Nanobodies targeting immune checkpoint molecules for tumor immunotherapy and immunoimaging (Review)

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Abstract. The immune checkpoint blockade is an effective strategy to enhance the anti-tumor T cell effector activity, thus becoming one of the most promising immunotherapeutic strategies in the history of cancer treatment. Several immune checkpoint inhibitor have been approved by the FDA, such as anti-CTLA-4, anti-PD-1, anti-PD-L1 monoclonal antibodies. Most tumor patients benefitted from these antibodies, but some of the patients did not respond to them. To increase the effectiveness of immunotherapy, including immune checkpoint blockade therapies, miniaturization of antibodies has been introduced. A single-domain antibody, also known as nanobody, is an attractive reagent for immunotherapy and immunoimaging thanks to its unique structural characteristic consisting of a variable region of a single heavy chain antibody. This structure confers to the nanobody a light molecular weight, making it smaller than conventional antibodies, although remaining able to bind to a specific antigen. Therefore, this review summarizes the production of nanobodies targeting immune checkpoint molecules and the application of nanobodies targeting immune checkpoint molecules in immunotherapy and immunoimaging.

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1. Introduction

Tumor immunotherapy has shown great application prospects by stimulating the body autoimmune system to balance the immunosuppressive microenvironemnt, thereby improving the antitumor effect (1). Immunotherapy includes adoptive cell immunotherapy, immune checkpoint inhibitors, cancer vaccines, costimulatory receptor agonists, monoclonal antibodies (mAbs), and oncolytic virus therapy (2). In recent years, research on immune checkpoint blockade has become a hotspot study in tumor immunotherapy (3). Immune checkpoint molecules are receptors on the surface of immune cells that, after binding with their ligand, transduce inhibitory signals or stimulatory signals (4). Drugs that target immune checkpoint molecules, which can transduce the inhibitory signals, are called checkpoint inhibitors. Among these receptors, the most studied ones are 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), and lymphocyte-activation

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gene 3 (LAG-3) (4,5). Blocking tumor immune escape and tolerance mechanisms through immune checkpoints is an effective way to enhance antitumor effect. An immune checkpoint inhibitor, especially the mAb-based one, as an immunoregulatory factor, can specifically bind to T cells or tumor cells, thus enhancing the antitumor ability of T cells (6-8).

The traditional mAb-based immune checkpoint blockade is the main method of tumor treatment and detection. Results of preclinical studies and follow-up of clinical trials led to the approval of various checkpoint inhibitors by the Food and Drug Administration (FDA) for the treatment of renal cell carcinoma, melanoma, lymphoma, classic Hodgkin lymphoma, pancreatic ductal adenocarcinoma, cervical cancer, non-small cell lung cancer, squamous cell carcinoma of the head and neck, and breast cancer. Some immune checkpoint inhibitors approved, include mAbs targeting PD-1 such as nivolumab (Opdivo®), pembrolizumab (Keytruda®), cemiplimab (Sanofi, Regeneron); mAbs targeting PD-L1 such as atezolizumab (Tecentriq®), avelumab (Bavencio®) and durvalumab (Imfinzi®); and mAb targeting CTLA-4 is represented by ipilimumab (4,9-14). Most tumor patients have benefited from these antibodies, however some have not responded to them (15). In addition, the characteristics of mAbs such as poor stability, high production costs, poor tissue penetration, and immune-related adverse events has limited their utility (16-18).

To improve the therapeutic effect of antibodies for immune checkpoint blockade, immune checkpoint expression should be analyzed in patients before and during treatment. Due to the heterogeneous and highly dynamic expression of the immune checkpoint molecules in primary or metastatic tumors, traditional immunohistochemical methods are limited because they cannot detect the dynamic information of immune checkpoint molecules in the tumor environment (19-30). Therefore, a real-time, dynamic, and accurate detection method with high sensitivity resolution is urgently required.

Methods involving imaging of labeled antibody molecules have been reported, but the poor tissue penetration of these antibodies, the long circulation time, and the high-contrast imaging are obstacles that prevent them from becoming ideal imaging agents (16,19,31). Thus, the development of tracers with faster kinetics is of utmost importance.

To increase the effectiveness of immunotherapy, including immune checkpoint blockade therapy, miniaturization of antibodies, has been introduced. Nanobodies have a small molecular weight conferring them a strong tissue penetration where they bind their antigens quickly and specifically, while unbound nanobodies can be quickly cleared through renal excretion. Therefore, compared to mAbs, nanobodies produce higher target-to-background signals soon after their administration (18).

Similar to mAb-based immune checkpoint inhibitors, nanobodies that target immune checkpoint molecules have been developed as effective tools for studying tumor immunotherapy and immunoimaging (32). In this review, some of the recent advances in the development of nanobodies and nanobody-based immune checkpoint inhibitors for immunotherapy and immunoimaging (Table I) were examined, as well as the challenges faced to achieve successful use.

2. Biophysical properties of nanobodies

In 1993, a special antibody was revealed in the blood of camels (camel, alpaca, llama) and sharks (33). This antibody is different from the traditional one with a tetrapeptide chain structure because it lacks a light chain, thus, it is called a heavy chain antibody. Due to the absence of the CH1 domain and the light chain, the antigen-binding region of a heavy chain antibody consists only of the heavy chain variable region of the heavy chain antibody (34). For this reason, it is called single domain antibody (sdAb), or VHH antibody or nanobody (Fig. 1), and can be obtained through cloning and expression. The special structure and unique biological properties of nanobodies have attracted the attention of numerous scholars, and several research institutions are screening new nanobodies as new drugs for cancer treatment (35-37) and diagnosis (38).

Small molecular weight and low immunogenicity. The crystal structure of nanobodies is similar to a rugby ball with a diameter of approximately 2.5 nm and a length of approximately 4.2 nm. The relative molecular mass is approximately 15 kDa, which is one-tenth of the size of conventional antibodies. In fact, it is the smallest antibody with fully functional properties that currently exists (39-41). Some methods using nanobodies have better results than conventional antibodies, such as imaging tracer agents (42-46), microscopic imaging (47), enzyme inhibitors (48,49), and electrochemical biosensors (50-57). Due to their small size, the binding region between the nanobody and the epitope forms a high-density binding, providing a significant advantage in increasing the sensitivity of the binding signal.

Nanobody backbone regions have more than 80% sequence homology with human VH regions, and their three-dimensional structures can overlap. The camel VHH germline gene sequence is highly homologous to the human VH3 family sequence. Thus, it has the advantages of weak immunogenicity and good biocompatibility, and humanizing VHH is relatively simple (58-61).

High stability. Nanobodies are markedly smaller than traditional antibodies and they are characterized by the presence of a disulfide bond, rendering their structure more stable and making them more resistant to heat and an acid environment (62,63). Under extreme environmental conditions, such as high temperatures or extreme acid and alkaline environments, the structure of the traditional polyclonal antibody changes, exposing its hydrophobic surface; the exposed hydrophobic molecules aggregate with each other to form large molecules that precipitate, losing their original function (64). Unlike the traditional antibody, the nanobody forms different conformational patterns to protect the stability of amino acids. After chemical and thermal denaturation, the nanobody refolds and forms a disulfide bond between the complementarity determination region-1 (CDR1) and CDR3 to improve the stability of its structure and ensure the stability of its functional activity (65-68). The stability of nanobodies establishes them as a potential drug in the treatment of gastrointestinal diseases, and their excellent characteristics render them an effective probe molecule for biosensor applications (38).

Improved solubility. There are some important differences between the VHH and the VH of the traditional antibodies.
The VH structure of the conventional antibodies easily form inclusion bodies when expressed alone, or when the exposed hydrophobic regions adhere to each other, making the antibody markedly poor in water solubility. The four hydrophilic amino acids in FR2 of the nanobodies replace the hydrophobic amino acids of conventional antibody FR2, such as the 42nd amino acid in the VH of traditional antibodies is often Val, while in VHH it is often Phe or Tyr. The 49th amino acid in the VH of the traditional antibodies is often Gly, while in VHH it is often Glu. The 50th amino acid in the VH of the traditional antibodies is often Leu, while in VHH it is often Arg or Cys. The 52nd amino acid in the VH of the traditional antibodies is often Trp, while in VHH it is often Gly. These 4 amino acids in VHH are hydrophilic, thus rendering the surface of the nanobody more hydrophilic and increasing its water solubility (40,65,69-72).

High affinity and cavity binding. Similar to traditional VH, VHH includes 4 FRs and 3 CDVs. CDV1 and CDV3 of the VHH are longer than the ones of the VH, which makes up for the lack of antigen-binding ability caused by the deletion of the light chains, at least to a certain extent (40). The cysteine in the VHH CDV3 also forms disulfide bonds with the cysteine in CDV1 or FR2. These increased sequences and loop structures expand the area of antibody-antigen binding and the diversity of antibodies, and concurrently lead to a markedly stable structure that tolerates high temperatures and harsh extreme environments (67,70-73). In addition, the nanobody does not have a traditional Fc segment, thereby avoiding complement reactions caused by this segment (74).

Conventional Fab fragments and typical ScFv have concave or planar antigen-binding sites, thus, only surface antigens can be identified. The nanobody has CDV3 loops that are generally longer than conventional VH, allowing it to bind to unconventional epitopes, such as protein clefts and some hidden epitopes, which are not recognized by traditional antibodies (46,75). Therefore, the nanobody is more suitable than the ScFv antibody binding site to bind to the recessed portion of the antigen surface, such as the catalytic reaction site of the enzyme, thereby blocking its catalytic activity (70,71,76,77).

Strong tissue penetrability. Nanobodies are small and highly soluble, thus, they have strong and fast tissue penetration capabilities, and can enter dense tissues such as solid tumors to play their role (72,78). In addition, nanobodies can penetrate the blood-brain barrier (71,79,80) and become potential new treatments for brain diseases such as dementia. Studies have revealed that camel-derived nanobodies immunize cerebrovascular endothelial cells and they can be released on the outer side of vascular endothelial cells through transcytosis. Their small size allows better penetration through the tissue- and immune-like synaptic cell interface. Furthermore, nanobodies

| Target | Nanobody name | Target species | Application | Referred studies |
|--------|---------------|----------------|-------------|-----------------|
| CTLA-4 | Nb16          | Human          | Immunotherapy | (112)           |
| CTLA-4 | Nb36          | Human          | Immunomaging  | (135,136)       |
| PD-L1  | B3            | Murine         | Immunotherapy | (116)           |
| PD-L1  | KN035         | Human          | Immunotherapy | (117)           |
| PD-L1  | Nb97          | Human          | -            | (118)           |
| PD-L1  | -             | Human          | -            | (119)           |
| PD-L1  | Nb109         | Human          | Immunomaging  | (132)           |
| PD-L1  | C3/E2         | Murine         | Immunomaging  | (133)           |
| PD-L1  | sdAb K2       | Human          | Immunotherapy/Immunomaging | (115,134) |
| TIM3   | -             | Human          | -            | (124)           |
| TIM3   | -             | Human          | -            | (125)           |
| LAG3   | 3131/3206     | Murine         | Immunomaging  | (131)           |

Figure 1. (A) Traditional antibody and single-chain antibody fragment (ScFv). (B) Camel heavy-chain antibody (HcAb) and nanobody (Nanobody).
are easily filtered by the glomerulus, and the blood clearing rate is fast so that the excess of free nanobodies is quickly removed without adversely affecting the body due to long-term retention. Compared with the shortcoming of monoclonal antibodies, which have poor penetrating power and are not easily removed, such characteristics are more useful in the diagnosis of diseases (31,39,46). Currently, several nanobody-based imaging technologies, such as radionuclides, optics, and ultrasound, have been used to visualize target protein expression levels in multiple types of disease models (31,39).

High expression yields. Nanobodies are markedly simpler in chemical composition and shape than traditional antibodies, which render nanobodies easily cloned, chemically or genetically modifiable, and recombiantly produced in various cells (18,40,71,72). It is easier to obtain them from prokaryotic cells and a soluble expression (81). Recombinant nanobodies are usually highly expressed in E. coli, reaching 10 mg/l-200 mg/l (82-84). A biopharmaceutical company (Ablynx) reported that they greatly increased the production of nanobodies produced by the yeast reactor to one gram per liter (85). Nanobodies are easily genetically manipulated to form monovalent, bivalent, bispecific, and multivalent antibodies, and they can also form fusion proteins for targeted therapy (31).

Easy modification and functional modification. Nanobodies are VHH genes cloned from the camel or alpaca blood by genetic engineering and then expressed by prokaryotic or eukaryotic cells. Therefore, nanobodies are easily modified or genetically modified (86). Wang et al (87) added heptamers with these added peptides.

3. Construction of library and panning of nanobodies

The merits of molecular properties such as affinity and specificity of nanobodies depend on two factors: The capacity and diversity of the library. The preparation and screening of nanobodies are commonly used in a phage display library (40), which contains three library types: Natural, immune, and synthetic (91,92). The natural library is formed by amplifying the variable region genes of heavy chain antibodies from camel peripheral blood mononuclear lymphocytes that have not yet been immunized, then these variable region genes are recombined into phagemid vectors, and transformed into host bacteria to form antibody libraries (93,94). The immune library is an antibody library obtained by immunizing an alpaca or camel with an antigen protein, using peripheral blood mononuclear cells of the camel to amplify the variable region gene of the heavy chain antibody, and then recombining this variable region gene into a phagemid vector (94).

The capacity of the immune library is lower than that of natural libraries, but numerous functional antibodies in the library recognize specific antigens for immunity, making screening of high-affinity antibodies a reality. For the construction of synthetic libraries (95), a certain VHH framework (such as cAbBC110) is generally selected as the backbone structure. Trinucleotide cassettes are used as a unit of raw materials to generate CDRs sequences. Then, the DNA of each region of VHH is assembled by PCR to form a complete VHH gene. The gene is cloned into a phagemid vector and transformed into an E. coli TGI cell to form a library. The synthetic library, as an artificial library constructed by genetic engineering technology, has a great complementary role in the immune libraries and natural libraries and is an important source for screening high-affinity antibodies, with significant importance for the development of antibody drugs (91,95).

The phage display technology is used to screen binders for various targets from diverse and large libraries and is widely employed by various research teams. Since the difference between VHH and VH is mainly due to the absence of the CH1 region, the work of building an immune and natural library focuses on isolating antibodies lacking CH1 from IgG, which can be achieved by one-step (96) or two-step nested PCR amplification (97). The key of PCR is to design PCR primers using the conserved nucleotide sequence of the antibody backbone region. With regard to the one-step PCR, the sequence can be amplified from the FR1 to the hinge region, and restriction sites are introduced on the primers. For two-step nested PCR, two bands are obtained through the first step of PCR amplification, and the sizes are 700 and 900 bp, respectively. Among them, the 700-bp band encodes the VHH-H-CH2 fragment of the heavy chain antibody. This fragment is recovered using a Gel Extraction kit and used as a template for the second PCR. The VHH fragment is amplified by the second PCR (98).

The amplified antibody sequence is ligated to the phagemid vector, such as pMECS (95,97), PHEN (99), pAX50 (100), and pComb3 (96,101). The remaining steps are the same as the ordinary process of constructing an antibody library (98). The specific protocol is presented in Fig. 2. In general, the nanobody library capacity can easily reach 1x10^8 or 1x10^9, and diversity is greater than 95%. Furthermore, because VHH has only one variable region, specific antibodies can be screened at 1x10^6. However, in ordinary antibody Fab or ScFv libraries, the library needs to be large enough to more easily screen for antibodies (93,96,97,102,103).

In addition to phage display, some other techniques are applied to screen nanobodies, such as mRNA (104), ribosome (105-107), yeast (108,109) and bacterial surface displays (110,111). Salema et al (112) reported an E. coli display system, which combines the advantages of both a phage display and yeast display system. Flow cytometric analysis of nanobodies on the surface of E. coli during the screening process can monitor the selection process in real-time and identify antigen-binding characteristics.

Nanobodies are generally screened from libraries using specific immobilized antigens. A specific antigen is coated in a microtiter plate to specifically bind VHH-displayed phages in the library, then unbound phages are removed, and the bound phages are eluted, and are used for further amplification. After several rounds of panning and enrichment, VHH with high
affinity is obtained, and a positive VHH is identified by indirect ELISA (98). Or, as an alternative, it can also be panned in the liquid phase (96). After blocking, binding, elution and amplification, specific binding between streptavidin-coated magnetic beads and biotin-labeled antigen can also be used to obtain antigen-specific antibodies. Cells expressing specific antigens are similar to protein molecules and can also be used for nanobody panning (113). The panning step is the same as the panning step for protein molecules. To improve the affinity of the antibodies obtained by panning, during the panning process, the concentration of immobilized antigen or the number of cells in each panning can be continuously reduced, and the number of washings can be gradually increased.

4. Nanobodies targeting immune checkpoints for immunotherapy

The large size of mAbs limits their penetration and distribution in tumor tissues in certain clinical situations. Compared to mAbs, the unique structure and biological activity of the small nanobody molecule make it an effective tool for successful immunotherapy (60).

**CTLA-4.** Ipilimumab (10,114-117), a fully human IgG1κ anti-CTLA-4 mAb, was the first immune checkpoint inhibitor against CTLA4 approved for non-small cell lung carcinoma, renal cell carcinoma, prostate cancer, and metastatic melanoma by the FDA in 2011. Tremelimumab is another fully human IgG2 anti-CTLA-4 mAb, which is used in clinical trials (10).

Considering that mAbs have some drawbacks, such as a markedly poor tissue penetration, production cost and unstable behavior, Tang et al (118) immunized the camel using the recombinant human CTLA4 protein, constructed a VHH library and screened nanobodies using phage display technology. Four CTLA-4-specific nanobodies were obtained from the repertoire of an immunized dromedary camel using phage display technology. These nanobodies recognized unique epitopes of CTLA-4 and exhibited high binding ability.
The Nb16 treatment for melanoma-bearing mice could reduce tumor growth and prolong their survival time. However, the study revealed that both the Nb16 and mAb groups have antitumor effects, with no difference between these two groups. Thus, the antitumor mechanism of Nb16 should be analyzed to facilitate the clinical application of antibodies.

**PD1/PD-L1.** The world’s first PD-1 inhibitor Opdivo® was approved for use in 2014. By the end of 2018, the FDA had approved 6 PD-1/PD-L1 mAbs such as nivolumab (Opdivo®; Bristol-Myers Squibb), pembrolizumab (Keytruda®; Merck), and cemiplimab (Libtayo®; Sanofi and Regeneron), and three PD-L1 inhibitors, such as atezolizumab (Tecentriq®; Roche), avelumab (Bavencio®; Pfizer and Merck) and durvalumab (Imfinzi®; AstraZeneca) (9,10,119). To increase the effectiveness of immunotherapy, including the immune checkpoint blockade therapy, miniaturization of antibodies has been introduced. Some nanobody-based PD1/PD-L1 inhibitors were developed (19).

Broos et al developed a PD-L1 specific sdAb called K2, which blocks the interaction between PD1 and PD-L1 (120). This property enhances the ability of dendritic cells to stimulate T-cell activation and cytokine production. sdAb K2 combined with dendritic cell vaccine treatment may be more beneficial than PD-L1 mAb against cancer diseases. The reason that PD-L1 mAbs fail to enhance T-cell activation may be that they have low efficacy in binding to PD-L1 on DCs, while sdAb K2 has a high ability to bind PD-L1 on both immune and non-immune cells.

Nanobodies can also be used as a carrier for cytokines to remodel the tumor microenvironment. In one context, Fang et al (121) developed a functional chemokine-VHH fusion protein using a PD-L1 specific nanobody B3 and a model chemokine CCL21 to deliver CCL21 to a PD-L1-positive environment and recruit the relevant leukocyte for improving immunotherapy.

KN035 (122), an anti-PD-L1 nanobody, was screened using a camel immunological library. It is the first nanobody research project used in the field of immunotherapy in the world. Its binding surface of PD-L1 is smaller than other PD-L1 antibodies, and its affinity is similar to other antibodies (3 nm). KN035 can bind the PD-L1 molecule with a high affinity and effectively block the action between PD-L1 and PD1. Similar to other PD-L1 antibodies, it can effectively compete for the five hotspot sites where PD-L1 binds to PD-1. In addition, KN035 can effectively activate PBMCs in vitro and induce interferon secretion. As an antitumor drug, its preliminary results regarding its efficacy are favorable.

Xian et al (123) screened anti-PD1 nanobody Nb97 by phage display, and then Nb97 was used to develop the Nb97-Nb97-Human serum albumin fusion protein (MY2935), which exhibited a more efficient blocking effect to that of a humanized Nb97-Fc (MY2626), and Human serum albumin fusion extended the serum half-life of nanobody Nb97. Li et al (124) obtained three anti-PDL1 nanobodies from a high quality dromedary camel immune library by phage display, and analyzed the binding activity and affinity of the three nanobodies, but did not research the PD1/PDL1 pathway blocking effect.

**TIM3 and LAG3.** Some cancer patients do not respond to PD1/PDL1 and CTLA4 inhibitors. To obtain a greater number of patients benefiting from immune checkpoint blockade immunotherapy, some other immune checkpoint molecules have been developed such as TIM3 (125,126) and LAG3 (127,128).

Homayouni et al (129) immunized a six-month Camelus dromedarius with a human TIM3 protein and developed a novel anti-human TIM-3 (CD366) nanobody from an immune library. This nanobody exhibited a high binding capacity to TIM-3, and a high antiproliferative effect on the acute myeloid leukemia cell line HL-60 by blocking the galectin/TIM-3 signal, with an inhibitory effect comparable to or better than that of anti-TIM3 antibodies. However, the researchers did not detect the difference in tissue penetration between nanobodies and mAbs. Ma et al (130) immunized a camel, constructed the phage display library, and then screened ten anti-TIM3 nanobodies with high specificity and high affinity using flow cytometry. However, the researchers did not detect the function of the anti-TIM3 nanobody, thus, it is not known whether blocking the TIM3 inhibitory signal by anti-TIM3 nanobodies can activate the antitumor function of T cells. Nevertheless, the aforementioned studies provide the basis for the development of specific nanobody drugs blocking TIM-3.

There are seven anti-LAG3 mAbs and two bispecific antibodies targeting LAG3 (131-133), which are at different stages of clinical development. However, studies on the application of anti-LAG3 nanobodies in antitumor research have yet to be published.

5. Nanobodies targeting immune checkpoints for immunooiaging

Molecular imaging can intuitively detect changes at the molecular level during and after the treatment of diseases, therefore it is one of the important methods for evaluating the effect of tumor therapies. With the continuous development of targeted therapies, it is becoming increasingly important to visualize the expression of tumor antigens and the level of immune infiltrations to predict the course of the treatment. Molecular imaging based on mAbs has been extensively studied, however the poor tissue penetration and long half-life severely hinder the development of successful molecular imaging (60). Nanobody molecular probes bind to the target with high specificity, and the unbound part is quickly excreted by the kidney. In addition, nanobody molecular probes have a deep tumor penetration and high tumor to background ratio soon after their administration, which clearly reveals the dynamic changes of target molecules (60,134,135). Therefore, nanobodies have recently become a powerful tool for in vivo and in vitro imaging diagnostics.

**Imaging of LAG-3.** In 2019, Lecocq et al reported an anti-LAG3 nanobody used for noninvasive imaging (136). They immunized alpaca with mouse LAG3 protein, bio-panned the phage-displayed library, and obtained nine nanobodies 3132, 3134, 3141, 3204, 3206, 3208, 3209, 3210, and 3366, which exhibited high specificity and high affinity validated by ELISA, flow cytometry, and surface plasmon resonance methods. These nanobodies were labeled with Technetium-99m ($^{99m}$Tc) at the His tail, and then, these nanobodies were injected into naive C57BL/6 mice intravenously. The results revealed...
the nanobody 3132 exhibited specific uptake by LAG3-low expression immune peripheral organs, such as the spleen and lymph nodes. To detect whether the nine 99mTc-labeled nanobodies bind to the tumor overexpressing LAG-3, SPECT/CT imaging was used to detect the biodistribution of 99mTc-labeled nanobodies in mice harboring a subcutaneous tumor modified to overexpress mouse LAG-3. The result revealed that it was possible to visualize the nanobody uptake using SPECT/CT imaging with high contrast levels immediately, even 1 h after injection, and this result was confirmed by flow cytometry and immunohistochemistry. The results demonstrated that the nanobodies 3132 and 3206 are both effective diagnostic tools for noninvasively evaluating LAG-3 expression within the tumor environment before and during immunotherapy.

Imaging of PDL1/PD-L1. Lv et al developed a nanobody tracer using the PD-L1 targeted nanobody (Nb109) and the radionuclide 68Ga through the chelator 1,4,7-triazacyclononane-1,4,7-triaetic acid (NOTA), and 68Ga-NOTA-Nb109 exhibited a high affinity for PD-L1 with a KD of 2.9x10^-8 M (137). The competitive binding assay demonstrated a different binding epitope between Nb109 and PD-L1 or Pd1 mAb, suggesting no impact on the tumor uptake of 68Ga-NOTA-Nb109 before and after the treatment with KN035. The 68Ga-NOTA-Nb109 tracer specifically accumulated in mice model harboring the A375-human PD-L1 tumor, with a maximum uptake of 5.0±0.35% ID/g at 1 h as determined through the PET imaging, biodistribution, immunohistochemical staining, and autoradiography assay, indicating that 68Ga-NOTA-Nb109 is a promising nanobody tracer for noninvasive PET imaging of PD-L1 in the tumor microenvironment and promising in evaluating the effectiveness of immune checkpoint blockade immunotherapy in real-time.

In 2017, Broos et al developed an anti-PD-L1 nanobody for noninvasive imaging of the murine PDL1 (138). They immunized 10 million PD-L1 high-expressing mouse macrophage RAW264.7 cells, and 37 mouse PD-L1 nanobodies were identified by biopanning of the Nb-phage display library. Among those Technetium-99m (99mTc)-labeled nanobodies, four nanobodies were selected to evaluate their biodistribution in PD-L1-knockout and wild-type mice using SPECT/CT. According to the results, Technetium-99m (99mTc)-labeled nanobodies C3 and E2 were used to image PD-L1 in a syngeneic mouse model because C3 and E2 have high specific antigen binding and beneficial biodistribution. Their work demonstrated that 99mTc-labeled nanobody tracers identified PD-L1-expressing tumors, but not in the PD-L1-knockout tumors, suggesting that these 99mTc-labeled nanobodies can be used in SPECT/CT imaging to assess the PD-L1 expression markedly soon even one hour after injection. Owing to the fast tumor-penetrating properties of the nanobodies, the results confirmed that a 99mTc-labeled nanobody tracer is a good method to image PD-L1 inhibitory signals during the treatment of the tumor environment.

In 2019, Broos et al (139) developed another tracer for noninvasive imaging of the human PDL1. They generated a new panel of sdAbs by alpaca immunizations, biopanning, and screenings on recombinant human PD-L1 protein, and they obtained a nanobody called sdAb K2, which binds to the same epitope on the PD-L1 molecule as the mAb avelumab. Thus, this nanobody is able to block the PDL1 inhibitory signal resulting in activated T cells and enhanced antitumor activity. The nanobody tracer also labeled with Technetium-99m (99mTc) to develop 99mTc-labeled sdAb K2 tracer, was intravenously injected into mice bearing melanoma and breast tumors to detect the PD-L1 by SPECT/CT imaging. This assay revealed a 99mTc-labeled sdAb K2 tracer with a high signal-to-noise ratio and a strong ability to image PD-L1. Collectively, sdAb K2 has a dual function as a diagnostic and therapeutic agent, offering broad prospects in a variety of tumor immunotherapy and immunoimaging techniques.

 Imaging of CTLA4. In 2018, Wan et al reported four CTLA-4-specific nanobodies from a camel immune library by phage display technology (140). One of these nanobodies called Nb36 was conjugated to the carbon quantum to synthesize a CTLA-4-specific nanobody-fluorescent carbon quantum dot complex (QDs-Nb36) (141) in 2019 by the same group. Because anti-CTLA-4 nanobodies specifically bind to CTLA-4+ T cells and QDs provide a sensitive fluorescent signal for accurate detection, the QDs-Nb36 complex revealed a high sensitivity detection of CTLA-4+ T cells by flow cytometry and immunofluorescence staining. Owing to the small size of the nanobody, the QDs-Nb36 complex was superior to mAbs in detecting the positive cells. Thus, nanobody-QDs is a promising method for the detection of some other biological targets, although this method cannot monitor the dynamic changes of the target molecule in real-time.

6. Conclusion

In view of the past few decades, monoclonal antibodies have shown considerable success in cancer treatment and diagnosis (44). However, the large and complex structure of the monoclonal antibodies limits their clinical utility (44). As revealed in this review, nanobodies have a small molecular weight conferring them a strong tissue penetration where they bind their antigens quickly and specifically, while unbound nanobodies can be quickly cleared through renal excretion, resulting in high target-to-background signals soon after their administration (134,135). Therefore, the introduction of nanobodies has demonstrated that they can overcome certain shortcomings of monoclonal antibody-based immunotherapy and immunoimaging. Currently, nanobodies targeting immune checkpoints are mainly concentrated in PDL1/PD1. In the future, it is necessary to develop more research on nanobodies targeting other immune checkpoints, including TIM3, LAG3, OX40 and VISTA. These immune checkpoints and their corresponding nanobodies should be characterized for clinical application. In addition, in order to prolong the half-life of nanobodies in vivo, nanobody dimers and multimeric nanobodies have been produced. The moderate relative molecular mass can better meet the requirements of deep tissue penetration, targeted aggregation, and blood clearance. With in-depth research on nanobodies, the application of nanobodies in tumor immunotherapy and immunodiagnosis is promising.

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SD and XB conceived and designed this review. GX and SZ researched and wrote sections 1 and 2 of the present review. SY researched and wrote section 3 of the present review. YT and HT researched and wrote section 4 of the present review. KW, HL and KL researched and wrote sections 5 and 6 of the present review. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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