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Development of tularemic scFv antibody fragments using phage display

Communication

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Abstract: Polyclonal antibodies, as well as monoclonal antibodies are efficacious in providing protective immunity against *Francisella tularensis*. This study demonstrates the application of phage display libraries for the construction of monoclonal antibodies against *F. tularensis*. Novel single-chain fragment variable (scFv) antibodies were generated against a whole bacterial lysate of *F. tularensis* live vaccine strain using the human single fold scFv libraries I (Tomlinson I + J). A total of 20 clones reacted with the bacterial cell lysate. Further, the library contains two clones responsive to recombinant lipoprotein FTT1103Δsignal (*F. tularensis* subsp. *tularensis* Schu S4), which was constructed without a signal sequence. These positively-binding scFvs were evaluated by scFv-phage enzyme-linked immunosorbent assay (ELISA). Then, positive scFvs were expressed in a soluble form in *Escherichia coli* HB2151 and tested for positive scFvs by using scFv-ELISA.

Keywords: *Francisella tularensis* • *Single-chain variable fragment* • *Monoclonal antibody* • *Phage display* • *Hypothetical lipoprotein FTT1103*

1. Introduction

1.1 Phage display

Monoclonal antibodies have been useful in research, diagnostics, prophylaxis and as therapeutics of infectious diseases. Several methods to produce human monoclonal antibodies are based on technology utilizing antigen-specific human B cell hybridoma [1], generation of chimeric and humanized antibodies by genetic engineering [2], or developing of transgenic mice for the production of human monoclonal antibodies [3]. Phage display is a technique for screening and identifying protein-protein interactions, first introduced in 1985 by George Smith as an expression vector used to present a foreign amino acid sequence capable of binding an antibody [4]. Since then, a large number of phage-displayed peptide or protein libraries have been constructed [5-8]. Phage display is an efficient production system that ensures an alternative antibody preparation together with working without animal immunization or hybridoma production. This technology can be used for preparing various types of antibodies or antibody fragments without using the specialized procedures or tools [9,10]. Phage display fuses a foreign DNA sequence to the phage genome and, as a result, the foreign protein is expressed at the surface of one of the phage major coat proteins.

Phage display-generated antibodies include a large spectrum of formats. The most popular format appears to be the single chain variable fragment (scFv) and its variants or Fab fragments of antibodies containing variable domains plus the first constant domains or $F(ab')_2$ consisting of two disulfide-linked Fab fragments. ScFv molecules are the smallest antibody fragments which contain a complete binding site, and are composed of the individual human immunoglobulin heavy chain variable (V_H) gene segments and human immunoglobulin light chain variable (V_L) gene segments with a typical flexible, hydrophilic polypeptide linker. The

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gene encoding single chain variable fragment (scFv) was produced by random combining V_H and V_L gene segments by using PCR [11,12].

With regard to antigenic specificity, three types of phage display libraries are currently being used for the selection of antibodies and they include immunized, naive, and synthetic phage [13-16]. The strength of phage display technology is in its ability to identify interactive regions of proteins and other molecules without pre-existing notions of the nature of the interaction. In contrast to naïve libraries enabling isolation of human antibodies to self-, non-immunogenic or immunogenic antigens, synthetic libraries have a major potential to resume diversity of the library. Isolation of an advisable phage from a phage antibody library may require a combination of different selection methods, which include biopanning, biotinylation, and affinity columns. Antibody fragments are expressed on the surface of the filamentous bacteriophage. Bacteriophages (also referred to as phages) mostly used in phage display technology are single-stranded DNA viruses that infect a wide variety of gram-negative bacteria. The filamentous phage particles of the Ff class mostly used for display purposes include strains M13, f1, Fd and ft. This class of phages can be extensively used as a reagent to detect antigens, tumor cells, viruses or toxins. Most of the currently used phage display vectors use the N-terminus of pIII protein or pVIII protein to display the foreign peptide or protein [7]. The pIII libraries display 3-5 copies of each individual peptide [7], whereas pVIII libraries can display up to 2700 copies of small peptides [17].

In general, the fundamentals steps of workflow with phage display library comprise preparation of a primary library or amplification of an existing library, exposure of the phage particles to a target (immobilized protein/cell surface) for which specific ligands are planned to be discovered, removal of non-specific binders (washing), phage elution and infection a suitable bacterial host. Consequent steps are phage antibody screening and preparing different antibody formats. The repeated cycling of these steps is referred to as 'panning'. This affinity selection ligand procedure can be repeated several times until a population is enriched in binding partners. By sequencing the phage genome encoding the displayed protein or protein fragment, one can determine and reproduce its sequence and finally determine specific and selective relevant ligands to target receptors.

1.2 Novel essential virulence factor of *F. tularensis*

Francisella tularensis is a facultative intracellular bacterium that can infect and proliferate inside a variety of host cell types including hepatocytes, macrophages, endothelial cells, and fibroblasts [18-20]. An attenuated *F. tularensis* subsp. *holarctica* live vaccine strain (*F. tularensis* LVS) is used for human vaccination [21], however is fully pathogenic for specific animals and lethal for mice [22,23]. Among the most virulent strains, the strain Schu S4 of *F. tularensis* subsp. *tularensis* (*F. tularensis* Schu S4), is a highly virulent human pathogen. As few as 10 organisms injected subcutaneously or 25 inhaled organisms can lead to various manifestations of tularemic infection [24,25]. Recently, a novel locus, FTT1103, *F. tularensis* Schu S4 has been identified [26]. Conserved hypothetical protein FTT1103 is composed of 365 amino acids and specified by MW 38,72 kDa and pI 5,11. According to the LipoP prediction program (http://www.cbs.dtu.dk/services/LipoP/, January, 2010), FTT1103 seems to be a membrane lipoprotein [27]. It is highly probable that protein FTT1103 belongs to a group of periplasmatic proteins [26], because its N-terminal signal peptide is eliminated *via* signal peptidase II [27]. The locus FTT1103 is predicted to encode a hypothetical lipoprotein which shares some similarity to DsbA proteins that catalyze disulfide bond formation. The activity of DsbA has been described to be important for the formation of various virulence factors including the bacterial type IV pili biogenesis, efflux pumps and function of type III secretion system [28-33]. However, the function of the protein FTT1103 remains unknown. The FTT1103 mutant strain was found to be defective in intracellular growth and it was observed that in BALB/c monocyte/macrophage cell line J774A.1 has a decreased ability to escape the phagosome [26]. Despite its requirement for virulence, this protein is not an essential protective antigen.

It has been reported that 83% of mice that were challenged intranasally or *via* aerosol infection with *F. tularensis* SchuS4 survived [26]. The FTT1103 lipoprotein has been also shown to be capable of inducing toll-like receptor 2 (TLR-2)-dependent proinflamatory chemokine production operating within innate immunity [34].

Very little is known about how *F. tularensis* causes disease at a molecular level and only a few potential virulence factors have been identified so far. Thus monoclonal anti-FTT1103 antibody, which could be prepared using the phage display method, might be

used to neutralize this bacterial protein, to confirm the subcellular localization, and elucidate its pathogenicity effect.

2. Experimental Procedures

2.1 Bacteria

Escherichia coli (E. coli) TG1Tr (K12, Δ(*lac-pro*), *sup*E, *thi, hsd*Δ5/F'*tra*D36, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15) was obtained from the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK) and kindly provided by Juraj Ivanyi`s lab. The strain was maintained on M9 minimal media agar plates, at 4°C containing 200 ml of 10x salts (0.22 M Na_2HPO_4 $7H_20$, 0.22 M KH_2PO_4, 0.09 M NaCl, and 5.35 M NH_4Cl/l dH_2O), 20 ml 20% glucose (filter sterilized), 1 ml of 1 M $MgCl_2$ (autoclaved), 500 µl thiamine (100 µg/ml,filter sterilized) and 15 g agar in 750 ml dH_2O at pH 7.0 [35]. *E. coli* TG1Tr single colonies were picked from M9 Minimal agar plates to 10 ml 2TY (16 g tryptone, 10 g trast extract, 5 g NaCl/l and pH 7.4) and incubated overnight at 37°C to produce working culture.

The strain of *E. coli* HB2151 was also kindly provided by Juraj Ivanyi`s lab which was obtained from the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK).

The strain of *F. tularensis* LVS (ATCC 29684, Rockville, MD, USA, batch No. 89-05 MED 192) has been used for preparing of bacterial lysate.

2.2 Library

The recombinant scFvs were issued from only one human single fold scFv library (Library I provided by MRC Geneservice). Library I is based on a single human framework for VH (V3–23/DP-47 and J_H4b) and Vκ (O12/O2/DPK9 and Jκ1) that can be selected and affinity matured without knowing the sequences of selected clones. The phagemid vector is derived from pHEN1 and contains a lac promotor and a pelB leader sequence, which is then followed by His6, myc tags, an amber codon, and the gene encoding the pIII phage coat protein. This vector is designed for the insertion of heavy and light chain variable domain for the production of a single-chain Fv antibody on the surface of M13 bacteriophage. The library I has been pre-tested for binding to Protein A or Protein L and constructed in the pIT2 vector (derived from pHEN1). The library size is about 1.48 x 10^8 different scFv fragments.

2.3 Chemicals and reagens

The helper phage KM13 was kindly provided by Juraj Ivanyi´s lab (Prof. Juraj Ivanyi, Clinical and Diagnostic Sciences Group, Kings College London, UK), recombinant protein FTT1103Δsignal (Apronex, Czech Republic), Mueller-Hinton agar (Difco, USA), IsoVitaleX (Becton-Dickinson, USA), Trypsin, from bovine pancreas (Sigma, USA), mouse anti-M13 monoclonal antibody (GE HealthCare, UK), anti-mouse immunoglobulins/HRP (Dako Cytomation, Denmark), ImmunoPure Protein L-Peroxidase conjugated (Pierce, USA), 3,3´,5,5´´-tetramethylbenzidine (TMB; Cell Signaling Technology, USA), Isopropyl β-D-1thiogalactopyranoside (IPTG) (Sigma, USA), Carbenicillin disodium salt (Sigma, USA), Kanamycin sulfate from *Streptomyces kanamyceticus* (Sigma, USA), Tetracyclin (Sigma, USA). Other commonly used reagens are all of analytic purification grade and made in Czech Republic.

2.4 Preparation of bacterial lysate

As the basal medium, Mueller-Hinton agar supplemented with 2% (vol/vol) IsoVitaleX, 0.1% (wt/vol) glucose, and 1% (vol/vol) fetal bovine serum (FBS) was used in these studies. *F. tularensis* LVS was routinely grown in broth at 36.8°C in an atmosphere of 100% air. The strain was cultured in iron-rich chemically-defined medium prepared according to Chamberlain (1965), containing 0.0002% of ferrous sulfate heptahydrate. *F. tularensis* LVS was inoculated from a deep-frozen prestock onto McLeod agar plates supplemented with bovine haemoglobin and IsoVitaleX and grown at 36.8°C for 48 h. This stock culture was stored at 4°C up to four weeks. Before each experiment, the stock culture was inoculated into iron-rich medium and grown overnight with mild shaking at 36.8°C. The next day, the culture (750 ml, optical density $OD_{600\ nm}$ 0.1) was prepared and incubated for 10 h until $OD_{600\ nm}$ 1.3-1.5. After incubation, bacterial cultures were harvested by centrifugation at 6,500 x g for 15 min at 4°C, cell pellets were resuspended in 10 ml of PBS and centrifuged again at 6,500 x g for 15 min at 4°C. The washing step with PBS was repeated twice. After last washing step, bacterial cultures were resuspended in 13 ml of PBS and 240 µl of protease inhibitors EDTA-free coctail was added. The cells were disrupted using a French press (Hermo IEC, Needham Heights, USA) twice at 16,000 psi and unbroken cells were removed by centrifugation at 12,000 x g for 30 min. The protein concentration of the cell preparation was determined by the bicinchonic acid assay (BCA), 70 µg of protein was loaded into each well for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

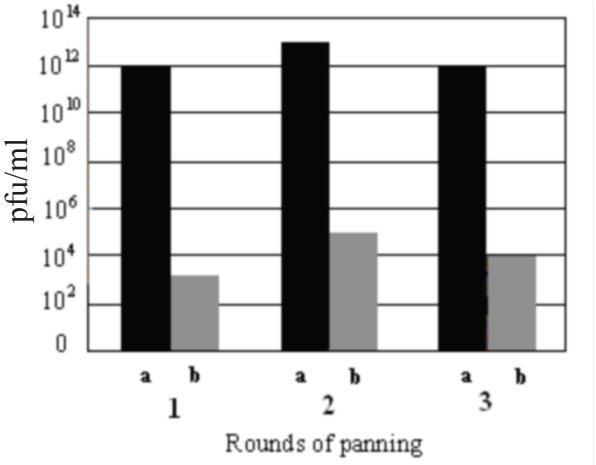

Figure 1. Number of amplified scFv-phages after each round of selection with *F. tularensis* LVS lysate. (a) The number of amplified scFv-phages after each panning assay, which were subsequently used in another panning round. (b) The number of eluted scFv-phages from each panning round. The titer was determined as a plaque forming unit pfu/ml (with using Tomlinson I Library).

2.5 Preparation of recombinant protein for panning

F. tularensis Schu S4 recombinant lipoprotein, FTT1103Δsignal, was constructed without a signal sequence by Apronex Biotechnologie, Czech Republic. This protein was obtained in a storage buffer containing 20 mM HEPES, 300 mM NaCl, 0.01% Tween and 5% glycerol with a protein concentration of 0.2 ml per vial. This storage buffer was not suitable for panning procedure, thus we used the Mini Dialysis Kit (Amersham Biosciences, UK) for buffer exchanging the sample into PBS. The protein concentration was determined by the BCA and 70 µg of protein was loaded onto SDS-PAGE.

2.6 Phage library preparation

Tomlinson Library I contains about 10^8 different single polypeptides with the VH and Vκ domains joined to one another by a flexible glycine-serine linker. These human scFv fragments are cloned in an ampicilin resistant phagemid vector pIT2 and transformed into *E. coli* TG1 cells. The human DNA fragments are amplified by PCR with using compatible primers. The library in the phagemid/scFv antibody format was already tested for positive binding to generic ligands, Protein A and Protein L thus, suggesting the presence of binding sites for these proteins.

Phage library (100 µl) was incubated in 200 ml of 2TY medium containing 50 µg/ml carbenicillin and 1% glucose at 37°C with shaking until OD_{600nm} 0.4. Next, 50 ml of the library culture was infected with 2 x 10^{11} M13KO7 helper phage and incubated at 37°C for 30 min without shaking followed by centrifugation at 3,000 x g for 10 min. The pellet was resuspended in 100 ml 2TY containing 50 µg/ml carbenicillin, 50 µg/ml kanamycin and 0.1% glucose. Resuspended pellets were incubated at 30°C overnight with shaking. Next day, overnight culture was centrifuged at room temperature at 3,300 x g for 30 min. After centrifugation, 20 ml of PEG/NaCl was added to 80 ml of supernatant to mix and incubate on ice for one hour. The step of centrifugation was repeated, PEG/NaCl was discarded and pellet was resuspended in 8 ml of PBS and 2 ml of PEG/NaCl to stay on ice for 20 min. Culture was subsequently centrifuged at 3,300 x g at 4°C for 30 min, all remaining PEG/NaCl was aspired and pellet was resuspended in 4 ml of PBS before final spin at 11,000 x g at 4°C for 10 min, in order to remove any bacterial debris.

2.7 Titering phage

Filamentous phage, M13K07, is a non-lytic phage. Plaque-forming unit (pfu) of phage was obtained by counting phage infected *E. coli* TG1Tr colonies growing on TYE (15 g agar, 8 g NaCl, 10 g tryptone, 5 g yeast extract/l). Phage solutions were titrated by diluting with TY and incubated with log-phase *E. coli* TG1Tr at 37°C for 30 min and subsequently plated on TYE+tet plates [36].

2.8 Panning assay

In total, we used three rounds of selection during the panning procedure. For the first round of the selection, 100 µg/ml of antigen per 4 ml PBS was coated, for the second round, coating was 50 µg/ml of antigen per 4 ml

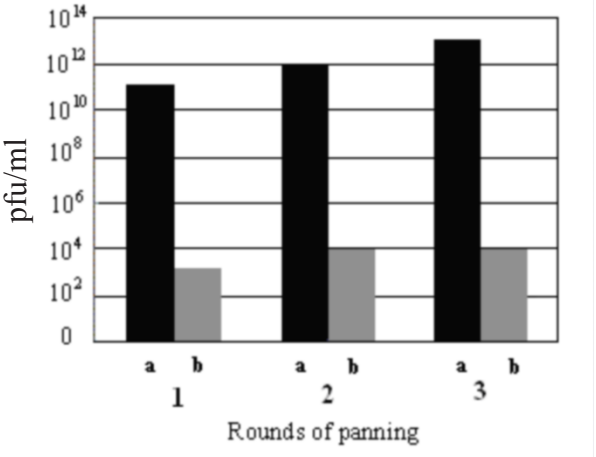

Figure 2. Number of amplified scFv-phages after each round of selection with FTT1103Δsignal protein. (a) The number of amplified scFv-phages after each panning assay, which were subsequently used in another panning round. (b) The number of eluted scFv-phages from each panning round. The titer was determined as a plaque forming unit pfu/ml (with using Tomlinson I Library).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.060	0.058	**0.403**	0.077	**0.209**	**0.382**	**0.256**	**0.241**	0.064	0.088	0.074	**0.344**
B	0.061	0.055	0.057	**0.176**	0.109	0.074	0.068	0.058	0.062	0.071	**0.335**	0.050
C	0.063	0.059	0.062	0.075	0.092	0.064	0.064	**0.311**	0.060	0.056	0.050	0.060
D	0.060	0.052	**0.186**	**0.201**	0.060	0.089	0.063	**0.326**	0.099	0.074	0.061	**0.199**
E	0.060	0.083	0.087	0.060	0.063	0.058	0.066	0.065	0.068	0.079	0.065	0.050
F	0.062	**0.296**	**0.228**	0.087	0.074	0.080	0.085	0.075	0.067	0.065	**0.287**	0.092
G	0.090	0.068	0.093	**0.252**	**0.263**	0.060	0.054	0.062	0.065	0.058	**0.406**	0.064
H	0.056	0.057	0.069	0.055	0.050	0.071	0.057	0.062	0.063	0.059	0.064	**0.402**

Table 1. The scFv-ELISA of positive clones carrying scFv. OD values of scFv samples of bacterial lysate panning (positive scFvs are highlighted in bold).

PBS, and for the last round of the selection, 10 µg/ml of antigen per 4 ml PBS was used. During the first two rounds of selection, Eppendorf tubes were coated with an equivalent concentration of *F. tularensis* LVS bacterial lysate and the recombinant protein, FTT1103Δsignal. The tubes were blocked overnight at 4°C. Next day, we inoculated 20 ml of 2TY with a single colony of *E. coli* TG1Tr from Minimal media and incubated at 37°C with shaking until OD_{600nm} 0.4-0.5. The tubes were then washed three times with PBS, filled with 2% skimmed milk powder (MPBS) and blocked for 2 h at room temperature. Tubes were washed three times with PBS after the blocking solution was removed. Next, 200 µl of the primary library solution (10^{12} to 10^{13} phages in 2% MPBS) were added and incubated at room temperature for 1 h with rotation and 1 h without rotation. The nonspecific binding phages were removed by washing the tubes with a 0.1% Tween PBS (PBST) 10 times (20 times in the second and third panning round) and the retained phages were eluted with 500 µl of trypsin-PBS (dilute trypsin with PBS 1:10). Subsequently, 1.75 ml of *E. coli* TG1Tr (OD_{600nm} 0.4-0.5) cultured in 2TY was infected with 250 µl of eluted phages for 30 min at 37°C without shaking, then a portion of the infected *E. coli* TG1Tr was serially diluted into 10^0, 10^1, 10^2, 10^3 and 10^4, and 5 µl of each dilution was spotted separately on TYE plates containing 50 µg/ml carbenicillin and 1% glucose. The plates were incubated overnight at 37°C to titer the eluted phage. At the same time, the remaining infected *E. coli* TG1Tr was centrifuged at 11,600 x g, for 5 min at 4°C and the pellet was resuspended in 2 ml of 2TY all spread on TYE plate (including 50 µg/ml carbenicillin and 1% glucose). The next day, bacteria on the plate were scrapped using a spreader and added into 10 ml of 2TY (including 50 µg/ml carbenicillin and 1% glucose) for amplification. Then 25 ml of 2TY was inoculated with 100 µl of scraped bacteria and incubated at 37°C until OD_{600nm} 0.5. After that, the phages amplified in *E. coli* TG1Tr were rescued by helper phage KM13 and 10^{12} rescued phages were added into 2% MPBS according to the titer result for the next round of selection (Figures 1, 2).

3. Results

The use of the phage library for production of monoclonal antibodies reacting with *F. tularensis* antigens comprise the firts such attempt to obtain tularemic scFv fragment antibody. With all different protocols, we have selected the experimental conditions of undermentioned procedures according to the obtaining maximum positive phage clones. After three rounds of the panning assay, 96 clones were randomly picked out and assessed for binding ability to bacterial lysate or recombinant protein FTT1103Δsignal by phage ELISA. Of these 96 clones,

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.027	0.023	**0.283**	0.021	0.031	0.022	0.035	0.032	0.038	0.033	0.037	0.045
B	0.028	0.020	0.017	0.024	0.030	0.027	0.026	0.035	0.029	0.034	0.043	0.044
C	0.024	0.019	0.024	0.023	0.035	0.028	0.029	0.035	0.034	0.034	0.052	0.044
D	0.025	0.015	0.016	0.021	0.026	0.019	0.021	0.025	0.026	0.017	0.028	0.037
E	0.030	0.026	0.026	0.031	0.039	0.031	0.027	0.035	0.034	0.036	0.038	0.048
F	0.029	0.028	0.021	0.021	0.035	0.020	0.026	0.028	0.031	0.039	0.044	0.046
G	0.029	0.024	0.025	0.033	0.038	0.030	0.033	0.032	0.033	0.037	**0.223**	0.042
H	0.043	0.034	0.030	0.028	0.036	0.032	0.036	0.028	0.034	0.039	0.026	0.065

Table 2. The scFv-ELISA of positive clones carrying scFv. OD values of scFvs samples of tularemic recombinant protein panning (positive scFv are highlighted in bold).

twenty with tularemic bacterial lysate binding activity were observed and two with FTT1103Δsignal protein binding ability (Table 1, 2).

3.1 Rescue of single clones for phage enzyme-linked immunosorbent assay (ELISA)

After the last panning and selection round, 96 single clones were randomly selected from large Bio-Assay TYE dishes using pipette tips and placed separately into wells (100 µl 2TY including 50 µg/ml carbenicillin and 1% glucose was added in advance) of a 96-well ELISA plate. Plates were incubated at 30°C overnight with shaking at 125 rpm. After culturing, 5 µl of inoculum was transferred to another plate containing 100 µl 2TY in each well. The master plates were stored at 4°C. Transfer plates were shaken for 5-6 h at 37°C. Phages were rescued in *E. coli* TG1Tr by adding 10^9 helper phages to each well, shaken for 1 h at 37°C before spinning plates at 2000 × g for 15 min. Pellets were resuspended in 100 µl 2TY (including 50 µg/ml carbenicillin and 50 µg/ml kanamycin with no addition of glucose) and incubated overnight at 30°C with shaking to produce phage particles. The next day, replica plates were centrifuged at 2000 × g for 10 min and 100 µl of the supernatants containing phage were used in monoclonal phage ELISA.

3.2 Monoclonal phage-ELISA

Wells from a microtitre plate were coated with 100 µl of antigen diluted in PBS prepared as for the Panning assay described in this paper. The control plate was coated with PBS without presence of antigen. The next day, the ELISA plates with coated antigens were removed from 4°C and incubated for 1 h at 37°C. The coating material was discarded and plates were washed three times with PBS. After washing, plates were blocked with 150 µl 2% MPBS for 1 h. At the same time, 100 µl of each culture supernatant from replica plates were transferred to plates containing 50 µl of 9% MPBS, mixed and incubated at room temperature for 30 min. Subsequently, 50 µl of each blocked phage was transferred to each of the washed antigen-coated and control plates and incubated for 2 h at room temperature. After washing the plates three times with PBS and three times with PBST, 50 µl of horseradish peroxidase (HRP)-anti M13 antibody diluted to 1:5000 in 2% MPBS was added. All plates were incubated for 1 h at room temperature and then washed again three times with PBS and PBST. After incubation and washing, the 100 µl TMB substrate solution was added to each well. The reaction was stopped by adding 100 µl 2 M H_2SO_4. The absorbance at OD_{450nm} was determined.

3.3 Production of soluble antibody fragments

Selected phages were used to infect *E. coli* HB2151 (a non-suppresor strain) which was induced to produce soluble expression of antibody fragments. The selected scFv-phages were inoculated into *E. coli* HB2151 for the expression of a soluble scFv antibody. *E. coli* HB2151 was grown in 2TY culture medium at 37°C until OD_{600nm} 0.4. Then 50 µl of the culture was infected with 2 µl of phages obtained from an individual positive clone. Subsequently, the culture was incubated for 1 h at 37°C without shaking and plated on TYE agar with 50 µg/ml carbenicillin. From this plate, a single colony was picked out and inoculated into 25 ml 2TY (including 50 µg/ml carbenicillin and 1% glucose) and incubated with shaking at 250 rpm overnight at 37°C. The next day, 50 µl of the overnight culture was transferred to 10 ml 2TY and incubated at 37°C with shaking at 250 rpm until OD_{600nm} 0.9. IPTG with final concentration 1 mM was added into the culture and incubation was continued with shaking at 30°C.

3.4 Purified scFv ELISA

The scFv-ELISA was used for testing of positive clones carrying scFvs. The day before the scFv-ELISA assay, different antigens (*F. tularensis* LVS lysate and the recombinant protein FTT1103Δsignal) were coated in wells of 96-ELISA plates overnight at 4°C. The control plate was coated with PBS. Next day, ELISA plates with different coated antigens were removed from 4°C and incubated for 1 h at 37°C. Then the coating material was discarded, washed three times with PBS and blocked with 2% MPBS for 1 h at 37°C. Subsequently, plates were washed for three times with PBS before adding purified scFv (diluted in 9% MPBS). After incubation for 2 h at 37°C, the plates were washed three times with PBST. Next, 50 µl Protein L-HRP, which was diluted 1:5000 in 2% MPBS, was added into each well, followed by 1 h incubation at room temperature and three times washing with PBS. After incubation and washing, the 100 µl TMB substrate solution was added to each well. The reaction was finished by adding 100 µl 2 M H_2SO_4. The absorbance at OD_{450nm} was read on ELISA reader.

4. Discussion

Microbial epitope mapping has significance in infectious disease studies, especially for the novel vaccine development [7]. Phage display is mainly useful for mapping potential bacterial vaccine candidates, tracking the subcellular localization of microbial components or intracellular traficking of bacteria themselves. Human monoclonal antibody research has advanced the

development of biological pharmaceuticals. Antibodies might have more fuctions and usefulness than presently characterized.

A new approach for preparing human monoclonal antibodies was developed with the Human Single Fold scFv library I (Tomlinson I+J). For the selection of human tularemic specific scFv antibodies, tularemic bacterial lysate and one successful antigen classified as a novel important factor of *F. tularensis* virulence was used. After three rounds of panning, 20 clones with tularemic bacterial lysate binding activity were observed and two with FTT1103Δsignal protein binding ability. These two clones were chosen for further analysis. Future research will include designing the antibody with suitable isotype that will be selected according to the knowledge of the molecular mechanism of tularemic infection.

Recently is clear, that the localization of protein molecules in individual subcellular compartments has direct relation to the microbe pathogenicity [37]. The importance of FTT1103 protein as the crucial factor of virulence has been described [26], the subcellular distribution of this important molecule is still a matter of debate A novel proposed anti-FTT1103 monoclonal antibodies prepared *via* phage display will be used for the demonstration of subcellular localization of this protein by employing immunoelectron microscopy and other similar techniques. As for the verification of protectivity of the proposed anti-FTT1103 monoclonal antibody, the protective function and the degree of protectivity during tularemic infection in an animal model could be validated according to the designed antibody isotype, the dose, and the route of infection.

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