

Development of Anti-human T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT) Monoclonal Antibodies for Flow Cytometry

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Immune checkpoint inhibitors targeting programmed cell death-ligand 1 (PD-L1), programmed cell death-1 (PD-1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) recently made a significant survival rate improvement in cancer treatment. T cell immunoreceptor with Ig and ITIM domains (TIGIT) is expressed in T and NK cells related to their activities. It has a single extracellular immunoglobulin domain, a type 1 transmembrane domain, and a single intracellular ITIM. TIGIT binds with poliovirus receptor (PVR) or PVR2, resulting in suppressing T and NK cell activities. Some studies showed that the combined use of a TIGIT inhibitor with another immune checkpoint inhibitor enhanced antitumor activities more strongly than their single use. Therefore, TIGIT should be a new target for immunotherapy. In this study, we developed new anti-human TIGIT (hTIGIT) monoclonal antibodies (mAbs) using the Cell-Based Immunization and Screening (CBIS) method. Mice were immunized with hTIGIT-overexpressed Chinese hamster ovary (CHO)-K1 cells (CHO/hTIGIT), and hybridomas were screened by flow cytometry. One of the mAbs, TgMab-2 (IgG₁, kappa), specifically and sensitively detects hTIGIT in CHO/hTIGIT and NK cells. The dissociation constants (K_D) of TgMab-2 for CHO/hTIGIT cells were determined to be 3.5×10^{-9} M. These results suggest that TgMab-2, which was developed by CBIS method, is useful for analyzing the function of hTIGIT by flow cytometry.

Keywords: TIGIT, monoclonal antibody, hybridoma, flow cytometry, TgMab-2

Introduction

T CELL IMMUNORECEPTOR WITH IG AND ITIM DOMAINS (TIGIT) is an immune checkpoint molecule expressed on T and NK cells. TIGIT consists of a single extracellular immunoglobulin domain, a type 1 transmembrane domain, and a single intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM).⁽¹⁾ ITIM motifs are commonly used by inhibitory receptors to recruit SH2 domain-containing tyrosine phosphatases SHP1 and SHP2.⁽²⁾ TIGIT binds to poliovirus receptor (PVR, also known as CD155) with high affinity and PVR-like protein 2 (PVRL2, also known as CD112) with low affinity.⁽¹⁾ These interactions cause suppression of T cell activation and NK cell cytotoxicity.^(1,3,4) DNAM-1 (CD226) competes with TIGIT to bind with PVR, although the affinity of PVR-DNAM-1 is much lower than PVR-TIGIT.^(1,5) PVR is expressed at low levels in many cell types of epithelial origin. In contrast,

PVRL2 is expressed in various cells, especially in epithelial cells, neurons, and fibroblasts.⁽⁶⁾ PVR and PVRL2 are over-expressed in cancer cells, including colorectal,⁽⁷⁾ pancreatic,⁽⁸⁾ breast or ovarian⁽⁹⁾ cancers, and melanoma.⁽¹⁰⁾

Recently, therapies targeting immune checkpoint molecules, such as programmed cell death-ligand 1 (PD-L1), programmed cell death-1 (PD-1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), have significantly improved the prognosis of cancer patients.⁽¹¹⁾ Johnston *et al.* reported that the co-blockade of TIGIT and PD-L1 was more effective in enhancing CD8⁺ T cell effector function than that of PD-L1 blockade alone, resulting in a remarkable tumor reduction in mouse cancer models.⁽¹²⁾ Another study has shown that both TIGIT and PD-1 blockade increased the function of CD8⁺ T cell function in melanoma patients,⁽¹³⁾ suggesting that TIGIT could be expected as a new target for immunotherapy.

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In this study, we developed a novel anti-human TIGIT (hTIGIT) monoclonal antibodies (mAbs), useful for flow cytometry using the Cell-Based Immunization and Screening (CBIS) method. We further characterized the established clone (TgMab-2) by flow cytometry.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Synthesized DNA encoding hTIGIT (Accession No. NM_173799) was purchased from OriGene Technologies, Inc. (Rockville, MD, catalog No: RC221447). hTIGIT open reading frame without signal sequence and with anN-terminal MAP16 tag were subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The MAP16 tag comprises 16 amino acids (aa) (PGTGDGMVPPGIEDKI) and is recognized by PMAb-1.^(14,15) A hTIGIT plasmid was transfected using a Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan) and cultivated in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, and CHO/hTIGIT were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator at 37°C, 5% CO₂, and 95% air.

NK cells (Donor Lot. 4022602 NK1602) for flow cytometry were purchased from Takara Bio, Inc. (Shiga, Japan).

Antibodies

An anti-hTIGIT mAb (clone A15153G; mouse IgG_{2a}, kappa) was purchased from BioLegend (San Diego, CA). Secondary Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Animals

All animal experiments were performed according to relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit No.: 2019NiA-001). Mice were monitored daily for health during the full 4-week duration of the experiment. A reduction of >25% of total body weight was defined as a humane endpoint. Mice were euthanized by cervical dislocation, and death was verified by respiratory and cardiac arrest. Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions.

Hybridoma production

To develop mAbs against hTIGIT, we employed the CBIS method.⁽¹⁶⁾ In brief, two BALB/c mice were immunized with CHO/hTIGIT cells (1×10^8) by the intraperitoneal route together with Imject Alum (Thermo Fisher Scientific, Inc.).

The procedure included three additional immunization followed by a final booster intraperitoneal injection administered 2 days before spleen cells were harvested. Harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The hybridomas were grown in RPMI medium supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B (Nacalai Tesque, Inc.), and hypoxanthine, aminopterin, thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were screened by flow cytometry.

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were washed with 0.1% bovine serum albumin in phosphate-buffered saline and treated with primary mAbs for 30 minutes at 4°C. Then, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc.). Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.) and analyzed using FlowJo (BD Biosciences, Franklin Lakes, NJ).

Determination of binding affinity by flow cytometry

CHO/hTIGIT was suspended in 100 μ L of serially diluted anti-hTIGIT mAbs, and Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were collected using the BD FACSLytic (BD Biosciences). The dissociation constant (K_D) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Establishment of the anti-hTIGIT mAbs

To establish anti-hTIGIT mAbs, we employed the CBIS method (Fig. 1). First, we immunized two mice with CHO/hTIGIT cells and produced hybridomas by fusing splenocytes with P3U1 myeloma cells. Next, we selected hybridomas whose supernatants were positive for CHO/hTIGIT cells and negative for CHO-K1 cells by flow cytometry. The first screening approach identified strong signals from CHO/hTIGIT cells and weak or no signals from CHO-K1 cells in 11 of 958 wells (1.1%). The second screening approach identified strong signals from NK cells using 2 of the 11 hybridoma supernatants determined in the previous step (18%). After limiting dilution and several additional screenings, TgMab-2 (IgG₁, kappa), which reacted most strongly with CHO/hTIGIT, was finally established.

Flow cytometric analyses

TgMab-2 recognized CHO/hTIGIT but not CHO-K1 (Fig. 2A). Also, TgMab-2 reacted with endogenous hTIGIT in NK cells (Fig. 2A). The concentration of 0.01 and 0.1 μ g/mL were enough for recognition with CHO/hTIGIT and NK cells, respectively (Fig. 2A). As a positive control, clone A15153G from BioLegend reacted similarly with both cells (Fig. 2B).

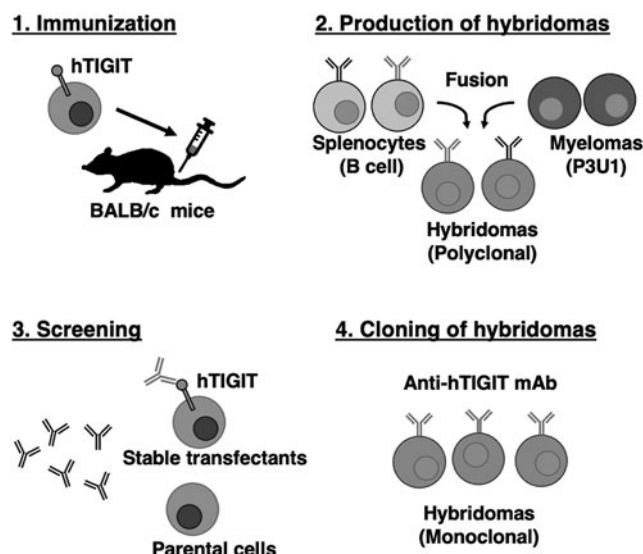


FIG. 1. Production of anti-hTIGIT mAbs. The CBIS method. CHO/hTIGIT cells were immunized into mice using intraperitoneal injection. The screening was performed by flow cytometry. CBIS, Cell-Based Immunization and Screening; CHO, Chinese hamster ovary; hTIGIT, human T cell immunoreceptor with Ig and ITIM domains; mAbs, monoclonal antibodies.

Determination of binding affinity using TgMab-2 on hTIGIT-expressed cells by flow cytometry

To assess the apparent binding affinity of TgMab-2 with CHO/hTIGIT, we conducted a kinetic analysis of the interaction of TgMab-2 with CHO/hTIGIT by flow cytometry. The geometric mean of fluorescence intensity was plotted versus the concentration of TgMab-2 and fitted

by one-site binding models. The K_D of TgMab-2 for CHO/hTIGIT was determined to be 3.5×10^{-9} M (Fig. 3), indicating that TgMab-2 possesses a high affinity for CHO/hTIGIT cells.

Discussion

In this study, we successfully developed a novel anti-hTIGIT mAb, TgMab-2, using the CBIS method. In the past studies, anti-hTIGIT mAbs were acquired by hybridoma technology or phage display method. Yu *et al.* generated mAbs by immunizing hamsters with fusion proteins of mouse TIGIT and selected mAbs, which cross-reacted to hTIGIT.⁽¹⁾ Stanietzky *et al.* and Chauvin *et al.* produced mAbs by immunizing mice with TIGIT-Ig fusion protein⁽⁴⁾ and TIGIT-Fc fusion protein,⁽¹³⁾ respectively. Han *et al.* acquired mAbs with a high-throughput strategy using a phage-displayed human single-chain antibody library and CHO/TIGIT cells.⁽¹⁷⁾ Compared with these methods, the CBIS method can produce mAbs more quickly and simply because it does not require purification of proteins for immunization and screening of phage libraries.

Supplementary Table S1 shows anti-hTIGIT mAbs available on the market. Their immunogens are peptides or proteins, so TgMab-2 is the first mAb produced by immunization with hTIGIT-expressing cells. There are 28 out of 31 clones that can be used for flow cytometry, and most of them are available for one or two applications. Only three clones, such as 4A10, 4A11, and 4A12, are useful for all applications, flow cytometry, Western blotting, and immunohistochemistry. Previously, we succeeded in establishing mAbs against various membrane proteins, such as CD44,⁽¹⁸⁾ CD133,⁽¹⁶⁾ and CD20,⁽¹⁹⁾ using the CBIS method. In each protein, we got some mAbs, useful for flow cytometry, Western blotting, and immunohistochemistry with the same clone. Therefore,

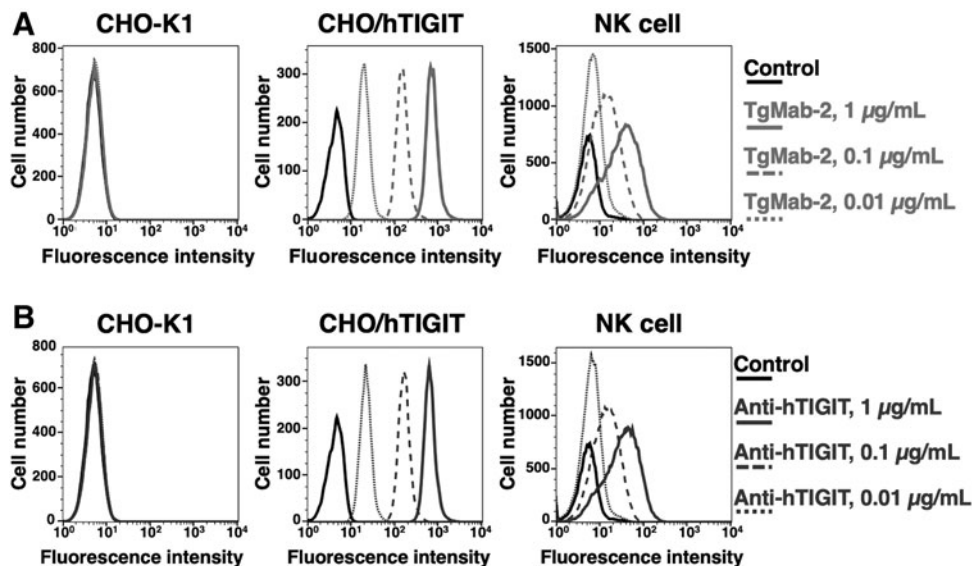


FIG. 2. Flow cytometry using anti-hTIGIT mAbs. (A) CHO/hTIGIT cells and NK cells were treated with 0.01–1 µg/mL of TgMab-2, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG; black line, negative control. (B) CHO/hTIGIT and NK cells were treated with 0.01–1 µg/mL of A15153G (BioLegend), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG; black line, negative control.

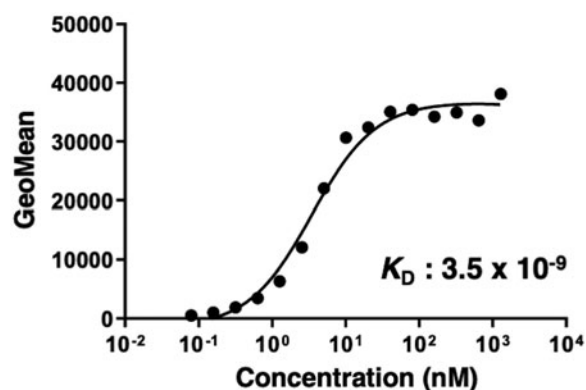


FIG. 3. Determination of the binding affinity of TgMab-2. CHO/hTIGIT was suspended in 100 μ L of serially diluted TgMab-2 (0.006–100 μ g/mL). Then, Alexa Fluor 488-conjugated anti-mouse IgG was added. Fluorescence data were collected using the BD FACSLyric.

in addition to investigating further characterizations of TgMab-2, such as the epitope and blockade of hTIGIT, we will produce another anti-hTIGIT mAb that can be used for all applications in the future.

Authors' Contributions

J.T., T.A., R.N., T.N., M.Y., H.Ho., T.T., and M.S. performed experiments; M.K.K. and H.Ha designed the experiments; J.T. and Y.K. wrote the article.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1

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