was conducted with 143 CH-C patients, 113 patients (genotype 1, $n = 86$; genotype 2, $n = 27$) who had previously completed PEG IFN-α-2b plus ribavirin combination therapy but had failed to attain SVR, and 30 patients (genotype 1, $n = 22$; genotype 2, $n = 8$) who had previously discontinued this combination therapy due to adverse events.

Treatment

For the previous treatment, patients had been treated with PEG IFN-α-2b (PEGINTRON; MSD, Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD). For re-treatment with PEG IFN plus ribavirin, patients were treated PEG IFN-α-2a (PEGASYS; Roche, Basel, Switzerland) plus ribavirin (COPEGUS; Roche) or PEG IFN-α-2b plus ribavirin. In principle, as a starting dose, PEG IFN was given once weekly at a dose of 180 μg of PEG IFN-α-2a and 1.5 μg/kg of PEG IFN-α-2b and ribavirin was given at a total dose of 600–1000 mg/day based on bodyweight (bodyweight, ≤60 kg, 600 mg; 60–80 kg, 800 mg; ≥80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients and the decision of the investigator at the participating clinical center. Dose modification followed, as a rule, the manufacturer's drug information on the intensity of the hematological adverse effects.

Laboratory tests and virological assessment

Examination of peripheral blood, transaminase and the serum HCV RNA level were tested at the start of treatment, weeks 4, 12 and 24, end of treatment (EOT), and 24 weeks after the treatment. Sequences of the IFN-sensitivity determining region (ISDR) and the core region of HCV were determined at start of the previous treatment, and the number of mutations in the ISDR, the amino acid substitutions at core 70 and 91, glutamine (Gln) or histidine (His) at core 70 and methionine (Met) at core 91, were analyzed. Genetic polymorphisms located near the IL-28B gene (rs8099917) and ITPA gene (rs1127354) were determined. As for the IL-28B gene, homozygosity for the major sequence (TT) was defined as having the IL-28B major allele, whereas homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having

the IL-28B minor allele. As for the ITPA gene, homozygosity for the major sequence (CC) was defined as having the ITPA major allele, whereas homozygosity (AA) or heterozygosity (CA) of the minor sequence was defined as having the ITPA minor allele. The serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test ver. 2.0 (detection range, 6–5000 KIU/mL; Roche Diagnostics, Branchburg, NJ, USA) or COBAS TaqMan HCV test (detection range, 1.2–7.8 $\log_{10}$ IU/mL) and qualitatively analyzed using the COBAS AMPLICOR HCV test ver. 2.0 (lower limit of detection, 50 IU/mL). When the serum HCV RNA level quantified by the COBAS TaqMan HCV test was less than 1.7 $\log_{10}$ IU/mL, which was equivalent to 50 IU/mL of HCV RNA, that case was judged as HCV RNA negativiation against the lower limit of detection of the COBAS AMPLICOR HCV test.

Definition of virological response

A rapid virological response (RVR) was defined as undetectable serum HCV RNA level at week 4, partial early virological response (p-EVR) as a more than 2-log decrease in the HCV RNA level at week 12 compared with the baseline, complete EVR (c-EVR) as undetectable serum HCV RNA at week 12, late virological response (LVR) as detectable serum HCV RNA at week 12 and undetectable at week 24, and SVR as undetectable serum HCV RNA at 24 weeks after the treatment. Relapse was defined as undetectable serum HCV RNA at the EOT but a detectable amount after the treatment. Patients without p-EVR or without clearance of HCV RNA at week 24 were considered to be showing non-response (NR), and treatment was stopped in both the previous treatment and this re-treatment. A patient who attained HCV RNA negativiation during the re-treatment continued to be treated for 48 weeks or 72 weeks according to response-guided therapy or the decision of the investigator at the participating clinical center.

Statistical analysis

Baseline data of the patients are expressed as means ± standard deviation or median values. In order to analyze the difference between baseline data or the factors associated with SVR, univariate analysis using the Mann–Whitney *U*-test or χ^2-test and multivariate analysis using logistic regression analysis were performed. A two-tailed *P*-value of less than 0.05 was considered significant. The analysis was conducted with SPSS ver. 17.0J (IBM, Armonk, NY, USA).

RESULTS

THE PATIENT FLOW in this study is shown in Figure 1. Among the patients who had previously discontinued PEG IFN-α-2b plus ribavirin combination therapy, two patients underwent splenectomy to increase platelet count prior to re-treatment, 25 completed re-treatment of PEG IFN plus ribavirin combination therapy and 15 achieved SVR (genotype 1, $n = 11$; genotype 2, $n = 4$).

All of the patients who completed previous treatment also completed re-treatment and the baseline characteristics of those patients are shown in Table 1. Of the 86 genotype 1 patients, 54 were relapsers and 32 had shown NR to previous treatment. Of the 27 patients with genotype 2, 25 were relapsers and two had shown NR to previous treatment. Thirty-seven patients with genotype 1 and 14 patients with genotype 2 were assessed as IL-28B genotype, and 27 patients with genotype 1 and 10 patients with genotype 2 were assessed as ITPA genotype. There was no significant difference in the baseline characteristics between the previous treatment and the re-treatment with respect to peripheral blood cell counts, amino transaminase level and serum HCV RNA at the start of treatment (Table 1).

The baseline characteristics of patients with genotype 1 according to antiviral efficacy of the previous treatment are shown in Table 2. Among those with NR in the previous treatment, the rate of the minor allele of IL-28B was significantly higher than those with relapse in the previous treatment ($P < 0.01$). For genotype 1, the HCV RNA negative rate on re-treatment was 20% (17/86) at week 4, 61% (52/85) at week 12 and 76% (65/86) at week 24, and the SVR rate was 48% (41/86). The factors associated with SVR were assessed by univariate analysis and the factors of relapse after previous treatment and the serum HCV RNA level at the start of re-treatment were selected as being significant (Table 3). The SVR

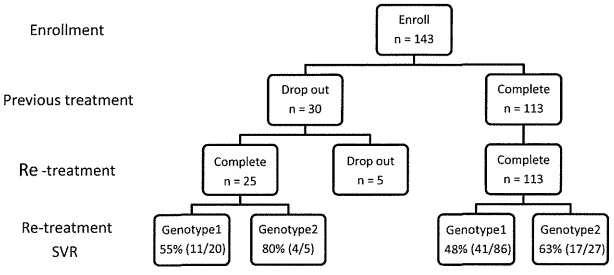

Figure 1 Patient flow for this study. SVR, sustained virological response.

Table 1 Baseline characteristics of patients and treatment factors in previous treatment and re-treatment

Factor	Genotype 1		Genotype 2	
No.	86		27	
Sex: male/female	46/40		15/12	
Effect of previous treatment: relapse/NR	54/32		25/2	
	Previous treatment	Re-treatment	Previous treatment	Re-treatment
PEG IFN type: α-2a/α-2b	0/86	41/45	0/27	6/21
Age (years)	58.1 ± 8.3	60.0 ± 8.5	58.9 ± 8.2	60.0 ± 8.1
White blood cells (/mm^3)	4779 ± 1383	4610 ± 1443	5195 ± 1473	4724 ± 1266
Neutrophils (/mm^3)	2478 ± 930	2355 ± 1071	2561 ± 827	2389 ± 941
Hemoglobin (g/dL)	13.7 ± 1.2	13.5 ± 1.7	14.4 ± 1.3	14.0 ± 1.2
Platelets (×10^4/mm^3)	16.0 ± 5.9	16.6 ± 6.2	18.0 ± 5.7	16.8 ± 5.2
ALT (IU/L)	75 ± 51	73 ± 72	57 ± 46	42 ± 32
Histology: activity, 0–1/2–3	29/29		11/7	
Fibrosis, 0–2/3–4	45/14		17/1	
Serum HCV RNA (KIU/mL)	1600	850	1500	700
IL-28B SNP: rs8099917; TT/TG	26/11		10/4	
ITPA SNP: rs1127354; CC/CA	20/7		9/1	
Core 70: wild/mutant	11/11			
Core 91: wild/mutant	15/7			
ISDR: 0–1/≥2	15/1			

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism.

rates of relapsers were significantly higher than those of patients with NR in the previous treatment (relapse, 67%, 36/54 vs NR, 16%, 5/32, $P < 0.0001$). As for the serum HCV RNA level at the start of re-treatment, although the SVR rate of those patients with 5 $\log_{10}$ IU/mL or more of HCV RNA was 38% (26/69), all patients with less than 5 $\log_{10}$ IU/mL of HCV RNA attained SVR (11/11) ($P = 0.0001$). As for the IL-28B genotype, among the patients with the major allele, the p-EVR rate was significantly higher and the EOT response rate showed marginal significance compared to that with the minor allele (p-EVR rate, 100%, 23/23 vs 30%, 3/10, $P < 0.0001$, EOT rate, 92%, 24/26 vs 64%, 7/11, $P = 0.05$). There was no significant difference of the SVR rate between major and minor alleles (major, 65%, 17/26 vs minor, 36%, 4/11, $P = 0.15$).

Figure 2(a) shows the result of stratified analysis according to the previous treatment response and HCV RNA at the start of re-treatment. The significant difference in SVR observed between high (≥5 $\log_{10}$ IU/mL) and low (<5 $\log_{10}$ IU/mL) baseline viral loads was still found in both previous relapsers ($P = 0.02$) and previous non-responders ($P = 0.02$). In patients with a high baseline viral load, previous relapsers achieved a higher SVR rate than previous non-responders ($P < 0.0001$). Next, the results of stratified analyses according to IL-28B genotype and previous treatment response or HCV RNA at the start of re-treatment showed no significant difference in SVR rates between the IL-28B genotype in patients with relapse after previous treatment ($P = 0.63$) (Fig. 2b). All patients with less than 5 $\log_{10}$ IU/mL of HCV RNA achieved SVR despite their IL-28B genotype and the SVR rates of patients with 5 $\log_{10}$ IU/mL or more of HCV RNA did not differ between IL-28B genotypes (Fig. 2c). Multivariate analysis among the factors of relapse to previous treatment response, HCV RNA at the start of re-treatment and IL-28B genotype showed that relapse after previous treatment response bore the most predictable relationship to SVR in re-treatment ($P = 0.074$).

As for the efficacy of re-treatment according to treatment duration among patients with HCV RNA negativity during re-treatment, the SVR rate of 72-week treatment was significantly higher than that of 48-week treatment (72 weeks, 73%, 29/40, vs 48 weeks, 52%, 12/25, $P < 0.05$). This significant difference was especially found in patients who attained c-EVR but not RVR on re-treatment (72 weeks, 73%, 16/22, vs 48 weeks,

Table 2 Baseline characteristics of patients and treatment factors according to the virological response in previous treatment among patients with genotype 1

Factor	Relapser in previous treatment		NR in previous treatment	
No.	54		32	
Sex: male/female	28/26		18/14	
	Previous treatment	Re-treatment	Previous treatment	Re-treatment
PEG IFN type: α-2a/α-2b	0/54	29/25	0/32	12/20
Age (years)	58.1 ± 8.1	60.3 ± 8.4	57.9 ± 8.9	59.6 ± 8.8
White blood cells (/mm^3)	4917 ± 1290	4692 ± 1035	4546 ± 1520	4462 ± 1993
Neutrophils (/mm^3)	2618 ± 846	2479 ± 805	2225 ± 1033	2105 ± 1454
Hemoglobin (g/dL)	13.9 ± 1.2	13.7 ± 1.6	13.5 ± 1.3	13.1 ± 1.9
Platelets (×10^4/mm^3)	17.1 ± 6.3	17.7 ± 6.1	14.1 ± 4.7	14.7 ± 6.2
ALT (IU/L)	75 ± 57	70 ± 76	75 ± 39	78 ± 64
Histology: activity, 0–1/2–3	20/18		9/11	
Fibrosis, 0–2/3–4	31/8		14/6	
Serum HCV RNA (KIU/mL)	1600	980	1550	800
IL-28B SNP: rs8099917; TT/TG	24/5		2/6	
ITPA SNP: rs1127354; CC/CA	15/6		5/1	
Core 70: wild/mutant	6/6		5/5	
Core 91: wild/mutant	9/3		6/4	
ISDR: 0–1/≥2	9/0		6/1	

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism.

38%, 5/13, $P < 0.05$) but not in patients who attained RVR or LVR (Fig. 3).

In genotype 2, the HCV RNA negative rate on re-treatment was 59% (16/27) at week 4, 85% (23/27) at week 12 and 93% (25/27) at week 24, and the SVR rate was 63% (17/27). The two patients with NR in previous treatment did not attain SVR with re-treatment. The factors associated with SVR were assessed by univariate analysis and only the factor of younger age at the start of re-treatment showed marginal significance ($P = 0.06$) (Table 4). Among the patients with RVR on re-treatment, the SVR rates were similar at 75% (6/8) to those with 24-week and 48-week treatment.

DISCUSSION

PAST STUDIES HAVE revealed that the factors of age, sex, progression of liver fibrosis, value of HCV RNA, number of mutations in the ISDR, amino acid substitutions in the core region, drug adherence and treatment duration show association with HCV eradication in PEG IFN plus ribavirin combination for naïve patients with CH-C.[3–5,25–33] Recently, the IL-28B genotype has been reported to be the most powerful factor associated with the antiviral effect of this combination therapy.[21–25] While the predictive factors for SVR in PEG IFN plus ribavirin combination therapy for naïve patients have been actively analyzed, those factors for patients who had already experienced this therapy are still unclear. Especially needing assessment is the correlation between IL-28B SNP or the previous treatment response and the antiviral effect in re-treatment. In this study, we tried to determine which factors could most effectively predict the antiviral effect in re-treatment.

In the present study, patients with relapse after the previous treatment and patients with a low serum HCV RNA level at the start of re-treatment showed significantly different results in this study of re-treatment of CH-C patients who had previously failed to attain SVR with PEG IFN plus ribavirin therapy. This result was similar to those of the EPIC[3] study on relapse and NR[17] and the SYREN trial of NR.[18] On the other hand, there was no significant difference between the influence of the IL-28B genotype and SVR. More specifically, if the previous treatment response was the same, there was no difference regardless of the IL-28B genotype. Considering this result, in re-treatment, the previous treatment response was a more effective predictive factor than IL-28B genotype. However, further investigation is needed to clarify the association between IL-28B

Table 3 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 1

Factor		SVR	Non-SVR	*P*-value
No. of patients		41	45	
Age (years)		60.2 ± 7.1	59.9 ± 9.6	0.71
Sex: male/female		24/17	22/23	0.40
Serum HCV RNA (log IU/mL)		5.8 ± 1.4	6.4 ± 0.6	0.11
Serum HCV RNA: <5 log/≥5 log		11/28	0/43	<0.001
White blood cells (/mm^3)		4656 ± 1029	4566 ± 1763	0.42
Neutrophils (/mm^3)		2443 ± 804	2259 ± 1301	0.16
Hemoglobin (g/dL)		13.5 ± 1.6	13.4 ± 1.8	0.80
Platelets (×10^4/mm^3)		16.9 ± 5.7	16.3 ± 6.7	0.36
ALT (IU/L)		68 ± 69	78 ± 75	0.43
IL-28B SNP: TT/TG		17/4	9/7	0.15
ITPA SNP: CC/CA		13/3	7/4	0.39
Core 70: wild/mutant		5/4	6/7	1.00
Core 91: wild/mutant		7/3	8/5	1.00
ISDR: 0–1/≥2		9/0	6/1	0.44
PEG IFN: α-2a/α-2b		16/25	25/20	0.14
PEG IFN dose (μg/kg per week)	α-2a	2.91 ± 0.77	2.74 ± 0.69	0.61
	α-2b	1.25 ± 0.39	1.20 ± 0.32	0.59
Ribavirin dose (mg/kg per day)		9.34 ± 2.72	9.64 ± 3.20	0.51
1st treatment virological response	Relapse/NR	36/5	18/27	<0.001

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism; SVR, sustained virological response.

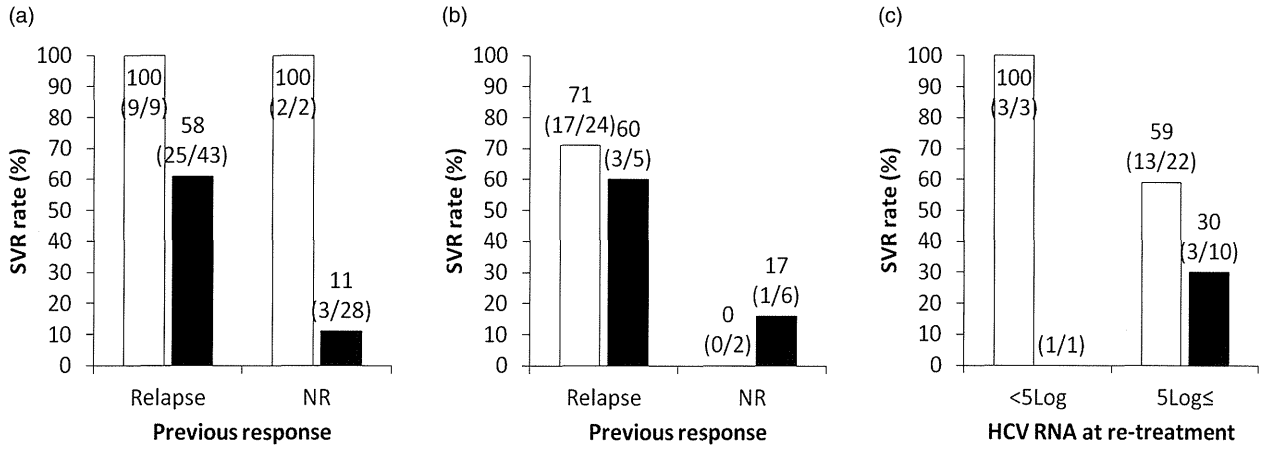

Figure 2 Sustained virological response (SVR) rates according to previous virological response, hepatitis C virus (HCV) RNA at start of re-treatment and genotype of interleukin (IL)-28B single nucleotide polymorphism (SNP) in patients with genotype 1. (a) Stratified analysis of previous virological response and HCV RNA at start of re-treatment. □, HCV RNA <5 log IU/mL at start of re-treatment; ■, HCV RNA ≥5 log IU/mL at start of re-treatment. (b) Stratified analysis of previous virological response and genotype of IL-28B SNP. □, Patients with major allele of IL-28B SNP; ■, patients with minor allele of IL-28B SNP. (c) Stratified analysis of HCV RNA at start of re-treatment and genotype of IL-28B SNP. □, Patients with major allele of IL28B SNP; ■, patients with minor allele of IL-28B SNP.

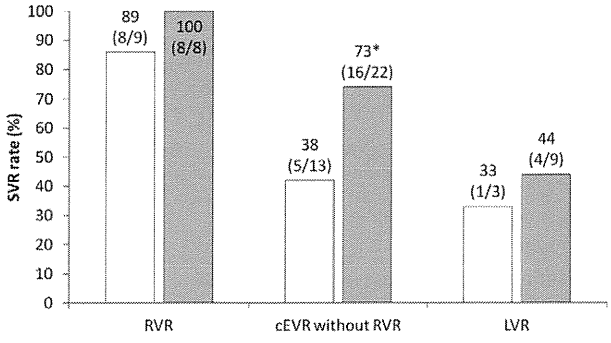

Figure 3 Sustained virological response (SVR) rates according to virological response in re-treatment and treatment duration in patients with genotype 1. □, Patients treated for 48 weeks; ▩, patients treated for 72 weeks. RVR, rapid virological response; cEVR, complete early virological response; LVR, late virological response. *$P < 0.05$; compared to 48 weeks of treatment.

genotype and antiviral effect of re-treatment because of their small number in this study. In this study, only one patient with the minor allele of IL-28B and NR in previous treatment could start and continue with the increased dose of PEG IFN (from 1.37 μg/kg in the previous treatment to 1.79 μg/kg in re-treatment) and ribavirin (from 10.3 mg/kg per day in the previous treatment to 11.1 mg/kg per day in re-treatment) and attained SVR by extended treatment. If the drug adherence does not improve, patients with the minor allele of IL-28B who show NR in the previous treatment should be treated with new drugs.

The next question is how the patients should be re-treated in order to attain SVR on re-treatment. In this study, the patients with a low serum HCV RNA level (<5 $\log_{10}$ IU/mL) at the start of re-treatment showed a significant rate of cure on re-treatment, and this is almost the same result as that previously reported.[16,17] In this study, the two patients with NR in the previous treatment and with less than 5 $\log_{10}$ IU/mL of HCV RNA level (20 KIU/mL and 52 KIU/mL of HCV RNA) at the start of re-treatment attained SVR. On the other hand, even if the previous treatment response was a relapse, the SVR rates were 58% (25/43) among the patients with 5 $\log_{10}$ IU/mL or more of HCV RNA. Because the HCV RNA level changed after the antiviral treatment, it is important to not miss the timing of when the HCV RNA level is low.

With respect to treatment duration among patients with HCV RNA negativiation during re-treatment, 72 weeks of treatment significantly increased the SVR rate compared to 48 weeks. This result was almost the same as that of the REPEAT study.[16] In our present study, the SVR rate among the patients with c-EVR but not RVR in re-treatment was significantly high by 72 weeks of treatment. On the other hand, the SVR rates among the

Table 4 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 2

Factor		SVR	Non-SVR	*P*-value
No. of patients		17	10	
Age (years)		57.7 ± 8.8	63.7 ± 5.1	0.06
Sex: male/female		7/10	8/2	0.11
Serum HCV RNA (log IU/mL)		5.4 ± 1.4	6.1 ± 0.8	0.15
Serum HCV RNA: <5 log/≥5 log		5/11	1/9	0.35
White blood cells (/mm^3)		5049 ± 1355	4171 ± 910	0.10
Neutrophils (/mm^3)		2556 ± 1064	1999 ± 404	0.24
Hemoglobin (g/dL)		14.1 ± 1.3	13.8 ± 1.6	0.51
Platelets (×10^4/mm^3)		17.9 ± 5.4	14.8 ± 4.3	0.17
ALT (IU/L)		38 ± 19	48 ± 47	0.71
IL-28B SNP: TT/TG		6/2	4/2	1.00
ITPA SNP: CC/CA		5/1	4/0	1.00
PEG IFN: α-2a/α-2b		4/13	2/8	1.00
PEG IFN dose (μg/kg per week)	α-2a	3.23 ± 0.34	2.24 ± 2.25	1.00
	α-2b	1.32 ± 0.28	1.18 ± 0.23	0.21
Ribavirin dose (mg/kg per day)		10.4 ± 2.21	10.1 ± 1.31	0.44
1st treatment virological response	RVR/non-RVR	4/13	3/7	1.00

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; PEG, pegylated; RVR, rapid virological response; SNP, single nucleotide polymorphism; SVR, sustained virological response.

patients with RVR in re-treatment were similar between the patients with 48 weeks and 72 weeks of treatment. Thus, patients with c-EVR but not RVR in re-treatment should be re-treated for a longer period. In order to attain better SVR, extended treatment duration is generally recommended for patients with on-treatment LVR, whereas standard treatment duration is considered to be sufficient for patients with on-treatment c-EVR. However, the present study revealed that, even if patients achieved c-EVR on re-treatment, 72 weeks of treatment seems to be better than 48 weeks for treatment-experienced patients. The majority of naïve patients showing on-treatment c-EVR could eradicate HCV with 48 weeks of treatment while some could not. In a treatment-experienced setting, patients who are able to respond early but not eradicate HCV would be selected, and therefore extended treatment may be needed.

With genotype 2, the SVR rate was relatively high (63%). The patients who could not attain SVR in re-treatment (two patients) showed NR in the previous treatment. Thus, the patients with genotype 2 and showing NR in previous treatment seemed to be difficult to treat and could be treated with other drugs. Among the patients with RVR in re-treatment, the SVR rates were similar among those with RVR in re-treatment between 24 weeks and 48 weeks of treatment. The effectiveness of extended treatment for the patients with genotype 2 in re-treatment could not be demonstrated because of their small number in this study. Further investigation is needed to clarify this.

In conclusion, this study shows that the efficacy of re-treatment for genotype 1 patients who failed to show SVR to previous treatment with PEG IFN plus ribavirin could be predicted from the previous treatment response and a low HCV RNA level at the start of re-treatment. Re-treatment for 72 weeks led to clinical improvement for genotype 1 patients with c-EVR and without RVR on re-treatment.

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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).

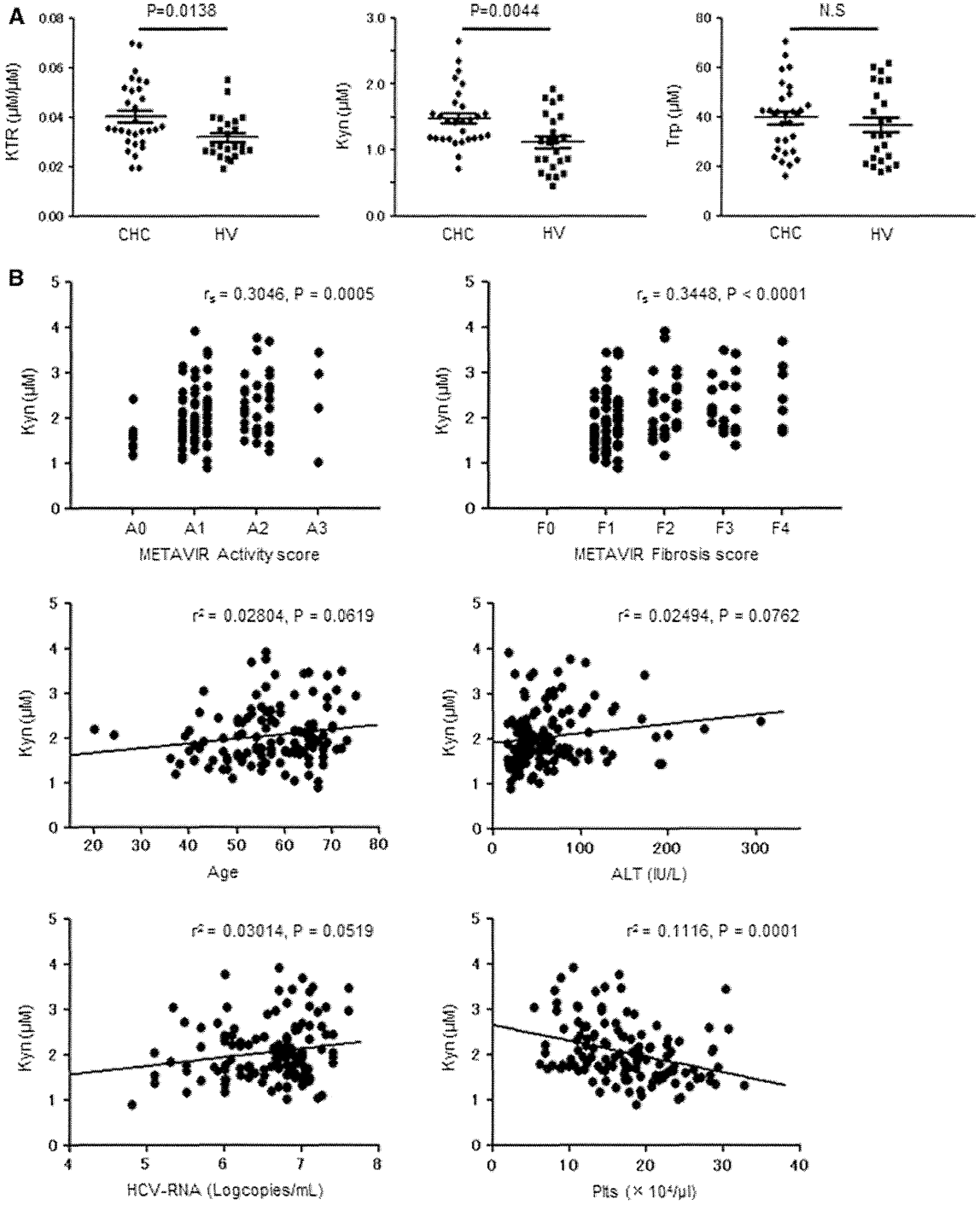


Fig. 1 Systemic indoleamine 2,3-dioxygenase (IDO) activity is enhanced in chronic hepatitis C patients. **a** Serum kynurenine (*Kyn*) and tryptophan (*Trp*) were assayed by HPLC as described in "Subjects, materials, and methods", and the kynurenine-to-tryptophan ratio (*KTR*) was calculated from their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

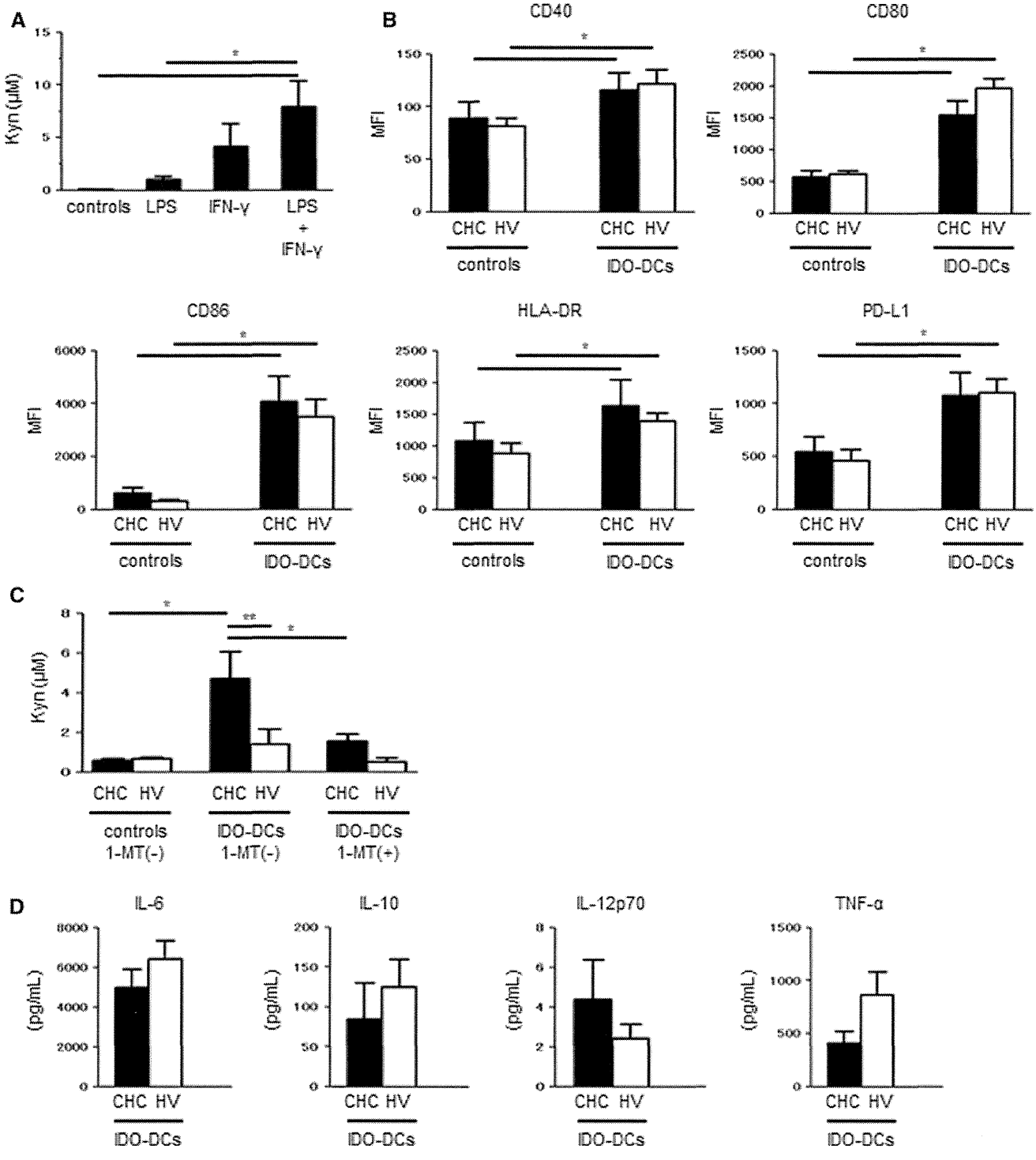
IDO is not involved in allogeneic T-cell proliferation and Th1/Th2 differentiation with DCs from chronic hepatitis C patients

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).

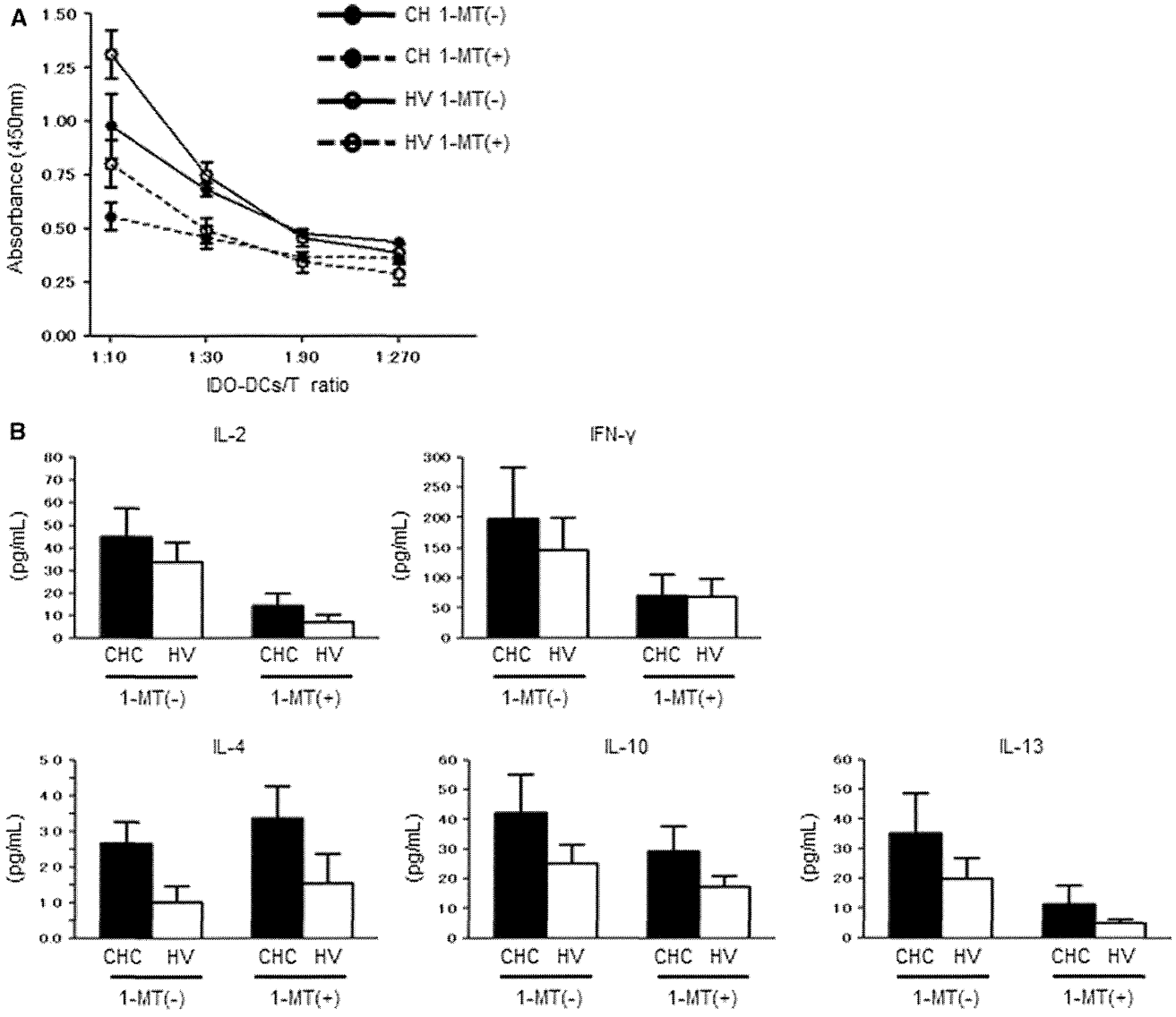


Fig. 3 IDO is not involved in lower allogeneic T-cell response and Th1/Th2 differentiation with DCs from chronic hepatitis C patients. **a** Allogeneic mixed lymphocyte reaction (MLR) with IDO-DCs was performed as described in "Subjects, materials, and methods". *Closed circles* are the 450-nm absorbance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

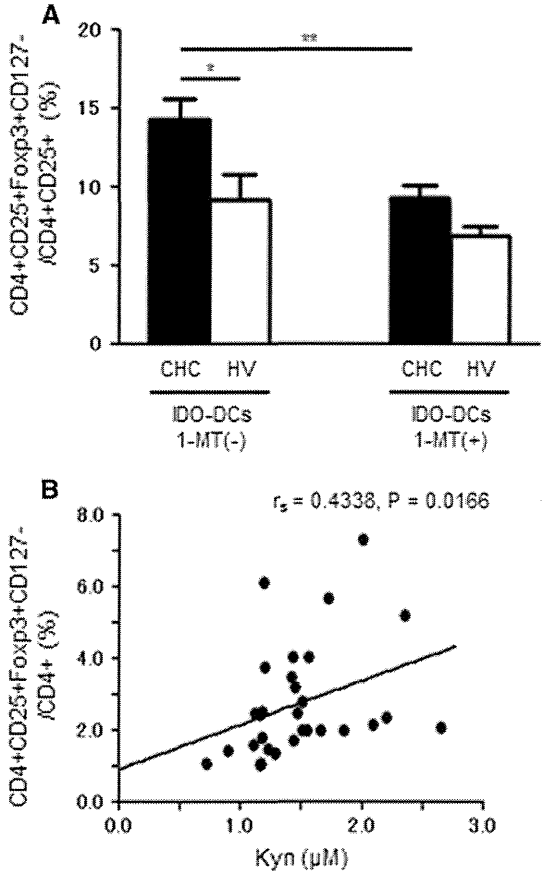


Fig. 4 IDO is involved in the induction of regulatory T cells. **a** After IDO-DCs were generated from the CHC or HV group, naive CD4+ T cells were co-cultured for 7 days with IDO-DCs in the presence or absence of 1-MT. The cultured T cells were stained with relevant antibodies (*Abs*) and analyzed with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