# Genetic Engineering of Primary Human Natural Killer (NK) Cells for Enhanced Cancer Immunotherapy

A DISSERTATION

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## Dedication

This thesis is dedicated to my son, Morrison Moody; my husband, Jason Moody; and my sister, Rachel Pomeroy. I love you all so much.

## Abstract

Natural killer (NK) cells are a critical component of the innate immune system due to their ability to kill a variety of target cells, including cancer cells. This innate anti-tumor phenotype has driven intense interest in the use of NK cells for cancer immunotherapy, but this has seen limited success in the clinic. Enhancing NK cell cytotoxicity by augmenting activating signals or eliminating inhibitory signals could significantly improve NK-based cancer immunotherapy.

We have developed highly efficient methods for editing the genome of human NK cells. Specifically, to target inhibitory signals for elimination, we have developed methods for CRISPR-Cas9-based gene knockout. We have also created platforms for delivery of activating signals using either CRISPR-Cas9 in combination with recombinant adeno-associated virus (rAAV) and a non-viral approach for engineering using DNA transposons.

We targeted relevant genes (ADAM17 and PDCD1) for knockout and delivering activating receptors CD16a and a CD19-specific chimeric antigen receptor (CAR). Importantly, we show direct functional consequences of engineering steps, using preclinical in vitro and in vivo models. Furthermore, we demonstrate the clinical scalability of all methods.

The focus of this work was to develop methods for engineering primary human NK cells, with the goal of creating clinical products to treat human disease. Future work will focus on combining approaches to generate NK cells expertly equipped to kill cancer.

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# CHAPTER 1: Non-Viral Genome Engineering of Natural Killer Cells[1]

## Summary

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system capable of immune surveillance. Given their ability to rapidly and effectively recognize and kill aberrant cells, especially transformed cells, NK cells are an attractive source for cancer immunotherapy. NK cells do not express a T cell receptor (TCR) and thus do not contribute to graft-versus-host disease (GvHD), nor do they induce the common toxicities observed with T cell-based therapies. The clinical efficacy of NK cell-based therapies has been hindered by limited in vivo persistence and the immunosuppressive tumor microenvironment (TME) characteristic of many cancers. Enhancing NK cell efficacy through genome engineering has the potential to improve NK cell persistence and restore cytotoxic capabilities in the TME. Alongside silencing NK cell inhibitor receptors, NK cell killing can be redirected or enhanced by the delivery of chimeric antigen receptors (CARs). However, NK cells face technical and biological challenges not seen in T cells, resulting in low genome editing efficiencies. Viral vectors are the most widely studied method for NK cell engineering, but they carry the concern of random insertional mutagenesis. As

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such, this review focuses on non-viral methods of gene transfer within the context of improving cancer immunotherapy using engineered NK cells.

## Introduction

Cancer therapy has been revolutionized through the adoptive transfer of tumor infiltrating lymphocytes (TILs) and genetically engineered T cells. However, clinical efficacy has been largely limited to blood cancers[1]. Natural killer (NK) cells are innate lymphocytes with cytotoxic and inflammatory effector functions in response to cancer and thus represent an additional cell type of interest for cancer immunotherapy. Moreover, NK cells have not been associated with some of the most detrimental side effects associated with T cell-based therapies, such as graft-vs-host disease (GvHD) and cytokine release syndrome (CRS), making NK cells an attractive candidate for cancer immunotherapy[2]. However, studies to date have shown minimal clinical efficacy with unmanipulated peripheral blood NK cells. Thus, improving NK cell function though genetic modification is of high interest, but primary NK cells display resistance to many gene editing methods that work well in T cells[3–5]. A current consideration for engineered T and NK cell therapy is the method of gene transfer. The vast majority of engineered lymphocytes used in the clinic are made using viral vectors to deliver genetic material[1,6]. However, high cost of production, long turnaround times, batch to batch variability, and safety concerns have motivated the field to search for non-viral gene delivery and modification approaches. Here, we review recent advances in the genetic modification of NK cells, with a focus on non-viral strategies.

## NK Cell Intransigence to Genetic Modification

Viral vectors including retrovirus and lentivirus have been used extensively to stably deliver genetic material to a broad range of cell types[7]. They have been especially useful in the cell therapy field for the generation of engineered T cell therapies and hematopoietic stem/progenitor cell (HSPC) transplant. In contrast to T cells and HSPCs, NK cells are notoriously difficult to transduce[3,8]. High expression of pattern recognition receptors (PRRs) activated in response to pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) may in part explain this phenomenon[9,10]. The RNA genome of retroviruses and lentiviruses is known to activate PRRs including TLR3, RIG-I, and MDA-5 in NK cells[8]. The result is poor NK cell viability and low transduction rates, which hinders the efficacy of this approach for therapeutic use[11].

## NK Cell Transfection Strategies

Transfection is a powerful tool for the deliberate introduction of nucleic acids into cells and can be used to deliver genome editing reagents for gene knockout or delivery of exogenous transgenes. Non-viral methods of transfection most often result in rapid, although transient, transgene expression when compared to viral-based methods[4,12]. A major concern with the viral modification of NK cells is the risk for insertional mutagenesis given the high viral titers necessary for transduction. Conversely, non-viral transfection-based methods do not carry the aforementioned risk, making them more favorable from a safety perspective, especially with an ultimate goal of developing novel immunotherapies.

The most common methods of gene transfer using transfection include lipofection and electroporation.

With lipofection, nucleic acids or proteins are encapsulated in cationic liposomes, which fuse with the target cell membrane[13]. Once fused, these liposomes release the cargo directly into the cell. While lipofection of NK cells has historically been used more sparingly, there are new bodies of research utilizing liposome-mediated transfection strategies[13]. One of the earliest studies using lipofection described the transfer of a murine IL-2 expressing plasmid into primary NK cells using the cationic lipid *N*-(1-(2,3-dimyristyloxypropyl)-*N*,*N*-dimethyl-(2-hydroxyethyl) ammonium bromide/dioleoyl phosphatidylethanolamine (DMRIE/DOPE)[14]. IL-2 promotes proliferation and enhances the cytotoxicity of NK cells, including the secretion of granzyme. Investigators found that melanoma xenograft tumors treated with transfected NK cells had significantly higher levels of granzyme A activity[14]. The transformed NK cell line NK-92 (derived from PBMCs of a non-Hodgkin's lymphoma patient)[15] was lipofected with stem cell factor (SCF) cDNA and found to have significantly greater proliferation and stronger cytotoxicity against a broad range of target tumors when compared to wild-type NK-92 cells[16]. Lipofectamine 2000 has been used to transfect primary NK cells with an activating chimeric antigen receptor (CAR) specific to HER-2, an oncogene frequently found overexpressed in a number of solid tumors. These HER-2 specific CAR-NK cells were selectively activated by HER-2 positive tumor cells and eradicated tumor cells *in vivo*[17]. Youness et al. identified miR-486-5p as a direct regulator of insulin-like growth factor-1 receptor (IGF-1R), which is a known modulator of hepatocellular carcinoma[18]. miR-486-

5p was lipofected into primary NK cells, resulting in improved NK cell cytotoxicity through an increase in NKG2D and perforin expression. Regis et al. found that miR-27a-5p negatively regulates $CX_3CR1$, which drives NK cells to peripheral tissues, including tumor sites[19]. Investigators utilized Lipofectamine 3000 to transfect primary NK cells with a miR-27a-50 inhibitor and achieved a transfection efficiency of ~30%. Most recently, Lipofectamine 2000 and 3000 were compared to a novel transfection reagent method known as charge-altering releasable transporters (CARTs). While lipofectamine-transfected primary NK cells had GFP detection below 1%, CART-transfected NK cells expressed GFP more efficiently (~10%) and showed improved viability with minimal changes to NK cell phenotype and function[20].

Electroporation-based methods are one of the earliest strategies used for nucleic acid delivery in NK cells. Electroporation is a method based on the generation of electrical pulses to induce small, temporary pores in the cell membrane[13]. These pores allow for charged molecules, such as DNA, RNA, and proteins, to move into the cell. Typically, electroporation requires target cells to be dividing in an exponential growth phase in order for optimal nucleic acids to have access to the nucleus. Previous studies demonstrate that primary NK cells require cytokine stimulation and/or expansion using feeder cell lines to allow for sufficient transfection efficiencies and post-electroporation viability[13,21]. Nucleofection-based methods were developed to allow for efficient gene transfer into the cell nucleus without relying on cell division for nucleic acid transfer into the nucleus. Nucleofection uses the physical methods of electroporation (induction of cell membrane

pores) but uses a unique device, known as a Nucleofector, as well as cell type-specific reagents[22].

## Transfection of NK Cells with *in vitro* Transcribed (IVT) mRNA

While nucleofection-methods are still employed, many investigators found that moving away from DNA-based cargo improved viability. Carlsten et al. electroporated primary NK cells with mRNA to introduce a high affinity CD16 and chemokine receptor CCR7[23]. Greater than 95% expression was achieved, and engineered cells showed substantial migration to chemokine, CCL19, as well as greater cytotoxicity against antibody-coated lymphoma cells. While electroporation-based methods have been utilized with high efficiencies, viral transduction remains heavily used due to its ability for stable gene transfer. A comparison of mRNA electroporation and lentiviral transduction of the NK-92 cell line showed significantly greater transfection efficiencies and cytotoxicity from mRNA electroporated cells[24]. Interestingly, cord blood NK cells had higher efficiencies when transduced virally, suggesting relevant differences between the NK-92 cell line and primary NK cells isolated from peripheral blood[24]. Li et al. found electroporation efficiencies greater than 80% when they introduced an mRNA encoding a CAR receptor against CD19 into rested (unstimulated) and expanded primary NK cells[25]. Both rested and expanded cells transfected with a CD19-CAR showed enhanced cytotoxicity against CD19+ targets when compared to untransfected cells. Similarly, NK-92 cells have also been transfected with mRNA to express a CD19-CAR, chemokine receptor CCR7, as well as other reporter genes such as eGFP, YFP, and Azuride[12]. Both viability and transfection

efficiencies achieved were between 50-60%, with CD19-CAR transfected cells showing improved cytotoxicity against CD19+ cell lines during *in vitro* killing assays. Importantly, delivery of activating receptors with mRNA or plasmid DNA results in transient expression, so alternative approaches are necessary to achieve stable expression of transgenes.

## NK Cell Engineering with DNA Transposons

A common strategy for stable, non-viral gene delivery is the use of DNA transposons. Transposons, also known as transposable elements, are naturally-existing repetitive DNA sequences that are capable of mobilizing from one location to another in the genome[26]. When used for gene delivery, DNA transposons are generally a two-component system, with a transposon vector containing sequence to be mobilized flanked by terminal inverted repeats (TIRs) and a transposase enzyme that identifies the TIRs and excises and re-integrates the transposon[27]. Since their discovery 70 years ago by McClintock, the "cut and paste" mechanism of transposons has been used as a genetic tool for multiple purposes, ranging from genetic screening to insertional mutagenesis and transgenesis[26,28]. As we enter the era of gene therapy and personalized medicine, transposons have been used extensively as an alternative to the viral vector system for engineering human cells. Recently, transposon-engineered iPSCs and T cells have been used in clinical trials, making the transposon system one of the most promising non-viral vector systems for stable gene transfer[27,29].

There are three major superfamilies of transposons commonly used for gene transfer in human cells, namely Tc1/mariner, piggyBac, and hAT[26]. The most extensively studied transposon system for gene transfer, the Sleeping Beauty (SB) transposon system, belongs to the Tc1/mariner superfamily[26]. Since its discovery and reconstruction from the genomes of salmonid fish[30], SB has undergone improvements through the generation of hyperactive mutants and transposon donor vector optimization[31,32]. The most hyperactive variant developed so far, SB100X, is shown to have a 100-fold improvement of efficiency compared to the original SB transposase, and is comparable to that of a viral vector system[32]. PiggyBac (PB) is another well-developed transposon system for stable gene transfer that was isolated from Cabbage Looper moth[26,33]. The PB system shares a similar transposition mechanism with SB, and its efficiency has also been significantly improved by hyperactive mutant screening and transposon/transposase vector optimization[33,34]. The current hyperactive PB (super-PB) also exhibits a comparable efficiency of transposition to viral vectors[33]. As a novel representative of the hAT family, the Tc Buster transposon originated from the red flour beetle and is a rising star for gene transfer[35]. Tc Buster was shown to be highly active in human cell lines, including HEK-293 and HeLa cells, and have a comparable transposition efficiency to PB and SB[36,37].

Engineering NK cells using transposable elements has gained fairly limited attention and even fewer publications so far. Among these published studies, NK-92 is almost exclusively used[24,38]. For instance, in collaboration with the Kaufman laboratory, we previously utilized NK-92 cells to screen mesothelin-specific CAR constructs to enhance NK cell activity[39]. We successfully expressed a panel of novel CAR architectures

in NK-92 cells using PB or SB systems and demonstrated improved anti-tumor activities of CAR-expressing NK-92 cells when co-cultured with mesothelin-expressing targets[39]. Another study focused on expressing a CD73-specific CAR in NK-92[40]. Using the PB system, Matosevic's group delivered a CD73-CAR construct to NK-92 cells and showed potent killing ability against both solid tumor target cells and humanized CD73+ lung cancer patient derived xenograft (PDX) models[40].

As an alternative to transfecting NK cells directly with transposon systems, transfecting iPSCs and differentiating them into NK cells allows for the circumvention of low transfection efficiency of plasmids in primary NK cells[39,41]. Using this approach, Li et. al. were able to express optimized mesothelin-CAR constructs using a super-PB system in human iPSCs, followed by differentiation into NK cells[39]. Like NK-92, stable expression of mesothelin-CAR was achieved in iPSCs, and subsequent functional assays indicated enhanced tumor specificity and killing in iPSC-derived CAR-NK cells[39].

Our finite understanding of NK cells in general and their notorious aversion to transgene uptake and expression are likely to blame for the limited study of transposable elements in primary NK cells. Transposons, especially SB and PB, have been used extensively in delivering CARs to T cells for treating both hematological and solid tumors, with targets including CD19, CD33, CD133, EGFR, mesothelin, and GPC3[42–47]. Among them, a CD19-CAR and a SLAMF7-CAR, both generated using the SB system, have already entered phase I/II clinical trial for treating leukemia or lymphoma[48,49] and multiple myeloma (MM) (NCT 04499339)[50], respectively. These achievements in T cells offer both insight and a basis for applying the concept in primary NK cells. With a better

understanding of NK isolation and expansion, as well as the improvements in SB technology through hyperactive mutant generation (SB11, SB100X) and transposon donor vector optimization (pT4)[31,32], it is feasible to expand and apply transposon systems for engineering primary NK cells. Our group performed proof-of-principle experiments delivering mRNA encoding SB11 or SB100X in combination with a minicircle DNA transposon encoding GFP to feeder cell-expanded primary human NK cells **(Figure 1)**. We show stable expression of GFP 21 days after electroporation, with 15% efficiency using SB100X, suggesting this is a viable approach for non-viral, transposon-based gene delivery to NK cells.

Because of their higher gene transfer efficiencies, viral vector systems, including lentivirus, adeno-associated virus (AAV), and retrovirus, are still the preferred vector systems used in NK cell engineering. Nevertheless, transposon systems have been shown to overcome many limitations of the viral vector systems. Larger cargo size is one of the most prominent features transposon systems have over viral vectors[51]. Transposons have demonstrated up to 200 kb of cargo capacity, with no known upper limit[52], while the largest carrying capacity for viral vectors has an 8 kb size limitation[51]. Moreover, as transposons do not have a tendency to integrate within 5' untranslated regions (UTRs) as retroviral vectors do[51,53], transposons have a better safety profile, and they are also more cost-effective and easier to produce and purify[26]. With the recent improvements of gene transfer efficiencies in transposon systems, especially that of SB and PB, the efficiencies are comparable to that of viral vectors[32,33], making transposon system a very promising gene editing tool for NK engineering.

## NK Cell Engineering with Cas9, Base Editors, and Prime Editors

The expression of an array of inhibitory receptors and checkpoint molecules that can be upregulated in tumor microenvironment (TME) has made CRISPR/Cas9-based gene targeting in NK cells a goal for scientists trying to augment NK cell anti-tumor efficacy[5]. Early efforts to use CRISPR/Cas9 in primary NK cells used Cas9 expressed via a DNA plasmid or mRNA. This often resulted in low transfection efficiencies[54]. Our group developed a platform to efficiently and reliably target genes with CRISPR/Cas9 using chemically modified guide RNAs and Cas9 mRNA[21]. We targeted NK inhibitory genes *ADAM17* and *PDCD1* for knockout and developed a method for targeted integration using recombinant adeno-associated virus as a donor template for homology-directed repair (HDR). Gene knockout and knock-in efficiencies in this study reached 90% and 75%, respectively, equivalent to reports of analogous approaches in primary T cells. Other groups have solved the problem of low transfection efficiency by delivering Cas9 and guide RNAs as ribonucleoprotein (RNP) complexes, as this approach has shown high editing rates in primary T cells[55]. Through the use of RNPs, Rautela et al. were able to achieve editing efficiencies of up to 75% across a number of genes in primary NK cells[56]. Others have used RNPs to efficiently knockout *TGFBR2* in primary NK cells, as TGFβ is a major NK cell inhibitor[55]. Electroporation of cells with RNP complex to knockout *TGFBR2* resulted in a 60% reduction in mRNA expression. Recently, Nguyen et al. developed methods to improve the efficiency of site-specific CRISPR/Cas9-based gene delivery, using Cas9-RNPs and DNA templates for HDR containing truncated Cas9 target sequences (tCTSs). The tCTSs associate with the Cas9-RNPs and are thus shuttled to the nucleus

which enhances HDR efficiency[57]. Using this approach, they achieved over 15% transgene delivery to NK cells.

Base editors (BEs) are another gene editing tool that takes advantage of the CRISPR-Cas system. BEs are composed of a catalytically inactive Cas9 protein fused to a DNA deaminase domain[58]. Unlike Cas9 nuclease, this feature enables precise introduction of targeted single nucleotide changes without introductions of double-strand breaks (DSBs)[59]. There are two types of base editors to date, adenine base editor (ABE) and cytosine base editor (CBE), and collectively they can achieve all possible transition mutations (A<->G for ABE and C<->T for CBE)[60,61]. Due to its relatively recent discovery, applications of BE in the immunotherapy context are very limited. Studies using BE in T cells have just started emerging, however, applications in NK cells are still lacking. Last year, our group reported a multiplex knockout of T-cell receptor alpha constant (*TRAC*), ß-2 microglobulin (*B2M*) and programmed cell death 1 (*PDCD1*) in CD19 CAR-T cells using both CBE base editors[62]. In this study, we achieved higher than 90% editing efficiency across all 3 target genes, at both DNA and protein levels[62]. Additionally, Zhang's group reported a down-regulation of PD-1 expression in CAR-T cells using ABE[63]. By altering the coding sequencing of N74 in the *PDCD1* gene, they reduced asparagine (N)-linked glycosylation of PD-1 protein. This modification reduced its inhibitory effect on CAR-T cells. These studies serve as a proof of principle for using both ABE and CBE in T cells, and potentially NK cells, given the shared characteristics and functioning mechanisms between these two cell types.

Last year, Liu's team reported prime editor (PE), further expanding our ability to precisely engineer DNA without inducing DSBs[58,64]. PE uses a reverse transcriptase fused to dCas9 and prime editing guide RNA (pegRNA) containing a sequence to be introduced. PE is capable of introducing all possible transversion and transition mutations, as well as small insertions and deletions[64]. At its current stage, PE has only been tested in a very limited numbers of mammalian cells, including 293T and K562, with up to 70% and 30% editing efficiency, respectively[64]. However, sharing a similar mechanism as BE, it is worth testing PE in NK cells.

## Materials and Methods

### *NK Cell Isolation and Expansion*

PBMCs from de-identified healthy human donors were obtained by automated leukapheresis (Memorial Blood Centers, Minneapolis, MN, USA). CD56+CD- NK cells were isolated by negative selection using the EasySep Human NK Cell Enrichment Kit (STEMCELL Technologies). After isolation, NK cells were expanded by co-culture with irradiated (100 Gray) mbIL21- and 41BBL-expressing K562 feeder cells as described previously[65].

### *Electroporation of Expanded NK cells*

Expanded NK cells were pelleted and resuspended at 3 x $10^7$ cells/mL in T buffer (Neon Transfection System Kit; Thermo Fisher Scientific). 1 $\mu$g minicircle plasmid DNA and 1 $\mu$g SB11 or SB100X mRNA were added to 10 $\mu$L (3 x $10^5$ cells) on ice. This mixture was

electroporated with the Neon Transfection System (Thermo Fisher Scientific) using two pulses of 1850 V and 10-ms pulse width. NK cells were re-expanded with feeder cells for 21 days.

### *Flow Cytometry*

The following antibodies were used: allophycocyanin (APC)-conjugated anti-CD56 (clone REA196; Miltenyi Biotec), phycoerythrin (PE)-conjugated anti-CD3 (clone SK7; BD Biosciences), SYTOX Blue dead cell stain (Thermo Fisher). Flow cytometry assays were performed on LSR Fortessa flow cytometers (BD Biosciences) and data were analyzed using FlowJo version 10.4 software (FlowJo).

## Figures

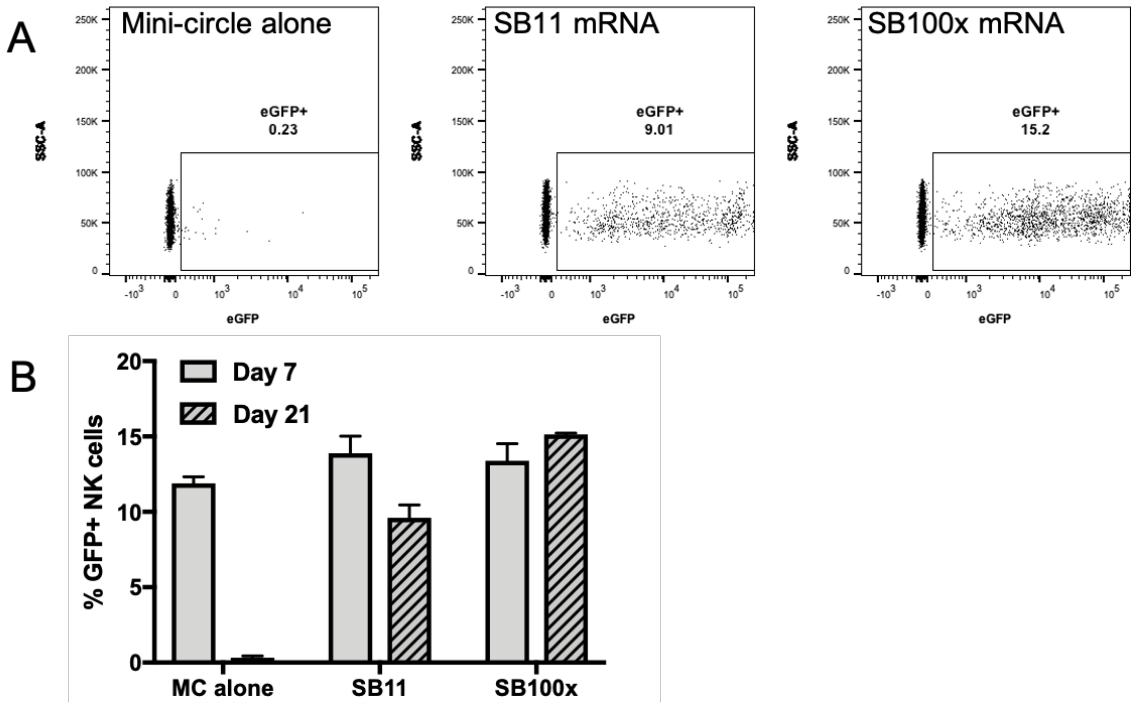



**Figure 1-1. SB mRNA and minicircle delivery of GFP to primary human NK cells**. Primary human NK cells (n=2 independent donors) were isolated from peripheral blood and expanded using mbIL21-expressing K562 feeder cells. After expansion, NK cells were electroporated with minicircle (MC) plasmid expressing eGFP alone or in combination with SB11- or SB100X-encoding mRNA. (A) Representative flow plots of eGFP expression 21 days after electroporation. (B) eGFP expression from 2 NK cell donors 7 and 21 days after electroporation.

## Concluding Remarks

NK cell-based therapies have shown great promise as a therapy for cancer, but there is much work to be done to augment their anti-tumor efficacy. In the second chapter of this thesis, I describe the development of methods for editing the genome of NK cells using CRISPR-Cas9. Using these methods, I targeted NK inhibitory genes *ADAM17* and *PDCD1* for knockout and demonstrate that engineered NK cells have enhanced anti-tumor activity. I also show efficient knock-in of non-cleavable CD16a (*FCGR3A*) using CRISPR-Cas9 in combination with a recombinant adeno-associated virus (rAAV) as a template for homologous recombination (HR).

The third chapter of this thesis details the process for engineering chimeric antigen receptor (CAR)-expressing NK and T cells using the Tc Buster transposon system. In developing this process, I optimized methods to avoid DNA toxicity and maximize efficiency and cell health. I demonstrate the target-specific anti-tumor activity of CAR-NK and CAR-T cells engineered using this method. Together, the advances in NK cell engineering described in this thesis may unleash the therapeutic potential of NK cells for cancer treatment.

# CHAPTER 2: A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy[2]

## Summary

Enhancing natural killer (NK) cell cytotoxicity by blocking inhibitory signaling could lead to improved NK-based cancer immunotherapy. Thus, we have developed a highly efficient method for editing the genome of human NK cells using CRISPR/Cas9 to knock out inhibitory signaling molecules. Our method efficiently edits up to 90% of primary peripheral blood NK cells. As a proof-of-principle we demonstrate highly efficient knock-out of *ADAM17* and *PDCD1* – genes that have a functional impact on NK cells, and demonstrate that these gene-edited NK cells have significantly improved activity, cytokine production, and cancer cell cytotoxicity. Furthermore, we were able to expand cells to clinically relevant numbers, without loss of activity. We also demonstrate that our CRISPR/Cas9 method can be used for efficient knock-in of genes by delivering homologous recombination template DNA using recombinant AAV6. Our platform represents a feasible method for generating engineered primary NK cells as a universal therapeutic for cancer immunotherapy.

[2] This work has been previously published: Pomeroy EJ, Hunzeker JT, Kluesner MG, Lahr WS, Smeester BA, Crosby MR, Lonetree CL, Yamamoto K, Bendzick L, Miller JS, Geller MA, Walcheck B, Felices M, Webber BR, Starr TK, and Moriarity BS. A genetically engineered primary human natural killer cell platform for cancer immunotherapy. *Molecular Therapy*. 2020 Jan 8;28(1)52-63. doi: 10.1016/jymthe.2019.10.009.

## Introduction

Natural killer (NK) cells are a critical component of the innate immune system due to their ability to kill a variety of target cells, including cancer cells. NK cell cytotoxicity is mediated by the integration of signals from activating and inhibitory receptors and cytokines.[66] The ability of NK cells to kill tumor cells, along with the ease with which they are isolated and expanded from peripheral blood, has made them an attractive source for immunotherapy.[67,68] Moreover, as NK cells do not induce graft versus host disease,[69] they can be generated from unrelated donors and, therefore, represent a potential 'off the shelf' therapeutic product. However, NK cell immunotherapy has seen limited success in the clinic, due in part to lack of persistence and expansion after transplant[70]. Changes in NK cell receptor repertoire and ligand expression in the tumor microenvironment can also lead to decreased NK cell activity.[71,72] Thus, it is predicted that targeting the receptor repertoire, specifically by decreasing inhibitory signals imposed upon NK cells, may lead to enhancement of their anti-tumor activity.[73] To date, multiple phase I and phase II clinical trials are ongoing testing NK cell checkpoint blockade against KIR (NCT01714739, NCT01750580) and NKG2A (NCT02331875) as a therapy for hematological and solid tumors.

One of the most potent activating receptors on NK cells is CD16a (FcγRIIIa). This Fc receptor binds the Fc portion of IgG-coated target cells and induces antibody-dependent cell-mediated cytotoxicity (ADCC), which is a key molecular mechanism of several clinically successful anti-tumor therapeutic mAbs.[74,75] After activation, CD16a is rapidly cleaved from the cell surface by A Disintegrin And Metalloproteinase-17 (ADAM17).[76]

Inhibition of ADAM17 leads to increased cytokine production by human NK cells,[77] suggesting that the use of tumor-targeting mAbs combined with inhibition of ADAM17 could enhance the anti-tumor response.

Various cells within the tumor micro-environment express immunosuppressive molecules that interfere with the complex array of receptors that regulate NK cells and their ability to expand.[79] For example, some tumor cells upregulate PD-L1, which engages PD1 on NK cells reducing cytotoxic activity.[81,82] This inhibition imposed on NK cells limits effective NK immunotherapy and overcoming this immunosuppression could improve therapeutic development. Strategies for overcoming these challenges have focused on releasing NK cell inhibition through the use of small-molecule inhibitors and monoclonal antibodies.[83–85] However, there are limitations to these strategies: off-target toxicity is common with these types of therapies and, importantly, no humanized antibodies or chemical inhibitors exist for some high interest targets.

An alternative approach is to use CRISPR/Cas9 to edit genes relevant to NK function in order to improve their utility as an immunotherapeutic agent. Changes made with this approach are confined to NK cells, reducing undesirable systemic effects. Additionally, design and optimization of CRISPR guide RNAs (gRNAs) is straightforward and cost-effective, making it easy to target any gene of interest. Here, we present a method for achieving high rates of gene editing in activated primary human NK cells. We can expand edited NK cells to clinically relevant numbers and demonstrate that gene-edited NK cells have enhanced function *in vitro* and *in vivo*. Our method is capable of knocking-out genes at rates up to 90% and site-specifically knocking in genes at rates approaching

80%. In summary, we present a rapid platform for generating high functioning genetically modified NK cells for use in cancer immunotherapy.

## Results

### *A platform for efficient gene editing of primary human NK cells*

To improve NK cell cytotoxicity, we developed an optimized CRISPR/Cas9 system capable of disrupting important regulatory genes in activated primary human NK Cells. Based on our previous work with primary human T cells and B cells,[86,87] we hypothesized that activating NK cells prior to electroporation would make them more amenable to nucleic acid delivery. We delivered nucleic acid in the form of eGFP mRNA to NK cells isolated from peripheral blood mononuclear cells (PBMCs) activated with irradiated feeder cells expressing membrane-bound IL21 (Clone9.mbIL21, C9)[65] and cultured with IL-2 for 7 days prior to electroporation (**Figure 1A**). Using this approach, we routinely achieve >98% transfection efficiency with >90% cell viability (**Supplemental Figure 1A**). Although other feeder cell and feeder cell-free expansion methods exist, we chose to use C9 feeder cells to activate and expand NK cells because this method has been shown to be safe and effective in patients with multiple myeloma.[88] However, the effectiveness of CRISPR editing is based on the activation state of the NK cells, and thus our electroporation parameters could potentially be used with NK cells expanded using different methods.

To test our optimized protocol we delivered Cas9 mRNA alone as a control, or in combination with chemically-modified gRNAs[89] targeting *ADAM17*[78] or *PDCD1*[82] to

activated NK cells from five independent human donors. Next-generation sequencing (NGS) of amplified target regions revealed gene editing efficiency of 80.9 ± 9.9% for *ADAM17* and 81.9 ± 7.4% for *PDCD1* without the use of any enrichment methods (**Figure 1B**). The differences in editing efficiency at the chosen target sites is attributed to efficiency of the specific gRNA and accessibility of the target locus.[90] We compared NGS and Tracking of Indels by Decomposition (TIDE) analysis to quantify gene editing efficiency in all samples. The TIDE web tool quantifies indel formation using Sanger sequencing reactions.[91] We observed no significant difference between editing efficiency calculated using TIDE versus NGS, in line with previous reports[92] (**Supplemental Figure 1B**), and as such we used TIDE for all further analysis of gene editing.

***Gene-edited NK cells can be expanded to clinically relevant numbers***

The dose of infused NK cells in the clinical setting ranges from 1 x $10^6$ – 1 x $10^8$ cells/kg.[93] Thus, a typical patient would require ~8 x $10^9$ NK cells at a high dose. Critically, we were able to maintain the gene edits at similar frequencies after multiple rounds of expansion during 21 days of culture using C9 feeder cells (**Figure 1C**) and gene knockout did not affect our ability to expand cells to the clinically relevant numbers referenced above (**Figure 1D**).

***Protein expression is significantly altered in gene-edited cells and minimal off-target activity is detected***

Protein expression of targeted genes was significantly decreased (89.8 ± 1.2% for ADAM17 and 86.9 ± 7.8% for PD1) and mRNA expression followed a similar pattern (**Figure 1E; Supplemental Figure 1C-F**). To assess clinical safety, the top 10 putative off-target sites for each gRNA were computationally identified and analyzed for off-target editing. Next-generation sequencing showed no indel formation at any of the predicted off-target sites (**Supplemental Figure 1G**). Furthermore, with the goal of developing clinical products, we sought to optimize cryopreservation of activated and gene-edited NK cells. Previous groups have shown low NK cell recovery after cryopreservation.[94] We found that freezing $1x10^7$ NK cells per mL using CryoStor CS10 preservation media yielded ~80% recovery after thaw, and that gene editing did not affect this process (**Supplemental Figure 1H**). The use of C9 feeder cells has been shown to maintain integrity of the NK cell receptor repertoire.[65] We compared expression of 13 NK cell receptors in control and CRISPR-edited samples (**Figure 1F; Supplemental Figure 2A**). While we found a reduction in KIR2DL1 expression in PD1 KO NK cells, and some donor variability in expression of CD16a, KIR2DL2,3, and KIR3DL1, overall expression levels were very similar in control and engineered cells.

***Efficient targeted gene knock-in using AAV***

In addition to successful gene knock-out (KO), we adapted our method for gene knock-in (KI) by co-delivering a DNA template for homologous recombination (HR) using

recombinant adeno-associated virus serotype 6 (rAAV6) along with Cas9 mRNA and gRNA. This approach has been used to achieve targeted integration of a transgene in primary human T cells and CD34+ hematopoietic stem cells.[95] This method has advantages over conventional delivery of a transgene using lentiviral-based methods, including maintenance of endogenous regulatory elements at the site of integration and preclusion of deleterious effects due to insertional mutagenesis.[96] As proof-of-principle, we delivered Cas9 and gRNA targeting the AAVS1 safe harbor locus downstream of the endogenous promoter-splice donor (**Figure 1G**). Co-delivery of a promoter-less EGFP targeting vector using rAAV6 resulted in successful HR in 75.6 ± 3.0% of NK cells based on junction PCR and EGFP expression (**Figure 1H-I**). Similar to gene KO, gene KI was stable through several rounds of expansion using C9 feeder cells (**Figure 1J**). Together, these data demonstrate that high-efficiency Cas9-mediated gene KO and KI is achievable in activated primary human NK cells.

### *Knockout of ADAM17 or modification of CD16 can prevent CD16 shedding and enhance ADCC*

ADAM17 is responsible for the rapid cleavage of the activating Fc-gamma receptor IIIa (CD16a) from the surface of NK cells after activation,[76,78,97] resulting in temporary inhibition ADCC events and the ensuing NK cytotoxicity. Small molecule inhibitors of ADAM17 are currently in clinical trials in combination with antibody treatments as a method of enhancing the therapeutic effect of NK cells (NCT02141451). We reasoned that targeting ADAM17 directly in the NK cell could avoid systemic toxicities associated with

off-target effects of chemical inhibitors. Using an artificial activation system (PMA)[78] we demonstrate that ADAM17 KO NK cells maintain significantly higher surface expression of CD16a compared to control NK cells (which received Cas9 mRNA alone) and are on par with what is observed when NK cells are pre-treated with the ADAM17 inhibitor INCB007839 (**Figure 2A; Supplemental Figure 3A**). Surface membrane levels of CD62L, an additional target of ADAM17,[78,98] were also undiminished in activated ADAM17 KO NK cells (**Supplemental Figure 3B**). To test if ADAM17 KO NK cells have enhanced ADCC we performed standard ADCC assays using the CD20-positive Burkitt's lymphoma cell line Raji. Raji cells were pre-treated with Rituximab, a monoclonal antibody targeting CD20. Rituximab-coated Raji cells induced cleavage of CD16a in 79.5 ± 1.3% of control NK cells, but there were no differences in CD16a cleavage in ADAM17 KO NK cells (**Figure 2B; Supplemental Figure 3C**). Moreover, ADAM17 KO NK cells displayed a significant increase in cytotoxic degranulation based on CD107a surface expression (**Figure 2C; Supplemental Figure 3D**) and IFNγ production (**Figure 2D; Supplemental Figure 3E**) in response to Rituximab-labeled Raji lymphoma. Importantly, ADCC was significantly enhanced when using ADAM17 KO versus control NK cells based on increased apoptosis as measured by cleaved Caspase 3 in Rituximab-coated Raji cells (**Figure 2E; Supplemental Figure 3F)**. Taken together, these data demonstrate that genetic KO of ADAM17 in NK cells prevents CD16a shedding comparable to ADAM17 inhibitors and leads to enhanced ADCC.

As an alternative approach, we used our gene KI strategy to deliver a non-cleavable version of CD16a (CD16 S197P)[97] to the AAVS1 locus (**Figure 2F**). Junction PCR

confirmed targeted integration (**Figure 2G**). Droplet digital PCR was performed to quantify a rate of 74.7 ± 5.53% targeted gene knock-in (**Figure 2H**), and CD16a expression was retained in CD16a S197P-KI NK cells after stimulation with PMA/ionomycin (**Supplemental Figure 3G**). We confirmed enhanced function of CD16a S197P-KI NK cells using the same co-culture methods described for ADAM17 KO cells. We observed retained CD16a on the cell surface (**Figure 2I**) and enhanced NK cell activity and target cell killing (**Figure 2J-L**). Interestingly, the enhanced ADCC effect of directly targeting CD16 was less drastic than that achieved by knockout of ADAM17. It is possible that this discrepancy is due to the slight difference in gene editing efficiency (82.7% ADAM17 KO vs. 74.7% CD16a KI). Alternatively, it could be due to retention of other targets of ADAM17 upon ADAM17 KO. This result warrants further investigation, but it is clear that altering CD16 cleavage can enhance ADCC.

***Knockout of PD1 enhances NK cell anti-tumor response***

NK cells are also capable of target cell killing independent of ADCC, through direct interaction between activating receptors on NK cells and cell surface proteins on target cells. However, tumor cells can block this cytotoxicity by inducing NK cell inhibitory signals through cell-surface protein interactions such as PD-L1:PD1.[81,82,99] We used our gene-editing method to generate PD1 KO NK cells and tested their ability to directly kill four cancer cell lines compared to control NK cells that were electroporated with Cas9 mRNA only. We selected four cancer cell lines with various expression levels of PD-L1 and PD-L2 (**Supplemental Figure 4A**) – the chronic myeloid leukemia line K562 (PD-L1

low), the acute monocytic leukemia line THP1 (PD-L1 high), the prostate carcinoma line DU145 (PD-L1 high), and the ovarian carcinoma line MA148 (PD-L1 low). In a co-culture killing assay, PD1 KO NK cells had significantly higher levels of degranulation (**Figure 3A; Supplemental Figure 4B)** and production of IFNγ (**Figure 3B; Supplemental Figure 4C**). Similar to our ADCC results, PD1 KO NK cells also led to increased apoptosis of target cells compared to all controls (**Figure 3C; Supplemental Figure 4D**).

Because there is limited data on the PD1:PD-L1 axis in NK cell function, there remains some discrepancy in the field as to its importance.[68,100,101] To confirm our results were due to PD1:PD-L1 interaction, we repeated the functional assays using wild-type (WT) and PD-L1 KO K562 (PD-L1 low) and DU145 (PD-L1 high) target cells. Importantly, control NK cells display degranulation, IFNγ production, and target cell killing at the level of PD1 KO NK cells when co-cultured with PD-L1-deficient target cells. (**Figure 3D-F**).

To test functionality *in vivo*, we used PD1 KO NK cells to treat an orthotopic mouse xenograft model of ovarian cancer[102] (**Figure 4A**). After xenograft establishment, mice were treated by a single intraperitoneal injection of rested control NK cells, donor-matched activated control NK cells which received Cas9 mRNA only, or donor-matched activated PD1 KO NK cells. Treatment with PD1 KO NK cells significantly prolonged survival (**Figure 4B**) and reduced tumor burden (**Figure 4C**) compared to both activated and rested control NK cells, indicating improved therapeutic efficacy. Supporting our hypothesis that NK cells are inhibited by PD1:PD-L1 interaction, we found that PD-L1 levels increase significantly on MA148 cells present in the ascites of mice treated with activated NK cells

during tumor progression (**Figure 4D**). At tumor endpoint, mice were sacrificed, and total ascites fluid was collected, total cells were counted and analyzed by flow cytometry. MA148 cells were identified by GFP expression and NK cells were identified as hCD45+/CD56+. We observed higher PD-L1 expression on MA148 cells from mice treated with activated NK cells (both wild-type and PD1 KO) (**Figure 4E**). We also observed a presence of NK cells in the ascites fluid at endpoint in mice treated with activated NK cells, which was significantly higher in mice treated with PD1 KO NK cells, both as a percentage and an absolute number (**Figure 4F, G**). These results are consistent with the hypothesis that the inflammatory environment created by activated NK cells stimulates PD-L1 expression on tumor cells and provides rationale for creating PD1-deficient NK cells for cancer therapy.

## Discussion

NK cell immunotherapy holds great promise as an off-the-shelf cell therapy that does not require antigen specificity. However, the full potential of NK cell cancer therapy has not been realized, due in part to negative regulation of NK cells in the tumor microenvironment. Here we present a feasible method for generating clinical quantities of gene-edited NK cells with enhanced cytotoxic capabilities.

We chose to target two genes predicted to have a functional impact on NK cells. We were able to effectively induce efficient indel mutations in targeted genes and gene editing was consistent across multiple independent human donors. Importantly, we show genetic changes are stable through multiple rounds of NK cell expansion, with the

generation of clinical doses in a short three-week time period. The method of expansion using C9 feeder cells has been deemed safe clinically and is currently being used in several clinical trials. We have also performed analyses for off-target editing across the top ten putative off-target sites for each gRNA. We observe no detectable indel formation at any of the top ten sites. We confirmed concurrent knockout of target genes at the mRNA and protein level.

In addition to gene knock-out, we demonstrate efficient gene knock-in using rAAV6 as a template for homologous recombination. We show knock-in of EGFP at the AAVS1 safe harbor locus that is also stable through expansion. Beyond proof-of-principle, we have shown a functional knock-in of a modified non-cleavable CD16a at this locus as well. This simple method for stable gene knock-in provides a platform for delivery of other relevant cargo including chimeric antigen receptors (CARs) and self-stimulating cytokine receptors.

*In vitro* assays show that ADAM17 KO NK cells are more reactive to antibody-labeled target cells and are capable of enhanced killing through ADCC. These data are reiterated upon stable integration of non-cleavable CD16a. These results are in line with previous studies using ADAM17 inhibitors, but likely come with reduced risk of unwanted off-target effects. NK cells engineered for enhanced ADCC, whether via KO of ADAM17 or modification of CD16a, will need to be tested in further *in vitro* assays using other cancer lines and mAb combinations, and in preclinical *in vivo* models.

*In vitro* and *in vivo* assays demonstrate that PD1 KO NK cells are capable of enhanced killing through non-ADCC pathways. Our data show enhanced NK cell

degranulation, cytokine production, and target cell killing across a panel of tumor cell lines. There has been some discrepancy about the importance of PD-L1:PD1 interaction in NK cell function, as this axis is much more well-studied in T cells than NK cells. Using PD-L1 deficient K562 or DU145 target cells, we show that enhanced activity of PD1 KO NK cells is indeed due to PD-L1:PD1 interaction.

We also delivered PD1 KO NK cells therapeutically using an *in vivo* model of ovarian cancer. Interestingly, we see upregulation of PD-L1 in these tumors upon treatment with activated NK cells, suggesting a response of the tumor cells to immune pressure. Accordingly, PD1 KO NK cells reduced tumor burden and enhanced survival in our model and persisted at higher numbers at endpoint in the ascites fluid of tumor-bearing mice. However, despite promising functional effects *in vitro*, our *in vivo* results show only a modest improvement in survival. We suspect this is due to a need to edit multiple regulatory genes at once, as tumors can escape NK cell killing through other mechanisms, or a need for multiple infusions of NK cells.

In summary, we demonstrate an easy-to-use method for efficient gene editing in primary human NK cells. In addition, we have shown functional consequence and therapeutic potential of knockout of two genes implicated in NK cell activity. Using these optimized methods, future work will focus on editing multiple genes simultaneously along with delivery of engineered receptors for enhancing NK cell immunotherapy.

## Materials and Methods

### *Donor NK Cell Isolation and Expansion*

Peripheral blood mononuclear cells (PBMCs) from de-identified healthy human donors were obtained by automated leukapheresis (Memorial Blood Centers, Minneapolis, MN) and further isolated on a ficoll-hypaque (Lonza) gradient. CD56+CD3- NK cells were isolated by negative selection using the EasySep Human NK Cell Enrichment Kit (Stemcell Technologies). After isolation, NK cells were either rested by culture with 1 ng/mL IL15, or activated by co-culture with Clone9.mbIL21 at a 2:1 or 1:1 (feeder:NK) ratio in B0 medium supplemented with 50 IU/mL IL2 (Peprotech), as described.[65] [86](Denman et al., 2012) Samples were obtained after informed consent with approval from the University of Minnesota Institutional Review Board (IRB 1602E84302).

### *Cell Culture*

NK cells were maintained in B0 medium[103] supplemented with 1 ng/mL IL15 unless otherwise noted. NK cells were cryopreserved at $1x10^7$ cells/mL in CryoStor CS10 (Sigma Aldrich). The transgenic Clone9.mbIL21 cell line, the human Burkitt's lymphoma cell line Raji, the human erythroleukemia cell line K562, the human prostate cancer cell line DU145, and the human monocytic leukemia cell line THP1 were maintained in RPMI 1640 (GE Healthcare Life Sciences) supplemented with 10% FBS (GE Healthcare Life Sciences) and 100 U/mL penicillin (Corning) and 100 U/mL streptomycin (Corning). The human ovarian cancer cell line MA148 was maintained in DMEM (GE Healthcare Life Sciences) supplemented with 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin. Cell

lines were authenticated by the University of Arizona Genetics Core (UAGC) using short tandem repeat profiling. All cell lines were routinely tested for mycoplasma contamination (Lonza) and found to be negative.

### *Guide RNA design*

Guide RNAs targeting ADAM17, PD1, AAVS1, and PD-L1 were designed using the CRISPR MIT webtool (http://crispr.mit.edu.ezp1.lib.umn.edu/) along with Cas-OFFinder. Six gRNAs per target gene were tested in HEK293T cells and the most efficient gRNA (Table 1) was purchased from TriLink Biotechnologies or Synthego with 2'-O-methyl and 3' phosphorothioate modifications to the first three 5' and the last three 3' nucleotides.

### *Electroporation of activated NK cells*

Activated NK cells were pelleted and resuspended at $3x10^7$ cells/mL in T buffer (Neon Transfection System Kit, ThermoFisher Scientific). 1.5 $\mu$g Cas9 mRNA (Trilink) and 1 $\mu$g chemically modified guide RNA (Trilink or Synthego) were added to 10 $\mu$L ($3x10^5$ cells) on ice. Cas9 mRNA alone, without guide RNA, was used as a control for all experiments. This mixture was electroporated with the Neon Transfection System (ThermoFisher Scientific) using 2 pulses of 1850 volts and 10 ms pulse width. NK cells were recovered in warm B0 medium containing 1 ng/mL IL15. For rAAV6 infection, rAAV6 (MOI=500,000) was added to NK cells 30 minutes after electroporation. rAAV6 particles were produced by the University of Minnesota Vector Core.

### *Analysis of gene editing efficiency*

PCR primers were designed to amplify a 400-500 bp region surrounding the gRNA target site (Table 2). Five days after electroporation, genomic DNA was PCR amplified using AccuPrime Taq DNA Polymerase (Invitrogen). For analysis by TIDE, PCR amplicons were Sanger sequenced (ACGT, Inc. or University of Minnesota Genomics Center) and Sanger chromatograms were uploaded to the TIDE webtool (https://tide-calculator.nki.nl). For Next Generation Sequencing (NGS), primers with Nextera universal primer adaptors (Illumina) were designed to amplify a 350-475 bp site surrounding the region of interest (Table 2). Samples were submitted to the University of Minnesota Genomics Center for subsequent amplification with indexing primers and sequencing on a MiSeq 2x200 bp run (Illumina). A minimum of 1000 read pairs were generated per sample. Raw .fastq files were analyzed against a reference sequence and gRNA protospacer sequence using the CRISPR/Cas9 editing analysis pipeline CRISPR-DAV, as previously described.[104]

### *Droplet digital PCR*

Targeted integration of MND-CD16 at AAVS1 was quantified using droplet digital PCR (ddPCR). Assays were designed using PrimerQuest software (Integrated DNA Technologies) using settings for 2 primers + probe qPCR. Each sample was run as a duplexed assay consisting of an internal reference primer + probe set (HEX) and an experimental primer + probe set (FAM) (Table 3). Reactions were set up in duplicate using the ddPCR Supermix for Probes (Bio-Rad) with 50 ng genomic DNA per assay according

to manufacturer's instructions. Droplets were generated and analyzed using QX200 Droplet-digital PCR system (Bio-Rad).

### *RT-qPCR*

RNA was extracted using the PureLink RNA Mini Kit (Ambion). cDNA was generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was conducted using exon-exon junction primer sets (Table 2) and SYBR Green Master Mix (Applied Biosystems) and analyzed using a CFX-96 (Bio-Rad). Gene expression levels were calculated relative to β-actin and expressed as a fold change compared to control.

### *Antibodies and Flow Cytometry*

The following antibodies were used: APC-, FITC-, PE-, or Biotin-conjugated anti-CD56 (clone REA196; Miltenyi Biotec), PE-conjugated anti-CD3 (clone SK7; BD Biosciences), PE/Cy7-conjugated anti-CD16A (clone 3G8; BioLegend), Brilliant violet 421-conjugated anti-CD16A (clone 3G8; BioLegend), Brilliant ultraviolet 395-conjugated anti CD158b (BD Biosciences), PE-conjugated anti CD336 (clone P44-8.1, BD Biosciences), Brilliant violet 421-conjugated anti CD337 (clone P30, BD Biosciences), FITC-conjugated anti NKB1 (clone DX9, BD Biosciences), PE-Cy7-conjugated anti CD314 (clone 1D11, BD Biosciences), APC-conjugated anti NKp46 (clone 9E2, BD Biosciences), FITC-conjugated anti CD226 (clone DX11, BD Biosciences), Brilliant violet 421-conjugated anti-IFNγ (clone 4S.B3; BioLegend), FITC-conjugated anti-CD107a (clone H4A3; BD Biosciences), Brilliant violet 605-conjugated anti-CD62L (clone Dreg56; BD Biosciences), PE-

conjugated anti-active caspase-3 (clone C92-605; BD Biosciences), SYTOX Blue dead cell stain (ThermoFisher), Fixable viability dye eFluor 780 (eBioscience). Detection of ADAM17 was performed using anti-human TACE ectodomain (R&D Systems) followed by biotinylated anti-mouse IgG (R&D Systems) and Brilliant violet 421-labeled streptavidin (BioLegend). Detection of PD1 was performed using optimized mild acid treatment followed by staining with PE-labeled anti-PD1 (clone PD1.3.1.3; Miltenyi Biotec). Flow cytometry assays were performed on LSRII or LSR Fortessa flow cytometers (BD Biosciences) and all data were analyzed with FlowJo version 10.4 software (FlowJo LLC).

***NK Cell Functional Assays***

For PMA stimulation, NK cells were pre-treated for 1 hour with 10 uM ADAM17 inhibitor (INCB007839) or DMSO control. NK cells were then stimulated with 1 ug/mL PMA for 1 hour and CD16a expression was measured by flow cytometry. For intracellular cytokine staining, NK cells were plated at $2.5x10^5$ cells per 100 $\mu$L B0 with no cytokines added. After incubation overnight, target cells were added at the indicated E:T ratios (2:1 for assays with ADAM17 KO NK cells, 1:1 for assays with PD1 KO NK cells). For ADCC assays, Raji cells were pre-coated with Rituximab (Genentech) at 10 ug/mL for 30 minutes. FITC-conjugated anti-CD107a was added to the culture and cells were incubated for 1 hour at 37 C. Brefeldin A and monensin (BD Biosciences) were added after 1 hour and cells were incubated for an additional 5 hours. Cells were stained with fixable viability dye and

for extracellular antigens and then were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences). Cells were then stained for intracellular IFNγ.

### *Target Cell Killing Assays*

NK cells were plated at $2.5x10^5$ cells per 100 $\mu$L B0 medium with no cytokines added and incubated overnight. Target cells were pelleted and labeled with CellTrace CFSE (ThermoFisher Scientific) for 10 minutes at room temperature, then washed in 10 mL FBS. For ADCC assays, CFSE-labeled Raji cells were pre-coated with Rituximab (Genentech) at 10 ug/mL for 30 minutes. CFSE-labeled target cells were added to NK cells (E:T=2:1 for ADAM17 assays, E:T=1:1 for PD1 assays). Co-cultures were incubated at 37C for 6 hours. Cells were stained with fixable viability dye and then were fixed and permeabilized with ice cold 70% ethanol for 30 minutes. Cells were then stained for active caspase-3.

### *In vivo tumor model*

All animal studies were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC 1610-34201A). NOD/SCID/$\gamma c^{-/-}$ (NSG) mice were purchased from Jackson Laboratories and used for all *in vivo* experiments. Mice were given $2x10^5$ luciferase-expressing MA148 ovarian carcinoma cells[102,105] via IP injection four days prior to NK cell delivery. One day before NK cell treatment, mice were sub-lethally irradiated (225 Centigray), and tumor burden was measured by bioluminescent imaging (BLI) using the Xenogen IVIS 50 Imaging System (Caliper Life Sciences). Mice were then grouped based on BLI to ensure each treatment group started with the same average tumor burden.

On day 0, NK cells ($1x10^6$ cells per mouse) were delivered IP. BLI was measured weekly. Mice received IP injections of IL-15 (5 μg/mouse) every Monday, Wednesday, and Friday for 3 weeks. Animal health was monitored daily, and mice were euthanized when moribund. Upon sacrifice, ascites fluid was collected by IP wash. Cells in the ascites fluid were washed, enumerated, and stained for flow cytometry for hCD45, CD56, CD3, and PD-L1. Total NK cells in ascites fluid were calculated by applying percent hCD54+CD56+ to the total ascites cell count.

***Predicted Off-Target Analysis and Next Generation Sequencing***

Putative off-target sites were predicted for each gRNA using the *offTargetAnalysis()* function from the CRISPRseek R package (version 3.8, PMID: 25247697) using UCSC hg19 and a maximum mismatch number of 4. Primers with Nextera universal primer adaptors (Illumina) were designed to amplify a 350-475-bp site surrounding either the on-target (OnT) or off-target (OT) region of interest using Primer3Plus (http://bioinfo.ut.ee/primer3-0.4.0/). Genomic DNA was PCR amplified in one step using AccuPrime Taq DNA polymerase, high fidelity according to the manufacturer's protocol (Invitrogen). Samples were run on a 1% agarose gel and gel extracted using QIAquick Gel Extraction Kit (Qiagen). Samples were submitted to the University of Minnesota Genomics Center for subsequent amplification with indexing primers and sequencing on a MiSeq 2x300-bp run (Illumina). A minimum of 1000 read-pairs were generated per OnT sample and 10,000 read-pairs per OT sample. Raw fastq files were analyzed against a reference sequence and gRNA protospacer sequence using the CRISPR/Cas9 editing analysis

pipeline CRISPR-DAV as previously described[104]. Output '*sample_snp.xlsx*' and '*sample_len.xlxs*' were compiled and analyzed using a custom R markdown script (RStudio v1.1.383, R v3.4.2). Raw .fastq files and custom script will be made available upon request.

### *Statistical Analysis*

The Student's t-test was used to test for significant differences between two groups. Differences between 3 or more groups were tested by one-way ANOVA analysis followed by Tukey's *post-hoc* test. All *in vitro* assays were repeated in 3-5 independent donors. Mean values +/- standard error of the mean (SEM) are shown. The level of significance was set at $\alpha = 0.05$. Statistical analyses were performed using GraphPad Prism 7.0.

## Tables

### Table 2-1. Guide RNA Target Sequences

| | |
|---|---|
| ADAM17 | GAACCACGCTGGTCAGGAAT |
| PD1 | CCTGCTCGTGGTGACCGAAG |
| AAVS1 | GTCACCAATCCTGTCCCTAG |
| PD-L1 | TTGAAGGACCAGCTCTCCCT |

### Table 2-2. Primer sequences

| Primer Name | Forward | Reverse |
|---|---|---|
| ADAM17 PCR | CCCGATGTGAGCAGTTTTCC | GAGACAGGCCCATCTCCTTT |
| PD1 PCR | GGGTGAAGGCTCTTAGTAGG | CAGGCTCTCTTTGATCTGC |
| AAVS1 PCR | ACTCCTTTCATTTGGGCAGC | GGTTCTGGCAAGGAGAGAGA |
| ADAM17 NGS | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGTAGAATCTTCCCAGTAG | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCAAACACCTGATAGACC |
| PD1 NGS | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACCTCTCTCCATCTCTCAG | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGGCTCTCTTTGATCTGC |
| ADAM17 RT-PCR | ACCTGAAGAGCTTGTTCATCGAG | CCATGAAGTGTTCCGATAGATGTC |
| PD1 RT-PCR | ACCTGGGTGTTGGGAGGGCA | GGAGTGGATAGGCCACGGCG |
| β-actin RT-PCR | CACAGGGGAGGTGATAGCAT | CTCAAGTTGGGGGACAAAAA |
| AAVS1-GFP Jxn PCR | GGACGAGCTGTACAAGTAACG | GAGACAGTGACCAACCATCC |
| AAVS1-CD16 Jxn PCR | TCTTGAGGGTCCTTTCTCCA | CTCTGTTCAGCCCTAAGAATCC |

**Table 2-3. ddPCR Sequences**

| | | |
|---|---|---|
| MND-AAVS1 Forward | Experimental | CTGAAATGACCCTGTGCCTTAT |
| MND-AAVS1 Reverse | Experimental | GCGATCTGACGGTTCACTAAA |
| MND-AAVS1 Probe | Experimental (FAM) | ACCAATCAGTTCGCTTCTCGCTTCT |
| B2M Exon 3 Forward | Reference | GGTTTCATCCATCCGACATTGAAGTTGAC |
| B2M Exon 3 Reverse | Reference | GGGTGAATTCAGTGTAGTACAAGAGATAG |
| B2M Exon 3 Probe | Reference (HEX) | GACCAGTCCTTGCTGAAAGACAAGTCTG |

# Figures

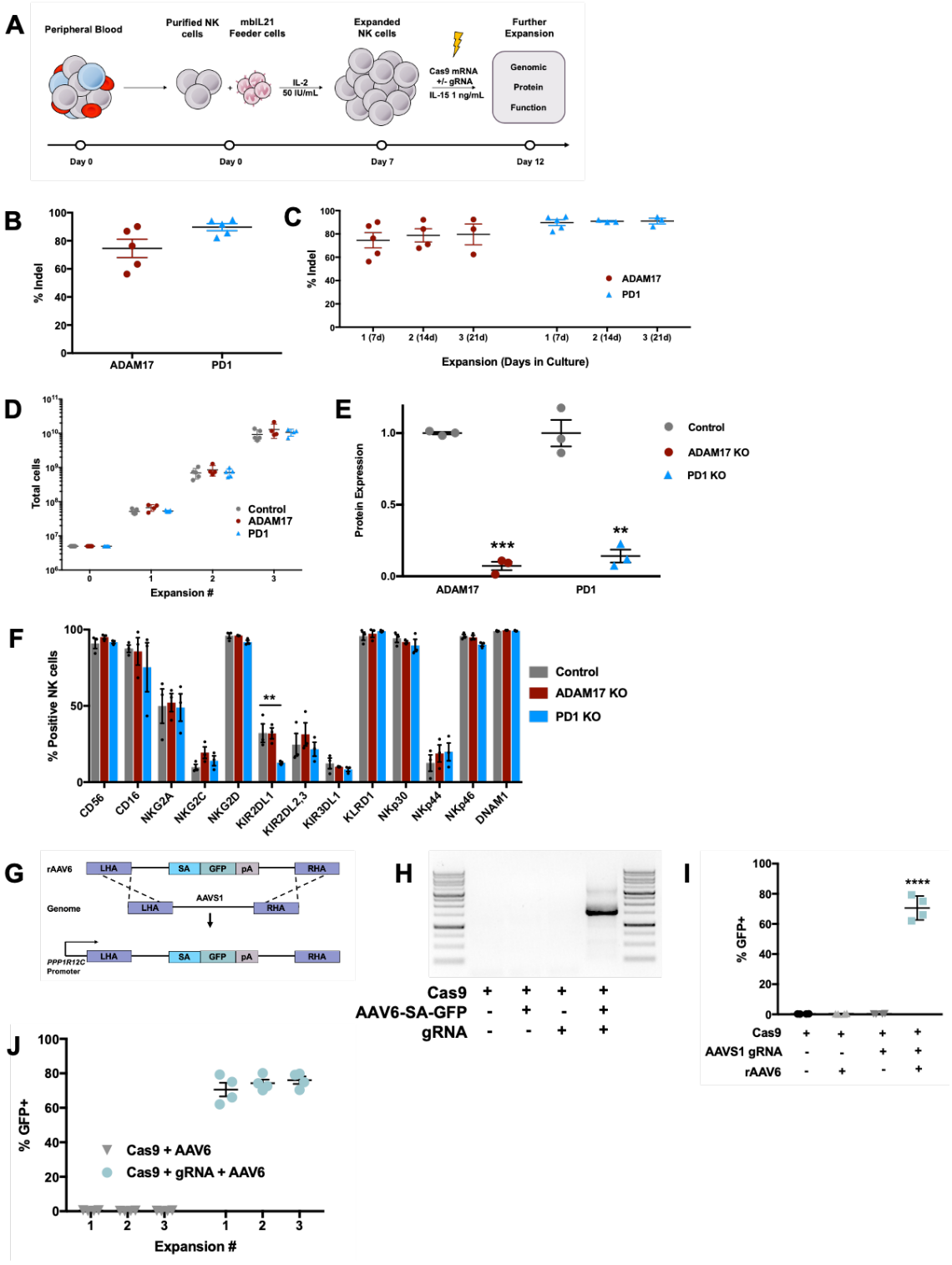

A
Peripheral Blood
Purified NK cells
mbIL21 Feeder cells
IL-2 50 IU/mL
Expanded NK cells
Cas9 mRNA +/- gRNA IL-15 1 ng/mL
Further Expansion
Genomic
Protein
Function
Day 0
Day 0
Day 7
Day 12
B
% Indel
ADAM17
PD1
C
% Indel
ADAM17
PD1
1 (7d)
2 (14d)
3 (21d)
Expansion (Days in Culture)
D
Total cells
Control
ADAM17
PD1
Expansion #
E
Protein Expression
Control
ADAM17 KO
PD1 KO
ADAM17
PD1
F
% Positive NK cells
Control
ADAM17 KO
PD1 KO
CD56
CD16
NKG2A
NKG2C
NKG2D
KIR2DL1
KIR2DL2,3
KIR3DL1
KLRD1
NKp30
NKp44
NKp46
DNAM1
G
rAAV6
LHA
SA
GFP
pA
RHA
Genome
AAVS1
PPP1R12C Promoter
H
Cas9
AAV6-SA-GFP
gRNA
I
% GFP+
Cas9
AAVS1 gRNA
rAAV6
J
% GFP+
Cas9 + AAV6
Cas9 + gRNA + AAV6
Expansion #

**Figure 2-1. A highly efficient method of CRISPR/Cas9-based engineering of primary human NK cells. (A)** Protocol for expansion and gene editing of primary human NK cells. Briefly, NK cells are isolated from PBMCs of healthy human donors and co-cultured for one week with mbIL21 (C9) feeder cells in the presence of IL2. Expanded NK cells are electroporated with Cas9 mRNA alone (control) or in combination with gRNA targeting a gene of interest. Electroporated NK cells are recovered in media containing IL15 and then analyzed for genomic editing, protein loss, and function. **(B)** Next generation sequencing (NGS) analysis of indel formation after delivery of Cas9 mRNA + gRNA targeting ADAM17 (maroon) and PD1 (blue) (n=5 independent donors). **(C)** Analysis of indel formation by TIDE over serial expansions with Clone9.mbIL21 feeder cells: x-axis shows expansion number and total days in culture (n=5 independent donors). **(D)** Expansion of engineered cells to clinically relevant numbers using multiple rounds of Clone9.mbIL21 expansion (n=5 independent donors). **(E)** Protein expression in knock-out NK cells (colored bars) compared to control cells (gray bars) normalized to 1. Flow cytometry analysis of % ADAM17+ NK cells (maroon, n=3 independent donors, ***$P$=0.0002, Student's t-test), flow cytometry analysis of % PD1+ NK cells normalized to controls (blue, n=3 independent donors, **$P$=0.0011, Student's t-test) after gene knockout. **(F)** Expression of a panel of NK cell markers measured by flow cytometry in control or gene modified NK cells (n=3 independent donors). **(G)** Gene knock-in strategy to integrate a splice-acceptor-EGFP cassette to the AAVS1 locus using rAAV6 and CRISPR/Cas9. **(H)** PCR primers were designed to span and amplify the 3' junction of genomic AAVS1 and EGFP. **(I)** EGFP expression 14 days after gene knock-in with rAAV6 (n=4 independent donors, ****$P$=0.0016, Student's t-test). **(J)** EGFP expression after 3 rounds of expansion with C9 feeder cells.

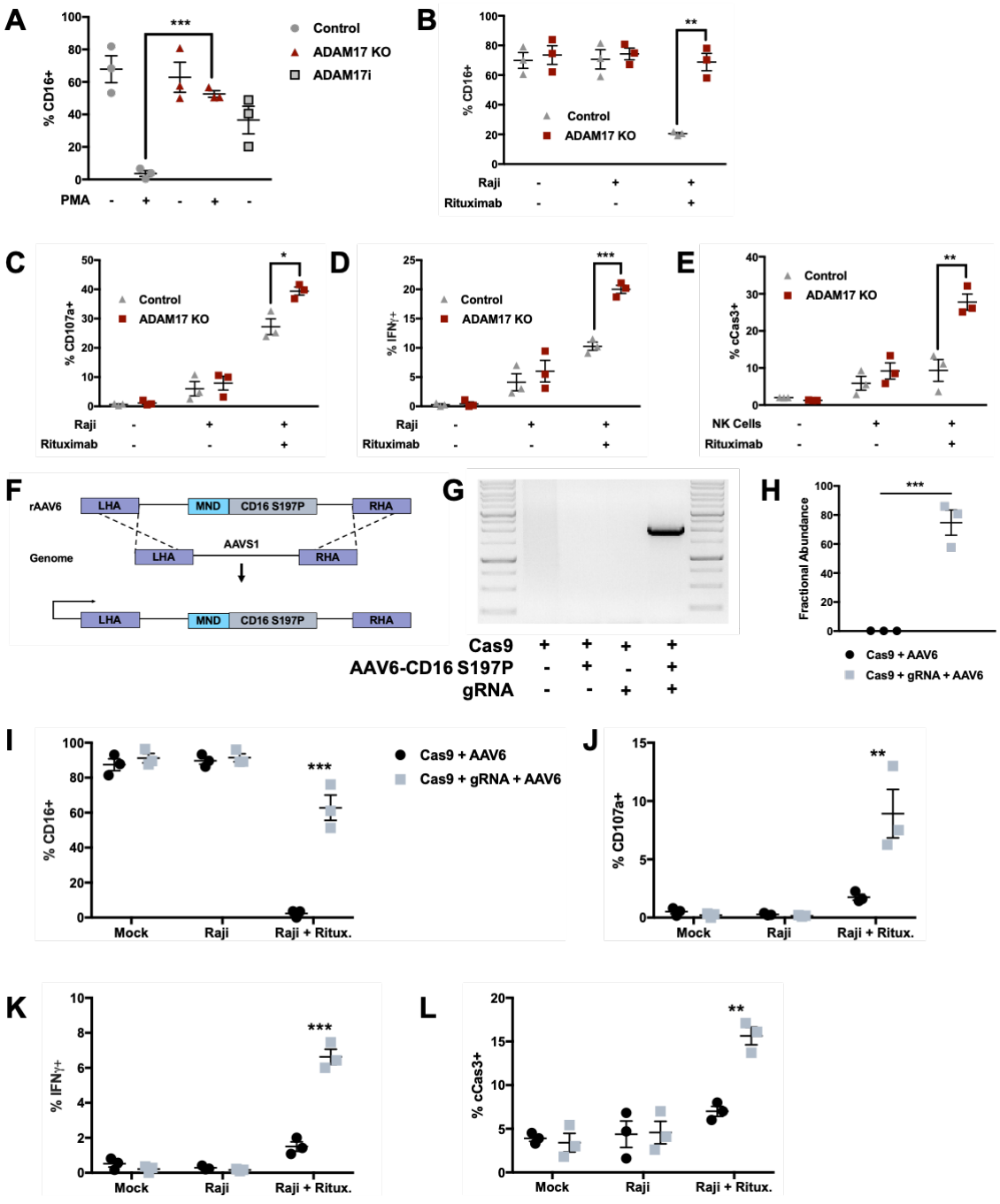



**Figure 2-2. ADAM17 KO and CD16 editing lead to enhanced ADCC. (A)** Control NK cells were treated for one hour with 10 $\mu$M ADAM17 inhibitor (light gray with dark outline) or DMSO (control = dark gray, ADAM17 KO = maroon). Cells were then stimulated with PMA/ionomycin (1 µg/mL) for one hour and CD16a expression was analyzed by flow cytometry (n=3 independent donors, ***$P$=0.0003, Student's t-test). **(B-**

**E)** Raji cells were labeled for 10 minutes with CellTrace CFSE then incubated for 30 minutes with 10 ug/mL Rituximab. Raji cells were then co-cultured at a 2:1 (E:T) ratio with control (dark gray) or ADAM17 KO (maroon) NK cells for 6 hours (n=3 independent donors). Flow cytometry assays detected increased NK cell CD16A expression (**B**) (***P*=0.0012, Student's t-test), increased NK cell degranulation (**C**) (mock = no target cells, **P*=0.0162, Student's t-test), increased NK cell IFNγ production **(D)** (mock = no target cells, ****P*=0.0006, Student's t-test), and increased Raji cell apoptosis **(E)** (mock = no effector cells, ***P*=0.007, Student's t-test). **(F)** Gene knock-in strategy to integrate an MND-driven non-cleavable CD16a (S197P) to the AAVS1 locus using rAAV6. **(G)** Primers were designed to span the 3' junction of genomic AAVS1 and CD16. **(H)** Targeted knock-in of MND-CD16 S197P was quantified using droplet digital PCR (n=3 independent donors, ****P*=0.001). **(I-L)** CD16 S197P-KI NK cells were co-cultured with rituximab-coated Raji cells and analyzed as described above for (**B-E**) (n=3 independent donors, ***P*<0.01, ****P*<0.001, two-way ANOVA with Tukey's *post-hoc* test).

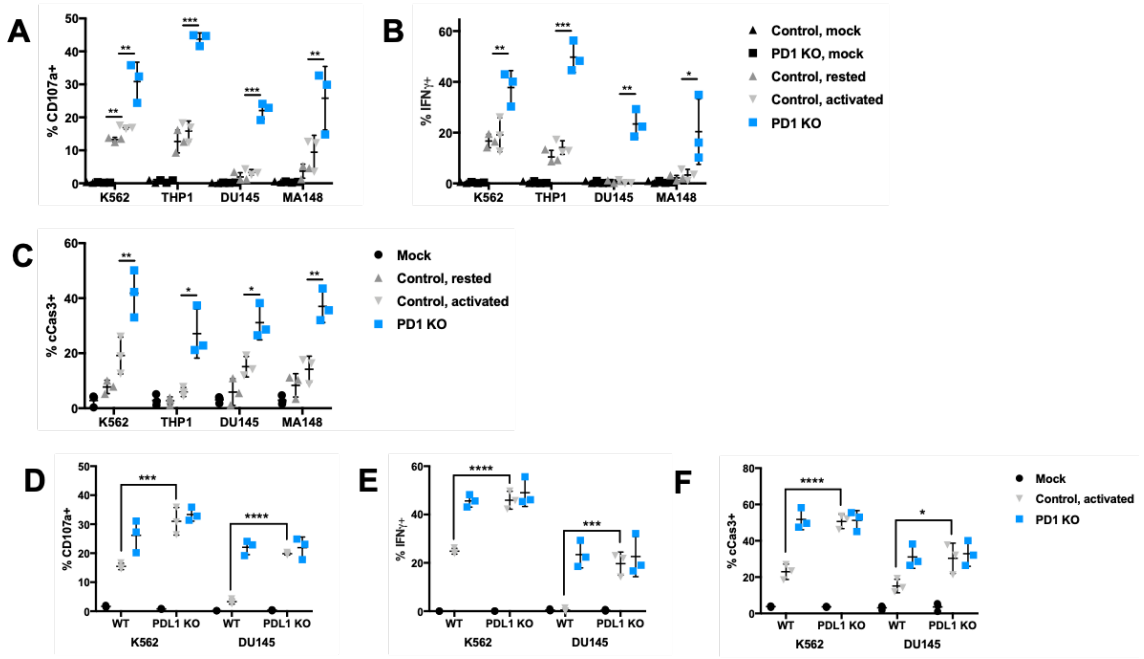



**Figure 2-3. PD1 KO NK cells demonstrate enhanced anti-tumor function *in vitro*.** **(A-C)** Control NK cells were rested in 1 ng/mL IL15 (light gray) to support survival as previously described[106]. Control (dark gray) and PD1 KO (blue) NK cells were activated for 1 week with C9 feeder cells. NK cells were then co-cultured at a 1:1 (E:T) ratio with target cell lines K562, THP1, DU145, and MA148 for 6 hours (n=3 independent donors, $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA with Tukey's *post-hoc* test). **(A)** NK cell degranulation (mock = no target cells), **(B)** NK cell IFNγ production (mock = no target cells), and **(C)** target cell apoptosis (mock = no effector cells). **(D-F)** Control (dark gray) and PD1 KO (blue) NK cells were activated with C9 feeder cells and co-cultured at a 1:1 (E:T) ratio with wild-type (WT) or PD-L1-deficient K562 or DU145 target cells for 6 hours (n=3 independent donors, $^{*}P<0.05$, $^{***}P<0.001$, $^{****}P<0.0001$, two-way ANOVA with Tukey's *post-hoc* test).

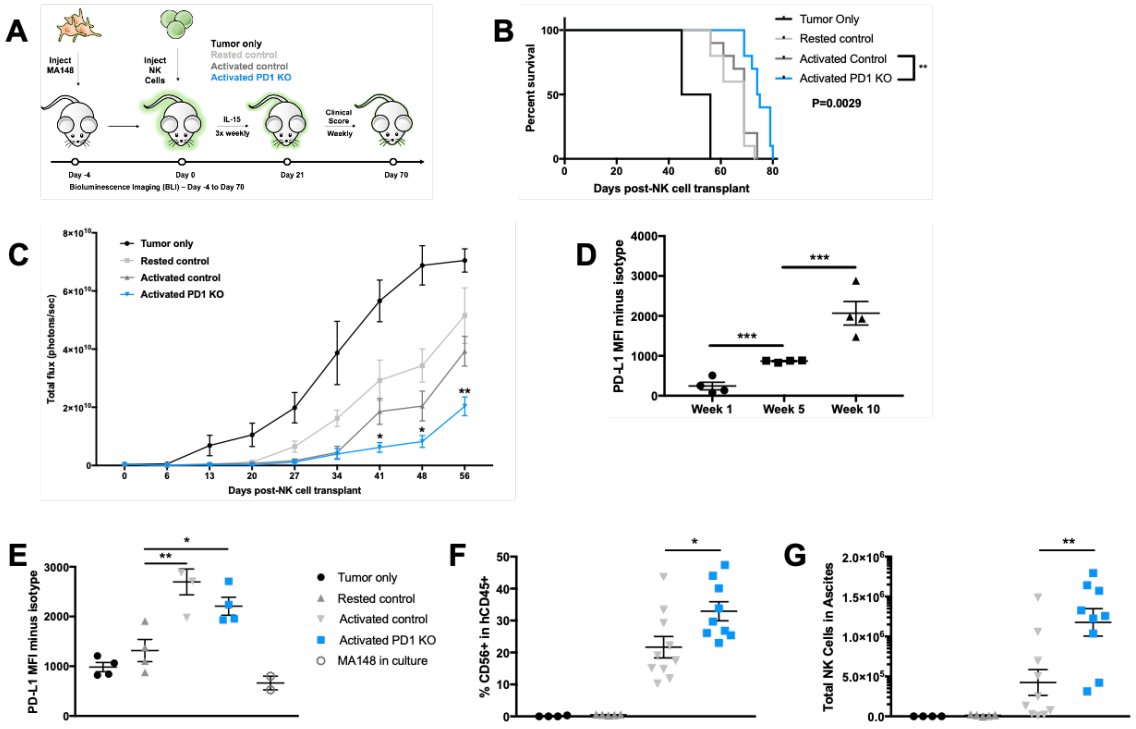
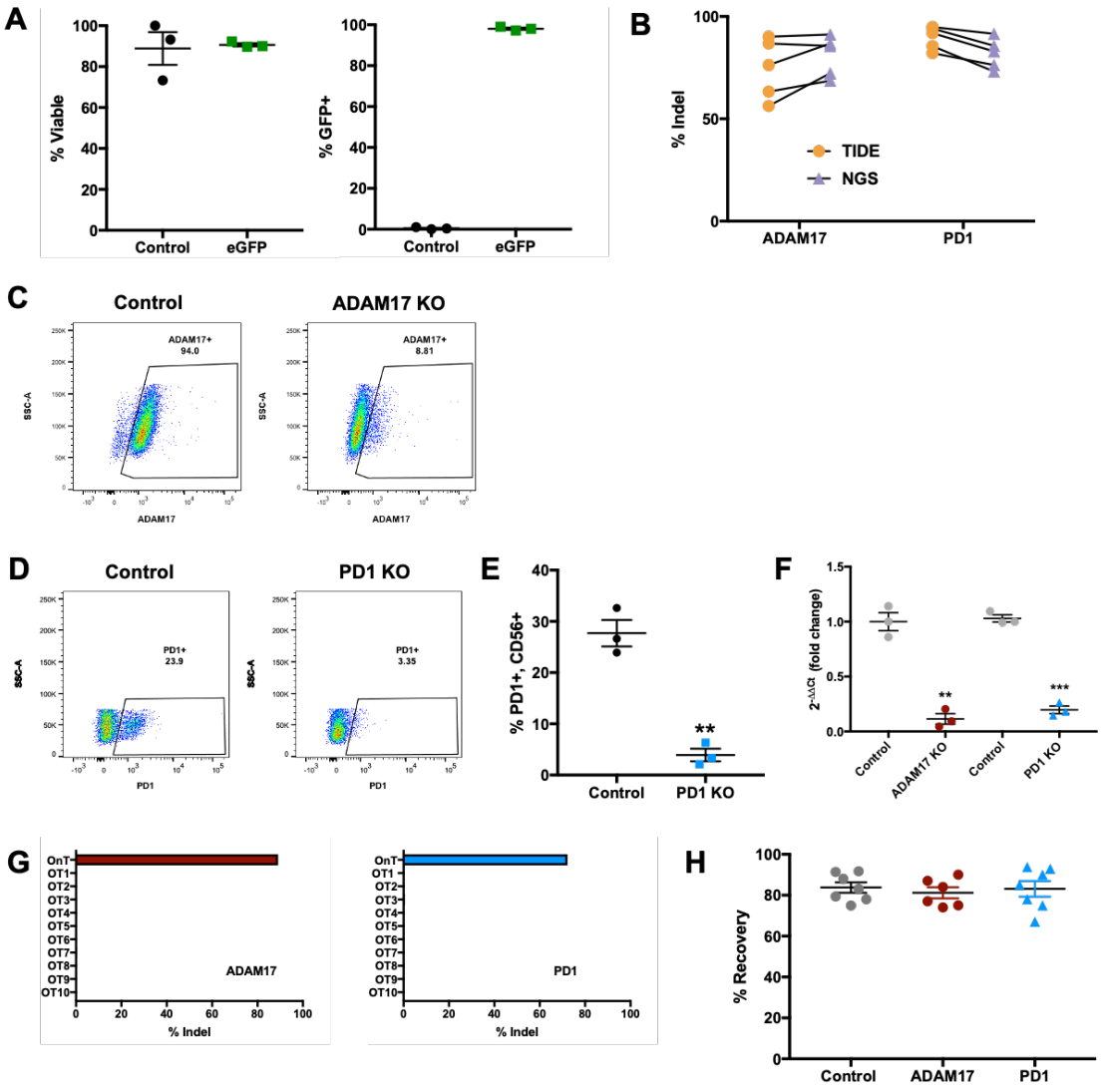
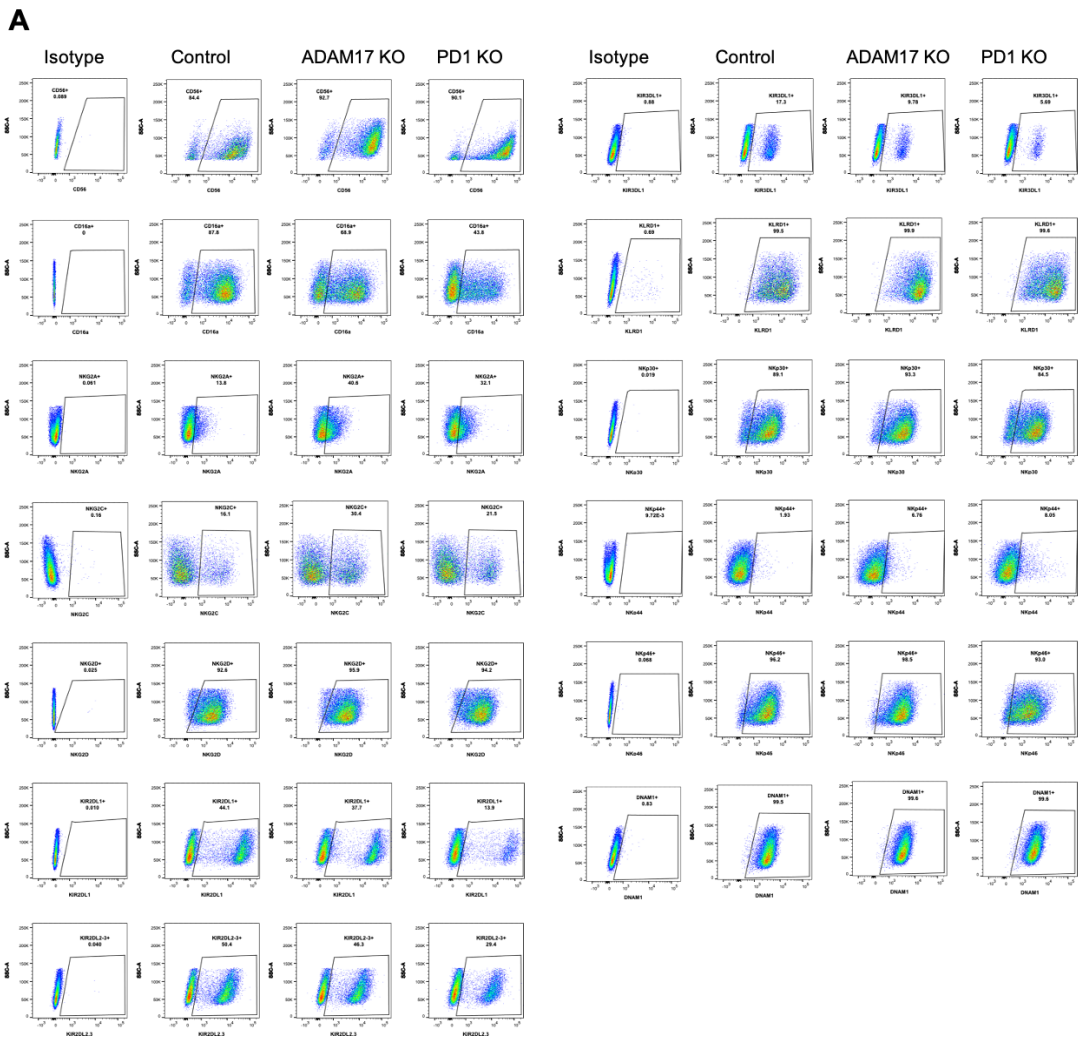



**Figure 2-4. PD1 KO NK cells demonstrate enhanced anti-tumor function *in vivo*.** **(A-C)** $2x10^5$ luciferase expressing MA148 cells were delivered IP to NSG mice. Four days after tumor injection, PBS (black), or $1x10^6$ rested control (light grey), activated control (dark gray), or activated PD1 KO (blue) NK cells were delivered IP. NK cells were supported with a 5 µg dose of IL15 3 times weekly for 3 weeks. Tumor burden was assessed with bioluminescence imaging weekly (n=8-10 mice per group). **(B)** Overall survival (***P*=0.0029, Log-rank test) and **(C)** tumor burden (**P*<0.05, ***P*<0.01, Student's t-test). **(D)** A subset of mice (n=4 mice per timepoint) treated with activated NK cells were sacrificed at week 1, week 5, and week 10 after treatment. MA148 cells were collected from ascites via IP wash and PD-L1 expression was measured by flow cytometry (****P*<0.001, Student's t-test). **(E-G)** Ascites fluid was also collected from all treatment groups at sacrifice. Cells from the ascites were washed, enumerated, and analyzed by flow cytometry for PD-L1 expression on GFP+ MA148 cells **(E)**, and for presence of hCD45+CD56+ NK cells **(F, G)** (n=4-10 mice per group, **P*<0.05, ***P*<0.01, One-way ANOVA with Tukey's *post-hoc* test).

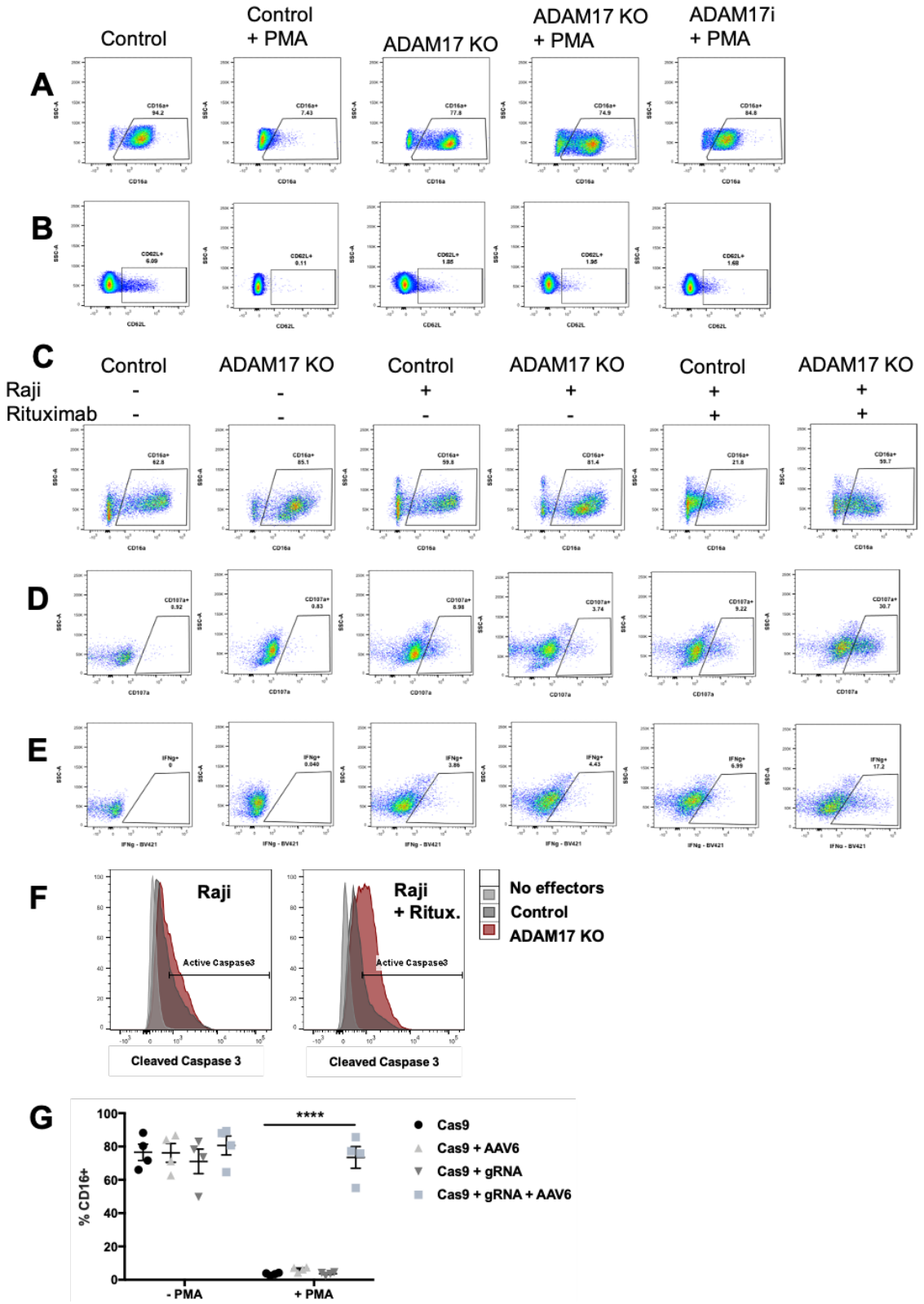



**Supplemental Figure 2-1. Analysis of gene knockout in activated NK cells.** (**A**) Activated NK cells were electroporated with PBS (gray) or mRNA encoding eGFP (green). Cell viability (left) was measured using Trypan blue exclusion and transfection efficiency (right) was measured by eGFP expression 48-hours after electroporation. (**B**) Indel formation in ADAM17 and PD1 measured by NGS and compared to TIDE (n=5 independent donors). (**C-E**) Representative flow cytometry plots for loss of ADAM17 and PD1. (**F**) mRNA loss after gene knockout was confirmed by RT-PCR. Target RNA expression was normalized to expression of *ACTB* (**G**) Percent of cells with on-target (OnT) and off-target (OT#) amplicons after PCR using primers designed to amplify the top 10 predicted off-target sites for each gRNA. Percentage based on Next Generation Sequencing of amplicons. Note: PD1 OT8 did not amplify. (**H**) Control or gene-edited NK

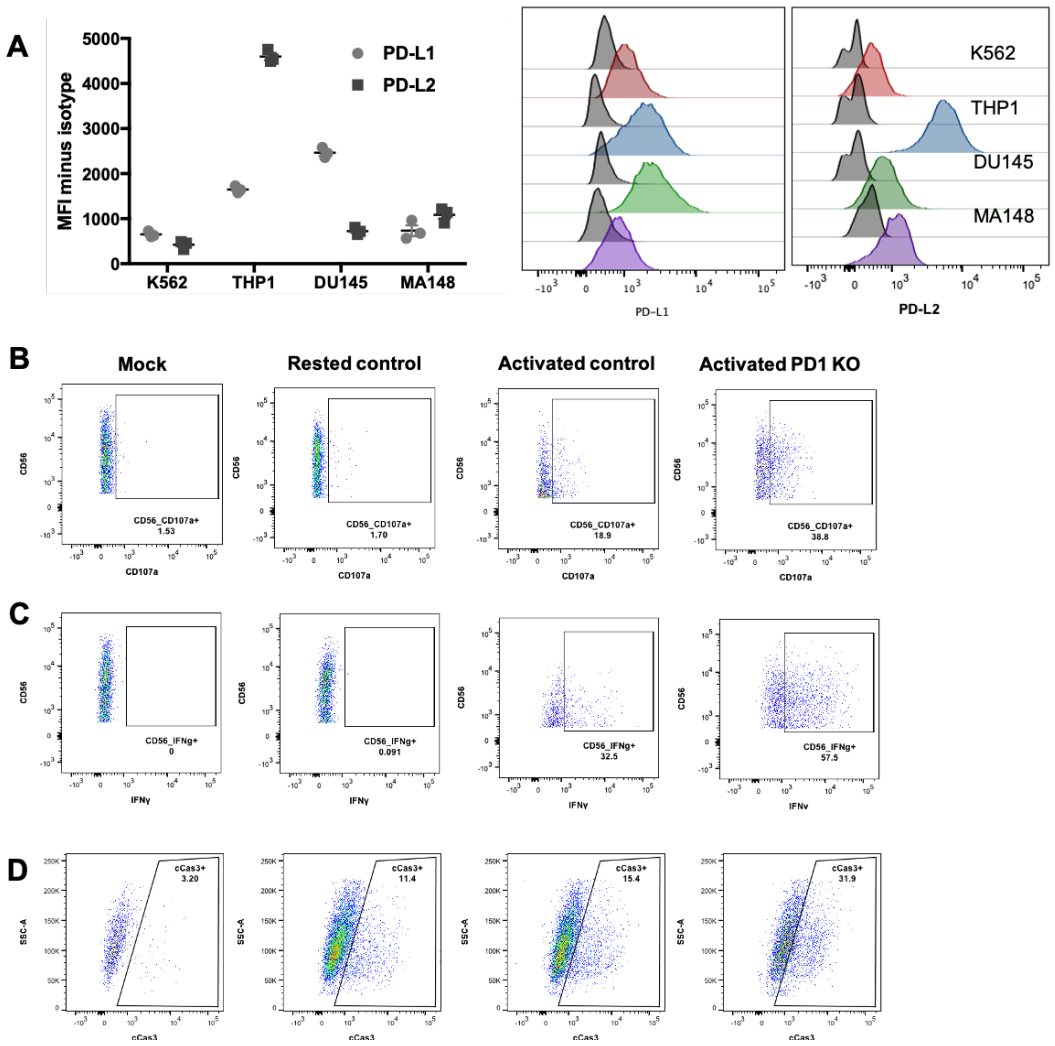
cells were frozen in CryoStor medium at $1x10^7$ cells/mL. Percent recovery was calculated by counting viable cells using Trypan blue exclusion 1 hour after thawing cells, after culture in 1 ng/mL IL15.




**Supplemental Figure 2-2. CRISPR-edited NK cells maintain expression of common NK cell receptors.** (**A**). Representative flow cytometry plots of expression of NK cell receptors in control or genetically modified NK cells. Gates were defined using fluorescently labeled isotype controls for each marker.


A
Control
Control + PMA
ADAM17 KO
ADAM17 KO + PMA
ADAM17i + PMA
B
C
Raji
Rituximab
Control
ADAM17 KO
D
E
F
Raji
Raji + Ritux.
Active Caspase3
Cleaved Caspase 3
No effectors
Control
ADAM17 KO
G
% CD16+
- PMA
+ PMA
****
Cas9
Cas9 + AAV6
Cas9 + gRNA
Cas9 + gRNA + AAV6

**Supplemental Figure 2-3. ADAM17 KO NK cells display enhanced ADCC. (A-B)** Representative flow cytometry plots of NK cell CD16a **(A)** and CD62L **(B)** expression after stimulation with 1 ug/mL PMA. **(C-F)** Representative flow cytometry plots of NK cell CD16a expression **(C)**, CD107a expression **(D)**, IFNγ production **(E)**, and target cell cleaved caspase 3 expression **(F)** after 6-hour co-culture of NK cells with Rituximab-coated Raji cells (E:T=2:1). **(G)** CD16a (S197P)-KI NK cells or controls were stimulated for 1 hour with PMA/ionomycin (1 μg/mL) and CD16a expression was analyzed by flow cytometry (n=4 independent donors, ****$P$=<0.0001, two-way ANOVA with Tukey's *post-hoc* test).




**Supplemental Figure 2-4. PD1 KO NK cells display enhanced antitumor activity. (A)** Target cell lines were incubated overnight with 500 IU/mL IFNγ and PD-L1 and PD-L2 expression was measured by flow cytometry (n=3 biological replicates). **(B-D)** Representative flow cytometry plots of NK cell CD107a expression **(B)**, NK cell IFNγ production **(C)**, and target cell cleaved caspase 3 expression **(D)** after 6-hour co-culture of NK cells with MA148 target cells (E:T=1:1).

# CHAPTER 3: Non-Viral Engineering of CAR-NK and CAR-T Cells using the *Tc Buster* Transposon System™

## Summary

Cancer immunotherapy using T cells and NK cells modified with viral vectors to express a chimeric antigen receptor (CAR) has shown remarkable efficacy in clinical trials. However, viral vectors are limited in their cargo size, carry the risk of insertional mutagenesis, and large-scale manufacturing for clinical use can be cost-prohibitive. Thus, CAR delivery via DNA transposon engineering has been pursued as an alternative due to convenient and cost-effective production and a better safety profile. Engineering via transposition is accomplished using a two-component system: a plasmid containing a gene expression cassette flanked by inverted terminal repeats (ITRs) and a transposase enzyme that binds to the ITRs and integrates the transposon into the genome.

Here, we used the newly developed Tc Buster™ (BioTechne) transposon system to deliver a transposon containing a CD19-CAR-DHFR-EGFP expression cassette to primary human peripheral blood (PB) NK cells and T cells. We optimized methods to avoid DNA toxicity and maximize efficiency. Our cargo contained a mutated dihydrofolate reductase (DHFR) which allowed us to select for stable transposon integration using methotrexate (MTX). We tested CAR-NK and CAR-T cells in functional assays against CD19-expressing Raji cells. CAR-expressing NK and T cells produced more cytokines than CAR-negative controls and efficiently killed target cells.

Our work provides a platform for robust delivery of multicistronic, large cargo via transposition to primary human NK and T cells. We demonstrate that CAR-expressing cells can be enriched using MTX selection, while maintaining high viability and function. This non-viral approach represents a versatile, safe, and cost-effective option for the manufacture of CAR-NK and CAR-T cells compared to viral delivery.

## Introduction

DNA transposons are natural DNA transfer vehicles that can be used for DNA delivery in a manner similar to integrating viruses. In nature, they exist as well-defined elements in which the transposase gene is flanked by inverted terminal repeats (ITRs) that encode transposase binding sites. They can be used as a tool for stable genomic insertion by surrounding a gene-of-interest with ITRs and co-delivering the transposase enzyme via an expression plasmid or mRNA.

### Transposon Families

Several DNA transposons have been used in such a manner in mammalian cells. In the 1990s, the Sleeping Beauty (SB) transposon system was molecularly reconstructed by eliminating inactivating mutations found in members of the Tc1/mariner family of transposons isolated from fish[30]. The reactivated transposon system has since been used for stable gene transfer and insertional mutagenesis in many vertebrate cell types including human cells. Subsequently, the *piggyBac* (PB) and *Tol2* transposable elements were isolated from insects and fish, respectively, and have been optimized for enhanced activity

in mammalian cells[107,108]. SB, PB, and Tol2 can be used as efficient non-viral tools for stable gene delivery, and each of these has been used for gene delivery in primary human lymphocytes[109]. The hAT superfamily of transposons (including Tol2) are found in diverse species[37]. A novel representative of this family is Tc Buster, originally isolated from the red flour beetle[35]. Tc Buster has been shown to be active in human cell lines and has a comparable transposition efficiency to SB and PB[36,37]. Bio-Techne (Minneapolis, MN) has developed a hyperactive mutant of Tc Buster to be used for the non-viral manufacture of cellular therapies.

Advantages of Transposons

Transposons have some meaningful advantages as an alternative to viral vectors for gene therapy. Several clinical gene therapy products have been developed using genetically modified CD34+ hematopoietic stem cells or T cells. The majority of these products rely on transduction of target cells with recombinant viruses, namely γ-retroviruses, and lentiviruses[110,111]. These delivery methods carry the risk of insertional mutagenesis via activation of proto-oncogenes or inactivation of tumor suppressor genes. In addition, large-scale manufacturing of these viral vectors for clinical use can be cost-prohibitive and impede progression through clinical trials. Thus, the use of transposon systems instead of viral vectors has been pursued as an alternative due to convenient and cost-effective production and a better safety profile[112–114]. Any vector that integrates into chromosomes poses the risk of insertional mutagenesis. A comparative study of the target site selection properties of SB and PB transposons as well as gammaretroviral and lentiviral systems in

primary human CD4+ T cells ranked their safety profiles based on multiple criteria. The group defined a genomic safe harbor as the intersection of five criteria: 1) the sequence is not evolutionarily conserved across species; 2) the locus is at least 300 kb from any cancer-related gene; 3) the locus is at least 300 kb from any miRNA; 4) the locus is at least 50 kb from any transcriptional start site (TSS); 5) the locus is not within any gene. Using these criteria, the likelihood of transposon integration in a genomic safe harbor is 21% while for viruses (retroviruses and lentiviruses) it is only 5%[115].

Plasmid-Based Transposon Systems

The use of transposon systems for gene delivery in human lymphocytes has been most widely studied as a method for generating human T cells engineered to express chimeric antigen receptors (CARs)[116,117]. Clinically, the SB system has been used to introduce CD19-specific CARs to patient- and donor-derived T cells[49,118]. Many preclinical studies and clinical trials thus far have introduced SB transposase and CAR by electroporation of bulk peripheral blood mononuclear cells (PBMCs)[47,119–122]. CAR-expressing T cells were subsequently expanded over several weeks in culture using feeder cells engineered to express the target antigen and co-stimulatory molecules[49]. Efforts are now being made to shorten the culture time before patient infusion. One such trial is underway in which PBMCs are transferred into the patient within 2 days after electroporation with SB transposase, CD19-CAR, and membrane-bound IL15 (NCT03579888). Signaling through the CAR and mbIL15 gives genetically-modified T cells a selective advantage after transplant, favoring their outgrowth[123].

Reduced-Toxicity Transposon Approaches

Another approach for shortening culture time ex vivo is to electroporate T cells directly, rather than as bulk PBMCs from which T cells need to be selected. This has been challenging as the delivery of transposons via plasmids leads to DNA toxicity in T cells due to a type I interferon (IFN) response[124,125]. Thus, efforts have been made to minimize the amount of DNA introduced to the T cell. Minicircle vectors are DNA delivery vehicles that do not carry a bacterial origin of replication or bacterial resistance genes, reducing the size of the vector to only that of the expression cassette[126]. This approach has been used to achieve stable expression of transgenes in primary human T cells with efficiencies over 50 percent[124,127,128]. However, minicircle vectors rely on the complex and laborious removal of the plasmid bacterial regions after plasmid purification, often leading to low plasmid yield. More recently, nanoplasmids have been developed that contain a small (<500 bp) backbone that encodes the R6K origin of replication and the RNA-OUT (antisense RNA) selectable marker. This small backbone is below the limit of susceptibility for DNA toxicity and thus nanoplasmids can be made with high yield and without complex purification methods[129,130].

NK Cell Modification with Transposons

Thus far, the use of transposons for NK cells has been mostly applied to the NK-92 cell line[40]. Recently, the SB transposon system has been used to deliver a CAR to cytokine-induced killer cells for targeting CD33 on chemoresistance acute myeloid leukemia (AML) in patient-derived xenografts[43]. However, lessons can be learned from T cells on the use of

transposons for CAR delivery to primary NK cells. The initial approach of electroporating PBMCs with transposon-based CARs suggests that NK cells could be selectively outgrown instead of T cells. indeed, some reports have shown outgrowth of NK cells reaching 50% of the PBMC population after co-culture with feeder cells[120]. Thus, this approach could be optimized for selection of CAR-expressing NK cells, or delivery of a mixed population of CAR-T and -NK cells might be advantageous as NK ells have been shown to produce inflammatory cytokines to help shape the adaptive immune response[131].

Alternatively, the use of minicircle or nanoplasmid vectors to deliver transposons directly to purified NK cells is an option. NK cells share many properties with T cells, and delivery of DNA to NK cells has been shown to induce similar toxicity[132]. Thus, reducing the amount of DNA delivery by using mRNA-encoded transposase in combination with minicircle- or nanoplasmid-encoded transposon may be ideal.

The use of transposons for engineering NK cells is not limited to the delivery of CARs. Other modifications have been proposed for enhancing aspects of NK cell activity including persistence, migration, and cytotoxicity. This includes the introduction of self-stimulating cytokine receptors[133], strong activating receptors or dominant negative versions of NK cell inhibitors[134]. This method could be used to non-virally introduce the non-cleavable CD16a described in Chapter 2 of this thesis. Such modifications could be used in combination to create an NK cell expertly equipped to kill a broad range of tumor types. Transposons provide a non-viral strategy to introduce these transgenes that could be scaled up for clinical use. Here we show such a method for clinically scalable production of CAR-NK and CAR-T cells using the Tc Buster Transposon System. We achieve manufacturing

of enriched (>99%+) CAR-expressing cells in a matter of 3 weeks and show functional efficacy of CD19 targeting CAR-NK and CAR-T cells.

## Results

### *Optimized Engineering of CAR-NK Cells using Hyperactive Tc Buster*

We sought to develop a robust platform for the manufacture and enrichment of CAR-expressing NK and T cells. We used the newly developed hyperactive Tc Buster™ (Hyp-TcB, Bio-Techne) system to deliver a nanoplasmid containing a CD19-CAR-DHFR-EGFP expression cassette (3.7 kb transposon, **Figure 3-1A**) to primary human peripheral blood (PB) NK cells.

In Chapter 2, I described a method for efficient RNA delivery to primary human PB NK cells. Briefly, we stimulated NK cells by co-culture with mbIL21- and 41BBL-expressing K562 feeder cells for 7 days before electroporating them with mRNA using the Neon Transfection System[21]. We initially tried using this same process to deliver the nanoplasmid transposon and mRNA Tc Buster transposase. As DNA toxicity has been observed in NK cells, we modified the protocol to include DNase I in the recovery media to prevent cell clumping and subsequent cell loss[135]. We observed low delivery efficiency as measured by GFP expression (2.07 ± 0.37%) and poor recovery (12.00 ± 0.58%) of electroporated cells (**Supplemental Figure 3-1A and 1B**).

We suspected that cytosolic DNA sensors may be upregulated in NK cells during the activation process leading to the poor delivery efficiency and cell recovery[136]. We surmised that by delivering DNA earlier in the activation process, we may circumvent the

upregulation of DNA sensors. Thus, we tested delivery of the transposon with Tc Buster (TcB) or the hyperactive Tc Buster mutant (Hyp-TcB) on day 2, 3, or 4 of feeder cell-mediated activation (**Figure 3-1B**). Indeed, we observed higher transposition efficiency in these earlier timepoints, with the highest efficiency on day 4 of activation (11.85 ± 1.26% for TcB and 24.05 ± 1.36% for Hyp-TcB).

As we were delivering the transposase as mRNA, and activation of NK cells is known to lead to the upregulation of ribonucleases[137], we tested the treatment of NK cells with a ribonuclease (RNase A, RNase B, and RNase T2) inhibitor for 5 minutes prior to electroporation with transposase mRNA (**Figure 3-1C – 1E**). We found that the addition of the RNase inhibitor enhanced transposition efficiency, and again observed the highest transposition efficiency when electroporation was performed on day 4 of NK cell activation (**Figure 3-1C**, 13.23 ± 1.89% for TcB and 49.63 ± 4.64% for Hyp-TcB). We re-expanded the NK cells 48-hours after electroporation for one week and compared viability and fold-expansion for each electroporation day. We observed minimal differences in cell viability (**Figure 3-1D**) but found that NK cells electroporated on day 4 of activation had significantly higher fold-expansion than those electroporated on day 2 or day 3 (**Figure 3-1E**). Thus, the optimal protocol for transposon delivery to NK cells includes electroporation on day 4 of expansion and pre-treatment with RNase inhibitor before electroporation.

### *Enrichment of CAR-NK Cells using Methotrexate*

The antifolate methotrexate (MTX) inhibits wild-type dihydrofolate reductase (DHFR), which is essential for cell growth and proliferation[138,139]. Our cargo contained an MTX-resistant DHF mutant, which allowed us to select for stable transposon integration. We incorporated this selection step into an additional round of feeder cell-mediated expansion and implemented this in our production timeline (**Figure 3-2A**). We tested three doses of MTX to determine the minimal dose needed to kill control cells and enrich engineered cells while maintaining high cell viability and recovery (**Figure 3-2B and 3-2C**). We found the dose of 250 nM MTX to completely kill control cells (**Figure 3-2B**) and enriched cells engineered with either TcB or Hyp-TcB to >99% GFP+ (**Figure 3-2C**). Our optimized protocol achieves manufacturing in 20 days and results in 99.2% (± 0.5%) CAR+ NK cells expanded 1380-fold (± 104.4) from electroporation input (**Figure 3-2D**).

### *Functional Validation of CAR-NK Cells*

We used NK cells engineered with Hyp-TcB and selected with 250 nM MTX for testing in functional assays against the CD19-expressing Burkitt's lymphoma cell line Raji. NK cells electroporated with the transposon nanoplasmid alone (without transposase) and expanded without selection served as CAR-negative controls. We co-cultured CAR-negative and CAR-positive NK cells with Raji cells for 5 hours at various effector-to-target (E:T) ratios. CAR-NK cells produced more inflammatory cytokines IFNγ and TNFα than CAR-negative NK cells after co-culture (**Figure 3-3A and 3-3B**). CAR-NK cells also expressed more CD107a on their surface, a marker of degranulation (**Figure 3-3C**). In a

luciferase-based killing assay, CAR-NK cells killed over 90% of Raji cells in 24 hours at the lowest E:T ratio of 1:3 (**Figure 3-3D**).

Our ultimate goal is for this platform to be used clinically, and thus cryopreservation and banking of manufactured CAR-NK cells will be necessary. Thus, we tested cytotoxicity of CAR-NK cells immediately after thaw or after overnight culture in media containing 100 IU/mL IL2 (**Figure 3-3D**). We observed reduced cytotoxicity of CAR-NK cells immediately after thaw compared to fresh cells. However, target cell killing was still efficient at higher E:T ratios (3:1). Cytotoxicity was restored after an overnight rest in media containing IL2. We did not observe a reduction in cell number after the overnight rest, but we did observe a slight reduction in cell viability (**Supplemental Figure 3-2A and 3-2B**).

***Efficient Engineering of CAR-T Cells using Tc Buster Transposition***

A long-term goal of our work is to develop a combination therapy of CAR-NK and CAR-T cells. Thus, we next wanted to apply this optimized production protocol for the manufacture of CAR-T cells. We stimulated CD3+ primary human T cells with αCD3/αCD28 DynaBeads for 2 days then electroporated them with the nanoplasmid alone or in combination with TcB or Hyp-TcB mRNA. We included DNase I in the recovery media to prevent cell clumping and then re-stimulated cells with DynaBeads. Three days after electroporation, we selected for transposon expression with 250 nM MTX for an additional 7 days, for a total production timeline of 12 days (**Figure 3-4A**). We observed successful enrichment of engineered cells with MTX while maintaining high cell viability

(Figure **3-4B – 3-4D**). We similarly tested CAR-T cells in a killing assay against CD19-expressing Raji cells and near complete killing of target cells at higher E:T ratios (3:1) (**Figure 3-4E**).

## Discussion

Current methods for the manufacture of CAR-T and especially CAR-NK cells for clinical use largely rely on viral transduction. The nature of NK cells as first responders to viral infection has likely led them to evolve resistance to viral infection and explains their resistance to viral transduction[10]. In addition, the use of viral vectors for CAR-T and CAR-NK production has several other downsides. Production and handling of viral vectors is time consuming and costly, and viral vectors are limited in the size and complexity of their cargo and carry the risk of insertional mutagenesis. Engineering using transposons is an attractive alternative approach as production is convenient and cost-effective, and transposons have been shown to have a better safety profile with reduced preference for integration near gene regulatory elements[115].

The ultimate goal of our work was to develop a protocol for manufacturing CAR-NK and CAR-T cells using the Tc Buster transposon system. The Tc Buster transposon is a member of the hAT superfamily of transposons and was originally isolated from the red flour beetle. Tc Buster has been optimized for transposition in mammalian cells and a hyperactive mutant (Hyp-TcB) has been evolved by Bio-Techne (Minneapolis, MN). We obtained wild-type TcB and Hyp-TcB mRNA from Bio-Techne and used them to deliver a model expression cassette containing a second generation CD19-CAR, mutant

dihydrofolate reductase (DHFR), and enhanced GFP (EGFP) flanked by Tc Buster inverted terminal repeats (ITRs) to primary human PB NK and T cells.

The use of transposons in NK cells has been limited by DNA toxicity. To avoid this, we delivered the transposase as modified mRNA[140] and the transposon via Nanoplasmid vectors which have a small backbone, high supercoiling, and are readily scaled under cGMP compliance[141]. We also optimized activation, electroporation, recovery, and expansion conditions to achieve 49.63% (± 4.64%) integration efficiency without selection (**Figure 1**). One such modification we made to our protocol included pre-treating NK cells with an RNase inhibitor prior to electroporation. This enhanced transposition efficiency from 24.05% (±1.36%) to 49.63% (±4.64%). This is likely due to degradation of the transposase mRNA by RNases produced by NK cells without RNase inhibition. However, there is very limited data available on the expression of ribonucleases in NK cells in resting or activated states. We used a broad RNase inhibitor (RNase A, RNase B, and RNase T2) based on the available limited data[137]. It would be useful to profile NK cells for expression of a full panel of RNases at resting state and during several activation timepoints. There may be other RNases produced by NK cells that could be inhibited to further enhance efficiency, or it may be unnecessary to inhibit some of the RNases included in the broad inhibitor.

We included a methotrexate (MTX)-resistant dihydrofolate reductase (DHFR) mutein (L22F, F31S) in our cargo[142]. This allowed us to enrich our engineered population using MTX, without the need for GMP-compliant cell sorting facilities or clinical-grade monoclonal antibodies. Further, MTX is approved for clinical use and the concentrations

we used for selection are clinically relevant (**Figure 2**). Overall, we developed a CAR-NK cell manufacturing protocol that includes 4 days of feeder cell activation before electroporation, re-expansion 2 days after electroporation, and a third expansion with MTX selection (**Figure 2A**). This process results in 1380-fold expansion from electroporation input when cells are engineered with Hyp-TcB (**Figure 2D**). Current clinical trials using CAR-NK cells have treated patients with doses ranging from $1x10^5$ – $1x10^7$ cells/kg of body weight[6]. Thus, the average North American patient (80 kg) would receive a dose of $8x10^6$ NK cells on the low end and $8x10^8$ NK cells on the high end. A single dose of $1x10^7$ cells/kg could be produced in 20 days from a starting number of just $5.8x10^5$ NK cells. We routinely obtain apheresis products from healthy donors containing an average of $1x10^9$ NK cells (n=12 donors). Thus, we could generate an average of 1724 high ($1x10^7$ cells/kg) doses from each donor.

Importantly, we validated the function of CAR-NK cells produced using our protocol in comprehensive *in vitro* assays. CAR-NK cells showed high levels of degranulation, inflammatory cytokine production, and target cell killing compared to CAR-negative controls (**Figure 3A – 3D**). It has been suggested that cryopreservation may lead to reduced cytotoxicity of NK cells. We understand the importance of producing a clinical product that can be cryopreserved and banked. We did observe reduced cytotoxicity of CAR-NK cells immediately after thaw, but this phenotype was rescued after an overnight rest in media containing 100 IU/mL IL2 (**Figure 3E**). However, it may not be feasible to include this type of overnight rest period in a clinical protocol. We observed efficient target cell killing immediately after thaw when we used higher E:T ratios. Therefore, instead of

implementing an overnight rest in a clinical protocol, it may be more practical to simply deliver a higher dose of cells. This is feasible as the administration of CAR-NK cells clinically has not been associated with cytokine release syndrome, neurotoxicity, or graft-versus-host disease (GvHD), and the maximum tolerated dose has not been reached[6].

Finally, we applied our protocol for the production of CAR-T cells. CAR-T cells have been generated for clinical use with Sleeping Beauty (SB) transposition. We demonstrated the feasibility of their production using Tc Buster and validated their function (**Figure 4**).

Our work provides a platform for robust delivery of multicistronic, large cargo via transposition to primary human PB NK and T cells. Importantly, we are currently using this platform to deliver even larger cargo of interest, that exceeds the carrying capacity of viral vectors. Our results demonstrate that CAR-expressing NK and T cells can be enriched using MTX selection at clinically relevant doses, while maintaining high viability and function, and we can manufacture a large number of doses in a matter of three weeks. This approach represents a versatile, safer, and more cost-effective option for the manufacture of CAR-NK and CAR-T cells compared to viral production methods. Future work will focus on the validation of CAR-NK and CAR-T cells in preclinical *in vivo* models. We will also explore the use of other CARs targeting solid tumors and combining CAR-NK and CAR-T cells in a single infusion.

## Materials and Methods

### *Vectors and Reagents*

Tc Buster (TcB) and Hyperactive Tc Buster (Hyp-TcB) sequences were obtained from Bio-Techne (Minneapolis, MN) and cloned into pmRNA production vectors. mRNA was produced commercially (Trilink Biotechnologies, San Diego, CA; and Aldeveron, Fargo, ND). CD19-DHFR-EGFP flanked by Tc Buster ITRs was cloned into a Nanoplasmid backbone (Nature Technology, Lincoln, NE).

### *Donor T and NK Cell Isolation*

Peripheral blood mononuclear cells (PBMCs) from de-identified healthy human donors were obtained by automated leukapheresis (Memorial Blood Centers, Minneapolis, MN). CD3+ T cells or CD56+CD3- NK cells were isolated from the PBMC population using the EasySep Human T Cell Isolation Kit or EasySep Human NK Cell Isolation Kit (STEMCELL Technologies, Cambridge, MA). T cells were frozen at 1-2 x $10^7$ cells/mL and NK cells were frozen at 5 x $10^6$ cells/mL in CryoStor CS10 (STEMCELL Technologies, Cambridge, MA) and thawed into culture as needed. Samples were obtained after informed consent with approval from the University of Minnesota Institutional Review Board (IRB 1602E84302)

### *T Cell Culture*

T cells were cultured in OpTimizer CTS T Cell Expansion SFM containing 5% CTS Immune Cell SR (ThermoFisher, Waltham, MA), L-Glutamine, Penicillin/Streptomycin,

N-Acetyl-L-cysteine (10 mM), IL-2 (300 IU/mL), IL-7 (5 ng/mL), and IL-15 (5 ng/mL). T cells were activated with DynaBeads Human T-Activator CD3/CD28 (ThermoFisher, Waltham, MA) at a 2:1 bead:cell ratio for 48 hours prior to electroporation. Following electroporation, T cells were re-stimulated with DynaBeads and maintained at ~1 x $10^6$ cells/mL.

### *NK Cell Culture*

NK cells were cultured in CTS AIM V SFM containing 5% CTS Immune cell SR (ThermoFisher, Waltham, MA), Penicillin/Streptomycin, and IL-2 (100 IU/mL). NK cells were activated by co-culture with X-irradiated (100 Gray) feeder cells (K562 expressing membrane-bound IL21 and 41BB-L) at indicated feeder:NK ratios (2:1 prior to electroporation, 5:1 48 hours after electroporation, or 1:1 for all subsequent expansions).

### *T Cell Electroporation*

After 48 hours of stimulation, DynaBeads were magnetically removed, and T cells were washed once with PBS prior to resuspension in electroporation buffer. The 4D-Nucleofector (Lonza, Basel, Switzerland) and P3 kit was used with 1 x $10^6$ T cells per 20 $\mu$L cuvette, 1 $\mu$g transposase mRNA, 1 $\mu$g transposon nanoplasmid, and the Nucleofector program FI-115. Transposon nanoplasmid alone was used as a control for all experiments. T cells were allowed to recover in antibiotic-free medium containing 1 ug/mL DNase I solution (STEMCELL Technologies, Cambridge, MA) at 37 C, 5% CO2 for 30 minutes

following gene transfer, and then were cultured in complete T cell medium and re-stimulated with DynaBeads.

***Electroporation of activated NK cells***

Feeder cell-activated NK cells were washed once with PBS and resuspended at 3 x $10^7$ cells/mL in electroporation buffer. Protector RNase inhibitor (Sigma Aldrich, St. Louis, MO) was added to the mixture at a concentration of 0.8 U/$\mu$L and incubated for 5 minutes at room temperature. The cell mixture was added to 1 $\mu$g of transposase mRNA and 1 $\mu$g transposon nanoplasmid on ice. Transposon nanoplasmid alone was used as a control for all experiments. This mixture was electroporated in a 10 $\mu$L tip using the Neon Transfection System (ThermoFisher, Waltham, MA) under the following conditions: 1850 volts, pulse width of 10 ms, two pulses. NK cells were allowed to recover at a density of 1.5 x $10^6$ cells/mL in antibiotic-free medium containing 1 ug/mL DNase I solution (STEMCELL Technologies, Cambridge, MA), and were then cultured in complete NK cell medium at a density of 6 x $10^5$ cells/mL. 48 hours after electroporation, NK cells were expanded with feeder cells at a 5:1 feeder:NK ratio.

***Antibodies and Flow Cytometry***

The following antibodies were used: APC- or PE-conjugated anti-CD56 (clone REA196; Miltenyi Biotec), PE-conjugated anti-CD3 (clone SK7; BD Biosciences), Brilliant violet 421-conjugated anti-IFNγ (clone 4S.B3; BioLegend), APC-labeled anti-TNFα (clone Mab11, BioLegend), Brilliant violet 650-conjugated anti-CD107a (clone H4A3; BD

Biosciences), SYTOX Blue dead cell stain (ThermoFisher), Fixable viability dye eFluor 780 (eBioscience). Flow cytometry assays were performed on a CytoFLEX S flow cytometer (Beckman Coulter) and all data were analyzed with FlowJo version 10.4 software (FlowJo LLC).

### *NK Cell Functional Assays*

For intracellular cytokine staining, NK cells were plated at 2.5 x $10^6$ cells/mL in NK cell medium without cytokines. After incubation overnight, the CD19+ Burkitt's Lymphoma cell line Raji was added at the indicated effector-to-target (E:T) ratios. Brilliant violet Anti-CD107a was added to the culture and cells were incubated for 1 hour at 37 C. Brefeldin A and monensin (BD Biosciences, San Jose, CA) were added and cells were incubated for an additional 4 hours. Cells were stained with fixable viability dye, then for extracellular antigens. Cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA) following manufacturer's instructions. Cells were then stained for intracellular IFNγ and TNFα and analyzed by flow cytometry within 1 hour.

### *Target Cell Killing Assays*

T cells or NK cells were cultured overnight in medium without cytokines. Luciferase-expressing Raji cells were seeded into a black round-bottom 96-well plate (3 x $10^4$ cells per well). T cells or NK cells were added to the wells in quadruplicate at the indicated E:T ratios. Target cells without effectors served as a negative control (spontaneous cell death) and target cells incubated with 1% NP-40 served as a positive control (maximum killing).

Co-cultures were incubated at 37C for 24 hours. After incubation, D-luciferin (potassium salt; Gold Biotechnology, St. Louis, MO) was added to each well at a final concentration of 25 ug/mL and incubated 10 minutes. Luminescence was read in endpoint mode using a BioTek Synergy microplate reader.

### *Statistical Analysis*

The Student's t-test was used to test for significant differences between two groups. Differences between 3 or more groups were tested by one-way ANOVA analysis followed by Tukey's *post-hoc* test. All assays were repeated in 3-5 independent donors. Mean values +/- standard error of the mean (SEM) are shown. The level of significance was set at $\alpha = 0.05$. Statistical analyses were performed using GraphPad Prism 8.0.

## Figures

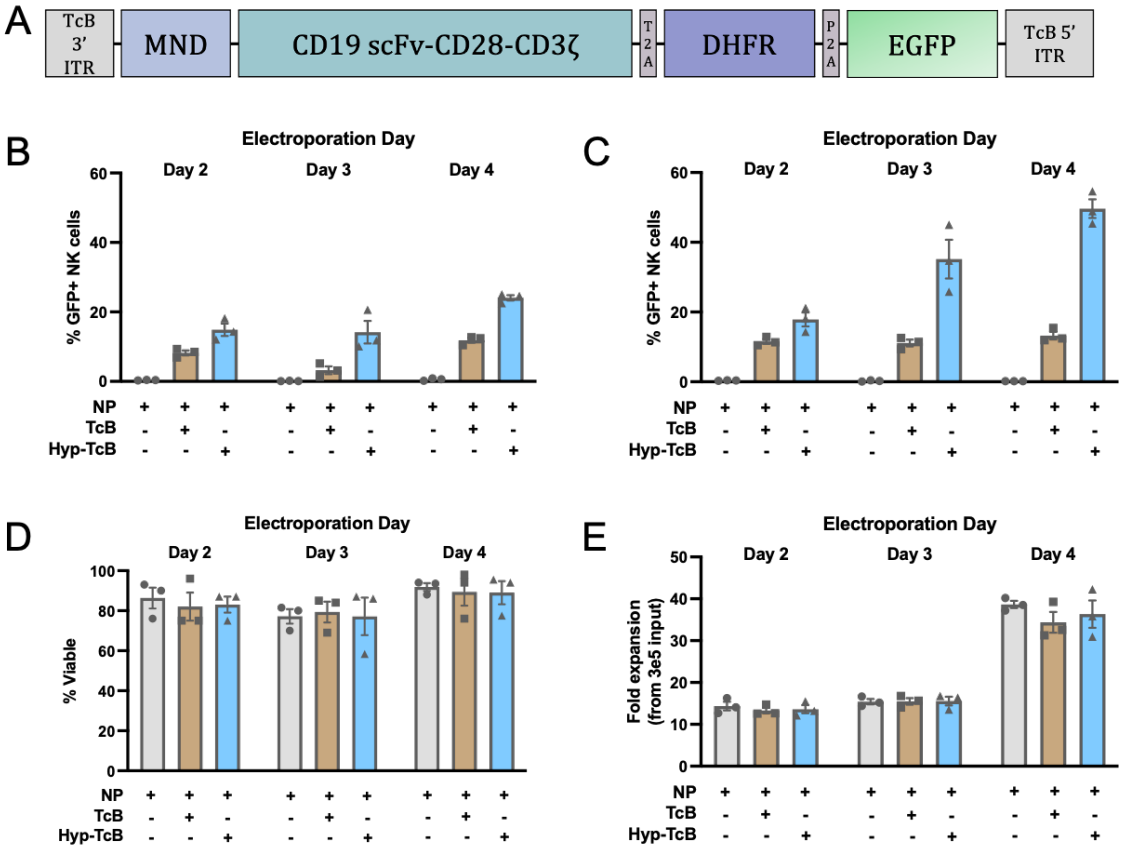



**Figure 3-1. Delivery of a CD19-CAR-DHFR-EGFP transposon to NK cells using the evolved hyperactive Tc Buster Transposon System. (A)** The 3.7 kb transposon flanked by Tc Buster ITRs, containing an MND promoter, second generation CD19 CAR, methotrexate resistant DHFR mutant, and enhanced GFP. Elements are separated by 2A self-cleaving peptides. This cargo was cloned into a Nanoplasmid (NP) backbone for delivery. **(B-E)** Primary human peripheral blood (PB) NK cells (n=3 human donors) were expanded for 2, 3, or 4 days with mbIL21- and 41BBL-expressing K562 feeder cells at a 2:1 (feeder:NK) ratio. NK cells were electroporated with the nanoplasmid transposon (NP) alone or in combination with mRNA encoding either Tc Buster (TcB) or the evolved hyperactive mutant Tc Buster (Hyp-TcB). Two days after electroporation, NK cells were expanded with feeder cells (5:1 feeder:NK ratio) for 1 week to allow for the loss of transient NP expression. After expansion, GFP expression was measured by flow cytometry, viability was measured by Trypan Blue exclusion, and fold expansion was calculated. **(B)** NK cells electroporated without pre-treatment with RNase inhibitor. **(C-E)** NK cells electroporated after 5-minute pre-treatment with RNase inhibitor.

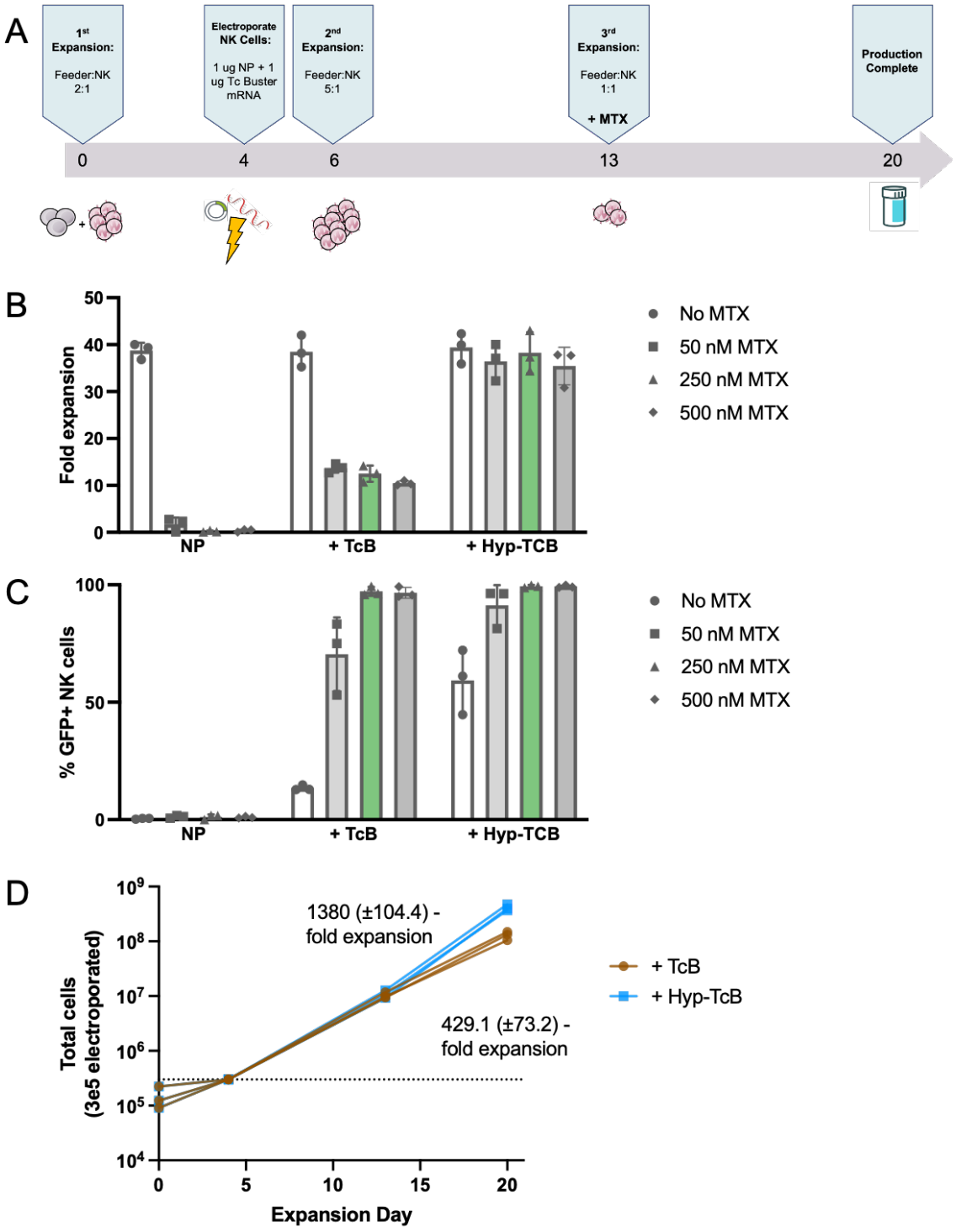



**Figure 3-2. Enrichment of engineered NK cells with methotrexate (MTX) selection.** **(A)** Timeline for production of CAR-NK cells: Primary human peripheral blood NK cells (n=3 human donors) are activated with mbIL21- and 41BBL-expressing K562 feeder cells

at a 2:1 (feeder:NK) ratio for 4 days. On day 4, NK cells are treated with RNase inhibitor and electroporated with transposition reagents. Two days after electroporation, NK cells are expanded for one week using a 5:1 (feeder:NK) ratio. NK cells are expanded again for one week at a 1:1 (feeder:NK) ratio in media containing methotrexate (MTX). After production, NK cells are cryopreserved. **(B)** NK cells were expanded a third time in media containing increasing doses of MTX and fold expansion was calculated from input into the third expansion. **(C)** GFP expression was measured by flow cytometry. **(D)** Total cells were tracked over the course of the full production timeline and fold expansion was calculated from electroporation input.

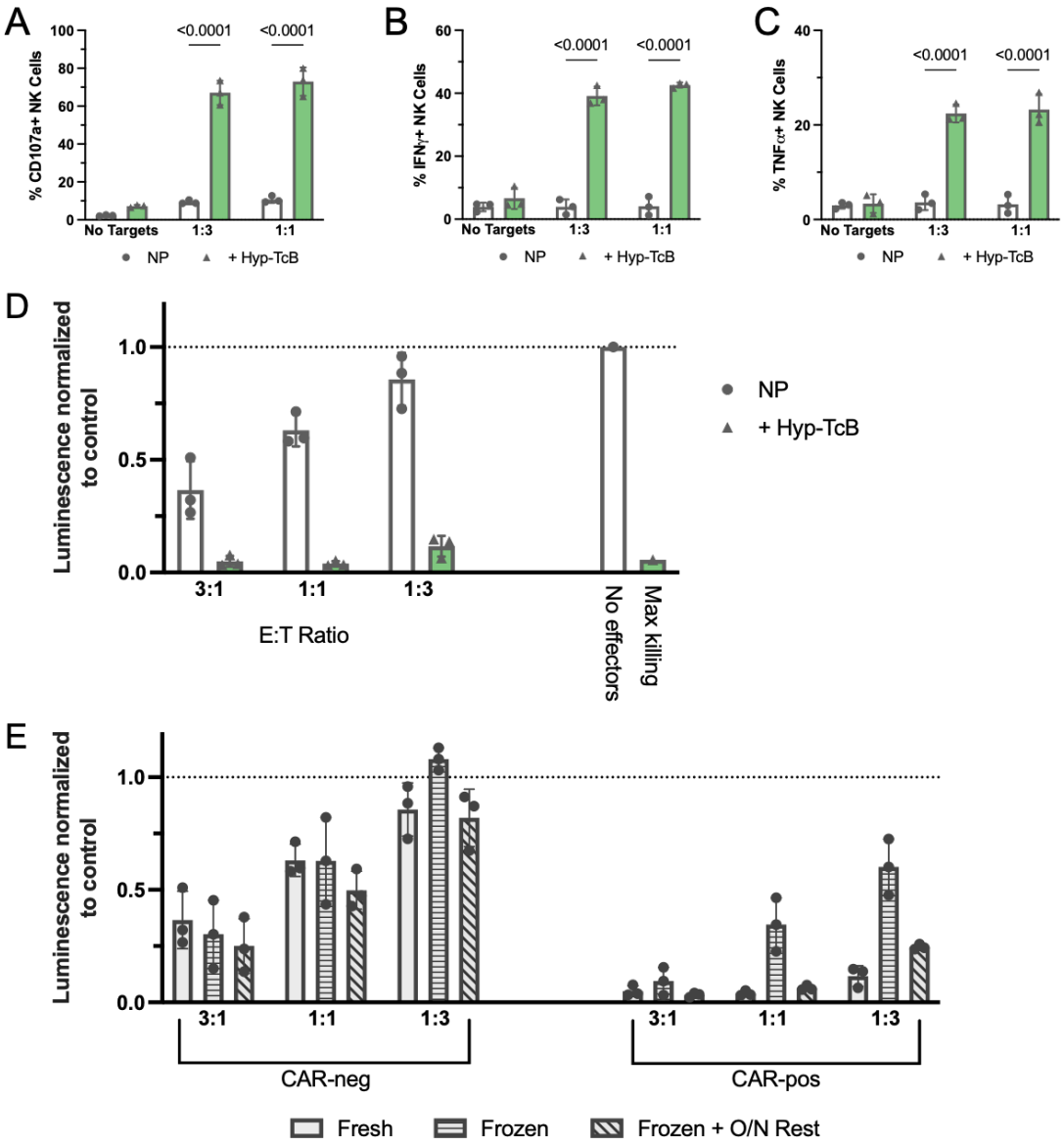



**Figure 3-3. CD19-CAR-expressing NK cells show enhanced activity and tumor cell killing.** CAR-positive cells were engineered with Hyp-TcB and selected with 250 nM MTX. CAR-negative control cells were electroporated with NP alone and were not selected. **(A-C)** CAR-positive and CAR-negative NK cells were co-cultured with CD19+ Raji cells at the indicated effector-to-target (E:T) ratios for 5 hours. Co-cultures were set up in triplicate. After co-culture, NK cells were analyzed by flow cytometry for the expression of CD107a **(A)**, IFNγ **(B)**, and TNFα **(C)**. **(D-E)** CAR-positive and CAR-negative NK cells were co-cultured with luciferase-expressing CD19+ Raji cells at the indicated E:T ratios for 24 hours. Co-cultures were set up in quadruplicate. After co-

culture, cells were incubated with d-luciferin for 10 minutes and luminescence was read on a plate reader. The assay was performed with fresh NK cells immediately after production and with cryopreserved NK cells immediately after thaw or after an overnight rest in media containing 100 IU/mL IL2.

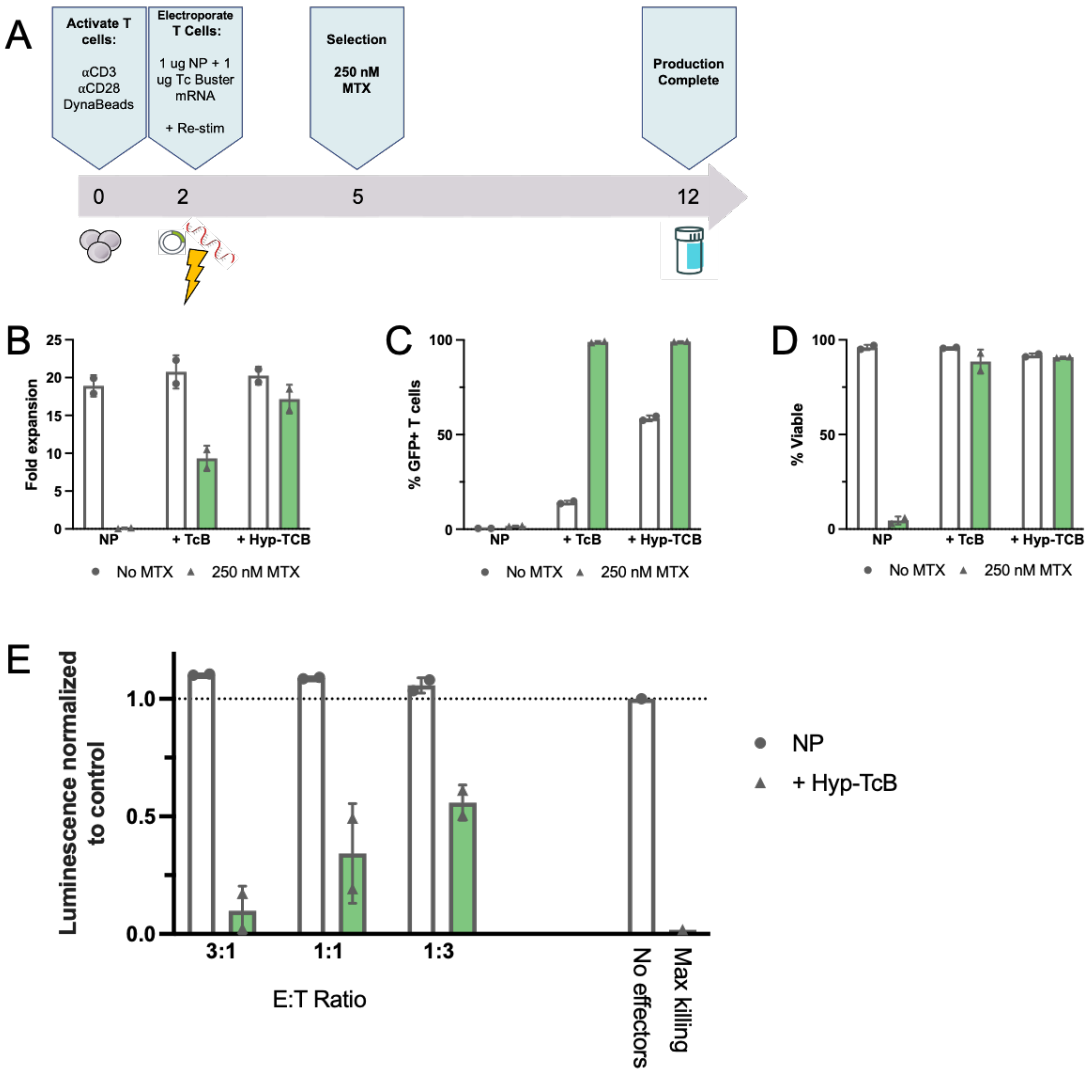
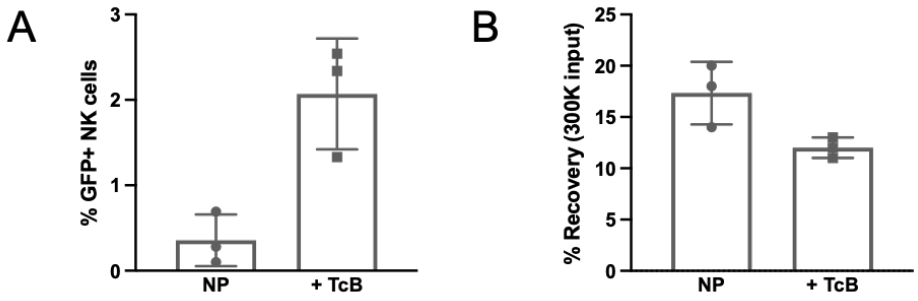
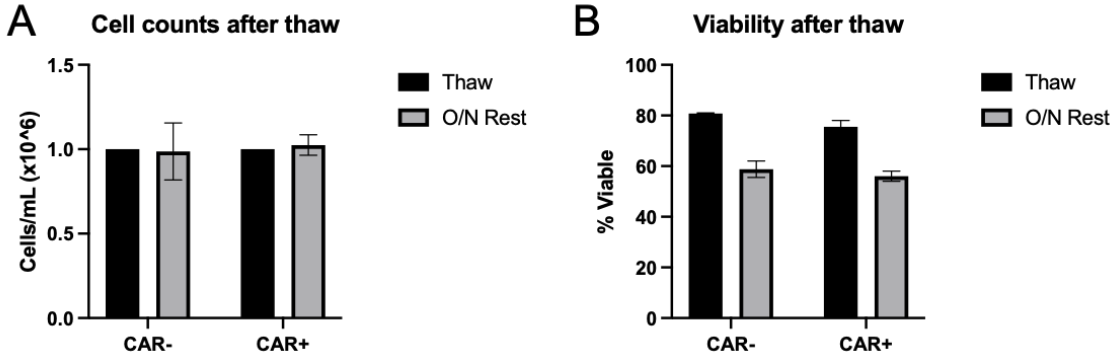



**Figure 4. Production of CD19-CAR T cells using Tc Buster transposition. (A)** Timeline for production of CAR-T cells: Primary human peripheral blood T cells are activated with αCD3/αCD28 DynaBeads for 2 days. On day 2, DynaBeads are removed, and T cells are electroporated with transposition reagents and then re-stimulated with DynaBeads after a 30-minute recovery in DNase I-containing media. 3 days after electroporation, methotrexate (MTX) is added to the media at a concentration of 250 nM. 7 days after MTX selection, production is complete and T cells are cryopreserved. **(B-D)** Production was performed with and without MTX selection. At the end of production, T cells were counted and fold expansion from electroporation input was calculated **(B)**, GFP expression was measured by flow cytometry **(C)**, and viability was measured by trypan blue exclusion **(D)**. CAR-positive cells were engineered with Hyp-TcB and selected with 250 nM MTX. CAR-negative control cells were electroporated with NP alone and were not selected. CAR-

positive and CAR-negative T cells were co-cultured with luciferase-expressing CD19+ Raji cells at the indicated E:T ratios for 24 hours. Co-cultures were set up in quadruplicate. Co-cultures were incubated with D-luciferin for 10 minutes and luminescence was read on a plate reader.




**Supplemental Figure 3-1.** Primary human peripheral blood (PB) NK cells were expanded for 7 days with mbIL21- and 41BBL-expressing K562 feeder cells at a 2:1 (feeder:NK) ratio. NK cells were electroporated with the nanoplasmid transposon (NP) alone or in combination with mRNA encoding Tc Buster (TcB). Two days after electroporation, NK cells were expanded with feeder cells (1:1 feeder:NK ratio) for 1 week to allow for the loss of transient NP expression. After this expansion, GFP expression was measured by flow cytometry and cells were counted to calculate recovery from electroporation input.




**Supplemental Figure 3-2.** CAR-positive and CAR-negative NK cells were cryopreserved after the 20-day production timeline. After thaw, cells were counted, and viability was measured by trypan blue exclusion. Cells were immediately plated in killing assays or plated at a density of $1x10^6$ cells/mL in media containing 100 IU/mL IL2 overnight. After overnight rest, cells were counted, and viability was measured again.

# CHAPTER 4: Conclusions and Future Directions

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that function to surveil the body and kill transformed cells. They are derived from the common lymphoid progenitor and reside in primary and secondary lymphoid tissues as well as lungs, liver, and peripheral blood[143]. They represent an attractive source for cancer immunotherapy because they can kill tumor cells with reduced or absent major histocompatibility complex (MHC) expression[144] and they induce a pro-inflammatory environment that primes other immune cells[145]. Beyond these functional attributes, they are readily available and easy to isolate from peripheral blood and can then be expanded *in vitro* to clinical relevant numbers using feeder cells engineered to express membrane-bound IL21 (mbIL21) and other co-stimulatory molecules such as 41BB-L[65].

These characteristics have driven interest in the use of NK cells for cancer therapy. This is underscored by the fact that endogenous NK cells in cancer usually have impaired function due to changes in receptor expression, including the downregulation of activating receptors and/or the upregulation of inhibitory receptors[146]. The first autologous NK cell transplant to treat cancer was initiated at the National Cancer Institute in 1980[147]. Since this pioneering work, NK cells have been used in many clinical trials as a treatment for a number of cancers. Despite these efforts, NK cell-based therapy has not enjoyed the success of T cell-based immunotherapies.

Changes in NK cell receptor repertoire and ligand expression in the tumor microenvironment (TME) lead to decreased NK cell activity. The same impairments

observed in endogenous cancer patient NK cells are often observed after exogenous NK cell transplant[9]. Tumor cells, stromal cells, and other cells in this environment can either secrete immunosuppressive molecules or express ligands to interfere with the complex array of receptors that regulate function of NK cells and their ability to expand *in vivo*[80]. This inhibition imposed on NK cells in the tumor microenvironment is likely the largest limiting factor in the success of NK immunotherapy; overcoming this immunosuppression is necessary for viable therapeutic development.

There are two general approaches for improving NK cell activity in the TME: 1) augmentation of activating signals and 2) blockade or removal of inhibitory signals. Several groups have worked toward the former, using chimeric antigen receptors (CARs) in NK cells or a modified antibody platform termed bi- or tri-specific killer engagers (BiKEs and TriKEs)[148]. Although traditional CARs with T cell-specific signaling components have activity in NK cells, our group has developed novel CAR constructs that contain signaling domains that mediate strong antigen-specific NK cell activity[39]. The activity of these CARs was demonstrated using iPSC-derived NK cells, but we have also generated preliminary data showing their activity after efficient delivery to peripheral-blood NK cells. Strategies for overcoming inhibition of NK cells in TME have focused on the use of pharmaceutical inhibitors and monoclonal antibodies against common immune suppressors termed immune checkpoints[83–85].

The focus of my work in the Moriarity Lab was to develop methods for engineering primary human NK cells, with the goal of creating a clinically scalable cellular product that could treat cancer. The ability to genetically engineer NK cells allows us to hardwire

changes that can both enhance activating signals and eliminate inhibitory signals. Future work will focus on identifying the optimal combinations of engineering steps to create living drugs for cancer patients.

In Chapter 2 of this thesis, I developed a method for editing the genome of primary human NK cells using CRISPR-Cas9. At the onset of this work, genetic modification of NK cells was challenging if not impossible – gene delivery was inefficient (<10%) and electroporation or nucleofection was toxic to the cells resulting in poor viability and recovery. Other methods for engineering (via viruses or lipofection) were also inefficient and NK cells were long considered intransigent to genetic modification. Previous work in T cells gave us a framework of what an engineering platform would look like: consisting of steps of cell activation, gene delivery, and recovery. We systematically tested conditions at each stage to identify an optimal protocol. NK cells can be activated by cytokines (namely IL2, IL12, IL15, IL18, and IL21), by antibodies that ligate activating receptors (namely NKp46 and CD2), or by culture with feeder cells engineered to express activating molecules, or a combination of these. The most robust method for NK cell activation uses feeder cells (K562) engineered to express membrane-bound IL21 and 41BB-L. NK cells are co-cultured with these feeder cells in the presence of one or more activating cytokines (usually IL15 or IL2). This is the method we used for NK cell activation prior to electroporation. The use of feeder cells to expand NK cells has since been approved and used clinically[6,149,150]. However, there is a desire in the field for a feeder-free method for NK cell activation. Ongoing work in our lab has focused on altering the NK engineering

protocol to use feeder-free expansion conditions, including the use of other cytokines and antibodies targeting activating receptors.

In our quest to develop the optimal engineering platform, we also tested multiple methods of nucleic acid delivery in NK cells, including viral transduction, electroporation, and nucleofection. In Chapter 2, we demonstrate that electroporation with the Neon Transfection System (ThermoFisher). We tested a range of voltages from volts to 2400 volts using this machine and found the optimized condition of 1850 volts, 2 pulses, at a pulse width of 10 ms. Our lab has since used the Neon Transfection System to engineer T cells for clinical use.

We also tested recovery conditions after gene delivery – including multiple media formulations and timing of re-activation. We have since modified this further, allowing engineered NK cells to recover in low-dose (100 IU/mL) IL2 for 48 hours before re-expanding them with a high feeder:NK ratio (5:1). Thus, we have methodically optimized all steps in the process to develop platform NK cell genetic modification.

Using this protocol, I wanted to modify NK cells to enhance their anti-tumor activity. I targeted *ADAM17* and *PDCD1* – genes that were predicted to have a functional impact on NK cells – for knockout with CRISPR-Cas9. Delivery of Cas9 mRNA in combination with guide RNA targeting these genes led to insertions and deletions at the target site and subsequent protein loss. Importantly, these changes were stable through repeated expansions and did not affect the NK cells' ability to expand, demonstrating the clinical scalability of this method (Figure 2-1).

I further modified the engineering protocol for targeted integration of a transgene. After delivery of Cas9 mRNA and a guide RNA targeting the safe-harbor site AAVS1, I transduced NK cells with recombinant adeno-associated virus serotype 6 (rAAV6) containing homology arms to the locus flanking a gene of interest. This allows for the delivery of activating signals such as non-cleavable CD16a (shown in Chapter 2) or chimeric antigen receptors.

To show a functional consequence of targeting ADAM17, I tested their ability to perform antibody-dependent cellular cytotoxicity (ADCC). This method of target cell killing is carried out through the CD16a receptor expressed by the majority of NK cells. CD16a recognizes the Fc-region of IgG antibodies bound to target cells. Ligation of CD16a leads to activation of the NK cell and subsequent degranulation and target cell killing. CD16a is cleaved by the metalloprotease ADAM17. In Chapter 2 (Figure 2-2), I demonstrated that ADAM17 knockout leads to retention of CD16a on the NK cell surface and enhances NK cell activity killing of antibody-bound target cells. Similar results were observed when rAAV6 was used to deliver non-cleavable CD16a to the AAVS1 safe-harbor locus. This *in vitro* data is promising and warrants further study of targeting this interaction, namely in other models of ADCC and *in vivo*.

To test the effect of targeting PDCD1 for knockout, I used a panel of cancer cell lines (K562, THP1, DU145, and MA148) to test activity *in vitro*. PD1-knockout NK cells displayed enhanced degranulation, cytokine production, and target cell killing. Testing against PD-L1 deficient cell lines suggested that this was indeed due to the PD1:PD-L1 interaction (Figure 2-3).

I further tested PD1 knockout NK cells *in vivo* in a model of ovarian cancer using luciferase expressing MA148 cells. Treatment of tumor-bearing mice with PD1 knockout NK cells led to modest increase in survival and control of tumor burden, although the mice eventually succumbed to their tumors (Figure 2-4).

During my time in the Moriarity Lab, the role of PD1 in NK cells has become a subject of intense debate in the field. One group demonstrated that NK cells lack PD1 expression under multiple conditions[151], while other groups insist it is expressed and important for regulating NK cell function[152]. Indeed, our group had challenges detecting PD1 on NK cells despite convincing functional evidence of its importance. We recently observed PD1 expression on NK cells by flow cytometry after antigen retrieval using mild citric acid treatment[153]. Still, there is much to be learned about how, when, and why PD1 is expressed on NK cells and the signaling pathways downstream of PD1 that lead to their inhibition.

A key learning from this work is that despite promising *in vitro* data with PD1 knockout NK cells, they were not as effective *in vivo*. This suggests an adaptation by tumor cells in response to NK cells pressure. We suspect that multiplexed NK cell editing, targeting multiple inhibitory genes for knockout in combination with delivery of activating signals such as CARs may be able to overcome this arm's race between NK cell and tumor. As such, we are currently using base editors to knockout out multiple genes in NK cells by targeting early exon splice sites, as has been demonstrated by our lab in other cell types[62,154].

In light of the need to efficiently deliver activating signals to NK cells, I developed a platform for the manufacture of CAR-NK and CAR-T cells in Chapter 3 of this thesis. Although this has been pursued using viral methods, there is a push in the field for non-viral engineering methods given the resistance of NK cells to viral transduction. As such, I pursued engineering via transposition using the Tc Buster Transposon System, including a hyperactive mutant evolved by Bio-Techne (Minneapolis, MN).

The transposon construct I used for proof-of-principle experiments contained a second generation CD19-CAR, methotrexate resistant mutant dihydrofolate reductase (DHFR), and enhanced GFP (EGFP) (Figure 3-1). Typical plasmids contain 1.5-2 kb of unnecessary bacterial regions that can lead to DNA toxicity in sensitive cell types like primary NK and T cells, and can also trigger plasmid silencing leading to reduced efficiency[126,141]. Several vectors, including minicircles, have been developed in which the bacterial regions can be trimmed after plasmid purification. In Chapter 1, I showed Sleeping Beauty transposition in NK cells using a minicircle to deliver an EGFP transposon (Figure 1-1). However, the trimming of these vectors after purification is a complex and laborious process. More recently, nanoplasmids have been developed that do not require this type of processing and instead contain a small (<500 bp) backbone that doesn't lead to the same DNA toxicity and plasmid silencing seen with traditional plasmid vectors. Importantly, since there is no post-purification processing, these vectors can be readily produced in our lab. Thus, I chose to use a nanoplasmid to deliver the transposon in combination with mRNA encoding the Tc Buster transposase.

I first wanted to optimize electroporation methods to achieve efficient transposition while maintaining high cell viability and recovery. In my previous CRISPR-Cas9 work using all mRNA, I found that activating NK cells for 7 days with mbIL21- and 41BBL-expressing feeder cells before electroporation led to efficient gene knockout. However, this approach was not successful for delivering DNA (Supplemental Figure 3-1). I tested delivery on earlier timepoint in activation and found day 4 to result in the best transposition efficiency, viability, and fold-expansion (Figure 3-1). In addition, pre-treatment of NK cells with RNase inhibitor prior to electroporation enhanced efficiency. This is likely due to secretion of RNases by NK cells that degrade the transposase mRNA, however, this needs to be systematically validated. Future studies should include assessing NK cells in resting states and during activation for expression of RNases.

I next wanted to enrich for CAR-expressing cells using methotrexate. Methotrexate (MTX) is an antifolate that inhibits the enzyme dihydrofolate reductase (DHFR), which catalyzes the production of tetrahydrofolate, a necessary factor for cell proliferation[155,156]. MTX is used clinically to treat a broad range of cancer types[157]. The mutant DHFR (L22F, F31S) in our transposon construct is resistant to MTX inhibition. Thus, cells that did not integrate the transposon vector will die in the presence of methotrexate while cells that have transposon integration will be enriched. I tested several doses of MTX in a third expansion and found 250 nM MTX to be the minimum effective dose needed to select for engineered cells (Figure 3-2). Thus, with the optimized production protocol detailed in Figure 3-2, I was able to expand nearly pure (>99%) CAR+ NK cells 1380-fold from electroporation input using Hyp-TcB.

I next validated the function of the CD19-CAR in comprehensive *in vitro* assays against CD19+ Raji cells (Figure 3-3) and applied the production protocol to T cells (Figure 3-4). Importantly, I tested the feasibility of cryopreservation of the engineered product. For the CAR-NK cells, I found decreased cytotoxicity after thaw, but was able to rescue this by increasing the dose of NK cells or by allowing for overnight rest in media containing IL2 (Figure 3-2). This suggests that simply giving a higher dose of NK cells to the patient may be a practical approach for clinical use, since NK cells are well tolerated by patients and large numbers of cells are easy to produce. This needs to be validated in preclinical *in vivo* models.

An important next step in this work is testing these CAR-NK and CAR-T cells *in vivo*. I am particularly interested in the delivery of a cellular therapy that contains both CAR-T and CAR-NK cells. CAR-T cells are the gold standard for cancer immunotherapy, but they can lead to severe toxicities including cytokine release syndrome, neurotoxicity, and graft-versus-host disease (GvHD)[158]. In addition, their effectiveness can be hindered by inhibition in the tumor microenvironment (TME) of solid tumors[159]. I envision giving a lower dose of CAR-T cells in combination with CAR-NK cells, which can produce inflammatory cytokines to help activate CAR-T cells and alter the inflammation state of the TME[2].

Another future direction of this work includes modifications to the transposon construct. We are currently working to deliver other CARs, specifically to target solid tumor antigens like mesothelin, HER2, B7-H3, and others. In addition, we are identifying other activating signaling molecules to include in the construct, such as secreted IL15 and

IL15 receptor alpha (IL15RA). The large cargo capacity for transposons means we can add multiple signaling molecules and even dual CARs (such as CD19 and CD20) to avoid tumor antigen escape which has been an issue in CAR-T cell therapy[160].

Finally, I would like to combine the methods developed in Chapter 2 and Chapter 3, delivering Tc Buster transposons for integration in combination with CRISPR-Cas9 or base editors for targeted gene knockout in a single electroporation event. This non-viral approach for multiplex editing of NK and T cells would be a versatile, safe, and cost-effective option for the manufacture of cellular therapies for cancer.

# Bibliography


1. Gauthier, J. & Yakoub-Agha, I. Chimeric antigen-receptor T-cell therapy for hematological malignancies and solid tumors: Clinical data to date, current limitations and perspectives. *Curr Res Transl Med* (2017) doi:10.1016/j.retram.2017.08.003.
2. Fang, F., Xiao, W. & Tian, Z. NK cell-based immunotherapy for cancer. *Semin Immunol* **31**, 37–54 (2017).
3. Freund-Brown, J., Chirino, L. & Kambayashi, T. Strategies to enhance NK cell function for the treatment of tumors and infections. *Crit Rev Immunol* **38**, 105–130 (2018).
4. Carlsten, M. & Childs, R. W. Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications. *Front Immunol* **6**, (2015).
5. Naeimi Kararoudi, M. *et al*. Genetic and epigenetic modification of human primary NK cells for enhanced antitumor activity. *Semin Hematol* **57**, 201–212 (2020).
6. Liu, E. *et al*. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. *New England Journal of Medicine* **382**, 545–553 (2020).
7. Buchholz, C. J., Friedel, T. & Büning, H. Surface-Engineered Viral Vectors for Selective and Cell Type-Specific Gene Delivery. *Trends Biotechnol* **33**, 777–790 (2015).
8. Sutlu, T. *et al*. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. *Hum Gene Ther* **23**, 1090–1100 (2012).

9. Hu, W., Wang, G., Huang, D., Sui, M. & Xu, Y. Cancer Immunotherapy Based on Natural Killer Cells: Current Progress and New Opportunities. *Front Immunol* **10**, 1205 (2019).

10. Lanier, L. L. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* **8**, 259–268 (2008).

11. Oberschmidt, O. *et al*. Development of Automated Separation, Expansion, and Quality Control Protocols for Clinical-Scale Manufacturing of Primary Human NK Cells and Alpharetroviral Chimeric Antigen Receptor Engineering. *Hum Gene Ther Methods* **30**, 102–120 (2019).

12. Ingegnere, T. *et al*. Human CAR NK Cells: A New Non-viral Method Allowing High Efficient Transfection and Strong Tumor Cell Killing. *Front Immunol* **10**, 957 (2019).

13. Deipolyi, A. R., Golberg, A., Yarmush, M. L., Arellano, R. S. & Oklu, R. Irreversible electroporation: evolution of a laboratory technique in interventional oncology. *Diagn Interv Radiol* **20**, 147–154 (2014).

14. Clark, P. R., Stopeck, A. T., Parker, S. E. & Hersh, E. M. Cationic lipid gene transfer of an IL-2 transgene leads to activation of natural killer cells in a SCID mouse human tumor xenograft. *Cell Immunol* **204**, 96–104 (2000).

15. Gong, J. H., Maki, G. & Klingemann, H. G. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* **8**, 652–658 (1994).

16. Zhang, J., Sun, R., Wei, H., Zhang, J. & Tian, Z. Characterization of stem cell factor gene-modified human natural killer cell line, NK-92 cells: implication in NK cell-based adoptive cellular immunotherapy. *Oncol Rep* **11**, 1097–1106 (2004).

17. Kruschinski, A. *et al*. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci U S A* **105**, 17481–17486 (2008).

18. Youness, R. A. *et al*. Contradicting interplay between insulin-like growth factor-1 and miR-486-5p in primary NK cells and hepatoma cell lines with a contemporary inhibitory impact on HCC tumor progression. *Growth Factors* **34**, 128–140 (2016).

19. Regis, S. *et al*. TGF-β1 Downregulates the Expression of CX3CR1 by Inducing miR-27a-5p in Primary Human NK Cells. *Front Immunol* **8**, 868 (2017).

20. Wilk, A. J. *et al*. Charge-altering releasable transporters enable phenotypic manipulation of natural killer cells for cancer immunotherapy. *Blood Adv* **4**, 4244–4255 (2020).

21. Pomeroy, E. J. *et al*. A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy. *Molecular Therapy* (2019).

22. Schoenberg, K., Trompeter, H.-I. & Uhrberg, M. Delivery of DNA into natural killer cells for immunotherapy. *Methods Mol Biol* **423**, 165–172 (2008).

23. Carlsten, M. *et al*. Efficient mRNA-Based Genetic Engineering of Human NK Cells with High-Affinity CD16 and CCR7 Augments Rituximab-Induced ADCC against Lymphoma and Targets NK Cell Migration toward the Lymph Node-Associated Chemokine CCL19. *Front Immunol* **7**, 105 (2016).

24. Boissel, L. *et al*. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. *Leuk Lymphoma* **53**, 958–965 (2012).

25. Li, L. *et al*. Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Therapy* **17**, 147–154 (2010).

26. Muñoz-López, M. & García-Pérez, J. L. DNA transposons: nature and applications in genomics. *Curr Genomics* **11**, 115–128 (2010).

27. Tipanee, J., VandenDriessche, T. & Chuah, M. K. Transposons: Moving Forward from Preclinical Studies to Clinical Trials. *Hum Gene Ther* **28**, 1087–1104 (2017).

28. Kumar, A. Jump around: transposons in and out of the laboratory. *F1000Res* **9**, (2020).

29. Tipanee, J., Chai, Y. C., VandenDriessche, T. & Chuah, M. K. Preclinical and clinical advances in transposon-based gene therapy. *Biosci Rep* **37**, (2017).

30. Ivics, Z., Hackett, P. B., Plasterk, R. H. & Izsvák, Z. Molecular Reconstruction of Sleeping Beauty, a Tc1-like Transposon from Fish, and Its Transposition in Human Cells. *Cell* **91**, 501–510 (1997).

31. Kebriaei, P., Izsvák, Z., Narayanavari, S. A., Singh, H. & Ivics, Z. Gene Therapy with the Sleeping Beauty Transposon System. *Trends Genet* **33**, 852–870 (2017).

32. Mátés, L. *et al*. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nat Genet* **41**, 753–761 (2009).

33. Cadiñanos, J. & Bradley, A. Generation of an inducible and optimized piggyBac transposon system. *Nucleic Acids Res* **35**, e87 (2007).

34. Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A* **108**, 1531–1536 (2011).

35. Arensburger, P. *et al*. Phylogenetic and functional characterization of the hAT transposon superfamily. *Genetics* **188**, 45–57 (2011).

36. Li, X. *et al*. A resurrected mammalian hAT transposable element and a closely related insect element are highly active in human cell culture. *Proc Natl Acad Sci U S A* **110**, E478-487 (2013).

37. Woodard, L. E. *et al*. Comparative analysis of the recently discovered hAT transposon TcBuster in human cells. *PLoS One* **7**, e42666 (2012).

38. Suck, G. *et al*. NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. *Cancer Immunol Immunother* **65**, 485–492 (2016).

39. Li, Y., Hermanson, D. L., Moriarity, B. S. & Kaufman, D. S. Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. *Cell Stem Cell* **23**, 181-192.e5 (2018).

40. Wang, J., Lupo, K. B., Chambers, A. M. & Matosevic, S. Purinergic targeting enhances immunotherapy of CD73 + solid tumors with piggyBac-engineered chimeric antigen receptor natural killer cells. *Journal for ImmunoTherapy of Cancer* **6**, 136 (2018).

41. Knorr, D. A. *et al*. Clinical-scale derivation of natural killer cells from human pluripotent stem cells for cancer therapy. *Stem Cells Transl Med* **2**, 274–283 (2013).

42. Ptáčková, P. *et al*. A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21. *Cytotherapy* **20**, 507–520 (2018).

43. Rotiroti, M. C. *et al*. Targeting CD33 in Chemoresistant AML Patient-Derived Xenografts by CAR-CIK Cells Modified with an Improved SB Transposon System. *Mol Ther* **28**, 1974–1986 (2020).

44. Hu, B. *et al*. Nucleofection with Plasmid DNA for CRISPR/Cas9-Mediated Inactivation of Programmed Cell Death Protein 1 in CD133-Specific CAR T Cells. *Hum Gene Ther* **30**, 446–458 (2019).

45. Li, H. *et al*. Antitumor activity of EGFR-specific CAR T cells against non-small-cell lung cancer cells in vitro and in mice. *Cell Death Dis* **9**, 177 (2018).

46. Xu, J.-Y. *et al*. Mesothelin-targeting chimeric antigen receptor-modified T cells by piggyBac transposon system suppress the growth of bile duct carcinoma. *Tumour Biol* **39**, 1010428317695949 (2017).

47. Wang, P. *et al*. PiggyBac-engineered T cells expressing a glypican-3-specific chimeric antigen receptor show potent activities against hepatocellular carcinoma. *Immunobiology* **225**, 151850 (2020).

48. Magnani, C. F. *et al*. Sleeping Beauty-engineered CAR T cells achieve antileukemic activity without severe toxicities. *J Clin Invest* **130**, 6021–6033 (2020).

49. Kebriaei, P. *et al*. Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. *J Clin Invest* **126**, 3363–3376 (2016).

50. Wuerzburg University Hospital. *A Phase I/IIa Clinical Trial to Assess Feasibility, Safety and Antitumor Activity of Autologous SLAMF7 CAR-T Cells in Multiple Myeloma*. https://clinicaltrials.gov/ct2/show/NCT04499339 (2020).

51. Thomas, C. E., Ehrhardt, A. & Kay, M. A. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* **4**, 346–358 (2003).

52. Woodard, L. E. & Wilson, M. H. piggyBac-ing models and new therapeutic strategies. *Trends Biotechnol* **33**, 525–533 (2015).

53. Wu, X., Luke, B. T. & Burgess, S. M. Redefining the common insertion site. *Virology* **344**, 292–295 (2006).

54. Naeimi Kararoudi, M. *et al*. Generation of Knock-out Primary and Expanded Human NK Cells Using Cas9 Ribonucleoproteins. *J Vis Exp* (2018) doi:10.3791/58237.

55. Liang, X. *et al*. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* **208**, 44–53 (2015).

56. Rautela, J., Surgenor, E. & Huntington, N. D. Efficient genome editing of human natural killer cells by CRISPR RNP. *bioRxiv* 406934 (2018) doi:10.1101/406934.

57. Nguyen, D. N. *et al*. Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. *Nat Biotechnol* **38**, 44–49 (2020).

58. Anzalone, A. V., Koblan, L. W. & Liu, D. R. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol* **38**, 824–844 (2020).

59. Komor, A. C., Badran, A. H. & Liu, D. R. CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes. *Cell* **169**, 559 (2017).

60. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424 (2016).

61. Gaudelli, N. M. *et al*. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 (2017).

62. Webber, B. R. *et al*. Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nat Commun* **10**, 5222 (2019).

63. Shi, X. *et al*. Targeting glycosylation of PD-1 to enhance CAR-T cell cytotoxicity. *J Hematol Oncol* **12**, 127 (2019).

64. Anzalone, A. V. *et al*. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 (2019).

65. Denman, C. J. *et al*. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS ONE* **7**, e30264 (2012).

66. Murphy, W. J., Parham, P. & Miller, J. S. NK Cells—From Bench to Clinic. *Biology of Blood and Marrow Transplantation* **18**, S2–S7 (2012).

67. Davis, Z. B., Felices, M., Verneris, M. R. & Miller, J. S. Natural Killer Cell Adoptive Transfer Therapy: Exploiting the First Line of Defense Against Cancer. *The Cancer Journal* **21**, 486–491 (2015).

68. Davis, Z. B., Vallera, D. A., Miller, J. S. & Felices, M. Natural killer cells unleashed: Checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Seminars in Immunology* **31**, 64–75 (2017).

69. Podgorny, P. J. *et al*. Immune Cell Subset Counts Associated with Graft-versus-Host Disease. *Biology of Blood and Marrow Transplantation* **20**, 450–462 (2014).

70. Knorr, D. A., Bachanova, V., Verneris, M. R. & Miller, J. S. Clinical utility of natural killer cells in cancer therapy and transplantation. *Seminars in Immunology* **26**, 161–172 (2014).

71. Schmiedel, D. *et al*. The RNA binding protein IMP3 facilitates tumor immune escape by downregulating the stress-induced ligands ULPB2 and MICB. *eLife* https://elifesciences.org/articles/13426 (2016) doi:10.7554/eLife.13426.

72. Hofer, E. & Koehl, U. Natural Killer Cell-Based Cancer Immunotherapies: From Immune Evasion to Promising Targeted Cellular Therapies. *Front Immunol* **8**, (2017).

73. Chester, C., Fritsch, K. & Kohrt, H. E. Natural Killer Cell Immunomodulation: Targeting Activating, Inhibitory, and Co-stimulatory Receptor Signaling for Cancer Immunotherapy. *Front Immunol* **6**, (2015).

74. Keating, G. M. Rituximab. *Drugs* **70**, 1445–1476 (2010).

75. Ahlgrimm, M., Pfreundschuh, M., Kruez, M., Regitz, E., Pruess, K.D., & Bittenbring, J. The impact of Fc-γ receptor polymorphisms in elderly patients with diffuse large B-cell lymphoma treated with CHOP with or without rituximab. *Blood* **118(17)**, 4657-62 (2011).

76. Wu, J., Mishra, H. K. & Walcheck, B. Role of ADAM17 as a regulatory checkpoint of CD16A in NK cells and as a potential target for cancer immunotherapy. *Journal of Leukocyte Biology* **105(6)**, 1297-1303 (2019).

77. Mishra, H. K., Pore, N., Michelotti, E. F. & Walcheck, B. Anti-ADAM17 monoclonal antibody MEDI3622 increases IFNγ production by human NK cells in the presence of antibody-bound tumor cells. *Cancer Immunol Immunother* **67**, 1407–1416 (2018).

78. Romee, R. *et al*. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood* **121**, 3599–3608 (2013).

79. Vitale, M., Cantoni, C., Pietra, G., Mingari, M. C. & Moretta, L. Effect of tumor cells and tumor microenvironment on NK-cell function. *European Journal of Immunology* **44**, 1582–1592 (2014).

80. Effect of tumor cells and tumor microenvironment on NK-cell function - Vitale - 2014 - European Journal of Immunology - Wiley Online Library. http://onlinelibrary.wiley.com/doi/10.1002/eji.201344272/abstract;jsessionid=35BDC66F92538B0CA1758AFC15CDC67F.f04t03.

81. Guo, Y. *et al*. PD1 blockade enhances cytotoxicity of in vitro expanded natural killer cells towards myeloma cells. *Oncotarget* **7**, 48360–48374 (2016).

82. Ray, A. *et al*. Targeting PD1–PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* **29**, 1441 (2015).

83. Vey, N. *et al*. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood* **120**, 4317–4323 (2012).

84. Chan, C. J. *et al*. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nature Immunology* **15**, ni.2850 (2014).

85. Lesokhin, A. M., Callahan, M. K., Postow, M. A. & Wolchok, J. D. On being less tolerant: Enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation. *Science Translational Medicine* **7**, 280sr1-280sr1 (2015).

86. Osborn, M. J. *et al*. Evaluation of TCR Gene Editing Achieved by TALENs, CRISPR/Cas9, and megaTAL Nucleases. *Molecular Therapy* **24**, 570–581 (2016).

87. Johnson, M. J., Laoharawee, K., Lahr, W. S., Webber, B. R. & Moriarity, B. S. Engineering of Primary Human B cells with CRISPR/Cas9 Targeted Nuclease. *Scientific Reports* **8**, 12144 (2018).

88. Shah, N. *et al*. Phase I study of cord blood-derived natural killer cells combined with autologous stem cell transplantation in multiple myeloma. *Br J Haematol* **177**, 457–466 (2017).

89. Hendel, A. *et al*. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nature Biotechnology* **33**, nbt.3290 (2015).

90. Jensen, K. T. *et al*. Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. *FEBS Letters* **591**, 1892–1901 (2017).

91. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* **42**, e168–e168 (2014).

92. Sentmanat, M. F., Peters, S. T., Florian, C. P., Connelly, J. P. & Pruett-Miller, S. M. A Survey of Validation Strategies for CRISPR-Cas9 Editing. *Sci Rep* **8**, 1–8 (2018).

93. Veluchamy, J. P. *et al*. The Rise of Allogeneic Natural Killer Cells As a Platform for Cancer Immunotherapy: Recent Innovations and Future Developments. *Front Immunol* **8**, (2017).

94. Lapteva, N., Szmania, S. M., van Rhee, F. & Rooney, C. M. Clinical Grade Purification and Expansion of Natural Killer Cells. *Crit Rev Oncog* **19**, 121–132 (2014).

95. Bak, R. O. & Porteus, M. H. CRISPR-mediated Integration of Large Gene Cassettes using AAV Donor Vectors. *Cell Rep* **20**, 750–756 (2017).

96. Bak, R. O., Dever, D. P. & Porteus, M. H. CRISPR/Cas9 genome editing in human hematopoietic stem cells. *Nat Protoc* **13**, 358–376 (2018).

97. Jing, Y. *et al*. Identification of an ADAM17 Cleavage Region in Human CD16 (FcγRIII) and the Engineering of a Non-Cleavable Version of the Receptor in NK Cells. *PLOS ONE* **10**, e0121788 (2015).

98. Li, Y., Brazzell, J., Herrera, A. & Walcheck, B. ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. *Blood* **108**, 2275–2279 (2006).

99. Hsu, J. *et al*. Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. *J Clin Invest* **128**, 4654–4668 (2018).

100. Mazzaschi, G. *et al*. The circulating pool of functionally competent NK and CD8+ cells predicts the outcome of anti-PD1 treatment in advanced NSCLC. *Lung Cancer* **127**, 153–163 (2019).

101. Zotto, G. D. *et al*. Markers and function of human NK cells in normal and pathological conditions. *Cytometry Part B: Clinical Cytometry* **92**, 100–114 (2017).

102. Felices, M. *et al*. IL-15 super-agonist (ALT-803) enhances natural killer (NK) cell function against ovarian cancer. *Gynecologic Oncology* **145**, 453–461 (2017).

103. Pierson, B. A., McGLAVE, P. B., Hu, W.-S. & Miller, J. S. Natural Killer Cell Proliferation Is Dependent on Human Serum and Markedly Increased Utilizing an Enriched Supplemented Basal Medium. *Journal of Hematotherapy* **4**, 149–158 (1995).

104. Wang, X. *et al*. CRISPR-DAV: CRISPR NGS data analysis and visualization pipeline. *Bioinformatics* **33**, 3811–3812 (2017).

105. Yokoyama, Y., Dhanabal, M., Griffioen, A. W., Sukhatme, V. P. & Ramakrishnan, S. Synergy between Angiostatin and Endostatin: Inhibition of Ovarian Cancer Growth. *Cancer Res* **60**, 2190–2196 (2000).

106. Romee, R. *et al*. Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751–4760 (2012).

107. Wilson, M. H., Coates, C. J. & George, A. L. PiggyBac Transposon-mediated Gene Transfer in Human Cells. *Molecular Therapy* **15**, 139–145 (2007).

108. Ni, J. *et al*. Active recombinant Tol2 transposase for gene transfer and gene discovery applications. *Mob DNA* **7**, (2016).

109. Tsukahara, T. *et al*. The *Tol2* transposon system mediates the genetic engineering of T-cells with CD19-specific chimeric antigen receptors for B-cell malignancies. *Gene Therapy* **22**, 209–215 (2015).

110. Staal, F. J. T., Aiuti, A. & Cavazzana, M. Autologous Stem-Cell-Based Gene Therapy for Inherited Disorders: State of the Art and Perspectives. *Front Pediatr* **7**, 443 (2019).

111. Staal, F. J. T., Pike-Overzet, K., Ng, Y. Y. & van Dongen, J. J. M. Sola dosis facit venenum. Leukemia in gene therapy trials: a question of vectors, inserts and dosage? *Leukemia* **22**, 1849–1852 (2008).

112. Hacein-Bey-Abina, S. *et al*. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**, 3132–3142 (2008).

113. Hacein-Bey-Abina, S. *et al*. LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science* **302**, 415–419 (2003).

114. Cavazzana-Calvo, M. *et al*. Transfusion independence and *HMGA2* activation after gene therapy of human β-thalassaemia. *Nature* **467**, 318–322 (2010).

115. Gogol-Döring, A. *et al*. Genome-wide Profiling Reveals Remarkable Parallels Between Insertion Site Selection Properties of the MLV Retrovirus and the piggyBac Transposon in Primary Human CD4(+) T Cells. *Mol Ther* **24**, 592–606 (2016).

116. Singh, H. *et al*. Redirecting Specificity of T-Cell Populations For CD19 Using the Sleeping Beauty System. *Cancer Res* **68**, 2961–2971 (2008).

117. Jin, Z. *et al*. The hyperactive *Sleeping Beauty* transposase SB100X improves the genetic modification of T cells to express a chimeric antigen receptor. *Gene Therapy* **18**, 849–856 (2011).

118. Srour, S. A. *et al*. Long-term outcomes of Sleeping Beauty-generated CD19-specific CAR T-cell therapy for relapsed-refractory B-cell lymphomas. *Blood* **135**, 862–865 (2020).

119. Crossland, D. L. *et al*. Antitumor activity of CD56-chimeric antigen receptor T cells in neuroblastoma and SCLC models. *Oncogene* **37**, 3686 (2018).

120. Chicaybam, L. *et al*. CAR T Cells Generated Using Sleeping Beauty Transposon Vectors and Expanded with an EBV-Transformed Lymphoblastoid Cell Line Display Antitumor Activity In Vitro and In Vivo. *Human Gene Therapy* **30**, 511–522 (2019).

121. Bishop, D. C. *et al*. PiggyBac-Engineered T Cells Expressing CD19-Specific CARs that Lack IgG1 Fc Spacers Have Potent Activity against B-ALL Xenografts. *Molecular Therapy* **26**, 1883–1895 (2018).

122. He, J. *et al*. Engineered CAR T cells targeting mesothelin by piggyBac transposon system for the treatment of pancreatic cancer. *Cellular Immunology* **329**, 31–40 (2018).

123. Hurton, L. V. *et al*. Tethered IL-15 augments antitumor activity and promotes a stem-cell memory subset in tumor-specific T cells. *PNAS* **113**, E7788–E7797 (2016).

124. Clauss, J. *et al*. Efficient Non-Viral T-Cell Engineering by Sleeping Beauty Minicircles Diminishing DNA Toxicity and miRNAs Silencing the Endogenous T-Cell Receptors. *Human Gene Therapy* **29**, 569–584 (2018).

125. Nakazawa, Y. *et al*. Optimization of the PiggyBac Transposon System for the Sustained Genetic Modification of Human T-Lymphocytes. *J Immunother* **32**, 826–836 (2009).

126. Darquet, A.-M., Cameron, B., Wils, P., Scherman, D. & Crouzet, J. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Therapy* **4**, 1341 (1997).

127. Hudecek, M. *et al*. Minicircle-Based Engineering of Chimeric Antigen Receptor (CAR) T Cells. *Recent Results Cancer Res*. **209**, 37–50 (2016).

128. Cheng, C. *et al*. Bacteria-free minicircle DNA system to generate integration-free CAR-T cells. *Journal of Medical Genetics* **56**, 10–17 (2019).

129. Luke, J., Carnes, A. E., Hodgson, C. P. & Williams, J. A. Improved antibiotic-free DNA vaccine vectors utilizing a novel RNA based plasmid selection system. *Vaccine* **27**, 6454–6459 (2009).

130. Williams, J. A., Luke, J., Johnson, L. & Hodgson, C. pDNAVACCultra vector family: high throughput intracellular targeting DNA vaccine plasmids. *Vaccine* **24**, 4671–4676 (2006).

131. Innate or Adaptive Immunity? The Example of Natural Killer Cells | Science. https://science-sciencemag-org.ezp1.lib.umn.edu/content/331/6013/44.long.

132. Matosevic, S. Viral and Nonviral Engineering of Natural Killer Cells as Emerging Adoptive Cancer Immunotherapies. *J Immunol Res* **2018**, 4054815 (2018).

133. Imamura, M. *et al*. Autonomous growth and increased cytotoxicity of natural killer cells expressing membrane-bound interleukin-15. *Blood* **124**, 1081–1088 (2014).

134. Blocking NK Cell Inhibitory Self-Recognition Promotes Antibody-Dependent Cellular Cytotoxicity in a Model of Anti-Lymphoma Therapy | The Journal of Immunology. https://www.jimmunol.org/content/180/9/6392.long.

135. Crissman, H. A., Mullaney, P. F. & Steinkamp, J. A. Methods and applications of flow systems for analysis and sorting of mammalian cells. *Methods Cell Biol* **9**, 179–246 (1975).

136. Marcus, A. *et al*. Tumor-derived cGAMP triggers a STING-mediated interferon response in non-tumor cells to activate the NK cell response. *Immunity* **49**, 754-763.e4 (2018).

137. Ackermann, M. R. Chapter 3 - Inflammation and Healing1. in *Pathologic Basis of Veterinary Disease (Sixth Edition)* (ed. Zachary, J. F.) 73-131.e2 (Mosby, 2017). doi:10.1016/B978-0-323-35775-3.00003-5.

138. Ng, S. K. Generation of High-Expressing Cells by Methotrexate Amplification of Destabilized Dihydrofolate Reductase Selection Marker. in *Protein Expression in Mammalian Cells: Methods and Protocols* (ed. Hartley, J. L.) 161–172 (Humana Press, 2012). doi:10.1007/978-1-61779-352-3_11.

139. Affleck, J. G., Nowickyj, S. M. & Walker, V. K. Selection for methotrexate resistance in mammalian cells bearing a Drosophila dihydrofolate reductase transgene: Methotrexate resistance in transgenic mammalian cells. *Cell Biol Toxicol* **26**, 117–126 (2010).

140. Kocmik, I. *et al*. Modified ARCA analogs providing enhanced translational properties of capped mRNAs. *Cell Cycle* **17**, 1624–1636 (2018).

141. Suschak, J. J. *et al*. Nanoplasmid Vectors Co-expressing Innate Immune Agonists Enhance DNA Vaccines for Venezuelan Equine Encephalitis Virus and Ebola Virus. *Mol Ther Methods Clin Dev* **17**, 810–821 (2020).

142. Jonnalagadda, M. *et al*. Efficient selection of genetically modified human T cells using methotrexate-resistant human dihydrofolate reductase. *Gene Therapy* **20**, 853–860 (2013).

143. Freud, A. G., Yu, J. & Caligiuri, M. A. Human natural killer cell development in secondary lymphoid tissues. *Semin Immunol* **26**, 132–137 (2014).

144. Pahl, J. & Cerwenka, A. Tricking the balance: NK cells in anti-cancer immunity. *Immunobiology* **222**, 11–20 (2017).

145. Abel, A. M., Yang, C., Thakar, M. S. & Malarkannan, S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. *Front Immunol* **9**, (2018).

146. Guillerey, C., Huntington, N. D. & Smyth, M. J. Targeting natural killer cells in cancer immunotherapy. *Nature Immunology* **17**, ni.3518 (2016).

147. Fagan, E. A. & Eddleston, A. L. Immunotherapy for cancer: the use of lymphokine activated killer (LAK) cells. *Gut* **28**, 113–116 (1987).

148. Davis, Z. B., Vallera, D. A., Miller, J. S. & Felices, M. Natural killer cells unleashed: Checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Seminars in Immunology* **31**, 64–75 (2017).

149. Liu, E. *et al*. GMP-Compliant Universal Antigen Presenting Cells (uAPC) Promote the Metabolic Fitness and Antitumor Activity of Armored Cord Blood CAR-NK Cells. *Front Immunol* **12**, (2021).

150. Daher, M. *et al*. Targeting a cytokine checkpoint enhances the fitness of armored cord blood CAR-NK cells. *Blood* **137**, 624–636 (2021).

151. Judge, S. J. *et al*. Minimal PD-1 expression in mouse and human NK cells under diverse conditions. *J Clin Invest* **130**, 3051–3068 (2020).

152. Davis, Z. *et al*. Low-density PD-1 expression on resting human natural killer cells is functional and upregulated after transplantation. *Blood Adv* **5**, 1069–1080 (2021).

153. Poláková, K. *et al*. Mild acid treatment induces cross-reactivity of 4H84 monoclonal antibody specific to nonclassical HLA-G antigen with classical HLA class I molecules. *Human Immunology* **64**, 256–264 (2003).

154. Kluesner, M. G. *et al*. CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary cells. *bioRxiv* 2020.04.16.045336 (2020) doi:10.1101/2020.04.16.045336.

155. Hooijberg, J. H. *et al*. The role of multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular folate homeostasis. *Biochem Pharmacol* **65**, 765–771 (2003).

156. Huennekens, F. M. In search of dihydrofolate reductase. *Protein Sci* **5**, 1201–1208 (1996).

157. McGuire, J. J. Anticancer antifolates: current status and future directions. *Curr Pharm Des* **9**, 2593–2613 (2003).

158. Neelapu, S. S. Managing the toxicities of CAR T-cell therapy. *Hematol Oncol* **37 Suppl 1**, 48–52 (2019).

159. Marofi, F. *et al*. CAR T cells in solid tumors: challenges and opportunities. *Stem Cell Research & Therapy* **12**, 81 (2021).

160. Majzner, R. G. & Mackall, C. L. Tumor Antigen Escape from CAR T-cell Therapy. *Cancer Discov* **8**, 1219–1226 (2018).