

NK cell-produced IFN- γ regulates cell growth and apoptosis of colorectal cancer by regulating IL-15

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Abstract. Globally, colorectal cancer (CC) is the third leading cause of mortality associated with cancer. Natural killer (NK) cells are a major class of cells that are responsible for eliminating tumor cells and cytokine production. NK cell-mediated production of interferon gamma (IFN- γ) has antiviral, immunoregulatory and anti-tumor properties. IL-15 is important in linking inflammation with cancer. For instance, IL-15 promotes humoral and cell-mediated immune responses to inhibit tumor growth. IL-15 inhibits colitis-associated colon carcinogenesis by inducing antitumor immunity. However, the effect of NK cell-mediated IFN- γ on IL-15 expression in CC progression remains unknown. mRNA and protein level were detected using reverse transcription-quantitative PCR and western blotting, respectively. IFN- γ concentrations were detected using ELISAs. The cytotoxicity of NK-92 cells on SW480 cells was detected using cytoTox 96[®] non-radioactive cytotoxicity assays. Cell apoptosis and cell proliferation was detected using flow cytometry and CCK-8 assays, respectively. IL-2 was used for NK-92 stimulation, IL-15 antibodies were used to neutralize IL-15 bioactivity. For the present study, 21 patients with CC and 21 healthy volunteers were enrolled at the First Affiliated Hospital of Xi'an Jiaotong University. IL-15 mRNA and protein expression were significantly lower in NK cells isolated from the CC group compared with healthy volunteer group. IL-2 enhanced the production/secretion of IFN- γ in addition to enhancing NK-92 cell-mediated killing of SW480 cells. Compared with the control group, NK-92 cells treated with IL-2 alone significantly increased cell apoptosis, BAX expression levels as well as phosphorylated (p)-Janus kinase 2 and p-STAT1 protein levels, whilst reducing cell viability and Bcl-2 protein levels in SW480 cells. These observations were not made when treated with IL-2 and

polyclonal antibody (pAb) targeting IL-15. Taken together, NK cell-mediated IFN- γ served a pivotal role in CC by regulating IL-15. The effects of IL-2 induced IFN- γ were abolished by pAb IL-15 treatment. The mechanisms of action behind how IFN- γ regulates IL-2 is unclear, and is a promising area for future research.

Introduction

Globally, colorectal cancer (CC) is the third leading cause of mortality associated with cancer (1). Worldwide, the increasing incidence of CC is possibly caused by the modern life style which is characterized by increased fat intake and reduced physical activity (2). In CC, poor efficiency and lack of effective methods for treating metastasis are the main causes for mortality among patients (3). For patients with local disease, the five-year survival rate can be as high as 90.3%, but it declines to 70.4 and 12.5% for those with regional and distant metastasis, respectively (3). Despite advances in the medical science and technology area, the molecular mechanisms underlying CC progression and pathogenesis remain unclear, which is important to be elucidated.

The immune system is responsible for eliminating cancerous cells and foreign infections (4). In particular, natural killer (NK) cells are primarily responsible for eliminating tumor cells through contact-dependent cytotoxicity and cytokine production (5). For instance, NK-92 cells attack cancer cells and the tumors grown within the control of the organism (6). One of those cytokines, interferon gamma (IFN- γ), is secreted by NK cells and has been previously reported to promote the apoptosis and cytolysis of target tumor cells (4,7). IFN- γ has immunoregulatory, antiviral and anti-tumor properties (8). Additionally, in cancer cells, IFN- γ results in the inhibition of cell proliferation (8). In cancer cells, IFN- γ is expressed at higher levels and results in cell death or growth inhibition (9). Therefore, it is vital to study the molecular mechanisms behind the NK cell-mediated killing of CC cells.

Cytokines produced during the process of the innate immune response are important components linking inflammation with cancer (10). IFN- γ has previously been demonstrated to contribute to the antitumor activity of a number of interleukins (ILs) (11). IL-15 is a pleiotropic cytokine expressed and secreted by dendritic cells, macrophages, fibroblasts and epithelial cells (12). IL-15 has demonstrated

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the ability to suppress colitis-associated colon carcinogenesis through the induction of antitumor immunity (13). However, the effects of IFN- γ on IL-15 in regulating tumor progression remain unknown. Since the establishment of NK-92 cells in 1992, their anti-cancer activity has been widely tested in mouse models (14). Therefore, pAb-IL-15R was used to inhibit IL-15R signaling in NK-92 cells in the present study, we aimed to investigate the role of NK-mediated IFN- γ in CC progression and provide the potential molecular mechanism in this process.

Materials and methods

Participants. For the present study, 21 patients with CC (aged 55 \pm 5 years old, 15 males and 6 females) and 21 healthy volunteers (aged 53 \pm 7 years old, 15 males and 6 females) were enrolled in the First Affiliated Hospital of Xi'an Jiaotong University between February 2015 and October 2016. Patients who received any radio/chemo-therapy are excluded from the study. All study participants provided written informed consent and the present study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

Peripheral blood mononuclear cells (PBMCs) were obtained from the patients with CC and healthy volunteers using Lymphocyte Separation medium (MP Biomedicals, LLC) as described: In brief, venous blood (10 ml) was collected in the early morning. This was anticoagulated using heparin (10 IU/ml; Merck KGaA), mixed with Lymphocyte Separation medium and centrifuged at 1,600 \times g for 20 min at 4°C. Cells were separated into 4 layers, namely: i) plasma or tissue homogenate layer; ii) cyan milky lymphocyte or monocyte layer; iii) transparent separation layer; and iv) a red blood cell layer. Cells in the second layer were collected and washed three times with normal saline and centrifuged at 1,600 \times g for 10 min at 4°C, followed by removal of the supernatant. The cells were resuspended in 10% DMSO and 90% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) at a density of 5 \times 10⁷ cells/ml, and finally frozen and stored in liquid nitrogen. Cells were counted using a Nexcelom Cellometer Auto 2000 (Nexcelom Bioscience, LLC) by acridine orange (AO)/propidium iodide (PI) staining (AO/PI cell viability kit, Nexcelom Bioscience, LLC.) according to the manufacturer's protocol. PBMCs exhibited >95% viability before and after freezing.

NK cells were extracted from PBMCs using Human NK Cell Isolation kit (negative selection procedure of magnetic activated cell sorting; cat. no. 130-092-657; Miltenyi Biotec, Inc.) according to the manufacturer's protocol. In brief, non-NK cells including T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells, were magnetically labeled with the cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail. NK cells were isolated by depletion of the magnetically labeled cells; NK cells with >90% purity at the end was determined by flow cytometry.

Cell culture. NK-92 cells were (NantKwest, Inc.) cultured in minimum essential medium Eagle (Sigma-Aldrich; Merck KGaA) with Earle's salts and nonessential amino

acids, supplemented with 12.5% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 12.5% horse serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 μ M 2-mercaptoethanol (Sangon Biotech Co., Ltd.), 100 U/ml penicillin (Sangon Biotech Co., Ltd.) and 100 U/ml streptomycin (Sangon Biotech Co., Ltd.) at 37°C with 95% humidity and 5% CO₂. For the stimulation of NK-92 cells, they were incubated with IL-2 (100 U/ml; PeproTech, Inc.) for 24 h at 37°C. For the neutralization of IL-15 bioactivity in NK-92 cells, NK-92 cells were treated with anti-IL-15/IL-15R Complex Monoclonal Antibody (pAb IL-15; clone GRW15PLZ; eBioscience; Thermo Fisher Scientific, Inc.) for 12 h at 37°C.

Human colorectal carcinoma cell line SW480 was purchased from American Type Culture Collection (ATCC). SW480 cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sangon Biotech Co., Ltd.), and 100 U/ml streptomycin (Sangon Biotech Co., Ltd.) in an incubator at 37°C with a 95% humidified atmosphere and 5% CO₂.

Cytotoxicity assays. CytoTox 96[®] non-radioactive cytotoxicity assay (Promega Corporation) was used for the detection of SW480 cell killing by NK-92 cells according to the manufacturer's protocol as a previous report (15). SW480 cells were first washed with PBS, re-suspended in fresh NK-92 culture medium (fully supplemented as previously described) and seeded into 96-well plates (density, 5 \times 10³ cells/well). NK-92 media alone did not affect the SW480 cells (data not shown). Immediately, IL-2-induced NK-92 cells were subsequently added to the SW480 cells at an effector-to-target ratio of 16:1 and incubated at 37°C with 95% humidified atmosphere and 5% CO₂ for 4 h. The cytotoxic activity was measured by LDH release from the supernatant which was obtained and analyzed using the CytoTox 96 assay. The SW480 cell killing was calculated using the following equation: % cytotoxicity=(Experimental-Effector spontaneous-Target spontaneous)/(Target maximum-Target spontaneous) \times 100, according to the manufacturer's protocol. Maximum and spontaneous are both referring to LDH release.

Cell treatment. SW480 cells were randomly divided into 3 groups: i) Control group, SW480 cells co-cultured with non-stimulated NK-92 cells; ii) IL-12 group, SW480 cells co-cultured with IL-2-activated NK-92 cells; and iii) IL-12 + pAb IL-15 group, SW480 cells co-cultured with IL-2 + anti-IL-15/IL-15R Complex Monoclonal Antibody treated NK-92 cells.

Enzyme-linked immunosorbent assay (ELISA). Briefly, the NK-92 cells which were stimulated by IL-12 for 24 h at 37°C were washed with PBS and seeded into 96-well plates at a seeding density of 5 \times 10³ cells/well and cultured for 4 h at 37°C with 5% CO₂. They were then centrifuged at 200 \times g for 4 min at room temperature. Supernatants were subsequently collected for the quantification of IFN- γ concentration using the human IFN- γ ELISA kit (cat. no. EK0458; Signalway Antibody LLC) in accordance with the manufacturer's protocol. The absorbance at 450 nm was measured in each well using a microplate reader (Synergy[™] HT; BioTek Instruments, Inc.).

Cell Counting Kit (CCK-8) assay. SW480 cells were randomly divided into 3 groups, the control group, IL-12 group and IL-12 + pAb IL-15 group. Cell proliferation was detected by CCK-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. In brief, SW480 cells (seeding density, 1×10^5) were seeded in Biocoat™ 24-well plates (BD Biosciences) in DMEM, 10% FBS, 100 U/ml penicillin (Sangon Biotech Co., Ltd.) and 100 U/ml streptomycin (Sangon Biotech Co., Ltd.) in an incubator at 37°C with a 95% humidified atmosphere and 5% CO₂ for 24 h. Afterwards, 10 μl of CCK-8 solution was added into each well and incubated at 37°C for 2 h. The absorbance in each well was recorded at 450 nm by a microplate reader.

Flow cytometry analysis. SW480 cells were randomly divided into 3 groups, the control group, IL-12 group and IL-12 + pAb IL-15 group. To measure apoptosis, SW480 cells were seeded into 12-well plates at a density of 3×10^5 cells/well and cultured for 48 h at 37°C with 95% humidified atmosphere and 5% CO₂. Thereafter, the SW480 cells were collected using 0.025% trypsin (Thermo Fisher Scientific, Inc.). After washing with PBS, 5 μl fluorescein isothiocyanate (FITC)-labeled Annexin V and 5 μl PI from Annexin V-FITC apoptosis detection kit (Sigma-Aldrich; Merck KGaA) were added into each well and incubated in the dark for 15 min at 37°C with 95% humidified atmosphere and 5% CO₂. Cell apoptosis was analyzed using flow cytometry (BD Biosciences) within 1 h, and the data were analyzed using the FlowJo software (version 10.2; FlowJo LLC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using RNAiso reagent (Takara Biotechnology Co., Ltd.) in accordance with the manufacturer's protocol. The quality and quantity of RNA were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized from RNA using PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) in accordance with the manufacturer's protocol. qPCR was performed using a SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.) in a StepOne™ Plus Real-Time PCR System (Thermo Fisher Scientific, Inc.) for the measurement of the mRNA expression profiles. GAPDH was used as an internal control. The primers used were as follows: IL-15 forward, 5'-ATGTTTCATCAACACGTCCTGACT-3' and reverse, 5'-GCA GCAGGTGGAGGTACCTTAA-3'; and GAPDH forward, 5'-ACACCCACTCCTCCACCTTT-3' and reverse, 5'-TTACTC CTTGGAGGCCATGT-3'. The thermocycling conditions used to perform the qPCR were as listed: Initial denaturation at 95°C (30 sec), followed by 40 cycles of 95°C (5 sec) and 60°C (30 sec). Data were analyzed using the $2^{-\Delta\Delta C_t}$ method (16).

Western blot analysis. Cells were lysed using radioimmuno-precipitation assay buffer (Beyotime Institute of Biotechnology). After that, protein concentration was determined using the BCA method. Equal amounts of protein (10 μg/lane) were separated by SDS-PAGE (10% gel) and subsequently transferred onto polyvinylidene difluoride membranes for 2 h at 4°C. The polyvinylidene difluoride membranes were then blocked by 5% non-fat milk at 37°C for 1 h and incubated with primary antibodies for IL-15 (cat. no. ab7213; 1:1,000; Abcam), IFN-γ (cat. no. 8455; 1:1,000; Cell Signaling Technology, Inc.), BAX

(cat. no. 5023; 1:1,000; Cell Signaling Technology, Inc.), Bcl-2 (cat. no. 4223; 1:1,000; Cell Signaling Technology, Inc.), p-JAK2 (cat. no. 3776; 1:1,000; Cell Signaling Technology, Inc.), JAK2 (cat. no. 3230; 1:1,000; Cell Signaling Technology, Inc.), p-STAT3 (cat. no. 9145; 1:1,000; Cell Signaling Technology, Inc.), STAT3 (cat. no. 12640; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.) at 4°C overnight, where GAPDH served as the reference protein. The membranes were incubated further with anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 2 h at 37°C. All blots were visualized using an ECL reagent (Beyotime Institute of Biotechnology). Densitometry was performed using ImageJ software (version. 1.8.0; National Institutes of Health).

Statistical analysis. Each experiment was performed at least 3 times. Values are expressed as the mean ± SD. GraphPad Prism (GraphPad Software Inc.) was used for the analysis of experimental data. Statistical comparisons between 2 groups were performed using Student's t-test. Statistical comparisons between 3 groups were performed using one-way ANOVA followed by Dunnett's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IL-15 expression in NK cells isolated from patients. To measure IL-15 expression in NK cells from patients with CC compared with healthy volunteers, RT-qPCR and western blot analysis were performed to detect the levels of IL-15 mRNA and protein, respectively. IL-15 expression was significantly reduced in NK cells from the CC group compared with the healthy volunteer group (Fig. 1A). Likewise, IL-15 protein levels were markedly lower in CC group compared with in the healthy volunteer group (Fig. 1B).

IL-15 expression in NK-92 cells. Cells were randomly divided into two treatment groups: The control group and the IL-2 treatment group. IL-15 expression was measured in the NK-92 cell line in response to IL-2 stimulation. IL-15 mRNA (Fig. 2A) and protein (Fig. 2B) expression was markedly increased in NK-92 cells following IL-2 treatment compared with untreated control cells.

Activation of NK-92 cells by IL-2 affects the killing effect. In the present study, cells were randomly separated into two groups, including the control and IL-2 groups. It was found that IFN-γ secretion (Fig. 3A) and expression (Fig. 3B) following IL-2 stimulation were markedly higher compared with that of the control group, suggesting the enhancement of the activation of NK-92 cells.

The killing effect of NK-92 cells was subsequently detected using CytoTox 96 non-radioactive cytotoxicity assay. Compared with the control group, IL-2 stimulation enhanced the killing effect of NK cells on SW480 cells (Fig. 3C).

Activated NK-92 cells regulate SW480 cell apoptosis. In the present study, following the stimulation of NK-92 cells using IL-2, the effects of NK-92 cells on SW480 cell apoptosis were

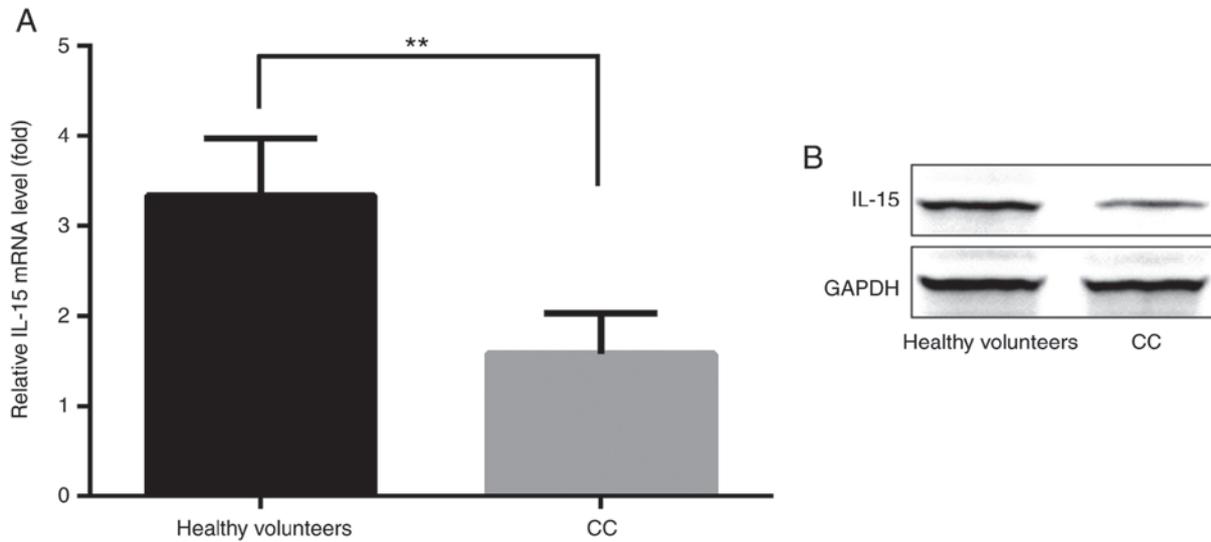


Figure 1. Measurement of IL-15 expression in NK cells from patients with CC. IL-15 (A) mRNA and (B) protein expression levels were reduced in NK cells isolated from CC patients compared with the healthy volunteer group. ** $P < 0.01$. NK, natural killer; CC, colorectal cancer; IL-15, interleukin-15.

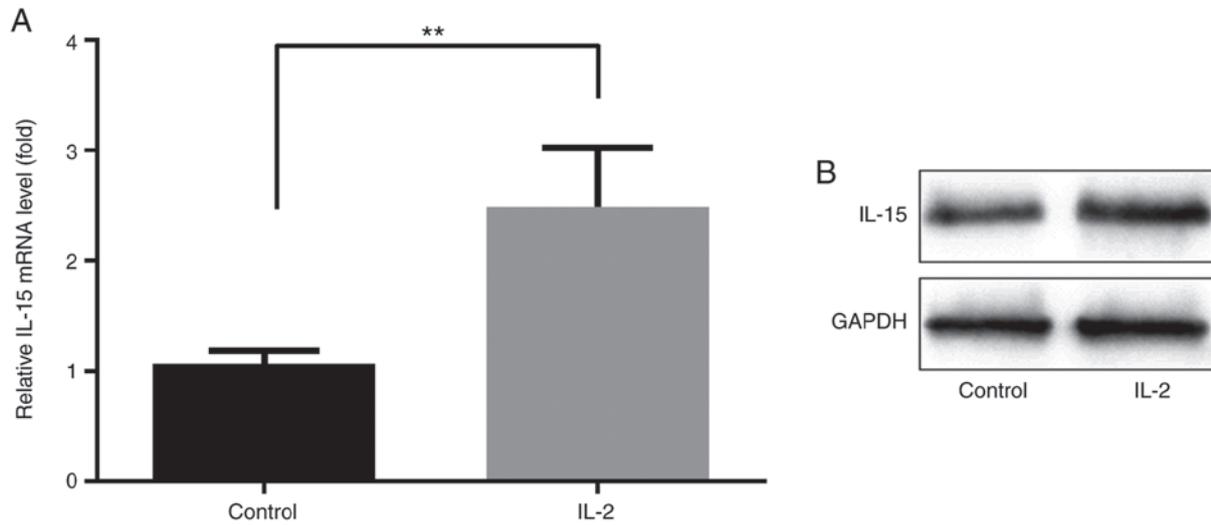


Figure 2. IL-15 expression in NK-92 cells following IL-2 treatment. Compared with untreated control cells, IL-2 stimulation increased IL-15 (A) mRNA and (B) protein expression in NK-92 cells. ** $P < 0.01$. IL, interleukin.

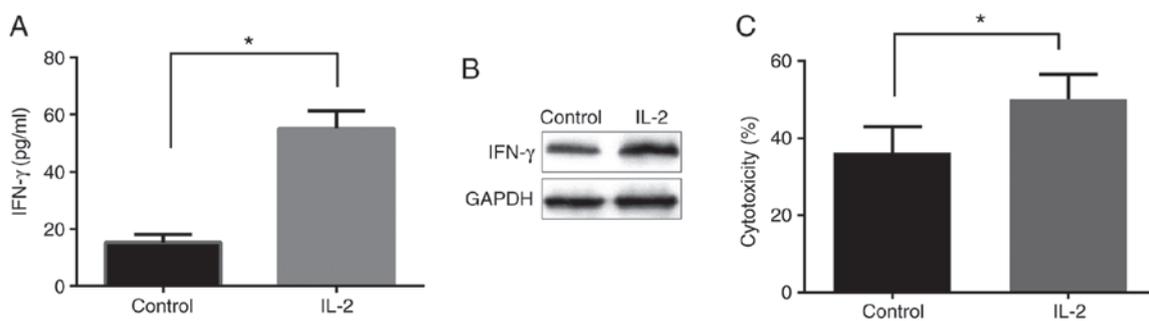


Figure 3. Activation of NK-92 cells by IL-2 induces the killing effect. The (A) production and (B) expression of IFN- γ by NK-92 cells after IL-2 stimulation were higher compared with control. (C) IL-2 stimulation enhanced SW480 cell killing by NK-92 cells. * $P < 0.05$. IL-2, interleukin-2; IFN- γ , interferon- γ .

determined using flow cytometry. Cells were randomly separated into three groups, including control, IL-2 and IL-2 + pAb IL-15 groups. In the IL-2 + pAb IL-15 group, SW480 cells were

co-cultured with NK-92 cells that were pre-treated with pAb IL-15 prior to IL-2 stimulation. Compared with the control group, NK-92 cells treated with IL-2 significantly increased

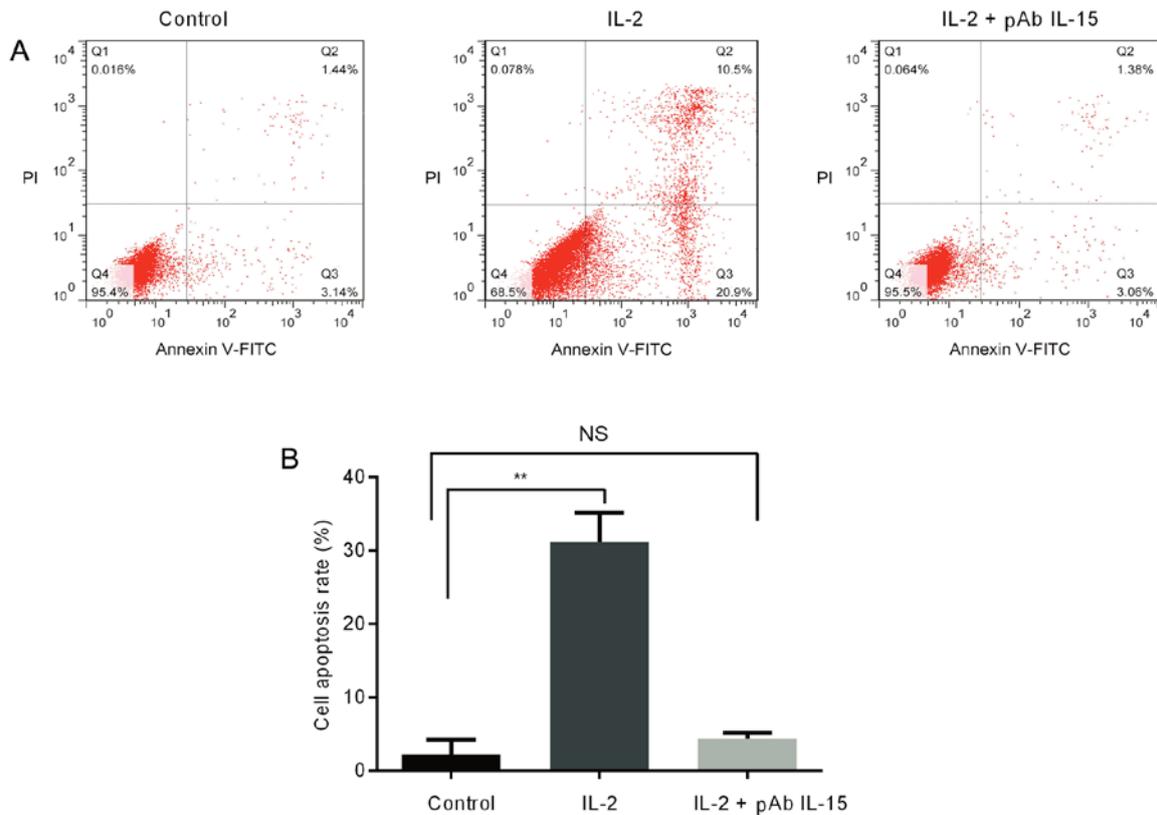


Figure 4. Activated NK-92 cells promote SW480 cell apoptosis. Flow cytometry (A) dot plots and (B) quantitative analysis. Compared with the control group, SW480 cell apoptosis was significantly higher when co-cultured with NK-92 cells stimulated with IL-2, but not when co-cultured with NK-92 cells co-treated with pAb IL-15 and IL-2. **P<0.01. IL, interleukin; pAb, polyclonal antibody; FITC, fluorescein isothiocyanate; PI, propidium iodide; NS, not significant.

SW480 cell apoptosis, but this effect was not observed in the IL-2 + pAb IL-15 group (Fig. 4A and B).

Activated NK-92 cells regulate SW480 cell proliferation. Therefore, the effects of NK-92 cells on SW480 cell proliferation were measured using CCK-8 assay. Accordingly, cells were randomly separated into three groups, including the control, IL-2 and IL-2 + pAb IL-15 groups. Compared with the control group, NK-92 cells treated with IL-2 significantly reduced SW480 cell proliferation, whilst no significant effects on SW480 cell proliferation were observed following co-culture with NK-92 cells co-treated with of IL-2 and pAb IL-15 (Fig. 5).

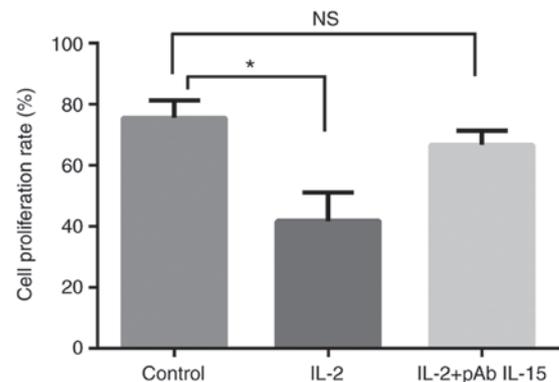


Figure 5. Activated NK-92 cells inhibit SW480 cell proliferation. Compared with the control group, NK-92 cells stimulated with IL-2 significantly reduced SW480 cell proliferation, but this effect was not observed following treatment with NK-92 cells co-treated with pAb IL-15 and IL-2. *P<0.05. IL, interleukin; pAb, polyclonal antibody; NS, not significant.

Activated NK-92 cells regulate the expression of proteins associated with apoptosis in SW480 cells. Compared with control group, IL-2 treated NK92 cells significantly increased BAX expression in SW480 cells, whilst significantly reducing Bcl-2 expression (Fig. 6A-C). IL-2 treated NK92 cells also significantly reduced the ratios of p-JAK2/JAK2 and p-STAT1/STAT1. The effects of IL-2 treated NK92 cells on SW480 cells were reversed when pAb IL-15 was included alongside IL-2 (Fig. 6A, D and E).

Discussion

Globally, CC is the third leading cause of mortality associated with cancer (1). Therefore, it is of utmost importance to investigate the molecular mechanism underlying the pathogenesis of CC.

NK cells are primarily responsible for the elimination of tumor cells (5). In particular, IL-15 is a pleiotropic cytokine secreted and expressed by dendritic cells, macrophages, fibroblasts and epithelial cells (12), in addition to being upregulated in macrophages during inflammation (17). In preclinical studies, IL-15 has been demonstrated to promote humoral and cell-mediated immune responses to inhibit tumor growth (18,19). Indeed, IL-15 inhibits colitis-associated colon carcinogenesis by inducing antitumor immunity (13) in addition to upregulating IFN- γ secreted by CD8⁺ T-cells, and

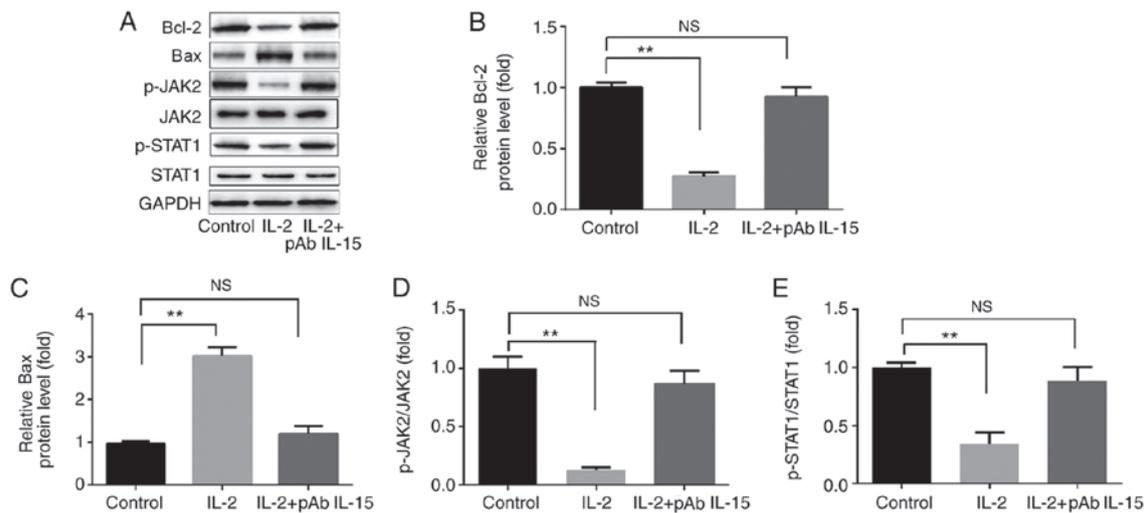


Figure 6. Activated NK-92 cells regulate the expression of proteins associated with apoptosis. (A) Representative western blots comparing the expression of Bax and Bcl-2 in addition to JAK2 and STAT1 phosphorylation in control, IL-2 and IL-2 + pAb IL-15 groups. Compared with the control group, IL-2 stimulation significantly (B) increased BAX expression, (C) reduced Bcl-2 expression, and reduced the ratios of (D) p-JAK2/JAK2 and (E) p-STAT3/STAT3. All of the aforementioned effects were reversed when pAb IL-15 was co-administered with IL-2. ** $P < 0.01$. NS, not significant; IL, interleukin; JAK2, Janus kinase 2; p-JAK2, phosphorylated JAK2; p-STAT3, phosphorylated STAT3; pAb, polyclonal antibody.

prolongs the survival of tumor-bearing animals (20). IL-15 has attracted increasing attention as a possible antitumor therapeutic agent in the immunotherapy of malignancies due to the reported enhancement of NK cell-mediated cytotoxicity (21). Consistently, in the present study, it was found that levels of IL-15 were significantly reduced in NK cells isolated from patients with CC compared with the healthy volunteer group. IL-15 expression was significantly increased in NK-92 cells following IL-2 stimulation compared with the control group.

IFNs are cytokines produced by host immune cells to non-specifically repress viral replication (22,23). Among the list of commercialized enzymes, vaccines, antibodies and antibiotics, IFNs exhibit remarkable therapeutic efficacy against a large number of diseases (24). In line with these previous reports, the present study found that IL-2 stimulation enhanced the production/secretions of IFN- γ by NK-92 cells which enhanced SW480 cell killing by NK-92 cells, suggesting a therapeutic potential of IFN- γ in CC patients.

In cancer cells, IFN- γ is secreted by NK cells to induce cell apoptosis (7) and growth inhibition (9). Compared with the control group, IL-2 stimulation significantly increased cell apoptosis whilst reducing cell viability, both of which were reversed when co-administered with pAb IL-15, suggesting that IL-15 is indispensable for the function of IL-2. However, the molecular mechanism involved in apoptotic progression require further study.

The Bcl-2 protein family involved in the process of apoptosis includes Bax, which is pro-apoptotic, and Bcl-2, which is anti-apoptotic (25). Compared with the control group, IL-2 stimulation significantly increased BAX expression whilst reducing Bcl-2 expression. The effect of IL-2 was reversed in the presence of pAb IL-15; however, the components lying upstream of BAX and Bcl-2 remain unclear.

During signal transduction, IFN- γ receptor binds to JAK2 binding domains (26), which is followed by the phosphorylation of STAT3 and transcription of IFN-stimulated genes

(ISG) (24) or IFN-regulated factor 1 (27). In the current study, compared with the control group, IL-2 treatment significantly reduced p-JAK2 and p-STAT1 levels, which was negated by the presence of pAb IL-15.

The present study investigated the role of NK-mediated IFN- γ in CC progression. However, the relationship between IFN- γ and IL-15, the molecular pathway linking JAK2/STAT1 with BAX/Bcl-2 and changes in the expression of ISGs remain unclear, and should be explored further in the future.

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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

FC and KN designed the study. FC, DQ, RS and MZ performed experiments. FC, DQ and KN interpreted the data. KN prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Shaanxi, China). All patients signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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