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**Optimization of culture conditions for high-level expression of soluble and active
tumor necrosis factor- α in *E. coli***

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Running title: Expression of recombinant TNF- α in *E. coli*.

ABSTRACT

Anti-TNF inhibitors exert their therapeutic effect by inhibition of the excessive amounts of TNF- α within the body. Recombinant TNF- α should be produced in a soluble refolded form to investigate the effectiveness and efficiency of anti-TNF- α compounds. In this research, the designed cassette was subcloned in the pET28a expression vector and expressed in *E. coli* BL21 (DE3). The identity of the protein was confirmed through SDS-PAGE and Western blotting. After optimizing expression conditions, protein purification was performed using native Ni-NTA affinity chromatography. The biological activity of the soluble recombinant TNF- α was investigated using MTT assay. Also, the affinity of an anti-TNF- α agent, Altebrel, was investigated against the expressed protein through ELISA. Optimization of TNF- α expression conditions represented that the highest expression could be achieved at 37°C using 0.5mM IPTG six hours post-induction. The recombinant protein represented an inhibitory effect on the L929 murine fibroblast cell line and was successfully detected by Altebrel in ELISA. Binding kinetics were also studied using Cimzia as an anti-TNF- α molecule and $7.2 \text{ E}^{-13}\text{M}$ was calculated as the equilibrium dissociation constant value (K_D). The significant expression level of the recombinant protein in the soluble form, its high purity, and assessment of its biological activity showed that the expressed protein could be used in tests of ELISA and MTT to assess the activity of anti-TNF- α agents.

Keywords: Affinity purification, recombinant, tumor necrosis factor- α .

1. Introduction

Tumor necrosis factor alpha (TNF- α) is a pluripotent inflammatory cytokine produced from macrophages, CD4 lymphocytes, neutrophils, mast cells, eosinophils, and natural killer (NK) cells in response to the inflammatory agents, including bacterial toxins [1]. Increased expression of this cytokine has been involved in the development of rheumatoid arthritis (RA), psoriatic arthritis, and Crohn's disease (CD) [2, 3]. Its cytotoxic activity is mediated by binding to specific receptors and induction of proto-oncogenes, such as c-fos [4] and c-myc [5], within fibroblasts. This circulating anti-tumorigenic cytokine is made up of 233 amino acids as a 26kDa transmembrane protein type 2, forming stable active homotrimers [reviewed in 6]. TNF- α immature protein loses a part of the molecule during metalloprotease activity and can be released as a 17kDa mature TNF- α [7] comprising 157 amino acids and a single disulfide-bond (Cys69-Cys101) [8]. The monomeric 17.35kDa protein is active, and a soluble protein without any glycosylation and its isoelectric pH is about 5.8. This fundamental molecule has attracted a great deal of attention during the last decades for its immunological, diagnostic, and medicinal applications. For instance, for detecting TNF- α in blood samples through immunochemical approaches, recombinant TNF- α has been expressed for rabbit immunization to obtain specific monoclonal antibodies [8, 9]. On the other hand, due to its suggested anti-tumor activities in combination with chemotherapy agents [10], several studies have been conducted to express the recombinant TNF- α protein [11, 12]. For neutralizing the excess amount of endogenous TNF- α in patients suffering from RA and CD, several TNF- α inhibitors have been developed and approved by FDA for clinical usage, raising due to the TNF- α double-edged signaling pathway. Biological activity assessment of these anti-TNF- α molecules needs the heterologous recombinant expression of TNF- α as well.

Escherichia coli (*E. coli*) expression system is one of the most widely used systems for heterologous expression of simple human proteins without glycosylation or other post-translational modifications. Preference of *E. coli* host is due to its fast growth, easy genetic manipulation, and simple scale-up for industrial production and purification [13]. This expression system, however, has its specific disadvantages, particularly in posttranslational modifications, codon bias, and, importantly, the formation of inclusion bodies, making protein purification more expensive, complicated, and time-consuming, especially in industrial scale. Besides genetic manipulations, critical strategies, including incubation temperature, post-induction duration, type of medium, and IPTG concentration, are the main parameters studied frequently to explore the best conditions for heterologous protein expression. A good balance between biomass growth conditions and criteria involved in protein production is, therefore, essential to achieve high productivity. In this regard, not only the choice of appropriate vector and promoter system but also the composition of the medium, cultivation temperature, and induction condition is important in the final destination of the desired protein in being correctly folded or aggregated as insoluble cytoplasmic or periplasmic inclusion bodies [13-19].

Here, we report the cloning, optimization of *E. coli* cytoplasmic expression, and native affinity purification of TNF- α , and evaluation of its biological activity using Altebrel as a biosimilar of Etanercept.

2. Methods

2.1. Materials

E. coli BL21 (DE3) and pET28a expression vector (Novagen, USA) were used for expression experiments. Restriction enzymes were purchased from Fermentas (Lithuania). Nickel

Nitrilotriacetic acid (Ni-NTA) agarose resin was purchased from ABT (Spain). CM sensor and other required materials for Surface Plasmon Resonance (SPR) were purchased from GE Healthcare Life Sciences (USA). All media, chemicals, reagents and antibodies required for experiments and buffers were purchased from Sigma Chemicals (USA) unless otherwise stated.

2.2. *TNF- α* gene cloning

The amino acid sequences encoding the active human TNF- α molecules were obtained from available data banks, including drug and NCBI protein data bank. The consensus sequence was selected, and the corresponding nucleotide sequence was codon-optimized for protein expression in *E. coli* expression host cells. Using *NcoI* and *HindIII* restriction enzymes under the T7 promoter, the synthesized gene fragment harboring N-terminal Histidine tag was subcloned in the pET28a expression vector.

2.3. *Expression of TNF- α* protein

Transformed *E. coli* BL21 (DE3) strain was inoculated into 5 ml LB broth medium supplemented with 15 μ g/ml Kanamycin on a shaker incubator at 37°C until the optical density at 600_{nm} reached 0.4. 0.5mM IPTG was used to induce protein expression. The tubes were incubated at 37°C for a further 4 hours. The bacterial pellet was collected by centrifugation at 9,000rpm (3min), resuspended in 2X loading buffer, and ran on 12% SDS-PAGE. The identity of the expressed protein was confirmed using western blotting by anti-His antibody or Altebrel, a TNF- α blocking biosimilar product (Aryogen, Iran). Briefly, protein bands were transferred on nitrocellulose membrane (GE Healthcare, USA) using a semi-dry blotting system (Bio-RAD, USA) under 18 volts for 35min. The membrane was blocked by 3% (w/v) skim milk in phosphate buffer saline (PBS) overnight at 4°C. Horseradish peroxidase (HRP)-conjugated anti-

His antibody was added to the membrane in a 1:1000 dilution and incubated for 2h at 30°C. After the washing step (5 times with 0.05% Tween 20 in PBS, one time with PBS alone), DAB (3, 3'-Diaminobenzidine) solution, HRP substrate, was added to the membrane until the desired bands appeared. In a further experiment, Altbrel was used as the first antibody (2mg/ml), and after 1 h incubation at 30°C and the washing step (5 times), 1:10,000 dilution of HRP-conjugated rabbit anti-human IgG (Invitrogen, USA) was added to the transferred membrane and incubated for 1h at 30°C. After the washing step (5 times with 0.05% Tween 20 in PBS, one time with PBS alone), DAB was added to the membrane until the bands appeared.

2.4. Optimization of protein expression

Two broth media (Luria Broth; LB and Terrific Broth; TB), different Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentrations (0.25, 0.5, 0.75 and 1mM) under various temperatures (25, 30 and 37°C), and different post-induction incubation times (2, 4, 6 and 24h) were examined in the optimization of TNF- α expression. Recombinant *E. coli* strain was inoculated into 50ml culture medium. After reaching to the appropriate optical density (OD) at 600nm, the culture medium was aliquoted in sterile tubes and subjected to different conditions mentioned above. At the end of each experiment, bacterial pellet was harvested, and the expression of recombinant TNF- α protein (~17kDa) was determined on 12% SDS-PAGE after staining with Coomassie Brilliant Blue G-250 dye. The ImageJ analyzer (National Institute of Health, USA) was used to measure the intensity of the expressed protein.

2.5. Solubility of the recombinant protein

Large scale protein expression (500ml) was performed under the optimized conditions. In the lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole; pH8.0), the bacterial pellet was

resuspended and sonicated by 9 pulses (30sec sonication with the same intervals at 25% amplitude). The suspension was centrifuged at 10,000 rpm (10min) at 4°C. The presence of the recombinant protein and its intensity was investigated separately in the pellet and supernatant of the sonicated bacteria through running on 12% SDS-PAGE.

2.6. Purification of His-tagged TNF- α under native conditions

The supernatant of sonicated bacteria was loaded on the Ni-NTA column after equilibration with the binding buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM Imidazole; pH8.0) in the FPLC purification system (Bio-RAD, USA) (under 1ml/min flow rate). The column was washed with the buffer I (50mM NaH₂PO₄, 300mM NaCl, 20mM Imidazole, 0.1% Triton-X114; pH8.0) (20 volumes of the column) to remove the residual endotoxin content. The next wash was done with the same washing buffer for removing Triton-X114 from the column (50 volumes of the column). His-tagged TNF- α molecules were eluted from the column using the elution buffer (50mM NaH₂PO₄, 300mM NaCl, 250mM Imidazole; pH8.0) under 1ml/min flow rate. The eluted soluble recombinant protein sample was dialyzed to remove imidazole. The final protein was equilibrated in 20mM phosphate buffer containing 100mM NaCl (pH7.2). Nanodrop was used to measure protein concentration at 280nm. Protein samples were stored at -70°C for further studies.

2.7. Binding activity of r-TNF- α through indirect ELISA

To investigate the affinity of anti-TNF- α molecules against the purified recombinant protein, the purified protein was coated in 96-well ELISA plates in a final 200 ng/ml concentration at 4°C overnight. After 3 times washing with PBS (pH7.2), the wells were blocked using the blocking buffer (PBS with 1% bovine serum albumin) for 2h at 30°C. Altebrel was used as the first

antibody (anti-TNF- α molecule) in a serial dilution for 1h at 30°C. After washing with PBS buffer (supplemented with 0.05% Tween 20), HRP-conjugated Rabbit anti-human IgG (1:10,000 dilution) was added to each well and incubated for 1h at 30°C. After the second washing, the TMB substrate was added to each well, and the reaction was stopped using 2M H₂SO₄. The optical densities were measured at 450_{nm} using an ELISA reader (BIOHIT, Finland).

2.8. Assessment of r-TNF- α binding activity by SPR

To evaluate the binding activity of the home-made recombinant TNF- α to Cimzia (anti-TNF- α inhibitor, Certolizumabpegol) (UCB, Belgium), surface plasmon resonance (SPR) experiments were accomplished by Biacore X100 (GE Healthcare) at 25°C using HBS-EP running buffer (10mM HEPES; pH7.4, 150mM NaCl, 3mM EDTA, 0.005% v/v p20 detergent; pH7.4) on CM5 sensor chip coated with Fab binder (Biacore Fab capture kit, GE Healthcare) and its corresponding amine coupling kit. Briefly, Cimzia was injected in an appropriate concentration at a flow rate of 30 μ l/min for 180sec with a surface density of about 300 resonance units (RU). The home-made recombinant TNF- α was injected at different concentrations (0.5, 16.6, 50.166, 150 and 450nM) in HBS-EP buffer at a flow rate of 30 μ l/min for 180sec. In order to remove all bound proteins, chip regeneration was performed with the recommended regeneration buffer (10mM glycine-HCl at pH2.1).

2.9. Biological activity of r-TNF- α (IC₅₀ determination of r-TNF- α)

The biological activity of r-TNF- α was assessed using the MTT assay. The L929 cell line (murine fibroblast cells obtained from Department of Cell Bank, Pasteur Institute of Iran) was seeded at a density of 20,000 cells/well in 96-well plate and incubated for 24h at 37°C under 5% CO₂. The medium was removed, and different concentrations of r-TNF- α and actinomycin-D

(1µg/ml) were added. Before adding MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), the plates were incubated at 37°C for further 24h. After 4h incubation, 1% SDS (Sodium dodecyl sulfate) was added to the wells, and the plates were incubated at 37°C overnight. The optical density was measured at 600_{nm}, and the IC50 value was calculated.

Further MTT test was performed to study the inhibitory effect of anti-TNF-α molecules, such as Altebrel, on the expressed recombinant TNF-α. Briefly, the initial 50ng/ml concentration of Altebrel was mixed with serially diluted TNF-α and incubated for 2h at 37°C. The mixture was added to the adherent L929 cells (20,000 cells/well) and incubated at 37°C (24h). The solution was removed, and the MTT substrate was added to the wells. After adding 1% SDS and incubating overnight at 37°C, the optical density was read at 600_{nm}. Then, the anti-TNFα inhibitory effect of Altebrel was calculated.

2.10. Statistical analysis

SPSS software (SPSS Inc., v. 17.0) was used to perform statistical analyses. Protein expression levels were expressed as mean ± SE, and differences were determined by nonparametric Mann Whitney U and two-sided Student's t-tests. P-values below 0.05 were considered statistically significant.

3. Results

3.1. Design of the contrast

The designed construct was synthesized by Biomatik Company (Canada) and subcloned into pET28a expression vector (Fig. 1). The designed vector was confirmed via restriction digestion

analysis using *NcoI* and *HindIII*, representing 506 and 5246bp fragments on 1% agarose gel (data not shown).

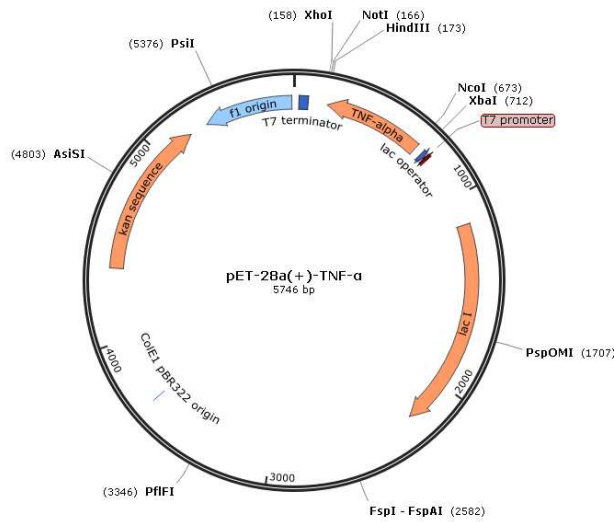


Fig. 1. Schematic design of the expression vector.

3.2. Expression of the recombinant protein

An approximately 17kDa protein was observed when the expression of the protein was induced in the LB medium using 0.5mM IPTG at 37°C (Fig. 2). The expressed recombinant protein (~17kDa) was detected by immunoblotting using an anti-Histidine monoclonal antibody and anti-TNF- α Altebrel (Fig. 3A and B).

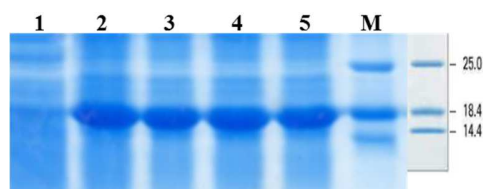


Fig. 2. Protein expression (12% SDS-PAGE). Lanes #1: Recombinant *E. coli* before induction (BI) sample; #2-5: Recombinant *E. coli* after induction (AI) samples; M: protein Molecular Weight (Mw) marker.

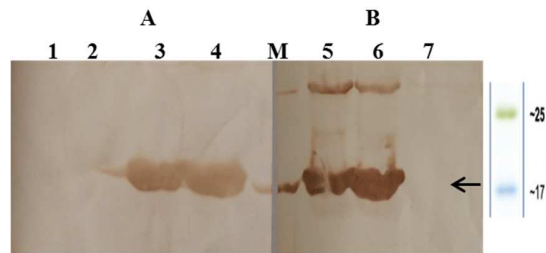


Fig. 3. Immunoblotting against r-TNF- α : A) anti-histidine antibody; B) Altebrel. Lanes 1, 7: *E. coli* BL21 DE3 harboring pET28a (Negative control); 2: Recombinant *E. coli* BL21 DE3 (before induction); 3, 5: Recombinant *E. coli* BL21 DE3 (clone#1, 4 h after induction), 4, 6: Recombinant *E. coli* BL21 DE3 (clone#2, 4h after induction); M: Protein Mw marker.

3.3. Optimization of TNF- α expression

Optimization of protein expression was performed under different conditions. Initial screening showed the highest expression of r-protein in LB medium at 37°C in 0.5mM IPTG 6h post-induction (approximately 56.6% of *E. coli* total protein), which was significantly different from other tested inducer concentrations (Fig. 4A). In contrast, within TB medium at the same incubation temperature, the highest expression was observed in 0.75mM IPTG 4h post-induction (58.2% of total host protein) (Fig. 4B). Further optimization was performed at different temperatures in several post-induction time points. It was observed that the highest expression level of recombinant protein in the LB broth medium was achieved at 30 or 37°C 6h post-induction (Fig. 4C). In TB medium, although the highest expression was achieved under 37°C

and 4 to 6h post-induction (Fig. 4D), its overall protein expression level was lower than the LB medium.

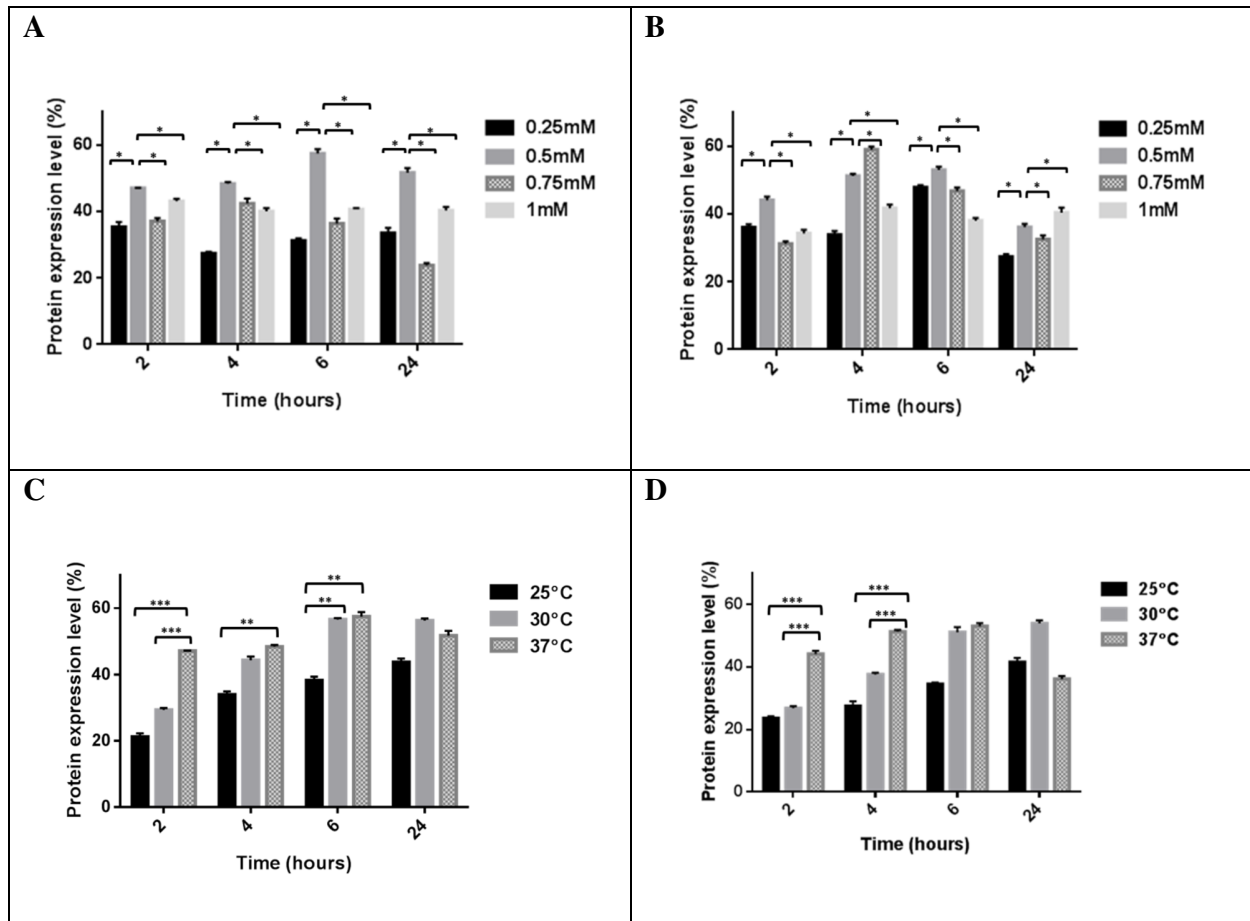


Fig. 4. Optimization of protein expression. A, B: LB and TB media at 37°C; C, D: LB and TB media using 0.5mM IPTG. Density of expressed protein bands was measured using Quantity One software. Bars represent the calculated percentage of protein expression level. Error bars indicate standard deviation of each triplicate. *** represents P values < 0.001 and ** represents P values < 0.05 . All corresponding SDS-PAGE figures have been provided in Figure S1(A-H).

3.4. Protein purification procedure

Assessment of protein solubility showed that recombinant TNF- α had been expressed in both soluble and inclusion body (IB) formats (data not shown), where the soluble form was used in the protein purification process. Protein expression was performed under optimum conditions. A soluble fraction of bacterial lysate was injected into Ni-NTA affinity chromatography. The eluted samples were dialyzed against phosphate buffer and analyzed on 12% SDS-PAGE, showing a purity of more than 95% (Fig. 5).

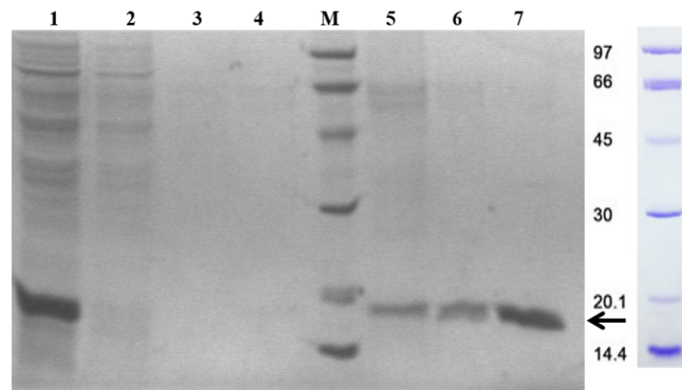


Fig. 5. Native Ni-NTA affinity purification. Lanes 1: Initial sample (IS); 2: Flow through (FT); 3, 4: Washing samples; M: Protein Mw marker; 5-7: Elution samples.

3.5. Measurement of r-TNF- α binding affinity through indirect ELISA

A home-made ELISA test using a constant concentration of purified recombinant TNF- α represented the affinity of Altebrel, an anti-TNF- α molecule, with an EC₅₀ value of 1.31 μ g/ml (Fig. 6).

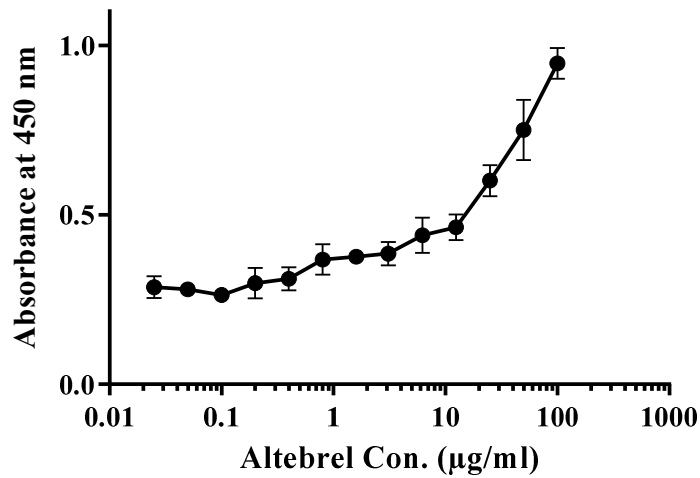


Fig. 6. TNF- α ELISA using altebrel. Serially diluted altebrel was used as the primary antibody. Results are the mean values \pm SEM of triplicates.

3.6. Measurement of r-TNF binding affinity through SPR

Biacore sensorgram was obtained using surface plasmon resonance and binding affinity of Cimzia as a TNF- α inhibitor to the developed r-TNF- α was shown (Fig. 7). The result revealed that home-made recombinant TNF- α was bound to Cimzia like commercial TNF- α proteins previously used for binding assessment of Cimzia antibody. In the present study, the calculated K_D value of this antibody molecule (Cimzia) towards the recombinant TNF- α was $7.281 \text{ E}^{-13}\text{M}$. This result confirmed that Histidine tag does not interfere with the binding kinetic of the TNF- α .

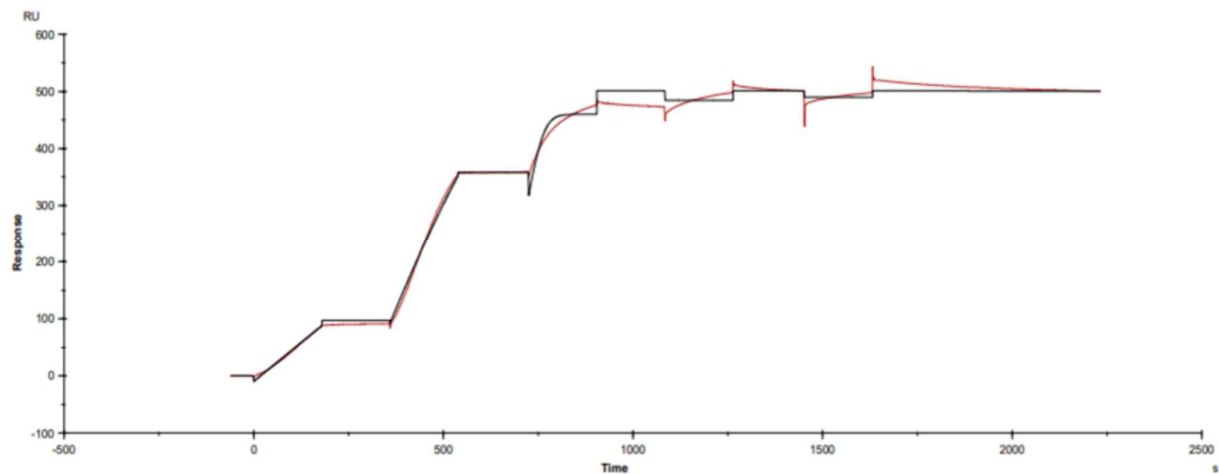


Fig. 7. Binding sensogram of Cimzia and r-TNF- α

3.7. In vitro neutralization of r-TNF- α cytotoxicity

The cytotoxic effect of TNF- α on the mouse fibroblast cell line L929 is due to the apoptosis induction following its attachment to the surface receptors. This apoptotic effect can be suppressed by anti-TNF- α molecules as Altebrel inhibiting TNF- α attachment to the corresponding receptors. In the present study, TNF- α cytotoxicity and its neutralization by Altebrel were investigated. Although actinomycin-D alone decreased the viability of the treated cells by 58%, recombinant TNF- α represented significantly increased cytotoxicity (up to 75%). Altebrel significantly decreased the TNF- α induced cytotoxicity in higher concentrations (in the range of 5 to 0.3125ng/ml) (Fig. 8).

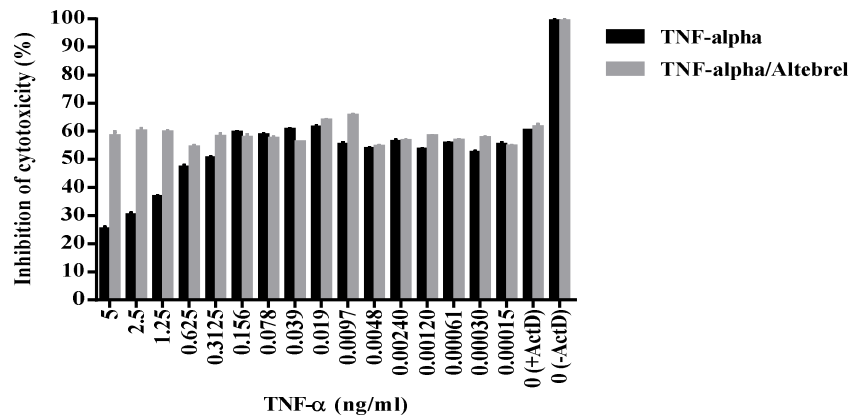


Fig. 8. *In vitro* neutralization of TNF- α in L929 cell-based assay. Neutralization of serially diluted TNF- α -induced cytotoxicity in the presence of actinomycin D using Altebrel. Results are the mean values \pm SEM of three replicates.

4. Discussion

Although the expression of TNF- α as inclusion bodies has been repeatedly reported in *E. coli* under different inducible promoters (T7 or tryptophan), its refolding strategy needed large volumes of buffers in which yield of the final protein could be dramatically reduced [8, 9]. Due to the difficulties industrial biotechnologists face in the commercialization of the proteins in the form of insoluble inclusion bodies, monitoring the effective extrinsic factors, including incubation temperature, IPTG concentration, and post-induction time, have been studied in details to achieve the most soluble format of recombinant proteins from *E. coli* host cells. Several studies have reported increased solubility of the examined proteins by decreasing the temperature [20-23]. On the other hand, the IPTG potential toxicity as the main inducer, besides its negative effect on host metabolism, growth, and viability, has been repeatedly reported [24-

27]. This chemical inducer is often consumed at 0.5-1.0mM final concentrations [24, 28, 29] that should be optimized in the case of each experiment. Thus, finding the best and the least IPTG concentration has been assumed as a key factor in preventing cytotoxicity within *E. coli* cells. In order to minimize this effect, *E. coli* host cells such as BL21 (DE3) or similar strains with the DE3 lysogen have commercially been developed and attracted too much attention in making a balance between the bacterial metabolic pathways [30, 31]. Zhang *et al.*, developed a tag-free form of TNF- α purified in a two-step purification protocol, representing a recovery of over 75% in 24h [11]. In an additional study, Alizadeh *et al.*, expressed a GST-tagged TNF- α fusion protein in *E. coli* host cells for achieving the higher yield of correctly folded recombinant protein [12]. Although the authors suggested the methodology as an effective and cost-benefit approach, using the GST tag requires a tag removal step that makes the procedure time-consuming and more expensive for large scale industrial production. Alternatively, affinity purification of the recombinant proteins using the fused poly-histidine tag can be performed under either native or denaturing conditions. The use of mild buffer conditions and imidazole as the eluent for the soluble cytoplasmic proteins, besides secreted proteins, often yields biologically active proteins. However, native purification conditions are not applicable in the case of insoluble inclusion bodies, and multi-subunit proteins possess a complicated tertiary structure in which poly-histidine affinity tag may be skinned. In such cases, proteins can be purified using the denaturing reagents such as 6M guanidine hydrochloride or 8M urea in sample preparation and purification buffers. Proteins purified under denaturing conditions should then be refolded into their active form by removing the denaturants [32].

The final goal of the present study was to produce TNF- α in its soluble format. Optimization of TNF- α expression, therefore, was performed, and high yield of the soluble recombinant protein

achieved in TB medium led to successful native affinity chromatography through its N-terminal histidine tag. A higher rate of protein expression in TB medium compared with LB can be explained by the additional source of carbon and energy within the TB medium due to the presence of glycerol. In contrast, LB does not have any additional carbohydrates, relying almost entirely on amino acids as the source of carbon and energy. On the other hand, the potassium phosphate components of the TB medium act as mild buffers in comparison to the NaCl content of LB [33].

The total yield of the r-TNF- α protein was measured as 7.2 mg/lit of bacterial medium, being significantly cost-effective in large scale production of this cytokine in assessing biological activity and affinity of TNF- α inhibitor molecules in *in vitro* and *in vivo* studies. Production of biologically active TNF- α was analyzed by cytotoxicity assessment via MTT assay on TNF- α sensitive L929 cell line. The activity of the produced recombinant TNF- α was neutralized by serial dilutions of the applied TNF- α inhibitors (Certolizumab pegol and Etanercept). Etanercept, a TNF- α inhibitor molecule, is a fusion protein that compromises the extracellular region of human TNFR2 and C-terminal human IgG1 Fc fragment. This protein has been approved for human use by blocking TNF- α induced inflammatory and cytotoxicity [34]. Certolizumab pegol (Cimzia) is another FDA approved anti-TNF- α inhibitor, which is structurally a Fab' molecule conjugated chemically to a PEG moiety [35]. SPR evaluated binding kinetics of the His-tagged TNF- α towards Cimzia re-confirmed that Histidine affinity tag does not have any effect on protein-protein interactions.

5. Conclusion

Although higher expression systems, such as yeast, Chinese hamster ovary (CHO), and Human embryonic kidney 293 (HEK293) cells, have been used in commercial production of TNF- α , the use of *E. coli* expression system takes less time and energy in the production of large protein amounts in which only one disulfide bond and no glycosylation modification have been reported. That is how the optimization of parameters involved in the soluble protein expression could significantly increase the yield of correctly folded TNF- α , which has been approved in biological assessments of the present study.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Y. T. and F. M. were the chief investigators. M.S. was involved in MTT assays. F. T. assisted in binding studies. E.B., F.B. and A.A. assisted in data collection. Sh. D. was responsible for the major experimental work and contributed in writing the main manuscript. Y. T., L. N. and H. J. contributed revising the main manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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