Biochimica et Biophysica Acta 1840 (2014) 378–386

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

T cells expressing VHH-directed oligoclonal chimeric HER2 antigen receptors: Towards tumor-directed oligoclonal T cell therapy

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ARTICLE INFO

Article history:
Received 23 March 2013
Received in revised form 22 August 2013
Accepted 20 September 2013
Available online 27 September 2013

Keywords:
Chimeric antigen receptor
HER2
Oligoclonal T cell therapy
Single domain antibodies (VHH)

ABSTRACT

Background: Adoptive cell therapy with engineered T cells expressing chimeric antigen receptors (CARs) originated from antibodies is a promising strategy in cancer immunotherapy. Several unsuccessful trials, however, highlight the need for alternative conventional binding domains and the better combination of costimulatory endodomains for CAR construction to improve the effector functions of the engineered T cells. Camelid single-domain antibodies (VHHs), which are the smallest single domain antibodies, can endow great targeting ability to CAR-engineered T cells.
Methods: We have developed a method to generate genetically engineered Jurkat T cells armed with a CAR comprising the anti-HER2 VHH as targeting moiety. From an immune camel library, five VHH clones were selected as a set of oligoclonal anti-HER2 VHHs that exhibited diverse binding abilities and joined them to CD28-CD3ζ and CD28-OX40-CD3ζ signaling endodomains. Jurkat T cells expression of VHH-CARs and cell functions were evaluated.
Results: The oligoclonal engineered T cells showed higher proliferation, cytokine secretion and cytotoxicity than each individual VHH-CAR-engineered Jurkat T cells.
Conclusions: The combination of superior targeting ability of oligoclonal VHHs with the third generation CAR can substantially improve the function of engineered T cells.
General significance: Antigen-specific directed oligoclonal T cells are alternatively promising, but safer systems, to combat tumor cells.

1. Introduction

Immunotherapy with adoptive cell transfer as an alternative to standard treatments is gaining increasing interest in many cancer types [1–3]. Adoptive transfer of expanded T cells with the desired specificity for intended antigens has demonstrated therapeutic efficacy in the treatment of patients with malignant tumors [3–5]. However, high affinity tumor-antigen specific T lymphocytes are often tolerized or anergized and exhibit a restricted life span [6,7]. T cell receptors (TCRs) need the MHC-restricted antigen presentation, whereas malignant cells down-regulate many of the molecules involved in the processing and presentation of peptides on MHC class I [3,8]. The dependency of TCR on antigen presentation on MHC molecule, can be overcome by antibody chimeric antigen receptor (CAR) engineering [4,8,9]. The first generation CARs were constructed by attaching a single chain variable fragment (scFv) derived from a monoclonal antibody directed against a tumor antigen, linked to the transmembrane and intracellular sequences of T cell signaling molecules that enabled the engineered T cells to specifically recognize and kill tumor cells [8,10,11]. The modular composition of most CARs is rather similar [4,8,12].

Many tumor cells, however, down-regulate their expression of the costimulatory molecules such as B7.1 and B7.2 required for optimal T cell activation [13]. To overcome this problem, second generation CARs were designed containing a costimulatory endodomain derived from CD28, 4-1BB or OX40 [6,14–17]. Subsequently, the incorporation of multiple costimulatory domains such as the combinations of CD28 and 4-1BB or CD28 and OX40 in CARs potentiated the costimulation of engineered T cells with superior activity compared with cells encoding a single

Abbreviations: CAR, chimeric antigen receptor; CDR, complementarity determining region; HCAb, heavy-chain antibodies; IPTG, isopropyl-β-D-thio-galactoside; MHC, major histocompatibility complex; TAG 72, tumor associated glycoprotein 72; TCR, T cell receptor; TMB, 3,3′,5,5′-tetramethyl benzidine; VHH, variable domain of camel heavy-chain antibody; VHH-CAR, VHH-chimeric antigen receptor

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http://dx.doi.org/10.1016/j.bbagen.2013.09.029

costimulatory domain [4,8,14,18,19]. Several studies have demonstrated that CD28 improves T cell effector functions, whereas OX40 in a combined CAR (OX40-CD28) mediates more secretion and proliferation of engineered T cells after binding to target cells [12,15,20–23].

To date, a variety of CAR-engineered T cells targeting various tumor antigens have been generated and investigated in clinical trials. However, most studies have resulted in immunogenicity and poor in vivo persistence of CAR-redirected T cells in phase I clinical trials [4,24,25]. The targeting moiety of CAR generally is a scFv derived from a murine monoclonal antibody that causes the human anti-mouse antibody responses, and this can potentially limit the life span of engineered T cells [4,25]. The humanization of mouse antibodies by CDR grafting have been exploited by many groups, but this approach does not fully abrogate immunogenicity and further more the generation of human antibodies is labor intensive [25]. One fascinating strategy is the utilization of camelid single-domain antibody as a binding domain of CAR instead of the scFv [26–28]. VHHs are the smallest single domain antibodies showing a high degree of homology to human VH sequences of the VH3 gene family and are easily generated due to their single domain entity [2,29,30].

Here we describe the generation, characterization and evaluation of a panel of epitope-distinct engineered model Jurkat T cells that express second and third generation CARs with HER2 specific VHHs as targeting moieties. The results showed that these oligoclonal VHHs are clearly able to target a range of different epitopes on HER2 antigen. The data further demonstrated the feasibility of employing epitope-distinct VHHs for generating the oligoclonal engineered T cells that are able to markedly recognize different epitopes on HER2 and specifically lyse HER2-expressing cells.

2. Materials and methods

2.1. Cell lines

BT-474 and SK-BR-3 (human breast cancer cell lines), HepG2 (human hepatocellular liver carcinoma cell line) and A431 (human epithelial carcinoma cell line) were cultured in DMEM (Gibco/Invitrogen, Carlsbad, CA). T47D (human breast cancer cell line), NIH3T3 (mouse embryo fibroblast cell line) and Jurkat T cell cl. E6.1 (human lymphoblast-like cell line) were grown in RPMI-1640 (Gibco/Invitrogen). All cells were purchased from the National Cell Bank of Iran, Tehran, Iran. NIH3T3^{HER2+} cells, HER2-expressing cell line, were also prepared as described previously [2]. All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Identification and expression of HER2 specific VHHs

A phagemid library carrying VHH genes from two immunized camels was used for the selection of phages displaying VHHs against the HER2-Fc chimera (R&D Systems, Minneapolis, MN, USA) and HER2-expressing cell lines. Five consecutive rounds of biopanning were performed on the VHH gene library. The phages carrying VHHs were also subjected to sequential negative and positive selection on HER2$^-$ A431 and NIH3T3^{HER2-} cells (negative selection) and HER2$^+$ SK-BR-3, T47D, BT-474 and NIH3T3^{HER2+} cells (positive selection), respectively. Panning on various target cells was separately performed for three rounds. After biopanning and cell panning, 12 positive clones were selected and sequenced, and their sequences were aligned by using the multiple sequence alignment program ClustalX version 2.1 with the default parameters and manually adjusted parameters. Sequence similarity and divergence among the selected VHHs were calculated by MegAlign of the DNASTAR program (DNASTAR Inc., Madison, WI, USA). Unique anti-HER2 VHH genes were re-cloned into the expression vector pSJ [2,31,32]. Transformants were confirmed by colony-PCR and DNA sequencing. The clones containing anti-HER2 VHH genes were grown in 1 L of M-9 medium. Following 24 h incubation, the expression of VHHs was induced by adding 1 mM isopropyl-β-D-thio-galactoside (IPTG). The bacterial extracts that contained soluble VHHs were released by sonication and then purified by immobilized metal affinity chromatography with 5 mL nickel nitrilo-triacetic acid (Ni^+-NTA) resin (Qiagen, Hilden, Germany). The final products were dialyzed against phosphate buffer (10 mM, pH 7.2) [2].

2.3. Characterization of HER2 specific VHHs

2.3.1. Assessment of VHHs binding to HER2-expressing cells

The ability of selected VHHs to recognize the HER2 antigen on HER2-expressing cells was evaluated using cell ELISA. Briefly, target cells (BT-474 and A431 as HER2 positive and negative cells, respectively) were incubated with the intended VHH (2 μg/mL). Following incubation with the HRP conjugated anti-c-Myc antibody (dilution 1:5000) (Roche, Mannheim, Germany) and adding TMB, the developed reaction was detected at $\lambda = 450$ nm [2].

2.3.2. Flow cytometry

The flow cytometry assay was performed as described by Jamnani et al. [2]. Briefly, HER2$^+$ BT-474 cells and HER2$^-$ A431 cells were incubated with the intended VHH. After washing, incubation was performed with the anti-c-Myc tag antibody. The binding of VHH was detected by staining with the goat anti-mouse (whole molecule) IgG-FITC antibody (Sigma, St Louis, MO, USA). Immunofluorescence was monitored using a FACS Calibur flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.3.3. Competition assay between trastuzumab and anti-HER2 VHHs

A microtiter plate was coated with the HER2-Fc chimera (100 ng/well) at 4 °C overnight. Trastuzumab (1 μg/mL) (Genentech, San Francisco, USA) was added and the plate was incubated at room temperature for 1 h. After washing with 0.5% (v/v) Tween 20 in PBS, the VHH was added at increasing concentrations (0.5, 2 and 5 μg/mL) and incubated at room temperature for 1 h. After washing, detection was performed with the goat anti-human Fc-HRP antibody (dilution 1:4000) (Sigma) and the HRP activity was detected with TMB.

2.4. Construction of VHH_{HER2}-CARs and generation of the oligoclonal engineered Jurkat T cells

Based on the results obtained from VHH characterization, five VHHs were selected for use as the extracellular domains *for constructing* recombinant pcDNA3.1 vectors encoding HER2-specific VHH-CARs: $pVHH_{HER2}$-H_{IgG3}-CD28-CD3ζ ($pVHH_{HER2}$-1H), $pVHH_{HER2}$-HH_{IgG3}-CD28-CD3ζ ($pVHH_{HER2}$-2H), $pVHH_{HER2}$-H_{IgG3}-CD28-OX40-CD3ζ ($pVHH_{HER2}$-1HOX) and $pVHH_{HER2}$-HH_{IgG3}-CD28-OX40-CD3ζ ($pVHH_{HER2}$-2HOX) [27]. In this respect, the VHH_{RR4}, VHH_{RR6}, VHH_{RR10}, VHH_{RR14} and VHH_{RR16} genes were PCR amplified, using VHH specific primers Vhfor2 forward, *5′-GACTAGTGCGGCCGCGTGAGGAGACGGTGACCTG-3′* which contains a *Not I* site and Vhbam reverse, *5′-CGCGGATCCAATGGCCGAKG TSGAGCT-3′* which contains a *BamHI* site. The PCR products were gel purified with NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany) and digested with *BamHI* and *Not I* enzymes (all restriction enzymes were purchased from Roche, Switzerland). The genes of VHHs were ligated into the similarly digested pcDNA 3.1/Hygro(+) vectors containing CAR constructs to generate $pVHH_{HER2}$-1H, $pVHH_{HER2}$-2H, $pVHH_{HER2}$-1HOX and $pVHH_{HER2}$-2HOX (Fig. 1A). The VHH-CAR constructs were constituted of a high or moderate affinity VHH, linked to the human IgG3 hinge, CH2 and CH3, the transmembrane and intracellular sequences of human CD28 molecule, and the intracellular sequence of human CD3ζ molecule. The constructs, referred to as $pVHH_{HER2}$-1HOX, was constituted with the same backbone but with the intracellular signaling sequence of human OX40 molecule between the CD28 and CD3ζ molecules. We also generated two vectors retaining the VHH fragment and CAR signaling sequences but with two repeats of the hinge region ($pVHH_{HER2}$-2H and $pVHH_{HER2}$-2HOX). All of these

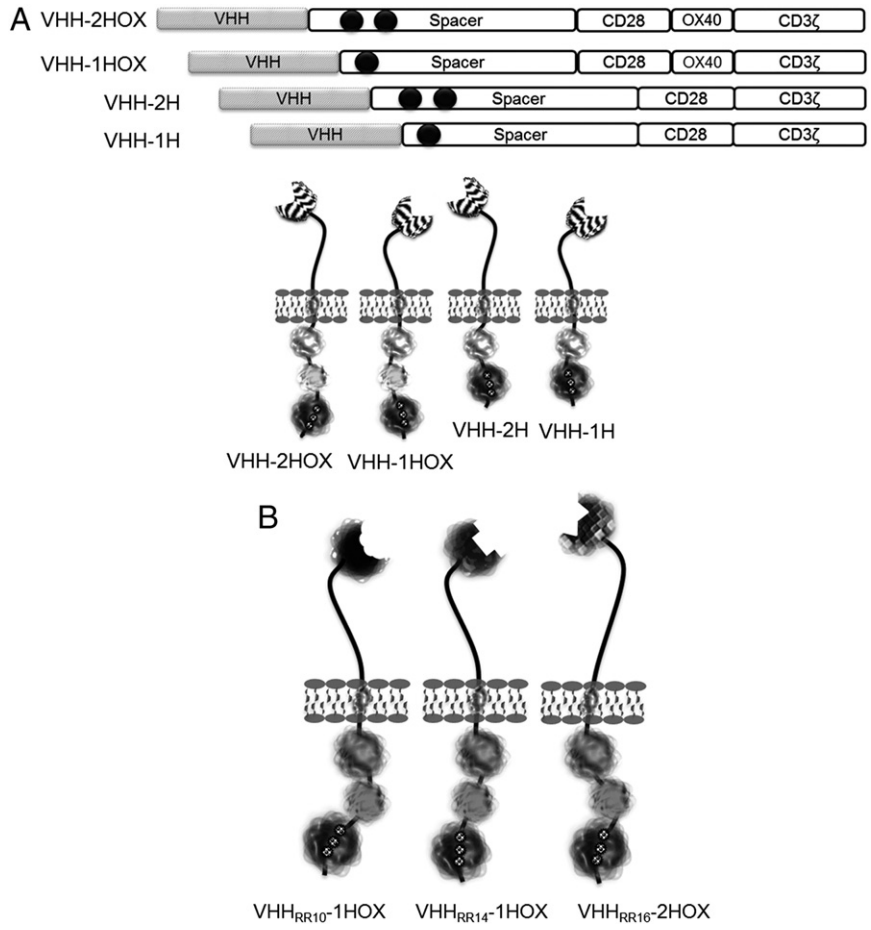


Fig. 1. Schematic representation of HER2 specific VHH-CARs. (A) Second and third generation CARs with different parts investigated in this study. Second generation CARs: VHH-H$_{IgG3}$-CD28-CD3ζ (VHH-1H) and VHH-HH$_{IgG3}$-CD28-CD3ζ (VHH-2H). Third generation CARs: VHH-H$_{IgG3}$-CD28-OX40-CD3ζ (VHH-1HOX) and VHH-HH$_{IgG3}$-CD28-OX40-CD3ζ (VHH-2HOX). (B) Schematic structure of three selected VHH-directed chimeric HER2 antigen receptors used to generate oligoclonal engineered Jurkat T cells. VHH$_{RR10}$-H$_{IgG3}$-CD28-OX40-CD3ζ (VHH$_{RR10}$-1HOX), VHH$_{RR14}$-H$_{IgG3}$-CD28-OX40-CD3ζ (VHH$_{RR14}$-1HOX) and VHH$_{RR16}$-HH$_{IgG3}$-CD28-OX40-CD3ζ (VHH$_{RR16}$-2HOX) CARs expressed on the cell membrane of engineered Jurkat T cells.

constructs were generated with the five VHHs (VHH$_{RR4}$,VHH$_{RR6}$, VHH$_{RR10}$, VHH$_{RR14}$ and VHH$_{RR16}$) as antigen binding domains (Table 1). Three pcDNA3.1/Hygro(+) vectors encoding VHH$_{TAG}$-2HOX (VHH-CAR against an irrelevant antigen TAG 72) [28], GFP and lacking any of the CAR sequences (empty vector), were used as negative controls (control Jurkat T cells). All constructs were confirmed by restrictive enzyme digestion, colony PCR and sequencing. To produce Jurkat T cells expressing VHH-CARs, the cells were transfected with constructs containing VHH-CARs using Lipofectamine™ LTX Reagent (Invitrogen) [33]. On the day of transfection, Jurkat T cells (2.5×10^6 cells/well) were cultured into a 6-well plate. The cells were individually transfected with 5 μg of different pVHH-CARs, pcDNA 3.1/Hygro(+) vector encoding green fluorescent protein and empty pcDNA 3.1/Hygro(+) vector (pcDNA) with 5 μl of PLUS™ Reagent and 14 μl of Lipofectamine™ LTX [2,34]. After 4 h, the medium was replaced with complete RPMI-1640 medium containing 10% FBS, PHA-L (Phytohemagglutinin-L) (1 μg/ml) and PMA (Phorbol myristate acetate) (50 ng/mL) and incubated at 37 °C. Following 48 h incubation, the expression of VHH$_{HER2}$-CARs on transfected Jurkat T cells was evaluated by reverse transcriptase-PCR and flow cytometry.

Table 1
The list of constructs containing VHH-CARs with different anti-HER2 VHHs, spacers and signaling endodomains.

VHHs	Constructs containing VHH-CARs
VHH$_{RR4}$	pVHH$_{RR4}$-H$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR4}$-HH$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR4}$-H$_{IgG3}$-CD28-OX40-CD3ζ
	pVHH$_{RR4}$-HH$_{IgG3}$-CD28-OX40-CD3ζ
VHH$_{RR6}$	pVHH$_{RR6}$-H$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR6}$-HH$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR6}$-H$_{IgG3}$-CD28-OX40-CD3ζ
	pVHH$_{RR6}$-HH$_{IgG3}$-CD28-OX40-CD3ζ
VHH$_{RR10}$	pVHH$_{RR10}$-H$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR10}$-HH$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR10}$-H$_{IgG3}$-CD28-OX40-CD3ζ
	pVHH$_{RR10}$-HH$_{IgG3}$-CD28-OX40-CD3ζ
VHH$_{RR14}$	pVHH$_{RR14}$-H$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR14}$-HH$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR14}$-H$_{IgG3}$-CD28-OX40-CD3ζ
	pVHH$_{RR14}$- HH$_{IgG3}$-CD28-OX40-CD3ζ
VHH$_{RR16}$	pVHH$_{RR16}$-H$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR16}$-HH$_{IgG3}$-CD28-CD3ζ
	pVHH$_{RR16}$-H$_{IgG3}$-CD28-OX40-CD3ζ
	pVHH$_{RR16}$-HH$_{IgG3}$-CD28-OX40-CD3ζ

2.5. Assessment of VHH$_{HER2}$-CARs expression

Total RNA from the transfected and non-transfected Jurkat T cells was extracted using RNeasy® Plus Mini kit (Qiagen) and used to generate cDNA using QuaniTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. The cDNA was amplified using two sets of primers; P2 forward, *5′-TGCTCTAGATGGCTGTTAGCGAGG-3′*, and P3 reverse, *5′-CCGCTCGAGTTTTGGGTGCTGGTGGTGGTTG-3′*, specific primers for CD28-CD3ζ chimera of transgene and β-act-forward, *5′-AGTAGGC TTTGTGGTTGATG-3′* and β-act-reverse, *5′-CTGTCAGGAAAGGAGAAATC-3′*,

Table 2
The binding affinity of anti-HER2 VHHs to the immobilized antigen. Based on the Beatty method, the VHH_{RR2}, VHH_{RR4}, VHH_{RR6}, VHH_{RR7} and VHH_{RR8} indicate K_{aff} ranging from 10^{10} to 10^{12} M^{-1} and the K_{aff} of VHH_{RR3}, VHH_{RR5}, VHH_{RR10}, VHH_{RR11}, VHH_{RR12} and VHH_{RR16} are approximately 10^9 M^{-1}.

VHHs	VHH_{RR8}	VHH_{RR6}	VHH_{RR4}	VHH_{RR2}	VHH_{RR7}	VHH_{RR11}	VHH_{RR16}	VHH_{RR12}	VHH_{RR3}	VHH_{RR5}	VHH_{RR10}	VHH_{RR14}
Affinity (K_{aff})(M^{-1})	8.6×10^{12}	**7.5×10^{12}**	**5.4×10^{12}**	3.2×10^{10}	2×10^{11}	8.0×10^{9}	**6.0×10^{9}**	5.3×10^{9}	5.2×10^{9}	3.2×10^{9}	**2.0×10^{9}**	**3.5×10^{8}**

specific primers for human β-actin housekeeping gene used as an internal control. PCR products were run on agarose gels and visualized by staining with SYBR Safe (Invitrogen).

The cell surface expression of VHH_{HER2}-CARs was measured by flow cytometry. The transfected and normal Jurkat T cells (6×10^5) were stained with the FITC-conjugated mouse anti-human IgG3 antibody

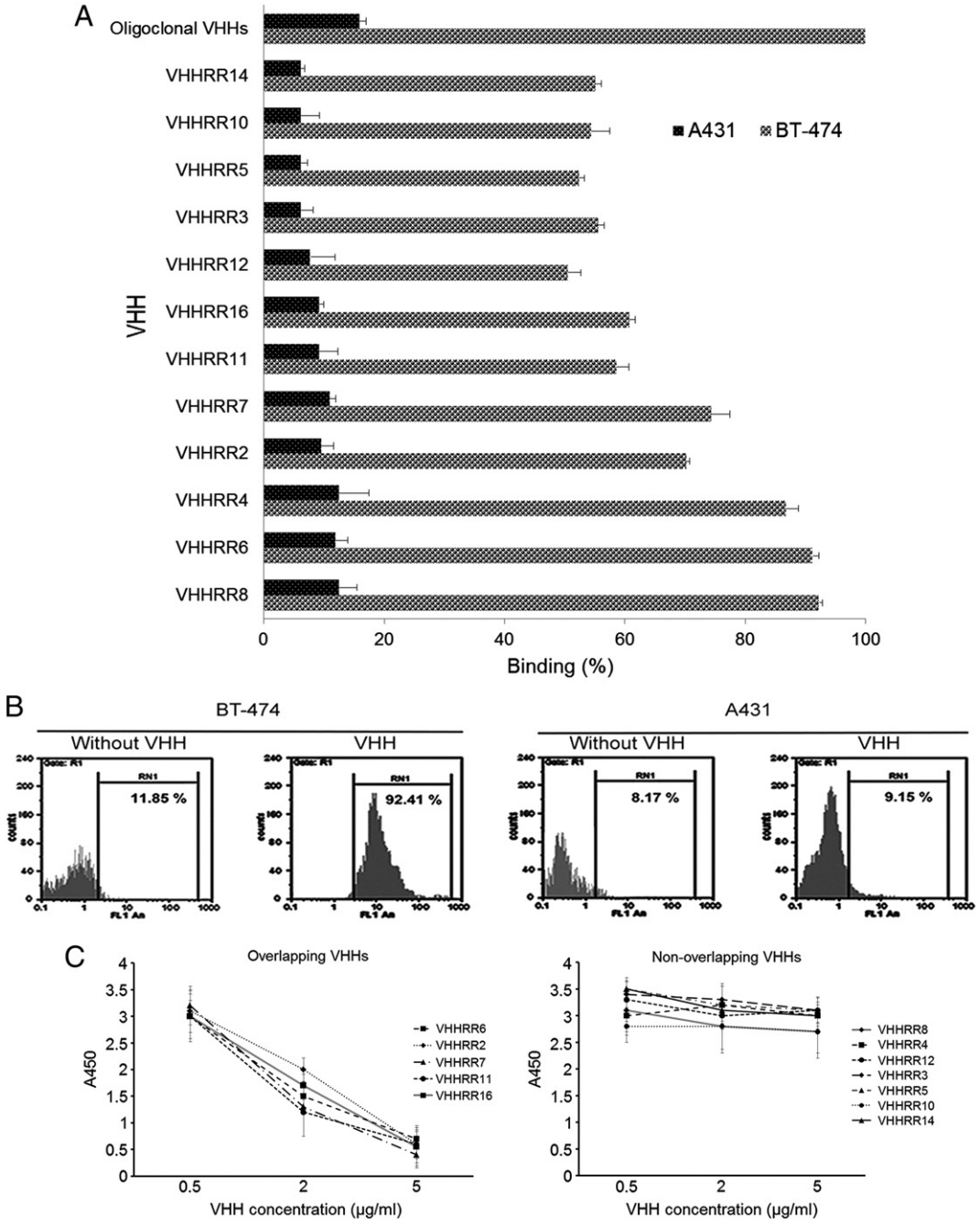


Fig. 2. The binding of HER2 specific VHHs to HER2 on target cells. (A) The high affinity VHH_{RR8}, VHH_{RR6} and VHH_{RR4} as well as the mixture of 12 VHHs as oligoclonal VHHs show excellent binding to $HER2^+$ BT-474 cells (gray bars) in comparison with HER2- A431 (black bars). Detection was performed by the HRP conjugated anti-c-Myc antibody. Data are means ± SEM of two separate experiments. Statistical analysis by Mann–Whitney test revealed significant differences between HER2-negative and HER2-positive cells ($p < 0.05$). (B) Flow cytometry of the VHH_{RR4} on $HER2^+$ BT-474 cell and $HER2^-$ A431 cells. The binding was detected by initially incubating the cells with the anti-c-Myc tag antibody and then immunofluorescence staining with the goat anti-mouse (whole molecule) IgG-FITC antibody was determined using a FACS Calibur flow cytometer. (C) Competition between trastuzumab and anti-HER2 VHHs. The increasing concentrations (0.5, 2 and 5 μg/mL) of VHH_{RR2}, VHH_{RR6}, VHH_{RR7}, VHH_{RR11} and VHH_{RR16} lead to decreasing OD values of trastuzumab (overlapping VHHs). The other VHHs (VHH_{RR3}, VHH_{RR4}, VHH_{RR5}, VHH_{RR8}, VHH_{RR10}, VHH_{RR12} and VHH_{RR14}) failed to affect the binding of trastuzumab to HER2 (non-overlapping VHHs). Detection was performed by the HRP conjugated goat anti-human Fc antibody. Data are means ± SEM of two separate experiments.

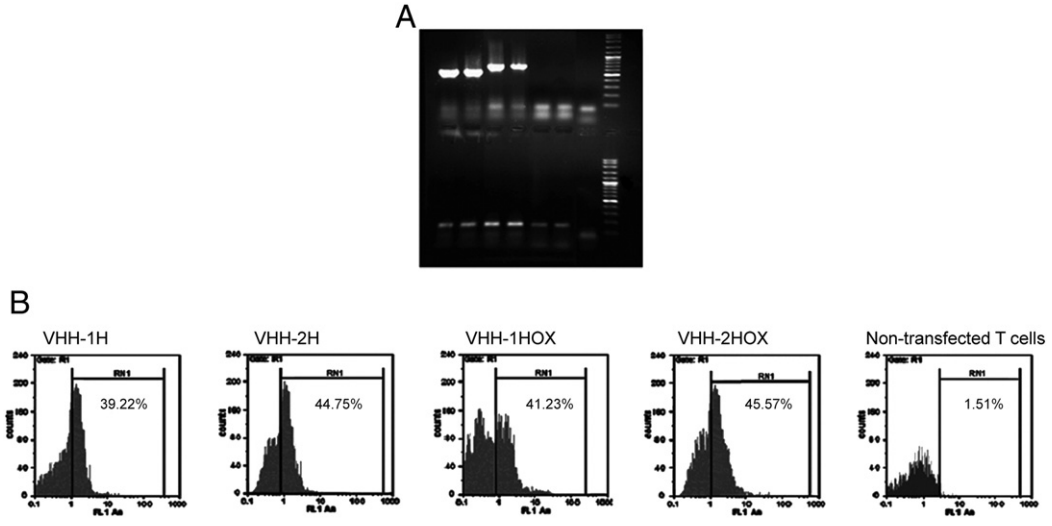


Fig. 3. Expression of VHH_{HER2}-CARs in engineered Jurkat T cells. (A) To assess the expression of VHH_{HER2}-CARs, reverse transcriptase-PCR analysis was performed. The β-actin mRNA expression was used as an internal reference. Amplified products were analyzed on 1% agarose gels using a 100-bp DNA ladder as reference. In upper panel, lanes 1 to 4 show the relative size of VHH_{RR4}-1H (568 bp), VHH_{RR4}-2H (568 bp), VHH_{RR4}-1HOX (690 bp) and VHH_{RR4}-2HOX (690 bp), respectively. Lane 5 is the pcDNA-transfected Jurkat T cells, lane 6 is non-transfected Jurkat T cells and lane 7 is lacking template. Lower panel shows the amplification products of β-actin (~200 bp). (B) Flow cytometry analysis of the transfected Jurkat T cells. The non-transfected and transfected Jurkat T cells (6×10^5) were stained with the FITC-conjugated mouse anti-human IgG3 monoclonal antibody (1 μg/mL), which recognized the IgG3 CH2-CH3 (spacer) of CAR constructs.

(1 μg/mL) (Sigma), which recognized the IgG3 CH2-CH3 (spacer) of VHH-CARs. The immunofluorescence was monitored using a FACS Calibur flow cytometer equipped with CellQuest software (BD Biosciences). The cell debris was eliminated from the analysis using a gate on forward and side light scatter.

2.6. IL-2 secretion and proliferation of the oligoclonal engineered Jurkat T cells in response to HER2

Jurkat T cells that expressed VHH_{HER2}-CARs containing VHH_{RR4}, VHH_{RR6}, VHH_{RR10}, VHH_{RR14} and VHH_{RR16} plus different repeats of hinge and combination of signaling domains were separately investigated in five panels. In this regard, Jurkat T cells with soluble HER2 (2 μg/mL) and Jurkat T cells with soluble TAG 72 (1600 U/mL) were separately plated on the HER2-coated microtiter plates and incubation was done for 24 h [2,28]. Jurkat T cells (transfected and non-transfected cells) (10^6 per well) were also co-cultured with HER2 positive (SK-BR-3 and NIH $3T3^{HER2+}$) and negative (HepG2 and $NIH3T3^{HER2-}$) cells for 48 h in 24-well plates (E:T of 10:1 ratio). The culture supernatants were harvested and assayed for human IL-2 using a Quantikine® Kit (R&D systems). To determine T cell proliferation, Jurkat cells (10^5 cells/well) were co-cultured with target cells (10^4 cells/well) in 96-well plates. After 48 h incubation, Jurkat T cells were harvested and viable cells were assessed by the MTT assay. In the sixth panel, IL-2 production and proliferation of the oligoclonal engineered Jurkat T cells expressing VHH_{HER2}-CARs with different VHHs (the mixture of VHH_{RR10}-1HOX, VHH_{RR14}-1HOX and VHH_{RR16}-2HOX CAR-engineered Jurkat T cells) were evaluated as described above (Fig. 1B).

2.7. Cytotoxicity assay

The cytotoxicity induced by Jurkat T cells was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following co-cultures of the transfected and non-transfected Jurkat T cells (5×10^5 cells/well) with target cells (10^4 cells/well) for 12 h in 96-well plates, the activity of LDH in medium was determined using a commercially available kit (Roche, Switzerland). Based on the results, this assay was also performed on the oligoclonal engineered Jurkat T cells (VHH_{RR10}-1HOX, VHH_{RR14}-1HOX and VHH_{RR16}-2HOX). The LDH concentrations were determined in 50 μl aliquots of cell-free culture supernatants and absorbance values were obtained in triplicate.

The percentage of specific cytotoxicity was calculated as follows:

$$\frac{\text{Experimental LDH release-Spontaneous LDH release}}{\text{Maximal LDC release-Spontaneous LDH release}} \times 100.$$

The experimental LDH release is the LDH released on co-culture of effector and target cells, whereas the spontaneous release is the LDH released from tumor cells in the absence of effector cells. The maximal LDH release represents the release after addition of Triton X-100 (100% LDH release) to cells.

2.8. Statistical analysis

The different experimental groups within the study were compared by using the Kruskal–Wallis test. The comparisons between the pairs of groups were performed with the Mann–Whitney test. A probability of less than 0.05 ($p < 0.05$) was used for statistical significance.

3. Results

3.1. Isolation and characterization of HER2 specific VHHs

To select HER2 specific VHHs, the VHH gene library was panned on the immobilized antigen and target cells. All of the input and output phages were screened by ELISA and the highest signals were observed in the fifth and third rounds of panning on the immobilized antigen and cells, respectively. Among the clones, 12 reacted specifically with the HER2 antigen, but not with the irrelevant proteins and these were selected. The binding affinity of the isolated VHHs to the HER2 antigen was also evaluated, based on the Beatty method that showed K_{aff} around 3.5×10^8 to 8.6×10^{12} M^{-1} (Table 2) [2,35].

To confirm whether the isolated VHHs were able to recognize the membrane-bound HER2 on target cells, a cell based ELISA and flow cytometry were performed. Strong signals were observed when the VHH_{RR4}, VHH_{RR6}, and VHH_{RR8} were incubated with $HER2^+$ BT-474 cells. Notably, all 12 VHHs concurrently targeted the HER2-expressing cells and exhibited higher signal intensity than each VHH alone. It is likely that the superior targeting of the oligoclonal VHHs results from a large

increase in the functional avidity of the mixture of VHHs (Fig. 2A). Flow cytometry analyses further confirmed that the selected VHH (VHH_{RR4}) bound substantially to $HER2^+$ BT-474 cells, but failed to bind to $HER2^-$ A431 cells (Fig. 2B). The competition between trastuzumab and VHHs for binding to HER2 was evaluated and based on these results, anti-HER2 VHHs were categorized in two groups of overlapping and non-overlapping VHHs. In the presence of trastuzumab and using the increasing concentrations of the VHH_{RR2}, VHH_{RR6}, VHH_{RR7}, VHH_{RR11} and VHH_{RR16} VHHs, the decreasing OD values of trastuzumab proved that these VHHs can displace trastuzumab from its epitope on HER2 (overlapping VHHs) (Fig. 2C, overlapping VHHs). The other VHHs (VHH_{RR3}, VHH_{RR4}, VHH_{RR5}, VHH_{RR8}, VHH_{RR10}, VHH_{RR12} and VHH_{RR14}) did not compete with trastuzumab for binding to the identical epitope on HER2 (Fig. 2C, non-overlapping VHHs). The results of the latter group indicated that these VHHs bound to variable regions of HER2 and targeted distinct epitopes, which are different with the one that is targeted by trastuzumab.

Based on the affinity for HER2 antigen and the ability in discriminating between HER2 positive and control cells, the two high-affinity VHH_{RR6} (K_{aff}: 7.5×10^{12} M^{-1}) and VHH_{RR4} (K_{aff}: 5.4×10^{12} M^{-1}) that showed highest activity against HER2 expressing cells (Genebank accession numbers JX576800 and JX576803, respectively) as well as three moderate affinity VHH_{RR16} (K_{aff}: 6.0×10^{9} M^{-1}), VHH_{RR10} (K_{aff}: 2.0×10^{9} M^{-1}) and VHH_{RR14} (K_{aff}: 3.5×10^{8} M^{-1}) (Genebank accession numbers JX576799, JX576801 and JX576802, respectively) that were able to distinguish between HER2 positive and negative cells, were selected and used as binding domains in VHH_{HER2}-CARs.

3.2. Construction and expression of HER2 specific VHH-CARs

To explore whether the VHH as a navigator redirects specificity of Jurkat T cells to HER2, a series of vectors were constructed encoding VHH_{HER2}-CARs comprising anti-HER2 VHHs as binding domains. A reverse transcriptase-PCR was performed for the transfected and non-transfected Jurkat T cells to demonstrate the expression of VHH_{HER2}-CARs in engineered Jurkat T cells (Fig. 3A). The β actin mRNA was used as an internal reference for reverse transcriptase-PCR to verify the expression stability in the assay. As shown in Fig. 3A, the expression of VHH_{HER2}-CARs was confirmed in the engineered Jurkat T cells, whereas these bands were not observed in the control Jurkat T cells. Also, the immunofluorescence intensity of GFP expression in the transfected Jurkat T cells confirmed the efficiency of transfection (data not shown). The cell surface expression of VHH_{HER2}-CARs on engineered Jurkat T cells was determined by flow cytometry using the FITC-conjugated mouse anti-human IgG3 antibody that recognized the "CH2–CH3" domain (spacer) of VHH_{HER2}-CARs. As shown in Fig. 3B, the HER2 specific VHH-CARs were expressed in 40–45% of engineered Jurkat T cells. The expression intensity of VHH_{HER2}-CARs containing the extended hinge VHH_{HER2}-2H and VHH_{HER2}-2HOX (Fig. 3B) was more than VHH_{HER2}-CARs lacking the extended hinge. Taken together, the highest expression was observed in the Jurkat T cells expressing VHH-2HOX CARs (Fig. 3B).

3.3. Substantial IL-2 secretion and expansion of oligoclonal engineered Jurkat T cells in response to HER2

The ability of VHH_{HER2}-CAR-expressing Jurkat T cells to secrete IL-2 and proliferate in response to the HER2 antigen was investigated in several panels. Jurkat T cells engineered with VHH_{HER2}-CAR specifically responded to the plate-bound HER2 antigen determined with IL-2 secretion (Fig. 4A). The specificity of target antigen recognition by the VHH_{HER2}-CAR-engineered Jurkat T cells was further confirmed by adding soluble HER2 and TAG 72. The soluble HER2 interfered with the interaction of VHH_{HER2}-CARs with the plate-bound HER2 and blocked the secretion of IL-2 by the Jurkat T cells expressing VHH_{HER2}-CARs that demonstrated the antigen dependency of the engineered T cell activation. As indicated in Fig. 4A, the control Jurkat T cells were not activated by the coated antigen. Furthermore, soluble TAG 72 did not block IL-2 secretion by VHH-CAR-engineered Jurkat T cells.

Next, Jurkat T cells were co-cultured with $HER2^+$ target cells (SK-BR-3 and $NIH3T3^{HER2+}$) and $HER2^-$ target cells (HepG2 and $NIH3T3^{HER2-}$). The culture supernatants were collected after 48 h to assess IL-2 release as a measure of Jurkat T cell activation. Jurkat T cells expressing VHH_{HER2}-CARs secreted significant amounts of IL-2 compared with control Jurkat T cells when co-cultured with SK-BR-3 and $NIH3T3^{HER2+}$ cells (Fig. 4B). The engineered Jurkat T cells showed no significant binding to HepG2 and $NIH3T3^{HER2-}$ cells lacking the HER2 antigen. Conclusively, the Jurkat T cells expressing VHH_{RR4}-1HOX and VHH_{RR6}-2HOX released higher amounts of IL-2 (630 ± 22 and 666 ± 20 pg/mL, respectively) in comparison with T cells transfected with VHH_{HER2}-CAR construct. To measure cell division, the Jurkat T cells were co-incubated with target cells for 48 h, then harvested and evaluated by a proliferative assay. The VHH_{HER2}-CAR-engineered T cells expanded extensively compared with the control Jurkat T cells (Fig. 4C). The VHH_{HER2}-CAR-modified T cells did not proliferate in the absence of antigen stimulation. The Jurkat T cells engineered with VHH_{RR6}-2HOX and VHH_{RR16}-2HOX (74 ± 8% and 62 ± 5%, respectively) and VHH_{RR4}-1HOX, VHH_{RR10}-1HOX and VHH_{RR14}-1HOX (72 ± 6%, 61 ± 7% and 53 ± 6%, respectively) demonstrated significant proliferation.

Although, the Jurkat T cell engineered with VHH_{HER2}-CARs containing VHH_{RR4} and VHH_{RR6} showed the highest IL-2 production and expansion, some extent of activity was observed when these cells were co-cultured with $HER2^-$ target cells (Fig. 4B and C). In contrast, the Jurkat T cells expressing VHH_{HER2}-CARs with moderate affinity VHHs (VHH_{RR10}, VHH_{RR14} and VHH_{RR16}) specifically targeted $HER2^+$ target cells and no cross-reaction was observed with the $HER2^-$ target cell. In this regard, the oligoclonal engineered T cells that exhibited high selectivity were co-cultured with target cells. This mixture of different VHH_{HER2}-CAR-engineered T cells operated as multi-targeting effectors and showed marked IL-2 release (690 ± 21 pg/mL) and expansion (74 ± 9%) compared with each individual VHH_{HER2}-CAR-engineered Jurkat cells (VHH_{RR10}-1HOX: 505 ± 25 pg/ml and 61 ± 7%, VHH_{RR14}-1HOX: 450 ± 13 pg/mL and 53 ± 6%, and VHH_{RR16}-2HOX: 540 ± 15 pg/mL and 62 ± 5%, respectively) (Fig. 4B and C).

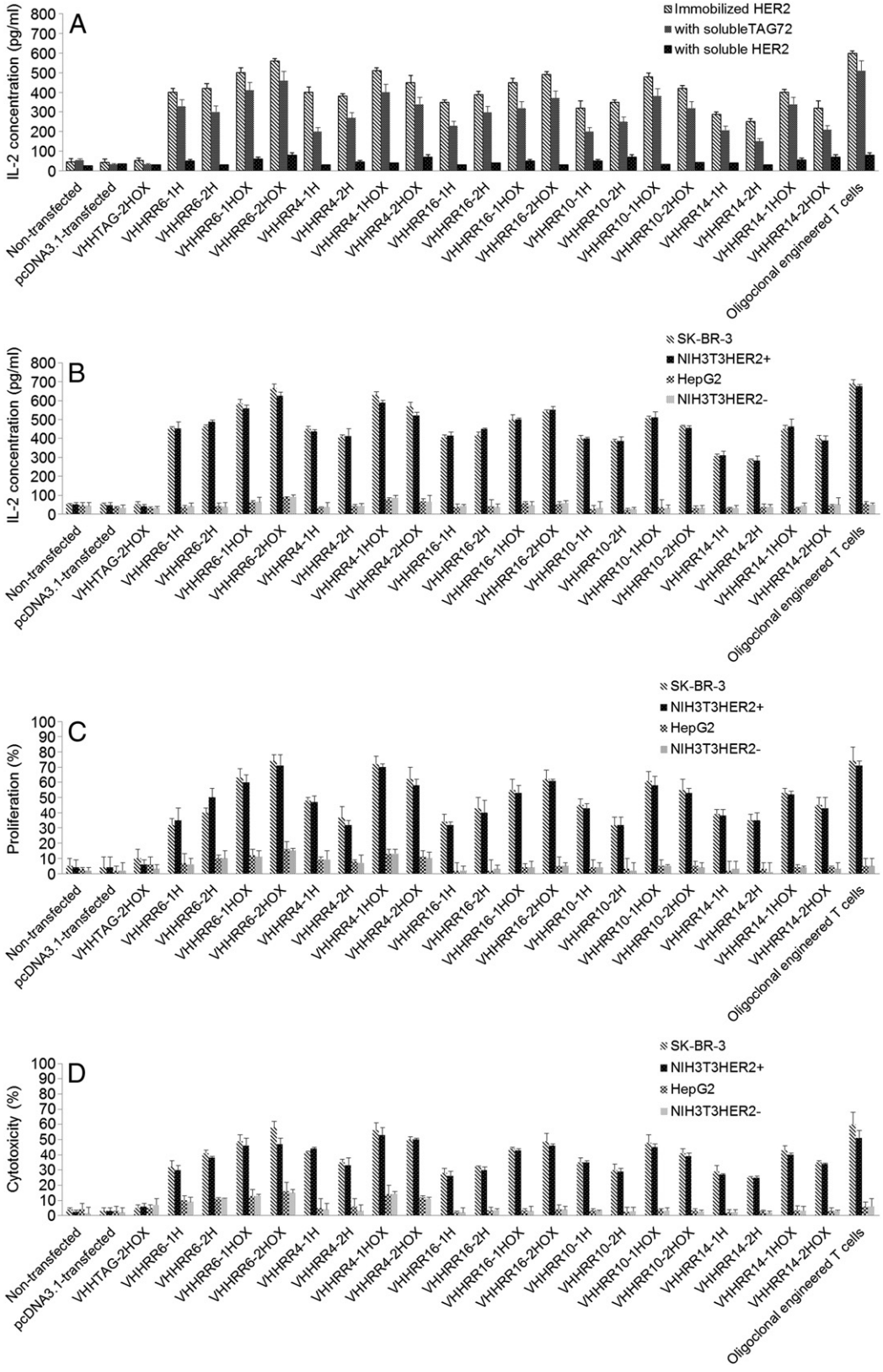
3.4. Oligoclonal engineered Jurkat T cells specifically lyse HER2-expressing cells

The cytotoxic activity of engineered Jurkat T cells against target cells was investigated by the LDH release assay. All VHH_{HER2}-CAR-engineered Jurkat T cells, containing or lacking the OX40 intracellular signaling sequence, showed cytotoxic activity and lysed $HER2^+$ target cells in a dose-dependent manner. The cytotoxicity of Jurkat T cells expressing VHH_{HER2}-CARs containing VHH_{RR4}, VHH_{RR10} or VHH_{RR14} with one repeat of hinge region was more than Jurkat T cells expressing VHH_{HER2}-CARs with these VHHs but with two repeats of the hinge region. On the other hand, VHH_{HER2}-CAR-modified Jurkat T cells with VHH_{RR6} and VHH_{RR16} as binding domains and the extended hinge in their structure showed significant cytolysis. Although the VHH_{RR4}-1HOX and VHH_{RR6}-2HOX CAR-transfected Jurkat T cells mediated the most cytotoxic activity against $HER2^+$ target cells (56 ± 6% and 58 ± 5% specific lysis in an E:T ratio of 50:1, respectively), $HER2^-$ target cells lysis was also observed (14 ± 5% and 16 ± 4%, respectively). In contrast, the VHH_{RR10}-1HOX, VHH_{RR14}-1HOX and VHH_{RR16}-2HOX CAR-transfected Jurkat T cells showed moderate cytotoxicity (48 ± 5%, 43 ± 3% and 49 ± 5%, respectively), but were not activated with $HER2^-$ target cells (4 ± 2%, 3.5 ± 1% and 4 ± 1%, respectively) (Fig. 4D). Notably, the co-culturing of the oligoclonal Jurkat T cell with $HER2^+$ target cells resulted in the superior cytolytic activity (60 ± 8%). Remarkably, the oligoclonal engineered T cells did not lyse $HER2^-$ target cells (6 ± 5%). The Jurkat T cells engineered with the VHH_{RR10}-2H, VHH_{RR14}-2H and VHH_{RR16}-1H CARs showed no significant cytotoxicity to $HER2^+$ target cells. Furthermore,

the control Jurkat T cells were unable to lyse any of target cells tested (Fig. 4D).

4. Discussion

To construct a series of VHH_{HER2}-CARs with different anti-HER2 VHHs as antigen binding domains, a panel of functional anti-HER2 VHHs was isolated and characterized. The oligoclonal VHHs showed great specificity and binding ability to the HER2 antigen. Based on a competition assay between trastuzumab and VHHs, anti-HER2 VHHs were classified into two overlapping and non-overlapping groups. The different binding affinities of overlapping and non-overlapping VHHs demonstrated that they recognize various epitopes on HER2. The natural antibody-mediated responses are elicited by the binding of the antibodies to several epitopes



and acting in synergy. The mAbs do not generate such actions in cancer therapy and remissions are only temporary because of the emergence of tumor cell escape variants. Moreover, mAbs compete for the identical antigenic epitope and therefore, the epitope density is a limiting factor. To improve efficacy, higher antibody doses are required and this may initiate adverse side effects. In contrast, by binding the poly and oligoclonal antibodies to several different epitopes on the antigen of the tumor cells, the emergence of escape variants may be minimized [36,37]. This generation of antibodies may act more efficiently than mAbs, because it is unlikely that a tumor cell loses its all target epitopes [36–38]. The use of non-competitive antibody combinations to engage multiple therapeutic mechanisms has been a striking strategy for enhancing drug efficacy [39–41]. In this respect, our study demonstrated why the binding of oligoclonal VHHs to distinct epitopes on HER2 resulted in the substantial targeting of the HER2-expressing cell [2].

In most CARs, IgG1 CH2CH3 (Fc) domain is generally used as a spacer [12,42,43]. These CARs with IgG1 Fc spacer can cross-react with FcγR on monocytes and NK cells and result in the unintended initiation of both an innate immune and an "off-target" T cell response. The 'off-target' T cell response occurs independently of scFv-mediated CAR binding to the cognate target antigen [12].

A side-by-side comparison of different combinations of costimulatory endodomains will help to design superior CAR constructs [21–23]. Several studies have showed prolonged in vitro proliferation, increased IL-2 production, and sustained a cytolytic function following repeated antigenic stimulation for a CD28-OX40-CD3ζ CAR [15,20,22,44]. Collectively, these approaches illustrated that stronger antitumor responses may be obtained by third generation CARs [4,8]. Therefore, we designed a series of second and third generation CARs with the epitope-distinct VHHs and different repeats of human IgG3 hinge. To minimize the toxicity of cationic lipids on T cells, gene transfer conditions were optimized and Lipofectamine LTX was used due to its lower toxicity than Lipofectamine2000™ [28]. We showed that the binding of VHH_{HER2}-CAR-engineered Jurkat T cells to HER2 either in its immobilized form or in a cellular context was sufficient to trigger the T cell activation. The VHH-CAR-modified Jurkat T cells with the HER2 specificity secreted IL-2 only in response to HER2-expressing cells and failed to recognize $HER2^-$ target cells. The activation of VHH_{HER2}-CAR-modified Jurkat T cells was blocked with the soluble HER2 protein, which indicated that the activation was fully antigen dependent and that the HER2 specific VHH acted as an efficient binding domain in VHH_{HER2}-CARs. Interestingly, the VHH_{HER2}-CAR-engineered Jurkat T cells exhibited efficient cytolytic effector functions toward the HER2-expressing cells. The CARs containing OX40 plus CD28-CD3ζ showed greater expansion and cytotoxicity in vitro when compared to the receptors containing CD28-CD3ζ domains. Jurkat T cells redirected by VHH_{RR4}-1HOX and VHH_{RR6}-2HOX CARs secreted the highest amounts of IL-2 in response to the HER2-expressing cells compared with cells transfected with other vectors, which is in accordance with the previous report [27]. Furthermore, the VHH_{HER2}-CAR-engineered T cells with the overlapping VHH_{RR6} or VHH_{RR16} and two repeats of hinge region showed superior activity than the VHH_{HER2}-CAR-engineered Jurkat T cells with these VHHs and one repeat of hinge region. It has been suggested that a spacer with the specific size and flexibility can have an effect on the optimal CAR function. In this way, the epitope position in relation to the target cell membrane plays a critical role [45]. Previously, the better membrane-proximal epitopes targeting and greater T cell activation were achieved when a spacer was inserted [46]. Here, we showed that overlapping VHH_{RR6} and VHH_{RR16} bind to the epitope, presumably within amino acids 529–627 on domain IV of the HER2 extracellular domain recognized by trastuzumab [2]. Hence, the T cell expressing VHH_{HER2}-CARs incorporating VHH_{RR6} or VHH_{RR16} with the elongated hinge targeted membrane-proximal epitopes and accessed new sites on HER2.

Our results also showed that Jurkat cells expressing VHH_{HER2}-CARs with the high affinity VHH_{RR4} or VHH_{RR6} had the highest activity against the HER2-expressing cells, although they slightly cross-reacted with target cells lacking the HER2 antigen. These data also indicated that the VHH_{HER2}-CARs comprising highly selective VHH_{RR10}, VHH_{RR14} or VHH_{RR16} with moderate binding affinity discriminated between HER2 positive and negative cells and prevented an unintended binding of engineered T cells to $HER2^-$ target cells. In a similar study, multiple CARs that targeted various epitopes on HER2 were designed [47]. This study showed that T cells with high affinity CARs had low selectivity compared to T cells with moderate affinity CARs [47].

Costimulatory CARs are currently investigated in phase I trials and some promising preliminary results are emerging. However, the administration of high numbers of engineered T cells and extensive costimulation can trigger a cytokine storm [5,48,49]. Different alternative approaches are still required to avoid adverse effects [4,24]. One striking approach is the using of oligoclonal engineered T cells that target different epitopes on the cognate antigen. Targeting a single epitope on a tumor antigen may result in the emergence of tumor cell escape variants. Furthermore, competition for the identical antigenic epitope may lead to a number of engineered T cells failing to bind their epitope on a cognate antigen, and subsequently cross-react with irrelevant antigens thereby initiating adverse side effects. Thus, the multi-targeting chimeric T cells that recognize various epitopes on an antigen can mount a more effective immunological antitumor response compared with T cells that largely rely on a specific epitope. Indeed, this study provided the proof of concept with Jurkat T cells and showed that oligoclonal T cell therapy targeting various epitopes of HER2 can provide superior specificity and cytotoxicity for target cells than each individual VHH_{HER2}-CAR-engineered T cells. The translation of our approach to primary T cells awaits further development, which could provide an efficient alternative approach for autologous T cell therapy.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgement

The authors express their gratitude to Prof. Michael Kershaw (Cancer Immunology Research Program, Peter MacCallum Cancer Centre, Melbourne, Australia) for kindly providing a retroviral vector encoding the full length of HER2, and Prof. Axel Ullrich (Department of Molecular Biology, Max-Planck-Institute of Biochemistry, Martinsried, Germany) for kindly providing a plasmid encoding the extracellular domain of HER2. This work was supported by the Pasteur Institute of Iran, Tehran, Iran and the Iran National Science Foundation (INSF)

Fig. 4. Characterization of VHH_{HER2}-CAR-engineered Jurkat T cells against HER2. (A) IL-2 secretion by the transfected and non-transfected Jurkat T cells incubated with the immobilized HER2. Incubation (24 h) of VHH_{HER2}-CAR-engineered Jurkat T cells with the immobilized HER2 in the presence of soluble HER2 protein (2 μg/ml) lead to the blocking of VHH_{HER2}-CAR-engineered cells. In contrast, the irrelevant TAG 72 antigen (control) can not affect to the function of VHH_{HER2}-CAR-engineered cells. (B) As data shows, there is specific IL-2 production by the VHH_{HER2}-CAR-engineered Jurkat T cells co-cultured with HER2 + target cells (SK-BR-3 and $NIH3T3^{HER2+}$), but not with $HER2^-$ target cells (HepG2 and $NIH3T3^{HER2-}$). Oligoclonal engineered T cells demonstrate superior IL-2 secretion than each VHH_{HER2}-CAR-engineered Jurkat T cells alone. (A and B) The differences between T cells expressing VHH_{HER2}-CARs with costimulatory signaling domain OX40 compared to T cells lacking OX40 were significant ($p < 0.05$, Mann–Whitney). (C) Jurkat T cells expressing VHH_{HER2}-CARs proliferate in response to HER2 on target cells. (D) Specific lysis of HER2-expressing cells by the VHH_{HER2}-CAR-engineered Jurkat T cells. The cytotoxicity of Jurkat T cells was determined as percentage of lysis (% lysis) of the different cell lines. The non-transfected Jurkat T cells, pcDNA-transfected, VHH_{TAG}-2HOX and $HER2^-$ cells served as controls. Oligoclonal engineered T cells were the mixture of epitope-distinct Jurkat T cells that were individually transfected with the VHH_{RR10}-1HOX, VHH_{RR14}-1HOX and VHH_{RR16}-2HOX CARs. In all panels, the differences between oligoclonal engineered T cells compared to each individual VHH_{HER2}-CAR-engineered T cells rather than VHH_{RR4}- and VHH_{RR6}-CARs ($p < 0.05$, Mann–Whitney) and VHH_{HER2}-CAR-engineered T cell groups compared to controls ($p < 0.01$, Mann–Whitney) were significant. Data are means ± SEM of three separate experiments.

reference 90006943. SMM acknowledges financial support from the Danish Agency for Science, Technology and Innovation (Technology and Production) reference 12-126894.

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