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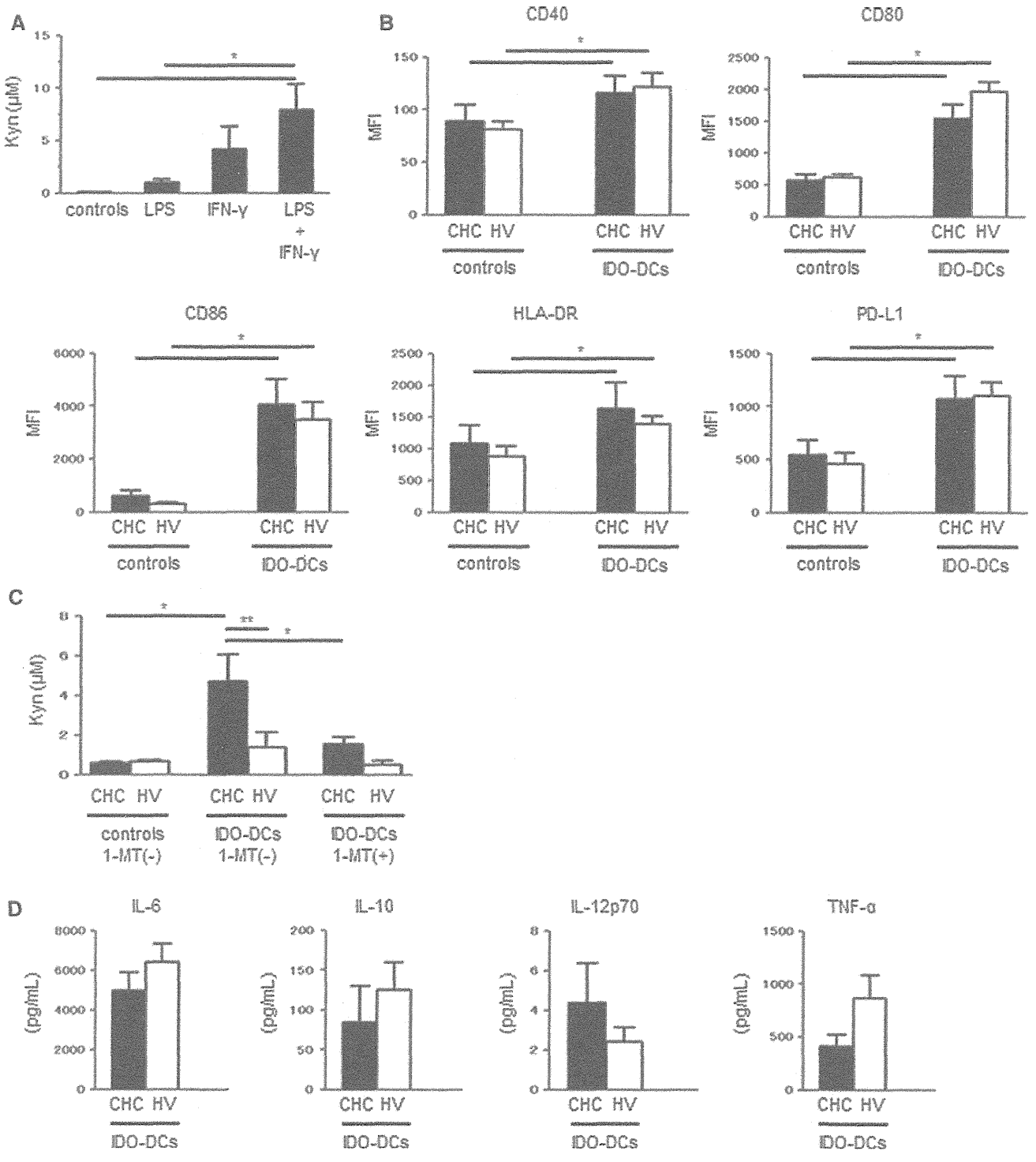

group, the frequency of Tregs after the co-culture was significantly higher than that with IDO-DCs from the HV group (Fig. 4a). Such Treg frequency from the culture of the CHC group was significantly reduced in the presence of 1-MT (Fig. 4a). These results show that functional IDO in DCs is partially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

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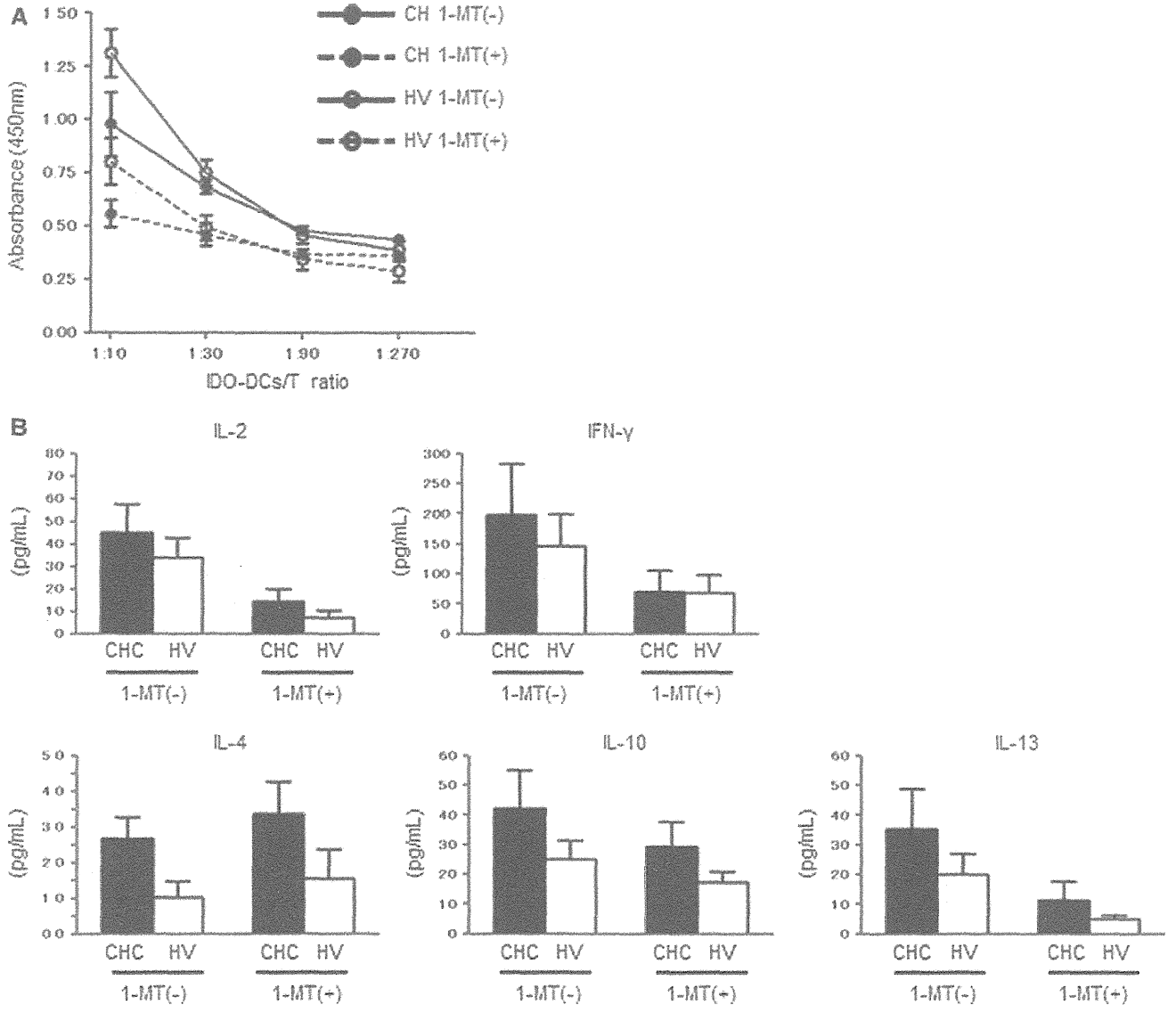

Fig. 3 IDO is not involved in lower allogeneic T-cell response and Th1/Th2 differentiation with DCs from chronic hepatitis C patients. **a** Allogeneic mixed lymphocyte reaction (MLR) with IDO-DCs was performed as described in "Subjects, materials, and methods". *Closed circles* are the 450-nm absorbance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

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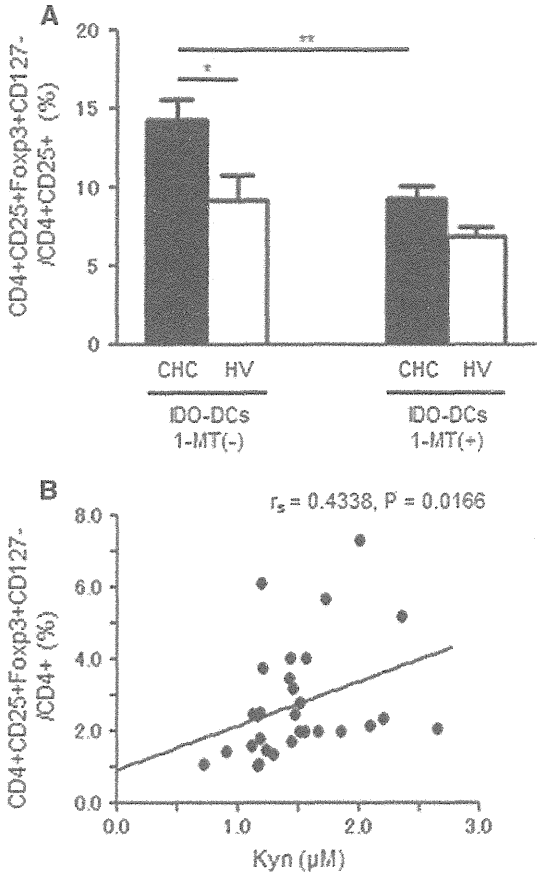

Fig. 4 IDO is involved in the induction of regulatory T cells. **a** After IDO-DCs were generated from the CHC or HV group, naive CD4+ T cells were co-cultured for 7 days with IDO-DCs in the presence or absence of 1-MT. The cultured T cells were stained with relevant antibodies (*Abs*) and analyzed with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

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

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

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

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

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

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

References

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

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

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

Tokuhiro Matsubara[1], Tatsuya Kanto[1,*], Shoko Kuroda[1], Sachiyo Yoshio[1], Koyo Higashitani[1], Naruyasu Kakita[1], Masanori Miyazaki[1], Mitsuru Sakakibara[2], Naoki Hiramatsu[1], Akinori Kasahara[1], Yoshito Tomimaru[4], Akira Tomokuni[4], Hiroaki Nagano[4], Norio Hayashi[3] and Tetsuo Takehara[1]

[1] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

[2] Department of Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari, Osaka, Japan

[3] Kansai Rosai Hospital, 3-1-69 Amagasaki, Hyogo, Japan

[4] Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

*Corresponding author: Tatsuya Kanto, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, 565-0871 Japan

E-mail: kantot@gh.med.osaka-u.ac.jp

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Telephone: +81-6-6879-3621, Fax: +81-6-6879-3629

Keywords

HCV, hepatitis C virus; MVD, microvessel density; AFP, α-fetoprotein; ROC, receiver operating characteristic; PIVKA-II, protein induced by vitamin K absence or antagonist II

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Abbreviations List

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

soluble vascular endothelial growth factor receptor-1; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, Tumor, lymph Node and Metastasis; CT, computed tomography; MRI, magnetic resonance imaging; BCLC, Barcelona-Clinic Liver Cancer.

Abstract

Angiogenesis is deemed to be a critical step in the development and progression of vascular-rich hepatocellular carcinoma (HCC). In this process, myeloid lineage cells, such as macrophages and monocytes, have been reported to act as vascular progenitor cells. TIE-2, a receptor of angiopoietins, conveys pro-angiogenic signals. We thus aimed to clarify the roles of TIE2-expressing monocytes (TEMs) in the clinical management of HCC patients. This study enrolled 168 HCV-infected patients including 89 with HCC and examined the frequency of TEMs, as defined as $CD14^+CD16^+TIE2^+$ cells, in the periphery and in the liver. The localization of TEMs in the liver was determined by immunofluorescence. Micro-vessel formation in the liver was quantified by counting $CD34^+$ cells. In HCC patients, the frequency of TEMs in the periphery was significantly higher than those in non-HCC groups, and also was higher in the liver than in the periphery. In patients who underwent local ablation or resection of HCC, the frequency of TEMs dynamically changed in parallel with the recurrence of HCC. Most TEMs were identified in the perivascular area of cancer tissues. A significant positive correlation was observed between micro-vessel density in HCC tissues and the peripheral or intra-tumor frequencies of TEMs, suggesting that TEMs are involved in angiogenesis in the liver. Receiver operating characteristic analyses revealed the superiority of TEM frequency to AFP, PIVKA-II and ANG-2 levels as a diagnostic for HCC. ***Conclusion:*** TEMs are increased in

patients with HCC and change in parallel with the therapeutic response or recurrence. The frequency of TEMs can be used as a diagnostic marker for HCC, potentially reflecting angiogenesis in the liver.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and the third leading cause of cancer-related deaths worldwide (1). Clinically, HCC frequently develops from liver cirrhosis, with most etiologies involving hepatitis B and C virus (HBV and HCV) infection (2, 3). Since the majority of HCC is characterized by a vascular-rich nature, vascular formation (i.e., angiogenesis) is deemed to be a critical step in the development and progression of HCC (4). Some clinical studies have demonstrated that the degree of vascularity in HCC tissues correlates well with the severity of the disease conditions (5), suggesting that the prevention of this process could have some beneficial impact on patient prognosis. However, the precise mechanisms of HCC-related angiogenesis in the liver remain obscure.

In general, two types of components are cooperatively involved in the progression of angiogenesis: humoral angiogenesis factors and vascular progenitor cells (4). Many studies have reported that angiogenesis factors produced from HCC drive vascularization that supports the development and progression of liver cancer, including invasion and metastasis (6). Among such factors, serum levels of angiopoietin-2 (ANG-2), macrophage migration inhibitory factor (MIF), vascular endothelial cell growth factor (VEGF) and soluble vascular endothelial cell growth factor receptor-1 (sVEGFR-1) have been reported to be higher in HCC patients than in those without HCC,

being strongly correlated with poorer prognosis or survival (7-11). As for the diagnostic value for HCC, such angiogenesis molecules often fail to show any advantage over other clinically available markers (12).

To support the growth of cancer cells, vascular or endothelial progenitor cells are considered to accumulate in the vicinity of cancer cells. Progenitors, such as hematopoietic stem cells or myeloid lineage cells, are reported to be involved (4). Tyrosine kinase with Ig and EGF homology domains 2 (TIE2) is a receptor of angiopoietins (ANGs); it is primarily expressed on endothelial cells and is capable of binding with all the known ANGs (ANG-1, ANG-2 and ANG-3/ANG-4). The TIE2-expressing monocytes (TEMs) are a novel subpopulation of peripheral and tumor-infiltrating myeloid cells presumed to be equipped with profound pro-angiogenic activity, which is found in both humans and mice (13-15). In the clinical setting of human cancers, TEMs are reported to be found in tumors of the kidney, colon, pancreas and lung, as well as in soft tissue sarcoma (15), where angiogenesis is known to be important for tumor progression. However, it is uncertain whether TEMs are increased or not in HCC patients and what their clinical impact is on the pathophysiology of the disease.

In this study, we aimed at clarifying the roles of TEMs in the clinical management of HCC patients by investigating their frequency, localization and correlation with clinical parameters and microvessel formation in the liver. Our findings indicate that TEMs could serve as a diagnostic

marker of HCC potentially reflecting angiogenesis in the liver.

Materials and Methods

Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 168 patients were enrolled (Table 1) according to the inclusion and the exclusion criteria (Supplementary table 1). They were categorized into three groups according to the stage of the liver disease: chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The clinical stage of HCC was determined according to the TNM classification system of the International Union against Cancer (7^{th} edition) or the BCLC staging classification system. The protocol of this study was approved by the ethical committee of Osaka University Hospital and Osaka University Graduate School of Medicine. At enrollment, written informed consent was obtained from all patients and volunteers. Some of the HCC patients in this study received radiofrequency ablation (RFA) therapy based on the therapeutic guidelines for HCC promoted by the Japan Society of Hepatology (16). After the RFA sessions, the efficacy of tumor ablation or HCC recurrence was evaluated by computed tomography (CT) or magnetic resonance imaging (MRI) scanning. With some of the HCC patients who underwent surgical resection, cancerous and adjacent non-cancerous tissues were obtained at operation for further analyses of TEMs. As controls, we examined healthy subjects (HS) without history of liver disease, HCC patients with HBV infection

(HBV-HCC group) and those without HBV or HCV (non-B, non-C [NBNC]-HCC group). The clinical backgrounds of the subjects are shown in Table 1.

Reagents

The fluorescence-labeled mouse or rat monoclonal antibodies against relevant molecules used in this study were: CD14 (M5E2), CXCR4 (12G5), CD40 (5C3), CD16 (3G8), CD34 (563), CD11b (ICRF44), CD49d (9F10), CD80 (L307.4), CD86 (2331), CD33 (WM53), CCR4 (1G1), HLA-DR (L243) and CCR5 (2D7/CCR5), which were purchased from Becton Dickinson (BD) Biosciences, San Jose, CA. Anti-human VEGFR2 (89106) or TIE2 (83715) Abs were purchased from R&D SYSTEMS, Minneapolis, MN; anti-human CD45 (HI30) from BioLegend, San Diego, CA; anti-human CX3CR1 (2A9-1) was from Medical & Biological Laboratories (MBL), Nagoya, Japan, and anti-AC133 (AC133) was from Militenyi Biotec.

Phenotype and frequency analysis of peripheral and tumor-infiltrating TEMs

After peripheral blood mononuclear cells (PBMC) had been separated from heparinized venous blood by Ficoll-Hypaque (Nacalai tesque, Kyoto, Japan) density gradient centrifugation, they were stained with a combination of fluorescence-labeled anti-human mouse mAbs against CD14, CD16 and TIE2. For the analyses of liver-infiltrated cells, fresh liver specimens were washed twice with

phosphate-buffered saline (PBS) and then diced into 5-mm pieces. After these pieces had been passed through a nylon mesh (BD Falcon, San Jose, CA), tumor-infiltrating and non-cancerous tissue-infiltrating leukocytes were isolated by density gradient centrifugation as described above. These cells were stained with fluorescence-labeled Abs (CD14, CD16 and TIE2) as done for PBMC. The stained cells were analyzed using FACS CantoII (BD) and FCS Express software (De Novo, Los Angeles, CA, USA).

Western-blot analysis

$CD16^+$ and $CD16^-$ monocytes were sorted using a FACS sorter. The sorted cells (10^5-5×10^5) were subjected to Western blot analysis for TIE2 expression as described elsewhere (15).

Immunofluorescence analysis

Tissue specimens were obtained from surgical resections of HCC from the patients. Five-micrometer sections were fixed in 4% paraformaldehyde (PFA) for 15 minutes and immunostained. Briefly, the sections were incubated with the following antibodies by detection with a polymeric labeling 2-step method as described (15): rabbit anti-human CD14 antibody (clone, HPA001887; Sigma), mouse anti-human CD16 (2H7; MBL) and mouse anti-human TIE2 (AB33; Upstate Biotechnology) antibodies and subsequently with secondary goat anti-rabbit Alexa

Fluor®488 or goat anti-mouse Alexa Fluor®594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-G™ (SouthernBiotech, Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Immunohistochemical analysis and assessment of microvessel formation (Microvessel density)

To evaluate microvessel density (MVD), immunohistochemical analyses were performed with anti-CD34 antibody (1/50 dilution; QB-END/10, Novo-castra, Newcastle, UK) using the avidin-biotin complex (ABC) method (Vectastain) as described (17).

Single microvessels were detected as any brown CD34-immunostained endothelial cells. MVD was evaluated according to the method described by Poon et al. (17). Sections were read by two double-blinded pathologists according to staining intensity.

Statistical analysis

Differences between two groups were assessed by the Mann-Whitney nonparametric U test, and multiple comparisons between more than two groups by the Kruskal-Wallis nonparametric test. Paired *t* tests were used to compare differences in paired samples using GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). To differentiate HCC and LC, receiver operating

characteristics (ROC) analyses were done using JMP software (SAS, Cary, NC, USA). In order to identify optimal cut-off point of TEM frequency for balancing the sensitivity and specificity, we defined the optimal point as those yielding the minimal value for $(1-\text{sensitivity})^2 + (1-\text{specificity})^2$. The correlation between two groups was assessed by Pearson's analysis. The recurrence-free survival rate in patients with HCC who underwent the treatment was compared using the Kaplan-Meier method, with the log-rank test for comparison. Associations among the variables were determined by χ^2 test of Fisher exact test and Student's *t* test. All tests were two-tailed, and a P value of less than 0.05 was considered statistically significant.

Results

TIE2 is selectively expressed on $CD14^+CD16^+$ monocytes

In order to examine which population of cells expresses TIE2, we stained PBMC obtained from HCC patients with the relevant Abs. Among them, $CD14^+$HLA-DR^+ monocytes express TIE2 (Fig. 1A), but the remaining $CD14^-$HLA-DR^- cells do not (Fig. 1A). More precisely, T cells, B cells, NK cells, NKT cells, dentritic cells did not express TIE2 (data not shown).

Monocytes are divided into two distinct subsets according to the expression of CD14 and CD16; they are $CD14^{++}CD16^-$ and $CD14^+CD16^+$, respectively (Fig. 1A). $CD14^+CD16^+$ monocytes express TIE2 to a degree higher than $CD14^{++}CD16^-$ monocytes (Fig. 1B). In Western blot, the band intensity of TIE2 in lysates from $CD14^+CD16^+$ cells was lesser than those of HUVEC as a positive control, whereas the intensity of $CD14^+CD16^+$ cells was much more than those in samples from $CD14^{++}CD16^-$ cells (Fig. 1C). Therefore, for the following sections of this paper, we define $CD14^+CD16^+TIE2^+$ cells as being TIE2-expressing monocytes (TEMs). As the control, $CD14^+CD16^-TIE2^-$ cells are TIE2-negative monocytes ($TIE2^-$ monocytes).

TEMs are phenotypically and functionally distinct from $TIE2^-$ monocytes or endothelial progenitor cells in myeloid lineage

TEMs are positive for CD45, CD11b, CCR4, CCR5, CX3CR1, CD40 and CD86, the expressions of which are greater than those for TIE2$^-$ monocytes (Fig. 1D, 1E). The expressions of CD33, HLA-DR, CD49d and CXCR4 are comparably high on monocytes regardless of CD16 or TIE2 expression. Since TEMs have been reported to be involved in the promotion of angiogenesis in some clinical settings (4, 13, 18). we next compared their phenotypes with those of endothelial progenitor cells (EPC) (19). We found that TEMs do not express AC133, VEGFR2 or CD34 (Fig. 1D). These results indicate that; TEMs are phenotypically and functionally distinct from TIE2$^-$ monocytes or EPC in myeloid lineage.

TEMs are significantly increased in the peripheral blood of HCC patients and their increase is associated with cancer occurrence and recurrence

We compared the frequency of TEMs in PBMC among healthy subjects and chronically HCV-infected patients with various stages of liver disease. With respect to the demographics of the subjects, no difference was found in the clinical and pathological characteristics among patient groups (Table 1). In HCC patients, the frequency of TEMs in the periphery was significantly higher than those in all other groups (Fig. 2A). In addition, the frequency of TEMs did not differ between patients at advanced HCC stages (TNM stages III and IV) and those at early HCC stages (stages I and II) (Fig. 2B). Similar results were obtained with the classification according to the BCLC