Design and Construction of a Novel Humanized Single-Chain Variable-Fragment Antibody against the Tumor Necrosis Factor Alpha

Running head: A Novel Humanized ScFv Antibody against TNF- alpha

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Abstract

The pro-inflammatory cytokine, TNF-α, which plays a major role in the development and persistence of diseases such as Crohn's disease, psoriasis, psoriatic arthritis, and rheumatoid arthritis, is the basis for the use of anti-TNF-α therapies. The neutralization of TNF-α or blockage of its binding to the corresponding receptor has mainly served as a therapeutic strategy against some inflammatory diseases. This study aimed to investigate the production of a humanized single chain antibody (scFv) against TNF-α. Therefore, a murine monoclonal antibody, D2 mAb, was selected for humanizing by the complementarity determining region (CDR)-grafting method. Briefly, the replacement of the CDRs from D2 mAb with the specific human single chain scaffold led to the production of a novel humanized single chain fragment variable mAb against human TNF-α (hD2). The subsequent cloning of hD2 into a suitable expression vector, pGEX-6P-1, resulted in the expression of a 52-kDa GST-fusion protein in *E. coli*, mostly in the form of inclusion bodies. The solubilization and refolding of GST-hD2 inclusion bodies was achieved with the addition of 4 M urea and subsequent dialysis to recover the fusion protein in soluble form. Then

the soluble GST-hD2 was purified by affinity chromatography through immobilized glutathione. The GST pull-down experiment showed a positive interaction between GST-hD2 and TNF-α protein. Moreover, the results of an MTT assay suggested that the purified GST-hD2 had the potential to be developed into a therapeutic TNF-α neutralizing antibody.

Keywords: TNF-α; Single chain antibody; Affinity chromatography; Pull down, MTT assay.

Introduction

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine, and its abnormal over-expression causes many inflammatory or autoimmune diseases (1). TNF-α neutralizing agents and inhibitors have being used extensively to treat inflammatory diseases such as Crohn's disease (CD), psoriasis, psoriatic arthritis (PA), and rheumatoid arthritis (RA), which result from the excessive production of TNF-□ in the body (2).

Various strategies including blocking of TNF-α mRNA synthesis, inhibition of its post-translational processing, and blocking of the activation of TNF-α receptors have been used to inhibit TNF-α biosynthesis (3). The utilization of monoclonal antibodies (mAbs) or soluble receptors has been reported to be commonly effective in the treatment of several TNF-α-related diseases. Infliximab (Remicade), adalimumab (Humira), golimumab (Simponi), certolizumabpegol (Cimzia), and etanercept (Enbrel), which are all currently approved by the Food and Drug Administration (FDA), are being used in humans to cure TNF-α-mediated diseases (4). However, a number of studies have reported some restrictions in the therapeutic uses of TNF-α inhibitors, including low stability, high production expenses, considerable treatment costs, and undesired side effects (5-7), as well as the broadly development of neutralizing antibodies against TNF-a inhibitors in a subset of treated patients. All of these may result in reduction or loss of therapeutic efficacy of these anti-TNF-α agents (8).

Of late, antibody engineering technology has been used to produce single-chain fragment variable (scFv) antibodies in which the genes encoding for V_H and V_L are joined together with a short flexible peptide linker (9). Occasionally, scFv antibodies are also manipulated by using the complementarity determining region (CDR) grafting method, replacing the murine content with the amino acid residues of human counterparts to generate a humanized version (10). In comparison with the parental antibody, the humanized scFv antibodies have several advantages in clinical uses, including better tissue penetration, more quick blood clearance, and lower retention times in non-target tissue. They also reduced immunogenicity as much as possible while they could retain the intact antigen binding site and then maintain the specificity and affinity toward the antigen (11).

The need for new TNF-α-blocking agent(s) with higher binding affinity and better neutralizing activity is always sought, because it provides the possibility of using antibodies at lower doses,

with less immunogenicity, and for a wider range of patients afflicted with TNF-α-related diseases.

The aim of the present study is to develop a humanized single chain antibody with anti-TNF-α activity suitable for the development of new therapeutic agents useful in inflammatory diseases.

Materials and Methods

Selection of anti-TNF alpha monoclonal antibody

To humanize an efficient murine anti-TNF-α antibody, the selection was carried out based on searching through all known antibodies (Table 1) with high specificity and binding affinity values at nM range.

Predicting antibody complementarity determining regions

The IMGT/V-QUEST program (version 3.3.1) was used to predict the complementarity determining regions (CDRs) on both heavy and light chains of the selected antibody (12). In addition, we aligned all publically available sequences of human anti-TNF-α scFv antibodies deposited in NCBI databank and then based on the alignment the CDR regions were assigned.

Design and Synthesis of human anti-TNF-□ scFv encoding gene

A humanized version of the selected anti-TNF-α scFv (13) was designed based on CDR grafting method. Briefly, the CDRs from the selected anti-TNF-α scFv mAb were used to replace those in the template scFv derived from the specific human single chain scaffold (Tomlinson I+J library) (14). The DNA sequence of the gene had been determined by reverse translation of the amino acid sequence of the designed scFv using the codons found in highly expressed *E. coli* genes (15) and taking into account the codon redundancy where appropriate. The sequence was then searched for potential restriction endonuclease sites. Based on the results, two restriction sites (*Bam*HI and *Eco*RI) were introduced at 5'- and 3'-UTR of the fragment, respectively. Sequences were checked to ensure the lack of formation of stem–loop structures and internal sequence similarities. The DNA sequence design was performed using BioEdit software (version 7.0, BioEdit Sequence Alignment Editor Software, Department of Microbiology, North California State University). The DNA sequence was synthesized and cloned into pGEX-6P-1 vector by Eurofins Genomics, Germany (http://www.eurofinsgenomics.eu/en/). The received construct was transformed into *E. coli* DH5□ for amplification. The amplified DNA was then used to verify

the accuracy of the designed scFv encoding gene by PCR, restriction enzyme digestion pattern, and sequencing by Bioneer, South Korea.

Expression of the synthesized human anti-TNF-□ scFv in E. coli

The recombinant plasmid carrying anti-TNF-□ scFv was transformed into *E. coli* BL21 (DE3) cells for the expression of scFv as a GST-fusion protein. The transformants were grown on LB agar supplemented with ampicillin (100 µg mL^{-1} final concentration) at 37 °C overnight and agitated at 180 rpm. The cultures were then diluted 1:100 with fresh LB medium plus antibiotic, and grown to OD_{600} value of 0.6 at 37 °C. The expression of the fusion protein was induced by the addition of isopropyl beta-D-thiogalactopyranoside (IPTG) at a concentration of 0.5 mM at 20 °C. The cells were harvested at intervals of 1, 3, 6, and 24 h after induction. They were re-suspended in 10 mM Tris-HCl (pH 8.0) and lysed by sonication. The cellular debris was pelleted by centrifugation at 12000 *g* for 15 minutes. Samples from both supernatant and pellet were analyzed by electrophoresis on a 12% SDS-PAGE under reducing conditions after staining with coomassie brilliant blue (16).

In vitro denaturation and refolding of the inclusion bodies

The cell lysate was centrifuged at 4 °C for 20 min at 30,000 g. The pellet was then re-suspended in a 10 mL wash buffer (50 mM Tris-HCl pH 7.5, 50–200 mM NaCl) containing 1% Triton X-10 and 1 M urea per gram cell wet weight and incubated at room temperature for 5 min. The cell lysate was centrifuged again at the above-mentioned condition, and the pellet was re-suspended in 10 mL wash buffer. Subsequently, it was centrifuged at 4 °C for 30 min at 15,000 *g*. In the next step, the inclusion bodies (IBs) were re-suspended in the extraction buffer (50 mM Tris-HCl pH 7.5, 4 M urea, 1mM PMSF, and 1mM DTT) at the final protein concentration of 1 mg mL^{-1} and incubated at room temperature for 60 minutes. Finally, the solution was dialyzed overnight against a 100-fold volume of wash buffer. This contained a gradient of urea concentration and the dialysate was centrifuged at 4 °C for 30 min at 15,000 *g* (17, 18).

Affinity purification of GST-hD2

Purification of the refolded fusion protein was achieved using Glutathione Sepharose 4B bulk matrix (GE Healthcare) according to the manufacturer's instructions (19).

Pull down assay

The TNF-α (the probe) and GST-hD2 fusion proteins (the target) are incubated together with glutathione-agarose beads and then the complex (TNF-α-GST-hD2) was recovered from the beads and analyzed using SDS-PAGE experiment. Briefly, 25 µg of the fusion protein was incubated with 25 µg of TNF-α and 50 µL of a 50% slurry of glutathione-agarose beads previously equilibrated with equilibration buffer (50 mM Tris (pH 8.0), 100 mM NaCl, 1.4 mM PMSF, 0.1% β-mercaptoethanol, and 1% Triton X100) for 2 h at 4 °C, while mixing by inverting at cold room. The mixture was centrifuged at 13,000 *g* for 2 min at 4 °C, and the supernatant was discarded. Then, beads were washed four times with 1 mL of ice-cold GST wash buffer consisting of 50 mM Tris (pH 8.0), 100 mM NaCl and 0.1% β-mercaptoethanol and then were centrifuged as before for 1 min at 4 °C and the supernatant was discarded. The samples of beads from each step were collected and analyzed by SDS-PAGE to determine the association between the fusion protein and TNF-α (20).

MTT assay

100 µL per well of murine fibroblast L929 cells in RPMI medium supplemented with 10% fetal bovine serum (FBS) were seeded in 96-well plates at 1×10^5 cells mL^{-1} and incubated for 20 h. Also several dilutions of GST- hD2 were prepared in medium containing actinomycin D (10 µg mL^{-1}) and TNF-α (2 mg mL^{-1}) and incubated at 37 °C for 2 h. After removing of the supernatants of the cultured L929 cells, different concentrations of GST- hD2 were added to the wells. Then the cells were incubated at 37 °C for 24 h, and the supernatants were removed again. To each well, MTT at 5 mg mL^{-1} concentration was added and incubation was continued at room temperature for 4 h. After removing supernatant, the solubilization buffer (Sorensen buffer 12.5% and DMSO 87.5%) was added to each well with shaking for 40 min at 25 °C. The plate was read in ELISA Reader for measuring OD in 570 nm (background was read at 630 nm wavelength) (21). Blank control (culture alone), TNF-α control (TNF-α alone), and antibody control (hD2 alone) were also included in the experiment.

Results

Selection of anti-TNF-□ mAb and determining its CDR regions

According to Table 1, murine D2 (mD2) single chain antibody was selected for humanization based on its high specificity and binding affinity (with Kd values at nM range) against TNF-α. We predicted the CDR regions on both heavy and light chains of the mD2 antibody using the

IMGT/V-QUEST tool. Three CDRs for each of the heavy and light chains were determined as shown in Figure 1. Similarly, the alignment of the sequences of all known anti-TNF-α scFv mAbs (Figure 2) revealed almost the same regions as the CDRs. As shown in Figure 2, a big portion of the aligned sequences is related to the conserved framework regions of the antibodies and the remaining segments are CDR1, CDR2, and CDR3 regions in both light and heavy chains, respectively.

Design, Synthesis and Expression of hD2 encoding gene

In order to construct the humanized D2 scFv, CDR grafting technique was used. To this end the sequences of CDRs from the mD2 mAb were used to replace the CDRs of the specific human single chain framework represented by Tomlinson I+J sequence (Figure 3). The designed DNA sequence (intended to encode human D2 scFv antibody) was synthesized and cloned into pGEX-6P-1 vector by Eurofins Genomics and called pGEXhD2. The result of PCR, digestion pattern and sequencing confirmed the accuracy of the hD2 coding gene inserted into pGEXhD2 plasmid. The sequencing showed that the size of the encoding region was 726 bp, composed of 366, 45, and 378 bp segments encoding the variable heavy chain, a $(Gly_4Ser)_3$ linker, and variable light chain, respectively as expected.

The transformation of pGEXhD2 into *E. coli* BL21 (DE3) cells was produced recombinant bacterial colonies which were resistant to ampicillin and capable to express GST-hD2 fusion protein. The SDS–PAGE analysis revealed that the transformants grown on LB medium supplemented with antibiotic express a protein with a molecular weight of almost 52-kDa in size (corresponded with the expected molecular size of the GST-hD2 scFv) as cytoplasmic insoluble aggregates (i.e., inclusion bodies) (Figure 4.A).

Solubilization and affinity purification of GST-hD2 inclusion bodies

The GST-hD2 inclusion bodies in the cytoplasm of *E. coli* were released by lysing the bacterial cells. To achieve the solubilized GST-hD2 fusion protein, the inclusion bodies were isolated by centrifugation due to the fact that inclusion bodies have a relatively high density. The highest recovery of pure inclusion bodies was achieved using 1% Triton X-10 and 1 M urea in a solublizing buffer. To solubilize the isolated inclusion bodies, the pellet was subjected to the different concentrations of urea. The optimized concentration of the urea to achieve the maximum GST-hD2 recovery into the soluble fraction was 4 M. To avoid any undesirable effect

of urea on the folding of GST-hD2 protein, the urea was removed from the solution by stepwise dialyzing the solubilized protein sample against buffers with progressively reduced urea concentrations. As shown in Figure 4.B, the purified soluble GST-hD2 protein gives rise to a major band at ~52-kDa in the SDS-PAGE analysis. Finally, the fusion protein GST-hD2 was further purified by the use of glutathione sepharose 4B bulk matrix (Figure 4.C). Affinity purification of fusion protein showed that GST domain of fusion protein is able to bind to the glutathione sepharose matrix, suggesting the proper refolding of the fusion protein.

Pull down assay

The result of pull down experiment was shown in Figure 5. In lane 4 of the figure, both TNF-α and GST-hD2 proteins are detectable with the expected molecular weights of 17 and 52-kDa, respectively, indicating an interaction between TNF-α and fusion protein GST-hD2 protein.

TNF-α cytotoxicity neutralizing activity of purified hD2 scFv

MTT assay was applied to determine the cell survival of L929 cells which were treated with 0 to 27 μg mL^{-1} of hD2 scFv antibody, in the presence of TNF-α (2 mg mL^{-1}) for 24 h. The results showed that 2 mg mL^{-1} of TNF-α completely let to L929 cells death. Twenty seven micrograms per milliliter of hD2 could completely neutralize TNF-α (2 mg mL^{-1}) mediated cytotoxicity in L929 cells. The results showed that anti-TNF-α hD2 scFv protects cells form TNF-α cytotoxicity in a dose-dependent manner (Figure 6). Antibody alone (anti-TNF-α hD2 scFv) did not show any significant effect on the survival of L929 cells.

Discussion

Glutathione-S-transferase (GST) fusion proteins have a range of applications in the detection, isolation, and purification of recombinant proteins. A GST pull-down experiment also is used to confirm suspected interactions between a probe protein and a known protein.

A relatively large molecular mass of antibody molecules is the major obstacle in their application as therapeutic agents due to the restricted distribution into the site of action (22). The use of considerably smaller genetically engineered antibody fragments has been shown to be a useful approach to resolve such problems (23, 24). In this regard, even though scFv antibody molecules have only one-sixth the molecular mass of the intact IgG antibodies, they can bind to the original epitope with the same affinity. Previous studies have demonstrated that scFv antibodies penetrate

better into tumor tissue and have improved pharmacokinetics and lower immunogenicity than intact antibodies (25, 26).

The aim of this study was to design a humanized scFv antibody against TNF-α with high affinity and low immunogenicity applicable in the development of therapeutic and diagnostic agents. Therefore, among the available twelve scFv murine antibodies listed in Table 1, the murine D2 (mD2) scFv antibody was selected to be humanized based on its high specificity and binding affinity (with Kd values at nM range) against TNF-α. The CDR regions were predicted on both heavy and light chains of the mD2 antibody based on aligning its sequence with all other anti TNF- scFv antibodies, as well as analyzing the mD2 sequence based on the knowledge-based algorithms implemented in the IMGT/V-QUEST program (12). The results of both multiple sequence alignment and sequence analysis by software were in close agreement and we have designed our humanized D2 using the identified CDRs of mD2 using the former approach. Although the software algorithm is based on the sequence alignment of the query with the reference sequences in the database, our multiple alignment was generated among scFv antibodies known to recognize a single target–that is, TNF-. Thus, we concluded that determining CDRs, based on aligning their sequences, was an efficient method that could be used in our study. To conclude, one may claim that the sequences of antibodies are so similar in their framework that the sequence variations are found only in the CDR regions; therefore, the regions with low homology can be easily determined by aligning the antibody sequences (27, 28).

Complementarity-determining region grafting technique is a well-known method for constructing novel antibodies that have improved characteristics (29). The most important application of this method is to humanize high-affinity antibodies developed in other species by inserting the appropriate CDR-coding segments into a human antibody scaffold (28, 30). In one study, the variable domain-resurfacing approach was employed to humanize the murine monoclonal antibody m357. They showed that the purified h357 antibody was capable of maintaining the high antigen-binding affinity and inhibiting disease progression significantly in a mouse model of antibody-induced arthritis in collagen (31).

In the present study, the CDRs from the mD2 anti-TNF- scFv antibody were used to replace the CDRs of the specific human's single-chain framework (Tomlinson I+J library scaffold) to

develop a humanized version of mD2 scFv (named hD2) with the sequence, as shown in Figure 3. Synthesis, cloning, and expression of hD2 scFv in reducing bacterial cytoplasm led to the forming of insoluble inclusion bodies, which were solubilized and refolded—by the adding and gradual removal of a denaturing agent (i.e., 4 M urea) —to ultimately obtain the properly folded and functional hD2. Many proteins fail to fold properly during the recombinant protein expression in *E. coli*. Therefore, the protein ends up as aggregates found in the inclusion bodies. This may be due to fast overexpression, absence of chaperones, or unfavorable oxidizing of the intracellular environment of the prokaryotic cell. Denaturing with chaotropic agents, such as 8 M urea or 6 M guanidine HCl, and then refolding in favorable conditions is a common technique used for isolating soluble-folded proteins from inclusion bodies (32).

In this work, salts and unwanted proteins of the host cell were eliminated by a two-step wash procedure. Removing these impurities and obtaining the IBs with high recovery and purity is required because impurities interfere with the refolding and significantly affect the yield and purity of the process. We used 1% Triton X-10 to solubilize the components of the bacterial cell wall that contaminate the inclusion-body preparation, and we used 1 M urea to remove any residual cell debris. In addition, we found that this method provided the most effective means for the refolding of aggregated proteins when the refolding procedure was carried out with a gradually decreasing concentration of urea in the extraction buffer. Thus, optimization of the early stages of the downstream process will impact the yield of the overall process and the purity of the final product.

Finally, the pull down assay showed the positive interaction between GST-hD2 and TNF-α, suggesting an affinity between the bait and predator proteins. Moreover, the results of an MTT assay suggested that the purified GST-hD2 has the potential to be developed into a therapeutic agent with TNF-α neutralizing activity. However, further investigation is needed to elucidate its *in vivo* neutralizing activity.

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Table 1

Anti-TNF-□ antibody	*K*d (M)	Reference
D2	nd	Zhu et al. 2006 (13)
CDP571	8.7×10^{-11}	Chang et al. 2007 (33)
TSK114	~5.3×10^{-12}	Song et al. 2008 (34)
2SD4	2.0×10^{-9}	Santora et al. 2001 (35)
D2E7	1.0×10^{-10}	Santora et al. 2001 (35)
Z12	0.1×10^{-9}	Qin et al. 2006 (36)
H357b	7.8×10^{-6}	Chiu et al. 2011 (31)
CA2	4.6×10^{-8}	Scallon et al. 1995 (37)
E6	nd	Zhu et al. 2006 (13)
F6	nd	Zhu et al. 2006 (13)

Figure Legends:

Figure 1. Prediction of complementarity determining regions (CDRs) of anti-human TNF-□ D2 immunoglobulin heavy and light chain variable regions using the IMGT/V-QUEST programme. A and B represent the heavy and light chain variable regions, respectively.

Figure 2. Determination of CDRs of anti-human TNF-□ D2 immunoglobulin heavy and light chain variable regions based on aligning all publically available sequences of human anti-TNF-α scFv antibodies.

Figure 3. Amino acid sequence of humanized version of anti-TNF-α scFv hD2.

Figure 4. SDS-PAGE analysis of the synthesized human anti-TNF-□ scFv expression in *E. coli*. (**A**) L, S and I represent protein molecular size marker (SM0671), supernatant and cellular debris of bacteria, respectively; 3h after induction by IPTG; **(B)** SDS-PAGE analysis of GST-hD2 fusion protein. The purified soluble GST-hD2 after solubilization of inclusion bodies; **(C)** Purified soluble GST-hD2 protein resulted from glutathione affinity purification.

Figure 5. Analysis of interaction between TNF-α and GST-hD2 fusion protein.

Figure 6. Neutralization of TNF-mediated cytotoxicity in L929 cells by the GST-hD2.

Figure 1

A.

LQQSGTVLARPGTSVKMSCKASG**YNFTSYW**MHWVKQRPGQGLEWIGA**LFPGNSDT**T
YKEMLKGR

CDR1 **CDR2**

AKLTATSASIAYLEFSSLTNEDSAVYYCA**RGDFGAMDY**WGQGTTVTVSS

CDR3

B.

DIQLTQSPAILSVSPGERVSFSCRAS**QSIGTS**IHWYQQRTNGSPRLLIK**YTS**ESISGLPSRFS
G

CDR1 **CDR2**

SGSGTDFTLTISSVESEDIADYYC**QQSYNWPTF**TFGGGTKLEI

CDR3

Figure 2

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          ....|....| ....|....| ....|....| ....|....| ....|....|
                10        20        30        40        50
D2 H        ------LQQS GTVLARPGTS VKMSCKASGY NFTS-YWMHW VKQRPGQGLE
CDP571 H    ---QVQLVQS GAEVVKPGSS VKVSCKASGY TFTD-YNVDW VKQAPGQGLQ
TSK114 H    ---QVQLVQS GPELKKPGET VKISCKASGY TFTH-YGMNW VKQAPGEGLK
Tomlinson   AMAEVQLLES GGGLVQPGGS LRLSCAASGF TFSS-YAMSW VRQAPGKGLE
a1         -MAEVQLVES GGGLVQPGGS LRLSCAVSGF TFSS-YAMSW VRQAPGKGLE
2SD4 H      ---QVQLVES GGGLVQPGRS LRLSCAASGF TFDD-YAMHW VRQAPGKGLD
D2E7 H      ---EVQLVES GGGLVQPGRS LRLSCAASGF TFDD-YAMHW VRQAPGKGAE
a3         -MAEVQLVES GGGVVEPGGS LTLSCTASGF TFTN-YWMHW VRQAPGKGLV
b3         -MAQVQLVES GGRVTRPGGS LRLSCSVSGF NLDD-HGMSW VRQVPGKGLE
Z12 H       ---QLELVES GGGLVKSGGS LKLSCAASGF AFNN-YDMSW VRQTPERRLE
H357b       ----VQLQES GGGLVQPGGS MRLSCIASGF TFSN-YWMNW VRQSPGKGLE
CA2 H       ---EVKLEES GGGLVQPGGS MKLSCVASGF IFSN-HWNNW VRQSPEKGLE
E6 H        --MQVQLLES GPGLVKPSQS LSLTCSVTGH SISSGYFWNW IRHFPGNKLE
F6 H        ------LQES GPGLVKPSQS LSLTCSVSGY SITSGYFWNW IRQFSGNKLE
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CDR1

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          ....|....| ....|....| ....|....| ....|....| ....|....|
                60        70        80        90       100
D2 H        WIGALFPGN- --SDTTYKEM LKGRAKLTAA TSASIAYLEF SSLTNEDSAV
CDP571 H    WIGNINPNN- --GGTIYNQK FKGKGTLTVD KSTSTAYMEL SSLTSEDTAV
TSK114 H    WMGWINTNT- --GEPRYDEE FKGRFAFSLE TSASTAYLQI NNLRREDTAT
Tomlinson   WVSXIXXXG- --XXTXYADS VKGRFTISRD NSKNTLYLQM NSLRAEDTAV
a1          WVSAISGSG- --GSTYYADS VKGRFTASRD NSKNTLYLQM NSLRAEDTAV
2SD4 H      WVSAITWNS- --GHIDYADS VEGRFAVSRD NAKNALYLQM NSLRPEDTAV
D2E7 H      HVSAITWNS- --GHIDYADS VEGRFTISRD NAKNSLYLQM NSLRAEDTAV
a3          WVSRINTDG- --SSTTYADS VQGRFAISRD NAKNTLYLQM NSLRAEDTAV
b3          WVSDVNMNG- --GHTGYASS VRGRFINSRD SAKNSLYLQM NNLRADDTAF
Z12 H       WVAYINTGG- --GYTYYPDT VKGRFTISRD NAKNTLYLQM SSLRSEDTAM
H357b       WVAEVRNQSD LFTTSHYAES VKGRFTISRD DSKSGVYLQM NNLGAEDTGI
CA2 H       WVAEIRSKS- INSATHYAES VKGRFTISRD DSKSAVYLQM TDLRTEDTGV
E6 H        WMGYISYDG- ---SNKYNPS LKNRISITRD TSENQFFLIL NSVTSEDTAT
F6 H        WMGYISYDG- ---SNNYNPS LKNRISITRD TSKNQFFLKL NSVTPEDTAT
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CDR2

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          ....|....| ....|....| ....|...
                110       120
D2 H        YYCARGDFG- ---AMDYWGQ GTTVTVSS
CDP571 H    YYCARSAFYN NYEYFDVWGQ GTTVTVSS
TSK114 H    YFCARYDSR- ---GFDCWGQ GTTLTVSS
Tomlinson   YYCAKX---X XXFD--YWGQ GTLVTVSS
a1          YYCARV---K RSLKGTRWGQ GTLVTSS-
2SD4 H      YYCTKASYLS TSSSLDNWGQ GTLVTVSS
D2E7 H      YYCAKVSYLS TASSLDYWGQ GTLVTVSS
a3          YYCARD-FDF WSVSLDHWGQ GTLVTV--
b3          YFCARE--SR FGYWFDSWGQ GPRSLSP-
Z12 H       YYCASERYDG LYYAMDYWGQ GTSVTVSS
H357b       YYCTP----- ----FAYWGQ GTTVTVSS
CA2 H       YYCSRN---Y YGSTYDYWGQ GTTLTVS-
E6 H        YYCARD---- DNWNFDVWGR HHGHRLL-
F6 H        YYCARD---- GDYYFDYWGQ GTTVTVSS
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CDR3

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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    10        20        30        40        50
D2_L_       DIQLTQSPAI LSVSPGERVS FSC-RASQSIG TS------IH WYQQRTNGSP
CDP571_L_   DIMNTQSPST LSASVGDRVT ITC-KSSQSLL YSNNQKNYLA WYQQKPGQAP
TSK114_L_   QIVLTQSPAI MSASLGERVT MTC-TASSSIS YN-----YFH WYQQRPGSSP
Tomlinson   DIQMTQSPSS LSASVGDRVT ITC-RASQSIS S------YLN WYQQKPGKAP
a1          EIVLTQSPSS LSASVTDRVT IQS-RPQSGHR ND------LG WYHHKPRKAP
2SD4        DIQMTQSPSS LSASIGDRVT ITC-RASQGIR N------YLA WYQQKPGKAP
D2E7_L_     DIQMTQSPSS LSASVGDRVT ITC-RASQGIR N------YLA WYQQKPGKAP
a3          DIQMTQSPSS LSASVGDRVT ITC-RASQGIS N------YLA WFQQKPGKAP
b3          EIVLTQSPAT LSLSPGQGAI LSC-RASQSVT SN-----YLA WYQQKPGQAP
Z12_L_      ELQMTQSPSS LAVSAGEKVT MSC-KSSQSLL NSRTRKNYLA WYQQKPGQSP
H357a       EIVLTQSPPT TSASPGERVT MTC-SASSSVS -------FMY WIQQKPGSSP
CA2_L_      DILLTQSPAI LSVSPGERVS FSC-RASQFVG SS------IH WYQQRTNGSP
E6 L        ---MTQSPSS LAMSVGQKVT MSC-KSSQSVL NSNTQKNYLA WYQKKPGQSP
F6 L        ---LTQSPSS LAMSVGQKVT MNC-KSSQSLL NSYTQKNYLA WYQQKPGQSP
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CDR1

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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    60        70        80        90        100
D2_L_       RLLIK-YTSES IS-GLPSRFSG SGSGTDFTLT ISS-VESEDI ADYYC-QQSYN
CDP571_L_   KLLIS-WASTR ES-GVPSRFIG SGSGTEFTLT ISS-LQPDDV ATYYC-QQYYD
TSK114_L_   KLWIY-SSSNL AS-GVPPRISG SGSGTSYSLT ISSSMEAEDA ATYYC-HQY-E
Tomlinson   KLLIY-XASXL QS-GVPSRFSG SGSGTDFTLT ISS-PQPEDF ATYYS-QQXXX
a1          NRLIY-AASSL QS-GVPSRFSG -HSIWDFHSH YQQ-PAPEDF ATYYC-LQHNI
2SD4        KLLIY-AASTL QS-GVPSRFSG SGSGTDFTLT ISS-LQPEDV ATYYC-QKYNS
D2E7_L_     KLLIY-AASTL QS-GVPSRFSG SGSGTDFTLT ISS-LQPEDV ATYYC-QRYNR
a3          KSLIY-AASSL QS-GVPSRFSG SGYGTDETLA ISS-LQPEDF ATYYC-LQDYN
b3          RLLIY-GASSR AT-GIPDRFSA SGSGTDSTLT ISG-LEPEDF AVYYC-QKYGD
Z12_L_      KLLIY-WASTR ES-GVPDRFTG SGSGTDFTLT ISG-VQAEDL AVYYC-KQSYN
H357a       ALLIY-DASIL AS-GVPVRFSG SGSGTSYSLT ISR-MEAEDV ATYYC-QQWSD
CA2_L_      RLLIK-YASES MS-GIPSRFSG SGSGTDFTLS IMT-VGSEDI AD-YC-QQSHS
E6 L        ELLVY-FASTR ES-GVPDRFMG SGSGTDFTLT ISS-VQTEDL ADYFC-QQHYR
F6 L        KLLVY-FASTR ES-GVPDRFMG SGSGTDFTLT ISS-VQTEDL ADYFC-QQHYR
```

CDR2 **CDR3**

```
          ....|....| ....|....
                    110
D2_L_       WPTFT-FGGGT KLEI-----
CDP571_L_   YP-WT-FGQGT KVEIKR---
TSK114_L_   RSPWT-FGG-T KLEIKR---
Tomlinson   XP-XT-FGQGT KVEIKRAAA
a1          YP-WT-FGQGT KVEIKRAAA
2SD4        AP-YA-FGQGT KVEIK----
D2E7_L_     AP-YT-FGQGT KVEIK----
a3          YP-FT-FGGGT KVEIKRAAA
b3          SPLYT-FGQGT KVEIKRAAA
Z12_L_      LP-WT-FGGGT KLEIK----
H357a       YSPRT-FGGGT KLEI-----
CA2_L_      WP-FT-FGSGT NLEVK----
```

E6 L **TP-FT**FGSGT KL-------
F6 L **IP-FT**FGSGT KLEI-----

Figure 3

EVQLLESGGGLVQPGGSLRLSCAASG**YNFTSYWMH**WVRQAPGKGLEWVS**ALFPGNSDTT YKEMLK**GR

CDR1 **CDR2**

FTISRDNSKNTLYLQMNSLRAEDTAVYYCA**RGDFGAMDY**WGQGTLVTVSSGGGGSGGGG SGGGGSTD

CDR3

IQMTQSPSSLSASVGDRVTIT**CRASQSIGTSIH**WYQQKPGKAPKLLIY**YTSESIS**GVPSRFSGS GSG

CDR1 **CDR2**

TDFTLTISSPQPEDFATYY**CQQSYNWPTF**TFGQGTKVEIKRAAA

CDR3

Figure 4

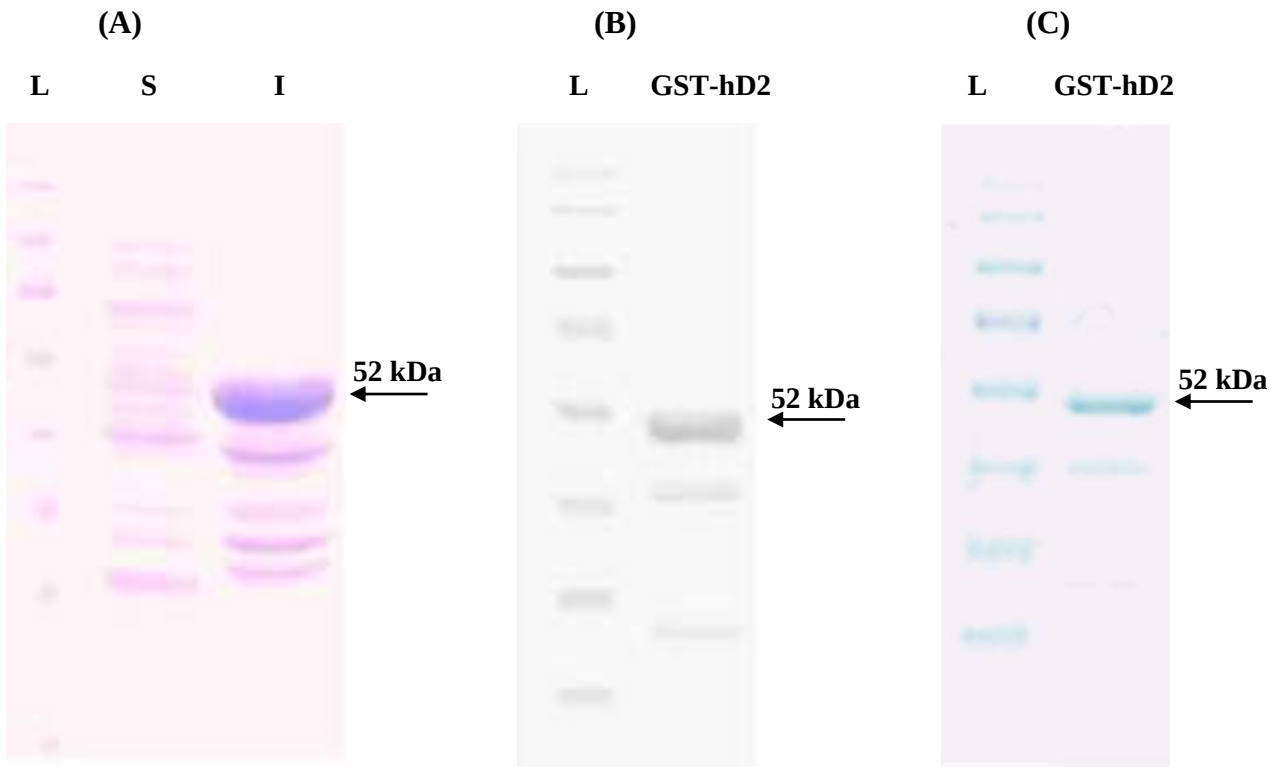


Figure 5

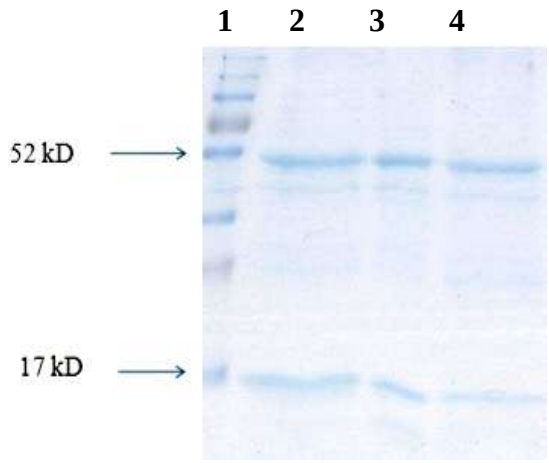
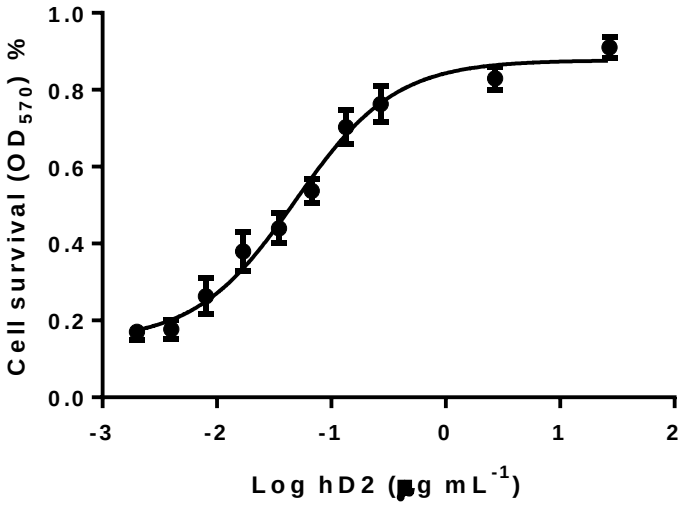


Figure 6



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