Avoidance of fratricide and improved efficacy of CD33-MSLN CAR-iNK cells in treating acute myeloid leukemia

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One Sentence Summary: Pluripotent stem cell-derived $CD33^{KO}$-LOOP CAR-iNK cells avoid fratricide and demonstrate superior cytotoxicity against $CD33^{+}MSLN^{+}$ leukemia cells.

Abstract

CAR-NK cell therapy has been investigated for treating acute myeloid leukemia (AML). CD33 is a well-established therapeutic target for AML. Conventional CD33 CAR-NK cells are prone to fratricide due to endogenous expression of CD33 on expanded NK cells. Mesothelin (MSLN), a tumor differentiation antigen, is expressed in approximately 36% of pediatric AML cases and 14% of adult AML cases. In this study, we designed a novel CD33-MSLN loop CAR (Loop CAR) and evaluated its anti-tumor efficacy in human umbilical cord blood-derived NK (UCB-NK) cells and human pluripotent stem cell-derived NK (hPSC-iNK) cells. The Loop CAR exhibited superior cytotoxicity against dual-antigen-positive tumor cell lines and primary AML cells. To further optimize this approach, we established a hPSC-derived cell line via knockout of endogenous CD33 gene ($CD33^{KO}$) and integration of the Loop CAR construct. Using organoid induction technology, we generated mature $CD33^{KO}$-Loop CAR-iNK cells, characterized by expression of endogenous CD16. This innovative strategy effectively mitigated NK cell fratricide and significantly enhanced CD33-mediated specific cytotoxicity. Moreover, the $CD33^{KO}$-Loop CAR-iNK cells demonstrated superior tumor-killing activity in AML xenograft mice and significantly prolonged survival of the treated animals. hPSC-derived $CD33^{KO}$-Loop CAR-iNK cells have translational potential for treating AML.

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy that demands urgent therapy (*1*). As new therapeutic targets are being identified, significant efforts have highlighted mesothelin (MSLN) as a promising target in AML. The MSLN is expressed in 36% of pediatric AML cases and 14% of adult AML cases, with 76% of MSLN-positive ($MSLN^+$) patients retaining MSLN expression at relapse. Furthermore, 4% of MSLN-negative ($MSLN^-$) AML patients acquire MSLN expression upon relapse (*2-4*). Preclinical data suggest that MSLN chimeric antigen receptor (CAR)-T cells effectively eliminate $MSLN^+$ AML cells in xenograft models (*5*). Therefore, MSLN is a promising target for AML immunotherapy.

Natural killer (NK) cells have demonstrated considerable potential in enhancing outcomes for AML treatment, particularly in the context of hematopoietic stem cell transplantation (*6*). The adoptive transfer of NK cells for AML therapy has been widely investigated in clinical settings (*7*). CAR-NK cells, which combine the target killing ability of CAR technology with the innate nonspecific cytotoxicity of NK cells, have shown promising results for tumor treatment (*8, 9*). CD33 CAR-NK cells, in particular, have been tested in relapsed/refractory AML therapies, with clinical data demonstrating their efficacy and safety (*10*). Additionally, pluripotent stem cell (PSC)-derived NK or CAR-NK cells have shown therapeutic potential in both preclinical and clinical settings for AML (*11, 12*). PSC-derived NK (iNK) cells provide a homogeneous alternative to NK cells isolated from human tissues (*11, 13, 14*).

Despite these advancements, challenges remain in CAR immune cell therapy, such as tumor antigen escape, on-target off-tumor toxicity, and unintended fratricide due to the shared expression of target antigens between CAR immune cells and tumor cells (*15-17*). To overcome these issues, dual-targeting CARs have been designed to simultaneously target two antigens expressed on tumor cells (*18, 19*). Inactivation of the target antigen in the CAR-engineered immune cells, or in healthy tissue cells, can mitigate fratricide and spare normal tissues. For

example, the disruption of CD7 in CD7 CAR-T cells prevents fratricide and allows for the expansion of these cells (*20*). Similarly, genetic inactivation of CD33 in hematopoietic stem cells enables CD33-deficient hematopoiesis to avoid the killing of CD33 CAR-T cells (*21*). Since CD33 is also expressed on NK cells, CD33 CAR-NK cells face similar fratricidal challenges, which can hinder their expansion (*17, 22*). Human umbilical cord blood NK (UCB-NK) cells, which exhibit a 1.4%-25.5% CD33 expression range in their resting state, show reduced cytotoxicity against K562 cells (*23*). Moreover, NK cells upregulate CD33 expression levels to nearly 50% during *in vitro* expansion (*17*).

In this study, we designed a bivalent loop CAR targeting both CD33 and MSLN antigens (CD33-MSLN loop CAR, denoted as Loop CAR). We engineered this Loop CAR into UCB-NK cells and hPSCs to generate Loop CAR-NK and Loop CAR-iNK cells. Both Loop CAR-NK and Loop CAR-iNK cells exhibited superior cytotoxicity against tumor cells expressing CD33 and MSLN antigens, including AML cell lines and patient-derived tumor cells. Transcriptomic analysis confirmed that the Loop CAR enhanced NK cell cytotoxicity. Additionally, we generated a CD33 gene knockout ($CD33^{KO}$) hPSC line expressing the Loop CAR ($CD33^{KO}$-Loop CAR-hPSCs) and successfully produced $CD33^{KO}$-Loop CAR-iNK cells. These $CD33^{KO}$-Loop CAR-iNK cells exhibited enhanced expansion and anti-tumor efficacy *in vitro* and effectively suppressed tumor progression in xenograft AML models. Our findings highlight the clinical potential of Loop CAR-NK and Loop CAR-iNK cells for the treatment of $CD33^{+}MSLN^{+}$ AML.

RESULTS

CD33-MSLN loop CAR-NK cells exhibit superior cytotoxicity against CD33- and MSLN-positive tumor cells

To enhance the efficacy of NK cells targeting mesothelin (MSLN)-expressing acute myeloid leukemia (AML), we employed a bivalent loop CAR strategy, designing a dual-target CAR

97 targeting both CD33 and MSLN. Specifically, the variable light (V_L) and heavy (V_H) chains of
98 anti-CD33 single-chain variable fragment (CD33 scFv) and anti-MSLN scFv (MSLN scFv),
99 the $(G_4S)_3$ linker (Linker 3), and the Whitlow linker (*24*) were sequentially fused to create a
100 bivalent scFv construct (CD33 V_L-linker 3-MSLN V_H-whitlow linker-MSLN V_L-linker 3-
101 CD33 V_H, CD33-MSLN loop). The CD33-MSLN loop scFv was then linked to the CD8α hinge,
102 the CD8 transmembrane domain (TMD), and the CD3 ζ signaling domain (SD) to construct
103 the final CD33-MSLN loop CAR (denoted as Loop CAR). We applied the MSLN CAR and
104 CD33 CAR as controls (**Fig. 1A**). To assess the potential of Loop CAR in enhancing tumor-
105 killing activity in NK cells, we first incorporated the Loop CAR element into the SFG vector,
106 followed by retroviral packaging as previously reported (*25*). After T-cell depletion, umbilical
107 cord blood NK (UCB-NK) cells were activated using K562-mbIL-21 feeder cells for 6 days,
108 followed by transduction with Loop CAR retroviral particles (MOI = 5). The transduced cells
109 were then expanded for 4 days in the presence of K562-mbIL-21 feeder cells (**Fig. 1B** and **fig.**
110 **S1A**). The proportion of Loop CAR^+ NK cells ($CD56^+$CD33 CAR^+MSLN CAR^+) reached 43.4%
111 two days after transduction, while control groups, including EGFP-NK, CD33 CAR-NK, and
112 MSLN CAR-NK, achieved transduction rates of 48.2%, 55.6%, and 52.1%, respectively (**Fig.**
113 **1C**). Loop CAR-NK cells showed comparable expression of typical NK cell activating and
114 inhibitory receptors, as well as cytotoxic granules, to the control groups (**fig. S1B**).
115 We next evaluated the cytotoxicity of Loop CAR-NK cells by sorting CAR^+ NK cells and
116 coculturing them with Nomo-1 ($CD33^+MSLN^+$), AGS ($CD33^-MSLN^+$), or HL-60
117 ($CD33^+MSLN^-$) tumor cells (**Fig. 1**, **D** and **E**, **fig. S1C**). Loop CAR-NK cells exhibited
118 significantly superior cytotoxicity against Nomo-1 cells compared to EGFP-NK, CD33 CAR-
119 NK, and MSLN CAR-NK cells across all effector-to-target (E:T) ratios (E:T = 0.2:1, 0.4:1,
120 0.8:1, 1.6:1) (**Fig. 1F**). In addition, Loop CAR-NK cells also demonstrated potent killing
121 activity against MSLN- or CD33-expressing tumor cells, such as AGS ($CD33^-MSLN^+$) and

HL-60 ($CD33^+MSLN^-$), with cytotoxicity comparable to MSLN CAR-NK or CD33 CAR-NK cells (**Fig. 1**, **G** and **H**). Mechanistically, Loop CAR-NK cells exhibited the highest expression of CD107a, a marker associated with NK cell functional activity, when cocultured with Nomo-1 cells (**fig. S1**, **D-F**). Furthermore, Loop CAR molecules accumulated at the immunological synapse (IS) formed between Loop CAR-NK cells and Nomo-1 cells, confirming that the Loop CAR participates in IS formation in a CD33-MSLN-dependent manner (**Fig. 1I**). Notably, Loop CAR-NK cells also demonstrated superior cytotoxicity against primary AML cells expressing both CD33 and MSLN. Mononuclear cells (MNCs) were isolated from the bone marrow (BM) or peripheral blood (PB) of three AML patients (**Fig. 1J**). All three patients' MNCs expressed CD33 (Patient #1: 87.5%, Patient #2: 95.6%, Patient #3: 98.0%) and MSLN (Patient #1: 41.3%, Patient #2: 23.5%, Patient #3: 57.8%) (**Fig. 1K**). The expression of MSLN was further confirmed by qPCR, which showed consistency with the protein expression levels (**Fig. 1L** and **table S1**). Loop CAR-NK cells (Effectors, E) were cocultured with these primary AML cells (Targets, T) for 20 hours at various E:T ratios (0:1, 0.5:1, 1:1, 2:1, and 5:1) (**Fig. 1M**). Loop CAR-NK cells exhibited significantly higher cytotoxicity against all patient-derived targets compared to CD33 CAR-NK and MSLN CAR-NK cells. In addition, both CD33 CAR-NK and MSLN CAR-NK cells killed more targets than EGFP-NK cells (**Fig. 1**, **N-P**). Collectively, these data demonstrate that Loop CAR-NK cells exhibit superior specific cytotoxicity against tumor cells expressing both CD33 and MSLN compared to CD33 CAR-NK or MSLN CAR-NK cells.

Loop CAR-NK cells exhibit a more pronounced activation status after incubation with Nomo-1 cells

To investigate the superior cytotoxicity of Loop CAR-NK cells, we performed single-cell RNA sequencing (scRNA-seq) on sorted Loop CAR-NK cells after incubation with Nomo-1 cells for 72 hours (**Fig. 2A**). UMAP analysis revealed that the transcriptional profiles of Loop CAR-

147 NK cells were similar to those of CD33 CAR-NK or MSLN CAR-NK cells. However, all CAR-
148 NK cells exhibited distinct transcriptional profiles compared to EGFP-NK cells (**Fig. 2B**). We
149 further examined the differentially expressed genes (DEGs) between Loop CAR-NK cells and
150 CD33 CAR-NK or MSLN CAR-NK cells. Gene Ontology (GO) enrichment analysis indicated
151 that genes upregulated in Loop CAR-NK cells relative to MSLN CAR-NK cells were primarily
152 involved in cell division, positive regulation of the Fc receptor-mediated stimulatory signaling
153 pathway, and positive regulation of the RIG-1 signaling pathway (**Fig. 2C** and **fig. S2**, **A** and
154 **B**). Similarly, genes upregulated in Loop CAR-NK cells compared to CD33 CAR-NK cells
155 were enriched in lymphoid cell differentiation, positive regulation of the RIG-1 signaling
156 pathway, and formation of immunological synapses. (**Fig 2D** and **fig. S2**, **C** and **D**). Among the
157 activation-related genes, several hallmarks of NK cell activation and cytotoxic function,
158 including *CD244*, *SLAMF7*, and *ITGAL* (LFA-1), were upregulated (*26-28*). Additionally,
159 genes involved in the RIG-I signaling pathway, such as *ANKRD17*, *PUM1*, and *PUM2* (*29, 30*),
160 and those associated with immunological synapse (IS) formation, including *NCAM1*, *PTK2B*,
161 and *DOCK8* (*31-34*), were also significantly elevated (**Fig. 2**, **E-G**). These findings suggest
162 that Loop CAR-NK cells exhibit enhanced activation following incubation with Nomo-1 cells,
163 which likely contributes to their superior cytotoxicity compared to CD33 CAR-NK or MSLN
164 CAR-NK cells.

165 **The fratricide of Loop CAR-NK and Loop CAR-iNK cells precludes their expansion**

166 The expansion ability of CD33 CAR-NK cells has been impaired due to fratricide mediated by
167 the CD33 CAR, as $CD33^+$ NK cells are eliminated by CAR-expressing NK cells (*17*). Here,
168 the expression of CD33 in UCB-derived Loop CAR-NK cells and hPSC-derived Loop CAR-
169 iNK cells also led to fratricide. In expanded UCB-NK cells, the expression of CD33 was
170 observed at 26.9% (**Fig. 3A)**. To assess fratricide, we sorted $CD33^-$ and $CD33^+$ NK cells as
171 targets and co-incubated them with Loop CAR-NK cells (**Fig. 3**, **B** and **C)**. As expected, Loop

172 CAR-NK cells exhibited specific cytotoxicity against $CD33^+$ NK cells at the indicated effector-
173 to-target (E:T) ratios, while no cytotoxicity was observed against $CD33^-$ NK cells (**Fig. 3D).**
174 We then sorted an equal number of EGFP-NK and Loop CAR-NK cells and cultured them
175 consecutively without K562-mbIL-21 stimulation. Over a 12-day period, the number of Loop
176 CAR-NK cells was lower than EGFP-NK cells, and the gap gradually widened, confirming
177 that fratricide impaired the expansion of Loop CAR-NK cells (**Fig. 3E)**. In our previous work,
178 we established a method for inducing iNK and CAR-iNK cells from pluripotent stem cells (*11,*
179 *35*). Here, we generated a Loop CAR-expressing hPSC line (Loop CAR-hPSCs) and then
180 conducted the induction of Loop CAR-iNK cells (**Fig. 3F**). Briefly, Loop CAR-hPSCs were
181 induced for two days to differentiate into Loop CAR-expressing lateral plate mesoderm cells
182 (Loop CAR-iLPM), which were then combined with OP9 cells to form organoid aggregates.
183 After 25 days of induction, Loop CAR-iNK cells were generated (**Fig. 3G**). The resulting Loop
184 CAR-iNK cells expressed similar levels of CD56 and CD16 as iNK cells, with 78.6% of the
185 cells expressing the Loop CAR (**Fig. 3H**). Moreover, Loop CAR-iNK cells showed comparable
186 expression of typical NK cell cytotoxic effectors to the iNK cells (**fig. S3A**). Notably, the CD33
187 expression level in iNK cells was 69.7%, which was significantly higher than that observed in
188 tissue-derived NK cells (**Fig. 3I**). Next, we sorted $CD33^-$ and $CD33^+$ iNK cells and co-
189 incubated them with Loop CAR-iNK cells (**Fig. 3**, **J** and **K**). The Loop CAR-iNK cells showed
190 significant cytotoxicity against $CD33^+$ iNK cells compared to $CD33^-$ iNK cells (**Fig. 3L**). As
191 observed with the Loop CAR-NK cells, the number of Loop CAR-iNK cells gradually declined
192 during a 12-day expansion period, reinforcing that fratricide significantly impaired the
193 expansion of Loop CAR-iNK cells (**Fig. 3M**). Taken together, the expression of CD33 in Loop
194 CAR-NK and Loop CAR-iNK cells led to fratricide, which precluded their expansion.

195 **CD33 inactivation in iNK cells preserves their broad-spectrum cytotoxicity and facilitates**
196 **the expansion of Loop CAR-iNK cells**

To address the fratricide caused by CD33 expression in Loop CAR-iNK cells, we generated a CD33 gene knockout hPSC ($CD33^{KO}$-hPSC) line using the CRISPR/Cas12i system. Vectors carrying Cas12i, sgRNA1 (5'-TTCAGAAGTGGCCGCAAGGGAAG-3'), sgRNA2 (5'-TTGCTAGTCATCCCCAAATATCC-3'), and a neomycin resistance gene were transduced into hPSCs (**Fig. 4A**). Following neomycin selection, single clones were isolated and genotyped using four pairs of PCR primers (**fig. S4**, **A** and **B**). Ultimately, we identified a $CD33^{KO}$-hPSC clone with the deletion of exon 3 and exon 4 in one allele of CD33 and the insertion of a loxp-NeoR-loxp sequence in the other (**Fig. 4B** and **fig. S4**, **C** and **D**). Subsequently, $CD33^{KO}$-hPSCs successfully generated CD33-deficient iNK cells ($CD33^{KO}$-iNK) with immunophenotype similar to iNK cells (**Fig. 4C**). Moreover, the yields of $CD33^{KO}$-iNK cells were comparable to those of iNK cells (**Fig. 4D**). Importantly, $CD33^{KO}$-iNK cells exhibited cytotoxicity against K562 cells equivalent to that of iNK cells, demonstrating that the loss of CD33 did not compromise their broad-spectrum cytotoxicity (**Fig. 4E**). We then introduced the Loop CAR into $CD33^{KO}$-hPSCs to establish the $CD33^{KO}$-Loop CAR-hPSC line. As anticipated, these cells successfully differentiated into $CD33^{KO}$-Loop CAR-iNK cells, which exhibited expression levels of CD56 and CD16 comparable to those of iNK cells, with a CAR expression rate of 89.3% (**Fig. 4**, **F** and **G**). Furthermore, the expression of typical activating and inhibitory receptors, as well as cytotoxic granules, was consistent with that of iNK cells (**fig. S4E**). Most notably, $CD33^{KO}$-Loop CAR-iNK cells demonstrated a similar expansion capacity to that of iNK cells, indicating that CD33 disruption effectively resolved the fratricide issue and restored the proliferation of Loop CAR-iNK cells **(Fig. 4H)**. In conclusion, we successfully mitigated the fratricide issue in Loop CAR-iNK cells by knocking out CD33 in hPSCs, thereby preserving their broad-spectrum cytotoxicity and restoring their expansion.

Genetic disruption of CD33 in Loop CAR-iNK cells enhances their specific cytotoxicity

222 To evaluate the specific cytotoxicity of $CD33^{KO}$-Loop CAR-iNK cells, we incubated them with
223 Nomo-1 cells or MSLN-expressing HL-60 cells ($MSLN^{+}$ HL-60) (**Fig. 5**, **A** and **B**). Notably,
224 $CD33^{KO}$-Loop CAR-iNK cells exhibited significantly enhanced cytotoxicity compared to Loop
225 CAR-iNK cells, suggesting that fratricide in the latter compromised their specific cytotoxicity
226 (**Fig. 5**, **C** and **D**). To further evaluate the durability of the cytotoxic response, we performed
227 serial killing assays by analyzing the cytotoxicity of $CD33^{KO}$-Loop CAR-iNK cells (**Fig. 5E**).
228 Compared to Loop CAR-iNK cells, $CD33^{KO}$-Loop CAR-iNK cells consistently demonstrated
229 superior specific cytotoxicity against Nomo-1 cells in each round killing assay (**Fig. 5F**).
230 Additionally, we assessed the expression levels of key effector molecules, including TNF-α,
231 IFN-γ, and CD107a, in $CD33^{KO}$-Loop CAR-iNK cells following incubation with Nomo-1 and
232 $MSLN^{+}$ HL-60 cells. The results revealed that $CD33^{KO}$-Loop CAR-iNK cells expressed
233 significantly higher levels of these molecules compared to Loop CAR-iNK cells, further
234 corroborating their enhanced effector function (**Fig. 5**, **G-O**). Collectively, these findings
235 underscore the critical role of CD33 disruption in restoring and strengthening the specific
236 cytotoxicity of Loop CAR-iNK cells, demonstrating its importance in overcoming fratricide-
237 induced functional impairments.

238 **$CD33^{KO}$-Loop CAR-iNK cells effectively suppress AML tumor development in xenograft**
239 **animals**

240 To further evaluate the anti-tumor efficacy of $CD33^{KO}$-Loop CAR-iNK cells, we employed an
241 AML xenograft mouse model, using iNK cells as control effectors (**Fig. 6A**). $MSLN^{+}$ HL-60
242 cells were intravenously injected into NCG mice (5×10^5 cells/mouse) on day -1 to establish
243 the AML xenograft models. Mice with comparable tumor burdens were randomly assigned to
244 three groups (Tumor only, iNK, and $CD33^{KO}$-Loop CAR-iNK). $CD33^{KO}$-Loop CAR-iNK cells
245 were administered intravenously at a dose of 1×10^7 cells/mouse on days 0 and 3 (**Fig. 6B**).
246 Tumor progression was monitored weekly using bioluminescence imaging (BLI). The results

247 showed that the tumor burden in mice treated with $CD33^{KO}$-Loop CAR-iNK cells was
248 significantly lower than in those treated with iNK cells, demonstrating the potent anti-tumor
249 activity of $CD33^{KO}$-Loop CAR-iNK cells (**Fig. 6**, **C** and **D**). Moreover, the enhanced tumor
250 suppression translated into a significant survival benefit, with $CD33^{KO}$-Loop CAR-iNK-treated
251 mice achieving a median survival of 42 days, compared to shorter survival in the control groups
252 (**Fig. 6E**). Collectively, these findings demonstrate that $CD33^{KO}$-Loop CAR-iNK cells
253 effectively eliminate AML tumor cells *in vivo* and prolong the survival of xenograft models,
254 underscoring their therapeutic potential for clinical application.

255 **DISCUSSION**

256 The dual CAR strategy is an effective approach to prevent tumor escape caused by antigen
257 heterogeneity or loss. Bivalent CAR, one CAR containing two scFvs, which can result in higher
258 transduction efficiency than bicistronic CAR, two single-targeted CARs. Bivalent CAR can be
259 categorized into tandem CAR and loop CAR (*36*). Previous studies have reported that loop
260 CAR exhibited superior anti-tumor potency than tandem CAR (*37, 38*). In this study, we
261 developed a bivalent loop CAR targeting CD33 and mesothelin (MSLN) (CD33-MSLN loop
262 CAR, denoted as Loop CAR). When the Loop CAR expression cassette was introduced into
263 umbilical cord blood NK (UCB-NK) cells, the resulting Loop CAR-NK cells exhibited superior
264 cytotoxicity against CD33 and MSLN-expressing tumor cells, including Nomo-1 and primary
265 $CD33^{+}MSLN^{+}$ AML cells. Furthermore, Loop CAR-NK cells demonstrated comparable
266 cytotoxicity against $CD33^{+}MSLN^{-}$ and $CD33^{-}MSLN^{+}$ tumor cells compared to CD33 CAR-
267 NK and MSLN CAR-NK cells. The accumulation of Loop CAR proteins at the immunological
268 synapse between Loop CAR-NK cells and Nomo-1 cells likely contributes to their enhanced
269 specific cytotoxicity (*39, 40*). Additionally, Nomo-1 cells stimulated Loop CAR-NK cells to
270 express higher levels of CD107a, a membrane protein associated with NK cell cytotoxic
271 activity (*41*). Transcriptome analysis revealed that Loop CAR-NK cells exhibited upregulated

signaling pathways related to cell division and activation after incubation with Nomo-1 cells. Genes such as *NCAM1*, *PTK2B*, and *DOCK8*, which are involved in immunological synapse formation and cytotoxic function, were significantly upregulated (*31-34, 42*).

Consistent with the previous report (*23*), a fraction of UCB-NK cells naturally express CD33. Our findings confirmed that hPSC-derived iNK cells exhibit even higher levels of CD33 expression compared to UCB-NK cells. This led to fratricide in both Loop CAR-NK and Loop CAR-iNK cells, ultimately impairing their expansion. Similar fratricide-induced expansion impairments have been previously reported in human tissue-derived CD33 CAR-NK cells (*17*), highlighting the necessity of addressing this challenge. To overcome the fratricide issue, we genetically disrupted CD33 in hPSCs ($CD33^{KO}$-hPSCs) using CRISPR/Cas technology. Previous studies have shown that CD33 knockout in hematopoietic stem cells allows for the development of a functional hematopoietic system without impairing differentiation potential (*21*). Our results demonstrated that $CD33^{KO}$-hPSCs successfully differentiated into iNK cells without affecting their induction efficiency or anti-tumor activity. Although CD33 has been considered an inhibitory receptor due to its cytoplasmic tyrosine-based inhibitory motifs (ITIMs) (*43*). We observed no significant differences in cytotoxicity against K562 cells between $CD33^{KO}$-iNK and iNK cells.

As expected, $CD33^{KO}$-Loop CAR-hPSCs efficiently generated $CD33^{KO}$-Loop CAR-iNK cells. The elimination of CD33 expression successfully prevented fratricide and restored the expansion capacity of Loop CAR-iNK cells. Interestingly, $CD33^{KO}$-Loop CAR-iNK cells demonstrated superior specific cytotoxicity against Nomo-1 and $MSLN^{+}$ HL-60 cells compared to Loop CAR-iNK cells, indicating that fratricide in the latter not only impaired their expansion but also reduced their specific cytotoxicity. Moreover, $CD33^{KO}$-Loop CAR-iNK cells significantly suppressed tumor progression and prolonged the survival of AML xenograft mice. We used $MSLN^{+}$ HL-60 cells, an M2 subtype of acute myeloid leukemia (AML), to

297 construct the tumor models, as these cells exhibit high tumorigenic potential *in vivo*.
298 Additionally, we attempted to use Nomo-1 cells, an M5a subtype of acute monocytic leukemia,
299 to establish a tumor model. However, the tumorigenic efficiency was suboptimal with Nomo-
300 1 cells. Given NK cells' relatively lower genome editing efficiency compared to T cells (*44*),
301 targeting endogenous CD33 at the pluripotent stem cell (PSC) stage represents a more practical
302 approach to avoid potential fratricide. With their amenability to genetic engineering, clonal
303 selection capabilities, and lack of T cell contamination, PSC-derived NK cells hold promise as
304 standardized, off-the-shelf immunotherapy products (*45, 46*).
305 Despite significant progress in identifying AML-specific antigens as therapeutic targets,
306 challenges remain due to their genomic and phenotypic heterogeneity and overlapping
307 expression with normal hematopoietic cells (*5*). CD33, expressed in over 80% of AML cells,
308 is absent from primitive stem cells and multipotent progenitor cells, making it an attractive
309 target for AML immunotherapy (*47, 48*). Recent clinical data have demonstrated the safety and
310 efficacy of functionally enhanced CD33 CAR-NK cells (*10*). MSLN, a well-established target
311 in solid tumors, is expressed in approximately 36% of pediatric AML cases and 14% of adult
312 AML cases, but is virtually absent in normal hematopoietic cells (*4*). MSLN CAR-T cells can
313 efficiently eliminate MSLN-positive ($MSLN^{+}$) AML cells in xenograft models derived from
314 cell lines and patients (*5*). However, MSLN is actively cleaved from the cell membrane and is
315 not highly expressed in AML cells. Strategies such as small molecules (e.g., INCB7839,
316 GM6001) and immunotoxins (e.g., RG7787, SS1P) have been developed to upregulate MSLN
317 expression and reduce shedding, thereby enhancing its viability as a therapeutic target (*5, 49,*
318 *50*). Our findings demonstrated that CD33-MSLN loop CAR-NK cells exhibited superior
319 cytotoxicity against primary AML cells expressing both CD33 and MSLN (23.5%-57.8%).
320 Moreover, $CD33^{KO}$-CD33-MSLN loop CAR-iNK cells displayed robust anti-tumor efficacy
321 both *in vitro* and in xenograft models. These results indicate that the CD33-MSLN loop CAR

design effectively enhances the tumor-killing potential of NK cells and supports the successful development of a dual-targeting CAR strategy. Additionally, by genetically inactivating CD33, we effectively addressed the fratricide issue, further improving the function of CD33-MSLN loop CAR-iNK cells. In conclusion, our study provides compelling preclinical evidence supporting the further evaluation of CD33-MSLN loop CAR-NK and $CD33^{KO}$-CD33-MSLN loop CAR-iNK cells as promising candidates for clinical trials in the treatment of $MSLN^{+}$ AML.

MATERIALS AND METHODS

Study design

The objective of this study was to develop a more efficient therapeutic CAR-NK cell for the treatment of acute myeloid leukemia (AML). This was achieved by designing a bivalent CD33-MSLN loop CAR (Loop CAR) and inactivating CD33, thereby generating Loop CAR-iNK cells that avoid fratricide and effectively target and kill $MSLN^{+}CD33^{+}$ AML cells. The construction of Loop CAR was described in the results. The functionality of the Loop CAR was validated by cloning its expression elements into the SFG vector and employing the RD114 retroviral system in conjunction with umbilical cord blood NK cells to generate Loop CAR-NK cells. The superior *in vitro* cytotoxicity of Loop CAR-NK cells compared to CD33 CAR-NK and MSLN CAR-NK cells was assessed through tumor killing assays against $CD33^{+}MSLN^{+}$ tumor cell lines (Nomo-1 and $MSLN^{+}$ HL-60). Furthermore, the expression of CD107a in CAR-NK cells stimulated by Nomo-1 cells was analyzed to evaluate degranulation capacity. Single-cell RNA sequencing (scRNA-seq) using the 10× Genomics platform further demonstrated the enhanced effector functions of loop CAR-NK cells at the transcriptomic level following co-incubation with Nomo-1 cells.

To extend this strategy, the Loop CAR was introduced into hPSCs to generate Loop CAR-iNK cells. However, functional assays revealed that both Loop CAR-NK and Loop CAR-NK iNK cells exhibited fratricide by targeting $CD33^{+}$ NK cells or iNK cells, leading to limited expansion. To address this challenge, the CRISPR/Cas12i system was employed to generate a CD33 knockout ($CD33^{KO}$) hPSC line. We first confirmed that CD33 disruption did not affect the immunophenotype or cytotoxic function of iNK cells through flow cytometric immunophenotypic analysis and tumor-killing assays against K562 cells. Subsequently, the Loop CAR was introduced into the $CD33^{KO}$-hPSCs to produce $CD33^{KO}$-Loop CAR-iNK cells. Flow cytometric analysis and *in vitro* expansion experiments confirmed that the loss of CD33

354 did not alter the immunophenotypic characteristics of Loop CAR-iNK cells but significantly
355 enhanced their expansion potential. To validate the improved *in vitro* effector functions of
356 $CD33^{KO}$-Loop CAR-iNK cells, tumor-killing assays and flow cytometry analysis of CD107a
357 expression and effector molecule secretion (IFN-γ and TNF-α) were conducted following
358 stimulation with Nomo-1 and $MSLN^{+}$ HL-60 cells. To assess the *in vivo* anti-tumor efficacy of
359 $CD33^{KO}$-Loop CAR-iNK cells, AML xenograft models were established using $MSLN^{+}$ HL-60
360 cells in NCG mice. Tumor-bearing mice were treated with either iNK or $CD33^{KO}$-Loop CAR-
361 iNK cells, and therapeutic efficacy was monitored through weekly bioluminescence imaging.
362 Blinding was not employed in the experiments. Each experiment was conducted independently
363 on different days using different batches of reagents. Experimental data were repeated to ensure
364 an adequate number of biological replicates. Details regarding sample sizes, replicates, and
365 statistical analyses are provided in the figures, figure legends, and supplementary materials.

366 **Cell culture**

367 Human embryonic kidney (HEK) 293T cells (ATCC) were cultured in Dulbecco's Modified
368 Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS,
369 HUANGKE). Human cervical cancer HeLa cells (Procell Life Science & Technology Co., Ltd)
370 were maintained in Minimum Essential Medium α (MEMα, Gibco) with 10% FBS.
371 Mesothelin-expressing ($MSLN^{+}$) AML cell lines, Nomo-1 (COBIOER), and luciferase-
372 expressing Nomo-1 were maintained in RPMI 1640 (Gibco) supplemented with 10% FBS.
373 Human gastric adenocarcinoma AGS cells (Procell Life Science & Technology Co., Ltd) were
374 cultured in Ham's F12 (Gibco) supplemented with 10% FBS. HL-60 (Procell Life Science &
375 Technology Co., Ltd) and luciferase-expressing $MSLN^{+}$ HL-60 ($MSLN^{+}$ HL-60 $luci^{+}$) cells
376 were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with
377 20% FBS. To generate MSLN-expressing HL-60 cell line, the cDNA encoding MSLN was
378 inserted into the PiggyBac expression vector (SBI) to generate a recombinant vector. The

379 MSLN-expressing PiggyBac vector and the transposase expression vector were electroporated
380 into HL-60 cells using Electroporator EX+ (Celetrix). Seven days after electroporation, HL-60
381 cells were sorted for two rounds to establish the MSLN-expressing HL-60 cell line. The PSC
382 line (Q380-ESC) was provided by the National Stem Cell Resource Center, Institute of Zoology,
383 Chinese Academy of Sciences. All hPSCs were kept in Essential 8 Medium (Gibco) on
384 Vitronectin-Recombinant Human Protein (rhVTN-N, Gibco) coated plates. The hPSCs
385 differentiation toward iNK and the related anti-tumor activity assessments of iNK cells in
386 animals were approved by the Biomedical Research Ethics Committee of the Institute of
387 Zoology, Chinese Academy of Sciences.

388 **Generation of CAR constructs and CAR-modified NK cells**

389 The monospecific CAR constructs included a signal peptide (SP), the anti-CD33 or anti-
390 mesothelin (MSLN) single-chain variable fragment (scFv) (*51, 52*), the CD8α hinge domain, a
391 transmembrane domain (TMD) of CD8, and a signaling domain (SD) of CD3ζ. The Loop CAR
392 construct was designed using the same backbone, with the scFv arrangement as follows: CD33
393 (V_L)-MSLN (V_H)-MSLN (V_L)-CD33 (V_H) were fused with the G4S linker and the whitlow
394 linker (*53*). The EGFP coding sequence or CAR constructs were cloned into SFG recombinant
395 retroviral vectors (*54*). Retroviruses carrying the CAR vectors were generated as previously
396 described (*25*). Umbilical cord blood (UCB) units were provided by Guangdong Cord Blood
397 Bank (Guangzhou, China). $CD3^-$ umbilical cord blood mononuclear cells (UCBMCs) were
398 isolated, and activated NK cells were produced as previously described (*25*). On day 0,
399 activated NK cells were transduced with retroviral supernatants at a multiplicity of infection
400 (MOI) of 5, following the Vectofusin-1 (Miltenyi Biotec) based transduction protocol. Infection
401 rates were assessed by flow cytometry on day 2. CAR-NK cells were harvested for use after 4

402 days of expansion. The CAR-NK cells were sorted and expanded for 12 days without
403 stimulation by K562-mbIL-21 cells (Hangzhou Zhongying Biomedical Technology Co., Ltd).

404 **Flow cytometry**

405 Transduced UCB-NK cells were stained with fluorochrome-conjugated antibodies against CD3
406 (UCHT1), CD56 (HCD56) (Biolegend), CD33 scFv, and MSLN scFv (ACROBiosystems) for
407 analysis of CAR expression using a BD LSRFortessa flow cytometer (BD Biosciences). Anti-
408 CD56, anti-CD33 scFv, and anti-MSLN scFv antibodies were used to sort CAR-NK cells using
409 Sony MA900 (SONY). Human anti-CD33 antibody (P67.6, Biolegend) and human anti-MSLN
410 antibody (Abcam) were used to determine the expression levels of CD33 and MSLN in tumor
411 cell lines and primary AML cells. The immunophenotypes of iNK cells were analyzed using
412 antibodies against CD45 (HI30, Biolegend), CD3, CD56, CD16 (3G8, Biolegend), CD33 scFv
413 and MSLN scFv. To evaluate the expression of NK receptors and effector molecules, we used
414 antibodies against CD56, NKp44 (P44-8), NKp30 (P30-15), NKG2D (1D11), CD69 (FN50),
415 CD16, CD319 (162.1), CD226 (11A8), CD96 (NK92.39), CD94 (HP-3D9), NKG2A
416 (S19004C), Perforin (dG9), GzmB (QA18A28) (all from Biolegend), CD33 scFv and MSLN
417 scFv. Cells were resuspended in DAPI (Sigma-Aldrich) or Dulbecco's phosphate-buffered
418 saline (BasalMedia Technologies) for analysis with the BD LSRFortessa cytometer (BD
419 Biosciences). Data were analyzed with FlowJo software (FlowJo_v10.8.1).

420 **Cytotoxicity assays**

421 For cytotoxicity assessment, target cells, labeled with eBioscience™ Cell Proliferation Dye
422 eFluor™ 670 (eFluor 670) (Invitrogen), were co-cultured with NK cells in the indicated
423 effector (E) to target (T) ratios (E:T). Target cell death was quantified using the BD
424 LSRFortessa flow cytometer or BD LSRFortessa X-20 cytometer (BD Biosciences) by

425 measuring the percentage of DAPI$^+$ among eFluor 670-labeled cells. In serial killing assays,
426 iNK cells received three rounds tumor challenge. Round 1: eFluor 670-labeled (eFluor 670$^+$)
427 Nomo-1 cells were mixed with relevant iNK cells and co-incubated for 12 h (E:T = 1:1).
428 Analysis of the cell lysis of eFluor 670$^+$ Nomo-1 cells at 12 h represents the Round 1
429 cytotoxicity of the relevant iNK cells. Round 2: Unlabeled Nomo-1 cells were mixed with
430 relevant iNK cells and co-incubated for 12 h (E:T = 1:1). Equal numbers of eFluor 670-labeled
431 Nomo-1 cells were added and co-incubated for the next 12 h. Analysis of the cell lysis of eFluor
432 670$^+$ Nomo-1 cells at 24 h represents the Round 2 cytotoxicity of the relevant iNK cells. Round
433 3: Unlabeled Nomo-1 cells were mixed with relevant iNK cells (E:T = 1:1). Equal numbers of
434 unlabeled Nomo-1 cells and eFluor 670-labeled Nomo-1 cells were added at 12 h and 24 h
435 respectively. Analysis of the cell lysis of eFluor 670$^+$ Nomo-1 cells at 36 h represents the Round
436 3 cytotoxicity of the relevant iNK cells. Specific cytotoxicity was calculated using the formula:
437 (percentage of specific death – percentage of spontaneous death) / (1 – percentage of
438 spontaneous death) × 100%.

439 **Assessment of CD107a, TNF-α, and IFN-γ by flow cytometry**

440 To evaluate CD107a expression, NK or iNK cells were co-cultured with or without Nomo-1 or
441 MSLN$^+$ HL-60 cells at an E:T ratio of 0.5:1 for 4 h. After incubation, cells were stained with
442 antibodies against CD56, CD107a (H4A3, Biolegend), CD33 scFv, and MSLN scFv. To
443 evaluate TNF-α and IFN-γ expression, iNK cells were co-cultured with or without Nomo-1 or
444 MSLN$^+$ HL-60 cells at an E:T ratio of 0.5:1 for 2 h, followed by adding BFA/Monensin
445 (MULTISCIENCES) for additional 2-h incubation. After incubation, cells were stained with
446 antibodies against CD56, CD33 scFv, and MSLN scFv. FIX &PERM Kit (MULTISCIENCES)
447 was used for fixation and permeabilization, followed by intracellular staining for TNF-α
448 (Biolegend, MAb11) or IFN-γ (Biolegend, 4S.B3). The cells were analyzed with BD

449 LSRFortessa X-20 cytometer (BD Biosciences). Data were analyzed with FlowJo software
450 (FlowJo_v10.8.1).

451 **Immunofluorescence imaging**

452 Immunofluorescence imaging of immune synapses of Loop CAR-NK cells by confocal
453 microscopy as previously described (*55*). Briefly, immune synapses of Loop CAR-NK cells
454 were formed by suspending NK cells at 5×10^6/mL and Nomo-1 target cells at 2.5×10^6/mL.
455 Equal volumes of Loop CAR-NK cell and Nomo-1 target cell suspensions were mixed and
456 incubated to form conjugates. Loop CAR-NK and Nomo-1 cell conjugates were permeabilized
457 using the FIX & PERM kit (MULTISCIENCES) and stained with antibodies against phalloidin
458 (Thermo Fisher Scientific), CD33 scFv, MSLN scFv, pericentrin (Abcam), and perforin
459 (Biolegend). Sequential scanning with a Leica TCS SP8 laser scanning confocal microscope
460 imaged NK cell immune synapses. The images were acquired using LASAF software (Leica).

461 **Quantitative real-time PCR**

462 The *MSLN* transcripts were reverse transcribed into cDNA using HiScript III All-in-One RT
463 SuperMix Perfect for qPCR (Vazyme) and quantified by quantitative real-time PCR using
464 Hieff® qPCR SYBR® Green Master Mix (Yeasen Biotechnology). *GAPDH* was used for
465 normalization. The relative gene expression levels were calculated as $2^{-\Delta Ct}$. All primers used in
466 this study were listed in **table S1**.

467 **Primary samples**

468 Primary AML cells were isolated from AML diagnostic bone marrow or peripheral blood
469 samples obtained from the State Key Laboratory of Experimental Hematology, National
470 Clinical Research Center for Blood Diseases, Institute of Hematology & Blood Diseases

471 Hospital, Chinese Academy of Medical Science & Peking Union Medical College, and
472 Department of Hematology, Guangdong Provincial People's Hospital. Primary AML cells were
473 thawed for immunophenotype analysis of MSLN expression and cultured in IMDM
474 supplemented with 20% FBS, 10 ng/ml GM-CSF (MedChemExpress), 100 U/ml DNase I
475 (Sigma Aldrich), and 50 uM GM6001 (Selleck) (a matrix metalloprotease inhibitor that
476 stabilizes MSLN expression on the cell surface (*5*) for cytotoxicity assays. *In vitro* killing
477 assays were performed as mentioned above. The use of patient samples was carried out by the
478 provisions of the Declaration of Helsinki. All patient samples were collected with patient
479 consent signatures and reviewed and approved by the Ethics Committee of the Hospital for
480 Hematology & Blood Diseases and the Guangdong Provincial People's Hospital, respectively.

481 **Single-cell RNA-sequencing and data analysis**

482 FACS-sorted EGFP-NK ($CD56^+EGFP^+$) and CAR-NK cells ($CD56^+CAR^+$) were activated
483 with eFluor 670 labeled (eFluor 670^+) Nomo-1 cells for 72 h. Fifty thousand cells sorted (eFluor
484 670^- and $CD56^+$) from activated NK populations underwent RNA-sequencing (10× Genomics).
485 Droplet-based scRNA-seq datasets were generated using a Chromium system (10× Genomics,
486 PN120263) following the manufacturer's instructions. The raw data were uploaded to the
487 public database of Genome Sequence Archive (HRA008163). Subsequently, these data sets
488 were aligned and quantified using the CellRanger software package (version 6.0.0) and
489 subjected to Seurat (version 3.2.3) for further analysis. To pass quality control, cells were
490 required to have at least 200 genes detected, less than 7,500 genes detected, and less than 20%
491 of aligned reads mapped to mitochondrial genes. 15,408, 17269, 15273, and 16734 cells from
492 EGFP-NK, CD33 CAR-NK, MSLN CAR-NK, and Loop CAR-NK scRNA-seq datasets that
493 passed quality control were merged and used for downstream analysis. The Unique Molecular
494 Identifier (UMI) value was normalized using Seurat's 'LogNormalize' and 'ScaleData'

495 methods. The Principal Component Analysis (PCA) used 2,000 highly variable genes (as
496 defined by Seurat), and clustering was performed on the most variable principal components
497 (PCs). The most variable PCs were determined by the Jackstraw method. Then, the 36 most
498 variable PCs were used for clustering and Uniform Manifold Approximation and Projection
499 (UMAP) analysis with resolution 0.2 and other default parameters. The median and dot plots
500 for gene expression were plotted using the Seurat VlnPlot function and the ggplot2 package.
501 Differential expression genes (DEGs) were identified using the neurinoma test with the
502 parameter 'min.pct = 0.25, logfc threshold = 0.25'. Gene ontology (GO) enrichment analysis
503 for DEGs was performed by the clusterProfiler package (version 4.2.2) with BH adjusted cutoff
504 value = 0.05. Box plots of the normalized expression of DEGs were plotted using the ggplot2
505 package. Volcano plots were plotted using ggplot2 and the ggrepel package.

506 **Regeneration of CAR-iNK cells**

507 CAR-expressing hPSCs were engineered using the piggyBac transposon system, as mentioned
508 above. CAR-iNK cells were regenerated from CAR-transfected hPSCs and harvested for use
509 as previously described (*11*). Briefly, CAR-hPSCs were performed a two-day induction to
510 produce CAR lateral plate mesoderm cells (CAR-iLPM). Then 2×10^4 CAR-iLPM were
511 combined with 5×10^5 OP9 cells to form organoid aggregates and seeded in the transwell
512 (Corning, 3450). Subsequently, the medium was changed every 2-3 days to induce NK cell
513 regeneration. After 25 days of induction, mature CAR-iNK cells were harvested for use.

514 **CRISPR/Cas12i-mediated CD33 knockout in hPSCs**

515 Two sgRNA sequences, sgRNA1 (TTCAGAAGTGGCCGCAAGGGAAG) and sgRNA2
516 (TTGCTAGTCATCCCCAAATATCC), targeting exon 3 and exon 4 of CD33 respectively,
517 were designed using a web-based guide-RNA designer (http://www.rgenome.net/) and

518 subsequently cloned into the Cas12i expression vector (a gift from Dr. Wei Li of the Institute
519 of Zoology, Chinese Academy of Sciences) (*56*). To create CD33 knockout hPSCs ($CD33^{KO}$-
520 hPSCs), the two Cas12i-sgRNA expression vectors and a donor vector carrying the neomycin
521 resistance gene (NeoR) were co-transfected into hPSCs using electroporation. Five days post-
522 transfection, G418 (InvivoGen) was introduced to select the targeted hPSCs for one week.
523 Following G418 selection, the cells were digested and single cells were seeded into 96-well
524 plates to form single-cell clones. PCR and sequencing were used to verify the mutation sites,
525 thereby establishing CD33 knockout clones.

526 **Genome PCR**

527 Single-cell clones of hPSCs resistant to G418 were harvested for DNA extraction using the
528 TIANamp Genomic DNA kit (TIANGEN). The CD33 locus target sequences were amplified
529 with the 2 × Rapid Taq Master Mix (Vazyme) and the primer pairs depicted in **fig. S4**.

530 **AML xenograft models with CAR-iNK cell treatment**

531 The $MSLN^{+}$ HL-60 $luci^{+}$ cells (5×10^{5} cells/mouse) were injected intravenously into NCG
532 mice (NOD/ShiLtJGpt-*Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/Gpt strain, GemPharmatech Co., Ltd.) to
533 establish human AML xenograft models on day -1. On day 0, bioluminescence imaging (BLI,
534 IVIS Spectrum PerkinElmer) was used to quantify the tumor graft burden in each group. After
535 irradiation (1.0 Gy, Rad Source RS2000), the animals were randomly assigned to treatment
536 groups and received an intravenous injection of the equivalent of 1×10^{7} fresh CAR^{+} iNK or
537 unmodified iNK cells. Three days later (day 3), the animals received the second intravenous
538 injection with the same cell count. Tumor aggressiveness was determined weekly by BLI. Mice
539 with a significant tumor burden were humanely euthanized. All animal experiments were

conducted under the Institutional Animal Care and Use Committee (IACUC) of the Institute of Zoology of the Chinese Academy of Sciences (IOZ-2021-182).

Statistical analysis

All quantitative analysis were performed with SPSS (IBM SPSS Statistics 25). The Shapiro-Wilk normality test assessed the normal distribution of the data. Quantitative differences (mean ± SD) between samples were compared using two-tailed Student's t-test, Mann-Whitney U test, one-way ANOVA test, and two-way ANOVA test. The *P values* were two-sided, and $P < 0.05$ was considered statistically significant. Survival curves for tumor-bearing models were plotted using the Kaplan-Meier method and compared to the treatment group using logarithmic rank (Mantel-Cox) tests. Statistical analysis was performed using the GraphPad Prism 9 software.

List of Supplementary Materials

Fig. S1 to S4 for multiple supplementary figures.

Table S1 for multiple supplementary table.

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Author contributions

Y.W. and X.J.Z. designed and conducted all experiments, performed data analysis, and wrote the manuscript. Z.Q.W., Z.Y.X., F.Z., Y.H.L., and P.C.L. conducted some experiments and performed data analysis. Y.Q.L. and Q.T.W. performed scRNA-seq and data analysis. L.Q.Z., C.X.X., D.H.H., L.J.L., Y.P.Z., T.J.W., M.Y.Z., W.Y.Y., and M.M.L. participated in multiple experiments. J.Y.W., X.F.Z., X.D., W.B.Q., and A.B.L. discussed the data and manuscript. F.X.H. designed the project, designed and guided all experiments, scrutinized all experimental data, and wrote the manuscript. J.Y.W. revised the manuscript and provided the final approval of the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

Data and materials availability

The scRNA-seq data of EGFP-NK, CD33 CAR-NK, MSLN CAR-NK, and Loop CAR-NK cells stimulated by Nomo-1 cells have been uploaded to the Genome Sequence Archive public database (HRA008163). Other relevant information or data is available from the corresponding

authors upon reasonable request.

Figures

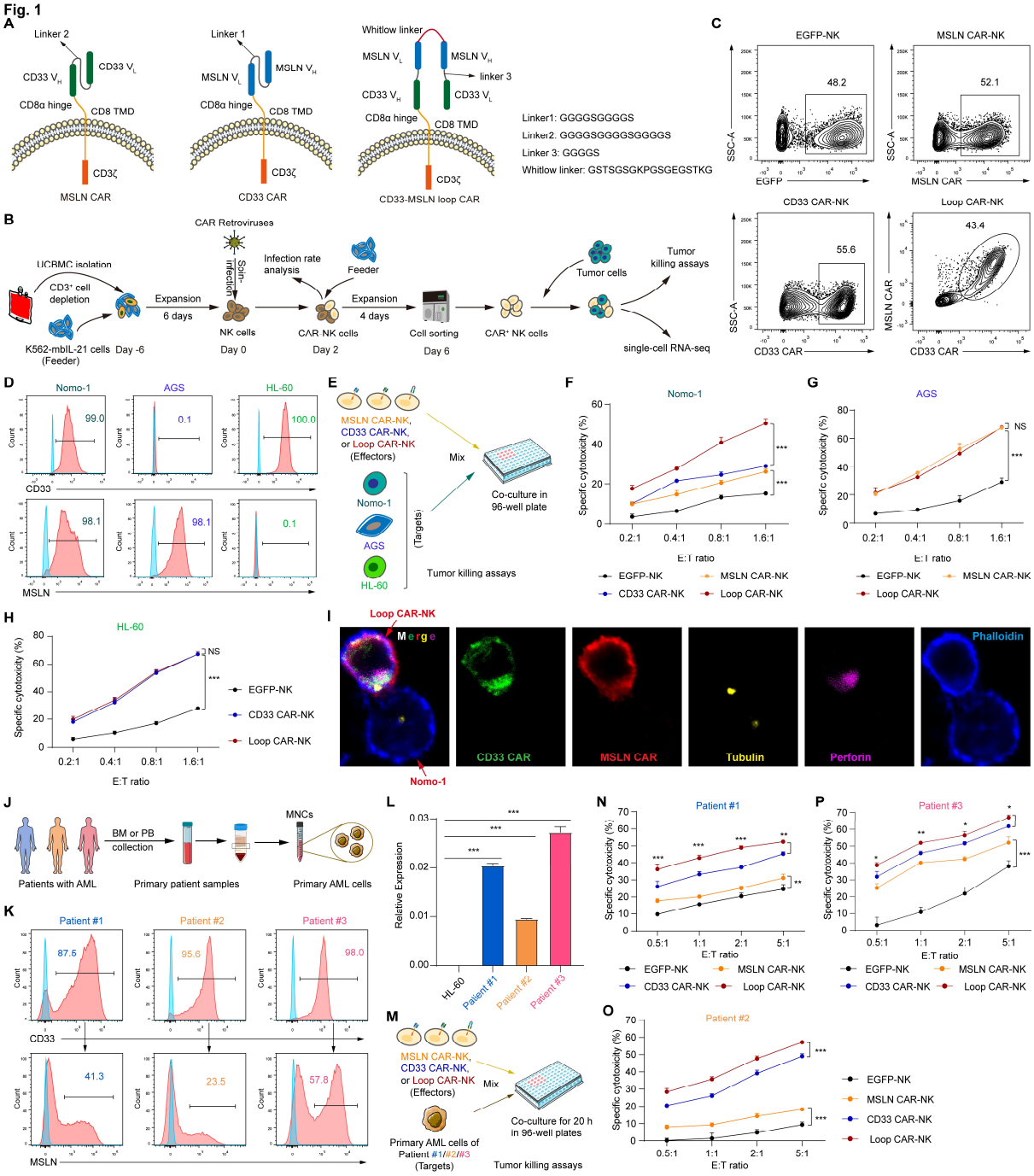


Fig. 1. Development and functional evaluation of Loop CAR-NK cells. (**A**) Schematic diagram of the design of Loop CAR. (**B**) Schematic diagram showing the generation of Loop CAR-NK cells procedures and evaluation their cytotoxicity. (**C**) Flow cytometric analysis of the transduction efficiencies of each group of CAR-NK cells. EGFP virus-infected NK cells were used as control. (**D**) Flow cytometry histograms showing the expression levels of CD33

760 and MSLN in target cell lines, including $CD33^{+}MSLN^{+}$ Nomo-1 AML cells, $CD33^{-}MSLN^{+}$
761 AGS gastric adenocarcinoma cells, and $CD33^{+}MSLN^{-}$ HL-60 AML cells. (**E**) Tumor killing
762 assay design. (**F-H**) Statistics of CAR-NK cells cytotoxicity against Nomo-1 tumor cells (F),
763 AGS cells (G), and HL-60 cells (**H**) at indicated E:T ratios. Nomo-1 cells and HL-60 cells were
764 incubated with effectors for 4 hours (n = 4 per group). AGS cells were incubated with effectors
765 for 6 hours (n = 4 per group). (**I**) Confocal microscopy representative images of immunological
766 synapse formed between loop CAR-NK cells and Nomo-1 cells. The conjugates were stained
767 with antibodies against perforin (Violet), phalloidin-F-actin (Blue), CD33 CAR (Green) and
768 MSLN CAR (Red). (**J**) Schematic diagram showing the collection of peripheral blood or bone
769 marrow cells from patients with AML. (**K**) Flow cytometric analysis of the expression of CD33
770 and MSLN in primary AML cells. (**L**) Real-time quantitative PCR analysis of MSLN transcript
771 expression levels in primary AML cells. The *GAPDH* gene was used as an internal control.
772 Relative gene expression levels were calculated as $2^{-\Delta Ct}$. (**M**) Schematic diagram showing
773 primary AML cells killed by CAR-NK cells *in vitro*. Each group of NK cells was co-cultured
774 with primary AML cells for 20 hours at indicated E:T ratios (n = 4 per group). (**N-P**) Specific
775 cytotoxicity of CAR-NK cells derived from UCB-NK against primary AML cells from three
776 patients (Patient #1: N, Patient #2: O, Patient #3: P). Statistical analysis: one-way ANOVA. NS:
777 not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig. 2

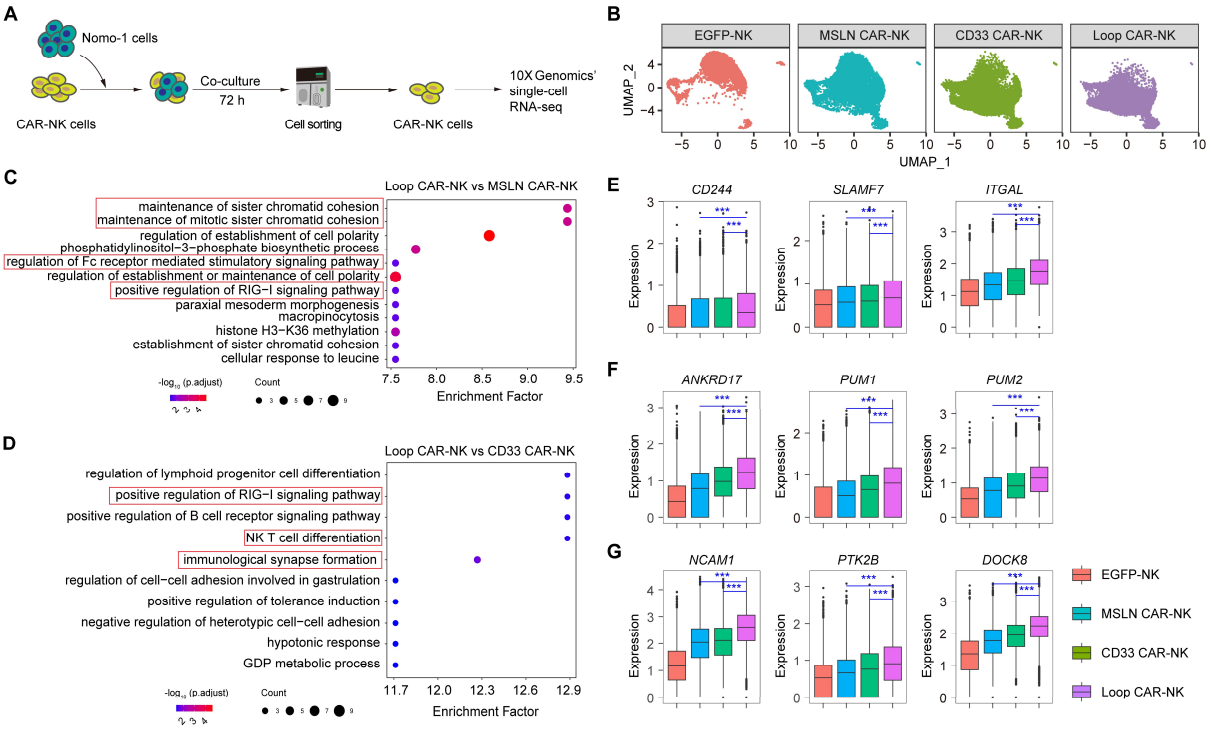
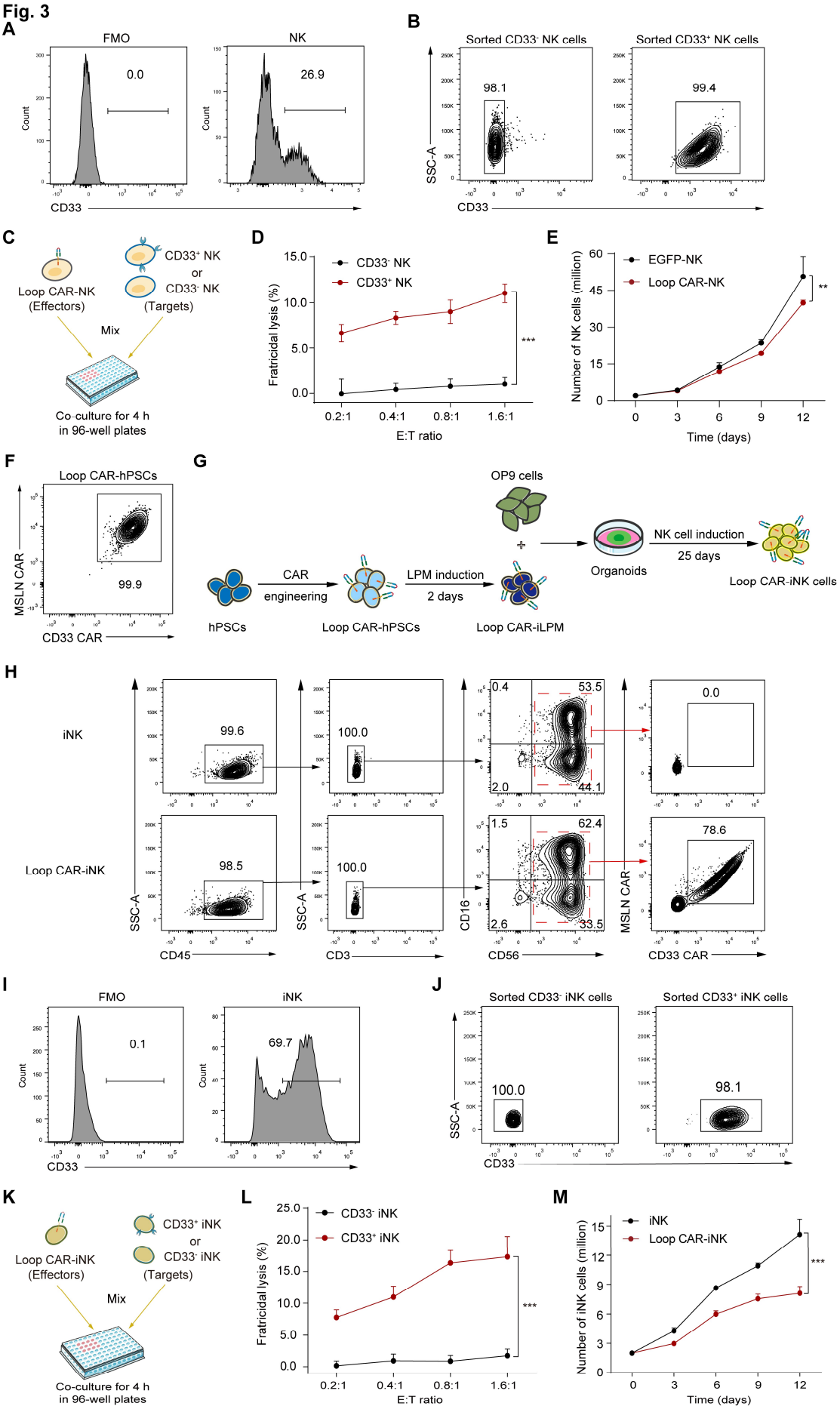


Fig. 2. Molecular characterization of Loop CAR-NK cells. (**A**) Schematic diagram of sorting CAR-NK cells for 10× Genomics' single-cell RNA-seq assay. CAR-NK cells were incubated with Nomo-1 cells (E:T = 1:1) for 72 h before sorting for RNA-seq. (**B**) UMAP visualization contrasting EGFP-NK, CD33 CAR-NK, MSLN CAR-NK, and Loop CAR-NK cells. EGFP-NK cells were used as control. (**C** and **D**) Gene Ontology (GO) analysis of differentially highly expressed genes in Loop CAR-NK cells compared with MSLN CAR-NK (C) and CD33 CAR-NK (D) cells, respectively. Each data point represented a distinct GO set. The color scale corresponded to $-\log_{10}$ transformed false discovery rate (FDR)– adjusted P values. The size of the dot was the number of genes enriched in one term. The enrichment factor was GeneRatio/BgRatio. (**E-G**) Box plots showed the expression profiles of differentially highly expressed genes related to activation and cytotoxicity of NK cells (E), participating in the RIG-1 signaling pathway (F), and involving in immunological synapse formation (G) in Loop CAR-NK cells compared with CD33 CAR-NK and MSLN CAR-NK cells.

Fig. 3


A
FMO
NK
0.0
26.9
Count
CD33
B
Sorted CD33⁻ NK cells
Sorted CD33⁺ NK cells
98.1
99.4
SSC-A
CD33
C
Loop CAR-NK
(Effectors)
CD33⁺ NK
or
CD33⁻ NK
(Targets)
Mix
Co-culture for 4 h
in 96-well plates
D
CD33⁻ NK
CD33⁺ NK
Fratricidal lysis (%)
E:T ratio
0.2:1
0.4:1
0.8:1
1.6:1
***
E
EGFP-NK
Loop CAR-NK
Number of NK cells (million)
Time (days)
**
F
Loop CAR-hPSCs
99.9
MSLN CAR
CD33 CAR
G
OP9 cells
hPSCs
CAR
engineering
Loop CAR-hPSCs
LPM induction
2 days
Loop CAR-iLPM
Organoids
NK cell induction
25 days
Loop CAR-iNK cells
H
iNK
Loop CAR-iNK
99.6
100.0
0.4
53.5
2.0
44.1
0.0
98.5
100.0
1.5
62.4
2.6
33.5
78.6
SSC-A
CD45
CD3
CD16
CD56
MSLN CAR
CD33 CAR
I
FMO
iNK
0.1
69.7
Count
CD33
J
Sorted CD33⁻ iNK cells
Sorted CD33⁺ iNK cells
100.0
98.1
SSC-A
CD33
K
Loop CAR-iNK
(Effectors)
CD33⁺ iNK
or
CD33⁻ iNK
(Targets)
Mix
Co-culture for 4 h
in 96-well plates
L
CD33⁻ iNK
CD33⁺ iNK
Fratricidal lysis (%)
E:T ratio
0.2:1
0.4:1
0.8:1
1.6:1
***
M
iNK
Loop CAR-iNK
Number of iNK cells (million)
Time (days)
***

792

Fig. 3. Expression of CD33 in NK or iNK cells led to fratricide and precluded expansion of Loop CAR-NK or Loop CAR-iNK cells. (**A**) Flow cytometry histograms showing the expression of CD33 in UCB-NK cells. (**B**) Representative FACS plots showing the purity of sorted $CD33^-$ and $CD33^+$ NK cells. (**C**) Schematic diagram of measuring the cytotoxicity of Loop CAR-NK cells against $CD33^+$/$CD33^-$ NK cells *in vitro*. The sorted NK cells were co-cultured with Loop CAR-NK cells for 4 h. (**D**) Statistics of the fratricidal lysis of CAR-NK cells Loop CAR-NK cells (Effectors, E) against $CD33^+$ and $CD33^-$ NK cells (Targets, T) at the indicated E:T ratios (n = 4 per group). (**E**) Statistical analysis of the cell number for EGFP-NK and Loop CAR-NK cells at indicated time points. An equal number of EGFP-NK and Loop CAR-NK cells were sorted for expansion *in vitro* (n = 3 per group). (**F**) Flow cytometric analysis of Loop-CAR expression in Loop CAR-hPSCs. (**G**) Schematic diagram of Loop CAR-iNK cells generated from Loop CAR-hPSCs. (**H**) Immunophenotypes ($CD45^+CD3^-CD56^+CD16^{+/-)}$ and Loop-CAR expression (CD33 CAR^+MSLN CAR^+) of Loop-CAR-iNK cells. (**I**) Flow cytometry histograms showing the expression levels of CD33 in iNK cells derived from hPSCs. (**J**) Representative FACS plots showing the expression levels of CD33 in sorted $CD33^-$ and $CD33^+$ iNK cells. (**K**) Schematic diagram of measuring the cytotoxicity of Loop CAR-iNK cells against $CD33^+$ and $CD33^-$ iNK cells *in vitro*. (**L**) Statistics of fratricidal lysis of Loop CAR-iNK cells (Effectors, E) against $CD33^+$ and $CD33^-$ iNK cells (Targets, T) at indicated E:T ratios (n = 4 per group). (**M**) Statistical analysis of the cell number for iNK and Loop CAR-iNK cells at indicated time points (n = 3 per group). An equal number of iNK and Loop CAR-iNK cells were started for culture. Data represent the mean ± SEM. Statistics analysis: one-way ANOVA test (D, L) and two-way ANOVA test (E, M). NS: not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

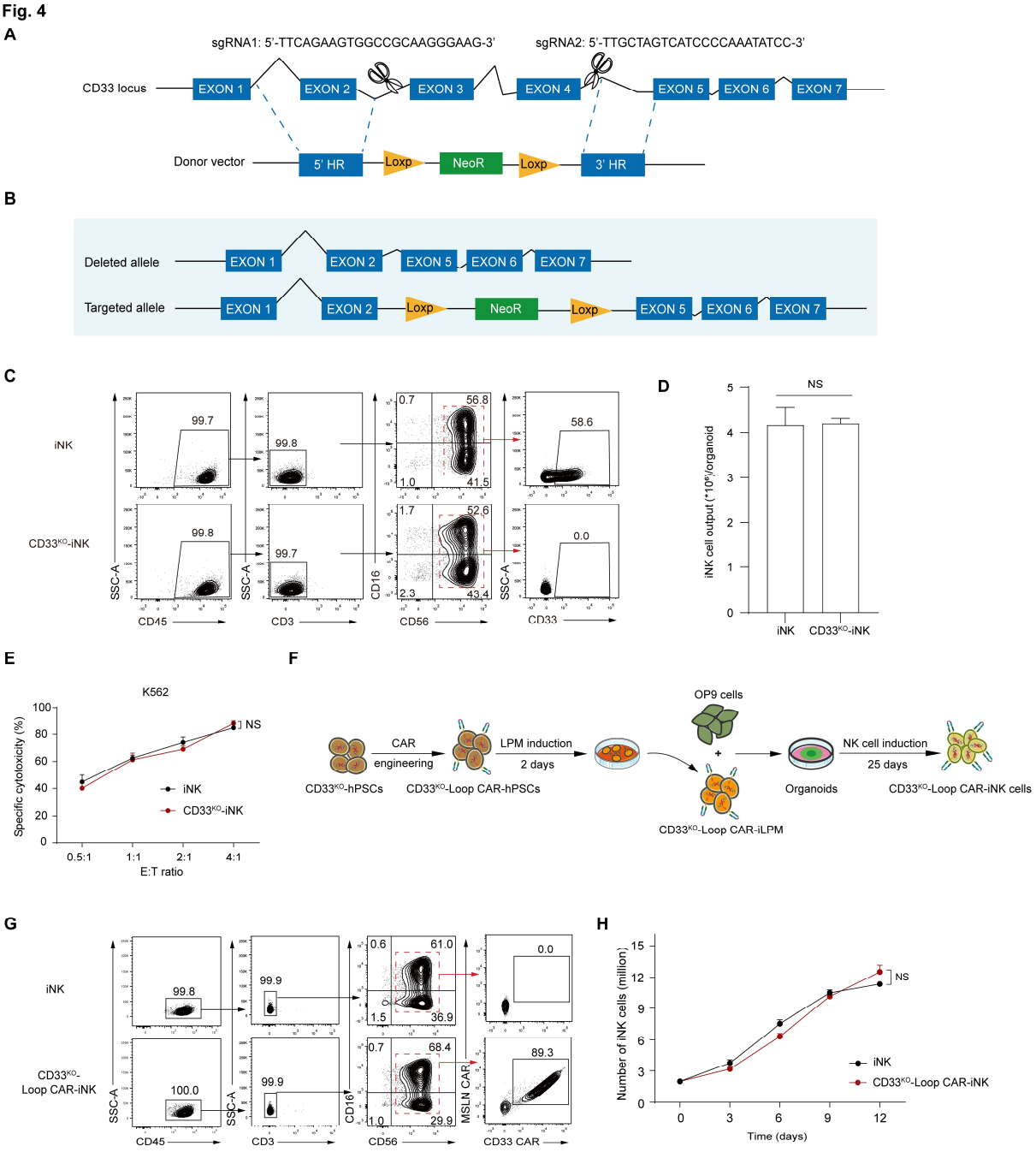
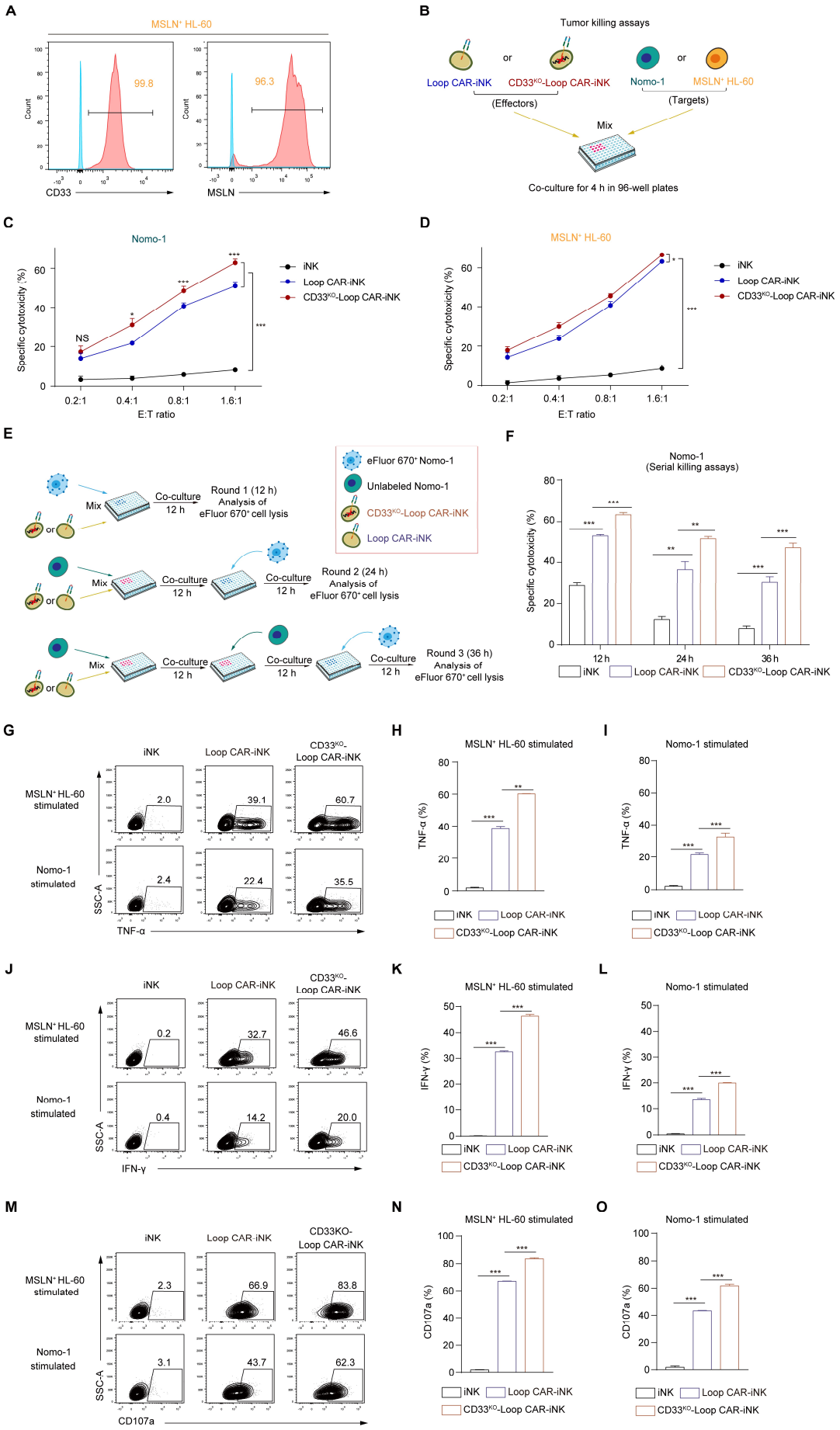


Fig. 4. Inactivation of CD33 restored the expansion of Loop CAR-iNK cells. (**A**) Schematic diagram of CRISPR/Cas12i-targeted CD33 gene strategy. sgRNA: single guide RNA; HR: homologous region; NeoR: neomycin resistance gene. (**B**) Schematic diagram showing the genotype of the established CD33^{KO}-hPSCs. (**C**) Flow cytometric analysis of iNK and CD33^{KO}-iNK cells. (**D**) Statistics of the yields of iNK and CD33^{KO}-iNK cell (Day 27) induced from hPSCs and CD33^{KO}-hPSCs (n = 3 repeats). (**E**) Statistics of cytotoxicity of iNK and CD33^{KO}-iNK cells (Effectors, E) against K562 cells (Targets, T) at the indicated E:T ratios (n

824 = 4 per group). (**F**) Schematic diagram of $CD33^{KO}$-Loop CAR-iNK cells induced from
825 $CD33^{KO}$-Loop CAR-hPSCs. (**G**) Immunophenotypes ($CD45^{+}CD3^{-}CD56^{+}CD16^{+/-}$) and Loop-
826 CAR expression in iNK and $CD33^{KO}$-Loop CAR-iNK cells. (**H**) Statistical analysis of the cell
827 number for iNK and $CD33^{KO}$-Loop CAR-iNK cells at indicated time points. An equal number
828 of iNK and Loop CAR-iNK cells were started for culture (n = 3 repeats). Data represent the
829 mean ± SEM. Statistics analysis: two-tailed Student's t test (D), one-way ANOVA test (E) and
830 two-way ANOVA test (H). NS: not significant.

Fig. 5


A
MSLN⁺ HL-60
99.8
96.3
Count
CD33
MSLN
B
Tumor killing assays
Loop CAR-iNK
or
CD33KO-Loop CAR-iNK
Nomo-1
MSLN⁺ HL-60
(Effectors)
(Targets)
Mix
Co-culture for 4 h in 96-well plates
C
Nomo-1
Specific cytotoxicity (%)
NS
iNK
Loop CAR-iNK
CD33KO-Loop CAR-iNK
0.2:1
0.4:1
0.8:1
1.6:1
E:T ratio
D
MSLN⁺ HL-60
E
eFluor 670⁺ Nomo-1
Unlabeled Nomo-1
CD33KO-Loop CAR-iNK
Loop CAR-iNK
Mix
Co-culture 12 h
Round 1 (12 h) Analysis of eFluor 670⁺ cell lysis
Round 2 (24 h) Analysis of eFluor 670⁺ cell lysis
Round 3 (36 h) Analysis of eFluor 670⁺ cell lysis
F
Nomo-1 (Serial killing assays)
12 h
24 h
36 h
G
iNK
Loop CAR-iNK
CD33KO-Loop CAR-iNK
MSLN⁺ HL-60 stimulated
Nomo-1 stimulated
2.0
39.1
60.7
2.4
22.4
35.5
SSC-A
TNF-α
H
MSLN⁺ HL-60 stimulated
TNF-α (%)
I
Nomo-1 stimulated
J
0.2
32.7
46.6
0.4
14.2
20.0
IFN-γ
K
MSLN⁺ HL-60 stimulated
IFN-γ (%)
L
Nomo-1 stimulated
M
2.3
66.9
83.8
3.1
43.7
62.3
CD107a
N
MSLN⁺ HL-60 stimulated
CD107a (%)
O
Nomo-1 stimulated

831

832 **Fig. 5. CD33^{KO}-Loop CAR-iNK cells exhibited superior tumor-killing abilities than Loop**
833 **CAR-iNK cells.** (**A**) Flow cytometry histograms showing the expression of CD33 and MSLN
834 in HL-60 cells engineered with MSLN (MSLN^{+}HL-60). (**B**) Schematic diagram showing
835 Nomo-1 or MSLN^{+} HL-60 cells killed by Loop CAR-iNK or CD33^{KO}-Loop-CAR-iNK cells
836 *in vitro*. (**C** and **D**) Statistics of specific cytotoxicities analysis of iNK, Loop CAR-iNK and
837 CD33^{KO}-Loop CAR-iNK cells (Effectors, E) against Nomo-1 (C) and CD33^{+}MSLN^{+} HL-60
838 cells (D) (Targets, T) at the indicated E:T ratios (n = 4 per group). (**E**) Schematic diagram of
839 the serial killing assays of iNK, Loop CAR-iNK, and CD33^{KO}-Loop CAR-iNK cells against
840 Nomo-1 cells. Each group of iNK cells was co-cultured with Nomo-1 cells for 12 h per round
841 at an E:T ratio of 1:1. The fresh Nomo-1 cells were added to the NK cell residues and incubated
842 for every other 12 h. The lysis of Cell Proliferation Dye eFluor 670-labeled (eFluor 670^{+})
843 Nomo-1 cells in each round represented the cytotoxicity. (**F**) Statistics of specific cytotoxicity
844 of iNK, Loop CAR-iNK, and CD33^{KO}-Loop CAR-iNK cells in serial killing assays (n = 4 per
845 group). (**G**, **J**, **M**) Representative flow cytometry plots showing the expression of TNF-α (G),
846 IFN-γ (J), and CD107a (M) in each group of iNK cells stimulated by MLSN^{+} HL-60 and
847 Nomo-1 cells. (**H** and **I**) Statistical analysis of the expression levels of TNF-α (n = 3 repeats).
848 (**K** and **L**) Statistical analysis of the expression levels of IFN-γ (n = 3 repeats). (**N** and **O**)
849 Statistical analysis of the expression levels of CD107a (n = 3 repeats). Data represent the mean
850 ± SEM. Statistics analysis: one-way ANOVA test. NS: not significant; *P < 0.05, **P < 0.01,
851 ***P < 0.001.

Fig. 6

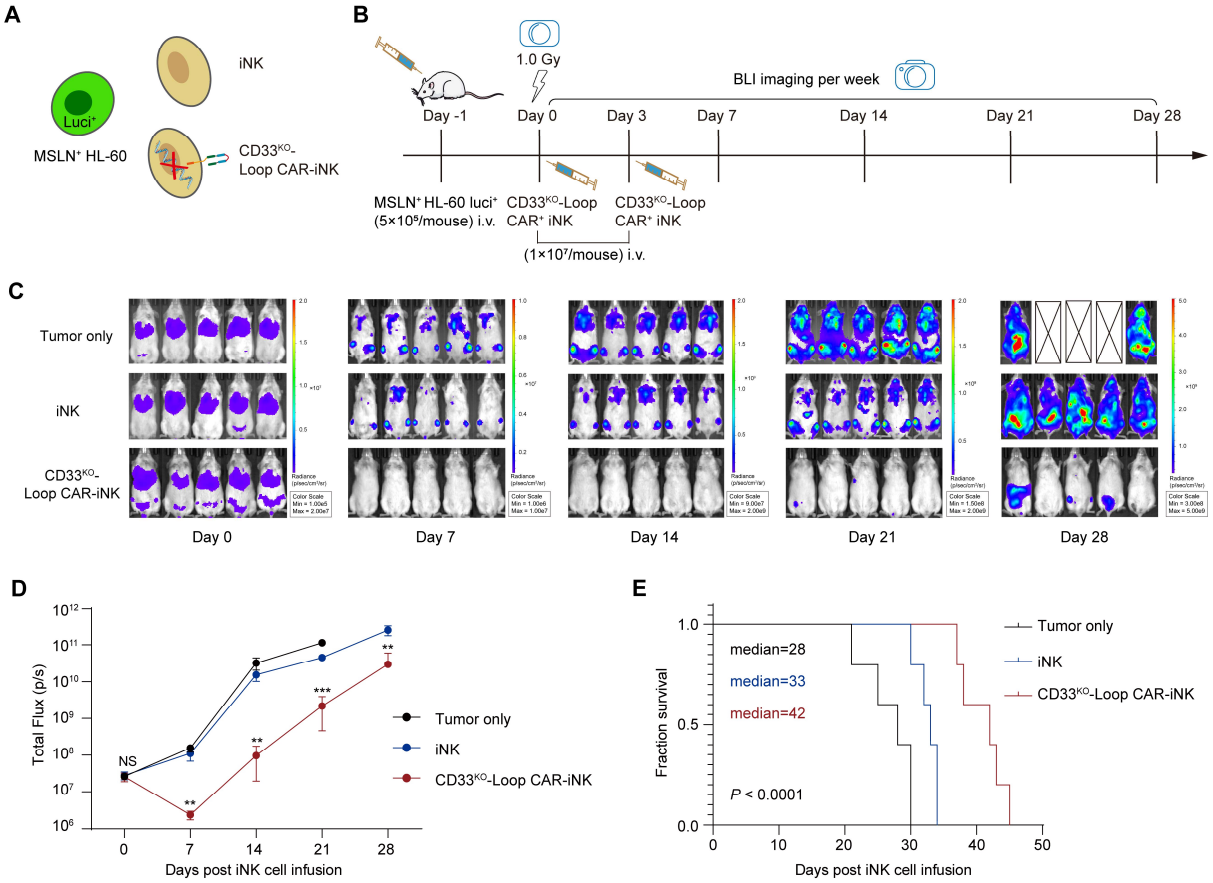


Fig. 6. CD33^{KO}-Loop CAR-iNK cells efficiently suppressed tumor progression *in vivo*. (**A**) Schematic diagram of the tumor cells and therapeutic cells used for *in vivo* assay. (**B**) Schematic diagram of *in vivo* studies. Luciferase-expressing (luci^{+}) MSLN^{+} HL-60 cells were used to generate mouse xenograft models (5 × 10^{5} cells/mouse, i.v.). Two doses of CD33^{KO}-Loop CAR-iNK cells (1× 10^{7} cells/mouse per dose) were injected into tumor-bearing mice for therapy. (**C**) Bioluminescence imaging (BLI) of xenograft models of each group (n = 5 per group). (**D**) Statistics of each group's total flux (p/s) (CD33^{KO}-Loop CAR-iNK vs. iNK). Statistics analysis: two-tailed Student's t-test. NS: not significant; $^{**}P < 0.01$, $^{***}P < 0.001$. (**E**) Kaplan-Meier survival curves of the experimental groups. Statistics analysis: two-tailed log-rank test. Tumor only vs. iNK, $^{**}P < 0.01$, CD33^{KO}-Loop CAR-iNK vs. iNK, $^{**}P < 0.01$.

Supplementary Materials

Fig. S1

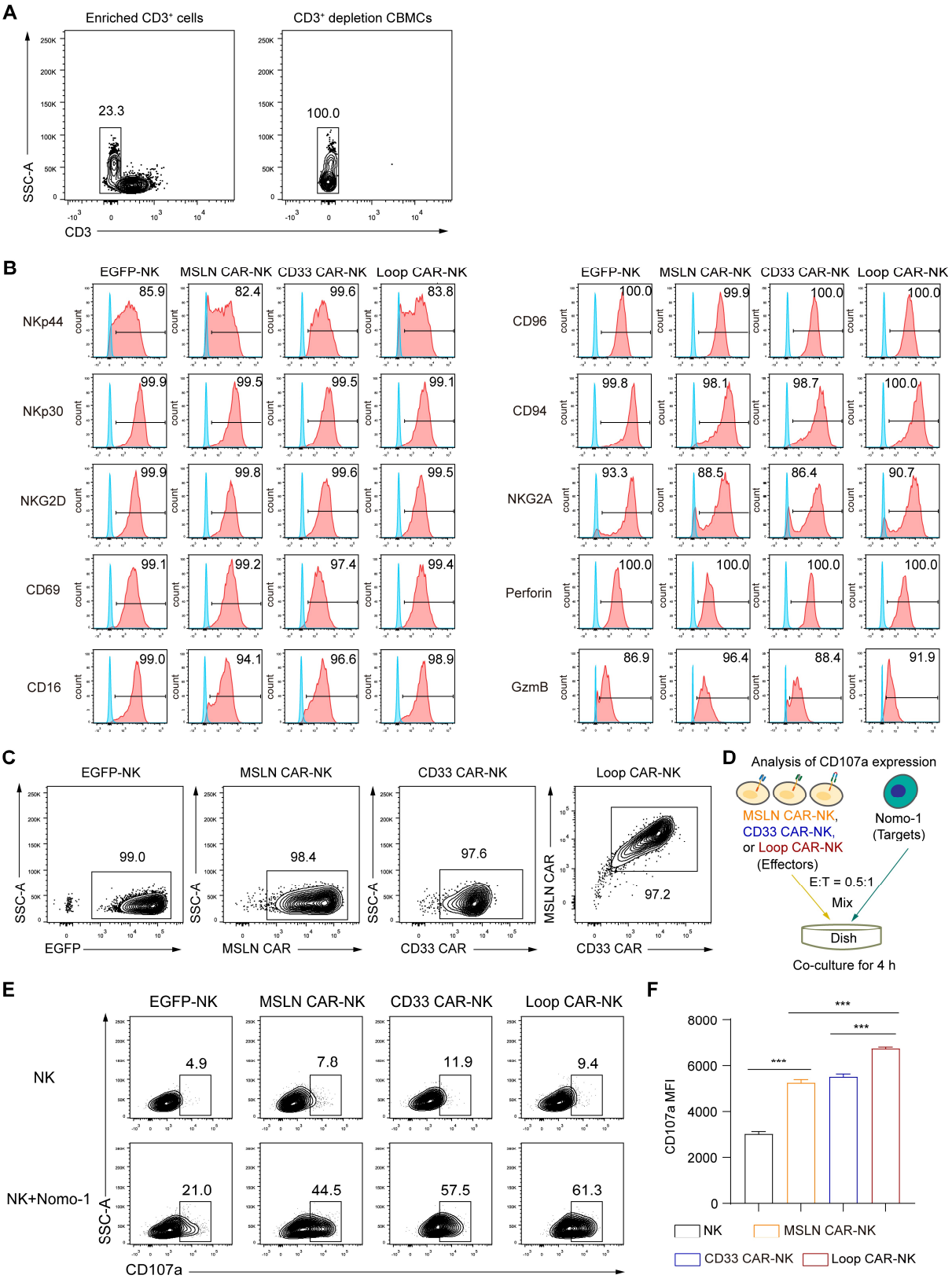


Fig. S1. Phenotypes and effector function of Loop CAR-NK cells. (A) Flow cytometric

analysis of the T cells ($CD3^+$) residual in UCBMCs after $CD3^+$ cells depletion on day -6. (B) Flow cytometry histograms showing the expression of typical NK cell receptors (NKp44, NKp30, NKG2D, CD69, CD16, CD96, CD94, and NKG2A) and cytotoxic granules (Perforin and GzmB) in respective NK cells. Control histograms represent FMO control. (C) The analysis of sorting purity for $CD3^-CD56^+EGFP^+$ (EGFP-NK) and $CD3^-CD56^+CAR^+$ (CAR-NK) cells post 24 h culturing. (D) Experimental design of the analysis of CD107a expression. EGFP-NK and CAR-NK cells were stimulated at an E:T ratio of 0.5:1 for 4 h. (E) Representative flow cytometry plots showing the expression of CD107a in EGFP-NK and CAR-NK cells in response to Nomo-1 cells. (F) Statistic analysis of the mean fluorescence intensity (MFI) of CD107a in (E), n = 3 repeats. Statistical analysis: one-way ANOVA, ***P < 0.001.

Fig. S2

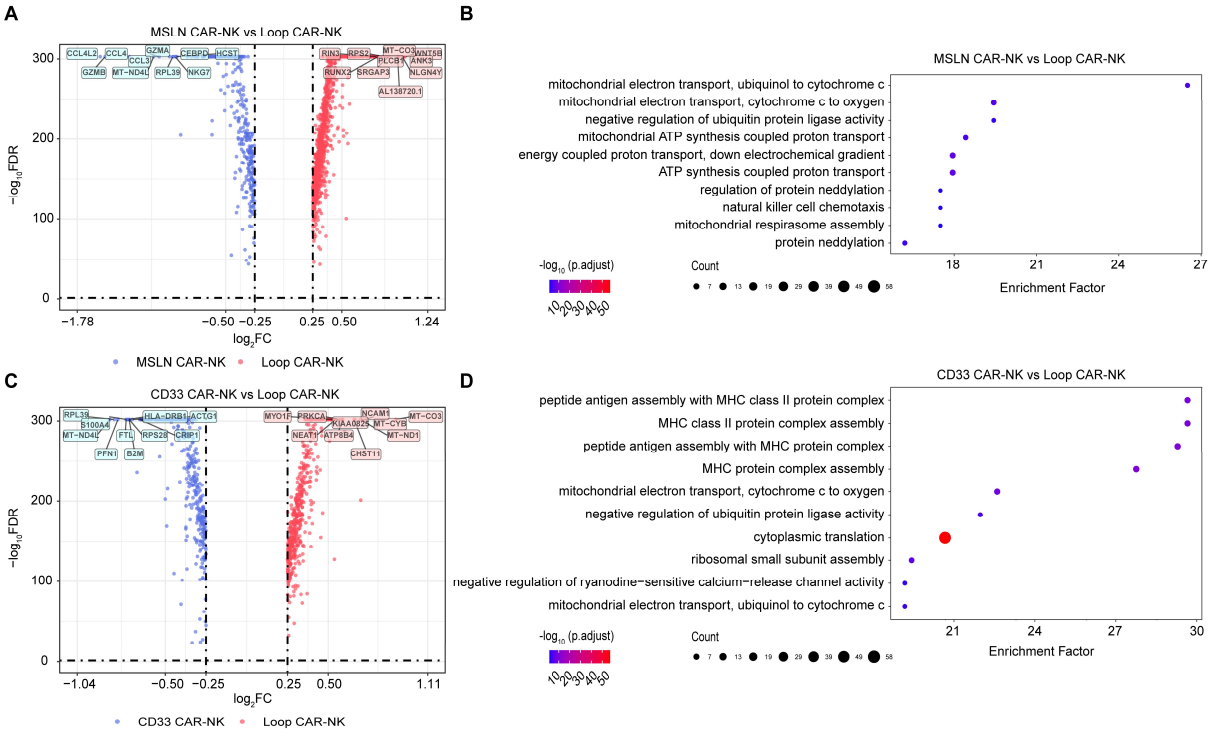


Fig. S2. Differentially expressed genes of Loop CAR-NK cells compared with CD33 CAR-NK and MSLN CAR-NK cells. (A) Volcano plot of differentially expressed genes between Loop CAR-NK and MSLN CAR-NK cells. (B) GO analysis of differentially down-regulated

genes in Loop CAR-NK cells compared with MSLN CAR-NK. Each data point represents a distinct GO set. Color scale corresponds to −log10 FDR (adjusted P values). Dot size was the number of genes enriched in one term. Enrichment Factor was the GeneRatio/BgRatio. (C) Volcano plot of differentially expressed genes between Loop CAR-NK and CD33 CAR-NK cells. (D) GO analysis of differentially down-regulated genes in Loop CAR-NK cells compared with CD33 CAR-NK cells. Each data point represents a distinct GO set. Color scale corresponds to −log10 FDR (adjusted P values). Dot size was the number of genes enriched in one term. Enrichment Factor was the GeneRatio/BgRatio.

Fig. S3

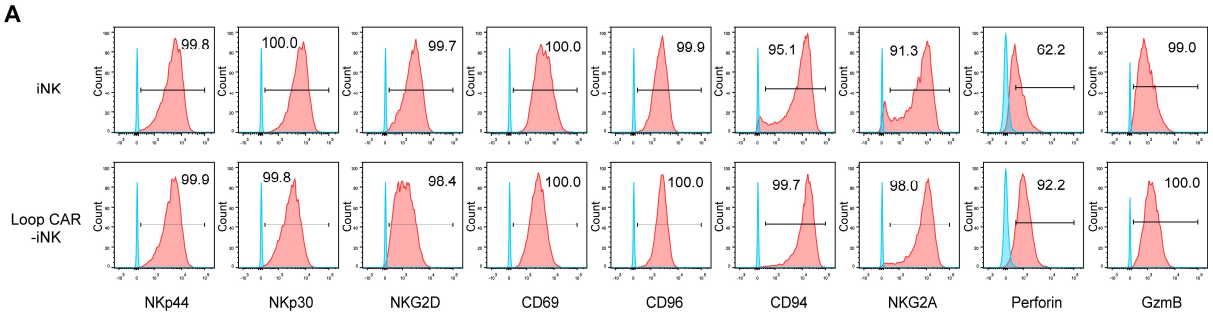


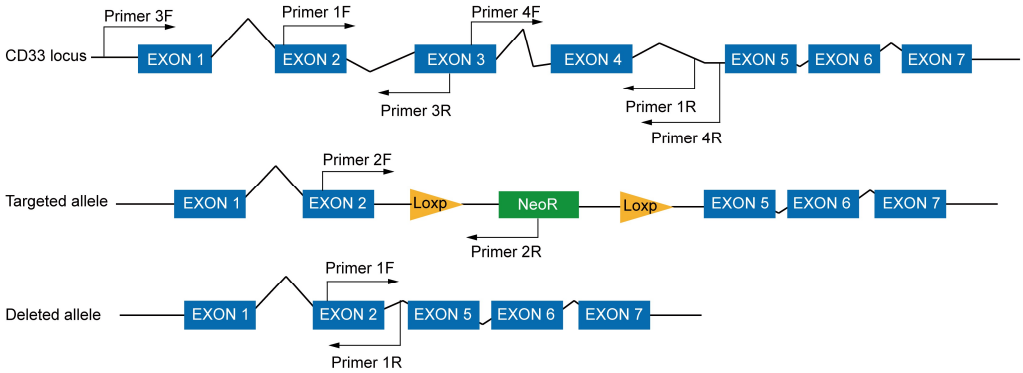
Fig. S3. Analysis of typical NK molecules of Loop CAR-iNK cells. (A) Flow cytometry histograms showing the expression of typical NK cell receptors (NKp44, NKp30, NKG2D, CD69, CD96, CD94, and NKG2A) and cytotoxic granules (Perforin and GzmB) in respective iNK cells. Control histograms represent FMO control.

Fig. S4

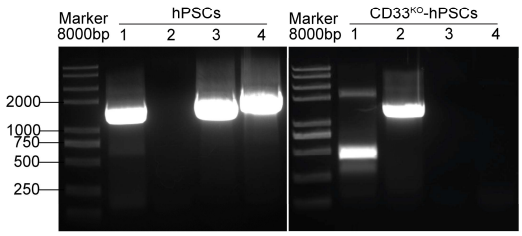
A

Primer No.	Forword primer sequence	Reverse primer sequence
1	5'-TCCCAGTAGGAACAACTGCTCCCTGAGCATCGTAGACGCC-3'	5'-TAAATGTTCAAGATGATGGATATGCTATTACCCTGATCTG-3'
2	5'-TCCCAGTAGGAACAACTGCTCCCTGAGCATCGTAGACGCC-3'	5'-GATGGATTGCACGCAGGTTC-3'
3	5'-CATGAGGCTAGAAAGGAAATTGGAACTTAAAGAGGCAGTG-3'	5'-GCAGCTGACAACCAGGAGAAGATC-3'
4	5'-CTCGGTGCTCATAATCACCCCACGG-3'	5'-CTATTCATCTGACAAGTGATTCATATCCAGAACATACGAG-3'

B



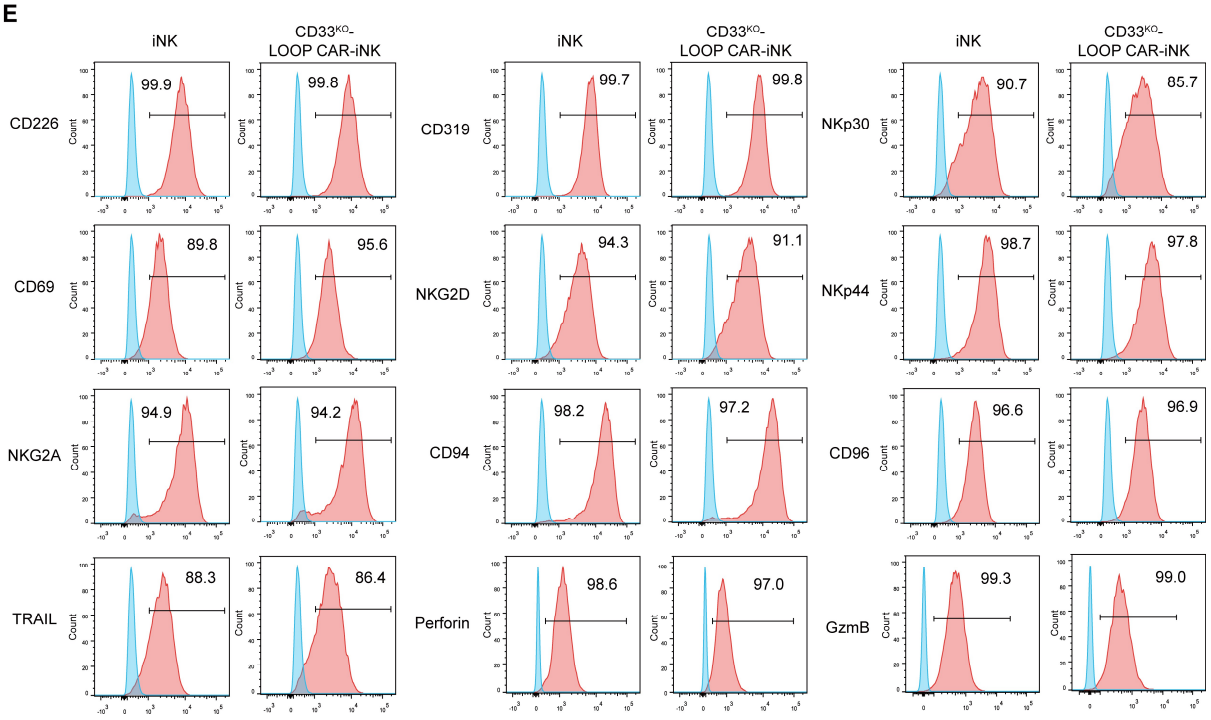
C



D

Primer NO.	hPSCs	CD33^{KO}-hPSCs
1	1241 bp	2124 bp, 302 bp
2	\	1282 bp
3	1437 bp	\
4	1673 bp	\

E



895

896 **Fig. S4. Identification of CD33 genotypes of CD33^{KO}-hPSCs and molecular features of**

897 **CD33^{KO}-Loop CAR-iNK cells.** (A) The sequences of four pairs of primers used for
898 genotyping CD33 knock-out PSC clones. (B) Schematic diagram showing the location of
899 primer pairs used for identifying CD33 genotypes. (C, D) PCR amplification results of each
900 fragment using the indicated primer pairs. (E) Flow cytometry analysis of typical NK cell
901 receptors (CD226, CD319, NKp30, CD69, NKG2D, NKp44, NKG2A, CD94, CD96, TARIL)
902 and cytotoxic granules (Perforin and GzmB) in respective iNK cells. Control histograms
903 represent FMO control.

Primer name	Primer sequence (5'→3')
GAPDH-F	CAAGAAGTGGGAGCTGGAAG
GAPDH-R	GTCTCCAGGGACGTCACATT
MSLN-F	ACCCAGAAGACTGTGGATGG
MSLN-R	TGCTGTAGCCAAATTCGTTG

904 **Table S1. Primer sequences for real-time quantitative PCR of selected genes.**