Received: 3 June 2023 | Revised: 1 November 2023 | Accepted: 7 November 2023

DOI: 10.1111/cas.16024

ORIGINAL ARTICLE

Cancer Science WILEY

Neuromedin U regulates the anti-tumor activity of CD8$^+$ T cells and glycolysis of tumor cells in the tumor microenvironment of pancreatic ductal adenocarcinoma in an NMUR1-dependent manner

Rui Zheng[1,2] | Si Wang[1] | Jia Wang[1] | Mengnan Zhou[1] | Qi Shi[1] | Beixing Liu[1]

[1]Department of Pathogenic Microbiology, School of Basic Medical Science, China Medical University, Shenyang, China

[2]Department of Physiology, School of Basic Medical Science, Shenyang Medical College, Shenyang, China

Correspondence
Beixing Liu, Department of Pathogenic Microbiology, School of Basic Medical Science, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang 110122, China.
Email: bxliu@cmu.edu.cn

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a poor prognosis, which is lethal in approximately 90% of cases despite advanced standard therapies. A typical feature of PDAC is the immunosuppressive tumor microenvironment with multiple immunosuppressive factors including neurotransmitters. Recently, neuromedin U (NMU), a highly conserved neuropeptide with many physiological functions, has attracted attention for its roles in tumorigenesis and metastasis in several types of cancers. However, whether NMU affects PDAC progression remains unclear. In this study, using an orthotopic mouse model of PDAC in combination with bioinformatics analysis, we found that NMU was upregulated in tumor tissues from the patients with PDAC and positively correlated with a poor prognosis of the disease. Interestingly, knockout of the *Nmu* gene in mice enhanced the anti-tumor functions of tumor-infiltrating CD8$^+$ T cells in an NMU receptor 1-dependent manner. Additionally, NMU promoted the glycolytic metabolism of mouse PDAC tumors. The activities of pyruvate kinase (PK) and lactate dehydrogenase (LDH), pivotal enzymes involved in the regulation of lactate production, were markedly reduced in tumor tissues from NMU-knockout mice. In vitro the presence of LDHA inhibitor can reduce the production of lactic acid stimulated by NMU, which can increase the anti-tumor activity of CD8$^+$ T cells. Moreover, treatment of the pancreatic cancer cells with a phosphoinositide 3-kinase (PI3K) inhibitor diminished NMU-induced lactate production and the activities of PK and LDH, suggesting that NMU might regulate glycolysis via the PI3K/AKT pathway.

KEYWORDS
CD8$^+$ T cells, glycolysis, neuromedin U, pancreatic ductal adenocarcinoma, tumor microenvironment

Abbreviations: HIF-1α, hypoxia-inducible factor 1α; IFN-γ, interferon-γ; LDHA, lactate dehydrogenase; NMU, neuromedin U; PDAC, pancreatic ductal adenocarcinoma; PKM, pyruvate kinase; TME, tumor microenvironment.

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma, accounting for 95% of pancreatic cancers, is the most aggressive malignant neoplasm with insidious onset, rapid disease progression, and poor prognosis.[1,2] Surgical removal of early localized lesions is the only potentially curative treatment.[3] However, patients with PDAC are usually diagnosed at an advanced stage with distant metastasis and are not suitable for surgery resection.[4] Although currently multimodal treatments involving radiotherapy, chemotherapy, and immunotherapy have contributed to improving the prognosis of the disease, long-term results are still not satisfactory.[5,6] Therefore, investigating more effective therapeutic targets and potential prognostic markers for patients with PDAC is meaningful.

The TME plays a key role in tumor progression and affects clinical treatment efficacy, especially the efficacy of immunotherapy.[7] Pancreatic cancer has a markedly immunosuppressive TME.[8] Multiple types of immunosuppressive cells, including tumor-associated macrophages, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) are present in the TME.[9] These cells secrete large amounts of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and increase the expression of immunosuppression-related receptors, such as cytotoxic-T lymphocyte-associated antigen 4 and programmed cell death protein 1 (PD-L1) on anti-tumor T cells.[10–12] Thus, despite rapid advances in the development of immunosuppressive factor/receptor-targeting agents, the efficacy of immunotherapy, (e.g., immune checkpoint inhibitors) is still limited in advanced PDAC, due to the inhibited anti-tumor immunity in the TME.[13]

Perineural invasion is a typical feature of pancreatic cancer and is associated with poor prognosis in PDAC patients.[14] Recent studies have shown that cholinergic signaling triggered by perineural invasion can decrease $CD8^+$ T-cell infiltration and reduce their IFN-γ production, resulting in an enhanced immunosuppressive status in the TME.[15] However, tumor cells and the immunosuppressive TME can change the structure and function of neuronal cells at the tumor site. Conversely, neurotransmitters released from neurons and peripheral glial cells can influence tumor progression in an autocrine and/or paracrine manner.[14] It has been reported that the level of serotonin, a neuromodulator with neurotransmitter and neuroendocrine functions, is increased in human PDACs and is associated with the growth of pancreatic tumors in mice.[16] In addition, the B-cell-derived neurotransmitter gamma-aminobutyric acid promotes monocyte differentiation into anti-inflammatory macrophages that secrete IL-10 and inhibit the function of $CD8^+$ T-cell killer.[17] Thus, exploring the effects of neurotransmitters on PDAC progression may help uncover new therapeutic targets for patients with PDAC.

Neuromedin U is a highly conserved neuropeptide with multiple physiological functions, including smooth muscle contraction, metabolism homeostasis, stress response, and inflammation.[18] NMU exerts its function via two main receptors: NMU receptor 1 (NMUR1) and NMU receptor 2 (NMUR2).[19,20] NMU has raised interest as a new player in the tumorigenesis and/or metastasis of many types of cancer.[18] This neuropeptide has been found to be highly expressed in the serum and tumor tissue of patients with hepatocellular carcinoma, and to be related to the increased percentage of M2 macrophages and the levels of Th2 cytokines IL-4, IL-10, and IL-13.[21] High expression levels of NMU can reduce the overall survival rate of patients with neuroblastoma by activating immunosuppressive Tregs and inducing macrophage M2 polarization.[22] NMU may also be a poor prognostic biomarker in lung adenocarcinoma.[23] Furthermore, NMU overexpression may contribute to drug resistance in human epidermal growth factor receptor 2 (HER2)-positive breast cancer, by increasing the expression of the immunosuppressive molecules TGF-β and PD-L1.[24]

So far, the role of NMU in the oncogenesis and development of PDAC remains unclear. In this study, we found that NMU is an independent prognostic factor for patients with PDAC and may regulate the anti-tumor activity of $CD8^+$ T cells in the TME in an NMUR1-dependent manner.

2 | MATERIALS AND METHODS

2.1 | PDAC murine model

All animal experiments were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of China Medical University. NMU-targeted knockout mice ($NMU^{-/-}$) on a C57BL/6 gene background were generated utilizing the CRISPR/Cas9 method by Cyagen Biosciences Co., Ltd. (Guangzhou, China). Mice had fresh water and autoclaved food and were kept at the Laboratory Animal Center, China Medical University throughout all the experiments. To establish a murine model of PDAC, 2×10^5 Panc02 cells mixed in a 1:1 cell suspension with Matrigel (Corning, USA) were injected into the pancreas or the right axilla of mice. Tumor volumes were calculated using the formula: $0.5\times\text{length}\times\text{width}\times\text{width}$.[25]

2.2 | Real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies). Quantitative PCR was performed using the SYBR Green Master Mix (Life Technologies). The primer sequences are reported in Table S1. Results are normalized to β-actin and presented as fold mRNA expression ($2^{-\triangle\triangle CT}$).

2.3 | Western blotting

Panc02 cells were stimulated in vitro with 10 ng/mL of recombinant NMU (Phoenix Pharmaceuticals) in the presence or absence of 1 μg/mL of anti-NMUR1 neutralizing antibody (EpiGentek, USA) for 24 h. Then, the signaling molecules were determined by western blotting.[26] Rabbit anti-NMU antibody/anti-NMUR1 antibody/anti-PI3K antibody/anti-*p*-PI3K antibody/anti-AKT antibody/anti-*p*-akt antibody (Affinity

Biosciences) were used for the experiments. Meanwhile, 30 μM inhibitor LY294002 (GlpBio GC15485, USA) was utilized for blockade of PI3K activity in the cultured Panc02 cells. The immunocomplexes were visualized with the BeyoECL Plus Chemiluminescent Substrate. Protein band intensity was quantified using ImageJ software.

2.4 | Immunohistochemistry assay

The tumor sections were cultured with the primary antibody against NMU as well as the biotinylated secondary antibody (Santa Cruz Biotechnology). Diaminobenzidine substrate was used to reveal immunoreactive products. The signal strength in tumor tissues was calculated using ImageJ software.

2.5 | Isolation and culture of $CD8^+$ T cells

In total, 1×10^5 of $CD8^+$ T cells were sorted from the spleens of tumor-bearing mice or tumor tissues using Miltenyi magnetically labeled beads (Miltenyi Biotec, Cologne, Germany), and cultured in a 24-well plate coated with 1 μg/mL anti-mouse CD3ε and 2 μg/mL anti-mouse CD28ε (BioLegend) for 72 h. Then these activated $CD8^+$ T cells were stimulated with 10 ng/mL of recombinant NMU in the presence or absence of 10 μM GSK2837808A (GlpBio GC13464, USA) for 24 h. The percentages of IFN-γ-, perforin- and granzyme B-producing $CD8^+$ T cells were collected by flow cytometry.

2.6 | Statistical analysis

All values were displayed as mean ± standard deviation (SD). One-way ANOVA was used to compare differences between three or more groups. Student's *t*-test was applied to analyze the difference between the two groups. *P*-values <0.05 were considered statistically significant.

Materials and methods also including bioinformatics analysis, cell culture, preparation of single-cell suspension from tumor mass, flow cytometry, detection of intracellular lactate dehydrogenase (LDH) and pyruvate kinase (PK), Determination of lactate concentration, and ELISA assay are described in Data S1.

3 | RESULTS

3.1 | NMU expression is upregulated in tumor tissue and positively correlated with poor prognosis in PDAC

To explore the expression of NMU in patients with PDAC, the transcriptome profiles of tumor tissues and non-carcinoma adjacent tissues were downloaded from the TCGA-PAAD and GTEx databases. The analysis revealed that expression of NMU was significantly increased in pancreatic tumor tissue compared to that in non-tumor pancreatic tissues (Figure 1A). Moreover, analysis of GEO data sets confirmed the high NMU expression of NMU in PDAC tissues (Figure 1B). We found the high expression of NMU to be negatively correlated with overall survival and disease-free survival, suggesting that NMU may have independent prognostic value (Figure 1C–E). Furthermore, NMU expression was associated with the pathological stage and grade of pancreatic cancer, in patients (Figure 1F,G).

To further verify the bioinformatic analysis results, NMU expression in mouse pancreatic implant tumors was detected. We found that the levels of NMU mRNA and protein in tumor tissues were significantly higher than that in normal pancreatic tissues (Figure 2A–C). NMU was mainly localized in the intercellular space of the pancreatic tumor tissue, suggesting a role as a secretory protein (Figure 2D,E). Knockout of the *Nmu* gene in mice could significantly improve the survival of tumor-bearing mice (Figure 2F,G) and reduce the tumor weight and volume (Figure 2H,I). These results indicated that NMU may be a poor prognostic factor for patients with PDAC and is associated with the outcome of pancreatic cancer.

Notably, Panc02 cells can express NMUR1, but cannot produce NMU protein (Figure S1A,B). However, the levels of NMU in the pancreas of NMU-knockout mice were almost undetectable compared with that in the wild-type control mice (Figure S1C), suggesting that Panc02 cells may be target cells for NMU but not the cellular source of NMU. Indeed, by using immunofluorescence staining, we found that neurons might be the main cellular source of NMU in the pancreatic tissue (Figure S1D).

3.2 | NMU impacts the abundance of immune cells, especially $CD8^+$ T cells, in the TME of PDAC

Using the CIBERSORT algorithm, we found that NMU might influence the infiltration of immune cells into the tumor site. The numbers of immune cells, particularly $CD8^+$ T cells in TCGA and GEO pancreatic cancer transcriptome database were different between high and low NMU expression groups (Figure S2A,B). In fact, knockout of the *Nmu* gene in mice resulted in a significant increase in the percentages and absolute numbers of $CD8^+$ T cells and $CD4^+$ T cells, but a decrease in the percentage and number of MDSCs in both tumor tissues (Figure 3A; Figure S3) and the spleen (Figure 3B) of tumor-bearing mice, suggesting that NMU may have a role in the regulation of the immune microenvironment in PDAC.

3.3 | NMU diminishes the biological function of $CD8^+$ T cells in TME of PDAC in a NMUR1-dependent manner

As NMU mainly impacted the percentage and numbers of T cells in the tumor tissues of PDAC (Figure 3A,B) and $CD8^+$ T cells play a critical role in anti-tumor immunity,[27] the effects of NMU on the biological function of $CD8^+$ T cells in TME were determined. On day 14 after tumor implantation, single-cell suspensions of tumor tissues were prepared and analyzed using flow cytometry. As shown

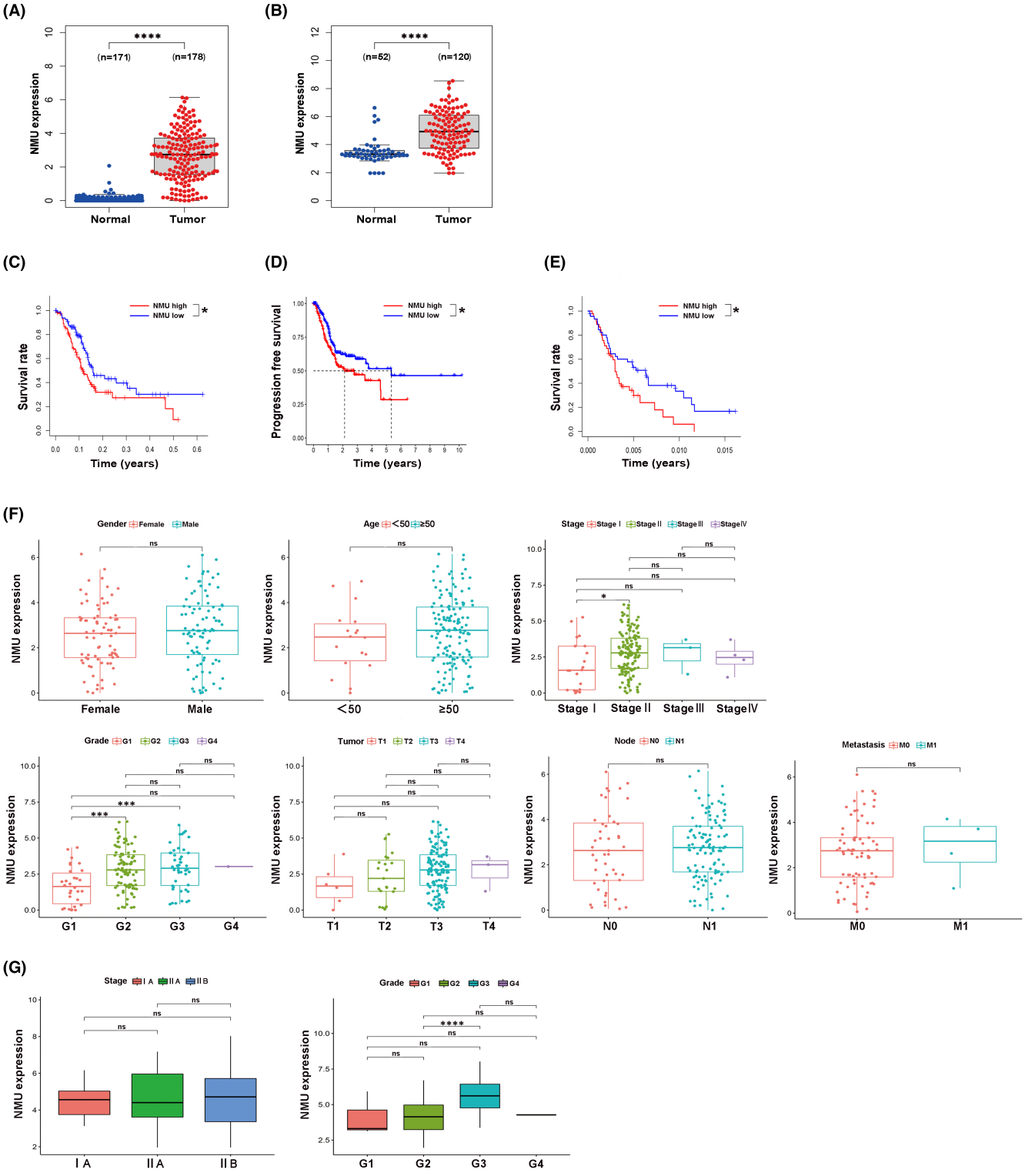

FIGURE 1 NMU is upregulated in tumor tissues and correlates with the poor prognosis of patients with (PDAC). (A, B) Boxplot showing the differential expression of NMU in normal and pancreatic cancer tissue in TCGA (A) and GEO (B) databases. (C, D) Comparison of OS (C) and DFS (D) in patients with PDAC expressing high (>median level) or low (<median level) NMU expression in the TCGA database. (E) OS in patients with PDAC with high (>median level) or low (<median level) NMU expression in the GEO databases. (F, G) Pearson's correlation analysis between NMU expression and clinicopathological variables in TCGA (F) or GEO (G) data. *$p<0.05$; ***$p<0.001$; ****$p<0.0001$; ns = not statistically significant. NMU, neuromedin U; PDAC, pancreatic ductal adenocarcinoma.

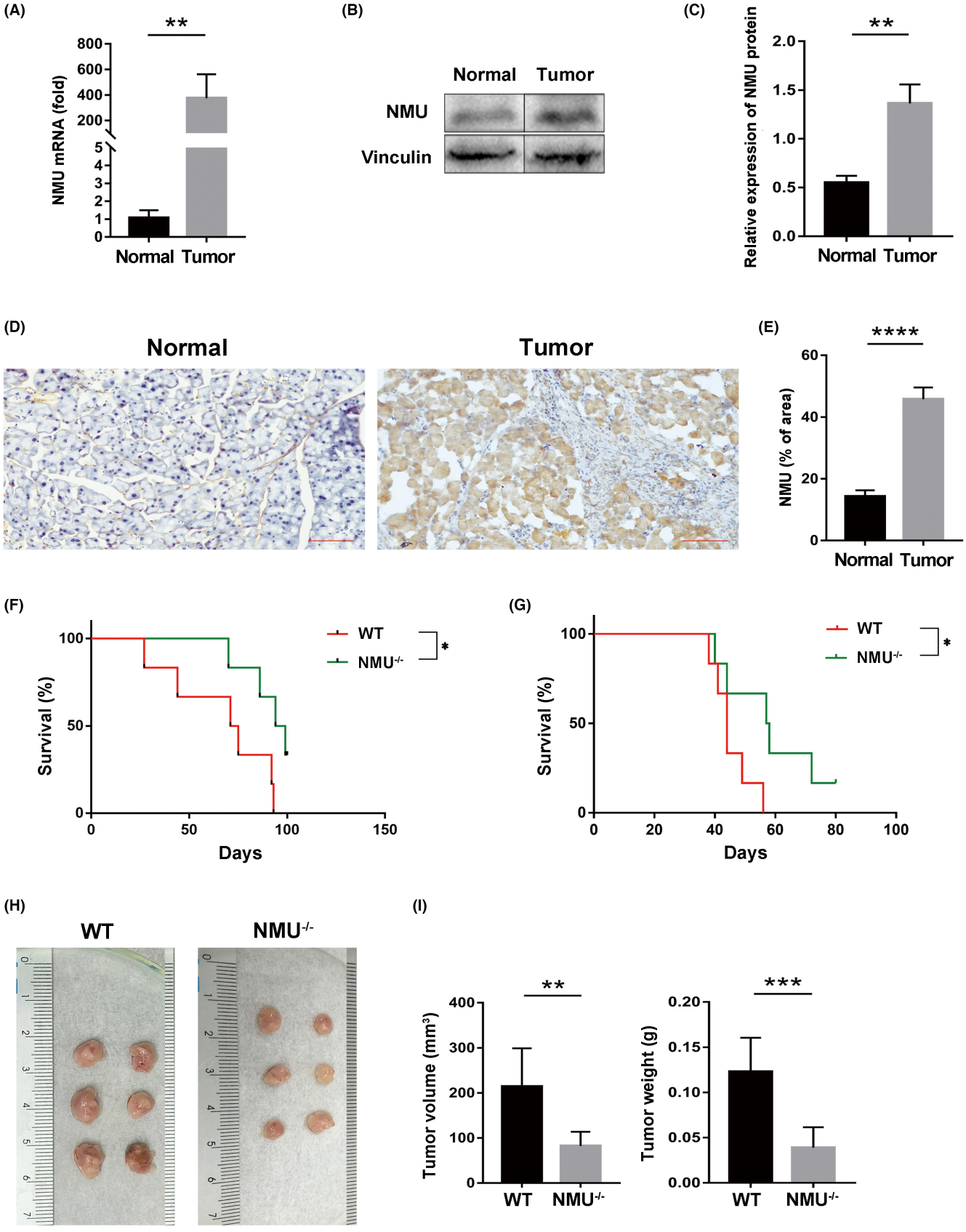

FIGURE 2 NMU contributes to the progression of PDAC. (A) Real-time PCR analysis of NMU expression in tumor tissues. (B, C) Western blot analysis and quantification of NMU protein levels in tumor tissues. (D, E) Immunohistochemical staining and quantification of NMU protein levels in pancreatic tumor tissues. (F, G) Kaplan–Meier survival curve showing the survival of tumor-bearing mice after subcutaneous (F) or orthotopic (G) implantation of Panc02 cells. (H, I) Pancreatic tumor image (H) and volume and weight (I) in tumor-bearing mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by Student t-test. Scale bars = 100 μm. NMU, neuromedin U; PDAC, pancreatic ductal adenocarcinoma.

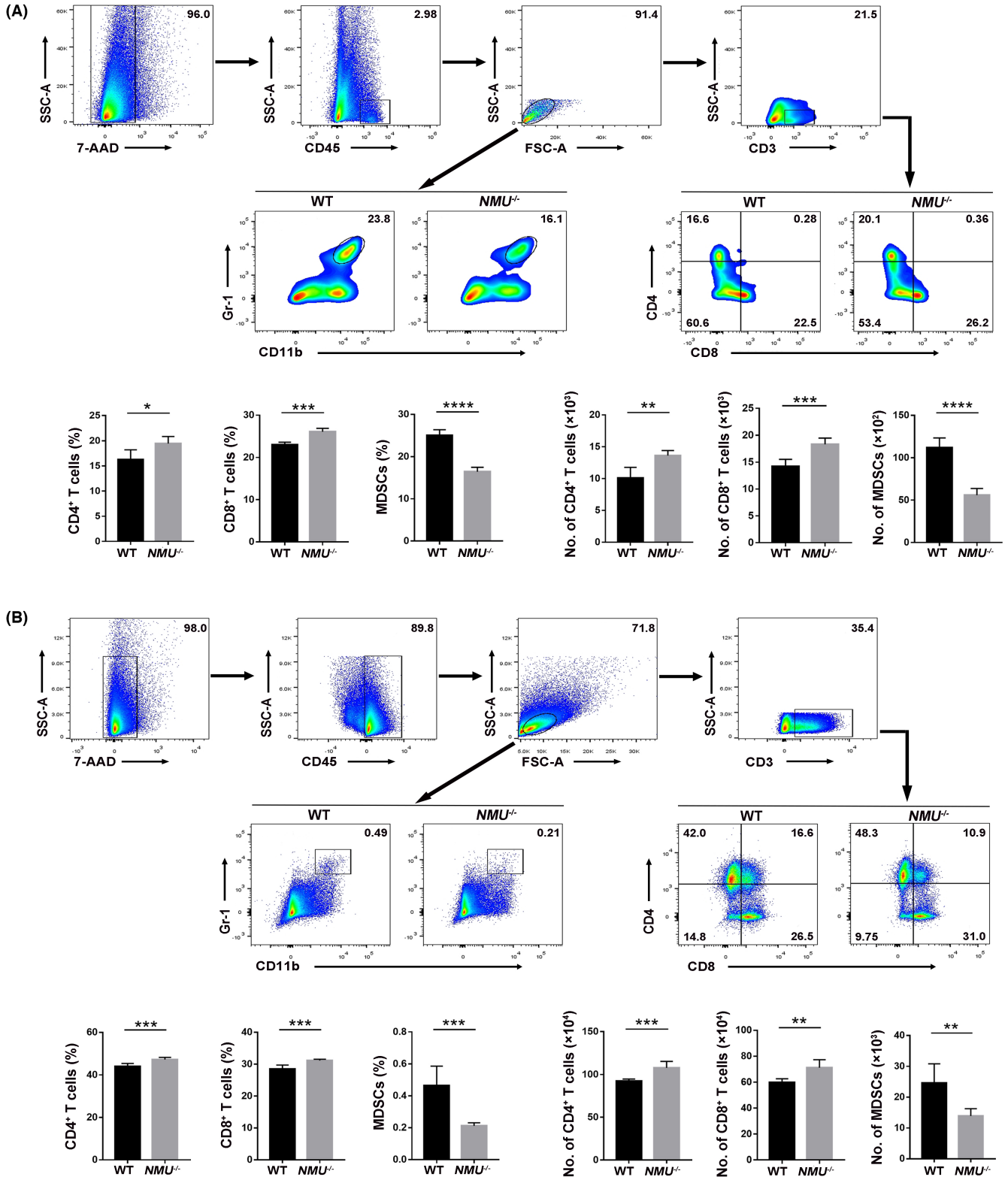

FIGURE 3 NMU affects tumor-infiltrating immune cells in tumor tissues from PDAC. (A, B) The percentage and absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells, and MDSCs in pancreatic tumor tissues (A) and spleens (B). $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ by Student *t*-test. NMU, neuromedin U; PDAC, pancreatic ductal adenocarcinoma.

in Figure 4, *Nmu* gene deletion in mice resulted in a significant increase in the numbers of anti-tumor $CD8^+$ T cells, including IFN-γ-, perforin-, and granzyme B-producing $CD8^+$ T cells in tumor tissues (Figure 4A). Moreover, the expression of mRNAs for IFN-γ, perforin, and granzyme B in $CD8^+$ T cells sorted from the tumor mass was increased in *Nmu*-knockout versus wild-type mice (Figure 4B). These

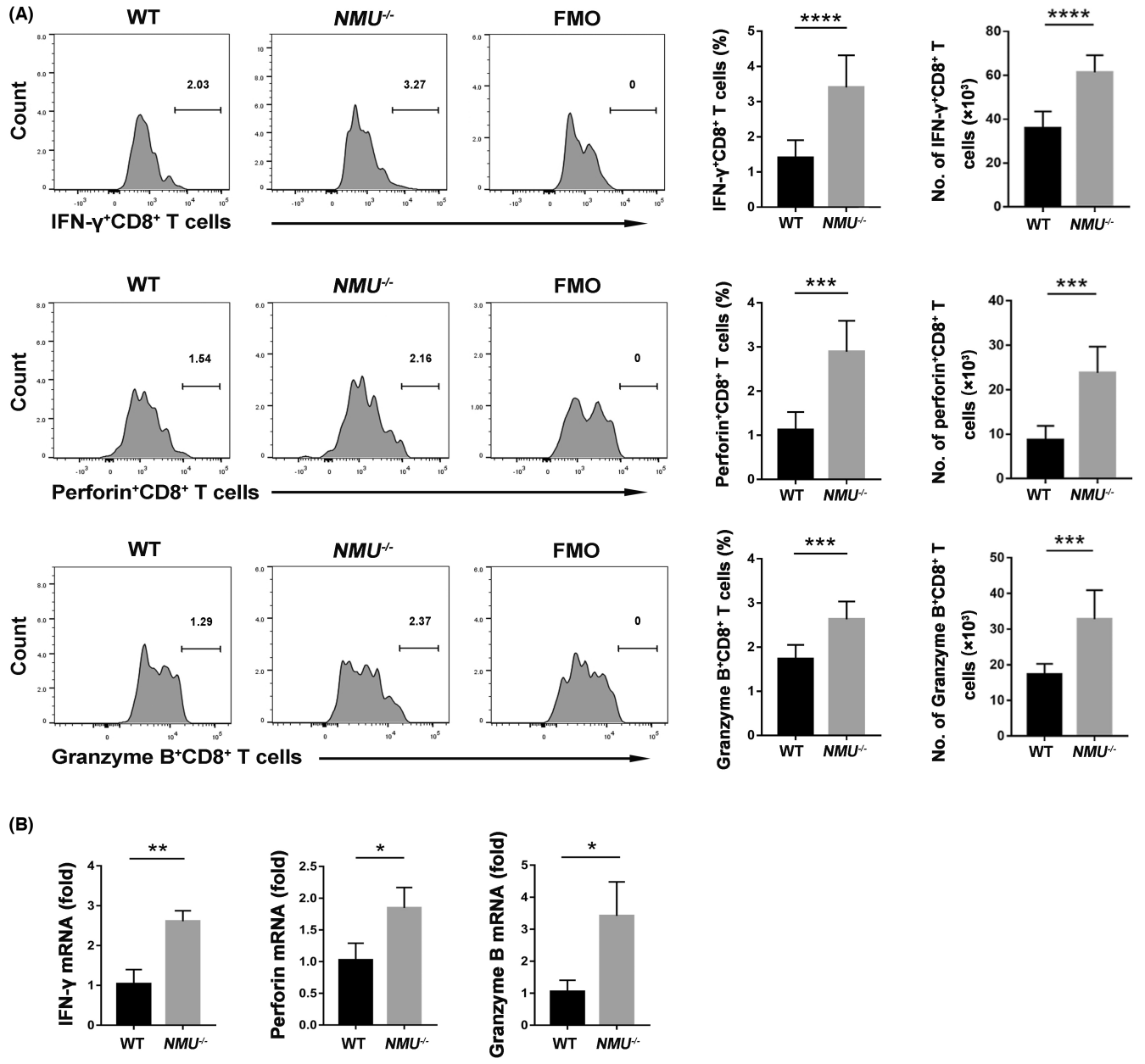

FIGURE 4 NMU reduces the anti-tumor activity of CD8$^+$ T cells in tumor tissues. (A) Flow cytometry analysis of the percentage and absolute numbers of IFN-γ$^+$CD8$^+$ T cells, perforin$^+$CD8$^+$ T cells, and granzyme B$^+$CD8$^+$ T cells in tumor tissues. (B) Real-time PCR analysis of the expression of mRNA for IFN-γ, perforin and granzyme B in the sorted tumor-infiltrating CD8$^+$ T cells. *$p<0.05$;**$p<0.01$; ***$p<0.001$; ****$p<0.0001$ by Student *t*-test. IFN-γ, interferon-γ; NMU, neuromedin U.

results suggest that NMU exerts an inhibitory effect on the anti-tumor activity of CD8$^+$ T cells.

Although multiple types of cells, such as Panc02 tumor cells, CD4$^+$ T cells and MDSC cells may express NMUR1 (Figure S4), tumor-infiltrating CD8$^+$ T cells expressed higher levels of NMUR1 on the cell surface (Figure 5A). In vitro stimulation of CD8$^+$ T cells, which were sorted from the tumor tissues of tumor-bearing WT mice by immunomagnetic beads (the purity >90%), with recombinant NMU protein reduced their anti-tumor activity, as indicated by the decreased percentage and number of IFN-γ-, perforin- and granzyme B-producing CD8$^+$ T cells following NMU treatment (Figure 5C). However, the NMU-induced effects in CD8$^+$ T cells were blocked by inhibition of NMUR1 with a neutralizing antibody (Figure 5B,C). These results suggest that NMU might inhibit the anti-tumor activity of tumor-infiltrating CD8$^+$ T cells in an NMUR1-dependent manner.

3.4 | NMU regulates the function of CD8$^+$ T cells by enhancing glycolysis in pancreatic tumor cells

NMU has been shown to enhance tumor glycolysis and impact immune cell functions in breast cancer TME.[28] Based on the expression

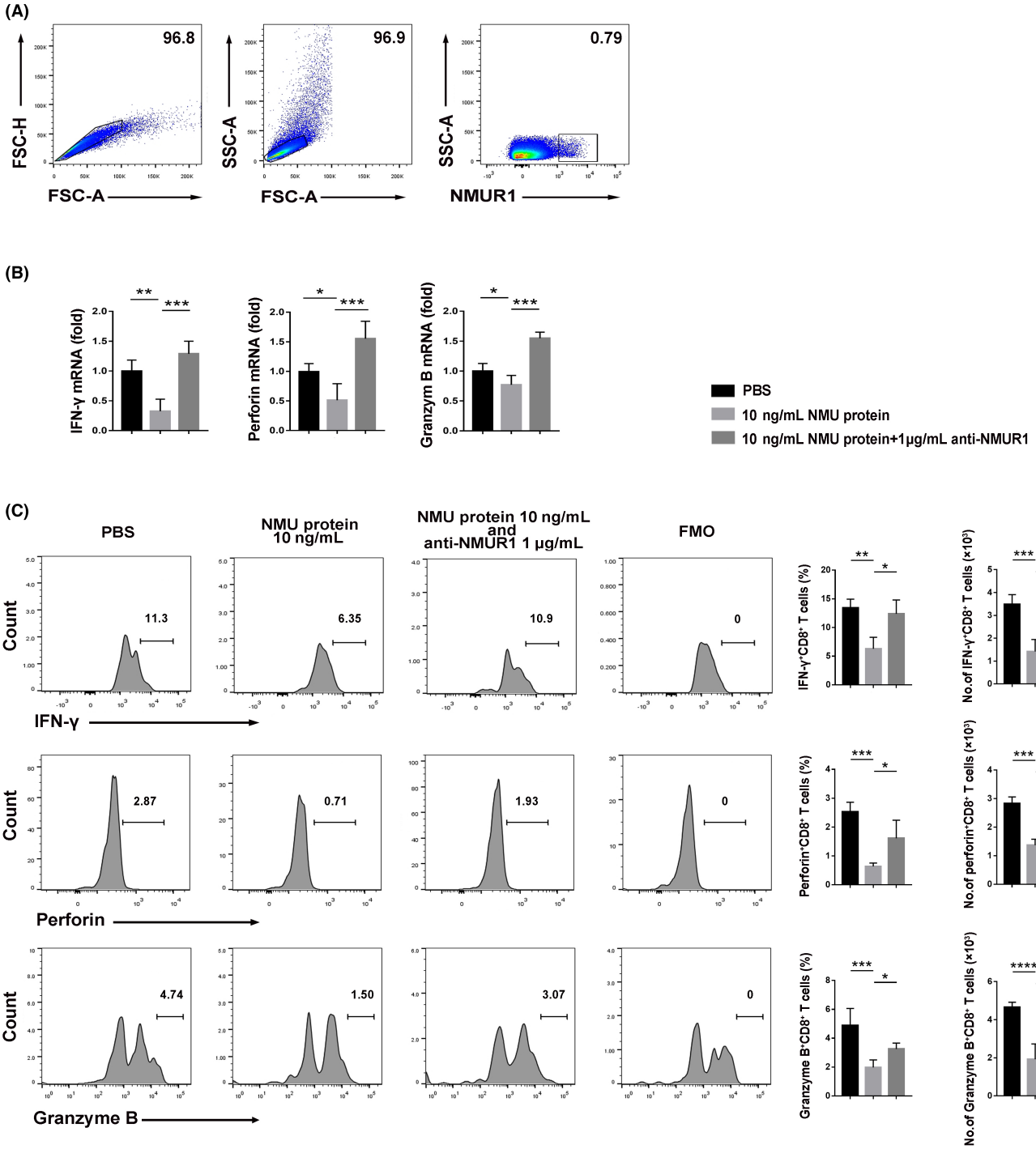

FIGURE 5 NMU diminishes the biological function of tumor-infiltrating $CD8^+$ T cells in a NMUR1-dependent manner. (A) Flow cytometry analysis of NMUR1 expression in $CD8^+$ T cells sorted from the tumor tissues. (B, C) Sorted $CD8^+$ T cells were in vitro stimulated with recombinant NMU (10 ng/mL) in the presence or absence of the anti-NMUR1 neutralizing antibody (1 μg/mL) for 4 h. Real-time PCR detected the relevant expression of IFN-γ, perforin and granzyme B mRNAs in the $CD8^+$ T cells (B). Flow cytometry analyzed the percentage and the absolute numbers of IFN-γ^+CD8^+ T cells, perforin$^+CD8^+$ T cells, and granzyme B^+CD8^+ T cells (C). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$by one-way ANOVA test. IFN-γ, interferon-γ; NMU, neuromedin U.

of NMU, data from TCGA databases were grouped for gene set enrichment analysis (GSEA) of glycolytic metabolism. Among them, the pentose phosphate pathway and the glucose catalytic process had differential expression in the high and the low NMU groups (Figure S5A). Moreover, NMU expression was positively correlated with the levels of several glycolysis-related genes. (Figure S5B–E). These data suggest that NMU expression affects glycolytic metabolism in pancreatic cancer cells.

To validate the bioinformatics analysis results, we observed that the expression of mRNAs for *Hk1*, *Hk2*, *Ldha*, *Glut-1*, *Pkm2*, *Mct-1* and

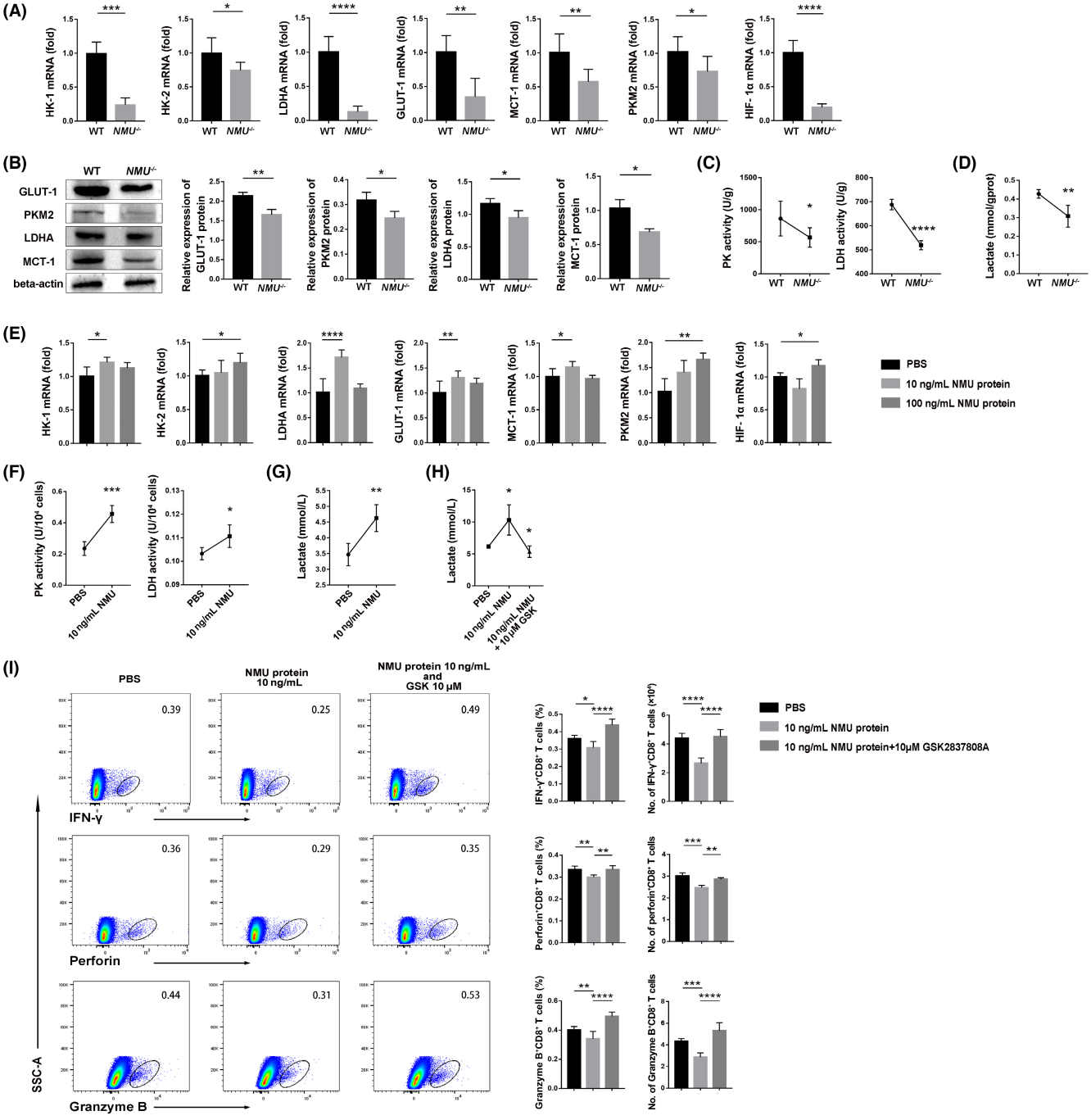

FIGURE 6 NMU regulates glycolysis in tumor tissues and affects $CD8^+$ T-cell function. (A) Real-time PCR detected the relative expression of mRNAs for glycolysis-related factors and *Hif-1α* in tumor tissues. (B) Protein levels of key glycolytic factors in tumor tissues were detected using western blot. (C) The activities of PK and LDH in tumor tissue. (D) The concentration of lactic acid in tumor tissues. (E) Panc02 cells were stimulated with different concentrations of recombinant NMU for 24 h, and the expression of glycolysis-related factors and *Hif-1α* mRNAs were determined using real-time PCR. (F, G) Panc02 cells were stimulated with 10 ng/mL of recombinant NMU for 24 h. The activities of PK and LDH in the cells (F) and the concentration of lactate in the supernatants of cultured cells (G). (H) Panc02 cells were stimulated with 10 ng/mL of recombinant NMU for 24 h in the presence or absence of 10 μM GSK2837808A. The concentration of lactic acid in the culture supernatants was determined. (I) $CD8^+$ T cells were sorted from the spleens of tumor-bearing mice and cultured in vitro with the supernatants from NMU-treated or NMU + GSK2837808A-treated Panc02 cells. Flow cytometry analyzed the percentages and absolute numbers of IFN-γ^+CD8^+ T cells, perforin$^+CD8^+$ T cells, and granzyme B^+CD8^+ T cells. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ by Student *t*-test or one-way ANOVA test. IFN-γ, interferon-γ; NMU, neuromedin U.

Hif-1α was significantly decreased in the pancreatic tumor tissue from *Nmu*$^{-/-}$ mice compared with those in WT mice (Figure 6A). In addition, the protein levels of LDHA, GLUT-1, PKM2, and MCT-1 in the tumor tissues from *Nmu*$^{-/-}$ mice were correspondingly reduced (Figure 6B). Therefore, the activities of PK and LDH, which are enzymes that regulate lactate production, were significantly inhibited in tumor tissues

from *Nmu*$^{-/-}$ mice (Figure 6C). Furthermore, the concentration of lactic acid was decreased significantly in the tumor tissues of NMU-deficient mice (Figure 6D). Additionally, we observed that in vitro stimulation of Panc02 cells significantly induced the expression for *Hk-1*, *Hk-2*, *Ldha*, *Glut-1*, *Pkm2*, and *Mct-1* (Figure 6E). Overall, these results demonstrated that NMU regulates glycolysis in PDAC tissues.

To explore the molecular mechanism associated with the NMU-mediated regulation of glycolysis, we assessed the enzymatic activities of PK and LDH in the Panc02 cells following recombinant NMU stimulation. We found that the activities of their enzymes were increased (Figure 6F), as was the concentration of lactic acid in the culture supernatant (Figure 6G). Conversely, inhibition of LDHA activity with the inhibitor GSK2837808A, decreased NMU-induced lactate production by tumor cells, and the levels of lactic acid in the culture supernatant were down to the levels of the control PBS group after GSK2837808A treatment (Figure 6H).

NMU-regulated tumor cell glycolysis and the anti-tumor activity of CD8$^+$ T cells were possibly correlated. The CD8$^+$ T cells were treated with the supernatants from NMU-stimulated Panc02 cells, which contained a high concentration of lactic acid (Figure 6G). This treatment resulted in a decrease in the percentage and numbers of IFN-γ-, perforin-, and granzyme B-producing CD8$^+$ T cells (Figure 6I). Conversely, treatment of CD8$^+$ T-cell cultures with NMU + GSK2837808A-treated Panc02 cell supernatants, in which NMU-induced production of lactate by tumor cells was inhibited by GSK treatment (Figure 6H), restored the anti-tumor function of CD8$^+$ T cells (Figure 6I). Suggesting that NMU-regulated glycolysis, especially the metabolic marker lactate in cancer cells, might affect the activity of CD8$^+$ T cells.

3.5 | NMU enhances the glycolytic metabolism in pancreatic cancer cells through the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway

Glycolysis is associated with tumor growth and immune evasion.[29] To reveal the correlation between NMU and glycometabolism in pancreatic cancer, the data from TCGA database was analyzed. We found that the expression of NMU was positively correlated with the PI3K/AKT signaling pathway in the pancreatic tumor (Figure 7A).

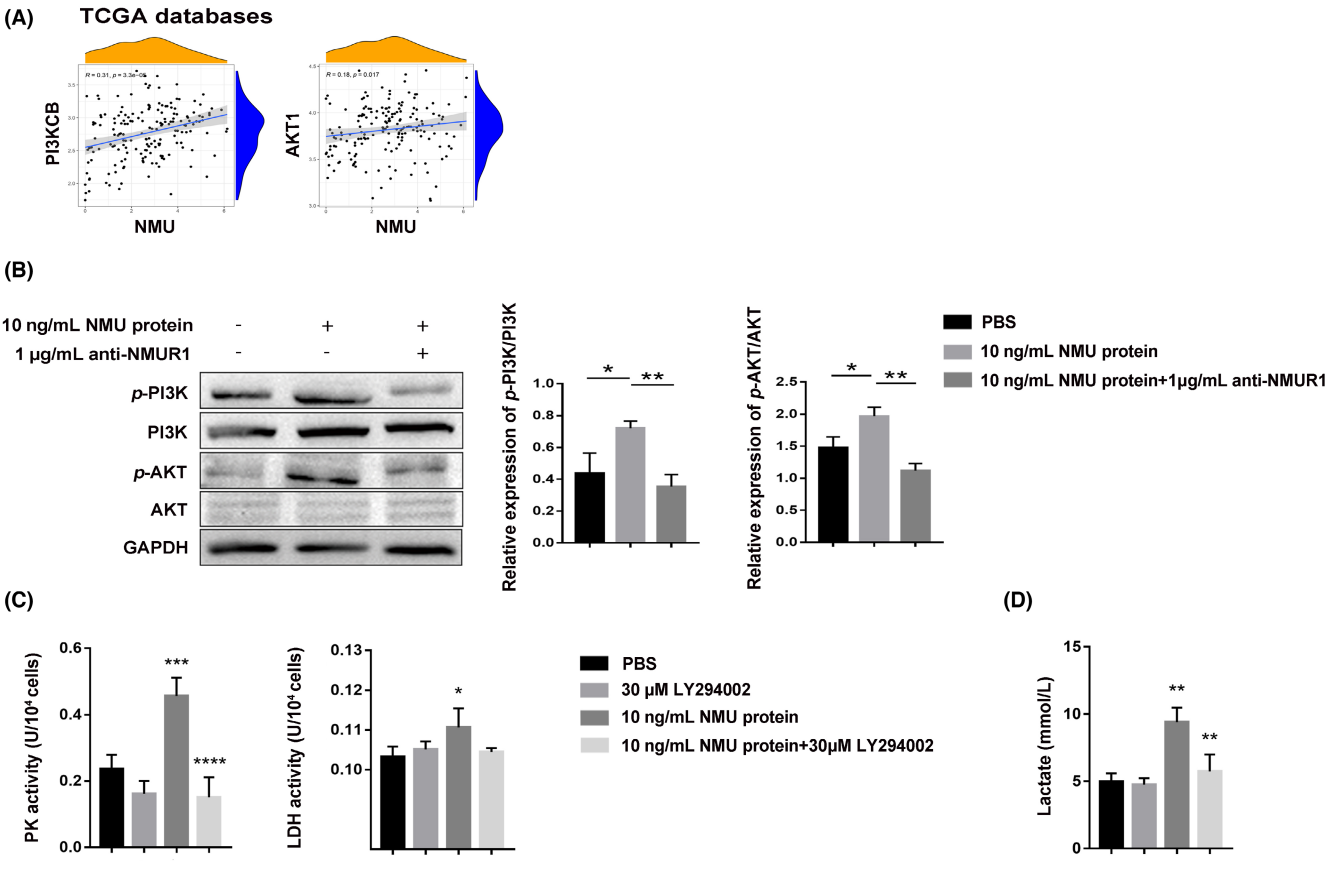

FIGURE 7 NMU regulates glycolytic metabolism in tumor cells through the PI3K/AKT signaling pathway. (A) Spearman correlation analysis between NMU and glycometabolism-related genes in TCGA database. (B) Panc02 cells were stimulated with 10 ng/mL recombinant NMU for 24 h in the presence or absence of 1 μg/mL anti-NMUR1 neutralizing antibody. Western blot detected the phosphorylated and total protein levels of PI3K/AKT in tumor cells. (C, D) Panc02 cells were stimulated with 10 ng/mL NMU recombinant proteins for 24 h in the presence or absence of 30 μM PI3K inhibitor. The activities of PK and LDH in tumor cells as well as the concentration of lactic acid in the supernatants were measured using detection kits. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$;****$p < 0.0001$ by one-way ANOVA test. NMU, neuromedin U.

In vitro stimulation of Panc02 cells with recombinant NMU significantly increased PI3K and AKT phosphorylation levels, in an NMUR1-dependent manner (Figure 7B). Conversely, the blockade of PI3K activity with the inhibitor LY294002 significantly inhibited NMU-induced activities of PK and LDHA in Panc02 cells (Figure 7C). In addition, NMU-induced elevation in lactic acid concentration in the supernatants of cultured tumor cells was significantly reduced by PI3K inhibitor treatment (Figure 7D). These results suggested that NMU regulates glycolysis in pancreatic cancer cells via aPI3K/AKT pathway-mediated mechanism.

4 | DISCUSSION

Pancreatic cancer is a highly aggressive gastrointestinal cancer, characterized by high mortality and low rates of early diagnosis.[30,31] Risk factors for PDAC are varied, including individual characteristics, genetic factors, and environmental factors.[32] Thus, a better understanding of the molecular mechanisms underlying the malignancy of pancreatic cancer assumes particular relevance. NMU has been known as a neuroactive peptide, named for its powerful contractile effect on the rat uterine smooth muscle.[33] However, studies on its biological function have revealed that NMU plays a role in the development of pancreatic tumors.[19] Liu et al. reported that NMU is involved in the invasion and metastasis of pancreatic cancer related to Yes-associated protein-1,[34] whereas Lee et al. found that NMU could be specifically labeled as a potential marker of pancreatic intraepithelial neoplasia.[35] Our bioinformatics data revealed that the high expression of NMU in pancreatic cancer tissues was associated with poor survival and primary clinical features of pancreatic cancer. These findings showed that increased NMU expression is an early and persistent event in pancreatic tumorigenesis that can be regarded as a potential marker for the early diagnosis of pancreatic cancer. Using a mouse model of pancreatic tumors, we were able to reproduce these observations. NMU expression was increased in tumors compared with non-tumor tissues. In contrast, knockout of *Nmu*$^{-/-}$ in mice bearing the tumors significantly slowed tumor growth and prolonged survival. These findings highlight the potential significance of NMU as a prognostic and therapeutic target in PDAC treatment.

In this study, we found that NMU expression negatively affected the abundance of immune cells in local tumors, reducing the number of immune-dominant T-cell populations and suppressing their immune functions. Here, a close relationship between NMU and the anti-tumor activity of CD8$^+$ T cells was observed. As CD8$^+$ T cells express the NMU receptor (NMUR1), it is possible that NMU might affect the function of CD8$^+$ T cells via NMUR1. Indeed, NMU may diminish the biological function of CD8$^+$ T cells in the TME of PDAC in an NMUR1-dependent manner (Figures 3, 4). In addition, as blocking the production of lactate by tumor cells may restore NMU-mediated suppression of anti-tumor activities of CD8$^+$ T cells (Figure 6), tumor-derived lactate-mediated inhibition in the function of CD8$^+$ T cells might also not to be ignored.

The lack of vascularization and the hypoxic environment in pancreatic tumors promote the Warburg effect in cancer cell metabolism, and the lactic acid generated is the main energy source for PDAC proliferation, invasion, and metastasis.[36,37] NMU induces metabolic reprogramming in HER2-positive breast cancer cells, enhances pyruvate dehydrogenase activity, and promotes cancer stemness and epithelial-to-mesenchymal transition.[28] Therefore, we investigated the oncogenic activity of NMU in PDAC metabolic rearrangements, showed differential enrichment in carbohydrate catabolic process-related genes, and positively correlated these with the expression of glycolysis-related factors. This finding suggests that NMU drives pancreatic tumor cells, leading to a switch to abnormal energy metabolism. Subsequently, we verified that the expression of key glycolysis genes and HIF-1α increased in a mouse model and in vitro recombinant NMU stimulation, resulting in increased activity of the key enzymes PK and LDH and promoting lactic acid production by tumor cells. Lactic acid can induce tumor-associated macrophages to differentiate into an M2-like phenotype by activating HIF-1α, which induces the expression of arginase 1 and vascular endothelial growth factor, contributing to tumor invasion, metastasis, and angiogenesis.[38,39]

Studies have shown that HIF-1α promotes bladder cancer cell proliferation by upregulating PKM2-mediated glycolysis.[40] Lactate increases the frequency of MDSC recruitment in TMEs with low pH and inhibits lymphocyte homing, which is a strong contributor to immunosuppression, interfering with perforin/granzyme B secretion by NK cells in an anti-tumor cellular pathway.[41,42] These results prompted us to explore how lactate production elicited by NMU affects the CD8$^+$ T-cell immune response in PDAC. An LDHA inhibitor was able to suppress lactic acid production by NMU-stimulated tumor cells, resulting in an enhanced ability of CD8$^+$ T cells to produce IFN-γ, perforin, and granzyme B. This finding can be explained by the promotion of tumor-derived lactate by NMU to help tumor cells escape immune surveillance; therefore, reducing lactic acid concentration in the immune TME can provide new approach for pancreatic cancer treatment.[43]

Based on the available evidence, we further explored whether NMU mediates lactic acid production in tumor cells through the PI3K/AKT signaling pathway. Genetic studies have shown that the most common missense mutation in pancreatic cancer is in *KRAS*, which mediates RAS signaling and affects PDAC proliferation and metabolism.[44] PI3K and AKT are major downstream effectors of RAS signaling activated by *KRAS* mutations in PDAC to induce cancer progression via protein kinase phosphorylation.[45,46] We found that *NMU* gene expression was positively correlated with key factors of the PI3K/AKT pathway in TCGA-PAAD database. Blockade of PI3K activity may inhibit NMU-induced activities of PK and LDHA, the key glycolytic enzymes in Panc02 cells, resulting in a decreased production of lactate by tumor cells (Figure 7), suggesting that then-PI3K/AKT pathway may be a pivotal signaling pathway for NMU-regulated glycolysis in the tumor cells. Indeed, it has been reported that the PI3K/AKT pathway regulates metabolism through a number of downstream transcription factors, including HIF1a, c-*Myc*, FOXO, ATF4, and SREBP, to control the expression of genes encoding metabolic enzymes.[47]

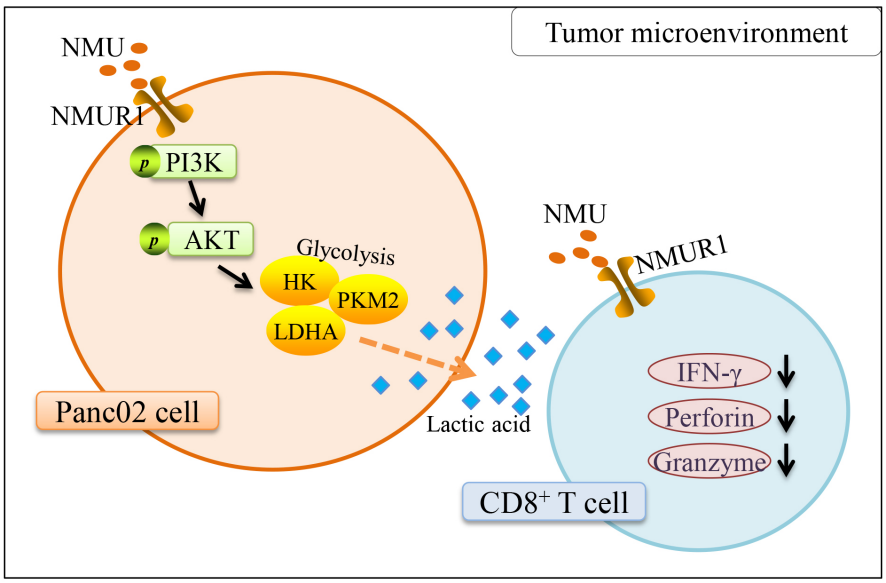

FIGURE 8 Proposed mechanism of NMU regulates the anti-tumor activity of $CD8^+$ T cells. NMU, neuromedin U.

Taken together, we concluded that NMU acts as a poor prognostic factor for patients with PADC, and it can inhibit the anti-tumor activity of tumor-infiltrating $CD8^+$ T cells directly in an NMUR1-dependent manner. NMU may also enhance glycolysis in tumors, resulting in increased lactate concentration in the TME, which further contributes to decreased $CD8^+$ T-cell anti-tumor activity (Figure 8).

AUTHOR CONTRIBUTIONS

Rui Zheng: Conceptualization; formal analysis; investigation; methodology; software; writing – original draft. **Si Wang:** Data curation; resources. **Jia Wang:** Investigation; visualization. **Mengnan Zhou:** Software; supervision. **Qi Shi:** Software; validation. **Beixing Liu:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing.

ACKNOWLEDEGMENTS

We are grateful to all members of our labs for their great support.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest for this article.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: This study was approved by the ethics committee of China Medical University.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal experiments were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of China Medical University (protocol ID: KT2022424).

ORCID

Si Wang https://orcid.org/0000-0001-9602-7555

Beixing Liu https://orcid.org/0000-0003-1855-2877

REFERENCES

1. MIzrahi J, Surana R, Valle J, Shroff RT. Pancreatic cancer. *Lancet*. 2020;395(10242):2008-2020. doi:10.1016/S0140-6736
2. Rawla P, Sunkara T, Gaduputi V. Epidemiology of pancreatic cancer: global trends, etiology and risk factors. *World J Oncol*. 2019;10(1):10-27. doi:10.14740/wjon1166
3. Zhang L, Sanagapalli S, Stoita A. Challenges in diagnosis of pancreatic cancer. *World J Gastroenterol*. 2018;19(24):2047-2060. doi:10.3748/wjg.v24
4. Park W, Chawla A, O'Reilly EM. Pancreatic cancer: a review. *JAMA*. 2021;326(9):851-862. doi:10.1001/jama.2021.13027
5. Zeng S, Pottler M, Lan B, Grutzmann R, Pilarsky C, Yang H. Chemoresistance in pancreatic cancer. *Int J Mol Sci*. 2019;20(18):4504. doi:10.3390/ijms20184504
6. Shukla SK, Purohit V, Mehla K, et al. MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. *Cancer Cell*. 2017;32(1):71-87.e7. doi:10.1016/j.ccell.2017.06.004
7. Zhao R, Pan Z, Li B, et al. Comprehensive analysis of the tumor immune microenvironment landscape in glioblastoma reveals tumor heterogeneity and implications for prognosis and immunotherapy. *Front Immunol*. 2022;13:820673. doi:10.3389/fimmu.2022.820673
8. Ho WJ, Jaffee EM, Zheng L. The tumour microenvironment in pancreatic cancer - clinical challenges and opportunities. *Nat Rev Clin Oncol*. 2020;17(9):527-540. doi:10.1038/s41571-020-0363-5
9. Yang M, Li J, Gu P, Fan X. The application of nanoparticles in cancer immunotherapy: targeting tumor microenvironment. *Bioact Mater*. 2021;6(7):1973-1987. doi:10.1016/j.bioactmat.2020.12.010
10. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunological effects of conventional chemotherapy and targeted anticancer agents. *Cancer Cell*. 2015;28(6):690-714. doi:10.1016/j.ccell.2015.10.012
11. Zhang M, Pan X, Fujiwara K, et al. Pancreatic cancer cells render tumor-associated macrophages metabolically reprogrammed by a GARP and DNA methylation-mediated mechanism. *Signal Transduct Target Ther*. 2021;6(1):366. doi:10.1038/s41392-021-00769-z
12. Henriksen A, Dyhl-Polk A, Chen I, Nielsen D. Checkpoint inhibitors in pancreatic cancer. *Cancer Treat Rev*. 2019;78:17-30. doi:10.1016/j.ctrv.2019.06.005
13. Fu T, Dai LJ, Wu SY, et al. Spatial architecture of the immune microenvironment orchestrates tumor immunity and therapeutic response. *J Hematol Oncol*. 2021;14(1):98. doi:10.1186/s13045-021-01103-4

14. Bapat AA, Hostetter G, Hoff DD, Han H. Perineural invasion and associated pain in pancreatic cancer. *Nat Rev Cancer*. 2011;11(20):695-707. doi:10.1038/nrc3131
15. Yang MW, Tao LY, Jiang YS, et al. Perineural invasion reprograms the immune microenvironment through cholinergic signaling in pancreatic ductal adenocarcinoma. *Cancer Res*. 2020;80(10):1991-2003. doi:10.1158/0008-5472.CAN-19-2689
16. Jiang SH, Li J, Dong FY, et al. Increased serotonin signaling contributes to the Warburg effect in pancreatic tumor cells under metabolic stress and promotes growth of pancreatic tumors in mice. *Gastroenterology*. 2017;153(1):277-291.e219. doi:10.1053/j.gastro.2017.03.008
17. Zhang B, Vogelzang A, Miyajima M, et al. B cell-derived GABA elicits IL-10(+) macrophages to limit anti-tumour immunity. *Nature*. 2021;599(7885):471-476. doi:10.1038/s41586-021-04082-1
18. Przygodzka P, Soboska K, Sochacka E, Boncela J. Neuromedin U: a small peptide in the big world of cancer. *Cancer*. 2019;11:1312. doi:10.3390/cancers11091312
19. Martinez VG, O'Driscoll L. Neuromedin U: a multifunctional neuropeptide with pleiotropic roles. *Clin Chem*. 2015;61(3):471-482. doi:10.1373/clinchem.2014.231753
20. Teranishi H, Hanada R. Neuromedin U, a key molecule in metabolic disorders. *Int J Mol Sci*. 2021;22(8):4238. doi:10.3390/ijms22084238
21. Li Q, Han L, Ruan S, et al. The prognostic value of neuromedin U in patients with hepatocellular carcinoma. *BMC Cancer*. 2020;20(1):95. doi:10.1186/s12885-020-6532-1
22. Yang D, Zhang X, Li Z, Xu F, Tang C, Chen H. Neuromedin U and neurotensin may promote the development of the tumour microenvironment in neuroblastoma. *PeerJ*. 2021;9:e11512. doi:10.7717/peerj.11512
23. Tang Y, Hu C. NMU is a poor prognostic biomarker in patients with lung adenocarcinoma. *Dis Markers*. 2021;2021:5031479. doi:10.1155/2021/5031479
24. Martinez VG, O'Neill S, Salimu J, et al. Resistance to HER2-targeted anti-cancer drugs is associated with immune evasion in cancer cells and their derived extracellular vesicles. *Onco Targets Ther*. 2017;6(12):e1362530. doi:10.1080/2162402X.2017.1362530
25. Tomayko M, Reynolds C. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol*. 1989;24(3):148-154.
26. Liu W, Wang S, Wang J, et al. Neuromedin U induces pulmonary ILC2 activation via the NMUR1 pathway during acute respiratory syncytial virus infection. *Am J Respir Cell Mol Biol*. 2023;68(3):256-266. doi:10.1165/rcmb.2022-0123OC
27. Reina-Campos M, Scharping NE, Goldrath AW. CD8+ T cell metabolism in infection and cancer. *Nat Rev Immunol*. 2021;21(11):718-738. doi:10.1038/s41577-021-00537-8
28. Martinez VG, Crown J, Porter RK, O'Driscoll L. Neuromedin U alters bioenergetics and expands the cancer stem cell phenotype in HER2-positive breast cancer. *Int J Cancer*. 2017;140(12):2771-2784. doi:10.1002/ijc.30705
29. Chen L, Huang L, Gu Y, Cang W, Sun P, Xiang Y. Lactate-Lactylation hands between metabolic reprogramming and immunosuppression. *Int J Mol Sci*. 2022;23(19):11943. doi:10.3390/ijms231911943
30. Wang Y, Yang G, You L, et al. Role of the microbiome in occurrence, development and treatment of pancreatic cancer. *Mol Cancer*. 2019;18(1):173. doi:10.1186/s12943-019-1103-2
31. Li S, Xu HX, Wu CT, et al. Angiogenesis in pancreatic cancer: current research status and clinical implications. *Angiogenesis*. 2019;22(1):15-36. doi:10.1007/s10456-018-9645-2
32. Cai J, Chen H, Lu M, et al. Advances in the epidemiology of pancreatic cancer: trends, risk factors, screening, and prognosis. *Cancer Lett*. 2021;520:1-11. doi:10.1016/j.canlet.2021.06.027
33. Malendowicz LK, Rucinski M. Neuromedins NMU and NMS: an updated overview of their functions. *Front Endocrinol (Lausanne)*. 2021;12:713961. doi:10.3389/fendo.2021.713961
34. Yoo W, Lee J, Jun E, et al. The YAP1-NMU Axis is associated with pancreatic cancer progression and poor outcome: identification of a novel diagnostic biomarker and therapeutic target. *Cancer*. 2019;11(10):1477. doi:10.3390/cancers11101477
35. Lee J, Snyder ER, Liu Y, et al. Reconstituting development of pancreatic intraepithelial neoplasia from primary human pancreas duct cells. *Nat Commun*. 2017;8:14686. doi:10.1038/ncomms14686
36. Cao L, Wu J, Qu X, et al. Glycometabolic rearrangements—aerobic glycolysis in pancreatic cancer: causes, characteristics and clinical applications. *J Exp Clin Cancer Res*. 2020;39(1):267. doi:10.1186/s13046-020-01765-x
37. Zhang Z, Zhang H. Glycometabolic rearrangements-aerobic glycolysis in pancreatic ductal adenocarcinoma (PDAC): roles, regulatory networks, and therapeutic potential. *Expert Opin Ther Targets*. 2021;25(12):1077-1093. doi:10.1080/14728222.2021.2015321
38. Colegio OR, Chu NQ, Szabo AL, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 2014;513(7519):559-563. doi:10.1038/nature13490
39. Wang JX, Choi SYC, Niu X, et al. Lactic acid and an acidic tumor microenvironment suppress anticancer immunity. *Int J Mol Sci*. 2020;21(21):8363. doi:10.3390/ijms21218363
40. Wang JZ, Zhu W, Jea H. The role of the HIF-1alpha/ALYREF/PKM2 axis in glycolysis and tumorigenesis of bladder cancer. *Cancer Commun (Lond)*. 2021;41(7):560-575. doi:10.1002/cac2.12158
41. Husain Z, Huang Y, Seth P, Sukhatme VP. Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *J Immunol*. 2013;191(3):1486-1495. doi:10.4049/jimmunol.1202702
42. Brand A, Singer K, Koehl GE, et al. LDHA-associated lactic acid production blunts tumor Immunosurveillance by T and NK cells. *Cell Metab*. 2016;24(5):657-671. doi:10.1016/j.cmet.2016.08.011
43. Certo M, Tsai CH, Pucino V, Ho PC, Mauro C. Lactate modulation of immune responses in inflammatory versus tumour microenvironments. *Nat Rev Immunol*. 2021;21(3):151-161. doi:10.1038/s41577-020-0406-2
44. Mann KM, Ying H, Juan J, Jenkins NA, Copeland NG. KRAS-related proteins in pancreatic cancer. *Pharmacol Ther*. 2016;168:29-42. doi:10.1016/j.pharmthera.2016.09.003
45. Stanciu S, Ionita-Radu F, Stefani C, et al. Targeting PI3K/AKT/mTOR signaling pathway in pancreatic cancer: from molecular to clinical aspects. *Int J Mol Sci*. 2022;23(17):10132. doi:10.3390/ijms231710132
46. Yang J, Ren B, Yang G, et al. The enhancement of glycolysis regulates pancreatic cancer metastasis. *Cell Mol Life Sci*. 2020;77(2):305-321. doi:10.1007/s00018-019-03278-z
47. Hoxhaj G, Manning BD. The PI3K-AKT network at the interface of oncogenic signalling and cancer metabolism. *Nat Rev Cancer*. 2020;20(2):74-88. doi:10.1038/s41568-019-0216-7

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Zheng R, Wang S, Wang J, Zhou M, Shi Q, Liu B. Neuromedin U regulates the anti-tumor activity of CD8$^+$ T cells and glycolysis of tumor cells in the tumor microenvironment of pancreatic ductal adenocarcinoma in an NMUR1-dependent manner. *Cancer Sci*. 2024;115:334-346. doi:10.1111/cas.16024