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RESEARCH

Quantification of Blood Dendritic Cells in Colorectal Cancer Patients During the Course of Disease

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Abstract Colorectal cancer is a malignancy with poor prognosis that might be associated with defective immune function. The aim of the present study was to investigate circulating dendritic cells in colorectal cancer patients, in order to contribute to elucidate tumor-escape mechanisms and to point out a possible correlation with the clinical condition of the disease. Therefore, we enumerated ex vivo myeloid and plasmacytoid dendritic cells, through multicolor flow cytometry, in 26 colorectal patients and 33 healthy controls. Furthermore we performed several analyses at determined time points in order to define the immunological trend of cancer patients after surgery and other conventional treatments. At the preoperative time point the absolute number of plasmacytoid dendritic cells in cancer patients was significantly reduced in comparison to controls, this result being mainly referred to stage III-IV patients. The number of myeloid dendritic cells did not show any significant difference compared to healthy controls; interestingly the expression of the tolerogenic antigen CD85k was significantly higher on cancer patients' myeloid dendritic cells than controls'. At the following samplings, circulating dendritic cell absolute number did not show any difference compared to controls. Conclusively the impairment of the number of circulating dendritic cells may represent one of the tumor escape mechanisms occurring in colorectal cancer. These alterations seem to be correlated to cancer progression. Our work sheds light on one of dendritic cell-based tumor immune escape mechanisms. This knowledge may be useful to the development of more effective immunotherapeutic strategies.

Keywords Circulating dendritic cells · Colorectal cancer · Immunosuppression · Flow cytometry

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Summary

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide, and 20 % of patients have metastases at time of diagnosis [1]. Like other solid tumors, CRC has long been considered poorly immunogenic, but several evidences have recently shown that also CRC expresses tumor associated antigens (TAAs), and that an antitumor immune response may take place in these patients [2]. In fact, it has been shown that the number of tumor-infiltrating CD8+ T lymphocytes (TILs) is associated with improved clinical outcome and survival in CRC patients, regardless of tumor stage [3, 4]. Moreover, it has been demonstrated a correlation between the number of TILs and of other tumor-infiltrating immune cells, especially dendritic cells (DCs) [5–7], the most important antigen presenting cells (APCs), playing a pivotal role in T-cell function and in the linkage between innate and adaptive immunity. As immature cells, DCs are able to recognize and capture antigens in peripheral tissues; the antigen uptake triggers the maturation process, during which they migrate to lymph nodes to prime both CD4+ and CD8+ specific T cell responses. Based on their lineage origins, peripheral blood dendritic cells (PBDCs), representing only the 0.1–1 % of mononuclear cells, can be divided into two major subsets, myeloid DCs (mDCs) and

plasmacytoid DCs (pDCs), that can be identified on the basis of phenotypic markers and different immunological activity [8].

Myeloid DCs lack the expression of lineage markers, but express HLA-DR, CD11c and CD33, require IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) for growth and function, and secrete large amount of IL-12, IL-6 and TNF-α upon stimulation [9]. Plasmacytoid DCs require fms-like tyrosine kinase 3 ligand (FLT-3) and IL-3 for growth and survival, and produce very high levels of type I interferons (IFN) upon stimulation with viruses; they lack the expression of lineage markers, and can be identified by the expression of the IL-3 receptor, or CD123 [10].

Because of their potential capacity to initiate and regulate a tumor-specific immune response, immunotherapeutic approaches focused on dendritic cells have long been considered in clinical trials, in order to develop anti-tumor vaccination protocols, with poor results [11].

Despite the rate of tumor-infiltrating immune cells, the immune system of cancer-bearing subjects is strongly deficient, unable to set up an efficient tumor-specific cytotoxic immune response. This is mainly caused by mechanisms established by tumor cells to escape from the immune control, such as the production of molecules inhibiting dendritic cells and other cells of the immune system, both in the tumor microenvironment and in the systemic circulation [12–14]. Several studies have demonstrated that DCs isolated from the peripheral circulation of patients affected by different types of cancer showed quantitative and functional abnormalities [15–21].

The infiltration rate and the local distribution of DCs in CRC tissue have been largely investigated [5–7, 22–26]. Conversely, few studies have been performed on the expression of DCs in CRC patients at the circulating level [27–29].

The aim of the present study was to investigate the frequency of circulating DCs and their subpopulations in early- and advanced-stage CRC patients directly ex-vivo, and to correlate their numbers with the stage of disease. Furthermore, we monitored the patients at specific times after surgical resection, in order to point out a possible correlation with the clinical condition of the disease.

Materials and Methods

Study Population

In this study we enrolled 26 CRC patients with newly histologically confirmed diagnosis of colorectal adenocarcinoma: 20 males and 6 females, aged between 42 and 86 years. A total of 5 patients had stage I disease (T1-2N0M0), 7 had stage II (T3-4N0M0), 12 had stage III (any T,N1-2M0) and 2 had stage IV (any T, any N, M1). We divided the population into groups based on the clinical stage; because of the small number of stage IV patients we combined stages III and IV to perform statistical analyses. None of these patients has previously experienced colorectal cancer or presented a history of neoplastic diseases; furthermore CRC patients were not treated with neo-adjuvant chemotherapy. Colorectal cancer patients who underwent adjuvant chemotherapy received the following regimen: 5-fluorouracil (5FU) + leucovorin (LV) + oxaliplatinum.

As control we enrolled a population of 33 healthy subjects (HCs; age range: 40–74 years old) without any history or diagnosis of neoplastic diseases. Cancerous or healthy subjects affected by immune system diseases, such as autoinflammatory, rheumatic and congenital immunodeficiencies, viral and/or bacterial infections, hematological diseases, diabetes or treated with immunosuppressive drugs were excluded from the study. Baseline CRC patients' and healthy controls' clinical characteristics are described in Table 1.

Blood samples were drawn from each CRC patient at the baseline, before surgical resection (first or pre-operative time point); the second blood sample was collected at the first follow-up medical examination, approximately 2 months after surgery and anyway before adjuvant chemo- and/or radiotherapy (second time point); the third one was drawn approximately 6-10 months after surgical resection and/or 2 months after the end of adjuvant chemotherapeutic treatment (third time point), and the last one (fourth time point) at a minimum of 12 months after surgery and/or 6 months after the end of adjuvant chemotherapy, both during routine follow-up examinations.

The study was carried out with the approval of the local Ethical Committee. All the CRC patients and healthy volunteers gave informed consent for the inclusion in the study.

Enumeration and Phenotypic Characterization of Peripheral Blood Dendritic Cells

Fresh peripheral blood samples were collected from both CRC patients and healthy controls, and analyzed within 24 h. Circulating dendritic cells were enumerated and phenotypically characterized in the two major subsets, myeloid and plasmacytoid DCs, directly on the whole blood, by three-colors flow cytometric method, as previously described [30].

Because of the lack of a specific marker to detect DCs, we used a mixture of monoclonal antibodies, specifically established to identify DCs, purchased by Immunotech (Beckman Coulter Inc.; Brea, CA, USA). Cells were stained with the following antibodies: CD14 and CD16 FITC (fluorescein isothiocyanate); CD85k PE (phycoerythrin); CD33 PC5 or CD123 PC5 (phycoerythrin linked to cyanine 5) for the myeloid and plasmacytoid DC subset, respectively. Dendritic cells were identified as $CD14^{lo/-}CD16^{lo/-}CD85k^{+}$ and $CD33^{+}$ or $CD123^{+}$ (Fig. 1).

Table 1 Baseline CRC patients and healthy controls clinical characteristics

	HCs	Total CRC	Stage I	Stage II	Stage III–IV
Number of subjects (n)	33	26	5 (19.23)	7 (26.92)	14 (53.84)
Age (years)					
Mean	55.15±1.81	68.85±2.14	70.00±2.41	70.57±5.94	67.57±2.69
Range	40–74	42–86	63–77	42–86	47–81
Sex					
M	14 (42.42)	20 (76.92)	4 (80)	5 (71.43)	11 (78.57)
F	19 (57.58)	6 (23.08)	1 (20)	2 (28.57)	3 (21.43)
Location of the tumor:					
Right side of colon		10 (38.46)	2 (40.00)	3 (42.86)	5 (35.71)
Left side of colon	/	5 (19.23)	3 (60.00)	1 (14.28)	1 (7.14)
Transverse colon		1 (3.85)	/	/	1 (7.14)
Rectum		10 (38.46)	/	3 (42.86)	7 (50.01)
Adjuvant chemotherapy					
Yes	/	6 (42.86)	0 (0)	1 (20.00)	5 (100.00)
No		8 (57.14)	4 (100.00)	4 (80.00)	0 (0)
WBCs (x10^3/μL)	6.80±0.34	7.02±0.39	5.86±0.27	6.76±0.87	7.56±0.55*
MNCs (x10^3/μL)	2.54±0.13	2.13±0.12*	2.21±0.16	2.23±0.31	2.05±0.15*

Data are shown as mean value ± SEM, in brackets percentage values

HCs, healthy controls; CRC, colorectal carcinoma; WBCs, white blood cells; MNCs, mononuclear cells

*$p < 0.05$ stage III–IV versus stage I CRC WBCs; total and stage III–IV CRC versus HCs MNCs

The antigen CD85k, known also as immunoglobulin-like transcript 3 (ILT3), is a transmembrane protein selectively expressed by APCs, such as dendritic cells, monocytes and macrophages [31]. We chose this gating strategy because the antigen CD85k is involved in antigen processing [32]; it is down-regulated following DC activation [33] and up-regulated in tolerogenic DCs, leading to the induction of T reg cells [34].

Estimates of absolute numbers of mDCs and pDCs were calculated multiplying the percent amount of mDCs and pDCs in the mononuclear cells gate by absolute PBMC count determined using a standard hemacytometer (Abbott Laboratories;

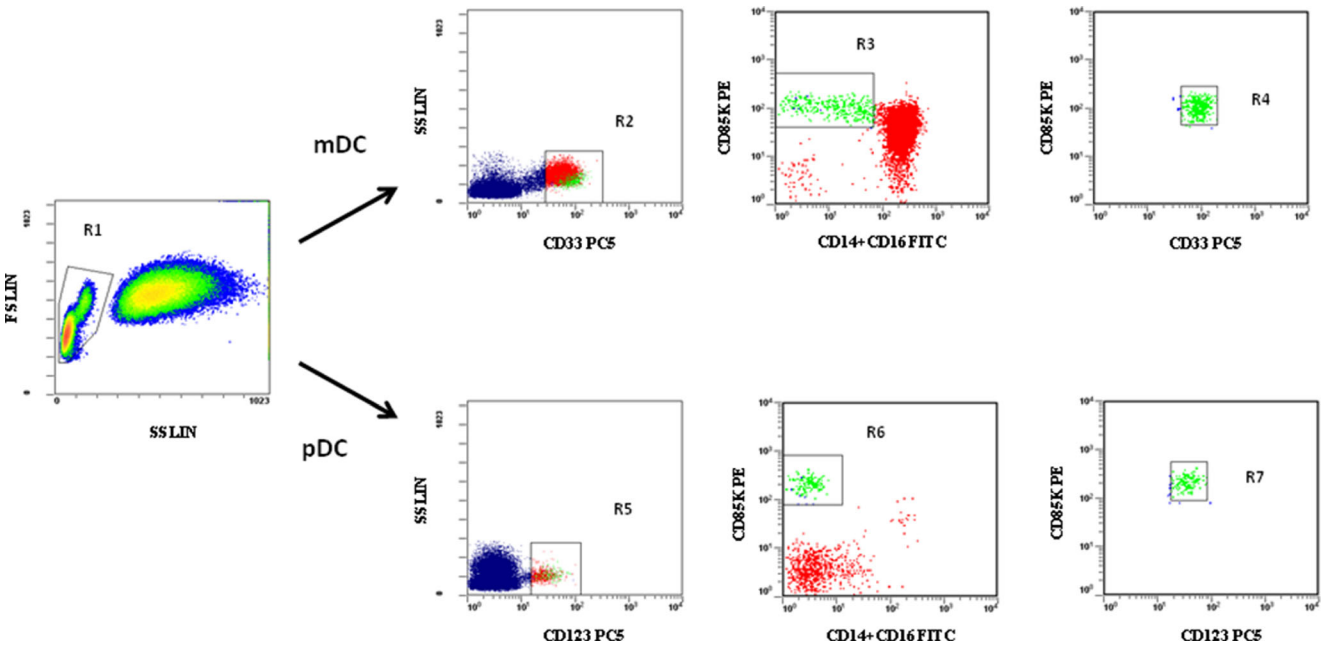

Fig. 1 Gating strategy for the identification of circulating mDCs and pDCs as percentage of PBMCs. Total PBMCs were gated based on their forward and side scatter (R1). CD33 (R2) or CD123 (R5) positive cells were selected for mDCs and pDCs, respectively. Then CD14/16$^{-/low}$ CD85k$^+$ cells were gated (R3 and R6). Regions R4 and R7 were created to define mDCs and pDCs as CD33$^+$CD85k$^+$ and CD123$^+$CD85k$^+$ cells, respectively. A representative dot plot relative to one CRC patient is shown

Abbott Park, Illinois, USA). Total DC number was obtained by summing mDC and pDC absolute values.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc.; La Jolla, CA, USA). Results were expressed as mean ± SEM. Comparison between tumor and healthy subjects was analyzed by using the two-tailed Student's *t*-test for unpaired data. *P*-values<0.05 were considered statistically significant.

Results

Patients' Characteristics

A total of 26 CRC patients were enrolled in this study. The average age (± SEM) of the patients was 68.85±2.14 years (median 70.50). One CRC patient died immediately after surgery, two patients presented distant metastasis at the time of diagnosis (stage IV); as one out of these two underwent continuous chemotherapeutic treatment after surgical resection, we could not perform any other blood samplings after the pre-surgical one. Fourteen of the 26 CRC patients had the minimal follow-up of approximately 2 months after the baseline collection of the blood specimen, whereas the others were lost after surgical resection. The third blood sample was obtained from 10 out of the 14 patients undergone the second blood collection: 43 % of these patients underwent adjuvant chemotherapy before sampling and 80 % underwent also the last sampling.

As shown by Table 1 the number of white blood cells (WBCs) was significantly higher in stage III–IV than stage I patients; a significant mononuclear cell (MNC) number reduction was also observed in stage I and stage III–IV patients compared with healthy controls.

Enumeration and Phenotypic Characterization of Peripheral Blood DCs pre-Surgery

Circulating plasmacytoid and myeloid dendritic cells were pre-operatively determined in all enrolled CRC patients and healthy subjects.

The number of total DCs, mDC and pDC subsets, and pDC/mDC ratio value obtained from CRC patients considered all together and stratified into three groups on the basis of the clinical stage (stage I, stage II and stage III–IV) was compared with those ones in the healthy group; furthermore statistical analyses were also performed within the three CRC clinical stage groups.

At the first time point plasmacytoid DCs, as absolute number and percentage of WBCs, were significantly lower in the peripheral blood of total CRC patients (6.40±0.83/μL and 0.10±0.01 %) compared with healthy controls (8.46±0.65/μL and 0.13±0.01 %; $p<0.05$). This reduction is presumably addressed to stage III CRC patients (5.92±0.94/μL and 0.09±0.01 %; $p<0.05$), because patients with early disease (stage I and II) did not show any difference compared to controls (Figs. 2b and 3b and Table 2).

On the contrary, we found no significant difference in myeloid DC absolute number between CRC patients and healthy donors, although subjects with advanced disease (stage III–IV) showed a marked decrease of mDCs (Fig. 2a), turning significant when mDCs were analyzed as percentage of WBCs (0.16±0.02 % vs 0.21±0.02 %, $p<0.05$; Fig. 3a). Therefore the total number of DCs observed in stage III–IV patients resulted significantly lower (16.38±2.15/μL and 0.24±0.04 %) than controls (22.46±1.38/μL and 0.35±0.02 %; $p<0.05$; Figs. 2c and 3c).

The value of pDC/mDC ratio was affected by the trend of pDC and mDC absolute numbers, in fact it was significantly lower in total CRC (0.48±0.04) and stage III patients (0.48±0.06) than healthy subjects (0.70±0.07; $p<0.01$ and $p<0.05$, respectively; Fig. 2d).

As previously reported [30], the mean fluorescence intensity of the antigen CD85k on pDCs of healthy controls was significantly higher than mDCs (159.10±10.38 vs 96.69±4.42, respectively; $p<0.0001$). Similar results were obtained in CRC patients (pre-surgery—pDCs vs mDCs: 175.60±9.37 vs 105.10±4.52; $p<0.0001$) as shown in Fig. 4a. Furthermore CD85k expression was significantly higher on mDCs, but not on pDCs, in patients with advanced disease (112.10±6.43) than in healthy controls (96.69±4.42; $p<0.05$; Fig. 4b).

Follow-up

During follow-up, no difference in mDC, as absolute number and WBC percentage, was found between CRC patients, at any stage of disease, and healthy controls, both at the second and the third sampling. However, at the third time point, a marked even though not statistically significant decrease of mDCs as percentage of WBCs was noticed in stage III-IV-CRC patients compared with stage I (0.16±0.02 % vs 0.21±0.02 %, respectively; Fig. 3a). When we analyzed pDCs as percentage of WBCs, at the second time point (2 months after surgical resection of the tumor), we observed a significant decrease in stage II-CRC patients (0.08±0.01 %) compared with controls (0.13±0.01 %; $p<0.05$). All these data were composed by unpaired data by including all CRC patients underwent blood samplings following the first one (Fig. 3b).

In order to observe the trend of DC absolute number in each patient throughout the overall follow-up, we performed a further analysis by including only CRC patients who underwent all the three blood samples, retrospectively sorting them by

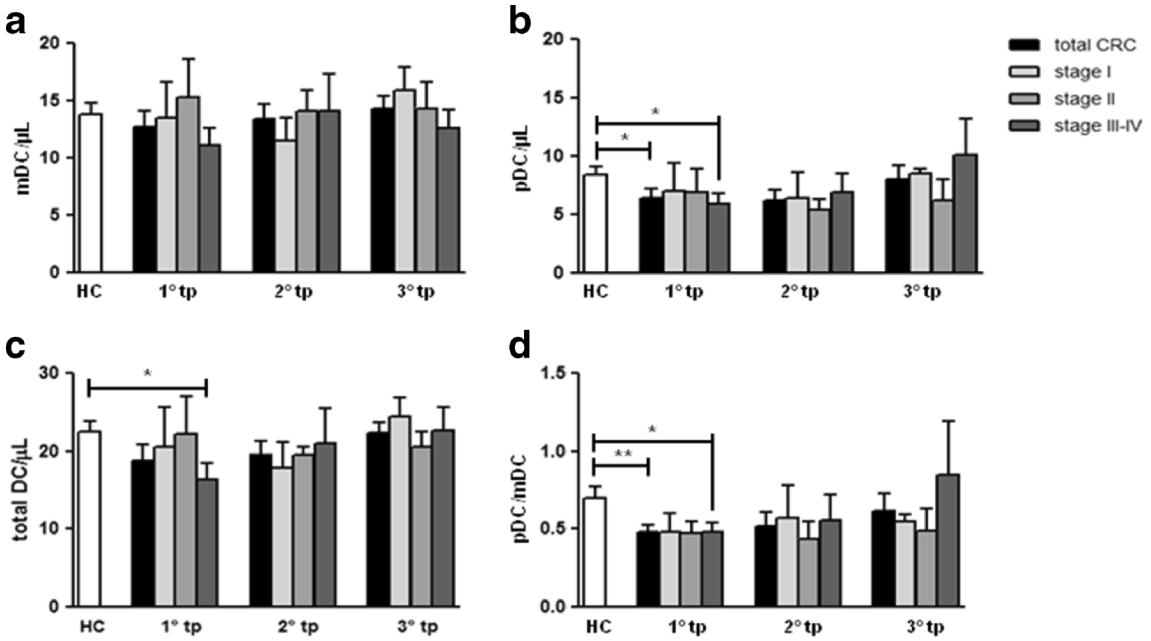

Fig. 2 Circulating DC absolute number in CRC patients and healthy controls. **a** Myeloid, **b** plasmacytoid, **c** total DC absolute numbers and **d** pDC/mDC ratio in healthy controls (HC) and CRC patients at the three time points are shown. CRC subjects were analyzed as total (*black*), stage I (*light grey*), stage II (*medium grey*) and stage III–IV patients (*dark grey*). Data are shown as mean ± SEM. *$p<0.05$; **$p<0.01$. 1° tp: pre-operative time point; 2° tp: two-months post-operative and/or before chemotherapy time point; 3° tp: six-months post-operative and/or standardize with previous expressions of time two months after adjuvant chemotherapy

those who had been treated with adjuvant chemotherapy. In Fig. 5, we also reported the results obtained at the fourth sample, although the number of chemotherapy-treated subjects was too small to allow statistical analyses.

We observed that, at the first time point, patients undergone adjuvant chemotherapy had a slightly greater number of myeloid DCs (18.98±3.97/μL) than untreated ones (13.78±2.06/μL); this difference was held also post-operatively (16.56±3.40/μL

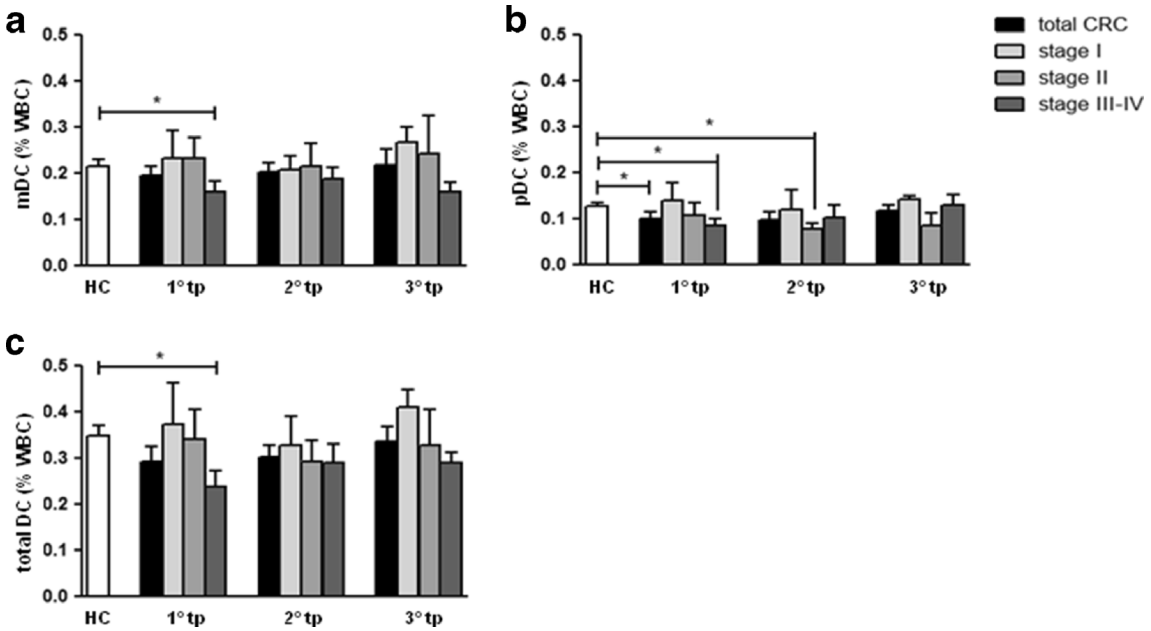

Fig. 3 Circulating DC number as WBC percentage in CRC patients and healthy controls. **a** Myeloid, **b** plasmacytoid and **c** total DC numbers as white blood cell (WBC) percentage in healthy controls (HC) and CRC patients at the three time points are shown. CRC subjects were analyzed as total (*black*), stage I (*light grey*), stage II (*medium grey*) and stage III–IV patients (*dark grey*). Data are shown as mean ± SEM. *$p<0.05$, 1° tp: pre-operative time point; 2° tp: two-months post-operative and/or before chemotherapy time point; 3° tp: six-months post-operative and/or standardize with previous expressions of time two months after adjuvant chemotherapy

Table 2 Circulating dendritic cells (DCs) in healthy subjects and pre-surgery colorectal cancer patients

	HCs	Total CRC	Stage I	Stage II	Stage III–IV
mDCs/μL	13.81±0.97	12.76±1.35	13.50±3.11	15.30±3.30	11.10±1.51
mDCs (% MNCs)	0.56±0.04	0.59±0.05	0.59±0.12	0.68±0.10	0.54±0.05
mDCs (% WBCs)	0.21±0.02	0.19±0.02	0.23±0.06	0.23±0.05	0.16±0.02*
pDCs/μL	8.46±0.65	6.40±0.83*	7.06±2.34	6.89±2.02	5.92±0.94*
pDCs (% MNCs)	0.28±0.04	0.28±0.04	0.31±0.12	0.24±0.06	0.28±0.06
pDCs (% WBCs)	0.13±0.01	0.10±0.01*	0.14±0.04	0.11±0.03	0.09±0.01*
total DCs/μL	22.46±1.38	18.84±1.98	20.56±5.06	22.19±4.76	16.38±2.15*
total DCs (% MNCs)	0.90±0.05	0.87±0.07	0.90±0.20	0.98±0.12	0.80±0.08
total DCs (% WBCs)	0.35±0.02	0.29±0.03	0.37±0.09	0.34±0.07	0.24±0.04*

Data are shown as mean value ± SEM

HCs healthy controls; *CRC* colorectal carcinoma; *WBCs* white blood cells; *MNCs* mononuclear cells

*$p < 0.05$; statistic significance is referred to HCs

versus 12.09±1.20/μL, respectively), but it was abrogated in the last two samplings (Fig. 5a). On the other hand, the absolute number of plasmacytoid DCs did not show any difference between untreated and chemotherapy-treated subjects in the first three time points, but at the fourth sample it showed a drastic decrease in the latter group in comparison with untreated patients (2.23±0.33/μL versus 7.31±1.56/μL, respectively; Fig. 5b). The analysis of pDCs as percentage of WBCs performed on chemotherapy-treated patients, at the second time point, showed a significant reduction compared with healthy donors (0.07± 0.02 % vs 0.13±0.01 %, respectively; $p < 0.05$; Fig. 5d).

The value of the ratio between pDC and mDC was affected by the results previously reported for absolute numbers of the two major DC subsets; in fact it was significantly lower in chemotherapy-treated patients compared with controls, both at first and second time point (first and second time point: 0.37 ±0.08 and 0.33±0.09 vs 0.70±0.07, respectively; $p < 0.05$; Fig. 5c).

Analyses performed within chemotherapy-treated and untreated CRC groups throughout the four time points did not show any significant difference, although this result could be influenced by the small number of patients who underwent the entire follow-up.

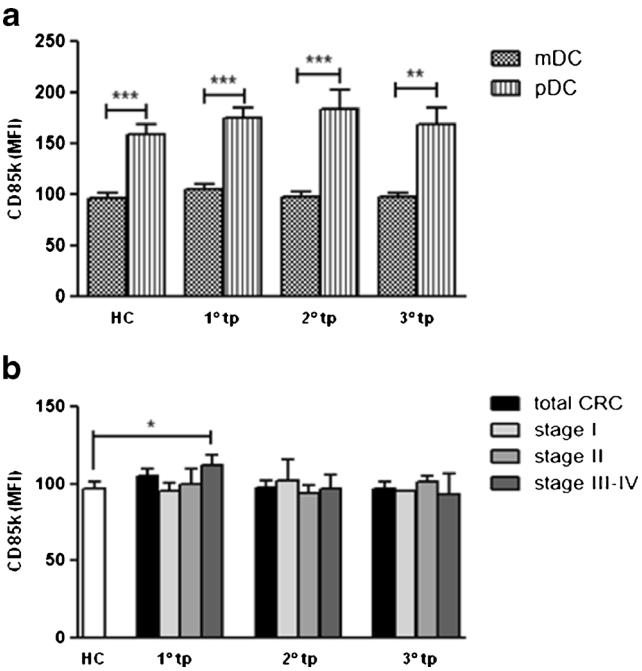

Fig. 4 Expression of CD85k on mDCs and pDCs in CRC patients and healthy controls. The expression of the antigen CD85k was evaluated by flow cytometry and compared between **a** mDCs and pDCs of healthy controls (HC) and CRC patients at the three time points. **b** CD85k expression on mDCs was compared between HC and CRC patients at the 1°, 2° and 3° time points. CRC subjects were analyzed as total (*black*), stage I (*light grey*), stage II (*medium grey*) and stage III–IV patients (*dark grey*). Data are shown as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, 1° tp: pre-operative time point; 2° tp: two-months post-operative and/or before chemotherapy time point; 3° tp: six-months post-operative and/or standardize with previous expressions of time two months after adjuvant chemotherapy

Discussion

The first choice therapy for colorectal cancer is represented by surgical resection, eventually followed by chemotherapeutic regimens; nevertheless the 5-year survival rate in advanced-disease patients is about of 25–40 % [1]. Therefore, several studies investigated the development and the biological characteristics of this type of cancer, focusing on the tumor microenvironment and the host immune system, in order to design new therapeutic strategies, pointed to improve the patient immune response against the primary tumor and eventually its metastases [35, 36]. Several immunotherapeutic clinic trials demonstrated hopeful results about the induction of tumor-specific immune responses, but these findings, favoring selective immunological aspects, did not necessarily imply patients' clinical improvements, suggesting a still poor knowledge of the biological mechanisms underlying tumors [37].

Therefore, in this study we focused on the quantification of circulating dendritic cells in CRC patients, categorized according to the stage of disease, at both pre- and post-operative time points, in order to shed light on the systemic

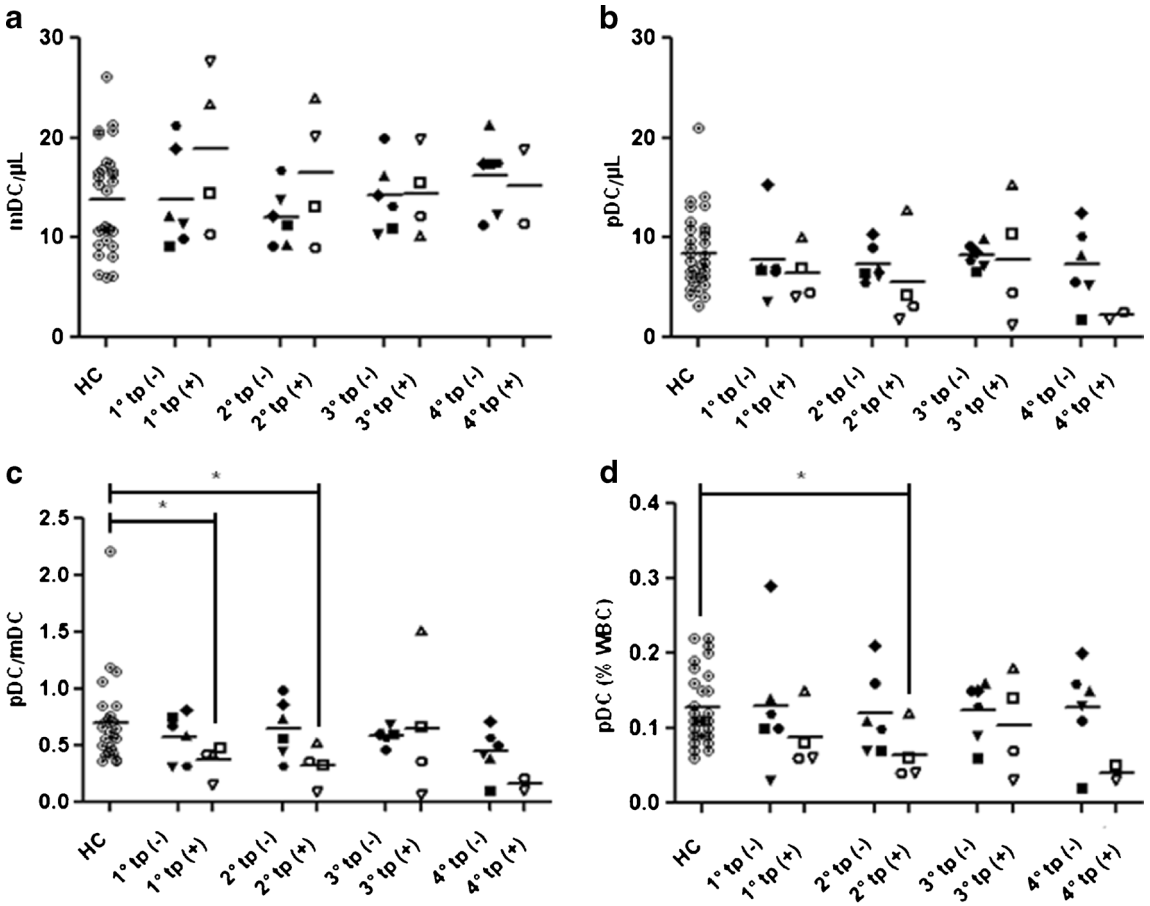

Fig. 5 Circulating DCs in CRC patients sorting by those who had been treated with adjuvant chemotherapy. Flow cytometry was used to assess **a** myeloid and **b** plasmacytoid in healthy controls (HC) and CRC patients at the 1°, 2°, 3° and 4° time points. The **c** ratio between pDC and mDC, and the **d** pDC number as percentage of white blood cells (WBC) are also shown. Each symbol in CRC patients' columns represents a different subject. White points refer to chemotherapy-treated patients (+), whereas black points to untreated ones (−). Data are shown as mean. *$p < 0.05$, 1° tp: pre-operative time point; 2° tp: two-months post-operative and/or before chemotherapy time point; 3° tp: six-months post-operative and/or standardize with previous expressions of time two months after adjuvant chemotherapy; 4° tp: twelve-months post-operative time point

immune status of CRC patients and contribute to the development of more effective immunotherapeutic strategies.

Our data showed a significant reduction of plasmacytoid DCs in total and advanced stage-CRC patients compared to healthy controls, both as absolute number and percentage of total leucocytes, affecting also the total number of DCs. Plasmacytoid DC absolute number impairment was totally recovered after complete tumor resection.

This stage-related defect may indicate a systemic immunosuppressive effect exerted by the tumor towards circulating DCs [38]. In fact our results showed that surgical resection can improve the systemic immune status of CRC patients, according to Takahashi et al. [39].

Schmidt et al. found a significantly higher number of both myeloid and plasmacytoid DCs in colorectal and breast cancer patients than healthy controls, arguing that it may be due to the removal of the tumor. However their analysis was performed in patients after tumor surgical resection and during neo-adjuvant irradiation but not at the pre-surgical time point [29]. Although maturational and functional status of pDC population in CRC patients have not been completely elucidated, the observed reduction of pDC number could lead to a diminished release of cytokines, such as type I interferon, necessary for an effective anti-tumor immune reaction. The low production of IFN-α could determine an altered innate immune response and myeloid DC activation, which are the major orchestrator of cell-mediated immune reaction, such as the anti-tumor one [40]. Hartmann et al. observed in squamous cell carcinoma of the head and neck (SCCHN) patients that plasmacytoid DCs held a reduced ability to secrete IFN-α, related to the tumor induced-down regulation of TLR-9 [41].

Based on the literature, our results are in line with a previous work on CRC subjects [27], even though the authors did not distinguish between the two DC subsets, and with another one on prostate cancer [20]. Contrarily, a study performed by Bellik et al. showed a higher percentage of circulating DCs in CRC patients before surgical excision [28], which may be attributable to the different

methodological approach, isolating PBMCs before the flow cytometric DC counting [42]. Studies performed on multiple myeloma showed an affection of both DC subtypes at diagnosis [43, 44], which persisted only for pDC subset during remission [44]. Other works performed on several types of cancer displayed an alteration of the myeloid component only [15, 17, 18, 21, 39, 45, 46]. Conversely Wilkinson et al. did not obtain any difference in the number and function of circulating DCs in patients affected by prostate cancer [47], whereas McCarter et al. found an increased number of this cell population in melanoma patients [19]. These contrasting results indicate that the type and the stage of the tumor could determine a different DC involvement.

Moreover, the DC gating strategy chosen in our experimental approach allowed us to analyze the expression of CD85k, a DC antigen related to immunosuppressive and tolerogenic functions [32, 48]. Interestingly, its expression on myeloid DCs was significantly higher in CRC patients compared with controls, suggesting a more immunosuppressive nature of this cell subtype in cancer subjects that may contribute to affect the host immune reaction against the tumor.

Evaluation of soluble immunosuppressive factors released in the plasma by the tumor might be helpful in order to find a possible correlation of cytokine pattern with circulating DC alterations [49, 50]. Furthermore these cytokines could affect DC differentiation from hematopoietic progenitor cells [51] and induce a DC redistribution between systemic circulation and the local peri- and intratumor tissue [27].

Further results obtained by sorting CRC patients into those who received or not adjuvant chemotherapy might suggest that the pharmacological treatment did not influence the absolute number of circulating DCs, excluding its long term toxicity on these cells. This observation is not in agreement with Bellik's work, which showed a decrease of circulating DC numbers in chemotherapy-treated patients instead [28]. However we performed this kind of analysis only on small patients' groups, therefore deserving further investigations. Nevertheless the pDC/mDC ratio was found significantly lower in CRC subjects who had undergone chemotherapy, both at pre- and post-operative time points, than healthy controls, due to the concurrent slight increase and decrease of mDCs and pDCs, respectively. This observation could be related to the more advanced clinical stage (mainly stage III) of CRC patients who received the chemotherapeutic treatment. Moreover the recruitment of a large cohort of patients can allow the evaluation whether the circulating DC number reflects and early points to the development of therapy resistance before the evidence of macroscopic alterations.

In conclusion, this work investigated the immune system in CRC patients, highlighting an impairment of circulating dendritic cells that could play a role in tumor-escape mechanisms. The severity of DC alterations seems to be related to the tumor stage, suggesting that the decline of host immune system efficiency is more marked as more serious the disease is, also at a systemic level. Taking account of the underlined relevance of the immune system in the tumor development and in the course of the disease, it has been recently proposed an immunological classification of CRC patients, based on type, density and localization of immune cells within the tumor, especially T lymphocytes, that could provide a prognostic factor superior to current criteria [2, 4, 52]. Therefore we think that both local and systemic DC evaluation in cancer subjects can offer an additional parameter useful for patients' classification based on the immune score.

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