# TOOLS FOR TIGIT RESEARCH

Background

Tumor cells have devised ways to evade recognition by immune cells or to suppress activation of the immune system, subverting mechanisms that normally protect the body against abnormal cells. Tumor cells achieve immune suppression by secreting or promoting the secretion of cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL10), by recruiting regulatory T cells, myeloid-derived suppressive cells, and type 2 macrophages. In addition, tumor cells can engage immune checkpoint inhibitors that inhibit the effector functions of T cells and natural killer (NK) cells.

Immune checkpoints consist of ligand/receptor pairs that block immune function upon interaction [1]. Thus, tumor cells often over-express one or several immune checkpoint ligands, which bind to immune receptors present on the surface of T cells resulting in the blocking of T cell activation. Monoclonal therapeutic antibodies that block checkpoint inhibitors have shown remarkable efficacy in some types of cancer, restoring T cell anti-tumor activity, and have become prominent anti-cancer drugs [2]. New checkpoint inhibitors are now under intense scrutiny [3-4].

NK and T cells are partially regulated by the checkpoint receptors CD155, CD226, and TIGIT (T cell immunoreceptor with Ig and ITIM domains, also known as WUCAM, VSIG9 and Vstm3). TIGIT is a member of the poliovirus receptor (PVR) family of immunoglobin proteins, highly expressed in NK cells and in some T cells including follicular B helper T cells, activated CD4+, CD8+, and regulatory T cells [5-6]. TIGIT binds with high affinity to CD155 (PVR) on dendritic cells and macrophages, and to CD112 (PVRL2) with low affinity.

Interaction between CD155 and CD226 normally stimulates the immune system through positive regulation of NK and T cells. TIGIT competes with CD226 for binding with CD155 expressed on
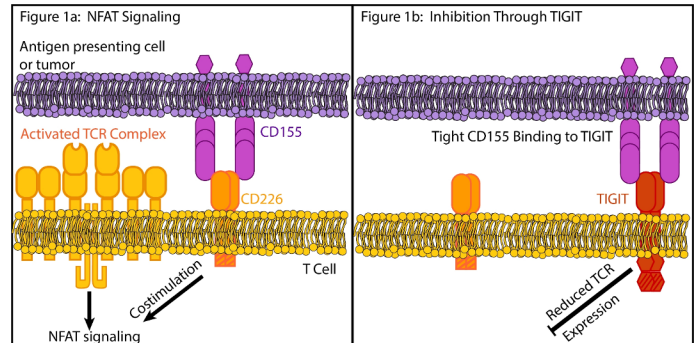
**Figure 1: Illustration of TIGIT mechanism of action**

antigen-presenting cells, and this results in immune suppression based on both competitive interaction and TIGIT-mediated intracellular signaling [7]. Indeed, upon binding to CD155, TIGIT recruits the Src homology (SH) domain-containing tyrosine phosphatases SHP1 and SHP2, or the Inositol phosphatase SHIP1 and SHIP2 to the TIGIT ITIM domain. This causes increased secretion of IL10 and decreased secretion of IL12B, suppresses NF-κB and NFAT T-cell receptor (TCR) signaling, and ultimately inhibits T cell proliferation and cytokine production.

Thus, TIGIT is an emerging checkpoint inhibitor [8] and blocking CD155:TIGIT interaction represents an attractive therapeutic strategy in cancer immunotherapy [9-10].
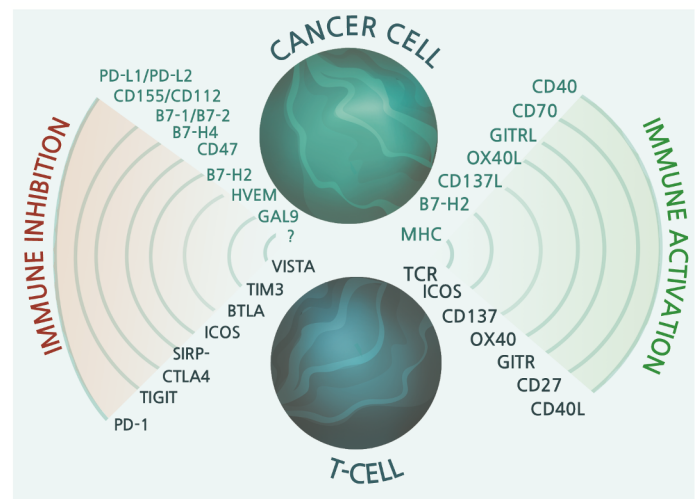
Recombinant cell lines

The TIGIT-HEK293 Recombinant Cell Line was generated to stably overexpress TIGIT. These cells are particularly useful to screen, characterize and validate antibodies against TIGIT, or to study the binding of TIGIT to partners such as CD155 (Poliovirus receptor, Nectin-like protein 5) or CD112. The expression of TIGIT was confirmed by flow cytometry (Figure 2).
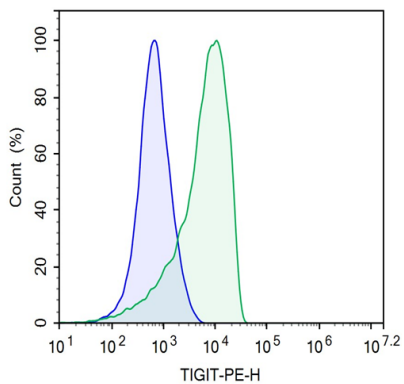
Figure 2. TIGIT cell surface expression in TIGIT HEK293 cells. TIGIT HEK293 cells (green) or control HEK293 cells (blue) were stained with PE-labeled anti-TIGIT Antibody (cat#71228) and analyzed by flow cytometry. Y-axis is the % cell number. X-axis is the intensity of PE.

Alternatively, CD155-expressing cells function as antigen-presenting cells to engage TIGIT-mediated responses in lymphocytes. CD155 is a transmembrane glycoprotein receptor highly expressed on dendritic cells, fibroblasts, and endothelial cells. It is also highly expressed in multiple tumor cell types including ovarian carcinoma, non-small cell lung cancer,
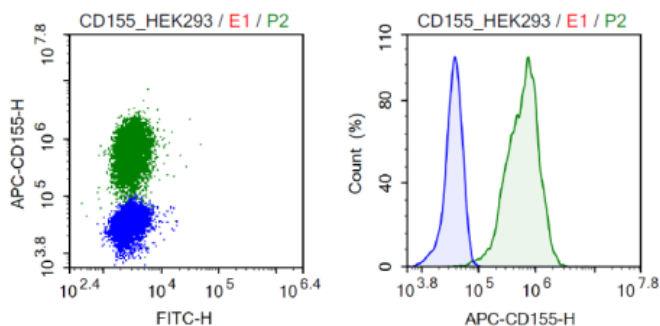

Figure 3: Expression of CD155 protein in CD155 HEK293 cells. CD155-positive cells (green) and parental HEK293 cells (blue) were stained with an APC-conjugated anti-human CD155 antibody and analyzed by flow cytometry. Y-axis is the % cell number. X-axis is the intensity of APC.

glioblastoma and colorectal carcinoma, playing a key role in tumor cell invasion and migration. CD155 binds to TIGIT with high affinity and to regulatory receptors CD226 (expressed on natural killer cells, monocytes and CD4+ T cells) and CD96. The expression of CD155 in CD155(PVR)-HEK293 Recombinant cells was evidence by flow cytometry (Figure 3).

Relevant resources include cell lines generated to express immunotherapy targets of interest such as PD-1, PD-L1, BCMA, CD19, CD20, BTLA, LIGHT, CD40, CD40L, among many other, in addition to primary cells such as CD4+ and CD8+ T cells and Normal Human Peripheral Blood Mononuclear Cells.

Of additional interest to the study of TIGIT are recombinant CHO cells overexpressing Nectin4. In contrast to other Nectins, which are abundant in adult tissues, Nectin4 is expressed during fetal development but declines in adult life. Its expression, however, returns specifically in lung, breast, pancreas and ovarian cancers. It has been shown that Nectin4 modulates the expression of proteins involved in epithelial–mesenchymal transition. In addition, Nectin4 is a cancer-specific ligand for TIGIT. Its expression is associated with poor prognosis, suggesting it could be a valid target for cancer immunotherapy.
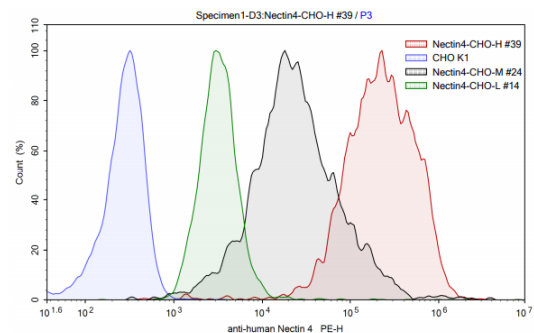

Figure 4: Expression of Nectin4 in Nectin4 CHO Recombinant cells. The cells were incubated with PE-conjugated anti-human Nectin4 antibody and analyzed by flow cytometry to detect surface expression of the protein. Red, black and green: high, medium and low expression, respectively. Blue: parental CHO cells.

The Nectin4-CHO Recombinant cell line was stably transfected to express full length human Nectin4, as confirmed by flow cytometry (Figure 4). Three clonal cell lines were selected for low, medium and high expression of Nectin4 to model heterogenous tumor cells expression.

2

# TIGIT CRISPR/Cas Lentiviruses (integrating and non-integrating)

To achieve efficient TIGIT knock-out in hard-to-transfect cells, BPS Bioscience has developed off-the-shelf integrating and non-integrating CRISPR/Cas9 lentiviruses. The lentiviruses are HIV-based pseudotyped virus particles, which means that the HIV envelope protein has been replaced with VSV-G (Vesicular stomatitis virus G), a protein that binds to the ubiquitously expressed LDL receptor (low-density lipoprotein). The viral particles are ready for infection and capable of transducing most types of dividing or non-dividing mammalian cells. None of the HIV genes from the backbone virus are expressed in the transduced cells, therefore these viral particles are replication-incompetent and can be used in a Biosafety Level 2 facility, which makes them accessible to many research laboratories.

Pre-validated TIGIT CRISPR/Cas9 Lentivirus (Integrating) and TIGIT CRISPR/Cas9 Lentivirus (non-integrating) allow for dual expression of Cas9 and TIGIT-directed single-guide RNAs. Upon infection of the target cells, a Cas9-NSL-Flag gene is transduced into the cells and expressed under the control of constitutive mammalian promoter, along with 4 sgRNAs driven by a U6 promoter and a puromycin selection marker (Figure 5).
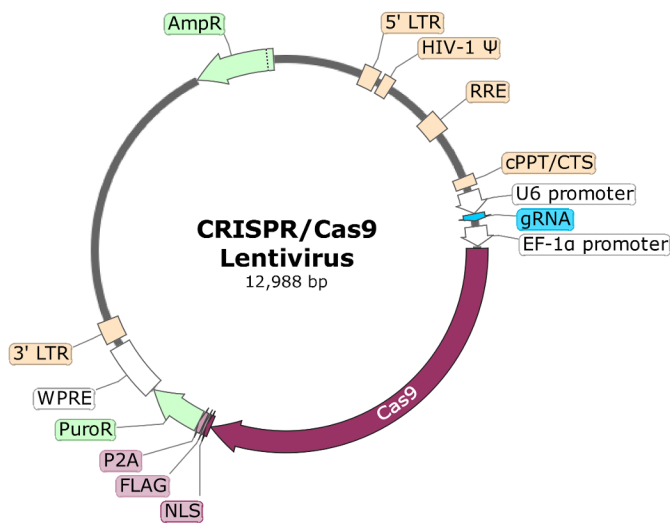
Figure 5: Diagram of the CRISPR/Cas9 lentiviral construct.

When using the integrating lentivirus, puromycin selection forces integration of the construct into the genome and results in sustained expression of both Cas9 and sgRNA, which increases knock-down efficiency (Figure 6).
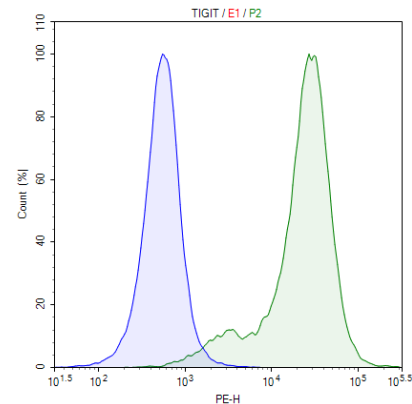

Figure 6: Knock-down of TIGIT in TIGIT-HEK293 cells. TIGIT over-expressing HEK293 cells (cat#79332) were transduced via spinoculation with 5,000,000 TU/well of TIGIT CRISPR/Cas9 lentivirus. 72 hours after transduction, cells were stained with PE-labeled anti-human TIGIT antibody (cat#71228) and analyzed by flow cytometry. Parental TIGIT over-expressing HEK293 cells are shown in green, and the KO transduced cells are shown in blue.

However, integration into the genome occurs randomly and may occasionally disrupt a biologically relevant gene, with unpredictable effects not imputable to Cas9 nuclease activity or to the function of the target gene. In addition, sustained expression of Cas9 and sgRNAs may lead to the accumulation of off-target cleavage events over time. This is a critical factor to consider in studies aiming at validating a new therapeutic target or the effect of a new drug. Our integrating/non-integrating virus pairs were explicitly designed to control for this. The non-integrating virus was engineered with the same plasmid construct as the integrating virus, but with a mutated integrase. An integrase-deficient virus permits transient Cas9 and sgRNA expression in the target cell, but not stable expression. This may result in lower knock-down efficacy compared to an integrating lentivirus; however, it eliminates the risk of random insertion into a biologically relevant gene while considerably diminishing the occurrence of off-target cleavage.

TIGIT/NFAT Reporter Jurkat Cell Line

Jurkat is an immortalized human T lymphocyte cell line established from a patient with leukemia. A firefly luciferase reporter, expressed under the control of a promoter containing an NFAT response element, monitors the activation of transcription factor NFAT (Nuclear factor of activated T cells) by upstream signals. This cell line has been validated for response to various NFAT agonists such as ionomycin, and for T cell activation through a variety of TCR activators including anti-CD3ε scFv/CHO cells. In addition, the cells were stably transfected to constitutively express TIGIT.
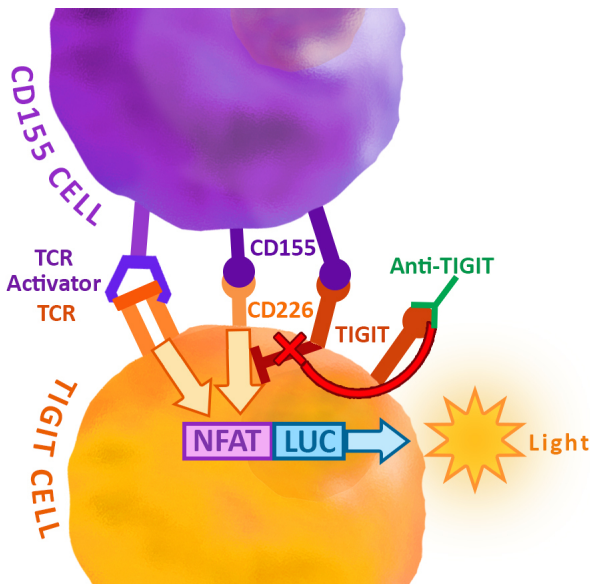
The TIGIT/NFAT-luciferase reporter Jurkat cell line is a double transfectant cell line that can be used as a surrogate T cell to develop and optimize functional cellular assays. Here, functional studies were performed in co-culture of TIGIT/NFAT Reporter Jurkat cells with CD155-presenting cells. The CD155/TCRa Recombinant CHO cells are stably transfected to
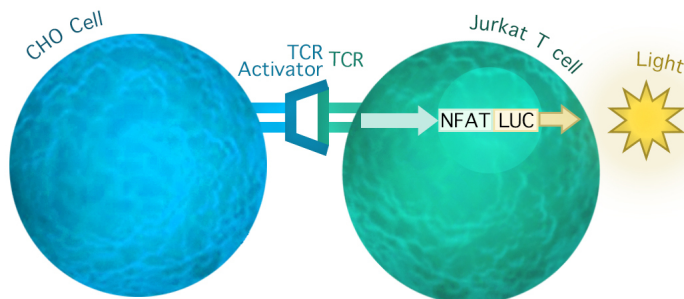

constitutively express human CD155 and an engineered T cell receptor (TCR) activator. TCRa CHO cells are capable of activating the TCR in T cells, leading to NFAT signaling and cell proliferation.

Applications:

- Develop/optimize functional assays
- Screen for compound effect on TIGIT signaling in a cellular context
- Characterize the biological activity of TIGIT and its interactions with ligands

High levels of CD155 expression were confirmed by flow cytometry (Figure 7).
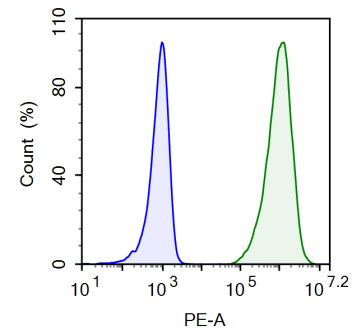
Figure 7: Cell surface expression of CD155 in CD155/TCR Activator CHO cells. CD155/TCR Activator cells (green) or control CHO cells (blue) were stained with PE-labeled anti-CD155 antibody and analyzed by flow cytometry. Y-axis is the % cell number. X-axis is PE intensity.

Co-culture of NFAT Reporter Jurkat cells with CD155-expressing cells capable of activating the TCR (T cell receptor) results in NFAT activation and expression of luciferase when TIGIT is absent. However, in TIGIT-NFAT reporter Jurkat cells, binding of TIGIT to CD155 suppresses the TCR activation signal through the recruitment of tyrosine phosphatases SHP1 and SHP2 to the ITIM domain of TIGIT, thereby preventing the NFAT-dependent expression of luciferase.

In the presence of TIGIT inhibitors, such as neutralizing antibodies, the interaction between TIGIT and CD155 does not occur. Therefore, no repression takes place and the TCR is activated, resulting in NFAT stimulation and induction of luciferase expression (Figure 8). The cells are ideal to screen and validate anti-TIGIT antibodies or compounds that are expected to block the interaction of TIGIT with its ligands.
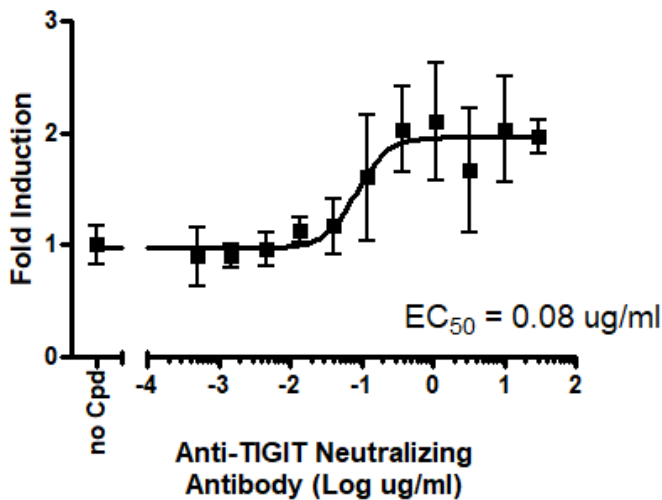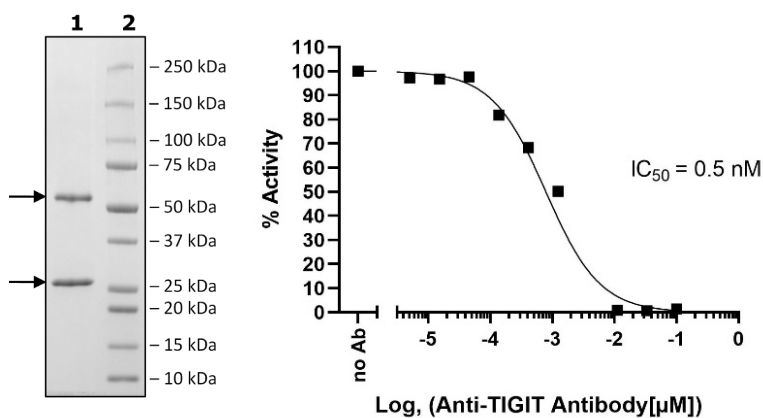

Figure 8: Blocking of TIGIT:CD155 interaction increases NFAT-dependent luciferase expression activated by the TCR. Increasing concentrations of TIGIT neutralizing antibody (cat#71340) were incubated with TIGIT-NFAT reporter cells for 30 min before adding them to a culture of CD155-TCRa CHO cells. Six hours later, luciferase activity was measured using the ONE-Step luciferase assay system (cat#60690).

Validation experiments indicated that anti-TIGIT antibodies induced the NFAT luciferase reporter activity in TIGIT/NFAT Reporter-Jurkat cells, but not in negative control NFAT Reporter Jurkat cells that do not express TIGIT, when co-cultured with CD155/TCR Activator-CHO cells (Figure 9, upper panel). Alternatively, anti-TIGIT antibodies induced the NFAT luciferase reporter activity in TIGIT/NFAT Reporter-Jurkat cells when presented with CD155-expressing cells but not when presented with parental TCR Activator CHO cells capable of TCR

activation but that do not express CD155 (Figure 9, lower panel).
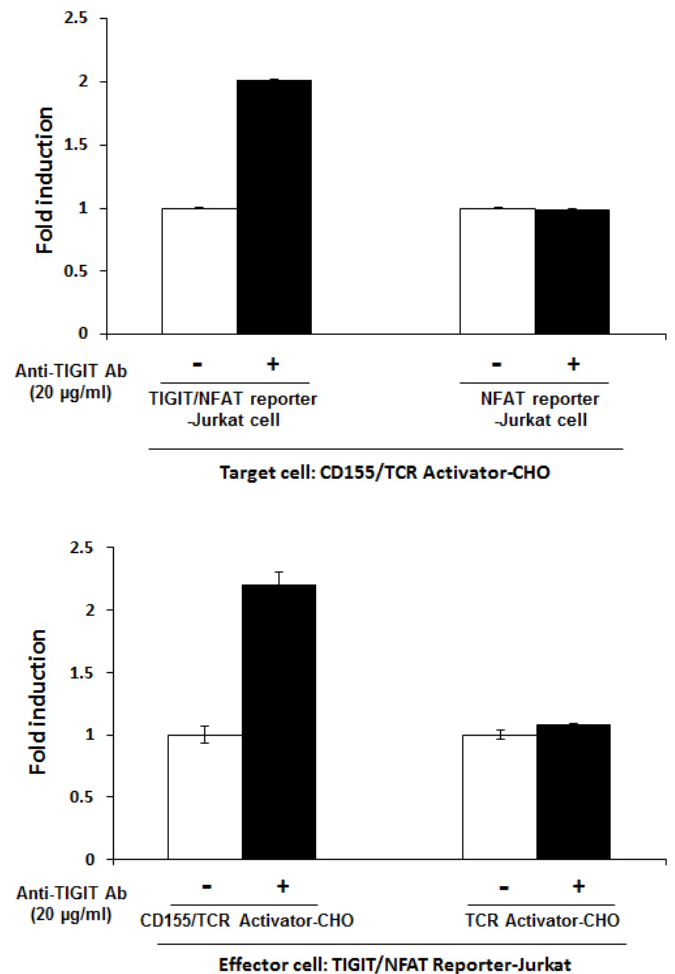
Figure 9: Blocking of TIGIT:CD155 interaction increases NFAT-dependent luciferase expression activated by the TCR. Upper panel: Anti-TIGIT neutralizing antibody was incubated with TIGIT-NFAT reporter cells or with parental NFAT-reporter cells for 30 min before adding the cell suspension to a culture of CD155-TCRa CHO cells. **Lower panel:** Anti-TIGIT neutralizing antibody was incubated with TIGIT-NFAT reporter cells for 30 min before adding the cell suspension to a culture of CD155-TCRa CHO cells or CD155-negative TCRa CHO cells.

IC$_{50}$ = 0.5 nM

Neutralizing Antibodies

The functional experiments described above used two neutralizing antibodies that block the interaction of TIGIT with CD155. These antibodies were affinity purified and validated for inhibition of TIGIT:CD155 interaction in a biochemical assay (Figure 10). Similar results were obtained with the other antibody (not shown, cat#71218).

BPS Bioscience's anti-TIGIT antibody was used in the study by He *et al.* entitled "CD155T/TIGIT Signaling Regulates CD8+ T-cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer" [11]. In this article the authors evaluated the role of TIGIT in gastric cancer. The antibody was used in co-culture assays to demonstrate that patient-derived gastric tissue or established cell lines, which express CD155, were able to engage TIGIT in stimulated CD8+ T cells. TIGIT binding to CD155 resulted in impairment of T cell metabolism and decreased production of IFNγ, an immuno-suppressive effect neutralized by the antibody.

The anti-TIGIT antibody was also used in the study from Lu *et al.* entitled "Co-inhibition of TIGIT, PD1, and Tim3 reverses dysfunction of Wilms tumor protein-1 (WT1)-specific CD8+ T lymphocytes after dendritic cell vaccination in gastric cancer" [12] to show that neutralization of TIGIT improved T cell function in a cellular model of gastric cancer.

A PE (Phycoerythrin)-labeled version of anti-TIGIT antibody was produced (cat#71228) to allow the labeling of TIGIT-expressing cells for flow cytometry (as shown in Figure 11) and immunofluorescence microscopy. These antibodies may also be used to select TIGIT-expressing cells by FACS (fluorescence-activated cell sorting).
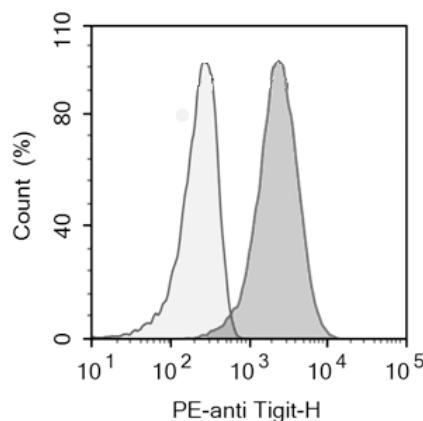
Figure 11: Cell surface expression of TIGIT assessed by flow cytometry. Control Jurkat cells (light grey) and TIGIT-expressing Jurkat cells (dark grey) were stained with human PE anti-TIGIT antibody and analyzed by flow cytometry.

Applications:

- Useful in assay development and optimization efforts
- Serve as reference when screening for TIGIT neutralizing antibodies in biochemical assays
- Useful to assess the biological function of TIGIT through inhibition of TIGIT:ligand binding
- Staining or sorting of TIGIT-positive cells

Biochemical TIGIT:Ligand binding assays

BPS Bioscience's TIGIT binding assays monitor the interaction of purified TIGIT with CD155 or CD112 in a homogeneous format using the fluorescence transfer principles of the Alpha® assay. Homogeneous, in-solution assays are ideal for high throughput screening since they are fast, can be performed in small volumes, and do not require washing steps.

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┌─────────────────────────────────┐
│   TIGIT-Fc-Biotin + compound    │
└─────────────────────────────────┘
              ↓ 30 min
┌─────────────────────────────────┐
│         Add His-CD155           │
└─────────────────────────────────┘
              ↓ 1 hour
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│   Add  Ni Chelate Acceptor      │
│            beads                │
└─────────────────────────────────┘
              ↓ 30 min
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│  Add Streptavidin-conjugated    │
│         donor beads             │
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│         Capture Data            │
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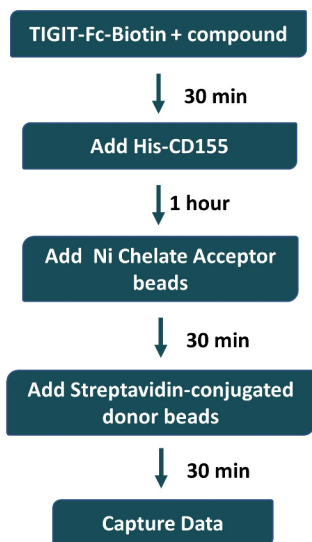
In these assays either the biotinylated TIGIT-Fc recombinant protein or the His-tagged CD155 or CD112 ligand is pre-incubated with the compound of interest (for example biotinylated TIGIT is pre-incubated with neutralizing anti-TIGIT antibody). The biotinylated TIGIT and His-tagged CD155 or CD112 are incubated together, then Nickel-chelate Acceptor beads and Streptavidin-conjugated Donor beads are added. Data is captured using a compatible Alpha® plate reader (PerkinElmer).

The beads bind to His-CD155 (or CD112) and biotinylated TIGIT, respectively. Interaction between TIGIT and its ligand brings the acceptor and donor beads into proximal range, enabling the energy transfer from the donor to acceptor beads after laser excitation. When the interaction is blocked by a compound of interest, energy transfer does not occur.
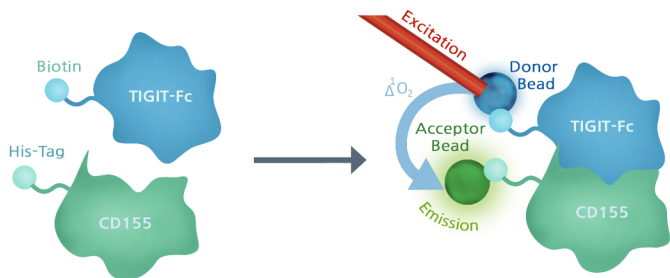

All assays have been validated using a neutralizing anti-TIGIT antibody, as shown for example in Figure 12 using the TIGIT:CD112 Homogeneous Assay Kit .

$IC_{50} = 0.001\mu M$

anti-TIGIT mAb, (Log [μM])
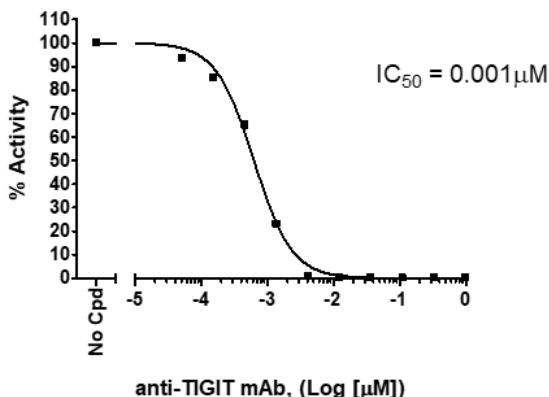
Figure 12: Inhibition of TIGIT:CD112 binding by neutralizing anti-TIGIT antibody. The biotinylated TIGIT-Fc recombinant protein was pre-incubated with increasing concentrations of neutralizing anti-TIGIT antibody for 30 min, and the complex was added to His-tagged CD112 protein for 30 min longer. Acceptor beads and donor beads were added to the reaction and data was captured using a plater reader compatible with AlphaLisa®. Results are expressed in term of Alpha-count. The negative control consisted of a no-antibody condition.

In addition, Human TIGIT:CD155 Homogeneous Assay Kit and Mouse TIGIT:Mouse CD155 (mTIGIT:mCD155) Homogeneous Assay Kit (Figure 13) are available to assess TIGIT:CD155 interaction.

$IC_{50}=4.6$ nM

a-TIGIT, log[nM]
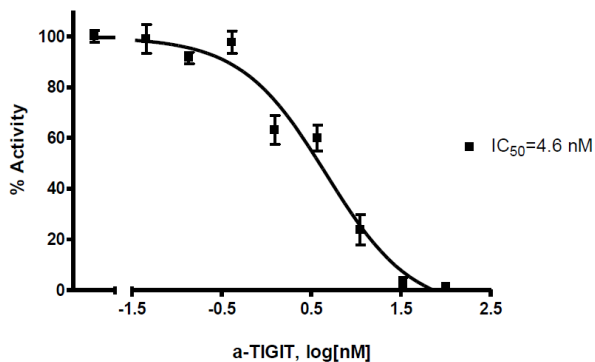
Figure 13: Inhibition of mouse TIGIT:CD155 binding by neutralizing anti-TIGIT antibody. The biotinylated mouse TIGIT-Fc recombinant protein was pre-incubated with increasing concentrations of neutralizing anti-TIGIT antibody for 30 min, and the complex was added to mouse His-tagged CD155 protein for 30 min longer. Acceptor beads and donor beads were added to the reaction and data was captured using a plater reader compatible with AlphaLisa®. Results are expressed in term of Alpha-count. The negative control consisted of a no-antibody condition.

Purified Recombinant Proteins

The quality of the homogeneous TIGIT binding assay kits depends heavily on the quality of the proteins used in the assay. BPS Bioscience has produced high-quality purified recombinant proteins for use in biochemical assays: human TIGIT-Fc fusion (cat #71186), biotinylated recombinant human TIGIT Avi-Tag™ as shown in Figure 14 (cat#71251), and biotinylated recombinant mouse TIGIT-Fc fusion AviTag™ (cat#79269).

In addition, purified recombinant proteins, labeled or unlabeled, containing either an N-terminal or a C-terminal tag are very desirable because they offer low-background, high-specificity staining options to detect a protein of interest on the cell surface.

To facilitate protein biotinylation, an AviTag™ is added at the N-terminal end of the recombinant protein. The recombinant proteins were enzymatically biotinylated using the Avi-Tag™ and affinity purified. BPS Bioscience's HiP™ indicates a high purity protein (≥90% pure) with less than 10% aggregation as measured by gel filtration.

Purified proteins were used in the study by Lin *et al.*
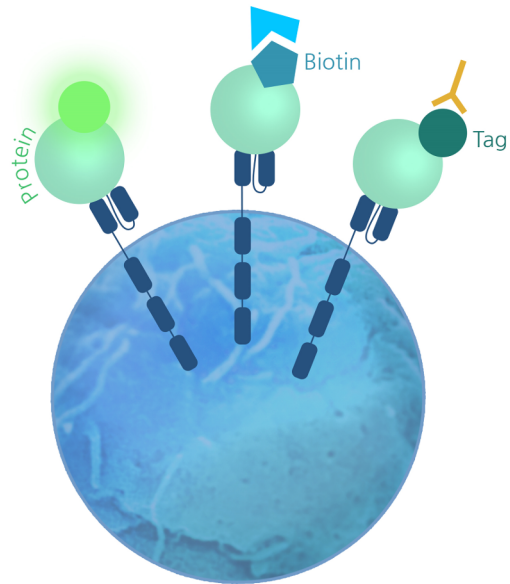
entitled "The N-linked glycosylations of TIGIT Asn32 and Asn101 facilitate PVR/TIGIT interaction" [13] in which the authors show that TIGIT glycosylation is essential for its proper interaction with CD155, leading them to hypothesize that TIGIT glycosylation sites may serve as target for neutralizing antibodies.
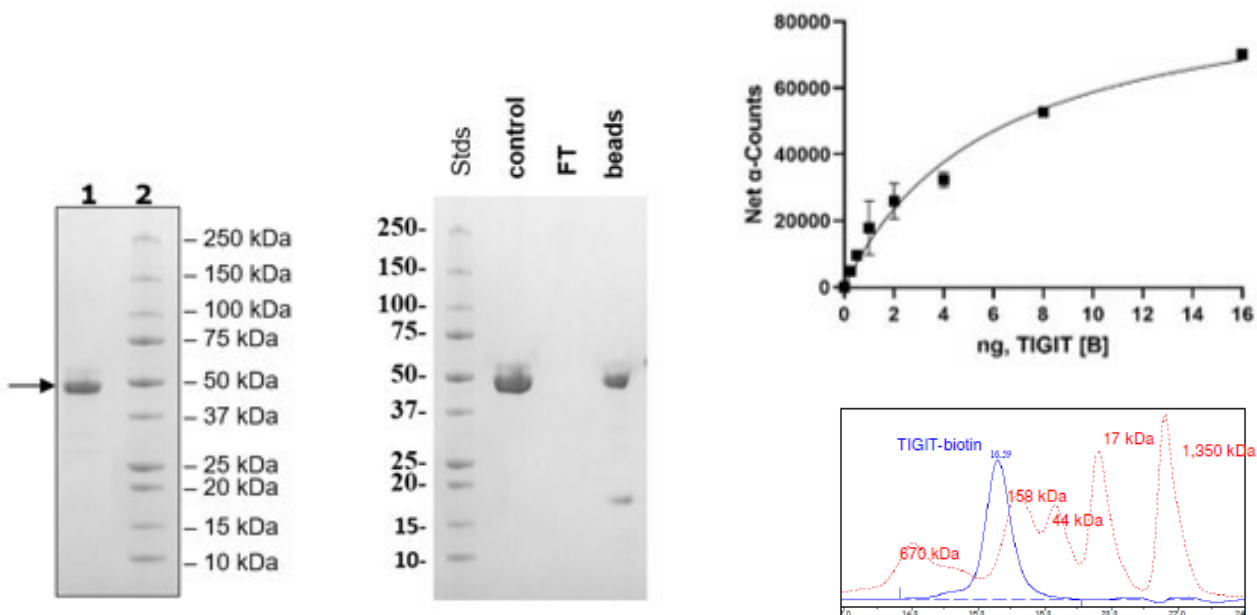
Figure 14: Validation of TIGIT-Fc, Avi-Tag, Biotin-Labeled (Human) HiP™ (cat#71251). Left panel: 4-20% SDS-PAGE Coomassie staining. **Middle panel:** Biotin-Avidin pull down of the labeled protein. **Upper Right panel:** Representative result of TIGIT:CD155 binding activity using the TIGIT:CD155 homogeneous Assay. The assay was performed according to the kit protocol with titration of TIGIT. **Lower panel:** Gel filtration trace of the purified protein.

Conclusion

In addition to TIGIT-focused tools, our portfolio of immunotherapy products includes a variety of recombinant proteins, lentiviruses, and reporter cell lines to support research on common targets of interest. Cytokine assay kits and reporter cell systems allow for functional validation of using biologically relevant cellular models. The design of new therapeutics and their translation into the clinic requires extensive validation and optimization at various steps of the process, which in turn necessitates the development of many types of assays. This can be time and resource consuming, therefore the availability of pre-validated tools and assay kits can significantly accelerate research efforts.

BPS Bioscience, scientist-founded and scientist-driven, supports researchers at all phases their research project to accelerate the clinical translation of new treatments for human diseases.

Immunotherapy screening services

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