Research and characterization of bispecific antibodies for cancer immunotherapies

Recent progress in cancer immunotherapy using monoclonal antibodies (mAbs) has spurred the investigation of alternative forms of therapeutic antibodies with enhanced specificity and potency. Bispecific antibodies (bsAbs) are a rapidly growing area of immunotherapy, attracting attention as a next-generation strategy for the treatment of cancer. BsAbs are recombinant molecules designed to simultaneously target two distinct antigens or epitopes. These antibodies have a traditional Y-shaped structure composed of dual heavy and light chains, with each arm having a unique active site that binds to a different target.

BsAbs can be categorized based on their mechanism of action (MoA). Many are engineered to bridge T cells and tumor cells by simultaneously binding to a tumor-associated antigen expressed on tumor cells and CD3 on T cells. This leads to the redirection of effector T cells' cytotoxic activity toward tumor cells. Research has also focused on bsAbs capable of blocking two interrelated signaling pathways through the targeting of two epitopes on tumor cells, as well as those that block immune checkpoints.

The distinct MoA of bsAbs has yielded promising results in preclinical and clinical studies for various types of cancers. Notably, eight cancer immunotherapy bsAbs have received approval by the FDA and/or EMA, including Catumaxomab, which was initially approved in 2009 but was later withdrawn from the EU market in 2017 (Table 1).

Table 1: Approved therapeutic bsAbs for cancer indications.

bsAb	Brand Name	Company	Target	Target	Mechanism of Action	Indications	Approved by	Approval Date
Catumaxomab	Removab	Neovii	CD3 (T cell)	EpCAM (cancer cell)	Recruitment and activation of T cells	Malignant ascites	EMA	Apr 2009 (withdrawn in Jun 2017)
Blinatumomab	Blincyto	Amgen	CD3 (T cell)	CD19 (cancer cell)	Recruitment and activation of T cells	B-cell precursor acute lymphoblastic leukemia (ALL)	EMA and FDA	Dec 2014
Amivantamab	Rybrevant	Janssen	EGFR (cancer cell)	c-MET (cancer cell)	Blocking of dual signal pathways	Non-small cell lung cancer (NSCLC)	EMA and FDA	May 2021
Tebentafusp	Kimmtrak	Immunocore	CD3 (T cell)	gp100 (cancer cell)	Recruitment and activation of T cells	Unresectable or metastatic uveal melanoma	EMA and FDA	Jan 2022
Mosunetuzumab	Lunsumio	Roche	CD3 (T cell)	CD20 (cancer cell)	Recruitment and activation of T cells	Relapsed or refractory follicular lymphoma	EMA and FDA	Jun 2022
Teclistamab	Tecvayli	Janssen	CD3 (T cell)	BCMA (cancer cell)	Recruitment and activation of T cells	Relapsed or refractory multiple myeloma	EMA and FDA	Aug 2022
Epcoritamab-bysp	Epkinly	Genmab	CD20 (B cell)	CD3 (T cell)	Recruitment and activation of T cells	Relapsed or refractory diffuse large B-cell lymphoma	FDA	May 2023
Glofitamab-gxbm	Columvi	Genentech	CD20 (B cell)	CD3 (T cell)	Recruitment and activation of T cells	Relapsed or refractory (R/R) diffuse large B-cell lymphoma (DLBCL)	FDA	June 2023

Various manufacturing approaches facilitate the production of bsAbs, including chemical conjugation, quadromas, and genetic/protein engineering. Over the past two decades, bsAb development has been revolutionized by advances in gene engineering and pharmaceutical techniques that enable the development of a wide variety of molecular structures.

BsAbs are characterized by their target specificity, format, binding affinities, MoA, and various other factors related to their structure, function, and potential therapeutic applications. The comprehensive characterization of bsAbs is crucial for their design and development, and to evaluate their efficacy and safety. It necessitates a multifaceted approach that combines a range of techniques to gain a comprehensive understanding of the antibody's properties, behavior, and ability to drive the immune system to target cancer cells. Here, we describe several functional approaches to characterize novel bsAbs targeting immune checkpoints and the tumor necrosis factor (TNF) superfamily.

Targeting immune checkpoints

Immune checkpoints, such as anti-programmed cell death protein-1 (PD-1), anti-programmed cell death protein ligand-1 (PD-L1), and anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), play pivotal roles in the immune evasion of cancer cells. Several mAbs targeting immune checkpoints have been approved by the FDA for the treatment of various cancer types, including ipilimumab which targets the CTLA-4 pathway, and atezolizumab, avelumab, durvalumab, nivolumab, cemiplimab, and pembrolizumab targeting the PD-L/PD-L1 pathway. Despite impressive outcomes, the response rate of solo administration of mAb checkpoint inhibitors remains relatively low in many cases.[1] Some patients respond initially and then lose benefit, while others do not respond at all. Consequently, the development of bsAbs that can target a checkpoint and a dominant signaling pathway or an alternative checkpoint is a promising avenue for advancing cancer treatment.

Evaluation of an anti-PD-1–GITR-L bispecific antibody

In a recent study, Chan *et al*. investigated the effects of a bispecific molecule consisting of an anti-PD-1 antibody fused with a multimeric glucocorticoid-induced TNF receptor-related protein-ligand (GITR-L) on immune cell activity.[2] GITR-L is a costimulatory receptor that plays a pivotal role in regulating the effector functions of T cells.

As part of their investigation, the researchers conducted PBMC co-stimulation assays to explore the effect of bsAb treatment on cytokine release. Studying cytokines during bsAb characterization is crucial for understanding the immunomodulatory effects of bsAbs *in vitro*. PBMCs were plated and treated with different dilutions of treatment antibodies in the presence of anti-CD3. After 48 and 96 hours of incubation levels of IFN-γ and IL-2 were detected in the collected supernatant using AlphaLISA™ kits and results were quantified using an EnSpire™ Alpha multimode plate reader.

AlphaLISA is a bead-based assay technology that can detect small amounts of analytes in microplates using donor and acceptor beads (Figure 1). If an acceptor bead is near a donor bead, energy is transferred to the acceptor bead resulting in light production. AlphaLISA cytokine assays use two unique antibodies specific for the cytokine of interest. One is biotinylated and binds to a streptavidin-coated donor bead while the other is directly conjugated to the acceptor bead. In the presence of a cytokine, the antibodies bind to the cytokine, bringing both the donor and acceptor beads into close proximity resulting in light emission. The generated AlphaLISA signal is proportional to the amount of cytokine present in the sample.

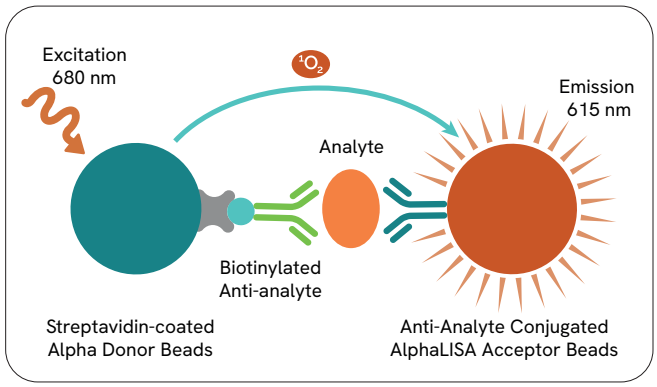

Figure 1: Principle of the AlphaLISA technology

The assay revealed that treatment with the bsAb (anti-PD-1–GITR-L) induced an enhanced dose-dependent proliferation of cytokines in comparison to single and 1:1 combination treatment (Figure 1). These data not only provide insights into how the bsAb influenced immune cell behavior but also highlight how the anti-PD-1–GITR-L bispecific had a different MoA compared to the effect of mono- and combination therapies.

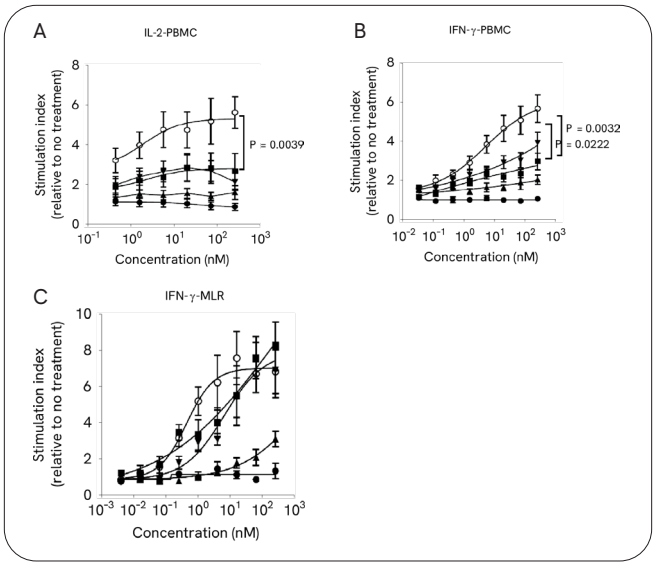

Figure 2: Human PBMC co-stimulation assay following the indicated treatments in the presence of anti-CD3. Cells and supernatants were harvested/collected for assessment of (A) IL-2, (B) IFN-γ, and (C) IFN-γ secretion in autologous CD4+ T cell MLR. Image credit: Chan, S., Belmar, N., Ho, S. *et al.* An anti-PD-1-GITR-L bispecific agonist induces GITR clustering-mediated T cell activation for cancer immunotherapy. *Nat Cancer* 3, 337-354 (2022). https://doi.org/10.1038/s43018-022-00334-9

Evaluation of an anti-PD-L1–VEGF bispecific antibody

Another group, led by Xiaopei Cui from Fudan University in China, developed a novel bsAb, named HB0025, which simultaneously targets PD-L1 and vascular endothelial growth factor (VEGF).[3] The bispecific was formed by fusing the domain 2 of VEGF receptor 1 (VEGFR1D2) with an anti-PD-L1 mAb. As part of their study, the researchers evaluated the ability of HB0025 to block the interaction between PD-1 and PD-L1 by measuring levels of cytokine secretion. They also examined the capacity of HB0025 to inhibit VEGF-mediated cell migration.

PBMCs were co-cultured with dendritic cells in 96-well plates and exposed to treatment with HB0025, atezolizumab (a PD-L1-targeting antibody), bevacizumab (a VEGF-targeting antibody), or a negative control (900543).
Following a five-day incubation period, a homogeneous time-resolved fluorescence (HTRF™) protocol was used to determine the concentration of secreted IL-2 in the collected supernatant. HTRF is a homogeneous method that combines standard fluorescence resonance energy transfer (FRET) technology with the time-resolved measurement of fluorescence (Figure 3). FRET is based on the transfer of energy between two fluorophores (a donor and an acceptor) when in close proximity and it can be used to detect cytokine secretion.

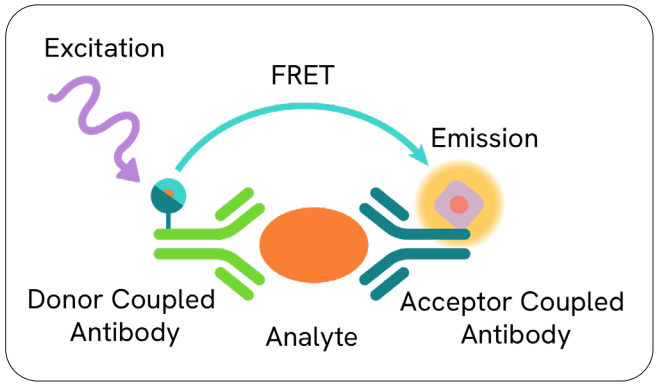

Figure 3: Principle of the HTRF assay

Analysis revealed that both HB0025 and atezolizumab prompted an increase in the secretion of IL-2 in a dose-dependent manner. Notably, the VEGF-targeting antibody bevacizumab did not have the same impact on IL-2 secretion (Figure 4).

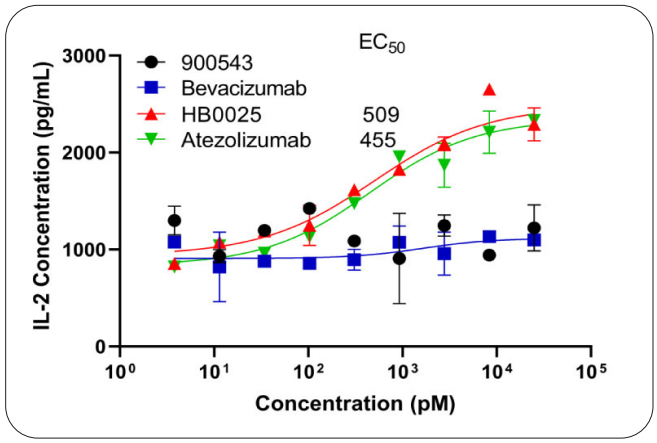

Figure 4: The dose-effect fitting curve of antibodies on the recovery of human IL-2 secretion, taking 900543 as the negative control. Image credit: Cui X, Jia H, Xin H, Zhang L, Chen S, Xia S, Li X, Xu W, Chen X, Feng Y, Wei X, Yu H, Wang Y, Zhan Y, Zhu X and Zhang X (2021) A Novel Bispecific Antibody Targeting PD-L1 and VEGF With Combined Anti-Tumor Activities. *Front. Immunol.* 12:778978. doi: 10.3389/fimmu.2021.778978

In contrast, when they evaluated the migration inhibition rate of human umbilical vein endothelial cells (HUVECs) using a HUVEC cell migration assay, both HB0025 and the bevacizumab prevented VEGF-mediated cell migration in a dose-dependent manner. Taken together, these findings demonstrate the antitumor effects of HB0025 and suggest that the novel bsAb effectively maintains the binding and blocking capacities of both its parent molecules.

TNF superfamily

The TNF superfamily pathway plays a pivotal role in controlling costimulatory signals that regulate immune cell survival, proliferation, differentiation, and effector functions. Comprised of 19 proteins and 29 receptors, the superfamily has been implicated in several inflammatory and autoimmune diseases, as well as cancer. Interactions between TNF ligands and their receptors can activate signaling pathways that mediate pro-tumor effects.

One member of the superfamily is CD30, which is known to be expressed in Hodgkin and Reed-Sternberg (HRS) cells – the malignant cells in Hodgkin lymphoma (HL). In 2011, the FDA approved an antibody-drug conjugate (ADC) targeting CD30, known as brentuximab vedotin, for the treatment of HL. Even so, the treatment has been associated with various toxicities, and more than 10% of patients relapse.[4]

Development of a bispecific antibody targeting CD30 and CD137 on HRS cells

HRS cells have been found to express another TNF receptor family member, CD137, with over 80% of HL cases featuring CD137-expressing cells. In light of this, Rajendran *et al.* decided to investigate whether simultaneous targeting of CD30 and CD137 with a bsAb could enhance the specificity of an antibody-based treatment for HL.[4] The bsAb was designed to selectively bind to CD30/CD137-double positive HRS cells.

To test the bsAb's killing efficiency, the group conducted an antibody-dependent cellular cytotoxicity (ADCC) assay using a DELFIA™ EuTDA cytotoxicity kit. DELFIA reagents offer a time-resolved fluorescence assay format that can be used to specifically label target cells with a ligand prior to performing ADCC co-culturing assays. For the study, target HRS cells were labeled with DELFIA BATDA reagent and seeded into 96-well plates along with bispecific and control antibodies. Effector cells (NK cells) were then added and allowed to incubate for three hours. The assay's readout was based on the measurement of fluorescence using a VICTOR™ Nivo™ multi-label microplate reader, with the measured signal correlating directly with the amount of lysed cells. Figure 5 describes the ADCC process utilizing the DELFIA cytotoxicity reagent.

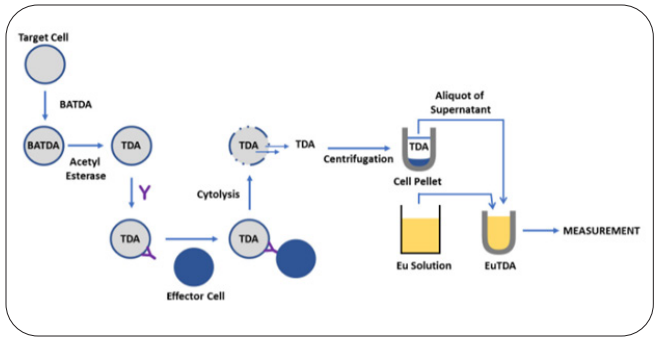

Figure 5: DELFIA EuTDA Cytotoxicity Assay for ADCC Determination. Target cells are first loaded with BATDA reagent then combined with antibody and co-cultured with chosen immune effector cell type. The antibody binds antigen on the target cell surface while the effector cells bind the Fc region of the antibody to form a complex with the target cells. Complex formation activates the immune effector cells which proceed to cytolyze the target cells releasing TDA into the supernatant. A supernatant is transferred to a fresh assay plate and europium solution is added to form the active lanthanide (EuTDA). A signal is detected using DELFIA time-resolved fluorescence (TRF) settings on a compatible plate reader (excitation at 340 nm and emission at 615 nm)

The results demonstrated that treatment with two bsAb clones led to a four-fold increase in the killing of double-positive ($CD30^+CD137^+$) cells compared to control antibodies (Figure 6). Additionally, the clones demonstrated comparable killing efficiency on single-positive cells when contrasted with spontaneous lysis levels. This indicates the selectivity of the bsAb towards double-positive cells, highlighting their potential to trigger ADCC against specific cell populations.

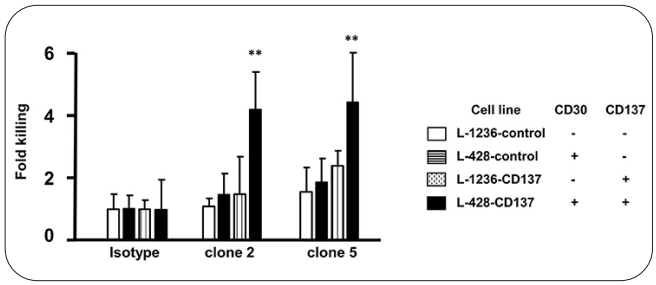

Figure 6: ADCC activity of bispecific antibodies. HL cells were labeled with 1 µl/ml of BATDA reagent and treated with 5µg/ml of bispecific antibodies (137A-30A, 137A-30B) or human IgG (Isotype) for 40min at 4°C. Effector NK cells were added at E:T of 20:1 and incubated for more than 3 hours. Means ± SD of fold killing are represented (**p < 0.01). Data are representative of three independent experiments. Image credit: Rajendran S, Li Y, Ngoh E, Wong HY, Cheng MS, Wang C-I and Schwarz H (2019) Development of a Bispecific Antibody Targeting CD30 and CD137 on Hodgkin and Reed-Sternberg Cells. *Front. Oncol.* 9:945. doi: 10.3389/fonc.2019.00945

Conclusion

Therapeutic bsAbs are a rapidly growing area of immunotherapy, gaining attention as a next-generation approach for combating cancer. So far, seven bsAbs have gained regulatory approval for oncology indications, with many promising candidates progressing through preclinical and early clinical phases. The success of bsAbs centers on their ability to overcome the limitations of current immunotherapies, as well as provide enhanced efficacy and safety.

Notably, bsAbs targeting immune checkpoints or the TNF superfamily have emerged as alternative approaches to current monotherapies. Given their dual targeting capabilities, both immune checkpoint and TNF superfamily-targeting bsAbs hold promise in overcoming the heterogeneity and adaptability of tumors, opening up new avenues for therapeutic intervention.

This paper underlines the multifaceted approach required for comprehensive bsAb characterization, leveraging an array of techniques and pioneering technologies. At Revvity, we have the tools to support and enhance your bsAb research. Our comprehensive suite of technology platforms, pre-configured kits, and customizable reagents have been developed to amplify your research, ensuring that you gain superior outcomes and results that matter.

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