

## Original Article

# Variable domain of the heavy chain of heavy-chain antibody of the Rv0733 antigen of mycobacterium tuberculosis panned and identified from a nonimmune llama VHH phage display library

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**Abstract:** The ability of *Mycobacterium tuberculosis* to cause disease is closely correlated with its ability to enter into and survive inside, what is currently thought to be, its permitted host cell, the macrophage. Rv0733 is one of the key factors for living *M. tuberculosis* to maintain intracellular nucleotide pools. Variable domain of the heavy chain of heavy-chain antibody (VHH) is one of the essential molecules for neutralizing antigens of the intracellular pathogen. Recombinant nonimmune llama VHH phage display antibody library was enriched after three rounds of panning with *M. tuberculosis* Rv0733-6His fusion antigen. The size of the VHH phage library was reduced from  $1.5 \times 10^9$  to  $4 \times 10^3$ , but the antigen-binding activity increased from 0.3-0.6 to 2.5-2.8. Eleven independent sequences were obtained from 1024 enriched clones by phage enzyme-linked immunosorbent assay and sequencing technology. The recombinant expression vector constructed by cloning Rv0733-VHH gene into the prokaryotic expression plasmid pET-22b-IFH harboring Fc portion of human IgG and 6His-tag gene was transformed into *Escherichia coli* for induction and expression of fusion antibodies to determine their potential in binding with Rv0733 antigen. The purity and the size of the expressed Rv0733-VHH-Fc-6His antibodies were confirmed by the Western blot. Immunofluorescence staining confirmed the specificity of the Rv0733-VHH-Fc-6His antibody binding to Rv0733 antigen. Treatment of macrophages with pcDNA3.1-Rv0733-VHH -6His led to reduced mycobacterial loads 0.31-fold ( $P < 0.05$ ) as compared with control cells by day 7 post-infection. These findings may provide an alternative approach for further studies on Rv0733-VHH antibody binding with intracellular *M. tuberculosis* antigen and improve the bactericidal ability of macrophages.

**Keywords:** *Mycobacterium tuberculosis*, expression, purification, variable domain of the heavy chain of heavy-chain antibody, phage display antibody library

## Introduction

Variable domain of the heavy chain of heavy-chain antibodies (VHHs) are fully functional antigen-binding fragments. These single variable domains of homodimeric antibodies lacking light chains within cartilaginous fish and camelids are easily cloned using genetic engineering approaches [1, 2]. The advantages of VHHs include their small size, high apparent stability, good tissue penetration in vivo, improved solubility, high expression levels in microorganisms, and the recognition of unique epitopes [3]. Multiple therapeutic intracellular

functional antibodies have been or are being developed from VHHs [4]. Tuberculosis (TB) is currently the second largest killer infectious agent after human immunodeficiency virus [5]. However, currently available commercial serodiagnostic tests provide inconsistent and imprecise findings. The World Health Organization issued a policy statement against the use of commercial serological tests for diagnosing active pulmonary TB [6]. Therefore, accurate, rapid and relatively inexpensive antibody-based tests for diagnosing TB and its treatment are needed urgently [7]. The sequence of Rv0733 (*adk*) from *Mycobacterium tuberculosis* enco-

des antigen adenylate kinase (20.09 kDa). It is essential for the bacteria in intracellular nucleotide metabolism [8]. The enzyme-linked immunosorbent assay (ELISA) plates coated with the purified Rv0733-6His fusion antigens were used to select VHHs specific for the Rv0733 antigens from the nonimmune Llama VHH phage display antibody library with a capacity of  $10^9$  [9, 10]. The cDNAs of the selected clones were re-cloned into a pET-22b vector containing C-terminal 6His-tags. The property of the VHH-6His fusion protein constructs could be detected by conventional immunotechniques.

## Materials and methods

### *Plasmids, antigens, bacterial strains, and cell lines*

Plasmid pCANTAB5E and M13K07 helper phage were purchased from Pharmacia Biotech, Uppsala, Sweden. The plasmids pET-22b carrying 6His-tag and BL21 (DE3) pLysS competent cells were purchased from Promega, Madison, US. *Escherichia coli* strains TG1 harboring the nonimmune Llama (*Lama glama*) VHH phage display antibody libraries (pCANTAB5E-VHH), 60 kDa-6His chaperonin from *Methylobacterium oxalidis*, Rv0733-6His antigen from *M. tuberculosis*, the plasmids pET-22b-IFH carrying human Fc fragment and 6His-tag were kept in our laboratory. The attenuated strain of *Mycobacterium bovis* BCG Pasteur, *Salmonella typhimurium* strain ATCC14028 and the THP-1 cell line, derived from human acute monocytic leukemia, was purchased from American Type Culture Collection, Manassas, VA, US.

### *Main reagents and instruments*

Mouse anti-M13 horseradish peroxidase (HRP)-conjugated antibody was purchased from Pharmacia Biotech, Uppsala, Sweden. Goat anti-human IgG (H+L) HRP conjugate secondary antibody was purchased from Thermo Fisher, Rockford, US. Restriction endonucleases were purchased from New England Biolabs, Nebraska, US. Plasmid preparation kit, DNA gel extraction kit, Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), His-Select<sup>®</sup> nickel magnetic agarose beads, phorbol 12-myristate 13-acetate (PMA) and saponin were purchased from Sigma, St. Louis, US. Polyoxymethylene 16% were purchased from Polysciences, Warrington, USA. Anti-his-tag-Alexa Fluor 488, goat anti-human Alexa Fluor 594-conjugated IgG, Image-iT<sup>®</sup> FX signal enhancer and Lipofectamine 2000

transfection kit were purchased from Invitrogen, Carlsbad, US. Amicon ultra-2 centrifugal filter unit with ultracel-3 membrane was purchased from Millipore, Bedford, US. 2 $\times$  Polymerase chain reactions (PCR) master mix was purchased from Takara, Dalian, China. Oberver. Z1 fluorescent microscope was purchased from CarlZeiss, Jena, Germany. ChemiDoc MP imaging system was purchased from Bio-Rad, Hercules, US.

### *Phage rescue of the naïve VHH library*

The construction of the phage library was described previously [9]. Briefly, *Escherichia coli* TG1 cells harboring the nonimmune Llama VHH phage display antibody libraries were propagated in 2 $\times$  yeast extract and tryptone growth medium with ampicillin and glucose (2YTAG) (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.2 supplemented with 100  $\mu$ g/mL ampicillin and 1% glucose) at 37°C until  $A_{600}$  reached around 0.6. M13K07 helper phages were added at a multiplicity of infection (MOI) of 20:1 (phage-to-cells ratio) to a final concentration of  $10^{10}$  pfu/mL, incubated at 37°C for 20 min without shaking, and then incubated for 1 h at 37°C with gentle shaking. Kanomycin (50  $\mu$ g/mL) and IPTG (0.1 mmol/L) were added and incubated for 10 h by shaking at 250 rpm at 30°C. The phage-containing supernatant was harvested, 1/5 volume polyethylene glycol/NaCl (20% polyethylene glycol 8000 in 2.5 mol/L NaCl in water) was added and mixed well, and precipitated on ice for 1 h. Phages were spun-down, the visible pellet was resuspended in phosphate-buffered saline (PBS: 1.47 mmol/L  $\text{KH}_2\text{PO}_4$ , 8.10 mmol/L  $\text{Na}_2\text{HPO}_4$ , 136.89 mmol/L NaCl, 2.68 mmol/L KCl) as VHH antibody libraries, and stored at 4°C.

### *Panning of the VHH library*

The VHH phage display libraries were panned against purified Rv0733-6His as previously described [11]. Briefly, flat-bottom 96-well Costar<sup>®</sup> plates were coated with 100  $\mu$ L per well of each purified Rv0733-6His antigen at a concentration of 10  $\mu$ g/mL in carbonate buffer (15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ , pH 9.6) overnight at 4°C. Wells were washed three times with PBS and nonspecific binding was blocked with a blocking buffer of 2% bovine serum albumin (BSA) (w/v) in PBS for 1 h at room temperature (RT). The VHH phage display libraries were mixed with an equal volume of blocking buffer

in the plates for 1 h at 37°C. Wells were washed 10 times with phosphate-buffered saline with Tween-20 (PBST) (0.05% Tween 20 in PBS) and 5 times with PBS. Bound phages were eluted with glycine buffer (0.1 mol/L HCl, 0.2 mol/L glycine, pH 2.2) for 10 min at RT by shaking, transferred to tubes, and immediately neutralized with a half volume of 1 mol/L Tris-HCl pH 7.4. The neutralized phages were collected and stored at 4°C. Subsequently, the neutralized phages were inoculated with 10-fold volumes of log-phase TG1 bacteria in 2× yeast extract and tryptone growth (2YT) medium for 30 min at RT, and then grown continuously by shaking at 250 rpm at 37°C for 1 h. After centrifugation, the cell pellets were resuspended in 1/10 original volume of fresh 2YT medium. Ten-fold serial dilutions of 10 µL cell suspension were used to establish the phage titer. The diluted cells were plated onto 2YT agar plates and incubated overnight at 37°C for determining the colony-forming unit (CFU). The remaining phage-infected cells were plated onto 2YT plates containing 50 µg/mL ampicillin and 10 µg/mL tetracycline, which were incubated overnight at 37°C. All grown colonies were scraped, one part was resuspended in 2YT containing 20% glycerol and stored at -80°C, and the other part was resuspended in 2YT for the next round of panning. In the second and third rounds of panning, the coating antigen concentration was decreased to 5 and 2.5 µg/mL to select affinity clones. After each round of panning, the enrichment was determined by the output/input ratio of phages, and 20 individual randomly picked clones were sequenced by Shanghai HuaGene Biotech Co. Ltd., Shanghai, China. Sequence analysis and alignment were performed by DNAMAN software.

#### *Detecting the binding activity of phage clones by indirect ELISA*

After three rounds of panning, single colony was randomly selected and inoculated with 300 µL 2YT in 96-deepwell plates. After growing overnight at 37°C without agitation, 50 µL cultures per well were inoculated with 300 µL fresh 2YT in new 96-deepwell plates. After incubating for about 1~1.5 h at 37°C with shaking until  $A_{600} \approx 0.6$ , 50 µL per well M13K07 helper phage (titer  $> 10^{12}$  pfu/mL) in 2YT was added, incubated for 1 h at RT, and then grown continuously by shaking at 250 rpm at 30°C for 1 h. The wells were supplemented with 100 µL 2YT containing 50 µg/mL kanomycin and 0.1

mmol/L IPTG and incubated overnight at 30°C. The phage-displayed plates were centrifuged for 30 min at 3300 rpm, and the supernatants were transferred to new plates. BSA and Tween-20 were added to a final concentration of 2% and 0.1%, respectively, for pre-block at 37°C for 15 min and the pre-block phage was named as Rv0733-VHH.

Flat-bottom 96-well Costar plates were coated with 100 µL per well of purified Rv0733-6His antigen at a concentration of 1 µg/mL in carbonate buffer as previously described [12]. The pCANTAB5E-phage (obtained by infecting *E. coli* TG1 with blank phagemid pCANTAB5E) and PBS served as negative and blank controls, respectively. After overnight incubation at 4°C, the plates were washed three times with PBST, blocked with blocking buffer for an hour at 37°C, followed by three washings. Subsequently, 100 µL of pre-block phage Rv0733-VHH was added. After incubation for 2 h at 37°C, the wells were again washed and filled with 100 µL of a 1:5000 dilution of HRP conjugated to mouse anti-M13 monoclonal antibody. After incubation for 1 h at 37°C, the plates were again washed with PBST. The wells were filled with 100 µL of substrate solution (1 mg/mL o-phenylenediamine in 0.1 mol/L citrate buffer, pH 4.2, containing 0.03%  $H_2O_2$ ). After incubation for 30 min at RT in darkness, the reaction was stopped by adding 50 µL per well of 2 mol/L  $H_2SO_4$ . The optical density (OD) of each well was measured at a wavelength of 490 nm in ELISA plate reader. If the  $OD_{490}$  ratio of the tested phages to the negative phages were higher than 2.1, the bacteria strains with the exactly equivalent codes were retained.

#### *Subcloning, prokaryotic expression and purification of Rv0733-VHH-Fc-6His fusion antibodies*

VHH fragments from camelid origin require polyhistidine-tags to be purified and detected by conventional immunological method. Hence a VHH-Fc-6His containing chimeric hexa histidine-tag engineered antibody was constructed as previously described [13] to investigate the binding efficiencies of the selected native VHH fragments. A human Fc fragment was inserted between VHH and 6His, as an anti-6His antibody was used as the secondary antibody, the signal observed might result from its known cross-reactivity with mycobacterial GroEL [14]. The plasmids of bacterial clones harboring positive phagemids displaying Rv0733-VHH were

extracted and used as a template to amplify the VHH fragments by PCR. The forward primer contained *Nhe* I recognition site (5'-TAATTA-GCTAGCGGAGACGGTGACCTGGGT-3'). The reverse primer contained *Bam*H I recognition site (5'-AATAAGGATCCGATGGCCCAGGTGCA GCT-3'). PCR amplification of the DNA was performed by using the 2× Polymerase chain reaction (PCR) master mix. The amplified DNA were digested with *Nhe* I and *Bam*H I and named Rv0733-VHH, and subcloned into a prokaryotic expression vector pET-22b-IFH containing Fc portion of human IgG and 6His-tag. The positive clones named pET-22b-Rv0733-VHH-IFH were confirmed by sequencing.

Positive pET-22b-Rv0733-VHH-IFH colonies were inoculated with 200 mL Lysogeny broth (LB) medium containing 50 µg/mL ampicillin and incubated at 37°C with shaking at 200 rpm to the OD value of approximately 0.6 at 600 nm. IPTG was added to a final concentration of 0.1 mmol/L for inducing expression. Incubation with shaking was continued at 30°C for 6 h. Bacterial cells were precipitated by centrifugation and resuspended in 20 mL lysis buffer containing 50 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 300 mmol/L NaCl, and 10 mmol/L imidazole, pH 8.0. The mixture was sonicated and centrifuged. The supernatant containing recombinant Rv0733-VHH-Fc-6His fusion antibodies named crude extracts were filtered through a 0.45 µm prefilter. His-select nickel magnetic agarose beads were uniformly suspended and 1 mL was added to the filtrate protein. The mixture was gently shaken overnight at 4°C and the affinity gel was washed with plenty of wash buffer containing 50 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 300 mmol/L NaCl and 20 mmol/L imidazole, pH 8.0. The sample was centrifuged and the supernatant containing unbinding contaminated proteins was removed. The His-Tag protein was eluted from the beads with elution buffer (50 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 300 mmol/L NaCl, 300 mmol/L imidazole, and pH 8.0). Eluted fractions were transferred to Amicon ultra-2 centrifugal filter unit with ultracel-3 membrane and centrifuged to remove imidazole and other small molecules. The ultrafiltered samples were sterilized using 0.22 µm filter. The purified Rv0733-VHH-Fc-6His proteins were stored in 40% glycerin at -80°C.

#### *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting*

The protein concentration of the crude extracts and the purified protein was measured using

the Bradford method. An equal amount of total protein content was loaded on each lane and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining at 37°C using Coomassie brilliant blue R-250 for assessing the effect of purity before and after purification.

For western blot analysis, purified Rv0733-6His and 60 kDa-6His chaperonin purified antigens were separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes by semi-dry apparatus, and nonspecific binding was blocked with PBS containing 3% BSA. The blots were probed with Rv0733-VHH-Fc-6His diluted at 1:1000, and the immune complexes were visualized using goat anti-human IgG (H+L) HRP conjugate secondary antibody, according to the instructions of the manufacturer. Blots were digitally photographed using the ChemiDoc MP imaging system.

#### *Rv0733-VHH-Fc-6His intracellular binding with M. bovis BCG assay using immunostaining*

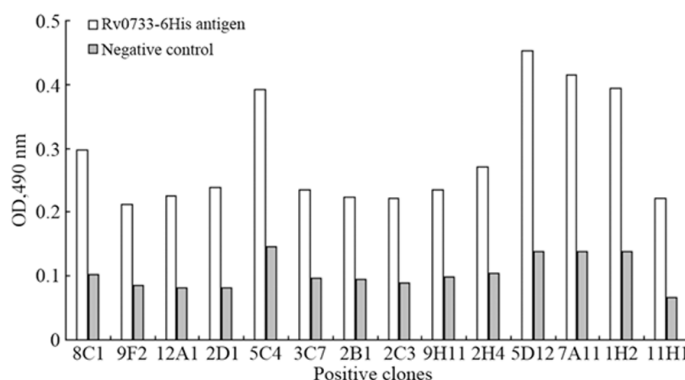
Human acute monocytic leukemia THP-1 cells were grown on sterile coverslips in Petri dishes. Three days before infection, PMA was added to the dish at a final concentration of 20 nmol/L to induce the differentiation of THP-1 cells into macrophages [15]. *M. bovis* BCG vaccine strain grown in Middlebrook 7H9 medium was washed with fresh drug-free 10% FBS/RPMI-1640 medium and vortexed vigorously with sterile glass beads. Infection was initiated by adding bacteria suspended in 10% FBS/RPMI-1640 medium to the monolayer at an MOI of approximately 10:1 and incubated for 10 h at 37°C in 5% CO<sub>2</sub>. The coverslips were washed three times with PBS, fixed in 4% paraformaldehyde for 30 min, washed with PBS, permeabilized in 0.5% Triton X-100 in PBS for 30 min, washed three times with PBS, and blocked with Image-iT FX Signal Enhancer for 30 min. Rv0733-VHH-Fc-6His were used as primary antibodies at a dilution of 1/500. Visualization was observed by staining with goat-anti-human IgG conjugated to Alexa Fluor 594. The coverslips were washed three times with PBS after each of the antibodies was incubated for 60 min at RT. Stained and dried coverslips were mounted onto glass slides using Gold antifade medium with DAPI. *Salmonella typhimurium* strain ATCC14028 [16] was used to mock infect differentiated THP-1 cells.



**Table 1.** Enriched effect of nonimmune llama variable heavy chain phage display antibody library

Item	Without selection	First round	Second round	Third round
Stop codon (%)	10	13	16	17
Library size	$1.5 \times 10^9$	$4 \times 10^5$	$7 \times 10^4$	$4 \times 10^3$
Ratio (output/input)	/	$3.6 \times 10^{-8}$	$7.9 \times 10^{-8}$	$3.7 \times 10^{-6}$
Supernatant OD <sub>490</sub>	0.3-0.6	1.3-1.7	2.0-2.3	2.5-2.8

OD, optical density.

**Figure 1.** Detection of the Rv0733-6His fusion protein-binding specificity to the molecules of variable domain of heavy chain of heavy-chain antibody through ELISA. ELISA, enzyme-linked immunosorbent assay; OD, optical density.

#### *M. bovis* BCG intracellular growth

The Rv0733-VHH was also subcloned into a prokaryotic expression vector pET-22b containing 6His-tag. The positive clones named pET-22b-Rv0733-VHH-6His were confirmed by sequencing. Then the Rv0733-VHH-6His fragments were cloned to a eukaryotic expression vector pcDNA3.1 to determine transfection efficiency by fluorescence microscopy following standard procedures [17]. The positive plasmid named pcDNA3.1-Rv0733-VHH-6His was transfected into differentiated THP-1 macrophages using Lipofectamine 2000 transfection kit as recommended by the manufacturer. The positive transfected cells named THP-VHH were infected by *M. bovis* BCG. The pcDNA3.1 transfected differentiated THP-1 macrophages named THP-C were used as controls. The infected THP-VHH cells were visualized with anti-his-tag-Alexa Fluor 488.

Several colonies of *M. bovis* BCG Pasteur were collected, washed twice with sterile phosphate buffer at pH 7.0, and dispersed in the positive transfected cells' culture complete medium for 4 h. MOI was adjusted to 10 using a standard-

ized calibration curve of OD<sub>600</sub>/CFU. Bacteria were added to host cells at 60-80% confluency in 12-well dishes. After 4 h infection, host cells were washed repeatedly with warm PBS to remove unbound BCG and further incubated in complete tissue culture medium without addition of antibiotics. At day 3 postinfection, cells were lysed in 0.1% saponin. Serial dilutions of cell lysates were plated on Middlebrook 7H11 agar plates and incubated at 37°C for determining CFU at 21 days.

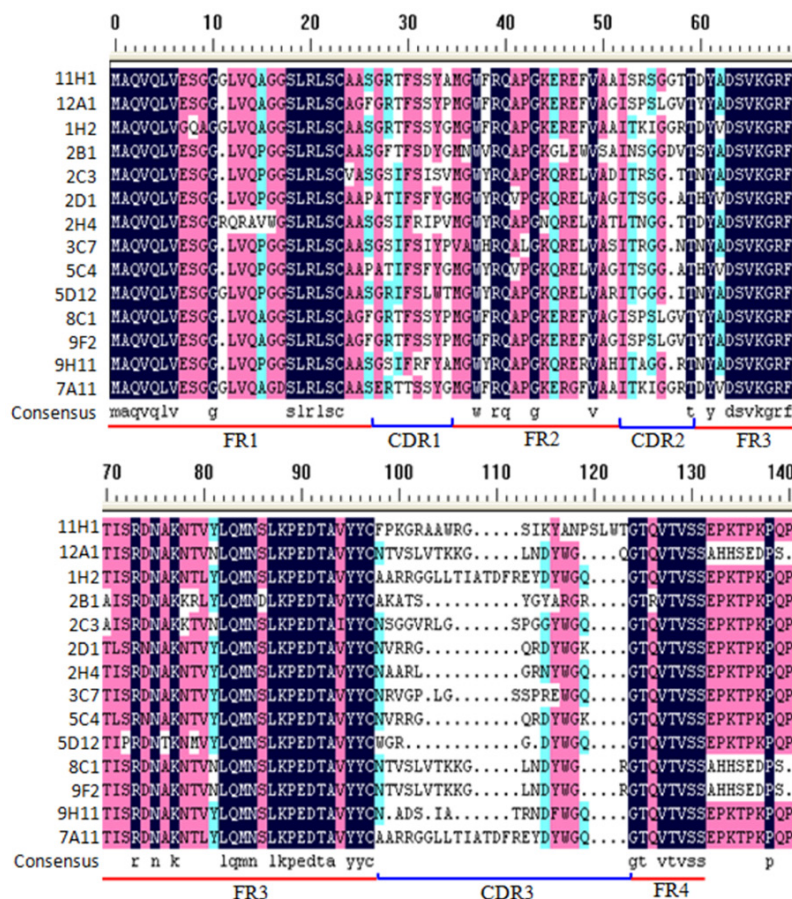
## Results

### *Enrichment of positive phages*

To enrich Rv0733-6His-binding phages from the nonimmune Llama VHH phage display antibody libraries, three rounds of selection were performed, with increasing panning stringency mediated by a progressive reduction in coating Rv0733-6His antigen. The size of the VHH phage library was reduced from  $1.5 \times 10^9$  to  $4 \times 10^3$ , but the output/input ratio of phages was increased by about 103-fold (from  $3.6 \times 10^{-8}$  to  $3.7 \times 10^{-6}$ ) after the third round of selection, and the antigen-binding activity was also increased from 0.3~0.6 to 2.5~2.8 (Table 1), which indicated an obvious enrichment for the specific binding of phages to Rv0733-6His antigens.

Twenty clones were randomly chosen from library-size titration plates for sequencing after each round of panning. If the number of positive clones having the target insert was > 10 and the number of the insert stop codons was < 10%, the plates were directly amplified, induced with IPTG and subjected to assay for binding VHH with Rv0733-6His antigen by ELISA. The OD<sub>490</sub> value of VHH-containing wells was divided by negative control wells and expressed as the "P/N ratio", positive wells with OD<sub>490</sub> values > 0.2 and negative wells with OD<sub>490</sub> values < 0.1 were considered as the threshold values. After three rounds of panning, approximately 14 positive clones were chosen from 1024 phage clones in this study (Figure 1). Of the 14 clones, 11 independent and distinct DNA sequences were obtained.

## Rv0733-VHH



**Figure 2.** Amino acid sequence alignment of 14 clones from Rv0733-VHH phage display library. VHH, variable domain of the heavy chain of heavy-chain antibody.

### Rv0733-VHH sequence analysis

Fourteen positive clones were determined by DNA sequencing. Corresponding amino acid sequences were deduced from DNA sequences using the DNAMAN software. Sequence alignment revealed 11 different strains of VHHs as shown in **Figure 2** (repeated sequences were deleted). The result suggested that the large diversity of Rv0733-VHH antibody fragments was due to the variations in length of complementarity determining regions (CDRs) 3. The positive single colony named 8C1 was used for prokaryotic expression.

### Prokaryotic expression, purification, and characterization of Rv0733-VHH-Fc-6His fusion antibodies

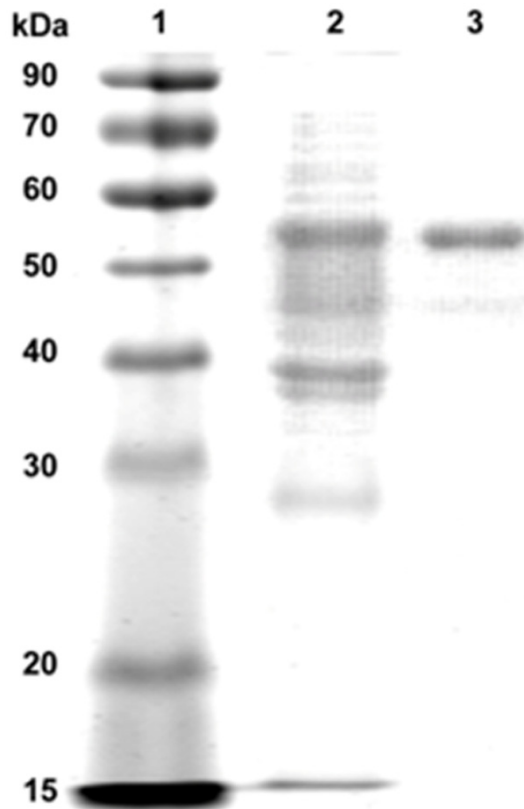
*E. coli* BL21 (DE3) pLysS competent cells were transformed using one of the recombinant plasmids named pET-22b-Rv0733-VHH-IFH whi-

ch containing Rv0733-VHH antibody fragment named 1H2 colony. Positive colonies were confirmed by DNA sequencing and the antibody expression was verified. Recombinant antibodies were induced with IPTG and purified using His-select nickel magnetic agarose beads. SDS-PAGE gels were used for visualizing Rv0733-VHH-Fc-6His sample by Coomassie brilliant blue R-250 staining. Taking the molecular weight of the 6× His-tag (0.84 kDa), human IgG Fc fragment (30.91 kDa) and that of Rv0733-VHH antibody (14.3 kDa) into account, the purified protein showed an expected size of protein band of 53.6 kDa (**Figure 3**). The final yield of purified Rv0733-VHH-Fc-6His was about 0.76 g/L of culture.

Based on the western blot assay, the purified Rv0733-6His fusion protein could be recognized by Rv0733-VHH-Fc-6His antibody and the expected band (20.93 kDa) was visualized by goat anti-human IgG (H+L) HRP (**Figure 4** lane 3). The 60 kDa-6His chaperonin could not be visualized by the 60 kDa band (**Figure 4** lane 2) as it could not bind the Rv0733-VHH-Fc-6His antibody. This meant that the purified Rv0733-VHH-Fc-6His antibody could bind very specifically to its target, and could not bind 6His-tag.

### Rv0733-VHH-Fc-6His binding with intracellular BCG

As the vaccine strain *M. bovis* bacille BCG could share with Rv0733 antigen present in *M. tuberculosis*, BCG was the economical choice in this study to validate the binding activity of Rv0733-VHH-Fc-6His. Human acute monocytic leukemia cell line THP-1 can be differentiated into macrophage-like cells by PMA. Differentiated THP-1 cells mimic native monocyte-derived macrophages and BCG was phagocytosed.

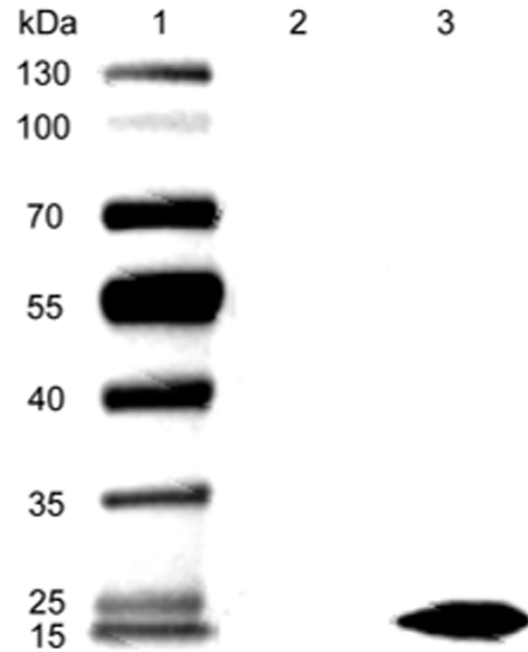


**Figure 3.** Expression and purification of Rv0733-VHH-Fc-6His fusion protein detected by SDS-PAGE. 1: Protein marker; 2: lysates of pET-22b-Rv0733-VHH-IFH/BL21 (DE3) pLysS with induction of IPTG; 3: purified native Rv0733-VHH-Fc-6His fusion protein. IPTG, Isopropyl- $\beta$ -D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VHH, variable domain of the heavy chain of heavy-chain antibody.

After 10 h of infection, cells were fixed and prepared for immunostaining. Representative merged images are shown in **Figure 5**. Fluorescence staining showed internalized BCG (red) co-localize with cell nucleus (blue). *Salmonella typhimurium* ATCC14028 strain was used as a control to mock infect differentiated THP-1 cells, but Rv0733-VHH-Fc-6His did not bind the strain (unspecific red fluorescence). The results suggested that Rv0733 antigen expressed by BCG could bind with the purified Rv0733-VHH-Fc-6His antibody.

#### *Rv0733-VHH-6His inhibits growth of M. bovis BCG*

To determine whether Rv0733-VHH-6His inhibits intracellular growth of *M. bovis* BCG in macrophages, differentiated THP-1 macrophages

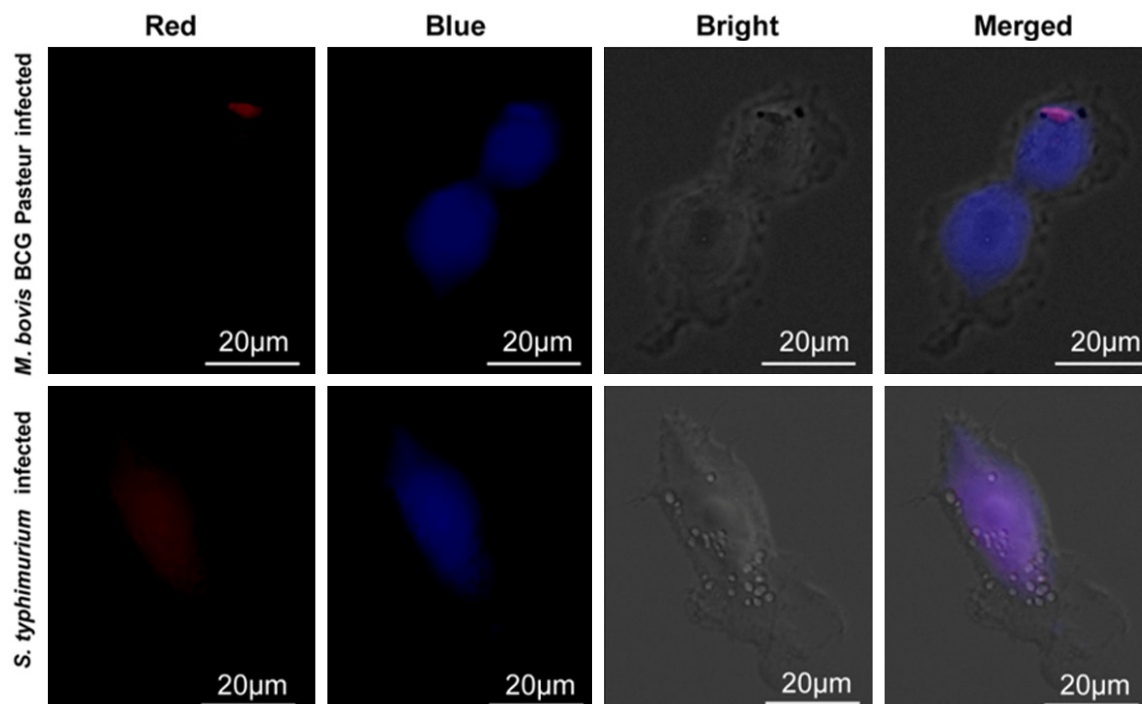


**Figure 4.** Analysis of the specificity of Rv0733-VHH-Fc-6His antibody binding with target antigen. 1: Protein marker; 2: Purified 60 kDa-6His chaperonin fusion protein; 3: Purified Rv0733-6His fusion protein. VHH, variable domain of the heavy chain of heavy-chain antibody.

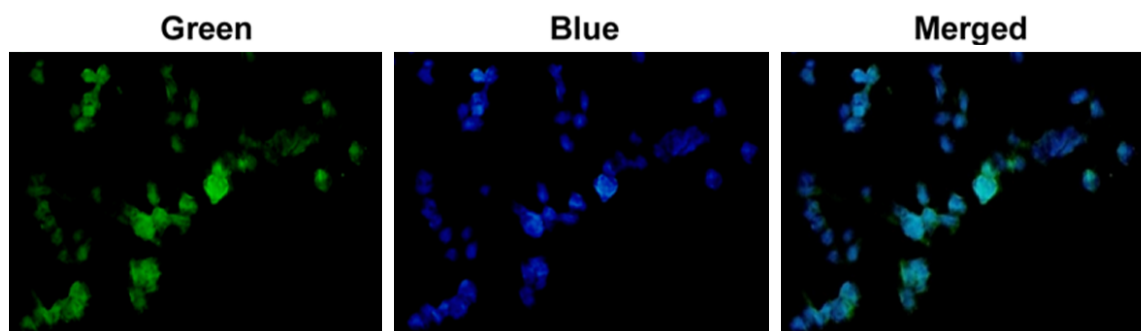
were transfected with pcDNA3.1-Rv0733-VHH and pcDNA3.1, respectively, as outlined in the Materials and Methods (**Figure 6**). Incubation of THP-VHH infected with *M. bovis* BCG at 3 d resulted in BCG CFU decreased 0.31-fold ( $t = 3.5355$ ,  $P = 0.0241$ ) as compared with infected THP-C cells (**Figure 7**).

#### Discussion

In the study by Pan *et al.* [7], there were no statistically significant differences ( $P = 0.306$ ) between the OD values obtained from the sera of 34 patients with active TB and 35 healthy control subjects with Rv0733-6His antigen-coated ELISA plates. This suggested that Rv0733 antigen (adenylate kinase) could not stimulate significant humoral immunity in patients with TB. The ability of *M. tuberculosis* to cause disease is correlated with its ability to enter into and survive inside, what is currently thought to be, its permitted host cell, the nonactivated macrophage [18, 19]. Rv0733 antigen is essential for the bacteria in intracellular nucleotide metabolism [8, 20]. Therefore, VHHs of Rv0733 were selected to screen. The advantageous features of VHHs are to bind antigens



**Figure 5.** Binding characteristic of Rv0733-VHH-Fc-6His in differentiated THP-1 cells. Red: *M. bovis* BCG Pasteur or *S. typhimurium* was visualized with Rv0733-VHH-Fc-6His Ab and Alexa 594-conjugated secondary Abs. BCG showed specific red fluorescence, *S. typhimurium* showed unspecific red fluorescence. For red fluorescence, the excitation filter was 575 nm and the emission filter was 624 nm; Blue: the infected cell nucleus were stained by DAPI. For blue fluorescence, the excitation filter was 340 nm and the emission filter was 488 nm; Bright: boundaries of differentiated THP-1 cell infected with BCG or *S. typhimurium* were visualized under bright field. Merged: fluorescence channels were merged with bright field. Purple areas indicate overlay of BCG and the cell nucleus. Ab, antibody; BCG, bacille Calmette-Guérin; DAPI, 4',6-diamidino-2-phenylindole; VHH, variable domain of the heavy chain of heavy-chain antibody.

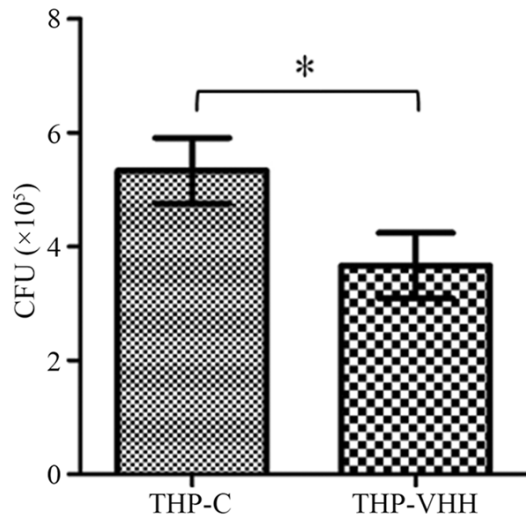


**Figure 6.** Rv0733-VHH-6His expressing in differentiated THP-1 cells. Green: pcDNA3.1-Rv0733-VHH-6His transfected cells were visualized with Alexa Fluor 488-conjugated 6His Abs (green fluorescence). For green fluorescence, the excitation filter was 488 nm and the emission filter was 519 nm; Blue: transfected cells were stained by DAPI. For blue fluorescence, the excitation filter was 340 nm and the emission filter was 488 nm; Merged: fluorescence channels were merged. Blue-green areas indicate overlay of Rv0733-VHH-6His and the cell nucleus. Ab, antibody; DAPI, 4',6-diamidino-2-phenylindole; VHH, variable domain of the heavy chain of heavy-chain antibody.

produced intracellularly by bacteria, which might help phagocytes clear intracellular pathogens.

A nonimmune Llama VHH phage display antibody library was constructed from Shanghai zoo. The nonimmune Llama peripheral blood





**Figure 7.** The intracellular survival of *M. bovis* BCG Pasteur in the transfected differentiated THP-1 cells at 3d. Results expressed as mean CFU  $\pm$  SD (standard deviation) for triplicate wells. Significant values are indicated as symbols. \*,  $P < 0.05$  (by *t* test). BCG, bacille Calmette-Guérin; THP-C, pcDNA3.1 transfected differentiated THP-1 cells. THP-VHH, pcDNA3.1-Rv0733-VHH-6His transfected differentiated THP-1 cells.

mononuclear lymphocytes were used in this study due to factors affecting the market price of Llama. The library has size of  $1.5 \times 10^9$  members, and titer of the phage library is calculated to  $1.3 \times 10^{11}$  transformation unit (TU)/mL. Because termination codon within the open reading frame of the VHH library was unpredictable in this study, the expression of phage gIII fusion gene could be stopped by unexpected excessive termination codon, and VHHs displayed on the phage surface could be interfere [21]. To control the massive increase in termination codon during the screening, the initial concentration of bacteria was adjusted  $A_{600}$  as  $\sim 0.05$ – $0.1$ , and the enrichment culture time of bacteria was designed to  $A_{600}$  achieving  $0.6$ . Termination codons were successfully controlled within 20% in the enrichment phage clones from the large nonimmune VHH library. Eleven independent DNA sequences with varied length were obtained from 1024 phage clones in this study. The major difference in the VHH fragments was variations in the length of CDR3. VHHs devoid of the CH1 domain (glycosylation sites) [22] were not only suitable for production by the prokaryotic expression system, but were also used to investigate the presence of binding motifs to related antigen in the CDR domain,

which was different from the conventional antibody. Therefore, in this study, the single *E. coli* colony named 8C1 containing Rv0733-VHH antibody fragment was selected for the prokaryotic expression. Results of the Western blot indicated that the prokaryotic-expressed Rv0733-VHH-Fc-6His fusion proteins with high purity and high yield were obtained. Evidence from immunofluorescence staining that Rv0733-VHH-Fc-6His could bind Rv0733, which was expressed by BCG in macrophages, was also provided. The result suggested that Rv0733-VHH screened from the nonimmune Llama VHH phage display antibody library had the potential feature of binding intracellular BCG antigen. Rv0733-VHH expressed in the pcDNA3.1-Rv0733-VHH transfected differentiated THP-1 macrophages could inhibit intracellular proliferation of *M. bovis* BCG.

It is still necessary to investigate the features of Rv0733-VHH antibody entering and binding with intracellular *M. tuberculosis* related antigen and improve the bactericidal ability of macrophages.

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#### Disclosure of conflict of interest

None.

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