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Characterization of lysate from NK-92 cells and its potential use as an immunotherapeutic modality

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ABSTRACT

Lysates from human cells represent biofluids that are used in the biotechnology field for a number of reasons such as biomarker identification and antibody detection. Lysate from human blood platelets is widely used in the clinical setting to control bleeding. We hypothesized that the lysate from the cytotoxic natural killer cell line NK-92® should contain perforin and proteolytic enzymes in addition to immunomodulatory cytokines, all of which have biological relevance and could be used for local treatment of cancer lesions. Here lysate from *NK-92 (aNK™)* cells, and its erIL-2 engineered variant *haNK™* was obtained by repeat freeze/thawing. Immunoblot, ELISA and cytokine immunoassay analysis confirmed the presence of perforin and the full spectrum of granzymes, as well as of various chemokines and cytokines known to be expressed in NK-92 cells. Lysate from haNK cells displayed cytotoxic and anti-proliferative activity against human and canine cancer cell lines after only a 15-min exposure in vitro. Importantly, under the same conditions the lysate did not affect primary cells. Intra-tumor injection of haNK lysate into intradermal tumors of immunocompetent C57BL/6 mice provided tumor control in 40 % of treated animals. When re-challenged with the same tumor line several weeks after primary tumor clearance, no growth occurred indicating that intra-tumor administration of haNK lysate can generate a vaccine-like effect.

1. Introduction

Using lysate from prokaryotic or eukaryotic cells for medical and laboratory purposes is not a new concept. Lysate from human platelets is produced on a large scale by ultrasonication or more commonly by freeze/thaw of platelet concentrate [1,2]. It has become a valuable additive for in vitro maintenance and expansion of primary human and animal cells as well as cell lines [3], and is also increasingly used as a therapeutic in regenerative medicine [4]. Lysate from human cancer cells and cell lines is being tested for its potential to induce a vaccine-like effect in patients or for ex vivo priming of dendritic cells [5,6]. Bacterial lysates elicit an immune response and have shown potential as an immunotherapeutic modality [7,8]

Cell lysate preparations are acellular, thereby reducing concerns regarding allogeneic rejection, and they can contain high concentrations of growth factors, cytokines, chemokines and cytolytic enzymes. The preparation of cell lysates is relatively straightforward, usually involving several freeze/thaw cycles or ultrasonication [9]. Subsequent centrifugation and filtration remove intact cells and membrane fragments, reducing the risk of aggregate formation and also depleting potential antigens. Importantly, cell lysates can be cryopreserved without affecting their activity [9,10].

Lysate from natural killer (NK) cells has not been as widely explored. It should be rich in perforin, granzymes and cytokines which can have a direct cytotoxic/anti-proliferative effect on tumor cells and modulate cells in the tumor microenvironment, in particular antigen-presenting cells and lymphocytes. To obtain sufficient lysate from blood-derived NK cells for therapeutic purposes, the cells require expansion on a feeder layer with subsequent harvest, a process that is logistically challenging and costly [11]. As an alternative, the off the shelf NK cell line NK-92 is a good candidate for a source of lysate that can be isolated under feasible conditions.

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The human NK-92 cell line has become the most widely used NK cell line for basic and translational research on NK cells [12, reviewed in 13]. It has also been administered to cancer patients in several phase 1 and 2 studies [13–18]. The original NK-92 cell line (also referred to as aNK) has been genetically modified to grow independently of IL-2, and expresses a high affinity CD16 Fc-receptor (termed haNK) [19]. A number of variants have been generated expressing chimeric antigen receptors (CAR) [20–26]. There are, however, two considerations with NK-92 cells when it comes to their clinical application: since the cell line has been generated from blood cells of a patient with lymphoma, they need to be irradiated prior to administration, which causes them to lose most of their cytolytic activity over 48 h. Secondly, NK-92 cells can induce an allogeneic MHC-triggered cellular immune reaction in the recipient especially after repeated administration. Due to its acellular nature, NK-92 cell lysate should not elicit either of these concerns.

We developed a GMP-compatible method to generate cellular lysate from NK-92 and haNK cells via repeated freeze/thaw and confirmed that the lysate contains perforin and the entire spectrum of granzymes (GrzA, GrzB, GrzH, GrzK, GrzM), in addition to immune-active cytokines and chemokines. Importantly, the lysate kills malignant cells after only a short exposure (15 min) and also inhibits their proliferation. The anti-tumor activity of the lysate was tested in an immunocompetent, syngeneic mouse tumor model. Compared to control, intratumor administration of haNK lysate provided a significant survival benefit with complete eradication of primary tumors in 40 % of mice. Importantly, these mice were protected from re-challenge with the same tumor line, indicating intra-tumor haNK lysate generates a robust and durable anti-tumor response. The lysate is also broadly cytotoxic/cytostatic to canine cancer cells opening up the possibility for an innovative cancer treatment for canines.

2. Material and methods

2.1. Cell lines and culture conditions

NK-92 and haNK cells were obtained from ImmunityBio Inc. (Culver City, CA). haNK cells are NK-92 cells that have been engineered to express the high affinity variant (158 V) of the CD16a Fc-receptor and erIL-2 that allows them to grow independently of exogenous IL-2 [20]. SKBR-3 (human breast adenocarcinoma, HTB-30) and SKOV-3 (human ovary adenocarcinoma, HTB-77) were obtained from the *American Type Culture Collection* (ATCC, Manassas, VI). MC-38 (murine colorectal cancer, CRC) was provided by the laboratory of Dr. J Schlom (NCI, Bethesda, MA). CTAC (canine thyroid carcinoma) was purchased from Millipore Sigma (Burlington, MA); OSCA-40 (canine osteosarcoma) was obtained from Kerafast Inc. (Boston, MA). Primary human lung fibroblasts and umbilical vein endothelial cells (HUVEC) were obtained from Sigma-Aldrich (St. Louis, MO) and Lonza (Walkersville, MD) respectively and cultured in fibroblasts and endothelial growth medium from Cell Applications (San Diego, CA).

NK-92 and haNK cells were cultured in X-VIVO™10 (Lonza, Walkersville, MA) with 5 % Human Serum (Access Biologicals, Vista, CA). NK-92 culture medium was supplemented with 500 IU/mL rhIL-2 (Prometheus, San Diego, CA). Human lung fibroblasts and HUVECs were cultured in fibroblasts and endothelial growth medium from Cell Applications (San Diego, CA). CTAC cells were cultured in RPMI-1640 GlutaMAX™ (Gibco - Thermo Fisher Scientific, Waltham, MA). SKBR-3 and SKOV-3 cells were cultured in McCoys' 5 A (Corning, Corning NY). OSCA-40 were cultured in DMEM high glucose GlutaMAX™ (Gibco) with 10 mM HEPES supplement without Sodium Pyruvate. Culture medium for those cell lines included 10 % Fetal Bovine Serum (Gibco). Nuclear localized red (NucLight™ Red) fluorescent-labeled versions of the SKBR-3, SKOV-3, CTAC and OSCA-40 cell lines were generated by transduction with Incucyte® Nuclight Red Lentivirus (Sartorius) in the presence of 8 μg/mL polybrene, followed by selection with 1.5 to 2.5 μg/mL puromycin (Invitrogen, Carlsbad, CA). For in vivo experiments, the murine MC-38 cell line was grown in DMEM supplemented with 10 % FBS (Hyclone), HEPES, 1 % Penicillin/Streptomycin, and non-essential amino acids (Gibco).

2.2. Preparation of cell lysate

NK-92 and haNK cells were expanded to a density of 1×10^6 cells/mL and harvested by centrifugation (500 x*g* for 5 min). The cell pellets were washed twice with PBS 1× without Ca^{2+}/Mg^{2+} (Corning, NY) and resuspended at a density of 1×10^8 cells/mL in a Balanced Salt Solution (BSS) composed of 150 mM NaCl (Ambion, Austin, TX) and 50 mM Tris-HCl pH 7.4 (Alfa Aesar, Ward Hill, MA). The cell suspensions were subjected to 3 cycles of freeze (30 s in liquid nitrogen) and quick thaw (37 °C water bath) to achieve cell lysis. Lysed cell mixtures were centrifuged at 3000 x*g* at 4 °C for 10 min to remove intact cells, insoluble cell debris and genomic DNA. The supernatants (i.e. the "lysate") were collected and used either fresh or cryopreserved at -80 °C until further use.

2.3. ImmunoBlot analysis

Total protein extracts from cultured cells were prepared using RIPA/SDS (Pierce, Waltham, MA) extraction buffer followed by centrifugation at 14,000 *g* for 20 min at 4 °C. Determination of the protein concentration in the lysate and extracts samples was performed using the Pierce™ bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Lysates and extracts were mixed with NuPAGE™ LDS sample buffer plus sample reducing agent (NuPage, Invitrogen) and equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12 % polyacrylamide Bis-Tris gel (Invitrogen™, Carlsbad, CA) with MOPS SDS Running buffer (NuPage, Invitrogen), and electro-transferred to polyvinylidene fluoride (PVDF) membranes using the iBlot™ 2 Dry Blotting system (Invitrogen™) according to manufacturer's instructions. Membranes were probed with specific primary antibodies followed by horse radish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). Primary antibodies against the following proteins were used at 1:1000 dilution: Perforin-1, GrzA, GrzB, GrzH, GrzK, GrzM, and Vinculin-1 from Cell Signaling Technologies. The activated and pro-form fragments of IL-16 were detected using an antibody against the IL-16C-terminus peptide antigen (R&D Systems). Protein bands were detected by enhanced chemiluminescence (ECL) using the Pierce™ Supersignal™ West Pico PLUS (Pierce). Images were acquired using the c-DiGit® blot scanner system and analyzed with Image Studio software Version 5.2 (LICORbio Biosciences, Lincoln, NE).

2.4. Quantification of perforin, granzymes, cytokines and chemokines in cell lysate

Quantification of granzymes, perforin, and granulysin in cell lysates and extracts of NK-92 and haNK was performed by ELISA, using commercially available kits for Granzyme A (Invitrogen), Granzyme B (Invitrogen), Granzyme H (Invitrogen), Granzyme K (Antibodies.com, Cambridge, UK), Granzyme M (Antibodies.com), perforin (Invitrogen), granulysin (Antibodies.com) and sFasL (Invitrogen). Absorbance was measured at 450 nm using an automated spectrophotometer (SpectraMax i3x; Molecular Devices, San Jose, CA). Quantification of a panel of cytokines and chemokines was performed by Meso Scale Diagnostics (MSD) Multi-Spot Assay System (Rockville, MD) using the V-Plex Plus Cytokine Panel 1 Human kit and Proinflammatory Panel 1 Human kit, according to manufacturer's instructions. Briefly, each MSD kit contains a 96 well- plate with microelectrode at the bottom of each well to enable it to produce high performance electrochemiluminescence with addition of MSD Read Buffer. These plates are pre-coated with either cytokine Panel 1 or Proinflammatory Panel 1 capture antibodies specific to the biomarkers on independent and well-defined spots. Readout was done

using MSD Quick Plex SQ 120 Reader using Methodical Mind software.

Quantification of Granzyme B Activity in cell lysate.

To determine the effect of cryopreservation on the enzymatic activity of granzyme, freshly prepared and frozen/thawed NK-92 and haNK cell lysates were assayed for granzyme B activity according to the manufacturer's instructions (Sigma-Aldrich) using a fluorimeter (SpectraMax i3x) at wave lengths of 380 nm (excitation) and 500 nm (emission).

2.5. Analysis of cytotoxic and cytostatic effect of cell lysate by Incucyte Live-Cell Assay

Nuclight Red-labeled human (SKOV-3, SKBR-3) and canine cancer cell lines (CTAC, OSCA-40) were plated in 96-well plates at a seeding density of 1,000–2,000 cells per well in 100 μL of their respective culture media and incubated at 37 °C in a 5 % CO_2 humidified incubator. After 24 h, culture media was removed, and 200 μL/well of either culture media, BSS buffer, or lysate was added to the target cells. The plates were incubated for 15 min in a 37 °C/5 % CO_2 humidified incubator. After co-incubation, the plates were briefly centrifuged at 500 x*g* for 2 min, supernatants were discarded and cells were washed once in 1× D-PBS. Finally, 200 μL of culture media containing Cytotox green reagent (Sartorius) was added to each well to identify dead/dying cells. Plates were placed inside an Incucyte S3 Live-Cell Imaging System device (Essen BioScience, Ann Harbor, MI), where four images per well were taken with a 20× objective every 2 h over 72 h in phase, green and red channels. Objects were counted by using the Incucyte Analysis Software (version 2020B), by masking cells and signals for Nuclight-Red (red fluorescence) and/or Cytotox green (green fluorescence). Cell proliferation was quantified by counting the number of red fluorescent objects over the course of the assay and expressed as cell population doubling time for intervals of 6 h, calculated using the formula ((t2-t1) x log(2))/(log(cell count at t2) - log(cell count at t1)), where (t2-t1) equals 6 h. The number of live cells after lysate exposure was calculated as a percentage of dead/dying objects over total object count.

2.6. Assessment of haNK lysate effects in murine tumor studies

Female C57BL/6 J mice were purchased from the Jackson Laboratory and were 7–8 weeks old at the start of experiments. Mouse studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals approved by the local Institutional Animal Care and Use Committee. Mice were injected intra-dermally (i.d.) in the left hind flank with 3×10^5 MC-38 tumor cells five days before study group enrollment. On the day of group enrollment (Day 0) mice with measureable tumors (volume range 20.1–44 mm^3) were randomly enrolled into treatment groups ($n = 10$/group). Tumors were measured 2–3 times per week using digital calipers by two opposing dimensions (*a, b; wherein b is the smaller dimension*) and tumor volume was calculated according to the formula $V = (a \times b^2)/2$. Mice were euthanized when tumor volume reached ≥1000 mm^3 or exhibited non-resolving tumor ulceration. Mice received six intra-tumor (i.t.) injections administered once daily every other day starting Day 0, in a 50 μL bolus of BSS or haNK lysate at the equivalent of 5×10^6 cells/ 50 μL dose. Mice that exhibited complete clearance of the primary tumor were re-challenged on Day 50 with 3×10^5 MC-38 tumor cells i.d. on the opposite hind flank. At the time of re-challenge, age-matched naïve controls ($n = 5$) were also implanted with MC-38 tumors on the right hind flank as a control for tumor implantation and growth. All surviving mice were euthanized at the termination of the study on Day 95. Results of primary tumor treatment are representative of two independent experiments.

2.7. Statistics

All statistical analyses were performed using GraphPad Prism (Version 10.1.1) software. Results for cellular growth rates in the Incucyte assays are expressed as average cell population doubling time ± standard deviation (SD). Statistical differences for in vitro assays were determined using an unpaired, two-tailed Student's *t*-test. For the in vivo tumor growth, unpaired t-test of growth rate as determined by exponential (Malthusian) growth were performed. Kaplan-Meier survival statistics were performed using Log-rank (Mantel-Cox) test relative to BSS controls or Naïve Controls. Statistical significance is indicated $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

3. Results

3.1. Perforin and granzymes are present in lysate preparations from NK-92 and haNK cells

To preserve both enzymatic activities and biological compatibility, we designed a buffer solution for lysate preparation that does not contain any detergent, chelating agents, or protease inhibitors. In order to determine how much of the cellular content is solubilized during cell lysate preparation, we prepared whole cell extracts using a conventional denaturing lysis buffer for comparison. We first focused on perforin and granzymes, since they are the main effector proteins that initiate and execute the cytotoxic and anti-proliferative effect of NK cells against cancer cells. Qualitative (immunoblot, Fig. 1) and quantitative (ELISA, Fig. 2) analyses did not show any difference in perforin, granulysin, GrzB, GrzK and GrzM between our cell lysates and the denatured cellular extracts. Some differences in GrzA and GrzH were observed, suggesting that the whole cell lysate preparation protocol enriches for these two proteins. We did not observe any detectable level of sFASL in any of the assays (data not shown).

3.2. Cytokine and chemokine concentrations in lysates from NK-92 and haNK cells

To determine the spectrum of cytokines and chemokines present in lysates from NK-92 and haNK cells, the MSD Quick Plex SQ 120 reader was used. Results show significantly elevated cytokine concentrations of IL-8, IL-10, IL-16, IFN-γ, TNF-β MIP1α and MIP1β in both NK-92 and haNK lysates (Fig. 3). No measurable concentrations were detected for: IL-1α, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL-15, IL-17, GM-CSF, TNF-α, VEGF, Eotaxin, Eotaxin-3, MCP-1, MCP-4, MDC, TARC.

As expected, elevated concentrations of IL-2 were found in the lysate of haNK cells that has been engineered with the erIL-2 gene. The IL-2 detected in NK-92 lysate is likely from residual exogenous IL-2 in the culture medium which contains IL-2 to stimulate cell expansion.

Because the MSD assay detected a high concentration of IL-16 in both lysates, Immunoblot analysis was performed to determine the presence of precursor and active forms of IL-16. Lysates from NK-92 and haNK contained both precursor and active forms of IL-16 (Fig. 4).

3.3. Lysate from haNK cells is cytotoxic and anti-proliferative to human and canine cancer cell lines

The Incuyte S3 device allows for real time live cell analysis of target cells over several days. Here we show that after only a short 15 min co-incubation with cancer targets, haNK lysate effectively induces lysis of *human* SKOV-3 (Fig. 5 A) and SKBR-3 (Fig. 5B), as well as of *canine* OSCA-40 (Fig. 5C) and CTAC (Fig. 5D) cancer cell lines. For control purposes, primary human lung fibroblasts (Fig. 5E) and endothelial vein cells (HUVEC, Fig. 5F) were tested with haNK lysate under the same conditions but no cytotoxic effect was noted. The short co-incubation with lysate also induced significant cytostatic effect on the same cancer cell lines, as evidenced by a longer cell population doubling time (Fig. 5G). haNK cell lysate did not affect the population doubling time of the normal primary cell controls.

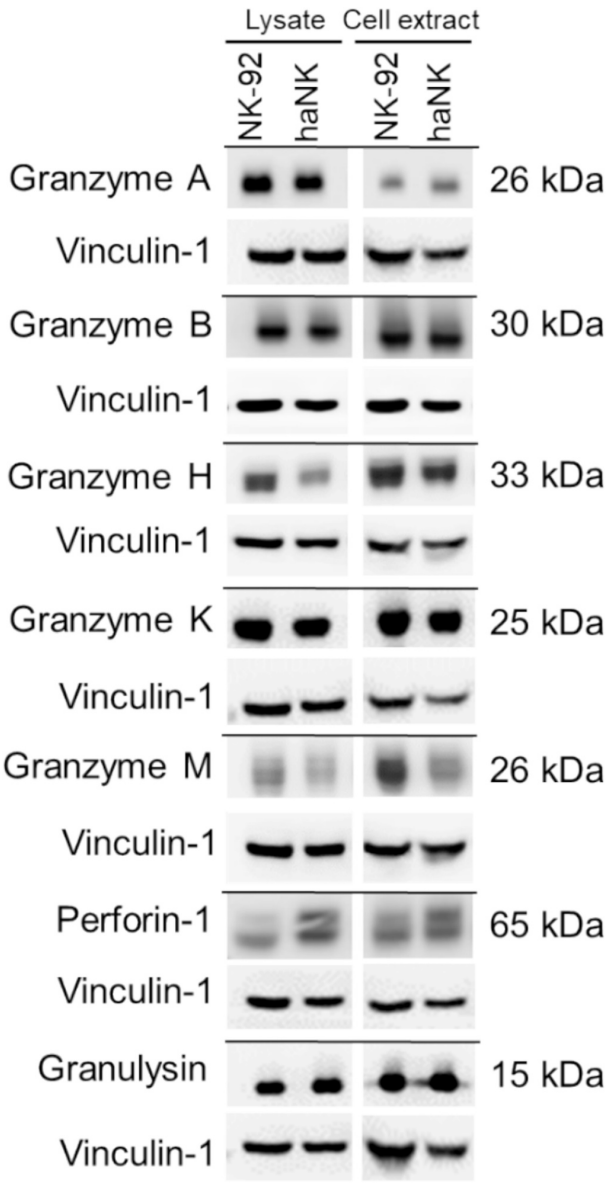

Fig. 1. Immunoblot analysis of perforin, granulysin and GrzA, GrzB, GrzH, GrzK, GrzM in lysates generated from NK-92 and haNK cells after repeated freeze/thaw cycles. The expression level of the vinculin-1 protein was used as a protein loading control. Whole cell extracts using a conventional denaturing lysis buffer was used for comparison.

3.4. Cryopreservation does not affect the biological activity of NK-92 and haNK cellular lysate

It is likely that NK lysate used clinically will have undergone previous cryopreservation, thus it is important to confirm activity is not diminished. As shown in Fig. 6 cryopreservation/ thawing of NK-92 or haNK lysate does not affect the activity of Granzyme B, a granzyme with key anti-tumor activity (Fig. 6A). Using the Incucyte device it was also confirmed that fresh and cryopreserved/thawed haNK lysate display similar cytotoxicity and anti-proliferative effects against canine OSCA-40 cancer cells (Fig. 6B and C, respectively).

3.5. Intra-tumor injection of haNK lysate inhibits tumor growth, extends survival, and protects against re-challenge in immunocompetent mice

Cellular lysate from haNK cells was administered intra-tumor (i.t.) to immunocompetent female C57BL/6 J mice bearing MC-38 tumors (Fig. 7A). Five days after intradermal tumor implantation, mice with measurable tumors were randomly assigned to the indicated treatment groups and received six i.t. injections of BSS or haNK lysate, administered once daily every two days starting on the day of enrollment. All mice that received i.t. haNK lysate had significantly reduced tumor growth (Fig. 7B) relative to BSS controls with 40 % of animals exhibiting complete tumor clearance. Mice that received haNK lysate also had prolonged survival as compared to BSS control (Fig. 7C).

To evaluate durability of the anti-tumor response, mice that exhibited complete eradication of the primary treated tumor ($n = 4$) were re-challenged on Day 50 with the same MC-38 tumor line on the opposite flank along with a group of age-matched naïve controls ($n = 5$). While tumors did develop in 4 of 5 naïve controls, none of the re-challenged mice developed tumors and all survived until the end of study on Day 95 (Fig. 7D). Importantly, the injections of lysate were well tolerated with no signs for adverse events (huntched posture, weight loss).

4. Discussion

The NK-92 cell line, isolated in 1992 from a patient with lymphoma, has been extensively used by investigators worldwide for studies to further define the characteristics of NK cells as they can be considered a suitable surrogate for blood NK cells [12,13]. NK-92 cells have been genetically engineered with IL-2 to make their ex vivo growth IL-2 independent [19,26]. A variant expressing the high affinity FcγRIIIa-receptor (haNK cells) can bind antibodies and execute ADCC [19]. The transgene construct for haNK cells also includes an erIL-2 sequence to allow haNK cells to grow and expand independently of exogeneous IL-2. Various CAR-expressing modifications of NK-92 have been generated: taNK [20,21] and t-haNK [21–26]. Patients with advanced cancers have been treated with NK-92, haNK, taNK and t-haNK cells with some encouraging responses and minimal side effects [reviewed in 13, 25]. Here we summarize our experience with a novel NK-92/haNK cell-derived product - cellular lysate - generated by repeat freeze/thaw cycles.

Our protocol for preparing cellular lysate yielded a product with very high concentration of perforin and granzymes, as well as cytokines such as IFN-γ or IL-16. We tested lysate from NK-92 cells and haNK cells side by side and showed essentially equivalent levels for perforin/granzyme and cytokine concentrations (Figs. 1 and 3). It has previously been reported that pre-stimulated NK-92 cells secrete higher concentrations of lytic enzymes which could be considered as an additional way of generating highly cytolytic lysate [41]. Since haNK cells do not require exogenous IL-2 for expansion, they would be preferred for any clinical use over unmodified NK-92 cells. Hence, in vitro and in vivo experiments presented here were performed with haNK lysate. Importantly, we show that the isolation process for lysate preparation yields a product with significant cytolytic and anti-proliferative activity. Since, for logistical reasons, in the clinical setting the use of previously cryopreserved lysate would be preferred over fresh material, we confirmed that repeated freezing/thawing does not affect the activity of Granzyme B and the cytotoxic and anti-proliferative activity of the lysate.

When considering potential clinical applications of NK-92/haNK lysate, the *intra-* and *peri- tumor* injection would be the most obvious modality which would also minimize any systemic side effects. The presence of perforin and the broad spectrum of granzymes in the lysate should allow for entry of cytolytic enzymes and cytokines into the tumor tissue. The anti-tumor activity of the lysate should further be augmented by the presence of TNF-β, IL-2, IL-16 and IFN-γ which will stimulate any immune cells present in the microenvironment. Some evidence for this mechanism is provided by the murine data showing that intra-tumor

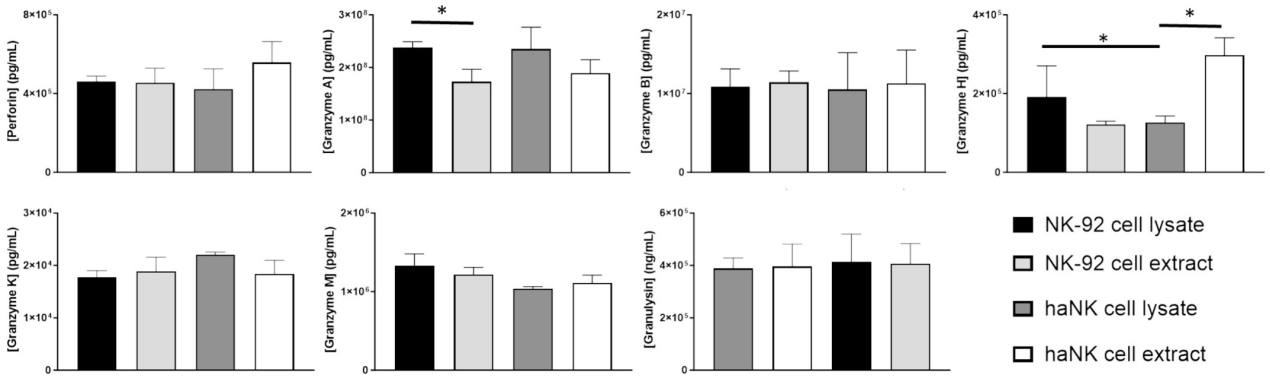

Fig. 2. ELISA for perforin, granzymes and granulysin in lysates and cell extracts from NK-92 and haNK cells. Mean and SEM are presented from three different experiment.

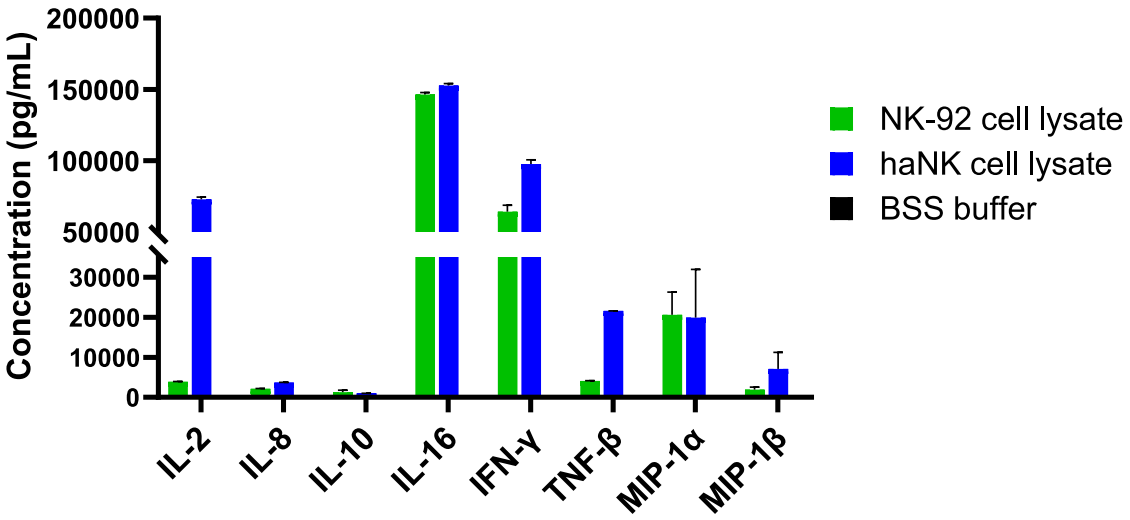

Fig. 3. MSD analysis of cytokine and chemokine concentrations in cell lysates from NK-92 and haNK cells ($n = 2$). Only detectable concentrations in the MSD panel are presented here.

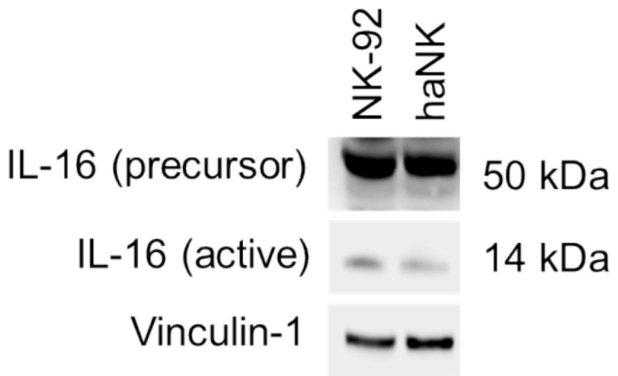

Fig. 4. Immunoblot analysis for IL-16 (precursor and active form) of cell lysate generated from NK-92 and haNK cells. The expression level of the vinculin-1 protein was used as a protein loading control.

injection of haNK lysate into MC-38 tumors of immunocompetent mice significantly reduced tumor growth and prolonged their survival. Importantly, this anti-tumor response was durable, as mice that had cleared the primary tumor after lysate injection did not develop tumors upon re-challenge. The presence of IL-16 in the lysate may support such a vaccine effect as the cytokine attracts CD4 T-cells [27] and also stimulates macrophages to secrete IL-1α, IL-6 and IL-15 creating a proinflammatory environment [28].

A vaccine-like effect with NK-92 administration has been observed before. When (murine) CD19-CAR engineered NK-92 were injected into A20 lymphoma of Balb/c mice, complete tumor elimination occurred in the majority of mice [23]. When those mice were re-challenged several weeks later with A20 lymphoma cells on the opposite site, no tumor growth/recurrence occurred. Similar data were presented by Wels et al. [21] who injected Her-2 CAR expressing NK-92 cells into immunocompetent mice with glioblastoma. Those mice that achieved a full remission did not develop cancer upon rechallenge.

Systemic cellular therapy of solid tumors, even with targeted CAR-T cells, has so far not achieved the same anti-tumor responses as seen for hematological malignancies [29–31]. This may be due to the fact that a cellular product faces the challenge of sufficiently infiltrating the solid tumor tissue due to its fibrous architecture in addition to the immunosuppressive tumor microenvironment [32]. Park et al. [33] recently described the negative impact of the peritumor glycocalyx on the cytotoxic function of NK-92 which, however, could be overcome by NK-92 cells engineered to degrade glycocalyx. The intra-or peri-tumor

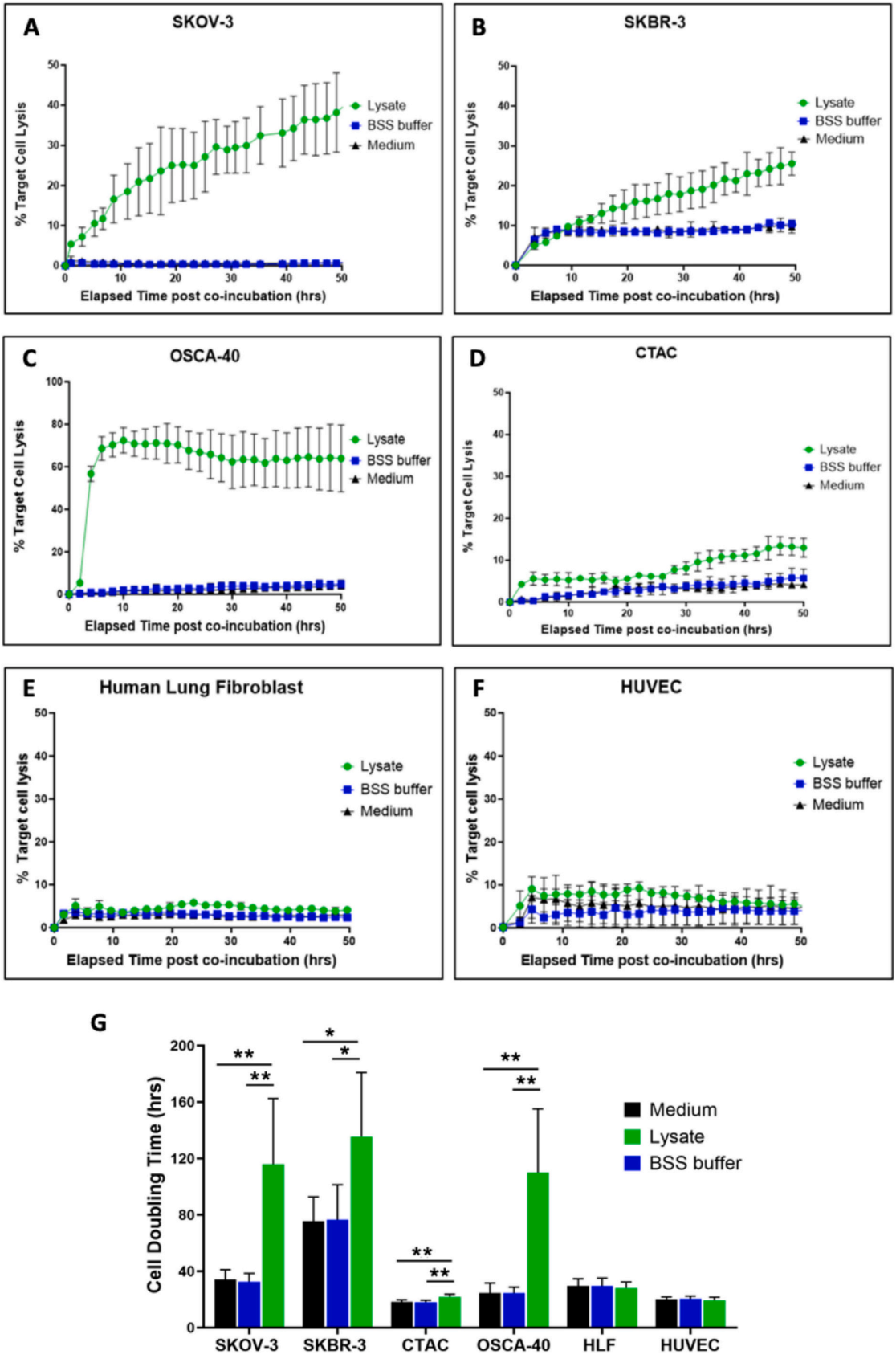

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Fig. 5. *Cytotoxicity and anti-proliferative activity of haNK cell lysate on cancer cell lines.* Percent target cell lysis by haNK lysate after 15-min exposure is shown for human cell lines SKOV-3 and SKBR-3; canine cell lines OSCA-40 and CTAC (A-D), as well as primary human non-malignant primary lung fibroblasts and umbilical vein endothelial cells (HUVEC), respectively (E,F)($n = 3$). Cell doubling time of each cell line was determined after exposure to cell medium, haNK lysate, or a balanced salt solution (BSS). Data graphed as the mean and SD. Statistical analysis performed using a student's *t*-test, $n = 2$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

administration of NK-92/haNK lysate could address this issue and make the anti-tumor activity independent of the presence of intact immune cell.

The testing of canine tumor cell lines here was deliberate, as cancer in dogs represents another possible indication for the use of NK-92/haNK lysate. This lysate was cytotoxic and cytostatic to *canine* cancer cells and would be more feasible than attempting to prepare cell lysate from NK cells derived from canine blood, as canine NK cells are not well described or functionally characterized [34]. Although expansion of canine peripheral blood mononuclear cells on an adherent layer may provide some cytotoxic cells, there is substantial donor to donor variability [35].

The observation that NK-92 lysate is cytolytic/cytostatic to canine cancer cells may not be entirely surprising considering that there is some 80 % cross-reactivity between human and canine proteins [36]. In fact, treatment of canines with human cytokines like IL-2 or IL-15 has shown anti - tumor effects [37,38]. Although there is concern that the xenogeneic proteins in the lysate may cause an immune response in the recipient, it is still uncertain how strong such an immune response would be. It is known from earlier studies that injection of the human TALL-104 cell line into dogs with cancer did not induce an immediate rejection [39,40]. Moreover, injection of NK-92 cells and as demonstrated herein, NK-92 cell lysates, into immunocompetent mice can elicit a long-lasting vaccine-like response possibly supported by the xenogeneic nature of lysate [21,23].

Importantly, the logistics of manufacturing clinical grade NK-92 lysate are relatively straightforward. NK-92/haNK cells can be expanded without the need for an adherent layer from 10^6 to 10^8 cells within 8–10 days. Extrapolating these numbers to a larger scale: a 50 L tank generating about 25 billion NK-92/haNK cells could yield about

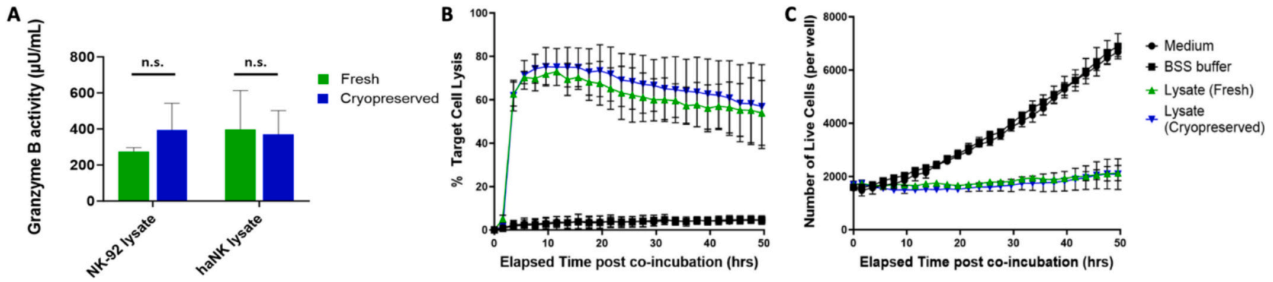

Fig. 6. *Granzyme B activity of fresh or previously frozen NK-92 and haNK cell lysates, and haNK cell lysate activity* in vitro. (A) Granzyme B activity (μU/mL) is shown for both fresh and previously cryopreserved NK-92 and haNK cell lysates ($n = 2$). Statistical analysis performed using a student's *t*-test; n.s. – not significant. Data graphed as the mean and SD. (B) Target cell lysis (% cytotoxicity) and (C) number of live cells of both fresh and previously cryopreserved haNK cell lysate against canine OSCA-40 cells in the Incucyte ($n = 3$). DMEM plus 10 % FBS ("medium") and Balanced Salt Solution (BSS) served as controls.

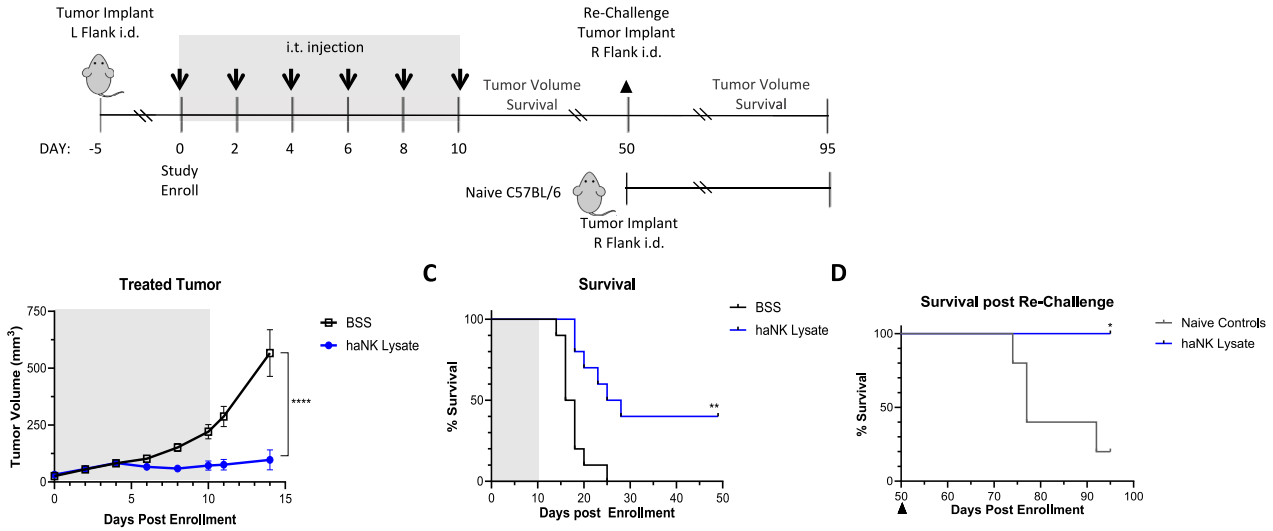

Fig. 7. *In vivo study design, tumor volume, survival and post-rechallenge of mice implanted with MC-38 tumors treated with haNK lysate.* (A) Shown is the study design; mice were implanted intradermally (i.d.) with MC-38 cells on Day −5, then treated by intratumor (i.t.) delivery of either haNK lysate or BSS ($n = 10$/group) on Days 0, 2, 4, 6, 8, and 10 (shaded in gray). Mice that survived and had no measurable tumor on Day 49 underwent rechallenge by i.t. injection on the opposite flank on Day 50. (B) Tumor volume (mm^3) over days post-enrollment measured up to Day 14; data graphed as the mean + SD. (C) Overall survival of mice with primary tumor up to Day 49. (D) Survival of rechallenged ($n = 4$) and age-matched naïve control ($n = 5$) mice, starting on Day 50 (arrow). Statistics performed using a student's *t*-test where $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{****}p \leq 0.0001$.

250 (1 mL) lysate doses. The fact that cryopreservation does not affect the activity of the NK-92/haNK cellular lysates makes it feasible to have the product readily available at the point of patient treatment.

In summary, cellular lysate from NK-92/haNK cells contains significant concentrations of perforin, granzymes, and immune-active cytokines/chemokines with a cytotoxic and growth inhibitory effect on both human and canine cancer cells. Intra-tumor injection of the lysate elicits a robust and durable anti-tumor response, significantly reducing tumor growth and extending survival of immunocompetent mice as well as preventing growth of re-challenge tumors. Hence, cellular lysate generated from NK-92/haNK cells has the potential as a therapeutic modality for certain human and canine cancers. It would be primarily for intra-and peri-tumor administration with the rationale to augment the immune-modulatory and anti-cancer effect of more systemic treatments like antibodies and CAR- T cells.

CRediT authorship contribution statement

Himani Chinnapen: Writing – review & editing, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Laurent Boissel:** Supervision, Methodology, Data curation. **Courtney Fleenor:** Supervision, Formal analysis, Data curation. **Thomas Bickett:** Data curation. **Zhimin Guo:** Conceptualization. **Vidya Godbole:** Data curation. **Manju Saxena:** Methodology, Data curation. **Patrick Soon-Shiong:** Validation, Resources, Funding acquisition. **Hans Klingemann:** Writing – original draft, Supervision, Conceptualization.

Informed Consent Statement

Not applicable.

Institutional Review Board Statement

This study was conducted in accordance with the National Institute of Health Guide For Care And Use of Laboratory Animals (NIH Publications No.8023, revised 1978), and approved by the ImmunityBio Colorado IACUC (protocol 2022-001 A approved 30 May 2022).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that has been used is confidential.

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