Neutralizing human anti-B-cell-activating factor of the TNF family (BAFF) scFv selected from phage antibody library

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Abstract
Elevated levels of B-cell-activating factor of the TNF family (BAFF) have been implicated in the pathogenesis of autoimmune diseases in humans. We now report the isolation by phage display of human single-chain antibody fragment (scFv) anti-BAFF. After four rounds of panning against BAFF, thirty-two out of 92 phage clones displayed BAFF binding activity. One of the positive clones, designated F8, bound to BAFF with relatively high affinity and neutralized BAFF bioactivity in vitro. F8 clone was expressed in soluble form in Escherichia coli HB2151 and purified by immobilized metal affinity chromatography (IMAC). The purified scFv recognized BAFF with the affinity constant (K\text{aff}) of 2.5 × 10^7 M$^{-1}$ without cross-reaction to APRIL. In addition to binding, the purified scFv could dose-dependently inhibit BAFF-induced mouse spleen B lymphocyte proliferation. Together with its fully human nature, F8 scFv may have therapeutic implications in therapy of autoimmune disorders mediated by BAFF.

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1. Introduction
B-cell-activating factor of the TNF family (BAFF), also known as BLyS, TALL-1, THANK, and TNFSF13B, is a novel member of the tumor necrosis factor (TNF) ligand family which is important in B lymphocyte maturation and survival. BAFF has been proposed to be a biomarker for systemic lupus erythematosus (SLE) disease activity [1–5]. Several lines of evidence suggest that elevated levels of BAFF may be involved in the pathogenesis of B cell-mediated autoimmune diseases. Firstly, constitutive overexpression of BAFF in transgenic animals results in anti-DNA antibodies, rheumatoid factor, circulating immune complexes, and deposition of immune complexes in the kidney leading to glomerulonephritis. These symptoms resemble those of SLE and some aspects of rheumatoid arthritis (RA) [6,7]. Secondly, elevated levels of BAFF have been found in other murine models of SLE, including MRL-\textit{lpr/lpr} and (NZB×NZWF)\textsubscript{1} strains [8]. Finally, elevated levels of BAFF have been observed in the serum of patients with SLE and RA as well as Sjögren’s syndrome [9–12]. Significant correlations between BAFF levels and autoantibody production were shown in these studies. The association of BAFF production with manifestations of several autoimmune diseases suggests that modulation of BAFF could be a novel therapeutic approach to the treatment of such diseases. Indeed, studies with soluble BAFF receptors as antagonists have shown efficacy in reducing disease manifestations in murine models of both SLE and RA [13,14]. Antibody antagonism of the effects of BAFF therefore has potentially important therapeutic value in the treatment of B-cell-associated autoimmune diseases.

However, the development of antibodies requires the use of animals, specialized cell culturing facilities, and an extensive commitment of time and labor. Advances in the field
of recombinant antibody technology provide an alternative means to engineer low-cost antibodies with desirable affinity and specificity by enabling one to manipulate the basic domain structure of the immunoglobulin molecule. One of the most successful approaches is to display single-chain antibody fragment (scFv) on filamentous phage [15–17]. scFv is an antigen-binding protein, composed of an immunoglobulin heavy-chain variable domain (VH) and a light-chain variable domain (VL) joined together by a flexible peptide linker. When expressed with phage protein p3 (ld g3 protein of phage VCSM13) as a fusion protein, a high affinity scFv-producing phage clone can be enriched by a procedure called panning [18,19]. In this report, we describe the panning, expression, and characterization of a functional scFv with high specificities and sensitivities to BAFF.

2. Materials and methods

2.1. Phage libraries, bacterial strains and recombinant human BAFF protein

The Griffin1 library is composed of human scFvs containing highly diverse CDR3s in both the VH and VL domains. This library was derived by recloning VH and VL from human synthetic Fab lox library vectors [20] into the phagemid vector pHEN2 [21]. scFvs can be displayed on the surface if bacteriophage when expressed in suppressor strains HB2151. Recombinant BAFF (residue A134-L285 of BAFF) was cloned and expressed in E. coli (including 1% glucose and 100 μg/ml ampicillin). The phage clone that was sequenced was selected after each round of panning were added into each well and incubated at RT for 90 min. The microtiter plate was washed with PBS-Tween three times, and anti-M13 antibody conjugated with horse radish peroxidase (HRP) (Amersham Pharmacia biotech, USA) was added into each well. The BM Blue POD Substrate (Roche, USA) was used to visualize the signal. The plate was analyzed at 450 nm with ELISA reader (Bio-Rad Molecular Biosciences Group, USA).

2.2. Panning for anti-BAFF phage displayed scFv

Affinity selection for BAFF-binding recombinant phages was performed by panning as described [20]. Briefly, Immunotubes (Maxisorp, Roskilde, Nunc, Denmark) were coated with BAFF diluted in coating buffer (50 mM NaHCO3, pH 9.6) for 12 h at room temperature (RT) and phages were eluted with 100 mM triethyramine and quickly neutralized with 0.5 ml 1 M Tris, pH 7.4. Four rounds of selection were carried out using stringency conditions which consisted in reducing progressively the BAFF concentration (80 μg/ml, 40, 20 and 10 μg/ml) for coating, and increasing from 10 to 20 the washing steps. The titer of the scFv-phae was estimated as follows: E. coli TG1 was grown in 2 x YT media at 37 °C until OD600 = 0.5, and 50 μl of the culture was infected with 10 μl of serial dilution of the eluted or amplified scFv-phages, incubated for 30 min at 37 °C without shaking and plated on TYE agar with 1% glucose and 100 μg/ml ampicillin. The titer of the scFv-phages was calculated by counting the number of grown colonies.

Each of the four-phae populations obtained was tested for specificity to BAFF by polyclonal phage ELISA, whereas individual bacterial clones expressing BAFF-reacting phages were selected by monoclonal phage ELISA.

2.3. Phage ELISA

A microtiter plates (High binding, Corning, USA) was coated with 10 μg/ml of bovine serum albumin (BSA) (Sigma, USA); BAFF or recombinant proliferation-inducing ligand (APRIL) (Peprotech, USA) in coating buffer overnight at 4 °C, and blocked with 2% skim milk in PBS (MPBS) for 2 h at 37 °C. One hundred microliters (about 1 x 10^12 pfu) of amplified phages after each round of panning were added into each well and incubated at RT for 90 min. The microtiter plate was washed with PBS-Tween three times, and anti-M13 antibody conjugated with horse radish peroxidase (HRP) (Amersham Pharmacia biotech, USA) was added into each well. The BM Blue POD Substrate (Roche, USA) was used to visualize the signal. The plate was analyzed at 450 nm with ELISA reader (Bio-Rad Molecular Biosciences Group, USA).

2.4. DNA sequencing analysis

Phagemid DNA was isolated from 3 ml overnight cultures of BAFF-specific clones using minipreparation kit according to the manufacturer’s protocol (Wizard Minipreps DNA Purification System, Promega, USA). The nucleotide sequence of the VH and VL genes of selected clone was determined using the primers FOR LinkSeq (GC-CACCTCCGGCCTGAACC) and pHEN-SEQ (CTA TGCG-GCCCCA TTCA). The variable region sequences obtained were analyzed using the Ig-BLAST (http://www.ncbi.nlm.nih.gov/blast/index.html) and V-BASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/pub/INTRO.html).

2.5. Expression and Western blotting

The phage clone that was sequenced was selected for induction of soluble scFv production. Briefly, plasmid minipreparations were made and transformed into the non-suppressor E. coli strain HB2151. One colony with an apparently intact scFv insert (checked by PCR) from each set was selected and inoculated into 3 ml 2 x YT (including 1% glucose and 100 μg/ml ampicillin), followed by shaking at 37 °C until OD600 = 0.9. Isopropyl-β-
<p>1% glucose and 100 μg/ml ampicillin) culture was transferred to 500 ml
YT (including 1% glucose and 100 μg/ml ampicillin) and shaken at
37°C until OD 600 = 0.9. IPTG (final concentration 1 mM)
was added into the culture, and shaking was continued after 0, 2, 4 and 6 h of adding IPTG. The bacteria samples obtained were boiled with loading buffer and subjected to electrophoresis in 13% SDS–PAGE. After that, the whole bacteria protein was transferred to nitrocellulose membrane (Amersham Pharmacia biotech, USA) from the gel 1.5 h at 150 mA in 50 mM Tris–HCl (pH 8.0), 150 mM glycine and 20% methanol. Next, the membrane was blocked with 5% MPBS for 1 h before washing with PBS. Then, 5 ml 1:2 20% methanol. Next, the membrane was blocked with 5%
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were kept uniformly at $1 \times 10^{13}$ pfu. After each round of panning, the titer of the eluted phage was measured to monitor the efficiency of the selection process (Fig. 1). Almost 200 times increase in phage recovery after the third round in comparison to the second round of selection, and minimal increase after the fourth round in comparison to the third, indicated that the library was already enriched in BAFF-specific binders.

The increasing number of the specific binders in the total phage population was confirmed with polyclonal phage ELISA, which was done with equivalent amounts of amplified phages (about $1 \times 10^{12}$ pfu) after each round of selection. Polyclonal phage ELISA after each round of panning gave higher values of absorbance than in the previous round (Fig. 2), verifying the continuous increase of the number of specific phage binders during selection.

The polyclonal character of the phage ELISA does not give a clear image of the antibody specificity because of different growth rates and expression levels between individual clones. Therefore, the eluted scFv-phages after the fourth round of selection were used for infection of \textit{E. coli} TG1, which were then plated to form single colonies. Next, 92 colonies selected at random from the fourth round of selection were picked and tested for binding to BAFF in monoclonal phage ELISA. From the fourth selection, 32 clones were found to be specific BAFF binders. The reaction was considered positive if the threshold value (mean of the background plus three times the standard deviation) was exceeded. For further binding analysis, nine representative colony A3, B5, B9, C7, F8, E7, E9, D8 and F1 were selected (Fig. 3).

PCR analysis confirmed that the selected clones contained an insert corresponding to the size of an scFv fragment (data not shown).

Mouse spleen B lymphocyte proliferation assay was used to select phage clone which can neutralize BAFF bioactivity. As shown in Fig. 4, clone F8 could inhibit BAFF-induced murine spleen B220$^+$ B cells proliferation strongly, compared as other eight clones.

The double-stranded phagemid DNA was isolated from the \textit{E. coli} cells of the F8 clone and DNA sequence of its scFv genes was determined. The deduced amino acid sequence of
Fig. 4. Selection of phage clone inhibiting BAFF bioactivity. 10^5 murine spleen B220+ B cell were cultured in triplicate in 96-well flat-bottom plates and stimulated with 2 μg/ml BAFF, 2 μg/ml F (ab’)2 fragment of goat anti-mouse IgM and 1 × 10^11 phage particles. A3, B5, B9, C7, F8, E7, E9 and D8 are monoclonal clones which give positive signal in monoclonal phage ELISA. P (primary library), H (helper phage VCSM13) and irrelevant phage clone F1 were used to negative control. Cell growth was then evaluated by MTT Assay. The experiment was triplicate and average value was shown in the figure.

F8 is shown in Fig. 5, and the predicated molecular weight of the whole soluble scFv about 32 kDa.

3.3. Expression and purification of soluble scFv

In the nonsuppressor strain E. coli HB2151, the amber stop codon between c-myc tag and p3 in scFv clones is recognized as a stop codon and soluble scFv fusion protein is produced as a consequence. Clone F8 was chosen for expression analysis, the samples aspirated according to induced time were subjected to 13% SDS–PAGE gel. Then proteins on the gel were transferred to nitrocellulose membrane and used for Western blotting analysis (Fig. 6A) according to the former described method. The result showed that scFv was expressed since c-myc tag was detected in Western blotting.

The soluble scFv (containing a polyhistidine tail) was purified from bacterial lysates by Ni^{II} -IDA His-bind resin and the purity of the obtained scFv was confirmed by 13% SDS–PAGE stained with Coomassie Brilliant Blue R-250 (Fig. 6B), where the purified scFv appeared as a single 32 kDa band at high purity (more than 95%, as estimated by absorbance scanning). The purified scFv with a yield of around 1.5 mg/l of the production culture was quantified by absorbance at 280 nm of the purity solution.

3.4. Antigen binding specificity of the purified scFv

A proliferation-inducing ligand (APRIL), which has the most similar structure as BAFF, could be used to detect the specificity of the scFvs [24]. As seen in Fig. 7, the soluble scFv specifically recognized BAFF with no cross-reaction to APRIL. The fact that the scFv bound to the epitope ran on the gel under denaturing conditions shows that the epitope region that the scFv recognizes is linear.

In order to investigate the functional potential of the selected scFv, a murine spleen B220+ B cell proliferation assay was used. As shown in Fig. 8, murine spleen B220+ B cells proliferate in response to the co-stimulation by BAFF
and anti-IgM signaling. In contrast, a dose-dependent inhibition effect was obtained with F8 scFv.

3.5. Antibody affinity constant measurement

The relative affinity constant for the A1 scFv against BAFF was calculated using a method based on the formation of antibody–antigen complex in a solid-phase EIA (Fig. 9). Based on four concentrations of BAFF used for coating on the immuno-plate (5, 2.5, 1.25 and 0.62 g/ml), six affinity constants (three for \( n = 2 \), two for \( n = 4 \) and one for \( n = 8 \)) were calculated for F8 scFv. The mean \( K_{\text{aff}} \) value for F8 scFv was \( 2.5 \times 10^{7} \) M\(^{-1}\).

4. Discussion

The aim of the study was the generation and characterization of scFv directed against BAFF, which could have therapeutic applications in autoimmune disorders such as SLE, RA, and SS. To the best of our knowledge, we are the first laboratory to screen antibody fragments from the Griffin. 1 antibody library. After four rounds of selection, 32 out of 92 phage clones displayed positive clones. Input/output ratio and affinity assays confirmed that all above improvements were effective. Phages binding to BAFF specifically were riched. Koivumen et al. found that some phages with high affinity could not be recovered by acid elution (1 M HCl, adjusted to pH 2.2 with glycine) [25]. In our study, it was observed that the retained phages after triethylamine elution instead of acid elution could be harvested by cytolysis. In this method, freshly diluted 100 mM triethylamine (pH 10.0) is incubated with the bound phage for 10 min. The pH of the eluted phage is then adjusted immediately with one-half volume 1 M Tris–HCl, pH 7.4. The phage can then be used to infect TG1 as normal. Care must be taken with this method because elution for longer than 10 min can damage the phage and affect infection. If there is still difficulty in eluting BAFF specific phage using this method, then it is possible to add the log-phase TG1 directly to the well (100 µl) or immunotube (2 ml with slow rotation) and incubating for 30 min at 37 °C. Amplification can then proceed as normal.

Western blotting analysis indicated that the purified F8 scFv exhibited the specificity to BAFF. The \( K_{\text{aff}} \) of F8 scFv for BAFF was determined by a series of non-competitive ELISA experiments. The affinity of scFv was usually less than that of the scFv-phage, this might be caused by residues at both ends of scFv displayed on phages, which stabilize the structure of scFv and reinforce its binding to BAFF. The scFv is an
artificial monovalent molecule with a different conformation at the antigen binding site. The reduced binding affinity of scFvs can be improved by changing the amino acid sequence [26] or by constructing a divalent form of the diabody [27] with similar binding affinities to those of the whole antibody.

Overexpression of BAFF has been associated with SLE, RA, and SS. The etiologies of these diseases are unknown, but they have the common features of B cell hyperactivity and autoantibody production, which are strongly implicated in the pathogenesis of the disease process. Specific antibodies against a diversity of antigen were developed to block the function of antigen or guide effective molecules to be applied research such as diagnosis and treatment of diseases since the foundation of antigen-antibody interaction [28].

Our functional assay also showed the purified scFvs could does-dependently inhibited BAFF-induced mouse spleen lymphocyte proliferation. Moreover, because of its single-gene structure, it will be very easy to conjugate this molecule with other effectors such as human Fc fragment and immunotoxin by fused with their genes. The construction of the scFv-Fc fusion will offer a potential candidate molecule for autoimmune disorders. Recently, a fully human monoclonal antibody against BlyS (LymphoStat-B) has entered clinical trials [29]. LymphoStat-B was generated from a native human Ig library, and only recognized soluble BAFF. Our recently flow cytometry analysis results (Cao et al., unpublished) showed F8 clone can recognize not only soluble BAFF, but also the membrane-bound BAFF. In fact, the extract nature of membrane-bound BAFF is not fully understood, so, it would be of interest to determine in vivo activity, and to compare the F8 clone with LymphoStat-B.

In conclusion, as to human BAFF, this report takes advantages over previous work in two aspects at least. Firstly, hybridoma technique was replaced with more convenient phage display technique. Secondly, human originated phage display library was used to avoid the immunoreaction between species. Our data indicate that by designing proper selection of antibodies from antibody library, specific antibodies toward BAFF can be achieve Further research work is in progress.

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References


