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# A T-cell engaging bispecific antibody with a tumor-selective bivalent folate receptor alpha binding arm for the treatment of ovarian cancer

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**ABSTRACT**

The use of T-cell engagers (TCEs) to treat solid tumors is challenging, and several have been limited by narrow therapeutic windows due to substantial on-target, off-tumor toxicities due to the expression of low levels of target antigens on healthy tissues. Here, we describe TNB-928B, a fully human TCE that has a bivalent binding arm for folate receptor alpha (FRα) to selectively target FRα overexpressing tumor cells while avoiding the lysis of cells with low levels of FRα expression. The bivalent design of the FRα binding arm confers tumor selectivity due to low-affinity but high-avidity binding to high FRα antigen density cells. TNB-928B induces preferential effector T-cell activation, proliferation, and selective cytotoxic activity on high FRα expressing cells while sparing low FRα expressing cells. In addition, TNB-928B induces minimal cytokine release compared to a positive control TCE containing OKT3. Moreover, TNB-928B exhibits substantial *ex vivo* tumor cell lysis using endogenous T-cells and robust tumor clearance *in vivo*, promoting T-cell infiltration and antitumor activity in mouse models of ovarian cancer. TNB-928B exhibits pharmacokinetics similar to conventional antibodies, which are projected to enable favorable administration in humans. TNB-928B is a novel TCE with enhanced safety and specificity for the treatment of ovarian cancer.

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Bispecific antibody; T-cell engager; folate receptor alpha; ovarian cancer; on-target, off-tumor toxicity

## Introduction

Ovarian cancer is the leading cause of gynecologic cancer mortality in women, and 95% of cases represent epithelial tumors (i.e. epithelial ovarian carcinoma; EOC).[1] Five-year overall survival in EOC decreases from 89% to 17% from stage I to stage IV, and approximately two-thirds of EOC patients present with stage III–IV disease.[2] High-stage disease patients frequently relapse following first-line treatment, which typically consists of a combination of platinum-based adjuvant or neoadjuvant chemotherapy and cytoreductive surgery. Recent therapeutic advances including the approval of poly (ADP-ribose) polymerase (PARP) inhibitors for patients with BRCA mutations have improved survival in a subset of patients,[3] but therapeutic options for recurrent disease, especially platinum-resistant disease, remain poor for most and there is a high unmet need for novel treatments.

Immunotherapy has emerged as a promising cancer treatment. In particular, T-cell therapies including chimeric antigen receptor T-cells (CAR-T) and CD3 T-cell engaging (TCE) bispecific antibodies that direct cytotoxic activity of T-cells in an MHC independent manner to specifically target and kill tumor cells have shown encouraging clinical results for treatment of chemotherapy-resistant and relapsed hematological malignancies.[4] Blinatumomab, a CD19xCD3 TCE, is approved for the treatment of B cell acute lymphoblastic leukemia. In support of immunotherapy for the treatment of EOC, tumor-specific T-cells have been detected in patients with advanced EOC disease,[5,6] and T-cell infiltration into the tumor microenvironment (TME) is associated with longer progression-free survival (PFS) and overall survival (OS),[7] suggesting that EOC may be amenable to treatment with a CAR-T or TCE. However, challenges of targeting solid tumors with a TCE include limited T-cell infiltration into tumors, an immunosuppressive TME that inhibits T-cell activity, and a lack of targets that are expressed only on the surface of tumor cells but not normal, healthy tissues.[8] Due to the potent mechanism of TCEs, any on-target, off-tumor activity from target engagement on normal tissue presents a toxicity challenge owing to destruction of vital tissues and secondary inflammatory cytokine release or immune related adverse events. For example, a phase 1 trial of an EpCAMxCD3 TCE resulted in severe gastrointestinal and liver dose limiting toxicities, consistent with EpCAM expression in the gastrointestinal tract and liver with strong lymphocyte infiltration observed in the duodenum.[9] Thus, when targeting solid tumors

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with TCEs, on-target, off-tumor toxicity remains a significant concern.

Folate receptor alpha (FRα, FOLR1) is a cysteine-rich GPI-linked membrane glycoprotein that mediates the cellular uptake of reduced folates and folic acid.[10] In normal tissues, the expression of FRα is limited and restricted to the luminal side of a subpopulation of epithelial cells primarily in kidney and lung tissue and is not accessible to FRα targeted agents in circulation.[11–14] In contrast, aberrant FRα overexpression is characteristic of several epithelial tumors including ovarian cancers, where the prevalence of FRα expression on tumors is up to 90%.[15–18] Several FRα targeted therapies have been evaluated in the clinic including folate-conjugates, monoclonal antibodies, and antibody-drug conjugates, which generally have tolerable safety profiles but have been hampered by limited efficacy.[19,20] Despite the observed safety of FRα targeted therapies in the clinic, significant preclinical on-target, off-tumor toxicities in the lung have been observed with FRα targeting TCEs in non-human primates (NHPs).[21,22]

Here, we describe a novel fully human bivalent FRαxCD3 TCE (TNB-928B) and the two-fold manner in which it is designed for enhanced safety by 1) utilizing avidity to avoid destruction of normal tissues that express low levels of FRα, therefore mitigating the risk of on-target, off-tumor toxicity; and 2) utilizing a unique anti-CD3 (F2B) that induces lower levels of cytokine release compared to a TCE containing a strong anti-CD3 (OKT3) to reduce the risk of cytokine release syndrome (CRS) in the clinic. Both of these attributes are anticipated to help achieve an expanded therapeutic index. Notably, TNB-928B engages both FRα and CD3 leading to the activation of resting polyclonal $CD4^+$ and $CD8^+$ T-cells, resulting in selective lysis of high FRα expressing tumor cell lines with markedly lower cytokine release. Moreover, TNB-928B displays potent antitumor activity in *in vitro, ex vivo*, and *in vivo* models of ovarian cancer.

## Materials and methods

### Immunizations, next-generation sequencing, clonotype analysis, and cloning

Methods used for immunization, NGS, and cloning were previously described.[23] Briefly, UniRats were immunized with recombinant FRα protein (Antibody Solutions, CA, USA). After 8 weeks, draining lymph nodes were harvested, and total RNA was isolated. Ig heavy chains were sequenced using next-generation sequencing (NGS) (Illumina MiSeq). The data were analyzed as previously described, and the top clonotypes were recombinantly expressed in HEK293 cells.[24]

### Protein expression and purification

Antibodies were expressed in ExpiCHO cells (Thermo Fisher Scientific). Clarified supernatants were harvested and affinity purified using CaptureSelect CH1-XL resin (Thermo Fisher Scientific) and cation exchange (Mono S). Antibodies were analyzed by size exclusion chromatography-ultra-high performance liquid chromatography (SEC-UPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### Cell lines

Tumor cell lines OVCAR-3, SKOV-3, HT-29, and HeLa were obtained from ATCC. LNCaP and MCF-7 were obtained from Millipore Sigma. IGROV-1 was obtained from NCI. Choroid plexus, retinal pigment, pulmonary alveolar, bronchial, and renal cortical epithelial cells were obtained from ScienCell Research Laboratories. All cell lines were maintained in a 5–8% $CO_2$ buffered incubator at 37°C and maintained as per manufacturer's recommendations.

### Thermal stress and stability characterization

Antibodies were formulated in 20 mM citrate and 0.1 M NaCl at pH 6.2. High-molecular-weight species were measured by SEC-UPLC (ThermoFisher UltiMate 3000 HPLC) before ($T_0$) and after temperature stress ($T_{30}$) for 1 month at 37°C.

### Quantification of FRα expression

Estimation of antibodies bound per cell (ABC) was described previously.[24] Briefly, cell lines were stained with a saturating concentration of a PE-labeled FRα antibody (BioLegend, clone LK26) and tested via flow cytometry using BD Quantibrite beads. Antigen density (or FRα molecules per cell) was calculated by multiplying ABC values by 2.

### Scatchard analysis

Methods for Scatchard analysis were described previously.[24] Briefly, the cell surface affinity of bivalent TNB-928B and monovalent FRαxCD3 TCEs was measured using IGROV-1. Antibodies were labeled with Alexa Fluor-488 (Thermo Fisher), and Quantum Alexa Fluor-488 MESF beads (Bangs Laboratories) were used to generate the standard curve. Affinity ($K_D$) was calculated as previously described.[24]

### Activation and proliferation of T-cells

To measure the activation of $CD4^+$ and $CD8^+$ T-cells, T-cells were incubated with IGROV-1 or SKOV-3 (E:T of 5:1) in the presence of an indicated antibody for 48–72 hours. Cells were harvested and incubated with LIVE/DEAD™ Fixable Near IR dye (Thermo Fisher Scientific) and Human TruStain FcX (Biolegend). The cells were washed with 1XPBS + 1% BSA + 0.1% $NaN_3$ and stained with antibodies for CD45 (Biolegend, clone 2D1), CD4 (Biolegend, clone OKT4), CD8 (Biolegend, clone RPA-T8) and CD69 (Biolegend, clone FN50). Cells were washed twice and analyzed by flow cytometry. T-cell proliferation assays were similar but with an incubation period of 3 d, and cells were stained via intracellular staining for Ki67 (Biolegend, clone Ki-67). $CD4^+$ Tregs were stained with antibodies for CD25 (Biolegend, clone BC96) and FOXP3 (Biolegend, clone 206D) using intracellular staining with the FOXP3 fix/perm kit (Biolegend).

### In vitro *cytotoxicity and cytokine assays*

Target cells were plated at a density of $0.1 \times 10^6$ to $0.2 \times 10^6$ in RPMI-1640 + 10% FBS + 1% P/S, and frozen PBMCs (STEMCELL Technologies) were thawed, washed, and rested overnight. After 24 hours, pan T-cells were isolated (Miltenyi Biotec). Pan T-cells were added to tumor cells at the E:T ratios indicated along with diluted antibody. After 48 hours, an aliquot of supernatant was removed, frozen, and subsequently analyzed for IL-2 and IFNγ release (MSD U-plex platform). Cytotoxicity was measured using Cell Proliferation Reagent WST-1 (Sigma) or CytoTox 96 LDH substrate (Promega). Percentage of cell lysis was calculated as previously described.[25]

### Ex vivo *cytotoxicity, cytokine, and cytotoxic granule measurement*

Fresh, de-identified, surgically removed ovarian tumor tissue (obtained under an MTA from Dr Chapman) was cut into small pieces, washed with PBS, and digested with 2.5 U/mL dispase II (STEMCELL Technologies) for 30 minutes at 37°C with manual agitation every 5 minutes. Dissociated cells were centrifuged and resuspended in DMEM with 10% FBS and 1% P/S at $0.4 \times 10^6$ cells/mL. Cells were incubated with the indicated antibodies for 48–72 hours. Cytotoxicity was measured using CytoTox 96 LDH substrate (Promega). Cytokines were measured as described above. Perforin and granzyme B were measured by ELISA (Cell Sciences and Thermo Fisher Scientific, respectively). T-cells were quantified, and the effector to target cell ratio in these samples was determined by measuring the subsets of live T-cells, positive for CD4 and CD8, vs. the target cells staining positive for FRα ($CD4^-/CD8^-$).

### *Analysis of FRα protein expression in tumor tissue*

Representative blocks of formalin-fixed, paraffin-embedded tumor were available for all nine patients that were tested ex-vivo. Briefly, 4-µm tissue sections were deparaffinized, rehydrated, and pretreated in appropriate buffer for antigen retrieval by using a Biocare Decloaking Chamber. Tissue slides were then incubated at room temperature with a primary antibody, anti-FRα (Abcam, EPR20277, 4 µg/ml) followed by an appropriate secondary antibody. FRα staining intensity was scored according to the following grading scheme: 0 = no stain; 1 + = weak (minimal) stain; 2+ = mild stain; and 3+ = intense stain.

### In vivo *mouse efficacy study*

NOD-*Prkdc*$^{em26Cd52}$*I2rg*$^{em26,d22}$ (NCG) mice (7–9 weeks, GemPharmatech Co., Ltd.) were subcutaneously injected with $5 \times 10^6$ IGROV-1 or SKOV-3 cells with 50% Matrigel and intraperitoneally injected with $10 \times 10^6$ resting human PBMCs (AllCells) on day 0. On day 3, mice were randomized into groups (n = 3) by body weight and injected intravenously (*i.v.*) with test article. Test article was injected every 3 d at respective doses for a total of 8–10 treatments. Twice per week, the length and width of each tumor was measured using calipers and the volume was expressed in $mm^3$ using the formula: V = (L × W × W)/2, where V is tumor volume, L is tumor length, and W is tumor width. In a similar follow-on study, NCG mice were injected with activated human PBMCs (AllCells). Prior to injection, PBMCs were activated for 3 d with 1 µg/mL plate-coated anti-CD3 (eBioscience, clone OKT3) and 1 µg/mL soluble anti-CD28 (eBioscience, clone CD28.2). At the end of the study, the tumors were harvested, processed into formalin-fixed paraffin-embedded sections, and stained with anti-human CD45 (Cell Signaling Technology, 13917, 1:800) and anti-human FRα (Abcam, EPR20277, 1:1000). The stained sections were scanned with NanoZoomer-2.0 HT Image System (Hamamatsu) at 40x magnification.

### *Mouse pharmacokinetic (PK) evaluation*

The PK of TNB-928B was evaluated in female BALB/c mice using the study design described previously.[24] Mice were injected via tail vein with 1 mg/kg or 10 mg/kg TNB-928B (n = 3/group * 6 groups). Serum samples were collected for 14 d post-dose at timepoints ranging from 30 minutes to 14 d. Serum TNB-928B concentrations were measured using human IgG4 AlphaLISA (Perkin Elmer). PK parameters were estimated using Phoenix WinNonlin version 7.0 software (Pharsight Corp).

### *Statistics*

Statistical analysis was performed using a two-tailed unpaired t-test using GraphPad Prism 9. P values <.05 were considered statistically significant.

## Results

### *FRα expression on normal cells and ovarian tumor cells*

FRα is expressed on normal healthy tissues including lung and kidney, and targeting FRα with a TCE could result in on-target, off-tumor toxicity. We sought to quantify cell surface expression of FRα on various ovarian and other solid tumor cell lines as well as normal primary cells by flow cytometry. The FRα antigen density on the ovarian tumor cell lines OVCAR-3, SKOV-3, and IGROV-1 ranged from $44 \times 10^3$ to $1{,}871 \times 10^3$ (Table 1), recapitulating the range observed in clinical samples

**Table 1.** FRα expression on normal and malignant cells. Antigen density of FRα was measured on the indicated human tumor cell lines and human primary epithelial cells by quantitative flow cytometry. Values are given as the mean and standard error of the mean (SEM) of 2–5 independent experiments.

| Cell line | FRα antigen density ($\times 10^3$) |
|---|---|
| IGROV-1 | 1,871 ± 838 |
| HeLa | 367 ± 5 |
| SKOV-3 | 82 ± 29 |
| OVCAR-3 | 44 ± 8 |
| HT-29 | 8 ± 1 |
| MCF-7 | 5 ± 1 |
| Choroid Plexus Epithelial Cells | 0.7 ± 0.6 |
| Retinal Pigment Epithelial Cells | 1.3 ± 0.2 |
| Pulmonary Alveolar Epithelial Cells | 0.1 ± 0.1 |
| Bronchial Epithelial Cells | 0.8 ± 0.4 |
| Renal Cortical Epithelial Cells | 6.7 ± 1.5 |

of relapsed ovarian cancer patients.[26] In contrast, normal primary alveolar and bronchial epithelial lung cells express $0.1 \times 10^3$ and $0.8 \times 10^3$ FRα/cell respectively, and renal cortical epithelial cells express $6.7 \times 10^3$ FRα/cell (Table 1). FRα expression has also been reported on choroid plexus and retinal epithelial cells;[13,14] however, we only measured FRα antigen densities of $0.7 \times 10^3$ and $1.3 \times 10^3$, respectively. Since the high end of expression on normal tissues reported to express FRα is in the range of $7 \times 10^3$, the cell lines HT-29 and MCF-7 with FRα antigen densities of $8 \times 10^3$ and $5 \times 10^3$, respectively, were selected as cell lines representing the FRα expression threshold at or below which no TCE-dependent cytotoxicity was desirable.

### *Generation and functional characterization of a FRαxCD3 TCE*

Fully human heavy-chain only antibodies (UniAbs) targeting FRα were generated by immunization of UniRats followed by a next-generation sequencing (NGS)-based antibody repertoire discovery approach.[23,27] FRα-specific UniAbs were identified by screening recombinant antibody candidates for cell surface binding to FRα$^+$ tumor cell lines and binding to FRα protein. Antibodies against CD3 were generated in OmniFlic animals, and the previously identified low-affinity anti-CD3_F2B arm that mediates tumor cell killing with minimal cytokine release was included in the creation of FRαxCD3 TCEs.[23,28]

We sought to improve the selectivity of a FRαxCD3 TCE to minimize the risk of on-target, off-tumor activity and increase the therapeutic window of a FRα targeting TCE. To achieve tumor selectivity, we utilized a reduced-affinity bivalent format to specifically target FRα overexpressing ovarian tumor cells. This 2 + 1 bispecific antibody format has previously been applied to TCEs for selective targeting of solid tumors overexpressing CEA and HER2.[29,30] FRαxCD3 TCEs were generated by pairing the lead anti-FRα VH (variable heavy region of the antibody) in either monovalent or bivalent formats with OKT3, a high-affinity CD3 binding arm (for a positive control, PC) or with CD3_F2B, a low-affinity CD3 binding arm (lead bivalent antibody, TNB-928B) using knobs-in-holes technology as previously described (Figure 1a).[28] TNB-928B showed robust expression (3.8 g/L) and low aggregation propensity after thermal stress (Supplemental Figure S1 and Supplemental Table S1). The cell surface affinity of the bivalent anti-FRα arm of TNB-928B was 3.7 ± 1.6 nM, and the monovalent FRαxCD3 was 123 ± 4 nM to IGROV-1 cells as determined by Scatchard analysis (Figure 1b and Supplemental Figure S2).

To determine the effect of FRα valency on the *in vitro* functional activity of FRαxCD3 TCEs, cytotoxicity assays were performed. The ability of TNB-928B to mediate the lysis of IGROV-1 (high FRα antigen density) was compared to a corresponding bispecific antibody containing the same VH, which is monovalent for FRα. Both TNB-928B and the bivalent

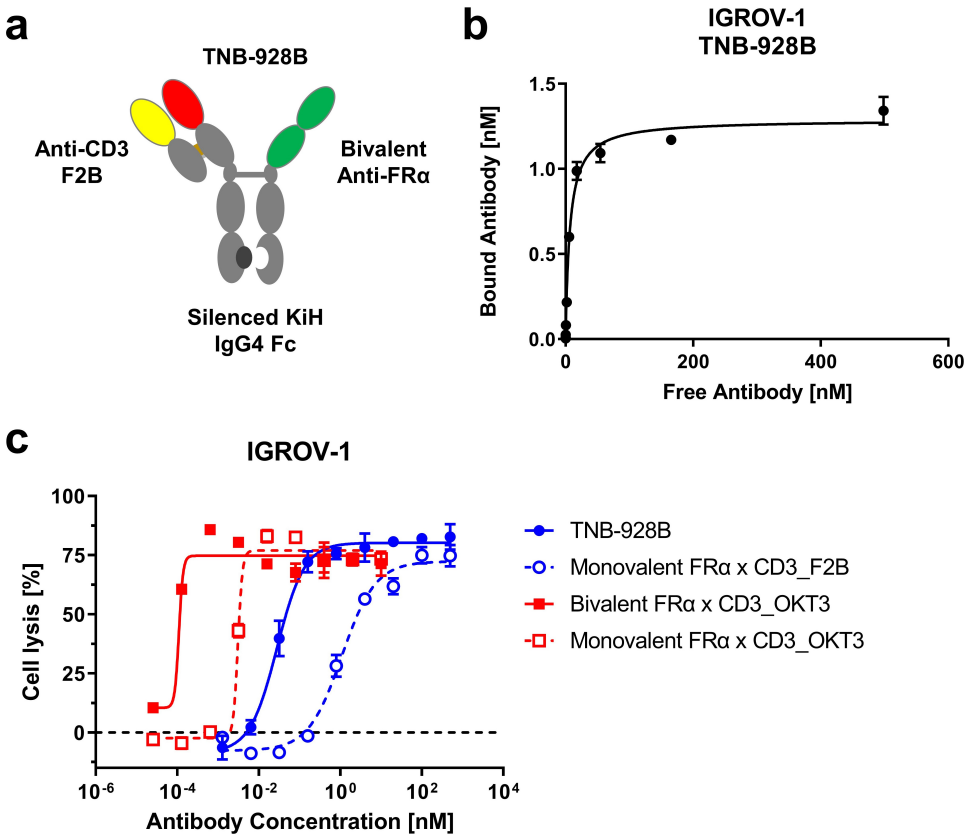


**Figure 1.** TNB-928B is a fully human bispecific antibody engaging FRα and CD3. (a) TNB-928B was constructed using knobs-into-holes technology. The schematic shows the format of the antibody. (b) Cell surface affinity of TNB-928B to FRα expressed on IGROV-1 cells was determined by Scatchard analysis. (c) Co-culture cytotoxicity assay with T-cells (E:T ratio of 10:1) showing cell lysis of IGROV-1 tumor cells after 48 h. Monovalent and bivalent FRαxCD3_OKT3 bispecific molecules are the positive controls for monovalent FRαxCD3_F2B and TNB-928B respectively.

PC antibodies exhibited an avidity effect observed in lower $EC_{50}$ values (increased potency) compared to the respective monovalent antibodies (Figure 1c and Supplemental Table S2). Importantly, TNB-928B exhibited similar maximum tumor cell lysis compared to the PC at saturating doses with ~75% lysis. Taken together, these data demonstrate that the bivalent format of TNB-928B exhibited a strong avidity effect for killing high FRα expressing tumor cells and TNB-928B achieves maximum activity comparable to the PC.

T-cell exclusion from solid tumors could present an obstacle for T-cell mediated therapies. To evaluate if TNB-928B can mediate tumor cell lysis at low effector to target cell (E:T) ratios, cytotoxicity assays were conducted with several E:T ratios ranging from 5:1 to 1:5. Substantial lysis of SKOV-3 and OVCAR-3 tumor cells was observed at E:T ratios of 1:5 and 1:3 with 44% and 61% maximum lysis, respectively (Supplemental Figure S3A). IGROV-1 was more resistant to lysis at low E:T ratios with minimal tumor cell lysis at 1:1 and below. These results demonstrated that TNB-928B is capable of tumor cell lysis at very low E:T ratios that reflect the predicted ratios in solid tumors.

Like other GPI-anchored proteins, FRα is cleaved from the cell surface. Soluble FRα protein (sFRα) is elevated in the serum of ovarian cancer patients, and in both early and advanced ovarian cancer patients, high sFRα is associated with shorter PFS.[31,32] Moreover, expression of FRα on tumor cells is strongly correlated with sFRα levels. To determine the effect of sFRα on TNB-928B mediated tumor cell lysis, a cytotoxicity assay was performed in the presence of exogenously added soluble recombinant FRα. A minimal decrease in potency of TNB-928B mediated SKOV-3 lysis was detected in the presence of sFRα up to 1,000 ng/mL, which is approximately 100-fold higher than sFRα measured in the serum of EOC patients,[31,33] but maximum cell lysis was unchanged (Supplemental Figure S3B). The measured levels of sFRα after 48 hours were comparable to the amount of exogenous sFRα added, suggesting sFRα was stable for the duration of the experiment (Supplemental Figure S3C). These data suggest that the cytotoxic activity of TNB-928B is not affected by sFRα levels comparable to physiological levels detected in the serum of ovarian cancer patients.

### TNB-928B activates resting T-cells and induces T-cell proliferation

To further characterize the mechanism of action of TNB-928B, we measured the upregulation of CD69 in subsets of $CD3^+$ T-cells as a marker of T-cell activation following co-culture of resting T-cells and IGROV-1 cells. TNB-928B induced dose-dependent activation of both $CD4^+$ and $CD8^+$ T-cells to a similar extent as the PC (Figure 2a). Approximately 73% of $CD4^+$ and 69% of $CD8^+$ T-cells were CD69-positive following co-culture of IGROV-1 cells and pan T-cells at saturating concentrations of TNB-928B. The $EC_{50}$ values for TNB-928B mediated $CD4^+$ and $CD8^+$ T-cell activation were 16.1 pM and 8.3 pM, respectively, compared to approximately 1 pM for the PC in both T-cell subtypes. Negligible CD69 upregulation was observed with treatment with a negative control (NC) with the same anti-CD3_F2B arm as TNB-928B but with an irrelevant tumor targeting arm. Similar T-cell activation results were observed with SKOV-3 cells (Supplemental Figure S4). To measure the relative activation of Tregs by TNB-928B, we measured the percentage of Tregs in the T-cell population by gating for $CD4^+CD25^+Foxp3^+$ cells. Importantly, TNB-928B induced Tregs approximately two-fold less than the PC at antibody concentrations saturating for T-cell activation compared to PC treated cells (Figure 2c), suggesting TNB-928B preferentially activates effector T-cells compared to Tregs.

To evaluate the ability of TNB-928B to induce T-cell proliferation, we measured the upregulation of Ki67 as a marker of proliferation following co-culture with IGROV-1 tumor cells. TNB-928B induced dose-dependent increases in Ki67 with approximately 43% of $CD4^+$ and 71% of $CD8^+$ T-cells (Figure 2b). The $EC_{50}$ values for TNB-928B mediated $CD4^+$ and $CD8^+$ proliferation were 48.9 pM and 17.8 pM, respectively. No Ki67 proliferation was detected with the NC, demonstrating target specificity of TNB-928B. Taken together, these data are consistent with the mechanism of action of TCEs.

### TNB-928B exhibits selective cytotoxicity of high FRα-expressing tumor cells

To evaluate the ability of TNB-928B to selectively lyse high FRα expressing cell lines while sparing low FRα expressing cell lines, we examined the tumor cell lysis activity of TNB-928B compared to a monovalent FRαxCD3_F2B (or a PC that contains CD3_OKT3) containing the same anti-FRα VH on SKOV-3, HeLa, HT-29, and MCF-7 cells. SKOV-3, and HeLa represent high FRα density cell lines, whereas HT-29 and MCF-7 are low FRα density cell lines with expression levels similar to normal cells (Table 1). While the monovalent FRαxCD3_F2B molecule was efficacious in lysing SKOV-3 (75% maximum lysis), this molecule also reached high levels of maximum lysis of HT-29 (60% maximum lysis, Figure 3 a and b), which likely would result in unacceptable *in vivo* on-target, off-tumor toxicity associated with targeting FRα with a monovalent TCE. In contrast, TNB-928B exhibited significantly reduced lysis of HT-29 and MCF-7 cells as compared to the bivalent PC (10% vs 74% maximum HT-29 lysis, respectively, Figure 3b and Supplemental Figure S5B). Nevertheless, TNB-928B exhibited similar maximum cytotoxicity of SKOV-3 cells as the PC with ~70% maximum lysis (Figure 3a). TNB-928B also mediated the lysis of IGROV-1, HeLa, and OVCAR-3 cell lines, demonstrating activity against tumor cell lines with high FRα antigen densities (Table 1 and Supplemental Figures S3A and S5A). Taken together, these results demonstrate that the bivalent format of TNB-928B has similar efficacy as that of a strong TCE on high-density FRα tumor cell lines but minimal activity on low FRα density cell lines, indicating that TNB-928B is selective for FRα overexpressing tumor cells due to its avidity-mediated activity.

### TNB-928B decouples tumor cell cytotoxicity from cytokine release

CAR-Ts and TCEs are frequently associated with CRS. To minimize the risk of CRS, TNB-928B contains the previously described and clinically validated anti-CD3_F2B (Figure 1a).[24,25,28,34] To evaluate TNB-928B-mediated

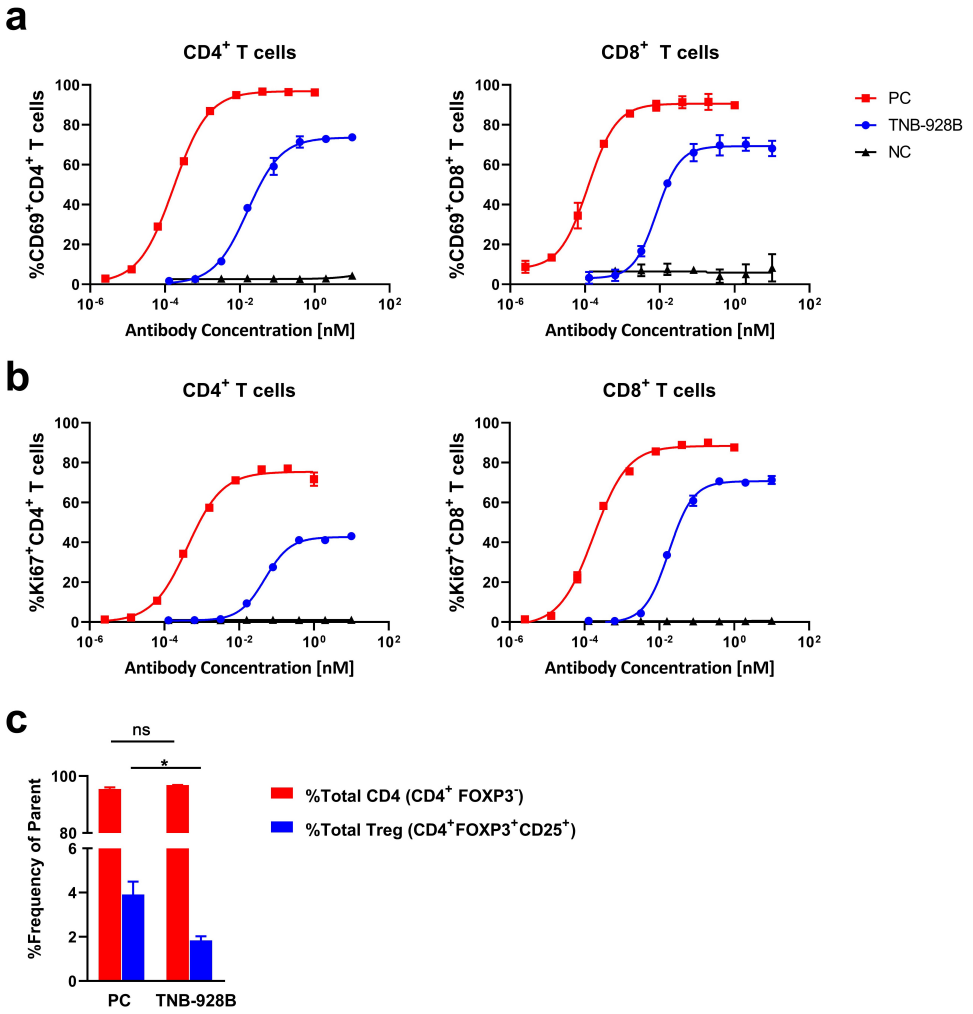


**Figure 2.** TNB-928B mediates preferential effector T-cell activation and proliferation. (a) Activation of $CD4^+$ or $CD8^+$ T-cells was measured by flow cytometric measurement of the activation marker CD69 after 72 h of co-culture of T-cells from a healthy donor and IGROV-1 tumor cells at an E:T ratio of 5:1. (b) Proliferation of $CD4^+$ or $CD8^+$ T-cells was measured by flow cytometric measurement of Ki67 after 72 h of co-culture with IGROV-1 tumor cells at an E:T ratio of 5:1. (c) $CD4^+$ T-cells were further gated on $CD25^+Foxp3^+$ expression to evaluate percentage of Treg cells induced 72 h after 8 or 16 nM of PC or TNB-928B treatment respectively. *p < .05; ns, not significant. PC, positive control; NC, negative control.

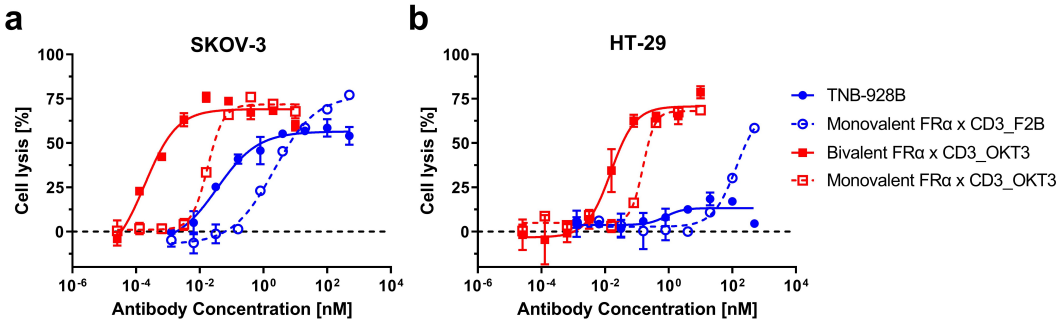


**Figure 3.** TNB-928B induces selective lysis of high FRα expressing tumor cells while sparing low FRα expressing cells. Co-culture cytotoxicity assay with T-cells from a healthy donor (E:T ratio of 10:1) showing cell lysis after 48 h of (a) SKOV-3 tumor cells as high FRα expressing tumor cells and (b) HT-29 as low FRα expressing cells.

cytokine release from several PBMC donors, aliquots of cell culture supernatant were collected from cytotoxicity assays and analyzed for IL-2 and IFNγ cytokine release. At concentrations of TNB-928B that mediate robust lysis of IGROV-1, SKOV-3, and OVCAR-3, the levels of IL-2 and IFNγ were lower than those induced by the OKT3 containing PC, irrespective of the donor (Figure 4). Furthermore, TNB-928B displayed no cytotoxicity nor IL-2 or IFNγ release when tested against the FRα-negative LNCaP cell line (Figure 4).

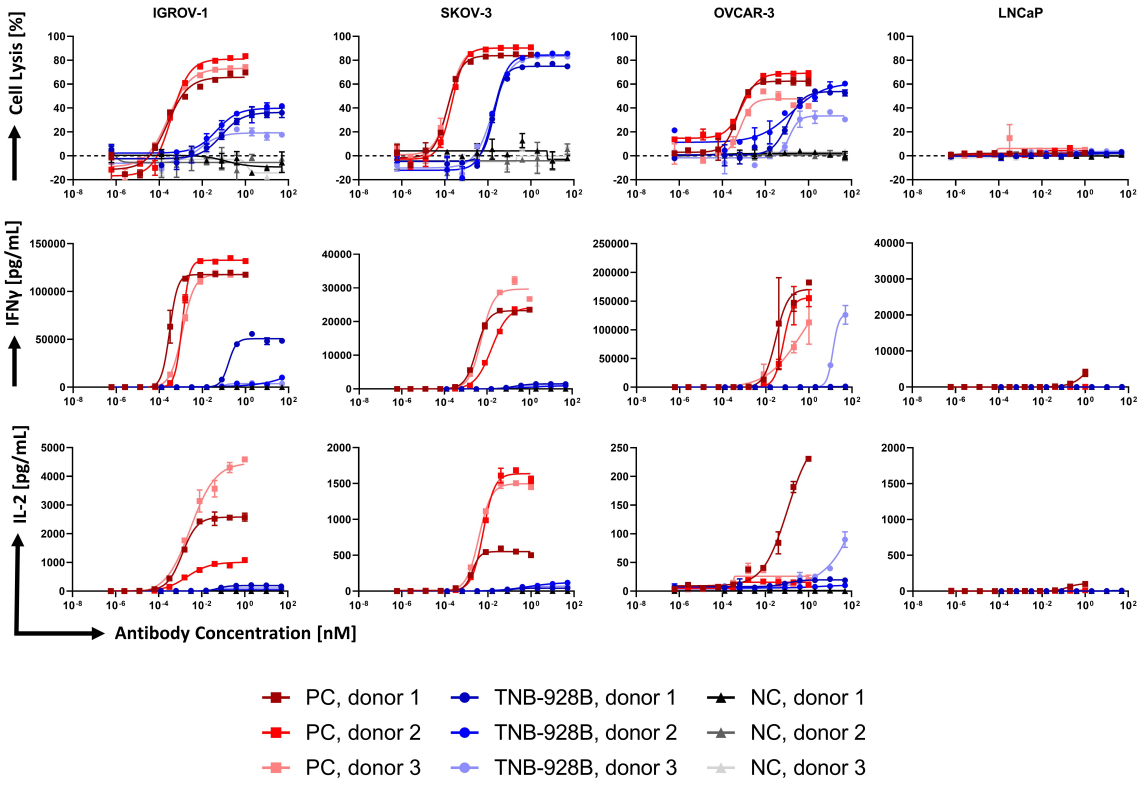


**Figure 4.** TNB-928B induced tumor cell lysis is accompanied by low cytokine release. Co-culture cytotoxicity assay with T-cells from three healthy donors (E:T ratio of 5:1) showing cell lysis after 48 h with IGROV-1, SKOV-3, and OVCAR-3 as high and medium FRα expressing tumor cells and LNCaP as FRα negative cells. IL-2 and IFNγ levels were measured from supernatants of the co-culture assay by MSD.

### *Activity of TNB-928B on* ex vivo *patient ovarian tumor samples*

We evaluated TNB-928B mediated cytotoxicity in patient-derived samples using fresh surgically removed ovarian tumor biopsies. *Ex vivo* ovarian tumor samples were incubated with TNB-928B, PC, or NC for 48–72 hours. TNB-928B mediated substantial *ex vivo* tumor cell lysis comparable to the PC in dissociated ovarian tumor samples from six of nine different patients (Figure 5a and Supplemental Table S3). No exogenous T-cells were added to these samples, and of the five samples that were immunophenotyped, four had E:T ratios <1, indicating that the few endogenous T-cells present in the tumor were sufficient for mediating tumor cell cytotoxicity. We measured FRα antigen density on seven of the nine patient samples (IHC expression identified in eight of nine patient samples; Supplemental Table S3) and found a trend between samples displaying <10% (non-responders) and >10% (responders) tumor lysis (Figure 5b). Two of the non-responders had FRα antigen densities $<1 \times 10^3$ (Supplemental Table S3) and therefore were not expected to show activity based on our *in vitro* results. In a representative dissociated ovarian tumor sample, the $EC_{50}$ of TNB-928B was 45.2 pM, consistent with our *in vitro* results (Figure 5c). Also, consistent with our *in vitro* cytotoxicity results, TNB-928B induced reduced levels of cytokine release as measured by IFNγ and IL-2 compared to the PC (Figure 5 d and e). TNB-928B mediated tumor cell lysis was accompanied by the release of cytotoxic granules, with levels of perforin and granzyme B in the supernatant comparable to the PC (Supplemental Figure S6A and B). Importantly, when dissociated ovarian tumor samples were incubated with patient-matched PBMCs at an E:T ratio of 1:1, TNB-928B induced approximately 2.5-fold less activation of Treg cells compared to the PC (figure 5f). These data suggest TNB-928B mediates robust ovarian tumor cell killing of primary patient tumor samples that express high levels of FRα, decouples cytotoxicity from cytokine release, and preferentially activates effector T-cells over Treg cells.

### In vivo *antitumor efficacy and pharmacokinetics (PK) of TNB-928B in murine models*

*In vivo* efficacy of TNB-928B was assessed in immunocompromised NCG mice implanted with IGROV-1 subcutaneously (*s.c.*) and resting PBMCs intraperitoneally (*i.p.*) in a prevention of tumor engraftment model. Mice were treated with TNB-928B administered intravenously (*i.v.*) every 3 d. TNB-928B displayed dose-dependent tumor growth inhibition (TGI) of 90.5%, 94.6%, or 96.4% with doses 1, 10, or 100 μg/animal, respectively (Figure 6a). In a separate study with activated PBMCs, both TNB-928B and the PC resulted in substantial anti-tumor activity of IGROV-1 and SKOV-3 tumors and human immune cell infiltration into IGROV-1 tumors as measured by IHC staining for human CD45 in tumors harvested at the end of the study (Figure 6b and Supplemental Figure S7A and B). Moreover, both TNB-928B and PC resulted in the elimination of nearly all FRα-

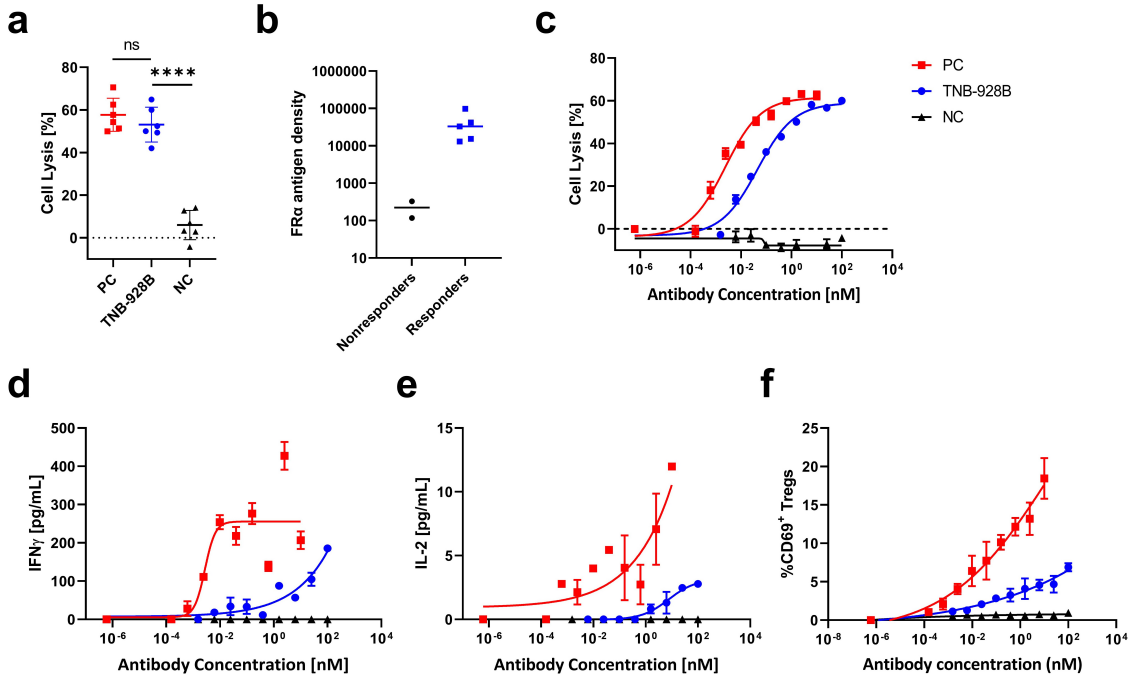


**Figure 5.** TNB-928B mediates tumor lysis and T-cell activity with patient-derived ovarian tumors. TNB-928B, PC, or NC were added to freshly dissociated ovarian carcinoma tissue and incubated without addition of exogenous PBMCs for 48 to 72 h. (a) Maximum cytotoxicity of ovarian carcinoma tissue derived cells from 6 different responding patients treated with 10 nM PC, 100 nM TNB-928B, or 100 nM NC. (b) Responders have higher expression of FRα than non-responders. (c) Representative dose curves of cytotoxicity as measured by LDH release, (d) IFNγ and (e) IL-2 release as measured by MSD. (f) $CD69^{+}$ Tregs were measured by flow cytometry following incubation for 72 h with patient matched PBMCs at an E:T ratio of 1:1. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; ns, not significant.

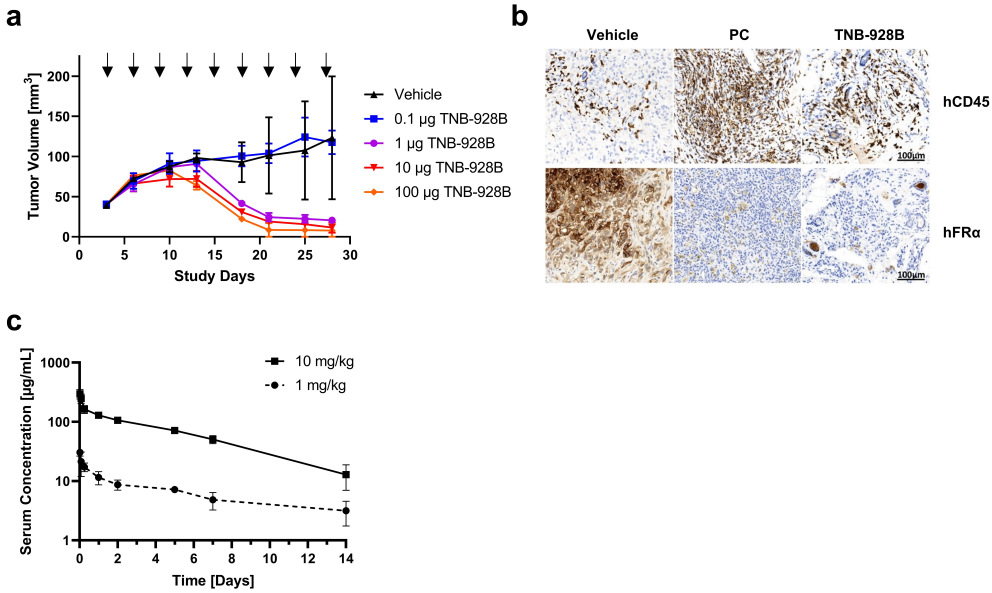


**Figure 6.** TNB-928B induces dose dependent anti-tumor activity in a NCG mouse xenograft model. (a) $5 \times 10^6$ IGROV-1 cells were injected *s.c.* and $10 \times 10^6$ resting hPBMCs were injected *i.p.* into NCG mice (n = 3 /group). Starting on day 3 post-implantation (pi), TNB-928B was injected *i.v.* at the respective doses (shown with downward arrows) every 3 d for a total of 9 doses. (b) A separate IGROV-1 study was performed with activated hPBMCs and mice were dosed with either 200 µg TNB-928B or 100 µg PC. Tumors were harvested on day 30 and stained by IHC with anti-human CD45 and anti-human FRα. Representative images are shown. (c) TNB-928B PK was evaluated in non-tumor bearing BALB/c mice at 1 or 10 mg/kg.

positive tumor cells by day 30 (Figure 6b). Taken together, these results suggest TNB-928B exhibits strong *in vivo* efficacy in multiple murine xenograft models.

The PK of TNB-928B was evaluated in BALB/c mice following a single tail vein injection at 1 and 10 mg/kg. TNB-928B clearance ranged from 7.9 to 10.3 mL/d/kg. The half-life ranged from 3.9 to 8 d for TNB-928B (Figure 6c). Since neither arm of TNB-928B cross-reacts with FRα or CD3 in rodent species, the observed linear PK was expected and is consistent with nonspecific clearance mechanisms. These

results suggest TNB-928B is stable *in vivo* with favorable PK and with a half-life similar to conventional antibodies.

## Discussion

FRα-targeted therapies for the treatment of solid tumors including ovarian have been explored with limited single-agent success in the clinic. Vintafolide, a folate–drug conjugate composed of a cytotoxic payload attached to folate via a linker, showed promising preclinical results but failed to improve PFS in a phase 3 trial in patients with platinum-resistant ovarian cancer.[35] A FRα targeted humanized IgG1 monoclonal antibody, farletuzumab, showed a promising overall response rate (ORR) of 75% in combination with carboplatin and a taxane in a phase 2 trial. However, in a randomized phase 3 trial, farletuzumab failed to meet the study's primary PFS endpoint.[19,36] Mirvetuximab soravtansine, an antibody–drug conjugate against FRα, showed single-agent activity in a phase 1 trial with an ORR of 26%; however, in a randomized phase 3 trial, it did not meet the primary endpoint of PFS except in a population of patients with high FRα expression.[20,37] Taken together, these hints of the efficacy of FRα targeted therapies in the clinic highlight the need for more effective ovarian cancer treatments and validate the use of FRα as a promising target in this population.

Bispecific TCEs are an emerging class of targeted immunotherapeutics capable of redirecting the cytotoxic activity of T-cells to kill cancer cells. As an alternative ovarian cancer target to FRα, a Mucin 16 TCE is being investigated in clinical trials.[38] An early attempt to target advanced ovarian cancer with a FRα TCE involved transfer of autologous peripheral T-cells activated with the FRα TCE *ex vivo* in combination with recombinant IL-2 and resulted in an ORR of 27% with complete responses lasting >1 y observed in 3 patients.[39] More recent FRα targeting TCEs have been described exhibiting robust preclinical activity, but these molecules displayed on-target off-tumor toxicity in NHP models related to FRα expression in the lung.[21,22] Lung toxicity from FRα targeting TCEs was surprising given the polarized expression of FRα toward the alveolar lumen, highlighting the need for safer FRα targeting TCEs to change the course of treatment available for ovarian cancer.[11,12,14] Current approaches for mitigating on-target off-tumor toxicity in TCEs broadly fall into two categories: an antibody format leveraging avidity-based binding for selective targeting of cells overexpressing the tumor associated antigen (TAA) or conditionally active antibody pro-drug formats consisting of a cleavable mask that prevents binding of the antibody until tumor-specific proteases cleave and activate either the TAA or CD3 arm, enabling efficient binding.[21,29,30,40,41]

To overcome the on-target off-tumor toxicity challenge associated with targeting FRα we developed TNB-928B, a novel bivalent TCE for selective lysis of tumors overexpressing FRα. Cells expressing low levels of FRα, such as healthy lung and kidney cells, are expected to engage TNB-928B with monovalent binding due to their low antigen density, whereas cells with high FRα antigen density, such as ovarian tumor cells, support sustained bivalent TNB-928B binding. Consistent with this, TNB-928B shows very low to no cytotoxicity on cell lines and *ex vivo* tumor samples with less than $8 \times 10^3$ copies of FRα on the cell surface. In contrast, TNB-928B is effective at inducing lysis of tumor cell lines and patient tumor samples with a broad range of high FRα antigen densities. Interestingly, the selectivity of TNB-928B is a result of both bivalent FRα binding and the unique anti-CD3_F2B, as the bivalent FRα binding arm paired with OKT3 does not show selectivity. Importantly, a monovalent FRα TCE does not show antigen density-dependent specificity and efficiently kills both high and low FRα density cell lines. In contrast to protease activated TCEs, TNB-928B is a simpler protein, which reduces the risk of immunogenicity.

In addition to selective tumor cell lysis, TNB-928B incorporates a low-affinity anti-CD3 to decouple cytotoxicity from cytokine release to further reduce the risk of CRS, which in severe cases can result in organ failure and death. An early TCE targeting FRα exhibited acute cytokine release in patients,[42] highlighting the importance of cytokine release mitigation. While reducing the incidence of CRS in the clinic by step-dosing and management of CRS by administering anti-IL6 antibodies and corticosteroids are effective in some settings,[43] next-generation TCEs with anti-CD3 binders optimized for low cytokine release show promising efficacy and safety targeting liquid tumors in the clinic without the need for such complex interventions.[34] TNB-928B exhibits similar levels of maximum tumor cell lysis as a PC containing OKT3 but with markedly lower levels of cytokines including IL-2 and IFNγ, both with tumor cell lines *in vitro* and patient-derived tumor cells *ex vivo*. An additional benefit of a TCE with a potentially favorable safety profile is that this enables combination therapies with the potential to further improve treatment of solid tumors. Several recent studies describe promising preclinical data combining TCEs with co-stimulatory molecules and immune checkpoint inhibitors.[44–47]

An additional challenge in the treatment of solid tumors with immunotherapy is overcoming the lack of T-cell infiltration in the tumor. In EOC, T-cell infiltration into the TME is associated with longer PFS and OS.[7] In the current study, IHC analysis of tumors harvested from the mouse xenograft model revealed TNB-928B induced substantial immune cell infiltration into the tumor. Interestingly, TNB-928B is effective at low E:T ratios, and furthermore, TNB-928B has promising cytotoxic activity in fresh *ex vivo* ovarian tumor samples that express high levels of FRα, particularly in high-grade serous carcinoma, in the absence of exogenous T-cells. Interestingly, TNB-928B did not have activity in a low-grade endometrial stromal sarcoma sample, which was negative for FRα by IHC. Furthermore, two *ex vivo* samples were positive for FRα by IHC but had very low FRα antigen density. While we cannot rule out the possibility that enzymatic digestion of the tumor samples may have removed FRα expressed on the cell surface, our cytotoxicity results are consistent with the observed antigen densities as both cytotoxicity and antigen density measurements were performed following digestion. Elevated levels of sFRα in the serum of ovarian cancer patients may present another challenge by competing with the binding of TNB-928B to tumor cells; however, *in vitro* tumor lysis activity of TNB-928B is not substantially impacted by the presence of sFRα at levels 100-fold higher than observed in the serum of

ovarian cancer patients.[31,33] Finally, while downregulation or loss of FRα expression could potentially be exploited by tumors as a potential resistance mechanism, partial tumor cell killing by TCEs has resulted in epitope spreading and secondary T-cell responses.[48,49] Further studies are required to determine if TNB-928B is capable of inducing FRα independent anti-tumor activity. Taken together, these results suggest that TNB-928B promotes T-cell infiltration into ovarian tumors, and this is sufficient for strong anti-tumor activity, even when the total number of available T-cells is low or in the presence of high levels of sFRα.

In summary, to mitigate potential on-target, off-tumor toxicity with a FRαxCD3 TCE, we developed TNB-928B for enhanced safety by selectivity targeting FRα expressing ovarian tumor cells while sparing normal tissue expressing low levels of FRα. The low cytokine release profile suggests TNB-928B could have less CRS than other TCEs. Ultimately, clinical studies will be required to fully characterize the therapeutic window of TNB-928B. Additionally, TNB-928B is anticipated to have a favorable dosing schedule due to a long half-life. In preclinical *in vitro, ex vivo*, and *in vivo* models, TNB-928B displays potent anti-tumor activity, strongly supporting the clinical development of TNB-928B for treatment of ovarian cancer.

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## Availability of data and materials

All data relevant to the study are included in the article or uploaded as supplementary information.

## Authors' contributions

B.C.A., K.P., P.D., N.D.T., and K.E.H. contributed to the study design, analysis of results, and manuscript preparation; NGS-based repertoire analysis was completed by B.C.A, A.A.B., N.D.T, and K.E.H.; molecular biology was conducted by B.C.A., A.B., and L.M.D.; expression, purification, and biophysical characterization of antibodies was performed by S. H., H.K.M.C., U.S., and H.S.U.; ELISA binding and flow cytometry assays were designed and run by B.C.A., K.P., and K.C.; *in vitro* cell-based assays were designed and conducted by K.P., H.K., K.C., V.K., U.S. R., and S.S.; fresh ovarian patient tumor samples were provided by W.P., G.P., and J.C.; *ex vivo* experiments were designed, conducted, and analyzed by K.P., P.D., and B.B.; IHC staining and evaluation was performed by R.D.Y. and K.L.W.; *in vivo* experiments were designed and analyzed by B.C.A., P.D., B.B., S.I., N.D.T. and K.E.H.; mouse PK studies were designed and analyzed by K.P., P.D., S.I., and V.K.; B.C.A., K.P., and P.D. prepared figures; B.B., S.I., U.S., R.B., N.D.T, and K.E. H. contributed to data analysis and critical review of the data; the manuscript was written by B.C.A. and K.E.H.; all authors read and approved the submitted version.

## Ethics approval and consent to participate

Human PBMCs were collected by STEMCELL Technologies and AllCells in accordance with scientific, ethical, and regulatory guidelines. Rat maintenance and immunizations were performed by Antibody Solutions (Sunnyvale, California, USA) with protocols reviewed by Institutional Animal Care and Use Committee (IACUC) boards. Mouse studies were reviewed and approved by the IACUC of CrownBio prior to execution, and studies were conducted in accordance with the regulations of the Association for Assessment and Accreditation of Animal Care.

## Disclosure statement

All authors except K.L.W., W.P., G.P., R.D.Y., and J.C. are current or former employees of Teneobio, Inc. with equity interests when the study was conducted and reported. B.C.A., K.P., P.D., S.H., A.B., A.A.B., L.M.D., V. K., K.L.W., H.K.M.C., U.S.R., S.S., H.S.U., R.D.Y., and K.E.H. are current or former employees of Amgen, Inc.

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