Th2 Dominance Might Induce Carcinogenesis in Patients with HCV-related Liver Cirrhosis

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Running title: Host Immune System in Carcinogenesis.

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Abstract. Background: It has been reported that type 2 helper T-cell (Th2) cytokines down-regulate antitumor immunity, while Th1 cytokines up-regulate it. We previously reported that hepatocarcinogenesis was associated with Th2 dominance in patients with hepatitis C virus (HCV)-related liver cirrhosis (LC), but we did not determine whether Th2 dominance induced carcinogenesis or carcinogenesis led to Th2 dominance. Aim: To clarify whether Th2 dominance induces the carcinogenesis or vice versa in patients with HCV-related liver diseases. Patients and Methods: The study population was 82 adult Japanese patients who had chronic inflammation due to HCV infection diagnosed by pathological examination of liver biopsy specimens, including 21 patients with early hepatocellular carcinoma (eHCC) and HCV-related LC. All patients were admitted to our hospital between 2008 and 2014. eHCC was treated by radiofrequency ablation (RFA). The non-HCC patients were divided into four subgroups based on the fibrosis score of Desment (stage F1-4). Blood samples were collected in the early morning before starting RFA and after 4 weeks of RFA. Flow cytometry was used to assess the percentages of $IFN\gamma^{+}$ and $IL4^{-}$ (Th1) cells and $IFN\gamma^{-}$ and $IL4^{+}$ (Th2) cells in $CD4^{+}$ T-cells of peripheral blood before the start of each RFA session. Results: There were 21 patients with fibrosis stage 1, 21 with stage 2, 18 with stage 3, 22 with stage 4, and 21 patients with eHCC. Before RFA, Th1 cells were significantly more frequent in the F4 and eHCC groups than in the F1 group, although

there was no significant difference between the HCC and F1 groups after RFA. Both before and after RFA, Th2 cells were significantly more frequent in the HCC group than in the F1 group. Conclusion: Th2 dominance was not altered by elimination of eHCC after RFA therapy. Therefore, Th2 dominance might induce carcinogenesis in patients with HCV-related LC rather than carcinogenesis leading to Th2 dominance.

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and causes 662,000 deaths worldwide annually (1). Chronic HCV infection of the liver is an important risk factor for the development of this type of cancer. Treatment of HCV infection has been revolutionized by introduction of direct-acting antiviral agents (DAAs) and the development of oral, interferon (IFN)-free DAA regimens that promise extremely high (>90%) sustained virological response (SVR) rates (2). In patients with all stages of liver fibrosis, achieving SVR is reported to lead to marked reduction of all-cause mortality (3), progression of fibrosis (4), need for liver transplantation (5), extrahepatic complications (6), and hepatocarcinogenesis. However, recent studies have shown that the cumulative incidence of HCC at 5 and 10 years after SVR ranges 2.3% to 3.1 and from 8.8% to 11.1%, respectively (7). Most HCC develops in patients with a natural history of ongoing inflammation and severe fibrosis or cirrhosis, and liver fibrosis is reported to be the main independent risk factor for HCC (8). These clinical observations suggest that chronic inflammation due to HCV infection simultaneously induces hepatic fibrosis and carcinogenesis (fibro-carcinogenesis) (9, 10). It has been reported that the recurrence rate is lower and 5-year survival rate is higher for patients who have small tumors with prominent T-cell infiltration than those who have larger tumors without such infiltration, and that most tumor-infiltrating lymphocytes are immunohistochemistry identified as T-cells with predominance of

$CD8^+$ cells over $CD4^+$ cells (11). It has also been reported that lymphocytic infiltration of the tumor and a high $CD4^+/CD8^+$ cell ratio are associated with a lower risk of recurrence following liver transplantation (12). Therefore, the immunological background of patients with HCC seems to be important. Immature dendritic cells (DCs) in the liver exhibit low expression of major histocompatibility complex class II and co-stimulatory molecules (CD80 and CD86), lack CD1a, and produce suppressive cytokines such as interleukin-10 (IL10) (13). Mature DCs release a variety of cytokines (IL12, TNFα, IL18, and IFNα) that act on natural killer T-cells. In addition, mature DCs prime naive T-cells (Th0) and induce inflammatory $CD4^+$ T-helper type 1 (Th1) cells and $CD8^+$ cytotoxic T-cells. A high serum level of IL6 was found to be an independent risk factor for HCC in female patients with chronic hepatitis C, but not in males (14). High IL10 expression in HCC tissues and elevated serum IL10 levels are also correlated with tumor progression (15). Furthermore, it has been reported that Th2 cytokines down-regulate antitumor immunity (16), while Th1 cytokines up-regulate it (17-20). We previously examined changes of peripheral blood $CD4^+$ T-cells related to carcinogenesis in patients with HCV-related chronic hepatitis and liver cirrhosis (LC). We found that carcinogenesis was associated with Th2 dominance in patients with HCV-related LC (21). However, we did not determine whether Th2 dominance induced carcinogenesis or carcinogenesis led to Th2

dominance. Therefore, in order to clarify the relation between Th2 dominance and carcinogenesis in HCV-related liver diseases, we evaluated the changes of host immunity in patients with early HCC receiving radiofrequency ablation (RFA) therapy.

Patients and Methods

Patients. The study population was 103 adults Japanese patients who had chronic hepatitis due to HCV infection diagnosed by pathological examination of liver biopsy specimens, including 21 patients who had HCV-related LC with early HCC. All early HCCs were less than 30 mm in diameter and all were solitary tumors (mean diameter = 16.5 ± 4 mm, range=10.8-20.0 mm). These cancers were treated by RFA therapy. We previously reported that the blood levels of Th1 and Th2 cells significantly increase with age in healthy volunteers. However, this change is not seen in patients with HCV-related chronic hepatitis (22), so we eliminated the influence of age by enrolling patients more than 55 years old. All patients were admitted to our hospital between 1997 and 2014. Those without HCC were divided into four subgroups based on the fibrosis score of Desment (stage 1-4). Blood samples were collected in the early morning before the start of IFN therapy. In the patients with early HCC, blood samples were collected in the early morning before starting RFA therapy and after 4 weeks of RFA therapy.

Serum parameters and HCV-RNA. The platelet count, white blood cell (WBC) count, and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standard procedures, while the HCV-RNA level was quantified with the Amplicor Ver 2.0 test (Roche Molecular Diagnostics, Pleasanton, CA, USA) or TaqMan Ver 2.0 test (Roche Molecular Diagnostics, Pleasanton, CA, USA).

Analysis of CD4-positive T-cell subsets. CD-4-positive T-cell subsets in 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.* (23, 24).

Flow cytometry was used to detect cytoplasmic expression of IFN-γ and IL4 by peripheral blood CD4-positive T-cells after culture and staining (23). The percentage of cytokine-producing cells in the $CD4^+$ T-cell population was determined separately for IFNγ+/$IL4^-$ (Th1) cells and IFNγ-/$IL4^+$ (Th2) cells (Figure 1). Regulatory T-cells (Treg cells) were identified as $CD25^{high}$/$CD127^{low}$ cells (Figure 2).

Evaluation of the response to RFA. Computed tomography was performed before

starting RFA and after 4 weeks of RFA, and the tumor response was assessed according to the modified Response Evaluation Criteria in Solid Tumors (25, 26). In all patients with early HCC, disappearance of the tumor was confirmed.

Statistical analysis. Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS version 11.0; SPSS, Chicago, IL, USA) and Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Japan). Results are expressed as the mean ± standard deviation (SD). Wilcoxon's signed-rank sum test was used to compare patient's characteristics within each group, while Dunnett's test was employed for comparisons between the control group and other groups. Normally distributed variables were compared between two groups with Student's *t*-test, while other variables were compared by the Mann-Whitney U-test. A probability of less than 0.05 was considered to indicate statistical significance.

This study was approved by Ethical Review Board of Toho University Medical Center, Omori Hospital (number 23-170).

Results

Clinical profile of the patients. The age and sex of patients grouped by staging are given in Table I. Among the 21 patients with early HCC, 17 patients were in Child-Pugh class A and four were in Child-Pugh B class, while the F4 group included 19 patients in class A and three in class B (p=0.635).

Laboratory data and HCV-RNA. Table II summarizes the serum levels of AST and ALT, platelet count, WBC count, differential WBC count, and HCV-RNA level in each group. There were no significant differences in the serum AST and ALT levels among the groups. The platelet count was significantly lower in the F4, and early HCC groups than in the F1 group (Figure 3), while there were no significant differences in the WBC count and differential WBC count among the groups. There were also no significant differences in serum HCV-RNA levels and HCV serotypes among the groups.

Laboratory data and HCV-RNA before and after RFA therapy. Changes of various parameters in the early HCC group were investigated by comparing data obtained before RFA and after RFA. There were no significant differences in serum AST, ALT, total bilirubin, albumin, prothrombin time, or platelet count between before and after RFA. There were also no significant differences in serum HCV-RNA levels between before and after RFA (Table III). With regard to the serum levels of various tumor

makers, such as α-fetoprotein (AFP), the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), and *des*-γ-carboxyprothrombin (DCP), there were also no significant differences between values before and those after RFA (Figure 4).

Peripheral blood Th1 and Th2 cells. In the F4 group and the early HCC group before RFA, the percentage of Th1 cells was significantly higher than in the F1 group ($p \leq 0.05$ by Dunnet's test), although there was no significant difference between the F1 group and the early HCC group after RFA (Figure 5). Both before and after RFA, the percentage of Th2 cells was significantly higher in the early HCC group than in the F1 group ($p \leq 0.05$ by Dunnet's test) (Figure 6). The proportion of Treg cells was significantly lower in the early HCC group after RFA compared with before RFA ($p \leq 0.05$ by Wilcoxon's signed-rank sum test) (Figure 7), although there were no significant differences in Th1 and Th2 cells between before and after RFA (Figure 3 and 4). The changes of Th1, Th2, and Treg cells were not correlated with counts (data not shown).

Discussion

We previously reported that both Th1 and Th2 cells were increased in the peripheral blood of patients with HCV-related chronic hepatitis and a high viral 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 serum ALT in the patients with HCV-related chronic hepatitis (22). In the present study, serum levels of HCV-RNA and aminotransferases did not significantly differ among patients with HCV-related chronic hepatitis, LC, and early HCC. These results corresponded with our previous findings and suggest that inflammation of hepatocytes and the serum HCV load does not influence peripheral blood Th1/Th2 cell balance.

In the present study, the platelet count was significantly lower in the F4 group and early HCC group before and after RFA than in the F1 group, although there were no significant differences in serum AST, ALT, WBC count, and differential WBC count. Moreover, the platelet count was not significantly correlated with Th1, Th2, or Treg cells. It was reported that combining splenectomy with hepatectomy for patients with HCC with cirrhosis led to an increase of the platelet count, increase of Th1 cells, and a decrease of Th2 cells (27). Although splenectomy might cause an increase of Th1 cells and a decrease of Th2 cells, it is difficult to consider that a higher platelet count would lead to changes of these cells. In our patients with fibrocarcinogenesis, the platelet count seemed to reflect liver fibrosis, and a decrease in platelet count might not be related to carcinogenesis in this study population.

Based on the cytokine production profile, helper T-cells can be divided into

two distinct populations: Th1 and Th2 cells. These cells cross-regulate their own development. It was reported that an imbalance between Th1-like cytokines and Th2-like cytokines in the tumor microenvironment plays an important role in recurrence of HCC. In patients with metastatic HCC, liver tissue levels of Th1 -cytokines are lower and levels of Th2 cytokines are much higher compared with the levels in patients without metastatic HCC (28). Of these cytokines, overexpression of IL2 and IL15 in peritumoral liver tissue, rather than in tumor tissue and serum, is significantly associated with a low incidence of intrahepatic recurrence and long overall survival (29). These reports indicate that Th2 dominance might be important for the occurrence of carcinogenesis. The response of T-cells to self- and non-self antigens is controlled by a network of Treg cells. $CD4^+$ cells that constitutively express CD25, the IL2 receptor α-chain, are generally considered to be natural Treg cells, and account for 5-10% of peripheral $CD4^+$ T-cells in healthy animals and humans (30-32). There are two distinct subsets of Treg cells in peripheral lymphoid organs, natural Tregs that develop in the thymus after recognition of high-affinity self-antigens, and induced Tregs that develop from conventional T-cells after peripheral exposure to antigens and cytokines such as Transforming growth factor β or IL10 (33). These Treg cell subsets form the Treg network and may act synergistically to maintain immune homeostasis or may have different targets;; these

cells possibly also have a developmental role (34). It has been reported that circulating and tumor-infiltrating forkhead box $P3^+$ Treg cells are increased in patients with HCC (35). We have already reported that carcinogenesis in patients with HCV-related LC is associated with Th2 dominance (21), but we did not investigate then whether Th2 dominance induced carcinogenesis or carcinogenesis resulted in Th2 dominance. In the present study, the elimination of early HCC by RFA therapy led to normalization of Th1 cells and a decrease of Tregs. These results indicate that natural Tregs were not exposed to HCC antigens and induced Tregs decreased by elimination of early HCC after RFA therapy, with Th1 cells being normalized after the host immune system no longer needed to act against early HCC. After RFA therapy led to the elimination of early HCC, the percentage of Th2 cells continued to increase despite a decrease in Treg cells. These results indicate that Th2 might induce carcinogenesis in patients with HCV-related LC rather than carcinogenesis leading to Th2 dominance. It was reported that the combination of depletion of Treg cells and concomitant stimulation of effector T-cells may represent an effective strategy for suppressing metastasis and recurrence of HCC (36, 37). Our findings in present study support this report. In patients with HCV-related LC, it might be important to suppress Treg cells and Th2 cells for the prevention of carcinogenesis. Interestingly, it was reported that the annual incidence of HCC after SVR is higher in patients

receiving IFN-free therapy than that in those receiving IFN-based therapy (38). In patients with HCV-related diseases, examination of host immunological changes may be important for the prevention of carcinogenesis.

In conclusion, Th2 dominance was not altered by elimination of early HCC after RFA therapy. This suggests that Th2 dominance might induce carcinogenesis in patients with HCV-related LC rather than carcinogenesis resulting in Th2 dominance. However, further studies will be needed to examine the changes of host immunity in liver tissue, because changes in serum parameters were observed in the present study.

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Figure legends

Figure 1. Flow cytometric detection of interferon (IFN)-γ and interleukin (IL)-4 in CD4-positive T-cells. Upper left: $IFN\gamma^-$ and $IL4^+$ cells (Th2); lower right: $IFN\gamma^+$ and $IL4^-$ cells (Th1); upper right: $IFN\gamma^+$ and $IL4^+$ cells (Th0).

Figure 2. Flow cytometric detection of CD25 fluorescein isothiocyanate and CD127 Phycoerythrin in $CD4^+$ T-cells. Upper left: leukocytes, monocytes, and lymphocytes; Upper right: $CD4^+$ lymphocytes; lower left: no reaction to a different protein (control); lower right: $CD4^+$ and $CD127^-$ lymphocytes (Tregs).

Figure 3. Comparison of the platelet count in different groups The platelet count was significantly lower in the stage 4 of fibrosis (F4), and early hepatocellular carcinoma (HCC) groups than in the F1 group.

Figure 4. Comparison of tumor markers. With regard to the serum levels of different tumor makers, such as α-fetoprotein (AFP), the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), and *des*-γ-carboxyprothrombin (DCP), there were also no

significant differences in values between before and after radiofrequency ablation (RFA).

Figure 5. Comparison of the percentage of peripheral blood type 1 helper T (Th1)-cells. In the stage 4 of fibrosis (F4) group and the early hepatocellular carcinoma (HCC) group before radiofrequency ablation (RFA), the percentage of Th1 cells was significantly higher than in the stage 1 of fibrosis (F1) group ($p \leq 0.05$ by Dunnet's test), although there was no significant difference in values between the F1 group and the early HCC group after RFA.

Figure 6. Comparison of the percentage of peripheral blood type 2 helper T (Th2)-cells. Both before and after radiofrequency ablation, the percentage of Th2 cells was significantly higher in the early hepatocellular carcinoma (HCC) group than in the stage 1 of fibrosis (F1) group ($p \leq 0.05$ by Dunnet's test).

Figure 7. Comparison of the percentage of peripheral blood regulatory T-cells (Tregs).

The proportion of Tregs was significantly lower in the early hepatocellular carcinoma (HCC) group after radiofrequency ablation (RFA) compared with that before RFA ($p \leq 0.05$ by Wilcoxon's signed-rank sum test).

Table I. **Clinical characteristics of the subject**

	F1	F2	F3	F4	HCC	*p*
No. of patients	21	21	18	22	21	
Mean age	62.7±4	63.0±5	62.7±4	63.5±6	67.7±6	*0.053*
Gender (M/F)	13/8	13/8	9/9	11/11	15/6	*0.591*
Child-Pugh class (A/B)				19/3	17/4	*0.635*

M: male; F: female

Table II. Comparison of clinical characteristics in patients with HCV infection

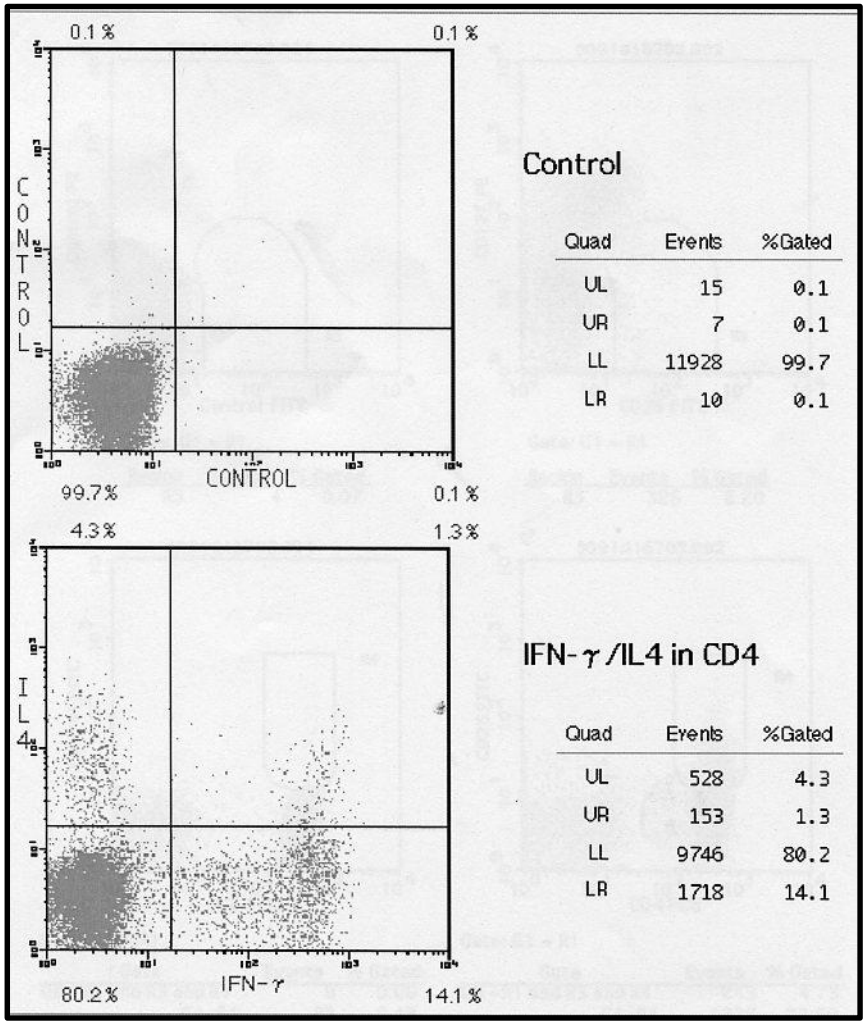
	F1	F2	F3	F4	HCC		*p*
					pre RFA	post RFA	
AST IU/l)	72.9±80	72.7±69	90.1±44	67.9±31	62.9±30	65.8±37	*0.266*
ALT (IU/l)	100.9±104	85.2±81	119.1±63	65.6±54	61.1±38	64.4±41	*0.052*
White blood cell (/mm^3)	5165.2 ± 1452	5157.1 ± 1235	4933.3 ± 1231	4268.8±1316	4717.7±1683	4200.3±1845	*0.288*
Lymphocyte (/mm^3)	1954.6±709	1784.9±525	2172.7±508	1541.8±801	1651.6±933	1488.7±351	*0.054*
Monocyte (/mm^3)	377.0±197	420.9±163	421.5±112	381.4±177	350.8±162	325.2±113	*0.414*
Platelet (/mm^3)	182190.5 ± 40641	158571.4 ± 45498	140666.7 ± 42759	106187.5 ± 43393	111647.1 ± 46733	112764.7 ± 50539	*0.001**
HCV-RNA (Log IU/ml)	5.28±0.8	5.51±0.8	5.33±0.6	5.38±0.6	4.10±2.3	4.33±2.4	*0.610*
Serotype of HCV (group 1 / group 2)	9/12	14/7	12/6	18/4	13/8		*0.126*

AST; aspartate aminotransferase , ALT; alanine aminotransferase

Table III. Change of various parameters in patients with early HCC treated by RFA

	HCC		*p*
	pre RFA	post RFA	
AST (IU/l)	62.9± 30	65.8± 37	*0.432*
ALT (IU/l)	61.1± 38	64.4± 41	*0.586*
Total bilirubin (g/dl)	0.89± 0.4	0.89± 0.4	*0.811*
Albumin (g/dl)	3.77± 0.6	3.73± 0.6	*0.607*
Prothrombin time (%)	85.7± 17	88.6± 13	*0.505*
Platelets (/mm^3)	111647.1± 46733	112764.7± 50539	*0.887*
HCV-RNA (Log IU/ml)	4.10± 2.3	4.33± 2.4	*0.138*

AST; aspartate aminotransferase , ALT; alanine aminotransferase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LR 1718 14.1

Fig. 1

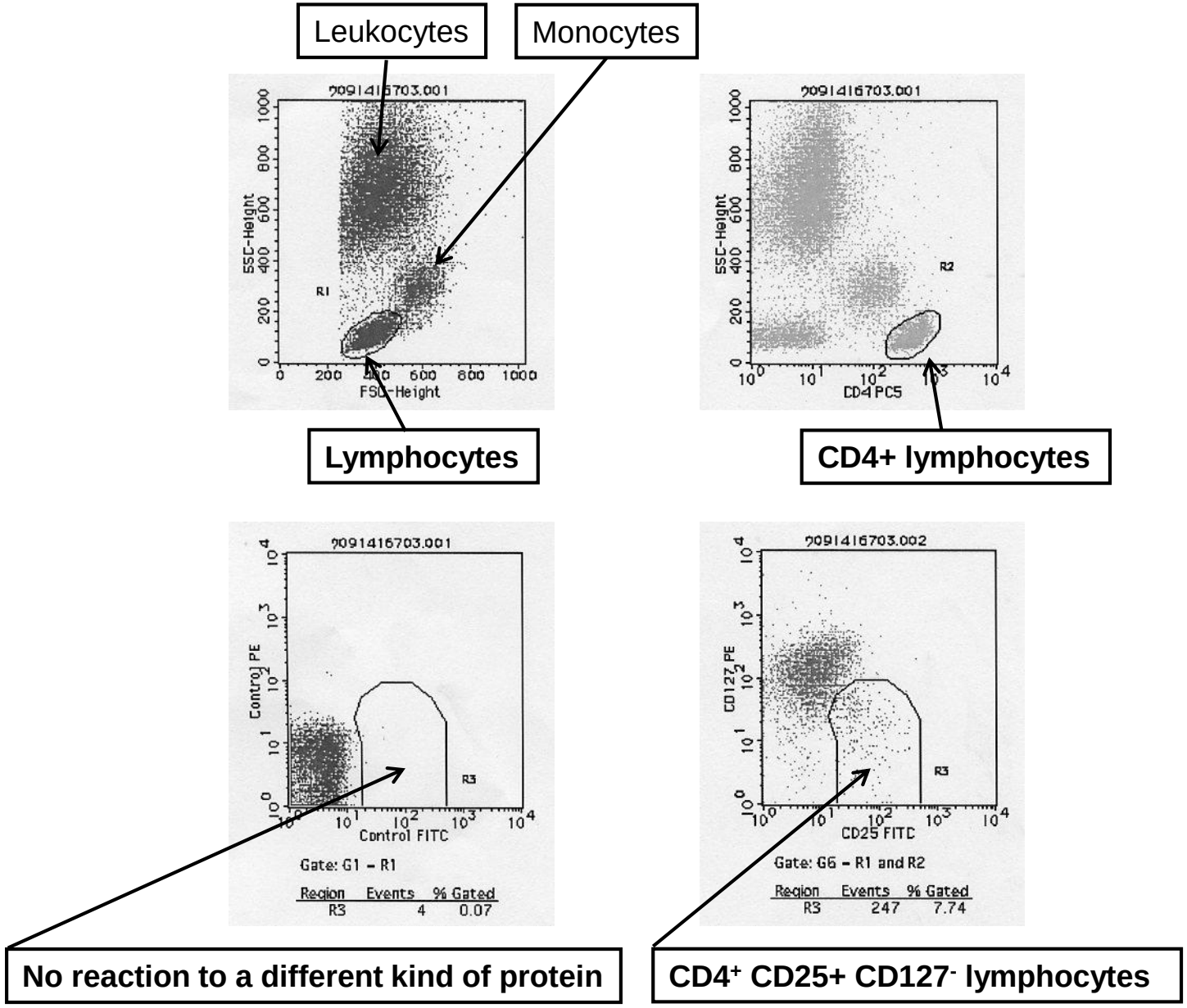
Leukocytes
Monocytes
9091416703.001
SSC-Height
FSC-Height
R1
Lymphocytes
9091416703.001
SSC-Height
CD4 PC5
R2
CD4+ lymphocytes
9091416703.001
Control PE
Control FITC
R3
Gate: G1 - R1
Region Events % Gated
R3 4 0.07
No reaction to a different kind of protein
9091416703.002
CD127 PE
CD25 FITC
R3
Gate: G6 - R1 and R2
Region Events % Gated
R3 247 7.74
CD4+ CD25+ CD127- lymphocytes

Fig. 2

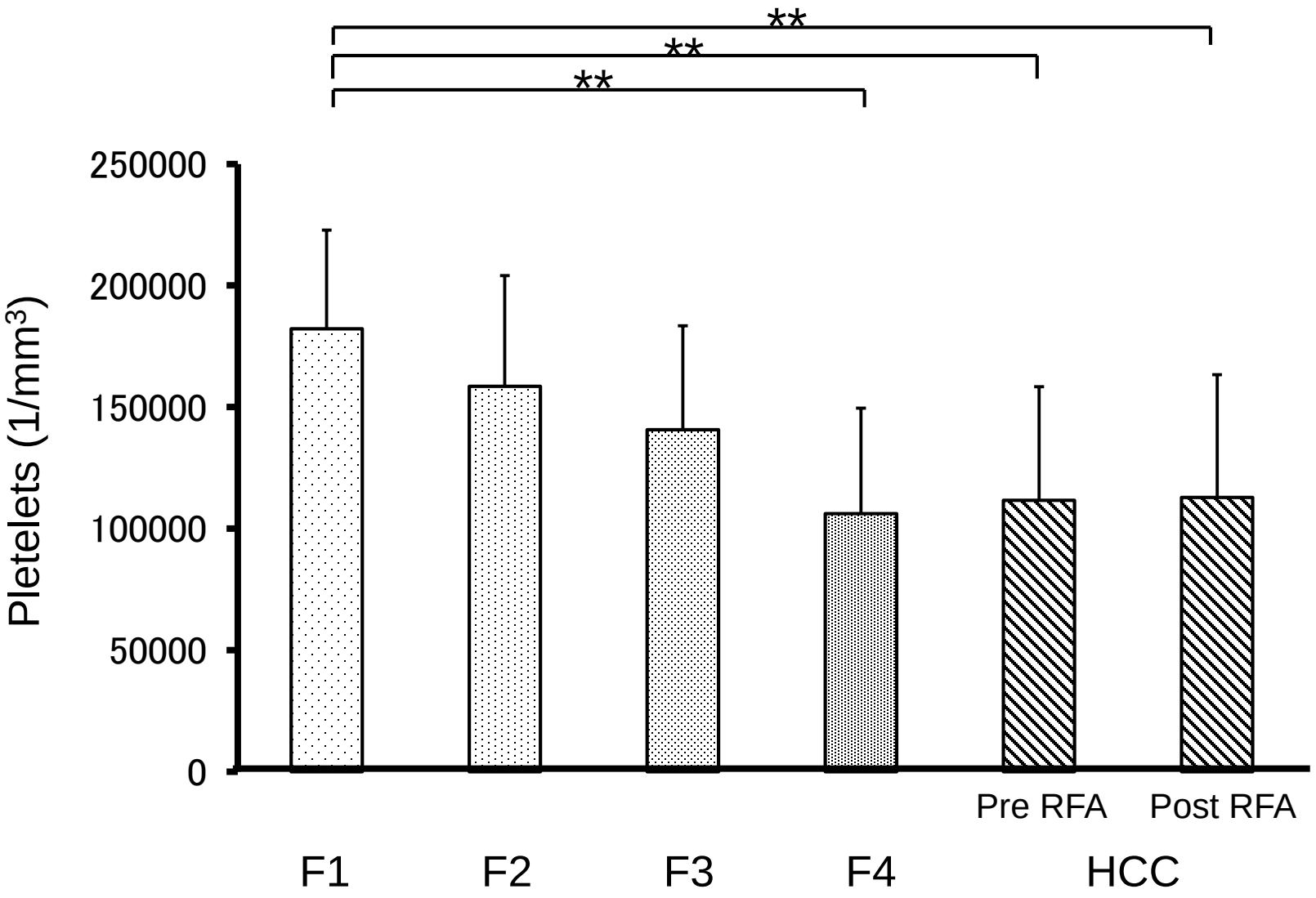
**
**
**
250000
200000
150000
100000
50000
0
Pletelets (1/mm3)
Pre RFA
Post RFA
F1
F2
F3
F4
HCC

Fig. 3

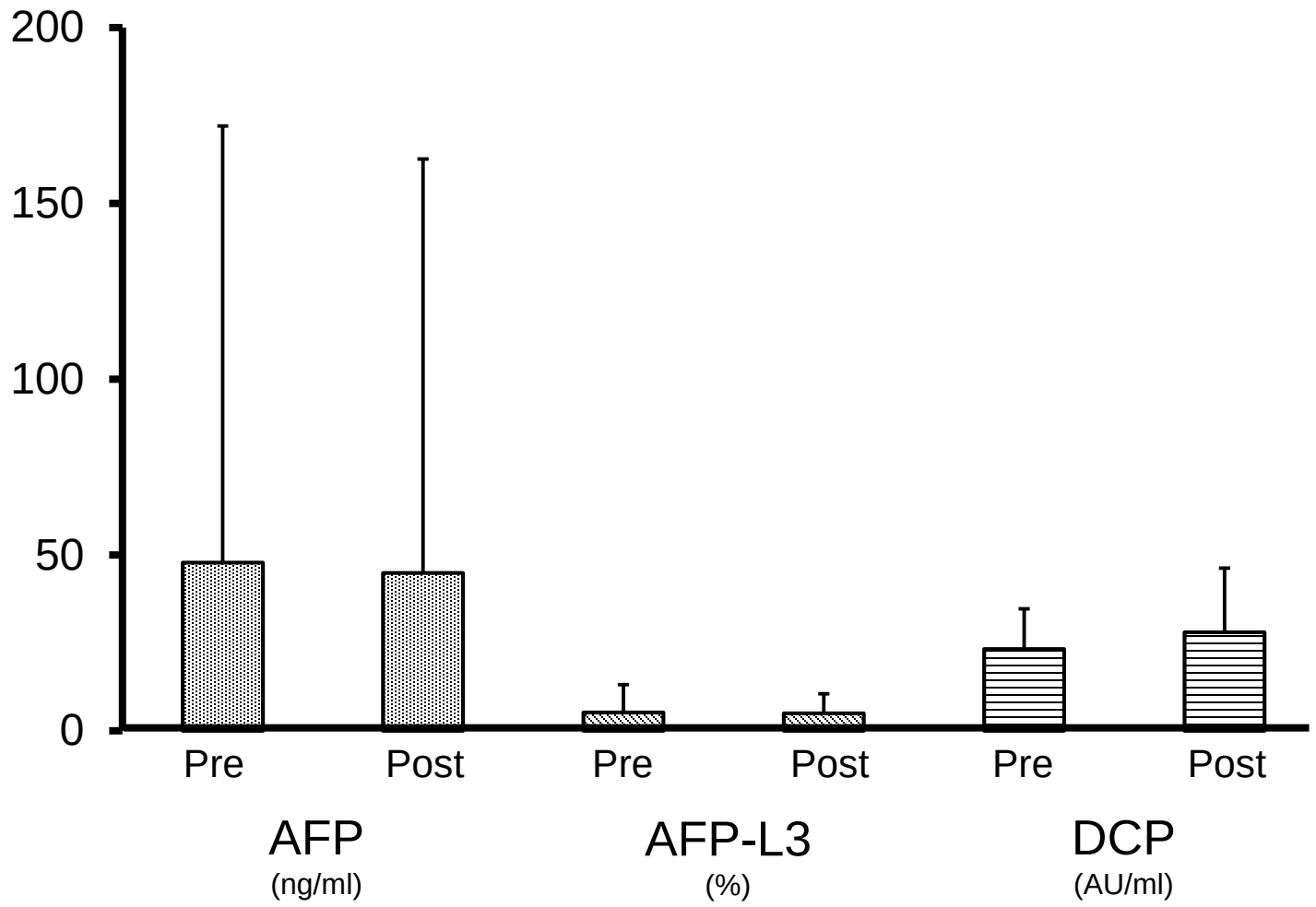
200
150
100
50
0
Pre
Post
Pre
Post
Pre
Post
AFP
(ng/ml)
AFP-L3
(%)
DCP
(AU/ml)

Fig. 4

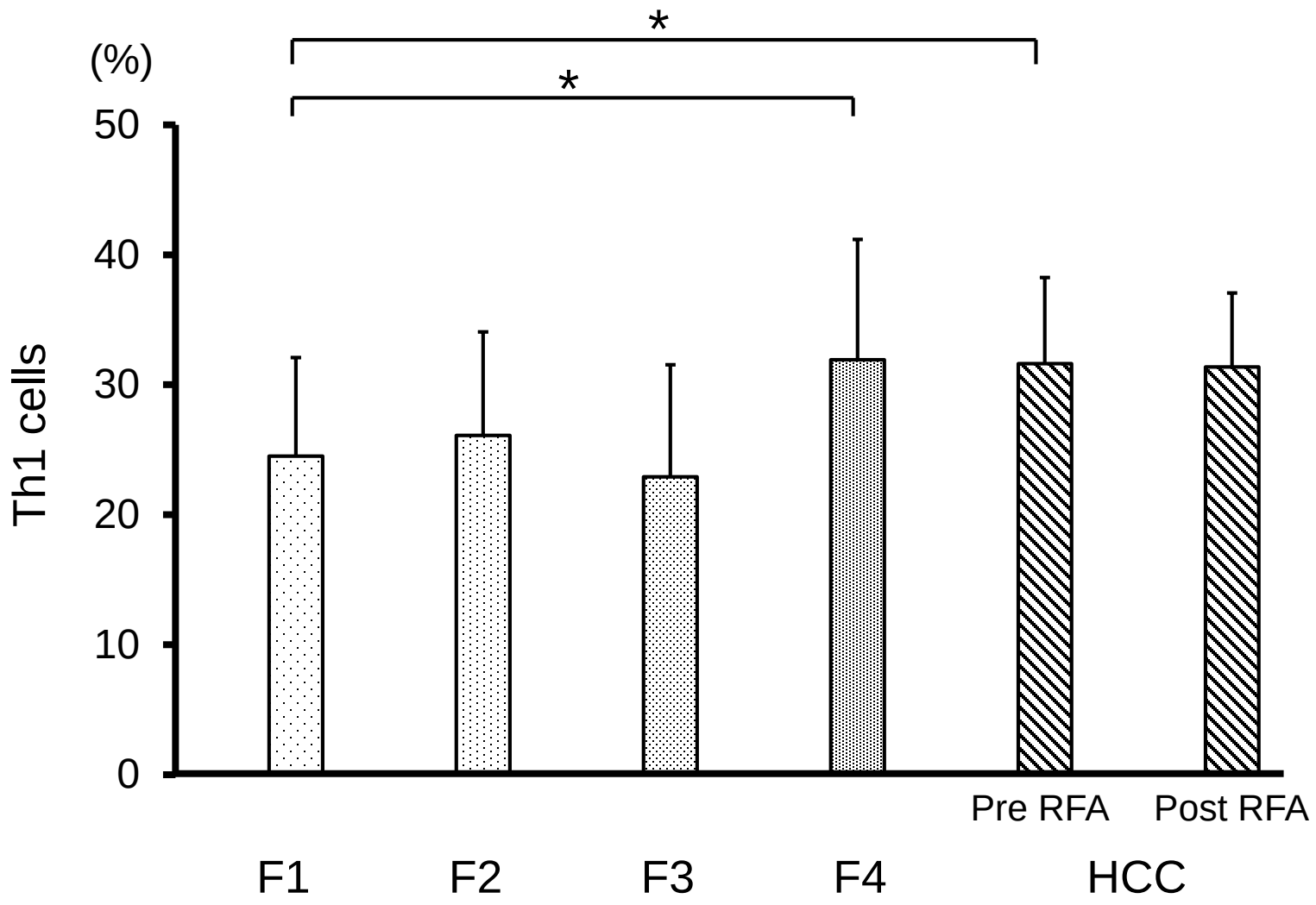

Fig. 5

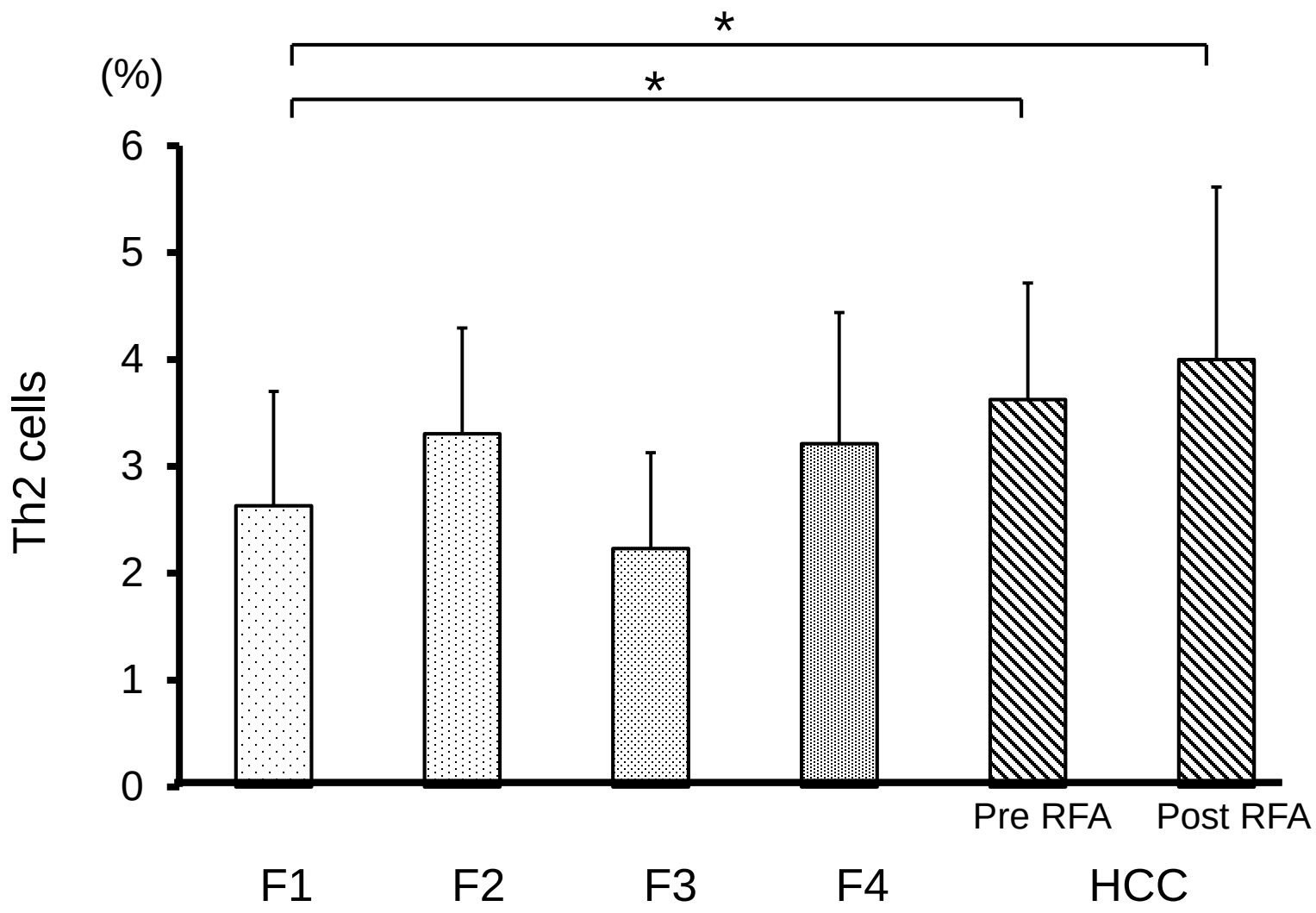

Fig. 6

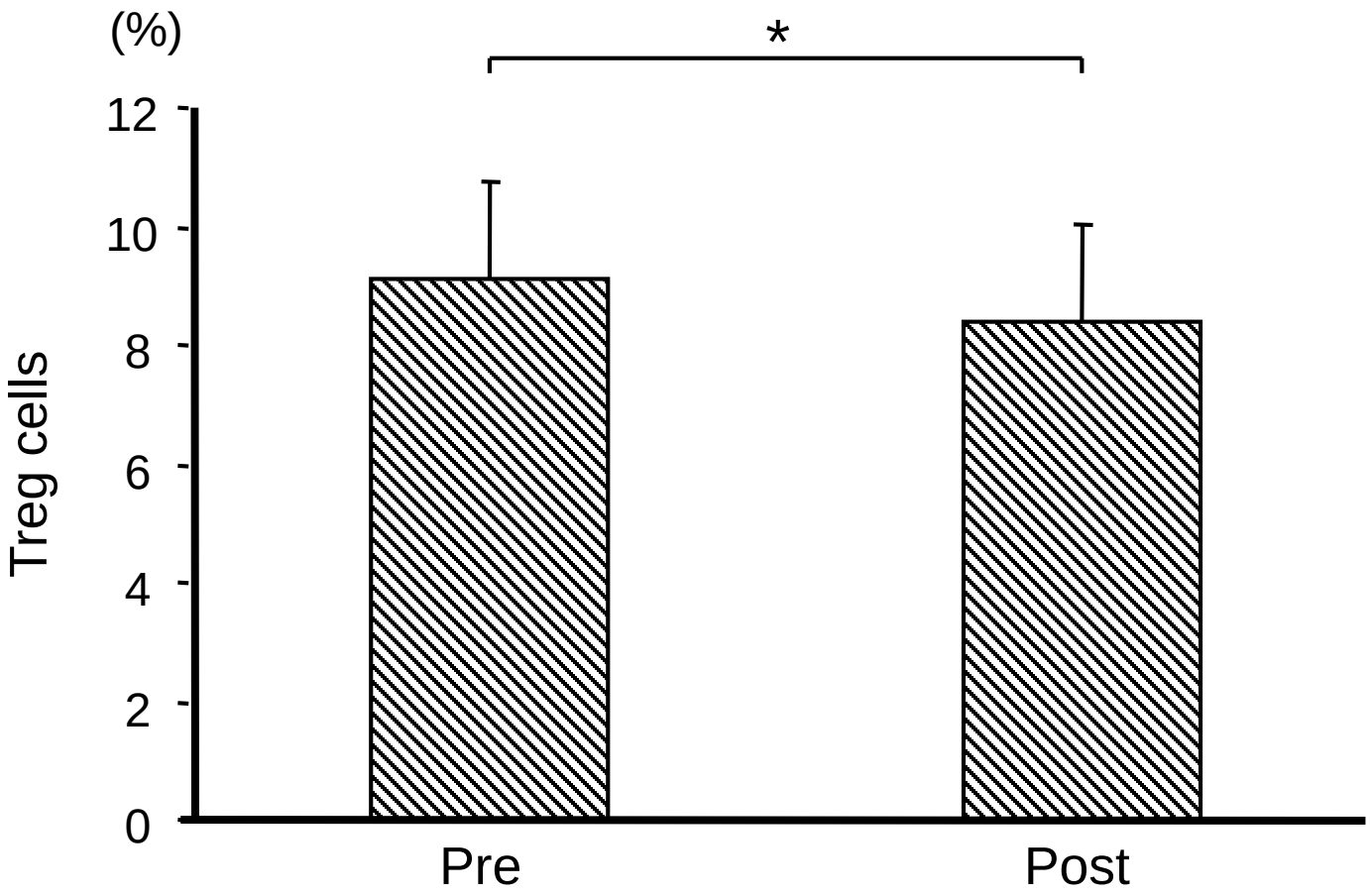

Fig. 7