

## Production Design of Efficient Recombinant Human Interleukin-4 (rhIL-4) under Specific Promoter in *Escherichia coli*

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### HIGHLIGHTS

- Interleukin-4 (IL-4) is a cytokine that regulates multiple biological functions.
- The recombinant Human IL-4 was cloned and produced under specific promoter in *Escherichia coli*.
- Through MTT assay, the exposed cells with rhIL-4 were proliferated in a dose dependent manner.

### Keywords:

Human IL-4

Cloning

Prokaryotic system


MTT assay

### ABSTRACT

Recombinant DNA technology plays a vital role in improving health conditions by developing new pharmaceuticals. Recently, some of the cytokines as other recombinant proteins could be produced using the recombinant DNA technology. The role of Recombinant IL-4 in allergy, autoimmunity, and cancer has been investigated. The present study was aimed to clone and produce the Human IL-4 under specific promoter in *Escherichia coli* and assessed its biological functions. The designed *hIL-4* gene construct was artificially synthesized; subsequently, it was sub cloned into the pcDNA3.1 (+) vector in HindIII restriction enzyme site. Recombinant plasmid was transferred and expressed in BL21 cells. The rhIL-4 protein was evaluated by SDS-PAGE and Western blotting. It was purified by Ni-NTA affinity chromatography. The purified protein concentration and also accuracy were determined by ELISA. MTT assay was applied to evaluate the biological activity of rhIL-4 on the erythroleukemic cell line proliferation. The rhIL-4 gene was successfully cloned and transformed into expression *E. coli* cells. As a result, a specific band was observed both on the SDS-PAGE and nitrocellulose membrane after Western blotting. The purified protein concentration was equal to 500 pg/ml. The MTT assay indicated that the exposed cells with rhIL-4 were proliferated in a dose dependent manner. The rhIL-4 gene under specific eukaryotic promoter was successfully cloned in the prokaryotic system and the transcription was carried out by T7 RNA polymerase. Therefore, mass production of IL-4 could be a great help in clinical trials and research studies. Additionally, prokaryotic system used in current work was less costly and less time-consuming.

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## Introduction

Cytokines are a large and heterogeneous group of secreted proteins that are produced by different types of cells (Werwie et al., 2017). About 180 genes in the human genome encode the proteins with structural property of the cytokines (Zhu and Stephens, 2017). Each cytokine is able to act on a variety of cells with several different biological effects (Abbas et al., 2014).

Interleukin-4 (IL-4) is a cytokine that regulates multiple biological functions; including proliferation, differentiation, and apoptosis in several cell types of haematopoietic and non-haematopoietic origin (Paul, 1991; Nelms et al., 1999). This cytokine is a growth and survival factor for lymphocytes. Although it was discovered as a B cell differentiation and stimulatory factor, its role in regulating T cell differentiation is critical during the immune response (Vitetta et al., 1985; Mosmann and Coffman, 1989; Seder et al., 1992; Zubiaga et al., 1992; Illera et al., 1993). This cytokine is a monomeric, approximately 13-18 kDa, Th2 cytokine that shows pleiotropic effects during immune responses. Human IL-4 is a polypeptide chain containing 153 amino acids (P05112 IL4\_HUMAN). It has a molecular mass of 17,492 Da as follows (Chomarat and Banchereau, 1998; Benczik and Gaffen, 2004).

It is a glycosylated polypeptide that contains three intrachain disulfide bridges and adopts a bundled four alpha-helix structure (Redfield et al., 1991). IL-4 exerts its effects through two receptor complexes (Nelms et al., 1999; Muller et al., 2002). The type I receptor, which is expressed on hematopoietic cells, is a heterodimer of the ligand binding IL-4 R alpha and the common gamma chain. The type II receptor on non-hematopoietic cells consists of IL-4 R alpha and IL-13 R alpha 1. The type II receptor also transduces IL-13 mediated signals. IL-4 is primarily expressed by Th2-biased CD4<sup>+</sup> T cells, mast cells, basophils, and eosinophils (Chomarat and Banchereau, 1998; Benczik and Gaffen, 2004). It promotes cell proliferation, survival, and immunoglobulin class switch to IgG4 and IgE in human B cells, acquisition of the Th2 phenotype by naïve CD4<sup>+</sup> T cells, priming and chemotaxis of mast cells, eosinophils, and basophils, and the proliferation and activation of epithelial cells (Paludan, 1998; Grone, 2002; Corthay, 2006; Morales et al., 2010).

IL-4 plays a dominant role in the development of allergic inflammation and asthma (Rosenberg et al., 2007; Morales et al., 2010). In contrast with allergic diseases, IL-4 acts as an anti-inflammatory cytokine and could be considered as a potential tool for the treatment of autoimmune diseases (Feldmann et al., 1996). The role of IL-4 in cancer has also been investigated (Margolin et al., 1994; Benedetti et al., 2000; Shintani et al., 2017).

Regarding the wide range of cytokines' biological

activities the production of recombinant form of these proteins is crucial for both the development of new protein drugs and the structural determination of drug targets. As such, recombinant protein production has a major role in drug development. Bacterial hosts are commonly used for the production of recombinant proteins, accounting for approximately 30% of current biopharmaceuticals on the market. The results of many previous studies indicated that genes of eukaryotic protein could be cloned in *E. coli* expression vectors as a transgenic prokaryote microorganism. *E. coli* is a suitable host for expressing stably folded, globular proteins from prokaryotes and eukaryotes. Also, the doubling time of *E. coli* is about 20 min and the high cell density cultures are easily achieved. Transgenic bacteria can be used for the production of valuable eukaryotic proteins that are hardly acquired (Bettelheim et al., 1991).

The present study was conducted to produce IL-4 cytokine by recombinant DNA technology methods under eukaryotic specific promoter in *Escherichia coli*, an efficient and low cost host, according to the necessity of self-sufficiency in our country and in order to benefit from them in clinical trials and research studies.

## Materials and Methods

### *Preparation of recombinant hIL-4 gene construct*

For design of recombinant hIL-4 gene construct has been used as a specific human promoter of the gene that was achieved from Uniprot data bank (ctcggtttcagcaattttaaatctatatagagatatcttggcagca TTGCATCGTTA). Also, the sequence of hIL-4 with signal peptide fragment was obtained from UniProtKB Databank with Entry no. P05112 and synthesized into pGH plasmid (GeneRay, China).

The IL-4 sequence fragment was sub-cloned into the expression vector to produce recombinant expression plasmid. So, the pGH plasmid was digested with HindIII (Fermentas, Lithuania). The desired fragment was extracted and purified using a DNA gel extraction kit (Qiagen, USA), then, it was cloned into the digested pcDNA3.1(+) vector (Invitrogen, USA) using T4 DNA Ligase (Fermentas, Lithuania).

Recombinant plasmids were confirmed with PCR by universal primers (Table 1). Amplification was carried out in 30 cycles of 30 sec at 94 °C, 40 sec at 50 °C, and 40 sec at 72 °C. The recombinant plasmids was also digested by NheI (Fermentas, Lithuania) and lastly, proved by nucleic acid sequencing.

### *Prediction of transcription factor binding sites by PROMO server*

PROMO is a virtual laboratory for the identification of putative transcription factor binding sites

**Table 1.** Universal Primers sequences.

Name	Sequence (5 to 3)
pcDNA3.1(+) <sup>F</sup>	TAATACGACTCACTAATG
pcDNA3.1(+) <sup>R</sup>	TAGAAGGCACAGTCGAG

(TFBS) in DNA sequences from a species. PROMO is using version 8.3 of TRANSFAC.

TRANSFAC database was used to construct specific binding site weight matrices for TFBS prediction (Messeguer, Escudero et al. 2002).

#### *Expression of recombinant hIL-4 gene*

The *E. coli* strain BL21 was transformed with the recombinant hIL-4 vector for expression of the protein and was cultivated in Luria Bertani agar supplemented by 100 µg/ml of ampicillin. The transformed colony was inoculated in 3 mL LB broth medium containing 100 µg/ml of ampicillin and incubated at 37°C in shaker incubator at 200 rpm, overnight. After 16 hours, the cultured bacteria were inoculated in a 50 mL flask and incubated for 3 hours at 37°C in shaker incubator at 200 rpm.

The culture in the logarithmic phase (at OD<sub>600</sub> = 0.6) was centrifuged at 4000rpm for 15 min and the sediment was resuspended in a lysis buffer (50 mM Tris base, 10% glycerol, 0.1% Triton X-100) (Merck, Germany). Subsequently, the suspensions of bacterial cells were lysed by sonication on ice. Then, the two volumes of acetone (Merck, Germany) were added and kept at -20 °C, overnight and finally, centrifuged at 4000 rpm for 15 min. The pellet was analyzed by 15% SDS PAGE electrophoresis stained with Coomassie brilliant blue and the quantity of the expressed protein was visually estimated by comparing the intensity of the protein bands.

#### *Recombinant hIL-4 protein analysis by western blotting*

Recombinant proteins were electrophoretically transferred to a nitrocellulose membrane (Wathman, UK). TBS buffer including Tris-Buffered Saline supplemented by 3% Bovine Serum Albumin (Sigma, USA) was used for blocking the membrane. The membrane immersed in 1:2000 dilution of ALP (alkaline phosphatase) conjugated anti S-tag monoclonal antibody (Abcam, UK) for 2 hours at room temperature. Subsequently, it was visualized for color after development in NBT/ BCIP substrate solution (Roche, Germany).

#### *Purification and identification of recombinant protein*

The rhIL-4 fused to S-tag peptide was purified from cell lysate by using protein S resin (Novagen, USA) as specified by the manufacturer's instructions. The purified protein was concentrated and desalted using Amicon Ultra-15 units with cut-off 10 kDa (Millipore, Germany). Human IL-4 Quantikine ELISA Kit (R&D systems, USA) was used to measure the concentrations of purified recombinant hIL-4.

#### *Cell culture and efficiency evaluation of rhIL-4 by MTT assay*

Erythroleukemic cell line (TF1, ATCC2031) was prepared from National Cell Bank of Iran (Pasteur Institute, Iran). This cell line was sub-cultured in RPMI 1640 (Gibco, Germany) media supplemented with 10% fetal bovine

**Table 2.** Statistical analysis of hIL-4 Proliferation.

Concentration (ng/ml)	OD	Mean	SD	P-value
0.001	0.035	0.032	0.002	0.009
0.003	0.041	0.045	0.002	0.006
0.01	0.1	0.097	0.002	0.025
0.03	0.139	0.145	0.008	0.011
0.1	0.18	0.181	0.003	0.003
0.3	0.21	0.249	0.020	0.005

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CTCGGTTTCAGCAATTTTAAATCTATATATAGAGATATCTTTGTCAGCATTGCATCGTTAATGGGTCTCACCTCCCAACTG
CTTCCCCCTCTGTTCTTCTCTGCTAGCATGTGCCGCAACTTTGTCCACGGACACAAGTGCATATCACCTTACAGGAGATC
ATCAAAACTTTGAACAGCCTCACAGAGCAGAAGACTCTGTGCACCAGTTGACCGTAACAGACATCTTTGCTGCCTCCAAG
AACACAACCTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCGGCAGTTCTACAGCCACCATGAGAAGGACACTCGC
TGCCTGGGTGCGACTGCACAGCAGTCCACAGGCACAAGCAGCTGATCCGATTCTGAAACGGCTCGACAGGAACCTCTGG
GGCTGGCGGGCTTGAATTCCTGTCTGTGAAGGAAGCCAACCAGAGTACGTTGGAAAACCTTCTTGGAAAGGCTAAAGACG
ATCATGAGAGAGAAATATTCAAAGTGTTCGAGCTGA
    
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**Figure 1.** The designed codon optimized human IL-4 sequence under gene promoter. Highlight sequence indicated IL-4 specific promoter, black sequence indicates IL-4 gene with signal peptide.

serum (FBS), 100 units/ml penicillin (Gibco, Germany) and maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

MTT (3-(4,(5-dimethylthiazol2-yl)-2,4-diphenylte trazoliumbromide)) as a Colorimetric assay was used to evaluate the biological activity of rhIL-4 on the cell proliferation. The cell suspension was prepared and then 2×10<sup>4</sup> cells were seeded in each well at 96-well cell culture plate. After 24 h of incubation, the purified recombinant hIL-4 at the concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 ng/ml were added to related groups.

The 96-well cell culture plate was incubated at 37 °C with a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 48 h. Then, 10 µl of MTT solution (0.5mg/ml in PBS) (Sigma, USA) was added per well and incubated for 4 h to form formazan crystals. Subsequently, the supernatants were slowly removed and 100µl of DMSO (Sigma, USA) was added to each well to dissolve the formazan crystals in the cells. The absorbance was measured at 570 nm with the reference filter of 620. The amount of color produced is directly proportional to the number of viable cells. All assays were performed in triplicates.

#### Statistical analysis

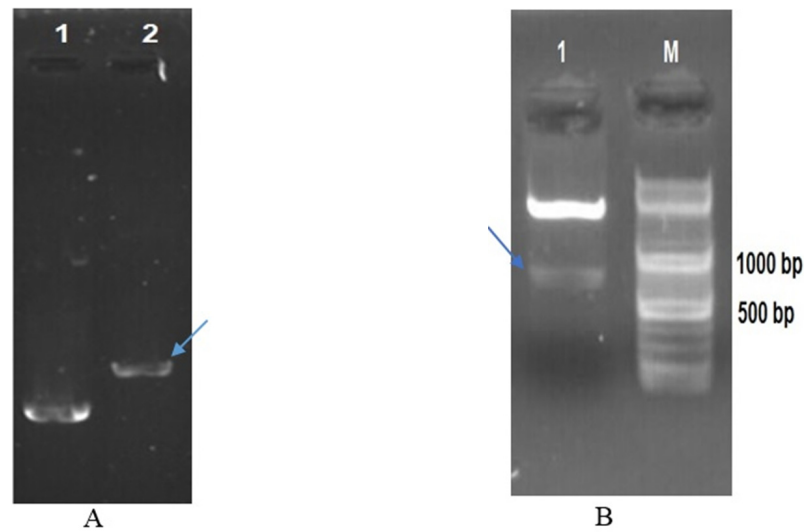
All experiments were performed at least three times. Descriptive statistics (means and standard deviations) were calculated using SPSS Software. P value of < 0.05 is considered to be statistically significant.

## Results and Discussion

The growth in the use of recombinant proteins has greatly increased in the recent years. Bacterial systems remain most attractive due to low cost, high productivity, and rapid use (Terpe 2006). In present study, we report the

cloning of human IL-4 cytokine in *E. coli* as a prokaryote host and the gene expression was evaluated by the specific promoter of the same gene under the control of the T7 RNA polymerase and we would eliminate the need for chemical inducers particularly in large-scale cultivation of recombinant bacteria. According to PROMO server analysis results of specific promoter, we found several binding sites for GR-beta, TFIID, HNF-3alpha, C-EBPbeta, XBP-1, and GR-1 that were recognized by *E. coli* DNA polymerase. Eukaryotic Promoters are much more complex and diverse than prokaryotic promoters. T7 RNA polymerase requires the associated protein sigma factor to bind the core promoter region stably and the transcription of the template strand can initiate. The specific IL-4 promoter is controlled by various DNA regulatory sequences including enhancers, boundary elements, insulators, and silencers. In fact, how T7 RNA polymerase locates this specific binding site in the large excess of non-promoter DNA remains a field of intense investigation. However, the rational choice of the adequate promoter system and host for a specific protein of interest remains difficult (Kanhere and Bansal 2005). *Escherichia coli* is one of the organisms of choice for the production of recombinant proteins. Its use as a cell factory is well-established and it has become the most popular expression platform (Rosano and Ceccarelli 2014). Large-scale protein expression trials have shown that <50% of bacterial proteins and <15% of non-bacterial proteins can be expressed in *E. coli* in a soluble form, which demonstrates the versatility of the system (Braun and LaBaer 2003).

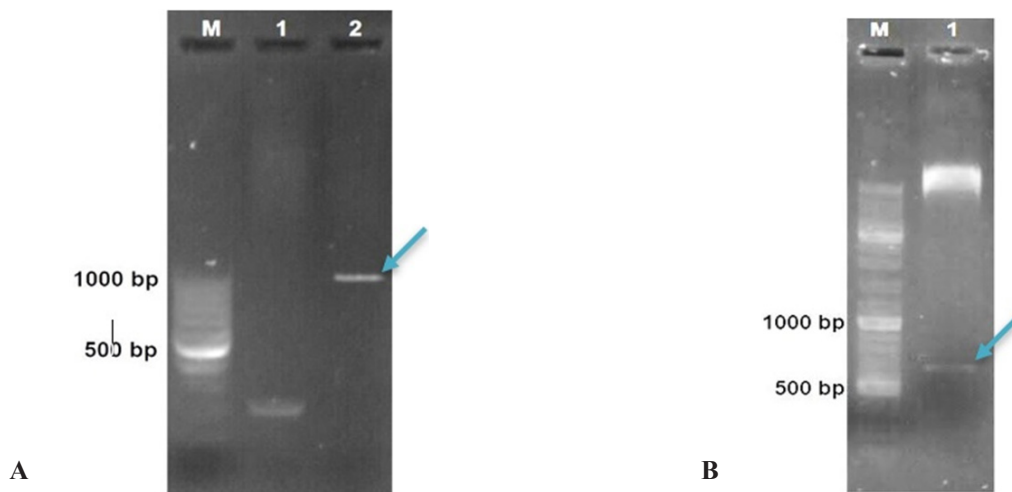
The detection and purification of proteins can be problematic. These processes can be generalized by using recombinant DNA technology to produce fusion proteins in which target proteins are fused to carrier polypeptides. The affinity of the carrier for a specific ligand enables



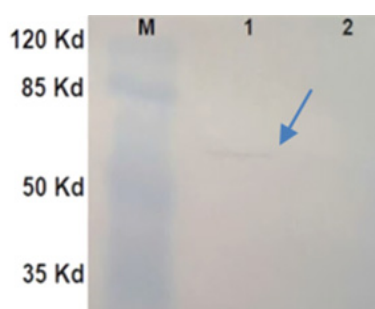
**Figure 2.** The Plasmids digestion by Hind III restriction enzyme. (A) lane1, intact pcDNA3.1 (+), lane2, pcDNA3.1 (+) digested by Hind III. (B), lane1, pGH- IL-4 digested by Hind III (812 bp), Lane M, DNA size marker.

the facile detection and purification of a fusion protein. S. Tag has several additional properties that are desirable in a carrier. The S. Tag carrier combines a small size (15 amino acid residues) with a high sensitivity of detection. S. Tag is composed of four cationic, three anionic, three uncharged polar, and five nonpolar residues. This composition makes S. Tag an excessively soluble peptide

with little structure and net charge near neutral pH. The S. Tag carrier is therefore unlikely to interfere with the proper folding or function of a fused target protein (Raines, McCormick et al. 2000). Accordingly, S tag was considered as fusion part for recombinant production and facile purification of recombinant IL-4, without need of post purification separation from IL-4.



**Figure 3.** (A) Confirmation of pcDNA3.1 (+)/hIL-4 cloning with PCR by plasmid universal primers; Lane1, universal PCR product of pcDNA3.1(+) as negative control. lane2; 988 bp PCR product of the cloned fragment; Lane M, DNA size marker 100-1000 bp (Fermentas, Lithuania). (B): Confirmation of pcDNA3.1(+)/hIL-4 cloning with digestion by NheI restriction enzyme; Lane1, 698 bp fragment of recombinant plasmid. Lane M, DNA size marker 100-3000 bp (Fermentas, Lithuania).



**Figure 4.** Western blot analysis of the recombinant protein expression. Lane 1, recombinant hIL-4 protein, Lane 2, BL 21 cell lysate as a negative control. Lane M, molecular weight marker.

Recombinant protein production has a major role in drug development accounting for approximately 30% of current biopharmaceuticals on the market (Overton 2014).

#### *Recombinant hIL-4 gene construct and promoter analysis*

Here, we showed the designed codon optimized sequence (JCAT server) under gene promoter (highlighted in Fig.1). According to PROMO server analysis results of specific promoter, several binding sites for GR-beta, TFIID, HNF-3alpha, C-EBPbeta, XBP-1, and GR-1 were recognized by *E. coli* RNA polymerase.

#### *Recombinant hIL-4 gene cloning into pcDNA3.1(+) vector*

pcDNA3.1 (+) vector and pGH plasmid were digested with HindIII restriction enzyme (Fig.2). The pcDNA3.1 (+)-rhIL-4 plasmid was confirmed by universal PCR and restriction analysis. Fig. 3 (A) shows PCR product of recombinant pcDNA3.1 (+)-hIL-4 plasmid (988 bp fragment) and recombinant plasmid digested by NheI restriction enzyme. Fig. 3 (B) shows 698 bp fragment of recombinant pcDNA3.1(+)-hIL-4 plasmid. Sequencing results confirmed the presence of hIL-4 gene in recombinant construct in an accuracy position.

#### *SDS-PAGE and western blot analysis*

The expression of recombinant hIL-4 protein was detected and compared with control forms of the bacteria by SDS-PAGE analysis and showed a specific protein band. Recombinant protein expression was confirmed by western blotting (Fig. 4).

#### *Purification of recombinant hIL-4 protein*

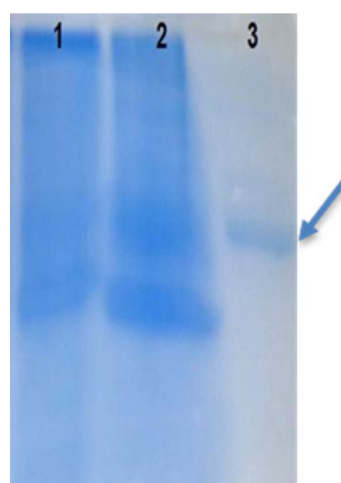
Purification of the recombinant protein was carried out

using S protein resin (Fig. 5). The purified protein was concentrated and its concentration was determined by Human IL-4 Quantikine ELISA Kit (R&D systems, USA) against the rhIL-4 specific antibody equal to 500 pg / ml.

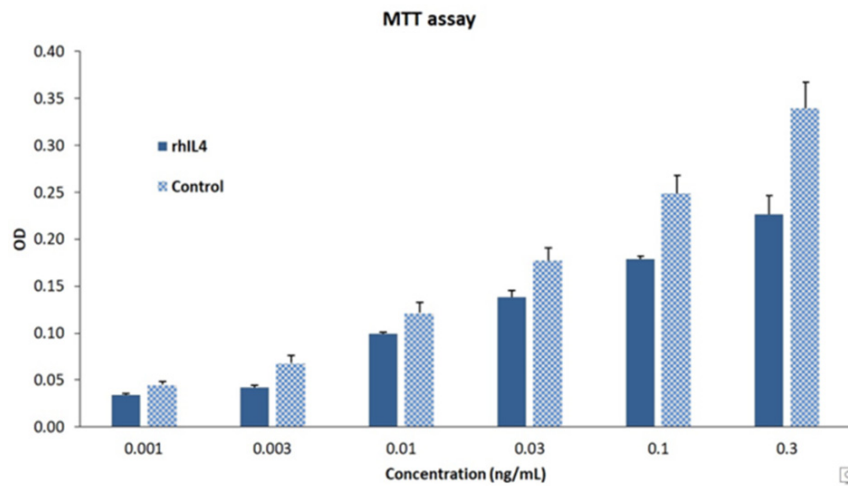
#### *Recombinant hIL-4 proliferative activity assay*

TF1 cell line was incubated in serum free RPMI 1640 with rhIL-4 for 48 hours. MTT assay demonstrated that the exposed cells with rhIL-4 were proliferated in a dose dependent manner in comparison to the control group (Fig. 6).

Most biotechnology pharmaceuticals are recombinant in nature which plays a key role against human lethal diseases. Recent advances in the cloning of cytokine genes and the availability of recombinant material have led investigators to conduct clinical trials with these agents (Khan, Ullah et al. 2016). For instance, Wong, Lotze et al. (1992) demonstrated that Administration of recombinant IL-4 to humans regulates gene expression, phenotype, and function in circulating monocytes. Monocytes taken from patients before therapy and cultured with and without LPS exhibited normal patterns of monokine-specific (IL-1 beta and TNF-alpha) mRNA expression, as well as secretion of peptide. The addition of rIL-4 to these cultures; however, resulted in the down-regulation of both gene expression and peptide release. Furthermore, monocytes from post-therapy patients exhibited reduced production of PGE2 and superoxide anion, compared with cells obtained before therapy. This effect persisted in culture independent of the



**Figure 5.** SDS-PAGE Analysis of the purified recombinant protein after purification. Lane 1, bacterial lysate negative control; Lane 2, bacterial lysate after 3h incubation; Lane 3, purified protein.



**Figure 6.** Recombinant Human IL-4 stimulates the TF-1 human erythroleukemic cell line Proliferation.

further addition of exogenous IL-4.

In other study Shintani, Ito et al. (2017) demonstrated that IL-4 administration at an acute phase of Myocardial Infarction enhanced cardiac repair through enhancing the M2-like macrophage-involved self-repair mechanism that represents IL-4 has great potential as a new (re-purposed) biological drug for the treatment of acute MI, encouraging the further development toward early clinical application.

As a multifunctional cytokine that can augment certain T cell responses, IL-4 is being evaluated as a possible therapeutic agent in the treatment of cancer patients. Benedetti, Pirola et al. (2000) demonstrated that retrovirus-mediated transfer of the gene for interleukin-4 was an effective treatment for rat brain glioblastomas. They transferred the gene for interleukin-4 into C57BL6J mouse primary neural progenitor cells and injected those cells into established syngeneic brain glioblastomas. This led to the survival of most tumor-bearing mice.

In phase II studies of recombinant human interleukin-4 in advanced renal cancer and malignant melanoma by Margolin, Aronson et al. (1994), rIL-4 was administered intravenously. They concluded that IL-4, when given as a single agent on this schedule at maximum tolerated dose, does not possess meaningful activity in renal cancer or melanoma. In similar study, Whitehead, Lew et al. (2002) have also used the recombinant human IL-4 by subcutaneous injection. The results of this study showed IL-4 in this dose and schedule is not useful for the treatment of patients with disseminated renal cell carcinoma. These findings clearly indicate that the effect of IL-4 in cancer is more complex.

Production of dendritic cells is another application of

the IL-4. Romani, Reider et al. (1996) used the recombinant IL-4 cytokine to produce dendritic cells from human peripheral blood monocytes. Bender, Sapp et al. (1996) also used the peripheral blood monocytes in the presence of recombinant IL-4 cytokine for the production of dendritic cells. Dauer, Obermaier et al. (2003) introduced a rapid production of dendritic cells from peripheral blood monocytes in the presence of recombinant IL-4 cytokine.

The molecular mechanisms involved in IL-4 signaling, and especially in the activation of STAT6, may be investigated as potential targets for the development of novel treatments (Zamorano, Rivas et al. 2003).

## Conclusion

Cloning and expression of the IL-4 proteins for a variety of applications were exercised by several researchers up to now (Struhl, Cameron et al. 1976; van Kimmenade, Bond et al. 1988). In current study IL-4 gene was successfully cloned in prokaryotic system and then biological activity of rhIL-4 measured through a cell proliferation MTT assay using TF-1 human erythroleukemic cells. Today, wide range of cytokine assays are available. As biologically active entities, cytokines cannot be fully characterized by physicochemical methods alone. Thus, biological assays have become increasingly important for their biological characterization and potency determinations. These bioassays include measurement of stimulation or inhibition of cell proliferation or cytotoxicity by MTT (Sachdeva and Asthana 2007). Kitamura, Tange et al. (1989) have found that it is possible to use TF-1 in bioassay systems for GM-CSF, IL-3, EPO, and some other cytokines. These

cytokines stimulate the proliferation of TF-1 cells and IL-4 and IL-6 extend the survival of TF-1 cells. The results of the present study similarly demonstrated that rhIL-4-S tag could stimulate the TF-1 human erythroleukemic cell line proliferation in a dose dependent manner.

Considering that rhIL-4 is not produced in our country and should be made from other countries at a high cost. Therefore, mass production of IL-4 can be a great help in clinical trials and research studies. On the other hand, in this study, the production of IL-4 was made in the prokaryotic system, which is less costly and less time-consuming than the eukaryotic system. Also, using prokaryotic system, we could produce more of IL-4 than eukaryotic ones, which are very important in large scale protein production.

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## Competing Interests

The authors declare that they have no conflict of interest.

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