# TriTCE Co-Stim: A next generation trispecific T cell engager platform with integrated CD28 costimulation, engineered to improve T cell function and antitumor responses in hard-to-treat cancers

Abstract # C091

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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]. By providing balanced activation of "Signal 1" (CD3) and "Signal 2" (CD28) in a single molecule, co-stimulatory trispecific TCEs (TriTCE Co-Stim) have the potential to induce more sustainable T cell responses in the tumor and increase therapeutic responses beyond that achievable with bispecific TCE. By enhancing T cell responses, TriTCE Co-Stim have the potential to increase the depth and durability of anti-tumor responses in patients with difficult to treat solid tumors with low T cell infiltration and poor T cell function.**

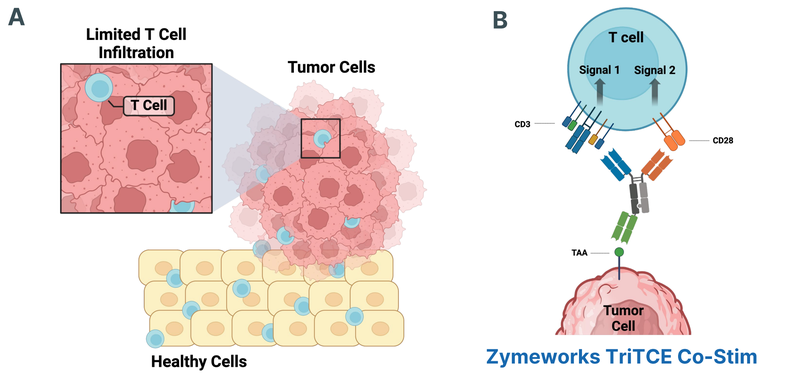



**Figure 1. Proposed mechanism of action for Zymeworks' TriTCE Co-Stim.** Schematic of limited T cell infiltration in solid tumors ¶ (**A**). Schematic of TriTCE Co-Stim-mediated T cell activation in solid tumors ¶ (**B**). TriTCE Co-Stim is designed to provide tumor-associated antigen (TAA) dependent agonism of Signal 1 (CD3) and Signal 2 (CD28) in a single molecule to increase T cell activation, fitness, and proliferation.

## TriTCE Co-Stim: From Concept to Platform

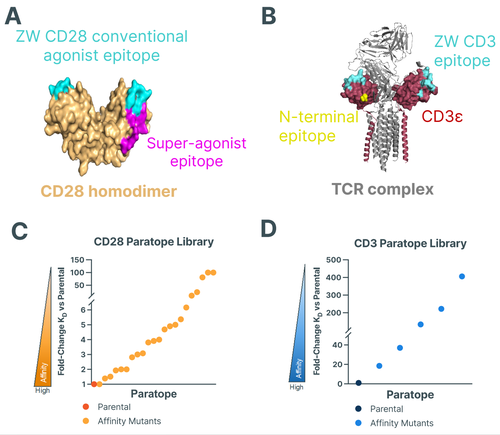
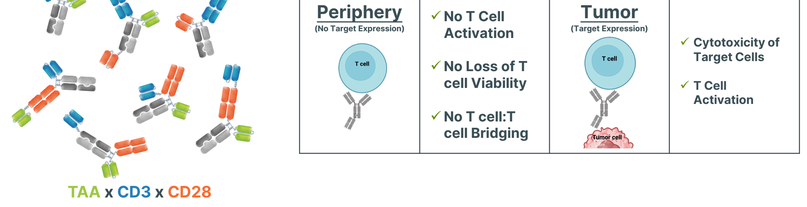



**Figure 2. CD3 and CD28 paratope engineering. (A)** CD28 homodimer structure (modelled using 1YJD) highlighting epitopes for Zymeworks' (ZW) conventional agonist vs super-agonist antibodies. **(B)** Full TCR complex (modelled using 7FJD) with surface representation of CD3ε domain, highlighting for ZW (conformational) vs the N-terminal (linear) epitopes. Libraries of conventional agonist paratope variants with a range of CD28 **(C)** and CD3 **(D)** binding affinities determined by surface plasmon resonance (SPR).

### TriTCE Co-Stim formats exhibit antibody-like developability with differential *in vitro* properties[2]




C

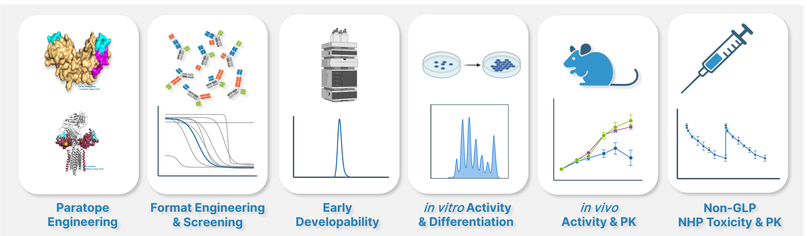
**Representative CLDN18.2 *in vitro* Screening to Assess Desired Functional Characteristics**

| Antibody Format | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Target-Dependent? | ✓ | ✓ | ✓ | x | ✓ | x | x | x | x | x |
| Cytotoxic Potency | | | | | | | | | | |
| Yield (mg/L) | | | | | | | | | | |
| Stability ($T_m$; °C) | | | | | | | | | | |

Low — High

**Figure 3. TriTCE Co-Stim antibodies with various paratope formats and geometries are engineered using the Azymetric™ and EFECT™ platforms to optimize the therapeutic window.** Schematic representation of a subset of formats tested using multiple tumor targeting TAA. (**A**) Summary of desired target-dependent properties of TriTCE Co-Stim achieved by optimized format design¶ (**B**). TriTCE Co-Stim formats that exhibit potent cytotoxicity of target cells, target-dependency, acceptable yield, and thermal stability are selected through extensive screening *in vitro* (**C**).

### TriTCE Co-Stim Lead Format Selection




**TriTCE Co-Stim platform tested with three targets including CLDN18.2[3] and DLL3[4]**

- **Assessed TriTCE Co-Stim relative to first generation CD3-engaging bispecific TCEs**
- **Interrogated mechanism of T cell engagement relative to benchmark trispecific TCEs**
- **Determined safety in NHP tox for CLDN18.2 lead TriTCE Co-Stim**

**Figure 4. Workflow Established for the Development of TriTCE Co-Stim Platform¶.**

## Enhanced T-cell mediated Cytotoxicity

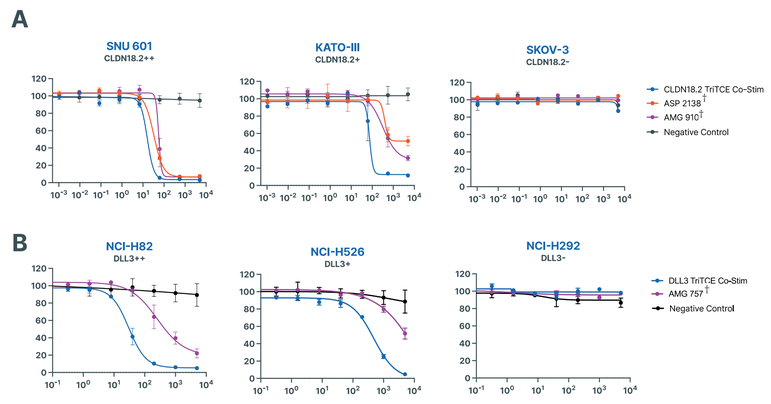



**Figure 5. TriTCE Co-Stim molecules display superior cytotoxic potency in long term low E:T co-cultures.** Test articles were incubated with human T cells co-cultured with CLDN18.2+ **(A)** or DLL3+ tumor cell lines for 7 days at low E:T and evaluated for cytotoxicity of target cells. TriTCE Co-Stim demonstrate superior cytotoxicity in a long-term low E:T ratio cytotoxicity assay and demonstrate no activity on TAA- cells.

## Sustained Tumor Cytotoxicity with Repeated Stimulations

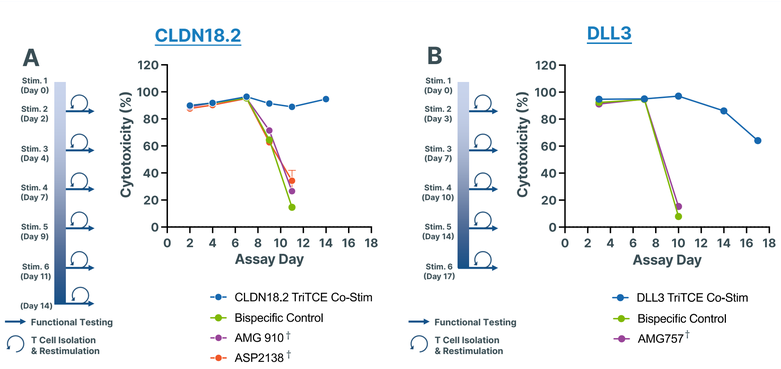



**Figure 6. TriTCE Co-Stim molecules display sustained T cell fitness and anti-tumor activity in a repeat challenge assay.** T cells were stimulated with CLDN18.2+ tumor cell line - SNU 601 cells (5:1 E:T) and test article (1 nM), and DLL3+ tumor cell line - NCI-H82 cells (5:1 E:T) and test article (5 nM). For each subsequent round of stimulation, T cells are isolated from the T cell:tumor cell co-culture, counted, and re-stimulated with fresh tumor cell and respective test article. Schematic of T cell restimulation ¶ (**A**). Following each round of stimulation, T cell:tumor cell co-cultures were then assessed for tumor cell cytotoxicity (**B**). Insufficient T cells for continued stimulation with CLDN18.2 Bispecific, AMG 910† and ASP2138† following stimulation 5 in SNU601 cells and similar results were observed with DLL3 bispecific control and AMG 757† following stimulation 3 for NCI-H82 cells

## Enhanced T-cell Proliferation and Survival

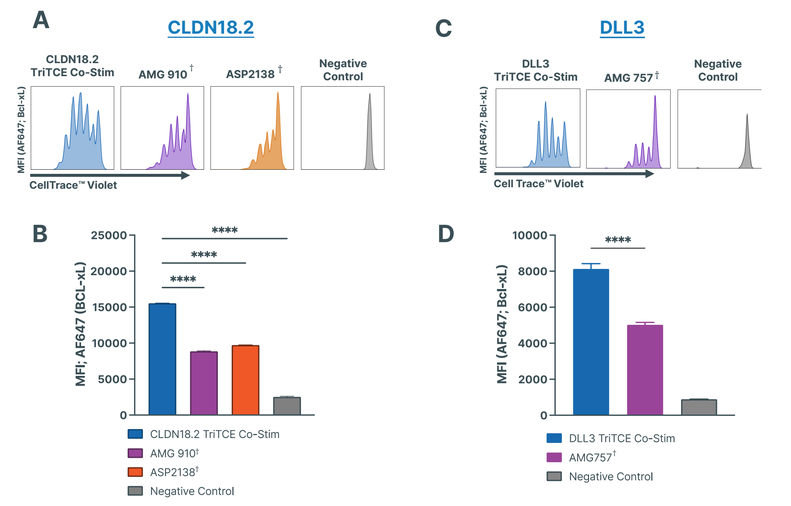



**Figure 7. TriTCE Co-Stim molecules Increases T cell proliferation and upregulation of anti-apoptotic marker Bcl-xL.** Test articles were incubated with CellTrace™ Violet-stained T cells co-cultured with tumor cells (SNU 601 cells for CLDN18.2 and NCI-H82 cells for DLL3 at 5:1 E:T) for 5 days and assessed by flow cytometry (**A, C**). Test articles were incubated with T cells co-cultured with SNU 601 cells for CLDN18.2 and NCI-H82 for DLL3 (2:1 E:T) and evaluated for Bcl-xL expression, an anti-apoptotic marker, by flow cytometry (**B, D**). Data are representative of two individual donors and are presented as mean ± SD.

## Enhanced Anti-tumor Activity in Humanized Xenograft Models

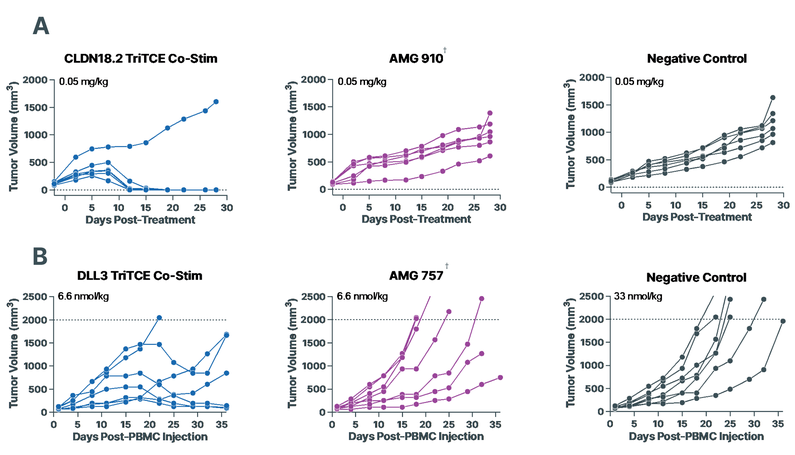



**Figure 8. *in vivo* efficacy following treatment with TriTCE Co-Stim molecules.** NCG mice (n=6) were injected SC with SNU 620 target cells, engrafted with human PBMCs, and treated intravenously with CLDN18.2 TriTCE Co-stim q1wx4 and full tumor regression is observed in 5/6 mice **(A)**. PBMC-engrafted SHP-77 (SC) xenograft mouse model used to evaluate DLL3 TriTCE Co-Stim *in vivo* efficacy. Tumor volume over time of mice treated intravenously with DLL3 TriTCE Co-Stim, AMG 757 and irrelevant mAb. Full or partial tumor regression is observed in 4/7 mice treated with DLL3 TriTCE Co-Stim when IV treated q1wx4. **(B)**.

## Conclusions

TriTCE Co-Stim antibodies with various paratope formats and geometries are engineered using the Azymetric™ and EFECT™ platforms. The evaluation of multiple formats, geometries, and paratope affinities allowed for optimization of selectivity and activity to promote a widened therapeutic index with enhanced anti-tumor activity. Zymeworks Co-Stim Abs using 2 different TAA's **CLDN18.2 and DLL3** show:

- **Greater *in vitro* cytotoxicity at low E:T ratios and improved T cell proliferation and survival** compared to bispecific TCEs
- **Sustained cytotoxicity, T cell viability and proliferation in serial challenge assays**
- **Obligate cis T cell binding to CD3 and CD28, and conditional CD28 binding requiring co-engagement of CD3**
- **Improved *in vivo* tumor regression** relative to clinical benchmark bispecific
- **No systemic cytokine release** in an *in vivo* CRS model study
- **CLDN18.2 TriTCE surrogate well tolerated in NHP[3]**

**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: A Novel Trispecific T Cell Engager Platform, with Integrated CD28 Costimulation, Engineered to Widen the Therapeutic Window for Treatment of Poorly Infiltrated Tumors. [Poster Presentation] SITC. San Diego, CA
3. Newhook et al., 2024. TriTCE Co-Stim: A next generation trispecific T-cell engager platform with integrated CD28 costimulation, engineered to improve responses in the treatment of solid tumors. (Poster Presentation) AACR. San Diego, CA.
4. Repenning et al., 2024. DLL3 TriTCE Co-Stim: A next generation Trispecific T cell engager with integrated CD28 co-stimulation for the treatment of DLL3-expressing cancers. (Poster Presentation) AACR. San Diego, CA.
5. 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).
6. 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
7. Türeci O., et al. 2011. Claudin-18 gene structure, regulation, and expression is evolutionary conserved in mammals. Gene. (Vol. 481, No 2, pp 83-92).

† Following molecules were produced in house: AMG 910 (CLDN18.2/CD3 BiTE), ASP2138 (CLDN18.2/CD3 2+1 bsAb) and AMG 757 (DLL3/CD3 BiTE)
ǂ TGN1412 replica produced in-house
§ CD3xCD28xTAA CODV Analog is a CD3xCD28xMSLN trispecific with the same format as the Sanofi Trispecific containing a CD3xCD28 CODV-Fab; produced in-house.
∗ Surrogate CLDN18.2 TriTCE Co-Stim exhibited ~10 fold increased cytotoxic potency vs. lead TriTCE Co-Stim and ~15-fold reduced cytotoxic potency vs. AMG 910 in cynomolgus T-cell dependent cytotoxicity asssays *in vitro*
¶ Image Created with BioRender.com

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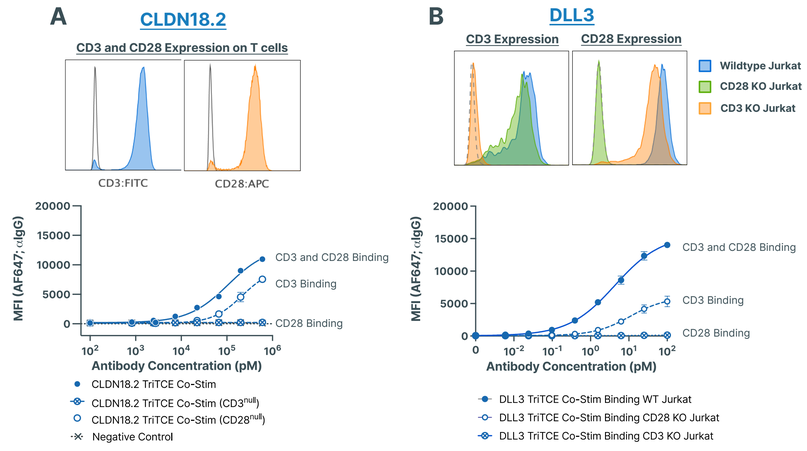
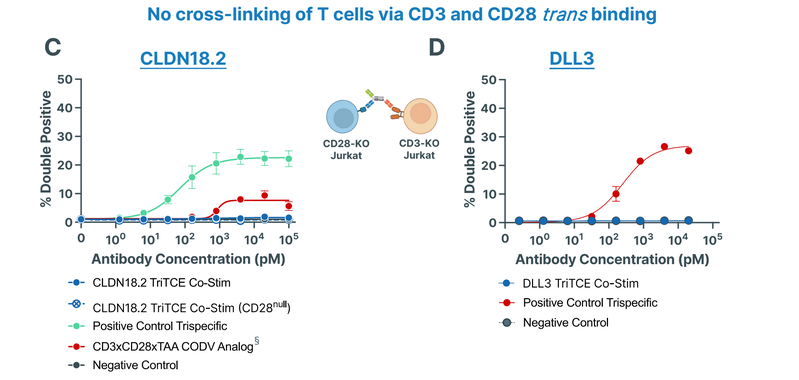
## TriTCE Co-Stim Facilitates Desirable T-cell Engagement

### Conditional binding of CD28 requiring CD3 co-engagement




### No cross-linking of T cells via CD3 and CD28 *trans* binding




### No reduction of T-cell viability

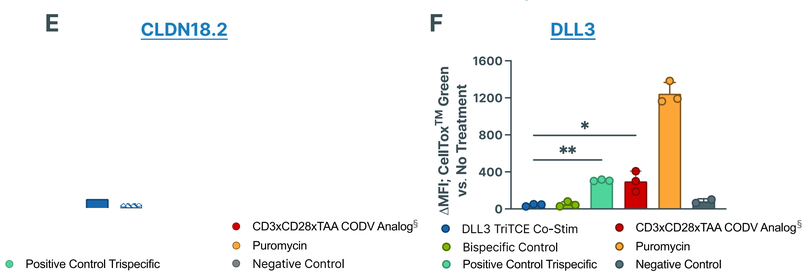



**Figure 9. TriTCE Co-Stim displays desirable T-cell engagement and does not cross link T cells in *trans*.** TriTCE Co-Stim molecules need CD3 engagement on T-cells in order to bind CD28, therefore CD28 engagement is conditional. No T-cell binding is observed with the CLDN18.2 TriTCE Co-Stim CD3 null **(A).** DLL3 TriTCE Co-Stim does not bind CD3 KO Jurkat T cells **(B)**. Cell bridging by immune cell engaging antibodies can induce fratricide and thus reduce therapeutic efficacy[4]. The ability of TriTCE Co-Stim molecules to cross link T cells was tested by incubating CD3 KO and CD28 Jurkat T cells fluorescently labelled with dye in the presence of TriTCE. No T-cell bridging is observed with both CLDN18.2 and DLL3 TriTCE Co-Stim molecules **(C,D)**. To assess effects of TriTCE Co-Stim molecules on T-cell viability, test article and T cells were incubated with CellTox™ green and signal was measured 48 hours post incubation. CLDN18.2 and DLL3 TriTCE Co-Stim show significantly less reduction in T-cell viability relative to positive control TriTCE and the CD3xCD28xTAA CODV Analog **(E,F)** . * p<0.05, ** p<0.01, **** p<0.0001.

## Lack of IL-2 production in solid phase CRS Assay

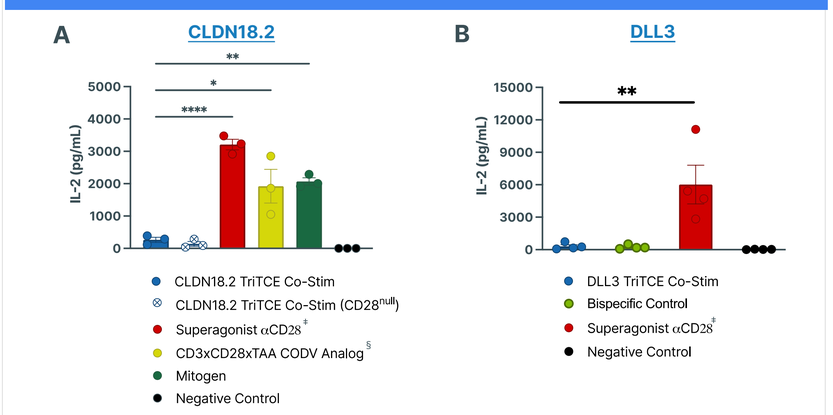



**Figure 10. Predictive *in vitro* model for cytokine release syndrome (CRS).** IL-2 production in a solid phase CRS assay is correlated with severity of CRS by TGN1412, an anti-CD28 superagonist antibody[5,6]. To test IL-2 production, immobilized test articles (1 $\mu$g/well) were incubated with PBMCs for 48 hours and cytokine levels were measured from supernatants via MSD. CLDN18.2 TriTCE Co-Stim **(A)** and DLL3 TriTCE Co-Stim **(B)** did not exhibit T-cell cytokine release. Superagonist $\alpha$CD28 is TGN1412 replica produced in-house. Mitogen is Staphylococcal enterotoxin B. Data presented are mean ± SEM of at least three individual PBMC donors. * p<0.05, ** p<0.01, **** p<0.0001.

## CLDN18.2 TriTCE Co-Stim Displays Favourable Safety Profile

**Cynomolgus monkeys (n=3) were administered a repeat dose of 3mg/kg of a cynomolgus surrogate CLDN18.2 TriTCE Co-Stim* on day 0 and day 8.**

- **Toxicology findings were mild and associated with the known mechanism of action of TCEs[3]**
- **No histopathological changes observed in the stomach, where CLDN18.2 is expressed[7]**
- **Other histopathological changes were secondary to decreased food consumption and body weight loss**

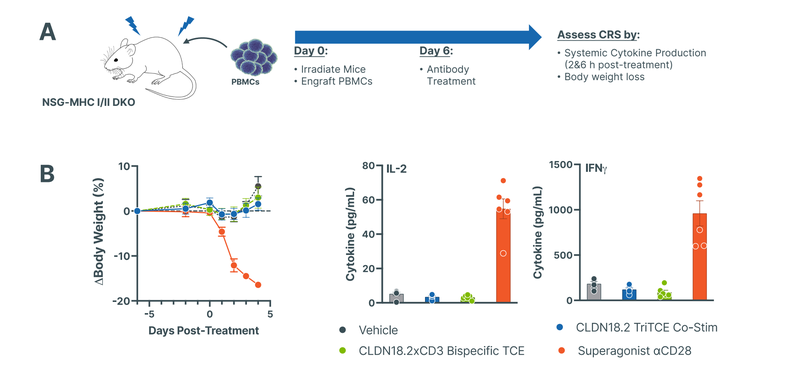



**Figure 11. No systemic toxicity is observed in *in vivo* models for cytokine release syndrome (CRS).** huPBMC-engrafted were treated with 1mg/kg test article and body weight loss was observed with superagonist $\alpha$CD28 (ANC28.1/5D10) **(A)** and IL-2 production is correlated with severity of cytokine release syndrome by TGN1412[5,6]. High levels of systemic cytokine production was also observed with superagonist $\alpha$CD28 6 h post-treatment when compared to CLDN18.2 TriTCE or Bispecific control **(B)**. Superagonist $\alpha$CD28 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).