TriTCE Co-Stim, Next Generation Co-Stimulatory Trispecific T cell Engagers for the Treatment of Solid Tumors

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Introduction

- Traditional bispecific T cell engager (TCE) therapies have exhibited clinical utility against hematological cancers, but limited success in solid tumors.
- Additional challenges posed by solid tumors that may limit the antitumor activity of CD3-bispecific TCEs include:
  - Immunosuppressive environments
  - T cell anergy
  - Low T cell infiltration
- Conventional T cell activation and sustained proliferation requires signaling via CD3 (signal 1) and co-stimulatory molecules (signal 2), such as CD28.

Lack of co-stimulatory ligand engagement in solid tumors may limit the activity and durability of bispecific TCE responses

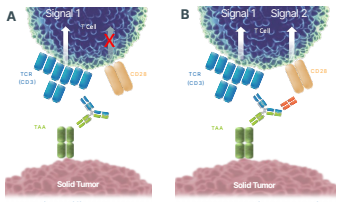

Figure 1. Schematic of T cell engager (TCE)-mediated T cell activation in solid tumors. (A) Activation of the T cell receptor (TCR) in the absence of co-stimulation may result in T cell anergy, limiting the activity and durability of bispecific TCE anti-tumor responses. **(B)** Activation of TCR with concomitant co-stimulation may enhance T cell activation, metabolism and fitness, cytokine production, and sustained proliferation.

Co-stimulatory trispecific TCEs (TriTCE Co-Stim) have the potential to provide more durable responses and re-invigorate 'cold' tumors with lower T cell infiltration

- The balance between signals 1 and 2 is critical for optimal T cell activation.
- Signal 1 in the absence of signal 2 results in T cell anergy.
- Overactivation via signals 1 and 2 can lead to T cell dysfunction and cytokine release.

Figure 2. Activation requires a balance of "Signal 1" and "Signal 2". Lack of Signal 2 co-stimulation leads to T cell anergy and no sustained T cell proliferation. Overactivation leads to T cell dysfunction and excessive cytokine release.

Zymeworks' Approach of Differentiated Trispecific TCE Engineering

- Novel approach of screening multiple trispecific geometries.
- Different CD3 and CD28 geometries, affinities interrogated in screening process.
- Opportunity to optimize Signal 1 and 2 in trispecific for optimal tumor specific T cell activation and tumor killing.

Core competency of protein engineering and flexibility of Azymetric™ platform enables extensive screening of multiple parameters in parallel

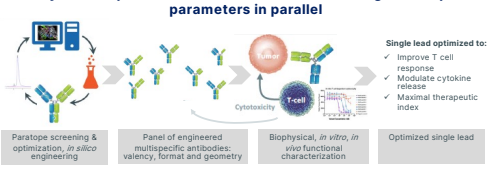

Figure 3. Azymetric™ platform enabled screening of multiple parameters in parallel. Paratope screening and *in silico* affinity engineering workflow for generation of a large panel of antibodies (Abs) with multiple parameters: format, valency, geometry and affinity. Initial biophysical and functional characterization (*in vitro* and *in vivo*) of multispecific antibodies enable optimization of a single lead multispecific antibody with optimal Signal 1 and 2 balance with maximized therapeutic index.

Paratope Engineering for Therapeutic Window Optimization

- Conventional anti-CD28 agonist with no super-agonist activity for potentially less risk of CD28 mediated toxicities.
- Library of anti-CD28 paratope with medium to low affinities for optimized co-stimulation signal (Signal 2).
- Anti-CD3 paratope engages CD3 at a different epitope than anti-CD3 antibodies that have high affinity for CD3.
- Library of anti-CD3 paratope with medium to low affinity for potentially less risk of toxicity related to cytokine release syndrome (CRS).

Protein engineering solutions employed to optimize signal strength for T cell activation and anti-tumor activity

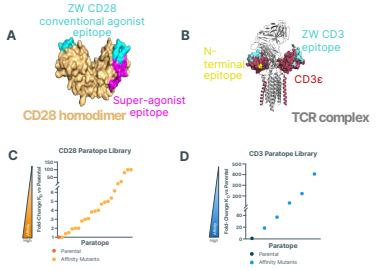

Figure 4. CD3 and CD28 paratope engineering. (A) Surface representation of CD28 homodimer structure (modelled using 1YJD) highlighting epitopes for Zymeworks' (ZW) conventional agonist vs super-agonist antibodies. **(B)** Cartoon representation of the full TCR complex (modelled using 7FJD) with surface representation of CD3-ε domain, highlighting for ZW (conformational) vs the N-terminal (linear) epitopes. **(C)** A library of conventional agonist paratope variants with a range of CD28 binding affinities determined by surface plasmon resonance (SPR). **(D)** A library of agonist paratope variants with a range of CD3 binding affinities determined by SPR.

TriTCE Co-Stim Antibodies Generated using Azymetric™ and EFECT™ Platforms

Azymetric™ allows screening of multiple trispecific formats and affinities for optimal T cell activation

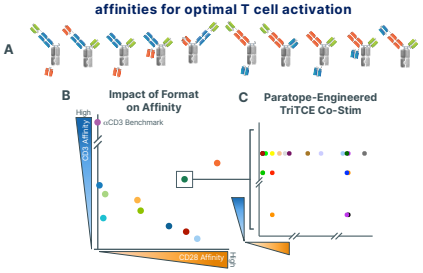

Figure 5. TriTCE Co-Stim antibodies with various paratope formats and geometries are engineered using the Azymetric™ and EFECT™ platforms. (A) Schematic representation of a subset of TriTCE Co-Stim antibody formats. **(B)** Representation of the impact of paratope format (scFv vs. Fab) and geometry on the binding affinities to CD3 and CD28 for a subset of formats with the same CD3 and CD28 paratopes. **(C)** Representation of affinities following CD3 and CD28 paratope engineering for one TriTCE Co-Stim format, which can be transferred among formats to create a large panel of TriTCE Co-Stim Abs.

Biophysical characterization highlighting stability of TriTCE Co-stim Abs

Antibody Format	1	2	3	4	5	6	7	8	9	10
T_m values (°C)	56.1 66.1 77.6	79.3	65.8 80.5	78.1	65.3 78.5	66.3 88.8	88.9	55.7 65.4 89.3	65.9 89.6	67.1 89.2

Figure 6. TriTCE Co-Stim antibodies display substantial thermal stability by differential scanning calorimetry(DSC). The table above shows the maximum melting temperatures (T_m) for each of the peaks in the thermograms of the subset of TriTCE Co-Stim antibody formats. Thermostability profiles are comparable to those of conventional IgG1 antibodies.

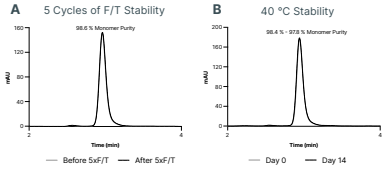

Figure 7. TriTCE Co-Stim antibody displays acceptable purity profiles post stress testing. (A) Freeze thaw (F/T) stress test shows no significant change in purity post 5 cycles freezing to -80 C followed by thawing to 4 C. **(B)** Accelerated stress test shows minimal change in purity post 14 days incubation at 40 °C. The data graphs are shown for one of the representative TriTCE Co-Stim antibody.

in vitro Screening of Multivalent Geometries and Affinities Enables Selection of Best-in Class Trispecific TCEs

Antibody Format	1	2	3	4	5	6	7	8	9	10
Cytotoxic Potency (IC$_{50}$; pM)										
TAA-Dependent?	✓	✓	✓	x	✓	✓	x	x	x	x

Figure 8. Lead TriTCE Co-Stim is selected through extensive screening *in vitro*. High throughput screening enables rapid assessment of a large panel of multivalent antibodies with various geometries and affinities. TriTCE Co-Stim antibodies are screened for T cell-dependent cytotoxic potency against target-expressing cells. A heatmap indicating cytotoxic potency is represented in the table above. TriTCe Co-stim antibodies are further assessed for target-dependent T cell activation by measuring the induction of cytokine in monocultures of T cells.

TriTCE Co-Stim screening process exhibits transferability across different TAA targets

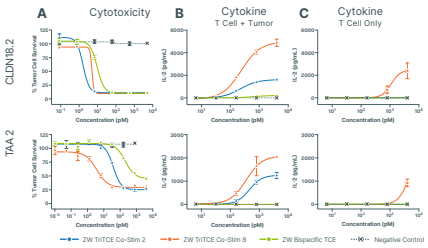

Figure 9. *in vitro* high throughput screening to assess TriTCE Co-Stim formats. Test articles were incubated with T cells co-cultured with TAA-expressing tumor cell lines **(A,B)** or in a monoculture of T cells **(C)** and evaluated for cytotoxicity of target cells **(A)** and IL-2 production by T cells **(B,C)**. Formats 2 and 8 from Figure 5 are depicted to exemplify formats exhibiting TAA-dependent or TAA-independent T cell agonism, respectively.

CLDN18.2 TriTCE Co-Stim (CLDN18.2 x CD3 x CD28) Molecules Support Enhanced T cell Mediated Activity *in vitro*

Reduced, Avidity-Driven T Cell Binding

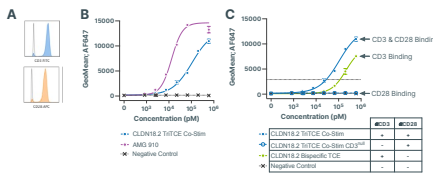

Figure 10. On-cell binding of TCEs to human CD3+ T cells. Test articles were incubated with T cells and assessed for binding by flow cytometry. **(A)** T cell expression of CD3 (top) & CD28 (bottom) by flow cytometry. **(B)** Reduced binding of CLDN18.2 TriTCE Co-Stim compared to the clinical benchmark. Affinity of the clinical benchmark for CD3 is multiple-fold higher than observed for CLDN18.2 TriTCE Co-Stim. **(C)** The CD28 paratope does not bind T cells without co-engagement of the CD3 paratope. Dashed line represents B_{max} of CD28xCLDN18.2 bispecific.

Enhanced long term cytotoxicity of CLDN18.2-expressing target cells co-cultured with T cells at low (1:5) E:T ratios

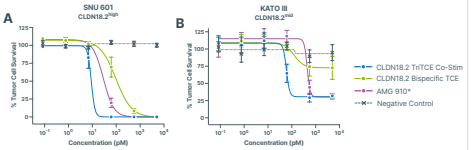

Figure 11. CLDN18.2 TriTCE Co-Stim displays superior cytotoxic potency of CLDN18.2-expressing cell lines in long term, low E:T co-cultures. Test articles were incubated with human T cells co-cultured with SNU 601 **(A)** or KATO III **(B)** cell lines for 7 days at low (1:5) E:T and evaluated for cytotoxicity of target cells. *AMG 910 (CLDN18.2/CD3 BITE) replica produced in-house.

CD28-related cytokine production only in the presence of CLDN18.2 expressing target cells

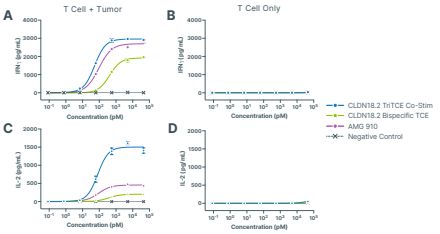

Figure 12. Assessment of IFNγ and IL-2 production following incubation with CLDN18.2 TriTCE Co-Stim. Test articles were incubated with T cells co-cultured with SNU 601 cells **(A,C)** or with a monoculture of T cells **(B,D)** and assessed for IFNγ **(A,B)** or IL-2 **(C,D)** production.

Improved T cell proliferation and survival

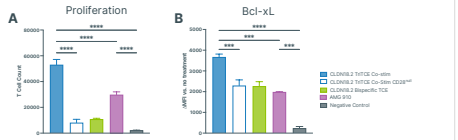

Figure 13. Assessment of T cell proliferation and Bcl-xL upregulation following incubation with CLDN18.2 TriTCE Co-Stim. (A) Test articles (200 pM) were incubated with T cells co-cultured with SNU 601 cells for 7 days and quantified by flow cytometry. **(B)** Test articles (20 nM) were incubated with T cells co-cultured with SNU 601 cells and evaluated for Bcl-xL expression by flow cytometry. (**** p<0.0001; *** p<0.001).

CLDN18.2 TriTCE Co-Stim Molecule Exhibits Superior *in vivo* Anti-Tumor Activity in a PBMC-Engrafted Xenograft Model

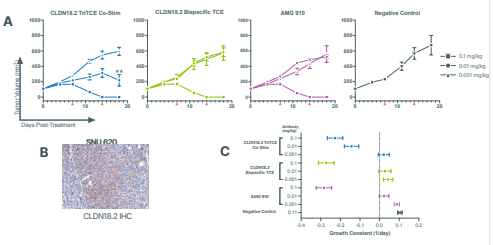

Figure 14. *in vivo* efficacy of CLDN18.2 TriTCE Co-Stim. (A) SNU 620 cells were injected s.c. in NCG mice. Following humanization with PBMCs, mice were treated IV with test article q1w (^ indicates dosing) and monitored for tumor volume (mean +/- SEM, ** p<0.01). **(B)** IHC of CLDN18.2 expression in established SNU 620 xenograft tumors. **(C)** Tumor growth inhibition constants.

Conclusions

Using our Azymetric™ and EFECT™ platforms, we have generated a panel of TriTCE Co-Stim Ab formats. The evaluation of multiple formats, geometries, and paratope affinities allowed **optimization of selectivity and activity** to **promote maximal therapeutic index and efficacy.**

Our lead CLDN18.2-targeting TriTCE Co-Stim exhibited **CLDN18.2-dependent T cell agonism**, with enhanced IL-2 – but similar IFNγ – production compared to bispecific TCEs. TriTCE Co-Stim induced **greater *in vitro* cytotoxicity** of CLDN18.2-expressing tumor cells and exhibited **improved T cell proliferation and survival** compared to bispecific TCEs. Furthermore, our lead TriTCE Co-Stim demonstrated avidity-driven T cell binding. Finally, TriTCE Co-Stim mediated **improved tumor regression** *in vivo* compared to bispecific TCE.

Taken together, these data suggest TriTCE Co-Stim has the potential to reduce T cell apoptosis and provide **more durable responses**, to **re-invigorate 'cold' tumors with lower T cell infiltration**, while **avoiding potential toxicity liabilities** such as **systemic cytokine release.** Taken together, TriTCE Co-Stim demonstrates key factors that may contribute to **improved clinical outcomes.**

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