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Functional characterization of interleukin-15 gene transduction into the human natural killer cell line NKL

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Background

Genetic modification of natural killer (NK) cells is a potential approach to gene-based immunotherapy of cancer. We created human interleukin-15 (hIL-15) gene-modified NKL cells and investigated their functional characterization in vitro.

Methods

A recombinant vector (pcDNA3-IL15) or control vector (pcDNA3) was transferred into NKL cells by an electroporation method. Standard reverse transcriptase-polymerase chain reaction (RT-PCR), flow cytometry and MTT methods were performed for NK cell proliferation, apoptosis, cytotoxicity assays and gene expression tests.

Results

Compared with parental NKL cells, hIL-15 gene modification promoted NK cell proliferation at low doses of IL-2 and inhibited cell apoptosis, which was associated with the up-regulation of anti-apoptosis genes Bcl-2, Bcl-xl and Mcl-1 as well as the down-regulation of apoptosis genes Bim and Noxa. Moreover, the anti-tumor activity of hIL-15 gene-transduced NKL cells against human hepatoma cancer cell line HepG2, H7402 and PLC/PRF-5 cells was enhanced, at least partly, through increasing expression of cytotoxicity-associated genes, including interferon (IFN)-γ, perforin and FasL.

Discussion

The hIL-15 genetic modification could improve the proliferation, anti-apoptosis and natural cytotoxicity of NKL cells against hepatocarcinoma cells. These data suggest that hIL-15 gene-modified NKL cells could be useful for clinical cancer immunotherapy in the future.

Keywords

gene transfer, interleukin-15, natural killer cells, natural killer cell line.

Introduction

Natural killer (NK) cells are a key component of the innate immune system that is involved in the early defense against virus-infected or transformed cells by non-major histocompatibility complex (MHC)-, non-T-cell receptor (TCR)-restricted mechanisms [1,2]. NK cell activation is thought to be regulated by the signaling balance between inhibitory and activating receptors, and is immediately followed by cytolysis against target cells and secretion of immunoregulatory cytokines if activated, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF and others [3–9]. NK cells not only mediate spontaneous anti-tumor effector functions but also regulate innate and adaptive immune responses. Adoptive transfer of activated or allogeneic NK cells is effective in the treatment of certain types of leukemia and solid tumors [10,11]. Given the importance of NK cells to immunity, a variety of approaches have been taken to try and selectively augment NK cell responses to tumors, including genetic modification.The cytokine interleukin-15 (IL-15) was originally identified in media conditioned by a monkey kidney epithelial cell line (CVI/EBNA) [12] and is present in a wide variety of tissues and cell types [13–16]. IL-15 shares many biologic activities with IL-2 and can directly induce $CD34^{+}$ cells to differentiate into NK cells in the absence of IL-2 [17,18]. This cytokine is also a potent regulator of NK cell proliferation, survival and cytolytic activity [19–21]. IL-15 signals through the β- and γ-subunits of the IL-2R and the unique private

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α-subunit [22]. Thus, based upon complex regulation, it is likely that the functions of IL-15 and IL-15Rα differ from those of IL-2 and IL-2Rα. In previous work, we have reported that IL-15 gene-modified NK-92 cells exerted more efficiency against cancer [23] but extensive utilization of this gene-modified human NK-92 cell line is limited by its tumor spectrum.

Compared with primary NK-92 cells, NKL cells appear to have a different anti-tumor spectrum. In recent work, some human cancer cells were more sensitive to NKL cell cytotoxicity than NK-92 cells, such as the human gastric carcinoma cell line SGC7901 [24]. NKL is one of six malignant NK cell lines that have currently been established and are sufficiently well characterized. The NKL cell line was established from the peripheral blood of a patient with $CD3^{-}$ $CD16^{+}$ $CD56^{+}$ large granular lymphocyte (LGL) leukemia and grows in the presence of IL-2 at a concentration of 100 U/mL. The morphology of NKL cells resembles that of normal activated NK cells, with a $CD16^{+}$ $CD56^{dim}$ phenotype and very similar natural killing, antibody-dependent cellular cytotoxicity (ADCC) and proliferative responses [25]. Among all the NK cell lines, NKL is probably the one that has retained the most original features of NK cells and could therefore potentially be used as effector cells in adoptive immunotherapy [26–28].

In this report, the human (h)IL-15 gene was stably transfected into NKL cells. We analyzed the characterization of hIL-15 gene-modified NKL cells and investigated the molecular mechanisms for IL-15 gene modification in improving the proliferation, anti-apoptosis activity of NKL cells and enhancing the natural cytotoxicity against hepatocarcinoma cells.

Methods

Cell culture and cell lines

Human hepatocellular carcinoma cell lines HepG2, H7402 and PLC/PRF-5, conserved in our laboratory, were cultured in RPMI-1640 medium (GIBCO/BRL, Grand Island, N.Y. USA) supplemented with 100 U/mL penicillin, 100 μg/mL treptomycin and 10% fetal bovine serum (FBS). NKL was gifted from Professor Jin BQ (Department of Immunology, Fourth Military Medical University, Xi'an, PR China), conserved in our laboratory and cultured in complete IL-2-containing (100 U/mL) RPMI-1640 medium. All these cells were incubated at of 37°C, 5% CO_2.

IL-15 gene transfection into the NKL cell line

A recombinant vector (pcDNA3-IL15) was constructed in our laboratory as described previously [18]. The vector was transferred into NKL cells by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA, USA) at 200 mV and a capacitance of 975 AFarads in a 0.4-cm electroporation cuvette [29]. At the same time, the vector pcDNA3 was transferred as a control. Transfectants were selected by incubation in the presence of 600 μg/mL G418 (GIBCO). The IL-15 gene-transferred NKL cells were then cloned and identified. The resulting cell line was called NKL-IL15, and the control cell line was called NKL-3.

CTLL-2 proliferation assay

CTLL-2 cells were used to identify the bioactivity of hIL-15 secreted by transfected NKL cells. Approximately 1×10^6 NKL-IL15 cells and NKL-3 cells were cultured in 10 mL IL-2-free medium after washing in phosphate-buffered saline (PBS) three times; 48 h later, supernatants were harvested. CTLL-2 cells were then seeded in 96-well culture plates at 1×10^4 cells/well and cultured in 200 μL culture medium containing two-fold dilutions of supernatants from NKL-IL15 cells. Negative controls contained supernatants from NKL-3, and two-fold dilutions of 500 U/mL rhIL-2 were used as a positive control. The cultures were incubated for 48 h at 37°C and pulse-labeled for another 3 h with 10 μL CCK (Dojindo Laboratories, Kumamoto, Japan)/well. Optical density (OD) was measured at 450 nm [30].

IL-15 enzyme-linked immunosorbent assay (ELISA)

Approximately 1×10^6 NKL-IL15 cells and NKL-3 cells were cultured in 10 mL IL-2-free medium after washing in PBS three times; 48 h later, supernatants were harvested. The concentrations of IL-15 in the culture supernatants were measured by ABC-ELISA (Biotechnology Systems, Davis, CA), following the manufacturer's instructions. Negative controls contained supernatants from NKL.

Semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized from 2 μg RNA using M-MLV reverse transcriptase (Promega, Madison, USA).

The concentration and quality of the extracted RNA were determined by measuring light absorbance at 260 nm (A_{260}) at a ratio of (A_{260}/A_{280}). PCR was performed on a 1:5 dilution of the cDNA and 2 μL were subsequently amplified. Each PCR cycle consisted of a denaturation step at 94°C for 1 min, annealing for 1 min (at a primer-specific temperature) and extension at 72°C for 1 min and, after completion of the cycles, one 10-min extension at 72°C. The PCR primers and their product lengths are listed in Table 1 and were synthesized by Shanghai Genecore Biotechnologies (Shanghai, China). Semi-quantitative RT-PCR was performed using β-actin as an internal control. The PCR products were electrophoresed on 2% agarose gels and the relative light intensities of bands were analyzed by AlphaEaseFC software.

Flow cytometric analysis of apoptosis

NKL, NKL-3 and NKL-IL15 cells underwent starvation in medium without serum and IL-2 for 2 or 4 h before harvest. Apoptosis cells were assessed using an apoptosis detection kit (Jingmei Biotech, Shenzhen, China) according to the manufacturer's protocol. In brief, cells (1×10^5) suspended in binding buffer were incubated with Annexin V–FITC and propidium iodide (PI) for 15 min at room temperature in the dark. The cells were then analyzed by flow cytometry performed on a FACScalibur cell analyzer using CellQuest software (Becton Dickinson, Mountain View, CA, USA) [11]. Cells undergoing apoptosis were defined as those positive for Annexin V.

Proliferation assay

All cellular growth assays were performed in 96-well plates (1×10^4 cells/well). NKL, NKL-3 and NKL-IL15 cells were cultured at 37°C in a 5% CO_2 incubator in complete 1640 medium at different concentrations of IL-2 and IL-15. The cell proliferation was determined with a colorimetric cell-counting kit (CCK-8; Dojindo Laboratories), according to the manufacturer's instructions, for 72 h. Cells

Table 1. Primers used for RT-PCR reactions

Transcripts	Product size (bp)	Sequences (5′–3′)	Annealing temperature, PCR cycle
β-actin	300	5′ATCATGTTTGAGACCTTCAACA3′ 5′CATCTCTTGCTCGAAGTCCA3′	58°C, 30 cycles
IL-15	594	5′CGGATCCGATGAGAATTTCGAAACCACAT3′ 5′GCGAATTCGTCAAGAAGTGTTGAT3′	58°C, 30 cycles
IFN-γ	494	5′ATGAAATATACAAGTTATATCTTGGCTTT3′ 5′GATGCTCTTCGACCTCGAAACAGCAT3′	58°C, 30 cycles
Perforin	436	5′AAAGTCAGCTCCACTGAAGCTGTG3′ 5′AGTCCTCCACCTCGTTGTCCGTGA3′	58°C,30 cycles
FasL	500	5′ATGTTTCAGCTCTTCCACCTACAGA3′ 5′CCAGAGAGAGCTCAGATACGTTGAC3′	58°C, 30 cycles
NKG2D	416	5′CTGGGAGATGAGTGAATTTCATA3′ 5′GACTTCACCAGTTTAAGTAAATC3′	58°C,30 cycles
NKG2A	325	5′CCAGAGAAGCTCATTGTTGG3′ 5′CCAATCCATGAGGATGGTG3′	58°C, 30 cycles
Bcl-2	364	5′CGACTTCGCCGAGATGTCCAGCCAG3′ 5′ACTTGTGGCCCAGATAGGCACCCAG3′	58°C, 30 cycles
Bcl-xl	258	5′GGTGGGAGGGTAGAGTGGATGGT3′ 5′GGAAAGCGTAGACAAGGAGATGC3′	55°C, 30 cycles
Fas	525	5′GCCATTAAGATGAGCACCAAGG3′ 5′CCCAAATAGGAGTGTATGCAGAGG3′	55°C, 30 cycles
Bim	204	5′GCCCCTACCTCCCTACAGAC3′ 5′ATGGTGGTGGCCATACAAAT3′	55°C, 30 cycles
Noxa	196	5′AGATGCCTGGGAAGAAG3′ 5′AGTCCCCTCATGCAAGT3′	55°C, 30 cycles
Mcl-1	496	5′CACGAGACGGTCTTCCAAGGCATGCT3′ 5′CTAGGTTGCTAGGGTGCAACTCTAGGA3′	61°C, 30 cycles

were treated with 10 μL CCK-8 solution/well and incubated for 4 h at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was measured by absorbance at 450 nm with a microplate autoreader (Bio-Rad).

Cytotoxicity assay

Human hepatoma cancer cell line HepG2, H7402 and PLC/PRF-5 cells, used as the target cells, were placed in 96-well plates at 10^4 cells/well. The cytotoxicity of NKL, NKL-3 and NKL-IL15 cells was determined by MTT assay. Effector cells were added to target cells at effector (E) to target (T) ratios of 20:1, 10:1, 5:1, 2.5:1 and 1.25:1. The cell mixtures were then incubated at 37°C for 12 h, after which 20 μL MTT (5 mg/mL) were added and incubated for a further 4 h. The absorbance (A) at 570 nm in each well was determined with a microplate autoreader (Bio-Rad).

Statistical analyzes

All data are expressed as mean ± SD and accompanied by at least three distinct experiments. Statistical analysis was performed using SPSS software (version 10.0; SPSS Inc., USA) and the significant difference was set at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Establishment of the hIL-15 gene-modified NKL cell line

Numerous studies have convincingly shown that IL-15 is critical for NK cell development [3,31]. It is the major physiologic growth factor responsible for NK cell ontogeny. In this study, the IL-15 gene was transfected into NKL cells by an electroporation method and the cells were selected by G418. Levels of hIL-15 gene expression in transfectant NK cells were evaluated by an RT-PCR method. As shown in Figure 1A, hIL-15 gene-modified NKL (NKL-IL15) cells expressed significantly higher IL-15 mRNA than parental

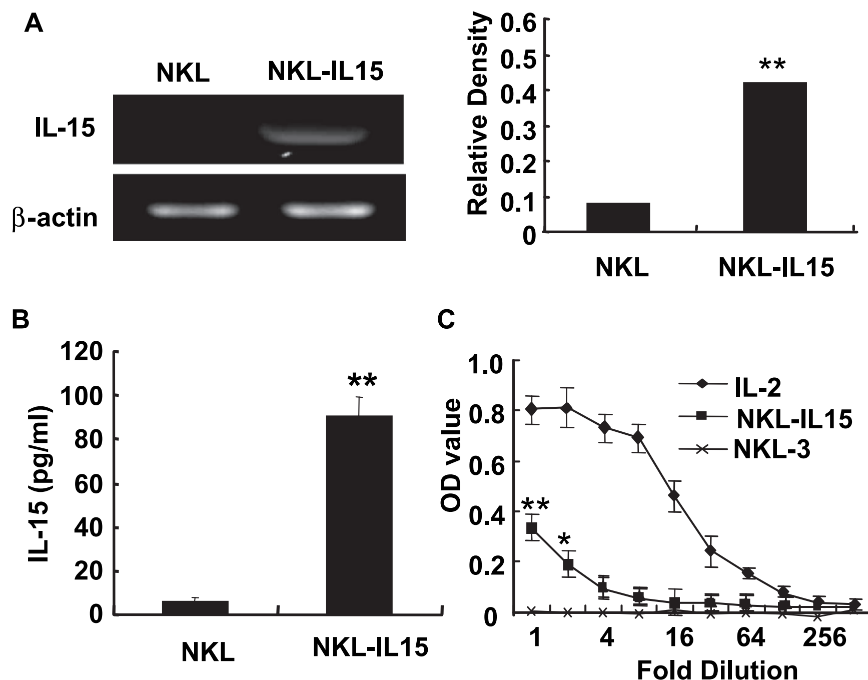


Figure 1. hIL-15 gene modification of the NKL cell line. As described in the Methods, NKL cells were transfected with hIL-15 gene and selected by G418, with pcDNA3 vector-transfected NKL cells (NKL-3) as a control. (A) The total cellular RNA was isolated and mRNA levels of the hIL-15 gene were detected using a RT-PCR method. (B) ELISA analysis for IL-15 in the supernatant of NKL-IL15 cells, which were cultured in free medium for 48 h. (C) Proliferation response of CTLL-2 cells to IL-2 or IL-15. After washing with PBS, NKL-IL15 and NKL-3 cells were cultured in IL-2-free medium for 48 h, and supernatants were harvested. CTLL-2 cell proliferation responses to these supernatants were tested, while hIL-2 was used as a positive control. Data shown are means ± SD from at least three separate experiments. $^{**}P < 0.01$ *by comparison with parental NKL cells.*

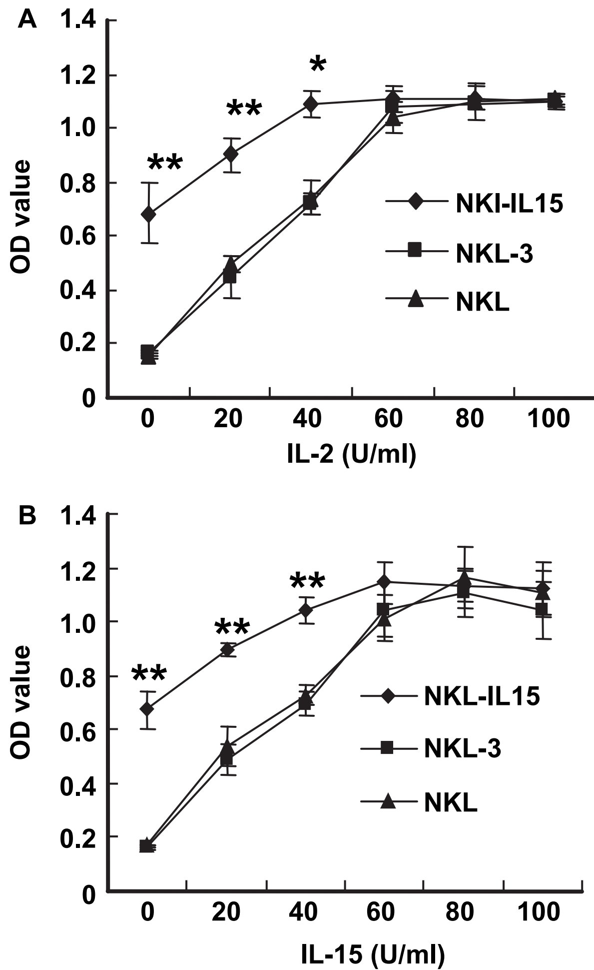


Figure 2. Enhanced proliferation of hIL-15 gene-modified NKL cells in culture with low levels of IL-2 or IL-15. NKL, NKL-3 and NKL-IL15 cells were exposed to different concentrations of IL-2 (A) or IL-15 (B), ranging from 0 to 200 U/mL. After 72 h, cells were treated with 10 μL CCK-8 solution/well. After incubation for 4 h, the OD values were measured. Data shown are means ± SD from at least three separate experiments. *P < 0.05, **P < 0.01 *by comparison with NKL-3 cells.*

NKL cells ($P < 0.01$). To identify whether IL-15-transfected NKL cells secret soluble IL-15, we used an ELISA to detect IL-15 protein levels in the supernatant of NKL-IL15 cells. The IL-15 concentration in NKL-IL15 cell supernatant was about 90 pg/mL (Figure 1B), which illustrated that IL-15 could be secreted by NKL-IL15 cells stably. To identify whether the IL-15 secreted by NKL-IL15 cells had bioactivity, we used the IL-2-dependent murine T-cell line CTLL-2 to detect IL-15 activity in the supernatant of NKL-IL15 cells. The OD value of IL-15 gene-transfected NKL supernatant was higher than that of vector-transfected NKL supernatant (Figure 1C), which meant the NKL-IL15 cells secreted active IL-15 stably. The hIL-15 gene-modified NKL cells exhibited similar morphologic characteristics with parent NKL cells and were suitable for long-term culture *in vitro*. The results indicate that the hIL-15 gene was stably transduced into NKL cells.

Improvement of proliferation and anti-apoptosis in the hIL-15 gene-modified NKL cell line

The NKL cell line is known to be IL-2-dependent and maintained in medium containing 100 U/mL IL-2 *in vitro*. To study the effect of hIL-15 gene on the growth of NKL cells further, NKL, NKL-3 (NKL cells transfected with pcDNA3 vector) and NKL-IL15 cells were incubated with different doses of IL-2 for 72 h. As shown in Figure 2A, the growth of NKL-IL15 cells was significantly increased by about 4.14-fold ($P < 0.01$), 2.01-fold ($P < 0.01$) and 1.50-fold ($P < 0.05$) at doses of 0, 20 and 40 U/mL IL-2, respectively, compared with NKL and NKL-3 cells. When exposed to low dosages of hIL-15 (< 40 U/mL), the proliferation of NKL-IL15 cells was markedly promoted

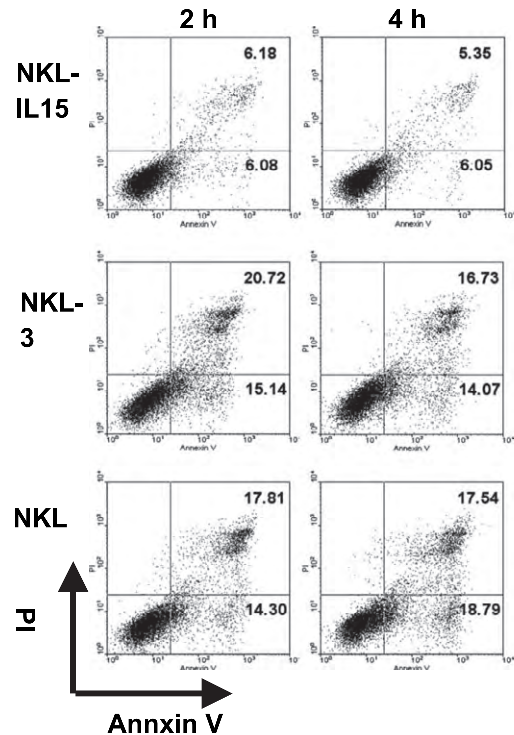


Figure 3. Anti-apoptotic effect of hIL-15 gene modification in NKL cells. NKL, NKL-3 and NKL-IL15 cells were plated in six-well plates; after serum and IL-2 starvation for 2 h or 4 h, the apoptotic cells were measured by flow cytometry using Annexin V–FITC and PI. hIL-15 gene-modified NKL cells have fewer apoptotic cells with a starvation background. One of three identical experiments is shown.

(Figure 2B). As under unsaturation status, the total concentration of IL-15, including exogenous and secreted IL-15 in NKL-IL15 cell supernatant, was higher than that in NKL cells. In contrast, the pcDNA3 vector did not impact on the growth of NKL cells.

As IL-15 was now viewed as a cytokine not only inducing proliferation but also reducing cell apoptosis, we next calculated the anti-apoptosis effects of the hIL-15 gene in NKL cells. As shown in Figure 3, after serum starvation for 2 h, flow cytometry analysis indicated that the apoptotic rate for NKL-IL15 cells was 12.26%, while NKL-3 cells and NKL cells were up to 35.86% and 32.11%, respectively. About 11.4% of NKL-IL15 cells were triggered to apoptosis after serum starvation for 4 h, compared with 30.08% of NKL-3 cells and 36.33% of NKL cells. These findings suggested that hIL-15 gene modification had effectively improved the proliferation and anti-apoptosis in NKL cells.

We further investigated whether the improvement of the hIL-15 gene on NKL cells was mediated by increasing the expression of anti-apoptotic genes. An RT-PCR assay was used to detect the mRNA levels of anti-apoptotic genes Bcl-2, Bcl-xl and Mcl-1 and apoptosis genes Fas, Bim, Noxa under serum starvation-induced apoptosis condition. As shown in Figure 4, NKL-IL15 cells expressed higher Bcl-2 ($P < 0.01$), Bcl-xl ($P < 0.05$) and Mcl-1 ($P < 0.05$) and lower Bim ($P < 0.05$) and Noxa ($P < 0.05$) levels than parental or NKL-3 cells. Another gene, Fas, was slightly down-regulated in NKL-IL15 cells, but to a much lower extent. Huntington *et al.* [31] recently suggested that bim, mcl-1 and noxa were major controllers of IL-15-dependent survival of murine NK cells, while Bcl-2 did not show any significant change when NK cells were withdrawn from IL-15. The different data for Bcl-2 may have been because the NKL cell line was derived from the peripheral blood of a patient with $CD3^-$ $CD16^+$ $CD56^+$ LGL leukemia,

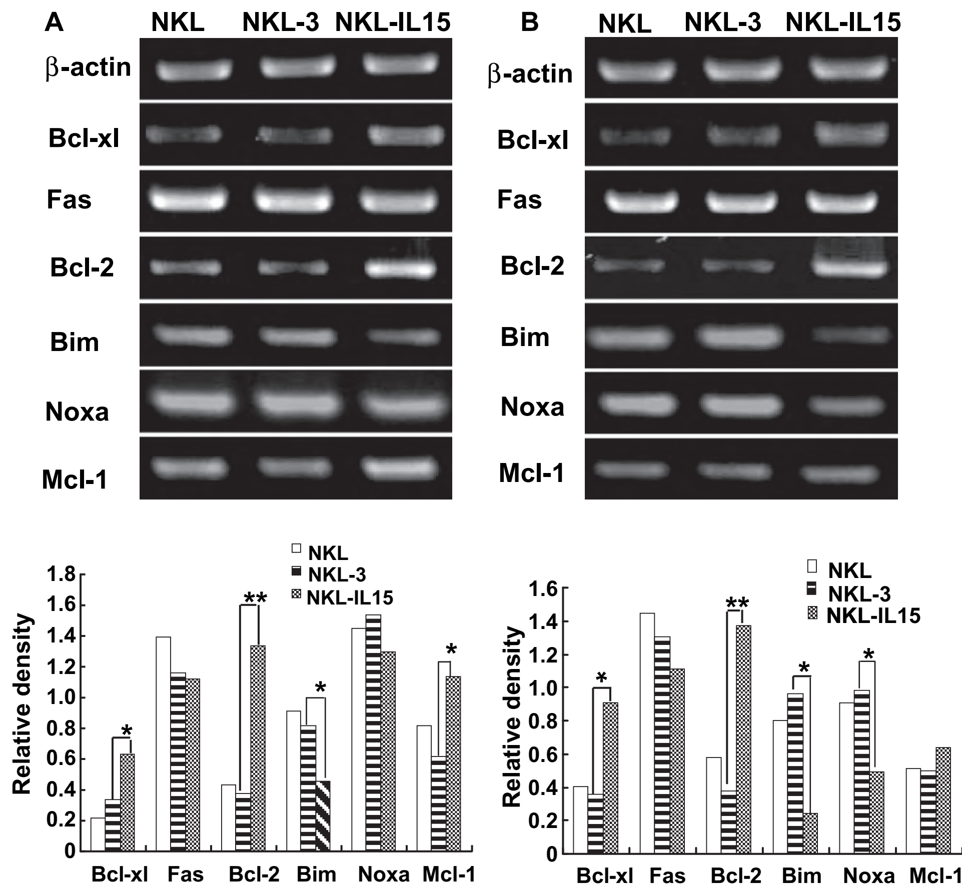


Figure 4. *Up-regulation of anti-apoptosis gene expression in hIL-15 gene-modified NKL cells. After serum and IL-2 starvation for 2 h or 4 h, NKL, NKL-3 and NKL-IL15 cells were harvested and total RNA was extracted using TRIzol. The expression levels of anti-apoptotic genes Bcl-2, Bcl-xl and Mcl-1, as well as apoptosis genes Fas, Bim and Noxa, were examined by RT-PCR and the products analyzed as described in the Methods. The experiment shown is representative of at least three repetitions.* *$P < 0.05$; **$P < 0.01$ *by comparison with NKL-3 cells.*

which is different from natural NK cells regarding some biologic properties. So the responses to IL-15 may be different, including apoptosis gene regulation.

Enhancement of natural cytotoxicity against hepatocarcinoma in hIL-15 gene-modified NKL cells

Because exogenous IL-15 is known to augment the cytotoxicity of NK cells, we wished to study the effect of endogenously secreted IL-15 on anti-tumor functions of transduced NK cells. Cytolytic activities of parental or transduced NKL cells were measured in 12-h MTT assays against human hepatoma cancer cell lines HepG2, H7402 and PLC/PRF-5. As shown in Figure 5, NKL-IL15 cells exerted a significantly stronger cytotoxicity against HepG2 cells than parental NKL cells, especially at E:T ratios of 10:1, 5:1 and 2.5:1. NKL-IL15 cells also showed greater cytotoxicity against H7402 and PLC/PRF-5 cells compared with NKL and NK-3 cells. In order to identify the viability of the NK cell lines during the cytotoxicity assays, flow cytometry analysis was performed to test the apoptosis of cells after co-culture with target cells for 12 h. The results showed that about 90% of the NK cells survived.

To investigate whether the enhancement of the hIL-15 gene on NK cells was correlated with expression of cytotoxicity-associated genes, RT-PCR was used to detect the mRNA levels of NKG2D, NKG2A, IFN-γ, FasL and perforin. The results showed that mRNA of IFN-γ, FasL and perforin were significantly elevated in NKL-IL15 cells ($P < 0.01$), while the expression of NKG2D revealed a little increase and NKG2A showed a little decrease (Figure 6A). Additionally, NKL cells were stimulated with IL-15 followed by investigation of IFN-γ and perforin mRNA levels. As shown in Figure 6B, both IFN-γ and perforin mRNA levels increased in a dose-dependent manner. Finally, we identified NK cell activation surface makers NKG2D and NKG2A via flow cytometry. Figure 6C shows that NKG2D elevated slightly and NKG2A decreased

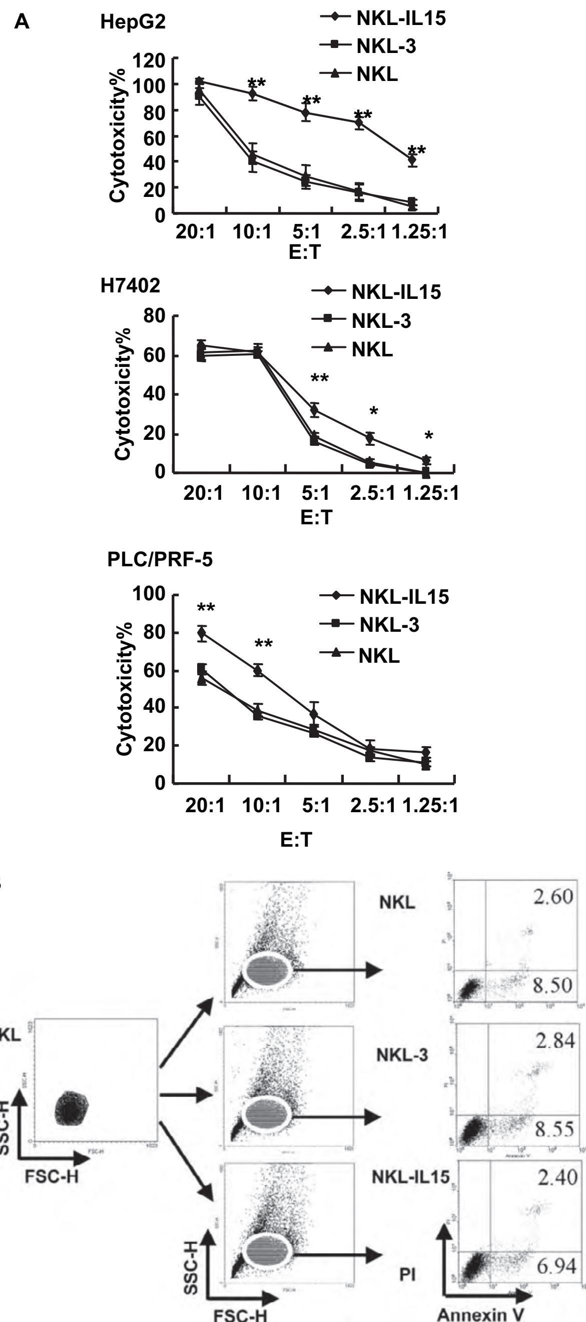
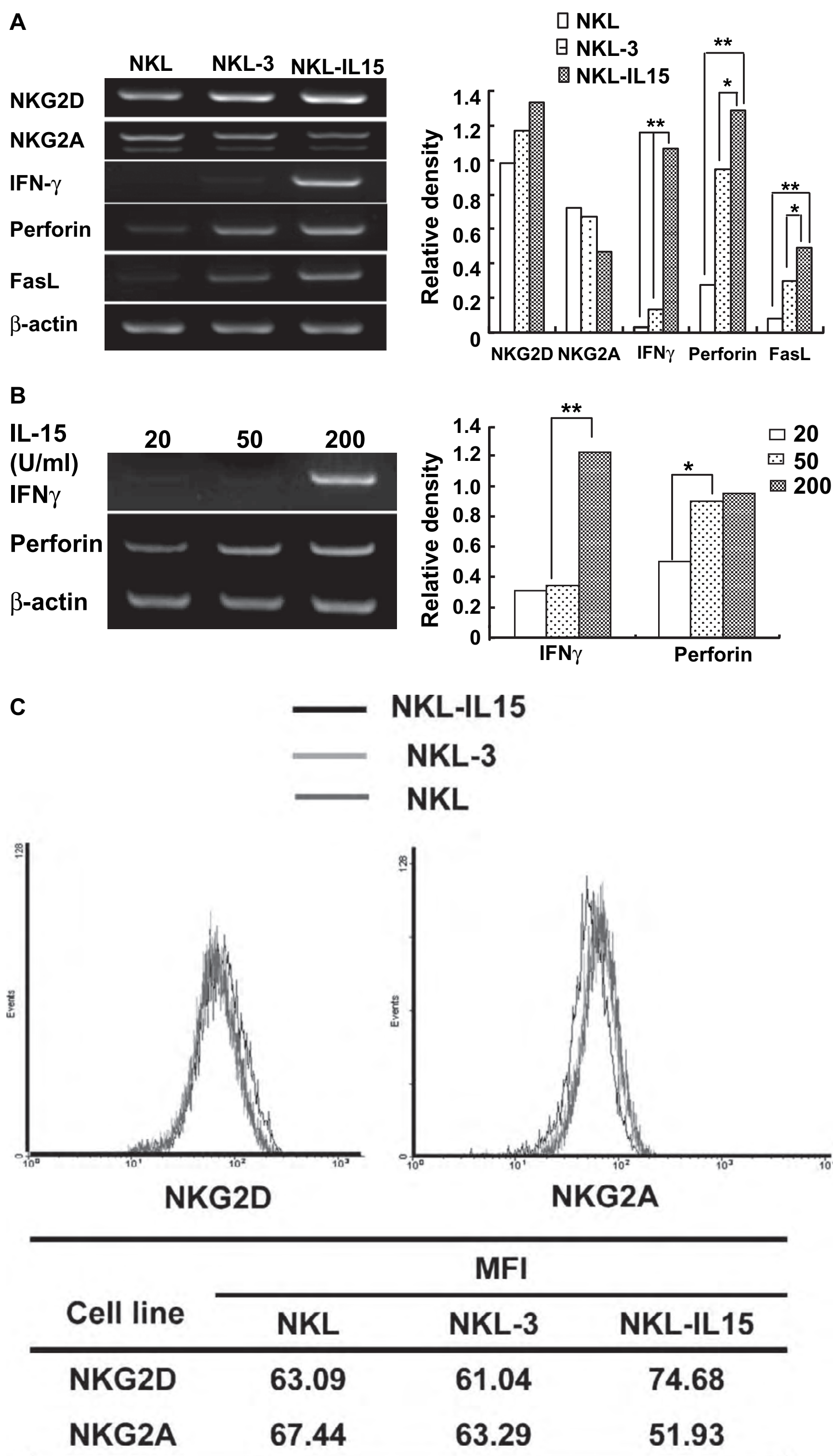


Figure 5 (*Continued*)

Figure 5*. Enhancement of cytotoxicity against hepatocarcinoma cells by hIL-15 gene-modified NKL cells. (A) Assays were performed using NKL, NKL-3 or NKL-IL15 cells that had been cultured in the presence of IL-2 (100 U/mL). NK cells were co-cultured in graded E:T ratios with human hepatoma cancer cell line HepG2, H7402 or PLC/PRF-5 cells for 12 h, and cellular cytotoxicity was tested by MTT assay. NKL-IL15 cells exerted stronger cytotoxicity than NKL and NKL-3 cells. Data shown are means ± SD from at least three separate experiments. (*P < 0.05, **P < 0.01 by comparison with NKL-3 cells and NKL cells) (B) After co-culture with cancer cells for 12 h, NK cells were harvested and the viability measured by flow cytometry using Annexin V–FITC and PI. As these NK cells can be suspended, they can be separated easily from human hepatoma cells, which are adhesive. In order to exclude HepG2 cells, NKL and HepG2 cells were gated by FSC and SSC signals separately, then NKL cells that fell in the region were analyzed. One of three identical experiments is shown.*



Cell line	MFI		
	NKL	NKL-3	NKL-IL15
NKG2D	63.09	61.04	74.68
NKG2A	67.44	63.29	51.93

Figure 6*. Up-regulation of cytotoxicity-associated gene expression in hIL-15 gene-modified NKL cells. (A) NKL, NKL-3 and NKL-IL15 cells were harvested, the total cellular RNA isolated using TRIzol reagent, and mRNA levels of NK cell cytotoxicity-associated genes detected by RT-PCR. (B) IFN-γ and perforin mRNA levels in NKL cells treated with IL-15 were analyzed by RT-PCR. (C) NK cell activation surface markers, NKG2D and NKG2A, were analyzed by flow cytometry. The experiment shown is representative of at least three repetitions.*

mildly compared with NKL and NKL-3 cells, but there was no significant difference. These results indicated that the hIL-15 gene modification of NKL cells could enhance the natural cytotoxicity against hepatocarcinoma cells, partly dependent on up-regulation of the cytotoxicity-associated gene expression.

Discussion

NK cells play a crucial role as a first line of defense against virus infection and tumor cells, and have been used as a strategic weapon in cancer cell therapy and hematopoietic stem cell transplantation, through activation of endogenous NK cells and adoptive transfer of *in vitro*-activated autologous NK cells or permanent NK cell lines among which the NK-92 cell line has already finished clinical trials [32,33]. Numerous studies have been conducted to improve the anti-tumor effect of NK cells, and gene modification is essential for many approaches to gene-based immunotherapy of cancer. Up to now, genes of IL-2, IL-15, SCF, CD20 and others have been used to modify the NK-92 cell line and exhibit more feasibility for clinical application [34,35].

To create more effective NK cells against human tumors, we have established hIL-15 gene-modified NKL cells with many original features of NK cells (Figure 1). Investigations have demonstrated that the hIL-15 gene modification has improved the NK cell proliferation potential at low concentrations of IL-2 and IL-15, and NKL-IL15 cells proliferate even in the absence of IL-2 (Figure 2), with less apopototic cells by serum starvation (Figure 3). These results indicate that IL-15 gene modification of NKL cells enhances their suitability for large-scale production *in vitro*.

The anti-apoptosis proteins Bcl-2 and Bcl-xl are anti-apoptotic members of the Bcl-2 protein family. Because IL-15 has been reported to maintain or increase Bcl-2 expression in human NK cells, Bcl-2 has been proposed as the critical factor by which IL-15 promotes the survival of NK cells [36,37]. It has also been reported that over-expression of Bcl-2 inhibits apoptosis of NK cells after their transfer into IL-15$^{-/-}$ mice. Huntington *et al.* [31] recently published bim, mcl-1 and noxa as major controllers of the IL-15-dependent survival of murine NK cells. We found that, with the improvement of anti-apoptosis in IL-15 gene-modified NKL cells, the anti-apoptosis gene Bcl-2, Bcl-xl and Mcl-1 expression was significantly increased and accompanied apoptosis gene down-regulation (Fas, Bim and Noxa) (Figure 4). These results indicate that the IL-15 gene-modified NKL cells improve the anti-apoptosis, which is more suitable for clinical application.

Because hepatocarcinoma is one of the most troublesome diseases world-wide, we tried to use the IL-15 gene-modified NKL cells as adoptive immunotherapy for hepatocarcinoma. We found that the natural cytotoxicity against hepatocarcinoma cells was significantly augmented in the IL-15 gene-transferred NKL cells (Figure 5). Furthermore, we explored the mechanism by which NKL-IL15 cells killed the tumor cell targets. The IL-15 gene-modified NKL cells expressed more IFN-γ, FasL and perforin than the parental cells, while the expression of NKG2D revealed a little increase and NKG2A showed a little decrease (Figure 6), The TNF-α level was almost unchanged (data not shown). These findings suggest that FasL and perforin might be responsible for the enhanced cell cytotoxicity of NKL-IL15, and IFN-γ for the inhibition of tumor cell proliferation.

IL-15 is a pleiotropic cytokine that plays an important role in both the innate and adaptive immune system. IL-15 promotes the activation of neutrophils and macrophages and is critical to dendritic cell function. Additionally, IL-15 is essential for the generation, homeostasis, function and survival of NK cells, NKT cells and CD8^{+} T cells [38]. In particular, in contrast to IL-2, IL-15 inhibits IL-2-induced AICD and stimulates the division and survival of memory-phenotype CD8^{+} T cells. Based on these properties, IL-15 gene-modified NK cells might be superior to IL-2 in the treatment of cancer.

Acknowledgements

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