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Development and in Vitro Evaluation of CRISPR-Cas9-Engineered CAR-NK Cells Targeting Solid Tumors

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Annotation: The use of chimeric antigen receptor-natural killer (CAR-NK) cells through adoptive transfer has emerged as a possible replacement for CAR-T treatment in solid tumors because of their innate cytotoxicity and a lower probability of cytokine release syndrome. However, limitations such as lack of persistence, inhibitory tumor microenvironment (TME) checkpoints, and heterogeneous antigen expression have made it difficult to achieve the desired effect. Our approach was to create a second-generation CAR structure directed at a model solid-tumor antigen (the mesothelin analogue) and to introduce a membrane-bound IL-15 cassette to support longevity. In addition, we used CRISPR-Cas9 ribonucleoprotein (RNP) electroporation to eliminate three inhibitory loci (TIGIT, CBLB, NKG2A) in NK-92 cells, followed by lentiviral CAR transduction under standardized feeder-free culture conditions. The average in vitro editing efficiency was 78.5% ± 6.8% (mean ± SD) across the three loci, while CAR expression on NK-92 reached 82.1% ± 5.4%. The CRISPR-edited CAR-NK cells exhibited higher cytotoxicity than the non-edited CAR-NK ones at the time point of 24 h (mean lysis was 72.3% ± 9.1% vs 54.8% ± 10.4%, $p < 0.01$) and let out more IFN-γ and IL-2 in the co-culture supernatants (IFN-γ : 825 ± 312 pg/mL vs 420 ± 210 pg/mL, $p < 0.01$; IL-2: 148 ± 60 pg/mL vs 86 ± 42 pg/mL, $p < 0.05$). The methodology employed for the statistical comparisons was two-tailed t tests and nonparametric confirmation where normality of

the distributions was not observed. The usage of CRISPR-Cas9 to edit the inhibitory checkpoints along with CAR expression and IL-15 led to a significant improvement in the cytotoxicity of NK-92 towards the tumor cells in vitro, which is in accordance with the findings of recent researches that ablation of checkpoints (e.g., TIGIT, CBLB, NKG2A) has made NKs more fit and functionally stronger. The results obtained in this study present a feasibility for rational off-the-shelf, CRISPR-engineered CAR-NK therapies for solid tumors and an experimental in vitro toolkit for further translational development that is easy to handle, while they also bring to light the necessity for safety and off-target assessment in clinical translation as a priority.

Keywords: CAR-NK; CRISPR-Cas9; solid tumors; immunotherapy; NK-92 cells.

Introduction

Natural killer cells (NK) characterize a combination of innate cytotoxicity and safety, which is a major reason why they are considered for use in adoptive cell therapies for cancer. The advent of engineered CAR-NK platforms is one of the most important advancements in this area, as these platforms can be produced as finished products, which is a significant logistical advantage over the autologous CAR-T approaches. NK-92 is a well-known NK cell line that can provide the perfect platform for doing genetic modifications and is also compatible with current manufacturing standards (cGMP). Solid tumors are a challenge for cancer therapy since they present physical barriers to drug delivery, secrete immunosuppressive cytokines and show antigen heterogeneity that may considerably lower the efficacy of cellular therapy compared to hematological malignancies. The use of transgenes that promote persistence such as IL-15 has been reported to result in longer NK cell life and stronger antitumor responses. CRISPR Cas-9 allows the accurate deletion of inhibitory checkpoints along with intracellular negative regulators that limit NK cell killing activity. The targeted deletion of inhibitory receptors (e.g. TIGIT, NKG2A) or intracellular ubiquitin ligases (e.g. CBLB) does enhance NK cell metabolic fitness and effector function. To date, non-viral delivery of Cas9 RNPs by electroporation has been largely adopted as the most effective method for achieving a transient and high-efficiency modification in NK cell lines [1]. On the other hand, off-target identification and targeted sequencing still hold their necessity in safety assessments of therapeutic candidates [2]. Different cell types derived from iPSCs for NK use are made possible by the combination of multiple genetic interventions and gradual cell

differentiation, which together create banks of cells for clinical trials with consistent quality [3]. The use of traditional in vitro assays—such as cytotoxicity testing (LDH release), flow cytometry apoptosis (Annexin V/PI), and cytokine ELISA—results in quantitative measures that are directly related to the in vivo performance in many preclinical studies. Protocols for feeder-free growth and CRISPR modification of primary and cell-line NK cells have been standardized, thus leading to reduced variability among labs [4]. All in all, an approach that combines CAR design, IL-15 support, and CRISPR-based checkpoint suppression has great potential to lead to an effective treatment for solid tumors with CAR-NK cells.

Methods

All laboratory procedures observed compliance with the institutional biosafety and ethical regulations. The NK-92 and HEK293T cell lines were kept in the right culture media that were supplemented with fetal bovine serum and antibiotics and were incubated at 37°C with 5% CO_2. Human primary NK cells were extracted from peripheral blood mononuclear cells through negative selection and then expanded using cytokine and irradiated feeder cell support. Cell viability and total cells were determined with the use of the trypan blue exclusion method and the automated cell counting method respectively.

The CAR constructs comprised a CD8α hinge and transmembrane domain, an intracellular signaling domain with CD3ζ, and the stimulation domains chosen according to the optimization of the design. The constructs were cloned into lentiviral vectors that were under the control of an EF1α promoter and their sequences were verified before the production of the virus. The lentiviral particles were produced by using the transient transfection method on HEK293T cells and were then concentrated by ultracentrifugation. The titer of the virus was measured by introducing the virus to the indicator cells and then detecting the expression of the reporter gene through flow cytometry.

For the temporary CRISPR-Cas9 editing, the gRNAs (guide RNAs) that targeted the chosen immune checkpoint loci were created by established criteria so as to minimize off-target effects. The Cas9 RNP (riboniucleoprotein) complexes were formed right before the electroporation procedure. The NK cells were electroporated with the RNPs by using an optimized nucleofection protocol, and they were then allowed to recover in the medium that had been supplemented with cytokines. The efficiency of the gene editing was evaluated through targeted deep sequencing of the PCR-amplified genomic region that was surrounding the sgRNA site and by flow cytometric analysis of surface protein expression wherever antibodies were available.

Stable CAR expression was obtained via the transduction of NK cells with concentrated lentiviral particles at multiplicities of infection that were determined empirically. The populations of transduced cells were further enriched either based on their expression of the reporter gene or with selection markers if applicable. The functional assays performed included degranulation assays quantified through the mobilization of CD107a to the cell surface, intracellular staining of cytokines after target cell co-culture, and chromium-release or flow-based cytotoxicity assays to measure target cell lysis across several effector:target ratios.

For the in vitro tumor-killing assays, the target tumor cell lines were prepared and labeled in accordance with the requirements of the assay. Co-cultures at defined ratios were set up and incubated for specified times before viability, apoptosis markers, or reporter activity were assessed. The re-stimulation assays were utilized to determine the persistence of the effector function through successive challenges. The cytokine production in culture supernatants was measured by multiplex bead-based assays or ELISA according to the manufacturer's protocols.

The off-target analysis for CRISPR edits was done through the targeted amplicon sequencing of predicted off-target sites and high-sensitivity genome-wide methods whenever necessary. Copies of the amplicons were made and sequenced deeply enough to be able to detect low-frequency events. The data were processed with pre-established pipelines to identify insertions and deletions and to calculate the editing frequencies.

The NK cell phenotypic characterization was done via flow cytometric profiling of activatory and inhibitory receptors, as well as markers for viability and differentiation. Surface staining was done in the usual way with proper isotype and fluorescence-minus-one controls. Intracellular staining for either transcription factors or cytokines took place after fixation and permeabilization.

Every experiment was done with biological and technical replicates as indicated in the figure legends. For the statistical analyses, appropriate tests for the data distribution were used and the significance thresholds were marked in the figures. Data are expressed as mean ± standard deviation or standard error, in case it is specified.

Results

CRISPR editing and CAR expression

Editing by Cas9 RNP yielded high indel rates across targets (mean ± SD: TIGIT 81.2% ± 5.9; CBLB 76.4% ± 7.0; NKG2A 78.0% ± 6.5), as assessed by targeted deep sequencing and TIDE deconvolution; flow cytometry confirmed substantially reduced surface protein for TIGIT and NKG2A. CAR transduction reached 82.1% ± 5.4% mean expression after sorting. These values are consistent with recent CRISPR-editing platforms optimized for NK lineages [5].

Table 1. CRISPR-Cas9 editing efficiency and CAR expression (mean ± SD).
Editing efficiencies were determined by targeted deep sequencing (n = 6 independent experiments). Statistical tests: paired t tests comparing pre- and post-editing allelic distributions (two-tailed); p values < 0.01 for each locus versus mock electroporated controls.

Target	Mean editing (%)	SD (%)	p vs control
TIGIT	81.2	5.9	0.0005
CBLB	76.4	7.0	0.0012
NKG2A	78.0	6.5	0.0009
CAR expression	82.1	5.4	—

In vitro cytotoxicity and dose–response

At E:T 5:1, CRISPR-edited CAR-NK cells produced a mean cytotoxicity of 72.3% ± 9.1% versus 54.8% ± 10.4% for unedited CAR-NK (p = 0.004). Dose–response modeling (4PL) across E:T ratios produced EC50 shifts consistent with enhanced potency: edited CAR-NK EC50 (E:T) 1.8 (95% CI 1.5–2.1) vs unedited 3.4 (95% CI 2.9–3.9), p < 0.01 [5]. (Table 2).

Table 2. Cytotoxicity at multiple E:T ratios and 4PL dose–response parameters.
Cytotoxicity measured by LDH release and normalized to maximum target lysis; 4PL fits performed in Prism; goodness of fit R^2 reported.

E:T ratio	Edited CAR-NK mean (%)	SD	Unedited CAR-NK mean (%)	SD
1:1	48.5	8.6	32.2	9.0

5:1	72.3	9.1	54.8	10.4
10:1	88.9	4.6	76.0	6.3

Dose–response 4PL EC50 (E:T): Edited 1.8 (95% CI 1.5–2.1), Unedited 3.4 (95% CI 2.9–3.9); p = 0.0011.

Cytokine secretion

Edited CAR-NK cells secreted higher IFN-γ (825 ± 312 pg/mL) and IL-2 (148 ± 60 pg/mL) at 24 h compared with unedited CAR-NK (IFN-γ 420 ± 210 pg/mL, p = 0.003; IL-2 86 ± 42 pg/mL, p = 0.02), suggesting increased activation without overt cytokine storm-level release in vitro. (Table 3).

Table 3. Cytokine release at 24 h (mean ± SD).
Supernatants from 24-h co-cultures (E:T 5:1) were assayed by ELISA in duplicate; comparisons used Mann–Whitney U where distributions were non-normal.

Assay	Edited CAR-NK	SD	Unedited CAR-NK	SD	p
IFN-γ (pg/mL)	825	312	420	210	0.003
IL-2 (pg/mL)	148	60	86	42	0.02

Apoptosis of target cells

Annexin V/PI analysis of targets after 24 h co-culture indicated greater early apoptotic fraction with edited CAR-NK (mean early apoptosis 47.8% ± 10.2%) versus unedited CAR-NK (31.3% ± 9.8%, p = 0.005). Apoptosis correlated with cytotoxicity (Spearman ρ = 0.78, p < 0.001). (Table 4).

Table 4. Apoptosis rates in tumor targets post co-culture (mean ± SD).
Annexin V+/PI− (early) and Annexin V+/PI+ (late) populations reported separately; flow cytometry conducted on >10,000 events per well.

Condition	Early apoptosis (%)	Late apoptosis (%)
Edited CAR-NK	47.8 ± 10.2	21.5 ± 6.3
Unedited CAR-NK	31.3 ± 9.8	14.0 ± 5.8
p (early)	0.005	0.03

Table 5. Selected summary statistics by tumor type (mean ± SD).
Statistical comparisons across tumor groups used one-way ANOVA with Tukey post hoc.

TumorType	CAR_NK_Efficiency (%)	Cytotoxicity (%)	IFN-γ (pg/mL)	IL-2 (pg/mL)	ApoptosisRate (%)
NSCLC	78.6 ± 6.9	66.2 ± 9.8	645 ± 220	125 ± 40	28.3 ± 9.2
Colorectal	74.1 ± 7.2	61.4 ± 10.1	560 ± 210	103 ± 38	31.7 ± 9.6
Pancreatic	70.4 ± 8.1	56.1 ± 11.5	482 ± 235	92 ± 35	37.8 ± 9.4

Ovarian	82.3 ± 5.6	73.2 ± 8.2	790 ± 275	160 ± 46	24.6 ± 8.1
Glioblastoma	68.9 ± 7.8	59.0 ± 10.8	410 ± 195	85 ± 34	39.6 ± 9.3
Melanoma	75.2 ± 6.7	66.9 ± 9.0	675 ± 250	132 ± 42	30.1 ± 8.8

Discussion

We introduce a flexible CAR-NK engineering pipeline that combines the production of a mesothelin-targeted CAR, expression of mbIL-15, and the use of CRISPR-Cas9 for the removal of inhibitory checkpoints in NK-92 cells. The reported editing efficiencies and functional gains are in line with those from the recent NK engineering studies [3]. The application of CRISPR for CBLB and TIGIT deletions has led to reports claiming the enhancement of NK cells' killing ability and their metabolic fitness; our method not only reproduces but also extends those results into CAR-armed NK-92 [6], [14]. The use of IL-15 as a non-soluble entity increases both persistence and autocrine support without making systemic cytokine administration a requirement, which has been the observation in various preclinical studies [4]. The results from the dose–response modeling reveal that the edited product reaches a lower EC50 and also shows a steeper efficacy slope; this pattern was also observed in CRISPR-enhanced NK studies and is in line with a rise in per-cell killing capacity [13], [3].

Our cytokine analysis shows a rising trend in IFN-γ and IL-2 that although still below the extreme levels of the in vitro cytokine storm, point to very high activation; these markers have to be verified in vivo with great care as high systemic levels of IL-15 or too much cytokine release could be problematic from the safety point of view. Off-target risks are the main issue: we applied GUIDE-seq and targeted deep sequencing to ascertain a safety envelope and discovered minor off-target indel rates at the chosen loci; however, clinical distribution necessitates the application of extensive genomic and transcriptomic surveillance, which has been illustrated in the recent methodological reviews [12], [8]. NK-92 is a good platform for pre-clinical development, but it is an irradiated, non-replicative cell line that cannot be used in clinical protocols; the transition to a long-lasting product may involve iPSC-derived or primary NK platforms with the same engineering strategies [9], [15].

The tumor microenvironment (TME) is one of the factors that imposes different gradients of chemokine, hypoxia, and stroma that may eventually limit the accumulation of cells within the tumor; therefore, future designs could incorporate the expression of chemokine receptors, such as CXCR2, and strategies aimed at remodeling the extracellular matrix along with the gene edits proposed here. The use of NK-92 in vitro models is mainly a constraint of the current study; while such studies provide valuable information, they still need to be supported by in vivo xenograft and syngeneic studies and humanized tumor models in order to carry out a comprehensive evaluation of the persistence, trafficking, and TME interactions of the cells. Before the first-in-human testing can be done, regulatory and manufacturing issues—scalability of electroporation, vector production, and robust off-target monitoring—must be resolved; some researchers suggest cGMP-compatible editing workflows that can be modified for this purpose [13].

The very first point could be the combination of checkpoint blockade, CAF modulation, or oncolytic vectors that might up the penetration and antigen spread; the strategy is further backed up by preclinical combinations that are facilitators of CAR-NK therapies.

The multiplexed CRISPR editing, coupled with rational CAR design and intrinsic IL-15 support, produces a CAR-NK product that is significantly superior in vitro potency against a wide range of

solid tumor models and has a clear translational path that is worthy of in vivo validation and safety profiling in GLP settings [8], [3].

Conclusion

An intricately designed CAR-NK platform that incorporates a mesothelin-targeted CAR, IL-15 that is membrane-bound, and CRISPR-Cas9 gene editing of TIGIT, CBLB, and NKG2A results in NK-92 cells showing very high in vitro antitumor effect against several solid tumor models that is supported by the fact that higher editing and CAR expression efficiencies, improved cytotoxic dose–response (lower EC50), elevated effector cytokine secretion, and increased tumor apoptosis have all been observed when compared to the unedited CAR-NK controls; these results are a strong backing for further GLP safety studies and in vivo validation in orthotopic and humanized models prior to the consideration of early phase clinical testing.

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