Up-regulation of proliferative and migratory genes in regulatory T cells from patients with metastatic castration-resistant prostate cancer

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Abstract

A higher frequency of regulatory T cells (Tregs) has been observed in peripheral blood mononuclear cells (PBMC) of patients with different types of solid tumors and hematological malignancies as compared to healthy donors. In prostate cancer patients, Tregs in PBMC have been shown to have increased suppressive function. Tumor-induced biological changes in Tregs may enable tumor cells to escape immunosurveillance. We performed genome-wide expression analyses comparing the expression levels of more than 38,500 genes in Tregs with similar suppressive activity, isolated from the peripheral blood of healthy donors and patients with metastatic castration-resistant prostate cancer (mCRPC). The differentially expressed genes in mCRPC Tregs are involved in cell cycle processes, cellular growth and proliferation, immune responses, hematological system development and function, and the IL-2 and TGF-β pathways. Studies revealed that the levels of expression of genes responsible for T-cell proliferation (C-FOS, C-JUN, and DUSP1) and cellular migration (RGS1) were greater in Tregs from mCRPC patients as compared to values observed in healthy donors. Increased RGS1 expression in Tregs from mCRPC patients suggests a decrease in these Tregs' migratory ability. Additionally, the higher frequency of CD4$^+$CD25^{high}CD127$^-$ Tregs in the peripheral blood of mCRPC patients may be the result of an increase in Treg proliferation capacity. Results also suggest that the alterations observed in gene expression profiles of Tregs in mCRPC patients may be part of the mechanism of tumor escape from host immune surveillance.

Keywords

regulatory T cells; prostate cancer; immune suppression

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Introduction

Prostate cancer is the second leading cause of cancer death in men and accounts for about 11% of cancer-related deaths in that population. In 2012, new cases of prostate cancer in the United States were estimated at 241,740, resulting in 28,170 deaths.[1]

Traditional cancer treatments, including surgery, radiation therapy, hormonal therapy, and chemotherapy, provide only partial cures. Therapeutic cancer vaccines aim to activate a specific antitumor response in the host, with lower toxicity than standard treatments.[2] However, tumor-induced immune suppression may hamper the effectiveness of immunotherapies and results in failure to arrest tumor progression.[3] Various mechanisms have been proposed for the tumor's ability to escape from host immune surveillance, including (a) the release of soluble factors that interfere with the host's immune response against tumor,[4] and (b) various immune cells that induce immunosuppression, including myeloid-derived suppressor cells,[5] tumor-associated macrophages,[6] type 2 natural killer T (NKT) cells,[7] mast cells,[8] and regulatory T cells (Tregs).[9]

Tregs play an important role in maintaining immunologic self-tolerance and controlling autoimmunity. However, in cancer, they contribute to tumor growth and progression by dampening the anti-tumor immune response. Although the Treg phenotype is debatable, $CD4^+CD25^{high}$ Tregs display strong immunosuppressive activity and are regarded as the functional representative of Tregs in humans.[10] $CD25^{high}$ cells represent 1% to 2% of $CD4^+$ T cells in peripheral blood. $CD4^+CD25^{high}$ Tregs express the transcription factor forkhead box P3 (FoxP3), which is the master regulator in the development and function of Tregs.[11] It has also been shown that CD127 expression is inversely correlated with FoxP3 expression, and that $CD127^-$ in the $CD4^+CD25^{high}$ population confers suppressive function.[12]

Tregs can suppress the immune response by a variety of mechanisms. They can affect both innate and adaptive immunity through (a) cytokine production; (b) activation, proliferation, and function of $CD4^+$ and $CD8^+$ T cells;[13, 14] (c) immunoglobulin production and response of B cells;[15] (d) maturation of dendritic cells;[16] and (e) the function of natural killer (NK)[17] and NKT cells.[18] Possible mechanisms of Treg suppression have been suggested. Some studies have shown that cell-to-cell contact between Tregs and target cells is required for suppressive function.[19] In particular, TGFβ and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) surface molecules on Tregs inhibit conventional T-cell proliferation.[20, 21] Other studies have demonstrated that Tregs exert a suppressive effect through the production of immunosuppressive cytokines such as IL-10 and TGFβ.[22]

In response to chemotactic stimuli, Tregs migrate selectively to inflammatory sites via chemokine integrin receptors.[23] Attraction of Tregs to tumor sites by CCL17 or CCL22 through CCR4 has been shown in breast and gastric cancers.[24] In addition, a higher percentage of Tregs isolated from cancer patients express the proliferation marker Ki67,[24, 25] suggesting increased proliferation of Tregs in cancer patients. Higher Treg numbers observed in the peripheral blood and tumor sites of patients with solid and hematologic malignancies correlated to poor prognosis.[26] Conversely, clinical studies have demonstrated that depletion of Tregs in cancer patients can improve vaccine-mediated antitumor responses.[27]

We have previously demonstrated an increased suppression of $CD4^+CD25^-$ cell proliferation by Tregs isolated from PBMC of mCRPC patients compared to Tregs from healthy donors.[28] Miller *et al.* found elevated Treg numbers in the tumors and peripheral blood of prostate cancer patients and detected CCL22 chemokine in the secretions of primary prostate tumors, malignant ascites, and prostate cancer cell lines.[9] Despite this information about the relationship between prostate cancer and Tregs, the molecular

mechanisms underlying the influence of prostate carcinomas on Tregs are still unclear. In this study, we investigated Treg characteristics beyond suppressive function by comparing the genome-wide expression profiles of Tregs with similar suppressive activity from mCRPC patients and healthy donors.

Materials and Methods

Patients and healthy donors

A concurrent multicenter randomized phase II prostate cancer vaccine trial[29] at the National Cancer Institute (NCI), National Institutes of Health, enrolled 32 patients with mCRPC who had not received any radiotherapy or chemotherapy within 6 months prior to blood draw. Of these 32 patients, 17 were evaluable for this analysis. At enrollment, all patients had evidence of disease progression as defined by (a) new metastases on bone scan and/or (b) disease progression on CT scan, or (c) increased serum PSA as determined by PSA consensus criteria.[30] All patients signed a consent form approved by the NCI's Institutional Review Board. The median age of the 17 evaluable patients was 63 (range, 46–78). The median age of healthy donors ($n = 33$) was 49 (range, 23–76).

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by density gradient separation using CAPPEL LSM Lymphocyte Separation Medium (MP Biomedicals, Solon, OH). Isolated PBMCs were washed 3 times with PBS and cryopreserved in liquid nitrogen at 5×10^7 cells/ml in heat-inactivated human serum with 10% DMSO.

Isolation of Tregs

PBMCs were collected from mCRPC patients pre-vaccination. CD4^{+} T cells were negatively selected from PBMCs by magnetic separation (CD4^{+} T-Cell Isolation Kit II, Miltenyi Biotec Inc., Auburn, CA). The CD4^{+} T cells obtained were stained with FITC-conjugated anti-CD25 and PE-conjugated anti-CD127 (BD Biosciences, San Jose, CA). CD25^{high}CD127^{−} cells, representing the Treg population, were isolated by a FACSDiva flow cytometer (BD Biosciences), and cell-sorting data were analyzed by FACSDiva software (BD Biosciences).

Immunosuppression assay

CD4^{+}CD25^{−} T cells (1×10^4 cells/ml) were cultured alone or cocultured with Tregs (1×10^4 cells/ml) with 1 μg/ml of anti-CD3 plate-bound antibody (clone OKT3; eBioscience, San Diego, CA) and irradiated (3,500 rad) T cell-depleted PBMCs (1×10^5 cell/ml) in a 96-well flat-bottomed plate at 37oC and 5% CO_2. Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin (Mediatech) and 2 mM of L-glutamine (Mediatech) in 10% heat-inactivated human AB serum (Gemini Bio-Products, West Sacramento, CA) at a total volume of 200 μl/well. T-cell proliferation was measured by [3H]thymidine (PerkinElmer, Waltham, MA) incorporation pulsed on day 4 at 1 μCi (0.037 MBq)/well and quantified 16 hours later using a liquid scintillation counter (PerkinElmer). All experiments were done in triplicate. Proliferation of CD4^{+}CD25^{−} T cells without coculturing with CD4^{+}CD25^{high} Tregs was defined as 100% proliferation.

RNA preparation

Total RNA was extracted from CD4^{+}CD25^{high}CD127^{−} Tregs using TRIzol® Reagent (Invitrogen, Carlsbad, CA). Briefly, 800 μl of TRIzol® Reagent was added to each CD4^{+}CD25^{high}CD127^{−} Treg sample, ranging from 3×10^5 to 2×10^6 Tregs. Samples were

vortexed briefly and incubated at room temperature for 5 minutes. Chloroform (200 µl) was added to each sample and vortexed for 15 seconds. After incubation at room temperature for 3 minutes, samples were centrifuged at 12,000 xg at 4oC for 15 minutes. The aqueous phase (300 µl) was collected in a new tube and 300 µl of nuclease-free water was added to the organic phase for re-extraction. The aqueous phases were pooled and mixed with 100 µl of 1M Tris-HCl, pH 8.0, and 700 µl of 70% ethanol. The mixture was loaded into an RNeasy MinElute spin column (Qiagen, Valencia, CA) for RNA purification according to the manufacturer's instructions. RNA contents and integrity were monitored by NanoDrop (Thermo Scientics, Rockford, IL) and Agilent Bioanalzyer 2100 (Agilent Technologies, Palo Alto, CA).

Microarray hybridization

From each sample, 100 ng of total RNA was amplified using the Two-Cycle Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA) and MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions. Biotin-labeled cRNAs were hybridized to GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix). Hybridization signals were obtained using the GeneChip Scanner 3000 (Affymetrix). Microarray data were deposited into the Gene Expression Omnibus database of the National Center for Biotechnology Information under accession number GSE38043.

Expression array and gene network analysis

Raw gene expression data were quantile normalized by a robust multiarray average algorithm and analyzed in Partek Genomics Suite 6.4 (Partek, St. Charles, MO). Statistical analyses were performed on log 2-transformed data. Differential gene expression was evaluated using one-way ANOVA. Within the ANOVA model, linear contrasts were used to compare the Tregs from cancer patients and healthy donors. A step-up Benjamini false discovery rate (FDR) adjustment was used to adjust for multiple testing errors. FDR was set at < 10% to produce a list of significantly differentially expressed genes for each contrast described above. An expression cutoff of 3-fold was set to identify genes whose expression was significantly differentially regulated. The integrated gene network analysis on the gene set with significant expression changes was generated by Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com), with an FDR of 5%. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Functional analysis identified the biological functions and/or diseases most significant to the data set. Fischer's exact test was used to calculate a p value, determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. A p value of < 0.05 was considered significant.

NanoString nCounter gene expression analysis

Analysis of gene expression was performed as previously described.[31] Briefly, 10 µl of reporter CodeSet reagent, 5 µl of capture ProbeSet reagent and 10 µl of hybridization buffer (NanoString Technologies, Seattle, WA) were mixed, and 100 ng of total RNA was added to the mixture. Nuclease-free water was used to adjust the final volume of each hybridization assay to 30 µl. The RNA was hybridized to the probes at 65oC for at least 12 hours. After hybridization, excess probes were washed away by a 2-step magnetic bead-based purification procedure on the nCounter Prep Station (NanoString Technologies). Purified target/probe complexes were immobilized in the cartridge for data collection. Barcodes, which represent gene expression level, were counted in the nCounter Digital Analyzer.

Flow cytometry

Cryopreserved PBMCs were stained with APC-Cy7-conjugated anti-CD4, PE-Cy7-conjugated anti-CD25, PerCP-Cy5.5-conjugated anti-CD127 (BD Biosciences), APC-conjugated anti-FOXP3 (clone 236A/E7, eBioscience), and PE-conjugated anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for phenotypic characterization. One million PBMCs were resuspended in 100 μl of FACS sheath solution (BD Biosciences) and stained with the antibodies for 45 minutes at 4oC. Cells were fixed and permeabilized with FoxP3 staining buffer (eBioscience) according to the manufacturer's protocol, then labeled with anti-FoxP3 and anti-c-Jun or isotype control antibodies. Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences) and 100,000 events were acquired for each sample. Data were analyzed by FACSDiva software (BD Biosciences).

$CD4^+CD25^-$ cells were isolated with a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit according to the manufacturer's instructions (Miltenyi). One million cells were surface stained with PE-Cy7-conjugated anti-CD4 and APC-conjugated anti-CD25 (BD Biosciences), then fixed and permeabilized as described above. Cells were then stained with rabbit anti-human RGS1 and mouse anti-human DUSP1, rabbit anti-human c-Jun and mouse anti-human c-Fos, or isotype controls, then with PE-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG secondary antibodies (Novus Biologicals, Littleton, CO). Flow cytometry was performed on an LSRII and 30,000 events were collected. Data were analyzed by FlowJo5 software (Treestar Inc, Ashland, OR).

Statistical analysis

The D'Agostino-Pearson omnibus normality test was performed in each group of data to check for normal distribution. An unpaired t test was used to assess differences among the groups for normally distributed data; otherwise, the Mann-Whitney test was performed. $p < 0.05$ was considered statistically significant.

Results

In order to best assess differences in the gene expression profile due to the presence of prostate cancer, Tregs that were as functionally and phenotypically similar as possible were selected for comparison. A representative example of the Treg analysis performed is shown in Figure 1. Functional activity was determined by the ability of Tregs to suppress autologous $CD4^+CD25^-$ T-cell proliferation (Fig. 1a). $CD4^+CD25^-$ T-cell proliferation was not significantly different between healthy individuals and cancer patients ($p = 0.84$, Supporting Information Table 1). Tregs from 3 healthy donors were analyzed by flow cytometry to confirm that $CD4^+CD25^+CD127^-$ Tregs were $FoxP3^+$ (Fig. 1b). Samples from 3 healthy donors and 3 mCRPC patients with similar Treg suppressive activity (Table 1) were chosen for gene expression profiling using the GeneChip Human Genome U133 Plus 2.0 Array. A principal component analysis model was constructed from the microarray data (Fig. 2). In the principal component analysis model, a ball represents an individual's global gene expression pattern. Of the gene array probes compared between the 3 healthy donors and the 3 mCRPC patients, 73% matched this principal component analysis model. The gene expression signature of the individuals within each group was highly similar, as illustrated by the close proximity of the balls within the same group in the principal component analysis diagram. The clear separation of these 2 groups of samples represented distinct differences in gene expression patterns. More than 38,500 genes were analyzed. At a false discovery rate of < 10% ($p < 0.0038$), 2,081 transcripts were differentially expressed. A more biologically relevant 3-fold change in expression level was set as the criterion for identifying genes that were differentially expressed. There were 384 transcripts that satisfied this criterion; 198 transcripts were up-regulated and 186 were down-regulated in mCRPC

patients compared to healthy donors (Supporting Information Table 2). Five functional categories of biological processes, including cell cycle, cellular growth and proliferation, immune response, hematological system development and function, and cancer, were identified by Ingenuity Pathway Analysis (IPA) to be over-represented in Tregs from mCRPC patients (Supporting Information Table 3).

Microarray data showed increased expression of C-FOS (113-fold; $p = 0.00015$), C-JUN (27.2-fold; $p = 0.00017$), DUSP1 (9.1-fold; $p = 0.00032$), and RGS1 (3.5-fold; $p = 0.00288$) in Tregs from mCRPC patients compared to healthy donors. The differential expression patterns of these 4 genes were verified by NanoString technique in a separate experiment with Tregs isolated from 9 mCRPC patients and 10 healthy donors. The average level of suppressive activity of Tregs from the mCRPC patients was 46%, compared to 47% for the healthy donors. Expression levels of these 4 genes were significantly higher in Tregs obtained from mCRPC patients, with a fold change of 1.8x ($p = 0.0093$) for C-FOS, 1.6x ($p = 0.009$) for C-JUN, 1.5x ($p = 0.0122$) for DUSP1, and 1.5x ($p = 0.0407$) for RGS1 (Fig. 3ad).

Expression of c-Jun at the protein level was evaluated in Tregs from 8 mCRPC patients and 8 healthy donors by flow cytometry. Expression of c-Jun was significantly higher in Tregs from mCRPC patients ($8.08 \pm 0.5\%$; range, 5.8–10.3%) than in Tregs from healthy donors ($4.83 \pm 0.53\%$; range, 2.7–7.4%) (Fig. 3e). Expression of c-Jun in CD4$^+$CD25^{high}CD127 FoxP3$^+$ Tregs was also higher in mCRPC patients ($5.2 \pm 0.41\%$; range, 3.3–6.8%) than in healthy donors ($3.51 \pm 0.58\%$; range, 1.7–5.8%) (Fig. 3f). To determine if the up-regulation of these genes was specific to Tregs, we examined protein expression of c-Fos, c-Jun, DUSP1 and RGS1 in CD4+CD25− cells from healthy donors and mCRPC patients. There were no significant differences between healthy donors and mCRPC patients in the percentage of cells positive (Fig. 4) or mean fluorescence intensity (data not shown) for any of the 4 proteins.

The differential expression of these genes in patients' Tregs may imply a change in the rate of cell proliferation. Therefore, we investigated whether the frequency of Tregs in CD4$^+$ T cells is different between 17 mCRPC patients and healthy donors. The percentage of Tregs in CD4$^+$ T cells in mCRPC patients ($6.92 \pm 0.89\%$; range, 2.3–15.8%) vs healthy donors ($4.97 \pm 0.35\%$; range, 2.6–7.8%) was significantly higher ($p = 0.0492$) (Fig. 5a). However, there was no significant difference in the percentage of FoxP3$^+$ cells in the CD4$^+$CD25$^+$CD127 compartment between mCRPC patients and healthy donors (Fig. 5b).

Discussion

This study showed that 384 genes had a 3-fold difference in expression between Tregs isolated from mCRPC patients and healthy donors. These genes fell into 5 functional categories: cell cycle, cellular growth and proliferation, immune response, hematological system development and function, and cancer. As Tregs are known to be poorly proliferative under most conditions, it was surprising to find that a number of genes involved in proliferation and the cell cycle were up-regulated in the Tregs from mCRPC patients. Although this study was focused on gene expression differences in Tregs, these effects did not appear to be applicable to the entire CD4$^+$ population, as there was no significant difference in CD4$^+$CD25$^-$ cell proliferation between healthy donors and cancer patients. These findings were further supported by a significantly higher frequency of Tregs in the peripheral blood of mCRPC patients than healthy donors. The up-regulation of four genes of interest, C-FOS, C-JUN, DUSP1 and RGS1, was confirmed by quantitative analysis of mRNA. C-FOS, C-JUN and DUSP1 are involved in various processes of cellular proliferation, while RGS1 regulates chemokine receptor signaling.

C-FOS and C-JUN form the heterodimer AP-1, which participates in the control of cellular responses to stimuli that regulate cell proliferation and differentiation, as well as cell death.[32] In T cells, C-JUN activation is critical for T-cell receptor signaling, T-cell activation, development, differentiation, and proliferation, as well as lymphokine production.[33–37] Studies have shown that transcriptional activation of C-JUN is inhibited in anergic T cells,[38, 39] which is one of the features of Tregs. Another study demonstrated impairment of the promoter DNA-binding activity of AP-1 in natural Tregs upon stimulation with anti-CD3 and anti-CD28, in which FOXP3 played an inhibitory role in C-JUN-based AP-1 transcriptional activity by interacting with C-JUN in Tregs to maintain Treg unresponsiveness.[40] Increased levels of C-JUN and C-FOS in Tregs from mCRPC patients could probably counteract the inhibitory effect of FoxP3 on AP-1 DNA-binding activity when the FoxP3 expression level remained unchanged. Consequently, the anergic characteristics of Tregs may be abrogated, leading to proliferation in response to activation signals in mCRPC patients.

Dusp1 is a phosphatase shown to be active in the mitogen i.e., "mitogen-activated protein" kinase (MAPK) pathway. MAPKs belong to an evolutionarily conserved group of serine/theronine protein kinases. In response to various stimuli, including growth factors, cytokines, and stress conditions, MAPKs play important roles in proliferation, differentiation, apoptosis, or immune responses such as production of inflammatory molecules.[41] MAP kinase-specific phosphatases (MKPs) are regulators of MAPK in both innate and adaptive immune responses.[42] Although the role of DUSP1 (MKP-1) in Tregs has not been addressed, a study of $CD4^+$ and $CD8^+$ T cells of DUSP1 knock-out mice demonstrated that DUSP1 was required for T-cell activation and proliferation.[43] The increase in DUSP1 gene expression in this study, together with the increased expression of C-JUN and C-FOS, indicate an augmented activation and proliferation of Tregs in mCRPC patients.

Chemokine function is mediated through binding to chemokine receptors, thus stimulating the respective pathways for cellular migration.[44] The expression of chemokine receptors on lymphocytes therefore determines the migration of immune cells into specific tissues.[45] Chemokine receptors belong to the G-protein-coupled receptor family,[46] for which signaling pathways can be controlled by the regulator of G-protein signaling (RGS) proteins.[47] RGS proteins affect chemoattractant-stimulated cell migration and adhesion. A T-cell migration study demonstrated that Tregs migrated less than naïve $CD4^+$ T cells *in vivo*, and that this phenomenon correlated with higher expression of RGS1.[48] Up-regulation of RGS1 in Tregs from the peripheral blood may make them more responsive to tumor-derived chemokines, such as CCL22. This may contribute to the accumulation of Tregs reported at the site of prostate cancer tumors.[49]

A recent study demonstrated an increase in the frequency of $CD4^+CD25^{high}$ Tregs in the peripheral blood of renal cell carcinoma patients.[50] In the same study, customized oligonucleotide microarray was employed to quantify 350 genes associated with Treg phenotype. Results showed that 49 genes were significantly up- or down-regulated, with a 1.5-fold change in gene expression as compared to healthy controls. Although IPA was not used to classify biological functions, genes related to cell cycle and the immune system were identified. Higher levels of C-JUN, DUSP1, and RGS1 expression were also observed in $CD4^+CD25^{high}$ Tregs from renal cell carcinoma patients, indicating that up-regulation of these genes is a common feature of Tregs affected by different types of cancer and is not cancer tissue-specific. In this same study, increased expression of the antiapoptotic genes LGALS3 and LGALS1 was confirmed by real-time PCR. The authors hypothesized that increased antiapoptotic activity might promote Treg survival and lead to the accumulation of Tregs in the periphery. While our study focused on the increased expression of proliferation

genes, together these studies suggest a shift in the balance toward Treg survival in cancer patients that may counteract host antitumor immunity.

Our IPA showed that C-JUN and DUSP1 were associated with each other as well as with other genes (Supporting Information Fig. S1a). Gene expression profile data generated from this study thus suggest that differential gene expression in Tregs from cancer patients is not confined to a few genes, but a network of genes affecting each other through positive or negative feedback mechanisms. Mapping differentially expressed genes in *de novo* pathways by IPA showed genes related to T-cell cycle, cytokinesis, and expansion of Tregs were affected by, and had interactions with, master controlling genes such as p38, MAPK and NFkB (Supporting Information Fig. S1b). Moreover, our mapping of the differentially expressed genes to canonical TGFβ pathways ($p = 0.0349$) by IPA (Supporting Information Fig. S1c) revealed that the TGFβ pathway may also be altered in Tregs from mCRPC patients. Controlling these master genes through enhancers or inhibitors, such as abiraterone and sunitinib, may modulate the pathways of cellular proliferation or expansion of Tregs, thus decreasing Treg proliferation in cancer patients and increasing the efficacy of active immunotherapy.

One caveat of this study is that in comparing Tregs with similar suppressive ability from cancer patients and healthy donors the healthy donors chosen had relatively highly suppressive Tregs, which were not representative of the average. However, we felt that matching suppressive abilities was necessary to focus on the effects of prostate cancer on the Tregs. Another caveat is the limited amount of PBMC available from patients. The small percentage of Tregs recovered from isolation prevented functional studies from being performed on the patient samples in this study. In future studies, we plan to investigate several more of the differentially regulated genes found in this study, perform functional studies, and determine the effect of a cancer vaccine on the gene expression profile of Tregs from metastatic prostate cancer patients.

The gene expression studies reported here from Tregs of mCRPC patients indicate that several biological processes, including proliferation (through C-JUN, C-FOS, and DUSP1), lymphocyte migration (through RGS1), cell cycle, cell growth and proliferation, immune response, and hematological system development and function, were affected by the presence of the prostate cancer. We also found that mCRPC patients have an increase in the percentage of Tregs from peripheral blood. Taken together, these data suggest that the mCRPC tumors can affect Tregs at the molecular level, perhaps by influencing their proliferative and migratory abilities. An accumulation of Tregs at the tumor site could dampen the anti-tumor response and potentially result in tumor escape from immune surveillance. These studies thus support the rationale of a strategy that combines a therapeutic cancer vaccine or other forms of immunotherapy with an agent that targets Treg proliferation; this may lead to improved clinical outcomes for patients with prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AP-1	activator protein 1
C-FOS	FBJ murine osteosarcoma viral oncogene homolog
C-JUN	JUN oncogene
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DUSP1	dual specificity phosphatase 1
FDR	false discovery rate
FoxP3	forkhead box P3
IPA	Ingenuity Pathway Analysis
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MKP	MAP kinase-specific phosphatase
mCRPC	metastatic castration-resistant prostate cancer
NCI	National Cancer Institute
NK	natural killer
NKT	natural killer T
PBMC	peripheral blood mononuclear cell
PCA	principal component analysis
RGS1	regulator of G-protein signaling 1
Treg	regulatory T cell

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Novelty

This is the first study to compare the global gene expression patterns of CD4$^+$CD25^{high}CD127$^-$FoxP3$^+$ regulatory T cells (Tregs) from healthy donors and metastatic castration-resistant prostate cancer (mCRPC) patients. We found that genes related to cell cycle, cellular proliferation, and immune responses were differentially expressed in CD4$^+$CD25^{high}CD127$^-$FoxP3$^+$ Tregs from prostate cancer patients. We confirmed the up-regulation of C-FOS, C-JUN, DUSP1, and RGS1 gene expression in Tregs from prostate cancer patients, and found that this up-regulation correlated with an increased frequency of CD4$^+$CD25^{high}CD127$^-$FoxP3$^+$ Tregs. These results suggest that the alterations observed in gene expression profiles of Tregs in mCRPC patients may contribute to tumor escape from host immune surveillance.

Impact

This study contributes to our knowledge of tumor-induced immune suppression in prostate cancer patients. These data show that Tregs from mCRPC patients have an up-regulation in genes involved in proliferation and migration, not seen in Tregs from healthy donors. This study indicates that inhibiting the influence of prostate cancer on Tregs function may enhance the efficacy of therapeutic cancer vaccines.
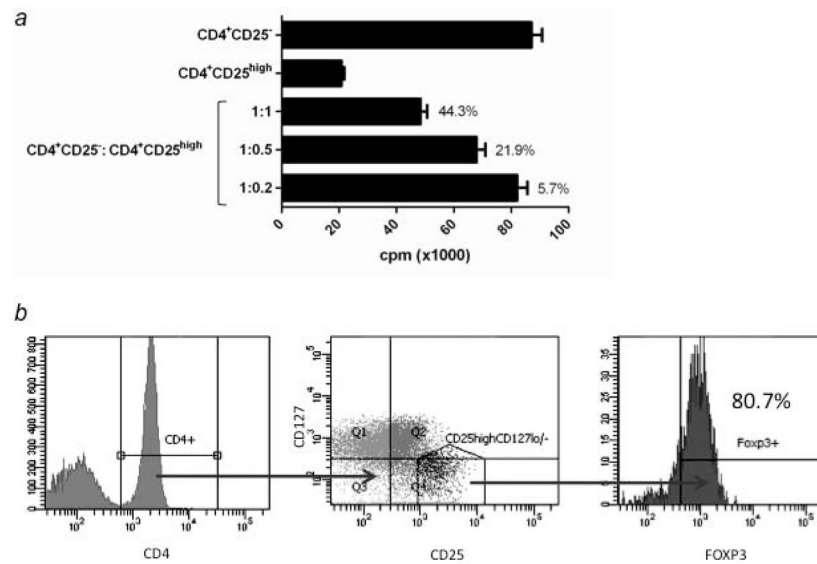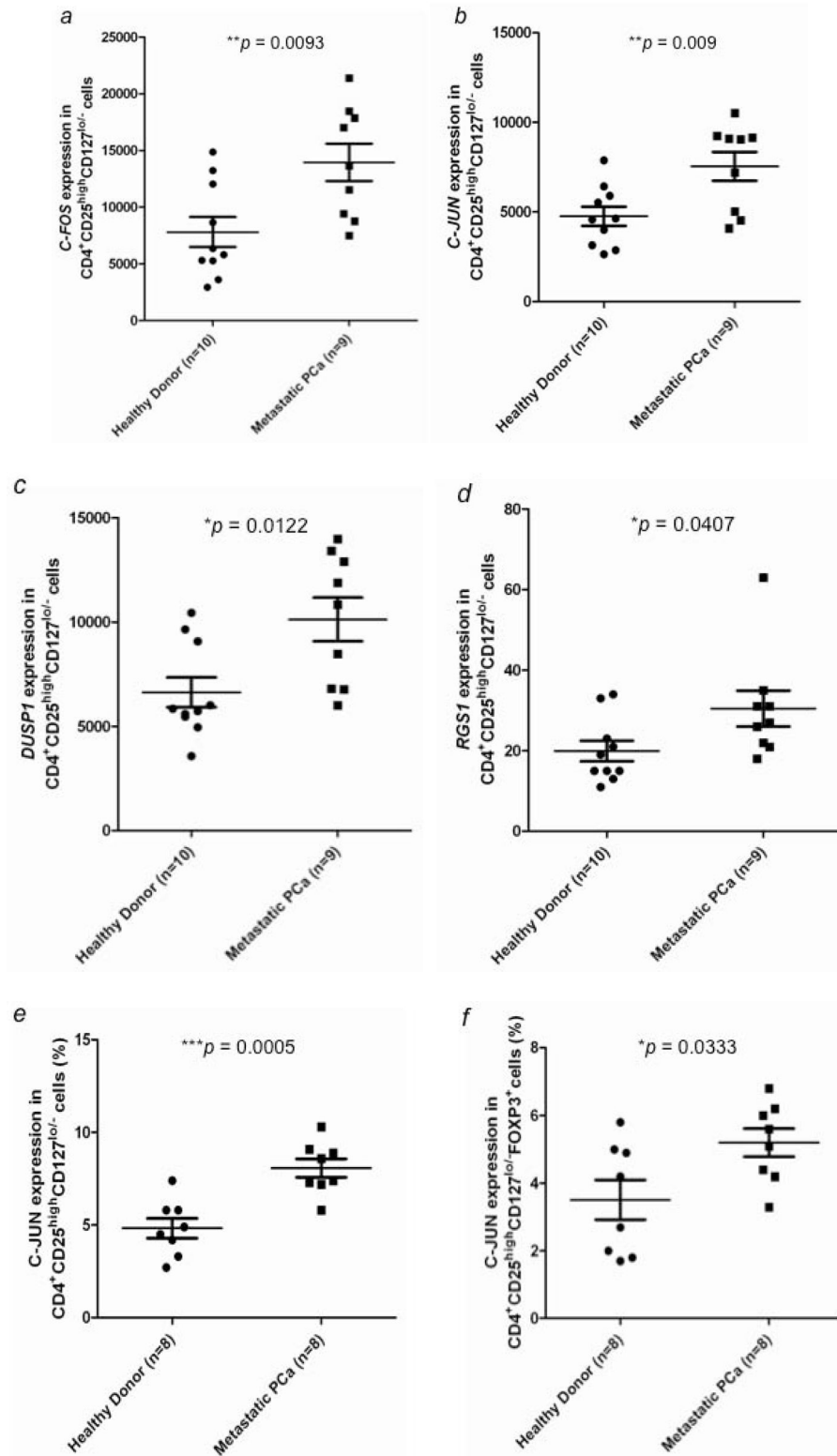
Figure 1.
Analysis of suppressive function of CD4+CD25high Tregs. (a) Example of suppression of CD4+CD25− T-cell proliferation by CD4+CD25high Tregs in a healthy donor. Isolated effectors (CD4+CD25) and Tregs (CD4+CD25high) were cultured separately, or cocultured at 3 different ratios (1:1, 1:0.5, and 1:0.2) upon stimulation with anti-CD3 and irradiated T cell-depleted PBMCs. Experiments were performed in triplicate. Columns: average count per minute (cpm); bars: SD. Percentages indicate level of inhibition of CD4+CD25− T-cell proliferation. (b) Expression of FoxP3 in CD4+CD25+CD127lo/− Tregs in a healthy donor. PBMCs from 3 healthy donors were analyzed by flow cytometry after cell surface staining with APC-Cy7-conjugated anti-CD4, PE-Cy7-conjugated anti-CD25, PerCP Cy5.5-conjugated anti-CD127, and intracellular staining with APC-conjugated FoxP3. The figure represents results from 1 healthy donor and similar results were also observed in other healthy donors and prostate cancer patients. Percentage indicates proportion of cells expressing FoxP3 in the CD4+CD25+CD127lo/− Treg population.
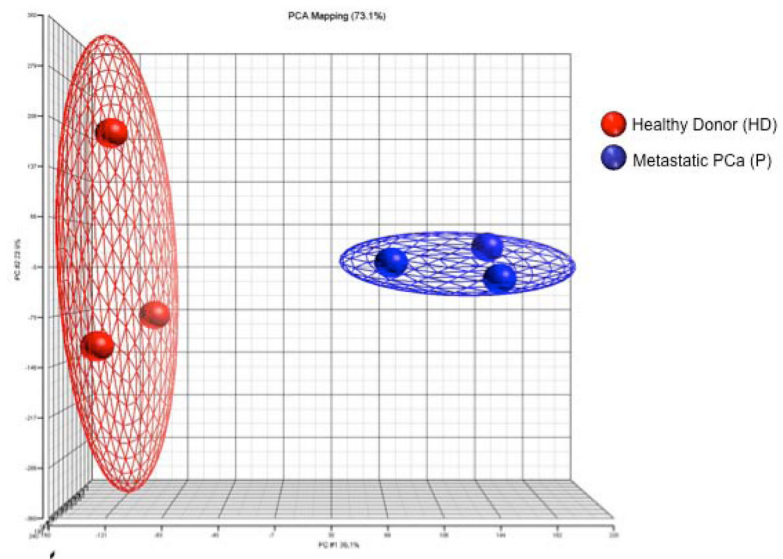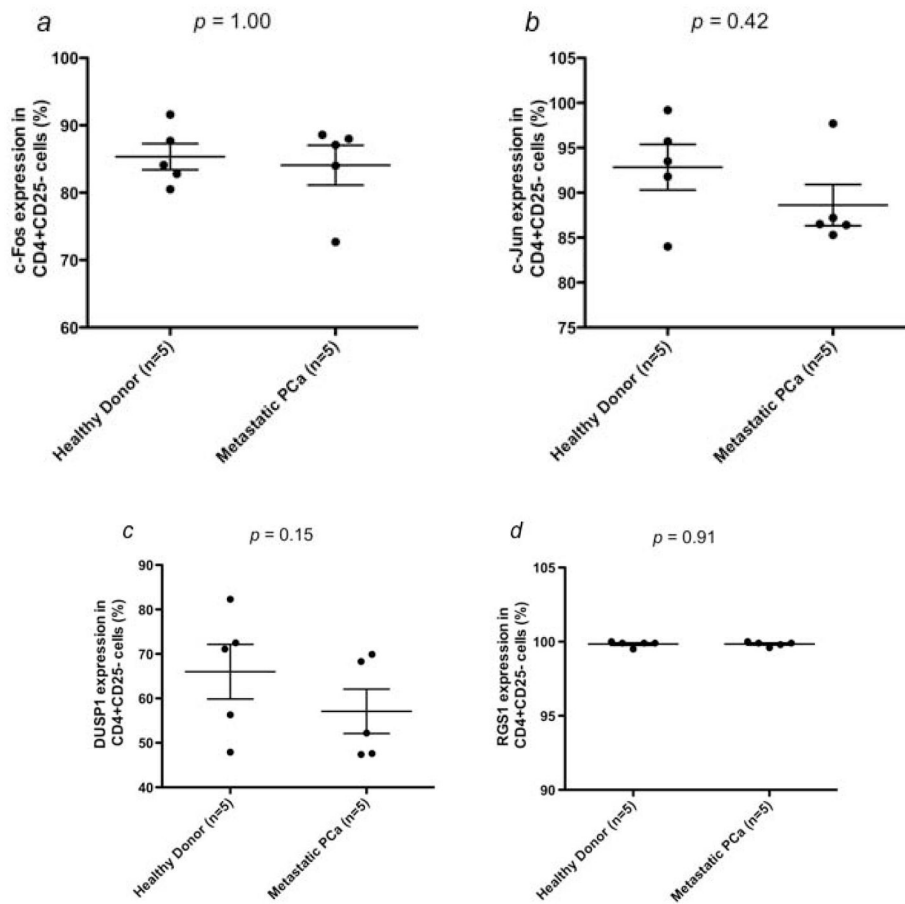
Figure 2.
PCA model showing the global gene expression difference between healthy donors (red balls; $n = 3$) and mCRPC patients (blue balls; $n = 3$).

Figure 3.
Verification of gene expression by NanoString analysis and flow cytometry. Expression of (a) C-FOS, (b) C-JUN, (c) DUSP1, and (d) RGS1 in $CD4^+CD25^{high}CD127^{lo/-}$ Tregs from
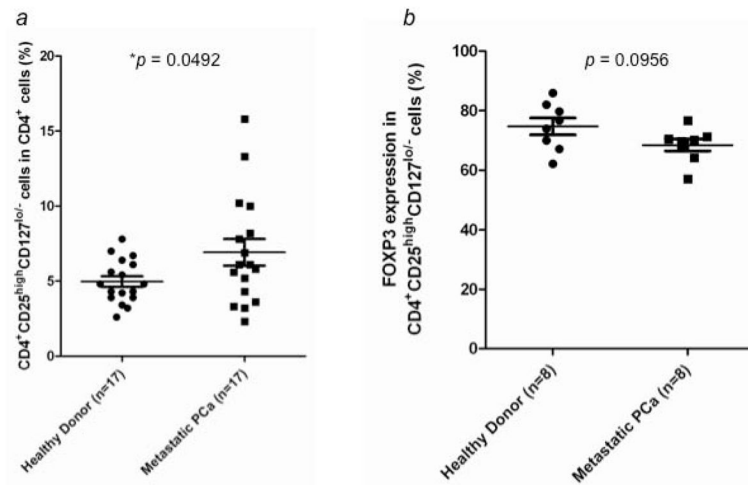
healthy donors ($n = 10$) and mCRPC patients ($n = 9$) was analyzed by NanoString analysis. C-JUN protein expression in CD4$^+$CD25^{high}CD127$^{lo/−}$ Tregs was further examined by flow cytometry analysis. (e) Percentage of c-Jun$^+$ cells from CD4$^+$CD25^{high}CD127$^{lo/−}$ Tregs was compared between healthy donors ($n = 8$) and mCRPC patients ($n = 8$). (f) Percentage of c-Jun$^+$ cells in CD4$^+$CD25^{high}CD127$^{lo/−}$ Foxp3$^+$ Tregs. A statistically significant difference was seen between healthy donors and mCRPC patients in all analyses. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Figure 4.
Differential gene expression between mCRPC patient and healthy donor Tregs is not applicable to all CD4^{+} cells. Expression of (a) c-Fos, (b) c-Jun, (c) DUSP1 and (d) RGS1 was examined at the protein level in CD4^{+}CD25^{−} cells isolated from mCRPC patients (n = 5) and healthy donors (n = 5). No significant differences were seen in the percentage of positive cells between the 2 groups (p > 0.05).

Figure 5.
Percentage of CD4$^+$CD25^{high}CD127$^{lo/-}$ Tregs in peripheral blood of healthy donors ($n = 17$) and mCRPC patients ($n = 17$). (a) Levels of CD4$^+$CD25^{high}CD127$^{lo/-}$ Tregs are presented as a percentage of total CD4$^+$ T cells. A statistically significant difference was observed between the 2 groups ($p < 0.05$). (b) Percentage of FoxP3 expression in CD4$^+$CD25^{high}CD127$^{lo/-}$ Tregs from healthy donors ($n = 8$) and mCRPC patients ($n = 8$). No statistically significant difference was observed between the 2 groups ($p > 0.05$).

Table 1

Suppressive activity of CD4$^+$CD25^{high} Treg samples

Samples	CD4$^+$CD25^{high} Treg suppressive activity
HD1	44.3%
HD2	44.1%
HD3	56.1%
PCa 1	44.0%
PCa 2	55.8%
PCa 3	51.7%

Results are expressed as % of suppressive activity of CD4$^+$CD25^{high} Treg against the proliferation of CD4$^+$CD25$^-$ effector T-cell.

HD = healthy donor; PCa = metastatic prostate cancer patient.