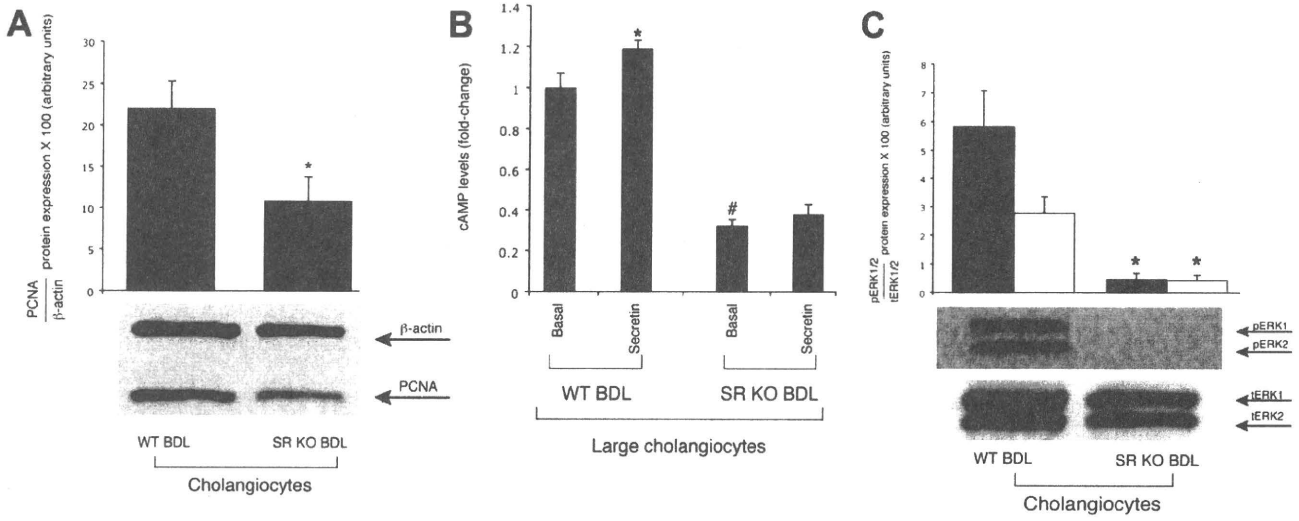

Fig. 4. Evaluation of (A) PCNA protein expression, (B) basal and secretin-stimulated cAMP levels, and (C) ERK1/2 phosphorylation in large cholangiocytes from WT and $SR^{-/-}$ 7-day BDL mice. (A) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus PCNA protein of large cholangiocytes from WT 7-day BDL mice. (B) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus 0.05 versus basal cAMP levels of large cholangiocytes from WT 7-day BDL mice. (C) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus 0.05 versus ERK1/2 phosphorylation of large cholangiocytes from WT 7-day BDL mice.

the corresponding levels of cholangiocytes from WT BDL mice (Fig. 4B). Secretin increased cAMP levels of large cholangiocytes from WT (but not $SR^{-/-}$) BDL mice (Fig. 4B). In large cholangiocytes from $SR^{-/-}$ BDL mice, there was a decreased ERK1/2 phosphorylation compared with large cholangiocytes from WT BDL mice (Fig. 4C).

Secretin Stimulates In Vitro Large Cholangiocyte Proliferation. Large (but not small) cholangiocytes proliferate after the administration of secretin (Fig. 5A). Secretin-stimulation of large cholangiocyte proliferation was blocked by H89 and partially by the MEK inhibitor, PD98059 (Fig. 5A). Secretin increased PCNA expression of large cholangiocytes, an increase that was blocked by H89 and PD98059 (Fig. 5B). There was increased PKA activity (Fig. 5C) and ERK1/2 phosphorylation (Fig. 5D) in large cholangiocytes treated with secretin compared to BSA-treated cells.

Silencing of the Secretin Receptor Gene Decreases the Proliferative Capacity of Large Cholangiocytes. The knockdown of SR protein expression by 50%, as demonstrated by FACS (Fig. 6B), was confirmed by way of western blot analysis (Fig. 6A). When we knocked down the gene for SR in large cholangiocytes, secretin did not increase cAMP levels (Fig. 6C) and proliferation (Fig. 6D, 48 hours of incubation) in these cells compared with the increase shown in large mock-transfected cholangiocytes. In support of the hypothesis that SR is a key trophic regulator in the regulation of biliary growth, there was a decrease in the basal proliferative capacity (Fig. 7) of SR-silenced large cholangiocytes compared with large mock-transfected cholangiocytes.

Discussion

In our study, we show that SR is an important trophic regulator sustaining large cholangiocyte proliferation during extrahepatic cholestasis. In the $SR^{-/-}$ mouse model, we show that proliferation of large cholangiocytes[12,14] is reduced (≈50%) during BDL compared with BDL WT mice, concomitant with elevation of biliary apoptosis. The reduction of cholangiocyte hyperplasia was associated with a decrease in both basal and secretin-stimulated cAMP levels and phosphorylation of ERK1/2 in large cholangiocytes compared with BDL cholangiocytes. *In vitro*, secretin increased the proliferation of large cholangiocytes by activation of cAMP→PKA→ERK1/2 signaling. Silencing of the SR gene induces a decrease in the basal proliferative capacity of large cholangiocytes compared with large mock-transfected cholangiocytes.

In our evaluation of SR expression, we found a time-dependent increase in the expression of SR in large cholangiocytes during BDL compared with normal large cholangiocytes. This finding was consistent with previous studies showing that: (1) in the rodent liver SR is only expressed by large cholangiocytes,[1,4,5,9,12] (2) SR expression is up-regulated following BDL ligation in large cholangiocytes,[14,17] and (3) the extent of secretin effects on cholangiocyte

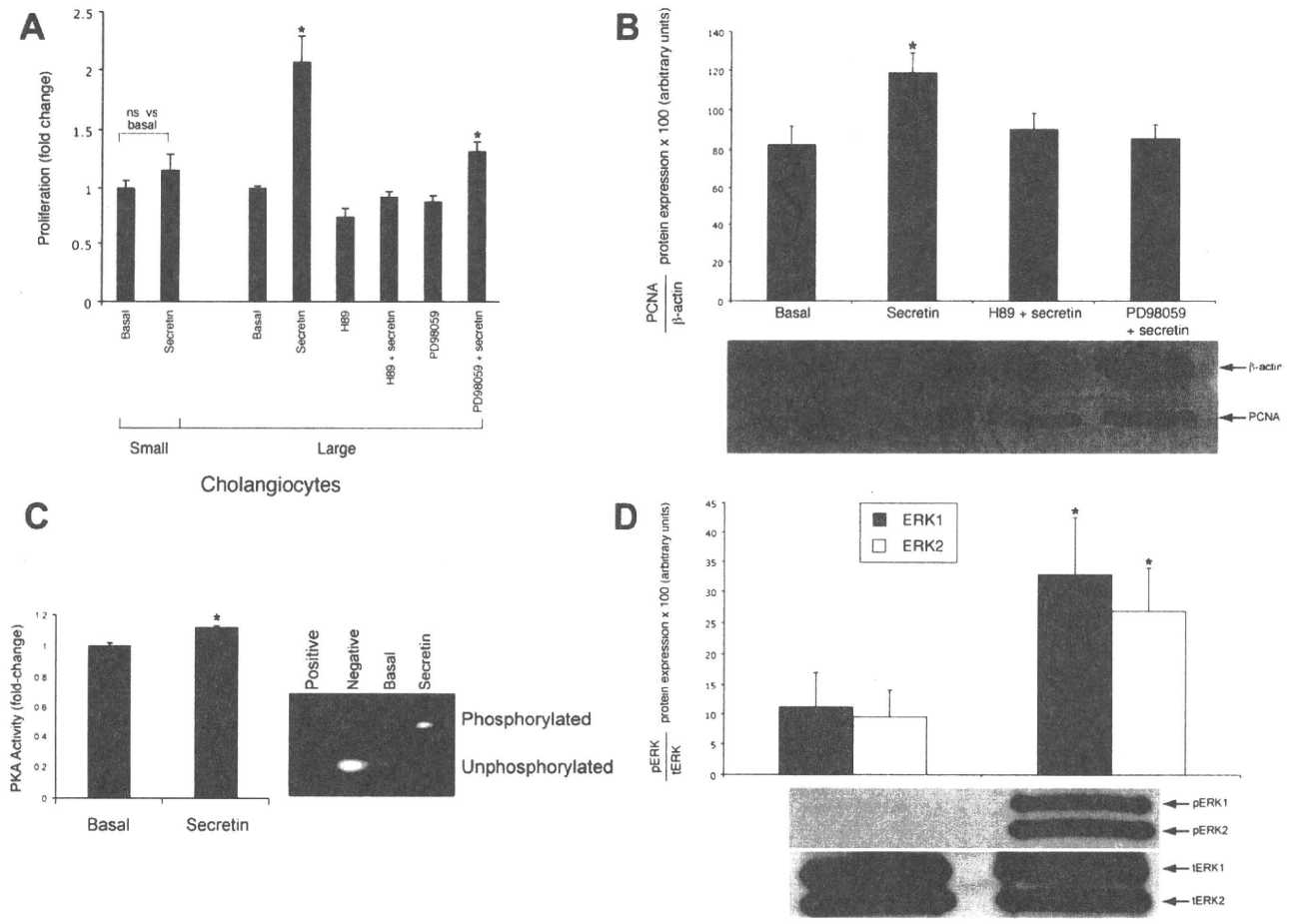

Fig. 5. (A) Effect of 0.2% BSA (basal) or secretin (100 nM) for 48 hours at 37°C on the proliferation of small and large cholangiocytes (MTS assay). Data are expressed as the mean ± SEM of 14 experiments. *$P < 0.05$ versus its corresponding basal value. (B) Data are expressed as the mean ± SEM of 14 experiments. *$P < 0.05$ versus its corresponding basal value. Secretin increased (C) PKA activity (n = 4) and (D) ERK1/2 phosphorylation (n =7) in large cholangiocytes compared with large cholangiocytes treated with BSA. *$P < 0.05$ versus its corresponding basal value.

functions parallel with the duration of BDL.[16] This finding parallels recent findings that mouse cholangiocytes share a similar heterogeneous profile as rat cholangiocytes[5] and freshly isolated and immortalized large mouse cholangiocytes are the only cell types to express the SR.[5,8,14] In human, SR expression is present in the biliary tract in normal bile ducts and ductules and the majority of cholangiocarcinomas, but is not present in hepatocytes or hepatocellular carcinoma.[26,27] Consistent with animal models of cholestasis, SR expression was up-regulated in ductular reactions in liver cirrhosis.[27]

In our *in vivo* model, the level of the reduction of cholangiocyte proliferation is consistent with the paradigm that cholangiocyte proliferation is regulated in autocrine and paracrine mechanisms by a number of stimulatory neurohormonal factors.[18,20,28] In a knockout mouse model for α-calcitonin gene-related peptide, the lack of circulating α-calcitonin gene-related peptide also reduces biliary proliferation during BDL to a similar degree as the lack of SR,[20] which indicates that the regulation of biliary proliferation during extrahepatic cholestasis is multifactorial and a complex regulatory system.[18,20,28]

The trophic effects of secretin were dependent upon the activation of the cAMP/PKA/ERK1/2 signaling. The second messenger system, cAMP, is a key factor for the function of large cholangiocytes.[1,4,7,9,13] Secretin stimulates bicarbonate secretion of large bile ducts through activation of cAMP-dependent CFTR→Cl^-/HCO_3^- anion exchanger 2.[1,4,7,9,13] Also, the activation of the cAMP/PKA/ERK1/2 pathway modulates cholangiocyte proliferation.[12,15,18,29] In fact, the direct stimulation of adenylyl cyclase activity by the chronic administration of forskolin stimulates normal cholangiocyte proliferation both *in vivo* and *in vitro*, which is associated with activation of the PKA/Src/MEK/ERK1/2 pathway.[29] Maintenance of cAMP levels by

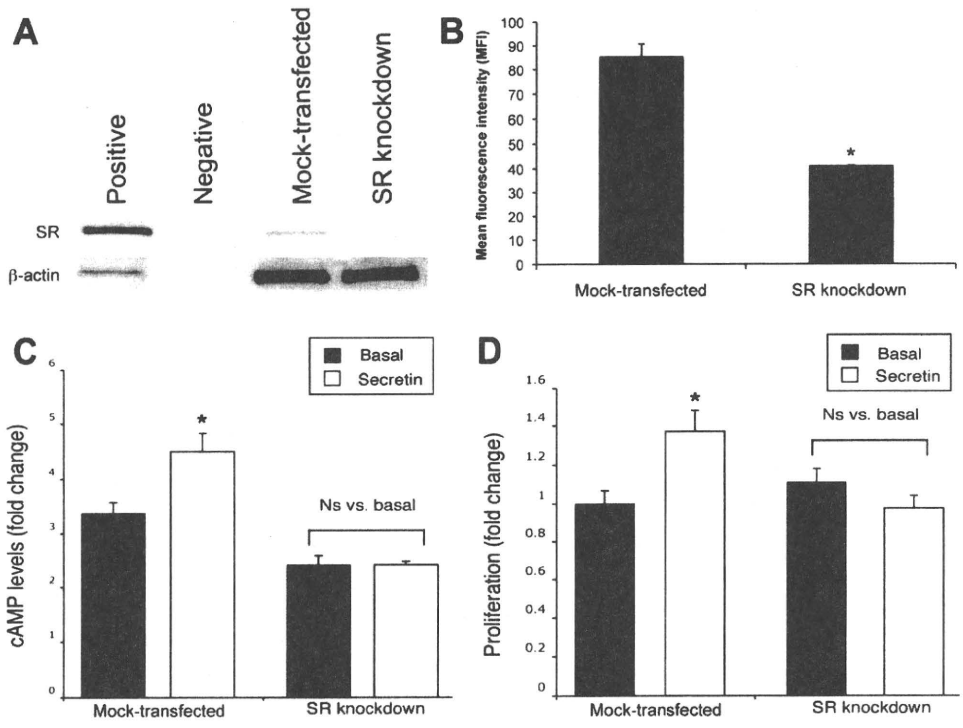

Fig. 6. Knockdown of secretin receptor protein expression in large cholangiocytes was evaluated by (A) western blotting and (B) FACS. Effect secretin receptor gene silencing on the effects of secretion on (C) cAMP levels, and (D) proliferation (by MTS assays) of large cholangiocytes. Data are expressed as the mean ± SEM of six experiments. *$P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.

forskolin administration prevents the impairment of cholangiocyte proliferation and enhancement of biliary apoptosis induced by vagotomy.[30] Furthermore, Banales et al. have shown[31] that cAMP stimulates cholangiocyte proliferation through two downstream effectors (i.e., PKA and Epacs) in an animal model of autosomal recessive polycystic kidney disease. Down-regulation of cAMP levels and cAMP-dependent signaling reduces biliary growth and increases cholangiocyte damage by apoptosis.[12,14,20,30] The involvement of the cAMP-dependent ERK1/2 pathway in secretin-dependent biliary proliferation during cholestasis was

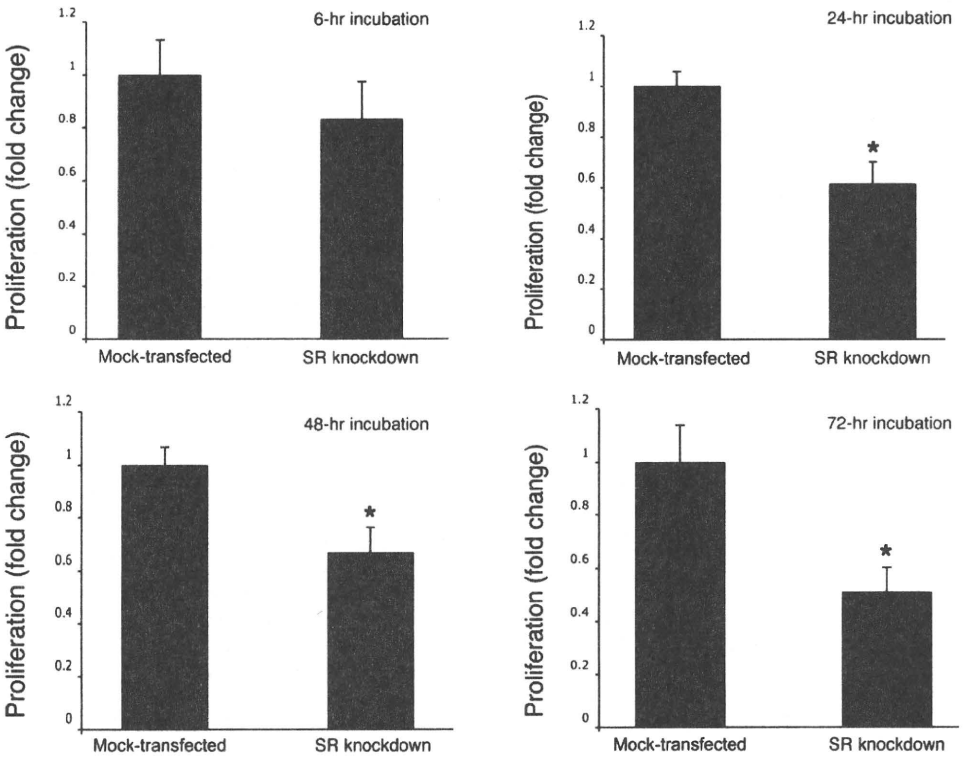

Fig. 7. Effect of secretin receptor gene silencing on the basal proliferative activity of large cholangiocytes following incubation for 6, 24, 48, and 72 hours with 0.2% BSA (MTS assay). Data are expressed as the mean ± SEM of four experiments. *$P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.

confirmed in BDL $SR^{-/-}$ mice, which had reduced levels of phosphorylated ERK1/2 in isolated large cholangiocytes. As expected, large cholangiocytes isolated from $SR^{-/-}$ did not respond to secretin, which was evidenced by lack of accumulation of intracellular cAMP levels.

Finally, we demonstrated that SR expression is critical for basal cholangiocyte proliferation in large mouse cholangiocytes that have stable knockdown of SR by transfection with short hairpin RNA for SR. These SR stable knockdown cells displayed decreased basal and secretin-stimulated proliferative capacity compared with control-transfected cholangiocytes. As expected, these stable knockdown SR cells lacked secretin-stimulated intracellular cAMP levels. Decreased basal proliferative rates that we observed in the cells with stable knockdown of SR compared with the mock-transfected controls are suggestive of the regulation of the basal proliferative rates by secretin perhaps in an autocrine mechanism. Consistent with our current study, we have previously shown that secretin stimulates the proliferation of two normal human cholangiocyte cell lines: H-69 and HiBEpiC.[26] Collectively, the findings of our study revealed that secretin is a trophic factor for cholangiocytes that differentially regulated the growth of large cholangiocytes by acting on the specifically expressed SR under normal and pathological conditions.

De novo SR expression in small cholangiocytes is often found in models of liver damage that alter the SR-dependent functional capacity of large cholangiocytes such as CCl_4 acute hepatoxicity.[14] We also have preliminary findings (unpublished data) that suggest that secretin has a protective role versus CCl_4-induced damage of large cholangiocytes.[14] These findings are consistent with the lack of secretin-dependent signaling resulting in an increase in the basal apoptotic activity in cells lacking SR that we observed in the SR knockdown cells. In addition, our other studies in which large cholangiocyte damage was prevented by administration of bile acids (such as taurocholate)[32] and cAMP agonists[30] suggest that secretin, a cAMP agonist, would have a role as a protective factor during large bile duct damage. Further studies are necessary to confirm this role, but are suggestive that secretin or other cAMP agonists could prevent biliary loss in ductopenia pathologies such as drug-induced vanishing bile duct syndrome or graft versus host disease.

The discovery of a novel proproliferative function of secretin in cholangiocytes, along with the demonstration that *in vitro* and *in vivo* molecular manipulations of the SR gene ablated the proliferative and apoptotic responses of large cholangiocytes, may shed light on the development of new therapeutic approach for the management of cholestatic liver diseases. Overexpression of SR or secretin administration might open new avenues for the treatment of ductopenic liver diseases.

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Branched Chain Amino Acids Enhance the Maturation and Function of Myeloid Dendritic Cells *Ex Vivo* in Patients with Advanced Cirrhosis

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An imbalance of plasma amino acids is observed in patients with advanced cirrhosis. The aim of this study was to investigate the influence of the extracellular amino acid imbalance on the function of myeloid dendritic cells (DCs) in patients with advanced cirrhosis. We made a serum-free culture medium consistent with the average concentration of plasma amino acids from healthy controls (HC, n = 25) or patients with advanced cirrhosis (LC, n = 43) to reflect more closely the actual environment of the living body. We compared the phenotypical and biological functions of blood dendritic cells antigen-positive dendritic cells (BDCA+ DCs) and monocyte-derived dendritic cells (MoDCs) from LC and HC with these media. After adding stimulants, the CD83 and CD86 expressions of DCs from LC were lower than those from HC. In both HC and LC, both CD83 and CD86 expressions of DCs stimulated under the cirrhotic medium were lower than under the control medium. This phenomenon was accompanied by a suppression of the mammalian target of rapamycin (mTOR)/S6K-signaling pathways. The interleukin 12 (IL-12) production in the cirrhotic medium was significantly lower than in the control medium and increased when valine or leucine was added to the medium. In patients with advanced cirrhosis, peripheral blood mononuclear cells stimulated in the autologous plasma after oral administration of branched-chain amino acid (BCAA) granules had significantly increased interferon gamma production. *Conclusion:* In advanced cirrhosis, there is impairment of the function and maturation of DCs, which has been shown to be related to an imbalance in the extracellular amino acid profile. Elevating the extracellular concentration of BCAAs *ex vivo* in patients with advanced cirrhosis improved the function of DCs. (HEPATOLOGY 2009;50:1936-1945.)

Cirrhosis makes it increasingly difficult for the liver to carry out its essential functions, such as detoxifying harmful substances and manufacturing vital nutrients. Cirrhosis progresses to decompensated cirrhosis and ultimately liver failure because of a lack of suitable treatment. Not only hepatocellular carcinoma but also nosocomical infections, such as spontaneous bacterial peritonitis (SBP) or pneumonia, are frequent clinical complications in these immune-compromised patients.[1] In patients with advanced cirrhosis, various metabolic disorders involving glucose, protein-amino acids, lipids, vitamins, and minerals might appear. Furthermore, an imbalance of plasma amino acids, with decreased levels of branched-chain amino acids (BCAAs)

Abbreviations: AAA, aromatic amino acid; ACM, advanced cirrhotic media; APC, antigen-presenting cell; BCAA, branched-chain amino acid; BDCA, blood dendritic cells antigen; DC, dendritic cell; HCM, healthy control media; IFN-γ, interferon gamma; IL, interleukin; MLR, mixed lymphocytes reaction; MoDC, monocyte-derived dendritic cell; mTOR, mammalian target of rapamycin; NKT, natural killer T; PBMC, peripheral blood mononuclear cell; SBP, spontaneous bacterial peritonitis.

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and increased levels of aromatic amino acids (AAAs), is commonly seen in patients with advanced cirrhosis.[2] In clinical situations, long-term nutritional supplementation with oral BCAA has been shown to be useful to prevent progressive hepatic failure and to improve surrogate markers and the perceived health status.[3,4] Moreover, the oral administration of BCAA granules was reported to inhibit hepatic carcinogenesis in patients with compensated cirrhosis.[5,6]

On the one hand, it has become clear that amino acids are not only important as substrates for various metabolic pathways but also activate a nutrient-sensitive signaling pathway in synergy with insulin.[7-10] The mammalian target of rapamycin (mTOR) signaling pathway is one of the most representative pathways, and this pathway has been shown to act as a major effector of cell growth and proliferation by way of the regulation of protein synthesis.[7-9] The phosphorylation of downstream effectors of mTOR is inhibited by rapamycin and activated by BCAA, especially by leucine,[11-13] although little is known about the impact of changes in the extracellular amino acid levels on the immune system.[14] Recently, we have shown that extracellular BCAAs, especially valine, regulate the maturation and function of monocyte-derived dendritic cells (MoDCs).[15] Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that stimulate innate and adaptive immune reactions by priming other types of blood cells. Typically, immature DCs migrate to lymphoid tissues and present antigenic peptides to naive T cells.[16] The mature DCs, which characteristically express CD83,[17] can rapidly activate other innate immune cells including natural killer (NK) cells and natural killer T (NKT) cells through the production of immunomodulatory cytokines such as interleukin (IL)-10 and IL-12. Several studies have reported that the immunological abnormalities occurring in cirrhosis,[18,19] such as a depressed reticuloendothelial system, neutrophil dysfunction, reduced serum complement, and low bactericidal function, account for the increased susceptibility of patients with cirrhosis to bacterial seeding and diffusion, and for the impaired functions of DCs in patients with liver cirrhosis.[15,20,21] However, it is not clear why the responses of immune cells, particularly DCs, are suppressed in patients with cirrhosis.

Roswell Park Memorial Institute medium 1640 (RPMI 1640) with human or bovine serum is typically used to culture peripheral blood mononuclear cells (PBMCs) or DCs and examine the function. The concentrations of almost all the amino acids in RPMI 1640 are higher than those typically found in the plasma of healthy adult humans. Accordingly, there are large differences between the amino acids of living bodies and those of culture systems. The concentration of amino acids except BCAAs in the medium used in our previous study was higher than that of plasma *in vivo*.[15] Furthermore, various types of amino acid imbalance actually appear in the plasma of patients with advanced cirrhosis. The aim of the study, therefore, was to investigate the influence of the extracellular amino acid imbalance observed in patients with advanced cirrhosis on the function of DCs using a serum-free culture medium consistent with the average concentration of plasma amino acids from healthy volunteers (healthy control media, HCM) or patients with advanced cirrhosis (advanced cirrhotic media, ACM) to reflect more closely the actual environment of the living body. Furthermore, we investigated whether oral administration of BCAA granules could enhance the responses of immune cells in patients with advanced cirrhosis.

Patients and Methods

Serum-Free Culture Media. The concentrations of the plasma amino acids from fasting healthy volunteers (n = 25), chronic hepatitis (n = 14), and patients with cirrhosis (n = 60) were measured by high-performance liquid chromatography (HPLC) in the early morning (Table 1). Briefly, sulfosalicylic acid was added to plasma to a final concentration of 5%. The samples were then placed on ice for 15 minutes followed by centrifugation to remove precipitated proteins. The extracts were then analyzed for the amino acid content with a JLC-500/V (Japan Electron Optics Laboratories, Tokyo, Japan). Also, these patients with cirrhosis were classified according to the Child-Pugh classification. We defined as Child-Pugh grade B or C the patients with advanced cirrhosis (n = 43: hepatitis c virus [HCV] n = 22; primary biliary cirrhosis [PBC] n = 5; alcoholic n = 3; nonalcoholic steatohepatitis [NASH] n = 3; hepatitis b virus [HBV] n = 2; primary sclerosing cholangitis [PSC] n = 2; HCV+HBV n = 1; autoimmune hepatitis [AIH] n = 1; Wilson's disease n = 1; Budd-Chiari syndrome n = 1; cryptogenic n = 2). A serum-free culture medium consistent with the average concentration of plasma amino acids from healthy volunteers was defined as the HCM; whereas that from patients with advanced cirrhosis was defined as the ACM (Table 2). Other components except amino acids were identical among media. We verified that there was no difference between the theoretical value and actual value in HCM and ACM. We cultured PBMCs under the two media with stimulant for 48 hours and measured the amino acid concentrations of these media. There was no difference in the concentrations of amino acids before and after culture in these media. The viability of PBMCs was determined using Annexin V^{FITC}, with dead cells identi-

Table 1. Aminogram for the Plasma in Chronic Hepatitis Patients and Patients with Cirrhosis

	HC (n=25)	CH (n=14)	Child A (n=17)	Child B (n=19)	Child C (n=24)
Glycine	225	250	205	234	313
Alanine	391	400	311	317	339
Serine	119	135	139	137	169
Threonine	142	139	137	135	165
Cystine	38	54	63	62	73
Methionine	29	31	40	60	68
Glutamine	564	585	616	642	739
Asparagine	51	57	62	58	77*
Glutamic acid	42	70	62	65	47
Aspartic acid	3	3	5	4	3
Valine	249	243	222	195†	164†
Leucine	132	141	120	110	93†
Isoleucine	76	71	63	56	51†
Phenylalanine	63	70	80	89	99*
Tyrosine	65	81	111	112	151*
Tryptophan	62	52	52	43	47
Lysine	183	223	219	199	179
Arginine	78	79	94	93	100
Histidine	83	90	77	81	93
Proline	204	163	142	165	202
Fischer's ratio	3.57	3.01	2.36†	1.95†	1.27†

The concentrations of plasma amino acids from fasting healthy volunteers (n=25), chronic hepatitis (n=14) and patients with cirrhosis (n=60) were measured by HPLC in the early morning after fasting. Also, these patients with cirrhosis were classified according to the Child-Pugh classification. Amino acid concentrations are expressed in nmol/mL.

*$P < 0.01$ increased. †$P < 0.01$ decreased. Fischer's ratio means: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine †decrease *increase $P < 0.01$ vs. CH (the data were analyzed with ANOVA and Dunnett's post-hoc procedure).

fied by propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit, BioVision, Mountain View, CA), according to the manufacturer's instructions. We confirmed the viability of PBMCs cultured in HCM and ACM equal to that of complete culture medium (CCM) and X-VIVO 10 (Cambrex Bio Science Walkersville, Walkersville, MD). The percentages of living cells were 78.7 ± 0.67, 77.7 ± 2.2, 71.7 ± 0.67, and 74.7 ± 0.33 for HCM, ACM, CCM, and X-VIVO10, respectively. The culture media, CCM, and other depleted media were made as described.[15]

Patients and Healthy Volunteers. We selected 15 patients with cirrhosis for *in vitro* or *ex vivo* studies (Table 3). All of these patients were inpatients. There were no significant differences on clinical and laboratory findings in this population compared to the 43 patients with advanced cirrhosis (Table 1): age 60.4 ± 12.8 versus 59.1 ± 11.3; aspartate aminotransferase (AST) 78.8 ± 45.4 IU/L versus 96.3 ± 65.0 IU/L; alanine aminotransferase (ALT) 47.6 ± 25.2 IU/L versus 54.3 ± 36.7 IU/L; total bilirubin 4.5 ± 5.36 mg/dL versus 3.94 ± 3.70 mg/dL; albumin 2.80 ± 0.51 g/dL versus 2.85 ± 0.55 g/dL; prothombin time / international normalized ratio (PT-INR) 1.54 ± 0.39 versus 1.37 ± 0.29; PLT 93.9 ± 68.7 × $10^3/\mu L$ versus 113.1 ± 54.2 × $10^3/\mu L$; Child Pugh score 9.0 ± 1.77 versus 8.6 ± 2.10; Model for End-Stage Liver Disease (MELD) score 11.9 ± 5.55 versus 11.2 ± 4.23; plasma Fischer's ratio 1.56 ± 0.77 versus 1.65 ± 0.57. The MELD score[22] was calculated by an online worksheet available on the Internet at www.mayoclinic.org/meld/mayomodel5.html. None of the patients had clinical or laboratory findings compatible with bacterial infection when we collected PBMCs from the patients. Written informed consent was obtained from each individual and the study protocol was approved by the Ethics Committee of Tohoku University School of Medicine (2003-326, 2008-337).

BDCA+ DCs Maturation and MoDCs Generation. PBMCs were separated from the peripheral blood of HC and LC by centrifugation on a density gradient. The blood dendritic cells antigen-positive dendritic cells (BDCA+ DCs) and the CD14-positive monocytes were isolated from PBMCs using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). BDCA1+ DCs were cultured at a density of 2.5 × 10^5 cells/well in 96-well flat-bottom plates (Corning, NY) for 48 hours with 1,000 U/mL GM-CSF (PreproTech, London, UK), 500 U/mL (hu) IL-4 in each media. At 24 hours culture,

Table 2. Serum-Free Culture Media Used in This Study (nmol/mL)

	CCM	HCM	ACM
Glycine	400	225	280
L-Alanine	400	391	307
L-Serine	400	119	151
L-Threonine	800	142	138
L-Cystine 2HCl	200	38	67
L-Methionine	200	29	75
L-Glutamine	4000	564	689
L-Asparagine	400	51	64
L-Glutamic Acid	400	42	53
L-Aspartic Acid	400	3	4
L-Valine	800	249	175
L-Leucine	800	132	100
L-Isoleucine	800	76	53
L-Phenylalanine	400	63	99
L-Tyrosine	400	65	133
L-Tryptophan	80	62	45
L-Lysine-HCl	800	183	184
L-Arginine-HCl	400	78	92
L-Histidine HCl-H2O	200	83	85
L-Proline	400	204	176
Fischer's ratio	3.00	3.57	1.42

Complete culture medium (CCM) contains 20 amino acids that are relevant to the make-up of mammalian proteins. HCM (healthy control medium): consistent with the average concentration of plasma amino acids from healthy volunteers (n=25). ACM (advanced cirrhotic medium): consistent with the average concentration of plasma amino acids from patients with advanced cirrhosis (Child-Pugh grade B or C, n=43). The amino acid concentrations are expressed in nmol/mL. Fischer's ratio means: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine.

Table 3. Characteristics of Study Participants

Patient Number	Disease	Sex	Age (years)	AST/ALT	Total Bilirubin	Albumin	PT-INR	PLT	Child-Pugh Classification	MELD Score	Plasma Fischer's Ratio	BCAA Medication
1	LC-C	M	71	116/61	0.8	3.3	1.09	149	A	6	2.49	-
2	LC-C+HCC	M	70	73/46	1.5	2.3	1.15	75	B	6	2.26	-
3	LC-C+HCC	F	80	72/55	1.3	2.8	1.19	144	B	9	NA	+
4	LC-C	M	42	52/38	4.2	1.8	1.79	79	C	16	0.99	+
5	LC-C+HCC	F	61	238/98	6.3	2.9	1.65	76	B	18	2.74	+
6	PBC	F	43	241/144	12.3	2.8	1.32	152	C	18	1.57	-
7	LC-C	M	56	71/45	2.2	3.7	1.24	81	B	10	1.90	+
8	LC-C	M	48	111/109	1.6	3.7	1.08	81	A	8	NA	-
9	LC-C	F	60	25/5	11.6	3.2	2.05	83	C	15	0.88	+
10	LC-C+HCC	F	69	68/40	1.3	2.8	1.17	132	B	7	1.81	-
11	non B non C	F	44	28/18	2.4	2.6	1.54	122	C	8	1.31	+
12	PBC	F	62	130/49	6.8	2.0	1.33	120	C	8	1.43	+
13	PBC	F	62	83/30	2.3	2.5	1.11	207	B	13	1.29	+
14	Alcoholic	M	54	53/24	2.5	3.1	1.60	219	C	14	1.24	+
15	LC-C+HCC	M	65	83/53	2.0	3.2	1.29	96	B	12	1.52	+

LC-C, liver cirrhosis due to HCV; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; NASH, nonalcoholic steatohepatitis; NA, not available; PLT, platelet counts ($x10^3/\mu L$); PT-INR, prothrombin time-international normalized ratio; AST/ALT, aspartate aminotransferase / alanine aminotransferase (IU/L); total bilirubin (mg/dL); albumin (g/dL); Fischer's ratio: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine.

DCs were stimulated by 500 ng/mL lipopolysaccharide (LPS; *Escherichia coli* 026:B6; Sigma, St. Louis, MO) or polyinosinic:polycytidylic acid (polyI:C) (30 μg/mL). Monocytes were cultured at a density of 3.0×10^5 cells/well with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 6 days in CCM. On day 6 we changed the medium from CCM to HCM or ACM with poly(I:C) and the culture was continued for an additional 48 hours.

Surface Marker Analysis. DCs were harvested and labeled with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) (antihuman CD14, CD40, CD83, CD86, CD98, HLA-DR, or the relevant isotype controls; BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Using a FACS Calibur (BD Immunocytometry Systems, San Diego, CA) flow cytometer, surface marker expressions were analyzed using the CellQuest (BD Immunocytometry Systems) program.

Phagocytosis Assay with Dextran. To evaluate the endocytosis potential of DCs, 1 mg/mL of FITC-dextran was supplied to 2.5×10^5 DCs that were then incubated for 30 minutes at 37°C. As a control, the DCs were given the same doses of FITC-dextran and stored for 30 minutes at 4°C. After the incubation the DCs were washed and subjected to FACS analysis.

Cytokine Analysis. BDCA1+ DCs were cultured at a density of 2.5×10^5 cells/well in 96-well flat-bottom plates for 48 hours with 1,000 U/mL GM-CSF, 500 U/mL (hu) IL-4 in each of the media. At 24 hours, 500 ng/mL LPS or poly(I:C) (30μg/mL) were added. The supernatants were collected after 48 hours and immediately IL-12 (p40+p70) and IL-10 were determined by specific cytokine enzyme-linked immunosorbent assay (ELISA) kits (Bender MedSystems) according to the manufacturer's instructions. For the interferon gamma (IFN-γ) production of PBMCs, PBMCs were cultured at a density of 2.5×10^5 cells/well in HCM or ACM for 48 hours, and at 5.0×10^5 cells/well in autologous plasma for 12 hours. IFN-γ was determined by specific cytokine ELISA kits (Bender MedSystems).

Mixed Lymphocytes Reaction (MLR). BDCA+ DCs were cultured at a density of 1.0×10^5 cells/well in 96-well round-bottom plates (Falcon) containing HCM or ACM with GM-CSF and IL-4 for 48 hours. At 24 hours culture, immature DCs were induced to mature using LPS or poly(I:C) for an additional 24 hours. The allostimulatory capacity of irradiated DCs (3,000 Rad) was tested in a one-way MLR with normal 2×10^5cells/well allogeneic CD4+ lymphocytes (isolated from PBMCs using magnetic beads) under CCM. Cocultured cells were maintained for 7 days and the proliferation rate of the cells was measured using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) Assay (CellTiter 96 aqueous one-solution cell proliferation assay; Promega, Madison, WI) according to the manufacturer's instructions. On carboxyfluorescein succinimidyl ester (CFSE) staining, cells were analyzed using a CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR). The staining methods followed the manufacturer's protocol.

Immunoblotting. DCs were cultured at a density of 3.0×10^5 cells/well in 96-well flat-bottom plates (Corn-

Table 4. Phenotypic Difference of BDCA1+DCs Derived from Patients with Cirrhosis and Healthy Volunteers

			CD40	CD83	CD86	HLA-DR
Isolated DC	Healthy control (n=4)		5 ± 1.4	6 ± 2.2	14 ± 3.1	166 ± 52.2
	LC patients (n=4)		12 ± 16.1	4 ± 1.4	12 ± 3.4	195 ± 79.3
Mature DC	Healthy control (n=5)	HCM	131 ± 54	240 ± 25	201 ± 67	910 ± 121
		ACM	121 ± 37	*190 ± 33**	*170 ± 53**	783 ± 90
	LC patients (n=5)	HCM	139 ± 44	*154 ± 48†*	*169 ± 37†*	*691 ± 112†*
		ACM	124 ± 47	*125 ± 45‡*	*122 ± 11‡*	625 ± 160

The MFI are presented for each marker as the mean ± SD of healthy controls and patients with cirrhosis (isolated DC: Patients 6, 7, 8, 10 / mature DC: Patients 8, 9, 10, 11, 12).

*Value of $P < 0.05$ vs. DCs of healthy control cultured under HCM (Wilcoxon t test).

†Value of $P < 0.05$ vs. DCs of healthy control cultured under HCM (Mann-Whitney U test).

‡Value of $P < 0.05$ vs. DCs of LC patients cultured under HCM (Wilcoxon t test).

ing) containing 200 μL medium supplemented with GM-CSF and IL-4 for 24 hours and the DCs were stimulated by poly(I:C) for 1 hour. The DCs were harvested and lysed using CelLyticTM-M Mammalian Cell Lysis/Extraction Reagent (Sigma). The lysed cells were centrifuged to pellet the cellular debris. Thereafter, these protein concentrations were determined by a Modified Lowry Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF (Immun-Blot PVDF Membrane; Bio-Rad, Hercules CA). After washing and blocking, immunostaining was performed with rabbit polyclonal primary antibody (PI3K, phospho-PI3K, mTOR, p70 S6K, phospho-p70 S6K; Cell Signaling Technology, Beverly, MA), followed by incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) (Sigma). Immunoreactive proteins were revealed with an ECL reagent (ECL advance; Amersham Biosciences, Little Chalfont, UK).

Oral Administration of BCAA to Patients with Advanced Cirrhosis and* Ex Vivo *Cytokine Production Assay. In the early morning we measured the fasting concentration of the plasma amino acids before and after oral administration of BCAA granules (30, 60, 120, 180 minutes) from healthy volunteers and patients with advanced cirrhosis. The BCAA granules: LIVACT (Ajinomoto Pharma, Tokyo, Japan) were composed of a mixture of valine, 1.144 g, leucine, 1.904 g, and isoleucine, 0.952 g. The concentrations of the plasma amino acids were measured by HPLC. We stimulated PBMCs from patients for 12 hours by LPS or poly(I:C) under autologous plasma, which was collected both before and after oral administration. After 12 hours we recovered the plasma and measured the IFN-γ by ELISA.

Statistical Analysis. The data were analyzed with analysis of variance (ANOVA) and multiple comparisons were performed with Dunnett's post-hoc procedure for the plasma aminogram. When two groups were analyzed, the differences between media were analyzed by the Wilcoxon t test. Frequencies of BDCA1+ DCs were compared between patient groups by the Mann-Whitney U test. All statistical analyses were performed with standard statistical software (SPSS 13.0 for Windows, Chicago, IL).

Results

Amino Acid Concentrations Similar to Those in Plasma of Patients with Advanced Cirrhosis Impaired the Maturation of Myeloid DCs from Healthy Controls. First we measured the cytokine production from PBMCs both under HCM and ACM. The IFN-γ production of PBMCs stimulated by poly(I:C) under ACM was significantly impaired (28.1 ± 7.3 pg/mL versus 16.7 ± 3.9 pg/mL; $P = 0.04$). Next, we cultured the BDCA+ DCs (purity >90%) for 48 hours under HCM and ACM and evaluated the phenotypes of DCs by flow cytometry. In ACM, the CD83 and CD86 expression of DCs was significantly impaired compared to that in HCM (Table 4). The HLA-DR expression had a tendency to decrease in ACM. This phenomenon was observed in MoDCs (Supporting Fig. 1). Next, The IL-12 production of BDCA+ DCs stimulated under ACM was significantly impaired (110.7 ± 8.6 pg/mL versus 79.9 ± 12.5 pg/mL; $P = 0.04$), although the IL-10 production of DCs was not different between HCM and ACM (31.0 ± 4.0 versus 32.4 ± 8.2; $P = 0.59$). Flow cytometric analysis revealed that the amount of FITC-dextran taken up by BDCA+ DC and MoDC did not differ between HCM and ACM (data not shown). The allostimulatory capacity of BDCA+ DCs cultured under ACM was significantly decreased as shown by the MTS assay (1.00 ± 0.15 versus 0.82 ± 0.13; $P = 0.04$; absorbance 490 nm), and this tendency was also confirmed by the CFSE assay.

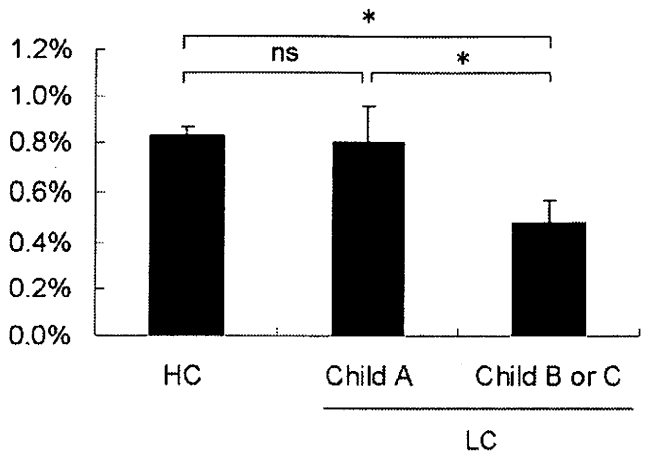

Fig. 1. The frequencies of DCs were significantly lower in the peripheral blood from patients with advanced cirrhosis compared with those from HC or early patients with cirrhosis. Percentages of BDCA+ DC in PBMCs were determined by flow cytometry. Significant differences in the percentages of DCs were observed between patients with advanced cirrhosis (Child-Pugh grade B or C: n = 10) and HC (n = 7). There was no difference between patients with Child-Pugh grade A (n = 7) and HC. Data are expressed as mean ± standard error of the mean (SEM).

Amino Acid Concentrations Similar to Those in Plasma of Patients with Advanced Cirrhosis Also Impaired the Maturation of Myeloid DCs from Patients with Cirrhosis. We first evaluated the frequency of BDCA+ DCs between HC and LC (Fig. 1). The frequencies of DCs were significantly lower in the peripheral blood from patients with advanced cirrhosis compared to those from HC or patients with early cirrhosis. Second, we determined the phenotype of BDCA1+ DCs from the LC before and after adding the stimulants. There was no difference regarding the mean fluorescence intensity (MFI) of isolated immature DCs expressing CD40, CD83, CD86, and HLA-DR between the HC and LC (Table 4). After adding the stimulants, the expressions of CD83 and HLA-DR by DCs from the LC were significantly decreased compared to those from the HC in both HCM and ACM (Table 4). The CD83 and CD86 expression of DCs was significantly impaired in ACM compared to that in HCM (Table 4).

Elevating the Concentration of BCAA Enhanced the IL-12 Production in BDCA+ DCs. As in the *in vivo* study, we confirmed that the plasma concentrations of BCAAs were significantly decreased and AAAs (except tryptophan) were increased along with the Child-Pugh grade (Table 1). Based on these data, to investigate which amino acid especially influenced the function of BDCA1+ DCs, we measured the cytokine production of DCs under HCM, ACM, and ACM supplemented with 800 nmol/mL of a single amino acid: valine, leucine, isoleucine, or AAAs. Interestingly, the IL-12 production of DCs stimulated under ACM plus valine or leucine was more increased than that under ACM, although there was no difference among ACM plus isoleucine, ACM plus AAAs, and ACM (Fig. 2A). Similar to the cytokine production, the allostimulatory capacity of DCs cultured under ACM plus valine or leucine had a tendency to be increased, as shown by the MTS assay (ACM: 0.71 ± 0.07, ACM plus valine: 0.88 ± 0.06; ACM plus leucine: 0.83 ± 0.03; absorbance 490 nm). Next, we determined the BDCA1+ DCs phenotype (CD14 and CD83) in CCM, BCAA-depleted, valine-depleted, leucine-depleted, and isoleucine-depleted media. In CCM, leucine-depleted and isoleucine-depleted media the DC phenotypes were similar (the percentages of CD83-positive cells were 33.7 ± 7.2%, 31.5 ± 5.4%, and 35.5 ± 7.9% for CCM, leucine-depleted, and isoleucine-depleted media, respectively). However, in BCAA-depleted and valine-depleted media, the CD83 expression of DCs was significantly impaired compared to that in CCM (BCAA-depleted media: 19.6 ± 3.0% and valine-depleted media 14.6 ± 1.8%; $P = 0.04$ versus CCM). After we cultured the DCs under depletion of valine for 2 days, we added valine to the medium and cultured the cells for an additional 24 hours. Then, the percentage of mature DCs was higher than that of valine-depleted media. Furthermore, to reflect more closely the actual environment of the living body, we induced DCs from LC to mature with either autologous plasma or autologous plasma supplemented with 100 nmol/mL valine for 12 hours. In all cases the DCs matured in the autologous plasma with valine had enhanced allostimulatory capacity and IL-12 production (Fig. 2B).

Amino Acid Concentration of Plasma in Patients with Advanced Cirrhosis Down-regulated the mTOR/S6K Signaling Pathway of BDCA1+ DCs. We hypothesized that the amino acid imbalance of the plasma in patients with advanced cirrhosis influence the mTOR/S6K signaling pathway of DCs and impaired their maturation. Under HCM with rapamycin, the percentage of CD14-/CD83+ mature DCs was higher than under HCM without rapamycin (Fig. 3A). BDCA+ DCs expressed similar levels of total PI3K, phospho-PI3K, mTOR, p70 S6K, and β-actin among all media. Interestingly, DCs cultured in ACM expressed lower levels of phospho-p70 S6K than those cultured in HCM (Fig. 3B). The expression of phospho-p70 S6K by DCs in ACM was partially recovered by adding 400 nmol/mL BCAA to the medium during stimulation. Isolated immature BDCA+ DCs expressed moderate levels of CD98 which modulate the amino acid transport functions and, after adding the stimulants, mature DC showed the up-regulation of CD98. There was no difference regarding

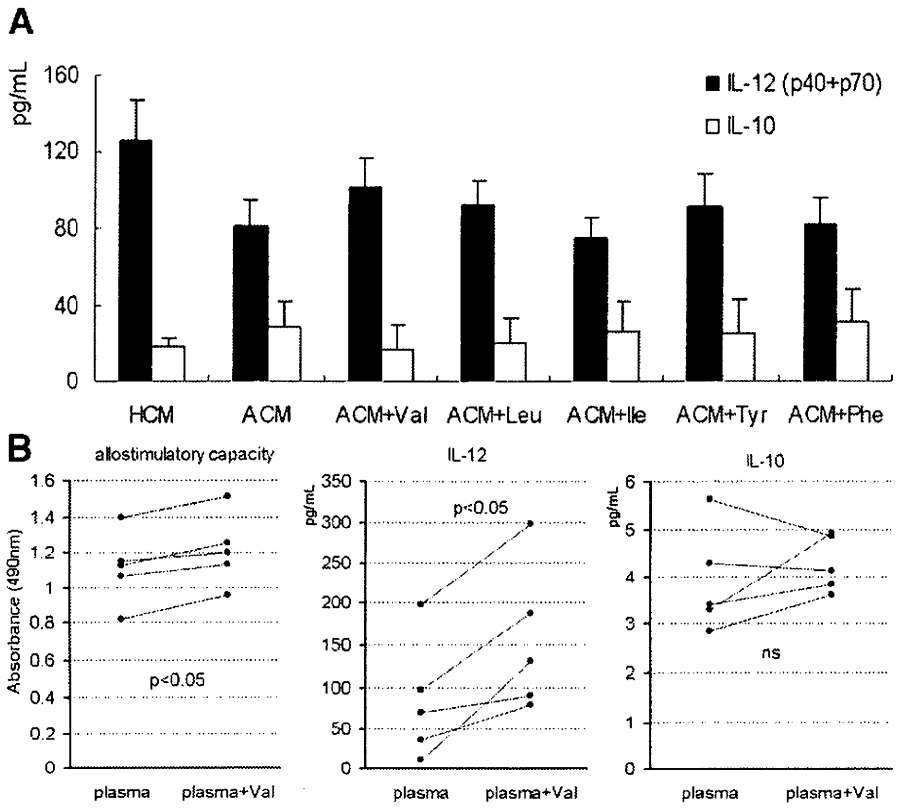

Fig. 2. Elevating the concentration of BCAAs enhanced the IL-12 production in BDCA1+ DCs. Isolated BDCA1+ DCs were cultured under HCM, ACM, and ACM supplemented with 800 nmol/mL single amino acid: valine, leucine, isoleucine, or AAAs. (A) After 48 hours the supernatants were assayed for cytokine concentrations. Mean ± SEM values from five different donors. (B) We induced BDCA1+ DCs from LC patients (Patients 1-5) to mature with either autologous plasma or autologous plasma supplemented with 100 nmol/mL valine for 12 hours. Supernatants were measured by ELISA. $P < 0.05$ (paired Student's t test, two-tailed).

the expression of CD98 between HCM and ACM (data not shown).

Oral Administration of BCAAs Enhanced the Production of IFN-γ by PBMCs from Patients with Advanced Cirrhosis Ex Vivo. Finally, we evaluated whether BCAAs have an effect on the immune response *ex vivo*. In healthy volunteers the concentration BCAAs of plasma was maximum 30 minutes after oral administration (Fig. 4A). Fischer's ratio increased from 4.78 ± 1.41 (standard deviation [SD]) to 13.39 ± 2.41 (SD). On the other hand, in the patients with advanced cirrhosis (Table 3: Patients 10-13), the concentration BCAAs of plasma was maximum 60 minutes after oral administration. Fischer's ratio increased from 1.37 ± 0.98 (SD) to 4.94 ± 0.99 (SD). AAAs decreased slowly during the following 3 hours. We stimulated PBMCs from the patients with advanced cirrhosis (Table 3: Patients 11-15) using either autologous plasma before and after 60 minutes oral administration. Interestingly, in all cases PBMCs stimulated by LPS in the latter had more IFN-γ production than the former (Fig. 4B).

Discussion

In this study we started by making two serum-free media (HCM and ACM) to be more representative of the human physiological environment and quantitatively measured the plasma amino acid profiles. First, we found that the amino acid imbalance of plasma in patients with advanced cirrhosis impaired the production of IFN-γ from PBMCs. IFN-γ is a dimerized soluble cytokine that is the only member of the type II class of interferons.[23] IFN-γ is secreted by Th1 cells, DCs, and NK cells. Although the commitment toward either the Th1 or the Th2 phenotype can be influenced by many signals active at the moment of naive Th cell priming, the levels of IL-12p70 (IL-12) produced by APC, especially DCs, are of major importance.[24,25] Therefore, we hypothesized that the impaired production of IFN-γ from PBMCs caused the dysfunction of DCs. Expectedly, the maturation and the IL-12 production of DCs were impaired in ACM. Furthermore, we confirmed that the allostimulatory capacity of DCs stimulated in ACM was impaired by MTS and CFSE assays. Previous studies have suggested an increase in IL-10 in cirrhosis and a potential link between high IL-10 and low HLA-DR expression in relation to immune dysfunction,[26] but in this study there was no difference in IL-10 secretion between DCs from ACM compared with HCM. Such differences were probably caused by (1) differences in the stimulation period of the immune cells (the former was *ex vivo*, this study was *in vitro*); (2) differences in the cell sources (the former was monocytes, this study was DCs); (3) other factors besides amino acids influence IL-10 production. Also in patients

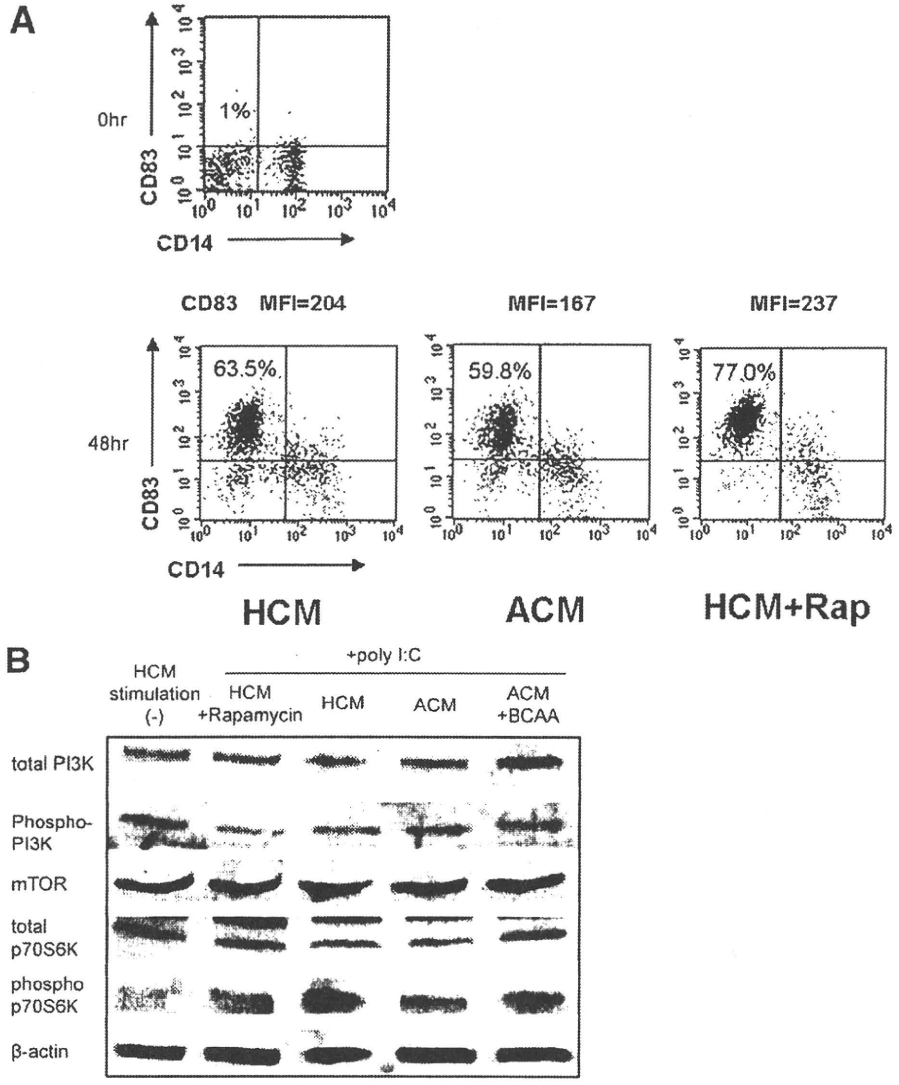

Fig. 3. Amino acid imbalance in plasma of patients with advanced cirrhosis down-regulated the mTOR/S6K signaling pathway of BDCA1+ DCs. (A) We stimulated BDCA1+ DCs under HCM, ACM, and HCM plus rapamycin (500 nM) for 24 hours with GM-CSF and IL-4, and exposed them to poly(I:C) for an additional 24 hours. We evaluated the phenotypes of DCs by flow cytometry. The percentages indicate the proportion of cells adopting the DC immunophenotype (CD14−/CD83+). (B) We cultured BDCA1+ DCs under HCM and ACM for 24 hours with GM-CSF and IL-4 and stimulated them with poly(I:C) for 1 hour. We also evaluated HCM plus rapamycin, and ACM plus BCAA. Equal amounts of protein were loaded and the levels of PI3K, phospho-PI3K, mTOR, p70 S6K, and phosho-p70 S6K were determined by Western blot analysis. (A,B) Data shown are representative of four independent experiments with cells from different donors.

with cirrhosis, the CD83 and CD86 expression of DCs stimulated under ACM was lower than that under HCM. When compared under the same medium, the CD83, CD86, and HLA-DR expressions of DCs from LC were lower than those from DCs of HC. To summarize these results, in advanced cirrhosis not only the DCs themselves but also the extracellular environments tend to impair the maturation of DCs.

Second, we examined which amino acids more strongly influences the function of DCs between HCM and ACM. We found that BCAA, especially valine and leucine, increased the BDCA+ DC allostimulatory capacity and IL-12 production. This confirms the findings of our previous study,[15] although the enhancement by a single amino acid was very subtle. To obtain greater enhancements, we may need to use combinations of other amino acids.

Concerning the mechanism that underlies these phenomena, we confirmed that the CD98 expression of DCs were not different between HCM and ACM. CD98 can regulate the expression and distribution of the light chains to modulate the amino acid transport functions. CD98hc is highly expressed on proliferating lymphocytes and on other rapidly growing cells.[27] Next, we examined whether the amino acid imbalance in the plasma of patients with advanced cirrhosis influenced the mTOR/S6K signaling pathway of the DCs. Recently, some studies reported the PI3K-mediated negative feedback regulation of IL-12 production in DCs,[28] and rapamycin-enhanced IL-12 production in LPS-stimulated DC.[29,30] In the present study, BDCA+ DCs stimulated in ACM impaired IL-12 production, even though the mTOR signaling was decreased. This paradox raises the possibility that the amino acid imbalance influences not only mTOR signaling but also other types of signaling such as GSK3 or NF-κB signaling. This issue should be evaluated in future studies.

Finally, we investigated whether elevating the level of plasma BCAAs enhances the immune response *ex vivo* in

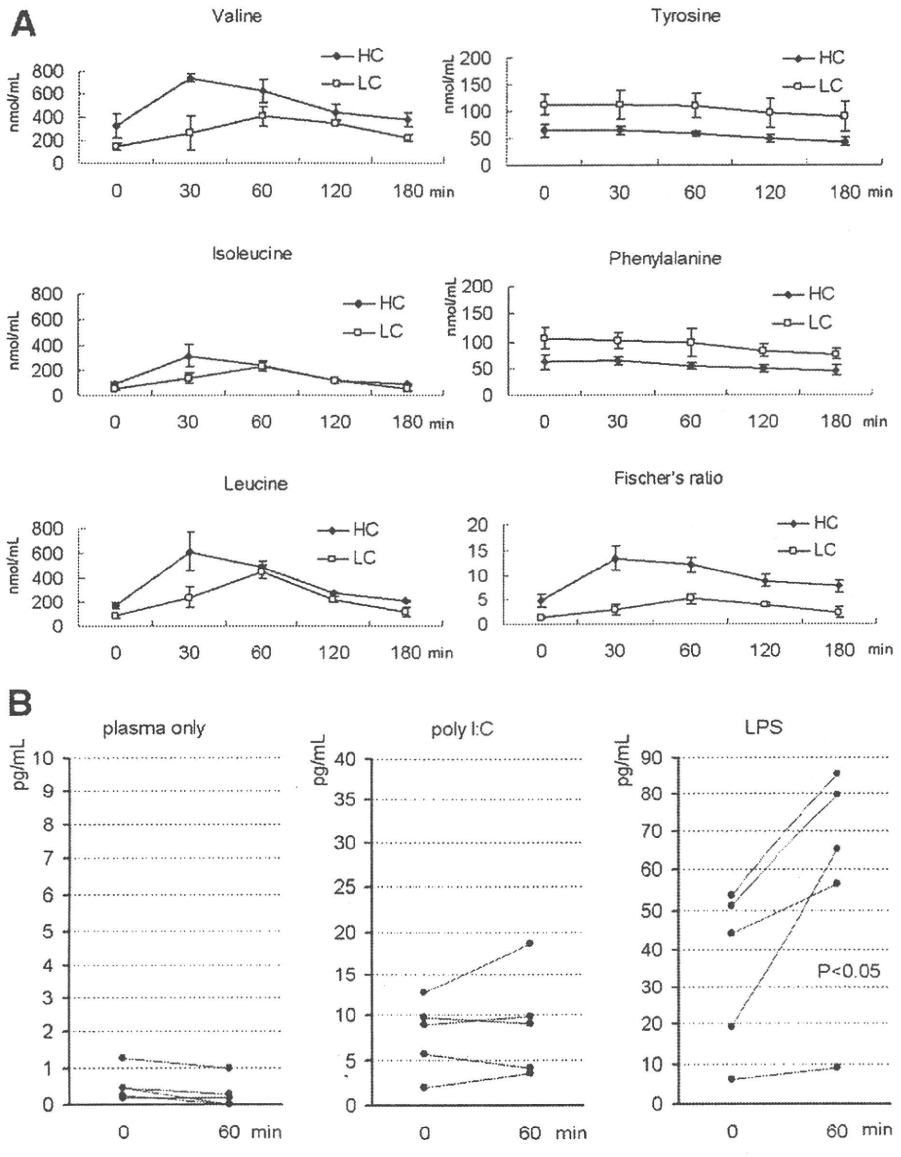

Fig. 4. Oral administration of BCAA granules enhanced the production of inflammatory cytokines from PBMCs stimulated by LPS *ex vivo*. (A) We analyzed the kinetics of the plasma amino acids after oral administration of BCAA granules. In the early morning while fasting, the concentrations of plasma amino acids were measured before and after oral administration of BCAA (30, 60, 120, 180 minutes). Mean ± SD values from three different HC and four patients with advanced cirrhosis (Patients 10-13). (B) We stimulated PBMCs from the patients using either autologous plasma before or after 60 minutes oral administration. After 12 hours we recovered the plasma and measured the IFN-γ by ELISA (Patients 11-15). $P < 0.05$ (paired Student's t test, two-tailed).

patients with advanced cirrhosis. BCAA granules have been used to effectively reverse the hypoalbuminemia and hepatic encephalopathy in patients with advanced cirrhosis.[31] In the preliminary investigation, we analyzed the kinetics of plasma amino acids after oral administration of BCAA granules. After oral administration, the BCAA concentration in plasma was maximal at 30 minutes in healthy volunteers. This was in contrast to patients with advanced cirrhosis, who had a slow increase in BCAA plasma concentrations that was maximal at 60 minutes. This difference was probably caused by the malabsorption of amino acids in the patients. In the *ex vivo* study, we could not use the medium to analyze the function of DCs of PBMCs because the concentration of the amino acids in medium influences the function. Thus, we stimulated cells in autologous plasma and analyzed the function over a short period of time. We found that oral administration of BCAAs enhanced the production of IFN-γ from PBMCs *ex vivo* in patients with advanced cirrhosis.

The results of this study still cannot be construed as conclusive evidence of a change in the functional clinical state in terms of lowering the risk of sepsis in cirrhosis or enabling consideration of such treatment for viral hepatitis. We need to perform a prospective, randomized, controlled trial in a well-characterized group of patients with appropriate immune mechanistic evaluation and determine the effects on the risk of sepsis in a longitudinal follow-up. In the present study we demonstrated at least that extracellular amino acids, especially BCAAs, influence the function of the immune system, and the amino acid imbalance in the plasma of patients with advanced cirrhosis impaired the maturation of DCs and the production of inflammatory cytokines from PBMCs or DCs.

In conclusion, the data from this study provide a rationale for future studies utilizing nutrition therapies that could be beneficial to immune function in patients with advanced cirrhosis.

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特集II 肝硬変・肝癌の栄養代謝異常とその対策

肝硬変・肝癌の栄養代謝異常とその対策「非代償性肝硬変におけるアミノ酸imbalanceが免疫機構に及ぼす影響」*

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Key Words : dendritic cell, decompensated cirrhosis, BCAA, nutrition, Immunology

はじめに

肝臓は栄養代謝をつかさどる最重要臓器であり，肝硬変では，糖・蛋白・アミノ酸・脂質・ビタミン・ミネラルなどきわめて多岐にわたる栄養代謝障害が出現する．特に非代償性肝硬変・肝不全へと進行するにつれそれらの栄養代謝異常は著明となる．アミノ酸に関しては，以前より肝硬変が進行するにつれ血漿中の分岐鎖アミノ酸(BCAA)が低下し芳香族アミノ酸(AAA)が増加することが知られている[1][2]．肝硬変でアミノ酸imbalanceが出現する詳細な機序は現在でも明らかではないが，AAAの上昇は肝臓での代謝能の低下による結果とされ，BCAAの低下は肝硬変では肝臓に効果的なグリコーゲンの貯蔵が行われず，骨格筋での糖の取り込みが低下し，BCAAがエネルギー器質として利用されることによる結果と考えられている．

非代償性肝硬変患者の三大死因は，①肝細胞癌，②食道静脈瘤破裂，③肝不全であり，肝不全に関しては肝性脳症と感染症が死因の大部分を占める．肝硬変患者は易感染性宿主であり，免疫機構の機能低下が出現していることは疑いようのない事実である．

免疫機構は多種多様な免疫細胞のネットワークにより構築される．自然免疫・獲得免疫の橋も重要な細胞の一つである樹状細胞(DC)は1973年にロックフェラー大学のスタインマン博士らによって発見された抗原提示細胞であり，全身の臓器・組織・器官に存在し病原体や腫瘍に対して免疫反応を誘導・仲介する．典型的には，未熟DCは抗原を捕獲しペプチドに分解し，主要組織適合性複合体(MHC)クラスIIもしくはI分子により提示し成熟化(活性化)する．通常，末梢組織に存在するDCは侵入抗原から刺激を受けたあと，所属リンパ節に移動して抗原をリンパ球に提示する[3]．最近の報告では，末梢組織から再度血中に戻り他の遠隔組織に移行するcirculating DCの存在が示唆されている[4]．現在，基礎的研究では分子生物学的手法を用いた樹状細胞の細胞内シグナル解析や共刺激分子を介した他細胞との詳細な抗原提示メカニズムまで解析が進められている．

非代償性肝硬変におけるアミノ酸imbalanceがひき起こす病態として，これまで肝性脳症，低アルブミン血症がよく知られているがこれらに関しては他の総説[5]を参考にされたい．本稿ではわれわれの研究室で中心的に研究を進めている非代償性肝硬変におけるアミノ酸imbalanceが免疫細胞，特に樹状細胞に与える影響を中心に解説する．

* An imbalance of plasma amino acids influences the immune mechanism in patients with decompensated cirrhosis.

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表1　各施設の肝硬変・肝臓癌患者の樹状細胞機能低下

発表者	機能異常	掲載
·Ninomiya T	リンパ球刺激能・IL-12産生低下・未熟DCのHLA-DR発現低下	J Hepatol 1999 ;31 : 323-31.
· Kakumu S	リンパ球刺激能・IL-12産生低下	J Gastroenterol Hepatol 2000 ; 15 : 431-6.
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· Li L	成熟化抑制	Immunol Lett 2007 ; 114 : 38-45.
· Ohno T	IFN-γの産生低下	Hepatol Res 2007 ; 37 : 276-85.
· Kakazu E	リンパ球刺激能・成熟化抑制(刺激後のCD40 CD83 HLA-DR低下)	J Immunol 2007 ; 179 : 7137-46.

非代償性肝硬変ではどのような樹状細胞の機能抑制が生じるのか

肝硬変ではさまざまな免疫細胞の機能低下がこれまで報告されている[1]~[9]．樹状細胞に関しては，1999年にKantoらが，慢性C型肝炎患者の樹状細胞機能低下を報告[1]して以降，各種肝疾患と樹状細胞機能低下について盛んに研究がなされた．肝硬変または肝臓癌では，樹状細胞の細胞数低下，分化・成熟抑制による共刺激分子・MHCクラスII発現低下，リンパ球刺激能の低下が生じることが各施設より報告され，肝硬変の進行・発癌により樹状細胞の数・機能低下が明瞭となる可能性が示唆される(表1)．われわれの施設での結果も，非代償性肝硬変患者の樹状細胞はLPSで刺激した場合に健常人よりも成熟化(活性化)が抑制されていることを報告した[10]．

非代償性肝硬変で出現するアミノ酸imbalanceが樹状細胞にいかなる影響を与えるのか

肝硬変とは，さまざまな慢性肝疾患の最終的な病態のことであり，その病因(HCV, HBV, アルコール，NASH, 肝臓癌の有無など)が樹状細胞に与える影響を解析することが優先される．しかし，肝硬変という病態がひき起こす細胞外環境の変化が樹状細胞に与える影響も合わせて解析する必要性がある．たとえば，樹状細胞の培養にはヒト・ウシ血清を10%程度添加した血清培地がよく用いられるが，そのロットにより解析の結果が大きく異なることをよく経験する．これは血清にはさまざまなサイトカインや未知の活性化物質が含まれ機能修飾を受けるからである．つまり，培地に添加する糖・アミノ酸・アルブミン・脂質・PH・電解質などさまざまな細胞外の要素により樹状細胞の機能は修飾を受けるため，この点を考慮しなければ肝硬変の病態が樹状細胞機能に与える影響を説明するのは困難である．現在われわれの施設では，非代償性肝硬変で出現するアミノ酸imbalanceに注目し，健常人と非代償性肝硬変の血漿中アミノ酸濃度に一致させた2種類の無血清培地(HCM：Healthy control medium, ACM：Advanced cirrhotic mediumu)を作製し(表2)，より生体内に近い環境で樹状細胞を中心とした免疫細胞機能の解析を進めている．非代償性肝硬変の血

表2　当研究で使用した無血清培地のアミノ酸組成(単位nmol/ml)

	CCM	HCM	ACM
Glycine	400	225	280
L-Alanine	400	391	307
L-Serine	400	119	151
L-Threonine	800	142	138
L-Cystine 2HCl	200	38	67
L-Methionine	200	29	75
L-Glutamine	4,000	564	689
L-Asparagine	400	51	64
L-Glutamic Acid	400	42	53
L-Aspartic Acid	400	3	4
L-Valine	800	249	175
L-Leucine	800	132	100
L-Isoleucine	800	76	53
L-Phenylalanine	400	63	99
L-Tyrosine	400	65	133
L-Tryptophan	80	62	45
L-Lysine-HCl	800	183	184
L-Arginine-HCl	400	78	92
L-Histidine HCl-H2O	200	83	85
L-Proline	400	204	176
Fischer's ratio	3.00	3.57	1.42

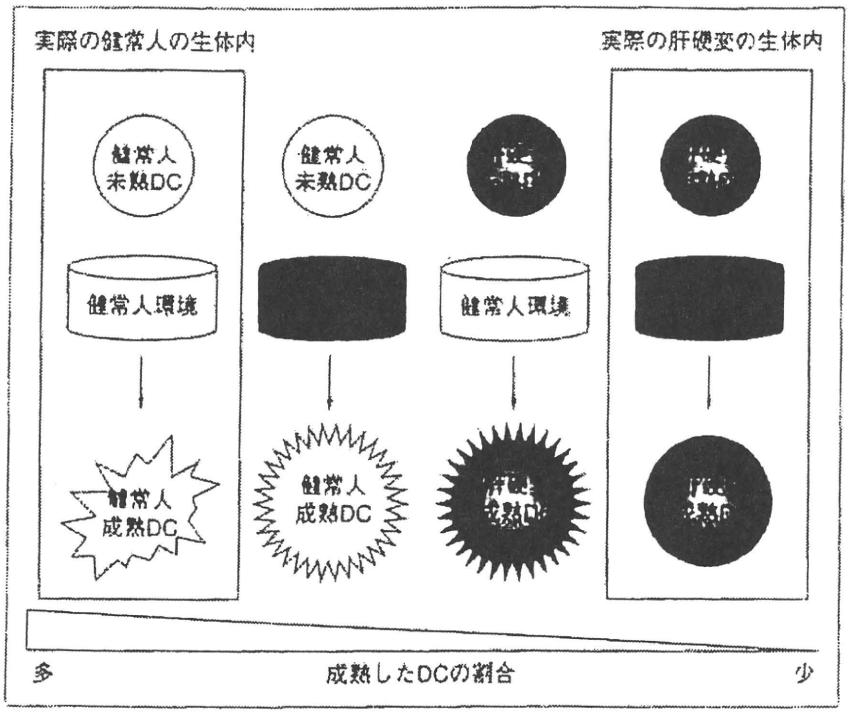

図1　非代償性肝硬変の生体内での結果
非代償性肝硬変では樹状細胞自体の成熟化が抑制されているだけでなく周囲のアミノ酸imbalanceが樹状細胞の成熟化を抑制する．

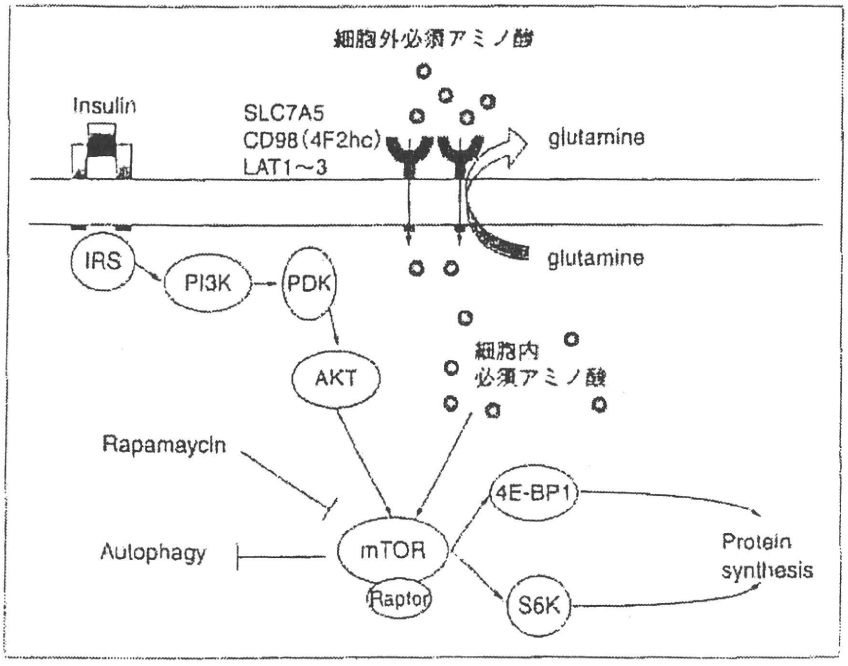

図2　mTORを中心とした栄養感受性シグナル

漿中アミノ酸濃度と一致した培地において，樹状細胞の成熟抑制(CD83の発現低下)・IL-12産生が低下すること，さらにこのことにより末梢血単核球(PBMC)からのIFN-γの産生が低下することが明らかとなった．これまでの結果から，非代償性肝硬変では樹状細胞自体の機能抑制が起きるだけ

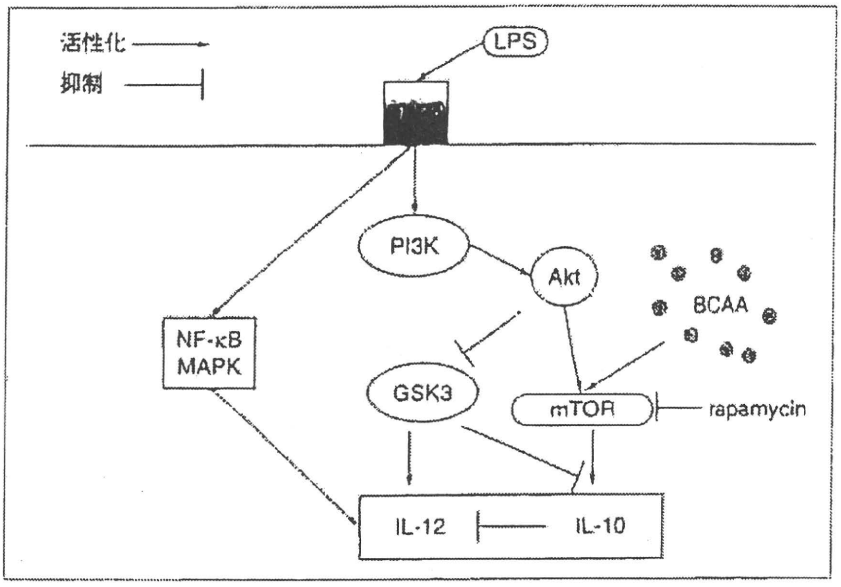

図3　樹状細胞のサイトカイン産生とmTORシグナルの関係

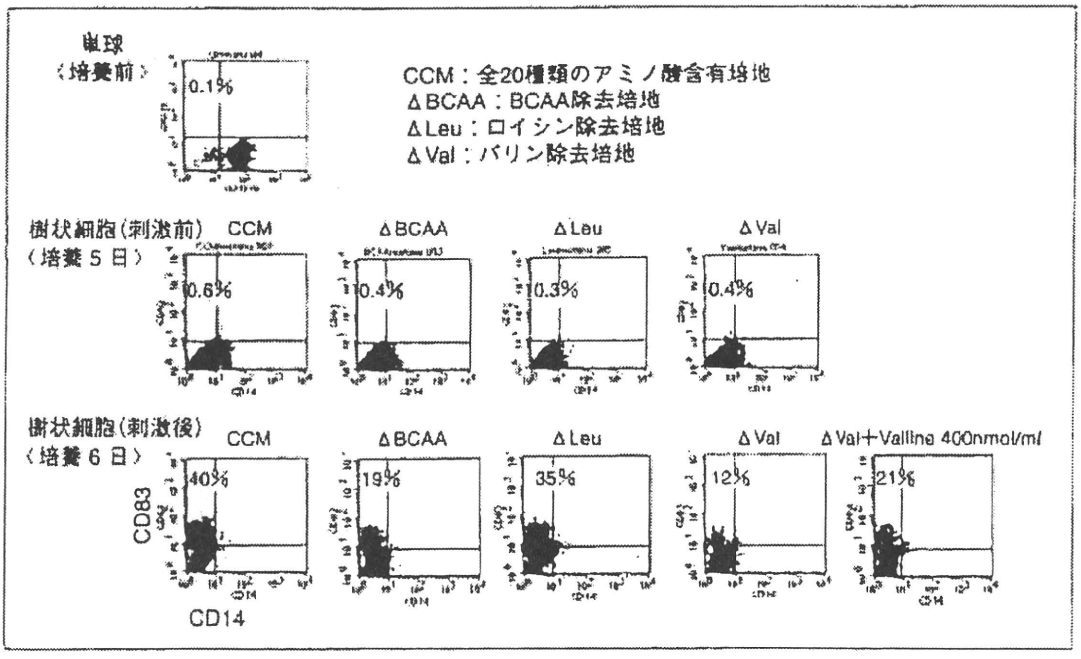

図4　分岐鎖アミノ酸の単球由来樹状細胞の分化・成熟に与える影響
%は成熟樹状細胞の割合を示す．(文献[10]より引用改変)

でなく，細胞外のアミノ酸imbalanceが樹状細胞機能を抑制することが明らかとなった(図1)．

非代償性肝硬変のアミノ酸imbalanceが樹状細胞機能を抑制するメカニズム

近年，アミノ酸はただ単に細胞骨格やさまざまな代謝経路の基質となるだけでなく，インスリンとともに栄養感受性シグナル経路に作用し，細胞機能を調節することが解明されてきている[11〜13]．その代表的なものとしてmTORシグナルが知られているが，mTORシグナルはDNAの転写・翻訳の制御，細胞の大きさや細胞骨格の再構成の制御，自食作用の制御などに関係している．これまでの報告では，分岐鎖アミノ酸の中でL-ロイシンが

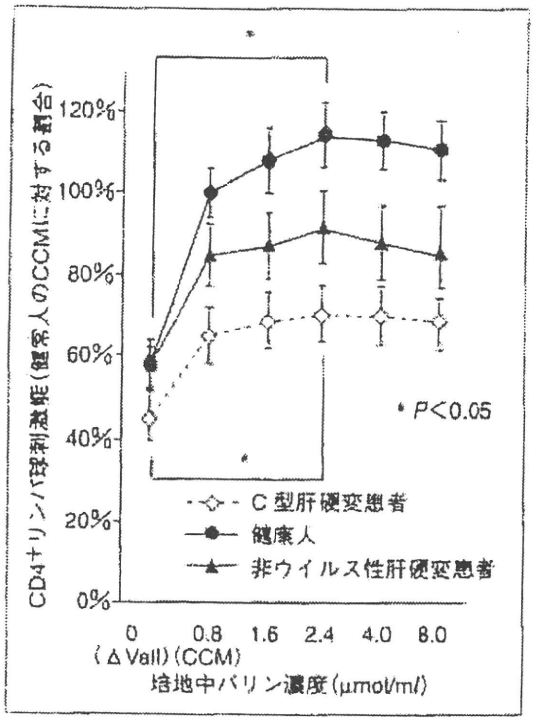

図5　培地中のバリン濃度に対する健常人と肝硬変患者の樹状細胞リンパ球刺激能
（文献[10]より引用改変）

mTORシグナルに深く関与しているとされており[14]，さらに最近の報告では，細胞内のグルタミンが細胞外に排出するのに合わせてロイシンを含めた必須アミノ酸が細胞内に流入しmTORを活性化することが明らかになっている[15]（図2）．一方で樹状細胞とmTORに関しては，mTORの阻害剤であるラパマイシンで樹状細胞を処理するとアポトーシスが誘導されたり[16]，LPSで刺激した際にIL-12産生が亢進しIL-10産生が抑制されるとの報告がある（17, 18）．われわれの検討では，非代償性肝硬変患者で出現するアミノ酸環境は樹状細胞のmTORシグナルを抑制すると同時にIL-12の産生が低下し，BCAAを添加することで部分的に改善することが明らかとなった．この結果はこれまでの報告と相反するが，BCAAはmTOR以外のシグナルにも影響している可能性がある（図3）．

非代償性肝硬変に対するBCAA製剤の免疫学的な有効性

肝硬変患者のアミノ酸代謝異常を補正するBCAA製剤は最も中心的な栄養療法の一つである．必須アミノ酸のL-ロイシン，L-イソロイシン，L-バリンから構成され，肝性脳症・低アルブミン血症・腹水出現予防などの効果が得られる．しかし，そのメカニズムに関しては依然として不明な点が多く，特に分岐鎖アミノ酸が免疫機構に与える影響については現在までほとんど知られていない[19]．過去の報告では，BCAAの制限食を与えたマウスと制限しないマウスに*Salmonella typhimurium*を感染させた場合に，前者で死亡率が有意に上昇す

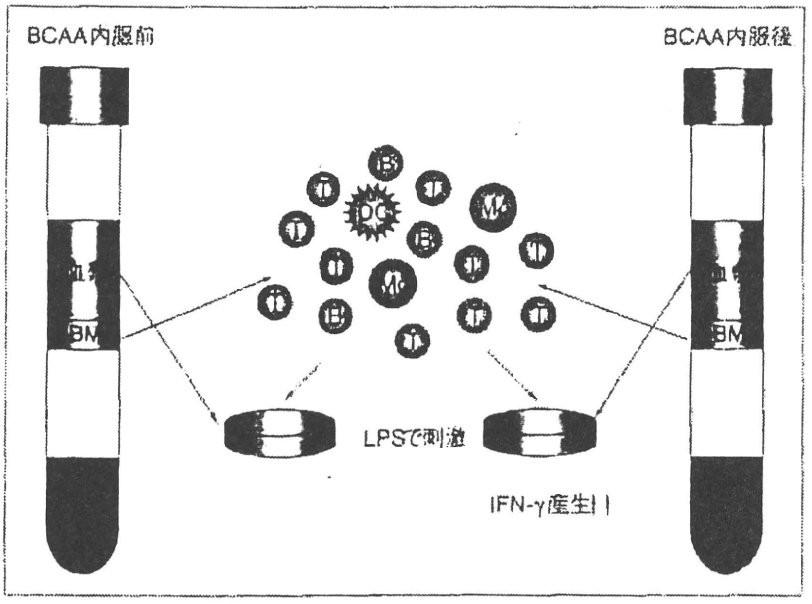

図6　*ex vivo*におけるBCAAの免疫賦活作用の検討