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Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy

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A B S T R A C T

Background and objectives. Natural killer (NK)-92 cells are effective against a broad range of malignant targets both *in vitro* and *in vivo*. Interleukin-15 (IL-15) is an important cytokine for NK cell development and differentiation. IL-15 gene-modified NK-92 cells need to be characterized and their clinical implications investigated.

Design and Methods. IL-15 cDNA was inserted into a pcDNA3 eukaryotic expression vector and the recombinant vector (pcDNA3-IL15) was tranfected into NK-92 cells. The IL-15 gene- modified NK-92 cells (NK92-IL15) were cloned and characterized with regard to their cytokine production, proliferation, cytotoxicity and surface phenotype.

Results. NK92-IL15 cells continuously produced a high level of IL-15 in culture supernatant, which made the cells proliferate significantly more rapidly in response to stimulation with low doses of IL-2 or IL-15; the cumulative number of cells in long-term culture was also significantly higher. NK92-IL15 cells became adherent to plastic and their expression of CD54 increased, which may explain their improved proliferating potential, like adherent NK cells. NK92-IL15 cells were more strongly cytotox against a broad range of target tumor cells than the parent NK-92 cells, and this increased cytotoxicity was correlated to the increased expression of cytotoxic effector molecules, such as perforin, Fas ligand and IFNγ, and up- or down-regulated expression of activating or inhibitory NK cell receptors (NKG2D or NKG2A/CD94).

Interpretation and Conclusions. These results demonstrate that NK92-IL15 cells are promising for adoptive cellular immunotherapy.

Key words: NK cells, cell line, interleukin-15, gene transfer.

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Given the important roles of natural killer (NK) cells in anti-tumor and anti-viral process, numerous studies have been conducted, and are still ongoing, to improve the antitumor effect of NK cells. Human NK cells comprise only about 10-15% of all circulating lymphocytes, and interleukin-2 (IL-2) was regarded as the natural activator for NK cells. NK cell-based cellular immunotherapy includes endogenous activation of the patient's own NK cells through administration of cytokines[1-3] or through adoptive use of *ex vivo*, expanded autologous[4,5] or donor-derived[6] adherent NK (A-NK) cell or lymphokine-activated killer (LAK) cells. NK-based immunotherapy has had some success in patients with renal-cell carcinoma and malignant melanoma,[6,7] as well as in patients with lung and hepatic cancers.[8] Affinity columns and new culture conditions have been developed to obtain more highly purified *ex vivo* population of NK cells, and a recent study describes the use of enriched NK cells to treat patients with breast cancer,[9] and most interestingly, to treat leukemia with donor allogeneic NK cells in mismatched hematopoietic transplants.[10] However, the isolation and large scale *ex vivo* expansion of NK cells free of contamination by other lymphocytes have proven to be technically difficult. So, establishment of NK cell lines has become a favored tool for immunotherapeutic purposes. Six malignant NK cell lines have currently been established and are sufficiently well characterized: NK-92, YT, NKL, HANK-1, KHYG-1, and NK-YS.[11] Their immunophenotype is remarkably similar and is as follows: $CD1^-CD2^+CD3^-CD4^-CD5^-CD7^+$ $CD8^-CD16^-CD56^+$ $CD57^-$. All these NK cell lines show natural cytotoxicity against a broad range of tumor cells, although the

NK-92 cell line has attracted more interest and generated much more basic data for further research than have the other cell lines.

NK-92 was established from a patient with large granular lymphoma in 1994.[12] The lack of apparent toxicity against allogeneic cells of the recipient,[13] the broad cytotoxic activity towards a wide range of tumor cells, as well as towards human leukemia and melanoma in mice,[14-19] and its ease of culture seem to make NK-92 an attractive alternative for use in adoptive cancer immunotherapy, and indeed these cells are now being tested in clinical trials. NK-92 is absolutely dependent on the presence of IL-2 for its survival, proliferation and function both *in vitro* and *in vivo.* The mechanism of dependence of NK-92 cells on IL-2 is closely related to IL-2 receptor expression. Large scale production of NK-92 cells *in vitro*, and adoptive transfer of NK-92 cells into the host are very expensive procedures, most of the cost being due to the IL-2 used in the *in vitro* cultures and the *in vivo* injections. Unfortunately, the efficacy of IL-2 has been offset by accompanying toxicity that is mediated by cytokines and other small molecules that are secreted by IL-2-activated effector cells following the injection with IL-2. In order to reduce or deplete the dependence of NK-92 cells on IL-2, two laboratories established IL-2 gene-modified NK-92 cells, which are independent of IL-2. The immunophenotype, proliferation and cytokine production of this gene-modified cell line are similar to those of the parent cell line and it has a strong antitumor effect both *in vitro* and in adoptive cellular immunotherapy of mice in the absence of IL-2.[20,21]

We transfected NK-92 cells with the IL-15 gene in order to explore the potential of NK-92 cells to respond to IL-15 rather than to IL-2. NK-92 cells have a $CD56^{bright}$ phenotype, similar to the $CD56^{bright}$ NK intermediate subset in peripheral blood, which responds to IL-15 stimulation by differentiating further into mature NK cells and exerting stronger cytotoxicity against tumor cells.

Design and Methods

Cytokines, antibodies, primers and cell lines

Endotoxin-free recombinant human IL-2 (2.1×10^7 U/mg), and human IL-15 (2.2×10^7 U/mg) were purchased from Genzyme (Cambridge, MA, USA). The following monoclonal antibodies (MAb) were used in this study: FITC-conjugated anti-human CD3 (IgG1) mouse mAb, PE-conjugated anti-human CD56 (IgG1) mouse mAb, FITC-conjugated anti-human CD16 (IgG1) mouse mAb, FITC-conjugated anti-human CD14 (IgG1) mouse mAb, FITC-conjugated anti-human CD19 (IgG1) mouse mAb, anti-human CD25 (IgG1) mouse mAb, anti-human CD132 IL-2R (IgG1) mouse mAb, anti-human CD48 (IgG1) mouse mAb, anti-human CD69 (IgG1) mouse mAb, anti-human CD94 (IgG1) mouse mAb, anti-human CD95 (IgG1) mouse mAb, anti-human CD54 (ICAM-1, IgG1)mouse mAb, anti-human NKG2D and NKG2A mouse mAb, biotinylated anti-mouse IgG (H+L) goat PCA, FITC- and PE-conjugated streptavidin and isotype control mAb: these were all obtained from Becton Dickinson.

We designed polymerase chain reaction (PCR) primers: these primers had to be 18–24 nucleotides long and to have 100% homology with the particular

Table 1. Sequence of primers and conditions for RT-PCR.

Transcript	*Sequence*	*Annealing temperature (°C)*	*Cycle*	*Product size (bp)*
IL-15	(F) 5'GCGGATCCGATGAGAATTTCGAAACCACAT			
	(R) 5'GCGAATTCGTCAAGAAGTGTTGAT	58	33	594
IFNγ	(F) 5'ATGAAATATACAAGTTATATCTTGGCTTT			
	(R) 5'GATGCTCTTCGACCTCGAAACAGCAT	58	33	494
IL-4	(F) 5'ATGGGTCTCACCTCCCAACTGCT			
	(R) 5'CGAACACTTTGAATATTTCTCTCTCTCAT	58	33	456
Perforin	(F) 5'AAAGTCAGCTCCACTGAAGCTGTG			
	(R) 5'AGTCCTCCACCTCGTTGTCCGTGA	58	33	436
Fas L	(F) 5'ATGTTTCAGCTCTTCCACCTACAGA			
	(R) 5'CCAGAGAGAGCTCAGATACGTTGAC	58	33	500
TNFα	(F) 5'CAGAGGGAAGAGTTCCCCAG			
	(R) 5'CCTTGGTCTGGTAGGAGACG	58	33	430
NKG2D	(F) 5'CTGGGAGATGAGTGAATTTCATA			
	(R) 5'GACTTCACCAGTTTAAGTAAATC	58	33	416
NKG2A	(F) 5'CCAGAGAAGCTCATTGTTGG			
	(R) 5'CCAATCCATGAGGATGGTG	58	33	325
β-actin	(F) 5'ATCATGTTTGAGACCTTCAACA3'			
	(R) 5'CATCTCTTGCTCGAAGTCCA3'	58	33	300

(F) Forward primer; (R) Reverse primer.

regions of the genes coding for characteristic extracellular regions of the molecules, according to the gene sequences. The gene sequences were obtained using the OLIGO Primer Analysis Software, Version 5.0 (NBA, Software and Research Services for Tomorrow's Discoveries, National Biosciences, Plymouth, MN, USA). PCR oligomers were produced at the Oligonucleotide Synthesis Facility, University of Science & Technology in China. The PCR primers and their product lengths are listed in Table 1.

K562, a human chronic myelogenous leukemia cell line purchased from ATCC, was used as the source of target cells for the cytotoxicity assays, and cultured in complete medium (CM) consisting of RPMI 1640 containing glutamine supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). Hep G2 (a human hepatocellular carcinoma cell line), HeLa (a human cervix adenocarcinoma cell line), 3AO (a human ovarian carcinoma cell line), PG-5 (a small cell lung cancer cell line) and, HT-29 (a human colorectal adenocarcinoma cell line) were purchased from ATCC and also used as target cells in cytotoxicity assays; these cell lines were cultured in the same medium as were the K562 cells.

Construction of IL-15 expression vector and transfection into NK-92 cells

Total RNA was extracted from lypopolysaccharide-stimulated adherent monocytes and IL-15 cDNA was obtained using RT-PCR method as described below. IL-15 cDNA was then inserted into a expression vector (pcDNA3), and the recombinant vector (pcDNA3-IL15) was transferred into NK-92 cells through LipofactAMINE (GIBCO). The IL-15 gene-transferred NK-92 cells were then cloned, screened and identified.

^{51}Chromium release cytotoxicity assay.

Assays were performed using NK-92 cells or IL-15 gene-transferred NK-92 (NK92-IL15) cells that had been co-cultured earlier in the presence or absence of cytokines (e.g. IL-2 or IL-15). The NK cells were mixed with ^{51}Cr-labeled K562 target cells (or any other target cells as indicated in experiment) for NK cytotoxicity assay at effector (E) to target (T) ratios of 20:1, 10: 1, 5:1, 2.5:1 and 1.25:1, as we have previously described.[22] After standard 4-h incubation, the supernatant were harvested and analyzed on a gamma counter (model 5500; Beckman Instrument, Irvine, CA, USA). The % specific lysis was calculated as followed:

$$\% \text{ specific lysis} = CPM_{exp} - CPM_{spontaneous} / CPM_{maximum} - CPM_{spontaneous} \times 100\%$$

Reverse transcription-PCR (RT-PCR)

RNA was extracted from NK-92 cells or NK92-IL15 cells (1×10^6 of each) as described above, using the acid-guanidinium phenol-chloroform method. RT-PCR was performed using an RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA) and a method that we have previously described.[23] Cellular RNA (100 ng) was reverse-transcribed into cDNA in a reaction mixture containing 5 mM $MgCl_2$, 1 mM dNTP, 2.5-μM oligo (dT) primers, 1 U RNase inhibitor, and 2.5 U reverse transcriptase. After incubation at 42°C for 15 min, the reaction was terminated by heating at 95°C for 5 min. PCR was performed on the cDNA using the sense/antisense primers listed above. The PCR reaction buffer (25 μL), consisting of 2 mM $MgCl_2$, 0.5 μM of each primer, and 1 U Ampli *Taq* DNA polymerase (5 μL of each reverse-transcriptase solution), was added to an amplification tube. The amplification was performed using the primers and conditions listed in Table 1. The PCR cycles were: 94°C (1 min) for melting, 58°C (1min) for annealing, 72°C (1 min) for extension, 33 cycles, with additional extension at 72°C for 10 min. Twenty-microliter aliquots of the amplified product were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. The band intensity of ethidium bromide fluorescence was measured using NIH Image Analysis Software Ver 1.61 (National Institutes of Health, Bethesda, MD, USA). The intensities of the bands were calculated as ratios to the intensity of β-actin.

Flow cytometry and immunofluorescent microscope analysis

For membrane staining, freshly harvested NK cells were suspended in ice-cold PBS containing 0.1% sodium azide and 1% FBS (PBS-AF). The cells (0.2×10^6/0.1 mL) were then incubated on ice for 30 min with unlabeled primary mAbs (10 μg/mL). Negative controls were cells incubated without antibodies, or incubated with isotype-matched non-reactive immunoglobulins. The cells were then washed twice with PBS and incubated on ice for 30 min in the presence of the appropriate biotinylated secondary antibodies (1/500 dilution). The cells were again washed and incubated on ice for 30 min in the presence of PE-conjugated streptavidin (1/20 dilution). Finally, the cells were washed twice in PBS, fixed with 4% (w/v) paraformaldehyde/PBS solution, and analyzed by flow cytometry using a FACScalibur.[24] For immunofluorescent microscope analysis, the recombinant human IL-15 was labeled by FITC using a standard method, and the FITC-labeled IL-15 was then used as a fluorescent probe to detect IL-15Ra on NK cells under a fluorescent microscope.

Proliferation assay and detection of cell proliferation by carboxyfluorescein diacetate succinimidyl ester (CFSE)

NK-92 or NK92-IL15 cells were cultured in triplicate at 37°C in a 5% CO_2 incubator in complete a-MEM

medium containing 2 mM L-glutamine, 0.2 mM I-inositol, 20 mM folic acid, 10^{-4} M 2-mercaptoethanol, 12.5% fetal calf serum, 12.5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in 24-well plates (1×10^6 cells/well) in the presence of IL-2 or IL-15 (100 u/mL of each cytokine). Culture medium was exchanged with 50% fresh medium every other day, and the cells were separated into two wells every four days. In order to analyze the proliferation capacity of NK cells in response to cytokines, the cell numbers were calculated every day by MTT colormetric analysis or under a microscope, and the cumulative number of cells was recorded.

For cell division analysis, CBMC were re-suspended in 1 mL PBS/1% BSA at a final concentration of 10^6/mL, then labeled for 10 min at 37°C with CFSE (10 mM, Sigma). Quench staining was performed on ice for 5 min by adding 5 vol of ice-cold RPMI 1640/10% FBS. Then the cells were washed three times with ice-cold PBS/1% BSA and cultured under appropriate conditions. At the indicated time points, cells were harvested, stained with the antibodies, and analyzed by FACS.

Enzyme linked immunosorbent assay (ELISA)

The supernatant of each NK cell culture was collected after 12 hours and examined by ELISA to measure concentrations of soluble cytokines and effector molecules (perforin and Fas ligand). The supernatants of the NK cell culture and cell protein extract were centrifuged for 10 minutes at 6000g, and stored at -70°C, according to routine procedures, until ELISA. Following the manufacturer's instructions (R&D systems), the ELISA kits were used to assay the cytokines IFN-γ (detection limit, 50 pg/mL), IL-4 (detection limit, 8 pg/mL), IL-10 (detection limit, 6.35 pg/mL), IL-13 (detection limit 10 pg/mL), perforin and Fas ligand. The data were analyzed with Origin Pro7.0 software.

Statistical analysis

All experiments were repeated at least four times and each sample was in triplicate in every experiment. The results were performed using the Wilcoxon's signed-rank pair and Mann-Whitney U tests. Differences were considered statistically significant when the p value was <0.05.

Results

Establishment of the IL-15 gene-modified NK-92 cell line

IL-15 can increase the differentiation and proliferation of NK cells in the same way as IL-2 does through the IL-15Rα/IL-2Rβγ complex, and in a different way from IL-2, which causes activation-induced cell death.[25] NK-92 cells are a typical immature NK cells with a standard $CD56^{bright}CD16^-$ phenotype. In order to cause further differentiation and more rapid proliferation of NK-92 cells we tried to transfer the IL-15 gene into these cells. As shown in Figure 1A, the morphologic characteristics of the parent NK-92 cells and IL-15-gene modified NK-92 cells (NK92-IL15) were totally different: cell aggregator forming potential disappeared and the cells became adherent to the plastic plate, an important feature usually observed in adherent NK (A-NK) cells, which are much more cytotoxic and more proliferative than non-adherent NK cells.[26] NK92-IL15 cells expressed significantly much more IL-15 transcripts (29.35 ± 4.84 fold, $p<0.01$) than the parent NK-92 cells, as assessed by RT-PCR assay (Figure 1B), which led to significantly greater production of IL-15 protein in the culture supernatant, tested by ELISA (9.76 ± 1.39 fold, $p<0.01$) (Figure 1C). The NK92-IL15 cell line was continuously cultured *in vitro* for more than six months, during which time the morphologic features and IL-15 production did not change (*data not shown here*). These results indicate that the IL15-gene modified NK-92 cell line had been established.

Proliferation and cytotoxicity of IL-15 gene-modified NK-92 cell line

In order to examine the proliferative potential, the NK92-IL15 cells were cultured in the presence of different doses of IL-2 (Figure 2A) or IL-15 (Figure 2B) for a total of 48 hours. The cells were then assayed by the MTT colormetric analysis. As shown in Figure 2A and 2B, the NK92-IL15 cells proliferated significantly more rapidly than did their parent NK-92 cells in the presence of low doses of each cytokine (below 125 U/mL), demonstrating that recombinant NK-92-IL15 cells produce more IL-15 to increase cell proliferation when the IL-15 concentration is inadequate. We then cultured these cells (1×10^4/mL in the beginning) for a long period (10 days) by replacing half of the culture with fresh cytokine-containing medium to keep the cytokine concentration at 10 U/mL. As shown in Figure 2C, the cumulative cell number, regardless of whether IL-2 or IL-15 was used as the activator, was significantly greater in NK92-IL15 cells after five to ten days of culture (5 day: $p<0.05$; any day more than five days: $p<0.001$). The fast cell proliferation in the presence of low doses of IL-2 was due to fast cell division of NK-92-IL15 as shown in Figure 2D, when the cells were cultured in 10 U/mL of IL-2 for 48 hours.

Next, we observed the cytotoxic ability of NK92-IL15 cells. As shown in Figure 3A, NK92-IL15 cells exerted significantly greater cytotoxicity against a variety of target tumor cells including leukemia (K562 cells) and solid tumors (HeLa, 3AO, PG5 and HT29 cell lines), the difference between NK92-IL15 and the parent NK-92

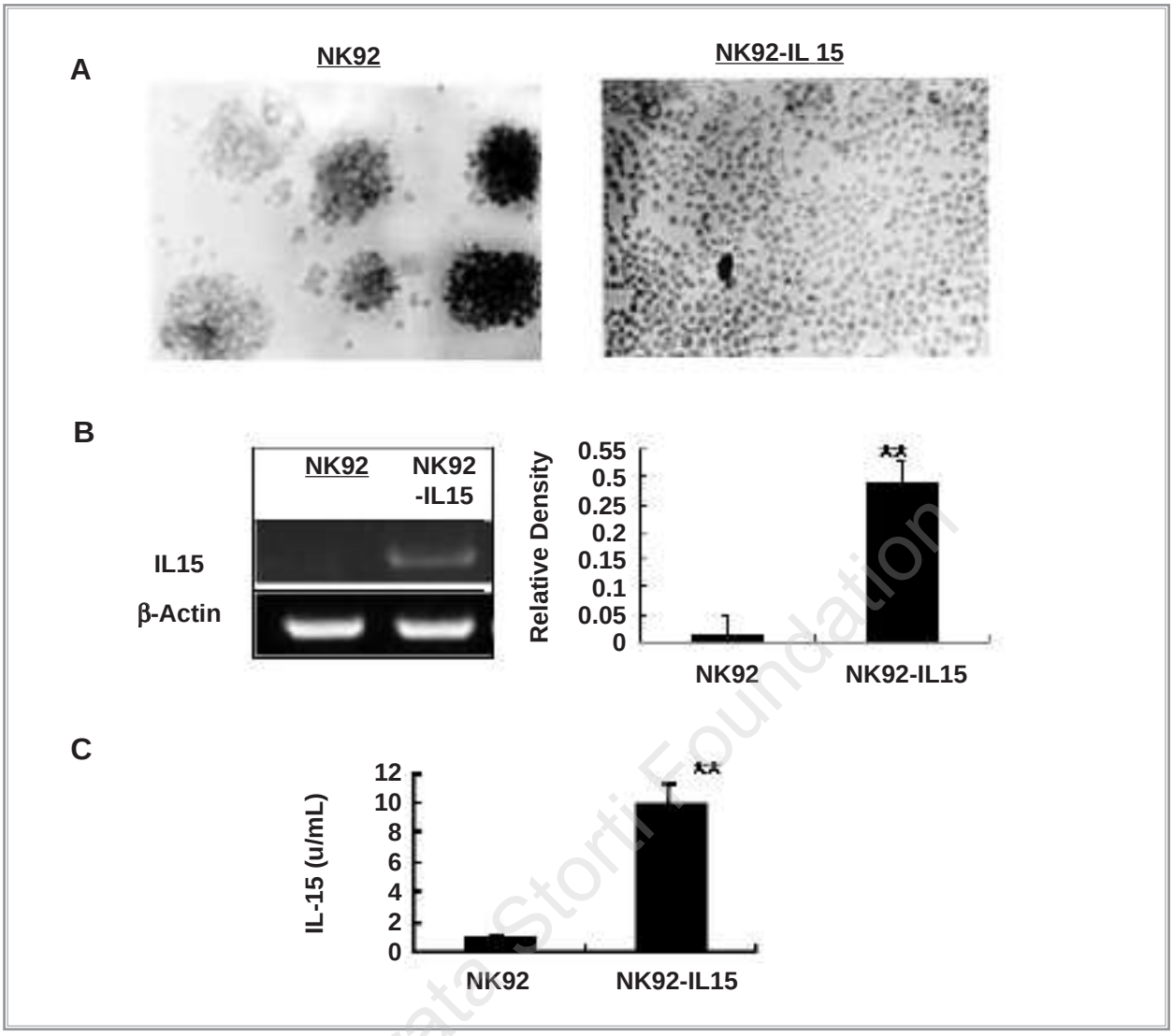

Figure 1. Establishment of IL-15 gene-modified NK-92 cell line. Total RNA was extracted from a LPS-stimulated monocytes and IL-15 cDNA was obtained using the RT-PCR method. IL-15 cDNA was then inserted into a eukaryotic expression vector (pcDNA3), the recombinant vector (pcDNA3-IL15) was transferred into NK-92 cells through LipofactAMINE (GIBCO). The IL-15 gene-transferred NK-92 cells (NK-92-IL15) were then cloned, screened and identified under pressure of G418 concentration. (A). Cell growth morphology under microscope. NK-92 cells transfected with IL-15 gene (NK92-IL15) displayed a stronger tendency to adhere to plastic without forming aggregators. (B). RT-PCR analysis to detect IL-15 gene expression level. (C). SCF concentration in the culture supernatant of NK92-IL15 cells using an IL-15-dependent CTLL2 cell proliferation assay.

cells being greater when the effector/target ratio was lower. This change of cell cytolytic function is at least partly through up-regulation of gene expression, which is thereafter able to code cytotoxic effector protein, of perforin, Fas ligand and IFNγ, whose gene expression in NK-92-IL15 cells was significantly higher than in the parents NK-92 cells ($p<0.01$, $p<0.01$, $p<0.01$), but not through that of TNFα (Figure 3B), which was further verified by analysis of perforin and Fas ligand protein from the cell extract using the ELISA method (Figure 3C).

Phenotypic analysis of IL-15 gene-modified NK-92 cells

After examining of the function of NK92-IL15 cells, we then began to test the phenotype of the IL-15 gene-modified cell line. Both T-cell (CD3, CD4, CD8) and B-cell markers (CD19 and sIgM) were negative, as they were in the parent NK-92 cells (data not shown here). CD16, an important marker for mature NK cells, was still negative (Figure 4A vs. 4B, Figure 4C), and CD56 expression was not changed and still approached nearly 100% (Figure 4A vs. 4B, Figure 4D). In addition to NK phenotypic markers, we also examined the other important surface func-

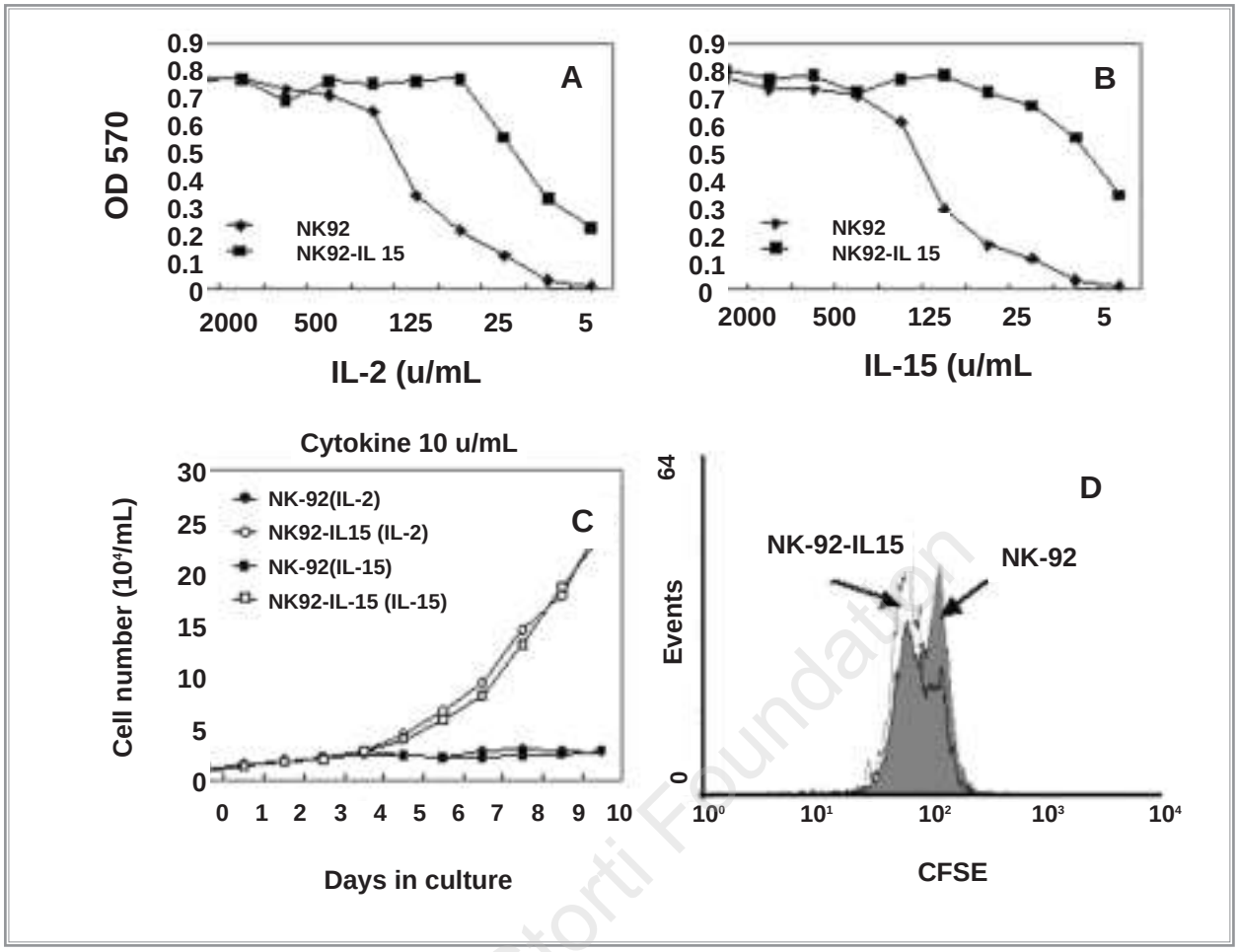

Figure 2. Proliferation of NK92-IL15 in response to IL-2 or IL-15 stimulation NK-92 or NK92-IL15 cells were cultured in triplicate at 37°C in a 5% CO_2 incubator in complete a-MEM medium containing IL-2 (100 U/mL). Culture medium was exchanged with 50% fresh medium and regulated to the same concentration of IL-2 as at beginning every other day, and the cells were separated into two wells every four days. In order to analyze the proliferative capacity of NK cells in response to cytokines, the cell numbers were calculated every day and the cumulative number of cells is presented. (A). Dose response of NK-92 or NK92-IL15 cells to IL-2. (B). Dose response of NK-92 or NK92-IL15 cells to IL-15 after 48 hours culture, respectively, using MTT colormetric analysis. NK92-IL15 cells proliferated more rapidly than their parent NK-92 cells, independently of the presence of IL-2 or IL-15. (C). Cumulative cell amount in a long-term culture in the presence of a low dose of IL-2 or IL-15 (10 U/mL). NK92-IL15 cells were more efficient than NK-92 in long-term culture in the presence of IL-2 or IL-15. (D). Cell division analysis. NK-92 cells or NK-92-IL15 cells were labeled for 10 min at 37°C with CFSE (10 μM, Sigma). The cells were then washed three times with ice-cold PBS/1%BSA and cultured with a low dose of IL-2 (10 U/mL) for 48 hours. The cells were analyzed directly by FACS.

tional molecules. As shown in Table 2, CD25, a specific receptor chain for IL-2 (IL-2Rα) was slightly increased, explaining the increased response to IL-2 stimulation. CD122, a common IL-2Rβ chain (IL-2Rβc), CD132, a common IL-2Rγ chain (IL-2Rγc) and IL-15Rα were not changed but the activation marker, CD69, was dramatically increased, suggesting the cells were in a state sensitive to stimulation. CD54, an important adherent molecule was markedly up-regulated, explaining the improved cell adherence. Interestingly, the activating receptor of NK cells, NKG2D, and the inhibitory receptor of NK cells, NKG2A/CD94 complex, were up- and down-regulated, respectively, indicating that NK92-IL15 cells become more efficiently activated by recognizing specific ligands on tumor cells through their receptors: being released from a *missing self* mechanism by decreasing inhibitory signal, and participating in a stimulatory mechanism by increasing activating signal.

Discussion

As outlined in Table 3, IL-15 gene-modified NK-92 cells can be characterized by seven features. The proliferation potentials improved after IL-15 gene transfection into the NK-92 cells, which at least partly resulted

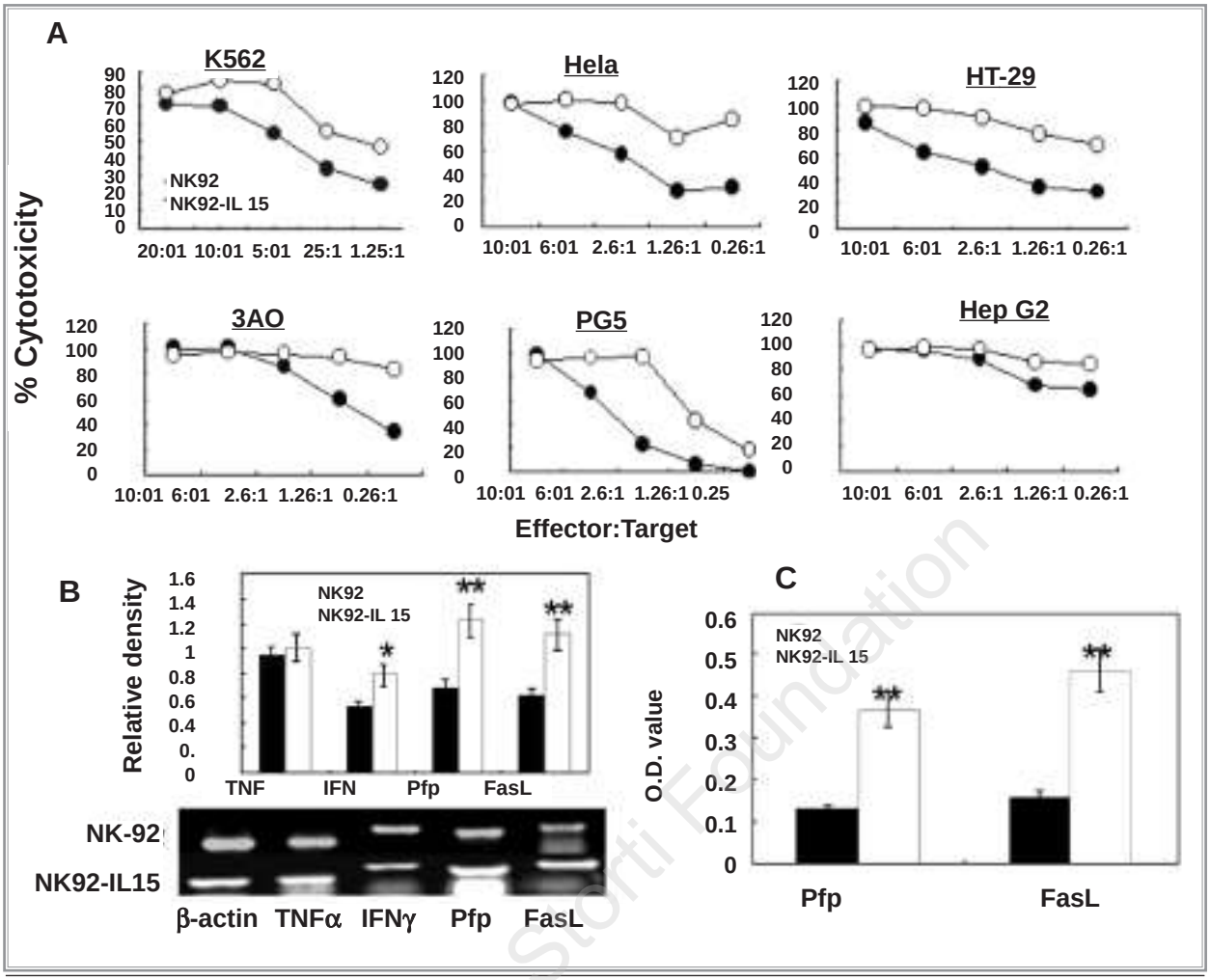

Figure 3. Cytotoxicity against target tumor cells of NK92-IL15 cells. Assays were performed using NK-92 cells or NK92-IL15 cells that had been co-cultured earlier in the presence of IL-2 (100 U/mL). (A). The NK cells were mixed with ^{51}Cr-labeled K562 target cells (or any other target cells as indicated in experiment) for the NK cytotoxicity test at effector (E) to target (T) ratios of 20:1, 10: 1, 5:1, 2.5:1 and 1.25:1. After standard 4-h incubation, the supernatant were harvested and analyzed on a γ counter (model 5500; Beckman Instrument, Irvine, CA, USA). The % specific lysis was calculated as followed: % specific lysis = CPMexp - CPMspontaneous/CPMmaximun - CPMspontaneous × 100%. NK92-IL15 cells exerted stronger cytotoxicity against K562, HeLa, HT-29, PG5 and 3AO cells than did NK-92 cells. (B). Total RNA was extracted from the cultured NK-92 or NK92-IL15 cells and transcripts of TNFα, IFNγ, perforin (Pfp) and Fas ligand (Fas L) were assayed by the RT-PCR method. NK92-IL15 cells transcribed more mRNA of IFNγ, Pfp and Fas L than did NK-92 cells. (C). ELISA analysis of Pfp and Fas L protein levels. Data are presented as O.D.

from the changes of adherence properties with up-regulated expression of CD54, activating states with over-expression of CD69 and responses to low dose of IL-2 or IL-15 with increased CD25 expression (Table 3). The cytotoxic activity against a broad range of hematologic and solid malignances was also enhanced, which most probably correlated with the increased production of effector molecules, such as perforin, Fas ligand and interferon-γ, and with up-regulation of NKG2D (an activating NK receptor) or down-regulation of NKG2A/CD94 (a inhibitory NK receptor complex) of those NK92-IL15 cells.

Briefly, human NK-cell development can be divided into two phases.[27-30] In the early phase, NK progenitor cells ($CD34^+Lin^-$) respond to early activating growth factors (e.g. Flt-3 ligand or stem cell factor) and develop into an NK-cell precursor with the basic phenotype $CD34^+IL\text{-}2R\beta^+$. IL-15 induces further differentiation into mature NK cells which are CD56 positive in the periphery. NK cells in peripheral blood can be divided into two subsets based on their surface expression of CD56 molecules: $CD56^{bright}$ NK cells and $CD56^{dim}$. $CD56^{bright}$ NK cells represent a minor subset (approximately 10%) of human NK cells that show low or no CD16 expression and are capable of marked proliferation but are less cytotoxic. The most important is that $CD56^{bright}$ cells keep their response to IL-15 stimulation, leading to greater proliferation and further differentiation, which is a feature

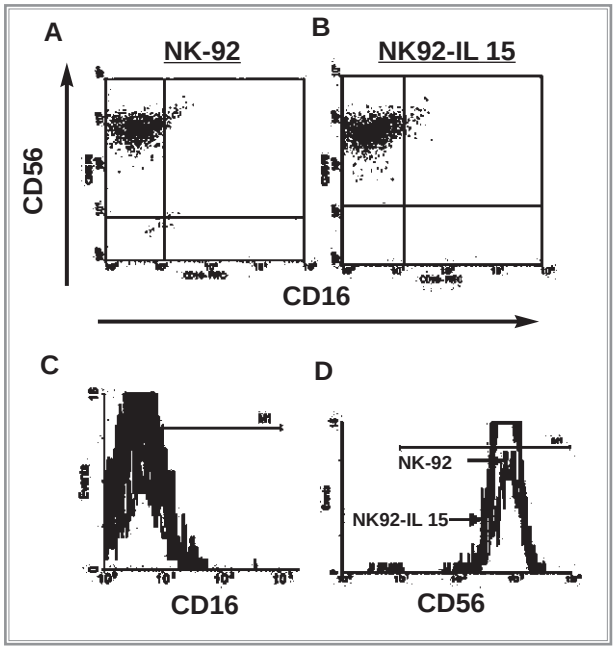

Figure 4. Surface NK marker expression of NK92-IL15 cells (A) and (B). Double staining of NK-92 and NK92-IL-15 cells with CD16 and CD56. (C) and (D). Histogram analysis of NK-92 or NK92-IL15 cells by CD16 and CD56 expression, respectively.

Table 2. Surface phenotype of IL-15 gene-modified NK-92 cells.

	NK-92		*NK92-IL15*	
	%	*MFI*	*%*	*MFI*
CD56	96.78	794.9	97.61	684.3
CD16	1.83	27.45	2.19	26.93
CD25 (IL-2Rα)	55.66	16.36	77.67	19.57
IL-15Rα	89.33		94.79	
CD122 (IL-2Rβ)	33.19	31.11	31.26	29.12
CD132 (IL-2Rγ)	64.12	134.56	71.23	162.43
CD48	96.83	403.21	98.82	435.94
CD69	14.30	13.63	64.25	16.62
NKG2D	94.36	222.35	100.00	367.45
NKG2A	31.35	26.54	22.13	20.12
CD94	54.37	79.99	42.19	63.23
CD54	67.33	79.67	100.00	230.45
CD95	93.81	45.28	90.66	54.67

Expression of cell-surface antigen was determined by direct or indirect immunofluorescence staining with the corresponding monoclonal antibody and flow cytometric analysis as described in Materials and Methods. For immunofluorescent microscope analysis of IL-15Rα on NK cells, the recombinant human IL-15 were labeled by FITC using a standard method, and the FITC-labeled IL-15 were then used as the fluorescent probe to detect IL-15Ra+ NK-92 cells under fluorescent microscope. "%": percentage of positive cells; "MFI": mean fluorescence intensity.

Table 3. Characterization of NK92-IL15 cells compared with NK-92.

	NK92-IL15 compared with NK-92
Proliferation	
IL-2Rα (CD25)	Increased
IL-15Rα	No change
IL-2Rβ (CD122)	No change
IL-2Rγ (CD132)	No change
To low dose IL-2	Markedly increased
To low dose IL-15	Markedly increased
CD95	No change
Adherence	
Morphology	No aggregator, adherent to plate
CD54 expression	Markedly increased
Cytotoxicity	
Lysis against K562 at low E:T	Increased
Lysis against solid tumors	Increased
Perforin mRNA & protein	Increased
Fas L mRNA & protein	Increased
TNFα mRNA	No change
NK Phenotype	
CD56	No change
CD16	No change
Cytokine production	
IL-4 mRNA	No change
IFNγ mRNA	Increased/Activation marker
CD69	Markedly increased
CD48	No change
NK receptors	
CD94	Slightly decreased
NKG2A	Slightly decreased
NKG2D	Increased

different from IL-2. Unfortunately, we did not observe an expression difference between NK-92-IL15 cells and the parent NK-92 cells, because NK-92 cells are a tumor cell line, in which almost all cells expressed CD56 molecules with a relatively high surface density. The majority (approximately 90%) of human NK cells in the periphery are $CD56^{dim}$, $CD16^{high}$, highly cytotoxic and with low proliferation. $CD56^{dim}$ NK cells might further derive from $CD56^{bright}$ cells under the control of factors including IL-15.[27-30] So, NK92-IL15 cells may proliferate more rapidly and differentiate further to exert strong cytotoxic effects.

Historically, IL-2 was regarded as the natural activator for NK cells. Recently, it was discovered that the IL-2 gene was not expressed in bone marrow (BM), and that IL-2 was produced primarily by antigen-activated T cells located in the periphery. In addition, mice deficient in IL-2 have functional NK cells, whereas mice and humans that lack the c subunit of the IL-2R or IL-2/15R lack NK cells.[31,32] Collectively, these data suggested that a factor other than IL-2 was produced in the BM and used signaling components of the IL-2R to induce NK-

cell development. Human IL-15 was first discovered from the IMTLH BM stromal cell line, and was observed to directly induce the differentiation of functional $CD56^+$NK cells from $CD34^+$ hematopoietic progenitor cells (HPC).[33] So, IL-15 is recognized as a natural physiologic regulator of NK cells.[34-37] The primary protein and cDNA sequences of human IL-15 showed little homology to IL-2, but IL-15 used the IL-2R subunit called common β- and γ-chain (IL-2Rβcγc).[38,39] Because IL-15 is a natural physiologic regulator in earlier stages of NK cell differentiation than IL-2 (a terminal activator of NK cells), IL-15 was compared with IL-2 in co-culture with NK92-IL15, which was characterized by immature NK markers. The results demonstrate that NK92-IL15 cells proliferated better in the presence of IL-15 or IL-2 than did their parent NK-92 cells (Figure 2A vs. 2B). The NK92-IL15 cells also became adherent to plastic plate without aggregators like NK-92 cells in culture, which was similarly reported in human adherent NK cells (A-NK cells),[26] with increased expression of CD54 adherent molecules (Figure 1A). Though it is difficult to explain the phenomenon, it seems that the change in growth benefits fast growth, which is possibly related to cell growth and differentiation status.

Implication of IL-15 gene-modified NK-92 cells in adoptive cellular immunotherapy

Natural killer (NK) cells are special lymphocytes that are able to lyse target cells without prior sensitization and without restriction by MHC antigens. Given the important roles of NK cells in anti-tumor and anti-viral process, NK cells have been recognized as the best choice to be a cellular therapeutic reagent.[40] There are several cellular therapeutic regimens, which have been used in the clinic, including lymphokine-activated killer cells (LAK cells), cytokine-induced killer cells (CIK cells) and adherent NK cells (A-NK cells).[41-45] Since human NK cells comprise only about 10-15% of all circulating lymphocytes, scientists have tried to establish isolation and culture models to harvest large amounts of activated and purified human NK cells for adoptive cellular immunotherapy. However, the isolation of NK cells free of contamination by other lymphocytes has proven to be technically difficult. The establishment of NK cell lines provided favorable tools for culture models. Two laboratories, one led by Dr. Klingemann and the other by Dr. Whiteside, were successful in establishing an IL-2 gene-transferred, IL-2-independent NK-92 cell line, in order to avoid the need for exogenous addition of IL-2 in the culture system.[20,21] IL-2-gene-transferred NK-92 cells kept all features of the parent NK-92 cells; the only improvement is independent of IL-2 or IL-15. Indeed, IL-2 gene modified NK-92 cells did not show any improvement in proliferation and cytotoxicity when compared with their parents NK-92 cells if the presence of sufficient amounts of cytokine (IL-2 or IL-15). In this study, we transferred IL-15 gene into NK-92 cells, finding that IL-15 may improve NK cell proliferation in response to IL-2 or IL-15. Because NK-92 cells are immature NK cells, based on their phenotype ($CD56^{bright}CD16^-$), and IL-15 is possibly a natural regulator of NK cell differentiation, the NK92-IL15 cells retain a stronger proliferative potential in the presence of IL-15 or IL-2 (Figure 2). This new NK cell line, NK92-IL15, that we have established can be expanded in long-term cultures in the presence of IL-15 or IL-2 in order to harvest large numbers of cells for adoptive transfer. In this way, we can manipulate the *ex vivo* protocol of NK-92 cells more efficiently for clinical utilization in future.

ZT designed the experiments to construct the recombinant vector containing the IL-15 cDNA and to compare the gene-modified NK-92 cells with wide-type cells, wrote the paper, together with JZ. JZ, RS, JHZ and HW performed almost all the experiments cluding the flow cytometry and Western Blotting analyses. HW and RS contributed to the novel ideas on the alternative method, co-designed and carried out some experiments including culture and activation of the NK cells with IL-15 or IL-2, substantial contributions to data interpretation and critical review of the draft. All authors approved the present version for publication and are listed according to a criterion of decreasing individual contribution to the work, with the exception of the last author (ZT) who had the most important role in the whole research and writing the paper.

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