Aberrant expression of p-STAT3 in peripheral blood CD4+ and CD8+ T cells related to hepatocellular carcinoma development

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Received December 16, 2013; Accepted July 7, 2014

DOI: 10.3892/mmr.2014.2510

Abstract. Hepatocellular carcinoma (HCC) is one of the most common cancer types worldwide. The signal transducer and activator of transcription 3 (STAT3) protein is a member of the STAT transcription factor family. Oncogenesis, invasion, and metastasis of HCC are associated with activation of STAT3. However, whether aberrant expression of phosphorylated STAT3 (p-STAT3) in peripheral CD4+ and CD8+ T cells relates to HCC pathogenesis remains unclear. In this study, the expression of p-STAT3 in CD4+ and CD8+ T cells, and the levels of interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-6 and IL-10 in the human hepatoma cell line Huh7 co-cultured with peripheral blood mononucleated cells (PBMCs) of healthy volunteers were measured. The correlations between p-STAT3 and IFN-γ/IL-4, IFN-γ, IL-4, IL-6 and IL-10 were then analyzed. Results showed that the p-STAT3 level is higher in CD4+ and CD8+ T cells in the peripheral blood of HCC patients, and in PBMCs co-cultured with Huh7 cells compared to controls. The cytokine (IL-4, IL-6 and IL-10) levels were increased and the IFN-γ level was decreased in the serum of HCC patients and in supernatants of PBMCs co-cultured with Huh7 cells. Correlation analyses demonstrated that the IFN-γ/IL-4 ratio and the IFN-γ level negatively correlate to the p-STAT3 level in CD4+ and CD8+ T cells in samples from patients and in cells cultured *in vitro*. By contrast, the levels of IL-4, IL-6 and IL-10 positively correlated to the p-STAT3 level. This study indicated that the expression of p-STAT3 is upregulated in peripheral CD4+ and CD8+ T cells of HCC patients, and which may result in abnormal immune surveillance and thereby, contribute to HCC pathogenesis.

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Key words: p-STAT3, peripheral blood, CD4+ T cells, CD8+ T cells, immune surveillance, pathogenesis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is a global health problem, ranking as the fifth most common cancer worldwide and the third leading cause of cancer-related mortality (1). It is the second cause of cancer-related mortality in China, and its prevalence is increasing, probably due to a high prevalence of hepatitis B (2,3). Surgical resection and liver transplantation are effective forms of therapy (4), but most patients have limited options and cannot afford these treatments. Therefore, improving our understanding of the HCC pathogenesis is especially important, since it may allow identifying effective, novel targets for therapy.

The signal transducer and activator of transcription 3 (STAT3) protein is a member of the STAT transcription factor family. STAT proteins mediate signal transduction induced by cytokines, growth factors, and oncogenes (5). In healthy cells, activation of the STAT3 protein is tightly controlled, in order to prevent deregulated gene transcription. STAT3 is constitutively activated in certain transformed cells (3). STAT3 activation is induced via phosphorylation of tyrosine (Tyr705), which allows STAT3 dimerization. The dimer translocates to the nucleus and directly regulates gene expression (5). Phosphorylated STAT3 (p-STAT3) contributes to malignant progression in various types of cancer, including carcinomas of the lung (6), breast (7), prostate (8) and melanoma (9). Oncogenesis, invasion, and metastasis of HCC are associated with activation of STAT3 (1,10).

Cellular immune responses play a critical role in the surveillance of malignancy and the control of HCC progression, with CD4+ and CD8+ T cells being the primary antitumor immune cells (11).It was previously shown that STAT3 is constitutively

activated not only in tumor cells, but also in tumor endothelial and myeloid cells, tumor-associated macrophages and dendritic cells (12). Other studies have suggested that active STAT3 is upregulated in tumor-infiltrating immune cells including dendritic cells, natural killer cells, and granulocytes (13,14). STAT3 signaling restrains natural tumor immune surveillance, and inhibition of hematopoietic STAT3 activity in tumor-bearing hosts elicits multicomponent therapeutic antitumor immunity (13). In addition, the expression of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells is increased in patients with multiple sclerosis (15), and p-STAT3 expression is induced by IL-10 in $CD4^+$ and $CD8^+$ T cells isolated from tumor-draining lymph nodes and tumors of mice bearing squamous carcinoma (16). However, whether the aberrant expression of p-STAT3 in peripheral $CD4^+$ T and $CD8^+$ T cells of HCC patients may affect their immune surveillance and immune tolerance and thus, contribute to HCC pathogenesis, remains unclear.

The T helper cells (Th) are a sub-group of lymphocytes that play a central role in immune protection (17). Th1 cells mediate antitumor reactivity through secretion of cytokines, including IFN-γ and tumor necrosis factor-α (TNF-α). IL-4, IL-6 and IL-10 are secreted by Th2 cells, and downregulate antitumor immunity (18,19). The Th1/Th2 balance is altered in HCC patients, and this event can lead to tumorigenesis (20). However, whether the Th1/Th2 imbalance is related to the abnormal expression of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells of HCC patients remains to be investigated.

STAT3 mediates cytokine signaling (21). IL-6 is a pro-inflammatory cytokine, playing an important role in regulating the immune response and other processes involved in the inflammatory response (10,22). IL-6 is one of the most important cytokines for STAT3 activation, and leads to STAT3 activation via the Janus-activated kinase (JAK) (22,23). IL-6 levels appear to be higher in HCC patients compared to healthy individuals (10). IL-10 is a potent immunosuppressive cytokine that downregulates the expression of Th1 cytokines and co-stimulatory molecules (24,25). IL-10 can also induce phosphorylation of STAT3 through activation of the JAK pathway (26). IL-10 expression is upregulated in HCC patients (27). IL-10 induces STAT3 phosphorylation in $CD4^+$ and $CD8^+$ T cells isolated from tumor-draining lymph nodes of mice bearing squamous carcinomas (16). However, whether IL-6 and IL-10 induce p-STAT3 expression in peripheral $CD4^+$ and $CD8^+$ T cells in HCC is still unclear.

In this study, we analyzed peripheral blood of HCC patients and healthy volunteers for the expression of p-STAT3. We also co-cultured healthy human peripheral blood mononucleated cells (PBMCs) with human hepatoma cells to investigate the expression of p-STAT3 in $CD4^+$ and $CD8^+$ T cells. Our data show that p-STAT3 is aberrantly expressed in $CD4^+$ and $CD8^+$ T cells of the peripheral blood in HCC patients, suggesting that activation of this transcription factor may contribute to the development of hepatocellular carcinoma.

Materials and methods

Study population. Patients with HCC and healthy controls were recruited from the Yuhuangding Hospital (Yantai, Shandong, China), from April, 2013 through August, 2013. Sample collection procedures for this study were approved by the University of Binzhou Medical College Ethics Committee, and informed consent was obtained from all patients. The study subjects included patients with HCC (n=10) and healthy controls (n=10). HCC was diagnosed on the basis of image findings [sonography, computed tomography (CT) scans, or magnetic resonance imaging (MRI) scans], biochemical tests [α-fetoprotein (AFP) levels ≥400 ng/ml], and histopathology, according to the guidelines of the American Association for the Study of Liver Diseases (28). Venous blood (4 ml) was collected from all subjects into two heparin tubes. Two ml of venous blood were used to isolate the serum. The serum was isolated by centrifugation at 1,734 g for 10 min at 4˚C, and was then stored at -80˚C. The remaining 2 ml of venous blood were lysed and fixed prior to flow cytometry. The erythrocytes were lysed and the leucocytes were fixed by immediately adding 30 ml of 1X pre-warmed Lyse/Fix Buffer (BD Biosciences, San Jose, CA, USA) and incubating in 37˚C for 10 min.

Co-culture. PBMCs were isolated from venous blood by Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Piscataway, NJ, USA) centrifugation at 771 x g, for 30 min. PBMCs were removed from the Ficoll/serum interface, washed twice in phosphate buffered saline (PBS), supplemented with 2% bovine serum albumin (BSA; Beyotime, Songjiang, Shanghai, China), and counted using an inverted microscope (OLYMPUS, Shinjuku, Tokyo, Japan).

The human hepatoma cell line Huh7 was purchased from the Cell Station of Shanghai Institute of Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Hyclone, Logan, UT, USA) in 5% CO_2 at 37˚C.

Huh7 cells were seeded into 12-well Corning Costar® plates (Corning, NY, USA) at densities of $1.25x10^5$, $2.5x10^5$ and $5x10^5$ cells/well. Samples were incubated for 4 h, and 0.5 ml of PBMCs ($5x10^5$/ml) were added to each well. After 24, 36, 48 and 60 h, PBMCs were harvested by centrifugation at 193 g for 5 min used 5 ml centrifuge tubes (Corning, NY, USA), washed in PBS supplemented with 2% BSA (Beyotime), and fixed prior to flow cytometry. The PBMCs were fixed by immediately adding pre-warmed Cytofix™ Fixation Buffer (BD Biosciences), and incubating at 37˚C for 10 min. In addition, the supernatants were collected, centrifuged at 1,000 x g for 5 min, and then stored at -80˚C.

Flow cytometry. Fixed cells were washed in Stain Buffer (BD Biosciences), vortexed and permeabilized by adding 1 ml of chilled Phosflow™ Perm Buffer III (BD Biosciences) and incubating for 30 min on ice. Samples were then washed and incubated with the mouse anti-human antibodies (all from BD Biosciences) anti-CD3-PE-Cy5 (clone UCHT1;), -CD4-FITC (clone RPA-T4), -CD8-FITC (clone RPA-T8), and -phosphorylated (p)-STAT3-PE (Phosflow™) at room temperature for 60 min. Then, the cells were washed and resuspended in Stain Buffer (BD Biosciences), and were subjected to flow cytometry analysis on a FACSCalibur instrument (BD Biosciences). To analyze the mean fluorescence intensity (MFI) of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells, cells were gated in $CD3^+$ T/$CD4^+$ T and $CD3^+$ T/$CD8^+$ T regions.

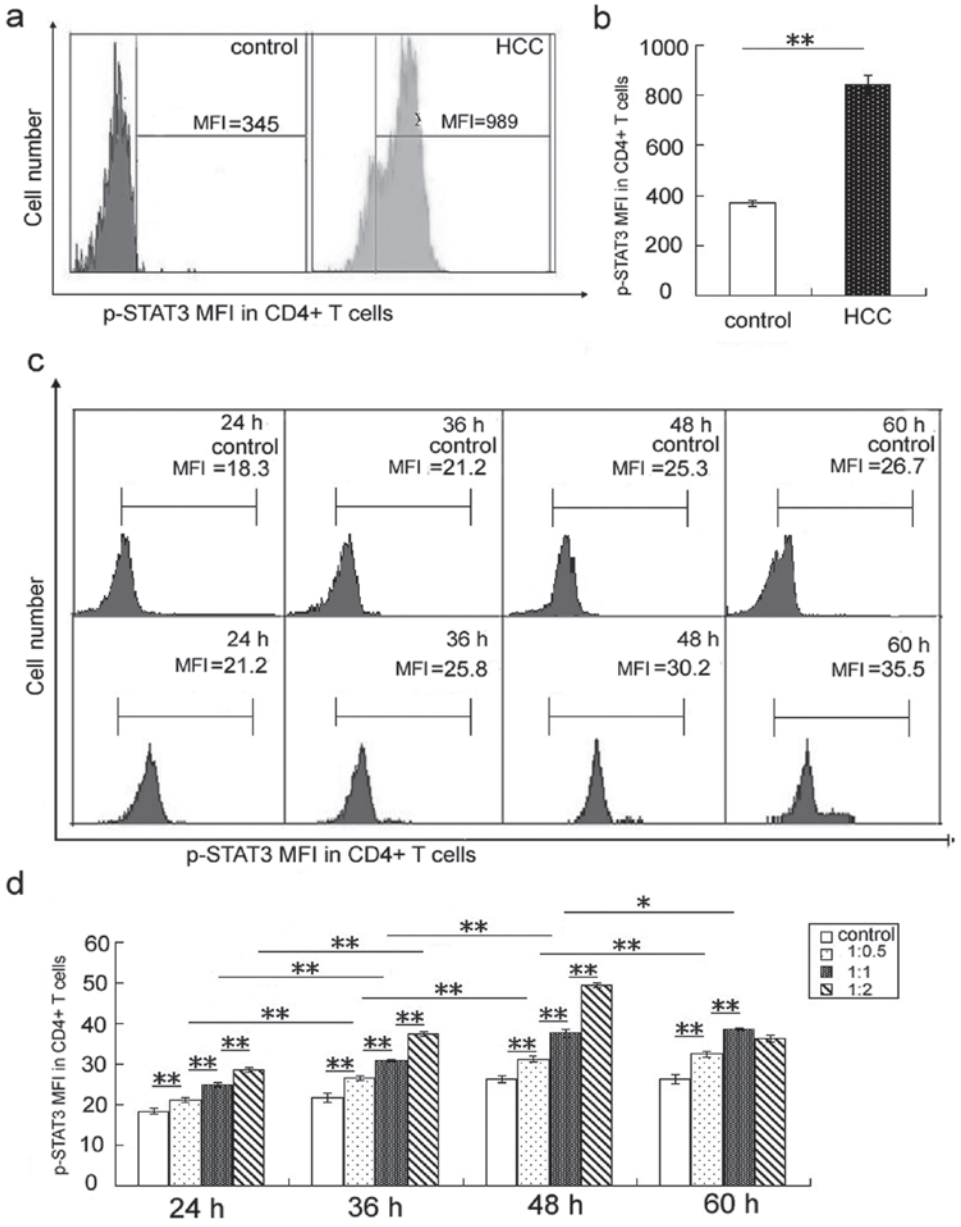

Figure 1. The level of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) is increased in peripheral CD4+ T cells in hepatocellular carcinoma (HCC) patients compared to healthy controls. (a) Representative images of flow cytometry analysis showing that the p-STAT3 level is increased in CD4+ T cells from peripheral blood of one HCC patient compared to that of a healthy control (P<0.001). (b) The average mean fluorescence intensity (MFI) of p-STAT3 in CD4+ T cells from peripheral blood is higher in HCC patients than in healthy controls (P<0.001). (c) Flow cytometry analyses show that the level of p-STAT3 in CD4+ T cells from a 0.5:1 PBMC:Huh7 cell co-culture is higher than the level observed in PBMCs cultured alone and increases with the time of co-culture (P<0.05). (d) The p-STAT3 level is increased in CD4+ T cells from PBMCs co-cultured with Huh7 cells as compared to PBMCs cultured alone. The p-STAT3 level increases with time and with the increasing ratio of Huh7 cells to PBMCs (P<0.05). *P<0.05 and **P<0.01.

The results were analyzed with the CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Cytokine measurements. The production of IFN-γ, IL-4, IL-6 and IL-10 was measured in the supernatant and the serum samples by enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN, USA) and following the manufacturer's instructions. All samples were assayed in triplicate.

Statistical analyses. Data are presented as mean ± standard error of the mean (SEM). Data were processed with the SPSS 17.0 statistical software (IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) was used to analyze the differences in the p-STAT3 mean fluorescence intensity (MIF) in CD4+ and CD8+ T cells, and in the cytokine levels between HCC patients or PBMCs co-cultured with Huh7 cells and their controls. To analyze the correlation between the levels of IFN-γ, IL-4, IL-6, IL-10 and p-STAT3, we performed a Spearman's correlation analysis. P-values <0.05 were considered to indicate statistically significant differences.

Results

The p-STAT3 level is higher in peripheral CD4+ and CD8+ T cells of HCC patients compared to healthy controls. The

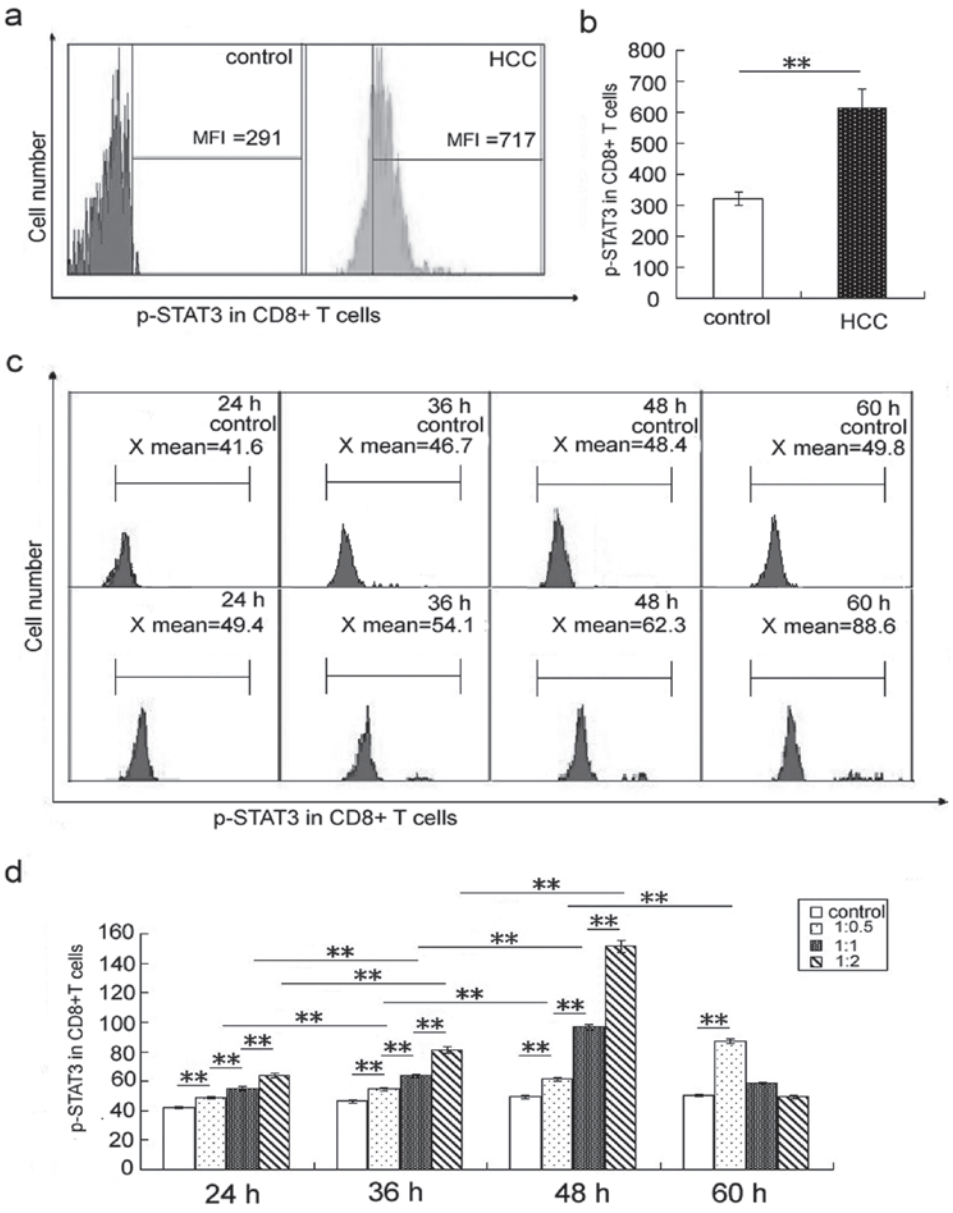

Figure 2. The level of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) is increased in peripheral CD8+ T cells in hepatocellular carcinoma (HCC) patients compared to healthy controls. (a) Representative images of flow cytometry analysis showing that the p-STAT3 level is increased in CD8+ T cells from the peripheral blood of a HCC patient compared to that of a healthy control (P<0.001). (b) The average mean fluorescence intensity (MFI) of p-STAT3 in CD8+ T cells from peripheral blood is higher in HCC patients than in healthy controls (P<0.001). (c) Flow cytometry analyses show that the level of p-STAT3 in CD8+ T cells from a 0.5:1 PBMCs:Huh7 cell co-culture is significantly higher than in PBMCs cultured alone, and increases with the time of co-culture (P<0.05). (d) The p-STAT3 level is increased in CD8+ T cells from PBMCs co-cultured with Huh7 cells as compared to PBMCs cultured alone. The p-STAT3 level increases with the time and with the increasing ratio of Huh7 cells to PBMCs (P<0.05). *P<0.05 and **P<0.01.

p-STAT3 level in peripheral CD4+ and CD8+ T cells of HCC patients and healthy subjects was measured using flow cytometry. The results showed that the expression of p-STAT3 in CD4+ T cells (Fig. 1a and b) and CD8+ T cells (Fig. 2a and b) is higher in HCC patients than in healthy controls.

Furthermore, the p-STAT3 level in CD4+ and CD8+ T cells from PBMCs co-cultured with Huh7 cells was higher than that of PBMCs cultured with medium alone (Figs. 1d and 2d). Expression of p-STAT3 in CD4+ and CD8+ T cells increased with the time of co-culture (Figs. 1c and d, 2c and d).

The IFN-γ level is decreased and the IL-4, IL-6 and IL-10 levels are increased in the serum of HCC patients. ELISA results showed that the IFN-γ levels are lower (Fig. 3a), whereas the IL-4 (Fig. 3b), IL-6 (Fig. 3c), and IL-10 (Fig. 3d) levels are higher in the serum of HCC patients than in controls.

Furthermore, the IFN-γ level was decreased (Fig. 3a and e), whereas the IL-4 (Fig. 3b and f), IL-6 (Fig. 3c and g), and IL-10 (Fig. 3d and h) levels were increased in the supernatants of Huh7 cells co-cultured with PBMCs compared to the controls. The IFN-γ level was decreased, while the IL-4, IL-6 and IL-10 levels increased with the time of co-culture and with the increasing ratio of PBMCs to Huh7 cells.

Correlation analysis between the the p-STAT3 level and the IFN-γ/IL-4 ratio, the IFN-γ, IL-4, IL-6 and IL-10 levels in

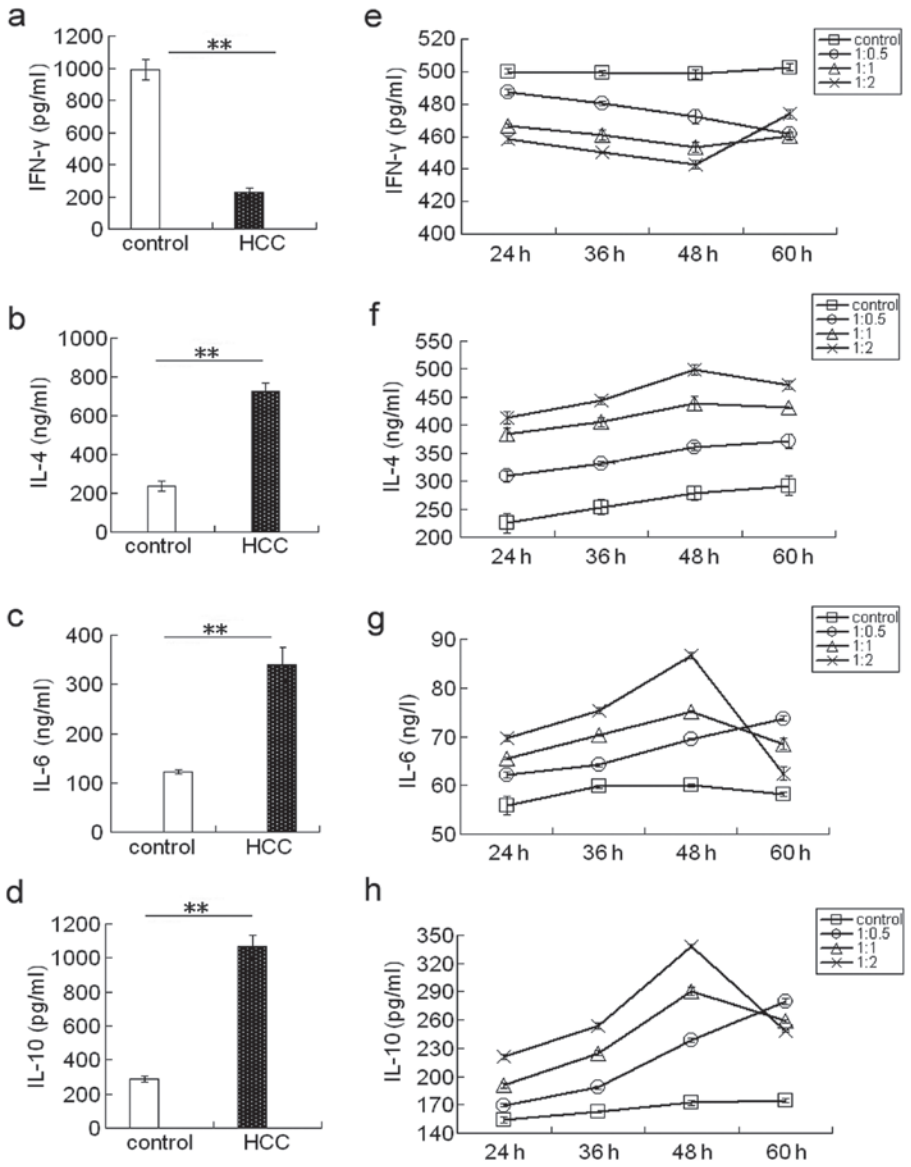

Figure 3. The interferon-γ (IFN-γ) level is decreased and the interleukin-4 (IL-4), IL-6 and IL-10 levels are increased in hepatocellular carcinoma (HCC) patients compared to healthy controls. The level of (a) IFN-γ is lower in HCC patients than in healthy controls (P<0.001), while the levels of (b) IL-4, (c) IL-6, and (d) IL-10 are all higher in the serum of HCC patients than in healthy controls (P<0.001). The level of (e) IFN-γ decreases with the time and with the increasing ratio of Huh7 cells to PBMCs in co-culture (P<0.01), while the levels of (f) IL-4, (g) IL-6 and (h) IL-10 increase with the time and with the increasing ratio of Huh7 cells to PBMCs in co-culture (P<0.01). *P<0.05 and **P<0.01.

peripheral CD4⁺ and CD8⁺ T cells from HCC patients. Spearman's correlation analysis was used to determine the correlation between the ratio of IFN-γ/IL-4, the levels of IFN-γ, IL-4, IL-6 and IL-10 and the p-STAT3 level in CD4⁺ and CD8⁺ T cells in patient samples and in the co-culture system. The ratio of IFN-γ/IL-4 and the level of IFN-γ negatively correlated to the level of p-STAT3 in peripheral CD4⁺ and CD8⁺ T cells (R=-0.923, P<0.001; R=-0.853, P<0.001; R=-0.926, P<0.001; R=-0.827 and P<0.001, respectively) (Fig. 4a, b, e and f). Similar correlations were found in data collected from CD4⁺ and CD8⁺ T cells from PBMCs co-cultured with Huh7 cells for 48 h (R=-0.870, P=0.001; R=-0.829, P=0.003; R=-0.916, P<0.001; R=-0.890, and P<0.001, respectively) (Fig. 4c, d, g and h). In addition, the IL-4, IL-6 and IL-10 levels positively correlated to the p-STAT3 level in peripheral CD4⁺ and CD8⁺ T cells from HCC patients (R=0.733, P<0.001; R=0.632, P=0.002; R=0.919, P<0.001; R=0.903, P<0.001; R=0.985, P<0.001; R=0.921 and P<0.001, respectively) (Fig. 4i, j, m, n, q and r) and from the co-culture system (R=0.947, P<0.001; R=0.934, P<0.001; R=0.980, P<0.001; R=0.966, P<0.001; R=0.955, P<0.001; R=0.938 and P<0.001, respectively) (Fig. 4k, l, o, p, s and t).

Discussion

HCC is one of the most common cancers worldwide; approximately 600,000 patients die of this disease each year in the world (29). Constitutively activated STAT3 has been shown to strongly correlate to the development and progression of a number of cancers, including HCC (30-34). A previous study suggested that the phosphorylation of STAT3 is upregulated in tumor-infiltrating immune cells, including dendritic cells, natural killer cells, and granulocytes, and that inhibiting STAT3 activity in hematopoietic cells triggers an intrinsic

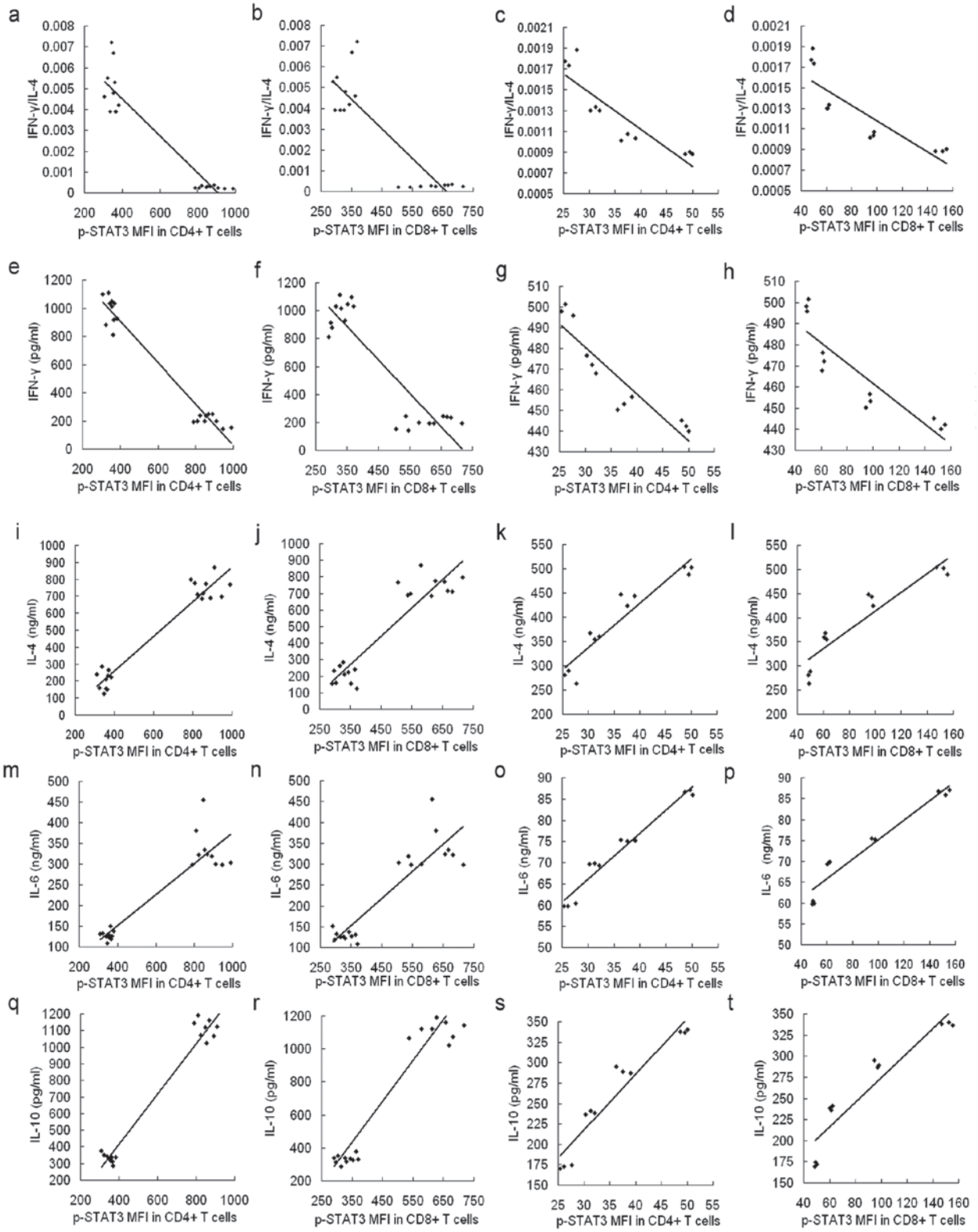

Figure 4. Correlations in the levels of interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-6, IL-10, and phosphorylated signal transducer and activator of transcription 3 (p-STAT3) in $CD4^+$ and $CD8^+$ T cells. (a and b) The ratio of IFN-γ/IL-4 and (e and f) the level of IFN-γ negatively correlate to the level of p-STAT3 in peripheral $CD4^+$ T cells and $CD8^+$ T cells from hepatocellular carcinoma (HCC) patients. The levels of (i and j) IL-4, (m and n) IL-6 and (q and r) IL-10 positively correlate to the p-STAT3 level in peripheral $CD4^+$ T cells and $CD8^+$ T cells from HCC patients. (c, d) The ratio of IFN-γ/IL-4 and (g and h) the levels of IFN-γ negatively correlate to the p-STAT3 level in $CD4^+$ and $CD8^+$ T cells from PBMCs co-cultured with Huh7 cells for 48 h. The levels of (k and l) IL-4, (o and p) IL-6 and (s and t) IL-10 positively correlate to the p-STAT3 level in $CD4^+$ and $CD8^+$ T cells from PBMCs co-cultured with Huh7 cells for 48 h.

immune-surveillance system that inhibits tumor growth and metastasis (13). The expression of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells is increased in active multiple sclerosis patients compared to healthy subjects, and the level of p-STAT3 is associated with the function of T cell responses in multiple sclerosis relapse cases (15). It is well established

that cellular immune responses, especially those mediated by $CD4^+$ and $CD8^+$ T cells, play a critical role in the surveillance of malignancy and the control of HCC progression (35). In this study, we found that the p-STAT3 level in $CD4^+$ and $CD8^+$ T cells from peripheral blood of HCC patients is higher compared to that of healthy controls, and that the p-STAT3 level in $CD4^+$ and $CD8^+$ T cells from PBMCs co-cultured with Huh7 cells is higher than that from PBMCs cultured with medium alone. In addition, we found that the expression of p-STAT3 increased with the time in co-culture and with the increasing ratio of Huh7 cells to PBMCs. These results suggest that the HCC microenvironment induces p-STAT3 expression in peripheral $CD4^+$ and $CD8^+$ T cells. High levels of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells may result in an abnormal immune response in HCC cells, or may decrease the levels of immune surveillance and induce immune tolerance to HCC. Therefore, these findings may enhance our understanding of the immunologic role of p-STAT3 in HCC progression.

Cytokines mediate numerous innate and adaptive immunity responses. We observed that the IFN-γ level is decreased and the IL-4 level is increased in the serum of HCC patients compared to healthy controls, in agreement with a previous study (36). Cytokine profiles indicating a deregulation of both Th1- and Th2-type cells have been previously associated with the development of HCC (18). Th1 cytokines (IFN-γ and IL-2) are related to cell-mediated immune responses in HCC (19). Our results showed that the ratio of IFN-γ/IL-4 and the IFN-γ level negatively correlate, while the level of IL-4 positively correlate to the p-STAT3 level in peripheral $CD4^+$ and $CD8^+$ T cells in patient samples and co-culture samples. These results indicate that the HCC microenvironment may induce the aberrant expression of p-STAT3 in peripheral $CD4^+$ and $CD8^+$ T cells, resulting in abnormal cytokine secretion and thereby, downregulating cell-mediated immune responses, which may overall contribute to the progression of HCC.

Cytokine signaling pathways involving transcription factors of the STAT family, and especially STAT3, play a key role in the pathogenesis of diseases. IL-6 was previously shown to be involved in STAT3 activation in HCC (22). In addition, STAT3 is constitutively activated in HCC (23). The serum level of IL-6 was also found to be elevated in HCC, and a higher serum level of IL-6 was associated with HCC progression (37). Here, we found that IL-6 expression is increased and positively correlates to the p-STAT3 level in the serum of HCC patients and in the supernatant of co-cultured PBMCs and Huh7 cells. This result indicates that IL-6 may activate STAT3 in $CD4^+$ and $CD8^+$ T cells of HCC patients. In addition, a previous study demonstrated that IL-10 is another cytokine that promotes STAT3 activation, the level of which is continuously increased in HCC (27). Another study showed that IL-10 induces p-STAT3 in PBMCs of healthy volunteers (38). In our study, the IL-10 level was also increased and positively correlated to the p-STAT3 level in the serum of HCC patients and in the supernatant of PBMCs co-cultured with Huh7 cells. Similar to IL-6, IL-10 may activate STAT3 in $CD4^+$ and $CD8^+$ T cells of the peripheral blood in HCC patients. Both cytokines may contribute to the immune tolerance observed in HCC patients.

The aberrant expression p-STAT3 in $CD4^+$ and $CD8^+$ T cells of the peripheral blood of HCC patients suggests that other factors in pathways that lie upstream of this transcription factor may result in the abnormal cell-mediated immune response to HCC cells and to their immune tolerance. Overall, our study may help to broaden the current view on the relationship between p-STAT3 expression in $CD4^+$ and $CD8^+$ T cells and HCC pathogenesis. It may also provide valuable data for the development of targets for therapeutic agents in the clinical treatment of hepatocellular carcinoma.

Acknowledgements

We thank Dr Jiang Wenjin, at the Yuhuangding Hospital, (Yantai, Shandong, China), for providing blood samples. This study was supported by funds from the Nature Science Foundation of Shandong Province (ZR2013HM050), the Foundation Project in Shandong Province Department of Education (J02K12), and the Science and Technology Planning Project of Binzhou City (2011ZC0917).

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