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RESEARCH ARTICLE

High expression of the checkpoint molecule PD-1 on conventional $CD4^+$ and regulatory T cells in the peripheral blood of Hepatocellular carcinoma patients

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ABSTRACT

Background: Regulatory T cells (Tregs), possess a suppression function leading to T cells exhaustion and tumor progression through serious of signaling pathways of inhibitory receptors under pathological conditions in particular cancer including hepatocellular carcinoma. Conventional helper $CD4^+$ T cells can be converted into regulatory T cells that may suppress anti HCC immunity through different mechanisms including expression of PD-1. PD-1 inhibitory checkpoint and its ligand PDL-1 emerge immune escape through interaction between T cell and tumor microenvironment. **Aim**: The aim of this study is to analyze the expression level of the regulatory checkpoint (PD-1) on T cells in HCC patients. **Materials and Methods**: The numbers of conventional and regulatory T cells and their expression of checkpoint receptor PD-1 were analyzed in the peripheral blood of HCC patients (n=20) as well as healthy control volunteers (n=15) using multi-parametric flow cytometry after staining with anti-CD4, anti-CD25, anti-CD127 for (T cells) and anti PD-1 (CD279). **Results**: As compared to healthy control volunteers HCC patients showed high relative numbers of Tregs expressing $CD4^+CD25^+CD127^-$ and decrease relative number of conventional $CD4^+$ defines as $CD4^+CD25^-CD127^-$. These cells showed high expression by increasing level of PD-1 where $CD4^+$ showed high expression than Tregs. Similar cells number of PD-1 expression were observed at the level of absolute numbers. **Conclusion:** HCC patients before treatment express high level of the checkpoint molecule PD-1 opening the door for further investigations.

Keywords: Cancer, CD4, Checkpoint, Hepatocellular carcinoma, immune, PD-1, Regulatory T cells

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INTRODUCTION

Lung cancer is known to be one of the most serious types of cancer in terms of both incidence and mortality. It accounts for more annual deaths than breast, prostate and colon cancers combined, representing approximately one-third of the total cancer-related mortalities (Wang, Guo et al. 2018). The most recent report estimated 2.1 million new lung cancer cases and 1.8 million deaths predicted in 2018 around the world (Ilbawi and Velazquez-Berumen 2018). Unfortunately, it holds a poor prognosis as its 5-year overall survival (OS) rate ranges between 5-15% (Black and Khurshid 2015). Despite the progress made in the treatment methodologies implied in lung cancer, it is primarily still treated by surgery, chemotherapy or radiation in different combinations. The limited success of these therapeutic interventions has been observed, as only 15%–30% of patients with NSCLCs respond to treatment highlighting the urgent need for novel therapeutic approaches (Aktaş, Öztürk et al. 2018).

Therefore, novel approaches for the treatment of lung cancer have been developed such as immunotherapy and

gene therapy (Lara-Guerra and Roth 2016). These new multimodal treatments have the ability to enhance cancer treatment in combination with conventional chemotherapy such as (1) Immune checkpoint inhibitors like PD1/PD-L1 or (2) Antigen-specific vaccines Mucin 1, L-BLP25, GVAX and GD3 or (3) Adoptive T cell transfer (CAR T-cell) all of which function by augmenting the immune system to kill cancer cells. While very promising, the presence of some limitations including tumor secretions, the physical barrier of trafficking and homing to the tumor, prevent the dependence on such treatments (Hiraki, Suzuki et al. 2016, Yang, Wang et al. 2016).

Recent studies have reviled that the development and progression of lung cancer are linked with the number and functionality of adoptive immune cell that leads to weakened antitumor immunity. Cytotoxicity plays a vital role in the immune system, which is carried out by various immune cells. Cytotoxic T cells CD8+ and natural killer cells (NK) conduct this process via two main pathways, in the first pathway CTLs use the ligands of the TNF superfamily present on their cell surface to bind and eliminate target cells (Paul and Lal 2017). Meanwhile, the second pathway depends on exocytosis of the cytolytic granular content of the immune cells (perforin, granzyme and interferon-gamma) into cancer cells leading to their immediate decomposition (Street, Hayakawa et al. 2004).

In lung cancer, as CTL, NK and NK T cells take part in the immune defense mechanism, the ability of cancer cells to resist these cytolytic mediators is essential for their survival and proliferation. This resistance can be attributed to the release of certain mediators from lung cancer cells such as prostaglandin E2 (PGE2) and transforming growth factor (TGF)-β, which play an important role in assisting tumor cell proliferation, anti-apoptotic properties, angiogenesis and chemotherapeutic resistance (Barrett, Millena et al. 2017). In addition, previous studies have correlated between the significant reduction of the cytolytic granzyme B levels in cancer tissues compared with non-cancer tissues, linking this finding with the decreased cytotoxic activity of immune cells and the establishment of a permissive tumor microenvironment (Hodge, Barnawi et al. 2014).

On the other hand, recent studies have reported that in non-treated lung cancer patient's levels of plasma cytokines and proteins play a key role in mediating the innate and adaptive immunity (Silva, Mariano et al. 2017). These cytokines were correlated with poor prognosis and increased risk of death by shaping different constituents of the tumor microenvironment (Mantovani, Barajon et al. 2018).

In this study, we aim to address the functionality of CD8+ T, NK and NKT cells by measuring GzB in early diagnosed lung cancer patients and correlate the findings with the cytokine and chemokine profile.

MATERIALS AND METHODS

Subjects' populations

In this study blood samples were obtained from volunteering healthy individuals (n=5) and early diagnosed lung cancer patients (n=5) by experienced oncologists at Oncology Department, Tanta University Hospital. All patients are given the informed consent under a protocol approved by Faculty of Medicine Ethical Committee Review Board, Tanta University. Patients were diagnosed according to world health organization (WHO) criteria based on TNM classification (Travis, Brambilla et al. 2015). Classification of subjects was performed

after detection of inclusion criteria and patient's performance.

Chemical and reagents

Lymphocyte separation medium (Ficoll paqué TM) was purchased from Lonza (Basel, Switzerland). Phosphate buffer saline (PBS) was obtained from (Verviers, Belgium). CD3 (perCP.Cy5•5), CD4 (Allophycocyanin APC-A), CD56 (FITC), granzyme B (GzB) Phycoerythrin (PE), FACS Perm were purchased from (BD Biosciences (BD), San Jose, CA, USA). Sheath Fluid was purchased from (Luminex Corp, Austin, TX, USA).

Preparation of samples PBMCs separation

Blood collection was on K2EDTA tubes for plasma separation, PBMCs were separated using Ficoll paquéTM technique in duplicate using break off centrifugation, washed twice using cold PBS and counted using a hemocytometer to check the viability and to determine the number of PBMCs in the sample to determine the actual number of cultured cells.

Surface and intracellular staining of PBMCs

After separation with Ficoll, cells were induced to surface staining of anti-CD3 (perCP.Cy5.5), anti-CD4 (APC-A) and anti-CD56 (FITC) and intracellular for granzyme B by using anti-GzB (PE).

Measurements of plasma cytokines and chemokine levels

Plasma specimens were evaluated with a total of 104 assays (consisting of 102 unique analytes), performed using Luminex immunobead assays as indicated below. All primary data points were collected on a Luminex FLEXMAP 3D® system. Analyte concentrations were calculated from a 7-point curve using a five-parametric fit algorithm (xPONENT® v4.0.3 Luminex Corp., Austin, TX). All data met minimum quality control thresholds defined by the kit manufacturer with a percent coefficient of variation (%CV) values ≤10%, all as previously defined (Fidler, Frankenberger et al. 2017).

Statistical analysis

Data were analyzed using SPSS software version 22 and Mann–Whitney or one-way analysis of variance (a nova) with post-hoc for non-parametric analyses. Correlations were performed using Spearman's rank test. Analyses were performed using SPSS software. p values < 0·05 were considered significant.

RESULTS

Decreased $CD4^+$ cells in HCC patients as compared to healthy control

Flow cytometric analysis in the peripheral blood of HCC patients revealed that the relative and absolute numbers of $CD4^+$ T cells population was significantly decreased in HCC patients as compared to healthy control volunteers (Figures 2,3). Of note, the numbers of CD4+ T cells population were variable among HCC patients.

Increase in the numbers of Tregs in peripheral blood of HCC patients as compared to healthy control volunteers

We analyzed the total numbers of Tregs in HCC patients as compared to healthy control volunteers Figure (4). We found that the percentages and absolute numbers of $CD4^+CD25^{High}$ $CD127^-$ Treg cells were significantly increased in HCC patients as compared to healthy control volunteers (Figure 5).

Investigation of PD-1 expression on conventional $CD4^+$ T cells

PD-1 expression level on conventional $CD4^+$ in the peripheral blood of patients were significantly increased in HCC patients as compared to healthy control volunteers as shown in Figure (6). Of note, higher level of PD-1 expression was more pronounced when the number of Conventional $CD4^+$T cells was decreased.

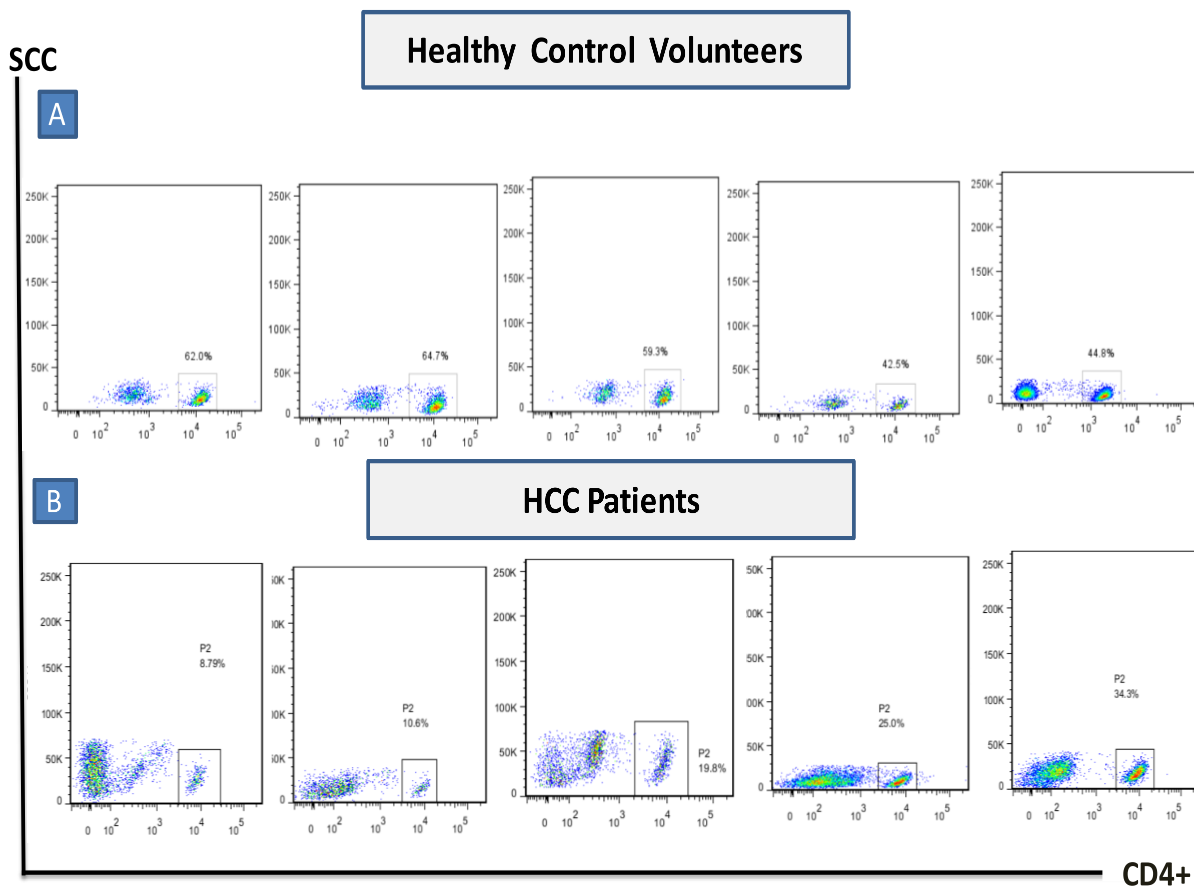

Figure 2. A representative flow cytometric analysis showing the relative number of CD4⁺ T cells in 5 out of 20 HCC patients as well as 5 out of 20 healthy control volunteers. CD4⁺ T cells numbers in healthy control volunteers (A) and in HCC patients' group (B).

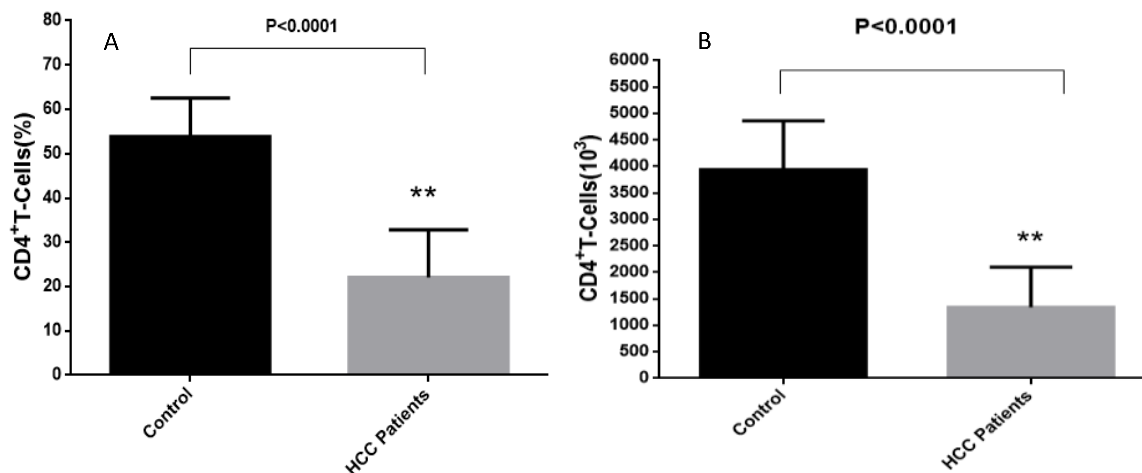

Figure 3. A representative statistical analysis showing the relative and absolute numbers of CD4⁺ T cells in HCC patients and healthy control groups. CD4⁺ T cells percentage (A) and absolute numbers (B) as compared to healthy volunteers. The absolute numbers of CD4⁺ T cells were calculated as: Total white blood cells [cells/μl]x CD4⁺ %) %100.

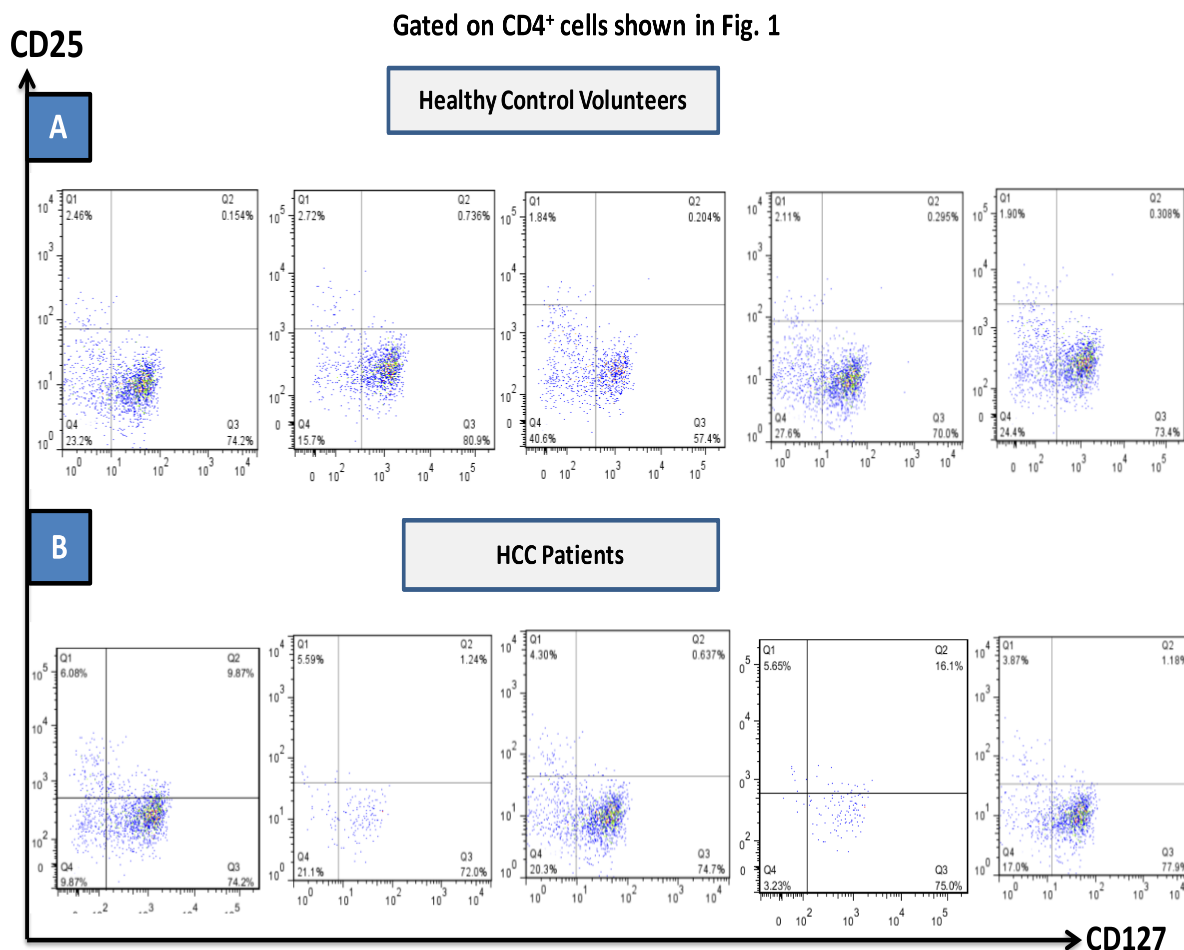

Figure 4. A representative flow cytometric analysis showing the relative number of $CD4^{+}CD25^{High}CD127^{-}$Tregs cells. $CD4^{+}CD25^{High}CD127^{-}$Tregs cells were analyzed using $CD25^{High}CD127^{-}$ markers gated on $CD4^{+}$ T cells populated in the two groups. $CD4^{+}CD25^{High}CD127^{-}$Tregs numbers in healthy control volunteers (A) and in HCC patients' group (B).

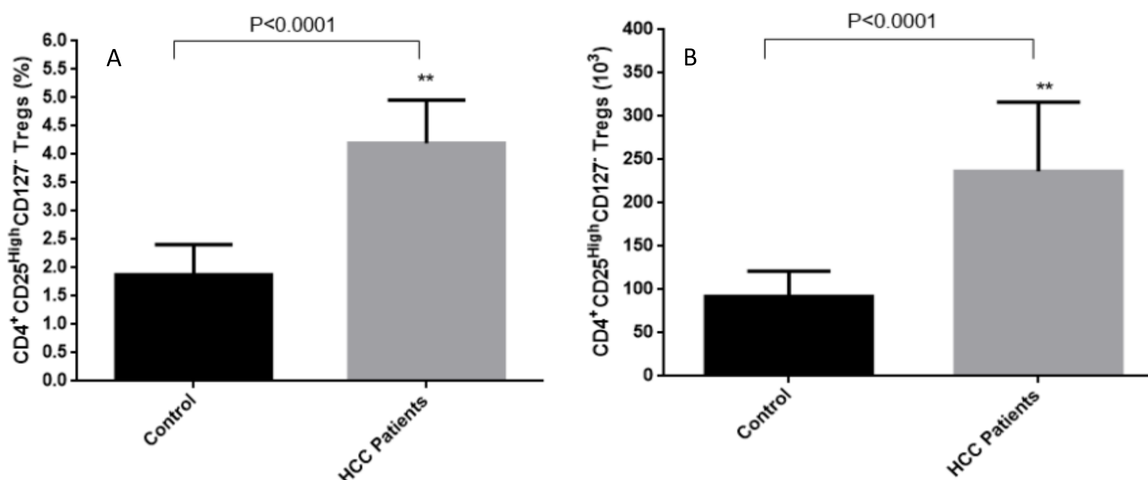

Figure 5. A representative statistical analysis showing the relative and absolute numbers of $CD4^{+}CD25^{High}CD127^{-}$Tregs in HCC patients' groups and healthy control volunteers. $CD4^{+}CD25^{High}CD127^{-}$Tregs cells percentage (A) and absolute numbers (B). The absolute numbers of Tregs were calculated as: Total white blood cells [cells/µl]x $CD4^{+}CD25^{High}CD127^{-}$Tregs%) %100.

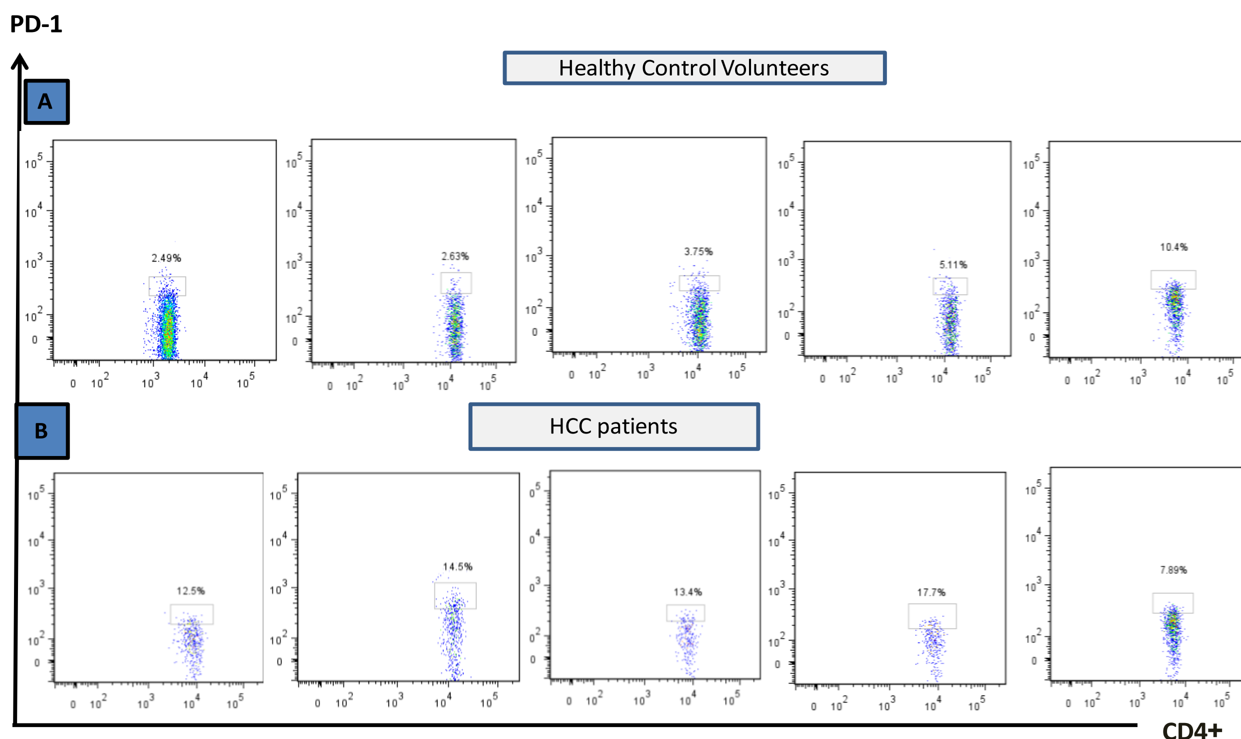

Figure 6. A representative flow cytometric analysis showing expression level of CD4+ T cells in healthy control volunteers (A) and in HCC patients' group (B).

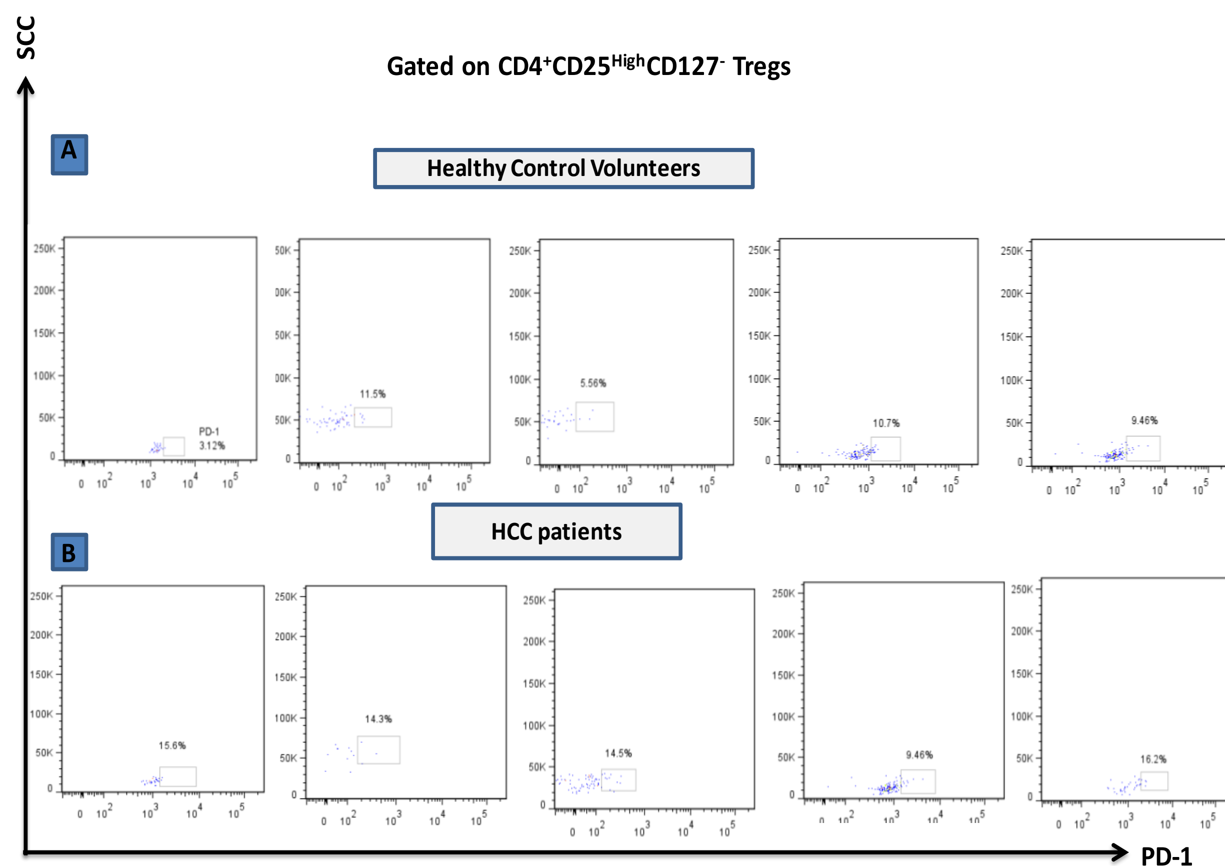

Figure 7. A representative flow cytometric analysis showing expression level of PD-1 gated on $CD4^{+}CD25^{High}CD127^{-}$ Tregs in healthy control volunteers (A) and in HCC patients' group (B).

Increased (PD-1) on Tregs in HCC patients

To check the phenotype of Tregs and also as inhibiting marker, we analyzed the expression level of PD-1 on $CD4^{+}CD25^{High}$ $CD127^{-}$Tregs. PD-1 showed higher expression on Treg cells in HCC patients as compared to healthy control volunteers (Figure 7). In control Treg cells expression ranged from 3.12 to 11.5 % while in HCC patients ranged from 9.48 to 16.2%.

DISCUSSION

In this study, the immunophenotypic analysis was performed on the peripheral blood of HCC patients in order to assess the number of conventional CD4 and Treg cells CD4+CD25HighCD127- and the expression of the inhibitory receptor PD-1. The analysis showed high and low number of Treg and conventional CD4+ T cells respectively, in peripheral blood of HCC patients as compared to healthy control volunteers. Our findings are consistent with previous studies (Sharma, Khosla et al. 2015) which showed that viral infection also associated in increase in the number of CD4+CD25HighCD127-Treg cells phenotype. In our study also, proportion and absolute number of CD4+CD25HighCD127. Tregs were significantly higher in HCC patients as compared to healthy control volunteers, Similar findings were reported (Huang, Wang et al. 2012).

In addition, our study showed inverse relation between number of CD4+ CD25HighCD127- Tregs and percentage of Conventional CD4+ helper T cells population. CD4+ helper T cells paly a fundamental role in activation, expansion and survival of immune protection through a coordinating and enhancing immune responses in sites of infection, inflammation and antitumor immunity through regulation the full categories of immune subtypes (Bevan 2004, Zhu and Paul 2008). Our results reported that, Decrease of CD4+ T cells assist in manifestation the immune suppressive function of regulatory T cells among HCC patients. The inverse relation between conventional CD4+ helper T cells and proportion of Tregs explain the significant increase in proportion of CD4+CD25High CD127- Tregs in HCC patients. Our results agreed with studies in explanation that a large number of Treg cells can infiltrate into tumor tissues of various cancer types and their ample presence is often associated with poor clinical prognosis (Tanaka and Sakaguchi 2017).

In the present study, PD-1 showed up-regulation on both $CD4^{+}$T cells and $CD4^{+}CD25^{High}CD127^{-}$Treg cells. Our results are consistent together with the result of (Xu, Chen et al. 2014, Sharma, Khosla et al. 2015) which suggest that the level of PD-1 on Treg cells in HCC patients occur regardless the cause of the disease. High expression of PD-1 among $CD4^{+}$ T cells and Treg cells in HCC patients group suggested that those inhibitory signals released from PD-1 expressed on both $CD4^{+}$ T cells and Tregs will with no doubt increase inflamed tumor microenvironment and help in tumor-immune escape which leads to tumor progression.

CONCLUSION

The data of this study spotted a light on the suppressive impact of regulatory T cells and not only they have immunosuppressive in numbers but also may contributed to patient's poor prognosis and clinical performance. Further studies are needed to characterize the impact of suppressive function of Tregs with or without PD-1 in HCC patients.

CONFLICT OF INTEREST

There is no conflict of interest.

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