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Double-negative T Cells Inhibit Proliferation and Invasion of Human Pancreatic Cancer Cells in Co-culture

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Abstract. *Background/Aim: Double-negative T (DNT) cells are phenotypically $CD3^+CD4^-CD8^-$T cells. This study aimed to investigate the anti-cancer activity of DNT cells against pancreatic cancer cells. Materials and Methods: DNT cells were isolated from human peripheral blood. The effect of DNT cells on proliferation and invasion of the human pancreatic cell line Panc-1 was assessed. Expression of Nrf2 and Fas in Panc-1 cells co-cultured with DNT cells was analyzed with RT-PCR. The supernatants of Panc-1 and DNT co-cultures were analyzed with ELISA for IFN-r and FasL levels. Results: The isolated DNT cell phenotype was $CD4^-CD8^-CD56^-CD3^+$TCR (T cell receptor) α/β^+ T cells with more than 90% purity. Panc-1 cell proliferation was significantly inhibited by co-culture with DNT cells. Panc-1 cells co-cultured with DNT cells showed significantly reduced cell invasion. Panc-1 cells co-cultured with DNT cells showed increased Nrf2 and Fas mRNA expression. Increased INF-r and FasL levels were detected in the supernatants of co-cultures of DNT and pancreatic cells. Conclusion: DNT cells inhibited proliferation and invasion of human pancreatic cancer cells. The INF-r, Fas/FasL pathway and Nrf2 may be involved in the anti-cancer effect of DNT cells against human pancreatic cancer.*

Pancreatic cancer is one of the most lethal human malignancies (1). The majority of patients with pancreatic cancer are diagnosed with metastasis or late stages of disease and are surgically inoperable (2). Pancreatic cancer has the lowest 5-year survival rate among other cancers, which is less than 5% (3). Many patients respond poorly to first-line gemcitabine monotherapy (4). It has been reported that therapy with immune checkpoint inhibitors is not sufficiently effective in patients with pancreatic cancer (5, 6). Therefore, there is an urgent need to find safer and more effective immunotherapies for pancreatic cancer patients.

Double-negative T (DNT) cells are phenotypically $CD3^+CD4^-CD8^-$T cells, which constitute a small but important fraction of T cells. Studies have suggested that DNT cells possess an immunoregulatory/suppressive function and can prevent graft-versus-host disease and autoimmunity (7, 8). Previous reports have demonstrated that DNT cells have significant anti-cancer effects in patient-derived xenograft (PDX) models of lung cancer and leukemia (9-12). Moreover, infusion of allogeneic DNT cells did not induce graft *versus* host disease (13). These unique features of DNT cells differentiate them from conventional T cells and support their potential use as a new adoptive cellular therapy for cancers (13). However, previous studies on DNT cell cytotoxicity and its underlying mechanism in pancreatic cancer are limited (14).

In the present study, we isolated DNT cells from human peripheral blood and investigated their activity against pancreatic cancer cells and the underlying mechanism.

Materials and Methods

Cancer cell line. The human pancreatic cancer cell line Panc-1 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Panc-1 cells were cultured in RPMI 1640 modified medium containing 10% fetal bovine serum, L-Glutamine (2.05 mM), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C.

Isolation of double-negative T cells. DNTs were isolated from the peripheral blood by using a double negative T cell isolation kit according to the manufacturer's instructions (130-092-614, Miltenyi, Germany). Firstly, $CD4^+$, $CD8^+$, and $CD56^+$ cells were labeled with the double-negative T cell biotin-antibody cocktail and anti-biotin MicroBeads. The magnetically labeled cells were

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Key Words: Double-negative T cell, pancreatic cancer, cell proliferation invasion, Nrf-2.

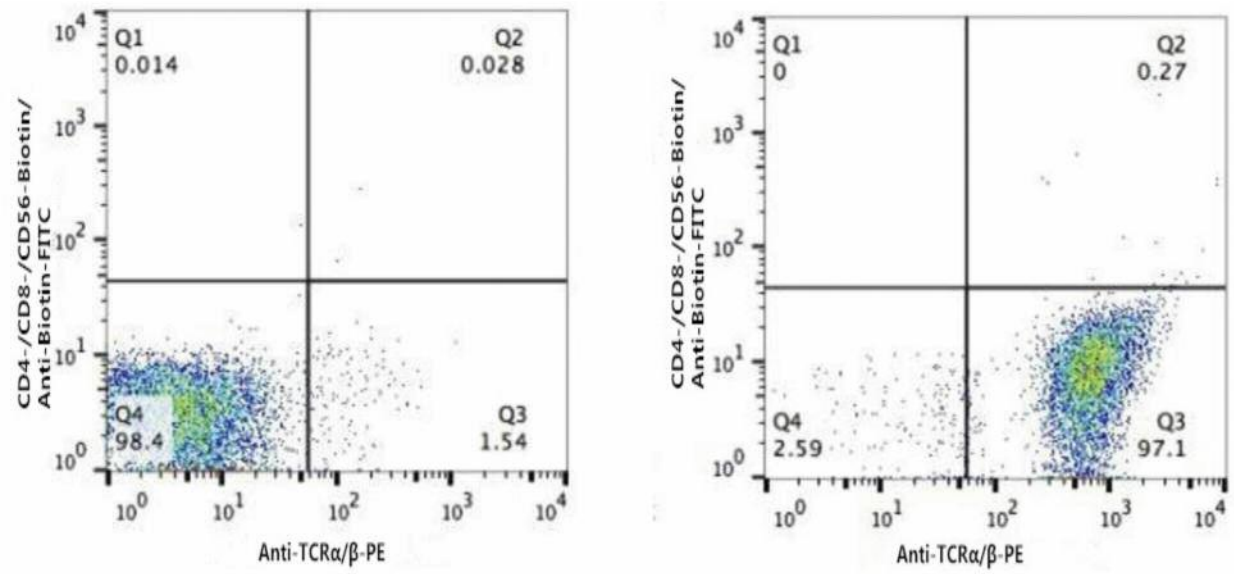

Figure 1. *Isolation and characterization of DNT cells. DNT cells were isolated from peripheral blood of healthy donors and characterized by flow cytometric analysis. The DNT cell phenotype was CD4⁻CD8⁻CD56⁻CD3⁺TCRα/β⁺ and their purity was higher than 90% purity.*

separated using an LD Column on the autoMACS Separator. Secondly, the selected TCR (T cell receptor) α/β⁺CD4⁻CD8⁻ cells present in the flow-through faction were incubated with Anti-PE MicroBeads and separated with MS column. The double-negative T cells were retained within the column and eluted by removing the column from the magnetic field.

Flow-cytometric analysis. Flow cytometric analysis was used to characterize the isolated DNT cells. Cells were digested with 0.25% trypsin and washed with PBS containing 0.5% (w/v) bovine serum albumin (BSA). Cells were then incubated with PERCP conjugated anti-CD3 (300325, Biolegend, San Diego, CA, USA), FITC conjugated anti-CD4 (357405, Biolegend), PE conjugated anti-CD8 (46-0567-41, eBioscience, Waltham, MA, USA), CD56 (43-0546-23, eBioscience) and TCRα/β (42-0567-41, eBioscience) antibodies. A fluorescence activated cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze the cells.

Cell-proliferation assay. Cell proliferation was examined by the CCK8 assay. Panc-1 cells (3×10^4 cells) were transferred to each well of a 96-well plate in RPMI 1640 containing 10% FBS and cultured overnight. DNT cells were added in each well at DNT to Pnc-1 ratios of 1:10, 1:1, 10:1 and 100:1. Five duplicate wells were set up for each ratio of co-incubation and the test was repeated 3 times. After 72 h incubation, CCK8 reagent (10 μl) was added to each well for 4 h. The optical density (OD) at 450 nm wavelength was measured using microplate reader (Bio-Rad, Hercules, CA, USA). The cell proliferation curves of each group were plotted.

Co-culture system. Panc-1 and DNT cells were co-cultured indirectly in a 6-well transwell co-culture plates (0.4 μm polyester film). The DNT cells were seeded in the upper layer and Panc-1 cells were seeded in the lower layer at DNT to Pnc-1 ratios of 1:10, 1:1, 10:1 and 100:1. The cells were cultured at 37˚C (RPMI-1640 with 10% FBS and 5% CO_2).

Wound-healing assay. For wound-healing assays, Panc-1 cells (1.5×10^5) harvested from Panc-1 and DNT co-cultures were plated in 12-well plates for 24 h. Similar-sized wounds were created in cell mono-layers by scraping a gap using a micro-pipette tip. Reference points were marked using a scalpel on the outer surface of the plate. After cell debris was removed by rinsing with phosphate-buffered saline, fresh medium containing <2% FBS was added. The cells migrated from both sides of the wound and repopulated the gap area. Wound closure was observed at 48h using a microscope and the images at each time point were captured (magnification, ×40). Image J Software was used for analysis and quantification of the healing rate. The wound area closure was measured in three-independent wound sites per group. The wound-healing rate was calculated as following: (0 h wound area -24 h wound area)/0 h wound area ×100%.

Transwell assay. After coating the upper chamber of an insert with 40 μl of 20% Matrigel (BD, Franklin lakes, NJ, USA) in RPMI1640 medium, Panc-1 cells (1×10^5 cells/well) harvested from Panc-1 and DNT co-cultures in 200 ul medium were seeded onto the upper chamber. A total of 600 μl of medium containing 20% FBS was added to the lower chamber. The cells were fixed with ethanol following 48 h incubation. The cells that had invaded through the membrane were stained *via* crystal violet. Under an inverted microscope (Olympus BX53, Tokyo, Japan), the stained cells on the back of the membrane were counted.

Isolation of RNA and reverse transcription-polymer chain reaction (RT-PCR). Panc-1 cells were harvested from Panc-1 and DNT co-cultures. Total RNA of the Panc-1 cells was isolated with Trizol reagent according to the manufacturer's protocol. One μg of the total RNA was reverse transcribed using PrimeScript RT-PCR kit (Takara, Kyoto, Japan). The following specific primers of each gene were used for PCR amplification: Nrf2 (sense, 5' TTTTCCGATGACCAG GACTTA 3', and antisense, 5' CAACCCTTGTCACCATCTCAG 3'), Fas (sense, 5'

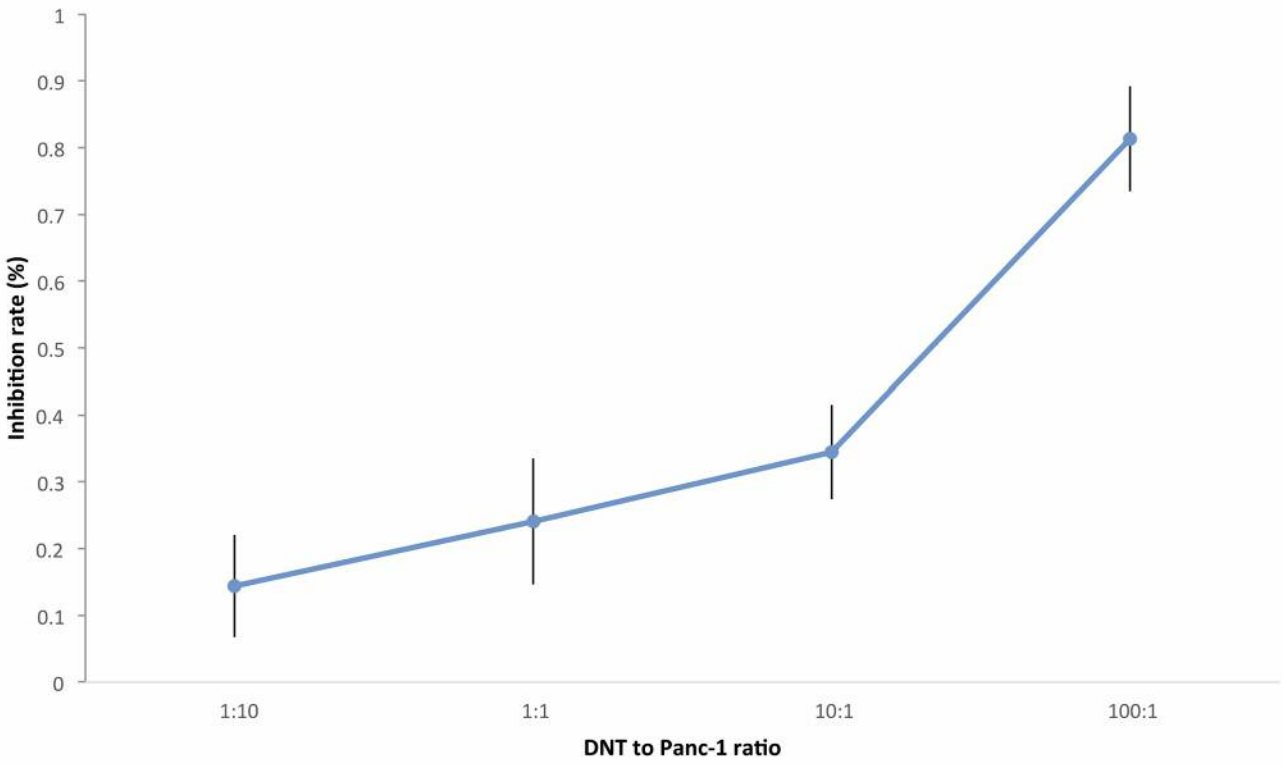

Figure 2. *Effect of DNT cells on pancreatic cancer cell proliferation. Cell proliferation was measured using the CCK8 assay. Panc-1 cell proliferation was significantly inhibited by co-culture with DNT cells in a ratio dependent manner.*

ACTTGGTGTTGCTGGTGAGTG 3', and antisense, 5' TGTGATGAA GGACATGGCTTAG 3') and GAPDH (sense, 5'-GC ACCGTCAAGG CTGAGAAC-3'and antisense, 5'-TGGTGAAGA CGCCAGTGGA-3').

Quantitative real-time PCR was carried out by using a 7300 Real-time PCR system (Applied Biosystems, Waltham, MA, USA). Data were analyzed by the relative standard curve method and normalized to that of GAPDH gene in the same sample. Finally, the relative RNA expression level in the gastric cancer cell lines or tumor tissues were calculated with the comparative $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA). Supernatants were collected from Panc-1 and DNT co-cultures. IFN-r and FasL levels in the supernatant were determined with ELISA kits (Abcam, Cambridge, MA, USA) according to manufacturer's instructions. To coat 96-well plates, anti- IFN-r or anti-FasL antibody was added and incubated overnight at 4˚C, followed by the addition of bovine serum albumin and incubating at 37˚C for 2 h for blocking. Then co-culture supernatant samples or standard (100 µl/well) was added to each well and incubated at 37˚C for 1 h. Then, anti-IFN-r or anti-FasL antibody was added for 1 h at 37˚C followed by the addition of horseradish-peroxidase-labeled avidin for 30 min at 37˚C. Afterwards, tetramethylbenzidine substrate (100 µl/well) was added to each well and incubated at 37˚C for 30 min. The reaction was stopped by adding H_2SO_4 (2 mol/l, 50 µl/well) and optical density (OD) was measured at 450 nm by a microplate-reader (ELx800uv; Bio-Tec Instruments, Winooski, VT, USA).

Statistical analysis. SPSS16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All results were expressed as mean±SD and a Student's *t*-test was used for comparisons between two groups. *p*-Value of significant positive expression was less than 0.05 ($p<0.05$).

Results

Isolation and characterization of DNT cells. To purify TCRα/β$^+$ DN T cells, we used a magnetic depletion system to eliminate CD4$^+$, CD8$^+$ and CD56$^+$. The isolated DNT cells were characterized with flow cytometric analysis. The DNT cell phenotype was CD4$^-$CD8$^-$CD56$^-$CD3$^+$TCRα/β$^+$. Purity was higher than 90% (Figure 1).

DNT cells inhibit pancreatic cancer cell proliferation. To investigate the effect of DNT cells on pancreatic cancer cell proliferation, we co-cultured DNT cells and pancreatic cancer Panc-1 cells at DNT to Panc-1 ratios of 1:10, 1:1, 10:1 and 100:1. Cell proliferation was measured using the CCK8 assay. As shown in Figure 2, Panc-1 cell proliferation was significantly inhibited by the co-culture with DNT cells in a ratio-dependent manner.

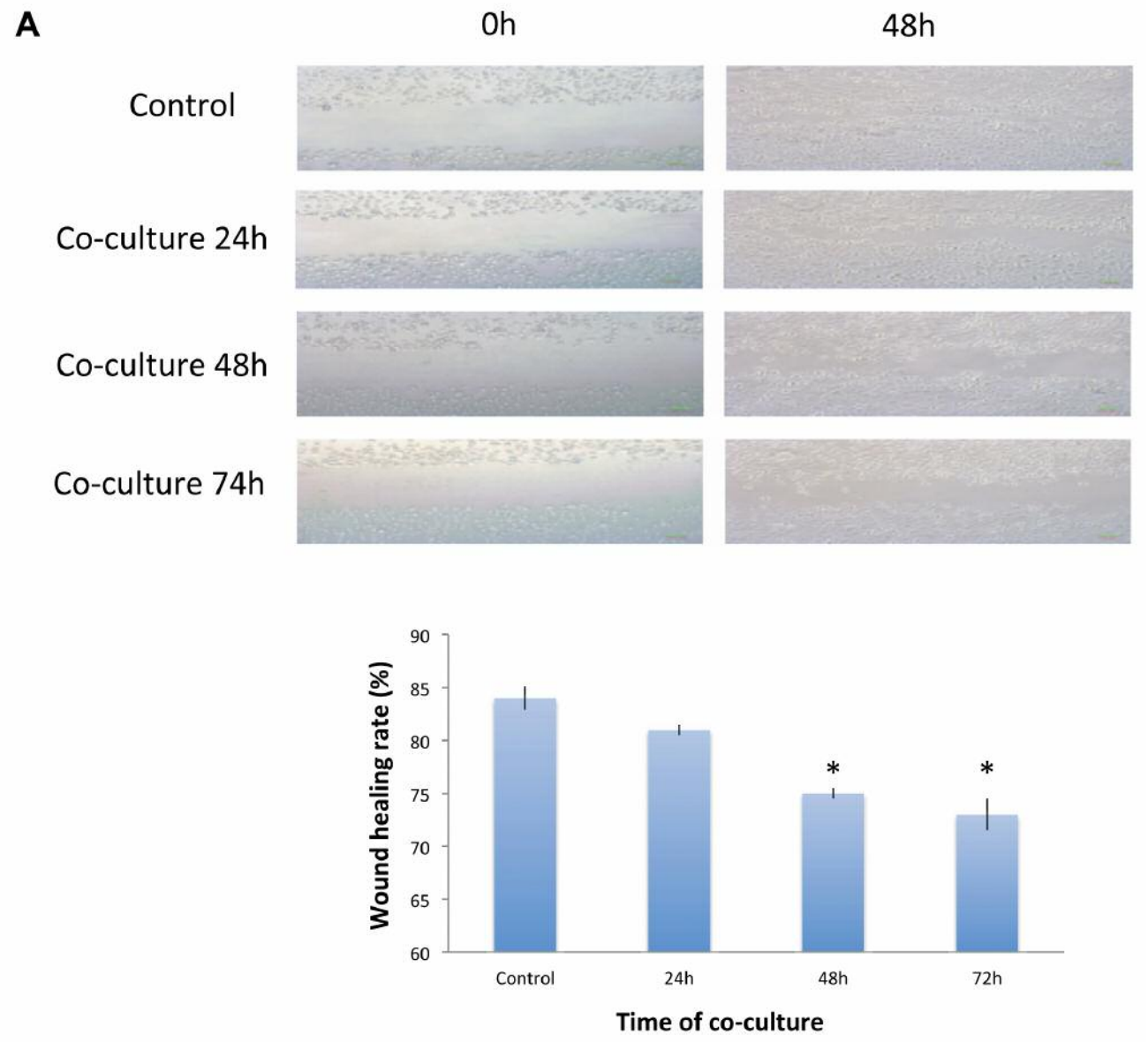

Figure 3. *Continued*

DNT cells inhibit pancreatic cancer cell invasion. Panc-1 cells were co-cultured with DNT cells at DNT to Panc-1 ratios of 1:10, 1:1, 10:1 and 100:1. Panc-1 cells co-cultured at 100:1 ratio for 24 h, 48 and 72 h were examined for their invasive ability by wound healing and transwell assays. As shown in Figure 3A, Panc-1 cells co-cultured for 48 h and 72 h with DNT cells showed significantly reduced cell migration compared to Panc-1 cell control ($p<0.05$) in the wound healing assay. Furthermore, in the transwell assay, the average number of cells migrating to the lower chamber was significantly decreased for Panc-1 cells co-cultured for 48 and 72 h with DNT cells compared to Panc-1 control cells ($p<0.05$) (Figure 3B).

DNT cells induce expression of Nrf2 and Fas in pancreatic cancer cells. Panc-1 cells were co-cultured with DNT cells at DNT to Panc-1 ratios of 1:10, 1:1, 10:1 and 100:1 for 24, 48 and 72 h. The Panc-1 cells were then analyzed for the expression of Nrf2 and Fas by RT-PCR. As shown in Figure 4A, Nrf2 mRNA expression was significantly increased in the Panc-1 cells co-cultured with DNT in a ratio-dependent manner compared to Panc-1 control cells ($p<0.05$). In addition, Panc-1 cells co-cultured with DNT for 24 and 48 h showed a significantly increased Fas mRNA expression in a ratio-dependent manner compared to the Panc-1 control cells ($p<0.05$). However, Fas mRNA expression was significantly

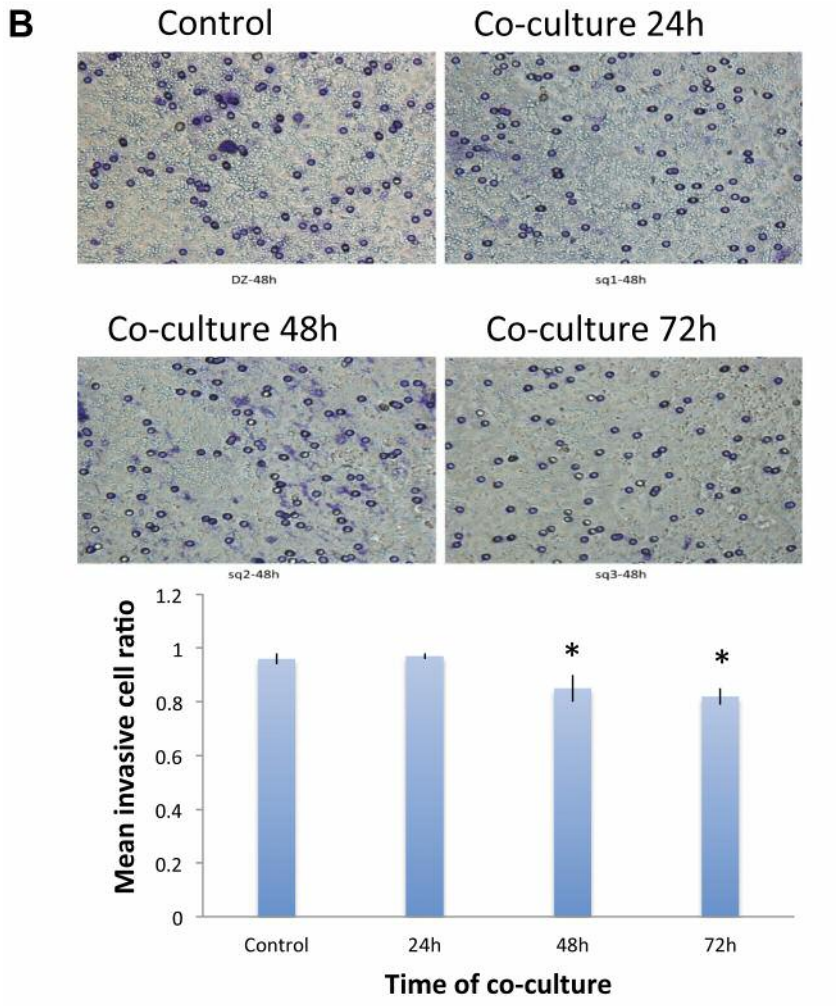

Figure 3. *Effect of DNT cells on pancreatic cancer cell invasion. Panc-1 cells co-cultured at a 100:1 ratio for 24, 48 and 72 h with DNT cells were examined for their invasive ability by wound healing and transwell assays. A. Wound healing assay. Panc-1 cells co-cultured with DNT cells for 48 and 72 h showed significantly reduced cell migration. *p<0.05, when compared to Panc-1 control cells. B. Transwell assay. The average number of cells migrating to the lower chamber was significantly decreased in the Panc-1 cells co-cultured with DNT for 48 and 72 h. *p<0.05, when compared to Panc-1 control cells.*

reduced in Panc-1 cells co-cultured with DNT cells for 72 h compared to Panc-1 control cells ($p<0.05$) (Figure 4B).

DNT cells induce secretion of IFN-r and FasL in pancreatic cancer cells. Panc-1 cells were co-cultured with DNT cells at DNT to Panc-1 ratios of 1:10, 1:1, 10:1 and 100:1 for 24, 48 and 72 h. The supernatants from Panc-1 and DNT co-cultures were collected and analyzed with ELISA for IFN-r and FasL levels. As shown in Figure 5A, increased IFN-r levels were detected in the supernatants from the co-culture at 100:1 ratio in a time-dependent manner compared to Panc-1 control cells ($p<0.01$) (Figure 5A). In addition, increased FasL levels were detected in the supernatants from the co-culture at 100:1 ratio compared to the Panc-1 control cells ($p<0.01$). However, FasL level was significantly reduced in the supernatants from the co-culture for 72 h compared to 24 h ($p<0.05$) (Figure 5B).

Discussion

Adoptive cellular therapy based on DNT cells has been considered for some malignancies (9, 11-13). In the present study, we obtained enriched DNT cells from human peripheral blood and co-cultured them with the human pancreatic cancer cell line Panc-1. We found that DNT cells significantly inhibited pancreatic cancer cell proliferation and invasion.

It has been reported that Fas/FasL pathway is involved in anti-cancer mechanism of DNT (15). The Fas/FasL

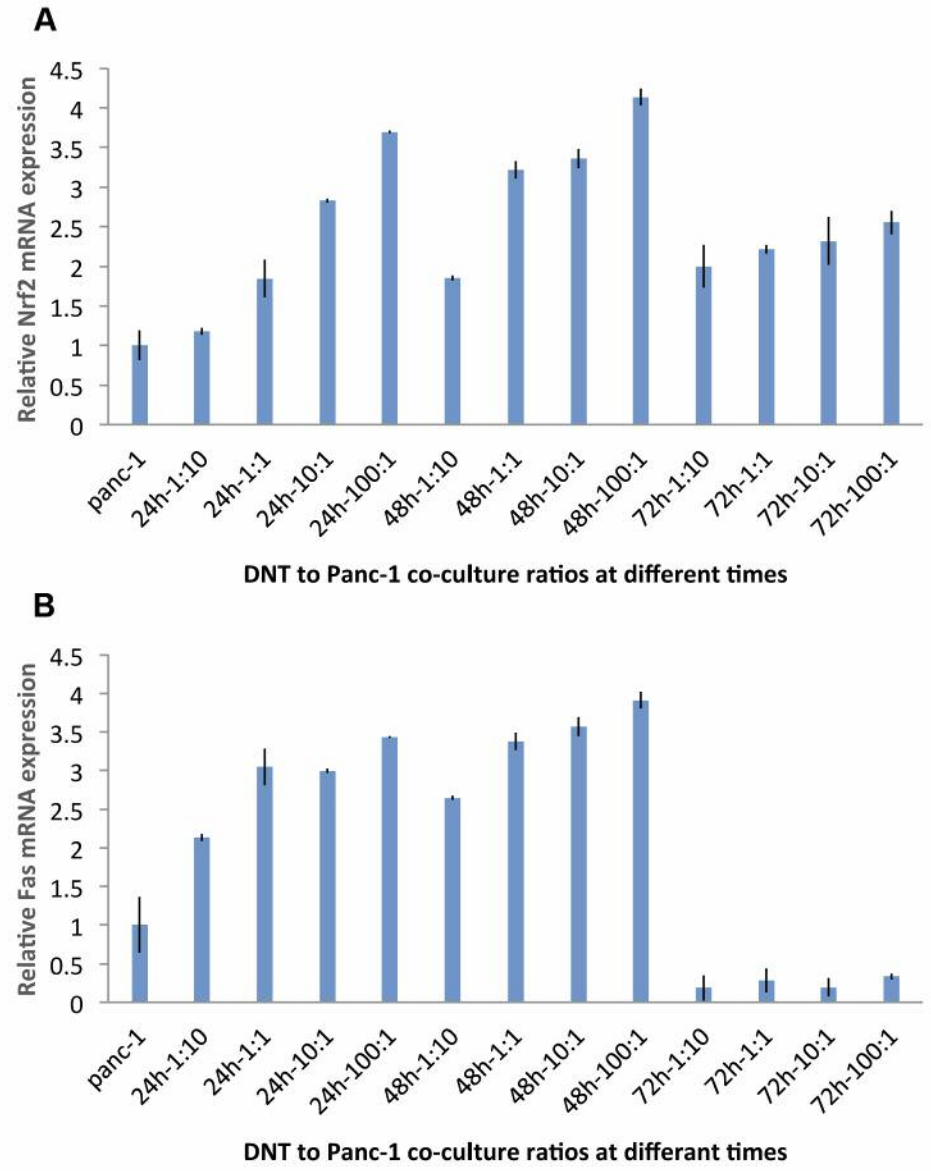

Figure 4. *Effect of DNT cells on the expression of Nrf2 and Fas in pancreatic cancer cells. Panc-1 cells were co-cultured with DNT cells at different DNT to Panc-1 ratios for 24, 48 and 72 h. The Panc-1 cells were then analyzed for the expression of Nrf2 and Fas by RT-PCR. A. Nrf2 mRNA expression was significantly increased in the Panc-1 cells co-cultured with DNT for 24h, 48 h and 72h in a ratio dependent manner compared to Panc-1 control cells. B. Panc-1 cells co-cultured with DNT for 24 and 48 h showed a significantly increased Fas mRNA expression in a ratio-dependent manner compared to Panc-1 control cells.*

pathway is associated with cell apoptosis (16, 17). When combining with FasL, Fas induces cell apoptosis by promoting the formation of death-inducing signaling complex (DISC) between the adapter protein Fas-associated death domain (FADD) and caspase-8, and activating caspase-3. Young *et al*. (15) have reported that DNT cells suppressed lymphoma growth through the Fas pathway. In the present study, we detected Fas and Fas L expression in co-cultures of DNT and Panc-1 cells. We found that Fas and Fas L expression levels were significantly increased, suggesting that the Fas/FasL pathway plays an important role in the inhibition of pancreatic cancer cell proliferation and invasion by DNT.

The transcription factor Nrf2 (Nuclear erythroid related factor-2) plays an essential role in response to oxidative stress, toxins and carcinogens (18). Nrf2 has been found to be involved in cancer development (19) and therapy resistance (20). It has also been reported that antioxidants

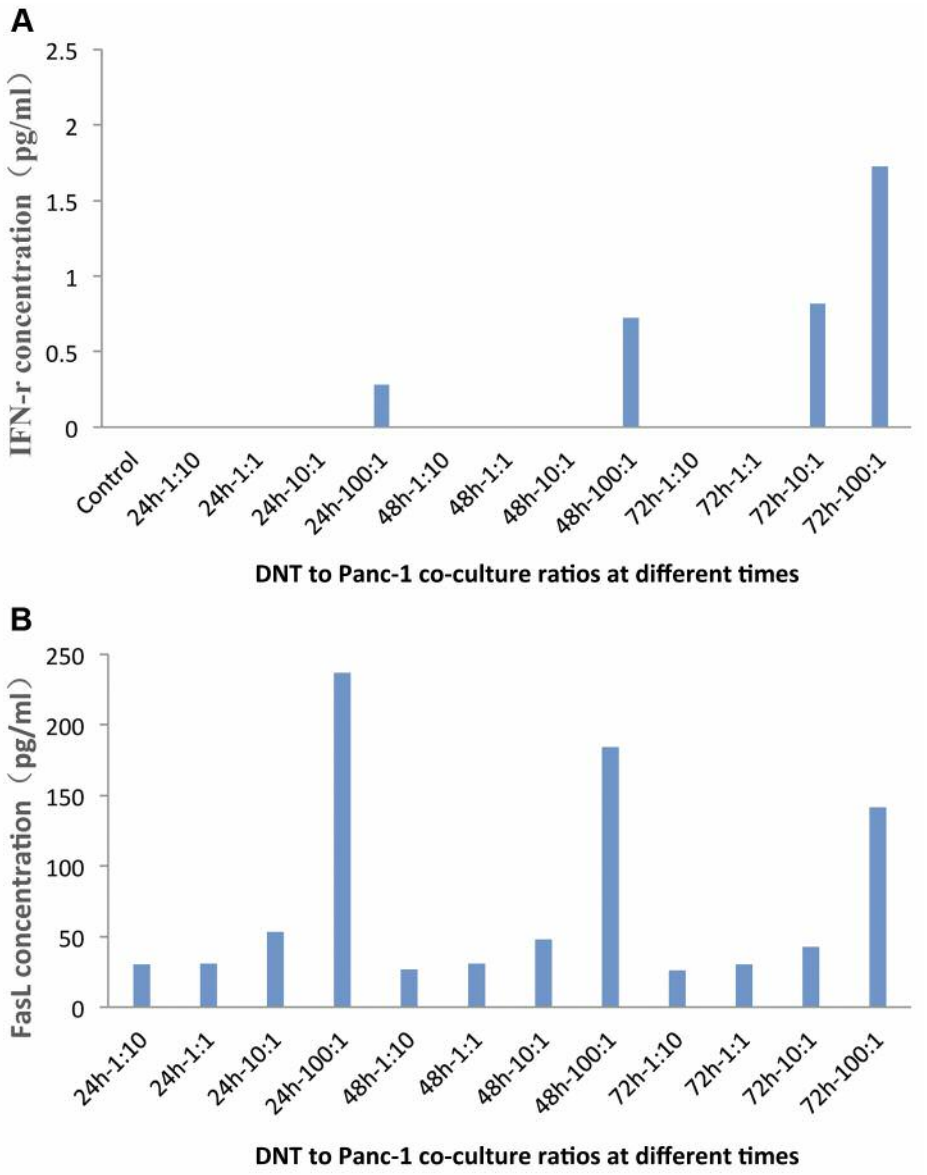

Figure 5. *Effect of DNT cells on the secretion of IFN-r and FasL in pancreatic cancer cells. Panc-1 cells were co-cultured with DNT cells at different DNT to Panc-1 ratios for 24, 48 and 72 h. The supernatants from Panc-1 and DNT co-cultures were collected and analyzed with ELISA for IFN-r and FasL levels. A. Increased IFN-r levels were detected in the supernatants from the co-culture at 100:1 ratio in a time-dependent manner compared to Panc-1 control cells. B. FasL levels were increased in the supernatants from the co-culture at 100:1 ratio compared to Panc-1 control cells.*

elicit accumulation and up-regulation of Nrf2 (21). In the present report, we showed for the first time that the co-culture of DNT with Panc-1 cells inhibited proliferation and led to up-regulation of Nrf2 in the latter, which indicates that Nrf2 may participate in the efficacy of DNT cell against pancreatic cancer.

In conclusion, DNT cells inhibit cell proliferation and invasion in human pancreatic cancer cells. INF-r, Fas/FasL pathway and Nrf2 may be involved in the efficacy of DNT cells against human pancreatic cancer.

Conflicts of Interest

None of the Authors have any conflict of interest with regard to this study.

Author's Contributions

Yin Lu designed the study, analyzed the data and wrote draft manuscript; Jiong Chen participated study design; Pibo Hu, Haibo Zhou and Yu Sun performed experiments; Zhijian Yang and Robert M. Hoffman revised the manuscript.

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