Natural killer cells inhibit metastasis of ovarian carcinoma cells and show therapeutic effects in a murine model of ovarian cancer

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Abstract. Ovarian cancer has the highest mortality rate and is the most common of all gynecologic malignancies. Novel treatments for ovarian cancer are urgently required to improve outcomes and the overall survival of patients. The present study investigated whether immunotherapy with natural killer (NK) cells affected the survival of mice with ovarian cancer. Results analysis identified adjunctive NK cells as a potential therapeutic method in ovarian cancer. Patient-derived ovarian cells were isolated, cultured and subsequently injected subcutaneously into immune deficient BALB/c-nude mice. Human NK cells were isolated from peripheral blood mononuclear cells and cultured for expansion *in vitro*. The present results demonstrated that ovarian cells in BALB/c-nude mice did not induce spontaneous ovarian cancer cell metastasis in the NK-treated group. In addition, NK cells activated immune cells in the immune system, which resulted in inhibition of ovarian tumor growth *in vitro* and in a murine xenograft model of ovarian cancer. The data also indicated that cytotoxic activity of NK cells prevented migration and invasion of ovarian cancer cells, which contributed to prevention of systemic metastasis and suggested that NK cells could be effective cells for therapy against ovarian cancer. Furthermore, NK cells induced apoptosis and increased the number of cluster of differentiation (CD)4$^+$, CD8$^+$ as well as cytotoxic T lymphocyte responses by intravenous injection in a murine xenograft model of ovarian cancer. These results suggested that NK cells inhibited the systemic metastasis for ovarian cancer cells. In conclusion, the present study suggested that NK cell immunotherapy inhibited systemic metastasis of ovarian cancer cells and improved the survival rate of mice. Sufficient supplementation of NK cells may serve as a promising immunotherapeutic strategy for ovarian cancer.

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Introduction

Worldwide, ovarian cancer is the most common, with the highest incidence and mortality, of all gynecologic malignancies (1). Ovarian cancer is a diverse disease and it presents high morbidity and mortality as it is often diagnosed at a late stage (2). Previous research has reported that the incidence rate of ovarian cancer is increasing worldwide and presents a rapid expansion trend (3). Women with ovarian cancer demonstrated continued symptoms of depression and anxiety for cancer survivors who had undergone chemotherapy treatment, according to a meta-analysis of prevalence rates (4). Ineffective or prolonged management of treatment could contribute to worsening of symptoms, treatment noncompliance and even reduced health-related quality of life (5).

Previous studies of ovarian cancer have been large-scale, multiregional and longitudinal cohorts for diagnosis, treatments and prognosis in preclinical and clinical trials (6-8). Increasing reports were engaged to research and improve treatment for patients with ovarian cancer, focusing on the improvement of diagnosis and the identification of novel therapies (3). In addition, the overall prognosis and therapy for patients with ovarian cancer remained poor despite increasing improvements in perioperative management, surgical techniques and other treatments (9,10). Therefore, effective therapeutic agents and protocols for patients with ovarian cancer are urgently required due to the high rate of occurrence and likelihood of metastasis following curative resection in clinics (11).

Treatments for ovarian cancer have been demonstrated to be poor at prevention and ineffective, resulting in a bad prognosis (12). The majority of newly diagnosed ovarian cancer cases are often at an advanced stage due to the lack of sensitive screening diagnosis at the early stage (13). Traditional treatments, including radiotherapy, chemotherapy and

surgery, are limited to palliative approaches for patients with advanced ovarian cancer (2,14). However, many patients with advanced ovarian cancer respond poorly to such treatments, with these traditional treatments demonstrating limited efficacy (15). Therefore, adjunctive therapeutic options are important for patients after receiving one or more anticancer treatments.

Immunotherapy is an efficient treatment for cancer due to its antitumor effects induced by stimulating host immune responses for cytotoxic lymphocyte activities against cancer cells in the microenvironment (16). Molecular mechanisms that inhibit systemic metastasis of ovarian cancer would be a novel therapeutic candidate for patients with ovarian cancer (17,18). Cellular immunotherapy exhibits immense potential to be a highly-targeted alternative to traditional treatments, which possesses the lowest or no toxicity to normal cells and demonstrates notable capacity to eradicate tumor cells (19-21). Cellular immunotherapy often employs active immunization with immune cells, including infiltrating T cells, effectors T cells and cytotoxic T cells, using adoptive transfer of T cells from the patients themselves to directly target antigens on malignant cells (22,23). The present study generated an efficient cellular therapy by using NK cells in an ovarian xenograft mouse model. The results suggested that natural killer (NK) immunotherapy inhibits the systemic metastasis of tumors in mice with ovarian cancer, which provided preclinical information of the potential role of NK cells for patients with ovarian cancer.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Weifang City People's Hospital (Weifang, China).

Cells culture and reagents. Ovarian cancer cells from one patient with ovarian cancer (54.2 years old) were collected with written informed consent on May 2015 in Weifang People's Hospital (Weifang, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biowhittaker; Lonza Group, Ltd., Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Biowhittaker; Lonza Group, Ltd.) at 37˚C and 5% CO_2 for 24 h. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy mice and cultured as described in a previous study (24). SKOV3, OVCAR3, CAOV-3 and A2780 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All tumor cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin under standard culture conditions (5% CO_2, at 37˚C) for 12 h.

Lactate dehydrogenase (LDH) assay. The ovarian tumor cells were cultured until a 90% confluency of monolayer cells was reached and then the media was removed. The ovarian tumor cells were washed with PBST three times and subsequently incubated with Triton X-100 (1%) for 30 min at 37˚C. LDH activity in the lysates was measured by using a Promega CytoTox 96 assay kit (Promega Corporation, Madison, WI, USA). The procedures were conducted according to the manufacturer's protocol.

Cell invasion and migration assays. Patient-derived ovarian cancer cells were treated with NK cells (effector:target ratios=15:1) for 24 h at 37˚C and non-treated cells were used as control. For the invasion assay, NK-treated cells were suspended at a density of $1x10^5$/ml cells in 500 μl serum-free DMEM. Matrigel-coated and uncoated Transwell inserts (8 μm pore size; Merck KGaA, Darmstadt, Germany) were used to evaluate cell invasion and migration, respectively. The ovarian tumor cells ($2x10^5$) were then subjected to the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA) for 48 h at 37˚C according to the manufacturer's protocol. For the migration assay, patient-derived ovarian cancer cells were treated with NK cells and PBS for 48 h using a control insert (BD Biosciences) instead of a Matrigel Invasion Chamber. DMEM and DMEM with 5% FBS was plated in the upper and lower chambers, respectively. Cells were then fixed in 4% paraformaldehyde for 15 min at 37˚C and stained with 0.1% crystal violet dye (Sigma-Aldrich; Merck KGaA) for 20 min at 37˚C. Tumor cell invasion and migration was counted in at least three randomly stained fields under a light microscope (Olympus Corporation, Tokyo, Japan) for every membrane (magnification, x40).

Expansion of NK cells in vitro. NK cell expansion was performed *in vitro* by using VarioMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and cultured in MEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 72 h at 37˚C. On day 5, MEM medium (Thermo Fisher Scientific, Inc.) was refreshed with the addition of autoplasma (1%) (Sigma-Aldrich; Merck KGaA) and interleukin-2 (500 IU/ml) (Sigma-Aldrich; Merck KGaA). Culture continued for 14 days at 37˚C. Cells were then fixed with 4% paraformaldehyde for 15 min at 37˚C and the viability of expanded NK cells was determined by 5% propidium iodide staining for 2 h at 37˚C as described previously (25). *In vitro*-expanded NK cells were stained with primary antibodies and analyzed using a flow cytometer with antibodies and FCS Express™ 4 IVD software (De Novo Software, Glendale, CA, USA), as described in a previous study (24).

Animal experiments. A total of 40 female specific pathogen-free female BALB/c (6-week old; body weight, 28-32 g) nude mice were purchased from Harbin Veterinary Research Institute (Harbin, China). All mice were feed under pathogen-free conditions. All rats were housed under controlled temperatures in 23±1˚C with a relative humidity of 50±5% and a 12 h light/dark cycle with *ad libitum* access to food and water. A density of $1x10^6$ CAOV-3 cells were injected into the right flank of female BALB/c nude mice in a total volume of 200 μl PBS. Therapy for tumor-bearing mice by NK cells was initiated when tumor diameters reached 6-8 mm on day 7 after tumor inoculation. Mice with ovarian carcinoma were randomly divided into two groups (n=20 in each experimental group) and injected intratumorally with $2x10^6$ NK cells or the same volume PBS as a control. The treatment was continued five times, with injections administered at intervals of every 2 days. Tumor diameters (n=6) were recorded once every 2 days and tumor volume was calculated using the following formula: 0.52 x smallest diameter^2 x largest diameter. Tumor metastasis was evaluated by tumor occurrence in other

subcutaneous sites. Mice were sacrificed when the tumor diameter reached 12 mm.

Splenocyte collection and cytotoxic T lymphocyte (CTL) responses. Splenocytes were obtained from the spleens of experimental mice following treatment. The monoplast suspension of splenocytes was then washed with PBST. Ovarian tumor cells were subsequently inactivated using ultraviolet ray for 2 h at 37˚C and then subjected to incubation with splenocytes for 48 h at 37˚C. Interferon (IFN)-γ release was determined using an ELISA kit (cat. no. MIF00; Bio-Rad Laboratories, Inc., Hercules, CA, USA) after culture for 72 h at 37˚C, according to the manufacturer's instructions. Meanwhile, T cells ($1x10^6$) from the splenocytes were purified (25) and co-cultured with fresh ovarian tumor cells in DMEM for 4 h at 37˚C at the effector: Target ratios of 5:1, 15:1 and 30:1. Specific CTL activity to the target cells was determined by LDH Assay Kit (cat. no. ab102526; Abcam, Cambridge, UK) (26).

Flow cytometry. Ovarian tumor cells were isolated from experimental mice as described previously (27) and washed three times with PBS. Cell suspensions were filtered through a 100 μm nylon strainer. Tumor cells were blocked with 2% BSA (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C and then washed with PBS three times at room temperature. Cells were then labeled with the following antibodies sourced from Abcam: CD69 (1:1,000; cat. no. ab202909), CD3 (1:1,000; cat. no. ab16669) and CD45 (1:1,000; cat. no. ab10558), plus CD4 (1:1,000; cat. no. ab183685) and CD8 (1:1,000; cat. no. ab22378) staining for 2 h at room temperature to determine the frequency of CD4 and CD8 cell subsets in the total infiltrated immune cells. Cells were washed with PBS three times at room temperature and then incubated with horseradish peroxidase-conjugated Goat Anti-Rabbit Imunnoglobulin G H&L (Alexa Fluor® 488; 1:2,000; cat. no. ab150077; Abcam) for 1 h at 37˚C. The stained cells were analyzed using FCS Express™ 4 IVD software (De Novo Software).

Immunohistochemistry. Paraffin-embedded tumor tissue sections (5 μm) were fixed using and 4% formaldehyde for 2 h at room temperature and epitope retrieval was performed by heating the samples to 100˚C for 30 min in a citrate solution (10 mmol/l; pH 6.0) followed by dewaxing in xylene and rehydration in graded ethanol solutions for further analysis. The paraffin sections were subjected to hydrogen peroxide (3%) for 10-15 min and subsequently blocked using a regular blocking solution (5% BSA; Sigma-Aldrich; Merck KGaA) for 10-15 min 37˚C. Finally, the sections were incubated at 4˚C for 12 h with the following primary antibodies sourced from Abcam: Anti-NK (1:100; cat. no. ab36388), anti-KI67 (1:500; cat. no. ab15580), anti-major histocompatibility complex (MHC) I (HLA-A; 1:500; cat. no. ab209541) and anti-leukomonocyte antibody (CD3; 1:1,000; cat. no. ab16669) and CEA (1:500; cat. no. ab33562). Sections were then washed three times with PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG monoclonal antibodies (1:2,000; cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) for 1 h at 37˚C and counterstained with hematoxylin or 4',6-diamidino-2-phenylindole. The relative expression of NK1.1, MHC I and lymphocytes were determined by the means of six random views under the microscope.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) analysis. Tumor tissue sections (5 μm) were fixed with 4% paraformaldehyde solution for 2 h at 4˚C. Sections were washed three times with PBS and then permeabilized by immersing cells slides in a 0.2% TritonX-100 solution with PBS for 14 min at 4˚C. Subsequently, sections were incubated with an equilibration buffer for 14 min at 4˚C and were then incubated with 50 μl reaction mixture at 37˚C for 60 min and washed 3 times with PBS. The tissues were washed with PBS three times at room temperature and then the TUNEL Apo-Green Detection kit (cat. no. A111-01-VAZ; Biotool, Stratech Scientific Ltd., Suffolk, UK) was used according to the manufacturers protocol to detect TUNEL-positive cells. Finally, tissue section was placed on glass slides and tissue section images were captured at 6 fields of view using fluorescent microscope (Olympus Corporation) at a magnification of x40.

Statistical analysis. All data were reported as the mean ± standard error of the mean. All data were analyzed using SPSS Statistics 19.0 (SPSS, Inc., Chicago, IL, USA). Unpaired data were analyzed by the Student's t-test. Comparisons of data between multiple groups were analyzed by one-way analysis of variance followed by a Bonferroni post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NK cells inhibit ovarian cancer cell growth, migration and invasion. Ovarian cancer cell lines (SKOV3, OVCAR3, CAOV-3 and A2780) were cultured and analyzed for inhibitory effects on growth and migration of NK cells. Cytotoxic effects of NK cells against SKOV3, OVCAR3, CAOV-3 and A2780 and were evaluated by LDH assays *in vitro*. SKOV3, OVCAR3, CAOV-3 and A2780 cells were co-cultured with NK cells (effector: Target=30:1, 15:1, 5:1 or 1:1) for 24, 48 and 72 h. As demonstrated in Fig. 1A-D, NK cells from patients with ovarian cancer lysed cancer cells in a dose-dependent and time-dependent manner.

Migration and invasion are key characteristics for metastasis of ovarian tumor cells. Therefore, the present study analyzed the inhibitory effects of NK cells on migration and invasive ability in a parallel assay. As expected, the capacities of tumor migration and invasion were significantly suppressed following treatment with NK cells (effector: Target=5:1) compared with the levels in control cells (Fig. 1E and F). These data suggest that effector NK cells are able to lyse ovarian SKOV3, OVCAR3, CAOV-3 and A2780 cells, and also inhibit migration and invasion of these ovarian cancer cells.

NK cell distribution in xenograft murine model of ovarian cancer and MHC I molecule expression in NK cell-treated tumors. The molecular mechanism of NK cells for xenograft mice has been elucidated and has demonstrated beneficial outcomes in preclinical trails (28). The present study investigated whether NK cells could increase tumor invasion and MHC I molecule expression in the tumors of xenograft mice.

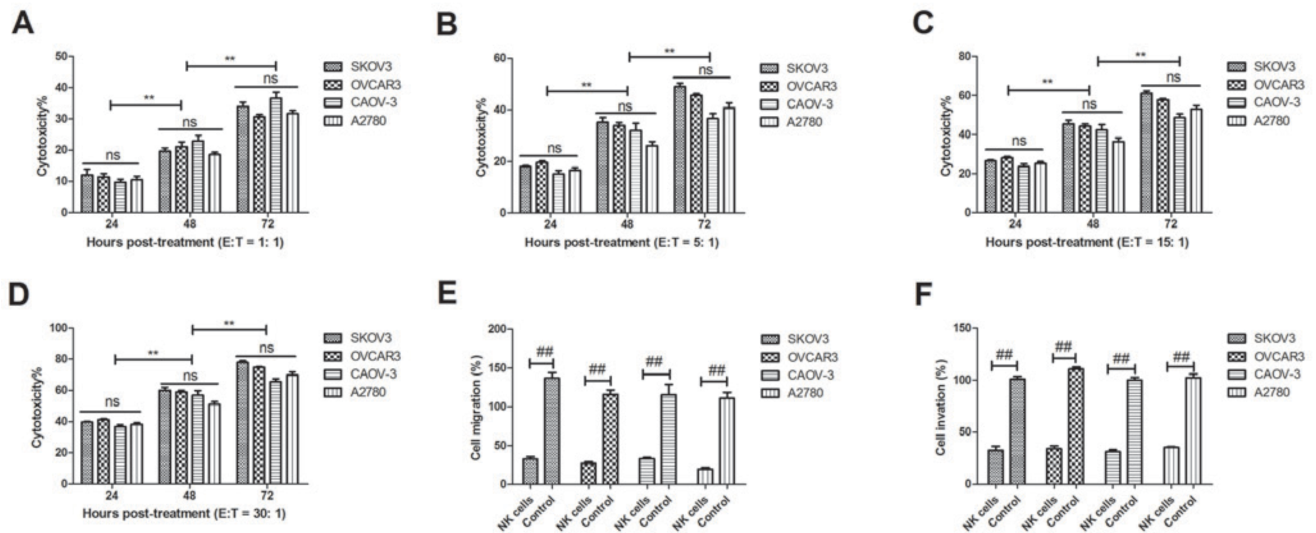

Figure 1. NK cells kill ovarian tumor cells at different E: T ratios and inhibit migration and invasion of ovarian tumor cells. Cytotoxicity of NK cells from patients with ovarian cancer at E: T ratios of (A) 1:1, (B) 5:1, (C) 15:1 and (D) 30:1 against ovarian tumor cells at the indicated time points. (E) Inhibitory effects of NK cells on migration of ovarian tumor cells following treatment for 48 h. (F) Inhibitory effects of NK cells on invasion of ovarian tumor cells following treatment for 48 h. n=3 repeats with similar results. Data are presented as the mean ± standard error of the mean. **P<0.01, 48 h vs. 24 h, **P<0.01, 72 h vs. 48 h, ##P<0.01 vs. controls. NK, natural killer; E:T, effector:Target; ns, no significance.

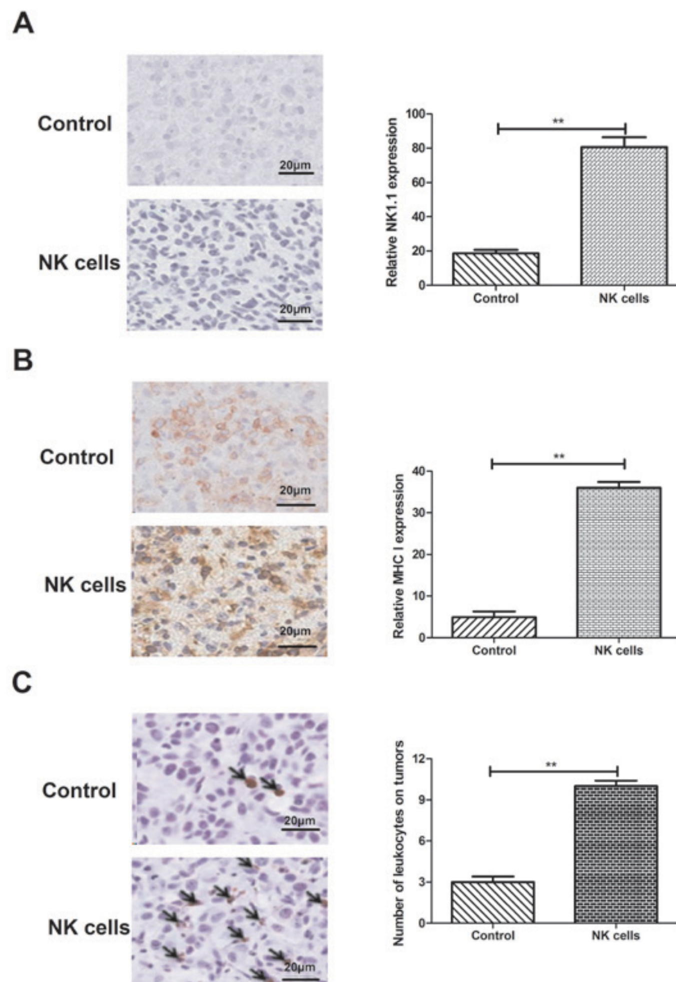

Figure 2. NK cells upregulate NK1.1 expression, MHC I molecules and lymphocytes in xenograft ovarian tumors. (A) NK1.1 expression was increased following treatment with NK cells in the xenograft mice with ovarian cancer. (B) MHC I molecule expression in xenograft mice with ovarian cancer was analyzed and the expression was higher compared to the levels in the control treatment group, according to immunohistochemistry. (C) The number of lymphocytes on tumor surfaces were upregulated following NK cell treatment, according to immunohistochemistry. Black arrows indicate lymphocytes. Scale bar, 20 μm. Data are presented as the mean ± standard error of the mean. **P<0.01 as indicated. NK, natural killer; MHC, major histocompatibility complex.

Table I. Number of tumors and tumor diameter in mice demonstrating >1 tumor on day 25.

Group	Tumor parameter	
	n	Diameter, mm
PBS		
1	2	4.02, 4.52
2	2	3.18, 3.86
3	2	4.14, 4.32
4	2	3.45, 3.66
5	3	4.42, 4.32, 3.45
6	2	3.22, 4.32
7	2	2.56, 4.35
8	2	4.56, 4.86
9	2	6.32, 4.04
10	3	5.21, 4.12, 2.18
Natural killer cell treatment		
1	2	3.02, 3.98
2	2	3.92, 4.28

NK cell distribution was analyzed using immunohistochemistry against NK cell marker NK1.1 in the xenograft ovarian tumors. As demonstrated in Fig. 2A, the level of NK1.1-positive cells was significantly lower in tumors from the BALB/c-nude mice without NK cell treatment compared with the level in those treated with NK cells. In addition, the present study evaluated MHC I molecule expression in the patient-derived xenograft murine model of ovarian cancer, as MHC class I molecules emit inhibitory signals for NK cells (29). The results in Fig. 2B demonstrate that MHC I expression was significantly higher in the xenograft mice in the presence of NK cells compared with the level in the control mice. Furthermore, the total leukocytes were analyzed in presence of NK cells in xenograft mice. As demonstrated in Fig. 2C, the total number of leukocytes was significantly upregulated in the NK cell treatment group compared to the number in the control group. These results indicate that NK cell treatment increased NK1.1 and MHC I molecule expression, which would increase the possibility that NK cells may recognize invasive and/or migrating ovarian tumor cells.

NK cells suppress metastasis of patient-derived ovarian cancer cells in a xenograft murine model. In the present study, BALB/c-nude mice were used to analyze suppression efficacy of NK cells on ovarian cancer cell metastasis. According to the hypothesis, immune deficient BALB/c-nude mice were implanted with patient-derived CAOV-3 cells (1×10^6 density) in a total volume of 200 μl by injection of the right flank. Compared with the control treatment mice, NK cell treatment demonstrated a significant inhibitory effect on tumor growth of ovarian cancer cells on day 25 (Fig. 3A; n=20/group). In addition, median survival of the mice in the NK cell treatment group was significantly longer than those of the PBS treatment group in a 180-day observation (Fig. 3B; n=10/group). These results indicate that treatment with NK cells had beneficial effects on the inhibition of ovarian cancer cell metastasis *in vivo* and tumor formation of patient-derived ovarian cancer cells.

It was observed that 10 experimental mice had >1 tumor in the control group, while only 2 experimental mice had >1 tumor in the NK group. The mean tumor diameters are demonstrated in Table I. Furthermore, formation of xenograft ovarian tumors was also confirmed by immunohistochemistry and pathology (Fig. 3C and D). These results indicate that metastasis of ovarian tumor cells was inhibited by circulating immune cells in the blood stream, which suggests that sufficient supplementation of NK cells in the circulating system may be a promising immunotherapeutic strategy for patients with ovarian cancer.

In vivo therapeutic effects of NK cells against xenograft mice with ovarian tumors. As it was observed that NK cells stimulated the immune system in xenograft mice with ovarian tumors and demonstrated antitumor effects, the activation status of T cells by the release of the T cell cytokine IFN-γ and CD69 was further analyzed. As demonstrated in Fig. 4A, CD69 expression was significantly increased following NK cell treatment compared with the expression level following the control treatment, as determined by the mean fluorescence intensity. Furthermore, the release of the T cell cytokine IFN-γ was also significantly increased in xenograft mice with ovarian cancer following treatment with NK cells compared with the level in the control mice (Fig. 4B). In addition, the percentage of $CD4^+$ and $CD8^+$ T cells was upregulated following NK cell treatment compared with the percentage in the control treatment (Fig. 4C). CTL responses were initiated by stimulation with NK cell-treated tumor cells. The present data indicate that stronger CTL responses were presented (~60%) following treatment with NK cells on day 7 (Fig. 4D). Furthermore, the number of NK cells was also increased in xenograft mice administrated with NK cells compared with the rate in the control treatment, as determined by TUNEL-positive apoptotic cells (Fig. 4E). The NK cells were evaluated in the tumors from experimental tumor-bearing mice that were treated with intravenous transplantation of 1×10^6 NK cells. The results in Fig. 4F indicate a significantly higher degree of NK cell infiltration in xenograft tumors from the NK cell-treated group compared with the level in the control treatment group. These results suggest that NK cells induced the infiltration of lymphocytes in the tumors of experimental mice, which suggests that *in vivo* treatment of supplementation with NK cells has positive effects against ovarian xenograft tumors.

Discussion

Currently, various strategies and medications for the treatment of human ovarian cancer have been studied and developed (30). Immunotherapy, as a main therapy or adjunctive, is an efficient treatment and serves an essential role in the modern healthcare system worldwide (31). The effects of immunotherapy represent a targeted treatment by activating the host immune cells stimulated by homologous cancer cells of ovarian tumors (32). The present study investigated immunotherapy of NK cells from patients with ovarian cancer in xenograft BALB/c nude mice. The experimental design demonstrated that NK cell treatment inhibited tumor growth and prolonged the survival of mice with ovarian cancer. The cytotoxic activities of these

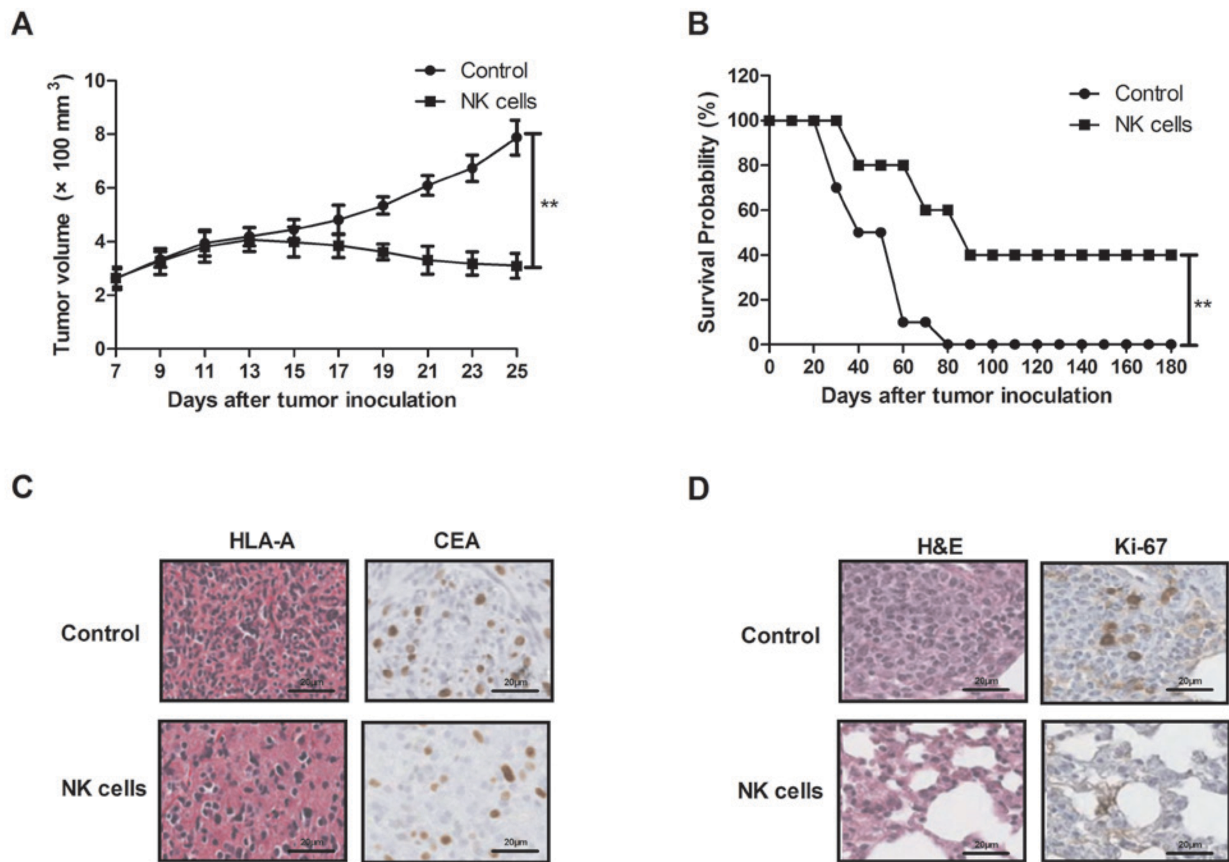

Figure 3. NK cells inhibit ovarian tumor growth and prolong survival of tumor-bearing mice. (A) Ovarian tumor volumes were evaluated and compared between the NK cell treatment group and PBS treatment group in an 18-day observation following tumor cell transplantation (n=20/group). (B) Survival rate of xenograft mice with ovarian cancer was studied in NK-treated mice using PBS-treated xenograft mice as a control (n=10 in each group). (C) Formation of xenograft ovarian tumor was inhibited following NK cell treatment by intravenous injection determined by immunohistochemistry. Scale bar, 50 μm. (D) Ovarian tumor cells were suppressed following treatment with NK cells determined by H&E and immunohistochemistry, using PBS treatment as a comparison. Scale bar, 20 μm. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. control. NK, natural killer; H&E, hematoxylin and eosin.

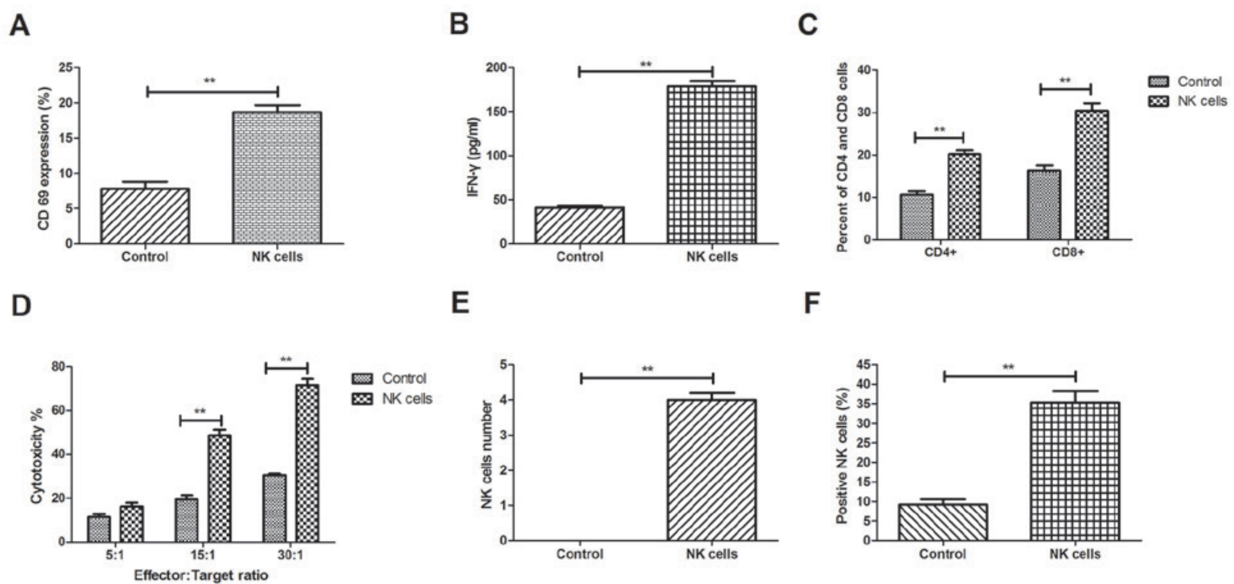

Figure 4. NK cells promote cytotoxic effects against ovarian tumor cells and enhance T lymphocyte infiltration. (A) CD69 expression was increased in T cell populations following NK cell treatment, as analyzed using flow cytometry. (B) IFN-γ release was assessed by ELISA following NK cell treatment. (C) Percentages of $CD4^+$ and $CD8^+$ cells were upregulated in NK-treated xenograft mice with ovarian cancer. (D) NK cells induced cytotoxicity on ovarian tumor cells at different effector: Target ratios after 7-day experiment. (E) The number of NK cells was analyzed and a higher number were presented on tumor surfaces in NK-treated xenograft mice than in the control-treated mice. (F) A higher degree of NK cell infiltration was observed in xenograft tumors from the NK cell-treated group compared with that in the control-treated group. Data are presented as the mean ± standard error of the mean. **P<0.01, vs. control. NK, natural killer; CD, cluster of differentiation; IFN, interferon.

NK cells killed ovarian cancer cells through inducing apoptosis, inhibiting proliferation and suppressing migration and invasion of ovarian tumor cells.

Immunotherapy has been demonstrated to alleviate gene therapy- and chemoradiotherapy-related side effects, enhance therapeutic effects of surgery and prolong the survival of patients with ovarian cancer (33). From previous reports, it has been concluded that the functions of the immune system are essential for eradication of ovarian malignant cells in cancer treatment of humans and animals (34-36). Failure of the immune cells to monitor tumor cells may lead to the emergence and development of cancer (37,38). Additionally, previous studies have elaborated on the various mechanisms used by tumor cells to escape from immune-mediated cytotoxicity (39-41). The most important of these escape factors was the lack of recognition of tumor antigens (42).

The present study investigated the effects of NK cell immunotherapy on inhibiting the growth of ovarian cancer cells, the survival rate and conditions of long-term survival in mice with ovarian cancer. The therapeutic effect on mouse survival was evaluated by analysis of immunotherapy of NK cells *in vivo*. Although a previous study has reported that NK cells inhibited systemic metastasis of glioblastoma cells and exhibited therapeutic effects against glioblastomas in the brain (24), to the best of our knowledge, no study has previously reported the therapeutic effects of NK cells for ovarian cancer. The present study demonstrated that NK cells from patients with ovarian cancer not only inhibited ovarian tumor cell growth, but also suppressed tumor cell migration and invasion.

To date, previous reports have clearly indicated that cellular immunity of NK cells may either provide strong antitumor effects or cure cancer patients in the advanced stage (43,44). In the present study, peripheral blood from patients with ovarian cancer was extracted and lymphocytes were sensitized by tumor cells from the patients themselves, which improved clinical protocols and inhibited immune escape and tumor-induced immune suppression, with little or no side effects. The present results showed that release of the T cell cytokine IFN-γ and CD69 expression were upregulated after NK cell treatment by intravenous injection. These data are similar to a previous report involving prescriptions of intravenous injection of immune cells for patients with ovarian cancer (45).

Notably, increasing levels of CD4, CD8, NK1.1 and IFN-γ release were observed in the present study in the NK cell treatment group. Expression of immune factors in plasma are biomarkers that are important mediators of CTLs following immune-related anticancer treatments, which contribute to the immunological memory of cancer patients (46,47). Changes in levels of various immune factors in the blood of mice suggested that the immune system was enhanced by the immunotherapy treatment at the indicated dose used in the present experiment. The present study also demonstrated that antitumor activities of immunotherapy significantly inhibited metastatic tumor cells. Notably, tumor shrinkage was observed in the majority of tumor-bearing mice treated with NK cells.

In conclusion, the beneficial outcomes of immunotherapy with NK cells on the inhibition of tumor growth and increasing the survival rate were observed following NK cell treatment in an 18-day period. The present data suggested that immunotherapy using NK cells is an effective treatment for mice with ovarian cancer and indicated that higher expression of immune factors contributed to long-term survival of mice with ovarian cancer. The present results indicate that the clinical relevance of immunotherapy for patients with cancer requires further development in prospective larger-scale investigations.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS perfomed most of the experiments. ZY, ZZ, HX, XM and ZX performed some experiments and analyzed the data. JX and CS designed the study. All authors have read and approved this manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Weifang City People's Hospital (Weifang, China). Written informed consent was obtained from the single patient for use of their cells.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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