Cancer Chemother Pharmacol (2008) 62:401–406
DOI 10.1007/s00280-007-0618-1

ORIGINAL ARTICLE

Relationship of peripheral blood CD4-positive T cells to carcinogenesis in patients with HCV-related chronic hepatitis and liver cirrhosis

Teppei Matsui · Hidenari Nagai · Yasukiyo Sumino · Kazumasa Miki

Received: 9 July 2007 / Accepted: 23 September 2007 / Published online: 12 October 2007

Abstract
Introduction It has been reported that Th2 cytokines down-regulate antitumor immunity, while activation of type 1 T cell responses promotes antitumor immunity. However, detailed information on the immunological background of patients with HCC is still unknown. The objectives of this study were to evaluate the Th1/Th2 balance and to investigate the relation between carcinogenesis and host immunity in patients with chronic hepatitis C, HCV-related liver cirrhosis (LC), or HCC.
Patients/methods The study population was 117 patients who had chronic inflammation due to HCV infection diagnosed from pathological examination of liver biopsy specimens, including 32 patients who had HCV-related LC with HCC. Apart from the patients with HCC, they were divided into the four subgroups based on the fibrosis score of Desment (stages 1–4). Blood samples were collected in the early morning before treatment. Flow cytometry was used to assess cytoplasmic IFN-gamma and IL-4 expression by peripheral blood CD4+ T cells, and the percentage of IFN-gamma+ and IL4− T cells (Th1) or IFN-gamma- and IL4+ T cells (Th2) was calculated before the start of each therapy.
Results There were 20 patients in F1, 25 patients in F2, 19 patients in F3, 21 patients in F4, and 32 patients with HCC. In the F4 and HCC groups, Th1 cells tended to increase depending on the extent of fibrosis, although there were no significant differences between these groups and the other groups. In the HCC group, Th2 cells showed a significantly higher percentage than in the F1 or F3 groups.
Conclusions These results suggest that Th1 dominance is lost due to an increase of Th2 cells in HCC patients and that carcinogenesis might occur in patients with chronic HCV infection and increased level of Th2 cells.

Keywords Th1/Th2 balance · CD4 · Cytokine · HCC · Carcinogenesis

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Introduction

Chronic HCV infection of the liver is a strong risk factor for the development of hepatocellular carcinoma (HCC). It has been reported that small tumors with prominent T cell infiltration show a lower recurrence rate and higher 5-year survival rate compared with a larger tumors that do not have such T cell infiltration, and that most of the lymphocytes infiltrating tumor tissue can be identified by immunohistochemistry as T cells with predominance of the $CD8^+$ over the $CD4^+$ subset [1]. It has also been reported that lymphocyte infiltration into the tumor and a high $CD4^+$:$CD8^+$ T cell ratios are associated with a reduced risk of recurrence following liver transplantation [2]. Helper T cell subsets are polarized by some cytokines and are believed to at least partly, regulate the immune response to viral infection. Helper T cells are divided into subsets depending on whether either interferon-gamma (IFN-gamma) or interleukin-4 (IL4) is produced along with other cytokines [3, 4]. Naive T cells (Th0) secrete both Th1 and Th2 cytokines in response to antigenic stimulation. Th1 cells (IFN-gamma+/IL4−), which produce IFN-gamma and interleukin 2 (IL2), play a pivotal role in cell-mediated immunity by activating cytotoxic T cells (CTL) and natural killer T cells (NK cells). Th2 cells (IFN-gamma−/IL4+), which produce IL4, interleukin 10 (IL10),

and other cytokines, are essential for the regulation of humoral immunity. In addition, Th1 and Th2 cells cross-regulate their own development [5, 6], while activated NK cells produce both IL4 and IFN-gamma and regulate Th1/Th2 cytokine responses [7]. In the present study, preoperative NK cell activity was not significantly related to any of the clinicopathologic variables, including liver function tests, and the underlying cirrhotic condition. It has been reported that patients with low NK cell activity have a higher risk of developing HCC [8]. Therefore, it is important to consider the immunological background of patients with HCC.

There have been reports indicating that Th2 cytokines down-regulate antitumor immunity [9], while activation of the Th1 response induces antitumor immunity [10–13]. However, detailed responses of various cytokines are still unclear in patients with chronic hepatitis (CH), liver cirrhosis (LC), and HCC. We examined the relative levels of the Th1 and Th2 subpopulations among CD4-positive T cells from patients with HCV-related CH, LC, and HCC using flow cytometric detection of intracellular IFN-gamma and IL4 expression. The objectives of the present study were to evaluate the Th1/Th2 balance in patients with HCV-related CH, LC, or HCC and to investigate the relations between carcinogenesis and host immunity in patients with HCV-related HCC.

Methods

Patients

The study population was 117 adult Japanese patients who had CH due to HCV infection (CH-C) diagnosed from pathological examination of liver biopsy specimens, and included 32 patients with HCC who had HCV-related LC. All of the patients were more than 55 years old and they were admitted to our hospital between 1997 and 2006. Except for the patients with HCC, the subjects were divided into four subgroups based on the fibrosis score of Desment (stages 1–4). Blood samples were collected from the patients in the early morning before the start of IFN therapy or chemotherapy.

Serum alanine aminotransferase, aspartate aminotransferase, platelet count, white blood cell count, and HCV-RNA

The platelet (PLT) count, white blood cell (WBC) count, and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to standard procedures, while the HCV-RNA level was quantified by the Amplicor Ver 2.0 assay (Roche) before the start of therapy.

Analysis of CD4-positive T cell subsets

CD4-positive T cell subsets in the peripheral blood were analyzed after nonspecific stimulation with phorbol 12-myristate 13-acetate (PMA), ionomycin, or brefeldin A (Sigma Chemical Co., St Louis, MO, USA), according to the modified method of Jung et al. [14, 15].

Flow cytometry was used to detect IFN-gamma and IL-4 expression in the cytoplasm of peripheral blood CD4-positive T cells after culture and staining, as reported previously [14]. Results were expressed as the percentage of cytokine-producing cells among the CD4-positive T cell population, with IFN-gamma-positive/IL4-negative cells being defined as Th1 cells and IFN-gamma-negative/IL4-positive cells as Th2 cells (Fig. 1).

Generation of effector cells for determination of NK cell activity

Effector cells were obtained by specific gravity centrifugation (Conray–Ficol: $d = 1.077$) with Ficol–Isopaque (IBL: American Research Products, Inc., USA). These cells were washed twice with PBS, seeded at a density of 1×10^6 ml^{-1} in U-shaped 96-well plates, and cultured in RPMI 1640 medium (IBL: American Research Products) containing 10% FBS (Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (F-RPMI). The ^{51}Cr-release assays were performed according to the previously reported method [16, 17]. In brief, K562 cells (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) were labeled at 37°C for 1 h with sodium chromate ($Na^{51}CrO_4$; NEN, Vienna, Austria) as the targets, and they were intensively washed with PBS three times. Then the target cells (1×10^6 ml^{-1}) were added to the wells with F-RPMI. Next, the effector cells (1×10^3) were added

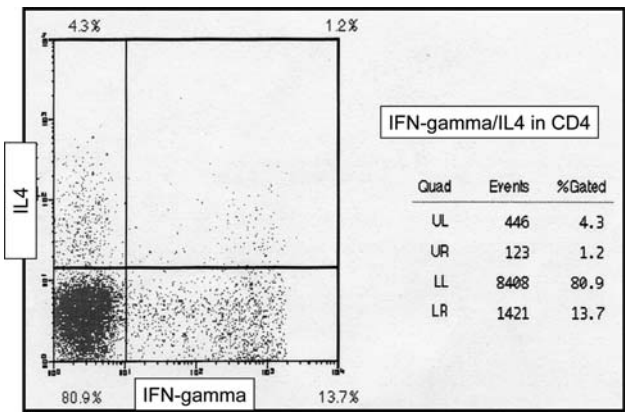

Fig. 1 Flow cytometric detection of interferon-γ (IFN-γ) and interleukin-4 (IL4) in CD4-positive T cells. *Upper left* IFN-γ negative and IL4 positive cells (Th2); *lower right* IFN-γ positive and IL4 negative cells (Th1); *upper right* IFN-γ positive and IL4 positive cells (Th0)

to the wells at an effector cell/target cell (E/T) ratio of 1/20. After 3.5 h of co-culture, supernatant was harvested and ^{51}Cr was counted with a gamma-counter (LKB-1272; LKB Wallac, Stockholm, Sweden). Results are expressed as a percentage of specific lysis according to the following formula: specific release = [(experimental ct/min) − (spontaneous ct/min)]/[(maximum ct/min) − (spontaneous ct/min) × 100], where spontaneous ct/min = ct/min released in the presence of 1 N HCl. Spontaneous ^{51}Cr-release was <18% for all NK cell assays.

Statistical analysis

Tukey's test was used to compare patient characteristics among the groups. Data were analyzed using Spearman's rank correlation coefficient method. Results are expressed as the mean + S.D. Probability values of less than 0.05 were considered to indicate statistical significance.

Results

The stage 1 group (F1) was composed of 12 men and 8 women within the age range of 56–68 years (mean ± SD, 61.6 ± 4 years), while the stage 2 group (F2) was composed of 17 men and 8 women aged between 56 and 70 years (mean ± SD, 63.5 ± 5 years). In addition, the stage 3 group (F3) contained nine men and ten women aged between 58 and 72 years (mean ± SD, 62.8 ± 4 years), and the stage 4 group (F4) was composed of 12 men and 9 women aged between 56 and 70 years (mean ± SD, 62.6 ± 4 years). The HCC group with LC comprised 28 men and 4 women aged from 57 to 73 years (mean ± SD, 65.3 ± 4 years) (Table 1).

Laboratory data and HCV-RNA

Figures 2, 3, 4, and 5 summarize the serum levels of ALT and AST, the PLT count, the WBC count, and HCV-RNA in each group. There were no significant differences of the serum levels of AST or ALT among the groups (Fig. 2). PLT counts were significantly lower in the F4 and HCC groups than in the F1 group (Fig. 3), while there were no significant differences of the WBC count among the groups (Fig. 4). There were also no significant differences of serum HCV-RNA levels among the groups (Fig. 5).

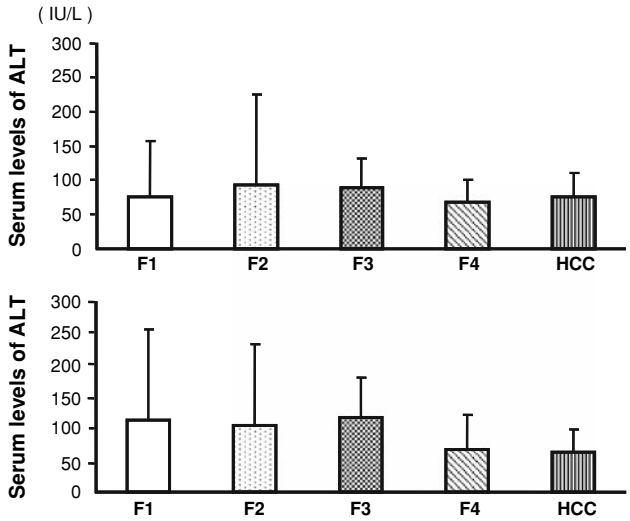

Fig. 2 Comparison of serum aminotransferase levels. There were no significant differences of the serum levels of AST or ALT among the groups

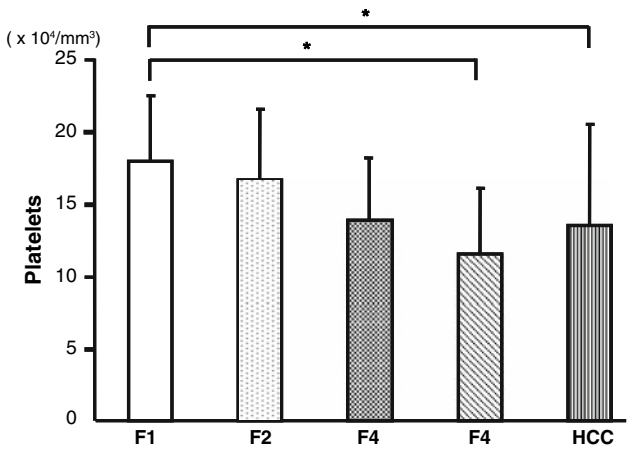

Fig. 3 Comparison of the platelet (PLT) count. In the F4 and HCC groups, the PLT count was significantly lower than in the F1 group ($p < 0.05$ by Tukey's test)

Table 1 Clinical characteristics of the subjects

	F1	F2	F3	F4	HCC
No. of patients	20	25	19	21	32
Mean age	61.6 ± 4	62.5 ± 5	62.8 ± 4	62.6 ± 4	65.3 ± 4
Gender (M/F)	12/8	17/8	9/10	12/9	28/4
AST (IU/I)	75.0 ± 82	92.3 ± 132	89.0 ± 43	68.0 ± 33	75.5 ± 34
ALT (IU/I)	112.6 ± 141	104.6 ± 128	117.5 ± 61	66.7 ± 55	63.5 ± 35
WBC (mm^{-3})	5,239	5,080	4,889	4,307	4,297
PLT ($\times 10^4$ mm^{-3})	17.9 ± 4.5	16.7 ± 4.8	13.8 ± 4.3	11.5 ± 4.6	13.5 ± 6.9

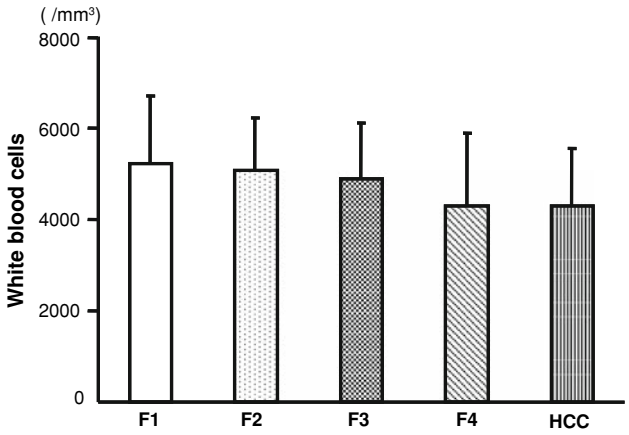

Fig. 4 Comparison of the white blood cell (WBC) count. There were no significant differences among the groups

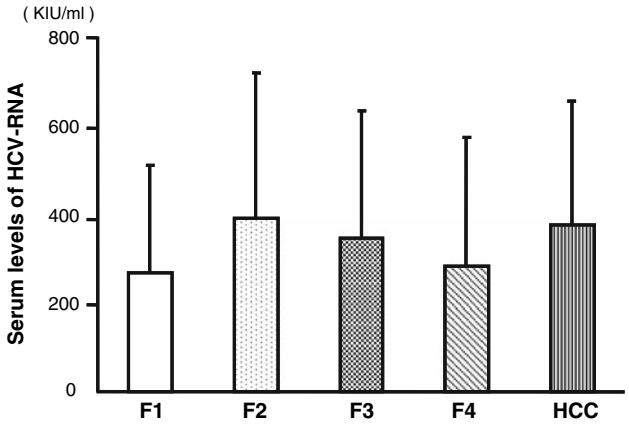

Fig. 5 Comparison of serum HCV-RNA levels. There were no significant differences among the groups

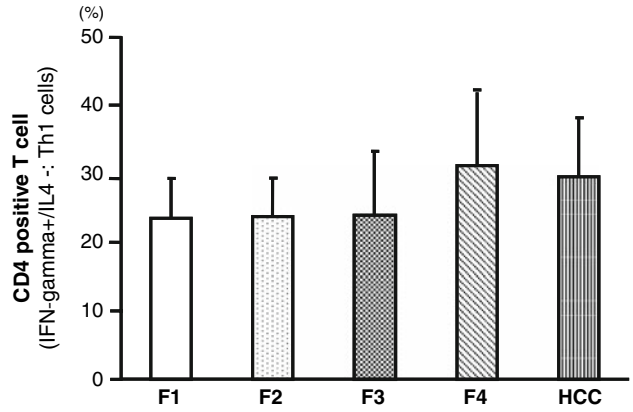

Fig. 6 Comparison of the percentage of peripheral blood Th1 cells. In the F4 and HCC groups, the percentage of Th1 cells tended to increase depending on the extent of fibrosis, although there were no significant differences between these groups and the other groups

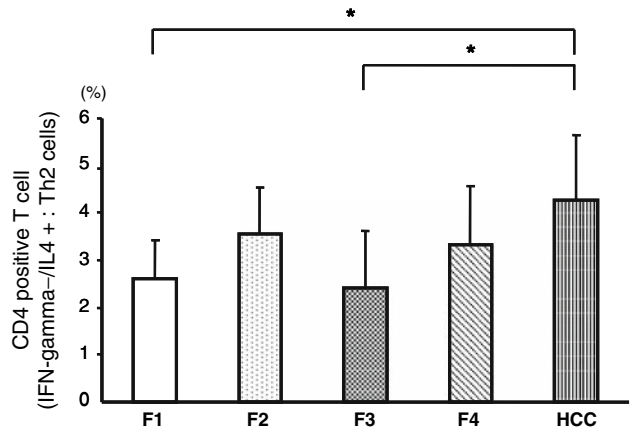

Fig. 7 Comparison of the percentage of peripheral blood Th2 cells. In the HCC group, the percentage of Th2 cells was significantly higher than in the F1 or F3 group ($p < 0.05$ by Tukey's test)

Peripheral blood Th1 and Th2 cells

In the F4 and HCC groups, the percentage of Th1 cells tended to increase depending on the extent of fibrosis, although there were no significant differences between these groups and the other groups (Fig. 6). In the HCC group, the percentage of Th2 cells was significantly higher than in the F1 or F3 groups ($p < 0.05$ by Tukey's test) (Fig. 7). However, there were no significant differences of the PLT count in relation to the Th1 or Th2 levels in the peripheral blood (Fig. 8).

Peripheral blood NK cells

Figure 9 compares the percentage of peripheral blood NK cells between each group. NK cells showed no significant differences among the groups, and NK activity was not higher in the HCC group than that in the F1, F2, F3, or F4 groups (Fig. 9).

Discussion

We previously reported that the relative levels of both Th1 and Th2 cells in the peripheral blood showed a significant increase with age in healthy volunteers. However, this relationship was not seen in CH-C patients [18], so we eliminated the influence of age by enrolling patients more than 55 years old. In addition, we reported that both Th1 and Th2 cells were increased in the peripheral blood of CH-C patients with a high virus load compared with healthy volunteers, and we also reported that the percentages of both Th1 and Th2 cells in the peripheral blood were not associated with the serum ALT level in the CH-C group [18]. In the present study, the levels of HCV-RNA and aminotransferases did not show any significant differences between CH, LC, and HCC patients. These results suggest that inflammation of hepatocytes in the liver or the HCV load in

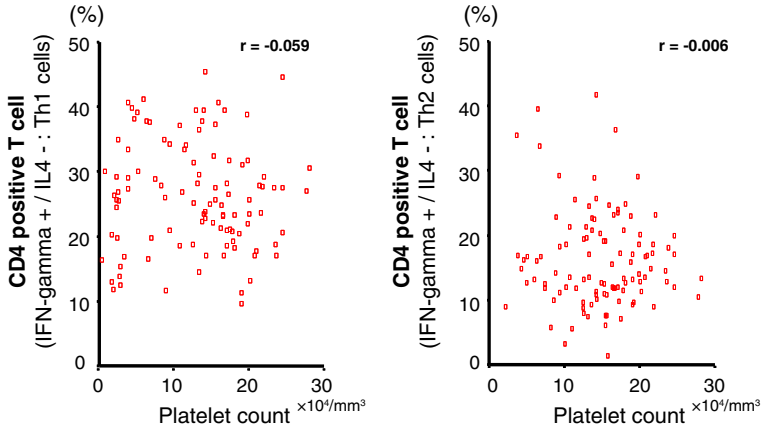

Fig. 8 Relation between the PLT count and the prevalence of Th1 or Th2 cells in the peripheral blood. There were no significant differences of the relative prevalence of Th1 or Th2 cells

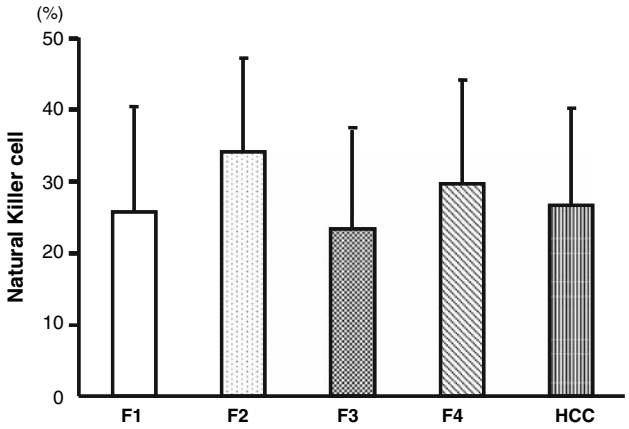

Fig. 9 Comparison of NK activity. There were no significant differences among the groups

the serum did not influence the Th1/Th2 cell balance in the peripheral blood.

There have been reports that Th2 cytokines down-regulate antitumor immunity [9], while activation of the Th1 response promotes antitumor immunity [10–13]. In the present study, the percentage of Th1 cells tended to increase depending on the extent of fibrosis in the F4 and HCC groups, although there were no significant differences between these and the other groups. On the other hand, the percentage of Th2 cells was significantly higher in the HCC group than in the F1 or F3 groups, while the PLT count of the F4 and HCC groups was significantly lower than that of F1 group. However, there was no significant relation between the percentage of Th1 or Th2 cells in the peripheral blood and the PLT count. It was reported that combining splenectomy with hepatectomy for HCC led to an increase of the PLT count, increase of Th1 cells and decrease of Th2 cells in cirrhosis patients [19]. Although splenomegaly certainly might induce an increase of Th1 cells and a decrease of Th2 cells, it is difficult to consider that a higher PLT count would increase Th1 and decrease Th2 cells. In this study, the PLT count seemed to reflect liver fibrosis and a decrease might not be related to carcinogenesis.

These results indicate that Th1 dominance is lost by an increase of Th2 cells in the HCC group and that carcinogenesis does not occur in patients with Th1 dominance. Also, the influence on the host immune response of the Th1/Th2 balance might be an important factor for the occurrence of carcinogenesis. Moreover, if Th1 dominance can be induced by down-regulating Th2 cells, we might be able to suppress carcinogenesis in patients with CH or LC. A change of the Th1/Th2 balance may be important to suppress or eliminate HCC in patients with HCV-related CH or LC. Another study, investigating the Th1/Th2 balance in patients with HCV-related LC concluded that Th1 cytokines (IFN-gamma and IL2) were lower in LC patients than in normal controls, although there was no significant difference of Th2 cytokines (IL10) between LC patients and a normal control group [20]. In the present study, we examined the percentages of CD4-positive T cell subsets, including Th1 (IFN-gamma+/IL4−) and Th2 (IFN-gamma−/IL4+) cells, in patients with HCV-related CH, LC, and HCC. Th1 cells produce IL2 or IFN-gamma, while Th2 cells produce IL4 or IL10, so it is possible that measurement of different cytokines (such as IL2 or IL4) might have led to different results compared with those of the other study.

In humans, NK cells appear to be involved in autoimmunity, and the innate responses to infections and tumors [21–24]. The human liver contains multiple populations of NK cells with distinct activities [25]. It has been reported that colorectal carcinoma with low preoperative NK activity shows a significantly increased risk of local recurrence [26]. In patients with HCV infection, however, NK cells have been suggested to contribute to pathologic as well as protective immune responses in the liver [27, 28]. In the present study, NK activity showed no significant differences among our groups, so the NK activity of the HCC group was not higher than that in patients who had HCV-related CH or LC without HCC. These results suggest that

chronic HCV infection continuously down-regulates host antitumor immunity, including NK activity, by increasing the number of Th2 cells.

Finally, we investigated the expression of Th1 and Th2 cytokines in patients with HCV-related CH, LC, and HCC. We found that Th1 dominance was lost due to an increase of Th2 cells in HCC patients and that carcinogenesis might occur in patients with chronic HCV infection who show an increase of Th2 cells, but it was unclear whether the change of Th2 cells was a cause or consequence of carcinogenesis. The immunological status of patients with HCV-related CH, LC, and HCC is complicated and the details are still unknown, so host immunity needs to be assessed by further studies.

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