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**Characterization of a single-chain antibody fragment (scFv) specific for  
*Plasmodium vivax* Duffy binding protein**

**Running title:** HUMAN ANTI-DBP scFvs

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1 **ABSTRACT**

2 Phage display of single-chain variable fragment (scFv) antibodies is a powerful tool for  
3 selecting important, useful, specific human antibodies. We constructed a library from three  
4 patients infected with *Plasmodium vivax*. Panning on recombinant PvRII enriched a  
5 population of scFvs that recognized region II of the *P. vivax* Duffy binding protein (DBP).  
6 Three clones of scFvs that reacted with PvRII were selected and their biological functions  
7 were analyzed. These scFvs inhibited erythrocyte binding to DBP. Clone SFDBII92 had the  
8 greatest affinity ( $K_D = 3.62 \times 10^{-8}$  M) and the greatest inhibition activity ( $IC_{50} \approx 2.9 \mu\text{g/mL}$ )  
9 to DBP. Thus, we demonstrated that human neutralizing antibody could be made from  
10 malaria patients using phage display and that these neutralizing scFvs should prove  
11 valuable for developing both passive and active immunization strategies based on DBP.

12

13

## 1 INTRODUCTION

2 Malaria caused by *Plasmodium vivax* is responsible for substantial morbidity in Asia and  
3 Central and South America (19). Merozoites of *Plasmodium* must attach to and invade RBC  
4 to begin asexual reproduction of the parasite, making this brief event a critical phase in the  
5 parasite life cycle. Invasion occurs quickly through a complex, multistep process that  
6 follows a distinct sequence of events involving numerous molecules expressed on the  
7 surface of the merozoite and in the apical organelles (1, 4, 6). This cascade of events  
8 represents potential targets for reducing or eliminating the blood stages of malarial parasites  
9 (21, 25, 31).

10 The Duffy binding protein (DBP) of *P. vivax* interacts with Duffy antigen receptor  
11 for chemokines (DARC) on the RBC during junction formation between the merozoite and  
12 RBC (1, 2, 16, 34). The *P. vivax* DBP (PvDBP) is a 140-kDa protein that belongs to a  
13 family of erythrocyte-binding proteins characterized by a functionally conserved cysteine-  
14 rich region (1, 6, 12). This cysteine-rich region is in DBP region II (DBPII), which contains  
15 the binding motifs necessary for adhering to DARC on the erythrocyte surface (9, 10, 29).  
16 The critical binding motif has been mapped to a 170-amino-acid segment between cysteines  
17 4 and 8 in the cysteines-rich region (26, 28, 29). Studies have shown that although the

1 cysteine residues are conserved, other regions of DBPII are highly polymorphic (3, 32, 36).  
2 However, the hypervariable region of DBP II is located on the sites remote from the  
3 DARC-binding site and does not alter the capacity of the protein to bind DARC-positive  
4 erythrocytes (28, 33).

5 Phage display antibodies offer a way to produce high-affinity single-chain variable  
6 fragment (scFv) derivatives of human antibodies of “natural host” origin (8). Our goal was  
7 to produce human monoclonal antibodies (mAbs) against the DARC-binding region of  
8 DBP II of *P. vivax* (PvRII). To do so, we constructed a combinatorial phage display library  
9 using peripheral blood mononuclear cells from three patients infected naturally with *P.*  
10 *vivax*. Subsequently, anti-PvRII human scFvs that had neutralizing activity against DBP  
11 binding to erythrocytes were identified. These neutralizing scFvs should prove valuable for  
12 developing both passive and active immunization strategies based on DBP.

## 1 MATERIALS AND METHODS

### 2 *Constructing the human scFv phage display library*

3 Peripheral blood mononuclear cells were collected from three patients infected by Korean  
4 strain of *P. vivax* using Ficoll-Paque. Total RNA was extracted from each sample using  
5 TRIzol (Gibco-BRL/Life Technologies, Gaithersburg, MD). First-strand cDNA was  
6 generated using Superscript II reverse transcriptase (RT; Invitrogen, Carlsbad, CA). Light-  
7 and heavy-chain genes were cloned using polymerase chain reaction (PCR) with the  
8 primers described by Barbas *et al.* (5). The variable regions of the light ( $V_L$ )- and heavy  
9 ( $V_H$ )-chain genes were amplified separately from each cDNA and recombined in a second  
10 round of PCR. A pool of gene fusions that encoded scFvs of the  $V_L$ -spacer- $V_H$  sequence  
11 was assembled. Following overlap PCR and gel purification, the amplified products were  
12 cloned into the phagemid vector. The ligation mixtures were electroporated into  
13 *Escherichia coli* ER2537 cells using Gene Pulser II (Bio-Rad Laboratories, Munich,  
14 Germany). Library phages were harvested from the culture supernatant of recombinant *E.*  
15 *coli* and precipitated with 20% PEG/2.5 M NaCl, as described previously (24). The phage  
16 pellet was reconstituted in 2 mL of 1% (w/v) bovine serum albumin in Tris-buffered saline  
17 (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH7.5) before being filtered through a 0.45- $\mu$ m

1 filter.

2

3 *Recombinant PvRII molecules*

4 Recombinant PvRII (C4-C7 cystein rich region of PvDBP II) protein of SK-1 strain isolated  
5 in South Korea was produced using a reported method (17). Briefly, the PvRII gene was  
6 amplified by PCR with C4-7-F (5'-CGA AGA TAT GAA TTC TGT ATG AAG GAA CTT-  
7 3') and C4-7-R (5'-ATT GAT TTC TCG AGC ACA TTT TTC TTT CAG-3') and cloned in  
8 the *E. coli* expression vector pET28a+. The expression constructs were transformed in *E.*  
9 *coli* BL21(DE3). The expression of recombinant PvRII was induced with isopropyl- $\beta$ -D-  
10 thiogalactopyranoside (IPTG) in shake flask cultures and purified by metal affinity  
11 chromatography using Ni-NTA matrix (Qiagen) from inclusion bodies that had been  
12 solubilized with 8 M urea. The recombinant proteins purified under denaturing conditions  
13 were refolded by rapid dilution and purified to homogeneity using ion-exchange  
14 chromatography with Toyopearl-SP (Sigma) and gel filtration chromatography with  
15 Superdex-75 (Amersham-Pharmacia).

16

1 *Panning the scFv library to select PvRII binder*

2 The phage library was panned for binders using 96-well enzyme-linked immunosorbent  
3 assay (ELISA) plates (Costar) coated with PvRII (1  $\mu\text{g}/100 \mu\text{L}$ ) at 4°C overnight. Blocking  
4 and negative-selection well coating were performed with 10% fetal bovine serum (FBS) in  
5 TBS. Antigen-binding clones were eluted by enzyme digestion with 10 mg/mL trypsin in  
6 TBS at room temperature for 30 min. The eluted phages were then allowed to infect *E. coli*  
7 ER2537 host cells to amplify the selected phages binding to PvRII. After amplification,  
8 phages were selected for three additional rounds using the same protocol. An aliquot of  
9 each of the polyclonal phages obtained after each round of selection was stored at 4°C until  
10 required. After four rounds of selection, 96 single clones were screened for binding to  
11 PvRII using ELISA.

12

13 *Enzyme-linked immunosorbent assay*

14 Microtiter wells were coated with PvRII, as described previously, and blocked with 10%  
15 FBS in TBS for 1 h at 37°C. Phages were incubated in the wells for 1 h at 37°C and then  
16 washed four times with TBS, 0.05% Tween 20. Bound phages were detected with a 1:3000  
17 dilution of horseradish peroxidase-conjugated mouse anti-M13 antibody (Amersham-



1 Pharmacia) for 30 min at 37°C and detected by adding ABST substrate (Kirkegaard and  
2 Perry).

### 3 4 *Production of soluble scFv antibody fragments*

5 We induced scFv expression as described previously (22). Briefly, the *E. coli* Top10F' was  
6 infected with selected phages before the cells were grown to the logarithmic phase and  
7 induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside overnight to produce soluble  
8 scFvs. The expressed scFvs were purified using immobilized metal affinity chromatography  
9 with a Ni<sup>2+</sup>-charged HP chelating column, following the manufacturer's protocol  
10 (Amersham-Pharmacia).

### 11 12 *DNA Sequencing*

13 The DNA that encoded the scFv was sequenced using a BigDye Terminator Ready Reaction  
14 Kit (Applied Biosystem) and the products were analyzed on an ABI 3130xl (Applied  
15 Biosystem). The sequences of the V<sub>H</sub> and V<sub>L</sub> genes were compared with the sequences  
16 present in the V base Sequence Directory to determine the closest germ line counterpart.

17

1 *Surface plasmon resonance*

2 A BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) was used to analyze the  
3 kinetic properties of the scFvs. PvRII was immobilized on a 1-ethyl-3-[3-  
4 dimethylaminopropyl] carbodiimide hydrochloride/N-hydroxysuccinimide-activated CM5  
5 sensor chip by injecting 10  $\mu\text{g/mL}$  PvRII in 10 mM sodium acetate (pH 4.0) to obtain 1000  
6 resonance units (RU). The scFvs were diluted with running buffer, HBS-EP buffer (0.01 M  
7 HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; Biacore AB), and  
8 were injected at 30  $\mu\text{L/min}$ . The rate constants of association ( $k_{\text{on}}$ ) and dissociation ( $k_{\text{off}}$ )  
9 were obtained at five different scFv concentrations (range: 1–100  $\mu\text{g/mL}$ ). All assays were  
10 repeated at least three times. The dissociation constant ( $K_{\text{D}}$ ) was calculated from the ratio of  
11 the rate constants of association and disassociation ( $k_{\text{on}}/k_{\text{off}}$ ). The sensorgrams were  
12 analyzed using BIAevaluation 3.2 software. The residual scFv was removed with 50 mM  
13 NaOH after each measurement.

14  
15 *Surface expression of PvDBPII in COS-7 cells*

16 The surface expression of region II of PvDBP was carried out as reported previously, with  
17 some modifications (14). Briefly, PvDBPII was amplified by PCR using the primers

1 PvDBPF (5'-ACAATTTGGTAATGTTAGAT-3') and PvDBPR  
2 (5'-TGATTTCCATTTTGACCATC-3') and cloned into the pDE vector using the *Bg*III and  
3 *Sac*II sites (pDE-PvDBPII). The plasmid DNA used for transfection was purified using an  
4 endotoxin-free plasmid MidiPrep kit (Qiagen); 1 µg of DNA was used to transfect COS-7  
5 cells in six-well plates (approximately  $5 \times 10^4$  cells/well) using FuGene6 (Roche). After 2  
6 days, the transfected COS-7 cells were used for fluorescence and erythrocyte binding-  
7 inhibition assays (EBIA). Fluorescence was detected using confocal microscopy (Carl-  
8 Zeiss 510Meta, Germany).

9  
10 *Erythrocyte binding-inhibition assay*

11 The EBIA was performed as described previously (14). After 48 h of transfection, COS-7  
12 cells were incubated with various concentrations of purified scFvs for 2 h at 37°C. After  
13 washing with PBS, a 10% human erythrocyte suspension of type “O” blood was added to  
14 each well, incubated for an additional 2 h, and washed three times with PBS. Binding was  
15 quantified by counting the rosettes observed over 20 fields of view at  $\times 100$  magnification.  
16 Each experiment was performed in triplicate and the data shown are from at least two  
17 separate experiments.

## 1 RESULTS

2 A human scFv antibody library was constructed using peripheral blood mononuclear  
3 cells from *P. vivax*-infected patients admitted to Busan Paik Hospital in August 2001. The  
4 library contained  $7.85 \times 10^8$  independent clones. The investigation of full-length inserts  
5 from 40 clones using PCR and *Bst*NI digestion showed that 70% had inserts and there were  
6 no identical digestion patterns in the inserted clones (data not shown).

7 In *E. coli* BL21(DE3), the recombinant PvRII accumulated mostly in inclusion  
8 bodies (Fig. 1). Purified recombinant PvRII showed a single 20.4 kDa band (Fig. 1C). The  
9 recombinant PvRII reacted with the pooled sera of the six patients with vivax malaria, but  
10 didn't react with an irrelevant antibody, anti-HBV preS1 scFv (Fig. 2) (22).

11 After the fourth round of selection, 96 clones were analyzed for PvRII binding using  
12 ELISA (Fig. 2). Clones which show the higher OD than anti-HBV pre-S1 scFv at least  
13 three times. Twelve clones were selected as positive and these were categorized into three  
14 groups according to absorbance: low, middle, and high. *E. coli* Top10F' (amber  
15 nonsuppressant) was infected with three clones (one from each group: SFDBII12,  
16 SFDBII58, and SFDBII92), and these were expressed as soluble scFv forms by IPTG  
17 induction for affinity measurements and EBIA. The soluble scFvs were purified using the

1 IMAC system and were about 34 kDa, as expected (Fig. 3A). In addition, the three clones  
2 were sequenced (Table 1). A comparison with the sequences of the germ line  $V_H$  genes  
3 shows that the clones use a  $V_H$  III family-derived germ line segment. Alignment with the  
4  $V_L$  germ line sequences showed that these clones use  $V_K$  I,  $V_K$  III, and  $V_\lambda$  I family  
5 segments. The kinetic parameters of these clones were measured using BIAcore (Fig. 3B).  
6 SFDBII92 had the greatest affinity ( $K_D = 3.62 \times 10^{-8}$  M vs.  $K_D = 1.54 \times 10^{-7}$  M for  
7 SFDBII12 and  $K_D = 1.26 \times 10^{-7}$  M for SFDBII58).

8 To evaluate the biological effects of anti-PvRII scFvs, region II of PvDBP was  
9 expressed on the surface of COS-7 cells in the form of an EGFP fusion protein. Following  
10 transfection, we confirmed the cell surface localization of green fluorescence protein and  
11 rosette formation under a confocal microscope (Fig. 4). The erythrocyte-binding activity to  
12 the transfected cells was measured by counting the number of rosettes. The rosette counts  
13 of transfected cells were  $53.6 \pm 3.5$ . Next, to determine whether the anti-PvRII scFvs  
14 blocked the adhesion of the PvDBP molecules to human erythrocytes, transfected COS-7  
15 cells were treated with anti-PvRII scFvs and reacted with erythrocytes to form rosettes. All  
16 anti-PvRII scFvs showed inhibitory activity, and the number of rosettes decreased as the  
17 amount of scFv was increased (Fig. 5). For a negative control, anti-pre-S1 human scFv (22)

- 1 was used and had no inhibitory activity. At 90  $\mu\text{g/mL}$  of scFvs, SFDBII92 showed the
- 2 highest inhibition (91.6% for SFDBII92, 88.4% for SFDBII12, and 80.9% for SFDBII58).
- 3 The 50% inhibition concentrations ( $\text{IC}_{50}$ ) were 2.9  $\mu\text{g/mL}$  for SFDBII12, 4.0  $\mu\text{g/mL}$  for
- 4 SFDBII58, and 4.5  $\mu\text{g/mL}$  for SFDBII92.

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## 1 DISCUSSION

2           The invasion of erythrocytes by *P. vivax* merozoites is thought to be as complex a  
3 process as the invasion of erythrocytes by other *Plasmodium* species, such as *P. falciparum*.  
4 Erythrocyte-binding capability of several proteins have examined, including merozoite  
5 surface protein 1, reticulocyte binding protein, and DBP, which have high binding affinity  
6 to erythrocytes (9, 10, 14, 26). For *vivax* malaria, DBP is considered an important vaccine  
7 candidate antigen due to its high binding affinity and its strong antigenicity in humans (20,  
8 36). The cysteine-rich region of DBP II, especially in the region between C4 to C8, has the  
9 binding motifs necessary for adherence to DARC on the erythrocytes; this region of the *dbp*  
10 gene is much more variable than other regions (32). Using site-directed mutation analysis,  
11 the DARC-binding site of DBP II consists of conserved amino acids (33) and is located on  
12 the surfaces distant from the hypervariable region (28). Therefore, we decided to make  
13 neutralizing antibody fragments against PvRII from a scFv library of naturally infected  
14 malaria patients.

15           Some human recombinant antibodies against *Plasmodium* have been reported using  
16 the MSP protein of *P. falciparum* or AMA protein of *P. chabaudi* as targets (13, 18, 27, 30).  
17 We constructed a phage display library from the peripheral blood of human patients, as

1 reported previously. Roeffen *et al.* (27) showed their biological activity and invasion-  
2 inhibition using competitive ELISA with neutralizing antibodies. Lundquist *et al.* (18)  
3 showed the neutralizing activity with an antibody-dependent cellular assay. Here, we  
4 demonstrated the neutralizing activities of recombinant antibody fragments using EBIA.

5 Two genotypes of *p. vivax* (SK-1 and SK-2) coexist in the endemic area of the south  
6 Korea. Comparing Sal-1, Belem, PNG (Papua New Guinea), COLV and COLT (Colombia)  
7 isolates, DBP variations of the SK-1 strain are considered not to affect the binding affinity  
8 of PvDBP to RBCs (17).

9 Using conventional panning, the O/I ratio increased continuously with each panning.  
10 After the fourth panning, 96 clones were tested with phage ELISA to determine whether  
11 they recognized the PvRII protein. Twelve positive clones showed OD values between 0.2  
12 and 0.8. We grouped these positive clones into three groups, selected three clones  
13 (SFDBII12, SFDBII58, and SFDBII92), and analyzed their antibody and functional  
14 characteristics.

15 All the heavy-chain genes of the scFvs were derived from the V<sub>H</sub> III germ line gene,  
16 whereas the light chains were derived from the kappa I, III, and lambda I germ line genes  
17 (Table 1). Surface plasmon response measurements showed that the three antibodies had



1 affinities ( $K_D$ ) in the nanomolar range. Previous work on an invasion-inhibiting anti-  
2 circumsporozoite antibody ( $K_D \approx 300$  nM) (35), HBV neutralizing antibodies ( $K_D \approx 160$   
3 nM) (22), and HIV neutralizing antibodies ( $K_D \approx 4.6$  nM) (11) showed that the SFDBII  
4 antibody series described here indeed has affinities within a biologically relevant range.  
5 Hans *et al.* (15) estimated that the binding constant of the PvRII-DARC is 8.7 nM. The  
6 affinity of the SFDBII antibodies is lower than that of the PvRII-DARC interaction. If an  
7 SFDBII antibody recognizes the binding site of DARC, the affinity can be increased using  
8 phage display techniques.

9 The EBIA results showed that the erythrocyte-binding inhibition activities of the  
10 anti-DBP II scFvs were correlated with their affinities. SFDBII92 had the greatest  
11 inhibition activity ( $IC_{50} \approx 2.9$   $\mu$ g/mL vs. 4.0  $\mu$ g/mL for SFDBII12 and 4.5  $\mu$ g/mL for  
12 SFDBII58). The neutralizing activities of SFDBIIs are correlated with ELISA signal and  
13 affinity. These suggest that PvRII, C4-C7 region of DBP II is a binding motif as previously  
14 reported.

15 Our anti-DBP antibodies can be candidates for passive immunization to prevent or  
16 treat *P. vivax* infection. Unfortunately, we cannot estimate the amount of scFv showing a  
17 neutralizing effect *in vivo*. For therapeutic purposes, SFDBII92 should have greater affinity

1 producing an effective neutralizing activity against vivax malaria, which can be facilitated  
2 by using error-prone PCR, chain shuffling, or complementarity determining region (CDR)  
3 mutagenesis of the variable regions of SFDBII92 (23, 24).

4 We successfully made human antibody fragments that neutralized DBP binding to  
5 the erythrocyte surface and demonstrated the neutralizing activity of these antibodies *in*  
6 *vitro* using an erythrocyte-binding inhibition assay.

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## 1 **FIGURE LEGENDS AND TABLES**

2 Figure 1. Expression and purification of recombinant PvRII protein. (A) Recombinant  
3 PvRII was expressed as a insoluble protein in *E. coli* BL21(DE3) and electrophoresed using  
4 12% SDS-PAGE (1, BL21[DE3] with pet28a; 2, Bio-Rad low-range protein marker; 3,  
5 whole lysates; 4, soluble fraction; 5, insoluble fraction of pet28a- PvRII). (B) The  
6 expression of PvRII was confirmed by Western blotting (15% SDS-PAGE) with anti-his  
7 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG (1, Bio-Rad  
8 low-range protein marker; 2, BL21[DE3]; 3, BL21[DE3] with pet28a; 4, pet28a-PvRII; 5,  
9 the soluble fraction of pet28a-PvRII; 6, the insoluble fraction of pet28a-PvRII). (C)  
10 Expressed PvRII was purified from the insoluble fraction with a Ni-NTA column under 8 M  
11 urea denaturing conditions (1, Bio-Rad low-range protein marker; 2, purified PvRII; 3,  
12 insoluble fraction of PvRII; 4, flow-through). Arrows at panel B and C indicate the PvRII,  
13 20.4 kDa.

14  
15 Figure 2. Selection of binders to PvRII. Each well of the ELISA plate was pre-coated with  
16 recombinant PvRII (1  $\mu\text{g}/\text{well}$ ) and then antibodies from each phage ( $10^{10}$  cfu/well) were  
17 added to each well. Bound phages were detected with horseradish peroxidase-conjugated

1 anti-M13 mouse antibody. Anti-HBV pre-S1 scFv (22) was used as an irrelevant antibody,  
2 and pooled sera of the six patients with vivax malaria were used for the reactivity of the  
3 purified recombinant PvRII. The results are the average of triplicate assays.

4  
5 Figure 3. Purification and analysis of the binding kinetics of soluble scFvs. (A) ScFvs were  
6 expressed in the form of soluble proteins without a p3 phage protein fused to the C-terminal  
7 of scFv. Monomeric scFvs were purified with an Ni-NTA affinity column using fast protein  
8 liquid chromatography and subjected to 12% SDS-PAGE. (B) The binding kinetics of the  
9 scFvs were measured using surface plasmon resonance on a BIAcore biosensor instrument.  
10 The graph shows an overlay plot of the sensorgrams obtained for SFDBII92 at six different  
11 concentrations (0.5–1  $\mu$ M) against immobilized PvRII.

12  
13 Figure 4. Erythrocyte-binding assay. PvDBPII was expressed on the surface of COS-7  
14 cells using the pDE-GFP surface display system. Transfected COS-7 cells were observed  
15 under confocal microscopy and reacted with a 10% erythrocyte suspension for rosette  
16 formation (A and C, pDE; B and D, pDE-PvDBPII).  
17 Microscope magnification is  $\times 200$ .

1 Interactions with receptor of erythrocytes and PvDBPII were observed as rosettes (arrows).  
 2  
 3 Figure 5. Erythrocyte-binding inhibition assay. Transfected COS-7 cells with pDE-  
 4 PvDBPII were incubated with various concentrations of soluble scFvs (2-100  $\mu$ g/mL) for  
 5 2 h at 37°C. After washing, a 10% erythrocyte suspension was added for rosette formation.  
 6 The binding inhibition activity was scored after counting the rosettes in 20 fields at a  
 7 magnification of  $\times 100$ . An irrelevant scFv was used as a negative control. The error bars  
 8 correspond to  $\pm$  standard error.

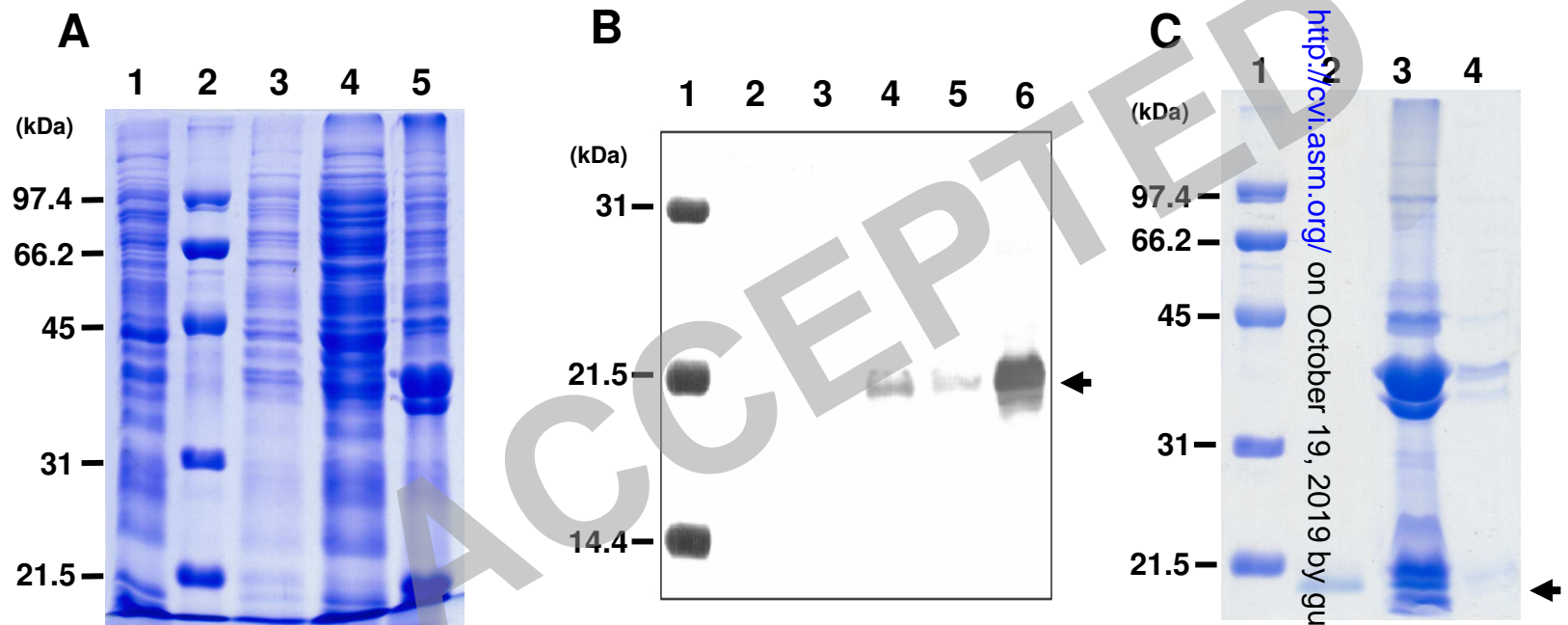
9  
 10 Table. 1. Analysis of the complementarity determining region (CDR) and human subgroup  
 11 assignment for the three anti-PvRII scFvs.

Clone	CDR1	CDR2	CDR3	Family
SFDBII12	GFTFGDYAMETH	GISWNSGSIG	GMETAANYYYYYGMETDV	V $\kappa$ III
V <sub>L</sub> SFDBII58	GFTFGDYGMETH	GISWNSGSIG	DIYSSSWYAAFDI	V $\kappa$ I
SFDBII92	GFTFSSYWMETS	NIKQDGSEKY	DCSSTSCYGYYYGMETDV	V $\lambda$ I
SFDBII12	RASQSVSSSYLA	GASSRAT	QQYGSSRLT	V <sub>H</sub> III
V <sub>H</sub> SFDBII58	RASQISYHLN	AASTLQS	QKYNAPLT	V <sub>H</sub> III
SFDBII92	SGSSNIGSNTVN	SNNQRPS	AAWDDSLNGWV	V <sub>H</sub> III

12 The V<sub>H</sub> and V<sub>L</sub> were searched against the Kabat database using the BLAST algorithm and

- 1 the scFvs groups were analyzed via the World Wide Web
- 2 (<http://acrmwww.biochem.ucl.ac.uk/abs/chothia.html>).
- 3

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Fig 1. SH Kim et al

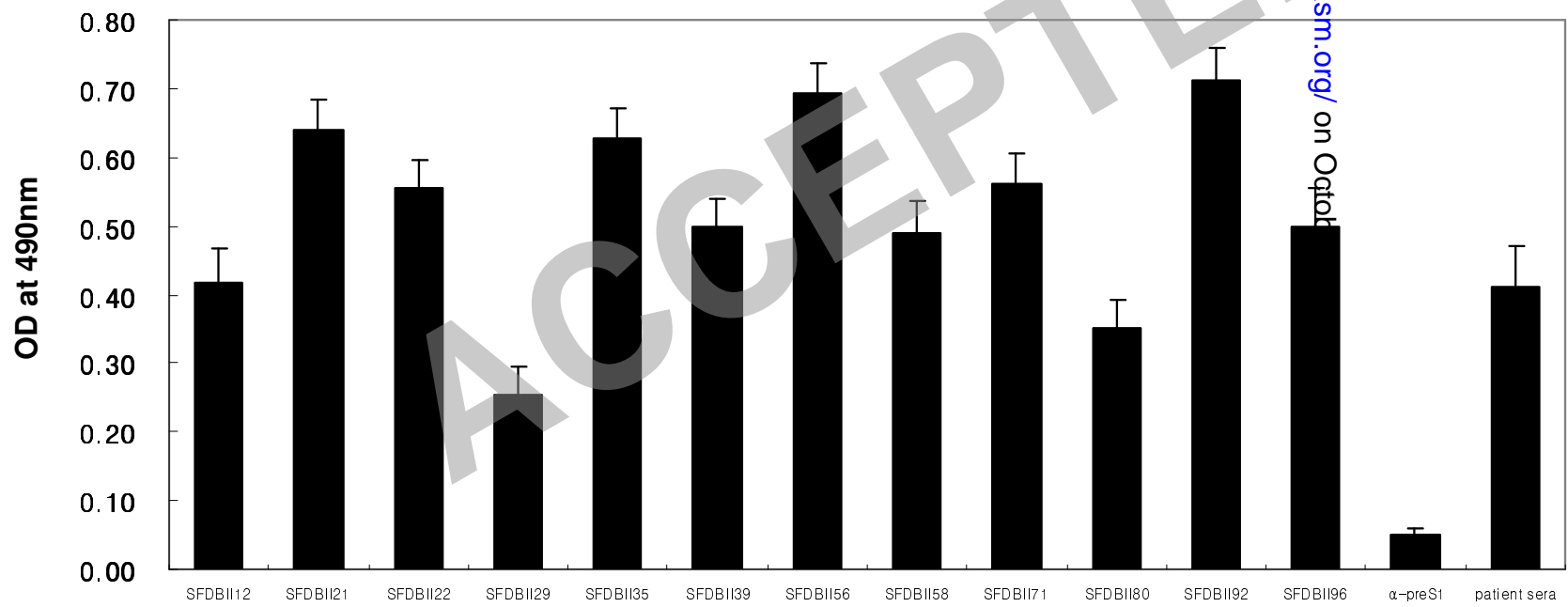
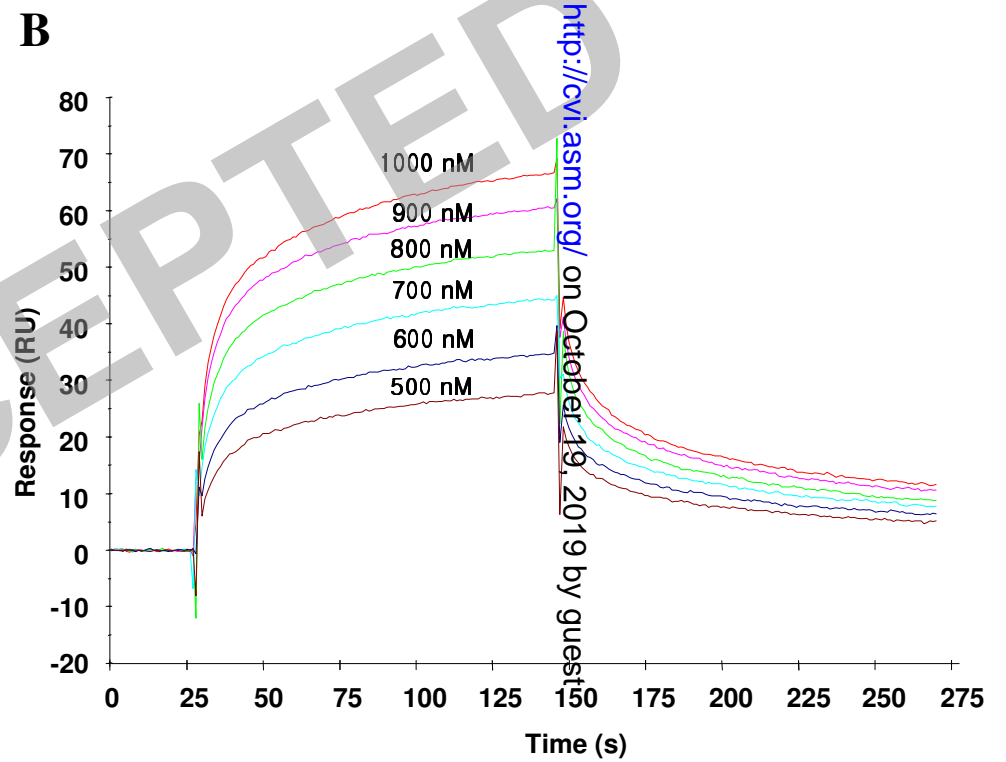
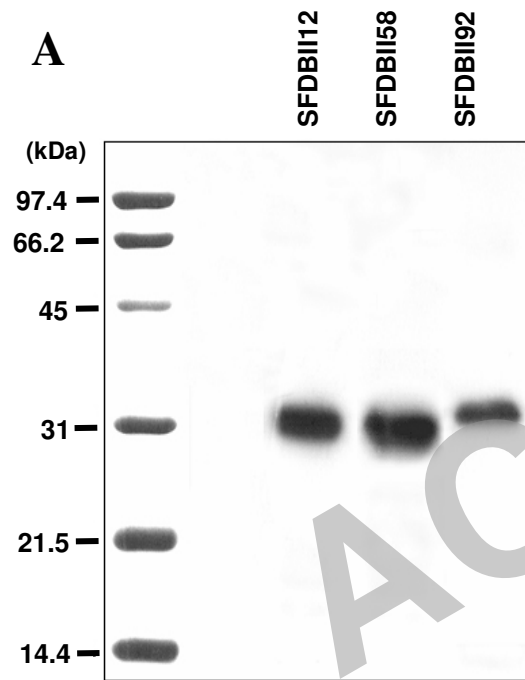


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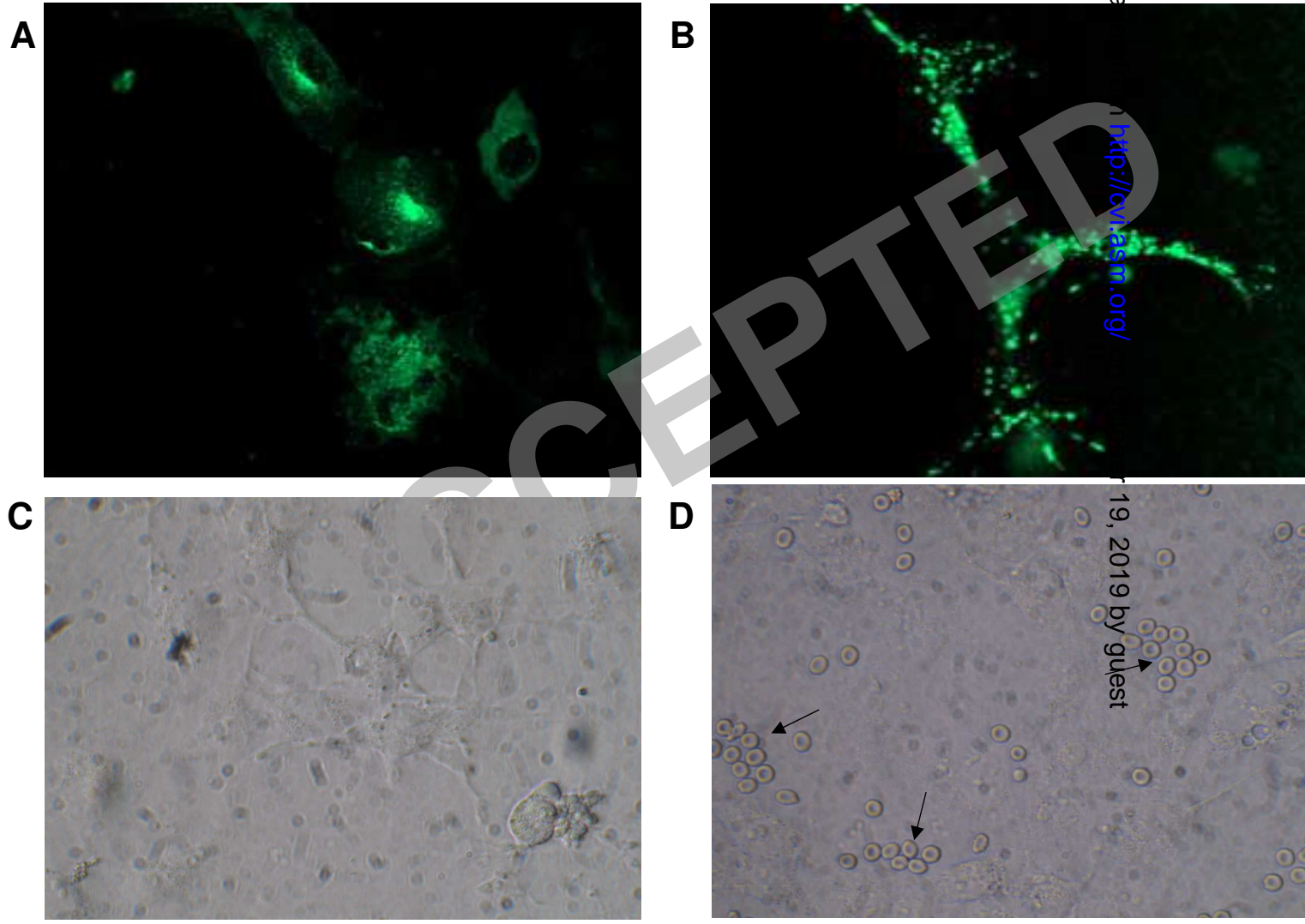
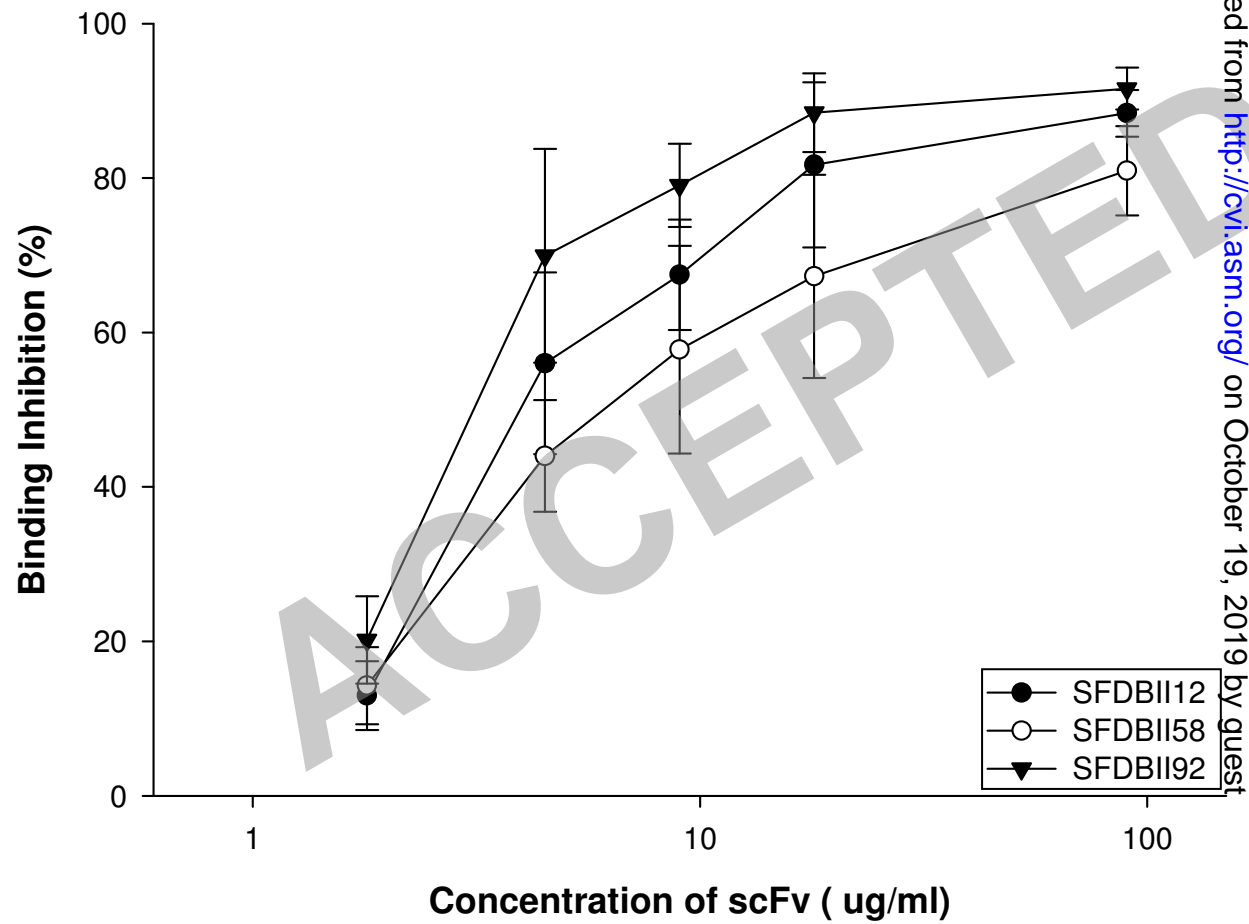


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