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REVIEW

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Nanobodies targeting the tumor microenvironment and their formulation as nanomedicines

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Abstract

Among the emerging strategies for cancer theranostics, nanomedicines offer significant promise in advancing both patients' diagnosis and treatment. In combination with nanobodies, nanomedicines can potentially enhance the precision and efficiency of drug or imaging agent delivery, addressing key limitations of current approaches, such as off-target toxicities. The development of nanomedicines will be further accelerated by the creation of smart nanoparticles, and their integration with immunotherapy. Obviously, the success of nano-immunotherapy will depend on a comprehensive understanding of the tumor microenvironment, including the complex interplay of mechanisms that drive cancer-mediated immunosuppression and immune escape. Hence, effective therapeutic targeting of the tumor microenvironment requires modulation of immune cell function, overcoming resistance mechanisms associated with stromal components or the extracellular matrix, and/or direct elimination of cancer cells. Identifying key molecules involved in cancer progression and drug resistance is, therefore, essential for developing effective therapies and diagnostic tools that can predict patient responses to treatment and monitor therapeutic outcomes. Current nanomedicines are being designed with careful consideration of factors such as the choice of carrier (e.g., biocompatibility, controlled cargo release) and targeting moiety. The unique properties of nanobodies make them an effective engineering tool to target biological molecules with high affinity and specificity. In this review, we focus on the latest applications of nanobodies for targeting various components of the tumor microenvironment for diagnostic and therapeutic purposes. We also explore the main types of nanoparticles used as a carrier for cancer immunotherapies, as well as the strategies for formulating nanoparticle-nanobody conjugates. Finally, we highlight how nanobody-nanoparticle formulations can enhance current nanomedicines.

Keywords Nanobody, Nanoparticle, Tumor microenvironment, Immunotherapy, Conjugational chemistry

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Introduction

Over the last few decades, oncology has rapidly advanced, offering cancer patients numerous treatment options, diagnostic opportunities, and preventive strategies. Among these, targeted therapies and immunotherapies have emerged as some of the most promising approaches. Targeted therapies rely on the identification of biomarkers within the tumor microenvironment (TME) that can be used as anchor points to specifically deliver therapeutic compounds, while immunotherapies harness the power and specificity of anti-tumor immune cells, both enhancing treatment efficacy while minimizing toxic side effects [1]. Depending on the specific cancer diagnosis, patients can benefit from a range of targeted or immunotherapeutic approaches, including antibody–drug conjugates, immune checkpoint inhibitors, adoptive cell transfer, cytokine therapies, and therapeutic vaccines, among others. However, despite the potential, the field still faces significant challenges, such as low response rates in certain cancers and potential toxicities [2].

In this context, the development of nanomedicines presents a promising solution, not only for enhancing drug delivery and improving the efficacy of these therapies, but also for predicting and monitoring the therapy response (Fig. 1). Nanomedicines can facilitate controlled delivery of therapeutic agents directly to specific sites within the TME, thereby most optimally affecting the target cell or strongly activating immune responses in localized tumor areas [2, 3]. In other words, to improve the biodistribution profile of drug payloads and circumvent the systemic toxicity to healthy tissues, these agents can be encapsulated into nanoparticles (NPs), carefully

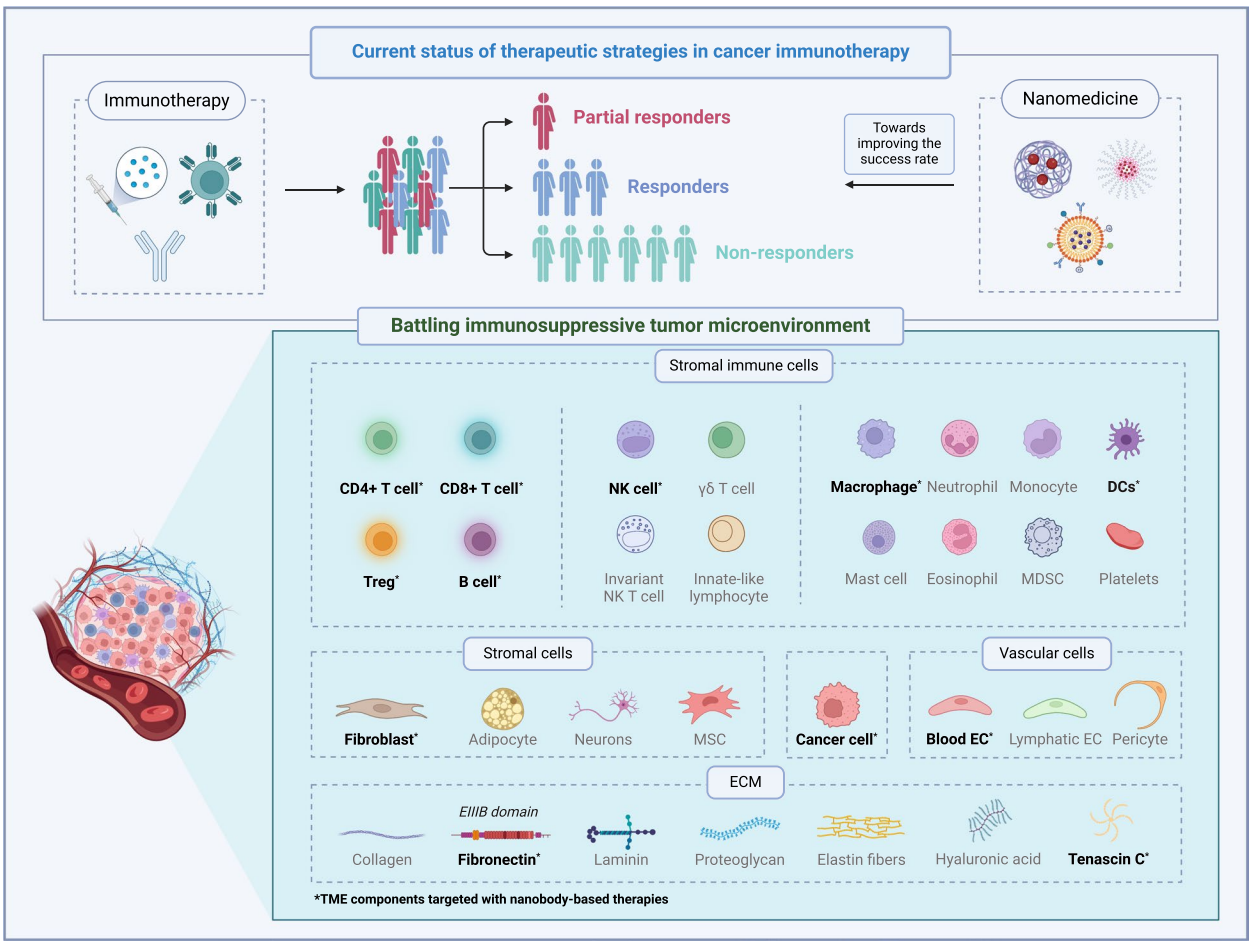

Fig. 1 Components of the tumor microenvironment that are targeted by nanobody-based therapies. Nanomedicines, encompassing encapsulated drugs that are delivered to a specific TME compartment in a targeted fashion, allow for the design of enhanced immunotherapeutic strategies. Moieties, like nanobodies, aid in the therapeutic targeting of selected cell types. Various immune, stromal, vascular and cancer cell molecules, as well as components of the extracellular matrix (ECM) have been assessed as therapeutic targets for nanobody-based therapies. Created in https://BioRender.com

designed to release their cargo under different environmental cues, such as pH, temperature, or electromagnetic fields. Nanoparticles can also deliver imaging agents for cancer diagnosis and patient stratification or combine diagnostic and therapeutic agents in a single formulation (theranostic nanomedicine). Furthermore, to achieve targeted delivery, nanoparticles can be functionalized with specific targeting moieties that bind with a high affinity to molecules within the TME. Monoclonal antibodies (mAbs) have often been used for this [4], but an attractive alternative is the single-domain antibodies (nanobodies, or Nbs), which offer unique advantages in terms of size, stability, conjugation chemistry, and specificity [5].

Nanobodies are small, single-domain moieties derived from the variable domain (VHH) of naturally occurring heavy-chain-only antibodies (HCAbs) found in camelids [6] (Fig. 2). Thanks to their unique properties, nanobodies have found diverse applications across various fields, being widely used in medical diagnostics and therapeutics, especially for oncology, neuro- and cardiovascular pathologies, and infectious diseases [7]. In the field of cancer therapy, nanobodies are particularly useful as they are roughly 10 times smaller (12–15 kDa) than conventional mAbs, which contributes to better tumor-penetrating characteristics [8, 9]. Moreover, Nbs bind to their target with high affinity within the nanomolar-picomolar range. Due to their convex paratope, Nbs can bind to cryptic epitopes, often inaccessible to conventional Abs, which, in contrast, possess flat or concave paratopes [10]. On top of that, Nbs are highly stable under proteolytic conditions and acidic pH (allowing them to withstand the harsh conditions of the TME), as well as high temperatures, which makes these molecules particularly suitable for modifications, such as radiolabeling (e.g., to deliver diagnostic and/or therapeutic radionuclides) and chemical conjugations (e.g., to fluorescent dyes, nanoparticles, photosensitizers, immunomodulatory molecules) [11]. Furthermore, the simple structure of Nbs and the relative ease of production allow for a wide range of molecular engineering strategies [12]. As such, Nbs can be generated in multivalent formats to increase their stability and avidity, fused with an Fc domain to gain an effector function, or purposefully engineered to have an extended half-life (e.g., by fusing to an albumin-binding Nb) [13–15]. Moreover, they can easily be engineered in the format of nanobody-drug conjugates, nanobody-based CAR-T cells, and bispecific T-cell engagers (BITEs) [16–18]. Although some nanobodies are reported to be able to penetrate the blood–brain barrier (BBB), they can be further engineered to facilitate the uptake in brain tissue, allowing for potential therapeutic application in various brain diseases, which represents another major advantage over conventional mAbs [19]. Finally, to address the risks of immunogenicity, Nbs can easily be humanized, although they are generally reported to be low- to non-immunogenic in mice and humans [20].

Thanks to their numerous advantages (Fig. 2), Nbs have made it to the clinic in recent years. The first nanobody-based drug, Caplacizumab, recognizing the von Willebrand factor, was approved by the FDA and EMA in 2018 for the treatment of thrombotic thrombocytopenic purpura [21]. In 2022, Ciltacabtagene autoleucel, a second-line therapy for multiple myeloma, was approved by the FDA. This therapy uses chimeric antigen receptor T-cells (CAR-T) engineered to target B-cell maturation antigen (BCMA) with nanobodies [22]. Additionally, Ozoralizumab, a trivalent anti-TNF Nb construct, was approved in Japan for rheumatoid arthritis in 2022 [23]. Aside from these clinically approved examples, many other nanobody-based therapies are currently being investigated at the stage of preclinical development or in clinical trials for the treatment of cancer. Nanobodies clearly hold great potential as a powerful therapeutic tool in the field of oncology. However, the development of a successful nanobody-based cancer therapeutic starts with the identification of potential targets within the TME (Fig. 1). Such molecular targets should either be directly involved in tumor progression (suggesting the targeting by antagonistic or agonistic compounds) or should be expressed on cells that contribute to tumor growth and invasion

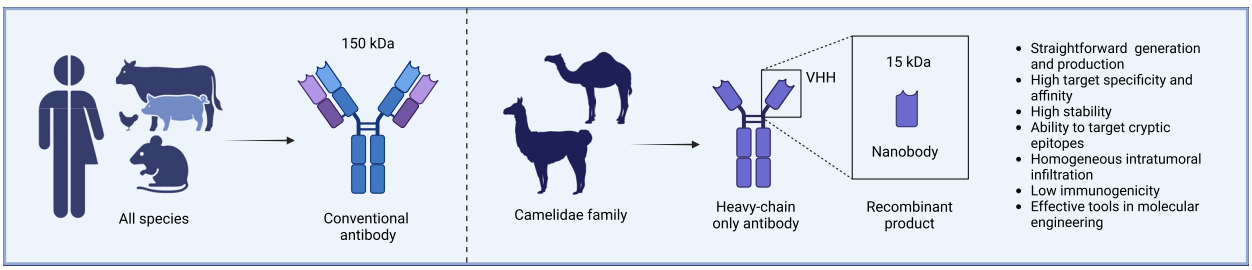

Fig. 2 Advantages of nanobodies. Nanobodies are derivatives of heavy-chain-only antibodies found in camelids. Their unique properties offer numerous advantages for designing targeted cancer immunotherapies and diagnostics. Created in https://BioRender.com

(suggesting the elimination or repolarization of such cells). Ideally, these targets should be uniquely expressed, or at least overexpressed, in the TME as compared to healthy tissues. This article reviews recent advances in the use of nanobodies to target cancer cells, immune cells, and stromal elements of the TME. We will also discuss the advantages of nanoparticles and the use of nanobody-based nanoparticle formulations for targeted drug delivery and therapeutic development, highlighting the potential for these technologies to revolutionize cancer treatment.

Molecular targets for Nanobodies in the tumor microenvironment

It is now evident that cancer initiation and progression are not only the consequence of the genetic alterations in the cancer cells, but they are also supported by the non-transformed tumor microenvironment, comprised of normal cells embedded in the extracellular matrix (ECM). In solid tumors, such normal cells generally include fibroblasts, endothelial cells, pericytes, and immune cells of lymphoid and myeloid origin [24]. Some tissue-specific cells, such as adipocytes and neurons, can also be present in the TME [25, 26]. Altogether, this complex cellular ecosystem, along with a plethora of soluble factors, contributes to immune suppression and therapy resistance. Targeting the TME is, therefore, becoming increasingly important for developing effective cancer therapies.

Nanobody-based targeting of immune cell function

The immune cell compartment of the TME consists of cells that, under normal conditions, help to maintain homeostasis. However, in the presence of pathological signals, these cells become immunosuppressive, aiding tumor growth (e.g., macrophages, regulatory T cells/Tregs). Furthermore, immune cells that typically engage in immunosurveillance may become exhausted, resulting in diminished antitumor responses (e.g., cytotoxic T lymphocytes, natural killer (NK) cells, and certain subsets of dendritic cells/DCs and B cells). To shift the phenotype of these cells towards an anti-tumoral state, nanobodies can be engineered for direct targeting and activation of immune cells, modulation of immune responses, and blockade of immune-inhibitory pathways (Fig. 3).

Targeting of immune checkpoints

One of the most effective strategies for boosting anti-tumor immune responses is the inhibition of immune checkpoint molecules (IC), which are often exploited by cancer cells to suppress anti-tumor T-cell activity [27]. ICs include cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed death receptor 1 (PD-1), programmed death ligand 1 (PD-L1), T-cell immunoglobulin and mucin domain-containing 3 (TIM-3), T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), and lymphocyte-activation gene 3 (LAG-3).

Since not all patients effectively respond to IC inhibitors, strategies for patient stratification, prediction, and follow-up of the therapy responses are required. Non-invasive molecular imaging techniques such as single-photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), optical imaging (bioluminescence and fluorescence), or their combination (multimodal imaging) can be used to identify those patients potentially benefiting from a specific treatment (Fig. 3). Due to their unique properties, Nbs are particularly well-suited for the development of PET and SPECT imaging probes. Broos et al. have generated a high-affinity anti-human PD-L1 specific nanobody, designated as K2, showing its use as a radiotracer for the SPECT/μCT-mediated in vivo detection of PD-L1 expression and its ability to block the PD-L1/PD-1 interaction [28]. Thanks to the rapid blood clearance and efficient tissue penetration, K2 allows for high-contrast imaging of PD-L1 with a high tumor-to-blood ratio one hour after injection, much faster than the clinically approved mAb atezolizumab (7 days post-injection). The anti-huPD-L1 Nb was further validated as a ^{68} Ga-labelled molecular tracer for in vivo PET imaging, more commonly used in clinical settings due to its better spatial resolution, showing favorable distribution patterns [29]. Along the same line, an anti-LAG-3 Nb-based tracer was developed to image human LAG-3 in mouse tumors (huLAG-3 transfected cancer cells), demonstrating its potential to be translated into a PET-tracer for clinical application [30]. It is important to note that the development of Nb-based PET and SPECT imaging probes is enhanced by the rapid blood clearance of Nbs, allowing the use of short-lived isotopes and thus reducing the radiation burden on patients. In contrast, radiolabeling of mAbs typically requires long-lived isotopes.

However, for therapeutic purposes, the fast clearance rate of Nbs from the circulation (half-life of 60–90 min) is one of the main drawbacks, necessitating measures to increase their lifetime [31]. This can be achieved by nanomedical features, e.g. a slow release of Nb K2 through encapsulation in a peptide hydrogel led to a higher accumulation of the Nb in melanoma tumors [32]. Another strategy that increases the in vivo lifetime of these biologicals is the generation of multimodal constructs, of which several examples are available that target ICs. Lentiviral delivery of K2 Nb-Fc fusions significantly outperformed the anti-PD-L1 antibody avelumab in tumor cell

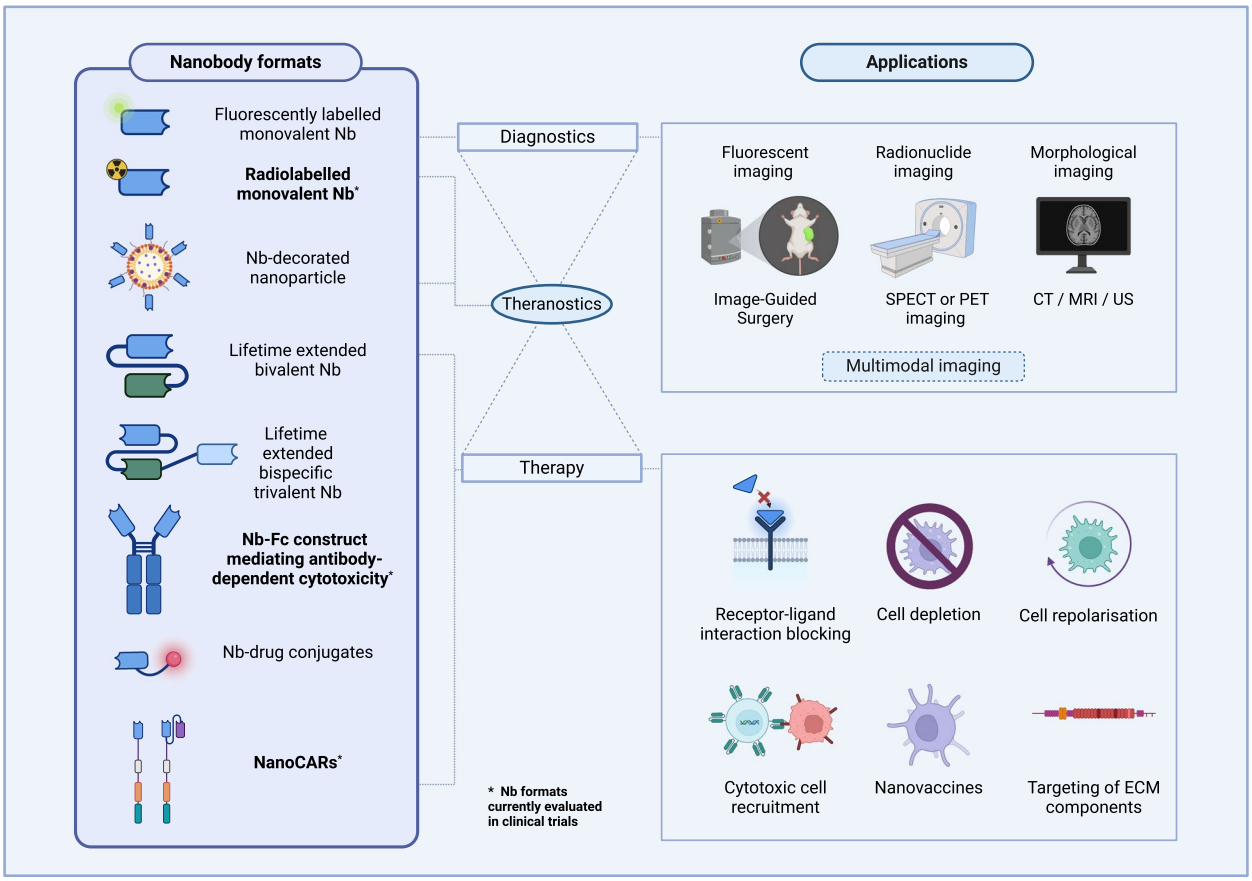

Fig. 3 Nanobody formats and their applications as cancer therapeutics and diagnostics. Nanobodies have been used to design imaging probes for different imaging techniques. The same nanobody can be engineered as a therapeutic to break the immunosuppressive pathways in the TME. Certain modalities can be used on the intersection of diagnostics and therapy, serving as theranostic tools in immuno-oncology. Created in https://BioRender.com

killing, as shown in a 3D melanoma model [33]. Fusing two Nbs to a human IgG1 Fc fragment not only prolongs their circulation time but also confers bivalency and enables effector functions, resulting in higher target affinity and enhanced antibody-dependent cellular cytotoxicity (ADCC). To enhance the efficacy of anti-PD-L1 therapy in "cold" tumors, Yu et al. designed a PD-L1/TLR7 dual-targeting nanobody-drug conjugate. The TLR7 agonist boosted the expression of PD-L1, promoting the anti-tumor efficacy of the anti-PD-L1 treatment and leading to T-cell memory activation [16]. Currently, an increasing number of studies are focusing on combination therapies using several IC inhibitors, aiming to target multiple disease pathways, potentially overcoming resistance mechanisms and enhancing synergistic antitumor immunity. Ma et al. generated a multivalent bispecific "antibody" (BsAb), consisting of tetravalent anti-PD-L1 Nb-Fc-fusions and tetravalent anti-TIGIT Nbs, which resulted in enhanced T-cell activity in vitro compared to the parental Nbs [34]. Compared to combination treatments with mAbs, BsAbs offer advantages in cost-effectiveness and enhanced targeting specificity, potentially reducing on-target off-tumor toxicity. Another example of the attempt to block two ICs at once is provided by Zeng et al., who developed a bispecific anti-PD-1/CTLA-4 nanobody called Z15-0 [17]. Administration of Z15-0-encoding mRNA, formulated in lipid nanoparticles, inhibited tumor growth in MC38 colon carcinoma tumor-bearing mice [17]. Preclinical studies of KN046, a humanized anti-PD-L1/CTLA-4 bispecific Nb-Fc fusion construct, demonstrated its superior effect on T-cell activation compared to the combination of monotherapies and a lower toxicity than that of the anti-CTLA-4 inhibitor. Furthermore, a Phase II study (NCT03872791) with KN046 in combination with nanoparticle albumin-bound paclitaxel (nab-paclitaxel), demonstrated good clinical efficacy and survival outcomes in patients with metastatic triple-negative breast cancer [35]. Finally, IC blockade can also be combined with other modalities, such as cancer cell targeting and T-cell activation. Indeed, the

Nb-based trispecific T-cell engager, Nb-TriTE, targets both PD-L1 and HLA-G on cancer cells along with CD3ε on T cells. Nb-TriTE showed better anti-tumor efficacy compared to monoclonal antibodies and bispecific T-cell engagers, inhibiting tumor growth and prolonging survival in a humanized orthotopic mouse lung cancer model [36]. Another example is provided by a 4-1BB/PD-L1 bispecific (PM1003) Nb construct, that combined potent inhibition of PD-L1 activity with 4-1BB agonism upon cross-bridging with PD-L1 in vitro. Antitumor activity of the single agent PM1003 was superior to the combination of anti-PD-L1 and anti-4-1BB Abs, with minimal toxicity found in vivo [37].

Targeting of specific immune cell types

Visualizing specific immune cell subsets in tumors has gained a lot of interest for the prediction of therapy efficacy and patient stratification, as well as for therapy follow-up. In this respect, effector memory $CD8^+$ cytotoxic T cells are mostly associated with a more favorable outcome. De Groof et al. developed a tracer against huCD8β, which was validated in huCD8 knock-in (KI) mice for the non-invasive imaging of $CD8^+$ T-cell dynamics as a perspective tool for immunotherapy follow-up. The tracer was evaluated via SPECT and PET imaging in naïve and tumor-bearing KI mice and in naive non-human primates, displaying a high sensitivity and specificity of $CD8^+$ T-cell detection since the CD8β chain is unique for this cell type, in contrast to CD8α [38]. This Nb was shown to be fully non-immunogenic. Similarly, huCD4 KI mice were employed to validate anti-huCD4 Nbs for non-invasive imaging [39]. It is important to realize that $CD4^+$ T cells can adopt different phenotypes, including immunosuppressive regulatory T cells (Treg) that support tumor growth. Interestingly, tumor-infiltrating (ti) Tregs express markers that discriminate them from effector $CD4^+$ T cells and peripheral Tregs, including CCR8 and IL1R2. Anti-CCR8 Nbs were shown to specifically bind and deplete (upon Fc functionalization) tiTregs, without signs of immune-related adverse events [40]. Of note, $CD8^+$ or $CD4^+$ T cells may also be exhausted, illustrating the need to detect activated T cells. Nb targeting huOX40 (CD134), a receptor expressed on activated T cells, was developed to monitor these cells in vivo. After binding to its ligand, OX40, a member of the TNF receptor superfamily, regulates T-cell survival, differentiation, and proliferation [41]. Nbs against 4-1BB, another T-cell activation marker, were mentioned before, and also Nbs against CD69, an early activation marker, are under consideration.

As for the myeloid cell compartment of tumors, tumor-associated macrophages (TAMs) comprise a major cell type of tumor stroma, that plays a significant role in tumor progression, metastasis, and drug resistance. Hence, targeting TAMs is a promising strategy in cancer immunotherapy, with the two main approaches currently focusing on the depletion of pro-tumoral TAMs or their modulation towards an anti-tumoral phenotype. Consequently, visualizing highly suppressive TAM subsets potentially carries a significant theranostic value in cancer. Movahedi et al. were able to target and visualize pro-angiogenic $CD206^+$ TAMs in hypoxic tumor regions using ^{99m}Tc-labeled anti-CD206 Nbs [42]. [^{68}Ga]Ga-NOTA-anti-CD206-Nb was then preclinically validated as a PET tracer, showing a high in vivo specificity for the target with no observed toxicity [43]. A phase I study confirmed the safety and specific uptake of the tracer in patients with solid tumors [44], leading to a currently running phase II trial (NCT04168528) in which tracer uptake is being correlated with CD206 expression through immunohistochemistry. Additionally, uptake of the [^{68}Ga]Ga-NOTA-anti-CD206-Nb will be evaluated in patients with macrophage-related pathologies in another phase II study (NCT04758650). Recently, Lauwers et al. developed a highly specific anti-CD163 PET tracer. Given the immunosuppressive role of $CD163^+$ TAMs, this tracer could serve as a valuable tool for predicting responses to macrophage-targeting therapies and as a follow-up approach for monitoring treatment progress [45]. Functionally important molecules on TAMs, that have been targeted by Nbs, include neuropilin-1 (NRP-1) and SIRPα. NRP-1 mediates the migration of TAMs into hypoxic areas, where they become highly pro-tumoral [46], and blocking NRP-1 with antagonistic anti-NRP-1 Nbs suppressed tumor growth in a colorectal carcinoma (CRC) model [47]. SIRPα inhibits macrophages' phagocytic capacity upon binding to the CD47 "don`t eat me" signal, so blocking this interaction would unleash macrophage killing by TAMs. In vivo imaging of $SIRP\alpha^+$ macrophages has been accomplished in mouse glioblastoma tumors [48], while a ^{64}Cu-hSIRPα-S36 Nb-based PET tracer visualized tumor-infiltrating macrophages in huSIRPα/CD47 KI mice [49]. In another study, introducing high-mannose glycans onto an anti-CD47 Nb (HM-nCD47) and displaying the Nb on cellular vesicles (CVs) extended its therapeutic half-life and activated the macrophage-mediated antitumor immunity in both subcutaneous and metastatic murine tumor models [50].

Currently, there are only few studies on the Nb-based targeting of DCs. However, some attempts have been made to utilize Nbs as a targeting moiety to deliver DC-specific vaccines (nanovaccines). Recently, Jung et al. have conjugated an anti-CD11c Nb to a magnetic core nanocarrier via a thiol-maleimide reaction and demonstrated the specific targeting of the functionalized nanoparticles to CD11c+cells in vivo. Nb-NP functionalization and

optimization, in that case, was a much more straightforward process than that for Ab conjugation [51].

Nanobody-based targeting of ECM components and stromal cells

The tumor stroma includes ECM components such as collagens, glycoproteins (fibronectin, laminin), and proteoglycans, as well as cellular components, such as cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and the tumor-associated vascular system with endothelial cells and pericytes [24]. Stromal cells and ECM are active participants in tumorigenesis and are essential components of the TME, which is usually tumor type-specific and very dynamic. During the early stages of cancer progression, stromal cells often act in an anti-tumorigenic fashion, however, over time, they transition to a pro-tumorigenic role and ultimately contribute to mechanisms that promote tumor growth (cancer cell survival and invasiveness, angiogenesis, immune suppression), metastasis and therapy resistance [52]. Therefore, tools to target these cells and improve therapy efficacy are needed.

Targeting of ECM components

Protein components of the ECM present an attractive target for the development of cancer therapies. Unlike cancer cells, the elements of ECM are rarely mutated and, therefore, are less susceptible to immune evasion [53]. Their stability and abundance in different types of solid tumors allow for the design of pan-cancer diagnostic, therapeutic, or imaging tools.

One of the main components of ECM and the neovasculature is fibronectin (FN). This glycoprotein is overexpressed in tumors and fibrotic tissue, but is nearly absent in normal human tissues. Jailkhani et al. developed a high-affinity nanobody, NJB2, targeting the alternatively spliced EIIIB (EDB) domain of fibronectin that is found in tumors. This nanobody was used to image primary tumors and metastases of human and mouse triple-negative breast cancer and melanoma. Moreover, it outcompeted conventional ^{18}F-2-fluorodeoxyglucose (FDG) PET/CT imaging in detecting pancreatic lesions of mice with pancreatic ductal adenocarcinoma (PDAC), demonstrating a higher signal-to-noise ratio and clarity [53]. Targeting CAR-T cells to the TME via an anti-EIIIB nanobody demonstrated anti-tumor efficacy in the B16 melanoma model [54].

Tenascin-C (TNC) is a multimodular ECM glycoprotein that is highly expressed in cancer and chronic inflammatory diseases and has been considered as a promising target for diagnostic and therapeutic approaches in anticancer treatments. Jailkhani et al. generated three picomolar-affinity Nbs against the EGFL (Gly23 – Pro625) domain of huTNC, which, upon ^{64}Cu-coupling, visualized mammary gland tumors and their lung metastases via PET/CT scan [55]. Other organs were rapidly cleared from this tracer. Dhaouadi et al. used the long isoform of recombinant huTNC as antigen, yielding two moderate-affinity Nbs ("Nb3" with Kd of 711 nM; "Nb4" with Kd of 537 nM) that bind to TNC FNIII-3–5. Interestingly, these Nbs were shown to block dendritic cell adhesion on TNC in conjunction with CCL21, thus opening the possibility to overcome TNC functions in immunosuppression [56].

Targeting of stromal cells

CAFs are a subpopulation of activated fibroblasts that obtained a myofibroblast phenotype and are characterized by the expression of various pro-tumorigenic factors, such as TGF-β and cytokines involved in ECM remodeling, cancer cell proliferation, suppression of immune responses, recruitment of MSCs, and initiation of angiogenesis. CAFs are heterogeneous depending on the cancer type, but commonly associated markers include fibroblast activation protein α (FAP), platelet-derived growth factor receptors alpha and beta (PDGFRα/β), vimentin, desmin, CD90, CD10, alpha smooth muscle actin (α-SMA), and podoplanin (PDPN) [57]. FAP is gaining attention as a target for cancer theranostics. FAP expression is elevated in CAFs from carcinomas, where it contributes to tumor growth and immunosuppression, but also in glioblastomas, melanomas, and sarcomas. Conversely, it is almost absent in healthy tissues. Dekempeneer et al. identified a FAP-targeting high-affinity cross-reactive human/mouse nanobody, 4AH29, and a [^{68}Ga]Ga-DOTA-4AH29 tracer was shown to specifically detect FAP-positive tumors in mice. Moreover, repeated administration of therapeutic [^{225}Ac]Ac-DOTA-4AH29 and [^{131}I]I-GMIB-4AH29 inhibited tumor growth and prolonged survival in mice bearing huFAP-positive U87-MG xenografts [58]. The same effect was observed in immunocompetent mice bearing a huFAP-expressing lung cancer model, with the anti-tumor activity even being enhanced in combination with anti-PD-L1 [59]. Recently, also Nbs against PDGFRβ were reported, whose binding results in a fast uptake and delivery of its cargo (e.g., a conjugated toxin) to the lysosome [60].

Cancer-associated endothelial cells form a vascular network responsible for supplying the tumor with nutrients and oxygen. However, due to a lower expression of adhesion molecules, the tumor vasculature is leaky and can easily be intravasated by cancer cells, which can migrate to other sites. This irregular and immature vasculature is the consequence of a high production of angiogenic factors, such as vascular endothelial growth factor (VEGF) [61]. Among the receptors for VEGF, VEGFR-2 is notably unregulated under pathological conditions, particularly

in tumor endothelial cells, and thus is a well-explored target for anti-angiogenic cancer therapy. In one study, an anti-VEGFR-2 nanobody was chemically conjugated to a truncated diphtheria toxin moiety. The co-incubation of this immunotoxin with PC-3 cells led to a decreased cell survival [62]. Karami et al. tested the inhibition of angiogenesis using an anti-VEGFR-2 nanobody in combination with an anti-NRP-1 nanobody in vitro and in vivo, a combination that led to a strongly diminished tumor growth [63]. NRP-1 is indeed another crucial factor for tumor vasculature development and metastasis [64, 65].

Nanobody-based targeting of cancer cells

Cancer cells have been the main target of cancer therapies for decades. However, classical cancer cell-directed therapies, such as chemotherapy and radiation therapy, often result in off-target systemic toxicities, providing a rationale to deliver drugs in a targeted fashion to cancer cells [1]. This can be achieved by using exquisitely specific targeting moieties, such as antibodies and nanobodies, provided that a suitable molecular target is identified on the surface of the cancer cells. A multitude of molecules have been reported as being overexpressed on cancer cells and have been targeted by nanobodies (Fig. 4). A list is provided in Table 1 and elaborated on below.

Targeting of growth factor receptors

Human epidermal growth factor receptor 2 (HER2) is one of the most crucial targets in immuno-oncology. HER2-targeted therapies have been under development for over twenty years, with the expression of this biomarker serving as a predictive indicator of patient response. A Phase I study of an anti-HER2 nanobody-based tracer demonstrated the safety of [^{68}Ga]Ga-NOTA-anti-HER2-sdAb PET/CT imaging with optimal image quality at 90 min post-injection [66]. A Phase II trial further assessed the repeatability of [^{68}Ga]Ga-NOTA-anti-HER2-sdAb uptake, confirming its similarity to [^{18}F]FDG. Most importantly, tracer uptake was observed in cancer lesions of breast cancer patients previously classified as HER2-low or -negative based on [^{18}F]FDG uptake, significantly enlarging the group of patients that would benefit from

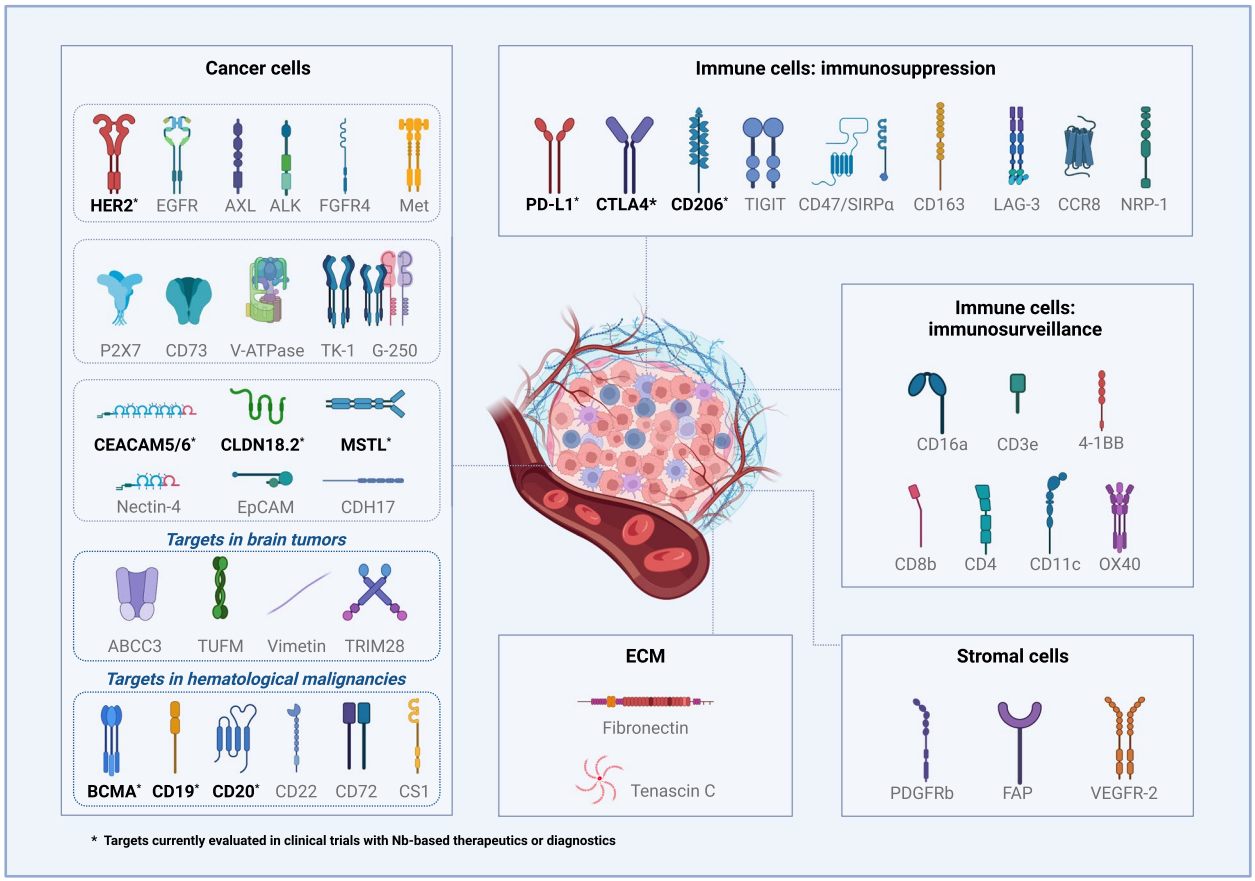

Fig. 4 Nanobodies have been used to target various components of the TME, including molecules expressed in cancer cells, immune subsets, stroma, and ECM components. A number of targets are currently investigated in clinical trials with Nb-based diagnostics and therapeutics and are highlighted in bold. Created in https://BioRender.com

Table 1 Targets of Nb-based biologics and diagnostics generated in recent years

Target	Nb format	Outcome	Cancer	Animal/human	Reference
Immune cell compartment of the TME					
PD-L1	[^{68}Ga]Ga-NOTA-(hPD-L1) Nbs	Preclinically validated PET-tracer; Low kidney uptake, imaging at an early timepoint after injection, high tumor-to-muscle and tumor-to-blood ratios	Melanoma (624-MEL)	Mice (human xenografts in nude mice)	[29]
	Nb-Fc fusion (Lentiviral delivery)	Better tumor cell killing shown in 3D melanoma model as compared to avelumab	Melanoma (624-MEL)	Allogeneic 3D model	[33]
	Nb release through hydrogel	Systemic presence of Nb for up to 72h after subcutaneous administration and enhanced tumor uptake in melanoma model	Melanoma (624-MEL)	Mice (human xenografts in nude mice)	[32]
	Nb-TLR7 agonist conjugate	Synergistic anti-tumor efficacy in "cold" tumors due to upregulated PD-L1 on APCs and effector function of cytotoxic T and NK cells	Lung (LLC) Melanoma (B16-F10) Colorectal carcinoma (CT26)	Mice	[16]
PD-L1/TIGIT	Tetravalent Nb-Fc fusion	Synergistic enhancement of T cell activity in vitro	Colon (MC38)	Mice	[34]
PD-L1/CTLA-4	Humanized bispecific Nb-Fc fusion	Positive clinical evaluation of safety and efficacy with manageable toxicity	Breast (TNBC)	Phase II study	[35]
PD-L1/ HLA-G/ CD3ε	Nb-TriTE	Inhibition of tumor growth and prolonged survival	Lung (NSCLC)	Mice bearing xenografts	[36]
PD-L1/4-1BB	Bispecific Nb	Antitumor activity in vivo, minimal toxicity	Colorectal carcinoma (CT26) Colon (MC38)	Mice	[37]
PD-1/CTLA-4	Bispecific Nb-encoding mRNA	Inhibition of tumor growth in MC38 model	Colon (MC38)	Mice	[17]
LAG-3	^{99}mTc-labelled hLAG-3 Nbs	Specific detection of LAG-3 in MC38, MO4, and TC-1 cancer models; potential translation for clinical application to predict therapy outcomes	Lung (TC-1)	Mice (human xenografts in nude mice)	[30]
CD8β	^{99m}Tc; ^{68}Ga-NOTA; ^{64}Cu-NOTA-CD8β Nbs	Non-invasive imaging of hCD8^{+} T cell dynamics by SPECT and PET during tumor growth via a tracer with high affinity and specificity and fast in vivo pharmacokinetics	Colon (MC38)—mice	Mice Cynomolgus monkey	[38]
CD4	^{64}Cu-NODAGA-hCD4 Nbs	Non-invasive imaging of CD4^{+} T cell tissues validated in huCD4 KI mice	Leukemia (HPB-ALL)	Mice	[39]
CCR8	Nb-Fc fusion	Specific depletion of highly-immunosuppressive tiTregs synergistically reduced tumor growth in combination with anti-PD-1 therapy	Lung (LLC-OVA) Colon (MC38)	Mice	[40]

Table 1 (continued)

Target	Nb format	Outcome	Cancer	Animal/human	Reference
OX40(CD134)	AF647-DBCO labelled Nb	Imaging probe validated in hOX40-expressing xenografts; Nb didn`t affect T cell viability and didn`t display agonistic effect on OX40 signaling	Fibrosarcoma (HT1080)	Mice	[41]
CD206	[^{68}Ga]Ga-NOTA-anti-CD206-Nb	Positive clinical evaluation of safety and tracer uptake in solid tumors, leading to two currently running Phase II trials (NCT04168528, NCT04758650)	NSCLC	Phase I study	[44]
CD163	^{99m}Tc; ^{68}Ga-NOTA-m/hCD163 Nbs	Imaging of CD163^{+} TAMs population via SPECT/CT and PET with potential application for patient stratification and immunotherapy follow-up	Lung (LLC-OVA)	Mice	[45]
NRP-1	antagonistic Nbs	Inhibition of NRP-1/Sema3A interaction led to the increase in proinflammatory TAMs and anti-tumor T cell responses, resulting in tumor growth suppression	Colon (MC38)	Mice	[47]
		Inhibition of EGFR upregulation and reversal of drug resistance in melanoma cells	Melanoma (A375, SK-MEL-28)	Mice	[71]
SIRPα	^{99m}Tc-labelled Nb	Non-invasive imaging with high signal-to-noise ratios of SIRPα^{+} macrophages in intracranial GBM tumors	Glioblastoma (GL261)	Mice	[48]
	^{64}Cu-hSIRPα Nb	Non-invasive PET imaging of SIRPα^{+} macrophages in huSIRPα/CD47 KI mice	Colon (MC38)	Mice	[49]
CD11c	Nanovaccine	Effective targeting of the Nb-functionalized nanoparticles to CD11c + cells in vivo	-	Mice	[51]
Stromal cells and ECM components of the TME					
EIIIB domain of FN	^{64}Cu-NOTA Nb	PET/CT imaging of PDAC tumors and early lesions with high clarity and signal-to-noise ration which outcompeted conventional imaging tools	Melanoma (B16F10) Breast (4T1)	Mice	[53]
	nanoCARs	Selective targeting of tumor ECM and vasculature led to the inhibition of tumor growth in vivo	Melanoma (B16F10)	Mice	[54]
Tenascin-C	^{64}Cu-NOTA-hTNC Nbs	PET/CT visualization of mammary gland tumors and their lung metastases	TNBC	Mice	[55]
	anti-huTNC Nbs	Inhibition of DC adhesion to TNC in combination with CCL21	Multiple	Human samples	[56]

Table 1 (continued)

Target	Nb format	Outcome	Cancer	Animal/human	Reference
FAP	[^{68}Ga]Ga-DOTA; [^{225}Ac]Ac-DOTA; [^{131}I]I-GMIB-huFAP Nbs	SPECT and PET/CT visualization and dose-dependent inhibition of tumor growth in mice bearing huFAP-positive U87-MG xenografts	Glioma (U87-MG)	Mice	[58]
		Synergistic inhibition of TC-1-hFAP tumor development in combination with anti-PD-L1	Lung (TC-1)	Mice	[59]
PDGFRβ	Toxin-conjugated Nbs	Faster uptake and delivery to the lysosome	Squamous cell carcinoma cell line (SCCVII)	Mouse cell line	[60]
VEGFR-2	Toxin-conjugated Nb	Concentration corelated cell death of prostate cancer PC-3 cell line	Prostate (PC-3)	Human cell line	[62]
VEGFR-2/NRP-1	Combination of Nbs	Significant inhibition of tumor growth in nude mice assay	Colon (HCT116)	Human cell line	[63]
Cancer cells					
HER2	[^{68}Ga]Ga-NOTA-anti-HER2 Nbs	Positive clinical evaluation of the safety and imaging quality with the PET tracer	Breast carcinoma	Phase I study	[66]
		Clinical evaluation of the tracer uptake in breast cancer patients showed higher sensitivity in detection of HER2^{+} positive lesions then [^{18}F]FDG imaging, enlarging the cohort of patients that could potentially benefit from trastuzumab therapy		Phase II study	[67]
EGFR	bispecific anti-EGFR-HAS-CD16 Nb	ADCC-mediated anti-tumor efficacy in A431 cell nude mouse xenograft model	Epidermoid carcinoma (A431)	Mice	[68]
	Nb-NK cell conjugate	Enhanced tissue penetration and anti-tumor efficacy in a solid tumor mouse model	Colon carcinoma (RKO)	Mice	[69]
	long-term retention photodynamic Nb conjugate	High anti-tumor efficacy and biosafety shown in tumor model in vivo	Epidermal (A431)	Mice	[31]
	Nb-drug conjugate	Targeted delivery of platinum prodrug to EGFR-positive cancer cells and MRI imaging of the tumors	Epidermal (A431)	Mice	[70]
AXL	^{99m}Tc-labeled Nb; Nb-Fc fusion	SPECT/CT imaging of AXL^{+} THP-1 and C1498 tumors; Synergistic anti-tumor efficacy of Nb-Fc and cytarabine in human AML cell lines	AML (THP-1)	Mice	[72]
ALK	Nb-based TCE	Cytolytic activity against ALK-expressing tumor cells	-	Cell line	[73]

Table 1 (continued)

Target	Nb format	Outcome	Cancer	Animal/human	Reference
FGFR4	nanoCARs	Improved safety and anti-tumor activity against HCC demonstrated in vivo	Liver (Huh-7)	Mice	[74]
Met (HGFR)	Nb-photosensitizer conjugate	Specific killing of Met-overexpressing MKN45 cancer cells	Gastric (MKN45)	Cell line	[92]
P2X7	AVV coding for blocking Nb-Fc	Tumor growth reduction in B16F10 melanoma and EG7 thymoma (in combination with an immunogenic chemotherapy)	Melanoma (B16F10) Thymoma (EG7)	Mice	[75]
CD73	AVV coding for bispecific CD73/PD-L1/HSA Nb	Tumor growth reduction in B16F10 melanoma and complete tumor rejection in EG7 thymoma	Melanoma (B16F10) Thymoma (EG7)	Mice	
V-ATPase	Bivalent Nb	Inhibited lung metastasis in the orthotopic 4T1-12B mouse model	Breast (4T1-12B)	Mice	[76]
TK1	Nb-Fc fusion	ADCC towards mTK1-expressing cancer cells mediated by human mononuclear cells	Lung (NCI-H460)	Cell line	[13]
G250	Nanobubbles	Multimodal imaging of G250 + PDX	Renal (786-O)	Mice	[77]
Nectin-4	Lifetime-extended trivalent Nb-drug conjugate	Anti-tumor efficacy in NCI-N87 human gastric cancer xenografts	Gastric (NCI-N87)	Mice	[15]
CEACAM5/6	nanoCARs	Anti-tumor efficacy in a human pancreatic xenograft model	Pancreatic (BxPC-3)	Mice	[78]
	Nb-drug conjugate	Tumor growth inhibition in BxPC-3 and MKN-45 xenografted mice	Pancreatic (BxPC-3) Gastric (MKN-45)	Mice	[79]
EpCAM	Monovalent	Inhibition of HCT116 tumor growth in nude mice	Colon (HCT116)	Mice	[80]
	Nb-toxin conjugate	Inhibition of MCF7 tumor growth in nude mice	Breast (MCF7)	Mice	[81]
MSTL	Nb-NP, Nb- IRDye conjugate	Non-invasive imaging of MSTL-positive lesions in mouse xenograft models of ovarian cancer via optical imaging and MRI	Ovarian (A1847)	Mice	[82]
	Fluorescent organic Nb-nanoassembly	Effective targeting and accumulation of the nanoassembly in MSTL + cancer cells with option of multimodal imaging	Mesothelioma (Meso34 and Meso13) lung (ADCA153)	Celli line	[83]
CLDN18.2	[^{68}Ga]-labelled Nb	Positive clinical evaluation of a PET tracer safety and efficacy	Gastric (AGS)	Mice Human (Phase I)	[86]

Table 1 (continued)

Target	Nb format	Outcome	Cancer	Animal/human	Reference
CDH17	Nb-IR800CW conjugate	Fluorescent imaging of CDH17-positive gastric cancer cells	Gastric (MKN45)	Mice	[87]
	Nb-toxin conjugate	Strong anti-tumor effect in cell-derived and patient-derived xenograft models	Gastric (PDX)	Mice	
	Nb-IR800CW conjugate	Fluorescent probe for visualization of CRC cancer cells and imaging-guided surgery in murine CRC models	Colon (HCT116)	Mice	[88]
	Nb-toxin conjugate	Synergistic anti-tumor effect with chemotherapy drug 5-FU in the CRC model			
CD47/ SIRPα	HM-CD47 Nb displayed on CVs	Increased therapeutic half-life and tumor uptake; macrophage-mediated antitumor immunity in subcutaneous and metastatic models	Colorectal carcinoma (CT26) TNBC (4T1)	Mice	[50]
	anti-PD-L1 CAR-T cells secreting blocking anti-CD47 Nb-Fc fusion construct	Local delivery limited the systemic toxicity and inhibited tumor growth in vivo	Colon (MC38)	Mice	[89]
	bispecific anti-CD47 Nb/CD20 Ab	Preferential binding to tumor cells and potent anti-lymphoma activity	Lymphoma (Raji cells)	Mice Cynomolgus monkey (tox study)	[90]
	Nb-aptamer conjugate	CD47-Met nanotamer enhanced tumor penetration and anti-tumor effect of chemotherapy	Colon (MC38)	Mice	[91]
CD19	Nb-based CAR-T cells	Tumoricidal capacity comparable to the scFv-based counterparts without impeding antigen-binding activity	Blood	Cell lines (Raji and Ramos)	[93], [94]
CD20	Nb-based radionuclide therapy	Delay in tumor growth and systemic anti-tumoral immune responses	Melanoma (B16-huCD20)	Mice	[95]
CD22	Nb-based CAR-T cells	Generation of CD22 CAR-T cells displaying cytotoxic activity towards human lymphoma xenograft cells	Lymphoma	Mice	[96]
CD72	Nb-based CAR-T cells	Elimination of xenograft tumors derived from patients relapsed after anti-CD19 CAR-T cell therapy	B-ALL	Mice	[98]
CS1	Nb-based radionuclide therapy	Targeting of residual MM cells led to a prolonged survival in mice	Myeloma (5T33MM)	Mice	[100]
ABCC3	Monovalent	Selective recognition of the transporter in glioblastoma xenograft mouse model	Brain (U-87 MG and U251)	Mice	[102]

Table 1 (continued)

Target	Nb format	Outcome	Cancer	Animal/human	Reference
TUFM	Monovalent	Inhibition of glioblastoma stem cell migration	Glioblastoma (U251MG and U87MG)	Cell line	[103]
Vimetin	Monovalent				
TRIM28	Monovalent	Inhibition of GSCs invasiveness and spread in zebrafish brains	Glioblastoma (U-87 and U373)	Zebrafish	[105]

HER2-targeted therapy with trastuzumab [67]. Mutations of EGFR, a key member of the human epidermal receptor (HER) or ErbB family that is often overexpressed in malignancies, are strongly associated with the development of carcinomas, including breast, colorectal, pancreatic, head and neck, and non-small cell lung. Xu et al. engineered a bispecific nanobody that targets the highly conserved dimerization interface of EGFR, which plays a crucial role in receptor dimerization with other family members and is linked to drug resistance. Anti-EGFR Nb was fused to anti-CD16a Nb (aiming to promote NK cell recruitment) and anti-HAS Nb (aiming to increase the construct half-life). This nanobody demonstrated significant tumor-suppressive activity both in vitro and in vivo [68]. Gong et al. developed an EGFR-targeting Nb-NK cell conjugate as an alternative to genetically engineered CAR-NK cells, which are often met with challenges like low transfection efficiency and mutagenesis. These Nb-NK cells demonstrated remarkable tissue penetration and potent cytotoxic activity against various tumors, exceeding the efficacy of non-functionalized NK cells. Noteworthy, the Nb recognized the mutant EGFR ectodomain, which is inaccessible to clinically approved mAbs [69]. Alternatively, Chen et al. achieved a high therapeutic efficacy against EGFR-expressing solid tumors by designing a long-term retention photosensitizer coupled to an anti-EGFR Nb via a cleavable linker. In that case, the photosensitizer is released at the tumor site, sustaining the effect of photodynamic therapy, whereas the Nb is cleared from the circulation, thus avoiding systemic phototoxicity [31]. Moreover, Huang et al. designed a Nb-drug conjugate fused with albumin- and Gd^{3+}-binding domains, enabling the targeted delivery of a platinum prodrug to EGFR-positive cancer cells and MRI of the tumors [70] Of note, NRP-1, previously mentioned as a target for immune cells, also plays a key role in promoting cancer cell growth through its interaction with EGFR. Anti-NRP-1 Nbs can inhibit EGFR upregulation and reverse acquired drug resistance in melanoma cells [71].

AXL, a tyrosine kinase receptor, is implicated in cancer cell proliferation, survival, migration, stemness, and chemoresistance in acute myeloid leukemia (AML) patients. Vandewalle et al. developed a tracer using ^{99m}Tc-labeled AXL-specific Nbs (sdAb20), which required the co-injection of cold sdAb20-Fc to enhance the tumor-to-background signal. Furthermore, combining sdAb20-Fc with cytarabine demonstrated a synergistic therapeutic effect, inducing apoptosis in human AML cell lines [72]. Another tyrosine kinase receptor, anaplastic lymphoma kinase (ALK, CD247), can drive cancer development when it gets rearranged and overexpressed, resulting in abnormal ALK signaling. Chen et al. developed nanobodies (VH20) targeting the extracellular domain of ALK to mitigate the risk of mutagenic escape associated with kinase-domain targeting therapies. Anti-ALK Nb-based bispecific T cell engagers (TCE) resulted in robust cytolytic activity against ALK-expressing tumor cells [73].

Finally, high-affinity Nbs targeting FGFR4, the fibroblast growth factor receptor 4 that is overexpressed in hepatocellular carcinoma (HCC) and contributes to epithelial-mesenchymal transition, were generated for the creation of Nb-derived CAR-T cells targeting this receptor. Such CAR-T cells demonstrated improved antitumor activity, enhanced safety, and effective cytokine secretion in vivo [74].

Targeting of metabolic pathways

Metabolism majorly affects the functionality and behavior of practically every cell, including cancer cells. When accumulating in the TME, adenosine triphosphate (ATP), can be catabolized by CD39 and CD73 to produce immunosuppressive adenosine, which promotes tumor growth through the P2X7 receptor. Injection of AAV vectors, coding for a P2X7-blocking Nb-Fc construct, reduced tumor growth in B16F10 melanoma and EG7 thymoma cancer models. A bispecific biologic targeting both CD73 and PD-L1 even more effectively inhibited tumor growth in both models, resulting in complete tumor rejection in EG7 [75]. ATP is also required to drive V-ATPase, a proton pump that contributes to the invasiveness of cancer cells, especially in breast cancer. A nanobody targeting the extracellular epitope of the mouse V-ATPase c subunit effectively inhibited tumor cell metastasis to the lungs in the orthotopic 4T1-12B mouse model. Notably, the selection of the Nb was performed in vitro to bypass the challenge of low immunogenicity, commonly associated with highly conserved epitopes, which presents a significant limitation in traditional antibody generation through animal immunization [76]. Thymidine Kinase 1 (TK1) is an enzyme involved in the pyrimidine salvage pathway, catalyzing the conversion of thymidine to thymidine-monophosphate. Elevated serum levels of TK1 are associated with tumor progression, patient response, and cancer recurrence. Nb-Fc fusion constructs have been shown to elicit potent ADCC responses towards mTK1-expressing cancer cells mediated by human mononuclear cells, suggesting that anti-TK1 Nbs hold great therapeutic potential [13]. G250 is an enzyme that belongs to the alpha carbonic anhydrase (CA) family of zinc metalloenzymes, also known as CA IX. G250 is induced by hypoxia and is involved in the formation of an acidic TME. Its overexpression is particularly linked to renal cell carcinoma. Anti-G250 Nbs were used to design nanobubbles for ultrasound/photoacoustic/fluorescence imaging of patient-derived xenografts, suggesting multimodal imaging might have an advantage in obtaining

more comprehensive and diagnostically relevant information on tumor localization and tissue structure [77].

Targeting of adhesion and cell–cell junction molecules

Cancer cells employ various adhesion molecules to ensure their mobility. Nectin-4 (PVRL4) is a transmembrane cell adhesion molecule that triggers the PI3K/AKT signaling pathway, promoting angiogenesis and cancer cell migration. Wu et al. designed an anti-Nectin-4 nanobody-drug conjugate, consisting of a lifetime-extended trivalent anti-Nectin-4 Nb conjugated to a drug via a cleavable maleimide linker. This conjugate exhibited strong anti-tumor activity in mice bearing NCI-N87 human gastric cancer xenografts [15]. Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) is another potential target for cancer therapies. Anti-CEACAM5/6 Nb-based CAR-T cells demonstrated potent anti-tumor efficacy in a human pancreatic xenograft model [78]. In another study, anti-CEACAM5 Nbs conjugated to a drug effectively inhibited tumor growth in BxPC-3 and MKN-45 xenografted mice without significantly affecting body weight [79]. Along the same line, epithelial cell adhesion molecule (EpCAM) is overexpressed in various cancer types, being crucial for cancer proliferation, invasion, and metastasis. Roshan et al. have isolated two Nbs that bind with high affinity to EpCAM, which inhibited the proliferation of MCF-7 cells in vitro and showed anti-tumoral efficacy in vivo [80]. The anti-EpCAM Nb was then genetically fused to a truncated form of diphtheria toxin, resulting in significantly reduced MCF7 tumor growth in nude mice [81]. Another molecule thought to be involved in cell adhesion is mesothelin (MSTL). Although the precise biological function of MSTL is unknown, this cell surface protein is anchored by glycosylphosphatidylinositol and is frequently overexpressed in several types of human cancers, including mesothelioma, ovarian cancer, and various adenocarcinomas. It is therefore vastly investigated as a target for cancer immunotherapy in preclinical studies and clinical trials. Prantner et al. have designed nanobody-based probes for fluorescent imaging and MRI of MSTL-positive lesions in mouse xenograft models of ovarian cancer [82]. However, the potential clinical implementation of anti-MSTL Nbs for non-invasive imaging will require its conversion into a tracer for imaging techniques that offer higher sensitivity, such as PET. For therapeutic applications, Briolay et al. have recently coupled anti-MSTL Nbs to fluorescent organic nanoassemblies (NA) via copper-free click chemistry, to enhance local drug delivery [83]. Anti-MSTL Nbs have also been used to design recombinant immunotoxins and CAR-T cells to potentiate therapy efficacy against MSTL-expressing tumors [84] [85].

Cell–cell junction molecules with an impact on tumor growth include claudin (CLDN)18.2 and cadherin (CDH)17. CLDN18.2 is expressed in primary tumors and metastatic lesions of 50–80% of gastric cancer patients. A Nb-based PET tracer [^{68}Ga]Ga-PMD22 was evaluated in a first-in-human study (NCT05937919) and demonstrated a good safety profile and clinical potential for detecting CLDN18.2 in patients [86]. Cadherin 17 (CDH17), also known as liver intestine (LI)-cadherin, is normally expressed in epithelial cells of the small intestine and colon but is often overexpressed in various cancers, including gastric and colorectal cancers. An anti-CDH17 Nb (E8) was used for rapid imaging of CDH17-positive gastric cancer cells and targeted delivery of a truncated Pseudomonas exotoxin A (PE38) to tumor tissues. In both cell-derived and patient-derived xenograft models, it demonstrated strong anti-tumor effects and significantly improved survival in mice [87]. Additionally, the E8-PE38 immunotoxin significantly enhanced the antitumor effects of the chemotherapy drug 5-FU in the CRC model. Furthermore, E8 was utilized to design a targeted fluorescent probe, enabling the visualization of CRC cancer cells and facilitating imaging-guided surgery in murine CRC models [88].

Targeting of "don't eat me" signals

As was mentioned before, disrupting the CD47/SIRPα interaction, generally known as a "don't eat me" signal, has been an extensively studied strategy in cancer therapy. As red blood cells also express CD47, the systemic delivery of CD47-targeting therapeutics remains a critical challenge due to the associated side effects. Local delivery of anti-PD-L1 CAR-T cells secreting blocking anti-CD47 Nb-Fc fusion constructs in the TME limited the systemic toxicity and delayed tumor growth in vivo more effectively than the CAR-T cells lacking the Nb [89]. Alternatively, Ma et al. used an anti-CD47 Nb and Rituximab to construct a bispecific anti-CD47/CD20 Ab, which showed preferential binding to tumor cells and potent anti-lymphoma activity. The safety profile of the BsAb is thus potentially higher than that of the single-agent treatments [90]. In another study, nanobodies were site-specifically conjugated to aptamers using microbial transglutaminase (MTGase) and click chemistry (compare conjugation chemistry below). The resulting 'CD47-Met nanotamer,' targeted to tumor cells, inhibited receptor function through steric hindrance and enhanced the anti-tumor effects of chemotherapy by improving tumor penetration [91]. The expression of Met (mesenchymal-epithelial transition receptor, or hepatocyte growth factor receptor (HGFR)) has been linked to multiple cancer types, and anti-Met Nbs were previously conjugated to a photosensitizer for the targeted killing of

Met-expressing cancer cells [92]. It was suggested that due to the short-lived nature of reactive oxygen species generated by the photosensitizer, using Nbs over Abs might potentiate the targeted photodynamic therapy by shortening the distance between the photosensitizer and the paratope of the target.

Nanobody-based targeting of specific malignancies: the case of blood and brain cancers

Brain tumors distinguish themselves from other solid tumor types by their unique location, with the brain being protected by various barriers that hamper the access of compounds to that site. Also hematological cancers pose different challenges as compared to solid tumors.

Targeting of hematological malignancies

In the context of blood cancers, CAR-T cells have shown particular success. However, the design of CARs typically relies on scFvs as the antigen-recognition element, which are prone to aggregation caused by inefficient folding. Replacing scFvs with nanobodies could mitigate the issues related to the immunogenicity and aggregation of CAR-T cell-based therapies. For instance, the previously mentioned Ciltacabtagene autoleucel, approved by the FDA for the treatment of multiple myeloma, is an Nb-based CAR-T cell engineered to target BCMA [22].

Several blood cancers originate from B cells, and, therefore, a lot of effort has been put into targeting B cell-expressed molecules. For example, high remission rates have been achieved with anti-CD19 CAR-T cell treatment. In one study, Nb-based CAR-T cells against CD19-positive cell lines were generated, and their tumoricidal capacity was comparable to their scFv-based counterparts [93]. Notably, a further humanization of anti-CD19 Nbs did not impede antigen-binding or antitumor reactivity of Nb-based CAR-T cells [94]. However, despite the promising results of anti-CD19 CAR-T cells for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma, many patients still relapse, prompting the need to discover novel targets and engineering approaches to overcome therapy resistance. Other B-cell antigens, like CD20 and CD22, have been targeted using Nb-based targeted radionuclide therapy and CAR-T cells [95, 96]. Nix et al. described an ITIM-bearing inhibitor of B-cell receptor signaling, CD72, overexpressed in B-ALL. They demonstrated that anti-CD72(NbD4) CAR-T cells can effectively eliminate tumor cells lacking CD19, suggesting their potential as a second-line treatment option [97]. Furthermore, they increased the anti-tumor potency of these nanoCARs by framework humanization of anti-CD72 Nbs, leading to a prolonged survival of mice implanted with KMT2Ar B-ALL tumors and the elimination of xenograft tumors derived from patients relapsed after anti-CD19 CAR-T cell therapy [98]. Recently, Hanssens et al. highlighted the importance of case-by-case selection of antigen-binding moieties in designing CAR-T cell therapies for optimal potency and lower risk of relapse. Moreover, they developed idiotype-specific Nb-based CAR-T cells, paving the way for personalized treatment of multiple myeloma (MM) [99]. The same group has previously used Nb-based anti-CS1 radionuclide therapy to target residual MM cells, showing a prolonged survival of syngeneic, immunocompetent 5T33MM mice [100].

Targeting of brain tumors

Due to their unique characteristics, nanobodies are considered an effective tool for targeting brain tumors [9]. One key reason for the low success rate of therapeutics targeting the central nervous system, particularly conventional antibodies, is the impenetrability of the blood–brain barrier (BBB). Nbs, however, have been shown to cross the BBB through receptor-mediated and adsorptive-mediated transcytosis. The latter allows Nbs with a high isoelectric point to spontaneously penetrate the BBB. Alternatively, Nbs can be delivered to the brain by fusing them with cell-penetrating peptides or by functionalizing them on nanoparticles or liposomes [101].

Glioblastoma is an exceptionally aggressive and heterogeneous primary brain tumor, with survival rates remaining low over the past several decades. In recent years, nanobodies against several glioblastoma targets emerged, potentially allowing for the development of novel glioblastoma theranostic procedures. Ruiz-López et al. generated Nbs against ATP Binding Cassette subfamily C member 3 (ABCC3), a transporter protein overexpressed in glioblastoma, as compared to normal tissue, and associated with an impaired response to temozolomide (TMZ) therapy. Upon systemic administration, two Nbs selectively recognized ABCC3 in glioblastoma xenograft mouse models [102]. In an earlier study, mitochondrial translation elongation factor (Tu translation elongation factor, mitochondrial; TUFM) and vimentin were identified as markers to differentiate between glioblastoma and normal brain tissue. Furthermore, anti-vimentin (Nb79) and anti-TUFM Nbs inhibited the growth and survival of glioblastoma stem cells without significant effects on astrocytes [103]. Nb79 was further investigated as a tool to target glioblastoma cell invasion, showing a reduction of up to 21% in vivo [104]. In another study, anti-TRIM28 Nb, targeting a biomarker overexpressed in therapy-resistant GB stem cells (GSCs), inhibited GSCs invasiveness and spread in zebrafish brains [105]. Further optimization of the delivery strategy is required to advance these Nbs as potential therapeutics. Of note,

also Nbs against stromal cells, such as anti-SIRPα against tumor-associated myeloid cells [48], were shown to penetrate mouse glioblastoma tumors, further illustrating the potential use of these small compounds to reach the brain.

Nanobody-decorated nanoparticles

While nanobodies themselves already hold great promise for cancer therapy applications, multivalency can further improve their properties and bring them to the next level. This is why the combination of nanobodies and nanoparticles, a pairing of biological specificity with nanotechnological innovation, might be able to transform the field of nanomedicine [106]. Though, for successful application of nanobody-carrier systems, different considerations must be made. For one, the correct orientation and the accessibility of the nanobodies' paratope are of concern to maintain affinities at a nanomolar range. Although there is more flexibility compared to the larger mAbs, random conjugation of the Nbs to the carrier can still result in a major loss of binding ability [107]. While random conjugation may still result in targeting capabilities, and its ease of manufacturing both on the proteins' as well as the carriers' side can render it attractive for certain applications, the advantages of site-specific conjugation have already been shown [108]. Additionally, combining the targeting capabilities of nanobodies with nanocarriers also results in the availability of a vast toolbox to incorporate additional molecules such as drugs, dyes and tracer molecules for theranostic purposes. Different chemical approaches on the particles' side also allow for the control of cargo release and improvements in blood circulation times, for example by PEGylation [109].

Depending on the specific target and desired application of the system in general, different particle platforms are favorable. These platforms can be mainly classified into four sections: inorganic nanoparticles, liposomes, biomaterials-derived carriers and synthetic organic nanoparticles. They each exhibit distinct features useful for different applications, like stability, biocompatibility and scalability of production.

Inorganic particles, such as gold, silica or iron oxide particles are often used for their high stability, robustness and long shelf life. They are easily prepared with precise size and shape control, resulting in scalability and reproducibility. Additionally, some carriers provide the intrinsic ability to respond to external triggers like light or magnetic fields. However, versatile modifications are mostly confined to the particles' surface and biocompatibility is largely dependent on size and surface properties, thus, competing with each other (e.g. unmodified silica particles are less compatible than their modified counterparts) [110–112].

Liposomes, composed of lipid bilayers of different compositions, offer intrinsic biocompatibility due to their structural similarity to biological membranes. Encapsulation of hydrophilic compounds in the core or lipophilic drugs in the lipid layer makes their application very broad and adaptable to a variety of diagnostic and therapeutic agents. By incorporating lipids with specific chemical motifs, responsiveness to triggers like pH or reactive oxygen species (ROS) can be integrated into the carrier. They are also easily scalable and easily produced. A downside of liposomal carriers is their limited shelf-life and susceptibility to structural disassembly in biological environments [110, 113–115].

Biomaterials-derived carriers like extracellular vesicles (EVs), protein-based and DNA-based nanoparticles offer great biocompatibility and low immunogenicity due to their origin. Their stability is balanced by biodegradability ensuring compatibility in biological systems. However, production and scalability are rather cost- and labor-intensive processes based on cumbersome purification procedures. Loading those carriers with small molecules adds further isolation and engineering steps, and maintaining structural integrity during further modifications makes EVs even more challenging in production [110, 116, 117].

Synthetic organic nanoparticles formed from various polymers provide the most versatile platform for nanocarriers. A broad selection of initiators and monomers allows for precise control over functionalities in the core and on the surface, payload encapsulation and control over the size of the later formed particle, as well as responsivity to external triggers like pH, temperature, ROS or enzymatic activity. There are also plenty of biocompatible and even biosimilar polymers enhancing the clinical potential. Generally, the stability of polymeric systems is rather sufficient in biological environments and can be fine-tuned by careful consideration, though, very dependent on the individual system. Multistep synthesis procedures for monomers and polymers and the need for sophisticated techniques for certain formulations make scalability an issue for these systems. Nevertheless, the vast library of different systems, their adaptability, broad range of applications and biocompatibility make them a valuable option for therapeutic and diagnostic strategies [102–104].

For all systems, several publications have elucidated individual advantages and disadvantages when combining nanobodies to each of these four carrier systems. The individual conjugation chemistries are illustrated in Fig. 5.

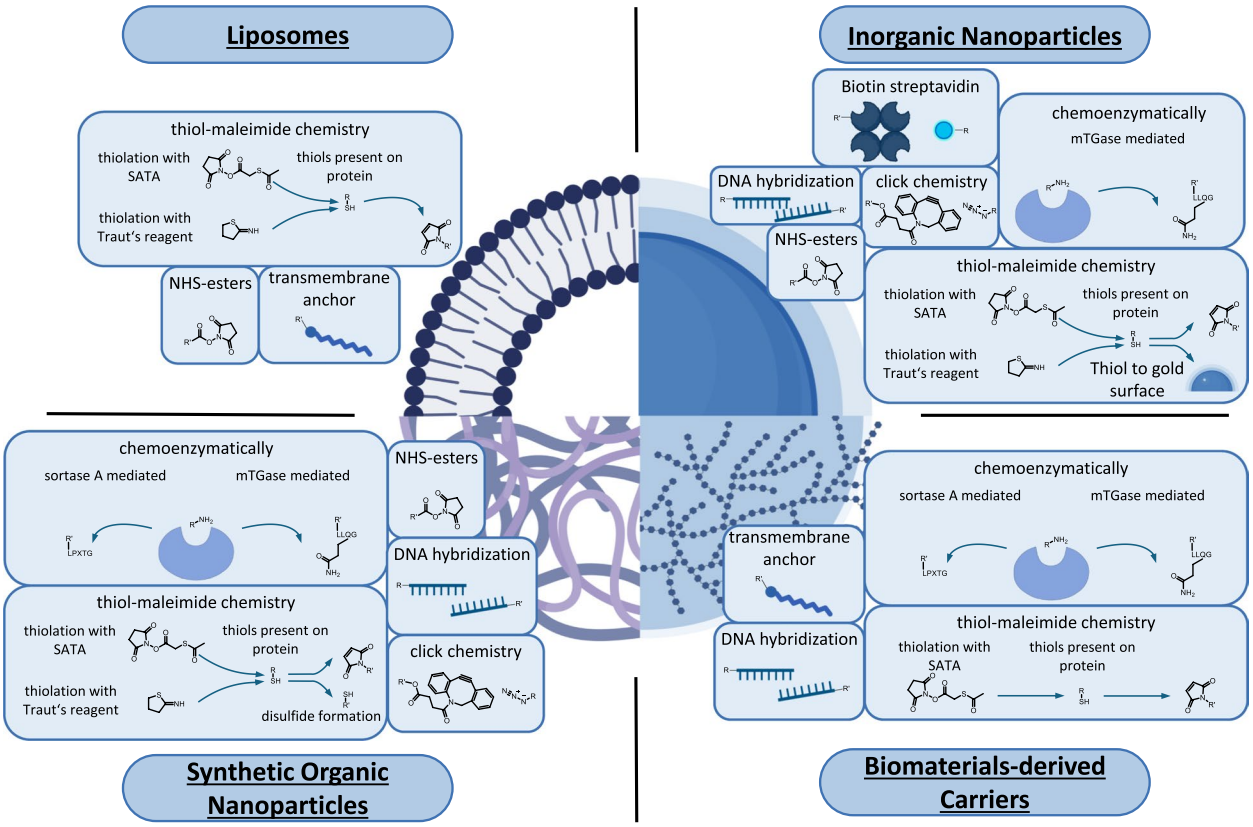

Fig. 5 The carrier platform can be subdivided into 4 categories. Depending on the sub-class of nanocarriers, different chemistries can be utilized to perform the modification of the carrier surface with nanobodies. R represents the protein while R' represents the respective carrier system. Created in https://BioRender.com

Inorganic nanoparticles

Inorganic nanoparticles (see Fig. 5, top right) and quantum dots (Q-dots) have emerged as pivotal tools in cancer therapy and diagnostics due to their unique optical, electronic, and structural properties. These materials enable high-resolution imaging and precise delivery of therapeutic agents, broadening the capabilities of modern nanomedicine [118].

Copper sulfide nanoparticles (CuS NPs) functionalized with bovine serum albumin (BSA) illustrate their versatility for photothermal therapy of cancer. The metal core facilitates efficient photothermal conversion, while the albumin shell serves as a functional platform for nanobody attachment using *N*-Hydroxysuccinimid (NHS) chemistry. Although this approach enabled targeting, its nonspecific conjugation can compromise targeting efficacy by modifying the nanobody's active site [119].

To overcome the limitations of random conjugation, site-specific strategies have been developed to preserve the biological activity of nanobodies while ensuring stable attachment. Engineering nanobodies with specific sequences at the C-terminus has proven effective. Song et al. utilized a GlyHis tag to introduce a terminal azide moiety, facilitating a click reaction with dibenzocyclooctyne (DBCO) modified single-stranded DNA (ssDNA). Hybridization of ssDNA-functionalized gold nanoparticles (AuNPs) with ssDNA-nanobody complexes yielded functionalized particles with enhanced tumor targeting properties [106].

Similarly, genetic engineering approaches have enabled the introduction of a terminal cysteine at the C-terminus, which is typically distal from the nanobody's active site. Van de Broek et al. demonstrated thiol-maleimide chemistry for nanobody conjugation, employing polyethylene glycol (PEG)-coated gold nanoparticles. The maleimide-functionalized PEG linker facilitated the attachment of anti-HER2 nanobodies via their terminal cysteine residues without loss of binding activity [120]. A comparable approach was used for Q-dots, where mal-PEG-*b*-PLGA–OH was conjugated to the Q-dot surface through a coupling reaction and subsequently functionalized with nanobodies bearing a C-terminal cysteine [121].

Protein ligation strategies also support precise functionalization. Stahl et al. employed intein-mediated

ligation to introduce alkyne functionalities on nanobodies, enabling copper-catalyzed click reactions with azide-modified PEG-Au-NPs [122, 123]. Genetic code expansion offers another avenue, allowing the incorporation of artificial amino acids containing azide groups at predetermined positions [108]. For instance, Yong et al. demonstrated how varying the conjugation site on an anti-EGFR nanobody influenced targeting efficacy. Nanobodies conjugated at position 13 near the C-terminus exhibited a sixfold increase in binding affinity compared to those randomly conjugated via NHS-chemistry, underscoring the superiority of site-specific approaches [107].

Silica nanoparticles have been extensively explored for targeted delivery applications. Site-selective conjugation has been compared with non-specific methods using NHS-PEG-N_3 and sortase-mediated ligation. The latter involves the enzyme Sortase A, which recognizes the LPXTG motif and catalyzes the formation of a peptide bond between a threonine residue and an N-terminal oligoglycine chain. This process enabled covalent nanobody attachment to siloxane nanoparticles' amino-functionalized surfaces, resulting in a precise nanobody orientation [124].

Silica nanoparticles have finally been modified for therapeutic delivery. For instance, their surface was functionalized with polyamidoamine polymers to create accessible amine groups. These were converted into DBCO functionalities using NHS-PEG-DBCO, enabling click chemistry with azide-modified nanobodies. This platform has shown potential for the delivery of small interfering RNA (siRNA) and doxorubicin, illustrating its adaptability for combination therapies [125].

A different technique, aiming for diagnosis using MRI, uses iron oxide nanoparticles. Large amounts of those particles at the target site are needed for high contrast and monitoring of cells, to compensate for the insensitivity of MRI as a diagnosis tool. To improve circulation times and biocompatibility, the particles were coated with PEGylated liposomes as well as targeting anti-HER2 nanobodies. This also reduced non-specific toxicity to normal cells and increased the quantity of contrast agent in the tumor [126]. Similar systems are currently undergoing clinical trial in phase I/II studies for the localization of sentinel lymph nodes in breast cancer and for the improved treatment of hepatocellular carcinoma (NCT05985551, NCT05359783, NCT04682847).

Iron oxide nanoparticles have also been investigated for multimodal imaging approaches. For this, superparamagnetic iron oxide nanoparticles were labeled with ^{68} Ga and magnetomotive ultrasound (MMUS) as well as PET/CT and MRI were used to detect the particles in sentinel lymph nodes (SLN) of rats [127]. All three techniques were able to track the particles, though MMUS only detected them in four out of six animals. This was in good accordance with MRI findings, as the by MMUS non-detected SLNs were confirmed to have the lowest nanoparticle concentration. The findings indicated that MMUS could complement PET and MRI as a radiation-free, real-time imaging technique. The authors also suggested that labeling nanoparticles with targeting moieties could enhance the potential of MMUS beyond SLN imaging and increase sensitivity and signal detection by increasing nanoparticle retention at the target site.

Liposomes

Liposomes (see Fig. 4, top left), as versatile nanocarriers, are formulated using diverse lipid compositions tailored to specific therapeutic or diagnostic needs. The incorporation of PEG-modified lipids with functionalized termini allows modular customization of liposome surfaces, enabling compatibility with various conjugation chemistries. One commonly used strategy integrates NHS-PEG-lipids into the liposomal structure, enabling conjugation to lysine residues on nanobodies via NHS-ester chemistry. This non-specific conjugation method is broadly applicable across protein types but may compromise nanobody targeting efficacy due to random lysine modification [128].

To overcome the limitations of non-specific attachment, maleimide-functionalized PEG -lipids are frequently employed. These selectively react with thiol groups, providing greater specificity for nanobody conjugation. For nanobodies lacking native thiol groups, thiolation can be induced using reagents like Traut's reagent [129–132] or *N*-succinimidyl *S*-acetylthioacetate (SATA) [126, 133–135]. These reagents modify lysine residues to generate sulfhydryl groups, which then react with maleimides on the liposome surface. While this approach enhances conjugation specificity compared to NHS chemistry, the reliance on lysine residues can still affect nanobody performance, as lysine modification may disrupt nanobody binding activity.

To achieve highly precise nanobody conjugation, genetic engineering strategies are therefore preferred. For instance, maleimide chemistry can be directed to a genetically engineered C-terminal cysteine on the nanobody. This site-specific conjugation minimizes interference with the nanobody's active binding region, preserving its targeting efficiency while ensuring stable attachment to the liposome surface [136–139].

Another innovative approach involves the attachment of a transmembrane domain to the nanobody's C-terminus. This domain spontaneously integrates into the liposomal lipid bilayer during formulation, yielding liposomes with nanobodies displayed on their surface. This strategy eliminates the need for additional

conjugation steps while enabling the targeted delivery of therapeutic or diagnostic agents, as demonstrated in recent studies [140].

Biomaterials-derived carriers

The integration of nanobodies into biomaterials (see Fig. 5, bottom right) provides versatile platforms for targeted therapeutic and diagnostic applications of biocompatible nanocarriers. While some approaches offer intrinsic biodegradability, others emphasize scalability, structural flexibility, and ease of manufacturing.

A straightforward yet complex strategy involves the use of biologically engineered EVs. Although production and scalability pose challenges, the inherent biocompatibility and universal applicability of this approach are highly advantageous. Nanobodies can be expressed by the same cells producing the vesicles, and fusion to the vesicle surface can be achieved by equipping the nanobody's C-terminus with a transmembrane domain [141, 142].

By combining the benefits of EVs with liposomal carriers, hybrid systems can be produced with reduced off-target delivery, and shielding against biomolecular interactions because of PEGylation. Functionalization of the surface can be achieved through PEGylated lipids, enabling diverse conjugation strategies. For example, Kooijmans et al. employed DMPE-PEGmaleimide (DMPE-PEG-mal) to conjugate thiolated- EGFR-targeting nanobodies to the liposomal EV surface [109].

Biopolymer platforms provide scalable and straightforward alternatives to the EVs as nanocarriers. Biomaterials-derived macromolecules such as polysaccharides, peptides, and DNA molecules can be readily isolated and synthetically processed [109, 143, 144]. Chitosan, a readily available polysaccharide derived from processed chitin, can be modified with nanobodies using simple NHS chemistry, which conjugates lysine residues to the particle surface [143].

Beyond carbohydrates, proteins such as ferritin and albumin are natural carriers offering functional versatility for nanobody integration. Ferritin, an iron-storage protein, presents functional groups for conjugation to nanobodies or polymers. Liu et al. employed free thiols on ferritin to attach mal-PEG-NH_2, followed by transglutaminase-mediated ligation of PEG-NH_2 to nanobodies. Alternatively, direct conjugation between Q-tag carboxamides in the nanobody and lysines on ferritin was achieved [145, 146]. Albumin nanoparticles, crosslinked with glutaraldehyde, were PEGylated and functionalized with maleimide end groups for nanobody attachment. Thiolated nanobodies targeting EGFR or HGFR were conjugated via SATA chemistry to maleimide-functionalized surfaces [144, 147].

Costa et al. developed an elastin-like polypeptide (ELP) recombinantly expressed in *E. coli* as a fusion protein with an amphiphilic peptide forming the nanocarrier. Although the structural similarity between the targeting unit and the carrier, both composed of amino acids, complicates any small-molecule cargo conjugation, introducing artificial amino acids enables click chemistry for this purpose [148].

Beyond carbohydrates and polypeptides, Wu et al. also investigated nucleic acids for nanobody conjugation and nanobody-directed delivery. They utilized DNA tetrahedrons as carriers and attached nanobodies via complementary DNA strands. Nanobodies with C-terminal cysteines were modified with succinimidyl-4-(*N*-maleimidomethyl)cyclohexan-1-carboxylate (SMCC) derivatized DNA oligos, allowing for a hybridization onto the tetrahedron surface [149].

Polymers such as poly(α-azido-ε-caprolactone) offer customizable functionalities. DNA strands were grafted onto azido groups of the polymer, while nanobodies functionalized with maleimide DNA strands were hybridized onto the polymer surface to create targeting nanoparticles [150]. These achievements already demonstrate the huge potential of synthetic macromolecules.

Synthetic organic nanoparticles

Organic nanoparticles (see Fig. 5, bottom left) offer arguably the most versatile carrier platform for nanobody-based therapeutics due to their precise modular design and the extensive library of monomer units available. These polymers provide flexibility in terms of conjugation chemistry, cargo delivery capabilities, and tunable physicochemical properties, making them adaptable to diverse applications [151].

Cationic polyamidoamines (PAMAM) and polyethyleneimines (PEI) have been PEGylated to reduce cytotoxicity and to improve the targeted delivery of plasmid DNA (pDNA). Thiol-maleimide chemistry was employed to attach nanobodies, either by leveraging C-terminal cysteines or by thiolating nanobodies with Traut's reagent prior to conjugation [152–154]. Röder et al. advanced this strategy by designing a sequence-defined polyamidoamide containing internal cysteines for crosslinking and nanobody attachment via disulfide linkages [155].

A wide variety of polymers, including PCL, PLA, PLGA, PLGHMGA, PS, PFPMA, and POx, often combined with PEG, have been employed in applications ranging from photothermal therapy to theranostics and drug delivery. These polymers were functionalized for targeting by attaching nanobodies using diverse chemistries, including click reactions (DBCO-azide), -maleimidethiol- conjugation, NHS-lysine chemistry, and chemoenzymatic methods [156–165].

Sabrina Oliveira's group developed micelles composed of PEG-ε-caprolactone block copolymers loaded with photosensitizers (0.5—10 wt%). Nanobodies targeting EGFR were conjugated via maleimide to C-terminal cysteines, enhancing uptake and photocytotoxicity in EGFR-overexpressing A431 cells. Compared to untargeted formulations, these micelles showed fourfold greater photocytotoxicity and superior circulation times for meso-tetra(hydroxyphenyl)chlorin (mTHPC) photosensitizer compared to its free form (e.g., Foscan) [165].

Debets et al. used PEG-polystyrene block copolymers to form polymersomes functionalized with anti-PlexinD1 nanobodies. Expressed protein ligation (EPL) enabled the introduction of thiol and azide moieties for multivalent conjugation, demonstrating the robustness of this approach. While in vitro and in vivo testing remains pending, the versatility of nanobody modification was highlighted [159].

Nanorods synthesized from poly(2-methyl-2-oxazoline)-*b*-poly(2-isopropyl-2-oxazoline) copolymers were functionalized with targeting nanobodies via strain-promoted azide-alkyne cycloaddition (SPAAC) reactions. Time- and size-dependent association studies revealed that smaller nanorods (< 225 nm) associated faster with cells, while targeting nanobodies improved cellular association by an order of magnitude. Internalization efficiency and localization were unaffected by size, underscoring the platform's versatility [161].

Nanogels based on reactive ester polymers have been demonstrated as highly versatile drug delivery systems. In our group, we can access such nanogels with amphiphilic polymers that were self-assembled and crosslinked to form stable nanogels with tunable degradability under acidic conditions [166]. Targeting nanobodies against CD206/MMR were conjugated via maleimide-DBCO chemistry. The resulting nanogels showed superior selectivity and multivalent targeting capabilities compared to degraded polymer chains, as confirmed by flow cytometry and confocal microscopy [162, 163].

Innovative strategies include directly modifying nanobodies with small molecules such as photosensitizers or immune adjuvants, as discussed already above. Such chemical modifications are most effectively achieved at the nanobody's C-terminus using self-immolative linkers for traceless drug release at the target site, preserving nanobody conformation and enhancing therapeutic efficiency [167, 168].

A list of the aforementioned nanocarriers, along with the chemistry used to conjugate the respective nanobodies and the nanobodies' target are mentioned in Table 2.

Clinical translation of Nb- and NP-based diagnostics and therapeutics

In the previous sections, we already mentioned clinically approved Nb- and NP-based therapies and discussed several clinical trials evaluating diagnostically or therapeutically relevant targets in immuno-oncology (e.g. CD206, HER2, CLDN18.2, PD-L1, CTLA-4). Here, we further highlight some of the promising clinical trials.

Clinical translation of Nb-based diagnostics and therapeutics

The previously described KN046, a humanized anti-PD-L1/CTLA-4 bispecific Nb-Fc fusion construct, has been vastly evaluated in multiple clinical trials and showed promising results in patients with various solid tumors, such as nasopharyngeal carcinoma and non-small-cell lung cancer [175]. In combination with Lenvatinib, a multiple kinase inhibitor, KN046 demonstrated an overall response rate of 51.9% and manageable toxicity in patients with advanced unresectable or metastatic HCC [176]. Along the same line, the combination of KN046 with KN026, a HER2-targeted bispecific antibody, has demonstrated promising results in a Phase II trial in patients with metastatic HER2-positive breast cancer [177] and in HER2-positive non-breast and non-gastric solid tumors [178]. Currently, there is an ongoing Phase II/III trial to evaluate the efficacy and safety of KN046 combined with Acitinib, a tyrosine kinase inhibitor, in resectable stage IB-IIIB NSCLC patients (NCT06020352).

There are currently also two active clinical trials at Phase I/II evaluating the efficacy and the optimal dose of Nb-based MSTL-targeting CAR-T cells in patients with various solid tumors (NCT03907852, NCT05451849). As an alternative approach, Zhang et al. engineered a variant of anti-MSLN CAR-T cells that secrete nanobodies against CD39, an enzyme driving adenosine production and acting as another immunosuppressive checkpoint in cancer. The therapy effectively eliminated or suppressed ovarian tumor xenografts and will be further evaluated in clinical trials [179].

As was already mentioned, Ciltacabtagene autoleucel has been previously approved by the FDA for the treatment of multiple myeloma. These nanoCAR-T cells are targeting BCMA and currently, there is one recruiting and two active Phase III trials aimed to evaluate the efficacy of Ciltacabtagene autoleucel as compared to other treatments of multiple myeloma (NCT05257083, NCT04923893, NCT04181827). Moreover, a Phase IV study is being conducted on patients previously treated with Ciltacabtagene autoleucel to collect data on delayed adverse events and to evaluate its long-term safety profile (NCT05201781).

Table 2 Summary of investigated carrier platforms, their classification by material, the used conjugation chemistry and the nanobodies' target

Carrier Material	Conjugation Chemistry	Target	Reference
Inorganic Particles			
Copper	NHS-Esters	HER2 (MDA-MB-231/HER2)	[119]
Gold	Thiol-Maleimide	HER2 (SKOV3 and CHO, athymic nude mice)	[120, 169]
	Click Chemistry	Survivin (HEK293T, HeLa Kyoto, A431-GFP-Survivin)	[122]
	DNA-Hybridization	SARS-CoV-2 (Vero6)	[106]
Quantum Dot	Thiol-Maleimide	EGFR (MDA-MB-468 human TNBC, athymic nude-Foxn1^{nu} mice)	[121]
	Click Chemistry	EGFR (A549)	[107]
	Biotin-Streptavidin	GFP (A549)	[108]
		SARS-CoV-2	[123]
Siloxane	Click Chemistry	EGFR (C4–2B)	[125]
		PSMA (C4–2B)	[125]
		PD-L1/CD47 (wild-type B16F10, hiPDL1-B16F10, C57BL/6 J mice)	[124]
	Site Selective Sortaging	PD-L1/CD47 (wild-type B16F10, hiPDL1-B16F10, C57BL/6 J mice)	[124]
	NHS-Esters	Nucleolin Protein (MCF-7)	[170]
Iron Oxide	Thiol-Maleimide via Traut's Reagent	HER2 (BT-474, MDA-MB-231)	[126]
Liposomes			
Different Lipid Compositions	Thiol-Maleimide	CD19 (Raji, CA 46, Ramos, Namalwa, Daudi, CCRM-CEM, SKW-3, K562)	[136]
		HER2 (TUBO, MDA-MB-231, female BALB/c mice)	[137]
		Met/HGFR (TFK-1, TOV112D, TOV + MET)	[139]
		CD169 (monocyte-derived dendritic cells,C57BL/6 wt mice, CD169-DTR mice, hDC-SIGN tg mice)	[138]
		CTLA4 (293 T, HepG2, MGC-803, A549, primary HCC, female SPF-grade NOD/SCID mice)	[171]
	Thiol-Maleimide via Traut's Reagent	HER2 (BT-474, SKBR3, MCF10A, MDA-MB-231, MCF-7	[126, 129–132]
	Thiol-Maleimide via SATA	EGFR (UM-SCC-14C, A431, MDA-MB-468, 3T3 2.2, female athymic Balb/c nude mice, male athymic NU/NU nude mice, female athymic NU/NU nude mice	[133–135]
	NHS-Esters	PDL1 (CT26, HUVEC, female Balb/c mice)	[128]
	Transmembrane Anchor on Nanobody	HER2 (SK-BR-3, MDA-MB-231, T24, HT-29, LS-174 T, NCI-N87, NCI-H838, NCI-H2170, SNU-5, RT4 cells, BALB/c mice)	[140]
Biomaterials-derived Carrier			
Extracellular Vesicles	Protein Fusion with PDGFRβ Transmembrane Domain	CDH17 (MKN45, 4T1, HEK-293, IM95, AGS, TMK1, GES-1, female BALB/c nude mice)	[141, 142]
	Nb-PEG-lipid Insertion to Membrane	EGFR (A431, female Crl:NU-Foxn1^{nu} mice)	[109]
Polysaccharide	NHS-Esters	MUC1 (MCF-7)	[143]
	Chemoenzymatically (Transglutaminase)	EGFR (A431, HeLa, MCF-7)	[172]
Proteins like Ferritin and Albumin	Thiol-Maleimide	PDL1 (B16F10, C57BL/6 mice)	[145]
	Chemoenzymatically (Transglutaminase)	EGFR (A431, MCF-7)	[146]
	Thiol-Maleimide via SATA	EGFR (UM-SCC-14C)	[144]
		Met/HGFR (TOV-112D, A549, A431, MKN45)	[147]
Elastine-like Protein	Protein Fusion	EGFR (A431, H69AR, HCT116, MDA-MB-468, SKOV-3, OVCAR-3)	[148]

Table 2 (continued)

Carrier Material	Conjugation Chemistry	Target	Reference
DNA Constructs	DNA-Hybridization	EGFR (A431, A549, MCF-7, A2780, BALB/c nude mice)	[149]
Synthetic Organic Carrier			
Cationic Dendrimers like PEI and PAMAM	Thiol-Maleimide	HER2 (MCF-10A, BT-474, MDA-MB-231, SK-BR-3, NIH3T3)	[152–154]
PEG-blockpolymer Micelles	Thiol-Maleimide	Met (TFK-1, EGI1, A431)	[156]
		EGFR (A431, HeLa, female Balb/c nude mice)	[165]
	Chemoenzymatically (Transglutaminase)	EGFR (A431, MDA-MB-231, A549, MCF-7, A2780, nude mice)	[173]
Polymersomes	Click Chemistry	PlexinD1 (mammacarcinoma tumor tissue)	[160]
	Thiol-Maleimide	HER2 (SKBR3, MCF10)	[159]
		GFP (SKBR3, MCF10)	[159]
Nanorod Crystals	Click Chemistry	EGFR (MDA-MB-231)	[161]
Blockcopolymer Nanogels	Thiol-Maleimide	HER2 (SKBR3-MDA-MB-231)	[157, 158]
	Cysteine-Cysteine Disulfide Linking	GFP (KB_*wt*, HeLa_PCNA-GFP, HeLa_Actin-GFP, HeLa_Tubulin-GFP)	[155]
	DNA-Hybridization	EGFR (A549, H460, H1299, female Balb/c nude mice)	[150]
	Click Chemistry	MMR ($CHO^{MMR\pm}$, female C57BL/6, 3LL-R Lewis lung carcinoma cells)	[162, 163]
		EGFR (A431, E98)	[164]
Nanobody as carrier			
	Thiol-Maleimide	MMR (3LL-R and LLC-OVA, female C57BL/6 mice, MMR-deficient (MMR-KO) C57BL/6 mice)	[168]
	Thiol-Disulfide-Exchange	MMR ($CHO^{MMR\pm}$, RAW-Blue™ macrophages)	[167]
	Click Chemistry	HER2 (PGI,Cal-27, SCID mice and oral cancer bearing male nude mice)	[174]

Clinical translation of NP-based diagnostics and therapeutics

For nanoparticulate systems, several approvals have been given already in the last 30 years. Liposomal formulations for intravenous application against fungal infections as well as cancer therapies paved the way in the form of Ambisome® (amphotericin B) and Doxil® (doxorubicin) formulations in the early 1990s [180, 181]. Also, in the field of vaccination, hepatitis A immunization formulations containing inactivated hepatitis A virus in liposomes were approved by the EMA, namely Epaxal® [182]. In the following years, similar formulations for cancer therapy, against fungal infections and different vaccinations were brought to the market. New lipid-based formulations continue to broaden the application spectrum as during the covid pandemic mRNA formulations in liposomes were approved by agencies like FDA and EMA. Besides liposomal formulations, in the 1990s and early 2000s different iron nanoparticles against anemia have been approved. In the same time frame, PEGylated proteins like Oncaspar® (L-asparaginase) and Pegasys® (Interferon alfa-2) were authorized by the FDA [183, 184]. The application of these PEGylated proteins ranges from leukemia to anemia and various other indications. Since the mid 2000s, polymeric nanoparticles have also been given approval by the FDA and MFDS as well as other drug agencies. These authorizations have so far been given for formulations of paclitaxel and docetaxel in polymeric micelles (block copolymers of either PEG or polyvinylpyrrolidone and polylactic acid) or bound to albumin [185, 186]. These are similar formulations as their liposomal predecessors from the early 1990s that were then adapted to a different carrier type. In the last decade, more and more formulations for cancer therapy have been approved by the EMA and FDA. Besides the aforementioned and still prominently featured liposomal formulations, a hafnium oxide nanoparticle (Hensify®) for radiotherapy against locally advanced soft tissue sarcoma as well as a formulation of paclitaxel bound to albumin as nanoparticle (Pazenir®) against metastatic breast cancer and other types of cancer, were approved in 2019 [187].

Currently, there are several clinical trials involving nanoparticle systems for cancer therapy. Since 2020, trials involving ferritin, iron oxide, and polysiloxane

nanoparticles in addition to several trials using albumin nanoparticles have been ongoing. Ferritin nanoparticles carrying the Epstein-Barr virus (EBV) gp350 protein were designed to be used as a vaccination [188]. EBV is an oncogenic virus linked to lymphoid and epithelial malignancies. The trial is a phase I study (NCT04645147) to evaluate the safety and immunogenicity of a 3-dose vaccination regimen by registering adverse events and measuring neutralizing antibody responses.

Iron oxide nanoparticles are currently investigated for use in breast cancer diagnostics (NCT05985551, NCT05359783) as well as for radiotherapy of hepatocellular carcinoma (NCT04682847). The studies for sentinel lymph node localization in breast cancer are currently an observational study and a phase Ib/II study aiming to provide an alternative for ^{99m}Tc- and blue dye-mediated localization of sentinel lymph nodes [189].

The polysiloxane nanoparticles with gadolinium chelates are being investigated in a phase I/II study in combination with radiotherapy and Temozolomide as treatment for glioblastoma (NCT04881032). The objectives of the study are to determine the recommended dose of the particle and to estimate the efficacy of the combination radiotherapy with the particle measured by the 6-month progression-free survival rate [190].

Based on the comprehensive experience that has been collected for various types of nanoparticles as well as nanobodies in several clinical trials, we also foresee a high potential for nanobody-decorated nanoparticles in the clinics.

Conclusion and outlook

In the face of rapidly increasing demands for alternative strategies in oncology, novel developments in the field of nanomedicine emerge as potential solutions. The successful design of effective targeted nano-immunotherapies and diagnostics will be built on three pillars: choice of the target, the targeting moiety, and the carrier.

Understanding the complexity of interactions between multiple components of the TME, as well as key mechanisms of immunosuppression and escape, is crucial for optimal target selection aimed at enhancing current nanomedicines. The choice of the target will be largely dictated by its accessibility to a particular therapy. Although nanobodies are well equipped to extravasate into tumor tissues and bind to cryptic epitopes, their effective therapeutic application will often require a carrier delivering a larger payload of therapeutics. This will, in turn, determine possible interactions with the components of the TME and the efficacy of the drug delivery. Another important consideration is the expression level of a targeted molecule on a specific cell type in the TME as compared to the healthy organs. In that respect, conclusions made from diagnostic observations of a given target molecule may not be fully translatable to a therapeutic approach due to low expression levels. Moreover, off-targeted binding is one of the major causes of low efficacy and toxic side effects of current therapies. Several targets are currently under extensive investigation at the preclinical stage, and various nanobody-based diagnostics and therapeutics have already entered clinical trials targeting some of them (e.g. HER-2, PD-L1, CTLA-4, MSTL, BCMA). A further increase in specificity could be approached by a combination of individual targets, e.g. by bi-specific nanobodies or a combination of nanobodies on the same nanocarrier. However, patient stratification, based on the biological tumor heterogeneity, remains essential to predict the clinical response of the therapy. For example, identifying specific immune cell subsets infiltrating the TME allows to distinguish between "cold" (immune-excluded or infiltrated with immunosuppressive cell types) and "hot" (with a higher percentage of effector or cytotoxic cell types) tumors, with the latter being more likely to respond to immunotherapies. This, in turn, allows for the selection of an appropriate therapeutic approach and design of therapeutic formulations targeted to cells expressing specific molecules of interest. The level of target expression, therefore, carries significant prognostic value. Furthermore, tumor type, morphology, the composition of the tumor margin, ECM density, and the infiltration with specific immune cells (e.g., macrophages) all determine the NP tumor entry, retention, distribution, and exit mechanisms [191]. Non-invasive molecular imaging techniques, like SPECT and PET, offer high sensitivity and quantitative information for a patient's diagnosis, and nanobody-based imaging modalities are particularly advantageous for this approach. Alternatively, nanobodies can be coupled to fluorescent dyes used in optical imaging and image-guided surgery. To obtain anatomical information, these techniques can be combined with CT, US, and MRI imaging, thus creating multimodal imaging modalities aimed at obtaining comprehensive diagnostic information.

Next, preventing off-target interactions of the nanocarrier should be taken into account. A proper stealth functionalization of nanoparticles followed by a specific decoration with nanobodies may hold great promise for the future of nanomedicine, particularly in cancer immunotherapy. Modification methods, however, must be carefully optimized to ensure the retention of nanobody activity and stability. These combinatory systems gain complementary benefits, such as: 1) the poor circulation properties of the nanobody can be improved by a long-circulating nanoparticle to enhance drug delivery; 2) the monovalent targeting properties of a single nanobody

can be enhanced by a multivalent presentation or even different types of nanobodies on the same nanoparticle; 3) the loading capacities of the nanoparticle can be used for potential theranostic applications. The encapsulation of multiple therapeutic agents or diagnostic molecules within the nanoparticle further boosts the overall efficacy and versatility of these platforms.

Nevertheless, significant challenges remain. Scalable nanoparticle synthesis, efficient and stable nanobody conjugation, and batch-to-batch reproducibility are hurdles that must be addressed for clinical translation. Tumor heterogeneity and resistance mechanisms, such as antigen loss, add complexity to the design of universal targeting systems. By changing the biodistribution profile of drugs, unintended accumulation in organs like liver, spleen and kidney can occur. Strategies to increase the tumor-to-organ ratio are therefore required for proper NP delivery. This difficulty might also be intensified by the disparity of animal models to human physiology. Preclinical models often fail to accurately predict biodistribution, tumor penetration and immune response [192]. Despite that, the field is already advancing rapidly, with the development of stimuli-responsive nanoparticles tailored to the tumor microenvironment and bispecific or multimodal systems that can target multiple antigens simultaneously or combine therapeutic approaches.

Additionally, pharmacokinetics, biodistribution, and long-term safety profiles need more comprehensive evaluation and the establishment of stringent quality control procedures before these systems can achieve widespread clinical adoption. As these characterization processes are time-consuming and expensive, cost of development can often exceed 1 billion dollars, further increasing treatment cost. The high financial burden together with the need to demonstrate clear benefits compared to already approved medicines regarding reduced toxicity, better efficacy or improved convenience are big challenges for widespread clinical approval of novel nanomedicines [193].

However, as personalized approaches leveraging tumor-specific biomarkers to customize treatment for individual patients, are emerging as cornerstones of the field, the integration of innovations in nanotechnology and immunology will likely push nanobody-decorated nanoparticles closer to clinical application, unlocking their potential as transformative tools in cancer therapy.

Abbreviations

ABCC3	ATP Binding Cassette subfamily C member 3
ADCC	Antibody-dependent Cell-mediated Cytotoxicity
ALK	Anaplastic Lymphoma Kinase
AML	Acute Myeloid Leukemia
APCs	Antigen-Presenting Cells
ATP	Adenosine Triphosphate
AuNPs	Gold Nanoparticles
B-ALL	B-cell Acute Lymphoblastic Leukemia
BBB	Blood-Brain Barrier
BCMA	B-cell Maturation Antigen
BITEs	Bispecific T-cell Engagers
BsAb	Bispecific Antibody
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase
CAFs	Cancer-associated Fibroblasts
CAR-T	Chimeric Antigen Receptor T-Cells
CDH	Cadherin
CEACAM	Carcinoembryonic Antigen-related Cell Adhesion Molecule
CTLA-4	Cytotoxic T-lymphocyte Associated Protein 4
CuS NPs	Copper Sulfide Nanoparticles
CV	Cellular Vesicles
DCs	Dendritic cells
DBCO	Dibenzocyclooctyne
ECM	Extracellular Matrix
ELP	Elastin-like Polypeptide
EpCAM	Epithelial Cell Adhesion Molecule
EVs	Extracellular Vesicles
FAP	Fibroblast Activation Protein α
Fc	Fragment Crystallizable
FDG	Fluorodeoxyglucose
FGFR4	Fibroblast Growth Factor Receptor 4
FN	Fibronectin
GSCs	Glioblastoma stem cells
HCC	Hepatocellular Carcinoma
HCAbs	Heavy-Chain-Only Antibodies
HER	Human Epidermal Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HLA-G	Human Leukocyte Antigen G
IC	Immune Checkpoints
KI mice	Knock-in Mice
LAG-3	Lymphocyte-Activation Gene 3
LI	Liver Intestine
mAbs	Monoclonal Antibodies
Met/HGFR	Mesenchymal-epithelial Transition Receptor/Hepatocyte Growth Factor Receptor
MMUS	Magnetomotive Ultrasound
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem Cells
MTGase	Microbial Transglutaminase
mTHPC	meso-Tetrahydroxyphenylchlorin
Nb-TriTE	Nb-based Trispecific T-cell Engager
Nbs	Nanobodies
NHS	*N*-Hydroxysuccinimid
NK cells	Natural Killer Cells
NRP-1	Neuropilin-1
PAMAM	Polyamidoamines
PCL	Polycaprolactone
PD-1	Programmed Death Receptor 1
PD-L1	Programmed Death Ligand 1
PDAC	Pancreatic Ductal Adenocarcinoma
PDGFRα/β	Platelet-derived Growth Factor Receptors Alpha and Beta
PEG	Polyethyleneglycol
PEI	Polyethylenimine
PE38	Pseudomonas Exotoxin A
PET	Positron Emission Tomography
PFPMA	Pentafluorophenyl Methacrylate
PLA	Polylactic acid
PLGA	Poly(Lactic Acid-co-Glycolic Acid)
PLGHMGA	Poly(Lactic Acid-co-Glycolic Acid-co-Hydroxymethyl Glycolic Acid)
POx	Polyoxazoline
PS	Polystyrene
PVRL4	Nectin-4
Q-dots	Quantum Dots
ROS	Reactive Oxygen Species
SATA	*N*-succinimidyl-*S*-acetylthioacetate
SIRPα	Signal Regulatory Protein α
siRNA	Small Interfering RNA
SLN	Sentinel Lymph Node
SMA	Alpha Smooth Muscle Actin

SMCC	Succinimidyl-4-(*N*-maleimidomethyl)cyclohexan-1-carboxylate
SPAAC	Strain-promoted Azide-alkyne Cycloaddition
SPECT	Single Photon Emission Computed Tomography
TAMs	Tumor-associated Macrophages
TCE	T-cell Engager
TGF-β	Transforming Growth Factor β
TIM-3	T-cell Immunoglobulin and Mucin Domain-Containing 3
TIGIT	T-cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-Based Inhibitory Motif Domain
TILs	Tumor Infiltrating Lymphocytes
TLC	Thin-Layer Chromatography
TLR7	Toll-like Receptor 7
TME	Tumor Microenvironment
TMZ	Temozolomide
TNBC	Triple Negative Breast Cancer
TNC	Tenascin-C
TNF	Tumor Necrosis Factor
Tregs	Regulatory T-cells
TUFM	Tu Translation Elongation Factor, Mitochondrial
US	Ultrasound
VHH	Variable Heavy Domain of Heavy Chain
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

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Declarations

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