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ORIGINAL ARTICLE – CANCER RESEARCH

hIL-15-gene modified human natural killer cells (NKL-IL15) exhibit anti-human leukemia functions

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Abstract

Purpose Natural killer (NK) cells can kill transformed cells and represent anti-tumor activities for improving the immunotherapy of cancer. In previous works, we established human interleukin-15 (hIL-15) gene-modified NKL cells (NKL-IL15) and demonstrated their efficiency against human hepatocarcinoma cells (HCCs) in vitro and in vivo. To further assess the applicability of NKL-IL15 cells in adoptive cellular immunotherapy for human leukemia, here we report their natural cytotoxicity against leukemia in vitro and in vivo.

Methods Flow cytometry, ELISA and MTT methods were performed for molecular expression, cell proliferation and cytotoxicity assays. Leukemia xenograft NOD/SCID mice were established by subcutaneous injection with K562 cells, and then treated with irradiated NKL cells.

Results We found NKL-IL15 cells displayed a significant high cytolysis activity against both human leukemia cell lines and primary leukemia cells from patients, accompanied with up-regulated expression of molecules related to NK cell cytotoxicity such as perforin, granzyme B and NKp80. Moreover, cytokines secreted by NKL-IL15 cells, including TNF-α and IFN-γ, could induce the expression of NKG2D ligands on target cells, which increased the susceptibility of leukemia cells to NK cell-mediated cytolysis. Encouragingly, NKL-IL15 cells significantly inhibited the growth of leukemia cells in xenografted NOD/SCID mice and prolonged the survival of tumor-bearing mice dramatically. Furthermore, NKL-IL15 cells displayed stimulatory effects on hPBMCs, indicating the immunesuppressive status of leukemia patients could be improved by NKL-IL15 cell treatment.

Conclusions These results provided evidence that IL-15 gene-modification could augment NK cell-mediated anti-human leukemia function, which would improve primary NK cell-based immunotherapy for leukemia in future.

Keywords NK cell · Interleukin 15 · Gene modification · Leukemia

Introduction

Natural killer (NK) cells are an integral component of the innate immune system and are characterized by their strong cytolytic activity against pathogen-infected cells and tumor cells via non-major histocompatibility complex (MHC)-, non-T-cell receptor (TCR)-restricted mechanisms (Sutlu and Alici 2009; Terme et al. 2008; Bray et al. 2011; Yang et al. 2017). NK cells also regulate innate and adaptive immune responses through secretion of immunoregulatory cytokines and cell-to-cell contact (Ljunggren and Malmberg 2007; Cheng et al. 2013; Xu et al. 2016). The usage of NK cells in leukemia immunotherapy has been proposed, and treatments based on NK cells have been recently entered clinical trials. Notably, in haploidentical transplantation, NK cell alloreactivity is triggered by mismatches between killer cell Ig-like receptors (KIRs) on donor NK cells and HLA class I molecules on recipient cells, which extends the usage of mismatched transplants for leukemia patients without an HLA-matched donor. In addition, alloreactive NK cells could become part of conditioning regimens to help engraftment and protect against T cell-mediated GVHD (Ruggeri et al. 2002; Miller et al. 2005).

For the purpose to improve the anti-tumor activity of NK cells to be more suitable for tumor immunotherapy, several cytokine genes including IL-2, IL-15 and stem cell factor

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(SCF) (Jiang et al. 2008; Zhang et al. 2004a, b; Nagashima et al. 1998), have been selected to modify NK cell lines. Among these cytokines, IL-15 is responsible for NK cell development, and provides signals necessary for both immature and mature NK cell survival, the expression of cell surface markers characteristic of mature NK cells, as well as NK cell-mediated lytic activity (Ohteki 2002; He et al. 2004; Kennedy et al. 2000). Because of the attractive unlimited proliferation ability, NK cell lines are more feasible for gene-modification than primary NK cells. Among all of the NK cell lines, the NKL cell line, generated from the peripheral blood of a patient with $CD3^{-}CD16^{+}CD56^{+}$ large granular lymphocyte (LGL) leukemia, is probably the one that has retained the most original features of NK cells and can therefore potentially be used as effector cells in adoptive immunotherapy (Robertson et al. 1996).

In previous reports, we have successfully established hIL-15 gene-modified NKL cell line (NKL-IL15) and confirmed its anti-HCC effect in vitro (Jiang et al. 2008) and in vivo (Jiang et al. 2014). Since NK cell-based cellular immunotherapy may be more favorable for particular benefit in blood-borne cancers, such as leukemias and lymphomas, we investigated the efficiency of NKL-IL15 cells against leukemia, including leukemia cell lines and primary leukemia patient cells. These data provided evidence that human IL-15 gene-modified NKL cells might also be a potential immunotherapy for leukemia.

Materials and methods

Mice and cell lines

Nod/LtSz-Prkdc^{scid}/Prkdc^{scid} (NOD/SCID) mice were obtained from Shanghai Experimental Animal Center (Chinese Academy of Sciences, Shanghai). All mice were housed in specific-pathogen-free conditions and mice 6 weeks of age were used in all experiments. The handling of mice and experimental procedures were conducted in accordance with experimental animal guidelines. The animal study was approved by the Institutional Review Board of Shandong University. Human chronic myelogenous leukemia cell line K562 and human promyelocytic leukemia cell line HL-60 conserved in our laboratory were cultured in RPMI-1640 medium (GIBCO/BRL, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS). NKL cells 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. NKL-IL15 and NKL-vec cells were constructed in our laboratory as described previously (Jiang et al. 2008). All these cells were incubated at 37 °C, 5% CO_2.

Patients

Ten patients with first visit or relapsed leukemia at Shandong Provincial Hospital, 4 male and 6 female, age range 15–68 years, median age 46 years. The patient group consisted of three CML patients, three AML patients and four ALL patients. This study was approved by the Institutional Review Board of Shandong University. Written informed consent was also obtained from each patient and the parents of minor participant.

Co-culture assay

K562, NKL-IL15/NKL-vec cells or PBMC cells were co-cultured using a 0.4 μm porous transwell system (Corning Costar, Tewksbury, MA, USA) of 12-well plates in a ratio of 1:1 for 24 h, and cells cultured in complete 1640 medium served as control.

Cytokine neutralization and receptor blockade

In neutralization assay, anti-IFN-γ mAb (R&D Systems) and anti-TNF-α mAb (R&D Systems) were used at a final concentration of 5 and 0.25 ng/ml, respectively, with 1 μg/ml actinomycin D. In NKG2D blocking assay, NK cells were treated with 10 μg/ml anti-NKG2D mAb (R&D Systems) or isotype control mAbs for 1 h before being co-cultured with K562 cells.

Isolation of human peripheral blood mononuclear cells (hPBMCs)

Human peripheral blood was collected from healthy donors, and hPBMCs were isolated by Isopaque–Ficoll gradient centrifugation. These cells were washed three times with PBS and resuspended in PBS.

Flow cytometry analysis

For cell surface molecule staining, cells were harvested and stained with the labeled mAbs at 4 °C for 45 min. For intracellular protein staining, cells were cultured in RPMI 1640 containing 10% FCS, and treated with monensin (Sigma) for 4 h to inhibit extracellular secretion of cytokines. The antibodies used were as follows: FITC-conjugated Ab to CD3 and Granzyme B (R&D System), FITC-conjugated Ab to perforin (eBioscience, San Diego, CA, USA), PE-conjugated Ab to NKp80, NKG2D or ULBP1-3 (R&D Systems), PE-conjugated MICA/B (eBioscience, San Diego, CA, USA), or FITC/PE-conjugated

anti-human IgG (eBioscience, San Diego, CA, USA). All stained cells were analyzed using a flow cytometer (FACScalibur, USA), and the data were processed with WinMDI 2.9 software (Scripps Research Institute).

Proliferation assay

K562 cells (3×10^5/well) were plated in 12-well plates cultured alone or co-cultured with NKL-IL15 cells/NKL-vec cells by transwell method at 37 °C in a 5% CO_2 incubator. 24 h later, the viable K562 cells in each well were collected and stained with Ki67 mAbs (Abcam) at 4 °C for 45 min. These stained cells were detected using BD FACSAria II instruments and then analyzed using flowJo software. Meanwhile, the viable K562 cells in each well were counted after trypan blue staining. Each sample was in triplicate, and each well was counted four times.

Cytotoxicity assay

The cytolytic activity of NKL cells against leukemia cell lines was determined via apoptosis assay. Briefly, 2×10^5 target cells (K562 or HL-60 cells) were collected and resuspended in Diluent C Solution containing PKH26 dye (Sigma, St. Louis, USA). Then NKL-IL15/NKL-vec cells were added to target cells at an effector/target (*E*/*T*) ratio of 2:1 and cultured at 37 °C with 5% CO_2. After 4 h, the co-cultured cells were harvested, and resuspended in binding buffer and incubated with AnnexinV-FITC (Apoptosis detection kit, BD Pharmingen) for 15 min at room temperature in the dark. The percentage of specific cytolysis rate (PKH26+Annexin-V+) of target cells was monitored by a BD LSR Fortessa cytometer.

The cytolytic activity of PBMCs or NKL cells against leukemia cells were determined via the MTT assay. Briefly, human leukemia cell lines K562 and HL-60 were used as the target cells and placed in 96-well plates at 1×10^4 cells/well. NKL-IL15/NKL-vec cells were added to target cells at effector/target (*E*/*T*) ratios of 10:1 and 5:1. As primary human leukemia cells were used as the target cells, 3×10^4 cells/well were placed in 96-well plates at the *E*/*T* ratios 5:1 and 2.5:1. The cytotoxicity of PBMCs treated with NKL-IL15/NKL-vec cells was evaluated at *E*/*T* ratio of 20:1. The effector and target cell mixtures were then incubated for 6 h at 37 °C in the 5% CO_2, and then 20 µl MTT (5 mg/ml) were added and incubated for a further 4 h. The absorbance (*A*) at 490 nm in each well was determined with a microplate autoreader (Bio-Rad). Cytotoxicity (%) = [1 − (*A* of target plus effector cells − *A* of effector cel1s)/*A* of target cells] × 100%.

ELISA

NKL cells (3×10^5/well) were plated in triplicate in 12-well plates with/not K562 cells by co-culture method, and the level of TNF-α and IFN-γ in cell culture supernatants was detected by ELISA kits (R&D Systems), following the manufacturers' instructions.

Tumor challenge and treatment

To evaluate local tumor growth, K562 cells (1×10^7) were injected into the right flank of NOD/SCID mice subcutaneously. The mice were then randomly assigned to three groups with six mice in each group (PBS treatment control, NKL-vec treatment group, and NKL-IL15 treatment group). After irradiation at 800 cGy (200 cGy/min), 1×10^8 NKL-IL15 or NKL-vec cells in 200 µl were injected intravenously on days 7 and 14 after inoculation, and a corresponding volume of PBS was injected into the control mice. The tumor growth was assessed every 1 week by measuring tumor volume, calculated as $V = lw^2/2$, where l = length and w = width. Four weeks later, the mice were killed, and tumors were weighed. Meanwhile, survival curve of mice peritoneal injected with 1×10^7 K562 cells (200 µl) and treated as above was evaluated.

Statistical analysis

All data are expressed as mean ± SD and accompanied by at least three distinct experiments. Statistical analysis was performed using a paired Student's *T* test. The significant difference was set at $^*P<0.05$, $^{**}P<0.01$. Mouse survival was analyzed using the Mann–Whitney *U* test.

Results

hIL-15 gene-modification enhanced NKL cell-mediated natural cytotoxicity against human leukemia cell lines and patient cells

To assess the application of NKL-IL15 cells in adoptive cellular immunotherapy, two human leukemia cell lines, K562 and HL-60, were used as target cells. As shown in Fig. 1a, b, hIL-15 gene significantly heightened NKL cell-mediated cytolysis against K562 cells and HL-60 cells at the *E:T* ratio examined by both MTT and apoptosis assays. Subsequently, we further evaluated the cytotoxicity of NKL-IL15 cells against primary leukemia cancer cells from first visit or relapsed leukemia patients, including three CML patients, three AML patients and four ALL patients. Compared with NKL-vec cells, NKL-IL15 cells displayed enhanced cytotoxicity. Especially at a high *E:T* ratio (5:1), the cytotoxicity

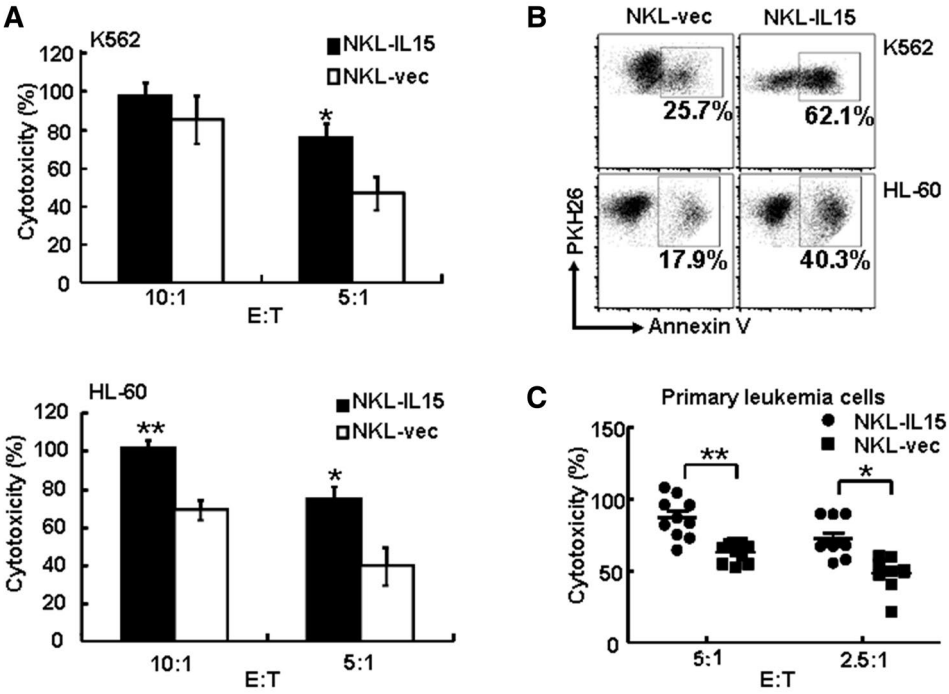

Fig. 1 hIL-15 gene-modification enhanced NKL cell-mediated natural cytotoxicity against human leukemia cells. NKL-IL15 cells or NKL-vec cells were used as effector cells, and human leukemia cells were used as target cells. **a** NKL cell-mediated lysis against human leukemia cell lines K562, HL-60 cells were assessed by the MTT assay at indicated *E:T* ratios. $^{*}P<0.05$, $^{**}P<0.01$ versus NKL-vec cells using the paired Student's *T* test. **b** NKL cell-mediated cytolysis against human leukemia cell lines K562 and HL-60 was detected by apoptosis assay at *E:T* ratio of 2:1. One representative of at least three independent experiments. **c** NK cell-mediated lysis against primary leukemia cells was assessed by the MTT assay at indicated *E:T* ratios. Data shown are means ± SD from at least three separate experiments. $^{*}P<0.05$ versus NKL-vec cells using the Wilcoxon–Mann–Whitney test

was increased from 62.9 ± 6.6% to 87.1 ± 14.1% ($P<0.01$) (Fig. 1c). These results indicated that hIL-15 gene modification could enhance NKL cell-mediated natural cytotoxicity against human leukemia cells.

hIL-15 gene-modification enhanced the responsiveness of NKL cells to leukemia cells

To clarify the characteristics of NKL-IL15 cell responsiveness to leukemia cells, NKL-IL15 cells or NKL-vec cells were co-cultured with K562 cells in a transwell system for 24 h, and then the level of IFN-γ and TNF-α in the supernatants was detected by ELISA. Meanwhile, the level of NKp80, a NK activating receptor, and cytolysis-related molecules perforin and granzyme B were analyzed by FACS. As shown in Fig. 2a, NKL-IL15 cells produced more IFN-γ and TNF-α than NKL-vec cells in response to K562 cell stimulation, the cytokine concentration was increased from 5.7 ± 3.2 to 205.9 ± 18.0 pg/ml for IFN-γ and from 13.3 ± 1.4 to 85.3 ± 12.0 pg/ml for TNF-α, respectively. Additionally, the levels of NKp80 and granzyme B were increased obviously in NKL-IL15 cells stimulated by K562 cells (Fig. 2b). These results indicated that hIL15 gene modification made NK cells with high response capacity against leukemia cells.

The products of hIL-15 gene-modified NKL cells disturbed the biological characterizations of leukemia cells

NK cells not only act as cytolytic effector cells against target cells, but also influence the target cells by producing cytokines under certain physiologic and pathological conditions. For instance, NK cells-produced IFN-γ and TNF-α could induce the apoptosis and decrease the proliferation of target cells. Here, after co-cultured with NKL-IL15 cells or NKL-vec cells by transwell system for 24 h, the apoptosis of K562 cells did not show significant difference (data not shown), but the absolute number of K562 cells was markedly decreased by co-cultured with NKL-IL15 cells compared to that co-cultured with NKL-vec cells (Fig. 3a), accompanied with the down-regulated expression of Ki67 (Fig. 3b).

Since NKG2D is an activating receptor for NK cell activation, both the expression of NKG2D in NK cells and NKG2D ligands in target cells could influence the cytotoxicity of NK cells. The expression level of NKG2D

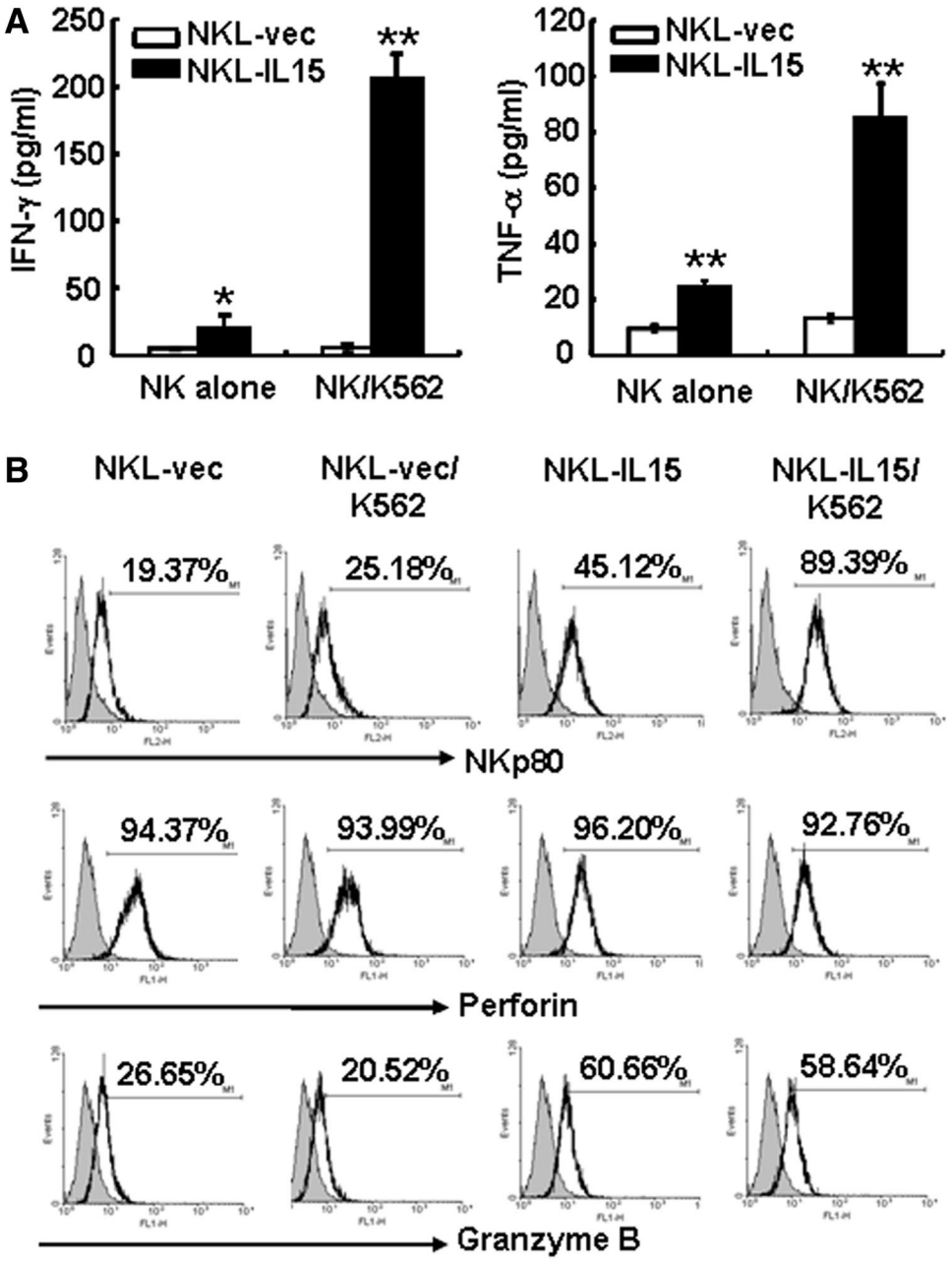

Fig. 2 hIL-15 gene enhanced the responsiveness of NKL cells to leukemia cells. NKL-IL15 cells or NKL-vec cells were co-cultured with K562 cells by transwell method for 24 h. **a** The levels of IFN-γ and TNF-α in supernatants were assessed by ELISA. Data shown are means ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NKL-vec cells. **b** NKL-IL15 and NKL-vec cells were harvested, the expression of cytotoxicity-associated genes NKp80, perforin and granzyme B were determined by flow cytometry. One representative of at least three independent experiments

in NKL-IL-15 cells showed no significant differences (Jiang et al. 2008), so, we wanted to understand whether the expression levels of NKG2D ligands on K562 cells would be affected by NK cells. As shown in Fig. 3c, compared with K562 cells co-cultured with NKL-vec cells, the expression of ULBP1-3 on K562 cells co-cultured with NKL-IL15 cells were significantly increased 5.21-, 5.67- and 5.26-fold, respectively. Meanwhile, the expression of MICA/B also increased 2.16-fold on K562 cells co-cultured with NKL-IL15 cells. These data demonstrated that factors produced by NKL-IL-15 cell decreased the proliferation of leukemia cells and induced the expression of NKG2D ligands on leukemia cells, which would augment the natural cytotoxicity of NKL cells against leukemia cells.

IFN-γ and NKG2D signaling contributed to IL-15 gene-induced the enhancement of NKL cell cytotoxicity

TNF-α and IFN-γ were the major effector molecules secreted by activated NK cells. To investigate whether these cytokines contributed to the up-regulation of NKG2D ligands on leukemia cells, NK cells were pre-treated with TNF-α or IFN-γ neutralizing mAb and then co-incubated with K562 cells. As shown in Fig. 4a, the cytotoxic activity

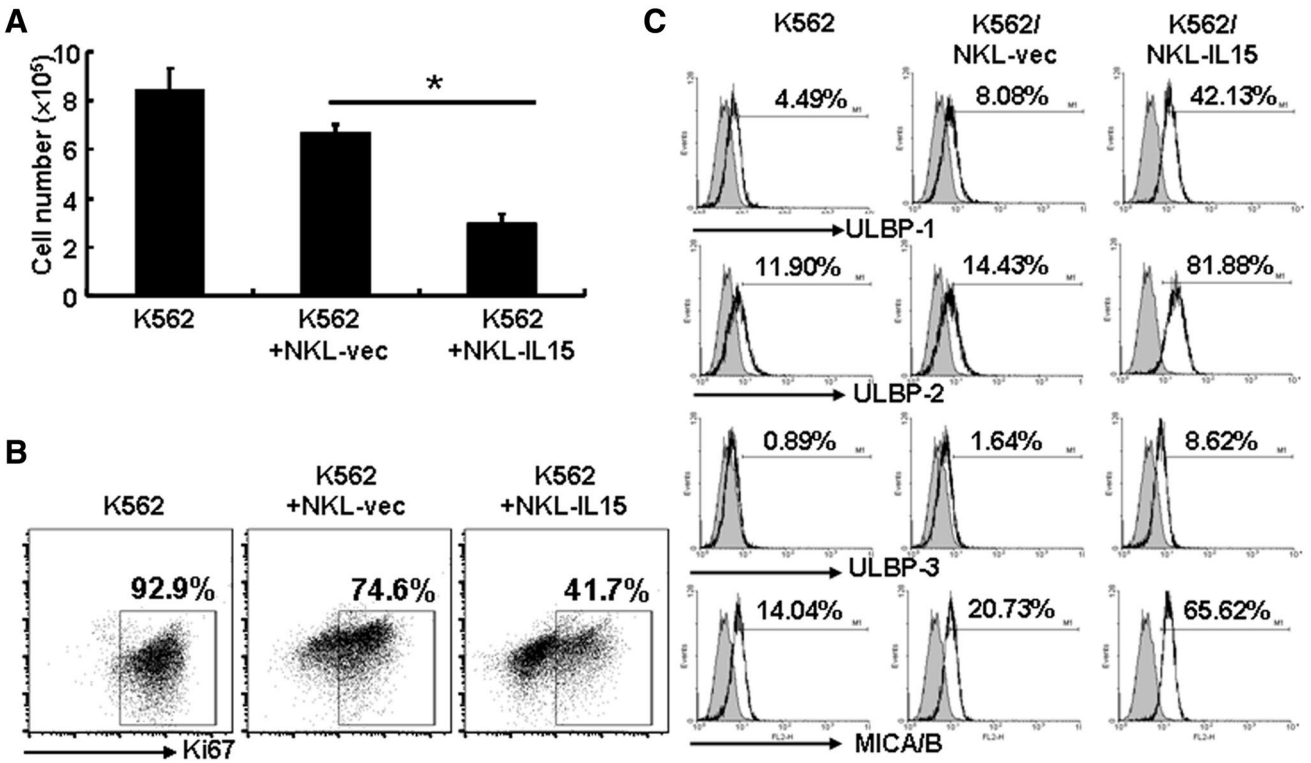

Fig. 3 The products of hIL-15 gene-modified NKL cells disturbed the biological characterizations of leukemia cells. K562 cells were co-cultured with NKL-IL15 cells or NKL-vec cells by transwell method for 24 h. **a** The absolute cell numbers of K562 cells based on trypan blue exclusion. Data shown are means ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. **b** The expression of the proliferative capacity marker Ki67 in K562 cells was assessed by intracellular flow cytometry. One representative of at least three independent experiments. **c** The protein levels of NKG2D ligands ULBP1-3 and MICA/B expressed on K562 cells were analyzed by flow cytometry. One representative of at least three independent experiments

of NKL-IL15 cells against K562 cells was not impacted by TNF-α neutralization. However, the neutralization of IFN-γ could significantly inhibit NKL-IL15 cell-mediated cytotoxicity, which was similar to the effect of NKG2D blockade. As neutralizing IFN-γ combined with blocking NKG2D, NKL-IL15 cell-mediated cytolysis was suppressed greatly. Meanwhile, neutralization of IFN-γ and TNF-α decreased the expression of NKG2D ligands on K562 cells significantly (Fig. 4b). These findings demonstrated that NKL-IL15 cell-mediated cytotoxicity against K562 cells was dependent on NKG2D signal and IFN-γ production.

IL-15 gene-modification improved the anti-leukemia effects of NKL cells in vivo

The in vivo anti-leukemia effect of NKL-IL15 cells was evaluated in NOD/SCID mice bearing K562 human leukemia xenografts. Firstly, mice were subcutaneously injected with 1×10^7 K562 cells. When the tumor models were established, the mice were randomly assigned into three groups, and treated with PBS, irradiated NKL-vec cells and NKL-IL15 cells, respectively, on days 7 and 14 post of inoculation. As depicted in Fig. 5a, NKL-IL15 cells exerted a significantly strong inhibitory effect on K562 cells compared with NKL-vec cells, and delayed the growth of K562 leukemia. After approximately 30 days, the tumor volumes in PBS control group and NKL-vec cell-treated group was 346 ± 26 and 198 ± 27 mm^3, separately, whereas the tumor volumes in NKL-IL15 cell-treated group was only 37 ± 11 mm^3, showing an 81.3% inhibition ratio ($P < 0.01$, compared with NKL-vec cells-treated group). Next, K562 cells were intraperitoneally injected into NOD/SCID mice and received treatments as above. As shown in Fig. 5b, compared to mice treated with PBS and NKL-vec cells, the lifetime of mice treated with NKL-IL15 cells was significantly prolonged, and over 80% leukemia-bearing mice survived longer than 80 days, whereas only 17% mice treated with NKL-vec cells survived, PBS-treated mice all died within 50 days after K562 cell inoculation. These results indicated NKL-IL15 cells were highly effective in anti-leukemia in vivo.

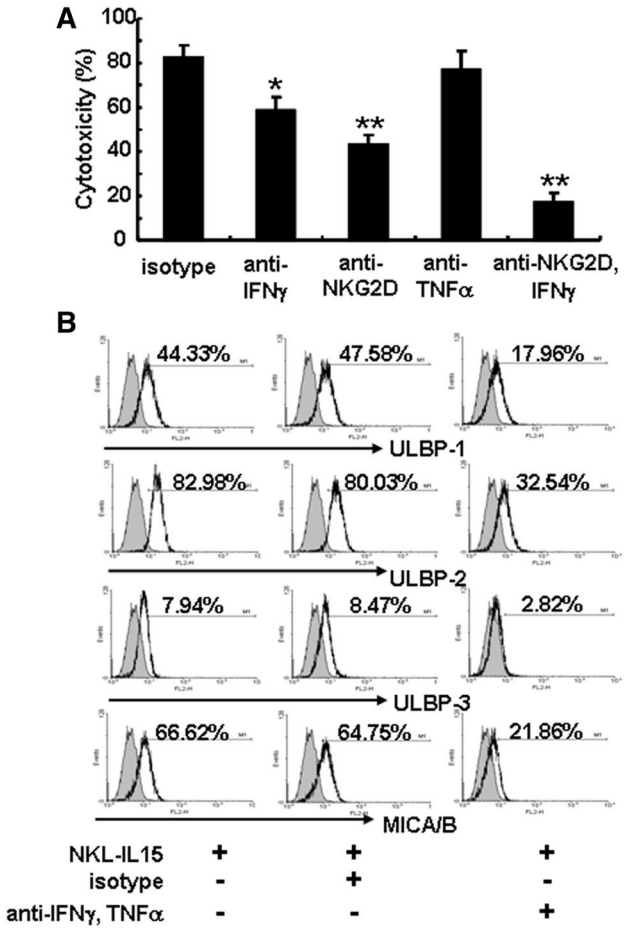

Fig. 4 IFN-γ and NKG2D signaling contributed to IL-15 gene-induced the enhancement of NKL cell cytotoxicity. NKL-IL15 cells and NKL-vec cells were pretreated with anti-NKG2D mAb, anti-TNF-α mAb and anti-IFN-γ mAb or isotype control. **a** The cytotoxicity of NKL-IL15 cells or NKL-vec cells against K562 cells was assessed by MTT method at the *E:T* ratio of 5:1. Data shown are means ± SD from at least three separate experiments. $*P < 0.05$, $**P < 0.01$ versus NKL-vec cells using the paired Student's *T* test. **b** K562 cells were incubated with NKL-IL15 or NKL-vec cells for 24 h, and then the expression of NKG2D ligands ULBP1-3 and MICA/B were determined by flow cytometry. One representative of at least three independent experiments

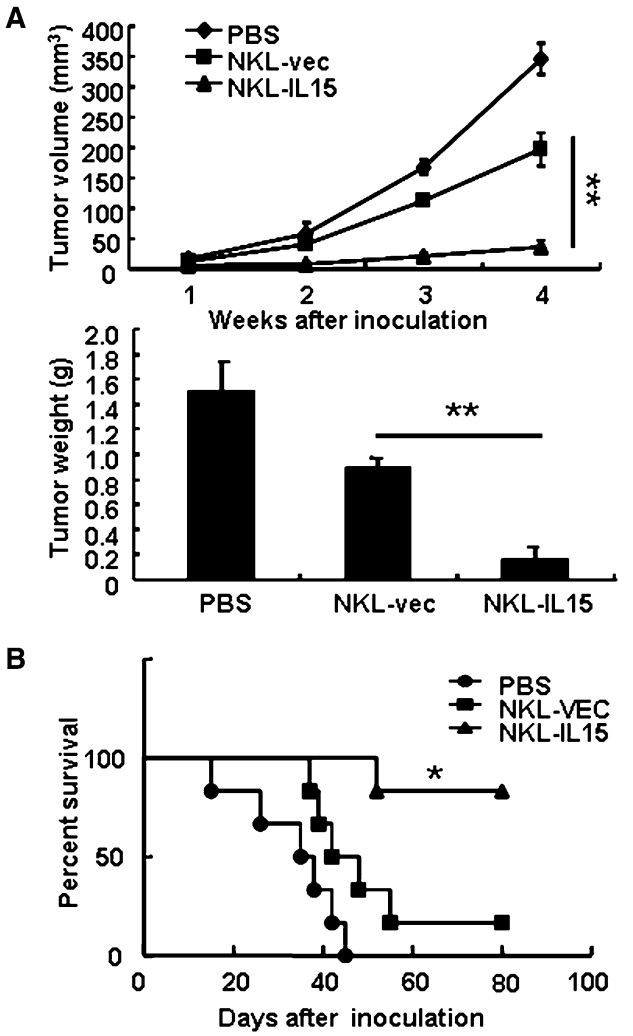

Fig. 5 IL-15 gene improved the anti-leukemia effects of NKL cells in vivo. **a** Nude mice were subcutaneously injected with 1×10^7 K562 cells, and then treated with 1×10^8 irradiated NKL-IL15 cells or NKL-vec cells on days 7 and 14 after inoculation. The tumor volumes were measured at the indicated days. On day 28, the mice were sacrificed and the tumors were weighed. $*P < 0.05$ versus NKL-vec cells using the paired Student's *T* test. **b** NOD/SCID mice were intraperitoneally injected with 1×10^7 K562 cells and treated with 1×10^8 irradiated NKL-IL15 cells or NKL-vec cells on days 7 and 14. The curves for the survival were shown as percentages of the initial number of animals per group. $*P < 0.05$ versus NKL-vec cells using a Mann–Whitney *U* test

NKL-IL15 cells could activate immune cell subpopulations in hPBMCs

IL-15 and some other cytokines such as TNF-α and IFN-γ, could notably induce the proliferation, survival, and effector functions of resting NK cells, T cells and NKT cells (Romee et al. 2014; Joyce 2001). To investigate whether NKL-IL15 cells also exert stimulatory effects on immune cells except for its direct anti-leukemia function, NKL-IL15 cells were co-cultured with hPBMCs by transwell method. As shown in Fig. 6a, the percentage of $CD56^+CD3^-$ NK cells in hPBMCs increased more than onefold in the presence of NKL-IL15 cells, concomitant with the up-regulation of cytolysis-related molecules, including NKG2D, TNF-α and IFN-γ (Fig. 6b). Furthermore, the cytotoxicity of NKL-IL15-stimulated hPBMCs against K562 cells was strengthened obviously (Fig. 6c), which was increased from 31.5 ± 5.7 to 66.1 ± 11.0% ($P < 0.01$, compared with NKL-vec cells). These results indicated that IL-15 gene-modified NK cells could display active effects on hPBMCs cells.

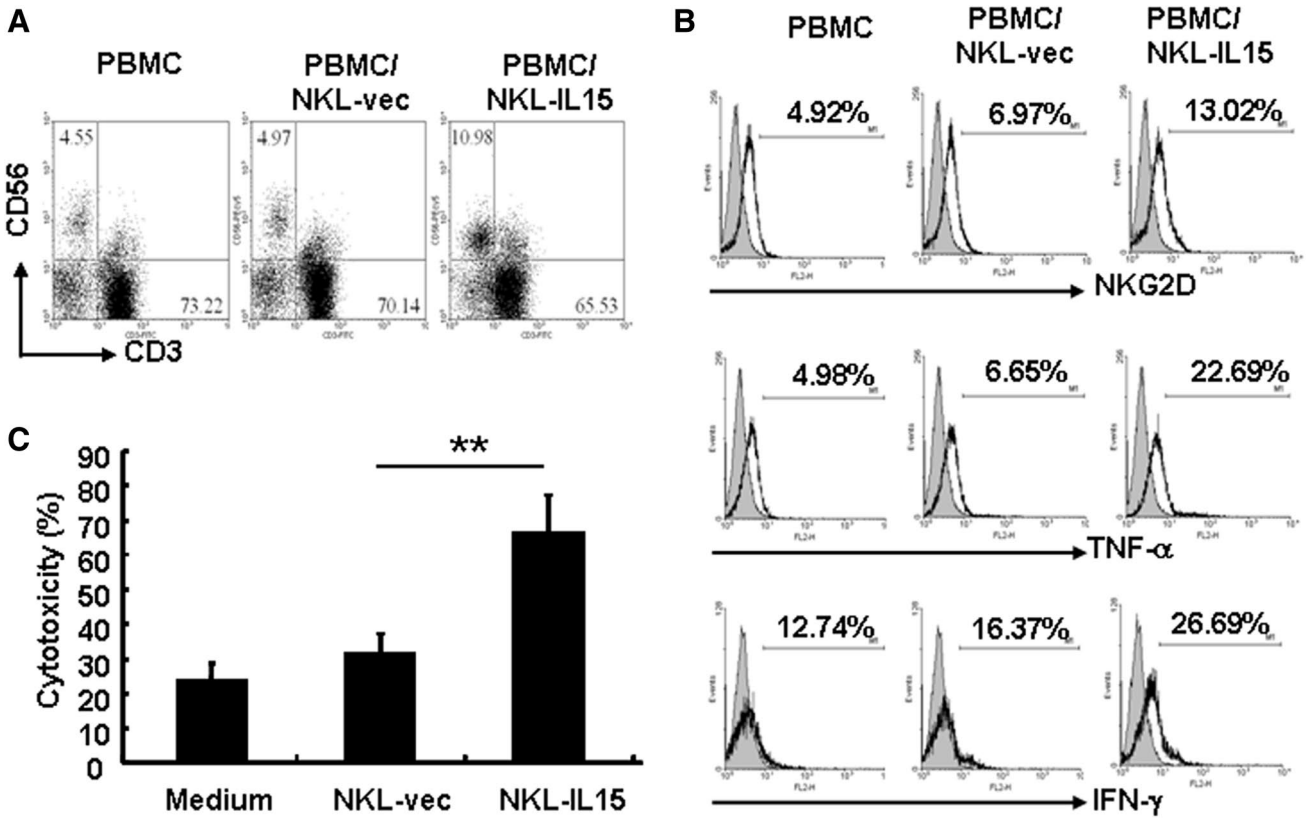

Fig. 6 NKL-IL15 cells could activate immune cell subpopulations in hPBMCs. Human PBMCs were isolated and stimulated with NKL-IL15 or NKL-vec cells in transwell systems for 24 h, and then the proportions of lymphocyte (**a**) and the levels of cytotoxicity-associated molecules NKG2D, IFN-γ and TNF-α (**b**) were determined by flow cytometry. One representative of at least three independent experiments. **c** PBMCs-mediated cytolysis against K562 cells was assessed by MTT assay at *E:T* ratio of 20:1. Data shown are means ± SD from at least three separate experiments. $*P < 0.05$, $**P < 0.01$ versus NKL-vec cells using the paired Student's *T* test

Discussion

Leukemia is the sixth most lethal cancer that occurs worldwide, accounting for 4% of all cancers, and 29% of all childhood cancers (Willard et al. 2017). High-dose chemotherapy and allogeneic hematopoietic stem cell transplantation (HSCT) offer the most effective methods for leukemia treatment, but relapses are frequent and often fatal (de Thé et al. 2017). NK cells display graft-versus-leukemia effect in allogeneic transplantation of hematopoietic stem cells without risk of initiating GVHD, indicating NK cells are implicated in the control and clearance of leukemia, which makes NK cells more attractive in the treatment of hematologic malignancies (Ruggeri et al. 2002; Crucitti et al. 2015). However, the number and activity of autologous NK cells against leukemic cells are frequently reduced and abnormal in patients with leukemia (Pierson and Miller 1996), which makes for immune evasion and results in leukemic relapse.

The levels of receptors associated with NK cell function and their ligands would influence the sensitivity of leukemic cells to NK cell-mediated cytolysis (Stanietsky et al. 2010). Genetic modification may improve the applicability of NK cells for cancer immunotherapy via a variety of approaches, including prolongation of NK cell survival by cytokine gene modification, conferring the specificity of NK cells targeting certain tissues or malignant cells (Cheng et al. 2013). In previous work, we have established the NKL-hIL15 cells by gene-modification and confirmed the efficiency of NKL-hIL15 cells against HCC in vitro and in vivo (Jiang et al. 2008, 2014). In view of the tendency that novel immunotherapeutic strategies utilizing the potentiation of NK cell functions have emerged in leukemia immunotherapy, here we further investigated NKL-hIL15 cell-mediated natural cytotoxicity against leukemia in vitro and in vivo. The results demonstrated that hIL-15 gene modification augmented the natural cytotoxic capability of NKL cells against human leukemia cell lines K562 and HL-60, as well as primary human leukemia cells from ten patients with CML, AML or ALL (Fig. 1), indicating the efficiency of NKL-IL15 cells against different kinds and progress of leukemia. Furthermore, the adoptive transfer of IL-15 gene-modified NKL cells could inhibit

the tumor growth and enhance the survival of tumor-bearing mice (Fig. 5). Studies have shown that NK cells combined with systemic administration of IL-15 could enhance the anti-tumor effect of NK cells in mouse model (Dubois et al. 2008), supporting our design philosophy of hIL-15 gene-modified NK cells. In addition to the high responsiveness of hIL15 gene modified NK cells against leukemia cells, NKL-IL15 cells have a greater proliferative capacity in vitro, simplifying the preparation of NKL-IL15 cells for adoptive immunotherapy. Furthermore, the use of hIL-15 gene modified NK cells could effectively avoid the potential side effects of systemic administration of IL-15.

Several mechanisms are involved in leukemia evasion from NK cell-mediated immune surveillance, including disturbing NK cell function directly, exhibiting immunosuppressive and -evasive characteristics (Lion et al. 2012; Wodnar-Filipowicz and Kalberer 2006). Here, we observed that IL-15 gene could up-regulate the cytolysis-associated molecules, including TNF-α, NKp80, granzyme B and IFN-γ, and promote the reactiveness of NKL cells to leukemia cells (Fig. 2). The expression of granzyme B in NKL-IL15 cells was not changed significantly by co-culturing with leukemia cells, indicating that the up-regulation of granzyme B in NKL cells is resulted by the genetic modification of IL-15, but not the specific activation by the target cells. The absence or downregulation of specific activating antigen is an important mechanism for tumor escape from immune surveillance. And evidences showed some certain types of leukemia are resistant to NK cells from allogeneic KIR ligand-mismatched donors, or negative for the MHC class I-related protein (MICA/B), ligands of NKG2D receptor, and lose or express some other NK activating ligands at low levels (Romanski et al. 2005; Pende et al. 2005). Interestingly, we found NKG2D ligands MICA/B and ULBP1-3 on leukemia cells could be induced by TNF-α and IFN-γ secreted by NKL-IL15 cells, which was favorable for NK cell recognition and activation (Fig. 3). Moreover, NKL-IL15 cell-mediated the rejection of leukemia was mainly dependent on NKG2D signal and IFN-γ production (Fig. 4). Furthermore, NKL-IL15 cells could display active effects on hPBMCs cells, which may destroy the tolerance of leukemia by interactions with other immune cells such as NK cells and T cells in PBMCs (Fig. 6).

Based on this research, hIL15 gene modification not only augmented NKL cell cytolytic activity directly, but also increased the expression of ligands on leukemia cells, which promoted the recognition and activation of NK cells, as well as improved immune response. Therefore, NKL-IL15 cells or hIL15 gene-modified healthy donor NK cells could provide more opportunities for future therapeutic trials, and could be a complement for allogeneic transplantation of hematopoietic stem cells in the treatment of leukemia.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Medical Ethics Committee of Shandong University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Institutional Review Board of Shandong University.

Informed consent Informed consent was obtained from all individual participants and the parents of minor participant included in the study.

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