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Original Article

Anti-androgen resistance in prostate cancer cells chronically induced by interleukin-1β

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Abstract: Chronic inflammation has been linked to cancer initiation and progression in a variety of tissues, yet the impact of acute and chronic inflammatory signaling on androgen receptor function has not been widely studied. In this report, we examine the impact of the inflammation-linked cytokine, interleukin-1β on androgen receptor function in prostate cancer cells. We demonstrate that acute interleukin-1β treatment inhibits the transcription of the androgen receptor gene itself, resulting in the reduction of androgen receptor protein levels. Interestingly, in cells subjected to chronic interleukin-1β stimulation, the transcription of the androgen receptor gene is restored within a few cell passages and the cells acquire the ability to grow in the presence of the anti-androgen, bicalutamide. Importantly, the changes that accompany this loss of androgen receptor regulation and gain of anti-androgen resistance are stably heritable since once established, the phenotype is maintained even in the absence of exogenously added interleukin-1β. Further, bicalutamide resistance correlates with increased transcription of androgen receptor target genes and histone H3K4 dimethylation at M-phase gene enhancers. Overall, our studies demonstrate a novel route to anti-androgen resistance upon exposure to an inflammatory cytokine and provide a new tool to further understand how anti-androgen resistance emerges under chronic inflammation.

Keywords: Interleukin-1β, inflammation, bicalutamide resistance, castration resistant prostate cancer, androgen receptor

Introduction

In males, maintenance of the prostate organ requires continuous androgen receptor (AR) signaling via androgen hormones, without which, the prostate regresses. For this reason, aggressive prostate cancer is treated with agents that block androgen synthesis and inhibit the action of AR. However, despite initial regression after androgen deprivation therapy, castrate-resistant prostate cancer (CRPC) is the inevitable consequence. A breakthrough in understanding recurring, resistant disease was the finding that prostate cancer cells become addicted to the AR pathway and that up-regulation of AR expression is the major determinate in CRPC [1]. Numerous mechanisms are likely to play a role in altered AR function in castration resistant disease [2-6], including altered cell signaling as a result of immune cell infiltration and local cytokine production [7-11]. However, the role that chronic inflammation plays in inducing CRPC is not known.

The contribution of inflammation to cancer initiation and progression has been demonstrated in tissues including the stomach [12], colon [13], liver [14, 15], pancreas [16] and lung [17]. In fact, many tumors exist in a milieu of cytokines secreted by infiltrating macrophages which may contribute as much as 50% of a solid tumor mass [18-20]. These macrophages secrete cytokines, among them TNFα and interleukin-1β (IL-1β), which are potent activators of the NF-κB pathway and can induce surrounding epithelial cells to secrete cytokines that further amplify cell signaling [11, 21]. In addition to activating the NF-κB pathway, TNFα and IL-1β also activate the stress kinases, including JNK, p38 and the mitogen activated protein kinase (MAPK) Erk1/2, which prime the cell to respond to infection or injury. In the con-

text of prostate cancer, a subset of proliferative atrophic lesions have increased inflammatory markers indicating that inflammation may play a very early role in the development of prostate cancer [22, 23]. Possible sources of inflammation in the prostate are complex and likely to include infection, urine reflux, chemical and physical injury and diet [10]. Inflammatory markers such as IL-1R1, IL-1α, IL-6, CXCL16 and MIC-1 are increased in prostate cancer samples compared to normal prostate controls [24-27]. Further, initial androgen blockade treatment can give rise to androgen-dependent cancer cell death where many cytokines are released into the surrounding cancer tissues, resulting in massive inflammation infiltration. This prostate cancer associated inflammation is a major cause for subsequent development of castrate-resistant cancer [28]. Therefore, inflammation appears to play an important role in prostate cancer development and particularly in CRPC. The characterization of the role of inflammation will lead to increased understanding of the disease.

Here we demonstrate that treatment with IL-1β, an inflammatory cytokine, can drive cells to become resistant to the AR antagonist, bicalutamide. This resistance to bicalutamide growth-suppression persists even in the absence of continued treatment with IL-1β and is associated with altered gene expression and histone modification, suggesting that exposure to IL-1β may promote castration resistant prostate cancer.

Materials and methods

Cell culture and preparation of protein extracts

The LNCaP human prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The LNCaP-abl cell line was a generous gift from Dr. Zoran Culig (Innsbruck Medical University, Austria) [29]. LNCaP and LNCaP-pCI cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). LNCaP-abl cells were cultured in RPMI-1640 media supplemented with 10% charcoal stripped FBS (cFBS). LNCaP-CI cells were generated and then maintained in RPMI media supplemented with 10% FBS and 1 ng/ml IL-1β (R&D Systems, Minneapolis, MN).

Extracts for immunoblotting were prepared from a subconfluent dish treated with 10 nM R1881 (Perkin Elmer, Waltham MA) reconstituted in ethanol, 10 ng/ml IL-1β reconstituted in PBS, or relevant vehicle. Cells were washed twice with ice cold phosphate buffered saline (PBS) and lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Trition X-100, 10% glycerol and additional protease and phosphatase inhibitors: 1 mg/ml aprotinin and leupeptin, 8 mM sodium pyrophosphate, 1 mM PMSF and 20 mM beta-glycerophosphate. Lysates were clarified by centrifugation at 4°C and soluble supernatants were normalized using the Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA) to determine total protein concentration. Samples were boiled in 6X SDS sample buffer.

Immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membrane (Millipore Corp., Bedford, MA). Membranes were incubated with 5% bovine serum albumin (BSA) in Tris-buffered saline pH 7.4 (TBS) blocking solution. Antibodies were diluted in blocking solution and incubated with membranes overnight at 4°C. The following antibodies were used at 1:1000: P-JNK, JNK, P-p38, p38, P-IkB, IkB, P-Erk, P-Akt, and Akt and were obtained from Cell Signaling Technology (Danvers, MA). The P-AR-S650 antibody was developed in the Logan laboratory. The affinity purified P-AR-S650 antibody was used at 1:1000. AR and IL-1R1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 and tubulin antibody (Covance, Denver, PA) was used at 1:5000. Erk antibody was used at 1:1000 and obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit HRP antibody (GE Healthcare, Piscataway, NJ) was diluted 1:5000 in TBS-Tween and goat anti-mouse HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:2000 in 5% milk in TBS. Membranes were incubated with secondary antibody prior to developing with enhanced chemiluminescence (ECL) according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ).

Dual-layer soft agar assay

6 cm plates were coated with a 2 mL layer of 0.7% agar in 50% media. Approximately 8,500

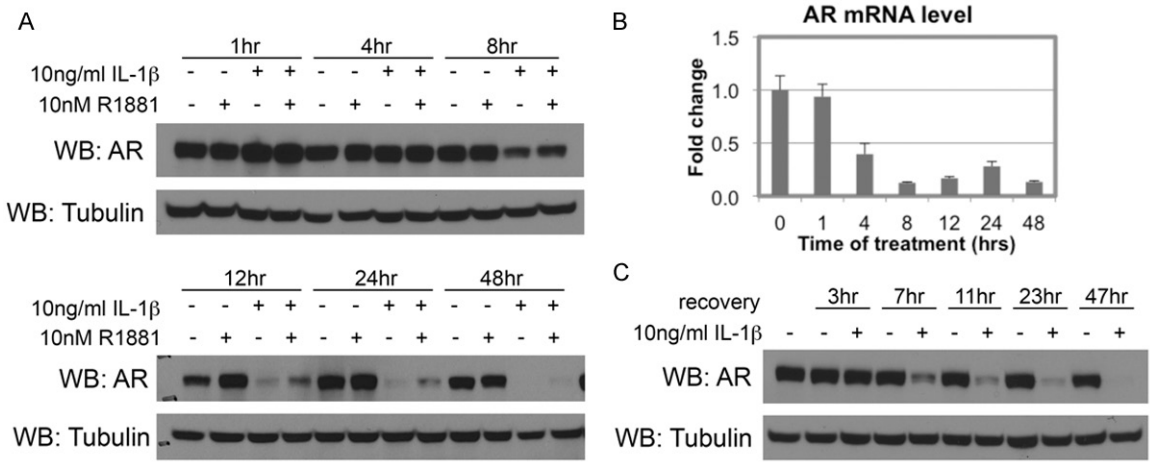

Figure 1. Acute treatment with IL-1β leads to a reduction of AR in LNCaP cells. A: LNCaP cells were steroid starved in cFBS overnight and then treated with vehicle, 10 nM R1881, 10 ng/ml IL-1β or R1881 and IL-1β for up to 48 hours. Protein was extracted at the indicated time points and analyzed by Western blot using antibodies against AR or tubulin (loading control). B: LNCaP cells were incubated in cFBS overnight and then treated with 10 ng/ml IL-1β for the indicated times prior to mRNA isolation. AR mRNA levels were determined by qRT-PCR. C: LNCaP cells were treated with a single dose of vehicle or 10 ng/ml IL-1β for 1 hour (indicated by +), washed once with PBS and then allowed to recover in fresh RPMI + cFBS without exogenous IL-1β for up to 47 hours. Protein was extracted at the indicated time points and analyzed by Western blot using antibodies against AR or tubulin.

cells per plate were suspended in 4 mL of 0.35% agar supplemented with 75% media. Once solidified 1.5 mL of media containing vehicle, IL-1β or bicalutamide (Sigma, St Louis, MO) was laid on top. Media was changed every 3-5 days for 3 weeks. Colonies were visualized using 0.005% crystal violet for 1 hr and images were acquired using a Canon PowerShot A470.

Neutral red growth assay

The growth of cells was assayed using neutral red dye. Cells were seeded in triplicate in 24-well plates. After 24, 48 or 72 hours of treatment, cells were incubated for 2 hours with neutral red (Sigma, St Louis, MO) to a final concentration of 2% in the media. After aspiration of the media cells were treated with extraction solution (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM DTT, 1% Triton X-100, 1% glacial acetic acid and 50% ethanol) for 5 minutes at room temperature. Absorbance was measured at 538 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA).

Quantitative RT-PCR

Total mRNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. cDNA was generated from 1 μg RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 2.5% (v/v) of the RT reaction was used for qPCR. qPCR was performed using SYBR green Taq ready mix (Sigma) and a MyIQ5 Real-Time PCR Detection System (BioRad). Data was analyzed via the ΔΔCT method (46) using RPL19 as a control gene, then normalized to untreated samples, arbitrarily set to 1.

Primers used (5' to 3'): AR-F, TACCAGCTCACCAAGCTCCT; AR-B, GAACTGATGCAGCTCTCTCG; UBE2C-F, TGGTCTGCCCTGTATGATGT; UBE2C-B, AAAAGCTGTGGGGTTTTTCC; CDK1-F, CCTAGTACTGCAATTCGGGAAATT; CDK1-B, CCTGGAATCCTGCATAAGCAC; CDC20-F, CCTCTGGTCTCCCCATTAC; CDC20-B, ATGTGTGACCTTTGAGTTCAG.

ChIP assay

Chromatin immunoprecipitation (ChIP) was performed as described previously [30]. LNCaP cells and LNCaP-CI cells were cultured until 80% confluence. The cells were grown in phenol red free RPMI supplemented with 5% cFBS for 4 days for hormone starvation. Proteins were double cross-linked with dithiobis (succinimidylproprionale) (DSP; Pierce, Rockford, IL) for 20 min and with 1% formalin for 10 min. Cells were lysed, nuclei collected, lysed in sonication buffer (1% SDS, 10 mM EDTA, 50 mM

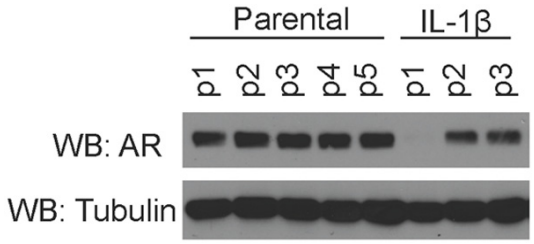

Figure 2. Chronic treatment with IL-1β normalizes AR levels to establish LNCaP-CI cells. LNCaP cells were cultured in media supplemented with 1 ng/ml IL-1β for at least 3 weeks. Protein was extracted at each passage (p1-p3) and subjected to Western blot analysis to determine the AR level. LNCaP-CI cells were subcultured 3 times whereas the LNCaP parental cells were subcultured 5 times in the same time period. Results are representative of three independent experiments.

Tris-HCl [pH 8.0]), and sonicated for 12 min (30s on, 30s off) in a Bioruptor sonicator (model XL; Diagenode, Denville, NJ). Sonicated lysates were pre-cleared for 2 h with protein A/G-agarose beads blocked with salmon sperm DNA (Millipore). Supernatants were then incubated overnight with 3 μg dimethyl-Histone H3 (Lys4) antibody (Millipore). Control ChIP was performed with 3 μg total Histone H3 antibody (Abcam, Cambridge, MA). Immunocomplexes were then washed and cross-linking was reversed. DNA was isolated with the Qiagen PCR purification kit and quantitative PCR (qPCR) was performed using 1 to 5 μl of DNA. Relative enrichment was calculated as a percentage of 4% input normalized to rabbit polyclonal Anti-H3 IgG. The ChIP primers used for UBE2C, CDK1 and CDC20 were described previously [31].

Results

Acute IL-1β treatment leads to decreased AR mRNA and protein

IL-1β is secreted by a variety of cell types such as macrophages and is associated with cellular inflammation thought to be linked to prostate carcinogenesis [10, 32, 33]. To mimic the effects of short-term and long-term inflammation on AR function, we treated the LNCaP prostate cancer cell line either acutely or chronically with IL-1β. AR protein and mRNA expression levels were examined and little change in AR protein was observed after 1 and 4 hours of IL-1β stimulation in either the presence or absence of the synthetic androgen, R1881. However, after 8 hours of IL-1β stimulation, a reduction in the steady state level of AR protein was apparent (**Figure 1A**). To determine if this effect persisted over time we extended the treatment time and examined AR protein expression in LNCaP cells stimulated with IL-1β for 12, 24 and 48 hours. A profound decrease in AR protein expression at later time points was evident, with minimal AR persisting at 24 and 48 hours (**Figure 1A**, bottom panel) and this effect was observed in both the presence and absence of R1881. To examine if loss of AR protein in response to IL-1β treatment occurred through alteration of AR transcription, we measured AR mRNA by qPCR at both early and late time points following incubation of the cells with IL-1β. Treatment with IL-1β led to a loss of AR mRNA beginning at 4 hours and this decrease continued throughout the 48-hour time course (**Figure 1B**). This is consistent with the loss of AR protein over time upon IL-1β treatment (**Figure 1A**). Thus, IL-1β appears to decrease AR protein levels through a mechanism that reduces the mRNA expression of the AR gene itself.

To determine if the effect of IL-1β on AR expression is reversible or persistent, cells were treated with a single acute dose of IL-1β for 1 hour, followed by incubation with media that did not contain exogenously added IL-1β for 3-47 hours. AR expression was monitored by Western blot analysis, which revealed that a 1 hour treatment with IL-1β resulted in dramatically decreased AR protein expression that persisted 7, 11, 23, and 47 hours (**Figure 1C**). Thus, the repression of AR by IL-1β signaling is rapid, robust, and does not require the continuous presence of IL-1β.

AR levels are re-established in cells chronically treated with IL-1β

LNCaP cells are androgen-sensitive for growth and as such, require both androgen and the AR for cell proliferation. Although AR mRNA and protein levels decrease with acute IL-1β treatment (**Figure 1**), over time cells were still able to proliferate suggesting that either AR levels were restored despite continued IL-1β treatment or that the cells no longer required AR for growth. To distinguish between these possibilities, we subjected cells to chronic IL-1β treatment and examined AR expression. LNCaP cells

Table 1. Generation conditions of cell lines utilized

Cell Line	Generation
LNCaP	Parental – acquired from ATCC
LNCaP-CI	Grown in 1.0 ng/ml IL1β for ~3 weeks
LNCaP-pCI	LNCaP-CI taken out of IL-1β for 2-10 weeks
LNCaP-abl	grown in cFBS for ~41 weeks – provided by Z. Culig

were cultured in media with or without 1 ng/ml IL-1β and continuously passaged. AR levels in cells chronically treated with IL-1β were examined at passages 1-3 (**Figure 2**, p1, p2, p3) and compared to control parental cells (**Figure 2**, Parental p1-p5). Since growth of IL-1β treated cells was initially slow due to very low levels of AR, passages p5 (parental) and p3 (IL-1β treated) were comparable with regard to the length of time in culture (approximately 3 weeks). The results demonstrate that cell passage number 1 shows negligible AR levels, with increasing AR protein exhibited in passages 2 and 3 in cells treated with IL-1β (**Figure 2**). This experiment was repeated with similar results three times. For simplicity, the population of cells chronically treated with IL-1β will be called LNCaP-CI for Chronic IL-1β treatment (**Table 1**). These results indicate that initially, IL-1β treatment results in growth suppression due to a reduction of AR expression, however over a relatively short time span, even in the continued presence of IL-1β, AR protein levels are restored and the cells resume a growth pattern similar to parental cells.

LNCaP-CI is resistant to growth inhibition by bicalutamide

Given the role of IL-1β as an inflammatory cytokine, we wondered if IL-1β affects prostate cancer cell growth and resistance to anti-androgen treatment. To test this, we utilized parental LNCaP cells, LNCaP-CI cells and a LNCaP cell derivative called LNCaP-abl [29] that was selected for its ability to grow in the absence of androgen. The cells were treated with the vehicle (EtOH) or the AR agonist, dihydrotestosterone (DHT) in the presence and absence of the AR antagonist, bicalutamide (**Figure 3**). In addition, to see if putative effects of IL-1β on cell growth persisted in the absence of chronic IL-1β stimulation, we generated a LNCaP-CI subline in which chronically treated LNCaP-CI cells were cultured in the absence of IL-1β for 2-10 weeks: LNCaP-post-CI (LNCaP-pCI; **Table 1**). After 72 hours, the effects of the various treatments became apparent. As expected, LNCaP cells grew in response to stimulation with 0.1 nM DHT and this growth was suppressed by in the presence of 1.0 μM bicalutamide. Proliferation in the presence of bicalutamide is similar to growth in the absence of hormone (vehicle only), with some initial growth before plateauing after 48 hours. LNCaP-abl cells were able to proliferate equally well in media with 0.1 nM DHT in the presence and absence of 1.0 μM bicalutamide, with no appreciable difference between the two treatments. While LNCaP-CI cells demonstrated a growth response stimulated by 0.1 nM DHT, growth was only slightly suppressed by the addition of 1.0 μM bicalutamide. Growth in the presence of bicalutamide did not plateau, in contrast to what was observed in parental LNCaP cells, suggesting a novel mechanism of bicalutamide-resistance. Further, the resistance to bicalutamide persists in the LNCaP-pCI cells where growth was also only slightly suppressed.

IL-1β treatment alters the anchorage independent growth properties of prostate cancer cells even after removal of IL-1β

Given the insensitivity of IL-1β treated cells to bicalutamide-mediated growth suppression, we sought to determine if other hallmarks of tumorigenicity, such as anchorage independent growth, were affected by IL-1β treatment. The effects of acute and chronic IL-1β treatment on anchorage independent growth were examined by using a dual-layer soft agar assay. Cells were grown in 0.35% agar supplemented with RPMI + FBS alone (untreated), RPMI + FBS + 1 ng/mL IL-1β (IL-1β) or RPMI+ FBS + 1 μM bicalutamide (bicalutamide). LNCaP cells supplemented with RPMI + FBS formed distinct colonies, while LNCaP cells supplemented with bicalutamide or IL-1β were unable to form colonies (**Figure 4**, top three panels). The inability of the cells to form colonies in the presence of IL-1β is likely due to the loss of AR expression. To investigate the effect of IL-1β preconditioning on anchorage independent growth, the same experiment was performed with LNCaP-CI cells. In contrast to LNCaP cells, the LNCaP-CI cells were able to form colonies supplemented with IL-1β or bicalutamide (**Figure 4**, bottom panels). In addition, LNCaP-pCI cells were able to form colonies in the presence of bicalu-

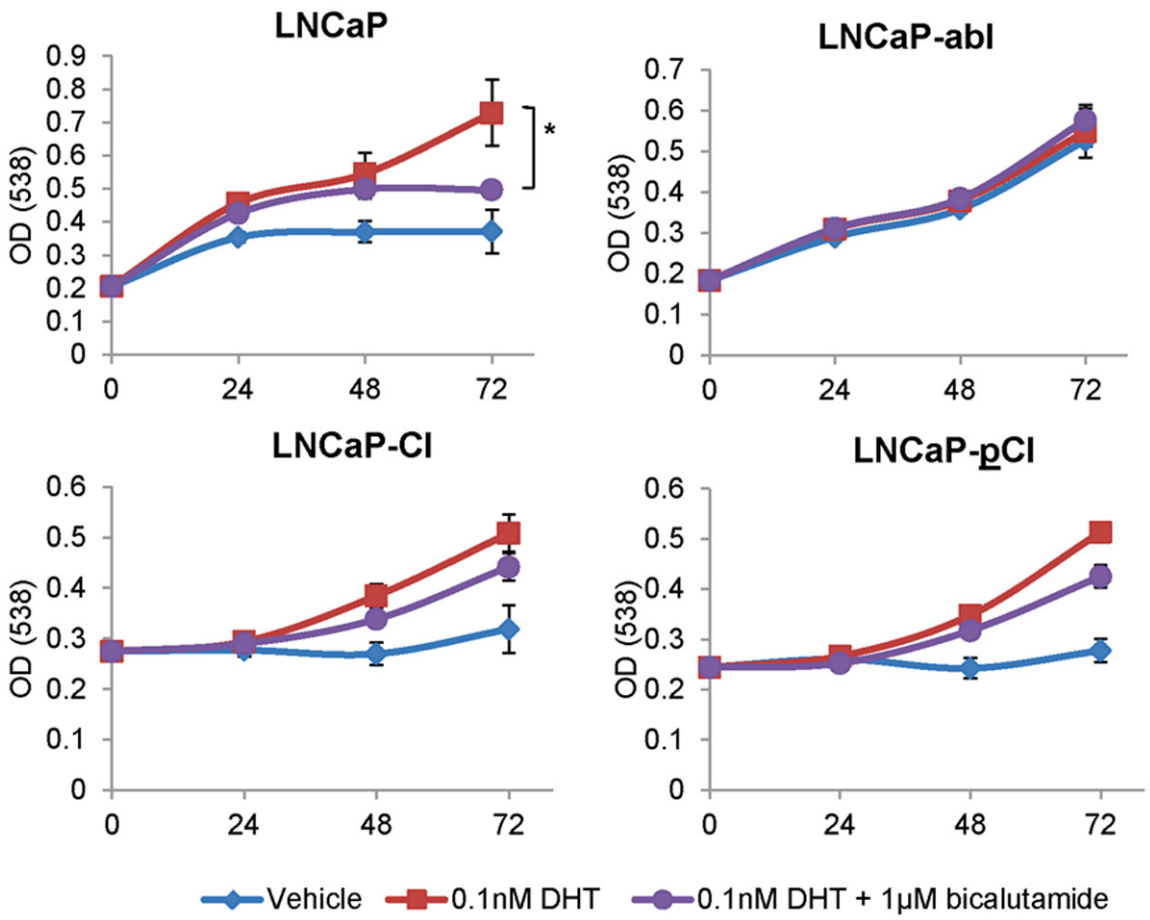

Figure 3. LNCaP-CI and LNCaP-pCI are resistant to growth inhibition by bicalutamide. Cells were treated with vehicle, 0.1 nM DHT, or 0.1 nM DHT and 1.0 μM bicalutamide. Growth was measured using neutral red dye and assayed every 24 hrs for 72 hrs. All treatments were conducted in triplicate and graphs are representative of three independent experiments performed with two distinct LNCaP-CI cell lines. Statistical significance was calculated using unpaired t-tests. $p<0.05$ (*).

tamide (data not shown). These results indicate that IL-1β naïve cells respond very differently from chronically treated IL-1β cells, despite comparable levels of IL-1 receptor (IL-1RI) seen in LNCaP and LNCaP-CI cells (**Figure 5**).

LNCaP-CI cells display a diminished capacity to respond to IL-1β signaling

To understand the changes in androgen and bicalutamide sensitivity observed in the LNCaP-CI and LNCaP-pCI cell lines we examined cellular pathways modulated in response to IL-1β including JNK, p38, IκB, Akt and Erk1/2. Since literature studies indicate that AR is phosphorylated on Serine (S)650 (S650) in response to activation of p38 and JNK kinases [34], we also examined AR S650 phosphorylation. Activation of these pathways was assessed by Western blot analysis of cell lysates procured following treatment of cell lines with IL-1β and/or R1881. Using a phosphorylation specific antibody developed and characterized in our laboratory, we show that AR is phosphorylated at S650 in response to PMA and IL-1β (**Figure 5A-D**). Specificity of the P-AR-S650 antibody is demonstrated by its detection of PMA-stimulated phosphorylation of wild type AR, but not an AR-S650A mutant (**Figure 5A**) and the sensitivity of the signal to phosphatase treatment (**Figure 5B**). We show that we can detect AR phosphorylated at S650 in response to stimulation with as little as 10 picograms of IL-1β (**Figure 5C**). In addition, we measured the activated, phosphorylated forms of JNK, p38, IkB, Akt and Erk1/2 (**Figure 5D**). IL-1β significantly increased activation of AR, JNK, p38, IκB and Erk1/2, in LNCaP cells, while treatment with

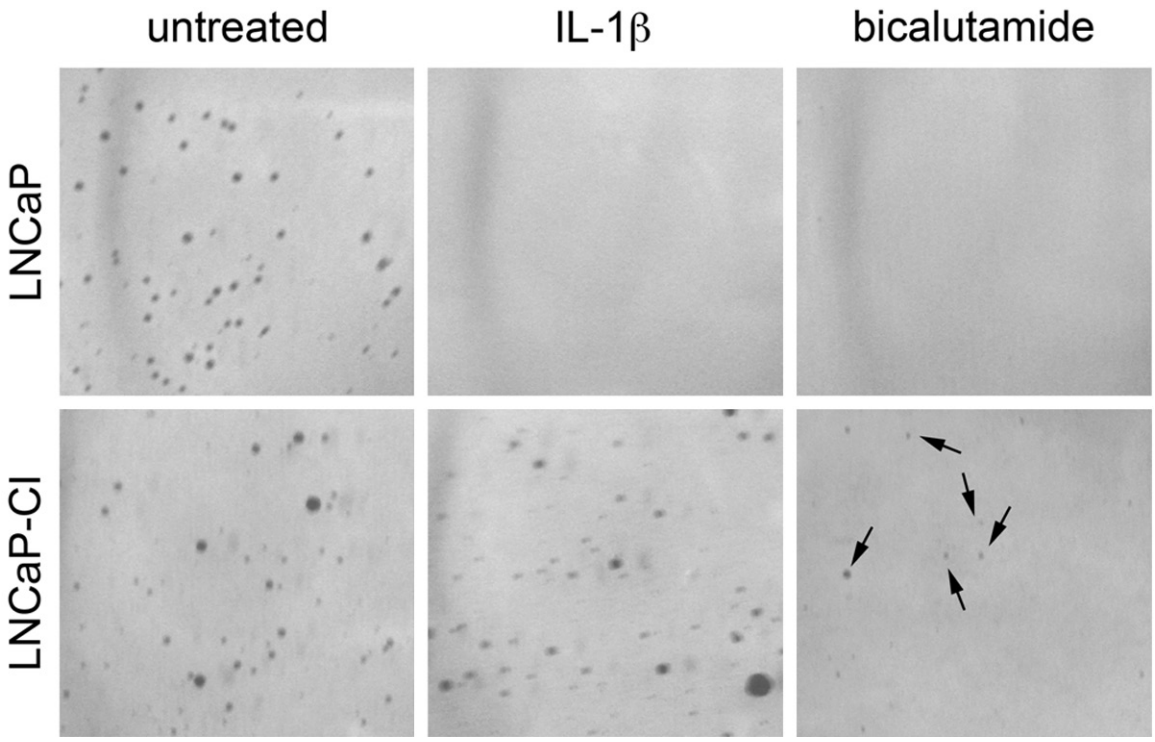

Figure 4. Anchorage independent growth of LNCaP-CI cells in the presence of IL-1β or bicalutamide. LNCaP or LNCaP-CI cells were grown in the top layer of the dual-layer soft agar for 3 weeks. 1 ng/ml IL-1β or 1 μM bicalutamide was added to the top layer of agar and media overlay. **Figure 4** is representative of 4 independent experiments, each performed in duplicate. Each panel represents approximately 45% of the surface area of a 6 cm plate. Arrows indicate colonies.

R1881 alone did not significantly affect activation of any of the pathways in LNCaP, LNCaP-CI, LNCaP-pCI or LNCaP-abl cells. All cell lines show a high degree of Akt activity, consistent with the mutation of PTEN in LNCaP cells [35]. In LNCaP-CI cells, treatment with IL-1β was insufficient to significantly activate the JNK or p38 stress kinase pathways. Further, consistent with previous studies [34], LNCaP-CI cells did not demonstrate AR S650 phosphorylation in the absence of activation of JNK and p38 (**Figure 5D**). While Erk1/2 was still phosphorylated in response to IL-1β in LNCaP-CI cells, the level of phosphorylation was significantly lower than in the parental LNCaP cells. Likewise, the phosphorylation of IκB was severely diminished compared to LNCaP cells and did not result in a significant decrease of IκB. The effect of IL-1β treatment on LNCaP-pCI cells was also examined. Unlike in LNCaP-CI, IL-1β treatment of LNCaP-pCI cells induces phosphorylation of JNK and p38, approaching levels seen in parental LNCaP and LNCaP-abl cells. The phosphorylation of IκB in LNCaP-pCI cells is intermediate between the LNCaP and LNCaP-CI cells and results in a moderate degree of IκB degradation. Finally, it is important to note that the changes presented here are not due to changes in overall protein levels, as the amount of IL-1RI, JNK, p38, Akt and Erk1/2 are consistent between the four cell types. Together these results indicate that the signaling downstream of IL-1β is decreased in LNCaP-CI while signaling in LNCaP-pCI cells is comparable to levels observed in parental LNCaP cells.

Elevated histone H3K4 dimethylation accompanies higher expression of M-phase cell cycle genes in LNCaP-CI

Long-term exposure of prostate cancer cells to IL-1β results in the ability of the cells to grow in the presence of bicalutamide, reminiscent of LNCaP-abl cells (**Figure 3**). To understand this phenomenon, we considered a literature report indicating that AR is preferentially recruited to M-phase cell cycle regulatory genes that are AR targets in hormone insensitive, LNCaP-abl cells and enhanced AR recruitment was accompa-

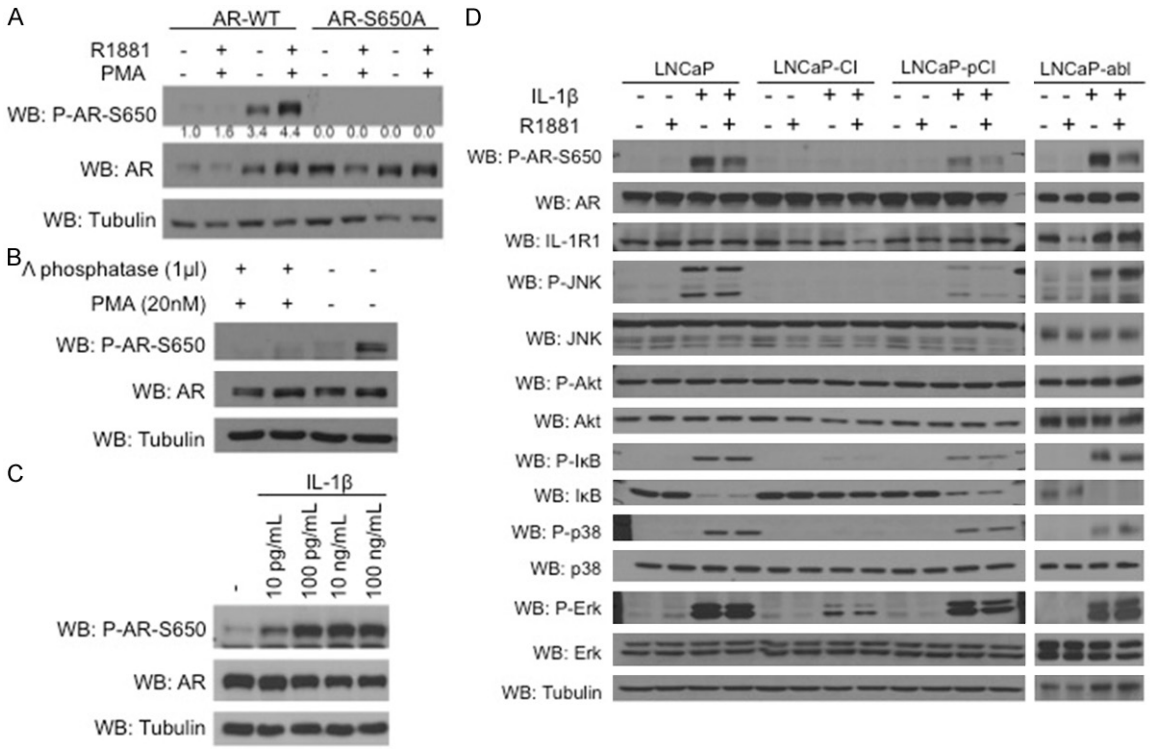

Figure 5. Antisera specifically recognize AR phosphorylated at S650. A: 293 cells were transfected with either WT or AR with a serine to alanine mutation at residue 650 (S650A). Cells were treated with R1881 or PMA or both. Antibody recognizes the WT receptor but not the S650A mutant. Phosphorylation increases with phorbol-12-myristate 13-acetate (PMA) treatment. There is no P-AR-S650 immunoreactivity to the S650A mutant transfected cells despite the presence of the receptor. Numbers represent change in phosphorylation relative to AR levels. B: LNCaP cells were treated plus or minus PMA. Lysates were then treated with 1 μl λ phosphatase. Immunoreactivity of P-AR-S650 is eliminated after lysates are treated with λ phosphatase. Cell signaling following IL-1β treatment. C: AR is phosphorylated at S650 in response to IL-1β. LNCaP cells were treated with increasing concentrations of IL-1β for 1 hour. Protein lysates were analyzed by Western blotting with the indicated antibodies. D: LNCaP, LNCaP-CI, LNCaP-pCI and LNCaP-abl cells were steroid starved overnight and then treated with 10 ng/ml IL-1β and/or 10 nM R1881 for 30 minutes. Protein lysates were analyzed by Western blot using the indicated antibodies.

nied by increased H3K4 methylation at the M-phase gene enhancers in LNCaP-abl, but not LNCaP cells [31]. The M-Phase cell cycle-related genes include CDC20, CDK1 and UBE2C. In particular, UBE2C (ubiquitin conjugating enzyme E2C) is critically important for cell growth as depletion of UBE2C by shRNA resulted in prolonged mitosis as evidenced by increased histone H3 serine 10 phosphorylation and decreased cell proliferation in LNCaP-abl cells [34]. To determine if exposure of cells to IL-1β promotes bicalutamide resistance through a similar mechanism, we treated LNCaP and LNCaP-CI cells with IL-1β in the presence and absence of R1881 and bicalutamide and examined levels of UBE2C and CDK1 by qRT-PCR and western blot (**Figure 6**). In agreement with previous studies [31], we show that compared to parental LNCaP cells, LNCaP-abl cells express higher levels of both UBE2C and CDK1 mRNA (**Figure 6A**, black bars) at 24 hours under all conditions tested. Further, in all the IL-1β-containing conditions, transcription of UBE2C and CDK1 mRNA is upregulated in LNCaP-CI, -pCI, and -abl compared to the parental LNCaP cells (**Figure 6A**). An examination of protein levels also show that at 24 hours expression of CDK1 and UBE2C is highest in LNCaP-abl cells compared to the other cell lines (**Figure 6B**, lanes 4, 8, 12, 16, 20 and 24). The expression level of CDK1 was similar in LNCaP -parental, -CI, and –pCI cells under all treatment conditions. However in the presence of IL-1β, UBE2C was increased in LNCaP-CI and LNCaP-pCI compared to parental LNCaP cells. We note that LNCaP-CI, LNCaP-pCI and LNCaP-abl cells all maintain responsiveness to IL-1β exhibited by decreased AR protein levels com-

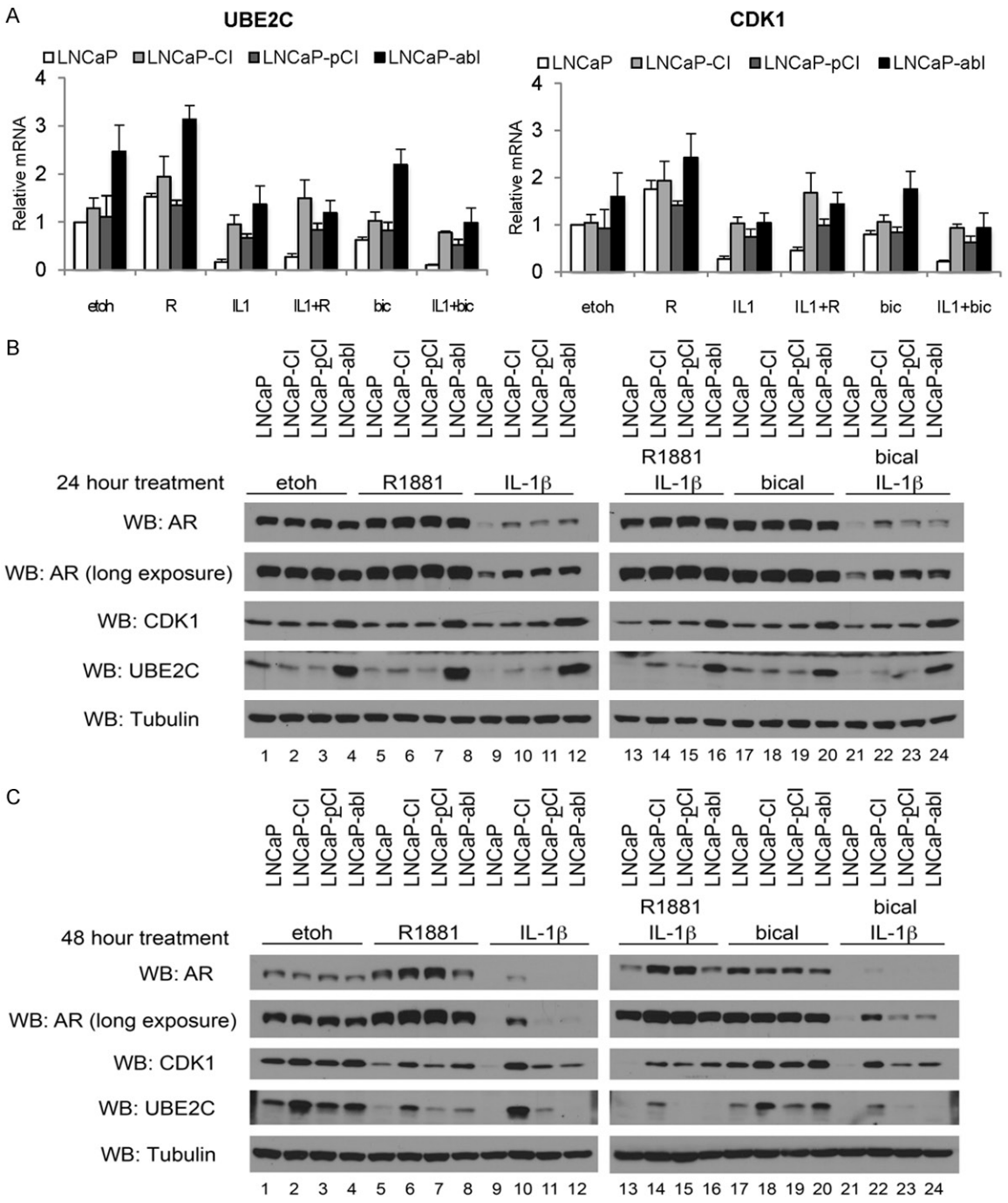

Figure 6. Regulation of UBEC2 and CDK1 in LNCaP-derived cell lines. The cells were steroid starved for 2 days prior to treatment with vehicle (ethanol), 10 nM R1881 (R), 10 ng/ml IL-1β and/or 10 μM bicalutamide (bic), as indicated. A: RNA was isolated after 24 hours for quantification using qRT-PCR. The mRNA results are expressed relative to vehicle-treated LNCaP cells and are the average of 3-4 independent experiments. Error bars represent SEM. B and C: Protein was extracted at 24 and 48 hours then analyzed for AR, CDK1, and UBEC2 expression by Western blot. Western blots are representative of three independent experiments.

pared to the controls, but importantly AR levels in LNCaP-CI and pCI are still higher than in parental cells and this correlates with higher levels of UBE2C protein (AR and UBE2C panels, compare lane 9 to 10-12, lane 13 to 14-16 and lane 21 to 22-24, **Figure 6B**).

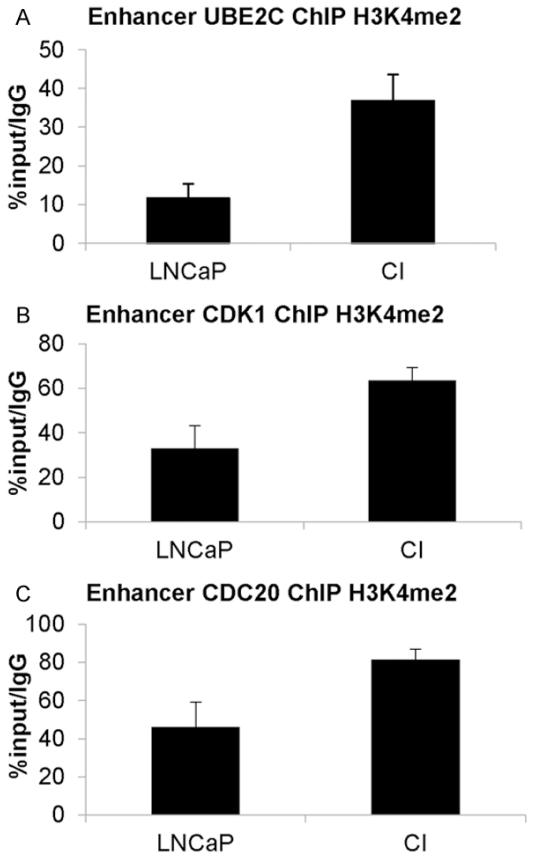

Figure 7. Higher levels of active epigenetic histone mark H3K4 dimethylation in LNCaP-CI cell than in LNCaP cell. Using specific antibody against H3K4 dimethylation, levels of H3K4 dimethylation (mean (3) ± S.E) on (A) UBE2C enhancer, (B) CDK1 enhancer and (C) CDC20 enhancer were determined by ChIP assays combined with quantitative PCR.

The impact of the various treatments became more pronounced at 48 hours (**Figure 6C**). While IL-1β treatment results in diminished AR protein with some stabilization in the presence of R1881 for all the cell types, AR is more abundant in CI cells than in the other cell types (**Figure 6C**, compare AR levels in lanes 10, 14 and 22 to the AR levels in the other cell types under the same conditions). In addition, LNCaP-CI cells exhibit higher levels of UBE2C than all the other cell types and this generally correlates with higher levels of AR under conditions that include IL-1β treatment. Of note, CDK1 and UBEC2 are increased in the presence of IL1-β and/or bicalutamide in LNCaP-CI cells (**Figure 6C**, lanes 18 and 22). Overall, the results suggest that while short term IL-1β treatment results in transcriptional down-regulation of AR, cells chronically exposed to IL-1β become resistant to AR down-regulation and as a consequence express higher levels of proteins that promote M-phase cell cycle progression.

To determine the effect of chronic exposure to IL1-β on AR regulation of M-phase gene enhancers, we measured H3K4 dimethylation on the UBE2C, CDK1 and CDC20 enhancers in LNCaP and LNCaP-CI cells (**Figure 7**). We did not examine the impact of IL-1β treatment in this experiment since acute treatment decreases AR levels. Interestingly, in the absence of hormone we observed significantly higher H3K4 dimethylation on the UBE2C, CDK1, and CDC20 enhancers in LNCaP-CI compared to parental LNCaP cells (**Figure 7A-C**). This did not reflect differences in total histone levels as ChIP for total H3 exhibited similar levels on the enhancers in both LNCaP and LNCaP-CI cells (not shown). H3K4 dimethylation enrichment on the UBE2C enhancer is consistent with upregulation of UBE2C mRNA and protein. Overall the data suggests that LNCaP-CI cells become bicalutamide resistant, likely through altered regulation of chromatin modification, such as H3K4 dimethylation enabling occupancy of AR on M-phase related cell cycle genes and expression of growth promoting genes.

Discussion

Inflammation is a well-characterized contributor to cancer initiation and progression in other tissues [12-14] and histological studies indicate proliferative inflammatory atrophy (PIA) lesions may be a precursor to a subset of prostate tumors [10]. Additional histological studies reveal that the inflammatory cytokines TNFα and IL-1β, as well as their corresponding receptors are upregulated in recurrent tumors compared to normal prostate [26, 27]. There is also a distinct increase of macrophage infiltrates that could produce inflammatory cytokines in prostate cancer tissue compared to benign tissue [36]. Tumor-associated inflammation also results from therapeutic intervention [37] whereby dying cells from androgen ablation therapy produce inflammatory mediators, and the recruitment of B cells and other immune cells. The B cells in the tumor release NF-κB-dependent cytokines including IL-1β, TNFα, IL-6 and IL-8 [37, 38]. However, the important role NF-κB-dependent cytokines play in driving CRPC and how AR function is affected are not well understood.

To mimic inflammation in prostate cancer we employed the inflammatory cytokine, IL-1β. We found that chronic IL-1β treatment is sufficient to impart bicalutamide resistance and anchorage-independent growth to cells over a short number of passages despite the fact that acute IL-1β treatment decreases AR mRNA and protein. In contrast to LNCaP-CI, bicalutamide-resistant LNCaP-abl cells, were selected by growth in hormone-free media for greater than 40 cell passages. Thus, LNCaP-CI cells represent an intriguing addition to model cell lines available for prostate cancer studies. Our data indicate that LNCaP-CI cells are not able to grow in the absence of androgen, perhaps suggesting that they are not sensitized to low levels of androgen like LNCaP-abl cells. However, LNCaP-CI are resistant to bicalutamide growth suppression, suggesting that sensitization to low levels of androgen and resistance to antagonist can be uncoupled and do not necessarily rely on the same mechanism.

Analogous to altered AR function in response to IL-1β, transcription of AR in response to TNFα is also altered and is different in androgen-sensitive versus androgen-insensitive cell lines [39]. Treatment of LNCaP cells with TNFα causes recruitment of a NF-κB repressor complex containing RelA, Sin3A and B-myb to the AR promoter, resulting in transcriptional inhibition but in androgen-insensitive LNCaP C4-2 cells, the repressor complex is no longer recruited and AR transcription is not repressed [39]. These experiments did not determine whether long-term treatment with TNFα resulted in eventual up-regulation of AR. However, AR is clearly needed for LNCaP cell survival as LNCaP cells stably expressing shRNA targeting AR survived only a few passages (unpublished data- JAS and SKL). IL-6, another NF-κB-dependent cytokine, is able to mediate the bicalutamide resistance in LNCaP cells by long-term exposure of more than 42 passages. The underlying mechanism is via increased expression of TIF2 but it is unclear whether mitotic cell cycle genes are altered [40]. In these experiments, it appears that IL-6 does not change AR levels, however, bicalutamide prevented DHT-mediated AR nuclear translocation in parental LNCaP cells but not in IL-6 treated cells [40].

Our results suggest that acute IL-1β action may have a protective role while chronic inflammation promotes cancer growth. In response to short-term IL-1β treatment AR is down-regulated and cells do not grow. Coupled with additional growth inhibitory or apoptotic signals this could be sufficient to prevent nascent tumor formation. There is some evidence this is the case since we were unable to generate a CI cell line with VCaP cells due to massive cell death in the presence of IL-1β, even at a 10-fold lower concentration (0.1 ng/ml). Unlike LNCaP, which have mutated PTEN resulting in constitutively active Akt, VCaP cells have wild type PTEN and tightly regulated Akt signaling. Thus VCaP lack the survival signal that might maintain LNCaP cells in the absence of AR. Similar to LNCaP, another prostate cancer cell line, LAPC-4 which has high Akt activity can proliferate in the presence of IL-1β (data not shown). Alterations in the PTEN/Akt signaling pathway are among the most common mutations found in prostate cancer. PTEN is mutated in 30-70% of primary tumors and approximately 20% of high-grade prostatic intraepithelial neoplasia [35, 39, 41]. Thus PTEN mutation occurs early in the progression of prostate carcinogenesis and may predispose cells to anti-androgen resistance with the addition of inflammatory signaling.

Taken together, chronic exposure of prostate cancer cells to IL-1β leads to reestablishment of AR levels and resumption of prostate cancer cell growth in the presence of bicalutamide. Bicalutamide resistance appears mediated by an increased prevalence of the active epigenetic mark, H3K4me2 on M-phase cell cycle genes. This process can recapitulate the biological properties of LNCaP-abl and CRPC clinical cases through upregulation of mitotic cell cycle genes, suggesting that chronic inflammation, induced by IL-1β and/or possible other NF-kB dependent cytokines characterizes the development of CRPC from androgen-dependent prostate cancer transformation. The data will lead to further investigation of potential therapeutic targets in the IL-1β pathway to prevent or prolong the process of this lethal transformation.

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Disclosure of conflict of interest

The authors declare no conflicts of interest.

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