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Nanobodies in cancer

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Abstract

For treatment and diagnosis of cancer, antibodies have proven their value and now serve as a first line of therapy for certain cancers. A unique class of antibody fragments called nanobodies, derived from camelid heavy chain-only antibodies, are gaining increasing acceptance as diagnostic tools and are considered also as building blocks for chimeric antigen receptors as well as for targeted drug delivery. The small size of nanobodies (~15 kDa), their stability, ease of manufacture and modification for diverse formats, short circulatory half-life, and high tissue penetration, coupled with excellent specificity and affinity, account for their attractiveness. Here we review applications of nanobodies in the sphere of tumor biology.

Introduction

In this review we capture developments in the application of antibody fragments, called nanobodies, to tumor biology, covering both diagnostics and therapeutics. Spontaneous or engineered, immune responses against cancers are seen as a powerful adjunct to other forms of treatment. The ensemble of antigen presenting cells (APCs), $CD4^+$ T cells, $CD8^+$ T cells and B cells regulate adaptive immunity. $CD4^+$ T cells (helper T cells) respond when they recognize antigen presented on class II major histocompatibility complex (MHC-II) molecules on the surface of APCs. Activated helper T cells and their products enhance the adaptive immune response through activation of B cells, NK cells and macrophages. B cells present antigen via MHC-II, which is recognized by helper T cells. Helper T cells then secrete signals to differentiate B cells into immunoglobulin (Ig)-secreting plasma cells. Secreted Ig serves various purposes, from neutralization of infectious agents to enhancement of phagocytosis or complement-assisted destruction of pathogens. These effector functions are attributable mostly to crosslinking of fragment crystallizable (Fc) receptors.

In most mammals, Igs are composed of a heavy chain and a light chain, each containing a variable and a constant region. A unique type of Igs, devoid of light chains, was discovered in sharks [1] and in camelid species in 1989 [2]. Engineering of the heavy chains of the camelid heavy-chain only antibodies (hcAbs) yields single-domain antibody (sdAb) fragments, also known as nanobodies (Nb) or VHHs (figure 1A). In select cases, it has been

possible to generate sdAbs from the heavy chain variable segments of human and mouse (conventional) Igs [3–7]. While such human or mouse V_H segments can be expressed in the absence of a light chain and retain proper solubility and antigen binding properties [8,9], this is not always the case. Therein lies the importance of the discovery and development of the camelid hcAbs.

Of late, sdAbs are having a major impact on how Igs and their derivatives are used in research and in practical applications. Despite being only ~1/10th the size of their full-sized counterparts, nanobodies retain the characteristics of antigen specificity and binding affinity. Other favorable attributes of nanobodies are their solubility [10] and stability [11], as well as ease of production in bacteria, thus enabling large-scale production [12]. Their small size (~15 kDa) endows nanobodies with excellent tissue penetration [13] and rapid clearance from the circulation ($t_{1/2}$ < 30 min) [14]. Because of their unique characteristics and relative ease of production, nanobodies are increasingly used in a variety of applications, such as delivery of drugs or radioisotopes, as well as imaging of tumors and other tissue types. The half-life of nanobodies can be extended at will, for instance by chemical modification with polyethylene glycol (PEG) [15], through fusion of the nanobody to serum albumin nanoparticles [16] or to a serum albumin-binding nanobody [14]. The field of nanobodies continues to advance rapidly. Several excellent reviews on the generation, properties and application of nanobodies across broad areas of biomedical interest have appeared [12,17–28]. The purpose of this review is to focus on recent applications of nanobodies in tumor immunology, primarily in the context of diagnostics, imaging, and therapeutics. We provide an overview of available nanobodies and the (tumor) targets they recognize, as well as their applications. While in many cases nanobodies are used in lieu of conventional antibodies, possibly to avoid intellectual property conflicts, it is helpful to think of nanobodies as immunological tools with unique properties.

Tumor-targeting nanobodies

Nanobodies have similar antigen-binding properties as conventional antibodies. However, because nanobodies employ a single Ig variable domain for antigen recognition, they can access epitopes that are beyond the reach of conventional antibodies or antibody derivatives such as single chain Fv fragments (scFvs). For example, nanobodies can penetrate into a cleft on a protein's surface or at a domain-domain interface. Currently available nanobodies for tumor-relevant targets are listed in Table 1. Figure 1B shows an overview of nanobody targets in relation to the tumor (microenvironment). In some cases, the nanobodies cross-react with homologous targets from other species. This may facilitate the transition from pre-clinical to clinical applications. Examples include cross-reactivity with human and murine antigen for the anti-EGFR nanobody 8B6 [29], the anti-HER2 nanobody 2Rs15d [30] and the nanobody directed against the EIIIB splice variant of fibronectin [31].

EGFR family

Members of the epidermal growth factor receptor (EGFR) family are often over-expressed on the surface of tumor cells of epithelial origin and play a role in their proliferation, survival, and in angiogenesis [32]. Antibodies that target the EGF receptor have been proven

successful in cancer treatment. An example is cetuximab, a full-size chimeric mouse/human monoclonal antibody specific for the EGFR [33]. Therefore, EGFR family members have been among the first tumor markers targeted by nanobodies. EGFR1-targeting nanobodies were identified by phage display, using competitive elution with the ligand EGF to identify specific binders [34]. Using the same EGFR phage nanobody repertoire and selecting for the EGFR extracellular domain, the nanobodies 7C12 and 7D12 [35] and 9G8 [34] were identified. The former competes with cetuximab, the latter does not. Multivalent nanobody molecules can be built by fusion of individual nanobody gene segments or through chemical conjugation methods. EGFR-specific nanobodies were formatted into bivalent molecules in different combinations, all of which inhibited tumor cell proliferation in an *in vitro* epidermoid cancer model. Specifically, the combination of 7D12–9G8 anti-EGFR nanobodies performed best in inhibiting EGFR signaling and reduced the growth of human epidermoid carcinoma A431 cells. When linked to Alb1, a serum albumin-binding nanobody, the construct was called CONAN-1, which strongly inhibited EGF-induced signaling, leading to tumor regression in A431 xenograft-bearing mice [36].

Using similar methods, the anti-EGFR nanobodies 8B6 and OA-cb6 were obtained [29,37]. Nanobodies that recognize HER2, another member of the EGFR family, specifically target $HER2^+$ SKOV3 ovarian cancer cell-derived tumors *in vivo* [30]. HER2-targeting nanobodies 11A4 [38] and 5F7GGC [39] have been used for a variety of (clinical) applications, described elsehwere in this review.

VEGFR2 and VEGF

Vascular epithelial growth factor receptor 2 (VEGFR2) is part of the human VEGFR family of receptors and is present on vascular endothelial cells. Its ligand, VEGF, is secreted by cell types such as macrophages and tumor cells, thereby inducing downstream signaling pathways involved in cell proliferation, angiogenesis and metastasis [40,41]. This makes VEGF and VEGFR2 appealing targets for nanobody-based therapies, for example to prevent the formation of new blood vessels on which tumors rely for nutrient and oxygen supply. The anti-VEGFR2 nanobody 3VGR19 was obtained by phage display on recombinant extracellular domains of the VEGFR2 receptor. It inhibits VEGFR2 signaling, thereby inhibiting the formation of capillary-like structures, as shown in an *in vitro* study on human umbilical vein endothelial cells (HUVEC) [42]. Ma et al. isolated an anti-angiogenic VEGFR2-D3 specific nanobody NTV1 from HuSdl™, a human single domain antibody library of ‘camelized’ human antibodies [43]. In similar fashion, nanobodies specific for VEGF were obtained. These inhibit endothelial cell proliferation in an *in vitro* angiogenesis assay using HUVECs [44]. A humanized version of one of these nanobodies, Nb42, has also been generated [45]. Lastly, the nanobody VA12, which specifically targets the binding domain of VEGF-A, showed anti-angiogenic potential in a chorioallantoic membrane assay [46].

c-Met and HGF

Hepatocyte growth factor (HGF) binds to the c-Met receptor [47], which activates pathways responsible for cancer progression, angiogenesis and metastasis [48]. For several different epithelial and nonepithelial cancers, overexpression of HGF and the c-Met receptor are

associated with a poor prognostic outcome [49,50]. Nanobodies against c-Met and HGF have been produced. The anti-cMet nanobody G2 competes with HGF for binding to the c-Met receptor [51]. Schmidt Slørdahl et al. used a bispecific nanobody, with one nanobody to target c-Met and the other nanobody to enable binding to human serum albumin for half-life extension. This bispecific anti-c-Met nanobody inhibited the interaction of c-Met with HGF and led to a reduction in cell migration and adhesion in multiple myeloma cells. This bispecific nanobody was even more efficient at inhibiting tumor growth than a conventional bivalent monoclonal anti-c-Met antibody [52].

The bispecific albumin- and HGF-specific 1E2-Alb8 and 6E10-Alb8 nanobodies showed a dose-dependent inhibition of HGF-induced proliferation of Bx-PC3 human pancreatic cancer cells. Nude mice bearing human glioma U-87 MG xenografts were treated with an anti-HGF nanobody, resulting in significant inhibition in tumor growth compared to the control group. Both 1E2-Alb8 and 6E10-Alb8 nanobodies show potential as a treatment option for multiple myeloma and other HGF-c-Met driven cancer types [53].

Other targets

In addition to the molecules described above, many other tumor-associated antigens have served as targets for nanobody development. Chemokine receptors, which are G-protein coupled receptors (GPCR), are overexpressed in a wide variety of malignancies [54]. Chemokines and their receptors drive migration and activation of a variety of cell types relevant for both innate and adaptive immune responses. If the goal is to interfere with cell migration, these molecules would appear to be ideal targets in view of the superior tissue penetration of nanobodies. Such nanobodies might neutralize the inhibition of chemorepellent signals, which would otherwise prohibit access of therapeutically efficacious immune cells to the tumor microenvironment. Conversely, immunosuppressive cells require chemoattractants to arrive at the site of the tumor. Nanobodies that target GPCRs and its ligands include reagents specific for human CXCR2 [55], CXCR4 [56–58], CXCR7 [59], CXCL11 and CXCL12 [60], and the viral GPCR US28 [61–63].

Furthermore, nanobodies have been identified that target human tumor-associated (trans)membrane proteins such as carcinoembryonic antigen (CEA) [64–66], prostate-specific membrane antigen (PSMA) [67–71], and human and murine macrophage mannose receptor (MMR) [72,73].

Other important targets are immune cell markers such as human CD7 [74,75], human and murine CTLA-4 [76,77], human and murine PDL-1 [78–82], murine CD8 [83] murine CD11b [19,84,85], human CD20 [86], human CD38 [87], mouse CD45 [84], mouse Ly-6C/Ly-6G [88], human and murine MHC-II [89,90]. Other targets include fibronectin [31], TUFM [91], CapG [92], CAIX [93,94], CD33 [95], human and murine CD47 [96,97], murine ARTC2 [98], and TNFα [99] (table 1).

Nanobodies for diagnosis through imaging

Molecular imaging has become an important tool in cancer research, both for understanding the underlying biology of a disease, as well as for diagnosis and therapy [100]. Molecular

imaging requires a targeting moiety labeled with a diagnostic radioisotope [101] or a suitable fluorophore. Radiolabeled monoclonal antibodies have been used extensively as targeting moieties, but their effectiveness is limited by the large size of full-sized Igs and their comparatively long circulatory half-life [102]. Notwithstanding their large size, conventional fully human monoclonal antibodies used for therapy have been converted into imaging agents. This strategy has the obvious advantage that agents approved for clinical use can be used with only slight modification for imaging purposes, and with minimal risk of immunogenicity and unexpected adverse outcomes, especially given the modest amounts of imaging agent administered. Only recently have nanobodies been used in first human trials [28]. Aside from the kidneys, uptake of radiolabeled nanobodies in non-targeted organs is usually low, resulting in a high target-to-background ratio shortly after administration. This allows same-day imaging and the use of shorter-lived radioisotopes, in contrast to the low target-to-background ratio found shortly after administration of ^{89}Zr-labeled full-sized monoclonal antibodies used for the same purpose [102,103].

These characteristics explain why nanobodies have been used in molecular imaging techniques such as positron emission tomography (PET) [104], single photon emission computed tomography (SPECT) [29], near-infrared fluorescence imaging (NIR) [105], and ultrasound-based molecular imaging [106] (figure 2A).

PET imaging

PET imaging uses positron-emitting radiotracers. Positrons collide with electrons in the tissue. This produces energy in the form of photons, which can be detected with a PET scanner [107]. Isotopically labeled Igs and Ig fragments used as PET imaging agents show exquisite specificity for select targets *in vivo* [108,109]. The EGFR-targeting 7D12 nanobody, radiolabeled with $^{68/67}$Ga or ^{89}Zr, was among the first nanobodies to be used for PET imaging. The PET images of A341 tumor-bearing mice show clearly visible tumors with good tumor-background contrast [104]. Some anti-HER2 nanobodies have also been used for imaging purposes, and the lead compound 2Rs15d has been studied in some detail. Coupled to ^{68}Ga-NOTA, the nanobody yielded high-contrast images of tumors in SKOV3 tumor-bearing rats [110]. The use of this nanobody has also successfully been translated to the clinic, with the first in-human phase I study of ^{68}Ga-NOTA-2Rs15d used in PET/CT scans of HER2-overexpressing cancer patients. The nanobody-based imaging agent showed favorable biodistribution and high accumulation in the primary lesions and/or metastases of the patients without side effects, indicating its safety and clinical potential [111]. Two phase II studies with this tracer have since been initiated, evaluating its potential to detect local and distant metastases in breast cancer patients (clinicaltrials.gov, NCT03331601 and NCT03924466). A similar approach with the anti-MMR nanobody 3.49 in 3LL-R tumor-bearing mice gave equally encouraging results, with promise for use in a phase I and II clinical trial (clinicaltrials.gov, NCT04168528) [112].

Labeling of biomolecules with ^{68}Ga requires a specific ^{68}Ge^{68}/Ga generator. The relatively short half-life of ^{68}Ga ($T_{1/2}$ < 68 min) [113] can result in low resolution PET images. These challenges can perhaps be overcome using ^{18}F for radiolabeling of nanobodies. ^{18}F has a half-life of ~109.8 min [114] and radiolabeling with ^{18}F provides better biodistribution and

tumor targeting, as has been shown *in vivo* in PET/CT images of $HER2^+$ SKOV3-tumor bearing mice when compared to labeling with ^{68}Ga [115]. ^{18}F labeling has also been performed on the anti-MMR 3.49 nanobody and resulted in specific visualization of the tumors of 3LL-R tumor-bearing mice [73].

Imaging of the myeloid compartment within the tumor microenvironment (TME) via PET is considered a desirable goal, as tumors are often infiltrated with myeloid-derived suppressor cells (MDSCs) [15]. Treatment with checkpoint blocking antibodies such as anti-PD-1 and anti-CTLA4 has changed the landscape of tumor therapy [116,117], and can likewise affect the distribution of myeloid cells within the tumor [118–120]. Thus, imaging the myeloid compartment within tumors can aid in understanding responses to cancer immunotherapies [15]. Nanobodies modified for use as PET imaging agents have now been applied to a variety of targets in pre-clinical models, directed against class II MHC (VHH7, VHH4), PD-L1, CTLA-4, fibronectin EIIIB (NJB2), CD8 (X118), CD11b (DC13), CD36 (DC20), and CD45 [15,31,82,89,90,121,122] labeled with ^{18}F, ^{64}Cu, or ^{89}Zr. Several tumor models have thus been examined, including the mouse B16 melanoma, PANC02 pancreatic adenocarcinoma, MC38 colorectal adenocarcinoma, and C3.43 human papillomavirus-induced cancer models. All of these agents visualize tumors by virtue of the fact that myeloid cells and lymphocytes are present in the TME [19].

SPECT with Micro-CT imaging

Single photon emission computed tomography (SPECT) imaging uses gamma-emitting radioisotopes. EGFR-targeting nanobodies 7D12 and 7C12, labeled with ^{99m}Tc, have been used in SPECT and micro-CT applications. Both nanobodies showed clear localization to the tumors of A431 xenograft-bearing mice [35]. SPECT imaging with the ^{99m}Tc-labeled anti-EGFR nanobody 8B6 also showed good tumor localization in mice bearing DU145 and A431 tumor xenografts [29]. When ^{99m}Tc-2Rs15d was evaluated for tumor accumulation by SPECT and Micro-CT, it showed clear accumulation at the tumor site of $HER2^+$ SKOV3 or LS174T xenograft-bearing mice, whereas no tumor localization of ^{99m}Tc-2Rs15d was observed in tumors of $HER2^-$ xenografted mice [30]. ^{99m}Tc-labeled NbCEA5, evaluated by total pinhole SPECT and Micro-CT, showed rapid clearance from the blood and efficient tumor targeting in LS174T xenografted mice [123]. The same held true for the ^{99m}Tc-labeled anti-MMR nanobody cl1 evaluated for tumortargeting potential in TS/A and 3LL-R tumor-bearing mice, imaged using pinhole SPECT and Micro-CT [72]. For diagnostic purposes, visualization of PD-L1 expression levels in patients can be valuable. SPECT imaging with ^{99m}Tc-labeled anti-PD-L1 nanobodies showed intense and specific uptake in PD-L1-overexpressing tumor models of melanoma and breast cancer in mice [79]. Moreover, these results were translated for human application in a phase I clinical trial on sixteen patients with non-small cell lung cancer (NSCLC), where an ^{99m}Tc labeled anti-PD-L1 nanobody showed clear visualization of the primary NSCLC tumors and metastases, while presenting favorable biodistribution and limited side-effects [81].

NIR fluorescence

The use of isotopically labeled imaging agents has as an obvious drawback the risk of radiation exposure for both patient and physician. Shorter lived isotopes with a high

positron yield such as ^{18}F in principle allow imaging shortly after administration of the ^{18}F-labeled agent, but this requires that tissue penetration and clearance from the circulation are compatible with visualization of the target of interest. Methods that do not rely on the use of radioisotopes therefore remain attractive alternatives, although these, too, have their limitations. Fluorescence-based methods suffer from absorption of light of the excitation and emission wavelengths by tissue and bodily fluids. Nonetheless, suitably labeled nanobodies have been used in these optical applications. The HER2-targeting nanobody 11A4 conjugated to a near-infrared fluorophore IRDye 800CW, localized specifically to the tumor site of $HER2^+$ SKBR3 xenograft-bearing mice, while maintaining good biodistribution. Near-infrared fluorescence imaging (NIR) has been exploited to enable image-guided surgery for the precise resection of $HER2^+$ tumors. In a clinical setting, this NIR-conjugated anti-HER2 nanobody should allow specific non-invasive classification of HER2-postive tumors and more precise surgical tumor resection [38]. A similar approach was used to label the EGFR-targeting nanobody 7D12. NIR fluorescence identified OSC-19 tongue tumors .*Ex vivo* fluorescence imaging of histology sections showed localization of the nanobody to cervical lymph node metastases [124]. The anti-carbonic anhydrase IX (CAIX) nanobody B9 has been exploited for the same purpose and yielded acceptable images in an orthotopic xenograft mouse model [94]. Because the tumor microenvironment is often hypoxic and CAIX is a marker enzyme of hypoxia, this approach should allow its non-invasive visualization. Kijanka et al. conjugated the 11A4 and B9 nanobodies to either IRDye 800CW or IRDye 680RD and injected both simultaneously into MCF10DCIS breast cancer xenograft-bearing mice. The results indicate the possibility of imaging and surgical resection of heterogeneous tumors at improved tumor-to-background ratios [38]. Using the 2Rs15d nanobody labeled with IRDye 800CW, NIR fluorescence image-guided surgery aided the precise debulking of ovarian tumors in SKOV3 xenograft-bearing mice [125]. The anti-ARTC2 nanobody S+16a has been conjugated to the fluorescent dye AlexaFluor-680 and was used for *in vivo* NIR imaging and *ex vivo* dissection of ARTC2-positive tumors in mice [126]. Combined, these examples show that fluorescence-based methods that exploit nanobodies as the targeting moieties have considerable potential, not only in the characterization of the tumor microenvironment, but also as an adjunct to surgery aimed at physical elimination of a tumor. Nevertheless, a study comparing the biodistribution of random and site-specific labeled 2Rs15d nanobodies shows the effect of different conjugation strategies on nanobodies' properties, which should be considered when developing nanobody-based fluorescent imaging agents [127].

Ultrasound-based molecular imaging

A wide branch of molecular imaging is ultrasound-based. Microbubbles or nanobubbles can be used as ultrasound contrast agents (Zhang et al. 2019). Nanobubbles can have various types of shells (polymers or phospholipids) and cores (gas, liquid, or solid) [129,130]. They can carry antibodies specific for tumor-associated antigens, aiding in the early diagnosis of different malignancies. The large molecular weight of full-sized antibody-particle complexes results in a limited number of nanobubbles that actually reach the intended target site. Therefore, the use of nanobodies may improve nanobubble performance [106] as tested with nanobubbles filled with C_3F_8 ultrasound imaging gas and carrying an anti-PSMA nanobody.

The modified nanobubble specifically adhered to prostate cancer cells and displayed high specificity in prostate cancer xenograft imaging *in vivo* [70].

Several issues must be addressed before nanobodies can be fully implemented for imaging in a clinical setting. Importantly, nanobodies show high renal retention due to reabsorption in the proximal tubules, caused by megalin receptors [131]. Kidney retention can lead to renal damage, especially when the nanobody is labeled with a radioisotope or equipped with a cytotoxic drug. Kidney retention also produces a strong signal in several imaging applications, possibly overshadowing the signal of the desired molecular targets when physically close to the kidneys. Several strategies have been pursued to address these issues, such as coadministration of gelofusin or positively charged amino acids, which interact with megalin receptors and thereby reduce kidney retention [131]. Modification of nanobody imaging agents with PEG can also mitigate this problem, as observed with the anti-CD8 nanobody X118, used to image T cell infiltration into mouse B16 and Panc02 tumors *in vivo* via PET [83]. Lastly, incorporation of a brush border enzyme-cleavable linker, a glycine-lysine dipeptide, between the ^{18}F-containing moiety and the 2Rs15d nanobody reduced renal activity levels as seen in micro-PET/CT images of SKOV-3 xenograft bearing mice [132].

Nanobodies for therapy

Nanobodies as checkpoint blockade therapies

Conventional checkpoint blockade therapies use monoclonal antibodies to bind to immune checkpoints such as PD-1 or CTLA-4 to improve the anti-tumor immune response [116,117,133,134]. The anti-PD-L1 nanobody KN035 fused to Fc (KN035-Fc) induced strong T cell responses and inhibited tumor growth of A375-PD-L1 cells in NOD-SCID mice *in vivo* (Zhang et al. 2017). The anti-CTLA-4 nanobody H11 alone failed to control B16 tumor growth in mice treated with the GVAX immunotherapy, but when linked to a murine Fc region, H11 resulted in better overall survival than an anti-mouse CTLA-4 monoclonal antibody [77]. CD47 is an antiphagocytic ligand (the “don’t eat me” signal) exploited by tumors. It does so by blunting antibody-mediated phagocytosis through binding to signal regulatory protein alpha (SIRPα) on phagocytes. The anti-CD47 nanobody A4 alone or in combination with a tumor-specific antibody fails to generate antitumor immunity against syngeneic B16 tumors, but CD47 antagonism substantially improved response rates against B16 tumors when used in combination with PD-L1 blockade [97]. Interestingly, administration of the A4 nanobody synergized with PD-L1, but not CTLA4 blockade [135].

Nanobody-drug conjugates

Specific tumor-targeted therapies include the use of antibody-drug conjugates (ADCs). ADCs exploit the targeting efficiency of antibodies combined with the action of the cytotoxic payload conjugated to it [136,137]. This ought to result in specific targeting of the cancer cells, thus alleviating off-target side-effects. The appeal of this approach is reflected by the large number of clinical trials that use ADCs (registered on clinicaltrials.gov), with almost 40 being completed and over 80 in progress. Popular targets for ADCs are HER2, c-MET, CD30, and PSMA.

Despite evidence for the effectiveness of ADCs, there are drawbacks to the use of monoclonal antibodies in cancer therapy. These include a limited capacity of antibodies to penetrate the tumor due to their relatively large size. Smaller antigen-binding fragments such as Fabs, scFVs, minibodies, and diabodies have therefore attracted attention as a platform for ADCs. Nonetheless, the efficiency of these smaller formats is often limited because of decreased stability, lower affinity, or difficulties in production [12]. Nanobodies can overcome most of these challenges, due to their shorter circulatory half-life, increased tissue penetration, stability and ease of production [136]. Figure 2B shows an overview of the described uses for nanobodies in cancer therapy.

Nanobody-drug conjugates under investigation include a nanobody-albumin nanoparticle (NANAP), which has an albumin core modified on its surface with EGFR-targeting nanobodies conjugated to PEG (EGa1-PEG). The NANAP is loaded with the multikinase inhibitor 1786. When internalized and digested in lysosomes, it causes the intracellular release of the kinase inhibitor and inhibition of proliferation of EGFR-positive 14C squamous head and neck cancer cells [16]. Furthermore, conjugation of the drug Mertansine (DM1) to an MHC-II targeting nanobody, VHH7, resulted in a reduction in liver metastases in mice engrafted with the A20 lymphoma [138]. The central role of MDSCs in driving cancer progression has raised interest in their depletion via ADCs for therapeutic benefit. In mice, CD11b is expressed on several myeloid cell types including monocytes, macrophages, and granulocytes, whereas Ly-6C is highly expressed on monocytes with lower levels on granulocytes, while Ly-6G is expressed on granulocytes [139,140]. Thus, the anti-CD11b nanobody DC13 and Ly-6C/Ly-6G-specific nanobodies (VHH16 and VHH21, respectively) were conjugated to *Pseudomonas* exotoxin A to deplete myeloid cells *in vitro* and *in vivo* [88]. All conjugates showed cytotoxicity *in vitro*. However, granulocytes were more sensitive than monocytes to Ly-6C/Ly6-G-specific immunotoxins *in vivo* despite similar binding of the nanobody-immunotoxins to each cell type, indicating the need to thoroughly characterize myeloid-specific ADC candidates.

Targeted radionuclide therapy (TRNT)

TRNT is an increasingly prevalent anti-cancer therapy, designed to deliver cytotoxic radiation to cancer cells, with delivery vehicles such as monoclonal antibodies, antibody fragments, or other small molecules equipped with a suitable radioisotope. Targeted delivery should limit exposure of healthy tissue to radiation. TRNT using antibodies has been approved by the FDA for Ibritumomab tiuxetan, a ^{90}Y-labeled CD20-targeting monoclonal antibody for radioimmunotherapy of non-Hodgkin's lymphoma [141–143], and the similar ^{131}I-tositumomab [144]. Furthermore, promising results in early clinical trials have been obtained for antibodies specific for CD33 [145,146], or preclinical results for a combination of CD20 and CD22 targeting antibodies [147,148]. Nevertheless, the targeting of (large) solid tumors remains a challenge, as shown in trials with antibodies specific for MUC1 [149], CEA [150–152], and CEA [153]. Because the poor penetration of labeled antibodies into solid tumor tissue is to a large extent due to their size, smaller labeled molecules such as peptides and nanobodies, have been explored as alternatives for TRNT, especially for the treatment of solid tumors.

D'Huyvetter et al. were the first to use a nanobody for TRNT, in a study with mice bearing HER2+ SKOV3 xenografts treated with the ^{177}Lu-DTPA-2Rs15d nanobody. The treated mice showed an almost complete arrest in tumor growth and significantly longer disease-free survival compared to the control group, while no evidence of renal inflammation or necrosis was observed [154]. The same nanobody, labeled with ^{131}I, has been used in a phase I clinical trial with breast cancer patients (NCT02683083) [155]. The 5F7GGC nanobody, labeled with the residualizing agent *N*-succinimidyl 4-guanidinomethyl 3-$^{125/131}$I-iodobenzoate (*I-SGMIB), designed to trap radioiodine inside a tumor cell [156], showed promising results in targeting HER2^{+} cancers with different radioisotopes useful for TRNT [157].

The promising results with Ibritumomab tiuxetan prompted researchers to repeat this strategy with CD20-specific nanobodies, which should limit the toxicity seen with mAbs in non-targeted tissues. The nanobody 9079, radiolabeled with ^{177}Lu, showed better disease-free survival when used for treating mice with B16 melanoma compared to controls. More importantly, minimal renal toxicity was seen when mice were treated with ^{177}Lu-DTPA-sdAb 9079 [86]. The results of these preclinical studies underscore how the unique characteristics of nanobodies could be leveraged perhaps also in a clinical setting. Further optimization to decrease renal retention is necessary to further reduce any possible adverse effects.

Nanobody-based carrier delivery systems

To increase tumor efficacy and decrease toxicity in non-targeted tissues, it is important to target the delivery of a drug or compound to the tumor. Nanoparticles used as carriers for targeted drug delivery include liposomes, polymeric nanoparticles, micelles, and albumin nanoparticles [158]. Despite their differences in structure and mechanism of action, they all depend on a targeting ligand at the surface of the nanocarrier to achieve adequate specificity.

Conjugation of the anti-EGFR nanobody EGa1 to PEGylated liposomes induced internalization and downregulation of EGFR in 14C cells, both *in vitro* and *in vivo* [159]. When formulated as a polymeric PEGylated micelle, similar receptor binding and internalization were observed, making micelles promising systems for active drug targeting [160]. To this end, EGa1-decorated micelles were loaded with temoporfin (mTHPC), a photosensitizer compound used in the clinic for photodynamic therapy (PDT) of head and neck squamous cell carcinoma (HNSCC). These micelles show prolonged circulation *in vivo* compared to free mTHPC, indicating a potential of these micelles to improve the selectivity and efficacy of PDT in EGFR+ tumors [161]. Extracellular vesicles (EV) are also being explored as nanoparticles for therapeutic purposes [162]. To be tumor specific, such EVs must be equipped with a targeting moiety. By anchoring EVs through a glycosylphosphatidylinositol (GPI) anchor to the EGa1 nanobody, the engineered EVs showed localization to and internalization in EGFR-expressing cells, but the conditions will require further improvement for pre-clinical use [163].

Tumor vaccination, lentiviral vector-based cancer therapy, and CAR-T cells

Vaccination against cancer would be a valuable prophylactic or therapeutic strategy and would benefit from specifically delivering tumor antigens to APCs. To this end, lentiviral vectors (LVs) have been used to deliver cancer autoimmune antigens to APCs [164]. Antibodies [165], and more importantly nanobodies, can be used to specifically deliver these LVs to APCs. LVs displaying the dendritic cell-targeting nanobody DC2.1 exclusively transduce only DCs and macrophages *in vitro* and *in vivo* [166]. Tropism of human adenovirus serotype 5 (Ad5), which can efficiently transduce human cells, can be altered by capsid modifications that incorporate a nanobody against human CEA (hCEA). These CEA nanobody-expressing Ad5 vectors successfully transduced murine MC38 cells that express hCEA [66]. In a similar manner, nanobodies can be used to improve the targeting and transduction of adeno-associated viral vectors, as shown by the successful transduction of myeloma cells with AAV1P5 displaying an anti-CD38 nanobody [167].

Another vaccination strategy focuses on activating cytotoxic $CD8^+$ T cells through targeted delivery of cancer antigens to APCs by anti-CD11b nanobodies [168]. This has been explored for HPV^+ tumors driven by the E6 and E7 genes of the oncogenic HPV type 16 strain. Vaccination based on anti-cd11b nanobodies conjugated to E7-peptide antigens elicited a strong $CD8^+$ T cell response *in vivo* and showed slower tumor growth and longer overall survival in an *in vivo* C3.43 cancer model [19]. These results highlight a new role for nanobodies in tumor vaccination strategies. In a similar approach, a strong Th1 immune response against the tumor-specific antigen MUC1 was generated by attaching a site-specifically glycosylated MUC1 peptide to the class II MHC-targeting nanobody VHH7 [122]. The enhanced production of antibodies in response to immunization with the nanobody-peptide adduct implied the induction of an adequate CD4 T helper response *in vivo*.

Adoptive cell transfer (ACT) employs a patient's own immune cells to target cancer cells. The T cells are engineered to express a cloned T cell receptor (TCR) or chimeric antigen receptor (CAR) that targets a tumor antigen of interest, the latter allowing for recognition of non-MHC restricted antigens. An ACT strategy using T cells engineered with a CAR comprised of an scFv against mouse VEGFR2 was effective in eliminating several different vascularized syngeneic tumors in mice [169]. Multiple CAR-T cells derived from antibodies or ScFvs are currently under investigation in a clinical setting. Some clinical trials show an immune response directed against the CAR-T cells [170–172], presumably due to immunogenicity to the non-human scFv component in the CAR constructs [173]. This problem might be solved by using humanized nanobody-based CARs. Albert et al. used their UniCAR system, a unique type of CAR T cell that can be redirected via simultaneously infused target modules (TM), allowing the UniCAR to be switched off in the absence of target modules. The UniCAR decorated with anti-EGFR nanobodies effectively target A431 cells *in vivo* [174], and showed an even better anti-tumor responses when formulated as a bivalent α-EGFR-EGFR nanobody-based UniCAR [175]. A VEGFR2-nanobody specific CAR showed promising results *in vitro*, with high concentrations of secreted IL-2 and IFN-γ by the CAR T-cells, as well as a cytotoxic activity measured by an LDH release assay in response to the VEGFR2 antigen on target cells [176]. Bispecific CAR-T cells that

target two antigens simultaneously might be effective to counteract potential antigen-escape in tumor cells. *In vitro* experiments show the great potential of a bispecific anti-CD20 and anti-HER2 nanobody-based CAR, which targets and kills Jurkat cells expressing either one or both antigens [177]. Targeting the TME rather than the tumor directly can be beneficial for targeting multiple tumor types. Anti-PD-L1-nanobody based CAR-T cells slow tumor growth rates *in vivo* in B16 and MC38 models. CAR-T cells based on a nanobody against the fibronectin splice variant EIIIB, which is exclusively expressed on tumor stroma and in the neovasculature, as found around tumors, significantly slowed B16 melanoma growth *in vivo* [178]. The anti-tumor efficacy of the EIIIB-nanobody CAR-T cells was improved in cells that simultaneously secreted nanobodies against PD-L1 or CTLA4, and their systemic cytotoxicity was reduced by secretion of a CD47 nanobody by the CAR T cells [179]. Because the sequence of the EIIIB splice variant is identical for mouse and man, there may be a future for the clinical use of human CAR T cells equipped with this nanobody as a recognition module.

These examples primarily focus on engineering the patient's autologous T cells. However, selecting non-malignant T cells is difficult for patients with T cell-specific cancer such as T-ALL. To overcome this problem, CAR-NK cells can be used. An anti-CD7 nanobody-based CAR on NK cells showed an inhibitory effect on tumor cells in a PDX mouse model [180]. Bispecific anti-CD38 nanobody-based CAR-NK cells effectively deplete CD38+ cells from patient-derived multiple myeloma bone marrow cells *in vitro* [181]. Nanobody-based CAR-T cell therapy is now being pursued in clinical trials for CD19/CD20 bispecific targeting in patients with B Cell lymphoma (NCT03881761) and BCMA targeting in multiple myeloma (NCT03664661).

Conclusions

Research has illuminated a valuable role for nanobodies in cancer diagnostics and therapy. Their biophysical properties are fundamentally distinct from those of their conventional two-chain counterparts. The small size, antigen specificity, binding affinity, and stability of nanobodies allows successful targeting of antigens in the tumor, the tumor microenvironment and of the immune cells that are recruited there. Nanobodies are increasingly being used as a diagnostic tool in molecular imaging techniques such as PET, SPECT and NIR fluorescence imaging, as evidenced also by successful early clinical trials. As therapeutic agents, nanobodies can aid delivery of drugs or radioisotopes and can be used for tumor vaccination strategies and CAR-T cell therapy. The full range of possible applications of nanobodies has yet to be explored, but as a complement or an alternative to conventional immunoglobulins: nanobodies are here to stay.

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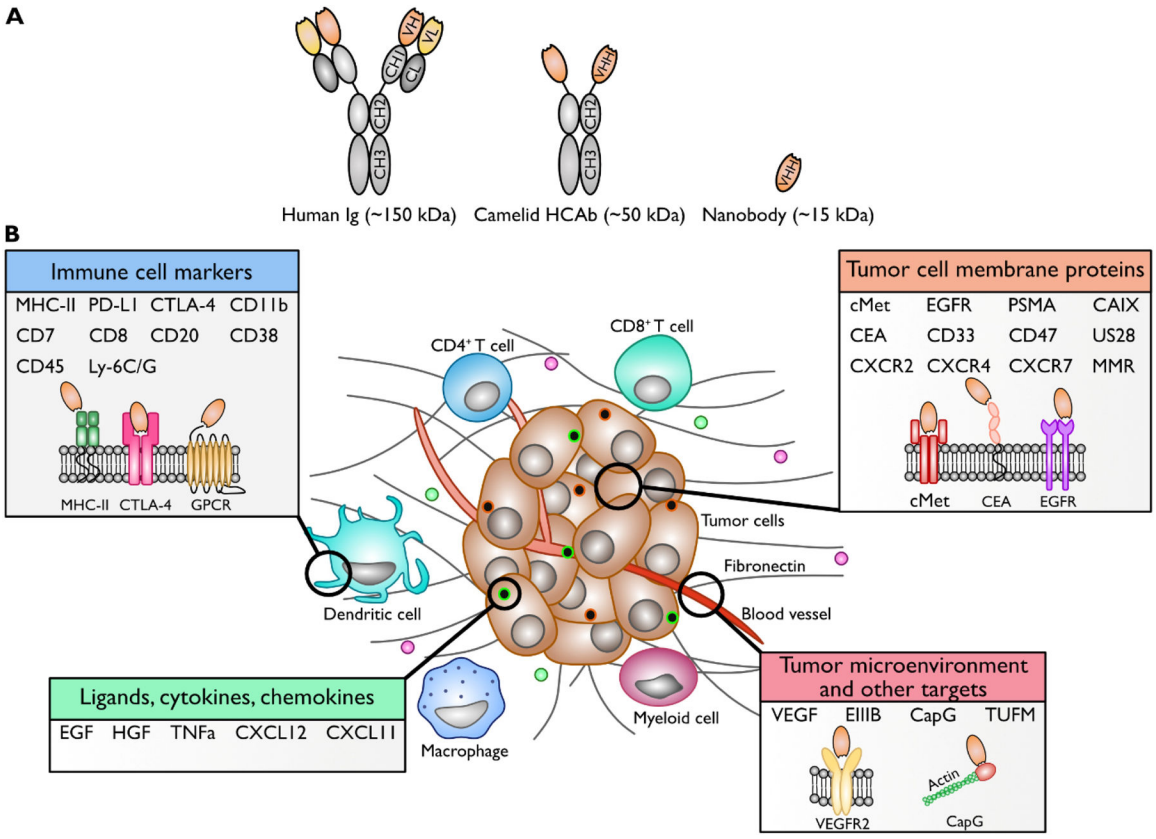

Figure 1. Nanobodies and their targets in relation to the tumor (microenvironment).

A. Schematic representation of a conventional human Ig, camelid HCab, and a nanobody. B. Schematic overview of the tumor-associated targets for which nanobodies have currently been established. Important targets are immune cell markers, tumor cell (membrane) proteins, receptor ligands, and proteins associated with the tumor microenvironment.

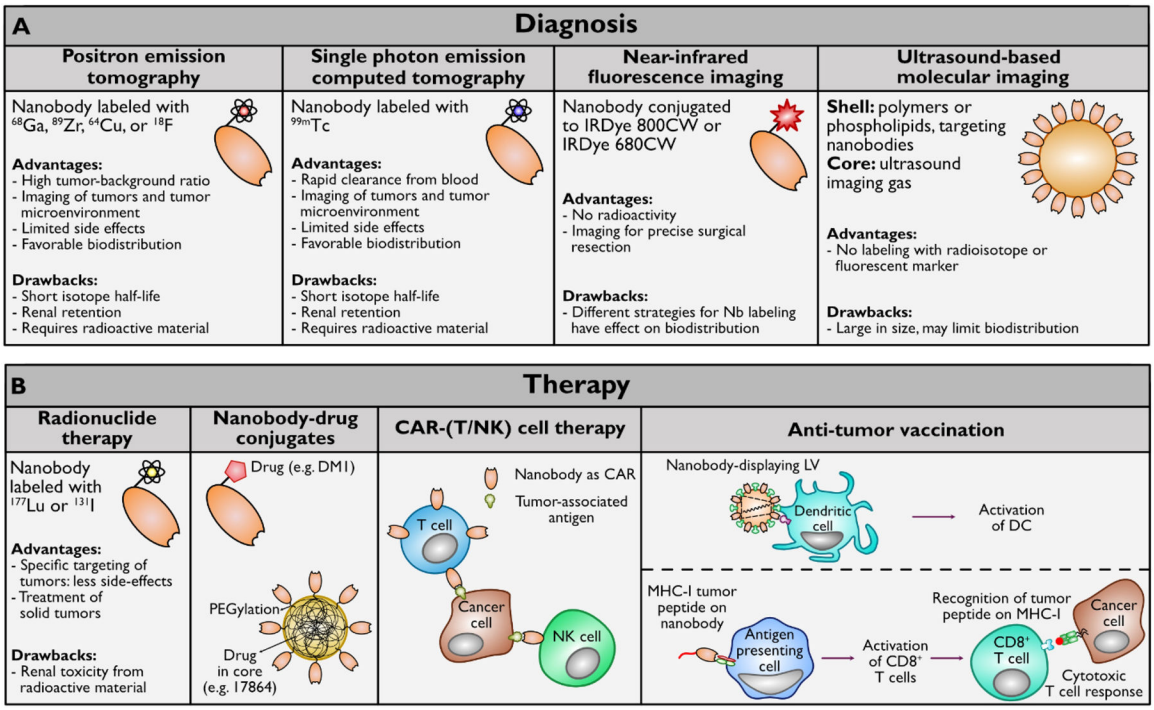

Figure 2. Overview of the applications of nanobodies in cancer diagnosis and therapy.

A. Nanobodies have been successful in diagnosis through molecular imaging techniques such as PET, SPECT, NIR, and ultrasound-based molecular imaging. B. Nanobodies can be used in a variety of tumor therapies, such as targeted radionuclide therapy, nanobody-drug conjugates, adoptive cell transfer, and vaccination.

Table 1

Currently available nanobodies for tumor-relevant targets.

Target	Disease examples	Origin	Model system tested	Nanobody name	References
ARTC2		Murine (ART2.2 in *Llama matahari*)	CD38 KO mice	S+16a	[98]
CAIX	Breast Cancer (ductal carcinoma)	rCAIX in *Camelus dromedarius*	PC3 and HeLa cell lines	K24	[93]
		Human (HeLa cells in *Llama glama*)	DCIS and CAIX xenograft-bearing SCID/beige mice	B9	[94]
CapG	Breast Cancer TNBC, melanoma, PDAC	Human (Recombinant CapG in *Llama glama*)	MDA-MB-231 cells, MDA-MB-231 cells in nude mice	CAPNb2	[92]
CD11b	Innate immune cell marker	Murine (BMDC in *Llama glama*)	BMDC and macrophage cell lines	V36, 76, 51, 81, B10 and 42	[84]
			HPV E7 xenograft bearing mice	VHH_{CD11b} (also known as VHH_{DC13})	[19]
CD20	B16 melanoma Melanoma, lung cancer, breast cancer	Human (hCD20-encoding plasmid and hCD20pos cells in *Llama glama*)	$hCD20_{pos}$ B16 xenograft-bearing mice	9077, 9079	[86]
CD33	AML	rCD33 in *Llama glama*	THP-1 tumor xenograft-bearing mice	Nb_7, Nb_21, Nb_22	[95]
CD38	Multiple myeloma	Human (rCD38 ectodomain, C-terminal domain, or cDNA expression vector for full-length CD38 in *Llama glama*)	LP-1, OPM2 and RPMI8226 myeloma cell lines, Primary malignant plasma cells	MU375, MU1053, MU551	[182]
			Human CD38-expressing DC27.10 cells in nude mice	WF211, MU1067, JK36, JK2, MU523, WF14 and MU738	[87]
CD45		Mouse (Mouse BDMC cells in *Llama glama*)	*In vitro* assays	G7 and 32b	[84]
CD47	AML, NHL, gastric, ovarian, colon and hepatocellular cancer	Mouse (Ig-like V-type domain (ECD) of mouse CD47 in alpaca)	Tubo-EGFR mouse breast cancer cell line, BALB/c BMDMs, B16F10 cells	A4	[97]
			BMDMs and B16F10 xenograft-bearing C57BL/6 mice	A4 fusion to IgG2a Fc (A4Fc)	[135]
		Human (hCD47(ECD)-Fc in *Camelus bactrianus*)	Raji cell lymphoma NOG mice, cynomolgus monkeys	HuNb1-IgG4	[96]
CD7	Leukemia	Human (CD7+ Jurkat cells in *Llama glama*)	Leukemia cell lines, CEM xenograft-bearing nude mice	VHH6	[74]
			T-ALL PDX model for humanized VHH6	Humanized VHH6	[75]
CD8	B16 melanoma, pancreatic cancer	Human and mouse (recombinant mouse CD8αβ heterodimer in alpacas)	C57BL/6 mice with B16 and B16 GVAX, MMTV-PyMT transgenic mouse model, human biopsy tumor sections	VHH-X118	[83]

Target	Disease examples	Origin	Model system tested	Nanobody name	References
CEA	Epithelial cancers (lung, thyroid, pancreas, uterus, breast, ovary, colorectal)	Human and murine (CEA in *Camelus dromedarius*)	LS174T cells and LS174T xenograft-bearing mice	cAb-CEA5	[64]
		Human (CEA in *Vicugna pacos*)	LS174T cells and MC38(CEA) mouse colon cancer cells	JJB-B2	[66]
			H460 xenograft-bearing nude mice	^{99m}Tc-nanobody	[65]
c-Met	Brain, liver, pancreatic and gastric cancer, multiple myeloma	Human (c-MET-Fc in *Llama glama*)	hMSCs	Anti-c-Met nanobody, bispecific	[52] Nb patent by Beste et al., WO 2012/042026 A1
		Human (A431 cells in *Llama glama*)	A549 cells, MKN-45 cells	G2	[51]
CTLA-4	B16 melanoma	Human (CTLA-4 protein in *Camelus dromedarius*)	B16/B6 melanoma cell injected C57BL/6 mice	Nb16	[76]
		Murine (CTLA-4 ECD fused to Fc domain in *alpaca*)		H11	[77]
CXCL11	Pre-B lymphoma	Human (Chemokine mixture in *Llama glama*)	HEK293T cells	11B1, 11B7	[60]
CXCL12				12A4	
CXCR2	Acute and chronic inflammatory diseases, cancer metastases	Human (CXCR2-expressing cells or pVAX1-hCXCR2 DNA in *Llama glama*)	CHO-CXCR2 cells	127D1, 163E3	[55]
CXCR4	HIV-1, tumor growth and metastasis, WHIM syndrome	Human (CXCR4-expressing HEK293T cells in *Llama glama*) 90% sequence identity with murine ortholog	Cynomolgus monkeys	238D2and 238D4 (mono- and biparatopic)	[56]
			HEK293T and CXCR4-R334X overexpressing K652 cell lines	10A10	[57]
		Human (CXCR4-expressing lipoparticles in *Llama glama*)	SUP-T1 and Jurkat cells	VUN400, VUN401, VUN402	[58]
CXCR7	Head and neck cancer	Human (CXCR7-expressing HEK293 cells or pVAX1-CSCR7DNA in *Llama glama*)	22A xenograft-bearing nude mice	NB1, NB2, NB3, NB4, NB5 (mono- and biparatopic)	[59]
EGFR	Epithelial cancers	Human (EGFRvIII peptide in *Camelus bactrianus*)	Ascites fluid of NSCLC	OR1–83, OR2–83	[183]
		Human (A431 cells in *Llama glama*)	Murine xenograft models	Ia1, IIIa3, L2–3.40, 9G8	[34]
				EGa1	[184]
				8B6	[29]
				aEGFR-aEGFR-aAlb	[14]
				7C12, 7D12	[35]
				CONAN-1 (7D12-9G8-Alb1)	[36]
				OA-cb6	[37]
				OR1–83, OR2–83	[183]

Target	Disease examples	Origin	Model system tested	Nanobody name	References
Fibronectin (EIIIB)	Mammary carcinoma	Mixture of ECM proteins, domains and peptides in *alpaca*	LM2 xenografts in NSG mice	NJB2	[31]
HER2	Breast cancer	Human (HER2-Fc recombinant fusion protein in *Camelus dromedarius*)	HER2+ SKOV3 tumor bearing mice	2Rs15d, 1R136d	[30,154]
		Human (MCF7 or BT474 cells in *Llama glama*)	SKBR3 xenograft-bearing mice	11A4	[38]
		Human (SKBR3 cells in *Llama glama*)	BT474M1 xenograft-bearing mice	5F7GGC	[39]
HGF	Glioma	Human (HGF in *Llama glama*)	U87 MG xenograft-bearing mice	1E2-Alb8, 6E10-Alb8	[53]
Ly-6C/Ly-6G	Myeloid cells in immune diseases and cancer	Mouse (mouse splenocytes in alpaca)	NUP98/HOXB4 cells and C57BL/6j mice	VHH16, VHH21	[88]
MHC-II	Pancreatic cancer	Murine (murine splenocytes in alpaca)	panc02-tumors in C57/BL6 mice	VHH7, VHHDC8, and VHHDC15	[90]
	Graft versus Host Disease	Human (Purified HLA antigen in *Vicugna pacos*)	Xenograft model of GvHD	VHH4	[89]
MMR	TAMs infiltrating tumors	Human (MMR EC in *Vicugna pacos*)	TS/A and 3LL-R tumor-bearing mice	Nb cl1	[72]
		Human and murine (recomb. Monomeric fusion proteins in *Vicugna pacos*)		3.49	[73]
PD-L1	NSCLC, colon, thyroid, uterus, pancreas, and ovary cancer	Human (PD-L1 Fc fusion protein in *Camelus bactrianus*)	PD-L1$^+$ A375 cells + hPBMCs xenograft-bearing nude mice	KN035	[118]
		Murine (RAW264.7 cells in *Camelus dromedarius*)	TC-1 (WT and PD-L1 KO) in WT or PD-L1 KO mice	C3, E2	[80]
		Human (PD-L1-Fc protein in *alpaca*)	PD-L1$^+$ MCF7 and 624-MEL xenograft-bearing nude mice	K2	[79]
		Human clinical trial	Human NSCLC patients	NM-01	[81]
PSMA	Prostate cancer	Human (Purified PSMA antigen in *Camelus dromedarius*)	*In vitro* binding predictions	C9, C24, N14, N50	[71]
		Human (rPSMA in *Camelus bactrianus*)	LNcaP and PC3 cells	C3	[69]
		Human (LNCaP cells, PSMA peptide, rPSMA EC in *Camelus dromedarius*)	PC-3 and LNCaP xenograft-bearing nude mice	PSMA30	[68]
		Human (4 different PCa cell lines in *Llama glama*)	PC-310 and PC-3 xenograft-bearing NMRI mice	JVZ-007	[67]
			LNCaP, C4-2 or MKN45 xenograft bearing BALB/c-nu nude mice		[70]
TNFα	Sarcomas, melanomas, carcinomas	DNA sequences encoding the camelidae antihuman TNFa single-domain)	MCF-7, T-47D and MDA-MB-231 cell lines, 4T-1 breast cancer mouse model	anti-TNF-VHH	[99]

Target	Disease examples	Origin	Model system tested	Nanobody name	References
TUFM	Glioblastoma	Human (GBM stemlike cells in *Alpaca*)	Several GBM cell lines and tissues	Nb206	[91]
VEGF(R-2)	Angiogenesis in solid tumors	Human (293KDR cells in *Camelus dromedarius*)	HUVEC cells	3VGR19	[42]
		Human ($VEGF_{121}$ in *Camelus dromedarius*)		Nb22, Nb23, Nb35, Nb42; Humanized Nb42	[44,45]
		Human sdAb from HuSdl™		NTV1	[43] through HuSdl™
			Chorioallantoic membrane	VA12	[46]
Viral GPCR US28	Glioblastoma	pVAX1-US28 DNA boosted with HEK293T-US28 expressing cells in *Llama glama*	U251 cells, intracranial GBM mouse model	(bivalent) US28 nanobody	[62]
		pcDEF3 vector encoding for VHL/E US28 in *Llama glama*	U251 cells	VUN100	[63]
			In silico	Nb7	[61]