Small is beautiful – VHHs enable multi-specific targeting for tandem-CAR therapies

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CAR-TCR Engineering A Disease-Free World

INTRODUCTION

Since the first anti-CD19 CAR-T therapies came into the clinic in 2017, the last 5 years have seen a global explosion in CAR research and development. A typical CAR construct consists of a cytoplasmic signaling domain, a transmembrane domain and a targeting moiety. The most conventional target moiety has been the single-chain variable fragment (scFv), which restricts development of more sophisticated CARs through vector capacities, potential for immunogenicity and T cell exhaustion. VHHs are an alternative, smaller, and simpler targeting format that can overcome these and other key challenges, exemplified by CARVYKTI™, approved by the FDA in March 2022.

Isogenica has developed a humanized VHH discovery platform that provides a neat solution to the discovery of new VHH moieties. Our VHH monomers bind tumour-associated antigens with a range of binding affinities and functional profiles. A panel of PD-L1 binding VHHs also demonstrated retained functionality when used in bi- and tri-specific formats, expected to translate well to CAR ectodomains.

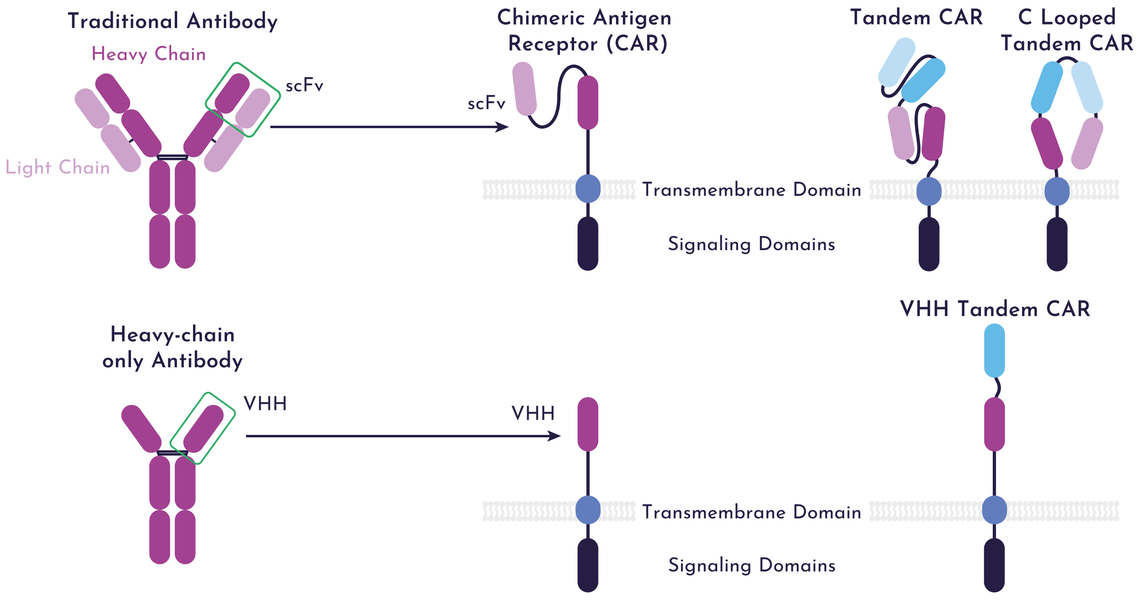

Fig. 1: VHH single domains are smaller than conventional antibody formats and can be easily engineered in mono- or multi-specific CAR ectodomains with multiple advantages over more complex scFvs. *This format is exemplified by the anti-BCMA VHH-based CAR-T therapy CARVYKTI™, FDA approved in March 2022.*

HUMANIZED VHH ANTIBODIES ADDRESS KEY ISSUES IN CAR ENGINEERING

	scFv	VHH
Size	~25kDa (800-900 bp) Requires heavy and light chains, limiting opportunity for multimerization due to viral vector capacity	~12-16kDa (<375 bp) Single domain format is easy to chain into multi-targeting ectodomains or more complex signaling domains within standard vector size
Chain mispairing	Risk of V_H-V_L and V_H-V_H chain mispairing, driving CAR aggregation and triggering T cell-exhausting signaling cascades[1]	Highly soluble single chain format avoids problems with mispairing
Immunogenicity	Mouse IgG-based antibodies can elicit non-self immune response, impairing efficacy and persistence[2]	Intrinsically low immunogenicity[3] further lowered through humanization
New epitopes	Access same pool of epitopes as conventional mAbs	Long, finger-like CDR3 can access cryptic epitopes on novel drug targets[4]

SYNTHETIC VHH LIBRARY SCREENING AGAINST TUMOR TARGET ANTIGENS

Isogenica uses three synthetic VHH libraries (LlamdA™, huLlamdA™, and V_{HH}antage™) with differing levels of humanization, making it possible to identify pre-humanized leads without the need for lengthy immunization campaigns or extensive lead engineering.

Synthetic library panning was conducted on a range of oncology targets, including the well-characterized checkpoint inhibitor PD-L1 as proof-of-concept, along with two additional antigens. Lead clones for all three targets displayed potencies in the nanomolar range in initial ELISA and cell binding assays (Figure 2), with PD-L1 clones progressing to further functional characterization studies (Figure 3). No binding was observed to non-expressing cell lines (data not shown).

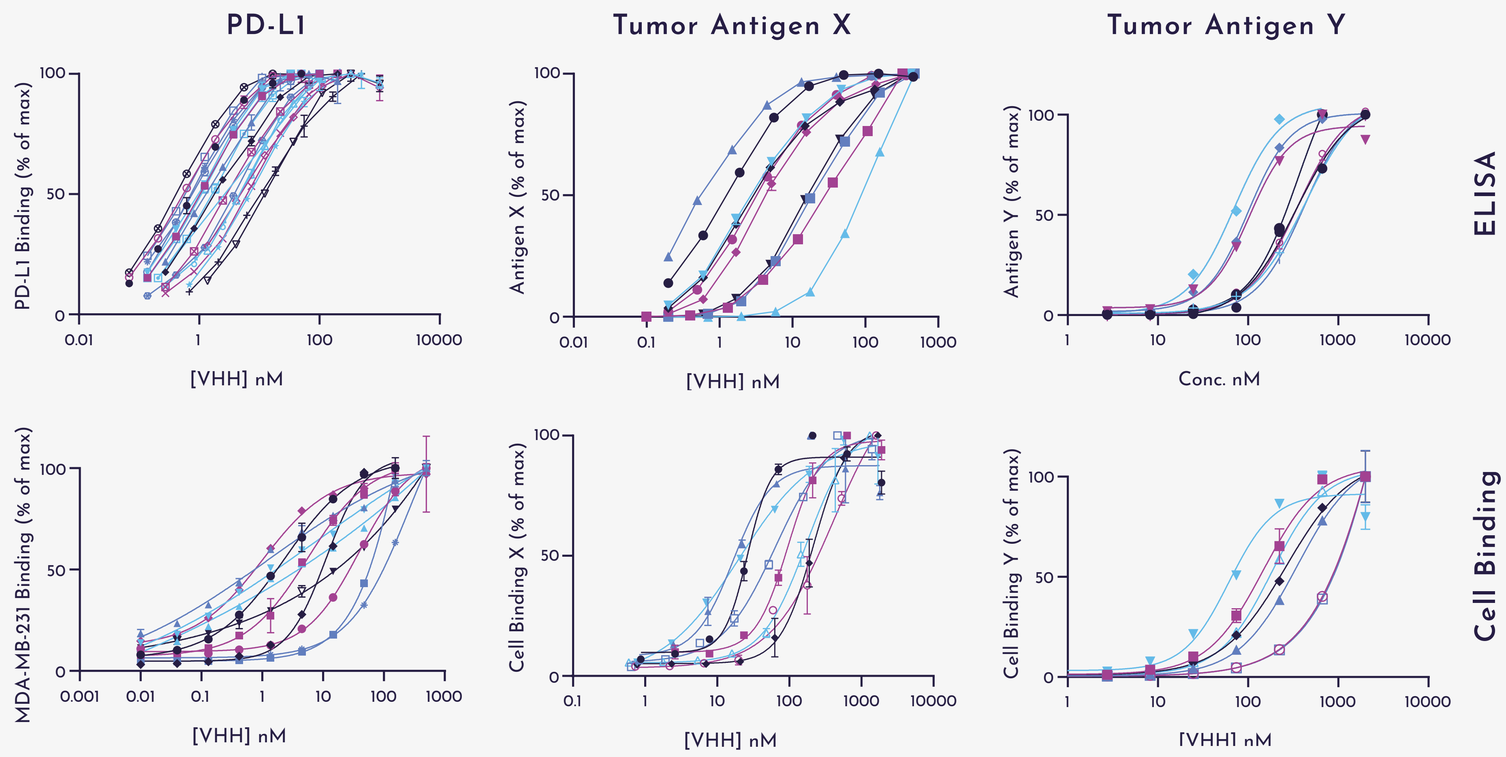

Fig. 2: ELISA (top) and cell binding (bottom) binding of initial VHH panels to tumor cell targets. *For ELISA, biotinylated antigen-bound VHHs were detected via anti-FLAG-HRP. For cell binding, bound VHHs were detected using anti-FLAG flow cytometry, represented as MFI. Antigens X and Y represent established TAAs.*

VHH ANTIGEN BINDING AND FUNCTIONALITY IS RETAINED IN BI- AND TRI- SPECIFIC FORMATS

Due to the simplicity and biophysical robustness of VHH domains, individual monomers can be easily linked to additional VHHs and/or other biomolecules such as receptor signaling domains. In a proof-of-concept experiment, anti-PD-L1 monomers were linked to a CD3-engaging scFv fragment with and without an additional VHH binding domain. These VHHs in a Bi-specific T cell Engager (BiTE) format targeted primary human T cells to PD-L1-expressing tumor cells, causing cell death (Figure 3). Feedback suggests that 'off-the-shelf' antibodies may not make ideal ectodomains. Therefore, a next-generation screening process is recommended for discovery, screening outputs earlier in the desired CAR format (Figure 4).

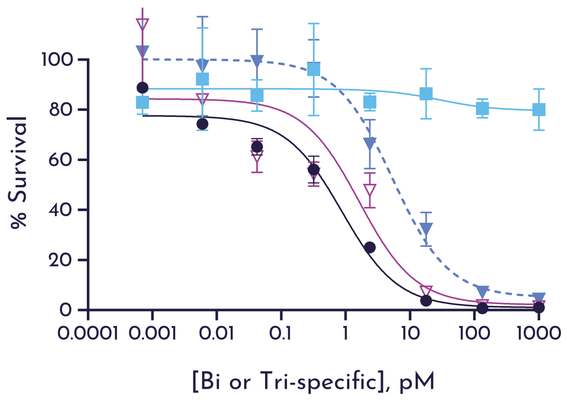

Fig. 3: Tumor cell killing in BiTE format. *Formatted VHHs were compared with an scFv of the clinical anti-PD-L1 mAb durvalumab. PD-L1+ MDA-MB-231 cells were co-cultured with human PBMCs over 72h at an E:T ration of 10:1. Data representative of 2 healthy donors, independently tested.*

CAR-OPTIMAL VHH DISCOVERY PIPELINE

Phage display selection on humanized synthetic VHH libraries

HTP screening of enriched polyclonal outputs for diversity and specificity

Cloning into CAR format

Lead selection in CAR format

Fig. 4: Multiple VHH antibodies identified from Isogenica's libraries can be screened directly within the desired CAR format for efficient identification and optimization of functional leads.

SUMMARY

Due to their small size and unrivalled engineerability, VHHs are an ideal moiety for next-generation cell therapies, especially where tandem formats are required. Highly specific humanized VHH monomers can be isolated quickly through synthetic library screening, avoiding the need for lengthy immunization and humanization. These VHHs share a formatting-tolerant framework which has demonstrated *in vivo* expression from a viral vector[5]. In addition to our discovery pipeline, we offer bespoke programs with strategic partners, allowing earlier screening of larger panels in their own cell therapy formats to identify the best possible leads for CAR therapies.

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