ANTICANCER RESEARCH *39*: 5541-5549 (2019)
doi:10.21873/anticanres.13747

Linking Infection and Prostate Cancer Progression: Toll-like Receptor3 Stimulation Rewires Glucose Metabolism in Prostate Cells

MARIA CHIARA MAGNIFICO, ALBERTO MACONE, MARINA MARANI, AMANI BOUZIDI, GIORGIO GIARDINA, SERENA RINALDO, FRANCESCA CUTRUZZOLÀ and ALESSIO PAONE

Department of Biochemical Sciences A. Rossi Fanelli, Sapienza University of Rome, Rome, Italy

Abstract. *Background/Aim: The connection between prostate cancer and inflammation has been proposed many years ago, but very little is known about the metabolic adaptations of prostate cells in case of infection or inflammation. The aim of this study was to examine the effect of the stimulation of Toll-like receptor 3 (TLR3) on the metabolism of prostate cancer (PCa) cell lines and benign prostate cells. Materials and Methods: Cytofluorimetry, qRT-PCR, western blot and Gas-chromatography/Mass-spectrometry were used. Results: Reprogramming of glucose utilization involving hypoxia-inducible factor 1-alpha (HIF-1α) and the extracellular adenosine axis was observed. TLR3 stimulation synergized with adenosine receptor A2b on PCa cells, and induced a strong production of lactate, exacerbating the Warburg effect. Moreover, stimulation of benign prostate cells with poly I:C reduced lactate secretion, a characteristic typical of the neoplastic transformation. Conclusion: TLR3 stimulation promotes metabolic adaptations likely involved in the mechanisms of disease onset and progression.*

In the last decades prostate cancer has been often classified as the cancer type with the highest incidence in the western population (1). Unfortunately, the present therapies are largely ineffective if the disease is diagnosed when the cancer is already metastatic (2). While different kinds of cancer-specific genetic mutations have been shown to determine tumor onset and progression, as for example for specific colon or breast cancers (3, 4), prostate tumors are genetically very heterogeneous and difficult to categorize. On the other hand, the metabolic adaptations of prostate cancer cells have been described in more detail and, although peculiar, seem to follow precise steps during onset and progression (5). Many types of cancer cells adapt their metabolism to their new needs of highly proliferative cells, for example they begin to consume large amounts of glucose under normoxic conditions. They up-regulate glucose transporters like GLUT1, enzymes directly involved in the glycolytic process like hexokinase 2 or enzymes that control the flux of metabolites during the production of biomass, reducing power and energy, and producing lactate as a waste product; this type of adaptation is commonly referred to as the Warburg Effect (WE) or aerobic glycolysis (6). A key role in the glycolytic flux control is played by pyruvate kinase muscle isozyme 2 (PKM2), an isoform of the rate limiting enzyme responsible for pyruvate production. The expression of the M2 isoform, typical of several cancer cells, slows-down the glycolytic flux leading to the accumulation of key intermediates that are then redirected for example in the Serine Synthesis Pathway or in the Pentose Phosphate Pathway producing molecules essential for DNA replication (7). Unlike other types of cancer such as lung, breast, colon, ovary etc, which activate this remodeling in the early stage of the disease (8), prostate cancer cells switch to the WE only when the process of metastasis begins, and this metabolic adaptation becomes an indicator of bad prognosis (5). The possible events favoring the onset of prostate cancer are largely debated: among others, chronic infection and inflammation have been often suggested (9). It has been observed that men with recurrent prostatitis are more likely to develop prostate cancer (10). Surprisingly there are very few data on metabolic adaptations of prostatic cells in case of infection or inflammation and the possible involvement of these processes in the progression of the disease. Among the receptors involved in inflammation, Toll Like Receptors (TLRs) play a key role. TLRs recognize specific molecules conserved in microorganisms and activate a signaling cascade that ultimately starts innate immunity (11). It has previously

Correspondence to: Francesca Cutruzzolà, Sapienza University of Rome Dept of Biochemical Sciences A. Rossi Fanelli, Piazzale Aldo Moro 5, 00185 Rome, Italy. Tel: +39 0649910955, e-mail: francesca.cutruzzola@uniroma1.it

Key Words: Inflammation, metabolism, Warburg effect, adenosine, CD73, A2b.

been shown that normal prostate cells and prostate cancer cell lines often express high levels of TLR3 (12–14) and that the stimulation of this receptor through a synthetic double-stranded RNA (poly I:C), mimicking a viral infection, induces HIF1-α activation with the consequent secretion of vascular endothelial growth factor (VEGF) (12). HIF1-α is notoriously involved in the metabolic remodeling of cells and its induction, specifically mediated by TLR-3 activation, is able to increase cancer cell proliferation and resistance to apoptosis (15–17). Recently, several studies clarify the metabolic remodeling that takes place in specialized cells of the immune system like monocytes or NK cells after stimulation with infectious agents and an interesting parallel between these and cancer cells has been proposed (18). The microenvironment has a fundamental importance for metastatic cells, as they must learn to survive in an alien environment and to do so they take advantage of what they find in their new niche. It is known that tumor cells and the other cells of the microenvironment interact producing and secreting specific molecules influencing each other and causing specific metabolic rearrangements that ultimately favor the survival of tumor cells that start using metabolites secreted as waste product by the other cells (5). Beyond HIF1-α activation, other signals from the inflammatory microenvironment have been described to participate in the metabolic remodeling of immune cells under conditions of infection or inflammation such as signals from extracellular adenosine. It has been previously reported that adenosine purinergic receptor signaling is able to synergize with TLR often by modulating the basic signaling induced by the canonical TLR stimulation (19). The extracellular adenosine is produced from ATP secreted during the inflammatory processes (20). ATP is converted to AMP by the enzyme ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and AMP in turn is transformed into adenosine by CD73. Adenosine binds to specific receptors (A1, A2a, A2b and A3) and activates cell type-specific signaling cascades (21). Human prostate cell lines, including PC3 and DU145 express high levels only of the functional adenosine receptor A2b and its inhibition reduces cell proliferation (22). Furthermore, overexpression of CD73 in prostate cancer is associated with lymph node metastasis (23). Herein, we demonstrate that similarly to immune cells, TLR3 stimulation induces a metabolic remodeling in PCA cells by inducing genes related to glycolysis and increasing HIF1-α–dependent lactate production and CD73. We also show that TLR3 and adenosine A2b receptor signaling synergize in the induction of lactate accumulation. Intriguingly poly I:C stimulation in benign prostate cells induces a reduction in lactate and pyruvate secretion suggesting a switch to the neoplastic transformation. Our data suggest that there is a link between inflammation and prostate cancer. The metabolic adaptation of cells in the event of prolonged exposure to infectious or inflammatory factors could produce an advantage selecting cellular clones responsible for the onset or the progression of the pathology to the metastatic state.

Materials and Methods

Cell lines and reagents. All cell lines used were from ATCC (Manassas, VA, USA) and maintained in a humidified atmosphere of 5% CO_2 at 37˚C. LNCaP, PC3, and DU145 cells were cultured in RPMI medium supplemented with 100 IU/ml penicillin/streptomycin, and 10% fetal calf serum (FCS; Sigma-Aldrich, St Louis, MO, USA). RWPE1 cells were grown in keratinocyte serum-free medium (K-SFM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/ml human recombinant epidermal growth factor (EGF) and 100 IU/ml penicillin/streptomycin. Where indicated, the cells were treated with poly I:C (high molecular weight) obtained from InvivoGen (San Diego, CA, USA) and 5'-(N-Ethylcarboxamido) adenosine (NECA) purchased from (Sigma-Aldrich).

RNA interference (iRNA). 1×10^5 cells/ml were seeded and simultaneously transfected for 18 h with the Qiagen AllStars RNAi Controls (scrambled sequences; Qiagen, Hilden, Germany) or the bulk of 4 sequences targeting HIF1-α (GeneSolution siRNA (Qiagen) using JetPrime (Polyplus transfection, Strasbourg, France) following the manufacturer instructions. The medium was then replaced with fresh medium and the cells eventually treated 48 h after transfection.

The target sequences of hif-α gene were:
HIF1-α_6 AGCCATTTACATAATATAGAA; HIF1-α_5 AGGAAGA ACTATGAACATAAA
HIF1-α_12 CACAGGCCACATTCACGTATA; HIF1-α_11TCGG CGAAGTAAAGAATCTGA

RNA extraction and real time qRT-PCR. Total RNA was extracted using TRIZOL reagent (Invitrogen) following the manufacturer instructions. One μg of RNA was used for reverse transcription (cDNA synthesis) by using the SuperScript First-Strand Synthesis System Kit (Invitrogen). qRT–PCR analysis was performed in triplicate for each sample using KAPA Sybr Fast universal premix following the manufacturer instructions. Each tube contained 10 μl of 2x qPCRBIOSyGreen Mix (KAPA), 0.8 μl of each primer, 1 μl of cDNA and 7.4 μl of water. Reactions were performed on a Stratagene MX3000P (Stratagene, La Jolla, CA, USA) using the following settings: 95˚C for 2 min, and 40 cycles for 95˚C for 5 sec and 60˚C for 25 sec; the dissociation curve was obtained using the standard machine settings. The primers used were:
Glut1 FWD. AACTCTTCAGCCAGGGTCCAC; Glut1 REV. CACAGTGAAGATGATGAAGAC; Hexokinase2 FWD. GAGCCA CCACTCACCCTACT; Hexokinase2 REV. CCAGGCATTCGGC AATGTG; PKM2 FWD. GAGGCCTCCTTCAAGTGCT; PKM2 REV. CCAGACTTGGTG AGGACGAT.

Lactate and pyruvate quantification. Lactic acid and pyruvic acid were analyzed as methoxime/tertbutyldimethylsilyl derivatives as previously described (24). Briefly, 300,000 cells were seeded in 6 well plates and eventually treated as described. For the intracellular analysis, cells were detached using trypsin, counted and the cells from 2 wells pooled together, collected by centrifugation at 1,200 rpm for

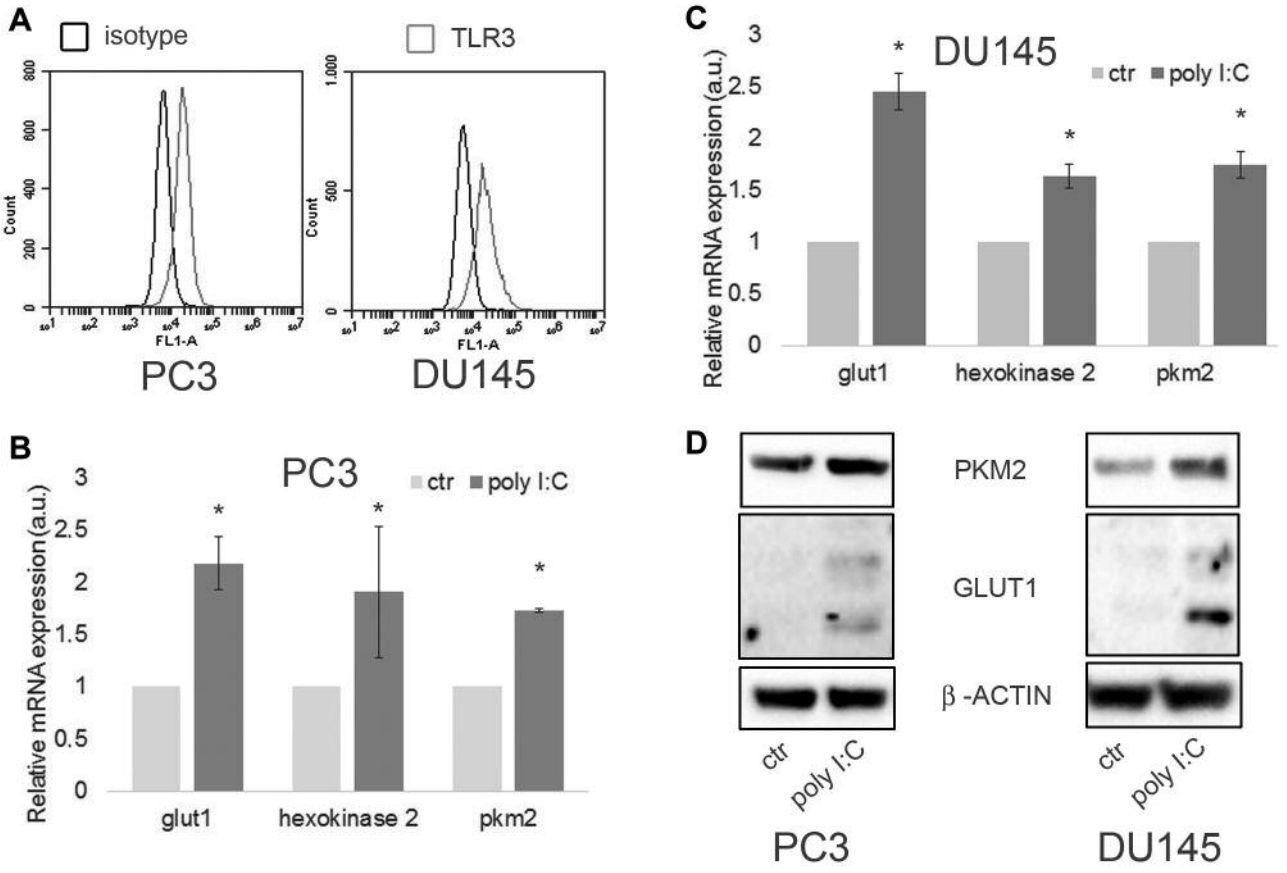

Figure 1. *Analysis of TLR3 expression and metabolic effect of its activation in PC3 and DU145 cells. (A) Analysis of TLR3 expression by flow cytometry in PC3 and DU145 cells. Effect of 1 μg/ml poly I:C treatment on the mRNA expression of the glucose transporter Glut1 and the enzymes Hexokinase2 and Pyruvate Kinase evaluated by qRT-PCR in PC3 (B) and DU145 cells (C). The data represent the average of three independent experiments (*p<0.05). Panel D shows the effect of 1μg/ml poly I:C treatment on Glut1 and PKM2 protein expression in PC3 and DU145 cells evaluated by western blot, β-Actin was used as loading control.*

10 min then washed with 1 ml PBS, and collected again by centrifugation. For the cell medium analysis, 20 μl of cell supernatant were used. Cell pellet or medium were deproteinized by adding 100 μl of acetonitrile and vortexed for 3 min. The mixtures were diluted 1:10 with distilled water and centrifuged at 15,000 rpm for 15 min at 4˚C to pellet proteins. The deproteinized supernatant was used to quantify extracellular L-lactate by Gas-Chromatography/Mass-Spectrometry (GC-MS) analysis. Aliquots of 0.25 ml of the supernatant layer spiked with the internal standard (IS) 3,4-dimethoxybenzoyc acid (final concentration 1000 ng/ml) were added to 0.7 ml of distilled water and adjusted to pH ≥13 with 7 M NaOH. Methoxymation was performed by adding to the reaction mix methoxyamine hydrochloride (5 mg) at 60˚C for 60 min. The samples were then washed with diethylether (3 ml × 2) and the aqueous phase was adjusted to pH < 2 with concentrated sulfuric acid. The mixture was saturated with NaCl and extracted with diethyl ether (3 ml) and ethyl acetate (2 ml). The organic extracts were combined in the presence of triethylamine (10 μl) and dried under reduced pressure. The samples were then suspended in 30 μl of toluene, subjected to the second derivatization step by adding 20 ml of N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (65˚C for 30 min), and analyzed by GC-MS. Results were normalized for cell number and expressed as fold change relative to control samples. GC-MS analyses were performed with an Agilent 7890B gas chromatograph coupled to a 5977B quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separations were carried out with an Agilent HP5ms fused-silica capillary column (30 m × 0.25 mm i.d.) coated with 5%-phenyl-95%-dimethylpolysiloxane (film thickness 0.25 μm) as stationary phase. Injection mode: splitless at a temperature of 280˚C. Column temperature program: 70˚C (1 min) then to 300˚C at a rate of 20˚C/min and held for 10 min. The carrier gas was helium at a constant flow of 1.0 ml/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy; ion source 280˚C; ion source vacuum 10 -5 Torr. MS analysis was performed simultaneously in TIC (mass range scan from m/z 50 to 600 at a rate of 0.42 scans/sec/) and SIM mode. GC-SIM-MS analysis was performed selecting the following ions: m/z 174 for pyruvate, m/z 261 for lactate and m/z 239 for 3,4-dimethoxybenzoic acid (internal standard). For the standardization of values, the number of cells was used.

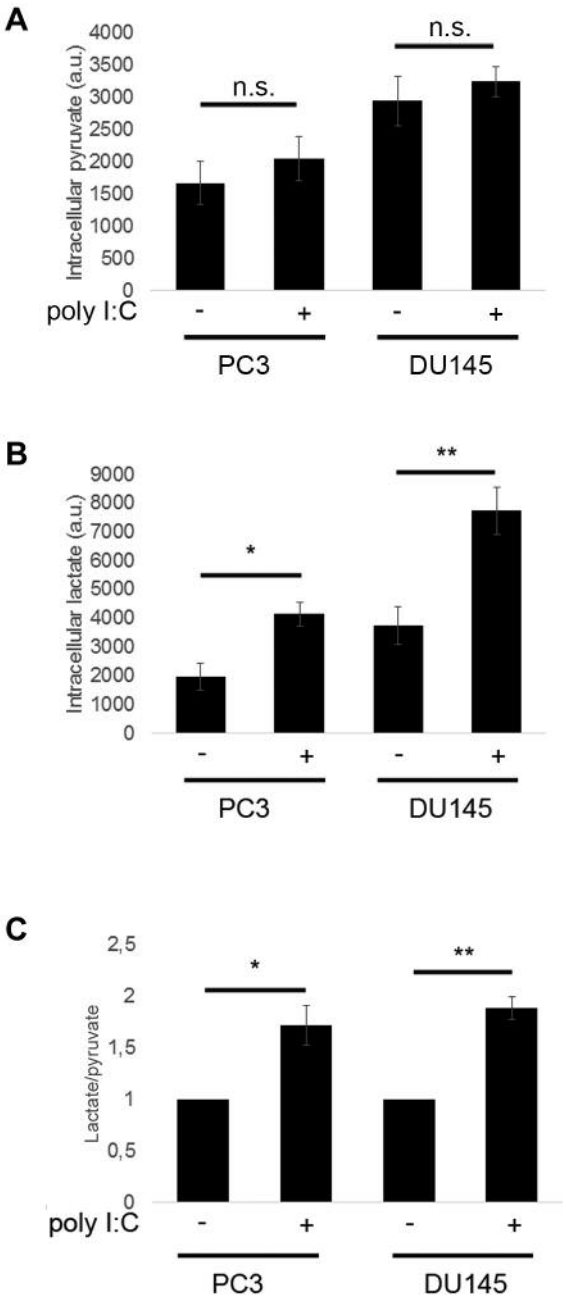

Figure 2. *Poly I:C treatment induces the modulation of specific metabolites in PC3 and DU145 cell lines. The intracellular amount of pyruvate (A) and lactate (B) produced were evaluated by gas chromatography/mass spectrometry. The ratio between lactate and pyruvate is represented in C. The data represent the average of three independent experiments. * p<0.05, **p<0.001, N.s.: Non-significant.*

Western blot. Cell lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, Leiden, The Nederlands) for 30 min on ice. Total protein concentration was determined using the BCA Kit (Thermo Fisher Scientific). A total of 15 μg of proteins were subject to SDS-PAGE and then transferred onto nitrocellulose. The membranes were saturated with 5% nonfat dry milk (Santa Cruz Biotechnology, Dallas, TX, USA) in PBS containing 0.1% Tween-20 (PBS-T) and then incubated overnight with the primary antibody at 4˚C. After three washes (10 min each) with PBS-T, blots were incubated for 1 h at room temperature with horseradish-conjugated secondary antibody. Membranes were washed again with PBS-T and developed using the chemiluminescence system (Luminata Crescendo Western HRP substrate, Millipore, Billerica MA, USA). Primary antibodies used were: anti PKM2 and anti HIF1-α, both from Cell Signaling Technology and anti GLUT1 and anti β-ACTIN, both from Santa Cruz Biotechnology. The secondary antibodies were goat anti-rabbit and goat anti-mouse, both from Santa Cruz Biotechnology. For densitometric analysis the software ImageJ was used.

Flow cytometry. Cells were detached from the culture dishes with trypsin/EDTA and washed with PBS plus 1% BSA. For TLR3 detection, the mouse immunoglobulin G fluorescein isothiocyanate-conjugated anti-TLR3 monoclonal antibody was used. As control fluorescein isothiocyanate-conjugated mouse IgG1κ Isotype Control was used (Adipogen Life Sciences, San Diego, CA, USA). After washings, cells were analyzed with an Accuri B6 cytometer. To exclude dead cells and cell debris, they were gated using forward versus side scatter. 104 cells/sample were acquired for quantification of the expression of the TLR3 and the mean fluorescence intensity was calculated.

Statistical analysis. Data presented as the mean±standard deviation of at least three independent experiments. Student's *t*-test was used for paired sample analysis, all the other statistical analyses were performed by one-way ANOVA followed by the Bonferroni *post-hoc* comparison test. $p<0.05$ was considered significant.

Results

TLR3 is expressed on human PCa cells and its stimulation increases the expression of genes involved in glucose metabolism. The expression of TLR-3 was initially examined on two of the most commonly used prostate cancer cell lines, PC3 and DU145. Figure 1A shows similar levels of expression of the receptor in the two different cell lines. We have previously shown that stimulation of human prostate cancer cells with poly I:C, the synthetic activator of TLR3, induces the transcription factor HIF1-α (12). Considering the ability of HIF1-α to modulate the expression of many genes involved in glycolysis, we decided to examine the expression of some of these genes after stimulation of PC3 and DU145 cells with 1 μg/ml of poly I:C. Figure 1B and C shows that, after 24 h of stimulation, the expression of genes coding for the glucose transporter GLUT1 and the enzymes hexokinase2 and PKM2 significantly increased in both cell lines. Western blot analysis was performed to validate the data obtained with the qRT-PCR experiments. Figure 1D shows that in both cell lines poly I:C stimulation increased GLUT1 and PKM2 expression also at the protein level. These data suggested that the activation of TLR3 is able to induce metabolic modulations, probably boosting glucose consumption.

Poly I:C treatment of PCa cell lines induces intracellular lactate accumulation. One of the main indicators of the WE is lactate accumulation. Mass spectrometry was used to study the concentration of lactate and pyruvate in the cell culture media of PC3 and DU145 cells stimulated or not with poly I:C 1 μg/ml. Poly I:C does not have a significant effect on the amount of lactate and pyruvate in the cell culture medium (data not shown). The same analysis was carried out on extracts from cells treated with and without poly I:C for 24 h. Poly I:C stimulation had no effect on pyruvate concentrations (Figure 2A), while it induced a two-fold accumulation of lactate in both cell lines (Figure 2B), as evident also by the analysis of the intracellular lactate/pyruvate ratio (Figure 2C).

Poly I:C-induced lactate accumulation in PCa cells is HIF1-α dependent. To demonstrate whether accumulation of lactate observed in PC3 and DU145 cells after TLR3 stimulation is dependent on the activation of HIF1-α, the cells were transfected with RNA interference (iRNA) oligos directed against hif1-α (iHif1-α) or a control iRNA (iscr) and then were stimulated with 1 μg/ml poly I:C. Initially, the efficacy of transfection with HIF1-α interfering oligos was confirmed. Figure 3A and B show that poly I:C treatment induced an increase of HIF1-α in cells transfected with the scramble sequence, while a significantly lower increase of HIF1-α was observed in cells transfected with oligos against HIF1-α. We then evaluated the metabolites concentration using mass spectrometry. Figure 3C shows that while iHif1-α or iscr oligos alone did not significantly modulate the lactate/pyruvate ratio in both cell lines, treatment with poly I:C reduced the ratio of lactate/pyruvate by about 50% in cells transfected with iHif1-α compared to cells transfected with iscr. These results indicated that, at least in part, the metabolic modulation described above is directly dependent on the activation of HIF1-α. Interestingly, in agreement with our working hypothesis, in the LnCap prostate cancer cell line that is unable to activate HIF1-α after stimulation with poly I:C (12), no modulation of the intracellular or extracellular concentrations of lactate and pyruvate was observed (Figure 3D and data not shown, respectively).

A2b and CD73 modulation after poly I:C treatment of PCa cell lines. It has been demonstrated that CD39 is constitutively expressed in prostate cells (25). To assess whether TLR3 stimulation modulates the extracellular adenosine axis, the expression of CD73 and A2b receptor was evaluated in PC3 and DU145 cells stimulated or not with Poly I:C. Figure 4 shows that treatment with 1 μg/ml poly I:C produced an 1.8-fold increase in the expression of CD73 in PC3 cells and a 7-fold induction in DU145 cells.

TLR3 and A2b signaling synergize in the induction of lactate accumulation in PCa cell lines. To evaluate a

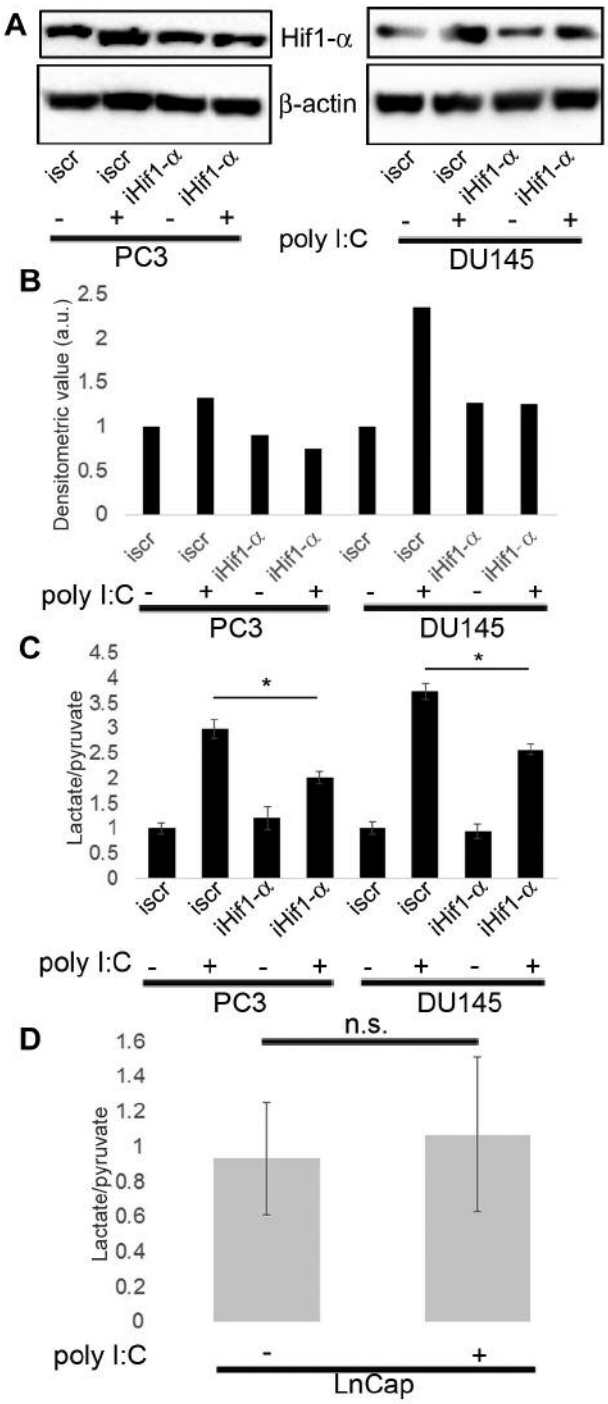

Figure 3. *Analysis of the interplay between HIF1-α expression and glycolytic metabolism in PC3 and DU145 cells. (A) Effect of 1 μg/ml poly I:C on the amount of HIF1-α protein evaluated by western blot. (B) Densitometric analysis of the western blot in A. The normalized ratio HIF1-α/β-actin is shown. (C) The intracellular ratio lactate/pyruvate evaluated by GC/MS in PC3 and DU145 cells transfected with a control iRNA (iscr) and an RNA interference directed against HIF1-α. (D) Effect of poly I:C treatment on the intracellular ratio lactate/pyruvate of LNCaP cells. The data in the graphs represent the average of three independent experiments. *p<0.05, N.s.: Non-significant.*

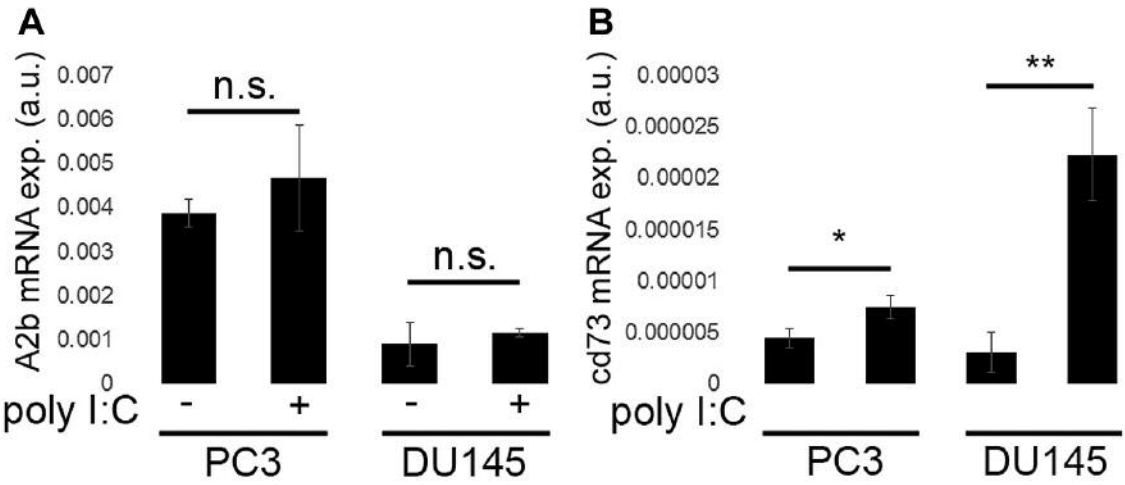

Figure 4. *qRT-PCR-based expression analysis of A2b (A) and CD73 (B) mRNA in prostate cancer cell lines PC3 and DU145 treated or not with poly I:C for 24 h. The data represent the average of 3 independent experiments. *p<0.05, N.s.: Non-significant.*

possible synergy in the onset of the WE between the signaling induced by TLR3 and that induced by the A2b receptor, PC3 and DU145 cells were treated with 5'-(N-Ethylcarboxamido) adenosine (NECA) a known A2b receptor activator, in combination or not with poly I:C. The data in Figure 5 show that the activation of the A2b receptor is able to induce a modest increase in the lactate/pyruvate ratio in both cell lines; however, the combination of the two treatments led to a 10-fold increase in the lactate/pyruvate ratio in both cell lines, highlighting an evident synergistic effect of the two pathways in the metabolic remodeling on cancer cells.

Effect of TLR3 and A2b stimulation in benign human prostate cells RWPE1. To better understand the meaning of the data reported above, our analysis was extended to the benign prostate cell line RWPE1.

Cytometric analysis was initially performed to examine whether expression of TLR3 in RWPE1 is similar to that observed in PC3 and DU145 cells (Figure 6A). RWPE1 stimulation with 1 μg/ml poly I:C induced an about 6 and 2 times decrease in the levels of pyruvate and lactate in the cell culture medium, respectively (Figure 6B). No effect was observed in their intracellular levels (data not shown). As observed in prostate cancer cells, the same stimulation does not alter the expression of A2b receptor but induces a fivefold increase in the expression of CD73 (Figure 6C). Surprisingly stimulation with NECA does not induce variations in either intra- or extra-cellular lactate or pyruvate concentration in the cells treated or not with poly I:C (data not shown and Figure 6C).

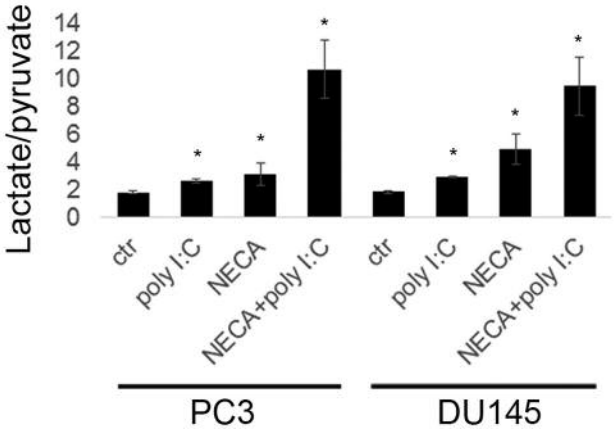

Figure 5. *Effect of poly I:C, NECA or the combined treatment on the intracellular lactate/pyruvate ratio in PC3 and DU145 cells. The data represent the average of three independent experiments. *p<0.05.*

Discussion

In recent years, several mechanisms of metabolic adaptation have been established in different cells of the immune system once activated. These adaptations, including increased glycolysis and reduced OXPHOS, are instrumental to support these cells in the production of large amounts of cytokines, antibodies and specific receptors as well as in the activation of rapid proliferation programs (26). Interestingly, cancer cells subjected to the same inflammatory stimuli are able to reprogram their metabolism, suggesting a parallelism that needs to be further explored (18).

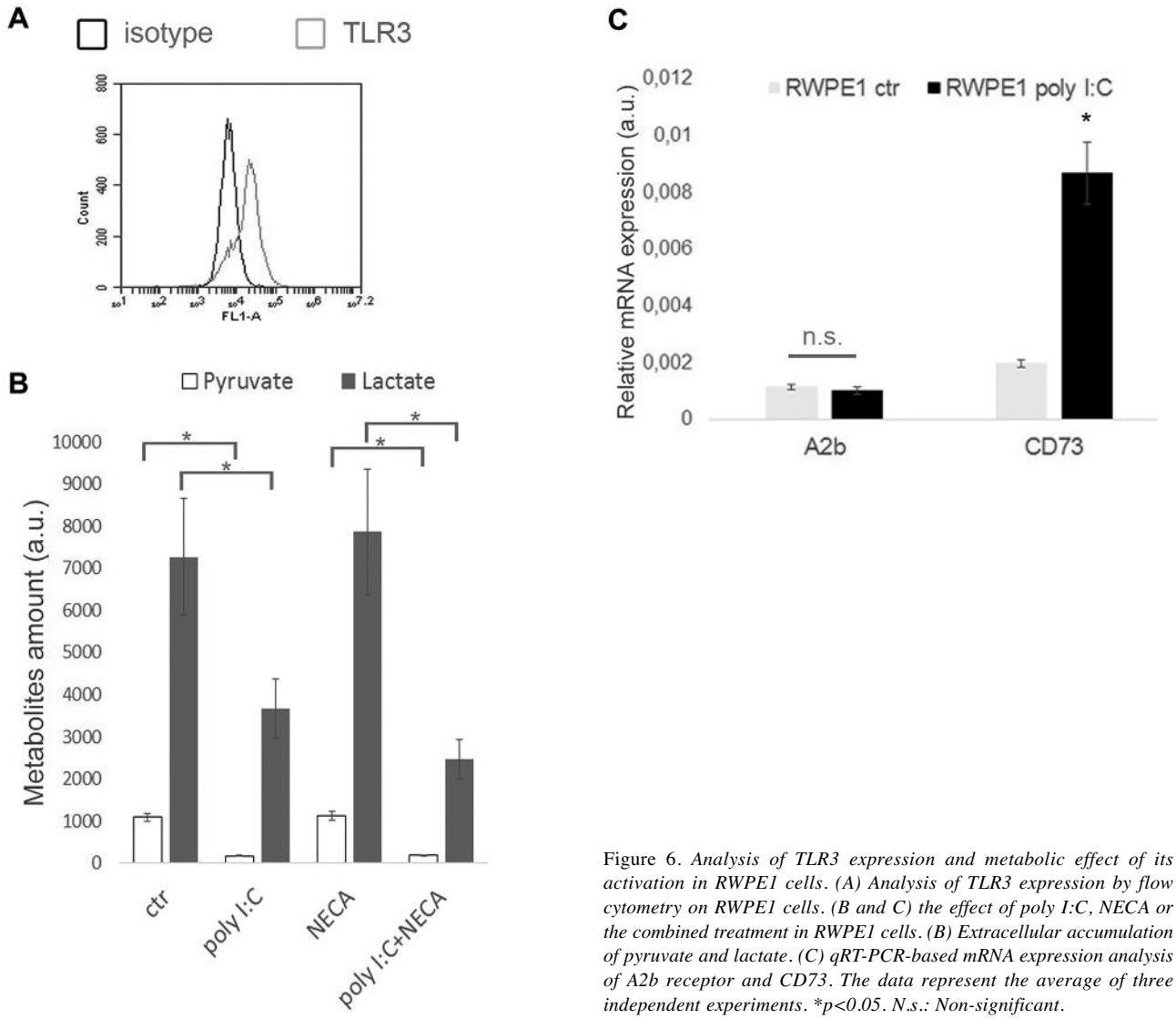

Figure 6. *Analysis of TLR3 expression and metabolic effect of its activation in RWPE1 cells. (A) Analysis of TLR3 expression by flow cytometry on RWPE1 cells. (B and C) the effect of poly I:C, NECA or the combined treatment in RWPE1 cells. (B) Extracellular accumulation of pyruvate and lactate. (C) qRT-PCR-based mRNA expression analysis of A2b receptor and CD73. The data represent the average of three independent experiments. *p<0.05. N.s.: Non-significant.*

Prostate inflammation is a very common pathology and, although the association between recurrent inflammation and prostate cancer onset and progression has been proposed many years ago and still debated, little is known about the metabolic adaptations of prostate cancer cells caused by infectious agents. The prostate has a peculiar metabolic profile and specific changes are commonly observed during the progression of prostate cancer (5); as an example, prostate cells activate the WE only when they become metastatic. For prostate cancer, therefore, the pattern of appearance or exacerbation of the WE suggests that this metabolic switch could facilitate the survival of cells in the stages of increased aggressiveness and the possible adaptation in microenvironments alien to the primary tumor sites. As clearly demonstrated in several models, the WE is not only a mechanism for fast production of energy, but also a mechanism that helps cancer cells to produce the building blocks and reducing units necessary for a high rate of cell proliferation (7).

Our data indicated that, mimicking a viral infection, TLR3 stimulation and subsequent HIF1-α activation directly induce the exacerbation of the WE in prostate tumor cells, by inducing expression of genes involved in glucose import and metabolism and the accumulation of lactate. We also demonstrated that poly I:C-induced lactate is not secreted but remains within cancer cells. Given the low efficiency of aerobic glycolysis in terms of energy production, the increased glucose utilization is probably related to the increased need of intermediate metabolites and/or reducing units. The observed increased

expression of PKM2 suggests, for example, a possible activation of the serine synthesis pathway or the pentose phosphate pathway (7). Additional experiments are needed to fully clarify which are the pathways driving the observed phenomenon under these specific inflammatory conditions. Interestingly, these cells, by blocking lactate secretion, avoid wasting it and allow its recycling to acetyl-coA which is then used in the TCA and offers an energetic gain. Among the various effects induced by pathogen-sensing receptors (including TLRs), ATP secretion has been also observed (27). In cells expressing CD39, secreted ATP is immediately transformed into AMP and then by CD73 into adenosine, which in turn activates specific receptors expressed differentially by the various cell types. The engagement of adenosine receptors has been shown to induce the inactivation (anergy) of the cells of the immune system. Indeed, this could be one of the mechanisms by which cancer cells producing large amounts of adenosine escape the immune system (28). However, adenosine is not bound only to receptors expressed on immune cells, but also to those expressed by tumor cells inducing an autocrine loop. Our data showed that TLR3 signaling synergizes with A2b receptor signaling [the only receptor expressed on the PC3 and DU145 cells (22)] suggesting a possible cooperation in the complex tumor microenvironment that could boost the WE further favoring these cells from a metabolic point of view.

In contrast to all other tissues, normal prostate epithelium produces and secretes citrate through a physiologic truncation of the TCA at the level of aconitase. Mitochondrial pyruvate import is, thus, critical to ensure an abundant supply of pyruvate to fuel citrate production. During oncogenic transformation, inactivation of aconitase restores the normal TCA cycle (5). Treatment of the RWPE1 cell line with poly I:C does not promote intracellular lactate accumulation, but dramatically reduces the amount of pyruvate and only partially that of lactate secreted in the culture medium suggesting a metabolic remodeling with increased consumption of these metabolites characteristicly observed during the pathology progression and related to the expression of GLUT1 (29). Surprisingly, while activation of TLR3 induces up-regulation of two CD73 gene, the stimulation of A2b receptor does not induce significantly different effect in terms of metabolic rearrangement in combination, or not with poly I:C.

Conclusion

Our data suggest that both normal prostate and prostate cancer cells gain an obvious advantage from prolonged exposure to agents that induce inflammation such as poly I:C. In benign cells treated with poly I:C, the use of metabolites increases, which is a typical behavior of cells at the beginning of the neoplastic transformation (29). On the other hand, in tumor cells, an exacerbation of the WE is observed, possibly, in order to sustain the metastatic cells in the new microenvironment. Future developments should also consider that the microenvironment of cancer cells is complex and includes many other cell types such as cancer associated fibroblasts, immune cells and others that could participate to metabolic reprogramming, by secreting several metabolites or molecules (including adenosine) that may synergize with those described here.

Conflicts of Interest

The Authors declare that there exist no conflicts of interest regarding this study.

Authors' Contributions

MCM, AM, MM, AB, AP, SR, GG performed experiments; AP and FC designed experiments; MCM, AP and FC wrote the paper, FC provided funding.

Acknowledgements

Funds from the AIRC (Grant n. IG16720) and the University of Rome La Sapienza (Grants n.RP11715C644A5CCE, RG118164 30AF48E1 and GA116154C8A94E3D) to FC are gratefully acknowledged.

References

1 Cleary J, Gelband H and Wagner J: Cancer: DiseaCancer statistics, 2016. Siegel RL, Miller KD and Jemal A (eds.). se Control Priorities. Dis Control Priorities 3rd Ed, pp. 1-363, 2015. PMID: 25246403. DOI: 10.1017/CBO9781107 415324.0042

2 Quinn DI, Sandler HM, Horvath LG, Goldkorn A and Eastham JA: The evolution of chemotherapy for the treatment of prostate cancer. Ann Oncol *28*: 2658-2669, 2017. PMID: 29045523. DOI: 10.1093/annonc/mdx348

3 Ryan BM, Wolff RK, Valeri N, Khan M, Robinson D, Paone A, Bowman ED, Lundgreen A, Caan B, Potter J, Brown D, Croce C, Slattery ML and Harris CC: An analysis of genetic factors related to risk of inflammatory bowel disease and colon cancer. Cancer Epidemiol *38*: 583-590, 2014. PMID: 25132422. DOI: 10.1016/j.canep.2014.07.003

4 Wood LD, Al E, Wood LD, Parsons DW, Jones S, Lin J, Sjöblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V and Nikolskaya T: The genomic landscapes of human breast and colorectal cancers. Science *318(5853)*: 1108-1113, 2007. PMID: 17932254. DOI: 10.1126/science.1145720

5 Cutruzzolà F, Giardina G, Marani M, Macone A, Paiardini A, Rinaldo S and Paone A: Glucose metabolism in the progression of prostate cancer. Front Physiol 8, 2017. PMID: 28270771. DOI: 10.3389/fphys.2017.00097

6 Feron O: Pyruvate into lactate and back: From the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol *92*: 329-333, 2009. PMID: 19604589. DOI: 10.1016/j.radonc.2009.06.0257

7 Dong G, Mao Q, Xia W, Xu Y, Wang J, Xu L and Jiang F: PKM2 and cancer: The function of PKM2 beyond glycolysis (Review). Oncol Lett *11*: 19801986, 2016. PMID: 26998110. DOI: 10.3892/ol.2016.4168

8 Zhu A, Lee D and Shim H: Metabolic PET imaging in cancer detection and therapy response. Changes *29*: 997-1003, 2012. PMID: 1000000221. DOI: 10.1016/j.biotechadv.2011.08.021

9 Sfanos KS, Isaacs WB and De Marzo AM: Infections and inflammation in prostate cancer. Am J Clin Exp Urol *1*: 3-11, 2013 PMID: 25110720.

10 Jiang J, Li J, Zhang Y, Zhu H, Liu J and Pumill C: The role of prostatitis in prostate cancer: Meta-analysis. PLoS One *8*: e85179, 2013. PMID: 24391995. DOI: 10.1371/journal.pone. 0085179

11 Barton GM and Medzhitov R: Toll-like receptor signaling pathways. Science *300*: 1524-1525, 2003. PMID: 12791976. DOI: 10.1126/science.1085536

12 Paone A, Galli R, Gabellini C, Lukashev D, Starace D, Gorlach A, De Cesaris P, Ziparo E, Del Bufalo D, Sitkovsky MV, Filippini A and Riccioli A: Toll-like receptor 3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor 1α. Neoplasia *12*: 539-549, 2010. PMID: 20651983. DOI: 10.1593/neo.9210613

13 Galli R, Paone A, Fabbri M, Zanesi N, Calore F, Cascionee L, Acunzo M, Stoppacciaro A, Tubaro A, Lovat F, Gasparini P, Fadda P, Alder H, Volinia S, Filippini A, Ziparo E, Riccioli A and Croce CM: Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RARβ and tumor regression. Proc Natl Acad Sci USA *110*: 9812-9817, 2013. PMID: 23716670. DOI: 10.1073/pnas.1304610110

14 Paone A, Starace D, Galli R, Padula F, De Cesaris P, Filippini A, Ziparo E and Riccioli A: Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-α-dependent mechanism. Carcinogenesis *29*: 1334-1342, 2008. PMID: 18566014. DOI: 10.1093/carcin/bgn149

15 Amelio I, Cutruzzolá F, Antonov A, Agostini M and Melino G: Serine and glycine metabolism in cancer. Trends Biochem Sci *39(4)*: 191-198, 2014. PMID: 24657017. DOI: 10.1016/j.tibs. 2014.02.004

16 Giardina G, Brunotti P, Fiascarelli A, Cicalini A, Costa MGS, Buckle AM, Di Salvo ML, Giorgi A, Marani M, Paone A, Rinaldo S, Paiardini A, Contestabile R and Cutruzzolà F: How pyridoxal 5'-phosphate differentially regulates human cytosolic and mitochondrial serine hydroxymethyltransferase oligomeric state. FEBS J *282*: 1225-1241, 2015. PMID: 25619277. DOI: 10.1111/febs.1321117

17 Karagiannis TC, Malik N, Ververis K, Ngo DC, Courtnay R and Tortorella SM: Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. Mol Biol Rep *42*: 841851, 2015. DOI: 10.1007/s11033-015-3858-x

18 Andrejeva G and Rathmell JC: Similarities and distinctions of cancer and immune metabolism in inflammation and tumors. Cell Metab *26*: 49-70, 2017. PMID: 28683294. DOI: 10.1016/j.cmet.2017.06.00419

19 Power Coombs MR, Belderbos ME, Gallington LC, Bont L and Levy O: The adenosine system modulates Toll-like receptor function: basic mechanisms, clinical correlates and translational opportunities. Expert Rev Anti Infect Ther *9*: 261-269, 2011. PMID: 21342073. DOI: 10.1586/eri.10.158

20 Dosch M, Gerber J, Jebbawi F and Beldi G: Mechanisms of ATP release by inflammatory cells. Int J Mol Sci 19, 2018. PMID: 29669994. DOI: 10.3390/ijms1904122221

21 Burnstock G: Purinergic signalling: Therapeutic developments. Front Pharmacol *8*: 661, 2017. PMID: 28993732. DOI: 10.3389/fphar.2017.00661

22 Wei Q, Costanzi S, Balasubramanian R, Gao ZG and Jacobson KA: A2B adenosine receptor blockade inhibits growth of prostate cancer cells. Purinergic Signal *9*: 271-280, 2013. PMID: 29045523. DOI: 10.1093/annonc/mdx348

23 Yang Q, Du J and Zu L: Overexpression of CD73 in prostate cancer is associated with lymph node metastasis. Pathol Oncol Res *19*: 811-814, 2013. PMID: 23653114. DOI: 10.1007/s12253-013-9648-7

24 Paik MJ, Cho EY, Kim H, Kim KR, Choi S, Ahn YH and Lee G: Simultaneous clinical monitoring of lactic acid, pyruvic acid and ketone bodies in plasma as methoxime/tertbutyldimethylsilyl derivatives by gas chromatography-mass spectrometry in selected ion monitoring mode. Biomed Chromatogr *22*: 450-453, 2008. PMID: 18254151. DOI: 10.1002/bmc.96625

25 Bastid J, Regairaz A, Bonnefoy N, Dejou C, Giustiniani J, Laheurte C, Cochaud S, Laprevotte E, Funck-Brentano E, Hemon P, Gros L, Bec N, Larroque C, Alberici G, Bensussan A and Eliaou JF: Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. Cancer Immunol Res *3*: 254-265, 2014. PMID: 25403716. DOI: 10.1158/2326-6066.cir-14-0018

26 Lachmandas E, Boutens L, Ratter JM, Hijmans A, Hooiveld GJ, Joosten LAB, Rodenburg RJ, Fransen JAM, Houtkooper RH, Van Crevel R, Netea MG and Stienstra R: Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. Nat Microbiol *2*: 16246. 2016. PMID: 27991883.DOI: 10.1038/nmicrobiol.2016.246

27 Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G and Rubartelli A: ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1 and IL-18 secretion in an autocrine way. Proc Natl Acad Sci *105*: 8067-8072, 2008. DOI: 10.1073/pnas.0709684105

28 Ohta A and Sitkovsky M: Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature *414*: 916-920, 2001. PMID: 11780065. DOI: 10.1038/414916a

29 Effert P, Beniers AJ, Tamimi Y, Handt S and Jakse G: Expression of glucose transporter 1 (Glut-1) in cell lines and clinical specimens from human prostate adenocarcinoma. Anticancer Res *24(5A)*: 3057-3063, 2004. PMID: 15517916.

30 Costello LC and Franklin RB: The Intermediary Metabolism of the Prostate: A key to understanding the pathogenesis and progression of prostate malignancy. Oncology *59*: 269-282, 2000. PMID: 11096338. DOI: 10.1159/000012183

Received August 7, 2019
Revised August 19, 2019
Accepted August 20, 2019