Human Immunology 80 (2019) 510–516

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

# Increased frequency and FOXP3 expression of human $CD8^+CD25^{High+}$ T lymphocytes and its relation to CD4 regulatory T cells in patients with hepatocellular carcinoma

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ARTICLE INFO

*Keywords:*
Hepatocellular carcinoma
Conventional $CD4^+$ regulatory T cells
$CD8^+CD25^{High+}FOXP3^+$T cells

ABSTRACT

The mechanism of action of $CD8^+CD25^{High+}FOXP3^+$ T cells in hepatocellular carcinoma (HCC) has not been fully understood. Herein, the role of $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC was compared with that of $CD4^+CD25^{High+}FOXP3^+$ regulatory T cells (conventional Tregs). Thirty-five patients with HCC and twenty age and sex-matched healthy adults (controls) were enrolled. The percentage of $CD8^+CD25^{High+}FOXP3^+$ T cells and conventional Tregs in peripheral blood was measured by flow cytometry. Our results revealed that the percentage of peripheral $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC patients was significantly higher than controls ($P = 0.005$). The conventional Tregs showed the same trend with a higher level in HCC than controls ($P < 0.0001$). FOXP3 expression of $CD8^+CD25^{High+}$ T cells is higher than that of $CD8^+CD25^{low+}$ and $CD8^+CD25^{Negative}$ T cells. The percentage of $CD8^+CD25^{High+}FOXP3^+$ T cells positively correlated with that of conventional Tregs in HCC patients but not in controls. The higher alpha-fetoprotein positively correlated with the higher $CD8^+CD25^{High+}FOXP3^+$ T cells and conventional Tregs ($R2 = 0.481$, $P < 0.0001$ and $R2 = 0.249$, $P = 0.001$, respectively). The frequency of both $CD8^+CD25^{High+}FOXP3^+$ T cells and conventional Tregs was significantly increased in HCC with multiple lesions compared with those with one or two lesions. In conclusion: $CD8^+CD25^{High+}FOXP3^+$ T cells similar to conventional Tregs might be used as biomarkers of HCC progression. Therapy targeting the peripherally expanded $CD8^+CD25^{High+}FOXP3^+$ T cells may provide a novel perspective for HCC treatment.

## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide and despite advances in diagnostic and surgical techniques, it is still considered the 3rd leading cause of cancer- related death universal [1–4].

The liver has been considered as an immunogenic organ and its tolerogenic role overweight its effector one [5–10]. Based on that theory, we hypothesise that regulatory T cells (Tregs)-based mechanism would be likely to be operated in our HCC patients, although several mechanisms may act role complementarily. In accordance, there is accumulating clinical pieces of evidence that Tregs play an important role in HCC [11,12].

In human, Tregs were conventionally reported to be $CD4^+CD25^{High+}FOXP3^+$ T cells (conventional Tregs) [13–19]. However, Churlaud's group and others have found the existence of $CD8^+$ T cells expressing $CD25^{High+}FOXP3^+$ as immunosuppressive cells associated with immune suppression in mouse and human subjects [20–24]. Thus, a question arose as to whether $CD8^+CD25^{High+}FOXP3^+$ T cells might play a key role in the mechanism of HCC, similar to the conventional Tregs. Some recent studies documented that $CD8^+CD25^{High+}FOXP3^+$ T cells, unlike conventional Tregs, more suppressive after stimulation in the presence of IL-2 [25]. However, the frequency of conventional Tregs predominates over $CD8^+CD25^{High+}FOXP3^+$ T cells in the peripheral blood of the human. Therefore, one may speculate that targeting $CD8^+CD25^{High+}FOXP3^+$ T

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https://doi.org/10.1016/j.humimm.2019.03.014
Received 11 November 2018; Received in revised form 11 March 2019; Accepted 19 March 2019
Available online 20 March 2019

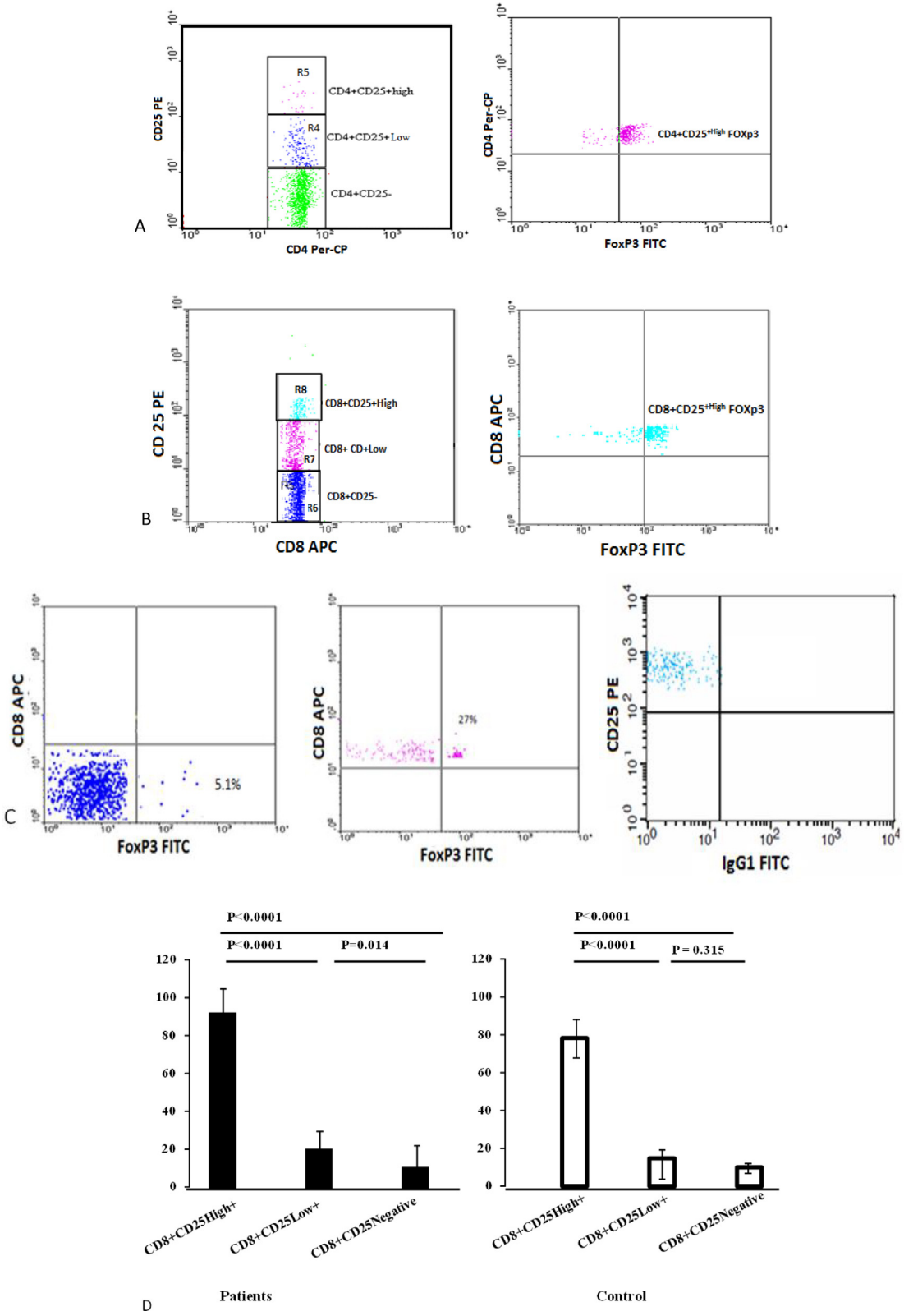


**Fig. 1.** The flow cytometric detection of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells. (A) The representative fluorescence-activated cell sorting (FACS) profiles of conventional Tregs ($CD4^+CD25^{High+}$) are shown (left); inside (CD4-gated T lymphocyte) CD25 versus CD4 are divided according to the fluorescent intensity and conventional Tregs represented the highest intensity. FOXP3 expression in conventional Tregs ($CD4^+CD25^{High+}$ gated T lymphocyte) is shown (right). (B) The representative FACS profiles of $CD8^+CD25^{High+}$ cells are shown (left); inside (CD8-gated T lymphocyte) CD25 versus CD8 are divided according to the fluorescent intensity and $CD8^+CD25^{High+}$ T cells represented the highest intensity. FOXP3 expression in $CD8^+CD25^{High+}$ gated T lymphocyte) is shown (right). (C) The representative FACS profiles of FOXP3 expression in $CD8^+CD25^{Negative}$ T cells (right) $CD8^+CD25^{Low+}$ T cells (middle), and a negative control (left) both cells show low expression of FOXP3 than that of $CD8^+CD25^{High+}$ T cells (D) The percentage of FOXP3 expression of $CD8^+CD25^{High+}$, $CD8^+CD25^{Low+}$ and $CD8^+CD25^{Negative}$ T cells in HCC patients (right) and controls (left) demonstrated an increase in FOXP3 expression of $CD8^+CD25^{High+}$ T cells when compared with that of $CD8^+CD25^{Low+}$ and $CD8^+CD25^{Negative}$ T cells in both HCC patients and controls.

cells by IL-2 therapy would be a new modality for HCC management. In addition, conventional Tregs, as well as $CD8^+CD25^{High+}FOXP3^+$ T cells, can be used as reliable prognostic markers in HCC patients for many reasons. First, its investigation is done by an easy noninvasive technique. Second, for patients who will not be operated, clinical outcomes can be assessed by measuring Tregs in the peripheral blood. Third, measuring Tregs in the lymphocyte in the blood can be reliably and accurately compared with measuring the tissue Tregs.

In this study, we examined the peripheral blood derived from thirty-five HCC patients for the frequency and FOXP3 expression of both conventional Tregs and $CD8^+CD25^{High+}$ T cells. Our data have suggested that $CD8^+CD25^{High+}FOXP3^+$ T cells may also play a crucial role in the pathogenesis of HCC beside the key role of conventional Tregs.

## 2. Patients, materials and methods

### 2.1. Patients

Thirty-five patients presented with HCC and 20 age and sex-matched healthy controls participated in this prospective case-control study. All patients were presented to AL-Rajhi Liver Center, Assiut University Hospitals in the period from February 2017 to January 2018. The study was performed after approval of the ethics committee of the Faculty of Medicine, Assiut University and in accordance with guidelines of the Declaration of Helsinki 1975 as revised in 2008. All patients had HCC as a consequence of HCV infection and liver cirrhosis that was diagnosed based on clinical presentation, biochemical and ultrasonographic findings. Child-Pugh score was used to estimate cirrhosis severity [26]. The number of hepatic focal lesions, as well as portal vein thrombosis, was investigated by computed tomography (CT) and magnetic resonance imaging (MRI). Alpha-fetoprotein (AFP) levels in the serum were measured by Access 2 (Beckman Coulter, USA). HCV infection was serologically investigated in all participants by Monolisa HCV Ag/Ab ULTRA (Bio-Rad, Marnes la Coquette, France). Quantitative determination of HCV RNA was analyzed by a reference method using Qiagen Gmbh, (Germany) and analyzed by 7500 fast real-time PCR, applied biosystem. All healthy controls did not reveal positive results.

### 2.2. Flow cytometric detection of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells

For identification of conventional Tregs fluoroisothiocyanate (FITC)-conjugated anti-FOXP3 (e Bioscience, USA), phycoerythrin (PE) conjugated anti-CD25 (IQ Product, The Netherland), peridinium-chlorophyll-protein (Per-CP)-conjugated anti-CD4 (Becton Dickinson, Bioscience, USA) monoclonal antibodies (mAbs) were used. For recognition of $CD8^+CD25^{High+}FOXP3^+$ cells FITC-conjugated anti-FOXP3 (e Bioscience, USA), PE-conjugated anti-CD25 (IQ Product, The Netherland), and allophycocyanin (APC)-conjugated anti-CD8 (Becton Dickinson, Bioscience, USA) mAbs were used. Surface staining was done first by incubating 100 μL of blood sample with 10 μL of APC-conjugated anti-CD8, 10 μL of Per-CP-conjugated anti-CD4 and 20 μL of PE-conjugated anti-CD25 for 30 min at 4 °C in the dark. Followed by red blood cell (RBC) lysis and washing with phosphate buffer saline (PBS). For intracellular staining of FOXP3, first fixation was done by incubation for 10 min at room temperature in a fixation solution, and then washed with a permeabilization solution; secondly, staining was done by incubating cells dissolved in 40 μL of permeabilization solution with 10 μL of FITC-conjugated anti-FOXP3 antibody for 30 min at 4 °C in the dark. FACSCalibur flow cytometric analysis using Cell-Quest software (Becton Dickinson Biosciences, USA) was used to measure the results. An isotype negative control using FITC-conjugated anti-human IgG (e Bioscience, USA) mAb was used with each sample. For data analysis; lymphocyte population were identified by forward and side scatter parameters. To identify conventional Tregs; $CD4^+$ (T-helper cells) were assessed in lymphocytes and were then gated for $CD25^{High+}$ and $FOXP3^+$ expression. For identification of $CD8^+CD25^{High+}FOXP3^+$ T cells; firstly, $CD8^+$ (T-cytotoxic) cells were gated, then $CD25^{High+}$ and $FOXP3^+$ expression were measured inside it. The expression of FOXP3 on conventional Tregs and $CD8^+CD25^{High+}$ T cells was expressed as the geometric mean of fluorescence intensity (MFI) as shown in (Fig. 1).

### 2.3. The statistical analysis

The data were presented by mean ± SD. The data were analyzed using the independent *t*-test, Chi-square test, Student's paired *t*-test, or Pearson's correlation coefficient when appropriate. P values less than 0.05 were considered significant. The Statistical Package for the Social Sciences for Windows statistical software was used for data analysis. (SPSS: An IBM Company, version 21, IBM Corporation, Armonk, NY, USA).

## 3. Results

### 3.1. The characteristics of the thirty- five HCC patients and controls

The details of the study's participants were listed in (Table 1). All patients had HCC as a squeal of HCV infection/liver cirrhosis. About half of them had splenomegaly, and 9 (26%) of HCC patients had portal

**Table 1**
Characteristic of 35 patients with hepatocellular carcinoma (HCC).

| | Patients 35 | Control 20 | P value |
|---|---|---|---|
| Age (mean ± SD) | 64.2 ± 8.6 | 63.5 ± 7.8 | > 0.05 |
| (Median, range, years) | (65, 45–85) | (63, 64–62) | |
| Gender; Male (%) | 32 (91.4%) | 18 (90%) | > 0.05 |
| Haemoglobulin g/dL | 8.97 ± 2 | 11.87 ± 1 | < 0.0001 |
| Platelet count ×10 * 9/L | 115.7 ± 70 | 255.2 ± 60.4 | < 0.0001 |
| Aspartate transaminase (IU/L) | 98.7 ± 55.4 | 13.8 ± 3.6 | < 0.0001 |
| Alanine transaminase (IU/L) | 90.9 ± 53.5 | 17.7 ± 2.7 | < 0.0001 |
| Albumin (g/L) | 3.5 ± 1 | 4.5 ± 1 | 0.05 |
| Total bilirubin (mg/dL) | 2.2 ± 2.7 | 4.6 ± 0.75 | < 0.0001 |
| Direct bilirubin (mg/dL) | 1.9 ± 2.4 | 0.9 ± 0.4 | 0.08 |
| WBC ×1000/$mm^3$ | 6.8 ± 2 | 7.1 ± 1.9 | 0.574 |
| Alpha-fetoprotein (ng/mL) | 5777 ± 7269 | NA | NA |
| Prothrombin time (s) | 13.8 ± 2.1 | 12.2 ± 1 | > 0.05 |
| INR (International Normalized Ratio) | 1.2 ± 0.2 | 1.1 ± 0.2 | > 0.05 |
| *Ascites* | | | |
| No | 19 | NA | NA |
| Mild | 2 | NA | NA |
| Moderate | 14 | NA | NA |
| He | | NA | NA |
| No | 28 | NA | NA |
| Yes | 7 | NA | NA |
| *Child-Pugh score* | | | |
| 5 | 10 | NA | NA |
| 6 | 7 | NA | NA |
| 7 | 4 | NA | NA |
| 8 | 6 | NA | NA |
| 9 | 3 | NA | NA |
| 10 | 1 | NA | NA |
| 11 | 4 | NA | NA |
| *Liver cirrhosis* | | | |
| Yes | 35 | NA | NA |
| *Enlarged spleen* | | | |
| No | 18 | NA | NA |
| Yes | 17 | NA | NA |
| *Portal vein thrombosis* | | | |
| No | 26 | NA | NA |
| Yes | 9 | NA | NA |
| *Survival* | | | |
| Dead/Alive | 6/29 | NA | NA |

vein thrombosis (PVT). Routine blood tests demonstrated that the haemoglobin level, platelet count, and albumin were significantly lower in patients compared with those in controls. On the other hand, alanine transferase, aspartate aminotransferase, and total bilirubin were significantly higher in HCC patients compared with those in controls.

### 3.2. The frequency of T lymphocyte subsets in HCC patients and controls

The frequency of the total T cells was significantly lower in HCC patients compared with that in controls ($P < 0.0001$). In contrast to the percentage of T helper ($CD4^+$ T) cells that was significantly decreased ($P < 0.0001$) in HCC patients, the percentage of T cytotoxic ($CD8^+$ T) cells was significantly increased ($P < 0.0001$). The result of that discrepancy was presented as a significant reduction in the $CD4^+/CD8^+$ ratio ($P < 0.0001$) in HCC patients compared to controls. The proportion of $CD4^+CD25^{High+}$ T cells was significantly raised in HCC patients than controls ($P < 0.0001$). Similarly, the frequency of $CD8^+CD25^{High+}$ T cells was significantly raised in HCC patients than controls ($P < 0.0001$). The frequency of the effector $CD4^+CD25^{Low+}$ T cells was higher in HCC patients compared to controls ($P = 0.001$). However, there was no significant difference in the level of effector $CD8^+CD25^{Low+}$ T cells (Table 2).

### 3.3. The frequency of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC patients and controls:

$FOXP3^+$ expression is higher in $CD8^+CD25^{High+}$ T cells when compared with that of $CD8^+CD25^{Low+}$ and $CD8^+CD25^{Negative}$ T cells (Fig. 1D) in both HCC patients and controls. In addition, the frequency of $CD4^+CD25^{High+}FOXP3^+$ (conventional Tregs) as well as $CD8^+CD25^{High+}FOXP3^+$ T cells was significantly increased in HCC patients compared with those in the controls ($P < 0.0001$ and $P = 0.005$, respectively) (Table 2).

While the Expression of FOXP3 in $CD8^+CD25^{Low+}$ and $CD8^+CD25^{Negative}$ T cells showed no significant difference between HCC patients and the controls [$CD8^+CD25^{Low+}$ $FOXP3^+$ T cells (HCC patients and control; $20.7 \pm 6.1$ and $16.9 \pm 5.8$, $P = 0.06$, respectively; $CD8^+CD25^{Negative}$ $FOXP3^+$ T cells (HCC patients and control; $12.1 \pm 7.8$ and $10.1 \pm 2.5$, $P = 0.408$, respectively].

### 3.4. The relation between conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC patients and controls

The frequency of $CD8^+CD25^{High+}$ T cells, as well as $CD8^+CD25^{High+}FOXP3^+$ T cells were significantly increased with that of $CD4^+CD25^{High+}$ and $CD4^+CD25^{High+}FOXP3^+$ T cells in patients with HCC but not in the control ($R2 = 0.281$, $P = 0.005$ and $R2 = 0.245$, $P = 0.035$, respectively) (Fig. 2 (A, B)). While measuring the percentage of $CD8^+CD25^{High+}FOXP3^+$ T cells and that of conventional Tregs found to be correlated using student paired *t*-test (Fig. 2C).

### 3.5. The relation between conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells and HCC pathological markers

The proportion of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells was significantly increased with the higher level of AFP ($R2 = 0.481$, $P < 0.0001$ and $R2 = 0.249$, $P = 0.001$, respectively) (Fig. 3(A, B)).

In addition, conventional Tregs were significantly increased in HCC patients with multiple lesions compared with that of one lesion, or two lesions less than 5 cm ($P = 0.006$, $P = 0.039$ and $P = 0.003$, respectively). While, $CD8^+CD25^{High+}FOXP3^+$ T cells were significantly decreased in HCC patients with one lesion less than 5 cm compared with that of two lesions more than 5 cm and multiple lesions ($P = 0.002$ and $P = 0.018$, respectively) (Fig. 3(C, D)).

Altogether, our data indicated that the frequency of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells was increased compared with those of healthy controls.

## 4. Discussion

We found that the proportion of $CD4^+CD25^{High+}$ T cells and the conventional Tregs were elevated in the peripheral blood of HCC patients compared with those in healthy volunteers. However, those data were not surprising because conventional Tregs in humans seem to be the counterparts to rodents' Tregs that showed the same tendency [27–33]. Of note, the frequency $CD8^+CD25^{High+}$ T cells and its FOXP3 level were elevated in HCC patients.

These observations were in accordance with the previous work demonstrating an altered number of $CD8^+CD25^{High+}FOXP3^+$ T cells in patients with systemic lupus, multiple sclerosis, other autoimmune diseases [34–40], asthma [41], and in malignancies [42,43]. Surprisingly, $CD8^+CD25^{High+}FOXP3^+$ T cells in our HCC patients exerted similar pattern like conventional Tregs. Thus, it would be important to conclude that both conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells play a role in HCC pathogenesis.

Although we did not measure the suppressive activity of $CD8^+CD25^{High+}FOXP3^+$ T cells, previous reports by Chaput et al. [42] and Kiniwa et al. [43], showed that $CD8^+CD25^{High+}FOXP3^+$ T cells from patients with colorectal and prostate cancers had strong immunosuppressive properties and played a role in disease progression. In further support of our hypothesis, the percentage of $CD8^+CD25^{High+}$ T cells shown to be increased with the increase of the proportion of $CD4^+CD25^{High+}$ T cells in our HCC patients ($P = 0.005$), but not in the controls ($P = 0.552$). In addition, we noticed that the percentage of

**Table 2**
The percentage of Lymphocytes subsets HCC patients and controls.

| Percentage % | Patients (35) | Control (20) | P value |
|---|---|---|---|
| $CD3^+$ T cells | $49.3 \pm 7.7$ | $58.6 \pm 8$ | $< 0.0001$ |
| $CD4^+$ T cells | $27 \pm 6.4$ | $39.1 \pm 6.5$ | $< 0.0001$ |
| $CD8^+$ T cells | $21.5 \pm 3.8$ | $17.1 \pm 3.3$ | $< 0.0001$ |
| $CD4^+/CD8^+$ ratio | $1.3 \pm 0.4$ | $2.3 \pm 0.5$ | $< 0.0001$ |
| Total $CD4^+CD25^+/CD4^+$ T cells | $25 \pm 4.6$ | $19.7 \pm 3.5$ | 0.005 |
| $CD4^+CD25^{Low+}/CD4^+$ T cells | $18.3 \pm 3.6$ | $15.7 \pm 3.1$ | 0.009 |
| $CD4^+CD25^{High+}/CD4^+$ T cells | $6.8 \pm 2.4$ | $4.2 \pm 0.9$ | $< 0.0001$ |
| $CD4^+CD25^{High+}FOXP3^+/CD4^+$ T cells | $2.1 \pm 0.4$ | $1.5 \pm 0.3$ | 0.001 |
| $CD4^+CD25^{High+}$ FOXP3 MFI | $147.2 \pm 39.6$ | $122.2 \pm 9.4$ | $< 0.0001$ |
| Total $CD8^+CD25^+/CD8^+$ T cells | $10 \pm 4.1$ | $8 \pm 2.5$ | 0.032 |
| $CD8^+CD25^{Low+}/CD8^+$ T cells | $6.7 \pm 4.3$ | $5.6 \pm 2.5$ | 0.311 |
| $CD8^+CD25^{High+}/CD8^+$ T cells | $3 \pm 0.88$ | $2.1 \pm 0.64$ | $< 0.0001$ |
| $CD8^+CD25^{High+}FOXP3^+/CD8^+$ T cells | $1.5 \pm 0.7$ | $0.9 \pm 0.3$ | 0.001 |
| $CD8^+CD25^{High+}$ FOXP3MFI | $94 \pm 10.9$ | $78.5 \pm 10.$ | $< 0.0001$ |

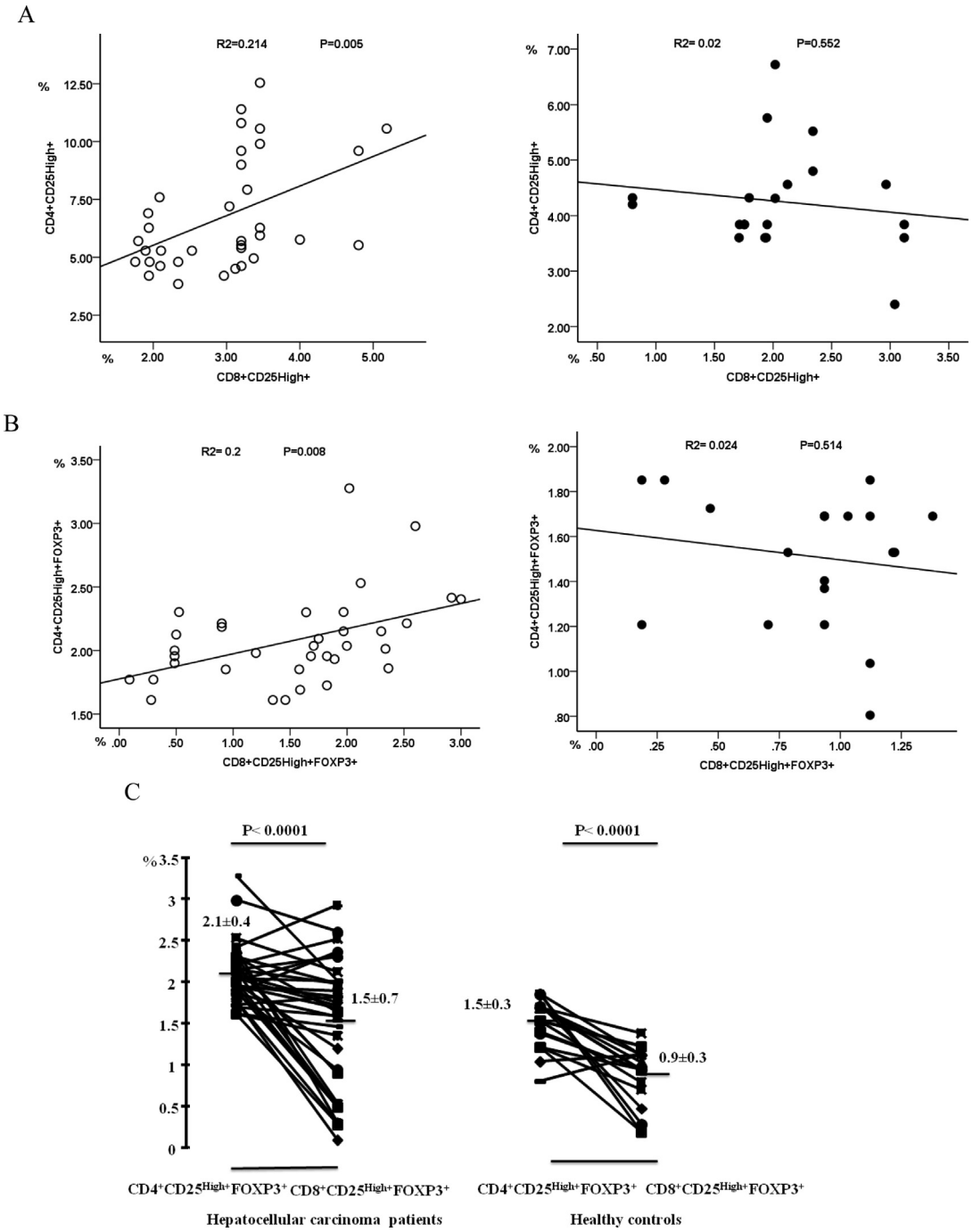


**Fig. 2.** The relation between conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells in patients with hepatocellular carcinoma (HCC) and controls. The frequency of $CD8^+CD25^{High+}$ T cells as well as $CD8^+CD25^{High+}FOXP3^+$ T cells were significantly increased with that of $CD4^+CD25^{High+}$ and $CD4^+CD25^{High+}FOXP3^+$ T cells in patients with HCC (right side) but not in the control (left side) (R2 = 0.214, P = 0.005 and R2 = 0.2, P = 0.008, respectively) (A, B). In addition, the proportion of $CD8^+CD25^{High+}FOXP3^+$ T cells correlated with that of $CD4^+CD25^{High+}FOXP3+$ T cells in HCC and controls using student paired *t*-test (C).

$CD8^+CD25^{High+}FOXP3^+$ T cells was higher than that of $CD8^+CD25^{Low+}FOXP3^+$ and $CD8^+CD25^{Negative}FOXP3^+$ T cells ($P < 0.0001$) among patients and controls. Moreover, the percentage of $CD8^+CD25^{High+}FOXP3^+$ T cells was positively correlated with the percentage of conventional Tregs in our HCC patients (P = 0.008) but not in controls (P = 0.514) (Fig. 2A, B). Hence, one can conclude that an expansion of $CD8^+CD25^{High+}FOXP3^+$ T cells in peripheral blood took place with the expansion of conventional Tregs. In vivo observation reported by Churlaud et al. [25] in which the administration of IL-2 resulted in an increase of both $CD8^+CD25^{High+}FOXP3^+$ T cells and conventional Tregs in the peripheral blood of non-human primates and healthy volunteers. Also, a similar finding reported in patients infected with tuberculosis [44] and in patients with type 1 diabetes mellitus after IL-2 treatment [45]. Altogether, our innovative findings may open a new direction that novel cancer treatment strategies (e.g., specific antibodies, low molecule compounds) may better target the smaller pool of $CD8^+CD25^{High+}FOXP3^+$ T cells, rather than targeting the larger pool of conventional Tregs that may lead to unwanted side effects.

Furthermore, in accordance with our results Klatzmann's group described that in a normal individual, FOXP3 protein expression within $CD8^+CD25^{High+}$ T cells was lower than that within $CD4^+CD25^{High+}$ T

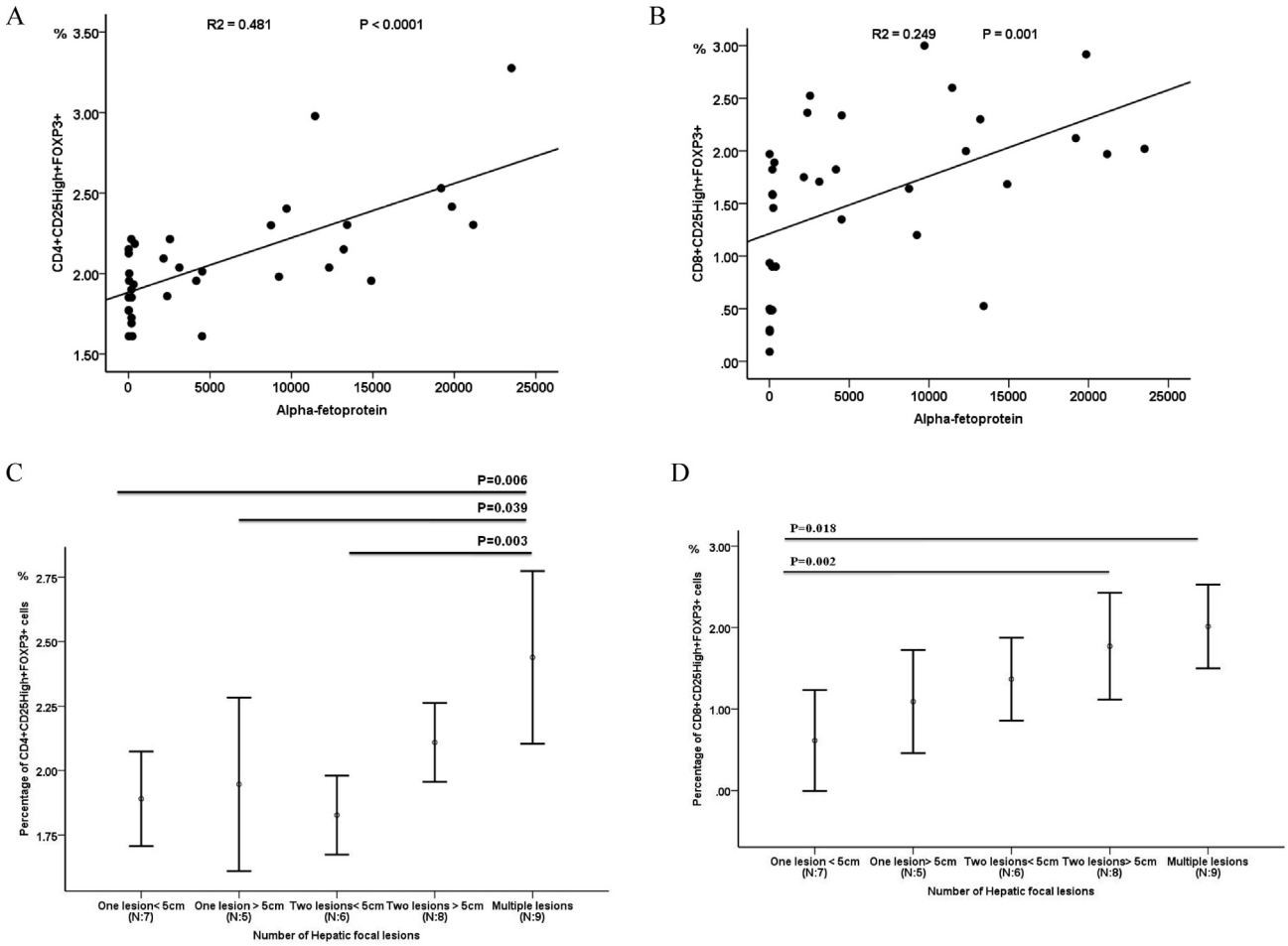


**Fig. 3.** The relation between conventional Tregs, $CD8^+CD25^{High+}FOXP3^+$ T cells and pathological markers in patients with hepatocellular carcinoma (HCC). The proportion of conventional Tregs and $CD8^+CD25^{High+}FOXP3^+$ T cells was significantly increased with the higher level of alpha-fetoprotein (R2 = 0.481, P < 0.0001 and R2 = 0.249, P = 0.001, respectively) (A, B). Conventional Tregs were significantly increased in HCC patients with multiple lesions compared with that of one lesion, or two lesions less than 5 cm (P = 0.006, P = 0.039 and P = 0.003, respectively). While, $CD8^+CD25^{High+}FOXP3^+$ T cells were significantly decreased in HCC patients with one lesion less than 5 cm compared with that of two lesions more than 5 cm and multiple lesions (P = 0.002 and P = 0.018, respectively) (C, D).

cells. However, in their study, once $CD8^+CD25^{High+}FOXP3^+$ T cells were activated by IL-2, they exert ed a potent suppressive activity [25,42]. The FOXP3 expression was up-regulated within $CD8^+CD25^{High+}$ T cells and conventional Tregs in HCC patients and this expansion had a positive correlation in patients rather than in controls (Table 2 and Fig. 2). This suggested that firstly, $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC were in an activated state, secondly, this specifically induced $CD8^+CD25^{High+}FOXP3^+$ T cells may be against HCC antigen. Further investigation is needed to elucidate the exact origin of $CD8^+CD25^{High+}FOXP3^+$ in the cancer setting. The frequency of $CD8^+CD25^{High+}FOXP3^+$ T cells and conventional Tregs was positively correlated with higher level of AFP and significantly increased in patients with multiple lesions compared with those of one or two lesions (Fig. 3) that was in accordance with previous reports [46–49] that found poor prognosis was associated with regulatory T cell expansion in HCC.

## 5. Conclusion

Up to our knowledge, this is the first report providing detailed evidence showing that frequency and FOXP3 expression of $CD8^+CD25^{High+}$ T cells were upregulated in the peripheral blood of HCC patients and this expansion is positively correlated with worse prognostic markers as AFP and hepatic focal lesions. Thus, it is incorrect to conclude that only conventional Tregs participated in HCC pathogenesis, but it may be that the role of $CD8^+CD25^{High+}FOXP3^+$ T cells is also critical. Further functional studies are warranted to fully understand the clinical involvement of $CD8^+CD25^{High+}FOXP3^+$ T cells in HCC settings.

## 6. Informed consent statement

The Human Research Ethics Committee of Assiut University approved this study. Informed consent was obtained from the guardians of patients and healthy volunteers in accordance with the Declaration of Helsinki.

## 7. Conflict-of-interest statement

All authors have no potential interest to declare.

## Author contributions

H.N. and A.Z. are equally contributed to this work All authors contributed to this work in research design, carrying out the research experiments, data analysis and writing the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.03.014.

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