VHH-based Tandem CARs enable OR-gated multi-targeting of pancreatic cancer

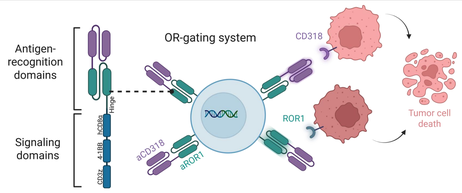
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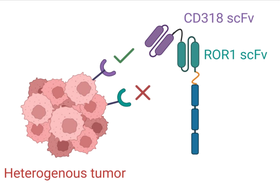
Introduction

Genetically engineered cellular products became a new pillar of cancer therapy. While cellular therapies have been unprecedentedly successful against leukemia, they still lack efficacy in solid tumors. A major obstacle is the limited T cell specificity in the face of tumor heterogeneity and potential antigen escape variants, leading to tumor recurrence after treatment with cells targeting a single antigen. Given the heterogeneous antigen expression in solid malignancies and the high medical need in pancreatic ductal adenocarcinoma (PDAC), we hypothesized that VHH-based bispecific tandem CARs (**TanCARs**) with OR-gated targeting could mitigate antigen escape and enhance CAR T cell activity. To enable the development of effective TanCAR constructs, we established an optimized screening workflow and evaluated multiple design parameters critical for TanCAR stability, flexibility and function.

Tandem CAR design



Misfolding issue in TanCAR



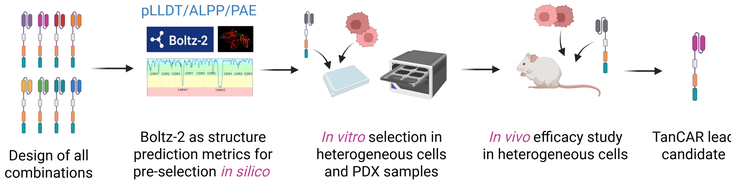
Methods

Contrary to expectations, established CAR designs could not be directly translated into effective TanCARs, highlighting fundamental differences in antigen engagement. TanCAR optimization requires systematic tuning of structural parameters. In particular, CAR flexibility and length critically affected dual-binding domain accessibility and cytotoxicity. To address this, we generated a library of 30 TanCAR constructs varying in 5 parameters, displayed alongside. We propose an optimized workflow for large-scale screening, combining Boltz-2-based structural stability prediction with *in vitro* functional assessment of cytotoxicity using PDAC cell lines and patient-derived xenografts representing target escape variants. Lead candidates were subsequently validated *in vivo* using mouse xenograft and bi-tumor models.

Tandem CAR screening parameters



Optimized screening workflow

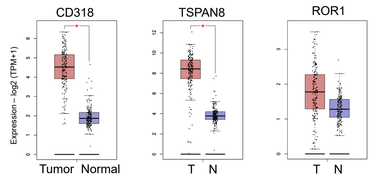


Results

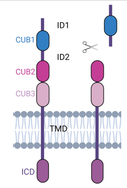
1 CD318, TSPAN8 and ROR1 provide complementary coverage of PDAC heterogeneity

To implement a combinatorial strategy for PDAC, we selected 3 relevant antigens: **CD318**, **ROR1** and **TSPAN8**. Multi-gene analysis showed increased expression of these antigens in primary tumor versus normal tissue. Beyond their success as mono-CAR targets[1,2], they are attractive for combinatorial targeting due to complementary expression patterns. CD318 is broadly expressed across pancreatic tumor cell lines but may enable immune escape via CUB1 domain cleavage, whereas ROR1 and TSPAN8 show heterogeneous expression in cell lines and patient samples. However, IHC analysis showed that every tumor expressed at least one target. Together, these data support a multi-target OR-gate CAR strategy to achieve broader PDAC coverage despite antigen heterogeneity and antigen escape risk.

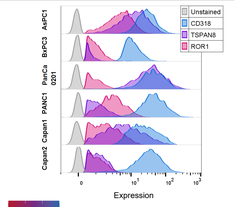
Gene expression in PDAC



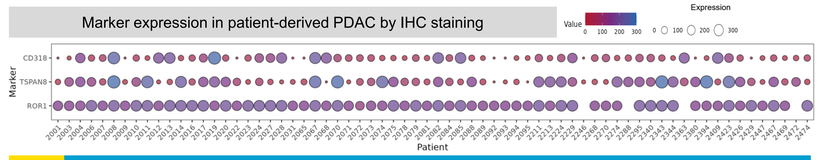
CD318 natural cleavage



Expression in PDAC cell lines



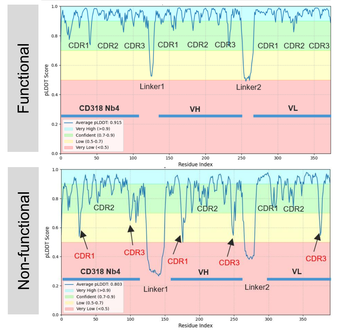
Marker expression in patient-derived PDAC by IHC staining



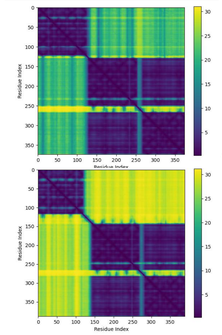
2 *In silico* structural predictions for TanCAR pre-selection

Candidates can be filtered *in silico* using Boltz-2[3] to identify potential misfolding or structural instability. Complementary predictive metrics were evaluated, including **pLDDT** (local structural confidence) and **PAE** heatmaps (domain positioning and inter-domain flexibility). In addition, the **ALPP** score is an internal metric that uses protein structures predicted by Boltz-2 to quantify the continuous electrostatic charge distribution across the surface of the predicted protein. Candidates with low pLDDT peaks in CDR regions, above-average ALPP scores, or increased yellow signal in PAE maps indicating uncertain domain positioning were deprioritized. While individual metrics could provide incomplete predictions, combining these parameters may improve the robustness of the pre-selection workflow. We identified 9 of 30 candidates with more stable predicted profiles (green dots in the ALPP graph).

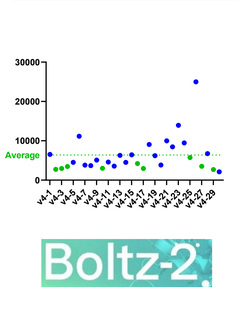
pLDDT score



PAE heatmap



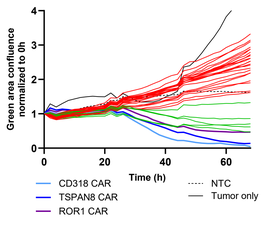
ALPP score



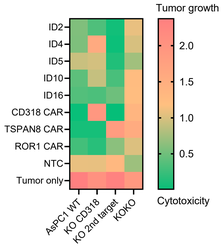
3 *In vitro* screening of the best TanCAR candidates in heterogeneous tumor cell lines

All candidates were functionally screened *in vitro* using WT and KO target cells. Five CARs showed moderate-to-strong cytotoxicity against WT cells and, except for ID5, had been prioritized by the *in silico* workflow. Only three constructs (ID2, ID10, and ID16) retained robust activity against escape variants, while **ID2** and **ID16** maintained sustained cytotoxicity across repeated killing rounds. Reduced linker length and the use of VHH-based binders emerged as key determinants of functionality, although optimal configurations varied across antigen combinations.

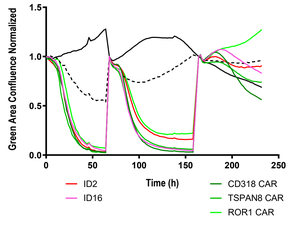
Screening in AsPC1 WT E:T 5:1



Endpoint with escape variants



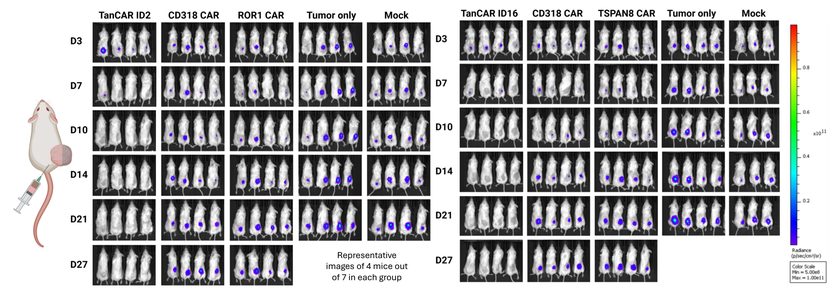
Repetitive killing assay in Capan1 E:T 5:1



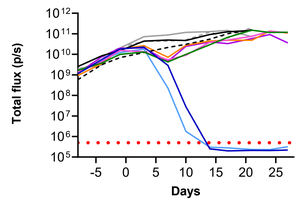
4 *In vivo* efficacy study in a bi-tumor xenograft mouse model

To evaluate *in vivo* efficacy of TanCAR lead candidates ID2 and ID16, a bi-tumor mouse model was established by co-injecting 40% AsPC1 CD318-KO cells and 60% AsPC1 cells lacking the second target, thereby mimicking tumor heterogeneity. Both TanCARs achieved complete tumor clearance within 14 days, whereas the corresponding single-target CARs induced only transient responses followed by outgrowth of antigen escape variants. *Ex vivo* analyses further confirmed the complementary escape phenotypes.

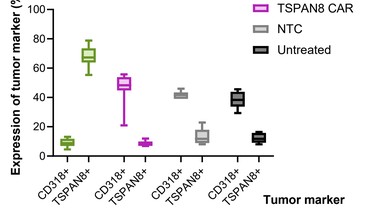
Bi-tumor	AsPC1 KO CD318 (40%) + KO ROR1 (60%)	AsPC1 KO CD318 (40%) + KO TSPAN8 (60%)



Median of all flux after 1e7 TanCAR treatment



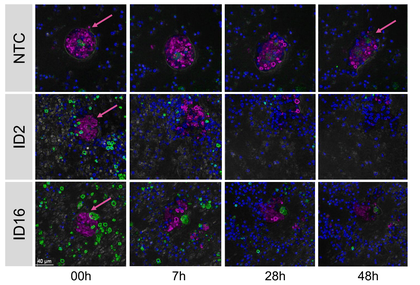
Ex vivo tumor analysis of CD318/TSPAN8 KO tumor



5 Evaluation of TanCAR lead candidate cytotoxicity in patient-derived organoids

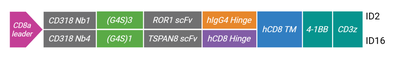
Finally, the ID2 and ID16 TanCARs were evaluated in patient-derived PDAC organoids (PDOs) displaying heterogeneous target expression, previously characterized. Organoid responses following CAR treatment were assessed by confocal imaging and quantified using cytotoxicity assays combined with the OrganoIDNet algorithm[4]. Despite pronounced tumor heterogeneity, both ID2 and ID16 markedly reduced organoid number and area across all 10 PDOs samples tested. A representative example with PDO 66T is shown alongside.

DAPI
T cells
Epcam



Conclusions

We demonstrate for the first time functional TanCARs targeting CD318 with TSPAN8 or ROR1 in heterogeneous PDAC tumors and organoids. These results highlight that effective TanCAR design is not straightforward and requires systematic optimization of structural parameters, including linker length and VHH incorporation. We further show that this screening process can be streamlined by *in silico* pre-selection using Boltz-2. Dual-targeting strategies against CD318, ROR1 and TSPAN8 enhanced antitumor activity by limiting antigen escape, with **ID2** and **ID16** emerging as lead candidates. Notably, ID2 (CD318+ROR1) appears most clinically translatable, as both targets have favorable safety profiles in healthy tissues and are already being evaluated in clinical mono-CAR trials.



(1) Schäfer *et al.*, 2021. (2) Tran *et al.*, 2024. (3) Passaro *et al.*, 2025 (BioRxiv). (4) Ferreira, Dourlens *et al.*, 2025 (BioRxiv). Results are part of a manuscript (planned submission in 2026). Visual contents were made with BioRender.com.

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