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Antipancreatic cancer effect of DNT cells and the underlying mechanism

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ABSTRACT

Objectives: This study aimed to explore double-negative T (DNT) cell cytotoxicity to pancreatic cancer and the effect of the Fas (CD95, APO-1)/FasL (CD178) signaling pathway on this process.
Methods: DNT cells from the peripheral blood of healthy volunteers were expanded in vitro. The inhibitory effect of DNT cells on pancreatic cancer cells was investigated using a CCK-8 assay and nude mouse tumor model. A mechanistic study was performed using pathway blocking assays.
Results: DNT cells were amplified in vitro with >90% purity, and the growth of pancreatic cancer in vitro was significantly inhibited by DNT cells. After coculture with DNT cells, Fas, caspase-8 and cleaved caspase-8 showed increased expression in pancreatic cancer cells. When blocking agent decoy receptor 3 (DcR3) was added, the antitumor effect of DNT cells and the expression of Fas, caspase-8 and cleaved caspase-8 were reduced in pancreatic cancer cells. In the nude mouse tumor model, the tumor volume and weight were lower in the DNT cell group and gemcitabine group than in the blank control group. Additionally, the expression of Fas, caspase-8 and cleaved caspase-8 was higher in the DNT cell group than in the blank control group. Moreover, DNT cells promoted apoptosis in cancer cells and animal model tissues.
Conclusion: DNT cells inhibited the growth of pancreatic cancer, and the Fas/FasL signaling pathway was involved in this process.

Introduction

Pancreatic cancer is a highly malignant tumor with a poor prognosis; despite a variety of available treatment methods, the desired treatment effect cannot be achieved, resulting in a five-year survival rate of less than 5% [1,2]. Currently, surgical resection is the most effective treatment, but pancreatic cancer is locally invasive and surrounded by a dense desmoplastic reaction, which could involve adjacent vital structures. Therefore, the opportunity for complete excision may be limited to 10–20% of patients who can be treated with surgery, but the five-year survival rate is only 15–25%. The failure of traditional therapeutic approaches to treat this devastating disease necessitates a new therapeutic approach for pancreatic cancer. Targeted therapies with agents such as antibodies, small molecule inhibitors or downstream signaling molecules have been clinically successful in various cancers.

Double-negative T (DNT) cells are phenotypically cell differentiation cluster 3+ ($CD3^+$) CD4(−) CD8(−) cells, which constitute a small but important proportion of T cells. Although the development of DNT cells has been extensively investigated [3,4], the origin of this cell type remains unclear. According to Thomson et al. [5], DNT cells account for 1–5% of the peripheral T cell population in mice and humans. DNT cells play an important role in the regulation of the immune response in models of transplant rejection, autoimmunity, and inflammatory diseases. Recent studies have demonstrated the antitumor effects of DNT cells. According to Young et al. [6], DNT cells can kill Ld + A20 tumor cells in vivo. Based on findings by Merims et al. [7], upon expansion in vitro with allogeneic donor lymphocytes, DNT cells can suppress the growth of lymphoma cells as well as the growth of allogeneic and autologous primary leukemic blasts in vivo and in vitro. However, reports on DNT cell cytotoxicity to pancreatic cancer are lacking, and the underlying mechanism remains unclear. Fas, a transmembrane protein that belongs to the tumor necrosis factor (TNF) receptor

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superfamily, has only one ligand, Fas ligand (FasL). FasL is expressed on the surface of a variety of lymphocytes, including DNT cells [6,8–10]. FasL binds to the Fas receptor of target cells and can induce apoptosis in target cells. According to the previous study, DNT cells are cytotoxic to lymphoma cells via the Fas/FasL signaling pathway [6]. More importantly, Fas was found to be highly expressed in pancreatic cancer cells compared to normal pancreatic cells [11,12]. Therefore, the Fas/FasL signaling pathway may also be involved in the mechanism of DNT cell cytotoxicity to pancreatic cancer.

In this article, we aimed to explore the therapeutic use of DNT cells and their mechanism of action in pancreatic cancer. Here, we used a novel method to efficiently expand human DNT cells ex vivo, performed DNT cell cytotoxicity and Fas/FasL pathway blocking experiments and established a nude mouse model of pancreatic cancer.

Materials and methods

Cells and reagents

To enrich the DNT cell population, we used the "antibody adsorption" method [7,13,14]. In this method, 10 ml peripheral blood was obtained from healthy volunteers, added to a CD4 Depletion Cocktail and CD8 Depletion Cocktail (Stemcell Technologies, Canada), and incubated for 10 min. Then, we used human blood lymphocyte separation medium (TianJinHaoYang, China) to extract lymphocytes. To further purify DNT cells, we incubated collected cells with an anti-CD3 antibody in complete medium supplemented with interleukin-2 (IL-2) and IL-4 (Zhongshan Golden Bridge Biotechnology, China). On day 12, we collected cells from wells, counted the cells and verified the phenotype by flow cytometry. Pancreatic cancer cell lines Panc-1 and SW1990 cells were cultured in Roswell Memorial Park Institute medium 1640 (RPMI 1640) supplemented with 10% fetal bovine serum (FBS, HyClone, USA). This research was approved by the Human Scientific Ethics Committee of the Affiliated Provincial Hospital of Anhui Medical University (Hefei, China).

Flow cytometry

DNT cells were first stained with CD4-phycoerythrin (PE), CD8-allophycocyanin (APC) and CD3-fluorescein isothiocyanate (FITC) antibodies (Biolegend, USA). Next, these cells were detected by flow cytometry. Cell phenotype was analyzed by FlowJo software.

CCK-8 assay

The cytotoxicity of DNT cells was assessed by a cell counting kit-8 (CCK-8) assay (BestBio, China). Panc-1 or SW1990 cells, which were used as the target cells, were seeded into 96-well plates. The number of cells per well was 1×10^3. DNT cells were inoculated with target cells with or without the blocking agent decoy receptor 3 (DcR3, 20 ng, R&D Systems, USA) [15]. Importantly, DNT cells were cocultured with DcR3 for 2 h before they were seeded into 96-well plates. The DNT/target ratio was 1:1. As a control, groups of target cells with or without DcR3 treatment were established. After cells were cultured for 12, 24, 36 or 48 h, a CCK-8 assay was performed. The spectrophotometric absorbance of each sample was measured at 450 nm by a microplate reader (Thermo Fisher Scientific, USA). Finally, the optical density (OD) values of each group were compared and analyzed.

Establishment of animal models

A total of 24 BALB/c male immunodeficiency mice (4–5 weeks) were used to establish an animal model as previously described [16]. Panc-1 cells were injected subcutaneously into the underarms of BALB/c mice (2×10^6 cells/mouse, Slac Laboratory Animal Co. Ltd, China). After subcutaneous tumors reached >0.5 cm in diameter, the possibility of tumor suppression was tested by injecting DNT cells into the tail vein of 8 mice every 2 days (5×10^6, 0.1 ml). As controls, 8 mice received no treatment, and another 8 mice received an intravenous injection of gemcitabine every 2 days

Table 1
Primers used for Fas, caspase-8 and GAPDH detection.

	Forward primer	Reverse primer
Fas	5′- TTGCTTAGGGTTCCCTCCTG-3′	5′-AAACTGGAGAGCAGACAGCA-3′
Caspase-8	5′-CGTCTATGGAACGGATGGGA-3′	5′-TACTTCCTTGGCAAGCCTGA-3′
GAPDH	5′-CAAGGCTGTGGGCAAGGT-3′	5′-GGAAGGCCATGCCAGTGA-3′

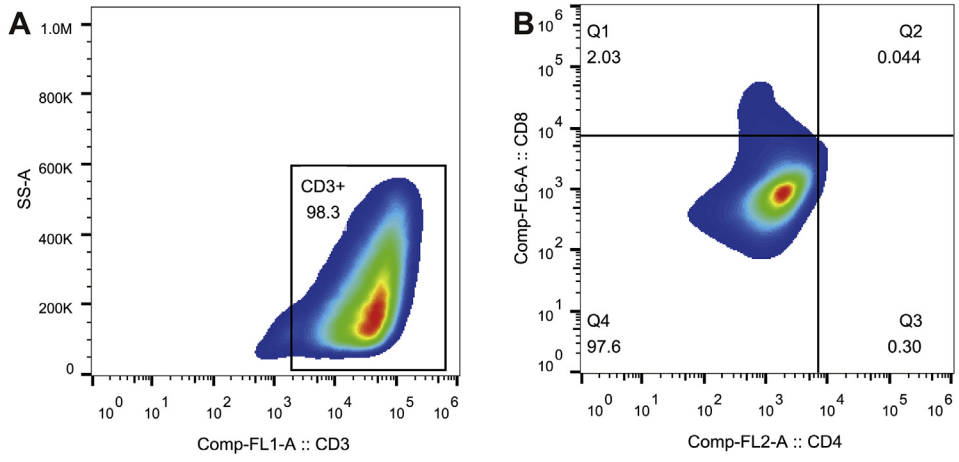

Fig. 1. Expression of CD3, CD4 and CD8 in DNT cells. The cell phenotype of DNT cells was $CD3^+CD4^-CD8^-$ with >90% purity.

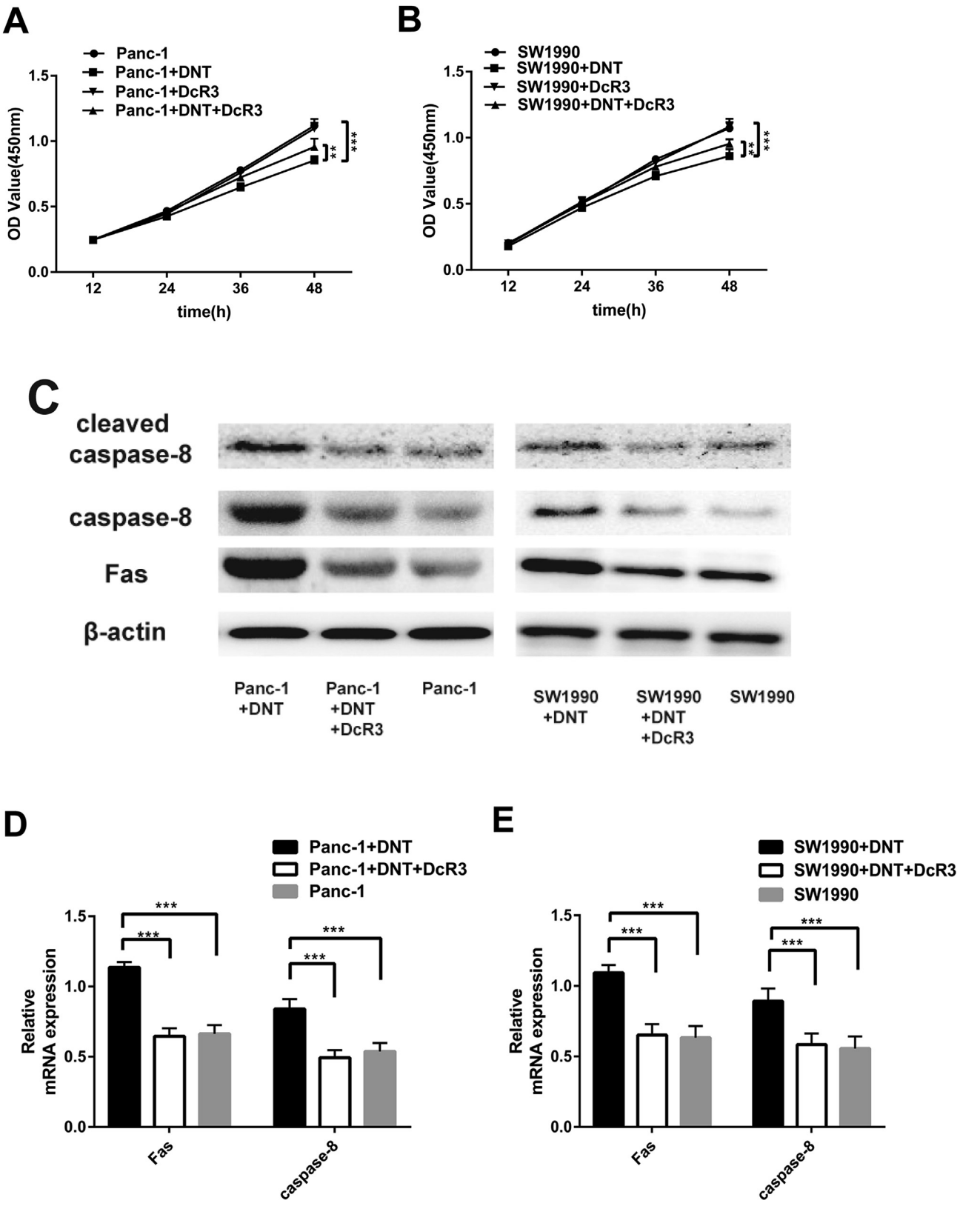

Fig. 2. DNT cells inhibited pancreatic cancer growth and via the Fas pathway in vitro. A, CCK8 assay showing the effect of DNT cells, DcR3-treated DNT cells and DcR3 on Panc-1 cells. B, CCK8 assay showing the effect of DNT cells, DcR3-treated DNT cells and DcR3 on SW1990 cells. C, Protein levels of Fas, caspase-8 and cleaved caspase-8 in the Panc-1 or SW1990 group, Panc-1 or SW1990 + DNT group and Panc-1 or SW1990 + DNT + DcR3 group. D, mRNA levels of Fas and caspase-8 in the Panc-1 group, Panc-1 + DNT group and Panc-1 + DNT + DcR3 group. E, mRNA levels of Fas and caspase-8 in the SW1990 group, SW1990 + DNT group and SW1990 + DNT + DcR3 group. $^{**}P < 0.01$ and $^{***}P < 0.001$.

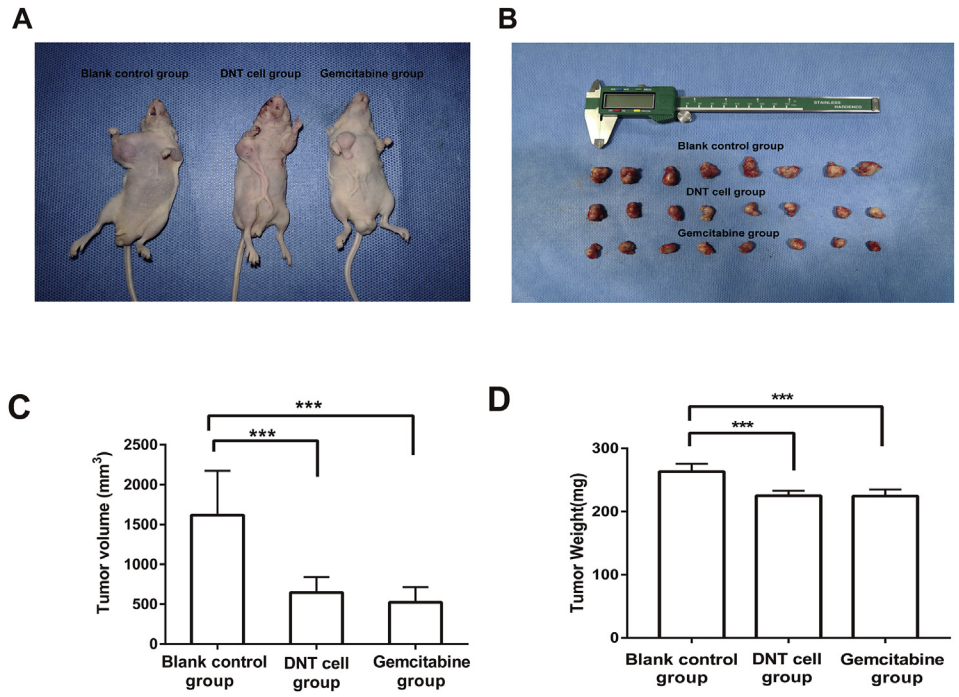

Fig. 3. DNT cells decreased the tumor size in animal models. A and B, Image of a tumor in the mouse pancreatic cancer model. C, Volume of tumor tissues in the three groups. D, Weight of tumor tissues in the three groups. ***P < 0.001.

(50 mg/kg) after the tumor size reached >0.5 cm. The growth and development of mice and their tumors were closely monitored each day. Nude mice were sacrificed at 50 days, and the weight and volume of tumor tissues were measured and analyzed. The tumor volume was determined by (length × width^2)/2. This experiment was approved by the Animal Scientific Ethics Committee of the Affiliated Provincial Hospital of Anhui Medical University (Hefei, China).

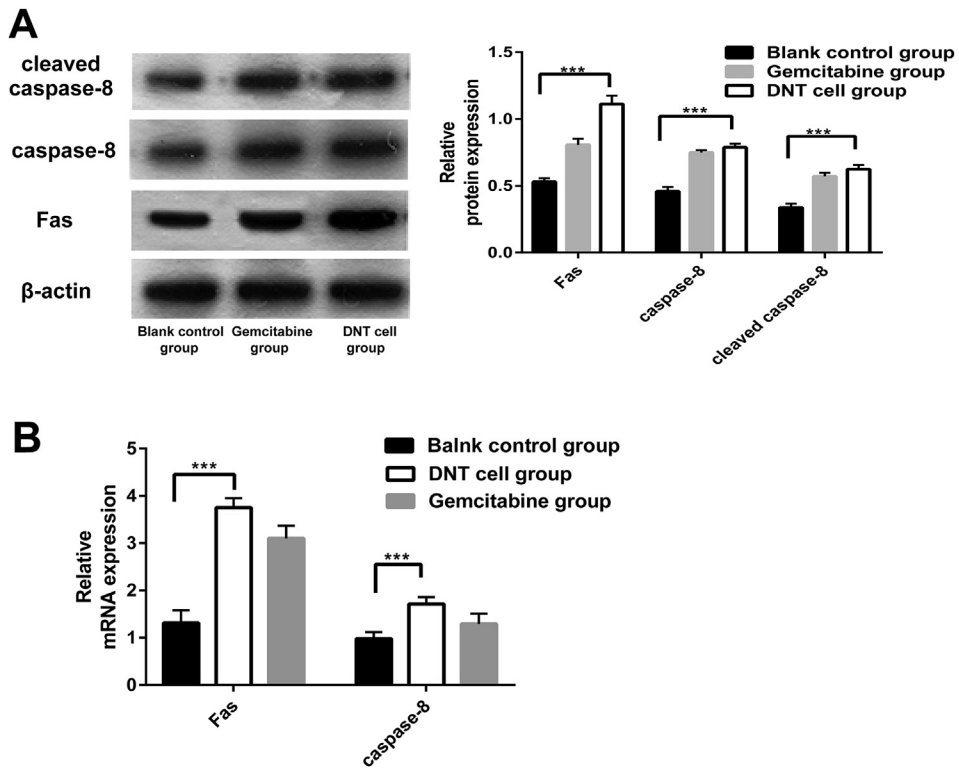

Fig. 4. DNT cells inhibited pancreatic cancer growth via the Fas pathway in vivo. A, Western blot analysis of Fas, caspase-8 and cleaved caspase-8 protein expression in tumor tissues from the three groups. B, qPCR analysis of Fas and caspase-8 expression in tumor tissues from the three groups. ***P < 0.001.

Western blotting

After 48 h of coculture with Panc-1 and SW1990 cells, the supernatant was discarded, cells were washed 3 times with phosphate-buffered saline (PBS), and suspended DNT cells were removed. Then, proteins were extracted from Panc-1 and SW1990 cells. In addition, proteins were extracted from murine tumor tissues. After full denaturation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), each sample was transferred to a polyvinylidene membrane. After the addition of skim milk for an appropriate dilution of anti-Fas, anti-caspase-8, anti-cleaved caspase-8 and anti-cleaved caspase-3 (1:500, Abcam, UK) antibodies, the membrane was incubated at room temperature for 2 h. After the membrane was washed, the secondary antibody was added at a dilution of 1:10,000, and the membrane was incubated in this solution at room temperature for 1.5 h. After another wash, detection reagent was added. Then, the membrane was exposed, developed in the dark and postfixed.

Quantitative real-time PCR

The levels of Fas and caspase-8 mRNA in pancreatic cancer cells and nude mouse tumor tissues were detected by quantitative polymerase chain reaction (qPCR). Cell processing before measurement was the same as that used for western blotting. Total RNA was extracted with TRIzol in accordance with the manufacturer's instructions. The OD260/OD280 was used to determine the purity and concentration of the RNA. cDNA was synthesized using a reverse transcription kit. According to the instructions of a real-time quantitative PCR sample kit, the following reaction conditions were used: 95 °C for 5 min and 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s for a total of 45 cycles. qPCR assays were repeated three times, and mRNA levels were determined according to the $2^{-\triangle\triangle Ct}$ method. The primers are shown in Table 1 (Invitrogen, USA).

TUNEL assay

Cocultured pancreatic cancer cells were fixed with Immunol Staining Fix Solution (Beyotime Biotechnology, China) for 30 min, and Enhanced Immunostaining Permeabilization Buffer (Beyotime Biotechnology, China) was added for 5 min at room temperature. After three washes with PBS, cancer cells were incubated with test solution from a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay kit (Beyotime Biotechnology, China). Animal model tumor tissue sections were first dewaxed and incubated with Proteinase K (20 μg/ml, Beyotime Biotechnology, China), and the remaining steps are the same as those used for cytology.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., USA). Data are expressed as the mean ± standard deviation (SD).

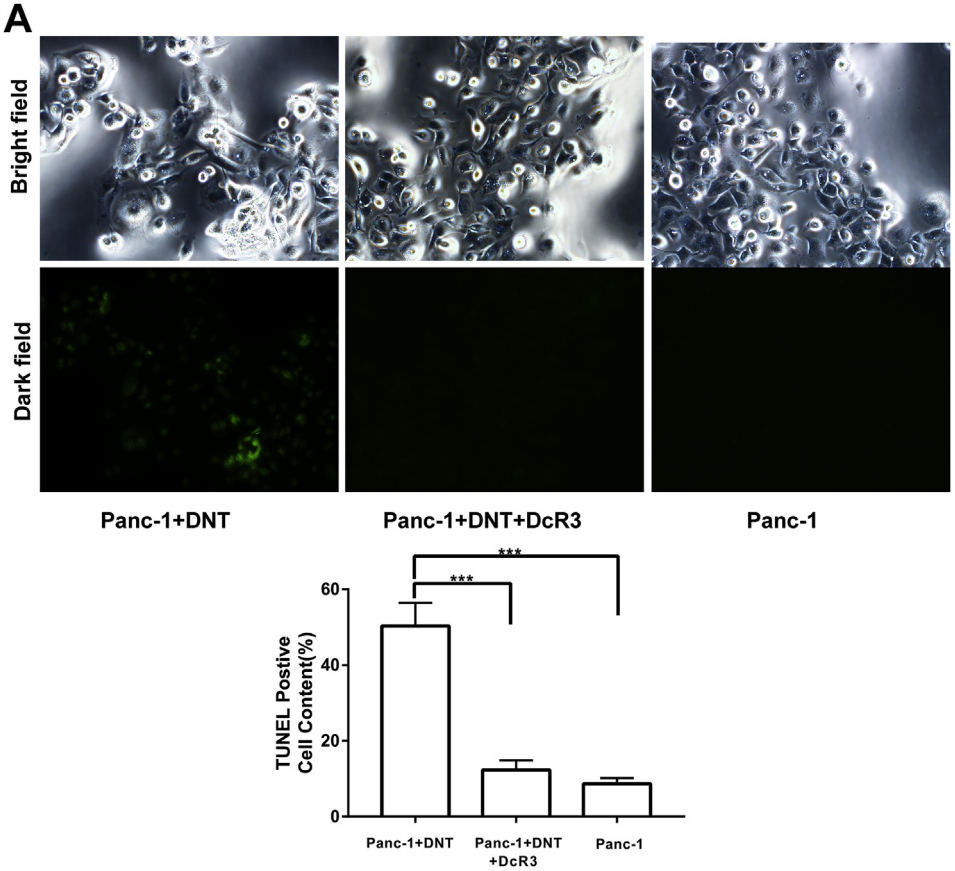

Fig. 5. DNT cells promoted pancreatic cancer cell and tissue apoptosis. A, TUNEL assays showing that the Panc-1 + DNT group had more apoptotic cells than did the other two groups. B, TUNEL assays showing that the SW1990 + DNT group had more apoptotic cells than did the other two groups. C, TUNEL assays showing that the DNT cell group and gemcitabine group had more apoptotic tissues than did the blank control group in the animal model. D, Western blot analysis of cleaved caspase-3 in cells from the three groups. E, Western blot analysis of cleaved caspase-3 in tumor tissues from the three groups. ***P < 0.001.

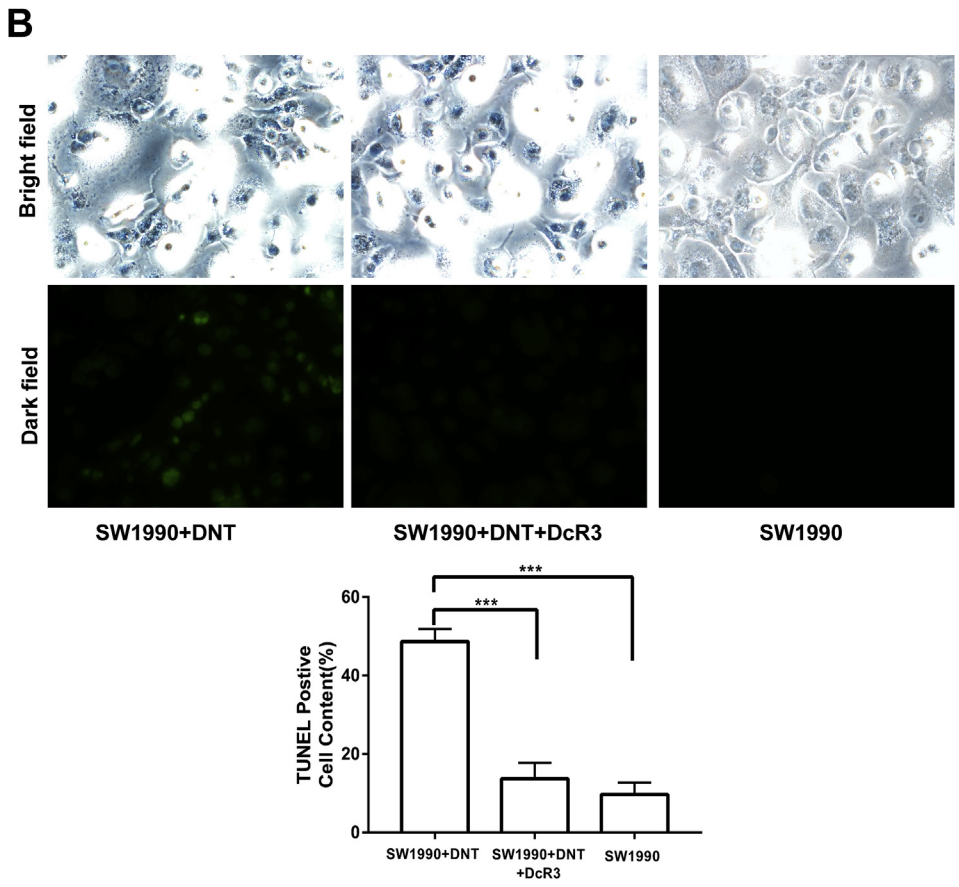

Fig. 5. (*continued*).

Comparisons between 2 groups were assessed using Student's t-test. Statistically significant differences among several groups were assessed using variance analysis. P values < 0.05 indicated statistical significance.

Results

DNT cell expansion

According to cell counts, the concentration of cells per well was as high as 1.0×10^6 cells/ml. DNT cells were also investigated by flow cytometry. The DNT cell phenotype was $CD3^+CD4^-CD8^-$ with >90% purity (Fig. 1A and B).

DNT cells inhibited pancreatic cancer growth via the Fas pathway in vitro

To ascertain the role and mechanism of DNT cells in pancreatic cancer growth, we cocultured DNT cells and pancreatic cancer cells and analyzed the effect. Panc-1 and SW1990 cell growth was significantly inhibited by DNT cells. However, treatment with DcR3 reduced the antitumor effect of DNT cells. Moreover, DcR3 had no effect on Panc-1 and SW1990 cell growth (Fig. 2A and B). Furthermore, the expression of Fas, caspase-8 and cleaved caspase-8 in the Panc-1 or SW1990 group, Panc-1 or SW1990 + DNT group and Panc-1 or SW1990 + DNT + DcR3 group was detected by western blotting and qPCR. Western blotting results showed that the protein levels of Fas, caspase-8 and cleaved caspase-8 were higher in the Panc-1 or SW1990 + DNT group than in the Panc-1 or SW1990 group, but these levels were decreased when blocking agent DcR3 was added (Fig. 2C). qPCR results revealed that Fas and caspase-8 mRNA levels in the three groups were similar to their protein levels (Fig. 2D and E).

DNT cells decreased the tumor size in animal models

The antitumor efficacy of DNT cells in vivo was evaluated by using subcutaneously xenografted Panc-1 cells. All 24 mice injected with tumor cells developed subcutaneous xenografted tumors and were sacrificed at 50 days (Fig. 3A and B). The tumor volume and weight of the DNT cell group ($646.5 \pm 68.6\ mm^3$, 225.0 ± 2.8 mg) and gemcitabine group ($523.3 \pm 66.9\ mm^3$, 224.7 ± 3.7 mg) were lower than those of the blank control group ($1618.0 \pm 196.1\ mm^3$, 263.2 ± 4.4 mg). However, no significant difference was observed in tumor growth between the DNT cell group and gemcitabine group (Fig. 3C and D), revealing their similar antitumor effects.

DNT cells inhibited pancreatic cancer growth via the Fas pathway in vivo

To identify the mechanism of DNT cell cytotoxicity in vivo, we used western blotting and qPCR to determine Fas, caspase-8 and cleaved caspase-8 expression. The protein levels of Fas, caspase-8 and cleaved caspase-8 in the DNT cell group were higher than those in the blank control group, and no significant difference was observed between the DNT cell group and gemcitabine group (Fig. 4A). Additionally, the mRNA levels of Fas and caspase-8 in the three groups were the same as the protein levels (Fig. 4B).

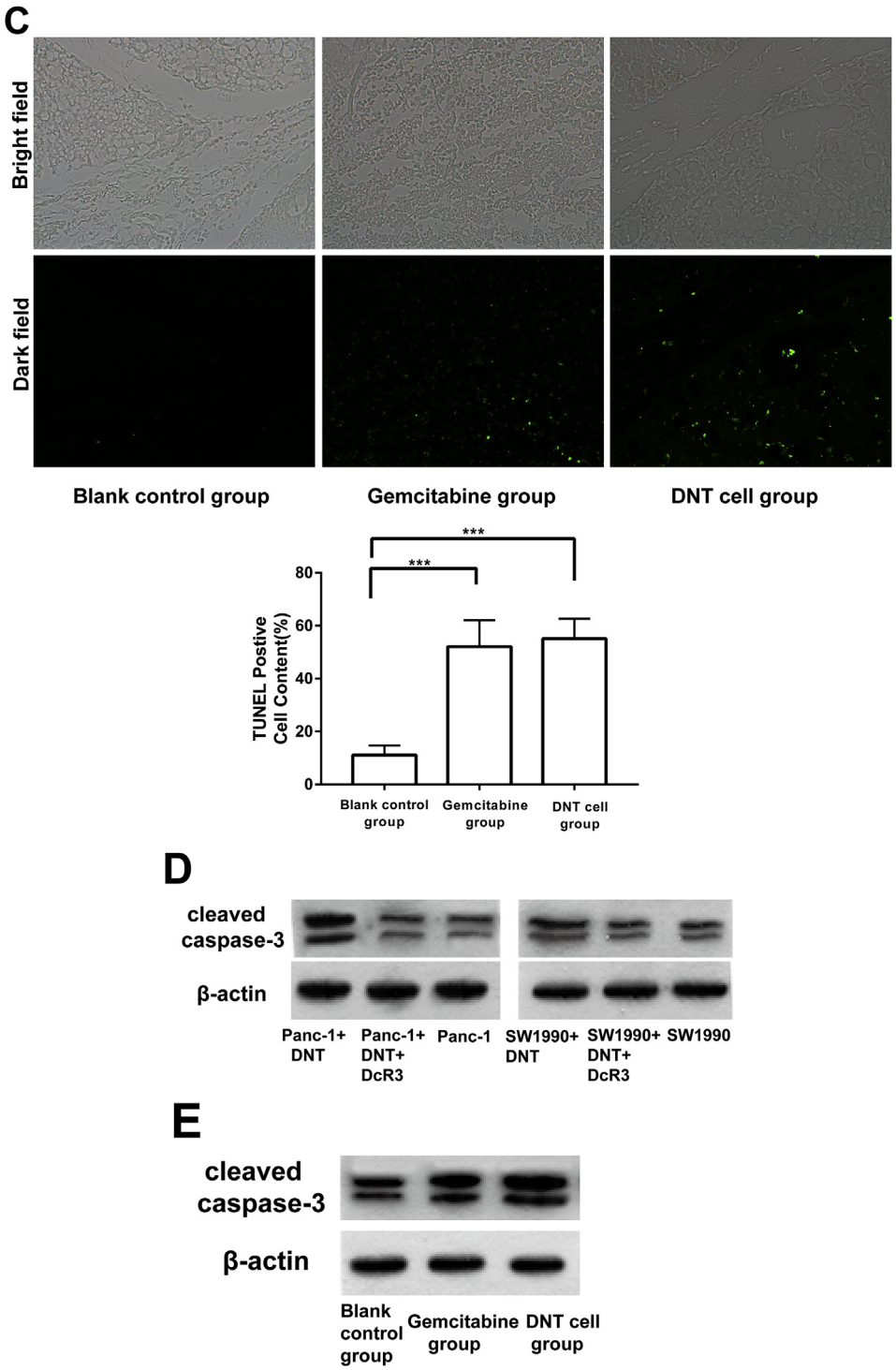

Fig. 5. (*continued*).

DNT cells promoted pancreatic cancer cell and tissue apoptosis

To evaluate whether apoptosis occurred in pancreatic cancer after treatment with DNT cells, we performed a TUNEL apoptosis assay and examined the expression of cleaved caspase-3. The activation of caspase-3 is a biochemical hallmark of apoptosis. Our TUNEL assay demonstrated that the cancer cell + DNT group had more apoptotic cancer cells than did the other two groups (Fig. 5A and B). In the animal tumor model, the DNT cell group and gemcitabine group had more apoptotic tissues than did the blank control group (Fig. 5C). Western blotting revealed that the expression of cleaved caspase-3 in the cancer cell + DNT group was higher than that in the other two groups (Fig. 5D). Moreover, the DNT cell group and gemcitabine group had higher cleaved caspase-3 expression than did the blank control group (Fig. 5E).

Discussion

Regulatory T cell populations play a critical role in the regulation of the tumor immunological response. Notably, recent studies have suggested that specific subsets of T cells (referred to as $CD3^+CD4^-CD8^-$ T cells) also play a role in this regulatory process. The majority of T cell receptor (TCR)+ lymphocytes in the peripheral blood and lymphoid organs of humans express CD4 or CD8 cell markers, whereas only 1–5% of T lymphocytes express TCR but not CD4 or CD8; these T lymphocytes are defined as DNT cells [17,18]. $CD8^+$ T cells, $CD3^+CD56^+$ T cells and NKT cells are antitumor cells with extensively investigated antitumor effects [19–21]. Unlike the aforementioned lymphocytes, DNT cells have not been widely reported in studies of cancer immunity, especially in solid cancers, because of their low purity and a lack of effective methods for their expansion. Merims et al. [7] developed a novel protocol by which DNT cells could be expanded ex vivo in 2 weeks. These ex vivo DNT cells effectively killed $CD34^+$ leukemic blasts, thus displaying antileukemic activity. By using the abovementioned procedures, Lee et al. [14] successfully amplified allogeneic human DNT cells that could be used as immunotherapy for acute myeloid leukemia. Here, we obtained enriched DNT cells from only 10 ml peripheral blood collected from volunteers using this novel protocol. In the present study, the growth of Panc-1 and SW1990 cells was inhibited when these cells were cultured together with DNT cells in vitro. In the nude mouse tumor model, DNT cells had an inhibitory effect on the growth of transplanted tumors in vivo. Moreover, apoptosis assays suggested that DNT cells could significantly promote cancer cell and tissue apoptosis. In addition, the antitumor effect of DNT cells was similar to that of the clinically used chemotherapy drug gemcitabine, which may provide further evidence for the clinical use of DNT cell immunotherapy for pancreatic cancer.

Recently, DNT cells specifically killing tumor cells has received increased attention from scholars [13,14], but the specific mechanism involved remains unclear. According to several studies, the Fas/FasL pathway is an important signal transduction pathway for apoptosis in cells and tissues [22–25]. When Fas is combined with FasL, Fas recruits the adapter protein Fas-associated death domain (FADD) and caspase-8, transforms into a death-inducing signaling complex (DISC), activates caspase-3 and finally induces cell apoptosis. Caspases can be cleaved into an activated form [26–28]. The abovementioned proteins all belong to the Fas-mediated pathway effector proteins. Based on several studies, Fas, caspase-8, cleaved caspase-8 and cleaved caspase-3 expression is increased when the Fas pathway is activated [29–32]. In a previous study by Young et al. [6], DNT cells inhibited the growth of lymphoma via the Fas pathway. In this study, the changes in pathway effector protein expression and the DNT cell antitumor effect were the most likely explanation for the involvement of the Fas signaling pathway in DNT cell antipancreatic effects in vitro. Here, the expression of the pathway effector proteins Fas, caspase-8, cleaved caspase-8 and cleaved caspase-3 in cancer cells cocultured with DNT cells was higher than that in untreated cancer cells. Blocking agent DcR3 competitively binds to FasL, preventing its binding to Fas receptors and reducing death signal transmission [33]. When DNT cells were pretreated with blocking agent DcR3 before coculture with cancer cells, the expression of pathway effector proteins in cancer cells was decreased, and the antitumor effect of DNT cells was significantly reduced. Therefore, the Fas pathway plays a positive role in the antitumor effect of DNT cells on pancreatic cancer cells in vitro.

In the nude mouse model experiment, western blotting and qPCR analyses showed that the expression of pathway effector proteins Fas, caspase-8, cleaved caspase-8 and cleaved caspase-3 in mouse tumor tissues was higher in the DNT cell group than in the blank control group. This result was similar to that of our in vitro experiment. This finding suggested that DNT cell cytotoxicity to pancreatic cancer in vivo may also be mediated via the Fas/FasL pathway. However, additional in vivo studies are needed for verification and to make our conclusions more convincing. Moreover, the result showed an interesting phenomenon that gemcitabine had similar effects on the activation of Fas pathway compared to DNT cells. But the studies on the inhibitory effects of gemcitabine on the growth of pancreatic cancer via the Fas signaling pathway are lacking. It is still unclear that the mechanism for both DNT cells and gemcitabine through Fas signaling pathway are different or the same. Gemcitabine is a commonly used chemotherapeutic drug. It may have the ability to directly activate the Fas pathway compared to DNT cells which rely on their own FasL. Therefore, further studies are needed to verify our hypothesis.

Inevitably, this study has limitations. For example, the animal injection method as well as the quantity and frequency of DNT cell and gemcitabine treatments was not based on previously published models.

In summary, our study demonstrated that pancreatic cancer growth is significantly inhibited by DNT cells both in vivo and in vitro and that the Fas/FasL signaling pathway is involved in the mechanism of DNT cell cytotoxicity. More importantly, this research may provide a preclinical basis for DNT cell therapy for pancreatic cancer.

Disclosure

None.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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