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Generation and Characterization of C305, a Murine Neutralizing scFv Antibody That Can Inhibit BLyS Binding to Its Receptor BCMA

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Abstract B-lymphocyte stimulator (BLyS) is a member of the tumor necrosis factor (TNF) family and a key regulator of B cell response. Neutralizing single-chain fragment variable (scFv) antibody against BLyS binding to its receptor BCMA has the potential to play a prominent role in autoimmune disease therapy. A phage display scFv library constructed on pIII protein of M13 filamentous phage was screened using BLyS. After five rounds of panning, their binding activity was characterized by phage-ELISA. Nucleotide sequencing revealed that at least two different scFv gene fragments (C305 and D416) were obtained. The two different scFv gene fragments were expressed to obtain the soluble scFv antibodies, then the soluble scFv antibodies were characterized by means of competitive ELISA and *in vitro* neutralization assay. The results indicated that C305 is the neutralizing scFv antibody that can inhibit BLyS binding to its receptor BCMA.

Key words phage display; neutralizing; scFv antibody; BLyS

B-lymphocyte stimulator (BLyS) [1], also known as BAFF [2], THANK [3], TALL-1 [4], or zTNF4 [5], is a member of the tumor necrosis factor (TNF) ligand superfamily. BLyS is synthesized as a 285-amino acid type II membrane protein and exists in both membrane and cleaved 152-amino acid soluble form [1,5–9]. BLyS is a key regulator of B lymphocyte development. Its biological role is mediated by the specific receptors: BCMA, TACI, and BAFF-R [10].

Several lines of evidence suggest that elevated levels of BLyS may be involved in the pathogenesis of B cell-mediated autoimmune disease. Overexpression of BLyS in transgenic animals results in manifestations of autoimmune-like symptoms, including anti-DNA antibodies, rheumatoid factor, and circulating immune complexes. These symptoms resemble those of systemic lupus erythematosus (SLE) and some aspects of rheumatoid arthritis (RA) [11,12]. Elevated levels of BLyS have been observed in the serum of patients with SLE and RA [13,14] as well as Sjogren's syndrome [15,16]. Reducing the level of BLyS may be a new therapeutic method in the treatment of B cell-mediated autoimmune disease. With the aim of developing a therapeutic agent for autoimmune disease, C305, a murine neutralizing single-chain fragment of variation (scFv) antibody against human BLyS, was generated.

We describe herein the generation of C305, a murine scFv antibody against BLyS, by phage display, as well as the characterization of C305 by competitive ELISA and *in vitro* neutralization assay.

Materials and Methods

Materials

Purified BLyS, BCMA-Fc (receptor of BLyS), the helper phage VCSM13, *Escherichia coli* XL-1 Blue and BL21

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(DE3) were stored in our laboratory. Horseradish peroxidase (HRP)-conjugated mouse anti-M13 was from Amersham Pharmacia Biotech (USA). Anti-Flag-HRP, anti-IgM and anti-Flag M2 affinity agarose were purchased from Sigma (USA). *Sfi*I and T4 DNA ligase were from TaKaRa (Japan).

Phage display library

The mouse semi-synthetic V_H+V_L scFv phage display library was prepared previously in our laboratory. The library was highly variable and contained approximately 10^6 different clones of scFv antibodies.

Rescue of scFv-displaying phage

To rescue scFv-displaying phages, 200 ml super broth (SB) medium (containing 2% glucose, 50 μg/ml carbenicillin, 10 μg/ml tetracycline) was inoculated with approximately 10^{12} cells from the glycerol stock library. The culture was then shaken at 37 °C until A_{600} reached about 0.5, and 10^{12} clone-forming units of VCSM13 and 200 μl of 1 M isopropyl β-*D*-thiogalactopyranoside (IPTG) were added. After 30 min incubation at room temperature, the culture was diluted in 400 ml SB (containing carbenicillin and tetracycline) and grown at 30 °C. After 2 h, 70 μg/ml kanamycin was added and the culture was allowed to grow overnight. Phage particles were purified and concentrated by polyethylene glycol (PEG)/NaCl precipitation.

Biopanning

The library was subjected to five rounds of panning. The immunotube was coated with 1 ml of 5 μg/ml BLyS in phosphate-buffered saline (PBS) overnight at 4 °C. After blocking with 3% BSA in PBS for 1 h at 37 °C, 1012 clone-forming unit phage particles in 1 ml PBS were added and incubated for 2 h at 37 °C. The tube was washed with water, PBS/0.5% Tween-20 and PBS 20 times. Bound phages were eluted from the tube with 1 ml of glycine-HCl (pH 2.2) for 10 min, then 60 μl of 1 M Tris was added to neutralize the glycine-HCl. Eluted phages were amplified by infection of fresh *E. coli* XL-1 Blue cells and the phage rescued as outlined above.

Enzyme-linked immunosorbent assay (ELISA)

Specificity of individual scFv phages and soluble scFv were assessed by ELISA. The 96-well plate was coated with 5 μg/ml BLyS in PBS at 4 °C overnight. After being washed 5 times with water, the wells were blocked with 3% BSA for 1 h at 37 °C. Then 50 μl/well scFv phage or soluble scFv was added and incubated for 1 h at 37 °C. After washing, for phage ELISA, 50 μl/well of HRP-conjugated mouse anti-M13 (Amersham Pharmacia Biotech) in 3% BSA with 1:1000 dilution was added for 30 min at 37 °C. For ELISA using soluble scFv, 50 μl/well of HRP-conjugated mouse anti-Flag M2 antibody (Sigma) in 3% BSA with 1:2000 dilution was added for 30 min at 37 °C. For detection, 50 μl/well of tetramethylbenzidine (TMB) substrate (Shenergy) was used and the absorbance was read at 450 nm.

Cross-reactivity with unrelated protein

The binding specificity of the phage scFv antibodies was evaluated by analyzing the reactivity to BCMA, TNF and eukaryotic cell lysate. 5 μg/ml of these antigens were coated respectively onto 96-well plates, and the phage-ELISA was conducted as described above.

Sequence analysis of scFv

Nucleic acid sequencing of non cross-reacting clone C305, D401, D405, D412, D416 and D419 were carried out by Biocolor Biological Science & Technology Company Limited (Shanghai, China). All sequences were analyzed with Primer (PRIMER Biosoft International, CA) and VectorNTI (InforMax, Inc.) software, and blasted and formatted to compare previously sequenced mouse V_H and V_L gene fragments.

Expression and purification of soluble scFv antibodies

The positive phage clones were digested with *Sfi*I and subcloned into the expression vector pTc, digested with the same restriction enzyme. Recombinants were transformed into *E. coli* BL21(DE3). The scFv expression was induced by growth in SB containing 1 mM IPTG overnight at 30 °C. The Flag-tagged scFv antibodies were purified on anti-Flag M2 affinity agarose from the periplasmic extracts. The purified proteins were then detected by ELISA.

Competitive ELISA with soluble scFv antibody

The range where the ELISA signal decreased with decreased BCMA-Fc concentrations was first determined by ELISA. The concentration near the top of this range was chosen for competitive ELISA. The 96-well plate was coated with 50 μl of 5 μg/ml BLyS in PBS at 4 °C overnight. After blocking and washing, soluble scFv antibodies were added simultaneously with the BCMA-Fc diluted in blocking buffer and incubated for 2 h at 37 °C. The soluble scFv antibody samples were diluted to a factor of 1:4. After washing, 50 μl of 1:1000 dilution of HRP-conjugated goat anti-human IgG was added and incubated for 30 min at 37 °C. For detection, 50 μl/well of TMB

substrate (Shenergy) was used and the absorbance was read at 450 nm. The inhibition rate was calculated as follows:

$$\text{Inhibition rate} = \frac{A_{450}(-\text{comp}) - A_{450}(+\text{comp})}{A_{450}(-\text{comp})} \times 100\%$$

where, A_{450}(–comp) is the A_{450} without competitor and A_{450}(+comp) is the A_{450} with competitor.

In vitro neutralization assay

Murine B lymphocytes were prepared in DMEM supplemented with 10% bovine serum and seeded in a 96-well plate at a concentration of 4.5×10^4 cells/well. Samples of BLyS (final concentration 4 μg/ml) and anti-IgM were mixed with series dilution of soluble scFv antibodies (C305 and D416, diluted to a factor 1:4, respectively) and added to the cells. After 72 h of incubation at 37 °C, 5% CO_2, 10 μl of 10 μg/ml MTT was added to the wells which were incubated at 37 °C, 5% CO_2 for another 5 h. After removing the solution from the wells, 100 μl of 15% SDS (15 mM HCl) were added and incubated overnight at 37 °C, 5% CO_2. The number of viable cells was determined by measuring the absorbance at 570 nm. The neutralization of the BLyS by soluble scFv antibodies was calculated using the following formula:

$$\text{Neutralization} = \frac{\text{B cell}_{\text{Con}}\ \text{prolif} - \text{B cell}_{\text{Ab}}\ \text{prolif}}{\text{B cell}_{\text{Con}}\ \text{prolif}} \times 100\%$$

where, B cell_{Con} prolif is the proliferation of B cell_{Con} and B cell_{Ab} prolif is the proliferation of B cell_{Ab}.

Results

Biopanning

The panning against BLyS was performed for five rounds with an enrichment of clone number from 9×10^3 in the first round to 1.1×10^8 in the fifth round (**Table 1**).

The pool of phages selected by five rounds of panning was used to infect XL-1 Blue cells, and subsequent plating on agar containing carbenicillin allowed individual phage clones harboring phagemid to grow. To verify that individual phage clones could bind to BLyS, 20 phage clones were picked up from each of the third, fourth and fifth rounds. Phages were rescued and tested for binding to immobilized BLyS by ELISA. Eleven clones obtained from the third round, 16 clones from the fourth round, and 20 clones from the fifth round exhibited binding activity to immobilized BLyS. The positive rate rose from 55% in the third round to 100% in the fifth round (**Fig. 1**).

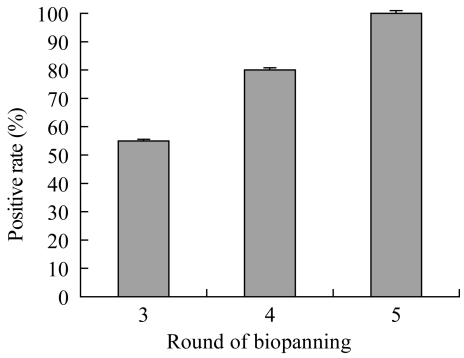

Fig. 1 The positive rate of different rond of biopanning
The positive rate rises from 55% of the third round to 100% of the fifth round.

The binding specificity of the positive phage clones that can bind to BLyS was evaluated by analyzing the reactivity to BCMA, TNF and eukaryotic cell lysate. The results showed that only clone C305, D401, D405, D412, D416 and D419 were specific to BLyS.

Table 1 Enrichment during screening of phage display library

Round	Number of input phages	Number of output phages	Ratio of enrichment
1	10^{12}	9×10^3	9×10^{-9}
2	10^{12}	1.7×10^5	1.7×10^{-7}
3	10^{12}	3.7×10^6	3.7×10^{-6}
4	10^{12}	1.4×10^7	1.4×10^{-5}
5	10^{12}	1.1×10^8	1.1×10^{-4}

Table 2 Complete protein sequences of clones C305 and D416

Clone	Heavy chain	Light chain
FR1		
C305	QVQLQHLGGGADEAWSLMKISCKATGYTFS	DIVMAOIPASLAVSLGQRATISC
D416	QVKLMESGGGLVQPGGSLRLSCATSGFTFT	DIQMTQSPLSLPVNLGDQASISC
CDR1		
C305	SYWIE	RASKSVSTSGYSYMH
D416	DYYMS	RSSQSIVHSNGNIYLE
FR2		
C305	WVKQRPGHGLEWIG	WYQQKPGQPPKLLIY
D416	WVRQPPGKAPEWLG	WYLQKPGQSPKLLIY
CDR2		
C305	EILPGSGSTNYDEKFKS	LASNLES
D416	FIRNKANGYTTDSTSASVKG	KVSNRFS
FR3		
C305	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCTR	GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC
D416	RFTISRDNAOSILYLQMNTLRPEDSATYYCAR	GVPDRFSGSGSGTDFTLKINRVEAEDLGVYYC
CDR3		
C305	GGSKNGLVVLP	QHSREL
D416	DRTYW	FQGSHV
FR4		
C305	GPRDSGHVSS	PYTFGGGTKLEIKR
D416	GQGTTVTVSS	PWTFGGGTKLEIKR

The table shows that the two clones have different protein sequences, especially in their complementary-determining regions. CDR, complementary-determining region; FR, frame work region.

Sequences of scFv clones C305 and D416

Analysis of the sequences of the six different clones that demonstrated specific to BLyS by ELISA revealed that two sequences (C305 and D416) were different in V_H and V_L (**Table 2**).

Purification and specificity of the soluble scFv antibodies

The scFv gene fragments of C305 and D416 were cut from the phagemid and inserted into the expression vector pTc-Flag. The expressed scFv antibodies were collected from the periplasm of the bacteria and purified with anti-Flag affinity chromatography. The specificity of the purified soluble scFv antibodies was assayed by ELISA. The results are shown in **Table 3**.

Table 3 Binding specificity of the purified scFv antibodies C305 and D416 to BLyS with ELISA

Concentration of samples (μg/ml)	A_{450} of scFv antibody	
	C305	D416
60.00	0.651	0.584
30.00	0.552	0.485
15.00	0.471	0.322
7.50	0.430	0.141
3.75	0.261	0.080
1.83	0.105	0.040
0.91	0.030	0.010

3% BSA was a negative control.

Competitive ELISA with purified soluble scFv C305 and D416

The neutralizing activity of purified scFv C305 and D416 was tested by competitive ELISA. The scFv antibodies were used at a range of concentration to inhibit

binding of BCMA-Fc to BLyS. Binding of BCMA-Fc was detected using goat anti-human IgG-HRP. The level of inhibition of C305 and D416 (expressed as a percentage) is shown in **Fig. 2**. C305 can inhibit the binding of BCMA-Fc to BLyS, while D416 can not.

In vitro neutralization assay

As shown in **Fig. 3**, neutralization of the proliferation of murine B cells stimulated by BLyS was determined by the MTT assay. It was found that C305 can inhibit the proliferation of murine B cells evidently.

The result of D416 in *in vitro* neutralization assay is different from that in competitive ELISA. The difference may be produced with lower neutralizing activity. The neutralizing activity of D416 should be characterized further. The results of C305 in *in vitro* neutralization assay is the same as that in competitive ELISA. Soluble scFv C305 is the neutralizing scFv antibody.

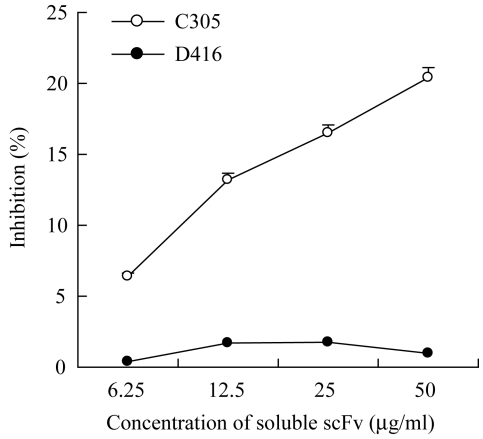

Fig. 2 Competitive ELISA: inhibition of binding of BCMA-Fc to BLyS by soluble scFv antibodies C305 and D416

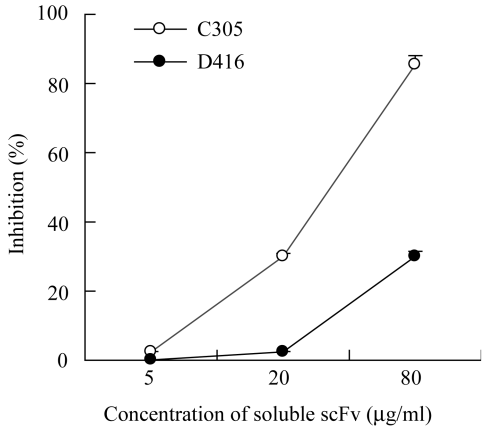

Fig. 3 *In vitro* BLyS neutralization assa y of the soluble scFv antibodies

Discussion

BLyS plays a critical role in the normal regulation of B cell development and immune response. This was demonstrated by the severely depleted B cell phenotype observed in BLyS-deficient animals [17,18]. As the case for other cytokines, BLyS levels must be carefully regulated in order to maintain "immune homeostasis".

Several lines of evidence suggest that elevated levels of BLyS may be involved in the pathogenesis of B cell-mediated autoimmune disease. Three kinds of transgenic mice overexpressing BLyS showed severe enlargement of spleen, lymph nodes and Peter's patches. They also developed autoimmune-like manifestations such as the presence of high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies, total immunoglobins and immunoglobin deposition in the kidneys [5,11,12], all of which are manifestations associated with autoimmune disease. Elevated levels of BLyS have been detected in the MZWB and MLR-lpr/lpr mouse models of spontaneous lupus. BLyS levels in these animals increase with age, in parallel with the severity and progression of the disease. Treatment with the soluble BLyS receptor significantly improves the survival of lupus mice [5]. These observations suggest that overexpression of BLyS may contribute to B cell hyperactivity and autoantibody production in multiple autoimmune diseases and reduction of the level of BLyS may provide a novel treatment for these diseases. Toward that end, we have generated a murine scFv antibody specific for BLyS that can inhibit BLyS binding to its receptor BCMA *in vitro* and in competitive ELISA.

The clone C305 was generated from an immune murine scFv antibody library using phage display technology. In competitive ELISA, C305 can inhibit BLyS binding to its receptor BCMA-Fc with low neutralizing activity, because the affinity of C305 is lower, and the concentration of C305 is lower than that of BCMA-Fc used in competitive ELISA; *in vitro*, C305 can evidently inhibit BLyS-induced stimulation of B cells from murine splenocytes. After humanization and affinity improvement, C305 may be used as a therapeutic agent for B cell-mediated autoimmune disease.

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