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hIL-15 gene-modified human natural killer cells (NKL-IL15) augments the anti-human hepatocellular carcinoma effect *in vivo*

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ABSTRACT

Genetic modification of NK cells may provide new possibilities for developing effective cancer immunotherapy by improving NK cell function and specificity. We previously established human interleukin-15 (hIL-15) gene-modified NKL cells (NKL-IL15) and demonstrated their therapeutic efficiency against human hepatocellular carcinoma (HCC) *in vitro*. To further assess the applicability of NKL-IL15 cells in adoptive cellular immunotherapy, we further investigated their natural cytotoxicity against HCC *in vivo* in the present study. NKL-IL15 cells exhibited strong inhibition on the growth of transplanted human HCC tumors in xenograft nude mouse models. Further investigation showed that NKL-IL15 cells expressed much higher levels of cytolysis-related molecules, including NKp80, TRAIL, granzyme B, IFN-γ, and TNF-α, than parental NKL cells in response to HCC stimulation. Moreover, soluble mediators secreted by NKL-IL15 cells decreased HCC cell proliferation; in particular, NKL-IL15-derived TNF-α and IFN-γ induced higher NKG2D ligand expression on target cells and resulted in the increased susceptibility of HCCs to NKL-mediated cytolysis. These results show that hIL-15 gene-modified human NK cells can augment the anti-tumor effect of NK cells on human HCC *in vivo* and suggest their promising applicability as a new candidate for adoptive immunotherapy against HCCs in the future.

Introduction

Natural killer (NK) cells are key components of the innate immune system that play important roles in the first line of defense against various types of tumors and virus-infected cells independent of pre-sensitization or major histocompatibility (MHC) restriction (Cerwenka and Lanier, 2001; Miller, 2001; Roder and Pross, 1982). Various studies demonstrate that NK cells are involved in eradicating experimentally induced and spontaneous tumors in mice by releasing cytolysis-associated molecules and cytokines (Smyth et al., 1998, 2001; van den Broek et al., 1995). The ability of NK cells to effectively respond to tumor cells makes them promising effectors for immunotherapeutic strategies (Kärre et al., 1986), and their application in immunotherapy against cancer has recently entered clinical trials (Ljunggren and Malmberg, 2007).

Although using highly purified primary NK cells would be ideal for immunotherapy, these cells present technical limitations, such as generating a large enough number of highly purified NK cells to meet clinical requirements. Thus, NK cell lines were established under GMP conditions to take advantage of their unlimited proliferation potential (Tonn et al., 2001). The NKL cell line, for instance, is a well-characterized and currently established malignant NK cell line from the peripheral blood of a patient with $CD3^{-}CD16^{+}CD56^{+}$ large granular lymphocyte (LGL) leukemia; as these cells maintain the most original NK cell features among all NK cell lines, they have strong potential for use in adoptive immunotherapy as an effector cell population (Maasho et al., 2004; Robertson et al., 1996).

Another distinct advantage of using NK cells lines in cancer immunotherapy is the relative ease of genetically modifying cell lines, which may provide new possibilities for improving NK cell function or endowing these cells with additional functions. Until now, several cytokine genes, including IL-2, IL-15, and stem cell factor (SCF) (Nagashima et al., 1998; Zhang et al., 2004a,b; Jiang et al., 2008), have been used to modify NK cell lines in order to augment NK cell activation and/or cytotoxicity against tumor cells. In particular, IL-15 plays various important roles in regulating NK cells,

Abbreviations: CIK, cytokine-induced killer; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; GMP, Good Manufacturing Practice; HCC, hepatocellular carcinoma; IFN-γ, interferon gamma; IL, interleukin; LAK, lymphokine activated killer; MHC, major histocompatibility; OD, optical density; PBMC, peripheral blood monocyte cell; SCF, stem cell factor; TNF-α, tumor necrosis factor-alpha.

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which is demonstrated best in IL-15Rα$^{-/-}$ and IL-15$^{-/-}$ mice that do not develop any functional NK cells (Kennedy et al., 2000; Lodolce et al., 1998). Indeed, IL-15 is the major physiologic growth factor responsible for NK cell ontogeny (Fehniger and Caligiuri, 2001) and is also a potent regulator of NK cell proliferation, survival, and cytolytic activity (Grund et al., 2004; He et al., 2004).

In a previous study, we successfully established an hIL-15 gene-modified NKL cell line (NKL-IL15) and confirmed that the addition of hIL-15 augmented the NKL cell-mediated anti-hepatocellular carcinoma (HCC) effect *in vitro* (Jiang et al., 2008). However, whether this anti-HCC effect could also be improved in an *in vivo* setting was unknown. Therefore, to further confirm the applicability of NKL-IL15 cells against HCC cells and HCC tumors *in vivo*, we evaluated the anti-HCC efficacy of NKL-IL15 cells in xenograft tumor models in the present study. Moreover, we identified the mechanisms underlying hIL-15 gene modification-induced enhancement of NKL cell activation.

Materials and methods

Cell culture and cell lines

The human hepatocellular carcinoma cell line HepG2 was maintained in our laboratory and grown 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). The NKL cell line was a gift from Professor Jin BQ (Department of Immunology, Fourth Military Medical University, Xi'an, PR China); it was maintained in our laboratory and cultured in complete IL-2-containing (100 U/mL) RPMI-1640 medium. NKL-IL15 and NKL-vec cells were established in our laboratory as described previously (Jiang et al., 2008) and cultured in the same conditions as NKL cells. All cells were incubated at 37 °C and 5% CO_2.

Animals

Female nude BALB/c mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and kept under specific pathogen-free conditions. All experimental procedures involving animals were conducted in accordance with the experimental animal guidelines approved by the State Science and Technology Commission, PR China. The animal study proposal and protocol were approved by the Ethical Committee of Shandong University.

Tumor challenge and treatment

To evaluate local tumor growth, HepG2 cells (5×10^6) were injected subcutaneously (s.c.) into the right flank of nude mice, or were administered intraperitoneally (i.p.). The mice were then randomly assigned to 3 experimental groups (PBS control, NKL-vec, and NKL-IL15) with 6 mice in each group. After 800 cGy (200 cGy/min) irradiation, 5×10^7 NKL-IL15 or NKL-vec cells were harvested and injected intravenously (i.v.) in a 200 μL volume on days 7 and 14 after tumor inoculation, while 200 μL of PBS was injected into the control mice. Four weeks later, the mice were sacrificed, and tumors, spleens, and livers were collected and weighed. Splenic lymphocytes were isolated by density gradient centrifugation and enumerated. To evaluate survival of xenograft mice, mice in each experimental group ($n = 6$/group) received 5×10^6 HepG2 cells (200 μL) i.p., followed by treatment with PBS control or 5×10^7 irradiated NKL-IL15 or NKL-vec cells on days 7 and 14 after tumor inoculation; mouse survival was evaluated twice a day.

Analysis of NKL cells in the tumor infiltrate

To evaluate NKL cells in the tumor infiltrate, HepG2 cells (5×10^6) were injected s.c. into the right flank of nude mice. After 7 days, 5×10^7 NKL-IL15 or NKL-vec cells were labeled with CFSE at 37 °C for 10 min and then inoculated i.v. into these tumor-bearing nude mice. Tumors were isolated 12 h later from xenograft mice, dissected into small pieces, and incubated in 0.25% trypsin at 37 °C for 20 min. Then, the upper portion of the cell suspension was carefully recovered and passed through a 70-μm cell sieve. After the cells were washed 3 times with PBS, the cells in the suspension were evaluated by FACS analysis.

Co-culture assay

HepG2 plus NKL-IL15 or NKL-vec cells were co-cultured using a 0.4 μm porous Transwell system in 12-well plates (Corning Costar, Tewksbury, MA, USA) in a 1:1 ratio for 24 h; NKL cells cultured in complete RPMI 1640 medium served as a control. To analyze their NK cell features, NKL-IL15 or NKL-vec cells were plated into the wells, while HepG2 cells were plated into the Transwell insert. To analyze HepG2 cell features, HepG2 cells were plated into the wells, while NKL-IL15 or NKL-vec cells were plated into the Transwell insert.

Cytokine ELISA

NKL cells (3×10^5/well) were plated in triplicate in 12-well plates with or without HepG2 cells. After 24 h of co-culture, TNF-α and IFN-γ levels in cell culture supernatants were evaluated by commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the manufacturer's instructions.

Flow cytometry analysis

To detect the expression levels of the NKG2D ligands (ULBP1-3, MICA/B), HepG2 cells were stained with PE-conjugated ULBP1-3 (R&D Systems), PE-conjugated MICA/B (eBioscience, San Diego, CA, USA), or isotype control (eBioscience) at 4 °C for 45 min. To detect NKp80 and TRAIL expression levels, NKL-IL15 and NKL-vec cells were stained with PE-conjugated NKp80 (R&D Systems) or PE-conjugated TRAIL (eBioscience) at 4 °C for 45 min. For intracellular staining of CD107a, perforin, and granzyme B, NKL-IL15 and NKL-vec cells were treated with monensin (Sigma) for 4 h to inhibit the secretion pathway, and then these NKL cells were harvested and labeled with PE-conjugated CD107a (eBioscience), FITC-conjugated perforin (eBioscience), FITC-conjugated granzyme B (eBioscience), or isotype control (eBioscience) at 4 °C for 45 min. All stained cells were analyzed using a flow cytometer (FACSCalibur, USA), and the data were processed using WinMDI 2.9 software (Scripps Research Institute).

Proliferation assay

HepG2 cells (3×10^5/well) were plated in 12-well plates at 37 °C in a 5% CO_2 incubator and cultured alone or co-cultured with NKL-IL15 cells or NKL-vec cells in a Transwell plate for 24 h. Viable HepG2 cells in each well were counted after trypan blue staining. Each sample was performed in triplicate, and each well was counted 4 times.

Cytokine neutralization and receptor blockade

The following antibodies were used to neutralize cytokines in culture: anti-IFN-γ mAb (R&D Systems) was used at a final concentration of 5 ng/mL, and anti-TNF-α mAb (R&D Systems) was used

at a final concentration of 0.25 ng/mL with 1 μg/mL actinomycin D. In the NKG2D blocking assay, NK cells were treated with 10 μg/mL anti-NKG2D mAb (R&D Systems) or isotype control mAb for 1 h prior to co-culture with HepG2 cells.

Cytotoxicity assay

NK cell-mediated cytotoxicity was determined by the MTT assay. HepG2 cells were used as the target cells and placed in 96-well plates at 1×10^4 cells/well. Effector cells were added to target cells at effector/target (E/T) ratios of 10:1 and 5:1. The effector and target cell mixtures were then incubated for 6 h at 37 °C in 5% CO_2. Then, 20 μL MTT (5 mg/mL) was added, and the plates were incubated for another 4 h. The absorbance (A) at 570 nm in each well was determined with a microplate autoreader (Bio-Rad). Cytotoxicity (%) = [1 − (A of target plus effector cells − A of effector cells)/A of target cells] × 100%.

Statistical analysis

All data are expressed as the mean ± SD from 3 independent experiments. Statistical analysis was performed using a paired Student's *t*-test, and the statistically significant differences were set at $^{*}p < 0.05$, $^{**}p < 0.01$. Statistical differences for mouse survival were analyzed using the Mann–Whitney *U* test.

Results

Adoptive immunotherapy of hIL-15 gene-modified NKL cells inhibits HCC tumor growth and prolongs survival in HCC xenograft mouse models

In order to provide possible new strategies for cancer immunotherapy by improving NK cell function, we successfully established the NKL-IL15 cell line and reported its therapeutic efficacy against HCCs *in vitro* in a previous study (Jiang et al., 2008). Since previous studies showed that IL-15 signaling was mediated through transpresentation by its IL-15Rα receptor, we verified the ability of NKL cells to transpresent IL-15 by detecting positive expression of IL-15Rα on both NKL-IL15 and NKL-vec cells (Fig. S1). In order to further assess whether NKL-IL15 cells can be successfully applied *in vivo* as an adoptive cellular immunotherapy, we adoptively transferred irradiated NKL-IL15, NKL-vec, or control PBS into nude mice bearing subcutaneous HCC tumors. We first analyzed NKL cell infiltration into the HCC tumors by enumerating CFSE-labeled NKL cells 12 h after adoptive transfer. As shown in Fig. 1A, both NKL-vec and NKL-IL15 cells efficiently infiltrated into the tumors. Subsequently, we established two separate HCC xenograft nude mouse model schedules that differed by the intraperitoneal or the subcutaneous route of tumor inoculation. In the first model, 6-week-old nude mice were inoculated i.p. with 5×10^6 HepG2 cells and then injected i.v. with irradiated NKL-vec cells, NKL-IL15 cells, or PBS on days 7 and 14. The mice were sacrificed 4 weeks later, and metastatic cells in the spleen, spleen weight, liver weight, and splenocyte numbers were evaluated. As shown in Fig. 1B, immunotherapy with irradiated NKL cells reduced the incidence of spleen metastases in xenograft mice, and NKL-IL15 cells exerted a stronger anti-tumor effect than NKL-vec cells. Moreover, NKL treatment also suppressed the increased spleen weight and splenocyte number that occurred upon HepG2 cell inoculation; compared to NKL-vec treatment, NKL-1L15 treatment significantly reduced both the spleen weight (0.448 ± 0.125 *vs.* 0.154 ± 0.030 g, respectively) and splenocyte number ($16.75 \pm 1.67 \times 10^4$ to $7.54 \pm 1.81 \times 10^4$ cells, respectively) (Fig. 1C and D). Additionally, NKL-IL15 treatment significantly prolonged the survival of tumor-bearing mice. As shown in Fig. 1E, more than 66% of the NKL-IL15-treated tumor-bearing mice survived longer than 90 days, whereas only 33% of NKL-vec-treated mice survived this long, and all PBS-treated mice died within 60 days.

In the second HCC xenograft model, we evaluated the effect of NKL-IL15 cell treatment on the growth of subcutaneously inoculated HepG2 cells in nude mice. As shown in Fig. 1F and G, NKL-IL15 cells significantly inhibited HepG2 tumor growth as compared to NKL-vec cells; while tumors weighed 0.252 ± 0.036 g in the PBS-treated group, they only reached 0.118 ± 0.046 g in the NKL-vec-treated group and 0.050 ± 0.024 g in the NKL-IL15-treated group. Additionally, NKL treatment prevented spleen enlargement, as the spleens in the PBS control, NKL-vec, and NKL-IL15 groups weighed 0.402 ± 0.020, 0.257 ± 0.045, and 0.123 ± 0.019 g, respectively (Fig. 1H). No obvious differences in the weight of the normal liver organs were observed among the different treatment groups in either tumor model (data not shown). Collectively, these results indicated that treatment with irradiated NKL-IL15 cells effectively suppressed tumor growth in, and prolonged survival of, HCC tumor-bearing mice in two different *in vivo* tumor models.

hIL-15 gene modification promotes NKL cell activation

The expression levels of cytolysis-related receptors and molecules are known to influence NK cell activation and cytotoxic ability. To investigate the mechanisms underlying how the hIL-15 gene modification improves the antitumor effects of NKL cells, we analyzed the IFN-γ, TNF-α, NKp80, TRAIL, NKp46, NKp30, FasL, CD107a, perforin, and granzyme B expression levels in and on NKL cells during *in vitro* co-culture with HepG2 cells. As shown in Fig. 2A, the IFN-γ and TNF-α levels produced by NKL-IL15 cells co-cultured with HepG2 cells by Transwell method were 171 ± 28 and 110 ± 14 pg/mL in the supernatant, respectively, which were significantly higher than that secreted by the NKL-vec cells (12 ± 1.1 and 11 ± 2.5 pg/mL). We also obtained similar IFN-γ and TNF-α levels after traditional co-culture of NKL and HepG2 cells that allowed for cell–cell contact (Fig. 2A), indicating that soluble molecules secreted by HepG2 cells induced IFNγ and TNFα production by NKL cells. We also tested the expression of cytolysis-related receptors and found that NKp80, granzyme B, and TRAIL expression levels were increased on NKL-IL15 cells as compared to NKL-vec cells in response to HepG2 cell stimulation (Fig. 2B and C). We did not, however, observe any changes between NKL-IL15 and NKL-vec cells in terms of CD107a, NKp46, NKp30, or FasL expression. These results indicated that hIL-15 gene-modified NKL cells were more sensitive to HepG2 cell stimulation, leading to the up-regulation of cytolysis-related molecules that likely augmented the NKL cell cytotoxic activity against HCC cells reported in our previous study and suggested here by our *in vivo* models.

hIL-15 gene-modified NKL cells secrete soluble mediators that decrease HCC cell proliferation

Not only can NK cells act as cytolytic effector cells against target cells, but they can also reciprocally influence the target cells themselves through the production of cytokine mediators under certain physiologic and pathological conditions. For example, NK cell-produced IFN-γ and TNF-α can induce apoptosis and inhibit the proliferation of target cells. After NKL-IL15 or NKL-vec cells were co-cultured with HepG2 by the Transwell method for 24 h, no significant differences in HepG2 apoptosis were observed (data not shown), but NKL-IL15 cells markedly suppressed HepG2 proliferation compared to the NKL-vec cells (Fig. 3). These results indicated that hIL-15 gene-modified NKL cells could decrease HCC proliferation, and that soluble cytokine mediators produced by NKL-IL15 might be involved in this process.

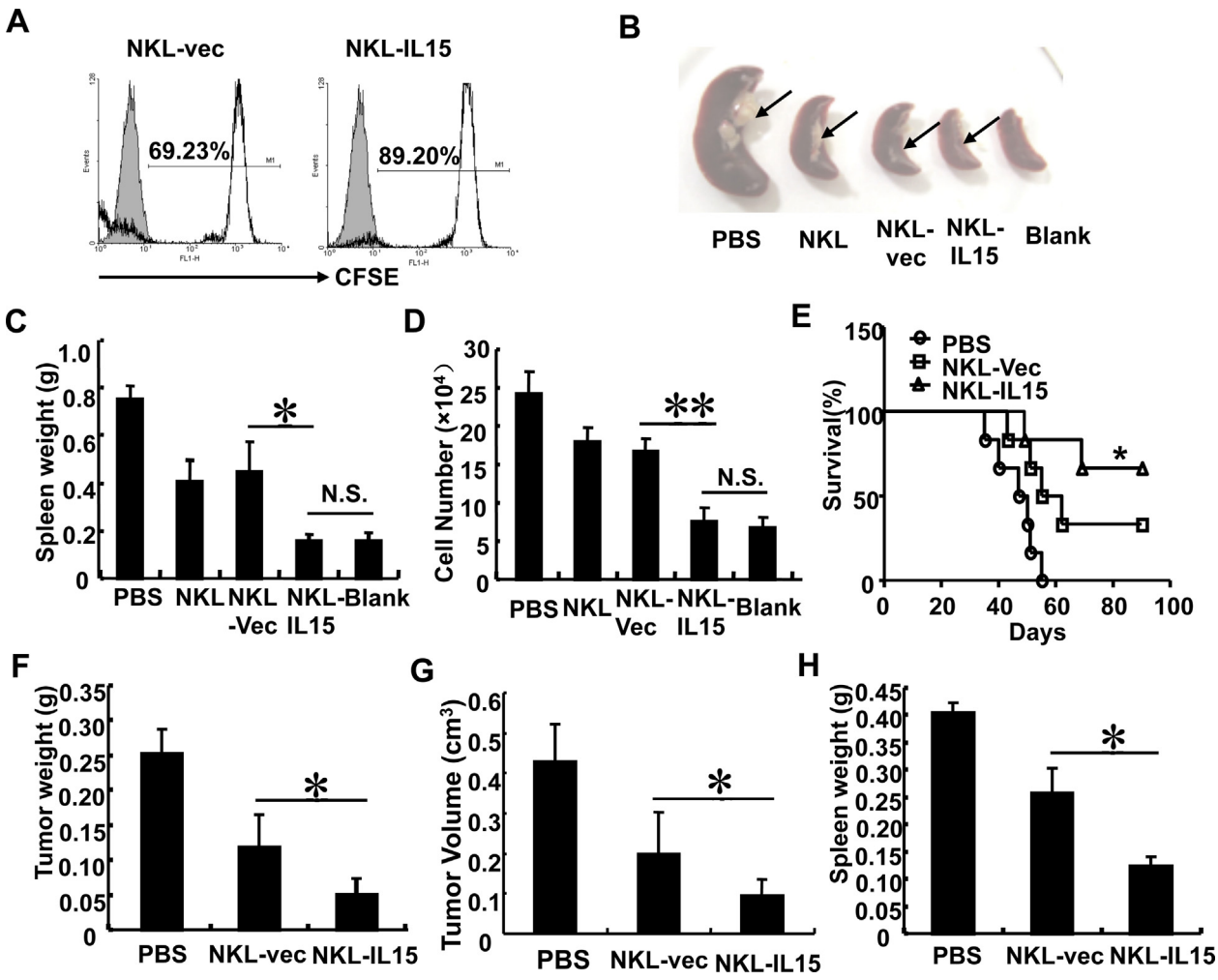

Fig. 1. Anti-HCC effect of IL-15 gene-modified NKL cells in tumor-bearing nude mice. (A) Irradiated NKL-IL15 or NKL-vec cells (5×10^7) were CFSE labeled and then adoptively transferred i.v. into nude mice 7 days after they were s.c. inoculated with 5×10^6 HepG2 cells. After 12 h, tumors were isolated from xenograft mice and evaluated for CFSE dilution by FACS analysis. (B–E) Nude mice were inoculated i.p. with 5×10^6 HepG2 cells and treated with 5×10^7 irradiated NKL, NKL-vec, or NKL-IL15 cells on days 7 and 14 after inoculation. On day 28, the mice were sacrificed and the tumors were removed. (B) Photographs of spleens from the tumor-bearing mice. (C) Weight of spleens from tumor-bearing mice. (D) Total splenocyte numbers. $^*p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test. (E) Survival curves for tumor-bearing mice; values are represented as the percentage of the initial number of animals per group. $^*p < 0.05$ *versus* NKL-vec cells using a Mann–Whitney *U* test. (F–H) A total of 5×10^6 HepG2 cells were inoculated s.c. into the right flank of nude mice. Then, 5×10^7 irradiated NKL-vec or NKL-IL15 cells were injected i.v. on days 7 and 14 after tumor inoculation, and mice were sacrificed on day 28. Tumor (F) weight and (G) volume as well as (H) spleen weight were measured from the tumor-bearing mice. $^*p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test.

TNF-α and IFN-γ produced by NKL-IL15 cells up-regulate NKG2D ligand expression on HCC cells

The NKG2D signaling pathway is known to mediate NK cell activation for the purpose of enhancing natural cytotoxicity. While IL-15 gene modification does not alter expression of the NKG2D activating receptor on NKL cells (Jiang et al., 2008), the NKG2D pathway could still be involved in this enhanced NKL-IL15 cell-mediated inhibition of HepG2 cell growth if NKL-IL15 cells induced up-regulation of NKG2D ligand expression on HepG2 cells. Indeed, as shown in Fig. 4, the expression of ULBP-1, ULBP-2, and ULBP-3 on HepG2 cells co-cultured with NKL-IL15 cells increased approximately 3.60-, 2.20-, and 3.34-fold, respectively, compared to HepG2 cells co-cultured with NKL-vec cells. In addition, NKL-IL15 cells induced approximately 20% higher expression than NKL-vec cells of another NKG2D ligand, MICA/B, on HepG2 cells. NK cells are known to induce up-regulated NKG2D ligand expression on some tumor cells through TNF-α and IFN-γ cytokine secretion (Zou et al., 2010). To investigate whether these cytokines also contributed to the observed up-regulation of NKG2D ligands on HCC cells, NKL cells were treated with TNF-α and IFN-γ neutralizing mAbs before co-culture with HepG2 cells. Indeed, neutralizing both TNF-α and IFN-γ significantly decreased NKG2D ligand expression on HepG2 cells, demonstrating that TNF-α and IFN-γ secreted by NKL-IL15 cells promoted NKG2D ligand expression on HepG2 cells, which could increase the sensitivity of HCC to NK cell cytolysis (Fig. 4). We did observe that ULBP-1 expression remained higher on HepG2 cells co-cultured with NKL-IL15 cells under cytokine neutralization conditions than in those co-cultured with NKL-vec cells, suggesting that residual TNF-α and IFN-γ function remained in the wells or that other unidentified factors in the system could also mediate up-regulation of some NKG2D ligands.

The enhanced NKL-IL15-mediated cytotoxicity is partially dependent on NKG2D recognition and TNF-α secretion

Based on the above results, we further investigated the molecular mechanisms underlying how NKL-IL15 cells mediate cytotoxicity against HepG2 cells. As shown in Fig. 5, neutralizing IFN-γ did not significantly affect NKL-IL15-mediated cytotoxicity

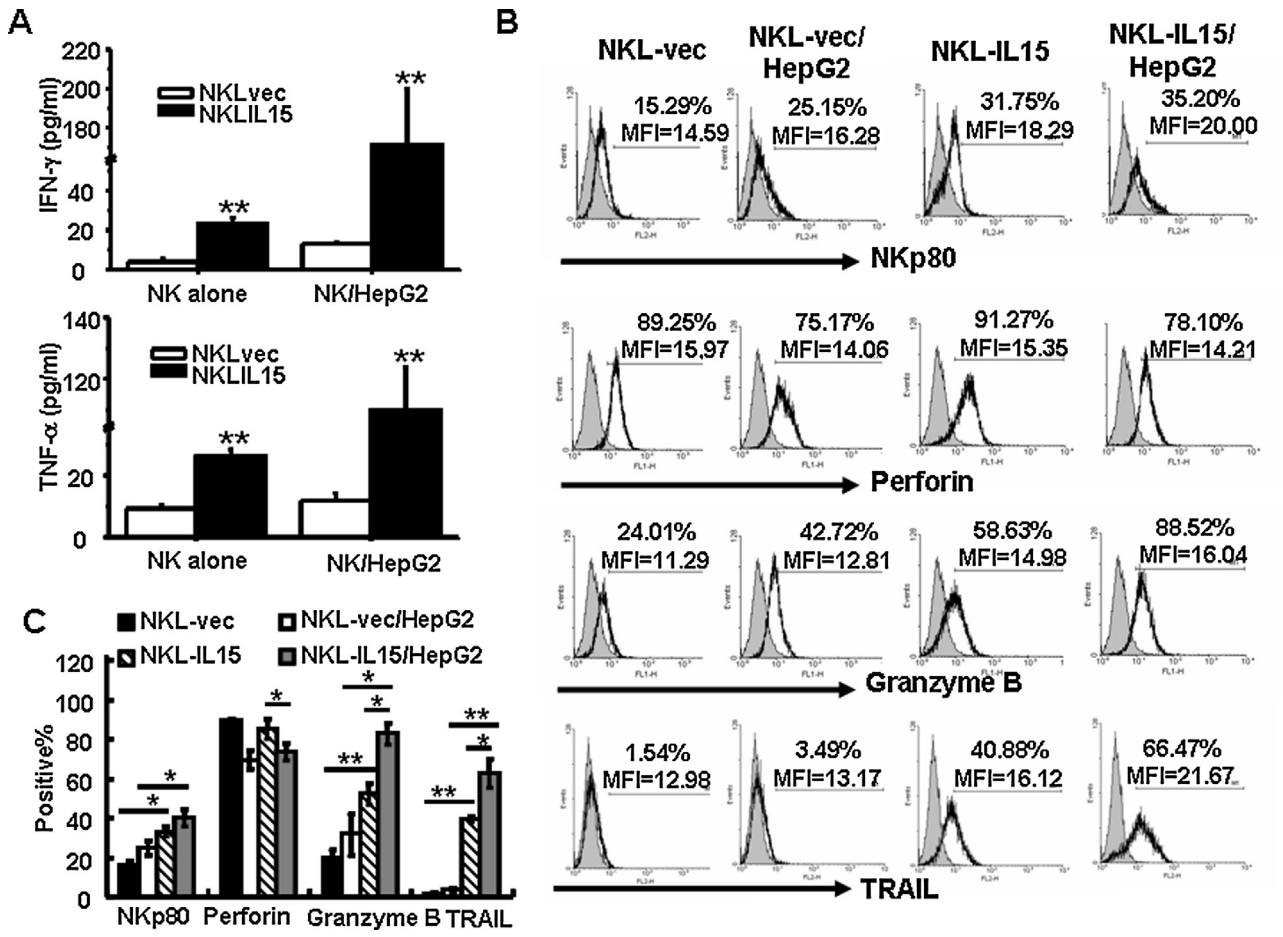

Fig. 2. Expression of cytotoxicity-associated molecules in IL-15 gene-modified NKL cells in response to HCC stimulation.
3×10^5 NKL-IL15 or NKL-vec cells were co-cultured with HepG2 cells by the Transwell or traditional cell–cell contact method for 24 h. (A) Supernatant IFN-γ and TNF-α levels were assessed by ELISA. Data are represented as the mean ± SD from 3 independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test. (B and C) NKL-IL15 and NKL-vec cells were harvested from the Transwell co-culture assay, and the expression of cytotoxicity-associated molecules was determined by flow cytometry. (B) Data were calculated from 1 representative experiment, and a total of 3 independent experiments were performed. (C) Diagrams show the mean percentage ± SD of cells expressing the indicated molecules. $^*p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test.

against HepG2 cells, suggesting that NKL-IL15-mediated cytotoxicity was independent of IFN-γ signaling. However, blocking NKG2D or neutralizing TNF-α in NKL-IL15 cell culture media significantly inhibited NKL-IL15-mediated cytotoxicity; this effect was especially pronounced upon NKG2D blockade. Importantly, NKL-IL15-mediated cytotoxicity was nearly abolished when NKG2D blockade and TNF-α neutralization were combined. These results indicated that NKG2D recognition and TNF-α production were important for NKL-IL15-mediated cytolysis against HepG2 cells.

Discussion

Genetic modification shows promise for redirecting the function of various immune cells for therapeutic use, including T cells (Kershaw et al., 2005), dendritic cells (Xu et al., 2007), monocytes (Biglari et al., 2006), and NK cells (Suck, 2006). Genetic modification of NK cells may provide new possibilities for cancer immunotherapy strategies by improving their natural cytotoxic ability or endowing them with additional capabilities. Genetic modification can be applied in a variety of approaches, ranging from modifying cytokine genes to induce NK cell survival to modifications that will specifically target NK cells to certain tissues or malignant cells (Sutlu and Alici, 2009). NK cells modified with cytokine genes enhance NK cell functions by directly providing them with the necessary cytokines. Indeed, NK cells modified to express IL-2, IL-15, and SCF have been shown to exhibit augmented NK cell activity and cytotoxicity to tumor cells.

Hepatocellular carcinoma is the fifth most common cancer worldwide and has ranked second in cancer-related mortality in China since the 1990s. Only surgical and local ablative treatments have shown therapeutic efficacy in HCC patients, and systemic chemotherapy has shown no benefit. Therefore, there is an impending need for the development of novel immunotherapeutics to complement these traditional treatments in order to effectively control HCC. Although only a limited number of patients have been enrolled in most immunotherapy trials conducted thus far, results from these studies clearly suggest that immunotherapy is safe for HCC patients. Several kinds of cells have already been used for adoptive cell transfer therapy for HCC, including LAK cells, CIK cells, and DCs (Bertelli et al., 2008; Greten et al., 2008; Iwashita et al., 2003).

In previous work, we established NKL-hIL15 cells by gene modification and demonstrated their therapeutic efficacy against HCC *in vitro*. Although the results suggested that NKL-IL15 cells exerted strong cytotoxicity against 3 human hepatoma cell lines at various ratios, this *in vitro* evidence was insufficient to confirm the applicability of NKL-IL15 cells in the clinic. Thus, in the present study, we further evaluated their ability to inhibit HCC tumor growth *in vivo* in 2 different tumor xenograft mouse models.

The experimental data showed that the adoptively transferred irradiated NKL cells could efficiently infiltrate into tumor tissues.

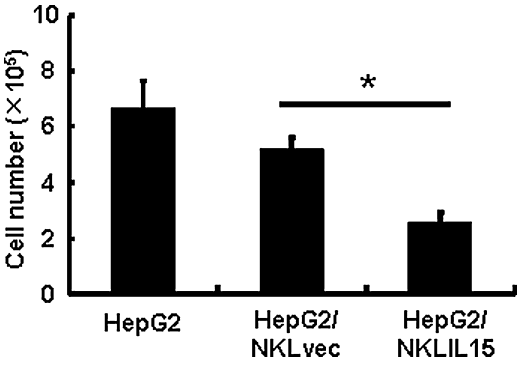

Fig. 3. Effect of soluble mediators from IL-15 gene-modified NKL cells in suppressing HepG2 cell proliferation. HepG2 cells were co-cultured with NKL-IL15 or NKL-vec cells by the Transwell method for 24 h. Live/dead HepG2 cell numbers were calculated based on trypan blue exclusion. Data are represented as the mean ± SD from 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test.

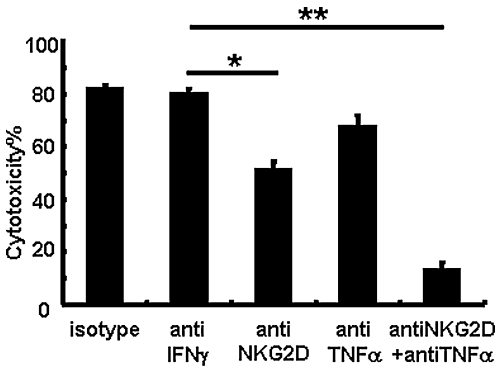

Fig. 5. The enhanced NKL-IL15-mediated cytotoxicity was dependent on NKG2D recognition and TNF-α secretion. Cytotoxicity against HepG2 cells by NKL-IL15 for NKL-vec cells was assessed by the MTT method at a 5:1 E:T ratio in the presence of anti-NKG2D mAb, anti-TNF-α mAb, anti-IFN-γ mAb, or isotype control. Data are represented as the mean ± SD from 3 separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test.

Moreover, NKL-IL15 cells significantly inhibited tumor growth, decreased the mortality rate, and prolonged survival of tumor-bearing nude mice (Fig. 1). The safety of adoptively transferring irradiated NKL cells was preliminarily examined both *in vitro* and *in vivo*, and the results showed 800-cGy irradiated NKL-IL15 or NKL-vec cells exhibit no ability to proliferate (Fig. S2) or form tumors in nude mice (data not shown). Further study of the underlying mechanism illustrated that cytotoxicity-associated molecules, including TNF-α, IFN-γ, and TRAIL, were up-regulated in NKL-IL15 cells co-cultured with HepG2 cells compared to NKL-vec (Fig. 2), but that there was no difference in CD107a expression. These results collectively suggested that NKL-IL15 cells exerted enhanced anti-tumor effects mainly through the TRAIL pathway, but less so by the perforin and granzyme pathway. Furthermore, the NKL-IL15 cell responses elicited against HCC cells were much higher than that of the control NKL cells. Importantly, TNF-α and IFN-γ secreted by NKL-IL15 cells induced NKG2D ligand expression on HCC cells, including MICA/B and ULBP1-3, which was favorable for enhanced NK cell recognition and activation (Fig. 4). Furthermore, NKL-IL15-mediated cytotoxicity against HCC was partially dependent on NKG2D recognition and TNF-α production based on our *in vitro* results (Fig. 5); whether these mechanisms are also required *in vivo* for the anti-HCC effect will be examined in future studies.

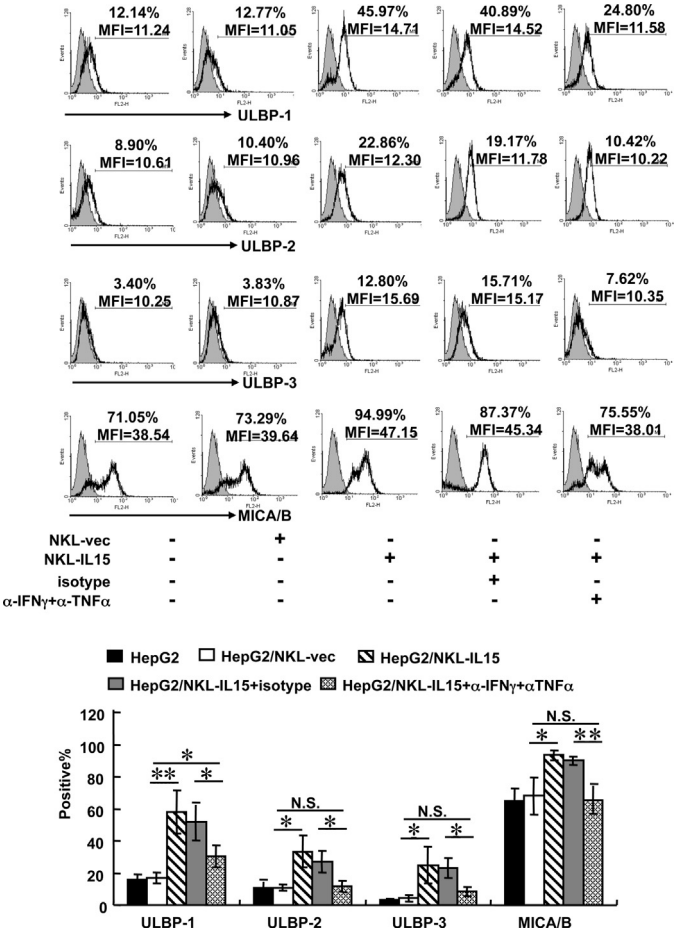

Fig. 4. TNF-α and IFN-γ cytokines produced by IL-15 gene-modified NKL cells induced NKG2D ligand expression on HepG2 cells. HepG2 cells were co-incubated with NKL-IL15 or NKL-vec cells in Transwell plates in the presence or absence of anti-TNF-α and anti-IFN-γ neutralizing mAbs for 24 h. NKG2D ligand expression levels of ULBP-1, -2, -3, and MICA/B on HepG2 cells were analyzed by flow cytometry. Data were calculated from 1 representative experiment, and a total of 3 independent experiments were performed (upper). Diagrams represent the mean percentage ± SD of NKG2D ligand-expressing cells (lower). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to NKL-vec cells using the paired Student's *t*-test.

Previous studies showed that IL-15-engineered human tumor cells exhibited decreased tumorigenicity in nude mice (Di Carlo et al., 2000; Suzuki et al., 2001), indicating that hIL-15 secreted by the NKL-IL15 cells might also enhance the host anti-tumor immune response by activating endogenous murine NK cells. Therefore, we can speculate that using NKL-IL15 cells in HCC patients may not only be able to directly kill tumor cells, but also indirectly augment host anti-tumor immune responses by breaking tumor immune tolerance *via* IL-15 secretion. These advantageous properties of NKL-IL15 cells indicate their potential suitability for use in adoptive immunotherapy against cancer in the clinic.

Based on this study, NKL-IL15 cells could thus provide opportunities to improve immunotherapy in future therapeutic trials. Of course, NKL-IL15 cells require extensive further assessment before they can be applied as a therapeutic in the clinic, including whether they may be leukemogenic in mice or whether the IL-15 modification may increase their leukemogenic potential. Moreover, the optimal dose of NKL-IL15 cells, the condition of the patient prior to therapy, the clinical context of therapy, and the appropriate adoptive cell transfer strategies will need to be considered in order to successfully develop NKL-IL15 cells as an effective human cancer therapy.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2014.03.007.

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