Nanobodies in cytokine-mediated immunotherapy and immunoimaging (Review)

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Abstract. Cytokines are the main regulators of innate and adaptive immunity, mediating communications between the cells of the immune system and regulating biological functions, including cell motility, differentiation, growth and apoptosis. Cytokines and cytokine receptors have been used in the treatment of tumors and autoimmune diseases, and to intervene in cytokine storms. Indeed, the use of monoclonal antibodies to block cytokine-receptor interactions, as well as antibody-cytokine fusion proteins has exhibited immense potential for the treatment of tumors and autoimmune diseases. Compared with these traditional types of antibodies, nanobodies not only maintain a high affinity and specificity, but also have the advantages of high thermal stability, a high capacity for chemical manipulation, low immunogenicity, good tissue permeability, rapid clearance and economic production. Thus, nanobodies have extensive potential for use in the diagnosis and treatment of cytokine-related diseases. The present review summarizes the application of nanobodies in cytokine-mediated immunotherapy and immunoimaging.

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1. Introduction

The incidence of malignant tumors has been increasing annually and has become a common issue faced by mankind. Traditional therapies such as surgery, conventional chemotherapy and radiation therapy have been playing a leading role in the treatment of cancer. However, the effects of these therapies are limited, particularly in the advanced stages of disease. Thus, it is difficult for patients to benefit from these treatments in the long term, and they cannot fundamentally solve or change their quality of life or survival. Therefore, researchers are increasingly focusing on the field of immunotherapy.

Immunotherapy has provided novel opportunities and hope for the treatment of tumors, in which immune checkpoint inhibitors, therapeutic antibodies, cytokine-immunomodulators, tumor vaccines and over-the-counter cellular therapies have markedly improved the prognosis of patients with tumors, and some patients with advanced-stage tumors have achieved long-term survival (1-3).

Cytokines are an integral part of the tumor microenvironment (TME) and those released in response to infection, inflammation and immunity can inhibit or promote tumor development. Thus, cytokines play a vital role in tumor pathogenesis. Numerous types of recombinant protein products are currently available, among which, antibody-cytokine conjugates are a class of cytokine drugs with immense value in clinical applications (4-6). With the continuous application of antibody drugs in more therapeutic fields, the structures of these agents and their therapeutic mechanisms have become increasing complex and diverse. Among these agents, nanobodies have attracted ample attention due to their small size and unique molecular structure, rendering them suitable

for a few applications, such as disease diagnosis and treatment (7-11). The present review summarizes the application of nanobody-related immunocytokines, agonists/inhibitors, engager cytokines and cytokine receptors in tumor immunotherapy and immunoimaging.

2. Cytokines and cytokine-based immunodrugs

Cytokines are a class of small proteins with a wide range of biological activities. These molecules are synthesized and secreted by immune cells [such as monocytes, macrophages, T-cells, B-cells and natural killer (NK) cells] and some non-immune cells (such as endothelial cells, epidermal cells and fibroblasts) under stimulation, and play a critical role in cell signaling (12,13). Cytokines are classified as interleukins (ILs), interferon (IFNs), tumor necrosis factor superfamily, colony-stimulating factors, chemokines and growth factors. They act through cell surface receptors and are particularly important in the immune system, regulating the balance between humoral and cell-based immune responses, as well as the maturation, growth, and responsiveness of specific cell populations. The effect of individual cytokines on immunity depends on the local cytokine concentration, the pattern of their receptor expression and the integration of multiple signaling pathways in the immune response cells (14-16). As molecular messengers, cytokines facilitate communication between immune cells, which perform regulatory and effector functions in a number of diseases. Consequently, cytokines and their receptors are increasingly being used in immunotherapy, particularly in host immune responses to inflammation (16), infection (17-21), tumors (4-6) and trauma (22-24).

As immunomodulators, numerous cytokines have been used in the form of recombinant, synthetic and natural agents for activation and suppression immunotherapy, including ILs (IL-2, IL-7 and IL-12) (25-30), chemokines (CCL3, CCL26 and CXCL9) (31-33) and other cytokines [IFN, granulocyte colony-stimulating factor (G-CSF)] (34-36).

During immunotherapy, cytokines directly stimulate immune effector cells and stromal cells at tumor sites to enhance immune effector cytotoxicity. Studies using animal tumor models have demonstrated that cytokines have broad antitumor activities, and many have been used in cancer therapy (4,6,37,38). Several cytokine drugs have been approved by the Food and Drug Administration (FDA), such as high-dose IL-2 for the treatment of melanoma and renal cell carcinoma in 1992 (39,40) and IFN-α for the adjuvant treatment of stage III melanoma (41). Several other cytokines, such as GM-CSF, IL-7, IL-12, IL-15 and IL-18, are also being investigated in trials with different clinical research statuses (NCT02978222, NCT04833504, NCT02451748, NCT01055522, NCT01189383, NCT05297084, NCT05307068) (39-44).

In activation immunotherapy, G-CSF (35,36) is used to stimulate peripheral blood stem cells to produce lymphocytes that are later co-cultured with tumor antigens *in vitro* and infused back into the patient. Combined with stimulating cytokines to enhance immunity, the tumor cells carrying the same antigens are destroyed to achieve the therapeutic effect. IL-7 and IL-2 can be used to restore the immune system in immunocompromised patients, and this approach is already being evaluated in trials with different clinical research statuses (NCT01339000, NCT03308786) (45,46). By contrast, the focus of suppressive immunotherapy is on reducing normal immune responses to prevent rejection in cell or organ transplantation (47,48) or suppressing abnormal immune responses in autoimmune diseases (49,50).

The potent immunomodulatory effects of cytokines, such as IL-2, IL-7, IL-15, IL-21 and IL-12, and IFNs have been used with some success in humans (51). However, the development of cytokine therapeutics in clinical practice is hindered by multiple issues, mainly polymorphisms in cytokine immunomodulation, the short half-life and toxicity due to the activation of off-target cells (52-55). These toxicities occur due to the uptake of large doses of free pro-inflammatory cytokines by surrounding tissues before reaching their intended destination and the lack of effective concentrations within the tumor. With a better understanding of the structural principles and functional signaling of cytokine-receptor interactions, artificial modifications of cytokines through methods, such as protein engineering have facilitated the generation of effective drugs (56). Engineered cytokines, including immunocytokines, cytokine agonists/inhibitors and engager cytokines with antibodies have been developed. Antibodies of engineered cytokines are directed mainly against tumor markers and the cytokines currently fused with antibodies include IL-2 (57), IL-10 (58), TNF-α (59), IL-12 (60,61), IL-15 (62), IL-21 (63) and IL-4 (64). This approach has exhibited great promise as a useful platform for the development of effective antitumor therapeutics. However, traditional antibodies have disadvantages, such as a long expression cycle, high costs, poor stability and cumbersome genetic engineering, thus limiting their application as cytokine drugs.

Since nanobodies are simple in structure, stable, soluble, easy to express and have a low immunogenicity, they have become the focus of research in the development of immunotherapy, widely used in various fields such as research, diagnosis, detection and drug development (65-70).

3. Nanobodies and their advantages in immunotherapy

Nanobodies were first reported by the Belgian scientist, Hamers-Casterman *et al* (71) in 1993; they, as well as others found that some antibodies in the blood of camelids (camels, alpacas and their relatives) existed as heavy chain antibodies with missing light chains (71,72). These naturally occurring light chain-deficient antibodies, also known as single-domain heavy chain antibodies, are found in Asian camels (*Camelus bactrianus*) and African dromedaries (*Camelus dromedarius*), as well as alpacas (*Vicugna pacos*), large alpacas (*Lama glama*) and llamas (*Lama guanicoe*) (71,72). These antibodies contain only two heavy chain variable regions (VHH) and two heavy chain CH2 and CH3 regions (Fig. 1). The VHH region retains intact antigen-binding capacity and is the smallest intact antigen-binding fragment, known as single-domain antibodies (73). VHH crystals are 2.5x4 nm in size, with a molecular weight of only 15 kDa.

Compared with traditional antibody fragments, such as the fragment of antigen binding (Fab) and single chain antibody fragment (scFv), nanobodies have significant advantages, such as weak immunogenicity, low production costs, good water solubility and tissue permeability, as well as good

Table I. Comparison of the properties of nanobodies and conventional antibodies.

Characteristics	Nanobody	Conventional antibody	(Refs.)
Stability	High stability and high temperature resistance	Low stability, easily inactivated, temperature sensitive	(65-67)
Immunogenicity	Low	High	(68)
Tissue penetration	High, penetrates the blood-brain barrier	Low	(66,68)
Half-life period	Short, fast serum clearance	Long half-life	(68,69)
Expression systems	Mammalian cell expression system, yeast expression system, *E. coli* expression system, low-cost, high water solubility of expressed antibodies	Mammalian cell expression system, high cost and long expression period	(68-70)

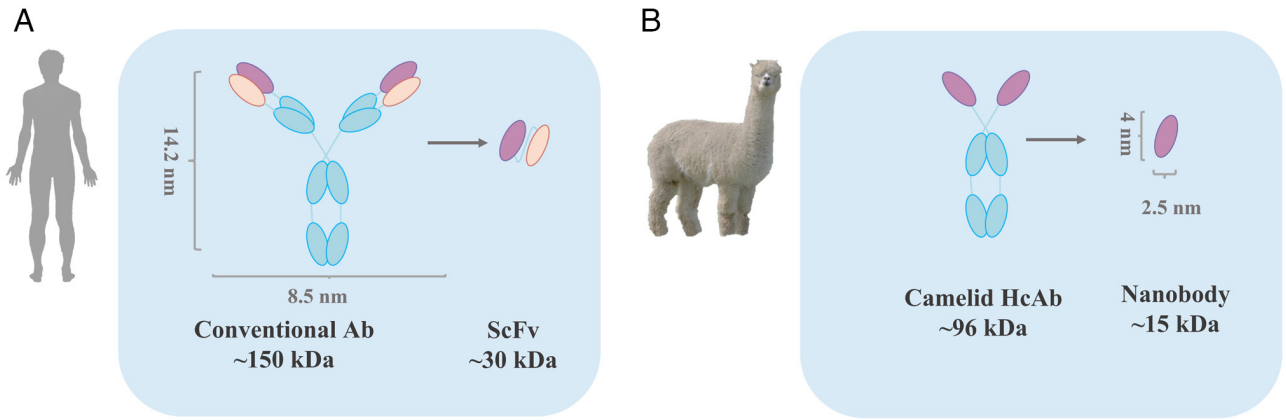

Figure 1. (A) Conventional antibody and single chain antibody fragment (ScFv). (B) Camel heavy chain antibody (HcAb) and nanobody.

stability (Table I). It is these excellent properties that have led to the widespread application of nanobodies in biotechnology (74-77). They have been widely used in multiple medical fields, such as infection and immunity (78-80), and oncology (81-83) and exhibit immense potential for new drug discovery (Fig. 2). A comparison of the properties of nanobodies and conventional antibodies is presented in Table I.

The present review summarizes the application of nanobodies in immunocytokines, cytokine agonists/inhibitors, engager cytokines and cytokine receptors.

4. Application of nanobodies in cytokine-mediated immunotherapy and immunoimaging

Immunocytokines (nanobody-cytokine fusion proteins). High concentrations of cytokines in the TME markedly enhance the effectiveness of tumor immunotherapy. Since cytokines lack targeting capacity, methods designed to direct cytokines to disease sites are critical for improving the therapeutic effectiveness of cytokine drugs. Antibodies or peptides against disease site-specific biomarkers may be ideal 'vehicles' for targeted cytokine delivery (84). Antibody-cytokine fusion proteins targeting tumor biomarkers have been shown to significantly increase the selective accumulation of corresponding cytokines at sites of tissue remodeling in many mouse models. These fusion proteins exploit the tumor-targeting ability of antibodies to specifically direct cytokines to tumor sites, where they can stimulate a more desirable antitumor immune response while avoiding the systemic toxicity that limits the potential efficacy of current cytokine doses (85-89). Such antibody-cytokine fusion proteins are referred to as immunocytokines (47,90), which have recently been redeveloped and

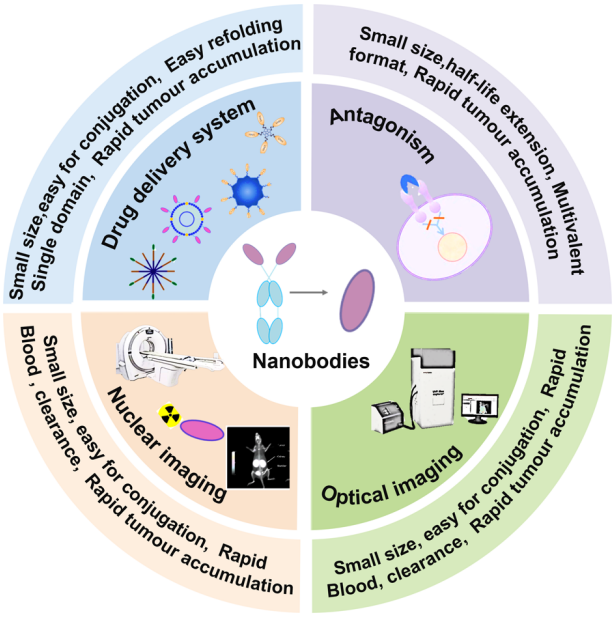

Figure 2. The advantages of nanobody characterization and the application of nanobodies in immunotherapy and immunoimaging.

Table II. Summary of immunocytokine-based nanobodies.

Immunocytokines	Cytokines	Fusion nanobody	Function	(Refs.)
CEA-IL15	IL-15	Anti-CEA nanobody	Antitumor	(92)
Ia1-TNFα	TNF-α	Anti-EGFR nanobody (Ia1)	Antitumor	(100)
IL-2 immunocytokine	IL-2	Nanobody of fibronectin EIIIB domain	Antitumor	(89)

described as a next-generation cytokine product. A summary of immunocytokine-based nanobodies currently under investigation is presented in Table II.

IL-2-based immunocytokines. As a water-soluble cytokine, IL-2 was the first IL to be discovered and cloned. In the 1980s and 1990s, the immune enhancing properties of IL-2 made it a promising immune-enhancing drug for the pharmaceutical market. Lutz *et al* (91) engineered IL-2 immunocytokines fused to nanobodies that specifically targeted the fibronectin EIIIB structural domain of tumors with nanomolar to picomolar affinity. This high affinity allows for the specific targeting of the immunocytokine to the tumor, where IL-2 exerts an antitumor effect in the TME. Moreover, the intra-tumoral administration improves the survival rates of tumor-bearing mice, indicating that this is a promising route for the targeted delivery of small molecules and cytokines.

IL-15-based immunocytokines. IL-15 belongs to the same cytokine family as IL-2. However, IL-15 has no immunosuppressive function and may exert a more potent antitumor effect than IL-2 (62,92). High doses of IL-15 are required to achieve biological function, but this carries the risk of toxicity (93). Therefore, the extension and enhancement of the therapeutic activity of IL-15 has been explored (93,94). Such approaches include targeting IL-15 to the TME to promote the specific chemotaxis of immune cells in the TME, thereby enhancing the antitumor function of IL-15.

Liu *et al* (95) constructed an anti-CEA-IL15 structure by fusing anti-CEA nanobody-Fc and IL15Rα-IL15. This fusion protein recognizes CEA-positive tumor cells, while possessing potent cytokine activity that activates and mobilizes the immune system against cancer cells. The anti-CEA-IL15 fusion protein promotes immune cell proliferation *in vitro* and targets the TME, where it exerts potent antitumor activity in a xenograft model *in vivo*. These data support the further development of the anti-CEA-IL15 immunocytokine for cancer immunotherapy, and highlight the potential application of this strategy to other cytokines and tumor-targeting molecules to enhance the antitumor efficacy.

TNF-α-based immunocytokines. TNF-α belongs to the TNF superfamily of cytokines (96-98) and is a multifunctional molecule that regulates biological processes, including cell proliferation (98), differentiation (98,99), apoptosis (98,99) and anti-tumor activity (100-103). TNF-α represents a key link in the cell signaling pathway and has thus become the target of numerous drugs. TNF-α-targeting inhibitors (TNF inhibitors) are mainly monoclonal antibodies, including infliximab (Remicade), adalimumab (Humira), certolizumab (Cimzia) and golimumab (Simponi), or the TNF receptor-antibody fusion protein etanercept (Enbrel). Clinically, therapeutic antibodies against TNF-α have been used successfully in the treatment of rheumatoid arthritis (RA) and Crohn's disease, and studies have shown positive efficacy in psoriasis and ankylosing spondylitis (101). However, the systemic administration of TNF-α inhibitors can lead to severe adverse effects, such as shock and organ failure, which greatly limits its clinical application (101,102).

Osaki *et al* (103) constructed an antibody-cytokine fusion protein (Ia1-TNF-α) consisting of a single-domain antibody (also called nanobody) Ia1 and a TNF-α domain. Ia1 targets the epidermal growth factor receptor (EGFR), which is overexpressed in epithelial tumors, while TNF-α has antitumor activity. By using the *E. coli* expression system, Ia1-TNF-α was produced in a soluble form, which exists mainly in a trimeric form that is consistent with the multimeric state of TNF-α. Flow cytometric analysis revealed the specific binding of Ia1-TNF-α to EGFR-expressing tumor cells, with higher binding activity than monovalent Ia1, indicating that the fusion protein binds multivalently to tumor cells. Taken together, these results suggest that the fusion of TNFα and single-domain antibodies Ia1 may be a cost-effective method to produce antitumor therapeutics (103).

Cytokine agonists/inhibitors. In diseases or defense states in which large amounts of cytokines are expressed, excessive levels of cytokine can cause systemic or local toxic responses, such as cytokine storms, and therefore inhibitors are required to limit their activity and tightly control cytokine levels (104-107). Antibodies obtained by immunizing animals with cytokines or their receptors as immunogens, exert poent inhibitory effects on cytokines and can be used to treat diseases caused by excess cytokines. In addition to inhibitors, agonists targeting certain cytokine receptors to mobilize agonistic activity can be used in tumor immunotherapy. If nanobodies targeting different cytokine receptors or different epitopes of the same cytokine receptor are designed as bispecific antibodies, their agonistic/inhibitor activity can also be enhanced by cross-linking. Bispecific nanobodies targeting two receptors concurrently were designed as antibody agonists for diverse purposes, such as nanobodies targeting IL-2 receptor and IL-15 receptor for tumor immunotherapy, IFN receptors for COVID-19 treatment, IL-2 receptor and IL-10 receptor for the activation of NK cells and T-cells (108).

Details of the cytokine agonists/inhibitors in the form of nanobodies currently under investigation are summarized in Table III.

IL-23 specific nanobody. IL-23 is a heterodimer consisting of two subunits, p40 and p19. In isolation, p19 has no biological

Table III. Summary of cytokine agonists/inhibitors in the form of nanobodies.

Nanobody-cytokine agonists/inhibitors	Cytokine	Function	Clinical trials: Stage and status	(Refs.)
Monovalent and multivalent anti-hIL-23 Nanobodies	IL-23	Treating inflammatory diseases	Preclinical	(112)
Anti-IL13 nanobody	IL-13	Inhibition of IL-13 function	Preclinical	(113)
Anti-IL-17A nanobody	IL-17A	Neutralization nanobody	Preclinical	(116)
M1095	IL-17A and IL-17F	Block IL-17A/ IL-17F	Clinical Phase IIb	(117,118)
BI-655088	Anti-CX3CR1	CX3CR1 biotherapeutic antagonist, inhibition of atherosclerosis	Clinical Phase Ib	(119)
V-L-R-H	TNF-α	Inhibit tumor cells migration and proliferation	Preclinical	(120)
TNF-α nanobody (NT-3)	TNF-α	Block TNF signaling	Preclinical	(121)
VHH-Zag	TNF-α	Prolonged VHH half-life and improved Pharmacokinetics	Preclinical	(122)
Ozoralizumab (ATN-103)	TNF-α	Treatment of patients with rheumatoid arthritis	Clinical Phase IIIb	(123)
Lactic acid bacteria secreting anti-murine TNF nanobodies	TNF-α	Treatment of chronic colitis	Preclinical	(124)

activity, but combines with p40 to form biologically active IL-23 (109), which plays a role in innate and adaptive immunity and is a key cytokine that promotes inflammatory responses in various target organs (110,111). Desmyter *et al* (112) prepared monovalent anti-human IL23 nanobodies comprising the nanobody 37D5, which blocks p19, and the nanobody 22E11, which blocks p40. The same research group also constructed multivalent IL23-specific blocking nanobodies by fusing the monovalent nanobodies 37D5, 22E11, 124C4 and the serum albumin antibody Alb1, thus providing improved hIL23 neutralization ability compared with the monovalent nanobodies. With an extended half-life that prolongs *in vivo* exposure, this multivalent nanobody construct represents an excellent drug candidate for the treatment of inflammatory diseases (112).

IL-13 specific nanobody. IL-13 is involved in allergies, inflammation and fibrosis in response to a number of different cell types, such as mast cells, B-cells and fibroblasts. The inhibition of IL-13 function has exhibited great potential in preclinical studies in animal models (113). However, targeting IL-13 with conventional monoclonal antibodies alone has failed to improve the therapeutic efficacy (113). Nanobodies targeting IL-13 were prepared by Gevenois *et al* (113) for combination therapy or for novel strategies, such as local (pulmonary) administration. They designed a multimeric structure resulting in a 36-fold increase in affinity and a 300-fold increase in biological activity, while maintaining high specificity for IL-13 (113). This approach therefore provides new insight for the development of cytokine nanobody drugs.

IL-17A specific nanobody. As a member of the IL-17 family, IL-17A plays a crucial role in host defense, autoimmune disease pathogenesis and tumorigenesis, particularly in promoting the inflammatory autoimmunity in diseases, such as psoriasis, psoriatic arthritis and ankylosing spondylitis (114,115).

To alleviate the immune system disturbance caused by cytokine overproduction, known as hypercytokinemia, Yao *et al* (116) constructed a novel immunosorbent containing anti-IL-17A nanobodies to remove IL-17A in the blood. Such nanobody-loaded immunosorbents represent a simple and cost-effective platform technology for the removal of single or even multiple cytokines from plasma.

M1095 (117) (also known as ALX-0761, Sonelokimab) is a trivalent bispecific nanobody targeting IL-17A and IL-17F. It consists of three single-domain antibodies comprising the anti-IL-17A domain, anti-IL-17F domain and anti-human serum albumin domain, which are coupled to enhance targeting and prolong the serum half-life.

Papp *et al* (117) and Svecova *et al* (118) conducted a clinical study of M1095 in patients with plaque psoriasis and evaluated the safety of M1095 in multiple dose increments in terms of susceptibility, tolerability and immunogenicity. Their results demonstrated a significant clinical benefit of M1095 at doses ≤120 mg for the treatment of moderate-to-severe plaque psoriasis, with rapid onset of action, durable improvement and an acceptable safety profile. Therefore, these data indicate the potential value of 17A/F nanobodies for clinical application.

C-X3-C motif chemokine receptor 1 (CX3CR1) specific nanobody. CX3CR1 is a multifunctional inflammatory chemokine, with diverse biological effects in the immune system and the TME. BI-655088, a nanobody targeting the CX3CR1 protein, is currently in phase I clinical studies (NCT02696616) for the treatment of chronic kidney disease. Low *et al* (119) reported that when added to standard-of-care treatments including statins, BI-655088 reduced inflammation in atherosclerotic plaques, which may contribute to the

Table IV. Summary of nanobody-engager cytokines.

Nanobody-engager cytokines	Cytokine-related factors	Nanobody-related factors	Function	(Refs.)
CD16-EGFR bispecific VHH	EGFR	Anti-CD16 nanobody C21	Tumor immunotherapy	(129)
CAM1615HER2	IL-15	Anti-CD16 nanobody	Tumor immunotherapy	(130)

significant reduction in atherothrombotic events in patients with existing cardiovascular disease.

TNF-α specific nanobody. The biological functions of TNF-α are diverse and its action mechanisms are complex. In addition to its anti-tumor effects, TNF-α plays a pathological role in a variety of autoimmune diseases, in which pro- and anti-inflammatory cytokines modulate the function of the immune system. Anti-TNF-α therapy has been used with varying degrees of success in arthritis, psoriasis, inflammatory bowel disease, psoriasis, Crohn's disease and non-infectious uveitis (102). In addition to tumor therapy, TNF-α nanobodies are also used for *in vivo* molecular imaging. However, certain side-effects involve rashes, transaminitis, anemia have also limited the clinical use of TNF-α inhibitors (102). Researchers at home and abroad have been exploring the mechanisms underlying the therapeutic effects of TNF-α and its inhibitors in order to 'target and pinpoint' more effective treatment strategies.

Ji *et al* (120) and Nie *et al* (121) developed an anti-TNF-α nanobody and experimentally demonstrated that the anti-TNF-α nanobody inhibited proliferation and promoted the apoptosis of tumor cells. However, since nanobodies are rapidly cleared from the circulation, novel strategies are required to extend their half-life for therapeutic use. Morais *et al* (122) innovatively designed novel nanobodies that couple a bacterial albumin binding structural domain from protein Zag and anti-human serum albumin antibody (123) to the TNF nanobodies, this fusion protein enhances the *in vivo* performance of TNF nanobodies and reduces blood clearance, renal retention and excretion.

Vandenbroucke *et al* (124) innovatively established lactic acid bacteria (*Lactococcus lactis*) that secrete anti-murine TNF (mTNF)-α nanobodies, which were shown to neutralize mTNF *in vitro*. Furthermore, the daily oral administration of the engineered *Lactococcus lactis* facilitated the local delivery of the anti-mTNF nanobodies to the colon and significantly reduced inflammation in model mice with dextran sodium sulfate-induced chronic colitis.

Engager cytokines. Bispecific immunoconjugates are designed with two arms, one targeting tumor antigens, while the other targets T-cells or NK cells. For example, bispecific T-cell engagers (BiTEs) are an engineered molecule that targets CD3 on T-cells via one arm and cancer cells through the other arm (80-83). Bispecific antibodies that bind CD3-activated T-cells have been successful in treating hematologic tumors, such as blinatumomab targeting CD3/CD19 (125), primarily by bridging T-cells to CD19-expressing tumor cells, while activating T-cells to release associated cytokines to kill tumor cells. In the context of bridging the activation of NK cells to kill tumor cells, an increasing number of studies are now adding cytokines, such as IL-15 and IL-2, to enhance the efficacy of these platforms. For example, IL-15 has been incorporated into the development of bispecific killer cell engagers (BiKEs) to promote the survival and expansion of NK cells (126-128). Details of the engager cytokines in the form of nanobodies currently under investigation are summarized in Table IV.

Toffoli *et al* (129) prepared a novel bispecific single-domain antibody (VHH) that binds to CD16 (FcRgammaIII) on NK cells and EGFR on epithelial-derived tumor cells. This bispecific VHH triggered CD16- and EGFR-dependent activation of NK cells and subsequent tumor cells lysis, independent of the tumor KRAS mutation status. The enhanced activation of NK cells by bispecific VHH was also observed when NK cells from patients with colorectal cancer (CRC) were co-cultured with EGFR-expressing tumor cells. Finally, higher levels of cytotoxicity against patient-derived metastatic CRC cells were found in the presence of bispecific VHH and autologous peripheral blood monocytes cells or allogeneic CD16-expressing NK cells. Thus, the antitumor activity of CD16-EGFR bispecific VHHs warrants further exploration.

Vallera *et al* (130) prepared the human epidermal growth factor receptor 2 (HER2) tri-specific killer cell engagers (TriKEs) CAM1615HER2, consisting of a camelid VHH antibody fragment recognizing CD16, a single chain variable fragment (scFv) recognizing HER2, and cross-linked human IL-15. This triple-specific killer attractor (TriKETM) has been shown to induce NK cell proliferation *in vitro* and exhibits strong cytotoxic activity against acute myelogenous leukemia (AML) cell lines and patient-derived AML cells.

Nanobody targeting cytokine-receptors. Cytokines generally exert their biological effects by binding to the corresponding cytokine receptors on the cell surface. This interaction initiates complex intracellular molecular changes that ultimately regulate cellular gene transcription. Cytokine receptors are capable of mediating a remarkable array of biological responses. Details of the nanobody-related cytokine-receptors currently under investigation are summarized in Table V.

Il-6 specific nanobody. IL-6 is a key pro-inflammatory cytokine that plays a crucial role in systemic inflammation and joint destruction in patients with RA (131). The biological activity of IL-6 is mediated through a hexameric signaling complex consisting of two molecules each of IL-6, IL-6R and glycoprotein (107,132). Unlike other cytokines, IL-6 exerts biological functions by binding to membrane-bound receptors (mIL-6R; classical signaling) or soluble receptors (sIL-6R; trans signaling) (132). Van Roy *et al* (133) developed Nanobody® ALX-0061 consisting of one domain that targets IL-6R and a second domain designed to improve the pharmacokinetic properties by binding to human serum albumin. In

Table V. Summary of nanobody-related cytokine-receptors.

Cytokine receptor nanobody	Target	Function	(Refs.)
ALX-0061	IL-6R	IL-6 signal blockade	(133)
VHH26 nanobody	G-CSF receptor (G-CSF-R)	Blockade of downstream G-CSF-R signaling	(138,139)
VEGFR2-specific nanobody (3VGR19)	Vascular endothelial growth factor receptor-2 (VEGFR2)	VEGFR2 signaling blockade	(141)
V21-DOS47	Anti-VEGFR2 nanobody (V21)	VEGFR site blockade with cytotoxicity	(142)
Second-generation CAR-T cell based on a VEGFR2-nanobody (anti-VEGFR2 CARs)	VEGFR2 nanobody	T cell immunotherapy for solid tumors	(143)
Anti-EGFR nanobody	EGFR	EGFR signal blockade	(146)
7D12, EgA1and 9G8	EGFR	EGFR signal blockade	(147)
7D12-IR	EGFR nanobody-7D12	Rapid preclinical optical imaging	(148)
7D12-800CW	EGFR nanobody-7D12	Head and neck cancer surveillance	(149)
(99m) Tc-D10	EGFR nanobody-D10	Specific and high contrast *in vivo* visualization of small human tumors overexpressing EGFR	**(152)**
OA-cb6-(99m) Tc	EGFR nanobody- OA-cb6	Tumor imaging in cancers with high EGFR expression	**(153)**
Tc radiolabeled EG2 (mTc-sdAb EG2)	EGFR nanobody EG2	EGFR detection for monitoring tumor cells	**(154)**
Multivalent antibodies EG2-RHCC and EG2-COMP	EGFR nanobody EG2	EGFR detection for monitoring tumor cells	**(157)**
68Ga labeled 7D12 [(68)Ga-nanobody]	EGFR nanobody 7D12	Tumor detection and imaging	**(155)**
DTPA-IRDye700DX-7D12	EGFR nanobody-7D12	Nuclear imaging and targeted photodynamic therapy of EGFR-positive tumors	**(156)**

The reference citations shown in bold font indicate immunoimaging studies.

cynomolgus monkeys, ALX-0061 exhibited the dose-dependent and complete inhibition of hIL-6-induced inflammatory parameters including plasma C-reactive protein, fibrinogen and platelet levels. It also exhibited a longer plasma half-life of 6.6 days, with albumin binding yielding the expected half-life extension technique (133). ALX-0061 is currently in clinical development with promising results obtained in a phase I/II trial in RA (NCT02518620). A number of preclinical pharmacological properties of ALX-0061 support its development as a clinical agent in RA.

G-CSF specific nanobody. G-CSF induces the proliferation and differentiation of hematopoietic precursor cells, and the activation of mature neutrophils (134). G-CSF is overexpressed in certain malignancies and the blockade of its binding to the receptor markedly reduces tumor growth, tumor vascularization and metastasis (135). In addition, targeting the G-CSF receptor has been shown to exhibit therapeutic efficacy in conditions such as RA (136), as well as progressive neurodegenerative diseases (137).

A nanobody known as VHH26 developed by Bakherad *et al* (138,139) specifically binds to the G-CSF-R on the surface of NFS60 cells and effectively blocks the downstream signaling pathway of G-CSF-R in a dose-dependent manner. Based on this, Bakherad *et al* (138,139) modified the G-CSF-R targeting nanobody (VHH1) to increase its affinity for G-CSF-R by redesigning the complementary determining region 3 domain of the VHH1 nanobody to mimic the interaction of G-CSF with its receptor. The newly engineered nanobodies exhibited dose-dependent binding to the G-CSF-R on the surface of NFS60 cells, as well as higher biological activity compared to the parental nanobodies. These newly developed nanobodies may be beneficial for tumor imaging and therapy and lay the foundation for the development of other engineered nanobodies. However, further studies are required to better characterize these nanobodies and evaluate their interaction with G-CSF-R both *in vitro* and *in vivo*.

Vascular endothelial growth factor receptor (VEGFR) specific nanobody. VEGFR2 is a critical tumor-associated receptor that is responsible for >85% of cancer-related deaths in solid tumors that require angiogenesis to promote their growth and metastasis. Targeting VEGFR2, which is overexpressed on tumor blood vessels, has emerged as a promising strategy for antiangiogenic therapy (140). Behdani *et al* (141) described the identification of the VEGFR2-specific nanobody, 3VGR19, isolated from dromedary camels immunized with cell lines expressing high levels of VEGFR2. Flow cytometric analysis

revealed that 3VGR19 specifically bound VEGFR2 on the cell surface of 293KDR and HUVECs, and effectively inhibited the formation of capillary-like structures (141). These data demonstrate the potential of nanobodies to block VEGFR2 signaling and provide a basis for the development of novel cancer therapies.

In addition to blocking VEGFR2 as receptors, nanobodies are also combined with other proteins to increase their destructive effects on tumor cells. Tian *et al* (142) prepared the fusion protein V21-DOS47, consisting of a VEGFR2-specific nanobody (V21) and urease, which converts endogenous urea to toxic ammonia. Thus, the V21-DOS47 immunocomplex not only blocks the VEGFR site, but also targets cytotoxic activity to the tumor. To improve the stability of the immunoconjugate, the research group generated two versions of the V21 antibody (V21H1 and V21H4) by adding several amino acid residues at the C-terminus to modulate the activity of the V21 antibody. In addition, different chemical cross-linkers were used to conjugate to urease. The heterofunctional cross-linker succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester was used to couple V21H1 to urease, whereas a homofunctional cross-linker 1,8-bis (male imide) diethylene glycol was used to conjugate V21H4. Bioactivity analysis revealed that V21H4-DOS47 was expressed at high levels, was easy to purify and produce, and retained higher binding activity than V21H1-DOS47 (142). This strategy for amino acid regulation of nanobodies and modification of complex cross-linker agents has highlighted new avenues for the development of cytokine nanobody-related drugs.

Taheri *et al* (143) developed second-generation CAR T-cells based on a nanobody (VHH) that targets VEGFR2-expressing tumor cells. This CAR T-cell was developed by linking the anti-VEGFR2 VHH to the signaling domains of CD28 and CD3 zeta. Following co-culture with VEGFR2-expressing cells, CAR T-cells exhibited a 41 and 48% expression of the activation markers, CD69 and CD25, and produced 470 and 360 pg/ml of IL-2 and IFN-γ, respectively. The CAR T-cells exhibited 30% cytotoxic activity at an effector-to-target ratio of 9:1. Thus, anti-VEGFR2 CARs are implicated as candidates for T-cell immunotherapy for solid tumors.

EGFR-specific nanobody for immunotherapy. EGFR, which plays a crucial role in cell growth, reproduction and differentiation, has long been recognized as a promising target for cancer diagnostic and therapeutic applications. EGFR tyrosine kinase-mediated signaling is closely related to tumor development, and the inhibition of this receptor activity can effectively suppress tumors (144). Anti-EGFR monoclonal antibodies block EGFR downstream signaling pathways, thereby inhibiting uncontrolled cell proliferation (145). Monoclonal antibodies and other large antibodies spread slowly in tumors, limiting their efficacy. To develop low molecular weight probes against EGFR and other tumor cell receptors, Gottlin *et al* (146) constructed a VHH phage library by immunizing llamas with the extracellular domain of EGFR, as well as the oncogenic mutant receptor EGFRvIII and tumor cell line extracts. The EGFR-specific nanobodies identified by screening were found to be cross-specific with existing anti-EGFR monoclonal antibodies, with a high affinity in the nM range (146).

Schmitz *et al* (147) described the X-ray crystal structures of three inhibitory nanobodies (7D12, EgA1 and 9G8) binding to the extracellular domain of EGFR. All three nanobodies inhibited EGFR activation, although via different mechanisms. 7D12 sterically blocked ligand binding to EGFR in a cetuximab-like manner, while EgA1 and 9G8 were shown to bind to epitopes near the EGFR domain II/III junction, thereby preventing the conformational changes of the receptor required for high-affinity ligand binding and dimerization. This epitope contacts the convex VHH paratope, but not with the flat paratope of the monoclonal antibody (147). Understanding the binding and inhibition modes of these VHH domains will aid their development in tumor imaging and/or cancer therapy.

To improve the performance for rapid preclinical optical imaging and visualization, Oliveira *et al* (148) developed a nanobody-based anti-EGFR probe consisting of the anti-EGFR nanobody 7D12 and cetuximab attached to the near-infrared fluorophore IRDye800CW. 7D12-IR allowed the visualization of tumors up to 30 min post-injection, whereas no signal above background was observed at the tumor site when cetuximab-IR was used, indicating that this nanobody-based anti-EGFR probe has excellent performance for rapid preclinical optical imaging and is expected to be applied as an auxiliary diagnostic tool in humans in the future (148).

Subsequently, van Driel *et al* (149) used a similar 7D12-800CW probe for head and neck cancer surveillance. Lymph node metastases in the neck were clearly detected following the injection of 75 μg 7D12-800CW (149). Applying this approach in clinical practice may thus improve the survival rate of radical surgical resection.

To block EGF-mediated EGFR activation van Lith *et al* (150) prepared a conjugate of a cell-penetrating peptide and 7D12 that specifically binds and internalizes EGFR cells. The VHH-CPP conjugate exhibited a combination of activities implemented through the application of a very powerful new design principle that blocks receptor activation by removing the receptor from the cell surface. To further apply 7D12 to antitumor therapy, van Lith *et al* (151) functionalized VHH 7D12 with the photosensitizer PS (IRDye700DX) to develop photoimmunotherapy (PIT). The use of low molecular weight camelid single-domain antibodies (VHHs, nanobodies) in PIT is preferred over full-size antibodies due to improved tumor penetration (151). Both VHH([PS]) and VHH([PS])-CPP conjugates specifically induced the death of cancer cells expressing high EGFR under near-infrared light, including tumor cell lines highly expressing EGFR and cells highly expressing EGFR extracted from the ascites of patients with high-grade plasmacytotic ovarian cancer. However, the VHH([PS]) were significantly more effective compared to internalized VHH([PS])-CPP, suggesting that cell surface binding is necessary for optimal therapeutic activity.

EGFR-specific nanobody for immunoimaging. Given that EGFR is overexpressed in several types of human epithelial cancers, the non-invasive molecular imaging of this receptor using specific monoclonal antibodies against EGFR as probes has important implications for the diagnosis of certain tumors. However, the long half-life of monoclonal antibodies in blood has prompted the development of smaller probes. Nanobodies are the smallest intact antigen-binding fragments (15 kDa), and the variable domains of heavy chain antibodies (VHHs) are valuable reagents for tumor diagnosis and therapy when combined with diagnostic probes and therapeutic compounds.

For example, EGFR nanobodies labeled with (99m) Tc (152-154) or Ga (155) were used in positron emission tomography (PET) to image EGFR expression in mouse tumors. In addition, EGFR nanobodies labeled with photosensitizers such as IRDye 700DX (152,156) and IRDye 800CW (149,150) were used for near-infrared (NIR) fluorescence imaging studies in tumor-bearing mice.

Krüwel *et al* (152) used the (99m) Tc-labeled EGFR Nanobody D10 [(99m) Tc-D10] for the visual detection of small tumor lesions <100 mm. EGFR-overexpressing small human tumors were visualized specifically and with high contrast *in vivo* by preclinical multi-pinhole SPECT following the intravenous injection of (99m) Tc-D10 (153).

Piramoon *et al* (153) synthesized and labeled the anti-EGFR nanobody, OA-cb6, with (99m) Tc(CO)3(+) and evaluated its specific targeting properties in the A431 human epidermal carcinoma cells. In their study, stable radiolabeled nanobodies were obtained with a high yield and radiochemical purity. Biodistribution analysis in nude mice revealed a good tumor-to-muscle ratio and the location of the tumor was visible 4 h after the injection of the radiolabeled nanobodies. Thus, the OA-cb6-(99m) Tc radiolabeled nanobody is a promising radiolabeled biomolecule for tumor imaging in cancers with a high EGFR expression (153).

Li *et al* (154) used a tricarbonyl kit to Tc radiolabel EG2, a nanobody targeting the EGFR, and single-photon emission computer imaging revealed that A431 tumor images were clearly visible as early as 1 h after the injection of (99m) Tc-sdAb EG2. Biodistribution analyses demonstrated that (99m) Tc-sdAb EG2 uptake by A431 tumors was blocked by ~51% 3 h following an overdose injection. This indicated that sdAb EG2 effectively targets EGFR and highlights its potential as a molecular probe for EGFR detection (154). To prepare more sensitive tracers, Li's group (157) fused the nanobody targeting the EGFR with a right-handed helical coil (RHCC) and human cartilage oligomeric matrix protein (COMP) to form multivalent antibodies EG2-COMP. SPECT imaging showed that A431 expressing high EGFR levels was clearly visible 6 h after injection of (99m) Tc-EG2-COMP. Therefore, EG2-COMP shows promise for clinical application in the real-time monitoring of tumor cells.

For PET imaging, Vosjan *et al* (155) used a novel bifunctional iron chelator for 68Ga labeling of the anti-EGFR nanobody 7D12. The 68 Ga nanobody was successfully prepared and exhibited high tumor uptake in nude mice with HoA431 xenograft tumors that were clearly visible in PET imaging studies. Thus, the 68 Ga nanobody conjugate represents a tool with clinical application for tumor detection and imaging.

Using chelating agents and photosensitizers, Renard *et al* (156) achieved dual labeling of EGFR nanobodies for nuclear imaging and the targeted photodynamic therapy of EGFR-Positive tumors. The site-specific binding of the chelator DTPA and photosensitizer IRDye700DX to the anti-EGFR nanobody 7D12 were optimized for nuclear imaging and photodynamic therapy applications. Using a dichlorotetrazine-conjugated platform, 7D12 was site-specifically equipped with the bimodal probe DTPA-tetrazine-IRDye700DX. The [111In] In-DTPA-IRDye700DX-7D12 was shown to bind specifically to A431 cells and efficiently induce their destruction under light exposure both *in vitro* and *in vivo*. In addition, SPECT and fluorescence imaging confirmed that dTPA-IRDye700DX-7D12 binds to A431 xenografts cells *in vivo*. The dichlorotetrazolium platform provides a feasible approach for site-specific dual labeling of nanobody 7D12, preserving its affinity and therapeutic efficacy. Moreover, the flexibility of this method facilitates modification of the properties of the probe for other combinations of diagnostic and therapeutic compounds (156).

5. Conclusions and future perspectives

Cytokines function as molecular messengers that allow communication between immune system cells to mediate regulatory and effector functions in a number of diseases. Therefore, cytokines and their receptors have important value in immunotherapy, and cytokines were the first tumor immunotherapy drug approved by the US FDA.

However, cytokine therapy is prone to various side-effects and has a narrow therapeutic window, which represents a challenge to the use of natural cytokines as clinical candidates. A growing number of research teams are developing new cytokine drugs, such as 'immunocytokines', cytokine agonists/inhibitors and engager cytokines. Compared with traditional antibodies, nanobodies are structurally different and exhibit different properties, and have broad application prospects in basic medical research and disease treatment. The introduction of nanobodies into the preparation of cytokine drugs has shown promise as an antitumor therapy.

Among the nanobody-related 'immunocytokine' drugs, nanobodies target tumor biomarkers by targeting the CEA, EGFR, the fibronectin EIIIB structural domain of tumors, while cytokines such as IL-15, TNF-α and IL-2 are introduced locally into the tumors simultaneously to achieve antitumor effects. Nanobody-engager cytokines, mainly in the form of BiKEs, are designed to activate the killing capacity of NK cells or deliver IL-15 in tandem to activate NK cells for efficient antitumor activity. Compared with cytokine agonists, greater focus has been placed on nanobodies-based inhibitors of cytokines, such as IL-23, IL-17, IL-13 and TNF-α, to neutralize and block inhibitory signals for the purpose of treating disease. Nanobodies to EGFR are used to block EGFR downstream signaling and also to generate probes as non-invasive molecular imaging for diagnostic imaging of tumors.

The main targets of the current nanobody probes include tumor membrane antigens and targets in the TME, including, but not limited to EGFR, HER, VEGF, VEGFR, lymphocyte activation gene-3 and programmed cell death ligand 1. Due to their small molecular weight and short circulation time, nanobody probes are rapidly metabolized by the kidneys; thus, the significant uptake of high affinity nanobody probes at the tumor site can be achieved within a short period of time. Nanobodies are therefore novel targeting molecules for the construction of molecular imaging probes, which, when labeled with radionuclides, have the advantage of obtaining high quality images in a short period of time, providing a comprehensive assessment of the disease and guiding individualized and precise treatment. However, persistent high uptake in the kidneys and short circulation times *in vivo* have

hindered the use of nanobody probes in oncology. To promote the clinical application of such probes for target-specific diagnosis and therapy, the pharmacokinetics of nanobody drugs and probes require further optimization to achieve improved target affinity, reduced renal uptake and an extended circulation time *in vivo*, thereby mitigating drug-induced adverse effects. and safeguarding patient safety.

Nanobody-related cytokine drugs will soon become a research focus in accurate tumor diagnosis and targeting therapy. The following issues are worth paying attention to: First, the selection of cytokines is the key to immunocytokine therapy. The same cytokine may have differential effects on different tumor types, or on different patients with the same tumor. Therefore, cytokines for antitumor treatment should be selected according to the characteristics of tumor type, tumor immune microenvironment and cytokine function. Second, the selection of nanobody targets is also a key point in cytokine-related immunotherapy. In addition to tumor antigens, anti-angiogenic factors, immune checkpoints, and TME factors are good choices for the preparation of 'immunocytokine drugs'. Third, the optimization and modification of cytokine drug structure and expression system are required to improve the quality of nanobody-related cytokine drugs. Fourth, a wider cytokine spectrum is still required. An increasing number of previously unidentified cytokines or new functions of cytokines are being discovered, which will lead to the development and application of unexpected cytokine drugs. Finally, for the successful application of emerging nanobody-based cytokine drugs in immunodiagnosis and therapy, a multidisciplinary team of researchers, clinicians and regulators needs to be established to construct an overall framework for clinical translation.

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Authors' contributions

XZ, JW, YT, CC, ST, SZ and QQ wrote the original draft of the manuscript. HH and SD designed and revised the manuscript. All authors contributed to the article and have read and approved the final version. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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