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Generation and functional characterization of a multigene-modified NK101 cell line exerting diverse mechanisms of antitumor action

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ABSTRACT

Clonal cell line-based, multigene-modified, off-the-shelf NK cell therapeutics are emerging as the new frontier of adoptive cellular immunotherapy. Here, we utilized a newly established NK cell line, NK101, as a backbone to derive multifaceted killer cells armored with various antitumor modalities through repeated cycles of genetic modification and clonal selection. First, NK101 cells were transduced with a tricistronic lentiviral vector expressing CD7, CD28, and cytosine deaminase (CD). The resulting cell line demonstrated enhanced cytotoxicity against $B7^+$ tumors and exerted bystander killing effects on neighboring tumor cells upon 5-FC treatment. Second, engineered NK101 cells were again transduced with a bicistronic vector expressing membrane-bound interleukin-15 (mbIL-15) and dominant negative TGFβ type II receptor (DNTβRII). Ectopic expression of mbIL-15 resulted in further augmentation of lytic activities against all tested target cells by inducing upregulation of multiple activating receptors, while that of DNTβRII allowed the cells to maintain heightened cytotoxicity in the presence of TGFβ. Finally, dual-transduced NK101 cells were modified to express chimeric antigen receptors (CARs) targeting either a solid tumor antigen (EpCAM) or a hematologic tumor antigen (FLT3). The final engineered products not only demonstrated antigen-specific killing activities in vitro but also exerted strong tumor-inhibitory effects in preclinical models of metastatic solid tumor and hematologic malignancy. Notably, combined treatment with 5-FC further enhanced antitumor efficacy of engineered NK101 in the solid tumor model. Our results demonstrate successful generation of multigene-modified NK101 cell therapeutics exerting diverse mechanisms of antitumor action – activation receptor-mediated innate killing, antigen-specific killing, and bystander effect-mediated killing.

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Introduction

The integration of genetic engineering technologies into cellular immunotherapy has facilitated rapid advancement of the immuno-oncology field, as represented by landmark approvals of patient-derived T cells modified to express a chimeric receptor that redirects cellular specificity to a tumor antigen.[1] Driven by the success of the first-generation therapeutics, growing efforts are being directed toward implementing multiple genetic modifications to alter or enhance various functional traits of antitumor immune cells and identifying an optimal cell source/type to apply such engineering.[1,2] As the insertion or deletion of multiple genes accompanies increased manufacturing and regulatory complexities, potentially necessitating longer ex vivo processing and more comprehensive characterization of the final products, primary immune cells that are prone to terminal differentiation/exhaustion with limited time frame for efficient genetic manipulation would be challenging targets for multigenic modification.[3–5] In this regard, cell line-based platforms, such as clonal master induced pluripotent stem cell (iPSC) lines and immortalized natural killer (NK) cell lines, serve as an attractive alternative source for multi-functional or multi-step engineering, as (i) they can be easily grown and repeatedly manipulated in vitro; (ii) single cells stably expressing multiple transgenes can be clonally selected for the creation of a master cell bank; and (iii) standardized and fully characterized products consisting of defined populations of therapeutic cells can be manufactured at clinical scale.[6]

Among the few cell line-based platforms established to date, human NK cell lines have been most popular source for adoptive cell transfer (ACT). Representatively, NK-92, the first and only FDA-approved NK cell line for clinical use, has been infused into nearly 40 patients with solid tumors or hematological malignancies.[7] Despite the favorable safety and tolerability of the therapy, the administration of unmodified NK-92 has provided limited clinical benefit in patients.[7] In response to these shortcomings, numerous efforts have been undertaken to improve the functionality of NK-92, as well as other NK cell lines, by adopting various genetic modification strategies as follows: (i) introduction of an activating or a co-stimulatory receptor to enhance natural cytotoxicity of NK cells; (ii) ectopic expression of a cytokine or a modified cytokine to improve proliferation/persistence as well as lytic activity of NK cells; (iii) insertion of a decoy receptor for suppressive molecules to counteract tumor microenvironment (TME); (iv) introduction

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of a chimeric antigen receptor (CAR) to redirect NK cell specificity toward tumor-associated antigens;[8] and finally (v) incorporation of a suicide gene to provide a kill switch that could complement or replace irradiation, which is required for all NK cell lines prior to human application.[9] To our knowledge, there have been more than 100 preclinical/clinical studies reported on gene-modified NK cell lines, but more than 90% of them were restricted in assessing the effects of a single transgene.[8,10] These facts implicate that the development of multigene-modified, NK cell line-based therapeutics is still in its early stages and a new generation of such therapeutics is worth investigating.

NK101 is the most recently discovered NK cell line derived from a patient with extranodal NK/T-cell lymphoma. As with the NK-92 cell line, NK101 demonstrates broad-spectrum cytolytic activities against tumor cells in a major histocompatibility complex (MHC)-unrestricted, activating receptor engagement-dependent manner and do not produce active EBV virions, thus possessing the potential for clinical use. Although distinct capacity to stimulate endogenous antitumor immunity and outstanding scalability provided a competitive edge to NK101, it exhibits relatively lower levels of cytolytic activities compared to NK-92.[7] In this study, we aimed to potentiate cytotoxicity of NK101 by incorporating multi-dimensional genetic engineering approaches and assess the efficacy and applicability of multigene-modified NK101 therapeutics in cancer treatment.

Methods

Plasmid construction and lentivirus production

Schematic diagram of transgene cassettes used in this study is shown in Figure 1a: (i) a tricistronic expression cassette, in which CD7, CD28, and cytosine deaminase (CD) open reading frames are linked via a P2A cleavage peptide and an internal ribosomal entry site (IRES) sequences, respectively; (ii) a bicistronic cassette, in which membrane-bound IL-15 (mbIL-15) and DNTβRII are connected by P2A; (iii) a CAR expression cassette consisted of a scFv derived from an anti-EpCAM monoclonal antibody (mAb) 12H8 or an anti-FLT3 mAb IMC-NC7,[11,12] linked to a spacer derived from hybrid Fc

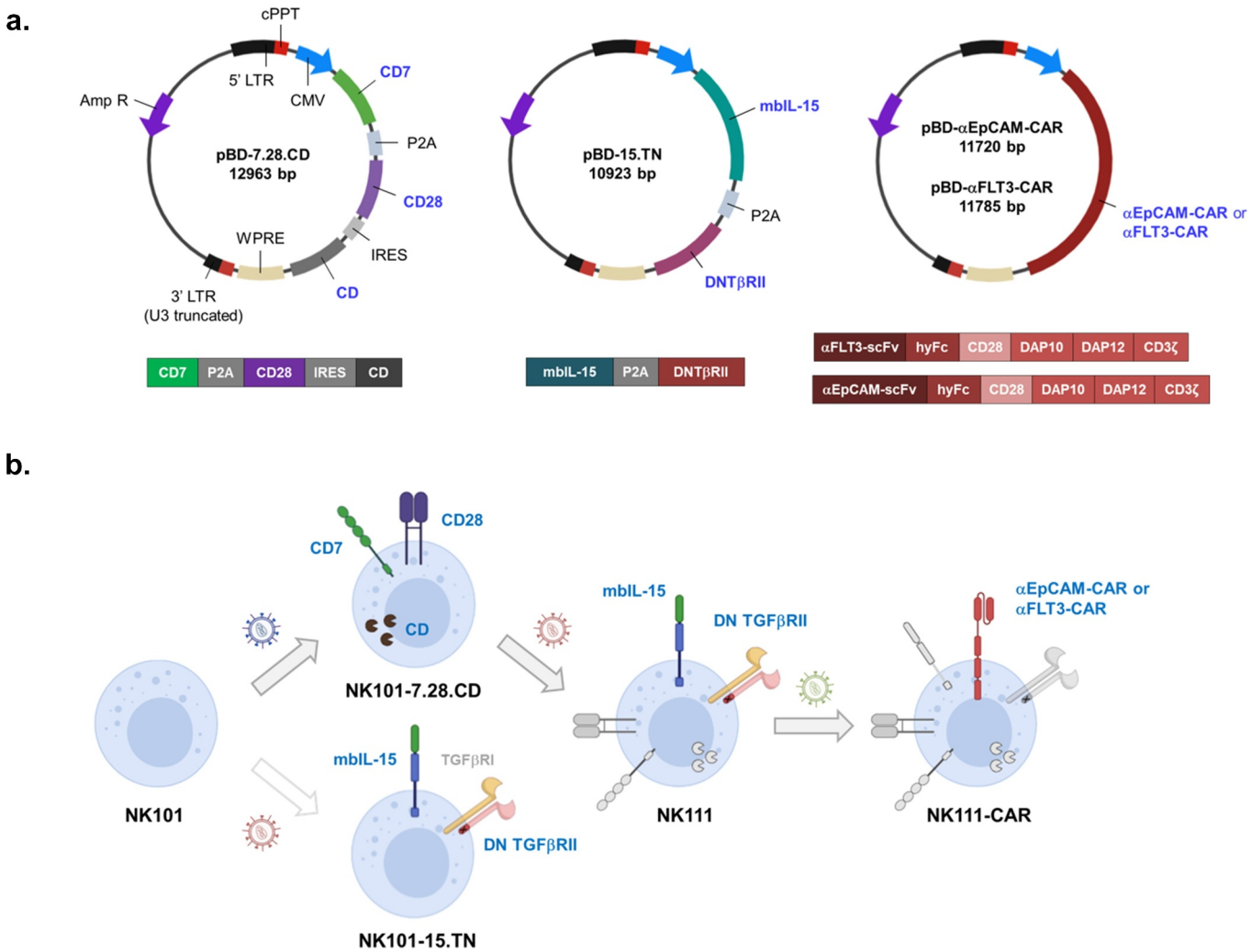

Figure 1. Schematic diagrams of lentiviral vectors and engineered cell lines used in this study. (a) Four different transgene expression cassettes (bottom panel) were constructed and cloned into pBD lentiviral transfer plasmids (top panel). LTR, long terminal repeat; cPPT, central polypurine tract; CMV, cytomegalovirus promoter; IRES, internal ribosomal entry site; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; Amp R, ampicillin resistance gene. (b) The process of genetic modification of the NK101 cell line. Newly introduced genes in each engineering step and the name of the resulting cell line are indicated.

(hyFc),[13] a CD28 transmembrane domain, and DAP10, DAP12, CD3ζ signaling domains. All transgene cassettes were codon optimized and chemically synthesized by GenScript (Nanjing, China), and then inserted into pBD lentiviral transfer plasmid (SL BiGen, Inc., Incheon, Korea).[14] The resulting plasmids were designated as pBD-7.28.CD, pBD-15.TN, and pBD-αEpCAM-CAR or pBD-αFLT3-CAR. Detailed procedures for lentivirus production are described in *supplementary methods*.

Lentiviral transduction and clonal selection of infected NK101 cells

Lentiviral transduction of NK101 cell line and its derivatives was performed as previously described with minor modifications.[15] Detection and purification of infected cells were performed using a FACSAria cell sorter and Cell Quest software (BD Biosciences, San Diego, CA). Sorting was carried out in "single-cell" mode into 96-well plates. Clones displaying stable growth profile, and at the same time, presenting consistently high transgene expression over multiple passages were selected for further analysis. Schematic diagram of engineered cell products generated in this study is shown in Figure 1b: A clone derived from NK101 cells transduced with lentivirus expressing pBD-7.28.CD or pBD-15.TN construct was designated as NK101-7.28.CD or NK101-15.TN, respectively. A clone derived from NK101-7.28.CD cells infected with lentivirus expressing pBD-15.TN was denoted as NK111. Finally, a clonal cell line derived from NK111 cells infected with lentivirus expressing pBD-αEpCAM-CAR or pBD-αFLT3-CAR construct was termed NK111-αEpCAM-CAR or NK111-αFLT3-CAR, respectively. Detailed procedures for lentivirus transduction, clonal selection of infected cells and their phenotypic and functional characterization are described in *supplementary methods*.

In vivo efficacy study

All animal experiments were conducted in compliance with the guideline of the Institutional Animal Care and Use Committee. Six- to eight-week-old female NOD/SCID IL2Rγ^{null} (NSG) mice were obtained from Genexine Co. Ltd. (Seongnam, Korea) and maintained under specific pathogen-free conditions. For the establishment of a solid tumor xenograft model, mice were intraperitoneally (i.p.) injected with 5×10^6 cells of RMG-1 human ovarian cancer cell line expressing enhanced green fluorescent protein (eGFP) and firefly luciferase (fLuc). Seven days after inoculation, mice received a single i.p. dose of 10 Gy-irradiated NK111-αEpCAM-CAR cells (1×10^7 or 3×10^7). For the repeated administration study, RMG-1-eGFP-fLuc tumor-bearing mice were injected with 3×10^7 cells of irradiated NK101, NK111, or NK111-αEpCAM-CAR, twice per week for 3 weeks. For the hematological tumor xenograft model, NSG mice were intravenously (i.v.) injected with 1×10^6 cells of EoL-1 human acute eosinophilic leukemia cells co-expressing eGFP and fLuc. Beginning on day 4 after tumor inoculation, 5×10^6 cells of irradiated NK101, NK111, or NK111-αFLT3-CAR were injected i.v. for 6 times, twice per week for 3 weeks. For both solid and hematologic tumor models, 5-FC treatment groups received daily i.p. injections of 500 mg/kg 5-FC from days 0 to 21 post NK cell infusion. In all experiments, tumor progression was regularly monitored by bioluminescence imaging (BLI) using Lago (Spectral Imaging Instruments, Tucson, AZ) after subcutaneous injection of 150 mg/kg D-luciferin (Goldbio, St Louis, MO).

Statistical analysis

Statistical analysis were carried out by the software SPSS v.26 for Windows (Chicago, IL). The Friedman test with post-hoc Wilcoxon signed ranks tests were used in the analysis of in vitro data. For in vivo studies, Kruskal–Wallis test followed by Dunn's post hoc test for multiple comparisons were performed to assess the differences in tumor volumes between different groups at the end of the study period. The data are presented as means ± standard error of the mean (SEM). In all cases, p values lower than 0.05 were considered statistically significant.

Results

Enforced expression of CD7 and CD28 promotes the cytotoxicity of NK101 against B7$^+$ tumor cells

We first examined expression levels of surface markers that are used to operationally define NK cell lines in weakly cytotoxic NK101 relative to those in KHYG-1 and NK-92, both of which possess potent killing activities.[16,17] Comparative flow cytometric analysis of 20 different markers identified two antigens – CD7 and CD28 – that are expressed in either one or both of control cell lines, but are completely absent in NK101 (Figure S1). As CD7 and CD28 are proposed to provide stimulatory signals in primary NK cells and NK cell lines, respectively,[18–20] we assessed whether enforced expression of CD7 and CD28 in NK101 could enhance its cytotoxic functions by establishing NK101-7.28.CD (Figure 1b). Unlike parental NK101 cells, NK101-7.28.CD cells showed high surface expression of CD7 and CD28 (Figure 2a). To determine the impact of CD7 and CD28 expression on NK101 cytotoxicity, unmodified NK101 and NK101-7.28.CD were then co-cultured with human tumor cell lines with varying degrees of surface expression of B7 molecules (CD80/CD86) and SECTM1, which are ligands for CD28 and CD7, respectively (Table 1). Compared to parental NK101, NK101-7.28.CD showed almost equal cytotoxicity toward B7$^-$ tumor cells, including SK-MES-1, K562 and A2780. On the other hand, NK101-7.28.CD exhibited significantly stronger killing activities against B7$^+$ tumor cells. The magnitude of increase in cytotoxicity upon CD7/CD28 insertion tended to correlate with (i) the presence of CD80 along with CD86 on tumor cell surface, as shown by a larger difference between percent specific lysis of NK101 and NK101-7.28.CD toward CD80$^+$ CD86$^+$ cell lines (HDLM2, Raji, IM9) than toward CD80$^-$ CD86$^+$ counterparts (KG-1, EoL-1, JeKo-1); and (ii) the intensity of surface CD86 on tumor cells, as NK101-7.28.CD exerted greater cytotoxic effects on HDLM-2 (MFI = 17,390.0 ± 606.4) than on Raji (MFI = 9,318.7 ± 348.3) or IM-9 (MFI = 8,998.0 ± 536.9). We could not find a clear correlation between surface SECTM1 expression levels on

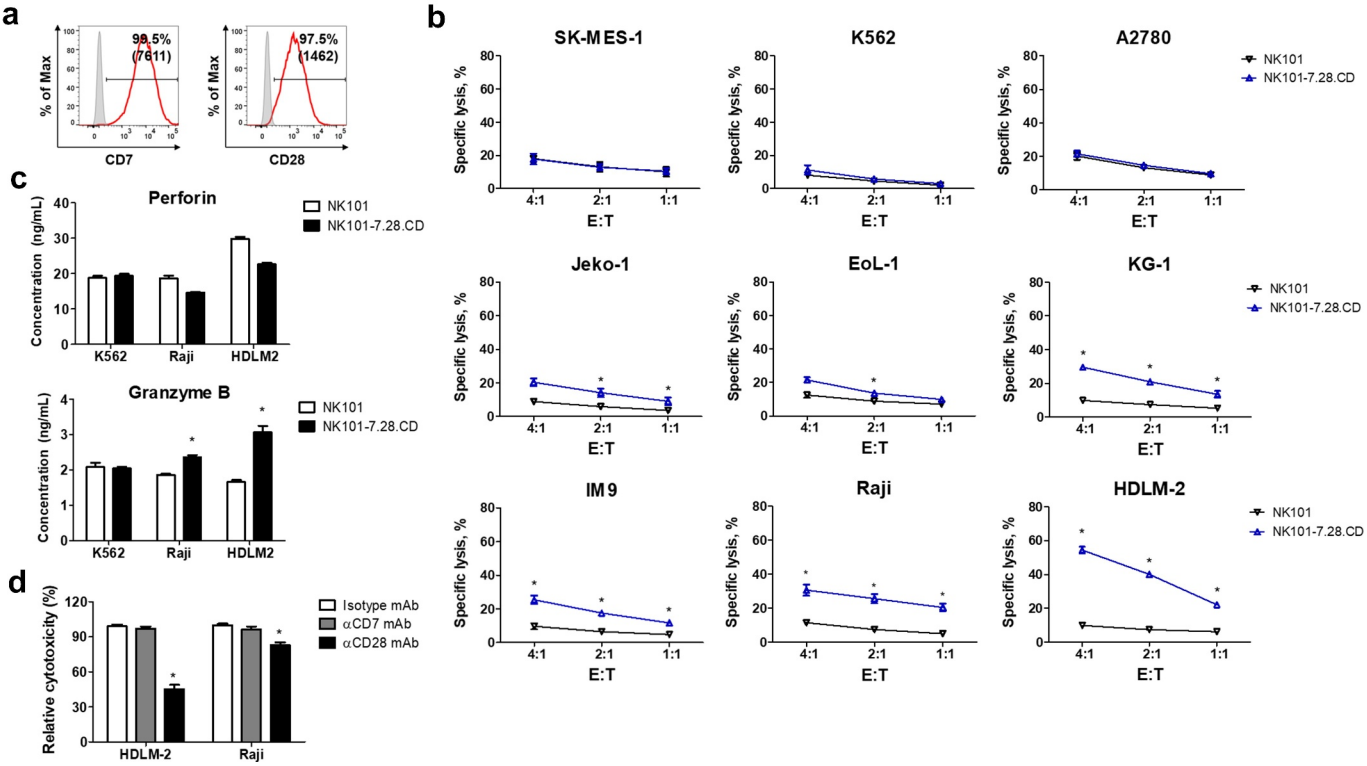

Figure 2. Effects of CD7 and CD28 expression on the cytolytic activity of NK101. (a) Representative histogram plots of CD7 and CD28 expression on NK101-7.28.CD. A gray-shaded line indicates isotype control and a solid line indicates the corresponding marker. Numbers in the histograms and parentheses indicate the percentages and mean fluorescence intensity, respectively. (b) Graphs showing lytic activities of NK101 and NK101-7.28.CD against indicated tumor cell lines at different effector-to-target (E:T) ratios after 24 h of co-culture. Apoptotic and dead cell population were discriminated by Annexin-V and fixable viability dye staining, followed by flow cytometric analysis. *$p < .05$ versus NK101 at indicated E:T ratio. (c) Bar graphs showing perforin and granzyme B concentrations in the 24 h co-culture supernatants quantified using ELISA. *$p < .05$. (d) Bar graphs showing the lytic activity of anti-CD7 mAb- or anti-CD28 mAb-treated cells relative to that of isotype mAb-treated cells (set as 100%). HDLM-2 and Raji cells were co-cultured with NK101-7.28.CD at E:T ratio of 4:1 in the presence of indicated antibodies (10 μg/mL) for 24 h. Harvested cells were stained with Annexin-V and fixable viability dye, and apoptotic tumor cells were analyzed by flow cytometry. *$p < .05$ versus isotype antibody-treated groups. All data represent mean ± SEM of 5 independent experiments.

Table 1. Surface expression of B7 (CD80/CD86) and SECTM1 in various tumor cell lines .

Cell line	CD80		CD86		SECTM1	
	Positive cells (%)	MFI	Positive cells (%)	MFI	Positive cells (%)	MFI
SK-MES-1	0.6 ± 0.2	97.0 ± 12.1	0.7 ± 0.2	94.5 ± 6.1	1.1 ± 0.3	105.7 ± 15.0
K562	2.1 ± 0.5	152.0 ± 4.9	0.8 ± 0.4	155.7 ± 6.3	12.0 ± 3.8	243.3 ± 50.2
A2780	0.4 ± 0.1	126.7 ± 9.0	0.3 ± 0.1	119.0 ± 12.5	34.3 ± 5.2	246.7 ± 29.2
Jeko-1	9.8 ± 1.4	176.3 ± 9.2	85.5 ± 4.8	1607.3 ± 185.6	14.1 ± 3.0	490.0 ± 37.9
EoL-1	1.7 ± 0.3	128.5 ± 19.0	99.1 ± 0.2	2443.7 ± 95.3	29.7 ± 0.7	283.0 ± 7.2
KG-1	0.4 ± 0.1	118.3 ± 12.2	95.7 ± 0.4	2947.0 ± 92.0	3.7 ± 1.1	190.0 ± 18.2
IM9	99.6 ± 0.1	5113.5 ± 374.4	99.9 ± 0.1	8998.0 ± 536.9	24.9 ± 1.8	340.7 ± 3.9
Raji	99.8 ± 0.1	5580.0 ± 225.1	99.9 ± 0.1	9318.7 ± 348.3	4.4 ± 0.8	186.7 ± 19.6
HDLM-2	99.4 ± 0.3	1916.6 ± 62.4	99.9 ± 0.1	17390.0 ± 606.4	24.8 ± 6.4	167.8 ± 32.1

Data are reported as the percentage of antigen-positive cells and mean fluorescence intensity (MFI). The results represent mean ± SEM from three independent experiments.

target tumors and the extent of target cell lysis (Table 1, Figure 2b). Since the principal mechanism of NK cell-mediated killing involves granule exocytosis,[21] we measured the secretion of perforin and granzyme B in co-culture supernatants to assess their relative contribution to cytotoxicity enhancement. The ELISA results revealed that NK101 and NK101-7.28.CD produced similar levels of perforin and granzyme B in co-cultures with K562. On the other hand, NK101-7.28.CD secreted significantly higher levels of granzyme B, despite lower perforin production, than NK101 in co-cultures with Raji and HDLM-2 (Figure 2c). These results suggest that enhanced cytotoxicity of NK101-7.28.CD against $B7^+$ target cells would be partly due to its heightened ability to secrete granzyme B and residual levels of perforin might be sufficient to support the delivery of granzyme B. Finally, to determine the potential involvement of CD7 or CD28 signaling in cytotoxic function of NK101-7.28.CD, we treated blocking antibodies against CD7 or CD28 in NK/tumor cell co-cultures and analyzed the changes in the proportion of apoptotic tumor cells. HDLM-2 and Raji cell lines were selected as targets based on their high susceptibility to NK101-7.28.CD cell killing. We found that anti-CD28 mAb, but not anti-CD7 mAb, inhibited the cytotoxicity of NK101-7.28.CD cells in a statistically significant manner (Figure 2d). The involvement of CD28 in MHC-unrestricted killing of a NK cell line has also been demonstrated in prior researches of YT and NK-92, where

blocking of CD28/B7 interactions resulted in the attenuation of cytolytic function.[20,22] Of note, the inability of CD7 to transmit a lytic signal in NK101 may be explained by a previous report by Rabinowich et al., where ligation of CD7 by either anti-CD7 mAbs or a CD7-specific hybridoma (7G5) failed to potentiate target cell killing activity of primary human NK cells.[23] Collectively, these results suggest that enhanced tumoricidal activity of NK101-7.28.CD is mainly mediated by the intercellular interaction between CD28 and its ligands expressed on target cells.

Incorporation of CD suicide gene enables self-elimination of transduced NK101 as well as bystander killing of neighboring tumor cells upon 5-FC treatment

Clinical application of the NK-92 cell line has required irradiation prior to infusion to prevent possible malignant expansion of transferred cells.[6] Since NK101 possesses higher intrinsic proliferative potential than NK-92,[7] CD suicide gene was incorporated into NK101 as a measure to provide an additional safeguard against irradiation-escapees. Gene expression in a CD-transduced cell line, NK101-7.28.CD, was first confirmed by reverse transcription PCR (Figure 3a). Next, we assessed the effects of 5-FC treatment on the proliferation of un-irradiated NK cells using the MTS assay. As expected, 5-FC treatment induced the apoptosis of NK101-7.28.CD in a dose-dependent manner, but not in parental NK101 (Figure 3b). Since 100 μg/mL 5-FC induced nearly complete growth inhibition in NK101-7.28.CD, this dosage was selected for the following experiments. High-performance liquid chromatography analysis revealed time-dependent increase of 5-fluorouracil (5-FU) with concomitant decrease of 5-FC in the supernatants from NK101-7.28.CD, while 5-FC concentration remained unchanged in that from parental NK101 (Table S1). These results provide direct evidence for CD activity in transduced cells and suggest the involvement of 5-FU in the induction of cellular apoptosis. To assess the contribution of converted 5-FU in bystander killing of neighboring tumor cells, lytic activities of NK101 and NK101-7.28.CD against IM-9 and K562 tumor cell lines in the presence or absence of 5-FC were evaluated. The results showed that the addition of 5-FC markedly enhanced cytotoxicity of NK101-7.28.CD, but not that of unmodified NK101 (Figure 3c). These results propose that CD/5-FC suicide gene system not only serves a potential safety switch to eliminate proliferating NK cells but also provides an effective anticancer modality to potentiate the therapeutic efficacy of engineered NK101.

Ectopic expression of membrane bound IL-15 enhances the cytotoxicity of NK101 against all tumor cells

IL-15 is a pleiotropic cytokine that increases the proliferation and effector function of NK cells. As a predominant physiological form of the cytokine, mbIL-15 is known to transmit a stronger activation signal than its soluble counterpart.[24] A previous report by Imamura et al. showed that ectopic expression of mbIL-15 not only promoted expansion and survival of NK cells in the absence of exogenous IL-2, but also increased the cytolytic activity of NK cells along with their activation receptor expression and lytic granule release. As they found that the principal mechanism of mbIL-15 stimulation was autocrine (*cis* activation), mbIL-15 engineering was suggested to be an effective approach to enhance the antitumor efficacy of NK cells while minimizing potential side effects

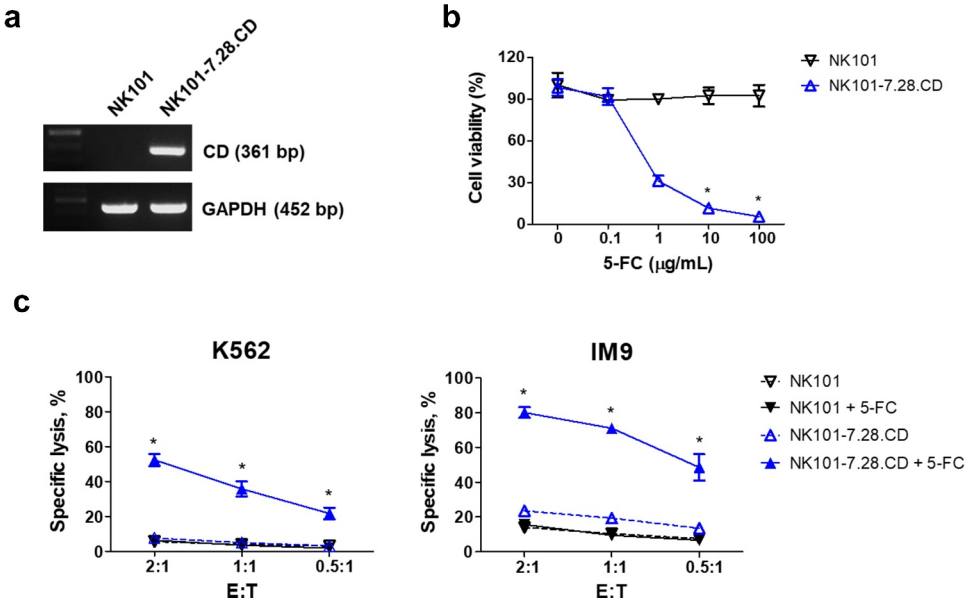

Figure 3. Effects of CD suicide gene expression on engineered NK101 cells and bystander tumor cells upon 5-FC treatment. (a) Expression of CD gene in NK101 and NK101-7.28.CD was analyzed by reverse transcription PCR. GAPDH was used as a control. (b) Various concentrations (0.1, 1, 10 or 100 μg/mL) of 5-FC were treated to NK101 or NK101-7.28.CD for 72 h, and cell viability was measured by CellTiter 96 Aqueous One Solution cell proliferation assay. Data represent mean ± SD of triplicate wells from two independent experiments. *$p < .05$ versus NK101 at indicated 5-FC concentration. (c) IM-9 and K562 tumor cell lines were co-cultured with NK101 or NK101-7.28.CD in the presence or absence of 5-FC (100 μg/mL) at different E:T ratios for 48 h. Harvested cells were stained with Annexin-V and fixable viability dye, and apoptotic tumor cells were analyzed by flow cytometry. *$p < .05$ for NK101-7.28.CD versus NK101-7.28.CD + 5-FC. All data represent mean ± SEM of 5 independent experiments.

caused by the interaction of IL-15 with cells other than NK cells.[24] Based on these observations, we transduced NK101 cells with mbIL-15-expressing lentivirus to generate NK101-15.TN (Figure 1b). Cell surface expression of IL-15 on NK101-15.TN was first confirmed by flow cytometry (Figure 4a). NK101-15.TN cells were able to grow stably without exogenous IL-2, while parental NK101 cells failed to expand in the absence of IL-2 supplementation (Figure S2). The population doubling time for NK101-15.TN without IL-2 was 35.0 ± 3.4 h, which was longer than that of NK101 cells (25.7 ± 2.2 h) but comparable to that of NK-92 cells (35.6 ± 6.1 h) grown in the presence of IL-2, reported in our previous study.[7] For following experiments, NK101-15.TN was maintained under IL-2 deprived conditions. We then performed parallel co-cultures of NK101 and NK101-15.TN with tumor cell lines described in Figure 2b and compared their tumoricidal activities. Remarkably, NK101-15.TN demonstrated stronger lytic activities than NK101 in all co-cultures conducted, albeit to varying degrees (Figure 4b). To elucidate the possible mechanisms underlying the enhanced cytotoxicity, we first compared the amount of secreted perforin and granzyme B by NK101 and NK101-15.TN in co-culture supernatants. The results showed that NK101-15.TN produced significantly higher levels of perforin, but much lower levels of granzyme B, in all co-cultures tested (Figure 4c). Although the capacity of perforin to induce direct lysis of target cells has been reported in numerous studies, further investigations are required to determine whether NK101-15.TN-mediated enhanced killing is due to heightened perforin secretion or by a mechanism distinct from perforin-induced lysis as shown in granzyme AB knockout cytotoxic T lymphocytes.[25] Next, we tested whether the changes in the expression of NK cell receptors contributed to the increased

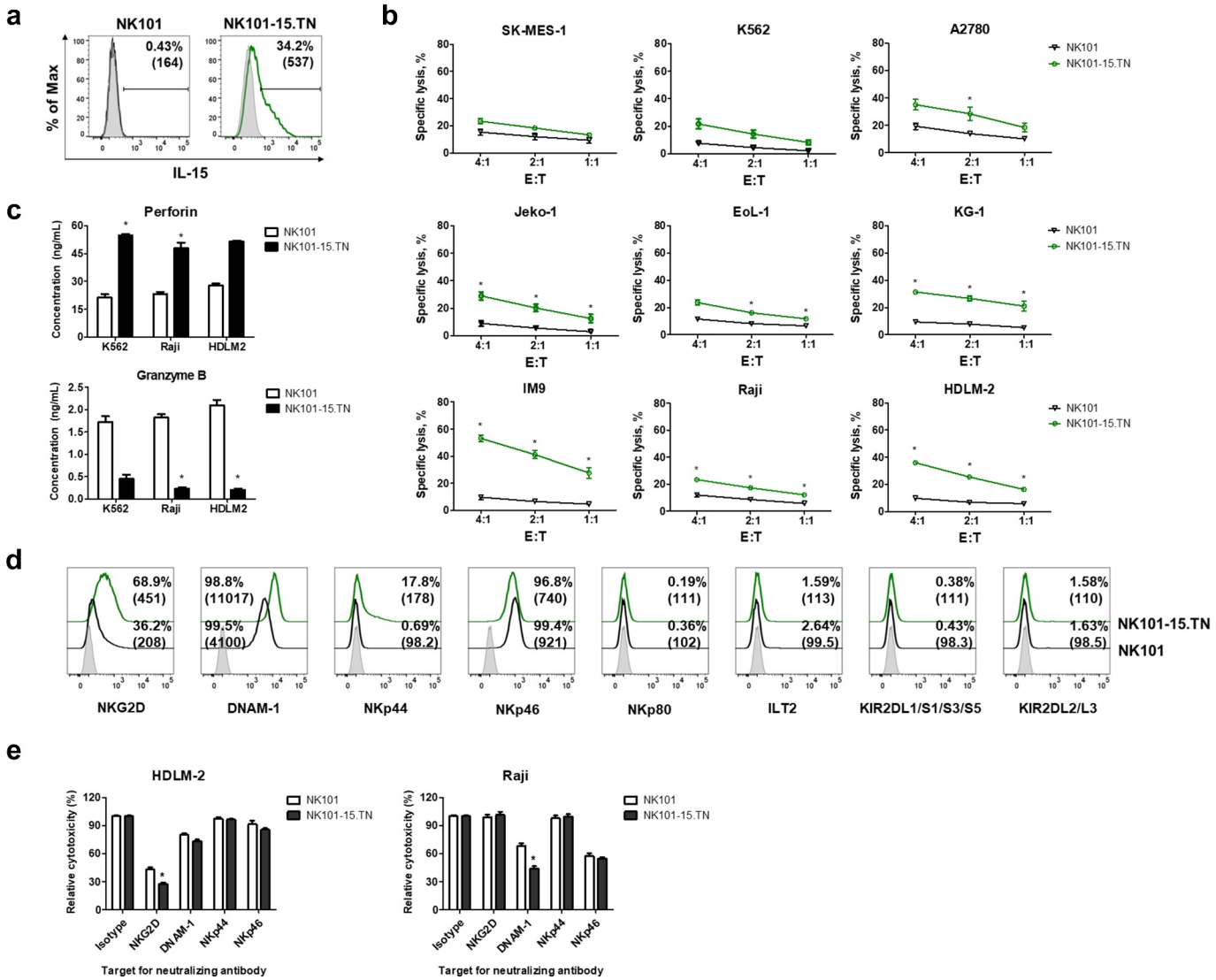

Figure 4. Effects of mbIL-15 expression on the effector function of NK101. (a, d) Representative histograms for cell surface IL-15 (a) and various activating/inhibitory receptor (d) expression on NK101 or NK101-15.TN cells. In each plot, gray-shaded, black, and green line indicates isotype control, NK101 and NK101-15.TN, respectively. Numbers in the histograms and parentheses indicate the percentages and mean fluorescence intensity of the gated population, respectively. (b) Lysis of indicated tumor cell lines mediated by NK101 or NK101-15.TN at different E:T ratios for 24 h was quantified by Annexin-V and fixable viability dye staining via flow cytometry. *$p < .05$ versus NK101 at indicated E:T ratio. (c) Perforin and granzyme B concentrations in the 24 h co-culture supernatants were determined by ELISA. *$p < .05$. (e) NK101 and NK101-15.TN cells were co-culture with HDLM-2 or Raji cells at E:T ratio of 4:1 in the presence of indicated mAbs (10 μg/mL) for 24 h. Harvested cells were stained with Annexin-V and fixable viability dye, and apoptotic tumor cells were analyzed by flow cytometry. Bar graphs represent the cytotoxicity of neutralizing antibody-treated cells relative to that of isotype antibody-treated cells (set as 100%). All data represent mean ± SEM of 5 independent experiments. *$p < .05$.

cytotoxicity of NK101-15.TN. Flow cytometric analysis revealed that mbIL-15 engineering resulted in increased NKG2D, DNAM-1, and NKp44 expression on NK101-15.TN cells, while expression levels of other activating receptors (NKp46, NKp80) and inhibitory receptors (ILT2, KIR2DL1/S1/S3/S5, KIR2DL2/L3) remained unchanged (Figure 4d). We then treated blocking antibodies against activating receptors in co-cultures of NK cells with HDLM-2 and Raji, as performed in Figure 2d. In NK cell/HDLM-2 co-cultures, anti-NKG2D and anti-DNAM1 antibodies inhibited cytotoxic activities of both NK cell lines but the inhibitory effects of NKG2D blockade was relatively greater in NK101-15.TN than NK101. In NK cell/Raji co-cultures, anti-DNAM1 and anti-NKp46 antibodies treatment effectively reduced the lytic activities of both NK cell lines but the inhibitory effects of DNAM1 blockade was greater in NK101-15.TN than NK101 (Figure 4e). These results suggest that the upregulation of NKG2D and DNAM1, albeit to varying degrees depending on target tumor cells, contributed to enhanced antitumor activity of NK101-15.TN.

Stable expression of DNTβRII confers resistance to TGFβ-induced suppression of cytotoxic functions in NK101

TGFβ is an immunosuppressive cytokine produced by tumor cells and other cell types in the TME that impairs cytotoxic function of NK cells.[26] As a measure to block TGFβ signaling in NK101 cells, DNTβRII lacking intracellular kinase domain was introduced into NK101 cells. Surface expression of DNTβRII on the resulting cell line, NK101-15.TN, was confirmed by flow cytometry (Figure S3A). Next, we compared lytic activities of NK101 and NK101-15.TN against HDLM-2 and IM-9 tumor cell lines in the presence or absence of recombinant TGFβ1. At all doses tested, TGFβ1 treatment diminished cytotoxic functions of NK101, while those of NK101-15.TN remained unaffected (Figure S3B). These results suggest that TGFβ signaling is effectively blocked by DNTβRII transfer and engineered NK101 cells are able to oppose immuno-suppressive effects of TGFβ.

Integration of multiple transgenes generates multifaceted killer cells exerting diverse mechanisms of antitumor action

The major advantage of utilizing NK cell lines would be their amenability to multiple rounds of genetic modifications. To assess the combined effects of integrating multiple transgenes on the overall antitumor activity of NK101 cells, NK101-7.28.CD cells were subjected to a second round of infection with lentivirus expressing pBD-15.TN to generate NK111 cells (Figure 1b). Surface expression of CD7, CD28, mbIL-15 and DNTβRII and CD gene expression on NK111 cells were first confirmed by flow cytometry and reverse transcription PCR, respectively (Figure S4). Comparative analysis of lytic activities of NK101-7.28.CD and NK111 demonstrated that NK111 possessed significantly higher cytolytic potential than NK101-7.28.CD against all cancer cell lines tested (Figure 5a). The ELISA analysis of co-culture supernatants revealed higher secretion of both perforin and granzyme B by NK111 than NK101-7.28.CD (Figure 5b). As with NK101-7.28.CD, NK111 cells exerted bystander effects on IM-9 and K562 cells upon 5-FC treatment (Figure 5c). It was worth noting that NK111 cells in combination with 5-FC killed nearly 90% of IM-9 cells at an E:T ratio of 0.5:1, inducing more cell death than NK111 cells alone at E:T ratio of 2:1. Moreover, unlike NK101-7.28.CD, NK111 cells were able to resist suppressive effects of TGF-β (Figure 5d). Collectively, the integration of multiple transgenes resulted in

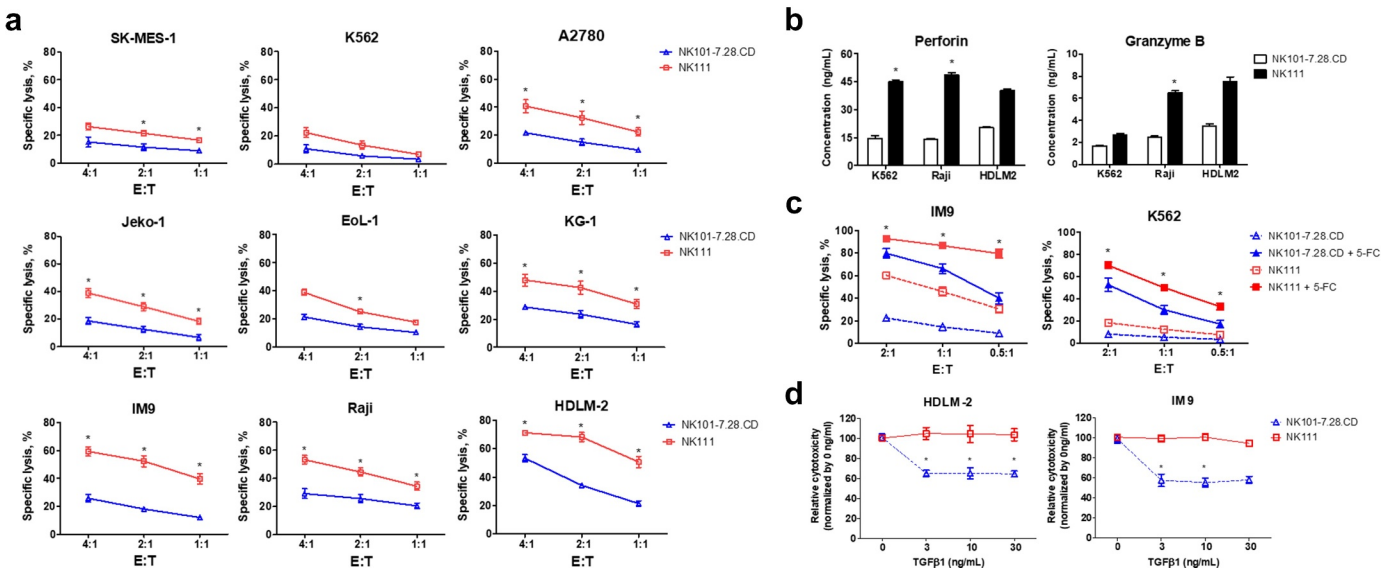

Figure 5. Combined effects of integrating multiple transgenes on the overall antitumor activity of NK101. (a) Specific lysis of indicated tumor cell lines mediated by NK101-7.28.CD or NK111 at different E:T ratios for 24 h was quantified by Annexin-V and fixable viability dye staining via flow cytometry. *p < .05 versus NK101-7.28.CD at indicated E:T ratio. (b) ELISA analysis of perforin and granzyme B secretion from the 24 h co-culture supernatants. *p < .05. (c) IM9 and K562 cells were co-cultured with NK101-7.28.CD or NK111 in the presence (100 μg/mL) or absence of 5-FC at different E:T ratios for 48 h. The percentages of apoptotic tumor cells were quantified by Annexin-V and fixable viability dye staining via flow cytometry. *p < .05 versus NK101-7.28.CD at indicated TGFβ1 concentration. (d) HDLM-2 and IM-9 tumor cell lines were co-cultured with NK101-7.28.CD and NK111 in the presence of recombinant TGFβ1 (3, 10 and 30 ng/mL) at E:T ratio of 4:1 for 24 h. Harvested cells were stained with Annexin-V and fixable viability dye, and apoptotic tumor cells were analyzed by flow cytometry. Lytic activities of TGFβ1-treated cells relative to the cytotoxicity of non-treated cells (set as 100%) are indicated. *p < .05 versus NK111 at indicated TGFβ1 concentration. All data represent mean ± SEM of 5 independent experiments.

the empowerment of NK101 cells with high baseline cytotoxicity and bystander killing potency as well as TGF-β insensitivity.

Introduction of CAR enhances antigen-specific killing activities of NK111

The introduction of CAR is the most widely adopted strategy for genetic manipulation of T cells as well as NK cells.[8] Hereafter, we tested the feasibility of NK111 as a base platform to derive suites of CAR-NK cells directed to different target antigens. For this purpose, we modified NK111 cells to express an EpCAM-specific or a FLT3-specific CAR bearing NK cell-specific signaling domains for targeting solid tumors or hematologic malignancy, respectively (Figure 1b). Surface expression of CARs on the resulting cell lines, NK111-αEpCAM-CAR and NK111-αFLT3-CAR, was confirmed by flow cytometry using an anti-IgG Fc antibody recognizing the spacer region (Figure 6a and 6b, left panels). Binding of EpCAM or FLT3 antigen by the corresponding CAR was also verified by flow cytometry using an EpCAM-Fc or a FLT3-Fc fusion protein (Figure 6a and 6b, right panels). Western blot analysis of NK101, NK111 and NK111-αEpCAM-CAR lysates using anti-CD3ζ antibody revealed that CAR is exclusively expressed by NK111-αEpCAM-CAR cells, mainly in dimer configuration (Figure S5). To assess the antigen-specific killing activity of CAR-modified NK111, human ovarian cancer cell lines with variable levels of EpCAM expression were first used to set up the co-cultures with NK101, NK111 or NK111-αEpCAM-CAR. The results showed that the magnitude of increase in cytotoxicity upon CAR insertion correlated with the expression levels of EpCAM on target tumor cells, as shown by the largest difference between percent-specific lysis of NK111 and NK111-αEpCAM-CAR against RMG-1 cell line with the highest EpCAM expression (Figure 6c). It was important to note that antigen-specific cytotoxic activity as well as surface phenotype of NK111-αEpCAM-CAR were well retained after γ-irradiation with 10 Gy (Figure S6 and S7). Analogous experiments were performed to evaluate FLT3-specific cytotoxicity of NK111-αFLT3-CAR using human blood cancer cell lines. As expected, NK111-αFLT3-CAR displayed superior killing activities against FLT3-expressing EoL-1 and REH cell lines when

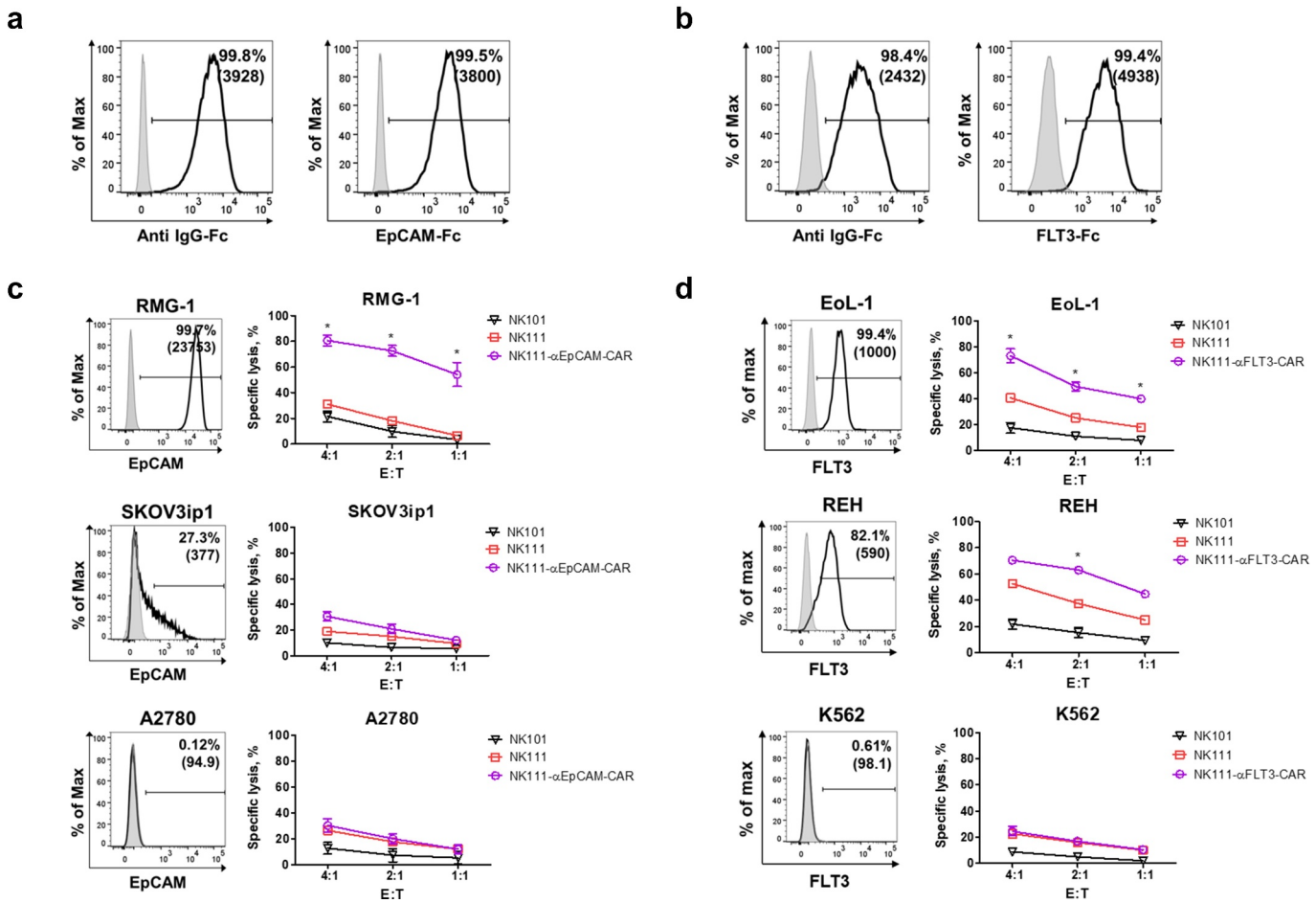

Figure 6. Effects of CAR expression on the cytolytic activity of NK111. (a, b) Representative histograms for cell surface CAR expression on NK111-αEpCAM-CAR (a) and NK111-αFLT3-CAR (b). Cells were either stained with a fluorochrome-conjugated antibody specific to hyFc spacer domain (anti-IgG Fc; left panel) and EpCAM-Fc or FLT3-Fc fusion protein followed by a fluorochrome-conjugated anti-human IgG Fc (right panel). (c, d) Representative histogram plots showing the frequencies of EpCAM-positive cells (c) and FLT3-positive cells (d) on target tumor cell lines (left panels) and corresponding graphs showing their apoptotic rates upon co-cultures with NK101, NK111 and NK111-αEpCAM-CAR or NK111-αFLT3-CAR at different E:T ratios for 24 h (right panels). For all FACS plots, open histograms indicate specific binding, while gray-filled histograms indicate respective controls. Numbers in the histograms and parentheses indicate the percentages and mean fluorescence intensity of the gated population, respectively. For co-culture experiments, apoptotic and dead cell population were discriminated by Annexin-V and fixable viability dye staining, followed by flow cytometric analysis. Data represent mean ± SEM of 5 independent experiments. *p < .05 for NK111-αEpCAM-CAR or NK111-αFLT3-CAR versus NK101.

compared to NK101 or NK111. On the other hand, NK111-αFLT3-CAR showed comparable cytolytic activity with NK111 against FLT3-null K562 cell line (Figure 6d). Antigen-specific killing activities of CAR-NK111 cells were further validated by co-culture experiments using Lenti-X 293 T cell line transduced with the lentiviral constructs encoding EpCAM or FLT3 as target cells. Cytotoxicity analysis against mock-transduced Lenti-X 293 T cells revealed that NK111-αEpCAM-CAR and NK111-αFLT3-CAR possess similar levels of baseline killing ability. The results also showed that NK111-αEpCAM-CAR exhibited higher cytotoxicity than NK111-αFLT3-CAR against EpCAM-transduced Lenti-X 293 T cells, while NK111-αFLT3-CAR demonstrated stronger killing activity against FLT3-transduced target cells (Figure S8).

CAR-NK111 cells exert potent therapeutic efficacy in both hematological and solid tumor xenograft models

Finally, we investigated the integrative effects of multi-dimensional engineering on the overall antitumor efficacy of NK101 in preclinical models of solid tumor and hematologic malignancy. For the assessment of the therapeutic potential of NK111-αEpCAM-CAR, we used a peritoneal metastatic xenograft model of RMG-1 that express high levels of surface EpCAM (Figure 6c). The i.p. route was selected for the administration of NK111-αEpCAM-CAR cells based on previous evidence showing superior efficacy of i.p.-delivered NK-92 over i.v.-delivered counterpart in the i.p. xenograft mouse model of human ovarian cancer.[27] Two preliminary studies were designed to determine the appropriate dose and interval for multiple dosing of irradiated NK111-αEpCAM-CAR. First, a single-dose escalation study revealed that 3×10^7 cells of NK111-αEpCAM-CAR were required to achieve noticeable tumor regression from day 7 post treatment (Figure S9). Second, a biodistribution/persistence study showed that irradiated NK111-αEpCAM-CAR cells were detected up to day 3 and cleared from most organs by day 7 (Table S2). These results led us to perform repeated administration of irradiated NK cells at a dosage of 3×10^7 cells every 3 to 4 days (for a total of 6 doses) (Figure 7a). In line with in vitro results, NK111-αEpCAM-CAR therapy was more effective at suppressing metastatic outgrowth than that of NK101 or NK111, as significant reduction in bioluminescence signal intensity relative to medium-treated control group was only shown in the

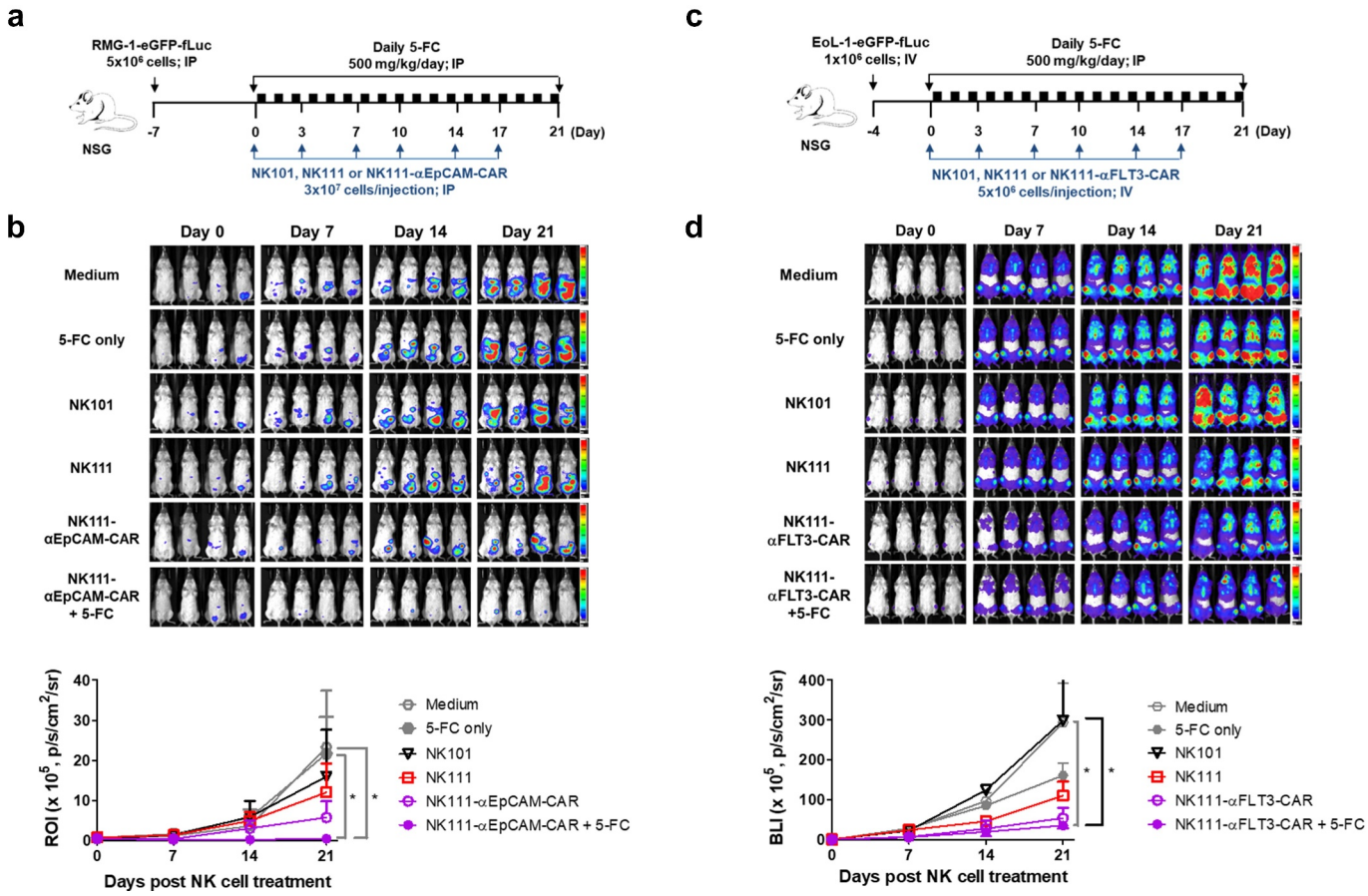

Figure 7. Anti-tumor effects of NK111-αEpCAM-CAR and NK111-αFLT3-CAR, with or without 5-FC, in a xenogeneic mouse model of metastatic solid tumor and hematologic malignancy. (a, c) Experimental schema: Female NSG mice were injected with 5×10^6 cells of RMG-1-eGFP-fLuc i.p. and tumors were allowed to grow for 7 days. Mice were then treated with multiple i.p. doses (twice per week for 3 weeks) of 3×10^7 irradiated NK101, NK111 or NK111-αEpCAM-CAR cells (a). Female NSG mice were injected with 1×10^6 cells of EoL-1-eGFP-fLuc i.v. Beginning on day 4 after tumor injection, mice were either treated twice a week i.v. 5×10^6 cells of 10 Gy-irradiated NK101, NK111 or NK111-αFLT3-CAR for a total of 6 doses (c). 5-FC treatment groups received daily i.p. injections of 500 mg/kg 5-FC till day 21 post NK cell treatment, starting on the day of NK cell injection. (b, d) Serial BLI was performed to assess tumor progression and representative images of mice taken at different time points are shown (n = 4 for each group; top panels). Bioluminescent signals were quantitated using Amiview and plotted in graphs (bottom panels). Data represent mean ± SEM of 4 mice per group. *p < .05.

NK111-αEpCAM-CAR-treated group ($p = .05$). Moreover, the addition of daily 5-FC to the regimen further potentiated antitumor activity of NK111-αEpCAM-CAR therapy that was sufficiently strong to control tumor burden to near baseline levels until the end of study (Figure 7b).

We expanded our studies to a hematologic tumor model to assess the therapeutic efficacy of NK111-αFLT3-CAR. For this purpose, a disseminated leukemia xenograft model was first established by i.v. injecting EoL-1 cells that expresses high levels of surface FLT3 antigen (Figure 6d). The maximum i.v. infusible dose for NK101 and its derivatives was set as 5×10^6 cells, as mice treated with 1×10^7 cells of NK101 cells by i.v. delivery showed signs of respiratory difficulty (due to probable pulmonary embolism) in our preliminary experiment. The dosing interval for NK111-αFLT3-CAR was the same as that for NK111-αEpCAM-CAR (Figure 7c). In agreement with in vitro results, repeated administration of irradiated NK111 showed better therapeutic effects than that of NK101. Furthermore, NK111-αFLT3-CAR therapy exerted even stronger effects in suppressing leukemic growth than did NK111 therapy. Unlike the results obtained in a peritoneal metastatic xenograft model, we did not observe significant improvement in the anticancer activity of NK111-αFLT3-CAR therapy upon combination treatment with daily 5-FC (Figure 7d). This might be due to the application of lower dose (5×10^6 cells/dose) of NK111-αFLT3-CAR cells along with the disseminated nature of disease, as opposed to RMG-1 tumors that remain localized in the peritoneal cavity.

Discussion

Given the importance of multiple genetic modifications to the success of ACT therapies and the challenges associated with the manufacture and testing of multigene-modified products, NK cell line platforms are gaining increasing attentions for their unlimited engineering potential and industrial scalability along with cellular uniformity, long-term stability and batch-to-batch consistency that enable extensive characterization of final products.[27,28] In this study, we used a novel human NK cell line, NK101, as a backbone to integrate six transgenes through three rounds of lentiviral transduction, representing the most extensive genetic modifications described so far for any NK cell line. As a result, we have successfully derived clonal, multigene-modified cell therapeutics capable of exerting various modes of antitumor actions: (i) activation receptor-mediated innate killing; (ii) tumor-directed, antigen-specific killing; and (iii) bystander effect-mediated killing. The resulting final products, NK111-αEpCAM-CAR and NK111-αFLT3-CAR, demonstrated significantly improved tumor-inhibitory effects in a preclinical model of metastatic solid tumor and hematologic malignancy, respectively. Although further investigations are required to translate the present results into human experimental settings, we believe that clinical and commercial value of CAR-NK111 cells of this study lie in their potential to overcome a number of limitations of conventional gene-modified cell therapies: First, the difficulties in manufacturing autologous cell therapies due to inadequate number or function of peripheral T or NK cells in heavily pretreated cancer patients could be circumvented by the use of off-the-shelf allogeneic NK cells. Second, antigenic loss and tumor heterogeneity that pose significant challenges to single antigen-targeting products, such as conventional CAR-Ts, could be overcome by CAR-NK111 cells possessing multiple CAR-independent killing mechanisms. According to the results from the current study and our previous study on NK101,[7] CAR-NK111 cells can utilize an array of activating receptors – DNAM-1, ICAM-1, CD28, and NKG2D – to recognize and kill tumor cells. Moreover, any residual tumor cells that escape destruction by CAR-NK111 cells can be eradicated by 5-FU produced upon 5-FC treatment. Third, immunosuppressive TME affecting the efficacy of immune effector cells can be better addressed by CAR-NK111 cells expressing DNTβRII. As the most potent suppressive factor regulating the effector function of cytotoxic T cells and NK cells in TME, TGFβ is secreted at higher levels in cancerous conditions and the average total TGF-β1 levels in the plasma of patients with solid tumors and hematologic malignancies ranged from 5 ng/mL to > 20 ng/mL.[29] Our findings revealed that a dose of 1 mg/mL TGFβ was sufficient to repress cytotoxic activity of parental NK101, while DNTβRII-modified NK101s could resist up to 30 mg/mL TGFβ. Taken together, the present study provides the rationale for further investigation of our first generation multi-engineered NK101 cell therapeutics in more clinically relevant models.

Therapeutic applicability of a NK cell line is continuously expanding based on its potential to serve as a carrier for targeted release of anticancer payloads. Representatively, recent studies have used NK-92 as a vehicle to deliver apoptosis-promoting agent, drug-loaded nanoparticles, and oncolytic viruses.[30–32] However, none of the previous research has attempted to introduce a prodrug-activating enzymes into NK cell lines for the purpose of inducing cancer cell death. Our current study is the first to utilize a (CAR-modified) NK cell line as a tumor-targeted suicide gene delivery vehicle and successfully demonstrate bystander tumor cell killing upon prodrug treatment that was translated to potentiation of antitumor efficacy of NK111-αEpCAM-CAR in an intraperitoneal RMG-1 xenograft model. Our novel finding might be explained as the result of a combination of the following factors: The first would be associated with the selection of an enzyme/prodrug pair. We utilized CD/5-FC over the most abundantly used herpes simplex virus thymidine kinase/ganciclovir, since CD/5-FC system is known to exert stronger bystander effects in a gap junctional intercellular communication-independent manner.[33,34] The second would be due to CAR-mediated enhancement of NK cell homing to antigen-expressing tumor cells. Previous studies of CAR-expressing NK92 cells showed a higher degree of tumor-infiltration by CAR-NK92 cells compared to unmodified counterpart in orthotopic xenograft models.[35,36] Third, irradiated NK111-αEpCAM-CAR seemed to persist for a certain period in vivo to allow adequate conversion of 5-FC to 5-FU and its diffusional export to take place. According to a previous study, the bystander effects can fail by inadequate 5-FC uptake, limited 5-FU export or rapid elimination of CD-expressing cells prior to sufficient 5-FU release.[37]

Our in vitro conversion analysis of NK111 revealed that over 50% of 5-FC was converted to 5-FU within 60 hours, while in vivo biodistribution study of NK111-αEpCAM-CAR showed that irradiated NK cells persisted up to 72 hours (Table S1 and S2). As 5-FU only targets cycling cells, cell cycle perturbation via irradiation would render NK111-αEpCAM-CAR cells resistant to 5-FU-mediated killing and sustain the duration of CD expression to achieve sufficient conversion of 5-FC to 5-FU that ultimately lead to tumor regression.[38] Future studies should explore strategies to maximize bystander killing effects of engineered NK101, including the use of an enzyme/prodrug system with a wider radius of action and the optimization of treatment regimens for practical clinical applications.[39]

Next-generation multigene-modified NK cell therapeutics are currently under active development and are generally categorized by the source of NK cells: (i) donor-derived NK cells; (ii) iPSC-derived NK cells; (iii) clonal NK cell lines.[40] The first successful application of multigene-modified NK cells was reported in patients with relapsed and refractory CD19-positive cancers, where a single infusion of allogeneic, cord blood-derived NK cells engineered to express anti-CD19 CAR, IL-15 and inducible caspase-9 induced durable complete remission (in 7 out of 11 patients) without severe adverse effects. Although this landmark study demonstrated clinical potential of multigene-modified primary NK cells, their manufacturing involved a complex process – adequate donor selection, NK cell purification, feeder-dependent expansion, viral transduction, and fresh cell infusion – that was repeated from batch-to-batch. Moreover, each patient lot displayed different purity and transduction rate, implicating the challenges associated with product qualification and standardization.[41] The derivation of engineered NK cells from a clonal master iPSC line can resolve some of manufacturing/regulatory complexities of individualized processing, as multi-patient doses are generated from a single starting material.[42] For instance, Bjordahl et al. employed multi-step engineering approach to construct a universal iPSC backbone – expressing surface-bound IL-15, high affinity CD16, HLA-G (human leukocyte antigen-G) but lacking HLA-class I – that could be further modified to express disease-specific CARs. Engineered iPSCs were then subjected to differentiation and expansion to produce ready-to-use banks of multi-functional NK cells with improved antitumor efficacy.[43] Although repeating this procedure allows consistent production of multi-engineered NK cells at industrial scale, the involvement of two-stage differentiation process – from iPSCs to $CD34^+$ hematopoietic lineage progenitor cells and then to NK cells – necessitating long production period and the liability of cells to spontaneous differentiation during this period could raise substantial technical challenges in manufacturing final products at desired quality.[40,44,45] It is also worth noting that clinical benefit of multi-engineered iPSC-NK cells is yet to be validated. Unlike the cases using primary cells or iPSCs as a source, the utilization of a NK cell line negates the need for complex processing. NK cell lines are easily grown in a feeder-free environment and not prone to differentiate, as they are characterized by differentiation arrest, enabling clinical scale production of NK cells with the streamlined process.[6,46] The fact that the final products derived from NK cell lines consist of homogeneous populations of multi-engineered NK cells expressing high levels of activating receptors but lacking inhibitory receptors provides an additional value to a NK cell line platform. Despite these advantages, multigene-modified NK cell lines have not yet demonstrated clinical benefits in patients. First-in-human trial of anti-CD33 CAR-engineered, IL-2-secreting NK-92 failed to show meaningful efficacy in relapsed and refractory AML, presumably due to limited in vivo lifespan of irradiated NK-92 cells.[47] Multi-engineered NK-92 cells co-expressing endoplasmic reticulum-retained IL-2, high affinity CD16 and PD-L1-targeting CAR are currently being investigated in a phase 1 clinical trial.[48] Since the present study unfolded immense engineering potential of a NK101 platform, we plan to incorporate other essential genetic modifications, including high affinity CD16 expression for mAb combination therapy and targeted disruption of HLA genes for minimizing alloimmune rejection, to maximize clinical efficacy of next generation engineered NK101 products. Overall, we envision that NK101 would become one of the major sources for generating diverse multi-engineered cell therapeutics for cancer immunotherapy.

Abbreviations

5-FC: 5-fluorocytosine; 5-FU: 5-fluorouracil; ACT: adoptive cell transfer; AML: acute myeloid leukemia; BLI: Bioluminescence imaging; CAR: Chimeric antigen receptor; CD: cytosine deaminase; DNTβRII: dominant negative TGFβ type II receptor; eGFP: enhanced green fluorescent protein; EpCAM: epithelial cell adhesion molecule; fLuc: firefly luciferase; FLT3: fms-like tyrosine kinase 3; HLA: human leukocyte antigen; hyFc: hybrid Fc; IL-15: interleukin-15; i.p.: intraperitoneally; iPSC: induced pluripotent stem cells; IRES: internal ribosomal entry site; i.v.: intravenously; mAb: monoclonal antibody; mbIL-15: membrane bound IL-15; MHC: major histocompatibility complex; NK: Natural killer; PD-L1: programmed death-ligand 1; SECTM1: secreted and transmembrane protein 1; scFv: single chain variable fragment; TGFβ: transforming growth factor beta; TME: tumor microenvironment

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Authors' contributions

IH designed the research and experiments, performed experiments, analyzed and interpreted the data, and wrote the manuscript. HTJ designed, supervised the research, and reviewed the manuscript. MCK and TYK designed, performed, and analyzed experiments. YCS designed, supervised the research and experiments, and reviewed the manuscript. SWK designed, supervised the research and experiments, analyzed and interpreted the data, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All the data and materials supporting the conclusions of this study are included within the article and the supplementary information. Any additional information will be available from the corresponding author upon reasonable request.

Disclosure statement

Authors from SL BiGen, Inc. and Progen. Co., LTD. are current or former employees and/or shareholders of the respective companies. The remaining author declares no competing financial interests

Ethics approval

Animal experiments were approved and performed in compliance with the guideline of the Institutional Animal Care and Use Committee.

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