DLL3 TriTCE Co-Stim: A next generation Trispecific T cell engager with integrated CD28 co-stimulation for the treatment of DLL3-expressing cancers

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Introduction

Small cell lung cancer (SCLC) is an aggressive neuroendocrine cancer with a poor prognosis and high unmet medical need[1]. DLL3 is a therapeutic target that is selectively expressed in SCLC and other neuroendocrine tumors[4]. Bispecific T cell engagers (TCE) targeting DLL3 have entered the clinic and demonstrated encouraging anti-tumor activity in SCLC patients[2,5]. However, SCLC is frequently characterized by an immunosuppressive microenvironment and poor T cell infiltration which may limit clinical activity of CD3 engagers[3].

DLL3 TriTCE Co-Stim is a trispecific T cell engager (TriTCE) designed to optimally engage CD3 and CD28 to redirect and enhance cytotoxic T cell responses to DLL3-expressing tumor cells while maintaining a desired safety profile. This approach has the potential to improve outcomes for patients, especially those with poorly infiltrated tumors, by increasing the depth and durability of response.

Key challenges and therapeutic goals

- **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)[3]**
- **Downregulation of HLA molecules in SCLC limits antigen presentation and responses to immunotherapy[3]**
- **Co-stimulatory trispecific TCEs (TriTCE Co-Stim) have the potential to provide enhanced T cell activation in the absence of TCR:pHLA recognition that may stimulate T cell proliferation in patients with poorly infiltrated tumors via optimization of "Signal 1" (CD3) and "Signal 2" (CD28)**

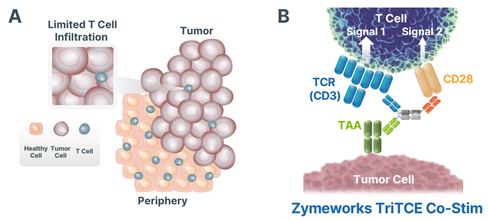
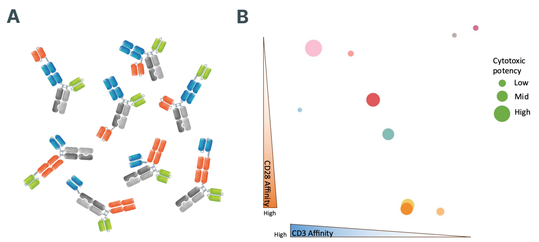


Figure 1. Proposed mechanism of action and therapeutic goals for DLL3 TriTCE Co-Stim. Schematic of limited T cell infiltration in solid tumors **(A)**. Schematic of TriTCE Co-Stim-mediated T cell activation in solid tumors **(B)**. TriTCE Co-Stim is designed to provide tumor-associated antigen (TAA) dependent agonism of Signal 1 (CD3) and Signal 2 (CD28) in a single molecule to increase T cell activation, fitness, and proliferation. This increased activity may enable treatment of a broader patient population than traditional bispecific TCEs by improving outcomes in patients with reduced tumor immune cell infiltration.

DLL3 TriTCE Co-Stim Is Engineered as a Best-in-Class Trispecific TCE

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



C

DLL3 Co-stim Molecule	1	2	3	4	5	6	7	8	9	10
Cytotoxicity										
Target-Dependent	✓	✓	✓	X	X	X	X	X	✓	✓

Cytotoxicity ↑

Figure 2. Geometries and CD3 and CD28 paratope engineering. Schematic representation of a subset of DLL3 TriTCE Co-Stim formats **(A)**. Correlation between paratope format (scFv vs. Fab), geometry, binding affinities to CD3 and CD28 (measured by surface plasmon resonance), and anti-tumor activity **(B)**. A panel of DLL3 TriTCE Co-Stim formats are screened for T cell-dependent cytotoxic potency against target-expressing cells. The same formats are further assessed for target-dependent T cell activation by assessing the induction of cytotoxicity and cytokine production in co-culture with DLL3-negative tumor cell lines and monocultures of T cells **(C)**.

DLL3 TriTCE Co-Stim Mediates Enhanced Anti-tumor Activity Compared to DLL3xCD3 Bispecific T Cell Engagers

Enhanced long-term, T cell-mediated cytotoxicity at low effector:target (E:T) ratios

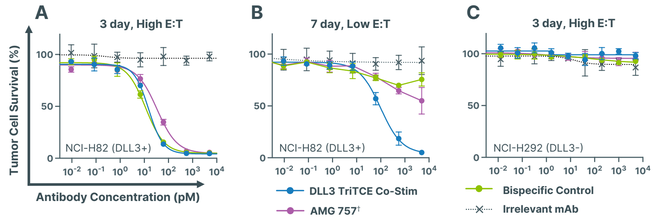


Figure 3. DLL3 TriTCE Co-Stim displays superior cytotoxic potency of DLL3-expressing cell lines in long term, low E:T co-cultures. Test articles were incubated with human T cells co-cultured with DLL3-expressing SCLC tumor cell line NCI-H82 for 3 days at high E:T **(A)** or 7 days at low E:T **(B)** or DLL3-negative NSCLC tumor cell line NCI-H292 for 3 days at high E:T **(C)** and evaluated for cytotoxicity. Bispecific control is a DLL3xCD3 format matched to DLL3 TriTCE Co-Stim.

Improved *in vitro* potency relative to bispecific clinical benchmarks across multiple DLL3-expressing SCLC tumor cell lines

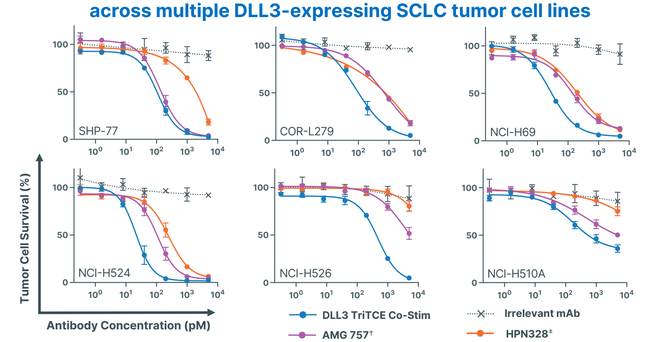


Figure 4. DLL3 TriTCE Co-Stim displays superior *in vitro* cytoxicity relative to clinical benchmarks across multiple DLL3-positive SCLC tumor cell lines. Test articles were incubated with T cells co-cultured with DLL3-expressing tumor cell lines for 7 days and evaluated for cytotoxicity.

Improved T Cell Function and Sustained In Vitro Cytotoxicity Relative to Bispecific T Cell Engagers

Enhanced T cell proliferation and survival

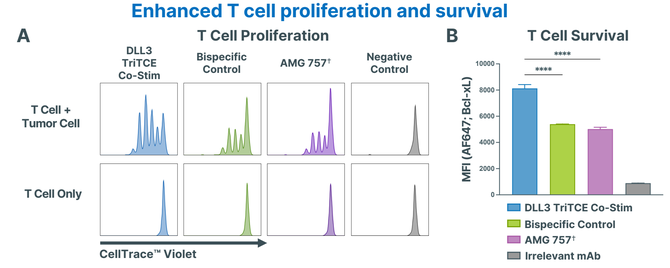


Figure 5. DLL3 TriTCE Co-Stim Increases T cell proliferation and upregulation of anti-apoptotic marker Bcl-xL. Test articles (5 nM) were incubated with CellTrace Violet™ labeled T cells alone or co-cultured with NCI-H82 cells for 5 days and assessed by flow cytometry **(A)**. Test articles (5 nM) were incubated with T cells co-cultured with NCI-H82 cells for 48 hours and evaluated for Bcl-xL expression by flow cytometry **(B)**. **** p<0.0001

Sustained T cell-mediated cytotoxicity over repeated T cell stimulations

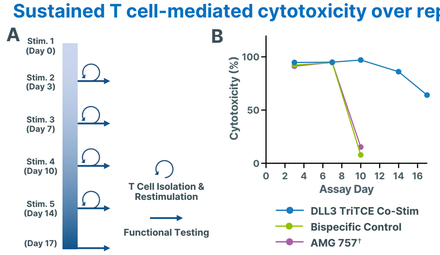


Figure 6. DLL3 TriTCE Co-Stim Displays sustained T cell fitness and anti-tumor activity in a serial, repeated challenge assay. T cells were incubated with NCI-H82 cells (5:1 E:T) and test article (5 nM). For each subsequent round of stimulation, T cells were harvested from the co-culture, counted, and re-stimulated with fresh NCI-H82 target cells (5:1 E:T) and fresh test article (5 nM). Schematic of T cell restimulation **(A)**. Following each round of stimulation, T cell: Tumor cell co-cultures were assessed for tumor cell cytotoxicity **(B)**. Following stimulation 3, bispecific control and AMG 757† had insufficient viable T cells for continued stimulation.

DLL3 TriTCE Co-Stim Design Facilitates Desirable T Cell Engagement

Exhibits obligate *cis* binding requiring co-engagement of CD3 to bind CD28

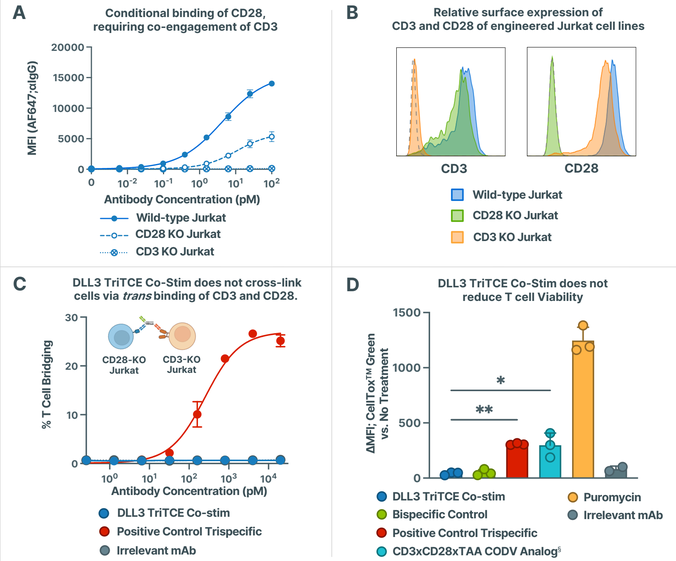


Figure 7. DLL3 TriTCE Co-Stim does not bind CD28 in the absence of CD3 or induce cross-linking of T cells via *trans* binding of CD3 and CD28. On cell binding of DLL3 TriTCE Co-Stim to wild-type (WT), CD28 Knockout (CD28-KO) and CD3 Knockout (CD3-KO) Jurkat cells. Test articles were incubated with Jurkat cells and assessed for binding by flow cytometry **(A)**. Histograms display relative surface expression of CD3 and CD28 in WT, CD28-KO and CD3-KO Jurkat cells assessed by flow cytometry, unstained cells displayed as dashed lines **(B)**. Cross-linking of CD3-KO and CD28-KO Jurkat cells. Test articles were incubated with pre-labelled Jurkat cells at 1:1 ratio (CD3-KO:CD28-KO) for 1h and evaluated for cross-linking via flow cytometry. Representative schematic of cell bridging (**inset**¶) **(C)**. T cell Viability. Test articles (50 nM) were incubated with monocultures of T cells in the presence of CellTox™ Green. After 48hs, fluorescence was detected using the Operetta and analysed for median fluorescence intensity (MFI) **(D)**. Cross-link positive control trispecific antibody is a DLL3xCD3xCD28 TriTCE format that binds CD3 and CD28 in *trans* and puromycin was included as a positive control for cell death. ** p<0.01, * p<0.1

Enhanced T Cell Activity is Target-dependent

Increased target dependent cytokine production relative to bispecific TCE control

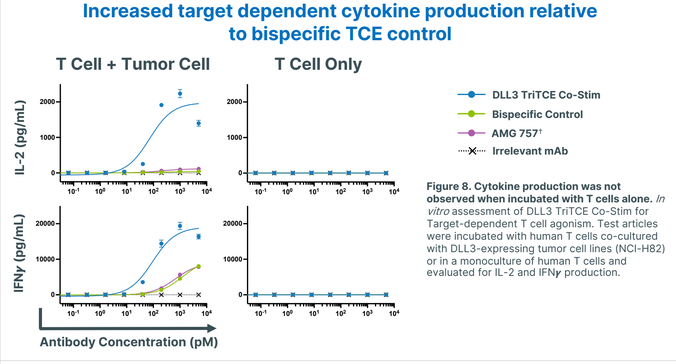


Figure 8. Cytokine production was not observed when incubated with T cells alone. *In vitro* assessment of DLL3 TriTCE Co-Stim for Target-dependent T cell agonism. Test articles were incubated with human T cells co-cultured with DLL3-expressing tumor cell lines (NCI-H82) or in a monoculture of human T cells and evaluated for IL-2 and IFNγ production.

Plate-bound DLL3 TriTCE Co-Stim does not mediate IL-2 production by PBMCs

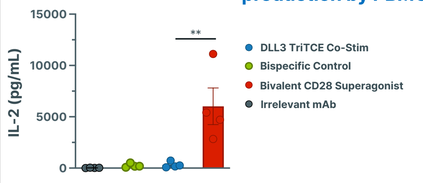


Figure 9. Predictive *in vitro* model for cytokine release syndrome (CRS). Immobilized test articles were incubated with PBMCs for 48 hours and assessed for IL-2 production. IL-2 production in solid-phase cytokine release assays is correlated with severity of cytokine release syndrome by bivalent CD28 superagonist. Bivalent CD28 superagonist is TGN1412 replica produced in-house. Data presented are mean ± SEM of four individual PBMC donors. ** p<0.01

Superior *in vivo* Anti-tumor Activity in an Established SCLC Humanized Xenograft Model

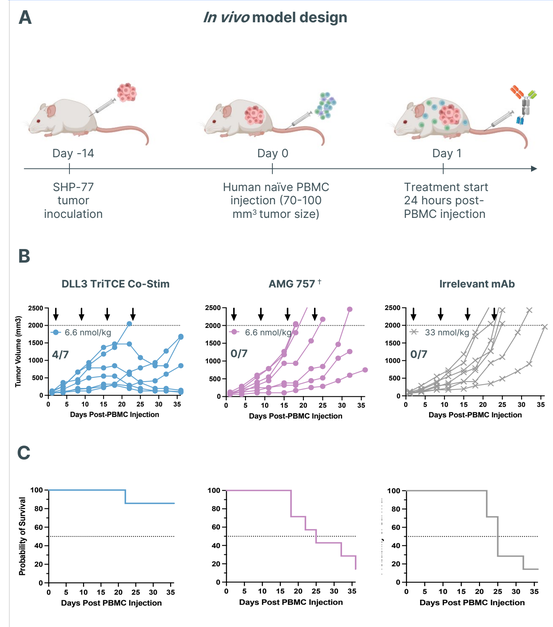


Figure 10. DLL3 TriTCE Co-Stim efficacy *in vivo*. Schematic¶ of the naïve PBMC-engrafted SHP-77 xenograft mouse model used to evaluate DLL3 TriTCE Co-Stim efficacy *in vivo*. SHP-77 cells were injected s.c. in NCG mice. Following PBMC humanization, mice were treated IV with test article q1w x 4 **(A)**. Tumor volume over time of mice treated with DLL3 TriTCE Co-Stim (6.6 nmol/kg), AMG 757 (6.6 nmol/kg) and irrelevant mAb (33 nmol/kg). Full or partial tumor regression was observed in 4/7 mice treated with DLL3 TriTCE Co-Stim. Arrows indicate treatment days **(B)**. Kaplan-Meier curves showing probability of survival of tumor-bearing mice treated with DLL3 TriTCE Co-Stim (blue), AMG 757 (purple, MS = 25 days) or an irrelevant mAb (grey, MS = 25 days). Death events represent euthanized animals due to reaching experimental endpoint (TV ≥ 2000 mm³)**(C)**. MS = Median Survival, TV = Tumor Volume.

Conclusions

Using Zymework's TriTCE Co-Stim platform in combination with our Azymetric™ and EFECT™ technologies, we have generated a panel of DLL3 TriTCE Co-Stim Ab formats. 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.

DLL3 TriTCE Co-Stim:

- Induces **greater *in vitro* cytotoxicity and improves T cell proliferation and survival** compared to bispecific TCEs.
- **Displays no cross-linking of T cells** and **exhibits obligate *cis* T cell binding of CD28, requiring co-engagement of CD3.**
- Exhibits **DLL3-dependent T cell agonism.**
- Mediates **improved *in vivo* tumor regression** relative to clinical benchmark bispecific TCE.
- Has the potential to provide **more durable responses by prolonging T cell-mediated cytotoxicity** as well as **increasing response rates by stimulating T cell proliferation in poorly infiltrated 'cold' tumors.**

DLL3 TriTCE Co-Stim demonstrates key factors that may contribute to **improved clinical outcomes.**

References:
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4. Yao, J. *et al.* 2022. DLL3 as an emerging target for the treatment of neuroendocrine neoplasms. Oncologist.
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† AMG 757 (DLL3/CD3 BiTE) produced in-house
‡ HPN328 (DLL3/CD3 TriTAC) 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.

¶ Image Created with BioRender.com