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).

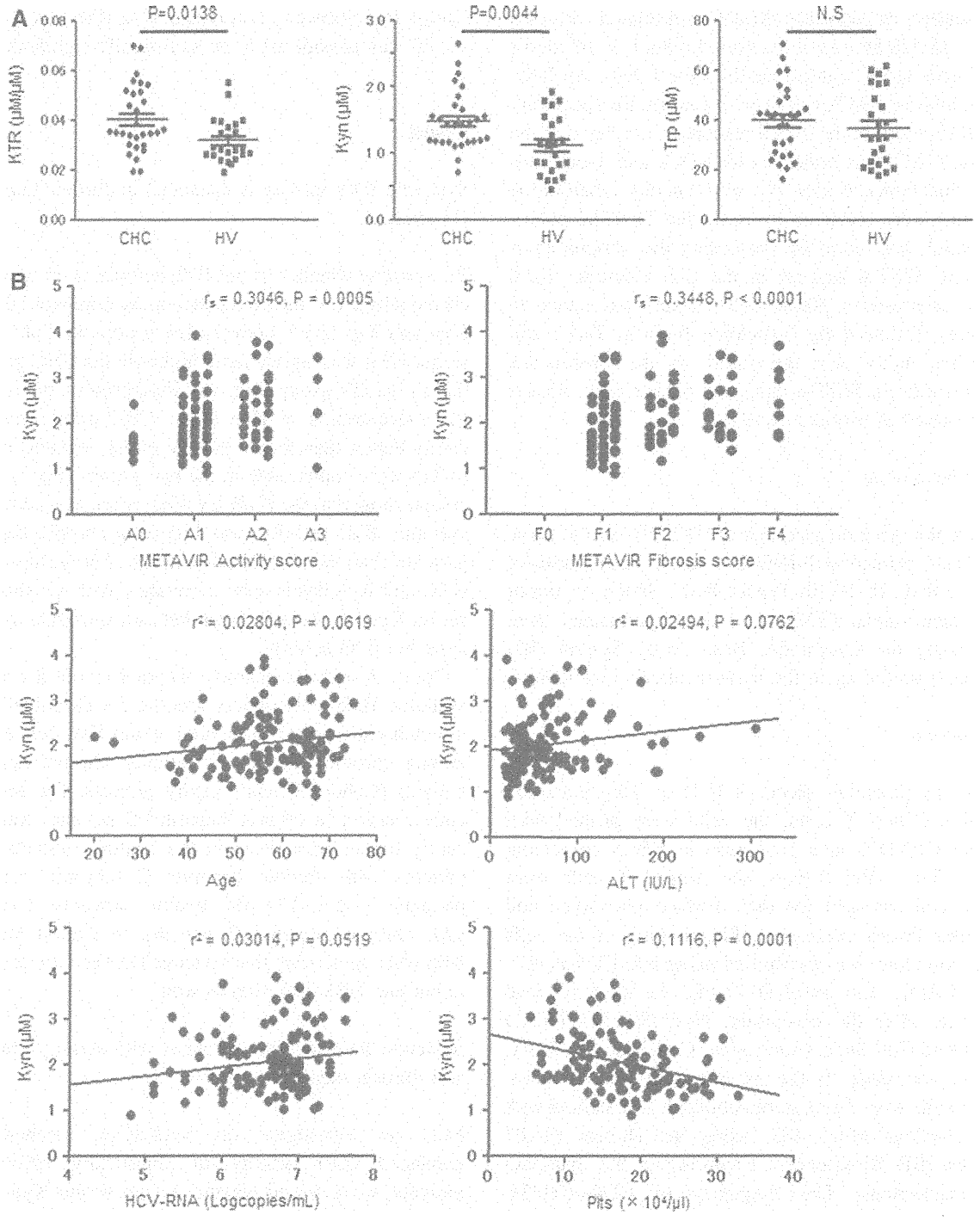


Fig. 1 Systemic indoleamine 2,3-dioxygenase (IDO) activity is enhanced in chronic hepatitis C patients. **a** Serum kynurenine (*Kyn*) and tryptophan (*Trp*) were assayed by HPLC as described in "Subjects, materials, and methods", and the kynurenine-to-tryptophan ratio (*KTR*) was calculated from their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

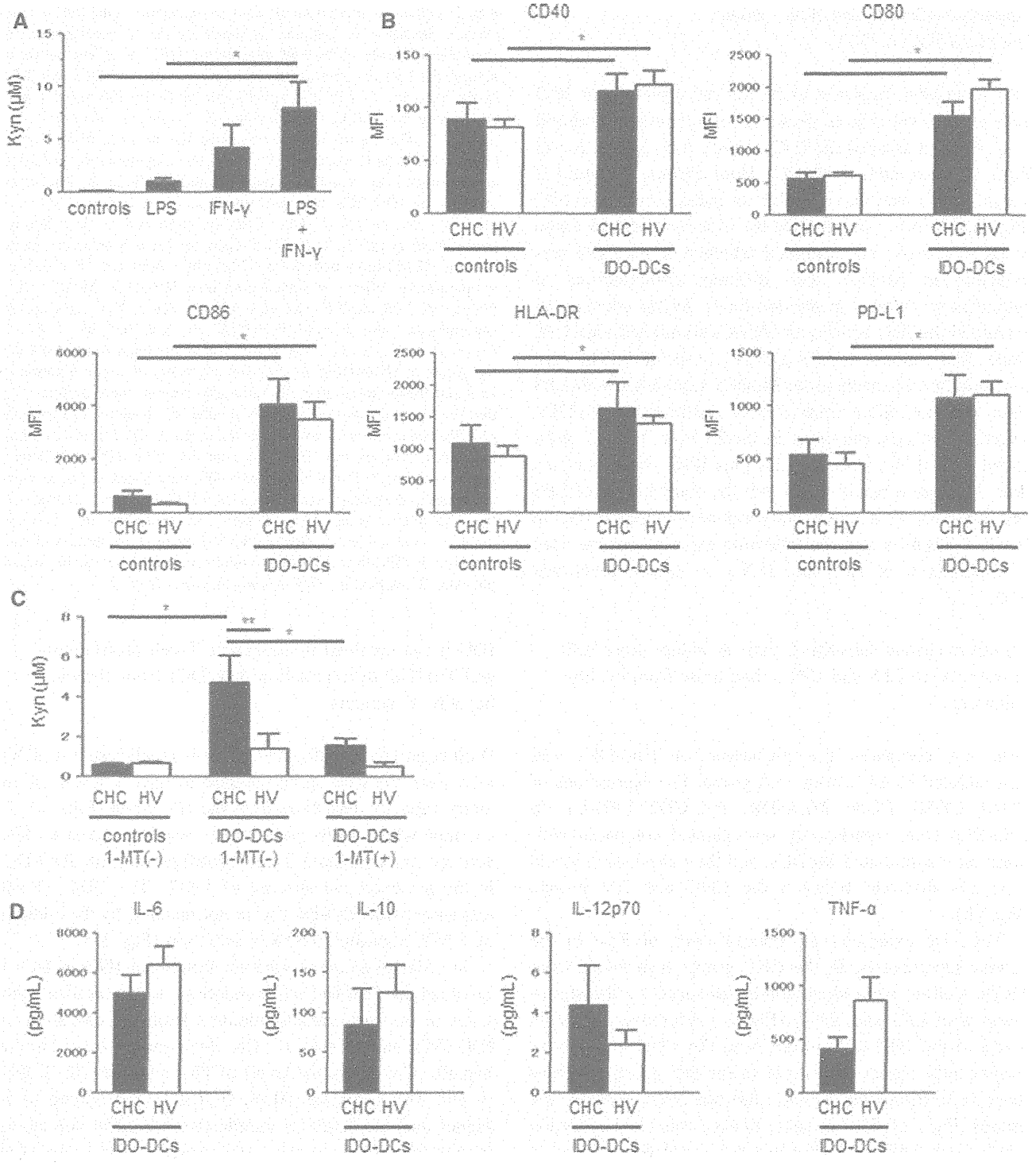
IDO is not involved in allogeneic T-cell proliferation and Th1/Th2 differentiation with DCs from chronic hepatitis C patients

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).

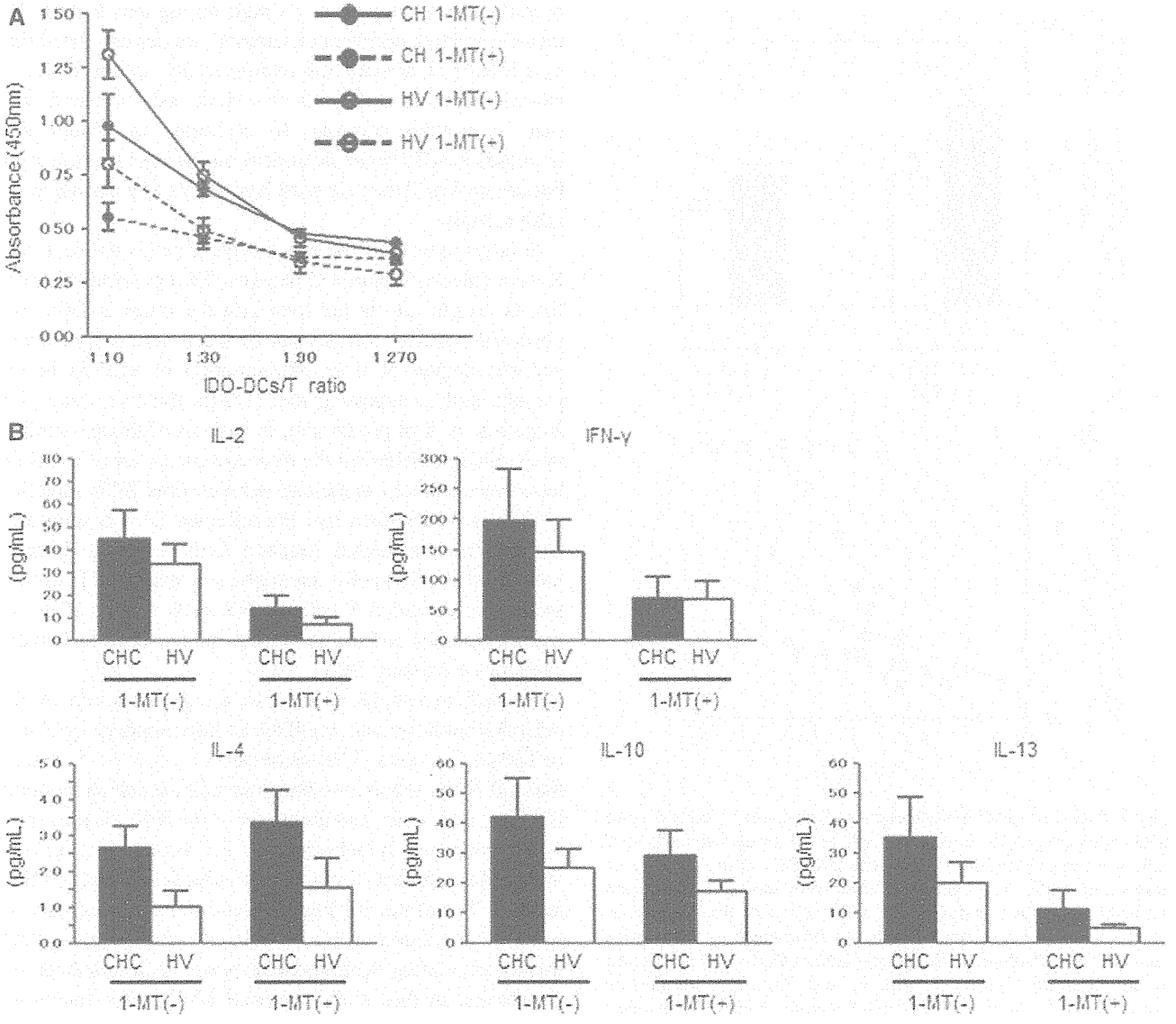


Fig. 3 IDO is not involved in lower allogeneic T-cell response and Th1/Th2 differentiation with DCs from chronic hepatitis C patients. **a** Allogeneic mixed lymphocyte reaction (MLR) with IDO-DCs was performed as described in "Subjects, materials, and methods". *Closed circles* are the 450-nm absorbance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

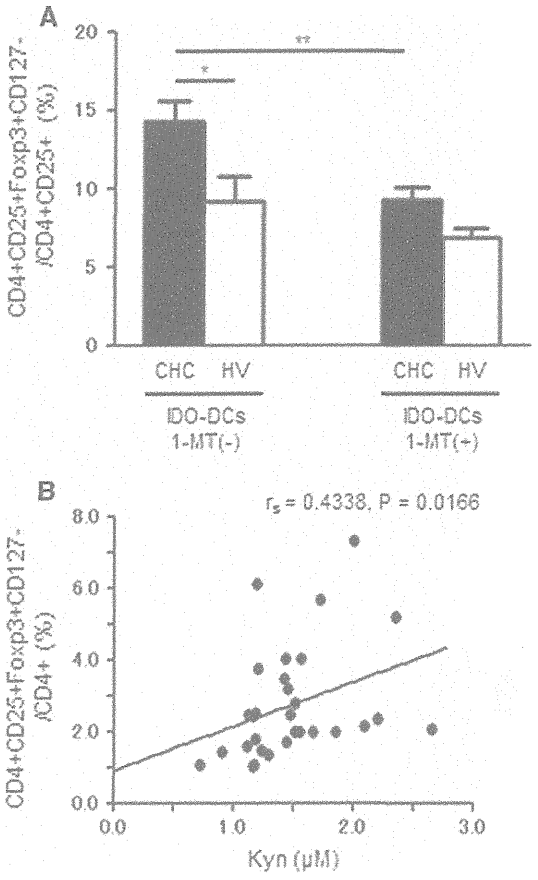


Fig. 4 IDO is involved in the induction of regulatory T cells. **a** After IDO-DCs were generated from the CHC or HV group, naive CD4+ T cells were co-cultured for 7 days with IDO-DCs in the presence or absence of 1-MT. The cultured T cells were stained with relevant antibodies (*Abs*) and analyzed with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

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The combination therapy of α-galactosylceramide and 5-fluorouracil showed antitumor effect synergistically against liver tumor in mice

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α-Galactosylceramide (α-GalCer) has been reported to be therapeutic against metastatic liver tumors in mice. However, little is known regarding the efficacy of combined chemo-immunotherapy using α-GalCer and anticancer drugs. In this study, we evaluated the antitumor effect of the combination therapy of α-GalCer and 5-fluorouracil (5-FU) against liver tumors of MC38 colon cancer cells. The liver weights of tumor-bearing mice treated with the combination were significantly lower than those of nontreated mice and of mice treated with 5-FU or α-GalCer alone. No toxic effects on the liver and renal functions were observed in any of the treatment groups. α-GalCer treatment induced significant activation of liver NK cells *in vivo*, but 5-FU treatment did not. 5-FU treatment resulted in a significant upregulation of NKG2D activating molecules (Rae-1 and H60) and DNAM-1 ligands (CD112 and CD155) on MC38 cells, but α-GalCer did not. The cytolytic activity of α-GalCer-activated liver mononuclear cells against 5-FU-treated MC38 cells was significantly higher than that against nontreated cells. The increase of the cytolytic activity induced by 5-FU partially depended on NKG2D-Rae-1 or H60 signals. Depletion of NK cells significantly inhibited the antitumor efficacy of 5-FU against MC38 liver tumors, which suggested that the antitumor effect of 5-FU partially depended on the cytolytic activity of NK cells. These results demonstrated that the combination therapy of α-GalCer and 5-FU produced synergistic antitumor effects against liver tumors by increasing the expression of NK activating molecules on cancer cells. This study suggests a promising new chemo-immunotherapy against metastatic liver cancer.

Tumor Immunology

Colon cancer is one of the most common cancers in the world. Despite recent progress in the development of treatment, the overall 5-year survival rate is only 50–60% due to local recurrence or distant metastasis.[1] In particular, patients with metastatic colon cancer have a median survival rate of only six months. 5-Fluorouracil (5-FU) remains key-drug in chemotherapy against colon cancer. However, colon cancer cells are becoming increasingly resistant to existing chemotherapies including 5-FU.[2] Therefore, novel strategies are needed especially for the treatment of advanced colon cancers including metastatic liver cancer.

A normal liver contains abundant lymphocytes that are usually enriched with NK and NKT cells in contrast to peripheral blood.[3,4] Thus, the effective activation of innate immune cells might be beneficial in the treatment of metastatic liver cancer. To date, however, immunotherapy has not yet been established against metastatic liver cancer. α-Galactosylceramide (α-GalCer) induces the activation of NKT cells in a CD1d-dependent manner.[5,6] Recently, α-GalCer has been attracting attention as a novel antitumor therapy. Systemic administration of α-GalCer has demonstrated antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma, and lymphoma) *in vivo* in animal models of hepatic and lung metastasis.[7,8] We and others have demonstrated that sequential activation of both NKT and NK cells could be observed in the liver after α-GalCer administration.[8–10] Although most NKT cells had disappeared from the liver within 12 hr of α-GalCer administration, strong activation and proliferation of liver NK cells could be

Key words: α-GalCer, 5-FU, NK cells, liver tumor

Abbreviations: 5-FU: 5-fluorourcil; Alb: albumin; ALT: alanine aminotransferase; Cr: creatinine; IFN-α: interferon-α; MICA: major histocompatibility complex class I-related chain A; MNCs: mononuclear cells; PBS: phosphate buffered saline; T-Bil: total bilirubin; α-GalCer: α-galactosylceramide

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What's new?
α-Galactosylceramide (α-GalCer) is effective against metastatic liver tumors in mice. In this study, the authors evaluated the antitumor effect of a combination therapy of α-GalCer plus 5-FU. They found that the combination therapy produced synergistic antitumor effects against liver tumors of colon cancer cells in mice, by both increasing the activation of natural killer (NK) cells and enhancing the sensitivity of the cancer cells to those NK cells. This combination may therefore represent a promising new chemo-immunotherapy against metastatic liver cancer.

observed, and the antitumor effect of the α-GalCer treatment against liver tumors depended primarily on NK cells. Based on the promising results of preclinical studies, several Phase 1 clinical studies using intravenous administration of α-GalCer have been conducted, but clinical responses of α-GalCer have been limited.[11] In view of future α-GalCer treatment of metastatic liver cancer, new strategies should be explored. We have previously reported that anticancer drugs enhance the expression of the human NKG2D ligand, membrane-bound major histocompatibility complex class I-related chain A (MICA), and the NK sensitivity of human hepatocellular carcinoma cells *in vitro*.[12,13] These findings suggest that the efficient activation of liver innate immunity after chemotherapy might represent a promising approach to the suppression of liver tumor growth.

In this study, we investigated the therapeutic potential of the combination of α-GalCer and 5-FU in the treatment of liver tumor of colon cancer cells. We found that 5-FU can enhance the NK sensitivity of colon cancer cells by increasing the expression of NK activating molecules. In addition, the combination therapy of α-GalCer and 5-FU showed synergistic antitumor effects against liver tumor of colon cancer cells. This study demonstrates a promising new therapeutic strategy for the treatment of metastatic liver cancer.

Material and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories Japan, INC (Yokohama, Japan) and were used at 6–10 weeks of age. The mice were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guidelines.

Cell lines

MC38, a mouse colon cancer cell line derived from C57BL/6 mice, was generously provided by Dr. Michio Imawari (Showa University School of Medicine, Tokyo, Japan). Colon26, a mouse colon cancer cell line derived from BALB/c mice, was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). This cell line was maintained in complete medium (CM, RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C.

Reagents

α-GalCer was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*[5] 5-FU was purchased from Kyowa Hakko Kirin (Tokyo, Japan) and dissolved in phosphate buffered saline (PBS). MC38 cell viability was determined 24 hr after the addition of 5-FU (used at 10 nmol/l to 2 μmol/l) or PBS by the WST assay using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan) as previously described (10).

Flow cytometry

MC38 cells were cultured with or without α-GalCer (100 ng/ml) or 5-FU (500 nmol/l) for 24 hr and evaluated for the expression of NK activating molecules. Treated and non-treated MC38 cells were incubated with PE-conjugated antibodies (Abs) against anti-Rae-1 (R&D Systems, Minneapolis, MN), H60 (R&D Systems), CD112 (Nectin-2) (Abcam, Cambridge, UK), and CD155 (BioLegend, San Diego, CA). Flow cytometric analysis was performed using a Canto II flow cytometer (Becton Dickinson, San Jose, CA).

Preparation of hepatic mononuclear cells from 5-FU- or α-GalCer-treated mice

C57BL/6 mice were administered 5-FU (20 mg/kg body weight)or PBS intraperitoneally (i.p.) for 3 consecutive days. Liver mononuclear cells (MNCs) were prepared as previously described.[8] In some experiments, C57BL/6 mice were administered α-GalCer (0.4 μg/mouse) or PBS i.p. on Day 0. On Day 3, hepatic MNCs were prepared. NK cells were identified as DX5+/TCRβ- by flow cytometry as previously described.[8] The expression levels of NKG2D and DNAM1 were evaluated with anti-NKG2D (R&D Systems) and anti-DNAM1 (BioLegend) Abs by flow cytometry.

Cytolytic assays

C57BL/6 mice were injected i.p. with α-GalCer (2 μg/mouse) for the preparation of activated NK cells as previously described.[8] Liver MNCs were prepared on Day 3 after α-GalCer injection. MC38 cells were cultured with or without 5-FU (500 nmol/l) for 1 day. α-GalCer-activated liver MNCs

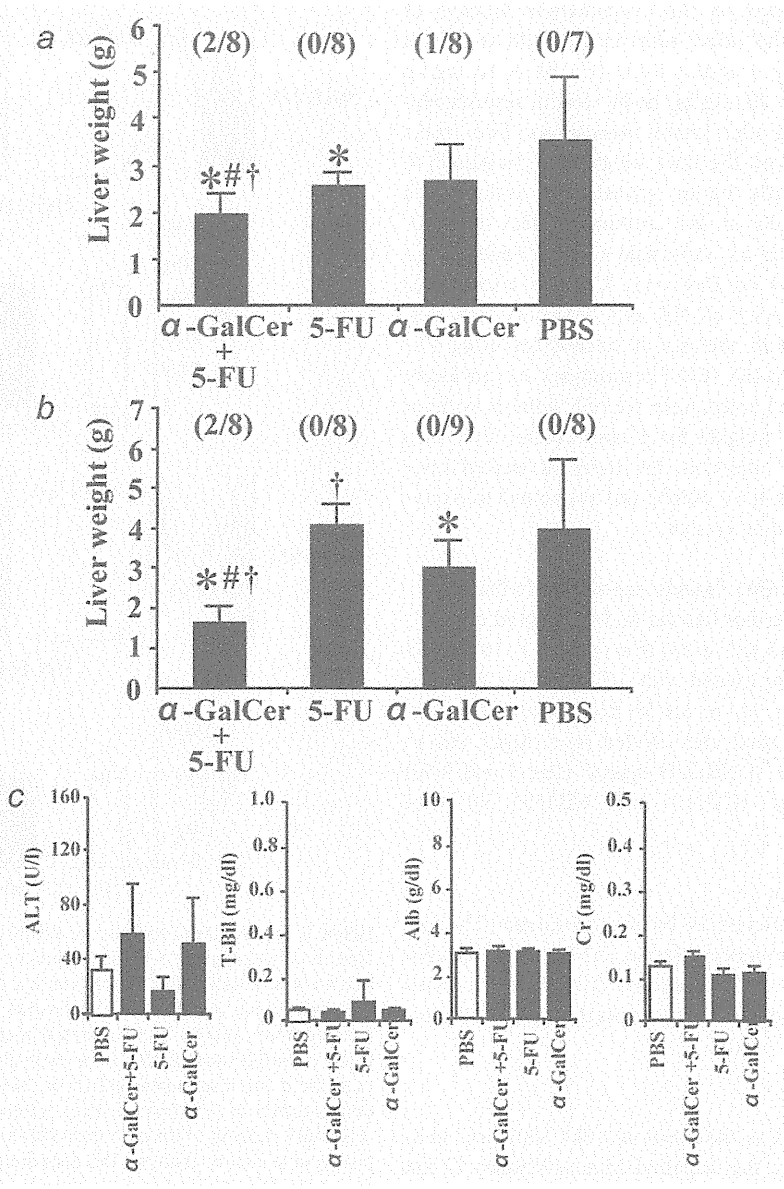

Figure 1. The antitumor effect of the α-GalCer and 5-FU combination therapy against MC38 liver tumors. (*a*, *b*) C57BL/6 mice or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0. To evaluate the efficacy of the α-GalCer and 5-FU combination therapy, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine intrahepatic tumor growth. $N = 7–9$ mice/group. Each data point represents the mean liver weight ± SD. The fraction of mice achieving tumor rejection in each treatment group is shown in parentheses. $*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* 5-FU group, $\dagger p < 0.05$ *versus* α-GalCer group. (*c*) Blood samples from treated C57BL/6 mice were obtained 1 day after the final injection of each treatment. The serum levels of ALT, T-Bil, Alb, and Cr were examined. $N = 3$ /group. No significant differences were observed between any of the groups.

were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated or nontreated MC38 cells as previously described.[12] The assays were performed in triplicate, and the spontaneous release of all assays did not exceed 25% of the maximum release. In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 (R&D Systems) or H60 (R&D Systems).

Animal experiments

C57BL/6 or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0.

To evaluate the efficacy of the combination therapy of α-GalCer and 5-FU, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight respectively) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine the intrahepatic tumor growth. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-asialo GM-1 (ASGM1) Ab (WAKO, Osaka, Japan) on Days −1, 4, and 9 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometric analysis of splenocytes using PE-conjugated anti-DX5 mAbs (BD-Pharmingen) as previously described.[8] NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of treated mice were removed, and the liver weight was measured to examine the intrahepatic tumor growth.

NKG2D lignads and DNAM1 ligands expression in MC38 tumor tissues and nontumor tissues in 5-FU-treated mice

C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry.

Blood biochemistry test

Blood samples were obtained 24 hr after treatment. The levels of serum alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin (Alb), and creatinine (Cr) were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Statistics

All values are expressed as the mean and SD. Statistical analyses were performed by the unpaired Mann–Whitney *U* test or one-way ANOVA unless otherwise indicated. When ANOVA analyses were applied, differences in the mean values among groups were examined by the Scheffe post hoc correction. We defined statistical significance as $p < 0.05$.

Results

The combination therapy of α-GalCer and 5-FU showed a synergistic antitumor effect against MC38 liver tumors

We examined the antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 liver tumors. C57BL/6 mice were injected intrahepatically with MC38 cells. The mice were treated with α-GalCer on Day 0 and/or 5-FU for 5 consecutive days after tumor inoculation. As shown in Figure 1*a*, the liver weights of the mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with 5-FU were significantly lower than those of nontreated mice, but treatment with α-GalCer did not produce this effect. We also examined the antitumor effect of α-GalCer plus 5-FU in a Colon26 liver tumor model. The liver weights of mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with α-GalCer were significantly lower than those of nontreated and 5-FU-treated mice (Fig. 1*b*). Tumor rejection in the MC38 liver tumor model was observed in 2/8 of the α-GalCer plus 5-FU-treated

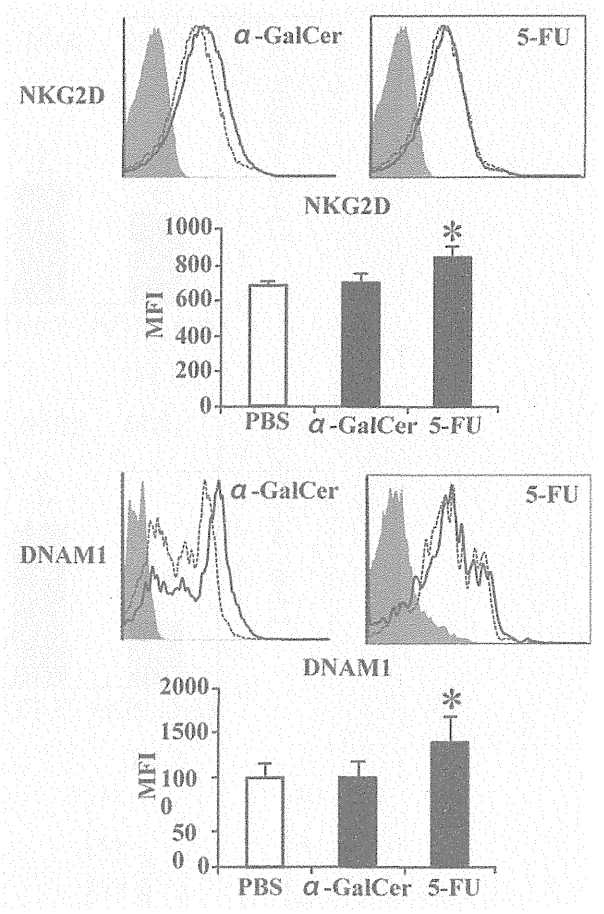

Figure 2. Expression of NKG2D and DNAM1 on liver NK cells isolated from α-GalCer- or 5-FU-treated mice. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or with 5-FU (10 mg/kg body weight) for 3 consecutive days. Liver NK cells were isolated from α-GalCer or 5-FU-treated mice, and the expression levels of NKG2D and DNAM1 were evaluated by flow cytometry. Black bold line histograms: NKG2D or DNAM1 staining of NK cells from α-GalCer or 5-FU-treated mice; dotted line histograms: NKG2D or DNAM1 staining of NK cells from PBS-treated mice; shaded/gray histograms: control staining. The data are represented as the average of the MFI obtained from 3 separate experiments. $^*p < 0.05$ *versus* PBS-treated group.

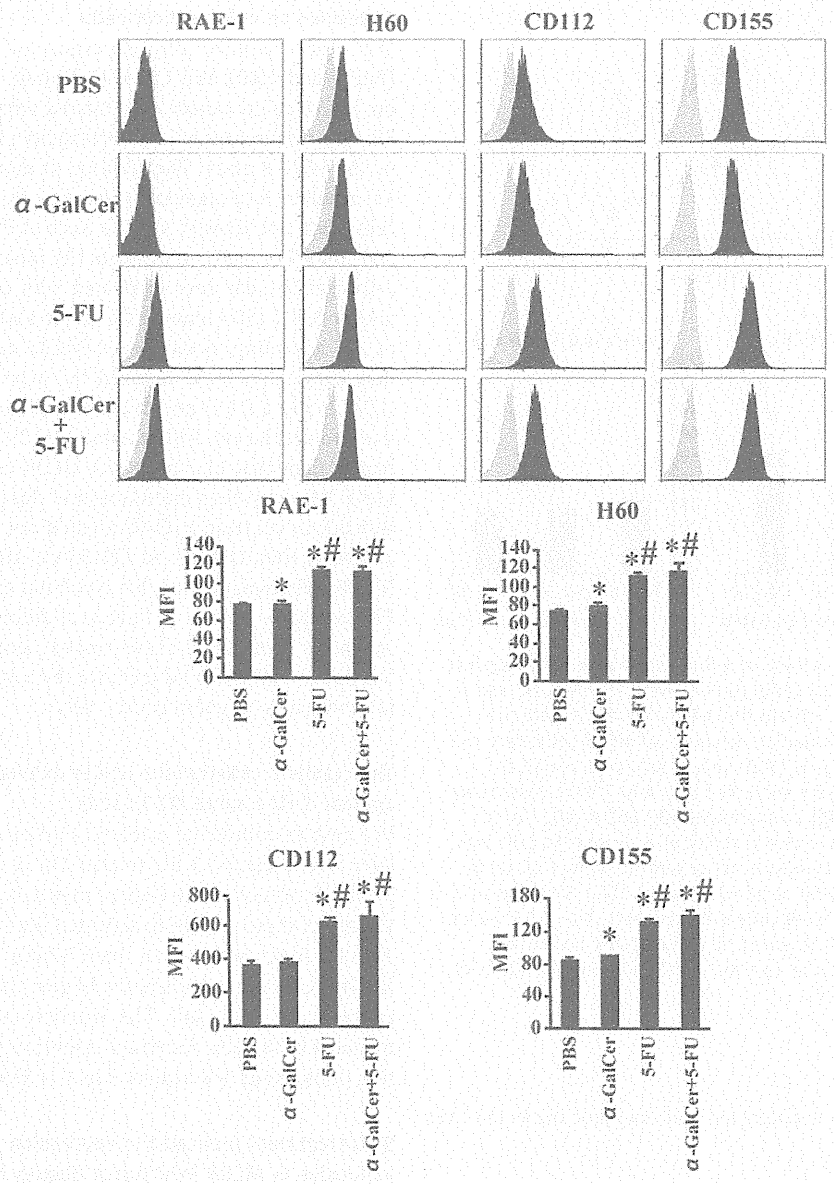

Figure 3. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells treated with α-GalCer and/or 5-FU. MC38 cells were cultured with or without α-GalCer (100 ng/ml) or 5-FU (500 nmol/l) for 24 hr. The treated cells were harvested and evaluated for the expression levels of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells by flow cytometry. Upper panel: representative data. Shaded/black histograms: NKG2D or DNAM1 ligand staining of α-GalCer or 5-FU-treated MC38 cells; shaded/gray histograms, control staining. Lower panel: data are represented as the average of MFI obtained from 3 separate experiments. *$p < 0.05$ *versus* PBS group, #$p < 0.05$ *versus* α-GalCer group.

mice, 0/8 of the 5-FU-treated mice, 1/8 of the α-GalCer-treated mice, and 0/7 of the PBS-treated mice (Fig. 1*a*). These results were consistent with those of another Colon26 liver tumor model in BALB/c mice, where tumor rejection was observed in 2/8 of the α-GalCer plus 5-FU-treated mice, 0/8 of the 5-FU-treated mice, 0/9 of the α-GalCer-treated mice, and 0/8 of the PBS-treated mice (Fig. 1*b*). These results demonstrated that the combination therapy of α-GalCer and 5-FU produced a synergistic antitumor effect against liver tumors in both the MC38 and Colon26 models. To evaluate the safety of this combination therapy, serum levels of ALT, T-Bil, Alb, and Cr were evaluated in C57BL/6 mice immunized with α-GalCer plus 5-FU, 5-FU, α-GalCer, or PBS. There was no toxic effect upon the ALT, T-Bil, Alb, or Cr levels for any of the treatment groups (Fig. 1*c*). These results demonstrated that the combination therapy of α-GalCer and

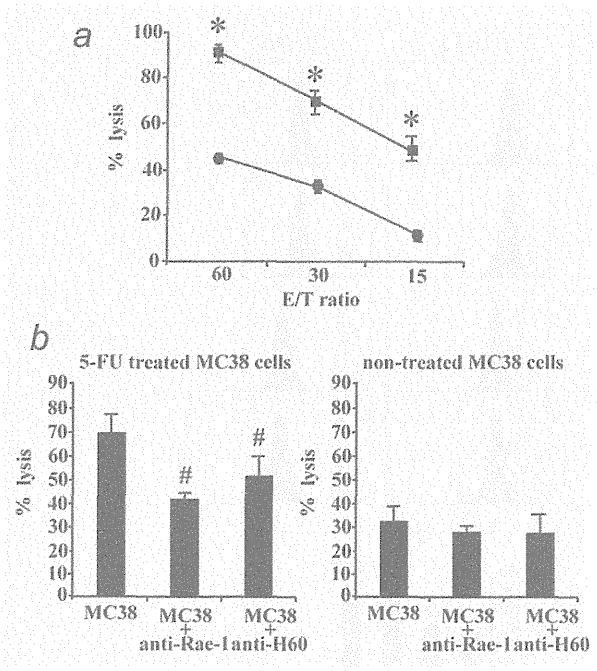

Figure 4. The cytolytic activity of α-GalCer-activated MNCs against 5-FU-treated MC38 cells. C57BL/6 mice were injected i.p. with α-GalCer (2 μg/mice) to activate NK cells. Liver MNCs were prepared on Day 3 after α-GalCer injection. (*a*) MC38 cells were cultured with or without 5-FU (500 nmol/l) for 24 hr. α-GalCer-activated liver MNCs were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated (■) or nontreated (●) MC38 cells. (*b*) In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 or H60 at an E/T ratio of 30:1. Similar results were obtained from 3 independent experiments. $*p < 0.05$ *versus* the cytolytic activity of activated NK cells against nontreated cells, $\#p < 0.05$ *versus* the cytolytic activity of activated NK cells against 5-FU-treated cells.

5-FU is not toxic to hepatocytes and does not harm the liver or kidney.

α-GalCer, but not 5-FU, treatment induced NK activating receptors on NK cells

We examined the expression levels of activating (NKG2D and DNAM1) receptors on liver NK cells. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or 5-FU (10 mg/kg body weight) for 3 consecutive days and liver NK cells were isolated from 5-FU- and α-GalCer-treated mice. As shown in Figure 2, the expression levels of NKG2D and DNAM1 on liver NK cells from α-GalCer-treated mice were significantly higher than those from PBS-treated mice. In contrast, the expression of NKG2D and DNAM1 on liver NK cells from 5-FU-treated mice was similar to that of PBS-treated mice. These results demonstrated that α-GalCer, but not 5-FU, could activate liver NK cells.

5-FU, but not α-GalCer, treatment induced NK activating molecules on colon cancer cells

We next examined the expression of the NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 colon cancer cells treated with α-GalCer and/or 5-FU. We first examined the cytotoxicity of 5-FU on MC38 cells by the WST-8 assay. The addition of more than 1 μmol/l of 5-FU resulted in a significant decrease in the growth of MC38 cells (data not shown). On the basis of these findings, we used 500 nmol/l of 5-FU to evaluate the biological effect on MC38 cells. MC38 cells were incubated with α-GalCer (100 ng/ml) and/or 5-FU (500 nmol/l) for 24 hr and the expression levels of NK activating molecules on MC38 cells were evaluated by flow cytometry. 5-FU induced the expression of Rae-1, H60, CD112, and CD155 on MC38 cells (Fig. 3). The expression of these molecules on 5-FU-treated MC 38 cells was significantly higher than that of nontreated MC38 cells. The induction of these NK activating molecules was dose-dependent (data not shown). In contrast, α-GalCer could not induce the expression of Rae-1, H60, CD112, or CD155 on MC38 cells. Even in the MC38 cells treated with this combination of α-GalCer and 5-FU, α-GalCer failed to induce additional expression of NK activating molecules. These results demonstrated that 5-FU, but not α-GalCer, could enhance the expression of NK activating molecules on colon cancer cells.

The cytolytic activity of α-GalCer activated liver MNCs against 5-FU-treated MC38 cells

We next examined the cytolytic activity of α-GalCer-activated liver MNCs against 5-FU-treated MC38 cells. We isolated liver MNCs from normal α-GalCer injected mice and the cytolytic activity of these α-GalCer-activated liver MNCs was measured. The cytolytic activity of liver MNCs against 5-FU-treated MC38 cells was significantly higher than that against non-treated cells (Fig. 4*a*). The cytolytic activity against 5-FU-treated MC38 cells decreased significantly following the addition of blocking Abs against Rae-1 or H60 (Fig. 4*b*).

5-FU treatment induced the expression of NK activating molecules in MC38 liver tumor tissues but not in MC38 nontumor tissues

We examined the induction of NKG2D ligand (Rae-1 and H60) and DNAM1 ligand (CD112 and CD155) expression in MC38 liver tumor or nontumor tissues of 5-FU-treated mice. As shown in Figure 5, the expression of Rae-1 in liver tumor tissues of 5-FU-treated mice was significantly higher than that of liver tumor and nontumor tissues of PBS-treated mice and that of nontumor tissues of 5-FU-treated mice. The expression of H60 and CD112 was similar to that of Rae-1. The expression of CD155 in liver tumor tissues of 5-FU-treated mice tended to be higher than that of PBS-treated mice, although the difference was not statistically significant. These results demonstrated that 5-FU treatment induced the expression of NK activating molecules in liver tumor tissues but not in non-tumor tissues consistent with the *in vitro* results.

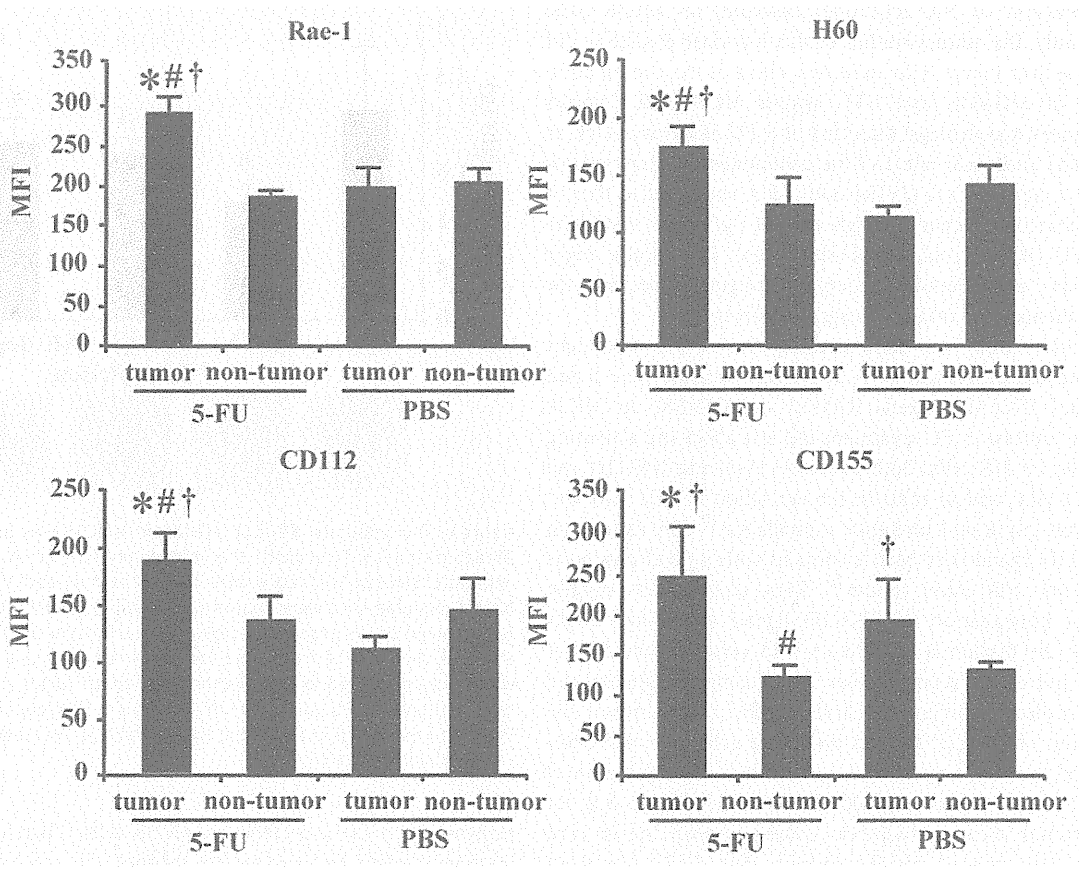

Figure 5. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 liver tumor tissues of mice treated with 5-FU. C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry. $N = 3$/group. *$p < 0.05$ *versus* nontumor tissues of PBS group, #$p < 0.05$ *versus* tumor tissues of PBS group, †$p < 0.05$ *versus* nontumor tissues of 5-FU group.

Tumor Immunology

The antitumor effect of 5-FU depended on both direct cytotoxicity and the cytolytic activity of NK cells in mouse colon cancer

The above results suggested that 5-FU could enhance the NK sensitivity of MC38 cells. To confirm that NK activity played a role in the antitumor effect of 5-FU, we examined the antitumor effect of 5-FU against MC38 liver tumors in NK depleted mice. As shown in Figure 6, the liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. Depletion of NK cells significantly inhibited the antitumor efficacy of 5-FU against MC38 liver tumors. These results suggested that the antitumor effect of 5-FU depended on not only on the direct cytotoxic effect of 5-FU but also on the cytolytic activity of NK cells. Therefore, NK activity plays a role in the antitumor effect of 5-FU in the liver which contains abundant NK cells.

Discussion

The lymphocytes in the liver are typically enriched with a higher number of NK cells than that found in the peripheral blood in a normal mouse.[3,4] Efficient activation of the abundant NK cells in the liver might be important in antitumor defense against liver tumors. Interferon-α (IFN-α) could activate liver NK cells efficiently.[14] Bui *et al.*[15] reported that IFN-α reduced the expression of H60 on MCA sarcoma cells, suggesting that IFN-α treatment may reduce the NK sensitivity of cancer cells. We and others have previously demonstrated that the systemic administration of α-GalCer can lead to antitumor effects against metastatic liver tumors through the efficient activation of liver NK cells.[8,16] Although α-GalCer has not yet been officially accepted for clinical application in cancer treatment, these previous results encouraged us to evaluate the antitumor effect of the combination of α-GalCer and 5-FU against MC38 liver tumors. In most reports, high dose (2 μg/mouse) α-GalCer was applied for the treatment of liver tumors. However, administration of these high dose resulted in liver injury.[9,17,18] In the present study, we used low dose (0.4 μg/mouse) α-GalCer in the combination therapy. The administration of low dose α-GalCer is enough to activate liver NK cells and did not affect

the expression of NK activating molecules on MC38 cells. Importantly, the administration of this low dose α-GalCer did not cause liver injury. The antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 and Colon26 liver tumors was stronger than that of 5-FU alone or α-GalCer alone. The antitumor effect of the combination therapy of low dose (0.4 μg/mouse) α-GalCer and 5-FU was equal to that of the combination therapy of high dose (2 μg/mouse) α-GalCer and 5-FU (Aketa *et al.*, unpublished data). Our results might offer new chemo-immunotherapy strategies, especially for those patients with advanced stages of cancer.

In this study, we demonstrated that 5-FU treatment enhanced the expression of both NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells. In contrast, 5-FU treatment did not affect the activating molecules on NK cells. Both pathways involving NKG2D and DNAM1 play critical roles in the activation of NK cells and have been implicated in tumor surveillance.[19] The expression of NKG2D ligands has been associated with a good prognosis in patients with colon cancer.[20] Thus, these results suggest that the upregulation of NKG2D ligand expression might improve the prognosis of patients with colon cancer. Gasser *et al.*[21] previously reported that DNA-damaging agents and DNA-synthesis inhibitors including 5-FU could induce the expression of NKG2D ligands on tumor cells. We also demonstrated that 5-FU treatment could induce the expression of NK activating molecules in MC38 liver tumor tissues but not in nontumor tissues, which was consistent with the *in vitro* results. Our present results suggest that 5-FU treatment might have strong immune-editing potential to enhance the NK sensitivity of colon cancer cells by regulating DNAM1 and NKG2D ligands.

In this study, we demonstrated that 5-FU treatment enhanced the susceptibility of MC38 cells to the cytolytic activity of liver MNCs *via* the NKG2D-NKG2D ligand pathway. Because the blocking antibody of the DNAM1-DNAM1 ligand is not commercially available, we could not evaluate the involvement of this pathway. We have previously demonstrated that membrane-bound MICA, an activating molecule of NK cells, on HCC cells is essential in the NK sensitivity of HCC cells.[12,13] The addition of both epirubicin and sorafenib enhanced the NK sensitivity of HCC cells by increasing the membrane-bound MICA.[12,13] This finding is consistent with this study of a colon cancer model. Interestingly, the expression of death receptors, such as FAS and TRAIL receptors, on MC38 cells was significantly increased by 5-FU treatment (Aketa *et al.*, unpublished data). This result may also explain the enhancement of the susceptibility of MC38 cells to the cytolytic activity of liver MNCs. We previously demonstrated that α-GalCer administration resulted in rapid and strong activation of liver NK cells and that the cytolytic activity of liver MNCs early after α-GalCer administration mainly depended primarily on liver NK cells and not on NKT or T cells.[8,10] Taken together, these results suggest that the addition of 5-FU enhanced the NK sensitivity of MC38 cells by increasing the

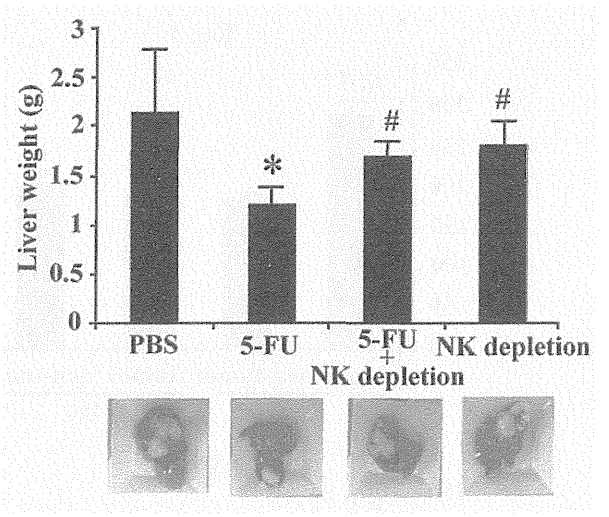

Figure 6. The antitumor effect of 5-FU against MC38 liver tumors in NK-depleted mice. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-ASGM1 Ab. NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine the intrahepatic tumor growth. $*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* 5-FU group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of Rae-1 or H60 on MC38 cells. Therefore, 5-FU treatment might be expected to enhance the susceptibility of MC38 cells to the cytolytic activity of NK cells by modifying the expression of NKG2D and DNAM1 ligands.

NK depletion decreased the antitumor effect of 5-FU against MC38 liver tumors, demonstrating that the antitumor effect of 5-FU depends on NK activity in addition to direct cytotoxicity. We also examined the antitumor effect of 5-FU against the Colon26 liver tumor model, derived from BALB/c colon cancer. The liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. The depletion of NK cells also significantly inhibited the antitumor efficacy of 5-FU against Colon26 liver tumors in BALB/c mice. A significant upregulation of Rae-1, H60, CD112, and CD155 could also be observed in 5-FU-treated Colon26 cells derived from BALB/c mice (Aketa *et al.*, unpublished data). These results were consistent with the results of C57BL/6 mice and suggest that the antitumor effect of 5-FU may always depend on NK activity in the liver. The liver contains abundant NK cells. In cancer tissues that are rich in NK cells, the combination therapy of α-GalCer and 5-FU might have a potential as a new chemo-immunotherapeutic strategy.

The liver is the most common site of metastasis of gastrointestinal cancers (*i.e.*, colorectal, gastric, and pancreatic cancers). Thus, new therapeutic approaches of cancer immunotherapy for metastatic liver cancer need to be developed. We have shown here that 5-FU can enhance the NK sensitivity of cancer cells by inducing the expression of NK activating molecules in

addition to the direct cytotoxicity of 5-FU to the cancer cells. In addition, the combination therapy of α-GalCer and 5-FU showed sufficient antitumor effects against MC38 liver tumors. These findings indicate that this new combination chemo-immunotherapy might represent a particularly promising approach for patients with metastatic liver cancer.

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