Changes in immune profile affect disease progression in hepatocellular carcinoma

Farshid Fathi1, Reza F Saidi2, Hamid Reza Banafshe3, Mohsen Arbabi4, Majid Lotfinia3 and Hossein Motedayyen5

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+CD127low 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

Date received: 16 June 2021; accepted: 17 January 2022
Introduction

Liver cancer accounts for the sixth most common cancer and the second leading cause of cancer-related deaths worldwide.\(^1\) 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.\(^7\) 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, γδ T 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.\(^13,14\) 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

\[
n = \frac{(Z_\alpha + Z_\beta)^2 \times (S_1^2 + S_2^2)}{(m_1 - m_2)^2}
\]

\(\alpha\) (study accuracy) = 95%

\(\beta\) (study power) = 80%

Mean difference between group 1 and 2 (\(m_1 - m_2\)) = 0.95

\(Z_\alpha = 1.96\)

\(Z_\beta = 0.83\)

\(S_1 = 1.3\)

\(S_2 = 1.2\)
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, 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.

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 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 |

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 | BI168 | BioLegend |
| PE anti-human FoxP3 antibody | Mouse IgG1, κ | 206D | BioLegend |
| Anti-Human/Mouse ROR gamma (γ) PE antibody | Rat IgG2a, κ | AFKJS-9 | eBioscience |
Circulating CD4+cell, Tbet+IFNγ+cell, GATA+IL-4+cell, IL-17+RORγt+cell, and Foxp3+CD127low 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+CD127low 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+CD127low cells had a significant increase in patients compared to healthy individuals (p < 0.0001, Figure 1 E and J).

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 IIA 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 3B and F). The frequency of Th2 cell was significantly associated with TNM stages of HCC (p < 0.05, Figure 3B and F), while

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%)        | Female: 15 (50%)       |
|                                | Male: 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%) | 0 (0.00%)              |
|                                | 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%)       |                        |
| 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.
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 countering 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 IFN\(\gamma\) 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+IFN\(\gamma\)+ cells and GATA+IL-4+ cells. Although some animal studies have revealed the increased expression of IFN\(\gamma\) in HCC,\(^{34}\) our data were consistent with other reports showing the reduced levels of IFN\(\gamma\) 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 IFN\(\gamma\).\(^{37,38}\) Gao et al. revealed diminished production of IFN-\(\gamma\) and TNF-\(\alpha\) in HCC subjects compared to a normal group.\(^{39}\) Similar studies on other cancers have mentioned decreased expression of IFN-\(\gamma\) 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 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.
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+CD127low 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+CD127low 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+CD127low 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 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.
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 II A (n = 15), and stage II B (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 < 0.001, **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. 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. Other studies have indicated the increased levels of Th1 cytokines in HCC. Moreover, Foerster et al. indicated the increased frequency of Th2 cells and the decreased numbers of Th17, cytotoxic cells, and γδ T cells in HCC tissue compared to the normal tissue. 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. 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.

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.

Acknowledgments

The authors would like to thank all individuals who helped to the study.

Ethics approval

Ethical approval for this study was obtained from the Ethics Committee of Kashan University of Medical Sciences (ethic code: IR.KAU.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.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Kashan University of Medical Sciences (grant number: 98186).

References

1. Bray F, Ferlay J, Soerjomataram I, et al. (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians 68(6): 394–424.
2. Research, EOF, Cancer, TO and Liver, EAFTSOT (2012) EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. Journal of hepatology 56(4): 908–943.
3. Siegel RL, Miller KD and Jemal A (2019) Cancer statistics, 2019. CA: A Cancer Journal for Clinicians 69(1): 7–34.
4. Pang RWC, Joh JW, Johnson PJ, et al. (2008) Biology of hepatocellular carcinoma. Annals of Surgical Oncology 15(4): 962–971.
5. Llovet JM, Zucman-Rossi J, Pikarsky E, et al. (2016) Hepatocellular carcinoma. Nature reviews Disease primers 2: 16018. Epub 2016/05/10. PubMed PMID: 27158749. DOI: 10.1038/nrdp.
6. Li N and Hua J (2017) Immune cells in liver regeneration. Oncotarget 8(2): 3628–3639.
7. Liaskou E, Wilson DV and Oo YH (2012) Innate immune cells in liver inflammation. *Mediators of Inflammation* 2012: 949157.
8. Ringelhan M, Pfister D, O’Connor T, et al. (2018) The immunology of hepatocellular carcinoma. *Nature Immunology* 19(3): 222–232.
9. Crispe IN (2009) The liver as a lymphoid organ. *Annual Review of Immunology* 27: 147–163.
10. Irving KM, Ratnasekera I, Powell EE, et al. (2019) Causes and consequences of innate immune dysfunction in cirrhosis. *Frontiers in Immunology* 10: 293.
11. Robinson MW, Harmon C and O’Farrellly C (2016) Liver immunology and its role in inflammation and homeostasis. *Cellular & Molecular Immunology* 13(3): 267–276.
12. Hernandez-Gea V, Toffanin S, Friedman SL, et al. (2013) Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 144(3): 512–527.
13. Chew V, Tow C, Teo M, et al. (2010) Inflammatory tumour microenvironment is associated with superior survival in hepatocellular carcinoma patients. *Journal of Hepatology* 52(3): 370–379.
14. Subramaniam A, Shanmugam MK, Perumal E, et al. (2013) Potential role of signal transducer and activator of transcription (STAT)3 signaling pathway in inflammation, survival, proliferation and invasion of hepatocellular carcinoma. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1835(1): 46–60.
15. He G, Yu G-Y, Temkin V, et al. (2010) Hepatocyte IKKβ/NF-kB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation. *Cancer Cell* 17(3): 286–297.
16. DVORAK HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England Journal of Medicine* 315: 1650–1659.
17. Shirabe K, Motomura T, Muto J, et al. (2010) Tumor-infiltrating lymphocytes and hepatocellular carcinoma: pathology and clinical management. *International Journal of Clinical Oncology* 15(6): 552–558.
18. Wada Y, Nakashima O, Kutami R, et al. (1998) Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. *Hepatology* 27(2): 407–414.
19. Xu X, Tan Y, Qian Y, et al. (2019) Clinicopathologic and prognostic significance of tumor-infiltrating CD8+ T cells in patients with hepatocellular carcinoma: a meta-analysis. *Medicine* 98(2): e13923.
20. Garnelo M, Tan A, Her Z, et al. (2017) Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. *Gut* 66(2): 342–351.
21. Liu H-Z, Deng W, Li J-L, et al. (2016) Peripheral blood lymphocyte subset levels differ in patients with hepatocellular carcinoma. *Oncotarget* 7(47): 77558–77564.
22. Chun YS, Pawlik TM and Vauthey J-N (2018) 8th Edition of the AJCC cancer staging manual: pancreas and hepatobiliary cancers. *Annals of Surgical Oncology* 25(4): 845–847.
23. Jia Y, Zeng Z, Li Y, et al. (2015) Impaired function of CD4+ T follicular helper (Tfh) cells associated with hepatocellular carcinoma progression. *PLoS One* 10(2): e0117458.
24. Chaoul N, Mancarella S, Lupo L, et al. (2020) Impaired anti-Tumor T cell response in hepatocellular carcinoma. *Cancers* 12(3): 627.
25. Picker LJ, Singh MK, Zdraveski Z, et al. (1995) Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86: 1408.
26. Yin Y, Mitson-Salazar A and Prussin C (2015) Detection of intracellular cytokines by flow cytometry. *Current Protocols in Immunology* 110(1): 6.
27. Axley P, Ahmed Z, Ravi S, et al. (2018) Hepatitis C virus and hepatocellular carcinoma: a narrative review. *Journal of Clinical and Translational Hepatology* 6(1): 79–84.
28. Macek Jilkova Z, Kurma K and Decaens T (2019) Animal models of hepatocellular carcinoma: the role of immune system and tumor microenvironment. *Cancers* 11(10): 1487.
29. Elsaed WM (2019) Amygdalin (vitamin B17) pretreatment attenuates experimentally induced acute autoimmune hepatitis through reduction of CD4+ cell infiltration. *Annals of Anatomy - Anatomischer Anzeiger* 224: 124–132.
30. Yamamoto S, Sato Y, Abo T, et al. (2002) Morphologic examination of CD3 - CD4 bright cells in rat liver. *Wound Repair and Regeneration* 10(4): 241–244.
31. Carol M, Lambrechts A, Van Gossum A, et al. (1998) Spontaneous secretion of interferon γ and interleukin 4 by human intraepithelial and lamina propria gut lymphocytes. *Gut* 42(5): 643–649.
32. Li S, Yang F and Ren X (2015) Immunotherapy for hepatocellular carcinoma. *Drug Discoveries & Therapeutics* 9(5): 363–371.
33. Hammerich L, Heymann F and Tacke F (2011) Role of IL-17 and Th17 cells in liver diseases. *Clinical & Developmental Immunology* 2011: 345803.
34. Chen C, Zhang C, Zhuang G, et al. (2008) Decoy receptor 3 overexpression and immunologic tolerance in hepatocellular carcinoma (HCC) development. *Cancer Investigation* 26(10): 965–974.
35. Budhu A, Forgues M, Ye Q-H, et al. (2006) Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 10(2): 99–111.
36. Liao J, Xiao J, Zhou Y, et al. (2015) Effect of transcatheter arterial chemoembolization on cellular immune function and regulatory T cells in patients with hepatocellular carcinoma. *Molecular Medicine Reports* 12(4): 6065–6071.

37. Lin Z-W, Wu L-X, Xie Y, et al. (2015) The expression levels of transcription factors T-bet, GATA-3, RORγt and FOXP3 in peripheral blood lymphocyte (PBL) of patients with liver cancer and their significance. *International Journal of Medical Sciences* 12(1): 7–16.

38. Xu J, Liu Z, He K, et al. (2021) T-bet transduction enhances anti-tumor efficacy of IFN-producing dendritic cell (IDKC) against hepatocellular carcinoma via apoptosis induction. *Biochemical and Biophysical Research Communications* 535: 80–86.

39. Gao J, Duan Z, Zhang L, et al. (2016) Failure recovery of circulating NKG2D+CD56dimNK cells in HBV-associated hepatocellular carcinoma after hepatectomy predicts early recurrence. *OncoImmunology* 5(1): e1048061.

40. Weidong Z, Bin L and Huaiping Z (2004) A study on the correlation between tumor escape in ovarian carcinoma and the expression of transcription factors T-bet/GATA3 [J]. *Acta Universitatis Medicine Nantai* 3(009).

41. Li P, Du Q, Cao Z, et al. (2012) Interferon-gamma induces autophagy with growth inhibition and cell death in human hepatocellular carcinoma (HCC) cells through interferon-regulatory factor-1 (IRF-1). *Cancer Letters* 314(2): 213–222.

42. Lu Y, Wu Z, Peng Q, et al. (2014) Role of IL-4 gene polymorphisms in HBV-related hepatocellular carcinoma in a Chinese population. *PLoS One* 9(10): e110061.

43. Wischhusen JC, Chowdhury SM, Lee T, et al. (2020) Ultrasound-mediated delivery of miRNA-122 and anti-miRNA-21 therapeutically immunomodulates murine hepatocellular carcinoma in vivo. *Journal of Controlled Release* 321: 272–284.

44. Bouhlel MA, Derudas B, Rigamonti E, et al. (2007) PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metabolism* 6(2): 137–143.

45. Yin G, Liu Z, Wang Y, et al. (2019) ZNF503 accelerates aggressiveness of hepatocellular carcinoma by downregulation of TATA3 expression and regulated by microRNA-495. *American journal of translational research* 11(6): 3426–3437.

46. Barnes P (2008) Role of GATA-3 in allergic diseases. *Current Molecular Medicine* 8(5): 330–334.

47. Zhou L, Lopes JE, Chong MMW, et al. (2008) TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function. *Nature* 453(7192): 236–240.

48. Qiu J, Che G, Liu F, et al. (2019) The detection and clinical significance of peripheral regulatory CD4+CD25hi/CD127low T-cells in patients with non-small cell lung cancer. *Clinical and Translational Oncology* 21(10): 1343–1347.

49. Li Q, Han Y, Fei G, et al. (2012) IL-17 promoted metastasis of non-small-cell lung cancer cells. *Immunology Letters* 148(2): 144–150.

50. Ivanov II, McKenzie BS, Zhou L, et al. (2006) The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* 126(6): 1121–1133.

51. Shen L-S, Wang J, Shen D-F, et al. (2009) CD4+CD25+CD127low/− regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. *Clinical Immunology* 131(1): 109–118.

52. Matsuo Y, Sawai H, Ma J, et al. (2009) IL-1α secreted by colon cancer cells enhances angiogenesis: The relationship between IL-1α release and tumor cells’ potential for liver metastasis. *Journal of Surgical Oncology* 99(6): 361–367.

53. Taniguchi K, Petersson M, Hoglund P, et al. (1987) Interferon gamma induces lung colonization by intravenously inoculated B16 melanoma cells in parallel with enhanced expression of class I major histocompatibility complex antigens. *Proceedings of the National Academy of Sciences* 84(10): 3405–3409.

54. Sachdeva M, Chawla YK and Arora SK (2015) Immunology of hepatocellular carcinoma. *World Journal of Hepatology* 7(17): 2080.

55. Lafià F, Miller AM, Ki SH, et al. (2010) Th17 cells and their associated cytokines in liver diseases. *Cellular & Molecular Immunology* 7(4): 250–254.

56. Budhu A and Wang XW (2006) The role of cytokines in hepatocellular carcinoma. *Journal of Leukocyte Biology* 80(6): 1197–1213.

57. Lin W, Zhang HL, Niu ZY, et al. (2020) The disease stage-associated imbalance of Th1/Th2 and Th17/Treg in uterine cervical cancer patients and their recovery with the reduction of tumor burden. *BMJ Women’s Health* 20(1): 126–127.

58. Yan J, Liu X-L, Xiao G, et al. (2014) Prevalence and clinical relevance of T-helper cells, Th17 and Th1, in hepatitis B virus-related hepatocellular carcinoma. *PLoS One* 9(5): e96080.

59. Foerster F, Hess M, Gerhold-Ay A, et al. (2018) The immune contexture of hepatocellular carcinoma predicts clinical outcome. *Scientific Reports* 8(1): 11.

60. Zhou J, Ding T, Pan W, et al. (2009) Increased intratumoral regulatory CD4+CD25+CD127low T cells and their impacts on cytokine profile of end-stage renal disease patients suffering from systemic lupus erythematosus.
63. Fathi F, Sami R, Mozafarpoor S, et al. (2020) Immune system changes during COVID-19 recovery play key role in determining disease severity. *International Journal of Immunopathology and Pharmacology* 33: 2058738419863238.

64. Zhou Y, Xu X, Ding J, et al. (2018) Dynamic changes of T-cell subsets and their relation with tumor recurrence after microwave ablation in patients with hepatocellular carcinoma. *Journal of Cancer Research and Therapeutics* 14(1): 40–45.

65. Keyhanmehr N, Motedayyen H and Eskandari N (2019) The effects of silymarin and cyclosporine a on the proliferation and cytokine production of regulatory T cells. *Immunological Investigations* 48(5): 533–548.

66. Sedaghat N, Motedayyen H, Alshehfosoul F, et al. (2019) Increased expression of lymphocyte activation gene-3 by regulatory t cells in multiple sclerosis patients with fingolimod treatment multiple siklero hastalarında fingolimod tedavisi sonucunda lenfosit aktivasyon gen-3 ekspresyonunda artış. *Turkish Journal of Immunology* 7(1): 31–39.

67. Sami R, Fathi F, Eskandari N, Ahmadi M, ArefNezhad R and Motedayyen H (2021) Characterizing the immune responses of those who survived or succumbed to COVID-19: Can immunological signatures predict outcome? *Cytokine* 140(2): 1–10.