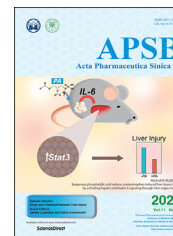




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ORIGINAL ARTICLE

# A reporter gene assay for determining the biological activity of therapeutic antibodies targeting TIGIT



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## KEY WORDS

TIGIT;  
Therapeutic antibodies;  
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Method validation

**Abstract** T cell immunoglobulin and ITIM domain (TIGIT) is a novel immune checkpoint that has been considered as a target in cancer immunotherapy. Current available bioassays for measuring the biological activity of therapeutic antibodies targeting TIGIT are restricted to mechanistic investigations because donor primary T cells are highly variable. Here, we designed a reporter gene assay comprising two cell lines, namely, CHO-CD112-CD3 scFv, which stably expresses CD112 (PVRL2, nectin-2) and a membrane-bound anti-CD3 single-chain fragment variable (scFv) as the target cell, and Jurkat-NFAT-TIGIT, which stably expresses TIGIT as well as the nuclear factor of activated T-cells (NFAT) response element-controlled luciferase gene, as the effector cell. The anti-CD3 scFv situated on the target cells activates Jurkat-NFAT-TIGIT cells through binding and crosslinking CD3 molecules of the effector cell, whereas interactions between CD112 and TIGIT prevent activation. The presence of anti-TIGIT mAbs disrupts their interaction, which in turn reverses the inactivation and luciferase expression. Optimization and validation studies have demonstrated that this assay is superior in terms of specificity, accuracy, linearity, and precision. In summary, this reliable and effective reporter gene assay may potentially be utilized in lot release control, stability assays, screening, and development of novel TIGIT-targeted therapeutic antibodies.

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## 1. Introduction

In the past 10 years, the first generation of immune checkpoint therapies (ICTs) for programmed cell death 1 (PD-1), programmed cell death ligand-1 (PD-L1), and cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) were developed. By 2018, James Allison and Tasuku Honjo received the Nobel Prize in Medicine for their discovery of cancer therapy by respectively inhibiting CTLA-4 and PD-1<sup>1,2</sup>. Despite this advancement, a large proportion of cancer patients did not benefit from anti-PD-1, PD-L1, or CTLA-4 antibodies, and there were also patients who did benefit from these treatments but eventually became refractory and their disease progressed<sup>3,4</sup>. Thus, it is imperative to develop a new generation of immune checkpoint inhibitors to expand the population benefited from immunotherapy and improved efficacy<sup>5</sup>. T cell immunoglobulin and ITIM domain (TIGIT), also known as Vsig9, Vstm3, or WUCAM, is an inhibitory receptor that is predominantly expressed by T and NK cells and discovered in 2009 by three independent groups using bioinformatics approaches<sup>6–8</sup>. TIGIT belongs to the family of poliovirus receptors (PVRs) that competes against CD226 (DNAM-1), which is the co-stimulatory receptor of T cells and NK cells, by binding to CD112 and CD155 (PVR) that are expressed on antigen-presenting cells (APCs) and various non-hematopoietic cell types, such as tumor cells<sup>9</sup>. The TIGIT/PVR axis imparts a co-inhibitory effect on both T and NK cells that lead to their exhaustion, in turn triggering a negative signal that suppresses their activities, resulting in tumor-immune evasion<sup>10,11</sup>. Preclinical tumor models have shown that blocking TIGIT reverses T and NK cell exhaustion, mediates anti-tumor immunity, and disrupts tumor growth<sup>12–16</sup>. Based on these findings, pharmaceutical companies have focused on the use of TIGIT-targeted antibodies for cancer treatment, either as monotherapy or in combination with anti-PD-1/PD-L1 mAbs<sup>17,18</sup>. Most recently, anti-TIGIT tiragolumab plus anti-PD-L1 atezolizumab as a first-line therapy for PD-L1-positive non-small cell lung cancer has been shown to provide superior clinical benefits relative to PD-L1 blockade alone, despite comparable toxicity profiles<sup>19</sup>. To confirm this finding, Genentech is currently conducting a registration-enabling trial consisting of 500 untreated patients with non-small cell lung cancer and PD-L1-high tumors. In addition, another phase 3 trial is assessing the same combination of anti-TIGIT tiragolumab and anti-PD-L1 atezolizumab for induction chemotherapy in 400 patients diagnosed with extensive small-cell lung cancer<sup>20</sup>.

Based on the highly heterogeneous structure and complicated structure–function correlation of protein therapeutics, the requirement for the control of biological activity has remained the same despite advances in physicochemical techniques. Biological activity is an essential parameter for testing in protein therapeutics, and it is also assessed in studies on stability, characterization, optimization, and process development<sup>21–23</sup>. Currently available bioassays used to investigate the biological activity of TIGIT-targeted protein therapeutics mainly utilize primary human T cells and examine functional endpoints, including T cell proliferation or cytotoxicity<sup>24–27</sup>, cytokine release test<sup>28</sup>, intracellular cytokine staining assay<sup>29,30</sup>, or profiling of mRNA expression levels using real-time PCR<sup>24</sup>. These cell-based assays are highly relevant to the elucidation of the mechanism of action of protein therapies targeting TIGIT, but these are mainly restricted to mechanistic studies instead of biological activity as these are largely complicated and time-consuming. In addition, these assays

are laborious and highly variable due to their reliance on donor primary T cells, complex assay protocols, and unqualified assay reagents. Thus, these assays are difficult to establish in a drug-development setting.

Here, we developed a reporter gene assay comprising two cell lines, namely, CHO-CD112-CD3 single-chain fragment variable (scFv), which stably expresses human CD112 as well as the membrane-bound anti-CD3 scFv as target cells, and Jurkat-nuclear factor of activated T-cells (NFAT)-TIGIT, which stably expresses human TIGIT as well as the luciferase gene that is controlled by NFAT response elements, as effector cells. The anti-CD3 scFv on target cells activates Jurkat-NFAT-TIGIT cells by binding to and crosslinking with CD3 molecules on the effector cell, whereas CD112 and TIGIT interactions disrupt activation. The presence of anti-TIGIT mAbs could prevent their interaction, thereby reversing the inactivation, as well as luciferase expression (Fig. 1). After optimization and validation, the luciferase reporter assay shows good performance characteristics such as specificity, accuracy, precision, and linearity, thereby allowing its application to lot release and stability testing as well as characterization and development of novel protein therapeutics that target TIGIT.

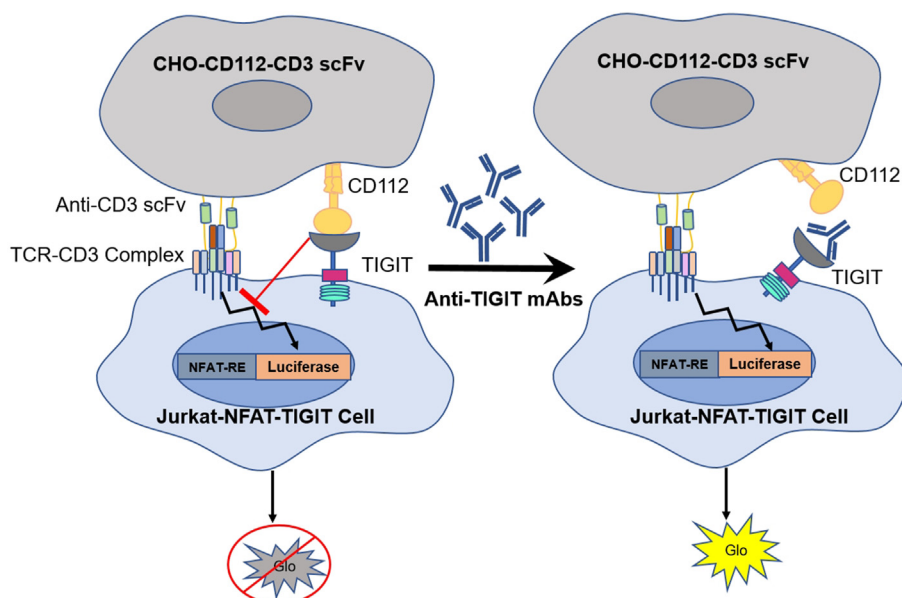
## 2. Methods

### 2.1. Cell lines and reagents

Jurkat cells and CHO-K1 cells were obtained from ATCC (Gaithersburg, MD, USA). The Jurkat cell line was cultured in RPMI-1640 medium that was supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (P/S). CHO cells were cultured in F-12 medium containing 10% FBS and P/S. Hygromycin B was obtained from Sigma (Saint Louis, MS, USA), puromycin and trypsin–EDTA were from Thermo Fisher Scientific (Waltham, MA, USA), and the stain buffer from BD Biosciences (San Jose, CA, USA). One-Glo Luciferase Assay System (Cat. No. E6130) and NFAT-Luc plasmid (Cat. No. E848A) were purchased from Promega (Madison, WI, USA). Purified anti-human CD3 (clone: OKT3, Cat. No. 317302) was purchased from Biolegend (San Diego, CA, USA). PE mouse anti-human CD112 antibody (Cat. No. 551057) was purchased from BD Biosciences. PE mouse anti-human TIGIT antibody (Cat. No. 12-9500-41) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). PE goat anti-mouse IgG antibody (Cat. No. abs20007) was purchased from Absin Bioscience (Shanghai, China).

### 2.2. Vector construction

The NFAT-Luc fragment was PCR amplified, digested using BamHI and PmeI, then cloned into multiple cloning sites of the CAGa plasmid with hygromycin B resistance, which was constructed from pPB-CAG-empty-pgk-hph vector (Addgene, Waltham, Massachusetts, USA). The anti-CD3 scFv fragment designed based on the anti-CD3 monoclonal antibody (OKT3) was PCR amplified and digested by NheI and SbfI, then cloned into multiple cloning sites of the CAGa plasmid. The human TIGIT fragment and human CD112 fragment were amplified by PCR and digested by NheI and SbfI, then cloned into the multiple cloning site of HXP plasmid with puromycin resistance, which was constructed from pLB2 CAG P2Gm vector (Addgene, Waltham, MA, USA).



**Figure 1** Schematic of a novel reporter gene assay for assessing the biological activity of TIGIT-targeted therapeutic antibodies. The reporter gene assay consists of two genetically engineered cell lines, namely, CHO-CD112-CD3 scFv cell line and Jurkat-NFAT-TIGIT cell line. Membrane-anchored anti-CD3 scFv and human CD112 were stably transfected into CHO cells, while human TIGIT and the luciferase gene, which is under the control of NFAT response elements, were stably transfected into Jurkat cells. During co-culture, anti-CD3 scFv of CHO cells crosslink and activate CD3 of Jurkat cells, inducing the expression of NFAT-controlled luciferase, whereas CD112 of CHO cells interacts with TIGIT of Jurkat cells, disrupting luciferase expression. The addition of anti-TIGIT mAb resulted in the reversal of the inhibition, and strong fluorescence was detected in a dose-dependent manner.

### 2.3. Generation of Jurkat-NFAT-TIGIT cell line

The plasmid CAGa-NFAT-Luc was introduced into Jurkat cells using electroporation, followed by selection with hygromycin B (400  $\mu\text{g}/\text{mL}$ ) to obtain a stable cell pool, then stable monoclonal cell lines were obtained by limited dilution. High-responsive clones of luciferase were screened by co-incubating cells with OKT3 for 6 h in a  $\text{CO}_2$  incubator, and luminescence signal was determined following the manufacturer's instructions. After that, one positive clone, named as the Jurkat-NFAT-D9 cell line, was selected and routinely cultured in selective media. Then, the plasmid HXP-hTIGIT was introduced into Jurkat-NFAT-D9 cells using electroporation, followed by selection with puromycin (1  $\mu\text{g}/\text{mL}$ ) to obtain a stable cell pool, and stable monoclonal cell lines were obtained by limited dilution. TIGIT expression on the surface of cell lines was determined using flow cytometry after labeling with PE mouse anti-human TIGIT antibody. After that, three positive clones were selected and named as Jurkat-NFAT-D9-TIGIT-3E7, Jurkat-NFAT-D9-TIGIT-3C9, and Jurkat-NFAT-D9-TIGIT-1D6.

### 2.4. Generation of CHO-CD112-CD3 scFv cell line

The HXP-CD112 and CAGa-CD3 scFv plasmids were mixed and then introduced into CHO-K1 cells by electroporation, followed by selection using puromycin (1  $\mu\text{g}/\text{mL}$ ) and hygromycin B (400  $\mu\text{g}/\text{mL}$ ) to generate a stable cell pool, then stable monoclonal cell lines were prepared by limited dilution. Then, the selected positive stable cells were pelleted and seeded into a 96-well plate at a density of 50,000 cells in 100  $\mu\text{L}$  of the assay medium (RPMI-1640 medium with 2% FBS) per well, and incubated at 37  $^\circ\text{C}$  with

5%  $\text{CO}_2$  overnight (16–24 h). Later, 100  $\mu\text{g}/\text{mL}$  of the TIGIT-targeted antibody in RPMI-1640 medium supplemented with 2% FBS was added into each well in the plate, and then Jurkat-NFAT-D9-TIGIT-3E7 cells were added into the plate at 50,000 cells per well and incubated for 6 h. Finally, 50  $\mu\text{L}$  per well of One-Glo Luciferase Assay reagent was added into the plate, and the positive clone cell was finally selected by relative luciferase units (RLU) signal scored by TECAN M1000 Pro plate reader. The positive clone CHO-CD112-CD3 scFv-1B5 that showed upregulated expression of CD112 and CD3 scFv on its cell surface was confirmed by flow cytometry after labeling with PE mouse anti-human CD112 and PE goat anti-mouse IgG antibodies, respectively.

### 2.5. Flow cytometry

Cells were dissociated using trypsin–EDTA, washed twice with cold stain buffer, and collected by centrifugation at 200 $\times g$  for 5 min followed by resuspension in stain buffer. PE mouse anti-human CD112, PE goat anti-mouse IgG, or PE mouse anti-human TIGIT antibody was diluted following the manufacturer's protocol, and then cells were incubated with the antibodies on ice for 30–45 min in the dark. Cells were then washed twice using stain buffer followed by resuspension in the same buffer for FACS analysis. Relative fluorescence units (RFU) signal was measured by FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA).

### 2.6. Reporter gene assay

The CHO-CD112-CD3 scFv cells were harvested and seeded into a 96-well assay plate with 50,000 cells in 100  $\mu\text{L}$  of the assay

medium (RPMI-1640 medium with 2% FBS) per well, followed by incubation at 37 °C with 5% CO<sub>2</sub> overnight (16–24 h). Then, the TIGIT-targeted antibodies were serially diluted at a ratio of 1:3 in assay medium (RPMI-1640 medium with 2% FBS) at a starting concentration of 30 µg/mL. After removing the media, the serially diluted antibodies were added into each well in the plate, then Jurkat-NFAT-TIGIT cell were added into the plate at 100,000 cells per well. After that, the plate was incubated at 37 °C in 5% CO<sub>2</sub> for 4–7 h. Then, One-Glo Luciferase Assay reagent (50 µL per well) was added into the plate. Relative luciferase units (RLU) were then scored using a plate reader (SpectraMax M5 or Tecan M1000 Pro).

### 2.7. Preparation of stressed anti-TIGIT mAb

Anti-TIGIT mAb was heat-stressed at 55 °C in an incubator for 0, 4, 7, 11, 14 days or stressed with strong base (pH 11.0) for 0, 2, 4, 6, 8 days. Then stressed samples were stored at 2–8 °C before analysis. The bioactivity of the stressed samples was determined with the RGA against the untreated counterpart.

### 2.8. Specificity

Here, specificity pertains to the ability of the assay to distinguish the analyte of interest from other unrelated analytes. Antibodies targeting other checkpoint members such as PD-1, PD-L1, BTLA, CTLA-4, and CD112R (PVRIG) were selected to evaluate the specificity of this assay. In addition, denatured TIGIT-targeted antibodies were also assessed. Denatured TIGIT-targeted antibodies were obtained by incubating native antibodies in a solution containing 0.06 mol/L dithiothreitol and 6.0 mol/L guanidine hydrochloride at 37 °C for 1 h. Sodium iodoacetate was then added into the solution, then incubated at room temperature for 45 min. A desalting column was used to remove small molecular components in the solution. All of the antibodies were serially diluted at a ratio of 1:3 in assay medium (RPMI-1640 medium with 2% FBS) at a starting concentration of 30 µg/mL, followed by the assay procedure.

### 2.9. Accuracy, linearity, and precision

Five potency levels, namely, 50%, 75%, 100%, 125%, and 150%, were used to validate linearity and accuracy, and the corresponding working concentrations of the anti-TIGIT mAb were 15, 22.5, 30, 37.5, and 45 µg/mL, and another independent 30 µg/mL was used as in-house reference. Each sample concentration was evaluated in this assay for four times at various dates. To evaluate inter-assay precision, each sample was assessed five times every single day on three different days. To evaluate the inter-analyst precision, three different analysts in one laboratory ran five repeat analyses of the same sample. For assessment of reproducibility, 15 runs of data analysis of the same sample was performed by two different laboratories.

### 2.10. Statistical analysis

We employed a four-parameter model to fit a dose–response curve that depicts the correlation between RLU and antibody concentration that was expressed as log<sub>10</sub>. EC<sub>50</sub> was estimated based on the fitting curve. The relative potency values were computed from the EC<sub>50</sub> ratio of the reference standard to the sample. Statistical techniques for method validation

involved the coefficient of variation (CV), recovery rate, and ordinary one-way ANOVA test. A *P*-value <0.05 was regarded as statistically significant. Analyses were accomplished using GraphPad Prism® software (San Diego, CA, USA).

## 3. Results

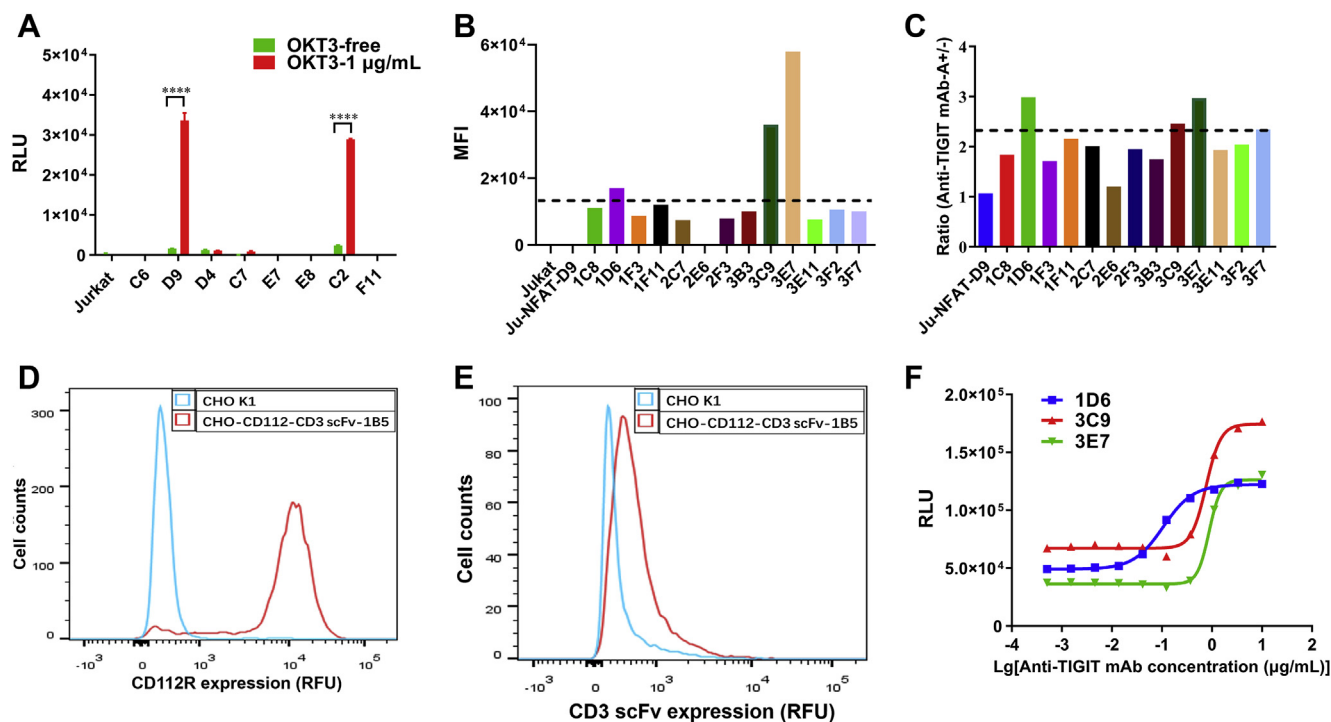
### 3.1. Generation of Jurkat-NFAT-TIGIT and CHO-CD112-CD3 scFv cell lines

To develop a cell-based assay to assess the biological activity of the anti-human TIGIT mAb, two stable cell lines were prepared. One was the Jurkat-NFAT-TIGIT cell line that stably expresses human TIGIT as well as the luciferase gene, which under the control of NFAT response elements. The second cell line was CHO-CD112-CD3 scFv, which stably expresses human CD112 as well as membrane-anchored anti-CD3 scFv. Anti-CD3 scFv, which is situated on the CHO cell membrane, generates an activation signal to Jurkat cells once it binds to CD3 located on the surface of Jurkat cells. Previous studies have shown that transfection of scFv that targets CD3 in tumor cells triggers strong T-cell activation, and thus we selected anti-CD3 scFv that was derived from an OKT-3 antibody to simulate the indirect activation by circumventing the interaction between T cell receptors (TCR) and tumor cell major histocompatibility complex (MHC)-peptide complexes<sup>31,32</sup>. The NFAT response elements were selected to induce luciferase expression as it has been extensively studied and validated to be activated by the CD3 pathway<sup>33–35</sup>.

For the establishment of Jurkat-NFAT-TIGIT and CHO-CD112-CD3 scFv cell lines, Jurkat-NFAT-TIGIT target cells were constructed by introducing the CAGa NFAT-Luc plasmid and HXP hTIGIT plasmid sequentially into Jurkat cells. The CAGa NFAT-Luc plasmid was introduced into Jurkat cells and then screened for luciferase expression. The stable cell clone (Jurkat-NFAT-D9) with the highest NFAT-luc expression was selected for subsequent construction (Fig. 2A). Similarly, HXP TIGIT plasmid was electrotransfected into Jurkat-NFTA-D9 cells. Different cell clones were assessed by FACS for TIGIT expression on the cell surface, and the top three clones (1D6, 3C9, and 3E7) were used in subsequent bioactivity screening (Fig. 2B). Also, after the development of stable cell line CHO-CD112-CD3 scFv-1B5, the high RLU ratio of with anti-TIGIT mAb/without anti-TIGIT mAb confirmed TIGIT expression on the cell surface of these three positive clones (Fig. 2C).

CHO-CD112-CD3 scFv target cells were generated by co-transfection of the HXP-CD112 and CAGa-CD3 scFv plasmids into CHO-K1 cells by electrotransfection, and then selection with puromycin and hygromycin B was performed to identify the positive clone CHO-CD112-CD3 scFv-1B5. Then, flow cytometry analysis were performed to confirm the ectopic expression of CD112 (Fig. 2D) and anti-CD3 scFv (Fig. 2E) on the cell surface of the stable cell line CHO-CD112-CD3 scFv-1B5. Finally, three clones showing upregulated TIGIT expression (3C9, 1D6, and 3E7) were selected to test the performance of the dose–response curve using the reporter gene assay. The 3C9 and 1D6 cell lines showed high background and relative narrow signal to noise window, and thus the stable cell clone 3E7 was selected for reporter gene assay optimization and validation (Fig. 2F).





**Figure 2** Development of Jurkat-NFAT-TIGIT and CHO-CD112-CD3 scFv stable cell lines. After electrotransfecting the CAGA NFAT-Luc plasmid into Jurkat cells, different cell clones were analyzed by luminescence signal determination for NFAT-Luc expression by co-incubating different clones with OKT3, and the stable cell clone (namely, Jurkat-NFAT-D9) showed the highest NFAT-luc expression (A). After electrotransfection of the HXP-hTIGIT plasmid into Jurkat-NFTA-D9 cells, various cell clones were analyzed by FACS for TIGIT expression, with the top three clones (3C9, 1D6, and 3E7) exhibiting the highest TIGIT expression (B). RLU ratio of with anti-TIGIT mAb/without anti-TIGIT mAb (+/-) was determined to confirm TIGIT expression on the cell surface of the three positive clones (3C9, 1D6, and 3E7) (C). After co-transfecting the HXP-CD112 and CAGA-CD3 scFv plasmids into CHO-K1 cells, the selected positive clone CHO-CD112-CD3 scFv-1B5 was analyzed by FACS for the upregulated expression of CD112 (D) and anti-CD3 scFv (E). Dose–response curve performance was assessed using the reporter gene assay with three different positive clones highly expressing TIGIT (3C9, 1D6, and 3E7) (F).

### 3.2. Reporter gene assay optimization

To determine the optimal anti-TIGIT mAb dose–response curve of this reporter gene assay, several critical parameters, including effector cell/target cell ratio, working concentration of FBS, and incubation time, were examined. To determine the optimal ratio of Jurkat-NFAT-TIGIT/CHO-CD112-CD3 scFv cells, anti-TIGIT mAb was diluted three-fold at a starting concentration of 30 μg/mL, and the Jurkat-NFAT-TIGIT/CHO-CD112-CD3 scFv cell ratios were set to 1:1, 2:1, 5:1, and 10:1. The results demonstrate that the lower asymptotes of the dose–response curve increased with the increasing of effector cell/target cell ratio, while the signal/noise ratio did not show a positive correlation. Meanwhile, effector cell/target cell ratios of 1:5 and 1:10 showed no sufficient points on the slope and poorly dose–response curve, and the effector cell/target cell ratio of 1:1 showed a narrow signal/noise ratio. Thus the optimal effector cell/target cell ratio was finally determined to be 1:2 and was used in the subsequent experiments (Fig. 3A).

No significant difference among the four working concentrations of FBS (1%, 2%, 5%, and 10%) was observed, so thus we selected the routine 2% working concentration of FBS for this reporter gene assay (Fig. 3B). Furthermore, 5 different incubation times (4, 5, 6, 7, and 24 h) were investigated. The performance of dose–response curve showed no significant difference when the incubation time was set at the 5, 6, and 7 h, while incubation time of 4 or 24 h showed significantly poor signal value or narrow

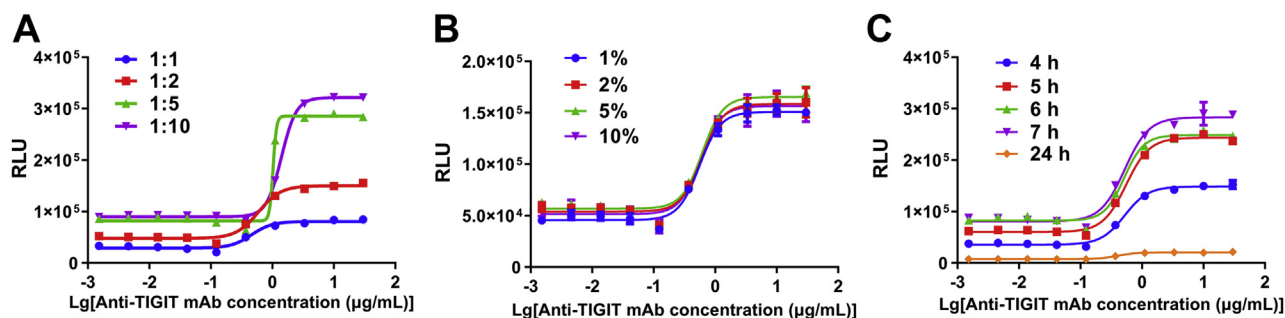
signal/noise ratio, so the optimal incubation time was determined to be 4–7 h (Fig. 3C). Finally, the initial working concentration of anti-TIGIT mAb in the plate and serial dilution ratio was determined to be 30 μg/mL and 3-fold, respectively (data not shown). The optimized conditions of all of the subsequent experiments are summarized in Table 1.

### 3.3. Validation of reporter gene assay

After optimization of the critical parameters described above, we validated the reporter gene assay following the International Conference on Harmonization Q2 (R1) guidelines, including evaluation of specificity, accuracy, precision, linearity, and stability of cell lines.

#### 3.3.1. Specificity

In this reporter gene assay, specificity described the ability of this assay to assess anti-TIGIT antibodies unequivocally when all expected components, including excipients and salts or contaminated related antibody therapeutics, are present. As expected, native but not the denatured anti-TIGIT mAb restored luciferase activation, with an EC<sub>50</sub> of about 0.5 μg/mL. Additionally, other immune checkpoints targeted antibodies, including anti-PD-1 mAb, anti-PVRIG mAb, anti-PD-L1 mAb, anti-CTLA-4 mAb, and anti-BTLA mAb, were incapable of restoring luciferase activation with the dose–response curve



**Figure 3** Optimization of the conditions of the reporter gene assay. Optimization of the effector cell/target cell ratio and the dose–responsive curve using various effector cell/target cell ratios (1:1, 2:1, 5:1, and 10:1) (A). Optimized working FBS concentration and dose–responsive curve are shown using various FBS concentrations (range: 1%–10%) (B). Incubation time optimization, after 100,000 effector cells per well and the serially diluted antibodies were added into each well that was pre-seeded with 50,000 target cells; RLU was measured, and the dose–responsive curve using different incubation times from 4 to 24 h are shown (C).

**Table 1** Optimized parameters of the cell-based gene reporter assay.

Parameter	Value
Target cell seeding density	50,000 cells/well
Effector cell seeding density	100,000 cells/well
Initial concentration of anti-TIGIT mAb	30 $\mu\text{g}/\text{mL}$
Dilution ratio	1:3
Working concentration of FBS	2%
Incubation time	4–7 h

(Fig. 4A). We expected that the reporter gene assay we developed could assess all mAbs targeting TIGIT. To this end, anti-TIGIT mAb A and other two similar anti-TIGIT mAbs provided by two manufacturers were assessed in parallel in one plate, and all three anti-TIGIT mAbs exhibited good dose-dependent response (Fig. 4B). Hence, this reporter gene assay was determined to be specific in measuring the biological activity of anti-TIGIT antibodies.

In addition, forced degradation investigations of therapeutic proteins are often employed to demonstrate and validate the specificity of stability-indicating methods, which contributes to the development of therapeutic proteins. To this end, the relative potency changes of the anti-TIGIT mAb exposed to high temperature (55 °C) for 14 days or strong base (pH 11) for 8 days were analyzed. Fig. 4C and D show that the relative potency of the anti-TIGIT mAb measured by this reporter gene assay gradually decreased over time in the presence of forced degradation conditions. Specifically, the relative potency of anti-TIGIT mAbs decreased to ~20% after 14 days of exposure to high temperature and was reduced to ~60% after 8 days in the presence of a strong base. These results hence depict that our developed assay is highly specific to the anti-TIGIT antibody and may be potentially be developed as a stability-indicating method.

### 3.3.2. Accuracy and linearity

In this reporter gene assay, we determined accuracy by recovery, which reflects concordance between measured and expected values, while linearity pertains to its ability to generate measured values that are directly correlated to the expected values. To assess relative potency in this investigation, five potency levels with various starting reference concentrations (50%, 70%, 100%,

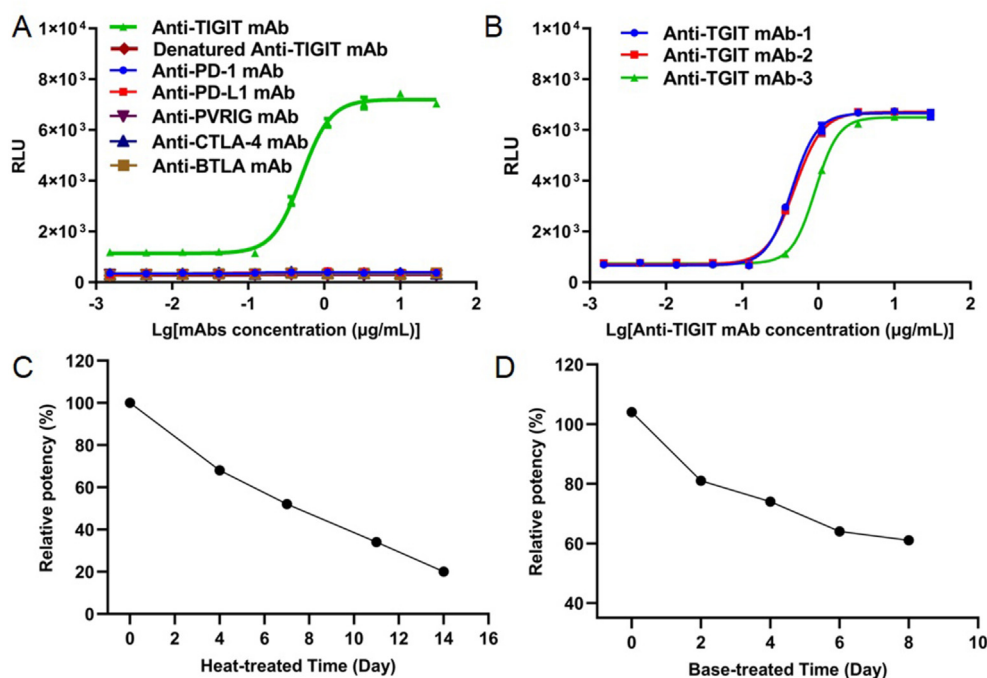
130%, and 150%) for anti-TIGIT mAb were employed. Using the sample with 100% potency as reference, each sample concentration was tested in this experiment four times by two analysts on different days. Table 2 shows a recovery between 96.2% and 109.2%, while CV was <8% for each potency level. Fig. 5A shows concordance between measured and expected values, implying sufficient linearity of the measurements. Taken together, these findings demonstrate that our established reporter gene assay has excellent accuracy and linearity.

### 3.3.3. Precision

Precision assessment was performed at three levels, namely, inter-assay CV (day-to-day variations), inter-analyst CV (analyst-to-analyst variations), and reproducibility (lab-to-lab variations). On three specific days, each sample was analyzed five times each single day to determine inter-assay precision. Fig. 5B shows that there was no significant difference by ordinary one-way ANOVA test ( $P = 0.4018$ ), and that inter-assay CV was <5.2%. To evaluate the inter-analyst precision, three different analysts in one laboratory analyzed each sample for five times. Fig. 5C shows that there was no significant difference by ordinary one-way ANOVA test ( $P = 0.4894$ ), and that inter-assay CV was <5.4%. For reproducibility assessment, 15 repeated analysis data of the same sample from two laboratories were analyzed, and no significant difference was shown by unpaired t test ( $P = 0.2606$ ), and inter-assay CV was 4.7% (Fig. 5D). These findings indicate that the reporter gene assay we developed had excellent precision.

### 3.4. Assessment of cell line stability

Jurkat-NFAT-TIGIT and CHO-CD112-CD3 scFv cell line stability influences assay robustness and consistency. Various passages of the CHO-CD112-CD3 scFv and Jurkat-NFAT-TIGIT cells were simultaneously assessed in terms of stability. Table 3 shows the similarity of signal to noise ratio (between 3.5 and 4.7) and relative potency (between 97.0% and 111.4%) between different passages of the cell line up to passage 30, indicating that these cell lines were relatively stable. In addition, the dose–response curve of this assay using various passages revealed a similar and excellent slope efficiency ratio (range: 0.9 and 1.2) and  $R^2$  ( $r$ -squared, >0.99). These cell line stability tests revealed that our reporter gene assay is highly robust and repeatable.



**Figure 4** Specificity of the reporter gene assay. Dose–response curve of the native and denatured anti-TIGIT mAb and immune checkpoint antibodies as indicated (A). Dose–response curve of three different anti-TIGIT mAbs as indicated (B). The relative potency changes in anti-TIGIT mAbs exposed to high temperature (55 °C) for 14 days (C) and strong base (pH11.0) for 8 days (D).

**Table 2** Results of accuracy and precision validation.

Expected potency (%)	Mean potency (%)	Recovery (%)	Intermediate precision (CV, %)
50	54.6	109.2	7.7
75	74.6	99.4	7.9
100	101.1	101.1	3.3
125	123.9	99.1	2.7
150	144.3	96.2	3.0

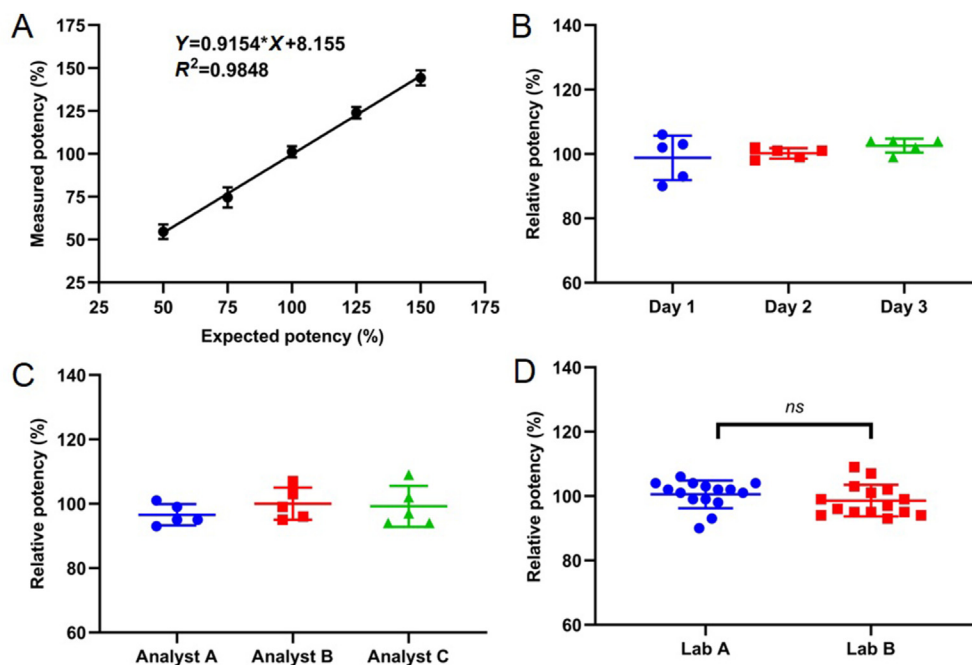
CV, coefficient of variation.

#### 4. Discussion

T cell stimulation requires binding to the TCR/CD3 complex, which in turn triggers a cascade of signal transduction events, inducing the activation of T cells, and eliciting an immune response. Furthermore, a costimulatory signal is necessary to initiate cytotoxic activity, which is usually generated by the interaction between CD28 molecules on the T cell surface with members of the B7 ligand family situated on the surface of APCs. The TCR/CD3 signal transduction pathway stimulates three nuclear transcription factors, namely, NFAT, nuclear factor kappa B, and activator protein 1. Following activation, NFAT is translocated to the nucleus and induces the expression of various cytokines such as interleukin 2. In addition, NFAT in the nucleus also interacts with other transcription factors that promotes T cell differentiation, proliferation, and survival, thereby contributing to tumor cell elimination<sup>36</sup>. Several earlier studies have reported that transfection of scFv targeting to CD3 into tumor cells could induce strong T-cell activation that mimics the process of T cell activation<sup>31,32,37</sup>. The present study engineered CHO cells as target cells that constitutively express the membrane form of scFv that was derived from OKT3, which is a murine mAb, targeting

the CD3 antigen of human T cells. TIGIT comprises a short intracellular domain with a single immunoreceptor tyrosine-based inhibitory motif (ITIM) and a immunoglobulin tyrosine tail (ITT)-like motif. After binding to ligand CD155 or CD112, the ITT-like motif or ITIM motif is phosphorylated and then recruits SH2-containing inositol phosphatase-1, resulting in the inhibition of the TCR/CD3 signal transduction pathway<sup>17</sup>. Thus, NFAT-driven luciferase expression can reflect the activation status of Jurkat T cells. Here, we established a Jurkat-NFAT-TIGIT cell line as effector cells that stably expresses the luciferase gene under the control of the NFAT response elements.

The development of bioassays for therapeutics, particularly those involving proteins that are heterogeneous in structure, entails the elucidation of a mechanism of action<sup>21,22</sup>. TIGIT binds to at least two ligands, CD155 and CD112<sup>17</sup>. In the study reported by Yu et al., although poor curve fitting in the radioligand assay, they did find specific binding of CD112 to TIGIT<sup>6</sup>. Just recently, the comprehensive structural and biophysical analysis of the TIGIT/CD112 interaction, especially the crystal structure of TIGIT bound to CD112 determined by X-ray with resolution of 3.1 Å, as well as its detailed molecular interactions has been reported by Deuss et al.<sup>38</sup>. Firstly, SEC-MALS data in this study suggested that the affinity of TIGIT for CD112 was relatively high. Secondly, to formally assess the strength of the TIGIT/CD112 interaction, they performed direct binding studies using surface plasmon resonance. The results showed that CD112 bound robustly to TIGIT with an apparent affinity (KD (app)) of 5.8 μmol/L. Although this affinity was only slightly lower than that observed for the binding of TIGIT to CD155 (3.2 μmol/L), it was at the higher end of the range typically observed for NK cell receptor/ligand interactions, suggesting that CD112 represents a *bona fide* TIGIT ligand. Thirdly, after overlay of TIGIT/CD112 and TIGIT/CD155 structures, they found that the vast majority of the structural elements at the interface overlaid closely, suggesting



**Figure 5** Accuracy, linearity and precision of the reporter gene assay. The closeness of agreement of the measured and expected values, with each point representing the average of six replicates (A). On three different days, analysis of the same sample for five times was repeated each single day under the same operating conditions to validate its precision (B). Three analysts repeated this reporter gene assay of the same sample five times under the same operating conditions to validate its precision (C). Fifteen repeated analysis data of the same sample from two laboratories were analyzed to validate its reproducibility (D). The mean  $\pm$  SD from 5 or 15 independent experiments is shown in the curves. *ns*, not significant.

**Table 3** Stability performance of the RGA cell line at different passage.

Cell passage	<i>S/N</i>		Slope efficiency ratio	<i>R</i> <sup>2</sup>		<i>EC</i> <sub>50</sub> (ng/mL)		Relative potency (%)
	RS	Sample		RS	Sample	RS	Sample	
P3	4.7	4.5	1.2	0.996	0.995	350.1	334.6	104.6
P8	3.5	3.5	0.9	0.996	0.995	473.8	488.6	97.0
P18	4.0	4.0	1.2	0.994	0.994	416.9	374.1	111.4
P28	3.9	3.9	1.0	0.995	0.991	302.5	310.1	97.6
P30	4.4	4.4	1.0	0.995	0.994	577.3	553.5	104.3

RGA, reporter gene assay; *S/N*, ration of signal to noise; RS, reference standard of anti-TIGIT mAb.

CD112 can compete with CD155 to binding TIGIT. Moreover, similar to CD155, CD112 is expressed on APCs and various non-hematopoietic cell types including tumor cells of different histological types<sup>9,17</sup>. Furthermore, the binding of CD112 to TIGIT results in the phosphorylation of Tyr225 within the ITT-like motif of TIGIT, which triggers intracellular signaling cascades that serve to limit NK cell activation<sup>39</sup>. Therefore, it is reasonable to select ligand CD112 in this reporter gene assay, which can completely represent the mechanism of action of TIGIT blockade. To mimic tumor cell immune evasion status, we developed CHO-CD112-CD3 scFv and Jurkat-NFAT-TIGIT cell lines that stably express CD112 and TIGIT, respectively. Anti-CD3 scFv on target cells binds to and crosslinks with CD3 molecules on effector Jurkat-NFAT-TIGIT cells, thereby resulting in the activation of the cells, whereas interactions between CD112 and TIGIT disrupts this activation. As expected, the addition of anti-TIGIT mAbs blocks their interaction, which in turn reverses the inactivation and luciferase expression. Therefore, the reporter gene assay that we developed can experimentally demonstrate the *in vivo*

mechanism of action of anti-TIGIT mAbs. The recent studies also developed several reporter gene assays using genetically engineered Jurkat T cells with luciferase reporter expression that was driven by an NFAT-response element for the assessment of the bioactivity of various immune checkpoint antibodies such as anti-PD-1/anti-PD-L1 mAbs and anti-LAG3 mAbs<sup>35,40,41</sup>. Hence, all of these investigations provide good reference for establishing methods of bioactivity determination in other immune checkpoint antibodies.

This study constructed CHO cells with stable expression of CD112 and membrane-bound anti-CD3 scFv, and Jurkat T cells with the expression of TIGIT and NFAT-Luc luciferase reporter gene (Fig. 2). Jurkat-NFAT-TIGIT was used as the effector cell and CHO-CD112-CD3 scFv as the target cell for the biological activity determination. After optimizing the ration of the effective cell and the target cell and incubation time, this reporter gene assay demonstrated full dose–response curve with complete upper and lower asymptotes, satisfied signal to noise ratio, sufficient points on the slope, and other appropriate characteristics (Fig. 3).



To apply this bioassay to routine anti-TIGIT mAb analysis, we optimized its conditions and validated our complete methodology based on the ICH-Q2. Our results showed that this developed reporter gene assay possesses excellent specificity, linearity, accuracy, and precision. A number of points should be noted in this study. First, this bioassay is highly relevant and specific to anti-TIGIT mAbs because the biological activity of the samples treated under stressed conditions markedly decreased, and the denatured sample exhibited complete inactivation (Fig. 4). We hypothesize that deamidation or isomerization of the asparagine residue in the anti-TIGIT mAb CDR region could have occurred, which reduced the interaction with human TIGIT. Second, this novel bioassay is highly reliable and accurate in measuring the biological activity, as revealed by the good linear dose–response correlation curve within the concentration range of 50%–150% with a high recovery rate (Fig. 5A). Third, this bioassay has excellent precision, which was demonstrated by low coefficients of variation within the concentration range of 50%–150%, minimal day-to-day and analyst-to-analyst variations, and high reproducibility between two laboratories (Fig. 5B–D). In addition, the cell line stability tests demonstrated that our bioassay is highly robust and reliable (Table 3), and testing various anti-TIGIT mAbs revealed that the bioassay can be utilized in assessing the activity of a wide range of anti-TIGIT mAbs (Fig. 4B).

In recent years, the successive listing and huge sales of antibody drugs have triggered an upsurge in the research and development of antibody biotherapeutics globally. Biological activity testing identifies the effective components, which is an important quality control indicator that ensure the effectiveness of antibody drugs. Therefore, a simple and effective method for biological activity is essential. Traditional methods used to measure the biological activity of protein therapeutics targeting TIGIT mostly rely on primary human T cells and measurement of functional endpoints, such as T cell proliferation or cytokine release during activation. Obviously, these assays are not only difficult to operate, but also time-consuming, as well as highly unstable due to their reliance on primary donor T cells, complex assay protocols, and unqualified assay reagents<sup>24–27</sup>. Therefore, these are not suitable for routine testing in the drug-development setting. Similarly, this established reporter-based bioassay serves as a simple, reliable, reproducible, and pathway-specific cell-based alternative option in measuring the biological activity of anti-TIGIT antibodies.

## 5. Conclusions

Current available bioassays for measuring the biological activity of therapeutic antibodies targeting TIGIT are restricted to mechanistic investigations because donor primary T cells are highly variable. In the present study, we developed a reporter gene assay comprising two cell lines, CHO-CD112-CD3 scFv and Jurkat-NFAT-TIGIT. Optimization and validation studies have demonstrated that this assay is superior in terms of specificity, accuracy, linearity, and precision. Our study shows that this reporter-based bioassay can be utilized in the bioactivity determination of anti-TIGIT antibodies with high accuracy and specificity. It can also be potentially used in antibody screening and determination of pharmaceutical dosage form, which in turn accelerates research and development of novel TIGIT-targeted therapeutic antibodies.

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## Author contributions

Zhihao Fu and Hongchuan Liu contributed equally to this work. Junzhi Wang, Lan Wang and Meiqing Feng designed the project. Zhihao Fu, Hongchuan Liu and Yalan Yang performed the experiments. Zhihao Fu, Hongchuan Liu and Chuanfei Yu wrote the manuscript. All of the authors reviewed the final version of the manuscript.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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