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# Conditionally active CD28xVISTA bispecific antibodies induce myeloid-driven tumor-specific T-cell co-stimulation for improved cancer immunotherapy

**Thomas Thisted, Zhi-Gang Jiang, Zuzana Biesova, Adejumoke Onumajuru, Yuliya Kleschenko, Kanam Malhotra, Vikas Saxena, Arnab Mukherjee, F. Donelson Smith, Edward H. van der Horst**
**Sensei Biotherapeutics Inc., 1405 Research Blvd., Suite 125, Rockville MD 20850**

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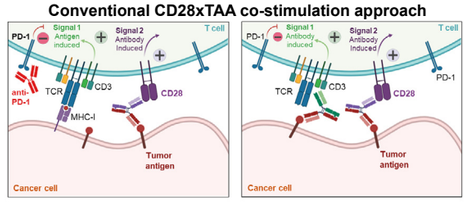
## BACKGROUND

Tumor-specific recruitment of co-stimulatory bispecific antibodies (bsAbs) is emerging as a promising therapeutic strategy. We developed pH-selective CD28xVISTA bsAbs to act within the acidic tumor microenvironment (TME). These bsAbs are designed for selective tripartite "*trans*-activation" of CD28 in the TME, aiming for enhanced T-cell-mediated cancer cell killing while minimizing systemic T-cell activation and Cytokine Release Syndrome (CRS) risk.

The *trans*-activation mechanism relies on engagement of VISTA on myeloid cells, where this immune checkpoint acts to suppress T-cell activation in the low pH environment (~pH 6) found in many tumors[1]. We and others previously developed pH-selective monoclonal antibodies (mAbs) to inhibit this checkpoint[1,2]. Here we exploit these findings to develop CD28xVISTA bsAbs for tumor-targeted CD28 agonism and T-cell co-stimulation.

**Conventional CD28xTAA co-stimulation approach**




**CD28xVISTA co-stimulation approach**

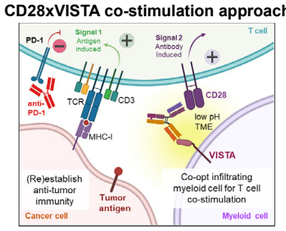



**Figure 1. CD28xVISTA bsAb mechanism of action**

- Takes advantage of the abundant tumor infiltration by VISTA⁺ myeloid cells
- pH-selective VISTA binding of bsAb ensures CD28 clustering on T-cells in the low pH tumor microenvironment with minimal risk of systemic CRS
- Bypasses the requirement for specific tumor associated antigen (TAA)
- A similar *trans*-activation concept has been utilized for 4-1BB agonists in the form of tumor stroma antigen FAPx4-1BB bispecific constructs[3]

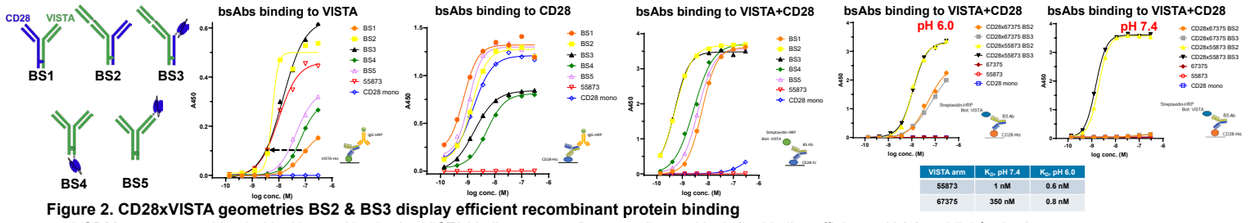
## EXPERIMENTAL PROCEDURES

CD28xVISTA bsAbs were generated containing mutations silencing FcγR interactions. BsAbs were tested for induction of luciferase expression from Jurkat-IL-2-luciferase reporter cells (Promega) in the presence of HEK293 cells expressing membrane bound OKT3-scFv (anti-CD3) or OKT3-scFv+VISTA and CHO cells expressing human VISTA. xCelligence-based human T-cell mediated killing of LNCaP prostate cancer cells was analyzed by co-culturing LNCaP cells with PBMCs and VISTA⁺ Kasumi-3 cells, in the presence of bsAb alone or in combination with a CD3xPSMA bispecific T-cell engager (BPS Bioscience). T-cell activation and proliferation were measured using flow cytometry with CD3, CD4, CD8 and CD25 markers. Tumor growth inhibition (TGI) of a MC38 cell population overexpressing VISTA was tested in a humanized CD28 mouse model (genOway) in combination with anti-murine PD-1 (anti-mPD-1; n=10/group). Cytokine release from human PBMCs, co-cultured with human umbilical vein endothelial cells (HUVECs; n=6) and treated with the indicated bsAbs (or TGN1412 as a positive control; n=3) was examined. *Ex vivo* cytokine release in human whole blood from 6 healthy donors was tested using the ID Flow circulating blood platform (Immuneed) with the controls anti-CD28 (ANC.28.1; 1 µg/ml), Alemtuzumab (3 µg/ml) or Cetuximab (250 µg/ml). In all experiments cytokines were measured using bead-based multiplex immune assays.

## REFERENCES

1. Johnston *et al.* Nature (2019) 574:656-570
2. Thisted *et al.* Nature Comm. (2024) (accepted)
3. Claus *et al.* Sci. Transl. Med. (2019) 11, 496-507

## RESULTS




| VISTA arm | $K_D$, pH 7.4 | $K_D$, pH 6.0 |
|---|---|---|
| 55873 | 1 nM | 0.6 nM |
| 67375 | 350 nM | 0.8 nM |

**Figure 2. CD28xVISTA geometries BS2 & BS3 display efficient recombinant protein binding**

- A CD28 arm was combined with either a pH-selective VISTA binding arm or a "surrogate" arm with similar binding affinity at pH 6.0 and 7.4 for *in vitro* assays
- BS2 & BS3 formats with bivalent VISTA engagement shows efficient simultaneous target binding in ELISA assays
- BS2 & BS3 formats with the pH-selective VISTA arm shows pH-dependent simultaneous binding to both targets

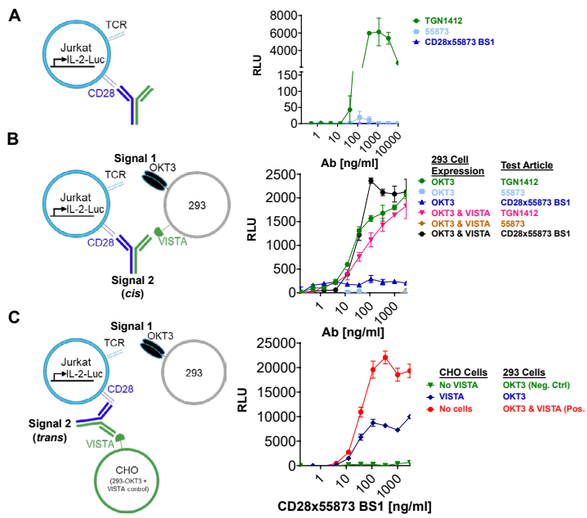



**Figure 3. CD28xVISTA bsAb induces IL-2–luciferase reporter expression both in *cis* and in *trans***

- CD28xVISTA bsAb with monovalent CD28 binding does not display superagonism (A; TGN1412 pos. ctrl.; VISTA mAb 55873 neg. ctrl.)
- CD28xVISTA BS1 provides dose dependent stimulation of IL-2-luc expression in *cis* (B) and in *trans* (C)

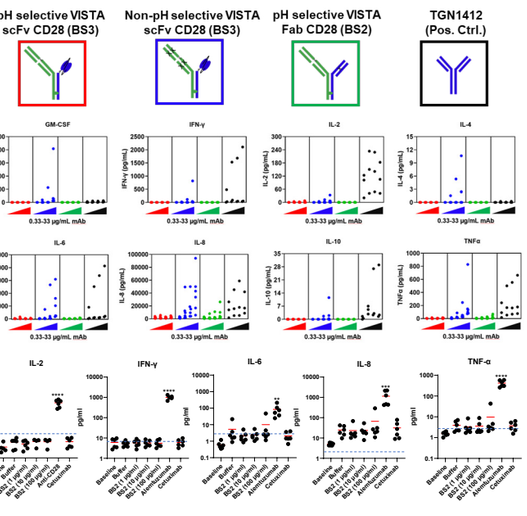
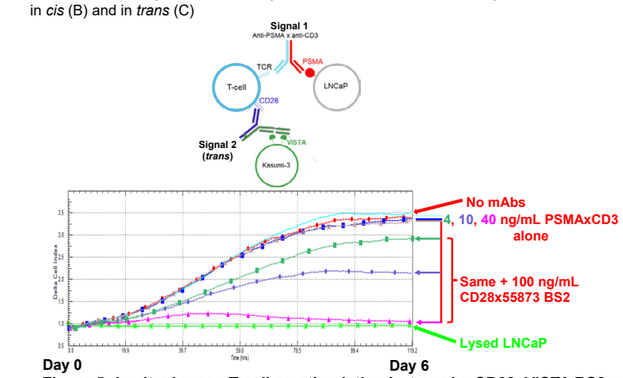



**Figure 4. pH selective CD28xVISTA bsAb's do not induce cytokine responses**

- Favorable safety profile in HUVEC:PBMC co-culture due to pH-selective VISTA engagement (A). Each point represents the results from one donor
- Cytokine release by CD28x67375 BS2 not significantly different from formulation buffer in sensitive *ex vivo* whole blood ID.Flow assay (B; Immuneed; LLOQ in dotted lines; mean values in red line; **p<0.01; ***p<0.001; ****p<0.0001 comparison to buffer by Paired Student's t-test with Holm-Sidak correction)



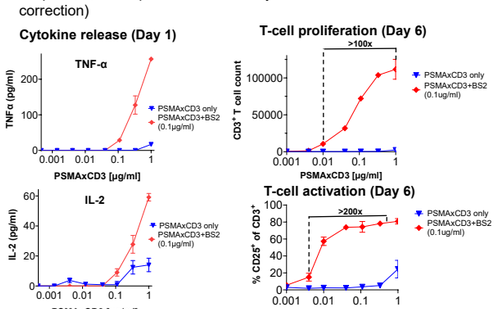
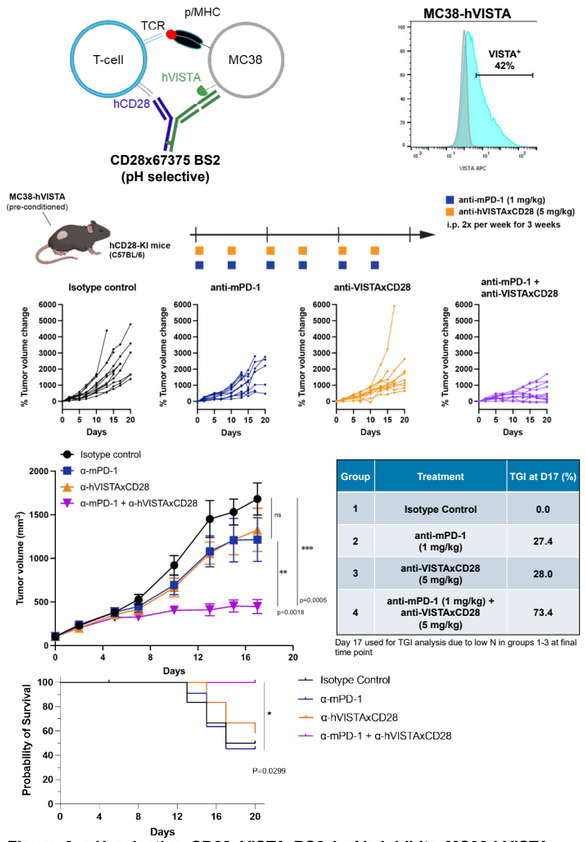




**Figure 5. *In vitro* human T-cell co-stimulation in *trans* by CD28xVISTA BS2 potentiates LNCaP killing by a CD3xPSMA T-cell engager**

- Enhanced killing was accompanied by increased T-cell activation, cytokine release and proliferation
- No effect in the absence of CD3xPSMA ("Signal 1"), i.e. no superagonistic properties of this CD28xVISTA bsAb




| Group | Treatment | TGI at D17 (%) |
|---|---|---|
| 1 | Isotype Control | 0.0 |
| 2 | anti-mPD-1 (1 mg/kg) | 27.4 |
| 3 | anti-VISTAxCD28 (5 mg/kg) | 28.0 |
| 4 | anti-mPD-1 (1 mg/kg) + anti-VISTAxCD28 (5 mg/kg) | 73.4 |

Day 17 used for TGI analysis due to low N in groups 1-3 at final time point

**Figure 6. pH selective CD28xVISTA BS2 bsAb inhibits MC38-hVISTA tumor growth in hCD28 KI mice in combination with anti-PD-1**

- CD28xVISTA BS2 with pH-selective VISTA engagement
- Natural "Signal 1" enhanced by CD28 co-stimulation in *cis*
- Significant tumor growth inhibition and enhanced survival despite highly heterogeneous tumor cell population (only 42% hVISTA⁺ cells)

## CONCLUSION

- Selected CD28xVISTA bsAb formats show dual engagement of target proteins and CD28 activation in Jurkat-reporter assays
- pH selective BS2 format with FcγR null mutations minimizes CRS risk
- CD28xVISTA bsAb (BS2 format) potentiates LNCaP cancer cell killing by a CD3xPSMA T-cell engager *in vitro*
- In hCD28 KI mice, BS2 with pH-selective VISTA binding arm significantly inhibits MC38-hVISTA tumor growth inhibition in combination with anti-PD-1
- Developing of a hCD28xhVISTA double knock-in mouse model for *in vivo trans*-activation testing
- A CD28xVISTA bsAb could complement PD-1/PD-L1 inhibitors or enhance bispecific T-cell engagers' selectivity and efficacy by targeting dual/orthogonal antigens on tumor and myeloid cells

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