IL-36β promotes anti-tumor effects in CD8$^+$ T cells by downregulating micro-RNA let-7c-5p

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Background: The anti-tumor effect of interleukin (IL)-36β-mediated activation of CD8$^+$ T cells has been reported, but the molecular mechanism is largely undefined.

Methods: The levels of IL-36β in pancreatic cancer were examined by quantitative real-time PCR (qRT-PCR) and immunohistochemical staining. Cytology and animal experiments were performed to study the effects of IL-36β on the growth of pancreatic cancer cells. We then examined the changes of CD8$^+$ T cells and natural killer (NK) cells in the tumor by flow cytometry. The microRNA expression profiles were determined by microarray analysis.

Results: The results revealed decreased levels of IL-36β in pancreatic cancer tissues. In addition, IL-36β inhibited tumor growth and promoted CD8$^+$ T and NK cell proliferation in the tumor microenvironment (TME). Moreover, IL-36β stimulated CD8$^+$ T cells to synthesize high amounts of interferon-gamma (IFN-γ) and IL-2. Microarray analysis showed that IL-36β administration to human and mouse CD8$^+$ T cells consistently downregulated the miRNA, let-7c-5p. Downregulation of let-7c-5p resulted in IFN-γ and IL-2 upregulation in CD8$^+$ T cells, whereas its upregulation had the opposite effect. Further experiments demonstrated that IL-36β downregulated IFN-γ in let-7c-5p$^+$ CD8$^+$ T cells.

Conclusions: These findings suggest IL-36β promotes IFN-γ and IL-2 production in CD8$^+$ T cells, as well as anti-tumor effects in CD8$^+$ T cells by downregulating let-7c-5p.

Keywords: Interleukin-36β (IL-36β); CD8$^+$ T cells; let-7c-5p

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Introduction

There are many bottlenecks in tumor immunotherapy, including tumor immune escape in the tumor microenvironment (TME) (1,2). The main manifestation of immune escape is the weakening and blunting of the proliferative capacity of T lymphocytes. Tumor cells cannot be cleared in time, and the secretion of cytokines is reduced; consequently, immune effector cells cannot be activated (3). Studies have shown that high levels of Th1 and $CD8^+$ T cells in the tumor significantly improve the prognosis of patients (4). This suggests reversing immune suppression in the TME is critical for successful immunotherapy in cancer. Some cytokines can reverse the inhibitory TME, so that disabled helper T cells (especially Th1 cells), cytotoxic T cells ($CD8^+$ T cells), etc. regain anti-tumor functions and exert anti-tumor effects (5).

The immune microenvironment of pancreatic cancer is composed of tumor cells, pancreatic astrocytes, immune cells, and extracellular matrix, and expresses a variety of tumor antigens, such as mucin 1 (MUC1), mesothelin (MSLN), CEA, etc. In different pancreatic cancer tissues, tumor progression is divided into three stages: clearance, balance, and escape. The elimination phase is mainly anti-tumor immunity, the escape phase is mainly immunosuppression, and the equilibrium phase has both. Immune cells infiltrated in the microenvironment of pancreatic cancer are characterized by immunosuppression. Pancreatic cancer cells can edit the immune system so that it cannot be recognized by the immune system. At the same time, it can recruit and activate various immunosuppressive cells such as pancreatic stellate cells, myeloid-derived suppressor cells, tumor-associated macrophages and regulatory T cells, etc., then secrete immunosuppressive molecules to inhibit the host's anti-tumor immune response, cause tumor immune escape, and promote the progression of pancreatic cancer. Hamanaka *et al.* (6) found that pancreatic cancer patients with positive serum MUC1-IgG had a better prognosis, and the level of serum MUC1-IgG was related to the survival of patients. With the development of technologies such as next-generation sequencing and single-cell sequencing, it is helpful to the study of the heterogeneity of malignant tumors and the screening of immune-related biomarkers of pancreatic cancer.

Interleukin (IL)-36 represents a group of pro-inflammatory cytokines, including IL-36α, IL-36β, and IL-36γ. IL-36's receptor is IL-36R, formerly termed IL-1 receptor-associated protein 2 (IL-1Rpr2) (7). IL-36R expression was detected on $CD4^+$ T, $CD8^+$ T, natural killer (NK), and γδT cells (8). IL-36 regulates dendritic cell (DC) and $CD4^+$ T cell functions, and promotes INF-γ and IL-2 secretion (9,10). B16 and 4T1 cells with high IL-36γ expression can significantly inhibit tumor cell proliferation and lung metastasis, promote interferon-gamma (IFN-γ) secretion, and increase $CD8^+$ T, NK, and γδT cell infiltration into tumors (11). Our previous research found that IL-36β promotes $CD8^+$ T cell activation by inducing mammalian target of rapamycin complex 1 (mTORC1) via PI3K/Akt (12). Therefore, we hypothesized that IL-36β can also stimulate Th1-type immune responses to play an anti-tumor role.

MicroRNAs (miRNAs, i.e., non-coding single-stranded RNAs averaging 22 nt) mostly regulate genes via binding to the 3'UTRs of the mRNA targets, promoting mRNA degradation or suppressing translation (13). Abnormal miRNA expression is implicated in multiple pathologies, including numerous human malignancies, including lung, gastric, and breast cancers (14-16). Park *et al.* (17) reported that microRNA-449a knockdown results in protective effects, downregulating catabolic genes and restoring the expression of anabolic genes, via SIRT1 in IL-1β-associated cartilage degeneration. MiRNAs are also found in immune cells, and may have critical functions in various immune responses. Zitzer *et al.* (18) reported that miR-155 expression is essential in the filtration of donor T cells into multiple target organs. Adoro *et al.* (19) reported that IL-21 directly inhibits human immunodeficiency virus (HIV)-1, and identified microRNA-29 as an antiviral molecule that is upregulated by IL-21 in helper T cells. However, the changes of miRNAs caused by IL-36β in $CD8^+$ T cells have not been reported.

The present study assessed IL-36β at the protein and gene levels in pancreatic cancer and adjacent noncancerous tissue samples by immunohistochemistry and quantitative real-time polymerase chain reaction (PCR), respectively. Next, IL-36β's anticancer activity was examined by establishing a mouse tumor model. The changes of cell populations in the TME were assessed by flow cytometry. In mechanistic studies, microarray assays showed that the miRNA let-7c-5p was consistently downregulated after induction by IL-36β of human and mouse $CD8^+$ T cells. Furthermore, IL-36β was found to regulate the biological activity of $CD8^+$ T cells by downregulating let-7c-5p, which is the innovation of this study. We present the following article in accordance with the ARRIVE reporting checklist

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Methods

Specimens

The pancreatic cancer tissue samples were derived from cases confirmed by pathological findings that were surgically treated in the General Surgery Department of the First Affiliated Hospital, Soochow University. These patients received no adjuvant treatment preoperatively. The tumors were extracted, and paired adjacent noncancerous tissue samples from these cases were obtained simultaneously. The samples were stored at –80 ℃ for qRT-PCR detection, or underwent fixation with 10% formalin and paraffin embedding for immunohistochemistry.

Spleen surgical samples were obtained from three individuals who received splenectomy without spleen diseases in the above-mentioned hospital. C57BL/6 mice provided by the Jackson Laboratory (Shanghai) underwent housing in the specific pathogen-free facility of Soochow University. Fresh spleens from mice and humans were immediately treated, and purified $CD8^+$ T cells were obtained using magnetic bead-based methods (Miltenyi Biotec, Auburn, CA, USA). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of The First Affiliated Hospital of Soochow University (approval No. 2015683). Informed consent was taken from all the patients.

Cell culture

Panc02 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS) When cells reached approximately 30% confluency, appropriate amounts of IL-36β lentivirus (GeneChem Co., Shanghai) were used to transfect them according to a multiplicity of infection (MOI) of 10. Transfection of an empty vector was performed in the negative control group. After 24 hours of transfection, cell lines were selected using the medium containing 10 μg/mL puromycin.

Animals and tumor model establishment

BALB/c mice, provided by the Zhao Yan (Suzhou) New Drug Research Center, were housed in a pathogen-free facility at the Jiangsu Institute of Clinical Immunology, Soochow University. Animal experiments were performed under a project license (No. 2016597) granted by institutional ethics committee of Soochow University and in compliance with the guidelines of Institutional Animal Care and Use Committee of Soochow University (http://dwzx.suda.edu.cn/). Mouse pancreatic cancer cells were administered subcutaneously into BALB/c mice, and tumor sizes were examined every other day. The tumor diameter was derived according to the formula D = (L + W)/2 (mm), where L and W are length and width, respectively, to plot the tumor growth curve.

Tumor vaccination

Panc02 cells were administered by subcutaneous injection into BALB/c mice. On the 7^{th} day, IL-36β adenovirus was injected in mouse tumors to observe its effect, monitoring the animals for tumor growth every 2 days.

Tumor-infiltrating lymphocyte (TIL) assessment

Each tumor was cut into 3–4 mm pieces and rinsed with Hank's balanced salt solution (HBSS). Next, the pieces were digested with collagenase IV at 37 ℃ for 2 h and passed through a 40-mm cell strainer. TILs underwent further purification by Ficoll-Paque gradient centrifugation according to the manufacturer's instructions, followed by washing and resuspension in HBSS with 1% FCS. Finally, flow cytometry was performed to detect cell populations on a FACS flow cytometer (BD Biosciences, San Diego, CA, USA).

Overexpression and interference lentiviral vectors

A pair of oligos (NP7179 and NP7180) were designed for gene synthesis according to the mmu-let-7c-5p (MIMAT0000523) mature body sequence (UGAGGUAGGUUGUAUGGUU) (*Table 1*). After annealing into double-stranded DNA, the lentiviral backbone vector PDS134_pL_shRNA_mKate2 (enzyme cutting site, BsmBI) was generated. The mmu-let-7c-5p overexpression lentiviral vector was constructed and confirmed by sequencing, and the constructed vector was named let-7c-$5p^+$. The interference lentiviral vector was constructed by the same method, and named let-7c-$5p^-$. The oligo sequences are shown in *Table 1*.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol according

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Table 1 Oligo sequences for overexpression and interference

Name	Sequence, 5'-3'
Overexpression	
Mmu-let-7c-5p-F	CACCGTGAGGTAGTAGGTTGTATGGTTCGAAAACCATACAACCTACTACCTCA
Mmu-let-7c-5p-R	AAAATGAGGTAGTAGGTTGTATGGTTTTCGAACCATACAACCTACTACCTCAC
Interference	
Mmu-let-7c-5p-TUD-F	CAACAACCATACAACCGATCTACTACCTCA
Mmu-let-7c-5p-TUD-R	CTTGTGAGGTAGTAGATCGGTTGTATGGTT

Table 2 Primers for qRT-PCR

Primer name	Sequence, 5'-3'
Q-mmu-let-7c-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACCATAC
Q-mmu-let-7c-5p-F	ACACTCCAGCTGGGTGAGGTAGTAGGTTGT
Q-mmu-let-7c-5p-R	TGGTGTCGTGGAGTCG
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA

qRT-PCR, quantitative real-time PCR.

to the manufacturer's instructions (ambion, USA). RNA quantitation was performed on a NanoDrop 2000 (Thermo Scientific, USA). Reverse transcription was carried out from 1.0 μg RNA with oligo-dT primers and avian myeloblastosis virus reverse transcriptase. Next, qRT-PCR was performed with SYBR Green Real-time PCR Master Mix (Invitrogen, USA) on a real-time PCR machine (ABI ViiA7 Sequence Detection System, Life Technologies, USA) to examine let-7c-5p, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. The primers are listed in *Table 2*. Amplification was carried out at 95 ℃ (15 s), followed by 45 cycles of 95 ℃ (5 s) and 60 ℃ (30 s). The $2^{-\Delta\Delta Ct}$ method was utilized for data analysis in triplicate assays.

Microarray analysis

Naive $CD8^+$ T cells, obtained from spleens of human and mice, were cultured with medium alone or medium containing IL-36β (100 ng/mL) for 48 h. The above two groups of cells were harvested separately, and their microRNA expression profiles were determined by microarray analysis (Agilent, USA). MicroRNAs with expression fold change ≥2 and $P<0.05$ were selected. A heat map was generated using R software with the pheatmap language package (https://www.bioconductor.org/).

Immunoblotting

Cell lysis was performed on ice. The bicinchoninic acid (BCA) method was used for protein quantitation. Equal amounts of total protein underwent separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by electro-transfer onto polyvinylidene difluoride (PVDF) membranes. After blocking, primary antibodies were added for overnight incubation at 4 ℃. This was followed by washing steps and incubation with secondary antibodies at room temperature for 2 h. An enhanced chemiluminescence (ECL) assay kit (Epizyme Biomedical Technology Co., Ltd., Shanghai) was used for development, and protein band intensities were semi-quantitatively assessed with Image J (NIH, USA). Triplicate assays were performed.

Immunohistochemistry

Paraffin-embedded clinical tissue samples sectioned at 4-μm underwent incubation with rabbit anti-human IL-36β polyclonal antibodies (1:400) overnight at 4 ℃. IL-36β signals appeared as brown staining. The samples were

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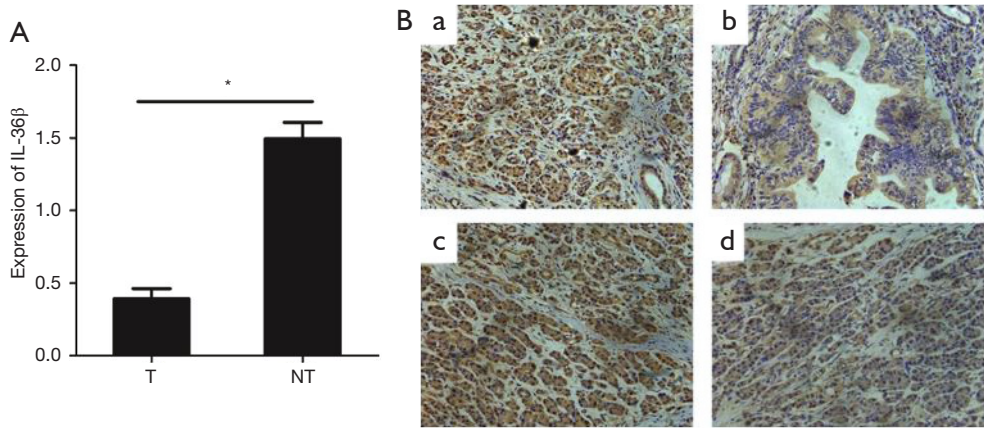

Figure 1 IL-36β in cancer tissues is downregulated. (A) IL-36β mRNA levels in cancer tissue (T) samples were 0.392±0.070, while the levels in paracancerous tissue specimens (NT) were 1.493±0.113, and the difference was statistically significant (P<0.05). (B) Immunohistochemistry was performed for IL-36β protein detection in tissue samples (magnification, 200×). Positive signals in pancreatic cancer tissue samples (a). No expression in pancreatic cancer tissue specimens (b). Positive signals in paracancerous tissue specimens (c). No expression in paracancerous tissue samples (d). *, P<0.05. IL, interleukin.

Table 3 Positive expression rates for the IL-36β protein in cancerous and paracancerous tissue samples

Tissue	Cases	Positive	Negative	χ^2	P
Cancer tissue	40	16	24	10.026	<0.05
Paracancerous tissue	40	30	10		

IL, interleukin.

scored based on cytoplasmic staining (0, 1, and 2 for no, weak, and strong signals, respectively) and positive area percentage (0, 1, and 2 for <10%, 10–50%, and >50%, respectively). The total score was the product of both subscores, and a value >1 was considered to indicate positive staining.

Enzyme-linked immunosorbent assays (ELISA)

Naive $CD8^+$ T cells were initially administered anti-CD3 mAb or anti-CD28 mAb (2.5/1.25 μg/mL). Subsequently, IFN-γ and IL-2 levels were assessed using specific human and mouse ELISA kits (ExCell Bio, China) according to the manufacturer's instructions.

Statistical analysis

The Student's *t*-test, the Mann-Whitney test, or one-way analysis of variance (ANOVA) were carried out for data analysis as indicated. Two-sided unpaired P<0.05 indicated statistical significance.

Results

IL-36β is downregulated in cancer tissues

IL-36β amounts in 40 pairs of pancreatic cancer and paracancerous tissue specimens were examined by qRT-PCR and immunohistochemical staining. As shown in *Figure 1A*, IL-36β mRNA levels in paracancerous tissue specimens were 3.8 times those of cancer tissue samples. Immunohistochemical staining revealed that IL-36β was mainly localized in the cytoplasm of pancreatic cancer cells (*Figure 1B*). The positive expression rates for the IL-36β protein were 40% and 75% in cancer and paracancerous tissue specimens, respectively, indicating a statistically significant difference (χ^2=10.026, P<0.05; *Table 3*). This suggested that IL-36β may be critical in the development of pancreatic cancer.

High IL-36β expression inhibits tumor growth

To further clarify IL-36β's function in the TME, we stably transfected Panc02 cells with IL-36β overexpression

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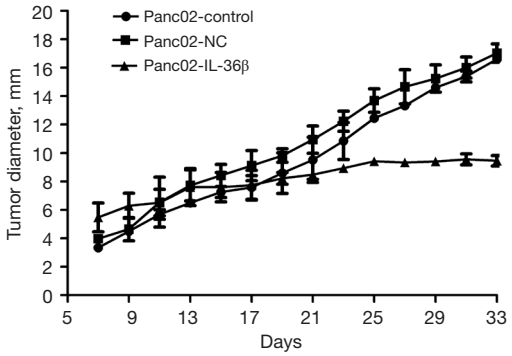

Figure 2 IL-36β overexpression in tumor cells inhibits tumor growth *in vivo*. In total, 1×10^6 Panc02-Control, Panc02-NC, and Panc02-IL-36β cells were injected subcutaneously into C57BL/6 mice, and the tumor sizes were monitored every other day. Data (mean ± SEM) are from three independent experiments (n=5/group). Until the end of the study, the tumor diameter was markedly reduced in the Panc02-IL-36β group (9.45±0.36 mm) compared with the Panc02-Control (16.59±0.08 mm) and Panc02-NC (17.01±0.65 mm) groups (P<0.05). IL, interleukin; NC, negative control; SEM, standard error of mean.

lentivirus and performed *in vivo* experiments in mice to assess IL-36β's effects on tumor growth after overexpression in the TME. Panc02 pancreatic cancer cells were subcutaneously transplanted into the left abdomen of mice; after 3 days, soybean sized-nodules were detected. There were differences in growth rate among the three transplanted tumor groups from the beginning of tumorigenesis. The IL-36β group initially grew rapidly, which may be due to inflammatory reactions caused by the proinflammatory cytokine IL-36β, resulting in tissue edema and increased tumor size. However, with prolonged observation time, the tumor size in the Panc02-IL-36β group began to lag behind those of the Panc02-negative control (NC) and Panc02-Control groups. Until the end of the study, the tumor diameter was markedly reduced in the Panc02-IL-36β group compared with the other two groups (*Figure 2*). The above findings indicated that IL-36β overexpression inhibited tumor growth in the mouse subcutaneous xenograft model.

IL-36β overexpression elevates the levels of CD8⁺ T and NK cells

To explore the basic mechanism by which IL-36β exerts anticancer effects, we examined the changes of TILs in the tumor by flow cytometry. First, the percentage of $CD45^+$ cells in tumor single cell suspensions was markedly elevated in the Panc02-IL-36β group compared with the blank and negative control groups (*Figure 3A*), suggesting that IL-36β increased the inflammatory response in the tumor. It is known that type 1 lymphocytes, such as $CD8^+$ T and NK cells, have the ability to inhibit tumor cells in tumor immunity. Therefore, we quantified various types of $CD45^+$ TILs, $CD8^+$ T cells, and NK cells. The results revealed that the levels of $CD8^+$ T cell were higher in the Panc02-IL-36β group compared to the other two control groups. Meanwhile, there was no significant difference in the NK cell content between the IL-36β group and both control groups. However, due to the increase of $CD45^+$ TILs in the tumor, the total NK cell levels were also elevated (*Figure 3B*,*3C*). These findings suggested that overexpression of IL-36β increases $CD45^+$ TILs as well as anti-tumor effector cells, including $CD8^+$ T and NK cells.

Adenovirus with IL-36β overexpression reduces tumor growth after intratumoral injection

The previous experiments basically demonstrated that high expression of IL-36β in the TME can promote $CD8^+$ T and NK cell aggregation to exert anti-tumor effects and inhibit tumor growth. We hypothesized that tumor immunogenicity can be increased by intratumoral injection of IL-36β adenovirus, recruiting immune cells to inhibit tumor growth. Mice were subcutaneously transplanted with wild-type Panc02 cells and randomized into three groups of five. Tumor growth was comparable in all groups in the first week. On the 7^{th} day, the three groups of mice were injected separately with IL-36β overexpression adenovirus, adenovirus empty vector, and phosphate-buffered saline (PBS). Afterwards, the tumor growth rates in different groups varied. Until the end of the study, the tumor size was markedly reduced in mice injected with IL-36β adenovirus compared with the control (PBS and adenovirus empty vector) groups (*Figure 4*).

Adenovirus with IL-36β overexpression causes CD8⁺ T, NK, and γδT cell aggregation

To further analyze the effect of intratumoral injection of adenovirus expressing IL-36β on the TME, the changes of TILs were assessed by flow cytometry. The results showed that the percentage of $CD45^+$ TILs was markedly elevated in the IL-36β adenovirus group compared with the negative and blank control groups (*Figure 5A*). In addition, the IL-

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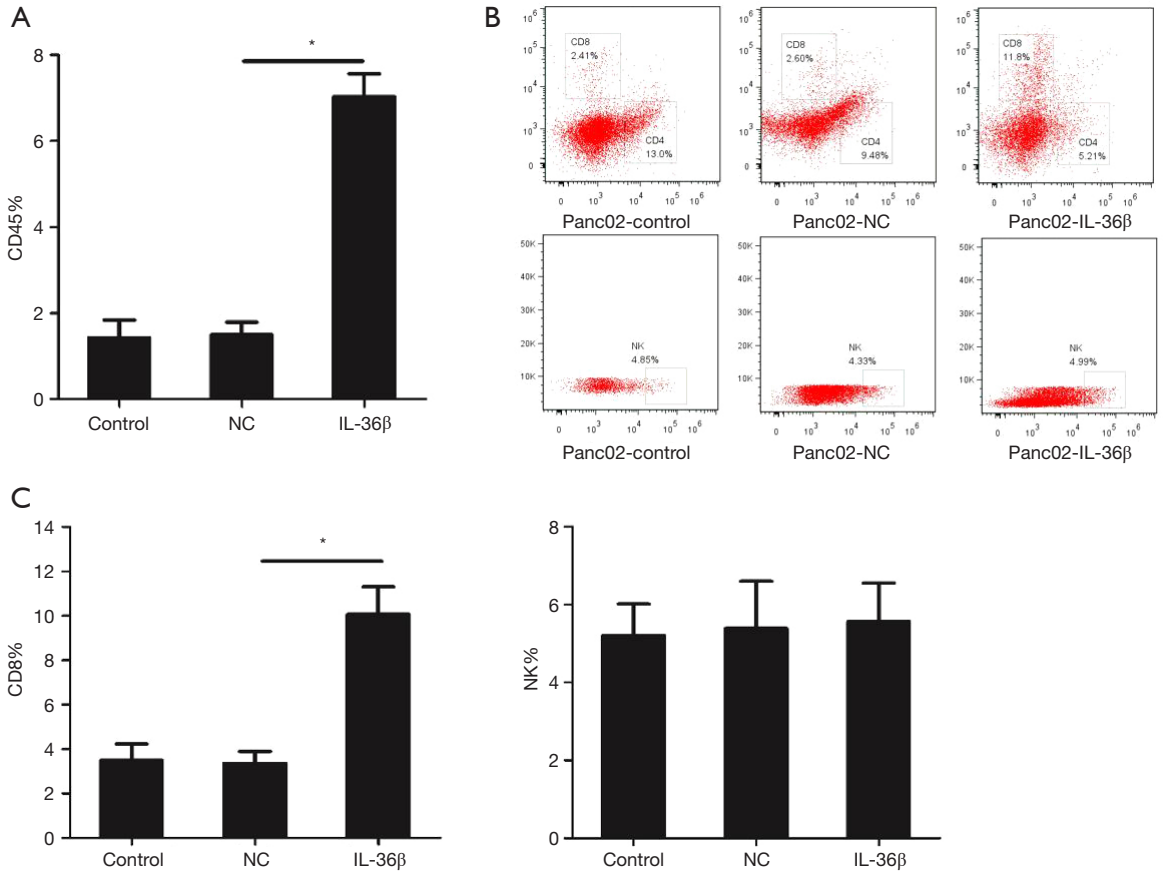

Figure 3 Tumoral IL-36β overexpression enhances type 1 immune responses in the tumor microenvironment. On day 33, tumor samples were obtained to generate single-cell suspensions. (A) Percentages of $CD45^+$ cells in tumor cell suspensions. (B,C) Representative flow-cytograms and $CD8^+$ T or NK cell rates within tumor $CD45^+$ cells. *, P<0.05. IL, interleukin; NK, natural killer.

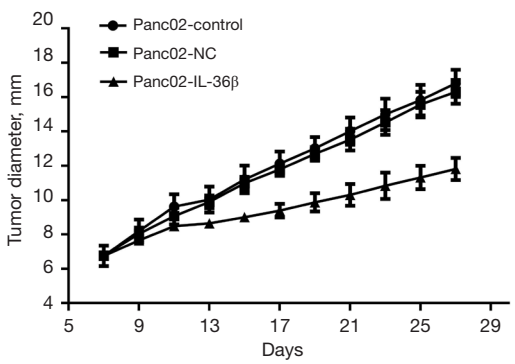

Figure 4 Adenovirus with IL-36β overexpression inhibits tumor growth after intratumoral injection. In total, 1×10^6 Panc02 cells were administered by subcutaneous injection into C57BL/6 mice. On the 7^{th} day, the animals were randomized into three groups and injected separately with 1×10^9 PFU IL-36β overexpression adenovirus, 1×10^9 PFU adenovirus empty vector, and 100 μL PBS. Tumor sizes were monitored every other day. Data (mean ± SEM) are from three independent experiments (n=5/group). Until the end of the study, the tumor diameter was markedly reduced in mice injected with IL-36β adenovirus (11.82±1.28 mm) compared with the control PBS (16.8±1.57 mm) and adenovirus empty vector (16.31±1.40 mm) groups (P<0.05). IL, interleukin; PFU, plaque-forming units; PBS, phosphate-buffered saline; SEM, standard error of mean.

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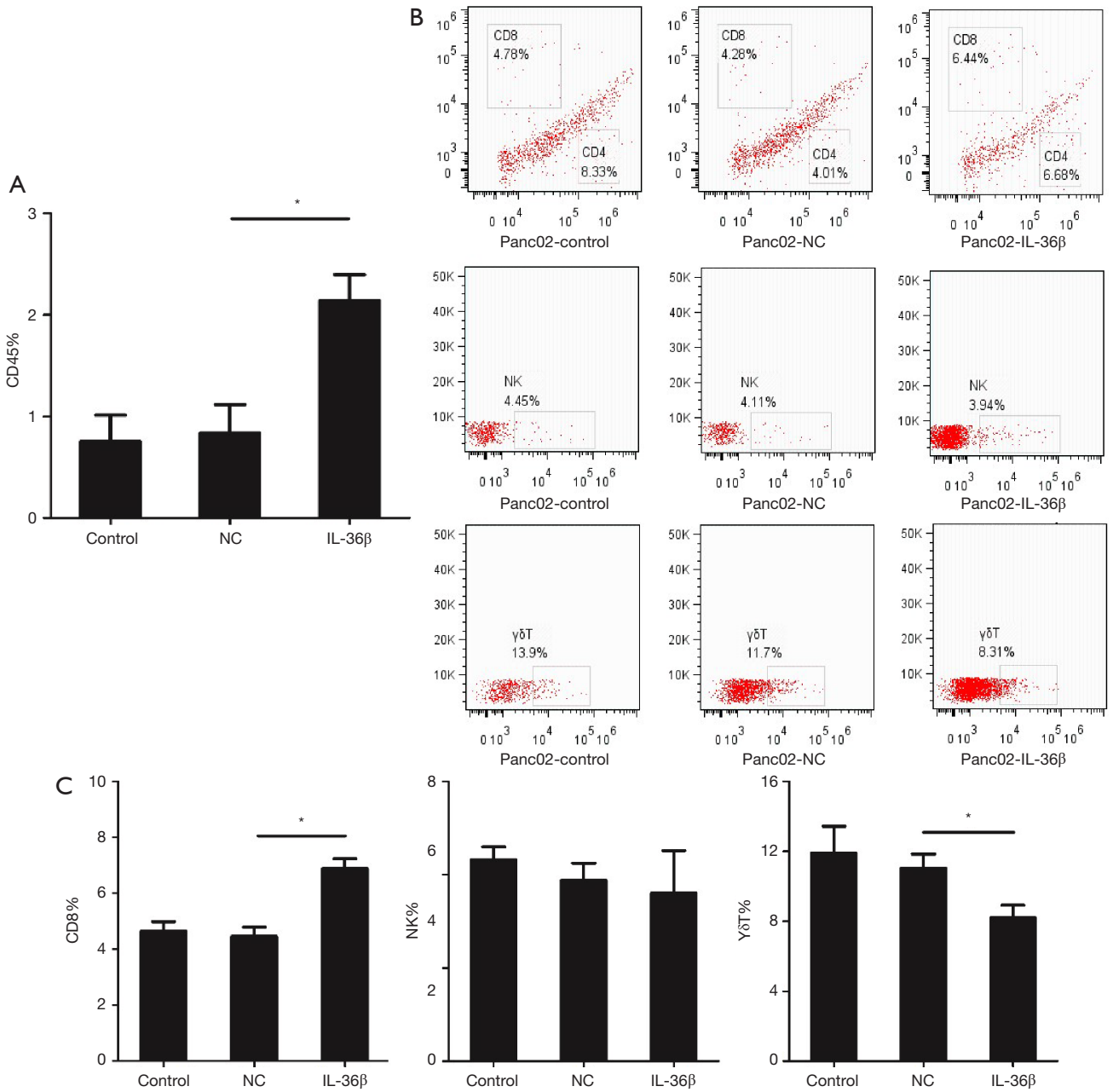

Figure 5 Adenovirus with IL-36β overexpression induces $CD8^+$T, NK, and γδT cell aggregation. On day 27, tumor resection was performed to generate single-cell suspensions. (A) Percentages of $CD45^+$ cells in tumor cell suspensions. (B,C) Representative flow-cytograms, and $CD8^+$ T, $NK1.1^+$, or γδT cell rates among tumor $CD45^+$ cells. *, P<0.05. IL, interleukin; NK, natural killer.

36β adenovirus group showed increased $CD8^+$ T cell content compared with the two control groups. NK cell contents in the IL-36β and both control groups were comparable, but the total NK cell number was increased in the IL-36β adenovirus group due to increased $CD45^+$ TILs in the tumor. The IL-36β group had reduced γδT cell content compared with both control groups, but the total γδT cells were also increased in the tumor, given that the $CD45^+$ TILs in the IL-36β group were increased by approximately two-fold (*Figure 5B,5C*). Together, the above findings suggested that adenovirus expressing IL-36β can be used as an immune enhancer to induce the accumulation of $CD8^+$ T, NK, and γδT cells in the

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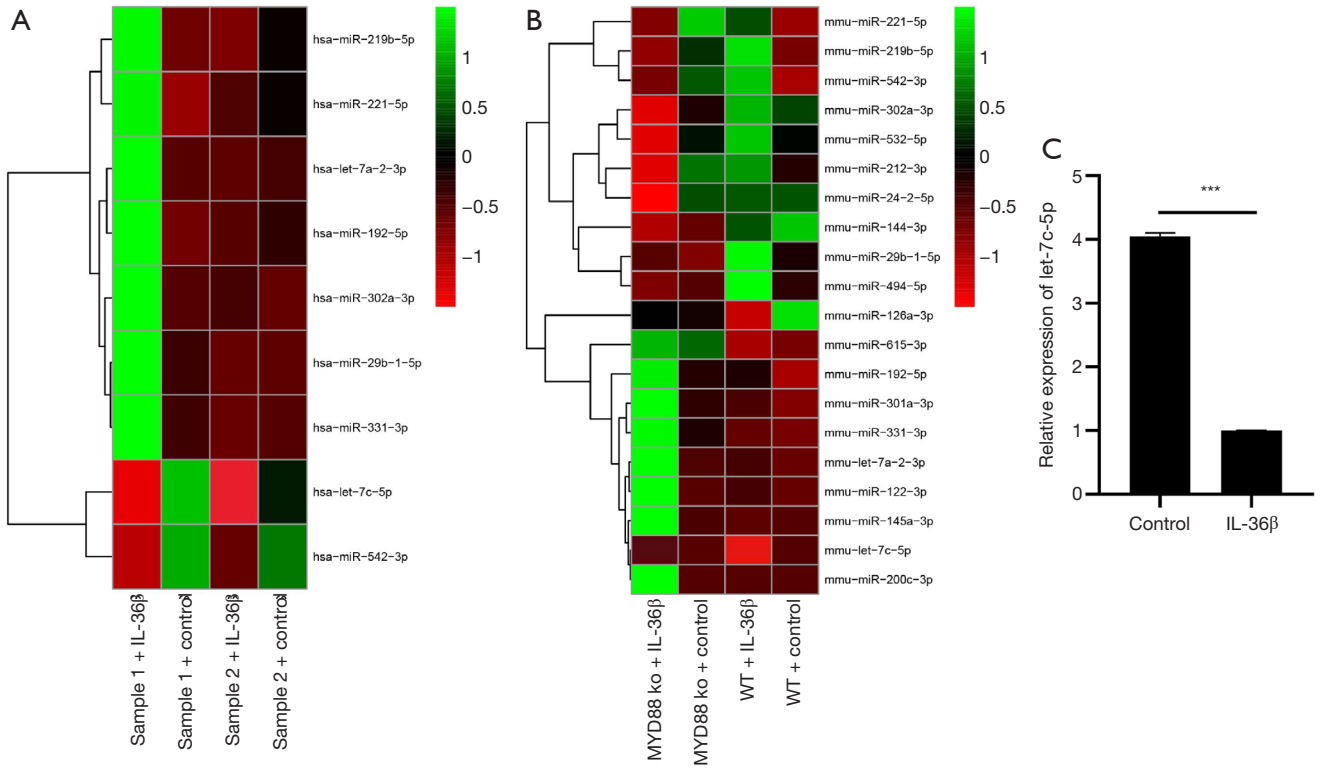

Figure 6 Heat map of 21 miRNAs generated with the R software (pheatmap package). (A) Human; (B) mouse. Myeloid Differentiation Factor 88 (MYD88, a critical upstream effector of IKK/NF-κB signaling). (C) The results of qRT-PCR indicated that let-7c-5p downregulation was the most consistent. Control group, WT CD8$^+$; IL-36β group, WT CD8$^+$/IL-36β stimulation (***, P<0.0001). KO, knockout; qRT-PCR, quantitative real-time PCR; IKK, IKappaBbeta kinase; NF-κB, nuclear factor-kappaB; IL, interleukin; WT, wild type.

cancer microenvironment and exert antitumor effects.

Reduced let-7c-5p expression after IL-36β treatment stimulates effector CD8$^+$ T cells

Naive CD8$^+$ T cells were obtained from spleens of humans and mice by magnetic bead-based assays (Miltenyi Biotec). The effector CD8$^+$ T cell culture was performed with medium alone or medium containing IL-36β (100 ng/mL) for 48 h. The above two groups of cells were harvested separately, and the microRNA expression profiles were determined by microarray analysis (*Figure 6A,6B*). Twenty-one miRNAs were changed in both humans and mice (*Table 4*). Subsequently, the changes in miRNA and mRNA expression levels were detected by qRT-PCR [including wild type (WT) CD8$^+$ and WT CD8$^+$/IL36β stimulation]. As shown in *Figure 6C*, let-7c-5p downregulation was the most consistent. Therefore, we proposed the hypothesis that IL-36β regulates the biological function of CD8$^+$ T cells by downregulating let-7c-5p.

Successful generation of let-7c-5p overexpression and let-7c-5p RNAi lentiviruses

Mmu-let-7c-5p overexpression and RNA interfere (RNAi) lentiviral vectors were successfully constructed. Thus, mmu-let-7c-5p overexpression lentivirus (let-7c-5p$^+$) and mmu-let-7c-5p RNAi lentivirus (let-7c-5p$^-$) were successfully prepared. QRT-PCR showed mmu-let-7c-5p upregulation after transfection of the overexpression lentivirus let-7c-5p$^+$ into the target cells (*Figure 7A*). The luciferase assay showed that the mmu-let-7c-5p RNAi vector successfully downregulated mmu-let-7c-5p (*Figure 7B*).

Downregulation of mmu-let-7c-5p upregulates IFN-γ and IL-2 in CD8$^+$ T cells, whereas its upregulation exerts the opposite effect

Naive CD8$^+$ T cells were firstly administered anti-CD3 mAb and anti-CD28 mAb at 2.5 and 1.25 μg/mL, respectively. Next, the above mmu-let-7c-5p constructs

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Table 4 The miRNAs changed in both mice and humans

miRNA
let-7a-2-3p
let-7c-5p
miR-122-3p
miR-126a-3p
miR-144-3p
miR-145a-3p
miR-192-5p
miR-200c-3p
miR-212-3p
miR-219b-5p
miR-221-5p
miR-24-2-5p
miR-29b-1-5p
miR-301a-3p
miR-302a-3p
miR-331-3p
miR-494-5p
miR-532-5p
miR-542-3p
miR-615-3p
miR-98-5p

(let-7c-5p$^+$ and let-7c-5p$^-$) were respectively transfected into mouse CD8$^+$ T cells; negative lentivirus transfection was performed in the control group. Subsequently, cell supernatants were collected for IFN-γ and IL-2 detection by ELISA. IKappaBbeta (IK-B) protein amounts were assessed by immunoblotting. In this study, downregulation of mmu-let-7c-5p increased the levels of IFN-γ and IL-2 in CD8$^+$ T cells, and its upregulation had the opposite effect (*Figure 8A*,*8B*). Western blotting detected no significant changes in IK-B protein levels (*Figure 8C*).

IL-36β downregulates IFN-γ in let-7c-5p$^+$ CD8$^+$ T cells

To further explore the link between IL-36β and let-7c-5p, we divided the cells into four groups, including the control, IL-36β, IL-36β + let-7c-5p$^+$, and IL-36β + NC groups. All CD8$^+$ T cells in the above four groups were administered anti-CD3 mAb (2.5 μg/mL) and anti-CD28 mAb (1.25 μg/mL). Next, IFN-γ was detected by ELISA, and the results showed IL-36β upregulated IFN-γ in CD8$^+$ T cells. Interestingly, IL-36β decreased the levels of IFN-γ in the let-7c-5p$^+$ group (*Figure 9*). Therefore, we speculated that IL-36β affected the function of let-7c-5p through a particular signaling pathway.

Discussion

As a proinflammatory cytokine, IL-36 is involved in many inflammatory reactions and plays an active role in immunity.

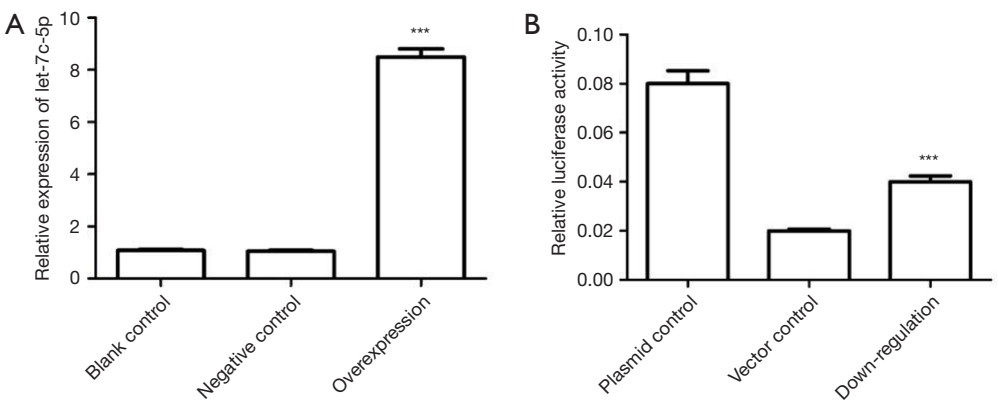

Figure 7 Successful generation of let-7c-5p overexpression and let-7c-5p RNAi lentiviruses. (A) qRT-PCR showed that mmu-let-7c-5p was upregulated after transfection of the overexpression lentivirus let-7c-5p$^+$ into the target cells. (B) CD8$^+$ T cells were co-transfected with the internal control and recombinant vectors along with miR-NC, and relative luciferase activity was assessed 48 h after transfection (***, P<0.0001). qRT-PCR, quantitative real-time PCR; NC, negative control.

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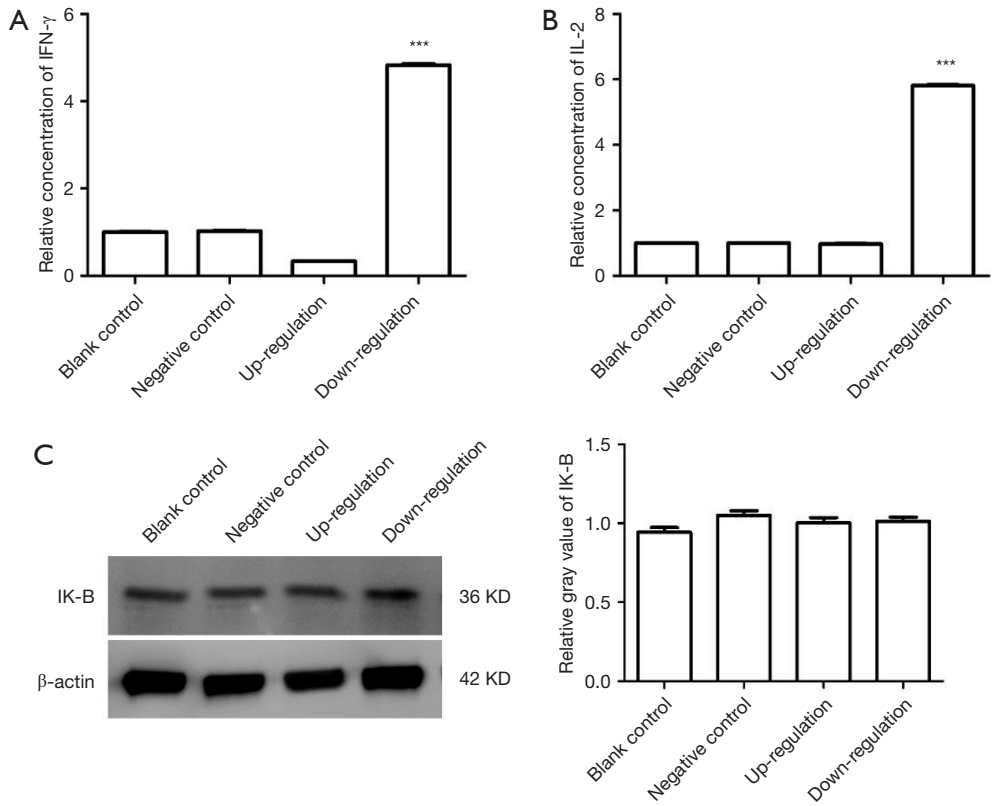

Figure 8 IFN-γ (A) and IL-2 (B) levels secreted by $CD8^+$ T cells were assessed by ELISA after downregulation and upregulation of mmu-let-7c-5p in mouse $CD8^+$ T cells, respectively. Data are mean ± SEM. ***, P<0.001 (two-tailed unpaired Student's *t*-test). (C) Western blot detected no significant changes in IK-B protein amounts. Blank control, $CD8^+$ T control; NC, $CD8^+$ T cells transfected with negative control lentivirus; Upregulation, $CD8^+$ T cells transfected with the let-7c-$5p^+$ lentivirus; Downregulation, $CD8^+$ T cells transfected with RNAi the let-7c-$5p^-$ lentivirus. IFN, interferon; IL, interleukin; SEM, standard error of mean; NC, negative control.

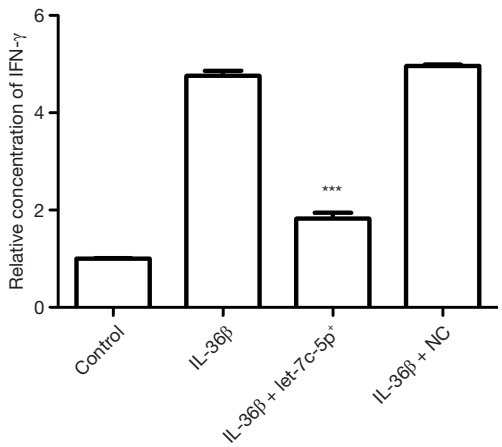

Figure 9 IL-36β downregulates IFN-γ in let-7c-$5p^+$ $CD8^+$ T cells. Data are mean ± SEM. ***, P<0.001 (two-tailed unpaired Student's *t*-test). IL, interleukin; IFN, interferon; SEM, standard error of mean.

Therefore, excessive IL-36 levels often means that the body has a certain degree of damage (20). Previous reports on IL-36 have mostly assessed its involvement in various chronic inflammatory and immuno-pathological processes mediated by Th2 cells, including psoriasis, rheumatoid arthritis, inflammatory colorectal diseases, etc. (21-23). Recent evidence suggests that IL-36 also promotes $CD4^+$ T cell-dependent type 1 immune reactions. Binding to the mouse bone marrow-derived dendritic cell (BMDC) surface receptor IL-36R, IL-36β upregulates CD80, CD86, and MHC-II molecules, and stimulates the activation of $CD4^+$ T cells and splenocytes to synthesize IFN-γ, IL-4, and IL-17 (10). IL-36 directly acts on $CD4^+$ T cells to increase cell division and IL-2 secretion; in addition, IL-36 synergizes with IL-12 in promoting Th1 polarization of naive $CD4^+$ T cells (8).

The present study revealed that IL-36β was markedly

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downregulated in pancreatic cancer tissue samples compared with adjacent tissue specimens, which indicates that IL-36β has a potential function in antitumor immunity and may be related to tumor progression. This might be because IL-36β is mainly secreted by immune cells. However, tumor cells are predominant in the microenvironment of tumor tissues, and immune effector cells are in an immunosuppressive state, resulting in fewer immune cells infiltrating; therefore, tumor IL-36β levels are reduced compared with normal tissues. The above mouse experiments illustrated that tumor IL-36β overexpression inhibited tumor growth, and IL-36β exerted antitumor effects by inducing the proliferation of $CD8^+$ T and NK cells in the TME. To further validate the antitumor effect of IL-36β, we transfected tumor cells *in vivo* with IL-36β adenovirus to increase the levels of IL-36β in tumors, and found that the IL-36β overexpression adenovirus could act as an immunopotentiator to induce $CD8^+$ T, NK, and γδT cell aggregation in the TME, thereby exerting antitumor effects.

As shown above, IL-36β stimulated $CD8^+$ T cells to synthesize high IFN-γ and IL-2 amounts. Microarray analysis showed that after IL-36β stimulation of human and mouse $CD8^+$ T cells, let-7c-5p levels were decreased consistently. Subsequently, the expression of let-7c-5p in mouse $CD8^+$ T cells was respectively increased and decreased by the lentiviral technique. It was found that let-7c-5p silencing increased IFN-γ and IL-2 synthesis by $CD8^+$ T cells. Meanwhile, when IL-36β was used to stimulate $CD8^+$ T cells, these effects were further enhanced. High expression of let-7c-5p yielded the opposite results. These findings indicate IL-36β promotes the production of IFN-γ and IL-2 by downregulating let-7c-5p in $CD8^+$ T cells. Therefore, we hypothesized that IL-36β affects the function of let-7c-5p through a particular signaling pathway.

The let-7 family was one of the first miRNA groups to be found in *Caenorhabditis elegans* in 2000 (24). The human let-7 family consists of 13 miRNAs, including let-7c. Several reports have indicated let-7c is a tumor suppressor that is downregulated or absent in multiple human tumors, including lung, ovarian, prostate, and colon cancers (25,26). Let-7c-5p, which belongs to the 1et-7 family, also has an anticancer function. New research has shown that CDKN2B antisense RNA 1 (CDKN2B-AS1), an oncogenic long noncoding RNA (lncRNA) of hepatocellular carcinoma (HCC), promotes the nucleosome assembly proteins 1-like 1 protein (NAP1L1)-dependent phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway by sponging let-7c-5p (27). Zhao *et al.* (12) also found IL-36β promotes $CD8^+$ T cell activation by inducing mammalian target of rapamycin complex 1 (mTORC1) via PI3K/Akt, IκB kinase (IKK), and myeloid differentiation factor 88 (MyD88) signaling, thus enhancing antitumor immune responses. Fu *et al.* (28) found that let-7c-5p decreases cell proliferation and enhances apoptosis via excision repair cross complementing 6 (ERCC6) in breast cancer. Wells *et al.* (29) unveiled a new let-7-dependent mechanism acting as a molecular brake to control the degree of $CD8^+$ T cell responses. Mao *et al.* (30) reported that long noncoding RNA GM16343 promotes IL-36β to regulate tumor microenvironment by $CD8^+$ T cells. As shown above, IL-36β increased IFN-γ and IL-2 levels by downregulating let-7c-5p in $CD8^+$ T cells, which has not been previously reported. In summary, direct and indirect experiments have confirmed that IL-36β enhances the anti-tumor effects of $CD8^+$ T cells by downregulating let-7c-5p.

Interestingly, this study found that IL-36β overexpression inhibited tumor growth in the mouse subcutaneous xenograft model, but the role of IL-36β in pancreatic cancer tumorigenesis has not been reported. Pan *et al.* (31) investigated the expression, prognostic value, and the underlying antitumor mechanism of IL-36α in hepatocellular carcinoma (HCC), found that IL-36α expression and overall patient survival, concomitant with a negative correlation with tumor size, degree of differentiation, and tumor growth.

Current cancer treatments include surgery, chemotherapy, and radiotherapy. With in-depth assessment of tumor etiology and immune responses, tumor immunotherapy has become the fourth treatment modality (32). Programmed death 1 (PD-1) monoclonal antibody therapy has had great success in clinical practice for the treatment of melanoma (33). However, the response to this treatment approach for other solid tumors may be limited since it relies on the response of spontaneous T cells to the malignancy. It is known that some cytokines can enhance tumor immunogenicity, thereby helping active lymphocytes or reversing their incompetent state, so as to ultimately exert anti-tumor effects. This study suggests that IL-36β has an anti-tumor activity, and let-7c-5p plays an important role in IL-36β-induced $CD8^+$ T cell-mediated immune response; however, the specific mechanism requires further investigated experimentally.

Conclusions

Our findings suggest that IL-36β promotes IFN-γ and IL-2 production in $CD8^+$ T cells, and IL-36β promotes anti-tumor effects in $CD8^+$ T cells by downregulating the micro-

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RNA let-7c-5p.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. For human research, the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of The First Affiliated Hospital of Soochow University (approval No. 2015683). Informed consent was taken from all the patients. Animal experiments were performed under a project license (No. 2016597) granted by institutional ethics committee of Soochow University and in compliance with the guidelines of Institutional Animal Care and Use Committee of Soochow University (http://dwzx.suda.edu.cn/).

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