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Circulating $CD4^{+}CD25^{high}FoxP3^{+}$ T Cells in Non- metastatic Hepatocellular Carcinoma Related to Hepatitis C Virus Cirrhosis

Authors
Abeer Attia Saad[1], MD, Heba Mohamed Abdella[2], MD, Doaa G Eissa[1], MD, Marwa G. A Hegazy[3], PhD, Amal Farouk[1], MD, Marcel William Keddeas[4], MD, Nahla Fawzy Abouelezz[5] , MD, Ahmed Kamal Eldorry[6], MD, Mohamed Kamal Shaker[2], MD
[1]Dept of Clinical Pathology, [2]Tropical Medicine, [3]Biochemistry Dept, [4]Internal Medicine, [5]Community, Environmental and Occupational Medicine, [6]Interventional Radiology; Faculty of Medicine and; Faculty of Science, Ain Shams University, Cairo, Egypt
Corresponding Author
Dr Doaa Gamal Eissa
Dept of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt, PO: 11381. Fax and E-mail: *doaaeissa3@yahoo.com* Fax/Telephone Number: +2024824073

Abstract
Background: *T-regs play a role in suppression of the tumor immune response, including hepatocellular carcinoma (HCC). We studied circulating T-regs in non-metastatic HCC patients related to hepatitis C virus (HCV) cirrhosis by comparing and correlating levels in different stages of the disease with survival and various clinico-pathologic features.*
Methods: *76 non-metastasizing HCC patients and 76 healthy volunteers were assessed for circulating T-regs using flow cytometry in addition to complete blood picture, liver function test, alpha fetoprotein (AFP).*
Results: *Patients showed higher T-regs% and T-effs% than controls (P<0.001) with lower absolute number of both cells in patients (P= 0.001, 0.002 respectively). No significant differences were found between stage A and stage B patients (P> 0.05). The absolute numbers of both T-regs and T-effs cells were significantly higher in patients with tumor size of the largest nodule more than 3 cm (P= 0.02, 0.008 respectively). T-regs% was significantly higher in patients with higher serum AFP levels and multiple tumor foci (P= 0.004, 0.02 respectively). Patients with higher T-regs% had significantly shorter overall mean survival time (p=0.003).*
Conclusions: *HCC showed increased peripheral blood T-regs more in patients with larger tumor sizes, multiple tumor foci and higher serum AFP, and was linked to shorter overall mean survival time.*
Keywords: *circulating T-regs, Hepatitis C virus, cirrhosis, Hepatocellular carcinoma.*

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide[1]. The tumor immune microenvironment has been reported to play a crucial role in the establishment and progression of tumors. Infiltration of CD8+ T cells within the tumor foci has been strongly related to a low recurrence rate and good prognosis[2]. On the other hand, infiltration by a sub-population of CD4+ T cells (regulatory T cells; T-regs) has been

found to counteract the beneficial role of CD8+ T cells [3-4]. T-regs are defined based on their expression of CD4, CD25 and forkhead, or winged helix family of transcription factor P3 (FoxP3), which is important for their development and function [5]. Many evidences indicated that T-regs are increased in patients with various types of cancer[6-8]. T-regs play an important role in immunologic self-tolerance and suppression in the tumor immune response [3,9]. Elimination of T-reg cells leads to a more effective antitumor immune reaction and causes better tumor rejection, mainly in the early stage of tumor growth [10]. Therefore, we aimed to study circulating T-regs in non-metastatic HCC patients related to HCV cirrhosis. With comparing their levels in different stages of the disease, and correlating them with various clinico-pathologic features and survival study, we could gain further insight into their role in the immune response during disease progression.

Subjects and Methods

Subjects: This study was conducted in Ain Shams University Hospitals and outpatient clinic of HCC unit. It included 76 non-metastasizing HCC patients: 64 males and 12 females. Seventy six healthy subjects of matched age and gender and negative for antibodies to HCV, HBV and HIV were included as a control group. Informed consent was obtained from the studied individuals. All patients had cirrhotic liver disease due to HCV infection and were newly diagnosed as HCC. Patients with hepatitis B virus infection were excluded. All patients were subjected to: Full history taking and clinical examination, Laboratory investigations: Complete blood picture using Beckman Coulter LH700 Series (USA), prothrombin time using SYSMEX CA-1500 (Germany), aspartate aminotransferase (AST), Alanine transaminase (ALT), serum albumin, serum bilirubin, serum creatinine (Synchron CX-9, Beckman, USA) and Alpha fetoprotein (AFP) (Cobas E411, Roche Diagnostics), Imaging studies: Abdominal ultrasound and Abdominal triphasic spiral computed tomography (CT). Diagnosis and staging of HCC was done according to The Barcelona Clinic Liver Cancer (BCLC) staging system [11]. In view of that, 44 patients were BCLC stage A, Group1 (G1) and 32 patients were stage B, Group2 (G2). None of the studied patients received any previous medical treatment or any interventions for HCC.

Flow cytometry analysis: Two ml of peripheral blood (PB) were collected using vacutainer containing anticoagulant potassium ethylene diamine tetraacetate (EDTA) in a final concentration of 1.5 mg/ml. Fifty µL of whole anti-coagulated blood was lysed using 1 mL IQ test lysing reagent (Beckman Coulter, USA) followed by washing with phosphate buffer saline (PBS) (Oxoid, England). After that, the cells were stained with combinations of anti-CD25-PE and anti-CD4-FITC, and isotype controls (FITC and PE) (five µl each): (Beckman coulter, USA). The test tubes were then incubated in dark for 20 minutes followed by washing with PBS. Intracellular staining FoxP3-PE-Cy5 and its isotype control (eBioscience, US) was according to Attia et al.[12]. Data acquisition and analysis were performed on EP-ICS XL flow cytometry (FCM) using SYSTEMII version 3 software with a standard 3-color filter configuration. A total of at least 10,000 cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. Discrimination of $CD25^{high}$ T-regs, $CD25^{low}$ activated effector-memory T cells (T-effs) were acquired after gating the non-$CD4^{+}$ T-lymphocyte population as previously described by Zhang et al.[13]. The $CD25^{high}$ population was determined relative to the low intensity of CD25 staining found on non-$CD4^{+}$ T cells; cells expressing CD25 at levels above those of the isotype control and at higher expression levels than the $CD25^{low}$ cells were considered as T-regs (Figure 1).

Statistical Analysis: Analysis of data was done by IBM computer using Statistical Program for Social Science version 15 (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to assess normality of the data distribution. Data were expressed as medians for quantitative measures, and both numbers and percentages for

categorical data. Mann Whitney test was used to compare non-parametric quantitative variables. Chi-Square test was used for inter-group comparisons. Spearman's correlation test was used to measure the correlation between the quantitative variables. Overall survival (OS) was estimated from the time of diagnosis to the date of death or last visit. The OS was determined using Kaplan- Meier curves. Log-rank test was used to calculate P value. The probability of error less than 0.05 was considered significant.

Results

The prevalence of T-reg cells was analyzed in the PB of 76 patients with non metastatic HCC and 76 healthy subjects as a control group of matched age and gender. The population of T-reg cells was identified by flow cytometry and expressed as a percentage of total CD4+ T-cells. The patients group aged between 40 and 68 years, median 56 years, whereas the control group aged between 41 and 65 years, median 53.5 years.

Comparison between patients and control group (Table 1): The comparison between patients and control group revealed a significantly higher T-regs% and T-eff% in patients group than control group (P<0.001) with lower absolute number of both cells in patient group (P= 0.001, 0.002 respectively). Although FoxP3 expression% on T-reg cells were also higher in patients than in controls, yet it did not show significant difference (p=0.7).

Comparison between G1 & G2 (Table 2): Statistically insignificant differences were found between both groups as regards T-regs%, T-regs absolute, T-reg foxP3%, T-eff % and T-effs absolute cells.

Correlation analysis: T-regs% was inversely correlated with direct bilirubin, whereas T-effs% was inversely correlated with total bilirubin (P= 0.01, 0.04 respectively), and T-reg FoxP3 expression was positively correlated with both total and direct bilirubin (P= 0.002, 0.04 respectively). Both T-regs% and T-effs% were inversely correlated with hemoglobin (Hb) concentration (P= 0.03, 0.04 respectively). However, both the absolute number of T-reg and T-eff cells were positively correlated with platelet count (P< 0.001, 0.004 respectively). T-regs FoxP3 expression was inversely; while T-effs% was positively correlated with prothrombin concentration (P= 0.008, 0.01 respectively).

Impact of several factors on FCM parameters (Table 3): Males had significantly higher T-regs% than females (P= 0.03). Patients with performance status 1 and 2 had higher T-effs% and absolute number but lower T-regs FoxP3 expression than patients with performance status zero (P< 0.05). The absolute numbers of both T-regs and T-effs cells were significantly higher in patients with tumor size of the largest nodule more than 3 cm in maximum diameter (P= 0.02, 0.008 respectively). T-regs% was significantly higher in patients with higher serum AFP levels and multiple tumor foci (P= 0.004, 0.02 respectively).

Survival studies (Figure 2): Kaplan–Meier analysis for estimation of mean OS time according to the studied T-cells, showed that patients with higher T-regs% had significantly shorter mean OS time (p=0.003).

Table 1: Comparison between patients and control group

Parameters	Control group	Patients	Test of significance	P
Gender: Males Females	52 24	64 12	X^2=2.621	0.1
Age: median (years)	53.5	56	Z=1.343	0.1
T-regs%: median	2.5	3.6	Z=3.957	<0.001
T-regs absolute/µl: median	50	32.4	Z=3.224	0.001
T-reg foxP3 %: median	56.2	65.2	Z=0.312	0.7
T-effs%: median	8.9	12	Z=4.39	<0.001
T-effs absolute/ µl: median	203	119.2	Z=3.078	0.002

T-regs:T regulatory cells, T-effs:T effector cells

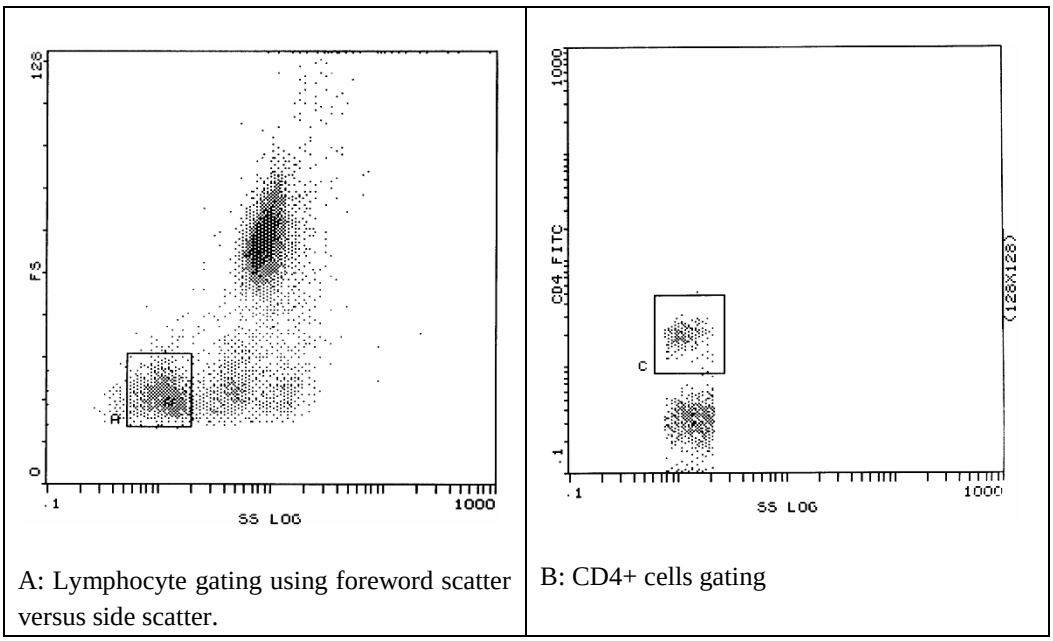
Table 2: Comparison between G1 & G2 patients as regards T-regs and T-effector cells

Parameter: median	G1 N=44	G2 N=32	Z	P
T-regs%	3.4	4.2	1.959	0.052
T-regs absolute/ µl	31.5	35.9	0.237	0.8
T-reg foxP3%	65.4	60.4	0.355	0.7
T-effs%	10.8	13.5	0.651	0.5
T-effs absolute/ µl	119.2	110.7	0.829	0.4

Table 3: Impact of several factors on T-regs and T-effector cells

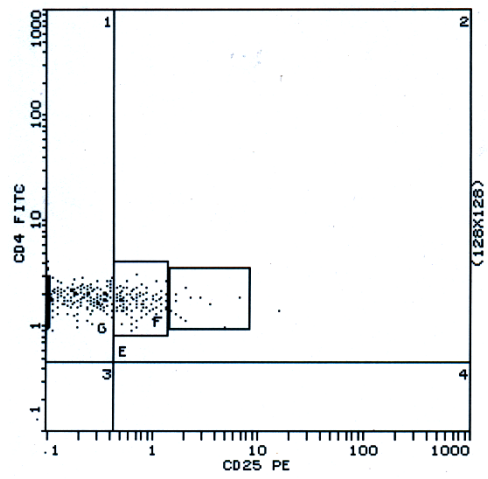
Parameter (No)	T regs %	T-regs absolute/ µl	T-reg foxP3%	T-effs%	T-effs absolute/ µl
Gender: Male (64) Female (12)	3.7 2.4	35.9 19.2	65.3 54.4	11.4 14.9	120.9 119.2
	Z=2.089 P=0.03	Z=1.924 P=0.054	Z=0.401 P=0.6	Z=1.123 P=0.2	Z=0.0 P=1
Stage: Child-PughA (20) Child-Pugh B, C (56)	3.8 3.4	39.5 31.9	49.5 65.1	18.3 11.4	91.5 125.8
	Z=1.397 P=0.1	Z=0.531 P=0.5	Z=1.594 P=0.1	Z=1.262 P=0.2	Z=1.026 P=0.2
PS: 0 (54) 1,2 (22)	3.5 3.8	30.7 45.6	67.5 55.5	10.6 17.7	98.7 265.5
	Z=0.732 P=0.4	Z=0.929 P=0.3	Z=2.191 P=0.02	Z=3.187 P=0.001	Z=3.318 P=0.001
Size of the largest nodule <3cm (16) ≥3cm (60)	2.4 3.8	15.7 39.5	65.9 65.2	10.2 13	52.9 124.8
	Z=1.814 P=0.07	Z=2.286 P=0.02	Z=0.286 P=0.7	Z=1.236 P=0.2	Z=2.666 P=0.008
Nodules: Single (40) Multi-nodular (36)	3.2 4.6	30.7 39.5	66.4 55.7	11.4 13	118.1 124.8
	Z=2.876 P=0.004	Z=0.858 P=0.5	Z=0.0 P=1.0	Z=0.293 P=0.7	Z=0.702 P=0.4
AFP: <44 (µg/L) (38) ≥44 (µg/L) (38)	3.4 4.1	28.8 56.1	55.5 66.4	14.1 10.6	117 155.8
	Z=2.315 P=0.02	Z=2.923 P=0.003	Z=0.409 P=0.6	Z=0.76 P=0.4	Z=1.227 P=0.2

PS: performance status, AFP: alpha feto protein

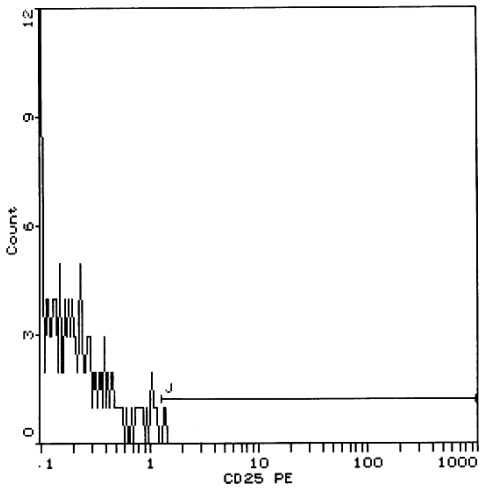


A: Lymphocyte gating using foreword scatter versus side scatter.

B: CD4+ cells gating



C: T-regs (Gate F region 2), T-effs (Gate G region 2)



D: CD25 expression on non-CD4+ T cells to discriminate between CD4+/CD25+ high and low cells

Figure (1): The gating strategy; $CD4^+$ cells were acquired after gating lymphocyte population by forward- and side-scatter properties. Gating approach; for discrimination of $CD25^{high}$ (T-regs) and $CD25^{low}$ (T-effs) cells were set using CD25 expression levels on non-$CD4^+$ T cells (low expression only), as described in Subjects and Methods section.

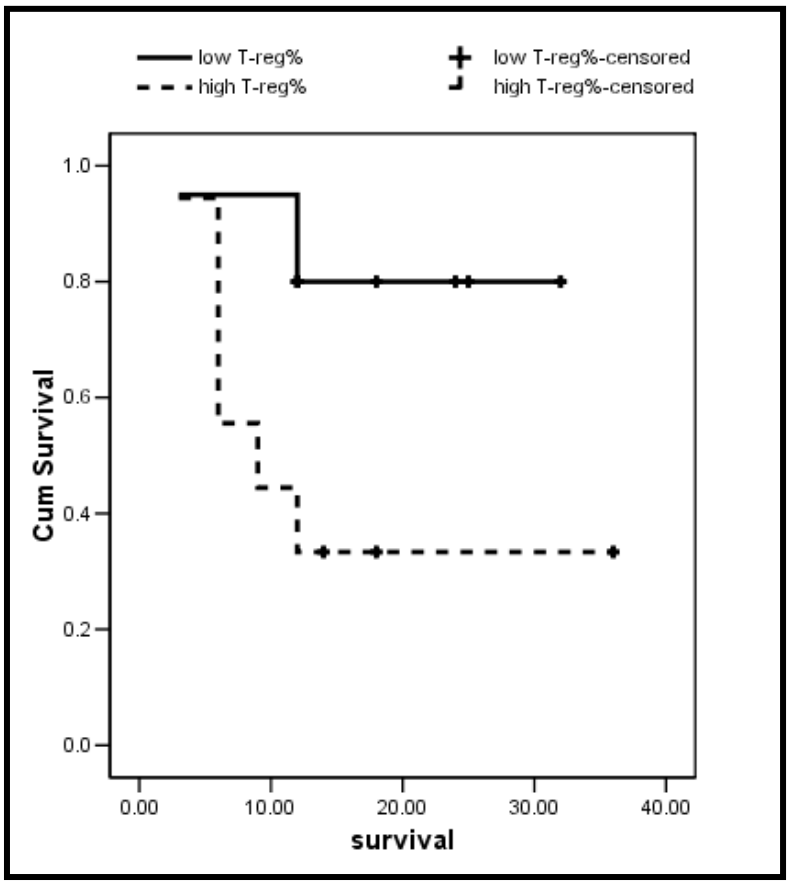


Figure (2): T-reg % and probability of survival

Discussion

T-regs have been related to chronicity and progression of viral hepatitis by down-regulating hepatitis virus-specific T cell response [14-16]. In established HCC, T-regs infiltration play an important role in tumor progression and clinical behavior as a result of T-regs-mediated suppression of anti-tumor immunity that hinders an effective immune response [17].

Cao et al.[3] reported a significantly higher percentage of circulating T-regs in HCV-related HCC when compared to healthy controls. This is consistent with our results as we found higher circulating T-regs% and effector% in our HCV-related HCC patients. However, Yang et al. [18] who studied 60 HCC most of them were as consequence of HBV found that the proportion of T-regs in the PB of HCC patients was significantly decreased. Unitt et al. [4] who studied 25 HCC as a consequence different predisposing factors reported that circulating T-regs in HCC patients was not increased compared to controls, or patients with chronic hepatitis or liver cirrhosis. Too few samples and different patient inclusion criteria has potentially led to conflicting results in these previous studies. On the other hand, unlike Ormandy et al.[19] who found that in HCC patients the absolute number of T-regs in the PB was significantly increased, our patients showed lower absolute count of both T-regs and T-effs, which could be explained by that our patients had low total leukocyte count as they developed HCC on top of chronic HCV infection.

In our study, PB T-regs% was insignificantly higher (with borderline P=0.052) in G2 than G1, which denoted active accumulation of both cells in the PB of patients during stage progression. Liu and Li [20] admitted that HCV- activation of T-regs may be associated with chronicity of infection and subsequent predisposition to development of HCC by the integrated viral genome. Subsequently, persistence of T-regs activation may lead to immune evasion of cancerous cells and thus persistence of the carcinomatous state.

A few previous studies investigated the significance of T-regs with various clinico-pathologic features. Our results demonstrated that PB T-regs% was not correlated with serum albumin, ALT, AST, however, prothrombin time was inversely correlated with circulating T-regs FoxP3 expression and directly correlated to T-effs%. Ormandy [20]; Unitt et al. [4] and Yang et al. [21] reported that the proportion of T-regs was not correlated with hepatitis and cirrhosis markers in their studies. Interestingly, T-regs% and T-effs% were inversely correlated to direct and total bilirubin respectively, while T-regs FoxP3 expression was directly correlated to total and direct bilirubin. Bilirubin has been recognized as a powerful immunomodulatory agent. In animals, bilirubin has a suppressive effect on T cell responses through various mechanisms, including inhibition of TCR signaling, down-regulation of costimulatory activity, suppression of immune transcription factor activation, and induction of reactive T cell apoptosis when used at high concentrations [22]. Therefore, further correlation studies are warranted on larger populations with different clinico-pathological features.

In our study, PB T-regs% and T-effs% were inversely correlated to Hb levels while their absolute numbers were directly correlated to platelets count. In patients with squamous cell carcinoma of the head and neck, T-regs count was increased while Hb level was decreased with early recurrence [23] for further studies.

Kobayashi, [24]; Shen et al.[25] and Zhou, [26] indicated that the increased frequency and expanded function of T-regs in the tumor microenvironment of HCC correlated with cancer stage. On the other hand, in accordance with Kobayashi,[24] and Zhou,[26] no significant differences between number of circulating T-regs and tumor stage were observed in our HCC patients. Thus, the informative value of monitoring of T-regs kinetics in the PB for staging and subsequent clinical outcome is limited.

In accordance with Yang et al.[21], circulating T-regs% in our study was significantly associated with high serum AFP, tumor size and multiple tumor foci. Our data at this point denoted that increased PB T-regs in HCC is related to tumor

burden and disease progression. However, it remains to be clarified; how the increase in PB T-regs contributes to immune tolerance or inhibition of effective antitumor immune responses in HCC.

In our study, patients with higher PB T-regs% had significantly shorter mean OS time. Similarly, Kobayashi et al.[24] showed that HCC patients with high T-regs had a significantly lower OS and lower disease free survival. In accordance, it was reported that a high frequency of T-regs in HCC predicted a low survival rate[17] and was an independent predictive factor of tumor recurrence after therapy[27].

In summary, our data suggested that increased circulating T-regs in our non- metastatic HCC related to HCV cirrhosis patients, particularly those with larger tumor sizes, multiple tumor foci and higher serum AFP, and was related to shorter overall mean survival time. Such finding is likely to prevent affective antitumor immune responses. Thus, it is important to consider T-regs in designing immunotherapy to HCC. We recommend further studies of circulating and intra-tumoral regulatory T-cells in HCC patients as a consequence of different reasons and in comparison to advanced carcinoma stage.

"All procedures performed in studies involving human participants were in accordance with the ethical standards of Ain Shams University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards"

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