Combined inhibition of $JAK_{1,2}$/Stat3-PD-L1 signaling pathway suppresses the immune escape of castration-resistant prostate cancer to NK cells in hypoxia

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Abstract. Castration-resistant prostate cancer (CRPC) is difficult to treat in current clinical practice. Hypoxia is an important feature of the CRPC microenvironment and is closely associated with the progress of CRPC invasion. However, no research has been performed on the immune escape of CRPC from NK cells. The present study focused on this subject. Firstly, when the CRPC cell lines C4-2 and CWR22Rv1 were induced by hypoxia, the expression of the UL16 binding protein (ULBP) ligand family of natural killer (NK) group 2D (NKG2D; ULBP-1, ULBP-2 and ULBP-3) and MHC class I chain-related proteins A and B (MICA/MICB) decreased. NKG2D is the main activating receptor of NK cells. Tumor cells were then co-cultured with NK cells to conduct NK cell-mediated cytotoxicity experiments, which revealed the decreased immune cytolytic activity of NK cells on hypoxia-induced CRPC cells. In exploring the mechanism behind this observation, an increase in programmed death-ligand 1 (PD-L1) expression in CRPC cells induced by hypoxia was observed, while the addition of PD-L1 antibody effectively reversed the expression of NKG2D ligand and enhanced the cytotoxic effect of NK cells on CRPC cells. In the process of exploring the upstream regulatory factors of PD-L1, inhibition of the Janus kinase $(JAK)_{1,2}$/signal transducer and activator of transcription 3 (Stat3) signaling pathway decreased the expression of PD-L1 in CRPC cells. Finally, it was observed that combined inhibition of $JAK_{1,2}$/PD-L1 or Stat3/PD-L1 was more effective than inhibition of a single pathway in enhancing the immune cytolytic activity of NK cells. Taking these results together, it is thought that combined inhibition of the $JAK_{1,2}$/PD-L1 and Stat3/PD-L1 signaling pathways may enhance the immune cytolytic activity of NK cells toward hypoxia-induced CRPC cells, which is expected to provide novel ideas and targets for the immunotherapy of CRPC.

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Introduction

With continuous promotion of castration and anti-androgen therapy, clinical treatment of androgen independent protate cancer or castration-resistant prostate cancer (CRPC) has become difficult. It is not uncommon that CRPC develops metastases that chemotherapy and radiotherapy have limited effects on, which seriously affects patients' quality of life. Therefore, research on mechanisms of CRPC progression seems particularly important (1-3). The tumor microenvironment is essential for tumor genesis and tumor development (4,5), with hypoxia a strong research topic in recent years. Hypoxia can induce vascular formation in tumors, and is also widely involved in tumor formation, development, metastasis and recurrence (6-8). Hypoxia accelerates epithelial-mesenchymal transition, invasion, and metastasis in prostate cancer. Also, hypoxia may lead to a decreased sensitivity to radiotherapy and chemotherapy in prostate cancer treatment (9-12). However, there have been scant studies on hypoxia-induced immune evasion in prostate cancer. Therefore, we carried out this study to discover the role of hypoxia in tumor immune regulation.

Hypoxia is involved in immune evasion of a variety of tumors (13) involving many types of immune cells, including T cells, natural killer (NK) cells, macrophages and dendritic cells, that can inhibit or kill tumors (13). Hypoxia may lead to upregulation of the expression of stem cell marker Nanog and transforming growth factor beta 1, resulting in low immune killing capacity of T lymphocytes and macrophages against tumor cells (14). It was discovered in a lung cancer and melanoma study that hypoxia could induce miR-210 expression, which decreased tumor cell susceptibility to antigen-specific cytotoxic T lymphocytes and led to tumor formation and development (15). The NK

cell mediated immune response can kill tumor cells directly with no dependence on antibodies or complements, which is a unique advantage in tumor immunity. By improving immune killing ability of NK cells against tumor cells, tumor formation and development can be effectively controlled. Suppression of expression of NK cell activating receptors MICA and MICB on the tumor cell surface by hypoxia can cause immune evasion from NK cells in pancreatic cancer, osteosarcoma, multiple myeloma and other malignant tumors (16-19). The role of hypoxia regarding NK cell immune evasion in prostate cancer is rarely reported. In a study of DU145 and PC3 in prostate cancer cells, hypoxia inhibited the expression of NKG2D ligands on the surface of the tumor cells, thereby inhibiting the killing of tumor cells by activated NK cells (20,21).

The mechanism of hypoxia-mediated immune evasion is unknown. Many studies have indicated that programmed cell death ligand 1 (PD-L1) plays an important role in tumor immune evasion (22,23). A study of non-small cell lung cancer showed that tumor cells overexpress PD-L1, thereby binding PD-1 receptors on the surface of T cells and inhibiting T cell immune attack, resulting in immune evasion (24,25). Studies of ovarian cancer, melanoma, bladder cancer, laryngeal squamous cell carcinoma and other malignant tumors have indicated that downregulation of PD-1/PD-L1 improves tumor susceptibility to immune cells (26-30). PD-1 is expressed in multiple immune cells, including T cells, B cells, NK cells and dendritic cells (31,32). The effect of PD-L1 in immune evasion of CRPC from NK cells, which is rarely studied, became the research direction of these experiments.

NKG2D is an important receptor for activation of NK cells. The upregulation of expression of NKG2D ligands, MHC class I chain-related proteins A and B (MICA and MICB) and UL16-binding proteins ULBP-1, ULBP-2, ULBP-3, can promote immune cytolytic activity of NK cells to tumors (33). This receptor has become the subject of enhancing the anti-tumor immunity of NK cells in this study. Previous work has shown that the combination of PD-1 and PD-L1 constitutes the PD-1-PD-L1 signaling pathway; in addition to this pathway's immunosuppressive effects through T cells (34), it can also inhibit the anti-tumor activity of NK cells by inhibiting the function of NKG2D ligands on the surface of tumor cells (35). Interestingly, based on a review of the literature, we found that the $JAK_{1,2}$/Stat3 signaling pathway is the upstream regulator of PD-L1, which also includes other widely studied regulators such as Akt, MAPK, MEK, NFκB, and mTOR (36-40).

We selected CRPC cell lines C4-2 and CWR22Rv1 as subjects of study. After induction of the cells by hypoxia, we assayed NK cell mediated cytotoxicity and colony formation to determine whether there were any changes in CRPC cell immune susceptibility to NK cells. We investigated the underlying molecular mechanisms of the changes to seek an effective way to enhance the killing ability of NK cells against CRPC cells, with the aim of inhibiting or killing tumors and offering a new approach to CRPC immunotherapy.

Materials and methods

Cell culture. The C4-2 cells used in the assays were generously donated by the China Center for Type Culture Collection (CCTCC). The CWR22Rv1 and NK92 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the cell culture medium was RPMI 1,640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% charcoal stripped fetal bovine serum (Thermo Fisher Scientific, Inc.). We used an anoxic incubator (1%O_2, 5%CO_2, 94%N_2) (Sanyo, Osaka, Japan) and a normoxic incubator (21%O_2, 5%CO_2, 74%N_2) (Sanyo). C4-2 and CWR22Rv1 lines of CRPC cells grown inananoxic incubator for 24 h served as anoxic cells. NK92 cells were cultured in Minimum Essential Medium containing sodium bicarbonate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), IL-2 (Bio-Techne, Minneapolis, MN, USA), inositol, folic acid, 12.5% horse serum (Sigma-Aldrich; Merck KGaA), 2-mercaptoethanol (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and 12.5% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA).

NK cell mediated cytotoxicity assay. Tumor cells were seeded at 2,000 cells/well in a 96-well plate and cultured overnight. After aspirating all the medium, NK cells were added at a ratio of tumor cells to NK cells of 1:1, 1:5, or 1:15; the inhibitors PD-L1 Ab (329710; BioLegend, Inc., San Diego, CA, USA), JAK inhibitor 1 (CAS457081-03-7; EMD Millipore, Billerica, MA, USA), and Stattic were added at a ratio of 1:1,000 at the same time. Cells were cultured for 4 h, then a 50 μl aliquot of medium was used in a LDH cytotoxic assay using the LDH cytotoxic assay kit (88954; Thermo Fisher Scientific, Inc.). The experimental release was corrected by subtracting the amount released spontaneously in cells at corresponding dilutions. The percentage cytotoxicity was expressed as

$$\frac{\text{Experimental value} - \text{Effector cells spontaneous control} - \text{Target cells spontaneous control}}{\text{Target cell maximum control} - \text{Target cells spontaneous control}} \times arg$$

and used to calculate the immune killing ability of NK cells against tumor cells.

Colony formation assay. Using the gradient dilution cell-count method, we seeded 200 cells in 6 cm dishes (REF353002; Corning Incorporated, Corning, NY, USA). After overnight culture, tumor cells and NK cells were incubated for 4 h at 1:1, 1:5 and 1:15 ratios. After aspirating all the medium and removing NK cells, tumor cells were continuously cultured for 14 days after replacing the conventional culture medium. Then the target cells were fixed with formaldehyde for 15 min, stained with crystal violet for 30 min, and observed and photographed under the microscope. The protocol was repeated three times in each group and the results were averaged.

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). When the adherent tumor cells had grown over about 70% of the surface, PD-L1 Ab, JAK inhibitor 1, Stattic, LY294002, SB203580, and U0126 were added separately at a ratio of 1:1,000, then cells were cultured for 6 h, and total RNA was extracted. We used Superscript III transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) to reverse-transcribe total RNA (1 μg). Following primer setup, (the primer solutions included RT buffer, dNTPs, RT random primers, MultiScribe reverse transcriptase and RNase-free water), we conducted qPCR with a Bio-Rad CFX96 reaction system (reactions included cDNA,

RNAase-free water, SYBR Green and primers; Table I). Using GAPDH as a reference, the expression of target gene mRNA was measured by the intensity of green fluorescence.

Western blot analysis. Tumor cells were collected after centrifugation and the supernatant removed. Then cell lysate was added, the protein concentration measured, sodium dodecyl sulphate-polyacrylamide gel electrophoresis performed, and the protein transferred to polyvinylidene difluoride membranes (EMD Millipore). After adding blocking solution and incubating with primary antibodies (1:1,000), the membranes were incubated with horseradish peroxidase labeled secondary antibody (1:5,000), followed by capture of images with an ECL imaging system (Thermo Fisher Scientific, Inc.). Primary antibodies used in the assay included p-HIF-1α (EP1215Y; ab210073; Abcam, Cambridge, UK), p-PD-L1 (MAB1086; R&D Systems, Inc., Minneapolis, MN, USA), JAK_1 (pY1022, A7125; Assay Biotech, Fremont, CA, USA), p-JAK_2 (pY1007 + 1008, 601-670; Abbomax, Inc., San Jose, CA, USA), p-MAPK (9101S; Cell Signaling Technology, Inc., Danvers, MA, USA), p-MEK (Ser 217/221, 9121; Cell Signaling Technology, Inc.), p-Akt (S473, 9271; Cell Signaling Technology, Inc.), p-Stat3 (Y705, ab76315; Abcam), p-NFκB (S536, ab86299; Abcam), and GAPDH (2118S; Cell Signaling Technology, Inc.).

Signaling pathway inhibitors. We added appropriately diluted inhibitors of various cell signaling pathways, JAK inhibitor 1 (5 μM) (CAS457081-03-7; EMD Millipore), Stattic (10 μM) (CAS19983-44-9; EMD Millipore), PD-L1 Ab (329710; BioLegend, Inc.), LY294002, SB203580 (both Sigma-Aldrich; Merck KGaA) and U0126 (Cell Signaling Technology, Inc.), which respectively inhibit JAK1, JAK2, Stat3, Akt, MAPK and MEK, to hypoxia-treated cells before co-incubation with tumor cells.

Statistical analysis. All data were reported as the mean ± standard deviation of 3 experimental repeats., and were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The differences between two groups were analyzed by a two-tailed Student's t-test. One-way analysis of variance followed by Fisher's least significant difference post hoc test were used for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia-induced C4-2 and CWR22Rv1 cells showed changes in shape and increased expression of hypoxia-inducible factor-1α (HIF-1α). After culture of C4-2 and CWR22Rv1 lines of CRPC cells in a hypoxic incubator for 24 h, the expected changes in cell morphology, such as to spindle-shaped and polygonal cells, and an increase in cell refractive index were observed (Fig. 1A). Normoxic cultured cells were the control group. We observed significantly upregulated expression of HIF-1α in tumor cells exposed to hypoxia. While HIF-1α can be easily degraded by the intracellular oxygen-dependent ubiquitin-proteasome pathway under normoxic conditions, under hypoxic conditions, its expression is stable (33), suggesting that the assayed C4-2 and CWR22Rv1 cells were effectively induced by hypoxia (Fig. 1B).

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence
GAPDH	Forward	5'-AACGGATTTGGTCGTATTGGG-3'
	Reverse	5'-CCTGGAAGATGGTGATGGGAT-3'
PD-L1	Forward	5'-GCTATGGTGGTGCCGACTAC-3'
	Reverse	5'-TTGGTGGTGGTGGTCTTACC-3'
MICA	Forward	5'-ACTGCTTGAGCCGCTGAGA-3'
	Reverse	5'-GAGGTGCAAAAGGGAAGA TGC-3'
MICB	Forward	5'-GGGGCGCAGGTGACTAAAT-3'
	Reverse	5'-CCTACGTCGCCACCTTCTCA-3'
ULBP-1	Forward	5'-CAGCAGACGATGAGGACATT-3'
	Reverse	5'-GACAGAAAGTGGCAGAAG GTG-3'
ULBP-2	Forward	5'-CATTACTTCTCAATGGGAGAC TGT-3'
	Reverse	5'-TGTGCCTGAGGACATGGCGA-3'
ULBP-3	Forward	5'- ATTCTTCCGTACCTGCTATT-3'
	Reverse	5'-GCTATCCTTCTCCCACTTCT-3'

PD-L1, programmed death-ligand 1; MICA, MHC class I chain-related protein A; MICB, MHC class I chain-related protein B; ULBP, UL16 binding protein.

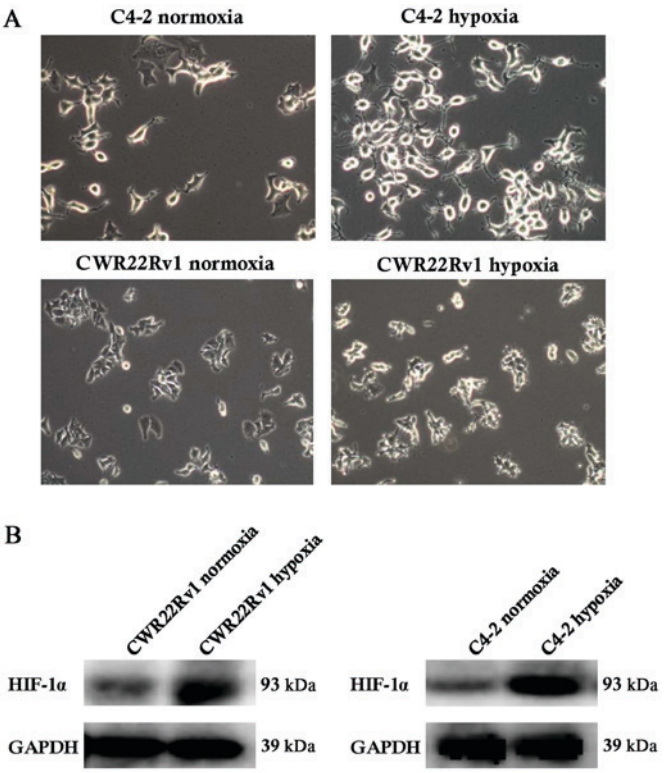

Figure 1. Hypoxia induction in the C4-2 and CWR22Rv1 lines of castration-resistant prostate cancer cells. (A) A total of 24 h following hypoxia induction, the cells appeared polygonal and spindle-shaped, and the refractive index of the cells increased (magnification, x40). (B) A significantly increased expression of HIF-1α was detected by western blotting following hypoxia induction. HIF, hypoxia-inducible factor.

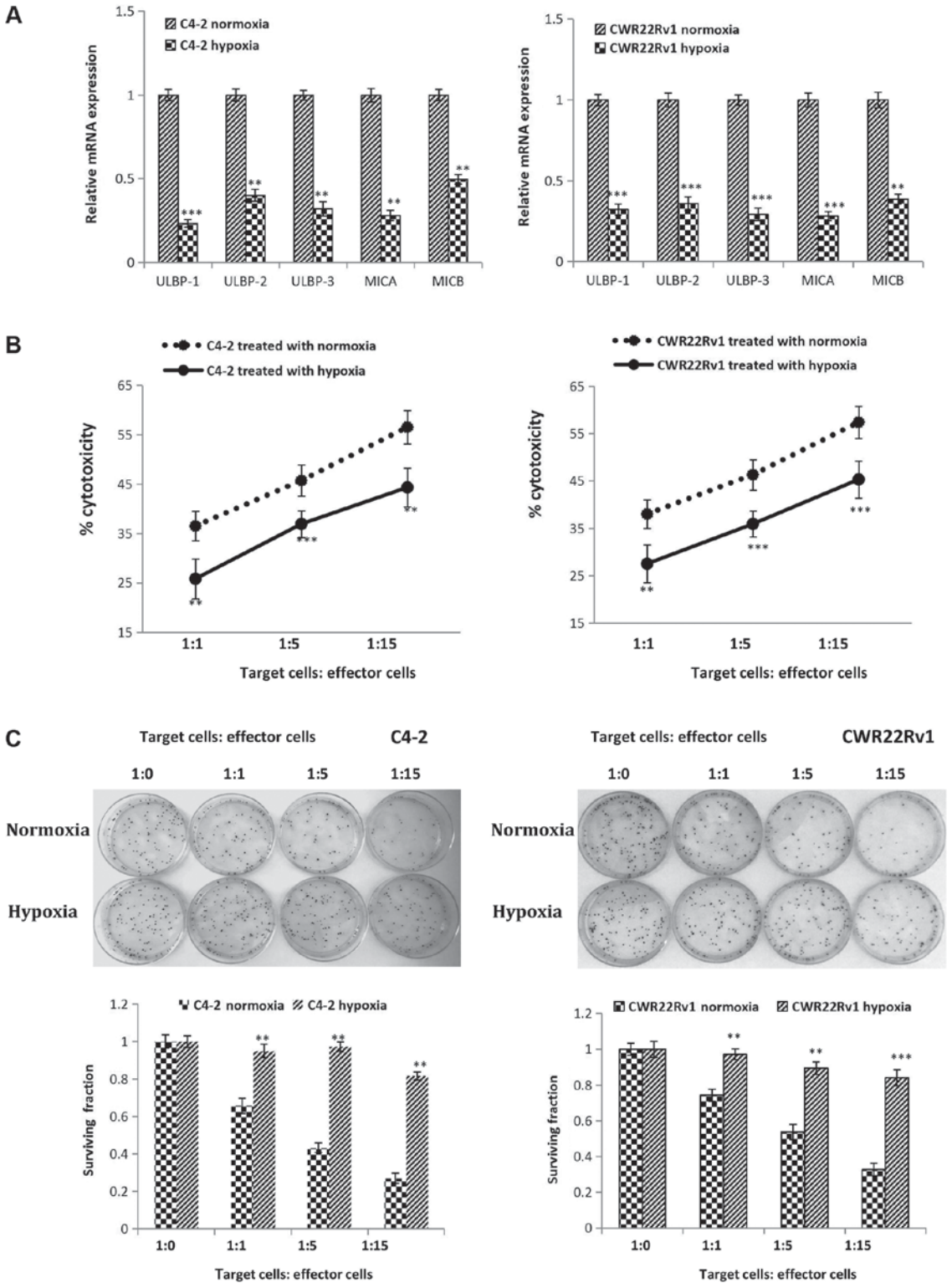

Figure 2. NKG2D ligand gene expression in the hypoxia-induced CRPC cell lines C4-2 and CWR22Rv1, and the effects of hypoxia on NK cell mediated cytotoxicity and clone formation. (A) Following hypoxia induction, gene expression of NKG2D ligand, which promotes NK cell activation, was significantly decreased. (B) Following hypoxia induction, the killing ability of NK cells against CRPC cells was significantly decreased. (C) The ratio of co-cultured NK cells to tumor cells and the CRPC cell clone-forming ability under hypoxia. **P<0.01 and ***P<0.001 vs. normoxia. NKG2D, natural killer group 2D; NK cells, natural killer cells; CRPC, castration-resistant prostate cancer.

Hypoxia reduced expression of NKG2D activating ligandsin C4-2 and CWR22Rv1 cell lines and led to increased tolerance to immune killing of NK cells. We also detected changes in expression of genes encoding NKG2D ligands in hypoxia-induced C4-2 and CWR22Rv1 cells. Gene expression of these ligands (ULBP-1, ULBP-2, ULBP-3, MICA and MICB, all found on the surface of target cells) significantly decreased (Fig. 2A). In hypoxia-induced CRPC cells co-cultured for 4 h with various ratios of NK cells, lactate dehydrogenase release showed that the killing ability of NK cells significantly decreased against

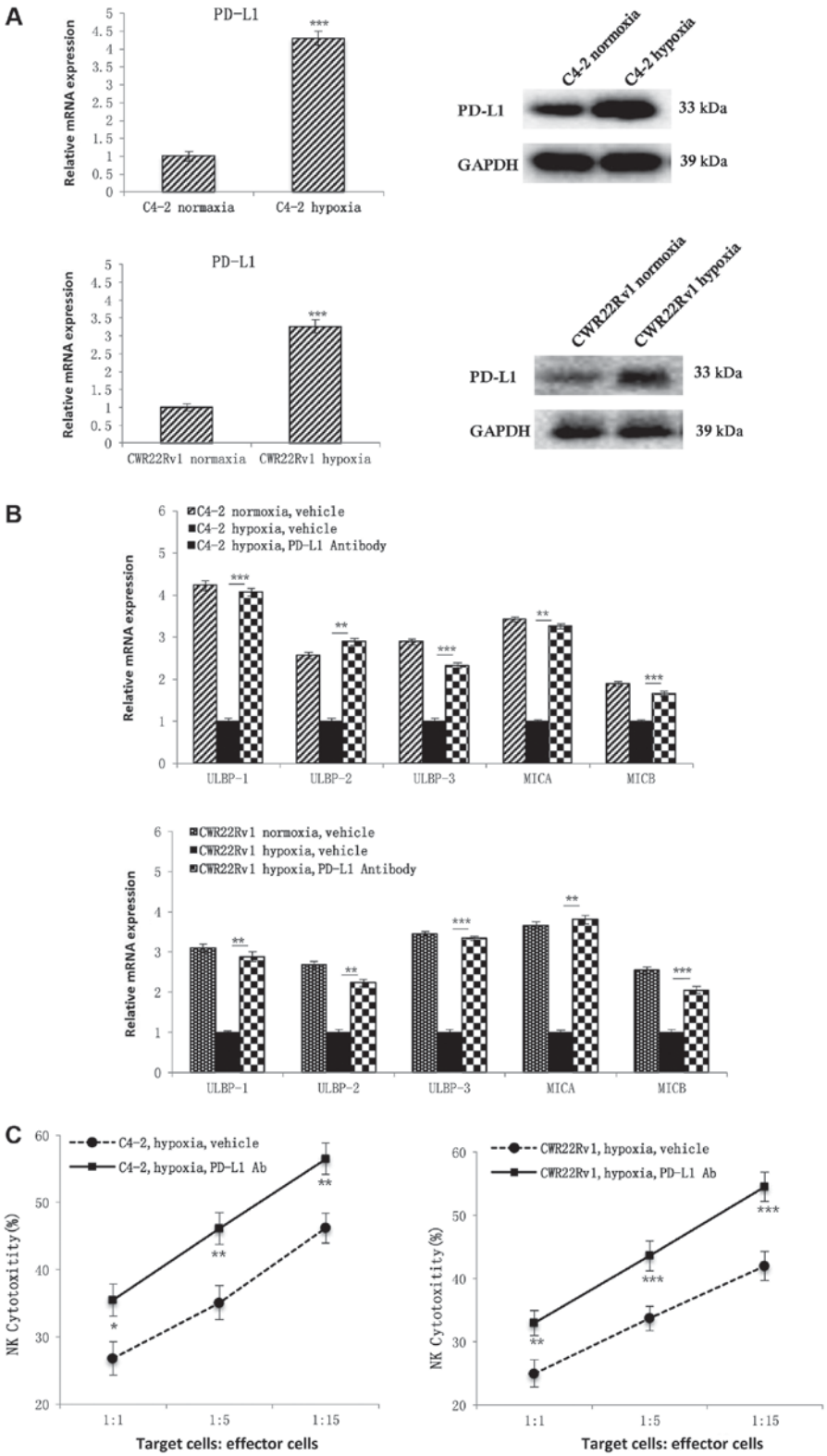

Figure 3. Expression of PD-L1 gene in the hypoxia-induced castration-resistant prostate cancer cell lines C4-2 and CWR22Rv1, and alterations in NKG2D ligand gene expression and NK cell mediated cytotoxicity following the addition of PD-L1 antibodies. (A) Following hypoxia induction, the expression of PD-L1 significantly increased. In the hypoxia-induced group, adding PD-L1 antibodies (B) reversed the expression of natural killer group 2D ligand genes and (C) enhanced NK cell immune killing ability against tumor cells. *P<0.05, **P<0.01 and ***P<0.001 vs. normoxia and as indicated. NK cells, natural killer cells; PD-L1, programmed death-ligand 1; MICA, MHC class I chain-related protein A; MICB, MHC class I chain-related protein B; ULBP, UL16 binding protein.

hypoxia induced target cells (Fig. 2B). The colony formation assay suggested that as the ratio of co-cultured NK cells to tumor cells increased, the clone-forming ability of the hypoxia group showed no obvious changes, while a significant decrease was observed in the normoxia group. These results indicated that hypoxia increased survival of target cells co-cultured with

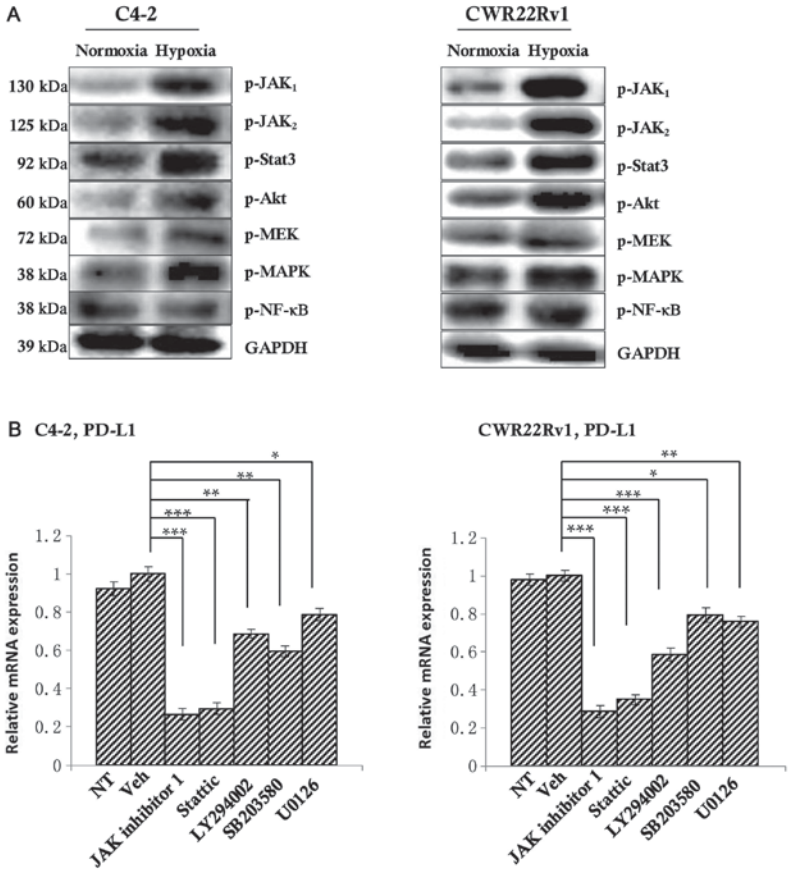

Figure 4. Detection of the upstream regulatory factors of PD-L1. (A) Western blotting indicated an upward tendency in the expression of p-JAK_1, p-JAK_2, p-Akt, p-Stat3, p-MAPK and p-MEK in the hypoxia group. (B) Once the corresponding inhibitors were added, PD-L1 expression in all of the above signaling pathways decreased to varying degrees, with significant decreases observed following the addition of JAK inhibitor 1 and Stattic, suggesting that the inhibition of the $JAK_{1,2}$/Stat3 signaling pathway may inhibit PD-L1 expression. *P<0.05, **P<0.01 and ***P<0.001, as indicated. PD-L1, programmed death-ligand 1; JAK, Janus kinase; Akt, protein kinase B; Stat, signal transducer and activator of transcription; NF, nuclear factor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; p-, phosphorylated; NT, not treated.

NK cells, which was consistent with the findings in the NK cell mediated cytotoxicity assay (Fig. 2C). In conclusion, hypoxia can lead to CRPC cell immune tolerance to NK cells.

Hypoxia-induced C4-2, and CWR22Rv1 CRPC cells had increased PD-L1 expression, resulting in immune escape from NK cells. In further study on the mechanisms affecting expression of PD-L1, both PD-L1 gene and protein expression in C4-2 and CWR22Rv1 cells were higher after hypoxia induction than under normoxic conditions (Fig. 3A). To determine whether hypoxia induces CRPC cells to evade NK cell immunity, we added PD-L1 antibodies to hypoxia-induced CRPC cells and detected upregulation of NKG2D ligand expression (Fig. 3B). We assayed NK cell mediated cytotoxicity, and discovered that addition of PD-L1 antibodies significantly increased the susceptibility of hypoxia-induced CRPC cells to NK cell immunity (Fig. 3C), which confirmed that PD-L1 protein is involved in hypoxia-induced CRPC cell immune evasion.

PD-L1 expression is inhibited by blocking the $JAK_{1,2}$/Stat3 signaling pathway. To explore the mechanism behind PD-L1 regulation of CRPC cells against NK cell immunity, we selected well-studied signaling pathways of PD-L1 regulation, including JAK_1, JAK_2, Stat3, PI3K/Akt, MAPK, MEK, and NFκB (34-39). We first detected differences in expression of these molecular markers in both normoxia and hypoxia groups by western blotting, and found that the expression of JAK_1, JAK_2, Stat3, Akt, MAPK, MEK in hypoxia group was increased compared with the normoxia group (Fig. 4A); thus, we think that these proteins may be involved in the regulation of PD-L1 under hypoxic conditions in the tumor. To observe their regulation of PD-L1, we added inhibitors of these proteins' corresponding signaling pathways JAK inhibitor 1 (which can simultaneously inhibit the expression of JAK 1 and JAK 2), Stattic, LY294002, SB203580 and U0126 at a ratio of 1:1,000 to hypoxic tumor cells. After 6 h, the level of PD-L1 was detected by RT-qPCR. The untreated group served as a control. The results showed that JAK inhibitor 1 and Stattic significantly down-regulate PD-L1 expression. PI3k inhibitor LY294002 could also significantly reduce the expression of PD-L1, but JAK inhibitor 1 and Stattic inhibited PD-L1 more. So we chose JAK inhibitor 1 and Stattic as the research subjects. Of course, given this result, PI3k inhibitor LY294002 will also be our future research direction (Fig. 4B).

$JAK_{1,2}$/Stat3 signaling pathway inhibition increases the susceptibility of CRPC cells to NK cell immunity, with a combination of PD-L1 antibodies and JAK inhibitor 1/Stattic

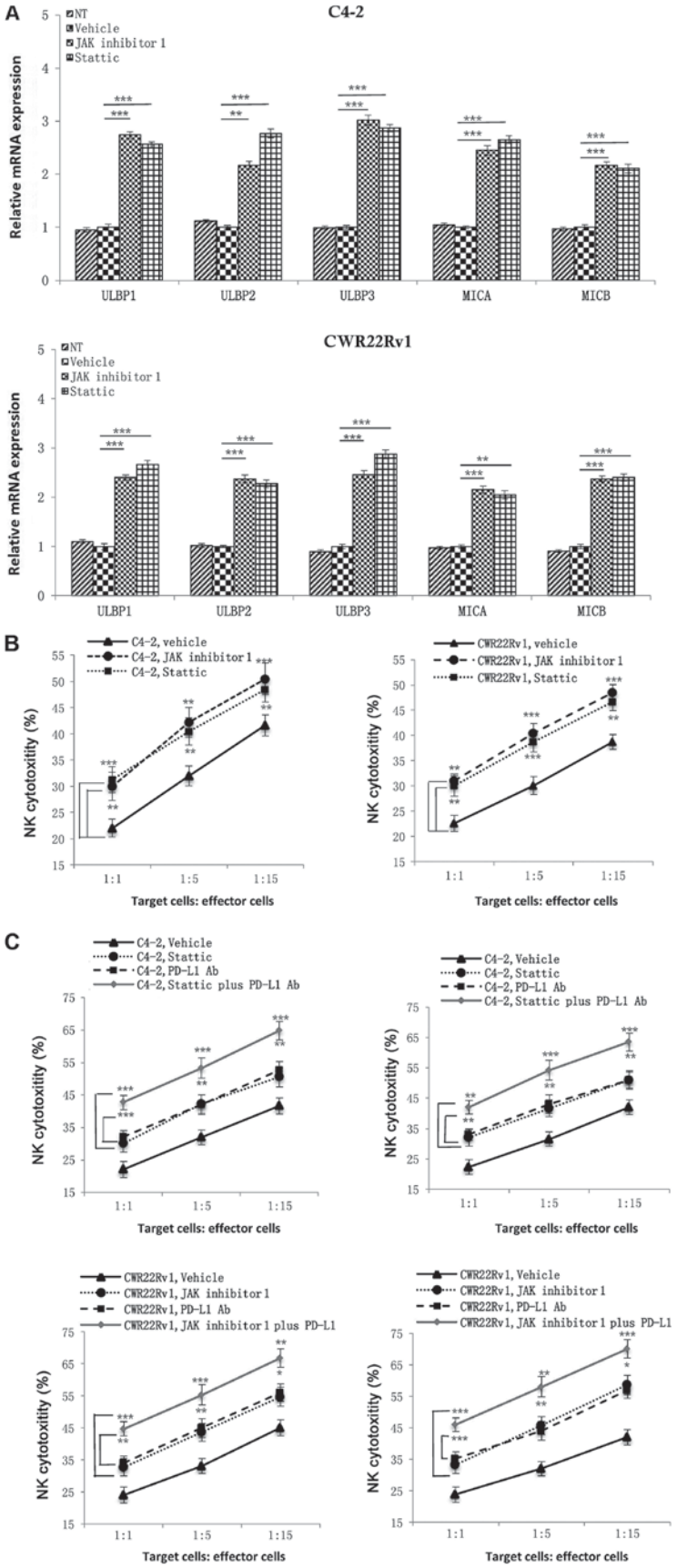

Figure 5. Effects of $JAK_{1,2}$ and Stat3 single inhibition and $JAK_{1,2}$/PD-L1 and Stat3/PD-L1 combined inhibition on NK cell mediated cytotoxicity. (A) Inhibition of the expression of $JAK_{1,2}$ or Stat3 upregulated NKG2D ligand expression in castration-resistant prostate cancer cells under hypoxia. (B) NK cell cytotoxic effects on tumor cells were significantly increased following the addition of JAK inhibitor 1 or Stattic in the hypoxia group. (C) Combinations of JAK inhibitor 1/PD-L1 antibody or Stattic/PD-L1 antibody were superior to single applications, indicating that combined inhibition of the PD-L1 and $JAK_{1,2}$/Stat3 signaling pathways enhanced NK cell immune killing ability against hypoxia-induced CRPC cells. *P<0.05, **P<0.01 and ***P<0.001, as indicated. JAK, Janus kinase; Stat, signal transducer and activator of transcription; NKG2D, natural killer group 2D; NK cells, natural killer cells; PD-L1, programmed death-ligand 1; MICA, MHC class I chain-related protein A; MICB, MHC class I chain-related protein B; ULBP, UL16 binding protein; NT, not treated.

more effective either alone. CRPC cells in the hypoxia group were used as the study subject, and the $JAK_{1,2}$ inhibitor JAK inhibitor 1 or the Stat3 inhibitor Stattic were added. The untreated group was used as the control. RT-qPCR showed increased expression of NKG2D ligands (Fig. 5A), suggesting that inhibition of the $JAK_{1,2}$/Stat3 signaling pathway may improve the immune cytolytic activity of NK cells toward CRPC cells under hypoxic conditions.

Since blocking the $JAK_{1,2}$/Stat3 signaling pathway can inhibit PD-L1 expression in hypoxic CRPC cells and may upregulate expression of NKG2D ligands of CRPC cells, to test whether this also enhances the immune killing function of NK cells against tumors by down-regulating PD-L1, we used hypoxic CRPC cells as targets and added the same $JAK_{1,2}$/Stat3 signaling pathway inhibitors to an NK cell mediated cytotoxicity assay. Both JAK inhibitor 1 and Stattic significantly increased susceptibility of CRPC cells to NK cell immunity compared to an untreated group (Fig. 5B). Finally, we added combinations of PD-L1 antibody/JAK inhibitor 1 and PD-L1 antibody/Stattic to the NK cell mediated cytotoxicity assay and discovered that combined treatments were superior to each single application. Combined treatments achieved better results in enhancing NK cell immune killing function against CRPC cells, which could provide new approaches to CRPC targeted therapy (Fig. 5C).

Discussion

In clinical practice, the incidence of both local progression and distant metastasis in CRPC patients has significantly increased, seriously affecting patients' quality of life (40,41). Although the mechanisms behind this increase are not yet fully understood, hypoxia, as an important feature of the tumor microenvironment, plays an essential role (42,43). In this study, CRPC cell lines C4-2 and CWR22Rv1 were grown in an incubator under conditions of hypoxia. We observed changes in the morphology of these cells to polygonal and spindle shapes, and high expression of HIF-1α that was dependent on the oxygen concentration, consistent with hypoxia-induced changes in the cells, which may provide fundamental data for further exploration of the effects of hypoxia on tumor immunity. In the hypoxic microenvironment of prostate cancer, HIF-1α and related proteins present a major research direction. Our focus on the $JAK_{1,2}$/Stat3-PD-L1 signaling pathway has not been studies before, and our results suggest this pathway was upregulated in a hypoxic microenvironment, resulting in immune escape of CRPC from NK cells.

The occurrence and development of tumors are closely related to the immunity of the human body, in which the direct killing function of NK cells against tumors has a unique advantage; however, there are scant studies on the mechanisms of NK cell immunity against CRPC. Our study took this as a starting point to explore the mechanisms of NK cell immunity against CRPC in a hypoxic microenvironment. Firstly we examined the expression of ligands of NKG2D, a major activating receptor of NK cells, which include the ULBP family (ULBP-1, ULBP-2 and ULBP-3) and MICA/MICB (44). Expression of all these markers showed a downward trend, consistent with NKG2D playing an important role in tumor immunity of NK cells. CRPC cells also had stronger immune tolerance to NK cells in a hypoxic microenvironment, confirming a stronger immune evasion function under these conditions.

To explore the mechanisms of immune evasion, we selected PD-L1, a relatively well-studied protein in tumor targeted therapy (45,46), as a research subject. PD-L1 expression of hypoxia-induced CRPC cells significantly increased, and by adding PD-L1 antibodies, the expression of NKG2D ligands was reversed, while the immune killing ability of NK cells against tumor cells was significantly enhanced. The combination of PD-1 and PD-L1 constitutes the PD-1-PD-L1 signaling pathway, thus affecting immune cytolytic activity of immune cells to tumor cells, which is mainly reflected in the following aspects: (1) Inhibition of function of TIL cells by (a) inhibiting the activation of TIL cells, (b) influencing Th cell differentiation, (c) inhibiting the production of effector cytokines, (d) promoting the secretion of suppressive cytokines, and (e) increasing TIL apoptosis, thus resulting in tumor immune escape (34); (2) Inhibiting the function of NK cells, thereby reducing their anti-tumor effect (35); (3) Expression of PD-L1 on TAM, which can cause synergistic stimulation, inhibiting the immune susceptibility of tumor cells (47).

During examination of PD-L1 upstream regulatory factors, we found that the $JAK_{1,2}$/Stat3 signaling pathways played an important role, and by adding appropriate antibodies (JAK inhibitor 1 or PD-L1 antibody), PD-L1 expression of CRPC cells was inhibited under hypoxic conditions. The $JAK_{1,2}$/Stat3 signaling pathway is widely involved in proliferation, differentiation, migration and other processes of cells (48). It plays an important role in occurrence and development of many tumors including prostate cancer (49-53). The results of our study also indicated that in a hypoxic microenvironment, the $JAK_{1,2}$/Stat3 signaling pathway promotes immune evasion of NK cells by CRPC cells. The current targeted immunotherapy of PD-1/PD-L1 against tumors has achieved good results, effectively reducing tumor size and prolonging the survival of patients. However, clinical observation shows that there is still some degree of immunosuppression, which may be related to the complex immune escape mechanism of the tumor itself. Combined administration of drugs through multiple ways may be an effective choice for tumor targeted immunotherapy (54). Thus, we studied the combined inhibition of $JAK_{1,2}$/PD-L1 and Stat3/PD-L1, and observed that a combined inhibition was more effective in enhancing NK cell cytotoxicity toward hypoxia-induced CRPC cells. Given these results, we may develop a combination inhibitor of $JAK_{1,2}$/Stat3 and PD-L1, and conduct related animal studies and clinical trials, thereby providing a more effective means of clinical immunotherapy of CRPC.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

LJX, QM and JZ performed the experiments and statistical analyses, and created the figures. JL and BXX contributed to the generation of the knockdown cell lines. JG and CYS provided and performed the staining of human tissues. YCZ and YBZ assisted with the interpretation of data and reviewed the manuscript. DRY and YXS conceived the idea and wrote the manuscript. All authors reviewed and agreed to the information in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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