World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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# Determination of function of regulatory T cells and associated cytokines in colorectal cancer patients at the local site and peripheral blood: A comparative study

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World Journal of Advanced Research and Reviews, 2023, 19(01), 736–743

Publication history: Received on 24 May 2023; revised on 12 July 2023; accepted on 15 July 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.19.1.1356

## Abstract

Colorectal cancer (CRC) is a common lethal cancer worldwide as genetic as well as environmental factors influence it. CRC is heterogeneous, with a diverse and plastic immune cell infiltration. Among the numerous tumoral antigens that could induce an immune response in CRC, the carcinoembryonic antigen is the most studied, but its immunogenicity remains low. Infiltrate of $CD3^+$ and $CD8^+$ T cells into CRC tumors has been validated worldwide as a valuable indicator of patient prognosis. Even though a high T cell infiltrate is a better indicator of patient survival than traditional histologically based staging methods, the immune response in colorectal cancer (CRC) has not yet been reliably and effectively harnessed to treat patients of all stages and types of disease. The management of colorectal cancer is fraught with challenges, and the lack of reliable biomarkers and laboratory tests is a major bottleneck in existing treatment strategies. This study explores and attempts to resolve some of these issues.

**Keywords:** Colorectal cancer (CRC); Treg cells; Cytokines; Peripheral blood; Immunomodulation

## 1. Introduction

Colorectal cancer (CRC) is the third most common cancer in terms of detection (6.1%) and second in mortality rate (9.2%) (1). Wherein males have higher rates of both mortality and incidence of CRC. Depending on the economic development of the country, the statistics may vary. Hence, CRC is generally known as an indicator of a country's socioeconomic status (2). Environmental factors and physical health play a key role in this disease. Furthermore, it has been observed that consumption of red and processed meat and alcohol increases the risk of developing disease [2, 3]. Thus, concluding that dietary patterns, lifestyle, and body fatness are the important factors in influencing the morbidity of the disease (4).

In recent years, immunotherapy has been in highlights for the treatment of cancer as a substitute for traditional treatments like chemotherapy, radiotherapy, or surgery. Immunotherapy has made possible to generate a positive immune response in metastatic melanoma by adoptive transfer of antigen-specific T cells (5). It has shown an impressive way of decreasing mortality in cancer patients (6). Regulatory T (Treg) cells were first defined by Shimon Sakaguchi in 1995. Since then, Treg cells have been studied to play key roles in most immunological processes elicited by self or non-self antigens in oncological, parasitic, inflammatory, and autoimmune diseases (7). These cells, as a subpopulation of T cells, were first characterized by the expression of CD25 (alpha chain receptor for IL-2), which is typically expressed on activated T cells. Later, it was shown that forkhead box p3 transcription factor (Foxp3) is a specific marker for Treg cells that confer suppressor activity on these cells [8, 9]. Therefore, Foxp3 is known as a major regulator of Treg cells (10).

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Treg cells suppress immune responses through various mechanisms, such as the manipulation of antigen-presenting cells by inducing a "tolerant phenotype" through Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), and the Lymphocyte Activation Gene-3 (LAG-3) to induce the Indoleamine 2,3-dioxygenase (IDO) enzyme, which in turn reduces the availability of tryptophan in the environment along the kynurenine pathway [11–13] the use of CD39 and CD73 ectoenzymes for the release of extracellular adenosine, which is a strong immunosuppressant [14–16], the secretion of suppressor cytokines such as IL-35 [17, 18], TGF-α [19, 20] and IL-10 (21), and deprivation of IL-2 by its IL-2 (CD25) high-affinity receptor [22–25]. Treg cells have also been reported to use granzyme and perforin-like molecules as a suppressive mechanism [26, 27].

Interestingly, a higher level of expression of Treg cell suppression molecules like TGF-β, CTLA-4, Tim-3, CD-25, IL-10, and LAG-3 is observed in CRC patients (28). It is also shown in a study that a higher level of Treg cell infiltration happens in CRC patients as compared to healthy individuals (29). Furthermore, the lymphatic invasion was found to be associated with the frequency of Foxp3+ cells in patients (30). The increased lymph node metastasis and the degree of tumour malignancy have been shown to have a correlation with an increase in the expression of Foxp3 in peripheral blood as well as colorectal tissue (31). Additionally, a higher Treg cell percentage has been demonstrated in mesenteric lymph nodes and peripheral blood in CRC patients than in healthy controls (32).

Hence, further evidence for the role of Treg cells in the development of tumours and immunosuppression in patients with CRC should be described. So, in this study, we have inspected Treg cells frequency in peripheral blood as well as colorectal tissue in CRC patients. Moreover, the pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-10, TGF-β and IL-4) cytokine specific cells and correlation of these cytokines with disease severity has been observed.

## 2. Material and methods

### 2.1. Study Participants

We undertook the present study among newly diagnosed colorectal patients. These patients were recruited respectively from the Out Patient Department (OPD) of the Department of Surgical Oncology, IRCH, All India Institute of Medical Sciences (AIIMS), New Delhi. In this study, we recruited patients who were considered for biopsy/surgery either for diagnosis or treatment. Written informed consent was taken from all the study participants before their enrolment in the study. The participation of study subjects was voluntary, and they were free to withdraw at any time. Ethical clearance for this study was obtained from Institutional Ethics Committee, AIIMS (Ref. No.: ICE-547/02.08.2019, RP-05/2019), New Delhi.

Colorectal cancer patients aged 40 years or above at the time of diagnosis and past cases over 40 years but previously diagnosed when aged 40 years or below were included. The cases (n=22) have documentary evidence of pathologically confirmed adenocarcinoma of the colon or rectum. All consecutive patients of colorectal cancer, either visiting the outpatient clinics or admitted to the inpatient wards of the department of pathology, AIIMS, were enrolled in the study after proper informed consent and assessment.

### 2.2. Sample collection and cell isolation

Both peripheral venous blood (in heparinized vacutainers, 6-8 ml) and tissue specimen (in complete media) were collected from all the study participants. Peripheral Blood Mononuclear Cells (PBMC) were isolated from heparinized blood using Ficoll histopaque gradient centrifugation and suspended in RPMI-1640 supplemented with 2 mM Glutamine, HEPES, antibiotics, Penicillin, 100 units/ml & Streptomycin, 100 µg/ml and 10% heat-inactivated fetal calf serum (FCS). After washing with RPMI supplemented with FCS, the cells were used for subsequent immunophenotyping and *in vitro* cell culture. Trypan blue dye exclusion test was used to measure live/dead cells.

### 2.3. *In vitro* Cell Culture

Culturing of T cells was done in 96 well U-bottom pre-coated with purified anti-CD3 and anti-CD28 antibodies, 10 µg/ml each for 2 hrs at 5% $CO_2$ & 37 ºC. The wells were then washed twice with sterile PBS. PBMCs were then seeded in these wells for 48 hrs along with Brefeldin-A (supplemented at the last 24 hrs of cell culture) for the phenotypic and functional characterization of Th1, Th2, and Treg cells using Flow Cytometry. Collected serum samples were used for ELISA based quantification of IL-10 and IFN-γ.

### 2.4. Flow Cytometry Based Detection

Cells were used for subsequent immunophenotyping and functional characterization of various cell types using polychromatic flow cytometry (BD LSR Fortessa X-20). Treg (FoxP3) Staining of PBMCs was done using an anti-FoxP3 antibody.

For the detection of intracellular cytokines, cells were stimulated *in vitro* with anti-CD3/CD28 for 48 hrs at 5% $CO_2$ at 37 ºC, and then Brefeldin-A was added for the last 24 hrs. Cells were harvested and stained for T cell surface markers using anti-CD4 and intracellular cytokines using anti-IFN-γ, anti-TNF-α, and anti-IL-2.

## 3. Results and discussion

We enrolled 22 participants (colorectal cancer patients) for the current study. The blood and tissue samples obtained from these participants were used for the isolation of mononuclear cells. These cells were then used for *ex vivo* enumeration of the percent frequency of $CD4^+CD25^+Foxp3^+$ T cells (regulatory T cells) in blood and at the local site (tissue) (Figure-1).

### 3.1. Significantly high frequency of Treg cells at the local site in patients with colorectal cancer

Our aim was to evaluate the frequency of Treg cells ($CD4^+$ $CD25^+$ $Foxp3^+$). We observed that the percentage frequency of Treg cells at the local site was significantly high (N=22; mean ± SD: 6.37 ± 4.0%) compared with autologous peripheral blood sample (N=22; mean ± SD: 3.76 ± 2.2%) of patients. {p= 0.003; non-parametric Mann-Whitney U test} (Figure-4A). The activated T cell frequency ($CD4^+CD25^+$) was also higher at the local site (Figure- 2-I & II). Further, we also compared the percentage Treg frequency in the peripheral blood of the patient and compared it with healthy controls, wherein we observed a non-significant change in peripheral blood (Figure.4A).

### 3.2. Significantly high frequency of $CD4^+$TNF-α and IFN-γ cells at local site in patients with colorectal cancer

We delineated the pro-inflammatory specific cells (mainly TNF-α and IFN-γ) and observed that the frequency of $CD4^+$ IFN- γ was significantly high at the local site in patients (N=22; mean ± SD: 2.58 ± 0.44%) compared with the peripheral blood (N=22; mean ± SD: 2.24 ± 0.34%) {p = 0.01; non-parametric Mann-Whitney U test, two-tailed for unpaired data} (Figure 4B). Further, we compared the same between the peripheral blood of patients with healthy study subjects and observed that the frequency was significantly high in patients' peripheral blood (p =0.042). Similar trends were observed for $CD4^+$ TNF-α cells in patients' PBL vs. local site (p = 0.013) and PBL of patient vs. PBL of healthy controls (p =0.042) (Figure- 4C). Furthermore, we have also evaluated the soluble level of IFN-γ in the serum of patients with that of healthy controls. We observed a significantly increased level of IFN-γ in the serum of patients (N=22; mean ± SD: 26.54 ± 11.97pg/mL) compared with the peripheral blood (N=18; mean ± SD: 19.10 ± 5.29pg/mL) (Figure- 4D).

### 3.3. No significant change in IL-10 level in patients with colorectal cancer

After the evaluation of pro-inflammatory cytokine-specific cells and levels, we were interested in evaluating the immune suppressive/anti-inflammatory cytokine level; for that, we assessed the soluble level of IL-10 in patient serum and compared it with the serum of healthy controls. However, we did not observe any significant change in IL-10 level (p =0.57) (Figure- 4E).

### 3.4. No clear correlation was seen in pro-inflammatory and anti-inflammatory/immunosuppressive cytokines and cells in patients with colorectal cancer

It is well known that if one arm of the immune system is overactive, then that arm suppresses the other arm; in the current pilot study in colorectal cancer patients, the pro-inflammatory cytokine milieu was observed to be higher than the anti-inflammatory cytokine milieu. However, we did not observe any significant and negative correlation between pro-inflammatory cytokines and suppressive cytokines produced by Treg cells (Figure- 5A, 5B & 5C)).

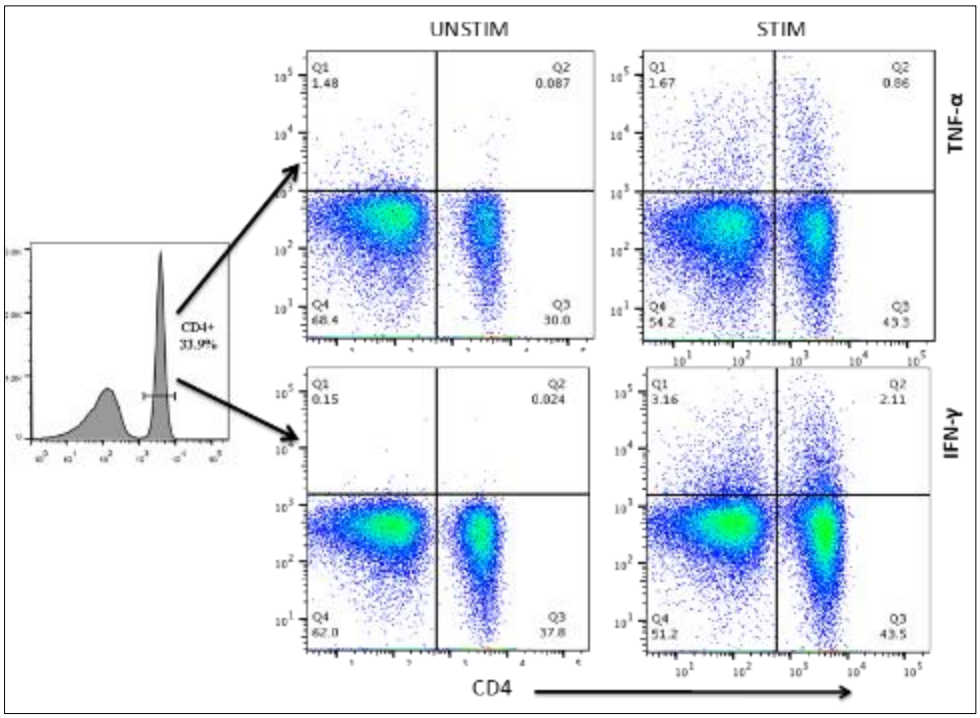



**Figure 1** Representative flow cytometry plot of CD4⁺ T Cells producing TNF-α & IFN-γ. Different panels were used for surface and intracellular staining for cells specific markers; Panel-1 (Treg cells) shows CD4, CD25, Foxp3; Panel-2 (Pro-inflammatory cytokines) shows CD4, IFN- γ & TNF-α; and Panel-3 (Treg specific IL-10) shows CD4, Foxp3 & IL-10

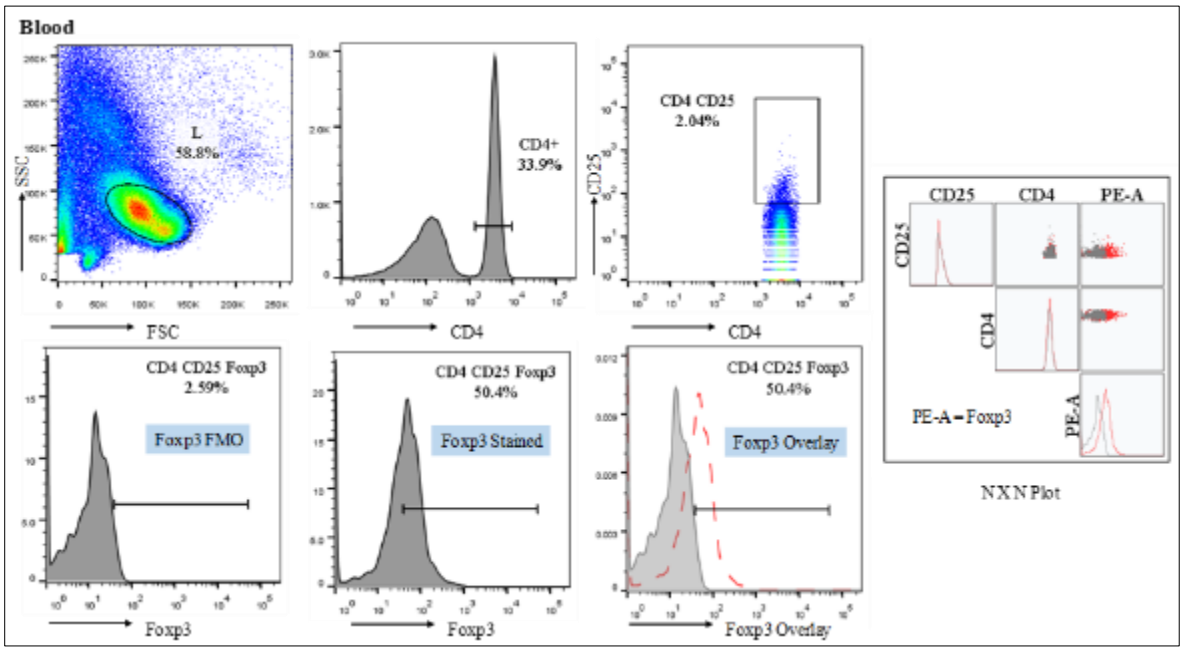



**Figure 2-I** Representative flow cytometry plot of Foxp3 expression on CD4⁺ CD25⁺ T cells in PBL

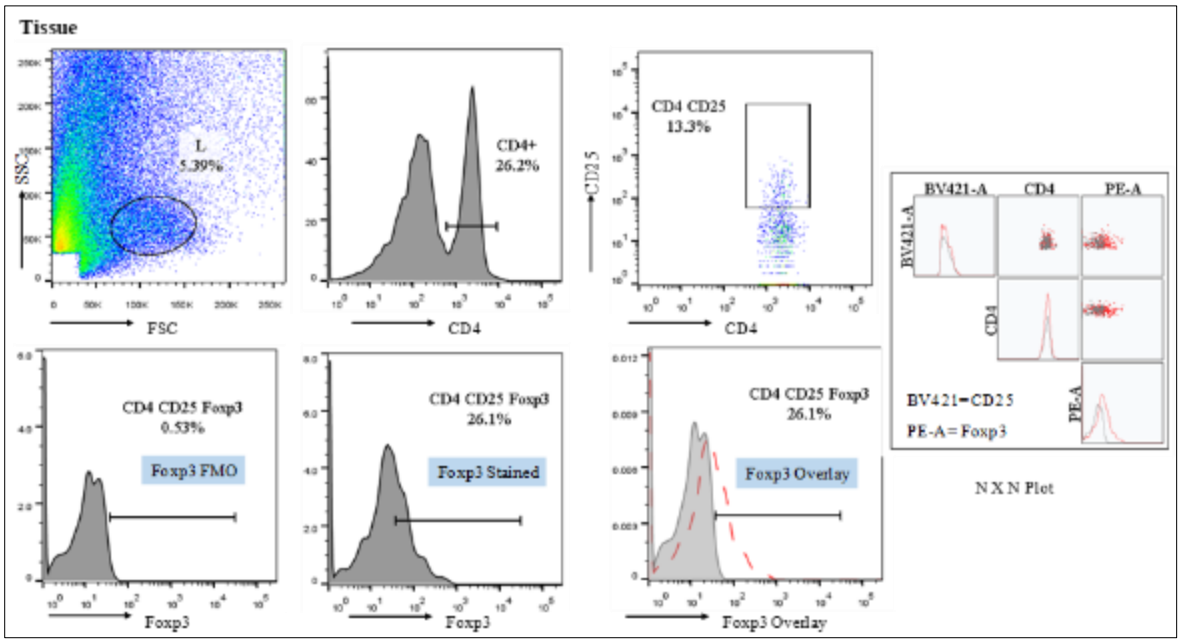



**Figure 2-II** Representative flow cytometry plot of Foxp3 expression on $CD4^+$ $CD25^+$ T at a cancer patient's local site (tissue) sample

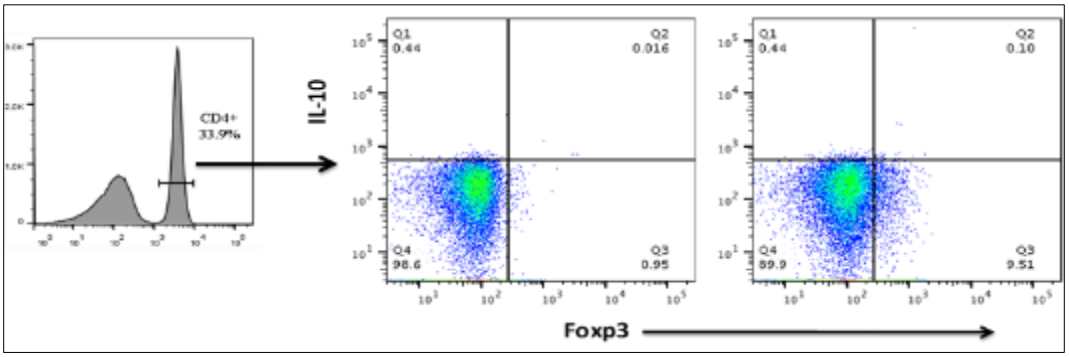



**Figure 3** Representative flow cytometry plot of Foxp3 & IL-10 in the PBL of patient

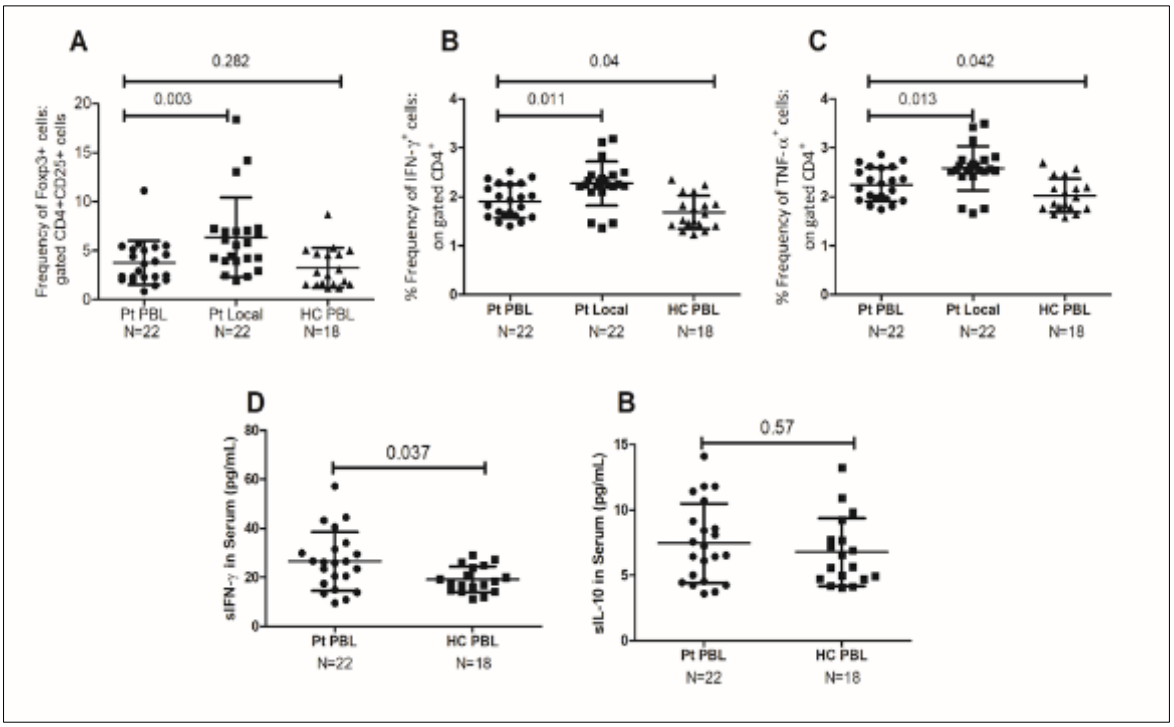
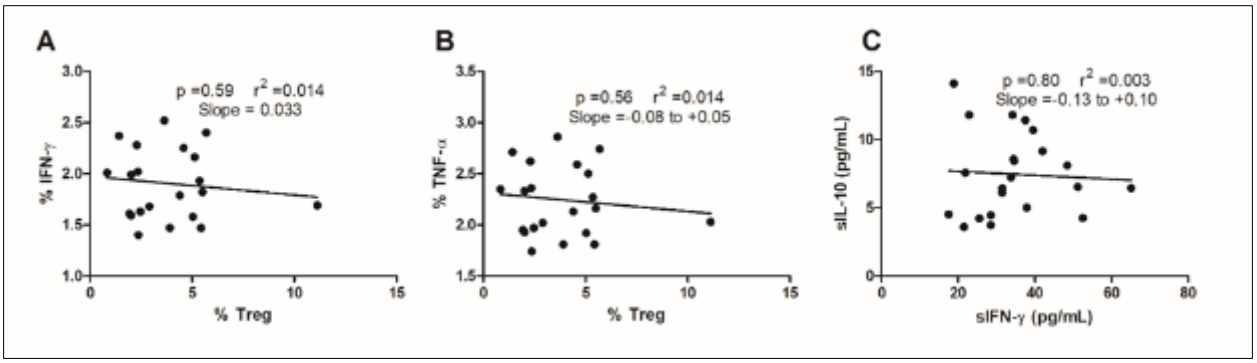



**Figure 4** Cumulative data of recruited study subjects: A) Treg frequency ($CD4^+CD25^+$ $Foxp3^+$); B) $CD4^+$ IFN-$\gamma^+$ cells; C) $CD4^+$TNF-$\alpha^+$ cells; D) Soluble level of IFN-γ and in blood serum and E) Soluble level of IL-10 in blood serum




*Correlations are of peripheral blood samples of patients only

**Figure 5** Correlation between A) % Treg ($CD4^+CD25^+$ Foxp3) frequency vs. % IFN-$\gamma^+$ $CD4^+$ cells B) % Treg ($CD4^+CD25^+$ Foxp3) frequency vs. % TNF-$\alpha^+$ $CD4^+$ cells C) Soluble level of IFN-γ vs. soluble level of IL-10.

## 4. Conclusion

Colorectal cancer is a state of inflammatory condition, especially at the local site; the T cells are hyperactive for eliciting an effective and dominant Th1 response (IFN-γ). Our results demonstrated that the frequency of Treg and activated T cells at the local site was much higher than in patients' peripheral blood. No significant change was observed in Treg cell frequency in the peripheral blood of patients and healthy control. In conclusion, Treg cell frequency in peripheral blood can't be used to detect CRC patients. Furthermore, the peripheral blood of patients showed a higher frequency of $CD4^+$ IFN-γ TNF-α than healthy control. The evaluation of soluble IFN-γ in the serum of patients and healthy control also showed a similar trend. Hence, the frequency of $CD4^+$ IFN-γ TNF-α in peripheral blood and levels of IFN-γ in the serum of patients can be further used for analysis and detection of CRC. However, no significant change was observed in levels of IL-10 in the serum of patients and healthy controls. Therefore, no correlation between pro-inflammatory and anti-

inflammatory cytokines can be determined. Further study is warranted to explore the functionality of the suppressive arm of the immune system in colorectal cancer patients.

## Compliance with ethical standards

### *Disclosure of conflict of interest*

The authors declare no potential conflicts of interest.

### *Statement of ethical approval*

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

### *Statement of informed consent*

Informed consent was obtained from all the individual participants included in the study.

### *Financial support*

All India Institute of Medical Sciences, New Delhi.

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