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Chapter 2:

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 sharks³⁰⁰ and in camelid species in 1989³⁰¹. 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) Igs³⁰²⁻³⁰⁶. 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^{307,308}, 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 solubility³⁰⁹ and stability³¹⁰, as well as ease of production in bacteria, thus enabling large-scale production³¹¹. Their small size (~15 kDa) endows nanobodies with excellent tissue penetration³¹² and rapid clearance from the circulation ($t_{1/2} < 30$ min)³¹³. 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)³¹⁴, through fusion of the nanobody to serum albumin nanoparticles³¹⁵ or to a serum albumin-binding nanobody³¹³.

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^{195,311,316–326}. 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 8B6³²⁷, the anti-HER2 nanobody 2Rs15d³²⁸ and the nanobody directed against the EIIIB splice variant of fibronectin³²⁹.

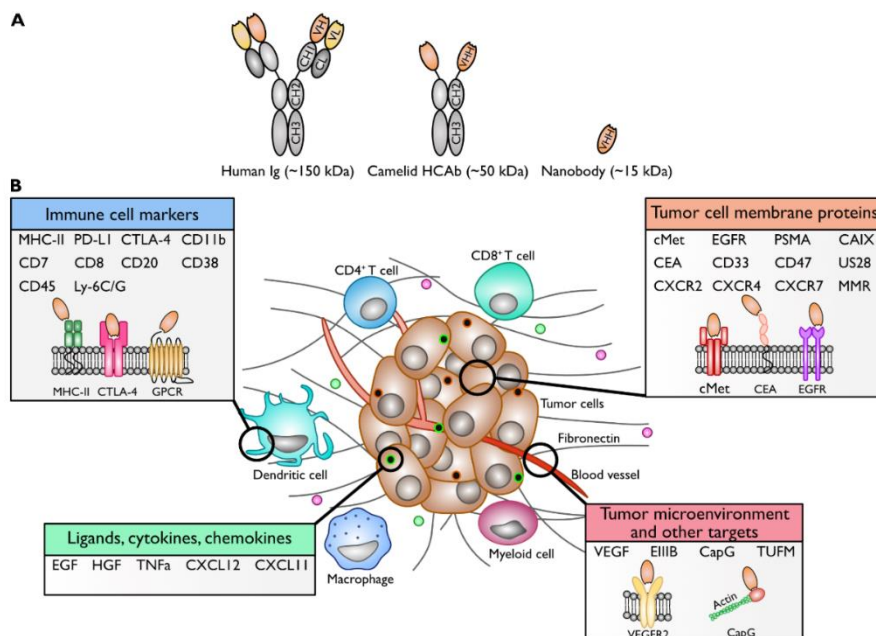


Figure 1. Nanobodies and their targets in relation to the tumor (micro-environment). (A) Schematic representation of a conventional human Ig, camelid HCAb, and a nanobody. (B) Schematic overview of the tumor-associated targets for which nanobodies have currently been established. Important targets are immune cell markers, tumor cell (membrane) proteins, receptor ligands, and proteins associated with the tumor microenvironment.

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 angiogenesis³³⁰. 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 EGFR³³¹. 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 binders³³². Using the same EGFR phage nanobody repertoire and selecting for the EGFR extracellular domain, the nanobodies 7C12 and 7D12³³³ and 9G8³³² 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 mice³³⁴.

Using similar methods, the anti-EGFR nanobodies 8B6 and OA-cb6 were obtained^{327,335}. Nanobodies that recognize HER2, another member of the EGFR family, specifically target HER2⁺ SKOV3 ovarian cancer cell-derived tumors *in vivo*³²⁸. HER2-targeting nanobodies 11A4³³⁶ and 5F7GGC³³⁷ have been used for a variety of (clinical) applications, described elsewhere 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^{338,339}. 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)³⁴⁰. Ma et al. isolated an anti-angiogenic VEGFR₂-D₃ specific nanobody NTV1 from HuSdlTM, a human single domain antibody

library of 'camelized' human antibodies³⁴¹. In similar fashion, nanobodies specific for VEGF were obtained. These inhibit endothelial cell proliferation in an *in vitro* angiogenesis assay using HUVECs³⁴². A humanized version of one of these nanobodies, Nb42, has also been generated³⁴³. Lastly, the nanobody VA12, which specifically targets the binding domain of VEGF-A, showed anti-angiogenic potential in a chorioallantoic membrane assay³⁴⁴.

c-Met and HGF

Hepatocyte growth factor (HGF) binds to the c-Met receptor³⁴⁵, which activates pathways responsible for cancer progression, angiogenesis and metastasis³⁴⁶. For several different epithelial and nonepithelial cancers, overexpression of HGF and the c-Met receptor are associated with a poor prognostic outcome^{347,348}. Nanobodies against c-Met and HGF have been produced. The anti-cMet nanobody G2 competes with HGF for binding to the c-Met receptor³⁴⁹. 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 antibody³⁵⁰.

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 types³⁵¹.

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 malignancies³⁵². 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 CXCR2³⁵³, CXCR4³⁵⁴⁻³⁵⁶, CXCR7³⁵⁷, CXCL11 and CXCL12³⁵⁸, and the viral GPCR US28³⁵⁹⁻³⁶¹.

Furthermore, nanobodies have been identified that target human tumor-associated (trans)membrane proteins such as carcinoembryonic antigen (CEA)³⁶²⁻³⁶⁴, prostate-specific membrane antigen (PSMA)³⁶⁵⁻³⁶⁹, and human and murine macrophage mannose receptor (MMR)^{370,371}.

Other important targets are immune cell markers such as human CD7^{372,373}, human and murine CTLA-4^{374,375}, human and murine PDL-1³⁷⁶⁻³⁸⁰, murine CD8³⁸¹, murine CD11b^{325,382,383}, human CD2³⁸⁴, human CD38³⁸⁵, mouse CD45³⁸², mouse Ly-6C/Ly-6G³⁸⁶, human and murine MHC-II^{387,388}. Other targets include fibronectin³²⁹, TUFM³⁸⁹, CapG³⁹⁰, CAIX^{391,392}, CD33³⁹³, human and murine CD47^{394,395}, murine ARTC2³⁹⁶, and TNF α ³⁹⁷ (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 therapy³⁹⁸. Molecular imaging requires a targeting moiety labeled with a diagnostic radioisotope³⁹⁹ 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-life⁴⁰⁰. 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 trials³²³. 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^{400,401}. These characteristics explain why nanobodies have been used in molecular imaging techniques such as positron emission tomography (PET)⁴⁰², single photon emission computed tomography (SPECT)³²⁷, near-infrared fluorescence imaging (NIR)⁴⁰³, and ultrasound-based molecular imaging⁴⁰⁴ (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 scanner⁴⁰⁵. Isotopically labeled Igs and Ig fragments used as PET imaging agents show exquisite specificity for select targets *in vivo*^{406,407}. The EGFR-targeting 7D12 nanobody, radiolabeled with $^{68/67}\text{Ga}$ 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 contrast⁴⁰².

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 rats⁴⁰⁸. 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 potential⁴⁰⁹. 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)⁴¹⁰.

Labeling of biomolecules with ^{68}Ga requires a specific $^{68}\text{Ge}/^{68}\text{Ga}$ generator. The relatively short half-life of ^{68}Ga ($T_{1/2} < 68 \text{ min}$)⁴¹¹ 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 $\sim 109.8 \text{ min}$ ⁴¹² 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 ⁴¹³. ^{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 mice³⁷¹.

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)³¹⁴. Treatment with checkpoint blocking antibodies such as anti-PD-1 and anti-CTLA4 has changed the landscape of tumor therapy^{414,415}, and can likewise affect the distribution of myeloid cells within the tumor^{416–418}. Thus, imaging the myeloid compartment within tumors can aid in understanding responses to cancer immunotherapies³¹⁴. 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^{314,329,380,387,388,419,420} 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 TME³²⁵.

SPECT with Micro-CT imaging

Single photon emission computed tomography (SPECT) imaging uses gamma-emitting radioisotopes. EGFR-targeting nanobodies 7D12 and 7C12, labeled with $^{99\text{m}}\text{Tc}$, have been used in SPECT and micro-CT applications. Both nanobodies showed clear localization to the tumors of A431 xenograft-bearing mice³³³. SPECT imaging with the $^{99\text{m}}\text{Tc}$ -labeled anti-EGFR nanobody 8B6 also showed good tumor localization in mice bearing DU145 and A431 tumor xenografts³²⁷. When $^{99\text{m}}\text{Tc}$ -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 $^{99\text{m}}\text{Tc}$ -2Rs15d was observed in tumors of HER2⁻ xenografted mice³²⁸. $^{99\text{m}}\text{Tc}$ -labeled NbCEA5, evaluated by total pinhole SPECT and Micro-CT, showed rapid clearance from the blood and efficient tumor targeting in LS174T xenografted mice⁴²¹. The same held true for the $^{99\text{m}}\text{Tc}$ -labeled anti-MMR nanobody cl1 evaluated for tumor-targeting potential in TS/A and 3LL-R

tumor-bearing mice, imaged using pinhole SPECT and Micro-CT³⁷⁰. 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 mice³⁷⁷. 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-effects³⁷⁹.

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 ¹⁸F in principle allow imaging shortly after administration of the ¹⁸F-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-positive tumors and more precise surgical tumor resection³³⁶. 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 metastases⁴²².

The anti-carbonic anhydrase IX (CAIX) nanobody B9 has been exploited for the same purpose and yielded acceptable images in an orthotopic xenograft mouse model³⁹². 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 ratios³³⁶. Using the 2Rs15d nanobody labeled with IRDye 800CW, NIR fluorescence image-guided surgery aided the precise debulking of ovarian tumors in SKOV3 xenograft-bearing mice⁴²³.

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 mice⁴²⁴. 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 agents⁴²⁵.

Ultrasound-based molecular imaging

A wide branch of molecular imaging is ultrasound-based. Microbubbles or nanobubbles can be used as ultrasound contrast agents⁴²⁶. Nanobubbles can have various types of shells (polymers or phospholipids) and cores (gas, liquid, or solid)^{427,428}. 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 performance⁴⁰⁴ as tested with nanobubbles filled with C₃F₈ 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*³⁶⁸.

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 receptors⁴²⁹. 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 retention⁴²⁹. 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 PET³⁸¹. Lastly, incorporation of a brush border enzyme-cleavable linker, a glycine-lysine dipeptide, between the ¹⁸F-containing moiety and the 2Rs15d nanobody reduced renal activity levels as seen in micro-PET/CT images of SKOV-3 xenograft bearing mice⁴³⁰.

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^{414,415,431,432}. The anti-PD-L1 nanobody KNo35 fused to Fc (KNo35-Fc) induced strong T cell responses and inhibited tumor growth of A375-PD-L1 cells in NOD-SCID mice *in vivo* [78]. 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 antibody³⁷⁵. 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 (SIRP α) 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 blockade³⁹⁵. Interestingly, administration of the A4 nanobody synergized with PD-L1, but not CTLA4 blockade⁴³³.

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^{434,435}. 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 production³¹¹. Nanobodies can overcome most of these challenges, due to their shorter circulatory half-life, increased tissue penetration, stability and ease of production⁴³⁴. 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 cells³⁴⁵. 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 Azo lymphoma⁴³⁶. 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^{437,438}. 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*³⁸⁶. All conjugates showed cytotoxicity *in vitro*. However, granulocytes were more sensitive than monocytes to Ly-6C/Ly-6G-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 lymphoma⁴³⁹⁻⁴⁴¹, and the similar ^{131}I -tositumomab⁴⁴². Furthermore, promising results in early clinical trials have been obtained for antibodies specific for CD33^{443,444}, or preclinical results for a combination of CD20 and CD22 targeting antibodies^{445,446}. Nevertheless, the targeting of (large) solid tumors remains a challenge, as shown in trials with antibodies specific for MUC1⁴⁴⁷, CEA⁴⁴⁸⁻⁴⁵⁰, and CEA⁴⁵¹. 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 observed⁴⁵². The same nanobody, labeled with ^{131}I , has been used in a phase I clinical trial with breast cancer patients (NCT02683083)⁴⁵³. 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 cell⁴⁵⁴, showed promising results in targeting HER2⁺ cancers with different radioisotopes useful for TRNT⁴⁵⁵.

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 9079³⁸⁴.

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 nanoparticles⁴⁵⁶. 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*⁴⁵⁷. When formulated as a polymeric PEGylated micelle, similar receptor binding and internalization were observed, making micelles promising systems for active drug targeting⁴⁵⁸. 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⁺ tumors⁴⁵⁹. Extracellular vesicles (EV) are also being explored as nanoparticles for therapeutic purposes⁴⁶⁰. To be tumor specific, such EVs must be equipped with a targeting moiety. By anchoring EVs through a glycosyl-phosphatidylinositol (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 use⁴⁶¹.

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 APCs⁴⁶². Antibodies⁴⁶³, 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*⁴⁶⁴. 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 hCEA³⁶⁴. 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 nanobody⁴⁶⁵.

Another vaccination strategy focuses on activating cytotoxic CD8⁺ T cells through targeted delivery of cancer antigens to APCs by anti-CD11b nanobodies⁴⁶⁶. 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 model³²⁵. 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 VHH7⁴²⁰. 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 mice⁴⁶⁷. 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 cells⁴⁶⁸⁻⁴⁷⁰, presumably due to immunogenicity to the non-human scFv component in the CAR constructs⁴⁷¹. 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*⁴⁷², and showed an even better anti-tumor

responses when formulated as a bivalent α -EGFR-EGFR nanobody-based UniCAR⁴⁷³. 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 cells⁴⁷⁴. 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 antigens⁴⁷⁵. 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*⁴⁷⁶. 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 cells⁴⁷⁷. 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 model²¹⁰. Bispecific anti-CD38 nanobody-based CAR-NK cells effectively deplete CD38⁺ cells from patient-derived multiple myeloma bone marrow cells *in vitro*⁴⁷⁸. 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).

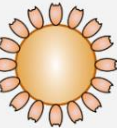
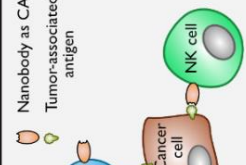
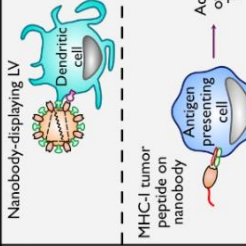
Diagnosis				
A	Positron emission tomography	Single photon emission computed tomography	Near-infrared fluorescence imaging	Ultrasound-based molecular imaging
	 Nanobody labeled with ^{68}Ga, ^{89}Zr, ^{64}Cu, or ^{18}F Advantages: - High tumor-background ratio - Imaging of tumors and tumor microenvironment - Limited side effects - Favorable biodistribution Drawbacks: - Short isotope half-life - Renal retention - Requires radioactive material	 Nanobody labeled with $^{99\text{m}}\text{Tc}$ Advantages: - Rapid clearance from blood - Imaging of tumors and tumor microenvironment - Limited side effects - Favorable biodistribution Drawbacks: - Short isotope half-life - Renal retention - Requires radioactive material	 Nanobody conjugated to IRDye 800CW or IRDye 680CW Advantages: - No radioactivity - Imaging for precise surgical resection Drawbacks: - Different strategies for Nb labeling have effect on biodistribution	 Shell: polymers or phospholipids, targeting nanobodies Core: ultrasound imaging gas Advantages: - No labeling with radioisotope or fluorescent marker Drawbacks: - Large in size, may limit biodistribution
Therapy				
B	Radionuclide therapy	Nanobody-drug conjugates	CAR-(T/NK) cell therapy	Anti-tumor vaccination
	 Nanobody labeled with ^{177}Lu or ^{131}I Advantages: - Specific targeting of tumors; less side-effects - Treatment of solid tumors Drawbacks: - Renal toxicity from radioactive material	 Drug (e.g. DM1)  PEGylation Drug in core (e.g. 17864)	 Nanobody as CAR Tumor-associated antigen	 Nanobody-displaying LV Activation of DC MHC-I tumor peptide on nanobody Activation of CD8⁺ T cells Recognition of tumor peptide on MHC-I Cytotoxic T cell response

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.

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.

Target	Disease examples	Origin	Model system tested	Nanobody name	Refs
ARTC2		<u>Murine</u> (ART2.2 in <i>Llama matahari</i>)	CD38 KO mice	S+16a	479
CAIX	Breast Cancer (ductal carcinoma)	rCAIX in <i>Camelus dromedarius</i>	PC3 and HeLa cell lines	K24	480
		<u>Human</u> (HeLa cells in <i>Llama glama</i>)	DCIS and CAIX xenograft-bearing SCID/beige mice	B9	481
CapG	Breast Cancer TNBC, melanoma, PDAC	<u>Human</u> (Recombinant CapG in <i>Llama glama</i>)	MDA-MB-231 cells, MDA-MB-231 cells in nude mice	CAPNb2	482
CD11b	Innate immune cell marker	Murine (BMDC in <i>Llama glama</i>)	BMDC and macrophage cell lines	V36, 76, 51, 81, Bio and 42	483
			HPV E7 xenograft bearing mice	VHH _{CD11b} (also known as VHH _{DC13})	484
CD20	B16 melanoma Melanoma, lung cancer, breast cancer	<u>Human</u> (hCD20-encoding plasmid and hCD20pos cells in <i>Llama glama</i>)	hCD20 _{pos} B16 xenograft-bearing mice	9077, 9079	485
CD33	AML	rCD33 in <i>Llama glama</i>	THP-1 tumor xenograft-bearing mice	Nb_7, Nb_21, Nb_22	486
CD38	Multiple myeloma	<u>Human</u> (rCD38 ectodomain, C-terminal domain, or cDNA expression vector for full-length CD38 in <i>Llama glama</i>)	LP-1, OPM2 and RPMI8226 myeloma cell lines, Primary malignant plasma cells	MU375, MU1053, MU551	487
			Human CD38-expressing DC27.10 cells in nude mice	WF211, MU1067, JK36, JK2, MU523, WF14 and MU738	488
CD45		<u>Mouse</u> (Mouse BDMC cells in <i>Llama glama</i>)	<i>In vitro</i> assays	G7 and 32b	483
CD47	AML, NHL, gastric, ovarian,	<u>Mouse</u> (Ig-like V-type	Tubo-EGFR mouse breast	A4	489

	colon and hepatocellular cancer	domain (ECD) of mouse CD47 in alpaca)	cancer cell line, BALB/c BMDMs, B16F10 cells		
			BMDMs and B16F10 xenograft-bearing C57BL/6 mice	A4 fusion to IgG2a Fc (A4Fc)	490
		<u>Human</u> (hCD47(ECD)-Fc in <i>Camelus bactrianus</i>)	Raji cell lymphoma NOG mice, cynomolgus monkeys	HuNb1-IgG4	491
CD7	Leukemia	<u>Human</u> (CD7+ Jurkat cells in <i>Llama glama</i>)	Leukemia cell lines, CEM xenograft-bearing nude mice	VHH6	492
			T-ALL PDX model for humanized VHH6	Humanized VHH6	493
CD8	B16 melanoma, pancreatic cancer	<u>Human and mouse</u> (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	494
CEA	Epithelial cancers (lung, thyroid, pancreas, uterus, breast, ovary, colorectal)	<u>Human and murine</u> (CEA in <i>Camelus dromedarius</i>)	LS174T cells and LS174T xenograft-bearing mice	cAb-CEA5	495
		<u>Human</u> (CEA in <i>Vicugna pacos</i>)	LS174T cells and MC38(CEA) mouse colon cancer cells	JJB-B2	496
			H460 xenograft-bearing nude mice	^{99m} Tc-nanobody	497
c-Met	Brain, liver, pancreatic and gastric cancer, multiple myeloma	<u>Human</u> (c-MET-Fc in <i>Llama glama</i>)	hMSCs	Anti-c-Met nanobody, bispecific	498 Nb patent by Beste et
		<u>Human</u> (A431 cells in <i>Llama glama</i>)	A549 cells, MKN-45 cells	G2	499
CTLA-4	B16 melanoma	<u>Human</u> (CTLA-4 protein in	B16/B6 melanoma cell injected	Nb16	500

		<i>Camelus dromedarius</i>)	C57BL/6 mice		
		<u>Murine</u> (CTLA-4 ECD fused to Fc domain in <i>alpaca</i>)		H11	501
CXCL11	Pre-B lymphoma	<u>Human</u> (Chemokine mixture in <i>Llama glama</i>)	HEK293T cells	11B1, 11B7	502
CXCL12				12A4	
CXCR2	Acute and chronic inflammatory diseases, cancer metastases	<u>Human</u> (CXCR2-expressing cells or pVAX1-hCXCR2 DNA in <i>Llama glama</i>)	CHO-CXCR2 cells	127D1, 163E3	503
CXCR4	HIV-1, tumor growth and metastasis, WHIM syndrome	<u>Human</u> (CXCR4-expressing HEK293T cells in <i>Llama glama</i>) 90% sequence identity with murine ortholog	Cynomolgus monkeys	238D2 and 238D4 (mono- and biparatopic)	504
			HEK293T and CXCR4-R334X overexpressing K652 cell lines	10A10	505
		<u>Human</u> (CXCR4-expressing lipoparticles in <i>Llama glama</i>)	SUP-T1 and Jurkat cells	VUN400, VUN401, VUN402	506
CXCR7	Head and neck cancer	<u>Human</u> (CXCR7-expressing HEK293 cells or pVAX1-CXCR7DNA in <i>Llama glama</i>)	22A xenograft-bearing nude mice	NB1, NB2, NB3, NB4, NB5 (mono- and biparatopic)	507
EGFR	Epithelial cancers	<u>Human</u> (EGFRvIII peptide in <i>Camelus bactrianus</i>)	Ascites fluid of NSCLC	OR1-83, OR2-83	508
		<u>Human</u> (A431 cells in <i>Llama glama</i>)	Murine xenograft models	Ia1, IIIa3, L2-3.40, 9G8	509
				EGa1	510
				8B6	511

				aEGFR- aEGFR-aAlb	512
				7C12, 7D12	513
				CONAN-1 (7D12-9G8- Alb1)	514
				OA-cb6	515
				OR1-83, OR2- 83	508
Fibro- nectin (EIIIB)	Mammary carcinoma	Mixture of ECM proteins, domains and peptides in <i>alpaca</i>	LM2 xenografts in NSG mice	NJB2	516
HER2	Breast cancer	<u>Human</u> (HER2- Fc recombinant fusion protein in <i>Camelus dromedarius</i>)	HER2+ SKOV3 tumor bearing mice	2Rs15d, 1R136d	517,518
		<u>Human</u> (MCF7 or BT474 cells in <i>Llama glama</i>)	SKBR3 xenograft- bearing mice	11A4	519
		<u>Human</u> (SKBR3 cells in <i>Llama glama</i>)	BT474M1 xenograft-bearing mice	5F7GGC	520
HGF	Glioma	<u>Human</u> (HGF in <i>Llama glama</i>)	U87 MG xenograft-bearing mice	1E2-Alb8, 6E10-Alb8	521
Ly-6C/ Ly-6G	Myeloid cells in immune diseases and cancer	<u>Mouse</u> (mouse splenocytes in <i>alpaca</i>)	NUP98/HOXB4 cells and C57BL/6j mice	VHH16, VHH21	522
MHC-II	Pancreatic cancer	<u>Murine</u> (murine splenocytes in <i>alpaca</i>)	panco2-tumors in C57/BL6 mice	VHH7, VHHDC8, and VHHDC15	523
	Graft versus Host Disease	<u>Human</u> (Purified HLA antigen in <i>Vicugna pacos</i>)	Xenograft model of GvHD	VHH4	524
MMR	TAMs infiltrating tumors	<u>Human</u> (MMR EC in <i>Vicugna pacos</i>)	TS/A and 3LL-R tumor-bearing mice	Nb cl1	525
		<u>Human</u> and <u>murine</u> (recomb. Monomeric		3-49	526

		fusion proteins in <i>Vicugna pacos</i>)			
PD-L1	NSCLC, colon, thyroid, uterus, pancreas, and ovary cancer	<u>Human</u> (PD-L1 Fc fusion protein in <i>Camelus bactrianus</i>)	PD-L1 ⁺ A375 cells + hPBMCs xenograft-bearing nude mice	KN035	527
		<u>Murine</u> (RAW264.7 cells in <i>Camelus dromedarius</i>)	TC-1 (WT and PD-L1 KO) in WT or PD-L1 KO mice	C3, E2	528
		<u>Human</u> (PD-L1-Fc protein in <i>alpaca</i>)	PD-L1 ⁺ MCF7 and 624-MEL xenograft-bearing nude mice	K2	529
		<u>Human</u> clinical trial	Human NSCLC patients	NM-01	530
PSMA	Prostate cancer	<u>Human</u> (Purified PSMA antigen in <i>Camelus dromedarius</i>)	<i>In vitro</i> binding predictions	C9, C24, N14, N50	531
		<u>Human</u> (rPSMA in <i>Camelus bactrianus</i>)	LNcaP and PC3 cells	C3	532
		<u>Human</u> (LNCaP cells, PSMA peptide, rPSMA EC in <i>Camelus dromedarius</i>)	PC-3 and LNCaP xenograft-bearing nude mice	PSMA30	533
		<u>Human</u> (4 different PCa cell lines in <i>Llama glama</i>)	PC-310 and PC-3 xenograft-bearing NMRI mice	JVZ-007	534
			LNcaP, C4-2 or MKN45 xenograft bearing BALB/c-nu nude mice		535
TNFα	Sarcomas, melanomas, carcinomas	DNA sequences encoding the camelidae antihuman TNF α single-domain)	MCF-7, T-47D and MDA-MB-231 cell lines, 4T-1 breast cancer mouse model	anti-TNF-VHH	536

TUFM	Glioblastoma	<u>Human</u> (GBM stem-like cells in <i>Alpaca</i>)	Several GBM cell lines and tissues	Nb206	537
VEGF/VEGFR	Angiogenesis in solid tumors	<u>Human</u> (293KDR cells in <i>Camelus dromedarius</i>)	HUVEC cells	3VGR19	538
		<u>Human</u> (VEGF ₁₂₁ in <i>Camelus dromedarius</i>)		Nb22, Nb23, Nb35, Nb42; Humanized Nb42	539,540
		<u>Human</u> sdAb from HuSdI™		NTV1	541
			Chorioallantoic membrane	VA12	542
Viral GPCR US28	Glioblastoma	pVAX1-US28 DNA boosted with HEK293T-US28 expressing cells in <i>Llama glama</i>	U251 cells, intracranial GBM mouse model	(bivalent) US28 nanobody	543
		pcDEF3 vector encoding for VHL/E US28 in <i>Llama glama</i>	U251 cells	VUN100	544
			<i>In silico</i>	Nb7	545

Table 1. Currently available nanobodies for tumor-relevant targets.

Outline of this thesis

In this thesis, we describe the targeting of tumor-specific proteins for cancer diagnosis and therapy. The thesis is divided into two parts.

Part 1, chapter 3 goes into detail on the establishment and characterization of nanobodies targeting MICA. These nanobodies, VHH-A1 and VHH-H3, show specific recognition of the most common alleles of MICA on cancer cells (MICA*008 and MICA*009). Therapeutically, we produced a nanobody-drug conjugate (NDC) by fusion of VHH-A1 to the Mertansine derivative molecule DM1. We treated the T-cell lymphoma cell line “EL-4” - stably transfected to express MICA - with the NDC in an *in vitro* model. We see excellent cytotoxicity of MICA⁺ cells compared to WT cells, with a clear reduction in IC₅₀ and specific targeting of MICA⁺ cells. In **chapter 4**, we describe unpublished data on the nanobody-drug conjugate used for the *in vivo* treatment of EL-4 MICA⁺ tumors. Although the *in vitro* results of the DM1-based nanobody-drug conjugate showed promising results, we did not observe significant reduction in tumor growth in EL-4 MICA⁺ tumor-bearing mice treated with intraperitoneal VHH-A1 nanobody-drug conjugate. In **chapter 5**, we describe the construction of a chimeric antigen receptor (CAR), using VHH-A1 and VHH-H3 nanobodies as the targeting domains. We expressed the construct in human NK-92 cells. We confirmed the localization of the VHH-A1-based CAR NK cells to MICA⁺ tumors in a lung metastases model with PET imaging, using a ⁸⁹Zr-labeled nanobody targeting the transferrin receptor on the surface of the NK cells. Therapeutically, we confirm the ability of these CAR NK-92 cells to kill MICA⁺ cancer cells *in vitro* on MICA⁺ EL-4 and B16F10 melanoma cells, and *in vivo* on MICA⁺ B16F10 tumors. In **chapter 6**, we describe unpublished data on the production of nanobody-based CAR T cells, and their use in *in vitro* cytotoxicity experiments. We confirmed specific cytotoxicity of MICA⁺ B16F10 and EL-4 cells when co-cultured with VHH-based CAR T cells.

Part 2, chapter 7 goes into detail on the establishment and characterization of a monoclonal antibody which recognizes a unique 13-amino acid epitope in the cytoplasmic tail of HLA-E. The epitope is not found on other human proteins. The antibody should thus show no cross-reactivity to other MHC-I molecules, and can be used as antibody-epitope pair with the corresponding epitope. We modified the antibody to contain an LPETG motif (for sortase-

mediated modification) and a (His)₆-tag (to facilitate purification on a NiNTA matrix) on the C-termini of both heavy and light chains. We show that the antibody can be modified by a site-specific and efficient sortase-catalyzed transpeptidation reaction to install fluorophores or biotin. The antibody, either modified or unmodified, can be used for labeling HLA-E intracellularly in flow cytometry, immunofluorescence, immunohistochemistry, and immunoblot. The antibody is thus a great tool for diagnostic purposes, and the antibody-epitope pair can also be used for tagging non-HLA-E specific targets.

In **Chapter 8**, the results of the abovementioned projects are summarized and discussed, and future perspectives are described.