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Frequency of CD45RO$^+$ subset in CD4$^+$CD25^{high} regulatory T cells associated with progression of hepatocellular carcinoma

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ABSTRACT

The purpose of this study was to assess the properties of CD4$^+$CD25$^{high/low/negative}$ T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of CD45RO$^+$ cells in CD4$^+$CD25^{high} T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the CD45RO$^+$ subset in CD4$^+$CD25^{high} Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1–3].

CD4$^+$CD25^{high} Foxp3$^+$ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4–6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients with several different tumor types [3–12]. It has been reported that, in HCC patients, increased Tregs are correlated with CD8$^+$ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8,13–15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation [16,17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be

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implicated in the regulation of immune responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF-β1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of $CD4^+CD25^{high}$ $Foxp3^+$ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than $CD4^+CD25^{low/negative}$ cells. Furthermore, the proportion of $CD45RO^+$ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and healthy controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

Table 1
Clinical characteristics of hepatocellular carcinoma, liver cirrhosis, chronic hepatitis patients and healthy control.

Hepatocellular carcinoma (n = 62)	
Age (yrs)	68.9 ± 9.5
Gender (M/F)	37/25
Etiology of liver disease	
HBV/HCV/HBV + HCV/NBNC	19/34/2/7
TNM stages I/II/III/IV-A/IV-B	18/12/20/6/6
Largest tumor (mm)	
Child-Pugh A/B/C	37.6 ± 34.4
AFP (ng/mL)	41/8/3
DCP (mAU/mL)	10–35,093 (52)
Liver cirrhosis (n = 16)	
Age (yrs)	10–32,818 (34)
Gender (M/F)	58.3 ± 10.3
Etiology of liver disease	11/5
HBV/HCV/NBNC	4/7/5
Chronic hepatitis (n = 17)	
Age (yrs)	58.9 ± 10.4
Gender (M/F)	8/9
Etiology of liver disease	
HBV/HCV/NBNC	0/17/0
Healthy controls (n = 41)	
Age (yrs)	46.1 ± 19.1
Gender (M/F)	16/25

Note: Results except for AFP and DCP are expressed as means ± SD. AFP and DCP values are expressed as range (median). The reference range of normal values for the laboratory values: AFP < 10 ng/mL, DCP < 40 mAU/mL. M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

2.2. Isolation of PBMC and $CD4^+$ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma–Aldrich, St. Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. $CD4^+$ T cells were isolated from fleshly isolated PBMC by negative magnetic selection using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated $CD4^+$ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE, anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpha Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells " Plasmacytoid Subset " and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset " (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and intracellular staining

To determine the frequency of $CD4^+CD25^{high}$ T cells and the surface marker profile, $CD4^+$ T cells (at least 2×10^5 cells/tube) were stained with mAbs in the above described panel for 30 min on ice. Apropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2×10^5 $CD4^+$ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37 °C in a humidified CO_2 incubator for 4 h. The activated cells were first incubated with anti-CD4-PerCP for 15 min on ice, followed by fixation and permeabilization of the activated cells for 20 min at room temperature with BD Cytofix/Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD

Biosciences) for 15 min at room temperature. Apropriate isotype controls were included for each sample.

2.5. Flow cytometric analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6. Statistical analysis

Data are indicated as means ± SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric U test. $P < 0.05$ was considered significant.

3. Results

3.1. Frequencies of $CD4^+CD25^{high}$ T cells

To evaluate the frequencies of $CD4^+CD25^{high}$ T cell subsets that contain Tregs, MACS-sorted $CD4^+$ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and B). Although the frequencies of $CD4^+CD25^{high}$ T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that $CD4^+CD25^{high}$ T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and cytokine production of the $CD4^+CD25^{high}$ T cell subset in HCC patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22–24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was larger than that of $CD4^+CD25^{low/negative}$ subset, and it was also significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients (Fig. 2B). Thus, not only is the number of $CD4^+CD25^{high}$ T cells in HCC patients larger, but also the frequency of $Foxp3^+$ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of $CD4^+CD25^{high}$-$Foxp3^+$ T cell subset was quantitated following the stimulation with PMA/ionomycin using the specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the $CD4^+CD25^{high}$ subsets. In addition, the levels of IL-4 and IL-10 were high in the $CD4^+CD25^{high}Foxp3^+$ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the $CD4^+CD25^{high}Foxp3^+$ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.3. Phenotypes of the $CD4^+CD25^{high}$ T cell subset in HCC patients

To determine the phenotypical properties of $CD4^+CD25^{high}$ T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RA, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of $CD45RO^+$, HLA-DR^+ and $GITR^+$ cells were higher in the $CD4^+CD25^{high}$ T cell subset in all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$) (Fig. 3A and B). The percentage of $CD45RO^+$ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the $CD4^+CD25^{high}$ T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

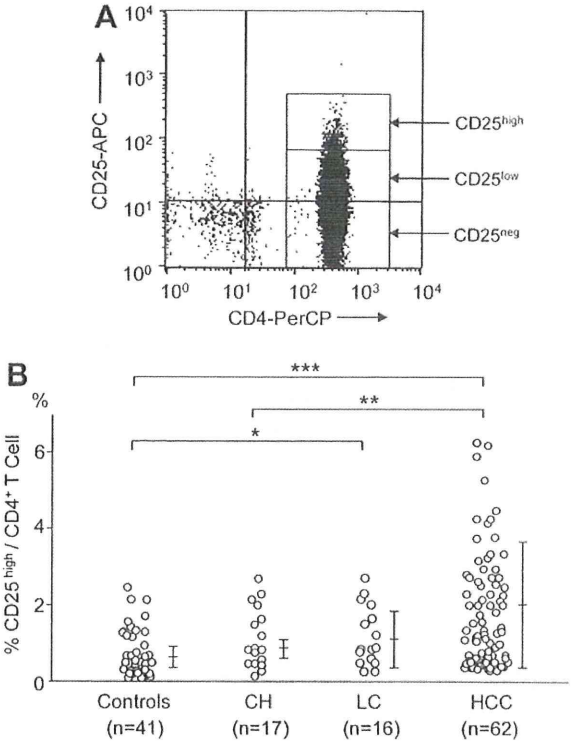

Fig. 1. Frequencies of $CD4^+CD25^{high}$ T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of $CD4^+CD25^{high}$ T cells in the peripheral blood of HCC ($n = 62$), LC ($n = 16$), CH ($n = 17$) patient, and healthy controls ($n = 41$). Percentages for individual patient analyzed are shown. The percentages represent the proportions of $CD4^+CD25^{high}$ T cells in total $CD4^+$ cells. The prevalence of $CD4^+CD25^{high}$ T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

3.4. $CD4^+CD25^{high}$ T Cell subset and dendritic cells of HCC patients

Several reports have suggested that the $CD4^+CD25^{high}$ T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells "Plasmacytoid Subset" and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset". HCC patients were divided into two groups according to the frequencies of $CD45RO^{positive}$ cells in $CD4^+CD25^{high}$ T cell subsets ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained >83.8% positive cells in $CD4^+CD25^{high}$ T cells. The frequencies of $CD123^+$ plasmacytoid DCs were significantly higher in $CD45RO^{++}$, group ($P < 0.05$) (Fig. 5A and B), although those of $CD33^+$ myeloid DCs were not correlated with the subsets in $CD4^+CD25^{high}$ cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of $CD4^+CD25^{high}$ T cells.

3.5. $CD4^+CD25^{high}$ T cell subset and tumor progression

To evaluate the association between $CD4^+CD25^{high}$ T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child-Pugh scores

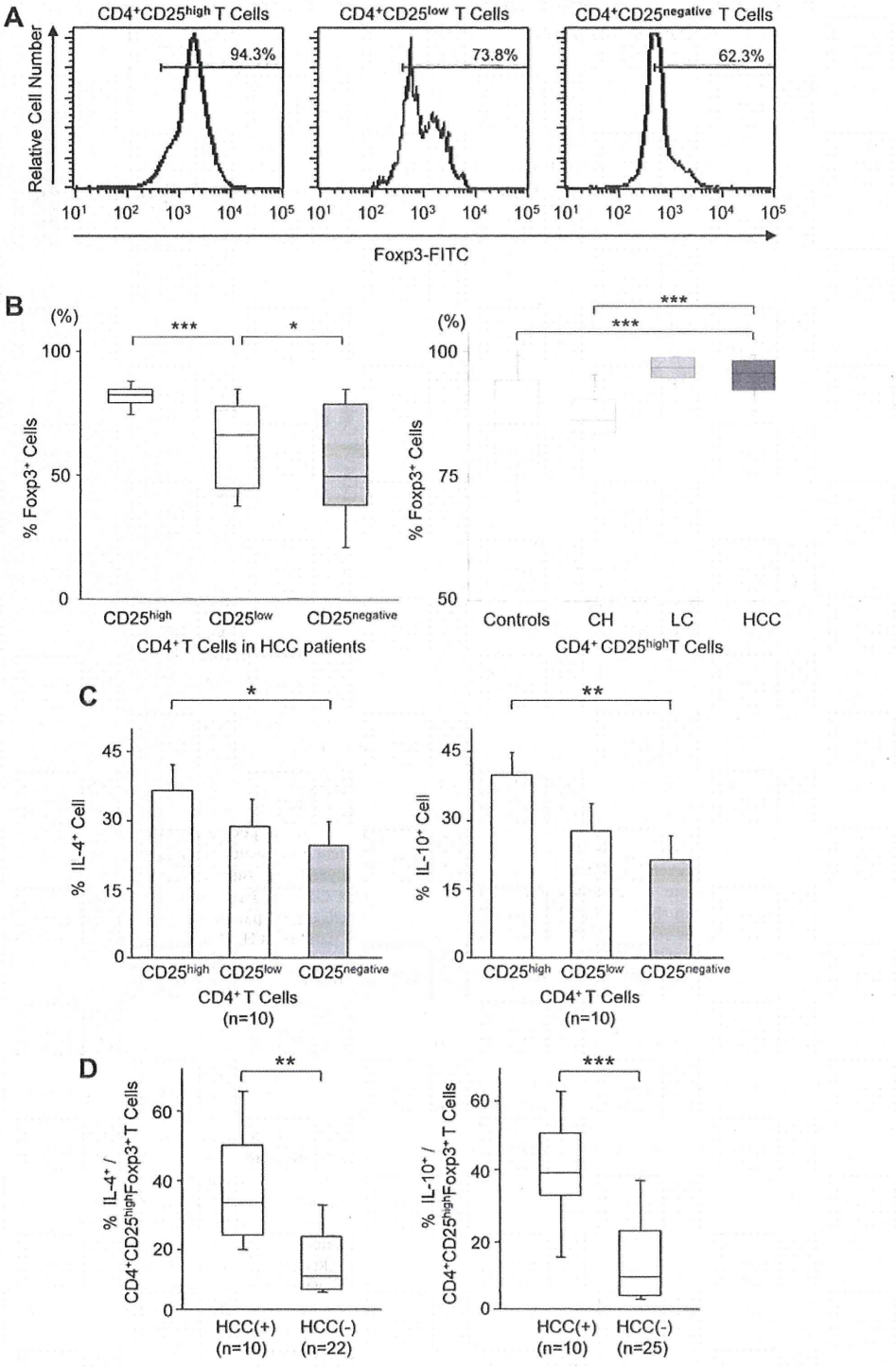

Fig. 2. Analysis of intracellular Foxp3 expression and cytokine production in $CD4^+$ $CD25^{high/low/negative}$ T cell subsets in HCC patients. (A) Representative expression of Foxp3 in $CD4^+$ T cells from an individual subject. Intracellular Foxp3 was stained following membrane permeabilization. Intracellular Foxp3 was detected by the specific mAb. (B) Statistical analysis in the left side panel shows that the percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{low/negative}$ T cell subsets, and in the right side panel shows that that of $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients. (C) Statistical analysis shows that the levels of Th2 cytokines IL-4 and IL-10 were remarkably high in the $CD4^+CD25^{high}$ T cell subset. (D) Comparison of intracellular cytokine production in $CD4^+CD25^{high}$ T cell subsets between patients with and without HCC. Healthy controls, patients with chronic hepatitis and liver cirrhosis were included in the HCC (–) column. IL-4 and IL-10 levels were higher in the $CD4^+CD25^{high}$ T cell subset in HCC patients. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

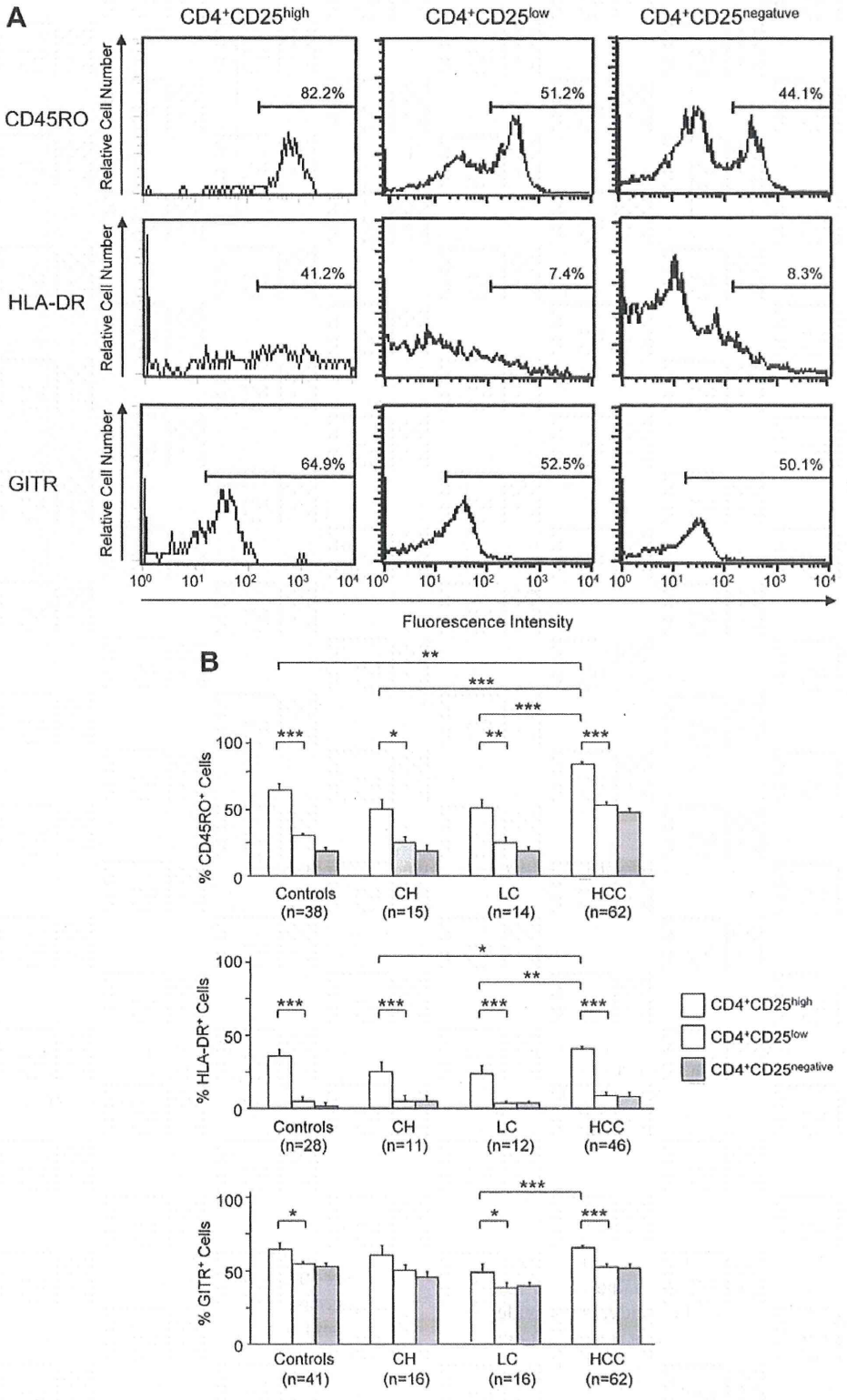

Fig. 3. Phenotypic analysis of $CD4^+CD25^{high/low/negative}$ T cell subsets in HCC patients. Fleshly isolated $CD4^+$ T cells (at least 2×10^5 cells/tube) from HCC patients were labeled with anti-CD4, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR7, anti-CTLA-4, anti-HLA-DR and anti-GITR mAbs. (A) Representative CD45RO, HLA-DR, and GITR expression profiles in $CD4^+$ T cell subsets that differ in CD25 expression. (B) Statistical analysis shows that the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ were elevated in the $CD4^+CD25^{high}$ T cell subsets of all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$). The percentage of $CD45RO^+$ cells in HCC patients was elevated compared to the patients with advanced liver diseases and healthy controls. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

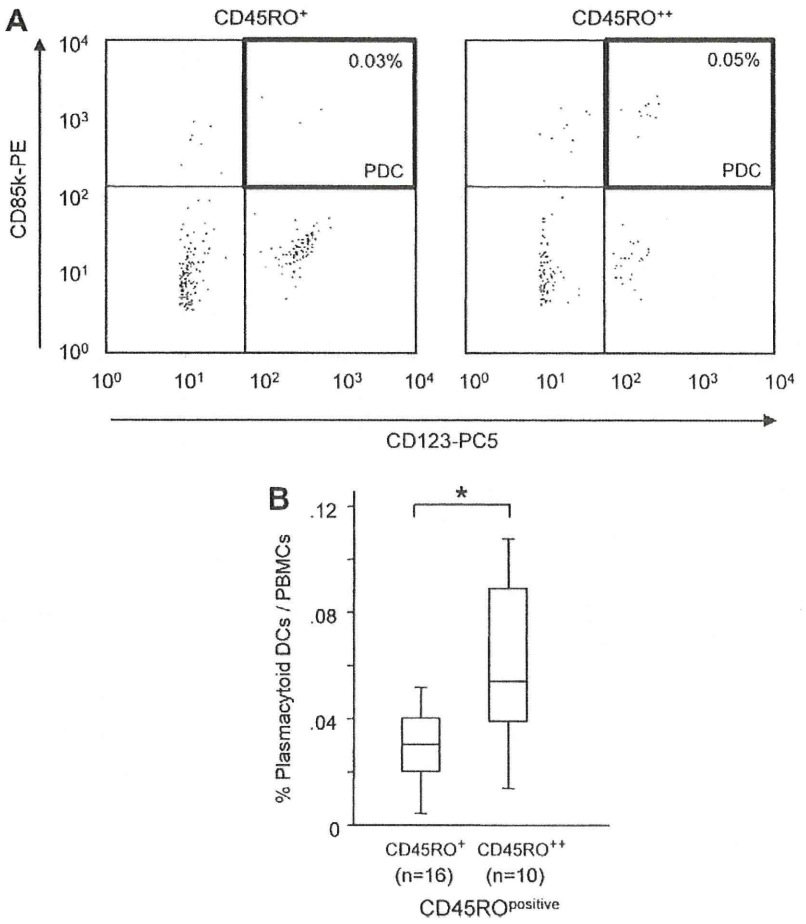

Fig. 4. Frequencies of plasmacytoid DCs in peripheral blood of HCC patients. Whole blood cells were analyzed by flow cytometry following staining with a combination of the mAbs. HCC patients were divided into two groups according to the frequencies of $CD45RO^{positivee}$ cells in $CD4^+CD25^{high}$ T cell subset ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained > 83.8% positive cells in $CD4^+CD25^{high}$ T cells. (A) Representative dot plots of plasmacytoid DCs. Plasmacytoid DCs of $CD45RO^+$ group are shown in the left panel and $CD45RO^{++}$ group in the right panel. (B) Statistical analysis shows that the frequencies of plasmacytoid DCs were significantly higher in $CD45RO^{++}$ group. *Indicates $P < 0.05$.

and fibrosis stages between two groups as described above. The levels of serum AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ group (Fig. 4). Others were not significantly different between two groups. These results imply that a subset of Tregs may contribute to the progression of liver tumors.

4. Discussion

$CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells have been shown to increase in patients with malignancies to suppress the immune responses. In this study, we provide evidence that patients with HCC have increased frequencies of $CD4^+CD25^{high}$ T cells in their peripheral blood compared to healthy controls and chronic hepatitis patients. A large proportion of $CD4^+CD25^{high}$ T cells expressed Foxp3 and produced Th2 cytokines. We also showed that $CD4^+CD25^{high}$ T cells expressed high levels of CD45RO, HLA-DR and GITR, and, interestingly, the T cell frequencies expressing these surface molecules were associated with plasmacytoid DC numbers and maximum tumor diameters in HCC patients.

There are several reports of elevated numbers of Treg cells in the peripheral blood and tumor tissues of patients with different types of cancer [3–12]. The study of Unitt et al. provided the first report of increased $CD4^+CD25^+$ T cell frequency within tumor tissue compared to non-tumor tissue in HCC patients [13]. Ormandy et al. showed that the frequency of $CD4^+CD25^{high}$ T cells in peripheral blood of patients with HCC was significantly higher (3.92 ± 3.3%) than in healthy donors (1.17 ± 0.87%) and liver cirrhosis patients (0.78 ± 0.43%) [3]. Our data revealed that a minimal increase in $CD4^+CD25^{high}$ T cells was detected in LC patients and more pronounced changes were found in HCC patients.

We showed that higher percentages of $CD4^+CD25^{high}$ T cells produced Th2 cytokines IL-4 and IL-10 in HCC patients. Tregs were recently observed to produce IL-10 [25–27], which can be a major mediator of immune suppression [28–30]. Voo et al. reported that Tregs in the peripheral blood of healthy donors secreted IL-10 but not IL-2, IFN-γ, or IL-4 [31]. Schmitz-Winnenthal et al. demon-

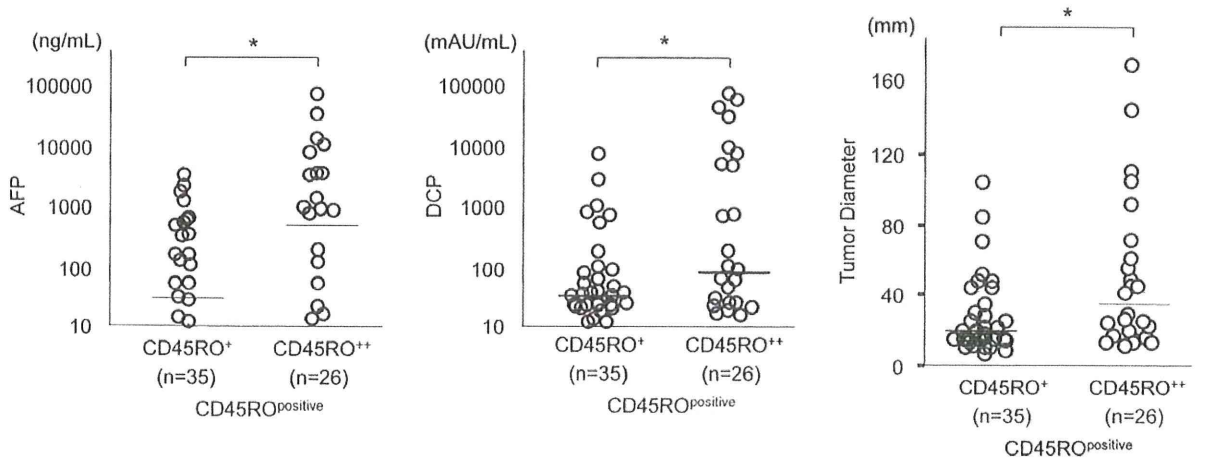

Fig. 5. Prevalence of $CD4^+CD25^{high/low/negative}$ T cell subsets and tumor progression. The levels of AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ groups. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyl prothrombin. *Indicates $P < 0.05$.

strated the presence of Treg secreting IL-10 but not IL-4 or IFN-γ upon antigen recognition in chronic pancreatitis patients [32]. The present data demonstrated that larger numbers of Tregs produced not only IL-10 but also IL-4 in HCC patients, which may contribute to the strong immunosuppressive properties of the T cells in liver malignancies.

It appears that Tregs consists of heterogenous populations within $CD4^+$ T cells, and that a subset of $CD4^+CD25^{high}$ T cells could be subdivided into different functional subsets based on the expression of various surface molecules [6]. The proportions of Tregs expressing these molecules are reported to be different in the various forms of cancer. The prevalence of $CD45RO^+$ and $GITR^+$ Treg cells is higher in $CD4^+CD25^{high}$ T cells than in $CD4^+CD25^{low/negative}$ T cells in renal cell carcinoma [4]. In head and neck squamous cell carcinoma, however, $CD4^+CD25^{high}$ T cells express CTLA-4, Foxp3, and CD62L but little GITR, and $CD25^{low/negative}$ T cells express intermediate to high levels of GITR and HLA-DR [8]. Our study showed that Tregs in HCC patients expressed significantly higher levels of CD45RO, HLA-DR and GITR compared to $CD4^+CD25^{low/negative}$ cells, suggesting that the activated populations of Tregs may contribute to the establishment of immunosuppressive microenvironments.

Little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated numbers of Treg cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells [20,33]. In peripheral blood, there are two distinct populations of DCs which can be distinguished based on phenotypical and morphological characteristics; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18,34]. Our data demonstrated that higher frequencies of $CD45RO^+CD4^+CD25^{high}$ T cells were associated with higher frequencies of pDCs in the peripheral blood of HCC patients. When the tumor antigens are assumed by pDCs through Toll-like receptor 9 (TLR9) via receptor-mediated endocytosis, secretions of pro-inflammatory cytokines, such as type I interferons (IFNs), would be caused. On the contrary, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape. They exhibit reduced IFN-α production upon TLR9 stimulation and can induce IL-10 producing $CD4^+$ and $CD8^+$ Treg [35,36]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the tumor and by limiting anti-tumor cytolytic activity through induction of $CD8^+$ Treg.

Concerning the association of Tregs and prognosis, it has been reported that an increased number of circulating Tregs predicts poor survival of patients with renal cell carcinoma [4], gastric and esophageal cancers [7], myelodysplastic syndrome [37] and HCC [11]. In addition, tumor-infiltrating Tregs were associated with reduced survival in ovarian cancer [12] and HCC patients [1]. In addition, we found that $CD45RO^+CD4^+CD25^{high}$ T cell subset was associated with larger tumor burdens, implying that a subset of Tregs may contribute to the promotion of tumor cell growth in the liver. However, it is also well possible that this just reflects stronger activation caused by a larger amount of antigen.

We performed the functional evaluation of Tregs derived from HCC patients by incubating with responder $CD4^+CD25^-$ T cells (Tresp). We observed that $CD45RO^+$ $CD4^+CD25^{high}$ T cells of HCC patients did not suppress the proliferation of responder T cells when co-cultured at Treg/Tresp ratios of 1:2 and 1:8 (data not shown). In contrast, Hoffmann et al. confirmed that the $CD45RA^+$ $CD4^+CD25^{high}$ T cells of healthy volunteers give rise to a homogeneous and highly suppressive Treg cell population, whereas $CD45RA\text{-}CD4^+CD25^{high}$ T cells generate cell lines with mixed phenotype and function [38]. Although the reasons of these conflicting data were not clarified in the current study, cell viability, apoptosis susceptibility, involvement of Th1 cytokines, and interaction to helper T cell subsets of Tregs obtained from HCC patients need to be evaluated in the future experiments.

This study may be helpful for a better characterization of Treg subsets in the peripheral circulation of patients with HCC, which may establish the immunosuppressive environment to promote tumor progression. Furthermore, to gain insights into changes in the Treg subsets

during the therapeutic option may lead to more effective immunotherapies against cancer and may improve prognosis.

Conflict of interest

None declared.

Acknowledgements

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Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

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BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS: Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.**

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1]

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,[2,3] and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment–induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.[4] In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome[4,5]; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

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primary hepatocytes[6] and cirrhotic rat liver[7] through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.[8] Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN-alfa–dependent antiviral responses.[9,10] Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.[4] All patients were administered Peg–IFN-alfa 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.[4] Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).[4]

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography–electrospray ionization–mass spectrometry, followed by derivatization.[11] Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g $NaHCO_3$, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22-μm filter (Millipore, Billerica, MA). Low amino acid media ($\times$1/5, $\times$1/10, $\times$1/30, and $\times$1/100 DMEM) were prepared by diluting $\times$1 DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.[12] Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti-forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μg/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488–conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN-alfa and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR	NR	Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
Patients, n	125	43		—	
Age and sex					
Age, y	57 (30–72)	56 (30–73)	.927	—	
Sex, male vs female	68 vs 57	24 vs 19	.872	—	
Liver histology					
F stage (F1–2 vs F3–4)	95 vs 30	20 vs 23	.001	6.35 (2.02–23.7)	.001
A grade (A0–1 vs A2–3)	68 vs 57	19 vs 24	.248	—	
Host gene factors					
IL-28B (TT vs TG/GG)[a]	109 vs 12	12 vs 31	<.001	19.7 (5.74–82.7)	<.001
ISGs (Mx, IFI44, IFIT1), (<3.5 vs ≥3.5)	103 vs 22	12 vs 31	<.001	5.26 (1.65–17.6)	.005
Metabolic factors					
BMI, kg/m^2	23.2 (16.3–34.7)	23.4 (19.5–40.6)	.439	—	
TG, mg/dL	98 (30–323)	116 (45–276)	.058	—	
T-Chol, mg/dL	167 (90–237)	160 (81–214)	.680	—	
LDL-Chol, mg/dL	82 (36–134)	73 (29–123)	.019	—	
HDL-Chol, mg/dL	42 (20–71)	47 (18–82)	.098	—	
FBS, mg/dL	94 (60–291)	96 (67–196)	.139	—	
Insulin, μU/mL	6.6 (0.7–23.7)	6.8 (2–23.7)	.039	—	
HOMA-IR	1.2 (0.3–11.7)	1.2 (0.4–7.2)	.697	—	
Fischer ratio	2.3 (1.5–3.3)	2.1 (1.5–2.8)	.005	8.91 (1.62–55.6)	.011
Other laboratory parameters					
AST level, IU/L	46 (18–258)	64 (21–283)	.017	—	
ALT level, IU/L	60 (16–376)	82 (18–345)	.052	—	
γ-GTP level, IU/L	36 (4–367)	75 (26–392)	<.001	—	
WBC, /mm^3	4800 (2100–11100)	4800 (2500–8200)	.551	—	
Hb level, g/dL	14 (9.3–16.6)	14.4 (11.2–17.2)	.099	—	
PLT, ×104/mm^3	15.7 (7–39.4)	15.2 (7.6–27.8)	.378	—	
Viral factors					
ISDR mutations ≤1 vs ≥2	80 vs 44	34 vs 9	.070	4.12 (1.25–15.9)	.019
HCV-RNA, KIU/mL	2300 (126–5000)	1930 (140–5000)	.725	—	
Treatment factors					
Total dose administered					
Peg-IFN, μg	3840 (960–7200)	3840 (1920–2880)	.916	—	
RBV, g	202 (134–336)	202 (36–336)	.531	—	
Achieved administration rate					
Peg-IFN, %					
≥80%	84	28	.975	—	
<80%	42	14			
RBV (%)					
≥80%	76	24	.745	—	
<80%	50	18			
Achievement of EVR	101/125 (81%)	0/43 (0%)	<.001	—	

BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; γ-GTP, gamma-glutamyl transpeptidase; Hb, hemoglobin; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; PLT, platelets; T-chol, total cholesterol; TG, triglycerides; WBC, leukocytes.

[a]IL-28B SNP at rs8099917.

instructions. The primer sequence for real-time detection PCR is given in the Supplementary Materials and Methods section. HCV RNA was detected as described previously[12] and expression was standardized to that of glyceraldehyde-3-phosphate dehydrogenase.

Reporter Assay

Construction of the interferon stimulated response element (ISRE)-luc reporter plasmid and Socs3-luc or Socs3 (FoxO binding element mutant [FBEmut])-luc reporter plasmids is described in the Supplementary Materials and Methods section.

Huh-7 cells were transfected with the ISRE-luc reporter plasmid 24 hours before IFN-alfa treatment. Cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid media. After 24 hours, luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI). For Socs3 promoter activities, Huh-7 cells were transfected with Socs3-luc or Socs3 (FBEmut)-luc reporter plasmids together with the Foxo3a expression plasmid, and luciferase activities were measured after 24 hours. Values were normalized to the luciferase activity of the co-transfected pGL4.75 Renilla luciferase-expressing plasmid (Promega).

Knockdown Experiments

Huh-7 cells were transfected with Ctrl (Stealth RNAi Negative Control Low GC Duplex #2; Invitrogen) or

targets (regulatory associated protein of mTOR [Raptor] and Foxo3a) (Supplementary Materials and Methods) small interfering RNA (siRNA) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were cultured in normal or low-amino-acid media for a further 24 hours. The knockdown effect was confirmed by Western blotting.

Chromatin Immunoprecipitation Assay

Detailed experimental procedures are described in the Supplementary Materials and Methods section.

HCV Replication Analysis

pH77S3 is an improved version of pH77S, a plasmid containing the full-length sequence of the genotype 1a H77 strain of HCV with 5 cell culture–adaptive mutations that promote its replication in Huh-7 hepatoma cells.[13] pH77S.3/Gaussia luciferase (GLuc)2A is a related construct in which the GLuc sequence, fused to the 2A autocatalytic protease of foot-and-mouth virus RNA, was inserted in-frame between p7 and NS2[14] (Supplementary Materials and Methods). A signal sequence in GLuc directs its secretion into cell culture media, allowing real-time, dynamic measurements of GLuc expression to be performed without the need for cell lysis.

A 10-μg aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA) and plated in fresh normal medium for 12 hours to recover. Cell medium was changed to ×1 DMEM without serum for 8 hours, then changed to low-amino-acid medium containing 0–8 mmol/L BCAA for a further 24 hours. Cells and culture medium were collected and used for GLuc assays, real-time detection PCR, and Western blotting. The number of viable cells was determined by a (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl] -2H-tetrazolium, inner salt) assay (Promega).

Continuously JFH-1–infecting Huh-7 cells were obtained by the infection of Huh-7 cells with JFH-1 cell culture-derived HCV at a multiplicity of infection of 0.01. Cells were maintained in normal medium by passaging every 3–4 days for approximately 6 months. About 20%–30% of the cells consistently were positive for HCV core protein (Supplementary Figure 4). Culture medium of JFH-1–infecting Huh-7 cells was changed to the low-amino-acid medium containing 0–8 mmol/L BCAA for 24 hours. Cells then were collected and used for assays.

Statistical Analysis

Results are expressed as mean ± standard deviation. Significance was tested by 1-way analysis of variance with the Bonferroni method, and differences were considered statistically significant at a P value of less than .05.

Results

Fischer's Ratio as a Predictive Factor for Treatment Response

The clinical characteristics of patients who received Peg-IFN and RBV combination therapy are shown in Table 1 and Supplementary Table 1, and explanations of these characteristics have been described previously.[4] All patients were infected with HCV genotype 1b and had a high viral load (>100 IU/mL). We compared patients with SVR + TR against those with NR, as assessed by the overall plausibility of treatment response groups using Fisher's C statistic as previously described.[4] We included data on the IL-28B polymorphism and plasma amino acid composition (aminogram).

Univariate regression analysis showed that no single amino acid was associated significantly with treatment response; however, using Fischer's ratio, the BCAA (Ile+Leu+Val)/aromatic amino acids (Phe+Tyr) ratio was associated significantly with treatment response (P = .005) (Table 1). Of the 121 patients with IL-28B major type, SVR, TR, and NR were observed in 53%, 37%, and 10%, respectively, and among 33 patients with IL-28B minor type, SVR, TR, and NR were observed in 15%, 17%, and 68%, respectively (P < .001) (data not shown). Fischer's ratio of SVR, TR, and NR was 2.35 ± 0.38, 2.30 ± 0.29, and 2.10 ± 0.31, respectively (P < .015) (data not shown).

We selected IL-28B polymorphism, hepatic ISG expression, fibrosis stage, HCV RNA, interferon sensitivity determining region mutation, and Fischer's ratio as factors for multivariate analysis. Multivariate analysis revealed that the minor type of IL-28B polymorphism (TG or GG at rs8099917) (odds ratio, 19.7; P < .001), advanced fibrosis stage of the liver (F3–4) (odds ratio, 6.35; P = .001), high hepatic ISGs (≧3.5) (odds ratio, 5.26; P = .005), low Fischer's ratio (continuous range, 1.5–3.3) (unit odds, 8.91; P = .011), and presence of ISDR mutation (≦1) (odds ratio, 4.12; P = .019) independently contributed to NR (Table 1).

The distribution of the Fischer's ratio according to fibrosis stage is shown in Supplementary Figure 1. The ratio decreased significantly in advanced fibrosis stage (F3–4) compared with early fibrosis stage (F1). No significant association between major or minor type of IL-28B polymorphism and different fibrosis stages of the liver was observed (Supplementary Figure 1*A*). In early fibrosis (F1–2) (Supplementary Figure 1*B*), 90% (80 of 89) of SVR+TR cases had the major type of IL-28B polymorphism, and 94% (16 of 17) of NR cases had the minor type. However, in the advanced fibrosis stage of the liver (F3–4) (Supplementary Figure 1*C*), 85% (23 of 27) of SVR+TR cases had the major type of IL-28B polymorphism and 50% (10 of 20) of NR cases had the minor type. Thus, in advanced fibrosis stages, factors other than the IL-28B polymorphism appear to contribute to NR. Interestingly, the Fischer's ratio was significantly lower in NR patients than SVR+TR pa-

tients in the advanced fibrosis stage of the liver. Therefore, Fischer's ratio could be an important predictor for NR that is independent of IL-28B polymorphism and histologic stage of the liver.

Fischer's Ratio and mTORC1 Signaling in CH-C Livers

Hepatic gene expression in 91 of 168 patients (Supplementary Table 1) was obtained using Affymetrix genechip analysis as described previously.[4] To examine the relationship between the plasma Fischer's ratio and mTORC1 signaling in the liver we evaluated the expression of key regulatory genes related to mTORC1 signaling. We found that expression of branched chain amino-acid transaminase 1 (BCAT1), an important catalytic enzyme of BCAA, was significantly negatively correlated with Fischer's ratio (Figure 1*A*). This indicates that the plasma Fischer's ratio is regulated in the liver as well as in peripheral muscle. Interestingly, the expression of c-myc, a positive regulator of BCAT1 (Figure 1*C*),[15] was correlated negatively with the Fischer's ratio (Figure 1*B*). The expression of PDCD4, a negative transcriptional target of ribosomal p70 S6 protein kinase (S6K), downstream of mTORC1, was correlated significantly with BCAT1 (Figure 1*D* and *E*). Thus, in CH-C livers, BCAT1 is induced with progressive liver disease and mTORC1 signaling is repressed, a process that might involve c-myc. Fischer's ratio of the plasma therefore can be seen to reflect mTORC1 signaling in the liver.

Impaired IFN Signaling in Huh-7 Cells Grown in Low-Amino-Acid Medium

Recent reports have shown the functional relevance of mTOR on IFN signaling and antiviral responses.[9,10] To evaluate IFN-alfa signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions (×1 DMEM, ×1/5 DMEM, ×1/30 DMEM, and ×1/100 DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in ×1/30 DMEM and ×1/100 DMEM (Figure 2*A*). Interestingly, the expression of the phosphorylated form of signal transducer and activator of transcription 1 (pSTAT1), an essential transducer of type 1 IFN signaling, also was decreased in these conditions (Figure 2*A*). Similarly, the expression of p-mTOR and pSTAT1 was repressed significantly in CH-C livers with a low Fischer's ratio compared with those with a high Fischer's ratio (Supplementary Figure 2, Supplementary Table 2).

To examine whether decreased pSTAT1 expression might be owing to repressed mTORC1 signaling, we knocked down the expression of Raptor, a specific subunit of mTORC1. We achieved more than 50% knockdown of Raptor by specific siRNA (Figure 2*B*). Under these conditions, the expression of p-mTOR and pSK6 were repressed, which is consistent with previous reports.[16] The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2*B*).

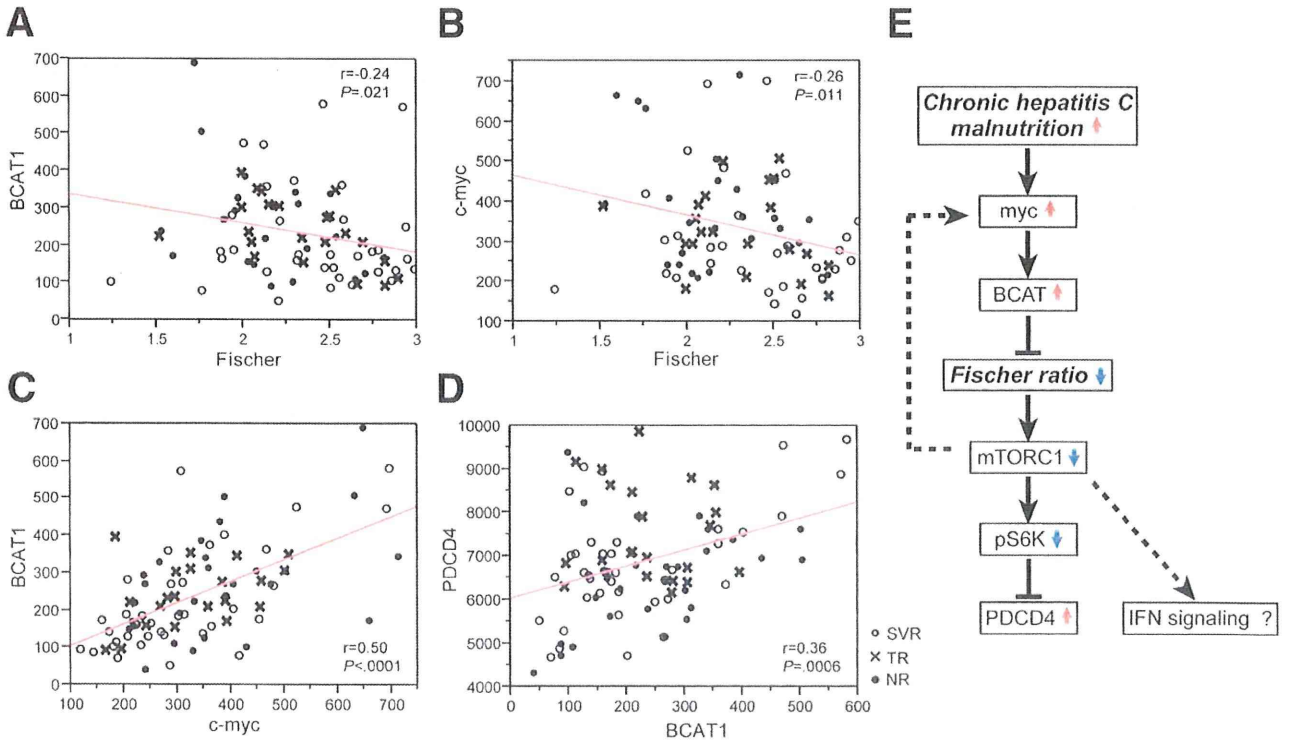

Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (*A*) BCAT1 and Fischer's ratio. (*B*) c-myc and Fischer's ratio. (*C*) BCAT1 and c-myc. (*D*) PDCD4 and BCAT1. (*E*) Putative signaling of mTORC1-related genes in CH-C.

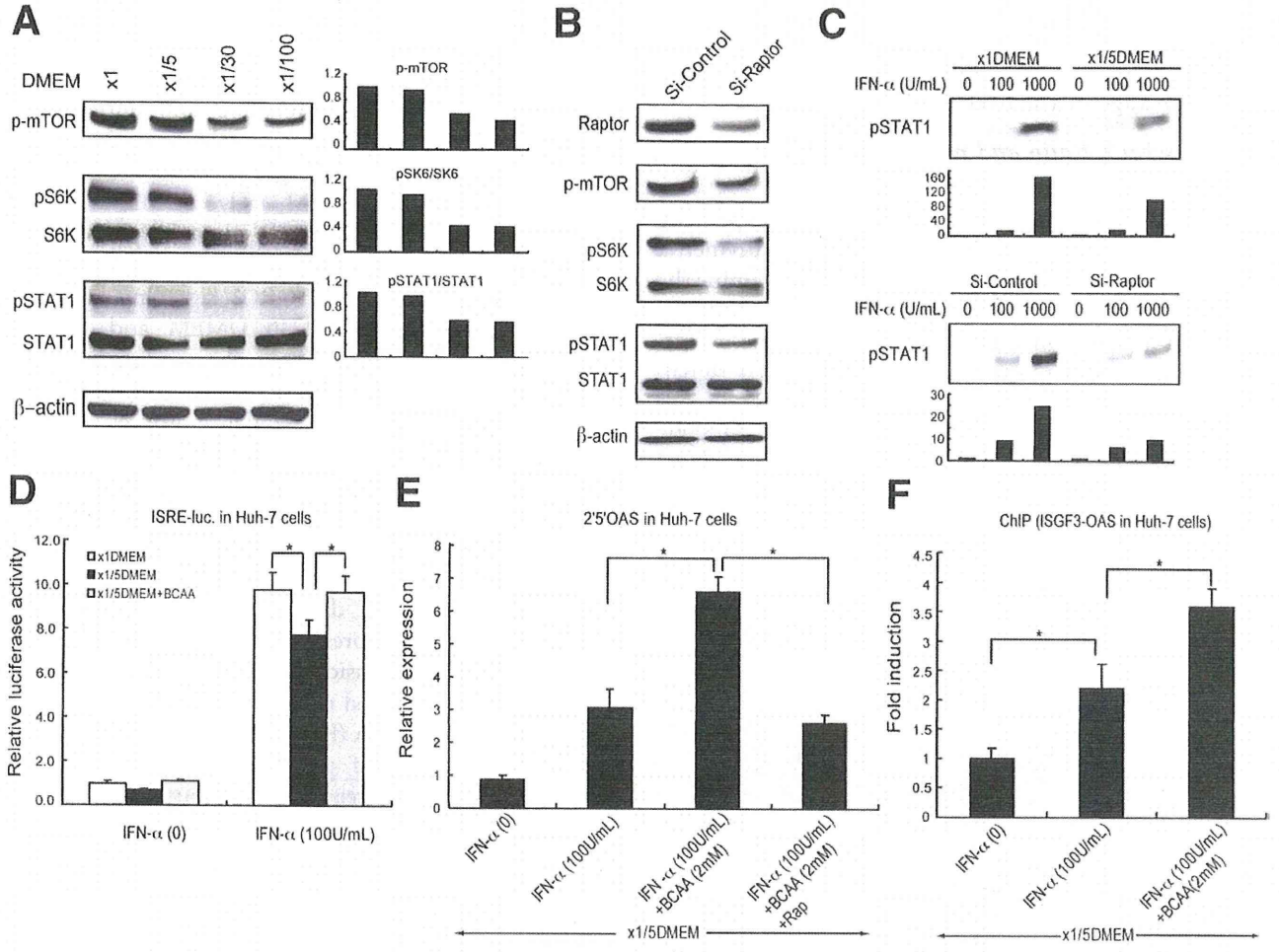

Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid medium. (*A*) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (*B*) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (*C*) IFN-alfa stimulation and pSTAT1 expression in low-amino-acid media or under Raptor knock-down conditions. (*D*) IFN-alfa stimulation and ISRE reporter activities in normal and low-amino-acid media. (*E*) IFN-alfa stimulation and 2'5'OAS expression supplemented with BCAA or rapamycin in low-amino-acid medium. (*F*) Chromatin immunoprecipitation of 2'5'OAS promoter region by ISGF3γ.

The induction of pSTAT1 by IFN-alfa (1000 U/mL) stimulation was impaired in ×1/5 DMEM or in Raptor knocked-down condition, compared with the control (Figure 2*C*). Consistent with these results, IFN-alfa–induced ISRE-dependent transcriptional activity, as measured using an ISRE-luciferase reporter assay, was impaired significantly in ×1/5 DMEM compared with ×1 DMEM (Figure 2*D*). However, this activity could be rescued by the addition of 2 mmol/L BCAA (Figure 2*D*). These results were confirmed by determining the expression of the endogenous IFN-alfa responsive gene, 2'5'OAS, using quantitative reverse-transcription PCR. Figure 2*E* shows that BCAA treatment augmented 2'5'OAS expression in low levels of amino acids, and that this could be reversed by the addition of rapamycin, an inhibitor of mTORC1 (Figure 2*E*). Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that transcriptional augmentation by BCAA was mediated by the binding of the IFN-alfa–inducible transcription factor, ISGF3γ, to the promoter region of 2'5'OAS (Figure 2*F*). These results indicate that amino acids in culture media play an essential role in IFN-alfa signaling through mTORC1 signaling, and that the addition of BCAA can overcome impaired IFN-alfa signaling in Huh-7 cells.

Induction of Socs3 in Low-Amino-Acid Medium in Huh-7 Cells

Besides being involved in mTOR signaling, Foxo transcriptional factors mediate another important branch of nutrition-sensing signaling pathway.[17] Therefore, we evaluated forkhead box O3A (Foxo3a) expression in low-amino-acid conditions in Huh-7 cells. After 6 hours culture in ×1/5, ×1/30, and ×1/100 DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in ×1/5 and ×1/30 DMEM, and the ratio of pFoxo3a to Foxo3a (pFoxo3a/Foxo3a) substantially decreased (Figure 3*A* and *B*). It has been reported that dephosphorylated Foxo3a is translocated to the nucleus before activation of its target genes.[18] In the present study, immunofluorescent staining

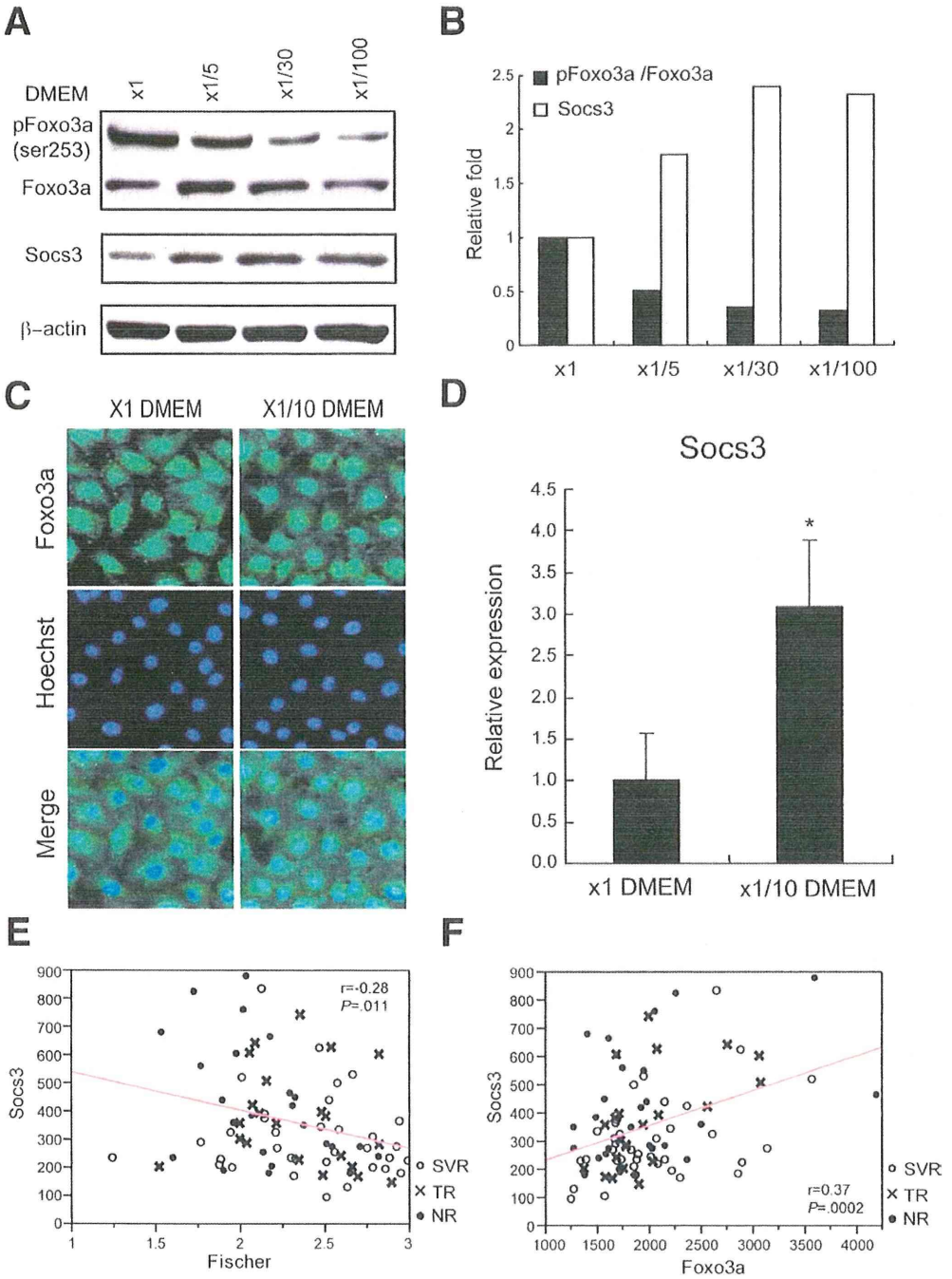

Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (*A*) Foxo3a and Socs3 expression in different amino acid media. (*B*) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (*C*) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal and low-amino-acid media. (*D*) Relative change of Socs3 messenger RNA in Huh-7 cells in normal and low-amino-acid media. (*E*) Regression analysis of Socs3 in liver and Fischer's ratio. (*F*) Regression analysis of Socs3 and Foxo3a in liver.

with an anti-Foxo3a antibody showed that Foxo3a diffused in both the cytoplasm and nucleus in normal amino acid medium, but localized in the nucleus in low-amino-acid medium (×1/10 DMEM) (Figure 3*C*).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3*A*, *B*, and *D*). The induction of Socs3 in a state of malnutrition also was confirmed in clinical samples. In CH-C livers there was a significant negative correlation between the plasma Fischer's ratio and Socs3 expression, implying that Socs3 expression increases during the malnutrition state induced by CH-C. There was also a significant correlation between Foxo3a and the transcriptional level of Socs3 in CH-C livers (Figure 3*E* and *F*), suggesting an in vitro and in vivo biological role for Foxo3a in the activation of Socs3 expression. These findings also were confirmed by Western blotting of CH-C livers (Supplementary Figure 2, Supplementary Table 2).

Socs3 Is a Transcriptional Target of Foxo3a

The significant correlation between Socs3 and Foxo3a in CH-C livers prompted us to analyze the Socs3 promoter sequence and, in doing so, we identified a putative Foxo binding element (FBE) (Figure 4*A*). To investigate the functional relevance of Foxo3a in the transcriptional regulation of Socs3, we constructed reporter plasmids containing a luciferase coding region fused to the Socs3 promoter region (Socs3-luc). Socs3-luc promoter activity was increased substantially by the overexpression of Foxo3a (Figure 4*B*). The mutations introduced in the putative FBE (FBEmut) in the Socs3 promoter significantly reduced Foxo3a-induced Socs3 promoter activation (Figure 4*B*).

Foxo3a then was knocked down by siRNA and Socs3 induction was evaluated. After suppression of Foxo3a (Supplementary Figure 3), Socs3 promoter activity was repressed significantly in low-amino-acid medium (×1/10 DMEM) (Figure 4*C*). Thus, Foxo3a appears to be indispensable for activating the Socs3 promoter under low-amino-acid conditions. Correlating with these results, ChIP assays using an anti-Foxo3a antibody showed a significant increase in the association between Foxo3a and the FBE of the Socs3 promoter in low-amino-acid conditions (×1/10 DMEM) (Figure 4*D*). Taken together, these results suggest that, besides mTORC1 signaling, the Foxo3a-mediated Socs3 signaling pathway might contribute to impaired IFN signaling in a state of malnutrition in CH-C. BCAA potentially restores this signaling (Figure 4*E*).

Effect of BCAA on HCV Replication in Huh-7 or Huh-7.5 Cells

Based on the earlier-described results, we used 2 HCV in vitro replication systems to examine whether BCAA affects HCV replication in Huh-7 or Huh-7.5 cells. The first system used a recombinant infectious genotype 1a clone, H77S.3/GLuc2A (Supplementary Materials and Methods, Supplementary Figure 4), including reporter genes, whereas the second used continuously JFH-1–infecting Huh-7 cells (Supplementary Materials and Methods).

The synthetic RNA transcribed from pH77S.3/GLuc2A was introduced into Huh-7.5 cells and replication of H77S.3/GLuc2A was evaluated in normal or low-amino-acid medium supplemented with BCAA. H77S.3/GLuc2A increased significantly by 2.6-fold in Huh-7.5 cells grown in low-amino-acid medium (×1/5 DMEM) compared with normal amino acid medium (×1 DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5*A*). In agreement with these results, the expression of Mx-1 was increased significantly by the addition of BCAA (Figure 5*B*). Similar findings were observed in JFH-1–infecting Huh-7 cells (Materials and Methods, Supplementary Figure 4). Although no obvious increase in HCV replication was observed in low-amino-acid medium (×1/5 DMEM) compared with normal amino acid medium (×1 DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5*D*). The expression of Mx-1 was increased substantially by the addition of BCAA (Figure 5*E*), suggesting that BCAA significantly repressed HCV replication in cells with either naive or persistent HCV infection. Importantly, there were no significant differences in cell viability between the conditions (Figure 5*C* and *F*).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6*A* and *B*). BCAA increased pS6K in a dose-dependent manner, implying its involvement in the activation of mTORC1 signaling. Related to this, expression of pSTAT1 was shown to be increased and the ratio of pSTAT1 to total STAT1 (pSTAT1/STAT1) increased 2.5- to 3-fold after the addition of BCAA. Thus, BCAA activated mTORC1 and the JAK-STAT signaling pathway in HCV-infected cells. In addition, the expression ratio of pFoxo3a to total Foxo3a (pFoxo3a/Foxo3a) increased 3- to 4-fold, indicating an increase in the cytoplasmic form of Foxo3a that is exposed to proteasome degradation. Concordant with these findings, we observed a decrease in the expression of Socs3. In addition, expression of the HCV core protein decreased as shown in Figure 6*A* and *B*. Thus, these results clearly show that BCAA repressed HCV replication through activation of IFN signaling and repression of Socs3-mediated IFN inhibitory signaling, as proposed in Figure 4*E*.

Discussion

Thompson et al[5] showed that the IL-28B polymorphism, HCV RNA, nationality (Caucasian/Hispanic vs African American), hepatic fibrosis stage, and fasting blood sugar level are all significant variables for achieving SVR in patients infected with genotype 1 HCV. However, the significance of variable factors for treatment response in conjunction with the IL-28B polymorphism has not been evaluated fully. In the present study, in addition to previously examined variables,[4] we included the plasma Fischer's ratio as a nutritional parameter. Multivariate analysis showed that the minor type of IL-28B polymorphism, advanced fibrosis stage, high hepatic ISGs, low Fischer's ratio, and ISDR mutation (≤1) independently contributed to NR (Table 1). Interestingly, among patients of similar fibrosis stage (F3–4), the Fischer's ratio was significantly lower in NR than SVR+TR cases. Therefore, the plasma value of Fischer's ratio was associated with the treatment response that was independent of the IL-28B polymorphism and histologic stage of the liver, although patients with advanced hepatic fibrosis are likely to be nutritionally affected.

As a nutrient sensor signaling pathway, the protein kinase mTOR plays an essential role in maintaining homeostasis and regulates protein synthesis in response to nutrient conditions. mTOR is the catalytic subunit of 2 distinct complexes, mTORC1 and mTORC2. In addition

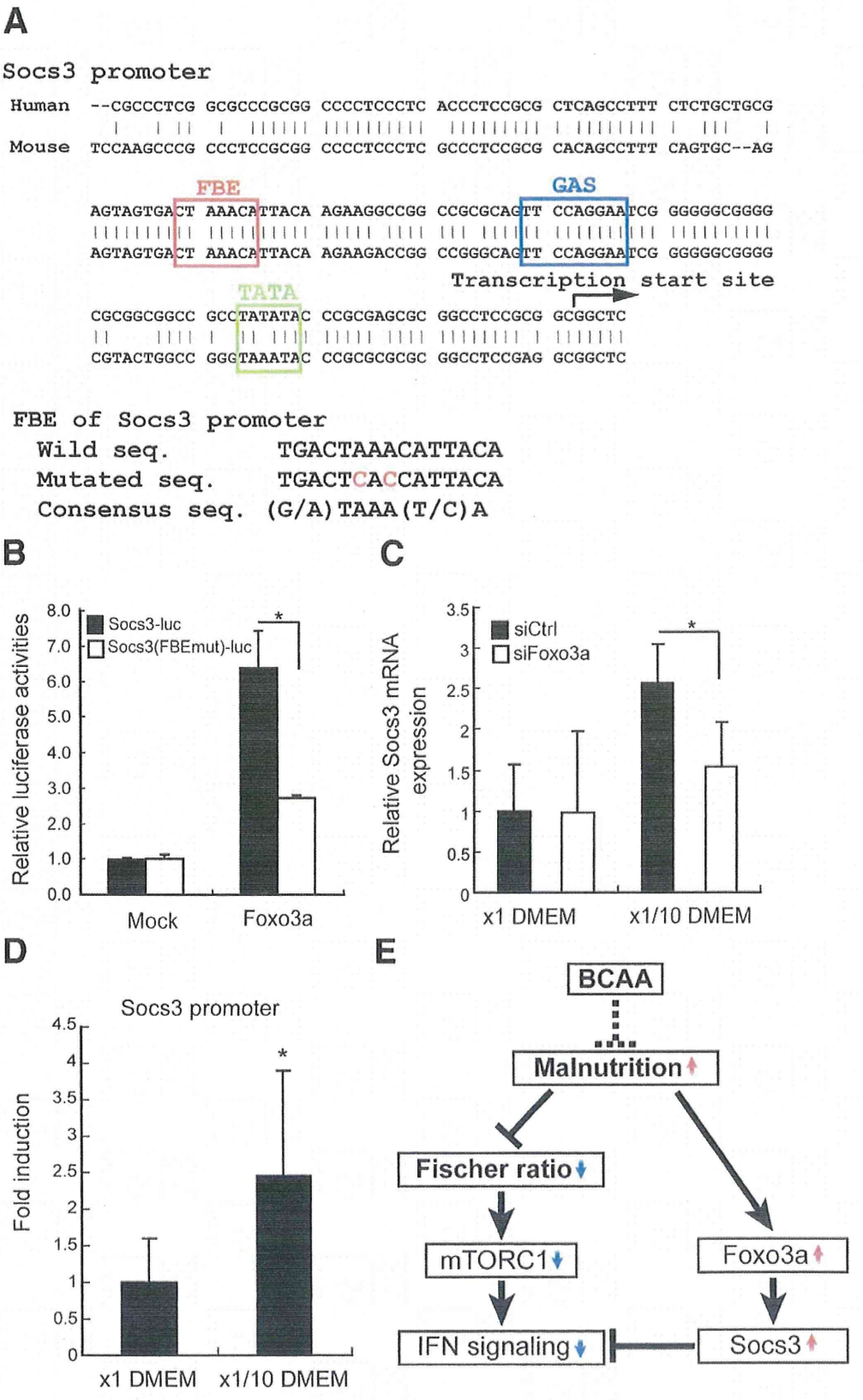

Figure 4. Socs3 promoter assay. (*A*) Primary structure of putative Foxo binding element in Socs3 promoter region. (*B*) Socs3-luc and Socs3 (FBEmut)-luc activities after overexpression of Foxo3a in Huh-7 cells. (*C*) Relative Socs3 messenger RNA (mRNA) expression after knockdown of Foxo3a in normal and low-amino-acid media. (*D*) Chromatin immunoprecipitation of Socs3 promoter region by Foxo3a in normal and low-amino-acid media. (*E*) Model of impaired IFN signaling by repressed mTORC1 signaling and increased Socs3 signaling under CH-C state of malnutrition.