TriTCE Co-stim: A differentiated T cell engager platform with conditional *cis* CD28 co-stimulation and transferability to diverse targeting strategies

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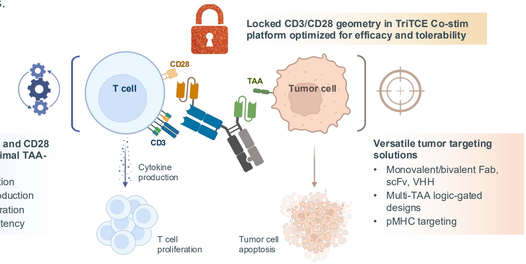
Abstract #1196

Introduction

TriTCE Co-stim is a modular and adaptable next generation T cell engager (TCE) platform developed to drive enhanced T cell activation, anti-tumor activity, and tolerability[1, 2]. Low T cell infiltration and T cell anergy remain as challenges in the treatment of solid tumors by conventional CD3-engaging bispecific TCEs. To overcome the lack of efficacy and durability of responses in solid tumors, concomitant CD28 co-stimulation provided by a trispecific T cell engager (TriTCE Co-stim) can be used to improve T cell responses[3].

Here we highlight the novelty and versatility of the modular TriTCE Co-stim platform enabling potent tumor-associated antigen (TAA)-dependent and tunable anti-tumor and autoimmune applications.

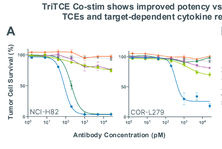


TriTCE Co-stim Platform Enables Widened Therapeutic Window

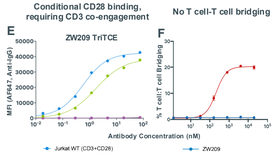
Case Study ZW209: DLL3 x CD3 x CD28 Trispecific T Cell Engager (TriTCE)

Efficacy ⟷ Therapeutic window ⟷ Safety

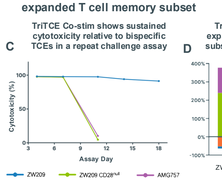
➢ Improved potency at low E:T ratios

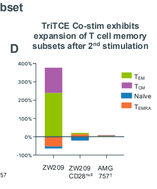


➢ Obligate *cis* binding to T cells



➢ Sustained cytotoxicity in repeat challenge assay and expanded T cell memory subset





➢ No T cell fratricide

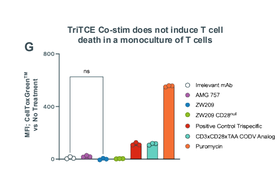


Figure 1. Features of TriTCE Co-stim platform that demonstrate efficacy advantage of adding CD28 compared to bispecific TCE (CD3xTAA) while maintaining safety profile equivalent to bispecific TCE. (A) Test articles were incubated with human T cells co-cultured with DLL3-expressing SCLC tumor cell lines 7 days at low E:T and evaluated for cytotoxicity. **(B)** 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 production. **(C)** T cells were incubated with NCI-H82 cells and test article. For each subsequent round of stimulation, T cells were harvested from the co-culture, counted, and re-stimulated with fresh NCI-H82 target cells and fresh test article. **(D)** T cell memory populations were assessed by flow cytometry staining for CD45RO and CCR7 expression 3 days after 2nd stimulation (day 7). **(E)** Test articles were incubated with Jurkat WT, CD28-KO or CD3-KO cells and assessed for binding by flow cytometry. **(F)** Test articles were incubated with pre-labelled Jurkat cells at (CD3-KO and CD28-KO) for 1h and evaluated for cross-linking via flow cytometry. **(G)** Test articles were incubated with monocultures of T cells in the presence of CellTox™ Green. After 48hs, fluorescence was detected using the Operetta and analysed for mean fluorescence intensity (MFI).

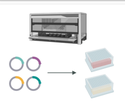
High Throughput Multispecific Antibody Screening Workflow Enables Rapid Selection of TriTCE Candidates

Multispecific antibody formats with varying TAA geometry and paratopes are screened while maintaining the locked TriTCE Co-stim platform CD3/CD28 geometry

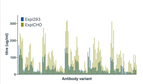
1. TriTCE panel design

- Rational paratope selection based on epitope and affinity
- Design 3- and 4-chain constructs using Azymetric™ HetFc and HetFab (κ-κ and κ-λ) solutions to drive proper chain pairing

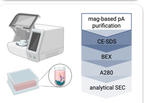
2. Cloning and expression



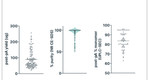
- Automated 96 deep well plate transfection and expression
- Capacity for 200-400 variants in parallel



3. 1-Step purification and analytics



- Automated 1-step pA purification of different antibody formats
- Antibodies analyzed by A280, CE-SDS, analytical SEC, DSF



4. Functional screen

- Automated high content imaging of HTP cytotoxicity assay for functional screening in 384 well plates
- Additional HTP assays for further screening (MSD, SPR)

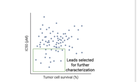
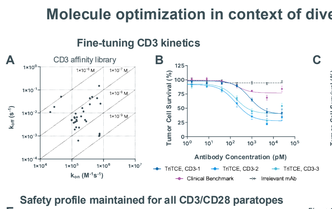
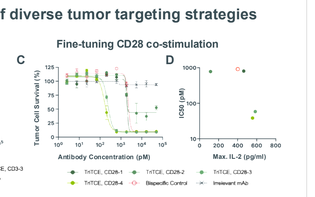


Figure 2. High throughput multispecific antibody screening workflow. TriTCE Co-stim panel designed using Azymetric™ HetFc and HetFab to drive heterodimeric Fc and correct light chain pairing. Automated high throughput expression methods used and antibody expression is verified by measuring titre. One-step purification and analysis by CE-SDS, A280, and analytical SEC to verify antibody yield and purity. High throughput functional screening is made amendable by using automation and robotics to identify multispecific antibodies that meet desired criteria.

Fine-tuning CD3/CD28 Affinity and Tumor-targeting Strategies Enable Broad TriTCE Co-stim Therapeutic Application

Molecule optimization in context of diverse tumor targeting strategies





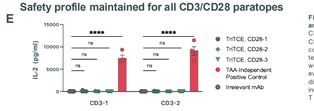


Figure 3. CD3 and CD28 paratopes can be fine-tuned for affinity to optimize cytotoxicity and cytokine production while maintaining desirable TCE safety profile. (A) Affinity plot of CD3 paratope library with varying kinetic parameters. **(B)** TriTCE test articles differing only in CD3 paratope (CD3-1-3) and a clinical benchmark were incubated with human T cells co-cultured with tumor cell line for 7 days at low E:T and evaluated for cytotoxicity. **(C)** TriTCE test articles differing only in CD28 paratope (CD28-1-4) and format-matched bispecific control were incubated with human T cells co-cultured with tumor cell line for 7 days at low E:T and evaluated for cytotoxicity and **(D)** maximum IL-2 production. **(E)** TriTCE test articles with two different affinity CD3 paratopes (CD3-1, CD3-2) and three CD28 paratopes (CD28-1-3) were incubated with human T cells and evaluated for IL-2 production to assess TAA-independent T cell activation.

Solid tumor

1+1+1 (TAA #1) Small cell lung cancer

2+1+1 (TAA #2) Colorectal cancer

scFv (TAA #3) Gastric cancer

Hematology

VHH (TAA #4) Multiple myeloma

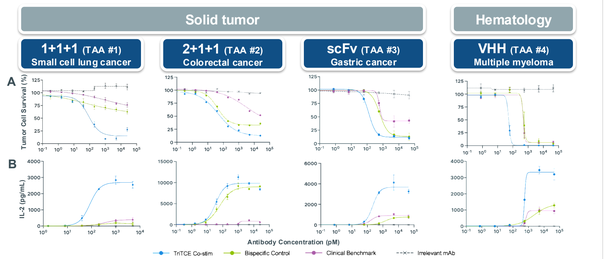
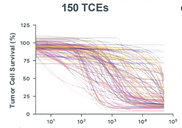
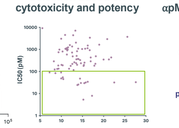


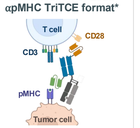
Figure 4. Versatility of TriTCE Co-stim tumor-targeting domain allows for broad therapeutic applications across diverse modalities. (A) Test articles were incubated with human T cells co-cultured with tumor cell lines for 7 days at low E:T and evaluated for cytotoxicity. **(B)** Test articles were incubated with human T cells co-cultured with tumor cell lines for 3 days and evaluated for IL-2 production.

Targeting Peptide-MHC with TriTCE Co-stim

Optimizing geometry for T cell activation is a key determinant in overcoming low density of pMHC targets in solid tumors







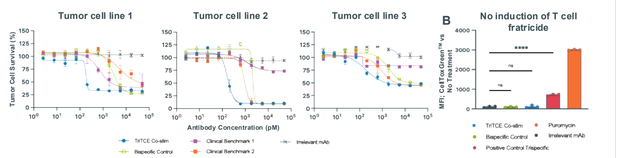


Figure 5. Lead αpMHC TriTCE Co-stim format induces increased T cell cytotoxicity compared to bispecific TCEs across three tumor cell-lines. (A) Test articles were incubated with human T cells co-cultured with tumor cell lines for 7 days at low E:T and evaluated for cytotoxicity. **(B)** Test articles were incubated with monocultures of T cells in the presence of CellTox™ Green. After 48hs, fluorescence was detected using the Operetta and analysed for MFI.

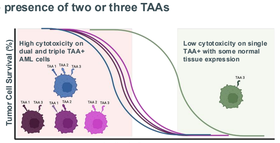
Applying Logic-gated Tumor Targeting with TriTCE Co-stim

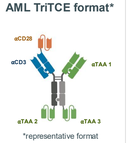
Multi-TAA AND/OR/NOT logic-gated designs to enable cancer cell selectivity, minimize off tumor/on target toxicity, and overcome antigen escape

Screened logic-gated TriTCE antibody formats for selective tumor cytotoxicity in the presence of two or three TAAs

TAA expression profile	TAA	Cytotoxicity
Triple positive	TAA 1 & TAA 2 & TAA 3	✔
Dual positive	TAA 1 & TAA 2 OR TAA 2 & TAA 3 OR TAA 1 & TAA 3	✔
Single positive	TAA 3	✖

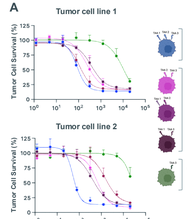
*TAA 1 and TAA 2 are expressed on LSCs and not HSCs; TAA 3 is expressed on LSCs and HSCs



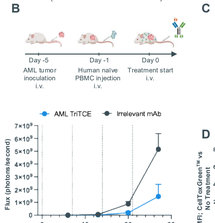


Lead logic-gated TriTCE demonstrates desired TAA-selective cytotoxicity

A



***In vivo* anti-tumor activity of AML TriTCE in disseminated AML xenograft model**
(TAA 1+/TAA 2++/TAA 3+++)



Antibody-like serum PK of AML TriTCE

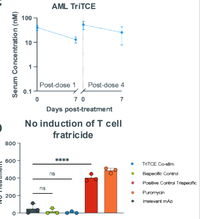


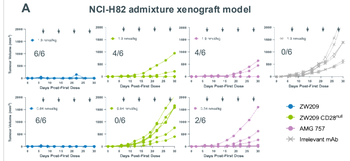
Figure 6. Lead logic-gated TriTCE format displays enhanced tumor cell cytotoxicity in the presence of two or three TAAs and demonstrates *in vivo* efficacy in disseminated AML xenograft model. (A) AML TriTCE was incubated with human T cells co-cultured with AML tumor cells containing zero, one, or two target TAAs knocked out by CRISPR for 3 days and evaluated for cytotoxicity. **(B)** THP-1 Luc cells were injected i.v. in NSG mice. Following PBMC humanization, mice were treated IV with test article (q1w x 4, 1.52 nmol/kg) as indicated by vertical dotted lines. Tumor burden was measured via administration of luciferin and bioluminescent imaging. **(C)** Serum samples (n=8) were collected 1 hour and 7 days post-dose 1 and 4 (q1w x 4, 1.52 nmol/kg). Serum concentration was measured by MSD. **(D)** Test articles were incubated with monocultures of T cells in the presence of CellTox™ Green. After 48hs, fluorescence was detected using the Operetta and analysed for MFI.

Preclinical Development Models Established for TriTCE Co-stim

Case Study ZW209: DLL3 x CD3 x CD28 Trispecific T Cell Engager (TriTCE)

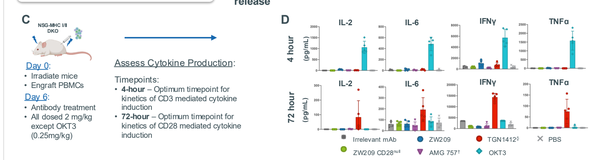
In vivo efficacy

➢ ZW209 mediates enhanced *in vivo* anti-tumor activity in humanized xenograft mouse models



In vivo cytokine

➢ No systemic cytokine induction observed in an *in vivo* model of cytokine release



NHP

➢ ZW209 exhibits transient, mild increases in serum cytokine expected of TCEs, and an antibody-like PK profile in non-GLP NHP

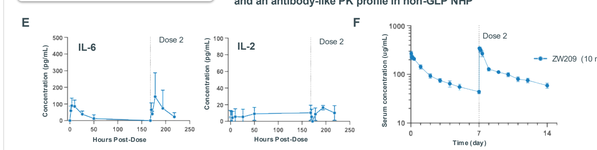


Figure 7. ZW209 demonstrates enhanced anti-tumor activity compared to bispecific TCEs in small cell lung cancer models and tolerability in toxicity models. (A) Tumor volume over time of mice engrafted SQ with a mixture of naïve T cells (one donor) and NCI-H82 tumor cells, and treated IV with ZW209, ZW209 CD28$^{hi/l}$, AMG 757, or an irrelevant mAb control at 1.9 and 0.64 nmol/kg, q.w. x 5 (arrows indicate dosing days). Number of mice where full tumor growth inhibition was observed is indicated per treatment group and donor. **(B)** Tumor volume over time of SHP-77 tumor bearing mice engrafted with human PBMCs and treated IV with ZW209, ZW209 CD28$^{hi/l}$, AMG 757, or an irrelevant mAb control at 1.9 or 0.64 nmol/kg, q.w. x 4 (arrows indicate dosing days). Data shown corresponds to the lowest dose where antitumor activity was observed per donor. **(C)** Schematic of huPBMC engrafted *in vivo* systemic cytokine release model. **(D)** Mice were assessed for systemic cytokine production at 4 hours and 72 hours post-treatment. **(E)** Cynomolgus monkeys (n=3) were given a repeat dose of 10 mg/kg ZW209 on day 0 and day 7. Toxicology findings were mild with transient, minor increase in serum cytokines observed and no histopathological changes. **(F)** ZW209 displayed antibody-like pharmacokinetics with exposure confirmed upon repeat dosing.

Conclusions

- TriTCE Co-stim platform CD3/CD28 geometry can be combined with diverse tumor targeting strategies including: **monovalent/bivalent Fab, scFv, VHH, 2+1+1, multi-TAA logic-gated designs, and pMHC targeting**
- These data highlight the flexibility of TriTCE Co-stim platform and potential to address unique biological problems across different disease settings
- ZW209 is a DLL3-targeting TriTCE Co-stim with favorable preclinical *in vivo* efficacy and tolerability and will be entering Phase 1 in H1-2026

References

1. Lau, D. (2025, Apr 25-30). ZW209, a DLL3 targeted trispecific T cell engager with integrated CD28 co-stimulation, demonstrates safety and potent preclinical efficacy in models of small lung cancer [poster presentation]. AACR, Chicago, IL.
2. Newhook, L. (2024, Apr 5-10). TriTCE co-stim: a next generation trispecific T cell engager platform with integrated CD28 co-stimulation, engineered to improve responses in the treatment of solid tumors. [poster presentation]. AACR, San Diego, CA.
3. Lobze, M.T. *et al.* 2024. CD28 co-stimulation: novel insights and applications in cancer immunotherapy. *Nat Rev Immunol* **24**(12):878-895.

†AMG 757 (DLL3/CD3 BITE) produced in-house, *BI 764532 (DLL3/CD3 bispecific TCE) produced in-house, *RG6524 (DLL3/CD3/CD137 trispecific TCE), ‡CD3xCD28xTAA CODV Analog is a CD3xCD28xMSLN trispecific with the same format as the Sanofi Trispecific containing a CD3xCD28 CODV-Fab; produced in-house, ††TGN1412 (hIgG4) biosimilar produced in-house.

All graphics created with BioRender.com

Acknowledgements

αpMHC paratope discovered by Alloy Therapeutics

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