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Research Article

Identification of Novel Single Chain Fragment Variable Antibodies Against TNF-α Using Phage Display Technology

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Abstract

Purpose: Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine, involved in both physiological and pathological pathways. Because of central role of TNF-α in pathogenesis of inflammatory diseases, in the current study, we aimed to identify novel scFv antibodies against TNF-α using phage display technology.

Methods: Using libraries composed of phagemid displaying scFv antibodies, four rounds of biopanning against TNF-α were carried out, which led to identification of scFvs capable of binding to TNF-α. The scFv antibody with appropriate binding affinity towards TNF-α, was amplified and used in ELISA experiment.

Results: Titration of phage achieved from different rounds of biopanning showed an enrichment of specific anti-TNF-α phages during biopanning process. Using ELISA experiment, a binding constant (Kd) of 1.11 ± 0.32 nM was determined for the phage displaying J48 scFv antibody.

Conclusion: The findings in the current work revealed that the identified novel scFv antibody displayed at the N-terminal of minor coat proteins of phagemid binds TNF-α with suitable affinity. However, the soluble form of the antibody is needed to be produced and evaluated in more details regarding its binding properties to TNF-α.

Introduction

Phage display technology, a combinatorial biology approach, introduced by G.P. Smith in 1985, is a powerful tool in drug discovery.[1-3] The concept is simple in principle: a library of phage particles displaying peptides, proteins and antibodies is used to select those that bind to desired target. The gene of interest is inserted into the genome of phage, which allows presentation or displaying of the encoded protein as a fusion with one of the coat proteins of phage creating a physical linkage between phenotype of phage and its genotype responsible for the encoded peptides or proteins.[1,3-5] In this technology, highly diverse libraries can be constructed allowing rapid identification and isolation of specific ligands for numerous protein targets such as enzymes, and cell surface receptors.[5,6] Structural analysis of the selected ligands bound to their targets could provide detailed information regarding ligand-target interaction which can be useful in drug discovery and development.

Recent advances in phage display technology and antibody engineering have paved the way for the development of phage-displayed antibody technology.[7,8] Various formats of antibodies, including Fab (fragment, antigen binding) and scFv (single chain fragment variable) have been cloned and displayed on phage to generate a library of antibodies which can be screened against almost any molecule.[9,10] An scFv antibody is a fragment of full antibody in which V_H and V_L are linked to each other by a peptide linker and can be displayed on the surface of bacteriophages enabling identification of antibodies with unique specificity in phage display technology.

Among different cytokines involved in inflammatory responses, TNF-α is known as the crucial cytokine, as it directly affects target cells and also activates the other inflammatory cytokines.[11] Various kinds of strategies can be used for alleviation of complications caused by high levels of TNF-α including monoclonal antibodies, soluble TNF-α receptor fusion proteins, recombinant TNF-α binding proteins, antagonists of TNF-α receptors, siRNA and nonspecific drugs such as pentoxyfylline and thalidomide and metalloproteinase inhibitors.[12-15] However, most of the specific anti- TNF-α therapeutics in the market are either anti-TNF-α antibodies (Infliximab (Remicade)®, adalimumab (Humira)®, and golimumab (Simponi)®) or soluble TNF-α receptors

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(etanercept (Enbrel)®, certolizumabpegol (CIMZIA)®).[16] The application of these inhibitors is limited partially because of their low stability, high production cost, immunogenicity, and potential side effects.[17-22] Such complexities necessitate development of new TNF-α inhibitors which are smaller than the marketed medicines but still have appropriate affinity towards TNF-α.

Therefore, the objective of the current study was to use phage display technology for the identification of anti-TNF-α scFv antibodies which are smaller in size while maintaining high blocking specificity.

Materials and Methods

Reagents

All of the chemicals used in this work were of biological grade. Tryptone, agar and glycerol were obtained from Applichem (Darmstadt, Germany). NaCl and polyethylene glycol (PEG) 8000 were purchased from Scharlau (Barcelona, Spain). Primers used in this work were supplied from Bioron (Germany) ordered via FAZA Biotech (Tehran, Iran). Gel purification and plasmid mini extraction kits were obtained from Bioneer (South Korea). Tomlinson Phage-display antibody library kit was purchased from MRC HGMP Resource Centre. Anti M13-HRP conjugated monoclonal antibody was prepared from Sino Biological Inc. (Beijing, P.R. China). TMB (3,3′,5,5′-Tetramethylbenzidine) was obtained from Sigma Aldrich (USA). Agarose was from Invitrogen Ltd (Paisley, UK). Sodium azide (NaN3) and methanol were from Merck (Darmstadt, Germany). PCR master kit was purchased from CinnaGen (Tehran, Iran). All other chemicals and reagents were of molecular biology grade. Ultra pure biology grade water (Milli-Q, Millipore Corporation, Bradford, MA, USA) was used for the preparation of all solutions.

Biopanning process using scFv antibody phage library

TNF-α solution with the concentration of 100 µg/mL in a buffer solution containing 50 mM Tris, 150 mM NaCl and 2.5 mM $CaCl_2$ at pH 8.0 was used to coat 96-well plates. The plate was incubated at 4 °C for overnight in an air-tight humidified box. The excess of TNF-α solution was discarded by slapping face-down the plate onto a clean towel. Then the wells were filled completely with blocking buffer (skim milk 2%) and incubated for 2 h at 4 °C. After incubation, the blocking buffer was aspirated and the wells were washed six times using TBS. Both of I and J libraries (scFv phage libraries) were used for biopanning. A total amount of 2×10^{11} phagemid particles in skim milk 2% were added to the wells and incubated for 2 h in room temperature. The supernatant was discarded and the wells were washed with TBST ten times. After the final wash, 200 µL trypsin in TBS with final concentration of 1mg/mL was added to the wells and incubated at room temperature for 10 min rotating on a rocker shaker. The eluate was used to infect *E.coli ER 2738* grown to OD_{600} of 0.4 with incubation for half an hour at 37 °C. For the titration, serial dilutions of the infected bacteria were prepared and 10 µL of each dilution was plated on TYE plates supplemented with ampicillin (100 µg/mL) and glucose (1%). The remainder of the infected *ER 2738* was centrifuged at 3000 *g* and the bacterial pellet was resuspended in 50 µL 2TY medium and plated on a TYE-ampicillin-glucose plate, incubated at 37 °C overnight. Onto the overnight plate, 2 mL of 2TY medium was added and the cells were completely loosen with a glass spreader. Fifty µL of scraped bacteria was used to inoculate 50 mL 2TY-ampicillin-glucose and grown while shaking at 37 °C. At OD_{600} 0.4, a 10 mL sample was taken and to which was added 5×10^{10} helper phage and incubated at 37 °C for 30 min. After incubation the bacterial culture was centrifuged at 3000 *g* and the pellet was resuspended in 50 mL 2TY-ampicillin-kanamycin-glucose (0.1%) medium, and grown with shaking overnight at 30 °C. The cells were harvested by centrifugation and to 80% of the supernatant, 1⁄6 of volume 20% PEG 8000 in 2.5 M NaCl was added and the mixture was incubated overnight at 4°C. Phage particles were precipitated by centrifugation at 8000 g for 20 min at 4°C. The supernatant was discarded and 1 mL of TBS was used to resuspend phage pellet. To purify further, repercipitation was performed by adding 1/6 of volume 20% PEG in 2.5 M NaCl and incubating at 4 °C for 1 h. Precipitated phage particles were harvested once again by centrifugation at 8000 g at 4 °C for 20 min. The pellet was suspended in 200 µL TBS containing 0.02 % NaN_3 and stored at 4 °C as the amplified phage.

Serial dilutions from the amplified phage were prepared for phage titration. The amplified phagemid was used for the next round of biopanning. Totally, four rounds of biopanning were performed.

ELISA experiment using phage displaying antibody

Individual colonies from each round of biopanning were used to inoculate 100 µL 2TY-ampicillin-glucose 1% in a 96-well plate and grown while shaking at 250 rpm overnight at 37 °C. The overnight cultures were diluted 1:100 in 200 µL 2TY-ampicillin-glucose 1% and grown at 37 °C for 2 h shaking at 250 rpm. To the cultures was added 25 µL of 10^9 helper phage and grown-shaking for additional 1h. After that, the cultures in the 96-well plate were centrifuged at 1800 *g* for 10 min and the bacterial pellet was resuspended in 200 µL 2TY-ampicllin-kanamycin-glucose 0.1% and grown overnight at 30°C shaking at 250 rpm. The cultures were spinned at 1800 *g* for 10 min and the supernatants were used for phage ELISA experiment according to the following protocol.

TNF-α at concentration of 100 µg/mL in a buffer containing 50 mM Tris, 150 mM NaCl and 2.5 mM $CaCl_2$ at pH 8.0 was used to coat a 96-well plate. The plate was incubated at 4 °C for overnight in an air-tight humidified box. The excess of TNF-α solution was discarded by slapping face-down the plate onto a clean towel and the wells were filled completely with blocking buffer (skim milk 2%) and incubated for 2 h at 4 °C. After incubation, the blocking buffer was aspirated and

the wells were washed six times using TBS. The amplified phagemid from each round resuspended in blocking buffer was added to the TNF-α coated wells and incubated for 2 h at room temperature with gentle shaking (TNF-α uncoated wells were used as controls). Following the incubation, the wells were washed six times with TBST. Subsequently, 100 µL of 1:5000 diluted HRP-conjugated anti-M13 monoclonal antibody in blocking buffer was added to each well and the plate was incubated for additional 2 h at room temperature with gentle shaking. After washing six times with TBST, the wells were treated with solution containing TMB 100 µg/mL, prepared in potassium acetate (100 mM, pH 6.0) and hydrogen peroxide (0.006 % v/v). After 15 min, the enzymatic reaction was terminated by adding 50 µL of 1 M H_2SO_4. The absorbance was measured at 450 nm using ELISA reader.

For each colony with positive result in ELISA experiment, an overnight culture was prepared in 2TY-ampicillin-glucose 1% and used in colony PCR reaction to confirm the existence of full length V_H and Vκ insert using LMB3 (CAGGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) primers. The colony PCR reaction mixture was prepared by mixing 10 µL Master Mix Pfu DNA polymerase, 1 µL from each forward (20 µM) and reverse (20 µM) primers, and made up to total volume of 50 µM by ultra-pure water. A small amount (about 2 µL) of the overnight culture was added to each mixture and the PCR reaction was performed according to the following program: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 8 min. The full length PCR products were sent out for sequencing using both LMB3 and pHEN primers.

The positive phagemid presenting the full length scFv antibodies was amplified and serially diluted for using in ELISA experiment. The results obtained from ELISA were used to calculate the binding constant (K_d) by Prism program (version 6.01, Graphpad Software Inc.).

Results

Identification of anti-TNF-α scFv using phage display

TNF-α protein was prepared to a high purity as described previously.[23] The Tomlinson I & J antibody displaying phage libraries composed of the phagemid particles each presenting an individual single chain fragment variable fused to N-terminal of pIII coat protein were used to isolate specific TNF-α binders. Biopanning procedure was carried out in four consecutive rounds. The output of each round was amplified and used for the next round of biopanning. The observed increase in titration values (output/input) from the first round through the fourth round of biopanning indicates the enrichment of specific binders during the biopanning process (Table 1). Figure 1 shows the result of phage titration throughout the enrichment process. As it can be seen, the increase in phage titration occurred during the biopanning process.

Table 1. Biopanning efficacy of Tomlinson I and J libraries against TNF-α

		First round	Second round	Third round	Fourth round
Input	Library I	2.0E+11	2.0E+11	2.0E+11	2.0E+11
	Library J	2.0E+11	2.0E+11	2.0E+11	2.0E+11
Output	Library I	2.6E+05	1.98E+07	1.0E+07	3.0E+07
	Library J	3.0E+05	3.5E+07	4.0E+07	4.0E+07
Output/input	Library I	1.3E-06	9.9E-05	5.0E-05	1.5E-04
	Library J	1.5E-06	1.7E-04	2.0E-04	2.0E-04

Input, titration values (colony forming unit, CFU) per 1 mL for phages in the input solution; output, titration values per 1 mL for phages in the elution solution; output/input, ratios of values in each round of panning.

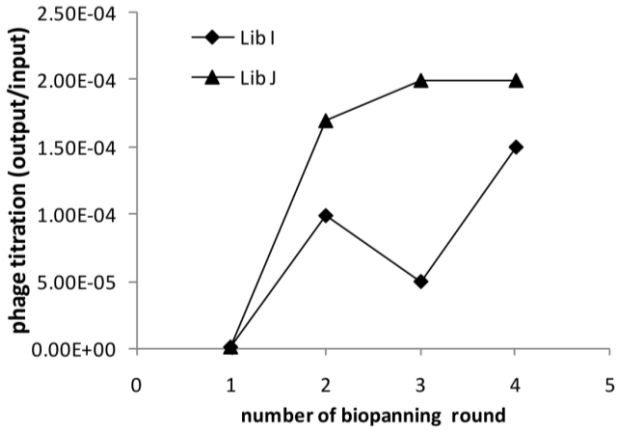

Figure 1. Representation of increase in phage titration through biopanning process for I and J Libraries.

Functionality assessment of anti-TNF-α scFv antibody displayed by phage

To identify specific binders to TNF-α, individual colonies from the each round of biopanning were randomly selected and amplified, and then were used to perform ELISA experiment in order to isolate those phagemids presenting scFv antibodies specific towards TNF-α.

Presence of a band at 935 bp in the PCR reactions on the ELISA positive colonies indicates the existence of a full length scFv (V_H and Vκ inserts) displaying phagemid in the reaction, while a band at 638 is observed when the phagemid displays aberrant scFv antibody (either V_H or Vκ insert)(Figure 2). Based on ELISA and PCR results the bacterial colony harbouring phagemid presenting full scFv antibody (i.e. J48) was selected for further evaluations.

The phagemid J48 presenting the anti-TNF-α antibody was amplified and serially diluted to the concentrations ranging from 0.026 nM to 1.66 nM, and then the solutions were used in ELISA experiment. The results of ELISA were analyzed by fitting the data into one-site binding curve and the Kd was estimated to be 1.11 ± 0.32 nM (Figure 3).

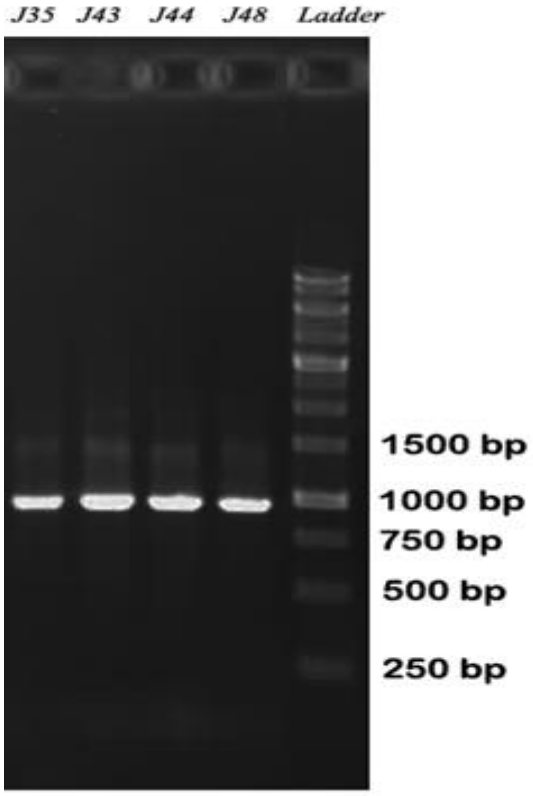

Figure 2. Results of 1% (w/v) agarose gel electrophoresis on PCR products from the isolated positive colonies of bacterial cells infected with phagemid presenting scFv against TNF-α using LMB3 (CAGGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) primers. The presence of bands with the size of 930 bp and 650 indicate the presence of full length (VH and Vκ) and aberrant (either of VH or Vκ) inserts, respectively.

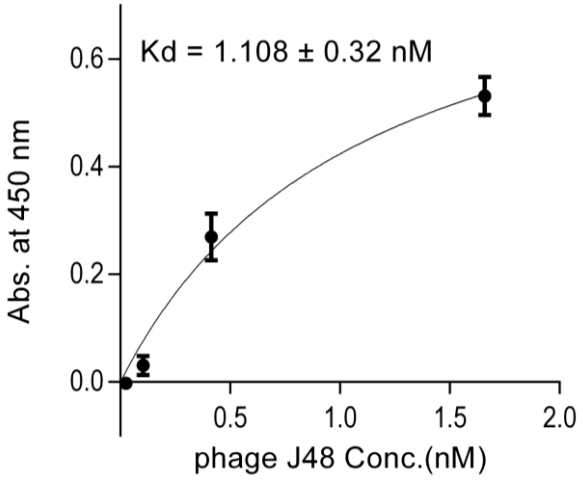

Figure 3. ELISA experiment using different concentrations of phagemid displaying J48 scFv ranging from 0.026 nM to 1.66 nM.

Discussion

TNF-α elicits many different physiologic roles in the body and acts as an important mediator of growth-modulation, differentiation, and inflammation. However, it has been shown that excessive amounts of TNF-α is involved in the pathways of the associated inflammatory responses. Such complicated diseases require targeting of TNF-α for alleviating such awkward conditions.[16,24-26] The clinical and diagnostic use of antibodies as TNF-α inhibitors are widespread due to their high specificity. Until now, several anti-TNF-α antibodies have been introduced to the market and are still being used as therapeutic agents in inflammatory conditions. However, the side effects of these huge macromolecules and also their high production cost have restricted their application in patients stricken by inflammatory diseases.[17-22] In the current study, we aimed to identify antibodies against TNF-α, which are fully humanized and small enough with manageable physicochemical properties.[27] Tomlinson I&J libraries are non-immunized human phage libraries composed of scFv antibodies displaying phagemids that provide an enormous source of individual antibodies to be screened against any target of interest. Through four rounds of biopanning against TNF-α using these libraries, phagemids displaying scFvs capable of binding to TNF-α were isolated. Furthermore, the identified phagemids were examined for the presence of the sequence required for coding the full length (V_H and V_L inserts) scFv (Figure 2). Based on the ELISA experiment, phage displaying J48 scFv antibody was chosen for further examination due to its appropriate affinity towards TNF-α. Different concentrations of phage displaying J48 scFv antibody were prepared and used in ELISA experiment in which the phage J48 showed Kd value of 1.11 ± 0.32 nM.

As antibodies are specific binders of their corresponding antigens, therefore, using antibodies to target pathologic agents is of great importance. Up to now, different anti-TNF-α scFv antibodies have been recognized using phage display technology. Okamoto et al. identified scFv antibodies specific towards TNF-α using scFv antibody library constructed from non-immunized sources. However, the origin of the scFv antibody library employed for screening against TNF-α was B lymphocytes taken from mouse bone marrow and spleen,[28] which poses possible limitations for their use in human diseases. Isolation of a humanized scFv antibody against TNF-α have been reported elsewhere, but the constructed scFv antibody library was originated from immunized human, which lacks the presence of all potential antibodies for screening against TNF-α. [29] It is of great interest to identify antibodies which are in small formats with human origin.[27] To this end, in the current study, we attempted to isolate a single chain variable fragment anti-TNF-α antibody from non-immunized human scFv antibody libraries. The identified anti-TNF-α scFv antibody can be produced in high degree of purity in its isolation (not displayed on phagemid) and its interactions with TNF-α can be investigated using

biophysical studies.[30] Based on such results, J48 may require optimization to produce refined anti-TNF-α antibody. For example, a previously identified parent murine anti-TNF-α antibody was humanized and structurally modified to generate a human anti-TNF-α scFv antibody namely TSA1 with higher binding ability to TNF-α compared to the original antibody.[31] Such structural modifications can be performed on the identified scFv antibody based on the detail information about its mode of interaction with TNF-α obtained from biophysical experiments as well as *in silico* scFv-TNF-α docking studies.

Conclusion

Briefly, the purpose of the current work was the identification of human single chain fragment antibody against TNF-α using phage display technology. Through four rounds of biopanning, a phage displaying scFv antibody with appropriate affinity towards TNF-α was isolated. Having shown that J48 displaying phagemid binds TNF-α with high affinity, it necessitates detailed investigations using pure J44 scFv antibody to demonstrate its functionality in various *in vivo* and *in vitro* tests, such as determining its TNF-α binding affinity and cell-based TNF-α toxicity inhibition.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

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