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# Alteration of serum cytokine balances among different phases of chronic hepatitis B virus infection

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**Abstract**

To understand the pathogenesis of chronic hepatitis B virus (HBV) infection, we examined the serum levels of IL-10, TNF-α IL-12 p70, and IL-12 p40 in 77 patients chronically infected with HBV and 19 controls. The patients were classified into four groups: asymptomatic carriers (ASC), patients with chronic hepatitis (CH), patients with liver cirrhosis (LC), and patients with hepatocellular carcinomas (HCC). The cytokine values among these groups were compared and their relations to clinical parameters were investigated. All these cytokine values were higher in the patient groups than in controls. IL-10 and TNF-α became higher in accordance with the progress of the disease phases, from ASC to LC, and lowest when the patients had HCC. IL-12 p40 was also lowest in HCC, however, the group with highest levels was CH. IL-12 p70 was unchanged among ASC, CH, and LC, but were raised in HCC. Serial analyses for the cytokine values in the same patients showed the similar tendencies. Regression analysis showed the significant correlations between ALT and IL-10. Serum cytokine values well reflected the pathological differences of the individual disease phases, and may become useful indices to understand the pathogenesis of chronic HBV infection.


*Keywords:* HBV; IL-10; IL-12 p70; IL-12 p40; TNF-α

## 1. Introduction

Hepatitis B virus (HBV) is an infectious agent, causing chronic infection, leading infected patients to various clinical conditions from asymptomatic status to liver cirrhosis, and sometimes to the condition complicating hepatocellular carcinoma (HCC). Since HBV is a noncytolytic virus, the most part of the pathology in HBV infection is thought to be mediated by the host immune response against the viral products [1–3].

Cytokines are produced by the various cell types including lymphocytes, macrophages, dendritic cells (DC), etc., and have been claimed to play pivotal roles in the pathogenesis of chronic HBV infection [1,4]. Inflammation in HBV-infected liver is proved to be mediated by cytokines, like IFN-γ and TNF-α, and the elimination of the virus is also caused by these cytokines [1,5–9]. Fibrosis in the liver is thought to be caused or accelerated by the cytokines, like TGF-β [10,11], and development or enlargement of HCC is suggested to be accompanied by the abnormality in immunity like cytokine profiles [12–16]. As mentioned, cytokines are believed to be important factors that organize pathology of the disease, however, the relation of cytokine levels to sequence of the disease phases or clinical factors are not well understood.

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In the present study, to elucidate the detailed roles of cytokines on the disease pathogenesis of HBV infection, we measured IFN-γ, IL-2, IL-4, IL-10, IL-12 p40, IL-12 p70, TNF-α, and TGF-β. IL-12 is known to be composed of two chains, a heavy chain (p40), and a light chain (p35), forming a disulfide-linked heterodimer (p70), and IL-12 p40 is thought to be a negative regulator of IL-12 p70, an active form of IL-12 [17]. Serum levels of these cytokines were measured in the chronically HBV-infected patients with various disease phases. They were compared among patient groups divided by the disease phases, and their relations to clinical parameters were investigated. The results suggested the deep association of these cytokines to inflammation, viral load, viral antigen load, fibrotic changes of the liver, and the complication of HCC in chronic HBV infection as described below.

## 2. Materials and methods

### 2.1. Patients and control subjects

Seventy-seven patients with chronic HBV infection and 19 healthy control individuals were tested for their serum levels of cytokines. Diagnosis for chronic HBV infection was done by detecting serum HBsAg and HBcAb. Serum ALT (IU/l), HBV-DNA (pg/ml), HBeAg titer (cut off index), and HBeAb titer (percent inhibition) were also measured in these patients. According to the activity of inflammation and the stage of fibrosis, patients were subdivided to four groups: ASC—asymptomatic carriers, CH—the patients with chronic hepatitis, LC—the patients with liver cirrhosis, and HCC—the patients with hepatocellular carcinoma. The patients consisted of 23 ASC, 34 CH, 12 LC, and 8 HCC (Table 1a). The diagnosis of each patient category was made by assessing the changes of biochemical tests, such as serum aminotransferase levels, and/or analyzing the shape of liver and spleen by ultrasonography (US) or computed tomography (CT), and/or examining the histology by liver biopsy. The diagnosis of ASC was made when the serum aminotransferase levels of the patients were persistently normal at least for six months. When the patients showed abnormalities in serum aminotransferase levels persistently or at least in two different time points during six months period, they were diagnosed as CH. Liver biopsies were done in 24 patients, and 22 of them were proved to have CH, 2 of them were diagnosed as LC. The extent of inflammation and fibrosis in the liver were evaluated according to the criteria suggested by Ludwig [18] and Ichida et al. [19] (Table 1b). The diagnosis of LC was made by the clinical parameters (albumin, prothrombin time, platelet count, etc.), morphological changes of liver and spleen (by US or CT), and clinical manifestation (esophageal varices, jaundice, acites, etc.), except for two cases by liver biopsies. The occurrence of HCC was diagnosed by US or CT, then, the tumor stages were classified by the TNM staging system suggested by the Liver Cancer Study Group of Japan (Table 1c). All had liver cirrhosis as background liver disease. Two of eight patients were operated, and both were diagnosed as moderately differentiated hepatocellular carcinoma. In the case of CH and LC, the blood was collected when the antiviral drugs, such as interferon or lamivudine were not used. In the case of HCC, the blood was drawn before the treatment for HCC had started. In the patients whose sera were available, the serum cytokine values at different phases of the

Table 1b
Histological findings in patients with chronic hepatitis

| | Mild | Moderate | Severe |
|---|---|---|---|
| Grade[a] | 4 | 14 | 4 |
| Stage[b] | 5 | 10 | 7 |

Liver biopsies were executed in 22 of 34 patients with chronic hepatitis. Histological findings were evaluated according to the criteria suggested by Ludwig [18] and Ichida et al. [19].

[a] Grade, activity of necro-inflammatory reaction in the liver; mild, mild limiting plate necrosis and/or focal lobular necrosis; moderate, moderate limiting plate necrosis and/or severe focal cell damage; severe, severe limiting plate necrosis and/or bridging necrosis.

[b] Stage, extent of liver fibrosis; mild, fibrous portal expansion; moderate, bridging fibrosis but intact architecture; severe, bridging fibrosis with architectural distortion. Numbers listed are the numbers of the patients categorized to each grade or stage.

Table 1a
Profiles of the patients with chronic HBV infection and the control subjects

| | Control ($n$ = 19)[a] | ASC[b] ($n$ = 23) | CH[c] ($n$ = 34) | LC[d] ($n$ = 12) | HCC[e] ($n$ = 8) |
|---|---|---|---|---|---|
| Age (years) | 34 ± 2 | 34 ± 3 | 37 ± 2 | 53 ± 2 | 56 ± 3* |
| Gender (M/F) | 13/6 | 10/13 | 19/15 | 10/2 | 6/2 |
| ALT[f] (IU/l) | – | 23 ± 1 | 153 ± 11 | 104 ± 47 | 61 ± 12* |
| HBeAg$^+$/HBeAb$^+$ | – | 9/14 | 22/12 | 6/6 | 2/6 |
| HBV-DNA (pg/ml) | – | 417 ± 193 | 783 ± 287 | 67 ± 48 | 2 ± 1 |

All the data are expressed as mean ± standard errors (S.E.).

[a] $n$, number of the patients.

[b] ASC: asymptomatic HBV carrier with persistently normal serum aminotransferase levels.

[c] CH: patient with chronic active hepatitis.

[d] LC: patient with liver cirrhosis.

[e] HCC: patients with hepatocellular carcinoma.

[f] ALT: alanine aminotransferase (normal range: <45 IU/l).

* $p < 0.001$ (ANOVA).

Table 1c
TNM stages of hepatocellular carcinomas

| TNM stage | Numbers of the patients |
|---|---|
| I | 2 |
| II | 5 |
| III | 1 |

Patients with hepatocellular carcinoma were classified by TNM staging system suggested by the Liver Cancer Study Group of Japan. Numbers indicated are the numbers of the patients categorized to each stage.

disease were analyzed (ASC and CH: 6 patients; CH and LC: 3 patients; LC and HCC: 6 patients). All the healthy control subjects were negative for hepatitis viral markers including HBsAg, HBcAb, and anti-HCV antibody. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the human research committee of Aichi Medical University School of Medicine. The profiles of the patients were summarized in Tables 1a–1c.

### 2.2. Detection of cytokines

Blood samples were collected under informed consent, and sera were stored at −80 °C until the measurement of cytokines. Commercially available ELISA kits (IFN-γ, IL-12 p70 + p40, and TGF-β by Amersham Pharmacia Biotech, U.S.A., IL-2, IL-4, IL-10, IL-12 p70, and TNF-α by BD Biosciences Pharmingen, U.S.A.) were used for the detection of serum cytokines, and assays were done according to the manufacturer's instruction. Briefly, the serum samples thawed just before the assay were applied to assay plate (96-well plate) that was precoated with monoclonal antibody (first antibody) for each cytokine. Then the plate was incubated at room temperature for 2–4 h, washed three times with 0.05% Tween 20 in PBS, and another monoclonal antibody for each cytokine conjugated with horseradish-peroxidase (second antibody) was applied to the wells. After 2 h incubation at room temperature, the plates were washed three times, and the enzyme reaction was performed with tetramethylbenzidine dissolved in substrate buffer and 33% $H_2O_2$. After 30 min of incubation at room temperature, the reaction was stopped with 1.8 M $H_2SO_4$, and the light absorbance at 450 nm was measured with an ELISA spectrophotometer. IL-12 p40 levels were calculated by subtracting the values of IL-12 p70 from the values detected by the kit for IL-12 p40 + p70. The assays for all the cytokines were done simultaneously to avoid frequent freeze-thawing of the serum samples.

### 2.3. Statistics

The data were presented as means ± standard errors (S.E.). The differences in clinical parameters among the patient groups and control group were analyzed by one-way analysis of variance between groups (ANOVA) or chi-square for independence test. The relationship of the cytokine levels among the controls and patient groups were analyzed by Student's *t*-test, paired *t*-test, and ANOVA. The regression analysis with clinical parameters and cytokine values were done using commercially available JMP software by SAS Institute Inc., U.S.A. The *p*-value below 0.05 was regarded as statistically significant.

## 3. Results

### 3.1. Clinical parameters in patient groups and control group

Since the patient groups were subdivided by the disease phases that show different activities of inflammation and the different fibrotic changes of the liver, there were significant differences in ALT levels and ages among the groups ($p < 0.001$, respectively, by ANOVA). The ages were almost equivalent among control, ASC, and CH, and higher in LC and HCC. The ages between the latter two groups were almost equivalent. Balances of gender and $HBeAg^+/HBeAb^+$ status were not significantly different among groups (by chi-square for independence test, Table 1a).

### 3.2. Serum cytokine levels of the patients and controls

Since serum levels of IL-2, IL-4, and IFN-γ were almost always under detection levels both in the patient groups and the controls, they were excluded from the analysis. Serum TGF-β levels were measured in the limited number of the patients, however, they were also excluded from the detailed analysis because of the instability of the data. The use of sera instead of plasma and the condition of preparing sera might cause the problem.

As shown in Fig. 1, all the detected cytokine values (IL-10, TNF-α, IL-12 p70, and IL-12 p40) in the patient groups were higher compared to those in the healthy controls.

IL-10 levels got higher as the disease phases progressed from ASC to LC (ASC: 11.2 ± 0.9 pg/ml; CH: 14.2 ± 0.9 pg/ml; LC: 17.0 ± 2.5 pg/ml; mean ± S.E., respectively), and declined when the patients complicated with HCC (9.4 ± 0.7 pg/ml). TNF-α levels showed similar tendency. They were lowest in HCC (89 ± 20 pg/ml), but in the patients without HCC, they went up with the advancement of the disease phases (ASC: 168 ± 37 pg/ml; CH: 193 ± 20 pg/ml; LC: 268 ± 39 pg/ml). IL-12 p40 levels were also lowest in HCC (89 ± 28 pg/ml), however, the group that had highest levels of the cytokine was CH (ASC: 138 ± 28 pg/ml; CH: 166 ± 31 pg/ml; LC: 115 ± 16 pg/ml). IL-12 p70 levels showed different tendency compared to the other cytokines. The changes of IL-12 p70 among the patient groups were not obvious, while their levels in HCC were tended to be higher than the other groups (ASC: 5.8 ± 1.4 pg/ml; CH: 6.2 ± 0.3 pg/ml; LC: 6.2 ± 0.3 pg/ml; HCC: 7.8 ± 0.6 pg/ml).

By ANOVA, significant differences in the individual cytokine levels were observed among all the groups including control group, and also among the patient groups, except in IL-12 p40 among the patient groups (Fig. 1).

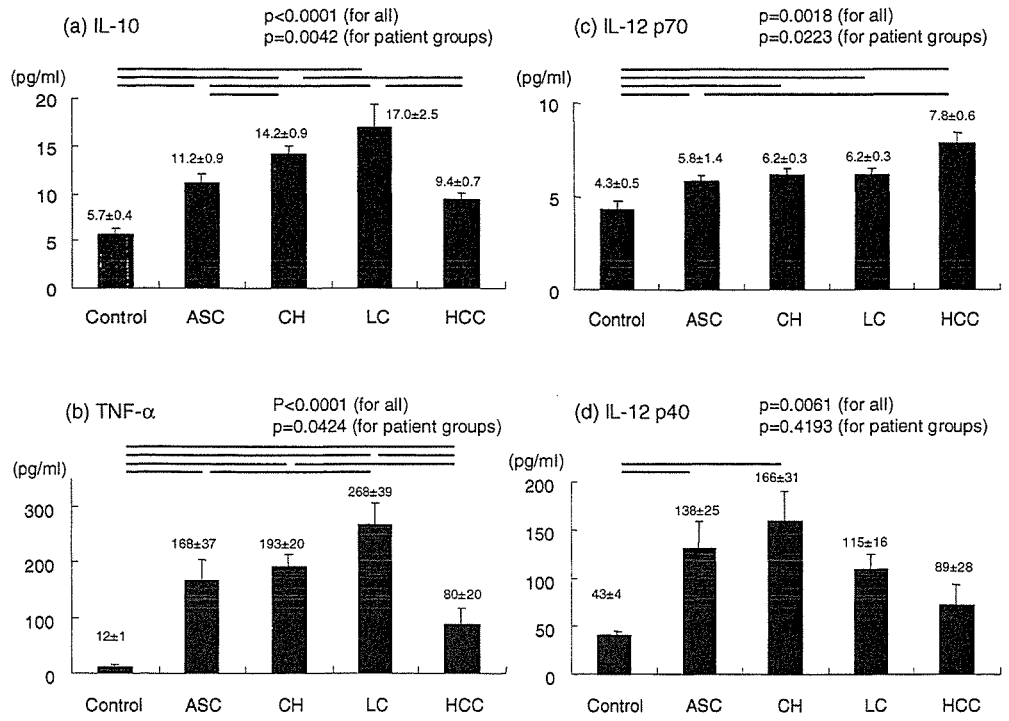


Fig. 1. Serum cytokine levels of the patient groups with chronic HBV infection. Serum cytokine levels in controls and patients were measured and calculated as described in Section 2. Numbers at the upper part of the vertical bars indicate mean ± standard error (S.E.) of the cytokine values in the individual groups. Statistical analysis was done using ANOVA and unpaired Student's *t*-test. *p*-Values calculated by ANOVA are described on top of the individual graphs. Horizontal bars on the upper part of the graphs indicate the existence of significant differences between the groups of both ends of the bars by *t*-test ($p < 0.05$). ASC, asymptomatic carriers with persistently normal serum aminotransferase levels; CH, patients with chronic active hepatitis; LC, patients with liver cirrhosis; HCC, patients with hepatocellular carcinomas.

### *3.3. Influence of HBeAg⁺/HBeAb⁺ status on cytokine balances*

To evaluate the influence of HBeAg⁺/HBAb⁺ status on cytokine values and clinical parameters, the patients were subdivided into HBeAg⁺ and HBeAb⁺ within each patient group.

In ASC, the patients with HBeAg⁺ were younger than the patients with HBeAb⁺ ($p = 0.0468$), and HBV-DNA levels of HBeAg⁺ group were higher than those of HBeAb⁺ group ($p = 0.0013$). With regard to cytokine values, TNF-α levels of HBeAg⁺ group were significantly higher than those of HBeAb⁺ group ($p = 0.0231$). IL-12 p40 levels showed similar tendency but without statistical significance ($p = 0.0786$, Table 2).

Different from ASC, the ages of the patients were almost equivalent between HBeAg⁺ and HBeAb⁺ in CH. However, HBV-DNA levels of HBeAg⁺ group were higher than those of HBeAb⁺ group ($p = 0.0478$). Although none of cytokine values showed significantly differences between two groups, TNF-α and IL-12 p40 levels seemed to be high in HBeAg⁺ group (Table 3). These results may suggest that HBeAg⁺/HBAb⁺ status and/or the amount of HBV-DNA influence the serum levels of these two cytokines in ASC and CH.

There were no statistical differences in clinical parameters and cytokine values between HBeAg⁺ and HBeAb⁺ when analyzed in LC and HCC (data not shown).

### *3.4. Influence of fibrotic changes of the liver on cytokine balances*

The patients who received liver biopsy were subdivided according to the extent of fibrosis. Then, the influence of liver fibrosis on cytokine values was evaluated among these groups and the group with LC. The significant difference was seen

Table 2
Comparison of clinical parameters and cytokine profiles between HBeAg⁺ and HBeAb⁺ group in asymptomatic carriers

| Parameters | HBeAg⁺ ($n = 8$)[a] | HBeAb⁺ ($n = 15$) | *p*-Value |
|---|---|---|---|
| Age (years) | 27 ± 4 | 39 ± 3 | 0.0468 |
| ALT (IU/l) | 26 ± 1 | 22 ± 1 | 0.0326 |
| HBV-DNA (pg/ml) | 1198 ± 452 | 0.8 ± 0.8 | 0.0013 |
| IL-10 (pg/ml) | 12.7 ± 1.7 | 10.3 ± 1.1 | 0.2252 |
| TNF-α (pg/ml) | 280 ± 89 | 109 ± 21 | 0.0231 |
| IL-12 p70 (pg/ml) | 5.6 ± 0.3 | 7.9 ± 1.9 | 0.3792 |
| IL-12 p40 (pg/ml) | 205 ± 69 | 102 ± 19 | 0.0786 |

All the data are expressed as mean ± S.E. *p*-Values lower than 0.05 were considered to be significant (Student's *t*-test).
[a] *n*, number of the patients.

Table 3
Comparison of clinical parameters and cytokine profiles between $HBeAg^+$ and $HBeAb^+$ group in the patients with chronic hepatitis

| Parameters | $HBeAg^+$ ($n=22$)[a] | $HBeAb^+$ ($n=12$) | $p$-Value |
|---|---|---|---|
| Age (years) | 37 ± 3 | 39 ± 3 | 0.6053 |
| ALT (IU/l) | 163 ± 11 | 136 ± 25 | 0.2717 |
| HBV-DNA (pg/ml) | 1120 ± 420 | 21.4 ± 10.4 | 0.0478 |
| IL-10 (pg/ml) | 15.3 ± 1.2 | 12.4 ± 1.2 | 0.1271 |
| TNF-α (pg/ml) | 208 ± 28 | 167 ± 23 | 0.3446 |
| IL-12 p70 (pg/ml) | 6.0 ± 0.2 | 6.6 ± 0.6 | 0.2976 |
| IL-12 p40 (pg/ml) | 194 ± 45 | 114 ± 28 | 0.2244 |

All the data are expressed as mean ± S.E. $p$-Values lower than 0.05 were considered to be significant (Student's $t$-test).
[a] $n$, number of the patients.

in TNF-α levels between the group with mild or moderate fibrosis and the one with LC ($p<0.05$). And TNF-α levels seemed to show sequential changes in accordance with the progression of fibrosis. The changes of other cytokine values among the groups seemed to be similar to those between CH and LC (Table 4).

### 3.5. Serial analysis of cytokine values in the same patients with different disease phases

In some patients whose sera at different phases of the disease were available, the cytokine values through the two phases were measured and the serial changes of them were analyzed (Fig. 2). There were six CH patients who were previously diagnosed as ASC, three LC patients previously diagnosed as CH, and six HCC patients previously diagnosed as HCC, respectively. The numbers of the patients were limited, however, the serial changes of cytokine values in the same patients showed similar tendencies to the changes observed among the patient groups of different phases (Figs. 1 and 2). IL-10 levels showed significant increase when the disease progressed from ASC to CH ($p=0.0001$), and significant decrease when HCC were complicated ($p=0.0169$). TNF-α levels of the LC patients who complicated HCC also showed significant decrease ($p=0.0009$). TGF-β levels were measured in the HCC patients before and after the complication of HCC. TGF-β levels in HCC phase (38.8 ± 21.5 ng/ml) seemed to be higher than those in LC phase (51.6 ± 20.9 ng/ml), but the difference between two phases was not significant ($p=0.0905$).

Table 4
Comparison of clinical parameters and cytokine profiles in patient groups with different severity of liver fibrosis

| Parameters | Extent of liver fibrosis | | |
|---|---|---|---|
| | ≤Moderate[a] ($n=15$) | Severe[b] ($n=7$) | Cirrhosis ($n=12$) |
| Age (years) | 31 ± 3[c,d] | 45 ± 4[c] | 53 ± 2[d] |
| ALT (IU/l) | 156 ± 21 | 115 ± 17 | 104 ± 47 |
| HBV-DNA (pg/ml) | 1380 ± 640 | 658 ± 508 | 67 ± 48 |
| IL-10 (pg/ml) | 14.8 ± 2 | 14.3 ± 1.1 | 17.0 ± 2.5 |
| TNF-α (pg/ml) | 149 ± 27[e] | 229 ± 46 | 268 ± 39[e] |
| IL-12 p70 (pg/ml) | 5.7 ± 0.3 | 6.0 ± 0.7 | 6.2 ± 0.3 |
| IL-12 p40 (pg/ml) | 119 ± 33 | 155 ± 33 | 115 ± 16 |

All the data are expressed as mean ± S.E.
[a] ≤Moderate, mild to moderate fibrosis, fibrous expansion of portal areas, with or without short fibrous septa and occasional bridging.
[b] Severe, fibrous expansion of portal areas, with marked bridging and architectural distortion.
[c] $p<0.01$.
[d] $p<0.0001$.
[e] $p<0.05$.

### 3.6. Regression analysis of clinical parameters and cytokine levels

The results above indicated that the cytokine levels were elevated even in ASC group without biochemical inflammation in the liver, and suggested that these cytokine levels might be influenced by the activity of the disease, the fibrotic changes of the liver, viral load, viral antigen load, and the complication of malignancy. Since the patterns of cytokine profiles were different among the patient groups, it was suggested that the production of the individual cytokines might be affected by the different factors. Thus, to analyze the relation of clinical parameters to cytokine levels, the regression analysis was done using laboratory data, such as ALT, HBV-DNA, and HBeAg levels as clinical parameters.

According to the analysis in all the patients, significant correlations were observed between ALT and IL-10 ($r=0.4095$, $p=0.0002$, Table 5; Fig. 3). In ASC, the significant correlations were observed between HBeAg and IL-12 p40/TNF-α (HBeAg and IL-12 p40: $r=0.4718$, $p=0.0230$; HBeAg and TNF-α: $r=0.4238$, $p=0.0439$). The correlation between HBV-DNA and IL-10 was observed when analyzed in CH ($r=0.4308$, $p=0.0110$) and LC ($r=0.6468$, $p=0.0230$). The correlation between HBV-DNA and IL-10 disappeared, when the patients had HCC. On the other hand, there was tendency of negative correlation between HBV-DNA and IL-12 p70 ($r=-0.6881$, $p=0.0592$) that was not seen in CH or LC (Table 5).

Thus, the analysis suggested that IL-10 levels closely related to liver inflammation and viral load, and that IL-12

Table 5
Regression analysis between clinical parameters and cytokine values in the patients with chronic HBV infection

| | Patient group | $n$ | $r$ | $p$-Value |
|---|---|---|---|---|
| ALT vs. IL-10 | All | 77 | 0.4095 | 0.0002 |
| HBeAg vs. IL-12 p40 | ASC | 23 | 0.4718 | 0.0230 |
| HBeAg vs. TNF-α | ASC | 23 | 0.4238 | 0.0439 |
| HBV-DNA vs. IL-10 | CH | 34 | 0.4308 | 0.0110 |
| HBV-DNA vs. IL-10 | LC | 12 | 0.6468 | 0.0230 |
| HBV-DNA vs. IL-12 p70 | HCC | 8 | −0.6881 | 0.0592 |

The combination of clinical parameter and cytokine value, with significant correlation within the indicated patient group, are listed. ASC: asymptomatic HBV carrier with persistently normal serum aminotransferase levels; CH: patient with chronic active hepatitis; LC: patient with liver cirrhosis; HCC: patients with hepatocellular carcinoma; $n$: number of the subjects; $r$: correlation coefficient.

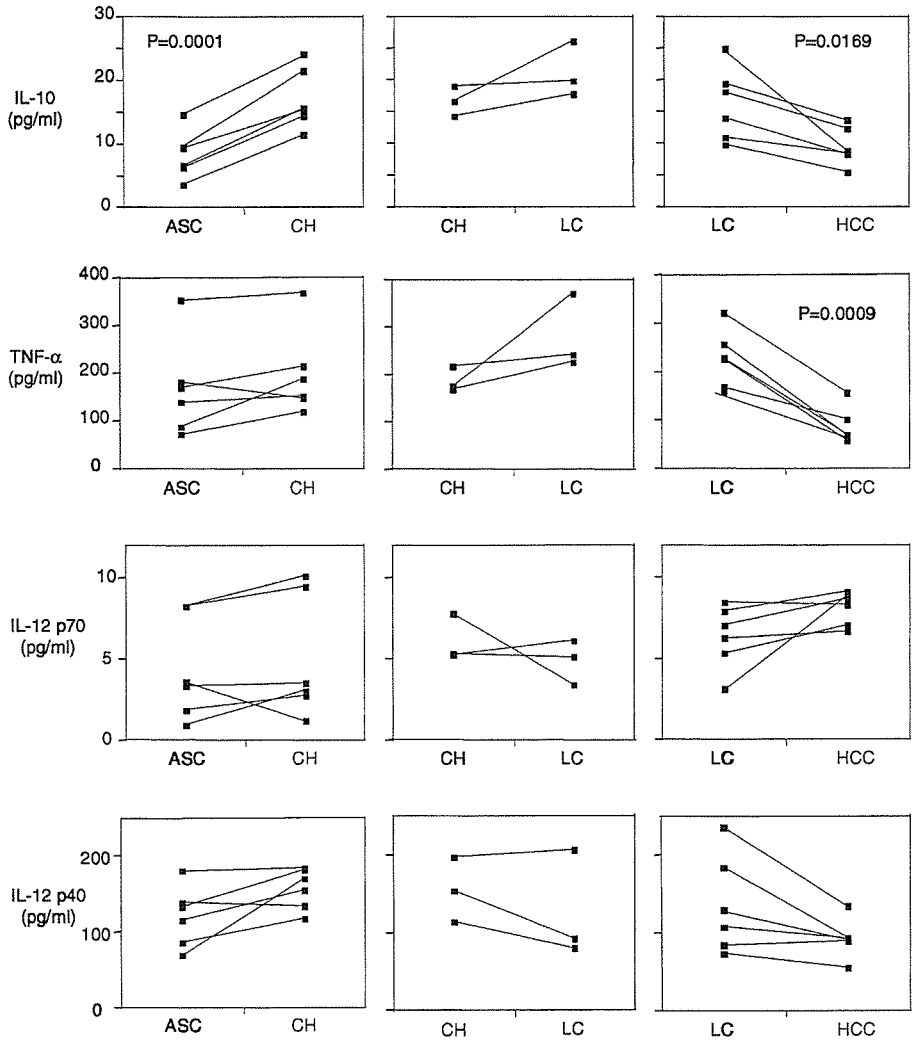


Fig. 2. Serial changes of cytokine values in the same patients in the different phases of chronic HBV infection. The patients analyzed were six with CH previously diagnosed as ASC (graphs in the right row), three with LC previously diagnosed as CH (graphs in the middle row), and six with HCC previously diagnosed as LC (graphs in the left row), respectively. The cytokine values of the same patient were connected by the lines. For statistical analysis, paired Student's *t*-test was applied. *p*-Values under 0.05 were considered to be significant and the actual numbers ($p < 0.05$) are described on the upper part of the graphs.

p40 and TNF-α levels were strongly affected by the amount of viral antigen.

## 4. Discussion

Liver inflammation caused by HBV infection is immune-mediated, and both acquired and innate immune responses are thought to play important roles on the pathogenesis of HBV-related hepatitis [1–3]. In the current study, we measured the serum levels of the cytokines in patients with chronic HBV infection in order to analyze the roles of the immune responses on the disease pathogenesis.

In the study, we could detect IL-10, TNF-α, IL-12 p70, IL-12 p40, and TGF-β, but not IFN-γ, IL-2, or IL-4 in the sera of the patients. There are some reports that could detect IL-2 [19], or the one that could detect only low levels of IL-10 [20]. Our data seem to be contradictory to these reports, however, we think that the differences are not substantial and that the sensitivity of the detection methods might be the main cause of this problem.

The serum levels of the cytokines were varied among the groups which represented the different phases of chronic HBV infection, and the changing patterns of the individual cytokines seemed to be peculiar to them (Fig. 1). The serial analysis of cytokine levels in the same patients through the different phases of HBV infection, showed the similar patterns seen in the analysis among the groups (Fig. 2). This suggests that the analysis among the groups well reflected the actual phenomena seen in the individual patients.

It was suggested from the analysis that the cytokine levels were influenced by various factors, such as inflammation, fibrosis, viral load, viral antigen load, and the occurrence of malignancy in the liver (Tables 2–5; Fig. 3). And it was also suggested that the factors relating to the changes of individual cytokine levels differed with the disease phases (Table 5). For example, IL-10 had correlation to ALT when analyzed in all the patients, but it had correlation to HBV-DNA in CH and in LC. HBV-DNA seemed to correlate to IL-12 p70 negatively in HCC, while no correlation was found between them in other phases of the disease. With regard to the relation of IL-10 to inflammation and viral load of HBV, there were the similar reports by others [19,21–23], however, the actual reasons for these correlations are difficult to explain. They might be indirect correlations, which were regulated by the unknown factors. Nonetheless, it may be important to know the changes of cytokine balances among the disease phases and the responsible factors for these changes, in order to understand the pathogeneses in the individual disease phases. These analyses may lead to the prediction for the transition of disease phase, for example, from LC to HCC, the complication of HCC.

We have to note that the levels of the measured cytokines except for IL-12 p70 were raised in ASC without visible inflammation in the liver. This clearly means that immune system recognizes and reacts against HBV even from immune tolerant phase.

ASC were usually regarded as 'healthy' because of the lack of biochemical abnormalities, however, our data suggest the reconsideration of this idea. It is well known that the occurrence of HCC is 100 times higher in HBV carriers than in healthy individuals without hepatitis virus infection [24], and HCC sometimes develops in the patients without cirrhosis or even in the patients with early stage of chronic hepatitis [25,26]. It is suggested that the development of HCC is caused by the accumulation of oxidative DNA damage in human study and in HBV transgenic mice model [27–30]. Our study may explain the high risk for HCC in the patients with chronic HBV infection, since it showed possible evidence of the activation of macrophages, the source of oxidant radicals, from early stage of infection. However, to confirm the speculation above, the liver biopsy in the patients including ASC to examine the DNA damage and the long-time follow-up of the patients, may be necessary.

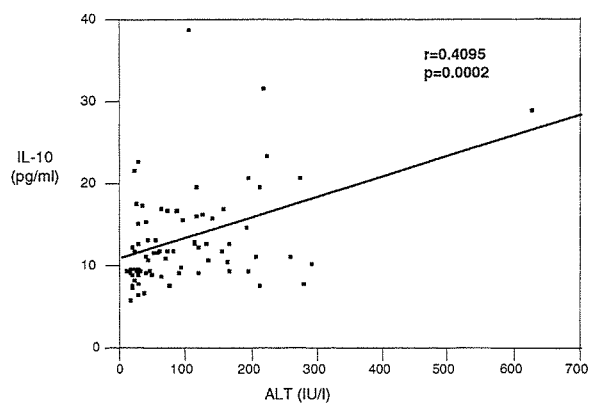


Fig. 3. Scatter plots with ALT and IL-10 in all the patients. ALT and IL-10 values of 77 patients were plotted. The line on the graph is the regression line. Correlation coefficient ($r$) and $p$-value are described on the graph.

The complication of HCC affected the cytokine levels dramatically. IL-10, TNF-α, and IL-12 p40 levels became lowest in HCC among the patient groups. It is reported that the patients with HCC are put in immuno-suppressive conditions and that the cancer cells themselves may contribute to the conditions by producing immuno-suppressive agents, such as TGF-β [13–15]. In the present study, TGF-β levels seemed to be raised when the patients complicated HCC, however, the data were not reliable because of the use of sera instead of plasma for the assay. More detailed study including the re-measurement of TGF-β, histological examination of TGF-β expression in HCC tissue, is necessary to elucidate the role of TGF-β and the conditions of immuno-suppression in HCC.

By measuring serum cytokine levels, we got the evidence that they well reflect the clinical manifestations, such as the activity of the inflammation, viral load, viral antigen load, the extent of fibrosis, complication of the cancer. The analysis of cytokine levels is helpful to understand the pathogenesis of the disease, and may become useful tool to predict the transition of the disease phase. However, more detailed analysis will be necessary for the purpose above.

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特集

# C型肝炎の発症機序とHCV持続感染

*Immunopathogenesis of hepatitis C and mechanism of HCV persistence*

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肝臓の臨床最前線

**Key words** 細胞障害性T細胞 ヘルパーT細胞 調節性T細胞 ナチュラルキラー細胞 サイトカイン

一般に肝炎ウイルスによる肝細胞障害は，ウイルス自身が直接肝細胞を障害するというよりも，患者自身の細胞障害性T細胞(CTL)などの免疫細胞がウイルス感染肝細胞を排除するために肝細胞を破壊する，という免疫応答の結果生じるとされている．肝炎ウイルス由来のペプチドを特異的に認識するCTLはウイルス肝炎患者の肝臓内や末梢血中に少なからず存在していることが以前から報告されているが，これら肝炎ウイルス特異的CTLはウイルス感染を終息させようとする生体防御にかかわる一方で，肝細胞を破壊して肝炎の慢性化や重症化にも関連していると考えられる．

HCV感染の持続化の機序については，HCVに対して生体の免疫機構に欠陥があることや，HCV自身が免疫逃避を起こす蛋白を産生していることなどが報告されている．また，小児や若年者ではHCV排除率が高く，逆に移植後の免疫抑制状態やアルコールなどにより免疫機構に異常がみられる状態では排除率は低下することがこれまでに知られてきており，患者の免疫応答の強さがウイルス肝炎の転帰を大きく左右すると考えられる．したがって，ウイルス肝炎に対する生体の免疫応答を観察し，ウイルス肝炎の発症機序やウイルスの生体免疫応答からの逃避機構を詳細に検討することは，ウイルスの排除や肝炎の終息を目的とした治療法の確立，さらにはウイルス感染の予防法の開発に大きな意味をもつ．

本稿では，これまでに免疫学的に解明，報告されているHCVに対する生体の免疫応答を中心に，C型肝炎における肝障害の発症機序や，HCVの生体免疫からの逃避機構などにつき述べる．

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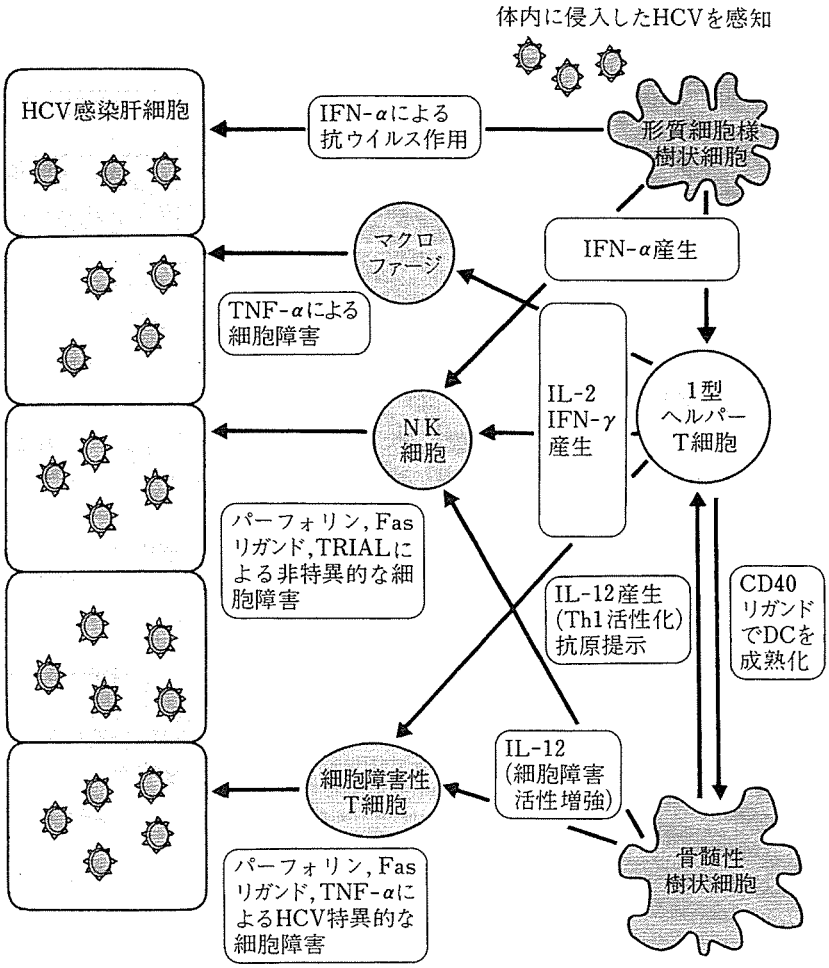


図1 HCV感染における生体の細胞性免疫応答

## Ⅰ. HCV感染に対する免疫応答

### 1. 非特異的免疫応答

HCV感染後，まず生体内では，他のウイルス感染と共通する非特異的応答が生じる(図1)．HCV感染初期は，HCVに感染した肝細胞や感染を認知した形質細胞様樹状細胞(plasmacytoid dendritic cell)などから産生されるインターフェロン(interferon ; IFN)-$\alpha/\beta$(Ⅰ型IFN)によりウイルスの増殖抑制が試みられる．Ⅰ型IFNは2'-5'オリゴアデニル酸合成酵素などを誘導しHCVの増殖を抑制するほか，樹状細胞など抗原提示細胞においてヒト白血球抗原(human leukocyte antigen ; HLA) Class Ⅰ分子の表出を増強させる作用や，ナチュラルキラー(natural killer ; NK)細胞，CTLなどの免疫細胞を活性化させる作用などの免疫応答増強作用を有する．IFN-$\alpha$により活性化したNK細胞はHCVに感染した肝細胞を認識し障害を起こす．肝細胞が障害を受けることにより刺激を受けた骨髄系樹状細胞(myeloid dendritic cell)は，NK細胞や，NK細胞とT細胞の両者の性質を持ち肝臓に多く存在するNKT細胞を活性化し，それらの細胞はIFN-$\gamma$を多量に分泌する．さらに，IFN-$\gamma$はマクロファージの活性化を増強し，局所の炎症反応を増強する．

### 2. HCV特異的細胞性免疫応答

HCV感染において，上記の非特異的免疫応答により肝炎ウイルスが十分に生体から排除できない場合，特異的免疫応答が誘導されさらなるウイルスの排除が試みられる．

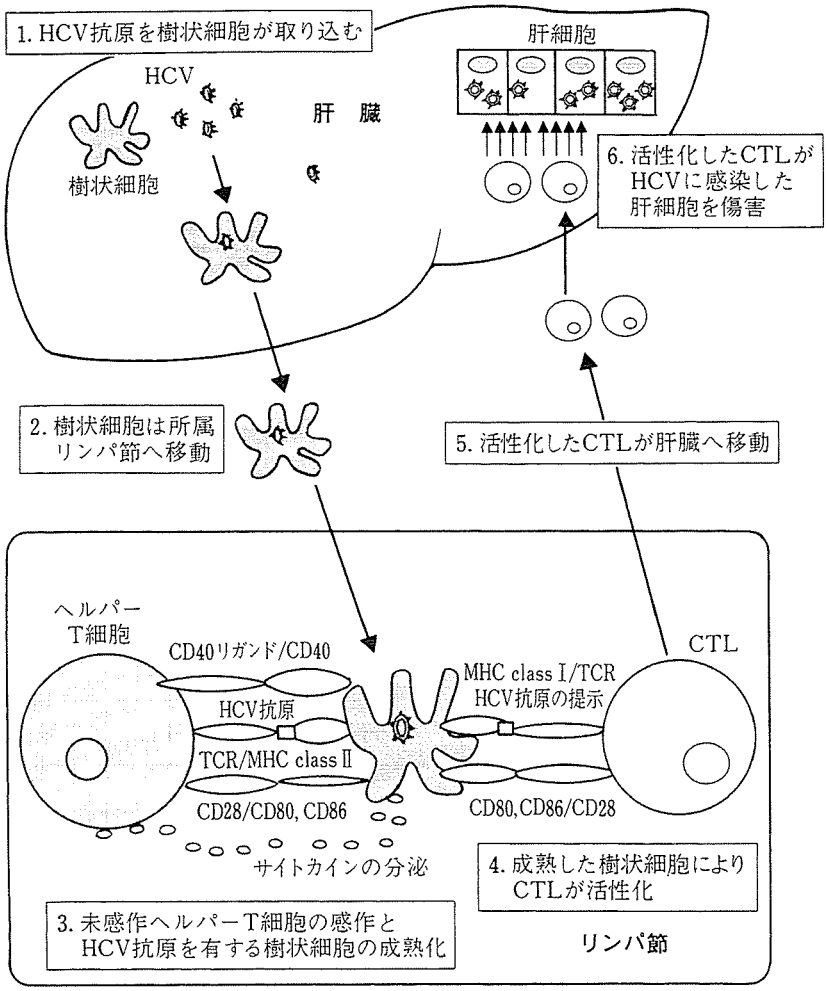


**図2　HCV特異的細胞障害性T細胞の誘導**
CTL；細胞障害性T細胞，TCR；T細胞レセプター，MHC；major histocompatibility complex

1型ヘルパーT細胞(type I helper T；Th1)は，ウイルス特異的CTLやNK細胞などの細胞性免疫の誘導や活性化に重要な役割を担う．骨髄系樹状細胞は，肝内で死滅したウイルス感染肝細胞などからHCV抗原を取り込むと所属リンパ節に遊走する．リンパ節で表面に共刺激分子などの発現を増強させて成熟した樹状細胞となり，T細胞を活性化する．樹状細胞は，表面のHLA class Ⅱ分子上に提示されたHCV抗原を認識する未感作ヘルパーT細胞(helper T；Th)を刺激し活性化させる．それにより活性化したTh細胞は，CD40リガンドを表出し，さらにサイトカインを分泌することで樹状細胞をさらに成熟化，活性化させる．主に骨髄系樹状細胞が産生するインターロイキン(interleukin；IL)-12は，感作されたTh細胞をTh1細胞に分化，誘導し，その後Th1細胞はIL-2やIFN-γを産生してCTLやNK細胞を刺激し，活性化や増殖を促す．それらにより未感作CTLは樹状細胞が提示するHCV抗原を認識し，初めて感作される．感作し活性化されたHCV特異的CTLはリンパ節を離れ末梢に到達して，HCVに感染した細胞の表面にあるHLA class Ⅰ分子上に提示されたHCV抗原を認識し，感染細胞の細胞死を誘導することによりウイルスを排除する(**図2**)．

HCV感染でCTL応答がウイルスの増殖を抑制していることが示唆されており，初感染時におけるウイルス排除にはウイルス特異的CTLの存在

が重要であると報告されている．HCV を排除した症例では，排除後35年という長期間にわたりHCV に対する $CD4^+$ や $CD8^+$ T 細胞の応答が認められることも報告されている[1]．このように，肝炎ウイルス感染時に適度な細胞性免疫応答が生じた場合には，ウイルスは完全に排除される．しかし，HCV 感染では生体での免疫応答が一般に弱いため，HCV による急性肝炎では肝障害の程度が軽く，感染が持続化しやすいと考えられている．それに対して，非常に強い免疫応答が誘導されると，劇症肝炎などの重症な肝障害を引き起こす可能性がある．このように，HCV 量と HCV 特異的CTL 活性のバランスによりさまざまな程度の肝障害が起こりうると考えられる．

また，C 型慢性肝炎で活性化したウイルス特異的CTL は肝臓に集積しており，ウイルスの増殖抑制と肝障害に関与していることが報告されている．また，HCV 量の少ないC 型慢性肝炎患者では末梢血中にも HCV 特異的CTL が検出され，多量のウイルスがT 細胞を抑制あるいは消費しているものと考えられている[2]．C 型肝炎治癒症例はTh1優位であるといわれているが，近年，C 型慢性肝炎の治療に用いられているリバビリン(商品名レベトール®)は，患者の免疫応答をTh2からTh1優位に変化させることが，抗ウイルス効果の一つの機序と考えられている．

## Ⅱ．CTL の HCV 感染肝細胞に対する障害機序

成熟した樹状細胞により刺激を受け活性化したウイルス特異的CTL は表面のT 細胞受容体により，ウイルス感染細胞の表面に存在するHLA class Ⅰ分子とその上に提示されている8から11個のアミノ酸よりなるウイルス抗原ペプチドを認識し，細胞表面に孔を形成するパーフォリンや，細胞をアポトーシスに陥らせる蛋白分解酵素グランザイムを，標的であるウイルス感染細胞に放出して細胞死を引き起こす．さらに，活性化したCTL は Fas リガンドや tumor necrosis factor (TNF)-$\alpha$の発現も増強し標的細胞の障害に携わる．パーフォリンはほとんどすべての細胞に細胞障害活性を示すが，Fas リガンドや TNF-$\alpha$はそれらの受容体を持ち感受性がある細胞のみに効力を発揮する．正常の肝細胞は Fas リガンドや TNF-$\alpha$に対して抵抗性を示すが，一方，肝炎患者の肝組織中の炎症が強い部位では肝細胞の Fas 抗原や TNF 受容体の発現が増強しており，Fas リガンドや TNF-$\alpha$に対しての感受性も高まっていることが考えられる．Fas 系や TNF-$\alpha$を介した細胞障害活性はパーフォリンと比較すると細胞障害効率は低いものの，活性化したCTL はこれらの系を介して感受性が高まったウイルス非感染細胞をも障害すると考えられる(**図3**)[3]．

## Ⅲ．HCV のアミノ酸変異による生体免疫機構からの逃避

HCV は，自らのアミノ酸に変異を起こさせやすい RNA ポリメラーゼの作用や高い複製能により，生体内にさまざまなタイプの HCV が存在するというクアシスピーシスを形成し，宿主の免疫監視機構からの逃避を試みている．抗体結合部やT 細胞が認識する抗原エピトープのアミノ酸を変異させることにより，抗体がウイルス自体に結合できなくなること，HCV 特異的CTL が感染細胞を認識できないようにすること，CTL にトレランスを誘導すること，あるいは未感作CTL を感作させにくくすることなどにより，生体の免疫応答から逃れていることが想定されている．さらに，クアシスピーシスは多様な細胞への感染を可能にすることや薬剤耐性の獲得にも影響を及ぼすと考えられる．

### 1．液性免疫からの逃避

一般に，ウイルス特異的中和抗体は体液中のウイルスの排除に作用するが，HCV の初感染から十分な中和抗体が産生されるまでには時間がかか

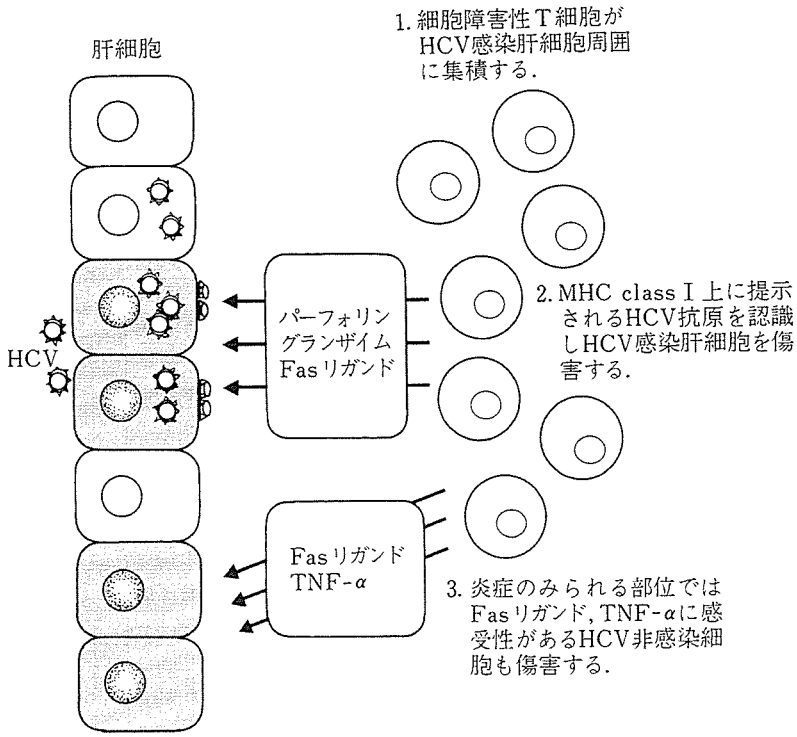


図3 HCV特異的細胞障害性T細胞による肝細胞傷害

ることから，HCV中和抗体はウイルス初感染におけるウイルス排除というより，二次感染の予防に関与していると考えられている．HCV E2領域のhypervariable region 1 (HVR1)のアミノ酸変異はきわめて多様であり，生体で産生される中和抗体による認識から逃れて持続感染に寄与しているとされている．しかし一方，チンパンジーでのHCV感染実験では，HVR1に変異はみられずに持続感染に発展しているとの報告もあり，E2領域の変異が感染の持続化に大きな役割を果たしているかを疑問視する意見もある．

### 2. 細胞性免疫からの逃避

HLA class Ⅰあるいはclass Ⅱ拘束性T細胞のエピトープの変異は，HCV感染肝細胞の排除を妨げることにより感染の持続化に寄与する可能性がある．これまでの研究では，感染HCVのクローニングにより，$CD8^+$ CTLの認識を妨げるアミノ酸変異が認められている．また，HLA-DRB1拘束性でNS3由来のペプチドを認識するTh1タイプのT細胞に対し，このエピトープ内の1つのアミノ酸に変異が起こると，分泌するサイトカインがTh1タイプからTh2タイプに変化したことが報告された[4]．

一方，HCV感染に対する初期のCTL応答は多様であり，一つのエピトープの変異のみで持続化は説明できないとの指摘もある．エスケープミュータントは持続感染の原因というより，持続感染の結果を見ている可能性も否定できない．

## Ⅳ．HCV感染による免疫細胞の機能抑制

抗HCV抗体は感染後2～4ヵ月間もの間出現せず，さらに抗体が現れてもHCV感染は持続し肝炎は進行する．また，細胞性免疫においても，急性期には多様なT細胞応答が認められるが，慢性化するとその応答は劇的に減弱してしまう．肝内には多数のHCV特異的$CD8^+$ T細胞が存在してはいるものの，HCVを排除できない．さらに，C型慢性肝炎患者にはB型肝炎ウイルスや細菌感染などの合併も多く，以前から生体での免疫力の低下が想定されてきた．近年，HCV自体が能

表1 HCV持続感染におけるHCV蛋白の作用

| 免疫細胞 | 作用 | 参考文献 |
|---|---|---|
| NK細胞 | CD81と結合しNK細胞の機能を抑制 | Tseng CT, J Exp Med 195：43, 2002 |
| T細胞 | アミノ酸変異により分泌するサイトカインがTh1からTh2に変化 | Wang JH, Hum Immunol 64：662, 2003 |
| | C型肝炎患者の末梢リンパ球でCD3ζ鎖の発現が低下 | Maki A, Hepatol Res 27：272, 2003 |
| | C型肝炎患者の肝浸潤リンパ球でT細胞レセプターδ鎖の発現が低下 | Leroy V, Hepatology 38：829, 2003 |
| | IL-2の欠乏によりCTLのエフェクター機能が低下 | Francavilla V, Eur J Immunol 34：427, 2004 |
| B細胞 | B細胞にもCD81分子が発現 | Zuckerman E, J Virol 77：10432, 2003 |
| 樹状細胞 | アロT細胞刺激能の低下 | Bain C, Gastroenterology 120：512, 2001 |
| | 形質細胞様樹状細胞におけるIFN-α産生能の低下 | Goutagny N, J Infect Dis 189：1646, 2004 |
| | HCV E1蛋白が樹状細胞の成熟化を抑制 | Sarobe P, J Virol 77：10862, 2003 |
| | HCV CoreとNS3蛋白が樹状細胞の分化とアロ刺激能を抑制 | Dolganiuc A, J Immunol 170：561, 2003 |
| | MIC A／B発現の抑制 | Jinushi M, J Immunol 171：5423, 2003 |
| | C型肝炎患者の樹状細胞にHCVが増殖 | Goutagny N, J Infect Dis 187：1951, 2003 |
| | チンパンジーのHCV感染実験では樹状細胞の機能低下は認められない | Larsson M, J Virol 78：6151, 2004 |
| 調節性T細胞 | $CD4^+$ $CD25^+$細胞が肝炎の持続化に関与 | Sugimoto K, Hepatology 38：1437, 2003 |
| | C型肝炎患者の末梢血からHCV Core蛋白特異的調節性T細胞を分離 | MacDonald AJ, J Infect Dis 185：720, 2002 |
| | C型肝炎患者の肝内に調節性T細胞が存在し，CTLの機能を抑制 | Accapezzato D, J Clin Invest 113； 963, 2004 |
| | NS4蛋白は健常者の末梢単核球からIL-10の産生を促進 | Brady MT, Eur J Immunol 33：3448, 2003 |
| その他 | Jak-STAT系の抑制 | Blindenbacher A, Gastroenterology 124：1465, 2003 |
| | Fasを介したアポトーシスを抑制 | Moorman JP, Virology 312：320, 2003 |
| | MHC class Ⅰ分子の発現抑制 | Konan KV, J Virol 77：7843, 2003 |
| | MHC class Ⅰ分子の発現増強 | Herzer K, J Virol 77：8299, 2003 |

動的に生体の免疫機構を抑制している可能性が考えられており，それを示唆する報告が多数なされるようになった．HCV感染による免疫細胞の機能抑制を表1に示す．

### 1. 自然免疫に対する抑制

HCVのE2タンパクは細胞表面上のCD81と高い親和性を持ち，CD81はHCVが細胞感染する際の受容体となりうると考えられている．HCVのE2タンパクはNK細胞上に発現するCD81と結合し，直接NK細胞の機能を低下させる作用があることが報告された[5]．NK細胞はIFN治療一週間後には肝内への浸潤が観察されるが，治療有効群と治療不応群との間でNK細胞の細胞障害活性は異なり，IFN治療の有効性を予知する指標となるとされている．このことからも，NK細胞を中心とした自然免疫系もHCV排除には重要な役割を果たすと考えられるが，HCVが直接NK細胞の活性を抑制することは感染の持続化に対し大きな意味を持つ．また，HCVコア蛋白はp53依存性にtransporter associated with antigen processing 1(TAP1)の発現を増強することで，MHC class Ⅰ発現を増強するとも報告されている．この中で筆者らはMHC class Ⅰの発現増強は，NK細胞のHCV感染肝細胞に対する細胞障害活性を低下させ感染の持続化に寄与すると推論している．

### 2. 液性免疫に対する抑制

C型肝炎患者の末梢リンパ球は，CD81分子が

強発現しており，HCVが感染しやすい状態になっていることが考えられ，感染を介して抗体産生などに影響を及ぼしている可能性がある．また，他の感染実験でも，中和抗体の抗体価は低く再感染を防止することはできなかったため，B細胞応答もHCVにより抑制されていることが想定されている．

### 3. T細胞に対する抑制

HCV特異的CTLのエフェクターとしての機能は明らかに低下している．C型慢性肝炎患者の末梢リンパ球においてはCD3ζ鎖の発現が，また肝浸潤リンパ球においてはT細胞レセプターδ鎖やCD56の発現が低下していることが報告されている．また，HBV特異的CTLに比しHCV特異的CTLは明らかにパーフォリンの発現量が少なく，これは機能低下を示す一つの例とされているが，このような免疫細胞自体の機能低下もC型肝炎の持続化に関与することが想定されている．また，C型急性肝炎時には$CCR7^-$ $CD8^+$ T細胞(メモリー・エフェクター細胞)は細胞障害活性が低下しているが，これにIL-2を加えると完全にエフェクター機能を有する細胞になることから，T細胞が活性化する際のIL-2の欠乏がCTLの機能低下主な原因であるとの報告もある[6]．循環しているコア蛋白がIL-2産生のシグナル伝達の抑制に関与していることも想定されている．

C型慢性肝炎患者において，HCV特異的$CD4^+$ T細胞の存在は認められるものの抗原特異的な増殖能は抑制され，さらに抗原刺激に特異的なIL-10やTGF-β産生も有意に認められており，HCV持続感染の一因となりうる．

また，HCV感染肝細胞より遊離し末梢血中に存在するHCVコア蛋白は，T細胞のgC1qRと結合することで，T細胞の増殖や活性，IFN-γ産生能を阻害することが報告された．HCVコア蛋白は血中にナノグラムの単位で存在しており，gC1qRと結合するには十分量と考えられるが，肝組織内ではさらに高濃度のコア蛋白が存在していると想定され，肝浸潤リンパ球に少なからず影響を与えていると推測される．

HCV NS4A/B蛋白は，細胞内で小胞体からゴルジ体への輸送を妨げることにより，major histocompatibility complex (MHC) class Ⅰ分子の細胞上への発現を抑制することが報告された[7]．これにより，HCV特異的$CD8^+$ T細胞がHCV感染肝細胞を認識しにくくなり，HCVの感染持続化に繋がることも考えられる．

さらに，肝臓には類洞内皮細胞やKupffer細胞といった免疫に関与する細胞が存在するが，それらは成熟した樹状細胞とは異なり，ウイルス抗原は提示するもののCD80やCD86といった共刺激分子に乏しいためT細胞を十分に刺激できないばかりか，かえって免疫寛容を誘導してしまうことも考えられている[8]．

### 4. 樹状細胞に対する抑制作用

樹状細胞は免疫を誘導するうえで，重要な役割を担っている．C型慢性肝炎患者においては，樹状細胞のallogeneicのT細胞を刺激する能力が低下していることや，形質細胞様樹状細胞の減少によりインターフェロンα産生が低下していること，さらに，HCVコアとE1蛋白は樹状細胞の成熟化を抑制することでT細胞刺激能を減弱させていたことがこれまでに指摘されている．また，Jinushiらは，IFN-α刺激後に樹状細胞は，その表面にMHC class Ⅰ-related chain A and B (MICA/B)を発現させNK細胞を活性化することに着目し，C型慢性肝炎患者においてNK細胞が有効に活性化できないのは，type Ⅰ IFNによるIL-15産生能が低下しており，MICA/B発現の増強が抑制されていることが原因であると報告している[9]．

HCVはE2蛋白が樹状細胞上に発現するDC-SIGNに結合することより樹状細胞にも感染することや，soluble E2蛋白も樹状細胞と結合が可能であることも報告され，それらにより樹状細胞は機能低下に陥る可能性も考えられている．

一方，チンパンジーへの感染実験では，樹状細胞の機能低下は認められないとする結果や，C型慢性肝炎患者の検討で樹状細胞の成熟化やアロ刺激能は正常であることも報告されており，樹状細胞のC型肝炎への関与は今後さらなる検討が必要である．

### 5. HCV蛋白による細胞に対するその他の影響

HCVトランスジェニックマウスの系で，HCV蛋白がインターフェロンによりもたらされる細胞内伝達シグナル(Jak-STAT系)を抑制することが示唆され，これがインターフェロン不応性の一因となることが想定されている[10]．また，HCV蛋白は感染した肝細胞のFasを介したアポトーシスを抑制しており，これも持続感染を誘導するのに重要であると指摘している．一方，HCVコア蛋白はJurkat細胞に対しFasを介したアポトーシスの経路を活性化させるとの報告もある[11]．細胞内コア蛋白は，TNFレセプターの細胞内ドメイン(TNFRI)あるいはFasと結合し，細胞にアポトーシスを誘導することが証明され，肝細胞やリンパ球のアポトーシスに関与する可能性も考えられている．

## V．HCV特異的調節性T細胞の関与

生体の免疫応答を抑制する要因の一つとして，抗原を特異的に認識してIL-10やTGF-$\beta$を産生する調節性T細胞(Tr)が注目されている．C型慢性肝炎患者においては，Tr細胞と考えられる$CD4^+$ $CD25^+$ T細胞のfrequencyが高く，この細胞集団は直接T細胞の機能を抑制し，これがHCV特異的細胞性免疫の質的，量的な抑制を引き起こして，肝炎の持続化に寄与していると想定されている[12]．HCVコア蛋白に特異的なTr細胞がC型慢性肝炎の末梢血から分離誘導され，この細胞が産生するIL-10がHCV感染の持続化に関与すると報告された[13]．また，C型肝炎患者の肝内にはIL-10を産生するHCV特異的$CCR7^-$ $CD8^+$調節性T細胞が存在し，肝内に多数集積しているHCV特異的$CCR7^-$ $CD8^+$メモリーT細胞の機能を抑制することも報告された[14]．さらに，HCV NS4蛋白は，C型肝炎患者のみならず，健常者の末梢単核球からもIL-10の産生を促しIL-12の分泌を抑制させ，さらに樹状細胞の分化成熟化を抑制するとの報告もあり，細胞性免疫の活性化抑制の一つの機序として興味深い[15]．

以上のようにTr細胞のHCV感染持続化への関与が強く示唆されているが，まだ不明な点も多くさらなる検討が必要である．

## Ⅵ．その他のHCV持続感染の機序

非構造タンパク領域であるNS5AのC末端側に存在する40個のアミノ酸からなる部分(IFN-sensitivity determining region)の変異は，IFNにより誘導され抗ウイルス効果を発揮するプロテインキナーゼと結合しその活性化を阻害することによりIFN治療抵抗性を得るとされている．また，HCV NS3/4のセリンプロテアーゼは，細胞が抗ウイルス効果を発揮するうえで重要なinterferon regulatory factor-3を抑制するといわれている．また，分子レベルではウイルスが感染細胞に，ウイルス遺伝子発現抑制，抗原のプロセッシング抑制といった影響を与えていることも想定されている．

## おわりに

肝炎動物モデルやの臨床検体の解析などにより，ウイルス肝炎における免疫応答が長年にわたり研究されてきた．肝細胞障害にはCTLを中心とした細胞性免疫応答の関与が明らかになり，その障害機序や生体免疫応答の抑制機序も徐々に解明されてきている．免疫応答を適切にコントロールすることは肝炎ウイルス排除あるいは肝炎の鎮静化に重要であるが，これから解明していかなければならない問題も多々ある．これらの問題点を

生体免疫反応のみならず，ウイルス側からも詳細に解明していくことで，将来，肝炎ウイルスを完全に生体から排除できる治療法の開発が可能になると考えられる．今後のウイルス学，免疫学のさらなる発展を期待する．

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**ORIGINAL ARTICLE**

# Dendritic cell therapy with interferon-α synergistically suppresses outgrowth of established tumors in a murine colorectal cancer model

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*Both dendritic cell (DC)-based immunotherapy and interferon (IFN)-α therapy have been proved to have potent long-lasting antitumor effects. In anticipation of synergistic antitumor effects, we performed combination therapy with DCs and IFN-α gene-transduced murine colorectal cancer MC38 cells (MC38-IFN-α). DCs incubated with MC38-IFN-α, but not neomycin-resistance gene-transduced MC38 cells (MC38-Neo), effectively enhanced proliferation of allogeneic splenocytes* in vitro. *In 12 of 17 mice, DCs in combination with MC38-IFN-α prevented the development of a parental tumor, while DCs and MC38-Neo did in only three of 17 mice (P = 0.008). In a therapeutic model of an established parental tumor, inoculation of DCs and MC38-IFN-α suppressed the growth of the established parental tumors significantly compared with the administration of DCs with MC38-Neo or naive splenocytes with MC38-IFN-α (P = 0.016 and 0.024, respectively). Analyses of immunohistochemistry and tumor-infiltrating mononuclear cells showed that CD8⁺, CD11c⁺, and NK1.1⁺ cells markedly infiltrated the established tumors of mice treated with DCs and MC38-IFN-α. From the results of observation of parental tumor outgrowth in immune cell-depleted mice, CD8⁺ cells, and asialo-GM-1⁺ cells were thought to contribute to the antitumor effects induced by the combination therapy. Furthermore, MC38-specific cytolysis was detected when splenocytes of mice inoculated with DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α cells* in vitro. *Since DC-based immunotherapy in combination with IFN-α-expressing tumor cells induces potent antitumor cellular immune responses, it should be considered for clinical application.*
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**Keywords:** *dendritic cell; maturation; interferon-α; immunotherapy*

## Introduction

Although therapies for malignant tumors including operation, chemotherapy, and radiation therapy have developed remarkably, the complete conquest of malignant tumors has not been achieved yet. In addition, in many cases, patients suffer from side effects of these therapies such as fatigue, anorexia, pyrexia, infection, and suppression of bone marrow. Thus, new anticancer therapies that have more efficacy and fewer side effects are required. The cellular immune response is considered not to work functionally and sufficiently in patients with advanced malignant tumors, and it has been reported that some tumors escape immune surveillance by several mechanisms.[1] To overcome immune suppression or immune escape in patients with advanced malignant tumors, many biologic therapies that aim at inducing potent antitumor immune responses have been tried.

Dendritic cells (DCs) are potent antigen-presenting cells that can elicit primary and secondary immune responses to foreign antigens.[2,3] Immature DCs express low levels of major histocompatibility complex (MHC) class I, class II, and costimulatory molecules (CD80, 86) that play important roles in T cell stimulation.[4] When immature DCs capture and take up antigens, DCs downregulate the function of antigen acquisition, but upregulate MHC and costimulatory molecules. As DCs mature, they will express higher levels of MHC and costimulatory molecules and present antigens to antigen-specific T cells and induce immune responses.[4–8] Since DCs are playing a crucial role in controlling immunity, the use of DCs may be ideal for cancer therapy.[4] Recently, various DC-based therapies have been tried to elicit antitumor responses,[6,8–15] and DCs have been used for therapy in patients with some malignant tumors such as malignant melanoma, lymphoma, renal cell carcinoma, pancreatic, and gastric cancer.[16]

Interferon (IFN)-α has been used to treat patients with not only viral infections such as hepatitis B and C but also some malignant tumors such as melanoma, renal cell carcinoma, and leukemia. The effects of type I IFN include antiviral function, enhancement of IFN-α/β production,[17,18] and inhibition of cell growth and angiogenesis.[19] In addition, IFN-α plays a crucial role in the immune system. IFN-α upregulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1-lymphocytes.[20] Previous studies emphasized the importance of IFN-α for the generation of cytotoxic T lymphocytes (CTLs) in specific antitumor

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immune responses.[21,22] In addition, we reported previously that IFN-α transduction of poorly immunogenic tumor cells reduces tumorigenicity and leads to a long-lasting tumor immunity,[23] and that IFN-α-expressing tumor cells promote the survival of tumor-specific CTLs by preventing apoptosis.[24]

IFN-α has been reported to induce the maturation of DCs. Santini *et al*.[25] demonstrated that IFN-α upregulated the expression of costimulatory molecules (CD80, 86) and MHC class II (HLA-DR) and induced CD83 expression, which is considered to be a marker of mature and activated human DCs. Then, they showed that DCs gained a greater capability to stimulate the proliferation of allogeneic lymphocytes in the presence of IFN-α.[25] Others have also reported the effects of IFN-α on enhancement of DC maturation.[26,27] However, it has also been reported that IFN-α fails to induce DC maturation, and that the presence of IFN-α prior to or during the differentiation of DCs from the monocyte precursors alters their response to maturation stimuli in the human system.[28]

In our previous studies, we demonstrated that IFN-α gene therapy in combination with CD80 transduction reduces tumorigenicity and the growth of established tumors in poorly immunogenic tumor models,[23] and that IFN-α has additive effects on suppressing tumor growth in cooperation with interleukin (IL)-12.[29] Costimulatory molecules such as CD80 are highly expressed on the surface of DCs, and IL-12 is produced mainly by DCs. Therefore, the combined use of DC-based immunotherapy with IFN-α gene therapy is considered reasonable. Recently, Tsugawa *et al*.[30] reported that combined use of DC with adenoviral vector encoding IFN-α elicits antitumor response in a murine intracranial gliomas model. In this study, as a preliminary investigation of the combined therapy, we investigated the effects of bone marrow-derived DCs and IFN-α-expressing colorectal cancer cells on the proliferation of allogeneic splenocytes. Then, we examined whether DCs and IFN-α-expressing tumor cells display synergistic effects on the induction of antitumor immunity in a therapeutic model to evaluate the possibility of applying this combined therapy to clinical trial.

## Results

### *IFN-α gene transduction does not affect growth of tumor cells* in vitro

Each of the tumor cells (wild type (WT), neomycin-resistance gene-transduced MC38 (MC38-Neo)-, murine IFN-α-overexpressing MC38 cells (MC38-IFN-α)) with or without γ-irradiation were seeded at $5 \times 10^5$ cells/well in six-well plates, and enumerated every day in duplicate to compare the growth *in vitro* of the genetically modified MC38 cells. The growth rates did not differ significantly between MC38-WT, MC38-Neo, and MC38-IFN-α cells within 72 h incubation (data not shown). Cell counts of each tumor cell were decreased to approximately one-third 48 h after 100 Gy γ-irradiation.

When nonirradiated tumor cells were incubated with DCs, cell growth of each tumor cell line was almost the same with that of MC38-WT without DCs (data not shown). DCs did not affect the cell growth of tumor cell lines.

With the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay (TUNEL), we found approximately 50% of the γ-irradiated (100 Gy) MC38 cells to be apoptotic 48 h after irradiation (data not shown). The proportion of apoptotic cells after γ-irradiation did not differ between the genetically modified MC38 cell lines.

### *Coincubation with MC38-IFN-α cells does not enhance cytokine production by DCs and phenotypic maturation of DCs*

The production of murine IFN-α by modified tumor cells or DCs was confirmed by enzyme-linked immunosorbent assay (ELISA). Nonirradiated MC38-IFN-α cells produced large amounts of IFN-α as shown in Table 1. On the other hand, 100 Gy γ-irradiated MC38-IFN-α cells produced about half as much IFN-α as nonirradiated MC38-IFN-α. There was no difference in IFN-α production between the irradiated MC38-IFN-α cells alone and DCs coincubated with irradiated MC38-IFN-α. IFN-α was not detected in supernatants of MC38-WT cells, MC38-Neo cells, DCs, splenocytes, and DCs with MC38-Neo cells.

IL-1β, IL-12, and TNF-α production by DCs was also confirmed by ELISA. Enhancement of these cytokine production was not observed when DCs were coincubated with parental or genetically modified tumor cells (data not shown). Expression of CD80, CD86, and I-$A^b$ molecules on DCs was compared by flow cytometry. Coincubation with genetically modified tumor cells did not enhance the expression of those molecules (data not shown). From these data, coincubation with parental or genetically modified tumor cells does not enhance the cytokine production by DCs as well as the phenotypic maturation of DCs in this system.

### *Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells*

To investigate proliferative effects of DCs and MC38-IFN-α cells on allogeneic splenocytes, we performed cell proliferation assays. As shown in Figure 1, stimulation by both DCs and MC38-IFN-α cells markedly enhanced the proliferation of allogeneic splenocytes compared with stimulation by DCs alone or by DCs and MC38-Neo ($P = 0.007$ or 0.020, respectively). When exogenous IFN-α was added to the culture of DCs and MC38-Neo cells, the proliferation of allogeneic splenocytes was

**Table 1** Production of IFN-α by genetically modified MC38 tumor cells and DCs

| *Cell* | *IFN-α production (ng/48 h)* |
|---|---|
| DC | Not detected |
| MC38-Neo | Not detected |
| MC38-IFN-α | 20.8±0.5 |
| MC38-IFN-α (irradiated) | 10.8±0.3 |
| DC+MC38-IFN-α (irradiated) | 11.0±1.1 |
| Splenocyte+MC38-IFN-α (irradiated) | 7.2±0.6 |

A total of $1 \times 10^6$ DCs or splenocytes were incubated with or without $1 \times 10^5$ γ-irradiated (100 Gy) MC38-IFN-α cells in 5 ml of CM in six-well plates. After 48 h incubation, concentration of IFN-α in the culture supernatant was confirmed by ELISA according to the manufacture's protocol.

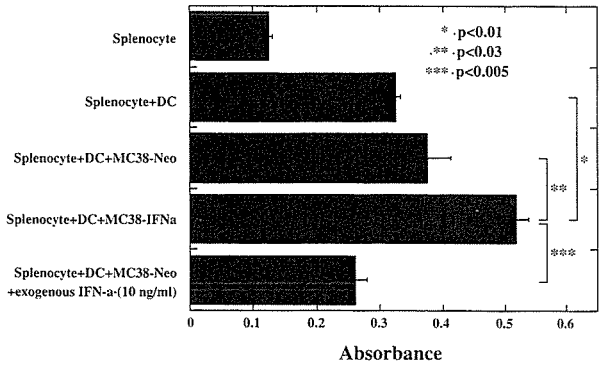


**Figure 1** Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Purified DCs were incubated with γ-irradiated (100 Gy) MC38-Neo or MC38-IFN-α cells at a DC to tumor ratio of 10 for 2 days. The cells were γ-irradiated (30 Gy) for the purpose of using as stimulator cells. In some wells, exogenous murine IFN-α was added at a concentration of 10 ng/ml. After allogeneic splenocytes ($5 \times 10^5$) were incubated with the stimulator cells ($5 \times 10^4$) for 3 days, cell proliferation assay was performed. This experiment was performed twice with similar results.

significantly suppressed compared with the stimulation by DCs and MC38-IFN-α cells ($P = 0.005$). The results suggested that MC38-IFN-α cells and DCs stimulate allogeneic splenocytes more effectively than control gene-transduced MC38 cells, and that continuous secretion of IFN-α may be responsible for the proliferative effect on allogeneic splenocytes in this system.

### *Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors* in vivo

We investigated the preventive effects of DCs and MC38-IFN-α cells on the development of parental MC38 tumors. At 1 week after the second intraperitoneal inoculation of DCs (or splenocytes) and the modified MC38 cells, MC38-WT cells were inoculated subcutaneously (s.c.). 12 of 17 mice inoculated with DCs+MC38-IFN-α cells did not develop parental tumors, although all mice injected with only DCs or splenocytes had growing parental tumors on day 28 as shown in Figure 2 and Table 2. All these 12 tumor-free mice rejected the subsequent parental MC38 cell challenge. DCs+MC38-Neo and splenocytes+MC38-IFN-α cells also had preventive effects on the development of parental tumors compared with splenocytes alone, although the preventive effects were less than those of DCs+MC38-IFN-α.

### *CD8⁺ cells and asialo-GM-1⁺ cells contribute to the antitumor effects induced by DCs and IFN-α-expressing tumor cells*

We depleted immune cells using anti-CD4, anti-CD8, and anti-asialo-GM-1 antibodies to explore the mechanism of the antitumor effects induced by DCs and IFN-α-expressing tumor cells. We depleted these immune cells *in vivo* after inoculation with DCs and IFN-α-expressing tumor cells. Then, MC38-WT cells were injected and the WT tumor development was measured. When $CD8^+$ cells or asialo-GM-$1^+$ cells were depleted, we observed obvious growing WT tumors in those mice, whereas

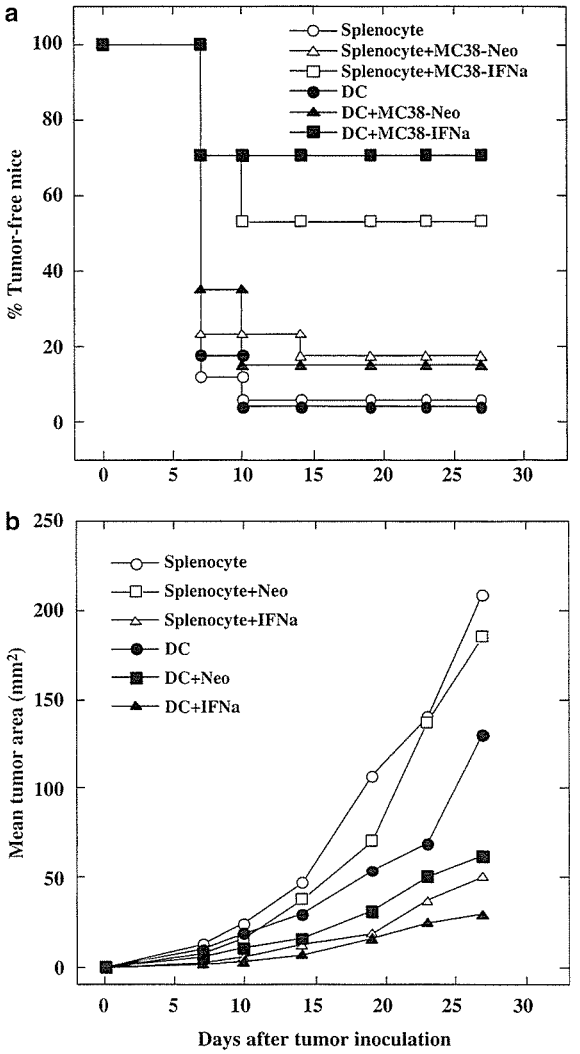


**Figure 2** Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors *in vivo*. Inoculation of both MC38-IFN-α cells and DCs prevents development of parental MC38 tumor. Mice were injected i.p. with $1 \times 10^6$ DCs or splenocytes and $1 \times 10^5$ the genetically modified tumor cells twice at a 7-day interval (days −14 and −7). At 1 week after the final vaccination (day 0), the mice were injected s.c. with $1 \times 10^5$ MC38-WT cells in the right flank. Each experiment involved five or six mice per group. Results are reported as (a) percent of tumor-free mice and (b) mean tumor area ($mm^2$). This experiment was performed three times with similar results.

we detected no tumor in mice not depleted of any immune cells ($P = 0.015$ or 0.023, respectively, Figure 3). On the other hand, in $CD4^+$ cell-depleted mice, the antitumor effects induced by DCs and IFN-α-expressing tumor cells were diminished marginally. Thus, DCs and IFN-α therapy seemed to stimulate $CD8^+$ cells and asialo-GM-$1^+$ cells mainly *in vivo*.

### *Therapeutic inoculation of DCs and MC38-IFN-α cells suppresses outgrowth of established parental MC38 tumors*

We evaluated the therapeutic effects of DCs and MC38-IFN-α on established parental MC38 tumors. As shown