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Llama-Derived Single-Chain Antibody for Targeting CD20 in B-Cell Lymphoma

*Lu Y[1], Li S[2,3] and Wu W[4]**

[1]Neonatal Intensive Care Unit, The Second Hospital of Tianjin Medical University, China

[2]College of Life Sciences and Health, Wuhan University of Science and Technology, China

[3]Beijing Novaboody Biotechnological Ltd, China

[4]Department of Blood Transfusion, The 960th Hospital of the PLA Joint Logistics Support Force, China

Abstract

Objective: Since the approval of rituximab for the treatment of B cell non-Hodgkin lymphoma, the development of monoclonal Antibodies (mAbs) for cancer treatment has been subject to intense investigations. However, rituximab is expensive and has limited availability. Llama-derived single-chain antibody fragments are small molecules with several advantages over conventional antibodies, which have been widely used for cancer treatment. The present study aims to obtain a single-domain anti-CD20 antibody from an immune Llama phage display library.

Methods: An immune Llama phage display library was constructed with phage display technology. The anti-CD20 single-domain Antibodies (sdAbs) were selected and the genes were cloned into expression vector pSJF2. The antibodies were efficiently expressed in *Escherichia coli*. The affinity and *in vitro* biological functions of different anti-CD20 sdAbs were measured by Biolayer interferometry.

Result: Three anti-CD20 single-domain antibodies (anti-CD20e7, anti-CD20b1 and anti-CD20f1) were obtained which were produced at high yield in *Escherichia coli* and showed high affinity.

Conclusion: These results, together with the ease of production, show that anti-CD20 VHHs are valuable candidates for the development of B-cell lymphoma therapies.

Keywords: B-cell lymphoma; Immune phage display library; Llama-derived single-chain antibody fragments

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Correspondence:
Wen Wu, Department of Blood Transfusion, The 960th Hospital of the PLA Joint Logistics Support Force, Jinan, China, Tel: +86-152-0132-1009

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Introduction

CD20, a non-glycosylated 33-kDa transmembrane phosphoprotein encoded by MS4A1 (Membrane-Spanning 4 domains, subfamily A), is involved in the regulation of B-cell growth and differentiation. CD20 is homogeneous or strongly positive, favoring diagnoses of lymphocyte-predominant HL, T-cell/histiocyte-rich large B-cell lymphoma [1], diffuse large B-cell lymphoma, primary mediastinal large B-cell lymphoma or grey zone lymphoma. Rituximab, applied as a monotherapy or in combination with chemotherapeutics, is the first monoclonal antibody licensed for Non-Hodgkin's Lymphoma (NHL) immunotherapy. Most of the clinical experience with anti-CD20 antibodies has been with rituximab [2]. The response rate, Progression-Free Survival (PFS), and Overall Survival (OS) of NHL patients has been improved with incorporation of rituximab. In aggressive lymphoma, patients who do not respond to rituximab-combined chemotherapy in a front-line setting represent individuals with poor clinical outcomes. Infusion-related symptoms (e.g., urticaria, fever, and chills) have been found in more than half of patients. Although these symptoms are usually modest, more serious reactions can occur, including hypotension, rigors, bronchospasm, and angioedema, in as many as 10% of patients [3-5]. Multiple next-generation anti-CD20 antibodies have been designed to improve the promise of B-cell lymphoma therapy [6-8].

An emerging alternative approach to immunotherapy is llama-derived VHH recombinant monoclonal antibodies, also known as “nanobodies”. Recently, the serum of came lids was found to contain not only the four conventional chain-Immunoglobulin G (IgG) molecules but also Heavy Chain Antibodies (HCAbs), which lack light chains and the CH1 domain. The VHH domain of approximately 15 kDa, which is much smaller than other recombinant antibody formats (60 kDa), is the smallest known antigen recognition site found in mammals. VHH has full binding capacity and affinity comparable to conventional antibodies. Monomeric Nbs have dimensions of 4 nm ×

2.2 nm and offer several advantages over antigen-binding fragments derived from classical antibodies [9]. Due to their small sizes, VHHs act as strong enzyme inhibitors, reaching enzyme pockets that are not accessible to common antibodies. When cloned and purified as Monomeric domains, VHHs demonstrate remarkable stability under a wide range of denaturing, temperature, and pH conditions [10]. Recombinant VHH antibody fragments are emerging as new versatile reagents for the diagnosis and treatment of tumors.

Here, we exploited the properties of VHH to develop a reagent that could be applied for the diagnosis or treatment of B cell lymphoma. We show that VHHs are broadly reactive reagents that can be engineered for use in immunodiagnostic tests for CD20 antigen. The results indicate that these new antibodies are promising as immunological tools for B cell lymphoma research and therapeutic development.

Materials and Methods

Llama immunization and immune response monitoring

A male llama of one year of age was subcutaneously immunized five times (days 0, 30, 50, 70, and 90) with 300 µg/per dose of CD20. For the first immunization, CD20 was emulsified in complete Freund's adjuvant. (Sigma-Aldrich, St. Louis, MO, USA). This study was approved by the hospital ethics committee [(2017) scientific research ethics review No. (03)]. Incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was used in subsequent immunizations. The antibody responses to CD20 in serum during the time course of immunization were monitored by ELISA. For the ELISAs, 96-well flat bottom Maxisorp ELISA plates (NUNC, Thermo Scientific, Waltham, MA, USA) were coated with 50 ng of CD20 diluted in 50 µL of PBS, pH 7.4, at 4°C. Then, 2% skimmed Milk Powder in Phosphate-Buffered Saline (MPBS) was used to blocked the wells, and the wells were incubated at 37°C for 2 h. Sera diluted with 2% MPBS were added at 100 µL per well and incubated at 37°C for 1 h. Subsequently, the Horseradish Peroxidase (HRP)-labelled goat anti-Llama antibodies were added to each well after three washes with PBS containing 0.05% Tween 20 (PBST, pH 7.4), and the plates were incubated at 37°C for 1 h. The ELISA was developed with commercial 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO, USA, added at 100 µL/well. The absorbance of each sample was read at 450 nm with an ELISA reader (Multiskan EX, Thermo scientific, Waltham, MA, USA), and averages of duplicate wells were used in the calculation of the values.

Antigen biotinylation

The EZ-Link Sulfo-NHS-Biotin (sulfosuccinimidobiotin, Thermo Fischer Scientific, Waltham, MA, USA) was dissolved in dimethyl sulfoxide (1.0 g/L), and 100 mg of CD20 antigen was biotinylated with EZ-Link Sulfo-NHS-Biotin. The antigen was reacted with a 10:1 molar excess of NHS-LC-Biotin buffer (succinimidyl-6-(biotinamido)-hexanoate, Thermo Fischer Scientific, Waltham, MA, USA). The biotinylated antigen was incubated at room temperature for 30 min and then dialyzed against PBS overnight.

Immunized llama sdAb library construction

From llama-immunized with CD20 antigen, 50 mL of blood were collected after the fifth dose. At day 90, the lymphocytes were collected from these Llama for the construction of phage libraries, as previously described. The VHH-repertoire was PCR amplified from the total RNA of the llama, and the PCR amplification products were purified, digested with SfiI and Pst restriction enzymes, and cloned into the corresponding sites of the phagemid vector pHEN-6. The recombinant plasmids were introduced into *Escherichia coli* strain TG1 by electroporation. A small amount of electroporated cells was diluted to determine the recombination rate, and the remaining cells were incubated with 2YT/Amp at 37°C. Phages were precipitated with PEG6000-NaCl solution (20%, sterile), and the phage titer was calculated.

Immunized llama sdAb library panning and phage Enzyme-Linked Immunosorbent Assay (ELISA)

Specific binders were enriched after three rounds of *in vitro* selection by "biopanning". M-280 streptavidin beads (Invitrogen, Carlsbad, CA, USA,) were incubated with 2% skim milk powder in PBS for 1 h at room temperature to block non-specific binding. The beads were drawn into 1.5 mL microcentrifuge tubes with a magnetic tube holder (Dynal Biotech, Oslo, Norway), and the supernatant was discarded. The magnetic streptavidin beads were mixed with biotinylated CD20 antigen (500 nM) and incubated with end-over-end rotation at room temperature for 30 min. Subsequently, the beads were incubated with phage for 2 h at room temperature. The magnetic streptavidin beads were rinsed 5 to 10 times. Bound phage was eluted with 200 µL of freshly diluted Triethylamine (TEA), and then neutralized with Tris-HCl. The unbound phage was discarded. Phages were purified with PEG (20% PEG6000-2.5 M NaCl). Three rounds of selection were conducted, and phage populations from the second and third rounds were tested for specificity to CD20 by ELISA. Next, 2 to 3 rounds of panning were performed with 100 nM, 20 nM, and 4 nM antigen concentration, and more than 100 clones isolated were after a single round. The isolated colonies were obtained from the second and third rounds of the bacteria, which was diluted and plated onto an LB/ampicillin plate. A single colony was selected and cultured in 96-well plates at 37°C for 5 h. Subsequently, M13K07 helper phage was added and incubated for 30 min without shaking at 37°C. The supernatant was collected and added to the 96-well plates, and a standard ELISA process was carried out with an HRP-anti-M13 antibody (GE Healthcare, Munich, Germany). Twelve positive phage clones were analyzed by DNA sequencing.

Expression and purification of anti-CD20 VHHs

The VHH cDNA was mobilized from the phage display vector to the soluble sdAb expression vector pSJF2 by BamHI/BbsI and re-sequenced prior to transfer to TG1 by electroporation. Individual clones were inoculated into 10 mL of 2YT (supplemented with ampicillin (100 mg/mL) and grown overnight at 37°C with shaking at 250 rpm. TG1cells were freshly transformed with the different plasmid constructs. VHH expression was induced with 1 mM IPTG for 8 h. After the cells were pelleted, the periplasmic proteins were extracted by osmotic shock. The VHH was purified from this periplasmic extract by using Ni-NTA (Qiagen, Duesseldorf, Germany), as recommended by the manufacturer.

According to the manufacturer's instructions, the sdAbs were assessed by size exclusion chromatography using a Superdex75 (GE Healthcare Bio-Sciences AB, Chicago, USA) column on an AKTA purifier 2000 system. The molecular weight was determined by using the Gel Filtration Calibration Kit HMW (high molecular weight, GE Healthcare), the purified proteins were assessed by SDS-PAGE, and the protein concentrations were determined by BCA (Pierce, Rockford, IL, USA).

Affinity measurements

Interaction studies between VHHs and CD20 were performed

by using BLI. CD20 antigen was biotinylated by using NHS-LC-biotin buffer, followed by dialysis with PBS. A standard coupling protocol was employed by BLI using the Octet RED system (Pall Forte Bio Europe, Portsmouth, UK) and Fortebio Acquisition Software. Biotinylated CD20 (20 μg/mL) coupled to Streptavidin (SA) biosensors was used to establish a baseline in PBST buffer (PBS pH 7.4, 0.1% BSA, albumin fraction and 0.02% Tween-20, both Merck KGaA, Darmstadt, Germany) prior to association at varying analyte concentrations. Serial dilutions of 200 μL were generated to produce 20, 10, 5, 2.5, and 1.25 μM concentration of sdAbs in appropriate wells of the assay plate. Octet Analysis Software version 6.4 was used for automatic data processing. Biosensor data were fit by using a 1:1 binding model.

ELISA competition assays

Ninety-six wells were coated with 100 μL CD20 (10 μg/mL in 50 mM $NaCO_3$ buffer, pH 9.5) and incubated overnight at 4°C. Non-specific binding was closed with 380 μL 3% BSA. Different concentrations (0, 50100, 250, 500 μg/mL) of anti-CD20e7, anti-CD20b1 and anti-CD20f1 were incubated with 50 μL CD20 (50 μg/mL) for 2 h at 37°C. Anti-BAFF sdAb was used as a negative control. After washing the microtiter wells three times with PBST, 100 μL HRP-labeled goat anti-human IgG (Sigma-Aldrich) was added and incubated for 1 h at room temperature. The wells were added substrate TMB after three washes with PBST, and incubated at room temperature for 10 min. Optical density was read at 450 nm.

Results

Anti-CD20 antibody titer test

Antibody titers to CD20 were monitored by ELISA to evaluate the llama immune response. The antiserum titer on day 90 was determined as 1×10^5 for CD20. The Llama serum was used as a control prior to immunization, and the Llama was immunized four times with CD20 antigen over three months. The antigen amount was approximately 1.5 mg. Prior to each immunization, 1~2 mL of blood was collected from the Llama jugular vein, and the anti-CD20 antibody level in the Llama was measured by enzyme immunoassay. Negative and immune sera were diluted 1:100, 1:1000, and 1:10,000. As shown in Figure 1, the level of anti-CD20 antibody in the serum increased with as the immune Llama interval lengthened, and the immune times increased after the fourth immunization. ELISA revealed an A450 value of 2.22 for the antibody in the Llama serum diluted 10,000 times, whereas an A450 value was 0.132 for the blank control, P/N>2 (positive results).

Table 1: Enrichment of specific phages during subsequent rounds of panning.

Round	Titre of input phage	Titre of output phage	Output phages/input
1	1.32×10^{12}	1.57×10^{6}	1.89×10^{-6}
2	7.51×10^{11}	4.76×10^{7}	5.33×10^{-5}
3	6.22×10^{11}	1.29×10^{7}	2.82×10^{-5}

Table 2: The relative affinity of selected antagonistic anti-CD20 sdAbs.

SdAbs	KD (M)	kon (1/Ms)	kdis (1/s)
Anti-CD20e7	3.19×10^{-7}	2.29×10^{3}	7.29×10^{-4}
Anti-CD20b1	3.18×10^{-6}	5.71×10^{3}	1.82×10^{-2}
Anti-CD20f1	8.23×10^{-7}	2.90×10^{3}	2.39×10^{-3}

All constants were calculated from five data sets obtained with different concentrations of VHHs (from 1.25-20 μM) by a global Langmuir 1.1 method.

The immunized llama library construction

Total RNA was extracted from 10^6 lymphocytes, which were collected from EDTA-treated blood, and cDNA was prepared and used as a template to amplify VHH genes. Antibody heavy chain observed in agarose gels was a 500-bp product, and the gel-purified VHH genes were amplified the variable domains as the template. PCR fragments were integrated into phage vector pHEN-6 by using the SfiI sites introduced with primers. The co-infection of the VHH-pHEN-6-transfected TG1 *E. coli* strain with the M13KO7 helper phage was performed in the phage rescue strategy, and a total of 8×10^6 phage particles were obtained. The library was approximately 3×10^9 clones with 24/24 unique clones having inserts at a ratio of nearly 2 VH:1 VHH, as determined by examining the amino acid composition of the framework. A total of 40 clones were randomly selected from the library to evaluate the percent of clones carrying VHH genes. Colony PCR showed that 90% of the VHH libraries contained phagemid inserts corresponding to the size of VHH.

Llama heavy chain variable domains (VHHs) selection

CD20 was used separately to select specific VHH phages. The immunized Llama phage display library for anti-CD20 sdAbs was panned over the course of three rounds of selection and amplification of the bound phage. The library was already enriched in CD20-specific binders after the last round of panning (Table 1).

Sequence alignment of VHHs

Sequencing of 24 ELISA-positive clones, junction sequences

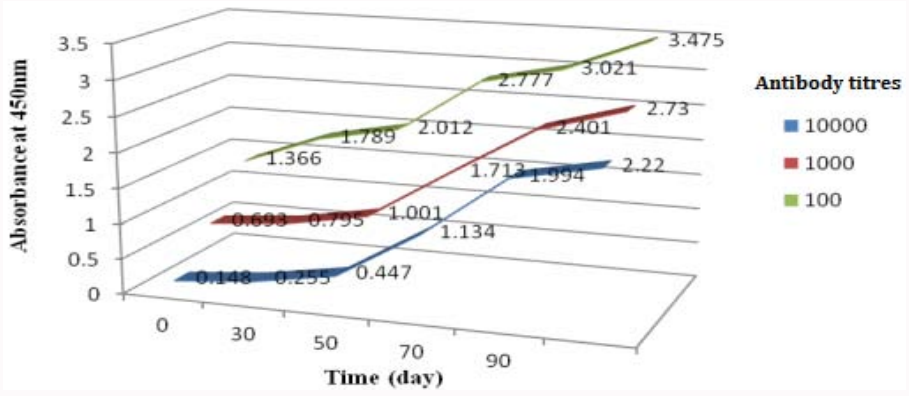

Figure 1: Llama immunization: The evaluation of the CD20 Ab response in serum during the time course of immunization is depicted. Antibody titers were measured by ELISA (lines).

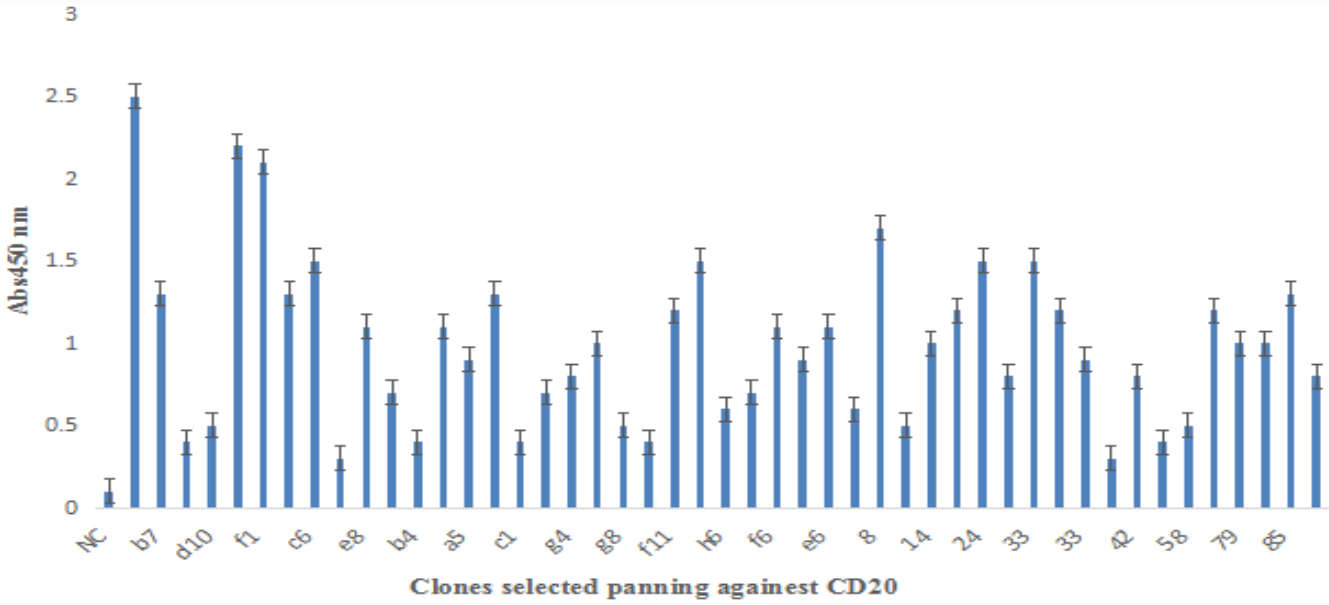

Figure 2: Selection of anti-CD20 VHHs. VHHs from the recombinant phage displayed library were respectively tested against immobilized CD20. After one round of panning, 96 clones selected asCD20 binding VHHs. ELISAs were performed to verify clone reactivity. Forty-eight clones recognized CD20. All measurements were performed in triplicate. The negative control was performed by using llama preimmune serum.

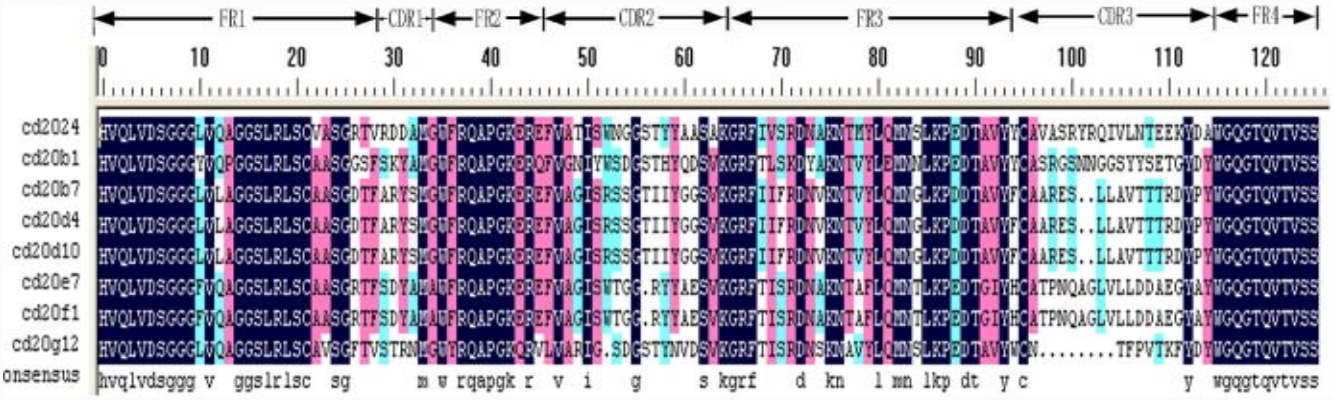

Figure 3: Comparison of the amino acid sequences of anti-CD20 sdAbs showed signature amino acid changes at positions F(Y) 37, E44, R45 and G47. The cysteine positions in the Complementary Decision Region 1 (CDR1) and CDR2 were 31-35 and 45-67, respectively. Cysteines were found to be multivariable in CDR3.

showed diversity and multiple sequence comparisons revealed eight sequence profiles of CD20 (Figure 2), which are stored in the GenBank database under the following accession numbers.: CD20-24, CD20b1, CD20b7, CD20d4, CD20d10, CD20e7, CD20f1, and CD20g2. Among these 8 clones, anti-CD20e7, anti-CD20b1 and anti-CD20f1 yielded the highest ELISA signals and were therefore selected for further characterization (Figure 3).

Expression of soluble VHH

As identified by phage ELISA, anti-CD20e7, anti-CD20b1 and anti-CD20f1 were expressed, and the antibodies were highly soluble and did not aggregate. Three sdAbs were highly pure and homogeneous by SDS-PAGE, which migrated at an expected size of 15 kDa. Size exclusion chromatography on a Superdex75 column generated single symmetric peaks at the expected elution position of a monomeric molecule with a molecular weight of 15 kDa, which is the average molecular weight of heavy chain variable domains. On a calibrated Superdex75 column, anti-CD20e7, anti-CD20b1, and anti-CD20f1 antibodies were eluted from 12.5 mL to 13.5 mL, which was equivalent to 15 kDa (Figures 4). The yield of purified anti-CD20f1 was approximately 28 mg/L, and the yields of the other antibodies were approximately 21 mg/L.

Antibody affinity constant measurement

The affinity between CD20 and the selected purified VHHs (anti-CD20e7, anti-CD20b1, and anti-CD20f1) was measured by BioLayer Interferometry (BLI) using Forte Bio's Octet System (Pall Forte Bio Europe, Portsmouth, UK). All obtained binding constants were in the nanomolar range (10-6 to 10-7). According to the kinetic model, anti-CD20f1 showed the highest affinity to CD20, which has a computed Kd of 8.23×10^{-7}. However, anti-CD20f1 VHH showed higher affinity to CD20 than anti-CD20e7 or anti-CD20b1 (Table 2).

Antigen binding specificity of the purified VHH

The three sdAbs were therefore tested for competition with CD20 to assess the affinity of the antibodies and the concentration of action. The inhibition ratios of the three sdAbs significantly increased with

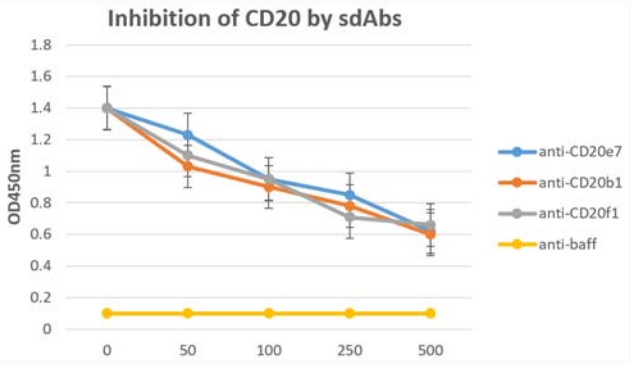

Figure 4: Competitive binding of VHHs for CD20. Anti-BAFF sdAb was the negative control.

sdAbs concentrations (Figure 4), which were compared with the negative control (anti-BAFF sdAb).

Discussion

With advances in antibody engineering, monoclonal antibody fragments have been able to precisely select antigenic epitopes to achieve rapid access to dense tissues and inhibit local damage progression [11-13]. Different types of nanobodies have been used clinically for disease diagnosis and therapeutic agents. Recently, we have explored "next generation" therapeutic approaches for B-cell lymphoma using dose-dense chemotherapy regimens and immunotherapy *via* monoclonal antibodies.

Three mechanisms of action of anti-CD20 antibodies have been proposed [14]. First, in Complement-Dependent Cytotoxicity (CDC), the activation of the complement cascade and cell lysis through the formation of Membrane Attack Complexes (MAC) was caused by the first component of Complement (C1) binding to the Fc portion of the anti-CD20 molecule [15]. Second, in Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), anti-CD20 antibodies bind to the CD20 molecule, and natural killer cells or macrophages, which release effector molecules, such as perforin, bind to the Fc portion of the anti-CD20 molecule through Fcγ receptors, resulting in cell lysis. Last, in direct cytotoxicity, the anti-CD20 antibody induces the internal signaling of apoptosis or other cell-death pathways within the tumor cells, causing antiproliferative effects or cell death [16]. Anti-CD20 antibodies bind to an extracellular portion of the CD20 molecule. Most anti-CD20 antibodies, including rituximab, Tositumomab, and Obinutuzumab, bind to the larger of two extracellular loops within the CD20 molecule, this loop includes the Alanine-N-Proline (ANP) residues at positions 170 to 172.

The nanobody has the characteristics of low molecular weight, low immunogenicity, high stability, high solubility, high affinity, high specificity and high permeability for tumor target antigen, and this molecule can identify the unusual and unexposed epitopes [17-21]. Therefore, the early diagnosis of nanobodies is particularly suitable for tumors or the preparation of special biological target tumor antigens to rituximab in drug treatments and is expected to make up for the lack of non-Hodgkin's lymphoma, provide new treatment options for the treatment of B cell lymphoma, and show broad application prospects.

In the present study, a novel therapeutic approach based onanti-CD20 VHH antibodies was developed. The purpose was to generate anti-CD20 sdAbs and to determine their biological effects. To our knowledge, the present study is the first report of the effects of anti-CD20 sdAbs on B-cell lymphoma cells. Bacterially expressed recombinant CD20 was successfully produced and conveniently purified. After immunizing llamas with recombinant CD20 and constructing an immune phage library displaying the VHH antibody repertoire, three VHHs that bind to CD20 were identified after multiple rounds of biopanning. Anti-CD20e7, anti-CD20b1, and anti-CD20f1 bound to CD20 when expressed and purified from *E. coli*. The recombinant protein was suitable for use as an antigen in phage bio-panning for selecting phage clones that bind to the protein from the established VH/VHH phage display library [22,23]. According to the set positive criteria of the assays, 24 VHH-phagemid-transformed *E. coli* clones were selected. DNA sequencing confirmed the high diversity of the obtained clones.

The immunized Llama VHH library was constructed. The choice of phage display strategy depends on the required affinity and the target application of the desired antibody [24-26]. The immune antibody library mainly originates from the specific antigens of the individual peripheral blood lymphocytes in the Llama *in vivo via* antigen stimulation of a highly mutated antibody, which increases the affinity by over ten or even several hundred-fold, so in the antibody library preparation and screening of the high positivity rate, it is easy to obtain better antibody specificity and high affinity [27-30]. Studies have attempted screening for anti-CD20-specific nanobodies from natural Llama-type nonimmune antibody libraries, but after four rounds of screening, the positivity rate of phage ELISA is relatively low, and it is difficult to select specific antibodies with high affinity, considering the main affected factors of nonimmune type antibody affinity and low output, despite subsequent affinity maturation. A series of methods are used to improve antibody affinity, but these methods are far less effective than the immune antibody library. Half of the antibodies in llama blood belong to VHH, which have light chains and CH1 regions of the heavy chain antibodies, while llamas aged 8 to 12 months are in the strongest stage to resist the original stimuli, through which it is easy to produce antigen-specific antibodies. Due to the specificity of the molecular structure of heavy chain antibody, its apparent molecular weight in reducing SDS-PAGE is only 43 kDa, which is less than that of the ordinary 55-kDa heavy chain antibody. Therefore, Protein G affinity chromatography can be used to observe the production of unique heavy chain antibodies in llamas. To select the best immune antigen dose for subcutaneous injection to induce an immune effect, a moderate amount of the antigen was mixed with Freund's adjuvant to enhance the immunogenicity of this molecule. The immune process and timely monitoring of serum antibody titers of Llamas can directly reflect the production of specific antibodies because of the direct impact of the quality of the antibody library. We fully considered the potential problems to focus on immune animals to ensure the quality of the anti-nanoscale antibody library.

The design of an appropriate selection strategy is equally important for the construction of immune phage library. Effective panning and gradual decreasing of the concentration of CD20 antigen resulted in the isolation of the most specific VHHs during each panning. The diversity of antibodies in the antibody library determines the quality of the antibody library. B lymphocytes undergo antigen stimulation, and most of these molecules differentiate into plasma cells that produce a large number of antibody molecules. The gene rearrangement mechanism is the basis of antibody molecular diversity. The random combination of antibody fragments at the DNA level can theoretically produce 108 different types of antibodies.

The variable regions of antibodies, particularly the different CDR regions, are the main reasons for the diversity of antibody molecules [31,32]. In the first round of PCR, we selected 11 different pairs of primers for VHH gene amplification by multiple degenerate primers to amplify Llama heavy chain antibody genes, which help to increase the diversity of the antibody gene. Moreover, the number of cycles in the two rounds of PCR amplification is controlled at 30 or more than 35 cycles to avoid repeated amplification of dominant antibodies, generate redundant PCR products, and ensure the diversity of antibody libraries.

The three selected sdAbs possess unique characteristics in terms of their different CDR structures. More than 80% of the sequences for the three sdAbs share identity with the human VH domain, the main differences in amino acid sequence between the llama VHH and human VH are located in the CDR regions. The spatial conformation of antibody- and antigen-binding sites is determined by the length and amino acid composition of the complementary region of the antigen (CDR region). CDR3 is the important factor determining the variable sequence of antibodies. The nanoscale antibody has a long CDR3 area, which makes it possible to identify different epitopes and increase the affinity of nanoscale antibodies. Three strains of nano antibody affinity are different, and the main reason lies in that the CDR zone and FR zone configuration is slightly different, with some residues changed, making three monoclonal antibodies anti-CD20 antibodies, namely, anti-CD20e7, anti-CD20b1, anti-CD20f1, that may recognize different epitopes, resulting in antibody affinity and biologically different functions.

In summary, three VHHs specifically recognize CD20 antigen. These clones could represent a new biotool for investigating the mechanisms of B-cell lymphoma and the development of alternative methods for CD20 diagnosis and may even be used as therapeutic agents to overcome the challenges of the current therapy.

Highlights

1. An immune Llama phage display library was constructed with phage display technology.

2. The anti-CD20 single-domain antibodies ((anti-CD20e7, anti-CD20b1 and anti-CD20f1) were selected from immune Llama phage display library.

3. Three anti-CD20 single-domain antibodies were produced at high yield in *Escherichia coli* and showed high affinity.

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References

1. Marshall MJE, Stopforth RJ, Cragg MS. Therapeutic antibodies: What have we learnt from targeting CD20 and where are we going? Front Immunol. 2017;8:1245-68.

2. Tsai PC, Hernandez-Ilizaliturri FJ, Bangia N, Olejniczak SH, Czuczman MS. Regulation of CD20 in Rituximab-Resistant Cell Lines (RRCL) and B-cell non-Hodgkin Lymphoma (B-NHL). Clin Cancer Res. 2012;18(4):1039-50.

3. Singh V, Gupta D, Almasan A. Development of novel anti-Cd20 monoclonal antibodies and modulation in Cd20 levels on cell surface: Looking to improve immunotherapy response. J Cancer Sci Ther. 2015;7(11):347-58.

4. Klein C, Jamois C, Nielsen T. Anti-CD20 treatment for B-cell malignancies: Current status and future directions. Expert Opin Biol Ther. 2021;21(2):161-81.

5. Bonavida B. Postulated mechanisms of resistance of B-NHL to rituximab treatment regimens: Strategies to overcome resistance. Semin Oncol. 2014;41(5):667-77.

6. Johnston HF, Xu Y, Racine JJ, Cassady K, Ni X, Wu T, et al. Administration of anti-CD20 mAb is highly effective in preventing but ineffective in treating chronic GVHD while preserving strong GVL effects. Biol Blood Marrow Transplant. 2014;20(8):1089-103.

7. Payandeh Z, Bahrami AA, Hoseinpoor R, Mortazavi Y, Rajabibazl M, Rahimpour A, et al. The applications of anti-CD20 antibodies to treat various B cells disorders. Biomed Pharmacother. 2019;109:2415-26.

8. Gnjatic S, Bronte V, Brunet LR, Butler MO, Disis ML, Galon J, et al. Identifying baseline immune-related biomarkers to predict clinical outcome of immunotherapy. J Immunother Cancer. 2017;5:44.

9. Van den Abbeele A, De Clercq S, Ganck AD, Corte VD, Loo BV, Soror SH, et al. A llama-derived gelsolin single-domain antibody blocks gelsolin-G-actin interaction. Cell Mol Life Sci. 2010;67(9):1519-35.

10. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. Nature. 1993;363(6428):446-8.

11. Hoelzer D, Walewski J, Döhner H, Viardot A, Hiddemann W, Spiekermann K, et al. Improved outcome of adult Burkitt lymphoma/leukemia with rituximab and chemotherapy: Report of a large prospective multicenter trial. Blood. 2014;124:3870-9.

12. De Greve H, Virdi V, Bakshi S, Depicker A. Simplified monomeric VHH-Fc antibodies provide new opportunities for passive immunization. Curr Opin Biotechnol. 2020;61:96-101.

13. Pothin E, Lesuisse D, Lafaye P. Brain delivery of single-domain antibodies: A focus on VHH and VNAR. Pharmaceutics. 2020;30;12(10):937.

14. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994;83(2):435-45.

15. Feugier P. A review of rituximab, the first anti-CD20 monoclonal antibody used in the treatment of B non-Hodgkin's lymphomas. Future Oncol. 2015;11(9):1327-42.

16. Keating GM. Rituximab: A review of its use in chronic lymphocytic leukemia, low-grade or follicular lymphoma and diffuse large B-cell lymphoma. Drugs. 2010;70(11):1445-76.

17. Chabrol E, Stojko J, Nicolas A, Botzanowski T, Fould B, Antoine M, et al. VHH characterization. Recombinant VHHs: Production, characterization and affinity. Anal Biochem. 2020;589:113491.

18. Rajabzadeh A, Rahbarizadeh F, Ahmadvand D, Salmani MK, Hamidieh AA. A VHH-based anti-MUC1 chimeric antigen receptor for specific retargeting of human primary T cells to MUC1-positive cancer cells. Cell J. 2021;22(4):502-13.

19. Muyldermans S. A guide to: Generation and design of nanobodies. FEBS J. 2021;288(7):2084-102.

20. Li D, Ren J, Ji F, Peng Q, Teng H, Jia L. Peptide linker affecting the activity retention rate of VHH in immunosorbents. Biomolecules. 2020;10(12):1610.

21. Caljon G, Caveliers V, Lahoutte T, Stijlemans B, Ghassabeh GH, Abbeele JVD, et al. Using microdialysis to analyse the passage of monovalent nanobodies through the blood-brain barrier. Br J Pharmacol. 2012;165(7):2341-53.

22. Hartmann L, Botzanowski T, Galibert M, Jullian M, Chabrol E, Zeder-Lutz G, et al. VHH characterization. Comparison of recombinant with chemically synthesized anti-HER2 VHH. Protein Sci. 2019;28(10):1865-79.

23. Roth L, Krah S, Klemm J, Günther R, Toleikis L, Busch M, et al. Isolation of antigen-specific VHH single-domain antibodies by combining animal immunization with yeast surface display. Methods Mol Biol. 2020;2070:173-89.

24. Clackson T, Hoogenboom HR, Griffiths AD, Winter G. Making antibody fragments using phage display libraries. Nature. 1991;352(6336):624-8.

25. Roth L, Krah S, Klemm J, Günther R, Toleikis L, Busch M, et al. Isolation of antigen-specific VHH single-domain antibodies by combining animal immunization with yeast surface display. Methods Mol Biol. 2020;2070:173-89.

26. Pekar L, Busch M, Valldorf B, Hinz SC, Toleikis L, Krah S, et al. Biophysical and biochemical characterization of a VHH-based IgG-like bi- and trispecific antibody platform. MAbs. 2020;12(1):1812210.

27. Cavallari M. Rapid and direct VHH and target identification by staphylococcal surface display libraries. Int J Mol Sci. 2017;18(7):1507.

28. Hoogenboom HR. Designing and optimizing library selection strategies for generating high-affinity antibodies. Trends Biotechnol. 1997;15(2):62-70.

29. Barthelemy PA, Raab H, Appleton BA, Bond CJ, Wu P, Wiesmann C, et al. Comprehensive analysis of the factors contributing to the stability and solubility of autonomous human VH domains. J Biol Chem. 2008;283(6):3639-54.

30. Dumoulin M, Conrath K, van Meirhaeghe A, Meersman F, Heremans K, Frenken LG, et al. Single-domain antibody fragments with high confrontational stability. Protein Sci. 2002;11(3):500-15.

31. Schut MH, Pepers BA, Klooster R, van der Maarel SM, el Khatabi M, Verrips T, et al. Selection and characterization of llama single domain antibodies against N-terminal huntingtin. Neurol Sci. 2015;36(3):429-34.

32. Wu W, Li S, Zhang W, Sun J, Ren G, Dong Q. A novel VHH antibody targeting the B cell-activating factor for B-cell lymphoma. Int J Mol Sci. 2014;15(6):9481-96.