Androgen-Regulated Expression of Arginase 1, Arginase 2 and Interleukin-8 in Human Prostate Cancer

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Abstract

Background: Prostate cancer (PCa) is the most frequently diagnosed cancer in North American men. Androgen-deprivation therapy (ADT) accentuates the infiltration of immune cells within the prostate. However, the immunosuppressive pathways regulated by androgens in PCa are not well characterized. Arginase 2 (ARG2) expression by PCa cells leads to a reduced activation of tumor-specific T cells. Our hypothesis was that androgens could regulate the expression of ARG2 by PCa cells.

Methodology/Principal Findings: In this report, we demonstrate that both ARG1 and ARG2 are expressed by hormone-sensitive (HS) and hormone-refractory (HR) PCa cell lines, with the LNCaP cells having the highest arginase activity. In prostate tissue samples, ARG2 was more expressed in normal and non-malignant prostatic tissues compared to tumor tissues. Following androgen stimulation of LNCaP cells with 10 nM R1881, both ARG1 and ARG2 were overexpressed. The regulation of arginase expression following androgen stimulation was dependent on the androgen receptor (AR), as a siRNA treatment targeting the AR inhibited both ARG1 and ARG2 overexpression. This observation was correlated in vivo in patients by immunohistochemistry. Patients treated by ADT prior to surgery had lower ARG2 expression in both non-malignant and malignant tissues. Furthermore, ARG1 and ARG2 were enzymatically active and their decreased expression by siRNA resulted in reduced overall arginase activity and l-arginine metabolism. The decreased ARG1 and ARG2 expression also translated with diminished LNCaP cells cell growth and increased PBMC activation following exposure to LNCaP cells conditioned media. Finally, we found that interleukin-8 (IL-8) was also upregulated following androgen stimulation and that it directly increased the expression of ARG1 and ARG2 in the absence of androgens.

Conclusion/Significance: Our data provides the first detailed in vitro and in vivo account of an androgen-regulated immunosuppressive pathway in human PCa through the expression of ARG1, ARG2 and IL-8.

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Androgens, ARG1, ARG2 and IL-8

in human PCa [6] and its inhibition, concomitant with iNOS, increases the activation of tumor-infiltrating lymphocytes (TILs) [7]. While the immunosuppressive properties of arginases through the metabolism of l-arginine are well documented (reviewed in [8]), the regulation of human arginase expression, however, is currently undefined.

Androgens are known to have immunosuppressive properties, which is illustrated by the intra-prostatic inflammation following ADT [9,10]. Gene expression analyses and murine studies suggest that androgens regulate the expression of ARG2 and other enzymes of the polyamine pathway [11,12,13]. Thus, considering the fundamental roles of androgens in prostate carcinogenesis and in the sculpting of the prostate’s microenvironment, we evaluated whether androgens could regulate the expression of arginases by PCa cells in vitro and in vivo.

In this study, we report that PCa cell lines express both functionally active ARG1 and ARG2. Interestingly, hormone sensitive (HS) and hormone refractory (HR) tissues expressed less ARG2 than non-malignant tissues. In the HS LNCaP cell line, androgen stimulation led to the increased expression of both ARG1 and ARG2 in an androgen receptor (AR) dependent manner. This androgen-regulated expression was also observed in the primary tumor of ADT-treated patients who expressed less ARG2 in both the non-malignant tissues adjacent to the tumor and the tumor tissues compared to control patients. Finally, we discovered that IL-8 was also regulated by androgens under the control of the AR, and participated in the regulation of ARG1 and ARG2 expression. Altogether, our data provides the first detailed in vitro and in vivo account of an androgen-regulated immunosuppressive pathway in human PCa.

Results

ARG1 and ARG2 expression in PCa

We first evaluated arginases expression from PCa cell lines and clinical samples. Our data demonstrate that PCa cell lines express both ARG1 and ARG2. Gene expression analyses by qPCR demonstrated that ARG1 mRNA was more abundant in the 22Rv1 cell line (Figure 1A), while ARG1 protein was slightly more expressed by the two HR PCa cell lines (Du145 and PC3) than in the LNCaP cells (Figure 1B, bottom panel). ARG1 protein expression did not correlate with the gene expression analysis suggesting a possible post-transcriptional regulation. As for ARG2 expression, the LNCaP cell line expressed the highest levels of ARG2 mRNA (Figure 1A). Low ARG2 mRNA expression was detected in the two HR cell lines DU145 and PC3 compared to the two HS PCa cell lines. ARG2 protein abundance correlated with the qPCR results with LNCaP cells expressing significantly more ARG2 than the other three cell lines (Figure 1B, bottom panel). Furthermore, LNCaP cells had the highest arginase activity suggesting that ARG2 is the predominant enzyme with regards to arginase activity of PCa cells (Figure 1B, top panel).

Expression of the ARG2 protein was evaluated in clinical samples by immunohistochemistry on three different tissue microarrays (TMAs) regrouping prostate samples from a cohort of 99 PCa patients and 50 normal prostate obtained from autopsies. We did not evaluate ARG1 protein expression as, in our hands, anti-ARG1 antibodies tested were not suitable for immunohistochemistry on archived formalin-fixed paraffin-embedded tissues. We observed that ARG2 expression was restricted to the prostate epithelium and absent from the stroma (Figure 1C). ARG2 was statistically significantly less expressed in tumor tissues compared to normal (p<0.001, Mann-U), to non-malignant normal adjacent (p<0.01, Mann-U) and to prostate intraepithelial neoplasia (PIN) tissues (p<0.001, Mann-U) (Figure 1D). HR tissues also expressed less ARG2, although only significantly different from PIN tissues (p = 0.033, Mann-U). There was no correlation between ARG2 expression within the normal adjacent and tumor tissues (Table S1). Finally, we evaluated if the ARG2 expression correlated with clinicopathological parameters such as Gleason Score, pre-operative prostate specific antigen (PSA) levels and biochemical recurrence. Our results show that ARG2 expression within the normal adjacent tissue inversely correlated with vesicle seminal invasion (Table S1). Altogether, these in vitro and in vivo data demonstrate the differential expression of ARG1 and ARG2 between various stages of PCa progression, independently of the HR status.

Androgen-regulated expression of ARG1 and ARG2

The differential expression of ARG1 and ARG2 between the HS and HR PCa cell lines led us to investigate the regulatory roles of androgens in arginase expression. To do so, we evaluated arginases gene and protein expression following androgen stimulation. ARG1 mRNA expression was not statistically significantly upregulated in either LNCaP or 22Rv1 cell lines following R1881 stimulation (Figure 2A). However, in LNCaP cells, ARG2 mRNA expression was increased at 48 hours (p = 0.002, Mann-U) and at 72 hours (p = 0.016, Mann-U) following the R1881 stimulation (Left panel, Figure 2B). The overexpression of ARG2 in 22Rv1 was not statistically significant (p = 0.248, Mann-U) (Right panel, Figure 2B). In fact, ARG2 expression correlated with the higher androgen sensitivity of LNCaP cells compared to 22Rv1 (Figure S1A). As such, LNCaP cells were used for further experiments. Corroborating the PCR data, Western blots from LNCaP cells demonstrated that the R1881 stimulation increased ARG2 protein expression (Figure 2C). Interestingly, although no significant changes were observed in ARG1 mRNA expression in LNCaP cells treated with R1881, ARG1 protein expression was significantly increased. We did not observe any increases in ARG1 or ARG2 protein expression in DU145 and PC3 stimulated with 10 nM of R1881 (Figure S1B).

The AR is implicated in ARG1 and ARG2 expression

As our results suggest that androgens regulate arginase expression, we evaluated the contribution of the AR. We inhibited AR activity with the non-steroidal anti-androgen bicalutamide (Casodex) (Figure 2D). We noted a decreased expression of ARG1 with the highest concentration (40 µM) of bicalutamide following R1881 stimulation. The androgen induction of ARG2 was not blocked, even at the highest concentration of bicalutamide. As previously documented [14], we observed that bicalutamide had AR-agonist activity in LNCaP cells cultured in the absence of androgens. There was an R1881-independent induction of PSA and ARG2 expression in LNCaP cells stimulated with 20 µM and 40 µM of bicalutamide in the absence of androgens. In this same condition, bicalutamide caused a decrease in ARG1 expression. These results suggest that ARG1 expression may be more sensitive to AR inhibition than ARG2, whose expression was induced by the anabolic effect of the AR inhibitor.

We decided to further inhibit the AR by blocking the AR expression in LNCaP cells using siRNA. The presence of siRNA against the AR resulted in a significant inhibition of AR expression and in a reduced PSA expression following R1881 stimulation (Figure 2E). Both the ARG1 and ARG2 induction following R1881 stimulation were inhibited by the siRNA treatment, which translated in the absence of an upregulation in arginase activity (Figure 2F). These results suggest that the AR regulates the
expression of ARG1 and ARG2 in vitro and that ARG1 and ARG2 have a different sensitivity to AR inhibition.

**Diminished ARG2 expression in PCa patients following ADT**

Based on our in vitro data, we hypothesized that androgens might modulate ARG2 protein expression in PCa patients as well. We observed that, compared to control cancer patients (surgery only), ADT-treated patients (ADT prior to surgery) had significantly lower ARG2 expression in both the non-malignant tissues adjacent to the tumor (46.4 vs 23.5 relative units; p<0.001, Mann-U) and the tumor tissues (41.7 vs 31.5 relative units; p<0.01, Mann-U) (Figure 3A). We also observed that androgen deprivation in vitro could decrease ARG2, but not ARG1 protein expression, in LNCaP and 22RV1 cells cultured for seven days in the absence of androgens (Figure 3B). Taken together, these results suggest that androgens regulate the expression of ARG2 in vivo in PCa patients as ADT reduces ARG2 expression.

**ARG1 and ARG2 are metabolically active**

To evaluate whether ARG1 and ARG2 expressed by LNCaP cells were metabolically active, we inhibited the expression of either ARG1 or ARG2 by siRNA. Compared to a siCTRL, both siRNA significantly inhibited ARG1 or ARG2 expression.
Figure 2. Androgen-regulated expression of ARG1 and ARG2. A-B) LNCaP cells (left panels) and 22Rv1 (right panels) were stimulated over a period of 72 hours with 10 nM R1881 following a 72 hour incubation period in charcoal-stripped media and the gene expression of A) ARG1 and B) ARG2 analyzed by qPCR. Control (light gray bars) and R1881-stimulated (black bars). *Statistically significant difference (p < 0.05, Mann-U). Mean relative expression (n = 4) with standard error (error bars). C) Increased protein expression of both ARG1 and ARG2 following R1881 stimulation by Western blot. LNCaP cells were stimulated with 10 nM R1881 as previously described. PSA served as positive control. Representative experiment,
Inhibition of AR activity by bicalutamide (Casodex). LNCaP cells were stimulated with R1881 for 72 hrs as previously described in the presence of increasing doses of bicalutamide (0, 10, 20 and 40 μM). ARG1 and ARG2 expression levels were evaluated by Western blot. Representative experiment is shown, (n = 6). D) Inhibition of AR activity with bicalutamide (Casodex). LNCaP cells were stimulated with R1881 for 72 hrs as previously described in the presence of increasing doses of bicalutamide (0, 10, 20 and 40 μM). ARG1 and ARG2 expression levels were evaluated by Western blot. Representative experiment is shown, (n = 6). Note the agonist effect of bicalutamide in the absence of R1881 illustrated by an increased PSA and ARG2 expression. E) Inhibition of AR expression by siRNA. LNCaP cells were transfected as previously described. AR, ARG1 and ARG2 expression levels were evaluated by Western blot. Representative experiment is shown, (n = 4). F) Arginase activity was quantified in mU/mg of proteins. LNCaP cells were transfected with siCTRL (light gray bars) or siAR (black bars) and then stimulated with R1881 for 72 hrs as previously described. Representative experiment, (n = 3).

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IL-8 induction of ARG1 and ARG2 expression

As arginases are well-documented to participate in the sculpting of the tumor immunological microenvironment [15] and that cytokines are known to induce arginase expression in murine models [16], we assessed whether cytokines could also regulate arginase expression in human PCa. The cytokine expression profile of LNCaP cells stimulated with 10 nM of R1881 was qualitatively evaluated using a Proteome Profiler cytokine array (R&D Systems) (Figure 5A). The proteomic data illustrated that the R1881-stimulated LNCaP cells had increased expression of IL-8 and Serpin E1 (Figure S2A). We further investigated the role of IL-8 in arginase expression as IL-8 has been recently linked to the expression of androgen-regulated genes in PCa [17]. We thus quantified the elevated expression of IL-8 in LNCaP cells following R1881 expression (Figure 5B). This IL-8 induction was dependent on the AR as the inhibition of AR expression by siRNA prevented IL-8 secretion following androgen stimulation (Figure 5C). We then evaluated whether inhibition of IL-8 could diminish ARG1 and ARG2 expression following R1881 stimulation. Using a siRNA against IL-8, we could significantly diminish

Figure 3. Reduced ARG2 expression following ADT. A) Analysis of androgen-regulated ARG2 expression in PCA patients by immunohistochemistry. Control patients (light gray bars, n = 40) and ADT-treated patients (black bars, n = 35). B) Decreased ARG2 protein expression in the absence of androgens in vitro determined by Western blot. Ran served as loading control. LNCaP cell lines (LNCaP, 22Rv1, DU145 and PC3) were maintained in RPMI 10% FBS or in RPMI supplemented with 10% charcoal stripped FBS for 7 days (n = 3). Note that ARG1 expression did not vary but that ARG2 was reduced in LNCaP and 22Rv1 cells in the absence of androgen.

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Figure 4. ARG1 and ARG2 are metabolically active. LNCaP cells were transfected with either a siCTRL or a cocktail of three siRNA against the ARG1 or ARG2. Post-transfection (24 hours), cells were plated in charcoal-stripped serum supplemented media for 72 hours and then stimulated for 72 hours with 10 nM R1881. A) siRNA inhibition of ARG1 and ARG2 expression was evaluated by Western Blot. Representative experiment is shown, (n = 4). B) Decreased arginase activity following transfection with siARG1 or siARG2 in LNCaP cells. The corresponding Western blot is shown in the
IL-8 secretion (Figure 5D). This reduced IL-8 production was associated with a reduction of ARG1 and ARG2 24 hrs following R1881 stimulation (Figure 5E). The treatment of LNCaP cells with siIL-8 also translated to a decrease in arginase activity (Figure S2B). Finally, we stimulated androgen-deprived LNCaP cells with increasing concentration of exogenous IL-8 for 72 hrs and monitored the expression of ARG1 and ARG2. By Western blot analysis, we observed that both 50 ng/ml and 100 ng/ml of IL-8 induced the expression of ARG1 and ARG2 when compared to control LNCaP cells (Figure 5F). The decrease in ARG1 and ARG2 protein expression with 250 ng/ml of IL-8 correlated with IL-8 induced cellular toxicity. We also observed an induction of ARG2, but not ARG1, gene expression after a 24 hr stimulation (Figure S2C). Finally, as it was previously reported that phenol red could activate the AR [18], we also stimulated LNCaP cells with IL-8 in phenol red-free RPMI media. These control experiments demonstrated that IL-8-dependent induction of ARG1 and ARG2 could occur in the absence of phenol red (Figure S2D). Taken together, the data clearly shows that androgens regulate the expression of IL-8, which on its own can induce the expression of both ARG1 and ARG2.

**Discussion**

A more thorough understanding of the prostate immunological microenvironment mechanisms may improve the clinical efficacy of current immunotherapies against PCa. We and others have shown that ADT leads to drastic changes in the prostate immunological microenvironment [9,10]. The arginase pathway participates in the development of an immunosuppressive state within the primary tumor of PCa patients [7]. However, the regulation of arginase expression by PCa cells remains undefined.

In this report, we observed that androgens induced the expression of both ARG1 and ARG2 in HS PCa cell lines. The AR was implicated in this regulation as both bicalutamide and siAR transfection prevented ARG1 and ARG2 overexpression following R1881 stimulation. Reciprocally, androgen deprivation and ADT reduced ARG2 expression *in vitro* and in the primary tumor of PCa patients, respectively. LNCaP cells expressed enzymatically functional ARG1 and ARG2 which, once their protein expression was inhibited, caused a decrease in cellular proliferation and in their immunosuppressive potential. Finally, we showed that IL-8 was also regulated by R1881 and could stimulate the expression of ARG1 and ARG2 independently of androgen.

Altogether, our results provide the first mechanistic evidence of an androgen-driven immunosuppressive pathway in PCa through the expression of ARG1, ARG2 and IL-8 by PCa cells. We demonstrate that PCa cells express both ARG1 and ARG2. ARG2 was predominantly expressed by HS PCa cell lines and by non-malignant prostate tissues. These results corroborate published data describing a lower ARG2 expression in androgen-insensitive PCa cell lines (DU145 and PC3) and in the tumor and HR tissues of PCa patients [6,19]. However, to our knowledge, we are the first group to study the expression of ARG1 by PCa cells and define mechanistic consequences leading to its androgen-regulated induction. Similar to ARG2, inhibition of ARG1 expression led to decreased tumor cell proliferation, reduced l-arginine metabolism and reduction of their immunosuppressive potential. Based on the protein expression (Figure 1B, bottom panel) and on the arginase activity of PCa cells (Figure 1B, top panel), our data suggest that ARG2 may nonetheless have a more prominent role than ARG1 in the arginase activity potential of PCa cells [20].

Furthermore, our data showed that ARG1 and ARG2 were differentially regulated by androgens. Contrary to ARG2 gene and protein expression, we clearly demonstrated that the gene and protein expression of ARG1 did not correlate. This suggests that androgens may influence a post-transcriptional regulation of ARG1 as it was previously reported in xenopus [21] and in yeast models [22]. Since ARG2 expression is localized to mitochondria, we evaluated whether cellular proliferation independent of androgens could induce ARG2 expression in LNCaP cells. In a proliferation assay with EGF instead of R1881, no ARG2 induction was observed (data not shown). Collectively, our results reveal that, although both induced by R1881, the signaling pathways leading to ARG1 and ARG2 expression differ for the two enzymes and need to be further examined.

The implication of an androgen-regulated expression of ARG1 and ARG2 in prostate carcinogenesis requires further investigation. Arginase expression and polyamine synthesis are elevated in PCa [23,24] and associated with tumor grade [25]. A high arginase activity correlates with increased proliferation of breast cancer [26], colon cancer [27] and kidney cell lines [28]. However, we observed that tumor or HR tissues express less ARG2 than non-malignant tissues. It is possible that tumor cells do not acquire the expression of these enzymes as a mean to further exploit their immunosuppressive potential, an aspect associated with tumor progression. In fact, since the prostate is the organ with the highest polyamine production, arginase expression by prostate cells may precede the development of cancer, as polyamine production is essential for the proliferation of prostate cells. Thus, the immunosuppressive advantage gained by prostate cells may be secondary to the proliferative role played by the arginases. From our data and that of others, we hypothesize that arginase may be implicated in the earlier hormone-sensitive stages of prostate carcinogenesis by promoting cancer cell proliferation and the development of an androgen-regulated immunosuppressive environment.

Finally, we observed that IL-8 was upregulated following androgen stimulation and could induce the expression of ARG1 and ARG2. IL-8 mediates its effects through the activation of two high-affinity G-protein coupled receptors, CXCR1 and CXCR2 [29], both of which are expressed by LNCaP cells [30,31]. It is important to note that expression of ARG1 and ARG2 following
Androgens induced Interleukin-8, which in turn promotes ARG1 and ARG2 expression. Evaluation of the cytokine expression profile of LNCaP cells following R1881 stimulation. A) Conditioned media of LNCaP cells stimulated as previously described were analyzed with a Proteome Profiler™ (R&D Systems). B) Conditioned media of LNCaP cells stimulated over time with either ethanol control (light gray bars) and R1881 (black bars) were analyzed for the production of IL-8 by ELISA. The representative experiment showed was performed with the same conditioned media used for the Proteome Profiler analysis in Sa, (n = 3). C) Quantification of IL-8 secretion by LNCaP cells transfected with siAR and stimulated with R1881 as previously described. Representative experiment is shown, (n = 3). D) Quantification of IL-8 secretion by LNCaP cells transfected with siIL-8 and stimulated with R1881 as previously described. Representative experiment is shown, (n = 3). For Sb and Sc, there was no IL-8 secretion detected in the absence of R1881 stimulation. E) Expression of ARG1 and ARG2 in LNCaP cells following transfection of siIL-8 and R1881 stimulation for 24 hours. Representative experiment is shown, (n = 3). F) LNCaP cells were plated in charcoal-stripped serum supplemented media for 72 hours and for 24 hours in serum-free RPMI. Cells were then stimulated for 72 hours with 10 nM R1881 or with 50, 100 or 250 ng/ml of IL-8 in serum-free RPMI. ARG1 and ARG2 expression levels were detected by Western blot. Representative experiment, (n = 3). Note the induction of both ARG1 and ARG2 at 50 and 100 ng/ml of IL-8 concentration in the absence of R1881.

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IL-8 stimulation was not as substantial as with R1881 stimulation suggesting that other androgen-regulated pathways could be involved. Altogether, this is the first indication that the expression of IL-8 is regulated by androgens and that arginases can be regulated by cytokine in human cancer cells.

Conclusion

Our data demonstrate that androgens regulate the expression of both ARG1 and ARG2 in HS PCa cell lines and in PCa patients in an AR-dependent manner. ARG1 and ARG2 are enzymatically active and their inhibition results in reduced l-arginine metabolism, cell growth and immunosuppressive potential. We found that IL-8 secreted by LNCaP cells was also regulated by androgens and could on its own promote the expression of ARG1 and ARG2. Collectively, the results presented in this report suggest that androgens actively participate in the development of an immunosuppressive microenvironment within the prostate through the expression of ARG1 and ARG2. A better understanding of the expression of immunosuppressive pathways at specific stages of PCa progression may eventually provide new insights for improving current immunotherapeutic strategies.

Materials and Methods

Cell Culture

LNCaP, 22Rv1, DU145 and PC3 cell lines were obtained from ATCC (MD, USA). All cell lines were maintained as previously described by our group [32]. For R1881 stimulation, cells were plated at 600,000 cells per 60 mm petri dish and incubated for an initial 72 hours in 10% (v/v) charcoal-stripped fetal calf serum (FCS)-supplemented RPMI 1640, which eliminates all steroid hormones from the serum. Afterwards, the cells were washed with PBS and cultured in fresh 10% charcoal-stripped FCS-supplemented RPMI 1640, with either 10 nM R1881 or ethanol (control) [32]. As control experiments, phenol red-free RPMI was purchased from Wisent (St-Bruno, QC) and was supplemented with 20 µM of L-glutamine (Wisent). Conditioned media, protein and RNA were extracted at 0, 24, 48 or 72 hours following the R1881 stimulation. For IL-8 stimulation, LNCaP cells were plated in charcoal-stripped serum supplemented media for 72 hours followed by 24 hours in serum-free RPMI. Cells were then stimulated for 72 hours with either 10 nM R1881 or with 100 ng/ml IL-8 (PeproTech, Rocky Hill, NJ) in serum-free RPMI. siRNA targeting the AR, ARG1, ARG2 and IL-8 as well as the control. Western blotting of proteins extracted in non-denaturing buffer was performed as previously described by our group [35].

Arginase Activity

Arginase activity was quantified as previously described [36]. Briefly, a solution of 10 mM MnCl2/50 mM Tris/HCl at pH 7.5 was added to whole cell extracts. Following an incubation at 55°C for 60 mins, 25 µl of 0.5 M arginine pH 9.7 was added to the samples and further incubated for 60 mins at 37°C. The arginine hydrolysis reaction was stopped by adding H2SO4/H3PO4/H2O at a ratio of 1:3:7, v/v/v. The samples were then boiled at 100°C for 15 mins following the addition of 9% ISPF and read at 540 nm. Using a standard curve, arginase activity was reported as mUnits/mg of protein.

Immunohistochemistry on PCa TMAs

Four different tissue microarrays (TMAs) were used in this study. The first TMA contained 50 normal prostate specimens obtained from 39 autopsied patients without PCa. The second TMA contained non-malignant tissue adjacent to tumor (n = 55), prostate intra-epithelial neoplasic (PIN) tissue (n = 32) and HS tumor tissue (n = 63) from 63 patients who had undergone radical prostatectomy [37]. The third TMA contained HR tumor tissues obtained by trans-urethral resection of the prostate (TURP) from 36 patients collected subsequent to hormone therapy failure [38,39]. Finally, the fourth TMA contained prostate specimens obtained from 35 patients who were treated by ADT prior to radical prostatectomy (ADT group) and 40 Gleason-matched control patients who were only treated by radical prostatectomy, as previously described [10]. For each patient, a total of four tumor cores and two normal adjacent cores were spotted on duplicate TMAs. Cell pellets of each PCa cell line (RWPE, LNCaP, 22Rv1, DU145 and PC3) were spotted on each array and served as internal staining controls. Ethics approval for this study was written consent was obtained from all participants involved in this study, including from the families of the autopsied patients.

Immunohistochemical staining was done as previously described by our group [39,40,41]. Briefly, the 90 min primary antibody incubation was followed by 30 min incubation with an anti-mouse HRP-coupled secondary antibody (sc-2005, Santa
Cruz). Positive signals were developed with diaminobenzidine (DAB) (Dako Cytomation, Mississauga, On, Canada) and the nuclei were counterstained with haematoxylin. High-resolution digital images of each TMA were generated using a whole-slide scanner (ScanScope XT automated high-throughput scanning system) from Aperio (Vista, CA). Two independent observers evaluated the intensity (0, 1+, 2+, 3+) and the percentage of positively stained cells. For each core a value corresponding to the intensity multiplied by the percentage of stained cells was calculated and reported for statistical analysis.

Quantification of l-Arginine concentration by HPLC
Perchloric acid (150 μl) was added to conditioned media (150 μl), which was then vortexed and shook for 10 min. The samples were then centrifuged (13,000 rpm) for 20 min and 240 μl of supernatant were transferred into an amber eppendorff tube. This solution containing the l-arginine was thus essentially cleared of cellular proteins [42]. The supernatant was then neutralized with 60 μl of 3 M NaOH and buffered to pH 7.0 using 100 μl of borate buffer. To this solution, 10 μl of 0.1 M NaCN and Naphthylene-2,3-dicarboxaldehyde (NDA) were added and shaken for 20 min before injection into the HPLC. All samples were run on a Varian Pursuit C18 column 250 x 4.6 mm with the following three solvents: Solvent A: 100 mM triethylammonium acetate (TEAA) buffered to pH 7.0 with 5% acetonitrile (ACN) in milli-Q water; Solvent B: 60% ACN in Solvent A; Solvent C: 100% ACN. A series of l-arginine standards were made ranging from 0 to 2.58 x 10^-4 g/ml. Each standard was done in triplicate and was functionalized with NDA to determine the retention time of l-arginine and the area under the peak corresponding to l-arginine at specific concentrations. Samples were monitored at 260 nM and 420 nM to identify which samples had been functionalized with the NDA. Peaks that appear at 420 nM correspond to substances that have a primary amine available to react.

Lymphocyte activation
PBMCs from healthy donors were isolated from whole blood by Ficoll gradient using lymphocyte-separating medium (Wisent, St-Bruno, Qt, Canada). PBMCs (150,000) were incubated in a 96-well flat-bottomed plate with 1 μg/ml of plate-bound anti-CD3 (OKT3, e Bioscience, San Diego, CA) or an isotype control. Supernatants were harvested for cytokine quantification by enzyme-linked immunosorbent assay (ELISA). For proliferation assays, bromodeoxyuridine (BrDU) was added in the last 12 hrs according to the manufacturer’s instruction. Informed written consent was obtained from all healthy donors involved in this study.

ELISA
The ELISA kit for IL-8 was purchased from R&D Systems (Minneapolis, MN) and the cell proliferation BrDU ELISA kit from Roche (Mississauga, ON, Canada). ELISAs were done according to the manufacturer’s instruction. The IFN-γ ELISA was completed as previously described [13].

Proteome Profiler Analysis
The proteome profiler Human Cytokine Array Panel A Array Kit (R&D Systems) was used according to the manufacturer’s instructions. Briefly, the membranes were incubated with conditioned media from LNCaP cells stimulated with R1881, as previously described, in the presence of the supplied antibody cocktail. Following washes and incubation with a Streptavidin-HRP buffer, positive signal was revealed using ECL reagent.

Statistics
Statistical analysis was performed using SPSS software 11.0 (SPSS Inc., Chicago, IL). The non-parametric Mann-Whitney U test was used to show statistically significant differences.

Supporting Information
Table S1 Correlations between ARG2 expression and clinico-pathological markers.
Found at: doi:10.1371/journal.pone.0012107.s001 (0.05 MB XLS)
Table S2 Correlations between ARG2 expression and immune cell infiltration.
Found at: doi:10.1371/journal.pone.0012107.s002 (0.05 MB XLS)
Figure S1 Androgen stimulation of PCa cells. A) LNCaP cells (left panels) and 22RV1 (right panels) were stimulated over a period of 72 hours with 10 nM R1881 following a 72 hour incubation period in charcoal-stripped media and the gene expression of Prostate-Specific Antigen (PSA), a positive control for R1881 stimulation, was analyzed by qPCR. Note that the ARG2 gene expression presented in Figure 1A correlated with the higher androgen sensitivity of LNCaP cells compared to 22RV1 as exemplified by the mRNA expression of PSA. B) Expression of ARG1 and ARG2 determined by Western blot in LNCaP, Du145 and PC3 cells stimulated with R1881 for 72 hours as previously described. Note the absence of ARG1 and ARG2 in the two HR PCa cell lines, Du145 and PC3.
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Figure S2 ARG1 and ARG2 induction following IL-8 stimulation. A) Positive signals from the Proteome ProfilerTM were quantified by densitometry using Quantity One software (Bio-Rad). Ethanol control (gray bars) and R1881 (black bars). B) Arginase activity of LNCaP cells transfected with siCTRL, siAR or siIL-8 and then stimulated with R1881 was quantified in mU/mg of proteins. Same representative experiment as presented in Figure 2F, (n = 3). C) Increased ARG2 gene expression at 24 hours following IL-8 stimulation. LNCaP were stimulated with IL-8 as previously described. Ran served as the loading control. D) LNCaP cells were plated in charcoal-stripped serum supplemented phenol red-free RPMI media for 72 hours and subsequently for 24 hours in serum-free, phenol red-free RPMI. Cells were then stimulated for 72 hours with 10 nM R1881 or with 5 nM of IL-8 in serum-free, phenol red-free RPMI. ARG1 and ARG2 expression levels were detected by Western blot. Representative experiment is shown, (n = 2).
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Author Contributions
Conceived and designed the experiments: POG JGE FS RL AMMM. Performed the experiments: POG MH ND MA AOP. Analyzed the data: POG MH ND MA AOP. Contributed reagents/materials/analysis tools: POG MAF. Wrote the paper: POG FS RL AMMM.
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