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Generating Anti-TIGIT and CD155 Monoclonal Antibodies for Tumor Immunotherapy

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Abstract

Many studies have confirmed that the human poliovirus receptor (PVR; CD155) is related to tumor cell migration, invasion, and thus tumor progression. A PVR receptor binds its ligand T cell Ig and the ITIM domain (TIGIT) to inhibit the function of T and NK cells, thereby allowing tumors to evade immune surveillance. In this study, two IgG1 monoclonal antibodies, anti-CD155 and anti-TIGIT, were expressed by the mammalian transient transfection system, then, antibody-dependent cell-mediated cytotoxicity, antibody-binding affinity, and antitumor efficacy were evaluated subsequently *in vitro*. In this work, protein A affinity chromatography was used for antibodies' purification. Analysis methods included Western blot, enzyme-linked immunosorbent assay, and flow cytometry. Our data suggested that both the two monoclonal antibodies have a purity of higher than 90%, and bound tightly to the antigen with dissociation constant (K_d) and 50% effective concentrations (EC_{50}) below micromolar range. Most notably, these antibodies promote antitumor activity of immune cells *in vitro*. Therefore, our study laid down the foundation for subsequent *in vivo* experiments for further evaluation.

Keywords

- monoclonal antibodies
- TIGIT
- PVR
- tumor immunotherapy
- antibody-dependent cell-mediated cytotoxicity

Introduction

Immunotherapies have become a pillar of cancer therapy, and the treatments involve use of immune checkpoint inhibitors (ICIs), chimeric antigen receptor T cell therapy, and cancer vaccine.[1,2] Of particular interest is the clinical development of PD-1/PD-L1 antibodies that have become a monument in cancer treatment with more indications for the use of them being increasingly approved in recent years.[3] However, only a few patients showed durable clinical efficacy from ICIs, because the inhibitory factors, such as cytokine IL-10 (interleukin-10), hypoxia, tumor metabolites, and other immune checkpoints, in the tumor microenvironment (TME) promote the dysfunction of tumor-infiltrating lymphocytes (TILs).[4] Therefore, combination of different immunotherapies will offer a promising revenue and improve clinical outcomes of cancer patients in the future.[5]

The T cell Ig and ITIM domain (TIGIT) is an emerging immune checkpoint. Its structure contains an extracellular immunoglobulin (Ig) variable domain, a type 1 transmembrane domain and a cytoplasmic tail that involves an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an Ig tail-tyrosine-like motif.[6,7] The TIGIT ligand is mainly expressed on the surface of NK (natural killer cell), T cells, and can bind to its receptor—poliovirus receptor (PVR, CD155)—a member of the nectin-like family of adhesion molecules, which is highly expressed by many tumor cells and associated with tumor progression.[8] The interaction between TIGIT and CD155 suppresses the immune responses of lymphocytes through increased secretion of IL-10 and

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transmits inhibitory signals in the cytoplasmic compartment.[9,10] The expression of TIGIT on TILs is upregulated in various types of malignancies, and positively correlated with the expression of other inhibitory receptors, such as PD-1. Thus, blockage of TIGIT and PD-1/PD-L1 may restore the function of TILs and enhance the secretion of the antitumor interferon-γ (IFN-γ).[11,12]

In addition, the immune checkpoint CD96 can also bind to CD155 receptor and generate immunosuppressive signals in NK or T cells. Therefore, blocking CD155 receptor may simultaneously inhibit the binding of TIGIT and CD96 to CD155.[13] Although there have been many studies and clinical trials on anti-TIGIT monoclonal antibodies (mAbs), few reports on the therapeutic effect of anti-CD155 mAb are available, thus targeting CD155 may have a great potential in anticancer therapy.[14]

Based on a previous research, expression plasmids for the light and heavy chains of anti-TIGIT and anti-CD155 mAbs were first constructed in this work.[15] Then a large amount of anti-TIGIT and CD155 antibodies were expressed transiently via the mammalian transient expression system. The products were purified through affinity purification and high-purity proteins were obtained for biological evaluations.[16] We also established a simple TME model to assess the content of TIGIT receptors on the surface of T cells after co-culture with tumor cells. Then, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody binding affinity, and the antitumor efficacy of the antibodies were evaluated *in vitro*.[17] Preliminary results showed that CD155 mAb has a more potent *in vitro* antitumor activity than TIGIT mAb. We also demonstrated that the *in vitro* antitumor effect of the combination of TIGIT mAb and CD155 mAb was comparable to the combination of TIGIT and PD-L1.

Materials and Methods

Reagents and Antibodies

The reagents and antibodies used in the study included: 25 kDa linear polyethyleneimine (PEI; Polyscience, United States); PrimeStar mix PCR polymerase, *Hind*III restriction enzyme, *Nhe I* restriction enzyme (TAKARA Bio, Japan); Ficoll-Paque (GE Healthcare, United States); CD155 and TIGIT recombinant protein (Sino Biological Inc., Beijing, China); Donkey Anti-Human IgG (H + L) Secondary Antibody (cat# 709–035–149; The Jackson Laboratory, United States); goat anti-human IgG (H + L) secondary antibody, FITC (cat# 31529; Thermo Fisher Scientific, United States); anti-TIGIT FITC (cat# 53–9500–42; eBioscience, United States); CFDA (cat# 40715ES25; Yeasen Biotechnology (Shanghai) Co., Ltd., China), SE Cell Proliferation Tracer Fluorescent Probe and propidium iodide (PI; cat#40710ES03; Yeasen Biotechnology (Shanghai) Co., Ltd., China); anti-CD3, anti-CD28, anti-CD4, and anti-CD8 PE antibody (Sino Biological); PD-L1 (Tecentriq) antibodies were expressed by 293F cell and preserved in our laboratory. Analytical reagents such as sodium chloride, citric acid monohydrate, and disodium hydrogen were purchased from Sinopharm Chemical Reagent Co., Ltd. ClonExpress MultiS One Step Cloning Kit (cat# C113–01) was obtained from Vazyme, China; AxyPrep DNA Gel Extraction Kit was purchased from Axygen, United States; Endotoxin-Free Plasmids Extraction Kit was obtained from Omega, United States; and human IFN-γ Enzyme-Linked Immunosorbent Assay (ELISA) Kit was obtained from Sino Biological, China. Freestyle 293, F12K, RPMI1640, and DMEM medium, as well as trypsin and fetal bovine serum (FBS) were purchased from Gibco (United States). Enhanced Chemiluminescent (ECL; cat# P10300) was purchased from New Cell & Molecular Biotech (Suzhou) Co., Ltd.

Cells

U251MG and A549 cells were purchased from the Chinese Type Culture Collection. HEK293F and huTIGIT-293T cells were maintained in our laboratory. All cell lines were cultured at 37°C with 5% CO_2 in the incubator. Fresh human peripheral mononuclear cells (PBMCs) were separated from healthy donors in Changhai Hospital of Shanghai; *Escherichia coli* DH5α was purchased from Suzhou NCM Biotech.

Plasmid Construction

The light and heavy chain variable region sequences of anti-TIGIT and anti-CD155 mAb are referred from two patents respectively.[18,19] When designing the antibody expression sequence, 15 bp of homologous sequences were added to the front end of the enzyme cutting site *Hind*III and the end of *NheI* respectively, and then delivered to Shanghai Sangon Biotech to synthesize the sequence. The synthesized light and heavy chain DNA vectors were cut from the original vector by restriction endonuclease, and then the target fragment was obtained by Gel Extraction Kit. The ADCC function was attenuated by using mutations at L234A, L235A, and P329G (LALA-PG) in the Fc region, then the heavy-chain variable regions of the two antibodies were linked with the mutated Fc domains by using homologous recombination.

Protein Expression and Purification

The six expression plasmids were extracted by an endo-free plasmid extraction kit, and all plasmids were sterilized by 0.22 μm filter. The light and heavy chain plasmids were mixed at a mass ratio of 1:1, then added PEI agent at a mass ratio of DNA:PEI = 1:4, and incubated at room temperature for 15 minutes. Lastly, the DNA blend was added to the HEK293F cell suspension and incubated in a 37°C shake incubator for 5 days. Then, cell supernatant was collected by centrifuge (4,000 *g*, 20 minutes), and filtered through a 0.45 μm filter. We connected the Protein A column on the AKTA Avant and equilibrated five column volumes with binding buffer, then loaded the sample via the Avant. After loading the sample, the impurities were washed with pH 5.0 citrate buffer and antibody was eluted with pH 2.7 citrate buffer and dialyzed overnight to remove the eluate buffer. The above-mentioned buffer formula is shown in ►**Table 1**.

Antibodies' Characterization

The antibodies were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and the molecular weight of the antibodies was analyzed by

Table 1 Buffers

Buffer	Formula
0.1 mol/L citric acid buffer, adjust pH to 5.0 and 2.7	21.014 g citric acid monohydrate + 1,000 mL H_2O
200 mmol/L NaH_2PO_4	23.996 g NaH_2PO_4 + 1,000 mL H_2O
200 mmol/L Na_2HPO_4	28.392 g Na_2HPO_4 + 1,000 mL H_2O
Binding buffer, adjust pH to 7.4	200 mmol/L NaH_2PO_4 (28 mL), 200 mmol/L Na_2HPO_4 (72 mL), 8.766 g NaCl + 900 mL H_2O

Coomassie brilliant blue and Western blot. Briefly, the antibodies and protein markers were added to gel holes of SDS-PAGE to run at a constant voltage of 90 V for 100 minutes. The gel was cut, stained with Coomassie brilliant blue, and then washed with destaining solution. Proteins on SDS-PAGE gel were transferred onto a PVDF membrane, and incubated with Donkey Anti-Human IgG (H + L) Secondary Antibody for 1 hour. Visualization was performed by chemiluminescence of membranes using ECL reagents. Purity of the two antibodies was determined by the size exclusion chromatography (SEC-HPLC) method.

Affinity Assay by ELISA Method

The ELISA method was used to assess the affinity of the antibodies to antigens. Briefly, TIGIT and CD155 recombinant proteins were coated overnight at a concentration of 25 ng/mL, and the two mAbs were serially diluted and added to the coated antigen. After 1 hour of incubation, added peroxidase-labeled donkey anti-human IgG (H + L) antibody to incubate and conducted a color reaction after the end of the incubation, and placed it in a microplate reader to measure the absorbance at OD_{450nm}. The EC_{50} value was determined as the concentrations of the antibody used for half-maximal of OD_{450nm}. The K_d values were determined as equilibrium dissociation constant of antigen/antibody complexes in solution.

Affinity Assay by Flow Cytometry

A549 and U251 cells were cultured in DMEM containing 10% FBS, while CHO-s cells were cultured in F12K medium containing 10% FBS. The A549 and U251 cells were digested with trypsin, and adjusted the cell count to 10^5 cells per tube, and anti-CD155 or anti-TIGIT mAb was added and incubated on ice for 30 minutes. After the incubation, the cells were incubated with goat anti-human IgG (H + L)-FITC antibody for another 30 minutes, and the samples were loaded and analyzed by a flow cytometer.

Tumor Cell and PBMC Co-culture Experiment

A549 and U251 cells were digested by trypsin. After centrifugation (300 *g*, 3 minutes), the supernatant was removed and the cells were resuspended (density 1.0×10^6 cells/mL). The cells were then seeded into a 96-well plate at 10^4 cells per well and cultured for 12 hours. After the cells have completely adhered to the wall, PBMCs were added at an effect-to-target ratio of 1:5. Blank wells contained PBMCs without target cells and positive controls were PBMCs activated by CD3/CD28 antibodies (CD3: 5 μg/mL, CD28: 2 μg/mL). After 24 hours of culture, the suspended PBMCs were gently collected. Anti-CD4 (PE), anti-CD8 (PE), and anti-TIGIT (FITC) antibodies were added to collected PBMCs, then were incubated at 4°C for 30 minutes. After the incubation, the cells were washed three times with 2% FBS-PBS buffer and evaluated by flow cytometry.

Antibody-Mediated Cytotoxicity against A549 and U251 Cells

CHO-s, A549 and U251 cells were digested with trypsin, and the cell count was adjusted to 10^6 cells/mL. CFDA was added to the cell suspension (the final concentration of CFDA was 5 μmol/L) and placed in a 37°C cell incubator for 30 minutes. After the incubation, the cells were washed three times with PBS, and then added to a 96-well culture dish at 10^4 cells per well. Human PBMCs are isolated by Ficoll density gradient centrifugation, and cultured with 10% FBS RPMI-1640. The next day, PBMCs were added to 96-well cell culture dishes at a ratio of 10:1 target cells and added different antibodies or different concentration to each group. After co-cultivation for 24 or 48 hours, the cell supernatant was collected by centrifugation (300 *g*, 3 minutes), and the adherent cells were detached and resuspended in PBS. Finally, PI dye (final concentration 0.3 mmol/L) was added to each sample and incubated for 15 minutes at room temperature for flow cytometry analysis. IFN-γ content in cell culture supernatant was determined by IFN-γ ELISA kit according to manufacturer's instructions. The cell killing rate was calculated according to Eqn. (1) and cell viability (%) = $CFSE^+ PI^-$ single positive cells.

$$\text{Killing rate (\%)} = \frac{(\text{percentage of CFSE without PBMC} - \text{percentage of CFSE})}{(\text{percentage of CFSE without PBMC})} \times 100\% \quad (1)$$

Statistical Analysis

Data were presented as the mean of at least two replicate samples and standard errors. Student's *t*-test was used to compare between groups with $p < 0.05$ being considered a statistically significant difference.

Results

Construction of Expression Plasmids for Anti-TIGIT and Anti-CD155 mAbs

The expression plasmid was pcDNA3.4 (►**Fig. 1A**), and the plasmids to be constructed in the study included the heavy

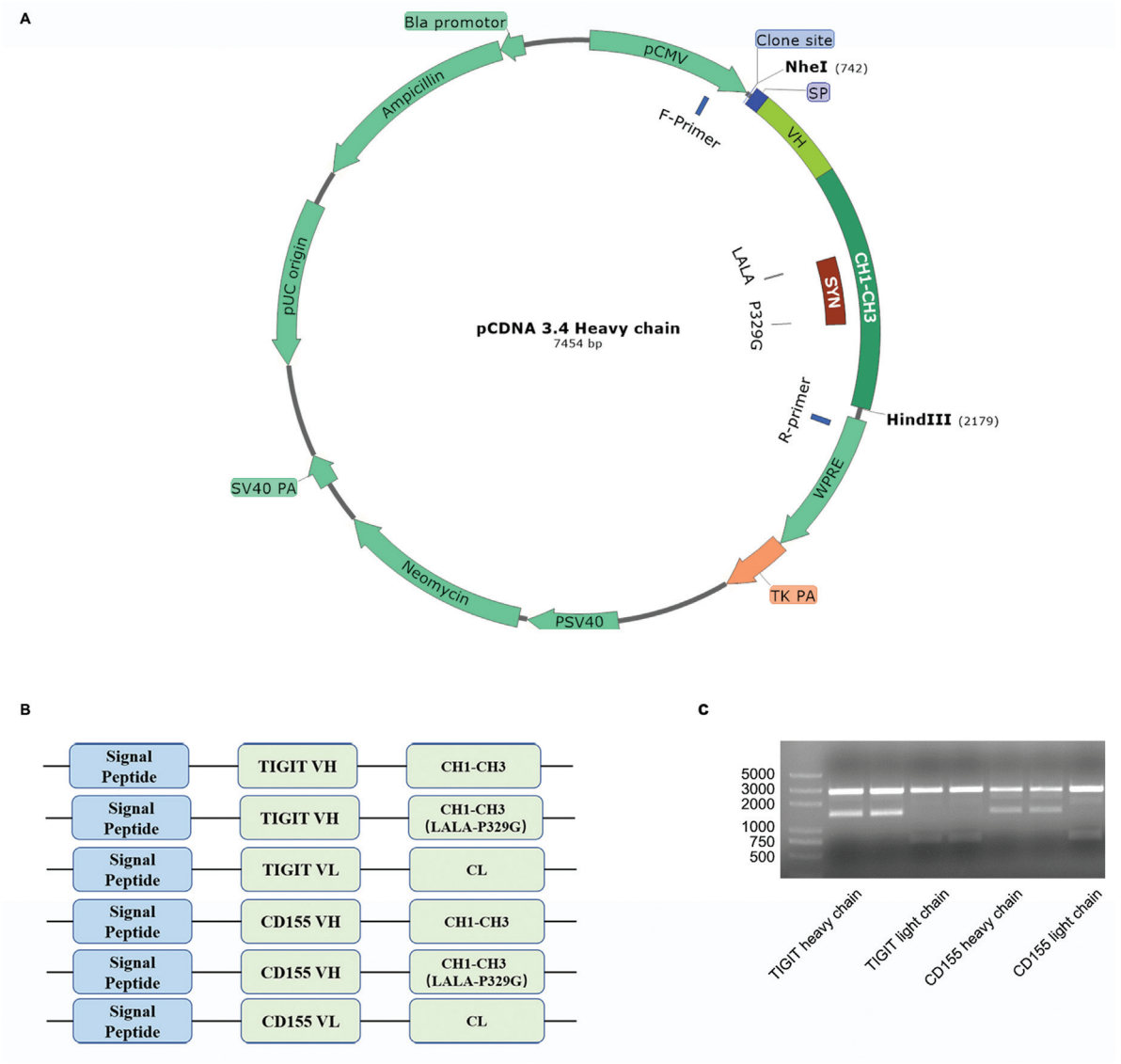

Fig. 1 Construction of expression vector. **(A)** pcDNA3.4 expression vector map. **(B)** Schematic diagram of different expression vectors. **(C)** Agarose gel images of light and heavy chains of TIGIT and CD155 antibody after digestion. TIGIT, T cell Ig and ITIM domain.

chain, the light chain, and the LALA-P329G mutant heavy chain of two antibodies (►**Fig. 1B**). The heavy chain and light chain of two antibodies were obtained by enzyme digestion of synthesized sequence fragments, and the size of the heavy chain was approximately 1,500 bp, and the light chain was approximately 700 bp (►**Fig. 1C**). The target fragments were purified by gel extraction. The fragments and the vector were linked through the homologous recombination. Finally, bacterial transformation was performed and positive clones were selected. The expression vectors were extracted and sterilized through a 0.22 μm filter.

Antibody Purification and SEC-HPLC Analysis

Protein A affinity chromatography was used for antibody purification. Results of nonreducing (►**Fig. 2Aa**) and reducing (►**Fig. 2Ab**) SDS-PAGE showed that the antibody band was approximately 180 kDa. The sizes of heavy chain and light chain were approximately 55 and 25 kDa, respectively. These bands of antibodies indicate that they were close to the theoretical molecular weight of the IgG-like antibody with high purity. ►**Fig. 2Ac** shows that the IgG-like antibodies we expressed were specifically bound by the anti-human IgG (H + L) secondary antibody. We measured the concentration of two purified mAbs using a BCA kit that showed CD155 mAb being 50 mg/L, and TIGIT mAb 20 mg/L.

SEC-HPLC analysis showed a high purity of the two mAbs (►**Fig. 2B, C**). We determined the purity by calculating the ratio of the main peak areas of the two antibodies with CD155 mAb and TIGIT mAb at 91.54 and 95.6%, respectively. The results of SEC-HPLC were consistent with the results of SDS-PAGE, and the purity of purified antibodies was greater than 90%. Therefore, the expression system of transient transfection of 293F cells readily produced mAb with high yield and purity.

Antibody Binding of TIGIT and CD155 Antigens

Flow cytometry was used to investigate CD155 expression on U251 and A549 cells according to reports.[20,21] We used anti-

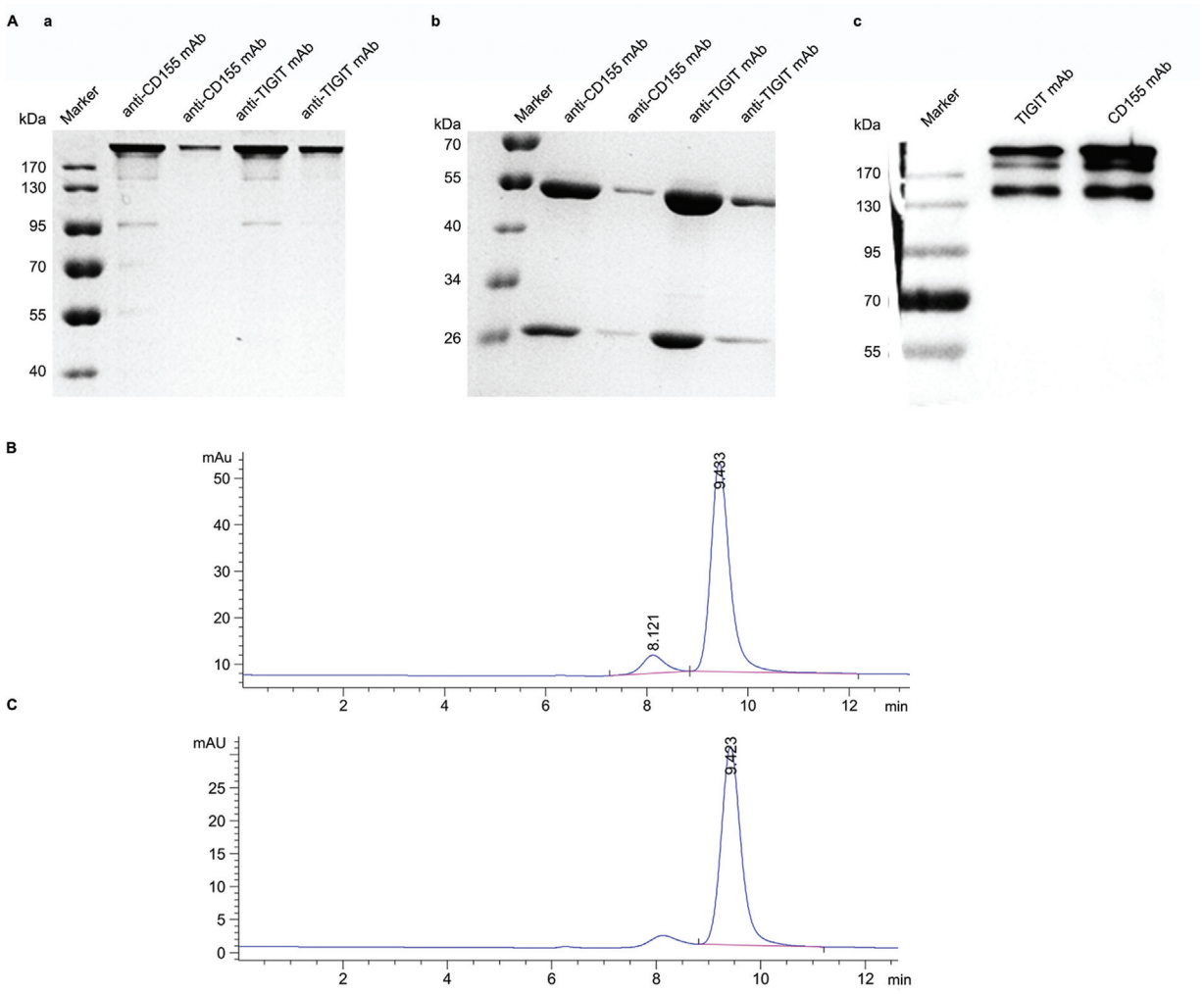

Fig. 2 Analysis of the molecular weight and purity of purified antibodies. **(A)** Analysis of antibody molecular weight by (**a**) nonreducing SDS-PAGE; (**b**) reducing SDS-PAGE; and (**c**) Western blot. **(B)** Absorbance chromatogram of CD155 mAb at UV 280 nm by SEC-HPLC. **(C)** Absorbance chromatogram of TIGIT mAb at UV 280 nm by SEC-HPLC. mAb, monoclonal antibody; TIGIT, T cell Ig and ITIM domain.

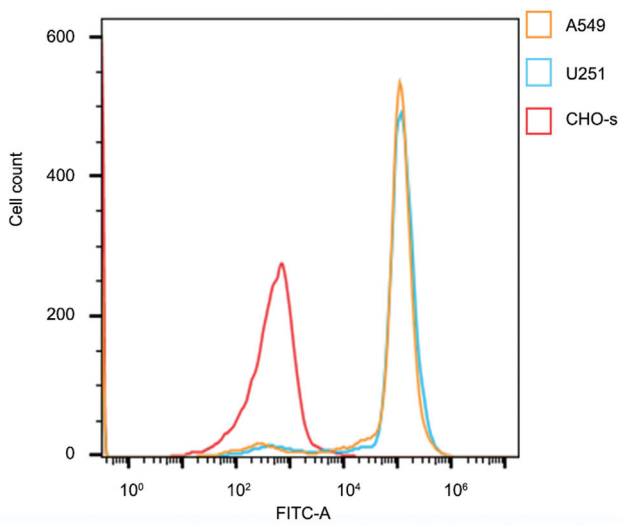

Fig. 3 Flow cytometry analysis showing CD155 expression on the surface of U251 and A549 cells.

CD155 mAb as a primary antibody to bind the CD155 receptor on the surface of U251 and A549 cells. Anti-human (H + L) FITC secondary antibody was added to capture primary antibodies. The data showed that both fluorescence intensities of U251 and A549 were shifted to the right, but the control CHO-s cells showed no shift (►**Fig. 3**). Therefore, we chose U251 and A549 as model cell lines in biological experiments.

Results from ELISA assay showed that both anti-TIGIT and anti-CD155 antibodies bound TIGIT and CD155 recombinant protein antigens with high affinity (►**Fig. 4A**). The EC_{50} of TIGIT mAb was 0.00714 µg/mL, and the EC_{50} of CD155 mAb was 0.01111 µg/mL (►**Table 2**). The affinity of TIGIT mAb appeared to be higher than that of CD155 mAb; therefore, subsequent experiments required adjustment of antibody concentration. Flow cytometry showed that the anti-CD155 mAb specifically targeted U251 cells and anti-TIGIT mAb bound to TIGIT overexpressing 293T cells, indicating theses mAbs bound cellular receptors with high affinity. Flow

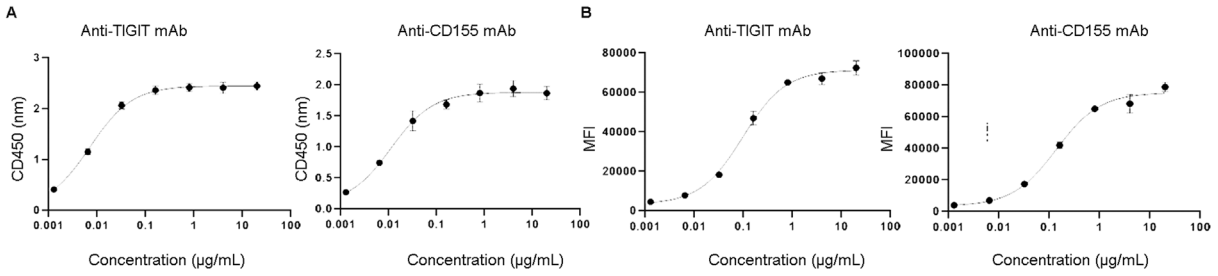

Fig. 4 The affinity of two antibodies to antigens and receptors detected by **(A)** ELISA and **(B)** flow cytometry. In flow cytometry, the affinity of TIGIT mAb for TIGIT receptor was detected on the surface of TIGIT-overexpressing 293T cells, while CD155 mAb for CD155 receptor was detected on the surface of U251 cells. ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; TIGIT, T cell Ig and ITIM domain.

Table 2 EC_{50} and K_d values for different antibodies to antigens measured by ELISA

TIGIT	TIGIT mAb	CD155	CD155 mAb
EC_{50} (μg/mL)	0.00714	EC_{50} (μg/mL)	0.0111
K_d (μg/mL)	0.00717	K_d (μg/mL)	0.0113

Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; TIGIT, T cell Ig and ITIM domain.

Table 3 EC_{50} and K_d values for different antibodies to antigens measured by flow cytometry

TIGIT	TIGIT mAb	CD155	CD155 mAb
EC_{50} (μg/mL)	0.09716	EC_{50} (μg/mL)	0.1377
K_d (μg/mL)	0.09280	K_d (μg/mL)	0.1377

Abbreviations: mAb, monoclonal antibody; TIGIT, T cell Ig and ITIM domain.

cytometry also showed (►**Fig. 4B**) that the EC_{50} of TIGIT mAb was 0.09716 μg/mL, and the EC_{50} of CD155 mAb was 0.1377 μg/mL (►**Table 3**). The difference between the cytometry result and the ELISA assay suggests that the counts of cell surface receptors were not homogeneous.

Upregulation of TIGIT on T Cells after Co-culture with Tumor Cells

A previous study demonstrated that the expression of TIGIT on the T cell surface was effectively enhanced after stimulating PBMC with CD3/CD28 antibodies.[6] Treating tumors with anti-TIGIT restored the function of immune cells against tumor cells.[22] We used activation antibodies (CD3/CD28) to treat PBMC and tested the expression of TIGIT on T cells. Our data suggest that after activation, the proportion of $CD4^+$ $TIGIT^+$ T cells increased from 2.71 to 4.09%, and $CD8^+$ $TIGIT^+$ T cells also increased from 2.39 to 10.1% (►**Fig. 5**). We then established a simple model by co-culturing A549 and U251 cells with PBMCs and using flow cytometry to detect TIGIT expression on immune cells. We collected PBMCs incubated for 24 hours from a 96-well plate, then used CD3 antibody to distinguish T cell populations, while PE-labeled anti-CD4 and CD8 antibodies were used to mark T cell subsets. FITC-labeled anti-TIGIT antibody was finally used to verify the expression level of TIGIT antibody.

After co-culture of PBMC with A549 and U251 cells, the TIGIT receptor on T cells was significantly upregulated and the proportion of $CD4^+$ $TIGIT^+$ T cells increased from 2.68 to 18.8 and 26.8%, respectively, while $CD8^+$ $TIGIT^+$ T cells also increased from 0.98 to 25.7 and 18.5% (►**Fig. 6**). The upregulation of TIGIT in this assay was higher than that of T cells activated with CD3/CD28, which might be due to the fact that activation with CD3/CD28 required a longer incubation time.[6] The inhibitory effect of tumor cells on T cells was

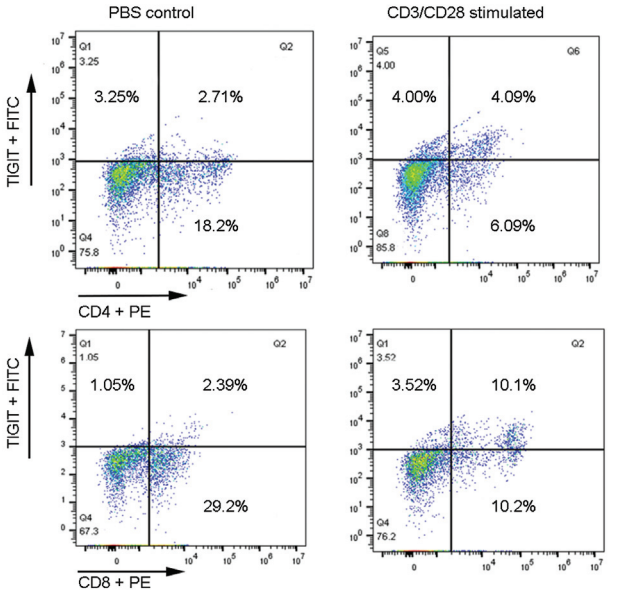

Fig. 5 Flow cytometry analysis showing surface TIGIT expression after CD3/CD28 antibody activation of PBMC. TIGIT, T cell Ig and ITIM domain.

related to the interaction between TIGIT and CD155 to a certain extent, and this part of the results provided a theoretical basis for the subsequent anti-TIGIT antibody-mediated antitumor experiments.

Antibody-Mediated Antitumor Effects

To directly measure tumor killing by lymphocytes, we stained tumor cells with CFSE before co-culturing with PBMCs.[12] Tumor cells were divided into separate populations by CFSE staining and tumor killing was revealed by PI staining of dead cells. As shown in ►**Fig. 7**, anti-TIGIT and

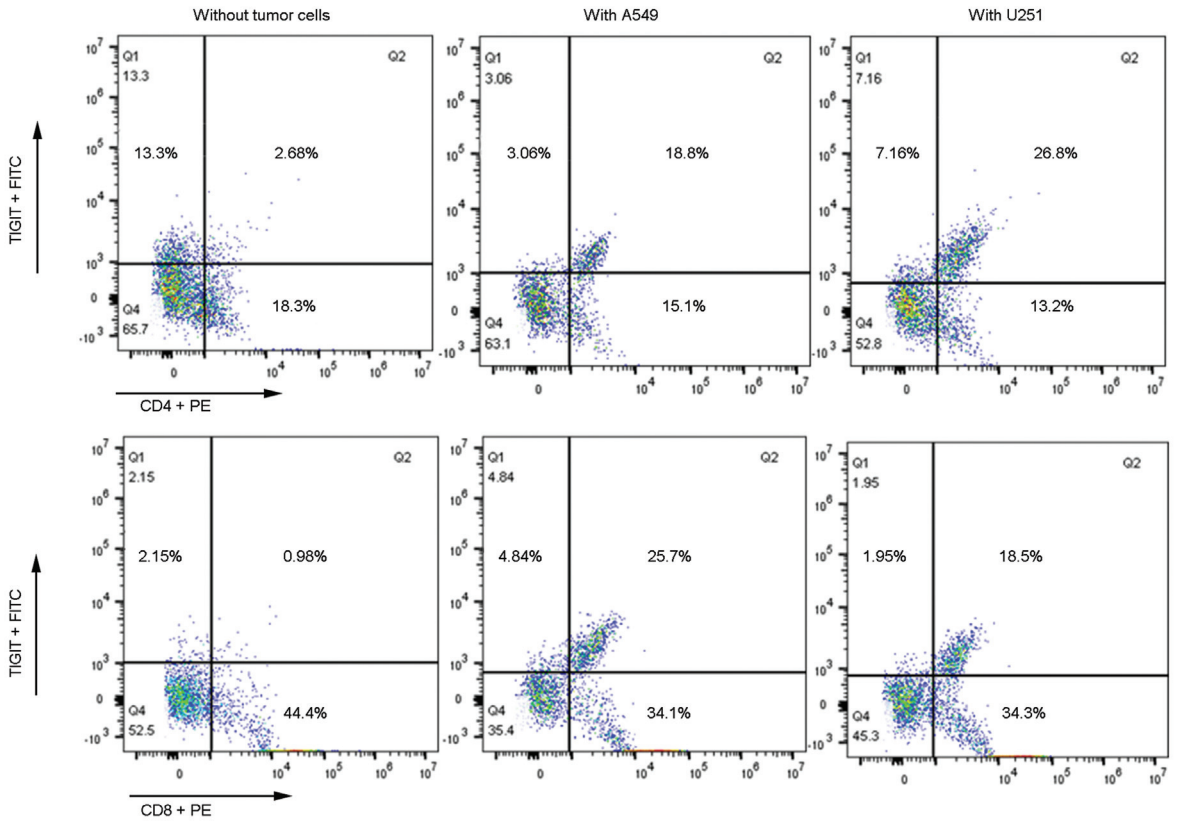

Fig. 6 Flow cytometry diagram of TIGIT expression on the surface of PBMC, A549, and U251 cells after co-culture for 24 hours. TIGIT, T cell Ig and ITIM domain.

anti-CD155 mAbs significantly enhanced the percentage of dead cells ($CFSE^+$ PI^+ positive cells) compared to the control samples (PBS + PMBC). We then used cell viability index (cell viability [%] = $CFSE^+$ PI^- single positive cells) to evaluate the killing effect of the antibody, because the results using the CHO-s cell line showed that the antibody did not trigger

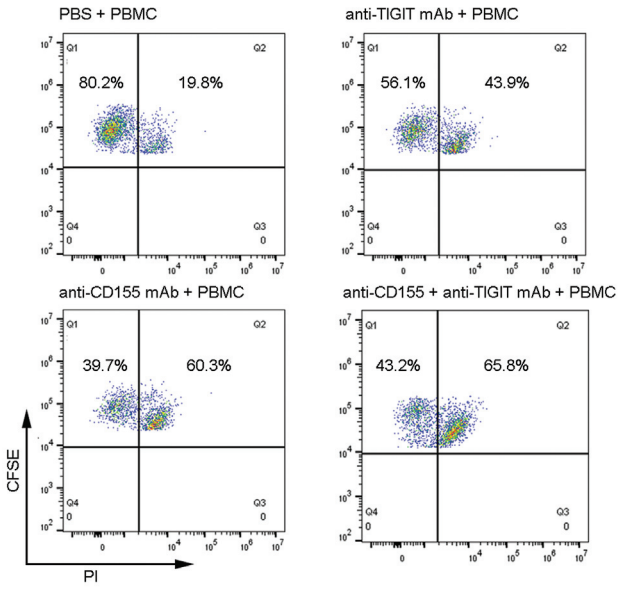

Fig. 7 Flow cytometry results of lymphocyte killing of U251 tumor cells mediated by different antibody combinations.

PBMC to produce killing effect on nontumor cell lines that did not express CD155 (►**Fig. 8**). Thus, the cell viability rate was used to compare the results more clearly. As shown in ►**Fig. 8**, the target cell viability of the anti-CD155 mAb group at different concentrations was lower than that of the anti-TIGIT mAb group, and the two antibodies showed a killing effect at a low dose of 2.5 μg/mL. Therefore, we believe that blocking the combination of TIGIT and CD155 may improve the PBMC killing effect on the CD155 high-expressing tumor cell line.

In addition, ADCC-enhancing antibodies were able to promote lymphocytes for killing tumor cells, while ADCC-impairing antibodies (Fc silence) only slightly enhanced killing of target cells (►**Fig. 9A**). The U251 cell line was more sensitive to PBMC than the A549 cell line. Therefore, we confirmed that blockade of TIGIT and CD155 targets increased the antitumor effect and the efficacy of CD155 mAb was superior to that of TIGIT mAb. Also, the combination of TIGIT and CD155 mAbs showed a potential anticancer effect, but the combination therapy did not show a difference from anti-CD155 mAb in the A549 cell line. We further validated the combination therapy of TIGIT and PD-L1 mAb. Since anti-PD-L1 did not possess ADCC function, we used CD155 mAb without ADCC function as a control. The results indicate that combination therapy of TIGIT + CD155 mAb generated the best antitumor activity on the U251 cell line (►**Fig. 9B**), while it did not show better effect than TIGIT + PD-L1 combination on A549 cells. In addition, the

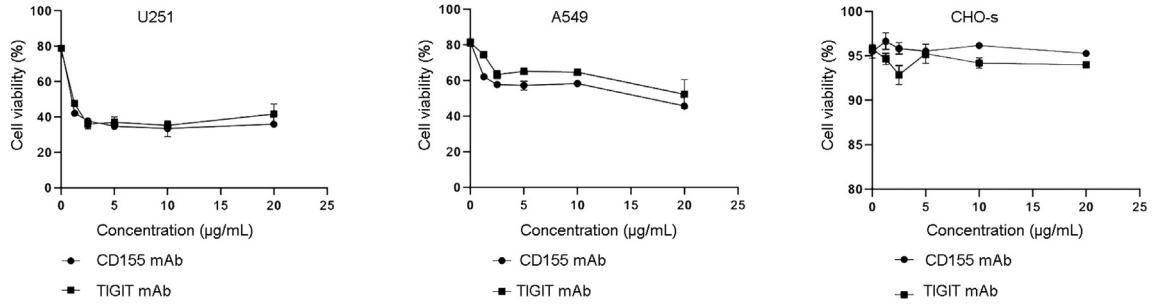

Fig. 8 The effect of different concentrations of TIGIT and CD155 mAb on the viability of U251, A549, and CHO-s cells in the presence of PBMC. mAb, monoclonal antibody; TIGIT, T cell Ig and ITIM domain.

killing rate of PD-L1 mAb was weaker than that of CD155 mAb, which may be due to the different expression levels of PD-L1 and CD155 on cells. Therefore, more models and experiments are required to determine whether the antitumor activity of CD155 mAb is more potent than PD-L1 mAb.

ELISA results showed that TIGIT mAb with ADCC function promoted IFN-γ release, and the effect was similar to CD155 mAb without ADCC function (►**Fig. 9C**). However, PD-L1 mAb without ADCC did not stimulate the release of a high level of IFN-γ compared with other groups. Overall, combined treatment of TIGIT + PD-L1 mAb and TIGIT + CD155 mAb promoted the release of a high level of IFN-γ to exert a more potent antitumor effect (►**Fig. 9C**). The result was similar to that obtained in killing experiments. These findings support that TIGIT and/or CD155 blockade increased cytokine production by lymphocytes with enhanced

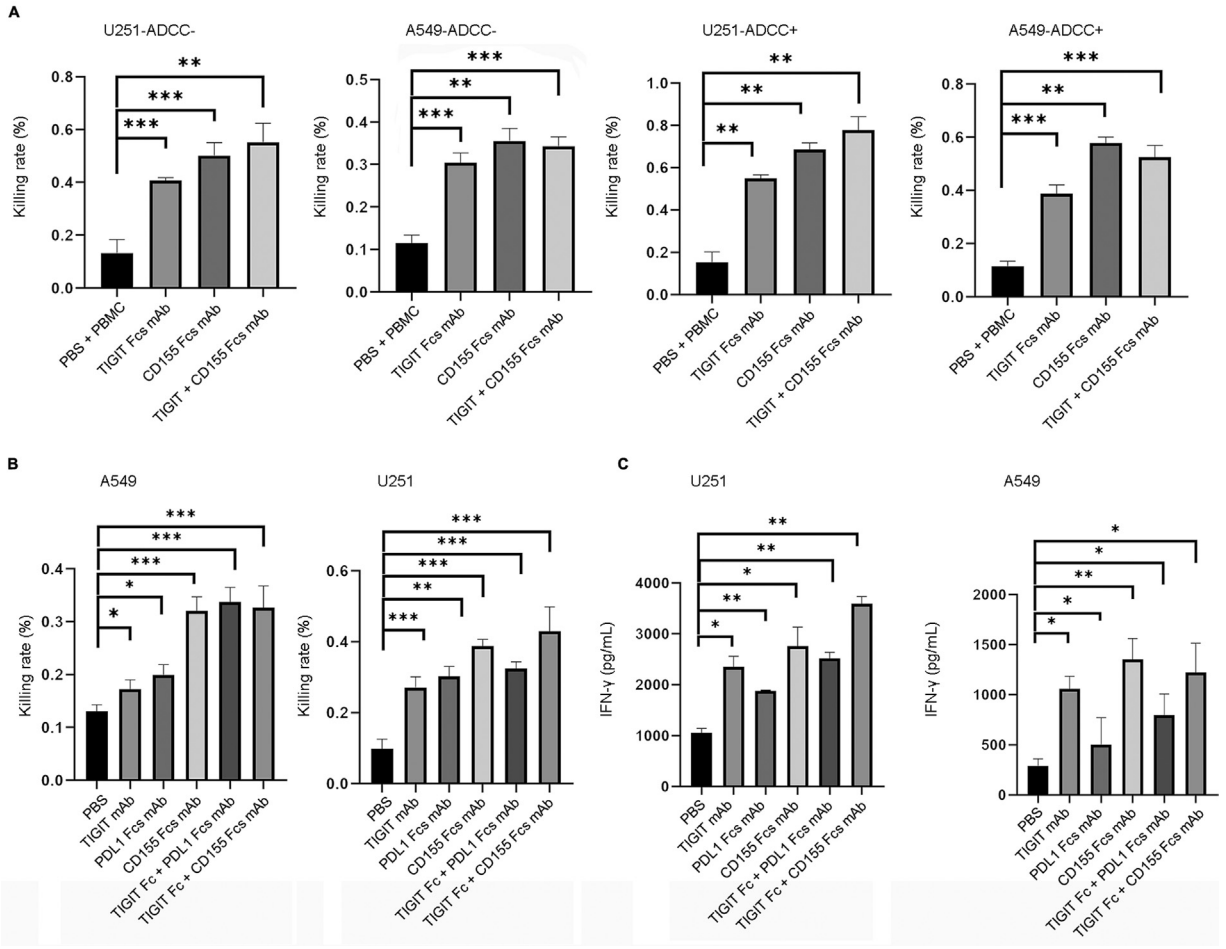

Fig. 9 The effect of TIGIT and/or CD155 on killing and IFN-γ production of U251 and A549 cell lines. Statistics of the killing rate of U251 and A549 tumor cells by (**A**) antibodies with or without ADCC effect and (**B**) different antibodies. All data shown are representative of independent experiments with at least two different PBMC donors replicated over three times. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. (**C**) ELISA results of IFN-γ release in A549 and U251 cell culture supernatants. Experiments were repeated three times. $^{*}p < 0.05$, $^{**}p < 0.005$. ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; TIGIT, T cell Ig and ITIM domain.

cytotoxicity to tumor cells. The reason for the ability of CD155 mAb without ADCC function to promote a high level of IFN-γ release is unclear. We speculate that CD155 mAb may block another immunosuppressive checkpoint CD96.

Discussion

How to extend the clinical benefits to the majority of patients with tumors and explore appropriate combination antitumor immunotherapies have been the key to this field.[5,11] As an emerging immune checkpoint, TIGIT has quickly entered the clinical trial; the combination therapy targeting the TIGIT-CD155 axis will become a novel direction of immunotherapy.[23]

There are thousands of mAbs in preclinical to early clinical development worldwide, to accelerate the progress required for development of efficient methods to express high yield and high purity mAbs. In our study, we expressed antibodies by transient transfection of mammalian cells, which can not only raise the protein yield, but also produce high-purity antibodies.[16] However, the transfection steps and purification conditions in the experiment need to be optimized, such as the ratio of plasmid to PEI during transfection and the use of different pH eluents to remove less pure proteins during the purification period. In the SEC-HPLC analysis of the two expressed antibodies, we found that the purity and yield were inconsistent, which may be due to different antibody variable region sequences. The higher yield of CD155 mAb compared to TIGIT mAb may be another reason for the lower purity of CD155 mAb. A higher expression efficiency may also generate more impurities.

The binding activity of the antigen–antibody was the key to the efficacy of mAbs. We have demonstrated that the expressed mAbs can bind to CD155 and TIGIT antigens measured by ELISA and flow cytometry. But ELISA experiments were not the most accurate method to determine the affinity constant values, so SPR (surface plasmon resonance) assay was needed to evaluate affinity-related parameters of antibody–antigen binding.[24]

Both CD155 on tumor cells and TIGIT on lymphocytes were upregulated in TME, and their interaction led to the depletion of immune cells.[25] Our study found that U251 and A549 cells have a high level of expression of CD155, which impaired the killing function of immune cells due to the inhibitory signal between TIGIT and CD155. We also demonstrated that TIGIT on the surface of T cells was significantly upregulated when co-cultured with tumor cells, which may explain why PBMCs have limited tumor-killing effect in the absence of anti-TIGIT and anti-CD155 mAbs. Therefore, when we added TIGIT and CD155 mAb into PBMC, lymphocytes promoted the lysis of tumor cells with the exception of the CHOs which did not express CD155 receptor. The effect of CD155 mAb is greater than that of TIGIT mAb, which led to our speculation that blocking CD155 may inhibit simultaneously the two immune checkpoints of TIGIT and CD96. Consistent with previous reports, we found that the ADCC effect contributed to the more potent antitumor effect of TIGIT mAb that may be an important effect concerning this therapy.[26]

A study indicated that the efficacy of anti-TIGIT mAb and anti-PD-L1 mAb alone in the treatment of a mouse tumor model was similar to the results of our *in vitro* experiments.[22] We confirmed the conclusion that single treatment of TIGIT mAb was less effective, and for the first time we observed that anti-CD155 mAb has higher *in vitro* antitumor activity than anti-TIGIT mAb and anti-PD-L1 mAb. Regardless of the ADCC function of anti-CD155 mAb, this mAb showed excellent *in vitro* antitumor effect, which was comparable to the combined use of TIGIT and PD-L1. Nevertheless, the current research on the antitumor therapeutic effect of CD155 mAb is not definite, and limited studies showed that blocking CD155 mAb decreases the invasion and migration of tumor cells.[27] Another research indicates that both anti-TIGIT mAb and anti-CD155 mAb were able to enhance cytotoxicity mediated by bispecific antibodies targeting EGFR and CD3 with similar therapeutic effects. However, the anti-TIGIT mAb and anti-CD155 mAb used in that study are commercial mAbs for proteomics, not for therapeutic purposes.[28] In addition, we evaluated the antitumor potential of various antibodies by measuring the content of IFN-γ in cell culture supernatant with results similar to those of the killing experiment. Surprisingly, CD155 mAb without ADCC function still promoted strong IFN-γ release, while the release amount caused by PD-L1 mAb without ADCC function was only stronger than that of the PBS group. We believe the reasons for this interesting phenomenon are worth further exploration.

At present, the combination therapy of anti-TIGIT and anti-PD-L1 mAb has entered the clinical trial, but with only limited studies on the therapeutic effect of anti-CD155 mAb.[29] Thus, our experiment indicates the potential antitumor effect of anti-CD155 mAb. Further animal experiments are required to examine whether the antibody may show functions other than blocking the TIGIT-CD155 signal.

Conclusion

We have demonstrated that anti-CD155 mAb and anti-TIGIT mAb stimulated T cells to release cytotoxic cytokines to kill tumor cells. *In vitro* experiments showed that anti-CD155 mAb was superior to the anti-TIGIT mAb in antitumor efficacy. Combination of the antibodies demonstrates promising potential as a novel cancer immunotherapy.

Ethics Statement

The separation of PBMCs from healthy donors abides by the relevant agreements of the Changhai Hospital of Shanghai, and it conforms to the provisions of the Declaration of Helsinki in 1995 (16). Informed consent was obtained from all individual participants included in the study.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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