CHAPTER 1

ISOLATION OF ANTI-TNFα scFv

INTRODUCTION

Inflammatory responses are generated by the immune system as a result of pathogenic invasion or tissue damage to clear the invaders by the cells of immune system. These responses are employed by both innate and adaptive immune system that results in release of a number of chemical mediators such as cytokines which bind to specific target receptors on the cellS that may increase vascular permeability, direct enzymatic activity, promote neutrophil chemotaxis, stimulate smooth muscle contraction, , induce pain and/or mediate oxidative damage (Coleman, 2002). Persistent chronic inflammation increases the development of the diseases such as autoimmune disease, heart disease, Alzheimers, asthma, etc. (Iwalewa, 2007).

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine mainly secreted by activated macrophages in response to various inflammatory stimuli. It is also produced by other cell types such as T lymphocytes, macrophages, NK cells, dendritic cells, endothelial cells, osteoblasts, mast cells, Kupffer cells, and smooth muscle cells. Large amount of secretory TNFα (sTNFα) is released in response to lipopolysaccharide and other bacterial products (Aggarwal, 2012).

TNFα is one of the most pleotropic cytokines involved in signalling a large number of physiological and biological responses. It induces different types of

signals, including activation of different cellular pathways, like apoptosis, NF-kB, extracellular signal regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun N-terminal kinase (Hsu, 1996; Aggarwal, 2012). It is chemotactic to monocytes and neutrophils. Stimulation of these cells with TNFα induces inflammatory responses that include phagocytosis, adherence of these cells to endothelial cells, and generation of free radicals of oxygen-superoxide anion and hydrogen peroxide. It also induces synthesis of an inflammatory cytokine, IL-8, and other chemotactic cytokines which regulate the migration, degranulation, and respiratory burst response of neutrophils (Morgan, 2010).

The malfunctioning of T-lymphocytes contributes to the pathogenesis of many autoimmune diseases. Naïve T cells get activated by the self-antigen that leads to production of IL-2,causes clonal expansion and induces the production of other pro-inflammatory cytokines, such as TNFα. Over- production of TNFα has been documented in rheumatoid arthritis, Crohn's disease, psoriasis, multiple sclerosis and other autoimmune disease in biopsy samples of the patients and many animal models (Kollias, 1999; Owens, 2001; Feldmann et al., 1995).

Autoimmunity is a condition that arises when cells of immune system fails to protect host adequately. It malfunctions by losing its sense of self and nonself antigen that result in immune attack upon the host. More than 3.5 million people

are affected by autoimmune diseases in India and approximately 5-8 % worldwide (US Census Bureau, International Data Base, 2004), most prevalent in women compared to men, which accounts nearly 75 % of affected population. (Davidson and Diamond, 2001; Marrak et al., 2001; Kindt et al., 2007). There are more than 80 different types of known autoimmune diseases and 40 other diseases based on autoimmunity such as rheumatoid arthritis, Crohn's disease, psoriasis, multiple sclerosis, type-1 diabetes, lupus and so on. These diseases are responsible for more than $100 billion in direct health care costs annually. The initiation of autoimmune diseases in most cases is unknown but a number of studies suggest that they are strongly associated with factors such as genetics, infections and /or environment (Graham et al., 2005).

Neutralization of TNFα is most effective therapeutic strategy for such diseases (Palladino et al., 2003) and for the traetmentTNFα inhibitors (antibodies and fusion proteins) are widely used. There are several FDA approved TNFα inhibitors including: infliximab, a chimeric IgG anti-human monoclonal (Remicade^{R}); Etanercept, a TNFR2 dimeric fusion protein, with an IgG1 Fc (Enbrel^{R}); Adalimumab, a fully human monoclonal antibody (mAb) (Humira^{R}); Golimumab, a fully human mAb (Simponi^{R}) and Certolizumab, a PEGylated Fab fragment

(Cimzia^{R}); which have been used successfully in clinic (Kavanaugh et al., 2009; Feldmann and Maini, 2001).

Since these drugs are very effective and ameliorate the disease conditions very well. Recently anti-TNFα drugs also have been approved by regulatory authorities for the management of ulcerative colitis. Clinical outcomes indicated that the drugs are efficacious for treatment of ulcerative colitis (Danese et al., 2013). Fleischmann et al., (2015) reported a 24-week, multicentre, randomised, double-blind, placebo-controlled study on 220 RA patients with certolizumab pegol 400 mg monotherapy for every 4 weeks; effectively reduced the sign and symptoms of active RA.

The clinical data from the patients showed that these anti-TNFα drugs are well tolerated and efficacious in remission of the disease but these drugs behave differently from patient to patient and in different diseases. As Etanercept therapy is not effective in Crohn's disease and sarcoidosis compared to the monoclonal anti-TNFα antibodies. All of these drugs have been reported with the anti-drug antibody in the serum of the patient. Similarly RA patients treated with either Adalimumab or Infliximab have a 7-17 fold higher risk of developing a *Mycobacterium tuberculosis* (TB) infection compared to RA patients treated with Etanercept (Sandborn et al., 2001; Papadakis, 2009; Utz et al., 2003; Doty et al.,

2005; Tubach et al., 2009). Uses of these large molecules (anti-TNFα drugs) as inhibitors were limited by high production cost and potential side effects (Keane et al., 2001). In addition, about one-third of patients with autoimmune diseases are refractory to these anti-TNF agents suggesting that there is an unmet medical need.

Invention of hybridoma technology (Kohler and Milstein, 1975) prompted the use of monoclonal antibodies and became the choice for the therapeutic and research use. Naturally derived monoclonal antibodies (mAb) met some of these demands but the complexity, cost and time required to generate them became a limitation for their use. Later on, advent of phage display technology revolutionized the world of monoclonal antibody production (McCafferty et al*.,* 1990). The strength of the technology lies with the linkage of genotype and phenotype of the selected phages. By phage display technology, cloning of the entire repertoire of antibody fragment genes become possible, from immunized or non-immunized animals, including humans (McCafferty et al., 1990). Selection, maturation and proliferation process of phage display mimics the B cell selection and maturation strategies (Winter et al., 1994). With the help of phage display technology, it is now possible to isolate antibodies against autologous antigens. The introduction and development of efficient antibody library display methods (e.g. phage display)

and selection methods (e.g. biopanning) have dramatically enhanced the development of recombinant antibodies. The antibody fragments obtained by phage display system can be easily affinity optimised, as improved binding correlates with improved clinical efficacy. Random mutation followed by display on yeast surface can be employed to achieve affinity maturation. Yeast display system was used successfully with a number of antibody fragments/proteins to improve their affinity or half-life (Beuckena et al., 2003; Boder et al., 2000; Graff et al., 2004).

Taking into account the merits of scfv for antibody generation and to produce therapeutic anti-TNFα in an effective and economic manner, this study has focused on the following objectives.

Objectives

1. Isolation of novel anti-TNFα specific scFv clones from scFv phage display libraries
2. Molecular and functional characterization of isolated scFv clones
3. Affinity optimization of selected clones

2. MATERIALS AND METHODS

2.1 Rescue of scFv displaying phages and precipitation using PEG/NaCl

Tomlinson I and Tomlinson J scFv phage display libraries from Greg Winter's lab at the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK) were used to isolate anti TNF α scFv. Each of these libraries has more than 10^8 clones. These libraries are constructed in a phagemid vector (pIT2). pIT2 vector has ampicillin selection marker and having 6xHis and c-myc affinity tags at C-terminal. The clones in these libraries have affinity for protein A and protein L, so either of these affinity reagents can be used for the detection and purification of the scFv fragmant. Both of these libraries were used separately for the isolation of the anti hTNF α scFv. scFv phage display library was grown in 200 ml 2XTY liquid medium containing 1% glucose and 100μg/ml ampicillin. It was grown at 37^0C until $OD_{600}$was 0.4. 50ml of grown culture was taken and 2×10^{12} helper phage were added to it and incubated at 37^0C for 30 min without shaking. Cells were pelleted down at 3,000 g for 10 min and resuspend in 100 ml of 2XTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose. The culture was incubated overnight at 200rpm at 30^0C.

The culture was transferred to sterile 50ml centrifuge tube and centrifuged at 10,000 g for 20 min at 4^0C. 20 ml PEG/NaCl (20 % Polyethylene glycol 6000, 2.5 M NaCl) was added to 80 ml supernatant and was left for 2h on ice. After incubation phage was pelleted down at 10,000 g for 20 min at 4^0C and re-suspended in 4 ml PBS. The resuspended phage was transferred to 1.5ml centrifuge tube and centrifuged at 13000 g for 10 min at 4^0C. The supernatant containing phage scFv was placed on ice and stored at 4^0C until required.

2.2 Titer determination of isolated Phage

To titre phage stock, 1µl phage was diluted in 100µl PBS and 6 subsequent dilutions were made. To each dilution 900µl of *E. coli* TG1 strain was added at an OD_{600} of 0.4 and incubated at 37°C for 30 min. 100µl of each dilution was plated on TYE plate containing 1% glucose and 100µg/ml ampicillin and was grown at 37°C overnight. The colonies were counted, and titer was determined.

2.3 Panning of scFv library to select hTNFα binders

Selection of phage-antibodies from the human combinatorial antibody library was performed according to the standard protocol. Immunosorbent tubes (Maxi-sorb, Nunc) were coated with 200µg hTNFα (50µg/ml in 50mM

$NaHC0_3$, pH 9.5) overnight at 25°C. The tubes were washed four times with PBS and blocked with 2% PBSM for 2h at room temperature. Simultaneously, 2ml of stock derived from the Tomlinson library, containing $2x10^{12}$ phages displaying scFv antibodies, was mixed with 2ml of PBSM-4% and pre-incubated for 1h (scFv-PBSM). After removing the blocking solution from the tubes and washing with PBS, 4ml of (scFv-PBSM) was added to the tubes. It was incubated for 1h at room temperature on an under and over turnable, followed by a one hour incubation without shaking. The unbound phages were removed by washing the tube (10 times for round one and 20 times for rounds two and 30 times for round three) with 4 mL of PBST-0.1% (PBS containing 0.1% (v/v) Tween-20). The bound phages were eluted with 0.5 mL of trypsin-PBS (trypsin 1mg/ml, (Sigma-Aldrich, St. Louis, MO), for 10 min at room temperature on an under and over turnable. 250µl of the eluted phages were infected into 1.75 mL of *E. coli* TG1 at an OD_{600} of 0.4. The infected *E. coli* was incubated for 30 min at 37°C in a water bath. Following incubation, infected cells were isolated by centrifugation at 13,000 g for 10 min at room temperature. Cells were suspended in 0.2 mL of 2XTY medium and plated on TYE plates containing 1% glucose and 100µg/ml ampicillin. The plates were incubated overnight at 37°C.

2.4 Preparation of scFv-phage for subsequent panning round

The bacterial lawn, derived from a previous panning round, were scraped from the plate and resuspended in 2 mL of 2XTY containing 15% glycerol of the pooled bacteria.50 µl of this was inoculated into 50 mL of 2XTY medium containing 1% Glucose and 100µg/ml ampicillin and grown at a 37°C shaking incubator until OD_{600} reached 0.4. 10 mL of this culture was infected with helper phage and incubated for 30 min at 37°C in waterbath. The culture was centrifuged at 13,000 g for 10 min at room temperature. The resulting pellet was suspended in 100 mL of 2XTY medium containing 0.1% (w/v) glucose, 100µg/mL ampicillin and 50µg/ml kanamycin and allowed to grow overnight at 30°C in a shaking incubator. Next day, culture was centrifuged at 13,000g for 10 min at 4°C. Phage-containing supernatant was precipitated with 1/5th volume of PEG/NaCl (20% Polyethylene glycol 6000, 2.5M NaCl) for 6h at 4°C. Precipitated phages were pelleted at 13000 g for 10 min at 4°C and resuspended in 2ml sterile PBS.

2.5 Polyclonal and monoclonal phage ELISA

ELISA plate was coated overnight with 100µl/well of hTNFα (in 50 mM $NaHC0_3$, pH 9.5) at the same concentration as used for the selection. Coating solution was removed and plate was washed 3 times with PBS. Plate was

blocked with 200µl of 2% PBSM for 2h. After washing, plate was incubated for 1h with 10µl of PEG precipitated phage from each round of selection in 2% PBSM at 100µl/well. After washing with 0.1% PBST, 100µl of 1/5000 dilution of a HRP conjugated anti-M13 antibody in 2% PBSM were added and plate incubated for 1h. Binding of HRP conjugated anti-M13 antibody was detected using TMB as substrate for HRP. This solution was left to develop for 10 min and reaction was stopped using 50 µL of 1M sulphuric acid. The absorbance was read at 450nm.

2.6 High throughput screening and small scale production of soluble Antibody Fragments (scFv antibodies)

10µl of eluted phage was taken from 2nd and 3rd round of selection to infect 200µl of exponentially growing HB2151 bacteria (OD_{600} of 0.4) for 30 min at 37°C. Different dilutions of it were plated on TYE plate containing 100µg/ml ampicillin and 1% glucose and allowed to grow overnight at 37°C. Next day individual colonies were picked with a sterile toothpick and swirled in 100µL of 2XTY containing 100µg/mL ampicillin, 1% (w/v) glucose, in a sterile 96-well polystyrene microtiter plate and grown in a shaker at 200 rpm, overnight at 37°C.. The next day, 2µl of grown culture was re-inoculated in 200µL of 2XTY containing 100µg/ml ampicillin and 0.1% (w/v) glucose, in a sterile 96-well

polystyrene microtiter plate. It was allowed to grow at 37^0C until the OD_{600} was approximately 0.9. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to it to a final concentration of 1mM. The plate was incubated overnight at 30°C at 200 rpm and on next morning was centrifuged at 4,000 g for 10 min at 20°C. Supernatants were analysed by ELISA.

2.7 Selection of Anti-TNF α scFvs based on specificity

Total protein from Yeast (*Yarrowia lipolytica*) and plant (*Lemna minor*) was extracted and 100µg/ml was coated on ELISA plate. Coating solution was removed and plate was washed 3 times with PBS. Plate was blocked with 3% PBSA (3% BSA in PBS buffer) at 200µl of for 2h. Supernatant of the induced culture was used as primary antibody for ELISA. 50µl of the supernatant was taken in PBSA (3% final concentration) at 100µl/well for 1h. After washing with 0.1% PBST, 100µl of 1/5000 dilution of a HRP- conjugated Protein-L in 2% PBSA were added and plate was incubated for 1h. Binding of HRP-conjugated Protein-L was detected using TMB as substrate for HRP. This solution was left to develop for 10 min and reaction was stopped using 50µL of 1M sulphuric acid. The absorbance was then read at 450 nm.

2.8 Selection of scFv clones based on avidity

ELISA plate was coated overnight in duplicate with 100µl/well of hTNFα (in

50 mM $NaHC0_3$, pH 9.5) at a concentration of 10µg/ml. ELISA was carried out as above except after adding primary antibody and washing with 0.1% PBST, one set of well were incubated with urea at different concentration and another set was incubated with PBS as a control for 30 min. Plate was washed with 0.1% PBST and secondary antibody was added. ELISA was developed and the avidity index was calculated by using formula: Avidity index (%) $=OD_{450/620}$ Urea/$OD_{450/620}$ PBS.

2.9 Screening of selected clones by PCR

Presence of full length VH (~520bp) and Vκ (~370bp) insert in the clones was confirmed by PCR using primers V_HF and V_HR for V_H region, and V_KF and V_KR for V_K region respectively (Appendix, Table 1). Annealing was carried out at 55 ^{0}C for 30s and extension at 72 for V_H or V_K was 30min and for V_H and $V_κ$ together, 1 min. to amplify full length scFv V_HF and V_KR primers were used with an extension of 1 min at 72^{0}C.

2.10 TNFα neutralization assay

Confluent monolayer murine L929 fibroblasts were trypsinized and

resuspended in DMEM culture media ($3.0x10^5$ cells/ml). 100 µL of cell suspension was seeded to each well of 96-well tissue culture plate. Cells were

allowed to grow until monolayer is formed (18h to 22h). The medium was discarded after overnight grown culture and replaced by fresh DMEM media. TNFα and actinomycin D to a final concentration of 8ng/ml and 2µg/ml respectively, were added per well. Different dilutions of adalimumab (Humira™) standard and in-house samples were added to the wells. Plate was incubated at 37°C for 20-24h. The supernatant from the well were discarded and MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazolium salt) to a final concentration of 0.5mg/ml was added to the wells. Plate was incubated at 37°C for 4h. Then supernatants in the wells were carefully discarded and 200 µl/well of dimethyl sulfoxide (DMSO) was added into plate to dissolve Formazan crystals for 10min. The absorbance was recorded at 570 nm.

2.11 Large scale expression of Recab in *E.coli*

E.coli strain HB2151 cells harbouring anti TNFα scfv clone were grown to an OD_{600} of 0.9 in 2XTY media containing 100µg/ml ampicillin and 2% glucose. The culture was centrifuged and resuspended in 2XTY media. The expression was induced by addition of isopropyl 1-thio-b-D-galactopyranoside (IPTG) to a final concentration of 1mM, with further overnight incubation at 30^0C . The supernatant and cell pellets were collected by centrifugation at 10,000 g for 10

min. Supernatant was concentrated by ultrafiltration giving a cut-off of 10kD. Protein from the pellet was extracted in the extraction buffer (25mM Tris, 150mM NaCl, pH7.4, 1.0 mM EDTA), lysozyme was added to a final concentration of 50μg/ml. It was kept on ice for 20min and was centrifuged at 10,000 g for 15 min and the supernatant was collected. As pellet and supernatant both have the protein of interest, so both were used for purification using protein A column (MabSelect Sure, GE Healthcare Ltd, Little Chalfont, Buckinghamshire).

2.12 Purification of anti-TNFα scFv (Recab) protein

Periplasmic extract and supernatant containing Recab were harvested at the end of production stage and was centrifuged and passed through 0.22 μm filter. After clarification, the clear protein mixture was reconditioned to adjust pH and salt concentration of protein solution. pH was adjusted to 7.4 ± 0.2 with 2M Tris solution and salt concentration was adjusted by adding NaCl to achieve final concentration of 150mM. After reconditioning, supernatant containing Recab (ScFv) protein was subjected to protein A affinity column chromatography. Purification by protein A column was carried out using medium pressure liquid chromatography system, AKTA purifier (GE Healthcare Ltd.). Protein A matrix (MabSelect Sure) was packed into C10/10

column (h 10 cm × id 1 cm) using AKTA purifier in a step-wise manner. Primary packing was carried out by passing Milli-Q water at a flow rate of 2 mL/min (~150 cm/h) followed by secondary packing at a flow rate of 4 mL/min (~300 cm/h). After packing, the column was equilibrated by passing 3 - 5 bed volumes of equilibration buffer containing 20mM Tris-Cl pH 7.4, 150mM NaCl at a flow rate of 2 ml/min. Reconditioned culture supernatant was loaded onto equilibrated protein A affinity column at a flow rate of 1 ml/min (~75 cm/h). After loading of protein solution, column was washed with 3 - 5 bed volumes of equilibration buffer to remove any unbound proteins. Further, column was washed with 3 - 5 bed volumes of a high ionic strength buffer containing 20mM Tris-Cl pH 7.4, 500mM NaCl to remove any non-specifically bound proteins. Column was re-equilibrated by passing 3 - 5 bed volumes of equilibration buffer. Elution of Anti-TNFα (ScFv) protein was performed at an acidic pH by passing 5 - 10 bed volumes of elution buffer containing 50mM Na-acetate pH 3.5-4.0, 100mM NaCl. Elution of protein is conducted at a flow rate of 2 ml/min (~150 cm/h). Eluted protein fraction was neutralized immediately to pH 7.0 by adding 2M Tris solution. Finally, protein A column was cleaned by passing NaOH and Milli-Q water, as per manufacturer's instructions. Small aliquots of samples were collected at each step of protein purification and stored at –20^{0}C for further analysis.

2.13 Stability assay for Recab

Strength of binding of the recombinant antibody fragment was also assayed qualitatively by performing ELISA at different concentrations of NaCl, urea, DMSO, guanidium HCl, NaSCN and at different pH. 1:100 dilution of periplasmic extract was made in PBS having different concentrations of NaCl (500mM to 4 M), urea (0.5–8 M), DMSO (0–50%), NaSCN (0-4M)and guanidium HCl (0–4 M). Diluted periplasmic extracts were added to antigen coated ELISA plates and bound scFv was detected with protein-L HRP. Similar experiments were performed at different pH with phosphate buffer at pH 6, 7.4, and 8. Carbonate buffer was used for pH 9.2, 10 and 11 and citrate buffer was used for pH 4.

2.14 Antibody affinity maturation

2.14.1 Construction of randomly mutated yeast scFv display vector

Amplified PCR product of Recab (750 bp) was digested with *Nhe*I and *Bam*HI, gel purified and ligated into the yeast surface display vector pCTCON2 (Addgene plasmid # 41843). A random mutant scFv library of Recab was created by adapting nucleotide analogue method of Zaccolo and Gherardi (1999). The expression cassette of Recab was amplified by PCR using YDF and

YDR primers (appendix, table 1). 2 µM dPTP and 8-oxo-dGTP, 1ng of Recab DNA, 200 µM each dNTP, 0.5 µM each primer, 1X Gibco PCR buffer supplemented with 2mM $MgCl_2$, YDF primer 0.5µM, YDR primer 0.5µM, 3 units Taq polymerase (Gibco). The reaction was cycled as follows: 94^0C 1 min, 60^0C 1 min, 72^0C 1.5 min, for 10 cycles final extensions was 72^0C for 10 min. PCR product was gel purified using 1% agarose gel and a product of about 900bp was excised from the gel, purified with a gel purification kit (Qiagen, Venlo, Netherlands). Purified PCR product was diluted to 1:10 for further amplification in the absence of nucleotide analogues for 30 cycles using Taq polymerase. PCR products were purified using a PCR purification kit (Qiagen, Venlo, Netherlands). 5 µg of pCTCON2 vector was digested with *Nhe*I, *Bam*HI and *Sal*I. Prepared Vector-insert DNA was transformed into yeast strain EBY100 (ATCC MYA-4941) by electroporation. Transformants were pooled in SD-CAA and aliquots were plated to determine library diversity.

2.14. 2 Primary screening of mutated Recab library using fluorescent microplate reader

Cells were grown at 30 ^{0}C at 200 rpm in sterile 96 well microtiter plate in

SDCAA medium. Next day, supernatants were discarded and cells were resuspended in SGCAA induction medium, allowed to grow for 20h at 20^0C at

200rpm. Supernatants were discarded and cells were stained using protocol of Boder and Wittrup (1998). In brief, yeasts cells displaying mutated Recab were incubated with chicken anti-c-Myc IgY (1:250) (Life Technologies, Carlsbad, CA) and different concentrations of biotinylated hTNFα. Recombinant hTNFα (Life Technologies, Carlsbad, CA) was biotinylated using 'Biotin-XX Microscale Protein Labeling Kit'(Life Technologies, Carlsbad, CA). Cells were washed with cold PBS–BSA (PBS containing 1 mg/ml BSA) and labelled with secondary reagents streptavidin–phycoerythrin (1:100) (Life Technology, Carlsbad, CA) and goat anti-chicken–Alexa flour 488 (1:100) (Life Technologies, Carlsbad, CA) on ice. Cells were washed with PBS–BSA and resuspended in PBS-BSA. The florescent signal for Alexa 488 was read by exciting at 485/20 and emission at 530/20. The fluorescent signal for phycoerythrin was red by exciting at 485/20 and emission at 590/20.

2.14.3 Microscopy

Inframe fusion of scFv with Aga2 protein was also confirmed by observing fluorescent signal of Alaxa 488 and phycoerythrin. All images were recorded with 63 X Zeiss LSM 700 confocal laser microscope systems (Carl Zeiss, LSM 700, Germany). The florescent signal for Alexa 488 was read by exciting at

485/20 and emission at 530/20. The fluorescent signal for phycoerythrin was read by exciting at 485/20 and emission at 590/20.

2.14.4 Flow cytometric analysis of yeast cells

Yeast surface display library was grown in SD-CAA. Cells were transferred to SG-CAA to OD_{600} = 0.5 and grown for 20h at 20^0C. The mutated Recab library was screened by equilibrium method of Boder and Wittrup (1998). Briefly recombinant hTNFα (Life Technologies, Carlsbad, CA) was biotinylated using 'Biotin-XX Microscale Protein Labeling Kit' (Life Technologies, Carlsbad, CA). Yeasts cells displaying mutated Recab were incubated with chicken anti-c-Myc IgY (1:250) (Life Technologies, Carlsbad, CA) and different concentrations of biotinylated hTNFα. Cells were washed with cold PBS–BSA (PBS containing 1 mg/ml BSA) and labelled with secondary reagents streptavidin–phycoerythrin (1:100) (Life Technology, Carlsbad, CA) and goat anti-chicken–Alexa flour 488 (1:100) (Life Technologies, Carlsbad, CA) and kept on ice for 10 min. Cells were washed with PBS–BSA and resuspended at concentration of 10^7cells/ml and sorted on a Becton Dickinson FACS ARIA III flow cytometer. Cells were collected with gate settings designed to collect the cells displaying the highest

PE fluorescent signal per FITC fluorescent signal.

3. RESULTS

3.1 Biopanning of hTNFα specific phage scFv particles

Two scFv phage display library, Tomlinson I and Tomlinson J were used to isolate scFv-antibody against hTNFα protein. The size of the library was $>10^8$, so it provided an opportunity to select rare clones in fewer rounds of selection. Three rounds of selection were carried out to obtain scFv antibody against hTNFα. During the selection process, the concentration of hTNFα for coating was reduced and the number of washes was increased. For first round 50 ug/ml of hTNFα was used for coating, in subsequent round (2^{nd} and 3^{rd}) the concentrations were reduced to 20 ug/ml and 5 ug/ml. Input and output titre for each round of selection was determined (**Table1**). Increase in stringency is important to ensure that positive clones are selected and reduces the levels of clones which have no specificity to the target antigen. After each round of selection eluted phages were used to infect *E. coli* TG1 cells. Infected TG1 cells were spread on ampicillin containing TYE plate.

A phage-titre, before and after selection on hTNFα-immobilised immunotubes, expressed as colony forming units (cfu), was calculated. As shown in table 1, 3000-fold increase in output was obtained after 2 rounds of panning in both the libraries. Clear enrichment of the positive clones were observed in panning rounds with increased washing stringency, enabling the selection of clones with specific-binding affinity. The 3rd panning round resulted in a 1000-fold enrichment over the second round in Tomlinson I and 7 fold in

Tolinson J library, as the binding clones became the dominant population in the library (**Table1**).

3.2 Polyclonal phage ELISA

Eluted phage from each round of selections was analysed by polyclonal phage ELISA. Results showed that ELISA signal were increasing in subsequent panning rounds of selection which is an indication that positive clones were selected **(Figure 8)**. Compare to second round the ELISA signals were higher in third round.

3.3 Screening for specific Binders to hTNFα

The pooled phages contain a collection of recombinant antibody fragments with a range of specificity and affinity for hTNFα and, therefore, individual clones were analysed by ELISA to determine the binding patterns of the clones. An experiment was performed which involve negative selection of the selected clones against yeast and plant proteins-. Both of these protein sources are not from animal origin, so there are very few chances of losing the positive clones. Antigen binding clones from 2nd and 3rd round of selection were transformed into non supressor HB2151 *E. coli* strain, for the soluble expression of the protein. Individual colonies were picked from TYE palate containing ampicillin and glucose. 188 colonies each from both libraries were grown in sterile 96 microwell plates and analysed by ELISA. Total protein from yeast, *Yarrowia lipolytica,* was coated on 96 microwell plate. Supernatant of the clones were analysed for binding to yeast protein coated ELISA plate and detected with an HRP conjugated Protein-L. Approximately 68 %

clones from Tomlinson I and 61 % from Tomlinson J showed binding to yeast protein coated to ELISA plate. These clones were discarded and remaining clones were again negatively selected against plant protein. A total of 112 clones from both the libraries showed specificity for hTNFα, were propagated further for the next stage of selection (**Table 2**).

3.4 Selection of anti-TNFα clones based on Avidity

The pooled phages contain a collection of recombinant antibody fragments with a range of affinities for hTNFα and, therefore, individual clones were analysed by ELISA to determine the binding strength of the clones. Clones obtained after specificity selection were subsequently propagated for the next stage of selection based on avidity, were allowed to bind to the antigen in presence of 4M urea and detected with an HRP-conjugated protein-L. 24 clones showed comparable OD 450/620 values to that of the control (PBS). These 24 clones were subsequently propagated for the next stage of selection. 6M urea was used and avidity index was specificity selection were allowed to bind to the antigen in presence of 4M urea and detected with an HRP-conjugated protein L. 24 clones showed comparable OD 450/620 values to that of the control (PBS). These 24 clones were subsequently propagated for the next stage of selection. 6M urea was used and avidity index was calculated using formula (%Avidity index= (OD450/620 in presence of urea / OD450/620 of control) x 100) for the individual clones (**Figure 9**).

Table 1: Selection of hTNFα specific antibody fragments.

A phage-titre, before and after selection on hTNF α immobilised immunotubes, expressed as colony forming units (cfu), is shown. hTNFα with different conecntrations was used for the selection for 1st round-50 µg/ml, 2nd round -20 µg/ml and for 3rd round – 5 µg/ml

% Bound = (output/input) x 100.

A.

Tomlinson J				
Panning round	**Phage input (CFU)**	**Phage output (CFU)**	**% bound ($x10^4$)**	**Enrichment**
1	$1.7x10^{12}$	$5.1x10^5$	0.3	
2	$1.1x10^{12}$	$1.05x10^9$	950	$3.166x10^3$
3	$2\ x10^{11}$	$1.35x10^9$	6750	7.10

B.

Tomlinson I				
Panning round	**Phage input (CFU)**	**Phage output (CFU)**	**% bound ($x10^4$)**	**Enrichment**
1	**$3x10^{12}$**	**$8x10^4$**	**0.026**	
2	**$2x10^{11}$**	**$1.32x10^7$**	**66**	**$2.538x\ 10^3$**
3	**$2.5x10^{11}$**	**$1.6x10^{10}$**	**64000**	**969.9**

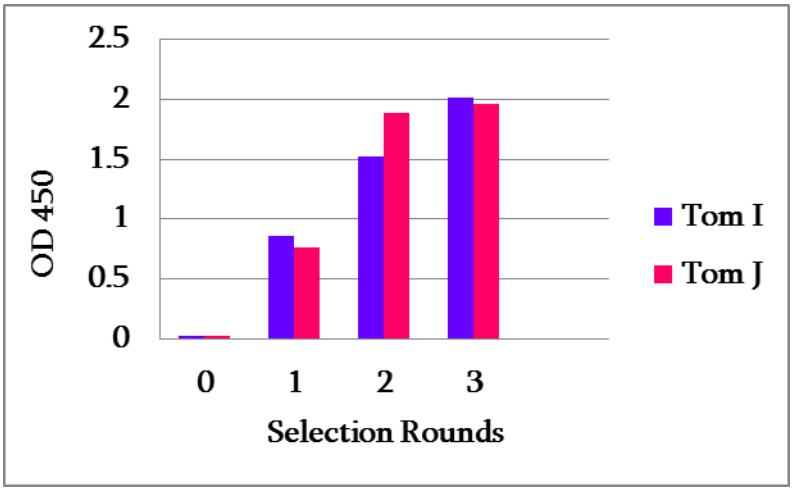


Figure 8. Polyclonal phage ELISA

Phage pools from the scFv library stock and the phage pool obtained after subsequent rounds of panning were tested for binding to hTNFα by ELISA. Bound phages were detected with an anti-M13 HRP-conjugated antibody and the absorbance read at 450nm. High affinity clones remained attach to the hTNFα coated surface and are subsequently propagated for the next stage of selection. A significant absorbance signal increase in pan three suggests the presence and enrichment of anti-hTNFα scFv phage fragments.

Tomlinson I				
Protein Source	Input Binders	Non specific	specific	%
Yarrowia lypolitica	188	128	60	31.9
Lemna minor	60	5	55	91.6

Tomlinson J				
Protein Source	Input Binders	Non specific	specific	%
Yarrowia lypolitica	188	116	72	38.2
Lemna minor	72	15	57	79.1

Table 2: Screening for specific Binders to hTNFα. Isolated pool of phage contain collection of recombinat antibody fragments with range of affinity and specificity. These pages were selected against yeast and plant proteins. scFv clones which did not bind were selected for further study A. Isolated clones from Tomlinson I B. Isolated clones from Tomlinson J lib

18 clones showing avidity index above 50% were propagated for further characterization.

3.5 Characterization of anti-TNFα scFv (Recab) clones

pIT2 phagemid vector was used to generate Tomlinson I & J phage display libraries (de Wildt et al., 2000). The heavy chain variable fragments were cloned downstream to the promoter followed by light chain (**Figure 10**). Three set of PCR reactions were carried out for amplification of the V_H, V_L and V_H+V_L. Plasmid DNA of selected clones was extracted and used for PCR amplification using V_HF, V_HR, V_KF and V_KR set of primers. Presence of DNA fragment approximately at 500 bp confirms the heavy chain, 370 bp the light chain and near 1kb is the combined DNA product of heavy and light chain variable regions (**Figure 10B**). Band pattern on agarose gel confirmed the presence of both light and heavy chain in the selected clones. Soluble expression of the scFv protein in HB2151 *E.coli* strain was analyzed by SDS-PAGE (**Figure 11A**) followed by western blot analysis (**Figure 11B**).

The binding of these clones to TNFα was also confirmed by western blot analysis (**Figure 11C**). Samples from uninduced and induced cultures, culture supernatants and periplasmic extract were checked for the presence of the antibody fragment on 12% SDS-PAGE. On SDS-PAGE a distinct protein band of approximately 28 kDa was distinguishable compared to the control culture protein. Antigen binding

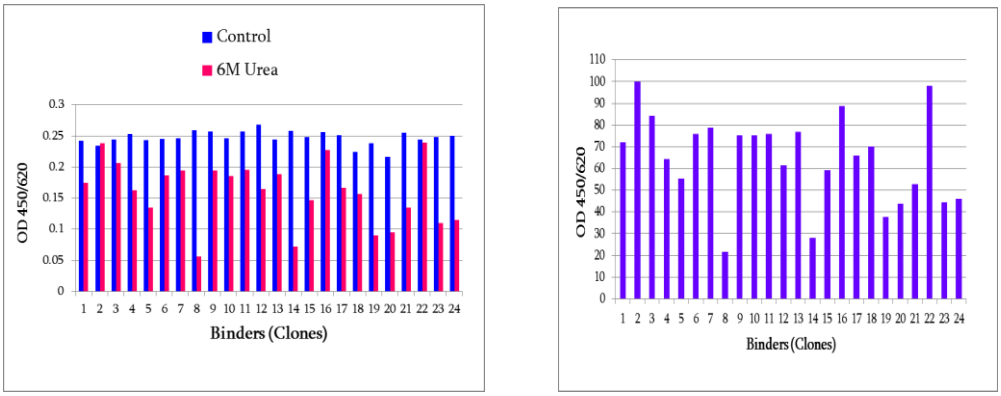


Figure 9: Selection of binders based on Avidity

Urea is known to destabilise the antigen antibody association. scFv with low affinity were eliminated using urea during the selection.

A. ELISA signal read at 450 nm. Twenty four clones obtained by selection on 4M urea, were further used to check for the avidity in presence of 6M Urea, in control PBS was used instead of urea.

B. Avidity index (%) was calculated according to formula Avidity Index = $OD_{450/620\ UREA}/OD_{450/620\ PBS}$. Clones showing avidity index above 50% were selected for further analysis.

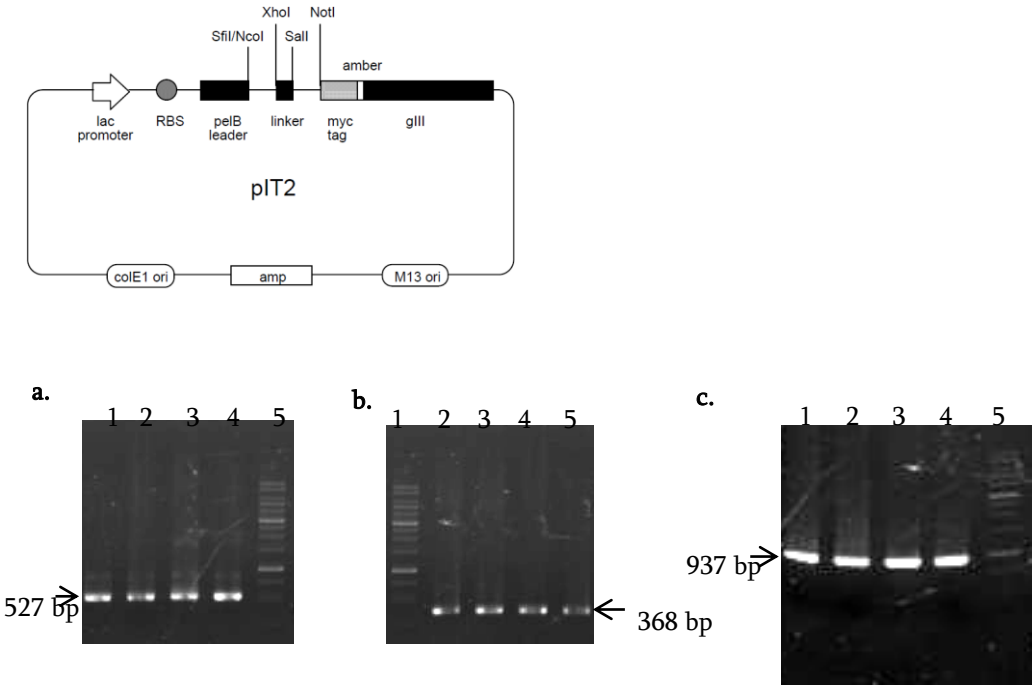


Figure 10: Map of phagemid vector pIT2

A. The phage display libraries, Tomlinson I and Tomlinson J were constructed in pIT2 vector. As shown in figure, this vector has two affinity tags 6xHis and c-myc. pelB leader sequence is to derive protein in periplasmic space of the bacteria

B. Confirmation of heavy and light chain in phagemid vector. Plasmid DNA was used for the amplification of V_H, V_L and V_H+V_L. Amplicon confirmed presence of both fragment (V_H and V_L) in the selected clones.

(a.)Lane1, 2, 3 and 4: V_H specific amplicon, Lane 5: 1 kb DNA marker

(b.)Lane 2, 3, 4 and 5: V_L specific amplicon, Lane 1: 1 kb DNA marker

(c.)Lane1, 2, 3 and 4: V_H + V_L specific amplicon, Lane 5: 1 kb DNA marker

activity from the periplasmic extract as well as supernatant was detected using ELISA as well as western blot analysis (**Figure 11**).

3.6 Neutralization of TNF-α-mediated cellular cytotoxicity by Recabs

The ability of recabs to inhibit soluble TNF-α activity was examined using cell based assay. The L929 mouse aneuploidy fibrosarcoma cell line is commonly used to assay the cytotoxic effects of TNFα, which causes apoptosis in cells sensitized by pre-treatment with actinomycinD. The MTT colorimetric assay was used to analyse the effect. The principle of MTT test involves the reduction of tetrazolium ring to Formazan crystals by mitochondrial enzyme succinate dehydrogenase. Survival of the cell can be evaluated by measuring the absorbance of Formazan crystals dissolved in solution at 570 nm. The number of surviving cells and the cytotoxicity of TNFα is proportional and reciprocally correlated with absorbance. Functional activity of the recabs was evaluated in L929 cell based assay by co-incubating with recombinant human TNF α. The concentration of TNF α and actinomycin D used was 5ng/ml and 2ug/ml, respectively. Humira (adalimumab) which is a commercially available anti TNF α antibody approved for the treatment of autoimmune diseases,was used as a positive control. Calculations and statistical analysis were performed using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Log values of serial concentrations were used as X-axis, value of OD at 570nm was set as Y-axis, sigmoid dose-response curves were plotted and the EC_{50} values of standard and sample were calculated automatically. Primary screening was done by taking the supernatant of the induced culture. A total of 42 recabs were used for the assay. Eight

clones which showed OD_{570} value above 0.85 nm were further selected for the assay (**Table 3**). Cultures were induced and protein was purified by using protein A resin column. Out of the selected 8 clones two (C2 and G2) showed OD at 570nm value comparable to that of the positive control, Humira (**Table 4**). EC_{50} of these two clones were calculated and a graph was generated (**Figure 12**). EC_{50} value for clone C2 was calculated as 10.93 µg / ml and for clone G2 it was 14.23 µg / ml. The results indicated that two clones neutralized hTNFα activity efficiently. hTNFα is a trimeric molecule for disruption of the binding of TNF α to its receptor the antibody should bind to the region where the ligand receptor binding takes place. It is possible that rest of the clones did not bind to the epitope of TNFα that is required for receptor ligand

binding or have low affinity for it. Dissociation of low affinity scFv molecules to their epitope takes place at a higher rate compare to high affinity, so they will not be able to neutralize toxic effect of hTNFα to the cell .Clone C2 which showed better EC_{50} value, was selected for further characterization.

Table 3: Neutralization of hTNF-α-mediated cellular cytotoxicity by Recabs

L929 cells were used for the assay. Humira™ was used as positive control

A. Well wise presentation of Recab clones

B. OD_{575} value obtained by MTCC assay

A.

A1	A5	B3	C1	C5	D4	E3	F2	G1	G5	H4	TOM 3(UNDILUTED)
A1	A5	B3	C1	C5	D4	E3	F2	G1	G5	H4	TOM 3(1/10)
A2	A6	B4	C2	D1	D5	E4	F3	G2	H1	H5	TOM 4(UNDILUTED)
A2	A6	B4	C2	D1	D5	E4	F3	G2	H1	H5	TOM 4(1/10)
A3	B1	B5	C3	D2	E1	E5	F4	G3	H2	CELL BLANK	TOM 5(UNDILUTED)
A3	B1	B5	C3	D2	E1	E5	F4	G3	H2	CELL BLANK	TOM 5(1/10)
A4	B2	B6	C4	D3	E2	F1	F5	G4	H3	AD+TNF BLANK	Ab BLANK
A4	B2	B6	C4	D3	E2	F1	F5	G4	H3	AD+TNF BLANK	Ab BLANK

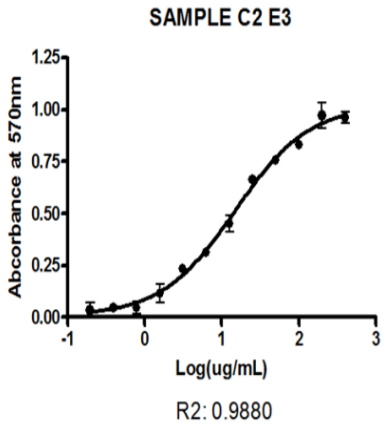
B.

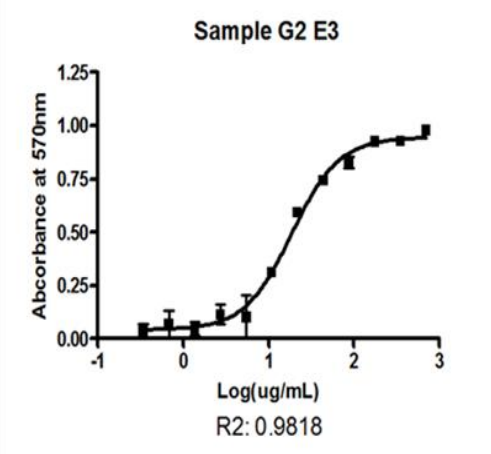
0.681	0.599	0.658	0.603	0.613	0.655	0.703	0.656	0.719	0.736	0.758	0.095
0.673	0.65	0.828	0.77	0.803	0.709	0.713	0.769	0.833	0.936	0.801	0.701
0.602	0.683	0.847	0.809	0.765	0.801	0.808	0.845	0.974	0.988	0.875	0.09
0.63	0.743	0.869	0.907	0.86	0.908	0.85	0.819	0.948	1.063	0.895	0.733
0.509	0.654	0.792	0.773	0.715	0.757	0.875	0.733	0.713	0.864	2.002	0.096
0.471	0.598	0.723	0.703	0.743	0.816	0.725	0.725	0.837	0.759	1.893	0.649
0.595	0.582	0.718	0.885	0.634	0.656	0.607	0.79	0.662	0.911	0.317	0.102
0.555	0.523	0.711	0.603	0.608	0.67	0.715	0.654	0.518	0.668	0.311	0.692

Table 4: Determination of EC_{50} value of anti-TNF clones

Purified scFv protein was used for the assay. Serial dilutions of the purified scFv protein were incubated with a constant concentration of hTNFα. Cell viability was cheched using MTT assay. Clone C2 and G2 neutralized of the cytotoxic effect of hTNFα on the cells.

SAMPLE ID	DILUTION No.											
	1	2	3	4	5	6	7	8	9	10	11	12
C2	2.148	1.915	1.777	1.692	2.067	1.444	1.587	1.575	1.149	1.21	1.202	1.168
C4	0.664	1.009	0.873	0.749	0.705	0.645	0.669	0.783	0.825	0.935	1.147	2.19
C5	0.622	0.886	0.645	0.588	0.579	0.814	1.022	1.151	1.269	1.362	1.098	1.153
D5	0.304	0.322	0.326	0.32	0.332	0.333	0.335	0.343	0.339	0.355	0.376	-
G2	2.004	1.949	1.93	1.881	1.776	1.646	1.334	1.013	1.098	1.046	1.016	0.957
H1	0.552	0.926	0.601	0.949	1.039	1.146	1.299	1.303	1.261	1.349	1.107	2.159
H3	0.398	0.639	0.506	0.501	0.525	0.648	0.784	0.804	0.839	0.815	0.401	0.943
H5	0.412	0.609	0.541	0.532	0.551	0.656	0.788	0.879	0.963	0.951	0.996	1.009
Humira®	2.001	1.779	1.617	1.285	0.464	0.37	0.358	0.356	0.348	0.345	-	-





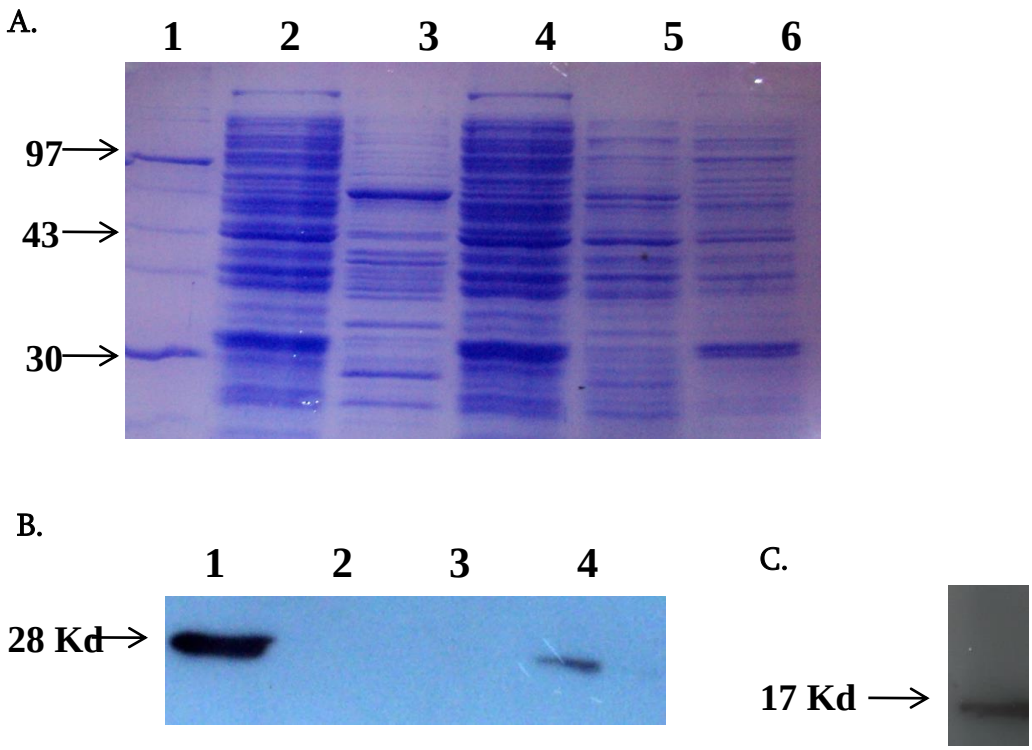


Figure 11: Expression of anti-TNF α scFv (Recab)

A. Expression of Recab protein checked on 12% SDS-PAGE

Lane 1- Protein Marker, Lane2 and 4: Induced culture periplasmic extract, Lane 3- Uninduced periplasmic extract

Lane 5- Supernatant of uninduced culture, Lane 6 - Supernatant of induced *E. coli* HB2151 strain. Band corresponding to 28 kDa is shown.

B. Western blot analysis of anti-TNF scFv. Band corresponding to 28 kDa was seen in both periplasmic extract as well as in culture supernatant of induced scFv clone. Periplasmic extract and supernatant of HB2151strain was used as control.

Lane 1- Periplasmic induced, Lane 2- Periplasmic Control (HB2151), Lane 3- Supernatant control, Lane 4- Supernatant induced

C. Western blot of hTNFα. Periplasmic extract of the induced scFv clone was used as primary antibody and was developed using HRP conjugated pritein L. Band corresponding to molecular weight of hTNF α (17 kDa) was obtained confirming the anti TNF α binding of the isolated clones.

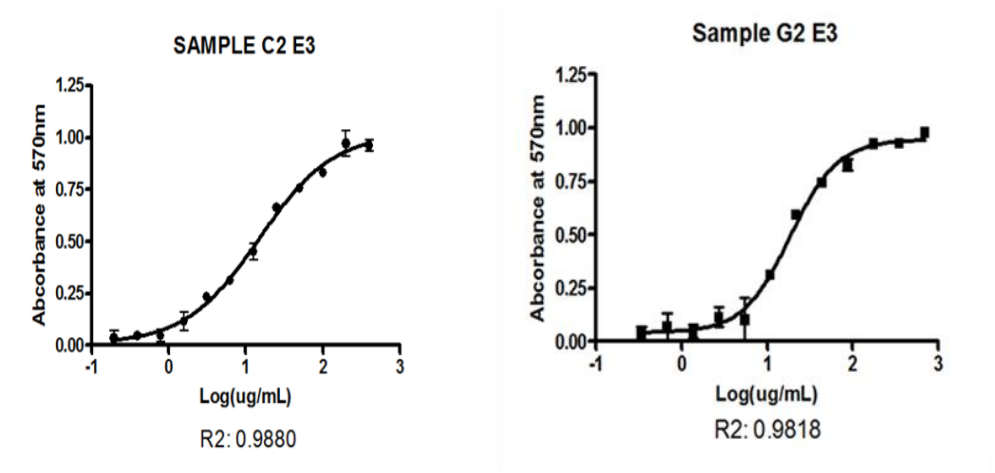


Figure 12: EC50 value of C2 and G2 clones

Serial dilutions of the purified scFv clones were added to the wells containg the L929 cells.Calculations and statistical analysis were performed using GraphPad Prism 4.0 software. Log values of serial concentrations were used as X-axis, value of OD at 570nm was set as Y-axis, sigmoid dose-response curves were plotted and the EC_{50} values of standard and sample were calculated automatically.

3.7 Large-scale expression of Recab and subsequent purification using Protein A affinity chromatography

The expression of recab was induced by addition of 1 mM IPTG to a log phase growing culture of HB2151 strain. The induction was done overnight at 30 ^{0}C and 200 rpm. After induction, Recab expression was confirmed using ELISA. Antigen binding activity was present both in periplasmic extract as well as in culture supernatant. Therefore both periplasmic extract and culture supernatant were used for protein purification. Protein was clarified by passing through 0.22 μm membrane. Staphylococcal protein A coupled sepharose medium was used to capture anti TNF scFv as the libraries were constructed using a single human framework for VH (V3-23/DP-47 and JH4b), which has affinity for it. Staphylococcal protein A selectively and efficiently binds antibodies in complex solutions such as crude protein extract, and it removes >99.5% of product impurities in a single step with high step yields and high throughput. Protein purification was monitored using AKTA purifier (GE healthcare Ltd). Non-specific binding was removed by washing the resin with washing buffer (500 mM NaCl,) and the recab protein successfully eluted using a low pH buffer (50 mM sodium acetate pH 4.0). Different fractions obtained during purification step were pooled, centrifuged and analysed by SDS-PAGE and Western blotting for presence of the Recab protein (**Figures 13A and B**). Bacterial lysate preparation is a critical step as the main purpose is to maximise protein extraction while minimising unwanted protein and possible contamination with genomic DNA, so secretory expression of the protein is advantageous as many of these contaminants are

either absent or present at low concentrations compared to the cytoplasmic expression. The eluted fractions were pooled and concentrated by centrifugation at 4,000 rpm at 4 ^{0}C using a 10 kDa molecular weight cut-off spin column. The protein samples were resolved on SDS-PAGE gels to verify that the expressed protein (Recab) exhibited the correct molecular weight (28 kDa) and to assess the purity of the isolated protein. While one gel was stained with Coomassie blue to visualise individual proteins expressed within the bacterial cell, the proteins on the other gel were transferred to a nitrocellulose membrane and subjected to Western blot analysis using an HRP labelled protein L. A large number of proteins were clearly visible in the lysate and flow-through with little or no proteins in the wash fraction for the Coomassie blue-stained SDS-PAGE gel. A single band at the correct molecular weight for the expressed scFv fragment was evident in **Figure 13B** indicating its purity. Yield of the purified protein was estimated by OD at 280 nm; the extinction coefficient of the recab was calculated and found to be 1.5, protein concentration was calculated using formula

Concentration (mg/ml) = Absorbance at 280nm / extinction coefficient (M)

Yield of the protein was estimated at 39.52 mg/ L. It has been reported that the scFv expression at periplasmic space is low. The expression of scFv at the

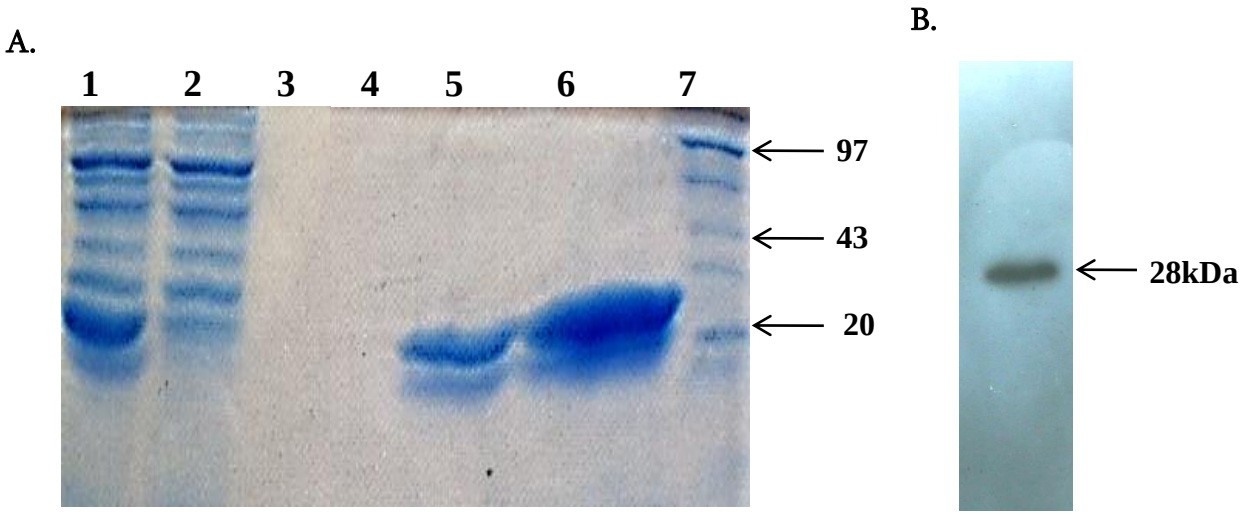


Figure 13: Protein-A affinity purification of Recab and western blot analysis

Protein A media was used to purify the scFv fragments. Protein A selectively binds to the scFv fragments in complex solutions such as crude protein extract, and it removes >99.5% of product impurities in a single step with high yields result in pure protein. As shown in the figure, single band corresponding to 28 kDa is obtained in the eluted fraction. scFv protein was efficiently binding to the protein A resin as no detectable band corresponding to 28 kDa was seen both in flow through and washes.

A. Purification of Recab protein with Protein-A resin. Lane 1 – Total protein, Lane 2 – Flow-through, Lane 3, 4 – wash, Lane 5, 6 – Elute, Lane7 – Protein Marker (kDa).

B. Western blot of purified Recab protein. A single band corresponding to 28 kDa was seen in the blot.

periplasmic space were low as reported by ~ 10mg/L (Tiwari et al., 2010), 2-6 mg/L (Raffai,R. et al.,1999) and 1.7–2.9 mg/L (Ossysek, K. et al., 2015).

3.8 Assay for stability of antigen–antibody association

To study the stability of the antigen–antibody complex formed by Recab and to understand the molecular forces involved in the complex formation, ELISAs were performed in presence of NaCl, urea, DMSO, GnHCl, NaSCN and different pH . NaCl is known to disturb the electrostatic interactions involved in protein–protein interactions. Recab showed stable binding even in presence of 4M NaCl (**Figure 14A**). Involvement of electrostatic interactions is more evident when ELISA is performed at different pH. Recab binding remained unaltered in the pH range of 6–8 with a sharp fall in binding towards the lower or higher side of the pH scale (**Figure 14B**). Binding of the Recab in presence of different concentrations of guanidium HCl was checked, it binds to the antigen even at 1M guanidium HCl (**Figure 14C**). DMSO is known to disturb the hydrophobic forces and at high concentration is also a denaturing agent. At 20% DMSO, binding of recab was stable, it fell at 30%, indicating that some critical hydrophobic interaction between the antigen and the scFv could be disturbed by DMSO (**Figure 14D**). Urea is a known destabilizing agent for antigen–antibody complexes had no significant effect on binding of Recab at 4M concentration (**Figure 14E**). Above 0.5M concentration of NaSCN, recab was not able to form stable association with hTNFα (**Figure 14F**). scFv molecules are prone to the denaturation and structural instability, their association in presence of these different chemical confirm their stabile association with hTNFα.

3.9 Affinity maturation for Recab using Yeast Display System

3.9.1 Construction of Recab surface display yeast strain

Phage display is a very powerful technology for the isolation of antibodies; most of these antibodies often require affinity optimisation for them to be used in clinical development, as improved binding correlates with improved clinical efficacy. A yeast surface display approach was employed to achieve affinity maturation. The display system comprises two protein components: AGA1 and AGA2. The N-terminal region of AGA2 binds to AGA1 on the surface of the yeast cell, by forming two disulfide bonds (Cappellaro et al. 1991). The EBY100 strain expresses the AGA1 peptide (which binds to β-glucan) on the surface of the yeast cell using the Gal1 promoter (Lu et al. 1995). The AGA2 subunit is encoded by the pCTCON2 vector and is expressed as an N-terminal fusion to the protein of interest (**Figure 15A**). The scFv antibody (Recab) was amplified using primer set YDF and YD R and cloned at *Nhe*I/*Bam* HI site of the pCTCON2 vector. The resulting vector pConC2 was confirmed by sequencing and transformed in EBY100 yeast strain. Transformants were selected on SDCAA media plate and presence of the vector DNA was confirmed by colony PCR (**Figure 15B**). Transformants showed a PCR amplicon of 368 bp that corresponded to the light

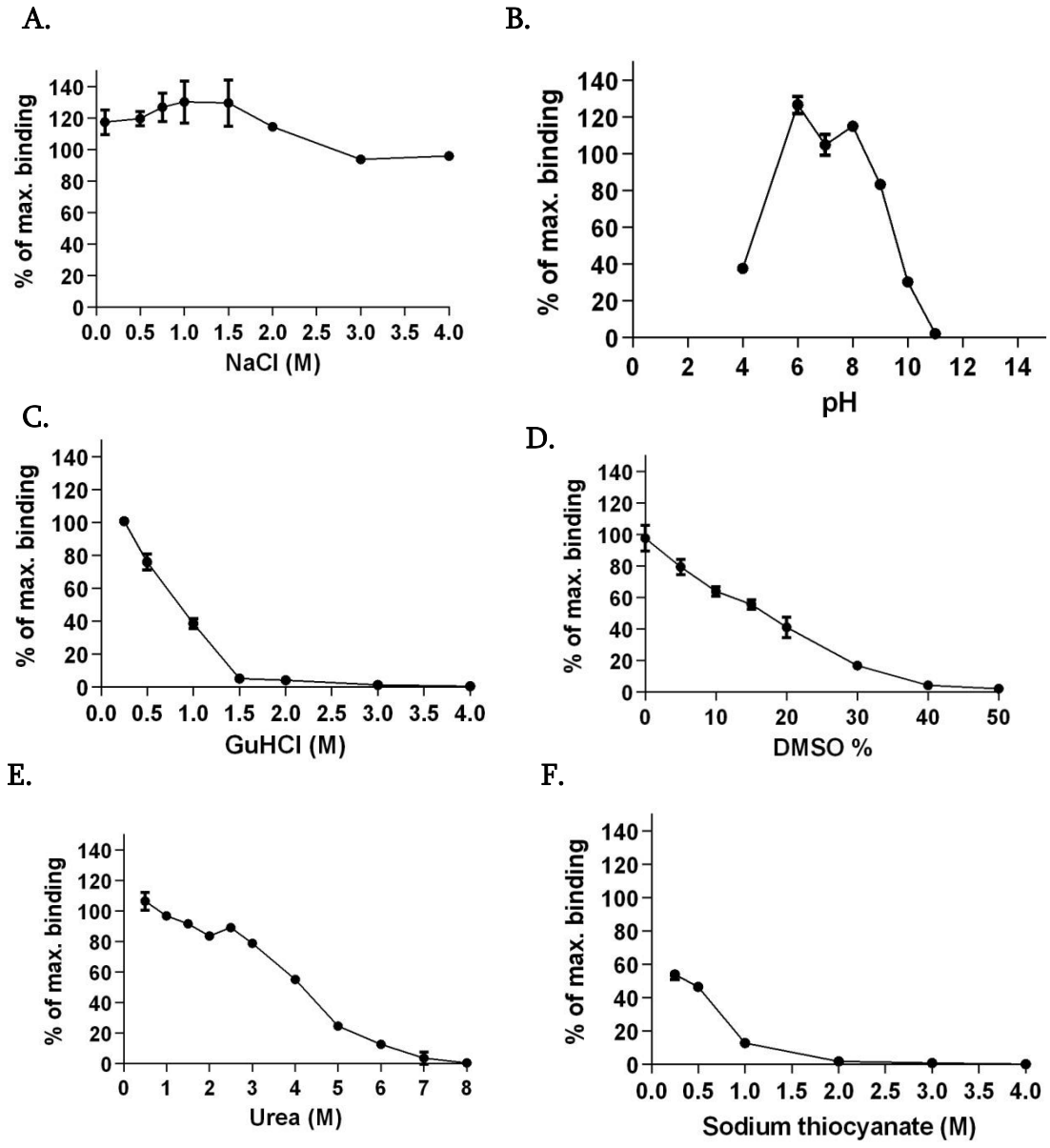


Figure 14: ELISA to investigate stability of binding of Recab to hTNF α at different concentration of NaCL, Urea, DMSO, NaSCN, and different pH on antigen binding

A. ELISA was done in presence of different concentration of NaCl.

B. different pH; **C.** NaSCN; **D.** DMSO; **E.** GuHCl; **F.** Urea

chain of the scFv. Control pCTCON2 vector transformant did not show PCR amplification.

3.9.2 Fluorescence microscopy imaging

Surface displayed Recab binding to biotinylated hTNFα was visualised using fluorescence confocal microscopy. Yeast cells displaying Recab were stained with Alexa-488. In FITC filter (FITC and Alexa 488 share the same excitation and emission wavelengths) yeast cells showed signal on cell surface, which are associated with the cell rims as shown in bright field image, confirming fusion of Aga2p/Recab (**Figure 15Ca**). The biotinylated antigen (hTNFα) was visualized for its binding to the antibody (Recab) using streptavidin phycoerytherin. The fluorescence signals for recab overlapped with biotinylated hTNFα on cell surface, which confirmed that the antigen binded to the antibody (**Figure 15Cb**). Control yeast cells EBY100, did not show any fluorescent signal, similarly cells which were transformed with vector (pCTCON2) only, did not show phycoerytherin signal, confirming the expression, specificity and binding of the Recab to hTNFα.

3.10 Generation of mutant antibody library in yeast

Error-Prone PCR method was used to generate random mutation in the Recab gene.

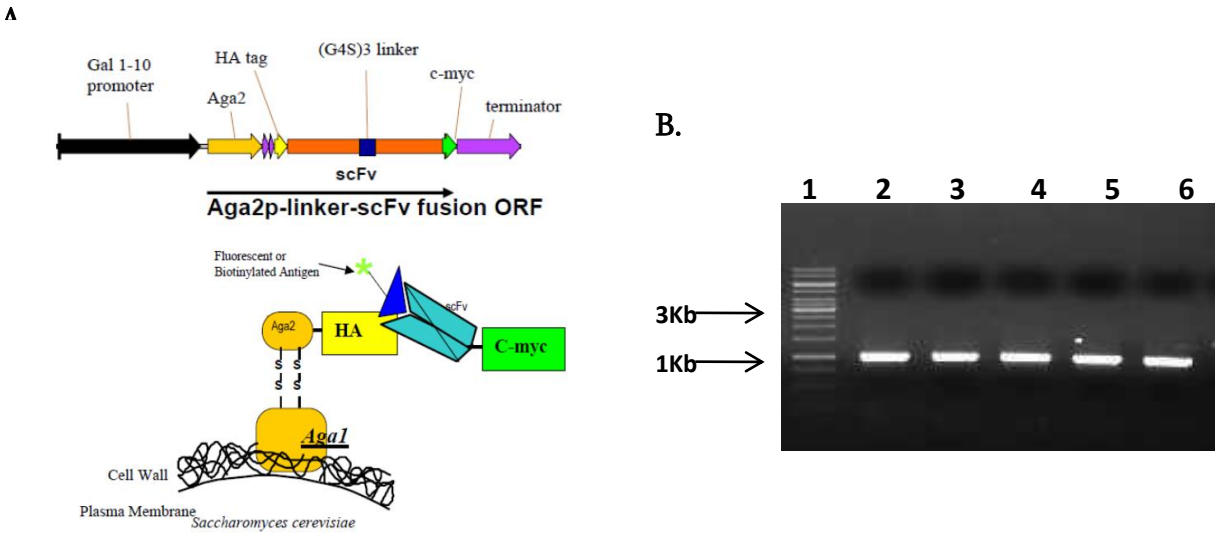


Figure 15: Affinity maturation for Recab using Yeast Display System

A. Schematic representation of construction of yeast surface display yeast strain. Recab was transnationally fused to the Aga2 protein at N-terminus followed by c-myc at C terminus. (Figure adapted from Yeast display scfv antibody library user's manual Pacific Northwest National Laboratory).

B. Colony PCR of recombinant yeast. Lane 1-DNA Marker, Lane2-6- Recab clones. A band of approximately 950 bp was obtained as shown near to 1 kb DNA marker.

C. Confirmation of expressed scFv on yeast surface by florescent confocal microscopy. Bar-5µm. Recab transformed yeast stained on its surface and gave signal for both Alexa 488 and SAPE. Whereas control EBY100 did not stained for both and pCTCON2 vector transformant stained only for Alexa488. a) EBY 100 control cell. b) pCTCON2 transformed yeast cell. c) Recab transformed yeast cells stained for both Alexa 488 and SAPE.

D. Colony PCR representative of mutated yeast library clones. Lane 1-5 and 8 – Recab mutated clones, Lane6 –DNA Marker, Lane 7- EBY100 control.

In this method two nucleotide analogues, 8-oxo-20-deoxyguanosine-50-triphosphate and 20-deoxy-pnucleoside-50-triphosphate (8-oxo-dGTP and dPTP, respectively), were used which create both transition and transversion mutations.

Nucleotide analogue mutagenesis allows the frequency of mutation to be tuned based on the number of PCR cycles and the relative concentration of the mutagenic nucleotide analogues used during PCR. The PCR conditions were used to give error rate of 0.5 -2 %. Yeast display library was made by following method of Chao G. et al., (2006). The method allowed direct cloning of the gene into *S. cerevisiae* by utilising gap repair method of the cell. Yeast cells were transformed and transformants were selected on SACAA agar plate. Serial dilutions of the transformed yeast were plated to calculate the library strength. A mutated library of 3 x 10^6 transformants was generated. Randomly representative clones were screened for insertion using colony PCR (**Figure 15D**).

3.11 Identification of clones with improved affinity

Transformants grown on SDCAA medium were picked and grown in SDCAA medium in sterile 96 well tissue culture microtiter plates. After overnight growth, culture was induced by shifting to SGCAA medium. Cells were induced for 20h. Biotinylated hTNFα was added at a concentration of 100nM to the induced cells. Cells were stained for c-myc tagged Recab and biotinylated antigen (TNFα). Alexa 488 was used to detect c-myc fusion to the Recab and streptavidin phycoerytherin for biotinylated hTNFα. Signals were read on Synergy HT Microplate reader (BioTek Instruments, Inc, Winooski, VT, USA). For

alexa-488, excitation was done at 485/20 nm and emission was read at 530/20 nm. Similarly for SAPE, excitation was done at 485/20 nm and emission was read at 590/20 nm. Values of 'SAPE signal / alexa 488 signal' were calculated and plotted on a graph. Clones which showed better signal compared to the parent clone (**Figure 16**) were further analysed using FACS. As shown in **Figure 16** most of the clones showed signal values comparable to that of the control (0.23), only two clones showed significantly better values compared to the parent one (0.43 & 0.53). These clones were selected for further analysis.

3.12 FACS analysis

Yeast cells carrying different scFv displaying plasmids were double-labeled with Alexa 488 and streptomycin phycoerytherin and were subjected for dual color flow cytometric scanning analysis using BD FACS ARIA III flow cytometer. Cells labelled with Alexa-488 were located in Q4. Yeast cells carrying pCONC2 scFv displaying plasmids had more than 60% of their populations located in the Q2 region, indicating that these scFvs were expressed and displayed on yeast surface and bound to hTNFα at antigen concentration of1200 nM. For the control, unstained Q2 region indicated no signals on Alexa-488 and SAPE channels. (**Figure 17**). Expression level of the recab on yeast surface varied with the florescence means (FM) ranging from 126 to 640, while the control cells had no detectable florescence signals (FM=0). Antibody clone 57 was isolated as a high affinity binder. FACS scanning of 57 clone showed 88.3 % of its population was positive for scFv expression and hTNF α binding, similarly for B3, it was 64.5 % whereas parent C2 the

value was 41.5 % confirmed that these clones were specific and improved Recab clones (**Figure 17**).

3.13 Determination of equilibrium dissociation constant

Yeast displaying scFv were incubated with varying amounts of hTNFα, from 1 to 100 nM. Data for ~10 000 events were collected. Binding to hTNFα was visualized during flow cytometry analysis using biotinylated hTNFα and SAPE. The median fluorescence intensity (MFI) of the population of cells was recorded (**Figure 18**). MFI waas plotted against the concentration of antigen and a nonlinear least square was used to fit the curve using Graphpad prism and K_D value was determined using equation: y=m1+m2*m0/(m3+m0)

The binding constant for the improved scFv clone 57 was calculated as 11nM and for B3 as 222 nM compared to the parent C2 430nM.

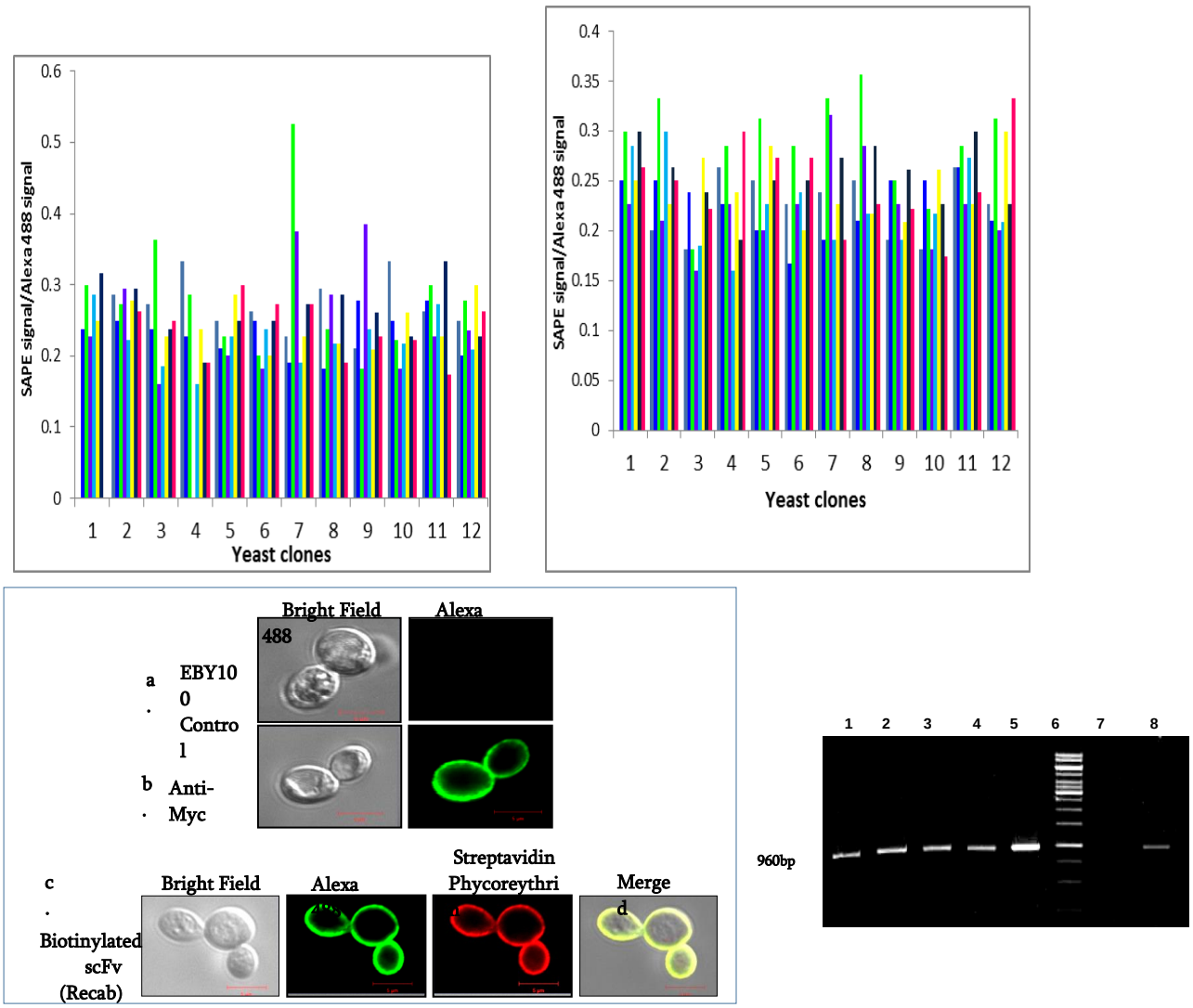
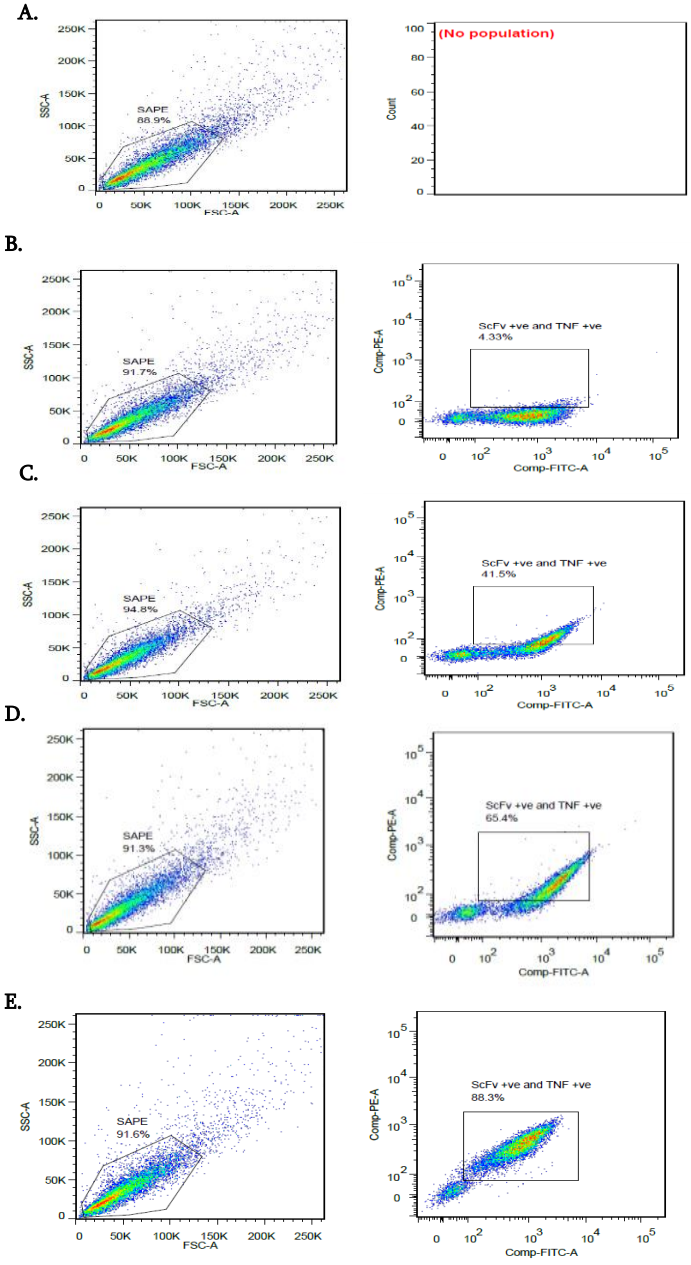


Figure 16 : Screening of mutated Recab clones

Transformants from mutant library were grown and induced in 96 well microtiter plate and stained with alexa 488 and SAPE for presence of Recab and binding of biotinylated hTNFα respectively. Signals were read and ratio of signal obtained with SAPE to Alexa 488 was plotted on the graph. Well 1a. contains unstained cells; 1b, containes only Alexa488 stained; well 1c, only SAPE and well 1d have control C2 transformed EBY100. Rest of the wells were having putative mutant.

A. Microtitre Plate 1 **B.** Microtitre Plate 2


A.
SAPE
88.9%
(No population)
B.
SAPE
91.7%
ScFv +ve and TNF +ve
4.33%
C.
SAPE
94.8%
ScFv +ve and TNF +ve
41.5%
D.
SAPE
91.3%
ScFv +ve and TNF +ve
65.4%
E.
SAPE
91.6%
ScFv +ve and TNF +ve
88.3%
SSC-A
FSC-A
Comp-PE-A
Comp-FITC-A
Count

F.

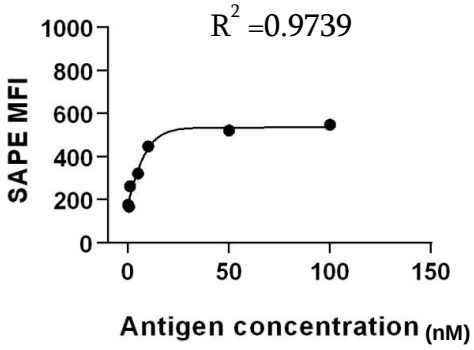

R^2 =0.9739
SAPE MFI
Antigen concentration (nM)
0
200
400
600
800
1000
0
50
100
150

Figure 17: Bivariate flowcytometric analysis

A. Representative flow cytometry data.

a. Unlabelled yeast cells.

b. Yeast cells labelled with chicken anti-c-Myc IgY followed by Alexa Fluor 488–conjugated goat anti-chicken (Alexa Fluor 488 control), compensated to reject crosstalk between the Alexa Fluor 488 and phycoerythrin channels.

c. C2, parent clone

d. B3 clone of Recab mutant library

e. 57 clone of Recab mutant library

B. K_D determination using flow cytometry and nonlinear least squares fit

To estimate the affinity of an scFv displayed on yeast the concentration of antigen in solution was titrated and the median fluorescence intensity (MFI) of antigen binding of only the scFv positive cells (SAPE) was plotted against the antigen concentration to obtain the estimated equilibrium binding constant (K_D).

K_D determination using flow cytometry and nonlinear least square fit

3.14 Multiple sequence alignment of Recab clones

The amino acid sequences of each of the selected recabs were determined to investigate similarities and differences in the fragments. Plasmids DNA from each clone was extracted and sequenced. The DNA sequences received were subsequently translated into their amino acid sequence using ExPASy translation tool. The amino acid sequences were aligned and the complementarity determining regions (CDR) of the antibody were evaluated using MEGA 6 and GeneDoc software. The diversity between the clones was evident from the sequence variation between the heavy and light chains highlighted in **Figure 18A.** The CDR H2, H3, L2 and L3 had variability in amino acid sequence whereas CDR H1 and L1 were highly conserved between the analysed fragments. Phylogenetic trees were created based on the alignment analysis conducted to the sequencing data of the heavy and light chains of the recabs (**Figure 18B**).

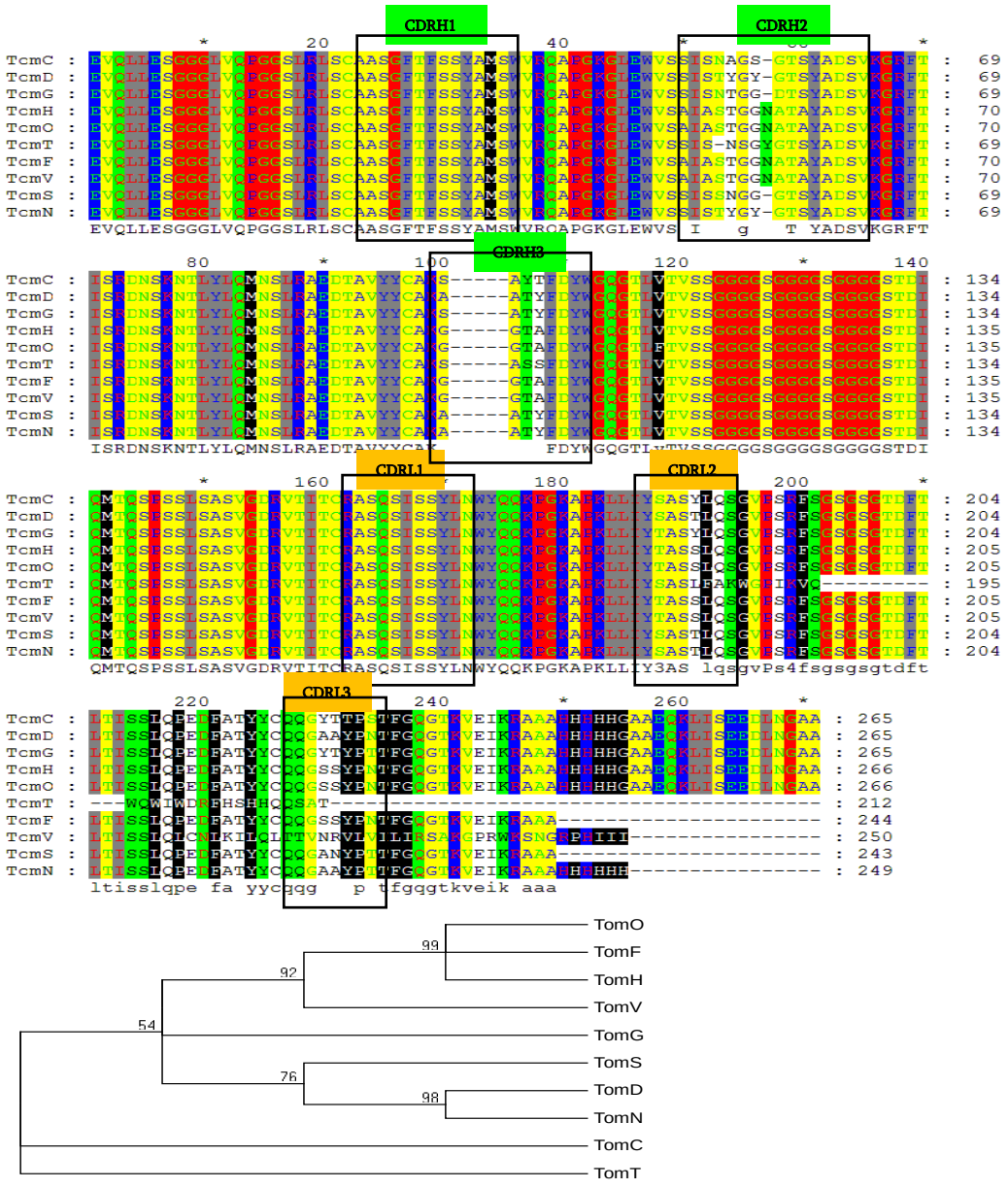


Figure 18: Multiple sequence alignment of anti-TNF clones

A. TOMC is parent clone (Recab). The CDR H2, H3, L2 and L3 had difference in amino acid composition whereas CDR H1 and L1 were highly conserved between the analysed fragments.

B. Phylogenetic tree was created Neighbour-Joining Method. The bootstrap numbers were indicated on clads

Discussion

TNFα is a homotrimeric, most important pro-inflammatory cytokines, produced by immune cells. It is present both in membrane bound form as well as in soluble form as homotrimeric. It plays an important role in induction of inflammation by immune cells and also significant in the development of lymphoid tissues, pathogenesis of immune disorders and tumor development. Increased level of TNF was reported at inflamed site in autoimmune disease. Neutralization of TNFα leads to the reduction of inflammatory symptoms. There are two type of anti TNFα drugs, monoclonal antibody and a TNFα receptor fused to Fc region of the immunoglobulin. The drug Certolizumab is a Fab fragment of a humanized TNF-α and clinically found very efficacious. However associated adverse effect and unresponsiveness of the patients open up new arena for the development of novel drugs. Phage display system has emerged a new and promising platform for the isolation and identification of monoclonal antibodies with desirable properties. To isolate a specific antibody fragment against human TNF α, Tomlinson I+J libraries were used. These libraries are very diverse; contain human antibody fragments in form of single chain variable fragment (scFv). TNF α was coated to the solid support (immunotube) and phages were isolated. These antibody phage display libraries contain more than 10^8 clones, enabling the isolation of high affinity

binders. Krebber *et al.* (1997) reported 3 rounds of selection were sufficient for the isolation of specific murine scFv generated library. As the libraries were very diverse with more than 10^8 clones, three rounds of selection were employed for the isolation of anti hTNFα clones. More antigen concentration may result in loss of high affinity binders, so antigen concentrations were reduced and stringency, but the efficiency was assured by increasing the frequency of washes in subsequent round of selections. Enhanced stringency levels are significant to ensure the selection of positive clones and also to eliminate clones having non-specific target towards the antigen. After each round of selection, infected *E. coli* TG1 cells were spread on ampicillin containing TYE plate to ensure that every clone gets an opportunity to get selected, as certain clones may grow more slowly than other clones due to the antibody toxicity within the cell. Therefore, this method increases chances of selecting high affinity clones and also allows enrichment of recombinant scFv phage against immobilized hTNFα.

After 3 rounds of selection on hTNFα coated immunotubes, polyclonal phage ELISA revealed the presence and enrichment of positively binding anti hTNF α phage-scFv clones. Augmented ELISA signal level and fold enrichment of eluted phage in subsequent rounds indicated that the isolated phages are enriched for hTNFα.

2nd and 3rd round isolated phages were transformed to non-suppressor HB2151 *E.coli* strain for soluble expression of the scFv protein. 94 representative clones from each round (total of 376 clones) were picked and analysed for the anti hTNF α binding property. Isolated pool of phage contains clones with different affinity and specificity, so yeast and plant proteins were used to eliminate the bispecific phages, or phages which were binding non-specifically. Yeast and plant proteins were selected to ensure that there should be no loss of the positive clone as these are non-animal sources. Result revealed that only 30 % of the isolated phage was specifically binding to only TNFα rest of them were binding to other proteins also. The term avidity (or functional affinity) denotes the net antigen binding force of an antibody. Binding strength of the selected clones to their specific Ag (hTNFα) was tested using an avidity ELISA. Avidity assay involves a protein-denaturing agent which is either included with the antibody, to prevent formation of the antigen-antibody complex, or in the post washing fluid to dissociate the antigen-antibody association. Urea was used as denaturing agent in the experiment. The binding of scFvs with less avidity to the antigen is disrupted at lower concentrations of urea than that of antibodies with greater avidity to the antigen. Avidity index were calculated and result showed that 16% of the clones were showed an index above 50%. Urea being a denaturing agent disrupts the association between antigen and antibody. The scFv are widely considered to be

relatively unstable and their folding free energy is indeed (Tan et al., 1998). A mechanistic study of scFv fragment folding was carried out and result showed that the folding intermediate vary in prolyl isomerization (Freund et al., 1997).

Heavy or light chain fragment individually can bind to the antigen. In order to confirm the presence of these fragments in the selected clones, PCR was carried out. Amplified PCR products confirmed the presence of both heavy and light chain fragments in the selected clones.

Large scale expression and purification of the Recab protein was carried out in order to characterize it further. V_{H3} domain of the antibody has affinity towards the *Staphylococcal* protein A (Tomlinson et al., 1992), so protein A conjugated resin were used to purify Recab protein. Both periplasmic extract and the culture supernatant were having Recab protein which was confirmed by ELISA, SDS PAGE and western blot hybridization. Yield of the protein was estimated at 39.52 mg/ L. The expression of scFv at the periplasmic space reported by previous researchers are ~ 10mg/L (Tiwari et al., 2010), 2-6 mg/L (Raffai et al.,1999) and 1.7–2.9 mg/L (Ossysek, K. et al., 2015) which indicated that protein yield from present study is in parity or more than the reported works.

Conformational stability of the scFv protein is always remained a matter of concern. scFv protein is highly susceptible to denaturation as well dimerisation on

prolonged incubation. Parameters used to asses above alterations are, varying concentrations of DMSO, Urea, NaSCN, GuHCl, NaCl and different pH used for the assay. The observations from denaturation and dimerisation studies confirmed the stability of Recab to different conditions studied.

The L929 mouse aneuploidy fibrosarcoma cell line is commonly used to assay the cytotoxic effects of TNFα, which causes apoptosis in cells sensitized by pre-treatment with actinomycin D. The MTT colorimetric assay was used to analyse the effect. hTNFα neutralizing ability of Recab was assayed on these cells. Two clones, C2 and G2, were able to efficiently neutralize the toxic effect of TNFα on cells. TNFα is a trimeric molecule, to inhibit the association of TNFα to its receptor; antibody should bind to the region where the ligand receptor binding takes place. The result indicated for rest of the clones that binding was happening to other epitopes which were not important for the ligand receptor binding. Similarly low affinity scFv will not be able to bind TNFα very strongly, thus unable to neutralize the same. Dissociation of low affinity scFv molecules to their epitope takes place at a higher rate compared to its high affinity, hence incapable to neutralize toxic effect of hTNFα to the cell.

Phage display is a very powerful technique for the isolation of antibodies, but lower affinity exhibited by the clones is the major drawback. Most of these

antibody fragments often require affinity optimisation for them to be used in clinical development, as improved binding correlates with improved clinical efficacy. Yeast surface display is a powerful technique for the isolation of high affinity clones. It allow application of all the stringencies which are applied to the phage display system but also allow to impart selection of high affinity clones during the sorting of the cells.

As a step towards affinity improvement, error prone PCR technique was used to mutagenize the antibody fragment and a library was generated with more than 10^6 clones. Proper expression of the antibody was checked using fluorescent microscopy by labelling the scFv with alexa-488. hTNFα was biotinylated and labelled with streptavidin phycoerytherin. Primary screening was done on fluorescence reader and clones showing higher signal were analysed by FACS. The binding of these clones was confirmed on FACS and the clones with higher binding affinity for the hTNFα showed better signal compared to the parent one. A nonlinear least square curve was made by titrating the hTNFα concentrations and K_D value was calculated. We observed that the mutated clones were having high affinity compared to the parent one. Calculated K_D value of mutated clone was recorded as 11 nM which is promising for Recab to be used as a therapeutic protein. K_D value of most of the marketed drugs is reported to be below 10 nM.

scFv is a monovalent binder, converting it into a bivalent form will result in increased in affinity or decrease in K_D.

Multiple sequence alignment of the protein sequence isolated scFv fragments was carried out. CDR H1 and CDR L1 regions are highly conserved, whereas rest of the CDR reagions showed variability.

To conclude, present study was to isolate scFv fragment against hTNFα using phage display technique. Clone obtained neutralised the effect of hTNFα effectively on the cell line. Affinity of the selected clone was enhanced by error-prone PCR mutations resulting in better affinity for hTNFα compared to the parent clone.

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