SUMMARY

PRODUCTION OF PHARMACEUTICALLY IMPORTANT PROTEIN IN NON-CONVENTIONAL YEASTS

In the present study, scFv phage display technology was used to obtain an anti-TNFα scFv. Two phage display libraries, Tomlinson I and Tomlinson J were used for isolating anti-TNFα scFv. Human TNFα (hTNFα) protein was coated on immunotubes and 10^{12} phages from each library were used for panning. Three rounds of selections were carried out for isolation and enrichment of anti-TNFα scFv. At the end of the third round 1.6 x 10^{10} and 1.35 x 10^{9} titer was obtained from Tomlinson I and Tomlinson J respectively.

Isolated phages from both the libraries were used for polyclonal phage ELISA. The ELISA signal indicated enrichment of the specific binders. For soluble expression of the antibody fragment, isolated phages were transformed in HB2151 *E. coli* strain. SDS-PAGE analysis of periplasmic extract and medium supernatant of induced culture confirmed presence of anti-TNFα as the protein band corresponding to 28 kDa which was further confirmed by western blot analysis. Binding of the scFv clones to TNFα was also checked by western blot analysis.

Specificity of the isolated anti-TNFα clones was checked on yeast and plant proteins by ELISA. 112 (30 %) clones did not show any signal on ELISA so these clones were further checked on hTNFα coated microtiter plate where they were

ELISA positive. These selected anti-TNFα clones were further screened based on the avidity in presence of urea. The avidity index in presence of 4M urea was determined and clones which showed index comparable to control (PBS) were further selected on 6M urea. In presence of 6M urea, 18 clones showed avidity index greater than 50%. DNA from the selected clones was extracted and PCR was performed to check presence of Heavy and light chain fragments. All clones showed expected band pattern on agarose gel.

The protein from the selected clones was isolated and used for the functional cytotoxicity assay on L929 cell line. Commercially available anti-TNFα antibody, Humira™, was used as positive control. Two clones, C2 and G2, showed neutralization of hTNFα and rescued the cells from the toxic effect. EC_{50} value of these selected clones was determined and found to be 10.93 μg/ml and 14.23μg/ml for C2 and G2 respectively. Clone C2 was selected for further analysis and herein referred as Recab.

Stability assays for Recab were carried out in presence of Urea, DMSO, GuHCl, NaSCN, NaCl and at different pH. NaCl at different concentrations did not affect binding of Recab to hTNFα whereas it is most affected in presence of NaSCN and GuHCl. Binding was stable between pH 6-9 but at higher and lower pH it was dissociated.

Affinity of the Recab was improved by random mutagenesis using error prone PCR. PCR products along with yeast surface display vector, pCTCON2, were used

to transform yeast. Clones that showed higher affinity than the parent Recab were analysed on FACS.

To determine the K_D values of the clone, different hTNFα concentrations were used to generate a nonlinear least square curve and K_D values were calculated. K_D value of the best binding clone was 11nM. Protein sequences of the selected clones were aligned using MEGA6 and GeneDoc softwares and CDR regions were compared. CDR H2, H3, L2 and L3 were highly variable while CDR H1 and L1 were conserved.

Anti-TNFα (Recab) was expressed in yeast, *Pichia pastoris*. In *P. pastoris*, the native alcohol oxidase (AOX1) promoter was successfully utilised to drive the expression of Recab. Single copy and two copy Recab expression transformants were generated and effect of copy number on Recab expression was analysed. Two *Pichia* strains were used for generation of the transformants, X33 and GS115. Transformants were characterized based on Southern blot, western blot and ELISA. Results showed that there is a negative relation between the increase in copy number and Recab expression levels. Two copy transformant was slow growing and sensitive to higher methanol concentrations. Single copy transformant obtained from X33 strain (CX4) was fast growing compared to the GS115 and the Recab expression levels. Total expression level of CX4 was higher in tested conditions. Temperature affected the expression level, lowering the temperature led to higher expression.

Yeast expressed protein was used for the functional assay on cell line wherein HeLa cells were taken and IL-6 level in the medium were monitored. There was a direct correlation between the hTNFα concentration and IL-6 expression levels. Recab protein successfully neutralised the hTNFα effect on the cells, confirming that it is functionally active.

Recab expression can be optimised for different parameter such as temperature and pH. It has been documented that both these parameter play an important role in expression and stability of recombinant proteins. Shake flask studies for growth and production optimization followed by scale-up, downstream processing can be targeted towards product development and commercialization.