Application of nanobody-based CAR-T in tumor immunotherapy (Review)

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Abstract. Chimeric antigen receptor (CAR) T cell therapy is a type of cellular immunotherapy showing promising clinical effectiveness and high precision. CAR-T cells express membrane receptors with high specificity, which enable them to identify certain target antigens generated by cancerous cells. The three primary structural elements of the CAR are the extracellular domain, transmembrane domain and cytoplasmic domain. Nanobodies are a type of antibody fragment derived from the variable domains of camelid heavy chain antibodies (VHH), which are the antigen-specific binding domains. They have high clinical applicability due to their tiny size, excellent target affinity, adaptable functions and guaranteed stability. Structurally pre-designed nanobodies were transduced in primary T lymphocytes, forming CAR-T cells and these have been demonstrated to have inhibitory effects on hematologic malignancy or solid tumor cells/tissues both *in vivo* and *in vitro*. At present, a number of novel nanobody-based modalities can include a single nanobody, a bi-valent nanobody and multivalent nanobody CAR-T cells with bispecific and multispecific characteristics, showing promising therapeutic efficacy that is similar to CAR-T cells modulated with a single-chain variable fragment. Intriguingly, CAR-T cells targeting the B-cell maturation antigen modified using an anti-B-cell maturation antigen single nanobody or bivalent nanobody have been shown to exhibit clinical efficacy comparable to scFv-modulated CAR-T cells. The application of nanobodies in CAR-T therapy has been well established from laboratory-based evidence to clinical application and they have great potential for developing advanced CAR-T cells for more complex employment.

Contents

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1. Introduction

Immune cellular therapy represents a large number of therapeutic modalities used in immune-oncology and that has been at the forefront of antitumor clinical strategies (1-3). Chimeric antigen receptor (CAR) T cell therapy has demonstrated consistent therapeutic advances for the treatment of patients, although there are challenges in the latter that need to be overcome (4,5). An illustration of clinical therapy employing CAR-engineered cells is adoptive T cell therapy (ACT), which is an example of passive immunotherapy that employs lymphocyte infusion to produce an anti-tumor response (6). CARs are expressed in genetically modified T cells using receptors with an external antigen-recognition domain derived from an antibody, a hinge domain, a transmembrane helix and an

intracellular signaling component that activates and enhances T cell function upon target antigen binding. Due to their size and complexity, monoclonal antibodies have low cellular penetrance and poor tissue infiltration, making them ineffective for cancer immunotherapy and thought to cause drug resistance (7). By contrast, nanobodies are 10-times smaller than monoclonal antibodies and can permeate cell membranes and tissues. Due to having >80% sequence homology with human IgG heavy chains and high similarity to the human VH3 family, in addition to their small size, nanobodies have low immunogenicity and excellent biocompatibility. Moreover, humanizing camelid heavy chain antibodies (VHH) domains is easy due to their structural commonality (8).

Nanobodies are different from single-chain variable fragments (scFvs), as they are comprised of heavy chain antibody domains. While scFvs do not always fold efficiently and can be prone to aggregation (9,10), nanobodies are small, persistent, single-domain antibody fragments produced from camelids, exhibiting binding affinities comparable to conventional scFvs (11,12). Furthermore, the small size of nanobodies allows them to target different epitopes from those recognized by scFvs and they are typically less immunogenic than murine scFvs (13). In addition, unlike scFvs, nanobodies do not require the extra folding and assembly stages associated with V-region pairing. They can undergo cell surface display without the need for substantial linker optimization or other forms of structural reconfiguration. In addition, some reports found that scFvs are associated with tumor recurrence in some individuals due to their large size, high immunogenicity, weak affinity, easy aggregation, tonic signaling and low efficiency (14,15).

Therefore, it is essential to employ novel antibody types that improve the efficacy of CAR-T cell therapy. The structural engineering of CAR-T cells requires the genetic modification of receptors that may recognize malignant cells expressing tumor-associated molecules and which avoid working in a major histocompatibility complex (MHC)-dependent pattern, through antibody-antigen binding. For example, the CAR-T cell products that target CD19 primarily consist of Yescarta, Kymriah, Tecartus and Breyanzi, all of which have received approval from regulatory authorities. The US FDA (Food and Drug Administration) has approved CAR-T cells as a therapeutic intervention for various malignancies, including B-cell precursor acute lymphoblastic leukemia (B-ALL), follicular lymphoma, multiple myeloma (MM) and mantle cell lymphoma (MCL). While their application within clinical studies has yielded encouraging results, there is often a tendency to cause various degrees of cytokine release syndrome (CRS) (16), immune effector cell-associated neurotoxicity syndrome (ICANS) (17) and various forms of special B-cell deficiency, such as infection and low toxicity (18,19). The present review summarized the limitations and risks of nanobody CAR-T-cell therapy and potential solutions to these problems.

2. Development of CAR-T

The origin of CAR. In the 1960s and 1970s, Jaques Miller discovered that the thymus was the origin of T cells, also referred to as T helper (TH) cells and that B lymphocytes (B cells) originated from the bone marrow (20,21). Two key interacting components primarily contribute to the exceptional cancer cell recognition capabilities of T cells, a compatible T cell receptor (TCR) associated with the cell membrane and a short peptide on the tumor cell that conveys tumor-associated antigens (TAAs). The MHC and human leukocyte antigen are both responsible for antigen presentation and helping the immune system recognize 'self' vs. 'non-self'. In the early 1980s, Steven Rosenberg isolated and described lymphokine-activated killer (LAK) cells from peripheral blood mononuclear cells (22).

The advancement of CAR-T design. In 1993, Klaus *et al* (23) observed that the TCR's α, β, γ and δ chains undergo rearrangement to form a clonal, non-specific TCR. Following this study, CD4-positive T helper cells, including regulatory T cells (Tregs) and CD8-positive cytotoxic T cells were identified (24). Subsequently, in 1989, Eshhar *et al* (25) proposed the existence of target-specific T lymphocytes using chimeric receptor molecules, opening up a new era of genetically engineered T cells. These targeted molecules consist of variable domains from antibodies and constant domains from TCRα or β, with a heavy variable (VH) domain from an anti-2,4,6-trinitrophenol (TNP) antibody and a light variable (VL) domain from an anti-αβ-TCR antibody switched out. Furthermore, Eshhar *et al* (25) redesigned an antibody-derived scFv into chimeric genes linked to the CD3ζ domain of the TCR. Fig. 1 shows that TCR has an antigen-binding domain, a transmembrane domain and a CD3ζ. CAR's binding domain is made up of a scFv, which contains the VL and heavy VH variable segments of a TAA-specific monoclonal antibody connected by a flexible linker. The scFv detects certain TAAs, which include proteins and glycoproteins (26). Fig. 2 shows how early CAR-T used monoclonal antibody protein-binding fragments to generate synthetic receptors, known as scFvs.

The designed structure of CAR-T. There have been five iterations of the CAR design, which have been designed mostly by altering the composition of the intracellular signaling environment of the cell, as illustrated in Fig. 3. In the genetically based production of CAR-T cells (Fig. 4), CAR-T cells are first multiplied *in vitro* before being administered into the peripheral circulation of the recipient patients.

The first generation of CARs was created by combining the scFv region of the anti-TNP antibody with a single ITAM region from CD3ξ or the Fc receptor γ-chain of the TCR. The first-generation CAR-T approach, also known as the 'T-body' technique, is used to genetically construct CAR-T cells (25). Despite the notable outcomes of first-generation CARs using *in vitro* and *in vivo* animal models or in the initial clinical trials, they demonstrated diminished cytotoxic potency and restricted persistence *in vivo* (27,28).

The second generation of CARs uses an extra-cytoplasmic signaling domain associated with the tumor necrosis factor receptor (TNFR) family, generated from co-stimulatory receptors such as CD28, CD134 (OX-40, TNFRSF4), 4-1BB (CD137, TNFRSF9) and ICOS (CD278) (29-31). These changes to the method improve T cell activation, cell proliferation *in vitro*, cytotoxic efficacy with increased persistence *in vivo* and anticancer activity (32). In addition, second-generation CAR-T cells possess the CD3ζ-activating domain, which

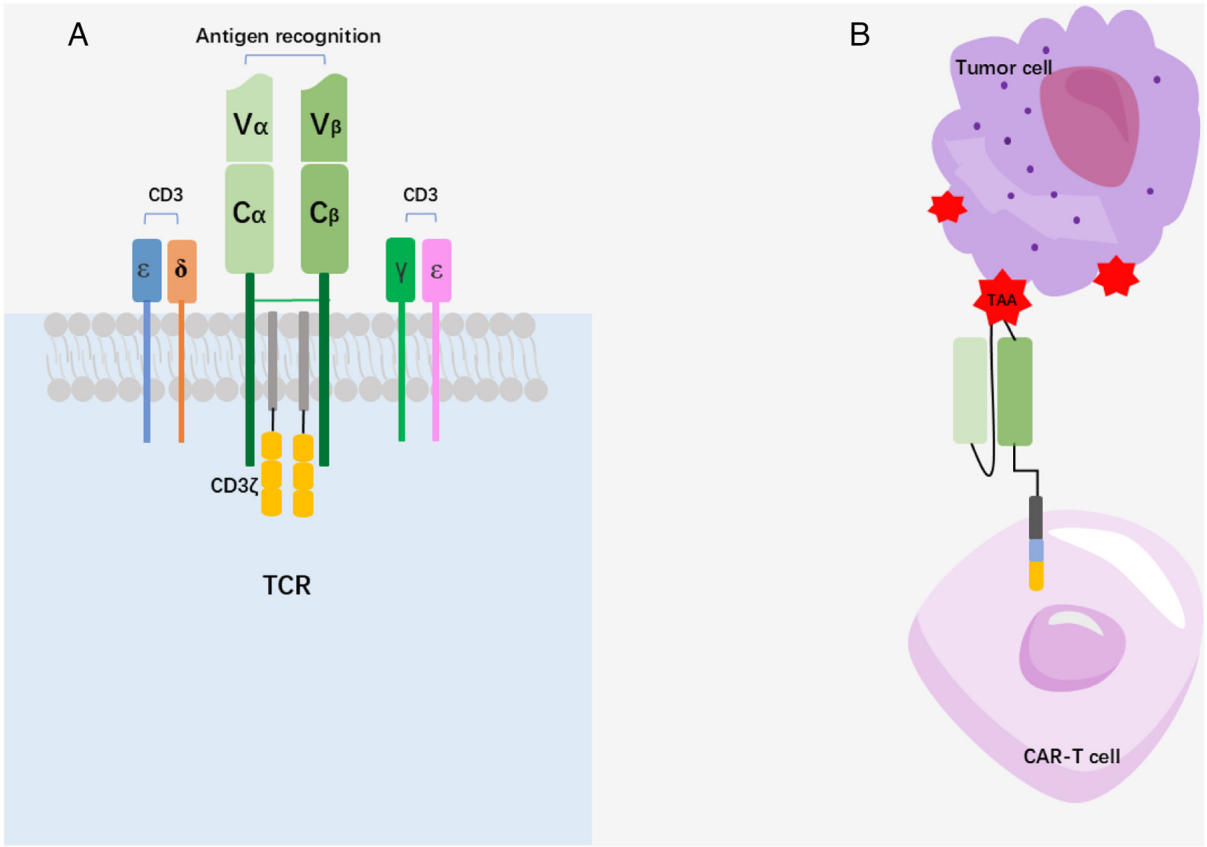

Figure 1. Graphical representation of different antibody structures. (A) The schematic figure of TCR. (B) The schematic figure of CAR-T therapy. TCR, T cell receptor; CAR-T, chimeric antigen receptor T cell; CD, cluster of differentiation.

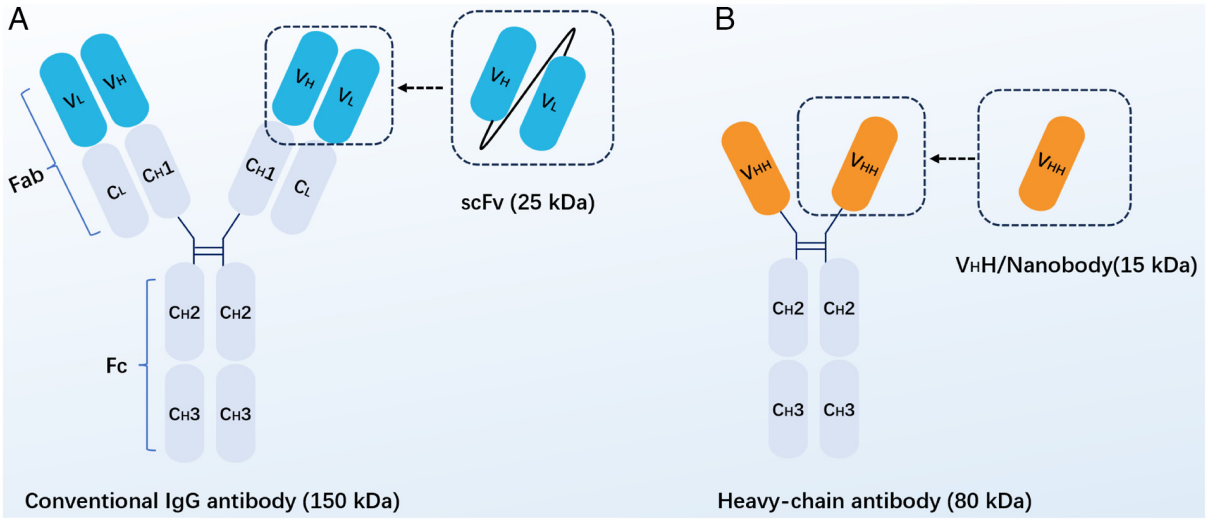

Figure 2. Two structures of antibodies are shown. (A) The scFv and a conventional IgG antibody. (B) An HcAb produced by llama, with an antigen-binding domain made up of a VHH or nanobody. HcAb, heavy-chain antibody; VHH, camelid heavy chain antibodies.

includes co-stimulatory receptors like CD28, 4-1BB and CD137 (33,34).

The third-generation CAR features more co-stimulatory motifs on the cytoplasmic side compared with the second-generation CAR, which boosts T cell activation, proliferation and malignant cell cytotoxicity (32). The cytoplasmic domains of third-generation CAR-T cells often combine CD28 with 4-1BB/CD3ξ or CD134/CD3ξ and are now being assessed in clinical trials (35-39). However, the immunosuppressive tumor microenvironment (TME) hinders T lymphocyte persistence, penetration and function, thereby hindering CAR-T cell cytotoxicity against target tumor cells (40-42).

The molecular design of fourth-generation CARs was developed by integrating components into additional T cell activation signals. For example, T cells redirected for antigen-unrestricted cytokine-initiated killing (TRUCKs) are

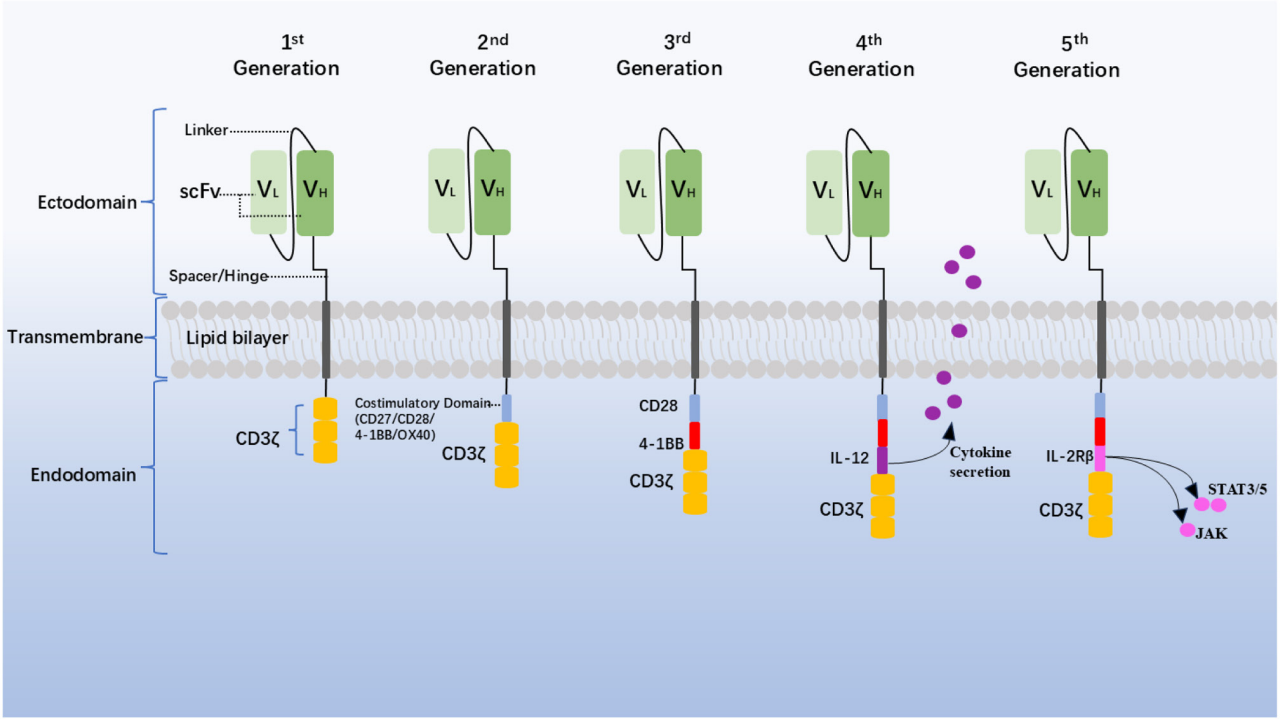

Figure 3. First-generation CARs comprised exclusively the intracellular CD3ζ signaling molecule, without a costimulatory domain. Second-generation CARs integrated the CD3ζ signal together with the costimulatory domains 4-1BB or CD28 fused with CD3ζ. Two distinct costimulatory domains, such as 4-1BB, CD28, or OX40, were added to CARs in the third generation. The fourth-generation CARs are distinguished by the incorporation of the IL-12 cytokine secretion domain, commonly referred to as TRUCKs. The fifth generation of CARs included an intracellular domain, featuring the engineering of the IL-2 receptors IL-2Rβ (β-chain) situated between 4-1BB/CD28 and CD3ζ, thereby enabling the stimulation of the JAK-STAT pathway through the IL-2Rβ domain. CAR, chimeric antigen receptor; CD, cluster of differentiation; TRUCKs, T cells redirected for antigen-unrestricted cytokine-initiated killing.

fourth-generation CAR-T cells that have the unique capacity to regulate cytotoxic activity in the absence of an antigen. These cells integrate the cytotoxic functions of CAR-T cells with the immunomodulatory potential to deliver cytokines, including IL-12, IL-7, IL-15, IL-21 and IL-23, as well as their combinations (43-49).

The fifth generation of CAR-T cells uses unique CAR designs to bind and activate the target antigen and induce cytokine signaling to achieve efficient antitumor effects with minimal off-target toxicity. Multiple CAR module designs should be improved to overcome challenges in their clinical application. For example, the iCasp9 'suicide gene' reduces the toxicity of CD19-targeted CAR-T cell therapy while maintaining its anti-tumor efficacy (50,51).

3. The antigen-binding structure of CARs

CARs typically comprise the antigen-binding fragment (Fab) of an antibody that targets surface antigens on tumor cells, together with an intracellular component that incorporates co-stimulatory signaling domains from CD3ζ along with supplementary co-stimulatory signals, including CD28 and 4-1BB. So far, the FDA has approved five CAR-T cell products for commercial use, four of which utilize scFv derived from the monoclonal antibody FMC63 and are designed to target CD19 (52). The VH and VL sections of these CARs are connected by a genetically encoded flexible linker, either (G4S)3 or (SG4)3, to produce an scFv for antigen recognition (53,54). The scFv exhibits superior tissue penetration compared with monoclonal antibodies (mAbs). The diminutive dimensions, enhanced affinity and prolonged specificity in recognizing target antigens make it a more suitable format for using antibodies in CAR-T applications and the modification of T cells (55). Nonetheless, the simple structure of the VH and VL domains in scFvs presents several constraints owing to the absence of an Fc region, which is present in a full immunoglobulin structure. In comparison to a full immunoglobulin, the scFv has similar instability and is prone to aggregation and misfolding. Therefore, scFv antigen binding stability and capacity are essential for CAR production and activity (56). Furthermore, the hydrophobic fragment of the scFv structure requires significant modification and further engineering to meet clinical safety requirements.

While the scFv can stably bind its target antigens, the molecular structure of scFvs leads to various unexpected challenges in practical applications. Fundamentally, a strong pairing linker is essential between the VH and VL domains. Products of CAR-T cells may stimulate host immune responses and, consequently, the arrangement of the linker and non-human protein structure can lead to an enhanced risk of immunogenicity, which, parallel to the generation of drug-resistant antibodies, may cause patients to develop mechanisms that enhance resistance to therapy (57). Should an antidrug reaction manifest in patients, the therapeutic efficacy of CAR-T will be reduced, ultimately leading to CAR-T cell death and treatment ineffectiveness (58). Humanized CAR-T cells improve survival, due to their decreased immunogenicity and are just as effective at cytotoxicity as murine CAR-T

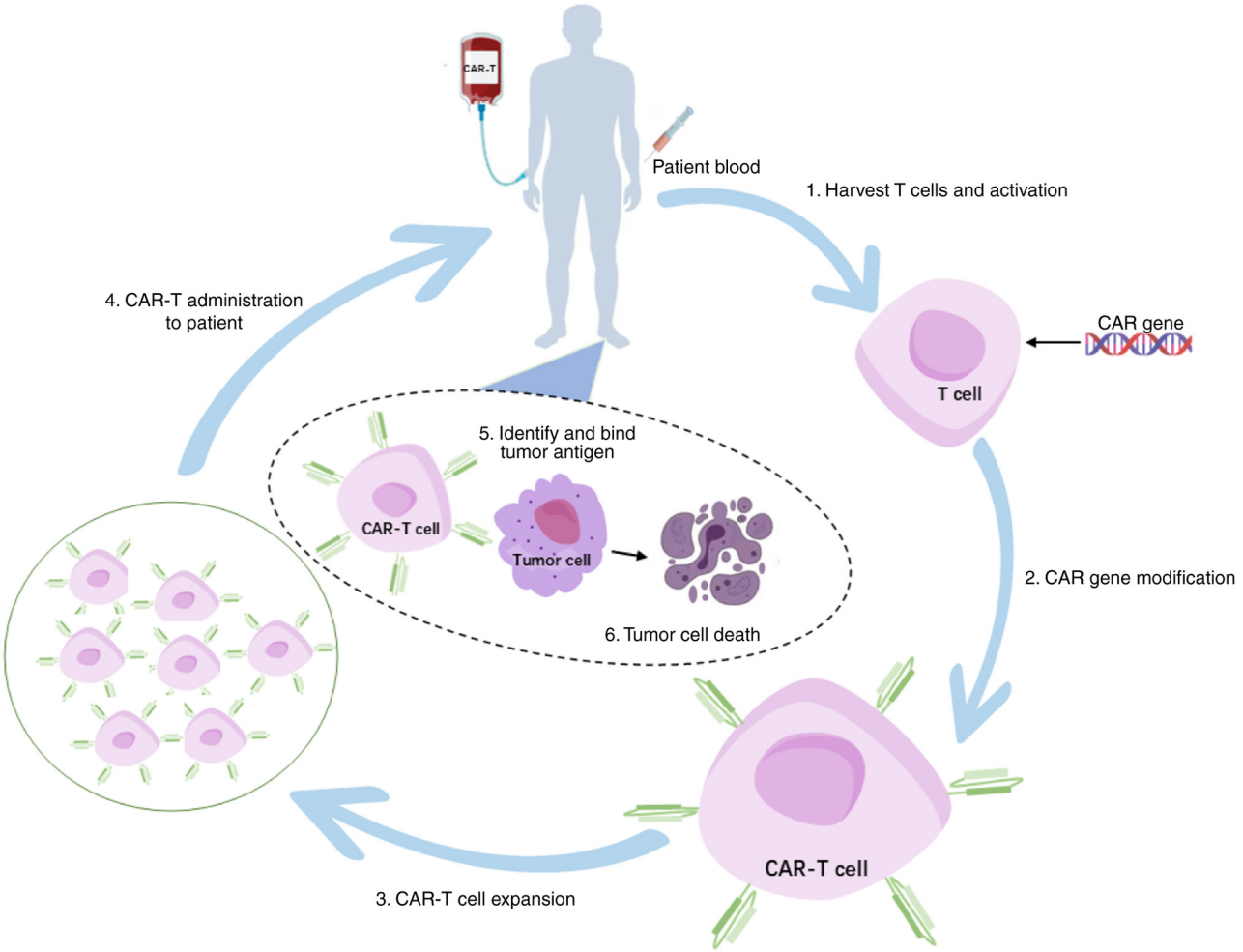

Figure 4. A brief diagram of CAR-T therapy including manufacturing and administration. CAR-T, chimeric antigen receptor T cell.

cells (59). The common occurrence of CAR-T cell loss during *in vivo* growth is attributed to the scFv format, which consists of natural dimeric variable sections linked by hydrophobic patches following their separation from constant domains (9). Prior research suggests that these hydrophobic structures are present on variable domains, increasing the propensity of recombinant scFvs for self-aggregation.

In addition, the antigen-independent self-aggregation of CAR-T cells promotes premature receptor clustering on the T cell surface, leading to tonic CD3ζ phosphorylation and subsequent cell exhaustion. The sustained activation-induced cell death resulting from this process leads to a progressive decline in functional CAR-T cell populations (60). Critically, this CAR-T cell depletion not only limits its therapeutic efficacy but also creates a permissive environment for immune escape, which is a key driver of tumor recurrence that has been observed in clinical CAR-T therapy. Moreover, some studies have shown that residual tumor cells that evade the initial CAR-T pressure often upregulate PD-L1 or downregulate the CAR-T target antigens, further exacerbating immune evasion (61-63).

On the other hand, a notable constraint is that the basic scFv structure restricts its further advancement in the use of complex CAR structures. The isolated Fv domain can only perform a function when the VH and VL domains are linked by a covalent bond, usually through a genetically programmed peptide, which leads to the formation of the scFv. scFvs were disregarded in prior research due to their instability, unfolding, domain shifting and aggregation (64). As such, simultaneously targeting two or more antigens may effectively reduce the likelihood of antigen escape variants in tumor cells. Dual targeting can be accomplished by employing two tandem antigen-binding regions to form bi-specific CARs. Two distinct tandem CARs (TanCAR) can precisely identify antigens or their epitopes. However, the production of TanCAR structures encounters significant obstacles because of the substantial inadequacy of scFv molecules, which depend on the potential right cross-pairing between VH and VL chains for both of the distinct scFvs (65). Furthermore, producing functional scFvs may be challenging because of the complexities involved in identifying the optimal linker (66). Alternatively, multiple CAR molecules can be expressed by several CAR-T cells. Triple CAR-T cells are derived from a tri-cistronic vector, as indicated by the relevant studies (67,68). This expanded alteration of T cells necessitates larger transgene inserts, which, in turn, hinders viral packaging and transduction effectiveness. In addition, multiple scFvs possess enlarged hydrophobic patches that encourage T cell surface receptor aggregation, which may result in overexpression of the CAR gene during dynamic domain swapping (69). Ultimately, scFv clustering

or misfolding may result from the poor folding stability of the VH and VL domains, as well as the exposure of hydrophobic components in the framework to solvents (70). Furthermore, the clustering of CARs can result from interactions within the scFv framework and the binding affinity affects the target's tonic excessive signaling, resulting in T cell exhaustion in the early treatment stage (71).

Recently, antigen-binding alternatives to scFvs have emerged, including nanobodies. The integration of nanobodies in CAR-T constructions may mitigate the previously noted limitations, which include antigen escape, off-tumor toxicity and immunogenicity. Hamers-Casterman *et al* (72) identified heavy-chain-only antibodies (HcAbs) are the source of the nanobody, or VHH, in 1993. Subsequently, it was also described by Camelidae (camel, llama, alpaca) and Sharks (73,74). A nanobody is a small antibody fragment, between ~12 and 15 kDa, which constitutes nearly half the size of a scFv (~25 kDa). These nanobodies are constructed with a pair of heavy chains lacking the constant heavy 1 (CH1) domain, while also expressing normal VH-only antibodies, including the CH2 and CH3 domains. Consequently, these HcAbs primarily rely on the variable domain, utilizing the DNA encoded within the VHH for sequencing. The nanobody functions by attaching and interacting with its target antigen without the VL or constant domains, utilizing the remaining complementarity-determining region 3, which is analogous to IgG. According to these characteristics, nanobodies can reach concealed and cryptic epitopes that are inaccessible to larger mAbs (75). Conversely, there exists a degree of comparison between nanobodies and traditional monoclonal antibodies. Nanobodies exhibit superior performance in four key aspects: Binding capacity, stability, penetrability and specificity. Notably, nanobodies exhibit robust structural integrity even when subjected to thermal stress (>60˚C), mechanical pressure (up to 500 MPa), acidic environments (pH<3) and protease-rich milieu (76).

Furthermore, besides the biochemical and functional properties, high binding affinity and adequate immunogenicity and stability of nanobodies, the variable domain VHH is cloned with minimal complexity and expressed easily while retaining its affinity capabilities (77). The sequence of the human VH gene family III, together with VH size, exhibits significant similarity, resulting in extensive applicability in patients. The immunogenicity of VHH is less than that of non-human antibodies due to the absence of the Fc domain and its optimal length. Furthermore, nanobodies necessitate little adjustment to their immunogenicity, in particular due to their short half-life, hence facilitating the humanization process (78). In addition, humanized or camelid VHH domains, or single motifs of human antibodies, can be engineered to exhibit binding diversity and selectivity akin to full VH and VL dual-domain antibodies, enhancing their use in CAR designs for tumor targeting (79).

Meanwhile, antibody variable domains exhibit significant variability due to their inherent dynamic stability and solubility, compared with conventional antibodies that include both VH and VL chains, while the VHH lacks light chains and CH1 domains. scFvs exhibit reciprocal interactions among variable domains and constant domains, in addition to their exposure to hydrophobic regions, due to their derivation from monoclonal antibodies, which imposes constraints on future engineering endeavors (80). The advantageous characteristics of the VHH, including diminutive size, exceptional stability, robust antigen-binding affinity, solubility and natural origin, render it appropriate for diverse CAR-T applications. There is a growing exploration of nanobody-based CAR constructs (81,82).

4. Nanobody-based CAR designs

The rising demand for candidate CARs to address therapeutic requirements has shifted the focus to nanobodies, which have been recognized for their notable properties and benefits in CAR-T treatments. Table I illustrates the structure of a synthesized nanobody.

Immunoglobulin superfamily members. CD38 is a membrane protein characterized by a single transmembrane region and a 256-amino acid catalytic carboxyl motif. The membrane protein CD38 is part of an evolutionarily conserved enzyme family and is expressed in several organs, particularly in mature immune cells. CD38 is notably appealing as a therapeutic target since multiple myeloma and other malignant tumors have exceptionally high levels of CD38 expression (83). CD38-specific conventional mAbs, including daratumumab, isatuximab and MOR202, were recently introduced in clinical settings to treat multiple myeloma (84,85). A set of recombinant C-terminal CD38-specific nanobodies was extracted from vaccinated llamas and which recognized CD38 at three distinct epitopes (86). The target domain comprises a nanobody against CD38, the CD8α transmembrane domain, the 4-1BB co-stimulatory domain and the CD3ζ-activating domain, to produce CD38 CAR-T cells, which efficiently lysed CD38-positive cells *in vitro*. Additionally, mice with non-obese diabetes or severe combined immunodeficiency that received subcutaneous injections of RPMI 8226 cells were found to benefit from the ability of the CD38 CAR-T cells to efficiently inhibit tumor growth (87). In addition, a separate study focused on targeting three distinct, non-overlapping alternative CD38 epitopes, resulting in the creation of humanized WF211, MU1067 and JK36 nanobodies (88). Using CRISPR/Cas9 technology, these nanobodies were transduced into NK-92 cells, which is an engineered human NK cell line that stably co-expresses GFP and CD16. The resulting CD38-directed CAR-NK cells demonstrated specific, effective and powerful cytotoxicity, as evidenced by their effects on multiple myeloma and Burkitt lymphoma cells taken from eight patients (89).

The checkpoint inhibitor protein programmed death-ligand 1 (PD-L1) is highly expressed in a number of cancers and immune cells, serving as a vital and complex regulator in the therapeutic application of CAR-T treatment (90). As such, B3 and A12 VHHs have been produced by immunizing an alpaca with the purified ectodomain of mouse PD-L1 and both exhibited binding to PD-L1 with overlapping epitopes (91). In addition, the second-generation CAR utilizing binder A12 was found to kill PD-L1-positive tumor cells in a dose-dependent way. Further to this, an anti-PD-L1 nanobody was extracted from a partially synthetic naïve shark V_{NAR} phage library (92). Following the incorporation of the B2 VNAR fragment as a cross-reactive binder for PD-L1 and PD-1, B2-based CAR-T

Table I. Nanobody-enhanced CAR-T cells.

Authors, year	Target	CAR generation	CAR structure				Functional type	(Refs.)
			Spacer	Transmembrane	Costimulatory	Signaling		
An *et al*, 2018	CD38	2nd	CD8	CD8α	4-1BB- CD3ζ	CD3ζ	Monospecific	(87)
Li *et al*, 2022	PD-L1	4th	IgG3	CD8α-hinge	4-1BB- CD3ζ	CD3ζ	Bivalent bispecific	(92)
Wang *et al*, 2021	CD20	2nd	IgG4 (LC)	CD8	4-1BB, P2A	4-1BB, CD3ζ	Bivalent bispecific	(96)
Schubert *et al*, 2023	CD19	3rd	IgG1	CH2-CH3	CD28-4-1BB-CD3ζ	CD3ζ	Bivalent bispecific	(171)
Ren *et al*, 2023	BCMA	4th	IgG1	CD28 CD28-hinge	CD28 CD28-IL5	CD3ζ	Bivalent bispecific	(172)
Hajari *et al*, 2019	VEGF 2	2nd	IgG1-Fc	CD28	CD28	CD3ζ	Bivalent monospecific	(110)
Sadeghi *et al*, 2020	VEGF	2nd	IgG2c	CD28	CD28	CD3ζ	Monospecific	(173)
Jamnani *et al*, 2014. Budi *et al*, 2022	HER	3rd	IgG3-Fc	CD28	CD28-CD3ζ or CD28-OX40-CD3ζ	CD3ζ	Oligoclonal	(112,174)
Sharifzadeh *et al*, 2013	TAG-72	3rd	IgG3-Fc	CD28	CD28-ζ or CD28-OX40-ζ	CD3ζ	Oligoclonal	(111)
McComb *et al*, 2022	EGFR	2nd	IgG1 Fc	CD28-hinge	CD28-CD3ζ or 4-1BB-CD3ζ	CD3ζ	Bivalent bispecific	(115)
You *et al*, 2016	MUC1	4th	IgD	CD28	4-1BB-CD3ζ, 4-1BB-CD3ζ-IRES-IL2	CD3ζ	Bivalent bispecific	(118)
Khaleghi *et al*, 2012		3rd	IgG3-Fc	CH2-CH3-hinge CH2-CH3-hinge-hinge CH2-CH3-hinge CH2-CH3-hinge-hinge	CD28 OX40-CD28 OX40-CD28	CD3ζ	Monospecific	(136)
Li *et al*, 2022	GPC 3	2nd	CD8 hinge	CD8α-hinge	4-1BB- CD3ζ	CD3ζ	Bivalent bispecific	(175)
Heitzeneder *et al*, 2022	GPC 2	2nd	CD8α	CD8α-hinge	CD28-4-1BB-CD3ζ	CD3ζ	Trivalent tri-specific	(176)
Xie *et al*, 2019	EIIIB	2nd	CD8	CD8	CD28	CD3ζ	Monospecific c	(130)
Hassani *et al*, 2020	PSMA	2nd	IgG1-hinge	CD28	CD28	CD28-ζ	Monospecific	(134)

CAR, chimeric antigen receptor; CD, cluster of differentiation; PD-L1, programmed death-ligand 1; BCMA, B-cell maturation antigen; VEGF, vascular endothelial growth factor; HER, human epidermal growth factor receptor; TAG, tumor-associated glycoprotein; EGFR, epidermal growth factor receptor; MUC, mucin; GPC, glypican; EIIIB, extra domain IIIB; PSMA, prostate-specific membrane antigen.

cells were found to effectively suppress tumor development in breast and liver cancer models.

The polytopic transmembrane protein CD20 has multiple membrane-spanning domains, which present substantial technical difficulties owing to its intricate structural organization. The extracellular domains of CD20 exhibit suboptimal folding attributed to the brevity of the hinge region. In pursuit of a long hinge CD20, researchers used an expression DNA

plasmid that encodes the complete CD20 for the purposes of immunizing a llama. Subsequently, anti-CD20 nanoCARs demonstrated strong CD20 expression and effectively killed $CD20^+$ B-cell lymphomas, including the RL and Raji cell lines. This confirms the potent cytotoxicity of anti-CD20 nanoCARs against non-Hodgkin lymphoma targets. Subsequently, a xenograft mouse model was created through the subcutaneous injection of RL cells. The engrafted mice then received intravenous injections of CD20 nanoCAR-T cells. In this model, the administration of CAR-T cells resulted in the complete eradication of subcutaneous RL tumors in under 20 days, in comparison to the PBS-injected control mice, greatly extending the lifespan of the mice (93). Moreover, Liu *et al* (94) aimed to develop bispecific single-domain antibodies against CD20 and CD3 in order to demonstrate their strong antitumor effect in killing B-cell lymphoma cells *in vitro*.

CD19 is a transmembrane glycoprotein antigen associated with the superfamily of immunoglobulins which represents a compelling therapeutic target. It is expressed by both healthy and malignant B cells, in addition to dendritic cells. In a research context, the isolation of the CD19 antigen occurred after the immunization of a camel. Following this, the vaccinated PBMCs were employed to establish a nanobody gene phage display system, facilitating the selection of specific nanobodies targeting CD19 on human B cells through phage display technology. The nanobody binder for CD19 exhibited a binding affinity characterized by a value ranging from 15 to 33 nM. One particular study identified CD19 and CD20 nanobodies from a naturally occurring nanobody-expressing phage display library, subsequently constructed CD19-, CD20- and bispecific nanobody CAR-T cells (95). These nanobody CAR T cells demonstrated a remarkable capacity to selectively identify and eliminate tumor cell lines expressing CD19 and CD20, such as Raji or Daudi cells (96). Furthermore, the lethal effects of nanobody CAR T cells have been verified using primary patient-derived (PD) malignant cells from ALL (PD-ALL), which exhibited CD19 expression and ~20% CD20 expression. The PD-ALL cells then underwent incubation with nanobody CAR-T cells for five days, leading to the lysis of the PD-ALL cells and the subsequent secretion of IL-2 (96). A diverse array of CD19 nanobodies has been developed, exhibiting significant therapeutic effectiveness in the field of CD19-expressing B cell tumors (33,97).

B-cell maturation antigen (BCMA) is a member of the tumor necrosis factor family of proteins, which is mainly produced by both healthy and cancerous plasma cells, in addition to certain mature B cells (98). Two distinct types of nanobodies, namely monovalent anti-BCMA nanobodies and bivalent nanobodies that recognize BCMA, are in the process of development and have exhibited significant therapeutic response rates in instances of recurrence or refractory multiple myeloma. One particular study demonstrated the successful engineering of a monovalent anti-BCMA nanobody, which integrates the human CD8α signal peptide and a BCMA nanobody, along with the 4-1BB and CD3ζ intracellular domains (99). This alternatively engineered anti-BCMA CAR was developed with a bispecific BM38 CAR, integrating anti-BCMA and anti-CD38 nanobodies in a bi-epitopic configuration (100). This has been approved to treat B-cell malignancies with T lymphocytes that carry CARs specific to CD19 and BCMA. In order to ensure stable expression of BCMA, Wei *et al* (101) targeted G protein-coupled receptor C5 family member D (GPRC5D) to form anti-BCMA/GPRC5D bispecific CAR-T cells and injected these CAR-T into relapsed/refractory multiple myeloma (R/R MM) patients with extramedullary disease who participated in a clinical trial of treatment for 3 months. Although the patient experienced four CAR-T cell expansions and developed Grade 3 CRS, the symptoms were well-controlled and the treatment was generally safe (101). The objectives and associated nanobody-based CARs underscore the prospective applications of these CARs, as demonstrated by cellular tests and mouse models (Table I).

Vascular endothelial growth factor (VEGF) family. Angiogenesis and tumor metastasis rely on VEGFs and their receptors (VEGFRs). Numerous VEGF- and VEGFR-targeted CAR-T cells have been engineered to suppress tumor proliferation, with the former functioning through the latter. VEGFR2 is well recognized to bind to fetal liver kinase-1 in mice and the orthologous kinase-insert domain receptor in humans. VEGFR2 is highly expressed in malignant endothelial cells and represents a promising particular target for anti-angiogenesis therapy (102). Various anti-angiogenic therapeutics and nanomedicines targeting VEGF or VEGFR have received market approval, yet certain undesirable effects persist in T cell antitumor treatments. For instance, VEGF possesses immunosuppressive properties, which include hindering Tregs and M2-like tumor-associated macrophage populations within the TME while augmenting T lymphocyte responses, dendritic cell maturation and facilitating effective T cell infiltration. The regulation of cellular immunity promotes angiogenesis and tissue healing in solid tumors (103,104). Additionally, there are encouraging outcomes from studies on VEGFR2 CAR-T cell therapies, which indicate that a uniform standardized dosage of VEGFR2 CAR-engineered T cells, administered alongside IL-2, can markedly suppress the proliferation of five distinct tumor types in experimental murine models, by targeting solid tumor vasculature (105). Furthermore, another relevant clinical investigation assessed the medicinal application of anti-VEGFR2 CAR-T cells in patients with solid tumors, although the findings on their responses were not entirely cohesive (106). In this study, not all participants exhibited favorable results, thus emphasizing the necessity for meticulously structured clinical trials using anti-VEGFR2 CAR-T cell therapy to reduce toxicities and explore the intricate mechanisms of CAR-T therapy within various tumor types (107).

VEGFR2-specific nanobodies have been extracted from the commercial humanized camelid single-domain antibody library HuSdLTM (Creative Biolabs), for example, the nanobody NTV1 selectively binds to VEGFR-2 D3. This recombinant nanobody was shown to be ~57 kDa, as assessed by surface plasmon resonance. NTV1 is an anti-VEGFR2 D3 candidate targeting human breast cancer cells. It was shown to almost completely reduce the development of capillary-like structures in human umbilical vein endothelial cells by blocking VEGFR2 activity (108). Based on this evidence, NTV1 provides an innovative strategy using nanobody-based therapeutic applications. In addition, CAR-modified T cells showed >50% surface expression of VEGFR2 in the context of breast cancers (109) and they produce IL-2 and IFN-γ,

thereby having a damaging effect on solid tumors that express VEGFR2 (110).

Tumor-associated glycoproteins. The tumor-associated glycoprotein-72 (TAG-72) antigen is a mucin-like transmembrane complex typically regarded as a biomarker indicative of poor prognosis in adenocarcinomas and is also a recognized target for selection. HER2 is a member of the human epidermal growth factor receptor family 1-4, which is highly expressed in individuals with breast or gastric cancer, thereby serving as an optimal target for antigen-specific guided CAR-T cells in these diseases. TAG-72 and HER2 nanobodies have been integrated into the second and third-generation CAR-Ts, respectively (111,112). A collection of 13 individual domains extracted from an immunized camel has been shown to produce TAG-specific CARs that activate against tumor cells expressing TAG-72 (LS-174T cells). The anti-TAG-72 CAR was engineered using a mixture of 13 VHHs that effectively activate in reaction to TAG-72-positive malignant cells, specifically LS-174T or MCF7 cells. These anti-TAG-72 CAR-T cells were shown to release IL-2 and also lyse cancerous cells. Following this, five oligoclonal HER2 VHHs were chosen from an immunological camel library to be incorporated into either the CD28-CD3ξ or CD28-OX40-CD3ξ signaling domains. As such, future studies on canine bladder cancer should take into account a research prediction analysis of the TAG72 and HER2 (113).

An attractive target for CAR-T therapy is epidermal growth factor receptor (EGFR), which is overexpressed in a number of solid tumors but expressed at lower levels in healthy tissues (114). McComb *et al* (115) used several camelid EGFR nanobodies as targets for CARs with high on-target activity against human EGFR *in vitro* and *in vivo*. As an approach, they gradually reduced the human CD8 hinge, a spacer region in several CAR constructs, to reduce their potent antigenic sensitivity. They proved that hinge-truncation can effectively reduce CAR antigenic sensitivity for epitopes near the target cell membrane. This approach could improve CAR selectivity for tumor-associated antigens and enable more complicated multi-antigen targeting CAR therapies. Hosking *et al* (116) used a human-derived HER2 targeting CAR with multiplex editing to overcome barriers and maximize efficacy in solid tumors. They used HER2-targeting CAR-T cells with multiplex editing to overcome barriers and maximize efficacy in solid tumors. By designing IL-7R-fusion, TGF-β-IL-18R and CXCR2, HER2-based CAR-Ts were able to persist and migrate to tumors.

Mucin A (MUC1) is a protein that can cross the cell membrane and is involved in immune system modulation, signal transduction and protecting mucous membranes. MUC1 can be selectively targeted by CAR-T cells without off-tumor effects, owing to its hypoglycosylated properties. In fact, the initial form of CAR-designed T cells incorporated a nanobody that uses anti-MUC1 VHH as the target attachment region (117). This restructured chimeric receptor design was integrated with human IgG3 or IgG3-Fc as the spacer, along with CD28 and CD3ζ for T cell activation purposes. Subsequently, CAR-transduced Jurkat cells had augmented proliferation after their co-cultivation with MUC1-positive MCF7 cancerous cells. In addition, these Jurkat cells demonstrated enhanced capabilities in IL-2 secretion and tumor cell lysis, despite the use of distinct hinges (IgG3-Fc and IgG3-Fc-hinge) within the CAR construct. However, this study demonstrated that using the FcγRII hinge in the CAR resulted in a distinct reduction in CAR production and IL-2 production (117). A separate study developed two anti-MUC1 CAR-T cell lines. The construct used SM3-CAR comprises an SM3 scFv that targets the MUC1 antigen and facilitates IL-2 secretion. This alternative CAR-T cell line, featuring the modified SM3 scFv (pSM3-CAR), displayed enhanced binding affinity to MUC1 and exhibited no IL-2 expression (118). Furthermore, to treat cholangiocarcinoma, researchers produced anti-MUC1-CAR T cells with PD-1-CD28 switch receptors that target MUC1 and activate the inhibitory signal of the PD-1/PD-L1 interaction (119).

With a molecular mass of 67 kDa, the PhiC31 integrase belongs to the resolvase/invertase family. The PhiC31 integrase system, derived from the phage of the *Streptomyces* bacteria, has the potential to enhance CAR expression efficiency (120). One study (121) employed the incorporation of donor plasmid (pCMV-γc-attB) and helper plasmid (pCAGGS-phiC31) DNA into targeted sites within mammalian genomes, leading to sustained and stable transgenic expression over time. The manifestation of anti-MUC1 on the surface of Jurkat cells was assessed using flow cytometry from 1-30 days post-transfection. In the initial phases, there was minimal distinction upon utilizing the PhiC31 integrase system. In the later stages, the expression of the anti-MUC1 CAR exceeded 50%. On day 30, the expression of the CAR was barely discernible in the control group. This suggested that the PhiC31 integrase system achieved optimal transfection efficiency and transgene stability. Rath *et al* (121) demonstrated strong expression of transgenes in stem cells and utilized recombinase-mediated cassette exchange at safe harbor loci. As such, the choice of recombinase enzyme is an important factor in ensuring the optimal efficiency and accuracy of the integration events.

Glypicans, including glypican-3 (GPC3), heparan sulfate proteoglycan (a biomarker for hepatocellular carcinoma) and glypican-2 (GPC2) (a related proteoglycan associated with neuroblastoma), are crucial in developmental morphogenesis and serve as modulators of the Wnt signaling pathway (122). For example, a llama VH single-domain antibody library was developed by screening llama-derived heavy chain antibody library against a target peptide antigen, followed by lead candidate production, purification and characterization by sdAb (123). The aforementioned operational procedures were conducted using recombinant antibody technology, yielding a phage-displayed library with specificities for target antigens separated through panning, including coating, blocking, binding, washing, elution and amplification (123). Using a comparable library, anti-GPC3 VH single-domain antibodies were identified through the phage display library and the leading nanobody demonstrated a specific binding capacity to a his-GPC3 antigen at a ratio of 1:3,000. In addition, Zhou *et al* (124) created bispecific CAR-T cells that target both FAP and GPC3 as a means of addressing tumor heterogeneity in HCC.

GPC2 represents an additional entity within the glypican family, characterized by a human single-domain demonstrating a high binding affinity (125). The CARs targeting

GCP2 were obtained from eight different individual volunteers. In the study, the efficacy of GPC2-specific CAR-T cells regarding their cytotoxic capabilities was assessed. With a ratio of effectors to targets of 8:1, these cells exhibited lytic efficacy against IMR5 GPC2-positive neuroblastoma cells, with a mean of 56% (range 44-71). Moreover, GPC2 CAR-T cells have been demonstrated to have a significant capacity for inhibiting tumor growth in neuroblastoma models.

Other specific biomarkers. Fibronectin Extra Domain IIIB (*EIIIB)* is a splice variant of fibronectin that is highly expressed in tumor cells and tumor-associated neovasculature, with negligible expression in normal tissues (126,127). A number of aggressive tumors are susceptible to CAR-T cell targeting because they rely on the extracellular matrix (ECM) and angiogenesis for nutrient sustenance. It is therefore relevant that EIIIB antigens, which are rarely found in healthy adult tissues, are prevalent in the tumor ECM and newly created blood vessels (128). NJB2 is a camelid-derived VHH antibody developed to specifically target the EIIIB isoform, which is a tumor-restricted epitope of fibronectin that results from alternative splicing in malignant tissues. As such, the use of the nanobody NJB2 was used to produce B2 CAR-T cells and facilitate the transduction of B2 CARs in order to demonstrate their specificity and cytotoxicity *in vitro*. Following this, B16 melanoma tumor-positive mice were administered with B2 CAR-T cells, then demonstrating mitigated tumor progression and an increase in the percentage of surviving mice (129). However, further assessment of the efficacy of these CAR-T cells in an MC38 colon cancer model found a decreased expression level of EIIIB, relative to B16 tumors. Markedly, NJB2 was found to act efficiently with fewer adverse effects in solid tumor animal models that lack innate adaptive immunity and when targeting the tumor microenvironment and treating (130).

Prostate-specific membrane antigen (PSMA), a type II transmembrane glycoprotein with folate hydrolase activity, is a well-known cell surface target in the treatment of prostate cancer. PSMA belongs to the Thy-1/Ly-6 family, which is markedly associated with poor-prognosis malignancies and has been investigated as both a biomarker for the activity of the disease and a particular therapeutic target that has gathered considerable interest in drug research (131). The binding capability of nanobodies to target antigens is categorized based on their affinities, whereby dissociation constants (K_D) ≤10 nM indicate high affinity, 10-100 nM represent moderate affinity and ≥100 nM is classified as low affinity. Another nanobody, designated JVZ-007, was isolated by immunizing a llama using four patient prostate carcinoma cell lines, demonstrating a K_D of ~27.4 nM (132). First-generation CARs with tumor cells expressing PSMA were found to promote the production of IL2 and INF-γ while increasing the levels of CD69 (133). Subsequently, the second-generation NBPII-CAR, derived from an anti-PSMA nanobody, was expressed in transfected Jurkat cells and the cells were activated by targeting PSMA on tumor cells. In addition, NBPII-CAR was engineered with a complex comprising ζ activating components, the CD28 transmembrane domain and a nanobody linked by a spacer and it was found to enhance IL-2 cytokine synthesis, CD25 expression and cellular proliferation (134). This novel anti-PSMA CAR may provide enhanced opportunities for PSMA-targeted immunotherapy for the treatment of patients with prostate cancer (135).

5. Nanobodies in optimized CAR

The utilization of scFvs in CAR-T cell design may inadvertently lead to excessive tonic signaling independent of their target antigens, ultimately culminating in the premature exhaustion of T cells, even though they are capable of promoting receptor clustering on T cell surfaces and inducing strong signaling activation (136). By contrast, as illustrated in Fig. 5, nanobodies emerge as a compelling and advantageous candidate for the construction of optimized and multi-generation CARs, offering a potential solution to mitigate these issues with tonic signaling, while enhancing the durability and efficacy of CAR-T cell therapies overall. To improve the ability of CAR-T cells to proliferate and fewer unanticipated adverse effects, researchers have endeavored to implement a caspase-8-induced suicide gene system switch by incorporating relevant protein molecules within *in vitro* assays (136).

Bispecific NanoCARs face the challenge of aggregation associated with two VHH-based CARs. To address the challenge posed by tumor cell antigen escape variants resulting from the absence of target epitopes, bispecific CAR-T cells have been posited to circumvent this obstacle. These more sophisticated CAR-T cells integrate the VHH antigen-binding domains while omitting light chains, potentially reducing the probability of domain interchange during the concurrent expression of numerous nanobodies (137). For example, nanobodies that selectively target CD20 and HER2 can be combined to create a bispecific CAR-T cell, demonstrating comparable efficacy in eliminating tumor cells that express CD20, HER2, or both antigens. This design presents an opportunity to avert antigen escape and circumvent tumor resistance, despite the absence of functional tests *in vitro* and clinical data (138). In one study (139), a bispecific CAR-T was developed using CD30 and CD5 nanobodies derived from an alpaca nanobody phage library. The authors presented potential bispecific CAR-T cell therapy approaches for B-cell lymphoma treatment, effectively avoiding antigen escape. As such, the authors illustrated that nanobody-derived bispecific CAR-T cells exhibit a marked enhancement in tumor-fighting efficacy when compared with both single-target and bispecific scFv-derived CAR-T cells.

Furthermore, TandemCAR encodes numerous scFvs, leading to possible cross-pairing across the various VL and VH chains of the scFvs. Nanobodies have demonstrated benefits and practicality over scFvs in bispecific CAR-T applications. For example, LCAR-B38M CAR-T cells, equipped with two VHHs, specifically target distinct epitopes located in the extracellular region of BCMA, as manufactured by Nanjing Legend Biotech Corporation. The LCAR-B38M CAR-T demonstrated efficacy in Phase II/III clinical trials (140).

The NanoCAR technique, using VHH-based CARs, produces CAR-T cells equipped with two VHHs that can concurrently activate two tumor-eradicating pathways (138). Most publications concerning the NanoCAR-T format are confined to preclinical research due to the aggregation issue associated with scFv-based CARs, which is known to result

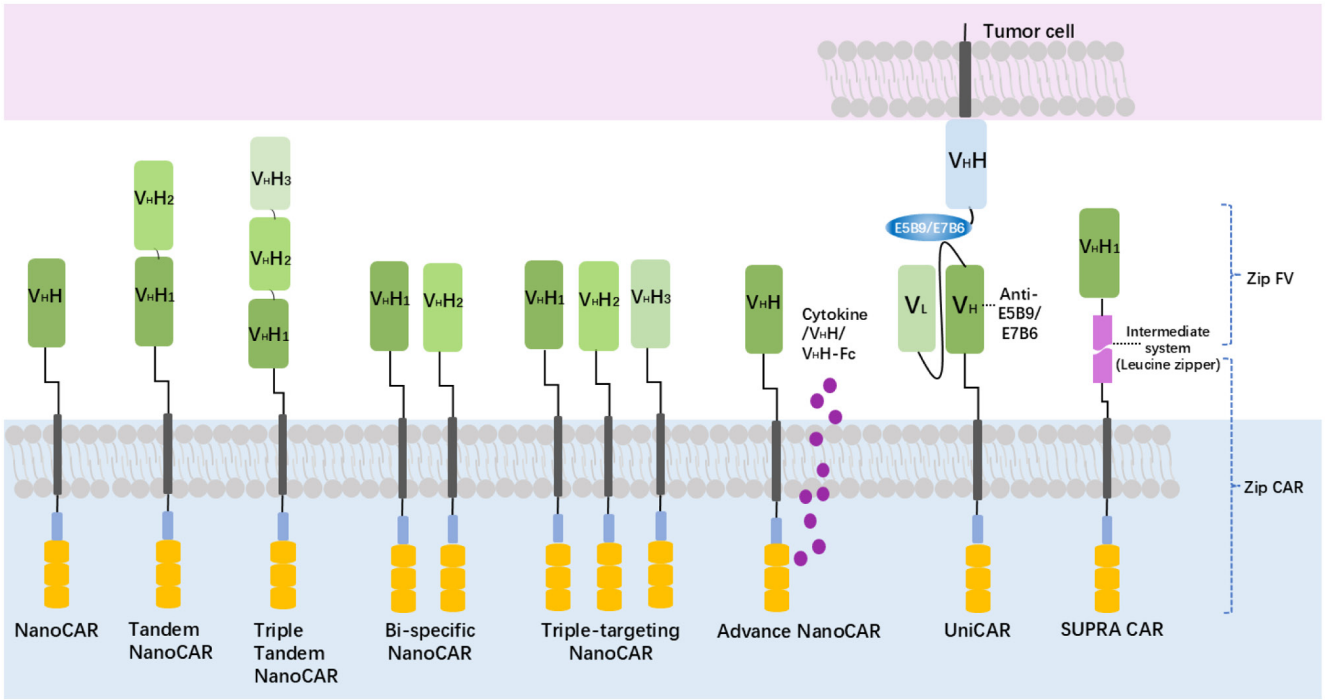

Figure 5. The schematic representation of VHH-based advanced CARs includes nanoCAR, bi-epitopic/specific/tandem CAR, cytokine-secreting CAR, SUPRA CAR and the E5B9-tagged tumor-specific target module, which facilitates the interaction between tumor cells and UniCAR via the anti-E5B9/E7B6 region. VHH, camelid heavy chain antibodies CAR, chimeric antigen receptor; SUPRA, split universal programmable.

in potential immunogenicity and unpredictability, therefore, investigating the a number of different forms of antibody release warrants greater focus (141). In one study (142), the engineered CAR-T cell structure was designed with a sophisticated dual-functional mechanism. This included an integrated release mechanism for anti-CD47 VHHs and an anti-PD-L1 inhibitory pathway-targeting mechanism. To evaluate the functional efficacy of this innovative CAR-T cell design, a killing assay was conducted using PD-L1-positive B16F10 cells. The results demonstrated that there were no statistically significant differences in either the killing activity or IFNγ production between CAR-T cells armed solely with the anti-PD-L1 mechanism and those co-expressing the anti-PD-L1 mechanism alongside the secreted anti-CD47 VHHs. B16F10 was injected into C57BL/6 PD-L1 knockout mice to assess its tumor-fighting efficacy on day one of the experiment, followed by therapy with varying quantities of anti-PD-L1 CAR-T cells on days 2, 6 and 12 (143). The cells that secrete anti-PD-L1 and target CD47 showed superior efficacy in preventing tumor growth and extending the life span of the mice, in contrast to anti-PD-L1 alone. Furthermore, the authors engineered EIIIB-target CAR-T cells that expressed anti-CD47 VHH to assess the *in vivo* production of anti-CD47 in a tumor model using C57BL/6 WT mice, which exhibited prolonged life compared with unmodified anti-EIIIB CAR-T cells. The results of this research confirmed that anti-CD47-expressing anti-PD-L1 CAR-T cells have enhanced effectiveness in combating cancer and can extend life expectancy via epitope spreading (143). Furthermore, two types of nanoCAR-T cells have undergone phase I clinical trials, BCMA-specific NanoCAR (NCT03664661) and CD19- or CD20-bispecific NanoCAR (NCT03881761).

By addressing the problem of cytokine release and precisely regulating cell activity to mitigate on-target and off-target undesirable effects, UniCAR, which is a switchable universal modular platform, has been designed to improve the safety and effectiveness of CARs (144). UniCAR consists of two components, UniCAR-T cells and an interchangeable tumor-specific targeting module. This UniCAR structure comprises an extracellular interaction portion originating from the antibody structure, a membrane region and an intracellular activation region featuring CD28 co-stimulatory and CD3ζ signaling components (145). In contrast to traditional CAR-T cells, the extracellular binding domain of the UniCAR fails to recognize a specific TAA on the target cells, but rather selectively binds to a shorter peptide, E5B6. At its C-terminal, this construct serves as a crucial connection between the α-EGFR-EGFR target module on tumor cells and has been demonstrated to guide UniCAR T lymphocytes to EGFR-expressing malignant cells both *in vitro* and *in vivo* (146). The authors of this study created a bivalent anti-EGFR antibody by fusing two α-EGFR nanobody domains using an E5B6 tag, which activates tumor cells when the UniCAR T cells are equipped with it (147). The E5B6 tag was incorporated into a lentiviral construct, which was then transduced into human primary T lymphocytes. In tumor-bearing murine models, the activation or deactivation of the UniCAR system occurs upon the fusion of the tumor-specific nanobody with the E5B6 tag, contingent upon the disruption of α-EGFR-EGFR (147). Whether UniCAR T cells can eradicate target-positive cells depends on the level of expression of EGFR by tumor cells and the binding ability of a bivalent EGFR-specific tumor marker.

6. Recent advances in various anti-BCMA CAR-T cells

Nanobody-based CAR-T cells have proven to be viable and promising for the advancement of immunotherapy. One monovalent anti-BCMA nanobody-based CAR-T cell, designated PRG1801, comprises CD8α, 4-1BB and CD3ζ and has been developed by Lu Han *et al* to target BCMA (99). At present, idecabtagene vicleucel (ide-cel or bb2121) and ciltacabtagene autoleucel (cilta-cel) are anti-BCMA CAR-T cell treatments developed by Memorial Sloan Kettering and Janssen (148,149), both of which obtained authorization for their clinical use from the US FDA in 2021 and 2022, respectively. Ide-cel consists of a murine anti-BCMA scFv split, which includes a 4-1BB co-stimulatory region, a CD3ζ signaling region, a CD8α hinge and a membrane region. The therapeutic effects of cilta-cel are analogous to those of ide-cel, which is known for its two OBAMA-binding scFv regions, transmembrane component, CD3ζ signaling region and 4-1BB co-stimulatory region (150). PRG1801 consists of a single anti-BCMA nanobody in addition to 4-1BB and CD3ζ co-stimulatory domains, whereas an alternative anti-BCMA CAR-T construct (Nanjing Legend Biotech) utilizes two distinct nanobodies for dual-epitope BCMA targeting (140,148,151,152). Certain clinical therapies utilize dual-targeted CAR-T cells aimed at BCMA for comparable clinical treatment efficacy, incorporating the recognition of single antigen scFv, bi-epitopic tandem VHHs, bispecific VHHs and single humanized VHH. Table II presents a comparison of the clinical data of four distinct anti-BCMA CAR-T cells.

According to the results presented in Table II, two anti-BCMA CAR-T cells, including ide-cel and cilia-cel, have obtained US FDA approval for the therapy of R/R MM, while PRG-1801 and orva-cel are under investigation. The clinical perfusion dosage for ide-cel and orva-cel ranged from 50 to $800x10^6$ cells, the aim for cilia-cel was $0.75x10^6$ CAR-positive T cells per kg, LCAR-B38M ranged from 0.5 to $0.75x10^6$ cells per kg and PRG1801 ranged from 2.5 to $10x10^6$ cells per kg. The trial results for ide-cel reported an objective response rate (ORR) of 85%, a complete response (CR) rate of 45.5% and a partial response (PR) rate of 48.5%. The orva-cel results indicated an ORR of 91%, a stringent CR (sCR) rate of 39% and a PR rate of 27%. As the CAR structure continues to be optimized, two VHHs have been incorporated into it more recently: For example, cilta-cel, which demonstrated an ORR of 97%, a sCR rate of 67% and a PR rate of 48%. According to the published clinical outcomes of LCAR-B38M, the number of clinical trials rose from 57 to 97, with ORRs between 87.8 and 97.7%, CRS rates from 68 to 82.5% and PR rates from 3.1 to 5%. According to the data for PRG1801, the sCR was 55.9% and the overall response rate was 88.2%.

Three primary adverse effects have been reported across these trials as follows: CRS, hematologic toxicity and neurotoxicity, with modest distinctions between them. CRS and neurotoxicity are the most prevalent side effects linked to CAR-T cell treatments (153,154) and neurotoxicity frequently occurs along with CRS, partly due to excessive cytokine release (155). The American Society for Transplantation and Cellular Therapy (ASTCT) Consensus Guidelines classify CRS into five distinct grades based on clinical severity: Grade 1 corresponds to mild symptoms, Grade 2 indicates moderate symptoms, Grade 3 signifies severe symptoms, Grade 4 represents life-threatening symptoms and Grade 5 denotes mortality (156). The incidence of CRS for ide-cel is 76%, with 6% classified as Grade 3 CRS, whereas neurotoxicity affects 42% of individuals and 3% experience Grade 4 CRS. Orva-cel-treated patients showed no significant difference and the frequency of ≥Grade 3 CRS was 2% lower than that of ide-cel, while the frequency of neurotoxicity ≥Grade 3 was 4%. Patients treated with cilta-cel exhibited more pronounced CRS, affecting 92 out of 97 patients, with 4% experiencing Grade 3 or 4 CRS and 1% Grade 5 CRS. The overall incidence of neurotoxicity was 21%, with 9% classified as Grade 3 or above. In three separate clinical trials with LCAR-B38M, the incidence of CRS ranged from 90-91.9%, with ≥Grade 3 CRS occurring in 7-9.5% of cases (151,152,157,158). Their neurotoxicity was modest, with an overall incidence ranging from 1-1.8%, which is considerably lower than the occurrence likelihood of other CAR-T therapies. The CRS of PRG1801 was seen in 29 individuals, with a total incidence of 85.3% and a Grade 3 CRS rate of 2.9%, with no instances of neurotoxicity reported. Together, nanobody-derived therapies using CAR-T are currently being employed in clinical settings and the safety data have bolstered trust in their potential. Updated discoveries may facilitate the comprehensive development of CAR designs that incorporate the unique and advantageous characteristics of nanobodies.

7. Challenges and mitigation strategies

Despite the success of nanobody-based CAR-T cell clinical therapy, reports indicate that CAR-T cells utilizing camel/mouse-derived antigen recognition structures can elicit neutralizing antibodies and induce hypersensitivity responses due to potential immunogenicity, potentially compromising the anti-tumor therapeutic efficacy of CAR-T cells (159,160). To address the risks of immunogenicity, nanobodies can easily be modified by humanizing the animal antibody-derived antigen-recognition domains, which may be a more reasonable option. However, in creating the native or humanized VHH-based CD19-redirected CAR-T cells, using fully human monoclonal antibody-derived antigen-recognition domains with a specific affinity range may not always be feasible (161,162). However, these humanized CAR-T cells exhibited comparable antitumor effects against tumor cells and produced comparable quantities of IFN-γ, IL-2 and TNF-α after co-cultivation.

Rapid blood clearance allows the use of short-lived isotopes in nanobody-based positron emission tomography imaging probes, decreasing patient radiation exposure (163). Conversely, mAb radiolabeling requires long-lived isotopes (164). However, for therapeutic applications, the rapid clearance rate of nanobodies from the circulation constitutes a significant limitation, requiring strategies to prolong their duration of action. One approach to mitigate this involves conjugating these single-domain antibodies into bivalent and trivalent forms (165). Furthermore, researchers have investigated the conjugation of specific VHHs with additional VHHs that target various antigens and epitopes, improving the remarkable circulatory half-life of these constructs (165). This advance is expected to facilitate more

Table II. Summary table showing clinical trials and adverse effects linked to four anti-BCMA CAR T cell therapies.

Authors, year	Different nanobody-based CAR-T	Antigen types	Dose of CAR-T cells	Response	CRS	≥Grade 3 hematologic toxicity	Neurotoxicity	(Refs.)
Raje *et al*, 2019	Idecabtagene Vicleucel (128/33 Cases)	A humanized scFv	Dose-escalation $50x10^6$, $150x10^6$, $450x10^6$ and $800x10^6$	ORR (85%) CR (45.5%) PR (48.5%)	Grade 1 or 2 (70%) Grade 3 (6%)	Neutropenia (85%) Leukopenia (58%) Anemia (45%) Thrombocytopenia (45%)	Grade 1 or 2 (39%); Grade 4 (3%)	(150)
Mailankody *et al*, 2020	Orvacabtagene autoleucel (100/51 Cases)		Dose-escalation $300x10^6$, $450x10^6$ and $600x10^6$	ORR (91%) sCR (39%) PR (27%)	≥Grade 3 (2%)	Neutropenia (55%) Anemia (21%) Thrombocytopenia (44%)	≥Grade 3 (4%)	(177)
Berdeja *et al*, 2021	Ciltacabtagene Autoleucel (113/97 Cases)	Two scFvs	$0.75x10^6$	ORR (97%) sCR (67%) PR (48%)	Grade 1 or 2 (91%) Grade 3 or 4 (4%) Grade 5 (1%)	Neutropenia (95%) Leukopenia (61%) Anemia (68%) Thrombocytopenia (60%) Lymphopenia (50%)	Grade 1 or 2 (12%); Grade 3 or 4 (9%)	(148)
Zhao *et al*, 2018	LCAR-B38M (57 Cases)	Bi-epitope tandem VHHs	Median $0.5x10^6$	ORR (88%) CR (68%) PR (5%)	Grade 1 or 2 (83%) ≥Grade 3 (7%)	Leukopenia (30%) Thrombocytopenia (23%)	Grade 1 (1.8%)	(151)
Zhao *et al*, 2022	LCAR-B38M (74 Cases)		Median $0.51x10^6$	ORR (87.8%) CR (73.0%)	Grade 1 or 2 (82.4%) ≥Grade 3 (9.5%)	Leukopenia (25.7%) Thrombocytopenia (18.9%)	Grade 1 (1.3%)	(152)
Martin *et al*, 2023	LCAR-B38M (97 Cases)		$0.75x10^6$	ORR (97.7%) CR (82.5%) PR (3.1%)	0	Neutropenia (97.9%) Thrombocytopenia (61.9%) Lymphopenia (99%)	Parkinsonism (1%)	(157)
Han *et al*, 2021	PRG-1801 (34 Cases)	A single humanized scFv	$2.5x10^6$-$10.0x10^6$	ORR (88.2%) sCR (55.9%)	Grade 1 or 2 (82.4%) ≥Grade 3 (2.9%)	Neutropenia (44.1%) Leukopenia (32.8%) Anemia (20.6%) Thrombocytopenia (38.2%) Lymphopenia (26.5%)		(158)

BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor ORR, objective response rate; CRS, cytokine release syndrome; CR, complete response; sCR, stringent CR; PR, partial response.

effective therapeutic applications. While this particular study highlights that nanobodies exhibit low immunogenicity owing to their small size, the humanization of selected VHHs remains a widely adopted strategy to mitigate the potential risks of immunogenicity. This humanization process typically involves fusing the chosen VHH with the Fc region of human IgGs (166). In addition, creating immune libraries with specialized nanobodies enables more efficient expression and purification operations. Under this approach, protein purification using PEG techniques can be used to purify the modified nanobodies, which may also be produced on a large scale, making them commercially viable. Furthermore, their enhanced penetration into solid tumors, owing to their small size and functionalization due to their low-fouling PEG coating, lowers transitory cellular interactions while increasing plasma circulation length (167,168). This can also be achieved via the slow release of nanobody, which is a high-affinity anti-human PD-L1 specific nanomaterial (also known as K2), within a peptide hydrogel, leading to enhanced accumulation of nanobodies in solid tumors to prolong their persistence (169,170).

8. Conclusion

The use of T cells in immunotherapy for neoplasms has achieved significant results in recent decades. Research has progressed from lacking an identifiable target to possessing a confirmed target and receiving clinical approval. The present review summarized a number of therapeutic targets that have become targetable by antibody-based CARs, which demonstrate anti-tumor effects analogous to those of scFvs. The diminutive size of nanobodies presents several significant advantages, including enhanced tissue penetration, the capability to cross the blood-brain barrier and access to epitopes that are otherwise difficult to reach (163). However, the nanobody structure requires considerable manufacturing complexity and exhibits the typical characteristics of reduced molecular weight (11,15). Furthermore, the small size of nanobodies results in a reduced circulating half-life and elevated renal clearance rates, which pose challenges for their therapeutic efficacy (78,165). Various innovative strategies have been developed to address these issues and improve the viability of nanobody-based therapies, such as fusing two nanobodies to a human IgG Fc fragment and utilizing PEG techniques to purify nanobodies (165,169,170).

In conclusion, as biotechnology advances, unique and useful nanobody-based CARs are being developed and have shown encouraging results, persuading researchers to investigate their therapeutic potential. Markedly, CAR-T cells produced with just one nanobody or bivalent nanobodies have demonstrated potential clinical efficacy. The viability of creating and producing bi-specific CARs in clinical trials has been demonstrated by LCAR-B38M bivalent nanobody CAR-T cells, paving the way for further advances in personalized cancer immunotherapy and potentially offering new hope for patients with previously challenging-to-treat malignancies.

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Availability of data and materials

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Authors' contributions

HL was primarily responsible for the writing, review and revision of this manuscript. XL, XZ, XH and SD compiled the references and developed the tables and figures. HF and YK developed the conceptual framework and design for the study. Data authentication is not applicable. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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