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REVIEW

Single-domain antibodies as therapeutics for solid tumor treatment

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Abstract Single-domain antibodies (sdAbs), initially identified in camelids or sharks and commonly referred to as nanobodies or VNARs, have emerged as a promising alternative to conventional therapeutic antibodies. These sdAbs have many superior physicochemical and pharmacological properties, including small size, good solubility and thermostability, easier accessible epitopes, and strong tissue penetration. However, the inherent challenges associated with the animal origin of sdAbs limit their clinical use. In recent years, various innovative humanization technologies, including complementarity-determining region (CDR) grafting or complete engineering of fully human sdAbs, have been developed to mitigate potential immunogenicity issues and enhance their compatibility. This review provides a comprehensive exploration of sdAbs, emphasizing their distinctive features and the progress in humanization methodologies. In addition, we provide an overview of the recent progress in developing drugs and therapeutic strategies based on sdAbs and their potential in solid tumor treatment, such as sdAb–drug conjugates, multispecific sdAbs, sdAb-based delivery systems, and sdAb-based cell therapy.

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1. Introduction

Traditional interventions for cancer treatment include surgery, chemotherapy, and radiotherapy. These methods are often poorly targeted and can cause damage to normal cells while killing tumor cells, thus leading to a spectrum of side effects. With the increasing understanding of tumor occurrence and progression, the significance and necessity of targeted therapy for cancer have been well recognized. Monoclonal antibodies (mAbs) are powerful tools in targeted tumor therapy and have been used in clinics for decades[1,2]. They can inhibit tumor cell proliferation by blocking the transmission of related signaling molecules such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) or can directly induce tumor cell death through cytolytic immune cell engagement[3]. To date, numerous mAbs have been approved by the US Food and Drug Administration (FDA) for the treatment of different tumors. Many of them, such as the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab, anti-EGFR antibody cetuximab, and anti-HER2 antibody trastuzumab, have been applied to the clinic for targeted tumor therapy and achieved superior effects[4,5]. However, certain inherent structural properties limit the potency of mAbs and traditional antibody-derived biologics for tumor therapy, especially in solid tumors with dense tissues[6]. The large size of mAbs (about 150 kDa) constrains their access to the cryptic epitopes in dense tumor tissues and can potentially compromise the therapeutic effectiveness[7,8]. Additionally, the long half-life (from several days up to 4 weeks) of mAbs may lead to safety concerns, further limiting their applications in some fields like imaging or radioimmunotherapy[9].

The discovery of naturally occurring heavy chain antibodies (hcAbs) in camelids by Hamers R. and his team in 1993 has opened new avenues for the development of anti-tumor therapeutics[10]. They analyzed serum samples from Arabian camels and found a kind of immunoglobulin G (IgG) lacking light chains. These heavy-chain-only antibodies have a molecular weight of about 90 kDa and show excellent binding affinity to their targets. The antigen recognition part of hcAbs belongs to a single variable domain, called VHH, or nanobody (Nb)[6]. Later, Flajnik and coworkers also found a class of heavy-chain-only antibodies in cartilaginous fish named immunoglobulin new antigen receptor (IgNAR)[11]. The variable domain of IgNAR is also referred to as VNAR. In general, VHH, Nb, and VNAR are collectively referred to as sdAb.

Compared with mAbs, sdAbs have a number of superior properties, making them an ideal choice for medical applications[12]. Firstly, they have outstanding physical properties such as good solubility and high thermal resistance, which are mainly attributed to their single-domain nature and a greater amount of hydrophilic amino acids on framework region two (FR2)[13-15]. Besides, the small size of sdAbs endow them with unique binding attributes. For example, the complementary determining region three (CDR3) loops of camelid VHHs are typically much longer than those of human VH domains, which may contribute to the increased interaction with the antigen[16]. Besides, sdAbs can bind to unique epitopes that are inaccessible by traditional antibodies[17-19]. This is mainly due to the extended CDR3 of sdAbs can form a finger-like extension that allows for binding to cavities and clefts of the target antigen[20-22]. Moreover, the long CDR3 of sdAbs may lower the binding entropy with antigens by forming a short helix[23-25]. Recent research has proven that sdAbs can across the blood−brain barrier (BBB) more efficiently than traditional antibodies, offering a good opportunity for the treatment of brain diseases[26-28].

Although with distinctive properties, the animal origin of sdAbs may introduce potential safety problems, hindering their clinical applications. Therefore, performing humanization has become a mainstream approach to solve the issue of sdAb immunogenicity. By using different humanization strategies, the camelid and shark-derived sequences of VHHs and VNARs can be replaced by human-derived sequences, generating humanized sdAbs or the recently emerged fully human sdAbs, which will be discussed in the following sections.

There are a number of therapeutic strategies for using sdAbs in the treatment of cancer. They mainly function by either directly targeting cytolytic immune cells such as NK cells and cytotoxic T lymphocytes (CTLs) to kill cancer cells or by inhibiting immunosuppressive immune cells including regulatory T cells (Tregs) and type II macrophages to revert tumor microenvironment (TME). Moreover, sdAbs can also target antigen-presenting cells (APCs) such as type I macrophages and dendritic cells (DCs) to enhance their antigen-presenting capability. All of these approaches give a lethal strike to cancer cells, bringing new hope for the treatment of cancer.

Here, we first describe the different strategies in sdAb humanization, particularly the new format of fully human sdAbs. In addition, we discuss various therapeutics and therapies based on sdAbs, focusing on their versatile diagnostic and therapeutic applications in cancer. At the same time, we also update the advances in this field, providing insight into the future development of sdAb-based anti-tumor therapies.

2. The humanization of sdAb

Due to their animal origin, sdAbs are typically recognized as foreign components by the human immune system, especially when conjugating with other foreign molecules such as chemical drugs or peptides. Thus, immunogenicity may be a stumbling block in the therapeutic application of sdAbs[29,30]. Humanization can minimize the immunogenicity of sdAbs, providing a safer choice for long-term treatments[31-35].

Antibody humanization is defined as the replacement of xenogeneic sequences with human sequences in the FRs of antibody variable domains[36]. The successful humanization of murine antibodies has paved the way for sdAb humanization (Fig. 1). According to the systematic review by Rossotti et al., there are mainly two types of strategies used in the humanization of sdAbs, which include CDR grafting and resurfacing (also known as veneering)[36]. CDR grafting is a process in which carefully selected CDRs from xenogeneic antibodies are grafted into human FRs, while resurfacing is achieved by replacing the only surface-exposed FR residues of nonhuman antibodies with corresponding residues from human antibody FRs[37-39]. By using the CDR grafting approach, Vaneycken et al. successfully engineered a carcinoembryonic antigen (CEA) specific dromedary VHH, named NbCEA5, into a previously identified universal scaffold[40]. Affinities of the humanized VHH to immobilized CEA protein was measured by surface plasmon resonance (SPR), showing an approximately 30-fold decrease than the parental VHH, despite the binding affinity was still in nanomolar range (∼10 nmol/L).

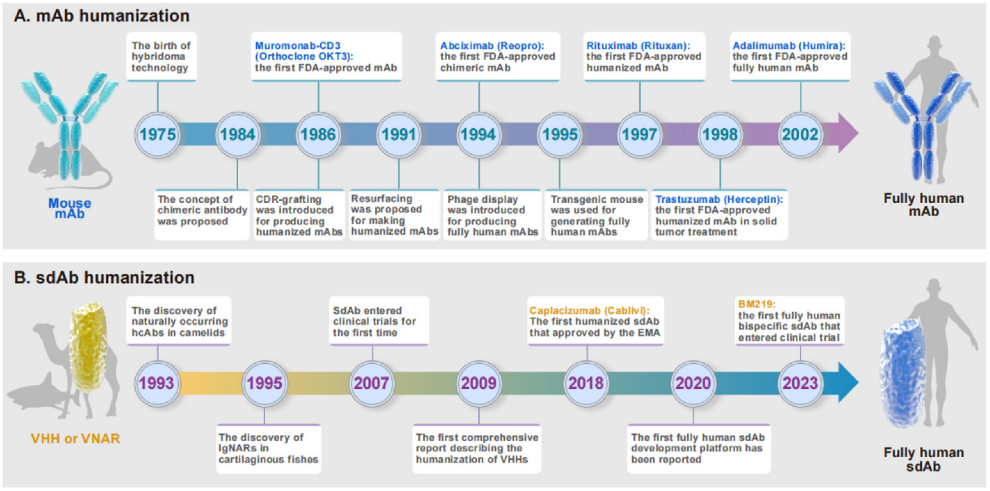

Figure 1 Schematic diagram of antibody evolution. (A) The development history of monoclonal antibody from mouse to human origin. The birth of hybridoma technology in 1975 marked a pivotal moment in mAb development, leading to FDA approval of the first mouse mAb Orthoclone OKT3 in 1984. Subsequent advances included chimeric and humanized antibodies, culminating in the approval of the first fully human mAb in 2002. (B) The evolution of sdAbs from camelid to human origin. In 1993, Hamers et al. discovered the sdAb in camelids for the first time. Rapid progress in the 2000s saw several sdAbs enter clinical trials. The breakthrough came in 2018 with the EMA approval for Caplacizumab (Cablivi), the first humanized sdAb. In 2020, the fully human sdAb development platform was reported, and by 2023, an inhalable bispecific fully-human sdAb entered clinical trials, marking a significant stride in sdAb therapeutic development. FDA, US Food and Drug Administration; EMA, European Medicines Agency.

They also validated the binding epitope between humanized NbCEA5 and its original version by competition binding assays and showed that both of them shared the same epitope. Furthermore, using tricarbonyl chemistry, Technetium-99 was labeled to humanized NbCEA5, forming a nanobody−radionuclide conjugate for tumor imaging. This sdAb-conjugated product exhibited efficient binding to both purified CEA protein and CEA-expressing CHO cells, and displayed low signals in all organs besides the kidneys, providing a noninvasive *in vivo* imaging method of tumors. Another study by Li et al.[41] also used CDR grafting to humanize an anti-CD16 and anti-Mucin 1 (Muc1) bispecific sdAb by using the human germline VH gene DP47 (IGHV3-23). The humanized version of the bispecific sdAb, named Muc-Bi-2, can bind to Muc1-positive cells and mediate significant cytotoxic activities against Muc1-positive tumor cells. Moreover, in a Muc1-positive LS174T grafted mouse model, both the original type of bispecific sdAb and the humanized Muc-Bi-2 demonstrated powerful inhibition of tumor growth than the control group, indicating successful implementation of this humanization strategy. Resurfacing is another critical strategy for sdAb humanization. Kazemi-Lomedasht et al. employed a conservative resurfacing approach to humanize Nb42, a dromedary anti-VEGF VHH[42,43]. Based on *in silico* prediction tools, a total of nine other sites in the FR regions were altered without changing the key camelid hydrophilic residues in the FR2 region. This humanized version of Nb43 showed no significant affinity alternation in ELISA assay and demonstrated a potent inhibition against the proliferation of human endothelial cells. Except for the traditional methods mentioned above, recently, with the rapid development of computational-based technologies, machine-learning (ML) or deep-learning (DL) guided strategies have been developed for antibody humanization. Using sequencing results of the Observed Antibody Space (OAS) library as training sets, Prihoda et al. developed Sapiens, a DL-based tool trained by language modeling for antibody humanization[44]. Moreover, in the same work, the authors also introduced another package for humanness evaluation (OASis). They combined the two to create an open-source platform, BioPhi, for *in silico* antibody design and optimization. Based on a test set of 177 humanized antibodies, Sapiens showed humanization results comparable to manual humanization by experts. This platform thus provided a convenient way for antibody humanization and contributed to the development of antibody therapeutics.

3. Fully human sdAb: A powerful platform for solid tumor treatment

Fully human antibodies are a class of antibodies composed entirely of human own sequences. Due to the absence of other heterogeneous sequences, fully human antibodies are considered to be non-immunogenic and unnecessarily humanized, making them suitable for long-term use as *in vivo* therapeutics, especially in the treatment of different types of cancers. According to the comprehensive review of Lyu et al., as of September 2022, there are a total of 55 fully human antibody therapies have been approved[45]. Adalimumab (Humira) was the first fully human IgG antibody for clinical use. It targeted TNF-α and was approved by the FDA in 2002 for the treatment of autoimmune-related diseases such as rheumatoid arthritis (RA) and ankylosing spondylitis (AS)[46]. Notably, since the invention of hybridoma technology in 1975, it took more than a quarter century for the approval of the first fully human mAb (Fig. 1A).

To date, several strategies have been developed to generate fully human antibodies. *In vitro* display technology, especially phage display technology, is one of the most popular ways for generating fully human antibodies. As one of the world's best-selling drugs, adalimumab was generated through this technique. Another significant way for producing fully human antibodies is

the use of immunoglobulin (Ig) loci-modified transgenic mice. By replacing the mouse Ig genes with those from humans, these genetically modified mice can produce fully human antibodies after immunization of the target antigen[47,48]. Single B cell screening is another convenient and efficient technique for generating fully human antibodies. It is well suited for high-throughput identification of antigen-specific clones with the help of different microarray-based methods such as micro-engraving and cell-based microarray chip systems[49,50]. Recently, with the rapid development in structure biology and the emergence of increasing numbers of protein-structure data, computational-based antibody humanization methods by homology modeling or artificial intelligence (AI) have also been designed for the generation of humanized or even fully human antibodies[51,52].

Compared with traditional mAbs, the smaller molecular weight of single-domain antibodies endows them with several distinct advantages, such as ease of engineering and the ability to access cryptic epitopes. Consequently, over the past decade, active attempts have been made for the generation of fully human sdAbs[53-55]. However, unlike VHH or VNAR, the human VH domain does not exist naturally, therefore, the stability and solubility may be key factors hindering its druggability. Several strategies have been developed for improving the bioproperties of sdAbs, such as introducing an extra disulfide bond at specific sites, panning phage-displayed sdAb libraries under harsh conditions to pick out the most stable candidates, and by grafting the less-stable donor CDRs into a highly stable scaffold[56-59]. However, while a few currently obtained human VHs exhibited relatively high antigen-binding affinity, methods for rapid and large-scale identification of fully human sdAbs with superior properties are still lacking.

Recently, with the rapid development in synthetic immunology and the emergence of increasing numbers of advanced techniques, there has been significant momentum in driving the development of fully human single-domain antibodies. Our group has made an active attempt to the generation of sdAbs with human immunoglobulin gene origin and successfully developed a platform for the rapid identification of fully human sdAbs with superior properties[60]. By analyzing 2391 camelid VHH sequences from a public database, we found the FR2s of these VHH are divergent. Besides, some human VHs identified by others also showed superior properties similar to camelid VHH, leading us to speculate that certain VH framework regions could compensate for the absence of light chains, resulting in soluble human single-domain antibodies[61,62]. Therefore, we used a highly soluble and stable HIV-1-neutralizing VH, named m36, as a reference, to search for human immunoglobulin heavy chain variable region (IGHV) alleles with the same FRs from the IMGT database. As a result, 17 human germline IGHV alleles were cloned and expressed in *Escherichia coli*, and evaluated for their biophysical properties[63]. Eight out of 17 alleles possessed high yields in bacterial culture (over 10 mg/L) and 10 out of 17 had protein-A-binding capabilities. The most notable one was germline 3-66*01, which demonstrated the most advantageous properties, including midpoint transition temperature (T_m) comparable to that of camelid VHH and the highest aggregation temperature (T_{agg}) among all tested antibodies. Using the CDR grafting approach, we successfully grafted the CDRs from the previously constructed libraries into the IGHV3-66*01 scaffold and built a naïve fully human sdAb library, generating a generalizable platform for rapid development of fully human sdAbs. N501, a fully human sdAb screened from the library, showed a nanomolar affinity with the oncofetal antigen 5T4 (K_D = 6.59 nmol/L) and exhibited exceptionally high stability[64]. We next compared the bioproperties of N501 with a camelid nanobody (VHH#3). As expected, N501 exhibited comparable thermal stability to camelid nanobody and can tolerate harsh storage temperatures and extreme pH conditions. Surprisingly, N501 still retained 80% of binding capability after 4 weeks at 37 °C and 70% of activity at 45 °C, while the camelid nanobody lost 50% of activity after 1 week at 37 °C and nearly all activity at 45 °C (<20%)[64]. Moreover, by coupling with 7-ethyl-10-hydroxycamptothecin (SN-38), the sdAb-drug conjugate demonstrated much deeper tumor penetration, significantly higher tumor uptake, and faster accumulation at tumor sites than conventional IgG1-based antibody−drug conjugate, highlighting the potential therapeutic applications of fully human sdAbs. Notably, using the same fully human sdAb platform, two fully human sdAbs that targeting distinct conserved regions of the SARS-CoV-2 S protein were screened and constructed into bispecific form by tandem linkage. The resulting product showed potent therapeutic efficacy *via* inhalation[65]. Furthermore, clinical trial data supported that it had a good safety profile and elicited limited immunogenicity similar to fully human mAbs.

4. Versatile formats of single-domain antibodies as therapeutics in solid tumor treatment

Tumorigenesis is an extremely sophisticated process driven by corrupted gene messages and is influenced by various factors, which affects the cell signaling pathway and reshape the immune status of the body. Reverting the immunosuppressive microenvironment in solid tumor tissues is a tough job, which often requires the synergistic effect of multiple factors to jointly curb the proliferation of tumor cells or activate immune effector cells. The distinctive features of small size, good stability making sdAbs easy to engineer and therefore extremely suitable for fusion with other proteins and effector domains to the National Medical Products Administration (NMPA) of China simultaneously act on tumor parts for better therapeutic efficacy. In 2021, the first sdAb-based Fc fusion protein named envafolimab (KN-035) was successfully approved by the National Medical Products Administration (NMPA) of China. It was the first subcutaneously administered anti-programmed cell death ligand 1 (PD-L1) sdAb for the treatment of microsatellite instability-high (MSI-H) or deficient MisMatch Repair (dMMR) advanced solid tumors in adults[66]. Envafolimab has also obtained the FDA's orphan drug designation for advanced biliary tract cancer[66]. Furthermore, a homogenous distribution of the anti-tumor therapeutics within the tumor sites is also a prerequisite for good therapeutic effects. The strong tissue penetration of sdAbs makes them outperform traditional antibodies, particularly suitable for the use of solid tumor therapeutics[67].

According to the different strategies used in antibody modification and various fusion moieties coupled to sdAbs, anti-tumor sdAbs can be mainly divided into the following categories (Fig. 2): sdAb−drug conjugates by fusion sdAb with toxins, peptides and chemicals; multispecific sdAbs having distinct antigen binding sites; sdAb-based delivery systems using nanocarriers, and sdAb-based cell therapy such as chimeric antigen receptor (CAR)-T cell therapy. Below, we describe and discuss them in detail according to their basic concepts.

4.1. *sdAb-based drug conjugates*

Antibody−drug conjugates (ADCs) consist of a mAb, a cytotoxic payload and a suitable linker. By targeting tumor antigens with

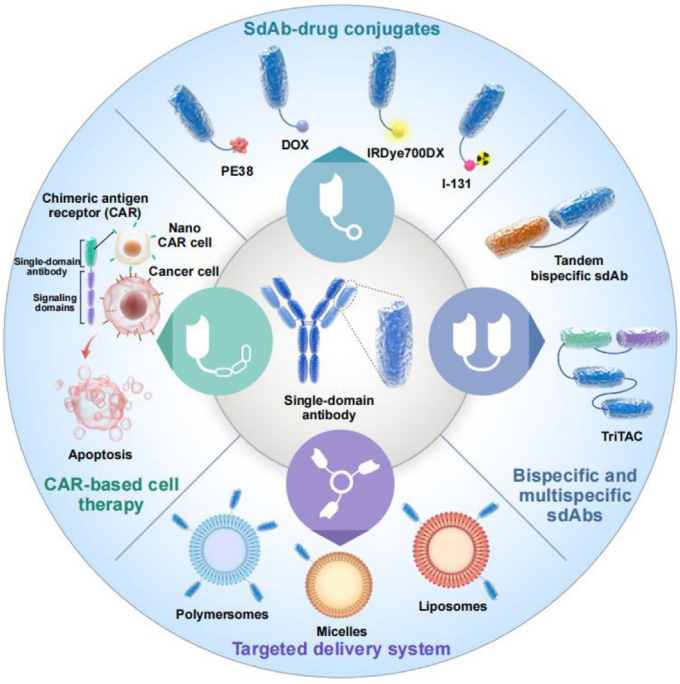

Figure 2 Schematic representation of the diverse formats of single-domain antibodies in solid tumor treatment. sdAb−drug conjugates are composed of sdAb that targets tumor antigens and payloads, including toxins, chemotherapeutic compounds, photosensitizers and therapeutic radionuclides. SdAbs that targeting different antigens or the same antigen with distinct epitopes can be assembled into bispecific or multispecific sdAbs. Moreover, sdAbs can be decorated on the surface of different types of materials including liposomes, micelles, albumin-based nanoparticles and polymeric nanoparticles, forming sdAb-based targeted delivery systems. By transducing the CARs with sdAb-based targeting domains into different immune cells such as T cells, NK cells and macrophages, the sdAb-based CAR-T/NK/M cell therapy can be generated. DOX, doxorubicin; I-131, Iodine-131; TAA, Tumor-associated antigen; TriTAC, Tri-specific T cell-activating construct.

mAbs, ADCs can specifically deliver potent cytotoxic drugs to the cancer sites and kill tumor cells efficiently. To date, 15 ADCs have been approved for cancer treatment worldwide, and more are in clinical or preclinical development[68,69]. Despite great achievements, the large molecular weight of traditional ADCs hinders their application in the treatment of solid tumors[6]. SdAb-based drug conjugates, also known as nanobody−drug conjugates (NDCs), are attractive alternatives to ADCs. Similar to ADC, NDC can selectively kill tumor cells by coupling cytotoxic payload to a high-affinity sdAb *via* a smart linker[70]. Due to the small size of sdAb, NDC can bind to antigens with high affinity and recognize unique hidden epitopes on them, leading to minimal off-target effects. These advantageous features broaden the application of sdAbs in solid tumor therapy, enabling them to be combined with various types of drugs such as toxins, chemotherapeutic compounds, photosensitizers and therapeutic radionuclides.

Immunotoxins, also termed as targeted-toxins, are a kind of fusion protein consisting of an antigen-targeting domain and a cytolytic effector domain. The toxin moieties mainly include pseudomonas exotoxin (PE38), ricin, diphtheria toxin or cucurmosin (CUS), among which the truncated form of PE38 is the most widely used one[6]. A few studies have coupled PE38 with tumor-targeting sdAbs and shown excellent treatment efficacy both *in vitro* and *in vivo*. For example, a PE38-conjugated dimeric anti-VEGFR sdAb has been shown to effectively inhibit the proliferation of VEGFR2-expressing tumor cells *in vitro*[71]. A similar Cadherin 17 (CDH17) specific sdAb named E8 fusing with PE38 also exhibited strong cytotoxic potency and significantly reduced cell proliferation in various types of CDH17-positive GC cell lines. The PE38-E8 immunotoxin demonstrated powerful anti-tumor efficacy and showed no significant body weight loss than PBS control group in both CDH17-positive CDX and PDX mouse models[72]. Moreover, an immunotoxin formed by the fusion of CUS and EGFR domain III-targeting sdAb 7D12, called rE/CUS, showed strong anti-proliferative activity in several solid tumor cell lines including A549, HepG2 and SW116[73].

Chemotherapeutic agents are another type of drug that can be coupled with sdAbs. By conjugating with different sdAbs, these chemical compounds can be specifically delivered to the tumor parts, avoiding damage to normal cells. Platinum-based drugs and doxorubicin (DOX) are the two most widely used chemotherapeutic drugs. A platinum-based multifunctional NDC was constructed by jointing four protein modules together which included an anti-EGFR sdAb, a gadolinium-binding protein domain for MRI imaging, a Cys3-tag for site-specific drug conjugation and an anti-albumin sdAb for extending half-life *in vivo*[74]. This multifunctional fusion protein can selectively induce apoptosis of EGFR-positive cancer cells, leading to delayed tumor growth in animal models and with little side effects when compared to classical treatment with cisplatin. DOX is another small molecule chemotherapy agent that commonly used in NDCs. By using phage display technology, a high-affinity sdAb ($K_D = 6.36 \times 10^{-10}$ mol/L) against CD147 was generated for tumor targeting. After conjugating with doxorubicin, the NDC showed a significant cytotoxicity effect in several CD147 positive cell lines and can inhibit tumor growth in 4T1-bearing mice[75]. Recently, our group has built a SN-38 conjugated

anti-5T4 fully human sdAb, called fully human single-domain antibody-drug conjugate (UdADC). This sdAb-based drug conjugate showed potent cytotoxicity in high 5T4-expressing cell line BxPC-3. Moreover, it also exhibited deep tumor penetration in both patient-derived organoids (PDOs) and tumor spheroids[64]. In addition to these classical compounds mentioned above, recently, other functional targeting drugs have also been introduced to the generation of NDCs. The programmed death-ligand 1 is a checkpoint molecule highly expressed in different tumor types. Yu et al. developed the first PD-L1/TLR7 dual-targeting NDC by conjugating the TLR7 agonist SZU-101 to PD-L1 targeting nanobodies[8,76]. The obtained NDC can promote PD-L1 expression on intratumoral APCs and tumor cells and elicit innate and adaptive immune responses simultaneously, exhibiting potent anti-tumor efficiency in both CT26 and B16-F10 mouse models.

Recently, sdAbs have also been introduced into the field of photodynamic therapy (PDT) as a promising strategy for selectively targeting photosensitizers to tumor sites. PDT works by hitting photosensitizers with specific wavelengths of light in an oxygen-containing environment to form reactive oxygen species (ROS). These ROS, in turn, destroy cellular components such as proteins, lipids and/or nucleic acids, leading to cell death[77]. IRDye700DX is a widely used photosensitizer in conjugation with sdAbs. A study using IRDye700DX-conjugated anti-EGFR monovalent and biparatopic sdAbs has shown that they can selectively induce cell death in EGFR-overexpressing cells at low nanomolar concentrations, whereas photosensitizer alone or in the absence of light do not induce cell death[78]. Another research using anti-HER2 sdAbs 1D5 and 1D5-18A12 fused with IRDye700DX to generate HER2-targeted nanobody−photosensitizer conjugates[79]. It was found that both nanobody−photosensitizer conjugates can effectively and selectively induce cell death of HER2-overexpressing cells, and showed specific accumulation in HCC1954 tumors in quantitative fluorescence spectroscopy. In addition, they can induce significant tumor regression of trastuzumab-resistant high HER2-expressing tumors while having little effect on normal tissues, providing a new treatment for HER2-positive breast cancer. A critical drawback of the porphyrin-based photosensitizers such as IRDye700DX is their tendency to aggregate due to the rigid planar and hydrophobic structures[80,81]. Therefore, new types of photosensitizers with aggregation-induced emission (AIE) characteristics have been generated to overcome this obstacle[82,83]. A new type of photosensitizer called AIEPS5 was designed and further conjugated to anti-HER2 sdAb for the treatment of oral cancer. The AIEPS5-sdAb conjugate demonstrated superior cytotoxicity in patient-derived PGI tumor cells with the IC_{50} value of 8.3 + 0.4 μg/mL[84]. Moreover, *in vivo* evaluation of the PDT efficiency for AIEPS5-sdAb conjugate was also performed using the PDX mouse model and showed potent tumor inhibition with a more than 90% tumor ablation under laser irradiation but low *in vivo* dark toxicity, demonstrating a promising approach in PDT for oral cancer treatment.

Targeted radionuclide therapy (TRT) is a systematic approach that can selectively deliver cytotoxic radionuclides to the tumor sites with minimal toxicity to normal healthy tissues. The cytotoxic radionuclides are mainly including β-emitting particles Yttrium-90 (^{90}Y), Iodine-131 (^{131}I), Lutetium-177 (^{177}Lu) and α-emitting isotopes Astatine-211 (^{211}At), Bismuth-213 (^{213}Bi), Actinium-225 (^{225}Ac)[85-89]. A ^{131}I-labeled anti-HER2 sdAb has been generated to treat HER2-overexpressing cancer and significantly improves the median survival time of BT474/M1 and SK-OV-3 tumor xenografted mice[88]. The same sdAb, 2Rs15d, has also been coupled with ^{225}Ac and co-injected with Gelofusin, a plasma extender, resulting in significantly reduced renal uptake in HER2-positive SK-OV-3 xenografts, despite a slight decrease in absorption at the tumor part[90,91]. Recently, a CD20-targeting sdAb radiolabeled with ^{225}Ac was developed for the treatment of melanoma[92]. The ^{225}Ac−sdAb complex can specifically accumulate in the tumor sites and exhibit strong tumor growth inhibition with elevated release of multiple cytokines such as IFN-γ, TNF-α and C−C motif chemokine ligand 5 (CCL5). Besides, the treatment showed no acute, systemic treatment-related toxicity with no significant body weight loss than control group in human CD20 and ovalbumin-expressing B16 melanoma-bearing mice[92]. However, when combined with α-TRT with PD-L1 blockade therapy, the combination treatment can induce enhanced adverse effects with severe body weight loss and kidney toxicity, although potentiated the anti-tumor efficacy. Except for the purpose of therapy, some radionuclides can also be used for tumor diagnosis *via* molecular imaging. When conjugating with tumor antigen-targeting antibodies, these diagnostic radionuclides can be applied for prognostic monitoring and tumor staging[93]. To date, several radiolabeled sdAbs for tumor diagnosis have passed through the preliminary stage of evaluation and are in middle-stage clinical trials for the treatment of different types of tumors (Table 1). All in all, these outcomes indicated the bright future of sdAb-based radionuclide diagnosis and therapy, offering a powerful tool for the treatment of solid tumors.

4.2. *Bispecific and multispecific sdAbs*

The immunosuppressive microenvironment at the tumor site is formed by the complicated interaction of many different factors. Therefore, targeting distinct epitopes located on the same or different cells is often necessary. This can be achieved by bispecific antibodies (bsAbs) or multispecific antibodies. As the name suggests, a bsAb is able to bind two distinct targets or two separated epitopes on the same target[97]. When compared with traditional antibodies, bsAbs or multispecific antibodies can improve the targeting abilities and reduce off-target effects. As reviewed by Chen et al., bsAbs can mainly function in the following ways: by the blockade of two receptors in tumors to enhance anti-tumor efficacy; by targeting T cells and tumor cells to redirect cytotoxic T cells to tumor parts; and by targeting innate effector immune cells such as NKs to enhance NK cell-mediated antitumor activity[98]. Due to the small size of sdAbs, they can be easily engineered into bispecific or multispecific formats by simply genetically fusing with other antibodies *via* a linker peptide. Such sdAb-based bispecific antibodies (termed bsNbs) typically exhibit superior properties including stability, good solubility and outstanding production yields, opening up a new avenue for the treatment of solid tumors. Vγ9Vδ2-T cells are a subpopulation of anti-tumor $\gamma\delta$T cells and their tumor infiltration have been associated with good prognosis in different types of tumors[99-101]. Therefore, using agonist antibodies to promote the proliferation and activation of Vγ9Vδ2 T cells seems to be a promising strategy[102]. De Bruin et al. screened several $\gamma\delta$T cells-activating sdAbs by phage display and used one of these to develop a bispecific sdAb combining anti-EGFR and anti-Vγ9Vδ2 TCR single-domain antibodies *via* a GS linker[103]. This bispecific sdAb can induce Vγ9Vδ2-T cell-mediated lysis of EGFR-positive tumor cells and cause improved survival in an *in vivo* mouse xenograft model, demonstrating the effective antitumor efficacy of this strategy[104].

Table 1 Summary of sdAb-based drugs that have been approved or are in middle/late-stage clinical trials for the treatment of solid tumors.

Drug	Target	Format	Disease	Clinical stage	Ref. [a]
Envafolimab	PD-L1	SdAb-Fc	MSI-H or dMMR advanced solid tumors	Approved	66
^{68}Ga-NOTA-anti-HER2 VHH1	HER2	Radiolabeled sdAb	Breast cancer	Phase II	94
KN046	PD-L1/CTLA4	Bispecific sdAb	Advanced pancreatic cancer	Phase III	95
ALX148	CD47	SdAb-Fc	Gastric cancer	Phase II/III	NCT05002127
^{68}Ga-NOTA-anti-MMR-VHH2	MMR	Radiolabeled sdAb	Non-small cell lung cancer	Phase II	NCT05933239
^{99m}Tc-NM-01	PD-L1	Radiolabeled sdAb	Non-small cell lung cancer	Phase II	NCT04992715
αPD-1-MSLN-CAR-T Cells	PD-1/MSLN	Novel sdAb-based CAR-T	Advanced solid tumors	Phase I/II	NCT05944185
Gavocabtagene autoleucel	MSLN	SdAb-based CAR-T	Advanced MSLN-expressing cancer	Phase I/II	96
TC-510	MSLN	sdAb-based CAR-T	Advanced MSLN-expressing cancer	Phase I/II	NCT05451849
BI 836880	VEGF/Ang-2	Bispecific sdAb	Advanced solid tumors	Phase II	NCT03697304

[a]Including the relevant articles or NCT numbers. NCT number: identifier in ClinicalTrials.gov (https://clinicaltrials.gov). PD-L1, Programmed death-ligand 1; MSI-H, Microsatellite instability-high; dMMR, Deficient mismatch repair; HER2, Human epidermal growth factor receptor 2; CTLA4, Cytotoxic T-lymphocyte-associated protein 4; Fc, Fragment crystallizable; ^{68}Ga, Gallium-68; MMR, Macrophage mannose receptor; ^{99m}Tc, Technetium-99 m; PD-1, Programmed cell death protein 1; MSLN, Mesothelin; CAR-T, Chimeric antigen receptor-T cells; VEGF, Vascular endothelial growth factor; Ang2, Angiopoietin-2.

NK cells are another type of cells targeted by bsAbs, since NK cell infiltration within tumors is a good prognosis in cancer patients[105]. CD16, also named FcγRIII, is a receptor for IgG1 and IgG3 Fc fragment expressed on NK cells, $\gamma\delta$T cells and macrophages. It can introduce antibody dependent cell-mediated cytotoxicity (ADCC) and phagocytosis of antibody-opsonized cells. The engagement of CD16 can promote NK cell proliferation and function independently of the ADCC effect through PI3K/MAPK pathways[106,107]. An anti-CD16 × anti-MUC-1 bsNb developed by Li et al. exhibited potent cytotoxicity in MUC1-overexpressing tumor cells by recruiting NK cells[41]. This bispecific sdAbs was constructed by tandem linkage of two single-domain antibodies through a GS linker. Additionally, in the presence of human peripheral blood mononuclear cells (PBMCs), this bsNb can significantly inhibit tumor growth in LS174T grafted mice. More recently, a study by our group has generated an inhalable bispecific single-domain antibody named bn03 by tandem linkage of two SARS-CoV-2 receptor binding domain (RBD)-targeting antibodies n3113v and n3130v[65]. Bn03 can simultaneously and synergistically bind to two conserved epitopes of a single RBD and can be effectively delivered to lung *via* inhalation administration, exhibiting potent neutralization breadth and therapeutic efficacy in SARS-CoV-2-infection mouse models[65]. The robust efficacy demonstrated by this inhalable bispecific sdAb validates the effectiveness of this delivery strategy, offering innovative insights for future drug design in the treatment of solid tumors.

Although the tandem format of bispecific sdAbs is in small size and easy to manufacture, their short half-life has become an obstacle to their application. A feasible approach for extending their half-life is to couple with another antibody targeting neonatal Fc receptor (FcRn) or human serum albumin (HSA) to generate a multispecific sdAb. A trispecific T cell-activating protein-based construct (TriTAC) named HPN536 was developed by Molloy et al., which consists of a humanized anti-mesothelin (MSLN) sdAb for tumor targeting, a humanized llama sdAb specific for HSA binding, and a humanized scFv specific for the human CD3ε activating[108]. In T cell-dependent cell cytotoxicity (TDCC) assays, co-cultures of MSLN-expressing OVCAR3 ovarian cancer cells and resting T cells with HPN536 showed efficiently lysis of OVCAR3 target cells with EC_{50} values ranging from 1.3 to 2.5 pmol/L. Furthermore, it also exhibited strong anti-tumor efficacy in three distinct xenograft mouse models and showed superior pharmacokinetics properties with high dose tolerance and no dose-limiting toxicities in cynomolgus monkeys, despite moderate, dose-dependent mesothelial hypertrophy along with a mixed immune cell infiltration and extracellular matrix deposition.

4.3. *sdAb-based targeted delivery systems*

Nanoparticles (NPs) are a kind of solid colloidal particles with a diameter of less than 200 nm, which mainly include liposomes, micelles, albumin-based nanoparticles and polymeric nanoparticles[109-111]. In nanoparticle-based targeted delivery systems, all NPs rely on a targeting ligand at the surface for adequate specificity[112]. Due to the small size and the absence of an Fc domain, sdAbs are suitable to be decorated on the surface of NPs. By using sdAb-based targeted delivery systems, the loaded drugs can be protected from oxide reduction and enzymatic reactions, maintaining the local drug concentration at a high level[113]. Furthermore, these systems can minimize the immunogenicity and avoid potential side effects of drugs, thus improving the therapeutic efficacy.

Among all delivery systems, liposomes are considered to be the most widely used drug carriers, with morphology and properties very similar to those of cell membranes[114,115]. To date, several liposomes with excellent properties have been approved for clinical application[116]. In early studies, sdAbs were coupled to empty liposomes without drug incorporation. An EGFR-targeting sdAb named EGa1 was conjugated to the surface of liposomes by PEGylation[117]. This sdAb−liposome conjugate induced a large amount of EGFR reduction (more than 90%) by receptor internalization and degradation both in 14C tumor cells and xenograft

mice. Soon afterwards, another modified version of EGa1−liposome conjugate was introduced by encapsulating an anti-IGF-1R kinase inhibitor (AG538) into the liposomes[118]. Compared to empty EGa1−liposome, the EGa1−AG538−liposome showed a more robust inhibition of tumor growth in EGFR-positive A431 and 14C tumor cell lines. Furthermore, contributing to the synergistic effect on the co-inhibition of EGFR and IGF-1R pathways, the EGa1−AG538−liposome conjugate exhibited strong antitumor efficacy in a 14C xenograft mouse model[119]. Another study by Chen et al. generated a multi-targeting liposomal system by modifying with a PD-L1 targeting sdAb and mannose ligands for codelivery of an mTOR inhibitor (rapamycin) and an anti-angiogenic drug (regorafenib)[120]. The multi-targeting liposomes (termed as t-LRR) can target both PD-L1 and mannose receptors overexpressing cancer cells and tumor-associated macrophages with high specificity. Moreover, t-LRR demonstrated strong anti-tumor activity in murine CT26 colon cancer cell line and CT26 peritoneal mouse model. Recently, some BBB receptors specific sdAbs were identified by Aguiar et al. using an *in vivo* phage display screening strategy[121]. The most promising sdAb, namely RG3, was conjugated at the surface of liposomes encapsulated with a model drug, panobinostat (PAN). The RG3−PAN−liposome conjugate induced an efficient brain endothelial barrier (BEB) translocation and showed a powerful antitumoral activity against LN229 glioblastoma cells without influencing BEB integrity.

Micelles are another type of nanocarriers with superior properties. Due to the amphiphilic attributes, micelles are suitable for the delivery of hydrophobic drugs. Early research found that an anti-EGFR sdAb (EGa1) coupling polymeric micelle exhibited enhanced recognition and uptake by EGFR-positive target cells[122]. Subsequently, this sdAb-micelle encapsulated a DOX, forming an EGa1−DOX−micelle conjugate[123]. *In vitro* experiments using EGFR-expressing squamous cell carcinoma cell line 14C showed that, compared with drug-free EGa1−micelles, the DOX-containing EGa1−micelles possessed higher cellular uptake and more effective tumor cell killing capability. Additionally, in a 14C tumor-bearing mouse model, treatment with DOX−EGa1−micelle conjugate exhibited strong tumor inhibition and significantly extended median survival time of mice. Another recent study loaded *meta*-tetra(hydroxyphenyl)chlorin (mTHPC), a clinically used photosensitizer for photodynamic therapy, to EGa1-decorated polymeric micelles, generating an EGa1−mTHPC−micelle conjugate[124]. This mTHPC-loaded targeted micelles demonstrated potent photocytotoxicity in EGFR-overexpressing A431 cells and displayed prolonged blood circulation kinetic in mice bearing human A431 tumor xenografts.

Polymer-based polymersomes, polyplexes and nanogels, are a group of NPs that have attracted much attention in recent years. Conjugation of these NPs with sdAbs provided a new strategy for targeted drug delivery. By using FDA-approved polymersomes (PEG-*b*-PCL), Zou et al. developed an anti-HER2 nanobody-polymersome conjugate and confirmed its specific binding to HER2-positive breast cancer cells by cell flow cytometry and microscopy[125]. A polyplexes-based targeted NP was also generated by using an anti-MUC1 sdAb decorated on polyethylenimine (PEI) polyplex[126]. This PEI polyplex was loaded with plasmids encoding a transcriptionally targeted truncated-Bid (tBid) killer gene, leading to apoptosis of the targeted cells. In caspase-3-positive T47D and SKBR3 cell lines, the engineered polyplexes elevated the expression level of Bid/tBid, and induced significant cell death. Nanogels are another polymer-based NP for drug delivery. Nuhn et al. used fully hydrophilic polymeric nanogels conjugated with anti-CD206 sdAbs, forming a CD206-targeted NP[127]. This resulting conjugate demonstrated highly specific targeting in CD206-expressing macrophages and 3LL-R tumor-bearing mice. Recently, several innovative forms of nanogel-based NPs have been generated, highlighting the promising future of this strategy[128,129].

Another type of tool used for targeted drug delivery is albumin-based NPs. Due to the high solubility and bio-safety of albumin, a number of sdAb-conjugated albumin NPs have been developed. An anti-EGFR sdAb-conjugated albumin NP was loaded with the multi-kinase inhibitor 17864 and showed specific targeting to EGFR-positive 14C squamous head and neck cancer cells[130]. A similar anti-hepatocyte growth factor receptor (c-Met) sdAb-conjugated albumin NP was generated by Heukers et al[131]. This resulting NP can be specifically taken up by Met-expressing cells and induced downregulation of the total Met protein. More recently, Zhang et al. developed an albumin-based NP by decorating the HSA with an anti-HER2 sdAb. Besides, Chlorin (Ce6) and catalase (CAT) were encapsulated to the HSA for PDT of ovarian cancer[132]. The generated complex has demonstrated synergistic effect with anti-CTLA-4 therapy to inhibit the progression of distant tumors and induce T cell infiltration in SK-OV-3 cells and SK-OV-3 tumor-bearing mouse model[132].

4.4. *sdAb-based CAR-cell therapy*

CAR-based cell therapies mainly include the well-studied CAR-T cell therapy, the CAR-NK cell therapy, and the most recent CAR-macrophage (CAR-M) therapy[133]. In the past decade, CAR-T cell therapy has achieved remarkable outcomes in the treatment of various types of tumors, especially in hematologic malignancies. CARs are conventionally composed of an antigen-binding extracellular domain, an anchored transmembrane domain and an intracellular domain for cell activation. Specifically, CAR-T cells recognize their target antigens *via* the targeting domain (typically a scFv) and become activated through the intracellular activation (usually CD3ζ) and co-stimulatory domain (4-1BB, CD28, OX40)[134,135]. The antigen-recognition part of CAR-T cells is of great significance, as an appropriate tumor-targeting antibody can specifically deliver T cells to the tumor site, minimizing side effects to normal tissues. The scFv-based CARs are the most widely used format of CAR-T cells in clinics, with five of these have been approved by FDA[136]. However, some studies have demonstrated that the potential immunogenic and unstable attributes of scFvs may limit the clinical efficacy because of the anti-CAR immune response and lead to premature T cell exhaustion[137-139]. Due to the stability and low immunogenicity of sdAbs, a number of sdAb-based CAR-T cells have been generated and exhibit the same function as traditional CARs.

A large number of solid tumor-associated antigens (TAAs) have been used as the targets of sdAb-based CAR-T cells, such as HER2 and MUC1. Besides, tumor extracellular matrix (ECM)-associated markers, such as the EIIIB fibronectin splice variant have also been targeted for solid tumor CAR-T cell therapies. Using Jurkat T cells, Jamnani et al. generated oligoclonal sdAb-based CAR-T cells by using a set of HER2-targeting nanobodies fused to CD28-CD3ζ and CD28-OX40-CD3ζ signaling domains[140]. These oligoclonal CAR-T cells can induce the secretion of IL-2 and show significant expansion in a proliferation assay. In addition, they also exhibited

potent cytotoxicity in HER2-positive tumor cell lines. CDH17 is a cell surface adhesion protein highly expressed in gastrointestinal cancers (GICs) and neuroendocrine tumors (NETs). Using a llama-derived sdAb named VHH1, Feng et al. generated VHH1-CAR T cells (CDH17CARTs)[141]. This resulting sdAb-based CAR-T cells exhibited highly specific cytotoxicity in both human and mouse tumor cells. Furthermore, CDH17CARTs can eradicate various types of CDH17-expressing cancers in either tumor xenograft or autochthonous mouse models, demonstrating their powerful therapeutic efficacy. Another study by Xie et al. constructed a PD-L1-targeting sdAb-based CAR-T cell and observed potent tumor cells killing and IFN-γ production in several different PD-L1-expressing tumor cell lines[142]. In addition, a significantly decreased tumor growth and prolonged survival rate were observed in B16 and MC38 transplanted mouse models, despite exhibiting low levels of immunogenicity. Another study using shark-derived VNARs as the targeting domain of CARs[143]. They built a semi-synthetic shark VNAR phage library and isolated several PD-L1-targeting sdAbs. Among these VNARs, a cross-reactive sdAb named B2 can block the PD-L1 pathway. These engineered B2-CAR-T cells can specifically lyse human breast cancer and liver cancer cells, and inhibit orthotopic breast cancer growth in mice. Furthermore, by combining with anti-Glypican-3 (GPC3) CAR-T cells, the combination treatment group exhibited a synergistic antitumor effect both *in vitro* and *in vivo*. Except for the great significance of the antigen recognition part in CAR-T cells, the hinge and transmembrane domains of CARs also have a great impact on CAR-T cell activity[144-146]. Most recently, Li et al. isolated a dromedary camel-derived Glypican-1 (GPC1) targeting sdAb named D4 and further constructed it into a sdAb-based CAR[147]. The generated sdAb-based CAR-T cells showed strong tumor inhibition effects with minimal toxicity in multiple types of GPC1-positive cancer cell lines and can induce strong tumor elimination in GPC1-expressing xenograft mouse model. Moreover, by replacing the traditional lengthy CD8 hinge (CD8H) with a short IgG4 hinge (IgG4H), the new version of sdAb-based CAR-T cells termed D4-IgG4H-CD28TM exhibited enhanced cytotoxicity than the original D4-CD8H-CD8TM in low GPC1-expressing tumor cells. Next, the research team investigated the role of IgG4H and CD28TM domains in the new version of sdAb-based CAR-T cells and found IgG4H-CD28TM can mediate D4 CAR dimerization by forming interchain disulfide bonds, leading to enhanced T-cell signaling and tumor regression in pancreatic cancer models. Moreover, the short rigid structure of IgG4H can facilitate the D4 antigen-binding domain dimerization, thus contributing to increased binding to GPC1. Together, this work highlights the huge impact of antigen density on the therapeutic efficacy of CAR-T cells, providing insights for future CAR development.

In addition to targeting cell surface antigens, some intracellular tumor antigen-targeting sdAb-based CAR-T cells have also been introduced in recent years. Targeting intracellular antigens with antibody constructs requires their binding to an HLA-restricted peptide epitope[148]. Using GPC3 and Wilm's tumor 1 (WT1) oncoprotein as panning antigens, Li et al. generated two novel TCR-like sdAbs including an anti-HLA-A2/GPC3 sdAb and an anti-HLA-A2/WT1 sdAb by 5 rounds of phage screening[149]. These TCR-like sdAbs were then engineered into second-generation CARs, forming TCR-like sdAb CAR T cells. The resulting CAR-Ts were tested for antigen-specific activation and proliferation by culturing with the target HepG2 (HLA-A2+/GPC3+) and OVCAR3 (HLA-A2+/WT1+) cells, and showed remarkable cell activation and pro-inflammatory cytokines production (including IFN-γ, IL-2, TNF-α, MIP-1α, and GM-CSF). In $GPC3_{144-152}$ peptide/$WT1_{126-134}$ peptide-loaded T2 cells or HepG2 and OVCAR3 cells, the TCR-like sdAb CAR T cells can mediate potent cytotoxic activity. Furthermore, these TCR-like sdAb CAR-T cells also demonstrated outstanding antitumor efficacy in HepG2 and OVCAR3 xenograft mouse models *via* inhibiting tumor growth and extending the survival time.

Except for traditional CAR-T cells, the recently emerging CAR-NK cells and CAR-macrophages have also been used as therapeutics in the treatment of various types of tumors. Compared with CAR-T therapy, CAR-NK has a number of advantages. CAR-NK cells can kill tumor cells through both CAR-dependent and NK cell receptor-dependent mechanisms, thus possessing potent cytotoxic activity. Besides, CAR-NK cells can eliminate MHC-deficient tumor cells and with minimal risk of side effects such as life-threatening graft versus host disease (GvHD) and cytokine release syndrome (CRS). Moreover, allogeneic CAR-NK cells can be generated from different sources, making them easier to obtain with lower production costs. To date, some sdAb-based CAR-NK cells have been generated for the treatment of solid tumors[150-152]. In a novel study by Hambach et al., the CD38-specific sdAbs were transduced into the engineered human natural killer cell line NK-92, forming the sdAb-based CAR-NK cells[150]. The resulting CD38-targeting CAR-NKs demonstrated potent cytotoxicity in both CD38-expressing tumor cell lines and patient-derived primary multiple myeloma cells. In another study, an EGFR-targeting nanobody conjugated CAR-NK was generated by glycoengineering and exhibited potent cytotoxic activity in multiple EGFR-overexpressing tumor cells. Furthermore, in tumor xenograft mice, the sdAb-based CAR-NK cells demonstrated superior therapeutic efficacy and outstanding safety. Recently, macrophages have also been proven to be armed with CARs, generating the CAR-M cells[153]. With their distinctive phagocytosis properties, CAR-M has become an ideal candidate for targeting solid tumors. According to the comprehensive review by Maalej et al., to date, four CAR-M therapies have been approved by the FDA for clinical trials[133]. However, no relevant research on sdAb-based CAR-M therapies have been reported so far.

5. Conclusions and future perspectives

The physical and physiological characteristics between solid tumors and hematological malignancies are quite different. Hematological malignancies involve circulating tumor cells in the bloodstream, surrounded by numerous effector cells, making them relatively accessible to therapeutic drugs and allowing for favorable therapeutic efficacy[154]. However, in solid tumors, tumor cells generally form dense clumps, posing challenges for drug infiltration. Although traditional mAb-based therapies have achieved remarkable outcomes in solid tumor treatment, certain inherent limitations of mAbs may restrict their applications to some extent.

One significant challenge for mAb-based therapeutics in the treatment of solid tumors is their difficulty in penetrating dense tumor tissues[64,155-159]. The disorganized vascular network and the absence of functional lymphatics in solid tumors can cause increased interstitial fluid pressure (IFP), preventing the drug transport from the blood compartment to the interstitium and compromising the therapeutic efficacy[155,160]. Moreover, the highly immunosuppressive and heterogenous properties of TME can further increase the difficulty of tumor immunotherapy.

Single-domain antibody, owing to its distinctive properties, becomes a potent alternative to traditional mAb in the treatment of solid tumors. To date, there are several sdAb-based drugs that have been approved or are in different stages of clinical trials for the treatment of solid tumors (Table 1). The low molecular weight of sdAbs endows them with superior ability to penetrate dense tumor tissues[161-163]. Moreover, due to their high stability and small size, sdAb-based therapies can be administered through inhalation, offering several advantages such as noninvasiveness, enhanced local concentration, and ease of self-administration for patients with lung cancer or other respiratory diseases[65].

SdAbs can be conjugated with various types of molecules including toxins, peptides or radionuclides, playing an important role in tumor imaging, diagnosis and therapy. As an object that has been investigated for decades, traditional ADCs have certain limitations in many aspects and have been well reported. The distinctive properties of sdAbs with high stability and solubility empower them as ideal targeting domains for targeted drug delivery. In addition, the cost-effectiveness of sdAbs in production and their ease of modification, coupled with the ability of NDCs to achieve homogeneous formulations through site-specific conjugation chemistry, suggest that NDCs are poised to emerge as formidable contenders in the future drug market, challenging traditional ADC drugs.

For tumor treatment, smaller drug molecular weight usually means a shorter half-life, which is a disadvantage of NDC compared to traditional ADC, as it can compromise the therapeutic efficacy to some extent. However, the half-life problem cannot be generalized. For example, for tumor imaging, the short half-life of NDC is an advantage because it typically means less *in vivo* toxicity. Therefore, it is necessary to combine practical application situations to ensure that NDC has an appropriate half-life. The increasing number of related clinical trials in recent years indicates the immense potential for the development of NDCs. As insights are gained from ongoing clinical trials, we anticipate that this field will witness a bright and promising future.

Moreover, sdAb can be decorated with many distinct types of NPs, generating the so-called targeted NPs. These sdAb-based targeted delivery systems enhanced drug accumulation at tumor sites while minimizing the risk of off-target side effects. Nonetheless, ensuring safety remains a pivotal consideration in the design of sdAb-based NPs. The future trajectory of this field should primarily concentrate on the research and development of novel NP variants with enhanced properties. This involves refining NP safety by mitigating drug leakage and preventing non-specific interactions, as well as bolstering NP stability and drug loading capacity. Additionally, optimizing the properties of sdAbs, serving as targeting domains, holds significant importance. For instance, augmenting sdAb affinity through techniques like affinity maturation may contribute to improved NP targeting for tumors and a reduction in off-target effects. Humanization can further reduce the immunogenicity of sdAb-based NPs, providing a safer option for clinical treatments.

Through various humanization strategies, many humanized sdAbs and even fully human sdAbs have been generated and exhibited powerful antitumor efficacy in different types of tumors. Some tumor-associated antigens can also be expressed on normal tissues, thus requiring a combination of different tumor antigen-targeting antibodies. Such sdAb-based bispecific or multispecific antibodies demonstrated highly specific cytotoxicity to both tumor cell lines and tumor xenograft mouse models. The marketing of the world's first sdAb-based bispecific antibody, Ozoralizumab, in 2022 marks a significant milestone, ushering in a new era for bsNbs. More and more bsNb drugs are in preclinical stages or undergoing different phases of clinical evaluation. One of the primary challenges in bispecific antibody development lies in determining the most suitable format. The optimal format not only enhances the functionality of bsNbs but also influences therapeutic efficacy. Presently, the predominant method for selecting the most suitable format involves constructing them one by one and determining the optimal form through a series of experimental validations. However, with the growing availability of protein-structure data and the continuous advancement of AI-based technologies, there is hope for corresponding technical means to assist in the future design of bsNbs.

SdAbs have also been used in the field of CAR-based cell therapy, forming sdAb-based CAR-T cells, CAR-NK cells, and the recently emerging CAR-macrophages. Ciltacabtagene autoleucel (cilta-cel, also termed Carvykti™) is a sdAb-based anti-B cell maturation antigen (BCMA) CAR-T cell product approved by FDA for the treatment of relapsed or refractory multiple myeloma. The antigen-binding extracellular domains of cilta-cel are composed of two tandem linked single-domain antibodies targeting distinct epitopes on BCMA. The successful application and commercialization of the first sdAb-based CAR-T therapy not only demonstrated the potential application value of this strategy[164], but also boosted the research and development on CAR-NK and CAR-M, bringing new opportunities to the cell therapy arena. It is crucial to acknowledge that, due to the unique attributes of various immune cells, insights gained from previous research and experiences with CAR-T cells may not seamlessly translate. While there have been limited reports on sdAb-based CAR-NK and CAR-M therapies to date, we anticipate that, with a progressively deeper understanding of diverse immune cells and immune system mechanisms, a broader range of products will become available in the near future.

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Author contributions

All authors conceived and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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