The Use of Self-delivering RNAi to Enhance NK Cell Cytotoxicity

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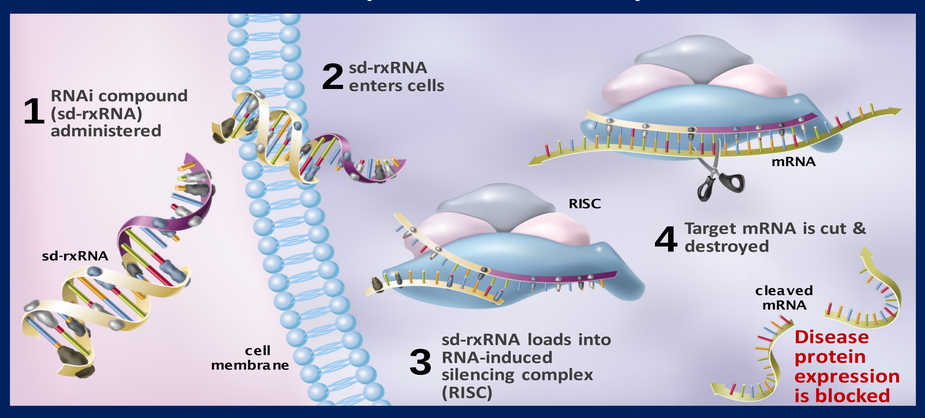
Abstract

NK cells are key players in a body's fight against cancer. They rapidly recognize and kill tumor cells without prior sensitization. NK cells are an attractive candidate for use in adoptive cell therapy (ACT) because they are not required to be matched to a specific patient, making an off-the-shelf NK therapy product possible. Therapeutic use of NK cells shows promise against hematological cancers but the cytotoxic activity of these cells is limited by inhibitory receptors and pathways. Overexpression of such receptors has been shown to reduce NK cell-mediated cytotoxicity. Overcoming this inhibition would allow for a more potent antitumor response following ACT. We have developed a new class of stable, self-delivering RNAi compounds or sd-rxRNAs® that incorporate features of RNAi and antisense technology. The work presented here shows that sd-rxRNAs are rapidly and efficiently taken up by immune effector cells without the use of transfection reagents. Using sd-rxRNA compounds against checkpoint inhibitors, we can suppress their expression levels by up to 95% in immune cells including T cells and NK cells. Furthermore, we demonstrate potent activity and stability in NK cells which is maintained through cryopreservation. By treating NK cells *ex vivo*, prior to ACT with sd-rxRNA reducing the expression of proteins such as Cbl-b, the anti-tumor response of these cells can be improved. Ongoing work expands these findings to include compounds for more NK specific targets, including NK specific inhibitory receptors, which could be used alone or in combination. Improved NK cytotoxic activity as a result of sd-rxRNA treatment during NK manufacturing is a promising approach towards more potent off-the-shelf therapy for hematological malignancies.

sd-rxRNA can penetrate immune cells, where antibodies fall short, and block the expression of disease proteins



RNA interference (RNAi) is a naturally occurring cellular process. Introduction of double stranded RNA into cells can result in association with the RNA-induced silencing complex (RISC) to target complementary mRNA sequences and degrade target genes. sd-rxRNAs are asymmetric RNAi compounds comprised of a small duplex region (≤ 15 base pairs) and a single-stranded phosphorothioate tail (4 to 12 nucleotides). In addition, sd-rxRNA compounds are chemically modified with stabilizing and hydrophobic modifications (e.g., sterol), which confer stability, efficient cellular uptake and reduced inflammatory response.

Figure 1: Mechanism of Cellular Gene Silencing using sd-rxRNA

Results

To test the uptake of sd-rxRNAs by immune effector cells without the use of transfection reagents, various human immune effector cells were transfected with 2uM fluorescently labeled sd-rxRNA in standard culture media for 24 hours. Cells were fixed with paraformaldehyde for 10 minutes and mounted for confocal microscopy. Results show rapid and efficient uptake in all cell types tested (Figure 2).

Broad Applicability - Empower existing clinical treatment paradigms *and* expand applicability of engineered cells

(sd-rxRNA shown in red, nucleus shown in blue)

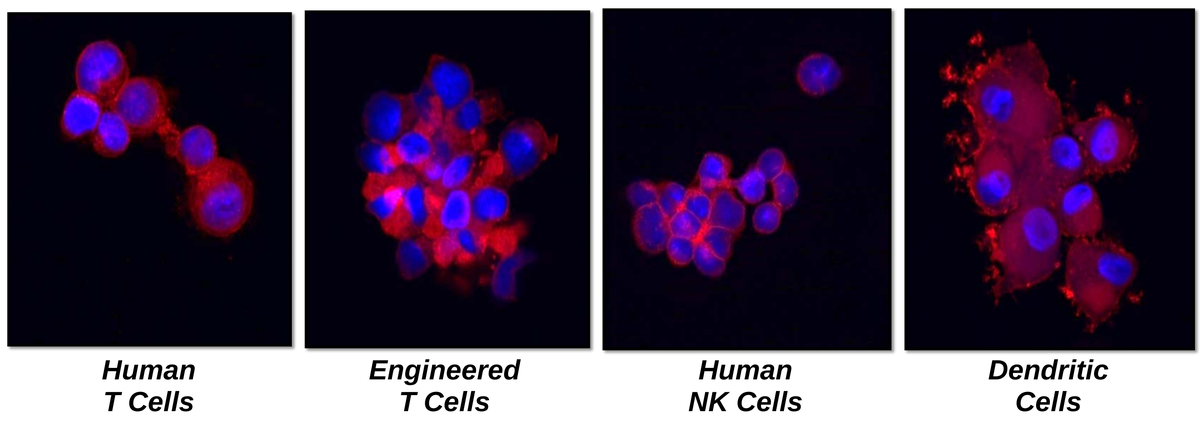
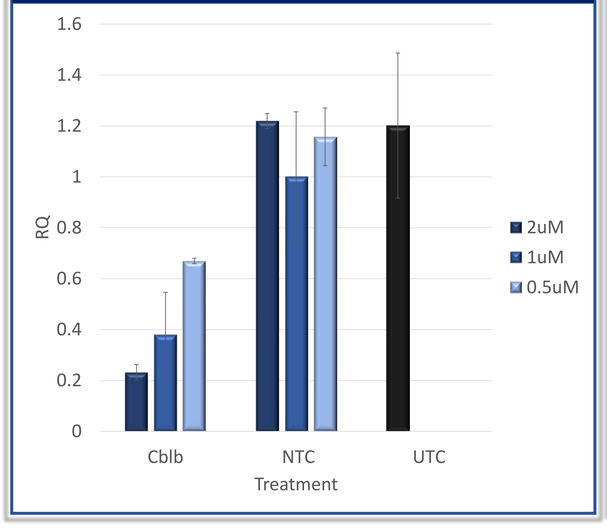


Figure 2: Fluorescently labeled sd-rxRNA is taken up efficiently within 24 hours

NK cell work included tests with RNAi against an immune checkpoint protein and an NK specific inhibitory receptor. Fresh human NK cells were isolated using negative selection and cultured in standard culture media containing IL-2. Twenty-four hours after isolation, cells were collected for transfection and the cell concentration was adjusted to ~1 x 10^6 cells/mL in RMPI media containing IL-2. Cells were seeded directly into 24-well plates containing chemically-optimized sd-rxRNA targeting Cbl-b ranging in final concentration from 0.5 µM to 2 µM. Taqman gene expression assays were used to determine expression levels of Cbl-b following the RNA to Ct 1-step protocol. Cbl-b targeting sd-rxRNA in human NK cells cause potent and long-lasting reduction of Cbl-b mRNA (Figure 3). Further analysis of the effects of siRNA on NK cells was performed on an NK-specific target. mRNA levels were measured after a 72 hour transfection, and surface expression of the target protein was analyzed by flow cytometry after 5 days (Figure 4).

A single dose of Cbl-b targeting sd-rxRNA is able to reduce Cbl-b mRNA levels by greater than 75% after 72 hours in freshly isolated human NK cells



The reduction of Cbl-b mRNA by >30% persists for up to 10 days with a single dose of sd-rxRNA

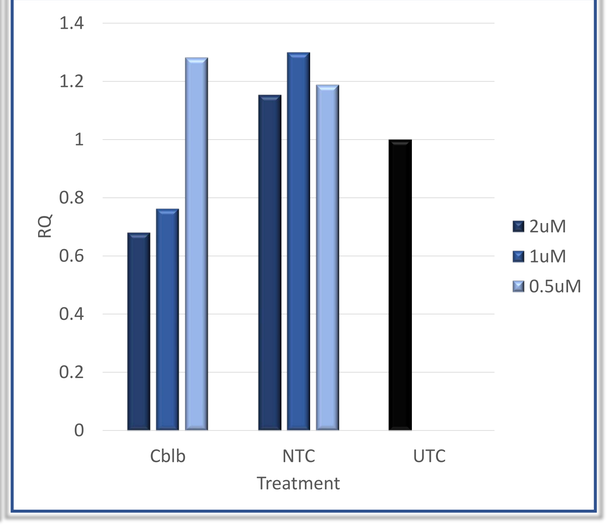
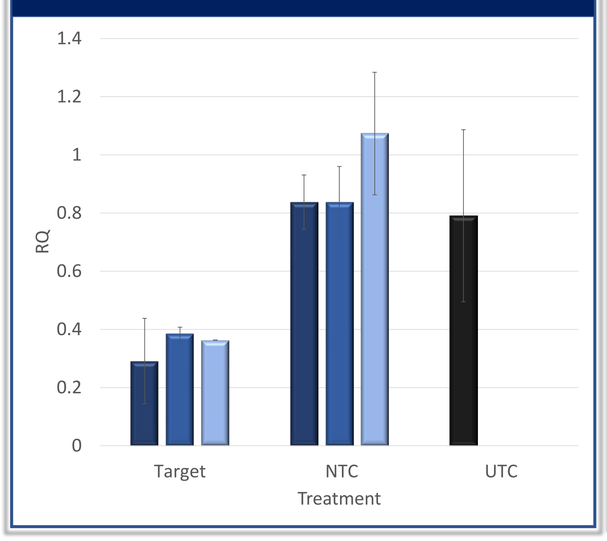


Figure 3: Cbl-b targeting sd-rxRNA in human NK cells cause potent and long-lasting reduction of Cbl-b mRNA

mRNA reduction of an NK-specific target is reduced after a 72 hour transfection with siRNA



Surface reduction of the target protein is seen 5 days after transfection with a targeting siRNA

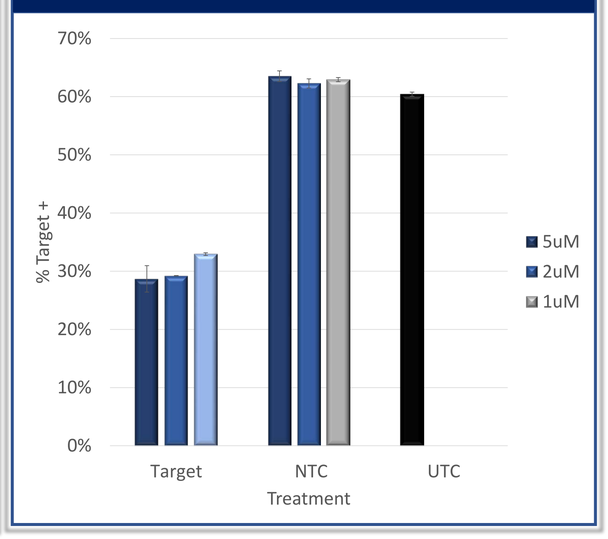
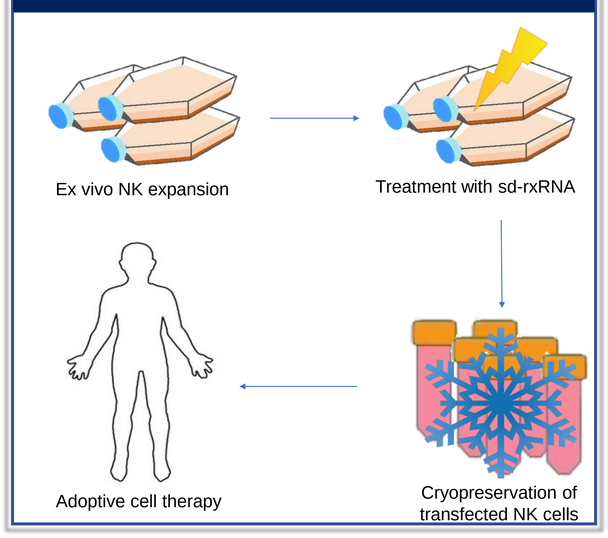


Figure 4: using siRNA in human NK cells cause reduction of target mRNA which leads to a reduction in surface expression of the target protein

To test whether a freeze/thaw cycle would negatively impact sd-rxRNA silencing of Cbl-b, freshly isolated human NK cells were transfected with Cbl-b-targeting sd-rxRNA for 48 hours. After 48 hours, cells were pelleted and resuspended in freeze medium containing 10% DMSO, cooled to -80ºC overnight then transferred to cryostorage for 72 hours. After 72 hours, the cells were thawed into standard culture medium and incubated an additional 24 hours. Silencing of Cbl-b expression after a freeze/thaw cycle was similar to that of cells that had been transfected for 72 hours without freeze/thaw cycle (Figure 5).

sd-rxRNA treatment is compatible with "off-the-shelf" (i.e. cryopreserved) NK product for adoptive cell therapy



Target protein silencing is not negatively impacted by freeze/thaw cycle (cbl-b data shown)

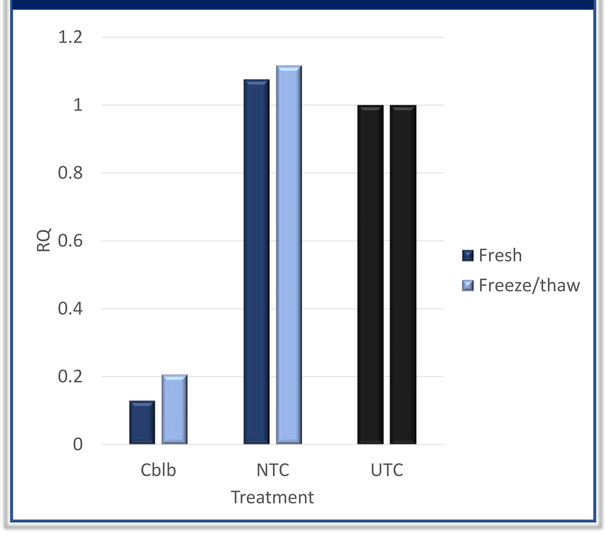


Figure 5: Cbl-b silencing is seen after freeze/thaw cycle making sd-rxRNA treatment compatible with current NK cell therapy protocols

Conclusions

These data demonstrate the potential of using sd-rxRNA to change NK cell phenotype for use in adoptive cell therapy. The use of these compounds is compatible with existing manufacturing paradigms (no need for transfection reagents, compatible with freeze/thaw cycles). Potent and long lasting reduction of target proteins can be achieved. By treating NK cells with sd-rxRNA targeting immune checkpoints - such as Cbl-b - or other inhibitory receptors, the anti-tumor response of these cells may be enhanced.

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