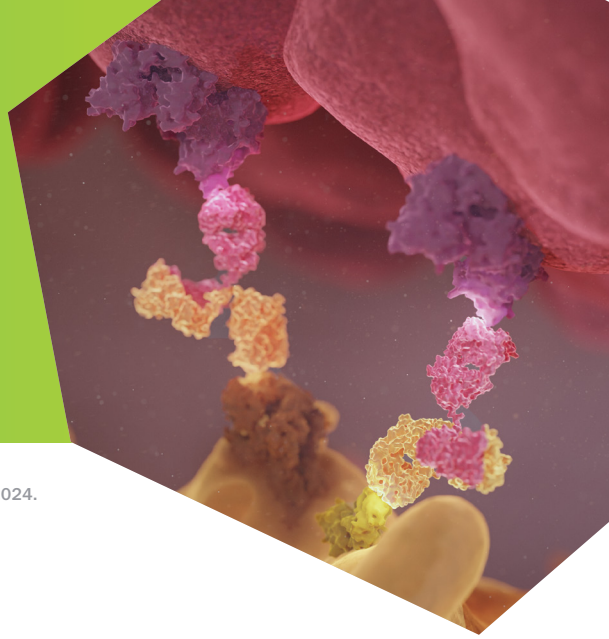
SCIENTIFIC CASE STUDY

# Diverse CD28-binding antibodies for costimulatory T-cell engager development



The following data was presented as a poster at the **American Association for Cancer Research® (AACR) Annual Meeting 2024.**

## BACKGROUND

### Optimal T-cell activation in the tumor microenvironment requires costimulatory signals

The anti-tumor activity of T cells, initiated via the T-cell receptor (TCR), can be enhanced through costimulatory engagement of the CD28 co-receptor.[1] This costimulation can be leveraged therapeutically using CD28-binding antibodies to improve the efficacy of T-cell activating strategies, such as CD3 T-cell engagers (TCEs), in the tumor microenvironment (Fig. 1).[2,3]

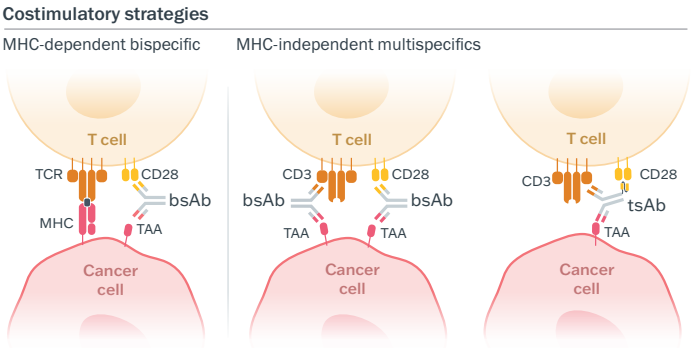



**FIGURE 1. CD28-binding antibodies can enhance anti-tumor activity in combination with T-cell activating therapeutics.**

## AIM

### Generate costimulatory molecules with potential for improved safety and efficacy profiles

CD28-costimulatory antibodies are a promising class of immuno-therapies for diverse tumor targets, yet clinical challenges remain.[4,5]

To address these challenges, our aim was to develop a diverse set of CD28-binding IgG and heavy-chain only antibodies (HCAbs) that:

- do not show superagonist activity — a property associated with CD28-mediated toxicities
- have diverse binding properties for the development of immuno-therapies across tumor targets and modalities

## OUTCOME

### Functionally diverse CD28-binding antibodies for T-cell engager development

We generated a panel of CD28-binding HCAbs and IgG antibodies. These antibodies bind a broad range of epi-topes, leading to diverse functional activity that is clearly differentiated from benchmarks with known toxicity issues.

**Combining these molecules with other T-cell activating strategies represents an opportunity to fine-tune novel costimulatory TCEs for diverse tumor targets.**

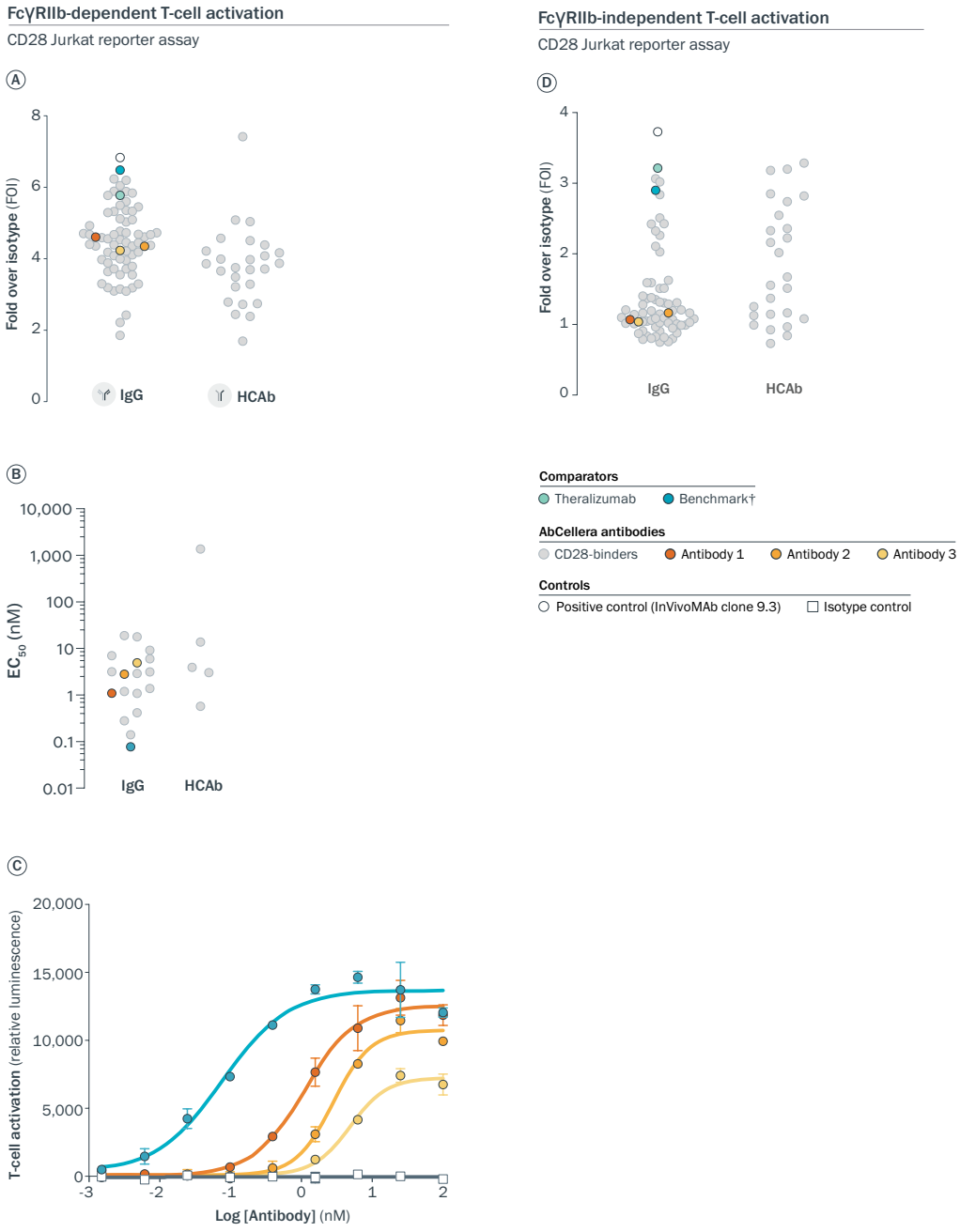
# CD28-binding antibodies with a broad range of costimulatory T-cell activities and differentiation from clinical benchmarks

We used our antibody discovery and development engine to generate hundreds of novel CD28-binding HCAb and IgG antibodies. Of these, we expressed 92 for functional characterization. We assessed crosslinking-dependent and -independent T-cell activation by measuring function in the presence and absence of cells expressing FcγRIIb, respectively. In the presence of FcγRIIb-expressing cells, we observed a broad range of potenties (Fig. 2A-C). In the absence of FcγRIIb-expressing cells, the majority of antibodies tested showed lower crosslinking-independent T-cell activation — a property that is associated with toxicities — compared to a known superagonist benchmark (Fig. 2D).

**FIGURE 2. CD28 activation in the presence or absence of FcγRIIb confirms that antibodies are conditional CD28 agonists, with the majority showing lower crosslinking-independent activation of effector cells than superagonist benchmarks.**

**(A)** 92 antibodies, generated with human IgG1 Fc, were tested for CD28 FcγRIIb-dependent activation. Antibodies were incubated with effector cells endogenously expressing TCR, CD3, and CD28, and CHO-K1 cells engineered to express FcγRIIb and a TCR-engaging protein (Promega JA9331). Relative luminescence for each antibody was normalized to the isotype control. Three example antibodies (1-3) with diverse functional responses are highlighted. **(B)** 22 antibodies were selected for a 1-in-4, nine-point titration series starting at 100 nM, $EC_{50}$ values are shown. **(C)** Relative luminescence for example antibodies, benchmark, and a human IgG isotype control are shown. **(D)** FcγRIIb-independent agonist activities were similarly assessed using CHO-K1 cells engineered to express a TCR-engaging protein, but not FcγRIIb.

*†Bivalent monoclonal CD28 benchmark antibody generated using sequences from REGN5678 patent.[6]*



# CD28-binding antibodies enhance T-cell activity without superagonism

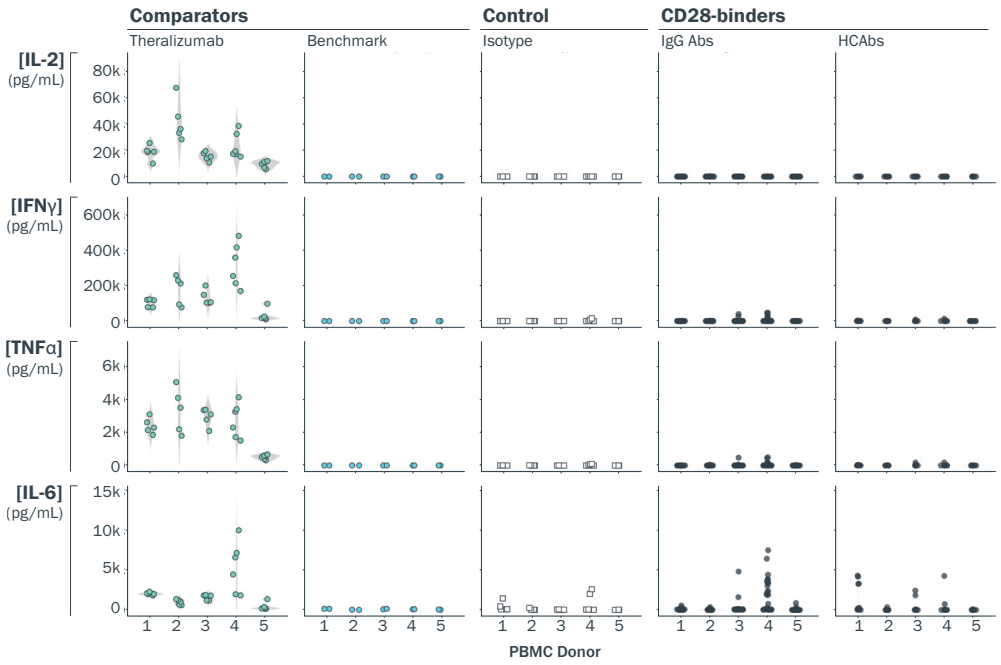
To further profile T-cell activation properties, we assessed antibodies for superagonism activity. In comparison to the superagonist molecule theralizumab (TGN1412), our antibodies showed lower levels of cytokine release across five peripheral blood mononuclear cell (PBMC) donors (Fig. 3A). To recapitulate T-cell activation in the context of a T-cell engager modality, we assessed MHC-independent costimulation in the presence of a CD3-binding antibody and observed activities within the range of clinical benchmarks (Fig. 3B).

**FIGURE 3. CD28-binding antibodies do not show superagonist activity when mixed with PBMCs from healthy donors.**

**(A)** Cytokine release profiles are shown for five PBMC donors that were cultured with wet-coated antibodies (five replicates/antibody, 22 total AbCellera CD28-binders), as previously described.[7] Values below the lower limit of detection are shown at y=0. Theralizumab is a known CD28 superagonist molecule. Antibodies were generated as a human IgG1 with a N297A mutation. **(B)** In a proof-of-concept study for MHC-independent costimulation (Fig. 1), PBMC donors were cultured with a combination of plate-bound CD28- and soluble CD3-binding (OKT3) anti-bodies. A single PBMC donor and representative cytokine, IFNγ, is shown. The costimulatory effect of CD28-binding antibodies was within the range of clinical benchmarks, as shown for three example antibodies.

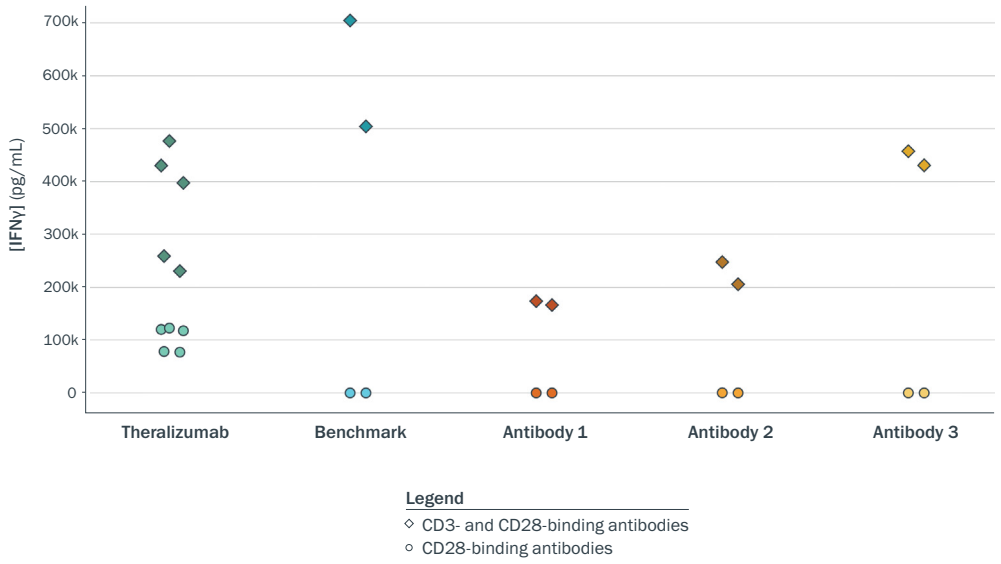
Ⓐ Cytokine release profiles

PBMCs cultured with CD28-binding antibodies




Ⓑ IFNγ release profiles

PBMCs cultured with CD3- and CD28- binding antibodies

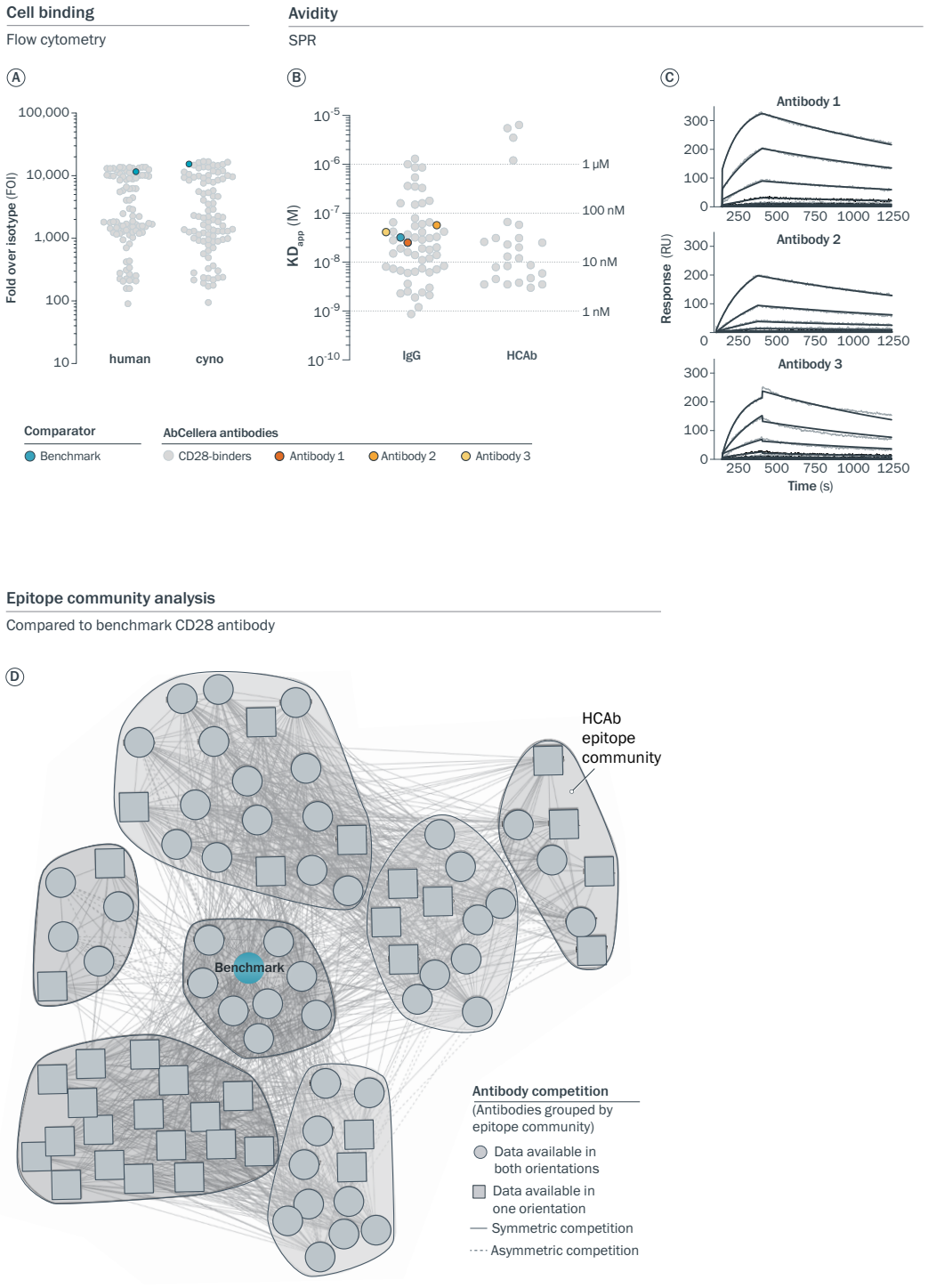


# Novel HCAb and IgG antibodies with diverse binding properties

We assessed a subset of cross-reactive antibodies (27 HCAbs and 65 IgGs) for binding to human and cyno CD28-expressing cells. Of the 92 antibodies assessed, 90 were validated as cross-reactive CD28-binders (Fig. 4A). Antibodies showed diverse binding avidities, ranging from 870 pM to 6.40 µM (Fig. 4B-C), and were clustered into seven epitope communities (Fig. 4D).

**FIGURE 4. Antibodies showed diversity in epitopes and binding avidities.**

**(A)** The median fluorescence intensity for each antibody was normalized to the isotype control, with positive binding (fold over isotype ⩾ 10) confirmed in 90 of the 92 antibodies. **(B)** Avidity was measured using the CD28 extracellular domain by surface plasmon resonance. **(C)** Sensorgrams show three example IgG antibodies (1-3) with diversity in on and off rate. **(D)** Epitope binning was performed, with communities determined by ward.D hierarchical clustering based on competition events. 81 antibodies clustered in seven epitope communities and the majority of antibodies compete with the benchmark for binding to CD28, consistent with the limited extracellular space.

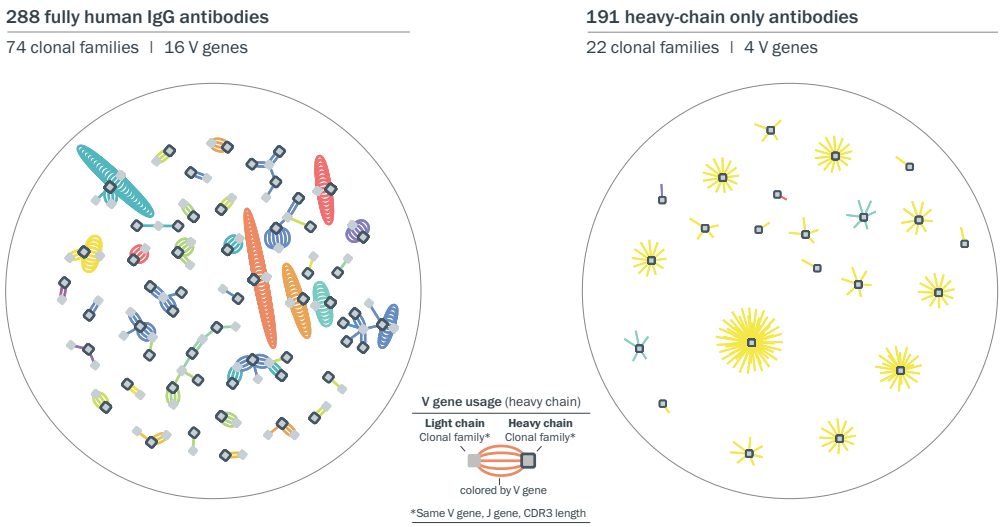


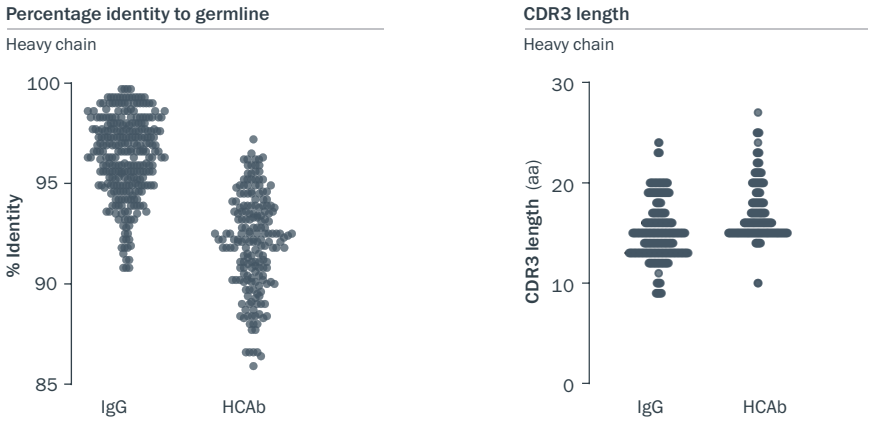
# Hundreds of diverse HCAb and IgG antibody sequences

The 92 antibodies profiled above were selected from a diverse panel of HCAb and IgG antibody sequences generated using our antibody discovery and development engine. Antibodies were discovered from camelids and humanized mice immunized against CD28 to identify HCAbs and IgGs, respectively. Antibody-secreting cells were analyzed using our proprietary single-cell screening platform to identify antibodies with cross-reactive binding to both human and cyno CD28. Bioinformatic analysis revealed a high degree of sequence diversity (Fig. 5).

**FIGURE 5. 479 CD28-specific HCAb and IgG antibodies were identified from immunization and deep single-cell screening.**

**(A)** Antibody sequence diversity was visualized using Celium™. **(B)** Antibodies showed a broad range of percentage identity to germline and CDR3 length, highlighting diversity in sequences obtained.



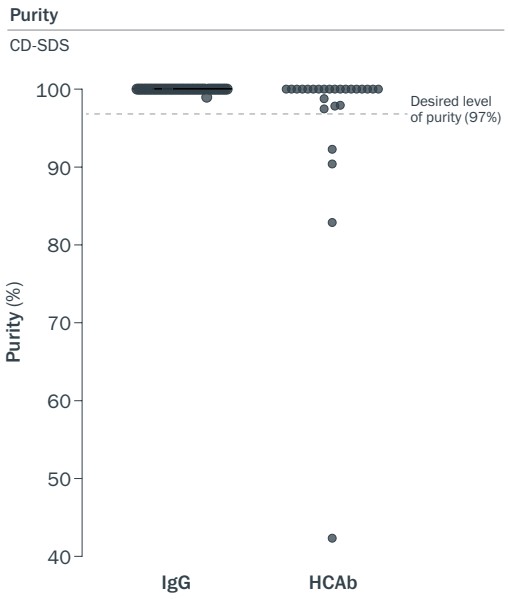



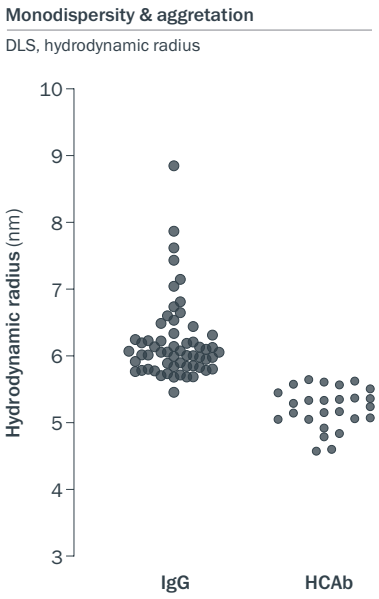
# CD28-binding antibodies with favorable preliminary developability properties

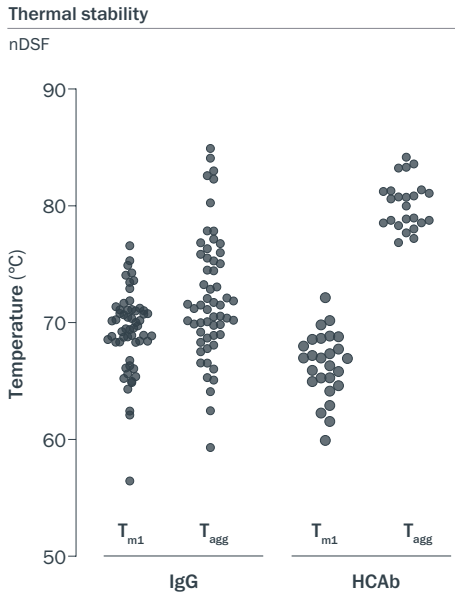
Prior to functional characterization, we performed preliminary analytical and biophysical characterization, revealing favorable early developability properties (Fig. 6).

**FIGURE 6. Preliminary analytical and biophysical characterization.**

The majority of antibodies showed a high level of purity (> 97%), a hydrodynamic radius within the expected range (IgGs: 5.3-6.5 nm; HCAbs: 4.7-6.3 nm), and typical thermal stability profiles.



## CONCLUSION

TCEs are among the most promising new modalities in cancer therapy, but limitations in efficacy and safety have been barriers to realizing their potential for patients with difficult-to-treat cancers. To address these challenges and expand the therapeutic window for this modality, we developed a TCE platform that includes novel CD3-binding antibodies that enhance potency while reducing cytokine release.

To enable development of molecules with enhanced potency for difficult-to-treat cancers, we have also integrated costimulatory building blocks into our TCE repertoire. TCEs that engage the CD28 costimulatory receptor can enhance T-cell activation, proliferation, and anti-tumor activities, particularly in solid tumors. The data shown here demonstrate that our HCAb and IgG CD28-binding antibodies activate T cells with a broad range of potencies and do not display superagonist activity — a property associated with toxicity.

We aim to leverage this platform to unlock the full potential of this modality by advancing a pipeline of internal programs and by engaging in strategic partnerships to bring powerful new cancer medicines to patients.

### REFERENCES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