Characterization of stem cell factor gene-modified human natural killer cell line, NK-92 cells: Implication in NK cell-based adoptive cellular immunotherapy

JIAN ZHANG[1,2], RUI SUN[1,2], HAIMING WEI[1], JIANHUA ZHANG[1] and ZHIGANG TIAN[1,2]

[1]School of Life Sciences, University of Science and Technology of China, Hefei City, Anhui 230027;
[2]Shandong Cancer Biotherapy Center, Shandong Academy of Medical Science, Jinan City, Shandong 250062, P.R. China

Received October 14, 2003; Accepted December 1, 2003

Abstract. NK-92 cells are effective against a broad range of malignant targets both *in vitro* and *in vivo*. Stem cell factor (SCF) is an important early acting cytokine for NK cell development. The characterization and implication in clinic of SCF gene-modified NK-92 cells need to be investigated. SCF cDNA was inserted into pcDNA3 eukaryotic expression vector and the recombinant vector (pcDNA3-SCF) was transfected into NK-92 cells. The SCF gene-modified NK-92 cells (NK92-SCF) were cloned and characterized by cytokine gene expression, proliferation potential, cytotoxic function and surface phenotype. NK92-SCF cells continuously produced a high level of SCF in culture supernatant, which made the cells proliferate significantly more rapidly in response to IL-2 or IL-15 stimulation, the cumulative amount of cells in long-term culture was significantly higher. NK92-SCF cells exerted stronger cytotoxicity against a broad range of target tumor cells than their parent NK-92 cells, which was correlated to the increased expression of cytotoxic effector molecules such as perforin and Fas ligand. NK92-SCF cells became more heterogeneous; the $CD56^{High}$ and $CD56^{Low}$ subsets appeared, which may, at least partly, explain the increased proliferating and cytotoxic potential of NK92-SCF cells. SCF gene-modified NK-92 cells (NK92-SCF cells) are more promising than their parent cells for adoptive cellular immunotherapy.

Introduction

Natural killer (NK) cells are special lymphocytes that are able to lyse target cells without prior sensitization and without restriction by MHC antigens. NK cells have important roles in anti-tumor and anti-virus processes. Human NK cells comprise about 15% of all circulating lymphocytes. IL-2 has been regarded as the natural activator for NK cells. Numerous studies have been conducted, and are still ongoing, to improve the anti-tumor effect of NK cells. These include 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) A-NK (adherent NK cells) or LAK cells (lymphokine-activated killer cells). Some successes have been demonstrated for patients with renal-cell carcinoma and malignant melanoma (6,7), as well as in patients with lung and hepatic cancer (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). However, the isolation and large scale of *ex vivo* expansion of NK cells free of contaminating other lymphocytes have proven to be technically difficult. So, establishment of NK cell lines provide favorable tools for this purpose. Currently, six malignant NK cell lines have been established and sufficiently well characterized: NK-92, YT, NKL, HANK-1, KHYG-1, NK-YS (10). Their immunophenotype is remarkably similar and described as follows: $CD1^-CD2^+CD3^-CD4^-CD5^-CD7^+CD8^-CD16^-CD56^+CD57^-$. All these NK cell lines showed natural cytotoxicity against a broad range of tumor cells, among which NK-92 cell line generated substantial basic data for further research.

NK-92 was established from a patient with large granular lymphoma in 1994 (11). The lack of apparent toxicity against non-malignant allogeneic cells (12), the broad cytotoxic activity towards a wide range of tumor cells, as well as towards human leukemia and melanoma in mice (13-18), and its ease of culture seem to make NK-92 an attractive alternative for use in adoptive cancer immunotherapy, and these cells are now being tested in clinical trials, as indicated by the founders of NK-92 cells (19-21). NK-92 has a $CD2^+CD3^-CD4^-CD8^-CD16^-CD56^{bright}$ phenotype and is absolutely dependent on the presence of IL-2 for its survival, proliferation and functioning including cytotoxicity and cytokine production both *in vitro* and *in vivo*.

Correspondence to: Dr Zhigang Tian, School of Life Sciences, University of Science and Technology of China, 443 Huangshan Road, Hefei City, Anhui 230027, P.R. China
E-mail: ustctzg@yahoo.com.cn

Key words: NK cells, cell line, stem cell factor, gene transfer

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

Transcript	Sequence	Annealing temperature (°C)	Cycle	Product size (bp)
SCF	(F) 5'-GGATCCATTATGCAACAGGGGGTAAC-3'			
	(R) 5'-GGTACCTGCCTTTCCTTATGAAG-3'	58	33	594
IFNγ	(F) 5'-ATGAAATATACAAGTTATATCTTGGCTTT-3'			
	(R) 5'-GATGCTCTTCGACCTCGAAACAGCAT-3'	58	33	494
IL-4	(F) 5'-ATGGGTCTCACCTCCCAACTGCT-3'			
	(R) 5'-CGAACACTTTGAATATTTCTCTCTCTCAT-3'	58	33	456
Perforin	(F) 5'-AAAGTCAGCTCCACTGAAGCTGTG-3'			
	(R) 5'-AGTCCTCCACCTCGTTGTCCGTGA-3'	58	33	436
Fas L	(F) 5'-ATGTTTCAGCTCTTCCACCTACAGA-3'			
	(R) 5'-CCAGAGAGAGCTCAGATACGTTGAC-3'	58	33	500
TNFα	(F) 5'-CAGAGGGAAGAGTTCCCCAG-3'			
	(R) 5'-CCTTGGTCTGGTAGGAGACG-3'	58	33	430
NKG2D	(F) 5'-CTGGGAGATGAGTGAATTTCATA-3'			
	(R) 5'-GACTTCACCAGTTTAAGTAAATC-3'	58	33	416
NKG2A	(F) 5'-CCAGAGAAGCTCATTGTTGG-3'			
	(R) 5'-CCAATCCATGAGGATGGTG-3'	58	33	325
ß-actin	(F) 5'-ATCATGTTTGAGACCTTCAACA-3'			
	(R) 5'-CATCTCTTGCTCGAAGTCCA-3'	58	33	300

(F), forward primer; (R), reverse primer.

The mechanism of dependence of NK-92 cells on IL-2 is closely related to the IL-2 receptor expression. Large scale of production of NK-92 cell *in vitro*, and adoptive transfer of NK-92 is expensive, mainly due to the use of IL-2 in *in vitro* culture and *in vivo* injection. Unfortunately, the efficacy of IL-2 has been offset by accompanying toxicities that are mediated by cytokines and other small molecules that are secreted by IL-2-activated effector cells when injection with IL-2. Attempts to reduce IL-2-mediated toxicity have been disappointing. In order to reduce or deplete the dependence of NK-92 cells on IL-2, several laboratories established IL-2 gene-modified NK-92 cells, which was independent on IL-2, with a similar characterization including immunophenotype, proliferation and cytokine production, and exerted a strong anti-tumor efficacy both *in vitro* and in adoptive cellular immunotherapy of mice in the absence of IL-2 (22,23).

In this study, NK-92 was transfected with SCF gene in order to explore the potential of NK-92 cells in response to IL-15 compared with IL-2. NK-92 cells have a $CD56^{bright}$ phenotype, similar to $CD56^{bright}$ NK intermediate subset in peripheral blood, which expresses c-kit (SCF receptor) and responds to SCF stimulation to up-regulate expression of IL-15Rα and IL-2R common ß chain (IL-2Rßc) (24,25). We speculate that SCF gene-modified NK-92 cells will be more sensitive to IL-2 or IL-15 stimulation, and will obtain stronger anti-tumor efficacy than their parent NK-92 cells.

Materials and methods

Cytokines, Abs, primers and cell lines. Endotoxin-free recombinant human IL-2 ($2.1x10^7$ U/mg), human stem cell factor (SCF, $2.1x10^7$ U/mg) and human IL-15 ($2.2x10^7$ U/mg) were purchased from Genzyme (Cambridge, MA). The following Abs 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 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 mAbs were all obtained from Becton Dickinson.

PCR primers were designed by us to be 18-24 nucleotides long and to have a 100% homology with the particular regions of the genes coding characteristic extracellular regions of the molecules, according to 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). PCR oligomers were produced at the University of Science and Technology of China Oligonucleotide Synthesis Facility. All utilized PCR primers and their product lengths are outlined in Table I.

K562, a human chronic myelogenous leukemia cell line purchased from ATCC, was used as target cells in cytotoxicity assays, and cultured in complete medium (CM) consisted of RPMI 1640 containing glutamine supplemented with 10% FCS, 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 assay, and cultured in the same medium as K562 cells.

SCF gene transduction into NK-92 cells

NK-92 cells. The NK-92 cell line was established from a patient with rapidly progressive non-Hodgkin's lymphoma (11) and was purchased from ATCC. The cells were maintained in myeloid long-term culture medium (MLTC; Terry Fox Laboratory, Vancouver, BC, Canada) containing 10% fetal calf serum (FCS), 10% horse serum, and 10^{-4} mol/l 6-mercaptoethanol and supplemented with 10^{-6} mol/l hydrocortisone (Sigma, St. Louis, MO) and 1,000 IU/ml IL-2 (Chiron, Emeryville, CA).

SCF expression vector. Total RNA was extracted from a hepatocarcinoma cell line (Hep G2B) and a 596-bp SCF cDNA fragment was obtained by RT-PCR method using the primers as below: sense primer 5'-GGT ACC TGC CTT TCC TTA TGA AG-3' and antisense primer, 5'-GGA TCC ATT ATG CAA CAG GGG GTA AC-3', which contained *Kpn*I and *Bam*HI restriction enzyme site. SCF cDNA and pcDNA3 were digested with the restriction enzymes *Bam*HI and *Kpn*I, linked and transferred in to bacterium DH5α. The recombinant SCF expression vector (pcDNA3-hSCF) was identified by PCR and sequencing.

SCF gene transduction into NK-92 cell lines. NK-92 cells cultured at a concentration of $4x10^5$ to $1x10^6$ cells/ml were supplemented with fresh MLTC medium 24 h prior to transduction. NK-92 cells were pelleted and resuspended in an aliquot of the Lipofectamine supernatant ($2x10^5$ cells/ml plus 10 μl Lipofectamine containing 3 μg recombinant vectors pcDNA3-hSCF extracted from DH5α/pcDNA3-hSCF bacterium) for 3 h. Aliquots (1 ml) of this suspension were pelleted, resuspended in fresh MLTC medium and then cultured for another 48 h in the presence of IL-2 (1,000 μ/ml). The neomycin resistance (*neo*^{r}) gene in pcDNA3 was a selectable marker. Transfectants were selected by culture in the presence of G418 (Gibco), and G418-resistant colonies were tested by enzyme-linked immunosorbent assay (ELISA), TF-1 cell proliferating bioassay and RT-PCR for the ability to produce SCF. Selection was accomplished by gradually increasing the concentration of G418 in the medium. Forty-eight hours after transduction, the cells were suspended in medium containing 400 μg/ml G418 and 1,000 IU/m IL-2 at a concentration of $2x10^5$ cells/ml and cultured for 14 days. Control cultures established in parallel contained parental (non-transduced) cells plated in the same medium and transduced NK cells cultured in the absence of G418. The cultures were centrifuged on Ficoll-Hypaque gradients to remove dead cells, washed, and replated at $2x10^5$ cells/ml in medium containing 200 μg/ml G418 and 1,000 IU/ml IL-2. During the selection process, cell viability was determined by the trypan blue exclusion method.

Subcloning of NK92-SCF cells. SCF gene transduced NK-92 cells were called as NK92-SCF cells. G418-resistante NK92-SCF cells were regulated to 10 cells/ml and cultured in 96-well plates (100 μl/well, e.g. 1 cell/well) in the presence of IL-2. The wells containing single cell were recorded for continuous observation. A single cells might grow to 10^3 cells/well after 10-day culture, the supernatant of which were bio-assayed using TF-1 cells, a standard bioassay cell line for SCF function. Eleven positive clones of NK92-SCF cells were obtained, which produced 6-8 μ/ml of SCF per 10^3 cells. All the positive clones were re-cloned and identified again. The NK92-SCF cells used in this study were continuously cultured for more than 1 year and freeze-stored for three times.

^{51}Chromium release cytotoxicity assay. Assays were performed using NK-92 cells or SCF gene transferred NK-92 (NK92-SCF) 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 the experiment) for NK cytotoxicity at an effector (E) to target (T) ratio of 20:1, 10: 1, 5:1, 2.5:1 and 1.25:1 as previously described by us (26). After standard 4-h incubation, the supernatants were harvested and analyzed on a gamma counter (model 5500; Beckman Instrument, Irvine, CA). The % specific lysis was calculated as followed: % specific lysis = CPMexp - CPMspontaneous/CPMmaximum - CPMspontaneous x 100%.

Reverse transcriptase-PCR (RT-PCR). The method was used as previously described by us (27). Briefly, RNA was extracted from NK-92 cells or NK92-SCF cells ($1x10^6$ of each) as described above. Extraction was performed by the acid-guanidinium phenol-chloroform method. RT-PCR was per-formed using RNA PCR kit (Perkin-Elmer, Norwalk, CT). 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 according to the conditions in Table I. PCR condition was: 94°C (1 min) for melting, 58°C (1 min) for annealing, 72°C (1 min) for extension, 33 cycles, additional extension at 72°C for 10 min. Aliquots (20 μl) 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

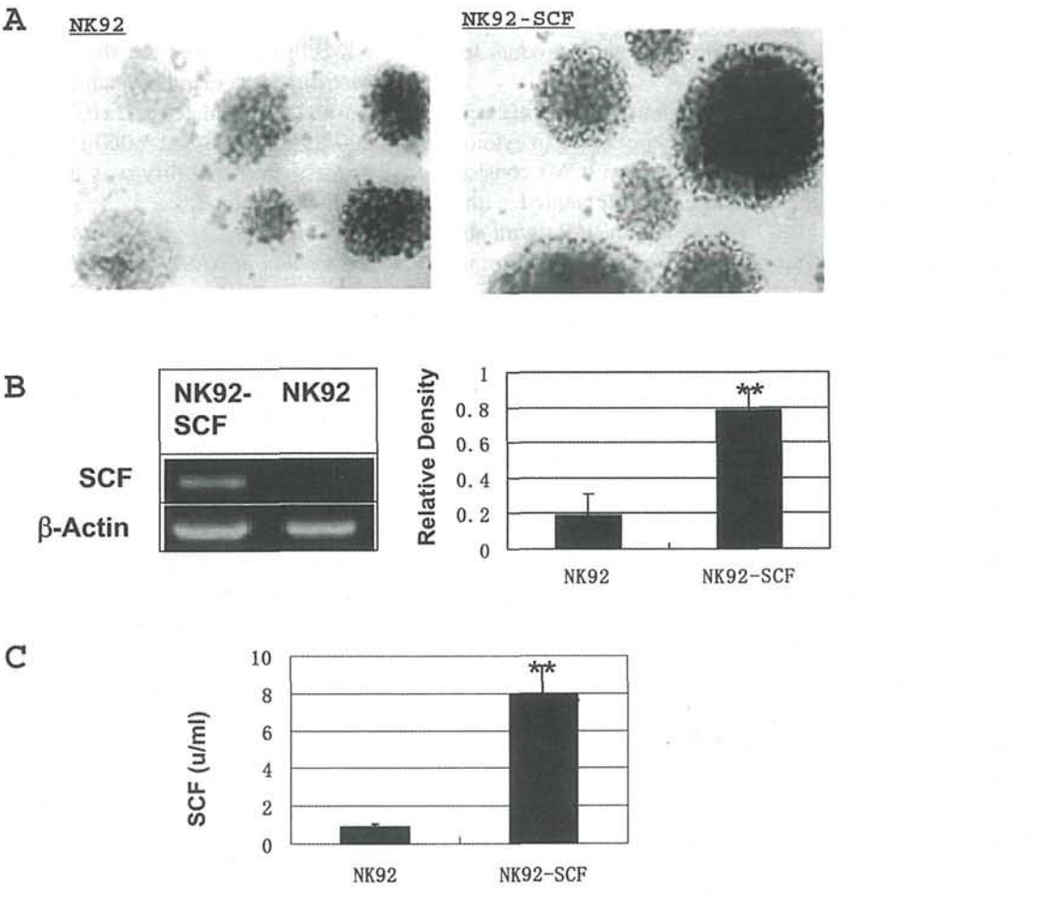

Figure 1. Establishment of SCF gene-modified NK-92 cell line. Total RNA was extracted from a hepatocarcinoma cell line (Hep G2B) and SCF cDNA was obtained using RT-PCR method. SCF cDNA was then inserted into a eukaryotic expression vector (pcDNA3), the recombinant vector (pcDNA3-SCF) was transferred into NK-92 cells through Lipofectamine (Gibco). The SCF gene-transferred NK-92 cells were then cloned, screened and identified under pressure of G418 concentration as described in Materials and methods. (A), Cell growth morphology under microscope. NK-92 cells transfected with hSCF gene (NK-92-hSCF) displayed a stronger tendency to form aggregates. (B), RT-PCR analysis to detect the SCF gene expression level. (C), SCF concentration in culture supernatant of NK92-SCF cells using SCF-dependent TF-1 cell proliferation assay.

measured using NIH Image Analysis Software Ver 1.61 (National Institutes of Health, Bethesda, MD). The intensities of the bands were determined with the use of the ratios to ß-actin.

Flow cytometry. 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 ($0.2x10^6$/0.1 ml) were then incubated on ice for 30 min with unlabeled primary mAbs (10 μg/ml). Cells incubated without Abs, or incubated with isotype-matched non-reactive Igs, were used as negative controls. The cells were then washed twice with PBS and incubated on ice for 30 min in the presence of appropriate biotinylated secondary Abs (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 FACScalibur (28).

Proliferation assay. NK-92 or NK92-SCF cells were cultured in triplicate at 37°C in a 5% CO_2 incubator in complete α-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 (FCS), 12.5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 24-well plates ($1x10^6$ cells/well) in the present of IL-2 or IL-15 (100 μ/ml for each cytokine). Culture medium was exchanged with 50% fresh medium every other day, and the cells were separated into 2 wells every 4 days. In order to actually analyze the proliferation capability of NK cells in response to cytokines, the cell numbers were calculated every day by MTT colorimetric analysis or under microscope, and the cumulative amount of cells was presented.

ELISA assays. The supernatant of NK cell culture was collected at 12 h and was then examined by ELISA method to detect soluble cytokine concentration. The supernatants of NK cell culture were centrifuged for 10 min at 6,000 g, and stored at -70°C for ELISA assays. Following the protocol of the kits,

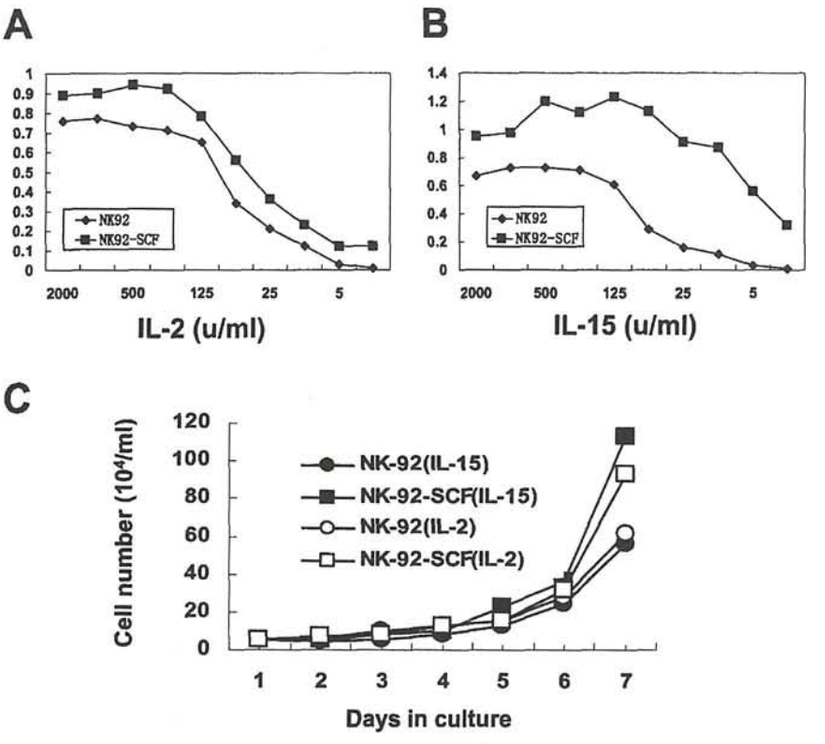

Figure 2. Proliferation of NK92-SCF in response to IL-2 or IL-15 stimulation NK-92 or NK92-SCF cells were cultured in triplicate at 37°C in a 5% CO_2 incubator in complete α-MEM medium containing IL-2 (100 μ/ml). Culture medium was exchanged with 50% fresh medium and regulated to the same concentration of IL-2 as at start of every other day, and the cells were separated into 2-well every 4 days. In order to actually analyze the proliferation capability of NK cells in response to cytokines, the cell numbers were calculated every day and the cumulative amount of cells is presented. (A and B), Dose response of NK-92 or NK92-SCF to IL-2 or IL-15 after 48-h culture, respectively, using MTT colormetric analysis. NK92-SCF cells proliferated more rapidly than their parent NK-92 cells, regardless of the presence of IL-2 or IL-15. (C), Cumulative cell amount in a long-term culture. NK92-SCF cells were more efficient in long-term culture in the presence of IL-2 or IL-15 than NK-92 cells, and more sensitive to IL-15 than IL-2.

the cytokines IFN-γ (detection limit, 50 pg/ml), IL-4 (detection limit, 8 pg/ml), IL-10 (detection limit, 6.35 pg/ml) and IL-13 (detection limit 10 pg/ml) were checked by ELISA kits (R&D Systems). The data were analyzed with software Origin Pro7.0.

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

Results

Establishment of stem cell factor (SCF) gene-modified NK-92 cell line. Since SCF can improve differentiation and proliferation of NK cells by up-regulating the IL-2Rßc expression of NK progenitor cells, and NK-92 cells are typical immature NK cells with a standard phenotype of $CD56^{bright}CD16^-$, we tried to transfer the SCF gene into NK-92 cells in order to improve the further differentiation and more rapid proliferation of NK-92 cells. As shown in Fig. 1A, the morphological manifestation under microscope was totally different in parent NK-92 cells and SCF-gene modified NK-92 cells (NK92-SCF), the cell aggregator-forming potential became stronger in NK92-SCF cells. The explanation is that the cell growth became faster in forming aggregators. NK92-SCF cell expressed significantly much more SCF transcripts (4.35±0.54-fold) than parent NK-92 cells using RT-PCR assay (Fig. 1B), leading to significantly more production of SCF protein in culture supernatant tested by ELISA method (7.67±0.89-fold) (Fig. 1C). NK92-SCF cell line was continuously cultured *in vitro* for more than 6 months, and the features of morphology and SCF production were not changed (data not shown). These results indicated that the SCF-gene modified NK-92 cells had been established by us.

Proliferation and cytotoxicity of the SCF gene-modified NK-92 cell line. In order to examine the proliferating potential, the NK92-SCF cells were cultured in the presence of different doses of IL-2 (Fig. 2A) or IL-15 (Fig. 2B) for 48 h. The cells were then assayed by MTT colormetric analysis. As shown in Fig. 2A and B, the NK92-SCF cells proliferated significantly more rapidly than parent NK-92 cells in each dosage of cytokine, the reason of more rapid proliferation is possibly the up-regulated expression of IL-2Rßc, which improved the utilization of the soluble growth factor, such as IL-2 or IL-15 as described before (21,22). We then cultured these cells ($1x10^4$/ml in the beginning) for 7 days (long-term) by changing 50% of the culture with fresh medium containing cytokine to keep the cytokine concentration at 100 μ/ml. As shown in Fig. 2C, the cumulative cell number, regardless of using IL-2 or IL-15 as the activator, was significantly greater in NK92-SCF cells after 5 to 7-days culture (5 day, $p<0.05$; 6 day,

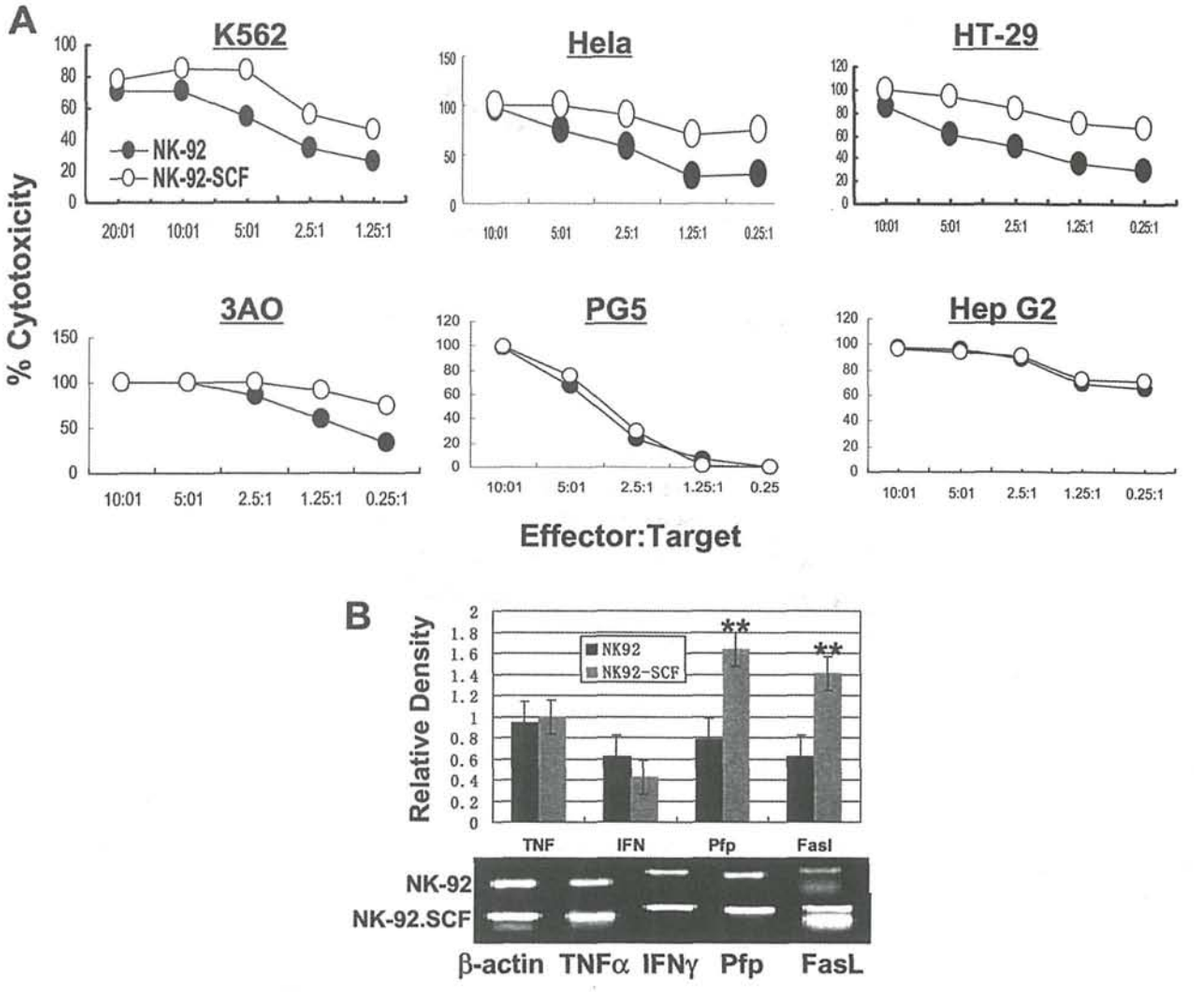

Figure 3. Cytotoxicity against target tumor cells of NK92-SCF cells. Assays were performed using NK-92 cells or SCF gene transferred NK-92 (NK92-SCF) cells that had been co-cultured earlier in the presence IL-2 (100 μ/ml). (A), The NK cells were mixed with ^{51}Cr-labeled K562 target cells (or any other target cells as indicated in the experiment) for NK cytotoxicity at an effector (E) to target (T) ratio 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 gamma counter. The % specific lysis was calculated as followed: % specific lysis = CPMexp - CPMspontaneous/CPMmaximum - CPMspontaneous x 100%. NK92-SCF cells exerted stronger cytotoxicity against K562, HeLa, HT-29 and 3AO cells than NK-92 cells. (B), Total RNA was extracted from the cultured NK-92 or NK92-SCF cells and transcripts of TNFα, IFNγ, perforin (Pfp) and Fas ligand (Fas L) were assayed by RT-PCR. NK92-SCF cells transcripted more mRNA of Pfp and Fas L than NK-92 cells.

$p<0.01$; 7 day, $p<0.001$). The precise mechanisms by which NK92-SCF cell proliferated more rapidly in short- or long-term culture remain elusive and need further investigation.

Next, we observed the cytotoxic ability of NK92-SCF cells. As shown in Fig. 3A, NK92-SCF cells exerted significantly greater cytotoxicity against a variety of target tumor cells including leukemia (such as, K562) and solid tumor (such as, HeLa and HT29), the difference between NK92-SCF and parent NK-92 cells was greater when the effector/target ratio was lower. These results suggest that SCF-gene modification causes the NK-92 cells to become potentially more cytotoxic, the change of this cell function is at least partly through up-regulating the gene expression and thereafter protein production of cytotoxic effector molecules including perforin and Fas ligand (Fig. 3B), but not through TNFα and IFNγ. There was no difference between the NK-92 cells when target cells were PG5 and Hep2, the reason for these differences remain unclear.

Phenotypic analysis of SCF gene-modified NK-92 cells. After examination of the function of NK92-SCF cells, we began testing the phenotype of the SCF gene-modified cell line. Both T cell (CD3, CD4, CD8) and B cell markers (CD19 and sIgM) were negative, same as their parent NK-92 cells (data not shown). CD16, an important marker for mature NK cells was still negative (Fig. 4A vs. B; and C), but CD56 expression was slightly changed (Fig. 4A vs. B; and D). Both $CD56^{bright}$ subset, a marker of immature NK cells, and $CD56^{dim}$ subset, a marker of mature NK cells, increased in NK92-SCF cell line, suggesting that SCF gene modification induces in parent NK-92 cells further differentiation, which has been verified previously by other laboratories (21,22).

In addition to NK phenotypic marker, we also examined the other important surface functional molecules. As shown in Table II, CD25, a specific receptor chain for IL-2 (IL-2Rα), and CD122, a common IL-2R ß chain (IL-2Rßc) were slightly and markedly increased, respectively, which explains the

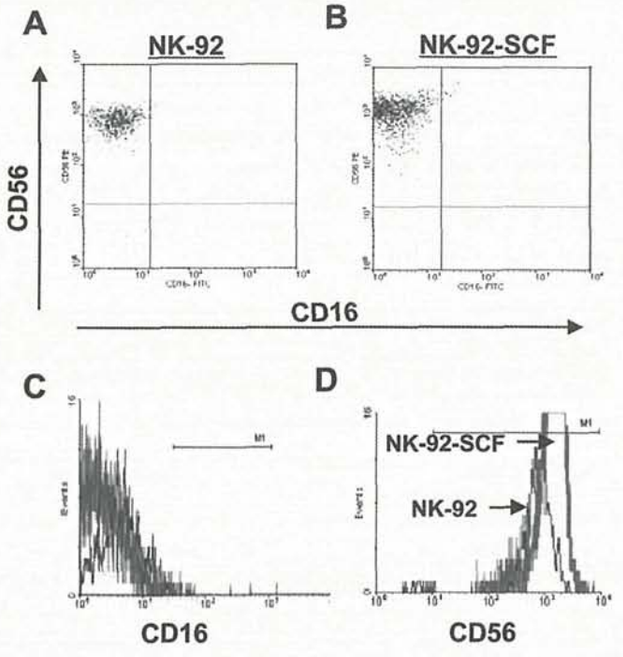

Figure 4. Surface NK marker expression of NK92-SCF cells. (A and B), Double staining of NK-92 and NK92-SCF cells with CD16 and CD56. NK92-SCF cells became more heterogeneous, e.g. new $CD56^{High}$ and $CD56^{Low}$ subsets appeared. (C and D), Histogram analysis of NK-92 or NK92-SCF cells by CD16 and CD56, respectively. CD16 expression was still negative, and the intensity of CD56 expression was increased.

Table II. Surface phenotype of SCF gene-modified NK-92 cells.

	NK-92		NK92-SCF	
	%	MFI	%	MFI
CD56	96.78	794.91	98.55	1409.1
CD16	1.83	27.23	1.45	36.2
CD25	51.66	16.36	67.67	21.00
CD122	37.89	33.21	77.56	54.12
CD48	96.83	403.21	93.82	300.54
CD69	18.30	12.63	14.88	13.72
NKG2D	93.56	223.45	96.78	334.56
NKG2A	33.25	24.34	26.78	21.22
CD94	56.27	78.91	47.89	69.87
CD54	84.78	79.67	100.00	116.40
CD95	93.81	45.28	93.66	34.37

Expression of cell-surface antigen was determined by direct or indirect immunofluorescence staining with the corresponding mAb and flow cytometric analysis described in Materials and methods. %, percentage of positive cells; MFI, mean fluorescence intensity.

Table III. Characterization of NK92-SCF cells compared with NK-92.

	NK92-SCF compared with NK-92
Proliferation	
IL-2Rα (CD25)	Slightly increased
IL-2Rß (CD122)	Markedly increased
To low dose IL-2	Slightly increased
To low dose IL-15	Markedly increased
Adherence	
Morphology	Bigger aggregator
CD54 expression	Increased
Cytotoxicity	
Lysis against K562 at low E:T	Increased
Lysis against solid tumor	Increased
Perforin mRNA	Increased
Fas L mRNA	Increased
TNFα mRNA	No change
NK phenotype	
CD56	Increased in density
CD16	No change
Cytokine production	
IL-4 mRNA	No change
IFNγ mRNA	No change
Activation marker	
CD69	No change
CD48	No change
NK receptors	
CD94	No change
NKG2A	No change
NKG2D	No change

Expression of cell-surface antigen (all CD molecules) was determined by direct or indirect immunofluorescence staining with the corresponding mAb and flow cytometric analysis, and mRNA level was examined by RT-PCR, as described in Materials and methods.

improved proliferation in the presence of IL-2 or IL-15. CD54, an important adherent molecule was also markedly up-regulated, explaining the cell aggregation. All other tested molecules including activating marker (CD69, CD48), death marker (CD95) and NK receptor (CD94, NKG2A and NKG2D) were not significantly changed in NK92-SCF cells.

Discussion

Summary of and possible mechanisms underlying SCF gene-modified NK-92 cells. As outlined in Table III, the characterization of SCF gene-modified NK-92 cells can be divided into seven features for description. The potentials of proliferation and cytotoxicity were improved after SCF gene

transfection into NK-92 cells (Table III), which at least partly resulted from NK-92 cells further differentiation (increased $CD56^{bright}$ cell subset for great proliferation and increased $CD56^{dim}$ cell subset for strong cytotoxicity) and IL-2 receptor (CD25 and CD122) up-expression for maximal utilization of IL-2 or IL-15 after SCF gene expression.

Human NK cell development can be briefly divided into two phases (29-32). In the early phase, NK progenitor cells ($CD34^+Lin^-$) respond to early activating growth factors (e.g. FL or KL) and develop into an NK cell precursor with the basic phenotype $CD34^+IL\text{-}2R\beta^+$. SCF increased the frequency of NK-cell precursors through up-regulation of expression of the IL-15Rα and IL-2Rß complex (24,25). IL-15 induces the further differentiation into mature NK cells that are CD56 positive in periphery. $CD56^{bright}$ NK cells represent a minor subset (approximately 10%) of human NK cells that are low or negative for CD16 and are capable of high proliferation and lower cytotoxicity. Most importantly, $CD56^{bright}$ cells keep their response to SCF stimulation through the surface SCF receptor, leading to high proliferation and further differentiation. The majority (approximately 90%) of human NK cells in the periphery is $CD56^{dim}$, $CD16^{High}$, highly cytotoxic and low proliferative. $CD56^{dim}$ NK cells might further derive from $CD56^{bright}$ cells or have a unique precursor under the control of other factors or cell contact signals (29-32). Thus, NK92-SCF cells may proliferate more rapidly and further differentiate into a $CD56^{dim}$ subset to exert strong cytotoxicity.

Historically, IL-2 was regarded as the natural activator for NK cells. Recently, it was discovered that IL-2 gene was not expressed at BM, and IL-2 was produced primarily by antigen-activated T cells located in the periphery. In addition, mice deficient in IL-2 contain functional NK cells, whereas mice and humans that lack the c subunit of the IL-2R or IL-2/15R lack NK cells (33,34). 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. The 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 (HPCs) (35). So, IL-15 is recognized as a natural physiological regulator of NK cells (36-39). The primary protein and cDNA sequences of human IL-15 showed little homology to IL-2, but IL-15 used the IL-2R subunit, common ß and γ chains (IL-2Rßcγc) (40,41). Because IL-15 is a natural physiological regulator in earlier stage 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-SCF, which was characterized by immature NK markers. The results demonstrate that NK92-SCF cells proliferated better in the presence of IL-15 than IL-2 (Fig. 2A vs. B). The NK92-SCF cells also became a bigger aggregator in culture, with increased expression of adherent molecules CD54 (Fig. 1A). Though it is difficult to explain the phenomenon, it seems that the change in growth manner benefits faster cell growth, which possibly is related to the cell growth regulatory function of SCF.

Implication of SCF gene-modified NK-92 cells in adoptive cellular immunotherapy. Natural killer (NK) cells are special lymphocytes that are able to lyse target cells without previous sensitization and without restriction by MHC antigens. Since the important roles of NK cells in anti-tumor and anti-virus processes, NK cells have been recognized as the best cellular therapeutic reagent (42). Several cellular therapeutic regimes have been practiced in clinic, including lymphokine-activated killer cells (LAK cells), cytokine-induced killer cells (CIK cells) and adherent NK cells (A-NK cells) (43-46). Since human NK cells comprise only about 10-15% of all circulating lymphocytes, the establishment of culture models to harvest activated and purified human NK cells on a large scale for adoptive cellular immunotherapy has been undertaken. However, the isolation of NK cells free of contaminating other lymphocytes has proven to be technically difficult. The establishment of NK cell lines provided favorable tools for this purpose. Currently, six malignant NK cell lines have been established and sufficiently well characterized: YT, NK-92, NKL, HANK-1, KHYG-1, NK-YS. Their immunophenotype is remarkably similar and described as follows: $CD1^-CD2^+CD3^-CD4^-CD5^-CD7^+CD8^-CD16^-CD56^+CD57^-$. These NK cell lines showed natural cytotoxicity, among which NK-92 cell line generated substantial basic data for further utilization.

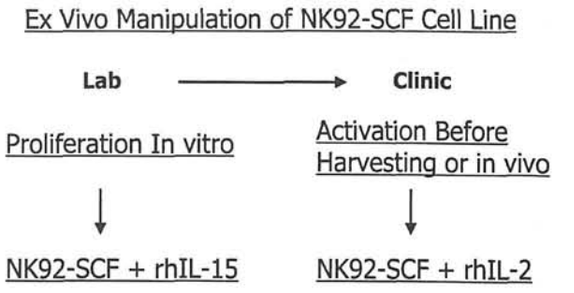

Figure 5. Schematic diagram for *ex vivo* manipulation of NK92-SCF cells for adoptive cellular immunotherapy. NK92-SCF cells are expanded for long-term culture in the presence of IL-15 in order to maximally harvest cells on a large scale for adoptive transfer. When enough cells are obtained, IL-2 may be added *in vitro* or injected *in vivo* in order to maximally activate the cell before or immediately after adoptive transfer into patients. The cells are then irradiated and transferred into patients.

NK-92 cell line was derived from a patient with non-Hodgkin's lymphoma in 1994. NK-92 has a $CD2^+CD3^-CD4^-CD8^-CD16^-CD56^{bright}$ phenotype and is absolutely dependent on the presence of IL-2 (or IL-15, unpublished data from our laboratory) for cytotoxicity and proliferation. NK-92 has a high cytolytic activity and is effective against a broad range of malignant targets *in vitro* and *in vivo*. Therefore, NK-92 has generated great interest both for its potential as an adoptive immunotherapy agent against cancer, as well as a tool to elucidate different aspects of NK cell biology, particularly the mechanisms of target recognition and killing. The absolute dependence on IL-2 stimulation and limited proliferating potential are major obstacles for the large scale production of the NK-92 cells for clinical utilization. Two laboratories were successful in establishing the IL-2 gene-transferred, IL-2-independent NK-92 cell line, in order to replace the exogenous addition of IL-2 in the culture system (22,23). IL-2-gene-transferred NK-92 cells kept all features

of their parent NK-92 cells; the only improvement is independent of IL-2 or IL-15. IL-2 gene modified NK-92 cells did not exert any improvement in proliferation and cytotoxicity when compared with their parent NK-92 cell in the presence of enough cytokine (IL-2 or IL-15). In this study, we transferred SCF gene into NK-92 cells, finding that SCF may improve NK cell proliferation in response to IL-2 or IL-15 stimulation by up-regulating the expression of IL-2 receptor system (especially IL-2Rα and IL-2Rßc). Our data demonstrated that SCF gene-modified NK-92 cells proliferated more rapidly in response to IL-15 than IL-2, which may be explained by the fact that immature NK cells are more sensitive to combined stimulations with SCF and IL-15 than SCF and IL-2. Because NK-92 cells are kind of immature NK cells based on their phenotype ($CD56^{bright}$ $CD16^{-}$), and SCF plus IL-15 are possibly natural combinations in NK cell differentiation, so, the NK92-SCF cells have stronger potential in proliferation in the presence of IL-15 (Fig. 2). We also found that IL-2 exerted greater effects on cytotoxicity of NK92-SCF cells than IL-15 (data not shown), which was observed by other laboratories (47), indicating IL-2 plays an important role in terminal activation for cytotoxic maturation.

As shown in Fig. 5, NK92-SCF cells may be expanded for long-term culture in the presence of IL-15 in order to maximally harvest cells on a large scale for adoptive transfer. When enough cells are obtained, IL-2 may be added *in vitro* or injected *in vivo* in order to maximally activate the cells before or immediately after adoptive transfer into patients. Thus, it will be possible to more efficiently manipulate the *ex vivo* protocol of NK-92 cells for clinical utilization.

Acknowledgements

This study was supported partly by Outstanding Young Scientist Award (#30125038) and Key Project (#30230340) by Natural Science Foundation of China, Chinese Key Basic Science Program by Ministry of Science and Technology of China (#2001CB510009), Chinese Hi-Tech Project (863 Plan) (2002AA216151) and Chinese Academy of Science (#KSCX2-2-08). The authors gratefully acknowledge the kindly reading by Dr Bin Gao from NIH and expert technical assistance provided by Mrs. Jinbo Feng and Xiaoqun Xu and post-graduates Anyuan Sun in the laboratory.

References

1. Maraninchi D, Vey N, Viens P, *et al*: A phase II study of interleukin-2 in 49 patients with relapsed or refractory acute leukemia. Leuk Lymphoma 31: 343-349, 1998.
2. Chang E and Rosenberg SA: Patients with melanoma metastases at cutaneous and subcutaneous sites are highly susceptible to interleukin-2-based therapy. J Immunother 24: 88-90, 2001.
3. Mulatero CW, Penson RT, Papamichael D, Gower NH, Evans M and Rudd RM: A phase II study of combined intravenous and subcutaneous interleukin-2 in malignant pleural mesothelioma. Lung Cancer 31: 67-72, 2001.
4. Benyunes MC, Massumoto C, York A, *et al*: Interleukin-2 with or without lymphokine-activated killer cells as consolidative immunotherapy after autologous bone marrow transplantation for acute myelogenous leukemia. Bone Marrow Transplant 12: 159-163, 1993.
5. Hayes RL, Koslow M, Hiesiger EM, *et al*: Improved long-term survival after intracavitary interleukin-2 and lymphokine-activated killer cells for adults with recurrent malignant glioma. Cancer 76: 840-852, 1995.
6. Rosenberg SA, Lotz MT, Yang JC, *et al*: Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst 85: 622-632, 1993.
7. Margolin KA: Interleukin-2 in the treatment of renal cancer. Semin Oncol 27: 194-203, 2000.
8. Semino C, Martini L, Queirolo P, *et al*: Adoptive immunotherapy of advanced solid tumors: an eight year clinical experience. Anticancer Res 19: 5645-5649, 1999.
9. Burns LJ, Weisdorf DJ, Defor TE, Repka TL, Ogle KM, Hummer C and Miller JS: Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer. Exp Hematol 28: 96-103, 2000.
10. Drexler HG and Matsuo Y: Malignant hematopoietic cell lines: *in vitro* models for the study of natural killer cell leukemia-lymphoma. Leukemia 14: 777-782, 2000.
11. Gong JH, Maki G and Klingemann HG: Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia 8: 652-658, 1994.
12. Maki G: *Ex vivo* purging of stem cell autografts using cytotoxic cells. J Hematother Stem Cell Res 10: 545-551, 2001.
13. Klingemann HG, Wong E and Maki G: A cytotoxic NK-cell line (NK-92) for *ex vivo* purging of leukemia from blood. Biol Blood Marrow Transplant 2: 68-75, 1996.
14. Klingemann HG and Miyagawa B: Purging of malignant cells from blood after short *ex vivo* incubation with NK-92 cells. Blood 87: 4913-4914, 1999.
15. Yan Y, Steinherz P, Klingemann HG, Dennig D, Childs BH, McGuirk J and O'Reilly RJ: Antileukemia activity of a natural killer cell line against human leukemias. Clin Cancer Res 4: 2859-2868, 1998.
16. Tam YK, Miyagawa B, Ho VC and Klingemann HG: Immunotherapy of malignant melanoma in a SCID mouse model using the highly cytotoxic natural killer cell line NK-92. J Hematother 8: 281-290, 1999.
17. Tonn T, Becker S, Esser R, Schwabe D and Seifried E: Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematother Stem Cell Res 10: 535-544, 2001.
18. Maki G, Klingemann HG, Martinson JA and Tam YK: Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J Hematother Stem Cell Res 10: 369-383, 2001.
19. Tam YK, Martinson JA, Doligosa K and Klingemann HG: *Ex vivo* expansion of the highly cytotoxic human natural killer-92 cell-line under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. Cytotherapy 5: 259-272, 2003.
20. Reid GS, Bharya S, Klingemann HG and Schultz KR: Differential killing of pre-B acute lymphoblastic leukaemia cells by activated NK cells and the NK-92 ci cell line. Clin Exp Immunol 129: 265-271, 2002.
21. Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann HG and Wels W: Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood 100: 1265-1273, 2002.
22. Tam YK, Maki G, Miyagawa B, Hennemann B, Tonn T and Klingemann HG: Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. Hum Gene Ther 10: 1359-1373, 1999.
23. Nagashima S, Mailliard R, Kashii Y, Reichert TE, Herberman RB, Robbins P and Whiteside TL: Stable transduction of the interleukin-2 gene into human natural killer cell lines and their phenotypic and functional characterization *in vitro* and *in vivo*. Blood 91: 3850-3861, 1998.
24. Colucci F and Di Santo JP: The receptor tyrosine kinase c-kit provides a critical signal for survival, expansion, and maturation of mouse natural killer cells. Blood 95: 984-991, 2000.
25. Fehniger TA, Carson WE, Mrozek E and Caligiuri MA: Stem cell factor enhances interleukin-2-mediated expansion of murine natural killer cells *in vivo*. Blood 90: 3647-3653, 1997.
26. Sun R, Wei H, Zhang J, Li A, Zhang W and Tian Z: Recombinant human prolactin improves anti-tumor effects of murine NK cells *in vitro* and *in vivo*. Neuroimmunomodulation 10: 169-176, 2002.
27. Tian Z, Shen X, Feng H and Gao B: IL-1 beta attenuates IFN-alpha beta-induced antiviral activity and STAT1 activation in the liver: involvement of proteasome-dependent pathway. J Immunol 165: 3959-3965, 2000.

28. Tian ZG, Woody MA, Sun R, *et al*: Recombinant human growth hormone promotes hematopoietic reconstitution after syngeneic bone marrow transplantation in mice. Stem Cells 16: 193-199, 1998.
29. Carayol G, Robin C, Bourhis JH, Bennaceur-Griscelli A, Chouaib S, Coulombel L and Caignard A: NK cells differentiated from bone marrow, cord blood and peripheral blood stem cells exhibit similar phenotype and functions. Eur J Immunol 28: 1991-2002, 1998.
30. Kalberer CP, Siegler U and Wodnar-Filipowicz A: Human NK cell development in NOD/SCID mice engrafted with cord blood CD34$^+$ cells. Blood 102: 127-135, 2003.
31. Carson WE, Fehniger TA and Caligiuri MA: CD56^{bright} natural killer cell subsets: characterization of distinct functional responses to interleukin-2 and the c-kit ligand. Eur J Immunol 27: 354-360, 1997.
32. Aiba Y, Hirayama F and Ogawa M: Clonal proliferation and cytokine requirement of murine progenitors for natural killer cells. Blood 89: 4005-4012, 1997.
33. Gilmour KC, Fujii H, Cranston T, Davies EG, Kinnon C and Gaspar HB: Defective expression of the interleukin-2/interleukin-15 receptor beta subunit leads to a natural killer cell-deficient form of severe combined immunodeficiency. Blood 98: 877-879, 2001.
34. Prlic M, Blazar BR, Farrar MA and Jameson SC: *In vivo* survival and homeostatic proliferation of natural killer cells. J Exp Med 197: 967-976, 2003.
35. Grabstein KH, Eisenman J, Shanebeck K, *et al*: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science 264: 965-968, 1994.
36. Briard D, Brouty-Boye D, Azzarone B and Jasmin C: Fibroblasts from human spleen regulate NK cell differentiation from blood CD34(+) progenitors via cell surface IL-15. J Immunol 168: 4326-4332, 2002.
37. Yu H, Fehniger TA, Fuchshuber P, Thiel KS, Vivier E, Carson WE and Caligiuri MA: Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. Blood 92: 3647-3657, 1998.
38. Carson WE, Fehniger TA, Haldar S, *et al*: A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest 99: 937-943, 1997.
39. Cooper MA, Bush JE, Fehniger TA, *et al*: *In vivo* evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood 100: 3633-3638, 2002.
40. Carson WE, Giri JG, Lindemann MJ, *et al*: Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med 180: 1395-1403, 1994.
41. Giri JG, Ahdieh M, Eisenman J, *et al*: Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J 13: 2822-2830, 1994.
42. Smyth MJ, Hayakawa Y, Takeda K and Yagita H: New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer 2: 850-861, 2002.
43. Kobari M, Egawa S, Shibuya K, Sunamura M, Saitoh K and Matsuno S: Effect of intraportal adoptive immunotherapy on liver metastases after resection of pancreatic cancer. Br J Surg 87: 43-48, 2000.
44. Nagayama H, Takahashi S, Takahashi T, *et al*: IL-2/LAK therapy for refractory acute monoblastic leukemia relapsing after unrelated allogeneic bone marrow transplantation. Bone Marrow Transplant 23: 183-185, 1999.
45. Tomita Y, Katagiri A, Saito K, *et al*: Adoptive immunotherapy of patients with metastatic renal cell cancer using lymphokine-activated killer cells, interleukin-2 and cyclophosphamide: long-term results. Int J Urol 5: 16-21, 1998.
46. Kimura H and Yamaguchi Y: A phase III randomized study of interleukin-2 lymphokine-activated killer cell immunotherapy combined with chemotherapy or radiotherapy after curative or non-curative resection of primary lung carcinoma. Cancer 80: 42-49, 1997.
47. Lai YG, Gelfanov V, Gelfanova V, Kulik L, Chu CL, Jeng SW and Liao NS: IL-15 promotes survival but not effector function differentiation of CD8$^+$ TCRalphabeta$^+$ intestinal intraepithelial lymphocytes. J Immunol 163: 5843-5850, 1999.