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Genetically stable multi-gene edited iPSCs-derived NK cells for enhanced cancer immunotherapy

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Chimeric antigen receptor (CAR)-T cell treatment is an innovative drug with excellent therapeutic effects against B cell blood cancer. However, multiple side effects and ultra-high treatment costs must be overcome. Off-the-shelf CAR natural killer (NK) cells can be a good alternative to patient-specific CAR-T cells. The purpose of this study was to combine cellular reprogramming, gene editing, and differentiation technologies to produce full-off-the-shelf NK cells and to verify their efficacy and safety. Genetically stable universal and potent CAR (upCAR)-induced pluripotent stem cells (iPSCs) showed biallelic insertions and deletions in the coding sequence and no off-target effects. upCAR-NK cells showed a very high differentiation yield and *in vitro* proliferation, and freezing/thawing was possible. In addition, upCAR-NK cells secrete interferon-γ when they meet cancer cells, showing cytotoxic effects *in vitro* and *in vivo*. upCAR-NK cells show no obvious toxicity *in vivo*. In conclusion, this study developed genetically stable upCAR-iPSCs and upCAR-NK cell platform technologies that are less likely to have side effects and can be more economically developed for B cell blood cancer than CAR-T cells. In the future, this technology could be useful in developing a full-off-the-shelf CAR-NK cells anti-cancer immune cell therapy with low side effects, high efficacy, and a low price.

INTRODUCTION

The emergence of patient-specific chimeric antigen receptor (CAR)-T treatments for relapsed/refractory CD19$^+$ B cell malignancies (B cell acute lymphoblastic leukemia and B cell non-Hodgkin lymphoma) is a huge breakthrough in the field of blood cancer treatment.[1–3] However, side effects, including cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), and ultra-high treatment costs are problems that must be addressed. Generally, when CRS occurs after CAR-T administration, it can be overcome in many cases after administration of tocilizumab, an interleukin (IL)-6 antagonist. However, ICANS is very difficult to overcome when it occurs.[4–6] In addition, the patient-specific treatment concept has limitations in terms of lowering treatment costs.

Natural killer (NK) cells, which are innate immune cells, are a good alternative to prevent fatal side effects of CAR-T cells that accompany the treatment of CD19$^+$ B cell malignancies (B cell acute lymphoblastic leukemia and B cell non-Hodgkin lymphoma).[7,8] NK cells are innate immune cells that belong to the same family of lymphocytes like T cells. The main cause of CRS is the vigorous clonal expansion of cells, which is characteristic of adaptive immune cells. However, NK cells do not have vigorous clonal expansion properties.[9,10] NK cells do not have a T cell receptor (TCR), when used off-the-shelf, no fatal graft-versus-host disease (GvHD) occurs. However, because human leukocyte antigen (HLA) class I is expressed, immune rejection can occur when used as off-the-shelf.[11] Recently, the lower-than-expected efficacy of off-the-shelf NK cell treatments has been attributed to immune rejection.[12]

To lower the treatment cost of anti-cancer immune cell therapy, it must be off-the-shelf and not patient specific. For this purpose, the editing of genes related to immune rejection through multiplex gene editing is essential, and induced pluripotent stem cells (iPSCs) can be the answer.[13] iPSCs have the advantage that pure iPSCs can be separated and cultivated after gene editing. Additionally, homogeneous and indefinite treatments can be produced by converting gene-edited iPSCs into master cell banks (MCBs). However, to implement this concept in practice, various element technologies, including cellular reprogramming technology for producing iPSCs and technology for differentiating genetically modified iPSCs into NK cells, must be supported.

What is needed for the development of CAR-NK cell therapy is the development of full-off-the-shelf treatments without immune rejection and increased efficacy. In the field of iPSC-NK cell therapy, the first-in-class study did not control immune rejection and the safety was confirmed, but the efficacy was unsatisfactory.[14] In a follow-up

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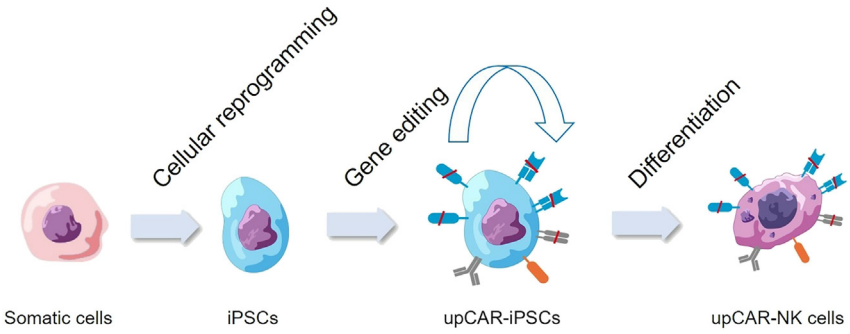


Figure 1. Study overview
By converging cellular reprogramming, gene editing, and differentiation technologies, upCAR-NK cells can be produced indefinitely. upCAR NK cells are pure, full-off-the-shelf therapeutics with improved efficacy, controlled immune rejection and blocked the possibility of GvHD.

study, HLA was knocked out, and the immune rejection was controlled by introducing the classical Don't eat me gene, *HLA-E*. It is currently in clinical trials, and good initial results have been derived.[15] In addition, studies have been reported to enhance efficacy by co-administration of anti-cancer immune cell therapy and immune checkpoint inhibitor.[16] Furthermore, research is being conducted to enhance efficacy by knockout of immune checkpoint gene from anti-cancer immune cell therapy.[17,18] Taken together, there is a demand for full-off-the-shelf cell therapy capable of host immune cell modulation and cancer cell modulation. Our previous study showed that we successfully generated iPSC-derived CD19 CAR-NK cells to specifically target pericytes in glioblastoma (GBM). The results showed that iPSC-derived CD19 CAR-NK cells in the GBM microenvironment targeting pericyte efficiently killed GBM both *in vitro* and *in vivo* assay.[19]

The purpose of this study was to produce multiplex gene-edited (*B2M*$^{-/-}$, *CIITA*$^{-/-}$, *TRA*$^{-/-}$, *PDCD1*$^{-/-}$, *CTLA4*$^{-/-}$, *CD19-CAR*$^{o/e}$, *CD24*$^{o/e}$) iPSCs/NK cells by the fusion of cellular reprogramming, gene editing, and differentiation technologies and to verify the efficacy and safety of these cells. In this study, we intend to establish a full-off-the-shelf CAR NK cell therapeutic platform that can be expanded to various cancer types by escaping host and cancer immune modulations.

RESULTS

Full-off-the-shelf CAR-NK cells can be produced by fusing various element technologies

By combining cellular reprogramming, gene editing, and differentiation technologies, upCAR-iPSCs and upCAR-NK cells are produced indefinitely (Figure 1). upCAR-NK cells are blocked by immune rejection and GvHD using multiplex gene editing (HLA class I/II gene knockout, Don't eat me gene overexpression, and TCR gene knockout). In addition, this function was improved by knocking out two immune checkpoint genes. These are the most advanced forms of allogeneic cells that can be used in off-the-shelf applications.

A multiplex gene editing system capable of producing genetically stable iPSCs has been established

upCAR-iPSCs with seven genes edited simultaneously (*B2M*$^{-/-}$, *CIITA*$^{-/-}$, *TRA*$^{-/-}$, *PDCD1*$^{-/-}$, *CTLA4*$^{-/-}$, *CD19-CAR*$^{o/e}$, *CD24*$^{o/e}$) were produced and verified. iPSCs were produced from fibroblasts, and upCAR-iPSCs were produced using CRISPR-Cas9 and gene overexpression technologies, and various analyses were performed at each stage (Figure 2A). Alkaline phosphatase^{+} SSEA4^{+} TRA-1-60^{+} TRA-1-81^{+} iPSCs were generated with high efficiency (Figure 2B). Five single guide RNA (sgRNA)/Cas9 (*B2M*, *CIITA*, *TRA*, *PDCD1*, and *CTLA4*) were simultaneously introduced into iPSCs through electroporation to secure clones in which insertions and deletions occurred biallelically in the coding sequences of the five genes (Table 1; Figure 2C). Ten potential off-target sites were selected (mismatch $\leq$3), five of which were genetic locus (intron), and five were intergenic regions (Table 2). Whole-genome sequencing showed that there were no off-target effects at the five gene loci (Figure 2D). Various CAR vectors were screened to identify those capable of CAR overexpression by more than 60% on the surface of iPSCs (Figure 2E). *CD24*, a new Don't eat me gene, with a similar level of function as *HLA-G*, was discovered (Figure 2F). The screened *CAR* (19-BBz) and Don't eat me (*CD24*) were subcloned into a dual plasmid containing the two promoters (Figure 2G). This dual plasmid was introduced into five types of gene-knockout iPSCs and secured a subclone with more than 90% CAR expression using G418 (Figure 2H). Microarray analysis confirmed that multiplex gene editing did not significantly alter the transcriptome (Figure 2I). upCAR-iPSCs maintained the normal karyotype (46, XX) (Figure 2J). upCAR-iPSCs confirmed that all nine mutation hotspots maintained a normal diploid (Figure 2K).

upCAR-NK cells derived from upCAR-iPSCs were differentiated with high efficiency

upCAR-NK cells in which seven genes were edited simultaneously (*B2M*$^{-/-}$, *CIITA*$^{-/-}$, *TRA*$^{-/-}$, *PDCD1*$^{-/-}$, *CTLA4*$^{-/-}$, *CD19-CAR*$^{o/e}$, *CD24*$^{o/e}$) were produced and verified. Differentiation from iPSCs to mesoderm, HSCs, and NK cells was induced for 50 days via cytokine cocktail-based differentiation (Figure 3A). As differentiation progressed, stage-specific surface markers were sequentially expressed (Figure 3B). upCAR-NK cells express various NK cell-specific markers; interestingly, the upCAR-NK cells were CD16^{high} and NKG2D^{high} (Figure 3C). As a result of RNA sequencing (RNA-seq), it was confirmed that the upCAR-NK cells had a very similar transcriptome to blood-derived NK cells (Figure 3D). Differentiated upCAR-NK cells did not express the knockout genes (B2M and PDCD1) or pluripotency-related markers (Figure 3E). The ratio of CAR^{+}, CD24^{+}, and CD56^{+}CD45^{+} upCAR NK cells exceeded 80%

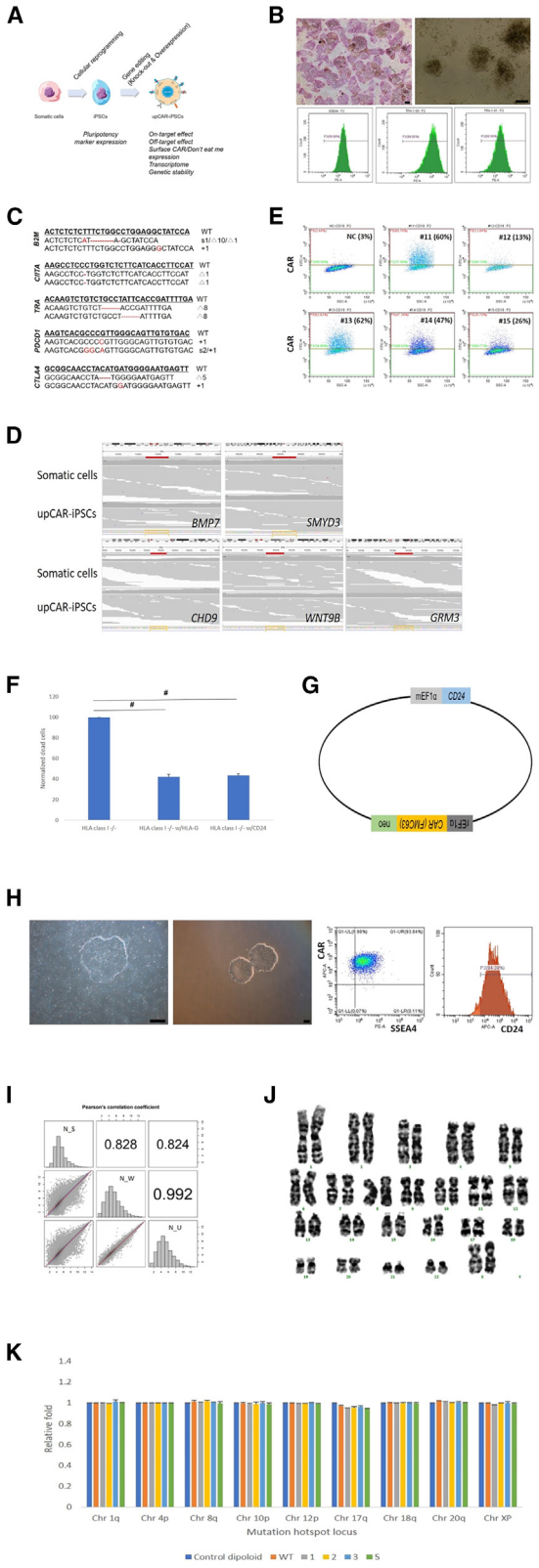


Figure 2. Production and characterization of upCAR-iPSCs with seven genes edited at the same time (five knockouts and two overexpressed)

(A) Using cellular reprogramming and gene editing techniques, iPSCs and upCAR-iPSCs were produced from somatic cells. Various analyses were performed for each step. (B) High-efficiency iPSCs generation and verification. (C) On-target effect verification on five genes. (D) Off-target effect verification on five sgRNA/Cas9 species (mismatch ≤3). (E) CAR vector screening with iPSCs surface expression. (F) Novel Don't eat me gene *CD24* screening. (G) Dual plasmid vector map with CAR (*FMC63*) and Don't eat me gene (*CD24*) simultaneously subcloned. (H) Dual plasmid introduction into five kinds of gene knockout iPSCs and start of G418 selection (left), completion of selection (right), and verification of surface marker expression. (I) Transcriptome analysis. S, somatic cells; W, WT-iPSCs; U, upCAR-iPSCs. (J) Karyotype analysis. (K) PCR-based mutation hotspot verification. Scale bar, 200 μm. Results in (B), (E), and (H) is representative image of three independent experiments. Results in (F) and (K) represent means ± SEM of 3 independent experiment. $^{\#}p < 0.01$ and $^{*}p < 0.05$.

in the selected upCAR-iPSC-derived NK cells (Figure 3F). When differentiation began with 2×10^3 upCAR-iPSCs, approximately $2.40 \times 10^7 \pm 5.29 \times 10^6$ upCAR-NK cells were finally obtained (Figure 3G). upCAR-NK cells expanded approximately 3-fold over 7 days under feeder-free conditions (Figure 3H). upCAR-NK cells maintained a surface marker similar to that of live cells even after freezing/thawing (Figure 3I).

upCAR-NK cells have the ability to attack cancer cells more powerfully

The efficacy and safety of upCAR NK cells, in which seven genes were edited simultaneously ($B2M^{-/-}$, $CIITA^{-/-}$, $TRA^{-/-}$, $PDCD1^{-/-}$, $CTLA4^{-/-}$, $CD19\text{-}CAR^{o/e}$, $CD24^{o/e}$), were confirmed. It was confirmed that upCAR-NK cells secrete a large amount of interferon (IFN)-γ when co-cultured with blood cancer cells (K562) (Figure 4A), and through *in vitro* lactate dehydrogenase (LDH) assay, that the upCAR-NK cells were cytotoxic to $CD19^+$ blood cancer cells (Nalm6 and Raji) (E:T = 10:1, 4 h) (Figure 4B). In addition, it was confirmed in real time that upCAR-NK cells have superior cytotoxicity against K562 cells compared to WT-NK cells and peripheral blood-NK cells (E:T = 10:1, 24 h) (Data not shown). A cancer model was created using NOG mouse and Nalm6, and upCAR-NK cells were administered once to confirm *in vivo* efficacy (Figure 4C). Significant inhibition of tumor growth and prolonged survival than positive control and WT-NK cells was confirmed when upCAR-NK cells were administered once in the Nalm6 cancer model ($p < 0.05$) (Figure 4D). In the dose escalation study, it was confirmed that significant tumor growth inhibition and survival were increased only in the high-dose group (5×10^6/head) ($p < 0.05$). In the low-dose group (1×10^6/head), no significant tumor growth inhibition and survival were not increased ($p > 0.05$) (Figures S1A and S1B). After IV of Nalm6 and upCAR-NK cells in the NOG mouse model, upCAR-NK cells were detected in the peripheral blood about a week (Figure S1C). upCAR-NK cells were detected in some mouse up to 30 days. All NOG mice that received 5×10^6 upCAR-NK cells once (S; day 0) or repeatedly (T; days 0, 7, and 14) survived for 3 weeks, and their weight was maintained without significant reduction (Figure 4E).

Table 1. sgRNA information for *B2M*, *CIITA*, *TRA*, *PDCD1*, and *CTLA4* gene knockout

Gene	Guide RNA sequence (5′ to 3′) without PAM	PAM	Orientation	Target locus	Potential off-target site
B2M	ACTCACGCTGGATAGCCTCC	AGG	(−)	Chr15 : 44,711,597	
CIITA	GATATTGGCATAAGCCTCCC	TGG	(−)	Chr16 : 10,895,741	
TRA	CTCTCAGCTGGTACACGGCA	GGG	(−)	Chr14 : 22,547,522	five intron and five intergenic region
PDCD1	ATGTGGAAGTCACGCCCGTT	GGG	(+)	Chr2 : 241,852,734	
CTLA4	GTGCGGCAACCTACATGATG	GGG	(+)	Chr2 : 203,870,730	

DISCUSSION

In this study, we developed a technology to produce iPSC-derived multiplex gene edited cells (upCAR-NK cells), which have a lower possibility of side effects than CAR-T cells and can be used to develop economical treatments. Genetically stable and pure upCAR-iPSCs can be produced using cellular reprogramming and gene-editing technologies. Through *in vitro* differentiation, NK cells can be manufactured with high efficiency and purity from upCAR-iPSCs. upCAR-NK cells have anti-cancer effects in *in vitro* and *in vivo* models using B cell malignant cancer cells (Figure 4). In addition, *in vivo* safety was confirmed by direct injection of upCAR-NK cells into an immunodeficient mouse model.

An off-target-effect-free multiplex gene-edited iPSCs production system was established. Gene editing can be used as one of the methods of imparting various functions to cells.[20,21] In this study, while maintaining the integrity of iPSCs using nonviral methods, immune rejection control, GvHD control, immune checkpoint inhibition, and CAR functions were added through multiplex gene editing (immune rejection control: $B2M^{-/-}$, $CIITA^{-/-}$, and $CD24^{o/e}$; GvHD control, $TRA^{-/-;}$ immune checkpoint inhibition, $PDCD1^{-/-}$ and $CTLA4^{-/-}$; and CAR, $CD19\text{-}CAR^{o/e}$). Five stable sgRNA sequences with very low off-target effect possibilities and a CAR/CD24 dual vector sequence with very high cell-surface expression were identified. Specifically, it demonstrated that innate/adaptive (cellular/humoral) immune escape is possible with HLA class I/II biallelic knockout and CD24 overexpression ($B2M^{-/-}$, $CIITA^{-/-}$, and $CD24^{o/e}$), which is a novel macrophage Don't eat me gene.[22] To prepare for the possibility of GvHD by mixing T cells with byproducts during the differentiation process, the TCR alpha locus gene was knocked out ($TRA^{-/-}$). In addition, two gene knockouts ($PDCD1^{-/-}$ $CTLA4^{-/-}$), which are targets of pembrolizumab and ipilimumab, can induce enhanced efficacy.[23] Multiplex gene editing is a possible approach because iPSCs are used, and this study is the most advanced form of full-off-the-shelf treatment research that can control immune rejection.[24]

An NK cell differentiation system with proven efficacy and safety has been established. The five-gene knockout in upCAR-iPSCs was permanent, and the overexpression of these two genes was maintained in upCAR-NK cells at a high rate. Interestingly, differentiated upCAR-NK cells highly expressed CD16 and NKG2D, which can be expected to have an antibody-dependent cellular cytotoxicity effect when co-treated with antibodies.[25] It is a very important point because the high expression of the key activating receptors CD16 and NKG2D of NK cells themselves can also enhance the efficacy of CAR-NK cells. Frozen upCAR-NK cells showed significant *in vivo* cytotoxicity only at a single dose (5×10^6/head) immediately after thawing. It was confirmed that upCAR-NK cells showed *in vivo* persistence for more than 1 week, which was comparable with general iPSC-NK cells. In addition, upCAR-NK cells were considered safe because no specific toxicity was observed 3 weeks after intravenous injection. In addition, even if differentiation is initiated at a small scale (1×10^5 upCAR-iPSCs), one batch of treatments (1×10^9 upCAR-NK cells) can be manufactured with differentiation.

Table 2. Potential off-target position

Chromosome	Position	Sequence	Strand	Mismatches	Gene/region
Chr1	246,005,573	CTCTCAGCTGGaAtACaGCA(GGG)	+	3	*SMYD3*/intron
Chr2	28,677,132	CaCTCAGCTGGTAagCGGCA(GGG)	–	3	-/intergenic
Chr5	145,407,350	*c*TGtGGCAACCTAtATGATG(GGG)	+	3	-/intergenic
Chr7	86,787,303	aTCTCgGCTGGTACACGaCA(TGG)	+	3	*GRM3*/intron
Chr11	67,581,044	*c*TGCGGCAACaTACATGtTG(TGG)	+	3	-/intergenic
Chr15	42,156,672	ACTCAgGCTGGATgGCCTgC(TGG)	+	3	-/intergenic
Chr16	53,081,166	CTCTCAGCTaGTACAgGGCA(TGG)	+	2	*CHD9*/intron
Chr17	20,852,557	ACTCcCGCTGGAaAGCCTgC(AGG)	+	3	-/intergenic
Chr17	46,867,608	GgTtTTGGCAaAAGCCTCCC(AGG)	–	3	*WNT9B*/intron
Chr20	57,188,868	CTtTgAGCTGGTcCACGGCA(AGG)	+	3	*BMP7*/intron

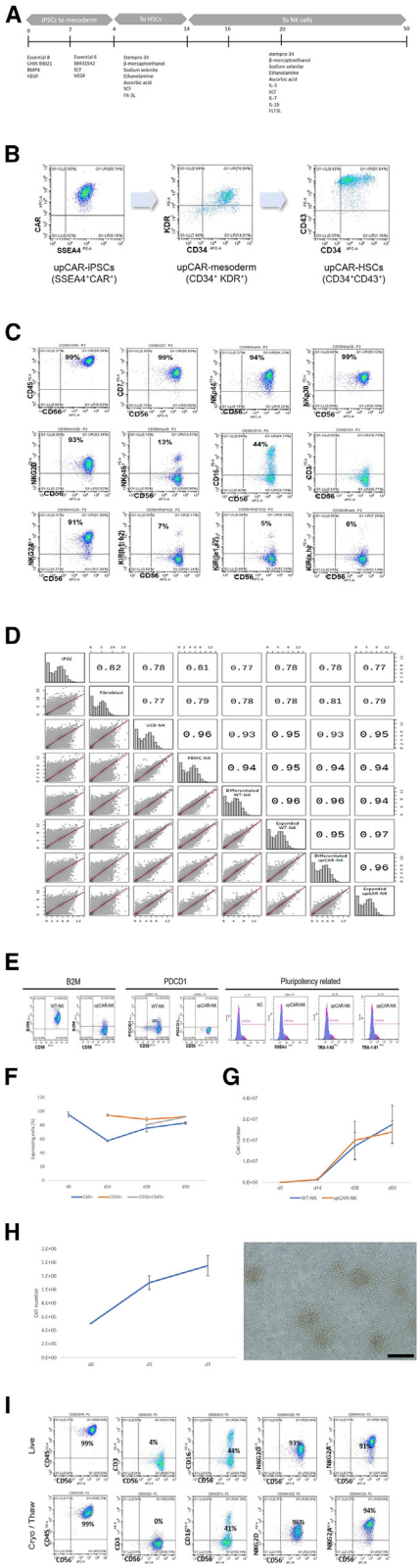


Figure 3. upCAR-NK cells production and characterization

upCAR-NK cells in which seven genes were edited simultaneously were produced and verified. (A) Differentiation was induced using various combinations of cytokine cocktails from iPSCs to mesoderm, HSCs, and NK cells. (B) Surface marker verification on iPSCs, mesoderm, and HSCs. (C) Verification of surface marker expression in differentiated NK cells. (D) Global transcriptome analysis of upCAR-NK cells by RNA-seq. (E) Verification of non-expression of surface marker (B2M, PDCD1, and pluripotency marker) in differentiated NK cells. (F) Time course changes in the ratio of $CD56^{+}CD45^{+}$, CAR^{+}, and $CD24^{+}$ cells during the differentiation of selected upCAR-iPSCs clones. (G) Time course differentiation yield analysis during NK cells differentiation ($p > 0.05$). (H) Feeder-free expansion of upCAR-NK cells and day 7 morphology. (I) Verification of surface marker maintenance of upCAR-NK cells after freezing/thawing. Scale bar, 200 μm. Results in (B), (C), (E), and (I) is representative image of three independent experiments. Results in (F), (G), and (H) represent means ± SEM of three independent experiment. $^{\#}p < 0.01$ and $^{*}p < 0.05$.

The upCAR-iPSCs developed in this study have characteristics of a platform that can be used in various ways. If only CAR is changed among the seven edited genes, the indications can be expanded to other cancer types. Making six-gene-edited iPSCs into MCBs can significantly reduce the production cost of homogeneous full-off-the-shelf CAR NK cell therapy. Since the TCR gene, which causes GvHD, has been knocked out, upCAR-T cells can also be produced by changing the differentiation method.[26,27] In addition, upCAR-macrophages with engulfing functions can be manufactured using upCAR-iPSCs.[28]

In conclusion, this study demonstrated that a safe and economical fully off-the-shelf allogeneic anti-cancer immune cell therapy platform can be implemented. Efficient expansion, GMP production, and detailed safety verification are required for future development of a treatment. During differentiation, 1×10^5 or more upCAR-NK cells can be obtained from one iPSC (Figure 3G); however, it is necessary to establish additional expansion protocols to secure more cells. iPSC-NK cells can be grown more than 100-folds efficiently through co-culture with engineered feeders overexpressed with several genes such as *41BBL*. If this system is applied to the expansion of differentiated upCAR-NK cells, fast translation will be possible. In addition, upCAR-NK cells should be able to implement a complex and difficult GMP process compared with existing blood-derived treatments. In addition, although *in vivo* safety was preliminarily verified (Figure 4E), a detailed safety verification process using good laboratory practices is required. If all these processes are implemented, they can be used as a full-off-the-shelf treatment platform for various types of cancer.

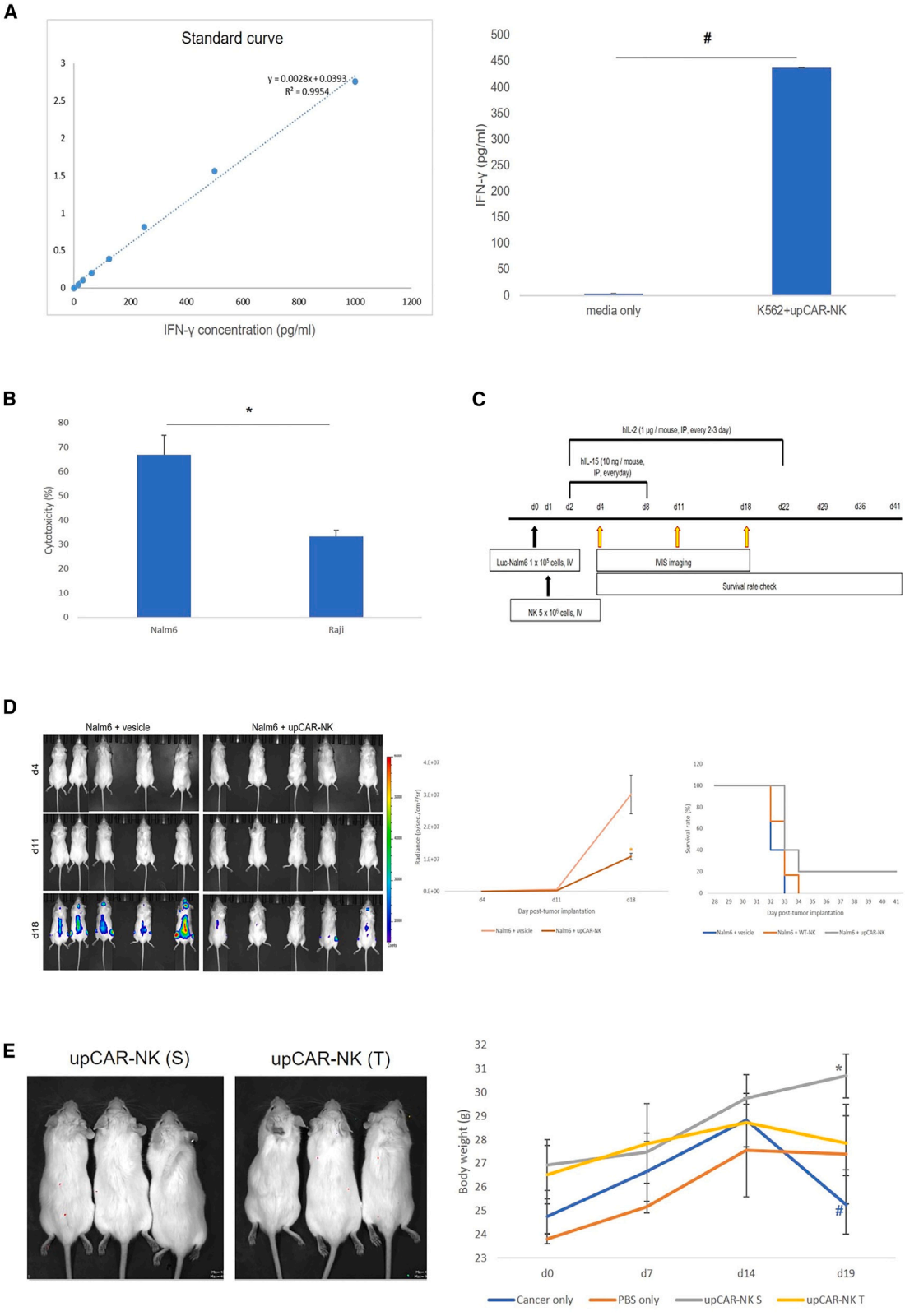
MATERIALS AND METHODS

Animal care

Male NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mice (Koatech, KR) were used for *in vivo* evaluation of efficacy and safety. All animal experiments were conducted in compliance with the guidelines approved by the Institutional Animal Care and Use Committee of Seoul National University.

iPSCs reprogramming

All human cell-related studies were approved by the Public IRB. iPSCs reprogramming experiments were performed at the LMO Research Facility (Grade 2). Human dermal fibroblasts (ATCC, US)



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were transduced with the *OCT4/SOX2/KLF-4/c-MYC* retrovirus at a multiplicity of infection of 10. Five days later, the cells were subcultured in iMatrix511 (Reprocell, US)-coated dishes and incubated in iPSCs medium (StemFit Basic04, Ajinomoto, JP) supplemented with 1× primocin (InvivoGen, US) for 2 weeks. iPSCs colonies were mechanically picked and subculturing was performed every week. The remaining iPSCs after picking were stained using the StemAB Alkaline Phosphatase Staining Kit II (Reprocell) and the degree of reprogramming was confirmed.

Gene editing I (knockout by sgRNA/Cas9)

The sgRNAs for five genes (*B2M*, *CIITA*, *TRA*, *PDCD1*, and *CTLA4*) were designed as shown in Table 1. The sgRNA/Cas9 complex prepared as a ribonucleoprotein-type was introduced into single iPSCs using a Neon transfection system (Thermo Fisher Scientific, US) (1,100 V, 30 ms, and 1 pulse). After culturing for 1 week, the colonies were mechanically picked. PCR amplification was performed using genomic DNA (gDNA) isolated from each colony and PCR primers (Table S1). The knockout locus was sequenced using PCR primers, and positive clones in which five types of genes were simultaneously knocked out were selected.

Gene editing II (overexpression by plasmid transfection)

The *CD19 CAR* gene (*FMC63*; Figure S2) and Don't eat me gene (*CD24*; GenBank: NM_001291737.1) were cloned into the pVITRO-Neo MCS dual plasmid (Invivogen) (Cosmogentech, KR). Five types of plasmids were produced by slightly different CAR gene sequences (signal peptide [sp], hinge [h], transmembrane domain [tm]; #11 CD8sp-V_L-$(GGGGS)_3$-V_H-CD8h-CD8tm-41BB-CD3z (#13 Q - > L); #12 CD8sp-V_L-$(GGGGS)_3$-V_H-CD8h (#11 C - > S)-CD8tm-41BB-CD3z; #13 CD8sp-V_L-$(GGGGS)_3$-V_H-CD8h-CD8tm-41BB-CD3z; #14 CD8sp-V_H-$(GGGGS)_3$-V_L-CD8h-CD8tm-41BB-CD3z; #15 CSF2RAsp-V_L-whitlow linker-V_H-CD28h-CD28tm-CD28cyto-CD3z). Each plasmid was introduced into five kinds of gene knockout single iPSCs using a Neon transfection system (Thermo Fisher Scientific) (1,100 V, 30 ms, and 1 pulse). Positive clones were selected by culturing in antibiotics free iPSCs medium for 3 days and adding G418 100–400 μg/mL for 9 days from the third day. After selection, colonies were mechanically picked. After treating iPSCs with APC-labeled human CD19 protein (Acro Biosystems, US) and Phycoerythrin (PE) anti-human SSEA-4 antibody (BD Biosciences, US), the expression levels of CAR and pluripotency markers were confirmed using a CytoFLEX Flow Cytometer (Beckman Coulter, US). *In vitro* and *in vivo* characterization analyses were performed using iPSC produced by introducing vector (#13). B2M-HLA-G heavy chain or CD24 was introduced into B2M-null cells to verify Don't eat me gene. The cells were stained with propidium iodide (PI), and the dead cell ratio was quantified after reacting with allogeneic innate immune cells for 4 h.

NK cell differentiation and expansion

Based on the schedule shown in Figure 3A, iPSCs were differentiated into NK cells (Xeno feeder-free). The cytokines used for differentiation were purchased from PeproTech (US). Differentiation days 0 (iPSCs), 4 (mesoderm), 14 (HSCs), and 50 (NK cells) were used to analyze the degree of differentiation by fluorescence-activated cell sorting (FACS) using antibodies (Table S2). The differentiation-completed NK cells were placed in STEM-CELLBANKER (NIPPON ZENYAKU KOGYO, JP) and slowly freezed. When thawing NK cells, fast thawing was performed in a 37°C water bath. upCAR-NK cell expansion was conducted for 7 days using CTS NK-Xpander basal medium (Gibco, US), 5% (v/v) GemCell U.S. Human serum AB, and 500–1,000 IU/mL IL-2 medium.

Evaluation of efficacy and safety of upCAR-NK cells (*in vitro* and *in vivo*)

After incubating upCAR-NK cells and K562 for 6 h, IFN-γ secreted by upCAR-NK cells was quantified using the human IFN-gamma ELISA Kit (R&D Systems, US). CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, US) was used to confirm *in vitro* efficacy. After 4 h of co-culture of upCAR-NK cells and Nalm6 or Raji cells (E:T = 10:1), the level of LDH in the medium was quantified using a SpectraMax ABS Plus microreader (Molecular Devices, US) at 450 nm. The K562 cells were stained with PI for real-time *in vitro* imaging. NK cells were placed on the upper side of the flow chamber, K562 cells were stained with PI on the lower side, and live imaging was performed during co-culture for 24 h (E:T = 10). For *in vivo* efficacy verification, upCAR-NK cells were administered as a single dose 1 day later by transplanting Nalm6-Fluc-Neo/eGFP-Puro (Luc-Nalm6; Imanis Life Sciences, US) into a fully immunocompromised NOG mouse (Koatech) (Figures 4C and S1A). upCAR-NK cells were administered by thawing frozen cells on the same day, washing them, and diluting them in 200 μL PBS (Hyclone, US). The IVIS Spectrum In Vivo Imaging System (PerkinElmer, US) was used to perform mouse live imaging/analysis about one week apart. For *in vivo* persistence analysis, Nalm6 and upCAR-NK cells were injected intravenously (Figure 4C), and approximately 20 μL of mouse venous blood was collected on days 6, 16, and 30. gDNA was isolated and PCR was performed with a *CAR*/*GAPDH*-specific primer (Table S1) to confirm the presence of upCAR-NK cells.

FACS

Live-cell FACS was performed using the antibody conditions listed in Table S2. The cell and antibody mixtures were incubated at 4°C for

Figure 4. Verification of efficacy and safety of upCAR-NK cells

The efficacy and safety of upCAR-NK cells were confirmed. (A) Quantification of IFN-r secretion by co-culture of K562 and upCAR-NK cells. (B) *In vitro* efficacy test (LDH assay) of upCAR-NK cells for $CD19^+$ blood cancer cells (Nalm6 and Raji). (C) *In vivo* efficacy test scheme (D) Confirmation of *in vivo* efficacy of upCAR-NK cells for the Nalm6 cancer model by single administration (left: live imaging photography; middle: live imaging quantified graph; right: survival rate graph). (E) Confirmation of safety of upCAR-NK cells in the mouse model by single administration (S; d0) and three repeated administration (T; d0, 7, and 14). Results in (A) and (B) represent means ± SEM of three independent experiment. Results in (D) and (E) represent means ± SEM (n = 3–5 mice). $^{\#}p < 0.01$ and $^{*}p < 0.05$.

30 min. After 30 min, excess antibody was removed by washing. The percentage of stained cells was measured using a CytoFLEX Flow Cytometer (Beckman Coulter) and this was analyzed as a negative control using an isotype control.

Microarray and off-target effect analysis

For the microarray analysis, RNA separation and cDNA synthesis were performed using somatic cells, wild-type (WT)-iPSCs, and upCAR-iPSCs. The sense cDNA was fragmented and biotin-labeled with terminal deoxynucleotidyl transferase using a GeneChip WT Terminal labeling kit. Approximately 5.5 μg of labeled DNA was hybridized to the Affymetrix GeneChip Array at 45°C for 16 h. Hybridized arrays were washed, stained on a GeneChip Fluidics Station 450, and scanned using a GCS3000 Scanner (Affymetrix, US). The probe cell intensity data computation was performed using Affymetrix GeneChip Command Console Software. The Cas-OFFinder iSAAC software (ver. iSAAC-04.18.11.09) were used for *in silico* potential off-target site screening (mismatch ≤ 3). Potential off-target sites located in the gene were experimentally verified using whole-genome sequencing.

RNA-seq analysis

For the RNA-seq analysis, RNA separation were performed using somatic cells, WT-iPSCs, blood-derived NK cells (UCB-NK and PBMC-NK), iPSC-derived differentiated NK cells (WT-NK and upCAR-NK), and iPSC-derived expanded NK cells (WT-NK and upCAR-NK). RNA is randomly fragmented into small pieces to be able to sequence in the appropriate length for sequencing. Fragmented RNA is reverse transcribed to cDNA. The sequencing adapters are ligated onto both ends of cDNA fragments. Sample-specific index sequences are labeled on cDNA fragments to allow identifying individual samples to be included in the same lane. The products are purified and enriched with PCR to create the final cDNA library. The quantification and qualification of the library are checked whether to run sequencing. The insert size of RNA-seq library is between 200 and 400 bp. In cases of paired-end sequencing, both ends of the product are sequenced to the read length.

Karyotyping and genetic stability analysis

Karyotyping of upCAR-iPSCs was performed at Cancerrop (KR), where 20 metaphase spreads were analyzed. gDNA was isolated from WT-iPSCs and upCAR-iPSCs using an AccuPrep gDNA Extraction Kit (Bioneer, KR). Control DNA from the hPSC Genetic Analysis Kit (STEMCELL Technologies, US) and isolated gDNA was amplified by PCR. Quantitative PCR amplification was performed using QuantStudio3 (Applied Biosystems, US). Relative gene expression levels were calculated using the ΔΔCt method.

Statistical analysis

All data were assessed with unpaired *t* test using GraphPad Prism online version (US), where $p < 0.01$ or $p < 0.05$ were considered significant.

DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

D.K.: Conceptualization, data collection, data analysis, writing, supervision. B.K.M.: Data collection, data analysis. M.H.: Data collection, data analysis. T.W.L.: Data collection, data analysis. J.L.: Data collection, data analysis. K.S.K.: Conceptualization, data analysis, supervision and final approval.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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