Androgen Deprivation Boosts Prostatic Infiltration of Cytotoxic and Regulatory T Lymphocytes and Has No Effect on Disease-Free Survival in Prostate Cancer Patients

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Running title: Immune events elicited by NHT in prostate cancer.

Key Words: prostate cancer, cytokines, hormonal control, lymphokines, chemokines and growth factors, tumor microenvironment.
Statement of Translational Relevance

The real value of neoadjuvant hormone therapy prior to radical prostatectomy has recently been questioned by urologists and oncologists, since its effectiveness is uncertain.

In the present paper, by means of immune-morphological and molecular biology analyses associated with patient’s clinico-pathological profiles, we identified novel immunological pathways/mechanisms regulated by androgen withdrawal in the prostate and lymphoid tissues and also stated their clinical impact.

These findings may help treatment decision-making, and the development of novel, less aggressive therapeutic approaches to localized disease.
Abstract

**Purpose:** The value of neoadjuvant hormone therapy (NHT) prior to radical prostatectomy (RP) as a means of restraining prostate cancer (PCa) and strengthening its immunotherapy is still uncertain. This paper asks whether it subverts immunoregulatory pathways governing tumor microenvironments, and has an impact on patient outcome.

**Experimental Design:** We microdissected epithelium and stroma from cancerous and normal prostate specimens from 126 prostatectomized patients, of whom 76 had received NHT, to detect cytokine/chemokine gene expression levels by real-time reverse transcription-PCR. Confocal microscopy was used to identify cytokine/chemokine cell sources, and immunostainings to characterize lymphocyte subsets whose prognostic effects were assessed by Kaplan-Meier analyses.

**Results:** NHT boosted the expression of IL-7 in the stroma and that of IFNγ-inducible protein (IP)-10/CXCL10 in the glandular epithelium of normal prostate tissue, and restored the CD8⁺ lymphocyte depletion occurring in PCa, while it significantly increased the CD4⁺ lymphocyte infiltrate. Lymphocytes, mostly with CD8⁺ phenotype, expressed the T-cell intracellular antigen-1, granzyme-B and perforin, typical of cytotoxic-effector T cells. NHT also induced thymus and activation-regulated chemokine (TARC)/CCL17 production by monocytes/macrophages in the prostate and draining lymph nodes, and increased the number of their Forkhead box P3 (Foxp3)⁺CD25⁺CD127⁻ T regulatory (Treg) cells. The chi-
The chi-square test disclosed the lack of association ($P=0.27$) between NHT and the high intra-tumoral CD8$^+$/$Treg$ ratio indicative of a good prognosis.

**Conclusions:** Androgen withdrawal regulates cytokine/chemokine gene expression in normal prostate and lymphoid tissues, and this probably favors both CD8$^+$ and $Treg$ infiltrates, leaves their intra-tumoral balance unchanged, and thus has no impact on disease-free survival.

**Introduction**

Since PCa is driven, in part, by androgens, hormone therapy has been used so far in its management (1). The value of neoadjuvant hormone therapy (NHT) prior to radical prostatectomy (RP), however, has recently been questioned by urologists and oncologists, since its effectiveness is uncertain. Its aim stems from its ability to shrink the tumor and reduce margin positivity so as to allow adequate surgical coverage of the cancerous area. Several studies have demonstrated an improvement in clinical and pathologic endpoints, but not a constant improvement in overall survival (2). On the other hand, some experimental data suggest that androgen deprivation improves the efficacy of PCa immunotherapy (3, 4), and could thus be an attractive alternative to RP in selected patients. Identification of the immunological pathways/mechanisms regulated by androgen withdrawal in PCa may help treatment decision-making, and the development of modern, less aggressive therapeutic approaches to localized disease.

We have recently shown that the lack of constitutive interleukin-7 (IL-7) gene expression in PCa, and related lymphocyte depletion, is a mechanism whereby PCa evades immunosurveillance (5). The present paper assesses the possibility that androgen deprivation may restore this pathway and interfere with other aberrant immunological
mechanisms regulating PCa and draining lymph node microenvironments and also have an impact on patient outcome.

Materials and Methods

Patients and samples. We collected biological samples (cancer and normal prostate samples, and draining lymph nodes), clinical and pathological data of 126 patients treated by RP for PCa between 2002 and 2009 at the "S.S. Annunziata" Hospital, Chieti, Italy. Seventy-six had received preoperative NHT for 3 months consisting of an androgen receptor blocker (flutamide, 250 mg orally three times/day), and one i.m. injection of a gonadotropin-releasing hormone (GnRH) agonist (leuprolide acetate, 7.5 mg depot, administered with the first dose of flutamide). The other 50 patients were selected by matching for age, Gleason score, pathological stage and PSA level at diagnosis, as shown in Table 1. In addition, we obtained normal prostates (histologically negative for PCa or benign prostatic hyperplasia) from 12 untreated patients, aged 57 to 63, prostatectomized for bladder cancer (control patients) and pelvic lymph nodes (control lymph nodes) from autopsies of 5 men, aged 59 to 65, who died for reasons other than cancer and were histologically free from PCa.

Details of assessment of the clinico-pathological profiles and tissue sample processing
are reported in Supplementary Methods.

Written informed consent was obtained from patients. The study was approved by the Hospital's Ethical Committee, and performed in accordance with the principles outlined in the Declaration of Helsinki.

**Immunohistochemistry and immunofluorescence stainings.** Single immunohistochemistry on formalin-fixed, paraffin-embedded samples, or on frozen samples sections was performed as described in Supplementary Methods. Details of double and triple immunohistochemistry and double immunofluorescent stainings are also provided in Supplementary Methods.

**Immune cell count.** Automated cell count was done by light microscopy using a Leica Imaging Workstation (Leica, Wetzlar, Germany) by applying a dedicated algorithm in Qwin image analysis software (version 2.7). CD4⁺, CD8⁺ and Foxp3⁺/CD25⁺ cells were counted by adding together the intraepithelial and stromal positive cells scattered in randomly chosen fields for the normal prostate samples (of both control and PCa patients), and in fields randomly chosen within neoplastic areas for the PCa samples. Values are represented as the mean ± SD of positive cells/field on single or double immunostained, formalin fixed, paraffin-embedded sections at x 400 in an 85,431.59 μm² field. Eight to 12 high-power fields were examined for each section and two sections per sample were evaluated.

**LCM and real-time RT-PCR.** We used the P.A.L.M. Micro Beam System (P.A.L.M. Microlaser Technologies, Bernried, Germany) for LCM of two 10-μm frozen sections from
each normal (of both control and PCa patients) and neoplastic prostate sample to obtain its epithelial and stromal components (details in Supplementary Methods).

The real-time RT-PCR was carried out, on the RNA extracted from microdissected cells, using the MiniOpticon System (Bio-Rad, Hercules, CA, USA) with SYBR Green fluorophore (details in Supplementary Methods).

**Statistical analysis.** Immune cell counts are reported as mean and SD. Between-group differences in immune cell count (in the prostate or lymph nodes), or the relative expression of cytokines/chemokines by real-time RT-PCR, were assessed by one-way analysis of variance (ANOVA). The difference between each pair of means was evaluated with the Tukey pairwise multiple comparisons test. Differences between groups of patients with different Gleason scores for the relative expression of cytokines/chemokines by real-time RT-PCR were assessed by ANOVA. The $\chi^2$ test and the Mann-Whitney $U$ probability test were used to examine the association between cytokine/chemokine expression levels, or cell counts in prostate samples, and the clinical and pathological characteristics. The three-year disease-free survival curves were constructed using the Kaplan-Meier method, and differences were analyzed with the log-rank test. The mean follow-up time was 43 months (range: 3 to 96 months). The Kruskal-Wallis test was used to determine whether there was a significant delay in disease recurrence between treated and untreated patients. The SPSS software, version 11.0 (SPSS Inc, Chicago, IL, USA) was employed, with $P<0.05$ as the significance cut-off.
Results

NHT up-regulates IL-7 gene expression in the normal prostate epithelium and stroma. To find out whether androgen blockade affects the lack of IL-7 production in PCa, and thus the related intra-prostatic lymphocyte depletion (5, 6), we first used LCM and real-time RT-PCR to determine IL-7 expression on cancer and normal prostate samples from untreated and NHT-treated patients.

Since both the epithelium and the fibromuscular stroma account for prostate gland
responsiveness to androgens (7), we isolated and analyzed both components from these samples.

Real-time RT-PCR corroborated our previous observation of a significant reduction (~53 times) of IL-7 mRNA expression level in neoplastic versus normal prostatic epithelium (5) (Fig. 1A). Both the epithelial and the stromal components of PCa samples from treated patients expressed IL-7 mRNA levels fully comparable to those in the untreated patients. By contrast, the histologically normal samples from treated patients displayed a considerable increase of IL-7 mRNA expression level in the stroma (~6 times) compared with those from the untreated patients (Fig. 1A). In addition, immunohistochemical examination of subsequent serial sections showed that IL-7 protein production was distinct in the stroma and bright in the glandular epithelium of normal samples from untreated patients, and scanty in PCa samples from both untreated and treated patients (Fig. 1B, a-d and g, h). IL-7 was moderately to strongly produced in the stroma and glandular epithelium of normal samples from treated patients (Fig. 1B, e and f). Double immunofluorescence and confocal analyses of these samples revealed that increased IL-7 production in the stroma was mainly attributable to vimentin+ fibroblasts and desmin+ αsma+ smooth muscle cells forming normal prostatic stroma (Fig. 1C, a-i).

Because androgen blockade fostered IL-7 production in the stroma of normal tissue surrounding PCa from the treated patients, we next investigated whether the lymphocyte content of their prostates was altered.

**NHT increases the intra-prostatic T cell population consisting of both cytotoxic-effector and regulatory T lymphocytes.** CD4+ T cells dramatically increased in both normal (~6 times) and neoplastic (~4 times) prostate tissues from treated versus normal prostatic epithelium.
untreated patients, while the mean number of CD8$^+$ cells significantly increased in neoplastic tissue following NHT (Table 2 and Fig. 2A, a-h). Thus, the increased IL-7 production was accompanied by intra-prostatic T lymphocyte accumulation (8, 9).

The expression of T-cell intracellular antigen-1 (TIA-1), which lies in the granules of cytotoxic lymphocytes (10), was more frequent and stronger in prostate samples from the treated patients, and co-localised with CD8$^+$ cells and CD4$^+$ cells to a lesser extent (Fig. 2B, a-d). Other molecules typically related to activated cytotoxic T effector (Teff) lymphocytes (11, 12), such as granzyme-B and perforin, were only detectable in prostate samples from treated patients and mostly co-localised with CD8$^+$ cells (Fig. 2B, e-h). The high density of intra-prostatic Teff lymphocytes promoted by androgen blockade prompted us to search for the production of specific Th1-type chemoattractants, such as monokines induced by IFN-γ (Mig)/CXCL9 and IFNγ-inducible protein (IP)-10/CXCL10 which have been associated with a significant Teff lymphocyte recruitment (13, 14). As shown in Fig. 2C and D, the relative expressions of IP-10/CXCL10 mRNA, not that of Mig/CXCL9, was significantly (~ 7 times) increased following androgen depletion in the normal prostate epithelium, whereas no significant alterations occurred in the epithelium and stroma from PCa samples. In keeping with these data, immunohistochemistry showed a stronger IP-10/CXCL10 production in the normal glandular epithelium from the treated patients (Fig. 2E, a and b).

Interestingly, one subset of mostly CD4$^+$ cells expressing surface CD25 and high levels of the transcription factor Forkhead box P3 (Foxp3), but lacking CD127 (as assessed by double immunostainings CD25/Foxp3 followed by single CD4 and CD127 staining of consecutive 3 μm serial sections), and thus identifiable as T regulatory (Treg) lymphocytes (15, 16), was also clearly increased in both normal and neoplastic prostate...
tissues from the treated patients (Table 2 and Fig. 2A, i-l). Monitoring of the expression of the cell-cycle-associated marker Ki67 to assess whether this increase was induced by stimulation of their proliferation revealed that most of them were not dividing inside the prostate (data not shown), as observed for other prostate infiltrating lymphocyte subsets (5). We thus explored the possibility that selective chemoattractants released in the androgen-depleted prostate microenvironment were involved in Treg cell accumulation.

**NHT induces TARC/CCL17 gene expression in normal prostate tissues and draining lymph nodes.** Since most intra-prostatic Treg specifically express the chemokine receptor CCR4 (Fig. 3A) (17), they should be responsive to macrophage-derived chemokine (MDC)/CCL22, and to TARC/CCL17 (18).

LCM followed by real-time RT-PCR analyses of prostate samples from the treated patients showed that in both the epithelial and the stromal component of their normal and neoplastic tissues MDC/CCL22 mRNA expression was low to undetectable, and not dissimilar to that in the untreated patients (Fig. 3B).

TARC/CCL17 mRNA expression (Fig. 3C) was undetectable in both the normal samples and the PCa epithelium of the untreated patients, while it was found in the neoplastic stroma. In the treated group, it was basically unchanged in PCa samples, whereas it was clearly evident in both the epithelium and particularly the stroma of their normal samples.

Immunohistochemistry, too, showed that TARC/CCL17 production was absent in the normal samples of the untreated patients and present in their PCa stroma (Fig. 3D, a and b), where it co-localized with CD68+ monocytes/macrophages, and with less differentiated CD11b+CD33+ myeloid cells (Fig. 3E, a) (19). TARC/CCL17 production was strong in the
normal samples and scanty to moderate in the PCa samples from the treated patients (Fig. 3D, c and d). Double immunohistochemistry revealed that CD68+ monocytes/macrophages were the major contributor to the substantial TARC/CCL17 production found in the normal prostatic stroma of the treated patients (Fig. 3E, b).

No significant differences of TARC/CCL17, IP-10/CXCL10 or IL-7 expression emerged between groups of patients with different Gleason scores (for both treated and untreated). Data about cytokine or chemokine production in normal samples from the untreated patients were not significantly different to those obtained in normal samples from controls. No significant association was disclosed between cytokine or chemokine expression levels and the clinical and pathological characteristics.

Because of its wide effects on the immune system (20), we next asked whether androgen blockade also regulates TARC/CCL17 production by macrophages homing to lymphoid tissues.

Immunohistochemistry was thus used to determine the production of TARC/CCL17 in pelvic lymph nodes removed during RP from both groups of patients.

The production of TARC/CCL17 (Fig. 3F, a-c), which co-localised with CD68+ monocytes/macrophages, and to a lesser extent, with CD1a+ dendritic cells (Fig. 3F g and h), was well represented in lymph nodes from the treated patients (Fig. 3F, c), whereas it was clearly restricted to fewer immune cells in those from the untreated patients (Fig. 3F, b), and almost absent in the control nodes (Fig. 3F, a).

Lymph nodes from treated patients showed a distinct accumulation of Foxp3+ cells (42 ± 13) (mean ± SD of positive cells/x400 field) (Fig. 3F, f), the vast majority expressing CCR4 (Fig. 3F, i), and crowding TARC/CCL17 producing immune cell foci. This Foxp3+ cell density was not significantly different than in lymph node from untreated patients (24 ±
NHT leaves the intra-prostate cancer CD8/Treg ratio unchanged and has no impact on disease-free survival. We asked whether somewhat opposite immunological events, namely recruitment of both Teff and Treg cells, elicited by androgen depletion in the PCa microenvironment, influence patient outcome and have clinical significance. Since most Teff were CD8⁺ lymphocytes, we first assessed the combined influence of low versus high number of intra-tumoral CD8⁺ and Treg on disease-free survival, and then used the chi-square test to assess the association between NHT and the intra-tumoral content of these subsets.

Using the median values as cut-off, patients were divided into groups with low and high intra-tumoral CD8⁺, Treg or CD8⁺/Treg ratio. Each group was represented by Kaplan-Meier biochemical disease-free survival curves constructed on the basis of patient’s PSA failure during the first 3-year follow-up after surgery.

Patients with higher frequencies of intra-tumoral CD8⁺ demonstrated a significant improvement of disease-free survival compared with patients with lower frequencies (Fig. 4A; median = 22.5, log-rank P=0.002). By contrast, patients with high versus low frequency of Treg showed a reduced disease-free survival (Fig. 4B; median = 2.3, log-rank P=0.019). Since it has been reported that the balance between CD8⁺ and Treg has a greater impact on outcome (21-23), than their number alone, we next performed survival curves of patients with high versus low CD8⁺/Treg cell ratio (Fig. 4C). Using the median value (10.65) as the cut-off, survival curves constructed with 3-year follow-up data, revealed that the group with a high ratio, 63/126 patients: 28 untreated (1 censored
enclosed) and 35 treated (7 censored enclosed), had a significant improvement of disease–free survival (log-rank $P=0.003$) compared with the low ratio group: 63 patients, 22 untreated (1 censored enclosed) and 41 treated (4 censored enclosed). Two patients in the first group displayed biochemical recurrence, (the first occurred 12 months after surgery), compared to 13 in the second group (the first occurred 3 months after surgery). The chi-square test showed a significant ($P<0.01$) association between NHT and the high intra-tumoral frequency of both CD8$^+$ (odds ratio [OR], 27.00, 95%; confidence interval [CI], 9.36–77.92) and Treg (OR, 11.93, 95%; CI, 4.95–28.74), and the absence of any association between NHT and the high or low intra-tumoral CD8$^+$/Treg ratio ($P=0.27$; OR, 0.67, 95%; CI, 0.32-1.37).

Lastly, though our cohort size is a limiting factor, we try to assess whether NHT had an impact on survival through mechanisms other than its own immunological effects. We thus compared the disease-free survival of treated versus untreated patients (Fig. 4D). At the end of the study, 80% (40/50) of the untreated patients were still disease-free (8 subjects displayed recurrence, 2 were censored) compared with 76% (58/76) of the treated patients (7 subjects displayed recurrence, 11 were censored). Thus, treated patients had no significant decrease in the frequency of recurrence (log-rank $P=0.291$), nor a significant delay in its occurrence ($P=0.344$, as assessed by the Kruskal-Wallis test), in line with data from most of the previous clinical studies (1).
Discussion

Androgen receptor (AR) signaling plays a crucial role in all steps of prostate carcinogenesis, as in normal prostate development and function (24), through both the epithelial and the mesenchyme/stroma compartments (7). These, in turn, cross-communicate and interact with prostate homing immune cells, and thus give rise to complex relationships that may be subverted by androgen ablation through the following pathways.

a. Up-regulation or induction of immunoregulatory cytokine/chemokine gene expression in “histologically” normal prostate tissue, epithelium and/or stroma, and in monocyte/macrophages infiltrating prostate and draining lymph nodes.

Histologically normal prostate tissue surviving NHT-dependent apoptosis (24), probably represented by AR- basal and a mix of AR- and AR+ basal-intermediate epithelial cells, thus seems to preserve important biological functions in (presumably indirect) response to androgen ablation, such as expression of IP10/CXCL10 and TARC/CCL17 by the glandular epithelia and that of IL-7 by stromal fibroblasts and smooth muscle cells. By contrast, PCa tissue remnants of the same apoptotic event, mostly composed of AR-cancer cells (24) equipped with fibroblasts/myofibroblasts (namely cancer associated fibroblasts or CAF) (25), are almost unresponsive.
Interestingly, monocyte/macrophages homing to normal prostate tissue and lymph nodes, not those infiltrating PCAs, account for most of the TARC/CCL17 produced following androgen deprivation. Whether TARC/CCL17 production is a direct (26) or indirect macrophage response remains to be investigated. It is only clear that androgen deprivation is unable to overcome macrophage conditioning by PCAs.

b. Alterations of prostate and draining lymph node microenvironments by means of key mediators of T cell survival, activation and trafficking, namely IL-7, IP-10/CXCL10 and TARC/CCL17.

IL-7 is a non-redundant trophic factor for T lymphocyte development that primarily acts by promoting lymphocyte survival and/or proliferation (27), and also displays T lymphocyte-mediated anti-tumor properties (28, 29). It has been identified from different cell types (30, 31), including smooth muscle cells (31) and fibroblasts (32). By means of prostatic fibroblasts and smooth muscle cells, regularly endowed with AR (24), androgen deprivation supplies the IL-7 lost in the prostate microenvironment at tumor onset (5), and likely offsets T cell depletion in PCAs, since both CD8+ and CD4+ lymphocytes are greatly increased in the prostate from treated patients.

IP10/CXCL10 directs the trafficking of activated effector CD8+ and CD4+ T lymphocytes by binding to its high-affinity receptor CXCR3 (13, 14), and displays antitumor and antimetastatic properties (33, 34). Its expression may be induced from a variety of cells (35), including epithelial cells (35, 36). Here, we show that androgen deprivation boosts IP-10/CXCL10 gene expression in normal epithelium in association with intra-prostatic infiltration of cytotoxic Teff lymphocytes endowed with anti-tumor activity (37).

TARC/CCL17 induces Treg cell recruitment by binding to the receptor CCR4 (17, 18). This chemokine is expressed by mature dendritic cells (18, 38), monocytes and activated
macrophages (39), and epithelial cells (40, 41). The present study reveals that androgen deprivation not only induces TARC/CCL17 gene expression in normal prostatic epithelium, but also boosts its expression by macrophages in normal prostate and lymph nodes, with a related influx of CCR4+Foxp3+ naturally occurring Treg cells (17, 42) able to suppress anti-tumor Teff lymphocytes (43, 44).

c. The concomitant increase of CD8+ and Treg cell populations, in both normal and neoplastic prostate tissues, related with changes of the cytokine/chemokine milieu.

The inflammatory environment and associated immune cell recruitment promoted by androgen withdrawal is consistent with emerging evidence of the reciprocal negative cross-talk between AR and transcription factor nuclear factor kB (45), which play a key role in the control of inflammatory and immune response genes (46), as also with the mutual antagonism documented between androgen and interferon signaling pathways (47).

Our data indicate that cytokine/chemokine gene up-regulation following androgen deprivation only involves normal prostate, whereas lymphocyte recruitment also involves cancerous tissues. This may be due to the fact that PCa consists of different foci (48) mingled with histologically normal areas. Thus, the effects of androgen deprivation on normal tissue may easily reflect on the nearby cancer foci. They, indeed, are invaded by both cytotoxic-effector CD8+ and immune-suppressive Treg cell populations. Thus, their intra-tumoral balance remains substantially unchanged in samples from treated versus those of untreated patients. This “double face” of the NHT-dependent immunological effects results in the lack of a significant clinical benefit, as revealed by the Kaplan-Meier disease-free survival curves, and the lack of association between NHT and a high CD8/Treg ratio which, as observed in other tumor types (21-23), is proving a good prognostic marker for PCa patients.
Taken as a whole, our findings provide mechanistic insights into some immunological pathways elicited by NHT inside the prostate and lymph node microenvironments, and offer new tools with which to assess its potential in the management of PCa patients.

The full exploitation of the immuno-stimulatory effects exerted by androgen blockade, probably via IL-7 and IP-10/CXCL10 induction and associated intra-tumoral cytotoxic-effector lymphocyte infiltration, requires to counteract the concomitant immune-suppressive effects likely mediated by TARC/CCL17 induction and associated intra-tumoral Treg lymphocyte accumulation.

References


Table 1. Percentages of patients with selected clinico-pathological profiles at time of diagnosis

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* PSA = prostate specific antigen.
### Table 2. Mean content of lymphocytes in normal and neoplastic prostate tissues from untreated and NHT-treated PCa patients

<table>
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<tr>
<th>Immune Cells</th>
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<td>CD8⁺</td>
<td>25.7 ± 5.0</td>
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<td>Foxp3⁺CD25⁺</td>
<td>2.1 ± 0.8</td>
<td>5.0 ± 1.7§</td>
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*Cell counts were performed by light microscopy, at ×400 in an 85431.59 μm² field, on single immunostained formalin-fixed, paraffin-embedded sections. Results are mean ± SD of positive cells/field in groups of normal and PCa samples from untreated and treated PCa patients.

†p<0.001 One-way ANOVA for comparisons between 4 groups.

‡Notably, data about cell counts obtained in normal samples from untreated PCa patients were not significantly different from those obtained in normal samples from control patients.

§p<0.05 Tukey-test compared with histologically normal prostate samples of untreated group of patients.

¶p<0.05 Tukey-test compared with PCa samples of untreated group of patients.

**No significant association was disclosed by Mann-Whitney U test or the χ² test between these counts and the different Gleason scores, clinical stages, PSA value and patient age.
Figure Legends

Fig. 1. Expression of IL-7 in normal and PCa tissues from NHT-treated and untreated PCa patients.

A, the histogram represents the relative expression ± SD of IL-7 in microdissected histologically normal epithelium and stroma, and their neoplastic counterparts from treated and untreated patients (groups of 12, graded as Gs 7), normalized with the housekeeping gene HPRT.

B, immunohistochemistry shows that, in normal prostate tissue from untreated patients (a), IL-7 production was distinct in the stroma and bright in the glandular epithelium (b), while in PCa samples from both untreated (c) and treated (g) patients it was barely detected (d and h). In normal prostate tissue from treated patients (e) IL7 was moderately to strongly expressed in the stroma and glandular epithelium (f). (a-h: x400).

C, double immunofluorescent staining and confocal microscopy of normal prostatic tissues from treated patients reveals that the stromal production of IL-7 (green stained in a, b and c), co-localised with vimentin+ fibroblasts (d) and with desmin+ (e), αsma+ (f) smooth muscle cells, as shown by the merge images (yellow-orange in g, h and i). (a-i: x400).

Fig. 2. Teff and Treg lymphocytes infiltrating normal and PCa tissues from NHT-treated and untreated PCa patients.

A, CD4+ lymphocytes were few in normal (a) and PCa (b) samples from untreated patients, whereas they massively infiltrated both normal (c) and PCa (d) samples from treated patients. CD8+ cells were reduced in PCa samples (f) when compared with normal prostate tissues (e) from untreated patients, whereas NHT favored their increase in both normal (g) and PCa (h) tissues. Foxp3+CD25+ were rare in normal prostate (i) and
increased in PCa (j) from untreated patients, whereas NHT favored their increase in both normal (k) and PCa (l) tissues. (a-l: x400).

**B,** in PCa tissue, TIA-1 molecules were usually scanty (a), whereas they were frequently and strongly expressed in PCa tissue from treated patients (b). Their expression (brown stained) was mostly attributable to CD8+ cells (red stained) (c) (inset shows TIA-1 colocalizing, indicated by arrow, and not with CD8+ cells) and, to a lesser extent, CD4+ cells (red stained) (d) (arrows indicating identifiable co-localization). Granzyme-B was undetectable in PCa from untreated patients (e), but clearly expressed in PCa from treated (f), and along with perforin (h, inset with clearer picture of the co-localization, while arrow indicates the lack of co-localization), it was mostly produced by CD8+ lymphocytes (g). (a-c and e-h: x400; d: x1000; inset in c: x630; inset in h: x1000).

**C,** the histogram represents the relative expression ± SD of Mig/CXCL9 in microdissected histologically normal epithelium and stroma, and their neoplastic counterparts from treated and untreated patients (groups of 12, graded as Gs 7), normalized with the housekeeping gene HPRT.

**D,** the histogram represents the relative expression ± SD of IP-10/CXCL10 in microdissected histologically normal epithelium and stroma, and their neoplastic counterparts, from treated and untreated patients (groups as above), normalized with the housekeeping gene HPRT.

**E,** immunohistochemistry shows that IP-10/CXCL10 production was stronger in the normal glandular epithelium from the treated (a) than the untreated (b) patients. (a and b: x630).

**Fig. 3. Expression of TARC/CCL17 in normal and PCa tissues and Foxp3+ cell infiltration in prostate-draining lymph nodes from NHT-treated and untreated PCa**
patients.

A, double immunohistochemistry shows that in PCa from treated patients most Treg (brown nuclei) expressed the chemokine receptor CCR4 (red stained). (x400).

B, the histogram represents the relative expression ± SD of MDC/CCL22 in microdissected histologically normal epithelium and stroma, and their neoplastic counterparts, from treated and untreated patients (groups of 12, graded as Gs 7) normalized with the housekeeping gene HPRT.

C, the histogram represents the relative expression ± SD of TARC/CCL17 in microdissected histologically normal epithelium and stroma, and their neoplastic counterparts, from treated and untreated patients (groups as above), normalized with the housekeeping gene HPRT.

D, immunohistochemistry shows that TARC/CCL17 was absent in normal prostate tissue (a) and moderately produced in PCa (b), whereas in the treated patients it was strongly produced in the normal prostate (c), particularly by the epithelial compartment, and moderately expressed in PCa (d). (a-d: x400).

E, triple immunohistochemistry (a) shows that the production of TARC/CCL17 (brown) in the stroma of PCa samples from untreated patients mostly co-localized with CD33+ (red) CD11b+ (blue) myeloid cells (inset with clearer picture of the co-localization at x1000 magnification).

Double immunohistochemistry (b) shows that the substantial TARC/CCL17 production (brown) found in the prostatic stroma of normal samples from treated patients mostly co-localized with CD68+ cells (red) (the inset shows the red and brown overlapping at x630). (a: x630; b: x400).

F, The production of TARC/CCL17 was almost absent in control lymph nodes harvested
from autopsies (a), whereas it was scarce but distinct in lymph nodes from untreated patients (b), and moderate to strong in prostate-draining lymph nodes from treated patients (c). Foxp3+ cells were scanty in the lymph nodes taken as control (d), more represented in lymph nodes from untreated patients (e) and clearly more numerous in lymph nodes from treated patients (f), where the vast majority of these cells expressed CCR4 (i). (a-f and i: x400).

In prostate-draining lymph nodes from treated patients, the production of TARC/CCL17 (brown) co-localised with CD68+ monocytes/macrophages (red) (g) and to a lesser extent with CD1a+ dendritic cells (red) (h). (g and h: x 630).

**Fig. 4. Prognostic significance of CD8+ and Treg lymphocytes in PCa and impact of the NHT-treatment on patient outcome.**

Kaplan-Meier graphical analyses of 3-year disease-free survival after RP in all patients (staged T1-T3 as indicated in Table 1), for (A) tumor-infiltrating CD8+, (B) regulatory T cells (Treg), and (C) their balance. (D) Kaplan-Meier graphical analyses of 3-year disease-free survival after RP in all patients (staged T1-T3 as indicated in Table 1), showed no significant decrease in the frequency of recurrence in NHT-treated versus untreated patients (log-rank P=0.291).
Figure 1

A) Expression Levels of IL-7 mRNA in the Prostate Tissue

B) Histological images of prostate tissue:
- Untreated:
  - Normal epithelium (a)
  - Neoplastic epithelium (d)
  - Normal stroma (g)
  - Neoplastic stroma (c)

- Treated:
  - Histologically Normal (b)
  - PCa (e)
  - Histologically Normal (f)
  - PCa (h)

C) Immunofluorescence staining:
- IL-7 (a, b, c, e, f, g)
- Vimentin (d, h, i)
- Desmin (g, h, i)
- α-smooth muscle actin (i)
Figure 2

A

Untreated vs Treated

B

Expression Levels of Hif-1α/CCL2 mRNA in the Prostate

C

Expression Levels of P-ERK/CCL2 mRNA in the Prostate

D

Expression Levels of P-ERK/CCL2 mRNA in the Prostate

E

Untreated vs Treated
Figure 4

A. 

B. 

C. 

D.
Androgen Deprivation Boosts Prostatic Infiltration of Cytotoxic and Regulatory T Lymphocytes and Has No Effect on Disease-Free Survival in Prostate Cancer Patients

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