Video Article

Generation of Knock-out Primary and Expanded Human NK Cells Using Cas9 Ribonucleoproteins

Meisam Naeimi Kararoudi[1], Hamid Dolatshad[2], Prashant Trikha[1], Syed-Rehan A. Hussain[3], Ezgi Elmas[1], Jennifer A. Foltz[1], Jena E. Moseman[1], Aarohi Thakkar[1], Robin J. Nakkula[1], Margaret Lamb[1], Nitin Chakravarti[1], K. John McLaughlin[3], Dean A. Lee[1]

[1]Center for Childhood Cancer and Blood Disease, Nationwide Children's Hospital

[2]Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford

[3]Center for Clinical and Translational Research, Nationwide Children's Hospital Research Institute

Correspondence to: Dean A. Lee at Dean.lee@nationwidechildrens.org

URL: https://www.jove.com/video/58237
DOI: doi:10.3791/58237

Keywords: Immunology and Infection, Issue 136, Human primary NK cells, Human expanded NK cells, CRISPR/Cas9, Cas9/RNPs, gRNAs, TGFBR2, TGFβ resistant NK cells

Date Published: 6/14/2018

Citation: Naeimi Kararoudi, M., Dolatshad, H., Trikha, P., Hussain, S.R., Elmas, E., Foltz, J.A., Moseman, J.E., Thakkar, A., Nakkula, R.J., Lamb, M., Chakravarti, N., McLaughlin, K.J., Lee, D.A. Generation of Knock-out Primary and Expanded Human NK Cells Using Cas9 Ribonucleoproteins. *J. Vis. Exp.* (136), e58237, doi:10.3791/58237 (2018).

Abstract

CRISPR/Cas9 technology is accelerating genome engineering in many cell types, but so far, gene delivery and stable gene modification have been challenging in primary NK cells. For example, transgene delivery using lentiviral or retroviral transduction resulted in a limited yield of genetically-engineered NK cells due to substantial procedure-associated NK cell apoptosis. We describe here a DNA-free method for genome editing of human primary and expanded NK cells using Cas9 ribonucleoprotein complexes (Cas9/RNPs). This method allowed efficient knockout of the TGFBR2 *and* HPRT1 genes in NK cells. RT-PCR data showed a significant decrease in gene expression level, and a cytotoxicity assay of a representative cell product suggested that the RNP-modified NK cells became less sensitive to TGFβ. Genetically modified cells could be expanded post-electroporation by stimulation with irradiated mbIL21-expressing feeder cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/58237/

Introduction

Cancer immunotherapy has been advanced in recent years. Genetically-modified chimeric antigen receptor (CAR) T cells are an excellent example of engineered immune cells successfully deployed in cancer immunotherapy. These cells were recently approved by the FDA for treatment against CD19 + B cell malignancies, but success has so far been limited to diseases bearing a few targetable antigens, and targeting such limited antigenic repertoires is prone to failure by immune escape. Furthermore, CAR T cells have been focused on the use of autologous T cells because of the risk of graft-versus-host disease caused by allogeneic T cells. In contrast, NK cells are able to kill tumor targets in an antigen-independent manner and do not cause GvHD, which makes them a good candidate for cancer immunotherapy[6,7,8,9].

CRISPR/Cas9 technology has been used recently in engineering immune cells, but genetically reprogramming NK cells with plasmids has always been challenging. This has been due to difficulties in transgene delivery in a DNA dependent manner such as lentiviral and retroviral transduction causing substantial procedure-associated NK cell apoptosis and the limited production of genetically engineered NK cells[4,9].

Many innate immune cells express high levels of receptors for pathogen-associated molecular patterns such as *Retinoic acid-inducible gene I* (RIG-I), which enable heightened recognition of foreign DNA. Suppression of these pathways has enabled higher transduction efficiency in NK cells when using DNA-based methods for genetic modification[10].

We describe here the method for using a DNA-free genome editing of primary and expanded human NK cells utilizing Cas9 ribonucleoprotein complexes (Cas9/RNPs). Cas9/RNPs is composed of three components, recombinant Cas9 protein complexed with synthetic single-guide RNA comprised of a complexed crRNA and tracerRNA. These Cas9/RNPs are capable of cleaving genomic targets with higher efficiency as compared to foreign DNA-dependent approaches due to their delivery as functional complexes. Additionally, rapid clearance of Cas9/RNPs from the cells may reduce the off-target effects such as induction of apoptosis. Thus, they can be used to generate knock-outs, or knock-ins when combined with DNA for homologous recombination[6,7]. We showed that electroporation of Cas9/RNPs is an easy and relatively efficient method that overcomes the previous constraints of genetic modification in NK cells.

TGFβ is a major immunosuppressive cytokine, which inhibits the activation and functions of NK cells. It has been suggested that targeting the TGFβ pathway can increase immune cell functions. We targeted the region encoding TGBR2 ectodomain which binds TGFβ[11]. The representative results show a significant decrease in the level of mRNA expression of this gene and further demonstrate that the modified NK cells become resistant to TGFβ. In addition, the modified cells retain viability and proliferative potential, as they are able to be expanded post-

electroporation using irradiated feeder cells. Therefore, the following method is a promising approach to genetically manipulate NK cells for any further clinical or research purposes.

Protocol

Healthy donor buffy coats were obtained as source material from the Central Ohio Region American Red Cross. This research was determined to be exempt research by the Institutional Review Board of Nationwide Children's Hospital.

1. Human NK Cell Purification and Expansion

1. Isolate PBMCs from Buffy Coat[12].
    1. Layer 35 mL of buffy coat sample on 15 mL of Ficoll-Paque.
    2. Centrifuge at 400 x g for 20 minutes without brake and collect the PBMC from the interface.
    3. Wash the recovered PBMCs three times by adding at least an equal volume of PBS, centrifuging at 400 x g for 5 minutes, and aspirating the PBC wash. NK cell can be isolated at this stage by RosettesSep[13].

2. Expand NK cells by stimulating with irradiated 10 x 10^6 mbIL21-expressing feeder cells at Days 0, 7, and 14. Replace media with fresh RPMI containing 10% FBS, 1% Glutamine, 1% Penicillin Streptomycin, and 100 IU/mL of IL-2 for the entire media volume every other day.

2. gRNA Design and Selection

1. Choose the specific genomic loci to target, using online tools, *e.g.,* NCBI, Ensemble.
   Example.
   Description: Transforming growth factor beta receptor 2 (TGFBRR2) ectodomain
   View record: PF08917
   View InterPro: IPR015013
   Position: 49 - 157 aa
   Targeted Sequence: Exon 4 of TGFBR2 gene (ENSG00000163513)
2. To design the gRNAs, use CRISPR design web tools such as http://crispr.mit.edu and 'Benchling.'
    1. Enter in the DNA sequence chosen in **step 2.1**. Choose human (hg 19) as a target genome. CRISPR guides (20 nucleotides followed by a PAM sequence: NGG) will be scanned from the sequence entered earlier. It also shows possible off-target matches throughout the selected genome.
    2. Choose the best three gRNAs which have the highest score, based on their on-target and off-target rates. For example, **Table 1** shows the designed CRISPR RNAs to target exon 4 of TGFBR2 gene suggested by CRISPR design web tools.

3. Order the CRISPR RNAs as synthetic sequence-specific crRNAs.
4. Order a conserved, transactivating RNA (tracrRNA) to interact through partial homology with the crRNA.

3. Design Deletion Screening Primers

1. Design primers spanning the gRNA cleavage sites for T7E1 mutation assay.
2. Use primers at least 100 bp away from the predicted cleavage site to ensure small insertion-deletion (indels) at the sgRNA target site will appear on 1.5% agarose gel following the mutation assay. **Table 2** shows the primers which are used to amplify the TGFBR2 ectodomain.

4. Transduction of Human Primary and Expanded NK Cells

Note: Transduction of Cas9/RNPs elements into NK is done by electroporation using 4D system as follows.

1. **Cell Preparation**
    1. **For primary NK cells**, incubate freshly isolated NK cells in RPMI medium in the presence of 100 IU/mL of IL-2 for 4 days and perform the electroporation at Day 5. Replace the media every other day as described earlier and the day before transduction.
    2. **For expanded NK cells**, stimulate the cells at day 0 with irradiated feeder cells at a ratio of 1:1 and perform the electroporation at Day 5 or 6 or 7. Replace the media every other day as described earlier and the day before transduction.
    3. On the day of electroporation, prepare a T25 flask filled with 8 mL of fresh RPMI containing 100 IU/mL of IL-2 for cells undergoing electroporation and pre-incubate flasks in a humidified 37 °C and 5% CO_2 incubator.
       Note: Thawed cells or cells that have undergone 2nd or 3rd stimulation can be electroporated at any time after their recovery as described.
    4. Take 3 - 4 × 10^6 cells per condition for 26 µL of transduction mix.
       Note: Very high concentration of NK cells in electroporation solution enhances the transduction rate.
    5. Wash the cells 3 times with PBS to remove all FBS, which commonly contains RNase activity. Spin them down each time at 300 x g for 8 minutes.
       Note: Consider 7 electroporation conditions for Cas/RNPs as single gRNA (gRNA1, gRNA2, gRNA3) and a combination of two gRNAs (gRNA1+gRNA2, gRNA1+gRNA3, gRNA2+gRNA3) and one control with no Cas9/RNPs.

2. **Form the crRNA:tracerRNA/complex**
    1. Resuspend crRNAs (gRNA1, gRNA2, and gRNA3) and tracerRNA in 1x TE solution to final concentrations of 200 µM. Mix 2.2 µL of each 200 µM gRNA with 200 µM tracerRNA as shown in **Table 3**.

2. Heat the samples at 95 °C for 5 min and allow to cool on the bench top to room temperature (15 - 25 °C). Store resuspended RNAs and crRNA: tracerRNA/complex at -20 °C for later use.

3. **Form the RNP complex**
   Note: To save time, form the RNP complex during the washing **step 4.1.5.**
   1. For single crRNA:tracrRNA duplex reaction, dilute Cas9 endonuclease to 36 µM as shown by the example in **Table 4**.
   2. For combination transduction of crRNA:tracrRNA duplexes, dilute Cas9 endonuclease to 36 µM as shown by the example in **Table 5.**
   3. Add Cas9 endonuclease to crRNA:tracrRNA duplexes slowly while swirling pipette tip, over 30 s to 1 minute.
   4. Incubate the mixture at room temperature for 15 - 20 min. If not ready to use the mixture after incubation, keep the mixture on ice until use.

4. **Electroporation**
   1. Add the entire supplement to the electroporation solution P3 and keep it at room temperature.
   2. Resuspend the cell pellet (3 - 4 × 10^6 cells from **step 4.1.5**) in 20 µL of P3 primary 4D electroporation solution. Avoid air bubbles while pipetting.
      Note: The cells should not be left for a long time in P3 solution.
   3. Immediately add 5 µL of RNP complex (Step 4.3) to the cell suspension.
   4. Add 1 µL of 100 µM Cas9 electroporation enhancer to the Cas9/RNPs/cell mix.
   5. Transfer Cas9/RNPs/cell mix into 20 µL electroporation strips.
   6. Gently tap the strips to make sure that the sample covers the bottom of the strips.
   7. Start 4D electroporation system and choose the **EN-138** program.

5. Post Transduction

1. Let the cells rest for 3 minutes in the strips.
2. Add 80 µL of the pre-equilibrated culture media to the cuvette and gently transfer the sample into flasks.
3. 48 hours after transduction, extract genomic DNA from 5 × 10^5 cells for the gene deletions screening.
4. Amplify the gene of interest using the primers designed in step 3.2 with Taq DNA polymerase kits.
5. Form PCR amplicon heteroduplexes for T7EI digestion and incubate the product for 30 - 60 minutes with a T7EI enzyme in 37 °C.
   Note: The T7EI assay is preferred for screening as it is fast, simple and provides clean electrophoresis results compared to using surveyor assay. However, this method cannot detect insertions and deletions of <2 bases that are generated by non-homologous end joining (NHEJ) activity in Cas9 RNPs experiments[14].
6. Run the digested DNA on 1.5% agarose gel at 110 V for 30 - 45 minutes, every 15 minutes visualize the gel.
7. Stimulate the rest of the cells with the mbIL21-expressing feeder cells at a ratio of 1:1.
8. Five days after stimulation extract the RNA for gene expression level using qPCR.
9. Conduct calcein assays as previously reported[12]. Briefly, load target cells with calcein AM (in the example shown, 3 µg/mL/1,000,000 DAOY cells was used). Prepare NK cells for cytotoxicity assays by resting overnight in IL2 (100 IU/mL) plus or minus 10 ng/mL soluble TGFβ. Conduct calcein assays in the same cytokines as the NK cells were rested in overnight.

Representative Results

Electroporation Efficiency

To optimize electroporation of Cas9/RNPs, we tested 16 different programs with transduction of GFP non-targeting siRNA and DNA plasmid into NK cells. Flow cytometry assay showed that the **EN-138** had the highest percentage of cell viability and transduction efficiency (35% live GFP positive cells) for both particles (**Figure 1** & **Figure 2**). Interestingly, the efficiency of using this program for Cas9/RNPs electroporation was higher as we saw 60% reduction in TGFBR2 mRNA expression level (**Figure 5**). Additionally, the genetically modified NK cells could be grown and expanded for 30 days and cryopreserved (data not shown).

Mutation assay

Cas9/RNPs containing gRNA2, gRNA1+gRNA2 and gRNA3 had successful TGFBR2 ectodomain gene knockout, but gRNA1 alone did not make any T7E1 detectable indels (**Figure 3**). Additionally, **Figure 4** indicates successfully knockout of Human HPRT1 *(hypoxanthine phosphoribosyltransferase 1)* in expanded human NK cells using commercially provided gRNAs. According to band densitometry, the proportional indel rates using gRNA1+gRNA2 resulted in 34% band for TGFBR2 modified NK cells, 25% for gRNA3 and 81% for the HPRT gene modified NK cells.

Gene expression level assay

As a representative of our result, **Figure 5** shows the effect of Cas9/RNPs (gRNA1+gRNA2) on mRNA production level of TGFBR2 ectodomain, analyzed by RT-PCR. As seen in the graph, the mRNA expression level of the targeted gene significantly decreased.

Cytotoxicity

As seen in **Figure 6**, after incubating gRNA1+gRNA2, gRNA2 and gRNA3 Cas9/RNPs modified cells with TGFβ1, co-cultured with DAOY cells; the modified cells did not show any significant decrease in their cytotoxicity level in comparison to the control group which had IL-2 in the media overnight. This result demonstrates that the Cas9/RNPs modified cells retain their cytotoxicity function in the presence of TGFβ1 and shows that the modified cells became TGFβ1 resistant.

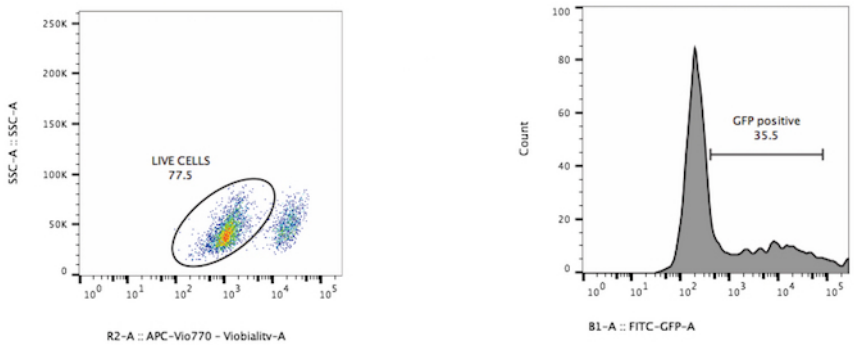

Figure 1. These figures show the electroporation efficiency of siRNA and plasmid DNA expressing GFP in NK cells using the EN-138 program. As seen here, the NK cell viability is 77.5%, and 35% of live cells were GFP positive. Please click here to view a larger version of this figure.

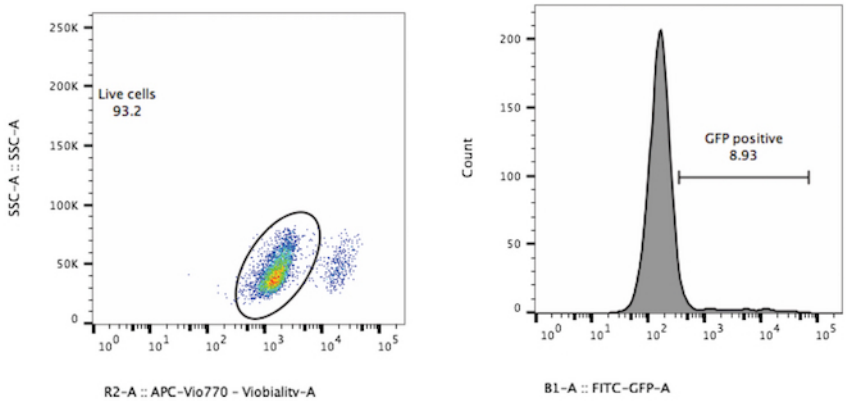

Figure 2. This figure shows viability and efficiency of another one of the 16 programs **(DN-100)** tested for electroporation optimization. Please click here to view a larger version of this figure.

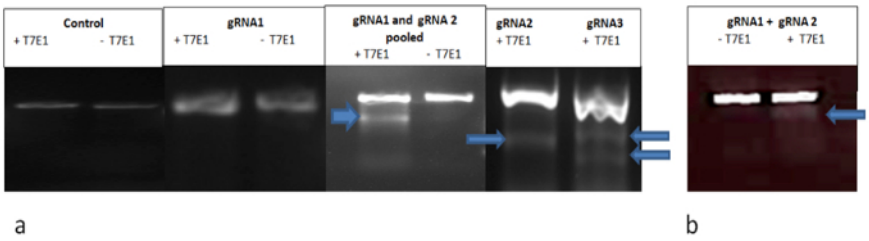

Figure 3. Cas9/RNPs-mediated TGFBR2 knockout in expended **(a)** Primary NK cells **(b)** measured by T7E1 mutation assay. T7E1 enzyme recognizes and cleaves mismatched DNA. Each small band (blue arrows) represents digested DNA fragments which carry an indel. Please click here to view a larger version of this figure.

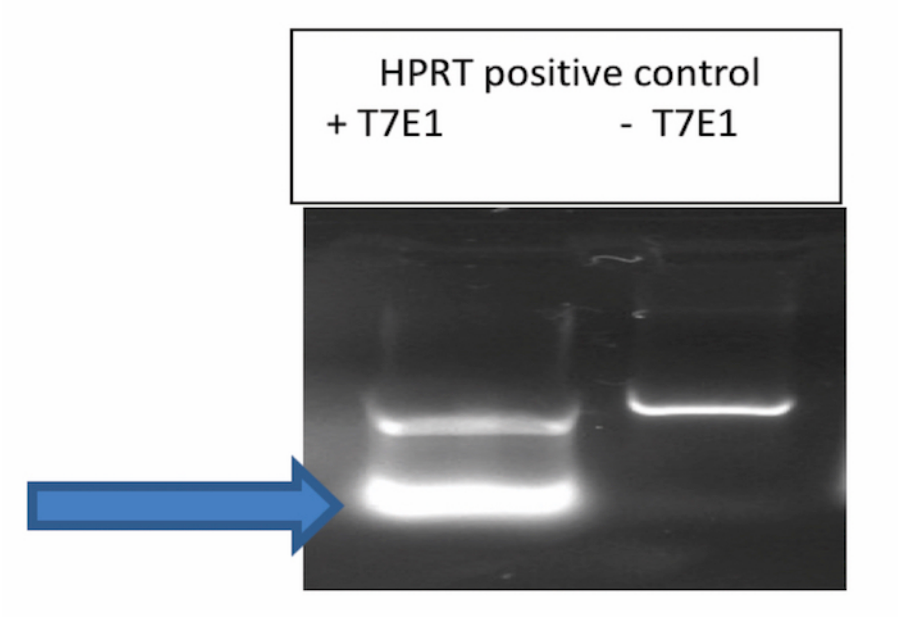

Figure 4. Cas9/RNPs - mediated HPRT disruption in expanded NK cells measured by T7E1 mutation assay. Please click here to view a larger version of this figure.

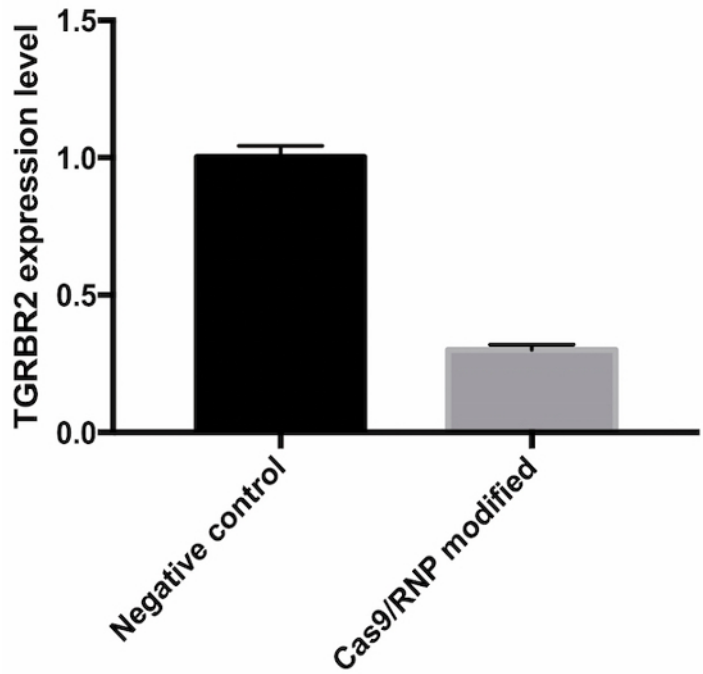

Figure 5. mRNA expression level of TGFBR2 ectodomain in CRISPR modified NK cells introduced by Cas9/RNPs (gRNA1+gRNA2) using RT-PCR. GAPDH was used as an endogenous control gene. The reduction in RNA levels indicates a disruption of TGFBR2 gene (mean ± SEM, P value <0.0001). Please click here to view a larger version of this figure.

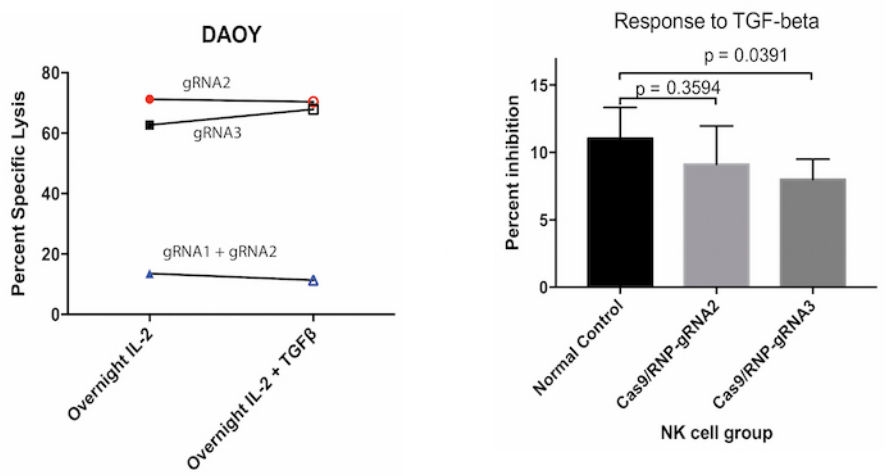

Figure 6. a. The cytotoxicity assay using a representative sample of Cas9/RNPs modified (gRNA1+gRNA2, gRNA2, and gRNA3) NK cells shows that overnight incubation of the cells with TGFβ1 does not decrease significantly their ability to lyse DAOY cells. **b.** When compared with non-modified NK cells, the Cas9/RNP modified NK cells (gRNA2 and gRNA3) are less sensitive to TGFβ1 (mean ± SEM). Please click here to view a larger version of this figure.

gRNA NO.	gRNA sequence	Ordered as synthetic crRNA
gRNA1	5 CCCCTACCATGACTTTATTC 3	/AltR1/ rArGrUrCrArUrGrGrUrArGrGrGrGrArGrCrUrUrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/ AltR2/
gRNA2	5 ATTGCACTCATCAGAGCTAC 3	/AltR1/ rArUrUrGrCrArCrUrCrArUrCrArGrArGrCrUrArCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/ AltR2/
gRNA3	5 AGTCATGGTAGGGGAGCTTG 3	/AltR1/rArG rUrCrA rUrGrG rUrArGrGrGrG rArGrC rUrUrG rGrUrUrUrUrA rGrArG rCrUrA rUrGrCrU/AltR2/

Table 1. Three designed gRNAs to target exon 4 of TGFBR2 ectodomain as synthetic crRNA.

TGFBR 2 ectodomain Primers FWD	5 GTC TGC TCC AGG TGA TGT TTA T3
TGFBR2 ectodomain Primer REV	5 GGG CCT GAG AAT CTG CAT TTA 3

Table 2. Primers used to amplify the TGFBR2 ectodomain gene

Component	Amount (uL)
200 µM crRNA	2.2
200 µM Tracer RNA	2.2
IDTE Buffer	5.6
Final product	10

Table 3. Form the crRNA:tracerRNA/complex using 200 µM RNAs

Component	Amount (µL)
PBS	1
crRNA:tracrRNA duplex (from step 4.2)	2 (200 pmol)
Alt-R Cas9 endonuclease (61 µM stock)	2
Total volume	5 ul

Table 4. For single crRNA:tracrRNA duplex reaction, dilute Cas9 endonuclease to 36 µM.

Component	Amount (µL)
PBS	1
crRNA:tracrRNA duplex (ex. gRNA1)	1 (100 pmol)
crRNA:tracrRNA duplex (ex. gRNA2)	1 (100 pmol)
Alt-R Cas9 endonuclease	2
Total volume	5 µL

Table 5. For combination transduction of crRNA:tracrRNA duplexes dilute Cas9 endonuclease to 36 µM.

Discussion

DNA-dependent modification of NK cells has been challenging[4,9]. We, therefore, introduced directly a synthetically preformed ribonucleoprotein (RNPs) complex and Cas9 protein as purified protein into primary and expanded NK cells[8]. This method allowed us to eliminate capping, tailing, and other transcriptional and translational processes started by RNA polymerase II, which may cause NK cell apoptosis associated with DNA-dependent transduction methods.

In addition, the method reported here uses purified Cas9 protein complexed as Cas9/RNPs, which are active immediately following electroporation and are degraded quickly, thereby increasing on-target and decreasing off-target effects over current protocols[5,6,7,16]. Furthermore, optimizing a new electroporation approach to transduce Cas9/RNP with high efficiency is another critical step introduced here, which are applicable to any other genes of interest. This method may be scaled up for modification of larger numbers of NK cells using commercially available larger electroporation cuvettes (data not shown).

In summary, Cas9/RNPs can be used to genetically modify human primary and expanded NK cells for cancer immunotherapy utilizing the above described method. Our results also demonstrated that a successful knockout of the *TGFBR2 ectodomain* gene leads to these modified NK cells becoming TGFβ1 resistant[11].

Combining RNP delivery with a source of template DNA (such as naturally recombinogenic adeno-associated virus (AAV) donor vectors) may enable site-specific gene insertion by homologous recombination[3,18,19].

Disclosures

DAL serves/has served as a consultant for Courier Therapeutics, Obsidian Therapeutics, Intellia Therapeutics, Merck Research Laboratories, and Miltenyi Biotec, and has equity/Leadership in CytoSen Therapeutics.

Acknowledgements

We acknowledge Brian Tullius for his kind help in editing the manuscript.

References

1. Guven, H. *et al.* Efficient gene transfer into primary human natural killer cells by retroviral transduction. *Experimental Hematology.* **33** (11), 1320-1328 (2005).
2. Alici, E., Sutlu, T., & Sirac Dilber, M. Retroviral gene transfer into primary human natural killer cells. *Methods in Molecular Biology.* **506** 127-137 (2009).
3. Carlsten, M., & Childs, R. W. Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications. *Frontiers in Immunology.* **6,** 266 (2015).
4. Mehta, R. S., & Rezvani, K. Chimeric Antigen Receptor Expressing Natural Killer Cells for the Immunotherapy of Cancer. *Frontiers in Immunology.* **9,** 283 (2018).
5. Zuris, J. A. *et al.* Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo. Nature Biotechnology.* **33** (1), 73-80 (2015).
6. Wang, M. *et al.* Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles. *PNAS.* **113** (11), 2868-2873 (2016).
7. Liang, Z. *et al.* Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications.* **8,** 14261 (2017).
8. DeWitt, M. A., Corn, J. E., & Carroll, D. Genome editing via delivery of Cas9 ribonucleoprotein. *Methods.* **121-122,** 9-15 (2017).
9. Rezvani, K., Rouce, R., Liu, E., & Shpall, E. Engineering Natural Killer Cells for Cancer Immunotherapy. *Molecular Therapy* . **25** (8), 1769-1781 (2017).
10. Sutlu, T. *et al.* Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. *Human Gene Therapy.* **23** (10), 1090-1100 (2012).
11. Viel, S. *et al.* TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Science Signaling.* **9** (415), ra19 (2016).
12. Somanchi, S. S., Senyukov, V. V., Denman, C. J., & Lee, D. A. Expansion, purification, and functional assessment of human peripheral blood NK cells. *JoVE.* (48) (2011).
13. Lee, D. A., Verneris, M. R., & Campana, D. Acquisition, preparation, and functional assessment of human NK cells for adoptive immunotherapy. *Methods in Molecular Biology.* **651** 61-77 (2010).

14. Vouillot, L., Thelie, A., & Pollet, N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda).* **5** (3), 407-415 (2015).
15. Eyquem, J. *et al.* Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature.* **543** (7643), 113-117 (2017).
16. Liang, X. *et al.* Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection.Journal of Biotechnology. **208** 44-53 (2015).
17. Kim, S., Kim, D., Cho, S. W., Kim, J., & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research.* **24** (6), 1012-1019 (2014).
18. Chmielecki, J. *et al.* Genomic Profiling of a Large Set of Diverse Pediatric Cancers Identifies Known and Novel Mutations across Tumor Spectra. *Cancer Research.* **77** (2), 509-519 (2017).
19. Schultz, L. M., Majzner, R., Davis, K. L., & Mackall, C. New developments in immunotherapy for pediatric solid tumors. *Current Opinion in Pediatrics.* **30** (1), 30-39 (2018).

