A Cytokine Cocktail Augments the Efficacy of Adoptive NK-92 Cell Therapy Against Mouse Xenografts of Human Cancer

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Abstract. *Background/Aim: Peripheral blood mononuclear cells (PBMCs) activated with immobilized monoclonal antibody against cluster of differentiation 3 (CD3) secrete cytokines in their culture supernatant (termed ACD3S). We examined the antitumor efficacy of ACD3S-activated NK-92 cells in vitro and in vivo. Materials and Methods: Interleukin (IL) 2-depleted NK-92 cells were reactivated with ACD3S, analyzed for their phenotype and tested for cytotoxicity, and perforin and interferon γ (IFNγ) production. Severe combined immunodeficient (SCID) mice xenografted with human melanoma and breast cancer cells were treated with ACD3S-activated NK-92 cells and tumor growth was monitored. Results: Brief activation of IL2-depleted NK-92 cells with ACD3S fully restored their in vitro cytotoxicity towards tumor cells. ACD3S-activated NK-92 cells were phenotypically similar to standard NK-92 cells, but exhibited prolonged cytotoxicity and produced higher levels of IFNγ. When adoptively transferred to tumor-bearing SCID mice, these cells retarded the growth of melanoma and breast tumors. Conclusion: Stimulation of NK-92 cells with ACD3S may be useful in clinical cancer therapy, as an alternative method for ex vivo natural killer cell activation.*

Adoptive transfer of *ex vivo*-activated immune cells marks an important advance in cancer immunotherapy. Several therapeutic strategies harnessing the power of natural killer (NK) cells to target multiple malignancies have been designed. Although *ex vivo*-expanded and -activated autologous NK cells have been reported to improve clinical responses, they often cannot exert their full cytotoxic capacity *in vivo* (1) and are not consistently effective in patients with cancer (2). Using NK cell lines as a source of allogeneic NK cells is considered potentially beneficial, as they have been shown to maintain their cytolytic functions *in vivo* and can be expanded in culture in large quantities under conditions of good manufacturing practice (GMP). Consequently, seven established human NK cell lines have been developed to date (3). Among these, NK-92 cells have reportedly shown superior antitumor activity against a broad spectrum of tumor targets and received US Food and Drug Administration (FDA) approval for testing in patients with advanced carcinomas, being the only human NK cell line that has entered clinical trials (4-6). NK-92 cells possess specific phenotypic and functional characteristics: i) they are positive for cluster of differentiation 56 (CD56), CD2, CD7, C11a, CD45, CD54 and negative for CD3, CD4, CD8, CD14, CD16 and human leukocyte antigen (HLA)-DR; ii) they are highly cytotoxic and their cytolytic activity is perforin- and granzyme B-mediated; iii) they lack all currently known killer-cell immunoglobulin-like receptors (KIRs), except for KIR2DL4, which is only expressed at a low level; iv) they express NK-activating receptors (*e.g.* NKp30, NKp44 and NKG2D); and v) their *in vitro* growth and expansion are interleukin (IL) 2-dependent (7,8). Most importantly, the antitumor activity of NK-92 cells can be amplified using appropriate cytokine combinations or conditioned media collected upon stimulation of specific immune-cell subsets (9,10).

Human lymphocytes, *in vitro*-activated with immobilized monoclonal antibody (mAb) to CD3 for 3 days, secrete immunomodulating agents, such as IL1, IL2, IL6, IL7, IL12, granulocyte-macrophage colony-stimulating factor, interferon-

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gamma (IFNγ) and tumor necrosis factor alpha (TNFα) into their culture medium which can be collected in the supernatant after centrifugation. These supernatants were previously termed allogeneic CD3 supernatants (ACD3S), whereas supernatants produced under the same conditions but in the absence of anti-CD3 (termed ACS), were much poorer in cytokine content (11). Brief exposure to ACD3S was shown to activate tumor-reactive lymphocytes of patients with cancer (11), promote stable maturation of dendritic cells differentiated from $CD14^+$ peripheral blood monocytes (12), and improve NK and lymphokine-activated killer cell-mediated cytotoxicity (13).

In this study, we investigated an alternative way for *in vitro* activation of NK-92 cells, substituting IL2 for ACD3S. Moreover, in order to test whether ACD3S-activated NK-92 cells maintain their antitumor effectiveness against solid tumors *in vivo*, we assessed their efficacy in immunotherapeutic protocols in severe combined immunodeficient (SCID) mice inoculated with human melanoma or breast cancer cells.

Materials and Methods

Cell lines. The human non-Hodgkin's lymphoma NK-92 cell line (ATCC CRL-2407; LGC Standards GmbH, Wesel, Germany) was grown in alpha minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY, USA) with 2 mM L-glutamine, adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 12.5% horse serum and 12.5% fetal bovine serum (FBS; Gibco-BRL), 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.002 mM folic acid (all from Sigma Chemical Co., St Louis, MO, USA), 50 μg/ml garamycin (Merck/Schering-Plough Pharmaceuticals, North Wales, Pennsylvania, USA; referred to hereafter as α-MEM-med), and 200 IU/ml human recombinant IL2 (rIL2; Cetus Corp., Los Angeles, CA, USA). The human chronic myelogenous leukemia K562, Burkitt's lymphoma Daudi and melanoma FM3 cell lines were grown in RPMI-1640 (Glutamax; Gibco-BRL) supplemented with 10% FBS, 2-mercaptoethanol (50 μM), Hepes (10 mM) (Sigma Chemical Co.) and 50 μg/ml garamycin (RPMI-med). Human breast cancer MCF-7 was grown in Eagle's minimum essential medium (EMEM; Gibco-BRL) supplemented with 0.01 mg/ml human recombinant insulin (Humulin; Eli Lilly and Co., IN, USA) and 10% FBS (EMEM-med). All cell lines grew at up to a density of $1\text{-}2\times10^6$ cells/ml in 25 cm^2 culture flasks (Greiner Bioline, Germany), in a total volume of 10 ml, in a CO_2 incubator (37˚C; 5% CO_2 in humidified air atmosphere). Passages in fresh medium were performed every 2-3 days.

Cell isolation. Peripheral blood mononuclear cells (PBMCs), isolated from heparinized blood or buffy coats obtained from healthy blood donors by standard Ficoll-HistoPaque density-gradient centrifugation (14), were suspended in RPMI-med and adjusted to $1\text{-}2\times10^6$ cells/ml. Prior to blood collection, individuals gave their informed consent according to the regulations approved by the Alexandra Hospital and Laikon Hospital Institutional Review Boards, Athens, Greece. All the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Preparation of ACD3S. The mAb against human CD3 molecule (anti-CD3-ε; BD Biosciences, San Jose, CA, USA), was diluted at 1 μg/ml in phosphate-buffered saline (PBS). Ten milliliters of this solution was then added to 25 cm^2 flasks and the mAb was immobilized by incubating the flasks for 2 hours at 37˚C in a CO_2 incubator. After washing the excess anti-CD3 from the flask, 10 ml of PBMC suspension (1×10^6 cells/ml) was added and flasks were incubated for 3 days at 37˚C. The resulting supernatant (ACD3S) was harvested and centrifuged (10,000×g; 10 min). Complete® protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany) was added to eliminate the action of endogenous proteases. ACD3S from 10 donors was first tested for NK-92 cytotoxicity induction *versus* K562 targets (see Cytotoxicity assay below) and those inducing the highest K562 lysis were pooled and stored in aliquots at −80˚C. Allogeneic control supernatants (ACS) were likewise collected under the same conditions, but in the absence of immobilized anti-CD3.

Determination of cytokines in ACD3S. Cytokine concentrations of IL2, IL4, IL6, IL10, TNF, IFNγ and IL17A in ACD3S and ACS were measured by the BD cytometric bead array (CBA) human Th1/Th2/Th17 Cytokine Kit (BD Biosciences), according to the manufacturer's instructions. Analysis was performed in a FACSCanto II instrument, using FACSDiva Software (BD Biosciences). Data were further processed and cytokine concentrations were calculated using the FCAP Array v3.0 Software (BD Biosciences).

Activation of NK-92 cells with ACD3S. To minimize the high spontaneous cytotoxic activity of NK-92 cells prior to activation with ACD3S, a 3-day protocol of gradual IL2 depletion was performed by daily substituting half of the α-MEM-med with fresh medium without rIL2. Reactivation of the cytotoxicity of NK-92 cells was induced upon 24-96 h incubation in 25% ACD3S-supplemented α-MEM-med (cell density 1×10^6 cells/ml). Standard NK-92 cultures (200 IU/ml IL2) and cultures treated with 25% ACS were used as controls.

Cytotoxicity assay. The cytotoxic activity of NK-92 cells was determined using the standard ^{51}Cr-release assay performed as previously described (15). The tumor cells K562, Daudi, FM3 and MCF-7 were used as targets. NK-92 cells were used as effectors upon extensive washing to remove excess ACD3S. The effector:target (E:T) ratios are indicated in the figure legends. Cultures were set in triplicates and the percentage cytotoxicity was estimated according to the formula: (experimental ^{51}Cr release−spontaneous ^{51}Cr release)/(total ^{51}Cr release−spontaneous ^{51}Cr release).

Flow cytometric analysis of NK-92 cells. ACD3S-treated, ACS-treated and standard NK-92 cells were stained for CD2/fluorescein isothiocyanate (FITC), CD11a/allophycocyanin (APC), CD56/Alexa Fluor 700, CD57/FITC, IL2Rα (CD25)/APC, IL2Rβ (CD122)/ phycoerythrin (PE), NKG2D (CD314)/PE-cyanine 7 (Cy7) and NKp30 (CD337)/PE (all from BD Biosciences). Cells were stained for 30 min on ice, washed and analyzed in a FACSCanto II instrument, using FACSDiva software. Perforin and IFNγ intracellular staining were performed as previously described (15). In brief, for perforin analysis, cells were treated with 1 μM brefeldin A (Sigma Chemical Co.) for 4 h. For IFNγ production,

cells were treated with brefeldin A, 0.2 μg/ml phorbol 12-myristate 13-acetate and 0.5 μg/ml ionomycin (both from Sigma Chemical Co.). Cells were then fixed with 4% paraformaldehyde in the dark for 15-20 min at room temperature. Saponin (0.1% in PBS) was used to permeabilize cell membranes. Phycoerythrin-labeled mAbs specific for human perforin, human IFNγ and mouse IgG2b κ (isotype control) were used (BD Biosciences). Cells were immediately analyzed.

In vivo immunotherapeutic protocol. Female SCID mice (Harlan, Derbyshire, UK), 6-8 weeks of age, were purchased from the Hellenic Pasteur Institute Animal Facility (License Number: ELBIO013) and maintained under pathogen-free conditions, in accordance with Presidential Decree 56/2013 and the Directive 2010/63/EU of the Council of Europe on Animal Welfare. The study was approved (09/02/2012) by the Ethics and Biosafety Committee, Subcommittee on Ethics, Faculty of Biology, University of Athens and experiments were conducted following the guidelines of the committee.

FM3 (1×10^6/mouse) or MCF-7 (8×10^6/mouse) cells were subcutaneously (*s.c.*) inoculated to the animals on day-0. For each cell line, mice were randomly assigned into four groups (7-10 animals/group): control, ACS, ACD3S and standard NK-92. On days 0, 4 and 11, mice were intraperitoneally (*i.p.*) administered PBS (0.5 ml), standard NK-92 cells or IL2-depleted NK-92 cells *in vitro* stimulated with ACS or ACD3S for 24 h (10×10^6 cells/mouse/0.5 ml PBS). Tumor growth was monitored every 3-4 days by measuring the two axes of the ellipse-like tumor formed and expressed as the product of the two perpendicular diameters of individual tumors (mm^2). Observation was terminated with euthanasia when the tumor mass reached ~250 mm^2 and overall survival was followed for a maximum of 80 days.

Statistical analysis. GraphPad prism v5 (GraphPad Software Inc., San Diego, CA, USA) was used. Data were analyzed by one-way ANOVA and statistical significance was presumed at a significance level of 5% ($p<0.05$). Tumor size was compared using the area-under-the-curve method, according to Duan *et al*. (16).

Results

ACD3S-activated NK-92 cells efficiently lyse various tumor targets. NK-92 cells lack KIR receptors and thus exhibit high cytolytic activity against a variety of tumor cells *in vitro* and *in vivo* (8). Although their cytotoxicity is IL2-dependent, it has been reported that depleting IL2 from their culture medium does not rapidly result in complete loss of their lytic ability (7, 17). Thus, to reduce high spontaneous NK-92 cell cytotoxicity prior to reactivation with ACD3S, we gradually depleted IL2 from α-MEM-med over 96 h (4 days). By determining NK-92 cell cytotoxicity *versus* the NK-sensitive K562 targets, we noticed that cells retained their lytic ability even at 48 h after IL2 depletion (Figure 1A, left vertical axis). However, at 72 h, lysis of K562 was greatly reduced compared to standard NK-92 cultures ($p<0.0001$), although viability was not considerably affected (Figure 1A, right vertical axis). NK-92 cell cytotoxicity was further reduced at 96 h (<10%), accompanied by very low cell viability

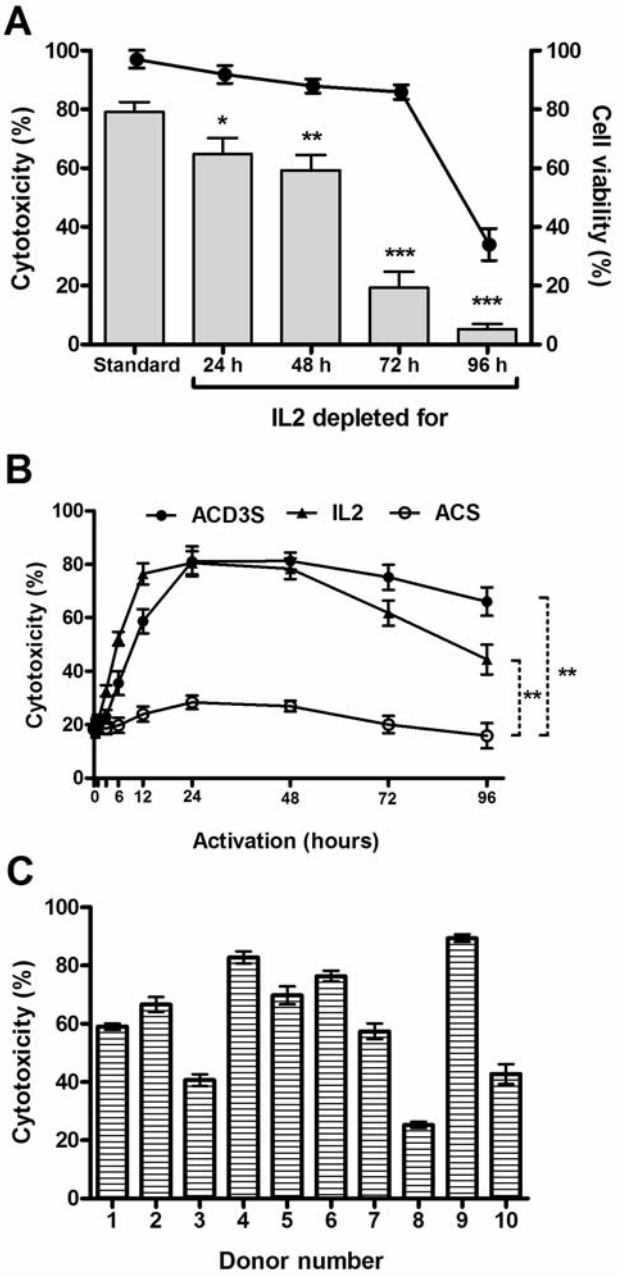

Figure 1. *Allogeneic CD3 supernatant (ACD3S) efficiently restores the cytotoxicity of interleukin (IL) 2-depleted NK-92 cells. A: NK-92 cells were gradually depleted of IL2 over 4 days, as described in the Materials and Methods section, and tested for cytotoxicity (columns) and viability (line) after 24 to 96 h. Standard NK-92 cells were cultured in the presence of 200 IU/ml IL2. B: The cytotoxic activity of NK-92 cells IL2-depleted for 72 h against K562 targets was restored after 24 h incubation with ACD3S and persisted up to 96 h. C: ACD3S preparations from normal donors (n=10) were screened for induction of NK-92 cytotoxicity. In all cases, the effector:target (E:T) ratio was 10:1. Data are mean values±SD from three independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared to standard NK-92 cells (A) and allogeneic control supernatants (ACS)-activated NK-92 cells (B).*

(<40%; Figure 1A). Therefore, for our subsequent experiments, we used NK-92 cells IL2-depleted for 72 h.

In a time-kinetic study, we determined the shortest period of efficient NK-92 cell reactivation by ACD3S. IL2-depleted NK-92 cells were incubated for 1-96 h with α-MEM-med supplemented with 25% ACD3S, 25% ACS or 200 IU/ml IL2, washed and tested for K562 target lysis (Figure 1B). In contrast to previously published results where PBMCs showed enhanced cytotoxicity as early as at 3 h post ACD3S treatment (11), NK-92 cells efficiently lysed K562 after 24 h stimulation with ACD3S (81.1%). NK-92 cell cytotoxicity was not further enhanced, whereas high K562 lysis was detected for up to 96 h post ACD3S stimulation (Figure 1B). Supplementation of α-MEM-med with IL2 also significantly enhanced NK-92 cell cytotoxicity in a shorter time period than ACD3S, but this was gradually reduced at 48 and 72 h, and was low at 96 h. No significant enhancement of K562 lysis over time was recorded in NK-92 cells treated with ACS. Finally, no further increase in NK-92 cytotoxicity was detected by supplementing the α-MEM-med with 50% or 75% ACD3S (data not shown).

The cytotoxicity assay of 24 h ACD3S-treated NK-92 cells *versus* K562 was used as readout to select the most stimulatory healthy donor-derived ACD3S. As shown in Figure 1C, variable levels of cytotoxicity (25.3% to 89.4%) were detected when NK-92 cells were activated with ACD3S preparations from 10 different healthy donors. To ensure the reproducibility of the results in subsequent experiments, we pooled and stored ACD3S that highly enhanced NK-92 cytotoxicity (donors 2, 4, 5, 6 and 9; Figure 1C). Additionally, using the CBA assay, we determined the cytokine content in individual ACD3S and ACS preparations. In agreement with previous reports, the concentration of all cytokines in ACD3S from different donors varied (Table I) (11-13). The concentration of IL17A, reported for the first time, was below the sensitivity levels of the assay in eight out of 10 ACS preparations and thus not determined, and was very low in ACD3S preparations compared to the concentrations of the other cytokines.

ACD3S-induced NK-92 cell killing was assessed at different E:T ratios against K562 (Figure 2A), Daudi (Figure 2B), as well as against human melanoma FM3 (Figure 2C) and breast cancer MCF-7 cells (Figure 2D). As shown, ACD3S-activated NK-92 cells demonstrated statistically significantly increased cytotoxicity against K562 and Daudi at all E:T ratios tested in comparison to ACS-treated NK-92 cells (in all cases, $p<0.05$). Furthermore, ACD3S-activated NK-92 cells demonstrated increased cytotoxic activity against FM3 (Figure 2C) and MCF-7 cells (Figure 2D), which was statistically significantly higher compared to ACS-treated NK-92 cell lysis (at most E:T ratios, $p<0.05$).

ACD3S activation of NK-92 cells increases IFNγ and perforin production. In order to test whether ACD3S activation induces phenotypic changes in NK-92 cells, we analyzed the expression of selected surface markers CD56, CD57, CD2, CD25 and CD122 (IL2Rα and β, respectively), the integrin CD11α, and the regulatory receptors NKG2D (CD314) and NKp30 (CD337). As shown in Table II, ACD3S up-regulated CD2 and NKp30 expression, but mean fluorescence intensity values were not statistically significantly higher compared to standard NK-92 cells ($p=0.584$ for CD2; $p=0.293$ for NKp30). No differences were noted in the expression of IL2Rα and β chains. ACS-treated cells in most cases presented lower expression levels of the same markers.

In order to reveal any functional changes, ACD3S-treated NK-92 cells were analyzed for intracellular perforin and IFNγ production. ACD3S-treatment induced increased perforin production in NK-92 cells, which was analogous to

Table I. *Concentrations of cytokines (ng/ml) in allogeneic cluster of differentiation 3 supernatants (ACD3S) and allogeneic control supernatants (ACS), as determined by cytometric bead array assay.*

Cytokine	ACS	ACD3S	Fold increase[#]
Interleukin 2	1.99±0.67	5.88±2.91	3.0
Interleukin 4	1.73±0.71	1.97±0.75	1.1
Interleukin 6	2.19±1.51	5.14±2.42	2.3
Interleukin 10	0.39±0.22	0.89±0.33	2.3
Tumor necrosis factor	1.96±0.91	3.56±0.87	1.8
Interferon γ	1.68±0.63	4.18±1.08	2.5
Interleukin 17A	ND	0.39±0.24	-

Data are means±SD from n=10 ACS and ACD3S preparations. [#]Mean fold increase of cytokine present in ACD3S relative to ACS. ND, Not determined.

Table II. *Flow cytometric analysis of surface and intracellular molecule expression in NK-92 cells cultured with interleukin 2 (IL2; standard), or stimulated with allogeneic cluster of differentiation 3 supernatants (ACD3S) or allogeneic control supernatants (ACS).*

Marker	Standard	ACS-stimulated	ACD3S-stimulated
CD11a	515±45	508±33	498±35
CD2	431±40	310±41	455±35.4
CD56	579±60	452±22	485±31
CD57	603±44	560±74	576±88
CD25 (IL2Rα)	240±11	188±10	231±14
CD122 (IL2Rβ)	80±6.9	71±4.1	75±7.4
CD314 (NKG2D)	305±23	110±14	243±25
CD337 (NKp30)	390±14.1	257±15	411±15.6
Perforin[#]	302±12	190±18	276±25

Data are means±SD mean fluorescence intensity values from two separate experiments performed. [#]Intracellular detection (see Materials and Methods).

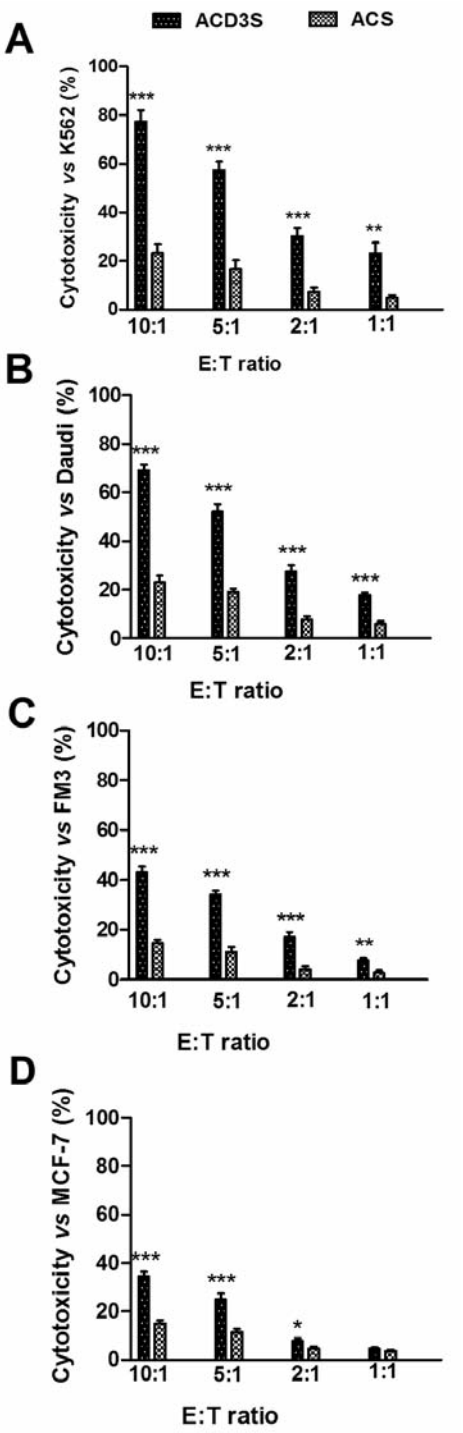

Figure 2. *Cytotoxicity of allogeneic CD3 supernatant (ACD3S)-activated NK-92 cells versus cancer cells. NK-92 cells were interleukin (IL) 2-depleted for 3 days, reactivated with ACD3S or allogeneic control supernatants (ACS) for 24 h, and used as effectors against K562 (A), Daudi (B), FM3 (C) and MCF-7 (D) cell lines, at the indicated effector:target (E:T) ratios. Co-incubation of effectors and targets was for 18 h. Data are mean values±SD from three independent experiments. *p<0.05, **p<0.01, and ***p<0.001 between ACD3S- and ACS-activated NK-92 cells.*

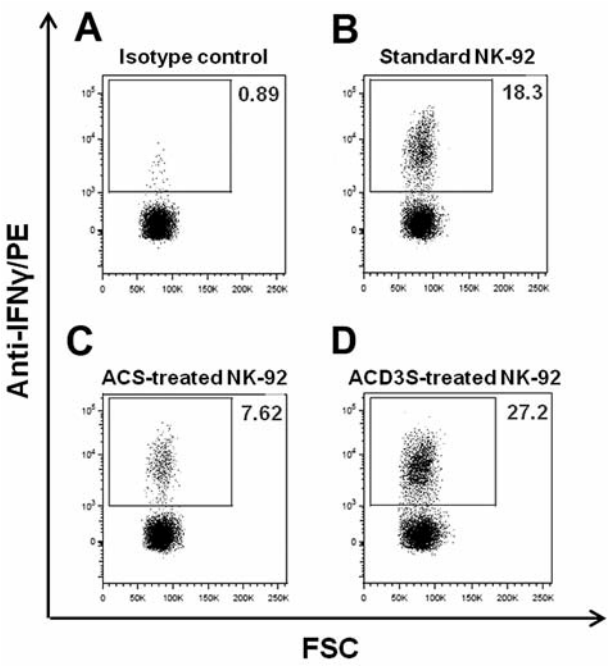

Figure 3. *Allogeneic CD3 supernatant (ACD3S)-activated NK-92 cells produce high levels of interferon γ (IFNγ). NK-92 cells grown with 200 IU/ml interleukin (IL) 2 (standard) (B), depleted of IL2 and reactivated with ACS (C) or ACD3S (D) for 24 h were intracellularly stained for IFNγ. The same cells stained with an isotype control are shown in (A). IFNγ-positive cells are gated and their percentage is shown on the dot plots. Representative dot plots from one out of two experiments performed with similar results are shown.*

that of standard NK-92 cells (Table II). Most importantly, the percentage of ACD3S-activated NK-92 cells producing IFNγ was increased compared to standard NK-92 cells (27.2% *versus* 18.3%) and this IFNγ up-regulation was statistically significant (p=0.022; Figure 3). NK-92 cells treated with ACS did not produce high levels of perforin nor of IFN-γ.

ACD3S-activated NK-92 cells retain their cytolytic ability in vivo and retard human melanoma and breast cancer cell growth. In order to evaluate the *in vivo* potency of ACD3S-activated NK-92 cells, we developed two adoptive immunotherapeutic protocols by inoculating human cancer cells in SCID mice, in which T-cells are deficient but NK cells are potentially active. SCID mice were *s.c.* injected with 1×10^6 FM3 or 8×10^6 MCF-7 cells. We selected these two specific numbers of cells in order to generate rapidly progressing models, where tumors would be palpable 12-16 days post inoculation. We administered three *i.p.* doses (10×10^6 cells/dose/mouse) of 24 h ACD3S-activated NK-92 cells on days 0, 4, and 11. As shown in Figure 4A (left column), tumor size in mice inoculated with FM3 cells and treated with PBS (control group) reached a maximum 44-48

days post inoculation. Melanoma tumors in mice administered ACS-activated NK-92 cells reached their maximum on days 48-52. Administration of ACD3S-activated NK-92 cells resulted in slower rates of tumor increase compared to controls, reaching maximum tumor size on days 52-72. A similar kinetics of FM3 tumor growth was recorded in mice treated with standard NK-92 cells, which were euthanized between days 56-72. Although some mice therapeutically treated with ACD3S-activated NK-92 cells experienced significant delay in melanoma tumor growth, the differences recorded between the four groups were not statistically significant (p=0.99).

Similarly, mice inoculated with MCF-7 cells and treated with ACD3S-activated NK-92 cells exhibited a slower rate of tumor increase compared to controls, reaching the maximum acceptable tumor growth on 56-68 days (Figure 4C, right column). Tumors in control mice reached the same size 48-56 days post inoculation, in mice administered ACS-activated NK-92 cells between 48-60 days, and in mice with adoptively-transferred standard NK-92 cells on days 60-72. Similar to the melanoma model, the difference in tumor growth between the four mouse groups was not statistically significant (p=0.96).

Discussion

NK cells are unique in anticancer immunity, as they recognize stressed cells in the absence of antibodies and major histocompatibility complex (MHC) molecules, allowing for a rapid immune effector response. However, NK cell paralysis caused by cancer, their short lifespan, the limited number of NK cells recovered from human blood and interdonor-related variations are major obstacles in NK cell use for cancer immunotherapy (3). In order to overcome these barriers, researchers turned to established human NK cell lines bearing several features of natural NK cells, but requiring cytokine supplementation for their growth. Moreover, stable allogeneic NK cell lines are reproducibly produced on a large scale under GMP conditions.

In this study, we treated NK-92 cells, that are highly cytotoxic towards a wide range of tumor cells *in vitro* and in xenografted SCID mice, with an alternative stimulation protocol to that using IL2. We activated NK-92 cells with ACD3S (11) and tested their efficacy against human tumor cells, both *in vitro* and *in vivo*. We selected NK-92 cells as it is the only human NK cell line approved by the US FDA for clinical studies. As shown in the phase I trials of Arai *et al*. (5) and Tonn *et al*. (6), NK-92 cells administered at doses up to $1\times10^{10}/m^2$ to patients with melanoma, advanced solid tumors or hematological malignancies, were well-tolerated and safe, and some encouraging responses were reported; currently, two phase I studies of NK-92 cell infusions in patients with hematological malignancies are ongoing (NCT00990717, NCT00900809). Furthermore, NK-92 cells acted beneficially as "biological purging agent" of peripheral blood stem cell grafts (17) and can be genetically engineered to express specific receptors for targeted tumor cell recognition (6, 18). Thus, determining the most favorable conditions for NK-92 cell activation for treating patients with cancer is still of clinical interest (3, 9).

In the aforementioned trials, NK-92 cells exhibited relatively short (48 h) persistence in patient circulation (6). To optimize *ex vivo* activation of NK-92 cells that might retain prolonged activity *in vivo*, we substituted IL2 with the naturally produced cytokine cocktail, ACD3S (11). Supernatants harvested from PBMC cultures, stimulated with anti-CD3, are reportedly rich in cytokines. Osband *et al*. cultured lymphocytes from patients with metastatic renal cell carcinoma for 5 days with anti-CD3-stimulated autologous cell supernatants and infused them back to the patients with concomitant administration of cimetidine; patients who received this treatment demonstrated a prolongation in survival (19). Baxevanis *et al*. reported that short-term incubation of lymphocytes from patients with cancer using ACD3S derived from anti-CD3 activated allogeneic healthy donor PBMCs resulted in non-MHC-restricted antitumor cytotoxicity against both autologous and allogeneic tumors and tumor cell lines (11). Interestingly, ACD3S from healthy donor PBMCs was richer in cytokines compared to ACD3S derived from PBMCs from patients with cancer, and thus was more effective in activating $CD56^+$ cells and, in long-term cultures, expanding $CD56^+CD3^+$ effectors (13). Moreover, similarly to other conditioned supernatants (*e.g*. monocyte-conditioned medium and T-cell-conditioned medium), ACD3S mediated the terminal maturation of human dendritic cells (12, 20, 21) and induced rapid expansion of $CD34^+$ progenitors (22), suggesting the pleiotropic activity of this preparation.

The stimulatory effect of ACD3S on NK-92 cells is due to its multicytokine content, and these cytokines, albeit at low concentrations, act in synergy to induce NK-92 cell cytotoxicity. Indeed, previous studies have indicated that several cytokines (IL2, IL15, IL12 and IFNα) enhance NK cell cytotoxicity (23-25); synergy between IL15 and IL2 or IL18 is known to activate $CD56^+$ effectors (26); IL2, IL15, IL12 and IFNα regulate the expression of granzymes specifically in NK-92 cells (27); IL21 enhances NK cell antitumor functions, particularly in combination with IL2 and IL12 (28), and although IL21 has not yet been detected in ACD3S, we could safely speculate that it is also present. In support of this synergistic cytokine effect and in agreement with previous reports (11, 13), the mean concentration of IL2 in our ACD3S preparations was 5.88 ng/ml, *i.e*. twofold lower than the recommended IL2 concentration supporting NK-92 cell propagation (200 IU/ml, corresponding to ~12 ng/ml). Considering 25% ACD3S supplementation of the culture medium, practically, NK-92 cells were stimulated with very low IL2 concentration (~27 IU/ml). Our ACD3S preparations also contained IL4, IL6, TNF and

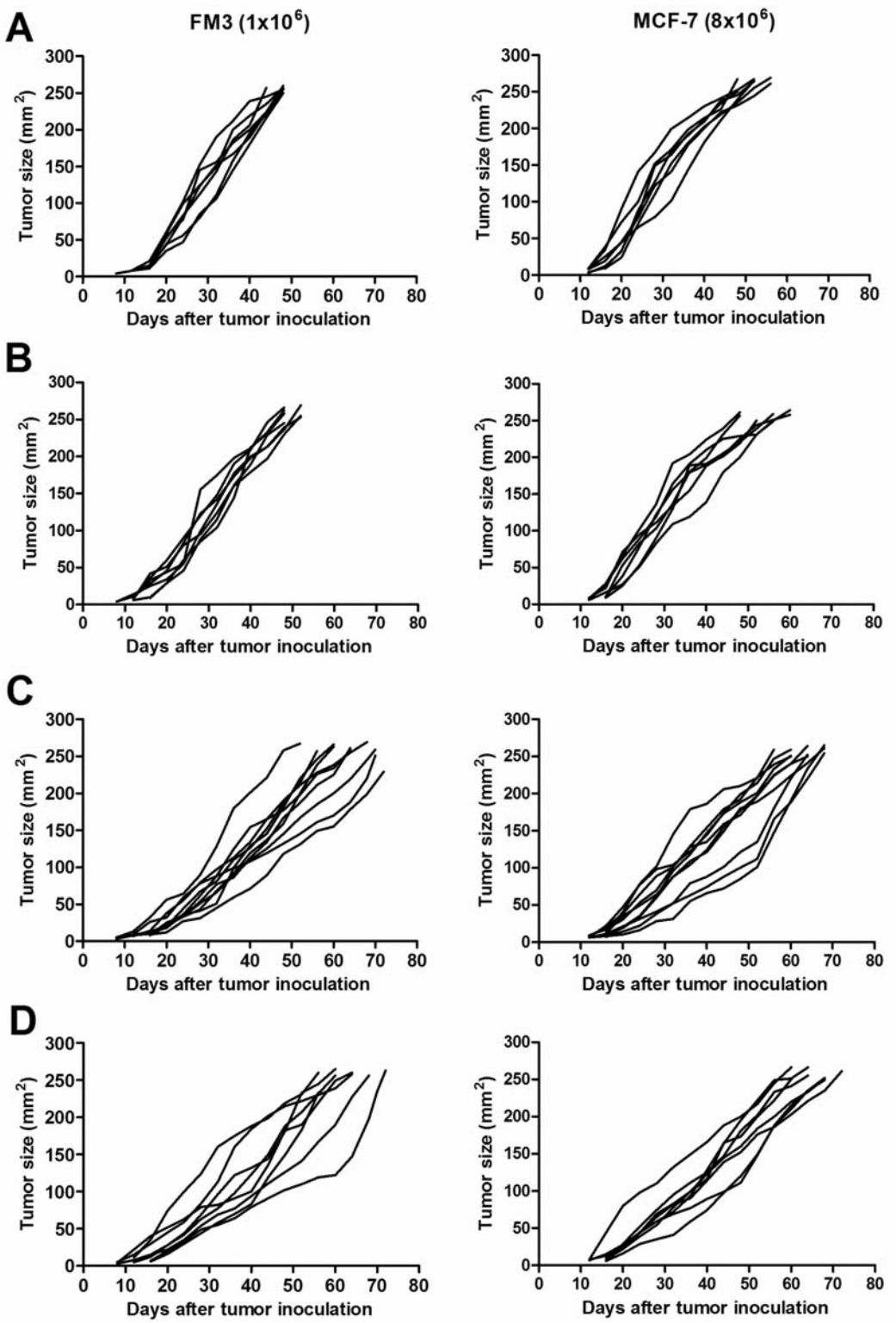

Figure 4. *Adoptive transfer of allogeneic CD3 supernatant (ACD3S)-activated NK-92 cells retards tumor growth in severe combined immunodeficient mice xenografted with human melanoma (FM3) or breast cancer cells (MCF-7). FM3 (1×10^6/mouse; left column) or MCF-7 (8×10^6/mouse; right column) were s.c. injected to the animals (n=7-10/group) on day 0. On days 0, 4 and 11,, mice were administered phosphate-buffered saline (A), 24-h ex vivo ACS- (B) or ACD3S-activated NK-92 cells (C), or standard NK-92 cells (D). Tumors were measured every 3-4 days and tumor growth was monitored until day 72 or until tumors reached ~250 mm^2. Growth of individual tumors is shown.*

IFNγ, at concentrations analogous to those previously reported (11, 13). Only IL10 levels were significantly higher [0.83 ng/ml, compared to 0.2 and 0.05 ng/ml (11, 13)], possibly due to the different detection method used. Nevertheless, IL10 concentrations of up to 1 ng/ml do not inhibit NK-92 cytotoxicity (29). Herein, we show that ACD3S additionally contains a very low concentration of IL17A, a cytokine reported to affect NK cell development (30), but having little or no impact on NK cell cytotoxicity (31).

In order to eliminate donor-related variability in the cytokine content of ACD3S, we used pooled supernatants from five healthy donors, thus ensuring the controlled expansion and stimulation of NK-92 cells. We further performed *in vitro* kinetics to determine the optimal incubation time for acquiring maximal NK-92 cell cytotoxicity. In contrast to the reported 3 h incubation of NK cells (11), we found that IL2-depleted NK-92 cells need to be activated for at least 24 h with ACD3S. This difference might be attributed to the different type of cells used [primary NK (11) *versus* an NK cell line (present report)], or to the IL2-depletion protocol we followed, which may delay NK-92 cell reactivation. Nevertheless, ACD3S-reactivated NK-92 cells adequately expanded in culture, were phenotypically similar to standard NK-92 cells, contained high perforin levels (Table II) and efficiently lysed various tumor targets (Figure 2). Most importantly, compared to IL2-reactivated NK-92 cells, ACD3S-treated cells retained their cytolytic activity for up to 96 h (Figure 1B) and, compared to standard NK-92 cells, a higher percentage thereof produced IFNγ (Figure 3). Both these observations are of crucial importance for the adoptive transfer of NK-92 cells, as ACD3S-activated effectors: i) will likely require less or even no exogenous cytokine supplementation *in vivo*; and ii) the IFNγ produced may support not only their *in vivo* activity, but it could also act beneficially on the host's overall immune competence (*e.g.* stimulation of endogenous NK cells). Although not tested herein, the possibility that ACD3S-activated NK-92 cells release more proinflammatory cytokines (*e.g.* TNFα, IL1β, IL6 and IL8) cannot be ruled out. In fact, in their recent most elegant report, Conlon *et al.* showed that NK cells highly activated with IL15 *in vivo* secreted a series of cytokines, including IFNγ, which resulted in visible clearance of tumor lesions in some patients (32).

In order to confirm that *ex vivo* ACD3S-activated NK-92 cells are effective under *in vivo* conditions, we adoptively transferred them to SCID mice, *s.c.* inoculated with the human tumor cell lines FM3 (melanoma) or MCF-7 (breast adenocarcinoma). We treated animals with 10×10^6 cells/mouse/dose administered in three *i.p.* doses on days 0, 4 and 11 post-tumor inoculation. The particular design of these protocols was based on the following rationale: i) the number of FM3 and MCF-7 cells inoculated (1×10^6 and 8×10^6, respectively) is reported to generate *s.c.* tumors within 12-16 days, according to previous studies (33-35); ii) the number of 10×10^6 NK-92 cells administered to mice was analogous to the maximum well-tolerated dose used in humans [$10^{10}/m^2$, (6)], as converted following FDA guidance (36); iii) as the cytotoxicity of ACD3S-activated NK-92 cells persists for more than 3 days, the intervals of administration were 4 and 7 days to allow NK-92 cells to exert their full activity *in vivo*; and iv) *i.p.* administration of NK-92 cells has been shown to allow their homing to the tumor site (33, 37). Compared to the control and ACS groups, a significant reduction of tumor growth rates was observed in several animals treated with ACD3S-activated NK-92 cells, suggesting that these cells retain their antitumor cytotoxicity *in vivo*. Since NK cells are not deficient in SCID mice, the possibility that NK-92 antitumor activity is assisted by endogenous murine NK effectors, *in vivo* activated by NK-92-produced IFNγ, should be further investigated.

Taking all the above into consideration, we propose that NK-92 cells activated with ACD3S could be considered for use in cancer immunotherapeutic protocols. The possibility that additional doses or intravenous or intratumoral injection of ACD3S-activated NK-92 cells would further hinder tumor growth needs to be explored. Nevertheless, the establishment of a short-term (24 h) protocol that enhances the functionalities (prolonged cytotoxicity and IFNγ production) of NK-92 cells and, concomitantly, reduces the risk of toxicity as a result of a single-dose of high cytokine (IL2) concentration, offers a promising alternative method for the *ex vivo* activation of competent effectors.

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References

1 Miller JS, Tessmer-Tuck J, Pierson BA, Weisdorf D, McGlave P, Blazar BR, Katsanis E, Verfaillie C, Lebkowski J, Radford J Jr. and Burns LJ: Low-dose subcutaneous interleukin-2 after autologous transplantation generates sustained *in vivo* natural killer cell activity. Biol Blood Marrow Transplant *3*: 34-44, 1997.

2 Burns LJ, Weisdorf DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SR, Panoskaltsis-Mortari A, Keever-Taylor CA, Zhang MJ and Miller JS: IL2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. Bone Marrow Transplant *32*: 177-186, 2003.
3 Cheng M, Chen Y, Xiao W, Sun R and Tian Z: NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol *10*: 230-252, 2013.
4 Tonn T, Becker S, Esser R, Schwabe D and Seifried E: Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. Hematother Stem Cell Res *10*: 535-544, 2001.
5 Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J and Klingemann H: Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy *10*: 625-632, 2008.
6 Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, Suttorp M, Seifried E, Ottmann OG and Bug G: Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy *15*: 1563-1570, 2013.
7 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.
8 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.
9 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.
10 Sutlu T and Alici E: Natural killer cell-based immunotherapy in cancer: current insights and future prospects. J Intern Med *266*: 154-181, 2009.
11 Baxevanis CN, Tsiatas ML, Cacoullos NT, Spanakos G, Liacos C, Missitzis I, Papadhimitriou SI and Papamichail M: Induction of anti-tumour lymphocytes in cancer patients after brief exposure to supernatants from cultures of anti-CD3-stimulated allogeneic lymphocytes. Br J Cancer *76*: 1072-1080, 1997.
12 Tsiatas ML, Gritzapis AD, Cacoullos NT, Papadhimitriou SI, Baxevanis CN and Papamichail M: A novel culture environment for generating mature human dendritic cells from peripheral blood $CD14^+$ cells. Anticancer Res *21*: 1199-1206, 2001.
13 Gritzapis AD, Dimitroulopoulos D, Paraskevas E, Baxevanis CN and Papamichail M: Large-scale expansion of CD3(+)CD56(+) lymphocytes capable of lysing autologous tumor cells with cytokine-rich supernatants. Cancer Immunol Immunother *51*: 440-448, 2002.
14 Skopeliti M, Iconomidou VA, Derhovanessian E, Pawelec G, Voelter W, Kalbacher H, Hamodrakas SJ and Tsitsilonis OE: Prothymosin alpha immunoactive carboxyl-terminal peptide TKKQKTDEDD stimulates lymphocyte reactions, induces dendritic cell maturation and adopts a beta-sheet conformation in a sequence-specific manner. Mol Immunol *46*: 784-792, 2009.
15 Voutsas IF, Baxevanis CN, Gritzapis AD, Missitzis I, Stathopoulos GP, Archodakis G, Banis C, Voelter W and Papamichail M: Synergy between interleukin-2 and prothymosin alpha for the increased generation of cytotoxic T-lymphocytes against autologous human carcinomas. Cancer Immunol Immunother *49*: 449-458, 2000.
16 Duan F, Simeone S, Wu R, Grady J, Mandoiu I and Srivastava PK: Area under the curve as a tool to measure kinetics of tumor growth in experimental animals. J Immunol Methods *382*: 224-228, 2012.
17 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.
18 Klingemann H: Are natural killer cells superior CAR drivers? Oncoimmunology *3*: e28147, 2014.
19 Osband ME, Lavin PT, Babayan RK, Graham S, Lamm DL, Parker B, Sawczuk I, Ross S and Krane RJ: Effect of autolymphocyte therapy on survival and quality of life in patients with metastatic renal-cell carcinoma. Lancet *335*: 994-998, 1990.
20 Reddy A, Sapp M, Feldman M, Subklewe M and Bhardwaj N: A monocyte-conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. Blood *90*: 3640-3646, 1997.
21 Kato K, Takaue Y and Wakasugi H: T-Cell-conditioned medium efficiently induces the maturation and function of human dendritic cells. J Leukoc Biol *70*: 941-949, 2001.
22 Cacoullos NT, Gritzapis AD, Tsitsilonis AE, Tsiatas ML, Baxevanis CN and Papamichail M: Efficacy of novel culture environments on the *ex vivo* expansion kinetics of cord blood progenitor cells. Haematologica *87*: 320-321, 2002.
23 Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R, Anderson D, Eisenmann J, Grabstein K and Caligiuri MA: Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells *via* components of the IL2 receptor. J Exp Med *180*: 1395-1403, 1994.
24 DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA and Bloom ET: IL12 synergizes with IL2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. Cell Immunol *165*: 33-43, 1995.
25 Gamero AM, Ussery D, Reintgen DS, Puleo CA and Djeu JY: Interleukin 15 induction of lymphokine-activated killer cell function against autologous tumor cells in melanoma patient lymphocytes by a CD18-dependent, perforin-related mechanism. Cancer Res *55*: 4988-4994, 1995.
26 Sotiriadou NN, Perez SA, Gritzapis AD, Mahaira LG, Salagianni M, Baxevanis CN and Papamichail M: Beneficial effect of short-term exposure of human NK cells to IL15/IL12 and IL15/IL18 on cell apoptosis and function. Cell Immunol *234*: 67-75, 2005.
27 Zhang B, Zhang J and Tian Z: Comparison in the effects of IL2, IL12, IL15 and IFNalpha on gene regulation of granzymes of human NK cell line NK-92. Int Immunopharmacol 8: 989-996, 2008.
28 Smyth MJ, Wallace ME, Nutt SL, Yagita H, Godfrey DI and Hayakawa Y: Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. J Exp Med *201*: 1973-1985, 2005.
29 Lee HM, Kim KS and Kim J: A comparative study of the effects of inhibitory cytokines on human natural killer cells and the mechanistic features of transforming growth factor-beta. Cell Immunol *290*: 52-61, 2014.
30 Bär E, Whitney PG, Moor K, Reis e Sousa C and Leibundgut-Landmann S: IL17 regulates systemic fungal immunity by controlling the functional competence of NK cells. Immunity *40*: 117-127, 2014.

31 Al Omar S, Flanagan BF, Almehmadi M and Christmas SE: The effects of IL17 upon human natural killer cells. Cytokine *62*: 123-130, 2013.

32 Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, Fleisher TA, Dubois SP, Perera LP, Stewart DM, Goldman CK, Bryant BR, Decker JM, Chen J, Worthy TA, Figg WD Sr, Peer CJ, Sneller MC, Lane HC, Yovandich JL, Creekmore SP, Roederer M and Waldmann TA: Redistribution, hyperproliferation, activation of natural killer cells and CD8 T-cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. J Clin Oncol *33*: 74-82, 2015.

33 Voutsas IF, Gritzapis AD, Mahaira LG, Salagianni M, von Hofe E, Kallinteris NL and Baxevanis CN: Induction of potent $CD4^+$ T cell-mediated antitumor responses by a helper HER2/neu peptide linked to the Ii-Key moiety of the invariant chain. Int J Cancer *121*: 2031-2041, 2007.

34 Ben-Batalla I, Seoane S, Macia M, Garcia-Caballero T, Gonzalez LO, Vizoso F and Perez-Fernandez R: The PIT1/POU1F1 transcription factor regulates and correlates with prolactin expression in human breast cell lines and tumors. Endocr Relat Cancer *17*: 73-85, 2010.

35 Zhang P, Zheng BB, Wang HY, Chen JH, Liu XY and Guo XL: DLJ14, a novel chemo-sensitization agent, enhances therapeutic effects of adriamycin against MCF-7/A cells both *in vitro* and *in vivo*. J Pharm Pharmacol *66*: 398-407, 2014.

36 www.fda.gov/downloads/Drugs/Guidances/UCM078932.

37 Zhou Y, Cheng Z, Zhu H, Feng D, Zhao W, Ling B, Wei H and Tian Z: Stable suppression of *HER2* gene expression using siRNA increases the lysis of human ovarian carcinoma cells mediated by NK-92 cell line. Oncol Rep *20*: 1425-1431, 2008.

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