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RESEARCH

INTASYL self-delivering RNAi decreases TIGIT expression, enhancing NK cell cytotoxicity: a potential application to increase the efficacy of NK adoptive cell therapy against cancer

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Abstract
Natural killer (NK) cells are frontline defenders against cancer and are capable of recognizing and eliminating tumor cells without prior sensitization or antigen presentation. Due to their unique HLA mismatch tolerance, they are ideal for adoptive cell therapy (ACT) because of their ability to minimize graft-versus-host-disease risk. The therapeutic efficacy of NK cells is limited in part by inhibitory immune checkpoint receptors, which are upregulated upon interaction with cancer cells and the tumor microenvironment. Overexpression of inhibitory receptors reduces NK cell-mediated cytotoxicity by impairing the ability of NK cells to secrete effector cytokines and cytotoxic granules. T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), a well-known checkpoint receptor involved in T-cell exhaustion, has recently been implicated in the exhaustion of NK cells. Overcoming TIGIT-mediated inhibition of NK cells may allow for a more potent antitumor response following ACT. Here, we describe a novel approach to TIGIT inhibition using self-delivering RNAi compounds (INTASYL™) that incorporates the features of RNAi and antisense technology. INTASYL compounds demonstrate potent activity and stability, are rapidly and efficiently taken up by cells, and can be easily incorporated into cell product manufacturing. INTASYL PH-804, which targets TIGIT, suppresses TIGIT mRNA and protein expression in NK cells, resulting in increased cytotoxic capacity and enhanced tumor cell killing in vitro. Delivering PH-804 to NK cells before ACT has emerged as a promising strategy to counter TIGIT inhibition, thereby improving the antitumor response. This approach offers the potential for more potent off-the-shelf products for adoptive cell therapy, particularly for hematological malignancies.

Keywords NK cells · TIGIT · RNAi · Adoptive cell therapy (ACT)

Introduction

Natural killer (NK) cells are the body's first line of defense against cancer cells. They are able to rapidly recognize and kill tumor cells without prior sensitization and play an essential role in antitumor immunity [1, 2]. Compared with T-cell-based therapies, natural killer (NK) cells are gaining popularity as a potential therapeutic platform because of their ability to target cancer cells without the need for major histocompatibility complex (MHC) antigen stimulation and without eliciting graft-versus-host disease [3, 4]. The use of NK cells in adoptive cell therapy (ACT) has shown promise for treating hematological cancers in preclinical models and clinical studies [5, 6], but the cytotoxic activity, proliferation, and survival of NK cells need to be enhanced to improve clinical efficacy. Ex vivo expansion, activation, and additional manipulation can be used to further increase the antitumor activity of NK cells [7, 8].

The ability of NK cells to differentiate between benign and neoplastic cancer cells is exquisitely regulated by complex interactions between activating and inhibitory receptors on the NK cell surface [9]. When this balance is tipped toward activation, NK cells release cytotoxic granules, proinflammatory cytokines, and chemokines into the tumor microenvironment. These molecules lyse and kill tumor cells and recruit additional immune cells into the microenvironment [10]. However, tumor cells are able to evade NK cells

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by employing different strategies. These strategies include downregulation of NK-activating ligands, such as MICA and MICB, or upregulation of NK inhibitory ligands, such as CD155 and PVR [11]. Tumor cells may also downregulate MHC-I expression as a mechanism to avoid recognition by T cells. Mutations that allow for escape from T-cell detection and elimination are favored during tumor progression and can give rise to antigen escape variants of T-cell recognition, severely limiting the success of T-cell-based immunotherapies [12]. An approach to overcome this resistance is the adoptive transfer of NK cells, which do not require antigen processing and presentation [13].

The inhibitory ligand CD155 is recognized by the inhibitory receptor T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT). TIGIT is expressed on T cells and NK cells and binds with high affinity for CD155. The malignant transformation of epithelial cells leads to the upregulation of CD155. Engagement of CD155 with TIGIT competes with the interaction between the activating receptor DNAM-1 and CD155. This results in the diminished cytolytic activity of NK cells [14, 15]. Blockade of TIGIT has been shown to prevent NK cell exhaustion and restore cytotoxic capacity [16, 17], suggesting that TIGIT is an attractive target for enhancing the fitness of NK cells for adoptive cell therapy.

Various approaches to modulating NK cell gene expression are explored to reduce the expression of inhibitory receptors, such as TIGIT, and to render NK cell-based ACT products more active in a therapeutic setting. Current methodologies for modifying NK cell gene expression include the use of both viral and nonviral techniques. However, these techniques have significant drawbacks. Viral transduction results in stable expression of the transgene of interest but has proven difficult in NK cells and leads to reduced cell viability, as well as the risk of uncontrolled long-term adverse effects due to the stable nature of transgene expression [18, 19]. Nonviral gene silencing methods, such as siRNAs, do not carry the risk of long-term adverse effects but require harsh transfection reagents or electroporation to deliver the compounds, which can lead to a significant loss of cell viability [20]. The ability to modify NK cell gene expression prior to ACT without the need for harsh delivery methods is desirable.

We have developed a class of stable, self-delivering RNAi compounds (termed INTASYL) that incorporate features of RNAi and antisense technology. INTASYL compounds demonstrate potent activity and stability and are rapidly and efficiently taken up by cells through their unique chemistry, obviating the need for deleterious delivery methods. INTASYL technology is in clinical development for the direct treatment of diverse indications, including oncological, dermatological, and ophthalmologic conditions [21] (NCT06014086, NCT02079168). Based on its self-delivering characteristics, it could be an interesting modality to reprogram cells used for ACT. Proof-of-concept work has been completed in T cells, where it was shown that the use of INTASYL against checkpoint targets (PD-1) could increase the cell-killing activity of T cells [22].

In this study, we present PH-804, an INTASYL compound designed to specifically target the inhibitory checkpoint receptor TIGIT. By treating NK cells with PH-804 ex vivo in an NK cell ACT product manufacturing workflow, a reduction in TIGIT expression can be achieved, potentially improving the antitumor efficacy of NK cell therapeutic products. Improved NK cytotoxic activity as a result of INTASYL treatment during NK manufacturing shows promise for increasing the potency of off-the-shelf NK cell-based ACT therapies for hematological malignancies.

Materials and methods

Selection and synthesis of the INTASYL compound

Sequences targeting TIGIT (NM_173799.4) were identified using a proprietary selection algorithm.

Therapure RNA phosphoramidite monomers were obtained from Thermo Fisher Scientific (Milwaukee, WI, USA). 2'-Fluoro-Adenine Controlled Pore Glass (CPG) solid support was obtained from LGC Biosearch Technologies, and the Cholesterol CPG solid support was obtained from Prime Synthesis. Bis-cyanoethyl-N, N-diisopropyl CED OP was purchased from ChemGenes Corporation. Xanthane hydride was purchased from TCI Chemicals. All the other synthesis reagents and solvents used were obtained from EMD Chemicals. RNA oligonucleotides were synthesized on a Mermade 12 DNA/RNA Synthesizer (LGC Biosearch) using standard oligonucleotide phosphoramidite chemistry, starting from the 3' residue of the oligonucleotide preloaded on the CPG support.

The synthesized oligonucleotides were cleaved from the support, and the protecting groups were removed by treatment with three parts ammonia (28–30%) and one part ethanol at 40 °C for 20 h (h). The crude antisense product was subsequently purified on a GE Akta Purifier UPC100 using ion exchange chromatography (source 15Q, 20 mM NaH2PO4, 15% CH3CN, 1 M NaBr, gradient 20–60% B over 30 column volumes), and the fractions were analyzed via reverse-phase ion pair chromatography on a Shimadzu HPLC. The sense strand was purified by ionic precipitation in 5X Phosphate Buffered Saline (PBS), fully dissolved at 60 °C, and then cooled to RT for 60 min, followed by cooling in an ice bath for 60 min. The purified oligonucleotides were desalted using Sartorios Vivaspin 15 R (5000 MWCO) centrifugal concentrators and evaporated to dryness on a GeneVac Personal Evaporator (SP Industries,

Warminster, PA, USA). The purity and MW were determined by HPLC analysis (Shimadzu Prominence, XBridge OST C18 column, 25 mM hexylammonium acetate-acetonitrile buffer system, 60 °C) and ESI-MS analysis using Promass Deconvolution for Xcalibur (Novatia, Monmouth Junction, NJ).

Cells and culture

The NK cell line KHYG-1 (Creative Bioarray) was cultured in Roswell Park Memorial Institute medium 1640 (RPMI; Corning) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (penicillin/streptomycin; Gibco), and 100 IU/mL recombinant human IL-2 (PeproTech).

Human peripheral blood NK cells (StemCell Technologies) were thawed according to the supplier's protocol and expanded using the ImmunoCult™ NK Expansion Kit protocol (StemCell Technologies). Briefly, cells were cultured for three days in ImmunoCult™ NK Cell Expansion Medium on plates coated with ImmunoCult™ NK Cell Expansion Coating Material. After 3 days, fresh medium was added to the culture. On day 7 and again on day 10, the expanding NK cells were harvested and replated on freshly coated plates. Following expansion, the cells were maintained in complete ImmunoCult NK Expansion media supplemented with 1% penicillin/streptomycin.

The chronic myelogenous leukemia (CML) cell line K562 (ATCC) was cultured in Iscove's modified Dulbecco's medium (IMDM; Corning) supplemented with 10% FBS and 1% penicillin/streptomycin. The SKOV3 human ovarian carcinoma cell line (ATCC) was cultured in McCoy's 5A medium (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

All the experiments were performed within one month of thawing.

INTASYL treatment

INTASYL compounds were diluted in 1X phosphate-buffered saline (PBS), and appropriate volumes of TIGIT-targeting INTASYL or a chemically matched Non-Targeting Control (NTC) INTASYL were added directly to the culture wells at final concentrations ranging from 0 to 5 µM. Vehicle (PBS)-treated cells were included as untreated controls (UTC). The plates were rocked gently to ensure thorough mixing of cells and compounds. Transfections were performed in triplicate for each condition. The cells were maintained at 37 °C and 5% CO_2 for the duration of the experiments.

Lead INTASYL compound identification

For screening of TIGIT compounds, $0.5e^6$ KHYG-1 cells/mL were seeded into 24-well plates containing prediluted INTASYL compounds at final concentrations of 0–2 µM. The cells were incubated for 72 h, at which point they were collected for analysis.

mRNA quantification by qPCR

Total RNA was isolated from triplicate wells for each condition using the PureLink RNA Mini Kit (Thermo Fisher) according to the manufacturer's recommendations. The isolated mRNA was quantified using an Epoch microplate spectrophotometer (BioTek), and ~ 75 ng of mRNA was used as an input for duplicate gene expression analysis via single-plex RT−qPCR using the TaqMan™ RNA-to-CT 1-Step Master Mix (Thermo Fisher) and TaqMan™ probes for the specific detection of human TIGIT-FAM (Thermo Fisher; Hs00545085_m1) and PPIB-FAM (Thermo Fisher; Hs00168719_m1) on a QuantStudio3 RT−qPCR machine (Applied Biosystems). Cycle thresholds (Ct) from TIGIT gene analytes were normalized to those of PPIB, and relative quantification (RQ; $2^{-\Delta\Delta Ct}$) was calculated as the fold change relative to the UTC condition.

Antibodies and flow cytometry

For phenotypic analysis, the cells were incubated with fluorochrome-conjugated mAbs in Flow Cytometry Staining Buffer (FCB; Fisher Scientific) at the manufacturer's recommended concentration for 30–60 min at 4 °C. The antibody details are provided in Table 1. Afterward, the cells were resuspended in FCB and analyzed via flow cytometry (NovoCyte flow cytometer and NovoExpress software from Agilent/ACEA Biosciences).

Cell viability assay

Cell viability following INTASYL treatment was determined by 7-AAD staining. Briefly, following the culture period, the cells were collected, washed once with PBS, resuspended in 100 µL of Cell Staining Buffer (BioLegend) with 1 µL

Table 1 Antibodies used for flow cytometry

Marker	Conjugate	Manufacturer	Clone
TIGIT	APC	R&D Systems	741,182
CD155	PE	R&D Systems	900,907
CD3	APC-Vio779	Miltenyi Biotec	BW264/56
CD56	PerCP-Vio700	Miltenyi Biotec	REA196

of 7-AAD staining solution (BioLegend), and incubated for 10 min prior to analysis by flow cytometry.

KHYG-1 in vitro antitumor cytotoxicity assay

To evaluate the ability of TIGIT-silenced KHYG-1 cells to kill target cancer cells, KHYG-1 cells were treated for 72 h with PH-804 or the appropriate controls for 72 h. Following 72-h treatment, the cells were collected by centrifugation and washed with culture medium to remove any treatment. The cells were then resuspended in fresh culture media and incubated at 37 °C for 72 h. Following the remainder of the incubation period, the cytotoxicity of KHYG-1 against K562 cells was evaluated using a DELFIA cytotoxicity assay (PerkinElmer). Briefly, the target K562 cells were washed with PBS and labeled with BATDA according to the manufacturer's protocol. The target cells were washed four times with PBS. PH-804-treated or control cells were cultured together with labeled target cells in 96-well round-bottom plates at an effector-to-target (E:T) ratio of 5:1 for 4 h. Controls for maximal TDA release were obtained by incubating target cells with lysis solution, and spontaneous release was measured from target cells incubated without effector cells. Following coculture, 20 µL of the supernatant was combined with 200 µL of DELFIA europium (Eu) solution and incubated for 15 min at room temperature with shaking. The time-resolved fluorescence of the Eu:TDA chelate, which corresponds to the number of lysed cells, was measured using a Victor Nivo plate reader (PerkinElmer). The background signal (media alone) was subtracted from all conditions, and the percentage of specific lysis was calculated relative to the maximum and spontaneous release controls. The supernatants from the coculture were stored for the determination of IFN-γ production.

Primary NK cell in vitro antitumor cytotoxicity assay

The cytotoxic potential of NK cells against tumor cells was also evaluated. After the 14-day expansion protocol, the cells were treated with PH-804 or a non-targeting control for 72 h, followed by washout and a 72-h rest period. For short-term cytotoxicity analysis, treated primary NK cells were then cocultured with K562 target cells in a DELFIA cytotoxicity assay as described above. Following coculture, the supernatants were collected for cytokine analysis.

For long-term cytotoxicity analysis, real-time cell analysis was performed using an xCELLigence assay (Agilent). Briefly, SKOV3 cells were seeded in 96-well E-plates (Agilent) at a density of 5000 cells per well and allowed to adhere for 24 h. Primary human NK cells that had been treated with PH-804 at 2µM were collected and added to the E-plates containing SKOV3 cells at an effector-to-target (E:T) ratio of 2:1. All conditions were seeded in triplicate. The xCELLigence system continuously monitored proliferation by measuring impedance every 15 min over a period of 80 h. Data were collected and analyzed using xCELLigence RTCA software. Cell killing was assessed by comparing impedance values of wells containing NK cells and SKOV3 cells to control wells with only SKOV3 cells and represented as a normalized cell index.

Cytokine secretion of INTASYL-treated NK cells

Cytokine production by NK cells in coculture experiments was measured using IFN-γ and Granzyme B Quantikine ELISA kits (R&D Systems) following the manufacturer's protocols. Supernatants were collected from the coculture experiments (~ 25,000 NK cells per well) and stored at − 80 °C until use.

Statistical analysis

The means of the biological replicates were assessed for each experiment and intercompared by one-way ANOVA and Tukey's multiple comparisons post hoc tests using Prism 9.4 (GraphPad). $P < 0.05$ indicated statistical significance.

Results

INTASYL compound identification and selection

The INTASYL compound PH-804 is a chemically modified asymmetric siRNA duplex comprising a 20-nucleotide antisense (guide) strand and a 15-nucleotide sense (passenger) strand with a 15-base pair duplex and a single-stranded phosphorothioate tail. The compound contains 2′-fluoro and 2′-O-methyl chemical modifications to confer stability, and the 3'-end is conjugated to cholesterol via a triethylene glycol (TEG) linker (Fig. 1A). The combination of stabilizing modifications, the presence of cholesterol, and the single-stranded phosphorothioate tail allows for rapid cellular uptake by endocytosis without the need for additional formulations or delivery vehicles [23].

To evaluate cellular uptake by natural killer (NK) cells, fluorescent Cy3-conjugated INTASYL was administered to primary human NK cells. After 24 h of exposure to INTASYL, greater than 95% uptake efficiency was detected via fluorescence microscopy (Fig. 1B). Flow cytometry analysis revealed complete and uniform uptake by NK cells (Fig. 1C).

To test which INTASYL sequences most effectively inhibit TIGIT expression in NK cells, four INTASYL compounds were designed and synthesized. The efficacy of mRNA silencing was examined in KHYG-1 cells using RT−qPCR. Three of the four TIGIT-targeting INTASYL compounds resulted in the silencing of TIGIT mRNA compared

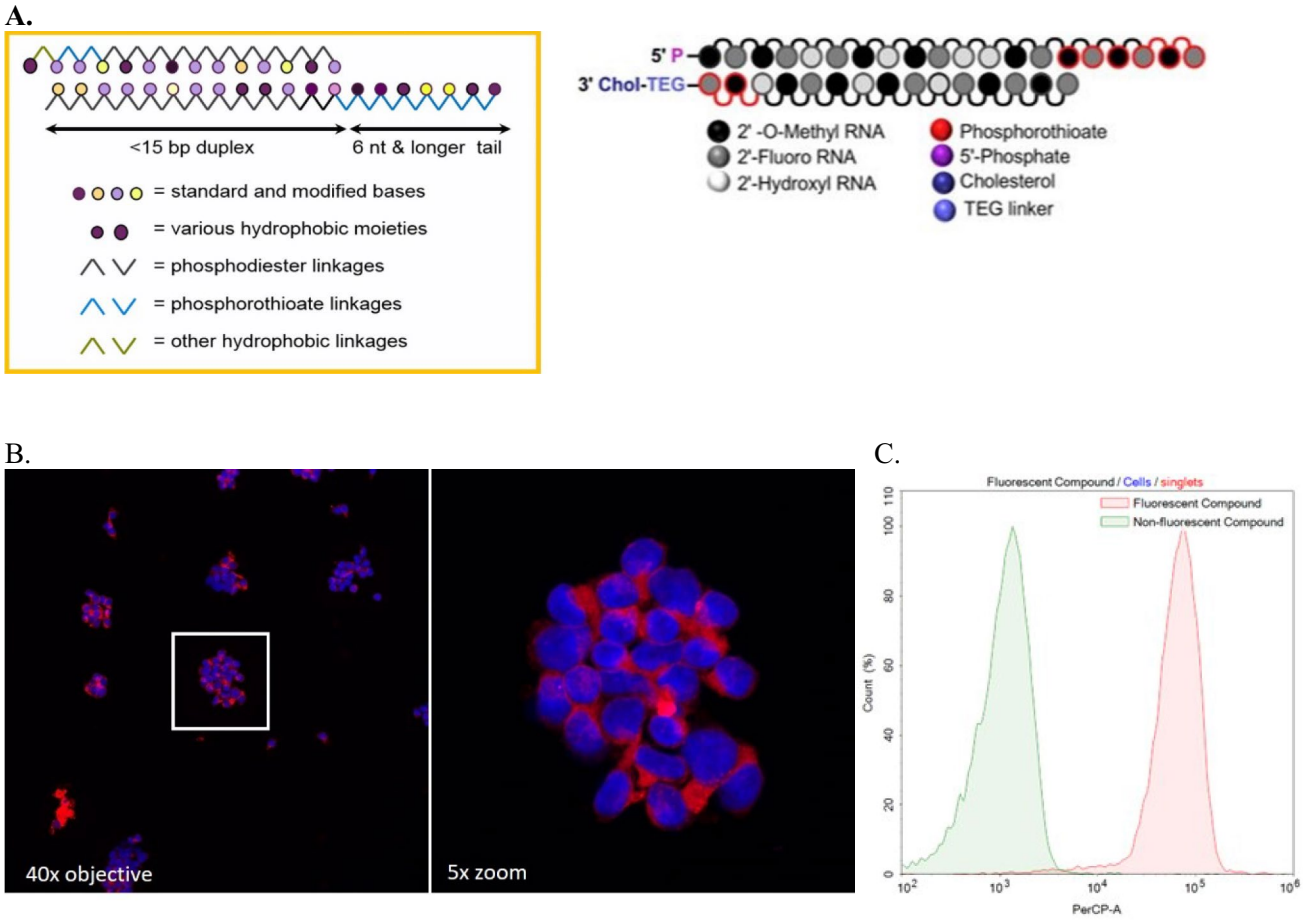

Fig. 1 INTASYL, a modified asymmetric self-delivering siRNA, is rapidly and efficiently taken up by NK cells. **A.** Schematic diagram of INTASYL self-delivering RNA. INTASYL compounds are asymmetric RNAi compounds comprising a small duplex region (< 15 base pairs) and a single-stranded phosphorothioate tail (≥ 6 nucleotides). In addition, INTASYL compounds are chemically modified by stabilizing and hydrophobic modifications (e.g., sterol), which confer stability, efficient cellular uptake and a reduced inflammatory response. **B.** Fluorescent INTASYL uptake by NK cells is shown. The cells were transfected for 24 h in RPMI medium supplemented with 10% FBS. Following transfection, the cells were incubated with Hoechst dye (blue), washed with PBS, and mounted for confocal imaging. Fluorescence images were captured on a Leica DM IRE2 confocal microscope using a 40× objective. Standard lasers and filters were used to image Hoechst and DY-547 fluorescently labeled INTASYL. A representative image is shown. **C.** Flow cytometry analysis was performed on NK cells that were treated with either a fluorescent or nonfluorescent INTASYL compound as described above. The cells were analyzed using a NovoCyte flow cytometer (ACEA) and analyzed using NovoExpress software (ACEA)

to that in the untreated controls. The lead compound PH-804 was able to silence TIGIT mRNA and protein by approximately 60% at 2 µM compared to that in untreated (UTC) controls (Fig. 2A **and** Fig. 2C). The IC50 values for the silencing of PH-804 for both TIGIT mRNA and protein were determined in KHYG-1 cells (Fig. 2B and Fig. 2D). The IC50 for TIGIT mRNA silencing by PH-804 in KHYG-1 cells was 0.5885 µM, and the IC50 for TIGIT protein silencing by PH-804 was 1.532 µM. Importantly, PH-804 treatment of KHYG-1 cells did not negatively impact cell viability (Fig. 2E).

To determine the duration of PH-804-mediated silencing of the TIGIT protein in the KHYG-1 cell line, the cells were treated with PH-804 or the appropriate control for 48 h. After the 48-h treatment period, the cells were collected, resuspended in fresh media without compound, and cultured for an additional 8 days with regular media addition to determine the duration of silencing. PH-804-mediated TIGIT protein silencing of approximately 30% was observed for up to 4 days following the washout of the compound (Fig. 3).

PH-804-mediated silencing of TIGIT mRNA and surface protein expression was confirmed in primary NK cells using RT−qPCR and flow cytometry, respectively (Fig. 4A, C). Treatment with 5 µM PH-804 for 72 h elicited a 40% reduction in TIGIT mRNA expression and a subsequent reduction in TIGIT$^+$ cells in primary NK cells compared to those in negative controls (Fig. 4D). INTASYL treatment did not negatively affect cell viability (Fig. 4B). To determine the

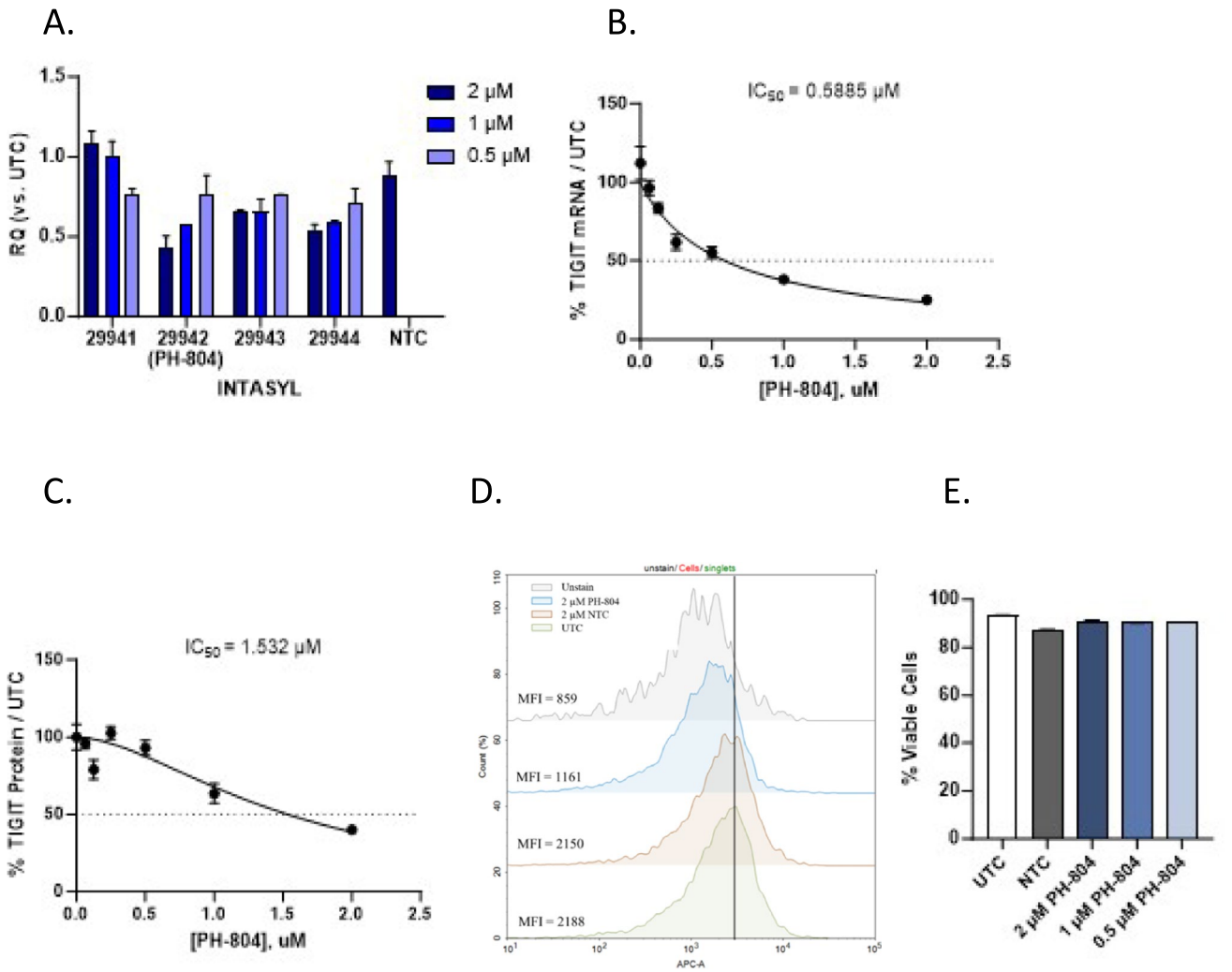

Fig. 2 Identification and confirmation of lead TIGIT-targeting INTASYL compound **A.** TIGIT silencing by the four candidate TIGIT-targeting INTASYL compounds. KHYG-1 cells were treated with INTASYL for 72 h. TIGIT mRNA silencing was detected by qPCR using TaqMan probes, and the results were normalized to the PPIB mRNA levels. **B.** mRNA IC_{50} calculation in KHYG-1 cells **C**. Protein IC_{50} determination in KHYG-1 cells by flow cytometry and representative histogram gated on live singlets (**D.**) E. Viability of KHYG-1 cells as determined by 7-AAD viability staining. $n = 3$ per experiment

duration of TIGIT protein reduction in primary NK cells after PH-804 treatment, PH-804 was washed out following 48 h of treatment, and the cells were cultured for up to 8 days. On day 6 after washout, a 35% reduction of TIGIT surface protein is observed in primary NK cells (Fig. 4E, F).

Silencing of TIGIT by PH-804 results in increased killing of tumor cells and release of IFNγ and granzyme B by KHYG-1 cells

To determine whether TIGIT silencing translated into increased killing of target K562 tumor cells, a DELFIA cytotoxicity assay was performed. Following 72 h of treatment and an additional 72 h of culture in the absence of the compound, KHYG-1 cells were cocultured with BATDA-loaded K562 target cells for 4 h at an E:T ratio of 5:1. Specific lysis of the target cells increased significantly with PH-804-mediated TIGIT silencing (Fig. 5A). Moreover, significantly greater levels of IFN-γ and granzyme B were detected in the supernatants from TIGIT-silenced KHYG-1 cells than in those from control cells (Fig. 5B and C).

Silencing of TIGIT by PH-804 results in increased killing of tumor cells by primary NK cells

The ability of PH-804 to improve the functionality of primary human NK cells was confirmed by testing the ability of PH-804-treated primary NK cells to kill K562 tumor cells via the DELFIA cytotoxicity assay, as described above. At all tested concentrations of PH-804,

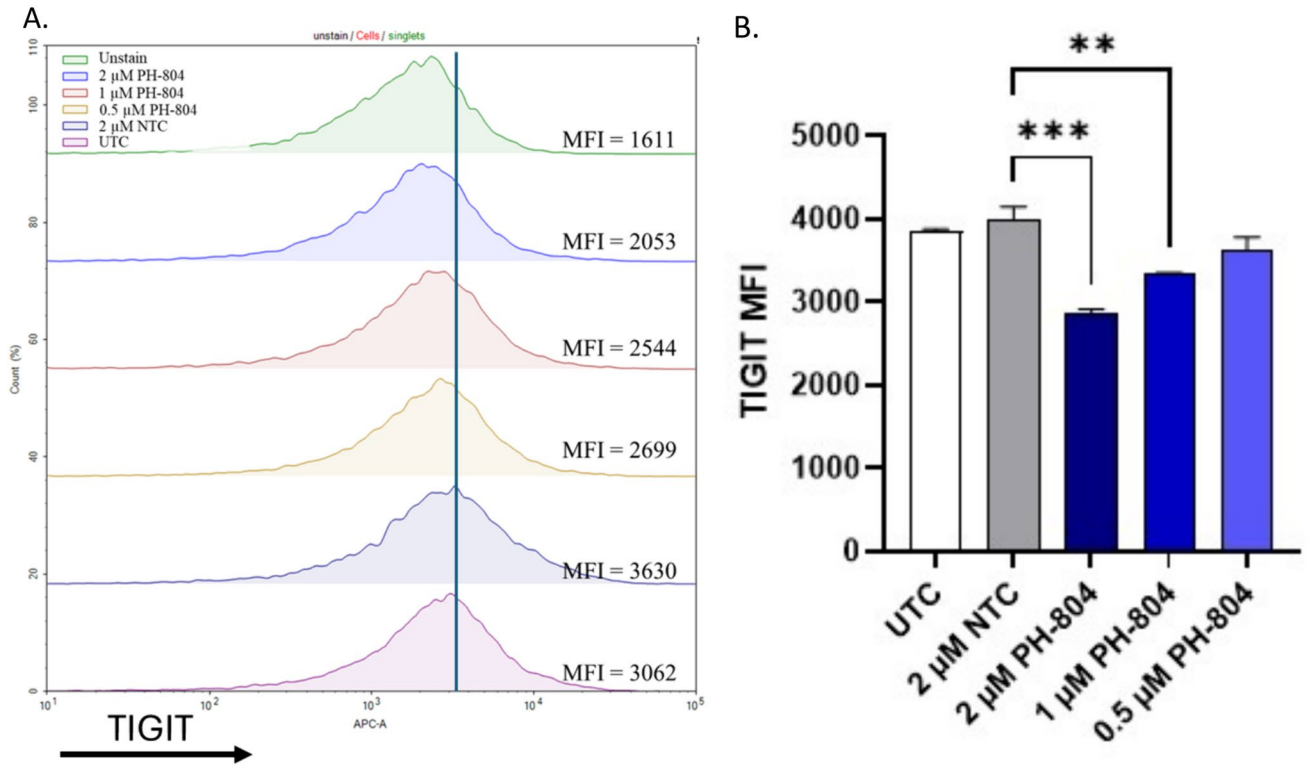

Fig. 3 TIGIT silencing by PH-804 cells lasts for at least 7 days in KHYG-1 cell line. Duration of TIGIT expression in KHYG-1 cells after PH-804 treatment. After 48 h of treatment, PH-804 was washed out and the cells were cultured for up to 10 days. (**A.**) Representative histograms gated on live singlets on day 10 (day 8 after washout); representative $N=3$ per experiment. (**B.**). Summary of $N=3$ per experiment. The bars are the mean fluorescence on day 10 (day 8 after washout). The error bars represent the SEMs. The statistical significance of differences in group means was compared by one-way ANOVA and Tukey's multiple comparisons post hoc tests. $^{***}p<0.001$; $^{*}p<0.05$

a significant increase in the specific lysis of K562 target tumor cells was observed compared to that in the control-treated cells (Fig. 6A). This increased lysis of target cells was accompanied by a significant increase in both IFN-γ and granzyme B levels in the supernatants collected following coculture, as detected by ELISA (Fig. 6B and C). Both 2 µM and 1 µM PH-804 induce similar levels of specific lysis, despite differences in IFN-γ production and granzyme release. The higher IFN-γ and granzyme levels at 2 µM may result from more comprehensive TIGIT silencing, leading to increased NK cell activation. This suggests that while both concentrations are effective in inducing cytotoxicity, 2 µM may offer additional immunological benefits by further enhancing NK cell function. Furthermore, in a long-term cytotoxicity analysis using the xCELLigence system, PH-804-treated NK cells exhibited enhanced killing of SKOV3 tumor cells compared to control-treated NK cells, as indicated by the normalized cell index (Fig. 7).

Discussion

We investigated the potential of improving NK cells for adoptive cell therapy by utilizing INTASYL, a novel proprietary self-delivering siRNA, to transiently silence the inhibitory protein TIGIT.

Efforts to use siRNA to improve the functionality of cells for adoptive cell therapy have largely focused on T cells and have been hindered by inefficient delivery methods. Common transfection methods, such as electroporation or lipid-mediated delivery, result in diminished cell viability and undesired activation of cells [19]. Emerging siRNA technologies, such as nanoparticles, show increasing promise but are still limited by poor water solubility, poor hydrophobicity, and limited bioaccumulation [24, 25]. INTASYL technology can circumvent the hurdles associated with other siRNA technologies. The chemical modification strategy employed by INTASYL allows rapid

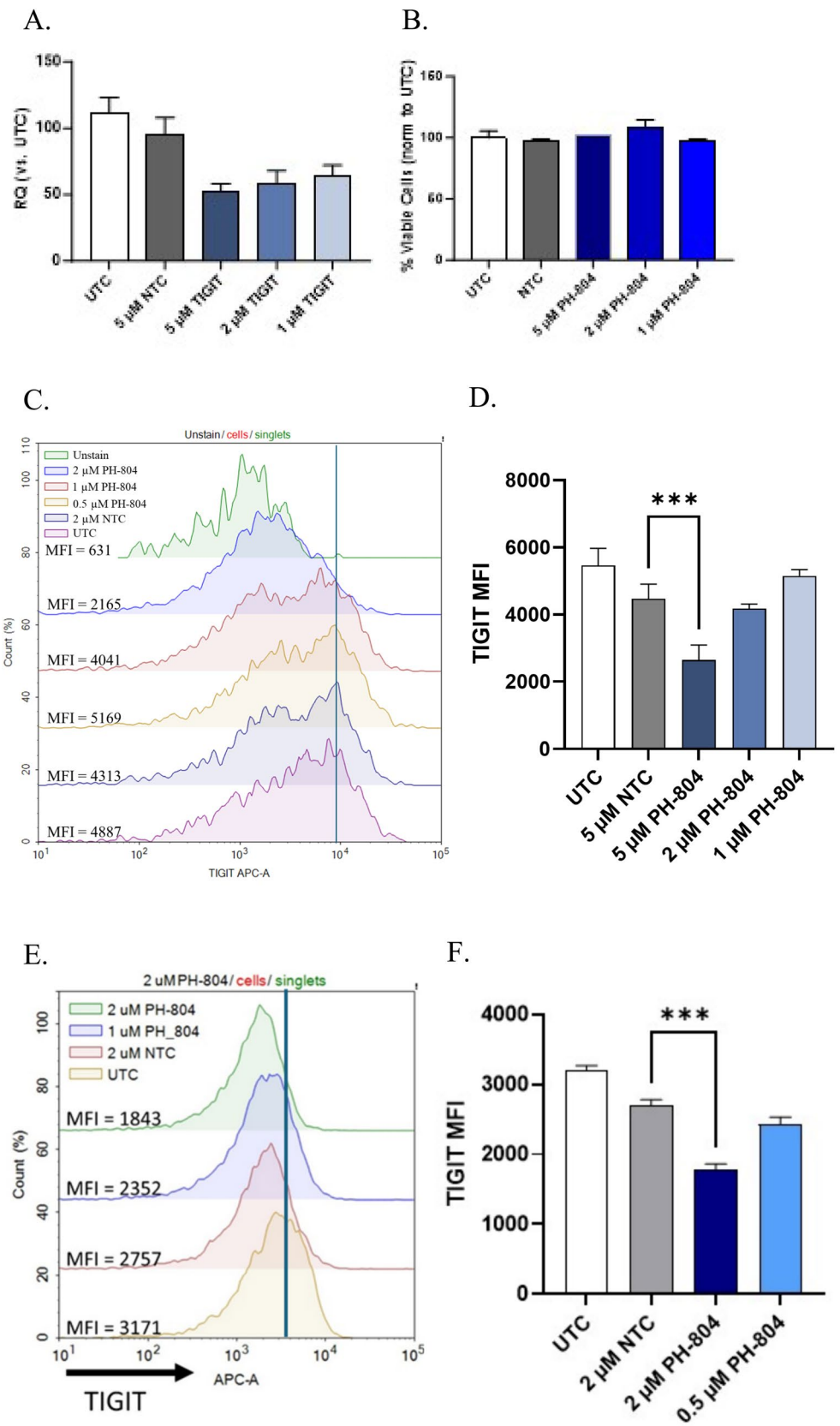

Fig. 4 PH-804 silences TIGIT mRNA and protein in primary NK cells without negatively impacting cell viability. **A.** TIGIT mRNA silencing in primary NK cells treated with PH-804 INTASYL for 72 h. TIGIT mRNA silencing was detected by qPCR using TaqMan probes, and the results were normalized to the PPIB mRNA levels. **B.** Viability of primary NK cells as determined by 7-AAD viability staining after 72 h of PH-804 treatment. **C/D.** TIGIT protein silencing in primary NK cells as determined by flow cytometry gated on live singlets. Summary of $N=3$ per experiment. The bars are the mean fluorescence on day 3 after different treatments, and the error bars represent the SEMs. To determine duration of TIGIT protein reduction in primary NK cells after PH-804 treatment, PH-804 was washed out following 48 h treatment and the cells were cultured for up to 8 days. (**E.**) Representative histograms gated on live singlets on day 8 (day 6 after washout); representative N = 3 per experiment. (**F.**). Summary of N = 3 per experiment. The bars are the mean fluorescence on day 8 (day 6 after washout). The error bars represent the SEMs. The statistical significance of differences in group means was compared by one-way ANOVA and Tukey's multiple comparisons post hoc tests. $***p<0.001$

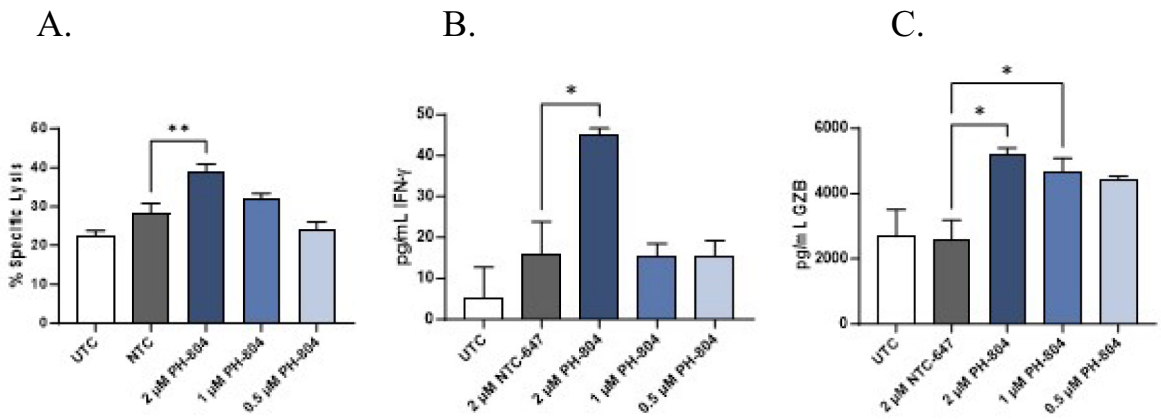

Fig. 5 Increased target cell killing and IFN-γ and granzyme B release in the KHYG-1 cell line. Treatment of KHYG-1 cells with PH-804 resulted in increased K562 cell killing **(A)** and IFN-γ **(B.)** and granzyme B release **(C.)** by KHYG-1 cells following 4 h of coculture with K562 cells. n = 3 per experiment. The statistical significance of differences in group means was compared by one-way ANOVA and Tukey's multiple comparisons post hoc tests. $^{**}p < 0.01$; $^{*}p < 0.05$.

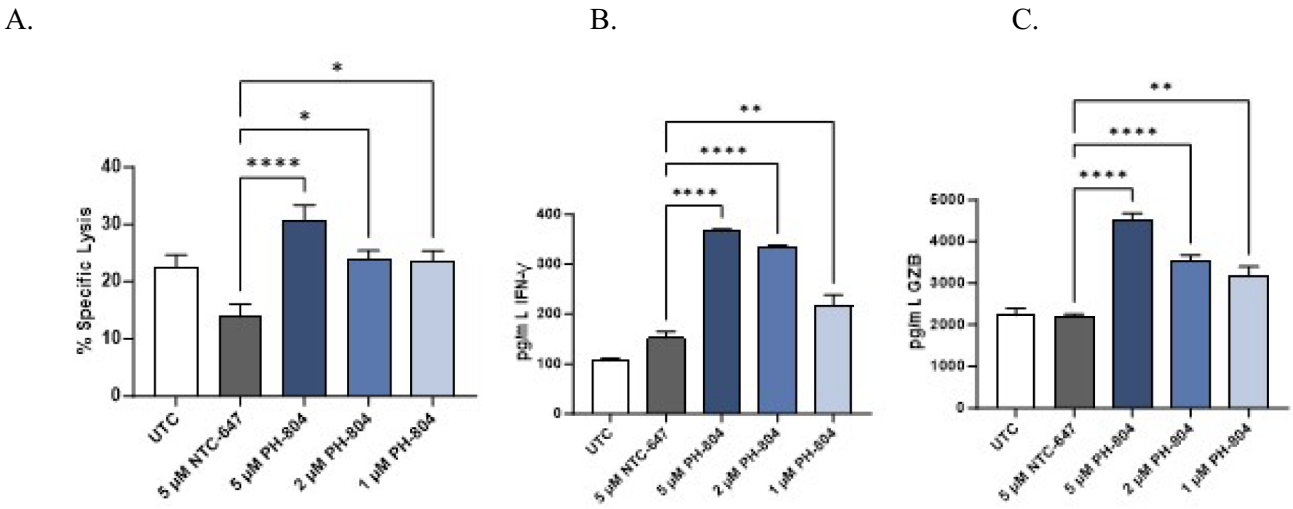

Fig. 6 Increased target cell killing, IFN-γ, and granzyme B release in PH-804-treated primary NK cells. Treatment of primary NK cells with PH-804 resulted in increased K562 cell killing **(A)** and IFN-γ **(B.)** and granzyme B release **(C.)** following 4 h of coculture with K562 cells. $n = 3$ per experiment. The statistical significance of differences in group means was compared by one-way ANOVA and Tukey's multiple comparisons post hoc tests. $^{****}p < 0.0001$; $^{**}p < 0.01$; $^{*}p < 0.05$

and efficient cellular uptake without the need for additional formulation or mechanical delivery. These modifications confer stability and result in long-lasting silencing of the target gene compared with other siRNA modalities [21].

INTASYL treatment of both an immortalized natural killer cell line (KHYG-1) and expanded primary human NK cells with PH-804 resulted in efficient TIGIT mRNA and protein silencing. This gene silencing has been shown to last for 7 days or longer in KHYG-1 cells and persist for at least 6 days in primary NK cells. Silencing of TIGIT by INTASYL in expanded primary NK cells represents an approach to more than double the killing of target cancer cells when administered in a coculture setting with K562 cells.

Current methods for blocking TIGIT are restricted to antibodies that are applied systemically [14], bearing the risk of peripheral immune-related adverse events, and have yielded lackluster results as monotherapies. Severe treatment-related adverse events (TRAEs) occurred in more than 20% of patients across several anti-TIGIT antibody trials, resulting in termination of the trials [26]. By treating NK cells ex vivo with PH-804, TIGIT can be directly inhibited on NK cells during the ACT cell product manufacturing process. This avoids the systemic administration of antibodies, provides

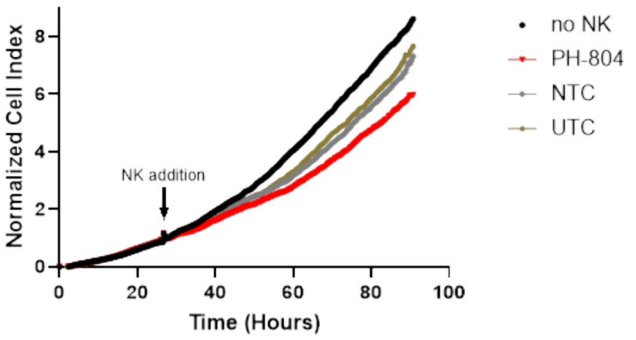

Fig. 7 Increased killing of SKOV3 by TIGIT-silenced primary NK cells. Treatment of primary NK cells with PH-804 resulted in increased SKOV3 cell killing over 80 h of coculture. The impedance values, represented as a normalized cell index, showed an increase in SKOV3 cell killing with TIGIT-silenced NK cells (PH-804) compared to the non-targeting control

more targeted inhibition, and reduces the risk of undesirable side effects. The targeted specificity of PH-804 toward a conserved region of the TIGIT gene diminishes the likelihood of cells developing resistance against its action.

Natural killer cells carry high levels of cytolytic vesicles with cytolytic proteins, such as granzymes, which are capable of inducing cell death upon release [27, 28]. Granule release is the first step in cytotoxic processes. Increased levels of Granzyme B were detected in coculture supernatants from NK cells treated with PH-804 compared with those from control cells. These findings suggested that PH-804-treated NK cells would have a greater capacity to kill tumor cells in an adoptive cell therapy setting. It was observed that both 2 µM and 1 µM PH-804 perform similarly for inducing specific lysis despite differences in IFN-γ production, and granzyme release suggesting that higher concentrations of PH-804 may offer additional immunological benefits by further enhancing NK cell function. This underscores the importance of considering both cytotoxicity and immune activation when evaluating PH-804 efficacy.

TIGIT silencing of NK cells could translate clinically to improved off-the-shelf NK products for adoptive cell therapy of hematological malignancies and potentially solid tumors. INTASYL is particularly attractive for this modality compared with the CRISPR−Cas9 system. CRISPR−Cas9 technology has several limitations, including a lack of editing efficiency, incomplete editing, low penetrance in the final cell product, and concerns regarding the inheritance of edited genes [29, 30]. INTASYL-based gene silencing in NK cells allows precise transient silencing of the target gene with nearly complete cellular uptake.

While NK-ACT is emerging as a monotherapy, NK cells are also attractive because ACT is complementary to T-cell therapy [31, 32]. Their cytotoxic activity does not require priming; therefore, their antitumor activity may precede that of T cells. Moreover, because their target recognition does not rely on antigen processing and presentation, NK cells can act on tumor cells to evade T-cell recognition by downregulating MHC class I and antigen expression. In addition to killing tumor cells, activated NK cells also secrete cytokines and chemokines to create a proinflammatory environment and recruit effector immune cells (T cells and DCs) to the tumor microenvironment, thereby enabling and enhancing the antitumor effects of the adaptive immune response [33, 34].

NK cells are the major initial source of IFN-γ prior to its production by T cells, which requires priming and activation events. Secreted IFN-γ plays an important role in modulating the immune response by increasing MHC expression to improve T-cell recognition by tumor cells [35, 36]. Treatment with PH-804 increased the release of IFN-γ by NK cells during coculture with tumor cells. In an adoptive cell therapy setting, increased IFN-γ could result in a more robust adaptive immune response to cancer cells, thereby potentiating the impact of ACT on both NK cells and T cells, creating an environment permissive for enhanced immune cell recruitment and activity.

Taken together, these data show that PH-804 can be used to silence the inhibitory checkpoint receptor TIGIT in NK cells. This inhibition leads to an increased cytotoxic capacity of NK cells and improved tumor cell killing. The incorporation of PH-804 into the NK cell manufacturing process for adoptive cell therapy could be a promising way to improve NK cell-based therapies for cancer treatment.

While our study provides compelling evidence for the therapeutic potential of TIGIT silencing using INTASYL, we acknowledge certain limitations. The current work is based solely on in vitro experiments, and additional studies are needed to confirm these findings in vivo. Moreover, while we have demonstrated enhanced NK cell function following TIGIT silencing, further research is required to explore the long-term effects of this approach on NK cell proliferation, exhaustion, and overall persistence within a tumor microenvironment.

Nevertheless, our study represents a pioneering effort to explore the use of INTASYL technology for TIGIT silencing in NK cells, and we believe it opens new avenues for research and development in the field of adoptive cell therapy. These findings should inspire further investigation into the therapeutic potential of this approach, particularly in combination with other immune-modulatory strategies aimed at enhancing NK cell-mediated cytotoxicity against solid tumors.

We anticipate that this novel application of INTASYL could lead to improved outcomes in NK cell-based therapies and encourage further exploration into its use in various cancer types, including those with solid tumor origins. Our work lays the groundwork for future studies aimed

at optimizing and translating this approach into clinical settings.

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Author contributions M.M. designed and carried out the experiments and wrote the manuscript. B.R. and A.B. contributed to sample preparation. D.Y. synthesized the INTASYL compounds. J.C. and E.N. contributed to the analysis of the results. All authors discussed the results and contributed to the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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