REVIEW

Nanobody: A Small Antibody with Big Implications for Tumor Therapeutic Strategy

This article was published in the following Dove Press journal:
International Journal of Nanomedicine

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Abstract: The development of monoclonal antibody treatments for successful tumor-targeted therapies took several decades. However, the efficacy of antibody-based therapy is still confined and desperately needs further improvement. Nanobodies are the recombinant variable domains of heavy-chain-only antibodies, with many unique properties such as small size (~15kDa), excellent solubility, superior stability, ease of manufacture, quick clearance from blood, and deep tissue penetration, which gain increasing acceptance as therapeutical tools and are considered also as building blocks for chimeric antigen receptors as well as for targeted drug delivery. Thus, one of the promising novel developments that may address the deficiency of monoclonal antibody-based therapies is the utilization of nanobodies. This article provides readers the significant factors that the structural and biochemical properties of nanobodies and the research progress on nanobodies in the fields of tumor treatment, as well as their application prospect.

Keywords: nanobody, drug delivery, immunotherapy, CAR-T, tumor treatment

Introduction to Antibody and Nanobody

Since 1975, monoclonal antibodies have been triumphantly applied to the diagnosis and management of various diseases. For example, chimeric antibodies, humanized antibodies, as well as all-human antibodies, and miniaturized genetic engineering antibodies, have manifested expanding application prospects. Nevertheless, the strengths of smaller size are, upon most occasions, counteracted by descending stabilization, leading to polymerization (especially single-chain variable fragment, scFv), lower affinity binding, and problems in mass production.[1] Therefore, nanobody (Nb) discovered in 1993 seems to be able to address the problems.

Nb is a naturally deleted light chain antibody in the peripheral blood of alpaca. The acquisition of Nb first needs to select a camelid animal for immunization, which will probably take 5–10 weeks. As shown in Figure 1A–F, the effector B cells are obtained from the plasma by extracting the peripheral lymph blood from the immunized camel, then the total RNA is extracted from the effector B cells and the cDNA sequence of the fragment is obtained by the reverse transcription-PCR technology, so primary PCR products can be used as templates for amplification. Phage display is employed to screen afterward and enrich Nb-phage with specific binding ability from the nanobody library which is subsequently established after the structure of the phage vector. Eventually, the Nb expression system is built to express proteins and function verification is carried out. Although the preparation process is relatively complicated, with the development of
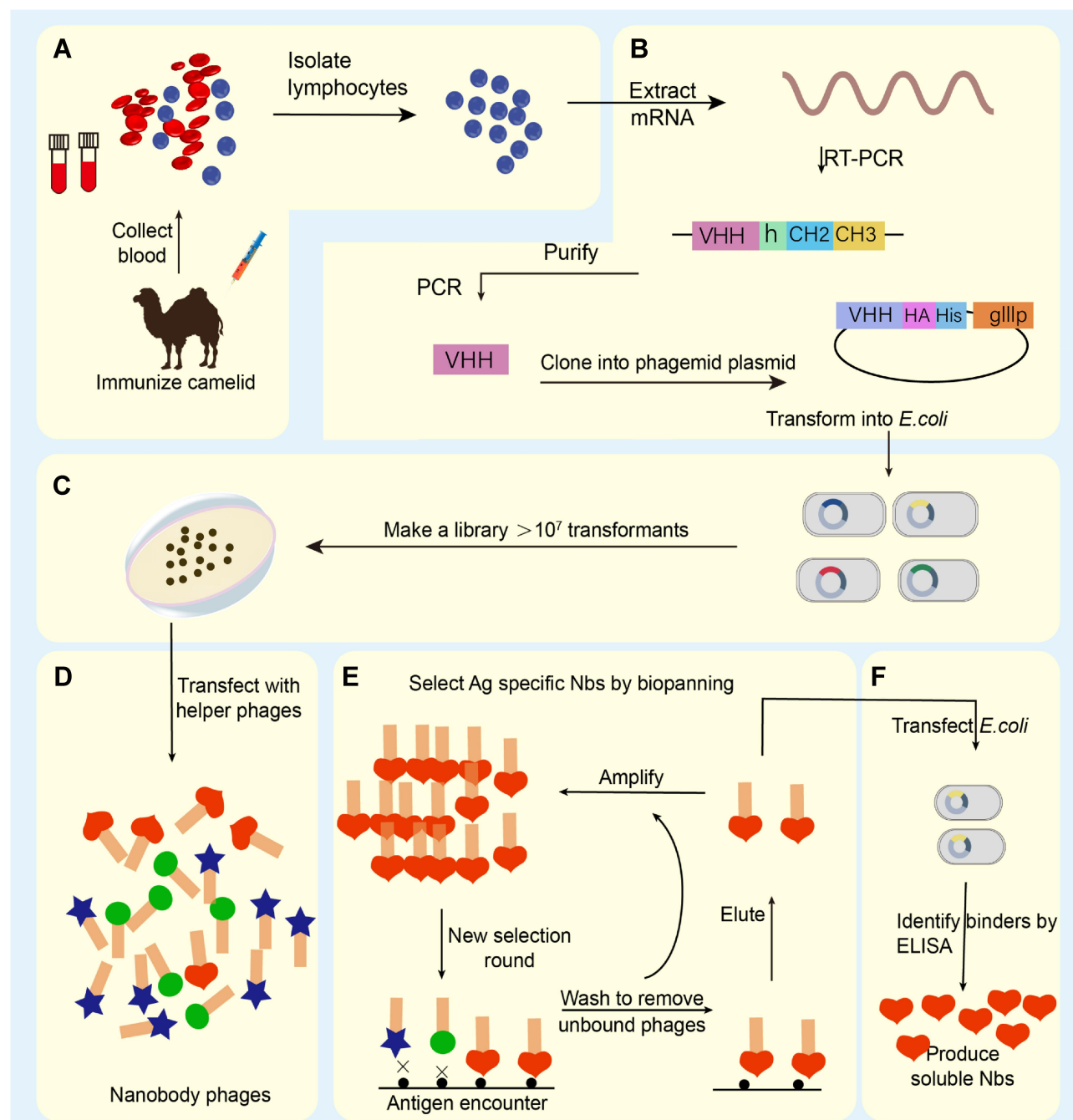
Figure 1 Schematic overview of nanobody generation. (**A**) Extracting a cDNA from peripheral blood obtained from a camelid after immunized. (**B**) Extracting Total mRNA and reversely transcribing into cDNA, then amplifying the VHH, the phage display vector and the VHH are digested by restriction enzymes, and the two fragments are connected. (**C**) Constructing natural or immune camelid-derived nanobody libraries. (**D–F**) Obtaining antigen-specific nanobody by multiple screening.
Notes: Adapted with permission of Future Science Ltd, from Novel therapy based on camelid nanobodies. Unciti-Broceta JD, Del Castillo T, Soriano M, Magez S, Garcia-Salcedo JA. *Ther Deliv.* 4(10):1321–1336, copyright 2013; permission conveyed through Copyright Clearance Center, Inc.[29]

molecular biology technologies such as phage display technology, the screening steps of Nb are being gradually optimized.[2]

Compared with the traditional antibody IgG, which includes one heavy chain variable region VHH connects with two traditional CH2 and CH3 constant domains, and its molecular weight is only half of that of the traditional antibody.[3,4] The cloned antibody containing only a heavy chain variable region named Nb or single-domain antibody (sdAb), has identical stability and excellent antigen binding vitality in comparison to the initial heavy chain antibody (HcAb).[5,6] Due to its high specificity, strong stability, and

top affinity, it has generated extraordinary accomplishments in the field of antibodies.[7,8] It is no exaggeration to say that Nb has been extensively used in biochemical mechanisms, structural biology, diagnosis and treatment of tumors and other diseases.[9,10] This article will evaluate the basic characteristics of Nb and its research progress in tumor treatment, and look forward to its application prospect.

Structure and Physicochemical Features of Nanobody
Basic Structure of Nanobody

Nb is the minimum known inartificial antigen-specific binding functional fragment, which consists of approximately 120 amino acids, with a crystal is 2.5 nm in diameter, 4 nm in length and merely 12~15 kb in weight (Figure 2D).[6] The molecular weight is smaller than the traditional monoclonal antibody mAb (~150 kb), Fab fragment (~55 kb) or HcAb (~90 kb) in Figure 2A–C. Furthermore, the variable region domain can form a domain antibody called nanobody by itself.[11] Previous research found that these nanobodies can be genetically engineered from the heavy-chain antibody derived from camelids or cartilaginous fish, whose immune systems had already evolved into high-affinity V-like domains so that intramolecular disulfide bonds with stability were not for the demand.[12–15] Therefore, Nb has a stronger and faster tissue penetration ability and can reach dense tissues such as solid tumors to play a role. At the same

time, due to renal filtration, the half-life of Nb in the blood is also relatively shorter, in some cases can avoid the accumulation of toxicity.

Nb consists of three antigenic complementary determining regions (complementarity determining region, CDR) and four frame regions (frame region, FR). Among them, three CDR are the binding regions of Nb to the antigen, while the traditional antibody needs six CDR to maintain the binding to the antigen. Compared with monoclonal antibodies, Nb showed considerable or even stronger antigen-binding ability.[16,17] In addition, the longer amino acid sequences of CDR1 and CDR3 of Nb make up for the loss of antigen-binding ability caused by the loss of light chain to some extent. After receiving antigen stimulation, the production of Nb mainly depends on somatic hypermutation, so a longer CDR sequence also means more antibody diversity. Crystallographic studies have shown that longer CDR3 regions give Nb a stronger ability to bind antigens, which can bind to antigenic epitopes that cannot be reached by traditional antibodies.[18] Sequence alignment showed that the VH domain of Nb was highly homologous to that of human immunoglobulin IgG, but there were significant differences between FR2 and CDR3 regions. Previous studies have shown that repeated administration of Nb will not cause any humoral and cellular immune response.[19–21] but whether long-term repeated use of Nb drugs will cause its immunogenicity to the body remains to be studied.[22]
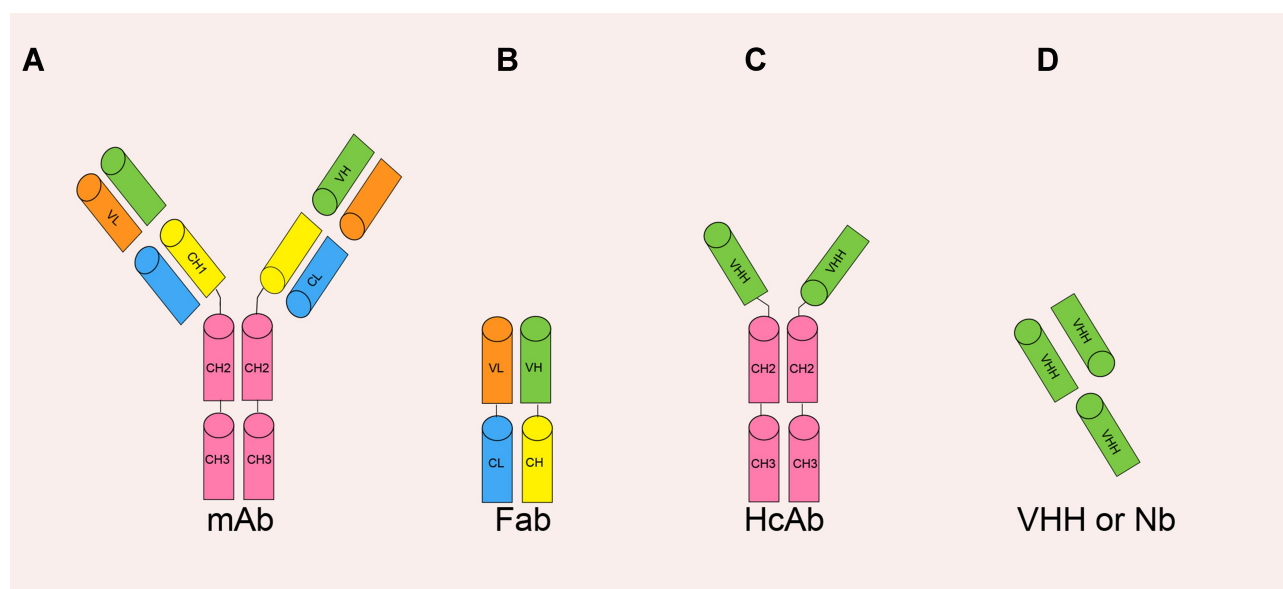
Figure 2 The various antibody formats: (**A**) mAb (monoclonal antibody); (**B**) Fab (fragment antigen binding); (**C**) HcAb (camel heavy-chain antibody); (**D**) VHH or Nb (nanobody). Adapted from Yang X, Xie S, Yang X, et al. Opportunities and Challenges for Antibodies against Intracellular Antigens. *Theranostics.* 2019;9(25):7792–7806. Available from: https://www.thno.org/v09p7792.htm. Creative Commons license and disclaimer available from: http://creativecommons.org/licenses/by/4.0/legalcode.[165]

Characteristics of Nanobody (Compared with Antibody)

A key feature of nanobody is its good solubility. In traditional antibodies, there are a large number of hydrophobic residues in the FR2 region where VH interacts with VL (Figure 3B).[23] In the absence of light chain VL, the hydrophobic residues on the surface of VH are exposed and aggregation is easy to occur in vitro.[24] These hydrophobic residues were replaced by hydrophilic residues in VHH, which reduced the aggregation ability of VHH and improved the water solubility of VHH (Figure 3B and C). Other characteristics of nanobody include, (1) high thermostability, Nb can maintain 80% activity at 37°C for one week.[25] After denaturation at 90°C, Nb can restore its natural active conformation.[26] Nb can also resist the effects of denaturants, proteases and extreme pH.12 (2) short development cycle, Nb can be manufactured in batch production of large quantities via microbial systems, whereas the production of traditional monoclonal antibodies requires a eukaryotic expression system, the process is technologically elaborate and expensive to maintain. (3) the various screening approaches, in addition to the traditional methods of immunization, the production of Nb, using natural antibody library and antibody library synthesized in vitro can overcome the shortcomings of immunization.[27,28] What is more, its low molecular weight, high affinity and strong antigen recognition ability, Nb has more advantages than traditional antibodies in tumor diagnosis and treatment. In recent years, Nb has indeed shown promising potentials in the treatment of tumors.[29]

Nanobody as Tumor Therapeutic Agent

The traditional antibody has played a great role in the field of tumor diagnosis and treatment and opened the era of targeted therapy. At present, it has already been widely used as one of the primary methods in the guide of crizotinib therapy and has created considerable socioeconomic benefits.[30] On condition that traditional antibodies contain Fc domains, and cancer cells can be killed by antibody-dependent cell-mediated cytotoxicity (ADCC)
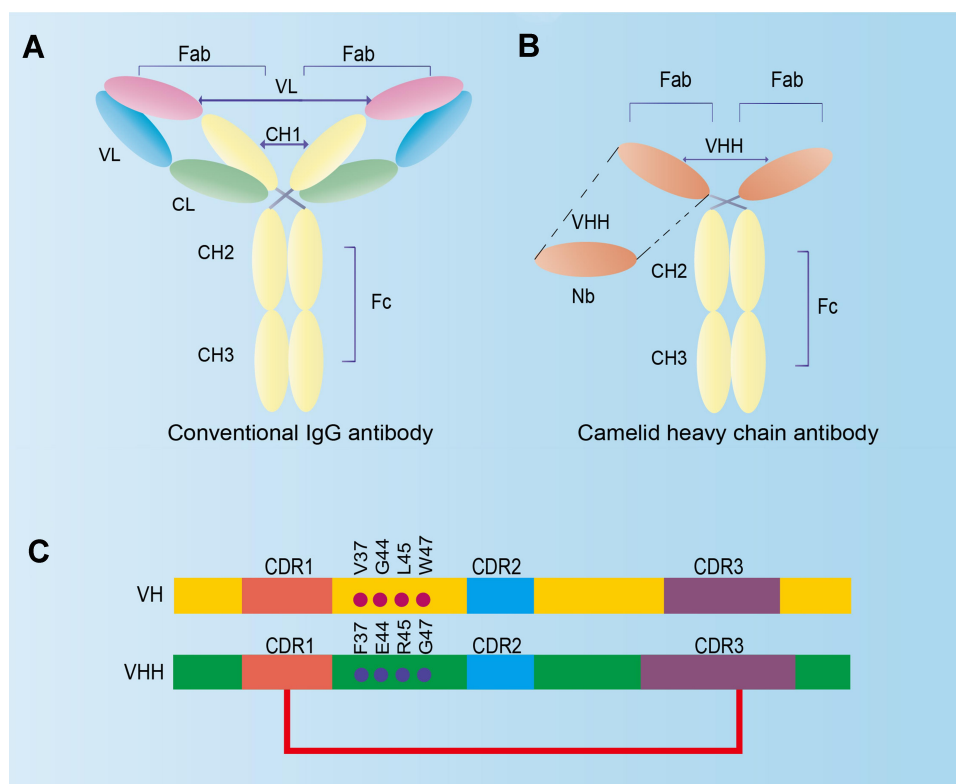
Figure 3 Comparison between traditional IgG antibody and camelid heavy-chain antibody. (**A**) Traditional IgG antibody has two identical heavy chains, consisting of VH, CH1, CH2, and CH3 domains, and two identical light chains, consisting of VL and CL domains. (**B**) Camelid heavy-chain antibody contains one VHH and two constant domains (CH2 and CH3). A Nb is the VHH domain of heavy-chain antibody obtained by recombinant gene technology. (**C**) The difference in amino acid sequence of VH and VHH. The average length of the CDR1 and CDR3 is better in the VHH domain than in the VH domain. The dotted line indicates the additional disulfide bond between CDR1 and CDR3 of the VHH domain. The Numbering of the international ImMunoGeneTics-IMGT information system is pointed out. Adapted from *J Biotechnol*, 74(4), Muyldermans S. Single domain camel antibodies: current status. 277–302, Copyright (2001), with permission from Elsevier.[196]

and complement-dependent cytotoxicity (CDC).[31] However, the molecular weight of a traditional antibody is as high as 150 kb,[32] and its penetration ability is poor, so it is sometimes difficult to reach the target tissue. Relatively speaking, the molecular weight of Nb is small, so it is easy to penetrate the barrier to reach the cancerous tissue. For the moment, according to the above-mentioned characteristics of Nb, Nb drugs have been developed for different tumors (B-cell lymphoma, multiple myeloma, malignant glioma, colon cancer, liver cancer and breast cancer, etc.),[33] which are widely related to tumor tracer diagnosis, direct targeted therapy, treatment carriers and so on.

Nanobody Targeting Classic Tumor-Associated Receptor

Nb can be used as a new generation of drugs for the treatment of human diseases because of their unique pharmacological and physicochemical properties. Nb is able to validly couple to the concaves on the appearance of protein.[34] Furthermore, Nb has the capability of functioning in cells to target intracellular antigens.[35,36] Nb has strong stability in extreme situations and can pass through the hemato-encephalic barrier.[37] Because of its insusceptibility to pepsin, oral administration may be used to enhance stomach function and improve gastrointestinal illness.[38,39] In addition, Nb can easily be engineered for the new development of particular and efficient cures for certain diseases such as rotavirus infection.[40] So far, apart from the application of Nb in microbial infection, treatment of neurodegenerative diseases, anti-inflammation and immune regulation, the research results in tumor diagnosis and treatment are particularly outstanding.

EGFR (Epidermal Growth Factor Receptor) is highly expressed in almost most tumors and monoclonal antibodies have been produced on therapeutic drugs to target EGFR through 20 years' endeavor in human malignancies.[41,42] However, mAb cannot easily penetrate tumor tissues due to the limitation of large molecular weights. Oliveira et al developed a probe based on anti-EGFR nanobody 7D12 which was linked with the near-infrared fluorophore IRDye800CW and made a comparison with an anti-EGFR mAb cetuximab. 7D12-IR allowed tumor microenvironment visualizable approximately only 30 minutes post injection, but no signal was detectable around the tumor site for cetuximab-IR.[43] Therefore, results highlight that the Nb is considered superior to the mAb in penetrating tumor tissue

for optical molecular imaging. Moreover, EGFR specific antibodies screened from the Nb library can block the binding of EGF to its receptors, thus inhibiting EGFR-mediated motility of signal transduction and proliferation of cells.[44,45] Its polyvalent antibody might suffice to further facilitate the appetency of EGFR Nb. Meanwhile, in order to prolong its half-life in vivo, the dual-valent Nb of EGFR or coupled with mouse serum albumin can better inhibit the growth of tumors.[46] Oliveira et al injected the nanobody targeting EGFR into mice, found that it specifically combined with EGFR in tumor cells, and detected that the expression of EGF was down-regulated.[47] Roovers et al and Sebastian et al have confirmed in animal experiments that the specific nanobody targeting EGFR can reach the tumor body more effectively, and the growth of solid tumor cells can be more effectively inhibited by competing with epidermal growth factor to bind EGFR site.[48,49] Tijink et al found that the bivalent nanobody targeting EGFR or mouse serum albumin was coupled. It can better inhibit the growth of tumor cells.[50]

Carcinoembryonic antigen (CEA) was highly expressed in epithelial-derived cancer cells, but not or at a low level in normal cells.[51] Moreover, in normal colon tissue, CEA is only expressed on the epithelium of the lumen, which is impenetrable by IgG antibody.[52] CEA Nb fuses with β-lactamases to localize β-lactamases inside the tumor and kill the tumor in situ by catalyzing β-lactam anti-tumor prodrugs to produce cytotoxic drugs.[53] This antibody-dependent enzyme prodrug therapy system can cause specific transplant tumor decline or cure, and has a strong targeting for tumor therapy.[54] It has been proved by experiment that this conjugate can establish a non-adverse effect tumor treatment method by selectively activating anticancer precursors.[53] In addition, CEA Nb is more sensitive in clinical diagnosis and treatment of lung cancer than traditional antibodies.[55]

Mucin 1 (MUC1) is not only overexpressed in a variety of tumor tissues but also glycosylation does not completely expose the concealed epitopes under normal conditions, which has become the target of immune diagnosis and therapy.[56,57] Rahbarizadeh F's team screened MUC1 Nb from the immune library for the first time.[58] The Nb was well expressed in Escherichia coli,[59] Pichia pastoris,[60] tobacco,[61] and the rest. Then, they used MUC1's Nb to construct chimeric antigen receptor modified T cells (CAR-T cells) and introduced the T cells into tumor cells to play the role of cellular immunity.[62]

Hypoxia-inducible factor-1 α (HIF-1α) is a crucial transcription factor that accommodates endosomatic oxygen homeostasis.[63,64] It promotes the proliferation and metastasis of tumor cells by regulating angiogenesis and glucose metabolism through downstream target genes.[65,66] The GROOT group screened the specific antibody against the oxygen-dependent domain (ODD) of HIF-1α from the unimmunized camel-derived Nb library.[67] The antibody down-regulated the transcriptional activity of HIF-1α to the target gene by binding to the ODD domain of HIF-1α. Since then, they have made an in-depth study on the conditional inactivation of HIF-1α with HIF-1α specific Nb. Through the binding of HIF-1α nanobody to the active site of PAS-B, the amount of HIF-1 (only the dimerization of HIF-1α and HIF-1β and tying up the target gene has transcriptional activation to the target gene) can be reduced, thus inhibiting the proliferation and metastasis of tumors.

Polyvalent antibodies targeting two or more different tumor-associated antigens at the same time can theoretically improve the anti-tumor efficacy to a certain extent. The multivalent Nb effect of anti-VEGF coupled with anti-angiopoietin 2 (ANG2) Nb was better than that of their monovalent Nb.[68] The polyvalent antibody MaAbNA formed by the coupling of anti-EGFR Nb with two anti-HER2 is being verified in vivo.[69] The recombinant protein formed by the fusion of anti-EGFR Nb and highly permeable cancer-specific associative peptide iRGD can enhance the anti-tumor clinical efficacy, and improve the permeability of tumor tissue.[70] Besides, through the guidance of signal sequence, direct targeted delivery of Nb in vivo has also become one of the methods to raise the efficacy of Nb. For example, linking anti-CapG Nb to the signal sequence T3s of EspF (*E. coli*-secreted protein F), intracellular delivery of tumor cells by bacteria with type III protein secretion, or targeted delivery of tumor cells through SP-neurokinin-1 receptor pathway have achieved good results.[71]

Nanobody Targeting New Tumor-Associated Receptor

In addition to the above common tumor targets. For example, the two G protein-coupled receptors (GPCRs) C-X-C chemokine receptor type 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3) are members of the class A chemokine GPCR family and embody biological targets for human immunodeficiency virus (HIV) infection,

cancerous growth, and auto-immune and inflammatory disorders.[72] Binding of synthetic nanobody to the intracellular transducer pocket of the receptor stabilized the great conformational change profiles of activated GPCRs.[73] ACKR3, which involved in stages of tumorigenesis, there is no reported description of mAbs against ACKR3 in the development of preclinical or clinical research currently, whereas the ACKR3 targeting nanobody NB1-NB3 effectively reduced tumor growth and improved overall survival in a xenograft model system by using the head and neck cancer 22A cell lines which highly express ACKR3. The preliminary experimental results suggested that the NB1-NB3 nanobody did not affect cell-cycle progression but restrained the excretion of angiogenic factor CXCL1.[74] Therefore, it demonstrated that nanobody is a potential alternative to mAb advancing GPCR targeting under the circumstances that there are still no selective antibodies approved against ACKR3, thus ACKR3 targeting nanobodies are expected to reverse the immunosuppressive cancer microenvironment and use as the new clinic therapeutics or research tools.

Other new targets also provide a basis for Nb therapy. CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) is a member of a family of immunoglobulin-related receptors and serves as a critical regulator of T cell responses to tissues.[75] Decreasing CTLA-4 function by nanobody blockade should activate self-reactive T cells and alter Treg homeostasis. Our previous study suggested that the CTLA-4 Nb16 induced CD8^{+} T cell activity with stronger cytotoxicity and anti-tumor effects against HepG2 cells for adoptive immunotherapy.[76] CXCR4 is the receptor of chemokine CXCL12, and CXCL12 is likewise understood as stromal cell-derived factor-1 (SDF-1).[77] CXCR4 Nb can block the formulation of CXCR4/CXCL12 axis,[78] thus imposing restrictions on the propagation of cancer cells;[79,80] Endothelial glycoprotein endoglin (CD105) Nb restricts endoglin-mediated signal transduction pathway and has an anti-proliferative effect on neovascular endothelial cells in tumor and neovascularization;[81,82] Heterogeneous nuclear ribonucleoprotein K (hnRNP K) Nb inhibits the invasion and metastasis of tumor cells by controlling the expression of genes related to tumor progression;[83–85] CapG Nb can control G-actin aggregation and strongly inhibit breast cancer metastasis;[71] The novel, orally administered anti-TNF agent, nanobody V565, having achieved Phase II trials for treatment of Crohn's disease.[86] The α3β1 integrin (VLA-3) Nb can inhibit VLA-3-connected cell-matrix adhesion.[87] The

increase of B cell-activating factor (BAFF) seemingly contribute to various B cell diseases. Anti-BAFF Nb can cut down extracorporeal cell proliferation in cancer tumors.[88] Anti-BAFF/CD20 bispecific Nb can improve the tissue targeting of B-cell disease and change the drug resistance of patients with chronic lymphoblastic leukemia (CLL) to CD20 antibody therapy.[89] CD38, as a cell surface antigen, can heavily be expressed in a number of hematological diseases, including multiple myeloma, and it has been verified to be a conducive target for immunotherapy strategy against this disease.[90] A few Nb can influence CD38 activity in a dose-dependent mode and specifically target tumor cell CD38 in mouse xenotransplantation model.[91,92] Because of its high solubility, Nb can form chimeric antibodies with hinge, CH2 and CH3 domains of human IgG1, this chimeric antibody can induce classical Fc-mediated cytotoxicity.[93] Although Nb has made much exciting progress in the tumor therapy side, the pharmacokinetics, targeting effect and immunogenicity of Nb drugs undergo further optimization.

Nanobody as Targeting Ligand for Tumor Drug Delivery

In the design of the targeted nano-drug delivery system, the antibody-drug conjugates (ADCs) coupled with the surface of nanoparticles (NPs) or conjugated directly with drug molecules can achieve the targeted delivery of traditional anticancer drugs.[94] ADCs are rather a concept of targeted drug design that has attracted much attention in clinical practice. Compared with small-molecule ligands such as folic acid and RGD (Arg-Gly-Asp) tripeptides, the most prominent advantage of antibodies is the excellent targeting performance of antibody-antigen binding.[95,96] Since Trasmzumab was used in the treatment of HER2$^+$ breast cancer in 1998, the development of mAb antibody drugs targeting the EGFR family has been gaining momentum.[97] The huge advantages in tumor clinical treatment, such as reducing toxic and side effects and overcoming multidrug resistance, make the research and application of antibody drugs in clinical treatment have a great prospect.[98,99] Nb, a star molecule, is expected to replace mAb and becomes a feasible strategy to improve the effectiveness of nanomaterials-based cancer diagnosis and therapy. As early as 2004, Cortez-Retamozo and her team fused Nb targeting human tumor-associated embryogenesis with Enterobacter Cloacae β-lactamase in the form of fusion expression to prepare nanobody

conjugations which had better target ability in animal model experiments and has the obvious effect of decreasing tumor size and even curing the animals.[53]

Traditional small molecule anticancer drugs are often limited in clinical application due to side effects and drug resistance. The concept of nanodrug delivery system (nanoDDS) reflected the new design philosophy for drug delivery methods.[100] Embedding small-molecule chemotherapeutics with nanoscale drug carriers can not only solve the problem of hydrophobic drug solubility, the problem of easy removal of drugs in the blood circulation process, and the accumulation of difficult drugs in the treatment target.[101] In addition, it is also possible to prepare an integrated diagnosis and treatment system, a multi-drug co-transport system, chemotherapy combined with light and hyperthermia, or a combined system of chemotherapy with other means of complex system.[102] The multifunctional DNA-based nano platinum-drug delivery system provided a promising strategy for the treatment of tumors, an anti-EGFR Nb was conjugated to DNA and assembled on to double-bundle DNA tetrahedron, which presented the first example of DNA intercalator and EGFR blockade.[103] By modifying nanoDDS with targeted functions of ligands, the enrichment of nanomedicine in tumor tissues can be enhanced so as to improve the distribution of drug system in vivo and increase the specific uptake of nanoDDS by tumor cells.[104,105] There are quite a few researches about modifying Nb on the outer layer of nanoparticles to enable nanoDDS actively targeted,[106] such as liposomes,[107] micelles,[108] protein nanostructures,[109] inorganic nanoparticles,[110] polymer composites,[111] etc.

Although Nb has no Fc effector and does not produce cytotoxic effects such as ADCC like traditional antibodies, it can be used as a targeting moiety on liposomes or nanoparticles encapsulating cytotoxic drugs to enhance the killing effect of tumor because of its high specificity and strong tissue penetration in Figure 4.[112,113] For example, VEGFR2 Nb-associated *Pseudomonas aeruginosa* exotoxin has a better anti-tumor effect,[114] and DM1, a microtubule polymerization inhibitor associated with major histocompatibility complex-II (MHC-II) Nb, also has a good therapeutic effect.[94] The formation and mechanism of nanobody-drug conjugates for targeting tumor cell with overexpressing EGFR is systematically elucidated in Figure 5 and the nanobody-based platform for targeted treatment system has been developed.[115] Neural stem cells (NSC) were transfected with recombinant proteins encoding bivalent anti-EGFR Nb and
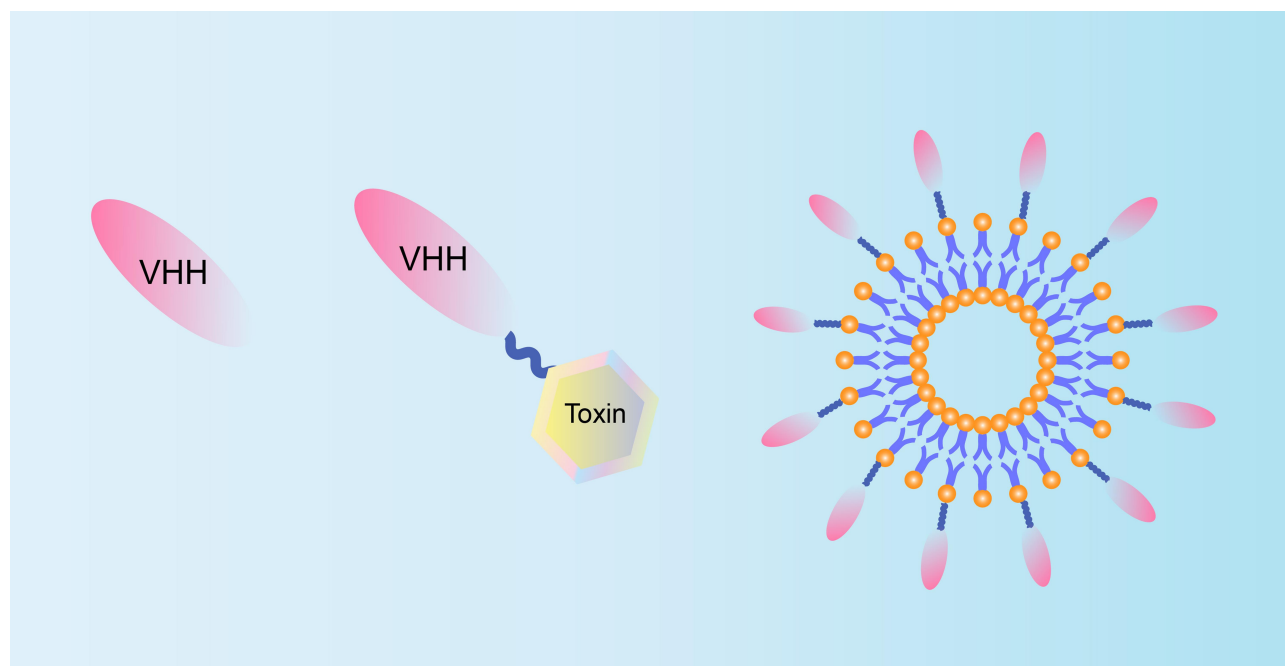
Figure 4 Diagrammatic sketch of nanobody-based therapeutic drug design. Left: Receptor antagonists, interfere with signal transduction and receptor activation; Middle: Targeting part of effector domain, such as medicine or toxic peptides; Right: Targeting molecules on the surface of nanoparticles, such as micelles or liposomes. Adapted from Bannas P, Hambach J, Koch-Nolte F. Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics. *Front Immunol*. 2017;8:1603. Creative Commons license and disclaimer available from: http://creativecommons.org/licenses/by/4.0/legalcode.[20]
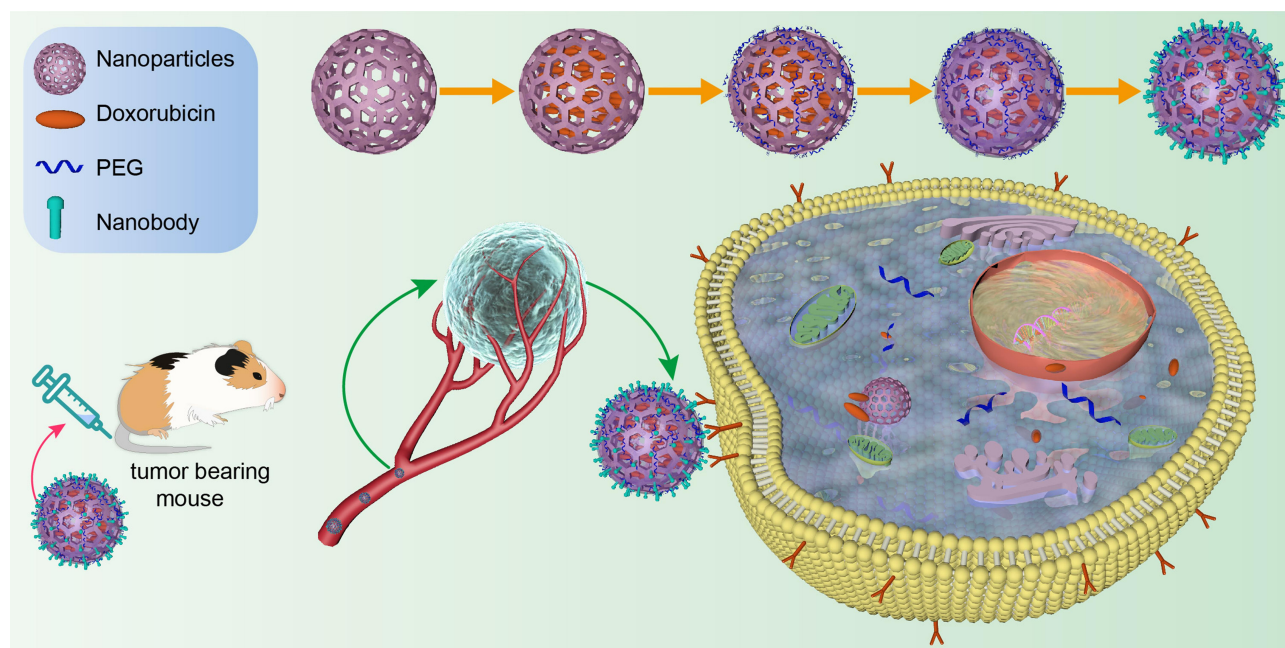
Figure 5 Schematic illustration of the construction and mechanism of nanobody-drug conjugates for targeting tumor cell. Nanobody targeting intracellular tumor marker to inhibit growth and proliferation after intravenous injection of tumor-bearing mouse: effective accumulation in tumor tissues; penetrating into tumor parenchyma; and internalized into cancer cells to produce combined chemotherapy and immunotherapy. Adapted with permission from Wang F, Zhang L, Bai X, et al. Stimuli-Responsive Nanocarrier for Co-delivery of MiR-31 and Doxorubicin To Suppress High MtEF4 Cancer. *ACS Appl Mater Interfaces*. 2018;10(26):22767–22775. Copyright (2018) American Chemical Society.[197]

cytotoxic TRAIL, which significantly reduced the viability of malignant glioma cells.[116] A HER2-specific Nb comes complete with a molecule that can recruit anti-dinitrophenyl antibodies that can cause tumor cell destruction.[117] Polymeric nanoparticles can serve as a vehicle for the delivery of cytotoxic proteins that have intracellular targets, that selective uptake can be attained by decorating the surface of the NPs with the 11A4 nanobody that is specific for the HER2 receptor.[118] The flexible nanobody-based system can be adapted to load pentamidine, and designed with components based on polyethylene glycol (PEG) and were conjugated with a nanobody that specifically recognizes the protozoan pathogen Trypanosoma brucei.[119] Simone and his group have pioneered the application of elastin-like polypeptides (ELPs) as the same category of peptide polymers that self-assembly into nanoparticles for drug delivery to optimize the ligand-nanoparticle interface to enhance cancer cell targeting by polymer micelles.[120] In the antibody-directed enzyme-catalyzed prodrug therapy (ADEPT) experiment, it was found that the Nb of anti-CEA delivered the coupled β-lactamases to the surface of CEA^+ tumor cells, activating the anticancer prodrug cephalosporin nitrogen mustard, thus improving the selectivity of anticancer drugs to tumor cells.[53] The relevant experimental results showed that the EGFR-Rapa-NPs system modified with EGFR antibody had a better tumor inhibition effect than the drug delivery particles without antibody conjunction, and at the same time had significant cellular selectivity.[121] The Nb of the target EGFR was acylated by biotin and coupled with the colloidal particles coated with streptavidin, which could directly target $EGFR^+$ liver tumor cells.[122] Targeting plexinD1 (a membrane-penetrating protein expressed in tumor vessels) Nb-modified tumor vascular targeted polymerase represented a promising approach in treating divergent carcinomatosis.[104,123]

Therefore, Nb can play an important role as a drug delivery system. The sealed drugs can be delivered to the action site by using the specificity of mass production of versatile nanobody repertoires.[124,125] The Belgian pharmaceutical firm Ablynx stepped into standard nanobody therapy which was tiny enough in volume that can be ingested, whereas the traditional antibody drug was given by injection to enter the bloodstream.[126] This method can avoid the systemic toxicity caused by common drug administration, and allow hydrophobic drugs to be transported in hydrophilic structures. In addition, large doses of drugs can be administered in this way, these drug delivery systems

incorporated into Nb can protect drugs against oxide-reduction and enzymatic reactions, so as to avoid the immunogenicity produced by multiple administration.[127–129] Coupled with the effect of other drugs and Nb itself can inhibit tumor growth, which is equivalent to the dual function of Nb.

Nanobody in Combination with Other Tumor Therapies
Nanobody Combining with Radioimmune Therapy (RIT)
At present, the main radionuclides used in clinic are β-decay nuclides, including ^{90}Y, ^{131}I and ^{177}Lu, but some α-decay nuclides have been used.[130] The radionuclide labeled anti-CD20 monoclonal antibody (^{90}Y-ibritumomab tiuxetan) approved by FDA has been put on the market,[131] and 44 kinds of these drugs in the treatment of solid tumors and hematological malignant tumors are in clinical trials.[132,133] When common radiopharmaceuticals are used in RIT, while the advantage of long half-life brings bad pharmacokinetic characteristics to radionuclides and poor permeability to tumors, which limits their targeting effect. By comparison, the radiolabeled Nb is more targeted and can be quickly removed from the blood.[134] ^{177}Lu-labeled targeted M protein (M protein is a monoclonal antibody produced by malignant plasma cells) Nb is being used in the treatment of multiple myeloma in order to inhibit the progression of the disease.[135] The 2Rs15d of ^{177}Lu labeled targeted HER2 was prepared. The metal binding region of the bifunctional joint 1B4MDTPA was connected with radionuclides and the chemically active region was connected with Nb.[136] In order to avoid radiation damage caused by the accumulation of Nb in renal tissue, co-administration with plasma expander succinyl gelatin (Gelofusin) could almost completely block the growth of tumor in $HER2^+$ tumor-bearing mice. There was critical variation among event-free survival (EFS) between the treated group and the non-treated group.[137]

Nanobody Combining with Photodynamic Therapy (PDT)
In contrast to radionuclides in RIT, PDT can damage irreparably tumor cells via motivating photosensitizer (PS).[138] On the basis of PDT preponderance for precise treatment and slight side effect,[139] currently at the forefront of research, PS takes on a shape of treelike gold nanoparticles (branched gold NP), because of its huge

absorption cross section, NP will destroy tumor cells through heat production after laser irradiation.[140] The junction which might selectively accumulate in vivo between Anti-HER2 nanobody and PS can give rise to striking inhibitory effect on especially against trastuzumab HER2$^+$ breast carcinoma with a single course of therapy, the tumor extinction was positive dose dependent, which will greatly enhance specifical targeting for photothermal anticancer agents.[141] The combination of molecular imaging and tumor therapy in the connection application of Anti-EGFR univalent or multivalent Nb and traceable PS (IRDye-700DX) is major progress in the clinical application of PDT technology.[142]

Nanobody-Modified CAR-T Therapy

Nb can be more than just steering anti-tumor function, medicine carrier system or radionuclide treatment, but act an adjustive character on macrophages which have long been considered to be important immune effector cells.[143] Cytokines are molecules which possess broad development prospect to be used in anti-tumor immune treatment on account of irritating the natural killer cells of both innate and acquired immunity.[144,145] According to the theory that FcγRIIIa (CD16a) on immune effector cells can mediate cytotoxicity, two Nb (C21 and C28) specifically binding to FcγRIIIa isolated from camel immune library indirectly mediate the ADCC effect of NK cells.[146] If the Nb is linked to anti-CEA Nb, it can dissolve CEA$^+$ tumor cells through NK cells in vitro models of human cells and tissues, and also suppress cancer cell growth in CEA$^+$ tumor transplanted mice, which fosters new thinking on the development of anti-tumor Nb.[147]

There was too much of a correlation between immune infiltration and tumor microenvironment (TME), which has turned out to be enormously successful in clinical cancer immunotherapeutic.[148,149] CAR-T cell therapy is one of the research hotspots of tumor immunotherapy and has a good prospect for the treatment of hematological tumors, which makes patients' own T cells genetically modified to better attack cancer cells (Figure 6B).[150,151,195] Compared with traditional antibodies, Nb has more advantages in the process of constructing CAR-T cell.[152] At present, the binding sites of antigens recognized in CAR are mostly designed based on the scFv (Figure 6A), which has high immunogenicity. Nb can reduce this kind of immunogenicity.[153] CAR-T cells obtained by using Nb as a connecting or targeting part is also one of the effective ways to avoid tumor escape. At

the same time, it was found that Nb-based CAR-T could fully exert its advantages of T cell immunotherapy, such as resultful tumor invasion and metastasis, induced the release of inflammatory cytokines and cytotoxic action. On the Nb treatment side, which also has relative comparative advantages in small size, decreased immunogenicity and strong specificity of related tumor antigens.[154] For instance, CAR targeting melanocyte differentiation antigen gp100 Nb can inhibit the development of melanoma in vivo.[155]

However, CAR-T cells are not good at treating solid tumors because it is difficult to find tumor-specific proteins that can be used as safe targets in solid carcinoma.[156] Given the current situation that CAR-T cell treatment has yet to be extrapolated to solid malignancies, researchers plan to solve this problem according to the characteristics of Nb, which can advance CAR-T cell technology for therapy of carcinoma.[149] One of the unique properties of Nb is its enhanced targeting ability to carry imaging agents so that metastatic tumors can be visualized accurately. Jailkhani and his team directed these Nb to the tumor extracellular matrix (ECM) or as targeted imaging agents for ECM, mainly targeting the tumor microenvironment that surrounds cancer cells.[157] The CAR-T cells constructed by some investigators are embedded with Nb, to recognize specific proteins in the tumor microenvironment, and Nbs usually carry signals that guide them to kill the cells they can bind to.[158,159] For example, a protein called EIIIB is a variant of fibronectin and is found only in neovascularization that provides nutrients for tumors.[160] The other is PD-L1, an immunosuppressive protein that is used by most tumors to silence neighboring T cells.[161] Use these relatively specific antigens to prepare Nb-based CAR-T cells. By testing in two different melanoma mouse models, these cells can kill tumor cells and significantly improved the survival of these mice without distinct side effects. Targeting EIIIB may damage blood vessels in a way that reduces the tumor's blood supply while making them more permeable to tumor drugs. The preliminary results show that the method may achieve ideal results for many solid tumors, such as pancreatic cancer and cholangiocarcinoma models.[149]

Although CAR-T can effectively induce T cells to attack tumor cells, it can also produce side effects such as cytokine storm syndrome.[162] In order to further reduce this side effect and improve the curative effect, some researchers developed a T cell antigen coupling agent, which can couple the chimeric antigen of endogenous
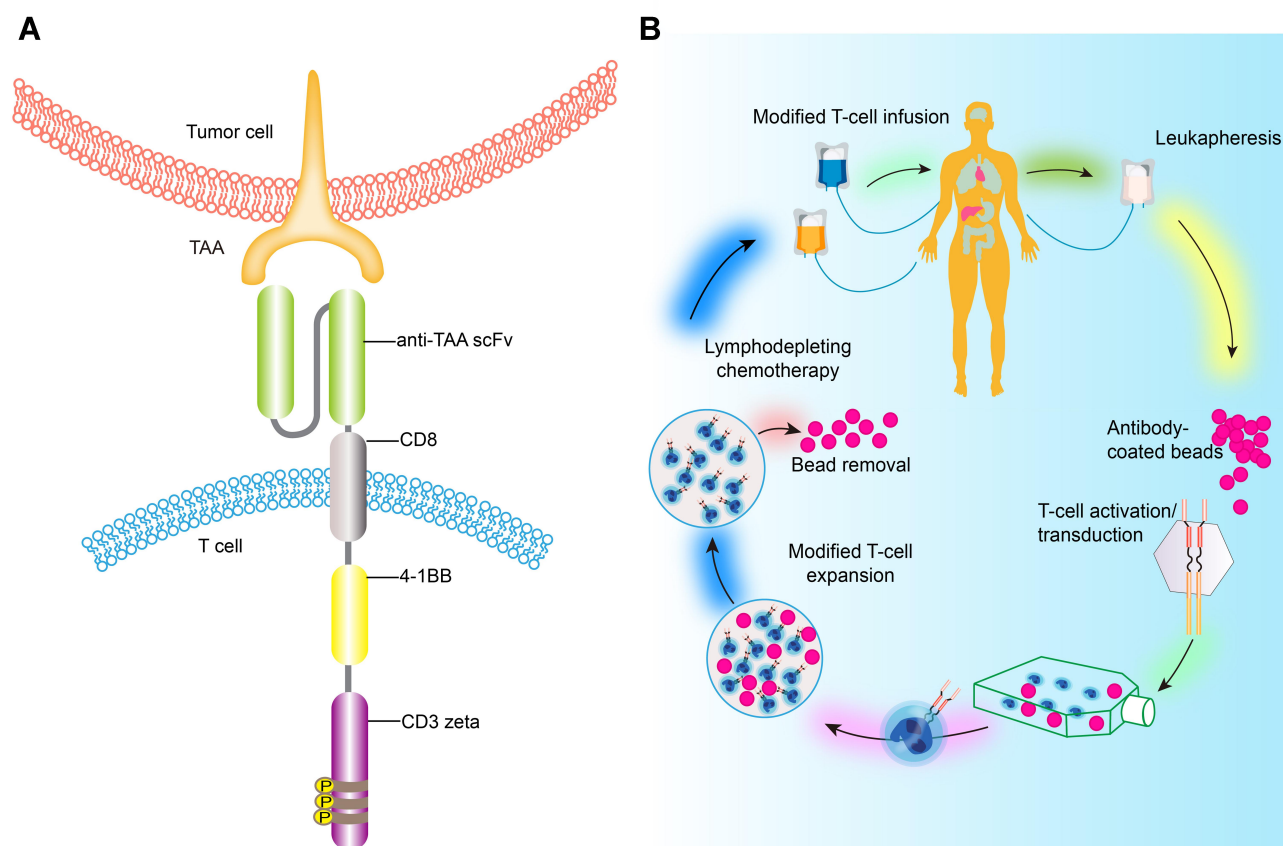
Figure 6 A brief flow chart of engineered-T cell therapy. (**A**) represents the CAR, which includes the single-chain variable fragment (scFv) that binds to tumor antigens, fused to a spacer and transmembrane domain. The intracellular domain contains costimulatory domains, such as CD28 and 4–1BB and the CD3ζ chain, which cause signal activation and amplification of CAR T cells. S–S denotes disulfide bond. Adapted from Tyagarajan S, Spencer, Smith J. Optimizing CAR-T Cell Manufacturing Processes during Pivotal Clinical Trials. *MOI Ther Methods Clin Dev.* 2020;16:136-144. Creative Commons license and disclaimer available from: http://creativecommons.org/licenses/by/4.0/legalcode.[194] (**B**) shows a process of CAR-T therapy, firstly apply the patient's own T lymphocytes, which have been reconstructed in the laboratory and loaded with receptors and co-stimulating molecules that recognize tumor antigens. After amplification in vitro, they are re-introduced to the patient to recognize and attack their own tumor cells. Adapted from Tyagarajan S, Spencer, Smith J. Optimizing CAR-T Cell Manufacturing Processes during Pivotal Clinical Trials. *MOI Ther Methods Clin Dev.* 2020;16:136-144. Creative Commons license and disclaimer available from: http://creativecommons.org/licenses/by/4.0/legalcode.[194]

TCR, namely chimeric TAC-T (T cell antigen coupler-T cell).[163] It mainly includes the design of three key parts: one is that the extracellular antigen binding domain is changed into Nb, the second part is extracellular anti-CD3 scFv, and the third part is the membrane-penetrating CD4 domain connecting protein kinase LCK. TAC-T represents a significant advance in CAR-T and TCR-T therapies because TAC is able to identify tumor cells more selectively, utilize natural activation and regulatory mechanisms to activate T cells through natural TCR, and lack tonic signaling, so that T memory cells persist (instead of being depleted).[164,165] Thus, TAC engineered T cells can reasonably hope to facilitate a treatable tumor-specific reaction that reduces toxicity and eliminates solid tumors.[166] In the solid tumor model, TAC-T cells showed stronger anti-tumor effect, lower cytotoxic side effects,

faster tumor penetration and higher therapeutic index than CAR-T cells activated by the second signal based on CD28. According to the efficacy of TAC-T cells and the biological characteristics of Nb, TAC-T-like cells based on Nb may also produce more ideal therapeutic effects.[167]

Challenges and Prospects in Nanobody-Related Tumor Therapy

Antibodies have been used more than decades for ectophylaxination to treat infected patients. While polyclonal antibodies produce superior neutralization and thus better protection under normal circumstances, monoclonal Nb selected for recognition of special epitopes might reach an exceedingly high-neutralization potency.[168] Although the current research results show that Nb has great potential

in tumor treatment,[169] the application of Nb still has some weaknesses: (1) The immunized animals such as camels are relatively expensive compared with the frequently used mice for the production of antibody.[113] (2) Their small dimension is also a disadvantage in cancer treatment as they are rapidly eliminated from the blood via individual kidney glomerular filtration, hence a small proportion of the total Nbs aggregate at the site of action, which might be settled by administration of standard and high dose regimens.[170] (3) The negative impact that Nb will not biodegrade immediately when they are released into the environment due to it yields higher stability,[171] therefore the risks of Nb need to be evaluated due to antibiotics release in groundwater can destruct agriculture.[172]

On account of nanobodies connect the salutary properties of micromolecule and monoclonal antibodies, an attractive agent for new therapeutic strategies is likely to emerge. Until now, biomedical engineering is turning into a level of subcellular, which is advanced with a rapid-developing field of research on nano diagnostics, nanomedicine and nano therapy in joint with the success of nanoparticles on sickness prevention and health promotion.[173] Cancer treatment has hit a bottleneck, tumor cells proliferate in complicated tumor microenvironments, thus causing heterogeneous diseases, usually with a fatal outcome in later stages for the patient. Therefore, nanobody emerges as the times require. Nanobody is a new and unparalleled class of multiple antigen-binding fragments, which originated from heavy-chain antibodies extracted from the serum of camelids naturally. Their favorable characteristics like small size, strong consistency, top antigen-binding affinity, natural formation and water-soluble material might generate the next cancer drug. Consequently, Nb has extraordinary potential in cancer care and control, and is anticipated to make a significant breakthrough technology in immunotherapy field. Their small volume acquires them conducive to target antigens residing in different tissues that are impotently vascularized and barely accessible.

The infiltration and growth of certain types of solid tumors typically hinge on the generation of new blood vessels and acceleration of their maturation and stabilization. Previous studies have shown that a few nanobodies take part in impacting this phenomenon of vascularization.[174] Up to now, potential targets applied to nanobody-based therapies are extracellular targets. For instance, ligand receptors or transmembrane proteins with

differential expression in major target cells. For this purpose, nanobodies against transmembrane growth factor receptors EGFR, VEGFR-2, c-Met and chemokine receptor type 7 (CXCR7) have been developed.[175–179] those receptors have been related to cancerous growths. Moreover, in oncologic therapeutic areas understanding the molecular biology of the disease increases; thus, the chances of developing specific targeted drugs increase. So nanobodies can be used either as antagonists, to prevent ligand binding and causing conformational changes that lead to activation of signaling cascades for the cancer therapy, their superior properties (small size, high antigen-binding affinity, low immunogenicity, high stability and solubility) allowed for several groups to conduct clinical trials.[180–182]

Based on the fact that nanobodies can bind to each protein and enzyme to form a combination with dual functions, the inactive prodrugs can be transformed into active drugs that can kill cancer cells, so as to focus on targeted chemotherapy of tumor cells, to achieve high efficiency and without any adverse reactions. It is envisaged that the construction of nanobodies and molecular drugs for the treatment of cancer will make use of the targeting and good tissue permeability of nanobodies. It will make antineoplastic drugs cease a more directly effective function especially in imaging and radionuclide therapy.[183]

The construction of multi-target nanocomposite antibody or multi-target combination of drugs will bring a new perspective to the treatment strategy of malignant diseases and play a better curative effect. With the in-depth study of biochemistry and human genomics and the exploration and development of nanobodies, an increase number of therapeutic targets for cancer will be found. Recently a shuttling-nanobody FC5 that targets immunological access linked to neuroinflammation for drug delivery technique of breaking brain barrier has been developed.[184] According to this, an ideal nanomedicine might have the following characteristics, which include tumor accumulation, deep penetration, long circulation, cellular internalization, and drug release for highly efficient tumor-associated therapeutic strategies (Figure 7). In the context of the global outbreak of COVID-19 pandemic and urgent demand of pressing development for effective prophylactics or therapeutics especially for immunocompromised patients. LQ050, the world's first inhaled COVID-19 neutralizing nanobody drug, due to its excellent stability profile, and superior nebulization,
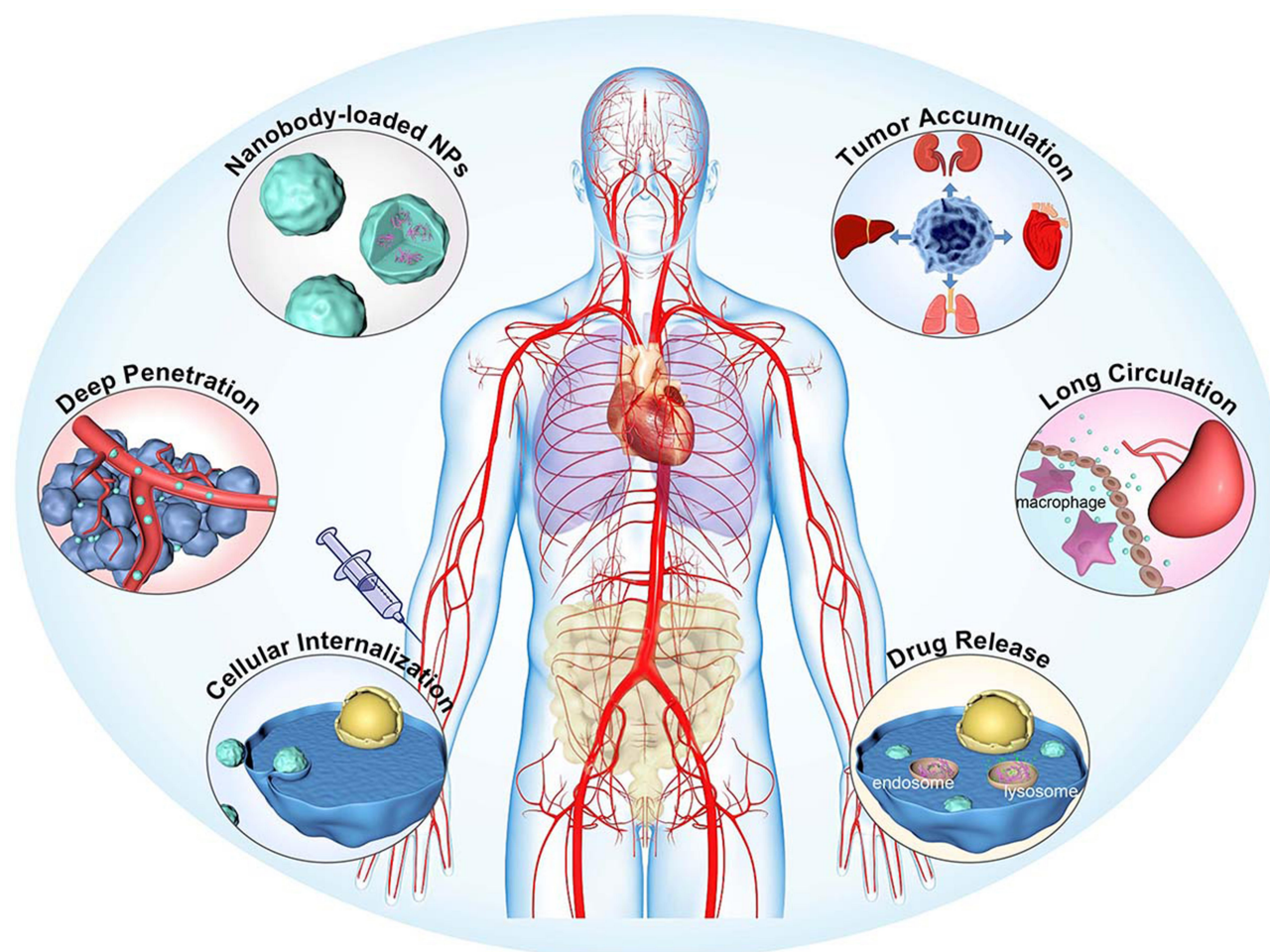
Figure 7 Nanoparticles as antitumor Drugs. The overall therapeutic efficiency of a nanobody-based compound medicine is determined by its efficiency in each step. During the transportation of blood lumen and tumor tissue, it must hold the drug tightly without burst release, and synchronously release the drug until at the intracellular site to exert its pharmaceutical action. Adapted with permission of Royal Society of Chemistry, *Chem Soc Rev*, from Influence of nanomedicine mechanical properties on tumor targeting delivery. Li Z, Xiao C, Yong T, Li Z, Gan L, Yang X. 49(8):2273–2290, copyright 2020; permission conveyed through Copyright Clearance Center, Inc.[198]

might be the first nanobody drug against SARS-COV with the delivery manner of respiratory drug.[185] As a result, the targeted therapy of nanobodies for tumor treatment has entered a new era.

The optimization of phage display technology will promote the construction of Nb library and the screening of specific Nb. The improvement of humanized Nb will further reduce the immunogenicity of Nb and improve the safety and effectiveness. Its strong tissue penetration will facilitate the intervention and treatment of solid tumors, especially the local microenvironment.[186] Nano-carriers carrying radionuclides, toxins or chemotherapeutic drugs will provide more treatments for tumor patients. CAR-T or TAC-T based on Nb will become the replacement of traditional CAR-T. The nanobody platform is now a main focus for immuno-oncology discovery research for pharmaceuticals giant and nanobody is now being tested in a series of clinical trials

which are shown in Table 1.[187] In a word, Nb will achieve a breakthrough in replacing traditional targeted antibody drugs, ADC drugs, fusion proteins, local tumor intervention therapy and oral antibody therapy.

Conclusion

To summarize, nanobodies are versatile molecules with favorable properties and have been evaluated for both therapeutic and diagnostic applications, as well as research tools. Nanobodies are featured by their small size, increased solubility as well as desirable stability opening up the gate towards previously inaccessible antigens in cancer immunotherapy. These tiny antibodies outperform conventional antibodies in quite a few respects and thrive in extreme conditions, ultimately, they will occupy a momentous portion of the antibody reagent market. After the initial research on functional heavy chain antibodies in alpaca, the versatility of antigen-binding

Table 1 Nanobody in Clinical Trials

Molecule	Target	Disease Examples	Current Status	References/ Sponsor
Caplacizumab (ALX-0081)	Von Willebrand factor	Acquired thrombotic thrombocytopenic purpura	Approved	[182,188]
Ozoralizumab (ATN-103)	TNF-α	Rheumatoid arthritis	Phase 2	Ablynx
M1095(ALX-0761)	IL-17A, IL-17F	Psoriasis	Phase 1	[189]
Vobarilizumab (ALX-0061)	IL-6R	Rheumatoid arthritis, systemic lupus erythematosus	Phase 2	[190]
ALX-0141	RANK ligand	Osteoporosis, bone metastasis	Phase 1	Ablynx
BCMA nanobody CAR-T cells	BCMA	Relapsed/Refractory Myeloma	Phase 1	Yongping Song
αPD1-MSLN-CAR T cells	MSLN	Non-small-cell Lung Cancer, mesothelioma, colorectal cancer, ovarian cancer	Early Phase 1	Xiaorong Dong
M6495	A Disintegrin and Metalloproteinase With Thrombospondin Motifs-5 (ADAMTS-5)	Osteoarthritis, Knee	Phase 1	[191]
CD20/HER2 Bispecific Nanobody-derived CAR-T Cells	CD20, HER2	B cell leukemias, lymphomas	Preclinical	[192]
ALX-0651	CXCR4	Healthy Volunteers	Phase 1	Ablynx
ALX-0171	RSV F-protein	RSV Infection	Phase 2	[193]

Notes: Ablynx, Clinicaltrials.gov, PubMed, FDA.
Abbreviations: TNF, tumor necrosis factor; IL, interleukin; RANK, receptor activator for nuclear factor-κ B; BCMA, B cell maturation antigen; MSLN, mesothelin; HER2, c-erbB-2; CXCR4, C-X-C motif chemokine receptor 4; RSV, respiratory syncytial virus.

proteins, strategies for their functionalization and methods for their cellular delivery lay a potent foundation for next-generation diagnostics and therapeutic tools with outstanding properties. The way is now open to further exploit the inexhaustible potential of nanobodies and to further position them as pharmaceuticals for use in daily clinical practice.

Abbreviations

scFv, single-chain variable fragment; Nb, nanobody; sdAb, single domain antibody; HcAb, heavy chain antibody; CDR, complementarity determining region; CH, constant domains of heavy chain; CL, constant domain of light chain; Fab, antigen-binding fragment; Fc, fragment crystallizable region; FR, frame region; VH, variable domain of heavy chain; VHH, variable domain of heavy chain antibodies; VL, variable domain of light chain; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen; MUC1, Mucin 1; CAR-T cells, chimeric antigen receptor modified T cells; HIF-1α, hypoxia inducible factor-1 α; ODD, oxygen-dependent domain; ANG2, angiopoietin 2; EspF, *E. coli*-secreted protein F; GPCRs, G protein-coupled receptors; CXCR4, chemokine receptor type 4; ACKR3, atypical chemokine receptor 3; HIV, human immunodeficiency virus; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; SDF-1, stromal cell derived factor-1; CD105, endoglin; hnRNP K, heterogeneous nuclear ribonucleoprotein K; VLA-3, α3β1 integrin; BAFF, B cell-activating factor; CLL, chronic lymphoblastic leukemia; ADCs, antibody-drug conjugates; NPs, nanoparticles; nanoDDS, nano-drug delivery system; MHC-II, major histocompatibility complex-II; NSC, neural stem cells; PEG, polyethylene glycol; ELPs, elastin-like polypeptides; ADEPT, antibody-directed enzyme-catalyzed prodrug therapy; RIT, radioimmune therapy; EFS, event-free survival; PDT, photodynamic therapy; PS, photosensitizer; TME, tumor microenvironment; ECM, extracellular matrix; TAC-T, T cell antigen coupler-T cell; CXCR7, chemokine receptor type 7; TNF, tumor necrosis factor; IL, interleukin; RANK,

receptor activator for nuclear factor-κ B; BCMA, B cell maturation antigen; MSLN, mesothelin; HER2, c-erbB-2; RSV, respiratory syncytial virus.

Ethics Approval and Consent to Participate

This manuscript is a review article that does not require prior approval.

Disclosure

The authors declare that they have no conflicts of interest.

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