IL-36β Promotes Anti-Tumor Effects in CD8$^+$ T Cells by Downregulating Micro-RNA Let-7c-5p

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Abstract

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

Methods: IL-36β amounts in pancreatic cancer were examined by 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. Then we examined the changes of $CD8^+$ T cells and NK cells in the tumor flow-cytometrically. MicroRNA expression profiles were determined by microarray analysis.

Results: The results revealed decreased IL-36β amounts in pancreatic cancer tissues. In addition, IL-36β inhibited tumor growth and promoted $CD8^+$ T and NK cell proliferation in the tumor microenvironment. Moreover, IL-36β stimulated $CD8^+$ T cells to synthesize high amounts of 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 effects. Further experiments demonstrated that IL-36β downregulated IFN-γ in let-7c-$5p^+$ $CD8^+$ T cells.

Conclusion: These findings suggest 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 micro-RNA let-7c-5p.

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 that the proliferative capacity of T lymphocytes is weakened and blunted. 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 amounts of Th1 and $CD8^+$ T cells in the tumor greatly 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 tumor microenvironment, 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].

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 (1L-1Rpr2) [6]. IL-36R expression was detected on $CD4^+$ T, $CD8^+$ T, NK and γδT cells [7]. IL-36 regulates dendritic cell (DC) and $CD4^+$ T cell functions, and promotes INF-γ and IL-2 secretion [8, 9]. B16 and 4T1 cells with high IL-36γ expression can significantly inhibit tumor cell proliferation as well as lung metastasis, promote IFN-γ secretion, and increase $CD8^+$T, NK and γδT cell infiltration into tumors [10]. 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 [11]. Abnormal miRNA expression is implicated in multiple pathologies, including many human malignancies, e.g., lung, gastric and breast cancers [12–14]. *Park KW et al.* [15] 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. *Ziter NC et al*. [16] reported miR-155 expression is essential in the filtration of donor T cells into many target organs. *Adoro S et al.* [17] reported IL-21 directly inhibits HIV-1 and identified microRNA-29 as an antiviral molecule 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 current work assessed IL-36β at the protein and gene levels in pancreatic cancer and adjacent noncancerous tissue samples by immunohistochemistry and quantitative real-time PCR, respectively. Then, IL-36β's anticancer activity was examined by establishing a mouse tumor model. Changes of cell populations in the tumor microenvironment were assessed flow-cytometrically. In mechanistic studies, microarray assays showed that after induction by IL-36β of human and mouse $CD8^+$ T cells, let-7c-5p was consistently downregulated. Furthermore, IL-36β regulated the biological activity of $CD8^+$ T cells by downregulating the miRNA let-7c-5p.

Materials And Methods

Specimens

Pancreatic cancer tissue samples derived from cases confirmed by pathological findings, who 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°C for qRT-PCR detection, or underwent fixation with 10% formalin and paraffin embedding for immunohistochemistry.

Surgical samples of spleen were obtained from 3 individuals administered splenectomy without spleen diseases in the above 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 by magnetic bead-based methods (Miltenyi Biotec). The clinical trial had approval from the ethics committee of The First Affiliated Hospital of Soochow University. Assays involving animals were also approved by the institutional animal care and use committee (IACUC).

Cell culture

Panc02 cells were cultured in DMEM plus 10% FCS. When cells reached about 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 by using the medium containing 10μg/ml puromycin.

Animals and tumor model establishment

BALB/c mice, provided by Zhao Yan (Suzhou) New Drug Research Center, underwent housing in a pathogen-free facility at the Jiangsu Institute of Clinical Immunology, Soochow University. Assays involving animals had approval from the IACUC of Soochow University, following NIH guidelines. 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 7th 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 assessment

Each tumor was cut into 3 to 4 mm pieces and rinsed with Hank's balanced salt solution (HBSS). Then, 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 as directed by the manufacturer, 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).

Overexpression and interference lentiviral vectors

According to the mmu-let-7c-5p (MIMAT0000523) mature body sequence (UGAGGUAGGUUGUAUGGUU), a pair of oligos (NP7179 and NP7180) were designed for gene synthesis (Table 1A). 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. 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 1B.

Table 1
A Oligo sequences for overexpression

Name	Sequence, 5’-3’
mmu-let-7c-5p-F	CACCGTGAGGTAGTAGGTTGTATGGTTCGAAAACCATACAACCTACTACCTCA
mmu-let-7c-5p-R	AAAATGAGGTAGTAGGTTGTATGGTTTTCGAACCATACAACCTACTACCTCAC
Table 1B Oligo sequences for interference	
Name	Sequence, 5’-3’
mmu-let-7c-5p-TUD-F	CAACAACCATACAACCGATCTACTACCTCA
mmu-let-7c-5p-TUD-R	CTTGTGAGGTAGTAGATCGGTTGTATGGTT

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

Total RNA extraction from cells utilized TRIzol, as directed by the manufacturer (ambion, USA). RNA quantitation was performed on a NanoDrop 2000 (Thermo Scientific, USA). Reverse transcription was carried out from 1.0μg RNA with oligo-dT primers and avian myeloblastosis virus reverse transcriptase. Next qRT-PCR was performed with SYBR Green Real-time PCR Master Mix on a real-time PCR machine to examine let-7c-5p, using GAPDH for normalization. The primers are listed in Table 2. Amplification was carried out at 95°C (15 s), followed by 45 cycles of 95°C (5 s) and 60°C (30 s). The $2^{-\Delta\Delta Ct}$ method was utilized for data analysis in triplicate assays.

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

Microarray analysis

Naive $CD8^+$ T cells, obtained from spleens of human and mice, were cultured with medium alone or containing IL-36β (100 ng/ml) for 48 h. The above two groups of cells were harvested separately, 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 the R software with the pheatmap language package (Bioconductor).

Immunoblot

Cell lysis was performed on ice. The 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°C. This was followed by washing steps and incubation with secondary antibodies at ambient for 2 h. The enhanced chemiluminescence (ECL) assay kit was used for development, and protein band intensities were semi-quantitatively assessed with Image J. Triplicate assays were performed.

Immunohistochemistry

Paraffin-embedded clinical tissue samples sectioned at 4-μm underwent overnight incubation with rabbit anti-human IL-36β polyclonal antibodies (1:400) overnight at 4°C. IL-36β signals appeared as brown staining. The samples were 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 firstly administered anti-CD3 mAb or anti-CD28 mAb (2.5/1.25μg/ml). Then, IFN-γ and IL-2 amounts were assessed by ELISA with specific human and mouse ELISA kits (ExCell Bio, China) as directed by the manufacturer.

Statistical Analysis

Student's t test, the Mann-Whitney test, or one-way ANOVA was carried out for data analysis as indicated. Two-sided unpaired $p < 0.05$ indicated statistical significance.

Results

IL-36β in cancer tissues is downregulated

IL-36β amounts in 40 pairs of pancreatic cancer and paracancerous tissue specimens were examined by qRT-PCR and immunohistochemical staining. As shown in Fig. 1A, IL-36β mRNA levels in paracancerous tissue specimens were 3.8 times those of cancer tissue samples. Immunohistochemical staining revealed IL-36β was mainly localized in the cytoplasm and nucleus of cells, mostly staining brown (Fig. 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 ($\chi 2 = 10.026$, $P < 0.05$; Table 3). This suggested that IL-36β may be critical in pancreatic cancer development.

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		

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 lentivirus and performed *in vivo* experiments in mice to assess IL-36β's effects on tumor growth after overexpression in the tumor microenvironment. Pancreatic cancer Panc02 cells were transplanted into the left abdomen of mice subcutaneously; after 3 days, nodules of soybean size 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, tumor size in the Panc02-IL-36β group began to lag behind those of the Panc02-NC and Panc02-Control groups. Until study end, tumor diameter was markedly reduced in the Panc02-IL-36β group compared with the other two groups (Fig. 2). The above findings indicated IL-36β overexpression inhibited tumor growth in the mouse subcutaneous xenograft model.

IL-36β overexpression elevates $CD8^+$ T and NK cell amounts

To explore the basic mechanism by which IL-36β exerts anticancer effects, we examined the changes of TILs in the tumor flow-cytometrically. 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 (Fig. 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 and NK cells. The results revealed $CD8^+$ T cell amounts were higher in the Panc02-IL-36β group in comparison with the other two control groups. Meanwhile, there was no significant difference in NK cell content between the IL-36β and both control groups. However, due to the increase of $CD45^+$ TILs in the tumor, total NK cell amounts were also elevated (Fig. 3B-C). 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 3 groups of 5. Tumor growth was comparable in all groups in the first week. On the 7th day, the three groups of mice were injected separately with IL-36β overexpression adenovirus, adenovirus empty vector and PBS. Afterwards, tumor growth rates in different groups differed. Until study end, tumor size was markedly reduced in mice injected with IL-36β adenovirus compared with the control (PBS and adenovirus empty vector) groups (Fig. 4).

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

To further analyze the effect of intratumoral injection of adenovirus expressing IL-36β on the tumor microenvironment, changes of tumor-infiltrating lymphocytes were assessed flow-cytometrically. 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 (Fig. 5A). In addition, the IL-36β adenovirus group showed increased $CD8^+$ T cell content in comparison with the two control groups. NK cell contents in the IL-36β and both control groups were comparable, but 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 total γδT cells were also increased in the tumor, considering that $CD45^+$ TILs in the IL-36β group were increased by about 2 fold (Fig. 5B-C). The above findings jointly 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 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 human and mice by magnetic bead-based assays (Miltenyi Biotec). Effector $CD8^+$ T cell culture was performed with medium alone or containing IL-36β (100 ng/ml) for 48 h. The above two groups of cells were harvested separately, and microRNA expression profiles were determined by microarray analysis (Fig. 6A-B). There were 21 miRNAs changed in both human and mice (Table 4). Subsequently, changes in miRNA and mRNA expression levels were detected by qRT-PCR (with into two groups, including WT $CD8^+$ and WT $CD8^+$/IL36β stimulation). As shown in Fig. 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.

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

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

Mmu-let-7c-5p overexpression and 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 opposite effects

Naive CD8^{+} T cells were firstly administered anti-CD3 mAb and anti-CD28 mAb at 2.5 and 1.25μg/ml, respectively. Then, the above mmu-let-7c-5p constructs (let-7c-5p^{+} and let-7c-5p^{-}), respectively, were 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. IK-B protein amounts were assessed by immunoblot. In this study, downregulation of mmu-let-7c-5p increased IFN-γ and IL-2 amounts in CD8^{+} T Cells, and its upregulation had opposite effects (Fig. 8A-B). Western Blot detected no significant changes in IK-B protein levels (Fig. 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 (2.5μg/ml) and anti-CD28 mAb (1.25μg/ml). Then, IFN-γ was detected by ELISA. The results showed IL-36β upregulated IFN-γ in CD8^{+} T Cells. Interestingly, IL-36β decreased IFN-γ amounts in the let-7c-5p^{+} group (Fig. 9). Therefore, we speculated that IL-36β affected the function of let-7c-5p through a certain signaling pathway.

Discussion

As a proinflammatory cytokine, IL-36 is involved in many inflammatory reactions and plays an active role in immunity. Therefore, excessive IL-36 level often means that the body has a certain degree of damage [18]. 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. [19–21]. Recent evidence suggests 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 [9]. 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 [7].

This analysis revealed that IL-36β was markedly downregulated in pancreatic cancer tissue samples compared with adjacent tissue specimens, which indicates 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 amounts are reduced compared with the normal tissue. The above mouse experiments illustrated that tumor IL-36β overexpression inhibited tumor growth, and IL-36β achieved 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 IL-36β amounts in tumors, and 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 amounts were decreased consistently. Subsequently, the expression of let-7c-5p in mouse CD8 + T cells was increased and decreased by the lentiviral technique, respectively. 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 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 certain signaling pathway.

The let-7 family was one of the first miRNA groups to be found in *Caenorhabditis elegans* in 2000 [22]. 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 [23, 24]. Let-7c-5p, belonging to the 1et-7 family, also has anticancer function. New research shows that CDKN2B-AS1, an oncogenic lncRNA of HCC, promotes NAP1L1-dependent PI3K/AKT/mTOR pathway by sponging let-7c-5p [25]. *Xin Zhao et al.* [26] also found IL-36β promotes CD8 + T cell activation by inducing mTORC1 via PI3K/Akt, IKK and MyD88 signaling, enhancing antitumor immune responses. *Fu X et al.* [27] found that let-7c-5p decreases cell proliferation and enhances apoptosis via ERCC6 in breast cancer. *Wells AC et al.* [28] unveiled a new let-7-dependent mechanism acting as a molecular brake to control the degree of $CD8^{+}$T cell responses. As shown above, IL-36β increased IFN-γ and IL-2 amounts by downregulating let-7c-5p in $CD8^{+}$ T cells, which has been reported by no previous studies. To summarize, direct and indirect experiments have confirmed IL-36β enhances the anti-tumor effects of $CD8^{+}$ T cells by downregulating the micro-RNA let-7c-5p.

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 [29]. PD-1 monoclonal antibody has had great success in clinical practice for the treatment of melanoma [30]. 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, 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, but the specific mechanism remains to be further investigated experimentally.

Conclusion

Our findings suggest 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 micro-RNA let-7c-5p.

Abbreviations

DC: Dendritic Cell; miRNAs: MicroRNAs; TME: Tumor Microenvironment; qRT-PCR: quantitative real-time PCR; IHC: Immunohistochemistry

Declarations

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Authors' contributions

Xin Zhao, Chen Xie and Jin Zhou proposed the study and were the guarantor. Dongbao Li , Yang Huang, Yanjin Bai and Jianglei Zhang wrote the first draft. Dr. Dongbao Li , Yang Huang, Yanjin Bai and Jianglei Zhang contributed equally to this article. Mingbing Sun, Chenrui Hu, Jin Wang, Zhe Zhang and Jun Ouyang contributed to the data analysis and interpretation. All authors contributed to the design and interpretation of the study and to further drafts.

Availability of data and materials

The data used to support the findings of this study are included within the article.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Soochow University and conducted according to the guidelines of the National

Health and Medical Research Council. All protocols were approved by the Ethics Committee of The First Affiliated Hospital of Soochow University.

Consent for publication

Not applicable.

Competing interests

The authors have no competing financial interests and non-financial competing interests to declare.

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Figures

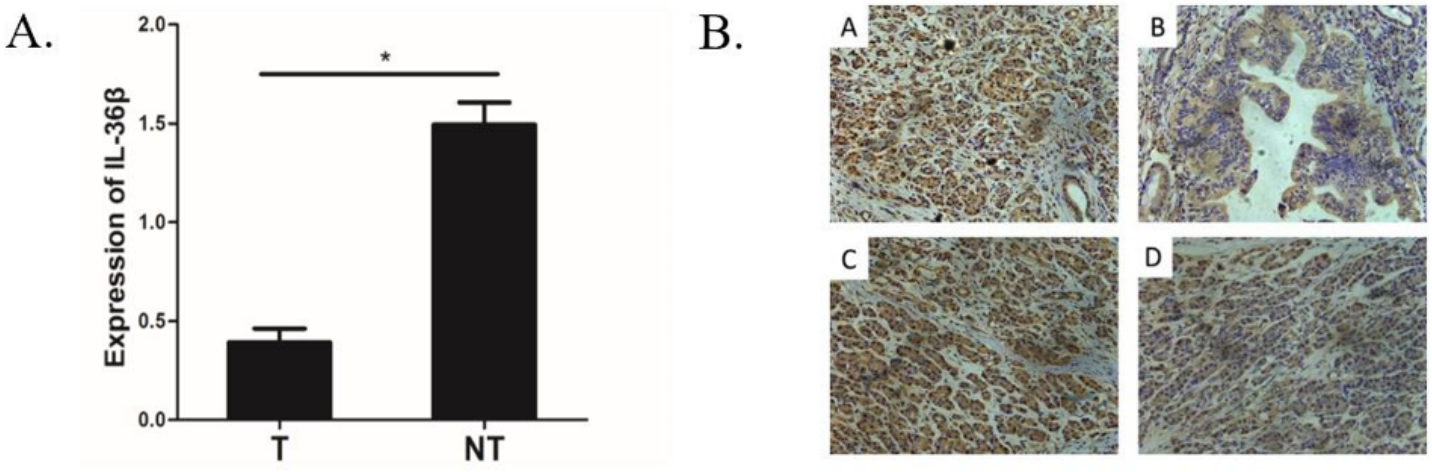

Figure 1

IL-36β in cancer tissues is downregulated. A. IL-36β mRNA amounts in cancer tissue（T）samples were 0.392 ± 0.070, while the levels in paracancerous tissue specimens（NT）were 1.493 ± 0.113, with statistical significance (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).

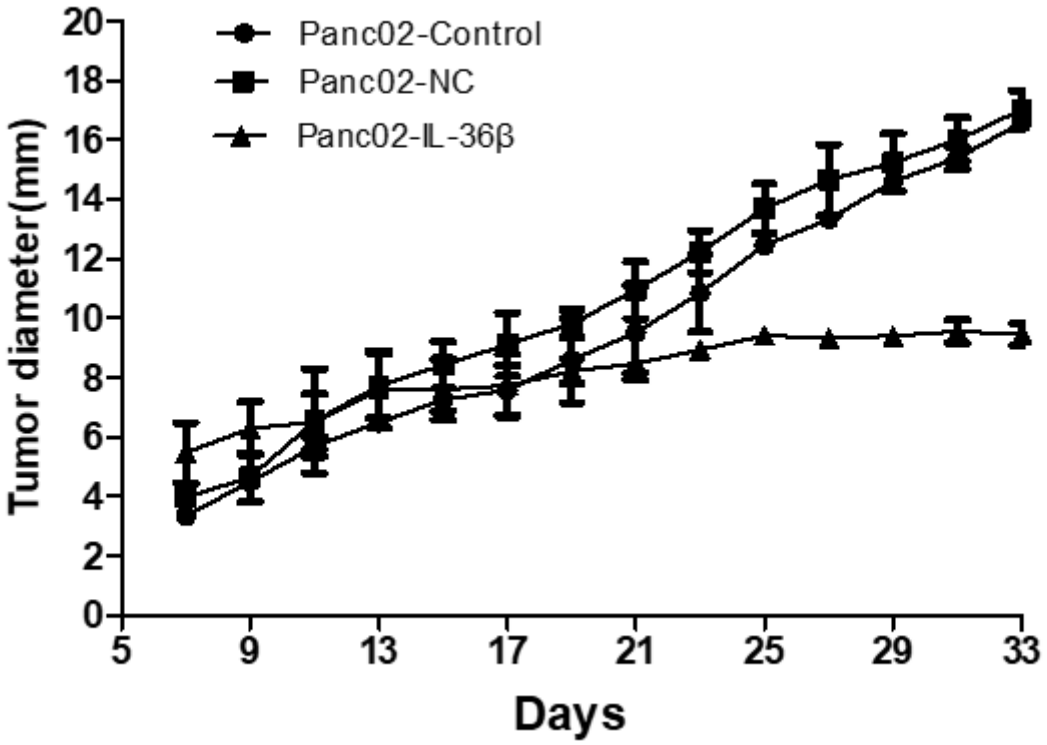

Figure 2

IL-36β overexpression in tumor cells inhibits tumor growth in vivo. Totally 1×106 Panc02-Control, Panc02-NC and Panc02- IL-36β cells were injected subcutaneously into C57BL/6 mice, and tumor sizes were monitored every other day. Data (mean±SEM) are from three independent experiments (n=5/group). Until study end, 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).

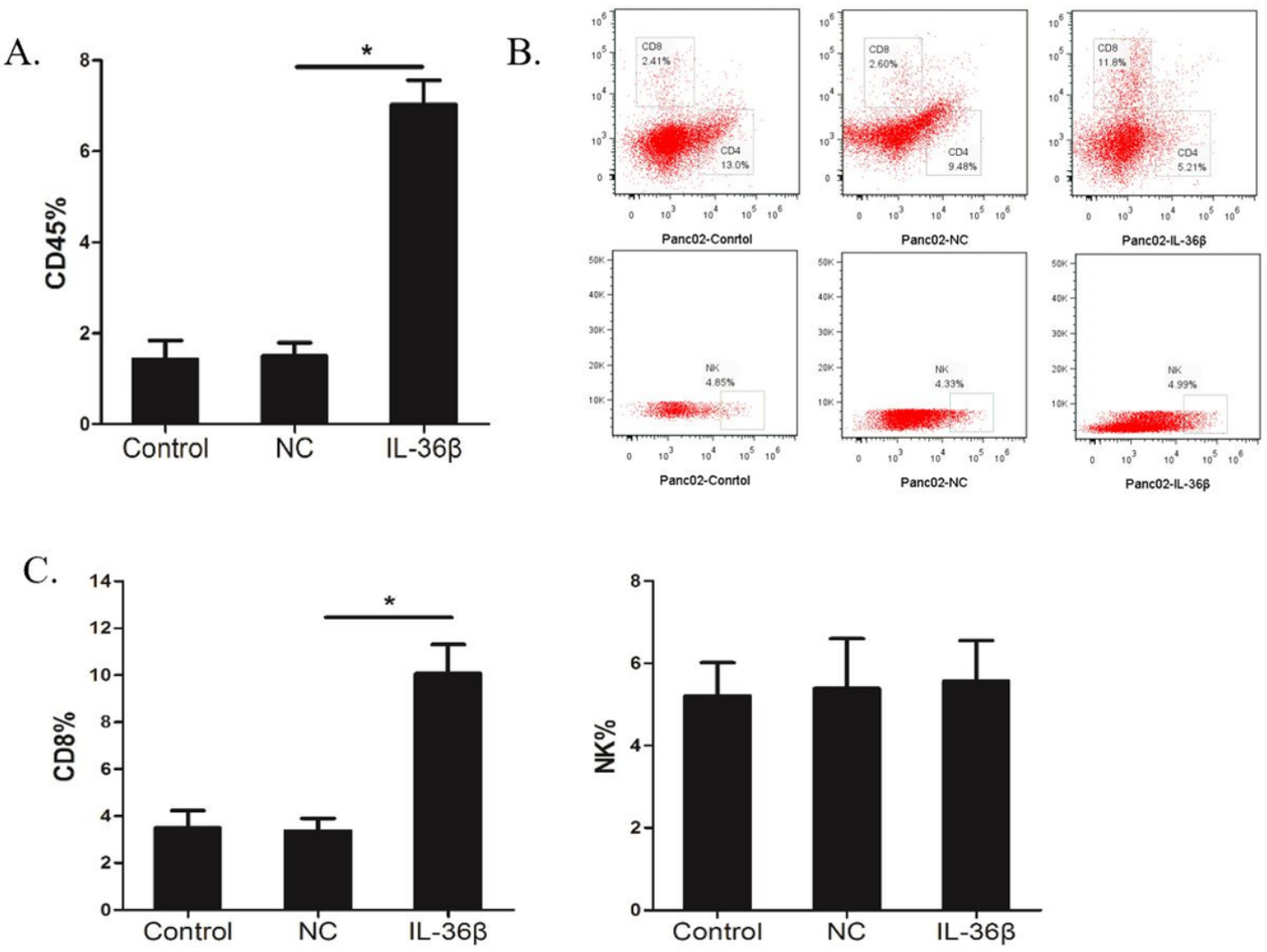

Figure 3

Tumoral IL-36β overexpression enhances type 1 immune responses in the tumor microenvironment. On day 33, tumor samples were obtained for generating single-cell suspensions. (A) Percentages of CD45+ cells in tumor cell suspensions. (B and C) Representative flow-cytograms and CD8+ T or NK cell rates within tumor CD45+ cells.

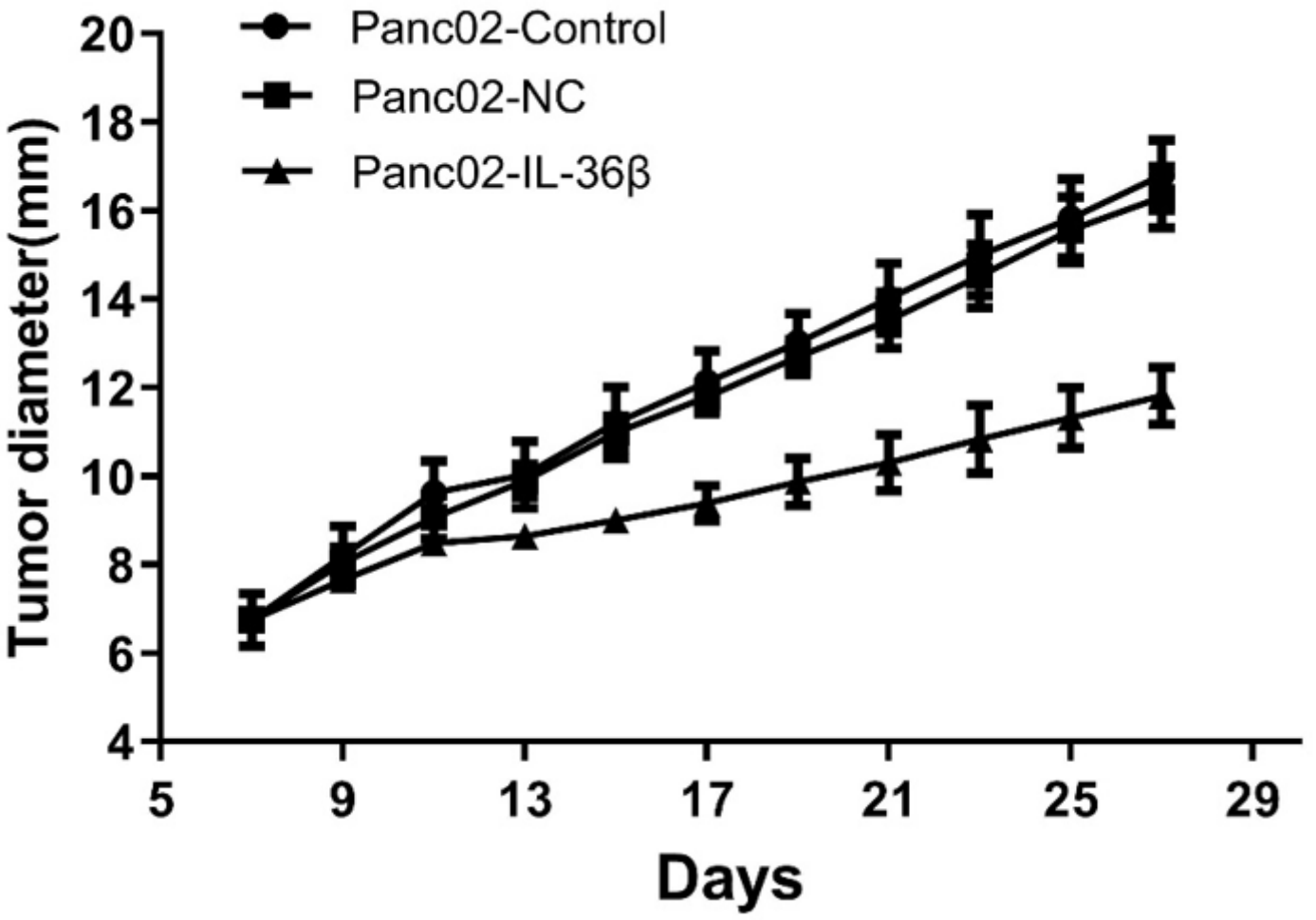

Figure 4

Adenovirus with IL-36β overexpression inhibits tumor growth after intratumoral injection. Totally 1×106 Panc02 cells were administered by subcutaneous injection into C57BL/6 mice. On the 7th day, the animals were randomized into 3 groups and injected separately with 1×109 PFU IL-36β overexpression adenovirus, 1×109 PFU adenovirus empty vector and 100μl PBS. Tumor sizes were monitored every other day. Data (mean±SEM) are from three independent experiments (n=5/group). Until study end, 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).

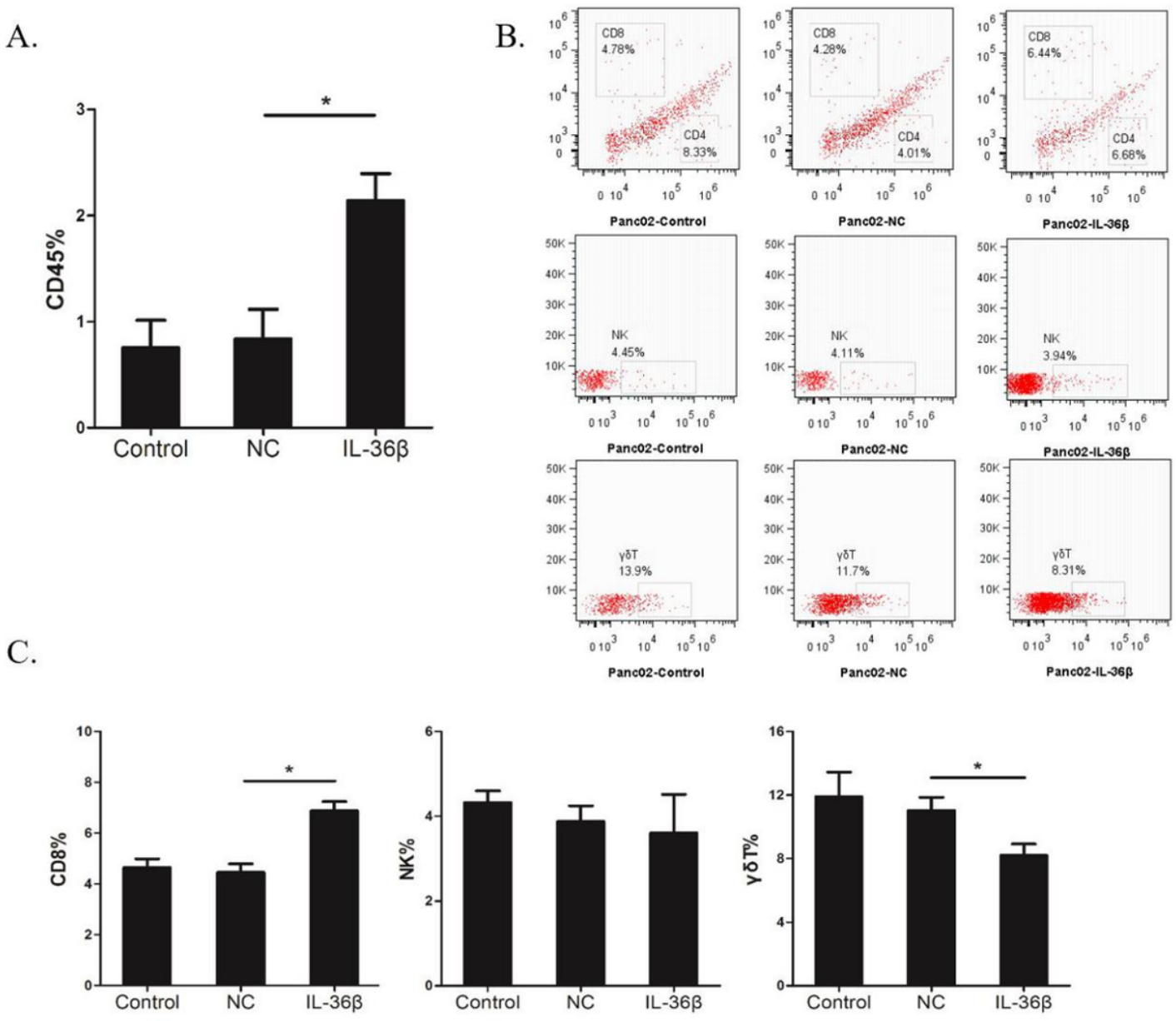

Figure 5

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 and C) Representative flow-cytograms, and CD8+ T, NK1.1+ or γδT cell rates among tumor CD45+ cells.

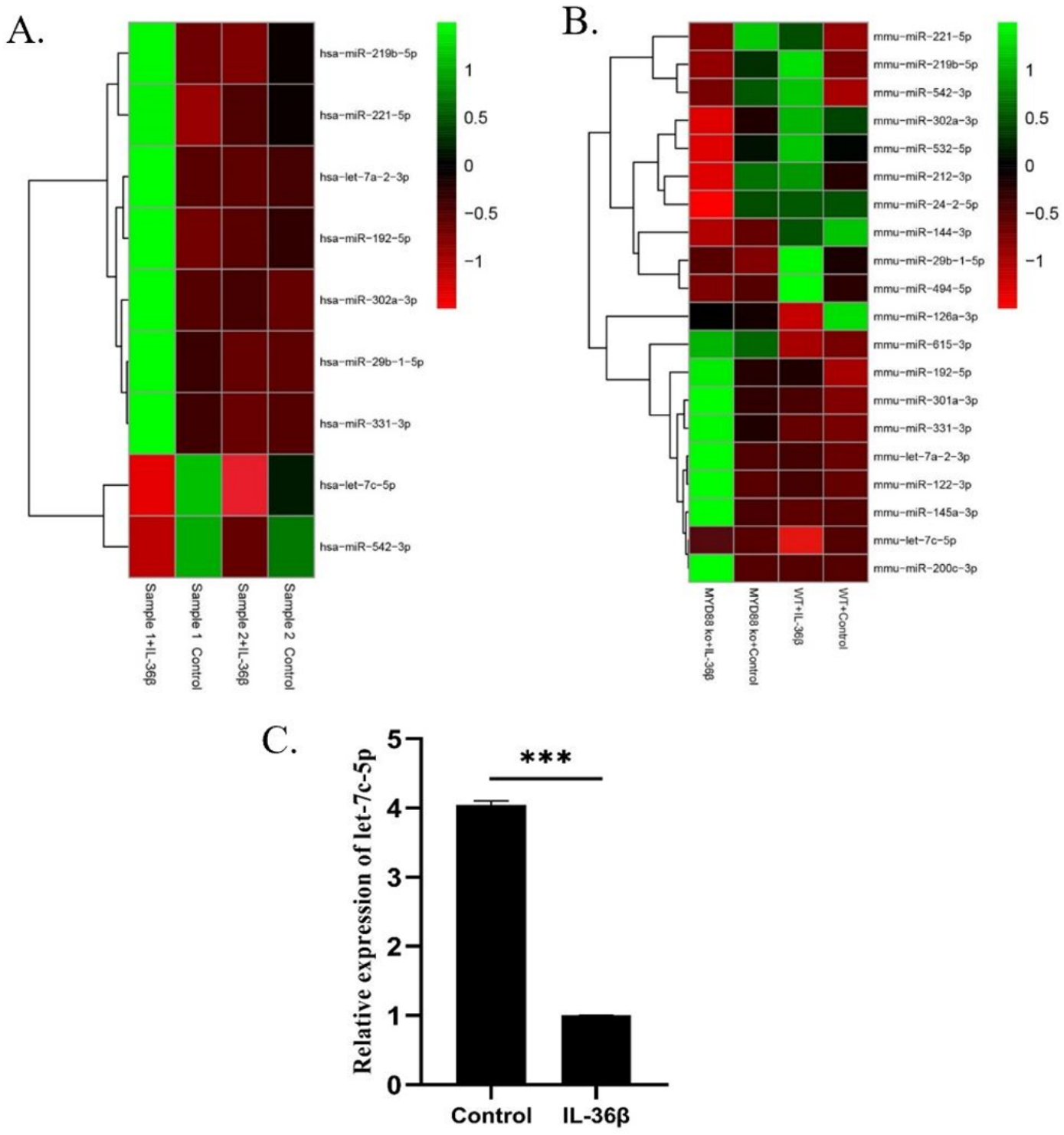

Figure 6

Heat map of 21 miRNAs generated with the R software (pheatmap package). (A) Human. (B) Mouse. MYD88, Myeloid Differentiation Factor 88 (a critical upstream effector of IKK/NF-κB signaling); KO, Knockout. 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).

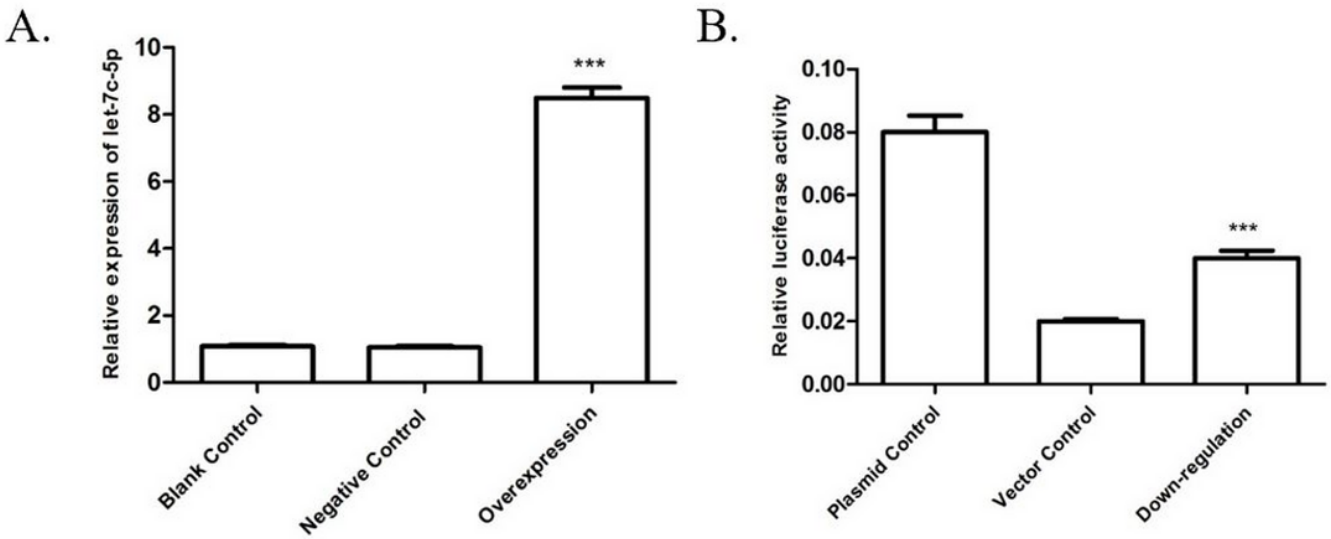

Figure 7

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).

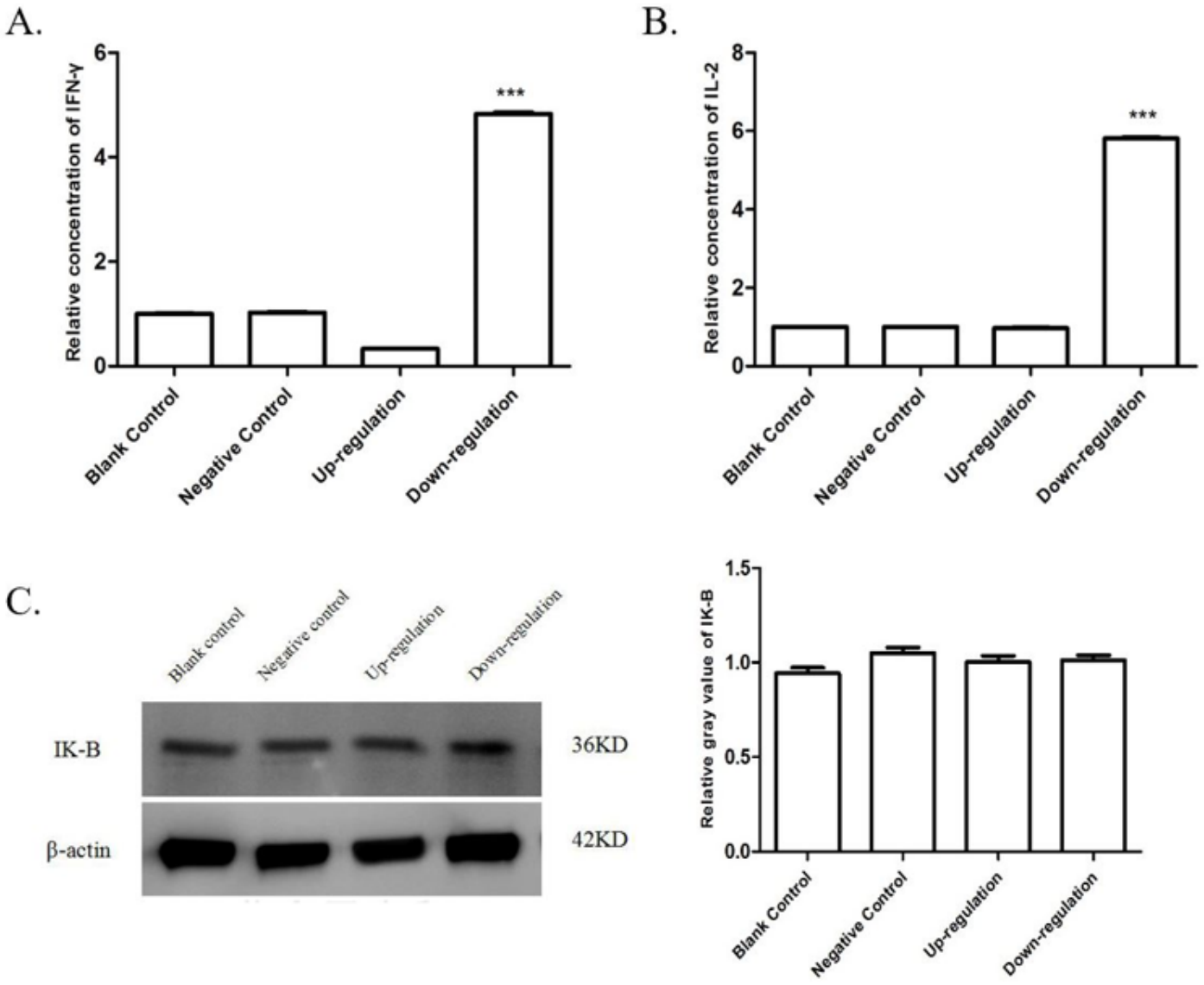

Figure 8

After downregulation and upregulation of mmu-let-7c-5p in mouse CD8+T cells, respectively, IFN-γ (A) and IL-2 (B) amounts secreted by CD8+ T cells were assessed by ELISA. 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.

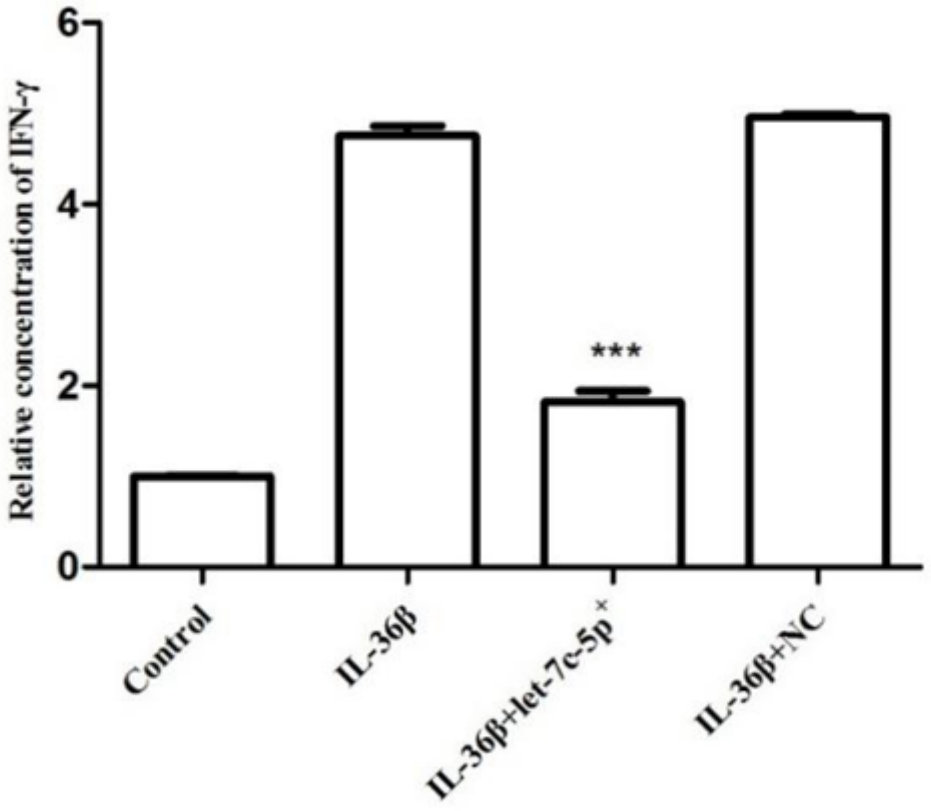

Figure 9

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).