MAKING A MURDERER

Retroviral and CRISPR/Cas9 genome editing of ADAPT-Natural Killer cells to increase cancer killing potential

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Table of Contents

Glossary

- Antibody Dependent Cellular Cytotoxicity (ADCC)
- Chimeric Antigen Receptor (CAR)
- Epithelial Growth Factor Truncated (EGFRt)
- guide RNA (gRNA)
- Human Leukocyte Antigen receptor class 1 (HLA-I)
- Killer Immunoglobulin like- Receptors (KIR)
- Knock-In (K.I.)
- Knock-Out (K.O.)
- Natural Killer (NK) cell
- Peripheral Blood Mononuclear Cells (PBMCs)
- Ribonucleoproteins (RNP)
- Stem Cell Growth Medium (SCGM)

Abstract

Natural killer (NK) cells have great potential as an immunotherapy platform due to their inherent cytotoxicity and favourable safety profile compared to T cell-based therapies. Recently, our group developed a method for expanding a particularly cytotoxic NK cell subset called ADAPT-NK cells. This study explores the enhancement of these ADAPT-NK cells trough genetic modification using retroviral transduction and CRISPR/Cas9 genome editing techniques. We demonstrate that introducing a CAR19 into the ADAPT-NK cells using retroviral transduction allows them to target CD19 positive cells that normally do not elicit a reaction. Furthermore, we show that by using CRISPR/Cas9 we can effectively knock out DNAM1 and NKG2C genes in ADAPT-NK cells, as well as up to three genes at the same time using multiplexed gene edits. Additionally, we also employ CRISPR/Cas9 knock-in strategies to prove that this technique can be used to introduce new genes into primary and ADAPT-NK cells. Our results suggest that, by using genome editing techniques, it is possible to expand the targetable space of ADAPT-NK cells and that both single and multiplexed gene edits are feasible in these cells. With this we lay the groundwork for future advanced immunotherapeutic strategies in ADAPT-NK cells, providing a robust platform for future clinical applications in cancer treatment.

Plain language summary

Immunotherapy is a type of cancer treatment where modified immune cells are used to fight of cancer in a patient. T cells are the most popular type of immune cell used in immunotherapy, but NK cells, another type of immune cell are also good at killing cancer and are less toxic to patients then T cells. Recently our group developed a method of growing a specific type of NK cell that is especially good at killing cancer, called the ADAPT-NK cells. In this study we looked at ways to make these ADAPT-NK cells even better at fighting cancer by modifying them using advanced genome editing techniques. We used two different gene editing techniques, retroviral transduction, which uses viruses to modify the DNA. And CRISPR/Cas9 which is an editing technique that uses the Cas9 protein to cleave the DNA. First, we added a CAR19 into the ADAPT-NK cells, this is a special receptor that allows the NK cells to attack cancer cells that they would normally not recognize that have a protein called CD19 on their surface. Next, we used CRISPR/Cas9 to both destroy specific genes in the ADAPT-NK cells. We were able to remove up to three genes at the same time, showing that this technique can be used to edit multiple genes in one go. Using CRISPR/Cas 9 we were also able to introduce new genes into both normal and ADAPT-NK cells. Our findings suggest that using these genetic modification techniques we are able to make the ADAPT-NK cells target a wider range of cancer cells and in the future improve them even further in killing cancer. With this we lay the groundwork for advanced cancer treatment using genetically modified ADAPT-NK cells.

Introduction

Over the past few decades, immunotherapy has emerged as a groundbreaking approach in cancer treatment, leveraging the body's immune system to target and eliminate malignant cells. Cellular immunotherapies can take many forms. Among these various strategies, chimeric antigen receptor (CAR)-T cell therapy has gained the most prominence, evidenced by the current approval of six CAR-T cell-based therapies by the U.S. Food and Drug Administration (1). However, NK cells, another type of cytotoxic immune cell, have not received comparable attention when it comes to immunotherapy, despite their promising features.

NK cells possess diverse mechanisms for targeting and destroying cancer cells. Unlike cytotoxic T cells which use a somatically rearranged T cell receptor to recognize specific antigens, the cytotoxicity of NK cells is regulated trough a balance of germline encoded stimulatory and inhibitory receptors. This allows them to target cells with high levels of stress-induced ligands and low levels of human leukocyte antigen class I (HLA-I). Furthermore, NK cells can also mediate antibody dependent cellular cytotoxicity (ADCC) through the CD16 Fc receptors they carry on their surface, consequently enhancing their cytotoxic potential in the presence of anti-tumour antibodies. In comparison T cell-based therapies often do not have any intrinsic cytotoxicity against tumour cells and therefore have to be modified with a CAR to be effective. NK cells can also be modified with a CAR giving them a broad arsenal for killing cancer cells, greatly decreasing the risk of the tumour becoming resistant to the therapy. Moreover, in clinical settings, both unedited and genetically modified NK cell-based therapies exhibit reduced toxicity compared to CAR-T cell-based therapies (2). With lower incidences of both cytokine release syndrome and neurotoxicity (3–5). Reasons proposed as to why NK cells are less toxic in this context include their reduced life span and different cytokine profile (6).

NK cells encompass many different subsets with a wide range of cytotoxic potential. One promising NK cell subset, adaptive NK cells, display features compatible with superior cytotoxicity and are naturally present in cytomegalovirus seropositive individuals. Notably, our group recently developed a method for in vitro expansion of this potent subset that is additionally characterized by the expression of a single- self- killer-cell immunoglobulin-like receptor (KIR), high levels of NKG2C. These precisely defined adaptive NK cells are called 'ADAPT-NK cells.

ADAPT-NK cells are made from NK-cells of super donors that naturally have a large subset of adaptive, NKG2C positive, and single self-KIR positive NK-cells at baseline. The NK-cells are then expanded using an HLA-E expressing K562 feeder cell line and IL-2. The resulting cell product is highly potent, showing high degranulation and efficient killing of cancer cell lines and primary leukemic blasts without signs of exhaustion. The unique single inhibitory KIR profile of ADAPT-NK cells makes them particularly effective against KIR-mismatched cancer cell lines, where the absence of a corresponding HLA-I ligand on the target cells invokes the "missing-self" response. Furthermore, HLA-E, which is expressed in a large fraction of solid tumours, serves as a ligand for both the inhibitory receptor NKG2A and the stimulatory receptor NKG2C (7). Since most NK cell subsets express NKG2A rather than NKG2C, HLA-E is often viewed as an inhibitory molecule. However, ADAPT-NK cells, with their high NKG2C expression, leverage this interaction for enhanced cytotoxicity. Lastly the ADAPT-NK cells retain their CD16 receptor, enabling ADCC, and express high levels of the stimulatory receptor DNAM1 (8). All these factors make ADAPT-NK cells an excellent platform for allogeneic cellular immunotherapy.

Despite the fact that ADAPT-NK cells are inherently very potent we aimed to further enhance their efficacy through genetic editing. While retroviral transduction is the current gold standard for genetic modification in immunotherapy, it also poses risks such as insertional mutagenesis and residual viral particles. As a result of this, the field is shifting towards virus- free editing techniques. Thus, in addition to viral transduction, we also wanted to explore the use of CRISPR/Cas9 technology for virus-free genetic modification. CRISPR/Cas9 offers precise genome editing capabilities through guideRNA (gRNA)-directed DNA cleavage, allowing for accurate gene knock-outs (K.O.) and knock-ins (K.I.). However, as of now, CRISPR/Cas9 gene editing is way less efficient than retroviral transduction and remains largely unexplored for creating NK cell immunotherapies. Our study investigates the feasibility of using both retroviral transduction and CRISPR/Cas9 techniques for enhancing the cytotoxic potential, extending cell persistence, and expanding the targetable space of ADAPT-NK cell-based therapies.

Results

Generation of CAR19 ADAPT-NK cells using retroviral transduction

To expand the targetable space of ADAPT-NK cells, we first introduced a CAR19 via retroviral transduction. CAR19 constructs have proven successful in many immunotherapies, enabling immune cells to bind to CD19, a protein highly expressed in most B cell malignancies (9,10). Our CAR19 construct included a 28z signalling domain and a truncated Epithelial Growth Factor (EGFRt) for tracking

transduction efficiency (Figure 1A). Five ADAPT-NK donors were transduced with a CAR19 retrovirus or a control on day eight of the ADAPT-NK expansion protocol. Flow cytometry analysis of the CAR19 ADAPT-NK cells showed heterogenous levels of EGFRt expression, in which we defined EGFRt-negative, EGFRt-dim, and EGFRt-bright populations. Comparatively, ADAPT-NK cells transduced with a control virus did not express any EGFRt (Figure 1B). Importantly, there was no significant difference in viability between the control and CAR19 ADAPT-NK cells (Figure 1C). This data indicates that retroviral transduction is an effective way of introducing a CAR19 into ADAPT-NK cells without affecting viability.

CAR19 transduced ADAPT-NK cells exhibit a broader cancer killing potential

In order to explore the functionality of the newly transduced CAR19 ADAPT-NK cells, we performed a degranulation assay against K562 and NALM6 cancer cell lines. These cell lines express different CD19 levels and some were modified to overexpress HLA-E on their surface. Using flow cytometry, the HLA-E and CD19 levels of the cell lines were analysed. K562 cell lines did not express CD19 while NALM6 cells lines expressed high levels. Additionally, only the HLA-E-Hi cell lines expressed HLA-E (Figure 1D). Both control and CAR19-transduced ADAPT-NK cells were incubated in a 1:1 effector-to-target cell ratio with the cancer cell lines for a functional assay. The control ADAPT-NK cells showed increased

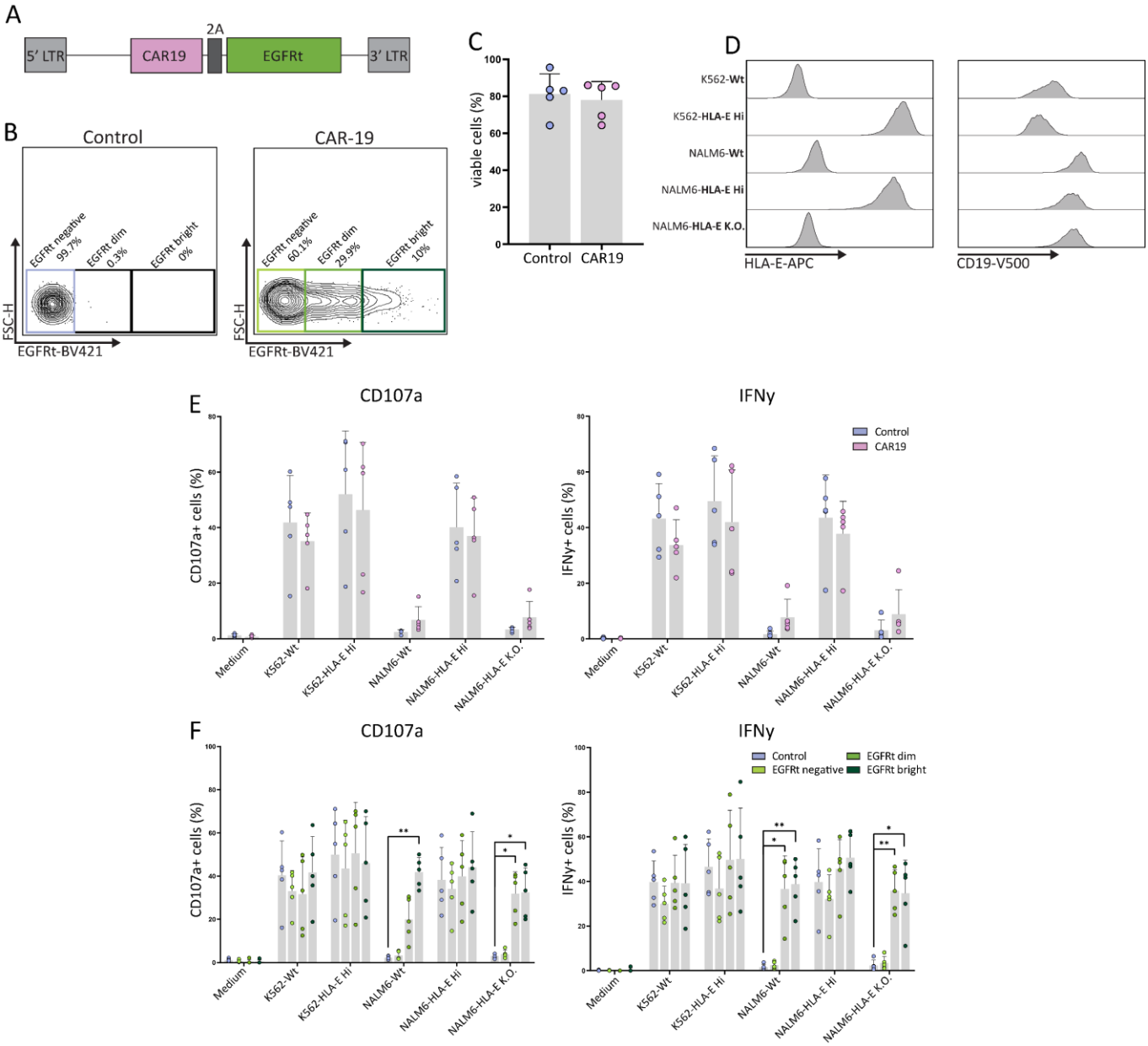

Figure 1: ADAPT-NK cells transduced with a CAR19 show improved degranulation against CD19 positive cells. (A) illustration of CAR19 construct used to transfect ADAPT-NK cells. (B) EGFRt levels of control and CAR19 retro-virus transduced ADAPT-NK cells. (C) Viability of control and CAR19 transduced ADAPT-NK cells. (D) HLA-E and CD19 expression of various K562 & NALM6 cell lines. (E) CD107a and IFNγ levels of control and bulk CAR19 ADAPT-NK cells exposed to K562 and NALM6 cell lines. (F) CD107a and IFNγ levels of control ADAPT-NK cells and EGFRt gated CAR19 ADAPT-NK cells exposed to K562 and NALM6 cell

degranulation markers CD107a and IFNγ against K562 cell lines as well as the NALM6-HLA-E high cell line but not against the NALM6-Wt and NALM6-HLA-E K.O. cell lines. The bulk ADAPT-NK cells transduced with the CAR19 behaved very similarly to the control ADAPT-NK cells except for a slightly increased degranulation against the NALM6-Wt and NALM6-HLA-E K.O. cells, though this increase was not significant (Figure 1E). To omit the effect of the untransduced cells from the analysis, the degranulation of the distinct EGFRt populations was compared to that of the bulk control ADAPT-NK cells. Here we found that for the NALM6-Wt and NALM6-HLA-E K.O. cell lines there was a significant increase in degranulation for all EGFRt bright and most EGFRt dim populations compared to the control. Demonstrating a CAR19 dependent activation of the ADAPT-NK cells against CD19 positive cells lines. Against the NALM6-HLA-E high cell line, though the EGFRt bright and dim populations show a slight increase in degranulation, this was not significant over to the already high reactivity of the control ADAPT-NK cells against this cell line (Figure 1F). We also measured TNF as a degranulation marker and while we saw the same trends the only significant difference was EGFRt bright versus control in NALM6-Wt (Supplementary Figure 1). Taken together these results show that ADAPT-NK cells can be effectively transduced with a CAR19, expanding their targetable space by allowing them to recognize CD19 positive cancer cells that would normally not elicit a response.

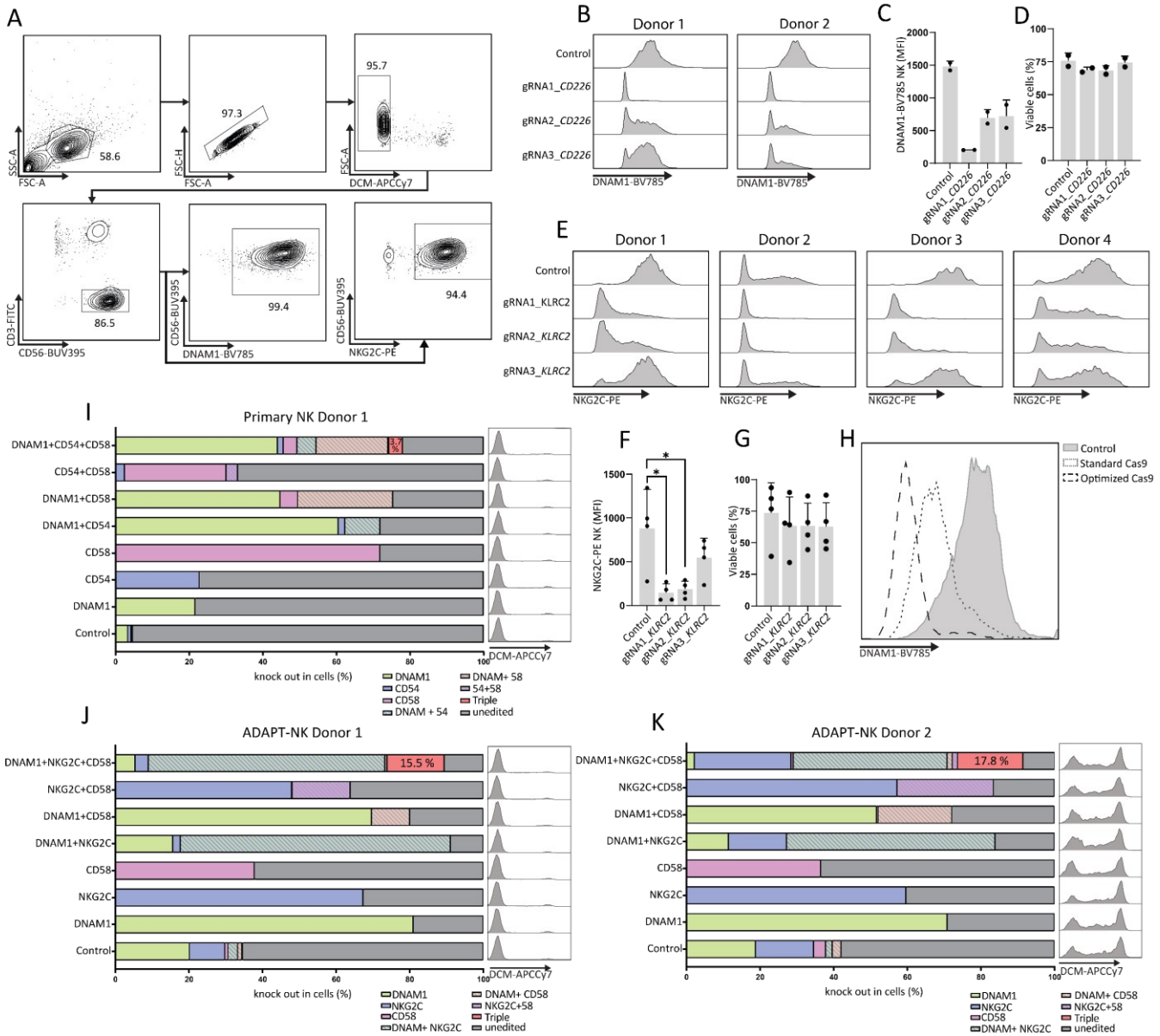

Figure 2: Single and multiplexed gene knock-outs in primary- and ADAPT-NK cells. (A) gating strategy for ADAPT-NK cells showing high and homogenous expression of DNAM1 and NKG2C. (B-C) DNAM1 levels in 2 primary NK cell donors after knockout with various gRNAs compared to control. (D) Viability of primary NK cells after knock-out with *CD226* and *CD14* gRNAs. (E-F) NKG2C levels in 4 ADAPT-NK cell donors after knockout with various gRNAs compared to safe harbour control. (G) viability of ADAPT-NK cells after knock-out with *KLRC2* and *CD14* gRNAs. (H) Histogram showing DNAM1 levels in primary NK cells after knock-out using our standard protocol compared to a protocol using less Cas9 and gRNA. (I-J) Viability and knock-out percentage of primary- and ADAPT-NK cells after knock-out with multiple gRNAs.

CRISPR/Cas9 mediated knock-out of DNAM1 and NKG2C in ADAPT-NK cells

To explore the possibilities of virus-free gene editing in ADAPT- and primary NK cells we targeted *CD226* (DNAM1) and *KLRC2* (NKG2C) for knock-out using CRISPR/Cas9. Flow cytometry confirmed that these proteins are highly and homogeneously expressed in ADAPT-NK cells (Figure 2A). We also confirmed that DNAM1 is highly expressed in primary NK cells (Supplementary Figure 2). A CRISPR/Cas9 K.O. was performed for *KLRC2* in four ADAPT-NK donors using three different gRNAs. Four to five days after the K.O. the NKG2C levels of the cells were measured using flow cytometry. A significant decrease was observed in NKG2C levels for gRNA1_*KLRC2* and gRNA2_*KLRC2* with the first guide performing the best in all donors. None of the gRNAs had a significant impact on the viability of the ADAPT-NK cells (Figure 2E-G). Subsequently, another K.O. was done for *CD226* in two primary NK donors. Primary NK cells were used for validation of the gRNAs since it is an easier material to acquire then ADAPT-NK cells. After four days DNAM1 levels were assessed using flow cytometry and while all gRNAs resulted in lower DNAM1 levels without effecting viability gRNA1_*CD226* performed by far the best (Figure 2B-D). These results demonstrate that that *KLRC2* and *CD226* can be knocked out in NK cells without affecting viability but choosing the right gRNA is crucial for achieving an optimal K.O.

Multiplexing Knock-outs is possible in primary NK cells but not very efficient

Some immunotherapy enhancing strategies, like extending cell persistence in patients, require the modification of multiple genes at the same time (11,12). Therefore, after succeeding in knocking out single genes we investigated the potential of multiplexed knock-outs in NK cells. Since NKG2C is not commonly highly expressed in primary NK cells we confirmed the best gRNAs for *ICAM1* (CD54) and *LFA-3* (CD58) (Supplementary Figure 3). With our three optimized gRNAs we performed a multiplexed K.O., the total amount of gRNA in all conditions remained the same. After seven days all markers were assessed using flow cytometry. While all K.O.s worked, single K.O. for DNAM1 and CD54 were inefficient, resulting in a triple K.O. of only 3.7%. Surprisingly, the K.O. efficiency DNAM1 actually went up when the amount of gRNA was decreased in the double K.O. conditions (Figure 2I). This prompted us to further optimize our K.O. protocol. A combination of modified Cas9:guide ratio and decreased amount of gRNA and Cas9 reagents used, resulted in an increased efficiency of our DNAM1 K.O. in primary NK cells (Figure 2H). We also attempted to increase K.O. efficiency using an electroporation enhancer and polyglutamic acid for better shielding of the Cas9 nucleoproteins, but this only resulted in minor improvements (Supplementary Figure 4).

Multiplexing CRISPR/Cas9 Knock-outs in ADAPT-NK cells is possible and does not affect viability

With the new optimized K.O. protocol we again attempted a multiplexed K.O. but this time in ADAPT-NK cells with guides for DNAM1, NKG2C and CD58. This time the single K.O.s performed significantly better, and we were able to achieve substantial double K.O.s as well as a triple K.O. of 15.5% in one donor and 17.8% in the other. The viability of the ADAPT-NK cells remained unaffected even if multiple gRNAs were added (Figure 2J-K). In conclusion, with our protocol we are able to successfully knock out up to three genes at the same time in NK cells using CRISPR/Cas9. This opens up the future possibilities of immunotherapy strategies requiring multiple edits in ADAPT-NK cells.

CRISPR/Cas9 can be used to introduce novel genes into primary- and ADAPT-NK cells

After confirming we can successfully generate knock-outs in ADAPT-NK cells we wanted to further explore the potential of CRISPR/Cas9 gene editing in ADAPT-NK cells by introducing genes using a knock-in strategy. The CLTA gene

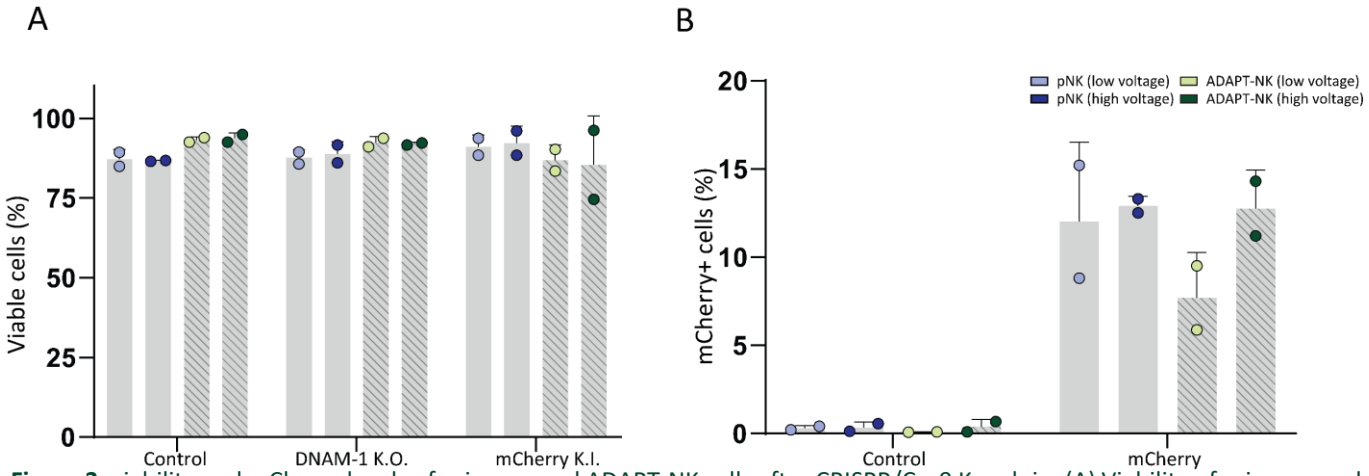

Figure 3: viability and mCherry levels of primary- and ADAPT-NK cells after CRISPR/Cas9 Knock-in. (A) Viability of primary- and ADAPT-NK cells after knock-out and knock-in compared to control at two different electroporation voltages. (B) Percentage of mCherry positive NK cells after knock-in using two electroporation voltages.

was chosen as the knock-in site since it is highly expressed in many cell types and has previously been shown to result in efficient gene insertion (13,14). The donor strand DNA was designed to insert the mCherry protein in-frame of the DNA. Using this template, a K.I. was performed on both feeder-stimulated primary-NK cells and ADAPT-NK cells, with either a low or a high electroporation voltage. Seven days after the K.I. viability and mCherry levels of the cells were analysed using flow cytometry. The introduction of the donor template into the NK cells did not significantly affect the viability compared to a control as well as a DNAM1 knock-out. Furthermore, the higher voltage electroporation program did not negatively affect the viability of the cells except for 1 ADAPT-NK donor (Figure 3A). for both the primary- and ADAPT-NK cells introduction of mCherry into the cells was successful, resulting in 5-15% mCherry expression. Interestingly, both cell types performed slightly better with the high voltage program than the low voltage one, suggesting there is room for further improvement. Thus, CRISPR/Cas9 can be used to introduce novel genes into feeder stimulated primary- and ADAPT-NK cells and the cells tolerate our method well.

Methods

NK cell isolation

Buffy coats were taken from healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated using lymphoprep (serumwerk) and sepmate tubes (stem cell technologies) according to manufacturer instructions. Subsequently, NK cells were isolated using negative magnetic bead selection with an NK cell isolation kit (Miltenyi biotec).

ADAPT-NK cell expansion

Isolated NK cells from super donors (cytomegalovirus positive, >20% NKG2C+ with single self-KIR2DL1 or KIR2DL3 expression) were co-cultured with K562 feeder cells transfected with HLA-E lentiviral construct in a 1:2 ratio in G-Rex24 plates (Wilson Wolf) at a total concentration of $0.5x10^6$ cells/mL. Cells were cultured in Stem Cell Growth Medium (SCGM, cell genix) supplemented with 10% human serum (HS, TCS biosciences or Access biologicals), 2mM L-Glutamine (Cytiva-FisherScientific), and 100IU/mL human recombinant IL-2 (Proleukin) for 11 days. 60% of medium was exchanged on day 7 and IL-2 was added on day 4, 7 and 10.

Retroviral transduction of NK cells

$5x10^6$ Human embryonic kidney (HEK)-cells (Phoenix Ampho) were seeded on a 10 cm plate in 7 mL of Dulbecco's modified eagle medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and cultured for 24 hours. Next 18uL X-tremeGENE (Sigma-Aldrich) and 600uL optimem (Thermo Fischer scientific) was added to 2.6µg JO119 (packaging DNA) and JO120 (packaging DNA) 0.2µg of JO108 (GFP DNA) and 2.6µg CAR19 construct DNA and incubated at 21°C for 15 minutes. For the control the construct DNA was not added. The medium of the HEK cells was refreshed with 7mL DMEM 10% FBS after which the DNA was added, finally cells were incubated at 37°C, 5% CO_2. The next day medium of the HEK cells was replaced by 7mL DMEM 1%FBS and incubated at 32°C, 5% CO_2. The following two days HEK cell supernatant was collected and replaced with fresh DMEM 1% FBS medium. Using a retrovirus concentrator (Takara biotechnology) retrovirus was concentrated four times and resuspended in SCGM 10% HS. At the same time 6-well plates were coated overnight at 4°C with 0.8mL 20ug/mL Retronectin (AH diagnostic). The following day coated plates were blocked with phosphate buffered saline (PBS), 2% FBS after which 2 million ADAPT-NK cells in 1mL SCGM (10% HS, 400IU/mL IL-2, and 20ng/mL IL-15 (prepotech)) and 1mL 4X concentrated retrovirus were added to each well. Plate was spun at 1800g for 60 minutes at 32°C after which the plates were placed in the incubator at 37°C, 5% CO_2. After 2-3 days the cells are ready for functional assays.

Degranulation assay ADAPT-NK cells

Transduced ADAPT-NK cells and target cancer line cells were combined in a 1:1 ratio in 200uL RPMI (Gibco) supplemented with 10% FBS, 2mM glutamine, 1000IU/mL Penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma-Aldrich), and a 1/4000 dilution Golgiplug (BD biosciences). Cells were incubated for 4 hours after which they were stained extracellularly with CD56-BUV395, CD107a-AF488, CD3-ACCCy7, EGFRt-BV421, DCM-Aqua, CD4-BV785 and NKG2A-PECy7. Subsequently cells were fixated and stained intracellularly with INFγ-APC and TNF-PE. Finally, cells were acquired using the FACSymphony A5 (BD biosciences).

CRISPR K.O. in NK cells

Isolated NK cells were stimulated overnight in SCGM (10% Human serum, 100IU/mL IL-2 & 10ng/mL IL-15). TracrRNA (200uM, Integrated DNA technologies) and crRNA (200uM, Integrated DNA technologies) were combined in equimolar concentrations and heated for 5 minutes at 95 degrees and cooled down to 21°C for 5 minutes to create gRNA. Next 1.2uL gRNA, 1.7uL Cas9 protein (61µM, Integrated DNA technologies) and 2.1uL PBS were combined per reaction and incubated for 15 minutes at 21°C to form ribonucleoproteins (RNP). For optimized CRISPR protocol 1.4uL gRNA, 0.85uL Cas9 protein and 2.75uL PBS were used per reaction. For multiplexing protocol amount of gRNA was divided equally by the number of guides used per condition. $0.25x10^6$ NK cells were resuspended in 16uL P3/1M buffer (Lonza) and transferred to 16-well nucleofection strip (Lonza). 5uL of ribonucleoproteins was added to the cells and

nucleofection was performed using CM-137 pulse program in the Lonza 4d nucleofector. Finally, cells were transferred to wells containing 200uL pre-warmed SCGM (10% HS, 100IU/mL IL-2, and 10ng/mL IL-15). After 4-7 days expression levels of proteins were measured using the FACSymphony A5 (BD biosciences).

CRISRP K.I. in NK cells

Primary NK cells were stimulated for 4 days using K562 feeder cells in a 1:2 ratio at a total concentration of $1x10^6$ cells/mL in SCGM (10% Human serum, 100IU/mL IL-2). ADAPT-NK cells were prepared according to standard protocol. RNPs were formed by adding DNAM1 or CLTA single guide RNA (120uM, Synthego) to Cas9 protein (60uM) in a 2:1 ratio and incubating at 21°C for 15 minutes. 1.67uL of RNPs was added to $1.25x10^6$ NK cells in 25uL SCGM (10% Human serum, 100IU/mL IL-2) in OC-25x3 nucleofection cartridges (Maxcyte). For the knock-in conditions 5uL mCherry HDRT at 300ug/mL was added. Next, cells were nucleofected using the ExPERT STx™(Maxcyte) at NK-4 and NK-5 programs. Lastly, cells were allowed to rest for 5 minutes after which they were transferred to wells containing 200uL pre-warmed SCGM (10% HS, 100IU/mL IL-2, and 10ng/mL IL-15). Cells were analysed after 7 days using FACSymphony A5 (BD biosciences).

Discussion

NK cell-based immunotherapies offer numerous advantages over T cell-based immunotherapies. They have shown to be safer in clinical settings and are able to recognize cancer through multiple cytotoxic pathways. Recently our group developed a method for culturing ADAPT-NK cells, a subset of NK cells with superior cytotoxic properties. These cells, characterized by the expression of a single self-KIR, NKG2C, and an adaptive phenotype are highly effective at killing certain cancer cell lines. To further enhance their immunotherapeutic potential, we explored the feasibility of genomic editing of these cells.

Our study demonstrates that by using genetic modification, it is possible to expand the targetable space of ADAPT-NK cells. By introducing a CAR19 using retroviral transduction we were able to redirect ADAPT-NK cells killing capacity to eradicate CD19 positive cells, a common marker of B cell malignancies. The enhanced degranulation of CAR19 ADAPT-NK cells against NALM6 cell lines underscores how CAR-mediated recognition can overcome limitations of the innate targeting mechanisms of ADAPT-NK cells. This finding aligns with previous studies exploring the use of CAR19 constructs in primary- and cord blood NK cells as well as T cells (2,15,16). Despite the promising results, our study also had some limitations. The observed heterogeneity in EGFRt and thus CAR19 expression indicates a variability in the transduction efficiency of NK cells. Furthermore, we were not able to see a statistically significant increase of degranulation against CD19 positive cell lines in the bulk CAR19 ADAPT-NK cells and against NALM6 HLA-E high cell lines in EGFRt gated ADAPT-NK cells compared to control. Future work should focus on optimizing transduction to achieve high and homogenous CAR19 expression in the ADAPT-NK cells. With an optimized protocol and more donors, we hope to be able to show a significant degranulation of bulk CAR19 ADAPT-NK cells against CD19 positive cells. This optimisation could also prove that the increased degranulation of CAR19 ADAPT-NK cells against CD19 HLA-E double positive cell lines is significant. Thereby indicating that a CAR19 and NKG2C can work synergistically, showing not only expansion of the targetable space but also increased cytotoxic potential of the CAR19 ADAPT-NK cells. Moreover, our current CAR19 construct utilizes a 28z structural and signalling domain, however different CAR domains exist and have shown to effect clinical outcomes in CAR-T therapy (17). Also, IL-15/IL-15Rα complexed to a CAR19 has shown to lead to improved proliferation and degranulation in NK92 cell lines (18). Future endeavours could explore whether other signalling domains, like those tailored specifically for NK cells, work superiorly for ADAPT-NK cells.

Additionally, we explored the use of CRISPR/Cas9 gene editing in ADAPT-NK cells. Achieving efficient single and multiplexed gene knock-outs without compromising cell viability. We found that for achieving an optimal knock-out choosing the best gRNA, the gRNA:Cas9 ratio, and the concentration of gRNA and Cas9 protein used are crucial factors. These results highlight the versatility and potential of CRISPR/Cas9 mediated K.O. in NK cell-based immunotherapies. DNAM1 and NKG2C are proteins critical for NK cell activation and signalling and are highly and homogenously expressed in ADAPT-NK cells. While knocking out activating receptors such as DNAM1 and NKG2C was not part on any translational approach, we used them for optimizing our CRISPR/Cas9 protocol. However, this also highlights the potential to modulate NK cell activity using CRISPR/Cas9. This ability to edit functional genes with high precision opens up future possibilities for tailoring therapeutically beneficial NK responses as well as investigating the contribution of specific genes to NK cell function. Our results also show that multiplexed gene knock-outs in primary- and ADAPT-NK cells are feasible, though some further optimisation may be required. Substantial triple K.O.s in ADAPT-NK cells without affecting the viability create the potential for the complex genetic modifications required in advanced immunotherapies. One example is the multiple edits required for immune-evasion strategies for allogeneic therapies, thereby extending cell persistence in patients (11,12).

Besides knocking-out genes we have also shown that CRISPR/Cas9 can be used to introduce novel genes into NK cells. The successful introduction of an mCherry reporter gene into both primary and ADAPT-NK cells indicates that knock-in strategies are viable for these cells. The capability to introduce new genetic constructs into ADAPT-NK cells opens up a host of therapeutic possibilities. In the future would like to experiment by introducing a CAR19 using CIRSPR/Cas9 mediated knock-in into ADAPT-NK cells. Afterwards we can compare the cytotoxicity of these cells to our current CAR19 ADAPT-NK cells created through retroviral transduction. Besides this, we also aim to combine CRISPR K.O. and K.I. strategies, thereby disabling detrimental genes to NK cell function while introducing beneficial gene constructs at the same time.

In conclusion, our study highlights the potential of ADAPT-NK cells modified either through retroviral transduction or CRISPR/Cas9 genetic editing. We have shown that with these methods we are able to expand the targetable space of our ADAPT-NK cells as well as open up future possibilities for enhanced cytotoxicity and extended persistence. While previous studies have shown the potential of CAR-NK cells, our work with CRISPR/Cas9 gene editing in ADAPT-NK cells is completely novel and represents considerable advancements in the field of NK cell-based immunotherapy. Continued optimisation and application of these techniques have the potential to lead to promising results for future NK cell-based clinical applications, ultimately contributing to more effective and precise cancer treatments.

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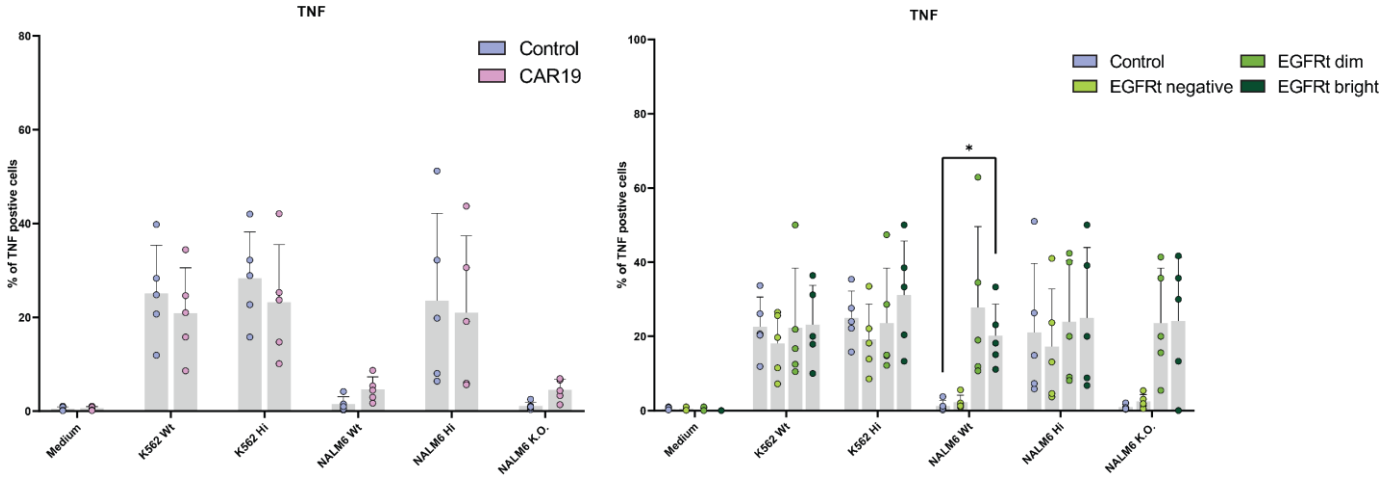
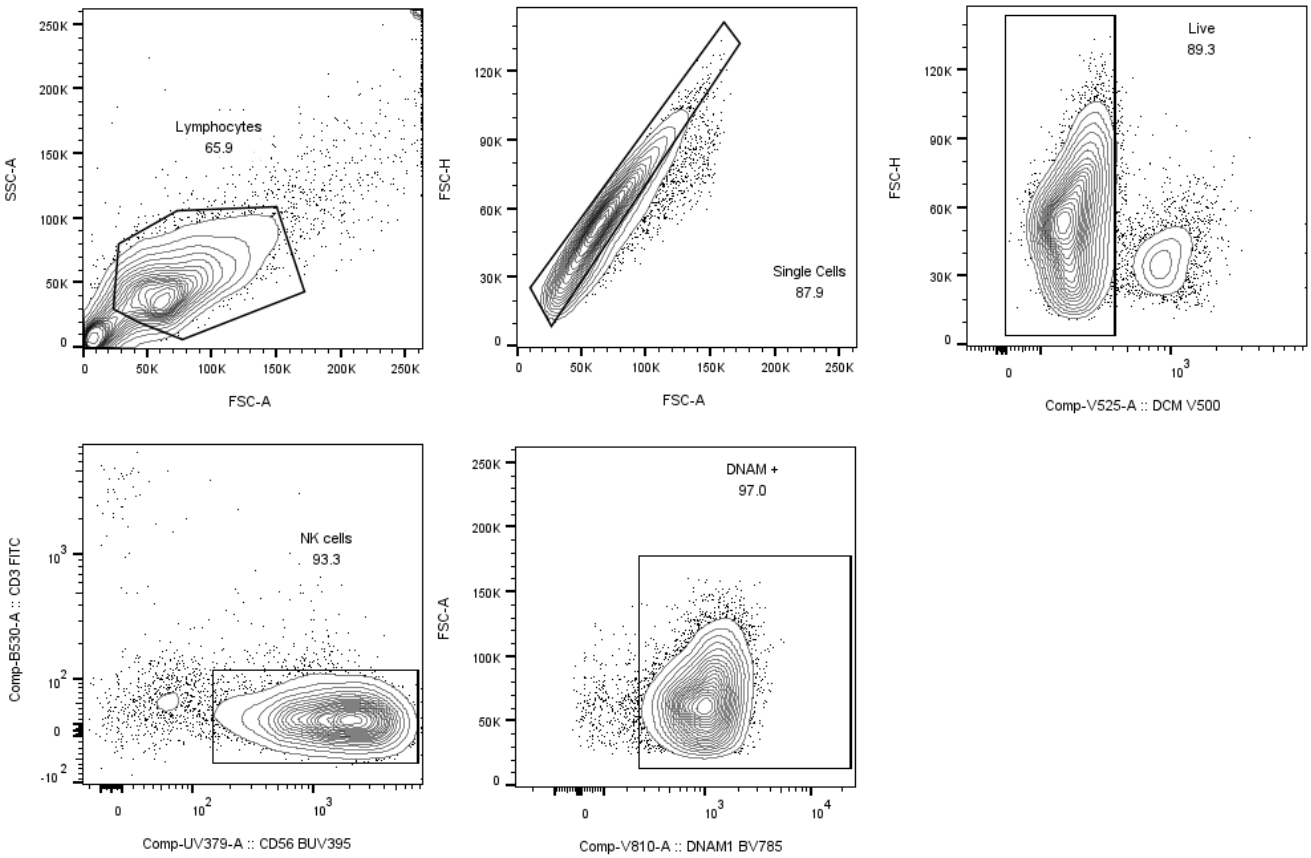
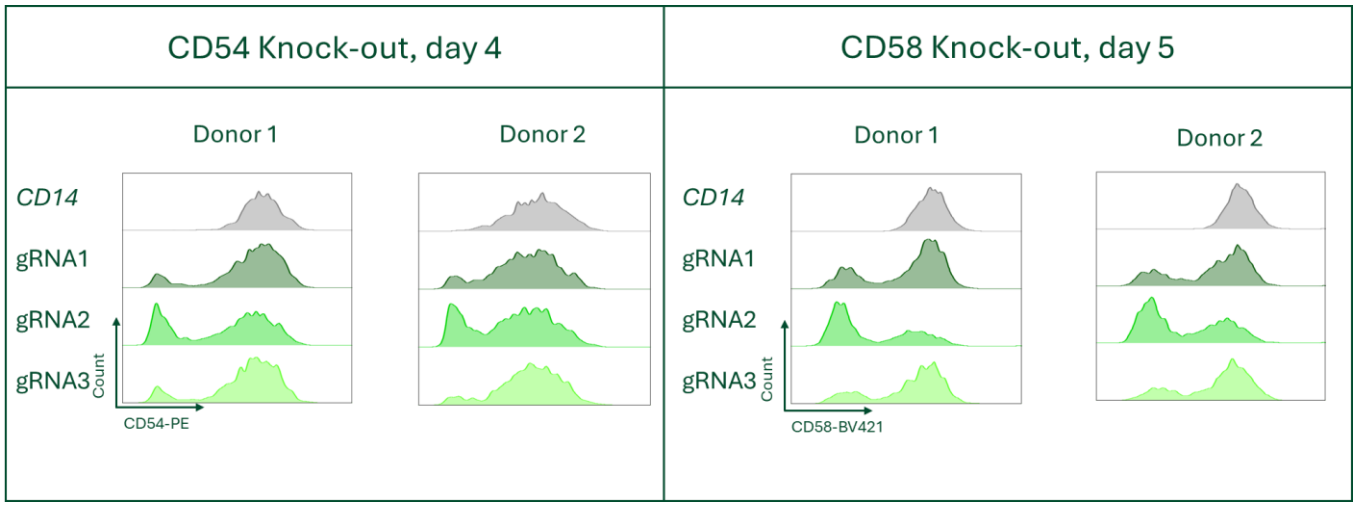
Supplementary Figures



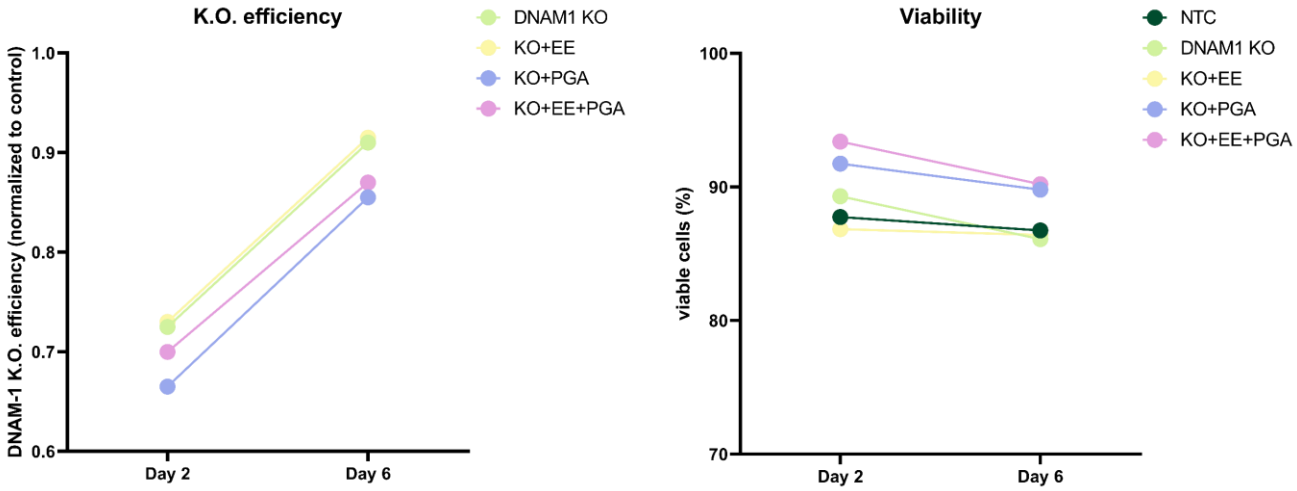
Supplementary figure 1: TNF of control, bulk and EGFRt gated CAR19 ADAPT-NK cells exposed to K562 and NALM6 cell lines.



Supplementary figure 2: Gating strategy for primary-NK cells showing high and homogenous expression of DNAM1



Supplementary figure 3: CD54 & CD58 levels in 2 primary NK cell donors after knockout with three different gRNAs compared to control. For both Genes gRNA2 was chosen as the best guide



Supplementary figure 4: Effect of electroporation enhancer (EE) and polyglutamic acid (PGA) on DNAM1 knock-out efficiency and viability of primary NK cells.