

Altered cytokine levels and increased CD4⁺CD57⁺ T cells in the peripheral blood of hepatitis C virus-related hepatocellular carcinoma patients

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Abstract. Although CD57⁺ lymphocytes are closely correlated with prognosis in various cancers, the role of subsets of CD57⁺ cells in hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) is unclear. In the present study, peripheral blood (PB) from HCV-related HCC patients was analyzed. Plasma cytokine levels and *in vitro* cytokine-producing capabilities were analyzed with enzyme-linked immunosorbent assays, and CD57⁺ cell subsets were studied using a multi-color FACS system. Interferon (IFN)- γ was undetectable in the plasma of patients with tumors at any stage, whereas the plasma levels of tumor necrosis factor (TNF)- α , interleukin (IL)-10 and

IL-18, but not that of IL-12, were significantly higher in stage IV patients compared to patients with earlier-stage tumors. In contrast, the IFN- γ -producing capability of PB was highest in stage I patients and gradually decreased with tumor progression. The IL-10-, IL-18- and IL-12-producing capabilities of PB increased from stage I to III. However, PB-TNF- α , IL-10- and IL-18-producing capabilities were reduced in stage IV patients, probably due to repeated anti-cancer treatments. The percentage of CD4⁺CD57⁺ $\alpha\beta$ TCR⁺ cells (CD4⁺CD57⁺ T cells) in peripheral blood lymphocytes (PBLs) increased with tumor progression. Moreover, the percentage of CD4⁺CD57⁺ T cells in PBLs and the ratio of CD4⁺CD57⁺ T cells to CD4⁺ $\alpha\beta$ TCR⁺ cells (CD4⁺ T cells), but not that of CD4⁺CD57⁺ T cells to CD57⁺ $\alpha\beta$ TCR⁺ cells (CD57⁺ T cells), showed a significant inverse correlation with PB-IFN- γ -producing capability. The present results suggest that an increase in CD4⁺CD57⁺ T cells controls the capability of PB to produce the anti-tumor cytokine IFN- γ and that PB-IFN- γ production is impaired with HCC tumor progression.

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Abbreviations: ALT, alanine transaminase; AFP, α fetoprotein; CD, cluster for differentiation; ELISA, enzyme-linked immunosorbent assay; FACS analysis, flow cytometric (fluorescence activated cell sorter) analysis; HAART, highly active anti-retroviral therapy; HBs Ag, hepatitis B surface antigens; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; NK, natural killer; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; RFA, radio-frequency ablation; TAE, transcatheter arterial embolization; $\alpha\beta$ TCR, T-cell receptor constructed with α and β chains; TIL, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor

Key words: hepatocellular carcinoma, hepatitis C virus, tumor immunology, peripheral blood lymphocyte, cytokine-producing capability

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the eighth most common cancer in women worldwide. HCC is highly prevalent in patients with chronic liver diseases resulting mainly from hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, and its incidence is now increasing. In Japan, HCC is the third most common cause of mortality, and the most frequent cause of HCC is chronic infection with HCV (1,2).

The clinical course of various cancers is influenced by host immune responses in which tumor infiltrating immunocytes, peripheral blood cells, and various cytokines play important roles. These immune cells and cytokines also correlate with tumor progression and prognosis in various cancers. Histochemical analyses of local tumor-infiltration by CD57⁺

lymphocytes and flow cytometric analyses (FACS) of circulating CD57⁺ lymphocytes in the peripheral blood (PB) of HCC patients have indicated that the number of CD57⁺ cells is closely correlated with the prognosis of these patients (3-5). Studies of the local tumor infiltration of CD57⁺ lymphocytes and the number of CD57⁺ lymphocytes in peripheral blood mononuclear cells (PBMC) and peritoneal exudate cells have also demonstrated a similar correlation with the prognosis of various types of cancers (6-8). Plasma levels of cytokines such as interleukin (IL)-10, IL-12, IL-18 and tumor necrosis factor (TNF)- α are prognostic markers of various cancers (9-11). Furthermore, the capability of peripheral blood lymphocytes (PBL) to produce the anti-tumor cytokine interferon (IFN)- γ is reduced in advanced gastric cancer patients (12). However, it is unknown if the production of these cytokines is under the influence of CD57⁺ lymphocytes in cancer patients including HCC patients.

A small but substantial number of CD57⁺ cells in PBMC express the $\alpha\beta$ TCR (CD57⁺ T cells). The proportion of these cells in PB increases with age in healthy volunteers (13-15). The CD57⁺ T cells that increase with age expand polyclonally, produce a large amount of IFN- γ , and express strongly cytotoxic activity; most of these cells express CD8 (15). The proportion of CD57⁺ T cells in PB is increased in patients who have received a bone marrow transplant (16,17) and in patients with HIV infection (18), rheumatoid arthritis (19), malaria infection (20), or gastric cancer (12,21). Also, many previous studies have demonstrated that CD8⁺CD57⁺ T cells are expanded in the PB from CMV-infected individuals (22-29). On the other hand, CMV seropositivity was associated with a dramatic increase in the expression of CD4⁺CD57⁺ T cells (30). Furthermore, CMV-specific CD4⁺ T cells are characterized by a CD45RO⁺CD27⁺ (CD28⁻) mature effector memory phenotype (31). Thus, the phenotypically mature CD4⁺CD57⁺ T cells exhibit functional properties that are different from CD8⁺CD57⁺ T cells and conventional CD4⁺ (CD57⁻) T cells. Furthermore, the expansion of CD4⁺CD57⁺ T cells in PB also occurs in patients with gastric cancer (12), HIV infection (32) rheumatoid arthritis (33) and atopic asthma (34).

In the present study, we examined the importance of CD57⁺ lymphocytes and their role in cytokine production in HCC patients. We analyzed subsets of CD57⁺ lymphocytes from HCC patients by using a multi-color FACS system. Patient plasma cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA), and the *in vitro* cytokine-producing capability of PB was determined by culture of PB with immunogenic lipopolysaccharide (LPS) followed by ELISA. We found that the capability of PB to produce IFN- γ significantly correlated with the proportion of peripheral blood lymphocytes (PBL) with the phenotype CD4⁺CD57⁺ $\alpha\beta$ TCR⁺ cells (CD4⁺CD57⁺ T cells) and also with the ratio of CD4⁺CD57⁺ T cells to CD4⁺ $\alpha\beta$ TCR⁺ cells (CD4⁺ T cells).

Materials and methods

HCV-related HCC patients. Ninety-six HCV-related HCC patients treated at Wakayama Medical University (Wakayama, Japan) between November 2006 and March 2008 were

enrolled in the present study. The patient group consisted of 62 men and 34 women with a mean (SD) age of 71.7 (8.2) years (range, 41-88 years). All patients were positive for anti-HCV antibodies and/or HCV-RNA and were negative for hepatitis B surface antigens (HBs Ag). The diagnosis of HCC was confirmed by dynamic computed tomography (Aquilion, Toshiba Medical Systems, Tokyo, Japan), ultrasonography (Aplio, Toshiba Medical Systems), and/or histopathology of a biopsied liver tumor specimen. Clinical staging was based on the Liver Cancer Study Group of Japan TNM Classification (35). The ethics committee of Wakayama Medical University approved the study protocol, which conforms to the provisions of the Declaration of Helsinki (1995, as revised in Tokyo 2004). Informed consent was obtained from all patients prior to initiation of the study.

ELISA assay of plasma cytokine levels. One milliliter of PB was drawn into a tube containing sodium heparin (Terumo Medical Products, Tokyo, Japan). The blood was maintained at room temperature and pressure, and then was processed within 24 h. Blood samples were centrifuged at 1200 x g for 15 min at room temperature, and the plasma supernatant was collected. The samples were stored at -80°C until used for ELISA. Plasma cytokine levels were measured by using ELISA kits for IFN- γ , IL-10, TNF- α , IL-12, (Beckman Coulter, Immunotech, Marseille, France) and IL-18 (Medical Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions.

Analysis of cytokine production from PB. PB (0.1 ml) was added to 0.1 ml of serum-free RPMI-1640 medium (R8758, Sigma, St. Louis, MO) in a 96-well plate (R Suspension Culture Treated, Sumitomo Bakelite, Tokyo, Japan) and cultivated with 1 μ g/ml of LPS (Invivogen, San Diego, CA) for 24 h under 5% CO₂ at 37°C. The supernatant was harvested and stored at -80°C until used for ELISA. Cytokine production (IFN- γ , IL-10, TNF- α , IL-12, and IL-18) was analyzed by using the above-described ELISA kits.

Flow cytometric analysis of CD57⁺ cells in PB. A 0.05-ml aliquot of PB was incubated with the following monoclonal antibodies (mAbs) for 30 min on ice. Fluorescein isothiocyanate (FITC)-conjugated mAb specific for CD57 (NC1), phycoerythrin (PE)-conjugated mAb specific for TCR β (BMA031) and allophycocyanin (APC)-conjugated mAb specific for CD4 (13B8.2) were purchased from Beckman Coulter. Peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated mAbs specific for CD8 (SK1), CD4 (SK3), and CD25 (M-A251) were purchased from BD Biosciences (San Jose, CA). An APC-conjugated mAb specific for Foxp3 (236A/E7) was purchased from eBioscience (San Diego, CA). Red blood cells were lysed by using a FACS Lysing Solution (BD Biosciences), and the PBMC were then fixed and permeabilized by using FACS permeabilizing Solution 2 (BD Biosciences). The PBMC were resuspended in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS), 1 mM disodium ethylenediaminetetraacetic acid (Na₂EDTA) and 0.1% sodium azide and then analyzed by flow cytometry (FACSCalibur, BD Bioscience) with Cell Quest software (BD Biosciences).

Table I. Clinicopathological parameters of HCV-related HCC patients

Stage	I	II	III	IV
Gender				
Male	11	18	11	22
Female	10	12	6	6
Age (years) ^a	70.5±8.0	71.7±8.8	72.0±8.9	71.0±7.0
Lymphocytes (cells/mm ²) in peripheral blood ^a	1484.0±688.5	1313.4±547.2	1499.9±555.1	1039.3±506.2 ^b
ALT (U/l) ^c in peripheral plasma ^a	39.0±27.8	41.6±26.8	46.1±44.7	48.7±39.9
AFP (ng/ml) ^d in peripheral plasma ^a	50.4±137.2	122.0±394.4	67.9±100.6	30076.2±112799.8
No. of treatments ^a	0.5±0.9	0.9±0.9	1.4±1.1	3.5±2.8 ^e

^aMean ± SD. ^bp<0.05 vs. stage II and <0.01 vs. stage I or III. ^cALT, alanine transaminase. ^dAFP, α fetoprotein. ^eP<0.01 vs. stage I, II, or III.

Statistics. All analyses were performed by using statistical software (SPSS 11.0 software package; SPSS Inc., Chicago, IL). Data are expressed as means ± standard deviation (SD). The Student's t-test was applied to determine the significance of differences between two groups. One-way ANOVA was applied to determine the significance of differences among overall groups, followed by the Tukey-Kramer test for multiple comparisons. The significance of correlations between each variable (such as plasma cytokines and lymphocytes) was determined by the Spearman correlation coefficients. P-values <0.05 were considered to be statistically significant.

Results

Clinicopathological parameters of HCV-related HCC patients. The clinicopathological parameters of the HCV-related HCC patients are shown in Table I. There was no significant difference in age, gender or serum alanine transaminase (ALT) levels between stage I, II, III and IV patients. In stage IV patients, the number of PBL was significantly reduced and the serum levels of α fetoprotein (AFP) were markedly increased as compared to the other stages. Anti-cancer treatments, such as arterial infusion of anti-cancer agents, radio-frequency ablation (RFA) or transcatheter arterial embolization (TAE), were administered significantly more frequently to the stage IV patients. Thus, it was presumed that the reduced number of PBL was probably caused by the repeated anti-cancer treatments.

Plasma cytokine levels of the HCV-related HCC patients. The plasma levels of IFN-γ, TNF-α, and interleukin IL-10 in the HCC patients were measured by using ELISA. The levels of TNF-α and IL-10 were significantly increased in the advanced stages of HCC as compared to earlier stages (Fig. 1a and b), whereas IFN-γ was undetectable in the plasma (<0.39 IU/ml) at any stage (data not shown). The plasma levels of IL-18 and IL-12, which are IFN-γ-inducing cytokines, were also analyzed by ELISA. The IL-18 level was significantly higher in stage IV patients as compared to patients at other stages (Fig. 1c). In contrast, there was no

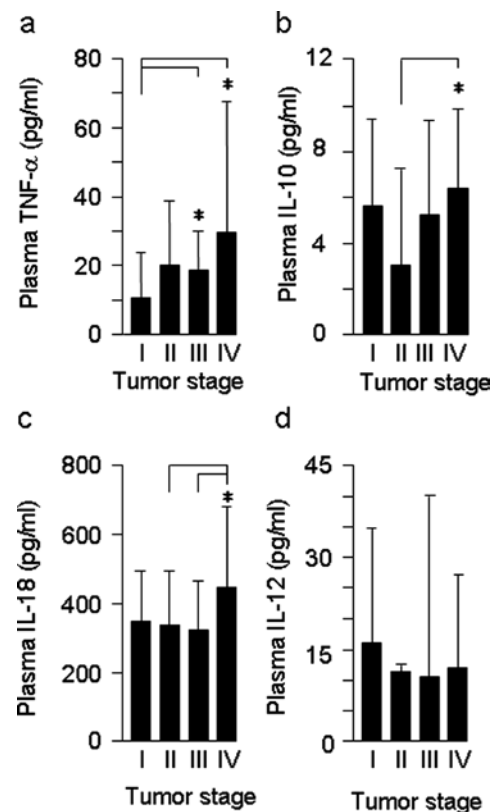


Figure 1. Plasma cytokine levels in HCV-related HCC patients. The plasma levels of TNF-α (a), IL-10 (b), IL-18 (c) and IL-12 (d) in patients at each stage of HCV-related HCC (stage I, n=21; stage II, n=30; stage III, n=17; stage IV, n=28) were determined by using ELISA. The IFN-γ level was lower than the ELISA detection limit (<0.39 international units (IU) /ml) at all stages. *p<0.05.

statistically significant difference in plasma IL-12 levels at any stage (Fig. 1d).

Cytokine-producing capabilities of PB from HCV-related HCC patients. The capability of PB to produce IFN-γ, TNF-α, IL-10, IL-18 and IL-12 when stimulated with the bacterial component LPS *in vitro* was determined by using ELISA. The IFN-γ-producing capability of PB was highest in

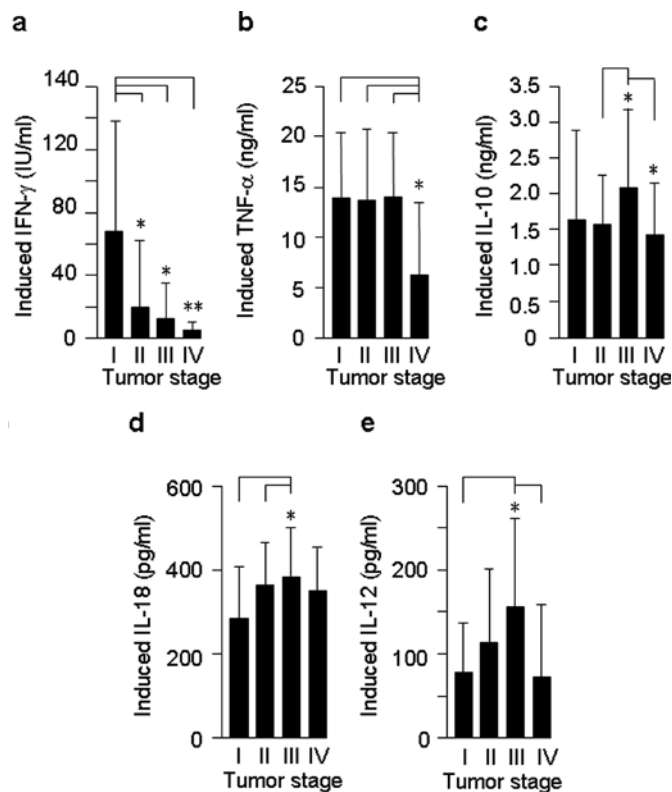


Figure 2. LPS-induced cytokine-production in PB from HCV-related HCC patients. PB from patients at the indicated tumor stage was stimulated with LPS for 24 h *in vitro*. Then, the induced IFN- γ (a), TNF- α (b), IL-10 (c), IL-18 (d) or IL-12 (e) in the culture supernatant was determined by using ELISA. * $p < 0.05$ and ** $p < 0.01$.

stage I patients and decreased in a step-wise fashion during the course of tumor progression up to stage IV (Fig. 2a). The PB-TNF- α -producing capability was significantly lower in stage IV patients (Fig. 2b), while the PB-IL-10-, -IL-18- and -IL-12-producing capability was significantly increased in stage III patients but not in stage IV patients (Fig. 2c-e, respectively).

CD4⁺CD57⁺ T cells in PB from HCV-related HCC patients. We next used FACS analysis of PBL to quantify CD57⁺-expressing cells (CD57⁺ cells), subsets of CD57⁺ cells that do (CD57⁺ T cells) or do not (CD57⁺ NK cells) express the $\alpha\beta$ TCR, and also CD57⁺ T cells that additionally express CD4 (CD4⁺CD57⁺ T cells) (Fig. 3a). The criteria used to define these PBL subsets are shown in Table II. The percentage of the total PBL represented by these various CD57⁺ cells and subsets (Fig. 3b), as well as the ratio of CD4⁺CD57⁺ T cells to CD57⁺ T cells and that of CD4⁺CD57⁺ T cells to CD4⁺ T cells (Fig. 3c) was then calculated. The percentage of CD4⁺CD57⁺ T cells in the PBL of the patients showed a gradual and significant increase with tumor progression from stage I to stage III, but decreased in stage IV patients. The percentages of CD57⁺ cells, CD57⁺ NK cells and CD57⁺ T cells in PBL showed no significant differences among the tumor stages. Furthermore, a significant increase in CD4⁺CD57⁺ T cells at stage III was clearly indicated when the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells was calculated but not when the ratio of CD4⁺CD57⁺ T cells to CD57⁺ T cells was calculated.

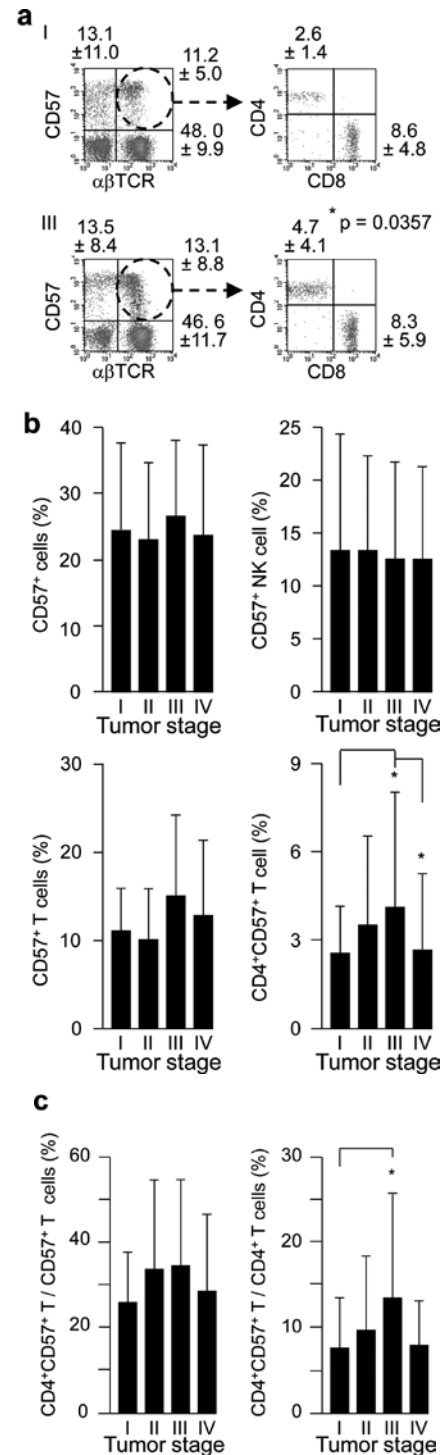


Figure 3. FACS analysis of CD4⁺CD57⁺ T cells in the PB of HCV-related HCC patients. (a) Representative FACS analyses of PB from patients at stage I (top) or stage III (bottom). Both CD57 (FITC) and $\alpha\beta$ TCR (PE)-expressing cells were gated and then analyzed by using anti-CD4 (PerCP-Cy5.5) and anti-CD8 (APC) antibodies. The numbers indicate the percentage (mean \pm SD) of each cell subpopulation, CD57⁺ NK, CD57⁺ T, CD4⁺CD57⁺ T and CD8⁺CD57⁺ T cells, in the total PB. The classification of the lymphocyte subsets is indicated in Table II. (b) The percentage of CD57⁺, CD57⁺ NK, CD57⁺ T and CD4⁺CD57⁺ T cells in the PBL of patients at each tumor stage was calculated. (c) The ratio of CD4⁺CD57⁺ T cells to CD57⁺ T cells and the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells in the PBL at each tumor stage were calculated and expressed as percentages.

Correlations between the cytokine-producing capabilities of PB and lymphocyte subsets. The cytokine-producing capabili-

Table II. Classification of lymphocyte subsets in peripheral blood lymphocytes.

Subset	Criteria	Phenotype
CD57 ⁺ cells	CD57-expressing cells including CD57 ⁺ NK and CD57 ⁺ T cells	CD57 ⁺
CD57 ⁺ NK cells	Cells expressing CD57, but not $\alpha\beta$ TCR	CD57 ⁺ $\alpha\beta$ TCR ⁻
CD57 ⁺ T cells	Cells expressing both CD57 and $\alpha\beta$ TCR	CD57 ⁺ $\alpha\beta$ TCR ⁺
CD4 ⁺ CD57 ⁺ T cells	Both CD57 and $\alpha\beta$ TCR-expressing cells bearing CD4	CD4 ⁺ CD57 ⁺ $\alpha\beta$ TCR ⁺
CD4 ⁺ T cells	Both CD4 and $\alpha\beta$ TCR-expressing cells, including CD4 ⁺ T and CD4 ⁺ CD57 ⁺ T cells	CD4 ⁺ $\alpha\beta$ TCR ⁺
Treg (Regulatory T cells)	Cells expressing intracellular Foxp3 and surface CD4, CD25 and $\alpha\beta$ TCR	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ $\alpha\beta$ TCR ⁺

Table III. Correlation between the cytokine-producing capability of PB and lymphocyte subsets.

Lymphocyte subset ^a	IFN- γ	TNF- α	IL-10	IL-12	IL-18
Proportion of CD57 ⁺ cells in PBL	-0.2109 (0.0593)	-0.0488 (0.6916)	0.2160 (0.0504) ^d	-0.0947 (0.4000)	0.0197 ^b (0.8610) ^c
Proportion of CD57 ⁺ NK cells in PBL	-0.0389 (0.7276)	0.0891 (0.4691)	0.2798 (0.0113) ^d	-0.1490 (0.1854)	0.1111 (0.3235)
Proportion of CD57 ⁺ T cells in PBL	-0.3763 (0.0008) ^d	-0.2975 (0.0156) ^d	-0.0013 (0.9905)	-0.0149 (0.8952)	-0.1516 (0.1779)
Proportion of CD4 ⁺ CD57 ⁺ T cells in PBL	-0.2882 (0.0099) ^c	-0.2705 (0.0280) ^d	-0.0946 (0.3916)	-0.1030 (0.3631)	-0.0827 (0.4624)
CD4 ⁺ CD57 ⁺ T cells/CD57 ⁺ T cells	-0.1502 (0.1790)	-0.1717 (0.1630)	-0.1713 (0.1208)	-0.1131 (0.3178)	-0.0383 (0.7337)
CD4 ⁺ CD57 ⁺ T cells/CD4 ⁺ T cells	-0.3290 (0.0037) ^c	-0.2568 (0.0369) ^d	-0.0331 (0.7702)	-0.0998 (0.3844)	-0.0475 (0.6807)

^aThe percentage of PBL represented by the indicated lymphocyte subset was used to evaluate the correlation. ^bCoefficient of correlation.

^cP-values are indicated in parentheses. ^dp<0.05; ^ep<0.01.

ties of PB were analyzed to determine if they correlated with the percentage of any of the lymphocyte subsets tested in PBL (Table III). The IL-10-producing capability of PBL was statistically significantly correlated with the percentage of CD57⁺ NK cells in PBL (p<0.05) and also showed marginal correlation with the percentage of CD57⁺ cells in PBL (p=0.0504). The IFN- γ -producing capability of PBL showed a statistically significant inverse correlation with the percentage of CD57⁺ T or CD4⁺CD57⁺ T cells in PBL (p<0.01). In addition, the TNF- α -producing capability of PBL showed a statistically significant inverse correlation with the percentage of CD57⁺ T cells or CD4⁺CD57⁺ T cells in PBL (p<0.05). Furthermore, the IFN- γ - or TNF- α -producing capability of PBL showed a statistically significant inverse correlation (p<0.01 and p<0.05, respectively) with the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells, but not with that of CD4⁺CD57⁺ T cells to CD57⁺ T cells in PBL.

Discussion

In the present study, we examined the cytokine levels and subsets of T lymphocytes in PB and the *in vitro* cytokine-

producing capabilities of PB from HCV-related HCC patients. The plasma levels of TNF- α , IL-10 and IL-18, but not IL-12, in PB from advanced-stage patients were significantly increased as compared to the levels in the PB of patients at the initial stage, while plasma IFN- γ was undetectable at any stage. These results are consistent with previous studies that found that plasma levels of TNF- α , IL-10 and IL-18 are increased in advanced-stage HCC (36-41). Similar results have also been reported for advanced-stage gastric cancer (42-45) and prostate cancer (46). These previous results combined with our present results strongly indicate that higher levels of plasma TNF- α , IL-10 and IL-18 correlate to a poor prognosis in cancer patients. On the other hand, in our study, the IFN- γ -producing capability of PB, which was analyzed *in vitro*, decreased with tumor progression in a step-wise fashion, and the IL-12-, IL-10- and IL-18-, but not the TNF- α -producing capability of PB from stage III patients was significantly increased as compared to earlier stages. The reduction in the capability of PB to produce IFN- γ , an anti-tumor cytokine, correlated both with tumor progression and with an increase in the percentage of CD4⁺CD57⁺ T cells in PBL. It has been

reported that IFN- γ production *in vitro* correlates with the proportion of CD8⁺CD57⁺ T cells in PBMC (15). However, in advanced gastric cancer patients, there was an increase in the ratio of CD4⁺CD57⁺ T cells to CD57⁺ T cells in PBL that was accompanied by a disappearance of the correlation between IFN- γ production from PBMC *in vitro* and the ratio of CD8⁺CD57⁺ T cells to CD57⁺ T cells (12). Furthermore, a large proportion of CD4⁺CD57⁺ T cells in PBL reduced the therapeutic efficacy of highly active anti-retroviral therapy (HAART) in patients infected with human immunodeficiency virus (HIV) (32). In the present study, a reduction in PB-IFN- γ -producing capability correlated with an increase in the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells, but not with that of CD4⁺CD57⁺ T cells to CD57⁺ T cells. The combined results strongly indicate the possibility that CD4⁺CD57⁺ T cells impair IFN- γ production and enhance tumor progression in HCC patients.

The total number of lymphocytes from PB of stage IV patients was significantly reduced following repeated anti-cancer treatments, such as arterial infusion of the anticancer agents RFA or TAE. In addition, the cytokine-producing capabilities of PB from stage IV patients were also reduced for all of the cytokines analyzed, among which the reduction in the three lymphocyte-related cytokines, TNF- α , IL-10, and IFN- γ , was significant. This result strongly indicates an involvement of repeated anti-cancer treatments in the observed alteration in the immune response of stage IV patients. Moreover, the percentage of CD4⁺CD57⁺ T cells in total PBL and the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells, but not that of CD4⁺CD57⁺ T cells to CD57⁺ T cells, were significantly reduced in the stage IV patients. These results suggest that CD4⁺CD57⁺ T cells are more sensitive than other lymphocyte subsets to anti-cancer treatments. However, despite the reduction in both the number and proportion of CD4⁺CD57⁺ T cells in PBL, the PB-IFN- γ -producing capability was still decreased at stage IV. These observations suggest the possibility that the anti-cancer treatments affected not only IFN- γ -producing cells, but also cells that produce IFN- γ inducers such as cytokines including IL-12.

An increase in CD4⁺CD57⁺ T cells in PB has been reported for patients chronically infected with HIV (32,47-48), mycobacterium tuberculosis (49) or malaria (20) and for patients with other chronic diseases such as rheumatoid arthritis (33,50,51), angina pectoris (22), colorectal cancer (52,53) or gastric cancer (12). These reports strongly support the possibility that HCC develops and progresses hand in hand with the generation and increase in the number of CD4⁺CD57⁺ T cells. Another possibility is that the CD4⁺CD57⁺ T cells were generated due to an effect of other diseases or of chronic HCV infection, and, as a result, HCC developed and advanced. There have also been reports of an increase in regulatory T cells in some of the disorders described above (54,55), which raises the possibility that CD4⁺CD57⁺ T cells are regulatory T cells (Treg). However, in the present study, CD4⁺CD57⁺ T cells from HCV-related HCC patients did not express surface CD25 or cytoplasmic Foxp3 markers, which are characteristic of Treg cells (data not shown). Further studies are required to elucidate the mechanisms by which the CD4⁺CD57⁺ T cells are generated and by which they increase in number in the

PB of HCV-related HCC patients, as well as the roles of this subset of T cells in the immune response.

In the present study, the plasma cytokine levels of HCV-related HCC patients were analyzed by using a minimal amount of PB (<1 ml), which is a very acceptable amount for HCC patients. In addition, the PB-IFN- γ -producing capability was analyzed by simple *in vitro* culture of 0.1 ml PB, and the ratio of CD4⁺CD57⁺ T cells to CD4⁺ T cells was analyzed by flow cytometry with only 0.05 ml PB. These procedures are quick and simple and do not require the separation of mononuclear cells from PB under aseptic conditions. In contrast, hepatic tumor biopsy of HCC patients is an uncomfortable and invasive procedure that is associated with the risk of bleeding or HCC dissemination (55). Thus, the histochemical analysis of hepatic tumor biopsy samples with specific antibodies for cell surface markers leaves much to be desired as a test for HCC patients, although some excellent studies have used this technique to determine the extent of tumor infiltrating CD57⁺ cells in breast (57), lung (58-60), and gastric cancers (61). Taking all these points into consideration, we conclude that the evaluation of cytokine levels and CD4⁺CD57⁺ T cells in PB and the IFN- γ -producing capability of PB by using flow cytometry and cell cultivation *in vitro* are safe and acceptable methods. The use of these methods would be beneficial for HCV-related HCC patients by providing an index of their immunological status and their prognosis.

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