Original Article

An immunosuppressive subtype of neutrophils identified in patients with hepatocellular carcinoma

Yasuhiro Tsuda,* Hideo Fukui, Akira Asai, Shinya Fukunishi, Katsuhiko Miyaji, Shinya Fujiwara, Kazuhisa Teramura, Akira Fukuda and Kazuhide Higuchi

Department of Gastroenteroloy and Hepatology, Osaka Medical College, Osaka 569-8686, Japan

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Functional disorders of various immune cells have been reported in hepatocellular carcinoma (HCC) patients. Recently, distinct subsets of neutrophils (polymorphonuclear leukocytes, PMN) have been identified in hosts with enhanced or impaired cell-mediated immunity. In this study, therefore, plasma factors and PMN from HCC patients were immunobiologically investigated. Plasma neopterin and CCL17 levels were measured by ELISA in 95 HCC patients. Peripheral PMN were isolated from each HCC patient and tested for CCL2 or CCL3 production by ELISA and flow cytometry. The results showed elevated plasma neopterin levels in HCC patients, while CCL17 levels decreased in correlation with tumor size. PMN from HCC patients produced CCL2, while PMN from healthy subjects did not. Moreover, CCL2 production by PMN was significantly increased in proportion to tumor load. When HCC patients were divided into two groups based on CCL2 produced by PMN, the survival rate of the CCL2 high group was significantly lower than that for other patients. While CCL3 production by PMN was also significantly increased in HCC patients, their CCL3 production did not correlate with tumor load and survival. The CCL2/CCL3 ratio in culture fluids of each PMN was also increased in proportion to tumor size. These results suggest that cell-mediated immunity may be impaired in advanced HCC patients. Moreover, distinct PMN subsets may exist in the peripheral blood of HCC patients. These PMN subsets, especially CCL2-producing PMN, may be involved in tumor extension and the survival outcomes for HCC patients.

***Key Words*: neutrophil, HCC, CCL2, CCL3, PMN**

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, and its incidence is increasing, especially in Japan.[1,2] Recent progress in diagnostic and therapeutic modalities has made it possible to detect small HCCs and to treat them curatively using hepatic resection or percutaneous ablation.[3–5] However, because of the high risk of recurrence, the long-term survival rate of HCC patients undergoing surgical or percutaneous ablation is far from satisfactory.[6] Various factors such as liver inflammation, fibrosis, oxidation stress or obesity have been reported as risk factors for liver carcinogenesis and recurrence,[7] but the mechanism behind frequent recurrence in HCC patients is yet to be elucidated. Most malignant tumors, including HCC, have developed different mechanisms to evade the host's immune response and generate a suppressive network. Functional disorders of various immune cells (T cells, NK cells or dendritic cells) have been reported in HCC patients.[8–10] Butterfield *et al.*[11] have reported that cytotoxic T cell activity was impaired in HCC patients. Kuang *et al.*[12] have demonstrated an increase in M2 macrophages in the peripheral blood of HCC patients. Thus, a change in the cell-mediated immunity of a host may be involved in the recurrence of HCC.

It has been suggested that polymorphonuclear neutrophils (PMN) are critical effector cells in the host's immune response against microbial invasion.[13] PMN enter inflamed sites first, phagocytose bacteria and kill them with reactive oxygen species and antimicrobial peptides.[14] PMN also have been found to produce various cytokines and chemokines.[15,16] Recently, three distinct subsets of PMN have been identified in mice with different resistance to methicillin-resistant *Staphylococcus aureus* (MRSA) infection: freshly isolated PMN from normal mice (PMN-N), PMN that produce IL-12 and CCL3 (PMN-I), and PMN that produce IL-10 and CCL2 (PMN-II).[17] PMN-I were from MRSA-resistant mice, while PMN-II were from MRSA susceptible mice.[17] In humans, several papers have reported that PMN are heterogeneous with regard to locomotion, phagocytosis, density, membrane depolarization and protein synthesis, but PMN subsets have not been well characterized.[18,19]

Thymus- and activation-regulated chemokine (TARC/CCL17), a CC chemokine, recruit Th2 cells into inflammatory sites through CCR4-expressing Th2 cells.[20] In a murine model, it was reported that CCL17 was produced by M2 macrophages and converted resident macrophages to M2 macrophages.[21] CCL17 is a useful marker of cell-mediated immunodeficiency. Neopterin belongs to the chemical group known as pteridines, and is one of the intermediate metabolites in the biosynthesis of tetrahydrobiopterin.[22] Neopterin is released from monocytes/macrophages stimulated by Interferon (IFN)-γ produced due to T-cell activation.[23] Neopterin has been used clinically to diagnose or monitor various diseases as a biological marker of increased cell-mediated immunity.[24]

In this study, first, we measured the plasma CCL17 (as a marker of impaired cell-mediated immunity) and neopterin (as a marker of increased cell-mediated immunity) levels in HCC patients to investigate the correlation between these markers and tumor extent, and clarified the degree of cell-mediated immunodeficiency in HCC patients. Second, we isolated PMN from the peripheral blood of these patients, and investigated their subsets by measuring their CCL2 or CCL3 production. We demonstrated that CCL2-producing PMN were involved in tumor size and poorer survival outcomes through impairment of host cell-mediated immunity.

*To whom correspondence should be addressed.
E-mail: in1258@poh.osaka-med.ac.jp

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Materials and Methods

Patients. A total of 95 Japanese HCC patients seen at the Department of Gastroenterology, Osaka Medical College (Takatsuki, Japan) were enrolled consecutively in this study. The diagnosis of HCC was established by spiral computed tomography (CT) with contrast enhancement, magnetic resonance imaging (MRI) with contrast enhancement or angiography with lipiodol injection and follow-up CT. Atypical tumors were evaluated by liver biopsy. Written consent was obtained from all patients before blood sampling and the Ethics Committee of Osaka Medical College approved the study protocol. This study is in full compliance with the principles of the Declaration of Helsinki. Data from clinical histories, laboratory examinations, and other diagnostic investigations were obtained before the commencement of therapy. Patients who were receiving immunosuppressive or immunomodulating agents were excluded from this study. Fifteen healthy subjects served as controls. Peripheral blood was drawn from all patients and plasma was stored at −70°C until neopterin or CCL17 assay. In HCC patients, tumor size, clinical stage and liver function were assessed based on CT findings or laboratory examination in accordance with the guideline of the Barcelona Clinic Liver Cancer (BCLC) staging system and Child-Pugh score.[25] Surgery was performed in 12 patients. Fourteen patients underwent local ablation and the others were treated with transcatheter arterial chemoembolization (TACE). All patients were regularly followed up and monitored for recurrence by assessment of serum α-fetoprotein and by ultrasonography or contrast computed tomography every 1 to 3 months. Follow-up was completed on June 30, 2009. The median follow-up period was 18 months (range: 1 to 48 months). To investigate the influence of PMN on survival, HCC patients were divided into two groups; those in whom PMN produced CCL2 and those in whom PMN produced CCL3. The cut-off value was mean + SE. The survival curve was analyzed using Kaplan-Meier methods and log-rank test.

Neopterin and CCL17 ELISA assay. Recombinant human CCL17, anti-human CCL17 monoclonal antibody (mAb) and biotin-conjugated anti-human CCL17 mAb were purchased from R&D Systems (Minneapolis, MN). Serum CCL17 levels were measured by ELISA as previously described.[26] Briefly, 96-well micro titer plates (Maxisorb F96, Nunc, Roskilde, Denmark) were coated with anti-human CCL17 mAb in a coating buffer at 4°C overnight. After performing a blocking process with PBS/10% fetal bovine serum (FBS), appropriate dilutions of culture supernatants or standard chemokine were added to each well and incubated at 4°C overnight. After incubation, biotin conjugated anti-human CCL17 mAb was added to each well and incubated for 2 h at room temperature (RT). Finally, wells were incubated with avidin peroxidase (Sigma-Aldrich, St. Louis, MO) for 30 min at RT and plates were subsequently developed with ABTS, containing H_2O_2 (0.0005%). Optical density was measured at 415 nm, and the chemokine concentration was calculated relative to recombinant CCL17 standard. Plasma neopterin levels were determined using a commercially available immunoenzymatic assay kit (NEOPTERIN Enzyme Immunoassay Kit, Immuno-Biological Laboratories GmbH, Hamburg, Germany).

PMN isolation. PMNs were isolated from whole peripheral blood using Ficoll-Hypaque and dextran sedimentations, as previously described.[16,17] Briefly, peripheral blood was withdrawn from each HCC patient with an anticoagulant, centrifuged with Ficoll-Hypaque (Wako Pure Chem. Ind., Ltd., Osaka, Japan) and precipitates were obtained as a PMN-rich fraction. The precipitates were suspended in 1% dextran (T-500, Pharmacia, Piscataway, NJ) and kept for 1 h at room temperature to allow the sedimentation of residual erythrocytes. The resulting PMN fraction was further treated with erythrocyte-lysing kits (R&D Systems, Minneapolis, MN) to eliminate small amounts of erythrocytes. The purity of the PMNs obtained was routinely more than 90%, when analyzed using Giemsa staining.

CCL2 and CCL3 production by PMN. PMN preparations (1×10^6 cells/ml) were cultured by stimulation with *Staphylococcus aureus* Cowan I bacteria (SAC, 0.0075%) for 48 h. SAC were purchased from Calbiochem-Behring Corporation. Culture fluids harvested were stored at −70°C until chemokine assay. Production of CCL2 and CCL3 by PMN was measured using ELISA kit (R&D Systems, Minneapolis, MN) and the correlation with tumor staging and size was analyzed. Moreover, to examine the dominance of CCL2 or CCL3 production by PMN in each HCC patient, the ratio of CCL2 to CCL3 was calculated and the correlation with tumor size was also analyzed. In some experiments, intracellular chemokine staining was performed. As previously described,[27] brefeldin A (Sigma, St. Louis, MO) was added to each well 6 h before the end of PMN cultivation. PMN were washed and fixed and permeabilized by a fixation buffer and permeabilization buffer, and then stained with the fluorescein isothiocyanate (FITC)-labeled anti-CCL2 mAb or phycoerythrin (PE)-conjugated anti-CCL3 mAb for 30 min at 4°C. The cells were washed, and CCL2 or CCL3 positivity was confirmed by flow cytometry and fluorescence microscopy.

PMN suppression assay. In some patients, PMN were isolated before (PMM-H) and after (PMN-NH) curative treatment of HCC. To investigate the suppressive ability of CCL2-producing PMN, peripheral blood mononuclear cells (PBMC) isolated from curatively treated HCC patients were cultured with the culture supernatant of PMN-H or PMN-NH from the same patients (10%, v/v) for 48 h and culture fluids harvested were tested for IFN-γ production by ELISA kit (R&D Systems, Minneapolis, MN). In some experiments, PMN-H culture supernatant previously treated with anti-CCL2 mAb or recombinant CCL2 was also used for PBMC stimulation.

Statistical analysis. Data are presented as mean values ± SE of the mean expressed as a percentage. The differences between the study groups were analyzed using the Mann-Whitney *U* test or Kruskal-Wallis (K-W) test. The survival curves were analyzed by Kaplan-Meier methods and the log rank test. Univariate and multivariate analyses were performed by the Cox proportional hazards regression models. Statistical analyses were performed using Stat-View 5.0 Software (SAS Institute, Cary, NC).

Results

Clinical characteristics of HCC patients. The clinical characteristics of the HCC patients in this study are summarized in Table 1: 65 males and 30 females, aged 59–82 years, comprising 70 HCV-related patients and 13 HBV-related patients. The others were not infected with HBV or HCV. When clinical stages were assessed in accordance with the BCLC staging system, Stages 0, A, B, C and D included 11, 32, 20, 24 and 8 patients, respectively. Surgery was performed in 12 patients. Fourteen patients underwent local ablation and the others were treated with transcatheter arterial chemoembolization (TACE). The clinical parameters of these patients were not significantly different among clinical stages except for tumor markers (data not shown).

Plasma CCL17 and neopterin levels in HCC patients. First, we investigated plasma markers to ascertain immune deficiency in advanced HCC patients. When they were divided into three groups by tumor size, plasma CCL17 levels were found to increase in proportion to tumor size (Fig. 1A). While plasma neopterin levels were significantly elevated in HCC patients compared with normal controls ($p<0.01$), no correlation with tumor size was found (Fig. 1B). When the neopterin/CCL17 ratio was analyzed to investigate the correlation between the plasma CCL17 and neopterin levels in each HCC patient, the ratio decreased in proportion to tumor size (Fig. 1C). Plasma CCL17 or neopterin levels were not correlated with any laboratory data in

Table 1. Clinical characteristics of patients in this study

	Parameters							
	Age	Sex (M/F)	Virus (B/C/N)	Tumor size (<3 cm/3–5 cm/>5 cm)	Tumor number (single/multiple)	Vin (N/Y)	Child-Pugh (A/B/C)	AFP (ng/dl)
HCC (*n* = 95)	69 ± 7.6	65/30	13/70/12	51/23/21	28/67	64/31	64/30/1	954 ± 3451
BCLC 0 (*n* = 11)	70 ± 6.1	5/6	4/7/0	11/0/0	11/0	11/0	9/2/0	34 ± 59
A (*n* = 32)	67 ± 7.6	18/14	6/23/3	27/5/0	14/18	32/0	20/12/0	809 ± 3835
B (*n* = 20)	70 ± 8.1	13/7	2/14/4	7/7/6	0/20	20/0	12/8/0	405 ± 1100
C (*n* = 24)	71 ± 7.7	21/3	0/19/5	6/10/8	3/21	1/23	19/5/0	617 ± 1585
D (*n* = 8)	71 ± 7.0	8/0	1/7/0	0/1/7	0/8	0/8	4/3/1	972 ± 1891

Data are expressed as mean ± SD. Virus (B: HBV, C: HCV, N: Others), Vin: vascular invasion (N: no vascular invasion, Y: positive vascular invasion by CT or MRI).

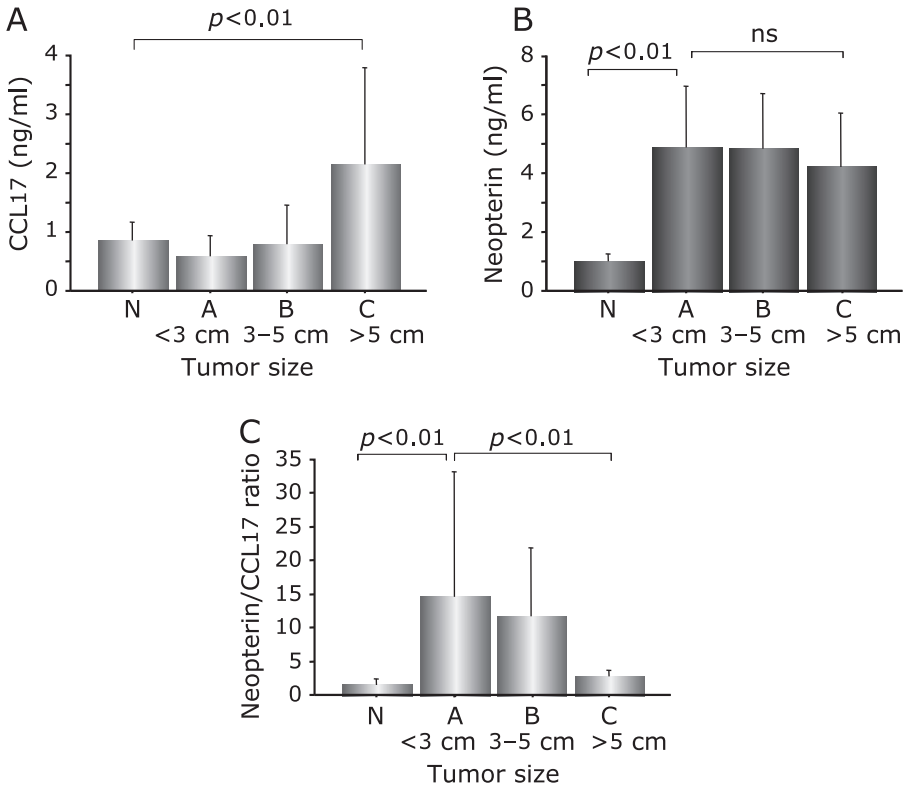

Fig. 1. (A) Plasma samples from HCC patients were tested for CCL17 by ELISA. Statistical analyses were performed by K-W test. (B) Plasma samples from HCC patients were tested for neopterin by ELISA. Statistical analyses were performed by K-W test. (C) The plasma nepterin/CCL17 ratio was calculated for each HCC patient. Statistical analyses were performed by K-W test. ns: normal subjects, HCC patients were divided into three groups by tumor extension; A: HCC patients whose tumor size was less than 3 cm, B: others, C: HCC patients whose tumor size was more than 5 cm.

the HCC patients. CCL17 is a known marker of impaired cell-mediated immunity while plasma neopterin levels indicate the enhancement of host cell-mediated immunity. These results suggest that cell-mediated immunity differed between small and advanced HCC.

CCL2 production by PMN isolated from HCC patients. Because distinct subsets of PMN, CCL2-producing PMN and CCL3-producing PMN, have been demonstrated in mice with different cell-mediated immunity, we analyzed the PMN subsets using the peripheral blood of each HCC patient.[17] From the results of analysis, CCL2 was detected in the culture fluid of PMN from HCC patients (Fig. 2A). The production of CCL2 by PMN was confirmed by intracellular CCL2 staining using a flow cytometer and a fluorescence microscope. Using flow cytometry, FITC-conjugated CCL2 intracellular staining (imaged with green fluorescent dye) showed a shift in fluorescence intensity compared with the isotype control (Fig. 2B). Moreover, CCL2 production increased in proportion to tumor size (Fig. 2C). These results suggest that CCL2-producing-PMN increased in advanced HCC patients.

CCL3 production by PMN isolated from HCC patients. CCL3 was also detected in the culture fluid of PMN from HCC patients (Fig. 3A). The production of CCL3 by PMN was also confirmed by intracellular cytokine staining using a flow cytometer and a fluorescence microscope. Using flow cytometry, PE-conjugated CCL3 intracellular staining (imaged with red fluorescent dye) showed in a shift in fluorescence intensity compared with the isotype control (Fig. 3B). However, CCL3 production was unrelated to tumor size (Fig. 3C). These results suggest that CCL3-producing PMN were also present in HCC

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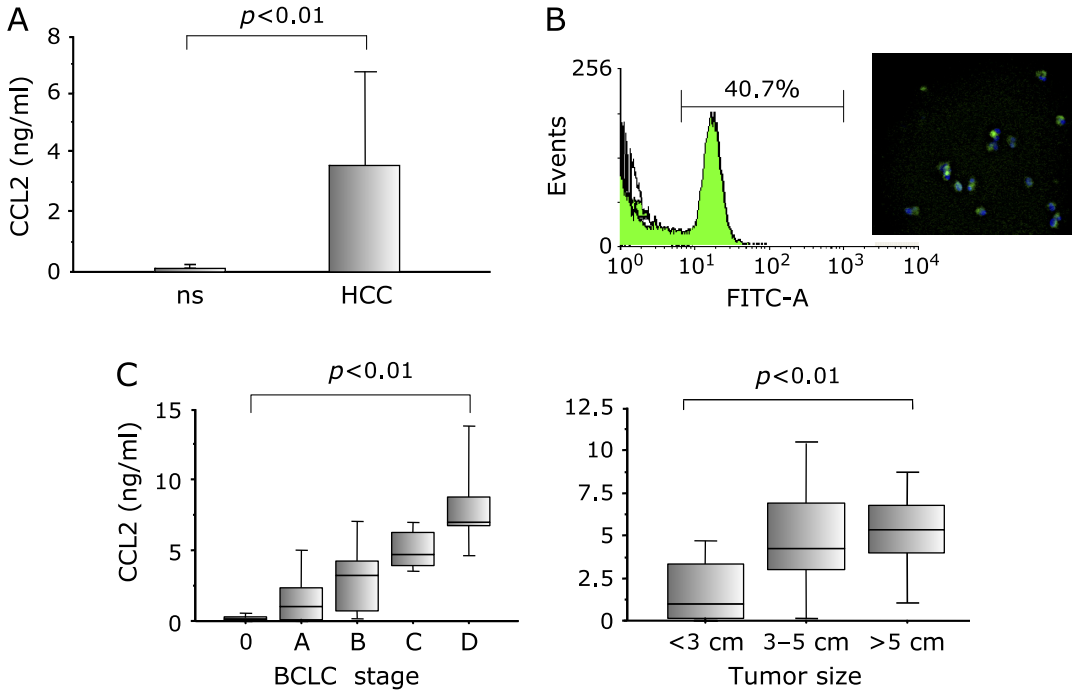

Fig. 2. (A) PMN were incubated with SAC (0.0075%) for 72 h at 37.0°C. Culture fluids harvested were tested for CCL2 by ELISA. Statistical analyses were performed by Mann-Whitney *U* test. ns: normal subjects, HCC: hepatocellular carcinoma. (B) PMN were incubated with SAC (0.0075%) for 72 h at 37.0°C. Intracellular cytokine in cells was stained for CCL2 with FITC- anti-CCL2 mAb. Data were analyzed by FACSaria and WinMDI software. CCL2 stained cells are shown by the green histogram and the open histogram is the isotype control. (C) PMN were incubated with SAC (0.0075%) for 72 h at 37.0°C. Culture fluids harvested were tested for CCL2 by ELISA. Statistical analyses were performed by K-W test.

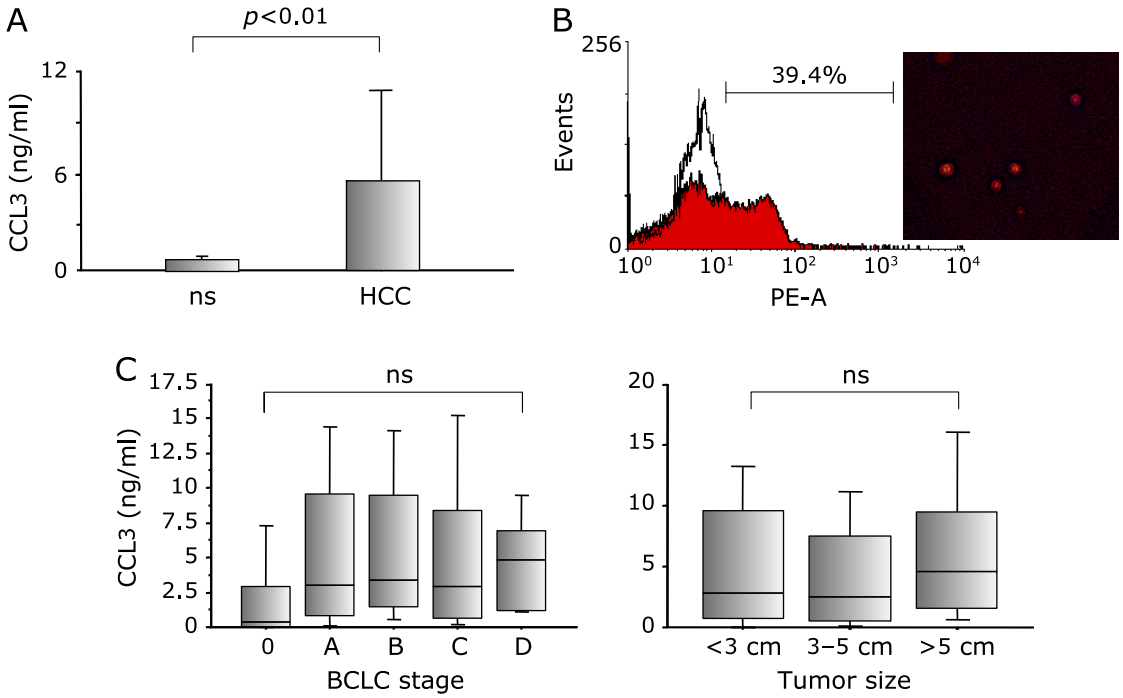

Fig. 3. (A) PMN were incubated with SAC (0.0075%) for 72 h at 37.0°C. Culture fluids harvested were tested for CCL3 by ELISA. Statistical analyses were performed by Mann-Whitney *U* test. ns: normal subjects, HCC: hepatocellular carcinoma. (B) Intracellular cytokine in cells harvested was stained for CCL3 with PE anti-CCL3 mAb. Data were analyzed by FACSaria and WinMDI software. CCL3 stained cells are shown by the red histogram and open histogram is the isotype control. (C) PMN were incubated with SAC (0.0075%) for 72 h at 37.0°C. Culture fluids harvested were tested for CCL3 by ELISA. Statistical analyses were performed by K-W test.

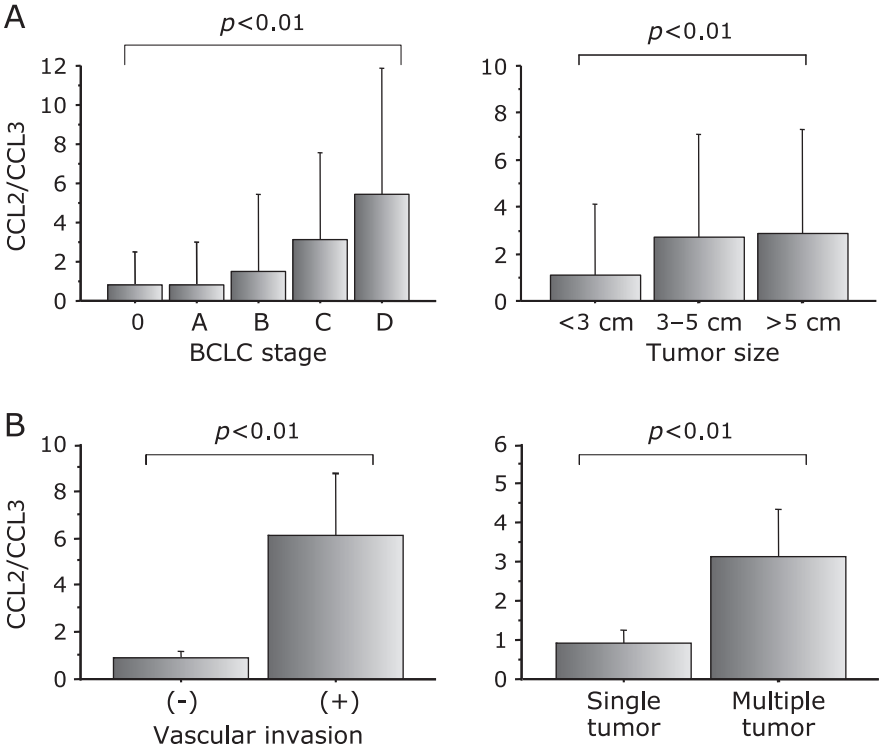

Fig. 4. (A) The CCL2/CCL3 ratio in culture fluids of PMN was calculated for each HCC patient, and the correlation with tumor staging and size was analyzed. Statistical analyses were performed by K-W test. (B) The CCL2/CCL3 ratio in culture fluids of PMN was calculated for each HCC patient, and the correlation with tumor number and vascular invasion was analyzed. Statistical analyses were performed by Mann-Whitney *U* test.

patients. However, in contrast to CCL2, CCL3 production from PMN tended to decrease in advanced HCC patients.

CCL2/CCL3 ratio of PMN culture fluid in each HCC patient. Because production of both CCL2 and CCL3 by PMN increased in HCC patients, we analyzed the ratio of CCL2 to CCL3 in each HCC patient. The CCL2/CCL3 ratio of PMN culture fluid increased in proportion to tumor size in HCC patients (Fig. 4A). Moreover, the CCL2/CCL3 ratio significantly increased in patients with multiple HCC or vascular invasion of HCC (Fig. 4B). These results suggest that CCL2-producing PMN were dominant in advanced HCC patients.

Affect on survival of CCL2 production by PMN in advanced HCC patients. By July 2009, 33 patients had died of HCC, and 3 patients were lost to follow-up. The median follow-up period was 18 months (range, 1 to 48 months). Because of the increase in CCL2 production from advanced HCC-derived PMN, we investigated the effect of PMN on overall survival (OS) and time to progression (TTP). TTP was defined as the time from study entry to disease progression. The OS curves are shown Fig. 5A. When all HCC patients are divided into two groups (those in whom PMN produced CCL2 and those in whom PMN produced CCL3), the high-CCL2-production group was shown to have significantly poorer survival than the low CCL2 group (Fig. 5A). However, there were no significant differences between the survival curves for the high- and low-CCL3-production groups (Fig. 5A). In the analysis of TTP, early progression of HCC tended to be higher in the high-CCL2-production group (Fig. 5B). Moreover, multivariate analysis revealed that the high CCL2 production from PMN was an independent prognostic factor of TTP (Table 2). These results indicate that CCL2-producing PMN (but not CCL3-producing PMN) had some influence on host survival.

Suppressor activity of CCL2-producing PMN on IFN-γ production by PBMC. To clarify the suppressor activity of CCL2-producing PMN, we isolated PBMC from curatively treated HCC patients and cultured these cells with the culture supernatants of PMN-H or PMN-NH. PMN-H is PMN isolated from the same HCC patients before curative therapy. PMN-NH is PMN isolated from the same HCC patients after curative therapy. IFN-γ production by PBMC decreased when they were cultured with PMN-H, but not PMN-NH, culture supernatants (Fig. 6A). The IFN-γ suppression activity of PMN-H on PBMC disappeared as a result of treatment with anti-CCL2 mAb (Fig. 6B). Moreover, IFN-γ production by PBMC also decreased as a result of stimulation with recombinant CCL2 (Fig. 6C). These results indicate that CCL2 from PMN may be involved in host immune suppression in HCC patients.

Discussion

In this study, we attempted to clarify the mechanism of tumor extension through an evaluation of cell-mediated immunity in HCC patients. In a previous study, we reported that plasma neopterin levels in HCC patients were elevated.[28] However, in this study, plasma neopterin levels tended to decrease in advanced HCC patients, while plasma CCL17 levels increased in proportion to HCC size. When we calculated the ratio of neopterin to CCL17 in each HCC patient, the neopterin/CCL17 ratio was found to be significantly increased in advanced HCC patients compared with small HCC patients. Neopterin has been used as a marker of activated cell-mediated immunity while CCL17 has been used as a marker for impaired cell-mediated immunity. These findings suggest that the cell-mediated immunity was reduced in advanced HCC patients compared with small HCC patients.

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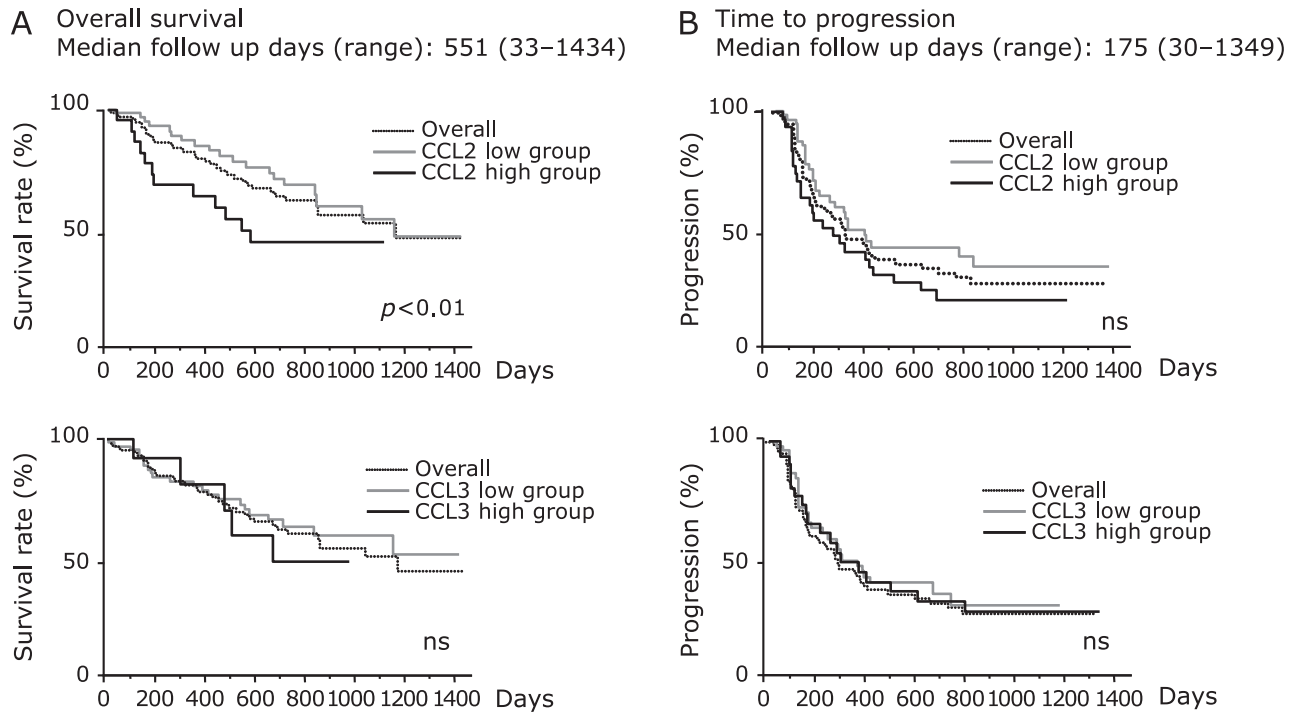

Fig. 5. HCC patients whose PMN produced more than 5.0 ng/ml of CCL2 were assigned to the CCL2 high group. The others were assigned to the CCL2 low group. Patients whose PMN produced more than 5.0 ng/ml of CCL3 were assigned to the CCL3 high group. The other patients were assigned to the CCL3 low group. Statistical analyses were performed by Kaplan-Meier methods and log-rank test.

Table 2. Univariate and multivariate analyses of factors associated with survival and recurrence

Variable	OS			TTP		
	Risk ratio	95% CI	*p*	Risk ratio	95% CI	*p*
Univariate analyses						
Age years (≥65 vs <65)	0.89	–0.46 to 0.37	0.79	1.03	–0.29 to –0.27	0.923
Sex (female vs male)	2.55	–0.02 to 1.08	0.06	1.51	–0.08 to 0.52	0.17
Etiology (viral vs no vial)	3.5	–0.14 to 2.36	0.12	1.86	–0.64 to 0.08	0.1
AFP, ng/ml (≥21 vs <21)	6.87	–0.42 to 1.69	0.001	1.47	–0.49 to 0.08	0.17
ALT, U/l (≥40 vs <40)	1.83	–0.10 to 0.73	0.14	1.31	–0.41 to 0.13	0.32
Child-Pugh score (A vs B or C)	0.92	–0.54 to 0.40	0.88	1.34	–0.142 to 0.42	0.31
Tumor size (≥5 cm vs <5 cm)	8.92	0.69 to 1.52	<.001	2.82	0.17 to 0.83	0.005
Tumor number (single vs multiple)	5.21	0.22 to 1.74	0.005	3.94	0.35 to 1.08	<.001
Vascular invasion (no vs yes)	5.06	0.40 to 1.27	<.001	2.73	0.22 to 0.78	0.001
CCL2 ng/ml (≥5 vs <5)	3.76	0.21 to 1.21	0.003	1.57	–0.04 to 0.50	0.1
CCL3 ng/ml (≥5 vs <5)	1.42	0.27 to 0.62	0.545	1.03	–0.028 to 0.30	0.91
Multivariate analyses						
AFP, ng/ml (≥21 vs <21)	6.57	0.34 to 1.71	0.002	1.25	–0.19 to 0.43	0.47
Tumor size (≥5 cm vs <5 cm)	4.64	0.27 to 1.31	0.003	1.92	–0.11 to 0.73	0.14
Tumor number (single vs multiple)	2.04	–0.57 to 1.84	0.49	3.76	0.19 to 1.17	0.005
Vascular invasion (no vs yes)	1.61	–0.33 to 0.88	0.42	2.64	0.08 to 0.91	0.02
CCL2 ng/ml (≥5 vs <5)	1.34	–0.58 to 0.94	0.704	2.36	–0.86 to –0.05	0.02
CCL3 ng/ml (≥5 vs <5)	1.38	–0.39 to 0.74	0.569	1.06	–0.42 to 0.28	0.67

Univariate and multivariate analyses were performed by the Cox proportional hazards regression model. Cut off value of CCL2 or CCL3 were decided according to ROC curve. Variables for multivariate analysis were adopted for their prognostic significance by uivariate analysis.

CCL17 has been reported to be produced by macrophages (M2 macrophages), dendritic cells, T cells, bronchial epithelial cells or keratinocytes, and to be elevated in sera in several allergic diseases.[21,29–32] Katakura *et al.*[21] reported that CCL17 was produced by M2 macrophages and converted resident macrophages to M2 macrophages in murine models. As the elevation of CCL17 has been reported to reflect a decline in host cell-mediated immunity, we used it as a marker of impaired cell-mediated immunity.[31] Neopterin belongs to the chemical group known as pteridines, and is one of the intermediate metabolites in the biosynthesis of tetrahydrobiopterin.[22] It is released from monocytes or macrophages upon stimulation with IFN-γ.[23] In *in vivo*

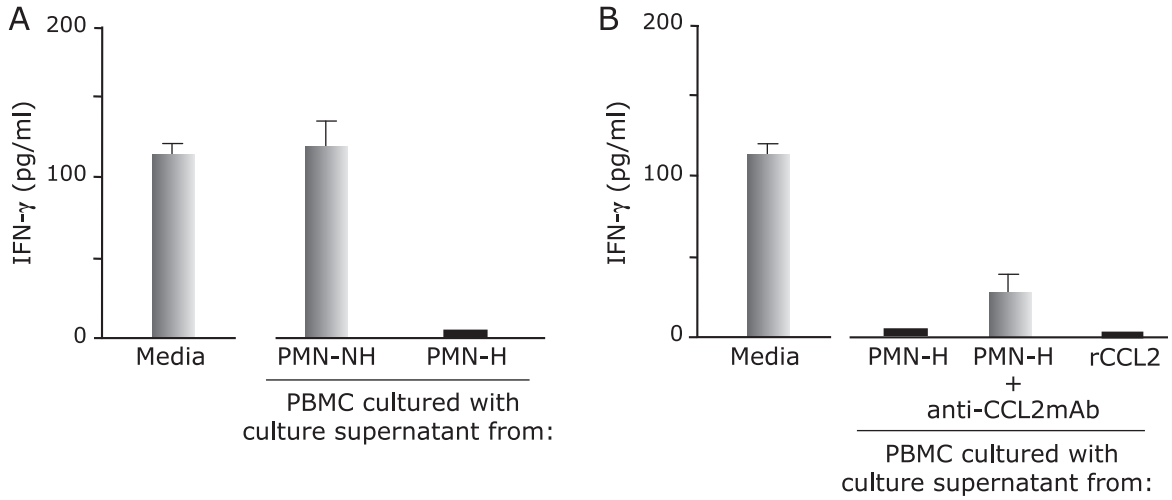

Fig. 6. (A) PBMC (1×10^6 cells/ml) isolated from a patient after curative HCC treatments were cultured with culture supernatant (30%, v/v) of PMN from the same patient with HCC (PMN-H) or without HCC (PMN-NH). The suppressor cell activity was determined by the inhibition of IFN-γ production by PBMC stimulated with LPS (1 μg/ml). (B) PBMC (1×10^6 cells/ml) isolated from a patient after curative HCC treatments were cultured with PMN-H culture supernatant treated with anit-CCL2 mAb (2.5 μg/ml) or recombinant human CCL2 (rCCL2; 25 ng/ml). The suppressor cell activity was determined by the inhibition of IFN-γ production by PBMC stimulated with LPS (1 μg/ml).

studies, it was revealed that serum and urinary neopterin levels increase in a number of disorders including viral infections, autoimmune diseases and graft rejection.[33–35] Therefore, neopterin has been used clinically to diagnose or monitor various diseases as a biological marker of activated cell-mediated immunity. Based on these findings, to investigate host cell-mediated immunity of each HCC patient, the Np/CCL17 ratio in each patient was evaluated. Our findings showed that the Np/CCL17 ratio significantly decreased in proportion to tumor load. Plasma CCL17 and neopterin levels were not correlated with any laboratory data or with liver function. These results suggest that cell-mediated immunity gradually decreases with tumor extension in HCC patients.

Next, distinct subsets of PMN have been reported in mice with different host cell-mediated immunity.[17] In this study, when we isolated PMN from each HCC patient, PMN from HCC patients produced CCL2. Moreover, CCL2 produced by PMN increased in proportion to tumor load. When patients were treated for their tumor, the level of CCL2 produced by PMN decreased (unpublished data). Moreover, it appears that CCL2 is related to their survival. Furthermore, multivariate analysis showed that CCL2 production from PMN was an independent predictor of early progression. CCL3 was also produced by PMN in HCC patients. However, there was no correlation between CCL3 production and tumor size or survival. Moreover, when we analyzed the ratio of CCL2 to CCL3 in each HCC patient, this ratio also increased in proportion to tumor size. These results suggest that PMN that produce CCL2 are predominant in advanced HCC patients while PMN that produce CCL3 are predominant in small HCC. In multivariate analysis, clinical parameters and Child-Pugh score were not correlated with CCL2 production from PMN. An increase in CCL2-producing PMN in particular may be involved in a poor prognosis for HCC.

Functional disorders of various immune cells (T cells, NK cells and dendritic cells) have been reported in HCC patients.[8–10] IFN-γ production by PBMC in HCC patients has been shown to be impaired.[36] Immunosuppressive macrophages increase in HCC patients.[12] Cytotoxic T cell activity against HCC is impaired.[8] In the peripheral blood of HCC patients, the number of dendritic cells is significantly reduced.[10] Furthermore, the majority of these cells are immature and cannot stimulate T cells.[10] $CD4^+CD25^+$ regulatory T cells increase in tumor tissue and malignant ascites from HCC patients.[37] The heterogeneity of PMNs has been demonstrated in human cells.[18,19,38,39] However, the influence of these PMN subsets on host immunity had remained unclear. In this study, we first reported the existence of distinct PMN subsets in the peripheral blood of HCC patients.

It is well known that CCL2 is a key chemokine acting on the development of Th2 responses.[40] In mice models, resident macrophages were converted to M2 macrophages by stimulation with CCL2.[41] PMN-II, one of the recently reported PMN subsets, produced CCL2 and IL-10, and generated M2 macrophages from resident macrophages.[17] An increase in CCL2-producing PMN in advanced HCC patients may result in increased M2 macrophages, and therefore play an important role in their impaired cell-mediated immunity. The increase in CCL2-producing PMN may impair host cell-mediated immunity, resulting in tumor extension. This study clarified that IFN-γ production from PBMC was decreased by cultivation with the culture supernatants of PMN from advanced HCC patients and their IFN-γ suppression activity disappeared as a result of treatment with anti-CCL2 mAb. In contrast, CCL3 has been recognized as an important soluble factor in the development of Th1 responses.[42] In this study, CCL3 production by PMN in HCC patients also increased compared with control patients. These results indicate that the cell-mediated immunity of HCC patients may be enhanced in the early stage.

It is a very difficult to ascertain if tumor extension causes an increase in CCL2-producing PMN or if an increase in CCL2-producing PMN results in tumor extension. However, Tsuda *et al.*[43] reported that PMN from normal mice were converted to PMN-II by stimulation with norepinephrine (NE). NE is a stress hormone and influences various immune cells.[44,45] Yamashiro *et al.*[16] was able to demonstrate CCL2 production from human PMN by stimulation with granulocyte macrophage colony-stimulating factor (GMCSF). These soluble factors are possible inducers of CCL2-producing PMN. Further studies are required to clarify the mechanism of tumor extension and PMN-II.

Tumors have evolved different mechanisms to evade the host's immune response and generate a suppressive network.[46] The appearance of CCL2-producing PMN is one of the factors that is involved in the generation of the suppressive network. Understanding the mechanisms of CCL2-producing PMN is also important to the success of immunotherapy protocols.

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Abbreviations

FITC	fluorescein isothiocyanate
GMCSF	granulocyte macrophage colony-stimulating factor
IFN	interferon
mAb	monoclonal antibody
NK cells	natural killer cells
PE	phycoerythrin
Th2 cells	T helper 2 cells

Conflict of Interst

None of the authors have any financial competing interests regarding this manuscript.

References

1 Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellularcarcinoma. *Cancer Cell* 2004; **5**: 215–219.
2 Okuda K. Hepatocellular carcinoma. *J Hepatol* 2000; **32** (Suppl 1): S225–S237.
3 Llovet JM. Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* 2005; **40**: 225–235.
4 Levy I, Greig PD, Gallinger S, Langer B, Sherman M. Resection of hepatocellular carcinoma without preoperative tumor biopsy. *Ann Surg* 2001; **234**: 206–209.
5 Lencioni R, Crocetti L, Pina MC, Cioni D. Percutaneous image-guided radiofrequency ablation of liver tumors. *Abdom Imaging* 2009; **34**: 547–556.
6 Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907–1917.
7 Watanabe N, Takai H, Imai K, *et al*. Increased levels of serum leptin are a risk factor for the recurrence of stage I/II hepatocellular carcinoma after curative treatment. *J Clin Biochem Nutr* 2011; **49**: 153–158.
8 Shibolet O, Alper R, Zlotogarov L, *et al*. Suppression of hepatocellular carcinoma growth via oral immune regulation towards tumor-associated antigens is associated with increased NKT and $CD8^+$ lymphocytes. *Oncology* 2004; **66**: 323–330.
9 Cai L, Zhang Z, Zhou L, *et al*. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. *Clin Immunol* 2008; **129**: 428–437.
10 Cicinnati VR, Zhang X, Yu Z, *et al*. Increased frequencies of $CD8^+$ T lymphocytes recognizing wild-type p53-derived epitopes in peripheral blood correlate with presence of epitope loss tumor variants in patients with hepatocellular carcinoma. *Int J Cancer* 2006; **119**: 2851–2860.
11 Butterfield LH, Meng WS, Koh A, *et al*. T cell responses to HLA-A*0201-restricted peptides derived from human alpha fetoprotein. *J Immunol* 2001; **166**: 5300–5308.
12 Kuang DM, Wu Y, Chen N, Cheng J, Zhuang SM, Zheng L. Tumor-derived hyaluronan induces formation of immunosuppressive macrophages through transient early activation of monocytes. *Blood* 2007; **15**: 587–595.
13 Verhoef J. Host defense against infection. In: Crossley KB, Archer GL, eds. *The Staphylococci in Human Disease*. New York: Churchill Livingstone, 1997; 213–232.
14 Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–395.
15 Romani L, Mencacci A, Cenci E, *et al*. Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. *J Immunol* 1997; **158**: 5349–5356.
16 Yamashiro S, Kamohara H, Yoshimura T. MCP-1 is selectively expressed in the late phase by cytokine-stimulated human neutrophils: TNF-α plays a role in maximal MCP-1 mRNA expression. *J Leukoc Biol* 1999; **65**: 671–679.
17 Tsuda Y, Takahashi H, Kobayashi M, Hanafusa T, Herndon DN, Suzuki F. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. *Immunity* 2004; **21**: 215–226.
18 Egger G, Burda A, Mitterhammer H, Baumann G, Bratschitsch G, Glasher A. Impaired blood polymorphonuclear leukocyte migration and infection risk in severe trauma. *J Infect* 2003; **47**: 148–154.
19 Borregaard N, Lollike K, Kjeldsen L, *et al*. Human neutrophil granules and secretory vesicles. *Eur J Haematol* 1993; **51**: 187–198.
20 Imai T, Nagira M, Takagi S, *et al*. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; **11**: 81–88.
21 Katakura T, Miyazaki M, Kobayashi M, Herndon DN, Suzuki F. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J Immunol* 2004; **172**: 1407–1413.
22 Fuchs D, Weiss G, Wachter H. Neopterin, biochemistry and clinical use as a marker for cellular immune reactions. *Int Arch Allergy Immunol* 1993; **101**: 1–6.
23 Huber C, Batchelor JR, Fuchs D, *et al*. Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. *J Exp Med* 1984; **160**: 310–316.
24 Reibnegger G, Fuchs D, Fuith LC, *et al*. Neopterin as a marker for activated cell-mediated immunity: application in malignant disease. *Cancer Detect Prev* 1991; **15**: 483–490.
25 Llovet JM, Di Bisceglie AM, Bruix J, *et al*. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698–711.
26 van Lieshout AW, van der Voort R, le Blanc LM, *et al*. Novel insights in the regulation of CCL18 secretion by monocytes and dendritic cells via cytokines, toll-like receptors and rheumatoid synovial fluid. *BMC Immunol* 2006; **7**: 23.
27 Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. *J Immunol Methods* 1993; **159**: 197–207.
28 Tsuda Y, Fukuda A, Kobayashi H, *et al*. Serum neopterin as a marker for screening of hepatocellular carcinoma. *Pteridines* 2004; **15**: 161–169.
29 Katoh S, Fukushima K, Matsumoto N, *et al*. Accumulation of CCR4-expressing $CD4^+$ T cells and high concentration of its ligands (TARC and MDC) in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia. *Allergy* 2003; **58**: 518–523.
30 Romagnani S. Cytokines and chemoattractants in allergic inflammation. *Mol Immunol* 2002; **38**: 881–885.
31 Mizukami Y, Kono K, Kawaguchi Y, *et al*. CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *Int J Cancer* 2008; **122**: 2286–2293.
32 Shimada Y, Takehara K, Sato S. Both Th-2 and Th1 chemokines (TARC/CCL-17, MDC/CCL-22 and MIG/CXCL-9) are elevated in sera from patients with atopic dermatitis. *J Dermatol Sci* 2004; **34**: 201–208.
33 Fuchs D, Hausen A, Reibnegger G, Werner ER, Dierich MP, Wachter H. Neopterin as a marker for activated cell-mediated immunity: application in HIV infection. *Immunol Today* 1988; **9**: 150–155.
34 Samsonov MY, Tilz GP, Egorova O, *et al*. Serum soluble markers of immune activation and disease activity in systemic lupus erythematosus. *Lupus* 1995; **4**: 29–32.
35 Reibnegger G, Aichberger C, Fuchs D, *et al*. Posttransplant neopterin excretion in renal allograft recipients—a reliable diagnostic aid for acute rejection and a predictive marker of long-term graft survival. *Transplantation* 1991; **52**: 58–63.
36 Kawarabayashi N, Seki S, Hatsuse K, *et al*. Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *Hepatology* 2000; **32**: 962–969.
37 Fu J, Xu D, Liu Z, *et al*. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; **132**: 2328–2339.
38 Krause PJ, Malech HL, Kristie J, *et al*. Polymorphonuclear leukocyte heterogeneity in neonates and adults. *Blood* 1986; **68**: 200–204.
39 Brown CC, Malech HL, Jacobson RJ, *et al*. Unique human neutrophil populations are defined by monoclonal antibody ED12F8C10. *Cell Immunol* 1991; **132**: 102–114.

40 Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of Th2 polarizations by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; **404**: 407–411.
41 Tsuda Y, Takahashi H, Kobayashi M, Hanafusa T, Herndon DN, Suzuki F. CCL2, a product of mice early after systemic inflammatory response syndrome (SIRS), induces alternatively activated macrophages capable of impairing antibacterial resistance of SIRS mice. *J Leukoc Biol* 2004; **76**: 368–373.
42 Taub DD, Turcovski-Corrales SM, Key ML, Longo DL, Murphy WJ. Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation *in vitro*. *J Immunol* 1996; **156**: 2095–2103.
43 Tsuda Y, Kobayashi M, Herndon DN, Suzuki F. Impairment of the host's antibacterial resistance by norepinephrine activated neutrophils. *Burns* 2008; **34**: 460–466.
44 Gosain A, Muthu K, Gamelli RL, DiPietro LA. Norepinephrine suppresses wound macrophage phagocytic efficiency through alpha- and beta-adrenoreceptor dependent pathways. *Surgery* 2007; **142**: 170–179.
45 Bergmann M, Gornikiewicz A, Sautner T, *et al*. Attenuation of catecholamine-induced immunosuppression in whole blood from patients with sepsis. *Shock* 1999; **12**: 421–427.
46 Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 2008; **27**: 5904–5912.

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