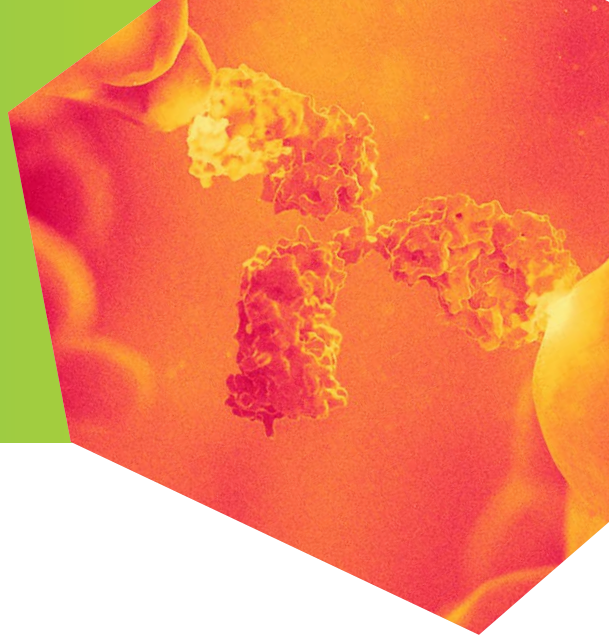
SCIENTIFIC CASE STUDY

Developing T-cell engagers for diverse tumor targets



The following data was presented as a poster at the **Society for Immunotherapy of Cancer Annual Meeting 2023**.

INTRODUCTION

Diverse CD3-binding antibodies for T-cell engager development

CD3 T-cell engagers (TCEs) function by recapitulating an immunological synapse between T cells and tumor cells to trigger tumor-cell killing. As a result, effective TCEs must balance potency with potential toxicities by maximizing tumor-cell killing and fine-tuning cytokine release. This mechanism is determined by the complex interplay between multiple independent factors that make up the bispecific-induced immune synapse, including binding properties of the CD3- and tumor-binding arms and target-dependent factors.[1]

Most TCEs in clinical development are derived from a small number of CD3-binding antibodies, such as SP34-2,[2] limiting the potential to generate optimal immune synapses. To address this challenge, we developed a TCE platform that includes hundreds of fully human CD3-binding antibodies with diverse binding properties.

In this case study, we present data on our largest panel of bispecific TCEs to date, providing insights into how CD3-binding properties impact function. We have now leveraged these learnings to identify promising TCEs for multiple tumor targets, two of which are shown here.

Optimization of antibody pairs for different tumor targets

To assess the impact of CD3-binding properties on TCE function, CD3-binders were paired with a single tumor-binding paratope to generate hundreds of bispecifics. We assessed function in high-throughput and performed a clustering analysis to group CD3-binding antibodies based on cell killing and cytokine release profiles. We then used these data to develop an efficient approach for optimizing antibody pairs for different tumor targets.

OUTCOME

Differentiated T-cell engagers for diverse tumor targets

We developed a comprehensive approach for generating TCEs against a wide range of tumor targets and are leveraging this to address key challenges in TCE development:

- Enabling generation of TCEs with high potency against difficult target classes, such as peptide-MHCs, by coupling rare tumor-binding antibodies with diverse CD3-binding antibodies
- Generating TCEs that balance potency and potential toxicities by diversifying immune synapse parameters
- Efficiently identifying optimal pairs of CD3- and tumor-binding antibodies for diverse tumor targets using a function-first approach

We identified TCEs against two tumor targets with distinct properties and target expression levels[3,4] — prostate-specific membrane antigen (PSMA) and peptide-MHC target, melanoma-associated antigen 4 (MAGE-A4). Selected antibodies showed desired functional profiles with differentiation from clinical molecules.

Diverse CD3-binding antibodies for TCE development

Proprietary immunization protocols using humanized mice yielded CD3-binding antibodies with diversity in sequence, epitope, kinetics, subunit specificity, and cross-reactivity (Figure 1). These antibodies were paired with a single tumor-binding arm using OrthoMab™, our proprietary bispecific platform. OrthoMab™ uses computationally designed, patented mutations to guide correct and stable pairing of multiple heavy and light chains, retaining the function and developability of the parental antibody (Figure 2). The resulting bispecifics were expressed and analyzed using high-throughput functional assays, including tumor-cell killing and release of cytokines IFNγ, TNFα, IL-2, and IL-6.

FIGURE 1. Fully human CD3-binding antibodies that are distinct from commonly used molecules

(A) Antibody sequence diversity was visualized using Celium™. Clusters represent clonal families based on V genes, J genes, and CDR3 lengths. The plot is colored using data from high-throughput SPR epitope binning, demonstrating that the majority of our CD3-binding antibodies bind epitopes that are distinct from that of SP34-2. **(B)** Binding kinetics to human CD3ε was measured by SPR-coupled kinetic analysis. On and off rates are plotted along the x and y axes with affinities displayed diagonally. **(C)** Subunit specificity was assessed by high-throughput SPR and species specificity was measured by binding to CHO-K1 cells expressing either human or cyno CD3.

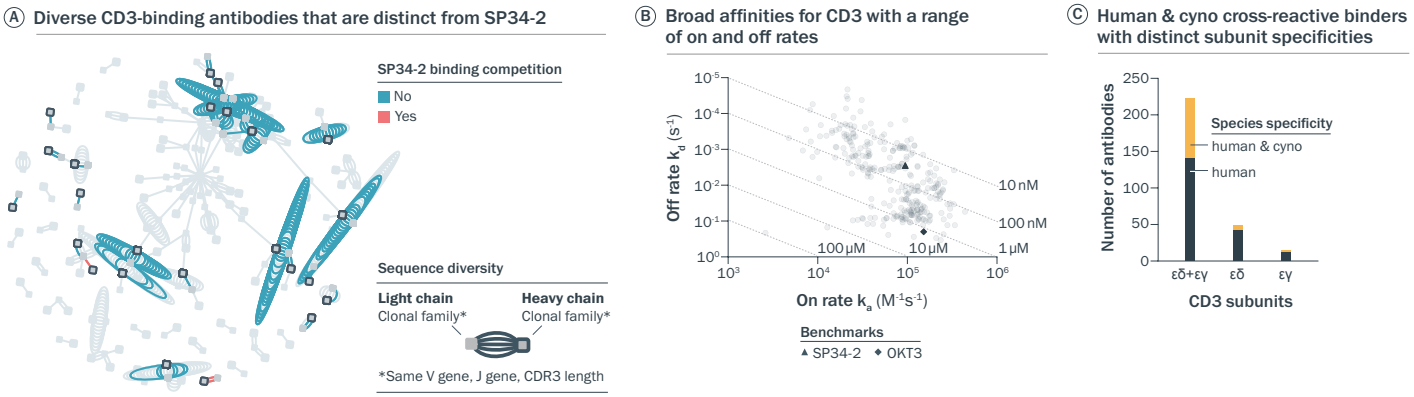
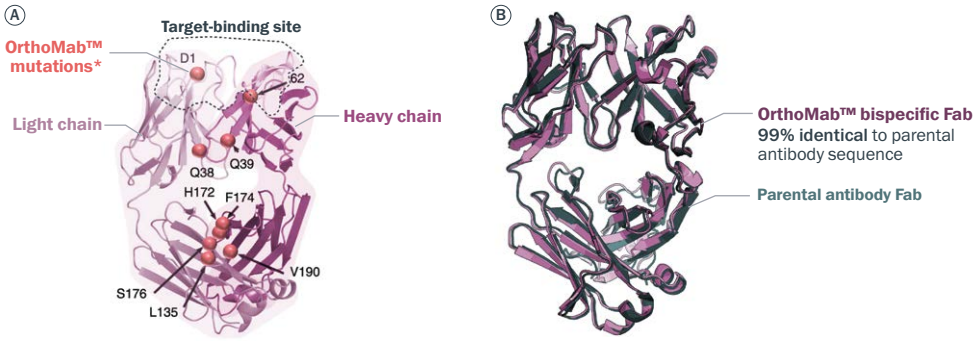


FIGURE 2. Stable and correctly paired bispecifics using OrthoMab™

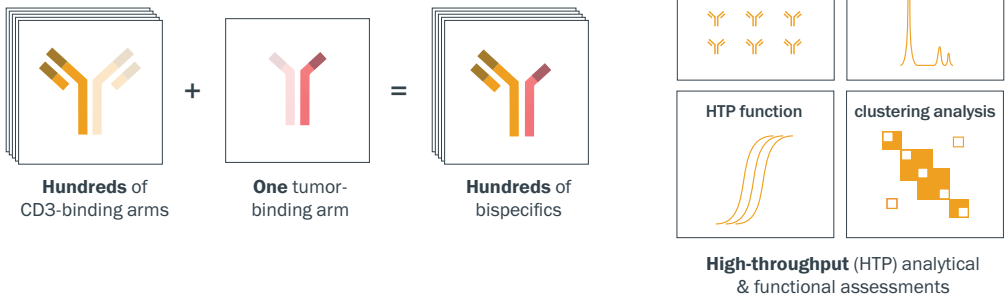
(A) A representative set of OrthoMab™ mutations in the VH/VL and CH1/CL interface. Mutations are clustered away from target-binding sites[5] to avoid potential impact on function. **(B)** Superposition of a parental Fab structure with and without the OrthoMab™ mutations applied shows the overall tertiary structure is not affected, and the CDRs are precisely overlaid.



Example of a representative set of OrthoMab™ mutations

FIGURE 3. Hundreds of TCEs generated to assess the impact of the CD3-binding arm on function

Hundreds of CD3-binding antibodies[6,7] were paired with a single tumor-binding VHH arm, then expressed and purified using standard protein A purification. The purity of the properly formed heterodimers were assessed using aSEC, CE-SDS, and intact mass spectrometry. Tumor-cell killing and cytokine release were assessed in a high-throughput imaging assay using unactivated human T cells incubated with target-expressing cells at a ratio of 10:1 for 48 hours. Clustering analysis is further described in Figure 6.



High-throughput (HTP) analytical & functional assessments

Multiple CD3-binding parameters impact TCE function

Binding kinetics for the human CD3εδ subunit were compared to TCE potency, tumor-cell killing, and cytokine release (Figure 4). Off rate was found to be a significant contributor to cytokine release and tumor-cell killing, but was not sufficient to fully describe these properties, particularly at faster off rates. In contrast, potency was not tightly correlated with off rate. Consistent with this observation, individual antibodies with similar binding kinetics displayed distinct functional profiles, while antibodies with different binding kinetics demonstrated similar functional behavior (Figure 5).

FIGURE 4. CD3-binding kinetics are a significant contributor to TCE function

Binding kinetics of the human CD3εδ subunit were compared to TCE potency, tumor-cell killing, and cytokine release. Off rate was identified as the primary driver of affinity and release of TNFα and IL-2 was consistent with IFNγ, as such these parameters are shown. CD3-binding antibody clonal family (defined as antibodies sharing the same V gene, J gene, CDR3 length, and animal origin for both heavy and light chains) is overlaid using color. All antibodies shown met a threshold of purity as described in Figure 3.

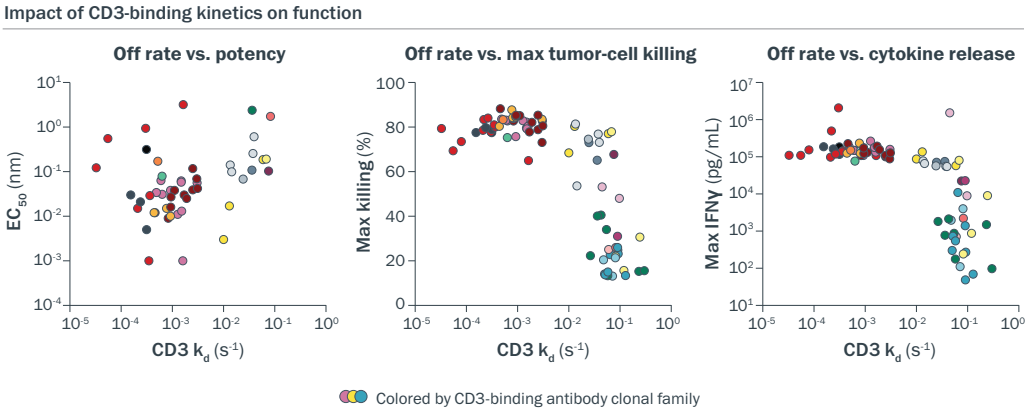
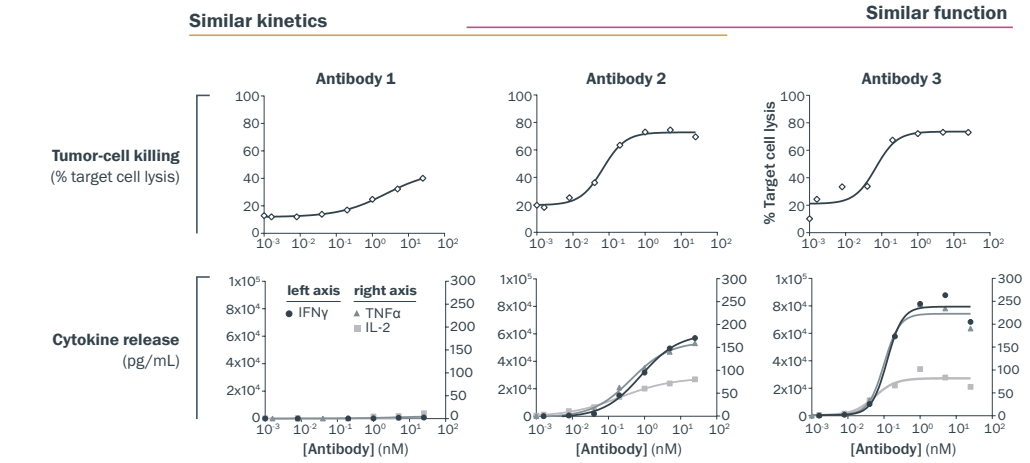


FIGURE 5. Diverse functional profiles were observed despite similar kinetic parameters

Tumor-cell killing and cytokine release curves for three example TCEs are shown. The CD3-binding properties and functional readouts for each are summarized in the table below. All three antibodies are from different clonal families.

*Functional clustering is described in Figure 6.



CD3-binding properties	Antibody 1	Antibody 2	Antibody 3
Affinity (nM)	320	600	38
Off rate (s^{-1})	4×10^{-2}	2×10^{-2}	3×10^{-3}
Subunit	epsilon	epsilon	epsilon

TCE properties			
Max killing (%)	40	75	73
Max IFNγ (pg/mL)	772	5.7×10^4	8.6×10^4
Max TNFα (pg/mL)	5	160	243
Max IL-2 (pg/mL)	12	81	102
Functional cluster*	2	3	3

A function-first approach to identifying optimal TCEs

Functional profiles for different tumor targets cannot be accurately predicted from the CD3-binding arm alone, as the tumor binding-arm is also known to impact TCE function.[1] To address this challenge, we explored a function-first approach for the selection of optimal CD3- and tumor-binding pairs for multiple targets.

CD3-binding antibodies were clustered into 19 groups based on multiple functional parameters (Figure 6). CD3-binders with desired properties (e.g., cyno cross-reactivity and subunit specificity) were selected from a range of functional clusters and paired with tumor-binding arms to generate a diverse panel of bispecifics (Figure 7). We then used high-throughput and high-resolution antibody assessment technologies to identify promising TCE candidates for further evaluation.

FIGURE 6. CD3-binding antibodies were clustered based on TCE functional profiles

(A) Tumor-cell killing and cytokine release data were normalized to a range (0, 1) and antibodies were categorized as high, medium, or low based on their distance from the mean. Example normalized EC_{50} data is shown. **(B)** Antibodies were then clustered based on functional profiles as shown in the white boxes along the diagonal of the heatmap. Example antibodies can be seen in Figure 5, where antibodies 1 and 2 are in different clusters and antibodies 2 and 3 are in the same cluster.

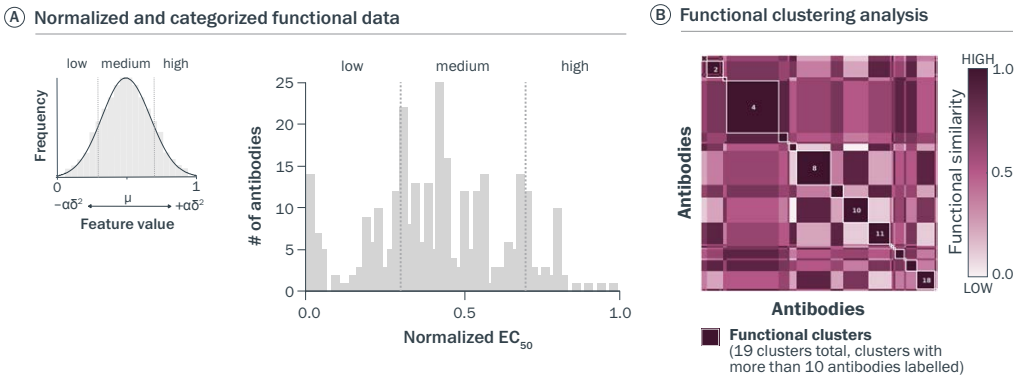
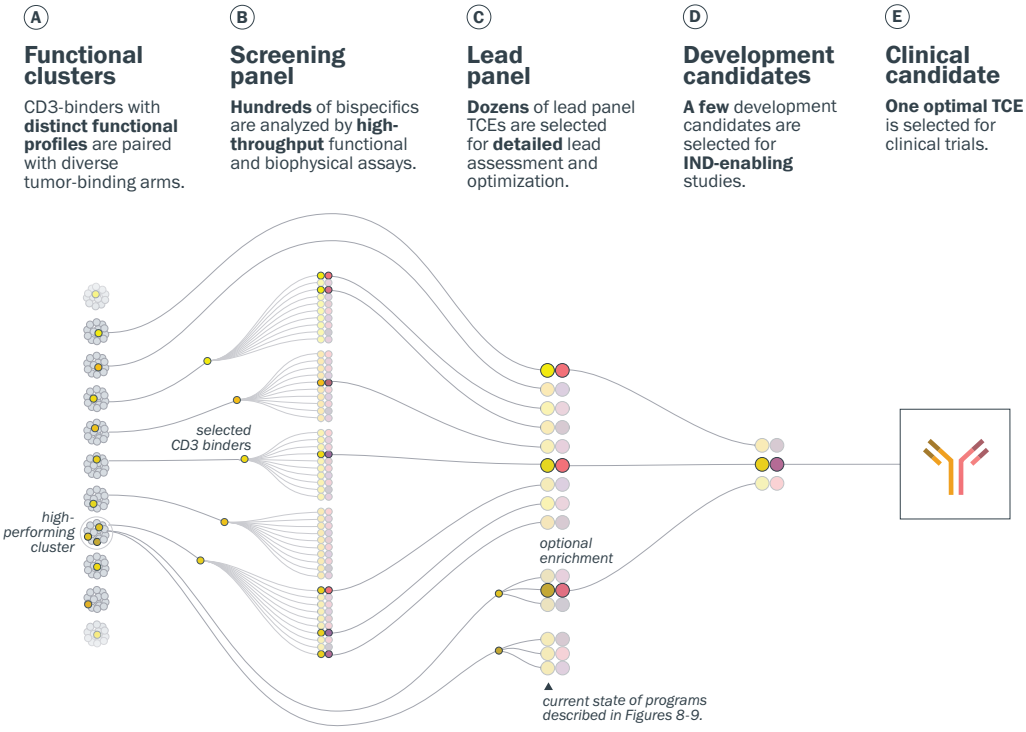


FIGURE 7. Functional clusters are used to select diverse CD3-binding antibodies for different tumor targets

(A) We maximize functional diversity upfront by pairing CD3-binders from distinct clusters with diverse tumor-binding arms, prioritizing desired properties such as cyno cross-reactivity and subunit specificities where needed. **(B)** We engineer hundreds of bispecifics using OrthoMab™ and analyze them in high-throughput functional and biophysical assessments. **(C)** We select dozens of lead panel antibodies based on function and developability. If needed, we can enrich the lead panel with CD3-binding arms from top-performing clusters. **(D)** We analyze selected antibodies with robust functional, biophysical, analytical, stability, and structural assessments, and select ~1-3 development candidates for IND-enabling studies. **(E)** Based on these studies, a final clinical candidate is selected for clinical manufacturing.



TCEs with desired properties

Our function-first approach to CD3-binder selection was applied to two tumor targets: PSMA and the peptide-MHC target MAGE-A4. Functionally diverse CD3-binders were paired with PSMA and MAGE-A4-specific arms based on desired properties for each program (Figure 8). Hundreds of TCEs were assessed in high-throughput functional assays (Figure 9) to guide selection of molecules for further assessment. Selected TCEs had functional profiles that spanned the range of clinical molecules, including potent tumor-cell killing and low cytokine release.

In each of the programs the TCEs selected for further assessment included a broad set of CD3-binders — six unique CD3-binders in the PSMA program and nine in the MAGE-A4 program (Figure 9) — demonstrating the value in engineering TCEs using multiple, diverse CD3-binders. Further, we showed that the same CD3 arm, when paired with different tumor arms, had differences in potency or cytokine release that can be attributed to the tumor arm.

FIGURE 8. Functionally diverse CD3-binders were used to engineer TCEs for two tumor targets

(A) CD3-binding antibodies selected for our PSMA x CD3 program were prioritized based on cyno cross-reactivity. In addition to having functional diversity, antibodies had different subunit specificities and affinities ranging from 1 nM to 12 µM. We selected nine developable PSMA-binding antibodies from five different epitope bins with affinities ranging from 46 pM to 31 nM. **(B)** Cyno cross-reactivity was not required for our MAGE-A4 x CD3 program, enabling selection from a broader range of functional profiles. The 19 selected CD3-binders ranged in affinity from 1 nM to 3 µM. We discovered developable antibodies with high specificity for a MAGE-A4 peptide-MHC[8] and selected six for bispecific engineering.

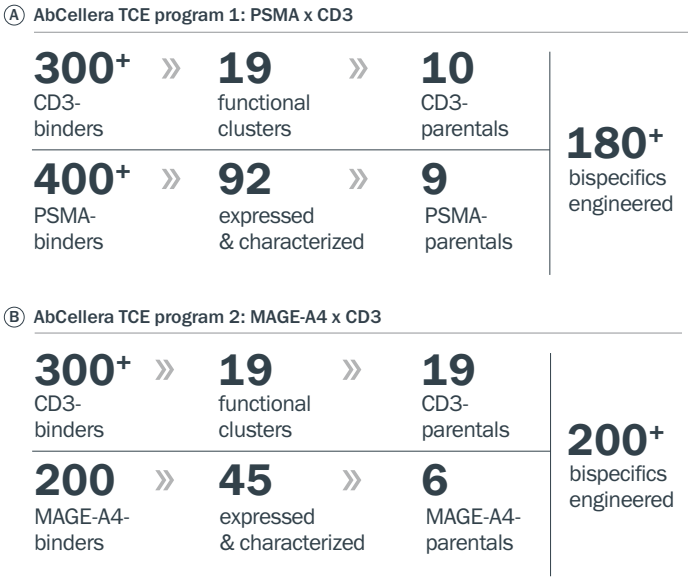
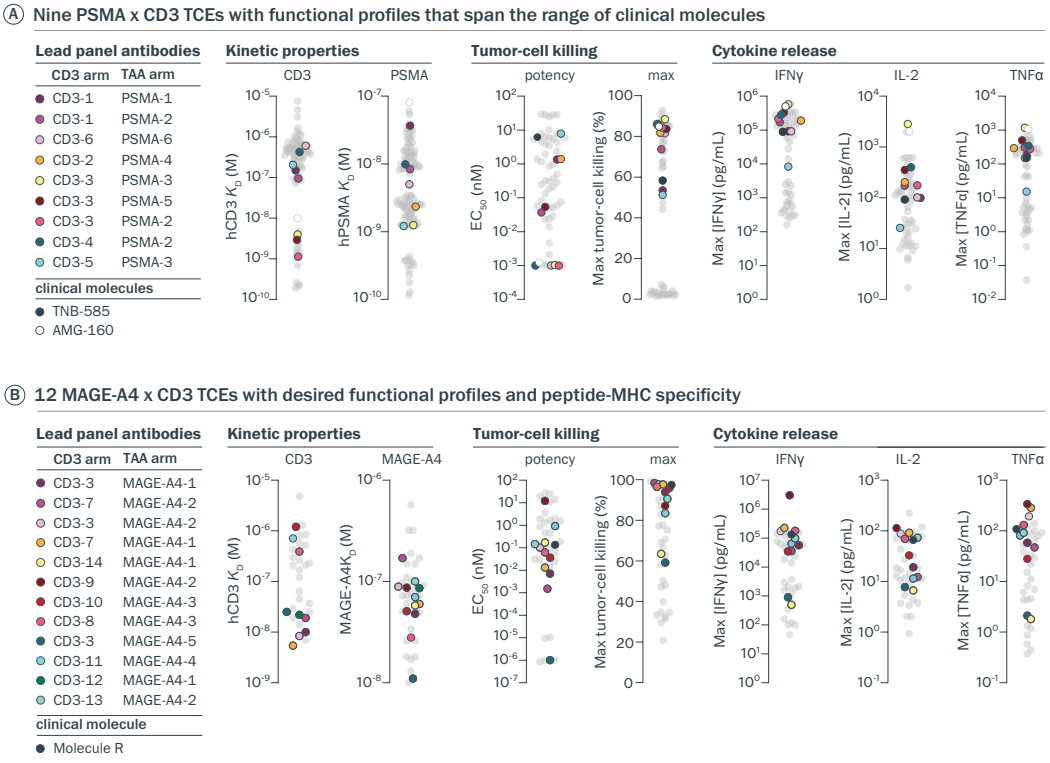


FIGURE 9. High-throughput assessment of TCEs enabled selection of molecules for further assessment

(A) 180+ PSMA x CD3 TCEs were assessed and compared to two clinical-stage molecules (TNB-585[9] and AMG-160[10]) in a high-throughput assay using unactivated human T cells incubated with PSMA-expressing LNCaP cells at a ratio of 10:1 for 48 hours. **(B)** 200+ 1x1 MAGE-A4 x CD3 TCEs were assessed and compared to Molecule R[11] (057D03 paired with V9 in a 2x1 bispecific format) in a high-throughput assay using unactivated human T cells incubated with wild-type or MAGE-A4 knockout A375 cells at a ratio of 10:1 for 48 hours.



CONCLUSION

A commonly used approach for TCE development is to modify the affinity of non-proprietary CD3-binding antibodies to mitigate the risk of cytokine release syndrome. Data presented here demonstrate that CD3 affinity alone is not sufficient to predict function. Based on these findings, our approach is to select functionally diverse CD3-binding antibodies for each tumor target. Our functional clustering approach enables us to efficiently broaden the range of CD3-binders selected for bispecific engineering, and our high-throughput platform supports streamlined identification of CD3- and tumor-binding pairs that result in the desired properties.

In addition to CD3- and tumor-binding properties, TCE function is also determined by target-dependent properties, including target density, cell type, and heterogeneity. We applied this approach to two distinct tumor targets with different target expression levels, PSMA and MAGE-A4-pMHC. For each program, we generated bispecifics with desirable functional profiles, including potent killing and desired cytokine release. These molecules were derived from diverse CD3- and tumor-binding arms, demonstrating the impact of optimal pairing on the immunological synapse. We are now leveraging this approach to develop TCEs for diverse tumor targets, unlocking this high-value class of antibody therapeutics.

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