網状細胞機能のアミノ酸依存性と肝癌患者における網状細胞機能回復の試み

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平成17～18年度科学研究費補助金 基盤研究(C)研究成果報告書

平成19年1月

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はしがき

肝硬変を含む慢性肝疾患においては樹状細胞機能など免疫能の低下が報告されており、これは特発性細菌性腹膜炎などの感染症の合併につながったり、QOLの低下や生命予後の悪化につながると考えられ、更には肝細胞癌の発生・再発にも関与していると考えられる。したがって、こうした状況を打開していくためには、低下した免疫能を回復される種の方策が重要と思われるが、今回我々は樹状細胞機能の回復の上でアミノ酸が重要な役割を担っているという点に着目し、核腫アミノ酸、特に吻示唆アミノ酸の血球凝集阻止と樹状細胞機能における役割、そのメカニズムの検討を進めていくとともに、実際臨床の場面でどのアミノ酸が、あるいはどのアミノ酸の組み合わせが樹状細胞機能回復の上で有用であるかを、実際の肝硬変患者の末梢血から採取・誘導した樹状細胞を用いて検討し、臨床につなげていくことを目的とした。

H17 年度の基礎検討により、分枝鎖アミノ酸が樹状細胞の生存と機能の療法に必要であることを見出した。特にバリンについては多くの生理作用を有していることが、表面マーカーの解析とリンパ球との混合培養といった点から明らかになった。

平成 18 年度は、更にその細胞内での機序を明らかにするため、mTOR などの分子に着目して、そのシグナル伝達いった点から検討した。

1)H17 年度確立した培養法を用いて、ヒト(健常者および肝疾患患者)より樹状細胞を分離して、様々なアミノ酸組成による培養を行うが、樹状細胞の成熟・分化の過程で、mTOR や細胞内のリン酸化酵素の状態を、例えば p70 Kinase assay などを用いて解析した。

2)慢性C型肝炎におけるインターフェロンを受けている患者における樹状細胞について検討し、アミノ酸がこれらの患者の樹状細胞にいかなる作用を持つかを検討した。

3)これらのアミノ酸投与による樹状細胞機能の回復がヒト肝癌の発症低下につながるかについて検討するための至適モデルの作成についての基礎検討をおこなった。

研究組織

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(3) **出版物**

無し

研究成果による工業所有権の出願・取得状況

出願中

研究成果

(次頁以降)

Abstracts

The functions of dendritic cells (DCs) are impaired in patients with liver cirrhosis. It is well known that cirrhotic patients show decreased levels of plasma branched-chain amino acids (BCAA). Although amino acids are associated with maintaining the cell structure and function in many organs, limited data are available regarding the role of amino acids including BCAA in the immune system. We aimed to investigate the roles of BCAA in the function of human monocyte-derived DCs (MoDC). CD14-positive monocytes (CD14 (+)) were isolated from PBMC from healthy volunteers and HCV cirrhotic patients. In medium deprived of BCAA or valine, monocytes were able to differentiate into immature DCs, but not into mature DCs, and showed their weak expression of CD83. The deprivation of leucine or isoleucine did not affect this process. The MoDC allo-stimulatory capacity was significantly decreased in medium deprived of BCAA or valine. Annexin V-FITC/PI staining showed the numbers of dead and apoptotic cells were not significantly different under any medium. Immunoblotting demonstrated that depletion of valine or leucine decreased P-S6 kinase expression. Valine increased dose-dependently the allo-stimulatory capacity and IL-12 production of MoDC from both healthy volunteers and HCV cirrhotic patients. An elevated extracellular concentration of valine could improve dendritic cell function in HCV cirrhotic patients. These data provide a rationale for nutrition therapy that could be beneficial to patients with cirrhosis.

Key Word: Dendritic cells, BCAA, Valine, liver cirrhosis, nutrition,

Introduction

Hepatitis C virus (HCV) induces chronic liver disease in hosts, which can eventually progress to liver cirrhosis and chronic liver failure. Combination therapy with peginterferon and ribavirin has been shown to result in a sustained virological response (SVR) in about 50% of patients (1, 2), but others continue to be viremic and progress to advanced fibrosis and liver cirrhosis. The number of advanced liver cirrhotic patients has been increasing, but combination therapy is poorly tolerated under cirrhosis and the response rate is low (3). Bacterial infection, such as spontaneous bacterial peritonitis and pneumonia, is one of the most frequent causes of death in immune-compromised cirrhotic patients. In such cirrhotic patients, a decrease in the levels of plasma branched-chain amino acids (BCAAs) is one of the characteristic features (4, 5). BCAAs comprise the three essential amino acids L-leucine, L-isoleucine and L-valine. BCAA granules (a mixture of L-leucine, L-Valine and L-isoleucine) have been used to effectively reverse the hypoalbuminemia and hepatic encephalopathy in patients with decompensated liver cirrhosis (6, 7), but little is known about the impact of changes in the BCAA levels on the immune system (8). In previous cohort studies, the BCAA supplemented groups demonstrated elevations of the absolute lymphocyte count (9, 10). In previous *in vitro* studies, the omission of a single BCAA from the medium of cultured lymphocytes resulted in the complete abolition of protein synthesis or proliferation (11-13). These bindings simply reflect the fact that BCAAs are essential cell components.

Recently, 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 (14). 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 effecter of cell growth and proliferation via the regulation of protein synthesis. The pathway is activated BCAA, especially leucine (15-17). mTOR was identified and cloned (18-20) shortly after the discovery of the two yeast genes, TOR1 and TOR2, in the budding yeast *Saccharomyces cerevisiae* during a screening for resistance to the immunosuppressant drug rapamycin. Rapamycin introduced to prevent allograft rejection, has been extensively studied for its effect on T lymphocytes, and is primarily known for its anti-proliferative effect (21). Some studies have described that dendritic cell functions (viability, antigen uptake, cytokine production and allo-stimulatory capacity) are impaired by rapamycin (22-24).

Dendritic cells (DC) are professional APC that initiate and mediate immune responses against pathogens and tumors. Typically, immature DC capture and process antigen to peptides which are then presented in the context of major histocompatibility complex (MHC) class II or class I molecules. They migrate to lymphoid tissues and present antigenic peptides to naive T cells. The mature DC, which characteristically express CD83 (25), can rapidly activate other innate immune cells including NK and NKT cells through the production of immunomodulatory cytokines such as interleukin IL-10 and IL-12. Human DC can be generated in vitro from peripheral blood CD14 positive monocytes, termed monocyte-derived DC (MoDC) (26). The ability of monocytes to differentiate into DC was originally demonstrated by Sallusto and Lanzavecchia (27), who reported the generation of DC from human peripheral monocytes after in vitro culture with GM-CSF + IL-4. Despite *in vitro* experimental evidence on the potential of monocytes to differentiate into DC, whether this process occurs under physiological conditions is still controversial. In patients with chronic hepatitis C, the functioning of

MoDC has been studied by various groups. These studies indicated that MoDC of patients had lower allostimulatory capacity and IL-12 production than MoDC of healthy subjects (28-30). It is considered that HCV virus proteins impair the hosts' dendritic cell function (31).

In this study, we demonstrated that branched-chain amino acids, especially valine, influenced the function of MoDC. Increasing the extracellular concentration of valine could improve the dendritic cell function in HCV cirrhotic patients.

Materials and methods

Patients and Healthy volunteers

Liver cirrhotic patients with chronic HCV infection were diagnosed for persistent positive HCV antibody and HCV-RNA in the serum. Also, five healthy volunteers were recruited to obtains MoDC. 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).

Monocyte isolation and dendritic cell generation

PBMC were separated from the peripheral blood of healthy volunteers and HCV cirrhotic patients by centrifugation on a density gradient (Ficoll-Paque Plus, Amersham Biosciences). The CD14-positive monocytes (CD14 (+)) were isolated from PBMC using magnetic microbeads (Miltenyi Biotec, Bergish Gladbach). CD14(+) were cultured at a density of 3.0×10^6 cells/well in 24-well flat-bottom plates (FALCON, Franklin Lales, NJ) for 5 days in the following culture medium: amino acid-free medium (D-MEM deprived of all amino acids) supplemented with 2 g/L bovine serum albumin (SIGMA, St. Louis, MO), 1,000 U/mL GM-CSF (PEPROTECH EC, London, UK), 500 U/mL (hu) IL-4 (PEPROTECH EC), 3.5 g/L glucose (SIGMA), 6 g/L HEPES (SIGMA), 1% Insuline-Transferin-Selenium-X (GIBCO, Grand Island, N.Y.), and variously conditioned amino acids. The culture medium containing all twenty kinds of amino acids was defined as the complete culture medium (CCM). The culture medium, in which all amino acids, BCAA, valine, leucine or isoleucine were removed from CCM, was defined as Zero, ΔBCAA, ΔVal, ΔLeu or ΔIle, respectively (Table I). At day 5, 20 ng/mL TNF-α (R&D systems, Minneapolis, MN) and 500 ng/mL LPS (Escherichia coli 026:B6 (SIGMA)) were added and the culture was continued for an additional 24 hours.

The differentiative capability of monocytes into mature dendritic cells under CCM was confirmed as previously reported. CD14 (+) monocytes expressed high levels of CD14, HLA-DR and CD86, and negligible levels of CD83. Immature DC expressed lower levels of CD14 and CD86, and higher levels of HLA-DR and CD40, but they did not express CD83. Mature DC showed the upregulation of costimulatory molecules (CD40, CD80 and CD86). These cells were also characterized by the induction of CD83 expression on their cell surface.

MoDC surface marker analysis and viability assay.

On day 5 or 6 of culture, MoDC were harvested, and labeled with FITC- or PE-labeled mAbs (anti-human CD14, CD40, CD80, CD83, CD86 HLA-DR, or relevant isotype controls: BD PharMingen, San Diego, CA), according to the manufacturer's directions. Briefly, cells (1×10^6) were incubated with 20 dL of Ab in a total volume of 100 dL (PBS) for 30 min at 4°C in the dark. Using a FACS Calibur (BD Immunocytometry Systems, San Diego, CA) flow cytometer, the cells were gated according to their size (forward light scatter), granularity (side light scatter), and surface marker expression and analyzed using the CellQuest (BD Immunocytometry Systems) programs. On 6 day, the viability of MoDC was determined using Annexin V^{FITC}, with dead cells identified by propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit, BioVision, Mountain View, CA), according to the manufacturer's directions.

Mixed leukocyte reaction and Cytokine analysis

CD14+ monocytes isolated by MACS were cultured at a density of 1.0×10^5 cells/well in 96-well round-bottom plates (FALCON) containing 200 dL of various concentrations of valine or leucine in medium supplemented with 1000 U/mL GM-CSF, 500 U/mL IL-4 and for the generation of immature DC. On day 5, immature DC were matured using

500ng/ml of LPS and 20ng/ml TNF-α for 24hr. On day 6, the allostimulatory capacity of 5.0×10^4 irradiated DC (3000 Rad) was tested in a one-way MLR with normal, allogeneic T lymphocytes (1×10^5cells/well) in duplicate or triplicate. Co-culture cells were maintained for 4 days at 37°C in a 5% CO2 humidified atmosphere. The proliferation rate of the cells was measured using an MTS Assay (CellTiter 96 aqueous one-solution cell proliferation assay, Promega; Madison, WI). Forty μL of CellTiter 96 aqueous one-solution were added to each well. After 2 h of incubation, UV absorbance of the solution was measured at a wavelength of 490 nm. All MTS assays were done in triplicate. Supernatants were collected on day 6 and immediately IL-12 (p40+p70) and IL-10 were determined by specific cytokine ELISA kits (Bender MedSystems) according to the manufacturer's instructions.

Immunoblotting

On day 6, MoDC were harvested and lysesd using CelLyticTM-M Mammalian Cell Lysis/Extraction Reagent (SIGMA). The lysed cells were centrifugated for 10 minutes at 12,000-20,000 × *g* to pellet the cellular debris. Thereafter, these protein concentrations were determined by a Modified Lowry Protein Assay Kit (PIERCE, Rockford, IL). The total 50μg of protein were loaded onto SDS-PAGE gel, and electro-transferred to PVDF (Immun-Blot PVDF Membrane, BIO-Rad, Hercules CA). After washing, the membranes were incubated in 25 ml of blocking buffer for 1 hour at room temperature. Immunostaining was performed with primary antibody (Cell Signaling Technology, Beverly, MA), followed by incubation with a secondary antibody conjugated to HRP (SIGMA). Immunoreactive proteins were revealed with an ECL reagent (ECL advance, Amersham Biosciences, Little Chalfont, Buckinghamshire). To confirm the equal protein loading in all samples, the blot was stripped for 30 min in

antibody stripping solution (Re-Blot Plus Western Blot Recycling Kit, Chemicon International, Temecula, CA), washed extensively, and relabeled with anti β-actin antibody and secondary HRP antibody (SIGMA).

Aminogram

Twenty-seven liver cirrhotic patients who were positive for both anti-HCV Ab and serum HCV-RNA were measured for their concentrations of peripheral amino acids by high performance liquid chromatography and classified according to the Child-Pugh classification.

Statistical Analysis

The data were analyzed with ANOVA, and multiple comparisons were performed with Dunnett's post-hoc procedure. When 2 groups were analyzed, the differences between groups were analyzed by the Wilcoxon t-test. All data are expressed as mean SEM. In all analyses, a P value of less than 0.05 was considered statistically significant. All statistical analyses were performed with standard statistical software (SPSS 13.0 for Windows, Chicago, IL).

Results

Depletion of extracellular BCAA did not influence the expression of costimulatory molecules on MoDC, but decreased the expression of CD83

Firstly, to investigate whether the depletion of extracellular BCAA influenced the generation of MoDCs, we cultured the monocytes for 6 days under CCM and ΔBCAA. We evaluated the expression of CD14, CD40, CD80, CD83, CD86 and HLA-DR on the surface of MoDC grown under either CCM or ΔBCAA (Fig. 1A) by flow cytometry. There was no difference in the percentage of MoDC expressing CD14, CD40, CD80, CD86 and HLA-DR between the two mediums. Negligible levels of CD14 and higher levels of HLA-DR, CD40, CD80 and CD86 indicated that the cells could differentiate into MoDC in both mediums. However, CD83 expression was decreased under ΔBCAA as confirmed by the single color staining. To investigate which amino acid in BCAA especially influenced the MoDC phenotype, we determined the MoDC phenotype (CD14, CD83 or CD86) in Δvaline, ΔLeucine and ΔIsoleucine. In CCM, ΔLeucine and ΔIsoleucine, the MoDC phenotype was similar. However, in ΔValine, the CD83 expression by MoDC was significantly impaired compared to that in ΔBCAA. CD86 expression was not significantly different in any medium (Table II). On microscopic appearance, depletion of BCAA also affected the morphological appearance and behavior of the cells in culture. Monocytes cultured under either CCM, ΔLeu, or ΔIle were adherent with little tendency to form aggregations. On day 6, cells formed large, firmly adherent clusters (Fig. 1B), which were typical of matured DC *in vitro*. In contrast, monocytes cultured under ΔBCAA and ΔVal formed much smaller clusters.

Depletion of extracellular BCAA or valine influenced MoDC maturation but not

differentiation.

To further investigate at which point in time that amino acids influenced the MoDC phenotype, we cultured monocytes under CCM, ΔBCAA, ΔVal or ΔLeu, and determined the phenotype of MoDC before (day 5) and after (day 6) adding LPS and TNFα by two color staining (Figure 2). Most monocytes (>95%), which were freshly isolated from PBMC, expressed the CD14 positive and CD83 negative phenotype. Before adding the stimulus (day 5), the MoDC phenotype was CD14 negative and CD83 negative in all medium compositions. These data indicated that the monocytes could differentiate into immature DC in almost any medium. Interestingly, after adding the stimulus (day 6), under ΔBCAA and ΔValine, the percentage of CD14(-)/CD83(+) mature DC was lower than that under CCM or ΔLeu. These data indicated that depriving BCAA, especially valine, influenced the MoDC maturation. After we cultured monocytes under ΔVal for 5 days, we added 400 nM/mL valine with stimulus to the medium and cultured the cells for an additional 24h, then, the percentage of mature DC was higher than that of ΔVal. We cultured the monocytes under CCM for 5 days, and with an additional 24hr under ΔVal, the percentage of mature DC was decreased compared to that of CCM (data not shown).

Depletion of extracellular BCAA does not influence MoDC viability

To elucidate the possibility that impaired MoDC maturation under ΔBCAA or ΔVal was caused by decreased cell viability, we evaluated the actual viability of MoDC on day 6-day by Annexin V/Propidium Iodide staining. The percentages of dead cells, living cells and apoptotic cells were not different under any medium (Figure 3).

BCAA, especially valine, modulated monocyte-derived DC allostimulatory capacity and cytokine production

From the result that MoDC maturation was suppressed by the lack of extracellular BCAA, especially valine, we hypothesized that the concentration of extracellular BCAA could influence the function of MoDC. To investigate this hypothesis, we cultured monocytes for 6 days under CCM, Zero, ΔVal, ΔLeu, ΔIle and ΔBCAA. The MoDC (5.0×10^4) were cocultured with normal allogeneic CD4(+) lymphocytes under CCM, and evaluated for their allostimulative capacity by mixed leukocyte reaction (MLR). Expectedly, the allo-stimulatory capacity of MoDC cultured under ΔBCAA and ΔVal was significantly impaired, although there was no significant difference between ΔLeu and ΔIle, and CCM (Fig.4A). Furthermore, to examine whether the addition of valine enhanced the function of MoDC, we cultured monocytes under various mediums that contained 0-800 nM/mL valine or leucine, and evaluated the allostimulatory capacity of the MoDC. The addition of valine increased the allostimulatory capacity of MoDC in a dose-dependent manner. However, the concentration of leucine did not influence the pharmacological effect (Fig4B). Cytokines play key roles in determining the strength and the phenotypes of the T cell response. Thus, on day 6, we measured the cytokine production from MoDC. The addition of valine increased the IL-12 production of MoDC in a dose-dependent fashion. Interestingly, the IL-10 production was not influenced by the concentration of valine (Fig4C).

Depletion of extracellular valine downregulated mTOR/S6K signaling pathway of MoDC

The mTOR signaling pathway, one of the most representative pathways, has become known as a major effecter of cell growth and proliferation via the regulation of protein synthesis. A previous study showed that the removal of extracellular amino acids, especially leucine, inhibited the ability of mTOR to signal to p70 S6 kinase (15-17).

We hypothesized that BCAA modulate the mTOR/S6K signaling pathway on MoDC and influence the maturation markers. First, to investigate whether the mTOR/S6K inhibitor rapamycin could influence the MoDC phenotype, we cultured monocytes under CCM for 5days and added LPS and TNF-α to medium with or without rapamycin. Under CCM with rapamycin, the percentage of CD14-/CD83+ mature DC was lower than under CCM without rapamycin (Fig5A). These data indicated that rapamycin could suppress MoDC maturation similarly as when depriving them of BCAA or valine. Secondly, on day 6, we determined the expression of mTOR, p70 S6K, and phospho-p70 S6K by immunoblotting. MoDC expressed similar levels of mTOR and β-actin among all mediums. MoDC cultured in ΔBCAA, ΔVal and ΔLeu expressed lower levels of p-S6K than those cultured in CCM. Interestingly, MoDC cultured in ΔBCAA and ΔVal showed decreased S6K expression compared to the other mediums (Fig5B).

Elevating extracellular valine concentration increased allostimulatory capacity and IL-12 production dose dependently in MoDC of liver cirrhotic patients.

The functions of dendritic cell are impaired in HCV patients with liver cirrhosis (28-30). First, we measured the concentrations of valine in the peripheral blood of 27 patients by high performance liquid chromatography (HPLC). We confirmed that the concentrations of valine in their peripheral blood were decreased according to the Child-Pugh grade (Fig 6A). In Child-Pugh B or C patients, the concentrations of valine were significantly decreased as compared with those of healthy subjects. As shown in Fig4, we cultured monocytes under the medium that contained 0-800 nM/mL valine and evaluated the cytokine production and allostimulatory capacity by MoDC. The addition of valine increased the IL-12 production of MoDC in a dose-dependent

manner, and the IL-10 production was not influenced by the concentration of valine in the culture medium (Fig 6B). These tendency was similar to that found in healthy controls. Regarding the allostimulatory capacity, the values were maximum under 2.4 μM/mL of valine, and those of cirrhotic patients were lower than those of healthy control (Fig 6C).

Discussion

In this study, we showed that branched-chain amino acids, especially valine, influenced the function of monocyte-derived dendritic cells. The culture of human monocyte-derived dendritic cells is typically performed in medium containing human or fetal calf serum supplements. Medium that contains serum varies in its concentration of amino acids according to each lot number, which can influence the phenotypes of the cells and their functional properties. Thus, the current study evaluated the concentrations of the amino acids strictly (detail in material and methods).

First, we found that depletion of extracellular BCAA did not influence the expression of costimulatory molecules (CD40, CD80 and CD86) on MoDC, but decreased the CD83 expression. Human CD83 is a 45 kDa glycoprotein which belongs to the immunoglobulin superfamily. CD83 is expressed on monocyte-derived DC after stimulation with inflammatory cytokines (29), and CD83 is considered as a maturation marker. Although the function of CD83 is still unknown, inhibition of CD83 expression, by interfering with a specific RNA exporting pathway, leads to a dramatic reduction of the DC-mediated T-cell stimulation (32). This study supports our hypothesis that the impaired allo-stimulatory capacity of MoDC cultured in medium deprived of valine was caused by the lower expression of CD83. We also found that during MoDC generation, depletion of extracellular BCAA, especially valine, did not influence the differentiation but impaired the maturation of MoDC. Moreover, this phenomenon were accompanied by a suppression of the mTOR/S6K signaling pathways. Monti P et al. recently reported that rapamaycin treated DC were less capable to up-regulate CD83 after exposure to CD40L (22). This observation partially supports our result, although the relation between mTOR/S6K signaling and CD83 expression

should be evaluated in different studies. In general, although the depletion of leucine is believed to suppress the mTOR/S6K pathway, in the current study the depletion of valine showed a greater suppression of P-S6K. Also, depletion of leucine suppressed P-S6k, whereas the depletion of valine decreased the expression of total S6K protein. This observation could have resulted from differences in the cell sources evaluated. We next showed that the addition of valine increased the IL-12 production by MoDC in a dose-dependent manner, and that the IL-10 production was not influenced by the concentration of valine in both healthy controls and patients. IL-12 is an interleukin that is naturally produced by macrophages, B-lymphoblastoid cells and dendritic cells in response to antigenic stimulation. It is involved in the differentiation of naive T cells into Th1 cells, which is important in the resistance to foreign pathogens. IL-10 is naturally produced by monocytes and type 2 helper T cells. It is believed to have important suppressive functions on immune responses and also may be involved in the maintenance of tolerance. Our results raised the possibility that elevating the extracellular valine concentration could modulate Th1/Th2 differentiation in both healthy subjects and patients. To examine this possibility, it was necessary to coculture MoDC and naive CD4 T-cells, and determine the phenotype of T-cells and cytokine production. In addition, we found that depriving extracellular valine decreased MoDC IL-12 production with impaired mTOR/S6K signaling. In a previous study, active S6K1 suppressed the PI3K-Akt pathway by inactivating insulin receptor substrate (IRS) (33), whereas PI3K negatively regulated IL-12 synthesis by DCs (34). These results permit us to speculate that valine influence MoDC IL-12 production through the PI3K/mTOR/S6K pathway.

In this study, we found that an increased concentration of valine could recover the

impaired function of DCs in cirrhotic patients. However, the degree of this improvement was very subtle, which lead to the speculation that persistent HCV infection itself could suppress the function of DCs in such patients. The allostimulatory functions of DCs were maximum at a considerably higher than physiological concentration in both normal subjects and patients. However, the concentrations of valine in either the liver, portal blood flow, or lymph nodes could be higher than that in the peripheral blood. This issue should be evaluated in different studies. Furthermore, the changes of this allostimulatory capacity demonstrated were most apparent at ranges near the physiological concentration in peripheral blood. Recently, Osugi et al. showed several differences between moDC and the myeloid DC present *in vivo* (35). On the other hand, monocytes could differentiate into DC in vivo (36-38). Further evaluations using circulating DC will be needed to clarify this issue. As previously described in a review (8) a BCAA study demonstrated that these amino acids were essential for the synthesis of proteins required for cellular proliferation. However, there is little information from cell-culture studies regarding the immunologic effect of variations in the BCAA concentrations at ranges that might occur physiologically or pathophysiologically. In this study, we have demonstrated, i)depriving extracellular BCAA for 6days does not influence the MoDC viability; ii) depriving extracellular isoleucine did not decrease the allostimulatory capacity of MoDC iii) CD40,CD80,CD86 and HLA-DR molecules were equally expressed in both CCM and ΔBCAA medium iv) IL-10 production was not influenced by the extracellular valine concentration v) mTOR signaling was associated with decreased DC function in valine or leucine depletion.

These data suggest that BCAA are important for cell function through a

nutrient-sensitive signaling pathway rather than through acting as substrates for various metabolic pathways and cell structures. Also, it is preferable to measure the intracellular concentration of amino acids, although their uptake was reported to be sodium dependent. Finally, we need to clarify why the depletion of valine itself had a more potent inhibition on the allostimulatory function compared to depletion of all three BCAA components.

In clinical situations, the administration of BCAA was reported to increase the number of peripheral lymphocytes and improve opportunistic infections or immune-functions (9, 10). In advanced cirrhosis, 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 (7).

Our data provide the rationale for future nutrition therapy, which could be beneficial to patients with cirrhosis.

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	medium					
	Zero	CCM	ΔBCAA	ΔVal	Δleu	ΔIle
Glycine	0	400	400	400	400	400
L-Alanine	0	400	400	400	400	400
L-Serine	0	400	400	400	400	400
L-Threonine	0	800	800	800	800	800
L-Cystine 2HCl	0	200	200	200	200	200
L-Methionine	0	200	200	200	200	200
L-Glutamine	0	4000	4000	4000	4000	4000
L-Asparagine	0	400	400	400	400	400
L-Glutamic Acid	0	400	400	400	400	400
L-Aspartic Acid	0	400	400	400	400	400
L-Valine	0	800	0	0	800	800
L-Leucine	0	800	0	800	0	800
L-Isoleucine	0	800	0	800	800	0
L-Phenylalanine	0	400	400	400	400	400
L-Tyrosine	0	400	400	400	400	400
L-Tryptophan	0	80	80	80	80	80
L-Lysine-HCl	0	800	800	800	800	800
L-Arginine-HCl	0	400	400	400	400	400
L-Histidine HCl-H2O	0	200	200	200	200	200
L-Proline	0	400	400	400	400	400

Table I: Concentrations of amino acids in medium.

'Complete culture medium(CCM)' contains 20 amino acids that are relevant to the make-up of mammalian proteins. 'Zero medium' is deprived of all amino acids. 'ΔBCAA medium' is deprived of branched-chain amino acids (L-Valine, L-Leucine and L-Isoleucine). 'ΔVal medium', 'ΔLeu medium', and 'ΔIle medium' each deprived of L-Valine, L-Leucine and L-Isoleucine, respectively. Amino acid concentrations are expressed in nM/mL. We verified that there was no difference between the theoretical value and actual value by high performance liquid

chromatography.

Table II. Depletion of BCAA, especially valine, decreased MoDC CD83 expression.

		CCM (n=5)	ΔBCAA (n=5)	ΔVal (n=5)	ΔLeu (n=5)	Δlle (n=4)
CD14	%Positive cell	8.5 ±7.6	7.9 ±9.8	8.1 ±6.6	5.3 ±6.5	8.4 ±7.0
	MFI	5.2 ±2.5	6.2 ±4.0	5.3 ±2.1	4.0 ±0.9	5.5 ±1.4
CD83	%Positive cell	34.1 ±7.8	16.0 ±11.6 *	13.7 ±3.8 *	29.5 ±8.1	30.4 ±4.0
	MFI	10.9 ±2.1	7.2 ±3.2	6.0 ±2.4 *	8.2 ±2.1	9.5 ±2.6
CD86	%Positive cell	61.1 ±11.3	45.9 ±6.2	59.6 ±23.5	57.4 ±11.7	56.8 ±16.8
	MFI	29.8 ±14.0	29.3 ±13.0	28.1 ±13.7	30.5 ±21.6	26.8 ±14.9

* $p<0.05$ vs MoDC cultured under CCM (one-way ANOVA and Dunnett's post-hoc procedure).

Figure Legends

FIGURE 1. MoDC cultured in BCAA deprived medium express low levels CD83. For 5 days we cultured monocytes under CCM or ΔBCAA medium in 24-well tissue culture plates and exposed them to LPS and TNF alpha for an additional 24h. (A) Cells were harvested on day 6 of culture, stained with different mAbs and analyzed using flow cytometry. Cells were stained with FITC-labeled anti CD14, CD40, CD80, CD83, CD86 and HLA-DR. For the histogram figure, filled traces represent isotype-matched control Ab staining; open traces indicate a marker-specific Ab; percentages indicate positive cells. Results are representative of three experiments with three different donors. (B) Influence of BCAA on microscopic appearance of monocytes undergoing DC differentiation under serum-free conditions. Day6, cells in loosely adherent clusters in CCM, ΔLeu and ΔIle, which are typical of DCs that mature in vitro. In contrast, cells in ΔBCAA and ΔVal formed much smaller clusters.

FIGURE 2. BCAA, especially valine, is necessary for MoDC maturation but not for differentiation. Monocytes were cultured under CCM, ΔBCAA, ΔVal, ΔLeu medium as described in Fig. 1. (A) Cells were harvested on day 5 and day 6 of culture, stained with different mAbs and analyzed using flow cytometry. Cells were stained with FITC-labeled anti-CD14 and PE-labeled anti-CD83. For the dot-plot figure, percentages indicate proportion of cells adopting DC immuno-phenotype (CD14-/CD83+). Results are representative of four experiments with four different donors. (B) On day 6, Annexin V–FITC/PI staining was performed to determine the cell viability. Data shown are representative of 4 independent experiments with different donors.

FIGURE 3. Depletion of extracellular BCAA doesn't influence MoDC viability.

Monocytes were cultured under CCM, ΔBCAA, ΔValine and ΔLeucine medium as described in Fig. 1. Cells were harvested on day 6 of culture. Annexin V–FITC/PI (Propidium Iodide) staining was performed to determine the cell viability. On quadrant statistics, PI negative and annexin-V negative indicated live cells. PI positive (upper) indicated dead cells. PI negative and annexin-V positive (lower right) indicated apoptotic cells. Data shown are representative of 4 independent experiments with different donors.

FIGURE 4. BCAA, especially valine, modulated monocyte-derived DC allostimulatory capacity. (A) For 5 days, we cultured monocytes under each medium (detailed amino-acid composition is shown in Table I) in 96-well tissue culture plates and irradiated MoDC after exposing them to LPS and TNF-alpha for additional 24h. The yielded MoDC (5.0×10^4) were cocultured with normal, allogeneic T lymphocytes (1×10^5cells/well) under CCM for 4days and evaluated for their allostimulative capacity by mixed leukocyte reaction (MLR). (B) We cultured monocytes in the same way under various mediums that contained 0-800 nM/mL valine or leucine. Zero nM/mL valine or leucine medium are represented by ΔVal or ΔLeu, respectively. CCM contained 800 nM/mL of valine and leucine medium. (C) After 6 days, the supernatants were removed and assayed for the cytokine concentrations. Mean ± (A) SEM values from five different donors are shown. (B)(C) Mean ±SEM values from four different donors are shown. Statistical significance for all conditions was determined by one-way ANOVA and Dunnett's post-hoc procedure. ** $P<0.01$, * $P<0.05$ vs. CCM (A)(B) or vs. ΔVal (C).

FIGURE 5. Depleting extracellular valine or leucine downregulated the mTOR/S6K signaling pathway of MoDC. (A) For 5 days, we cultured monocytes

under CCM in 24-well tissue culture plates and exposed them to LPS and TNF alpha for an additional 24h in CCM, ΔVal and CCM + Rapamycin (1μM). Representative results of one of the subjects are shown. (B) Cells were harvested on day 6 and lysed. Equal amounts of protein were loaded and the levels of mTOR, S6K and p-S6K were determined by Western blot analysis. Data shown are representative of 4 independent experiments with different donors.

FIGURE 6. Elevating extracellular valine concentration increased MoDC allostimulatory capacity and IL-12 production dose dependently in liver cirrhotic patients. (A) 27 liver cirrhosis patients were classified by Child-Pugh classification. The level of serum valine in these patients was measured using high performance liquid chromatography. The dots represent the results from patients and the circles represent averages. (B) In the same way as in Fig. 3C, cytokine production was measured with ELISA. (C) We cultured monocyte under various mediums that contained 0-8.0 μM/mL valine. Zero nM/mL valine is indicated by ΔVal. 8.0 μM/mL valine medium is identical to CCM. (B)(C) Mean ± SEM values from four different patients are shown. Statistical significance for all conditions were determined by one-way ANOVA and Dunnett's post-hoc procedure. ** $P<0.01$, * $P<0.05$ vs. (A) healthy, (B) ΔVal, (C) CCM.

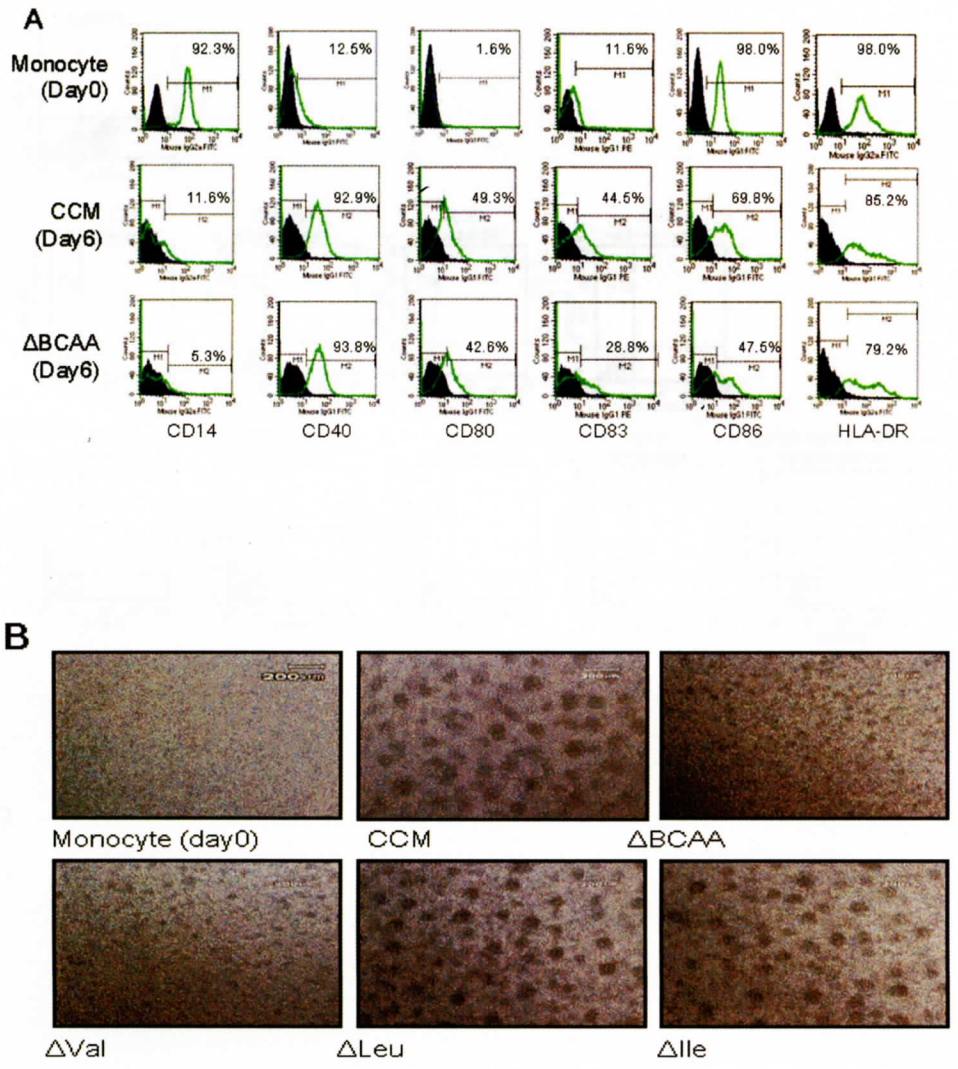
A
Monocyte (Day0)
CCM (Day6)
ΔBCAA (Day6)
92.3%
12.5%
1.6%
11.6%
98.0%
98.0%
11.6%
92.9%
49.3%
44.5%
69.8%
85.2%
5.3%
93.8%
42.6%
28.8%
47.5%
79.2%
CD14
CD40
CD80
CD83
CD86
HLA-DR
B
Monocyte (day0)
CCM
ΔBCAA
ΔVal
ΔLeu
ΔIle

Figure 1

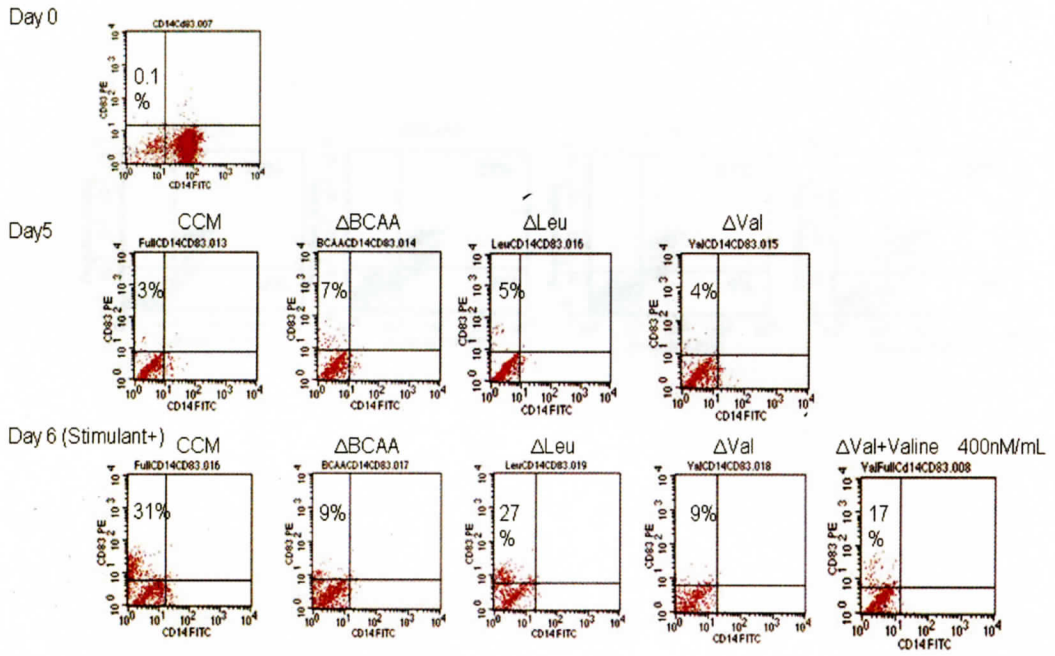
Day 0
0.1 %
Day5
CCM
ΔBCAA
ΔLeu
ΔVal
3%
7%
5%
4%
Day 6 (Stimulant+)
CCM
ΔBCAA
ΔLeu
ΔVal
ΔVal+Valine 400nM/mL
31%
9%
27 %
9%
17 %
CD83 PE
CD14 FITC

Figure 2

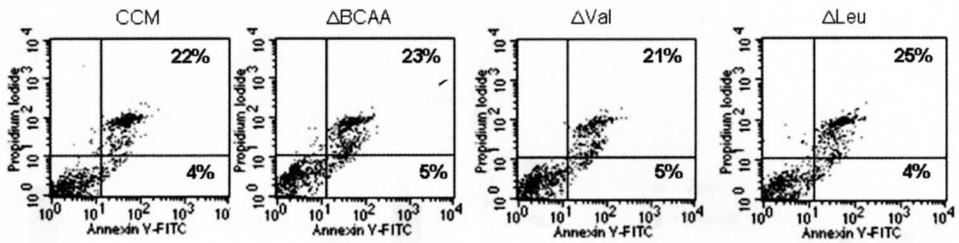
CCM
ΔBCAA
ΔVal
ΔLeu
22%
4%
23%
5%
21%
5%
25%
4%
Propidium Iodide
Annexin V-FITC

Figure 3

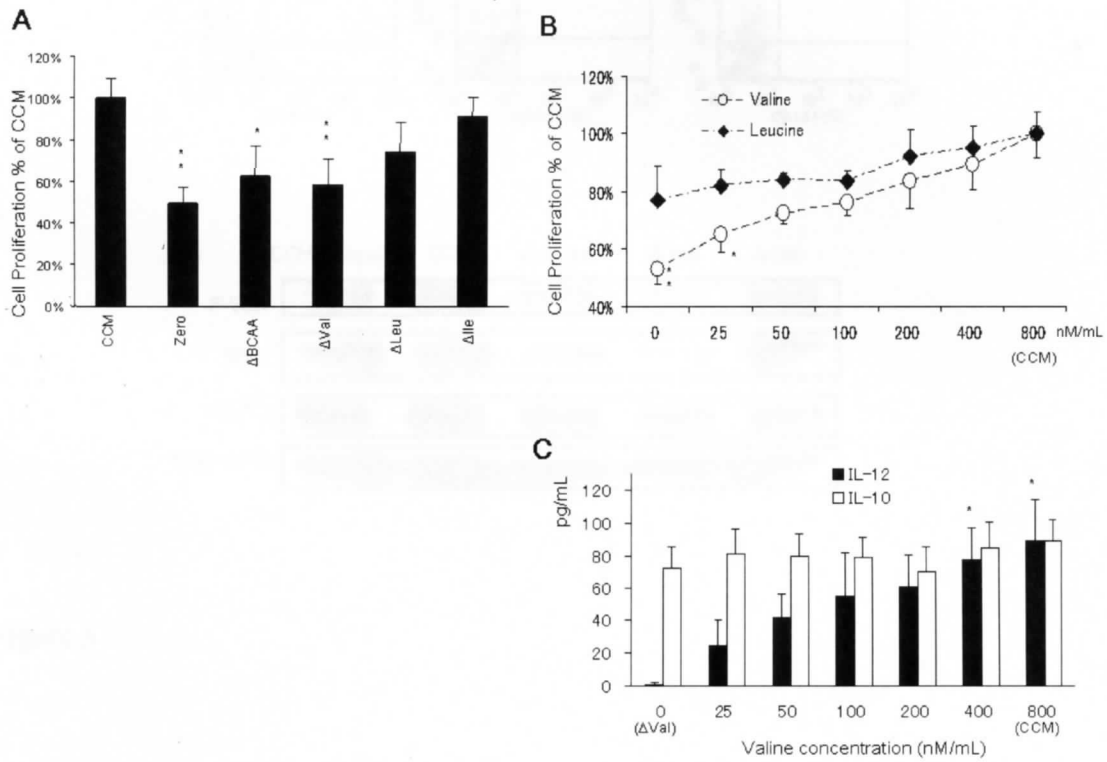
A
Cell Proliferation % of CCM
120%
100%
80%
60%
40%
20%
0%
CCM
Zero
ΔBCAA
ΔVal
ΔLeu
Δlle
B
Cell Proliferation % of CCM
Valine
Leucine
120%
100%
80%
60%
40%
0
25
50
100
200
400
800
nM/mL
(CCM)
C
pg/mL
IL-12
IL-10
120
100
80
60
40
20
0
0
(ΔVal)
25
50
100
200
400
800
(CCM)
Valine concentration (nM/mL)

Figure 4

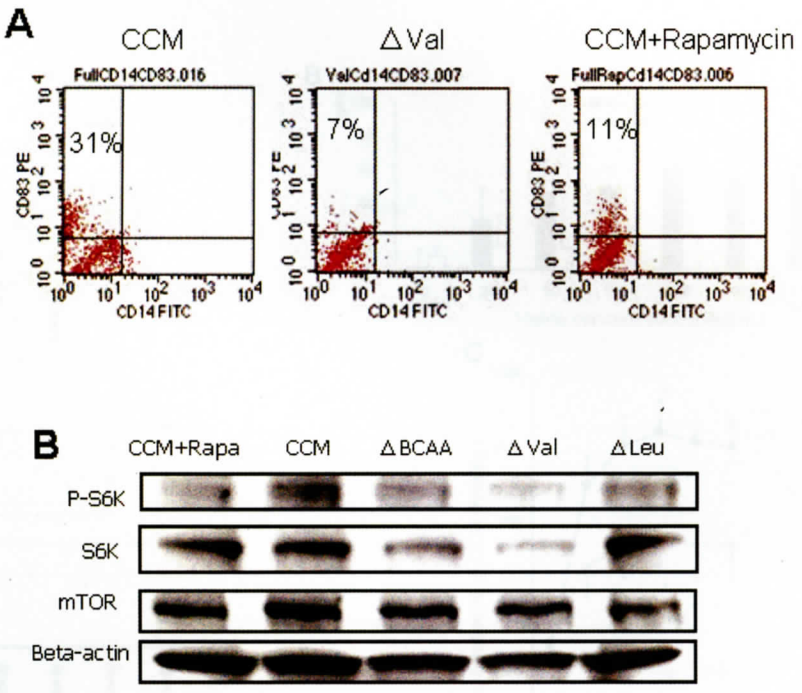
A
CCM
ΔVal
CCM+Rapamycin
FullCD14CD83.016
ValCd14CD83.007
FullRapCd14CD83.006
31%
7%
11%
CD83 PE
CD14 FITC
B
CCM+Rapa
CCM
ΔBCAA
ΔVal
ΔLeu
P-S6K
S6K
mTOR
Beta-actin

Figure 5

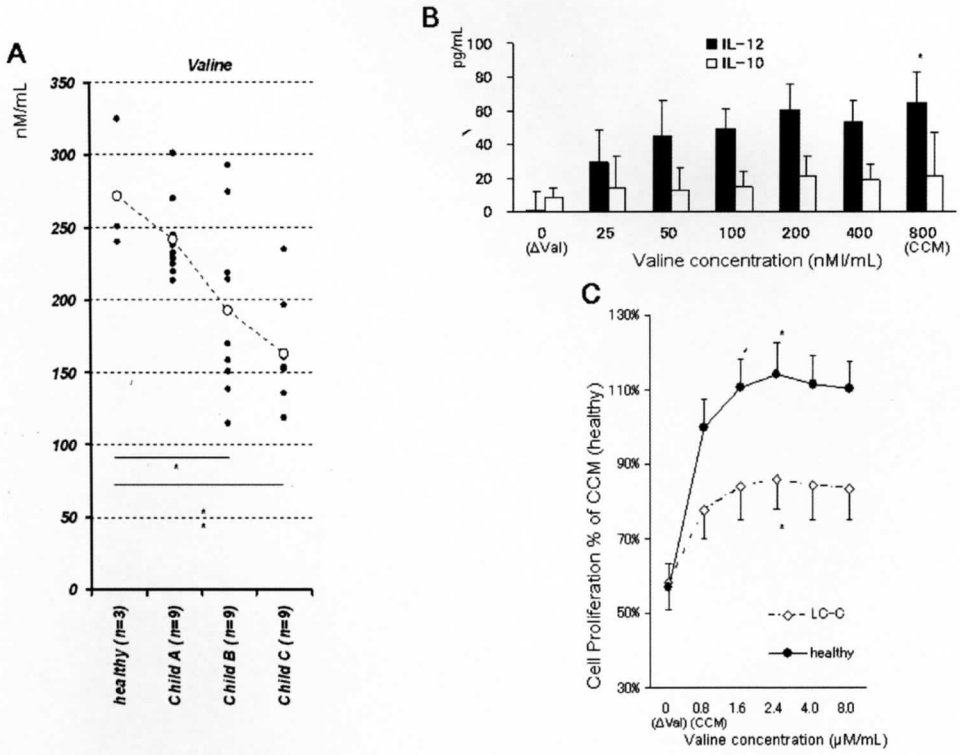
A
Valine
nM/mL
350
300
250
200
150
100
50
0
healthy (n=3)
Child A (n=9)
Child B (n=9)
Child C (n=9)
B
pg/mL
IL-12
IL-10
100
80
60
40
20
0
0 (ΔVal)
25
50
100
200
400
800 (CCM)
Valine concentration (nMl/mL)
C
Cell Proliferation % of CCM (healthy)
130%
110%
90%
70%
50%
30%
LC-C
healthy
0 (ΔVal)
0.8
1.6
2.4
4.0
8.0 (CCM)
Valine concentration (μM/mL)

Figure 6