Next-generation multi-specific and conditionally activated CD3 Switch-DARPins with CD2 co-stimulation to tackle current limitations of T cell engagers in solid tumors

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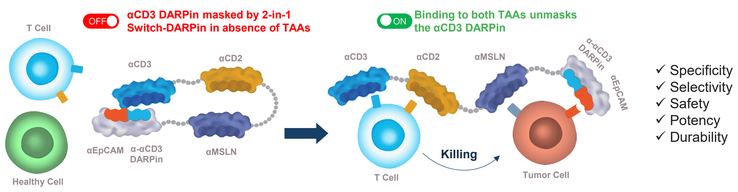
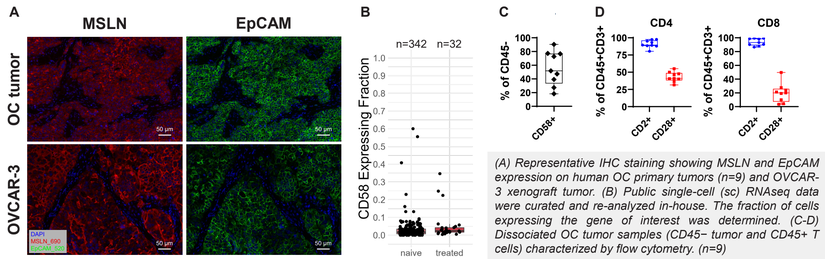
Introduction

Current therapies for ovarian cancer (OC) lack durable response. Targeted immunotherapy has had limited success due to the absence of clean tumor-associated antigens (TAAs), poor efficacy/toxicity profile, and the presence of dysfunctional T cells. To overcome these challenges, we generated a conditionally activated CD3 Switch-DARPin T cell engager (TCE) boosted by a CD2-engaging costimulatory domain. In cancer, loss of CD58, the ligand of CD2, facilitates immune evasion and is linked to poor prognosis.[1–3] The CD3 Switch-DARPin triggers, tumor-specific T cell activation (TCA) only in the presence of mesothelin (MSLN) and epithelial cell adhesion molecule (EpCAM), two TAAs highly co-expressed on OC tumor cells, while the CD2 DARPin provides a co-stimulatory signal for sustained and potent T cell responses.



- ✓ Specificity
- ✓ Selectivity
- ✓ Safety
- ✓ Potency
- ✓ Durability

MSLN/EpCAM are co-expressed in OC & CD2 is maintained on TILs

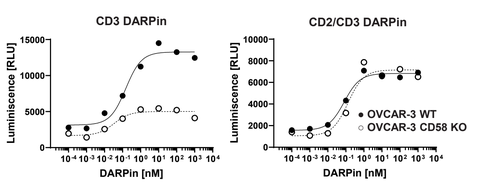


(A) High co-expression of MSLN and EpCAM in OC (scRNAseq data[4]) was confirmed by immunohistochemistry (IHC). (B-C) scRNAseq and flow cytometry data show that CD58, the ligand of CD2, is often down-regulated on OC tumor cells. (D) Analysis of tumor-infiltrating lymphocytes (TILs) shows that CD2 is expressed by >90% of CD4 and CD8 T cells.

(A) Representative IHC staining showing MSLN and EpCAM expression on human OC primary tumors (n=9) and OVCAR-3 xenograft tumor. (B) Public single-cell (sc) RNAseq data were curated and re-analyzed in-house. The fraction of cells expressing the gene of interest was determined. (C-D) Dissociated OC tumor samples (CD45− tumor and CD45+ T cells) characterized by flow cytometry. (n=9)

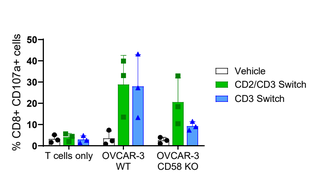
CD2 co-stimulation prevents loss of activity against CD58 KO cells

Jurkat T cells



Jurkat IL-2 cells co-cultured 1:1 with OVCAR-3 WT or CD58 knock-out (KO) cells for 24h. Activation measured by luminescence.

T cells from OC ascites

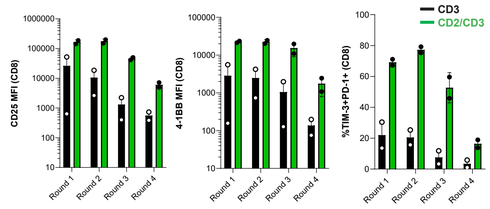


CD45+ cells from OC ascites co-cultured 10:1 with OVCAR-3 WT or CD58 KO cells with 1nM DARPins. Activation (CD107a) of CD8 T cells measured by flow cytometry after 16h.

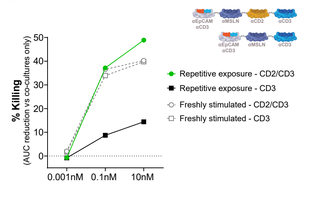
Loss of CD58 on tumor cells results in reduced efficacy of the CD3 Switch-DARPin whereas CD2/CD3 co-stimulation maintains efficacy, thus compensating for the lack of CD58.

CD2 co-stimulation enables continued polyfunctionality of T cells

Phenotype of T cells



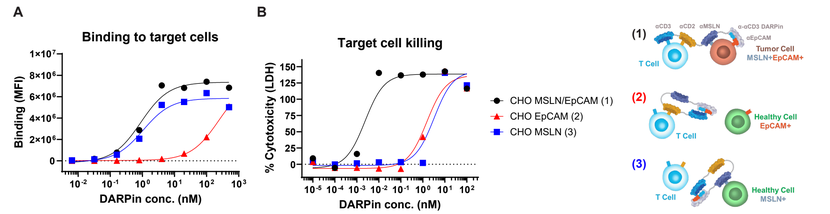
Redirected T Cell Cytotoxicity



(A) T cells were repetitively exposed to CD3 or CD2/CD3 DARPins on EpCAM/MSLN-coated plates. After each round of stimulation, a portion of cells were taken for analysis by flow cytometry. (B) After 3 rounds, T cells were co-cultured with NucLightRed (NLR) labeled Caov-3 cells and DARPins for 48h in an IncuCyte to measure the NLR signal over time.

T cells repetitively exposed to CD2/CD3 DARPin maintain a more activated phenotype as well as a higher cytotoxic capacity compared to exposure to CD3-only DARPin.

CD3 Switch-DARPin enables preferential targeting of tumor cells

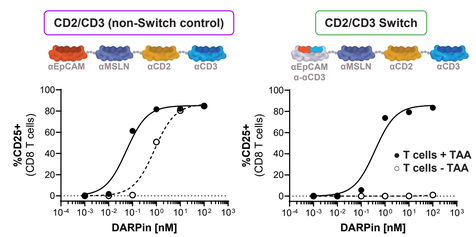


(A) DARPin binding to target cells (CHO) co-expressing MSLN and EpCAM or singly expressing MSLN or EpCAM. Measured by flow cytometry using an anti-DARPin detection antibody. (B) Pan T cells co-cultured with target cells (CHO) co-expressing MSLN and EpCAM, or just MSLN or EpCAM. Cytotoxicity measured by LDH release after 48h.

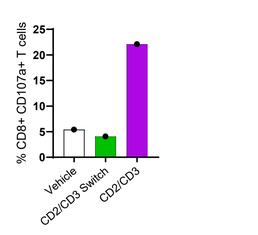
In absence of MSLN, binding to EpCAM is strongly reduced with the Switch-DARPin format. CD2/CD3 Switch induces selective targeting of MSLN/EpCAM co-expressing cells (AND-gate), while activity against single TAA-expressing cells is strongly reduced.

CD3 Switch-DARPin prevents TAA-independent T cell activation

T cells from healthy PBMCs



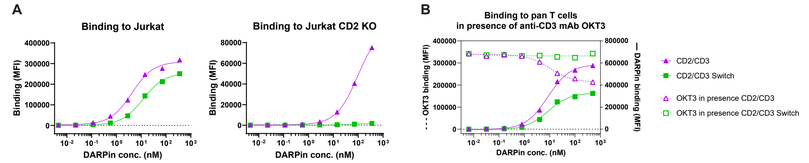
T cells from OC ascites



Pan T cells co-cultured ± TAA co-expressing target cells (OVCAR-3). Activation (CD25) of CD8 T cells measured by flow cytometry after 48h.

CD45+ cells isolated from OC ascites were incubated with 1nM DARPins. Activation (CD107a) of CD8 T cells measured by flow cytometry after 16h.

CD3 Switch-DARPin prevents binding to CD3 in absence of TAAs

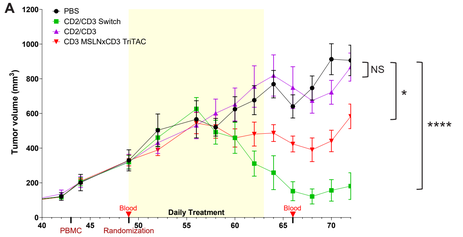


DARPin binding to (A) Jurkat T cells (WT or CD2 KO) or (B) pan T cells in the presence of the anti-CD3 mAb OKT3. Measured by flow cytometry using anti-DARPin and anti-mouse IgG detection antibodies.

In the absence of TAA-expressing target cells, binding to T cells is exclusively mediated by the CD2 DARPin. This does not trigger the opening of the Switch-DARPin, thus preventing binding to CD3. Only the non-Switched control DARPin can bind to T cells when CD2 is knocked out or compete with OKT3 for binding to CD3 in the absence of target cells.

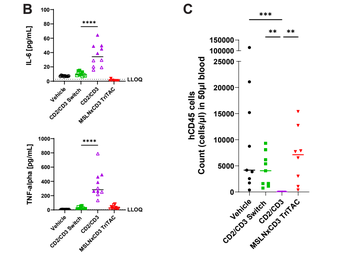
CD2/CD3 Switch-DARPin induces tumor regression *in vivo*

Tumor growth curves of OVCAR-3 s.c. model



*hPBMC engrafted in NXG mice at 100 mm³ OVCAR-3 tumor volume; 3.8 mg/kg DARPin or 0.25 mg/kg TriTAC daily (i.v.); average +/- SEM, two-way ANOVA. *p<0.05, ****p<0.0001. MSLN x CD3 TriTAC was used as a comparator.*

Safety of CD2/CD3 Switch



*(B) Cytokines measured in serum 2h post first-injection and (C) T cell count in blood at day 66; Kruskal-Wallis (median). **p<0.01, ***p<0.0001.*

(A) CD2/CD3 Switch-DARPin led to significant tumor regression (B) and lower cytokine levels in serum *vs.* the non-Switched control DARPin. (C) CD2/CD3 Switch-DARPin effectively prevented the loss of T cells in the periphery, unlike the non-Switch DARPin. This suggests that masking the CD3 domain allows the integration of a CD2 costimulatory domain, ensuring that the activity is targeted only in the presence of TAA-expressing tumor cells.

Conclusions

We present a preclinical proof-of-concept for a conditionally activated CD3 Switch-DARPin TCE with CD2 co-stimulation, designed to increase the therapeutic window against MSLN and EpCAM co-expressing tumors as in OC. Our findings demonstrate that:

- The CD3 Switch-DARPin activates T cells specifically in the presence of cells co-expressing MSLN and EpCAM, increasing tumor specificity.
- CD2 co-engagement leads to sustained T cell activation and cytotoxic capacity, preventing T cell dysfunction.
- The CD3 Switch-DARPin with CD2 co-stimulation effectively induces significant tumor regression *in vivo*, without signs of T cell activation in the periphery, indicating a favorable safety profile.
- Our CD3 Switch-DARPin platform provides a novel approach for sustained tumor-specific T cell engagement, by combining a logic-gated on/off Switch mechanism with CD2 co-stimulation.

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For any questions, please contact: info@molecularpartners.com / attention of M. Guzman.

References: 1. Cao et al., Oncotarget 2016; 2. Majzner et al., ASH 2020; 3. Shen et al., JITC 2022; 4. Robinson et al., SITC 2024.