BIO Integration

Brief Report

Low-intensity Pulsed Ultrasound Enhances NK Cell Adoptive Therapy by Modulating the Wnt/β-catenin Signaling Pathway

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Abstract

Natural killer cell adoptive therapy is a novel immunotherapy strategy for the treatment of multiple refractory tumors, including ovarian cancer. However, natural killer cells' anti-tumor effectiveness is limited by their viability and cytotoxicity. Our preliminary study suggested that ultrasound irradiation improves the therapeutic effects of natural killer cells on ovarian cancer through an unclear mechanism. The Wnt/β-catenin signaling pathway is a complex protein network system associated with the maturation and function of natural killer cells. Therefore, herein, to reveal the precise mechanism underlying the effects of ultrasound on natural killer cells, we measured the expression of Wnt/β-catenin signaling pathway proteins before and after ultrasound irradiation. The expression of therapeutic factors secreted by natural killer cells increased after ultrasound irradiation. Therefore, this study provides an additional strategy for optimizing the therapeutic efficiency of natural killer cells.

Keywords

Adoptive cell therapy, natural killer cells, ultrasound, Wnt/β-catenin signaling pathway.

Introduction

Ovarian cancer is a gynecological cancer with a high mortality rate [1]. Immunotherapy based on the immune recognition and immunological modulation mechanisms of cancer cells has garnered substantial attention because of its optimal therapeutic efficacy. Natural killer (NK) cells are intrinsic immune cells critical in killing malignant pathogens and solid tumor cells without major histocompatibility complex class I restriction and prior sensitization [2, 3]. Use of immunotherapy based on NK cells has increased and has become a major research direction in immunotherapy. Activated NK cells secrete various cytokines and enhance anti-tumor effects through adaptive immune responses [4]. The concentration of secreted cytokines is key in NK cells' killing of tumor cells via antibody-dependent cell-mediated cytotoxicity or inflammatory action [5]. In recent years, remarkable progress has been made in adoptive therapy based on NK cells. However, challenges in anti-tumor immunotherapy persist; for example, serial immunosuppressive factors in the tumor microenvironment inhibit the anti-tumor efficacy of NK cells, and consequently limit their anti-tumor efficacy *in vitro* and *in vivo* [6–8]. The number and killing activity of NK cells are significantly associated with their therapeutic effects on cancer treatment [9]. Consequently, increasing NK cell numbers and cytotoxicity has emerged as a major strategy for promoting NK cell-based immunotherapy.

Mechanical stimulation, such as electric, magnetic, or laser stimulation [10, 11], triggers cellular differentiation or alters cellular metabolic function [12]. However, low-energy laser irradiation promotes the proliferation of both normal cells and tumor cells [13]. Among various physical stimulations, low-intensity pulsed ultrasound (LIPUS) has shown promising application potential in cell proliferation, migration, and differentiation, because it is non-invasive, cost-effective, and easily performed [14, 15]. LIPUS is a form of ultrasound with a much lower energy intensity (<3 W/cm^2) than traditional therapeutic ultrasound [16]. However, the influence of LIPUS on the tumor-killing function of NK cells remains unknown. Two studies have shown that after treatment with high-intensity focused ultrasound, the number

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of NK cells, as well as the expression of granzyme B and perforin, increases in treated areas [17, 18]. Another study has indicated that the expression of perforin and granzyme B in NK cells significantly increases after activation of the Wnt/β-catenin signaling pathway, through inhibition of glycogen synthase kinase-3β (GSK3β) [19]. In addition, Tang et al. have shown that LIPUS activates the Wnt/β-catenin signaling pathway in osteoblasts [20]. Moreover, both LIPUS alone and the activated Wnt/β-catenin signaling pathway have been found to inhibit the NF-kB pathway [21, 22]. On this basis, we hypothesized that LIPUS might enhance the cytotoxicity of NK cells by activating the Wnt/β-catenin signaling pathway.

A series of clinical studies have been conducted on NK cell therapy (including NCT05528341, NCT05776355, and NCT05213195). This study was aimed at providing a safe, non-invasive, economical, and simple adjuvant enhancement strategy for NK cell relay therapy that can easily be translated and applied clinically. We preliminarily explored the mechanism underlying the effects of LIPUS on NK cell cytotoxicity and the Wnt/β-catenin signaling pathway. In a co-incubation system comprising NK cells and SK-OV-3 cells, we detected secreted amounts of granzyme A, granzyme B, and human perforin, as well as the expression levels of Wnt/β-catenin signaling pathway-associated proteins. Finally, to test the effect of LIPUS on the anti-tumor activity of NK cells, we examined various treatments in tumor-bearing mice, and assessed the anti-tumor activity of NK cells on the basis of changes in tumor volume and perforin levels (**Figure 1**).

Materials and methods

Cell lines

SK-OV-3 human ovarian cancer cells and NK-92 cells were obtained from ATCC. The SK-OV-3 cells were cultured in RPMI-1640 medium with 2 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin, whereas the NK-92 cells were cultured in NK-92 medium containing MEMα, inositol, folic acid, 0.1 mM β-mercaptoethanol, 12.5% horse serum, 12.5% FBS, and 1% penicillin/streptomycin (Thermo Fisher Scientific, Co., America).

Ovarian cancer model establishment

All animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines in EU Directive 2010/63/EU for animal experiments. All animal experiments were approved by the University of South China Animal Experiment Ethics Review. NOD-SCID IL2rγ null (NSG) female mice were obtained from the Guangdong Medical Laboratory Animal Center. To establish a tumor-bearing mouse model, we resuspended 6×10^6 SK-OV-3 cells in 0.2 mL PBS and subcutaneously injected them into the right anterolateral thigh skin in mice. After the tumor volumes reached 50–100 mm^3, tumor-bearing mice were used in experiments.

LIPUS treatment condition

We examined the cells and animals with a Sonovitro (Shengxiang Hi-Tech, Co., Ltd., China) ultrasonic transfection apparatus. The following ultrasound equipment parameters were applied in all experiments: focused ultrasonic probe located at the bottom of the orifice plate; ultrasonic head area of 5 cm^2; acoustic frequency of 1 MHz; duty cycle of 20%; pulsed repetition frequency of 100 Hz; and irradiation time of 1 min. All controls were subjected to the same conditions, except that the LIPUS apparatus was turned off.

NK cell activity, and granzyme A, granzyme B, and human perforin release assays

NK-92 cells were seeded into 96-well plates (1×10^4 cells per well) and incubated overnight. Subsequently, the cells were irradiated with LIPUS at an acoustic power of 0.2 W/cm^2, 0.4 W/cm^2, or 0.6 W/cm^2. Three replicate groups were analyzed for each group. After 6 and 12 hours of incubation following irradiation, the cells were collected. A CCK-8 kit (Beyotime Biotechnology Co., Shanghai, China) was used to detect NK cell activity. SK-OV-3 human ovarian cancer cells were seeded into 96-well plates and incubated overnight in 100 μL RPMI-1640 medium with 2 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin. First, the NK cells were irradiated with LIPUS at an acoustic power of 0.2 W/cm^2, 0.4 W/cm^2, or 0.6 W/cm^2. Three replicate groups were analyzed for each group. After LIPUS irradiation, the NK cells were co-incubated with SK-OV-3 cells in a 2:1 ratio in 96-well plates. After 6 or 12 hours of incubation, supernatants were collected for granzyme A, granzyme B, and human perforin detection with ELISA kits (DAKEWE Co., China), according to the manufacturer's protocol.

Detection of Wnt/β-catenin signaling pathway proteins

The LIPUS irradiation procedure and the co-incubation of NK cells and SK-OV-3 cells were as previously described. Supernatants collected from the co-culture system were centrifuged (at 178 *g*) for extraction of the NK cells. Total cellular proteins were extracted with RIPA lysis reagent (Cell Signaling Technology, Co., America) and used for detection of Wnt5a and β-catenin expression via western blotting. Quantitative expression levels were analyzed in BIO-RAD Image Lab Software.

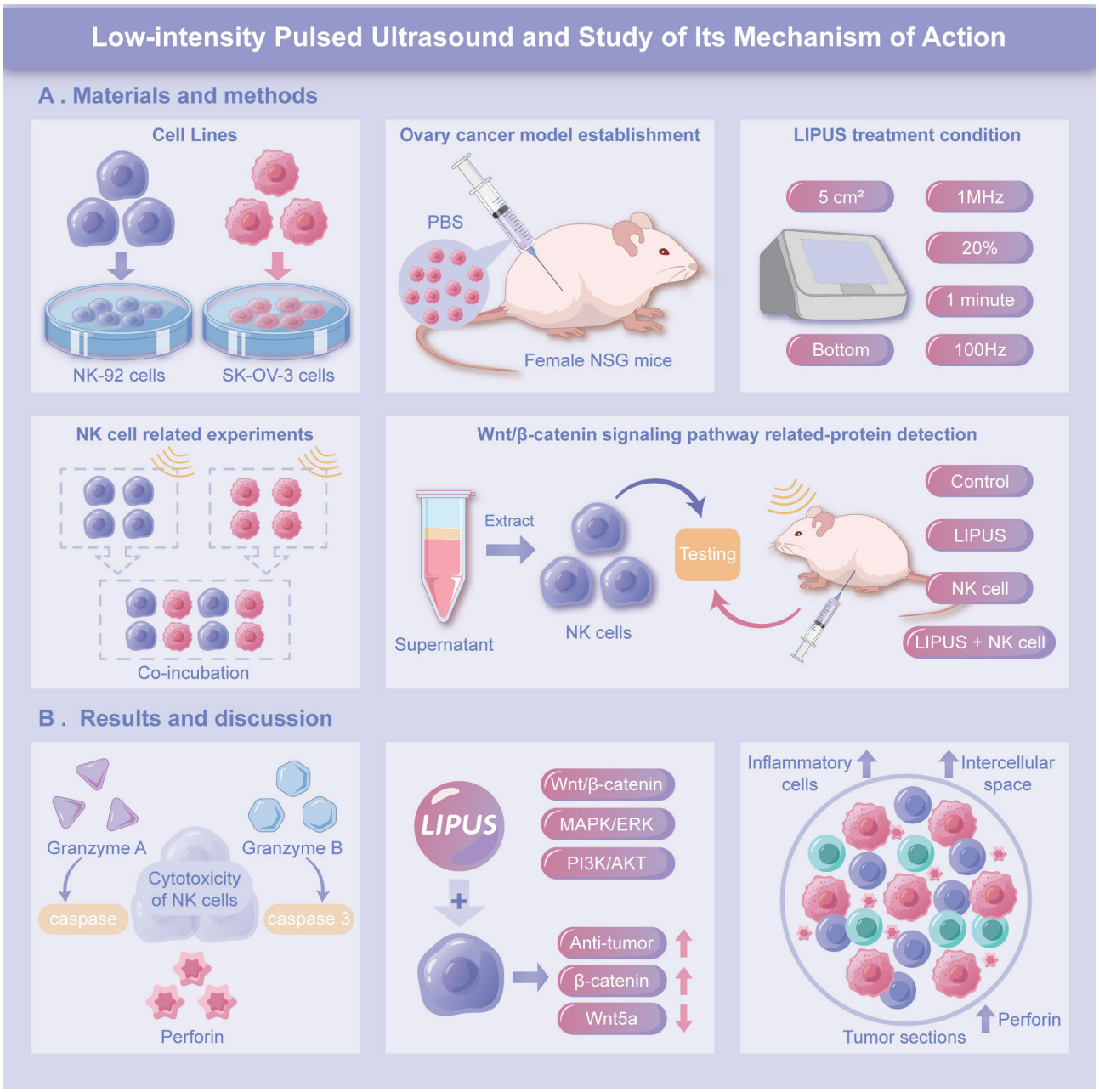

Figure 1 Schematic diagram of low-intensity pulsed ultrasound-enhanced NK cell anti-tumor therapy. Low-intensity pulsed ultrasound irradiation of NK cells activates the Wnt/β-catenin signaling pathway and subsequently promotes the expression and secretion of the cytokines granzyme A, granzyme B, and perforin by NK cells. These factors enhance the anti-tumor ability of NK cells, thereby enabling effective killing of SK-OV-3 tumor cells and inhibiting the growth of ovarian cancer.

Anti-tumor activity of NK cells combined with LIPUS against SK-OV-3 ovarian xenografts

Ovarian cancer xenografts were established with SK-OV-3 cells. After the tumor volume reached 100 cm^3, 20 tumor-bearing mice were randomly divided into four groups (control, LIPUS irradiation alone, NK cell treatment alone, and NK cells combined with LIPUS irradiation treatment), with similar tumor volumes in each group. For NK cell treatment, 1.0×10^7 NK cells per mouse were administered intraperitoneally on days 0, 3, 6, and 9. LIPUS irradiation was performed on days 1, 4, 7, and 10, with an acoustic power of 0.6 W/cm^2. Tumor volume was measured every 3 days with the following formula: volume = ((tumor length) × (tumor width)^2)/2. Twelve days after treatment, or after the tumor volume exceeded 2,000 mm^3, tumor-bearing mice were euthanized through anesthesia, and tumor tissues and sera were collected and used for perforin detection with ELISA. A portion of tumor tissue was subjected to H&E staining.

Statistical analysis

One-way ANOVA was used to analyze all quantitative results in this study in GraphPad Prism 8 (GraphPad Software Inc.). Expression levels of Wnt5a and β-catenin were quantified

in grayscale in ImageJ software. Quantitative data are presented as mean ± SD.

Results and discussion

Granzyme A, a factor with trypsin-like effects, induces apoptosis primarily by activating the independent caspase pathway [23]. Unlike granzyme A, granzyme B is a product derived from aspartic acid, which activates the caspase 3 and 7 pathways, and thereby causes DNA breakage [24]. The cytotoxic substances secreted by NK cells include perforin and granzyme. Perforin is a glycoprotein that promotes granzyme entry into the cytoplasm and causes cell apoptosis [25, 26]. Moreover, Cichocki has demonstrated that perforin expression is associated with Wnt/β-catenin signaling pathway activity [19]. Herein, we determined the proliferation rates of NK cells, as well as the concentrations of granzyme A, granzyme B, and human perforin, in a coculture system comprising irradiated NK cells and SK-OV-3 cells. No significant differences were observed in NK cell viability at 6 h and 12 h after irradiation with LIPUS intensities of 0.2 W/cm^2, 0.4 W/cm^2, or 0.6 W/cm^2 (**Figure 2A, E**, n=3, P>0.05), thus suggesting that LIPUS did not affect the NK cell proliferation rate. We subsequently subjected NK cells to 0.4 W/cm^2 LIPUS irradiation, after co-culture with SK-OV-3 cells for 6 hours. These cells showed higher expression of granzyme A (20.6% higher; n=3, P<0.05), granzyme B (20.2% higher; n=3, P<0.001), and perforin (30.6% higher; n=3, P<0.001) than observed in cells with no LIPUS irradiation (**Figure 2B-D**). After 12 hours of co-incubation, the group treated with a sonication intensity of 0.2 W/cm^2 showed a uniform increase in expression, comprising a 74.13% increase in granzyme A (n=3, P<0.01), 9.76% increase in granzyme B (n=3, P<0.001), and 24.78% increase in perforin (n=3, P<0.0001). In contrast, there was an inconsistent trend in the expression levels of each cytokine in the group treated with 0.6 W/cm^2 ultrasound intensity (**Figure 2F-H**). On the basis of these findings, in conjunction with previous studies, we hypothesized that the increased cytokine expression might be associated with activation of the Wnt/β-catenin signaling pathway, and that the degree of activation of this pathway might correlate with ultrasound intensity.

Consequently, we investigated whether the underlying mechanism might be associated with the Wnt/β-catenin signaling pathway. Tang et al. have demonstrated that LIPUS activates the Wnt/β-catenin signaling pathway in bone tissue and prevents bone loss [20]; therefore, LIPUS stimulation is associated with Wnt/β-catenin signaling pathway activity. In addition, ultrasound upregulates MAPK/ERK and PI3K/AKT pathway-related proteins, which in turn contribute to NK cell proliferation and cytokine secretion [27]. Our results indicated an increase in Wnt5a protein expression by 1.1-fold (n=3, P<0.001) and 1.4-fold (n=3, P<0.0001) 6 h after irradiation with 0.2 W/cm^2 and 0.4 W/cm^2

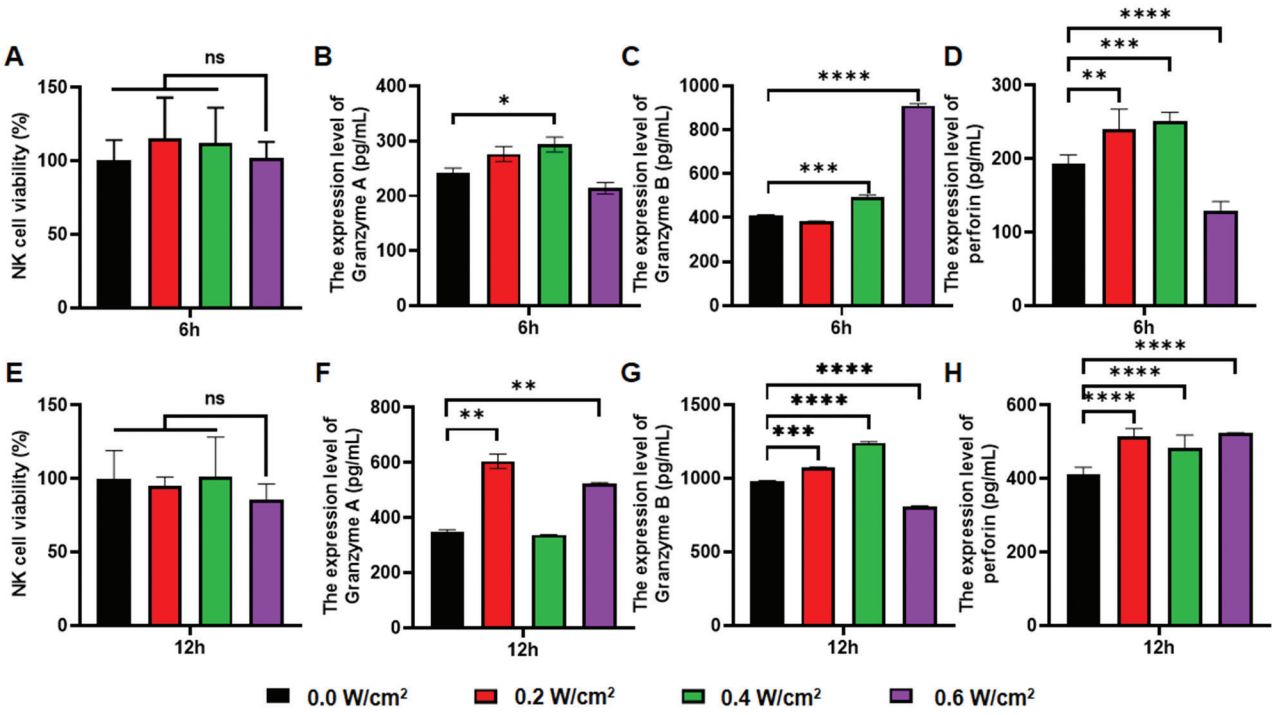

Figure 2 Viability of NK cells and secretion levels of functional proteins by NK cells after LIPUS irradiation at varying acoustic power. A, E. Viability of NK cells 6 h (A) and 12 h (E) after exposure to various LIPUS irradiation intensities. B, F. Secretion levels of granzyme A in SK-OV-3 and NK cell co-culture system supernatants 6 h (B) and 12 h (F) after exposure to various LIPUS irradiation intensities. C, G. Secretion levels of granzyme B in SK-OV-3 and NK cell co-culture system supernatants 6 h (C) and 12 h (G) after exposure to various intensities of LIPUS irradiation. D, H. Secretion levels of perforin in SK-OV-3 and NK cell co-culture system supernatants 6 h (D) and 12 h (H) after exposure to various intensities of LIPUS irradiation. All experiments involved sequential irradiation with ultrasound intensities of 0.0 W/cm^2, 0.2 W/cm^2, 0.4 W/cm^2, and 0.6 W/cm^2 (1 MHz, pulse repetition frequency 100 Hz, duty cycle 20%, time 1 min). The results were statistically analyzed with one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 were considered statistically significant (n=3 per group).

ultrasound, respectively, whereas no significant change in β-catenin protein expression was observed. However, with an ultrasound intensity of 0.6 W/cm^2, Wnt5a protein did not significantly increase (n=3, P>0.05), whereas β-catenin protein increased by 20.97% (n=3, P<0.0001) (**Figure 3A-C**). At 12 h after 0.4 W/cm^2 and 0.6 W/cm^2 ultrasound irradiation, Wnt5a protein content significantly decreased, whereas β-catenin protein expression increased by 24.39% (n=3, P<0.05) and 46.34% (n=3, P<0.01), respectively, thus suggesting that Wnt5a protein might be associated with the levels of activated β-catenin protein (**Figure 3D-F**). On the basis of the above results, we hypothesized that LIPUS might first activate the Wnt/β-catenin signaling pathway and Wnt5a protein expression; subsequently promote the expression of the downstream protein β-catenin; and finally activate the downstream expression of secretion of virulence factors, such as granzyme and perforin.

Furthermore, we verified that NK cells subjected to LIPUS irradiation achieved greater anti-tumor efficacy than NK cells without LIPUS irradiation or LIPUS irradiation alone. On day 12 after treatment, the tumor volume and weight in the NK cell combined with LIPUS treatment group were significantly lower than those in the Control group (tumor volume decreased by 39.52%, tumor weight decreased by 52.75%, n=5, P<0.05). (**Figure 4A-C**). According to tumor tissue histology, in the group with NK cells combined with LIPUS treatment, the intercellular space in the tumor tissue showed widening and contained abundant inflammatory cells. Simultaneously, the presence of vacuole-like structures indicated cancer cell necrosis and deformation (**Figure 4D**). Compared with the Control group, the NK cell combined with ultrasound treatment group showed a 70.41% increase in perforin secretion in tumor tissue (n=3, P<0.05) and a 21-fold increase in serum (n=3, P<0.01) (**Figure 5**).

Herein, LIPUS was found to enhance NK cells' anti-tumor efficacy by activating the Wnt/β-catenin signaling pathway. However, some prior studies have concluded that LIPUS significantly activates the Wnt/β-catenin signaling pathway, whereas others have reported that LIPUS suppresses the Wnt/β-catenin signaling pathway [28]. These discrepant findings might be associated with the applied ultrasound parameters. According to previous research, 0.5 W/cm^2 LIPUS enhances iPSC–NCSC proliferation ability, whereas 1.5 W/cm^2 LIPUS has opposite effects [29]. In addition, we observed inconsistency in the results obtained with various ultrasound intensities and sampling times; therefore, additional subgroups and longer observation times must be examined in subsequent studies to further optimize this treatment regimen. In addition, although LIPUS is safe and noninvasive, and has been demonstrated by several recent clinical studies to provide advantages of safety and noninvasiveness in short-term treatment [30–32], an in-depth examination of the effects on normal tissues as well as the long-term effects on the disease is lacking. Therefore, the mid- and long-term effects of LIPUS on normal tissues and disease sites must be explored to enable application of this technique in late clinical stages.

In conclusion, this study provides a precise, controllable, safe, and effective strategy for enhancing NK therapy through LIPUS. The combination of LIPUS was found to

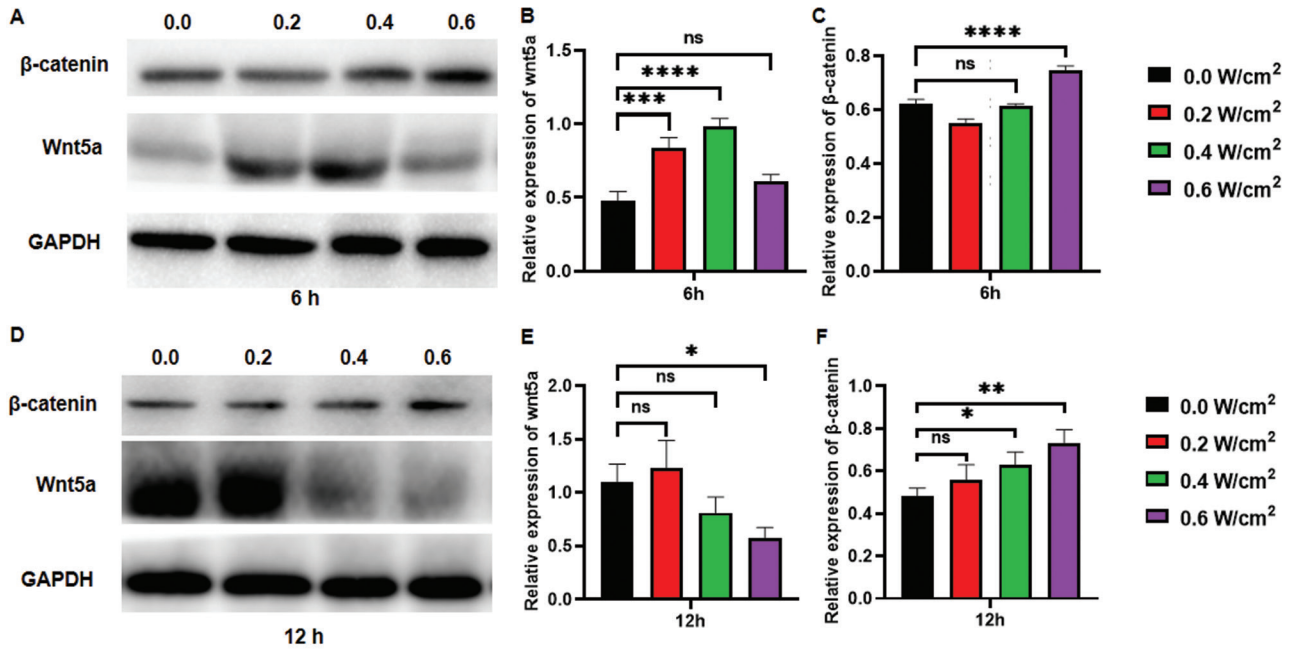

Figure 3 Expression of Wnt5a and β-catenin in NK cells after LIPUS irradiation. A. Western blot results of Wnt5a and β-catenin in NK cells 6 hours after LIPUS irradiation at various intensities. B, C. Quantitative grayscale analysis of western blot bands of Wnt5a (B) and β-catenin (C) in NK cells 6 hours after LIPUS irradiation at various intensities. D. Western blot results of Wnt5a and β-catenin in NK cells 12 hours after LIPUS irradiation at various intensities. E, F. Quantitative grayscale analysis of western blot bands of Wnt5a (E) and β-catenin (F) in NK cells 6 hours after LIPUS irradiation at various intensities. All experiments involved sequential irradiation with ultrasound intensities of 0.0 W/cm^2, 0.2 W/cm^2, 0.4 W/cm^2, and 0.6 W/cm^2 (1 MHz, pulse repetition frequency 100 Hz, duty cycle 20%, time 1 min). Expression levels of Wnt5a and β-catenin were quantified in grayscale in ImageJ. The results were statistically analyzed with one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 were considered statistically significant (n=3 per group).

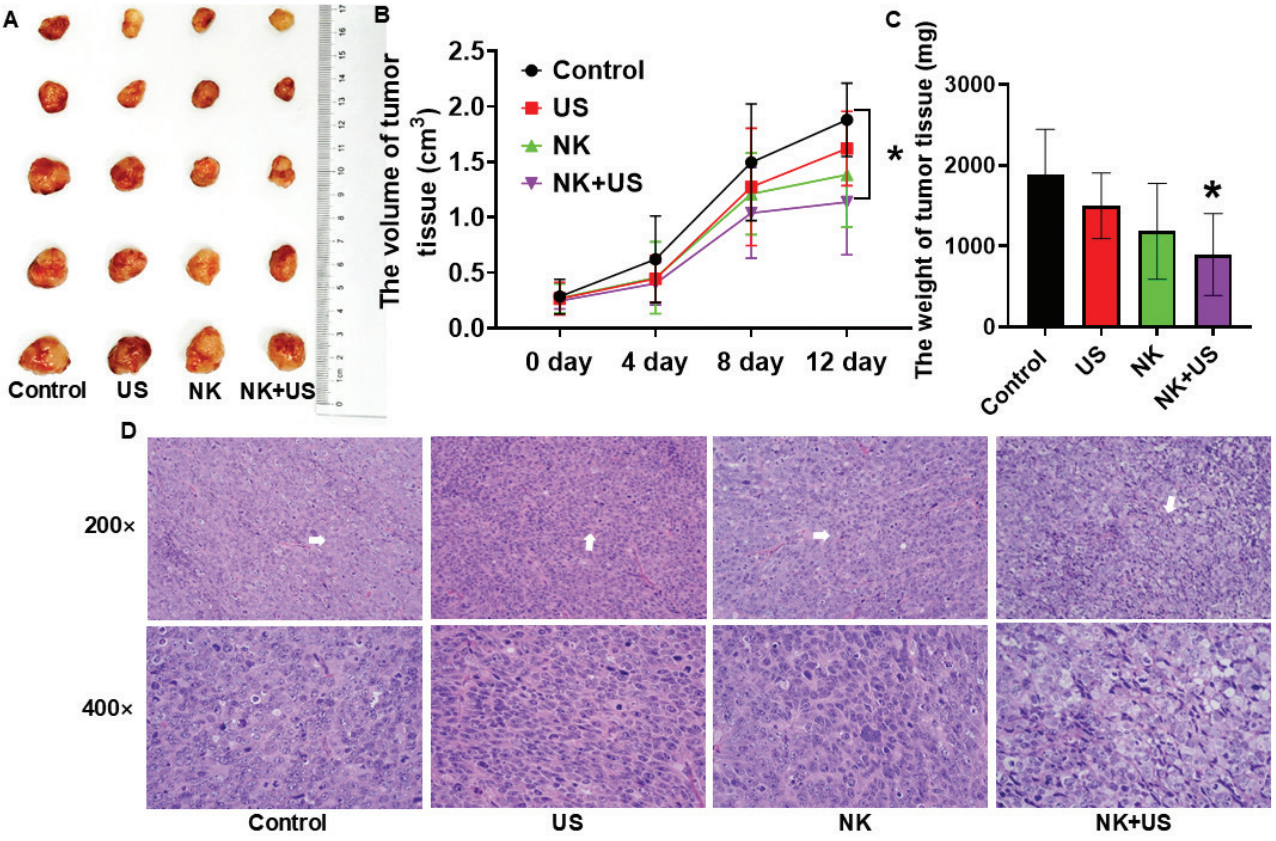

Figure 4 Anti-tumor activity of NK cells against ovarian cancer xenografts (scale bar: 50 μm). A. Photographs of tumor tissue *in vitro* after various treatments. B. Volume change curves of tumors under various treatments. C. Tumor tissue weights in the indicated groups. D. Histological slices of tumor tissues after various treatments. These results were statistically analyzed with one-way ANOVA. **P*<0.05 was considered statistically significant (n=5 per group).

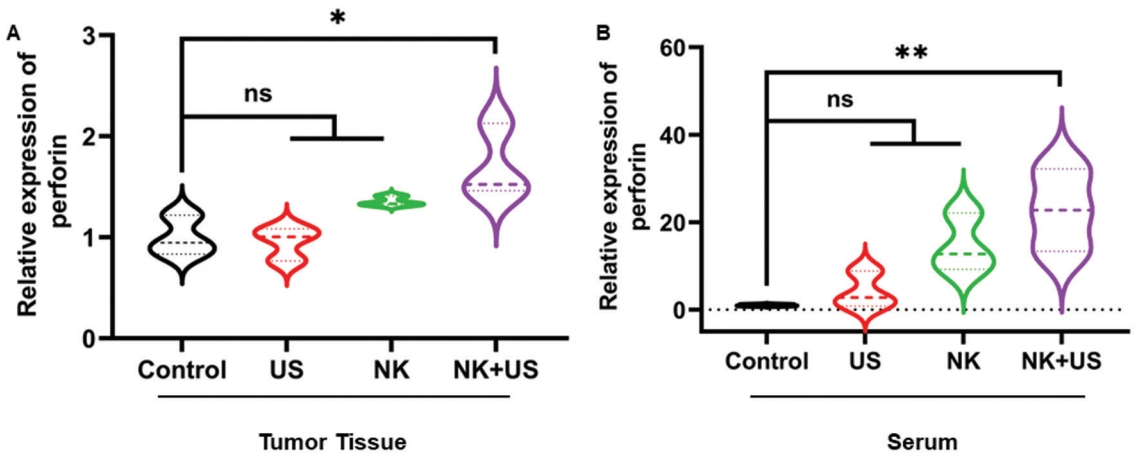

Figure 5 Secretion of perforin *in vivo* after various treatments. A. Secretion of perforin in tumor tissue after various treatments. B. Secretion of perforin in serum after various treatments. The results were statistically analyzed with one-way ANOVA. **P*<0.05 was considered statistically significant (n=3 per group).

increase the cytotoxicity and anti-tumor efficiency of NK cells in ovarian cancer. Future research is needed to clarify the detailed relationship between changes in the Wnt/β-catenin signaling pathway in NK cells and various ultrasound parameters, to further refine the mechanism.

Data availability statement

All data analyzed during this study are included in this published article. Additional data are available from the corresponding author on reasonable request.

Ethics statement

All animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines in EU Directive 2010/63/EU for animal experiments. All animal experiments were approved by the University of South China Animal Experiment Ethics Review.

Author contributions

Renjie Feng, Conceptualization, Writing—original draft, Writing—review & editing. **Meng Du**: Conceptualization, Supervision, Writing—review & editing, Funding acquisition. **Ao Gu**: Investigation, Methods. **Gökhan Zengin:** Conceptualization, Supervision, Writing—review & editing.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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