ORIGINAL CONTRIBUTION

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MODULATION OF *PD-L1* EXPRESSION IN PROSTATE CANCER CELLS THROUGH ANDROGEN RECEPTOR INHIBITION DIFFERS DEPENDING ON RECEPTOR STATUS

Background. Immune checkpoint blockade (ICB) therapy targeting the PD-1/PD-L1 axis results in poor outcomes in prostate cancer (PCa). PD-L1, the most commonly used predictive marker for the efficacy of PD-1/PD-L1-targeted immunotherapy, appears to be rarely or at low levels expressed in primary androgen-responsive PCa tumors, with higher levels in advanced PCa. PD-L1 expression has not yet been studied regarding the androgen receptor (AR) status. **Materials and Methods.** We investigated the effect of hormone stimulation by dihydrotestosterone (DHT) and AR inhibition by enzalutamide on $_{PD\text{-}L1}$ expression in LNCaP and LNCaP-AR+ cells, the latter overexpressing AR. Cells were grown for 24 h under hormone-free conditions and then for 24 h in the presence of DHT (10 nM) and/or enzalutamide (10 μM). Cell viability was assessed by Annexin V and propidium iodide staining. *PD-L1* expression was determined semiquantitatively at the mRNA level. ANOVA and independent *t*-tests were used to compare experimental results between different treatment modalities. **Results.** DHT treatment induced some degree of apoptosis in AR-overexpressing LNCaP-AR + cells, but not in parental LNCaP cells. We found low basal expression of *PD-L1* in both cell lines, with 2.7-fold higher levels in LNCaP-AR+ cells. DHT treatment increased *PD-L1* expression by approximately three-fold in LNCaP cells, while in enzalutamide-treated cells, the expression was lower than the basal level. In LNCaP cells treated concomitantly with DHT and enzalutamide, AR inhibition reduced DHT-induced *PD-L1*, suggesting an androgen-dependent expression of *PD-L1*. Unlike in LNCaP cells, androgen stimulation did not increase *PD-L1* expression in LNCaP-AR+ cells, and enzalutamide did not affect *PD-L1* expression either. **Conclusion.** Our data reveal that *PD-L1* is expressed in an AR-dependent manner in PCa cells, and its expression in AR-overexpressing cells is not modulated by receptor inhibition.

Keywords: prostate cancer, PD-L1, androgen, androgen receptor, enzalutamide.

Prostate cancer (PCa) is the most commonly diagnosed malignancy in men in most (118/185) countries and the fifth leading cause of cancer-related death in men [1]. Although immune checkpoint blockade (ICB) represents a promising new therapy in many cancer types, outcomes in PCa are

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poor [2, 3]. The high heterogeneity of prostate tumors and the increased immunosuppressive microenvironment in PCa tissues are thought to be responsible for the poor responses in PCa [3].

Inhibition of the PD-1/PD-L1 axis represents the most widely used type of ICB therapy [4]. PD-L1, the ligand of death domain PD-1, in tumor cells, is the most frequently studied biomarker for checkpoint inhibitors [5] and its expression status is associated with response to anti-PD-1/PD-L1 therapies in various cancers [6]. Different results have been reported regarding PD-L1 expression in PCa. LNCaP cells, which represent hormone-sensitive PCa, have no or very low PD-L1 expression while it is highly expressed in PC3 and DU145 cell lines [7, 8]. It is to be noted that PC3 and DU145 cell lines have been shown to have lower androgen receptor (AR) expression than LNCaP cells [9]. In a relatively large cohort comprising primary prostate tumors (n = 539) and metastatic castrate-resistant PCa (mCRPCa) (n = 57), Haffner et al. [10] showed that PD-L1 expression is rare in primary prostate tumors with an increased positivity rate in mCRPCa. Another study has also reported low PD-L1 expression in PCa tissues [11]. He et al. [12] described that PD-L1 expression was significantly higher in cancer tissues compared to benign tissues. PD-L1 positivity rates in primary prostate tumors may differ from those at different metastatic sites in the same patient [13]. PD-L1 is more frequently expressed in CRPCa than in hormone-sensitive PCa and is also associated with other adverse outcomes, including an advanced tumor stage and a high Gleason score (reviewed in [13]).

Since PCa is an androgen-dependent malignancy, to our knowledge, PD-L1 expression has not been investigated by androgen receptor (AR) status in PCa. Here, we have investigated the effect of hormone stimulation and AR inhibition on PD-L1 expression by receptor expression status using an in vitro model composed of two cell lines expressing AR at different levels.

Materials and Methods

Cells. We used two cell lines, LNCaP and LNCaP-AR+. LNCaP cells, originally obtained from lymph node metastases of a PCa patient [14], express AR and are therefore androgen-sensitive and were purchased from the American Type Culture Collection (ATCC# CRL-1740). The LNCaP-AR+ cell line is the genetically modified version of ancestral LNCaP cells to express higher AR [15] and was kindly provided by the Memorial Sloan Kettering Cancer Center (New York, USA). Standard cell culture protocols were used for the growth and handling of cells in the experiments as previously described [16].

Androgen stimulation and androgen receptor inhibition in prostate cancer cells. Before treating cells with androgen hormone, growth hormones present in growth media were removed using charcoal treatment as described previously [17]. Dihydrotestosterone (DHT), a more potent AR agonist than its precursor testosterone, was used to stimulate PCa cells. DHT was dissolved in ethanol to prepare a stock solution of 100 mM and stored at –20 °C in aliquots. We used DHT at a concentration of 10 nM, which is within the effective dose range commonly used in in vitro studies [18].

We used enzalutamide as an AR inhibitor. It is a non-steroidal anti-androgen approved for the treatment of patients with mCRPCa. Enzalutamide has multiple modes of action; it can block the binding of androgens to the AR and its transit into the cell nucleus or prevent the AR from binding to its binding sites in DNA [5]. Enzalutamide, kindly provided by Astellas, was stored as 1 mM stocks at –20 °C after dissolving in dimethyl sulfoxide (DMSO). We used 10 μM enzalutamide, the standard dose in in vitro studies [19]. For the experiments (androgen stimulation or AR inhibition), cells (2×10^5) were kept in hormone-free medium conditions for 24 h and were then further grown for 24 h in a medium containing DHT or enzalutamide at the indicated doses.

Cell viability analysis. To assess the viability of PCa cells treated with DHT or enzalutamide, we performed a flow cytometry analysis using Annexin V and propidium iodide (PI) staining assay. The cells treated with DHT or enzalutamide were harvested, and their number was adjusted to 1×10^6 cells per 100 μL. After washing, the cells were resuspended in 100 μL of annexin V binding buffer, 5 μL annexin V-FITC (Thermo Fisher Scientific. USA), and 1 μL PI from 100 μg/mL stock was added. The cells were incubated for 15 min in the dark and exposed to the fluorescence mea-

surement in a FACSCalibur flow cytometer (BD Biosciences, USA). Cell viability was assessed using the CellQuest software. Data were presented in two-dimensional scatter plots showing PI vs Annexin V-FITC.

Expression analysis in prostate cancer cells. *PD-L1* expression was evaluated in PCa cells treated with DHT or enzalutamide compared to control cells. The expression of long non-coding RNA ARLNC1 (androgen receptor-regulated long noncoding RNA 1) was used as evidence of the effectiveness of androgen stimulation and AR inhibition [16]. Total RNA was isolated from the cells using a TriPure RNA Isolation Solution (Roche Diagnostics GmbH, Germany) according to the instructions. Briefly, cells were treated with the RNA isolation solution to disintegrate nucleoprotein complexes. RNA was recovered from the aqueous phase by isopropanol precipitation. The mixture was centrifuged to obtain an RNA pellet, which was then washed with ethanol, dried, and resuspended in RNAse-free water. RNA integrity was checked using agarose gel electrophoresis.

The cDNA synthesis from total RNA was performed using a suitable kit (RevertAid First-Strand cDNA Synthesis Kit, Thermo Scientific, USA) according to the instructions. Quantitative PCR was performed using the primers indicated in Table 1 and SYBR Green (Roche Diagnostics, Switzerland) as a fluorescent dye in a LightCycler 480 instrument (Roche Diagnostics, Switzerland) according to the instructions. The *GAPDH* gene was used as an internal control to determine relative expression levels using the $2^{-\Delta\Delta Ct}$ formula. The results of RT-qPCR measurements from three independent experiments performed at different time points were used to determine the mean expression levels. The changes in the treated cells relative to control cells were expressed as fold changes, and mean values were statistically compared using the ANOVA and independent *t*-test between test groups where $p < 0.05$ was considered the level of statistical significance.

Results

Effect of androgen stimulation and AR inhibition on cell viability. The treatment of LNCaP cells for 24 h with DHT or enzalutamide did not meaningfully increase the rate of dead cells (Fig. 1, upper panel). In LNCaP-AR+ cells, a noticeable increase (approx. 8-fold in mean compared to control cells) in the rate of apoptotic cells was detected upon the DHT treatment (Fig. 1, lower panel). Also, in enzalutamide-treated LNCaP-AR+ cells, a small increase (2.4-fold in mean) in the number of apoptotic cells was detected.

Effectiveness of androgen stimulation and AR inhibition. Based on our previous finding that ARLNC1 (androgen receptor-regulated long noncoding RNA 1) is strongly induced by DHT treatment and that AR inhibition by enzalutamide reduces the DHT effect on ARLNC1 [16], in the present study, we considered ARLNC1 induction as evidence of effective androgen stimulation and reduced ARLNC1 levels as evidence of AR inhibition. As shown in Fig. 2, DHT treatment strongly induced ARLNC1 in both cell lines, whereas in the cells co-treated with DHT and enzalutamide, ARLNC1 expression reduced the DHT effect, suggesting an effective hormone stimulation and receptor inhibition.

PD-L1 expression in LNCaP and LNCaP-AR+ cells and the effect of AR inhibition. Compared to *GAPDH* as the internal control, we found a very low basal expression of *PD-L1* in PCa cells. The mean *PD-L1* expression in LNCaP cells was approximately 5,000-fold lower than that of *GAPDH* and 1,850-fold lower in LNCaP-AR+ cells, meaning that the basal *PD-L1* expression was on average 2.7-fold higher in LNCaP-AR+ cells than in LNCaP cells ($p = 0.04$, Fig. 3, *a*).

DHT treatment increased *PD-L1* expression in LNCaP cells by a mean of approximately threefold, although it did not reach statistical significance ($p = 0.3$), probably due to the relatively high variance between experiments. Treatment with enzalutamide significantly reduced PD-L1 expression below the basal levels ($p = 0.04$), evidencing that AR inhibition suppresses PD-L1 (Fig. 3, *b*). In the cells co-treated with DHT and enzalutamide, AR inhibition non-significantly reduced DHT-induced *PD-L1*, further confirming the suppressive effect of AR inhibition on *PD-L1*. Interestingly, in contrast to LNCaP cells, androgen stimulation by DHT did not increase but decreased PD-L1 expression in LNCaP-AR+ cells ($p = 0.2$; Fig. 3, *c*),

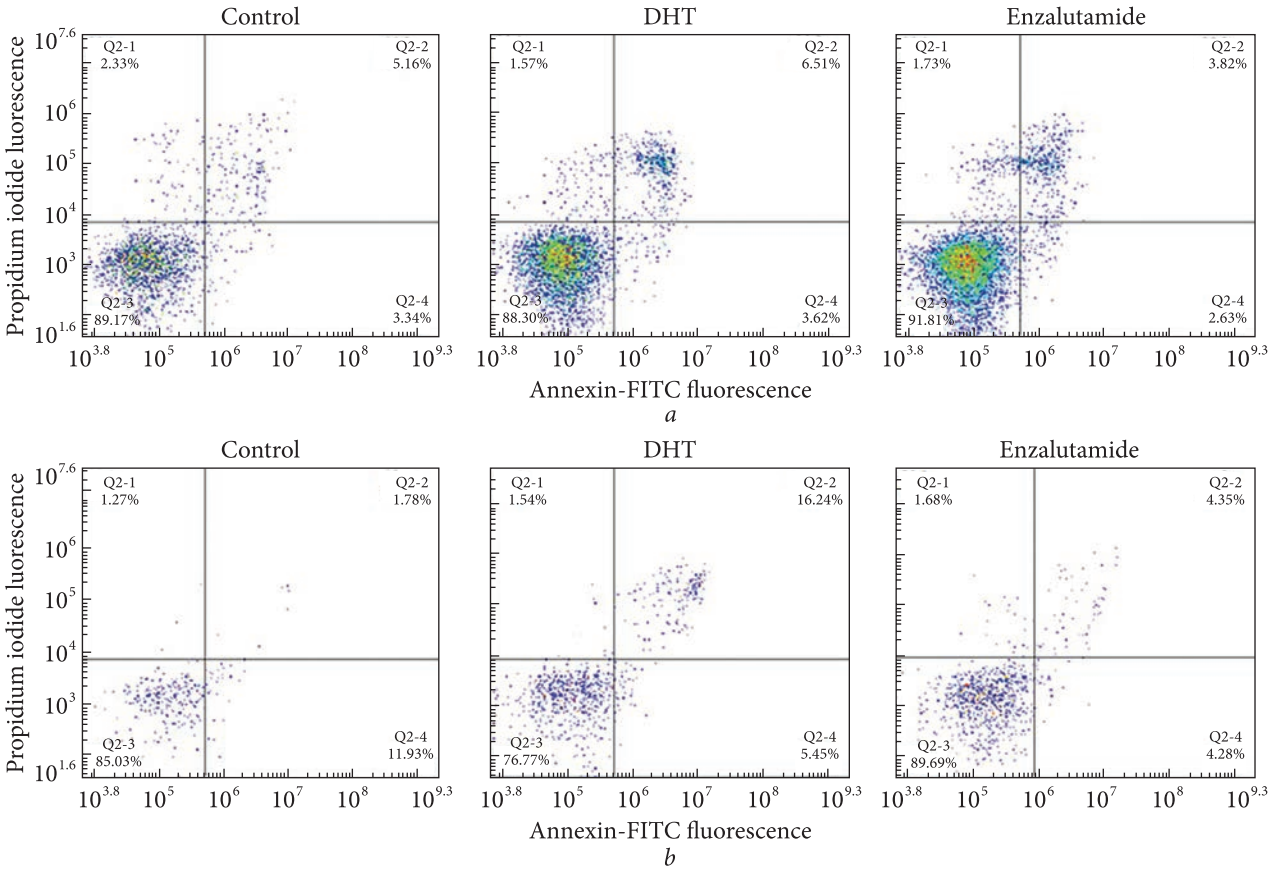

Fig. 1. Assessment of cell viability in PCa cells. The cells were grown in a hormone-free medium for 24 h, followed by treatment with 10 nM DHT or 10 μM enzalutamide for 24 h. After treatment, the cells were stained with Annexin V-FITC and propidium iodide. Following fluorescence measurement, propidium iodide (y-axis) was plotted against Annexin V-FITC (x-axis): (*a*) LNCaP cells and (*b*) LNCap-AR+ cells. DHT — dihydrotestosterone; Enz — enzalutamide

possibly affected by the cell death-inducing effect. Furthermore, enzalutamide also did not affect *PD-L1* expression ($p = 0.66$). These data reveal that *PD-L1* is expressed in an AR-dependent manner in PCa cells and its expression status can be modulated by AR inhibition in parental LNCaP cells. However, in cells overexpressing AR, *PD-L1* cannot be modulated by AR inhibition.

Discussion

The assessment of PD-L1 expression in cancer tissues has become a common practice for predicting the effectiveness of ICB therapy. It has been reported that PD-L1 expression is rare or low in primary prostate tumors [10, 11], but increased PD-L1 expression seems to be a hallmark of PCa progression [10, 13, 20]. In accordance with the low expression status of PD-L1 in primary hormone-sensitive prostate tumors, we found very low basal expression of PD-L1 in hormone-sensitive LNCaP cells. In our model, LNCAP-AR+ cells overexpressing AR had a higher basal PD-L1 expression than LNCaP cells revealing androgen-dependent expression of PD-L1 in PCa cells. However, the finding that the PC3 and DU145 cell lines, which display a lower AR expression [9], express higher levels of PD-L1 than LNCaP cells [7, 8] seems somewhat contradictory to our finding. However, it should be noted that there is some controversy regarding the level of the AR

Table 1. **Primer sequences used**

Gene	Primer sequences (5′-3′)
PD-L1	F: TATGGTGGTGCCGACTACAA R: TGGCTCCCAGAATTACCAAG
ARLNC1	F: TGAGAAGAGAAATCTATTGGAACC R: GGTTTGTCTCCGCTGCTTTA
AR	F: TATCCCAGTCCCACTTGTGTC R: CTTGTGCATGCGGTACTCATTG
GAPDH	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC

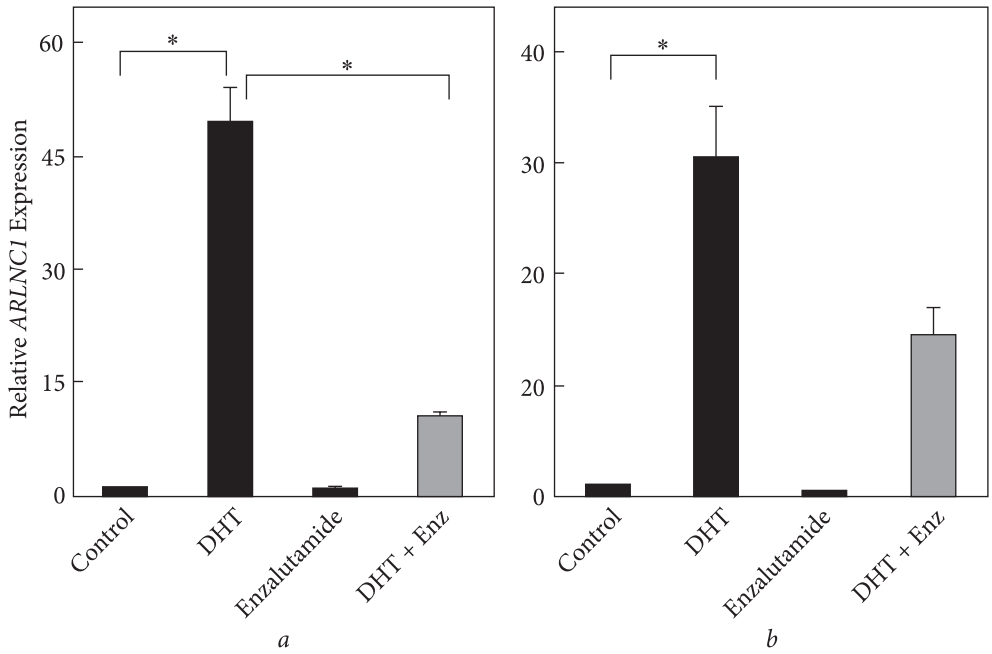

Fig. 2. *ARLNC1* expression in PCa cells. Gene expression analysis was done from the total RNA fraction isolated from control cells and the cells treated with DHT or enzalutamide and used for complementary DNA (cDNA) synthesis. The expression level of *ARLNC1* was determined semi-quantitatively using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the reference gene: (*a*) *ARLNC1* expression in LNCaP cells and (*b*) LNCaP-AR+ cells. Bar graphs show mean values and standard deviations. Comparisons shown with an asterisk (e.g. control vs. DHT and DHT vs. DHT + enzalutamide in *a* and control vs. DHT in *b*) were significant ($p < 0.01$). DHT — dihydrotestosterone; Enz — enzalutamide

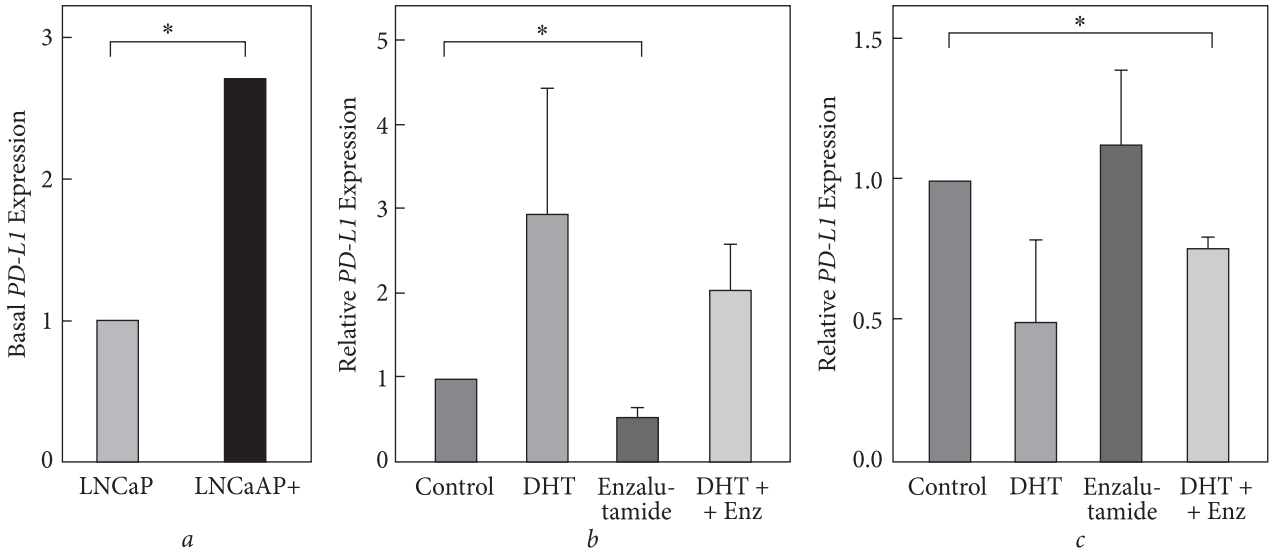

Fig. 3. *PD-L1* expression in PCa cells and the effect of hormone stimulation and receptor blockade. The *PD-L1* expression was determined semi-quantitatively with *GAPDH* as the internal control using the $2^{-\Delta\Delta Ct}$ method: (*a*) Basal expression of *PD–L1* in LNCaP and LNCaP-AR+ cells, with 2.7-fold higher expression in the latter ($p = 0.03$). (*b*) The effect of DHT or enzalutamide on *PD-L1* expression in LNCaP cells. Enzalutamide significantly reduced basal *PD-L1* level ($p = 0.04$). (*c*) The effect of DHT or enzalutamide on *PD-L1* expression in LNCaP-AR+ cells. The difference in *PD-L1* expression between the basal status and co-treatment (e.g. DHT and enzalutamide) was significant ($p = 0.04$). Bar graphs show mean values and standard deviations. DHT — dihydrotestosterone. Enz — enzalutamide

expression in PC3 and DU145 cells and that androgen stimulation with DHT does not result in stimulation of the activity of an AR-responsive reporter in these cells [9], suggesting differences in the functional AR status in different PCa cell lines. Furthermore, some additional factors, such

as the cell line origin, culture conditions, and cumulative number of cell doublings, likely influence the experimental results and can induce discrepancies in the AR and PD-L1 expressions between different studies.

We showed that the androgen stimulation strongly induces PD-L1 expression and the AR inhibition reduces the basal PD-L1 expression in the LNCaP cells, further proof of androgen-dependent expression of PD-L1. This is consistent with scientific data that reveal a positive correlation between AR expression and PD-L1 expression in prostate tumors [21]. Accordingly, a meta-analysis found that PD-L1 expression tends to be elevated in prostate tumors of AR-positive cases [22]. Also, in triple-negative breast cancer, PD-L1 expression was found to be significantly more common in cancers with AR expression [23]. The androgen-dependent expression of PD-L1 has also been demonstrated in bladder cancer cells, as both the anti-androgen treatment and AR knockdown effectively reduced membrane PD-L1 expression [24]. However, an inverse relationship has been demonstrated in some other cancer types. In hepatocellular carcinoma (HCC) cells, the AR expression was shown to negatively regulate PD-L1 by acting as a transcriptional repressor of PD-L1, and AR-expressing HCCs were more responsive to PD-L1 inhibitors [25]. Similarly, in tumor tissues from patients with muscle-invasive or metastatic urothelial carcinoma, PD-L1 expression decreased with increasing AR expression [26], and in patients with upper tract urothelial carcinoma, AR-negative tumors had significantly higher PD-L1 expression than AR-positive tumors [27]. All these findings suggest that the relationship between the androgen pathway and PD-L1 may be cancer-type-specific and vary depending on the androgen dependence of tumor growth in PCa. Accordingly, it was shown that enzalutamide-resistant PCa cells or patient-derived tumors that are AR-independent increase PD-L1 levels [20, 28], and this overexpression is independent of AR [28].

In our model, LNCAP-AR+ cells having higher basal PD-L1 expression than LNCaP cells may represent a more advanced phenotype of PCa in which the effects of androgen stimulation and AR inhibition on PD-L1 seen in LNCaP cells were not observed suggesting that the modulation of PD-L1 in AR-overexpressing cells through AR stimulation and inhibition may be affected by other mechanisms. AR not only regulates the cell survival effects of androgens but also plays an important role in mediating cell death, depending on the cellular context and extracellular stimuli [29, 30]. Since we found the increased apoptotic cell death rates in LNCaP-AR+ cells, but not in LNCAP cells, after hormone stimulation and AR inhibition, increased AR expression appears to increase susceptibility to cell death. This non-immunogenic pro-death activity of AR might influence the modulation of PD-L1 expression by androgen stimulation or AR inhibition in LNCAP-AR+ cells.

The weakness of our study is that it does not include different PCa cell lines and tumor samples. Nevertheless, this is the first study to investigate PD-L1 expression in PCa cells based on the AR status. Our data show that PD-L1 is expressed in an AR-dependent manner in PCa cells and its expression status can be suppressed by AR inhibition in parental LNCaP cells, but not in cells overexpressing AR. This differential finding should be investigated in tumor tissues for its clinical relevance as well as in the context of immunotherapy.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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МОДУЛЯЦІЯ ЕКСПРЕСІЇ *PD-L1* У КЛІТИНАХ РАКУ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ ЧЕРЕЗ ІНГІБУВАННЯ АНДРОГЕНОВОГО РЕЦЕПТОРА РОЗРІЗНЯЄТЬСЯ В ЗАЛЕЖНОСТІ ВІД СТАТУСУ РЕЦЕПТОРА

Стан питання. Терапія, спрямована на блокаду контрольних імунних точок через вісь PD-1/PD-L1, не приводить до бажаних результатів у хворих на рак передміхурової залози (РПЗ). PD-L1 як поширений предиктивний маркер ефективності імунотерапії, спрямованої на PD-1/PD-L1, експресується в клітинах первинного РПЗ, що відповідає на андрогени, лише зрідка і на низькому рівні. Експресія його підвищується в клітинах при прогресуванні РПЗ. Залежність експресії PD-L1 від статусу андрогенового рецептора (АР) не досліджувалась. **Матеріали та методи**. Ефект гормонального стимулювання дигідротестостероном (ДГТ) та інгібування АР ензалутамідом на експресію *PD-L1* досліджували в клітинах LNCaP та LNCaP-AR (останні характеризуються надекспресією АР). Клітини вирощували впродовж 24 год у середовищі, яке не містило гормонів, а потім — 24 год у присутності ДГТ (10 нмоль/л) та/або ензалутаміду (10 мкмоль/л). Життєздатність клітин оцінювали в тесті з анексином V та пропідій йодидом. Експресію *PD-L1* визначали напівкількісно на рівні мРНК. Дисперсійний аналіз та незалежний *t*-критерій Стьюдента застосовували для порівняння результатів, отриманих в різних варіантах експериментальних впливів. **Результати**. ДГТ індукував апоптоз у клітинах LNCaP-AR+ з надекспресією АР, але не в клітинах вихідної лінії LNCaP. Базальний рівень експресії *PD-L1* був низьким у клітинах обох досліджуваних ліній, хоча в клітинах LNCaP-AR+ він був у 2,7 рази вищим. ДГТ підвищував експресію *PD-L1* у клітинах LNCaP приблизно втричі. При цьому в клітинах, проінкубованих з ензалутамідом, експресія *PD-L1* була нижчою за базальний рівень. У клітинах LNCaP, проінкубованих із ДГТ разом з ензалутамідом, інгібування АР знижувало рівень *PD-L1*, індукованого ДГТ, що дозволяє припустити андроген-залежну експресію *PD-L1*. На відміну від клітин LNCaP, стимуляція андрогеном не підвищувала експресію *PD-L1* у клітинах LNCaP-AR+, і ензалутамід теж не впливав на рівень експресії *PD-L1*. **Висновки**. *PD-L1* експресується АР-залежно в клітинах РПЗ. Експресія *PD-L1* у клітинах з надекспресією АР не модулюється в разі інгібування рецептора.

Ключові слова: рак передміхурової залози, PD-L1, андроген, андрогеновий рецептор, ензалутамід.