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Construction of a Natural Human Fab Phage Antibody Library and Screening of Phage Antibody against PD-L1

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Abstract: Programmed Death Ligand-1(PD-L1) is an important target to drive T cell dysfunction when it connects with Programmed Death-1(PD-1), leading to immune escape of tumor cells, thus anti-PD-L1 antibody shows a promising prospect in the treatment of tumor. In order to construct a large natural antibody library, we collected a large number of lymphocytes of adults and children. The light chain and Fd genes of antibody were amplified by PCR, and the Fab phage antibody library with a library capacity of 4.27×10^9 was constructed. The insertion rates of the light chain library and the Fab library were 90% and 70%, respectively. The cloned sequences identified by PCR showed that all the sequences analyzed were unique, and the amino acid sequences of the CDR regions were diverse, which proved that there was good diversity in the antibody library. The positive clones that bind to PD-L1 were screened by phage ELISA, PCR identification and sequence analysis. In the end, two high-affinity positive clones were determined. The successful construction of this natural phage antibody library provided an effective method for screening human PD-L1 antibodies, which was expected to screen humanized antibodies against various antigens.

Keywords: PD-L1, Phage Antibody Library, Fab Antibody, Solid-Phase Screening

Introduction

PD-L1 is considered to be the ligand of the immunosuppressive molecule programmed cell death-1(PD-1) (Boland et al., 2013; Wang et al., 2013). PD-L1 is highly expressed in many tumor cells and can produce tumor immune escape by inhibiting the activation of T cells. PD-1 is highly expressed in activated T lymphocytes, especially in depleted T cells, which are common in tumor-infiltrating lymphocytes (Mellman et al., 2011; R et al., 2010; Shi et al., 2013). PD-1 / PD-L1 immune checkpoint is one of the important targets of tumor immunity therapy, which has been widely studied in recent years(Gurung et al., 2020; Li et al., 2020). When PD-L1 is bound to PD-1, it will induce a series of cascade reactions of phosphorylation and dephosphorylation, thus inhibiting the activity of $CD8^+$ T cells and producing immunosuppression (Bishop et al., 2009; Chikuma et al., 2009). Tumor cells can inhibit the activation of T cells by overexpression of PD-L1, resulting in immune escape of tumor cells. If the corresponding inhibitors are developed and the PD-1 / PD-L1 signaling pathway is blocked, the suppressive effect of tumor cells on T cells can be rescued. PD-1 / PD-L1 blocking antibody therapy has made great progress in tumor treatment (Bylicki et al., 2018; Kambayashi et al., 2019; Kwok et al., 2016). Nivolumab is the first blocking drug against PD-1. Clinical studies had shown that this treatment method could inhibit the growth of various tumor cells, including epithelial tumors, melanoma, renal cancer, and colorectal cancer (Brahmer et al., 2010). In patients with non-small cell lung cancer (NSCLC), the use of nivolumab treatment could be observed to reduce the tumor volume (Topalian et al., 2012). The results of subsequent clinical studies showed that there were significant effects in patients with refractory NSCLC (17%, n = 129), melanoma (31%, n = 107) and renal cell carcinoma (RCC) (27%, n = 34). Long-term observation to patients with these three kinds of cancer found that their overall survival time was 9.9 months, 16.8 months and 22.4 months, respectively (Topalian et al., 2014). FDA has approved Nivolumab for chemotherapy-resistant squamous NSCLC. Phase III clinical study of melanoma showed that compared with chemotherapy, nivolumab had better curative effect (Topalian et al., 2012). The first monoclonal antibody targeting PD-L1 was BMS-956559 (anti-PD-L1 antibody, human IgG4 subclass, Bristol Myers Squibb Inc) (Robert et al., 2015). A variety of tumors, such as bladder cancer (Armand et al., 2013), melanoma, head and neck cancer, renal cell carcinoma, and non-small cell lung cancer, had effectively responded to anti-PD-L1 antibody mpdl3280a. Med (anti-PD-L1 antibody, human IgG1 subclass) also showed a good therapeutic prospect (Powles et al., 2014).

Antibody drugs play an important role in tumor therapy (Cai et al., 2017; Zhang et al., 2021). In 1986, the United States FDA approved the world's first monoclonal antibody OKT3 (mouse source) (Aksentijevich and Flinn, 2002). Although OKT3 showed a certain curative effect on the treatment of transplant rejection, mouse source monoclonal antibody was easy to cause immune rejection in human body, which greatly limited the application of monoclonal antibodies in antibody drugs. Phage antibody library display technology was originated in the 1990s (Deantonio et al., 2014). With this technology, researchers can prepare full human antibodies. The corresponding antibody genes in human B lymphocytes can be obtained by PCR. Through phage display technology, the expression products of the antibody genes are fused with the capsid protein of the phage and displayed on the

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surface of the phage particle. The full human antibody molecule specifically bounds to the target antigen, which will be screened by the corresponding screening method. Since phage display technology combines phenotype and genotype, the required target genes will be directly screened out using specific antigen (Smith, 1985; Tajiri et al., 2007). After obtaining the gene sequence of antibodies, it can also be constructed into other types of genetically engineered antibodies. According to the type of antibody molecule, the antibody library can be divided into scFv antibody library and Fab antibody library. Fab was more stable than scFv, and it was not easy to form multimers (Fox et al., 2001). Fab was also easier to transform into full-length antibodies. Fab antibodies have stronger tissue penetrating ability due to their small particle size. In addition, Fab antibodies do not have an Fc terminal and will not produce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), making it have a wide application in clinical therapy (Liu et al., 2014).

In this study, we established a natural human Fab phage antibody library to evaluate its diversity and quality. The human Fab antibody against PD-L1 was screened by phage ELISA, PCR identification and sequence analysis. The high-affinity anti-PD-L1 antibody screened at this time was expected to be used in clinical diagnosis and treatment, and the constructed natural phage antibody library was expected to be used to screen antibodies against other antigens.

Materials and methods

RNA extraction and cDNA synthesis

Lymphocyte separation fluid (LTS1077, TBD, Tianjin, China) was used to separate Peripheral blood mononuclear cells (PBMC) from peripheral blood. The peripheral blood of eighteen children and sixty adults (all people were healthy) came from the Third Affiliated Hospital of Sun Yat-sen University and Guangzhou women and children medical center (the blood samples were obtained with the patient's informed consent). Total RNA was extracted from purified PBMC with TRIzol (15596018, Thermo Fisher, China), according to the manufacturer's instructions. And then, the RNA from PBMC was reversely transcribed into cDNA, using the reverse transcription kit (4366597, Thermo Fisher, China).

Construction of antibody library

Using cDNA as a template, the light chain κ and λ or Fd (including VH and CH1) were separately amplified with different primers. The primers used for amplification were designed according to Scripps Institute and relevant literature(Tian et al., 2000). The primer sequences were presented in Table 1. The light chain and the Fd genes repertoire were assembled with degenerate primers including *SacI*(1078S, Takara Bio, Shiga, Japan), *XbaI*(1093S, Takara Bio, Shiga, Japan) and *XhoI*(1094S, Takara Bio, Shiga, Japan), *SpeI*(1086S, Takara Bio, Shiga, Japan) restriction sites, using Polymerase Chain Reaction Kit (TSE101, TSINGKE, Beijing, China). For preparation of the light chain repertoire, the amplified products of light chain were purified and cloned into the *SacI*/*XbaI*site of pComb3XSS vector by T4 DNA ligase (M0202V, NEB, USA). The Fab library was obtained by cloning of Fd into pComb3XSS vector holding the light chain repertoire. The recombinant plasmids were transformed into competent *E. coli* XL1-Blue at 2.5 kV, 25 μF, 200 Ω, for 5 ms by electroporation (Gene pulser Xcell, BioRad,USA). To calculate the reservoir capacity, the gradient dilutions of the transformant were coated on the 2×YT medium (per liter contained tryptone,16.0 g;Yeast extract, 10.0 g; Sodium chloride, 5.0 g) plate, containing 50 μg/mL ampicillin, 10 ug/mL tetracycline, 2% glucose and 1.5% agar powder. To determine the insertion rate, ten isolated colonies were randomly selected to perform colony PCR. Colony PCR using the universal primer set for pComb3XSS (Table 1). Different colonies of Fab library were randomly selected to perform colony PCR and sequenced to estimate the diversity of the library.

Table 1 Primers for the construction of human phage-displayed Fab library

Primer name	Primer sequence (5′ to 3′)
primers for κ	
κF1	GAC ATC GAG CTC ACC CAG TCT CC
κF2	GAA ATT GAG CTC ACA CAG TCT CCA
κF3	GAY ATY GAG CTC ACY CAG TCT CCA
κR	GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA
primers for λ	
λF1	AAT TTT GAG CTC ACT CAG CCC CAC
λF2	TCT GYS GAG CTC CAG CCK SCC TCM GTG
λF3	TCT GAA GAG CTC CAG GAC CCT GTT GTG TCT GTG
λF4	TCT GAA GAG CTC CAG GAC CCT GTT GTG TCT GTG
λF5	CAG ACT GAG CTC ACT CAG GAG CCC
λR	CGC CGT CTA GAA TTA TGA ACA TTC TGT AGG
primers for Fd	
FdF1	SAG GTG CAG CTC GAG SAG TCT GGG

FdF2	SAG GTG CAG CTR CTC GAG TCT GG
FdF3	CAG GTG CAG CTR CTC GAG TSG GG
FdF4	CAG GTA CAG CTC GAG CAG TCA GG
FdR1	CTC GAC ACT AGT TTT GCG CTC AAC TGT CTT
FdR2	TGT GTG ACT AGT GTC ACC AAG TGG GGT TTT
FdR3	GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG
FdR4	GCA TGA ACT AGT TGG GGG ACC ATA TTT GGA
FdR5	GCT CAC ACT AGT AGG CAG CTC AGC AAT CAC
primers for L (PCR identification)	
LF	AAGACAGCTATCGCGATTGCAGTGG
LR	GGGCAGCGAGTAATAACAATCCAGC
primers for H (PCR identification)	
HF	ACCTATTGCCTACGGCAGCCG
HR	AGAAGCGTAGTCCGGAACGTC

Library amplification

A total of 2 mL Fab library was inoculated into 800 mL 2×YT liquid culture, containing 100 μg/mL ampicillin, 10 ug/mL tetracycline and 2% glucose, and the culture medium was incubated at 37℃ with shaking (250 rpm) until the OD600 to 0.5 was reached. Then, the bacterium was infected with VCSM13 helper phage at 37 °C aqueous bath for 30 min and then with shaking slowly for 30 min. Then, it was exchanged by medium (2×TY containing 100 μg/mL ampicillin, 10 μg/mL tetracycline, and 50 μg/mL kanamycin) and incubated overnight with shaking (250 rpm) at 30 ℃. Precooled PEG8000/NaCl precipitated Phage particles resuspended in 10 mL phosphate-buffered saline (PBS). A part of the library was used to determine the titer of the phage antibody library, and the rest could be used directly for subsequent screening.

Screening the Fab library against PD-L1

In order to select the specific Fab antibody against PD-L1, the specific protein was coated in a microtiter plate (JET BIOFIL, Guangzhou, China). In the first round of panning, each well was coated with 100 μL of 50 μg/mL PD-L1(C315, novoprotein, China) (in 0.05 M Na_2CO_3/$NaHCO_3$, pH 9.6). In the following rounds, the antigen concentration was halved in turn. After incubated overnight at 4 °C, the microtiter plate was blocked with 5% MPBST (PBS-T containing 5% skim milk, w/v). Next, the freshly amplified phage library mixed with 5% MPBST was added to the microtiter plate and incubated. After that, the microtiter plate was washed two times (five times in the second round, ten times in the third round and ten times in the fourth round) with PBS-T. Next, the bound phage-Fabs were eluted with Gly-HCl (pH 2.2) and then added 2M Tris for neutralization. Afterward, using the eluted phages (output) to infect the log-phase XL1-Blue cell (OD600=0.5) for 30 min at 37 ℃. The amplified output phages were rescued by VCSM13 and after PEG concentration and precipitation, it was used for the next round of screening.

Polyclonal phage enzyme-linked immunosorbent assay (ELISA)

To monitor the rate of enrichment of phages bound to PD-L1 following the panning cycles, ELISA was performed on polystyrene 96-well plates. ELISA plates was coated with 100 μL of 10 μg/mL PD-L1. Each output was amplified and diluted 10 times with PBS. 100 μL/well of the diluent were incubated for 2 h 37°C, and VCSM13 was a negative control. After 45-min incubation of HRP-labeled anti-M13 mouse monoclonal antibody (1:10,000,dilution) (11973-MM05T-H, Sino Biological, Beijing, China) at 37°C, the absorbance at 450nm was read by a microplate reader (BioTek, Highland Park, Winooski,VT, USA). The plates were washed with PBS-T between incubation steps.

Identification of Fab by phage ELISA

The single clone from the plate of the last round of screening was randomly selected. The method of amplifiing recombinant phage was the same as that of the library amplification described in the previous method. The recombinant phage supernatants(100 ul) were exploited for monoclonal phage-Fab ELISA as same as polyclonal phage ELISA as mentioned previously. When the OD was at least 2.1 times greater than the negative control (NC), the clones were regarded as positive.

Sequence analysis

The PCR analysis was performed to identify the presence of Fab genes inside plasmids using the primers described in the previous method (Table 1). The primers of HF and LF were used for sequencing. The sequencing data were analyzed by the abYsis database (http://www.abysis.org/abysis/) and igblast database (https://www.ncbi.nlm.nih.gov/igblast/).

Soluble induced expression and identification of Fab antibody

The single clone was inoculated into medium (2×TY containing 100 μg/mL ampicillin and 2% glucose) and cultured until OD600 to 0.9. Then IPTG was added to make the final concentration 0.1 mM, and the culture was grown 16 h at 30 °C with shaking (250 rpm). The supernatant was collected. Immunoreactivity of supernatants was tested by ELISA. ELISA was performed as described in the previous method except that the secondary antibody was anti-HA-tag antibody (1:500, dilution) (BEENbio-PR042, BEENbio,

Shanghai, China). The HRP-conjugated goat anti-mouse IgG (1:5,000 dilution) (405306, Biolegend, San Diego, CA) was added and incubated at 37°C for 30 min, and HBsAg (ANTY, TANGSHAN, China) was irrelevant control.

Results

Construction and identification of Fab antibody library

The design of Fab antibody library construction was shown in the figure (Fig. 1). A total of 120 mL of blood collected from 18 children and 60 adults was separated with human lymphocyte separation solution (The blood sample was approved by the relevant personnel of the hospital). Total RNA extracted from PMBC was measured quantitatively. The quality of RNA was analyzed by using agarose gel electrophoresis and the ratio of A260/A280. The ratio of A260/A280 for the RNA was 2.02, suggesting that the quality of RNA was high. Then, the quality of RNA was further verified by agarose gel electrophoresis. The results of agarose gel electrophoresis, 28S RNA, 18 S RNA, and 5S RNA can be clearly seen (Fig. 2B). The vector construction process of the antibody library was shown in the figure (Fig. 2A). Using cDNA as the template, the light chain and Fd genes were amplified. The electrophoresis result showed a brighter band between 500 bp and 750 bp (about 680 bp), which was consistent with the expected result (Fig. 2C,D). After the light chain library was generated, we estimated the reservoir capacity by the serial dilution method. The reservoir capacity of the light chain library was 2.5×10^9 cfu by counting the number of colonies. Ten colonies were randomly selected to identify the insertion rate by PCR. The electrophoresis result showed that 90% of the colonies contained the insert of the expected size for light chain gene (Fig. 2E). The efficient reservoir capacity of light chain library as follows: $2.5\times10^9\times90\%=2.25\times10^9$ cfu. The Fab library was obtained by cloning Fd genes into pComb3XSS vector holding the light chain genes. Serial dilution of the Fab library determined the reservoir capacity of 6.1×10^9 cfu. PCR analysis of ten isolated bacterial colonies indicated that the insertion rate of the Fab antibody library was 70% (Fig. 2F). The efficient reservoir capacity of the Fab library as follows: $6.1\times10^9\times70\%=4.27\times10^9$ cfu. The titer of helper phage VCSM13 was calculated by plaque assay. After packaging with the helper phage VCSM13, the titer of the Fab library is 5×10^{13} pfu/ml.

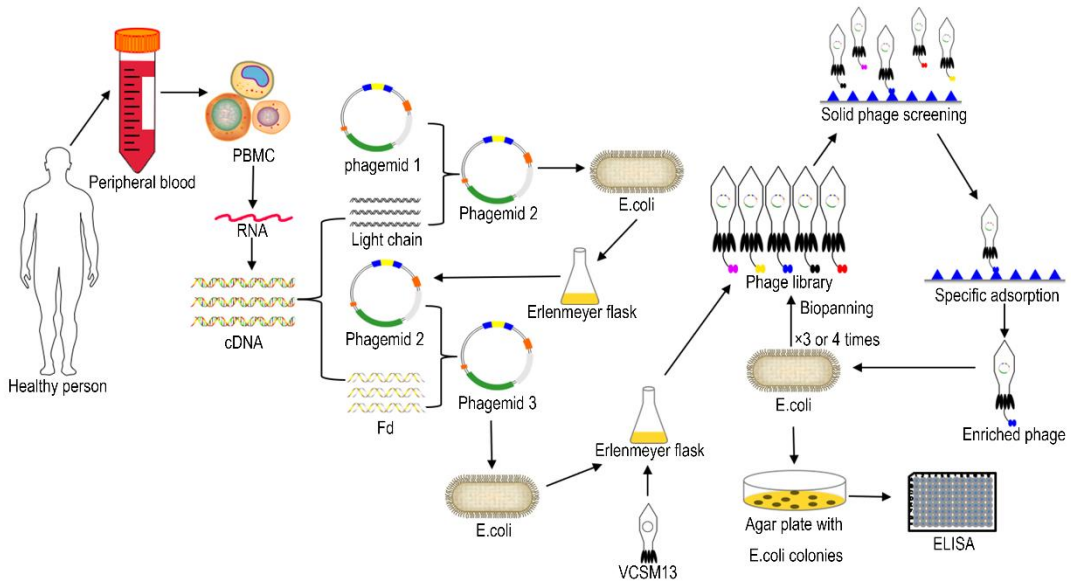

Fig. 1 Schematic diagrams of the preparation and construction of the natural human Fab phage antibody library and screening of phage antibody against PD-L1

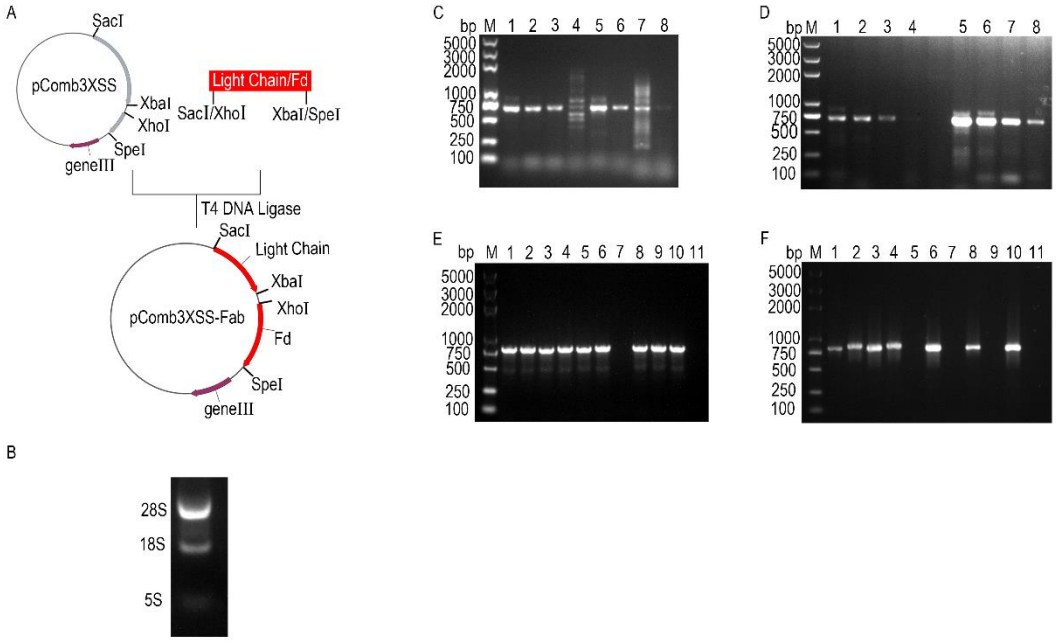

Fig. 2 The construction of phage antibody library

A) The schematic illustration of the recombinant phagemid of pComb3XSS-Fab. B) Agarose gel electrophoresis analysis of total RNA. C) PCR amplification of light chain genes. M: DNA marker; lane1-8, PCR products of light chain genes. D) PCR amplification of Fd genes. M: DNA marker; lane1-8, PCR products of Fd genes. E) Plaques were randomly selected to detect the percentage of phage containing an insert of light chain. Lanes 1–10, colony PCR products of ten recombinants picked randomly from the plates; lane 11, the negative control. F) Plaques were randomly selected to detect the percentage of phage containing an insert of Fd. Lanes 1–10, colony PCR products of ten recombinants picked randomly from the plates; lane 11, the negative control.

Diversity analysis of antibody library

In order to identify the diversity of the antibody library, the recombinant transformants were evaluated by sequencing. Different colonies were randomly selected from the agar plate for colony PCR. The twenty clones that were identified as positive were sent to Sangon Biotech for sequencing. Sequencing results showed that 18 light chain colonies and 19 Fd colonies were successfully identified. The sequences were evaluated using the Blast search on the abYsis database (Fig. 3A,B). The results showed that all the sequences were valid, without repetitive sequences and stop codons. The sequence in the red box was the CDR region, and the CDR region of the antibody was the central part of the antibody binding to the antigen. The analysis result showed that the amino acid sequence of the CDR region of VL and VH in different clones were quite different, indicating that the antibody had a rich diversity. The CDR3 region mainly determines the affinity and specificity of the antibody, so we conducted a statistical analysis of the amino acid length and amino acid composition of the CDR3 region. By analyzing the length of amino acids, it was found that the number of amino acids in the CDR3 region of VL was mainly concentrated in 9, accounting for more than 60% of all sequences (Fig. 3C). The number of amino acids in the CDR3 region of VH was mainly concentrated between 12-14. The 14 amino acid length sequences were the most, accounting for more than 20% of all sequences (Fig. 3D). Analyzing the amino acid composition of the CDR3 region (Fig. 3E,F), it was found that in CDR3-L, the frequency of alanine in the first position was higher. In comparison, the frequency of serine and glycine in the amino acid composition of CDR3-H was higher. Compared with CDR3-H, CDR3-L had fewer amino acid types at each position, but the overall amino acid types of CDR3-L were diverse. Next, we used the igblast database to analyze the homologous family information of antibody genes. The results showed that the variable regions of light chain belonged to Vκ1 (22.2 %) 、Vκ2 (5.6%) 、Vκ3 (27.8 %) 、Vκ4 (11.1%) gene family and Vλ1 (22.2%) 、Vλ3 (11.1%) gene families; and the variable regions of Fd belonged to VH1 (15.8%), VH3 (52.6%) and VH4 (31.6%) gene families respectively. (Fig. 3G,H).

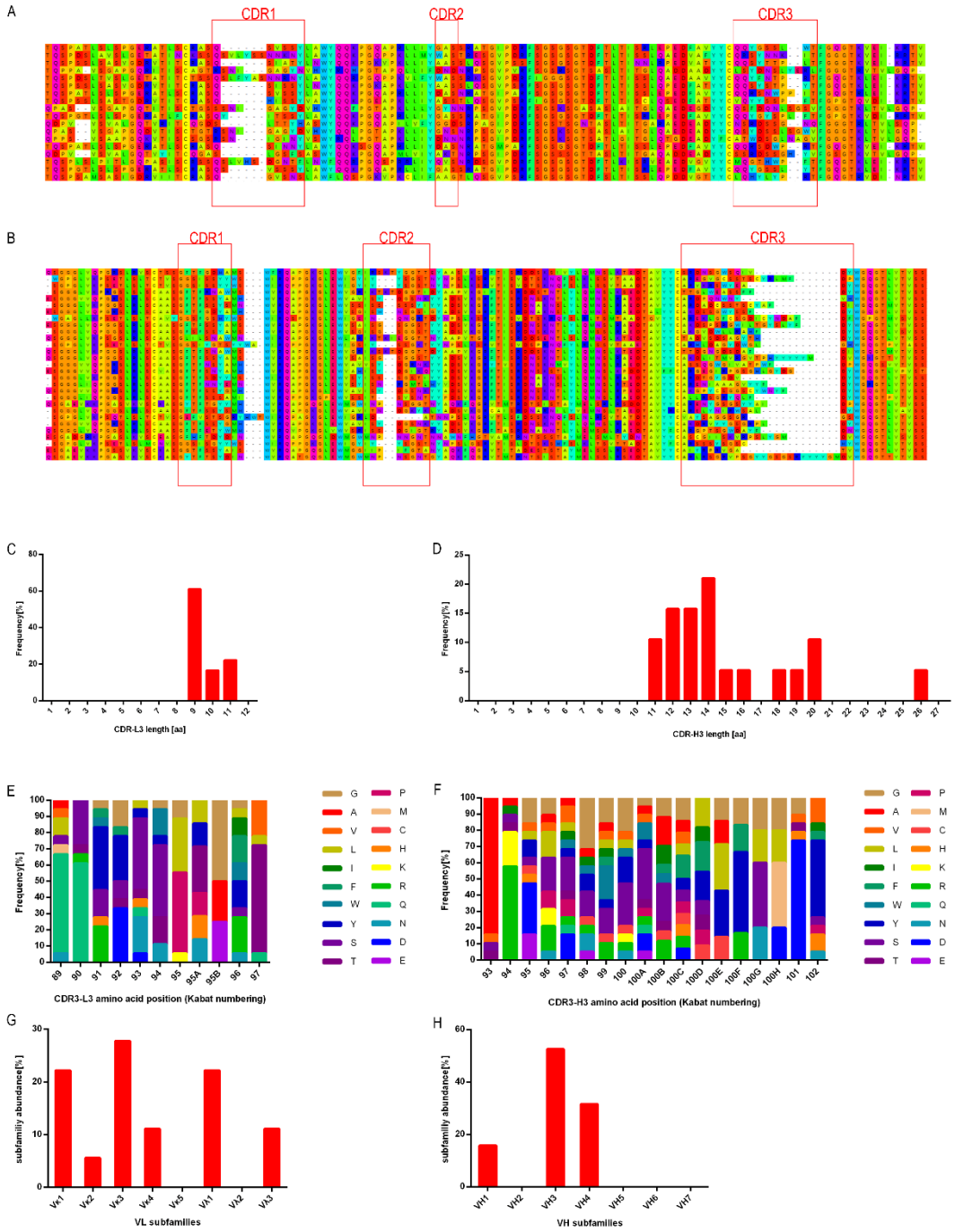

Fig. 3 Diversity analysis of antibody library

A) The amino acid sequences of the VL regions of the selected clones. B) The amino acid sequences of the VH regions of the selected clone. C) CDR-L3 length distribution in library as determined by DNA sequencing. D) CDR-H3 length distribution in library as determined by DNA sequencing. E) CDR-L3 amino acid distribution in library as determined by DNA sequencing. F) CDR-H3 amino acid distribution in library as determined by DNA sequencing. G) The distribution of the VL subfamily of antibodies in the library. H) The distribution of the VH subfamily of antibodies in the library.

Screening of anti-PD-L1 Fab antibody

Fab antibodies against PD-L1 were selected from the phage library. The anti-PD-L1 Fab library was screened through four rounds of PD-L1 adsorption-elution-enrichment. After four rounds of affinity selection, the harvest rate of phage antibodies showed an upward trend (Table 2), and the rate of harvest in the fourth round was 235.8 times that of the first round (Fig. 4A). After the four rounds of affinity selection, the result showed that PD-L1-specific phage

antibodies were effectively enriched. After four rounds of panning, polyclonal phage ELISA result indicated that numerous specific phage-Fab against PD-L1 was enriched (Fig. 4B). Forty-eight clones were randomly selected from the fourth round of coated plates and identified by monoclonal phage ELISA. In the phage-ELISA experiment, VCSM13 was used as a negative control. When the absorptive values of positive clones/the absorptive values of negative ones ≥2.1, it was judged as a positive clone. After identification by phage-ELISA, the results showed that seven clones (8, 9, 15, 25, 26, 28, 30) were positive (Fig. 4C).

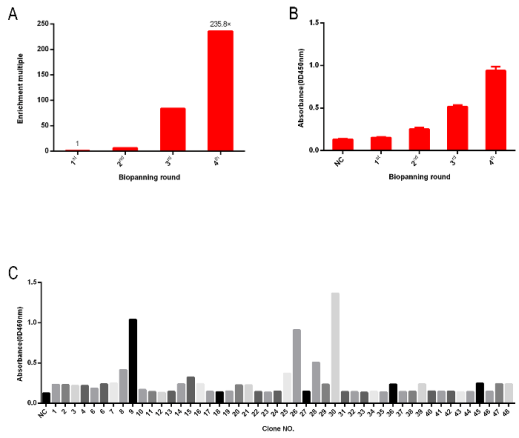

Fig. 4 The screening of phage antibody against PD-L1
A) After 4 rounds of biopanning for PD-L1 recombinant protein, the phage recovery rate was increased by 235.8-fold over that of the first round. B) Polyclonal phage ELISA for the PD-L1 specified phage particles. Binding of different phage outputs to PD-L1 as a target antigen，and VCSM13 as negative control. C) Selection of positive clones against PD-L1. 48 single clones from the plate of the last round of screening was randomly selected and detected by phage ELISA, and VCSM13 as negative control.

Table 2 The enrichment effect of PD-L1 as antigen on phage antibody library

Panning	Input pfu	Output pfu	Recovery rate (output/input)	Enrichment(rate(n)/rate(1))
1	2×10^{13}	1.51×10^{6}	7.55×10^{-8}	1
2	8×10^{12}	3.70×10^{6}	4.63×10^{-7}	6.1
3	3.6×10^{12}	2.28×10^{7}	6.33×10^{-6}	83.8
4	6.4×10^{12}	1.14×10^{8}	1.78×10^{-5}	235.7

PCR identification and sequence analysis

In order to identify whether the seven positive clones obtained through the preliminary screening of phage-ELISA had inserted the complete antibody light chain and Fd segment genes, PCR of bacterial liquid was carried out. The results of agarose electrophoresis showed that only four clones (9, 15, 26, 30) fully amplified the light chain and Fd genes (Fig. 5A,B). The four clones were sent to Sangon Biotech for sequencing and then the sequences were analyzed by IgBlast tool to identify any similarity between the sequences of Fabs and the human germline V, D, and J segments. The alignment results indicated that except for clone 15, which did not contain complete sequence of the antibody, the other three clones (9, 26, 30) all contain a complete antibody sequence. Among them, clone 9 and clone 30 had the same sequence, proving that the two clones are identical. The sequence analysis of the two positive clones sequenced correctly was as follows (Table 3). In addition, IgBlast analysis showed that the VJ genes in the light chain variable region of clone 9 came from the Vκ1 and Jκ4 gene families, and the VDJ genes in the heavy chain variable came from the VH3, DH4, and JH4 gene families. The VJ genes in the light chain variable region of clone 26 came from the Vλ1 and Jλ3 gene families, and the VDJ genes in the heavy chain variable region came from the VH1, DH5, and JH5 gene families.

Table 3 Sequence characteristics of positive clones

Fab	Germline genes	VL-CDR3	Length	Germline genes	HV-CDR3	Length
9	IGKV1-39*01	QQSYSVPLT	9	IGHV3-7*01	ARDSYGFDY	9
26	IGLV1-40*01	QSYDSLPSGSRV	12	IGHV1-69*04	ARGWDSGYEPSSWFDP	16

Soluble expression of positive clones and identification

The two clones identified as positive could be expressed by IPTG induction in XL1-Blue, producing a soluble Fab antibody. The binding activity of the antibody was verified by ELISA, the PD-L1 was coated, and HBsAg was used as an irrelevant control. Compared with the control, two positive clones had the highest absorbance at 450 nm (Fig. 5C).

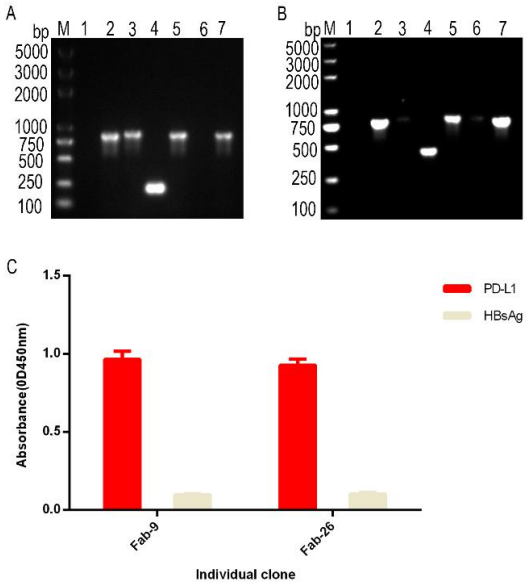

Fig. 5 PCR identification and Soluble expression of positive clones A) Agarose gel electrophoresis analysis of light chain genes of positive clones. M: DNA marker; lane1-7, PCR products of light chain genes. B) Agarose gel electrophoresis analysis of Fd genes of positive clones. M: DNA marker; lane1-7, PCR products of Fd genes. C) Soluble monoclonal ELISA in XL1-Blue strain. Binding of soluble monoclonal to PD-L1 as a target antigen, and HBsAg as negative control.

Discussion

The phage display technology has a wide potential application, particularly in the screening of humanized antibodies (Bostrom et al., 2009; Ebrahimizadeh and Rajabibazl, 2014; Weiner et al., 2010). Compared with scFv antibody, Fab antibody is more stable and not easy to form polymer (Fox et al., 2001). The binding activity of Fab fragment can maintain more effectively when the full-length IgG antibody is constructed (Schwimmer et al., 2013). In order to increase the diversity of antibody libraries, we collected peripheral blood from different age groups with different immune background. In our study, primers were designed according to Scripps Institute and related literature (Tian et al., 2000). According to the frequency of usage of antibody genes in the human body, the PCR products of antibody genes were mixed. After optimization, we finally determined that the molar ratio of vector to fragment was 1:3. To amplify the antibody library, the amount of helper phage (MIO) added was at least twenty times as bacterial (XL1-Blue) to ensure that each bacterium could be infected by helper phage. For rescuing the antibody library, it was necessary to take at least ten times as much as the library's capacity for amplification and rescue.

Using the peripheral blood of 78 individuals, we constructed a Fab antibody library with a capacity of 4.27×10^9, which was similar to the report by Jiao et al. (Jiao et al., 2005). The analyzed VH/VL sequences were derived from all sorts of VH/VL families. We found that VH3 (52.6%) accounted for the highest proportion in VH gene families, followed by VH4 (31.6%) and VH1 (15.8%), and in VL gene families, Vκ1 (22.2 %), Vκ3 (27.8 %) and Vλ1 (22.2%) was more common, which was consistent with the report by Tiller et al. (Kügler et al., 2015; Tiller et al., 2013). However, in the study of Zarei et al. (Zarei et al., 2020), the VH4 gene family was the most common. The amino acid length analysis showed that the number of amino acids in the CDR3 region of VL was mainly 9 and in the CDR3 region of VH was mainly 14, which was similar to the report by Kügler et al. (Bostrom et al., 2009). When analyzing the amino acid composition of the CDR region, we found that the frequency of alanine at the first position of CDR3-L was relatively high, while in the amino acid composition of CDR3-H, the frequency of serine and glycine were relatively high. The results were similar to the research of Kügler et al. (Kügler et al., 2015). Sequence analysis showed that the length of CDR3 in all sequences was different, particularly in heavy chains. In the heavy chain CDR3 region, the length was prolonged, up to 26 amino acids, and in the light chain CDR region, the length was shorter, with only 11 amino acids, which indicated that the heavy chain variable region was diverse. In the heavy chain CDR3 region, the sequences with 14 amino acids residue were accounted for more than 20% of all sequences. However, the study showed that the sequence with 16 amino acids residue in the heavy chain CDR3 region was accounted for the most by Zarei et al. (Zarei et al., 2020). In the light chain CDR3 region, the sequence with 9 amino acids residue was accounted for the most consistent with the report by Brígido et al. (Dantas-Barbosa et al., 2005).

We selected forty-eight individual clone to prepare recombinant phage antibodies, followed by identification using phage-ELISA. Subsequently, we obtained seven positive clones. Through further PCR identification and sequencing analysis, we finally obtained two positive clones . In addition, in our work, there was two clones (clone 9 and clone 30) showed the same sequence. The reason for selecting two identical clones might be that they were effectively enriched in the phage antibody library, which were resulted in a higher copy number and more accessible to be screened.

Conclusion

Based on the application prospects of the Fab phage antibody library, we constructed a natural human Fab phage antibody library with a library capacity of 4.27×10^9. Then, we analyzed the diversity and quality of the antibody library, hoping that the library could be used to screen humanized antibodies against various antigens.

Author contributions statement

Jiangtao Gu and Raoqing Guo designed and performed the experiments and drafted the manuscript. Ligang

Zhang revised the manuscript. Ning Deng supervised this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest
The authors declare that they have no conflict of interest.

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