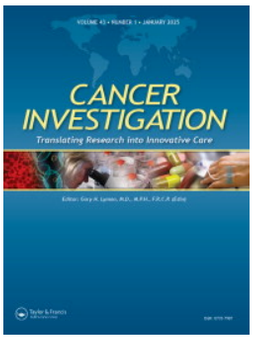

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LDHB Mediates Histone Lactylation to Activate PD-L1 and Promote Ovarian Cancer Immune Escape

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ABSTRACT

Background: To investigate the effects of LDHB on lactylation of programmed cell death 1 ligand (PD-L1) and immune evasion of ovarian cancer.

Methods: Ovarian cancer cells were transfected with LDHB siRNA and cultured with primed T cells. Cell proliferation and viability were measured by cell counting kit 8 (CCK-8) and colony formation assay. The production of immune factors was detected by enzyme-linked immunosorbent assay (ELISA). The histone lactylation and activity of PD-L1 promoter were measured by chromatin immunoprecipitation (ChIP)-qPCR assay and luciferase reporter gene assay, respectively.

Results: Knockdown of LDHB notably inhibited the growth, glucose uptake, lactate production, and ATP production of ovarian cancer cells. Knockdown of LDHB enhanced the killing effects of T cells, led to increased production of immune activation factors IL-2, TNF-α, and IFN-γ, as well as elevated the levels of granzyme B and perforin. Mechanical study identified that LDHB regulated the H3K18 lactylation (H3K18la) modification on PD-L1 promoter region to promote its expression. Overexpression of PD-L1 abolished the immune activation effects that induced by siLDHB.

Conclusion: The LDHB modulated lactate production and the histone lactylation on PD-L1 promoter, which ultimately regulated its expression and participated in the immune evasion of ovarian cancer cells.

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Ovarian cancer; lactate dehydrogenase B; lactylation; PD-L1; immune activation

Introduction

General screening biomarkers for ovarian cancer are insufficient in clinics. Currently, CA125 and HE4 are the only approved biomarkers available, yet they are insufficient for early detection. This limitation has also driven economic studies, with cost-effective strategies for early detection and prevention of ovarian cancer being a focus over the past decade. Treatment costs per ovarian cancer patient remain among the highest across all cancer types (1). In recent years, immunotherapy, primarily based on immune checkpoint inhibitors (ICIs), has been increasingly recognized in the comprehensive treatment of cancer, with research on programmed cell death 1 (PD-1) and its ligand PD-L1 standing out (2). Malignant tumors activate immune checkpoints to establish an immunosuppressive microenvironment. The interaction between PD-1 and PD-L1 can inhibit the proliferation of $CD8^+$ cytotoxic T lymphocytes, suppress the production of certain cytokines, alter the function of tumor-infiltrating T cells, and thus contribute to the immune tolerance of tumors (3). In the tumor microenvironment, inflammatory factors secreted by inflammatory cells, such as interferon-gamma (IFN-γ), can increase the expression of PD-L1 (4). Additionally, the myeloid-derived suppressor cells, T regulatory (Treg) cells, and tumor-associated macrophages constitute immunosuppressive

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cells present within the tumor microenvironment, which release reactive oxygen species (ROS) amongst other factors, effectively inhibiting (natural killer) NK cell response. Higher levels of fibroblasts secrete more metalloproteinases, resulting in further shedding of ligands that could link to NK cells. Fibroblasts even have a more direct impact on NK cells by preventing cytokine induced activating receptor upregulation. This immune repressive microenvironment severely impaired the therapeutic efficacy of cancers (5). For patients with advanced tumors, blocking the PD-L1/PD-1 signaling pathway has shown significant anti-tumor effects and is widely recognized as the gold standard for currently developed immune checkpoint inhibitors and combination therapies (6). Therefore, blocking immune checkpoints has become an important direction in tumor immunotherapy.

The integration of immune checkpoint inhibitors (ICIs) with radiotherapy facilitates the use of modified fractionation schemes, which minimize radiation exposure to circulating blood. This approach is beneficial as it conserves nearby lymphocytes and bolsters the efficacy of anti-PD-L1 antibody therapy, thereby amplifying the potency of ICIs. Furthermore, research suggests that the synergy between radiotherapy and ICIs can elicit an abscopal effect. This phenomenon occurs when localized treatment triggers a systemic anti-tumor immune response that extends beyond the irradiated area, targeting distant metastatic lesions that were not directly irradiated. This strategic combination has the potential to revolutionize cancer treatment by harnessing the immune system to combat metastasis (7). Relieving immune suppression, restoring immune system function, achieving immune attack and clearance of tumors, and thus achieving the goal of treating cancer (8). Due to the important role of the immune checkpoint PD-L1 in the tumor immune microenvironment and tumor therapy, more and more research is focusing on key signaling pathways and regulatory factors that control PD-L1 expression, using this as one of the strategies to improve PD-1/PD-L1 targeted therapy (9). To date, several upstream regulators of PD-1/PD-L1 have been identified, including p53, PTEN, AKT-mTOR, and NF-κB (10). However, the current PD-L1 blockade strategy is not quite efficient in clinical use for certain cancers, including ovarian cancer. Therefore, it is important to explore the molecular mechanism underlying this issue.

In tumor cells, the regulatory molecular mechanisms of PD-L1 mainly include transcriptional regulation, post-transcriptional regulation, post-translational modification regulation, and epigenetic regulation (11). The PI3K pathway is often overactivated in ovarian cancer, where it plays a crucial role in conferring chemoresistance and maintaining genomic stability. This pathway is integral to numerous aspects of DNA replication and cell cycle regulation. Inhibition of PI3K can result in genomic instability and mitotic catastrophe, primarily by reducing the activity of the spindle assembly checkpoint protein, Aurora kinase B. This reduction in activity can lead to an increased incidence of lagging chromosomes during prometaphase, thereby disrupting the normal cell division process (12). Histone modification-mediated chromatin remodeling and epigenetic regulation are one of the most important regulatory patterns for PD-L1 expression (13). As a crucial epigenetic regulatory mechanism, post-translational modifications of histones can regulate a multitude of biological processes, including gene transcription, chromatin dynamic structure, and development (14, 15). Histone modifications can adjust the genomic DNA into active euchromatin regions (easily transcribed) or inactive heterochromatin regions (difficult to transcribe), thereby regulating gene expression, which is a type of post-translational modification that controls gene expression (16, 17). The lactate shuttle hypothesis describes the role of lactate in the transfer of oxidative and gluconeogenic substrates and cell signal transduction (18), and there is growing evidence that lactate acts as an important regulator coordinating systemic metabolism (19). Lactate is no longer considered a waste product of anaerobic metabolism, and more and more people are beginning to regard it as a signaling molecule (20). A study published in Nature in 2019 elucidated the key role of lactate in promoting histone lysine lactylation modification and epigenetic regulation (21). Histone lactylation modification can participate in

transcriptional regulation and play a key role in a variety of diseases (22). This modification is thought to stimulate gene expression, particularly in macrophages, and may have broader implications for the regulation of gene transcription in different cell types (23). In the context of cancer, histone lactylation may promote the expression of genes that contribute to tumor growth and progression. Manipulating the levels of lactate within the tumor microenvironment might alter the pattern of histone lactylation, thereby affecting gene expression and potentially the behavior of cancer cells (23). As part of the glycolytic process, lactate dehydrogenase (LDH) is a key molecule in regulating lactate metabolism and lactylation modification (24). LDHB is a subunit of LDH that upregulated in certain cancer genotypes, dependent on aerobic glycolysis (25), however, the knowledge related to the LDHB regulation is not well established.

In this study, we explored the role of LDHB in ovarian cancer and determined the LDHB-mediated histone modification on PD-L1. Our study may provide a novel target for ovarian cancer and complete the regulatory mechanism for immune response during ovarian cancer development.

Materials and methods

Cell culture and treatment

Ovarian cancer cell line SKOV3 and OVCAR3 were obtained from Shanghai Cell Band of Chinese Science Academy and maintained in McCOY's 5A (16600082, Gibco, USA) with 10% fetal bovine serum (GIBCO) and RPMI 1640 (Gibco), respectively, in 37 °C incubator and 5% CO_2 atmosphere. For cell transfection, cancer cells were seeded into 6-well plates at a density of 40%, siRNA (50 nM) that targets LDHB was mixed with the RNAiMAX reagent (Invitrogen, USA) and added into the cell culture medium. After transfection for 48 h, cells were used for subsequent experiments. The LDHB inhibitor AXKO-0046 (1 μM) and PD-L1 antibody (20 nM) were added to the culture medium and treated for 24 h.

T cell priming

Peripheral blood mononuclear cells (PBMCs) were processed with percoll solution (Beyotime, China) to isolate the lymphocytes. Then T cells were primed with CD3 and CD28 antibody for 2 days and were used for subsequent experiments (26).

Cancer cell-T cell co-culture assay

Cancer cells and the primed T cells were added into 12 well plates at a ratio of 1:10. After co-culture for 24 h, the T cells, cancer cells, and the culture medium were collected for the following examination.

Cell viability and proliferation

Cell viability and proliferation were measured by cell counting kit 8 (CCK-8) (Beyotime, China) and colony formation assay. For CCK-8 assay, the cancer cells were placed in 96-well plates (5×10^6/well) and cultured for 24, 48, and 72 h. The CCK-8 reagent was then introduced into each well and incubated for an additional 2 h. Subsequently, the medium was removed and replaced with DMSO (150 μl). The absorbance values were measured at a wavelength of 450 nm. For the colony formation assay, cells were resuspended in culture medium as single cells and transferred into 12-well plates. After a 15-day incubation period, the colonies were stained with crystal violet (Thermo, USA) for 20 min and then visualized under a microscope (Leica, Germany).

Enzyme-linked immunosorbent assay (ELISA)

The cancer cells were gathered and lysed, and the concentrations of IFNγ, IL-2, and TNF-α were determined using an ELISA (Enzyme-Linked Immunosorbent Assay) with commercial kits from Thermo, USA, following the manufacturers' protocols.

Western blotting

Cells were lysed using RIPA buffer (Thermo, USA) to extract total proteins. Equal amounts of protein were loaded and separated using SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide

Gel Electrophoresis), then transferred onto nitrocellulose (NC) membranes. The membranes were blocked with 5% skim milk, followed by an overnight incubation at 4 °C with a primary anti-PD-L1 antibody (Proteintech, China). Subsequently, the membranes were probed with a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (Invitrogen, USA) at room temperature for 1 h. After the reaction with an ECL (Enhanced Chemiluminescence) reagent (Millipore, Germany), the protein bands were visualized using a gel imaging system.

qPCR assay

Total RNA was extracted from cells and tissues using Trizol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (TaKaRa, China), and the expression levels were quantified with a SYBR Green mix (TaKaRa, China). The relative levels of the target genes were determined using the $2-\Delta\Delta CT$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as an endogenous control.

Detection of glucose metabolism

The levels of glucose, lactate, and ATP in cancer cells were measured by Glucose detection kit (ab65333, Abcam, USA), L-lactate detection kit (ab65331, Abcam), and ATP detection kit (S0026. Beyotime, China), respectively, following the manufacturer's protocols.

Chromatin immunoprecipitation (ChIP)-qPCR assay

ChIP assays were performed using the EZ-ChIP kit (Millipore). After transfection with siLDHB, cells were exposed to formaldehyde and incubated for 10 min to create DNA-protein cross-links. Subsequently, the cell lysates were sonicated to produce chromatin fragments in the range of 200–300 base pairs (bp) and subjected to immunoprecipitation with antibodies specific for H3K18la or with IgG as a control. The level of PD-L1 in DNA from the precipitated chromatin was then analyzed using qPCR) assays.

Luciferase reporter gene assay

The PD-L1 promoter region construct was amplified from genomic DNA and cloned into pGL3-Basic. Then, SKOV3 and OVCAR3 cells were transfected with siLDHB and PD-L1 promoter constructs by Lipofectamine 2000 (Invitrogen, USA) for 48 h. The cells were then lysed and assessed by dual luciferase reporter assays (Promega, Madison, WI, USA). The results were expressed as the ratio of firefly luciferase activity to renilla.

Statistics

Data were presented as mean ± standard deviation (*SD*) and were analyzed using GraphPad Prism 8. Comparisons of means between groups were made using the two-tailed Student's *t*-test as indicated; a *p*-value of <0.05 was considered to indicate statistical significance.

Results

Knockdown of LDHB repressed the glucose metabolism of ovarian cancer cells

To determine the functions of LDHB in ovarian cancer, we performed LDHB knockdown using siRNAs. As shown in Figure 1(A), siLDHB-1 and siLDHB-2 both effectively downregulated the protein expression of LDHB in cancer cells, and siLDHB-2 was chosen for following experiments. Results from CCK-8 experiment indicated that knockdown of LDHB notably inhibited the growth of ovarian cancer cells (Figure 1(B)). Moreover, knockdown of LDHB reduced the glucose uptake (Figure 1(C)), lactate production (Figure 1(D)), and ATP production (Figure 1(E)). These data indicated that LDHB depletion in ovarian cancer repressed the *in vitro* growth and glucose metabolism.

Knockdown of LDHB enhanced the in vitro anti-cancer effects of T cells

To further explore the effects of LDHB in ovarian cancer cell immune evasion, we established a co-culture model using cancer cells and T cells. The results from colony formation assay suggested

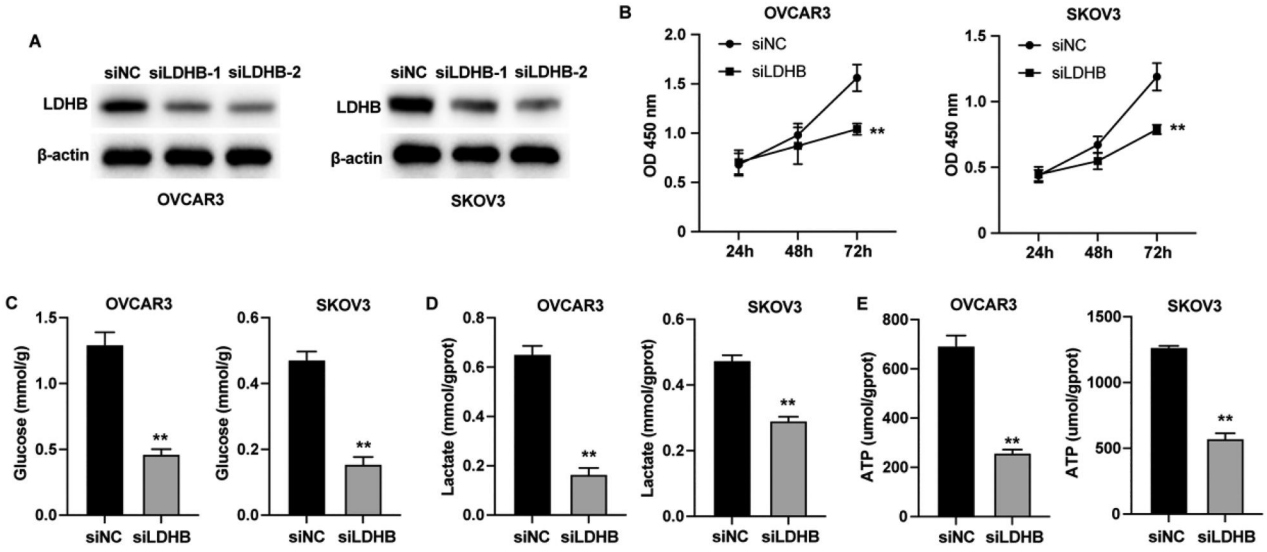

Figure 1. Knockdown of LDHB repressed the glucose metabolism of ovarian cancer cells. (A) OVCAR3 and SKOV3 cells were transfected with siLDHB. (A) Protein level of LDHB was detected by western blot. (B) Cell proliferation was detected by CCK-8. (C–E) The glucose uptake, lactate production, and ATP production. $^{**}p < 0.01$.

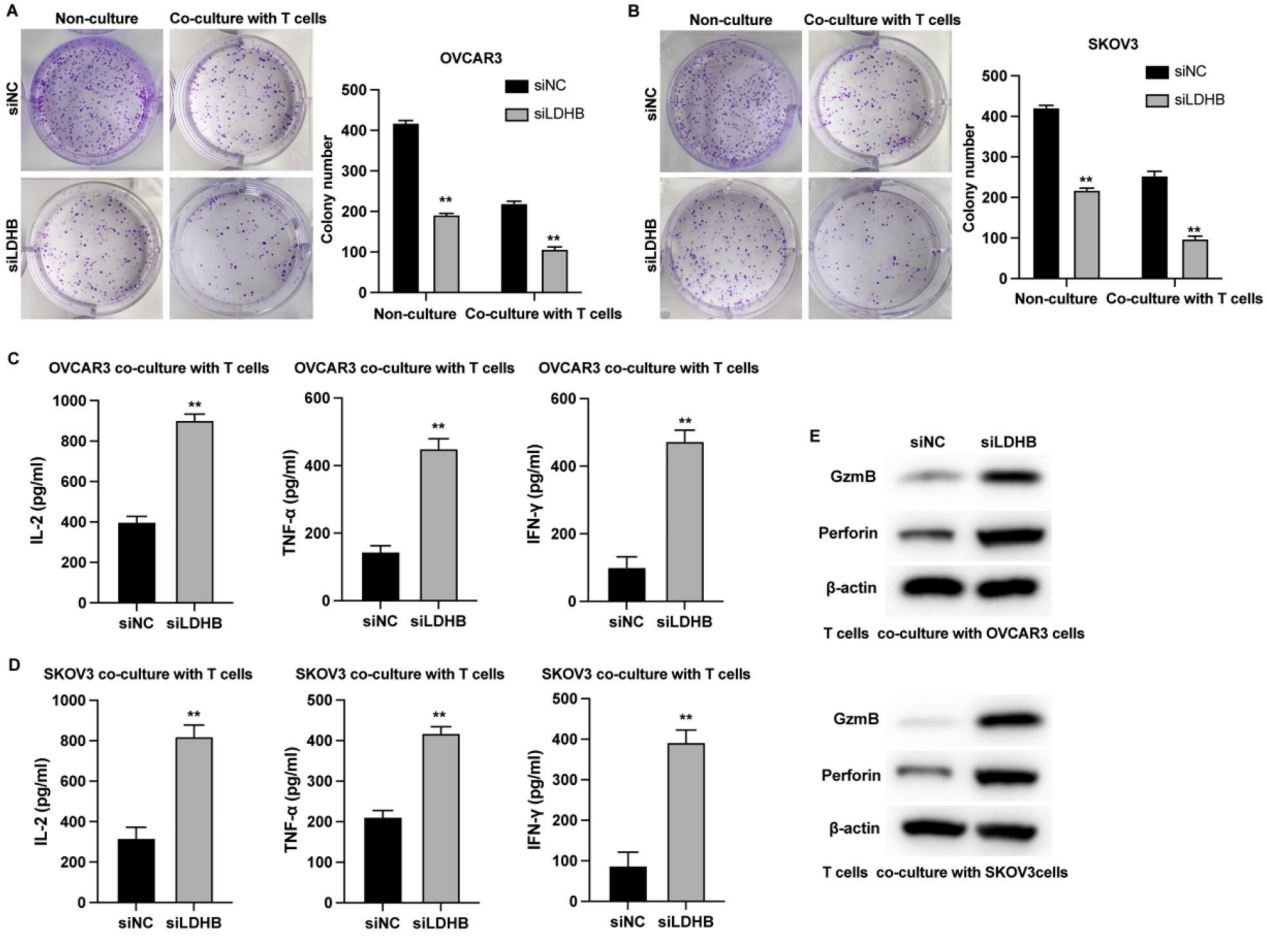

Figure 2. Knockdown of LDHB enhanced the *in vitro* anti-cancer effects of T cells. OVCAR3 and SKOV3 cells were co-cultured with T cells, respectively. (A,B) Cancer cell growth was measured by colony formation assay. (C,D) The production of immune factors by cancer cells was measured by ELISA. (E) The protein levels of granzyme B and perforin in T cells were detected by western blot assay. $^{**}p < 0.01$.

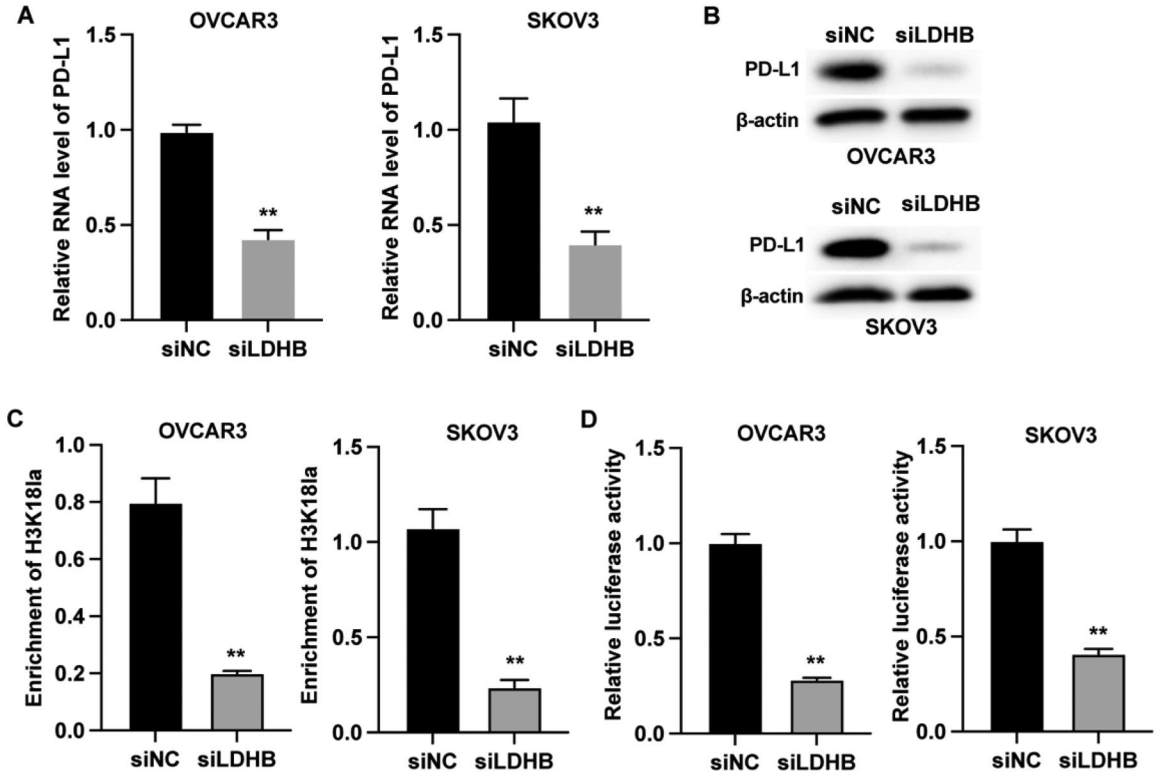

Figure 3. LDHB epigenetically regulates the PD-L1 expression in ovarian cancer cells. (A,B) RNA and protein level of PD-L1 in OVCAR3 and SKOV3 cells was measured by qPCR and western blot assay, respectively. (C) The enrichment of H3K18la modification on PD-L1 promoter was detected by ChIP assay. (D) PD-L1 promoter activity was measured by luciferase reporter gene assay. $^{**}p < 0.01$.

that knockdown of LDHB suppressed the *in vitro* growth of cancer cells and also enhanced the killing effects of T cells (Figures 2(A,B)). Knockdown of LDHB led to increased production of immune activation factors IL-2, TNF-α, and IFN-γ (Figures 2(C,D)). Moreover, the levels of granzyme B and perforin, the cytotoxic factors secreted by activated T cells, were notably elevated under stimulation of cancer cells that depleted of LDHB (Figure 2(E)). These data demonstrated that the knockdown of LDHB reduced the production of immune cytokines of T cells and their killing ability *in vitro*.

LDHB epigenetically regulates the PD-L1 expression in ovarian cancer cells

We next investigated the molecular mechanisms underlying LDHB's effects on immune evasion. We observed that the knockdown of LDHB suppressed the RNA and protein levels of PD-L1 in ovarian cancer cells (Figures 3(A,B)). Results from ChIP assay determined decreased H3K18la modification on PD-L1 promoter region (Figure 3(C)). Further luciferase reporter gene assay experiment indicated that LDHB depletion reduced the activity of PD-L1 promoter (Figure 3(D)). Their data indicated that LDHB possibly regulated PD-L1 expression *via* H3K18la modification. These data indicated that the knockdown of LDHB reduced the H3K18la modification and expression of PD-L1 in ovarian cancer cells.

LDHB modulates immune evasion through regulating PD-L1 expression

Subsequently, we determined the LDHB/PD-L1 axis in ovarian cancer immune evasion. In the cancer cell-T cell co-culture model, we observed that LDHB inhibitor AXKO-0046 enhanced the killing effect of T cells on cancer cells, whereas overexpression of PD-L1 reversed this effect (Figure 4(A)). Consistently, the levels of IL-2, TNF-α, and IFN-γ in cancer cells were increased (Figures 4(B,C)) and the production of granzyme

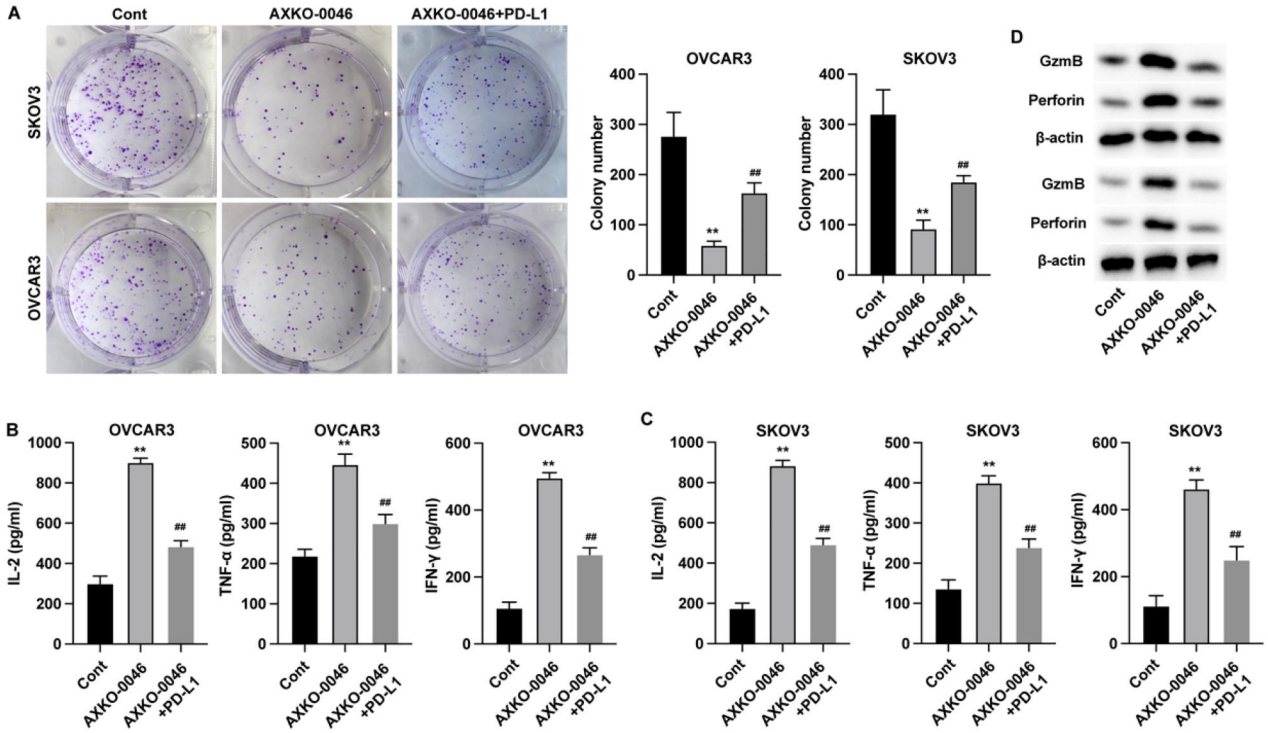

Figure 4. LDHB modulates immune evasion through regulating PD-L1 expression. OVCAR3 and SKOV3 cells were co-cultured with T cells, respectively, and treated with LDHB inhibitor AXKO-0046 and PD-L1 overexpression. (A) Cancer cell growth was measured by colony formation assay. (B,C) The production of immune factors by cancer cells was measured by ELISA. (D) The protein levels of granzyme B and perforin in T cells were detected by western blot assay. $^{**}p < 0.01$.

B and perforin was enhanced (Figure 4(D)) under LDHB inhibition, which was abolished by PD-L1 overexpression. These findings demonstrated that PD-L1 may mediate the LDHB-regulated production of immune cytokines by T cells.

Discussion

Approaches that focus on inhibiting the PD-1/PD-L1 pathway have earned regulatory approval in oncology, providing enduring benefits for cancer patients (27, 28). As such, uncovering the intricate molecular processes that control PD-1 and PD-L1 expression is essential. This exploration is crucial for the advancement of immunotherapy and could lead to the development of more precise and potent cancer treatments. For example, the integration of ICIs with PARP inhibitors is a therapeutic strategy that is partly based on the hypothesis that BRCA1/2-mutated, as well as wild-type BRCA1/2 homologous recombination (HR) deficiency tumors, exhibit a higher neo-antigen load compared to HR-proficient cancers, thereby potentially triggering a more effective anti-tumor immune response. This hypothesis is supported by evidence suggesting that BRCA deficiency can induce a STING-dependent innate immune response, characterized by the induction of type I interferon and pro-inflammatory cytokine production. Clinical models have demonstrated that PARP inhibition can lead to the inactivation of GSK3 and a dose-dependent upregulation of PD-L1. Consequently, this upregulation of PD-L1 can suppress T-cell activation, which may result in enhanced cancer cell apoptosis. This strategic combination therapy aims to leverage the immune system's ability to target cancer cells, particularly in the context of genomic instability associated with BRCA1/2 and HR deficiencies (29). In this study, we uncovered that LDHB facilitated cancer cell immune evasion *via* regulating the PD-L1 expression. Knockdown of LDHB reduced the glucose metabolism to suppress lactate accumulation, leading to decreased histone lactylation and downregulated expression of PD-L1. Overexpression of PD-L1 in ovarian cancer cells could attenuate the effects of LDHB inhibition. And LDHB modulated the lactylation and expression of PD-L1. Hence, we speculated

that LDHB1 participated in the immune evasion of ovarian cancer cells through epigenetically regulating PD-L1 expression.

The interaction between PD-1 and PD-L1 has been identified across a spectrum of cancers, and the levels of PD-L1 expression have become a valuable biomarker for anticipating patient outcomes with immunotherapies targeting this pathway (30). Immune checkpoint inhibitors (ICIs), including the PD-L1 inhibitors, represent a promising therapeutic strategy for patients with cancer of unknown primary (CUP), potentially capitalizing on their robust anti-tumor immune responses that may target the elusive primary lesion. Studies have indicated that ∼28% of CUP patients exhibit one or more predictive biomarkers for ICI treatment. This biomarker-driven approach holds promise for a disease that currently has a paucity of effective treatment options (31). Research into the control of PD-L1 expression encompasses a range of mechanisms, such as genetic factors, epigenetic modifications, transcriptional controls, and post-transcriptional adjustments (32). Investigations have uncovered that the expression of PD-L1 is subject to modulation by metabolites generated by tumors, with lactate being a notable example (33, 34). A recent work has reported that STAT5 promoted the lactylation of PD-L1 *via* promoting glycolysis and lactate accumulation (35, 36). Consistent with the previous studies, we identified that LDHB promoted glycolysis metabolism and enhanced lactate production, which consequently elevated PD-L1 lactylation and expression in ovarian cancer cells.

Lactate has been found to induce a unique form of post-translational modification on histones through a process termed lactylation, which involves the attachment of lactate to the lysine residues on histones (23). Tumor cells exhibit a pronounced metabolic adaptability, enabling them to activate anaerobic glycolysis and lactate fermentation as a response to oxygen deprivation (37–39). Key to this metabolic flexibility is the enzymes LDHA and LDHB, which catalyze the reversible reaction between pyruvate and lactate (24). Beyond their established metabolic roles in tumor cells, including their contribution to energy production and adaptation to hostile conditions, LDHA and LDHB are also implicated in the modulation of cellular life and death processes (40). Their influence extends to the regulatory mechanisms that govern cancer cell survival and immune surveillance, underlining their multifaceted impact on cellular physiology (41, 42). Here, we revealed that LDHB participates in the epigenetic regulation of cancer cells and affects the immune response. However, further studies should be performed to verify whether the LDHB-regulated immune evasion involves other regulatory mechanisms.

In conclusion, the LDHB regulated glycolysis and lactate production and ultimately drove lactylation on PD-L1 promoter and its expression. This LDHB-regulated PD-L1 expression could block the activation of T cells and impede their cytotoxicity against ovarian cancer cells. Our research illuminates the intricate interplay among metabolic processes, epigenetic modifications, and immune responses in the advancement of ovarian cancer. Specifically, it suggests that lactate, whose production is stimulated by LDHB, could potentially be harnessed as a target for therapeutic intervention in the clinical application of immunotherapy for ovarian cancer. This approach could offer a novel strategy for countering the disease by disrupting the metabolic pathways that support tumor growth and enhance immune evasion.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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