TriTCE Co-Stim: A novel trispecific T cell engager platform, with integrated CD28 costimulation, engineered to widen the therapeutic window for treatment of poorly infiltrated tumors

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Introduction

Low T cell infiltration and T cell anergy are challenges for the treatment of solid tumors with conventional CD3-engaging bispecific T cell Engagers (TCEs)[1]

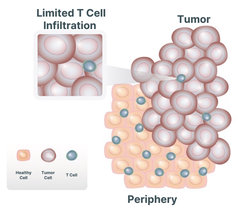


Figure 1. Schematic of T cell infiltration in solid tumors. T cell density positively correlates with prognosis in several solid tumor indications. The ability of conventional tumor-targeting, CD3-engaging, bispecific TCEs to induce anti tumor activity relies on high baseline T cell infiltration. Treatment of solid tumors with these TCEs can result in limited proliferation and recruitment to the tumor site, suggesting that treatment with conventional bispecific TCEs may be insufficient to inhibit the growth of poorly infiltrated, rapidly growing tumors.

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[2]

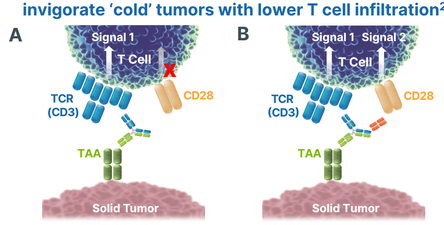


Figure 2. Schematic of TCE-mediated T cell activation in solid tumors. Lack of co-stimulatory ligand engagement in solid tumors can limit the activity and durability of conventional bispecific TCE responses. **(A)** Activation of the T cell receptor (TCR; signal 1) in the absence of co-stimulation can result in T cell anergy, limiting the activity and durability of conventional bispecific TCE anti-tumor responses. **(B)** Activation of TCR with concomitant CD28 co-stimulation (signal 2) may enhance T cell activation, metabolism and fitness, cytokine production, and sustained proliferation.

Therapeutic window optimized via paratope and format engineering

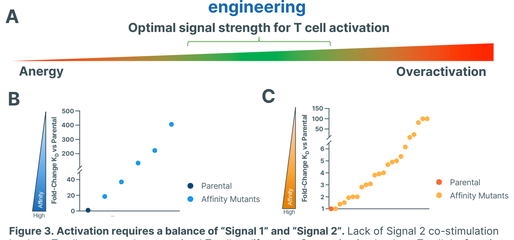


Figure 3. Activation requires a balance of "Signal 1" and "Signal 2". Lack of Signal 2 co-stimulation leads to T cell anergy and no sustained T cell proliferation. Overactivation leads to T cell dysfunction and excessive cytokine release **(A)**. A library of CD3 agonist paratopes **(B)** and conventional CD28 agonist paratopes **(C)** with a range of binding affinities determined by surface plasmon resonance (SPR) were generated to further optimize signaling via CD3 and CD28.

Design Criteria

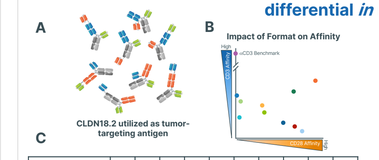
- ✓ **Trispecific that provides Signal 1 and 2 in one molecule**
- ✓ **Balanced αCD3 and αCD28 affinities and optimized format to sustain T cell function and expansion**
- ✓ **Target-dependent T cell activation, no T cell activity in the absence of target antigen**
- ✓ **Enhanced T cell functionality and anti-tumor activity compared to corresponding CD3 bispecific**
- ✓ **Optimal production characteristics (e.g., high purity, yield, stability)**

Exposure Condition	Monomer Purity (%)
5X Freeze/thaw	98.6
10 weeks; -80 °C	99.1
2 weeks; 4 °C	99.1
2 weeks; 40 °C	97.9
3h; pH 9.0	98.4
3h; pH 3.5	97.9
No control (no treatment)	99.8

Table 1. Lead CLDN18.2 TriTCE Co-Stim format exhibits high monomer stability. Lead CLDN18.2 TriTCE Co-Stim format was exposed to various conditions and remains highly stable with >95% monomer purity compared to the no treatment control.

Format Matters!

Various TriTCE Co-Stim formats exhibit antibody-like developability with differential *in vitro* properties



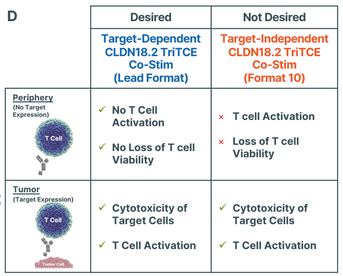


Figure 4. TriTCE Co-Stim antibodies with various paratope formats and geometries are engineered using the Azymetric™ and EFECT™ platforms. Schematic representation of a subset of TriTCE Co-Stim antibody formats **(A)** and the impact of paratope format (scFv vs. Fab) and geometry on the binding affinities to CD3 and CD28 (measured by SPR) for a subset of formats with the same CD3 and CD28 paratopes **(B)**. TriTCE Co-Stim formats that exhibit potent cytotoxicity of target cells, target-dependency, high yield, and thermal stability are selected through extensive screening *in vitro* **(C)**. Summary of properties of target-dependent and target-independent TriTCE Co-Stim formats **(D)**.

No binding of CD28 observed in the absence of CD3 / Binding of CD28 observed in the absence of CD3

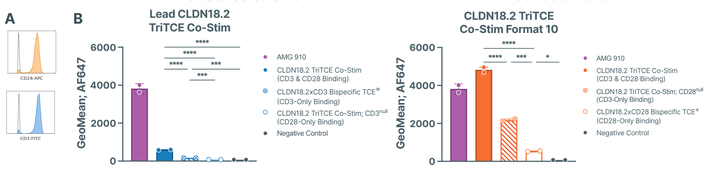


Figure 5. On-Cell Binding of TriTCE Co-Stim formats and format-matched single-arm binding controls. T cell expression of CD3 and CD28 **(A)**. GeoMean of Alexa Fluor 647 (AF647) fluorescence with 1 nM test article. **(B)**. Similar trends with CD28 binding observed up to 600 nM of test article (data not shown). AMG 910 (biosimilar; produced in-house) included as high affinity CLDN18.2xCD3 bispecific TCE. *CD3 and CD28 bispecific TCEs have same paratope geometry as lead TriTCE Co-Stim format (blue) and TriTCE Co-Stim Format 10 (orange), respectively. ****; p<0.0001, ***; p≤0.0005, *; p<0.05.

Lead CLDN18.2 TriTCE Co-Stim format is dependent on target expression to induce cytokine production by human immune cells and exhibits potent target cell lysis

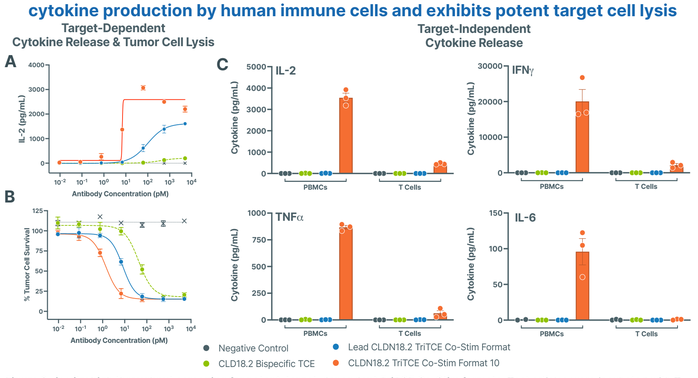


Figure 6. *in vitro* high throughput screening for potent, target-dependent TriTCE Co-Stim formats. Test articles were incubated with T cells co-cultured with CLDN18.2-expressing SNU 601 tumor cells and evaluated for IL-2 production **(A)** and target cell lysis **(B)**. Test articles (5 nM) were incubated with monocultures of PBMCs or T cells and assessed for production of cytokine **(C)**.

Lead CLDN18.2 TriTCE Co-Stim format does not reduce T cell viability

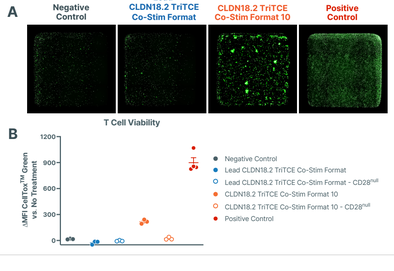


Figure 7. CellTox™ Green T cell viability assay. Test articles (45 nM) were incubated with monocultures of T cells in the presence of CellTox™ Green. After 48h, fluorescence was detected using the Operetta **(A)** and analyzed for median fluorescence intensity (MFI; **B**). Puromycin was included as a positive control for T cell death.

CLDN18.2 TriTCE Co-Stim Mediates No Systemic Toxicity or Peripheral Cytokine Release *in vivo*

TriTCE Co-Stim does not result in body weight loss or systemic cytokine production relative to superagonist αCD28

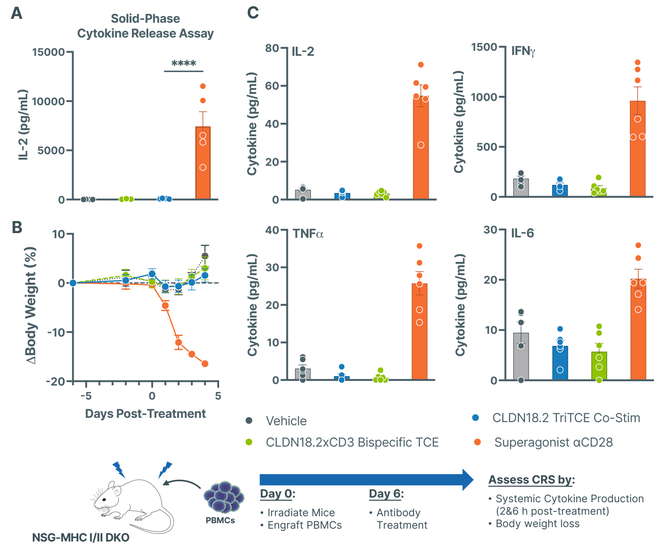


Figure 8. Predictive *in vitro* and *in vivo* models for cytokine release syndrome (CRS). Immobilized test articles (1 μg/well) were incubated with PBMCs for 48 hours and assessed for IL-2 production **(A)**. IL-2 production in solid-phase cytokine release assays is correlated with severity of cytokine release syndrome by TGN1412[3]. **** p<0.0001. huPBMC-engrafted mice were treated with 1 mg/kg of test article and assessed for changes in body weight **(B)** or systemic cytokine production 6 h post-treatment **(C)**. Similar trends were observed at 2 hours-post treatment and for IL-10 and IL-4 production (data not shown). Superagonist αCD28 used in *in vitro* assessment is TGN1412 (hIgG4; biosimilar produced in-house). Superagonist αCD28 used for *in vivo* assessment is ANC28.1/5D10 (mIgG1). CLDN18.2 TriTCE Co-Stim is cross-reactive with mouse CLDN18.2 (data not shown).

CLDN18.2 TriTCE Co-Stim Supports Enhanced T Cell Mediated Activity *in vivo*

TriTCE Co-Stim mediates an increase of T cells within the tumor, but not in the periphery

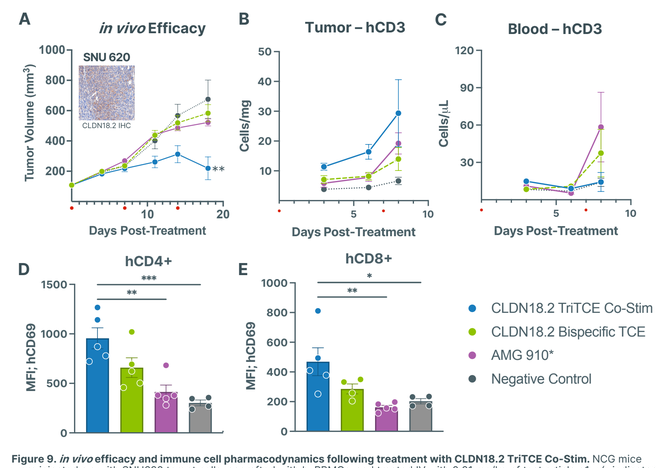


Figure 9. *in vivo* efficacy and immune cell pharmacodynamics following treatment with CLDN18.2 TriTCE Co-Stim. NCG mice were injected s.c with SNU620 target cells, engrafted with huPBMCs, and treated IV with 0.01 mg/kg of test article q1w (▪ indicates dosing). Mice were assessed for tumor volume **(A)**, CD3+ T cell numbers in the tumor **(B)** or blood **(C)**, and CD69 expression by tumor-infiltrating CD4+ **(D)** and CD8+ **(E)** cells. CD69 expression was assessed 1 day post-second dose. * p<0.05; ** p≤0.01, *** p<0.001.

CLDN18.2 TriTCE Co-Stim Exhibits Advantages Over Combination Therapy

Therapeutic strategies to provide Signal 1 (CD3) and Signal 2 (CD28)

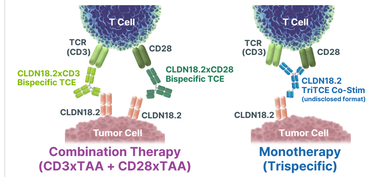


Figure 10. Schematic of combination therapy & monotherapy approaches to provide Signal 1 and Signal 2. **Left:** Signal 1 and Signal 2 are provided by conventional CD3xTAA and CD28xTAA bispecific TCEs. **Right:** CLDN18.2 TriTCE Co-Stim provides Signal 1 and Signal 2 within a single molecule.

TriTCE Co-Stim exhibits equivalent tumor cell lysis with decreased cytokine production

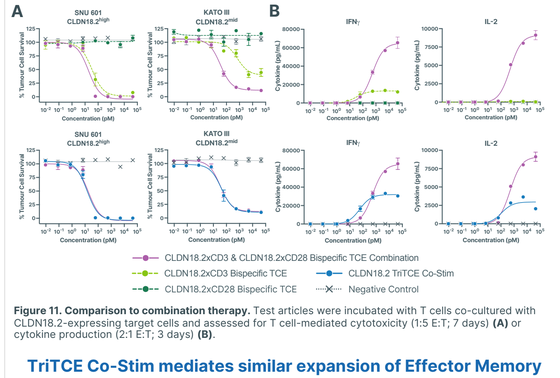


Figure 11. Comparison to combination therapy. Test articles were incubated with T cells co-cultured with CLDN18.2-expressing target cells and assessed for T cell-mediated cytotoxicity (1:5 E:T; 7 days) **(A)** or cytokine production (2:1 E:T; 3 days) **(B)**.

TriTCE Co-Stim mediates similar expansion of Effector Memory (T_{EM}) and Central Memory (T_{CM}) Cells

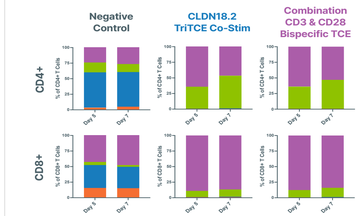


Figure 12. T cell memory subset expansion. Test articles were incubated with PBMCs co-cultured with CLDN18.2-expressing SNU 601 target cells (1:1 E:T) and assessed for expansion of CD4+ and CD8+ central and effector (T_{CM} & T_{EM}) memory cell subsets by flow cytometry.

Conclusions

- Panel of TriTCE Co-Stim Ab formats with various formats, geometries and paratope affinities generated using Azymetric™ and EFECT™ Platforms to optimize selectivity and activity
- Lead CLDN18.2 TriTCE Co-Stim exhibits target-dependent T cell agonism and no reduction of T cell viability
  - CD28 paratope of lead format does not exhibit binding in the absence of CD3 binding
- CLDN18.2 TriTCE Co-Stim mediates improved tumor regression with an increase of activated intratumoral T cells *in vivo*
- TriTCE Co-Stim exhibits equivalent cytotoxicity with reduced cytokine production compared to combination approach of CD3 and CD28-engaging bispecific TCEs
- TriTCE Co-Stim has the potential to provide more durable responses, re-invigorate tumors with low T cell infiltration, and avoid potential toxicity liabilities, such as systemic cytokine release, key factors that may contribute to improved clinical outcomes

References
1. Arvedson T., et al. 2022. Targeting Solid Tumors with Bispecific T Cell Engager Immune Therapy (Vol. 6, pp.17-34).
2. Newhook L., et al. 2023. TriTCE Co-Stim, Next Generation Co-Stimulatory Trispecific T cell Engagers for the Treatment of Solid Tumors. [Poster Presentation] AACR. Orlando, FL
3. Eastwood D., et al. 2013. Severity of the TGN1412 Trial Disaster Cytokine Storm Correlated with IL-2 Release. *Br J Clin Pharmacol.* (Vol. 76, No. 2, pp.299-315).

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