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Characterization of Circulating Blood Dendritic Cell Subsets DCI23+ (Lymphoid) and DCIIC+ (Myeloid) in Prostate Adenocarcinoma Patients

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PURPOSE. We verified whether prostate adenocarcinoma produces specific modifications in DC subsets count.

METHODS. Twenty-one untreated prostate adenocarcinomas were divided on the basis of clinical stage in localized and metastatic disease. As control we used a population of 18 healthy male subjects. For DCs enumeration, peripheral blood (PB) samples were obtained in all cases. A single-platform flow cytometric assay based on Tru-COUNT was used for the enumeration of the two DCs subsets, myeloid (mDCs) and plasmacytoid (pDCs).

RESULTS. We showed a statistically significant reduction in pDCs count in prostate cancer population when compared to healthy controls ($P = 0.002$). Comparing each clinical stage with healthy controls, significant differences were found between controls and the metastatic group in both pDCs and mDCs ($P = 0.005$ and $P = 0.023$ respectively) but not between controls and the localized group ($P = 0.055$ and $P = 0.829$ respectively).

CONCLUSIONS. We showed that DCs count in PB is significantly affected by prostate adenocarcinoma progression in a metastatic disease. *Prostate 9999: 1–7, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: **dendritic cells; prostate cancer; PSA; gleason score; immunotherapy**

INTRODUCTION

Among a test assay of novel therapies being developed for advanced prostate cancer, a number of immunotherapeutic approaches have started to reach clinical testing stages in the last decade [1–3]. Dendritic cell (DC) based modality is the center of intense investigation in the quest for on effective therapy.

Dendritic cells are bone marrow derived antigen presenting cells (APCs), that are responsible for the initiation and direction of immune responses. The ability of DCs is to take of and present antigens to stimulate T (and B) lymphocytes [4]. Two types of DCs are circulating in an immature form in the human blood: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) that can be identified on the basis of phenotypic markers and different function [5]. In particular mDCs express CD11c marker and they require granulocyte macrophage colony stimulating factor (GM-CSF) for growth and functions such as antigen uptake, T-cell activation and secretion of interlukin (IL)12 and (IL) 18. pDCs express CD123 marker, they are dependent on IL3 for survival and they produce high levels of interferon (IFN)-α [5,6].

Dendritic cells are considered the most potent professional APC for inducing anticancer immunity, both in vitro and in vivo [7,8,12]. Early clinical trials performed in human tumor systems have suggested favorable toxicity profiles and therapeutic efficacy for the administration of DCs in some cancer patients [8,9]. Human prostate cancer cells are generally considered to be poorly immunogenic despite the presence of antigens that are organ or tumor specific [3]. This lack of response to an immune stimulation may be overcome by enchancing the function of APC. DCs are potent APC that can both elicit primary or secondary immune responses [9–11].

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The turnover of DCs in human tissues substantially increases in relation to various inflammatory stimuli and blood DCs number raises to support this event. Recent studies using DCs counting methods have shown a significant changes not only in viral and bacterial infection such us HIV-infection [12] and tuberculosis [13], but also in cancer patients [14].

The identification and counting of DCs subsets in patients are important, allowing to understand the immunological activity and DCs role in different diseases. The ability to identify virtually every DC in the peripheral blood (PB) of the patient can provide a ''Snapshot'' view of the size of the various DC compartments and their changes over time [12–14]. Several methods for identifying and counting DCs have been previously proposed. These methods of absolute DCs count vary considerably, they produce conflicting data and are clearly dependent on the cell isolation procedures. The criteria used to define DCs subsets and the potential loss of DCs during the washing and centrifugation steps are practical issues and potential sources of variability. Despite the interest in DCs immunotherapy for prostate cancer there have been few studies on the expression of DCs in prostate cancer patients.

The aim of the present study was to characterize DCs subsets enumeration in PB samples of prostate adenocarcinoma patients, using a single-platform flow cytometric assay based on Tru-COUNT. In a previous study Vuckovic et al. underlined that the Tru-COUNT cytometric assay provides new methodological guidelines that could lead to the global standardization of DCs counting in clinical practice [14]. We also correlated DCs count with clinical and pathological characteristics of prostate cancer cases.

MATERIALS AND METHODS

Study Population

In this study we included 21 consecutive patients with newly histologically confirmed diagnosis of prostate adenocarcinoma obtained at prostate biopsy. None of these patients was previously treated for prostate adenocarcinoma. None of these patients presented a history of neoplastic diseases (except prostate adenocarcinoma) or infection diseases, others disorders, therapies or conditions known to interfere with DCs expression. In all cases, clinical stage (TNM 1997) of prostate cancer was obtained on the basis of digital rectal examination (DRE), transrectal ultrasonography (TRUS), CT scan, bone scan and serum PSA level determination. At biopsy pathological grading of the tumor was assessed using the Gleason system. Blood samples for PSA determination (RIA, Hybritech) were homogeneously and centrally collected in all cases before biopsy or other manipulations on the prostate gland. This population was divided on the basis of the clinical stage in two groups: localized versus metastatic prostate cancer. Nine cases showed a clinically localized (T1-T2 N0M0) whereas 12 cases an advanced metastatic (T1-T4 N0-1M1) prostate cancer. Moreover, 9/21 patients showed a tumor Gleason score ≤ 7 $(3+4)$, whereas 12/21 patients a Gleason score $\geq 7(4+3)$. Clinical and pathological characteristics of the 21 patients are described in Table I. As control we used a population of 18 age matched male healthy subjects without history or diagnosis of neoplastic diseases or other disorders or conditions know to influence DCs enumeration.

TABLE I. Clinical and Pathological Characteristics of Patients With Prostate Adenocarcinoma (mean ± SD, range)

Number of patients	21
Age (years)	70.2 ± 7.3(58–81)
PSA (ng/ml)	17.5 ± 6.2 (6.0–32.0)
Localized clinical stage	9
Metastatic clincal stage	12
Gleason score ≤ 7 $(3+4)$	9
Gleason score ≥ 7 $(4+3)$	12

Reagents

For DCs staining and count, Tru-COUNT^{TM} tubes, FACS Lysing Solution, and monoclonal antibodies (mAbs) were purchased from Becton Dickinson (BD Biosciences Pharmingen, Italy). Fluorescein isothiocyanate (FITC) and phycoerythrin (PE)—conjugate mAb were used; in particular mAb anti CD45-PerCP, anti-HLA-DR-APC, lineage-FITC cocktail, anti-CD123-PE and anti-CD11c-PE were used.

Quantification of Circulating pDCs and mDCs

DCs subsets were quantified using a single platform Tru-COUNT^{TM} assay. For DCs enumeration, PB samples were obtained after informed consent from all patients and all controls. All PB samples were homogeneously collected in the first morning using EDTA (citrate-phosphate-dextrose-adenine) tubes. Whole blood (0.1 μl) was directly stained in Tru-COUNT tubes that posses a known number of fluorescent beads released when the appropriate mAbs reagent and whole blood were added. To define DCs subpopulations, the following monoclonal antibodies were added: anti CD45-PerCP, anti-HLA-DR-APC, Lineage-FITC cocktail composed of anti-CD3 (SK7), anti-CD14 (MP9), anti-CD16 (3G8), anti-CD19 (SJ25C1),

anti-CD20 (L27), anti-CD56 (NCAM 16-2), and anti-CD123-PE, or anti-CD11c-PE. Relevant isotype controls (mAbs) were used. After adding the appropriate mAbs reagent to the whole blood and mixing gently, a 15 min incubation time in the dark, at room temperature (RT), was used. Each tube was added with 450 μl of FACS Lysing solution, vortexed and incubated for 15 min at RT. Samples were analyzed within 1–3 hr of staining, using a FACS calibur flow cytometer and Cell Quest 1.0 (Becton Dickinscon, MountainView CA). All data were collected using an identical instrument setting. Setting the threshold on PerCP fluorescence (FL3), we defined a R1 gate for lymphocytes and monocytes in a dot plot of CD45 versus side scatter (SSC). To determine a significant number of DCs, we have acquired 100,000 cellular events in R1 from 0.1 μl of blood.

These events were displayed in a second dot plot to identify lineage-negative (R2 gate). To define DCs subsets (mDC and pDC) we have created a contour plot of CD11c versus HLA-DR or CD123 versus HLA-DR respectively.

In this gating strategy, all HLA-DR positive, $CD11c^{hi}$ and $CD123^{hi}$ events were included (Fig. 1). To calculate absolute number of pDCs or mDCs (cells/ml blood) the following equation was used: (R3 events × known Tru-COUNT beads)/(R4 events × 0.1 μl), where the Tru-COUNT bead number (R4) was obtained in an ungated dot plot of FL1 versus FL2 and 22.000 bead events were collected on average.

Statistical Analysis

Statistical analysis was performed using Sigma Stat 2.2 (Jandel Scientific Software, San Rafael, CA). Statistical analysis was performed either in the whole population or dividing patients on the basis of clinical stage (localized vs. metastatic). The healthy population was always used as control. The parameter Gleason score was dichotomized in $\leq 7(3+4)$ and $\geq 7(4+3)$. Serum PSA was used as a continuous variable. Descriptive analysis were used to characterize adenocarcinoma prostate cancer patients as well as mDCs and pDCs determination in PB. Spearman coefficients were calculated to measure the association among parameters. Differences between groups were analyzed by the Mann–Whitney test. P-values <0.05 were considered statistically significant.

RESULTS

Using the Tru-COUNT method, pDCs and mDCs were effectively detected and enumerated in PB of all subjects analyzed. Gating strategy performed for the identification of DCs subsets, using the flow cytometric method, is described in Materials and Methods section; DCs subpopulations were easily distinguished from other cells in controls as well as in patients, adding appropriate mAbs for DCs staining.

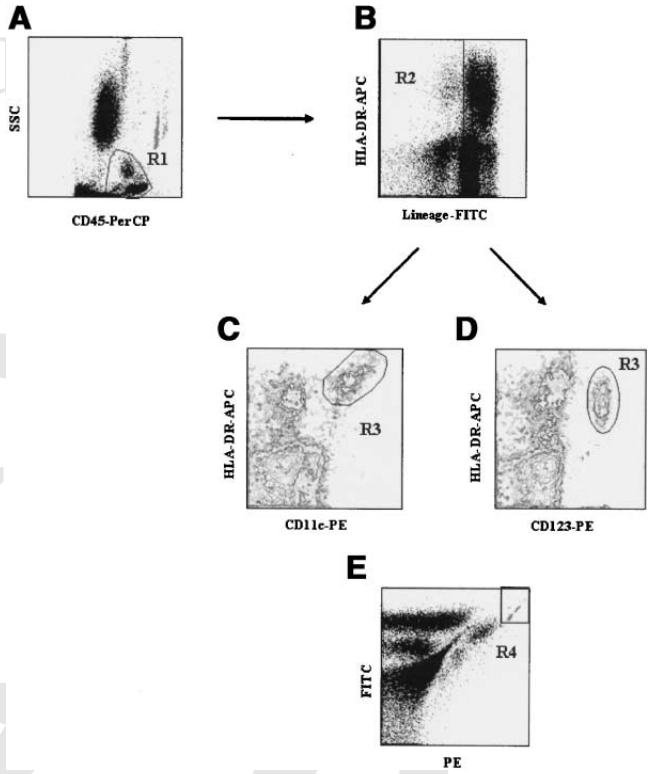

Fig. 1. Cytofluorimetric DC subsets definition by Tru-COUNT assay. Gate R1 was used for lymphocytes and monocytes. Beads appear at the extreme right of the dot plot (**A**). Gate R2 was created to identify lineage-negative cells (**B**). To define mDC and pDC, events from R1 and R2 were analyzed in a contour plot of CD11c versus HLA-DR (**C**) or CD123 versus HLA−DR (**D**). All $CD11c^{hi}$-HLA-DR^{+} and $CD123^{hi}$-HLA-DR^{+} were included in this gating strategy. Tru-COUNT beads (R4) were obtained in an ungated dot plot of FL1 versus FL2 (**E**).

DCs Subsets Enumeratios in Patients With Prostate Adenocarcinoma Versus Healthy Controls

Circulating pDCs and mDCs were determined in all 21 patients with prostate adenocarcinoma and all 18 healthy controls. In our prostate adenocarcinoma patients we showed a statistically significant reduction in pDCs count when compared to healty controls. The median value of pDCs in patients was 7497 cell/ml (range 208–19043) versus 11907 cell/ml (range 3875–37675) in healthy subjects ($P = 0.002$). Also for mDCs count a reduction in prostate adenocarcinoma patients when compared to healthy controls was found (median 12769 cell/ml; range 264–43014 vs. 13295 cell/ml; 8901–45917) but it did not reached statistical significance ($P = 0.100$) (Fig. 2).

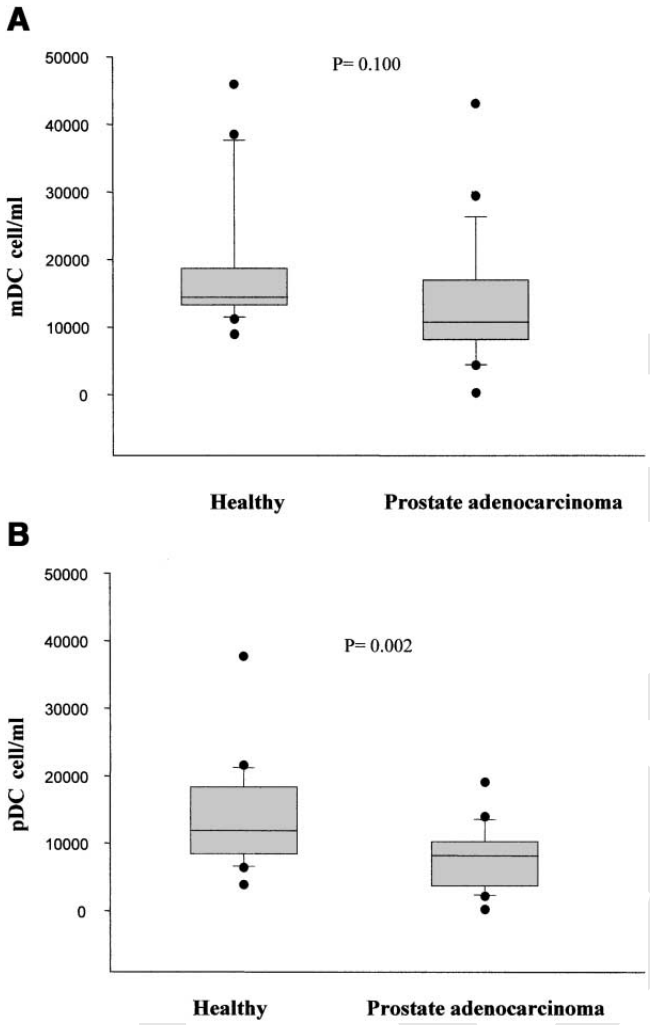

Fig. 2. DCs subsets, (**A**) mDCs and (**B**) pDCs, enumeration: prostate adenocarcinoma group versus healthy group (*P*-value: Mann–Whitney test).

DCs Subsets Enumeration in Localized and Metastatic Prostate Adenocarcinomas

Distinguishing our prostate adenocarcinoma patients, on the basis of clinical stage, pDCs and mDCs counts were lower in patients with metastatic when compared to localized disease. In fact in localized cancers the median value of pDCs and mDCs was 8168 cell/ml (range 3593–19043), and 16978 cell/ml (range 6536–29392) respectively versus 5967 cell/ml (range 208–13927) for pDCs and 10587 cell/ml (range 264–43014) for mDCs in metastatic cancers, but differences did not reached statistical significance ($P = 0.260$ and $P = 0.380$ respectively). However, comparing each group (localized and metastatic) with healthy controls, significant differences were found between healthy controls and the metastatic group in both pDCs and mDCs count ($P = 0.005$ and $P = 0.023$ respectively) but not between healthy controls and the localized group ($P = 0.055$ and $P = 0.829$ respectively) (Fig. 3). The cytofluorimetric analysis of two representative patients metastatic and localized were showed in Figure 4.

Association Between DCs Subsets Enumeration and Gleason Score or PSA Levels in Prostate Adenocarcinomas

Using the Spearman rank correlation test, in the prostate adenocarcinoma population a no statistically significant association between pDCs or mDCs counts and serum PSA levels ($r = -0.312$ $P = 0.166$ and $r = -0.356$ $P = 0.111$ respectively) was found (Fig. 5). Analyzing the correlation between DC subsets and Gleason score, only mDCs ($r = -0.502$ $P = 0.0206$) and not pDCs ($r = -0.327$ $P = 0.145$) were significantly and inversely associated with tumor Gleason score (Fig. 6).

DISCUSSION

The role of DCs in the initiation and control of innate and adaptive immune responses is well documented

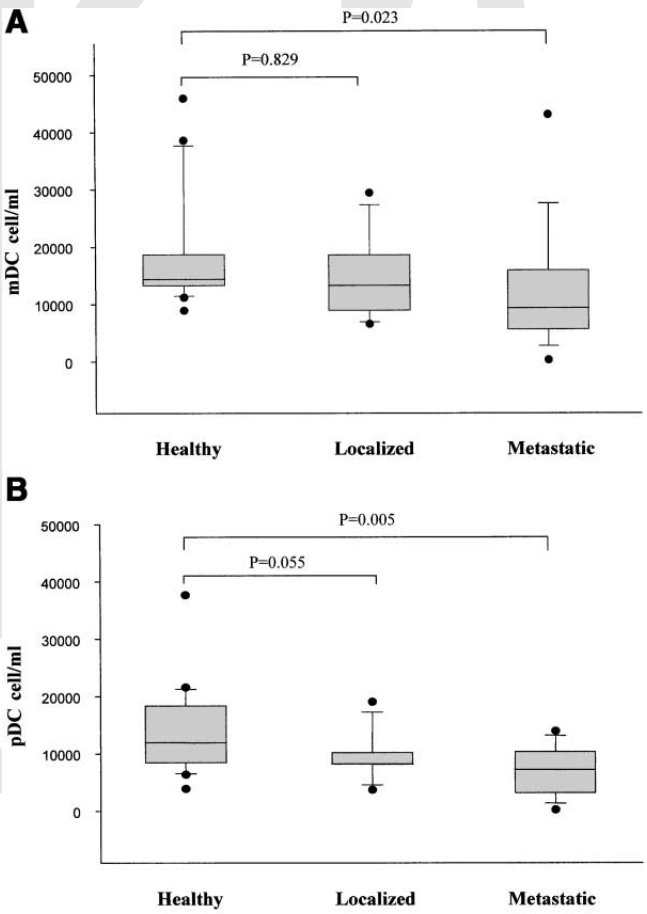

Fig. 3. DCs subsets, (**A**) mDCs and (**B**) pDCs, enumeration: metastatic versus localized prostate adenocarcinoma cases compared with healthy group. (*P*-value: Mann–Whitney test).

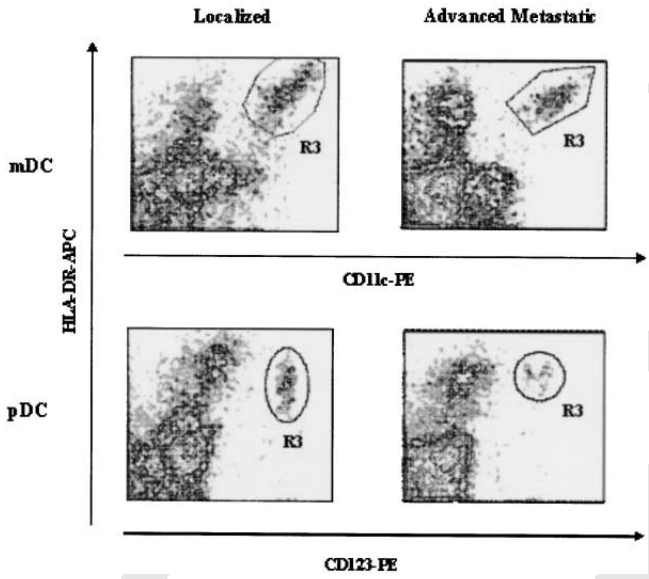

Fig. 4. pDC and mDC enumeration in one representative localized cancer patient, and one metastatic cancer patient. Gating strategy used to define DC subsets is shown in Figure I. Here contour plots are shown.

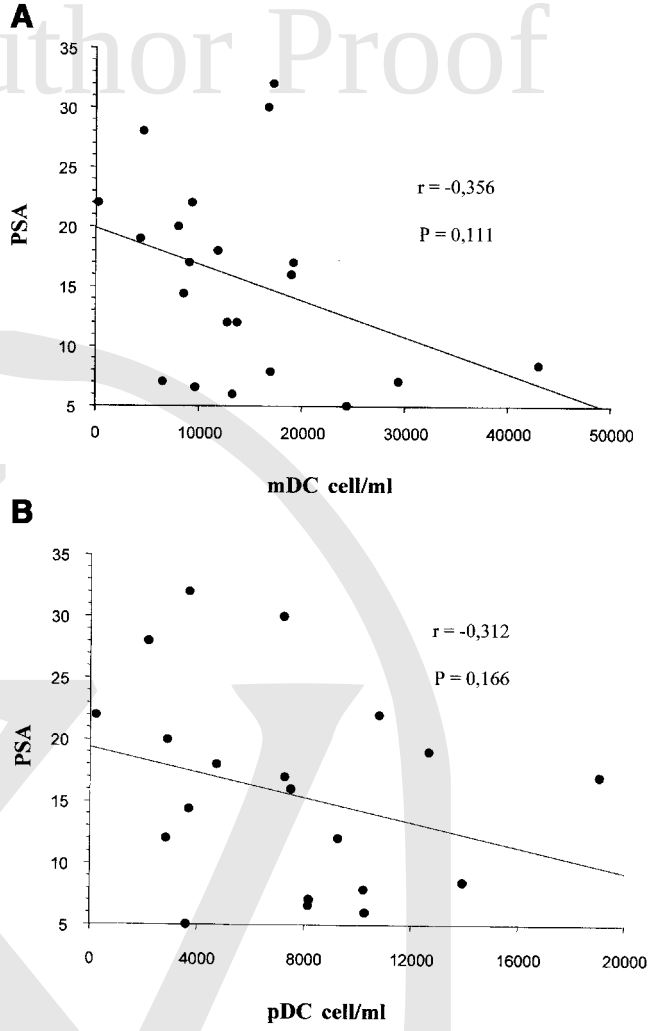

Fig. 5. DCs subsets, (**A**) mDCs and (**B**) pDCs, enumeration: association with PSA levels (Spearman's Coefficent).

[4]. In recent years there has been an increased interest in the evaluation of DCs subsets in PB, since an improvement in the enumeration methods has been obtained [14]. Several methods for identifying and counting DCs have been proposed. In the present study we used a single platform Tru-Count assay to directly evaluate and quantify circulating blood DCs subsets in prostate adenocarcinoma patients. This technique requires the use of whole blood and a flow cytometric analysis and it is considered to be a simple assay with a very precise counting [14]. The use of a single platform assay for DCs counting combines three key advantages: (1) mAbs that bind to antigens expressed on circulating DCs, (2) Tru-COUNT beads for precise cellular quantification and (3) a whole blood ''Lyse/No Wash'' flow cytometic protocol to eliminate the potential cell loss during washing steps. In this way, the single platform Tru-COUNT assay provides accurate and reproducible DCs counting and eliminates the problem of variability related to the method. Vurckovic et al. [14] affirmed that this assay provides new methodological guidelines that could lead to the global standardization of DCs counting for physiological, diagnostic and prognostic application in clinical practice.

In the present study, we characterized DCs subsets enumeration in PB of prostate adenocarcinoma patients. The utility of DCs preparation for immunotherapy is highlighted by the promising results of clinical trials using density gradient enriched DCs for metastatic prostate cancer [2]. Previous studies proposed the use of DCs to cross-present antigens derived from human prostate tumor cells and to obtain a better activation of antigen-specific T-cells [15]. Phase I and II clinical trials [7,16] examined the stimulation of autologous DCs in advanced prostate cancer cases. They also suggested the potential of autologous DCs as a vehicle to deliver specific target antigens, a crucial issue in prostate cancer vaccine development. However, none of these works specifically characterized DCs count in prostate cancer cases, so to define their basic role and possible modifications induced by the tumor. In vitro studies have shown that DCs development can be inhibited by the tumor itself [17] or factors such as PSA [18].

With a view to providing a better understanding of the role of DCs in prostate cancer, in the present work we verified wheather prostate adenocarcinoma produces specific modifications in DCs count and whether these modification, are related to clinical and pathological characteristics of the tumor. Our results

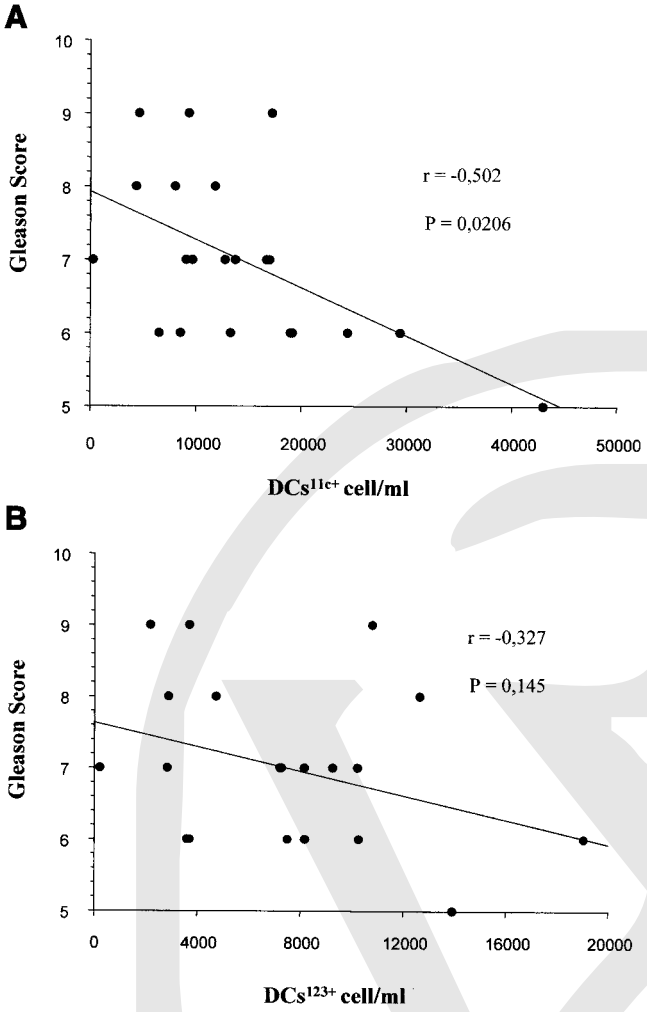

Fig. 6. DCs subsets, (**A**) mDCs and (**B**) pDCs, enumeration: association with Gleason Score (dichotomized in $\leq 7(3+4)$ and ≥ 7 $(4+3)$) (Speraman's Coefficient).

were always compared to a population of healthy adults. In our population we showed that, in particular metastatic cancer patients exhibited a significant decrease in the absolute number of circulating DCs when compared to healthy controls. The reduction affects both the two main subsets of DCs circulating in the PB (pDCs and mDCs), but the most significant reduction was seen for pDCs count. The decrease in circulating pDCs may have functional consequences on the production of cytokines and on antigen presentation to naïve T-cells, particularly through a reduction of type I IFN since pDCs are the natural IFN-producing cells in the immune system [4]. On the contrary, we found that localized prostate cancer patients showed lower, but not statistically significant, DCs counts when compared to healthy controls. Therefore, the progression of the tumor in an advanced metastatic stage seems to be a relevant factor able to influence DCs expression in PB.

Early studies using DCs counting methods have shown that DCs in the PB can be decreased by viral, parasitic, and bacterial infectious diseases [12,13]. Variations were also described as a function of other neoplastic diseases such as breast cancer, always in an advanced stage [14,19]. Generally, data analyzed in neoplastic diseases suggest that the degree and type of DCs dysfunction is likely to depend on the tumor type and disease stage. The clinical hypothesis is that any defects of DCs in vivo can be readily reversed by activation in vitro. In contrast with our results, recently Wilkinson et al. [20], in prostate cancer cases compared to healthy donors, reported no significant differences in any of the DCs subsets in PB, at any clinical stage of prostate cancer. They concluded that DCs development in vivo is preserved during prostate cancer progression. As in our study, also Wilkinson et al. [20] used a single platform Tru-COUNT assay to evaluate DCs in PB. However, authors [20] focused their work more on a functional analysis than on a specific quantification of DCs subsets in patients. In particular, the absolute number (cell/ml blood) of DCs subsets is not presented in the work of Wilkinson et al. [20] and it cannot be compared with our data. To specifically analyze the role of clinical stage progression on DCs enumeration in prostate cancer, we simply distinguished our population in localized and metastatic disease. On the contrary, in the study of Wilkinson et al. [20] patients were divided in ''Biopsy and PSA positive,'' hormone sensitive and hormone resistant cases. In this way authors [20] seem to analyze more the influence of androgen dependency than that of clinical stage progression on DCs expression. However, differences in results between our study and that of Wilkinson et al. [20] may also reflect a variability in DCs enumeration in prostate cancer disease.

If we analyze the results of our study in relation with other two prognostic parameters related to the aggressiveness of prostate cancer, such as serum PSA and tumor Gleason score, DCs count was inversely associated with both parameters. In other words, at the increase of PSA or Gleason score corresponded a decrease in DCs count in PB. However, in our experience, these associations did not reached statistically significance (with except of mDCs with Gleason score). Therefore, comparing different parameters related to prostate cancer aggressiveness and progression, the stage and the presence of a systemic metastatic disease, seems to be the most relevant factor influencing DCs count in the PB.

We must also underline that our results, as in most of the studies [20] in the literature, are on a limited population of prostate adenocarcinoma cases; therefore, a stratification in subgroups can reduce the statistical significance. We programmed to continue

our analysis on DCs in prostate adenocarcinoma, verifying a possible association of DCs enumeration with the use of different hormone therapies and with the hormone dependency or refractory status of the tumor.

CONCLUSIONS

With a view to providing a better understanding of the role of DCs in prostate cancer, we showed that in our population, DCs count in PB is significantly affected by prostate cancer progression when compared to healthy non-neoplastic cases. The presence of a systemic metastatic disease particulary affects DCs count. The increasing interest in DCs for cancer inmmunotherapy has hastened the clinical need for a reliable DCs counting and determination also in prostate cancer cases. Any conclusion on this topic can be obtained only from larger studies.

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