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Changes in immune profile affect disease progression in hepatocellular carcinoma

Farshid Fathi[1], Reza F Saidi[2], Hamid Reza Banafshe[3], Mohsen Arbabi[4], Majid Lotfinia[3] and Hossein Motedayyen[5]

Abstract

Objective: Hepatocellular carcinoma (HCC) as a chronic liver condition is largely associated with immune responses. Previous studies have revealed that different subsets of lymphocytes play fundamental roles in controlling or improving the development and outcome of solid tumors like HCC. Hence, this study aimed to investigate whether immune system changes were related to disease development in HCC patients. **Methods:** Peripheral blood mononuclear cells were isolated from 30 HCC patients and 30 healthy volunteers using Ficoll density centrifugation. The isolated cells were stained with different primary antibodies and percentages of different immune cells were determined by flow cytometry. **Results:** HCC patients indicated significant reductions in the numbers of $CD4^+$ cells, Tbet+IFNγ+cells, and GATA+IL-4+cells in peripheral blood in comparison with healthy individuals ($p < 0.05$). There was no significant change in IL-17+RORγt+cells between patient and healthy groups. In contrast, $Foxp3+CD127^{low}$cell frequency was significantly higher in patients than healthy subjects ($p < 0.0001$). The numbers of Th1, Th2, and Th17 cells were significantly lower in HCC patients than healthy control ($p < 0.0001$), although the reduction in Th2 cell numbers was not statistically significant. On the contrary, Treg percentage showed a significant increase in patients compared to healthy subjects ($p < 0.0001$). Other data revealed that Th1, Th2, and Th17 cell frequencies were significantly higher in healthy individuals than patients with different TNM stages of HCC, with the exception of Th2 in patients with stage II HCC ($p < 0.01–0.05$). Treg percentage was significantly increased in patients with different TNM stages ($p < 0.0001$). Among all $CD4^+$ T cells, the frequency of Th2 cell was significantly associated with TNM stages of HCC ($p < 0.05$). **Conclusion:** Our data provide further evidence to show that immune changes may participate in determining HCC progression and disease outcome. However, it should be mentioned that more investigations are needed to clarify our results and explain possible impacts of other immune cells on the pathogenesis of HCC.

Keywords

hepatocellular carcinoma, cellular immunity, T helper cells, immune system

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[1]Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[2]Division of Transplant Services, Department of Surgery, SUNY Upstate Medical University Syracuse, Syracuse, NY, USA
[3]Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran
[4]Department of Medical Parasitology, Kashan University of Medical Sciences, Kashan, Iran
[5]Autoimmune Diseases Research Center, Kashan University of Medical Sciences, Kashan, Iran

Corresponding author:
Hossein Motedayyen, Autoimmune Diseases Research Center, Shahid Beheshti Hospital, Kashan University of Medical Sciences, 5th Kilometer of Ravand Road, Kashan 8717765761, Iran
Email: hmotedayyen@gmail.com

Introduction

Liver cancer accounts for the sixth most common cancer and the second leading cause of cancer-related deaths worldwide.[1-3] Primary liver cancer consists of a heterogeneous group of malignancies with no metastasis to the liver from other sites. Hepatocellular carcinoma (HCC) is considered as the most frequent histologic type of primary liver cancer, originating from the epithelial liver cells known as hepatocytes.[4] Hepatocellular carcinoma is responsible for approximately 85–90% of all primary liver cancers.[5] Importantly, only a small number of patients with HCC are diagnosed at early stages when curative approaches are effective.

Liver resection and transplantation represent the gold standard approaches for the treatment of patients with HCC. However, the survival rate is poor even with the best treatment. There are great differences between incidence and mortality rates of HCC, highlighting the uneven distribution of major risk factors.[2] Hepatocellular carcinoma development depends on various risk factors such as cirrhosis and chronic inflammation.[5] Underlying chronic necroinflammation, the induction of fibrosis and/or subsequent cirrhosis, accounts for nearly 90% of HCC development. [5]

The liver harbors a wide variety of innate immune cells, including NK cells, macrophages, NKT cells, neutrophils, dendritic cells, γδT cells, innate lymphoid cells,[6,7] and adaptive immune cells, including T cells and B cells, affecting the status of immune tolerance, tumor progression, and pathogen clearance.[8,9] Chronic liver injury, which is mainly caused by viral infections, alcohol, and liver fat accumulation, leads to the activation of resident and infiltrating immune cells, which in turn results in progressive inflammation.[10] Complex interactions occurred among immune cells after liver injury regulate liver regeneration and repair. Defect(s) in control activated immune mechanisms results in pathological inflammation and disrupted tissue homeostasis marked by progressive fibrosis development. [11]

In chronic necroinflammation, constant cell death, compensatory regeneration, and non-parenchymal cell activation, together with a changed immune response, promote liver fibrosis and tumorigenesis.[8]

Hepatocellular carcinoma is a prototypical inflammation-related cancer which the immune microenvironment has a pivotal role in disease pathogenesis.[12] Inflammatory tumor microenvironment was demonstrated to be correlated to higher survival in patients with HCC.[13] Pro-inflammatory cytokines, IL-6, and TNF-α, play fundamental roles in the development and progression of HCC through inducing some transcription factors, such as STAT3 and NF-κB.[8,14] In addition, animal model of HCC has demonstrated that hepatocyte-specific inhibition of STAT3 leads to prevent HCC development.[15] It is documented that some tumors are largely infiltrated through different cells from the immune system showing inflammatory conditions in non-neoplastic tissues.[16] Previous studies have indicated that tumor-infiltrating lymphocytes, a type of immune cells, have an important role in recognizing and killing cancer cells through the migration from the bloodstream into various tumors.[17] Of note, the presence of immune infiltrates in fully developed HCC is related to a better prognosis, which is probably due to more effective antitumor immunity.[18,19] Functional interaction between tumor-infiltrating T cells and B cells was found to be correlated to an enhanced local immune activation and better prognosis for patients with HCC.[20] In addition, changes in circulating lymphocyte numbers may be useful for monitoring the immunological status in subjects with high HCC risk and targeted therapy of HCC.[21] In the current study, we investigated the frequencies of different subsets of lymphocytes in peripheral blood from HCC patients to determine how changes in the immune system have critical roles in determining HCC progression and disease outcome.

Materials and methods

Subjects

This work is an analytical observational (case-control) study performed on 30 patients with HCC, who were recruited among those referred to a surgery center of Sina hospital, Tehran, Iran, from May 2019 to June 2020, and 30 age- and sex-matched healthy volunteers without any health problems. Disease diagnosis was performed by the specialist according to the eighth edition of the Cancer Staging Manual by the American Joint Committee on Cancer (AJCC).[22] HCC was histologically confirmed following radiological and serological investigations. Blood sampling was performed after disease diagnosis and before any treatment. Patients with HCC were negative for metastasis and other malignancies. The study was approved by the Ethics Committee of Kashan University of Medical Sciences (ethic code: IR. KAUMS.-MEDNT.REC.1398.125) and performed based on the declaration of Helsinki. The informed consent was obtained from all participants prior to study initiation. Based on the SD values reported in previous studies,[23,24] sample sizes were calculated by the following statistical formula

$$n = \frac{(Z\alpha + Z\beta)^2 \times \left(S1^2 + S2^2\right)}{(m1 - m2)^2}$$

α (study accuracy) = 95%
β (study power) = 80%
Mean difference between group 1 and 2 (m1–m2) = 0.95
Zα = 1.96
Zβ = 0.83
S1 = 1.3
S2 = 1.2

Sample collections

EDTA-treated samples (10 mL) were obtained from participants. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque centrifugation following the manufacturer's guidelines (Lymphodex, Germany). The isolated cells were washed several times with phosphate-buffered saline (PBS) at 300 × g for 10 min. Cell count was done using a hemocytometer and its viability was determined by trypan blue dye exclusion.

Flow cytometry

To determine the percentages of $CD4^+$ cell, Tbet+IFNγ+ cell, GATA+IL-4+ cell, IL-17+RORγt+ cell, Foxp3+$CD127^{low}$ cell, Th1 cell, Th2 cell, Th17 cell, and Treg in peripheral blood of patient and healthy groups, one set of the isolated cells was stimulated by phorbol 12-myristat 13-acetate (PMA, 5 ng/mL, Sigma)/ionomycin (I, 1 μmol/L, Sigma) and incubated at the presence of Brefeldin A (BFA, 10 μg/mL final concentration) as described in previous studies.[25,26] After stimulation of T-cell cytokine production, the stimulated and unstimulated cells were stained. Isotype-matched control antibodies were used as negative controls. To stain some intracellular molecules, the cells were fixed and then permeabilized according to the manufacturer's guideline (eBiosciences, USA). Briefly, single cell suspensions were washed twice with PBS, mixed with 1 × working solution of fixation buffer, and incubated in the dark for 30 min at room temperature. The fixed cells were washed twice with 1 × working solution of permeabilization buffer. Afterwards, the cells were stained with different monoclonal antibodies or matching isotype control antibodies in permeabilization buffer for 25 min at 4°C. The cells were washed twice with PBS and centrifuged at 300 ×g for 10 min at room temperature. The frequencies of the stained cells were determined by the FACSCalibur system (Becton Dickinson, San Jose, CA) and then analyzed by the FlowJo software (v10.1, FlowJo, Ashland, OR, USA). The cell markers used to measure the percentages of Th1 cell, Th2 cell, Th17 cell, and Treg are indicated in Table 1. To measure the percentage of each cell population, lymphocyte population was gated using forward and side scatters to eliminate debris or dead cells from cell analyses. The gated cell population was used to determine the percentages of $CD4^+$ cells, Tbet+IFNγ+ cell, GATA+IL-4+ cell, IL-17+RORγt+ cell, and Foxp3+CD127low cell. Afterwards, the $CD4^+$ cell population was gated to assess the frequencies of Th1 cells, Th2 cells, Th17 cells, and Tregs. The monoclonal and their isotype control antibodies are revealed in Table 2.

Table 1. The cell markers used to analyze the frequencies of $CD4^+$ T cell subsets by flow cytometry.

T Cell subsets	Markers
Th1 cells	$CD4^+$, Tbet+, and IFNγ+
Th2 cells	$CD4^+$, GATA3+, and IL-4+
Th17 cells	$CD4^+$, RORγt+, and IL-17+
Tregs	$CD4^+$, FoxP3+, and CD127low

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) and the mean ± standard error of the mean (SEM) after analyzing by GraphPad Prism 6 (GraphPad software, San Diego, CA). The groups with normal distributions were compared by unpaired t-test, while Mann–Whitney test were used to compare those with non-normal distributions. p value < 0.05 was considered statistically significant.

Results

Patient descriptions

Thirty subjects with HCC (15 males and 15 females, mean age: 62.19 ± 2.75, mean ± standard deviation, range: 56–66 years) were participated in the study (Table 3). All patients had a primary tumor and 50% of them were in stage IIIA of HCC (Table 3). Table 3 shows the demographic and other features of patients and healthy individuals.

Table 2. Antibodies used to determine immune changes in HCC patients by flow cytometry.

Fluorochrome/Antibody	Isotype	Clone	Company (All from USA)
FITC anti-human CD4 antibody	Mouse IgG1, κ	SK3	BioLegend
PE/Cyanine5 anti-human CD127 (IL-7Rα)antibody	Mouse IgG1, κ	A019D5	BioLegend
PE anti-T-bet antibody	Mouse IgG1, κ	4B10	BioLegend
PerCP/Cyanine5.5 anti-human IFN-γ antibody	Mouse IgG1, κ	4S.B3	BioLegend
PE anti-GATA3 antibody	Mouse IgG2b, κ	16E10A23	BioLegend
PerCP/Cyanine5.5 anti-human IL-4 antibody	Rat IgG1, κ	MP4-25D2	BioLegend
PerCP/Cyanine5.5 anti-human IL-17A antibody	Mouse IgG1, κ	BL168	BioLegend
PE anti-human FoxP3 antibody	Mouse IgG1, κ	206D	BioLegend
Anti-Human/Mouse ROR gamma (t) PE antibody	Rat IgG2a, k	AFKJS-9	eBioscience

Table 3. The clinicopathological characteristics of participants.

	Patients group (n = 30)	Control group (n = 30)
Age (mean ± SD.)	62.19 ± 2.75	61.31 ± 4.21
Gender	Female: 15 (50%) Male: 15 (50%)	Female: 15 (50%) Male: 15 (50%)
Tumor type	HCC: 30 (100%)	
TNM*	T2: 9 (30%); T3: 15 (50%); T4: 6	———
Primary tumor	(20%)	
Regional lymph nodes	0 (0.0%)	
Distant metastasis	0 (0.0%)	
TNM stages	II: 9 (30%) IIIA: 15 (50%) IIIB: 6 (20%)	———
BCLC stages	Stage 0: 13 (43.4%) Stage A: 14 (46.6%) Stage B: 3 (10%)	———
CPT class	Class A: 27 (90%) Class B: 3 (10%)	
Smoking history	8 (26.6%)	7 (23.3%)
Etiology	Hepatitis C: 11 (36.6%) Hepatitis B: 7 (23.4%) Hepatitis B and Hepatitis C: 2 (6.6%) Fatty liver disease (ALD and NAFLD): 8 (26.7%) Unknown: 2 (6.6%)	0 (0.00%)
Alcohol consumption	6 (20%)	5 (16.6%)

* TNM staging system based on AJCC 8th edition.
Note: HCC: Hepatocellular carcinoma; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; BCLC: Barcelona Clinic Liver Cancer; CPT: Child-Pugh-Turcotte.

Circulating CD4+cell, Tbet+IFNγ+cell, GATA+IL-4+cell, IL-17+RORγt+cell, and Foxp3+CD127^{low} cell percentages in HCC patients

To assess the frequencies of CD4+cells, Tbet+IFNγ+cells, GATA+IL-4+cells, IL- 17+RORγt+cells, and Foxp3+C-D127^{low} cells among the circulating lymphocytes, the percentages of these cells in the gated lymphocytes were measured. As shown in Figure. 1,A–C and F–H, HCC patients showed significant reductions in the numbers of CD4^{+} cells, Tbet+IFNγ+cells, and GATA+IL- 4+cells in peripheral blood in comparison with healthy subjects (p < 0.05). There was no significant change in IL-17+RORγt+cells between patient and healthy groups (Figure. 1D and I). In contrast, Foxp3+CD127^{low}cells had a significant increase in patients compared to healthy individuals (p < 0.0001, Figure. 1 E and J).

Circulating Th1, Th2, Th17 cell, and Treg percentages in HCC patients

To evaluate the status of adaptive immunity in HCC patients, the numbers of circulating Th1, Th2, Th17 cells, and Tregs in patients were compared to those of healthy subjects. Th1 and Th17 cell numbers were significantly lower in HCC subjects than healthy controls (p < 0.0001, Figure. 2A, C, E, and G). Although a reduction was observed in Th2 cell percentage, it was not statistically significant (Figure. 2 B and F)

(Figure. 2 C and D). On the contrary, Treg percentage showed a significant increase in patients compared to healthy individuals (p < 0.0001, Figure. 2 G and H).

Correlations of immune changes with prognosis and TNM stages of HCC

To determine the relationships of immune changes with disease prognosis and stages, the percentages of Th1, Th2, Th17 cells, and Tregs in patients with different TNM stages of HCC and healthy subjects were investigated. The results revealed that the frequencies of Th1 and Th17 cells were significantly higher in healthy individuals than patients with different TNM stages of disease, unlike the reduced number of Tregs in healthy subjects (p < 0.0001–0.05, Figure. 3A, C, D, E, G, and H). The percentage of Th2 cell was significantly reduced in patients with stages IIIA and IIIB, however, there was no significant difference in Th2 cell number between patients with stage II HCC and healthy subjects (p < 0.01–0.05, Figure. 3 B and F). The frequency of Th2 cell was significantly associated with TNM stages of HCC (p < 0.05, Figure. 3 B and F), while

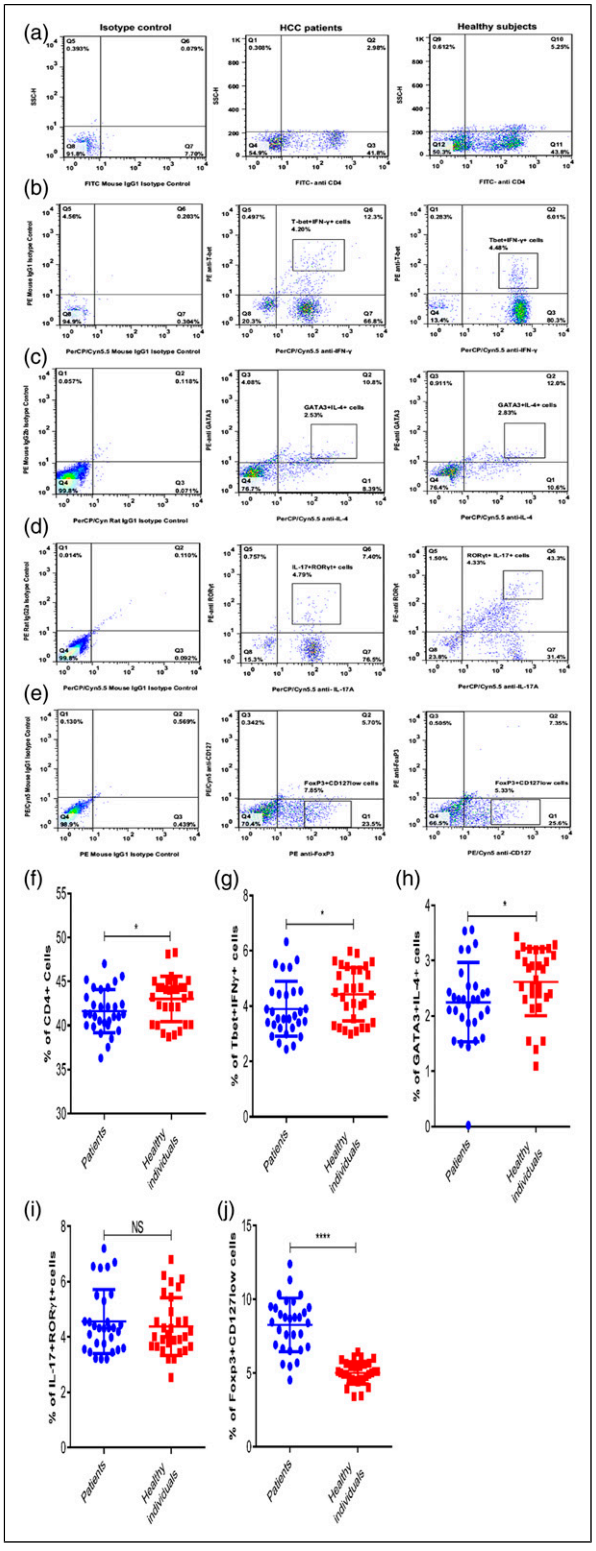

Figure 1. The frequencies of $CD4^+$, Tbet+IFNγ+, GATA3+IL-4+, RORγt+IL-17+, Foxp3+CD127low cells in peripheral blood of patient and healthy subjects. PBMCs of Hepatocellular carcinoma patients (n = 30) and healthy controls (n = 30) were stained with monoclonal antibodies. The frequencies of the stained cells were measured using flow cytometry (A, B, C, D, and E) and then analyzed (F, G, H, I, and J). Data are shown as mean ± SD. Two groups with non-normal distributions were compared by Mann–Whitney test, while unpaired t-test was used in case of normal distributions. ****$p < 0.0001$, *$p < 0.05$.

this correlation was not observed in the percentages of Th1, Th17 cells, and Tregs (Figure 3 A, C, D, E, G, and H).

Discussion

Hepatocellular carcinoma, as a chronic liver condition, has high morbidity and mortality throughout the world.[27,28] Immune agents of the liver take part in the inflammatory damages, liver fibrosis, and deteriorating toward HCC. Among different agents of the immune system, lymphocytes have a potential capacity for elevating or counteracting the development of solid tumors like HCC.[24] Hence, we evaluated the various subgroups of circulating lymphocytes in peripheral blood from HCC patients and normal individuals.

Regarding the role of $CD4^+$ cells in the initiation of inflammatory reactions, the frequency of these cells were studied. Our results revealed a significant reduction in $CD4^+$ cell number in peripheral blood of HCC patients compared to the control group. In agreement with our findings, some studies have indicated the decreased frequency of $CD4^+$ cells in HCC cases.[23,24] It is reported that $CD4^+$ cell has indispensable roles in the initial stages of liver damages. These cells can trigger cytokine responses involved in liver reactions which lead to liver injury.[29,30] In addition, $CD4^+$ cells produce IFNγ and IL-4 that have detrimental effects on the liver, such as pro-inflammatory cytokine inductions, and hepatocyte apoptosis. [31-33]

In the next step, the frequencies of other immune cells were determined. Similar to the frequency of $CD4^+$ cells, significant reductions were observed in the numbers of Tbet+IFNγ+ cells and GATA+IL-4+ cells. Although some animal studies have revealed the increased expression of IFNγ in HCC,[34] our data were consistent with other reports showing the reduced levels of IFNγ and IL-4 in HCC cases.[35,36] Lin et al. reported a significant reduction in Tbet expression which plays a pivotal role in regulating cytokine productions, especially IFNγ.[37,38] Gao et al. revealed diminished production of IFN-γ and TNF-α in HCC subjects compared to a normal group.[39] Similar studies on other cancers have mentioned decreased expression of IFN-γ and T-bet in ovarian carcinoma.[40] However, there are some reports pointing to the elevated level of IL-4 in metastatic HCC patients and increased expression of GATA3 in liver cancer patients.[33] Weidong et al. indicated the increased expression of IL-4, IL-10, and GATA3 in ovarian carcinoma.[40] It is thought that changes in the numbers of

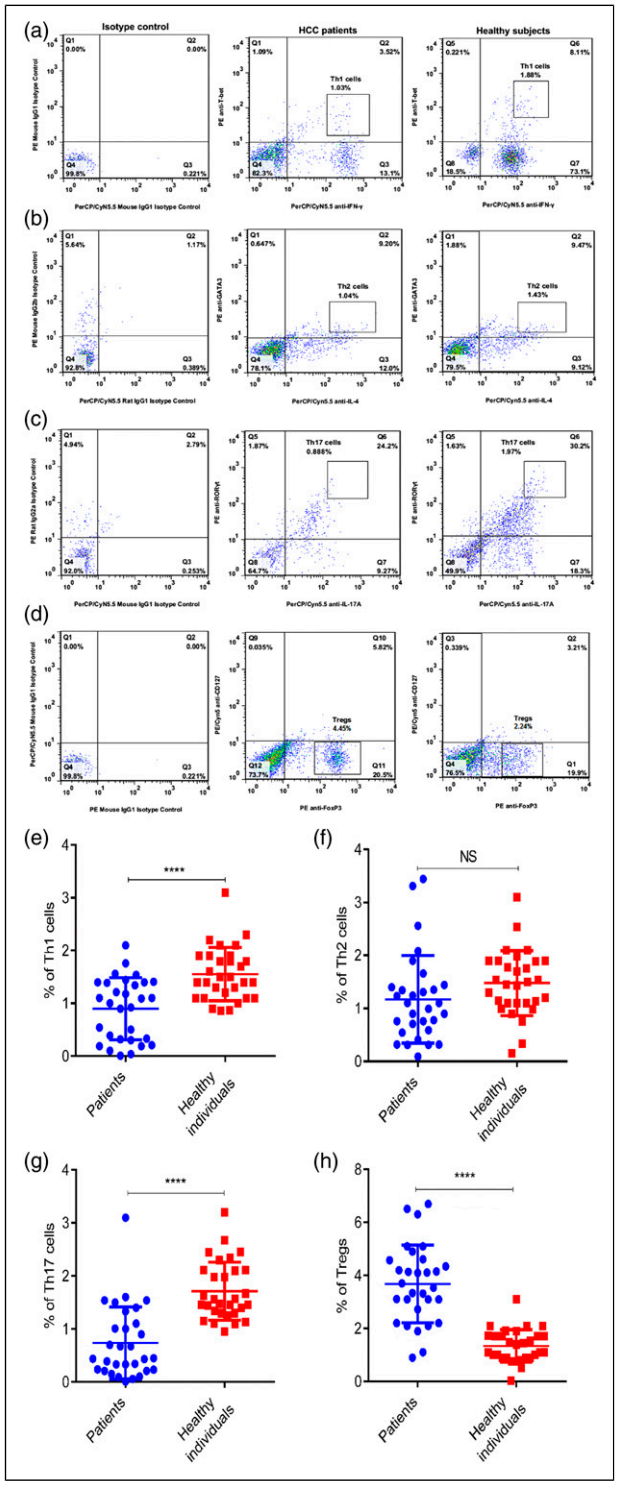

Figure 2. The frequencies of circulating different $CD4^+$ T subsets of patient and healthy subjects. PBMCs of Hepatocellular carcinoma patients (n = 30) and healthy controls (n = 30) were stained with monoclonal antibodies. The numbers of Th1, Th2, Th17 cells, and Tregs were measured using flow cytometry (A, B, C, and D) and then analyzed (E, F, G, and H). Values are shown as mean ± SD. Unpaired t-test and Mann–Whitney test were used to compare two groups with normal and non-normal distributions, respectively. ****$p < 0.0001$.

Tbet+IFNγ+ and GATA+IL-4+ cells may participate in HCC development. To support this notion, it is revealed that IFNγ exerts a protective impact on HCC progression through stimulating apoptosis of cancer cells and activation of macrophages and T lymphocytes.[41] Furthermore, some reports have revealed that IL-4 is linked to cell survival and proliferation in some cancers, such as breast, lung, and ovarian cancer.[42,43] Others have revealed that IL-4 along with other mediators, such as IL-13, contributes to polarization of M2 macrophage, which has anti-inflammatory and is associated with development of different tumors.[44] However, there are some studies indicating GATA3, as a transcription factor for IL-4 expression, plays an important role in inhibiting HCC progression.[45,46] These discrepancies in the roles of IL-4 and GATA3 in HCC propose that additional studies are required to explain the precise impacts of these agents on developing or inhibiting HCC.

In an attempt to determine the frequencies of IL-17+RORγt+ and Foxp3+$CD127^{low}$cells in HCC subjects, the percentages of these cells were investigated. Our data indicated no significant change in IL-17+RORγt+ cells in HCC subjects compared to healthy individuals. Besides, we observed that Foxp3+$CD127^{low}$ cell frequency was significantly increased in HCC subjects. These findings were consistent with the results of the studies revealing a significant increase in FoxP3 expression in HCC patients and HCC mice models.[34,47] Furthermore, Qiu et al. reported that patients with non-small cell lung cancer experienced an elevated expression of CD127 in comparison with healthy subjects.[48] Another study has indicated that the increased expression of CD127 was related to disease progression in lung cancer patients.[49] Based on the study conducted by Lin et al., the mRNA levels of FoxP3 and RORγt are increased in HCC cases compared to healthy subjects.[37] Regarding the fact that Foxp3, IL-17 and its transcription factor, RORγt, have critical roles in the development and outcome of various cancers,[50,51] it seems that the reduced numbers of IL-17+RORγt+ and increased frequencies of Foxp3+$CD127^{low}$cells may associate with HCC progression.

It is needless to say that different cells from the immune system have pivotal roles in inhibiting tumor growth and preventing tumor progression, however, some evidence suggests that pro-inflammatory cytokines produced by Th1 cells such as IL-1α, IFN-γ, and TNF-α, may participate in tumor development through potentiating angiogenesis, metastasis, and invasion.[52-54] In addition to the role of Th1 cell in HCC, it is shown that Th17 cells can elevate HCC growth through stimulating angiogenesis and secreting some pro-inflammatory cytokines like IL-22, which induces the proliferation of liver tumor cells.[55] Furthermore, some studies have shown that IL-4, IL-8, and IL-10 secreted from Th2 cells can exert anti-inflammatory impacts and thereby contribute to tumor progression.[56]

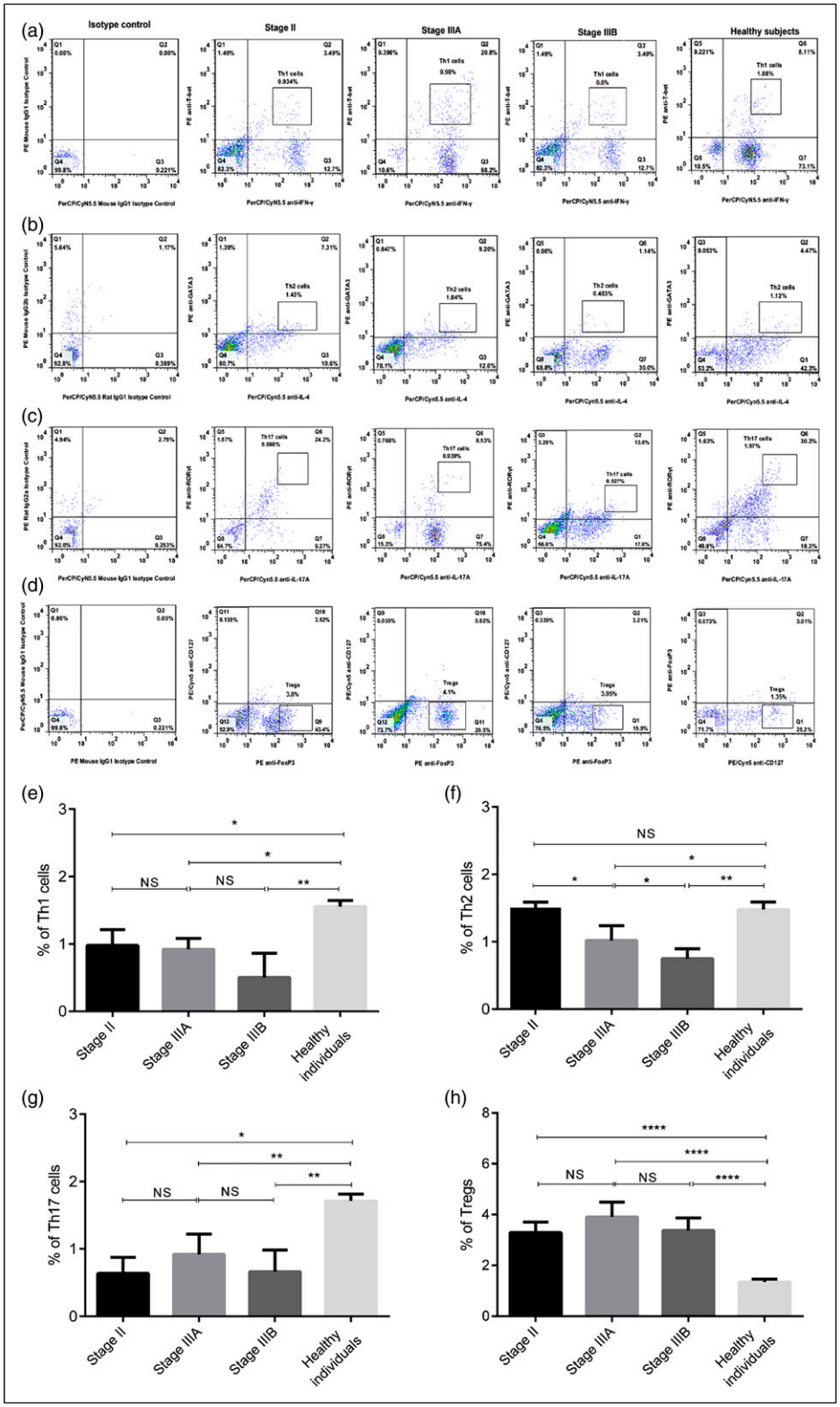

Figure 3. The frequencies of immune cells in different TNM stages of Hepatocellular carcinoma (HCC). The percentages of Th1, Th2, Th17 cells, and Tregs in PBMCs of HCC patients with stage II ($n = 9$), stage IIIA ($n = 15$), and stage IIIB ($n = 6$) and healthy controls ($n = 30$) were investigated by using flow cytometry (A, B, C, and D) and then analyzed (E, F, G, and H). Data are shown as mean ± SEM. Unpaired t-test and Mann–Whitney test were used to compare two groups with normal and non-normal distributions, respectively. ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$.

To discover possible impacts of immune cells in HCC subjects, the frequencies of Th1 cells, Th2 cells, Th17 cells, and Tregs were studied in patient and healthy individuals.[57] Our data indicated that Th1, Th2, and Th17 cell numbers were considerably decreased, although the reduced percentage of Th2 cells was not statistically significant. Furthermore, classification of patients with different TNM stages of HCC revealed that patients with different TNM stages had significant reductions in the frequencies of Th1, Th2, and Th17 cells in comparison with healthy subjects, with the exception of Th2 cell number in patients with stage II HCC. In addition to these observations pointing to the possible roles of immune changes in HCC prognosis, other data indicated that there was a significant correlation between the percentage of Th2 cells and TNM stages of HCC. However, the frequencies of Th1 and Th2 cells were not associated with disease stages, due perhaps to low sample size used in the study. In contrast with these findings, it is reported that the numbers of Th1 and Th17 cells are elevated in HCC compared to healthy individuals.[58] Other studies have indicated the increased levels of Th1 cytokines in HCC.[56] Moreover, Foerster et al. indicated the increased frequency of Th2 cells and the decreased numbers of Th17, cytotoxic cells, and Tγδ cells in HCC tissue compared to the normal tissue.[59] These discrepancies among our data and other studies may be attributed to the type of samples, subjects with different HCC stages, methods and sample size used in different studies. Nonetheless, the results of the present study along with previous reports suggest that an imbalance in the immune system and alterations in immune cell frequencies have fundamental roles in HCC developments.

Other results of the present study demonstrated a significant increase in circulating Treg number in patients compared to healthy subjects. Moreover, patients with different TNM stages of HCC showed significant increases in Treg frequencies, although there was no significant correlation between Treg numbers and TNM stages of disease. In agreement with this finding, there are studies showing the elevated number of Treg in HCC patients.[60] These cells have well-known roles in suppressing different cells from the immune system and inhibiting productions of pro-inflammatory cytokines leading to tumor progression via the increment of the tumor cell escape from the immune system and promotion of tumor growth.[61-66]

Conclusion

Taken together, the results of this study in along with other reports provide evidence to show that changes and imbalance in the immune system have critical roles in determining HCC progression and disease outcome. However, a limitation of the present study was the lack of assessments of other efficient immune cells, such as NK cells, B lymphocytes, macrophages, and their possible roles in the pathogenesis of the disease. Another limitation is low sample size selected for this study. Therefore, it should be mentioned that more robust studies with larger sample size are needed to determine the numbers of different immune cells and explain their possible impacts of the pathogenesis of HCC.

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Ethics approval

Ethical approval for this study was obtained from the Ethics Committee of Kashan University of Medical Sciences (ethic code: IR.KAUMS.MEDNT.REC.1398.125).

Informed consent

Written informed consent was obtained from all participants prior to study initiation.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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