CRISPR-Targeted CAR Gene Insertion Using Cas9/RNP and AAV6

Enhances Anti-AML Activity of Primary NK Cells

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Abstract

Human peripheral blood natural killer (NK) cells have intense antitumor activity and have been used successfully in several clinical trials. Modifying NK cells with a chimeric antigen receptor (CAR) can improve their targeting and increase specificity. Recently, we described an efficient method for gene targeting in NK cells using Cas9/ribonucleoprotein (RNP) complexes. Here we combined this approach with single-stranded (ss) or self-complementary (sc) Adeno-associated virus (AAV)-mediated gene delivery for gene insertion into a safe-harbor locus using a wide variety of homology arms for homology repair (HR) and non-homologous directed CRISPR-assisted insertion tagging (CRISPaint) approaches. For proof-of-concept, we generated mCherry-expressing primary NK cells and determined that sc vectors with 300bp homology arms had optimal transduction efficiency. Then, we generated CD33-targeting CAR NK cells with differing transmembrane and signaling domains (CD4/4-1BB+CD3 ζ and NKG2D/2B4+CD3 ζ) and expanded them on CSTX002 feeder cells. Expansion kinetics were unaltered and the expanded NK cells maintained high CAR expression (mean 68% CAR+). The CD33-CAR-NK cells showed increased activation markers and enhanced antileukemic activity with improved killing kinetics against CD33-positive acute myeloid leukemia (AML) cell lines and primary samples. Using targeted sequencing we demonstrated the accuracy of CAR gene insertion in human primary NK cells genome. Site-directed insertion using RNP and scAAV6 is an efficient method for stable genetic transfer into primary NK cells that has broad potential for fundamental discovery and therapeutic applications.

Keywords: CRISPR, NK, Cas9/RNP, AAV6, CRISPaint, HR, CAR, CD33CAR-NK

Author Contribution Statement:

M.N.K and D.A.L conceived of the project, S.L, K.Y, B.S.M, K.M, G.K.B, M.P and D.A.L designed the experiments. M.NK, S.L, K.Y, N.C, E.E, M.S, R.D.D, J.M.L and K.S performed the experiments. M.NK, K.Y, B.S.M, G.K.B and D.A.L analyzed and interpreted the data. M.NK, K.Y, B.S.M, M.S and D.A.L prepared the manuscript.

68 Introduction

69 Human primary NK cells have been tested in numerous clinical trials demonstrating a high safety profile
70 and evidence of clinical benefit for patients with cancer, which has been most widely applied to acute
71 myelogenous leukemia (AML). Enhanced targeting of NK cells to AML through CD33 targeting has been
72 shown with antibody Fc modifications^{1, 2} or fusion to alternative activation domains as bi- or tri-specific
73 NK cell engagers^{3, 4}. CD33-targeting by T cells has been enabled by CD33-targeting chimeric antigen
74 receptors (CAR)⁵⁻⁷, but clinical application has been hindered by concerns of long-term suppression of
75 hematopoiesis with CAR-T persistence.

76 Gene modification of NK cells to enable stable expression of a CAR can also improve their antitumor
77 activity⁸. However, gene modification of human peripheral-blood derived NK cells (PB-NK) using viral or
78 non-viral vectors has been challenging due to robust foreign DNA- and RNA-sensing mechanisms that limits
79 the efficiency of these gene delivery methods⁸. To overcome this limitation, mRNA-based gene delivery
80 has been tested in PB-NK cells, but only allows for transient expression of transgenes⁹. We recently
81 demonstrated highly efficient gene knockout in human primary NK cells by electroporating
82 Cas9/ribonucleoprotein complexes (Cas9/RNP)¹⁰⁻¹². After Cas9 introduces a DSB, two independent and
83 innate DNA repair mechanisms may be employed to repair the break: homologous recombination (HR) or
84 non-homologous end-joining (NHEJ). In the presence of a DNA template encoding a gene of interest, the
85 exogenous gene can be integrated into the Cas9-targeting site using either of these repair mechanisms¹³.

86 There are several ways to provide the DNA template, including viral and non-viral methods. In non-viral
87 approaches, the single-stranded or double-stranded DNA template is typically electroporated along with
88 Cas9/RNP¹⁴, however, it typically has a lower efficiency in comparison to viral transduction. For viral gene
89 delivery, adeno-associated viruses (AAV), including AAV6, have been used safely as delivery vectors in
90 clinical trials for primary immune cells, including T-cells^{15,16}.

The Cas9/RNP-AAV approach has not been described in human primary NK cells. Here, we set to optimize this approach wherein Cas9/RNP electroporation in primary human NK cells is followed by a DNA template encoding a transgene, with or without homology arms for Cas9 targeting site, delivered using single stranded or self-complementary AAV6. Using this approach, we achieved simple yet highly efficient generation of stable transgene-modified human primary NK cells, including two CAR-NK cells which showed enhanced anti-AML activity. The gene modified NK cells generated by the AAV-Cas9/RNP platform has utility for clinical applications such as CAR expression for antigen specific cancer immunotherapy, and for studying NK cell biology.

Results

Expansion of NK cells provides optimal conditions for gene insertion

The DNA modifying and repairing enzymes required for NHEJ and HR are different. NHEJ, which is essential for CRISPR-assisted insertion tagging (CRISPaint), is a LIG4-dependent process, while BRCA1 and BRCA2 are essential for HR^{31, 32}. We have shown that expansion of NK cells on feeder cells expressing membrane-bound IL-21 (FC21) induces broad changes in gene expression^{18, 19}. Therefore, we analyzed the expression level of genes essential for HR and NHEJ in freshly isolated NK cells and after seven days of stimulation (Day 7 expanded cells) to determine the timepoint at which these mechanisms would be most active. RNA-seq showed that the expanded NK cells had highly increased expression of BRCA1 and BRCA2 in comparison to naïve NK cells, and there was no significant change in LIG4 expression (Figures 1a and 1b), suggesting optimal conditions for either HR or NHEJ-directed gene insertion in expanded NK cells.

Targeting a genomic safe harbor for gene insertion

113 For gene insertion in NK cells, we chose the *adeno-associated virus integration site 1 (AAVS1)*, which is an
 114 exemplary genomic safe harbor locus within the *phosphatase 1 regulatory subunit 12C (PPP1R12C)* gene¹³,
 115 ³³. Chromatin accessibility of AAVS1 was similar in naïve and Day 7 expanded NK cells (n=2) as determined
 116 by ATAC-seq (Figure 1c). Next, AAVS1 was targeted by electroporation of Cas9/RNP into Day 7 expanded
 117 NK cells (Supplementary Figure 1a)¹¹. After 48 hours, the frequency of insertions and deletions (Indels) in
 118 CRISPR-edited NK cells was determined using Inference of CRISPR Edits (ICE) using primers flanking the
 119 AAVS1 locus (Supplementary Table 1)²². The ICE results showed that up to 85% of CRISPR modified NK
 120 cells had at least one indel at the AAVS1 Cas9-targeting site (Figure 1d). Using the Calcein-AM assay¹¹, we
 121 observed no difference between wild type and CRISPR-modified AAVS1^{KO} NK cells in their killing ability
 122 against Kasumi-1, an acute myeloid leukemia (AML) cancer cell line, suggesting that genome modifications
 123 at this locus do not interfere with the ability of NK cells to target cancer cells (Supplementary Figure 1b).

124 **Gene insertion in primary human NK cells using single-stranded AAV6 and Cas9/RNP**

125 To compare the efficiency of gene insertion across DNA-repair mechanisms, we generated a parallel series
 126 of AAV6 vectors suitable for HDR-mediated gene insertion (Figure 2a) using both single-stranded and self-
 127 complementary designs with varying homology arm lengths, and for NHEJ-mediated gene insertion with
 128 CRISPaint containing PAMgPAMg sequences (Figure 2b). To maximize HDR-mediated gene insertion, we
 129 identified homology arms (HA) for the right and left sides of the flanking regions of the Cas9 targeting site
 130 in the AAVS1 locus, cloned these together with the mCherry gene into the backbone of a single-stranded
 131 AAV plasmid, and packaged this construct into the AAV6 viral capsid (Figure 2a)^{16,34,35}. It has been shown
 132 that the efficiency of recombination increases as the length of HAs increases³⁶⁻⁴⁰. Therefore, for the ssAAV
 133 backbone, we used the longest possible length of the left and right HA for mCherry (800bp-1000bp of HAs).
 134 The constructs also contained a splice acceptor downstream of the transgene to improve the transcription
 135 of the mCherry gene (Figure 2a). Electroporation of the NK cells with Cas9/RNP targeting AAVS1 followed

30 minutes later by AAV transduction (Figure 2c)¹⁰ resulted in 17% (300K MOI) and 19% (500K MOI) mCherry positive NK cells (Figure 3a). We further expanded these cells for one week using FC21, enriched the mCherry positive cells by FACS sorting, and did not see any reduction in the expression level of mCherry during an additional 30 days of expansion (Figures 3a, 3b, and 3c) demonstrating stable integration.

Improved gene insertion by using self-complementary AAV6 and Cas9/RNP

After transduction, scAAV vectors can acquire the necessary double-stranded state in a shorter time frame than ssAAV, which may impact the efficiency of gene insertion. Due to the size limitation of packaging transgenes in scAAV, we designed HAs of varying lengths to minimize the size needed for scAAV backbones. Hence, HAs of 30bp, 300bp, 500bp, and 1000bp length for the right and 30bp, 300bp, 500bp, and 800bp for the left (Figure 2a) were cloned with mCherry into the scAAV backbone and packaged into AAV6 capsid. We then followed the same steps as for the ssAAV, above, to electroporate and transduce the Day 7 expanded NK cells. scAAV with HA $\geq$ 300bp showed markedly increased efficiency of gene transfer at >80% (Figures 3a and 3b). Stable mCherry gene expression was observed for at least 3 weeks of additional NK cell expansion. When we used the same approach in freshly isolated NK cells, the mCherry expression was significantly lower (1.13% for ss800bp AAV6; 2.9% for sc300bp AAV6, Supplementary Figure 2).

CRISPaint for gene insertion in NK cells

To overcome the complexity of HAs optimization seen in HDR-directed gene insertion, we tested a homology-independent gene insertion approach called CRISPaint. For the CRISPaint DNA templates, we incorporated double Cas9-targeting sequences of AAVS1 (PAMgPAMg) around the mCherry transgene but within the ITRs of scAAV and packaged it into AAV6 (Figure 2b). Two days after electroporation and

transduction we performed flow cytometry to assess mCherry expression in NK cells. The cells which were electroporated and transduced with 300K MOI of scAAV6 delivering CRISPaint PAMgPAMg were found to be up to 6% of mCherry positive. We further sorted and enriched these NK cells and expanded them for 30 days and saw no decline in the percentage that were mCherry positive (Figures 3b and 3c). Although we saw lower efficiency of gene integration using CRISPaint compared to HR-directed gene insertion, this method may still be useful because it allows integration into a user-defined locus without designing homology arms.

Generation of human primary CD33-CAR NK cells

We designed two CAR constructs comprising the same CD33-targeting scFv, but with a CD4 transmembrane domain and 4-1BB / CD3 ζ signaling domain (Gen2) or an NKG2D transmembrane domain and 2B4 / CD3 ζ (Gen4v2)⁴¹ (Figures 4a and 4b). The size of the CAR constructs were too large for suitable packaging into the scAAV backbone, therefore we cloned them into the ssAAV backbone with the largest possible HAs of 600bp. To improve the expression of the CARs, we also incorporated a murine leukemia virus-derived promoter (MND) before the start codon of the CARs instead of the splice acceptor. As with the mCherry vectors, these were packaged into the AAV6 capsid. Seven days after electroporation and transduction, we detected up to 78% CD33 CAR-expressing NK cells (mean 59.3% for Gen2 and 60% for Gen4v2). Of note, the CD33CAR-Gen2 resulted in a higher level of expression on NK cells compared to Gen4v2 (Figures 4c and 4d, Supplementary Figure 3a). We expanded the cells for another week (Day 14) and observed no significant reduction in CAR expression (Figure 4e, Supplementary Figure 3b) or proliferative potential (Figure 4f). Surprisingly, efficient CAR integration (> 60%) was observed with MOIs as low as 10K (Supplementary Figure 4).

Detection of the transgene at the targeted AAVS1 locus and unintended insertion sites

Using PCR with primers to flanking and inter-transgenic regions (Figure 5a, Supplementary Table 2), we confirmed the DNA integration of the transgenes (Figure 5b). Additionally, targeted locus amplification (TLA) was used for whole-genome mapping of CD33CAR-Gen2 integration in CAR-expressing NK cells with a sensitivity of detecting random integrations of more than 5%, which demonstrated high prevalence of vector integration at the targeted location in chromosome 19, with low level random integrations identified throughout the genome (Figure 5c, Supplementary Figure 5, Supplementary Table 3). There was no indication of a dominant secondary off-target integration site. One sequence variant (Supplementary Table 4) and four structural variants (Supplementary Figure 6, Supplementary Data 1) were detected.

Human primary CAR-NK cells have enhanced antitumor activity

To determine whether the CD33CAR enhanced NK cell killing of AML cells, we performed Calcein AM based cytotoxicity assay with two CD33-expressing AML cell lines (Kasumi-1 and HL60) and one patient-derived sample (AML10) (Supplementary Figure 7) using CD33CAR-NK cells generated from three different healthy individuals. CD33CAR-gen2 and CD33CAR-gen4v2 NK cells showed a significantly higher degranulation and target cell lysis when cocultured with Kasumi-1 or HL60 cell lines in comparison to wildtype or AAVS1^{KO} NK cells. (Figures 6a-6f). Importantly, we showed significantly higher antitumor activity of CD33CAR NK cells against AML-10, a primary human AML sample derived from a patient with relapsed and refractory AML (Figures 6g and 6h)^{5,19}. Overall, CD33CAR-Gen2 NK showed better cytotoxicity in comparison to CD33CAR-Gen4v2 NK cells. Using real-time assessment of cytotoxicity (xCELLigence), we showed that CD33CAR-NK cells kill the CD33-expressing AML cells more completely and with faster kinetics than the same donor WT NK cells (Figure 6f).

Mass Cytometry showed enhanced killing and specificity of CD33CAR-NK cells against AML

After co-culture of WT or CD33-CAR NK cells with primary AML, we could identify four main populations (Figure 6g) by both manual gating and SPADE clustering: live proliferating NK cells, quiescent NK cells, live proliferating AML cells, and dying AML cells. At 3 hours, control primary AML cells were ~66% viable, whereas viability decreased to 56% when co-cultured with WT-NK cells and only 21% when co-cultured with CD33CAR NK cells. At 24 hours, control primary AML cells recovered to 89% viability, compared to 76% when co-culture with WT-NK cells and only 42% when co-cultured with CD33CAR NK cells. At 3 hours, the surviving AML cells had a 20-fold reduction in CD33 expression when cultured with the CD33CAR NK cells (median of 104 counts down to 5 counts) while there was minimal change in CD33 expression in the WT NK cell co-culture (median of 104 counts down to 87 counts). This difference persisted at 24 hours, at which time the median CD33 counts were 25 for CD33CAR NK cells, 118 for WT NK cells, and 120 in control AML without NK cells. Co-culture with AML also increased NK activation markers (CD69, CD99, CD71, NKG2D, CD16, and CD45) at 24 hours compared to the NK cells cultured alone. The CD33CAR NK cells had lower levels of activation markers at baseline that increased more with co-culture. Together, these data show that CD33CAR-NK cells specifically target CD33-expressing AML and are more activated by the AML targets compared to WT-NK cells (Figure 6g).

Discussion

Gene modification in primary human NK cells has always been challenging; here, we report a successful, highly efficient site-directed gene integration into human primary NK cells using a combination of electroporation of Cas9/RNP and single-stranded or self-complementary AAV6 gene delivery through HR and homology-independent gene insertion (CRISPaint). Here for the first time, we showed how the expression level of genes regulating HR and NHEJ pathways in human NK cells alter during expansion with FC21 and provides an optimal condition for site-directed gene insertion. We also demonstrated that AAVS1

could host and express exogenous genes in a very highly efficient level, as shown previously in T cells and NK cells¹⁰. Furthermore, we showed that a range of HAs from 30-1000bp that can be used for gene insertion into the AAVS1 locus in NK cells, but that the shortest optimal length is at 300bp when used in scAAV6. This helps researchers to choose an optimal HA based on the size of their exogenous DNA that they will introduce in NK cells. CRISPaint gene insertion can be potentially used for tagging endogenous genes and be used for studying the biology of proteins in NK cells.

Transcripts that are delivered via AAV vectors can be packaged as a linear single-stranded (ss) DNA with a length of approximately 4.7 kb (ssAAV) or as linear self-complementary (sc) DNA (scAAV). The benefit of the scAAV vector is that it contains a mutated inverted terminal repeat (ITR), which is required for replication and helps to bypass rate-limiting steps of second strand generation in comparison to ssDNA vectors⁴². Due to the limitation in the packaging capacity of scAAV, we designed 30bp, 300bp, 500bp, and 800-1000 bps of HAs for the right and left side of the Cas9-targeting site to find the most optimal length of HAs and to provide possible lengths of HAs to be chosen based on the size of transgenes by researchers (Figure 2a). Additionally, due to limitations in packaging capacity compared to ssAAV, scAAV is not suitable for larger transgenes such as chimeric antigen receptor (CAR) targeting CD33⁴². Therefore, based on the size of transgenes, we designed and tested both ssAAV and scAAV, which provides a wide range of options for gene insertion in primary NK cells.

Since designing homology arms is a time-consuming procedure and requires multiple optimizations, we also investigated the CRISPaint approach, a homology-independent method for gene insertion or tagging. In this method, the same Cas9 targeting site, including the crRNA and PAM sequence, is provided in the DNA template encoding the gene of interest. Upon the introduction of the Cas9 complex, both template and genomic DNA will be cut simultaneously. As a result, the CRISPaint template will be presented as a linearized double-stranded DNA that can be integrated through non-homology repair machinery (Figure

2b)^{14, 31}. We also used the combination of Cas9/RNP and AAV6 gene delivery and generated two different human primary CD33CAR NK cells with enhanced anti-AML activity. Our results also showed that the gene-modified NK cells could be subsequently expanded with FC21, enabling the production of large numbers of gene-modified NK cells for cancer immunotherapy. Overall, our novel method can be used for several applications in immunology, cancer immunotherapy, and studying the biology of NK cells.

Material and Methods

Human NK Cell Purification and Expansion

NK cells were purified as previously described¹¹. Briefly, NK cells were isolated from PBMC collected from healthy individuals using RosetteSep™ Human NK Cell Enrichment Cocktail. Purified NK cells were phenotyped using flow cytometry as >90% CD3-negative/CD56-positive population (Supplementary Figure 1a). These cells were then stimulated with irradiated feeder cells (FC21) comprised of K562 transduced with 4-1BBL and membrane-bound IL-21 (CSTX002)¹⁷ at a ratio of 2:1 (feeder: NK) as previously described^{18,12 19}. The stimulated cells were cultured for 7 days in serum-free AIM-V/ICSR expansion medium containing 50 IU/mL of IL-2²⁰.

ATAC-seq assay

Freshly-isolated (naïve) and FC21-expanded NK cells from two donors were cryopreserved in aliquots of 100,000 viable cells/vial before processing for ATAC-seq. ATAC-seq was performed as previously described²¹. DNA libraries were sequenced using Illumina HiSeq 2500 at 50 bp paired-end reads.

Cas9/RNP electroporation for targeting AAVS1 in NK cells

AAVS1 was targeted using gRNA (crRNA: 5'GGGGCCACTAGGGACAGGAT) via electroporation of Cas9/RNP into Day 7 expanded NK cells as described before¹¹. Briefly, 3 x 10⁶ expanded NK cells were harvested and

274 washed twice with 13ml of PBS followed by centrifugation for 5 minutes at 400g and aspiration of PBS.
 275 The cell pellet was resuspended in 20ul of P3 Primary Cell 4D-Nucleofector Solution. 5ul of pre-complexed
 276 Cas9/RNP (Alt-R® CRISPR-Cas9 crRNA, Alt-R® CRISPR-Cas9 tracrRNA, and Alt-R® S.p. HiFi Cas9 Nuclease V3)
 277 (Integrated DNA Technologies, Inc., Coralville, Iowa), targeting AAVS1 and 1ul of 100uM electroporation
 278 enhancer (Alt-R® Cas9 Electroporation Enhancer) were added to the cell suspension. The total volume of
 279 26ul of CRISPR reaction was transferred into 4D-Nucleofector™ 16-well Strip and electroporated using
 280 program EN-138 (Supplementary Figure 1a). After electroporation, the cells were transferred into 2ml of
 281 media containing 50IU of IL-2 in a 12 well plate and incubated at 37 degrees and 5% CO₂ pressure. Two
 282 days post electroporation, cells were stimulated with 2 x 10⁶ feeder cells, and 8ml fresh media
 283 complemented with 50IU was added in cell suspension and kept in a T25 flask.

284 **Inference of CRISPR Edits (ICE) mutation detection assay**

285 To measure the indel rate in AAVS1^{KO} NK cells, the Cas9/RNP targeted site was PCR amplified using forward
 286 and reverse primers described in Supplementary Table 1. The amplicons were sequenced using Sanger
 287 sequencing, and results were analyzed using ICE ²² (Synthego, Menlo Park, CA).

288 **RNA-seq sample preparation and sequencing**

289 Total RNA was purified from naïve resting, expanded resting, naïve IL21-stimulated, and Day 14 FC21-
 290 expanded NK cells using the Total RNA Purification Plus Kit (Norgen Biotek, Ontario, Canada). The resulting
 291 total RNA was sequenced and analyzed as described before ¹².

292 **AAV6 production**

293 The transgenes cloned into ssAAV or scAAV plasmids were packaged in AAV6 capsids as described before
 294 ²³.

295 **Combining Cas9/RNP and AAV6 to generate mCherry and CAR NK cells**

296 A media change and resuspension at 5×10^5 cells per ml was performed on day 6 of NK cell expansion one
 297 day before experimental manipulation. The NK cells were electroporated with Cas9/RNP targeting AAVS1
 298 on day 7, as described above. Thirty minutes after electroporation, 3×10^5 live cells were collected and
 299 resuspended at 1×10^6 cells per ml in media containing 50IU IL2 (Novartis) in a 24 well plate in a total
 300 volume of 300ul. For each transduction condition with ssAAV6 or scAAV6 to deliver HR or CRISPAint DNA
 301 encoding mCherry or CD33CARs, we transduced 3×10^5 electroporated cells with 300K MOI (10-500K MOI
 302 if needed). Negative controls included as NK cells that were not electroporated, or were electroporated
 303 with Cas9/RNP but not AAV transduced, or were transduced with 300K MOI of AAV6 without
 304 electroporation of Cas9/RNP. The day after electroporation and transduction, we added 300ul of fresh
 305 media containing 50IU of IL2 to each well without changing the old media. The cells were kept in culture
 306 for 48 hours after electroporation and were then restimulated with 2×10^6 feeder cells and kept in a total
 307 volume of 2ml media containing 50IU in 12 well plate, without changing the old media. 48 hours later, 8ml
 308 fresh media supplemented with IL2 was added to cells, a total volume of 10ml was kept in a T25 flask. At
 309 day 7 post-transduction, cells were re-stimulated with feeder cells at a ratio of 1:1 and grown for one more
 310 week, every 2 days fresh media was added to the cells.

311 **Flow Cytometry for detection of CAR-NK cells**

312 7 days and 14 days following electroporation, 5×10^5 NK cells were washed twice with staining buffer
 313 containing 2% FBS in PBS. Next, 2.5ug of recombinant human siglec-3/CD33 Fc chimera protein, (CF; R&D
 314 systems #1137-SL-050) was added to cell suspension in a total volume of 80ul and incubated for 30
 315 minutes at 4C. Cells were washed twice with staining buffer before staining with 2ul of Alexa Fluor® 647
 316 affinipure goat anti-human IgG, Fcy fragment specific, (Jackson ImmunoResearch #109-605-098) at 1:100
 317 ratio in 200ul of staining buffer and kept at 4C for 30 minutes. Once stained, cells were washed twice with

318 staining buffer then acquired on MacsQuant flow cytometers. Flow cytometry data were analyzed using
319 FlowJo software (FlowJo, LLC).

320 **Cytotoxicity assay**

321 Cytotoxicity assays were performed for 3-4h as described previously using a calcein-acetoxymethyl-release
322 assay¹⁹. Cytotoxicity was assessed against Kasumi-1, HL60, or AML10 cells at different effector:target ratios
323 as defined in Figure 6a-6e.

324 **CD107a staining**

325 NK cells and cancer cells were cocultured at 10:1 ratio and supplemented with 20ul of PE mouse anti-
326 human CD107a antibody (BD Pharmingen™, #555801) in a total volume of 220ul in a 96 well plate at 37C
327 incubator for 90 minutes. The cells were washed with staining buffer once and analyzed on MacsQuant
328 flow cytometer.

329 **PCR-based detection of transgenes integration**

330 In-out PCR was performed using 2 pairs of primers (Figures 5a and 5b and Supplementary Table 2) designed
331 inside or outside of the CD33CAR constructs. We also added a set of primers to amplify 1200bp right and
332 left flanking region of Cas9 targeting and transgene integration site (Figure 5a). PCRs were performed using
333 the Platinum™ Taq DNA polymerase high fidelity kit (Thermofisher #11304011).

334 **Targeted Locus Amplification (TLA)**

335 For the whole-genome mapping of CD33CAR-Gen2 integration, we used the TLA technology (Cergentis
336 B.V.)²⁴. The genomic DNA from CD33CAR-expressing NK cells was isolated using Qiagene DNeasy Blood &
337 Tissue Kit and crosslinked, fragmented, and re-ligated using the kit provided by Cergentis, then submitted
338 to Cergentis for sequencing²⁴.

339 **Real-time potency assessment for suspension target cells killed by WT and CD33CAR NK cells.**

The xCELLigence RTCA MP instrument (ACEA Biosciences) was utilized as previously described²⁵. Briefly, the CD29 tethering reagent was coated on the plate (2 µg/mL, for 3 hours at 37°C) to immobilize the Kassumi cell line, seeded at 6 x 10⁴/well. After 20-24 hours, WT or CD33CAR NK cells were added at indicated E:T ratios. An “effector cell only” control was included in the presence of tethering reagent. Cell Index values reflecting viable target cell adherence were applied to xIMT software to plot percentage cytotoxicity.

AML-NK Cell Co-Culture for Mass Cytometry

Cells were grown in SFEM II (StemCell, Cambridge, MA) supplemented with the following cytokines; SCF, IL-6, TPO, FLT3, GM-CSF, G-CSF, IL-3 (20ng/ml) and EPO (10ng/ml). A de-identified primary AML sample was obtained from OSU Leukemia Tissue Bank consistent with Declaration of Helsinki. NK or CAR-NK cells at a target:effector ratio of 1:2 were cultured for either 6 or 24 hours. Following culture, cells were prepared for staining as previously described²⁶.

Mass Cytometry Staining and Analysis

Fixed cells were washed with CSM as previously described²⁷. Antibodies used for this study are listed in Supplemental Table 5. Samples were resuspended in 1:20 dilution of four elemental equilibration beads (Fluidigm) at a concentration of 1 million cells/ml. FCS generated files were normalized using a normalization tool developed by Finck et al and analyzed on Cytobank (www.cytobank.org)^{28,29}. Once the singlet gate was established, cells were identified using markers and analyzed using SPADE³⁰.

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370 Pharma, outside the submitted work; In addition, Dr. Lee has patents US62/825,007; US63/105,722; US62928,524; PCT-
371 US201/032,670; WO-2019/222,503-A1; PCT-US2020/018,384; US62/805,394; US62/987,935; US62/900,245; US62/815,625;
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374 Dr. Naeimi Kararoudi has patents US62/825,007; WO2019222503A1; USPTO63/105,722; PCT/US2020/02545; US63/018,108;
375 US62/928,524; US62/987,935; Self-driving CAR with royalties paid by Kiadis Pharma. Dr. Moriarity is founder of and has
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377 PCT/US2020/025454 with royalties paid by Kiadis. Dr. Chakravarti reports In addition, Dr. Chakravarti has a patent PM21
378 particles to improve bone marrow homin of NK cells licensed to Kiadis.
379

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Figure 1.

Figure 1: Efficient CRISPR targeting of AAVS1 in mbIL-21 expanded human primary NK cells

Relative gene expression level of NHEJ-related genes **(a)** and HR-related genes **(b)** in naïve and mbIL-21 expanded NK cells, ***P < 0.001 for all comparisons (n=4). **c.** ATAC-seq data shows that AAVS1 has a similar chromatin accessibility between freshly isolated (Naïve), mbIL-21 expanded NK cells (n=2). **d.** Efficiency of Cas9/RNP-mediated targeting of AAVS1 in NK cells. Data are shown as mean ± s.d. P values are from 2way ANOVA.

Figure 2.

Figure 2: Constructs of mCherry encoding DNA for insertion into AAVS1 through HR and CRISPaint

a, Cas9/RNP introduces DSB in AAVS1, DNA encoding gene of interest can be integrated into NK cells through HR with optimal length of HAs. The schematics show the constructs design for integration of DNA encoding mCherry with HAs between 30-1000bp for Cas9 targeting site in AAVS1 and cloned in ssAAV6 and/or scAAV6 backbone. **b**, Top, schematics of how CRISPaint gene insertion works through homology independent DNA repair pathway. Bottom, schematic of construct design for insertion of DNA encoding mCherry through CRISPaint and cloned in scAAV. **c**, Schematics of workflow to electroporate Cas9/RNP and transduce day seven mbIL21 expanded IL2-stimulated NK transduced with AAV6 for gene delivery.

Figure 3.

Figure 3. Combinations of AAV6 and Cas9/RNP results in efficient generation of mCherry expressing NK cells.

a, Representative flow cytometry of human primary NK cells expressing mCherry, 2 days after CRISPR electroporation and AAV6 transduction (MOI = 3×10^5), **b**, Efficiency of Cas9/RNP and AAV6-mediated mCherry expression in human primary NK cells through HR and CRISPaint (n=3). **c**, Stable mCherry expression in NK cells after enrichment and expansion using mbIL21 K562.

Figure 4.
a

b

c

d

e

f

Figure 4. Successful generation of CD33CAR expressing NK cells using combination of Cas9/RNP and AAV6

a and b, Schematic of anti-CD33 CAR constructs (Gen2 and Gen4v2) designs with HAs for AAVS1 targeting site and cloned in ssAAV. **c** Representative flow cytometry showing the expression level of CD33CAR on NK cells, 7 days after Cas9/RNP electroporation and AAV6 transduction (MOI = 3×10^5). **d**, MFI of CD33CAR expression of Gen2 was significantly higher than Gen4v2, $**P = 0.0014$, measured by unpaired t-test. **e**, CD33CAR expression level on NK cells seven and fourteen days after transduction and electroporation showed no significant reduction ($n=3$). **f**, Fold expansion of CD33CAR expressing NK cells on feeder cells for 14 days starting from 3×10^5 cells ($n=3$) was similar to wildtype NK cells. n.s are from multiple comparison 2way ANOVA.

Figure 5

Figure 5. Integration of the transgene in AAVS1 locus was confirmed by PCR and TLA.

(a), Schematic of PCR primers designed inside and outside of CD33CARs encoding DNA and integrated in AAVS1. **(b)**, Amplicons were amplified and visualized on 1% agar gel only in NK cells with successful CD33CAR gene insertion at AAVS1 locus (condition 1 and 2). The gene insertion in human primary NK cells also was seen when primers designed outside of the transgenes and were used to amplify AAVS1 locus in wildtype, mCherry or CD33CARs (condition 3, primers: Forward-1200bp (2) Reverse – 1200bp (1)) , Supplementary Table 2 . **(c)**, TLA sequence coverage across the human genome using designed primers to detect integration of CD33CAR-Gen2 in day 14 cells. The chromosomes are indicated on the y-axis, the chromosomal position on the x-axis. Identified integration site is encircled in red. The , Supplementary Table 3 indicates the primers used for the TLA sequencing

Figure 6.

Figure 6. CD33CAR NK cells have enhanced anti-AML activity

CD33CAR NK cells degranulate significantly higher than wildtype NK cells when cocultured with *Kasumi-1*, ** adjusted P value= 0.004 **(a)** and show higher killing ability **(b)**. The enhanced degranulation **(c)** and killing against *HL60*, * adjusted P value= 0.01 **(d)** as shown in representative cytotoxicity assay performed in different effector:target ratios **(b and d)** and in three donors, **** adjusted P value <0.0001., * adjusted P value= 0.01. CD33CAR-Gen2 and Gen4v2 significantly killed higher *AML-10* primary cells, **** adjusted P value <0.0001 **(e)**. P Values are from multiple comparison 2way ANOVA. CD33CAR-Gen2 NK cells also showed faster killing of AML cells in compare to WT-NK cells in a xCelligence killing assay **(f)**. We also observed the specificity and killing potency of CD33CAR-NK cells against CD33-expressing primary AML cells in CyTOF assay **(g)**.

Supplementary Figure 1.

a.

b.

Cytotoxicity of wildtype NK and AAVS1^{KO} NK cells against Kasumi

Supplementary Figure 1. Targeting AAVS1 in expanded CD3^{negative}CD56^{positive} NK cells does not alter normal function of the cells. **a**, Schematic of workflow for electroporation of Cas9/RNP into day 7 expanded human primary NK cells to target AAVS1. **b**, Cytotoxicity assay of AAVS1^{KO} NK cells does not show any suppression in their antitumor activity againsts AML cell line.

Supplementary Figure 3.

a

b

Supplementary Figure 3. Representative flow cytometry analysis of CD33CAR expression level 7 days (a) and 14 days (b) post electroporation and AAV6 transduction in human NK cells.

Supplementary Figure 4

a

b

Supplementary Figure 4. Representative flow cytometry (a) analysis of CD33CAR-Gen2 expression level in NK cells transduced with 10K-300K MOI of ssAAV6 encoding CD33CAR-Gen2 showed successful expression of CAR on NK cells isolated from three healthy donors (b).

Supplementary Figure 5

Supplementary Figure 5. TLA sequence coverage (in grey) across the vector integration locus, human chr19:54,550,476-55,682,266. The blue arrow indicates the location of the breakpoint sequences. Y-axes are limited to 20x and 100x resp. The coverage profile this figure shows that no genomic rearrangements have occurred in the region of the integration site.

From this data it is concluded that the vector has integrated as intended in human chromosome chr19: 55,115,754- 55,115,767. According to the RefSeq this is in intron 1 of PPP1R12C. Other integration sites were observed between chr19: 55,115,155-55,116,371. According to the RefSeq this is also in intron 1 of PPP1R12C.

Supplementary Figure 6

Supplementary Figure 6. NGS sequencing coverage (in grey) across the vector. Black arrows indicate the primer location. The blue arrows indicate the locations of the identified vector-genome breakpoint sequences (described below). The vector map is shown on the bottom. Y-axes are limited to 100x.

High coverage is observed across the region between the ITR sites, vector sequence Vector: 12-4,255. Low/no coverage is observed across the Vector: 0-11 and 4,256-6, 864 indicating the backbone has not integrated in a large proportion of this sample, potentially a small subset of the sample might contain the backbone as well. Also, coverage is observed at the ITRs, indicating that next to the integration through the homology arms also ITR based integrations occurred in the sample. Sequence variants and structural variants were called in the covered regions.

Sequence variants

A single sequence variants of the transgene was detected by this method as shown in Supplementary Table 4. The frequency of detection suggests this variant was present within the AAV6 vector itself.

Supplementary Figure 7

Supplementary Figure 7. CD33 expression level in different cancer cells.

Supplementary Figure 8

Expressing CD33CAR on NK cells also enhances antitumor activity of NK cells against *Kasumi-1* as shown in representative cytotoxicity assay performed in different effector:target ratios. This enhanced cytotoxic activity was observed against *HL-60* only in CD33CAR-Gen2 NK cells.

Supplementary Table 1: Primers used for Inference of CRISPR Edits (ICE) mutation detection assay

Forward primer	5' TTCTCCTGTGGATTCTGGGTCAC 3'
Reverse primer	5' CTCTCTGGCTCCATCGTAAGCA 3'

Supplementary Table 2: Primers used for PCR-based detection of transgenes integration

Condition 1	
Reverse – 1200bp (1)	TCCTGGGCAAACAGCATAA
Forward-CD33CAR (1)	GAGCTGCAGAAGGACAAGAT
Condition 2	
Reverse-CD33CAR (2)	CTCTGTGTCATCTGGATGTCTG
Forward- 1200bp (2)	CTTTGAGCTCTACTGGCTTCTG
Condition 3	
Reverse – 1200bp (1)	TCCTGGGCAAACAGCATAA
Forward- 1200bp (2)	CTTTGAGCTCTACTGGCTTCTG

Supplementary Table 3: Vector-specific primer sets used for targeted locus amplification (TLA).

Primer set	Name/View point	Direction	Sequence
1	600bp HA AAVS1	Reverse	GCGAGTGAAGACGGCATG
		Forward	GTCTGTGCTAGCTCTTCCAG
2	CD33CAR- Gen2	Reverse	GCGATGTCAGAAGGGTAAA
		Forward	GGCGGACACTCTGACTACAT

Supplementary Table 4: Transgene sequence variant identified

				Primer set 1		Primer set 2	
Region	Position	Reference	Mutation	Coverage	%	Coverage	%
600bp HA AAVS1	4,116	T	C	193	37	341	14

Supplementary Data 1

Identifying structural variants

4 vector-vector breakpoints were found. All fusions were located at the annotated homology arm. Due to the heterogeneous nature of the sample it is expected that these fusions are only present in a subset of the sample. It should be noted that three out of four fusions show 9-12 bp homology which might indicate technical bias.

Vector: 149 (head) fused to Vector: 4116 (tail) with 9 homologous bases
GGCATGGGGTTGGGTGAGGGAGGAGAGATGCCCCGAGAGGACCCAGACACGGGGAGGATC
CGCTCAGAGGACATCACGTGGTGACGCGCGCGCCGCCGAGAAAGGGAGTAGAGGCGG
CCACGACCTGGTGAACACCTAGGACGCACCATTTCTACAAAGGGAGTTTTCCACACGGACACC
CCCCCTCACCACAGCCCTGCCAGGACGGGGCTGGCTACTGGCCTTATCTC

Vector: 149 (head) fused to Vector: 4,113 (tail) with 12 homologous bases
GCGAGTGAAGACGGCATGGGGTTGGGTGAGGGAGGAGAGATGCCCCGAGAGGACCCAGAC
ACGGGGAGGATCCGCTCAGAGGACATCACGTGGTGACGCGCGCGCCGCCGAGGAAGG
GAGTAGAGGCGGCCACGACCTGGTGAACACCTAGGACGCACCATTTCTACAAAGGGAGTTTT
CCACACGGACACCCCCCTCCTCACCACAGCCCTGCCAGGACGGGGCTGGCTACTGGCCTTA

Vector: 155 (head) fused to Vector: 4,163 (tail) with 9 homologous bases
GCGAGTGAAGACGGCATGGGGTTGGGTGAGGGAGGAGAGATGCCCCGAGAGGACCCAGAC
ACGGGGAGGATCCGCTCAGAGGACATCACGTGGTGACGCGCGCGCAGAGAGGGAGTGCC
CAACTCCATCACTAGGGGTTCTGCGGCCGAGAAAGGGAGTAGAGGCGGCCACGACCTGGT
GAACACCTAGGACGCACCATTTCTACAAAGGGAGTTTTCCACACGGA

Vector: 158 (head) fused to Vector: 4,121 (tail) with 4 homologous bases
GCGAGTGAAGACGGCATGGGGTTGGGTGAGGGAGGAGAGATGCCCCGAGAGGACCCAGAC
ACGGGGAGGATCCGCTCAGAGGACATCACGTGGTGACGCGCGCGCAGAAAGGGAGTAGAG
GCGGCCACGACCTGGTGAACACCTAGGACGCACCATTTCTACAAAGGGAGTTTTCCACACGG
ACACCCCCCTCCTCACCACAGCCCTGCCAGGACGGGGCTGGCTACTGGCCTT

Supplementary Table 5: List of the antibodies used for CyTOF analysis

140Ce_cPARP
151Eu_CD123
153Eu_HLA-DR
154Sm_CD69
156Gd_CyclinB1
165Ho_pRb
175Lu_Perforin
142Ce_CD7
148Nd_CD34
152Sm_CD33
167Er_CD99
139La_CD41
143Nd_CD71
144Nd_CD94
149Sm_CD127
155Gd_PD-1
158Gd_Ki67
159Tb_CD38
160Gd_CD14
164Dy_CD15
166Er_NKG2D_CD314
168Er_CD13
169Tm_NKG2A_CD159
170Er_pH2AX
172Yb_CD10
174Yb_CD20
161Dy_CD16
146Nd_CD8a
150Nd_CD117
157Gd_CD45RA
162Dy_CD11b
163Dy_CD64
171Yb_CD200
173Yb_CD19
176Yb_pCREB
115In_CD45
194Pt_H3K27me3
147Pm_CD56
113In_CD3
209Bi_pHH3
89Y_CD235