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Immunosuppressive CD14^{+}HLA-DR$^{low/-}$ Monocytes in Prostate Cancer

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Abstract

Objective—To determine if the levels of circulating myeloid-derived suppressor cells increase with progression of prostate cancer (PCa); to determine if such cells could contribute to the relative inefficiency of PCa immunotherapy.

Materials and Methods—We analyzed peripheral blood mononuclear cells isolated from untreated PCa patients (uPCa; N = 18; mean age ± SD: 72.1 ± 6.9 years), tPCa (N = 22; 72.8 ± 9.8 years) and age matched controls (AMC; N = 12; 68.8 ± 7.5 years). We quantified surface marker phenotype, differentiation potential, effects on T cell proliferation and intracellular cytokines.

Results—We observed an unexpectedly high percentage of a type of myeloid-derived suppressor cells, CD14^{+}HLA-DR$^{low/-}$ monocytes, in tPCa (30.7 ± 15.0% of CD14^{+} cells) relative to AMC (4.1 ± 6.5%, $P < 0.0001$) and uPCa (10.6 ± 14.3%, $P = 0.0001$). The levels of CD14^{+} HLA-DR$^{low/-}$ cells were significantly correlated with circulating PSA levels and treatment with LHRH-agonist leuprolide in combination with either an antiandrogen or dexamethasone. Monocytes from tPCa inhibited autologous T cell proliferation statistically significantly more effectively than AMC monocytes and were defective in their ability to differentiate into phenotypically mature dendritic cells. Isolated CD14^{+}HLA-DR$^{low/-}$ cells expressed higher levels of intracellular interleukin-10 and suppressed T cell proliferation more effectively than isolated CD14^{+}HLA-DR^{+} cells.

Conclusions—This is the first report of CD14^{+} cells exhibiting reduced expression of HLA-DR molecules in PCa patients. These cells suppress immune cell function in vitro and, plausibly, in vivo, a finding that must be factored into the design of immunotherapy protocols for PCa patients.

Keywords

androgen suppression; CD14^{+} cells; dendritic cells; HLA-DR expression; monocytes; myeloid-derived suppressor cells; prostate cancer

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Additional Supporting Information may be found in the online version of this article.

Introduction

The lack of an effective cure of androgen-independent prostate cancer (PCa) has stimulated development of immunotherapeutic approaches to this disease. One approach has been the use of patient-derived ex vivo matured dendritic cells (DCs), the antigen-presenting cells unique in their ability to stimulate antigen-naïve T cells in addition to memory T cells [1]. While DC-based treatments have induced clinical responses in one half of 400 PCa patients studied in different clinical trials so far, the responses were only transient (recently reviewed in Ref. [2]). Listed among the reasons for the lack of durable clinical effects have been tumor-associated defects in patients' tumor-specific immunity, including tolerance to prostate-associated antigens, poor expression of HLA molecules by PCa cells, tumor-derived immunosuppression, increased levels of regulatory T cells, etc. (cf. Ref. [3]).

Recently, however, tumor-associated alterations of innate immunity have come into focus as well. For example, patients suffering from metastatic PCa contained fewer circulating myeloid DCs than their age-matched controls [4]. This finding indicates that in PCa patients monocytes do not develop into myeloid DCs as efficiently as they do in healthy subjects, a notion supported by observations that PCa patient serum inhibits monocyte differentiation into DCs and that the degree of inhibition is correlated with higher prostate specific antigen (PSA) levels [5]. Some evidence, though, for aberrant monocytes in PCa has been available for years. For instance, the cells isolated from PCa patient lymph nodes [6] and peripheral blood, later shown to be monocytes [7], could inhibit leukocyte proliferation in vitro [8].

T regulatory (T_{reg}) cells characterized as $CD4^+$ $CD25^{high}$ have recently been documented at an increased level in the blood and tumor tissue of *early-stage* PCa patients; these cells potently suppressed T cell proliferation in vitro [9]. Interestingly, $CD4^+CD25^+$ cells isolated from the blood of *healthy subjects* suppressed expression of cytokines and HLA class II molecules in monocytes [10]. T_{reg} cells, specified as $CD4^+CD25^+CD127^{lo}Foxp3^+$, directed monocyte differentiation into a phenotype characterized by anti-inflammatory effects and a role in "immune regulation, tissue remodeling, parasite killing, and tumor promotion" [11]. Such monocytes have been designated as "alternatively activated"; while devoid of the ability to express normal levels of proinflammatory molecules such as IL-1β, IL-6, IL-8, MIP-1α, and TNF-α, alternately activated monocytes expressed IL-10, IL-4, and IL-13, the likely mediators of immunosuppression [11]. Taken together, these data imply the possibility of complex mutual interactions of PCa and immunity, but the system has not been characterized within the context of natural history of the disease and the effects of treatment.

In an attempt to validate a standard method of DC maturation from monocytes derived from PCa patients, we found that these cells yielded fewer fully differentiated DCs than monocytes from healthy donors. To investigate the reasons underlying this phenomenon, we characterized phenotypic features of peripheral blood mononuclear cells (PBMC) isolated from newly diagnosed untreated PCa patients (uPCa), from PCa patients treated by standard adjuvant therapy with luteinizing hormone releasing hormone (LHRH)-agonists and an antiandrogen or dexamethasone (tPCa), and from non-cancerous age-matched control subjects (AMC).

Patients, Materials, and Methods

Patients

Patient blood and access to medical records were obtained with the approval of the Mayo Clinic Institutional Review Board. All study subjects received care at or came for second opinion to Mayo Clinic Rochester and participated in the study with informed consent. Subjects were identified for the study by review of medical records at the Mayo Clinic Prostate Cancer SPORE

registry (tPCa), Department of Urology (uPCa) and Division of Executive Health (AMC). Most tPCa underwent prostatectomy or radiation as first-line therapy and have since received the standard treatment by luteinizing hormone releasing hormone (LHRH) agonists (leuprolide acetate or goserelin acetate) with or without an antiandrogen (bicalutamide or nilutamide) or dexamethasone; one patient was orchiectomized instead. Subject demographics and pertinent clinical and laboratory data abstracted from patients' charts are shown in Supplementary Table I.

Cell Isolation

On average we drew 45 ml of blood on sodium heparin and an additional 6 ml on ethylenediamine tetraacetate. We isolated PBMCs by buoyant density separation using Lymphoprep separation medium (ICN, Aurora, OH) according to manufacturer's instructions. Cells were counted and assayed for viability by trypan blue exclusion. $CD3^+$ cells and $CD14^+$ cells were isolated from PBMCs by incubation with the pertinent immunomagnetic reagent (Miltenyi Biotec, San Diego, CA) according to manufacturer's instructions. After incubation and washing, we separated the labeled cells on an AutoMACS separator (Miltenyi Biotec) running the POSSEL program.

Cell Characterization by Flow Cytometry

We characterized the cells using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and fluorophore-conjugated monoclonal antibodies with specificity indicated in Table I. By multiple immunostaining we characterized $CD3^+CD4^+$ T helper cells, $CD3^+CD8^+$ cytotoxic T cells, $CD14^+$HLA-DR^+ monocytes, $CD19^+$ B cells, $CD56^+$ NK cells, $CD3^+CD4^+$ $CD25^+CD127^{low/-}$ T regulatory cells and $CD83^+$ DCs. We fixed the cells in 1.0% paraformaldehyde and then recorded 100,000 cytometry counts per sample. Data were analyzed with CellQuest software (BD Biosciences).

Fluorescence Activated Cell Sorting

To isolate $CD14^+$HLA-$DR^{low/-}$ and $CD14^+$HLA-DR^{high} cells, we stained the cells with fluorophore-conjugated monoclonal antibodies specific for CD14 and HLA-DR for 20 min at room temperature in the dark, washed and resuspended at $(7-10) \times 10^6$/ml in AIM V medium (Invitrogen, Carlsbad, CA) containing 10% human AB serum (HABS; Sigma–Aldrich, St. Louis, MO). Cells were sorted by the use of a FACS Aria fluorescence activated cell sorter (BD Biosciences) under sterile conditions.

Intracellular Staining of TGF-β1 and IL-10

Two hundred microliter of anticoagulated whole blood was distributed per 12 mm × 75 mm polystyrene tube. Cells were stained for CD14 and HLA-DR for 15 min at room temperature in the dark. Erythrocytes were lysed and white cells were fixed with Becton Dickinson (BD) Phosflow Lyse/Fix Buffer (1×) for 10 min at room temperature, centrifuged at 1,500 rpm and supernatant removed. The pellet was resuspended, washed with PBS, centrifuged, permeabilized in 1.0 ml of BD Phosflow Perm Buffer II for 30 min on ice and washed twice with Stain Buffer containing 2.0% fetal bovine serum (SB-FBS; BD Biosciences). Next, the cells were incubated in 100 μl of SB-FBS containing antibodies specific for TGF β1 and IL-10 for 30 min at room temperature in the dark, washed with 2.0 ml of SB-FBS and resuspended in equal volumes of SB-FBS and 4.0% paraformaldehyde and analyzed by flow cytometry for cells stained for CD14/IL-10/HLA-DR and CD14/TGF-β1/HLA-DR.

Preparation of Mature Dendritic Cells

We matured the cells by the modified two-step method we used earlier [12,13]. Briefly, we seeded $(2.5-3.0) \times 10^6$ $CD14^+$ cells in 1.0 ml of clinical-grade X-VIVO 15 cell culture medium

(Cambrex, East Rutherford, NJ) containing 1.0% pooled human AB serum. The first step to immature DCs took place in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF; Berlex, Montville, NJ; 2,800 IU/ml) and interleukin 4 (IL-4; R&D Systems; Minneapolis, MN; 1,000 IU/ml) for 3 days. Final DC maturation was induced by the addition of tumor necrosis factor-α (TNF-α; R&D Systems; 1,100 IU/ml) and prostaglandin E_2 (PGE_2; Sigma–Aldrich; 1.0 μg/ml) for two additional days. The final product was analyzed for the percent of mature DCs among all viable cells (by expression of CD83, a hallmark of mature DCs) and viable cell yield (the measure of survival calculated by dividing the measured amount of the product by the theoretical (maximal) yield multiplied by 100%; for example, [number of viable cells at the end of incubation/number of viable cells at the beginning of incubation] × 100).

Monocyte Effects on T Cell Proliferation

To assess the effect of autologous monocytes on T cell proliferation, we isolated $CD3^+$ cells from monocyte-depleted PBMCs by CD3-specific immunomagnetic adsorption (Miltenyi Biotec) to 95.8 ± 4.0% purity. Ten million freshly isolated T cells were resuspended in 1.0 ml PBS, combined with one vial of carboxyfluorescein succinimidyl ester (CFSE; Renovar, Madison, WI) and incubated for 10 min at 37°C. Cells were washed twice in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Mediatech) and 1.0% penicillin/streptomycin (Invitrogen) and resuspended in the same medium.

We plated one million CFSE-labeled T cells per well of 24-well plates (Costar, Corning, NY). We incubated the cells alone without or with 20 μl/well of anti-CD3/anti-CD28 bead suspension (Dynabeads, Invitrogen Dynal, Oslo, Norway). To some wells we added autologous $CD14^+$ cells in the ratio 0.1 to 2.0 $CD14^+$ cell per one T cell. The final volume of all wells was 2.0 ml. The cells were incubated for 4 days at 37°C in humidified atmosphere containing 5% CO_2. Subsequently, the cells were collected, each sample split in two, and incubated for 10 min at room temperature with allophycocyanin-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD4 or PE-conjugated anti-CD8 (all from eBioscience; San Diego, CA) according to manufacturer's instructions. The cells were washed, resuspended in PBS containing 1.0% propidium iodide (Renovar), and analyzed by a FACSCalibur flow cytometer and CellQuest software. Viable T cells were gated based on their characteristic position in the forward versus side scatter plot and absence of propidium iodide staining. To analyze the data, we compared the CFSE fluorescence distribution in histograms obtained from T cells incubated alone (negative control), with anti-CD3/anti-CD28 reagent (positive control) or with anti-CD3/anti-CD28 reagent and autologous monocytes.

The effects of isolated $CD14^+$HLA-$DR^{low/-}$ and $CD14^+$HLA-DR^{high} cells on proliferation of autologous T cells were assessed as above with some modifications due to the availability of fewer sorted cells in comparison to unsorted cells. $CD14^+$HLA-$DR^{low/-}$ or $CD14^+$HLA-DR^{high} cells were placed into round-bottom 96-well plates (Costar) in the 1:1 ratio with T cells activated by the anti-CD3/anti-CD28 reagent in the total volume of 200 μl of AIM V medium containing 1.0% penicillin/streptomycin. The cells were incubated for 60 hr when 125 μl of the medium was removed from each well and replaced by the same volume of the fresh medium containing 2.0 μCi [^{3}H]thymidine (Amersham–GE Healthcare, Mt. Pleasant, IL) and incubated for another 12 hr. The cells were harvested by the use of a semiautomatic cell harvester (Skatron Instruments, Sterling, VA) and the incorporated radioactivity was measured by a LS 6000SC scintillation counter (Beckman Coulter, Fullerton, CA).

Statistical Analysis

We report the data as mean ± standard deviation using 5% significance as the limit of statistical significance in all analyses. We assessed the significance of differences in the cells among age-

matched noncancerous controls (AMC), newly diagnosed untreated PCa patients (uPCa) and treated PCa patients (tPCa) by Kruskal–Wallis tests. For comparisons of data between any two groups we used the Mann–Whitney tests with Bonferroni correction for multiple comparisons. These nonparametric tests were more robust and thus preferred over their parametric counterparts. We computed the Spearman's correlation coefficients to explore the possible correlations between cell abundance, PSA values, Gleason scores and adjuvant treatment modes and Pearson's correlation coefficients in other cases. In addition, we used Mann–Whitney tests for comparisons of patient treatment effects on cell phenotype. We constructed regression models with an interaction term to investigate treatment effects after controlling for demographic and clinical variables.

We analyzed the T cell proliferation measurements in the following way: The effects of CD3/CD28 stimulation on T cells from tPCa and AMC and the levels of intracellular cytokines were compared by the two-sample *t*-test and Mann–Whitney test. To acknowledge the correlation between repeated measurements at various monocyte/T cell ratios for the same individual, we compared the effects of the monocyte/T cell ratio on T cell reactivity by repeated-measures ANOVA (separately for $CD4^+$ cells and $CD8^+$ cells). Finally, assuming a linear relationship between measured T cell activation and monocyte/T cell ratios, we used ANCOVA models treating the monocyte/T cell ratio as a continuous predictor. Hence, the interaction term of the experimental group (e.g., $CD4^+$ cells of tPCa) and the respective monocyte/T cell ratio are interpreted as the difference of slopes of the dependence of T cell activation on monocyte/T cell ratio.

Results

More HLA-$DR^{low/-}$ Monocytes Circulate in PCa Patients Than in Healthy Controls

Based on the published evidence for altered development of myeloid DC in cancer [14] and the presence of altered (HLA Class-II^-) monocytes in patients suffering from ovarian carcinoma [15] and melanoma [16], we hypothesized that monocytes in tPCa were also altered. Consequently, by flow cytometry first we determined the percentage of cells in the monocyte region of AMC, uPCa and tPCa; they were 17.6 ± 6.9%, 19.0 ± 14.3%, and 23.9 ± 6.1%, respectively ($P = 0.0223$ for AMC vs. uPCa and $P = 0.0144$ for uPCa vs. tPCa). Next, in these cells we analyzed the percent of $CD14^+$ cells; we found that 95.1 ± 4.4%, 92.3 ± 4.5%, and 94.4 ± 3.3% of cells were $CD14^+$ ($P > 0.05$ for all comparisons). Thus, the three groups did not differ in the amounts of $CD14^+$ cells in the scatter plots. Because of the preponderance of $CD14^+$ cells among monocytes, we use the terms “monocyte” and “$CD14^+$ cell” interchangeably.

To characterize the monocytes in PCa further, we measured the percentage of HLA-$DR^{low/-}$ cells in freshly isolated $CD14^+$ cells. In this analysis we included the cells from uPCa in addition to those from tPCa. We found that HLA-$DR^{low/-}$ cells comprised 4.1 ± 6.5% (range: 0.5–23.3%) of $CD14^+$ cells in AMC and 30.7 ± 15.0% (range: 5.1–62.4%) in tPCa, respectively ($P < 0.0001$; Fig. 1). Newly diagnosed untreated PCa patients exhibited 10.6 ± 14.3% (range: 0.1–50.4%) of HLA-$DR^{low/-}$ monocytes, that is, more than AMC ($P = 0.290$), but less than tPCa ($P = 0.001$).

Further we compared the percentage of $CD14^+$HLA-$DR^{low/-}$ cells in tPCa receiving a LHRH-agonist alone (tPCa/0, 7 patients) with patients additionally receiving an antiandrogen (tPCa/A, 8 patients) or dexamethasone (tPCa/D, 6 patients). The presence of such cells in tPCa/0 (24.3 ± 11.3%, range: 5.1–38.3%) was higher than in AMC and uPCA ($P = 0.0018$ and $P = 0.0131$, respectively), but was not statistically significantly different from the levels in tPCa/A (36.7 ± 16.9%, range: 8.4–62.4%) and tPCa/D (31.4 ± 16.1%, range: 7.4–56.0%). More

samples in each treatment groups are needed for a definitive resolution whether antiandrogens and dexamethasone contribute to the abundance of $CD14^+$ HLA-$DR^{low/-}$ cells.

Monocytes From Treated PCa Patients Yield Fewer Mature Dendritic Cells Than Monocytes From Age-Matched Controls

Our standard method for ex vivo DC differentiation includes isolation of $CD14^+$ cells by immunomagnetic adsorption followed by a two-step incubation. The first step requires the presence of GM-CSF, IL-4 and 1.0% human AB serum in X-VIVO 15 medium for 3 days followed by incubation with additional TNF-α and PGE_2 for two more days. Starting with $CD14^+$ cells from the blood of healthy blood donors not selected for age, we routinely obtain DCs that are 90.8 ± 7.9% mature (as measured by the expression of CD83) with a yield of 33.9 ± 13.5% (data not shown). To validate this procedure for a clinical trial of DC immunotherapy in androgen-independent tPCa, we prepared DCs from $CD14^+$ cells of patients treated with LHRH agonists and antiandrogens or dexamethasone and one orchiectomized patient (Supplementary Table Ic) and compared the DCs with those prepared from the cells of AMC. Data in Figure 2a show that $CD14^+$ cells from AMC matured into $CD83^+$ cells efficiently and rather uniformly (85.8 ± 6.1%, range: 73.6–92.8%); the cells isolated from tPCa matured less effectively and less uniformly (62.3 ± 30.2%, range: 3.7–98.1%; $P = 0.0541$). This difference was not related to different final cell yields because the final numbers of total viable cells for AMC and tPCa were indistinguishable, 25.2 ± 16.2% (range: 7.7–63%) for AMC and 25.2 ± 9.3% (range: 6.2–45.3%), respectively ($P = 0.543$; Fig. 2b).

Levels of HLA-$DR^{low/-}$ Cells Do Not Predict DC Maturity

To determine the relationship of HLA-$DR^{low/-}$ cells and $CD83^+$ DCs, we plotted the percentage of $CD83^+$ cells in the final DC product as a function of percentage of HLA-$DR^{low/-}$ cells among the $CD14^+$ cells. Interestingly, $CD14^+$ cells from AMC matured into $CD83^+$ cells efficiently and independently of the initial content of HLA-$DR^{low/-}$ cells ($R^2 = 0.038$, $P = 0.542$); for tPCa, the percentage of $CD83^+$ cells was lower and related to the percentage of HLA-$DR^{low/-}$ cells among the $CD14^+$ DC precursors ($R^2 = 0.551$, $P = 0.0003$; Fig. 2c). Interestingly, only in isolates containing more than 17 percent HLA-$DR^{low/-}$ cells we observed poor DC maturation; this indicates the possibility for the existence of a threshold density of HLA-$DR^{low/-}$ cells needed to impair differentiation into DCs. An analysis of CD83 expression measured as mean fluorescent intensity (MFI; Fig. 2d) is consistent with the conclusions drawn from Figure 2c: The higher percentage of HLA-$DR^{low/-}$ cells in the starting monocytes population, the lower CD83 MFI ($R^2 = 0.3948$, $P = 0.004$). In analogy to the data in Figure 2c, if CD83 MFI measured in AMC cells is included into the analysis, there is no correlation ($R^2 = 0.0471$, $P = 0.2411$). The percentage of cells surviving in vitro incubation appears independent of the percentage of HLA-$DR^{low/-}$ cells in the initial $CD14^+$ cell population ($P > 0.05$ both for AMC and tPCa; data not shown). Thus, solely the level of expression of HLA-DR on $CD14^+$ cells is not sufficient to predict the percentage of $CD83^+$ cells in the final DC preparation.

PCa Monocytes Suppress T-Cell Proliferation

HLA-$DR^{low/-}$ monocytes found in other pathological states suppress immunity [17–19]. Hence, we compared the effects of tPCa monocytes and AMC monocytes on proliferation of autologous T cells. We isolated monocytes from five additional AMC (containing 3.5 ± 1.0% HLA-$DR^{low/-}$ cells) and four additional tPCa (containing 8.4 ± 1.8% HLA-$DR^{low/-}$ cells); these tPCa contained more HLA-$DR^{low/-}$ cells than AMC ($P = 0.041$), but contained fewer such cells than tPCa in Figure 1 ($P = 0.010$). We isolated $CD3^+$ T cells from AMC (97.4 ± 0.4% purity) and tPCa (93.9 ± 2.8% purity, $P = 0.211$ relative to AMC), stimulated them by anti-CD3/CD28 reagent and analyzed their proliferation (Fig. 3a,b). Both AMC and tPCa

$CD4^+$ cells proliferated with high efficiency (87.9 ± 4.2% vs. 85.5 ± 5.7%; $P = 0.515$), as did $CD8^+$ cells (74.5 ± 7.0% vs. 74.5 ± 2.0%; $P = 0.995$).

To some T cell containing wells we introduced autologous monocytes from AMC (92.1 ± 2.5% purity) or tPCa (89.9 ± 2.2% purity, $P = 0.539$ relative to AMC) ranging from 0.1 monocyte to 2.0 monocytes per one T cell (the range was limited by the number of cells obtained from a minimally invasive blood draw). The presence of monocytes affected proliferation of T cells (Fig. 3a,b); at ratios of 0.1 and 0.2, monocytes stimulated $CD4^+$ and $CD8^+$ cell proliferation. At higher ratios AMC monocytes had little effect on proliferation of AMC T cells except for the suppressed proliferation at the ratio of two monocytes per one T cells. On the other hand, tPCa monocytes suppressed autologous T cell proliferation at much lower ratios, that is, they were more potent inhibitors than AMC monocytes ($P = 0.0036$ for $CD4^+$ cells, $P = 0.0044$ for $CD8^+$ cells). Thus, AMC and tPCa T cells did not differ in the response to CD3/CD28 stimulation, but tPCa monocytes inhibited autologous T cell proliferation more effectively than AMC monocytes.

$CD14^+HLA\text{-}DR^{low/-}$ Cells Suppress T Cell Proliferation More Effectively and Express More IL-10 Than $CD14^+HLA\text{-}DR^+$ Cells

In contrast to $CD14^+HLA\text{-}DR^{low/-}$ cells in tPCa patients (Fig. 1), myeloid-derived suppressor cells (MDSCs) in other systems often express little or no CD14 [20–23]. Because $CD14^+$ tPCa monocytes inhibited T cells similarly to such MDSCs [14,20,22,24], we hypothesized that this effect is due to $CD14^+HLA\text{-}DR^{low/-}$ cells. Hence, we isolated $CD14^+HLA\text{-}DR^{low/-}$ and $CD14^+HLA\text{-}DR^+$ cells from tPCa patients by fluorescence activated cell sorting (Fig. 3c) and measured the effects of isolated cells on DNA synthesis by activated autologous T cells. (Because of the rather low numbers of cells isolated from the available volume of patient blood, we conducted the experiments only at the 1:1 ratio and measured the effects by radioactive thymidine incorporation.) Data in Figure 3d show that $CD14^+HLA\text{-}DR^{low/-}$ cells inhibited T cell proliferation statistically significantly more potently than $CD14^+$ $HLA\text{-}DR^+$ cells.

The suppressive effects of MDSCs on T cell proliferation are often mediated by IL-10 and TGF-β, the "M2-type" cytokines [25]. To determine if the suppressive function of $CD14^+HLA\text{-}DR^{low/-}$ cells is mediated by such cytokines, we measured and compared the percentages of $CD14^+HLA\text{-}DR^{low/-}$ cells and $CD14^+HLA\text{-}DR^+$ cells that contained intracellular IL-10 and TGF-β1. We found that $CD14^+HLA\text{-}DR^{low/-}$ expressed statistically significantly more IL-10 than $CD14^+HLA\text{-}DR^+$ cells while the difference for TGF-β was not stastistically different (Table II). Interestingly, this effect was characteristic of the cells expressing high and low levels of HLA-DR, respectively, irrespective of whether they were isolated from tPCa or AMC. Although the paucity of available cells isolated by sorting precluded experiments whereby activity of IL-10 and TGF-β would be blocked, the higher level of inhibitory cytokine IL-10 in $CD14^+HLA\text{-}DR^{low/-}$ is compatible with their likely role in suppression of T cell proliferation.

Percentages of $CD4^+$ Cells, $CD8^+$ Cells and Regulatory T Cells in AMC and PCa Patients Are Largely Indistinguishable

T_{reg} cells have been documented to interfere with therapeutic vaccination [26]. For this reason, we analyzed $CD3^+$ T cells for the frequency of $CD4^+$ cells, $CD8^+$ cells and $CD4^+CD25^+CD127^{low/-}$ T_{reg} cells [27–29]. Figure 4a shows the ratios of $CD4^+$ cells and $CD8^+$ cells in AMC, uPCa patients and tPCa. There was no difference among the groups in the levels of $CD8^+$ cells, but there were fewer $CD4^+$ T cells as percentage of $CD3^+$ cells in AMC than in uPCa ($P < 0.05$). Figure 4b shows the percentages of $CD25^+CD127^{lo/-}$ cells among $CD4^+$ cells in the three groups. These percentages were low overall with fewer cells in uPCa than in tPCa ($P < 0.05$).

Frequency of HLA-DR$^{low/-}$ B Cells Is Not Changed in PCa Patients

High levels of HLA-DR$^{low/-}$ monocytes in tPCa (Fig. 1) prompted the question whether the loss of HLA-DR expression is specific for antigen presenting cells in general or whether it is restricted to monocytes. Thus, we quantified HLA-DR expression in CD19$^+$ B cells. Interestingly, the percentage of HLA-DR$^+$ cells in B cells was statistically indistinguishable among AMC, uPCa and tPCa (Fig. 5). An analysis of HLA-DR$^+$ cells in CD4$^+$ cells and CD8$^+$ cells of the same subjects revealed no difference among the groups either (data not shown). Apparently, diminished expression of HLA-DR in PCa patients appears particular to monocytes.

Androgen Suppression Is Associated With Increased Levels of CD14$^+$HLA-DR$^{low/-}$ Cells

To detect additional relations among the values measured in AMC, uPCa and tPCa, we undertook a search by the Mann–Whitney test. For example, PSA levels (Fig. 6 and Supplementary Table I) were significantly higher in uPCa and tPCa than in AMC ($P < 0.0001$ and $P < 0.0001$, respectively). Similarly, the percent of HLA-DR$^{low/-}$ cells among CD14$^+$ cells was higher in uPCa and tPCa than in AMC ($P = 0.2898$ and $P < 0.0001$, respectively); also, this percentage was higher in tPCa than in uPCa ($P = 0.0001$).

Next we searched by regression analysis for associations between the disease, treatment and percentages of CD14$^+$HLA-DR$^{low/-}$ cells among all CD14$^+$ cells. We found the levels of CD14$^+$HLA-DR$^{low/-}$ cells significantly and positively associated with treatment (leuprolide, $P = 0.0058$; antiandrogen, $P = 0.0381$), that is, receiving one or both drugs was associated with higher percentages of CD14$^+$HLA-DR$^{low/-}$ cells. In a regression analysis of the percentages of CD14$^+$HLA-DR$^{low/-}$ cells versus androgen suppression (all drugs taken into account) *and* PSA levels, treatment with LHRH-agonist and/or antiandrogen was still significantly and positively associated with percentages of CD14$^+$HLA-DR$^{low/-}$ cells ($P = 0.0027$ and 0.0222, respectively); however, in this analysis, PSA levels were not significantly associated with CD14$^+$HLA-DR$^{low/-}$ cell levels ($P = 0.1250$). This is different from the significant bivariate Spearman's correlation between CD14$^+$HLA-DR$^{low/-}$ cells and PSA above, but the nature of this study does not allow us to deduce the underlying reasons for the difference.

Discussion

Attempts to treat cancer by cellular immunotherapy have focused largely on the manipulation of immune effector cells and/or vaccination. These methods have contributed a steady progress in the effectiveness of immunotherapy and evolution of its concepts (cf. Ref. [30]), yet they still do not deliver a predictable and definitive cure [3]. Some have associated this failure with the findings of abnormally high levels of CD4$^+$CD25$^+$ T_{reg} cells [9] and CD8$^+$Foxp3$^+$ T_{reg} cells in tumor tissue [31] and peripheral blood of prostatectomized patients [9]. Others, though, found little difference in the levels of circulating T_{reg} cells between PCa patients and healthy controls, but observed that T_{reg} cells in PCa were more immunosuppressive [32]. We too found similar T_{reg} cell levels in PCa patients and AMC, but the significance of all these findings is uncertain as experiments in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice question whether T_{reg} cells are necessary for induction of T-cell tolerance for PCa [33].

Androgen suppression, the long-standing standard in the treatment of metastatic PCa, boosts thymopoiesis and T cell proliferation [34,35], yet its role in PCa immunotherapy remains unclear. In TRAMP mice, modified by the introduction of influenza hemagglutinin as an artificial prostate-restricted antigen, androgen ablation stimulated immunity by the purported increase of access to antigen [36]. However, it is difficult to extrapolate from the effects of acute androgen deprivation in young mice to elderly cancer patients on chronic androgen suppression. Consequently, the significance of our finding of similar percentage of T_{reg} cells

in tPCa patients and AMC needs to be evaluated in terms of possible effects of chronic androgen suppression on T cell function, not just numbers.

Lately there has been an increased interest in the heterogeneous group of myeloid cells that negatively regulate antigen-specific immunity, the myeloid-derived suppressor cells (cf. Ref. [20]). In humans, these cells express CD11b, CD13, CD15, CD33 and CD34, are devoid of lineage markers, CD14, and HLA-DR [20–23] and can suppress $CD4^+$ and $CD8^+$ cell function in vitro [14,24]. In this new area, there are few if any data on these cells in PCa and their role in regulation of human immunity. Similarly, the possible role of androgen suppression in generation and effects of these cells has not been studied.

Prostate Cancer and Androgen Suppression Affect Monocyte Phenotype

To prepare a cellular vaccine for administration to PCa patients, we isolated their $CD14^+$ cells and matured them into DCs by a standard method. In the process, we observed that PCa monocytes differentiated into mature DCs less efficiently than monocytes of age-matched controls. To better understand the reasons for the impairment, we characterized these monocytes and found them significantly different from controls, particularly in patients on androgen suppression therapy. We found that tPCa were higher in the ratio of $CD14^+$HLA-$DR^{low/-}$/$CD14^+$HLA-DR^+ cells than uPCa who, in turn, exhibited higher ratios than AMC. The increased percentage of HLA-$DR^{low/-}$ appears characteristic for PCa monocytes, as HLA-DR expression in B cells (and other hematopoietic cells; data not shown) was unchanged indicating that the phenomenon was specific for monocytes. (This, however, does not necessarily mean that B cells in PCa are normal; recently, normal HLA expression was found in CD19 cells associated with systemic plasmocytosis in patients with advanced melanoma, breast cancer, glioma and pancreatic cancer; Ref. [37].) The statistically insignificant increase of HLA-$DR^{low/-}$ monocytes in uPCa relative to AMC appears related to the disease itself; this increase is statistically significant in patients treated by androgen suppression. Importantly, DC precursors in this study were CD14-positive; hence, they are different from “immature myeloid cells” [24] or “myeloid-derived suppressor cells” [23] that have been defined as CD14-negative.

Despite the statistically significant correlation between HLA-$DR^{low/-}$ monocytes and androgen suppression, the conclusion that the effects in tPCa are predominantly iatrogenic must await further corroboration. Namely, it is possible that in tPCa the significantly longer time since diagnosis itself contributes to the impairment of monocytes. Additional factors, such as diabetes [38], could affect the phenotype of circulating monocytes as well; however, as only three of the 22 tPCa in this study were diabetic, it is unlikely that diabetes contributed significantly to the present results.

To get a measure of the functional potential of tPCa monocytes in the absence of a technically feasible method in vivo, we compared in vitro the effects of these cells and normal controls on activated autologous T cells. We found that monocytes from tPCa patients inhibited T cell proliferation more effectively than AMC monocytes. The reason for the more pronounced reduction of T cell proliferation by HLA-$DR^{low/-}$ cells than by HLA-DR^+ cells is likely related to higher levels of IL-10 expressed by HLA-$DR^{low/-}$ cells. IL-10, an M2-type cytokine, suppresses T cell function [25] and, expressed by $CD14^+$ cells, predicts poor outcome in stage IV melanoma [39]. Thus, the lack of HLA-DR expression and IL-10 expression could signify that HLA-$DR^{low/-}$ cells in PCa patients are similar to M2-type cells observed in other systems.

Malignant Diseases, Sepsis and Trauma Are Associated With Elevated Levels of HLA-DR$^{low/-}$ Monocytes

The observation that PCa patients exhibit monocytes different from healthy individuals is not new. For example, such monocytes can inhibit the mixed leukocyte reaction [6–8] in analogy to our results. Aberrant monocytes have been observed in ovarian cancer [15], malignant melanoma [16,40], hepatocellular carcinoma [41] and a complex disease such as inflammatory bowel disease [42]. These diseases are associated with increased occurrence of HLA-DR$^{low/-}$ monocytes that have often been documented for expression of IL-10 and TGF-β, but not IL-12 and tumor necrosis factor-α (TNF-α); the presence of IL-10 and TGF-β and absence of IL-12 and TNF-α can contribute to the ability of these cells to inhibit T cell proliferation [15,25]; our findings of elevated expression of IL-10 in HLA-DR$^{low/-}$ monocytes and their inhibition of T cell proliferation are fully in line with these observations. In view of these findings it is noteworthy that sera of androgen-independent tPCa patients contained increased levels of IL-4, IL-6 and IL-10 (but not of IL-1β, IL-2, IL-12, and IFN-γ) compared to androgen-dependent patients and noncancerous controls [43]. Similarly, elevated levels of TGF-β have been associated with androgen-deprivation in rats [44] and humans after prostatectomy [45]. Despite these insights, the primary factors responsible for elevated levels of HLA-DR$^{low/-}$ monocytes and the role of such cells in modulation of immunity and outcome of immunotherapy remain poorly understood.

Although comparison of the natural history of solid tumors with the short-lived sepsis and trauma is tenuous, it is informative to consider the better understood role of HLA Class II^{-} cells in these states [17–19,46,47]. The overall conclusion from sepsis and trauma is that the CD14^{+}HLA-DR$^{low/-}$ cells are strongly immunosuppressive and predict poor outcome [17, 19,47]. A recent study in sepsis established that the early loss of HLA-DR molecule is due to the reduced levels of Class II transactivator and consequential reduction in HLA-DR transcription; the reduction of Class II transactivator correlated with the increased cortisol levels in patients and is also caused by glucocorticoids in vitro [19]. However, a recent study demonstrated that the levels of cortisol in prostatectomized PCa patients are physiological [48] pointing to the likely role of androgen suppression in increased levels of HLA-DR$^{low/-}$ monocytes.

Androgen Suppression and Prostate Cancer Immunotherapy

The preceding discussion alludes to a rather complex and poorly understood role of androgen suppression in immunotherapy of prostate cancer. On one hand, expanded thymopoiesis and T cell proliferation can stimulate tumor-specific immunity [35,36]. On the other, the immunosuppressive role of HLA Class II$^{low/-}$ monocytes has been corroborated by studies of endotoxin-resistant cells [49]. Similar to the HLA-DR$^{low/-}$ cells isolated from cancer patients [15], endotoxin-resistant HLA-DR^{-} cells express IL-10 and cannot stimulate antigen-specific T cells [49]. In addition, other mechanisms whereby HLA-DR$^{low/-}$ monocytes suppress immunity are likely involved. For example, this article and others [50] have shown that HLA-DR$^{low/-}$ monocytes mature less effectively into functional DCs; this can lessen the stimulation of naïve and memory T cells [1,50]. It is also plausible that HLA-DR$^{low/-}$ monocytes cannot develop into functional macrophages as effectively as normal monocytes; this could reduce the ability of macrophages to re-present the antigens released from tumor cells previously killed by T cells or NK cells [51] and thus maintain the length, amplitude and range of T cell attack on tumor cells. Consequently, the sum total of the effects of androgen suppression on the natural history of disease and development of therapeutic immunity must be evaluated in clinical studies and considered when designing immunotherapeutic strategies aimed at eradication or control of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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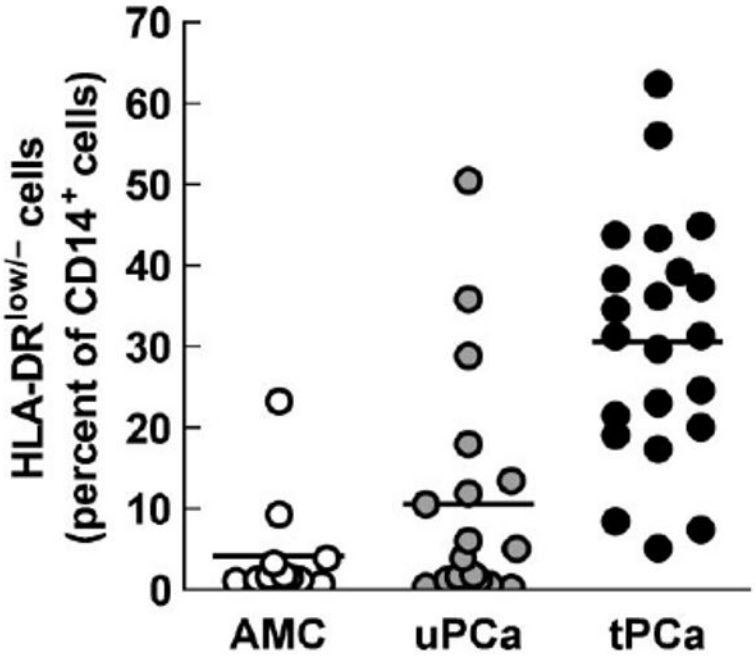

Fig. 1.

Percentage of HLA-DR$^{low/-}$ cells among CD14^{+} cells in peripheral blood of AMC subjects (white circles), newly diagnosed untreated prostate cancer patients (uPCa; gray circles) and tPCa patients (black circles). Horizontal lines indicate mean values. The differences between AMC and tPCa and between uPCa and tPCa are statistically significant ($P < 0.0001$ and $P = 0.001$, respectively).

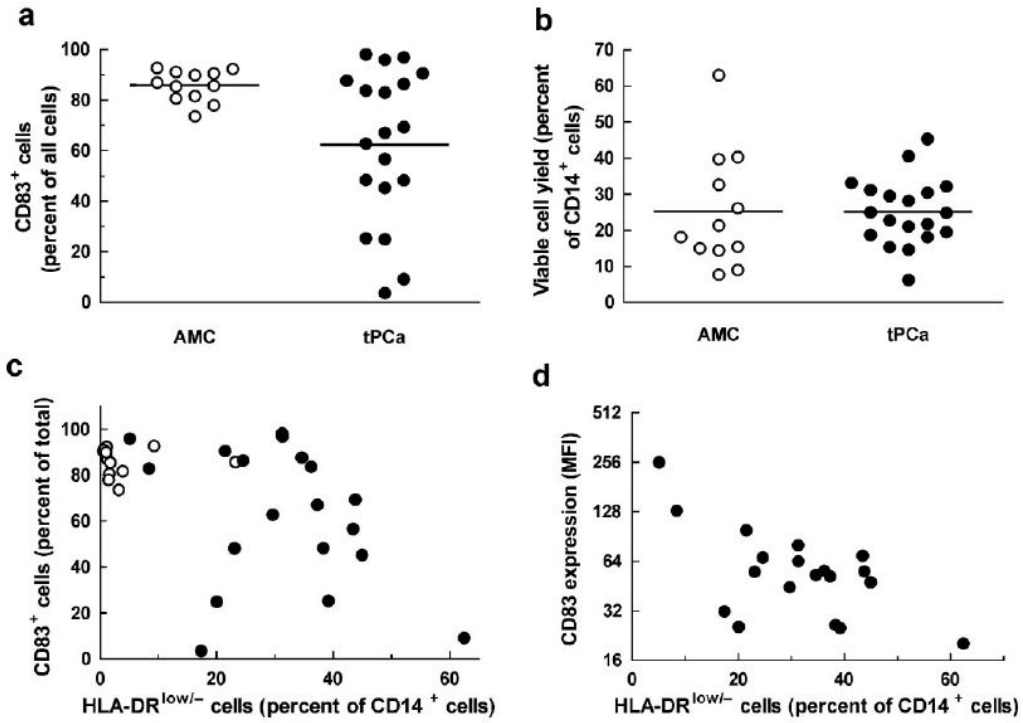

Fig. 2.

a: Percentage of mature CD83$^+$ dendritic cells in the final preparation and (**b**) viable cell yield expressed as percentage of the initially plated CD14$^+$ cells for age-matched non-cancerous controls (AMC; white circles) and prostate cancer patients treated by androgen suppression (tPCa; blackcircles). Horizontal lines indicate mean values; the difference between CD83$^+$ cell levels from AMC and tPCa in panel a is statistically significant ($P = 0.0541$); the difference in yields (b) is not ($P = 0.543$). **c:** Percentage of CD83$^+$ cells and (**d**) CD83 mean fluorescence intensity (MFI) of cells after DC maturation from the CD14$^+$ cells plotted as functions of the percentage of HLA-DR$^{low/–}$ cells among CD14$^+$ cells.

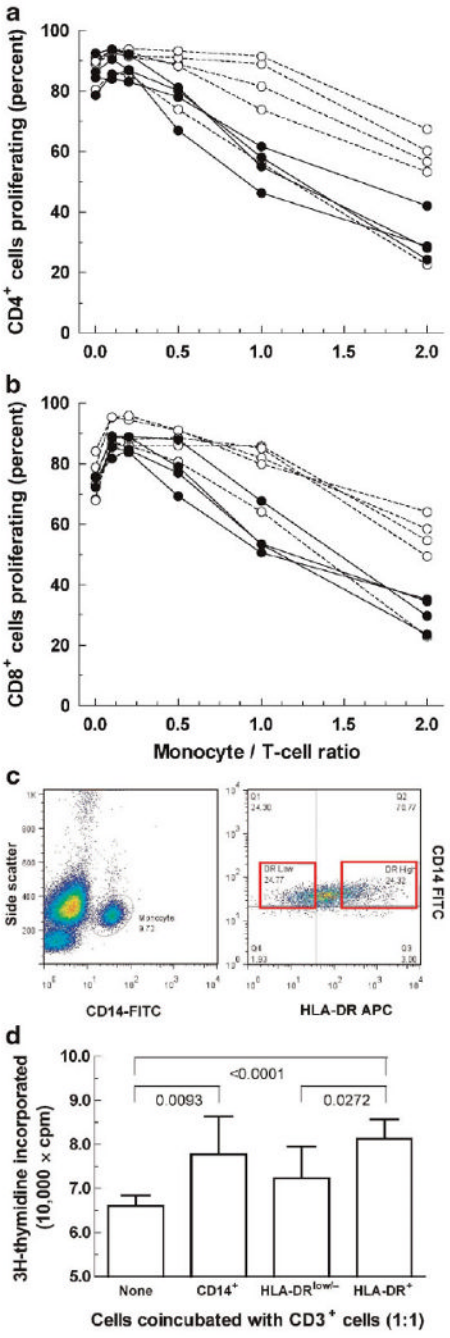

Fig. 3.

T cell proliferation in the presence of autologous monocytes. **a:** $CD4^+$ cells; (**b**) $CD8^+$ cells. White circles, AMC; blackcircles, tPCa. T cells were stimulated by CD3/CD28 ligation in the absence or presence of autologous monocytes. All data were corrected by subtracting the corresponding values measured in unstimulated T cells in the absence of monocytes. There was no difference in proliferation of $CD4^+$ cells ($P = 0.515$) or $CD8^+$ cells ($P = 0.995$), but tPCa monocytes inhibited proliferation of autologous $CD4^+$ cells and $CD8^+$ cells more potently than AMC monocytes ($P = 0.0036$ and $P = 0.0044$, respectively). **c, Left panel:** A representative scattergram of mononuclear cells isolated from the blood of a treated PCa patient (P29 in Table II). $CD14^+$ cells in the rightmost peak (monocytes) were sorted into HLA-

$DR^{low/-}$ cells and HLA-DR^{+} cells (**right panel**). **d:** Comparison of the effects of sorted HLA-$DR^{low/-}$ cells and HLA-DR^{+} cells (shown in panel c) on proliferation of autologous T cells (measured as DNA synthesis) at the 1:1 ratio. Numbers in the panel designate the *P*-values for differences between bracketed pairs of groups (two-sided Student's *t*-test).

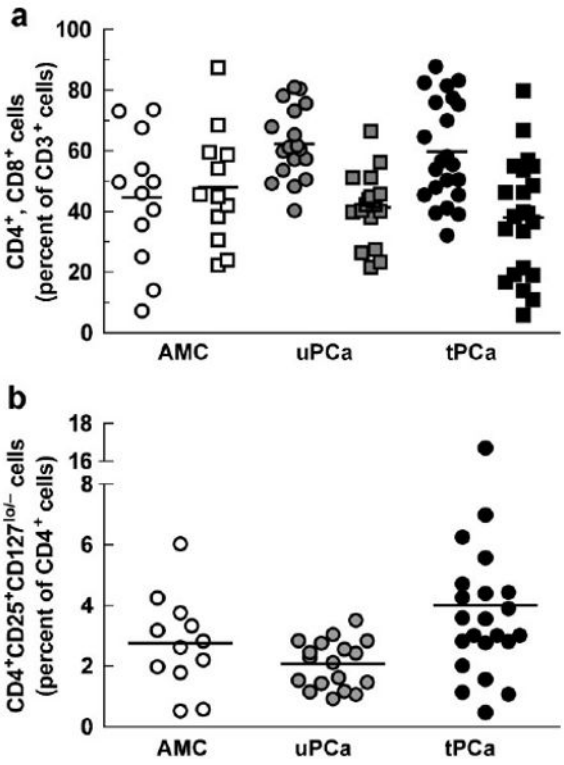

Fig. 4.

T cells in PBMCs of subjects described in the legend to Figure 1. **a:** Percent of $CD4^+$ cells (circles) and $CD8^+$ cells (squares) among $CD3^+$ cells. The percentage of $CD4^+$ cells in AMC differed statistically from uPCa($P < 0.05$). **b:** $CD4^+CD25^+CD127^{low/-}$ T regulatory cells as percent of $CD4^+$ cells. Horizontal lines indicate mean values. The percentage of cells in uPCa differed statistically from tPCa ($P < 0.05$).

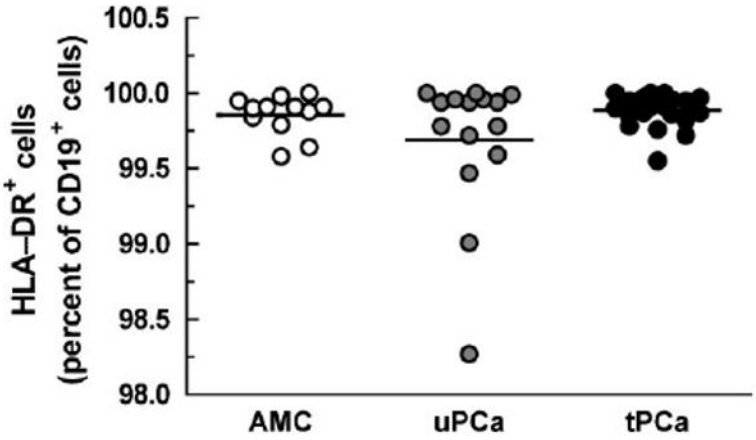

Fig. 5.

HLA-DR expression on $CD19^+$ B cells from subjects described in the legend to Figure 1. Horizontal lines indicate mean values; these values did not differ statistically.

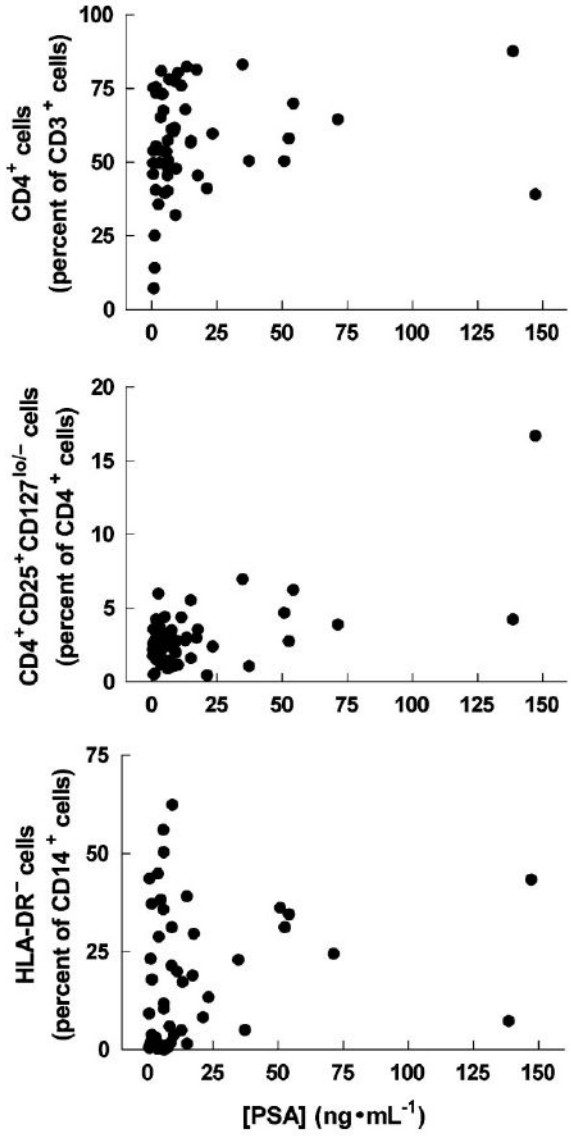

Fig. 6.

Percent of $CD4^+$ cells among $CD3^+$ cells (**upper panel**), $CD4^+CD25^+CD127^{lo/-}$ cells among $CD4^+$ cells (**middle panel**) and HLA-$DR^{low/-}$ cells among $CD14^+$ cells (**lower panel**) plotted as a function of PSA concentrations measured in the blood of all subjects in this study. For statistical details, see the text.

TABLE I

Immunoreagents Used in This Study

Antibody specificity	Fluorescent label	Manufacturer
CD3	FITC, PE, APC	eBioscience
	PE-Cy5	Pharmingen
CD4	FITC, PE	eBioscience
CD8	FITC, PE, APC	
CD14		eBioscience
CD19	PE	
CD25	APC	Pharmingen
CD56	FITC	eBioscience
CD83	PE	Beckman Coulter
CD127	PE	Pharmingen
HLA-DR	FITC	Biosource
	PerCP	Pharmingen
IgG	PE	Biosource
TGF-β[a]	PE	R&D Systems
IL-10[a]	PE	R&D Systems

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; Cy-5, cyanine 5.

[a]Used for intracellular staining.

TABLE II

Expression of Intracellular IL-10 and TGF-β1 by $CD14^+HLA\text{-}DR^{low/-}$ Cells and $CD14^+$ $HLA\text{-}DR^+$ Cells From Treated Prostate Cancer Patients (tPCa) and Age-Matched Controls

		IL-10 expressing cells			TGF-β1 expressing cells		
Subject	**$HLA\text{-}DR^{low/-}$ cells, percent of $CD14^+$ cells**	**$HLA\text{-}DR^{low/-}$ cells**	**$HLA\text{-}DR^+$ cells**	***P*-value[a]**	**$HLA\text{-}DR^{low/-}$ cells**	**$HLA\text{-}DR^+$ cells**	***P*-value[a]**
[b] P27	14.0	90.4[c]	73.4		43.6	33.6	
P28	20.8	40.6	13.5		25.9	18.6	
P29	24.3	89.2	69.0	0.0244	44.3	41.2	0.5405
A18	1.7	70.0	13.8		8.8	15.7	
A19	5.0	40.4	26.4		47.2	50.2	

[a] Two-sided values calculated by paired Student's *t*-test for paired samples.

[b] P denotes tPCa patients, A the age-matched controls (both indicated by ** in Supplementary Table I).

[c] Percentage of $CD14^+HLA\text{-}DR^{low/-}$ cells and $CD14^+HLA\text{-}DR^{high/+}$ cells, respectively, expressing IL-10 or TGF-β1.