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Original Article

USP18 promote tumor immune evasion in pancreatic cancer through enhancing autolysosome-mediated degradation of MHC-I

Yan Fang[1*], Jiaqiang Wang[1*], Baoliang Fu[2], Yuanchun Yu[1], Qinrong Mao[1], Meizhen Yao[3], Xin Yu[4], Jing Cai[1]

[1]Department of Oncology, The Second Affiliated Hospital of Nanchang University, Nanchang University, Nanchang, Jiangxi, China; [2]Department of General Surgery, Yushan County Hospital of Traditional Chinese Medicine Affiliated to Jiangxi University of Traditional Chinese Medicine, Shangrao, Jiangxi, China; [3]Department of Gynaecology and Obstetrics, The Second Affiliated Hospital of Nanchang University, Nanchang University, Nanchang, Jiangxi, China; [4]Hepatopancreatobiliary Surgery Division, Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang University, Nanchang, Jiangxi, China. [*]Equal contributors.

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Abstract: Pancreatic cancer, a lethal malignancy with a 5-year survival rate below 9%, is characterized by an immunosuppressive tumor microenvironment that facilitates immune evasion. Despite the clinical urgency, the molecular mechanisms driving the scarcity of cytotoxic T lymphocyte (CTL) infiltration, a hallmark of its immunologically 'cold' phenotype, remain poorly defined, which represents a critical barrier to developing effective immunotherapies. In this study, we identify ubiquitin-specific peptidase 18 (USP18) as a critical regulator of major histocompatibility complex I (MHC-I) degradation that enables immune evasion in pancreatic ductal adenocarcinoma (PDAC). In clinical PDAC samples, USP18 protein levels were significantly elevated and inversely correlated with MHC-I expression, independent of transcriptional regulation. Functionally, USP18 knockdown enhanced MHC-I surface expression, promoted $CD8^+$ T cell activation, and sensitized PDAC cells to immune-mediated killing, while USP18 overexpression suppressed MHC-I expression and facilitated immune escape. Mechanistically, USP18 accelerated the lysosomal degradation of MHC-I through selective autophagy, a process dependent on the neighbor of BRCA1 gene 1 (NBR1). USP18 directly bound and stabilized NBR1 by deubiquitinating it, thereby inhibiting its proteasomal degradation. Collectively, our findings unveil the USP18-NBR1-MHC-I axis as a central mechanism driving immune evasion in PDAC and highlight USP18 as a promising therapeutic target for overcoming resistance to immunotherapy.

Keywords: Pancreatic cancer, immune evasion, USP18, MHC-I, NBR1

Introduction

Pancreatic cancer (PC), particularly pancreatic ductal adenocarcinoma (PDAC), is one of the most common and highly lethal malignancies of the digestive system worldwide [1]. Statistics indicate that the 5-year survival rate for pancreatic cancer patients remains below 9%, posing significant challenges in clinical management [2]. Although surgery, chemotherapy, and radiotherapy remain standard treatments, the majority of PDAC patients do not experience significant clinical benefits from these conventional therapies [3]. In recent years, immunotherapy has achieved breakthrough progress in various cancers [4]. Immune checkpoint blockade (ICB) therapies targeting CTLA-4, PD-1, and PD-L1, as well as monoclonal antibody-based immunotherapeutic strategies, have notably delayed disease progression in tumors such as lung, gastric, and liver cancers [5]. However, in contrast to responses in these malignancies, PDAC patients generally exhibit poor responses to immunotherapy. PDAC is recognized as a prototypical immunosuppressive "cold" tumor, characterized by limited T cell infiltration and functional suppression within its microenvironment that impede effective antitumor immune responses [6, 7]. Consequently, immunotherapeutic strategies for PDAC face unique and complex obstacles. Currently, the molecular mechanisms regulating PDAC progression and

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T cell-mediated immune responses remain incompletely understood. In-depth exploration of these underlying regulatory mechanisms and their interactions is critical for overcoming the immunosuppressive state of PDAC, enhancing the efficacy of immunotherapy, and developing more effective therapeutic approaches.

Major Histocompatibility Complex class I (MHC-I) plays a pivotal dual role in anti-tumor immunity [8, 9]. As an antigen presentation system encoded by the HLA-A, HLA-B, and HLA-C genes, MHC-I presents intracellular protein fragments (neoantigens) to cytotoxic T lymphocytes (CTLs), thereby bridging the gap and enabling immune surveillance of abnormal cells [10]. However, tumor cells often downregulate or completely lose MHC-I expression as a strategy for immune evasion [11]. This phenotypic alteration significantly impairs CTL-mediated recognition and cytotoxic function, thereby promoting tumor progression. Notably, the polymorphism and high mutation frequency of HLA class I genes, particularly at the HLA-A locus, may directly determine a tumor cell's capacity for immune evasion [12]. Unraveling the molecular mechanisms underlying MHC-I loss has become a research priority. This not only provides theoretical foundations for developing novel immunotherapeutic targets but also opens new avenues for enhancing CTL-mediated anti-tumor immunity through strategies aimed at restoring MHC-I expression.

Ubiquitination, a crucial post-translational modification mechanism, regulates various cellular functions and plays a pivotal role in tumorigenesis [13, 14]. This reversible process is dynamically controlled by deubiquitinating enzymes (DUBs), which specifically remove ubiquitin moieties from substrate proteins [15, 16]. Emerging evidence highlights that DUBs not only maintain protein homeostasis but also significantly influence anti-tumor immune responses and the efficacy of immunotherapy [17, 18]. Among DUBs, ubiquitin-specific peptidase 18 (USP18) has garnered particular attention due to its pleiotropic regulatory functions [19, 20]. USP18 exhibits oncogenic roles across multiple cancers by modulating cellular signaling, stress responses, and tumorigenic pathways [21, 22]. For instance, Ge et al. demonstrated that knockdown of USP18 in paclitaxel-resistant breast cancer cells substantially enhances their chemosensitivity [23]. Diao et al. research group revealed that USP18 promotes cervical cancer progression via AKT pathway activation, driving proliferation while suppressing apoptosis [24]. Notably, elevated USP18 expression strongly correlates with tumor progression and poor prognosis in pancreatic ductal adenocarcinoma. However, the precise molecular mechanisms underlying USP18-mediated immune evasion in PDAC remain poorly understood. Elucidating USP18's immunomodulatory network within the PDAC microenvironment will provide critical insights for developing novel combination immunotherapy strategies.

In this study, we discovered a significant protein-level negative correlation between USP18 and MHC-I in pancreatic cancer, independent of transcriptional regulation. Functionally, USP18 facilitated immune evasion by decreasing the expression of MHC-I on the surface of PDAC tumor cells and impeding the activation of $CD8^+$ T cells. Furthermore, USP18 accelerated the loss of MHC-I through enhancing NBR1-mediated autophagy-lysosomal degradation of MHC-I in PDAC. Mechanistically, USP18 stabilizes the NBR1 protein by antagonizing its ubiquitin-proteasome pathway-mediated degradation through deubiquitination activity. Our findings unveil the USP18-NBR1-MHC-I axis as a central regulator of PDAC immune evasion and highlight USP18 as a promising therapeutic target for overcoming immunotherapy resistance.

Materials and methods

Clinical samples

The study utilized surgically resected pancreatic ductal adenocarcinoma specimens and matched adjacent normal tissues collected from the Department of General Surgery, The Second Affiliated Hospital of Nanchang University ([2019] No. (053)). Written informed consent was obtained from all participants through an institutional review board-approved protocol. All experimental procedures strictly adhered to the ethical guidelines of the Declaration of Helsinki and were conducted in compliance with China's Regulations on the Management of Human Genetic Resources.

Cell culture

Human pancreatic adenocarcinoma cell lines (AsPC-1, PANC-1, MIA PaCa-2, SW1990, BxPC-

3) and human normal pancreatic ductal epithelial cells (HPDE) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, with all cell lines authenticated by short tandem repeat (STR) profiling. Cells were cultured in two distinct media: AsPC-1 and BxPC-3 were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), whereas PANC-1, MIA PaCa-2, SW1990, and HPDE cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% FBS. All cell lines were incubated under standardized conditions at 37°C with 5% CO_2 in a humidified atmosphere.

RNA extraction and qRT-PCR

Total RNA was isolated from PC tissues or cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. RNA concentration and purity were determined by spectrophotometric measurement, with purified RNA samples subsequently stored at -80°C to prevent degradation. Quantitative real-time PCR analysis was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, USA) with TB Green Premix Ex Taq (Takara Bio, Japan). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH as the endogenous control gene.

Flow cytometry

The cultured cells were first digested with trypsin and washed with PBS. Human HLA-ABC and murine H-2Kb antibodies diluted in FACS buffer (PBS containing 2% FBS) were then incubated with the cells at 4°C in the dark for 30 minutes. The staining was terminated by adding 1 mL FACS buffer, followed by centrifugation at 5000 rpm for 5 minutes at 4°C. The cell pellet was resuspended in 300 μL FACS buffer and transferred to flow cytometry tubes. Samples were analyzed using a BD Fortessa flow cytometer, with subsequent data processing performed through FlowJo software version 10.8.

Immune escape assay

Peripheral Blood Mononuclear Cells (PBMCs) were provided by ZEYI Primary Cells (Catalog No.: PB00010C; Batch No.: 2024071201). All experiments involving human biological materials were conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from the donor prior to blood collection. PBMCs were co-cultured with PC cells for 24 hours. Cell proliferation was assessed using the CCK-8 assay. Following co-culture, PD-L1 expression was analyzed by Western blot with an anti-PD-L1 antibody (1:1000 dilution, Product No. 66248-1-Ig).

Immunoprecipitation and western blotting

Cells were lysed in cell lysis buffer. For Western blot analysis, a protease inhibitor cocktail was included in the lysis buffer to preserve protein integrity. Pre-clearance: Lysates were pre-incubated with Protein A/G magnetic beads (Santa Cruz, USA) to remove non-specific binders. Antigen-Antibody Binding: Pre-cleared lysates were mixed with IgG (control) or a target-specific primary antibody and incubated overnight at 4°C. Protein A/G magnetic beads were added to the lysate-antibody mixture and incubated for 120 minutes at 4°C to immobilize the antigen-antibody complexes. Immunoprecipitated proteins or whole-cell lysates were resolved by SDS-PAGE. Separated proteins were electroblotted onto PVDF membranes (Millipore, USA). Membranes were blocked with 5% skimmed milk to prevent non-specific binding. Primary Membranes were probed with primary antibodies overnight at 4°C. HRP-conjugated anti-rabbit/mouse secondary antibodies (Zsbio, China) were applied for target detection. Protein signals were captured using a chemiluminescence kit (GE, USA) following the manufacturer's guidelines. The primary antibodies included anti-USP18 (1:1000, Proteintech, Cat No. 12153-1-AP), anti-NBR1 Monoclonal antibody (1:1000, Proteintech, Cat No. 68062-1-Ig), anti-MHC-I (1:1000, abcam, ab134189), anti-HA (1:1000, Sigma, SAB1306082), anti-Flag (1:1000, Sigma, F1804), anti-GAPDH (1:1000, Abcam, ab8245), and anti-ubiquitin (1:1000, Santa Cruz, sc-53509).

Molecular docking analysis

The structures of the compounds and proteins were optimized using the Discovery Studio 2019 client for docking analysis. Water molecules were removed from the model structures of USP18 and NBR1 receptors, and the force field and hydrogen structures were optimized. The ZDOCK module in Discovery Studio was then used to perform protein-ligand docking

with an induced-fit approach. A 20 Å cavity was selected as the docking active site, and standard parameters were used for docking calculations. The highest-scoring docking models were subsequently examined and validated to assess their binding modes and reliability.

Animals and treatment

For immunotherapy experiments, six-week-old C57BL/6 mice were obtained from Shanghai Model Organisms Center, Inc. Pretreated Panc02 cells (1×10^6 per mouse) were injected into the right flank of these mice. After 4 days of subcutaneous injection, 100 μg/mouse anti-PD-L1 antibody (BioXCell, BE0101, New Hampshire, USA) were intraperitoneally administered twice per week until the completion of the study. Tumor volume was calculated using the following equation: volume = length × $(\text{width})^2$ ×1/2. All animal experiments were conducted in accordance with the protocols approved by the Animal Experimental Ethics Committee of Nanchang University (NCULAE-20250901006) and the procedures set forth in the NIH Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Pearson's and Spearman's correlation analyses were performed to measure correlations between two factors in samples from cancer patients. The correlation was considered significant at $P < 0.05$ and Pearson's $R > 0.30$. The data were derived from at least three independent experiments and are presented as mean ± standard deviation (SD). Statistical analyses were performed with GraphPad Prism 9.0 software (GraphPad Software, La Jolla, CA, USA), and two-tailed Student's t test and analysis of variance (ANOVA) were used to compare data between two groups and among more than two groups, respectively. Survival analysis was estimated using the Kaplan-Meier method, and statistical differences were assessed by the Log-rank test. A *P*-value of < 0.05 was considered statistically significant.

Results

USP18 and MHC-I proteins are negatively correlated in pancreatic cancer tissues

To investigate the expression patterns and clinical relevance of USP18 and MHC-I in pancreatic cancer, we analyzed their expression in 50 pancreatic cancer tissue samples and paired adjacent nontumor tissues using qRT-PCR and Western blotting. The qRT-PCR results demonstrated that USP18 mRNA expression was significantly upregulated in tumor tissues compared to adjacent nontumor tissues (**Figure 1A**), while MHC-I mRNA levels were significantly downregulated (**Figure 1B**). However, no significant correlation was observed between USP18 and MHC-I mRNA expression levels in pancreatic cancer tissues (**Figure 1C**). Furthermore, Western blotting analysis revealed that USP18 protein was highly expressed in pancreatic cancer tissues, while MHC-I protein was strongly expressed in tumor tissues compared to nontumor tissues (**Figure 1D**, **1E**). These data indicate that USP18 protein expression is significantly upregulated, while MHC-I protein expression is downregulated in pancreatic cancer tissues. Notably, scatter plot analysis demonstrated a negative correlation between USP18 and MHC-I protein expression levels in pancreatic cancer tissues ($r = -0.42$, $P < 0.01$; **Figure 1F**). These results suggest that USP18 and MHC-I exhibit a protein-level negative correlation in pancreatic cancer, independent of their transcriptional regulation.

USP18 downregulates MHC-I expression to promote immune escape in pancreatic cancer cells

MHC-I plays a crucial role in mediating antitumor immunity and $CD8^+$ T cell cytotoxicity [25]. To determine whether USP18 is involved in the immune infiltration process, we performed GO biological process and Reactome pathway analyses of differentially expressed genes (DEGs) between the High-USP18 and Low-USP18 groups. Multiple immune-related pathways, such as cytokine-mediated signaling, tumor necrosis factor superfamily cytokine production, regulation of chemokine production, T cell apoptotic process, and interferon gamma signaling, were enriched (**Figure 2A**, **2B**). Furthermore, by co-culturing PANC-1 cells with peripheral blood mononuclear cells (PBMCs), we observed that knockdown of USP18 profoundly altered tumor-immune dynamics. CCK-8 viability assays demonstrated a near doubling of cancer cell death rates under conditions of USP18 knockdown, directly implicating USP18 in promoting immune escape in pancreatic cancer cells (**Figure 2C** and

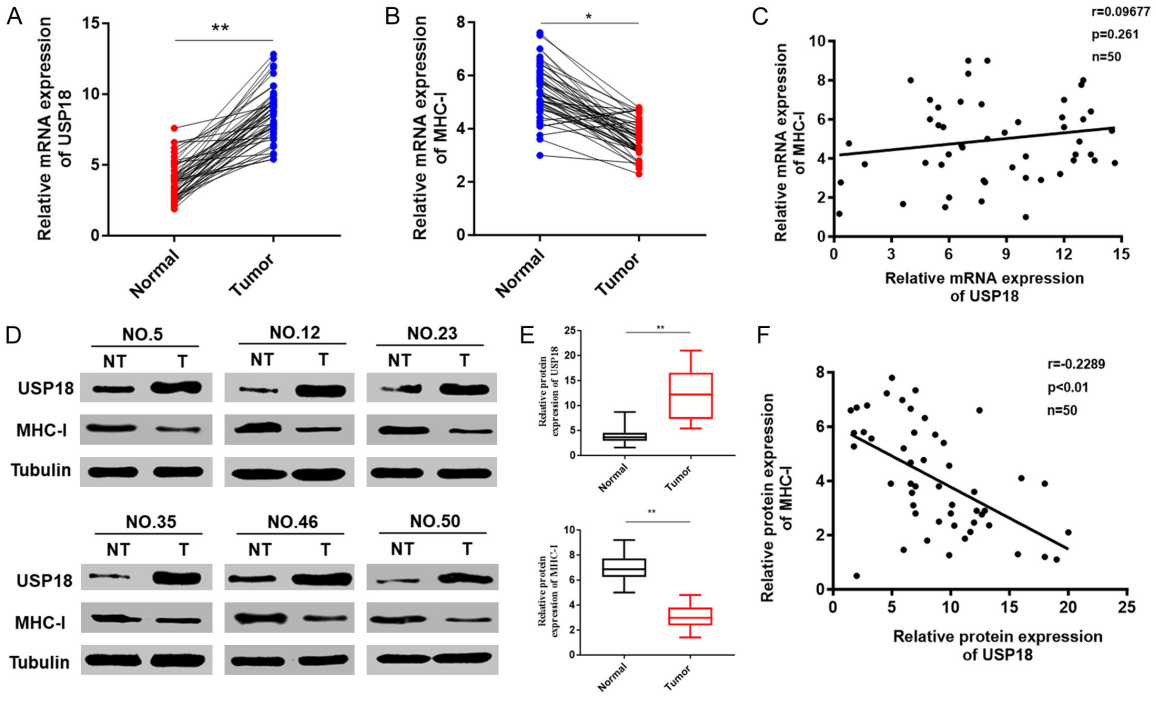


Figure 1. USP18 and MHC-I exhibit a protein-level negative correlation in pancreatic cancer. A, B. qRT-PCR analysis of USP18 and MHC-I mRNA expression in PC tumours and paired normal tissues, n = 50, *P < 0.05, **P < 0.01. C. Scatter plots show a positive correlation between USP18 and MHC-I at the mRNA level in PC, n = 50. D, E. qRT-PCR analysis of USP18 and MHC-I protein expression in PC tumours and paired normal tissues, n = 50. F. Scatter plots show a positive correlation between USP18 and MHC-I at the protein level in PC, n = 50, **P < 0.01.

Supplementary Figure 1A, 1B). In the absence of PBMCs co-culture, knockdown of USP18 did not significantly affect the baseline viability of PANC-1 cells (Supplementary Figure 2A). This enhanced cytotoxicity correlated with robust $CD8^+$ T cell activation, as evidenced by elevated Granzyme B (GZMB) and Perforin (PFN) levels in the co-culture supernatants (**Figure 2D**, **2E**). Meanwhile, without PBMCs co-culture, knockdown of USP18 did not significantly induce GZMB or PFN expression within the PANC-1 cells themselves (Supplementary Figure 2B, 2C). In addition, Flow cytometry analysis showed that co-culturing PANC-1 cells with USP18 deficiency and $CD8^+$ T cells resulted in a marked elevation in the release of IFN-γ and GzmB by $CD8^+$ T cells compared to control cells (Supplementary Figure 3). Finally, we investigated the effects of anti-PD-L1 treatment in PDAC using subcutaneous models. As shown in Supplementary Figure 4A-C, the combination of anti-PD-L1 treatment and USP18 knockdown resulted in significant inhibition of tumor growth and a reduction in tumor weight. Collectively, USP18 deficiency promotes $CD8^+$ T-cell-mediated antitumor immunity *in vitro* and *in vivo*.

Next, to explore the potential relationship between USP18 and MHC-I, we measured the expression of MHC-I on the surface of tumor cells by flow cytometry after the knockdown and overexpression of USP18. The expression of MHC-I on the surface of tumor cells increased upon USP18 knockdown (**Figure 2F**), while overexpression of USP18 significantly reduced the expression of MHC-I on the surface of tumor cells (**Figure 2G** and Supplementary Figure 1C, 1D). Furthermore, the total protein level of MHC-I was enhanced in PANC-1 cells with USP18 knockdown, as detected by Western blot (**Figure 2H**), while it was reduced in cell lines with overexpression of USP18 (**Figure 2I**). These results indicate that USP18 negatively regulates MHC-I expression in pancreatic cancer.

USP18 accelerates the degradation of MHC-I which is dependent on the lysosomal pathway

To investigate the mechanism by which USP18 mediates impairment of MHC-I expression, we assessed protein stability using cycloheximide (CHX)-based protein synthesis blockade.

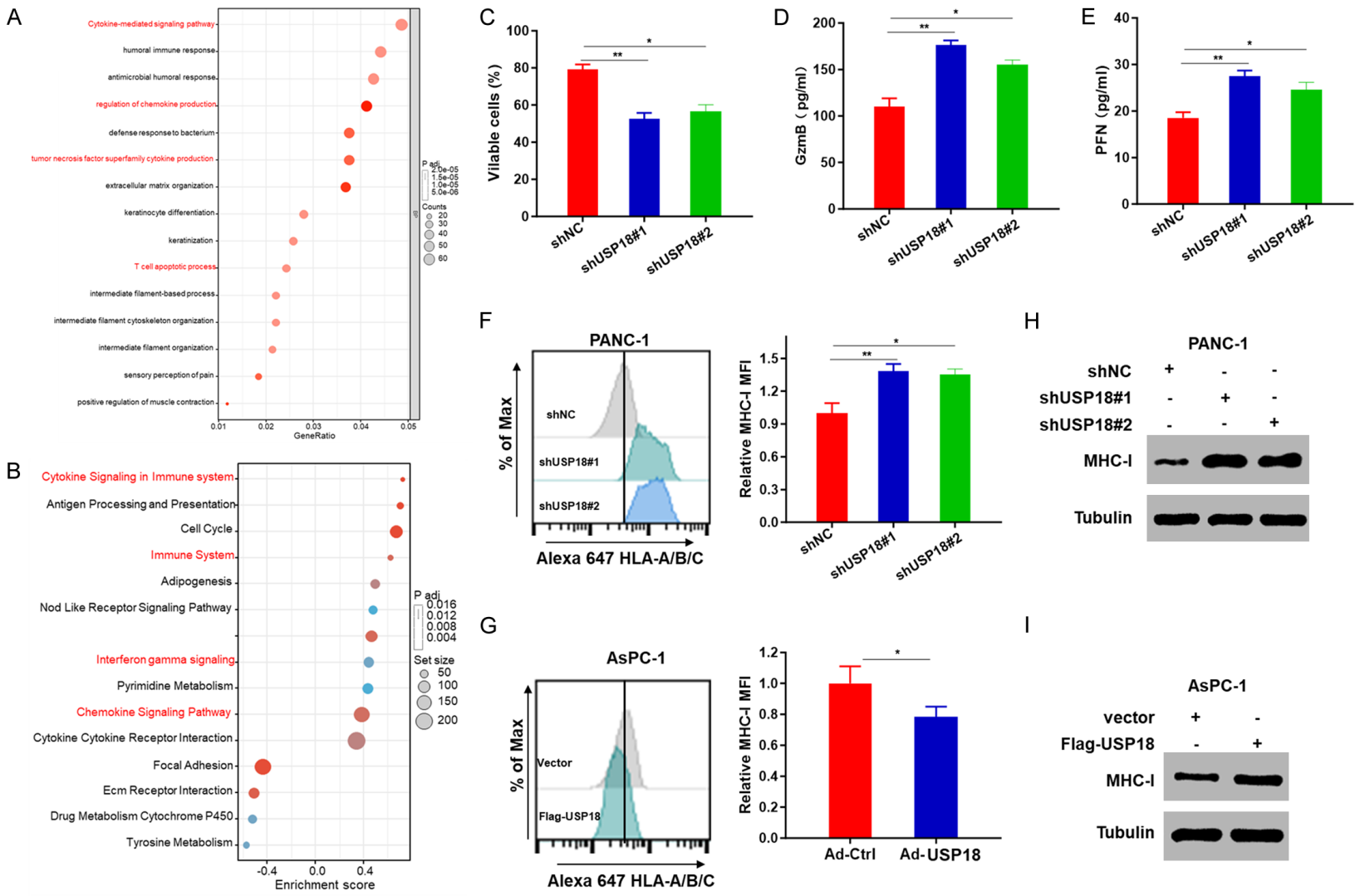


Figure 2. USP18 negatively regulates MHC-I expression in pancreatic cancer. (A, B) GO Biological Process analyses (A) and Reactome pathway analyses (B) of differentially expressed genes between High-USP18 and Low-USP18 groups from PAAD-TCGA database. The High-USP18 and Low-USP18 groups were defined based on the median expression level of USP18 across all samples in the analyzed cohort. (C) CCK-8 viability assays demonstrated a near doubling of cancer cell death rates under conditions of USP18 knockdown. *P < 0.05, **P < 0.01. (D, E) ELISA quantification of Granzyme B (GZMB) and Perforin (PFN) levels in co-culture supernatants. (F, G) The expression of HLA-A/B/C on the surface of PDAC cell lines was measured by flow cytometry in PANC-1 cells with USP18-knockdown (F) and AsPC-1 cells with USP18-overexpression (G). *P < 0.05, **P < 0.01. (H, I) Western blot analysis of MHC-I in PANC-1 cells with USP18-knockdown (H) and AsPC-1 cells with USP18-overexpression (I).

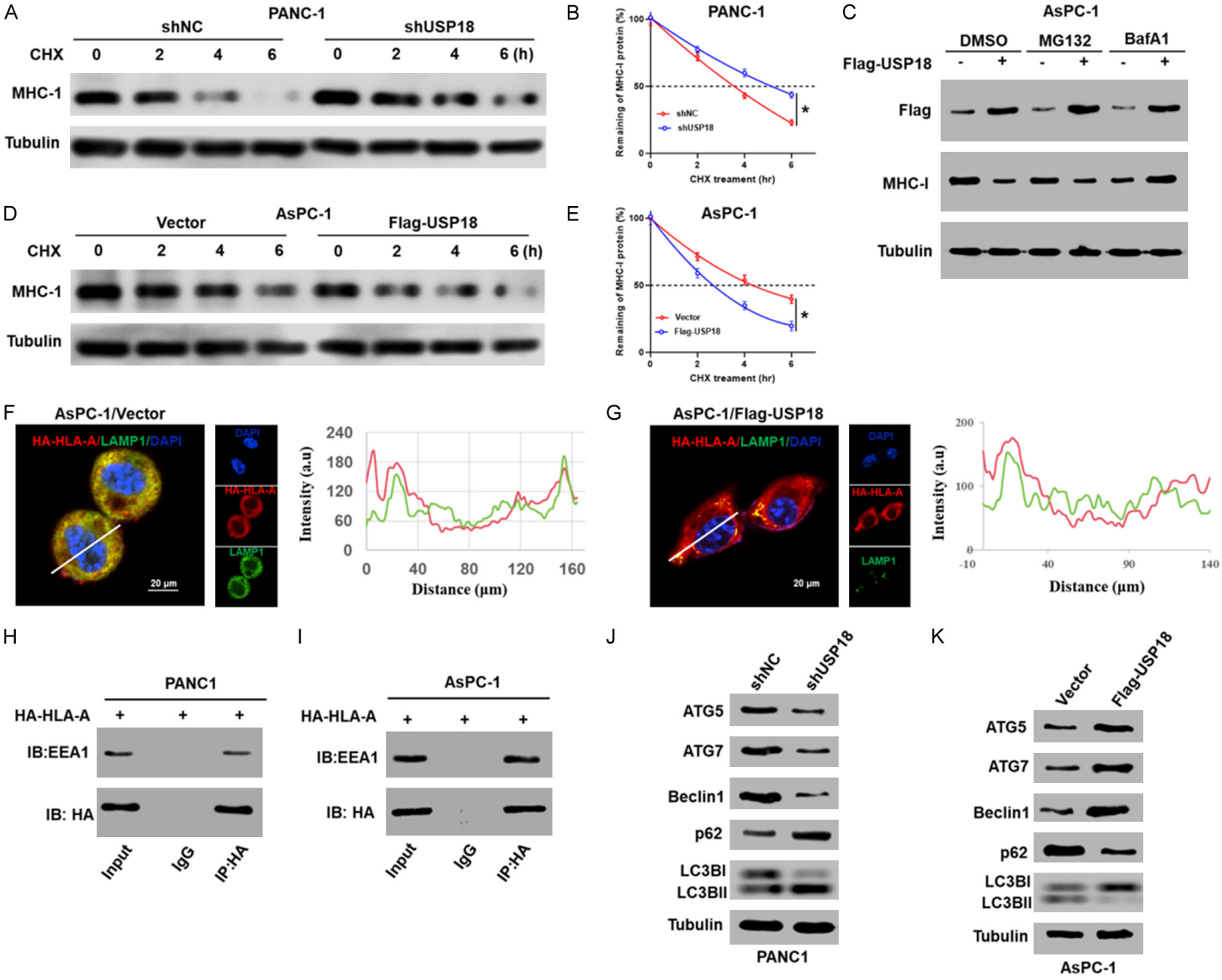
In USP18-silenced PANC-1 cells, MHC-I degradation showed delayed kinetics relative to controls (**Figure 3A**, **3B**). Conversely, Flag-USP18-overexpressing AsPC-1 cells demonstrated accelerated MHC-I depletion compared to vector-transfected counterparts (**Figure 3C**, **3D**), confirming that USP18 mediates the destabilization of MHC-I in pancreatic cancer. Given the dual degradation pathways involving proteasomes and lysosomes, mechanistic studies were conducted using the lysosomal inhibitor bafilomycin A1 (BafA1) and proteasomal inhibitor MG132. Western blot analysis revealed that USP18 overexpression consistently reduced MHC-I levels in AsPC-1 cells, regardless of MG132 treatment, excluding proteasomal involvement. In contrast, BafA1 administration restored MHC-I expression in USP18-overexpressing cells, implicating lysosomal degradation as the primary pathway (**Figure 3E**). Immunofluorescence imaging further confirmed enhanced colocalization between HA-tagged HLA molecules and lysosomal marker LAMP1 in USP18-overexpressing cells (**Figure 3F**, **3G**). Coimmunoprecipitation assays verified a physical interaction between HLA-A and early endosome marker EEA1 in both PANC-1 and AsPC-1 cells, with USP18-EEA1 colocalization observed specifically in AsPC-1 cells (**Figure 3H**, **3I**). This suggests that USP18 facilitates the trafficking of HLA-A through early endosomes to lysosomes. Further investigation into autophagy regulation revealed that USP18 silencing reduced the expression of autophagy-related proteins (ATG5, ATG7, Beclin1) and autophagosome marker LC3 in PANC-1 cells (**Figure 3J**). Conversely, USP18 overexpression upregulated these markers in AsPC-1 cells (**Figure 3K**). Collectively, these findings establish that USP18 promotes the lysosomal degradation of MHC-I through coordinated regulation of endosomal sorting and autophagy pathways.

USP18 promotes MHC-I trafficking to lysosomes via NBR1

To further uncover the mechanism of MHC-I degradation promoted by USP18. The interaction of USP18 and HLA-A was further verified by Co-IP assay in AsPC-1 cells (**Figure 4A**). Furthermore, we found that USP18 colocalized with HLA-A in AsPC-1 cells (**Figure 4B**). The Co-IP assay indicated that the ubiquitination level of HLA-A was higher in PC cells with expressing Flag-USP18 than control cells (**Figure 4C**). This result showed that USP18 promoted the ubiquitination and degradation of MHC-I in PDAC. Neighbor of BRCA1 gene 1 (NBR1), a canonical selective autophagy receptor, mediates the degradation of ubiquitinated substrates through autophagic-lysosomal pathways [26]. Therefore, we reasoned that the MHC-I deficiency was largely caused by NBR1-mediated autophagy-lysosomal degradation. To confirm the hypothesis, we initially examined whether USP18 can bind to HLA-A and NBR1 using immunoprecipitation assays. In accordance with the hypothesis, we verified the interaction of USP18 with HLA-A and NBR1 in AsPC-1 cells by Co-IP assay (**Figure 4D**). Immunoblot analysis demonstrated a direct correlation between USP18 expression and NBR1 protein abundance: ectopic USP18 expression in PANC-1 cells significantly elevated NBR1 levels, while USP18 depletion markedly reduced them (**Figure 4E**). Functional rescue experiments showed that NBR1 knockdown effectively reversed MHC-I suppression in USP18-overexpressing AsPC-1 cells (**Figure 4F**). These coordinated findings establish that USP18 regulates MHC-I surface expression by controlling NBR1-mediated substrate degradation through autophagy-lysosomal mechanisms.

USP18 interacts with NBR1

To investigate the regulatory mechanism of USP18 on NBR1 expression, we first assessed NBR1 transcript levels using qRT-PCR in pancreatic cancer cells with USP18 overexpression or depletion. Quantitative analysis demonstrated no significant alterations in NBR1 mRNA abundance across experimental groups (**Figure 5A**, **5B**), suggesting that USP18-mediated regulation occurs post-transcriptionally rather than through transcriptional modulation. Subsequent proteomic interrogation of the USP18 interaction network through co-immunoprecipitation (co-IP) coupled with mass spectrometry identified NBR1 as a direct binding partner (**Figure 5C**). This physical association was biochemically validated through reciprocal co-IP experiments under endogenous conditions, demonstrating that USP18 interacts with NBR1 in pancreatic cancer cells (**Figure 5D**, **5E**). Collectively, these multimodal approaches confirm a direct molecular interaction between USP18 and NBR1, which underlies post-translational regulation.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LC3BII
Tubulin
AsPC-1

Figure 3. USP18 accelerates the degradation of MHC-I which is dependent on the lysosomal pathway. (A-D) The half-life of MHC-I was evaluated in PANC-1 cells with USP18-knockdown (A, B) and AsPC-1 cells with USP18-overexpression (C, D). *P < 0.05. (E) AsPC-1 cells with overexpression of USP18 were treated with dimethyl sulfoxide (DMSO), proteasome inhibitor MG132 (20 μM) and lysosomal inhibitor BafA1 (20 μM) for 4 h, and the expression of MHC-I was evaluated by Western blot. (F, G) AsPC-1 cells stably expressing HA-HLA-A were transfected with Flag-USP18 and Vector and were stained for HA-HLA-A (red) and LAMP1 (green). The cell nucleus (blue) was stained with DAPI. The colocalization was visualized by confocal microscopy. (H, I) The relationship between HLA-A and EEA1 was analyzed by Co-IP analysis. (J, K) The expression levels of ATG5, ATG7, Beclin1, p62 and LC3 (LC3-I and -II) were detected by Western blot analysis.

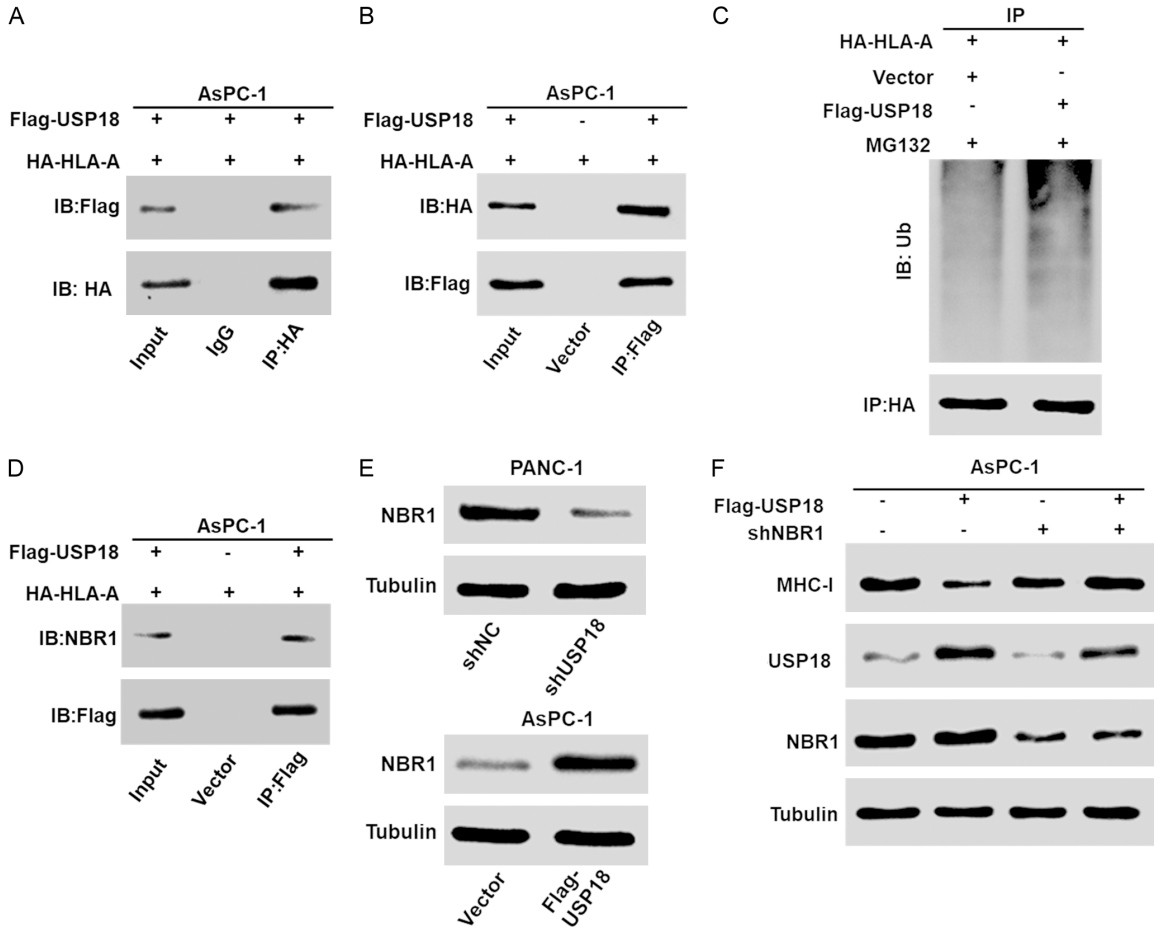

Figure 4. USP18 promotes MHC-I trafficking to lysosomes via NBR1. A, B. The interaction of USP18 and HLA-A was analyzed by Co-IP analysis. C. Vector and Flag-USP18 were transfected in PC cells with HA-HLA and treated with MG132 (20 μM) for 4 h. Cellular extracts were immunoprecipitated with anti-HA and followed by Western blot with anti-ubiquitin (Ub) antibody. D. AsPC-1 cells expressing Flag-USP18 and HA-HLA-A were analyzed by Co-IP and Western blot. E. The expression levels of NBR1 and Tubulin were detected by Western blot analysis. F. The expression levels of MHC-I, USp18, NBR1 and Tubulin were detected by Western blot analysis.

USP18 stabilizes NBR1 protein expression via suppressing NBR1 degradation mediated by proteasome

Given that USP18 functions as a deubiquitinating enzyme, we hypothesized its potential regulatory role in modulating proteasomal degradation and ubiquitination of NBR1 in pancreatic cancer. Experimental evidence revealed that MG132, a proteasome inhibitor, significantly attenuated NBR1 degradation (**Figure 6A**), confirming the proteasome-dependent regulation of NBR1 turnover in pancreatic cancer cells. To investigate USP18's involvement in this process, genetic manipulation studies were performed. Depletion of USP18 markedly reduced NBR1 protein levels, an effect that was fully rescued by co-treatment with MG132

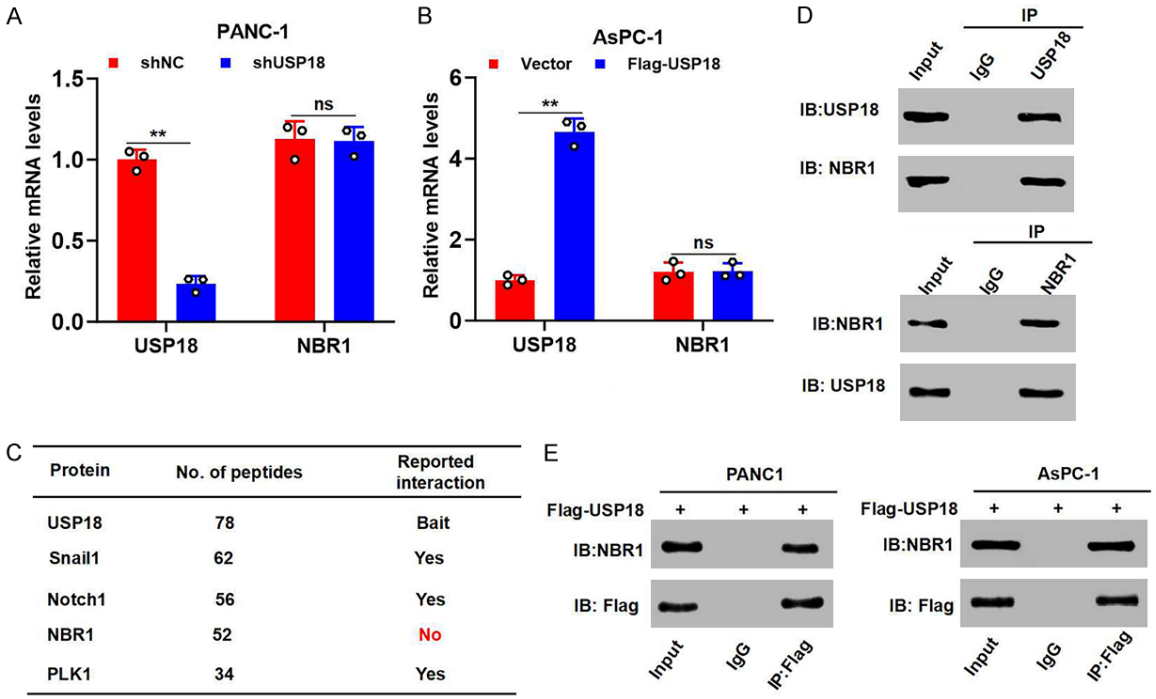

Figure 5. USP18 interacts with NBR1. A. qRT-PCR analysis of USP18 and NBR1 at the mRNA level in USP18 knockdown PC cells. **P < 0.01. B. qRT-PCR analysis of USP18 and NBR1 at the mRNA level in Flag-USP18 PC cells. **P < 0.01. C. The proteins that co-precipitated with USP18 were identified via LC-MS/MS. #PSMs with matching peptide profiles. D, E. The interaction of USP18 and HLA-A was analyzed by Co-IP analysis.

(**Figure 6B**). Conversely, ectopic USP18 expression elevated NBR1 abundance, which was counteracted by proteasome inhibition (**Figure 6C**). Cycloheximide (CHX) chase assays further demonstrated that USP18 silencing accelerated NBR1 protein decay, while USP18 overexpression substantially extended its half-life (**Figure 6D-G**). Importantly, ubiquitination profiling via co-immunoprecipitation (Co-IP) showed that USP18 deficiency amplified endogenous NBR1 polyubiquitination signals, while USP18 overexpression reduced ubiquitin conjugation on NBR1 (**Figure 6H**). Collectively, these results establish that USP18 stabilizes the NBR1 protein by antagonizing its ubiquitin-proteasome pathway-mediated degradation through deubiquitination activity in pancreatic cancer.

Discussion

Pancreatic cancer is among the most aggressive malignancies. It exhibits profound invasiveness and suboptimal treatment outcomes [27]. Despite immunotherapy advances, immune checkpoint blockade (ICB) shows limited clinical efficacy in pancreatic cancer patients. Most patients fail to achieve durable responses [28]. This therapeutic impasse stems from the tumor's intrinsic ability to orchestrate immunosuppressive reprogramming of the tumor microenvironment. Elucidating key molecular mediators of PC-driven immune evasion is therefore critical for developing targeted strategies that restore anti-tumor immunity. This study elucidates a novel USP18-NBR1-MHC-I regulatory axis that drives immune evasion in pancreatic cancer. We demonstrate that USP18, a deubiquitinating enzyme overexpressed in pancreatic cancer, orchestrates MHC-I degradation via NBR1-mediated selective autophagy, establishing its pivotal role in shaping the immunosuppressive tumor microenvironment. These findings provide mechanistic insights into PC's resistance to immunotherapy and identify actionable targets for intervention.

USP18, a member of the USP subfamily of deubiquitinating enzymes (DUBs), plays a pivotal role in regulating various cellular processes through the removal of ubiquitin from target proteins [29-31]. Beyond its protease activity, USP18 also serves as a crucial negative regula-

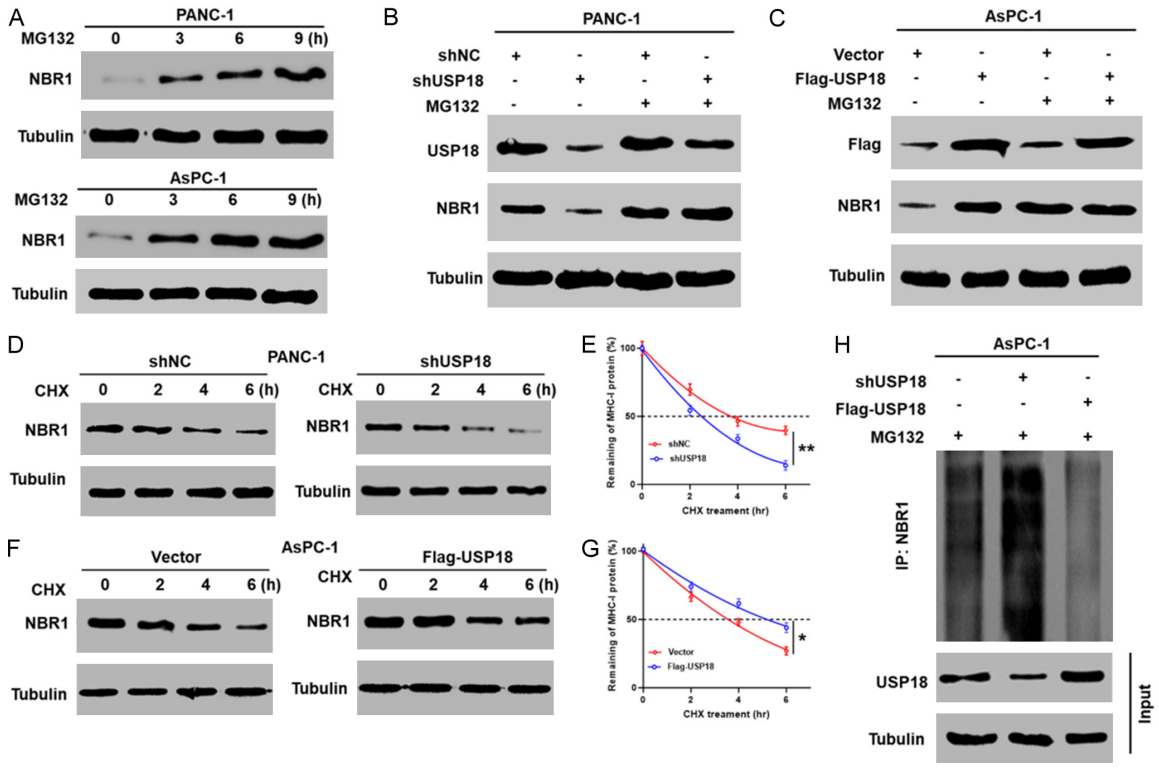

Figure 6. USP18 stabilizes NBR1 protein expression via suppressing NBR1 degradation mediated by proteasome. A. NBR1 protein levels at various times were measured by western blotting after MG132 addition (10 μM) to AsPC-1 and PANC-1 cells. B, C. Western blot analysis of USP18 and NBR1 protein expression in PANC-1 cells transfected with shUSP18 or shNC and AsPC-1 cells transfected with exogenous USP18 or a control vector, with or without treatment with 10 μM MG132. D-G. PANC-1 cells transfected with shUSP18 or shNC and AsPC-1 cells transfected with exogenous USP18 or a control vector were subjected to treatment with 20 μg/mL CHX, followed by assessment of NBR1 protein levels using western blotting. **P < 0.01. H. Lysates from PC cells transduced with shUSP18 or Flag-USP18 were immunoprecipitated with the anti-Ub and immunoblotted with the anti-NBR1. Cells were treated with MG132 for 6 h before collection.

tor of the type I interferon response, highlighting its bifunctional nature. Recent studies have shown that USP18 is upregulated in several tumor types, where it acts as an oncogene. For example, Tan et al. demonstrated that USP18 promotes breast cancer progression by enhancing EGFR expression and activating the AKT/Skp2 signaling pathway [32]. Additionally, elevated USP18 levels have been observed in lung cancer, and lower USP18 expression correlates with improved cancer-specific survival in patients with muscle-invasive bladder cancer [33]. These findings underscore the potential oncogenic role of USP18 in various cancers. Our prior research revealed high USP18 expression in pancreatic cancer tissues. In this context, USP18 functions as an oncogene. However, its precise role in immune evasion within pancreatic cancer remains poorly understood. In our current study, co-culturing PANC-1 cells with peripheral blood mononuclear cells (PBMCs) revealed that knocking down USP18 significantly altered tumor-immune interactions. CCK-8 viability assays indicated a nearly twofold increase in cancer cell death under USP18 knockdown conditions, implicating USP18 in promoting immune escape in pancreatic cancer cells. This enhanced cytotoxicity was associated with robust $CD8^+$ T cell activation, as evidenced by elevated levels of GZMB and PFN in the co-culture supernatants. Notably, USP18 knockdown restored $CD8^+$ T cell-mediated cytotoxicity. This suggests therapeutic targeting could convert immunologically "cold" pancreatic cancer into "hot" tumors. Such "hot" tumors are responsive to checkpoint inhibitors. These results suggest that USP18 drives immune evasion in pancreatic cancer cells, contributing to their resistance to immune-mediated destruction.

MHC-I molecules play a critical role in antigen presentation by displaying tumor-derived peptides on the surface of cells for recognition by $CD8^+$ T cells [34, 35]. However, tumor cells often evade immune surveillance by downregulating MHC-I expression. This reduction in MHC-I is strongly associated with diminished $CD8^+$ T cell infiltration, a feature that becomes increasingly evident during cancer progression. While mutations in the MHC-I gene are rare in pancreatic cancer, the downregulation of MHC-I appears to be a key mechanism driving immune escape in pancreatic cancer. A recent study identified a ternary complex containing SUSD6, TMEM127, and MHC-I. This complex recruits WWP2, which facilitates ubiquitination and lysosomal degradation of MHC-I molecules [11]. This process ultimately leads to reduced $CD8^+$ T cell infiltration in the tumor microenvironment. Another study showed that silencing or pharmacologically inhibiting the glucocorticoid receptor (GR) enhances MHC-I expression. This increase not only boosts $CD8^+$ T cell infiltration but also improves pancreatic cancer's responsiveness to immune checkpoint blockade [36]. In this study, we demonstrated that USP18 protein expression is significantly upregulated, while MHC-I protein expression is downregulated, in pancreatic cancer tissues. Scatter plot analysis showed a negative correlation between USP18 and MHC-I protein expression levels in pancreatic cancer tissues. However, no significant correlation was observed between USP18 and MHC-I mRNA expression levels in these tissues. Clinical pancreatic cancer specimens show an inverse correlation between USP18 and MHC-I protein levels. This highlights a post-translational regulatory mechanism independent of transcriptional control. Further studies revealed that the expression of MHC-I on the surface of tumor cells was increased upon USP18 knockdown, while overexpression of USP18 significantly reduced MHC-I expression on the surface of tumor cells. Our results indicate that USP18 downregulates MHC-I expression to promote immune escape in pancreatic cancer cells. Subsequently, we demonstrated that USP18 accelerates the degradation of MHC-I in a manner dependent on the lysosomal pathway.

Next, we explored the molecular mechanisms by which USP18 modulates MHC-I expression. NBR1, an autophagy receptor, is crucial for selective autophagy, and its stability is essential for its role in MHC-I degradation [37, 38]. A key mechanistic insight from our study is the identification of the USP18-NBR1-MHC-I axis as a central pathway driving immune evasion in PDAC. Specifically, we demonstrate that USP18 regulates MHC-I degradation by promoting its selective autophagy. Selective autophagy targets damaged or superfluous proteins and organelles for degradation via autophagic vesicles. Tumors hijack this critical cellular process to evade immune surveillance. In PDAC, USP18 accelerates the lysosomal degradation of MHC-I molecules through this pathway, ultimately reducing the availability of MHC-I for presenting tumor antigens to CTLs. Importantly, this degradation is dependent on the autophagy receptor NBR1, which recognizes the ubiquitinated MHC-I molecules and directs them for autophagic degradation. The role of NBR1 in this process emphasizes the selective nature of this autophagic degradation, distinguishing it from general cellular protein turnover. These findings suggest that USP18 regulates MHC-I surface expression by controlling NBR1-mediated substrate degradation through autophagy-lysosomal mechanisms.

Finally, we examined the mechanism by which USP18 modulates NBR1 expression. Hou et al. demonstrated that USP18 positively regulates innate antiviral immunity by promoting K63-linked polyubiquitination of MAVS [19]. Song et al. showed that USP18-mediated deubiquitination increases FTO protein stability [39]. Additionally, Song et al. revealed that USP18 directly binds ZEB1 and decreases its ubiquitination, enhancing the protein stability of ZEB1 in esophageal squamous cell carcinoma cells [40]. In this study, we reveal that USP18 directly binds to NBR1 and stabilizes it by removing ubiquitin moieties, which would otherwise target NBR1 for proteasomal degradation. This interaction between USP18 and NBR1 represents a key regulatory step in the USP18-mediated immune evasion mechanism. USP18's ability to deubiquitinate and stabilize NBR1 highlights the dynamic interplay between deubiquitination and selective autophagy in regulating immune evasion. By preventing the proteasomal degradation of NBR1, USP18 ensures that this receptor remains available to facilitate the autophagic targeting of MHC-I molecules. This mechanistic insight under-

scores the importance of ubiquitination and deubiquitination processes in regulating immune responses within the tumor microenvironment.

Taken together, our findings provide a comprehensive model of how USP18 facilitates immune evasion in PDAC by regulating MHC-I degradation through a NBR1-dependent selective autophagy pathway. By stabilizing NBR1 through deubiquitination, USP18 enhances the efficiency of MHC-I degradation, ultimately promoting tumor immune escape. This mechanism highlights USP18 as a promising therapeutic target for overcoming resistance to immunotherapies in PDAC, with potential for broader implications in other malignancies exhibiting similar immune evasion strategies.

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Disclosure of conflict of interest

None.

Address correspondence to: Jing Cai, Department of Oncology, The Second Affiliated Hospital of Nanchang University, Nanchang University, No. 1 Minde Road, Nanchang 330006, Jiangxi, China. Tel: +86-791-86297662; Fax: +86-791-86297662; E-mail: caijinefy5566@126.com; ndefy11072@ncu.edu.cn; Xin Yu, Hepatopancreatobiliary Surgery Division, Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang University, No. 1 Minde Road, Nanchang 330006, Jiangxi, China. Tel: +86-791-86297662; Fax: +86-791-86297662; E-mail: yuxinefy2007@163.com; ndefy06069@ncu.edu.cn

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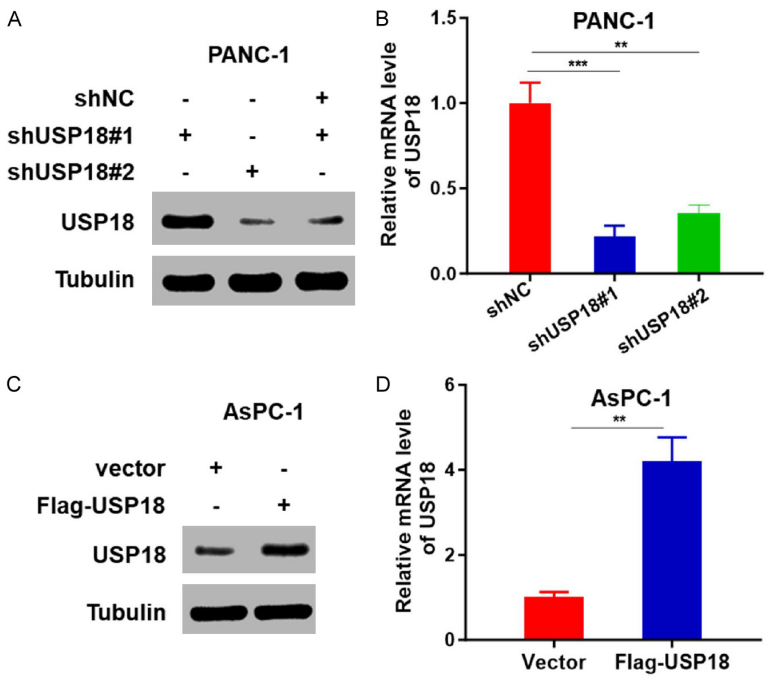
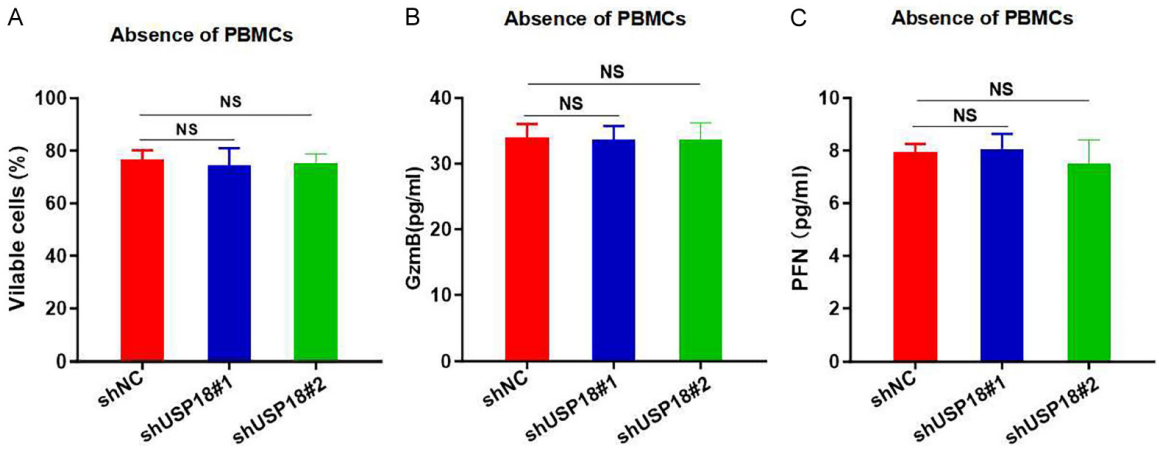
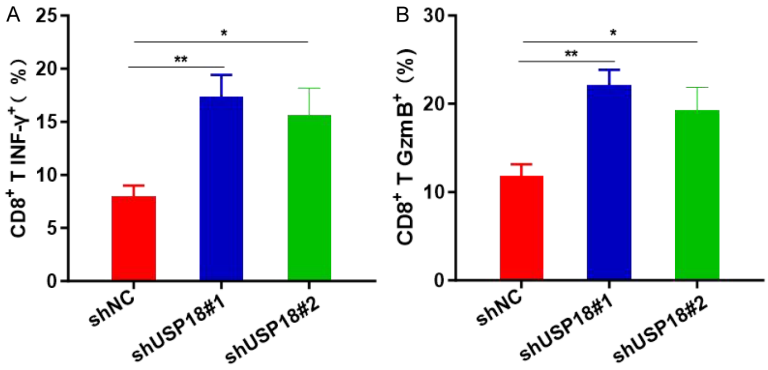


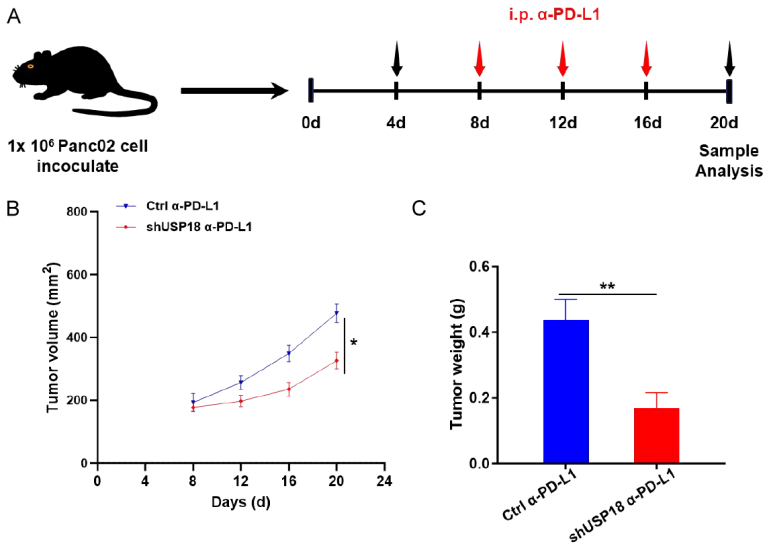
Supplementary Figure 1. USP18 Expression Profiling in Engineered Pancreatic Cancer Cells. A, B. The protein (A) and mRNA levels (B) of USP18 were detected in PANC-1 cells stably transfected with the USP18-silenced vector. Tubulin was used as a loading control. C, D. The protein (C) and mRNA levels (D) of USP18 were detected in USP18 overexpression PC cells. Tubulin was used as a loading control. $^{**}p< 0.01$, $^{***}p< 0.001$.



Supplementary Figure 2. USP18 knockdown does not affect PANC-1 cell viability or cytotoxic molecule expression without immune co-culture. A. In the absence of PBMC co-culture, USP18 knockdown (KD) did not significantly affect the baseline viability of PANC-1 cells. B, C. Without PBMC co-culture, USP18 KD did not significantly induce Granzyme B (GZMB, B) or Perforin (PFN, C) expression within PANC-1 cells themselves. NS (not significant).



Supplementary Figure 3. USP18 deficiency promotes CD8+ T-cell-mediated antitumor immunity in vitro. shNC or shUSP18 PANC-1 cells were co-cultured with purified CD8+ T cells for 48 hours, Flow cytometry was used to detect the expression of IFN-γ (A) and GzmB (B) in CD8+ T cells. $^{*}p<0.05$, $^{**}p<0.01$.



Supplementary Figure 4. Anti-PD-L1 treatment combined with USP18 knockdown inhibits tumor growth in a PDAC subcutaneous model. (A) Tumor growth curves of PANC-1 xenografts in mice treated with control, anti-PD-L1 alone, or the combination of USP18 KD and anti-PD-L1. (B) Representative images of resected tumors at the experimental endpoint for each treatment group. (C) Tumor weights at the endpoint for each treatment group. $^{*}p<0.05$, $^{**}p<0.01$.