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# Dendritic and lymphocytic cell infiltration in prostate carcinoma

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**Summary.** We examined the distribution of $CD1a^+$ cells and $CD8^+$ and $CD4^+$ T lymphocytes in prostate cancer (PCa) and correlated these with clinicopathological parameters. We also investigated whether the distribution of these cells was related to the expression of the cell membrane protein B7-H3, a putative negative regulator of the immune response expressed on PCa cells.

A cohort of 151 PCa patients treated with radical prostatectomy (RP) was followed prospectively from 1985 until 2006 with a median follow-up of 9 years. Whole-mount sections of PCa specimens were immunostained to identify immune cells.

A low number of $CD1a^+$ cells was significantly associated with a high Gleason score and high pathological stage of pT3. The number of $CD1a^+$ cells correlated significantly with the number of intratumoral and stromal $CD8^+$ and stromal $CD4^+$ lymphocytes. Kaplan-Meier analysis showed a tendency toward impaired biochemical progression-free survival in patients with few $CD1a^+$ cells within their RP specimens. The expression of B7-H3 correlated inversely with the number of $CD1a^+$ cells and intratumoral $CD4^+$ lymphocytes; there was a trend for a similar inverse relationship between B7-H3 expression and the number of $CD8^+$ lymphocytes.

Our findings suggest that high-grade prostate carcinoma cells manipulate the immune system and that these changes contribute to the mechanism underlying tumor escape from immune surveillance.

**Key words:** Prostate cancer, CD1a, Dendritic cells, T lymphocytes

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## Introduction

Prostate cancer (PCa) is the most common type of cancer among males in the USA and other Western countries. PCa is the second leading cause of cancer mortality and accounted for more than 32,050 deaths in the USA in 2010.

Immune cell infiltration into the tumor environment occurs during the host response to a malignant tumor and has been reported for various tumor types [for review see (Pages et al., 2010)]. The presence of tumor-infiltrating lymphocytes (TILs) is an independent prognostic factor for many tumor types (Galon et al., 2012). Bronte and coworkers (2005) reported that, among TILs, $CD8^+$ cells comprise the major proportion and $CD4^+$ cells a minor proportion of infiltrating cells.

Langerhans cells (LCs) were initially described as antigen-presenting cells present in the epidermis. In a study comprising 38 PCa cases, the presence of LCs was associated mainly with low-grade prostate carcinoma (Bigotti et al., 1991). Professional antigen-presenting cells such as dendritic cells (DCs) and LCs express CD1a on their cell surface. Several studies have indicated that LCs are involved in the primary immune response in several types of malignancy [for review see

(Coventry and Heinzel, 2004)]. The presence of DCs is associated with a favorable prognosis in some cancers such as gastric cancer (Ananiev et al., 2011) and colorectal cancer (Gulubova et al., 2012). However, the opposite relationship has also been reported. In breast cancer, the presence of DCs is associated with poor prognosis (Coventry and Morton, 2003), and one publication on colorectal cancer has reported an association between the numbers of DCs and $CD4^+$ and $CD8^+$ T lymphocytes (Dadabayev et al., 2004).

The B7 family of immune costimulatory cell surface proteins comprises four members [for review see (Thompson et al., 2009)], all of which are involved in cross talk between immune cells. Surprisingly, one of these proteins, B7-H3, is expressed as a surface protein on carcinoma cells and is thought to be involved in cell-cell interactions through its ligand being expressed on neighboring cells. Aberrant expression of B7-H3, especially in high-grade PCa, has been reported (Roth et al., 2007; Zang et al., 2007). We hypothesized that B7-H3 expression contributes to a malignant PCa phenotype that might negatively influence the immune response. However, B7-H3 has also been shown to play a nonimmunological role in cancer. B7-H3-knockdown experiments have shown that certain cancer cell lines display increased chemosensitivity and reduced potential for metastasis [for review, see (Nygren et al., 2011)].

Research from the past decade has provided insight into different molecular mechanisms used by cancer cells to escape the host's immune system [for review see (Janikashvili et al., 2011)]. The exact mechanisms behind the interplay between tumor, tumor stroma, and nontumor cells are not understood fully and need further detailed investigation.

The aim of the present study was to explore the underlying immune mechanisms in PCa and to examine the frequency, distribution, and composition of $CD1a^+$ LCs and $CD8^+$ and $CD4^+$ T lymphocytes. We examined the relationships between these variables and B7-H3 expression in prostate carcinoma cells and clinicopathological parameters in a group of 118 Norwegian patients with PCa treated with radical prostatectomy (RP). We found a significant correlation between the numbers of $CD1a^+$ cells and the grade and stage of PCa, but a scarcity of these cells in benign prostate tissue.

## Materials and methods

### *Clinical features and radical prostatectomy*

From 1985 to 2006, 151 consecutive patients with clinically localized PCa were treated with a radical retropubic prostatectomy in Arendal Hospital, Norway. The hospital serves approximately 100.000 inhabitants. In three patients, the carcinoma diagnosis could not be confirmed histologically, and they were excluded from further analyses. All patients were followed in an outpatient unit every 3 and later every 6 months for the first 5 years, and annually thereafter. Prostate-specific antigen (PSA) concentration was measured from 1991. Clinical failure was defined as disease progression with clinical local recurrence or evidence of distant metastasis by skeletal scintigraphy. An elevated PSA concentration above 0.20 ng/ml in two consecutive measurements was defined as biochemical failure. The patient characteristics are presented in Table 1.

The ethical committee of the Health Region South-East of Norway approved the study (REK no 2.2007.219).

### *Histopathological features*

The prostate specimens were processed as described previously (Servoll et al., 2010). Gleason score assessment and histological classification were performed according to the International Society of Urological Pathology Consensus on Gleason grading of prostate cancer (Table 1) (Lotan and Epstein, 2010). The surgical margin, seminal vesicle invasion, and extraprostatic extension were also recorded. All cases were reexamined by a senior genitourinary pathologist (LV) without prior knowledge of the clinical data. In challenging cases, the Gleason score was reexamined together with a second genitourinary pathologist (JMN) until final agreement was reached.

### *Immunohistochemical examinations*

Thirty cases were lost during immunohistochemical analysis because of limited tumor tissue availability. One hundred eighteen cases were included in the final evaluation and clinicopathological correlations.

Formalin-fixed, paraffin-embedded 5-$\mu$m-thick sections were processed for immunohistochemistry using the Dako EnVision™ Flex+ System (K8012, Dako Corporation, Carpinteria, CA, USA) and Dako Autostainer. Deparaffinization and unmasking of epitopes were performed using PT-Link (Dako) and EnVision™ Flex target retrieval solution, low pH. To block endogenous peroxidase, the sections were treated with 0.03% hydrogen peroxide for 5 min. The sections were incubated for 30 min with antibodies against CD1a, CD4, and CD8 (Novocastra, Leica Microsystems A/S, Ballerup, Denmark). The slides were then incubated with EnVision™ Flex+ rabbit linker (15 min) and EnVision™ Flex/HRP enzyme (30 min). The tissue was stained for 10 min with 3,3'-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, dehydrated, and mounted in Diatex (Dako). All series included positive controls. Negative controls were made by replacing the antibody with the corresponding nonimmune normal IgG at the same concentration as the antibody used. The immunohistochemical staining procedure for B7-H3 has been described (Liu et al., 2012).

All slides were evaluated without prior knowledge of the patients' outcomes. For each case, infiltrating

immunoreactive cells were counted in 10 randomly selected 40x magnification fields. The number, distribution, and composition of infiltrating immune cells were recorded for further clinicopathological analyses.

### *Statistics*

Nonparametric tests were used because of the nonnormal distribution of data. Continuous data were compared between groups using the Mann-Whitney U test. Correlations were calculated using Spearman's rho. The end points of the study were biochemical failure, clinical failure, PCa-specific mortality, and overall survival. Kaplan-Meier analyses were used to compare survival curves for patients with high and low numbers of CD1a$^+$ LCs using a median value of 17 as the cutoff. The relationship between CD1a$^+$ LC numbers and clinical outcomes was analyzed as a continuous variable in a univariate Cox proportional hazards regression analysis and a multivariate Cox proportional hazards regression analysis after controlling for pathological T-stage, Gleason score, extraprostatic extension, and seminal vesicle invasion. Patients who died of unrelated causes without developing biochemical or clinical failure were treated as censored cases. A two-sided p-value of less than 0.05 was considered significant. All analyses were performed using SPSS 18.0 (SPSS, Chicago, IL, USA).

## Results

### Clinicopathological features and clinical outcomes

The mean age of the patients at surgery with RP was 61 years (44-72). The median follow-up period for the whole cohort analyzed was 108 months (14-296), i.e. 9 years. Five of 118 patients (4%) died of PCa. The overall

**Table 1.** Patient characteristics.

| Variable | Frequency (%) |
|---|---|
| Age, yrs., mean (range) | 61 (44-72) |
| Preoperative PSA, ng/mL | |
| <10 | 72 (61%) |
| 10-20 | 26 (22%) |
| >20 | 20 (17%) |
| Gleason score | |
| <7 | 35 (30%) |
| 7a | 48 (41%) |
| 7b | 18 (15%) |
| >7 | 17 (14%) |
| Pathological stage | |
| ≤T2 | 64 (54%) |
| ≥T3 | 54 (46%) |
| Extraprostatic extension | 56 (48%) |
| Seminal vesicle invasion | 14 (12%) |
| Positive surgical margins | 70 (59%) |
| Lymph node involvement | |
| N0 | 82 (70%) |
| N1 | 5 (4%) |
| Nx | 31 (26%) |
| Biochemical failure | 51 (46%) |
| Clinical failure | 43 (36%) |
| Prostate cancer-specific mortality | 5 (4%) |
| Overall mortality | 22 (19%) |

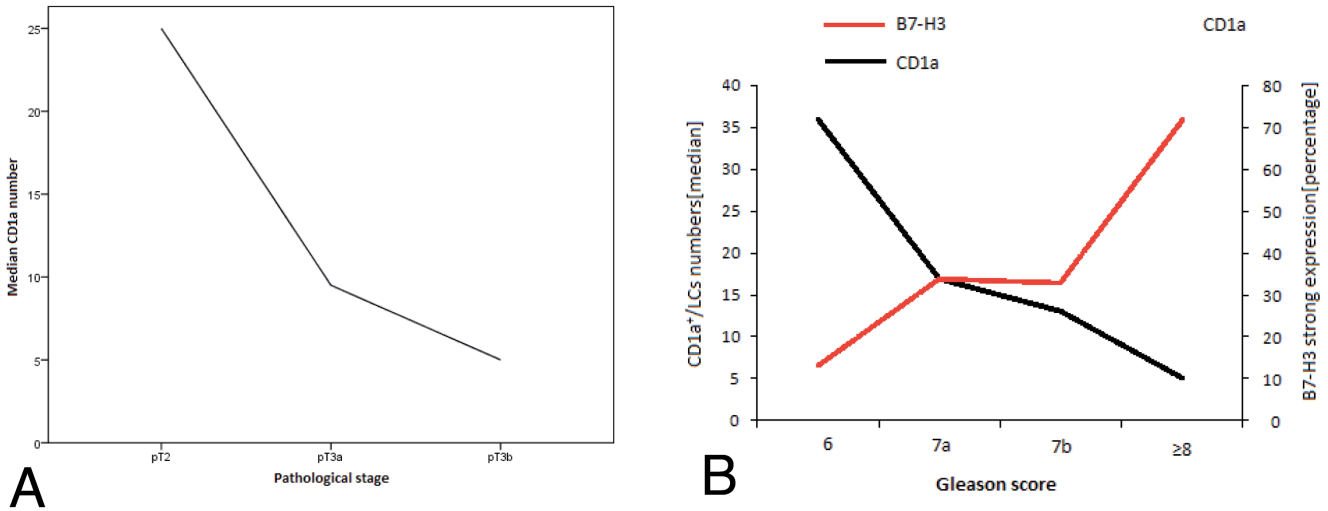


**Fig. 1. A.** Median CD1a$^+$/Langerhans cells number according to pathological stage. **B.** Relationship between Gleason score, CD1a$^+$/Langerhans cell numbers and B7-H3 expression. The correlation between Gleason score and B7-H3 is statistically significant positive (Spearman's rho 0.341, p-value <0.005), whereas the correlation between Gleason score and CD1a$^+$/Langerhans cell numbers is statistically significant negative (spearman's rho -0.202, p-value <0.028).

death rate was 22 of 118 patients (19%). Fifty-one patients (46%) developed biochemical relapse, and 43 patients progressed clinically (36%). The pathological characteristics and clinical outcomes of the 118 RP specimens are listed in Table 1.

### *CD1a+ LCs in PCa and in relation to clinicopathological parameters*

The total numbers of CD1a+ LCs ranged from 0 to 286 cells/10 selected magnification fields, with a median value of 17. The median number of CD1a+ cells in benign prostate hyperplasia specimens was 2 cells/10 selected magnification fields. CD1a+ cell number correlated inversely with the Gleason score (Spearman's rho=-0.202, p=0.028). Prostatic carcinomas with a Gleason score of 6 or 7a (3+4=7) had significantly more CD1a+ LCs compared with prostatic carcinomas with a Gleason score of 7b (4+3=7) or above (Table 2 and Fig. 1A). The number of CD1a+ cells correlated inversely with the pathological stage of prostate carcinomas (r=-0.245, p=0.008). Prostate carcinomas that extended beyond the prostate capsule (pT3a) had fewer CD1a+ LCs than did organ-confined carcinomas (pT2), and tumors invading the seminal vesicles contained even fewer CD1a+ cells (Table 2, Figs. 1A, 2A).

**Table 2.** Association between numbers of CD1a+/Langerhans cells and clinical-pathological characteristics.

| | CD1a+ low numbers | CD1a+ high numbers | Correlation coefficient (p-value) |
|---|---|---|---|
| Pathological stage | | | r=-0.245 |
| pT2 | 27 (42%) | 37 (58%) | p=0.008 |
| pT3a | 24 (60%) | 16 (40%) | |
| pT3b | 10 (77%) | 3 (23%) | |
| Gleason score | | | r=-0.202 |
| <7 | 15 (43%) | 20 (57%) | p=0.028 |
| 7a | 24 (50%) | 24 (50%) | |
| 7b | 10 (56%) | 8 (44%) | |
| ≥8 | 12 (71%) | 5 (29%) | |

CD1a+ low numbers: ≤ 17(median); CD1a+ high numbers: >17(median). The p-value was calculated with the Spearman's correlation coefficients and p-values.

### *Correlation between CD1a+ LCs and stromal and intratumoral CD4+ and CD8+ T lymphocyte numbers*

We also analyzed the relationships between the number of CD1a+ LCs cells and the frequency and distribution of CD8+ and CD4+ T lymphocytes in the stromal and intratumoral compartments. As shown in Table 3, the number of CD1a+ cells correlated significantly with the numbers of CD8+ cells in both the tumor and surrounding tumor stroma (Fig. 3). The

**Table 3.** CD1a correlation coefficient and p-values. Correlation with CD8+ and CD4+ T lymphocytes in tumor and surrounding stroma in whole mount slides of RP specimens.

| | Tumor CD8 | Stroma CD8 | Tumor CD4 | Stroma CD4 |
|---|---|---|---|---|
| Coefficient | 0.377 | 0.312 | 0.121 | 0.185 |
| P-value | 0.000 | 0.000 | 0.192 | 0.045 |

Numbers of cells are the median of the sum of ten visual fields (x10 magnification) in whole mount slides. The p-value was calculated with the Spearman's rho-test.

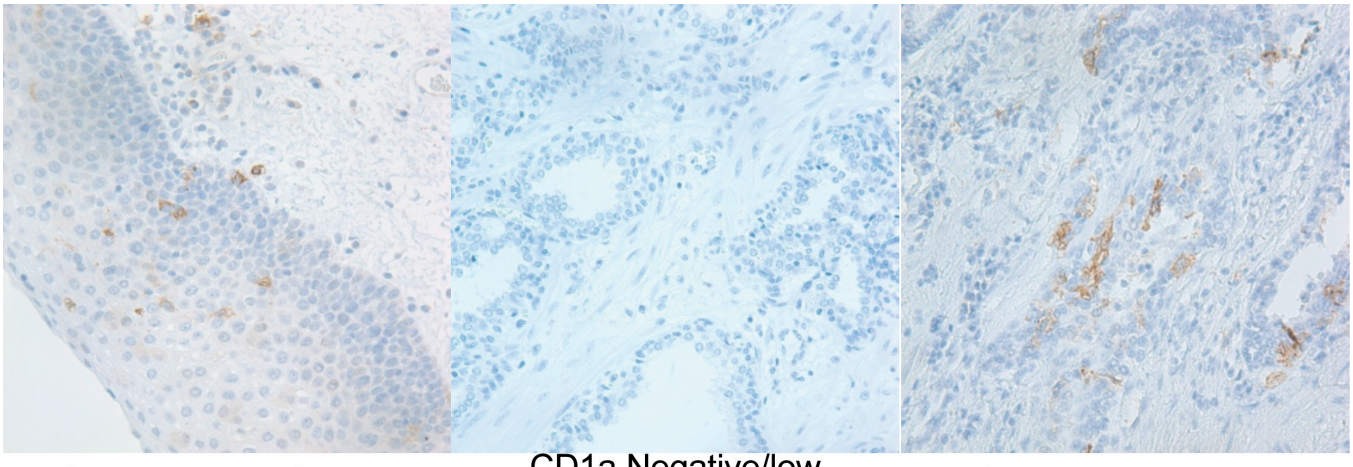


**Fig. 2.** Immunohistochemical staining of prostate cancer specimens for CD1a+ cells. x 40

number of CD1a$^+$ cells also correlated significantly with the number of CD4$^+$ T lymphocytes in the surrounding stroma, but not with the number of intratumoral CD4$^+$ T lymphocytes (Fig. 4).

### *Inverse association between CD1a$^+$ LCs and CD8$^+$ and CD4$^+$ T lymphocytes with B7-H3 expression in prostate carcinoma cells*

B7-H3 is a cell surface protein that downregulates the immune response. We analyzed the expression of this protein in relation to the numbers of CD1a$^+$ cells and CD8$^+$ and CD4$^+$ T lymphocytes in the tumor area. Prostate carcinomas with strong expression of B7-H3 had significantly fewer CD1a$^+$ cells (Table 4) and fewer intratumoral CD4$^+$ T lymphocytes. There was a tendency toward fewer intratumoral CD8$^+$ T lymphocytes in prostate carcinomas expressing high B7-H3 levels. We found no association between the number of stromal CD4$^+$ or CD8$^+$ T lymphocytes and immunostaining intensity of B7-H3 in prostate carcinoma cells.

**Table 4.** Median cell numbers of CD1a$^+$ cells, and CD8$^+$ and CD4$^+$ T lymphocytes counted in tumor and stroma stratified by B7-H3 immunostaining intensity of prostatic carcinoma in whole mount RP specimen slices.

| T-cell (median) | B7-H3 none-moderate | B7-H3 strong | p-value |
|---|---|---|---|
| CD8 tumor | 151 | 107 | 0.067 |
| CD8 stroma | 155 | 131 | 0.229 |
| CD4 tumor | 165 | 131 | 0.040 |
| CD4 stroma | 399 | 351 | 0.259 |
| CD1a | 32 | 10 | 0.025 |

The p-value was calculated with the Mann-Whitney U-test. Missing values: 30

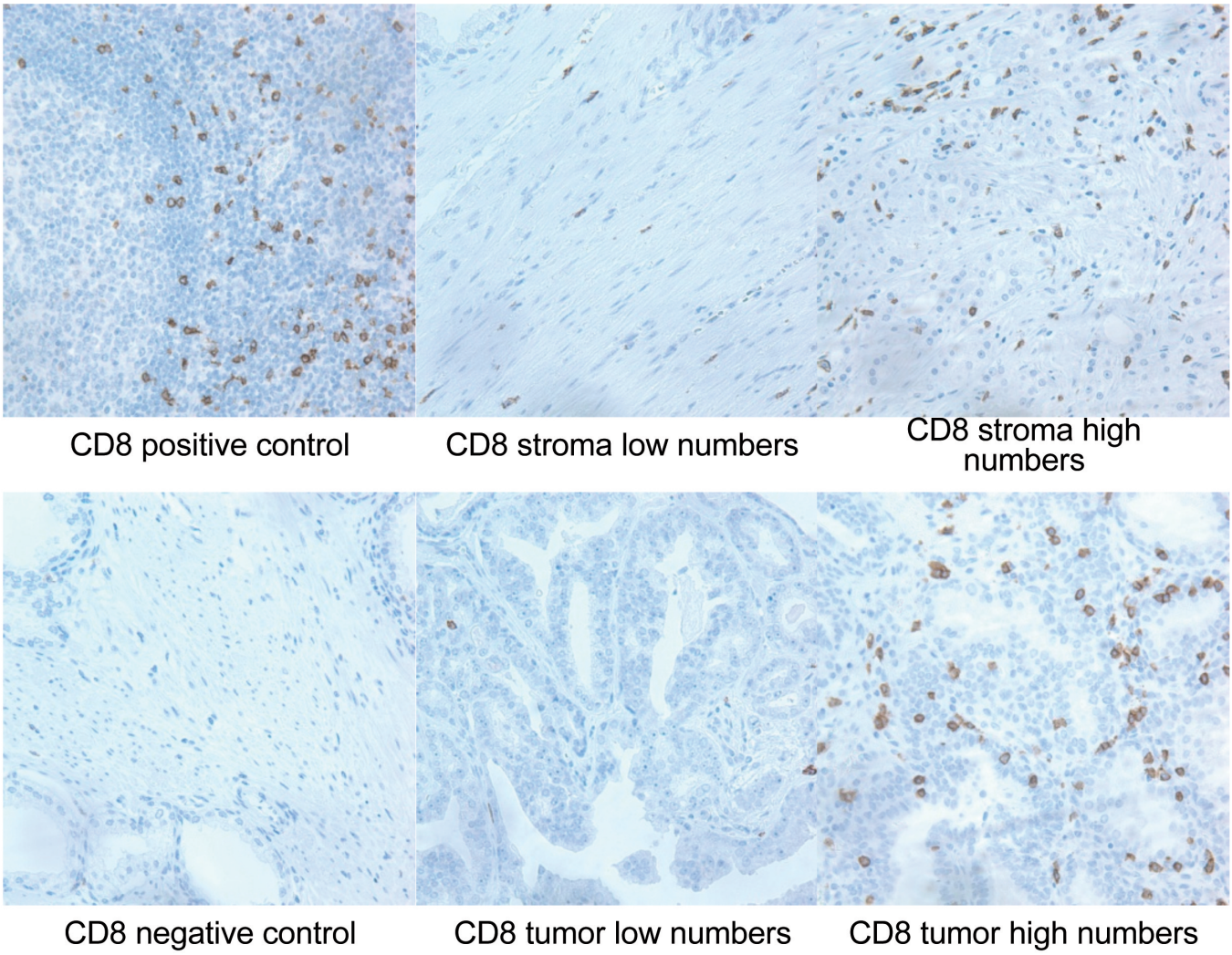


**Fig. 3.** Immunohistochemical staining of prostate cancer specimens for CD8$^+$ lymphocytes. x 40

### *Presence of CD1a+ LCs in prostate carcinoma and relationship with clinical outcome*

In univariate Kaplan-Meier analyses, the number of CD1a+ cells in prostate carcinoma specimens was not related significantly to the prognostic factors biochemical failure, clinical failure, prostate cancer-specific mortality, or overall survival (Table 5). In the univariate and multivariate Cox proportional hazards regression analyses, the number of CD1a+ LCs was not related to the clinical outcome. However, there was a tendency toward longer biochemical-free survival in patients with high numbers of CD1a+ cells.

## Discussion

To our knowledge, the present study is the first to demonstrate a direct and significant association between the numbers of antigen-presenting cells, in this case CD1a+ cells, and a progressive malignant carcinoma phenotype. We found a significant correlation between low numbers of CD1a+ cells and a high Gleason score and pathological stage pT3. By contrast, the numbers of

**Table 5.** Association between CD1a+ cell numbers in RP specimens and correlation to clinical outcome.

| Clinical outcome | CD1a low numbers | CD1a high numbers | P-value |
|---|---|---|---|
| Biochemical failure | 31 (53%) | 20 (39%) | n.s. |
| Clinical failure | 23 (38%) | 20 (35%) | n.s. |
| Prostate cancer specific mortality | 3 (5%) | 2 (4%) | n.s. |
| Overall survival | 13 (21%) | 9 (16%) | n.s. |

CD1a+ low numbers: ≤17(median); CD1a+ high numbers: >17(median). The p-value was calculated with the Log-rank test. n.s.: non-specific.

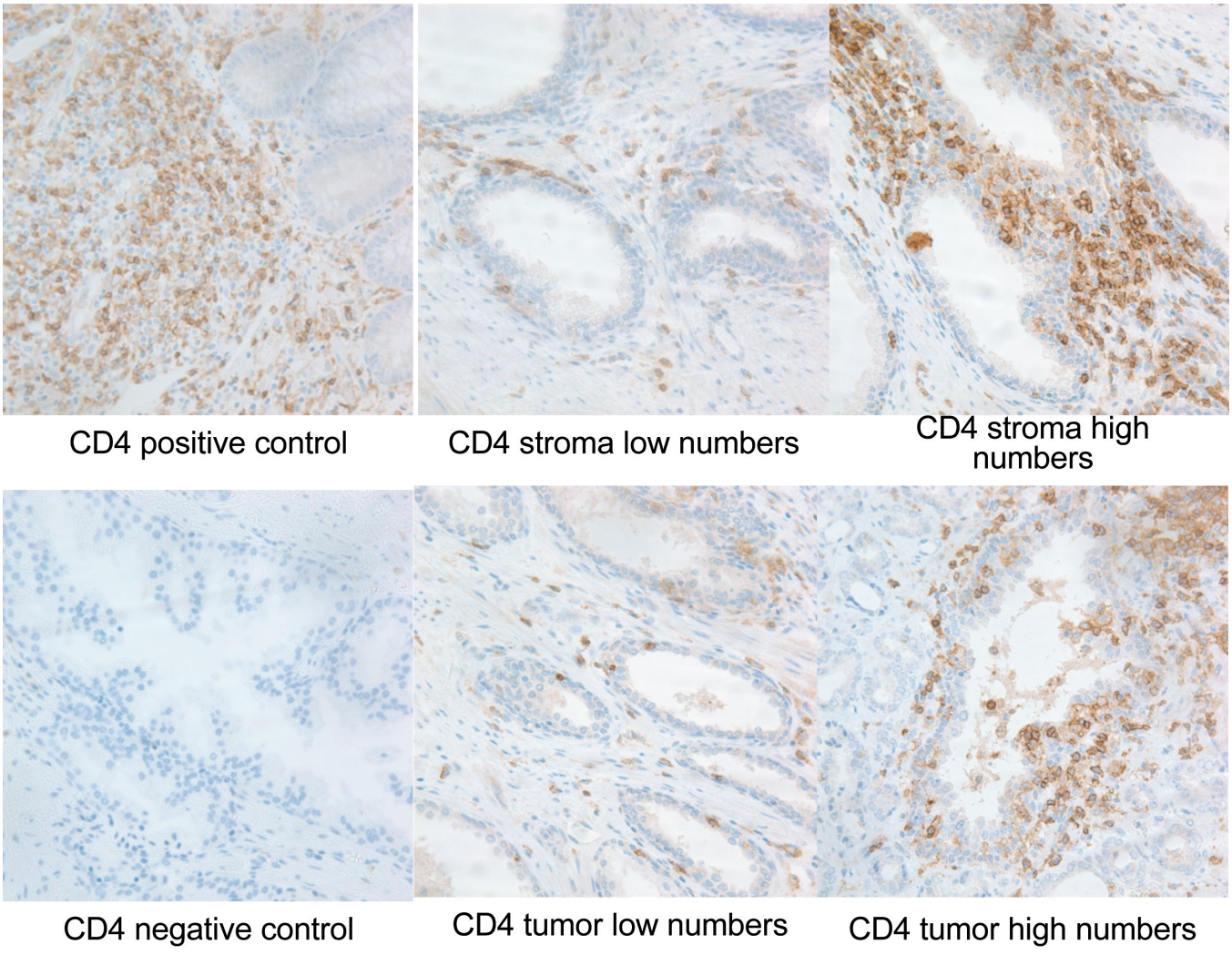


**Fig. 4.** Immunohistochemical staining of prostate cancer specimens for CD4+ lymphocytes. x 40

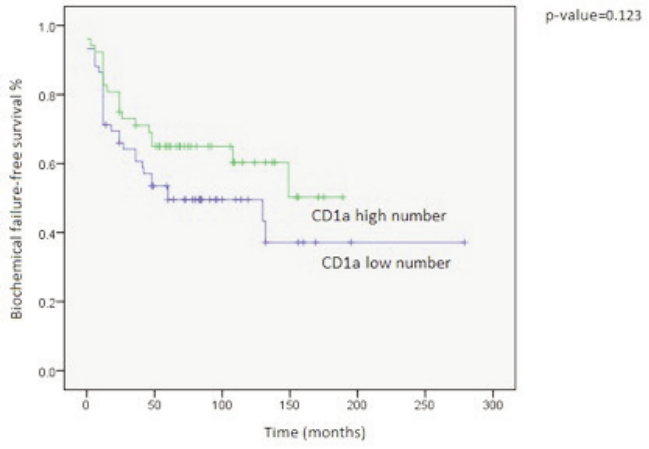


**Fig. 5.** Kaplan Meyer analysis of numbers of CD1a positive in relation to biochemical failure-free survival in prostate cancer.

$CD1a^+$ cells were very low in normal and benign prostate tissue. Interestingly, for prostate carcinoma with a Gleason score of 3+3=6, which is considered a slow growing and not a metastasizing carcinoma, we found a high number of $CD1a^+$ cells, indicating an ongoing immune response. The more malignant PCa phenotypes with a Gleason score 4+4=8 or 4+5=9 had significantly fewer $CD1a^+$ cells. The fact that $CD1a^+$ cell numbers are very low in benign prostate tissue but high in low-grade prostatic carcinomas indicates that these cells have been actively recruited to the tumor tissue. The correlations between the numbers of $CD1a^+$ cells and stromal $CD4^+$ and intratumoral and stromal $CD8^+$ T lymphocytes probably reflect cross talk between prostatic carcinoma cells and the immune system. Our results extend and confirm earlier data on the role of $CD1a^+$ cells in PCa in a smaller number of patients (Bigotti et al., 1991).

Our knowledge of the immune system's function has increased significantly during the past decades. Of major importance is the interplay between cancer cells and stromal components. Cancer cells often express an immune-modulating phenotype; these cells also express immune-modulatory cell surface molecules such as the programmed death-1 ligand (Topalian et al., 2012) and they secrete immune-modulating substances such as cytokines and growth factors [for review see (Rabinovich et al., 2007)].

$CD4^+FoxP3^+$ cells, also called regulatory T cells, are associated with aggressive breast cancer phenotypes (Bohling and Allison, 2008). The presence of immunosuppressive $CD8^+FoxP3^+$ T lymphocytes has been reported in human PCa (Kiniwa et al., 2007). Our present finding of significantly fewer intratumoral lymphocytes, both $CD4^+$ and $CD8^+$ T lymphocytes, in high-grade PCa is intriguing because it suggests that these cell populations are suppressed in the tumor, but the stromal T cell compartment is unaffected. Thus, the three cell types-$CD1a^+$, $CD4^+$, and $CD8^+$ cells-constitute a local antitumor immunological microenvironment that may keep the tumor in a stable state, during which time, the number of these cells decreases as the disease progresses. Gannot et al. demonstrated recently that the content of $CD8^+$ T lymphocytes was significantly lower in regions with prostate carcinoma compared with normal prostate tissue (Gannot et al., 2011). $CD8^+$ cell number decreases and their function is inhibited in aggressive prostate carcinoma probably because of immune-inhibitory effects exerted by the tumor microenvironment. Other molecules such as B7-H3 are likely to exert such an inhibitory effect (Nygren et al., 2011).

The findings in this study are compatible with the concept of immunoediting of cancer developed by Schreiber's group (Dunn et al., 2002; Koebel et al., 2007). The Gleason grading system is a histopathological concept describing a progressive malignant phenotype; this concept could be explored further in an experimental setting, e.g. how the different malignant carcinoma phenotypes might influence immune cells. Currently, several groups are working on methods to establish in vitro cultures of prostate carcinoma cells, and these approaches should lead to experimental models for studying the mechanisms underlying immunoediting.

Parallel with increased research on the interplay between the tumor microenvironment and the immune system, different kinds of immune therapy have evolved in the past two decades (Drake and Antonarakis, 2012). These clinical protocols include vaccination with peptides, and protocols involving immune therapy based on T cells and DCs are ongoing. The US Food and Drug Administration has approved sipuleucel-T for PCa (Kantoff et al., 2010). Another example is the DC vaccination strategy developed at The Norwegian Radium Hospital (Mu et al., 2005). In this context, it is interesting that the generation and maturation of DCs are inhibited in the presence of the PCa cell line LNCaP (Aalamian et al., 2001). Even though the $CD1a^+$ cells present in PCa specimens do not display an activated phenotype (Troy et al., 1998), a difference in numbers between the various PCa phenotypes suggests an active role of these cells.

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