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A ROR1 targeted bispecific T cell engager shows high potency in the pre-clinical model of triple negative breast cancer

Fan Wang[1†], Weina Li[2†], Guohui Han[1], Jun Xie[3] and Xiangdong Bai[1*]

Abstract

Background Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype characterized with poor prognosis and high metastatic potential. Although traditional chemotherapy, radiation, and surgical resection remain the standard treatment options for TNBC, bispecific antibody-based immunotherapy is emerging as new strategy in TNBC treatment. Here, we found that the receptor tyrosine kinase-like Orphan Receptor 1 (ROR1) was highly expressed in TNBC but minimally expressed in normal tissue. A bispecific ROR1-targeted CD3 T cell engager (TCE) was designed in IgG-based format with extended half-life.

Method The expression of ROR1 in TNBC was detected by RT-qPCR and immunohistology analysis. The killing of ROR1/CD3 antibody on TNBC cells was determined by the in vitro cytotoxicity assay and in vivo PBMC reconstituted mouse model. The activation of ROR1/CD3 on T cells was analyzed by the flow cytometry and ELISA assay. Pharmacokinetics study of ROR1/CD3 was performed in mouse.

Results The ROR1/CD3 TCE triggered T cell activation and proliferation, which showed potent and specific killing to TNBC cells in ROR1-depedent manner. In vivo mouse model indicated that ROR1/CD3 TCE redirected the cytotoxic activity of T cells to lyse TNBC cells and induced significant tumor regression. Additionally, the ROR1/CD3 bispecific antibody exhibited an extended half-life in mouse, which may enable intermittent administration in clinic.

Conclusions Collectively, these results demonstrated that ROR1/CD3 TCE has a promising efficacy profile in preclinical studies, which suggested it as a possible option for the treatment of ROR1-expressing TNBC.

Keywords TNBC, ROR1, T cell engager, Immunotherapy

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Background

Triple negative breast cancer (TNBC) is one of the mostly frequent malignancies and the leading cause of cancer-related death among women worldwide [1, 2]. High invasiveness, proneness to relapse, and poor prognosis have been the typical hallmarkers of TNBC [3–5]. Due to lacking of human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR), TNBC patients are not sensitive to endocrine therapy or therapies targeting HER2. Chemotherapy and radiotherapy are generally employed for the treatment of TNBC, however, the 5-year survival rate, especially for those diagnosed in advanced stage, is still poor [6, 7]. Because the stage of cancer determines the treatment strategy and outcome, biomarkers that specifically or aberrantly expressed in TNBC that can be targeted for drug design need to be investigated.

Over the past decade, immunotherapies are emerging outstanding clinical benefits beyond the traditional treatments in TNBC [8–12]. In particular, anti-programmed death-ligand 1 (PD-L1) monoclonal antibody atezolizumab and Sacituzumab Govitecan targeting Trop 2 show impressive response and are changing the paradigm of TNBC treatment, even for patients in advanced stage [13]. Nevertheless, the dark side is that only very limited patients can benefit from the current immunotherapy. Bispecific antibody that recognizes two targets or one target but different epitopes has drawn wide attention and shown exciting perspective in clinic [14, 15]. The T cell-redirecting bispecific antibody specifically engages CD3 on T cells and antigens on tumor cells, leading to the activation of T cells and killing of cancer cells. These T cell engagers are considered more potent because T cells can be redirected to the tumor microenvironment regardless of the antigen specific T cell receptors [16]. Blinatumomab, a TCE targeting CD19 and CD3, has been approved by the U.S. Food and Drug Administration for the treatment of hematological malignancies [17]. However, the application of TCEs in solid tumors calls for more attention and are still in clinical development to overcome the "on-target off-tumor" toxicity, which is caused by the low expression of antigens in normal tissues [18, 19]. To avoid the insufficient tumor selectivity, antigens that have high tumor specificity are required for the safety and efficacy of TCE in clinical trials.

ROR1 is a transmembrane glycoprotein member of the Receptor Tyrosine Kinase (RTK) superfamily, which consists of extracellular region, transmembrane domain and intercellular parts [20]. Besides the physiological roles of ROR1 in embryonic development, aberrant expression of ROR1 has been found from hematological malignancies to solid tumors [21]. Overexpressed ROR1 promoted cancer cell proliferation, migration and drug resistance [22, 23]. Interestingly, ROR1 was highly expressed in breast cancer and associated with the advanced aggressive phenotypes, including TNBC [24]. Currently, ROR1 has emerged as a potential target for the development of anticancer drugs [23, 25–27]. So far, monoclonal antibodies, chimeric antigen receptor T cells (CAR-T cells), antibody-drug conjugates, and T cell engagers by targeting ROR1 have been investigated in preclinical studies in multiple settings [23, 25–27]. ROR1 targeted antibody-drug conjugates induced the growth inhibition of mantle cell lymphoma and diffuse large B-cell lymphoma [28]. Recent study reported that a ROR1/CD3 bispecific antibody in scFv-Fc format induced T-cell derived cytokine release, and recruited the infiltration of $CD4^+$ and $CD8^+$ T cells in the tumor tissues of NSCLC [29]. The anti-cancer efficacy of the ROR1/CD3 biAb was also validated in the xenograft mouse models. These evidences suggested that it would be necessary to expand the role of ROR1 in TNBC by determining the function and exploring the possibility to target ROR1 for TCE as a strategy in TNBC treatment.

In this study, we generated a ROR1/CD3 T cell engager in an IgG-based format containing two chains that were covalently linked via disulfide bonds to the Fc hinge region. The biophysical properties of ROR1/CD3 antibody and killing potency of TNBC cells were investigated by in vitro assays and in vivo xenograft mouse models. Additionally, the pharmacokinetics study of ROR1/CD3 was also performed in mouse. These results suggested the potential application of ROR1/CD3 antibody for the treatment of ROR1-positive TNBC.

Methods

Tissues and cell culture

Paired TNBC tissues and adjacent non-cancer tissues ($n = 50$) were obtained from TNBC patients that were collected at the Shanxi Province Cancer Hospital. TNBC cell line (MDA-MB-231, BT-549, HCC-1937, HS-578T) and normal MCF10A cells were provided by the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 (cat#12633020, Gibco) or Leibovitz's L-15 medium (cat#11415064, Gibco) with 10% fetal bovine serum (FBS, cat#A5669801, Gibco). Cells were maintained at a 37 °C incubator with 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were purchased from the AllCells (Shanghai, China). T cells were cultured in the CelThera™ GMP T Cell Expansion Medium (GMP-CM3101, Acro Biosystems, Beijing, China).

ROR1/CD3 bispecific antibody engineering

The sequence of the ROR1 and CD3 binders for ROR1/CD3 TCE molecule were obtained using the hybridoma platform after several rounds of primary screening based on binding and specificity. ROR1/CD3 bispecific antibody was generated using the Knob-in-hole strategy to

make sure heavy chain heterodimerization. Briefly, the TCE molecule consists of three chains: anti-ROR1 antibody heavy chain (chain 1), anti-ROR1 antibody light chain (chain 2) and an anti-CD3 antibody ScFv-Fc fused chain (Chain 3). In chain 3, the ScFv fragment was linked to the Fc region of the lgG1 backbone via a 15 amino-acid long (glycine4serine)3 linker. The Fc domain of the antibody was engineered by introducing P329G/L234A/L235A mutations to abolish the Fc function. To get heterodimerized bispecific antibody, chain 1 was designed as the "Knob" arm and chain 3 was designed as the "hole" arm. The DNA sequences of these three chains were constructed into the pcDNA3.1 expressing vector, respectively. CHO cells were transfected with these three protein-encoding vectors with the molar ratio of 1:1:1. Cell culture supernatants were collected and filtered through a 0.22 um filter. The solution was purified through a protein A column. To remove the contamination of hole-hole dimer, the solution was further purified by Mono S cation exchange column (GE healthcare) and the final products were verified by non-reducing SDS-PAGE (Supplementary Fig. 1A).

Cell-based binding assay

TNBC cells or human T cells isolated from PBMC using the EasySep™ Human T Cell Enrichment Kit (STEMCELL) were incubated with serially diluted ROR1/CD3 antibody for 30 min at 4 °C. Cells were washed three times with PBS and stained with a secondary PE-conjugated anti-hIgG. After washing, cells were prepared and the median fluorescence intensity (MFI) was determined by the flow cytometry analysis.

RNA extraction and qPCR analysis

Total RNA was extracted from TNBC tissues and adjacent non-cancer tissues using the Trizol reagent (DP424, TIANGEN, Beijing, China). RNA was quantified with the NanoDrop 2000 (Thermo Fisher Scientific, Shanghai, China) and then 0.5 ug RNA was taken for reverse transcription with the SuperScript™ IV First-stand Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's guide. The quantitative real-time PCR analysis was performed using the SYBR Green qPCR Master Mix (11201ES08, Yeasen, Shanghai, China) with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Expression of GAPDH was also detected to normalize the level of ROR1. The thermo cycles was set as follows: 95°C for 1 min and 42 cycles at 95°C for 15s, 58°C for 25s and 72°C for 25s. Primers used in this study included ROR1: forward, 5'-GTTTCCCAGAGCTGAATGGA-3' and reverse, 5'- GGATGTCACACAGATCAGACTT-3'. The fold change of ROR1 expression was calculated according to the formula of $2^{-\Delta\Delta CT}$.

ROR1 knockout cell generation

ROR1 knockout MDA-MB-231 cells were obtained via the CRISPR/Cas9 technology as previously described [30]. Briefly, the one-plasmid knockout system was established with the Lenti-Cas9-gRNA-GFP plasmid backbone ("All- in- one vector", Addgene, 124770). Guide RNAs (gRNAs) targeting ROR1 were designed using the Synthego (https://www.synthego.com/products/bioinformatics/crispr-design-tool) (gRNA_1: forward oligo: 5'-CACCGTCATTGGTTCATCGAGGGTC-3', reverse oligo: 5'-AAACGACCCTCGATGAACCAATGAC-3'; gRNA_2: forward oligo: 5'- CACCGGTTATTCATTGGTTCATCGA-3', reverse oligo: 5'- AAACTCGATGAACCAATGAATAACC-3';) and cloned into the All-in-one vector by restriction digestion and ligation. The ligation products were transformed into competent *E.coli* cells. After cultured overnight at 37 °C, three clones per gRNA were picked, and cultured overnight in 2 ml LB medium containing 100 ug/ml ampicillin. The plasmid vector was purified using the TIANprep Mini Plasmid Kit (DP104, Beijing, China) and the plasmid was verified by sequencing using the U6 primer. To generate the lentivirus vector, the CRISPR plasmid DNA was transfected into the HEK293T cells by calcium phosphate transfection involving two viral packaging plasmids (PsPAX2.0 and VSVG). ROR1 knockout cell was established by transducing the lentivirus carrying the cloned all-in-one vector into the MDA-MB-231 cells in 6-well plate with the help of 10 ug/ml polybrene (40804ES76, Yeasen, Shanghai, China). The transduction efficiency was monitored under the fluorescence microscope. Cells were harvested and diluted into single clone in the 96-well plate and continue expanding in 24-well plate until enough cells were obtained for validation. Genomic DNA was prepared from control and candidate ROR1 knockout cells, followed by confirmation via western blot (Supplementry Fig. 1B).

Western blot

TNBC cells were lysed with NP-40 buffer containing protease inhibitor (P1005, Beyotime, Shanghai, China) and the protein concentration was determined via the BCA assay (Beyotime, Shanghai, China). 20 ug of total protein was loaded and separated by 15% SDS-PAGE followed by transferring onto the NC membrane (cat#1620112, Bio-Rad). After blocking with 5% of non-fat milk, the membrane was incubated with anti-ROR1 antibody (PA5-50830, 1:1000, ThermoFisher Scientific.) overnight at 4 °C. Membrane was washed three times with PBST (PBS plus 0.1% Tween 20) and then incubated with the secondary goat anti-rabbit IgG H&L (HRP) antibody (ab6721, 1:3000, abcam, Shanghai, China) for 50 min at room temperature (RT). The blot signaling was visualized

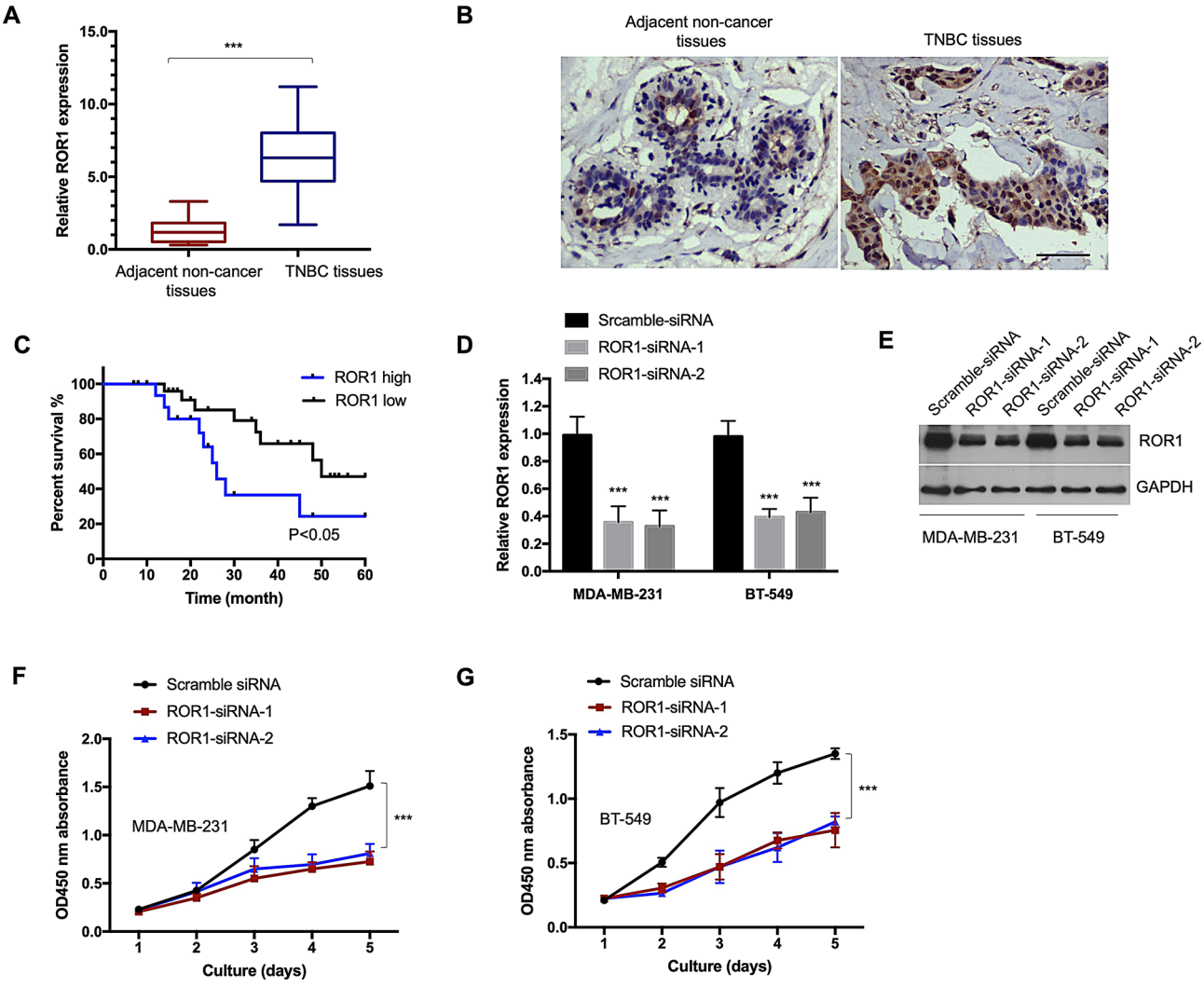


Fig. 1 ROR1 was highly enriched in TNBC and essential for TNBC cell growth. (**A**) ROR1 expression in TNBC and adjacent normal tissues was detected by RT-qPCR, which showed the significantly increased expression of ROR1. The PCR reaction was performed in duplicates. Data analysis was done by student's *t* tset. (**B**) Representative IHC staining of ROR1 in TNBC and adjacent normal tissues. Scale bar, 100 μm. (**C**) High levels of ROR1 were correlated with the shorter 5-year survival rate of TNBC patients. Patients were classified as ROR1-high ($n=29$) or -low ($n=21$) according to the median expression value of ROR1 in these 50 TNBC tissues. Log rank test was performed for data analysis. (**D**, **E**) TNBC cells were transfected with siRNA-control or siRNA-ROR1, and the knockdown efficacy was confirmed by RT-qPCR after transfection for 48 h. The experiment was repeated three times and PCR reaction was performed in triplicates. One-way ANOVA test was performed for data analysis (**D**) and western blot analysis (**E**). (**F**, **G**) CCK-8 assay showed the significantly reduced TNBC cell proliferation upon ROR1 down-regulation. The experiment was performed with three technological replicates and validated by other two biological replicates. Two-way ANOVA was performed for data analysis

with the SuperSignal West Pico Chemiluminescent Substrate Kit (cat#34579, Pierce).

Immunohischemistry (IHC) staining of ROR1 in tumor tissues

The paraffin-embedded tissue sections were deparaffinized in xylene 1, ll and Ill for 5 min, respectively. The samples were then rehydrated gradually in serial dilutions of ethanol (100%, 95%, 85%, 70%) once for 5 min followed by washing with ddH_2O. Antigen retrieval was done by incubating the samples at 92 °C for 5 min in 10 mM citrate buffer (pH = 6.0). To reduce the background noise, the endogenous peroxidase activity was quenched by treating the samples with 3% H_2O_2 solution for 10 min at RT. After washing with PBS for three times, the tissue sections were pre-blocked with goat serum for 15 min at RT and then incubated with the rabbit anti-ROR1 antibody (PA5-50830, 1: 50 dilution) or normal rabbit lgG (negative control) at 4 °C overnight. After washing, tissue sections were stained with horseradish peroxidase-labeled anti-rabbit IgG (H&L) (1:50 dilution, ZSGB, Beijing) at RT for 30 min. The reaction was visualized using the DAB^+ substrate-chromogen solution (K3467, Agilent Technologies, Inc., Santa Clara, CA, USA) for

5 min at RT. Finally, the counterstaining of the section was performed with hematoxylin. The IHC staining of ROR1 was independently evaluated by two experienced pathologies.

In vitro functional assays

T cells were specifically enriched from PBMC donor using the human T-cell enrichment kit (cat#19051, STEMCELL Technologies). For the cytotoxicity assay, PBMCs and TNBC cells were incubated at the ratio of 10:1 with increasing gradient concentration of bispecific antibody. After cultured at 37 °C for 24 h, the lactate dehydrogenase content released into the supernatants by the dead cells was determined using the CytoTox96 cytotoxicity assay kit (cat#G1780, Promega). For T cell activation, PBMC and tumor cells were co-cultured with the antibody for 24 h. Cells were centrifugated and stained with these following antibodies: anti-hCD3 (300434, 1:100), anti-hCD4 (300519; 1:100), anti-hCD8 (300924, 1:100), anti-hCD25 (356140, 1:100), anti-hCD69 (310906, 1:100) for 30 min and then detected by the FACSVerse. For the detection of T cell proliferation, $CD4^+$ or $CD8^+$ T cells were isolated from PBMC and cultured with TNBC cells in the presence of ROR1/CD3 antibody for 96 h. After centrifugation, cells were incubated with FITC-labeled anti-hCD4 (1:100), or phycoerythrin-labeled anti-hCD8 (1:100) antibodies for 30 min at 4 °C. Cells were washed twice with PBS and detected by the flow cytometer.

In vivo PBMC reconstituted mouse model

Female NOG mice aged 5–7 weeks were provided by the Vital River Laboratories (Shanghai, China). After acclimatized for 7 days at the SPF-level laboratory animal facility, TNBC cells were subcutaneously implanted into the flank of mice. For the MDA-MB-231 xenograft, 15 days later after cell implanating ($3*10^6$ cells/mouse), mice were injected intravenously with 0.5 million PBMCs. On day 20, when tumor volume reached around 200 mm^3, mice were grouped randomly on the basis of tumor volume and body weight ($n=8$ for each group). For the HCC1937 xenograft, $3*10^6$ cells were implanated on day 0 and PBMC were injected on day 2. Mice were randomly divivded when the tumor volume reached around 200 mm^3 ($n=8$ for each group). In both model, ROR1/CD3 bispecific antibody (1 mg/kg) or vehicle was intraperitoneally administrated once a week. The tumor size and mouse body weight were measured twice a week. The tumor volume was calculated with the formula: $V = 1/2*ab^2$, "a" is the long diameter and "b" is the short diameter of the tumor. The experiment was approved by the Ethics Committee of Shanxi Province Cancer Hospital.

Tumor infiltrating lymphocytes (Tils) analysis

In addition to the tumor growth assay, MDA-MB-231 xenograft was also prepared and the tumors were isolated after 7 days of the first dose of ROR1/CD3 antibody. The Tils ($n=5$ for each group) were isolated using the Ficoll-Paque density gradient centrifugation as previously described [31]. Flow cytometry was analyzed using the anti-human CD45 (1: 100, 304014), anti-human CD4 (1:100; 300519), anti-human CD8 (1:100, 300924), and anti-human granzyme B (1:100, 372222) that were purchased from BioLegend Inc (San Diego, California, USA).

Cell proliferation analysis

The proliferation of TNBC cells transfected with siRNA-control or siRNA-ROR1 was detected by the Cell-counting kit-8 assay (CCK-8, Dojindo, Japan). Cells were seeded into the 96-well plate with the density of 2,000 cells in each well. At the interval of 24 h, 10 ul of CCK-8 reagent was added into the medium and cultured in the CO_2 incubator for 3 h. The absorbance values at 450 nm were detected using the plate reader.

Cytokine release measurement

Human PBMCs and TNBC cells were co-cultured at the ratio of 10:1 in the 96-well plate. Different concentrations of ROR1/CD3 antibody were added and incubated for 24 h. The medium was collected and the levels of cytokines in the supernatants were determined using the ELISA kit (RD) (cat# D2050, D4050, D6050, D1000B, DTA00D, DIF50C) according to the guidelines. The assay was performed with three replicates.

Pharmacokinetics (PK) of ROR1/CD3 TCE in mouse

10 mpk ROR1/CD3 antibody was administrated intravenously into the wild-type BALBc mouse (7–8 weeks, female). Bleeding was performed at the time points of 5 min, 0.5-, 2-, 6-, 24-, 48-, 168-, 336- and 504 h post dosing. Three mice was used for each time point. Total serum was obtained by centrifugation at 3,000 rpm for 15 min and diluated according to the experience of preliminary experiment. The concentrations of ROR1/CD3 was determined via ELISA by pre-coating with ROR1 (RP-88153, ThermoFisher Scientific) or CD3 antigen (CDE-H8224, ARCO). In briefly, ROR1 protein was coated and the TCE was detected via the binding of a recombinant His-CD3 protein and then detected with the anti-his-HRP. Additionally, another ELISA method was established by coating CD3 antigen, and then detected with anti-human lgG Fc conjugated with HRP. The concentration obtained from these two methods was compared and validated.

Statistical analysis

Data was presented as means ± standard deviation and statistical analysis was performed using the SPSS

software (SPSS Inc., Chicago, Illinois, USA). Student's *t* test or one-way ANOVA was employed for the comparison between two or more groups. $P < 0.05$ was statistically significant.

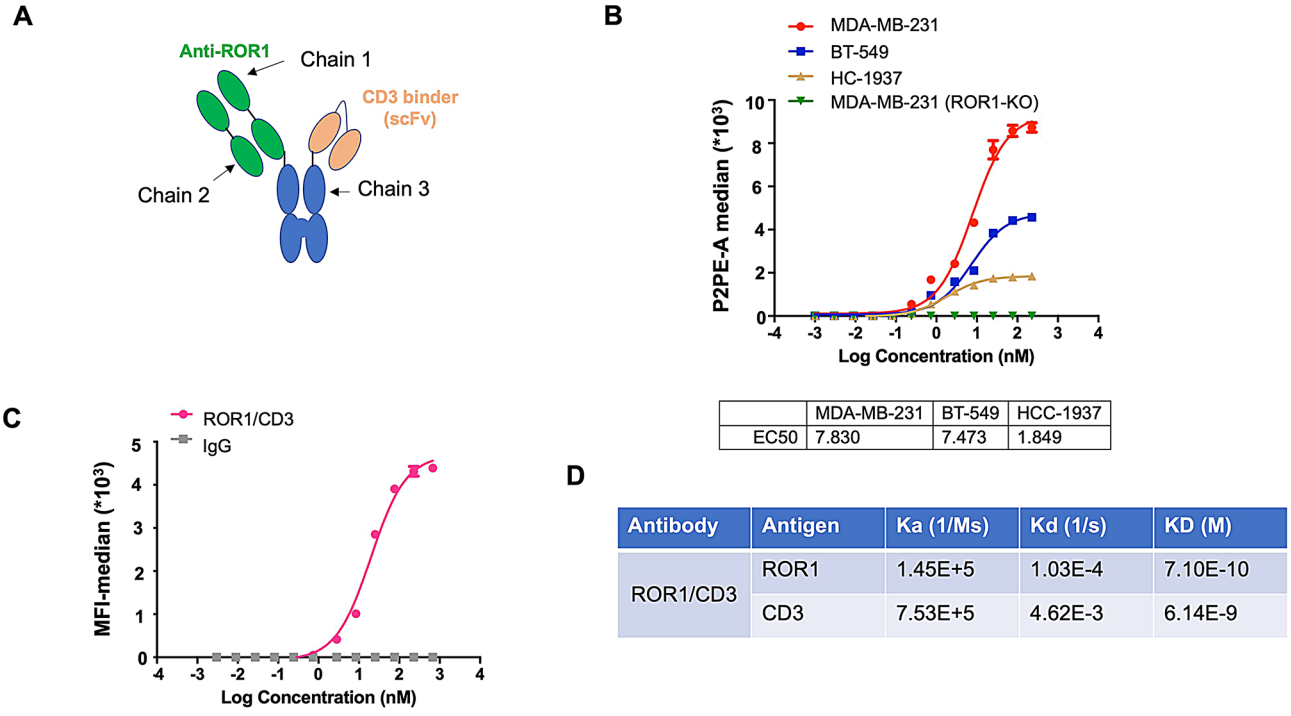
Results

ROR1 was overexpressed in TNBC and correlated with the worse prognosis of TNBC patients

To determine whether ROR1 was a drugable target for TNBC, ROR1 expression in TNBC and normal breast tissues was detected by RT-qPCR. The data showed that ROR1 was highly expressed in TNBC but lowly in normal samples (Fig. 1A). Consistently, the immunohistochemical staining showed the increased expression of ROR1 in TNBC tissues (Fig. 1B). Additionally, the correlation of ROR1 abundance with the 5-year overall survival of TNBC patients was also analyzed by log-rank test. Patients were classified as ROR1-high ($n = 29$) or -low ($n = 21$) according to the median expression value of ROR1 in these 50 TNBC tissues. As shown in Fig. 1C, higher level of ROR1 was significantly correlated with the shorter survival time of TNBC patients. To test whether ROR1 was essential for TNBC cell growth, MDA-MB-231 and BT-549 cells were transfected with siRNA-ROR1. The down-regulation of ROR1 was validated by RT-qPCR and western blot, respectively (Fig. 1D and E). Depletion of ROR1 significantly inhibited TNBC cell proliferation (Fig. 1F and G). Collectively, these results suggested that highly expressed ROR1 might be involved in TNBC progression and a possible drug target for TNBC.

ROR1/CD3 TCE showed high binding affinity to ROR1 and CD3

To evaluate whether the differential expression pattern of ROR1 in tumors and normal breast tissues could translate to a potential therapeutic window, ROR1 targeted TCE antibody was generated as the proof of concept. The antibody frame was designed as asymmetrical IgG-like format, which was composed of an anti-ROR1 fab domain and anti-CD3 single-chain variable fragment (ScFv) fused to the Fc domain to extend the half-life (Fig. 2A). The Fc constant region is IgG1 subtype but the Fc function was silenced by introducing PGLALA mutation. The binding affinity of ROR1/CD3 TCE with cell surface expressed ROR1 and CD3 was determined by flow cytometry analysis. To assess this, ROR1-high



	MDA-MB-231	BT-549	HCC-1937
EC50	7.830	7.473	1.849

D

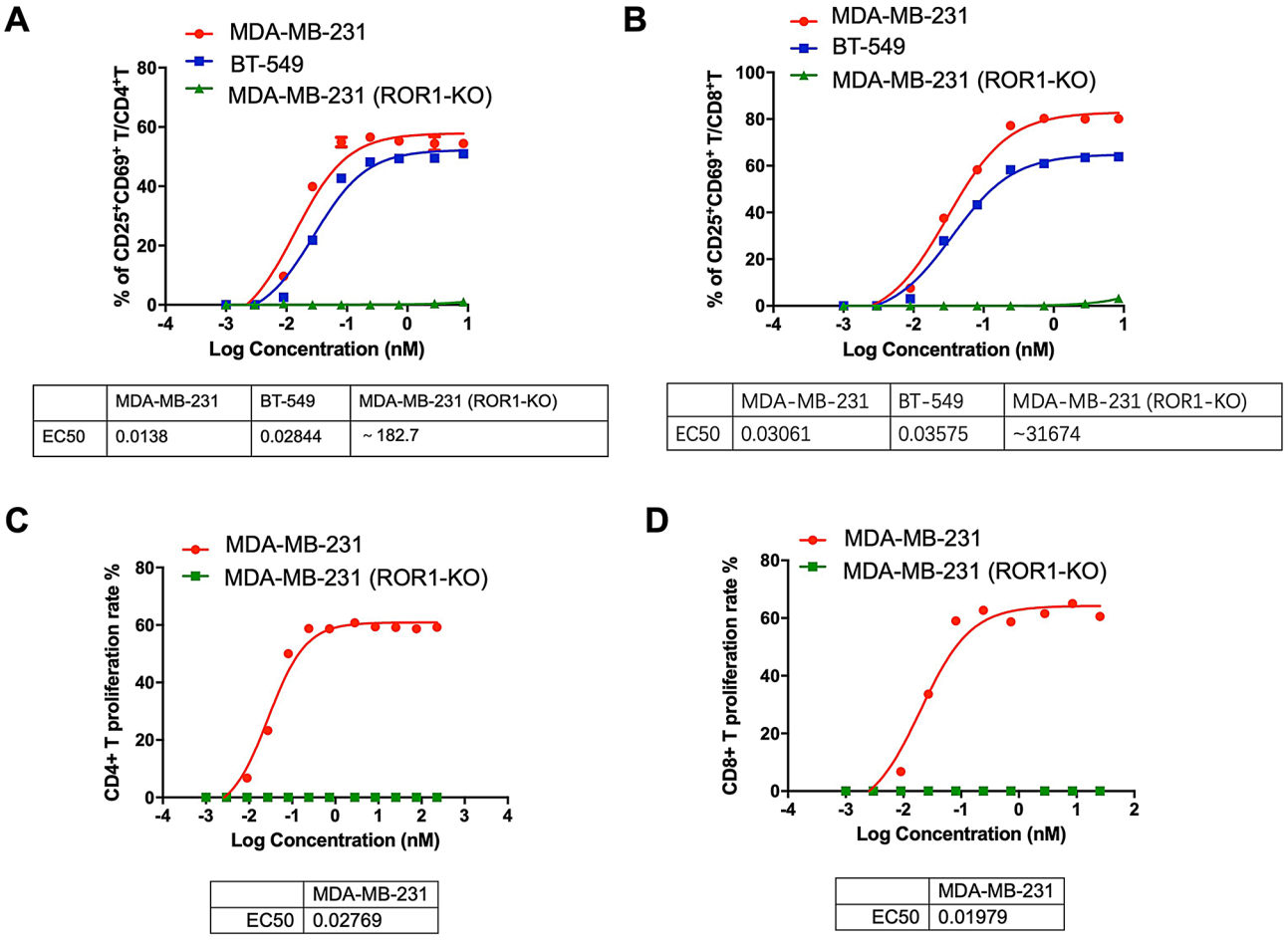
Antibody	Antigen	Ka (1/Ms)	Kd (1/s)	KD (M)
ROR1/CD3	ROR1	1.45E+5	1.03E-4	7.10E-10
	CD3	7.53E+5	4.62E-3	6.14E-9

Fig. 2 ROR1/CD3 TCE shows high binding affinity to ROR1 and CD3. (**A**) The schematic diagram of ROR1/CD3 antibody. The green part is the Fab targeting ROR1, and the right yellow part is the single-chain variable fragment (scFv) targeting CD3. ROR1/CD3 TCE molecule consists of three chains: anti-ROR1 antibody heavy chain (chain 1), anti-ROR1 antibody light chain (chain2) and an anti-CD3 antibody ScFv-Fc fused chain (Chain 3). (**B**, **C**) Cell-based binding of ROR1/CD3 TCE molecule to ROR1-expressing MDA-MB-231 cell and CD3-expressing human T cells. Cells were incubated with a serially diluted ROR1/CD3 antibody followed by staining with PE-conjugated anti-human IgG. The MFI was determined by flow cytometry. The experiment was performed with three technological replicates and validated by other two biological replicates. (**D**) The binding affinity of ROR1/CD3 to ROR1 and CD3 was evaluated by the surface plasma resonance. The experimemnt was performed with three technological replicates. K_D, binding dissociation equilibrium constants; K_a, kinetic association rate; K_d, kinetic dissociation rate

MDA-MB-231 and low-expressed HCC-1937 cell line, as well as MDA-MB-231 cells with ROR1 knockout were applied. These results showed that ROR1/CD3 TCE bound to ROR1 positive cells regardless of the expression level of ROR1, while no binding was observed with ROR1 negative MDA-MB-231 cells (Fig. 2B). Moreover, ROR1/CD3 antibody also showed dose-dependent cell surface binding to human T cells (EC_{50} = 20.37 nM), suggesting its high binding potency to CD3 (Fig. 2C). The parnetal ROR1 and CD3 binder on cells was shown in supplementary Fig. 1C and 1D. The binding kinetics were further confirmed by BiaCore, which revealed an equilibrium binding constant (KD) for ROR1 and CD3 were 0.71 nM and 6.14 nM, respectively (Fig. 2D). Taken together, these data demonstrated the high binding affinity of ROR1/CD3 TCE with both ROR1 and CD3.

ROR1/CD3 TCE induced T cell activation in a ROR1-dependent manner

To determine the in vitro activity of ROR1/CD3 TCE on T cell activation, human PBMCs and ROR1-expressing TNBC cells were co-cultured and the activation of $CD4^+$, $CD8^+$ T cells was determined via FACS analysis. The percentages of $CD25^+CD69^+$ double positive T cells were significantly increased in both $CD4^+$ T and $CD8^+$ T populations followed by the addition of ROR1/CD3 (Fig. 3A and B). To test the essential role of ROR1 antigen in mediating the function of ROR1/CD3, ROR1 knockout cells were generated via the CRISPR Cas9 technology, and the knockout out results of ROR1 was validated by western blot (Supplementary Fig. 1B). Notably, the activation of $CD4^+/CD8^+$ T cells by ROR1/CD3 depends on the antigen recognition, as no T cell activation was observed in ROR1-negative TNBC cells (Fig. 3A and B). In addition to T cell activation, the proliferation of $CD4^+$ and $CD8^+$ T cells was also significantly enhanced after



	MDA-MB-231	BT-549	MDA-MB-231 (ROR1-KO)
EC50	0.0138	0.02844	~182.7

	MDA-MB-231	BT-549	MDA-MB-231 (ROR1-KO)
EC50	0.03061	0.03575	~31674

	MDA-MB-231
EC50	0.02769

	MDA-MB-231
EC50	0.01979

Fig. 3 ROR1/CD3 TCE induced T cell activation in a ROR1-dependent manner. (**A**, **B**) The activation of $CD4^+$, $CD8^+$ T cells were determined by flow cytometry via analyzing the activation marker CD25 and CD69. (**C**, **D**) The proliferation of $CD4^+$ and $CD8^+$ T cells was measured after co-cultured with a serially diluted ROR1/CD3 antibody for 96 h. The experiment was performed with three tecnological replicates, and validated by another PBMC donor

the stimulation of ROR1/CD3 in ROR1-dependent manner (Fig. 3C and D).

ROR1/CD3 TCE triggered the cytotoxic activity of T cells against TNBC cells

In theory, T cell engager can trigger the cross-linking of cancer cell and T cell to form immunological synapse and kill cancer cells. To assess the in vitro cytotoxic activity of ROR1/CD3 TCE against ROR1-expressing TNBC cells, tumor cells were co-cultured at the ratio of 10:1 with human PBMCs for 48 h. As shown in Fig. 4A, ROR1/CD3 molecule induced the significantly cell killing of MDA-MB-231 and BT-549 cells (Fig. 4A). No cell death was found when MDA-MB-231 ROR1-negaitve cells were cultured with ROR1/CD3 (Fig. 4A), indicating the killing by ROR1/CD3 TCE of TNBC cells depended on ROR1 antigen. Inflammation cytokine release is a marker of T cell mediated cytotoxicity. To determine this, TNBC cells and PBMCs were treated with increasing doses of ROR1/CD3 and the cytokine production was tested. The result showed that ROR1/CD3 TCE molecule dramatically up-regulated the cytokine release, such as IL-2, interferon-g (IFNg), IL-10, IL-4, IL-6 and TNFa (Fig. 4B-G). These findings demonstrated the robust in vitro potency of ROR1/CD3 TCE in killing ROR1-positive TNBC cells.

In vivo killing potency of ROR1/CD3 TCE against ROR1-expressing TNBC cells

To provide proof of concept evidence for the in vivo antitumor efficacy of ROR1/CD3 TCE molecule, xenograft mouse model was established by intraperitoneally injecting human PBMCs as effector cells. MDA-MB-231 cells were inoculated into the NOG mice and 15 days later, T cell reconstitution was established by injecting PBMC. Five days after T cell reconstitution, when the tumor volume reached around 200 mm^3, mice were randomly grouped and administrated with 1 mpk of ROR1/CD3 antibody or isotype IgG. Compared with the isotype control, ROR1/CD3 treatment significantly suppressed the tumor growth (Fig. 5A). The body weight of the mice remained normal until the end of this study (Fig. 5B), suggesting the acceptable safety of ROR1/CD3 TCE in this model. Additionally, to provide more evidence to confirm the anti-tumor potency of ROR1/CD3, HCC1937 cells were also applied to establish the in vivo model. As shown in Fig. 5C and D, administration of ROR1/CD3 induced dramatical tumor shrinkage in this model. And no significant body weight loss was found in comparison with the control group. These results demonstrated the promising in vivo tumor-killing efficacy of ROR1/CD3 TCE in TNBC.

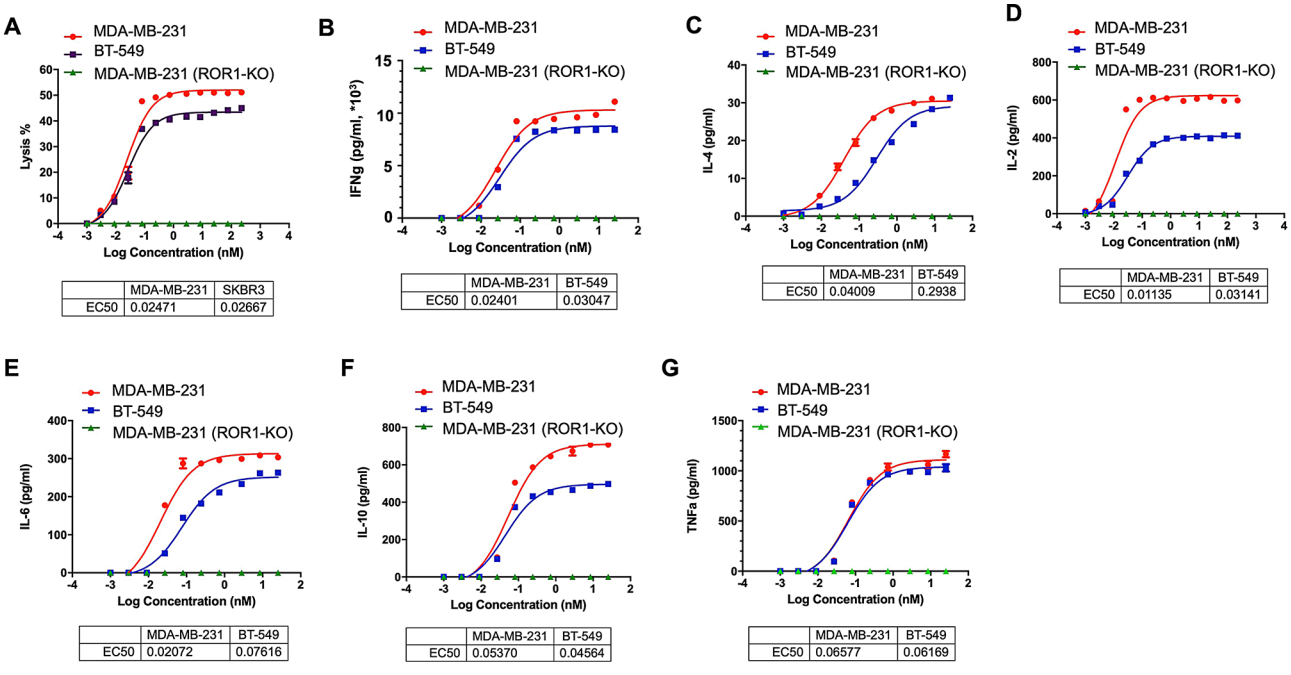


Fig. 4 ROR1/CD3 TCE killed ROR1-expressing TNBC cells. (**A**) The cytotoxic lysis of ROR1/CD3 antibody to TNBC cells were detected by measuring the lactate dehydrogenase release. (**B**-**G**) Human PBMCs were incubated with ROR1/CD3 antibody molecule and the cytokine release in the supernatants was detected by ELISA. Both the cytotoxic and cytokine release experiments were performed with three tecnological replicates, and validated by another PBMC donor

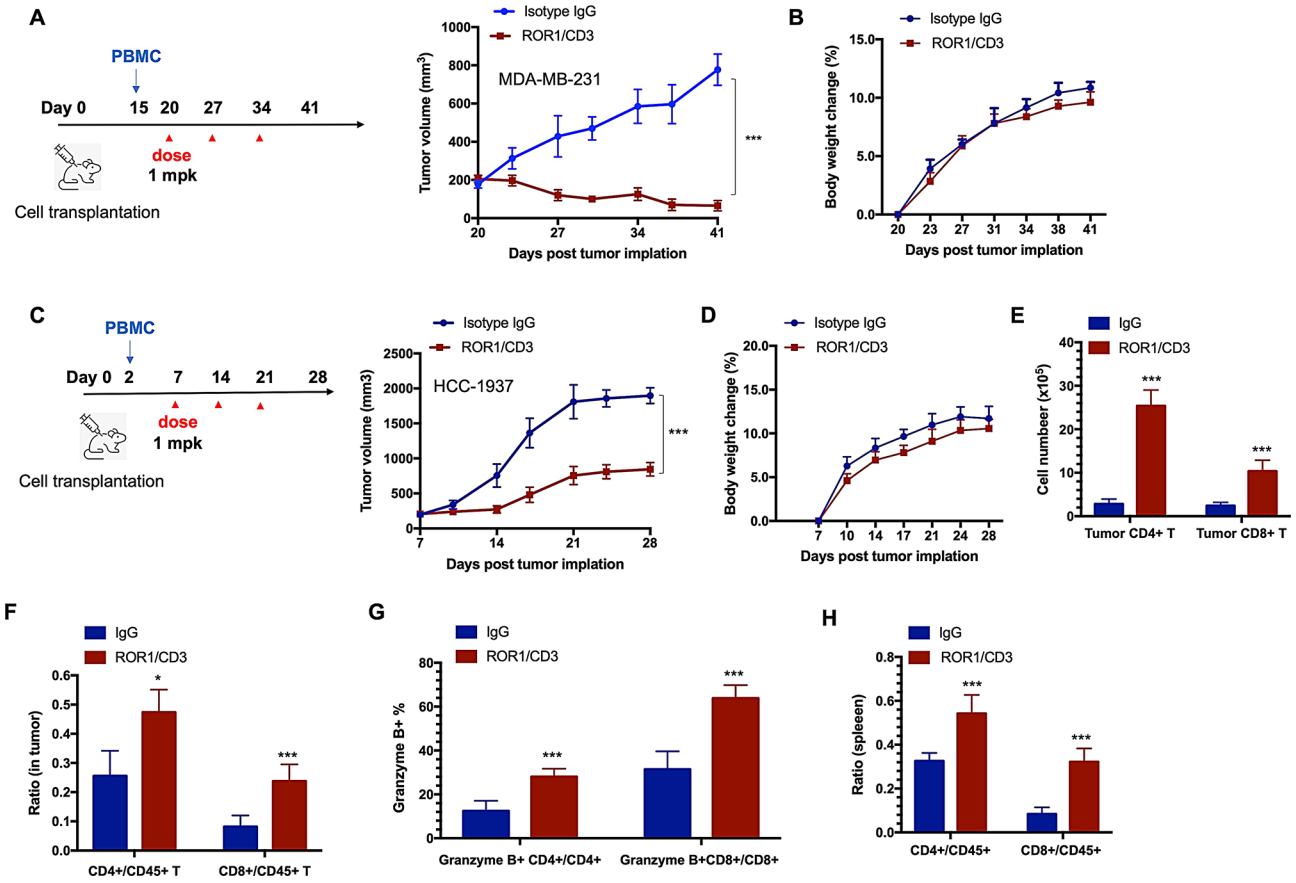


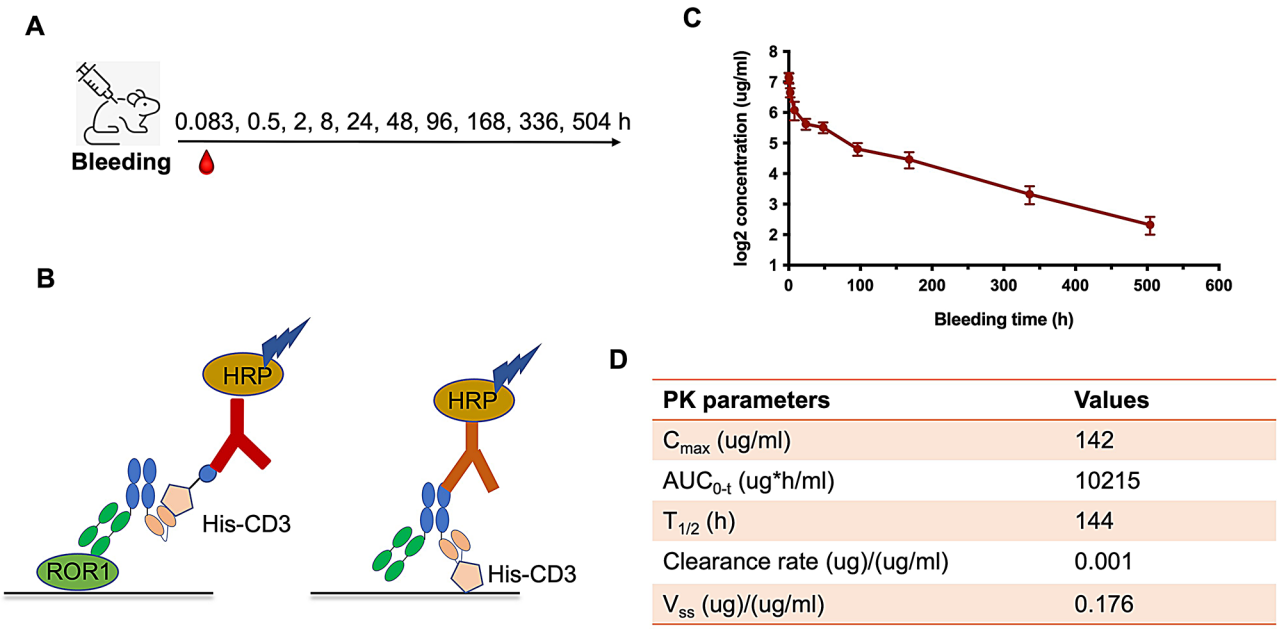
Fig. 5 ROR1/CD3 inhibited tumor growth in TNBC xenograft mouse model. (**A**) The schematic diagram for the in vivo efficacy study (left panel). Administration of 1 mpk ROR1/CD3 antibody induced significantly tumor growth of NOG mice bearing MDA-MB-231 compared with mice treated with control IgG (right panel). *N* = 8 mice for each group. Two-way ANOVA was performed for data analysis. (**B**) No body weight loss was observed within mice treated with ROR1/CD3 antibody. (**C**, **D**) ROR1/CD3 suppressed the tumor growth of HCC1937 model on NOG mice. *N* = 8 mice for each group. Two-way ANOVA was performed for data analysis (**C**). No significant body weight loss was observed with the administration of ROR1/CD3 molecule (**D**). ROR1/CD3 engaged tumor-infiltrating T cells. Mice were treated with 1 mpk ROR1/CD3 after 20 days' of tumor implantation. At day 27, tumors were isolated and analyzed for T cell activation and infiltration by flow cytometry. The percentage of $CD4^+$, $CD8^+$ T cells in tumors (**E**), the ratio of $CD4^+$ or $CD8^+$ in $CD45^+$ T cell populations (**F**), granzyme B^+ positive $CD4^+/CD8^+$ in $CD4^+$ or $CD8^+$ T cell (**G**), the ratio of $CD4^+$ or $CD8^+$ T cells in $CD45^+$ population of the spleen (**H**) was determined. S student's *t* test was performed for data analysis

ROR1/CD3 TCE engaged tumor-infiltrating T cells

In principle, T cell engager plays tumor-killing role via recruiting and activating T cells into the tumor microenvironment. Thus, to deep understand the pharmacodynamic effect of ROR1/CD3, tumors were isolated at day 7 after antibody injection and the infiltrated $CD4^+/CD8^+$ T cells were quantified via FACS analysis. The result showed that the absolute account of both $CD4^+$ and $CD8^+$ T cells, which was normalized to tumor weight, was statistically significantly enhanced after a single dose of ROR1/CD3 antibody (Fig. 5E). Higher ratios of $CD4^+/CD45^+$ and $CD8^+CD45^+$ T was also observed with the administration of ROR1/CD3 (Fig. 5F). Additionally, the effector granzyme B-positive $CD4^+$ and $CD8^+$ T cells was also obviously enriched in tumors followed ROR1/CD3 treatment (Fig. 5G). $CD4^+/CD45^+$ and $CD8^+CD45^+$ T in spleen tissues was also significantly increased after ROR1/CD3 administration (Fig. 5H). These findings indicated that ROR1/CD3 TCE reshaped the immune microenvironment, which driven T cell priming and activation in TNBC.

ROR1/CD3 TCE shows acceptable pharmacokinetics characteristics in mouse

Compared with other TCE with low molecular weight, ROR1/CD3 was designed to fused to effector-function-less Fc fragment to increase its serum stability and reduce dosing frequency. To characterize the pharmacokinetics (PK) of ROR1/CD3, BALB/c mice were administrated with a single dose of 10 mpk ROR1/CD3 intravenously. Bleeding was performed at the time points of 5 min, 0.5-, 2-, 6-, 24-, 48-, 168-, 336- and 504 h after dosing (Fig. 6A). The serum concentrations of ROR1/CD3 were detected by ELISA with plate-coated ROR1 or CD3 antigen (Fig. 6B). The blood concentration of ROR1/CD3



PK parameters	Values
C_{max} (ug/ml)	142
AUC_{0-t} (ug*h/ml)	10215
$T_{1/2}$ (h)	144
Clearance rate (ug)/(ug/ml)	0.001
V_{ss} (ug)/(ug/ml)	0.176

Fig. 6 ROR1/CD3 TCE shows acceptable PK parameters in mice. (**A**) The schematic diagram of dosing and bleeding schedule of the PK study. (**B**) The format of ELISA used for detecting the blood concentration of ROR1/CD3. *N* = 3 mice for each time points. (**C**) The PK profiles of ROR1/CD3. BALB/c mouse was intravenously injected with ROR1/CD3 as mentioned in panel (**A**) and the serum concentration of ROR1/CD3 was detected at the indicated time points after dosing. (**D**) The PK parameters of ROR1/CD3 molecule in BALB/c mouse

TCE decreased overtime with the maximum concentration of 142 ug/ml (Fig. 6C and D). The mean clearance was 0.001 ug/(ug/ml)/h and the area under the concentration-time curve was 10,215 ug*h/ml. Half-life of ROR1/CD3 was around 144 h (Fig. 6D). These data demonstrated the good PK parameters and stability of ROR1/CD3 antibody in mice.

Discussion

Identification of clinically targetable cell-surface molecules in TNBC that meet the stringent criteria for optimal therapeutic safety and efficacy is a big challenge. ROR1 is a type I single-pass transmembrane protein, which is normally expressed in embryonic tissues and abnormally in a variety of cancers [26]. The undetectable expression of ROR1 in adult tissues makes it a promising target for cancer therapy and drug design. Highly expressed ROR1 was reported to be correlated with the aggressive features, poor prognosis and chemotherapy resistance of TNBC [32], suggesting ROR1 as a potential drug target of TNBC. Although monoclonal antibody targeting ROR1 has been developed, its clinical anti-tumor potency was insufficient [33]. Therefore, novel modalities that is created upon the naked antibody of ROR1, such as TCE, ADC may generate promising clinical outcome for cancer patients. In this study, we generated a proof of concept ROR1/CD3 TCE molecule and demonstrated that ROR1/CD3 engaged T cells to trigger the cytolysis of TNBC cells with acceptable PK parameters.

Although some mutated or oncogenic proteins are overexpressed in TNBC, their detectable levels in normal tissues make them impossible as drug targets due to possible on-target off-tumor toxicity. T cell engager molecules induced the formation of cytotoxic synapse by linking T cells and tumor cells via recognizing the antigen, which consequently release cytotoxic granules and triggers tumor cell death [34, 35]. Therefore, therapeutic approaches targeting these antigens need to be monitored closely to avoid on target off tumor adverse effects. It has been reported that blinatumomab, a CD19/CD3 bispecific antibody, induced cytokine release syndrome (CRS) and neurotoxicity in early clinical experience [17]. These side effects are mainly due to the redirecting of T cells against B cells. Overactivation of T cells by high affinity CD3 arm contributes to the poor tolerance because of extensive cytokine release. Reduced the cytokine release but not changed the maximum cytotoxic activity of ROR1/CD3 antibody is the emphasis of our future work. In this study, CD3 arm with high affinity was selected to guarantee the strong killing capacity of the TCE molecule. Meanwhile, increasing evidence has shown that the intermembrane distance and the strength of the synapse formed between T cell and tumor cells greatly impact the TCE potency and cytokine release. A recent study found that t cell engager binds to the membrane distal domain of ROR1 has the potential to decouple cytotoxicity and cytokine release [36]. In this study, ROR1 binder that targets the epitope of distal membrane

domain was selected, and we are now working on the optimization of the antibody to reduce the IFNg release but not affect the maximum killing potency. It should be noted that the adverse effects of central nervous system of ROR1/CD3 could not be evaluated by the preclinical mouse model, that calls for further investigation in cyno monkeys and clinical trials.

Despite remarkable achievements have been made in the clinical practice of immune checkpoint antagonists including PD-1 monoclonal antibody (mAb), a majority of patients with advanced cancers respond poorly and could not benefit from this treatment [37]. The immunosuppressive microenvironment may complicate the clinical development of ROR1/CD3, while combination with PD-1 antibody is a rational approach. Chemotherapy has been demonstrated to induce the lysis of tumor cells and trigger T cell activation. Because the fundamental role of chemotherapy in TNBC treatment, the efficacy of ROR1/CD3 combo with chemo is also worth expecting. Additionally, since ROR1 was overexpressed in a variety of cancers, the potency of ROR1/CD3 TCE molecule in other solid tumors deserves more studies.

Improvements in protein engineering technology have enabled the generation of bispecific antibodies with extended half-life, greater specificity, flexibility and efficacy. Blinatumomab, the CD19/CD3 bispecific antibody, was designed as single-chain Fv format with small molecule size [17]. Because of the short half-life, administration of blinatumomab requires continuous intravenous dosing to maintain the efficacy [17]. There are several literatures reporting ROR1/CD3 TCE in solid tumors [29, 38, 39]. Gohil and colleagues generated a ROR1/CD3 bispecific antibody, in which both anti-CD3 and anti-ROR1 are ScFv in a heavy chain-linker-light chain format [39]. This molecule showed efficient T- cell mediated killing in a range of solid tumor cell lines. Additionally, Qi and colleagues reported that they created a ROR1/CD3 TCE, in which both ROR1 and CD3 binding moieties were placed as the ScFv on each arm that fused to the Fc fragment [38]. In this study, the bispecific antibody was designed in lgG-like format with one Fab targeting ROR1 and a CD3 binder in ScFv format, which is possible to have an extended half-life and low immunogenicity in human. ROR1/CD3 administration in mice demonstrated a PK characteristic of full-length IgG1 antibody, which enables reasonable dosing schedules in clinic. To further support this conclusion, the PK parameters in non-human primate cynomolgus monkeys are necessary to be confirmed. Based on the preclinical data of ROR1/CD3 in TNBC, more modalities of bispecific antibodies by co-targeting ROR1 and other specific cell surface antigens are also new avenues to be explored. Additionally, unlike the published design, ROR1 binder that targets the membrane distal domain of ROR1 was selected in this study, which has the potential to decouple the cytotoxicity and cytokine release under further optimization. The current ROR1/CD3 antibody showed good killing efficacy in both ROR1-high and -low preclinical mouse models, which indicate the possible to cover a larger population of patients. However, the safety and therapeutic window of this format still calls for more deep investigation, especially in cyno monkeys and clinical trials.

Conclusions

Our study proposed a novel design of TCE molecule for TNBC by targeting ROR1 and engaging T cell function. The specificity, potency and stability of this ROR1/CD3 bispecific antibody were evaluated by in vitro and in vivo analyses. Characterization of ROR1/CD3 safety profile in cyno monkeys and clinical assessment for TNBC are needed.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13058-025-02005-w.

Supplementary Material 1: Supplementary Fig.1. (A) The purification of ROR1/CD3 antibody was validated by 10% non-reducing SDS-PAGE. (B) ROR1 knockout MDA-MB-231 cell line generated by the CRISPR Cas9 method was validated by western blot with anti-ROR1 antibody. (C, D) Cell based binding of parental ROR1 or CD3 binders on TNBC or human T cells.

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Author contributions

F. W., WN. L., and XD. B.: Conceptualization, Supervision, Investigation, Writing-original draft. GH.H., J.X.: Methodology, Validation, Data curation.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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