https://doi.org/10.31925/farmacia.2023.2.17 **ORIGINAL ARTICLE**

OPTIMIZATION OF A FLOW CYTOMETRY PROTOCOL FOR PD-1/CTLA-4 IMMUNE CHECKPOINTS RECEPTORS DETECTION IN COLORECTAL CANCER TUMOUR MICROENVIRONMENT

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Abstract

Considering the individual heterogeneity of the tumours, a personalized approach in oncology is crucial to design a particular dynamic treatment for the individual, from the diagnostic, to monitoring and therapy. In particular, the cellular makeup of the tumour microenvironment consists of diverse immune cells, which include tumour-infiltrating lymphocytes (TILs), as well as tumour-associated macrophages (TAMs) and tumour-associated neutrophils (TANs). These immune cells express various immune checkpoint molecules such as PD-1 and CTLA-4 that play a crucial role in modulating the colorectal cancer microenvironment, controlling the disease's development and response to therapy. Considering the huge potential of PD-1/PD-L1 and CTLA-4 as actionable targets for immunotherapy in colorectal cancer as well as the advantages of the flow cytometry technique, we propose here a flow cytometry method to characterize the colorectal cancer microenvironment in terms of cells populations and their associate immune checkpoint molecular profile.

Rezumat

Având în vedere heterogenitatea individuală a tumorilor, o abordare personalizată în oncologie este esențială pentru a construi un tratament dinamic individual, pornind de la diagnostic, până la monitorizare și terapie. În mod particular, micromediul tumoral este format din diferite tipuri celulare, printre care și următoarele celule imunitare: limfocite care infiltrează tumorile (*tumor infiltrating lymphocytes* - TILs), macrofage asociate tumorilor (*tumor associated macrophages* - TAM) și neutrofile asociate tumorilor (*tumor associated neutrophils* - TAN). Aceste celule imunitare exprimă diferite molecule de control imun, cum ar fi PD-1 și CTLA-4, care joacă un rol crucial în modularea micromediului tumoral în cancer colorectal, controlând dezvoltarea bolii și răspunsul la terapie. Având în vedere potențialul uriaș al PD-1/PD-L1 și CTLA-4 ca ținte probabile pentru imunoterapia în cancerul colorectal, precum și avantajele tehnicii de citometrie în flux, propunem aici o metodă pentru a caracteriza micromediul tumoral în cancerul colorectal prin citometrie în flux, pentru a evidenția subpopulațiile celulare și profilul lor molecular asociat.

Keywords: flow cytometry, immune checkpoints, PD-1 and CTLA-4, colorectal cancer, tumour microenvironment

Introduction

Colorectal cancer (CRC) is widely spread in the population of the developed countries being the third most frequently diagnosed malignancy worldwide and the second cause of cancer related death after lung cancer [1]. Several risk factors have been identified for colorectal cancer, including age, family history, inflammatory bowel disease, a diet high in red and meat, physical inactivity, as well as tobacco and alcohol use [2]. Prevention and early detection of risk factor modifications through screening *via* colonoscopy, faecal immunochemical test or sigmoidoscopy are essential for reducing the burden of this disease. Early detection is crucial in reducing the burden of colorectal cancer, as it is highly treatable in early stages [2]. The current therapeutic approach of CRC includes surgery, radiotherapy and several regimens of pharmaco-therapy [3].

While the genetic and epigenetic status of colorectal cancer cells is crucial for the overall progression of CRC, the tumour microenvironment (TME) that include stroma and resident immune cells, is a constantly changing and dynamic phenomenon that also plays a key role in the initiation and progression of this disease [4]. The TME of CRC is characterized by a unique and complex interplay between cancer cells and immune cells that evolves over time. While immune cells intend to protect the body against the tumour, the tumour try to escape take turn immune reaction to its advantage. As tumours grow, various components of the TME trigger angiogenesis and degradation of the extracellular matrix, which ultimately leads to invasion and metastasis [5].

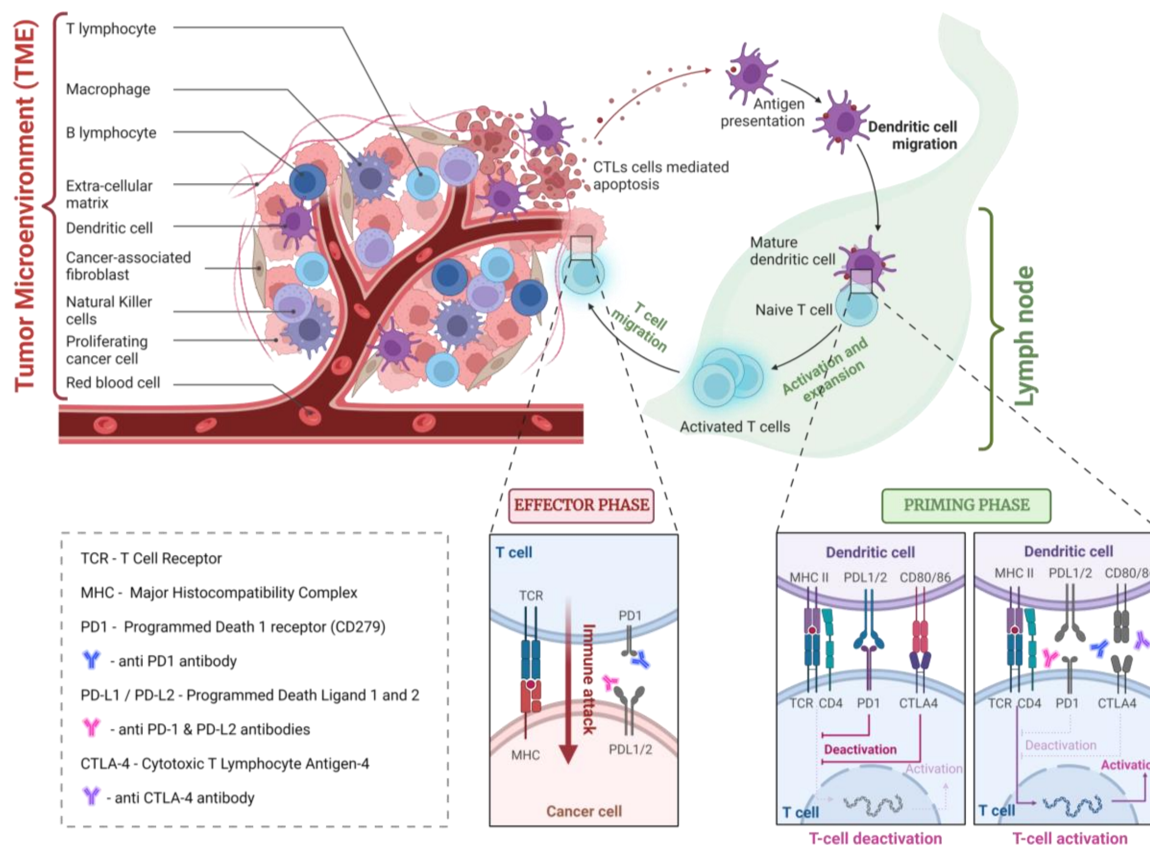

Figure 1.

Graphical representation of: (i) the colorectal cancer tumour microenvironment, (ii) the priming and effector phases of anti-tumour immune responses, as well as (iii) the role of PD-1 and CTLA-4 in the checkpoint signalling. Created with BioRender.com

The cellular makeup of the TME consists of diverse immune cells (Figure 1), which include tumour-infiltrating lymphocytes (TILs), as well as tumour-associated macrophages (TAMs) and tumour-associated neutrophils (TANs). In particular, TILs are a heterogeneous population of lymphocytes consisting of T cells, along with B and NK cells. Their primary function is to protect the host against tumour formation by recruiting, maturing, and cooperating in repressing the tumour growth [7]. With respect to T cells subsets, $CD8^+$ cytotoxic T cells in the TME can directly induce cytotoxicity or generate inflammatory interleukins triggering cell death in tumour cells, while $CD4^+$ helper T cells in the TME are involved in activating $CD8^+$ T cells against tumour cells [8-10]. At some stage of activation, T cell express checkpoint receptors that play a crucial role in down modulating their response, controlling the disease's development [6]. On the other hand, cancer cells have developed several strategies to evade immune recognition and destruction, including the upregulation of immune checkpoint ligands such as the co-inhibitory receptor programmed death-1 (PD-1), also known as CD279 and the cytotoxic T lymphocyte antigen-4 (CTLA-4) which serve to annihilate the defence of the T cells within the TILs [11, 12].

PD-1 and CTLA-4 are two immune checkpoint molecules that are upregulated on T cells in the CRC TME (Figure 1).

Despite the effort in validating new molecules for CRC treatment [13, 14], the development of immune checkpoint inhibitors (ICI) that target PD-1/PD-L1 (*e.g.*, nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab) and CTLA-4 (*e.g.*, ipilimumab,

tremelimumab) has revolutionized the treatment of several different types of cancer, including CRC [15]. ICI, also known as immune checkpoint blockers (ICB) block the interactions between PD-1 or CTLA-4 and their ligands, thereby allowing T cells to attack cancer cells more effectively.

To understand the functional diversity of immune cells that infiltrate tumours and advance in cancer immunotherapy, several multiplexed technologies have been developed. Single-cell RNA sequencing and mass cytometry or multiplex immunohistochemistry/ immunofluorescence are valuable methods used for identifying specific proteins as they can provide standardized quantitative data [16, 17]. On the other hand, flow cytometry has been traditionally used for (i) diagnostic purposes in haematology, (ii) characterization of immune status in the peripheral blood of patients suffering from different diseases [18] and also for (iii) liquid biopsy approaches [19]. Besides the quantitative data displayed, this analysis method has the potential to highlight subpopulations of cells for further analysis. Considering all the above, flow cytometry could be successfully used for other applications such as TME phenotyping after the design and optimization of a proper sample preparation protocol.

In this context, we propose here a standardised flow cytometry protocol as a multiplex assay of detecting PD-1 and CTLA-4 receptors along with other TME specific proteins in CRC fresh tissue samples. One of the most important advantage of this method is the highlight of the cellular populations within the TME along with their specific protein expression.

Materials and Methods

Tissue samples & Ethics

Fresh tumour tissue fragments were harvested from 7 patients undergoing curative colorectal surgery for tumour removal and placed immediately in sterile MACS Tissue Storage Solution (Miltenyi Biotec, Germany). All the intraoperative specimens were harvested in accordance with the medical procedures and regulations as a result of a medical indication and served primarily for curative and diagnostic purposes and secondary for the study. All the patients were informed about the study and the samples used were collected and further processed in this study after obtaining the patients written consent regarding their participation in the research. This study was developed under a Collaboration Agreement between the University of Bucharest and Monza Oncology Hospital (10763/ 25.05.2022) and was conducted under the approval of the Ethical Committee of Research within the University of Bucharest, Romania.

Sample processing

The whole sample processing protocol was illustrated in Figure 2. After arrival in the research lab, the tubes containing fresh tissue immersed in MACS Tissue Storage Solution (Miltenyi Biotec, Germany) were centrifuges for 10 min at 1500 rpm using a swing out rotor for 15 mL tubes (Hettich, Universal 320R, Germany). The supernatant was discarded, and the remaining tissue was placed in a Petri dish and cut in small pieces using a sterile blade. The resulting tissue was further processed using the Tumour Dissociation Kit (Miltenyi Biotec, Germany) that has been developed for obtaining of single-cell suspensions from human tumour tissue. The kit is optimized for high yield of tumour cells and TILs. Considering that the colon tumours are considered soft, we applied the tumour dissociation protocol for soft tissues as recommended by the manufacturer. Briefly, we performed a combined enzymatic digestion of the ECM using the mix of enzymes provided and a 30 minutes mechanical dissociation step at 37ºC. The obtained suspension was filtered using MACS Smart Strainers of 70 µm (Miltenyi Biotec, Germany) and the resulting cells suspension was centrifuged for 10 minutes at 1500 rpm. In cases where red blood cells were present an erythrocytes lysis was performed using the Red Blood Cell Lysis Solution (10×) (Miltenyi Biotec, Germany).

Cells labelling and flow cytometry characterization of the isolated cells

The cells previously obtained were re-suspended after the final centrifugation step in 115 µL PBS buffer supplemented with 2% bovine serum albumin (BSA) containing the following panel of antibodies: (1) anti EpCam - FITC (CD136, clone VU-1D9, StemCell Technologies, Canada), (2) anti CTLA-4 - PE (CD152, clone BNI3, Exbio, Czech Republic), (3) anti PD1 - PerCP-Cy 5.5 (CD279, clone EH12.2H7, Exbio, Czech Republic), (4) anti CD4 - APC (clone MEM-241, Exbio, Czech Republic), (5) anti CD8 - PacoRANGE (clone MEM-31, Exbio, Czech Republic) and (6) anti CD3 - APC-C750 (Cytognos, Spain). After staining for 30 minutes at room temperature and in drak, the samples were centrifuged 5 minutes at 1500 rpm, re-suspended in 500 µL PBS, transferred in 10 mm flow cytometry tubes and acquired in a CytoFlex Cytometer (Beckman-Coulter; Fullerton, CA) using the CytExpert Soft-ware (Beckman-Coulter; Fullerton, CA).

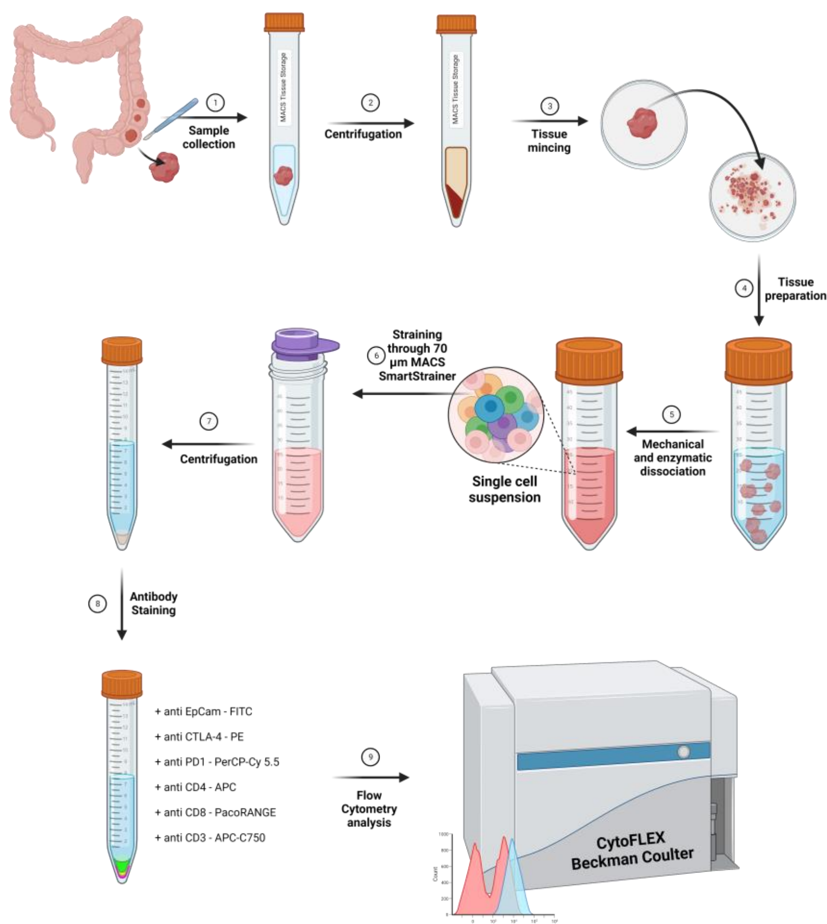

Figure 2.
Sample preparation workflow: from tissue sample collection to flow cytometry analysis. Created with BioRender.com

Data analysis
All the samples were acquired 3 times in the CytoFlex cytometer using the CytExpert Software version 2.0. and analysed using CytExpert Software version 2.3. Statistics of the quantitative data were performed using GraphPad Prism Software, version 6. Data are expressed as Mean ± Standard deviation (SD).

Results and Discussion

Patients group description
In this preliminary study, 7 patients undergoing CRC surgery for tumour removal were enrolled. Following histopathology and immunohistochemistry tests, all the participants were confirmed with CRC adeno-carcinoma. The age of the enrolled patients ranged from 54 to 79 years with a mean of 62.86 years. The mean value of the body mass index (BMI) was 26.04, with no obese patients, 14.28% normal weight patients and 85.72% overweight patients. Most of the volunteers were males (71.43%). 57.15% of the patients were smokers and 42.85% acknowledged moderate alcohol consumption. Lastly, only 2 patients, representing 28.57%, underwent neo-adjuvant radiotherapy before surgery. The detailed characteristics of the patient's cohort are described in Table I.

Table I
Description of the patient's analysis group

Number	7
Age (mean ± SD), (range - years)	62.86 ± 10.87 years, (54 -79)
Gender	
Female	2 (28.57%)
Male	5 (71.43%)
IMC (mean ± SD)	26.04 ± 1.321
Normal	1 (14.28%)
Overweight	6 (85.72%)
Obese	0 (0%)

Smoke	
Smokers	4 (57.15%)
Non smokers	3 (42.85%)
Alcohol	
No alcohol	4 (57.15%)
Moderate	3 (42.85%)
Treatment	
Naive (pre-treatment)	5 (71.43%)
Neo adjuvant radiotherapy	2 (28.57%)

PD-1/CTLA-4 receptors expression

The flow cytometry analysis was done using a complex gating strategy. For demonstrative purposes, some of the representative dot plots and histograms within the flow cytometry protocol were shown in Figure 3 for one of the patients within the study group. First, a forward scatter (FSC)/ side scatter (SSC) dot plot was created to display all the cells populations within the sample and also, a SSC/Time dot plot was created to monitor the sample acquisition process. Next, a FITC histogram was displayed to reveal the expression of EpCam and then a FSC/FITC dot plot was created to reveal the EpCam$^+$ cells and FSC-A / FSC-H to gate tumour derived EpCam$^+$ singlets. Then, a SSC/APC-C750 dot plot was created to display and gate the CD3 positive cells (the T cells population). Next, a Pacific Orange /APC dot plot, gated to the T cells population, was created to display and quantify CD8$^+$ and CD4$^+$ T subsets. This dot plot includes specific gates for: (i) double negative, (ii) CD8$^+$ and (iii) CD4$^+$ T subsets. The last step consisted in highlighting the expression of PD1 and CTLA-4 on T subsets and also on EpCam$^+$ cells. For this, the following dot plots were created: PerCp-Cy5.5/PE gated on: (i) EpCam$^+$ cells to determine the expression of PD-1 and CTLA-4 on EpCam$^+$ cells, (ii) CD4$^+$ cells to determine the PD-1 and CTLA-4 on CD4$^+$ subset, (ii) CD8$^+$ cells to determine the PD-1 and CTLA-4 on CD8$^+$ subset and (iii) double negative T cells to determine the PD-1 and CTLA-4 on these cells.

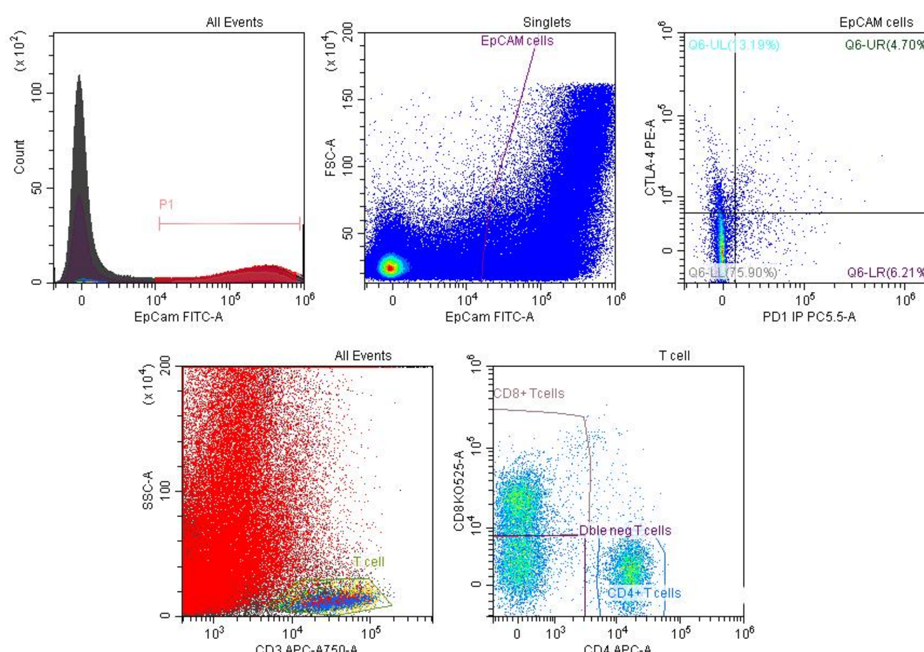

Figure 3.

Flow cytometry dot plots and histograms revealing the quantitative expression of specific molecules (PD-1, CTLA-4, EpCam, CD3, CD4 and CD8) in the CRC TME

After data analysis, our results showed that the mean number of EpCam$^+$ cells was 12.22% (minimum 3% and maximum 22%), with the lowest values obtained for the patients that underwent neo adjuvant radiotherapy. The mean number of T cells (CD3 positive cells) in our cohort was about 6.8% of which 3.41% revealed PD-1 positive expression and 2% expressed CTLA-4. Despite the low number of patients enrolled in this preliminary study, our data is in accordance with the available literature [20], sustaining the potential interest of flow cytometry for the CRC TME characterization.

Interestingly, 4.1% of the EpCam$^+$ cells also expressed PD-1 and 24.6% of the EpCam$^+$ cells also expressed

CTLA-4. Previous studies have described this intrinsic expression of PD-1 in melanoma and hepatocarcinoma [21, 22] and also highlighted its major impact on combinatorial immunotherapy. Moreover, the The Cancer Genomic Atlas (TCGA) project [23] listed several types of cancer cells potentially expressing PD-1, including CRC and many other types of cancer [21]. Considering all the above, besides the TME populations and the molecular profile of PD-1 and CTLA-4 in TILs, our data also evidenced intrinsic expression of PD-1 and CTLA-4 on tumour derived EpCam$^+$ cells.

More, to confirm the staining and also the flow cytometry protocols, we labelled with the same antibody panel a peripheral blood sample and compared the obtained results with the ones from fresh tissue. The flow cytometry analysis of the blood sample presented in Figure 4 revealed the presence of CD3$^+$ cells with well represented CD4$^+$ and CD8$^+$ subsets but no EpCam expression, as expected.

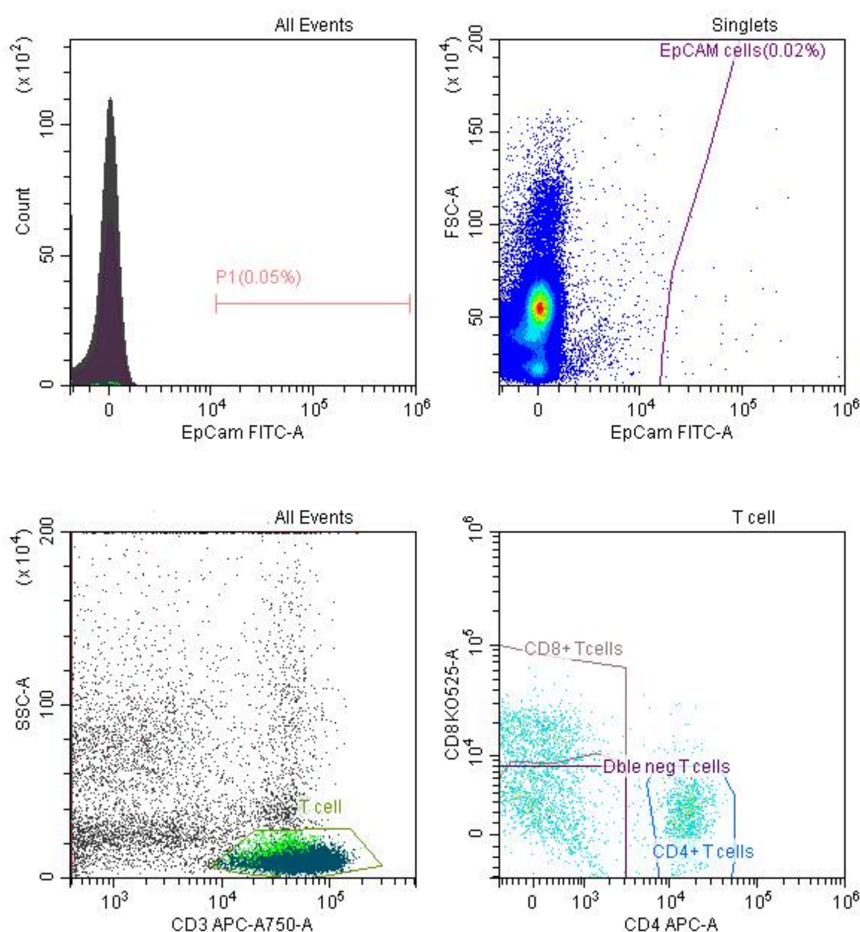

Figure 4.

Flow cytometry dot plots and histograms revealing the quantitative expression of specific molecules (PD-1, CTLA-4, EpCam, CD3, CD4 and CD8) in the peripheral blood

Undoubtedly, the upregulation of immune checkpoint molecules on infiltrating immune cells in the CRC TME is an important mechanism by which cancer cells evade immune recognition and destruction. PD-1 is expressed on the surface of T cells and binds to its corresponding ligands: PD-L1 (also known as B7-H1) and PD-L2 (also known as B7-DC), which are expressed on cancer cells and other cells in the microenvironment. This interaction downregulates the function of the T cells, preventing them from attacking cancer cells. Similarly, CTLA-4 is a check-point molecule expressed on T lymphocytes and plays an inhibitory role by T cell activation after binding to CD80 (also known as B7-1) and CD86 (also known as B7-2), which are located on the antigen-presenting cells (APCs) [4]. Knowing the expression of this pathway may help in indication of check point inhibitor therapy. Furthermore, recent studies [24] revealed that PD-L1 expression in CRC TME is a directly correlated with the recurrence-free survival and related with cancer-associated immune responses such as: IFN-gamma response, IL-2-STAT5 signalling and the IL-6-STAT3 signalling pathway, which makes it a valuable prognostic marker in early-stage CRC.

Conclusions

In conclusion, by using a standardized tumour tissue processing protocol and a flow cytometry acquisition and analysis protocol we were able to underline the CRC TME molecular pattern, to identify and quantify the cellular subpopulations and to associate them with a specific expression of PD-1 and CTLA-4 immune checkpoints receptors, with high impact on the targeted therapy modulation and personalized treatment of the patients.

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Conflict of interest

The authors declare no conflict of interest.

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