

## ORIGINAL RESEARCH ARTICLE

# mRNAs encoding NKG2D ligand-targeted bispecific T-cell engagers confer robust anti-tumor activity

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## Abstract

Natural killer group 2, member D (NKG2D) ligands are highly expressed in various tumor cells and therefore represent an attractive target for cancer immunotherapy. A bispecific T-cell engager (BiTE) with a fused extracellular domain of NKG2D and an anti-CD3 single-chain fragment variable (scFv) has shown significant anti-tumor potency. To circumvent the manufacturing challenges and short serum half-life of BiTEs, we developed lipid nanoparticle-encapsulated messenger RNA (mRNA) encoding  $\alpha$ -CD3-mNKG2D fusion proteins. *In vitro* functional analysis revealed that the BiTE-encoded mRNA induced T cell activation and cell cytotoxicity against tumor cells. An *in vivo* study using an A20 lymphoma mouse model showed that BiTE-encoding mRNA significantly inhibited tumor growth. Notably, BiTE constructs without an antibody Fc domain exhibited markedly higher anti-tumor efficacy than BiTE constructs with an Fc fragment. Additionally, we designed a human version of the BiTE mRNA encoding an NKG2D and an anti-CD3 (OKT3) scFv fusion protein. Human BiTE mRNA treatment significantly upregulated CD69 expression on T cells, promoted cytotoxicity against tumor cells *in vitro*, and suppressed tumor growth in a peripheral blood mononuclear cell-engrafted, tumor-bearing mouse model. Based on these preclinical findings, NKG2D BiTE mRNA holds promise as a potential anti-tumor treatment meriting further clinical development.

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**Keywords:** Messenger RNA; NKG2D; CD3; Bispecific T-cell engager; Cancer immunotherapy

## 1. Introduction

T cell-mediated anti-tumor immunity can be achieved using bispecific T-cell engagers (BiTEs), which bind simultaneously to tumor antigens on the surface of cancer cells and to cluster of differentiation (CD)3 on T cells.<sup>1</sup> BiTEs allow cytotoxic T cells to engage with tumor cells, thereby inducing target-dependent T cell activation and tumor cell lysis. Typically, a BiTE consists of two single-chain variable fragments (scFvs), with one pair binding to the tumor antigen and the other pair binding to CD3.<sup>2</sup> Each scFv pair comprises heavy and light chain variable fragments connected by a flexible serine-glycine linker to form a single chain. Blinatumomab, a CD19 and CD3 bi-(scFv)<sub>2</sub>, is a BiTE approved for the treatment of acute lymphoblastic leukemia.<sup>2-4</sup> Several other BiTEs targeting CD20, B-cell maturation protein (BCMA), CD33, delta-like protein 3 (DLL3),

glypican-3 (GPC3), epithelial cell adhesion molecule (EpCAM), and other molecules are either approved or currently in clinical development.<sup>5-9</sup>

A spectrum of BiTE architectures has emerged from distinct engineering strategies.<sup>10</sup> Blinatumomab adopts an immunoglobulin G (IgG)-free format that minimizes molecular weight and maximizes tumor penetration.<sup>11</sup> Conversely, mosunetuzumab and glofitamab, both CD20 and CD3 BiTEs, incorporate modified Fc domains to confer high stability and extended half-life.<sup>12,13</sup> These structural differences translate into divergent pharmacokinetic and pharmacodynamic profiles, making format selection disease- and context-specific.<sup>14</sup>

Natural killer (NK) group 2, member D (NKG2D) is an activating receptor expressed on NK cells that interacts with NKG2D ligands expressed on cancer cells and other stressed cells.<sup>15</sup> The ligands of NKG2D receptors in mice include Rae1, Mult1, and H60, and in humans include major histocompatibility complex class I chain-related proteins A (MICA) and B (MICB) and UL16-binding proteins.<sup>16,17</sup> These ligands are preferentially expressed on tumor cells and cells under stress compared with normal tissue. Various types of tumor cells, including lymphoma, leukemia, hepatocellular carcinoma, colon cancer, and breast cancer, express NKG2D ligands.<sup>18,19</sup> Consequently, NKG2D ligands represent potential targets for cancer immunotherapy. To target these ligands in cancer treatment, NKG2D chimeric antigen receptor T cells and NKG2D BiTEs have been developed.<sup>20-23</sup>

Messenger RNA (mRNA)-lipid nanoparticle (LNP) technology has successfully delivered genes *in vivo* and has been used in vaccine development.<sup>24</sup> In addition to delivering vaccine antigens, LNP has also been used to deliver mRNA encoding therapeutic antibodies.<sup>25-27</sup> The manufacturing process of mRNA is less complex than that of antibody drugs, and the mRNA *in vivo* half-life is greater than that of antibodies.<sup>28</sup> A previous study revealed that the plasma level of BiTEs translated from the administered mRNA could be sustained for more than 3 d, and its levels were barely detectable after 24 h when the recombinant BiTE protein was injected.<sup>25</sup> Although recombinant NKG2D BiTE proteins have previously been generated and functionally characterized,<sup>21,22</sup> the mRNA-encoded NKG2D BiTE has not yet been reported. Here, we reported—for the first time, to our knowledge—the design of mRNA encoding an NKG2D-anti-CD3 scFv fusion protein and demonstrate its anti-tumor activity both *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. mRNA design, synthesis, and formulation

The scFv of the anti-CD3 antibody was constructed by linking the amino acid sequences of the heavy chain and light chain variable domains with a peptide linker (GGGGSGGGSGGGSGGGGS). The scFv was then fused to the extracellular domain of NKG2D using a short GS linker (GGGGS), producing the  $\alpha$ -CD3-NKG2D construct. The Fc domain of the antibody (GenBank ID: AAH02121.1) was also inserted between the short GS linker (GGGGS) and NKG2D fragments to create another variant,  $\alpha$ -CD3-Fc-NKG2D. In order for the protein to be secreted by cells, the human interleukin (IL)-2 signal peptide (MYRMQLLSIALSLALVTNS) was fused to the N-terminus of the BiTE constructs. The amino acid sequence of the anti-mouse CD3 $\epsilon$  scFv was derived from the 145-2C11 monoclonal antibody,<sup>29</sup> and that of the anti-human CD3 $\epsilon$  scFv was obtained from the OKT-3 monoclonal antibody.<sup>30</sup> Mouse NKG2D (Uniprot O54709) and human NKG2D (Uniprot P26718) sequences were used for the constructs. The protein sequences were back-translated, and the corresponding DNA sequences were optimized for enhanced protein expression using a population immune algorithm, an *in-silico* analysis algorithm that leverages principles from both population genetics and immunology. The optimized open reading frames, flanked by 5'UTR, 3'UTR, and poly-A sequences, were subsequently integrated into plasmid vectors.

The NKG2D BiTE mRNA was produced and encapsulated in LNP using the Liverna Therapeutics platform (China patent ZL201911042634.2), as described previously.<sup>31-33</sup> Briefly, the mRNA was synthesized through *in vitro* transcription and subsequently purified using an oligo-dT affinity column (Monomix dT20, Sepax Technologies, Inc., United States of America [USA]) followed by tangential flow filtration (KR2i, Repligen Corporation, USA). The purified mRNA was then encapsulated in LNPs as described previously.<sup>31-33</sup> Specifically, the ionizable lipids MC3, DSPC, cholesterol, and DMG-PEG2000, at a molar ratio of 49.1:6.9:41.1:2.9, were dissolved in ethanol to achieve a final concentration of 20 mM. The lipid mixture was rapidly mixed with a 10 mM citrate buffer (pH 4.0, with 130 mM NaCl) containing 0.2 mg/mL of mRNA at a ratio of 1:3 (lipid/mRNA, v/v), using a microfluidic device (Benchtop, Precision Nanosystems Inc., Canada). The product was analytically characterized by assessing the particle size, polydispersity, and encapsulation efficiency. The mRNA concentration in

the final product was determined using a UV spectrometer (TU-1810PC, Persee Inc., China).

## 2.2. Demonstration of NKG2D bispecific T-cell engager expression

To evaluate the *in vitro* expression of the NKG2D BiTE, 5 µg of LNP-encapsulated mRNA encoding α-CD3-Fc-mNKG2D, α-CD3-mNKG2D, or α-CD3-Fc-hNKG2D was individually transfected into Jurkat cells ( $1 \times 10^6$  cells; American Type Culture Collection, USA) and cultured in RPMI 1640 medium (Cat. #11875093, Gibco, USA) supplemented with penicillin/streptomycin (Cat. #15140-122, Gibco, USA) and 10% fetal bovine serum (FBS; Cat. #FSP500, Excell Bio, China) at 37 °C with 5% CO<sub>2</sub>. Empty LNPs without mRNA served as the negative control. Cellular protein secretion was inhibited with 5 µg/mL of brefeldin A (Cat. #420601, BioLegend, USA) 18 h post-transfection. The transfected cells were incubated for an additional 6 h and subsequently subjected to intracellular staining with fluorescein-conjugated anti-mouse NKG2D (Cat. #115711, BioLegend, USA) or allophycocyanin-conjugated anti-human NKG2D (Cat. #320807, BioLegend, USA) antibodies using a Cytofix/Cytoperm kit (Cat. #554714, BD Biosciences, USA) according to the manufacturer's instructions. The samples were then assessed using a CytoFLEX flow cytometer (Beckman Coulter, USA), and the flow cytometry data were processed using FlowJo software v10 (Tree Star Inc., USA).

To quantify the NKG2D BiTEs, Jurkat cells were transfected with increasing doses (0.156, 0.313, 0.625, and 1.25 µg) of LNP-encapsulated mRNA encoding either α-CD3-Fc-mNKG2D or α-CD3-mNKG2D and cultured in RPMI 1640 medium supplemented with penicillin/streptomycin and 10% FBS at 37 °C with 5% CO<sub>2</sub>. Empty LNPs served as the negative control. Culture supernatants were harvested 24 h post-transfection and analyzed using a customized enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated overnight with 1 µg/mL of recombinant mouse CD3 protein (Cat. #CDG-M58D2, AcroBiosystems, USA) diluted in phosphate-buffered saline (PBS), then blocked with 2% FBS for 2 h. Supernatants were diluted 1:10 in PBS buffer and incubated in the CD3-coated wells for 1 h. After three washes with PBS containing 0.05% Tween 20, plates were incubated sequentially with 1 µg/mL of rat anti-mouse NKG2D antibody (Cat. #12-5882-82, Thermo Fisher, USA) for 1 h and 1 µg/mL of horseradish peroxidase-conjugated anti-rat IgG secondary antibody (Cat. #7077, Cell Signaling Technology, USA) for 1 h. BiTE concentrations were determined by measuring absorbance at 450 nm.

## 2.3. *In vitro* bioactivity analysis

To measure the bioactivity of α-CD3-Fc-mNKG2D and α-CD3-mNKG2D, Jurkat cells were initially transfected with the corresponding LNP-encapsulated BiTE mRNA. Empty LNPs served as the negative control. The cells were incubated for 24 h, and then the culture supernatant was collected and used to stimulate mouse splenocytes in the presence of the mouse tumor cell line MC38 at an effector-to-target cell ratio of 10:1. After 24 h of stimulation, the co-cultured cells were harvested and stained with fluorescein-conjugated anti-mouse CD3 (Cat. #100204, BioLegend, USA) and BD Horizon BB700-conjugated anti-mouse CD69 (Cat. #566500, BD Biosciences, USA). The cells were then analyzed using flow cytometry according to the manufacturer's instructions.

To analyze the bioactivity of the human NKG2D BiTE mRNA, the same procedure was used, but human peripheral blood mononuclear cells (PBMCs) were used as the T cell source, and the tumor cell lines Caski and HepG2 served as sources of NKG2D ligands. Allophycocyanin-conjugated anti-human CD3 (Cat. #555335, BD Biosciences, USA) and brilliant violet 605-conjugated anti-human CD69 (Cat. #310938, BioLegend, USA) antibodies were used for staining, followed by flow cytometry analysis.

## 2.4. *In vitro* cytotoxicity analysis

To analyze the *in vitro* cytotoxicity, Jurkat cells were transfected with NKG2D BiTE mRNA, and the resulting culture supernatant was collected and used to stimulate T cells and tumor cells at an effector-to-target cell ratio of 10:1, as described in Section 2.3. The mouse tumor cell line MC38/Luc and the human tumor cell line HepG2/Luc, both stably expressing the luciferase gene, were used as target cells in a luciferase-based cell cytotoxicity assay. After 24 h of stimulation, the supernatant from the T cell and tumor cell co-culture was collected for lactate dehydrogenase (LDH) analysis (CyQUANT™ LDH cytotoxicity assay kit, Cat. #C20301, Thermo Fisher, USA) according to the manufacturer's instructions. The LDH activity of co-culture supernatant was normalized to that of the positive control (supernatant of Triton X-100-treated tumor cells) to calculate specific tumor cell lysis. A higher amount of LDH reflects higher cell cytotoxicity. Additionally, the co-cultured cells were subjected to luciferase analysis (luciferase reporter gene assay kit, Cat. #11401ES76, Yeasen, China) following the manufacturer's instructions. The luciferase signal of the co-culture cells was normalized to that of the negative control (tumor cells alone) to calculate relative luminescence. A lower luciferase signal indicates lower cell viability.

## 2.5. *In vivo* pharmacokinetics and anti-tumor efficacy analysis

BALB/c mice (female, 6–8 weeks old, 18–23 g) were purchased from Zhuhai BesTest BioTech Co., Ltd. (China), and M-NSG mice (NOD.Cg-PrkdcscidIl2rgem1Smoc) were purchased from Shanghai Model Organisms Center, Inc. (China). A total of 32 BALB/c mice and 12 M-NSG mice were used in this study.

To characterize the pharmacokinetics of the NKG2D BiTEs, BALB/c mice received a single intravenous injection of 25 µg LNP-encapsulated NKG2D-BiTE mRNA. Serum samples were collected at 6, 24, 48, and 72 h post-injection, diluted 1:100, and analyzed using ELISA, as described in Section 2.2.

Syngeneic lymphoma tumor models were established in the BALB/c mice through subcutaneous injection of A20 cells ( $1 \times 10^6$  in 100 µL of Dulbecco's phosphate-buffered saline [DPBS]) in the right flank. A humanized immune system mouse model was created using M-NSG mice, intravenously grafted with human PBMCs ( $1 \times 10^7$  cells in 200 µL of DPBS). Seven days post-PBMC grafts, Huh7 cells ( $5 \times 10^6$  in 100 µL of DPBS) were subcutaneously injected into the right flanks of mice. The reconstituted human T cells were verified using flow cytometry analysis of mouse peripheral blood samples collected 20 days after PBMC engraftment.

The width (smaller diameter) and length (larger diameter) of the tumors were measured two to three times each week using calipers. The tumor volumes were calculated using the following equation:

$$\text{Tumor volume} = \frac{\text{Length} \times \text{Width}^2}{2} \quad (1)$$

Mice were randomly grouped for *in vivo* drug administration when the average tumor volume reached 80–100 mm<sup>3</sup>. LNP-encapsulated NKG2D BiTE mRNA was intratumorally injected using a fixed volume (50 µL) at the indicated dose once a week for three consecutive weeks. Empty LNP without encapsulated mRNA served as the negative control. The day the first dose was administered was designated day 0. Mice were monitored daily for adverse clinical reactions. The tumor size and body weight of each mouse were measured two to three times per week. Tumor growth inhibition (TGI) index was calculated using the equation:

$$\text{TGI} = \left(1 - \frac{\text{Change in tumor volume in treatment group}}{\text{Change in tumor volume in control group}}\right) \times 100\% \quad (2)$$

Survival events were recorded when mice died or underwent euthanasia due to their tumor volume exceeding the limit or adverse effects occurring. In the Huh7 tumor model, mice were euthanized on day 26, and the tumors were excised and weighed.

## 2.6. Statistical analysis

Data are presented as mean  $\pm$  standard error of the mean or mean  $\pm$  standard deviation, as specified. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Inc., USA). Statistical analysis was conducted using unpaired two-tailed Student's *t*-tests or two-way ANOVA with Sidak's multiple comparison test, as indicated in the figure captions. An adjusted  $p \leq 0.05$  was considered statistically significant. Statistical differences are denoted as \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , and \*\*\* $p \leq 0.001$ .

## 3. Results

### 3.1. Construction and *in vitro* expression of mRNA encoding NKG2D bispecific T-cell engager

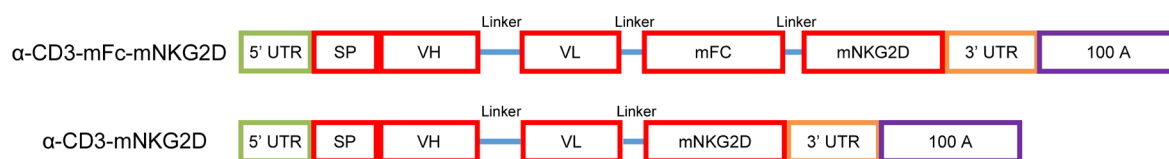
To specifically target NKG2D ligands expressed on tumor cells, we created mRNA encoding BiTEs with an N-terminal anti-CD3 scFv and a C-terminal extracellular NKG2D domain. The Fc domain of an antibody typically facilitates antibody dimerization and extends its half-life. Therefore, we generated two constructs for comparison,  $\alpha$ -CD3-mFc-mNKG2D with a mouse Fc fragment and  $\alpha$ -CD3-mNKG2D without the Fc fragment (Figure 1A). The mRNA was encapsulated in LNPs. The characterization of the mRNA-LNP revealed an average particle size of 70.90 nm for  $\alpha$ -CD3-mFc-mNKG2D and 76.34 nm for  $\alpha$ -CD3-mNKG2D. The zeta potential was  $-0.661$  mV for  $\alpha$ -CD3-mFc-mNKG2D and  $-0.095$  mV for  $\alpha$ -CD3-mNKG2D. The encapsulation rate was 95% for  $\alpha$ -CD3-mFc-mNKG2D and 94% for  $\alpha$ -CD3-mNKG2D.

The BiTE-encoding mRNAs were transfected into the human leukemia Jurkat cell line. Expression of the murine NKG2D BiTEs was verified by intracellular staining (Figure 1B) and quantified using ELISA (Figure 1C). Notably, no significant difference was observed in the expression levels between  $\alpha$ -CD3-mFc-mNKG2D and  $\alpha$ -CD3-mNKG2D.

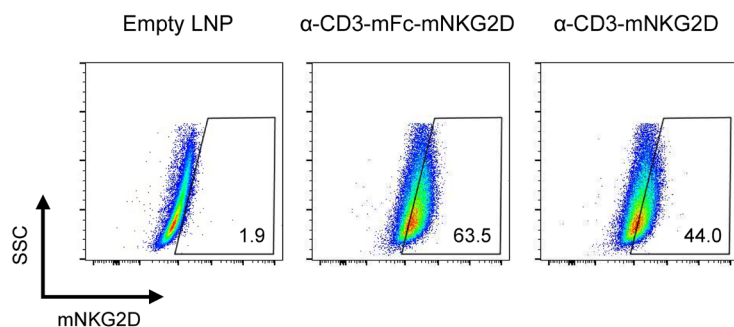
### 3.2. *In vitro* bioactivity of NKG2D bispecific T-cell engager mRNA

To evaluate the bioactivity of the mouse NKG2D BiTE mRNA, we co-cultured MC38 tumor cells (murine colon adenocarcinoma cell line) expressing NKG2D ligands<sup>34</sup> with mouse splenic T cells and stimulated the cells with supernatant from mRNA-LNP-transfected cells. The supernatant from cells transfected with  $\alpha$ -CD3-mFc-mNKG2D and  $\alpha$ -CD3-mNKG2D mRNA significantly induced CD69 expression on T cells, with the  $\alpha$ -CD3-mNKG2D supernatant-treated group exhibiting slightly higher T cell induction (Figure 2). Furthermore, both  $\alpha$ -CD3-mFc-mNKG2D and  $\alpha$ -CD3-mNKG2D BiTE enhanced T cell-mediated cytotoxicity against MC38/Luc tumor cells in a dose-dependent manner (Figure 3A & B).

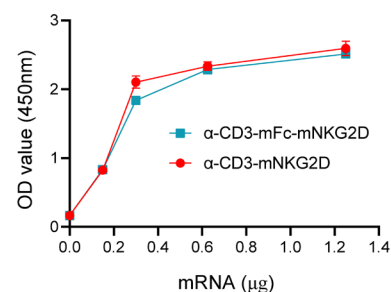
A



B



C

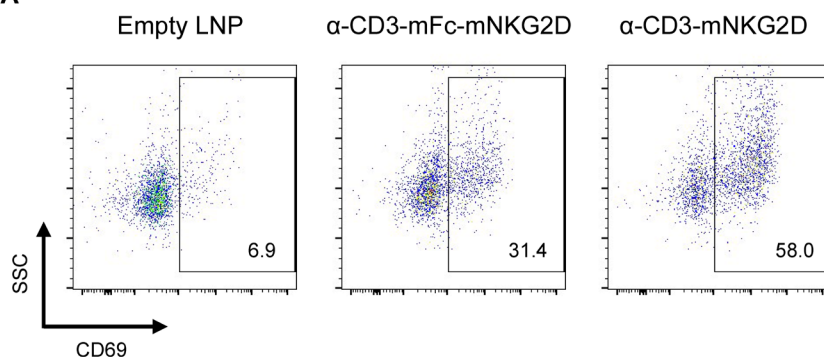


**Figure 1.** NKG2D BiTE mRNA expressed *in vitro*. (A) Schematic of the NKG2D BiTE mRNA constructs. (B) Jurkat cells were transfected with NKG2D BiTE mRNA or empty LNP and subjected to intracellular staining with anti-mouse NKG2D antibody, followed by flow cytometry analysis. (C) NKG2D BiTEs secreted from Jurkat cells transfected with indicated doses of mRNA were measured using an enzyme-linked immunosorbent assay.

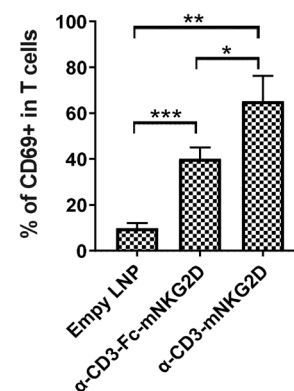
Notes: Data are presented as mean  $\pm$  standard deviation.  $n = 3$ . Data in (B) and (C) are representative of two independent experiments.

Abbreviations: BiTE: Bispecific T-cell engager; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D; OD: Optical density; SSC: Side scatter.

A



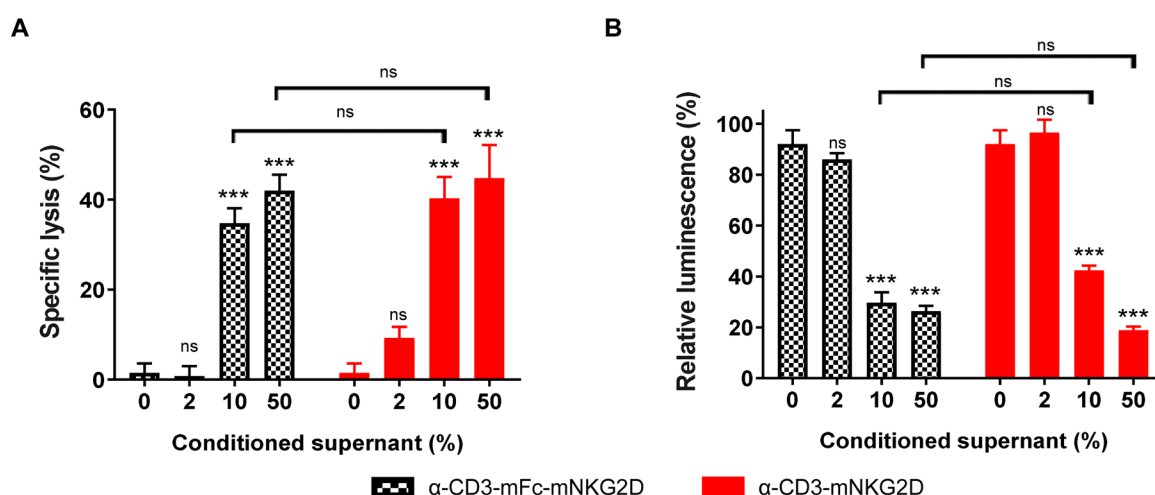
B



**Figure 2.** NKG2D BiTE mRNA induces T cell activation. Supernatant derived from α-CD3-mFc-mNKG2D-, α-CD3-mNKG2D mRNA-, or empty LNP-transfected Jurkat cells stimulates CD69 expression on splenocytic T cells in the presence of MC38 cells. (A) Representative flow cytometry plots. (B) Quantification of flow cytometry data.

Notes: Data are presented as mean  $\pm$  standard deviation.  $n = 3$ . Statistical analysis was conducted using an unpaired, two-tailed Student's *t*-test;  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ . The data are representative of two independent experiments.

Abbreviations: BiTE: Bispecific T-cell engager; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D; SSC: Side scatter.



**Figure 3.** NKG2D BiTE mRNA induces T cell-mediated cytotoxicity against tumor cells. The supernatant derived from α-CD3-mFc-mNKG2D-, α-CD3-mNKG2D mRNA-, or empty LNP-transfected cells was used to stimulate splenocytic T cells in the presence of MC38/Luc cells. The supernatant from co-cultured T cells and tumor cells was collected for (A) LDH analysis, and the co-cultured cells were subjected to (B) luciferase analysis to determine the cell cytotoxicity. A higher amount of LDH reflects higher cell cytotoxicity, while a reduced luciferase signal indicates reduced cell viability.

Notes: Data are presented as mean ± standard deviation.  $n = 4$ . Statistical analysis was conducted using an unpaired, two-tailed Student's  $t$ -test; \*\*\* $p \leq 0.001$ . The data are representative of two independent experiments.

Abbreviations: BiTE: Bispecific T-cell engager; LDH: Lactate dehydrogenase; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D.

Collectively, the mouse NKG2D BiTEs translated from the mRNA were biologically functional, induced T cell activation, and enhanced T cell-mediated cytotoxicity.

### 3.3. NKG2D bispecific T-cell engager mRNA treatment inhibits tumor growth in a syngeneic murine model

Building on the *in vitro* evidence of expression and potency, we examined the *in vivo* expression and therapeutic activity of the mRNA-encoded NKG2D BiTEs. *In vivo* pharmacokinetic analysis confirmed robust NKG2D BiTE expression in serum. The α-CD3-mFc-mNKG2D construct peaked at 6 h post-injection and remained stably detectable for at least 72 h (Figure 4A). By contrast, α-CD3-mNKG2D also peaked at 6 h but was cleared rapidly, becoming barely detectable beyond 48 h.

The *in vivo* anti-tumor efficacy of murine NKG2D BiTE mRNA was assessed in a subcutaneous, syngeneic A20 B-cell lymphoma model, in which tumors constitutively express NKG2D ligands.<sup>34</sup> Repeated intratumoral administration of α-CD3-mFc-mNKG2D or α-CD3-mNKG2D mRNA significantly inhibited tumor growth. α-CD3-mNKG2D mRNA exhibited higher efficacy, with a TGI index of 57.2% on day 17 after the first treatment, than α-CD3-mFc-mNKG2D mRNA (20.5% TGI on day 17; Figure 4B & C). Notably, NKG2D BiTE mRNA treatment was well tolerated, with no notable weight loss in the mice

(although weight gain tended to slow down) (Figure 4D) or obvious adverse reactions during the treatment period.

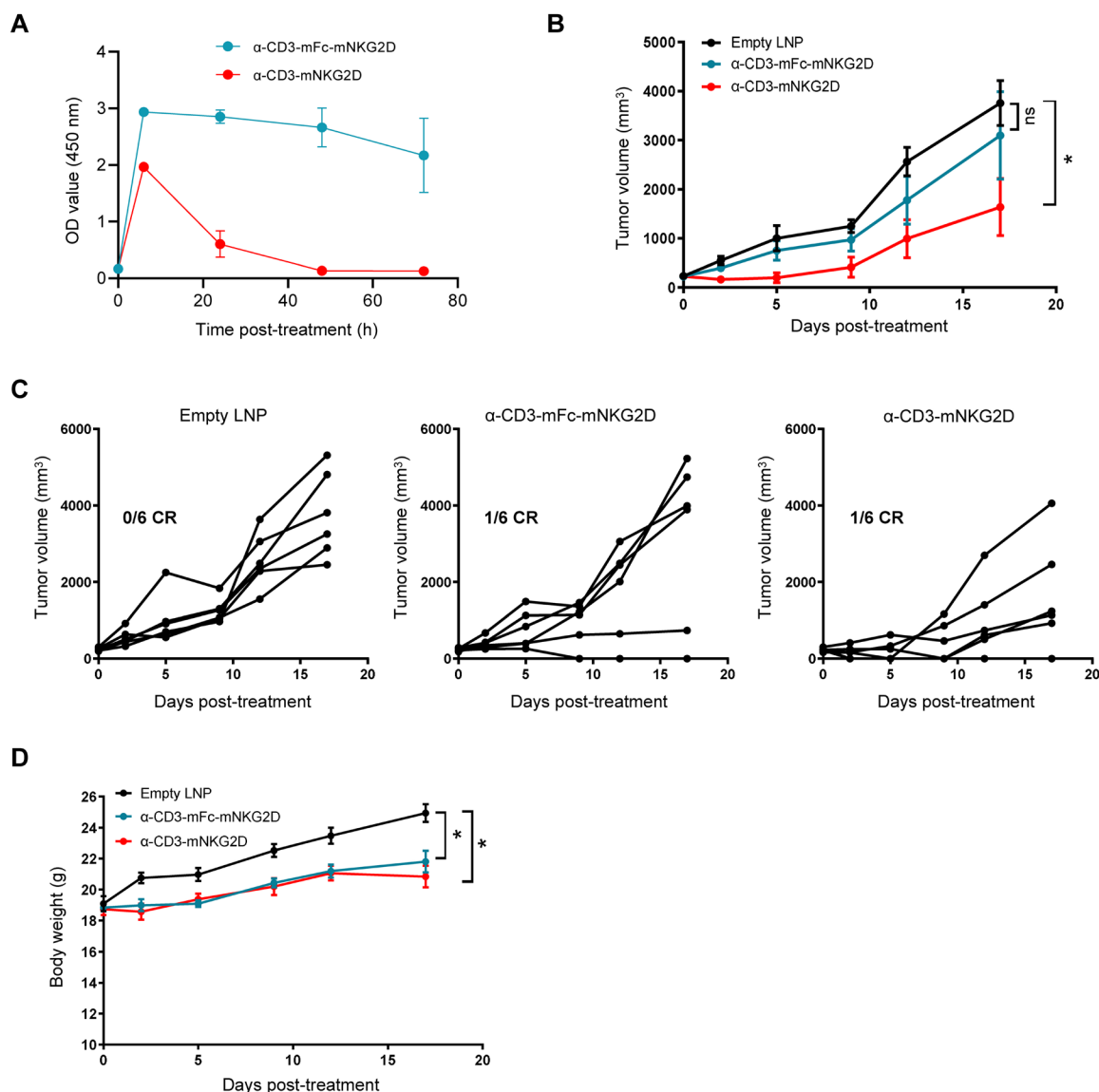
### 3.4. Human NKG2D bispecific T-cell engager mRNA induces T cell activation *in vitro* and inhibits tumor growth in a humanized mouse model

Based on the remarkable anti-tumor efficacy demonstrated by NKG2D BiTE mRNA, particularly α-CD3-mNKG2D mRNA, in syngeneic models, we developed a human NKG2D BiTE mRNA, designated α-CD3-hNKG2D. This mRNA consisted of the scFv of the anti-human CD3 antibody OKT3 fused directly to the extracellular domain of human NKG2D, without the antibody Fc domain (Figure 5A). Expression of α-CD3-hNKG2D was validated through intracellular NKG2D staining and flow cytometry of transfected cells (Figure 5B).

Tumor cells expressing NKG2D ligands, including human cervical cancer cell line Caski<sup>35</sup> and hepatocellular carcinoma cell line HepG2 cells,<sup>36</sup> were cultured in the supernatant of Jurkat cells transfected with α-CD3-hNKG2D mRNA, which significantly induced CD69 expression on the T cells (Figure 5C & D). Additionally, the supernatant markedly enhanced T cell-mediated cytotoxicity against HepG2/Luc cells (Figure 5E & F).

To investigate the *in vivo* anti-tumor activity of α-CD3-hNKG2D mRNA, we established a humanized immune system mouse model by grafting human PBMCs into highly



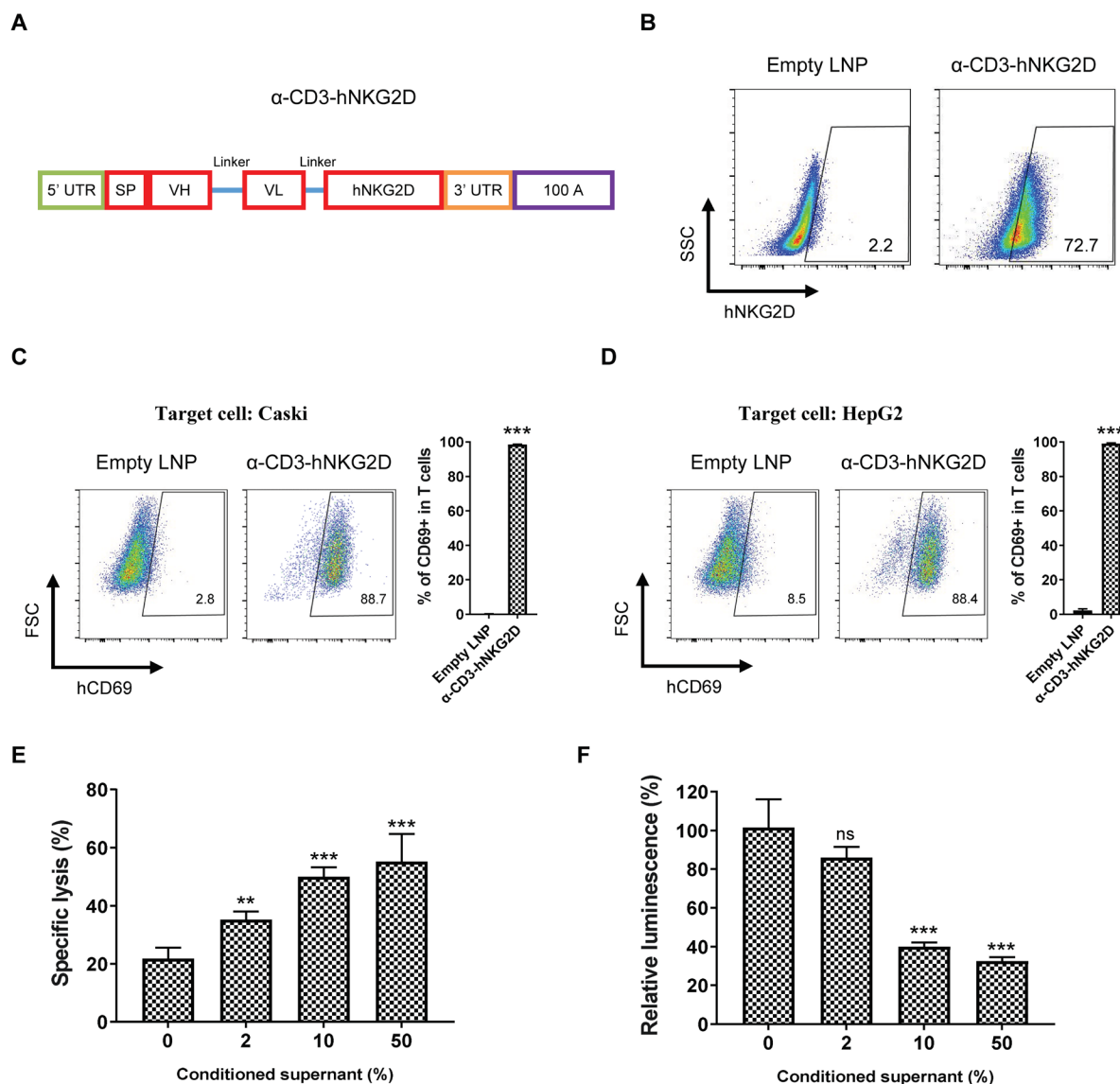


**Figure 4.** NKG2D BiTE mRNA treatment inhibits tumor growth in a syngeneic A20 tumor-bearing mouse model. (A) BALB/c mice received a single intravenous injection of 25  $\mu$ g LNP-encapsulated  $\alpha$ -CD3-mFc-mNKG2D or  $\alpha$ -CD3-mNKG2D mRNA, and their serum BiTE levels were quantified using enzyme-linked immunosorbent assay. (B–D) Mice bearing subcutaneous A20 tumors were treated with 25  $\mu$ g of either  $\alpha$ -CD3-mFc-mNKG2D,  $\alpha$ -CD3-mNKG2D mRNA, or empty LNP once a week for three consecutive weeks, and their (B, C) tumor volumes and (D) body weights were measured. Data in (A) are presented as mean  $\pm$  standard deviation ( $n = 5$ ). Data in (B and D) are presented as mean  $\pm$  standard error ( $n = 6$ ). Comparisons between BiTE-mRNA and empty-LNP groups (B, D) were performed using two-way ANOVA; ns: Not significant;  $*p \leq 0.05$ .

Abbreviations: BiTE: Bispecific T-cell engager; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D.

immunodeficient M-NSG mice, followed by subcutaneous implantation of human hepatocellular carcinoma cell line Huh7 cells. Administration of 20  $\mu$ g of  $\alpha$ -CD3-hNKG2D mRNA significantly inhibited tumor growth, with TGI index of 82.6%, on day 26 following the initial treatment (Figure 6A). This efficacy was corroborated by reductions

in both tumor size (Figure 6C) and tumor weight (Figure 6D). Notably,  $\alpha$ -CD3-hNKG2D mRNA treatment was well tolerated, with no notable weight loss in the mice (Figure 6B) or obvious adverse reactions during the treatment period. In summary, human NKG2D BiTE mRNA treatment produced anti-tumor activity both *in vitro* and *in vivo*.



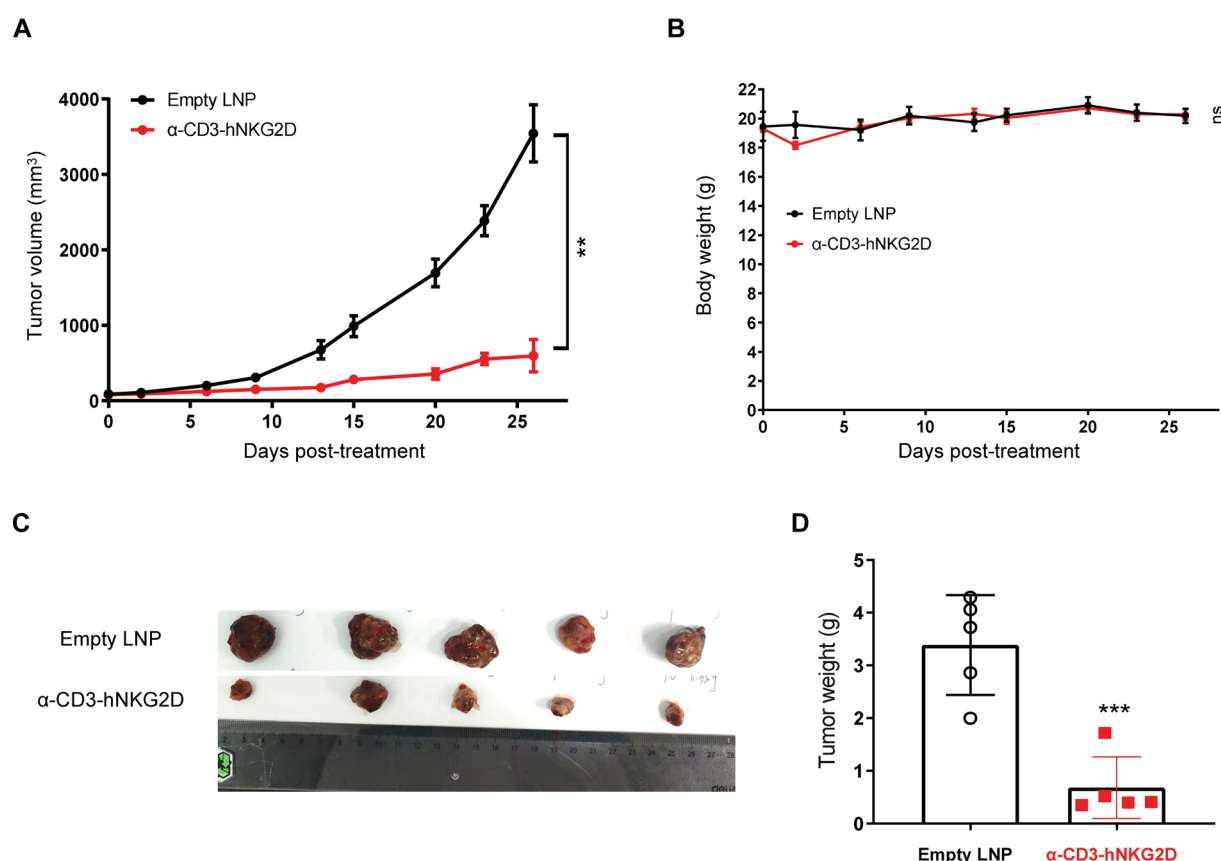
**Figure 5.** Human NKG2D BiTE mRNA treatment induces T cell activation *in vitro*. (A) Schematic diagram of α-CD3-hNKG2D mRNA construction. (B) Jurkat cells were transfected with α-CD3-hNKG2D BiTE mRNA or empty LNP and subjected to intracellular staining with an anti-human NKG2D antibody, followed by flow cytometry analysis. (C & D) The supernatant derived from α-CD3-hNKG2D mRNA- or empty LNP-transfected cells stimulated CD69 expression on human PBMCs in the presence of (C) Caski and (D) HepG2 cells. Left, representative flow cytometry plots; right, quantification of flow cytometry data. Data are presented as mean ± standard deviation ( $n = 3$ ). (E & F) The supernatant derived from α-CD3-hNKG2D mRNA or empty LNP-transfected cells was used to stimulate human PBMCs in the presence of HepG2/Luc cells. The supernatant from co-cultured PBMCs and tumor cells was collected for (E) LDH analysis, and the co-cultured cells underwent (F) luciferase analysis to determine cell cytotoxicity. A higher amount of LDH reflects higher cell cytotoxicity, while a reduced luciferase signal indicates reduced cell viability. Data are presented as mean ± standard deviation ( $n = 4$ ). Comparisons between BiTE-mRNA and empty-LNP groups (C & D) or between supernatant-treated and untreated conditions (E & F) were performed using an unpaired, two-tailed Student's *t*-test. ns: Not significant; \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . The data are representative of two independent experiments. Abbreviations: BiTE: Bispecific T-cell engager; LDH: Lactate dehydrogenase; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D; PBMC: Peripheral blood mononuclear cell.

## 4. Discussion

In this study, we generated different NKG2D BiTE mRNA constructs and demonstrated their therapeutic potential *in vitro* and *in vivo*. NKG2D BiTE mRNA treatment

induced T cell activation and promoted T cell-mediated cytotoxicity against tumor cells expressing NKG2D ligands. Intratumoral administration of NKG2D BiTE mRNA significantly inhibited tumor growth in murine tumor





**Figure 6.** Human NKG2D BiTE mRNA treatment inhibits tumor growth in a humanized mouse model. M-NSG mice reconstituted with human PBMCs were subcutaneously implanted with Huh7 cells. Mice with established tumors were intratumorally injected with 20  $\mu$ g of  $\alpha$ -CD3-hNKG2D mRNA or an equal volume of empty LNP once a week for three consecutive weeks. Their (A) tumor volumes and (B) body weights were measured. Mice were euthanized on day 26, and the tumors were (C) excised and (D) weighed. Data are presented as mean  $\pm$  standard error of the mean ( $n = 5$ ). Statistical analysis was conducted using two-way ANOVA; ns: Not significant;  $**p \leq 0.01$ ,  $***p \leq 0.001$ .

Abbreviations: BiTE: Bispecific T-cell engager; LNP: Lipid nanoparticle; mRNA: Messenger RNA; NKG2D: Natural killer group 2, member D; PBMC: Peripheral blood mononuclear cell.

models. Notably, eliminating the antibody Fc domain produced a BiTE with markedly higher anti-tumor efficacy than its counterpart containing an Fc fragment.

Through flow cytometry analysis, we observed slightly lower expression levels of  $\alpha$ -CD3-mNKG2D mRNA compared to  $\alpha$ -CD3-mFc-mNKG2D, despite the molar concentration of  $\alpha$ -CD3-mNKG2D being higher under the same conditions. *In vitro*, ELISA revealed comparable expression levels of  $\alpha$ -CD3-mNKG2D and  $\alpha$ -CD3-mFc-mNKG2D in the culture medium of transfected cells. *In vivo*, however, pharmacokinetic analysis showed a markedly longer serum half-life for  $\alpha$ -CD3-mFc-mNKG2D, most likely attributable to Fc-mediated stabilization. Paradoxically, functional analysis revealed that  $\alpha$ -CD3-mNKG2D induced significantly higher T cell activity and greater anti-tumor efficacy than  $\alpha$ -CD3-mFc-mNKG2D. This discrepancy can be rationalized by

structural considerations. The Fc region in  $\alpha$ -CD3-mFc-mNKG2D elongates the molecule, increasing the spatial distance between T cells and tumor cells and thereby diminishing synaptic efficiency. Moreover, Fc-mediated antibody effector functions—such as antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity—can produce nonspecific cell cytotoxicity and unintended *in vivo* effects.<sup>1</sup> Finally, the smaller size of the Fc-deleted BiTE may facilitate better tumor penetration. Collectively, these factors may contribute to the reduced functional activity of the Fc-containing NKG2D BiTE construct. Notably, in antibody drug development, the Fc fragment is often mutated or removed entirely to eliminate these effector functions, thereby improving specificity and reducing off-target effects.<sup>10,37,38</sup>

The function of the mouse NKG2D BiTE protein was

previously investigated.<sup>39</sup> In that study, MC38 tumor cells, genetically modified to exogenously express  $\alpha$ -CD3-NKG2D, exhibited reduced tumor growth *in vivo*. We confirmed this result using LNP-mRNA, which has greater clinical applicability than genetically modified MC38 tumor cells. Additionally, making genetic modifications to tumor cells may lead to off-target effects in genes associated with cell proliferation, rendering the results of some studies unpredictable and difficult to interpret. The *in vitro* function of the human NKG2D BiTE protein was previously explored.<sup>22,23</sup> Our results both confirmed NKG2D BiTE protein *in vitro* activity and extended these previous investigations to include a humanized immune system mouse model.

Although NKG2D ligands are compelling targets for cancer immunotherapy, they are not restricted to malignant cells.<sup>18</sup> Low-level expression has been documented in some human fetal tissues and in stressed normal cells, raising legitimate on-target/off-tumor safety concerns. Moreover, proteolytic shedding of these ligands can attenuate therapeutic efficacy.<sup>40,41</sup> Achieving an optimal balance between potency and safety will therefore be essential for clinical translation. Notably, early-phase trials have reported a manageable safety profile and encouraging anti-tumor activity with NKG2D ligand-targeted therapies.<sup>20</sup> Nonetheless, rigorous safety studies—including evaluation in non-human primate models—must be completed before advancing mRNA-encoded NKG2D BiTEs into the clinic.

Drug delivery methods using gene-encoding adenoviruses, oncolytic viruses, or plasmid DNA have been explored extensively.<sup>42</sup> However, these approaches may produce deleterious genomic rearrangements and elicit anti-viral vector immunity. In contrast, LNP-mRNA therapy ensures transient gene expression without compromising the integrity of the host's genomic DNA.<sup>28</sup> Furthermore, compared with antibody proteins, LNP-mRNA technology eliminates the complicated and costly processes of protein engineering and purification.<sup>28</sup> *In vivo* mRNA translation has the potential to prolong the half-life of a drug and maintain its concentration within a therapeutic window.

## 5. Conclusion

In summary, we explored the immunotherapy potential of LNP-encapsulated mRNA encoding BiTEs targeting NKG2D ligands. Our results highlight the substantial anti-tumor activity of NKG2D BiTE mRNA treatment, both *in vitro* and *in vivo*. Therefore, NKG2D BiTE mRNA is a promising anti-tumor therapeutic meriting further clinical development.

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## Conflict of interest

All authors are employed by the company Liverna Therapeutics Inc. The authors declare no conflict of interest.

## Author contributions

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## Ethics approval and consent to participate

The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The mouse studies were approved by the Research Ethics Committee of Zhuhai BesTest BioTech Co., Ltd. (IAC202201003).

## Consent for publication

Not applicable.

## Availability of data

Data are included in the article. Further inquiries can be directed to the corresponding author.

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