RESEARCH

NDR1 mediates PD-L1 deubiquitination to promote prostate cancer immune escape via USP10

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Abstract

Prostate cancer (PCa) is one of the most common male genitourinary system malignancies. Despite the significant benefits of anti-PD-L1 immune checkpoint inhibitor therapy in other cancers, the reasons for its poor therapeutic efficacy in prostate cancer (PCa) remain unclear.NDR1 plays an important role in innate immunity, but its role in tumor immunity and immunotherapy has not been investigated. The role of NDR1 in the immune microenvironment of PCa and the related mechanisms are unknown. Here, we found a positive correlation between NDR1 and PD-L1 expression in PCa. NDR1 significantly inhibits CD8+T cell infiltration and function, thereby promoting immune escape in prostate cancer.More importantly, NDR1 inhibition significantly enhanced CD8+T cell activation, which enhanced the therapeutic effect of anti-PD-L1. Mechanistic studies revealed that NDR1 inhibits ubiquitination-mediated PD-L1 degradation via the deubiquitinase USP10, upregulates PD-L1, and promotes PCa immune escape. Thus, our study suggests a unique PD-L1 regulatory mechanism underlying PCa immunotherapy failure. The significance of NDR1 in PCa immune escape and its mechanism of action were clarified, and combined NDR1/PD-L1 inhibition was suggested as an approach to boost PCa immunotherapy effectiveness.

Keywords Prostate cancer, NDR1/STK38, PD-L1, Immune escape, Immune checkpoint Blockade, Anti-PD-1 therapy

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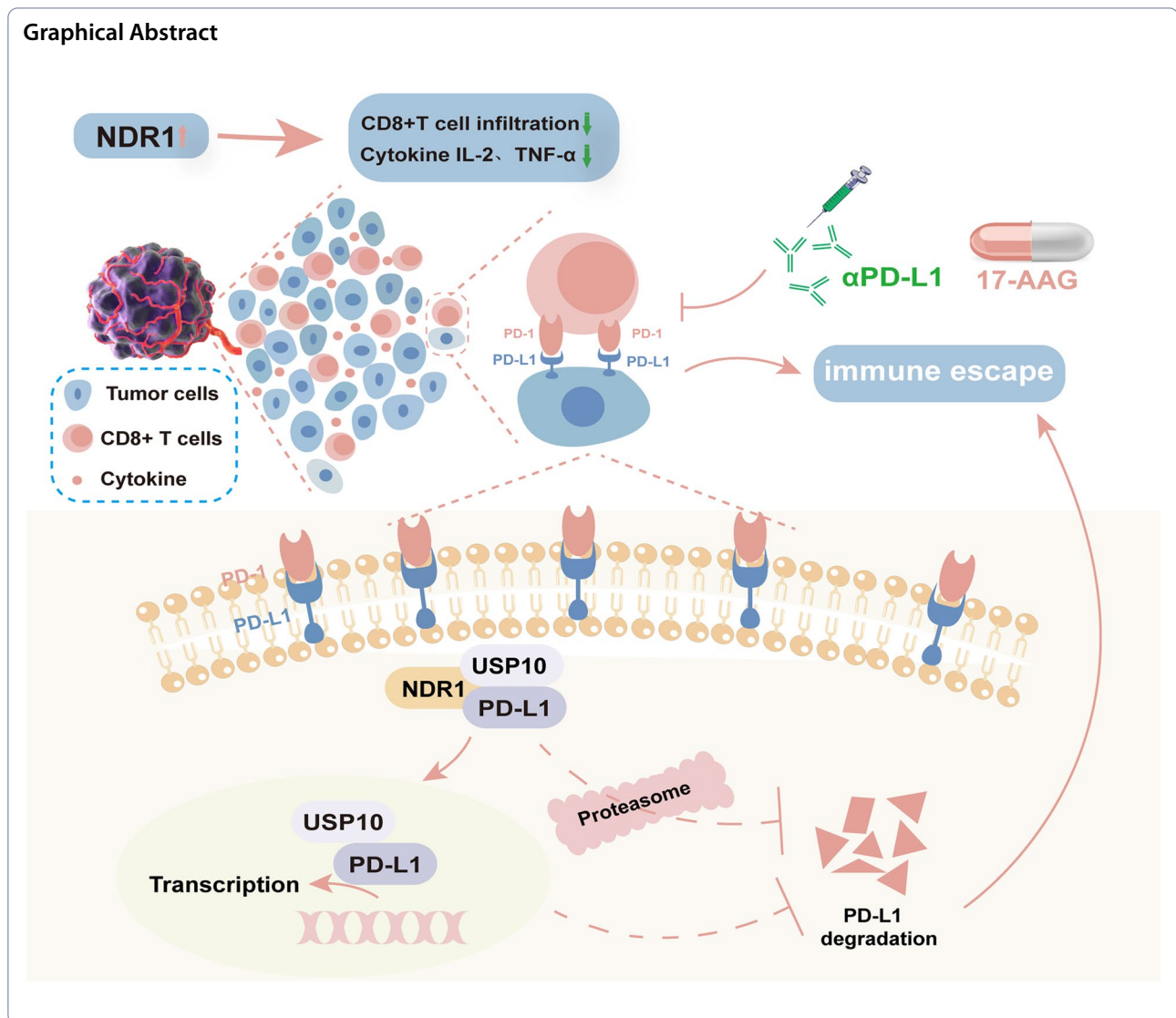*Correspondence:
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Graphical Abstract

Introduction

Prostate cancer (PCa) ranks as a frequently occurring malignant tumor within the male genitourinary system, holding the position of the second most prevalent cancer among men [1]. Surgery, radiotherapy and androgen deprivation therapy (ADT) are the mainstays of conventional PCa treatment [2], with ADT serving as the primary therapy for advanced PCa [3]. Prostate cancer patients ultimately failed ADT treatment due to drug resistance and developed metastatic castration resistant prostate cancer (mCRPC) [4]. Moreover, in vivo research suggests that ADT therapy may increase side effects such as neuroendocrine differentiation (NED) and PCa cell invasion [5].

In recent years, immunotherapy, particularly immune checkpoint inhibitors (ICIs) like anti PD-1/PD-L1, has shown promise in treating various solid tumors [6], including lung [7] and kidney cancer [8]. However, using anti PD-1/PD-L1 alone hasn't yielded satisfactory results in prostate cancer treatment [9]. PCa is a "cold tumor" that is mainly characterized by poor T-cell infiltration, low tumor mutation load and immunosuppressive tumor microenvironment, so immunotherapy is not effective in PCa [10]. In addition recent insights in cancer immunotherapy suggest that PD-L1 expression levels in cancer cells may influence the response to anti-PD-L1 therapy [11]. Investigating PD-L1 regulation in prostate cancer may uncover why immunotherapy isn't effective and could be crucial in finding complementary treatments to enhance its response.

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NDR1, part of the AGC kinase family, is also known as serine-threonine protein kinase 38 (STK38) [12]. As a part of the Hippo signaling system, NDR1 can work in tandem with NDR2, LATS1, and LATS2 [13] and is primarily engaged in cellular mitosis, embryonic development, and centrosomal replication [14]. The role of NDR1 in cancers is complicated. Previous studies have shown that NDR1 can be used as the proto-oncogene of tumors to promote the occurrence and development of tumors, including breast cancer [15] and in small cell lung cancer [16], etc. Some studies have also shown that NDR1 can be used as the tumor suppressor gene to inhibit the progress of tumors, including gastric cancer [17], skin cancer [18], etc. Especially in prostate cancer, there have been reports of both cancer suppression [19] and cancer promotion [20]. NDR1 is necessary for thymocyte export and T-cell migration, according to certain research, and in vivo tests have revealed that NDR1 exert a vital role in the innate immune response [21]. Liu et al. found through bioinformatics research that NDR1 is closely related to cancer immunity [22]. However, the significance of NDR1 in the immunological microenvironment of PCa and the related underlying mechanisms are unclear.

In our research, we confirmed that NDR1 increases PD-L1 expression and inhibits T cell infiltration, promoting immune escape in prostate cancer. Crucially, inhibiting NDR1 significantly improves the effectiveness of anti-PD-L1 therapy, offering a fresh approach to enhance PCa immunotherapy. This study aimed to unveil NDR1's role in regulating PD-L1 and its contribution to immune evasion in PCa, while investigating combination therapy options to enhance immunotherapy response.

Methods and materials
Cell line and cell culture
All cells, including prostate cancer cells (PC3, 22rv1, and DU145), murine prostate cancer RM-1 cells, and HEK293T, were obtained from the ATCC.

All cells were cultured in complete medium (1640 medium [Gibco, China], then supplemented with 10% heat-inactivated fetal bovine serum [Gibco, China]), 1% penicillin/streptomycin (Macklin, China) were routinely cultured. All cells applied in this study were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Plasmids and transfection
Transfer plasmids or siRNAs to the destination according to the requirements of the transfection reagent.

Plasmids were purchased from Sino Biological: pCMV3-STK38-Myc (HG12319-NM), pCMV3-PD-L1-His (HG10084-CH), pCMV3-USP10-HA (HG17833-CY).

Western blot assay
Place cells in a lysate containing proteasome inhibitors for lysis and obtain a protein solution by denaturing the lysate. After electrophoresis, membrane transfer, room temperature sealing for 1 h, overnight incubation of the corresponding first antibody, and room temperature incubation of the second antibody for 1 h, the obtained protein solution can be imaged through chemiluminescence.

Immunoprecipitation
For Co-IP determination, cell lysis uses IP specific lysis solution (Thermo Fisher Scientific, MA, USA), while others are similar to regular cell lysis. Divide the final obtained protein solution into two parts, one part is directly added to the loading buffer for boiling denaturation as the input group, and the other part is antibodies according to the requirements of the magnetic bead (Bimake, USA) instruction manual. For continuous IP samples, anti HA antibodies and magnetic beads are added to the first Anti Myc IP sample for a second IP.

Protein half-life assay
After applying cycloheximide (CHX, 100ug/ml) to the culture medium, cells were collected at 4.8.12.24 h for Western blot assay.

Ubiquitination analysis
After transfection of Myc-NDR1, HA-Ub, His-PD-L1 or HA-USP10, Myc-Ub, His-PD-L1 into HEK293T cells for 48 h. After 48 h of transfection, the cells were treated with MG132 (40 ng/ul) for 6 h, followed by immunoprecipitation with anti His antibodies.

LDH toxicity test and T-cell killing test
Co-culture PC3 cells transfected with plasmids (NDR1 and pCMV) in a 1:10 ratio with human peripheral blood monocytes (activated by co-stimulation with anti-CD3/CD28 magnetic beads T (ThermoFisher, USA)) for 48 h. The supernatants of the acquired cells were assayed for LDH content according to LDH kit (Beyotime Biotechnology, China) to detect the LDH content.

Establishment of mouse tumor model
All animal experiments received approval from the Ethics Committee of Xiamen University School of Medicine. We obtained 6–8-week-old male C57BL/6 and C57BL/6-nu mice from Xiamen University's Experimental Animal Center.

Inject tumor cells(RM-1 control cells and RM-1-shNDR1 or RM-1 NDR1 cells (1×10^6)) subcutaneously into mice and measure the tumor volume every 5 days.

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After the experiment is completed, tumors were obtained from mice and weighed for subsequent experiments.

For treating mice, we randomly divided them into four groups: control group, 17AAG (NDR1 inhibitor) treatment group, anti PDL1 treatment group and combined treatment group. Every three days, mice were treated with intraperitoneal injection of anti PD-L1 (dose 200ug/animal, Bio X Cell, USA) and 17AAG (dose 75 mg/kg, MedChemExpress, China) to simultaneously measure tumor volume and mouse weight. After five treatments, all mice were euthanized, and subsequent experiments were conducted.

Mouse tumor and human lymphocyte extraction

After digesting mouse tumor tissue with collagenase IV (C8160, Solarbio, China), tumor infiltrating lymphocytes were isolated from percoll cell separation solution(P8370, Solarbio, China).

Normal human peripheral blood was extracted according to Human Peripheral Blood Single Nucleated Cell Isolation Reagent (P8610, Solarbio, China).

RT-qPCR assay

According to the requirements of the kit, extract total RNA from cells (AC0202-A, SparkJake, China) and reverse transcription (RK20400, ABclonal, China) for RT-PCR.

Perform RT-PCR using complementary DNA and SYBRGreen II (RK21203, ABclonal, China). Primers sequences were in Table S1.

Antibodies

NDR1 (A-8) (sc-365555, Santa Cruz), PD-L1 (#13,684,cst), USP10 (#8501,cst), H3 (#4499,cst) His-tag (AE068, ABclonal), HA-tag (AE008, ABclonal), Myc-tag (AE010, ABclonal). CD25 (ab227834, Abcam). PD-L1 (2B11D11, proteintech). anti-human antibody(Biolegend): PE-CD3 (317,307), PE-PD-L1 antibody (329,705);anti-mouse antibody(Biolegend):FITC-CD45 (157,214), perCP- CD45 (103,129), APC-CD8 (126,614), BV421-CD69(124,529).

Statistical analysis

The data was statistically analyzed with GraphPad Prism 8 software from GraphPad Software Inc. in San Diego, CA, USA. Statistically significant results were represented as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$), while 'ns' indicated no significant difference.

Results

NDR1 promotes tumor growth by suppressing the immune response

To investigate the effect of NDR1 on PCa, we first created cell lines with stable NDR1 knockdown and transient NDR1 overexpression with the mouse PCa cell line RM-1 as the parental cell line (Fig. 1A, B). Following that, control and NDR1 overexpression (RM-1-NDR1)/NDR1 knockdown (RM-1-shNDR1) cells were implanted subcutaneously into C57BL/6 mice and T-cell-deficient C57BL/6-nu nude mice to create subcutaneous transplantation tumor models respectively. The procedures used for tumor generation and detection are shown in Fig. 1C. The results showed that in C57BL/6 mice, the tumor weight and volume in the RM-1-NDR1 group were significantly higher than those in the control group (Fig. 1D-F), but the tumor weight and volume in the RM-1-shNDR1 group were significantly lower (Fig.S1A-C). The findings imply that NDR1 promotes the growth of PCa in immunocompetent C57BL/6 mice. However, the tumor weight and volume of the RM-1-NDR1 group in immunodeficient C57BL/6-nu nude mice were significantly lower than those of the control group (Fig. 1G-I). Contrary conclusions were observed in immunocompetent and immunocompromised mice. In immunocompromised mice,NDR1 may exert tumor suppressive effects, while in immunocompromised mice,NDR1 may promote the progression of prostate cancer. The close relationship between NDR1 and immunity may be used to partially explain the complexity of both pro cancer and anti cancer reports of NDR1 in prostate cancer.

NDR1 suppresses immune response by inhibiting T cell response

To ascertain whether NDR1 suppresses the immune response to promote tumor growth. First, We conducted a pathway enrichment analysis using TCGA data, revealing an association between NDR1 and immunity (Fig. 2A). We then examined immune cell infiltration in PCa cells with altered NDR1 expression and observed a negative correlation between NDR1 and CD8 + T-cell infiltration into tumors (Fig. 2B). Therefore, we proposed that NDR1 inhibits T-cell responses to promote tumor growth. By performing flow analysis on the subcutaneous tumors of the C57BL/6 mice mentioned above (Fig. 1D and S1A), we found that the percentage of CD8 + T cell infiltration in subcutaneous tumors of mice injected with RM-1 NDR1 cells was significantly lower compared to the control group (Fig. 2C), while it was significantly higher in mice that had received injections of RM-1 ShNDR1 cells (Fig.S2A). Moreover, our immunohistochemical staining results were consistent with the flow cytometry results (Fig. 2E). CD69 is a CD8 + T-cell activation marker [23]. These results indicate a substantial decrease in the CD8 + T cell population expressing CD69 within the tumors of mice
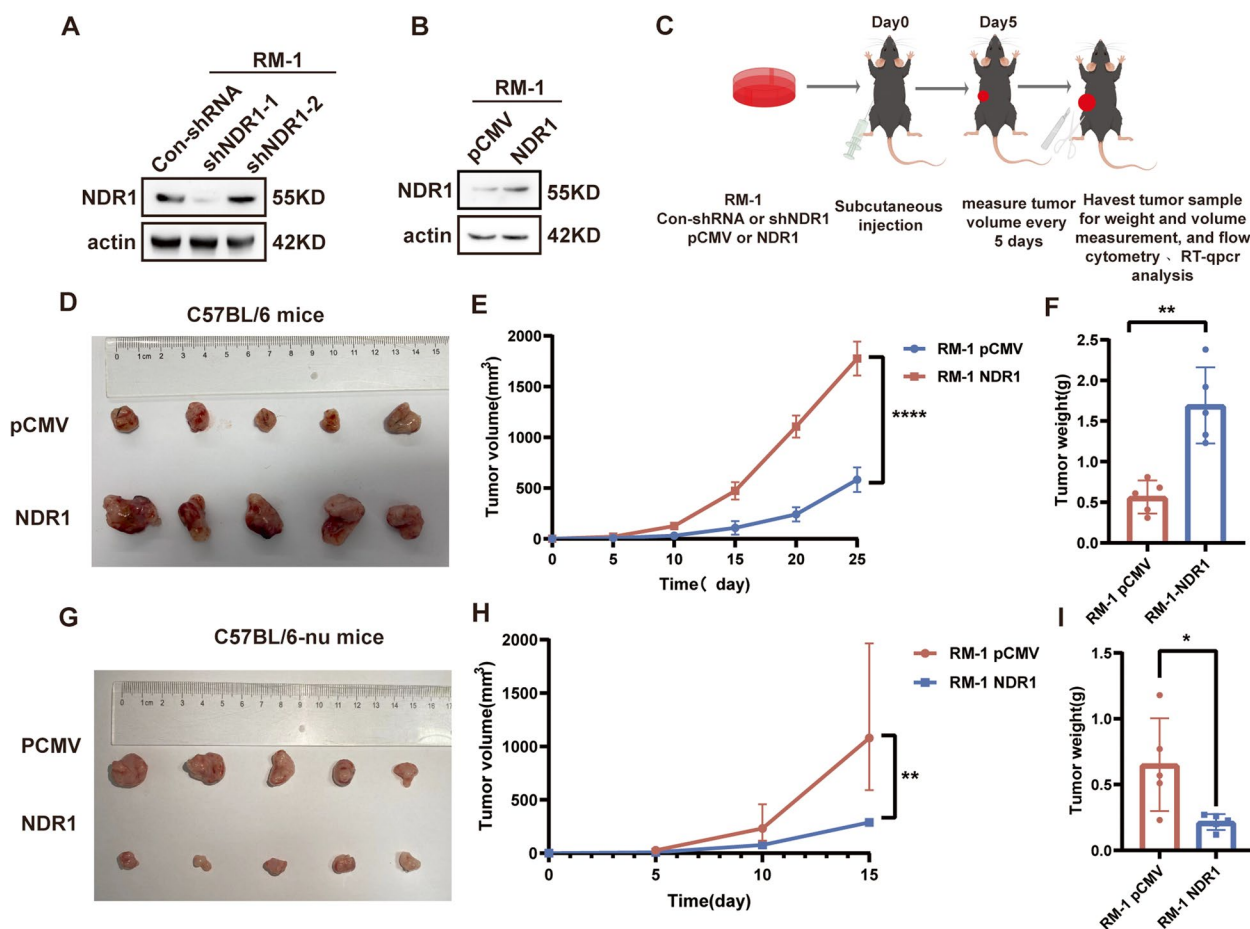
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Fig. 1 NDR1 promotes tumor growth by suppressing the immune. **A-B** Stable cell lines with overexpression and knockdown of NDR1 was constructed in RM1 cell line. **C** The diagram illustrates the subcutaneous injection of either control RM-1 cells or RM-1 cells with silenced NDR1 into C57BL/6 mice. **D-F** Display representative images (**D**), tumor growth curve (**E**), and tumor weight (**F**) of control or RM-1 NDR1 cells injected into C57BL/6 mouse tumors. Measure the tumor at the designated time point and dissect the tumor at 25 days (*n* = 5). **G-I** Display representative images (**G**), tumor growth curve (**H**), and tumor weight (**I**) of control or RM-1 NDR1 cells injected into C57BL/6-nu mouse tumors. Measure the tumor at the designated time point and dissect the tumor at 15 days (*n*=5).

in the RM-1 NDR1 group as opposed to the control group (Fig. 2D). However, in subcutaneous tumors of mice injected with RM-1 ShNDR1 cells, there was a notable increase in the percentage of CD8 + T cells expressing CD69. (Fig.S2B). The important growth factor for T-cell activation, IL-2, is a crucial part of T cells [24]. Moreover, activated CD8 + T cells can generate IL-2 and TNF-α, which enhance the cytotoxicity

of CD8 + T cells [25], so we detected IL-2 and TNF-α in tumor tissues by RT–qPCR, and compared with the control group, The results showed that the expressions of IL-2 and TNF-α were decreased in the over-expression group(Fig.S2C). At the same time, we demonstrated once again with immunohistochemical staining that the overexpression of NDR1 resulted in a decrease in IL-2 expression (Fig. 2F). The above results indicate

(See figure on next page.)
Fig. 2 NDR1 suppresses immune response by inhibiting T cell response. **A** Gene enrichment analysis of NDR1 in the KEGG database in PRAD. **B** NDR1 expression is associated with immune cell infiltration based on CIBERSORT. **C-D** Flow cytometry was used to analyze tumor-infiltrating lymphocytes (*n* = 5). **E-F** IHC assessed CD8 and CD25 expression in mouse tumor tissues (×200 above;×400 below), with a scale bar of 100 mm or 50 mm. **G** Schematic diagram showing co-culture of tumor cells and activated T cells. **H** Co-culture PC3 cells overexpressed with control or NDR1 with activated T cells, and detect the apoptosis of PC3 by flow cytometry. **I** Representative images and statistical analysis results of T cell killing test. **J** Statistical analysis results of LDH toxicity test
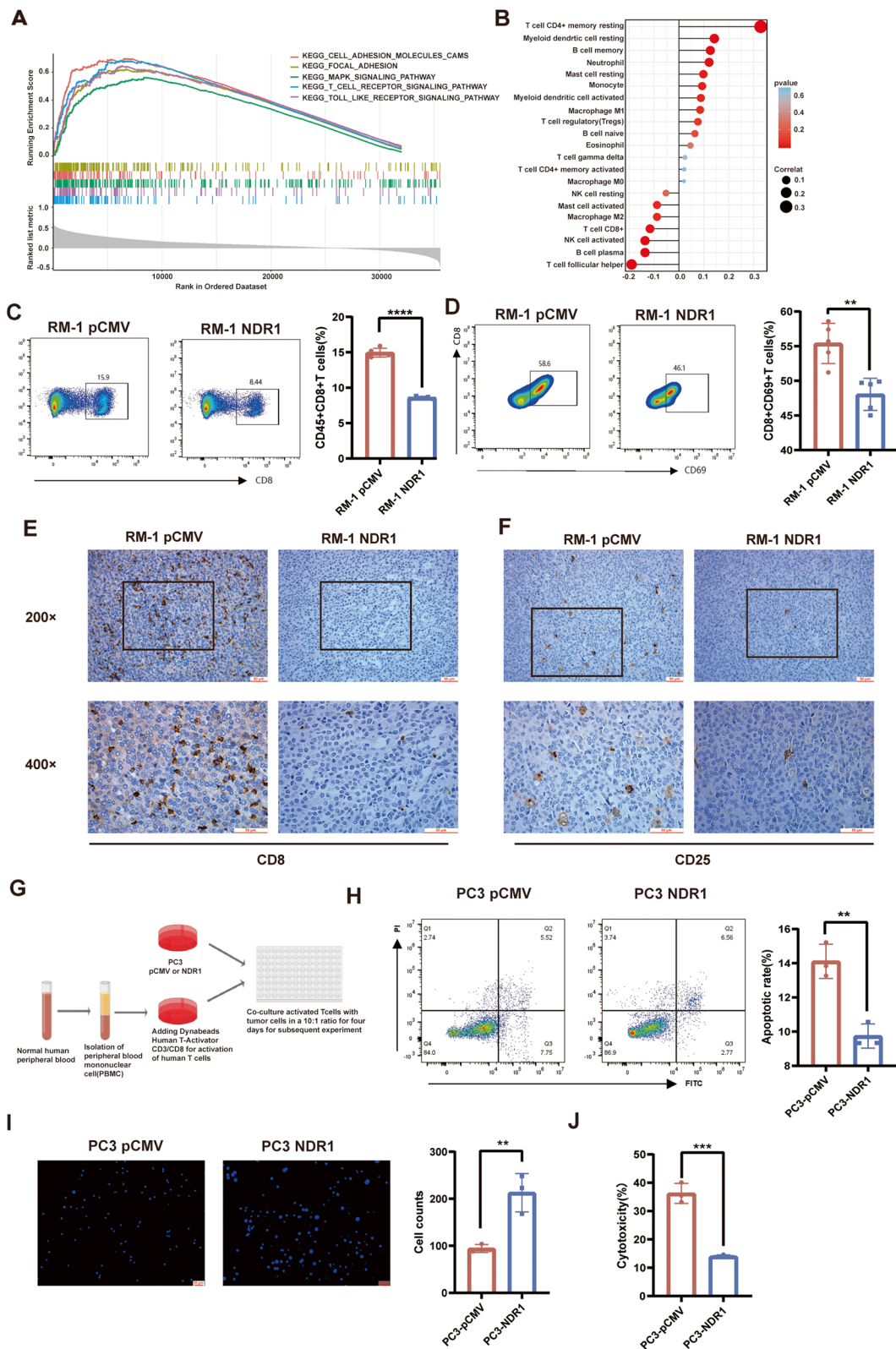
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Fig. 2 (See legend on previous page.)

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that NDR1 may inhibit CD8 + T-cell function and promote tumor immune escape by inhibiting CD8 + T-cell infiltration and activation in vivo.

In addition, we co-cultured human PCa cells with anti-CD3/CD28 magnetic bead-activated T cells (see Fig. 2G for schematic) and the results showed that tumor cells overexpressing NDR1 reduced apoptosis (Fig. 2H), the cytotoxity of T cells on tumor cells was attenuated (Fig. 2I). Lactate dehydrogenase (LDH) is an enzyme that is stable in the cytoplasm, and normally exists only in the cell. When the cell membrane is harmed, it's swiftly released into the culture medium. Through the determination of the content of LDH in cell supernatant on the degree of damage can be judged cells [26]. We found that LDH levels in the supernatant were significantly lower when NDR1 was overexpressed than in the control group, indicating that the cytotoxicity of T cells against these cells was reduced (Fig. 2J). Due to our concern that the killing effect on tumor cells is caused by cytokines secreted by T cells in the supernatant, we co cultured the supernatant with tumor cells (Fig. S2D). The results showed that the cytokines in the supernatant had no killing effect on tumor cells (Fig. S2E-F), further confirming that NDR1 may promote tumor growth and immune escape by inhibiting T cell responses.

To summarize, these findings indicate that NDR1 inhibits cytotoxic T cells in both in vivo and in vitro settings, thereby significantly promoting PCa cell growth and immune evasion.

NDR1 is positively correlated with PD-L1 expression in PCa
To explore the relationship between NDR1 and PD-L1, we overexpressed NDR1 in PC3 cells, resulting in a corresponding increase in PD-L1 expression (Fig. 3A). Knocking down NDR1 in DU145 cells led to downregulation of PD-L1 (Fig. 3B). We used flow cytometry to detect PD-L1 expression on the cell membrane, and the results were consistent (Fig. 3C). Next, we utilized the TIMER2.0 database, the analysis demonstrated a positive correlation between NDR1 and PD-L1(Fig. 3D). Moreover, WB and IHC of tumor tissues from mice described above (Fig. 1D) showed that PD-L1 increased with the increase of NDR1 (Fig. 3E, F). Finally, we used samples from prostate cancer patients for further validation. The immunohistochemical staining results showed that as the tumor progressed, the expression level of NDR1 increased, and the expression level of PD-L1 also increased (Fig. 3G, H). In summary, our research findings further confirm the positive correlation between the expression of NDR1 and PD-L1 in prostate cancer.

NDR1 binds to PD-L1 and enhances PD-L1 stability
Next, we explored the mechanism of action of NDR1 and PD-L1. First, we investigated the interaction between NDR1 and PD-L1. The Co-IP results showed that NDR1 coprecipitated with PD-L1, and the same results were found for endogenous IP(Fig. 4A, B). Immunofluorescence experiments showed colocalization of NDR1 and PD-L1 in the spatial structure (Fig. 4C). The above results indicate that NDR1 can bind to PD-L1 in PCa cell lines. Since knockdown of NDR1 resulted in an opposite trend of PD-L1 at the mRNA level and protein level (Fig. 3A, B), we considered that it might be due to the effect of NDR1 on the stability of PD-L1. To test whether NDR1 regulates the stability of PD-L1 protein, we examined the expression level of PD-L1 in PCa cells treated with the protein synthesis inhibitor CHX. Overexpression of NDR1 in PC3 significantly increased the stability and half-life of PD-L1, whereas knockdown of NDR1 in DU145 cells significantly destabilized the PD-L1 protein (Fig. 4D, E). This indicates that NDR1 inhibited PD-L1 degradation and increased PD-L1 stability. The autophagy-lysosomal pathway and the ubiquitin–proteasome pathway are the major pathways of protein degradation [27]. We treated DU145 cells with the ubiquitin–proteasome pathway inhibitor MG132 and the autophagy–lysosome pathway inhibitor chloroquine diphosphate (CQ) and found that MG132 could reverse PD-L1 regulation by NDR1 to some extent, but not after CQ treatment (Fig. 4F). Moreover, we found that overexpression of NDR1 can inhibit PD-L1 ubiquitination (Fig. 4G). In summary, the above results indicate that NDR1 binds to PD-L1 and enhances its stability by inhibiting the ubiquitination pathway.

USP10 binds to PD-L1 and mediates its deubiquitination
Promoting protein stability can be achieved by removing the ubiquitin pathway from proteins, which is achieved through deubiquitinases (DUBs) [28], namely deubiquitination. Similar to ubiquitination, deubiquitination mediated regulation requires specific DUB.We speculate that NDR1 affects the stability of PD-L1 by affecting its deubiquitinase. To identify the potential deubiquitinase of PD-L1, we intersected the mass spectrometry results of NDR1 with those of PD-L1. The highest-scoring deubiquitinase identified was USP10 (Fig. 5A). First, we checked whether USP10 and PD-L1 could bind. The Co-IP showed that USP10 coprecipitated with PD-L1, the same results were found for the endogenous IP experiment (Fig. 5B, C). Immunofluorescence experiments showed colocalization of USP10 and PD-L1 in the spatial structure (Fig. 5D). These results suggest that USP10 and PD-L1 bind in PCa.

Then, we detect the expression of USP10 in several PCa cell line. As shown (Fig. 5E), USP10 was highly expressed in DU145 cells but expressed at low levels in PC3 and
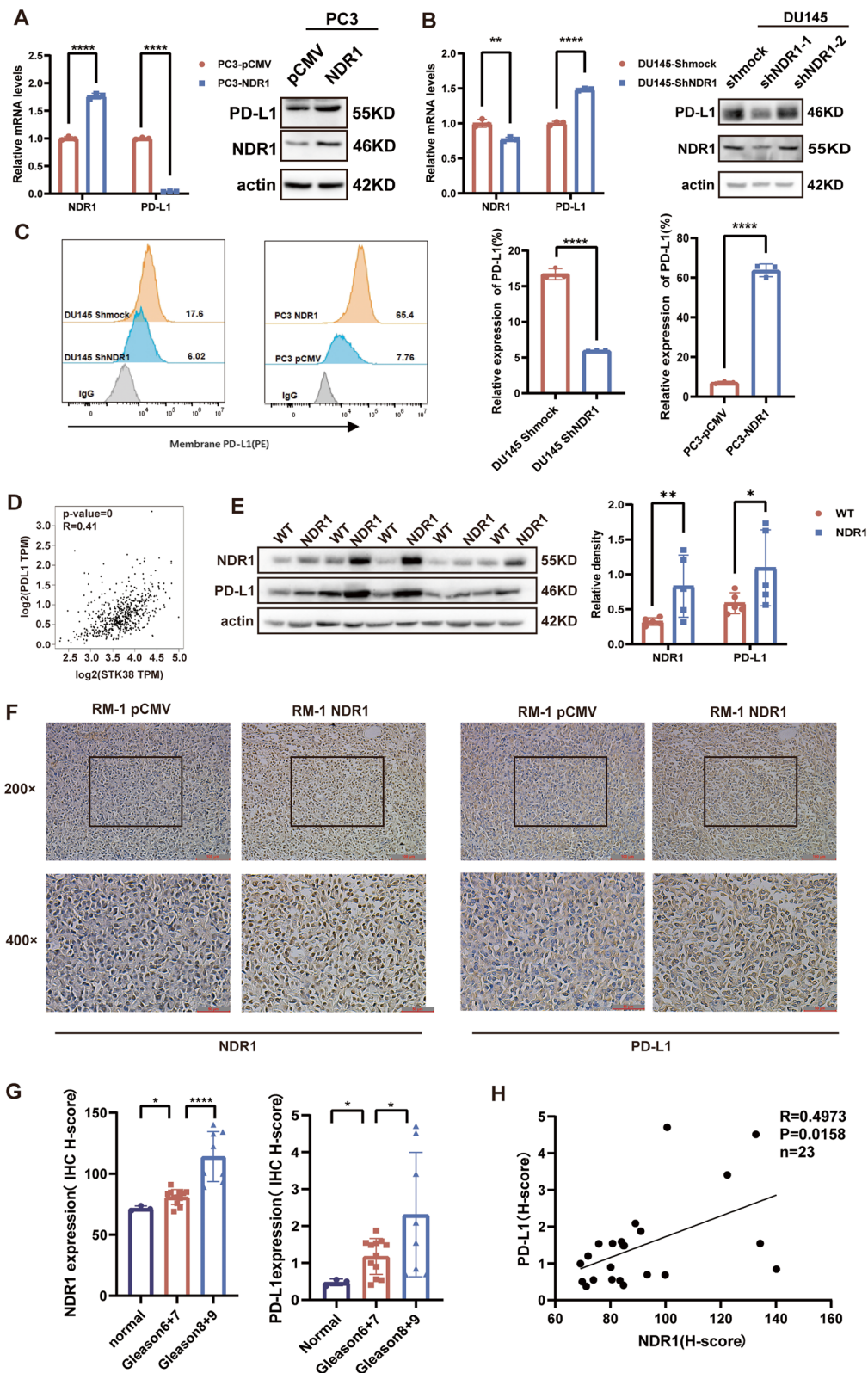
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Fig. 3 NDR1 is positively correlated with PD-L1 expression in PCa. **A** In NDR1-overexpressing PC3 cells, we assessed NDR1 and PD-L1 expression using WB. **B** In NDR1 knockdown DU145 cells, we examined NDR1 and PD-L1 expression via WB. **C** Evaluation PD-L1 expression on the cell surface. **D** Analyzing the correlation between NDR1 and PD-L1 using TIMER2.0 database. **E-F** Detection the levels of NDR1 and PD-L1 in mouse tumor tissues via WB and IHC. **G**-**H** IHC staining and statistical results of NDR1 and PD-L1 in patients ($n = 23$)

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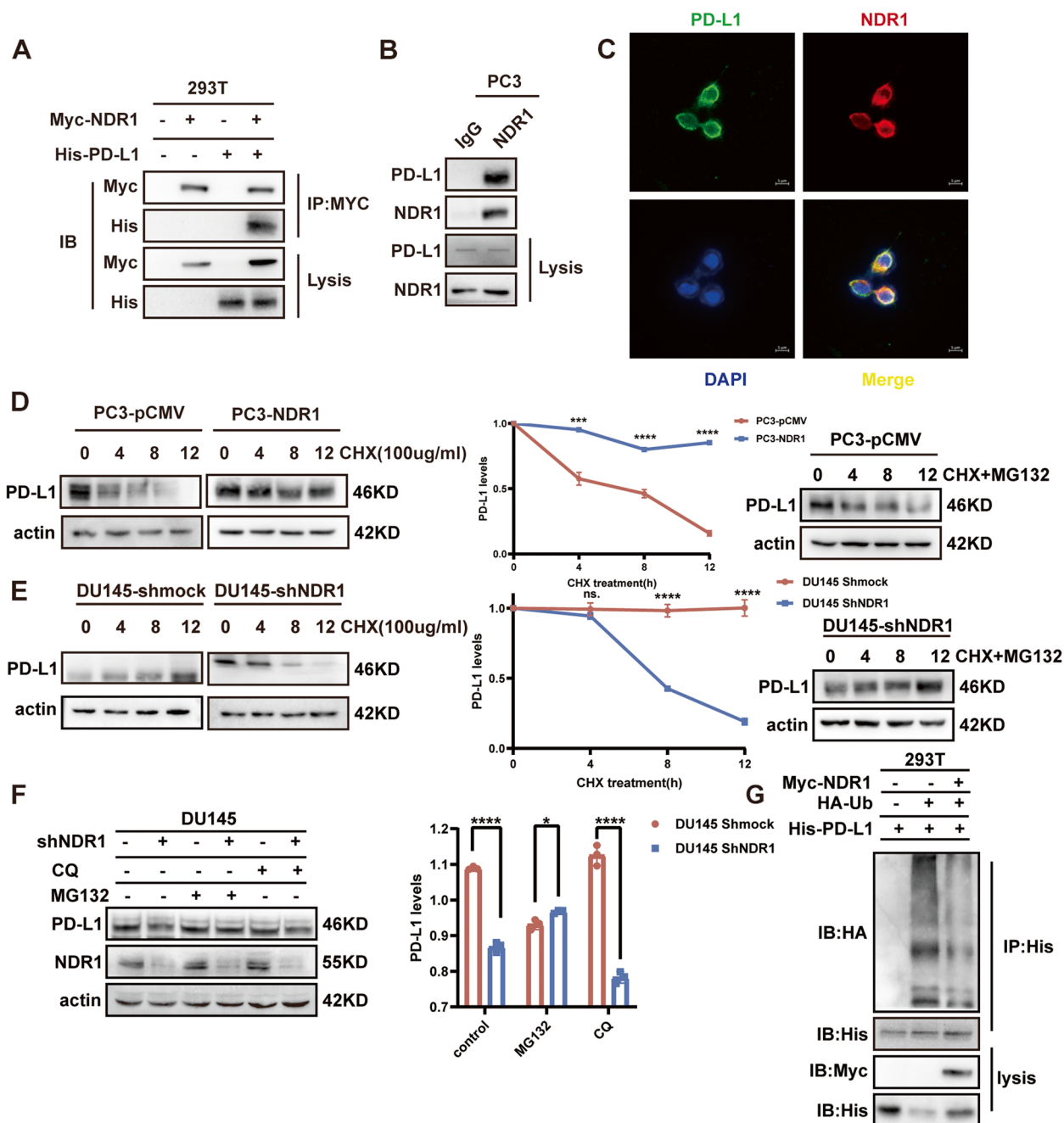
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Fig. 4 NDR1 binds to PD-L1 and enhances PD-L1 stability. **A** Exogenous PD-L1 coimmunoprecipitated with NDR1 in 293 T cells. **B** NDR1 was coimmunoprecipitated with PD-L1 in PC3. **C** IF experiment to determine the spatial localization of NDR1 and PD-L1. **D** PC3/pCMV and PC3/NDR1 cells were subjected with CHX (100 μg/mL) or MG132 at specified intervals to detect PD-L1-degrading protein levels. **E** DU145/shmock and DU145/shNDR1 cells were subjected with CHX (100 μg/mL) or MG132 at specified intervals to detect PD-L1-degrading protein levels. **F** PD-L1 level in NDR1 knockdown cells subjected with MG132 and CQ by WB. **G** The impact of NDR1 on PD-L1 ubiquitination in 293 T cells was detected

22rv1 cells. Thus, we overexpressed USP10 in PC3 and 22rv1 cell lines, the expression level of PD-L1 increased with the increase of USP10 (Fig. 5F and S3A). Knockdown of USP10 using siRNA technology (Figure.S3B) or USP10 inhibitor (Spautin-1 [29]) in DU145 cells resulted in a decrease in PD-L1 expression level and a dose-dependent

decrease (Fig. 5G, H and S3C). We examined the expression level of PD-L1 in PCa cells treated with the protein synthesis inhibitor CHX, and overexpression of USP10 prolonged the half-life of PD-L1 (Fig. 5I). A ubiquitination assay showed that USP10 in PC3 cells remarkably increased the stability and half-life of PD-L1(Fig. 5J). In
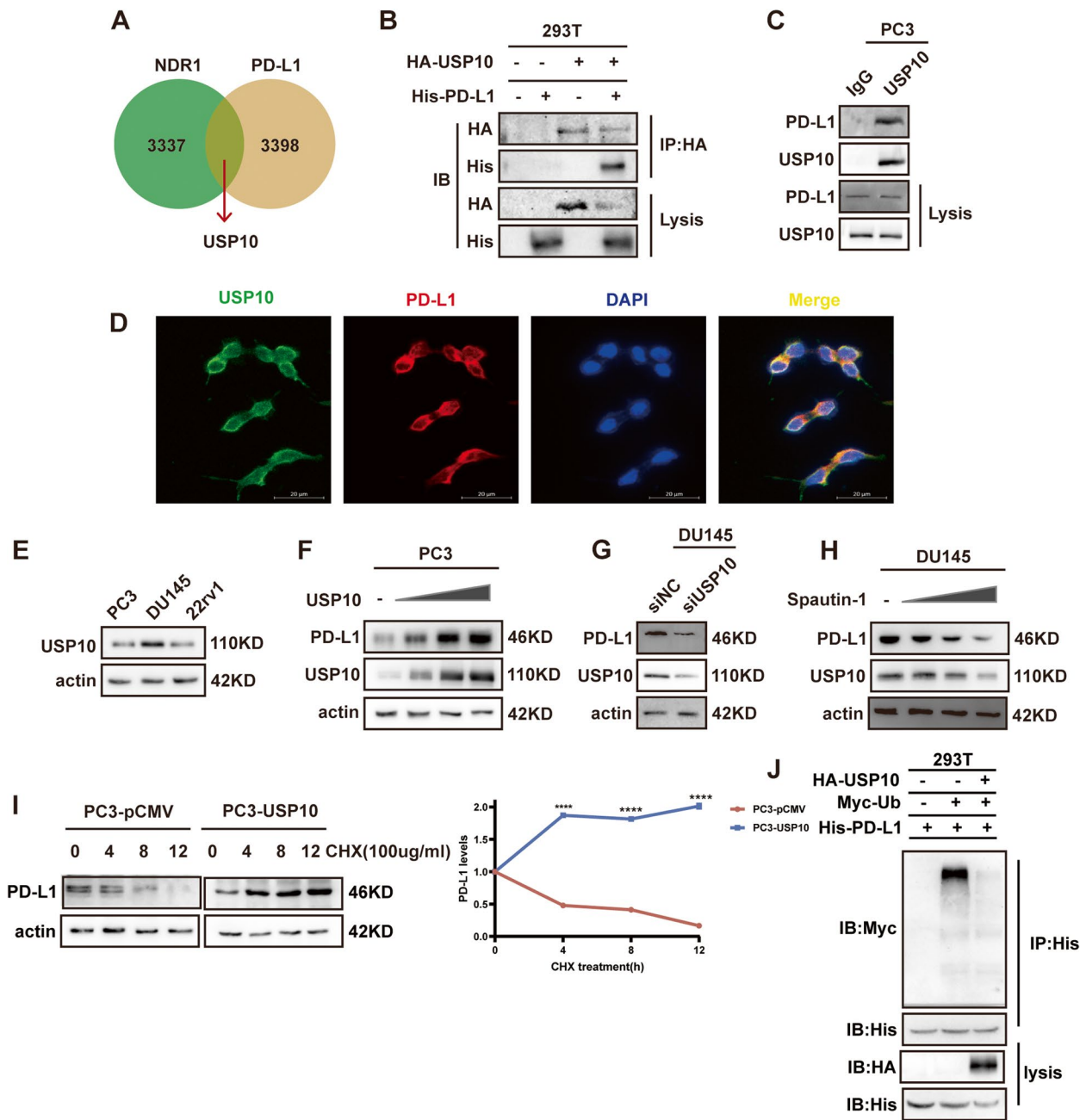
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Fig. 5 USP10 binds to PD-L1 and mediates its deubiquitination. **A** Identification of the number of candidate proteins interacting with NDR1 and PD-L1 in PC3 cells through CO-IP-MS analysis. **B** Exogenous PD-L1 coimmunoprecipitated with USP10 in 293 T cells. **C** USP10 was coimmunoprecipitated with PD-L1 in PC3 cells. **D** IF experiment was conducted to determine the spatial localization of USP10 and PD-L1. **E** USP10 expression in prostate cancer cells. **F** Detection of PD-L1 expression level in PC3 cells after concentration gradient increase of USP10 by WB. **G** Detection of PD-L1 expression in DU145 cells with siUSP10 gene knockdown by WB. **H** PD-L1 expression levels in DU145 cells were assessed using a concentration gradient of the USP10 inhibitor (Spautin-1) via immunoblot analysis. **I** PC3/pCMV and PC3/USP10 cells were subjected with CHX (100 μg/mL) at specified intervals to detect PD-L1-degrading protein levels. **J** The impact of USP10 on PD-L1 ubiquitination in 293 T cells was detected

brief, these findings indicate that USP10 binds to PD-L1, promoting its deubiquitination.

NDR1 promotes binding of USP10 and PD-L1 and inhibits PD-L1 ubiquitination degradation

Following this, we wanted to further explore whether NDR1 regulates PD-L1 through USP10. The Co-IP results showed that NDR1 coprecipitated with USP10, and the same results were found with endogenous IP experiments (Fig. 6A-B and S4A). Immunofluorescence experiments showed colocalization of USP10 and NDR1 in the spatial structure (Fig. 6C). The above results suggest that USP10 and NDR1 bind in PCa.

Since both USP10 and PD-L1 can interact with NDR1, Continuous IP analysis confirmed that NDR1, PDL1 and USP10 can form trimers (Fig. 6D). Subsequently, we wanted to explore whether NDR1 regulates PD-L1 by affecting USP10. We discovered that although NDR1 did not affect the RNA and protein levels of USP10 (Fig. 6E, F and S4B), immunoprecipitation experiments and immunofluorescence experiments showed that overexpression of NDR1 enhanced the interaction between USP10 and PD-L1 (Fig. 6G, H). Interestingly, we found that overexpression of NDR1 resulted in an increase in USP10 in the nucleus and an increase in PD-L1 (Fig. 6I), while an intranuclear ubiquitination assay showed that overexpression of USP10 inhibited the ubiquitination of PD-L1 (Fig. 6J). Research has indicated that nuclear PD-L1 can activate diverse immune response pathways, facilitating immune evasion [30, 31]. Knocking out NDR1 in DU145 cells resulted in a decrease in PD-L1 expression, but overexpressing USP10 resulted in an upregulation of PD-L1 expression. These results indicate that USP10 plays an important role in the regulation of PD-L1 by NDR1(Fig. 6K). Moreover, We examined PD-L1 expression in PCa cells treated with CHX. In NDR1 knockdown DU145 cells, PD-L1 degraded rapidly, but its degradation was slowed down when USP10 was overexpressed. (Fig. S6C). These results showed that NDR1 promotes the binding of USP10 and PD-L1, inhibits the ubiquitination degradation of PD-L1 and upregulated PD-L1.

Inhibiting NDR1 significantly enhances the effectiveness of anti-PD-L1 therapy for PCa

The above findings demonstrate that NDR1 increases PD-L1 expression in prostate cancer and suppresses T cell infiltration in the tumor microenvironment. To further confirm whether combining NDR1 inhibition with an anti-PD-L1 antibody can inhibit tumor growth and enhance T cell infiltration, we subcutaneously injected RM1 cells into mice and randomly divided them into four groups: control, NDR1 inhibitor (17AAG [32]), anti-PD-L1, and combination treatment (Fig. 7A). The experimental results revealed that the combination treatment group reduced tumor burden compared to the monotherapy groups (Fig. 7B-D). Moreover, there were no significant changes in the mice's body weight (Fig. 7E) and HE staining indicated no noticeable tissue damage or lesions in the liver and kidneys (Fig. 7F). This suggests that 17AAG and anti-PD-L1 are relatively safe in in vivo tests. Additionally, the combined treatment group exhibited increased infiltration of CD69 + CD8 + T cells (Fig. 7G). These findings imply that NDR1 inhibitors enhance the effectiveness of anti-PD-L1 therapy in prostate cancer by boosting CD8 + T cell activity.

Discussion

Tumor immune escape refers to the evasion of tumor cells from the recognition and attack of the immune system through various mechanisms. This promotes tumor growth, metastasis, and is a vital strategy for tumor survival and development [33]. Tumor cells evade immune recognition, in part due to the development of an immunosuppressive tumor microenvironment [34]. The PD-L1/PD-1 signaling pathway is a vital element of tumor immunosuppression [35]. Inhibiting T cell cytotoxicity allows tumors to escape, which is a major contributor to tumor immune evasion. Therefore, understanding the mechanism of PD-L1 expression regulation is of great significance for further improving the effectiveness of PD-1/PD-L1 targeted therapy.

However, PD-L1 expression has complex regulatory mechanisms, such as gene transcription [36] and posttranscriptional and posttranslational modifications [37]. Evidence suggests that PD-L1 protein expression is generally regulated by proteasomal degradation pathway [37–39]. In non-small cell lung cancer, OTUB1 acts as a ubiquitin enzyme for PD-L1, which boosts PD-L1 expression and facilitates immune evasion [40]. In OSCC, USP9x was discovered as a deubiquitinating enzyme for PD-L1 [41]. Moreover, CSN5 exerts a role

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Fig. 6 NDR1 promotes binding of USP10 and PD-L1 and inhibits PD-L1 ubiquitination degradation. **A** Exogenous NDR1 coimmunoprecipitated with USP10 in 293 T cells. **B** USP10 was coimmunoprecipitated with NDR1 in PC3 cells. **C** IF experiment revealed the spatial localization of USP10 and NDR1. **D** Sequential IP assay for NDR1-USP10-PD-L1 interaction. **E**-**F** NDR1 knockdown in DU145 cells was assessed for its impact on the protein and mRNA expression of NDR1 and USP10. **G-H** co-IP assays and IF analysis to explore the relationship between USP10 and PD-L1 in 293T cells, with or without NDR1 overexpression. I: Intranuclear USP10 and PD-L1 expression levels in PC3 cells following a concentration gradient increase of NDR1. **J** The ubiquitination of intranuclear PD-L1 was examined in 293T cell by WB. **K** The expression of USP10,NDR1and PD-L1 was detected in NDR1 knockdown or overexpressed DU145 cells with or without Transfected USP10
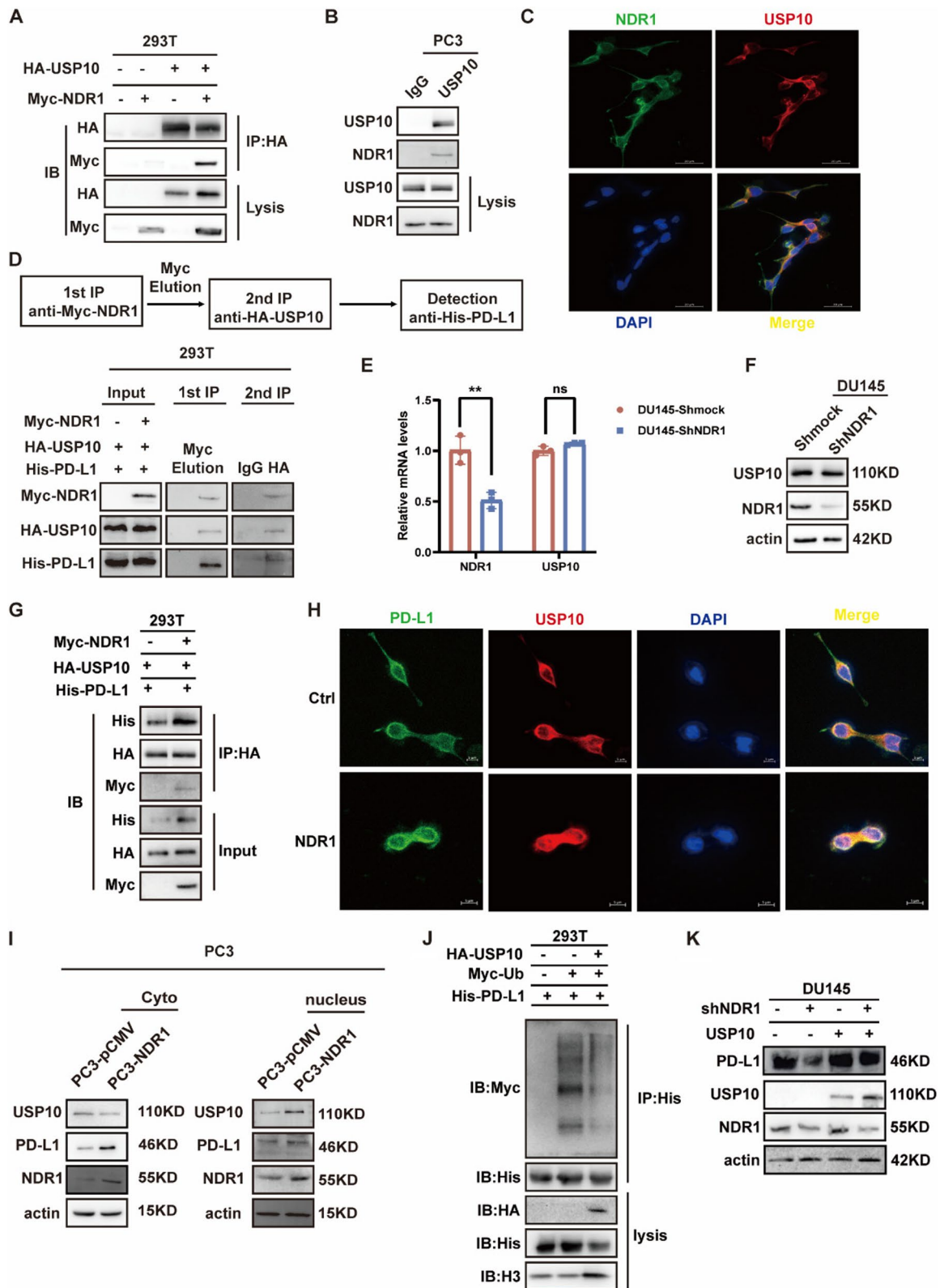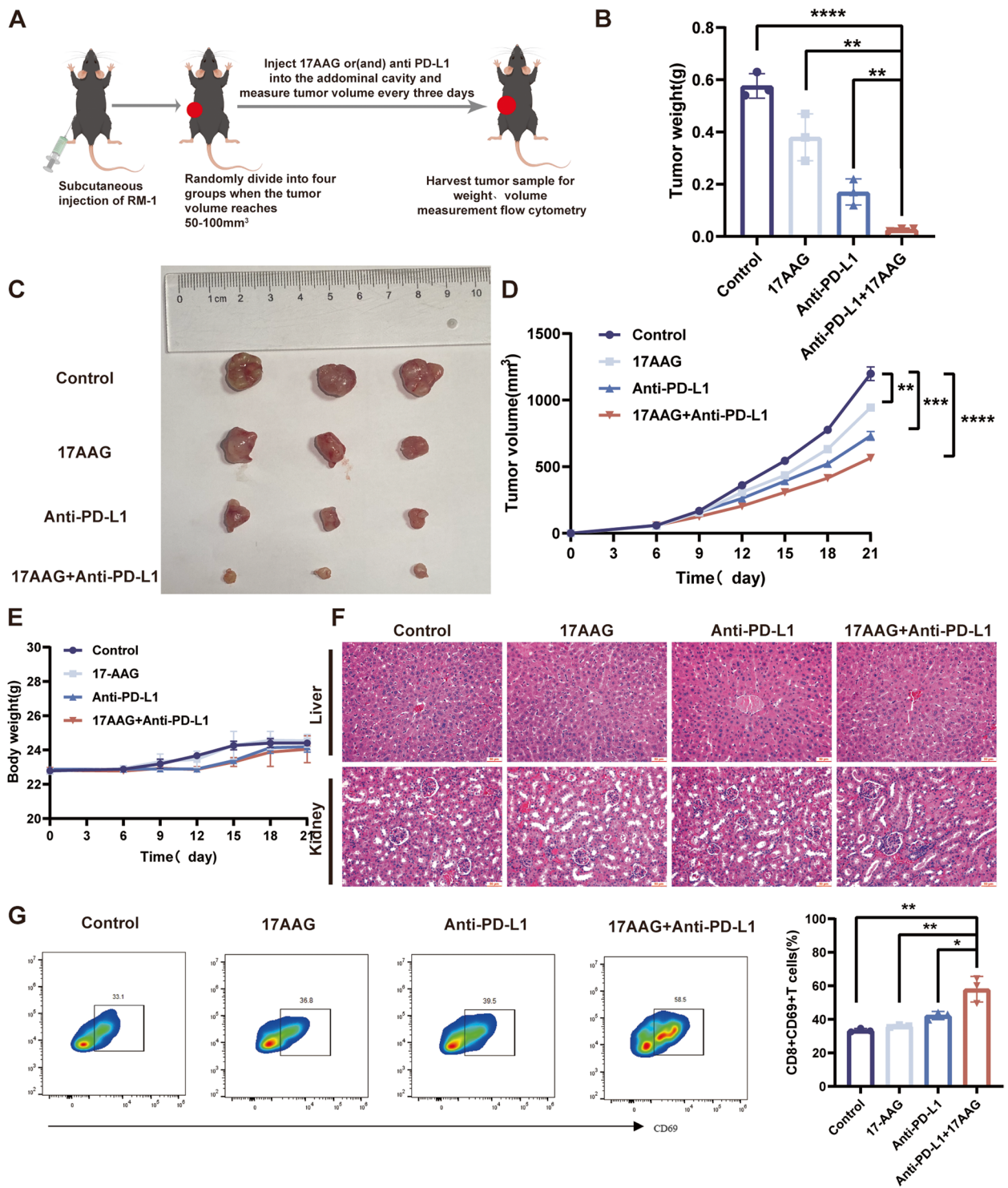
Fig. 6 (See legend on previous page.)

Fig. 7 Inhibiting NDR1 significantly enhances the effectiveness of anti-PD-L1 therapy for PCa. **A** The process of treatment strategies. **B-D** tumor weight (**B**),Tumor representation images (**C**) and tumor growth curves (**D**) (*n*=3) in four groups of mice. **E** Weight of four groups of mice. **F** HE staining of liver and kidney tissues of four groups of mice. **G** The tumor infiltrated lymphocytes were analyzed by flow cytometry (*n*=3)

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as a deubiquitinating enzyme of PD-L1 in a variety of tumors, including hepatocellular carcinoma [42], colorectal cancer [43], and breast cancer [37]. In PCa, USP22 was shown to act as a deubiquitinating enzyme for PD-L1 [44]. However, it's essential to conduct further screening of deubiquitinating enzymes in PCa to delve deeper into the regulation of PD-L1 expression and advance tumor immunotherapy.

Ubiquitination and deubiquitination are reversible processes regulated by E3 ligases and deubiquitinating enzymes, and abnormalities in deubiquitinating enzymes are associated with poor disease prognosis [45]. USP10 is abundantly expressed in PCa and significantly correlates with a negative patient prognosis [46]. It has been shown that USP10 promotes AR signaling and stabilizes G3BP2 protein, inhibits p53 activity, and promotes PCa growth [46]. Significantly, Spautin-1, an inhibitor of USP10, hinders EGFR phosphorylation and its downstream signaling. In vivo experiments have demonstrated that Spautin-1, whether used alone or combined with enzalutamide, markedly enhances the anticancer effects in PCa [47]. A recent study illustrated that USP10 may promote tumor growth by affecting immunity, but the exact mechanism is still unclear [48].

Immune checkpoint inhibitors, a common form of tumor immunotherapy, combat tumors by inhibiting immune checkpoints and reactivating T cell responses against them [49]. For "cold tumors" such as PCa, T cell infiltration within the tumor is small, and immune checkpoint inhibitors have limited effects [50]. Transforming 'cold tumors' into 'hot tumors' is crucial to enhancing immunotherapy effectiveness for PCa. Studies indicate this can be achieved by boosting T-cell responses, like CAR-T therapy, or blocking inhibitory signals, such as MDSC depletion. However, potential side effects must not be overlooked [51]. Enhancing immune checkpoint expression and fostering effector T cell infiltration can also shift 'cold tumors' towards becoming 'hot tumors' [52]. In this study, we observed that NDR1 upregulated PD-L1 expression, while suppressing CD8 + T cell infiltration and activation. Inhibition of NDR1, conversely, enhanced effector T cell infiltration, potentially transforming PCa from a 'cold tumor' to a 'hot tumor' and improving anti-PD-L1 therapy efficacy.

Mechanistically, NDR1 increases the expression level of PD-L1 in PCa (Fig. 3) and promotes immune escape from the tumor.These findings fill a gap in the immunomodulatory role of NDR1 in prostate cancer. Next, we used mass spectrometry to screen and analyze USP10, the deubiquitinating enzyme of NDR1 that stabilizes PD-L1 (Fig. 5). Subsequent experiments demonstrated that NDR1 didn't impact USP10 expression but instead hindered PD-L1 degradation by enhancing the interaction between USP10 and PD-L1 (Fig. 6G). Simultaneously, we observed that NDR1 facilitates the nuclear translocation of USP10 (Fig. 6I), enhancing its nuclear presence. Nuclear USP10 acts as a deubiquitinating enzyme, preventing ubiquitination-mediated PD-L1 degradation (Fig. 6J) and thus elevating PD-L1 stability. These findings reveal the mechanism of a novel signaling axis, NDR1/USP10/PD-L1, in immune escape and provide new insights into how NDR1 promotes immune escape in PCa.

Conclusions

In summary, this study identified that NDR1 promotes PCa growth and immune escape, which aids in clarifying the mechanism of PCa development and filling the gap in the related research field. Deeper exploration of its regulatory mechanisms unveiled the significance of the NDR1/USP10/PD-L1 signaling axis in PCa immune evasion. Moreover, we observed that inhibiting NDR1 can enhance the in vivo effectiveness of anti-PD-L1 treatment.Thus, NDR1 inhibitors may become candidates in PCa immunotherapy in the future. These findings will guide the development of new strategies for PCa treatment and provide more effective treatments for patients.

Limitations of the study

However, our research on NDR1 in the immune microenvironment is only the tip of the iceberg, more research is needed to explore, such as using single-cell analysis to explore the entire immune microenvironment and exploring the mechanisms of other immune checkpoints (such as CTLA4). Although we found that NDR1 can stabilize PD-L1 through deubiquitination, its transcriptional level and specific action sites still need further investigation. Overall,our research indicates that NDR1 serves as a potential molecular target for prostate cancer.

Supplementary Information

Additional file 1: Fig. S1. NDR1 promotes tumor growth by suppressing the immune. C: Display representative images (A), tumor growth curve (B), and tumor weight (C) of C57BL/6 mouse tumors injected with control or RM-1 shNDR1 cells. Measure the tumor at the designated time point and dissect the tumor at 25 days (*n*=5).

Additional file 2: Fig. S2. NDR1 suppresses immune response by inhibiting T cell response. A-B: Flow cytometry was used to analyze tumor-infiltrating lymphocytes (*n*=5).C: Detection of IL-2 and TNF- α expression in tumor tissue through RT-Qpcr. D: Schematic diagram showing co-culture of tumor cells and incubate T cell supernatant. E: Representative images and statistical analysis results of T cell killing test. F: Statistical analysis results of LDH toxicity test.

Additional file 3: Fig. S3. USP10 binds to PD-L1 and mediates its deubiquitination. A: PD-L1 expression in 22rv1 cells with USP10 gene overexpressing by WB. B: The knockdown efficiency of siUSP10 in DU145

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cells through WB. C: PD-L1 expression levels in DU145 cells using USP10 inhibitor(Spautin-1) by WB.

Additional file 4: Fig. S4. NDR1 promotes binding of USP10 and PD-L1 and inhibits PD-L1 ubiquitination degradation. A: Exogenous NDR1 coimmunoprecipitated with USP10 in 293T cells. B: USP10 expression were detected in NDR1 overexpressing PC3 cells by WB.C: Overexpressing USP10 was observed to mitigate the degradation of PD-L1 protein induced by NDR1 knockdown.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments were conducted in accordance with the protocol approved by the Experimental Animal Welfare and Ethics Committee of Xiamen University (No. XMULAC20200039).

Consent for publication
All authors agree for publication.

Competing interests
The authors declare no competing interests.

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